Peptidomimetic GnRH Antagonist AEZS-115 Inhibits the Growth of Ovarian and Endometrial Cancer Cells

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Abstract. *Background: AEZS-115 (Aeterna Zentaris GmbH, Frankfurt/M, Germany) is an orally active peptidomimetic antagonist of gonadotropin-releasing hormone (GnRH). In various tumors, an autocrine growth-promoting loop has been described for GnRH. The current study evaluates the antitumor activity and mechanism of action of AEZS-115 in models of ovarian and endometrial cancer. Materials and Methods: Human A2780, Acis2780, OAW-42, Ovcar-3, SKOV-3, Hec1A and Ishikawa cells were analyzed for GnRH receptor expression by reverse transcription polymerase chain reaction (RT-PCR). These cell lines were incubated with AEZS-115 at 1, 10 and 100 μM for 24 h, 48 h, and 72 h and the number of viable cells was determined. Fluorescence activated cell sorting (FACS) cell cycle analyses were performed with increasing concentrations of AEZS-115. Co-treatment experiments of cancer cells with GnRH antagonist cetrorelix and peptidomimetic GnRH antagonist AESZ-115 were carried out. Results: A2780, Acis2780, OAW-42, Ovcar-3, SKOV-3, Hec1A and Ishikawa cells expressed GnRH receptors as demonstrated by RT-PCR. GnRH antagonist AEZS-115 inhibited growth of all cell lines in a dose- and time-dependent manner. Half maximal inhibitory concentration (IC_{50}) values at 48 h of incubation were between 7 and 17.5 μM and for 72 h between 4.5 and 12.5 μM. IC_{50} values for ovarian and endometrial cancer cells were rather similar. These results were obtained by tetrazolium salt [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] assay and confirmed by additional crystal violet staining. Cell cycle FACS analysis revealed that AEZS-115 dose-dependently increased the fraction of apoptotic cells. Co-treatment experiments carried out with AEZS-115 and peptidic GnRH-antagonist cetrorelix suggest that the antitumor effect of AEZS-115 is not mediated by blockade of the GnRH receptor. Conclusion: GnRH antagonist AEZS-115 exhibited substantial antitumor activity in ovarian as well as endometrial cancer cell lines. However, this antitumor effect was not mediated by the tumoral GnRH receptors. To identify the mechanism of action of this compound, further research is warranted. Its in vitro antitumor activity makes AEZS-115 a promising candidate for in vivo studies of ovarian and endometrial cancer.*

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Gonadotropin-releasing hormone (GnRH) antagonists induce an immediate suppression of sex steroids by competitively blocking pituitary GnRH receptors, thus abolishing the secretion of the gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (1). This fully reversible pharmacological castration is clinically used in the field of reproductive medicine (2), namely in controlled ovarian hyperstimulation and for the treatment of sex steroid-sensitive tumors, such as prostate and breast cancer. In recent years, it has become evident that a part from the endocrine effect, native GnRH and its analogs also have direct auto and paracrine effects mediated by GnRH receptors on cells of benign and malignant tissues (3). While these peripheral GnRH receptors are mainly confined to benign cells of the reproductive system, they are also found in an increasing number of cancer types, thus representing a potential structure for targeted tumor therapy (4, 5). In fact, extensive work by Schally has identified an autocrine stimulatory loop based on GnRH and its respective receptor in a number of tumor types and also in ovarian and endometrial cancer (6, 7). As a matter of fact, 60-80% of endometrial and ovarian carcinomas express receptors for GnRH, and it has been shown *in vitro* and *in vivo*, that these tumors can be successfully treated with the peptidic GnRH-antagonist cetrorelix (4). Currently, there are two peptidic GnRH antagonists clinically available, which are extensively used in the field of reproductive medicine. The main disadvantage of peptidic antagonists is that oral application of these compounds is not possible. Therefore, a variety of nonpeptide GnRH antagonists which are orally active have

been developed in order to facilitate treatment (8). However, these compounds are still in varying degrees of preclinical and clinical development. In the current study, the antitumor effect of nonpeptide GnRH antagonist AEZS-115 was evaluated in preclinical models of human ovarian and endometrial cancer.

Materials and Methods

Reagents and cell lines. Peptidomimetic GnRH antagonist AEZS-115, as well as decapeptide cetrorelix, were kindly provided by Aeterna Zentaris GmbH (Frankfurt, Germany). Human ovarian cancer cell lines A2780, Acis2780, Ovcar-3, SKOV-3 and human endometrial cancer cell lines Hec1A and Ishikawa were obtained from the American Type Culture Collection (Manassas, VA, USA). The human ovarian cancer cell line OAW-42 was obtained from Cell Line Services, Heidelberg, Germany. All cells were grown in RPMI-1640 medium (PAA, Cölbe, Germany) containing 10% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin and 0.5% sodium pyruvate solution. The broad-spectrum caspase inhibitor (benzyloxycarbonyl-val-ala-asp(OMe)-fluoromethylketone) z-VAD-FMK was from Bachem (Weil am Rhein, Germany); Necrostatin was from Enzo Life Sciences (Farmingdale, NY USA). Propidium iodide (PI) and all other reagents, unless indicated otherwise, were purchased from Sigma (St. Louis, MO, USA).

Cytotoxicity MTT assay and photometric evaluation of cell growth. To quantify the cytotoxicity of AEZS-115 and cetrorelix, the viability of cells was measured with a non-radioactive cell counting assay. Cells were cultured in 96-well flat-bottom plates, in humidified atmosphere with 5% CO_2 at 37˚C. The cell density was initially adjusted to 2×10^5 cells/ml in a final volume of 50 μl/well. Cells were treated with 0.1 μM-100 μM AEZS-115 or with 1 μM-200 μM cetrorelix for 24 h, 48 h and 72 h, respectively. For the last 4 h of incubation, cells were pulsed with 10 μl of tetrazolium salt [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT] labelling reagent (Roth, Karlsruhe, Germany) at a final concentration of 0.5 mg/ml. The colorimetric assay is based on the cleavage of yellow MTT to pure violet formazan crystals by metabolically active cells. The crystals were solubilized by addition of 100 μl 10% sodium dodecyl sulphate (SDS) in 0.01 M HCl to each well. Absorbance was measured spectrophotometrically using a 540-nm wavelength ELISA reader (Tecan, Männedorf, Switzerland) and Magellan software (Tecan, Männedorf, Switzerland). The experiments were performed sixfold, and at least three independent experiments were performed for each cell line.

Determination of GnRH receptor I (GnRHRI) mRNA content by reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from cells by RNeasy kit (Qiagen, Hilden, Germany). The quality and quantity of RNA preparations was assessed with NanoDrop ND-1000 (PEQLAB Biotechnologie GmBH, Erlangen, Germany). Generation of cDNAs by reverse transcription and RT-PCR reaction was performed in one step (Qiagen OneStep RT-PCR kit; Qiagen) according to the manufacturer's instructions. The gene for the constitutively expressed ribosomal protein L13A (rpL13A) was used as housekeeping gene (9) in order to monitor RNA quality and cDNA synthesis, and to ensure that equivalent amounts of cDNA were used in all PCR amplifications. Oligonucleotides were synthesized by Sigma-Aldrich (Taufkirchen, Germany): *rpL13A* f: 5'-TACGCTGTGAAGGCATCAAC-3', rpL13A r: 5'-CACCATCCGC TTTTTCTTGT-3'; *GnRHRI* f1: 5'-TCTAGCAGACAGCTCT GGACA-3'; GnRHRI f2: 5'-TGGCAAACAGTGCCTCTCCT-3'; and GnRHRI r: 5'-GAGTCTTCAGCCGTGCTCTT-3'. All PCR products were analyzed by separation on a 2% agarose gel stained with GelRed (BIOTIUM, Hayward, CA, USA).

Flow cytometry. For cell cycle analysis, cells were treated with AEZS-115 as indicated, harvested, fixed and permeabilized overnight in ice-cold 70% ethanol (Merck, Darmstadt, Germany). The cells were washed twice with PBS. RNA was digested with RNase A (Gibco Life Technologies, Paisley, UK). The DNA was stained with PI (50 μg/ml). Fluorescence was recorded in a FACSCalibur instrument (Becton Dickinson, Heidelberg, Germany). Instrument settings were adjusted to move the G_0/G_1 peak to 200 relative fluorescence units. Cells to the left of this peak appeared to have a DNA content below 2n, indicative of cell death. Aggregated cells were gated out. A total of 10,000 events per condition were recorded.

Results

Effects of AEZS-115 on the growth of human endometrial and ovarian cancer cells. To investigate whether the peptidomimetic GnRH antagonist AEZS-115 would lead to reduced growth or to cell death in human endometrial and ovarian cancer cells, cancer cell lines were treated with increasing concentrations of AEZS-115 or with solvent only for 24, 48 and 72 h.

While the solvent DMSO did not affect cell growth, AEZS-115 exhibited strong growth-inhibitory effects that became more pronounced with increasing treatment time.

While IC_{50} values at 24 h were broadly variable, we found IC_{50} values at 48 h incubation ranging from 7-18 μM. IC_{50} values after 72 h were slightly lower than after 48 h as shown in Table I.

Comparison of peptidomimetic GnRH antagonist AEZS-115 to the decapeptide GnRH antagonist cetrorelix. The two chemically different GnRH antagonists, AEZS-115 and cetrorelix, were compared with respect to their ability to inhibit the growth of two ovarian and two endometrial human cancer cell lines. In all these cell lines, AEZS-115 proved to be superior, with IC_{50} values of at least two-fold less than the concentrations needed of cetrorelix (Table II).

Detection of GnRH I receptor mRNA by RT-PCR in endometrial and ovarian cancer cell lines. We performed an RT-PCR analysis of GnRH I receptor-specific mRNA and were able to detect the receptor-specific mRNA in all the cell lines we tested. Thus, specific bands at the expected size of 794 base pairs were present (Table III). Of not, Acis2780 cells showed only a very weak expression for the GnRH receptor.

Table I. *IC_{50} values for AEZS-115 in μM at 24 h, 48 h and 72 h of incubation.*

Cell line	24 h	48 h	72 h
Ovarian cancer			
A2780	9	7	7
Acis2780	8	9	9
SKOV3	50	15	11.5
Ovcar-3	100	18	12
OAW42	>100	13.5	4.5
Endometrial cancer			
Ishikawa	30	17.5	12.5
Hec1A	25	11	5

Table II. *Comparison of IC_{50} values for AEZS-115 and cetrorelix in μM in two ovarian (SKOV-3, Ovcar-3) and two endometrial (HEC1A, Ishikawa) cancer cell lines.*

Cell line	AEZS-115	Cetrorelix
SKOV-3	21.5	48.46
Ovcar-3	11.09	64.97
HEC1A	8.885	61.66
Ishikawa	6.223	≥200

Table III. *GnRH receptor expression measured by RT-PCR.*

Cell line	GnRH receptor expression
Ovarian cancer	
A2780	+
Acis2780	–/+
SKOV3	++
Ovcar-3	++
OAW42	+
Endometrial cancer	
Ishikawa	+
Hec1A	++

Expression is very weak (–/+), normal (+) or strong (++).

Co-treatment of human ovarian and endometrial cancer cells with AEZS-115 and multicaspase inhibitor z-VAD-FMK as well as with AEZS-115 and necrostatin. We incubated A2780 ovarian and endometrial cancer cells with increasing concentrations of AEZS-115 and the multicaspase inhibitor zVAD-FMK (30 μM) for 24 h. In addition, A2780 ovarian and endometrial cancer cells were incubated with increasing concentrations of AEZS-115 and necrostatin, an inhibitor of necroptosis (50 μM) for 24 h (Figure 1). There was no difference in the AEZS-115-induced cytotoxicity regardless of caspase inhibition by z-VAD-FMK or necrosis inhibition by necrostatin in A2780 ovarian cancer (Figure 1) and endometrial cancer cells (data not shown).

DNA cell cycle analysis by FACS. In order to assess whether AEZS-115 arrested cell growth in a specific phase of the cell cycle or rather caused cell death, we performed a flow cytometric DNA cell cycle analysis on AEZS-115-treated OAW42 and A2780 human ovarian (Figure 2), and Ishikawa human endometrial cancer cells (data not shown). Cancer cells were treated with two different concentrations of AEZS-115 or not for 48 h, fixed, permeabilized, stained with PI and analyzed by flow cytometry. This revealed a dose-dependent decrease of cells in the G_0/G_1, S and G_2 phases of the cell cycle, whereas the fraction of hypodiploid cells appearing to the left of the G_0 cell population was concomitantly increased (Figure 2). Since this subG_0 population is indicative of cell death, it can be concluded that AEZS-115 not only arrests the growth of human endometrial and ovarian cancer cells, but actually kills tumor cells, irrespective of their current state in the cell cycle.

Co-incubation of ovarian cancer cell line A2780 with GnRH antagonist cetrorelix and peptidomimetic GnRH antagonist AEZS-115. We co-incubated A2780 ovarian cancer cells with GnRH antagonist cetrorelix and with increasing concentrations of AEZS-115. The cells were saturated with 200 μM cetrorelix leading to growth inhibition by 70%. The remaining cells were then treated with increasing doses of AEZS-115 to demonstrate competition for the same receptor. However, the IC_{50} of AEZS-115 remained virtually unchanged and was in the low micromolar range (Figure 3).

Discussion

We demonstrated that all ovarian and endometrial cancer cell lines studied here did express mRNA for the GnRH receptor, however, to varying degrees. AEZS-115 was consequently effective in suppressing cell growth in all cell lines, with IC_{50}s in the low micromolar range. Additionally, it was shown by flow cytometric analysis of the cell cycle that AEZS-115 dose-dependently increased the fraction of apoptotic cells. However, the antitumor effect of AEZS-115 was not reduced with inhibitors of classical apoptosis and necroptosis. This observation is in accordance with novel findings concerning programmed cell death. Thus, it was demonstrated that there are various caspase-dependent and -independent mechanisms which can induce apoptosis (10). Additionally, necroptosis, a form of programmed cell death resembling necrosis, was recently discovered (11). Thus, as observed in our study, if one way of programmed cell death is inhibited, cells can still undergo alternative forms of cell death.

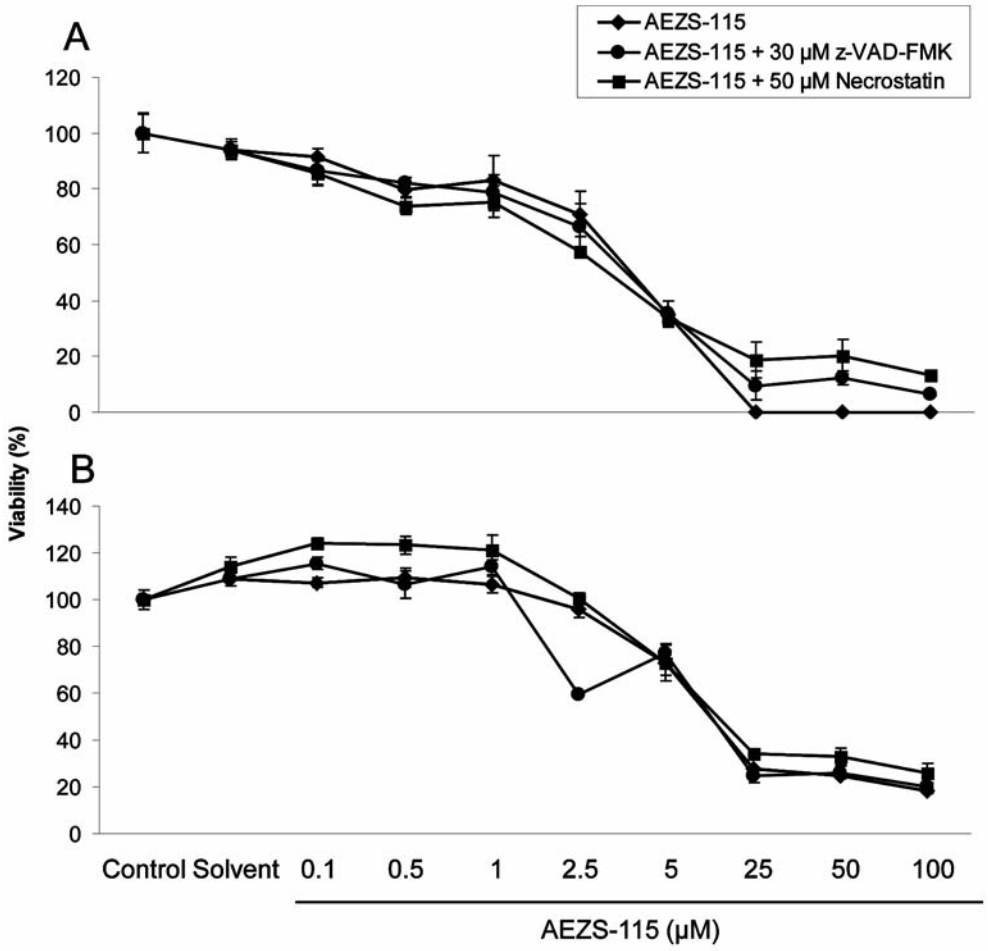

Figure 1. *Co-treatment of human ovarian cancer cells A2780 (A) and human endometrial cancer cells HEC1A (B) with AEZS-115 alone and in combination with multicaspase inhibitor z-VAD-FMK, as well as, with necrostatin an inhibitor of necroptosis.*

As previously shown, peptidic antagonist cetrorelix effectively suppressed tumor growth in ovarian and endometrial cancers (12-19). Surprisingly, growth inhibition was substantially stronger subsequent to treatment with AEZS-115 as compared to cetrorelix. Millar and co-workers demonstrated that tumoral GnRH receptors may display different affinities to GnRH and its analogs, and may use different post receptor signal transduction pathways as compared to pituitary GnRH receptors (20, 21). This group also demonstrated that signal transduction of the GnRH receptor is somehow determined by the cellular context, i.e. in different cells, the same receptor may use different signal transduction pathways (21). This theoretic concept could explain the lack of correlation of GnRH receptor expression with the oncostatic effect of AEZS-115 observed here. Thus, we were prompted to speculate that the GnRH receptors on tumor cells display distinct affinities for cetrorelix and AEZS-115 and performed co-treatment experiments with cetrorelix and AEZS-115. GnRH receptors on A2780 ovarian cancer cells were saturated with 200 µM cetrorelix leading to a growth inhibition by 70%. The remaining cells were then treated with increasing doses of AEZS-115 to demonstrate competition for the same receptor, however the IC_{50} of AEZS-115 remained virtually unchanged in the low micromolar range. As most of the GnRH receptors were saturated with cetrorelix, at least a shift towards a higher IC_{50} would be expected. These findings suggest that the observed growth inhibition subsequent to treatment with AEZS-115 was not mediated by blockade of the GnRH receptors. However, endocrine activity at nanomolar concentrations has been demonstrated for AEZS-115 both *in vitro* and *in vivo* (Engel *et al*. unpublished data). Additionally, when tested in 130 G-protein-coupled receptors, affinity of AEZS-115 was only shown for the GnRH receptor (Engel *et al*. unpublished data). However, due to its molecular structure, AEZS-115 can freely permeate cell membranes and may thus target intracellular components of growth-promoting signal-transduction pathways at adequate concentrations.

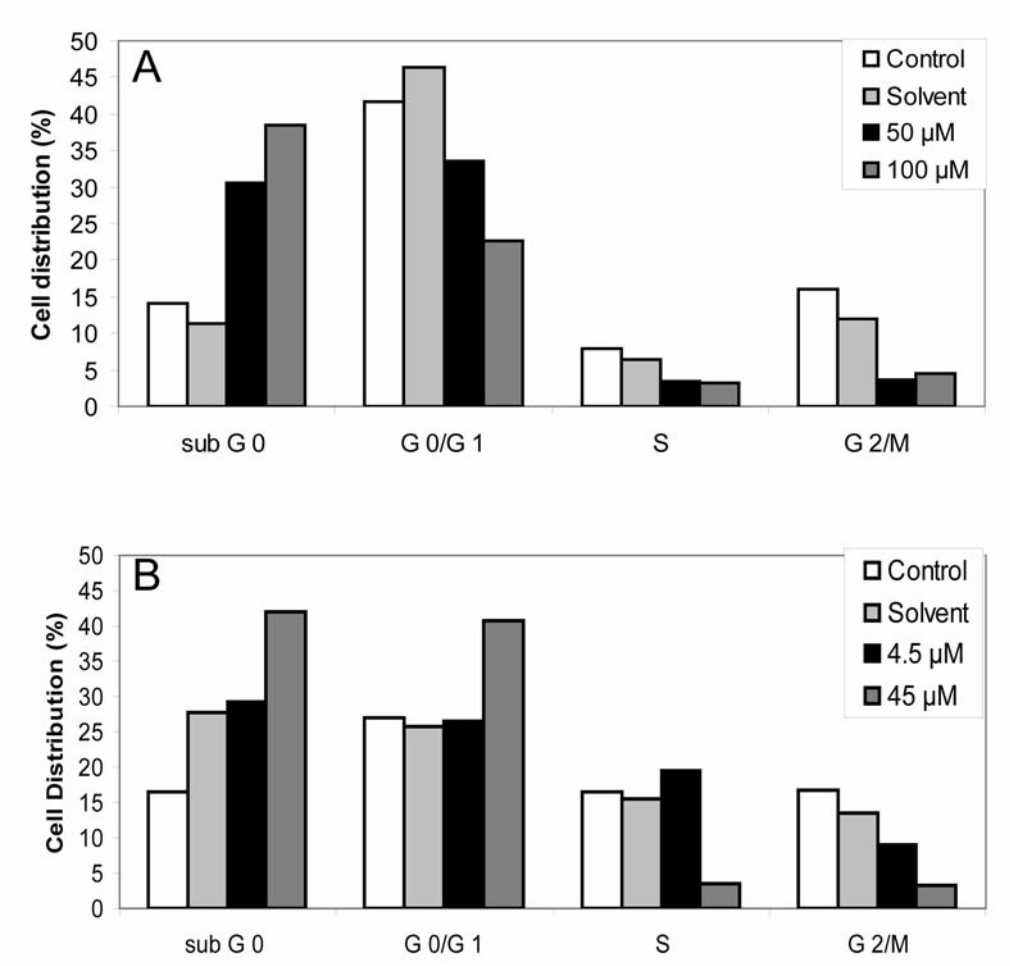

Figure 2. *Effect of AEZS-115 on the cell cycle distribution of human endometrial and ovarian cancer cells. OAW42 (A) and A2780 (B) human ovarian cancer were treated with AEZS-115 (A: 50 µM, 100 µM) (B: 4.5 µM, 45 µM) or not for 48 h, fixed, permeabilized, stained with PI and analyzed by flow cytometry. The figure shows the distribution of the cells in the different phases of the cell cycle.*

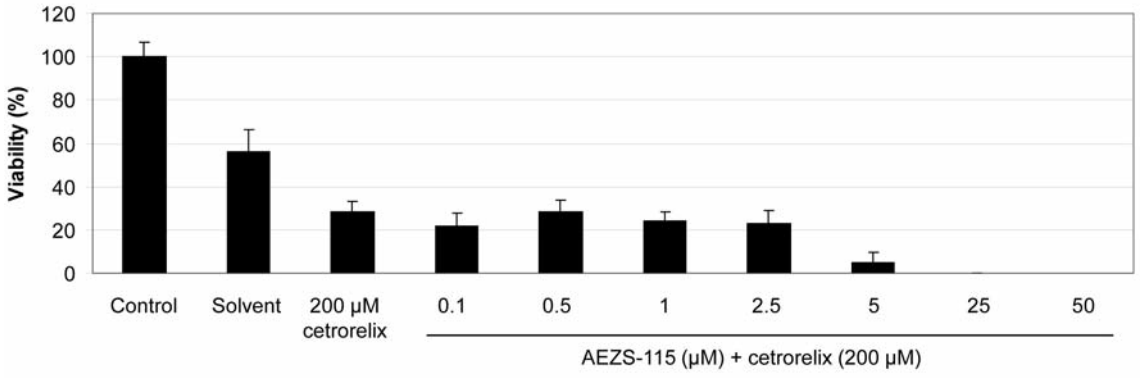

Figure 3. *Co-incubation of ovarian cancer cell line A2780 with GnRH antagonist cetrorelix and peptidomimetic GnRH antagonist AEZS-115.*

Thus, the current study demonstrated good antitumor activity of peptidomimetic GnRH antagonist AEZS-115 in human ovarian and endometrial cancer cell lines. However, this effect was most likely not mediated by tumoral receptors of GnRH and further research is warranted to clarify the mechanism of tumor inhibition of AEZS-115.

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