

Treatment with luteinizing hormone-releasing hormone antagonist SB-75 decreases levels of epidermal growth factor receptor and its mRNA in OV-1063 human epithelial ovarian cancer xenografts in nude mice

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Abstract. The aim of this study was to investigate the effect of administration of LH-RH antagonist SB-75 and agonist [D-Trp⁶]LH-RH on receptors for epidermal growth factor (EGF) in OV-1063 human epithelial ovarian cancer. Female athymic nude mice bearing xenografts of OV-1063 human epithelial ovarian cancer were treated for 3 weeks with the modern LH-releasing hormone (LH-RH) antagonist [Ac-D-Nal(2)¹, D-Phe(4Cl)², D-Pal(3)³, D-Cit⁶, D-Ala¹⁰] LH-RH (SB-75, Cetrorelix), the agonist [D-Trp⁶]LH-RH, or bombesin/gastrin-releasing peptide antagonist RC-3095. SB-75 and [D-Trp⁶] LH-RH were injected s.c. at doses of 100 µg/day, and RC-3095 was injected at a dose of 40 µg/day. Tumor growth, as measured by percentage change in tumor volume, was significantly inhibited by the treatment with SB-75, but not by [D-Trp⁶] LH-RH or RC-3095. Treatment with SB-75 greatly decreased the levels of mRNA for EGF receptor and reduced the number of EGF binding sites on tumor membranes. Effects of SB-75 on EGF

receptors might be related to inhibition of tumor growth. Our findings support the view that LH-RH antagonists such as SB-75 could be considered for possible hormonal therapy of epithelial ovarian cancer.

Introduction

Various findings suggest that epidermal growth factor (EGF) plays a role in the growth of epithelial ovarian cancer (1-4). Elevated levels of transforming growth factor- α (TGF- α), an EGF-related polypeptide that binds to EGF receptor (EGFR), were reported in ascites (1) from patients with disseminated ovarian cancers and EGFR is commonly expressed in human ovarian cancers (2). In addition, expression of mRNA for EGFR has been demonstrated in primary human ovarian adenocarcinomas (3,4). EGFR positive status has been shown to be correlated with poor survival in patients with ovarian cancer (2).

High levels of gonadotropins have been implicated in the growth of human epithelial ovarian cancers (5,6). Luteinizing hormone-releasing hormone (LH-RH) agonists have been used for the treatment of women with epithelial ovarian cancer (5,7,8). In addition to its function as a key hormone in the regulation of the pituitary-gonadal axis, LH-RH affects human extrapituitary tissues (9). The existence of a binding site specific for LH-RH has been demonstrated in 80% of human epithelial ovarian cancers (5). LH-RH and mRNA for LH-RH are present in human ovarian cancers (10). These data indicate that an autocrine system involving LH-RH might exist in ovarian cancer which could be a point of attack for new therapeutic approaches based on the direct action of LH-RH analogues.

Previously, we demonstrated that inhibition of growth of estrogen-dependent MXT mammary cancer in mice by LH-RH antagonist SB-75 and agonist [D-Trp⁶] LH-RH was associated with a significant down-regulation of EGFRs (11). We have also shown that inhibition of growth of some hormone-dependent cancers by the bombesin/gastrin-

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Abbreviations: LH-RH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; [D-Trp⁶]LH-RH, D-tryptophan-6-luteinizing hormone-releasing hormone; EGF, epidermal growth factor; TGF- α , transforming growth factor α ; EGFR, epidermal growth factor receptor; Ac, acetyl; Nal(2), 3-(2-naphthyl)alanine; Phe(4Cl), 4-chlorophenylalanine; Pal(3), 3-(3-pyridyl)alanine; cit, citrulline; Tpi, 2, 3, 4, 9-tetrahydro-1H-pyrido[3, 4-b]indol-3-carboxylic acid; cDNA, complementary deoxyribonucleic acid; IP₃, inositol 1, 4, 5-triphosphate; DAG, diacylglycerol

Key words: LH-RH analogs, ovarian carcinoma, nude mice, epidermal growth factor receptor, Northern blot analysis

releasing peptide (GRP) antagonist RC-3095 could be linked to a major decrease in EGFR levels in tumor membranes (for review, see ref. 12).

Recently, we have found that LH-RH antagonist SB-75 (Cetorelix) and agonist [D-Trp⁶] LH-RH inhibit proliferation of OV-1063 human epithelial ovarian cancers *in vitro* (13) and that SB-75 inhibits growth of this cancer *in vivo* (14). In this study we investigated the effect of administration of SB-75, [D-Trp⁶]LH-RH and RC-3095 on LH-RH receptor and EGFR in OV-1063 human epithelial ovarian cancer xenografted in nude mice.

Materials and methods

Chemicals. LH-RH antagonist SB-75 [Ac-D-Nal(2)¹, D-Phe(4Cl)², D-Pal(3)³, D-Cit⁶, D-Ala¹⁰]LH-RH(Cetorelix) and bombesin/GRP antagonist D-Tpi⁶, Leu¹³ Ψ[CH₂NH]-Leu¹⁴ bombesin (6-14)(RC-3095), originally synthesized in our laboratory by solid-phase methods (9-12), were made by Asta Medica (Frankfurt/Main, Germany). LH-RH agonist [D-Trp⁶] LH-RH was supplied by Debiopharm S.A. (Lausanne, Switzerland).

Animals. Five to six-week old female athymic Ncr nu/nu nude mice were obtained from the Frederick Cancer Research Facility of the National Cancer Institute (Frederick, MD). The mice were housed in sterile cages under laminar flow hoods in a temperature-controlled room with a 12 h light/12 h dark schedule, and were fed autoclaved chow and water *ad libitum*.

The institutional guidelines for the use and care of animals were followed.

Cells and tumors. OV-1063 cells were kindly provided by the late Dr Shoshana Biran (Hadassah University, Jerusalem, Israel) and were maintained in phenol-red free RPMI 1640 medium, supplemented with 10% heat-inactivated and dextran-coated charcoal-treated fetal bovine serum (13,14). The medium also contained 25 mM N-[2-hydroxyethyl] piperazine-N' [2-ethanesulfonic acid] buffer, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin-B (0.25 μg/ml). Cells were cultured in Costar T-75 flasks in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. OV-1063 cells growing exponentially were transferred into nude mice by s.c. injection of 1 x 10⁷ cells into the right flank of 5 mice (14).

In vivo experimental protocol. Xenografts of 1 mm³ OV-1063 tumor pieces were transplanted subcutaneously into the flanks of nude mice. Treatments were started 2 weeks after tumor transplantation, when the tumors measured approximately 10 mm³, and continued for 3 weeks (14). The mice were divided into three groups (10 animals per group). The control group was injected s.c. with 0.9% sodium chloride only. Group 2 received SB-75 dissolved in distilled water containing 5% mannitol by daily s.c. injections at a dose of 100 μg/day/animal. Group 3 was given [D-Trp⁶]LH-RH dissolved in 0.9% sodium chloride at a dose of 100 μg/day/animal s.c. Group 4 received RC-3095 dissolved in 0.1% dimethyl sulfoxide and 0.9% sodium chloride by daily

s.c. injections at a dose of 40 μg/day/animal. Tumors were measured weekly with a microcaliper, and tumor volume was calculated by using the following formula: Length X Width X Height X 0.5236. Percentage change in tumor volume from the start of the treatment was used herein as a parameter of growth rate. Complete procedures for measuring tumor growth inhibition are described in detail elsewhere (14). At the end of the experiment, the mice were sacrificed under light methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, NJ) anesthesia (14). Tumors were removed, cleaned and weighed. Tumor pieces were stored at -80°C for receptor studies and in liquid nitrogen for RNA extraction.

RNA extraction and Northern blot analysis. Total RNA was extracted from the tumor tissues using acid guanidinium thiocyanate-phenol-chloroform (15). Total RNA (40 μg) was subjected to electrophoresis through 1.2% agarose gels containing 2.2 M formaldehyde. After electrophoresis, the RNA was transferred to Nytran nylon membrane (Schleicher & Schuell, Keene, NH). The membrane was heated for 2 h at 80°C. Following the prehybridization in 50% formamide, 5 X Denhardt's reagent (50 X Denhardt = 10 g/liter Ficoll, 10 g/liter polyvinylpyrrolidone, 10 g/liter bovine serum albumin), 200 μg/ml denatured salmon testis DNA, 5 X SSPE (20 X SSPE = 175.3 g/liter NaCl, 27.6 g/liter NaH₂PO₄, and 7.4 g/liter EDTA, pH 7.4), 50 g/liter dextran sulfate, and 10 g/liter sodium dodecyl sulfate at 42°C for 4 h, hybridization was performed for 18 h using a ³²P-labeled human EGF receptor cDNA probe. The membrane was then washed in stringent conditions and was autoradiographed using Kodak XAR-2 film at -80°C for 3-6 days.

A human EGF receptor cDNA probe (16) constructed from 2.4 kilobase (kb) pair sequence of the *Cla*I-digested fragment was purchased from American Type Culture Collection (Rockville, MD). The probe was labeled with deoxycytidine 5'-[α-³²P] triphosphate (Amersham, Arlington Heights, IL) using random primers per vendor's protocol (United States Biochemical, Cleveland, OH) to a specific activity of approximately 5-10 x 10⁸ cpm/μg DNA. Densitometric analysis of the results was performed using Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

EGF and [D-Trp⁶] LH-RH binding studies in tumor membranes. Preparation of tumor membranes and receptor binding studies of EGF and [D-Trp⁶] LH-RH were performed as previously described (11,12). The LIGAND-PC computerized curve fitting program of Munson and Rodbard was used to determine the types of receptor binding, dissociation constant (K_d), and maximal binding capacity of receptors (B_{max}).

Statistical analyses. All data are expressed as the mean ± SEM, and statistical analyses were performed by using Duncan's new multiple range test or Student's t-test.

Results

The OV-1063 tumors in the control group grew rapidly and continuously throughout the treatment period (Fig. 1).

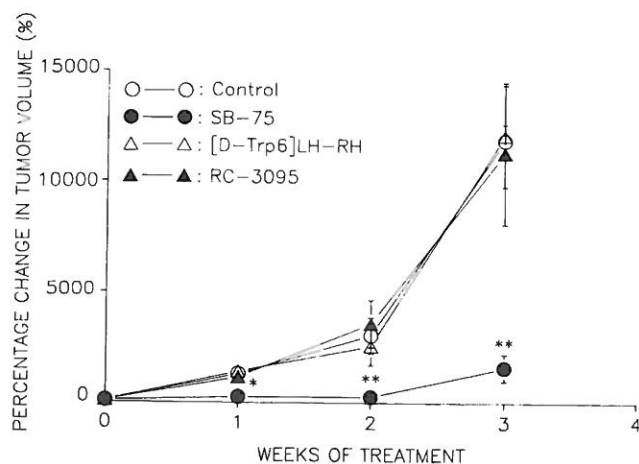


Figure 1. Percentage change in tumor volume in nude mice bearing OV-1063 human ovarian cancer xenografts during treatment with LH-RH antagonist SB-75, agonist [D-Trp⁶]LH-RH, and bombesin/GRP antagonist RC-3095. Vertical lines indicate the SEM. * $p < 0.05$, ** $p < 0.01$ vs. control by Duncan's new multiple range test.

Percentage change in tumor volume in mice receiving LH-RH antagonist SB-75 was significantly decreased following 1 week of therapy ($p < 0.05$) and this inhibition continued until day 21 when the experiment was ended ($p < 0.01$). Complete results on tumor volume, tumor weight and other parameters of tumor growth are reported elsewhere (14). Treatment with the agonist [D-Trp⁶]LH-RH or bombesin/GRP antagonist RC-3095 had no significant effect on tumor growth.

The results of receptor assays for EGF are shown in Table I. ¹²⁵I-labeled EGF was bound to one class of high affinity binding sites ($K_d = 0.78 \pm 0.04$ nM). Maximal binding capacity (B_{max}) of EGFR was 289.4 ± 11.3 fmol/mg of membrane protein. Treatment with SB-75 significantly increased the binding affinity ($K_d = 0.37 \pm 0.11$ nM, $p < 0.05$), and decreased maximal binding capacity (66.1 ± 7.7 fmol/mg of membrane protein, $p < 0.01$) of EGFR. Treatment with [D-Trp⁶]LH-RH or RC-3095 did not change the binding affinity and capacity of EGFR. In our study, shortage of tumor tissue did not allow us to repeat the displacement assays for LH-RH receptors on membranes from the treated groups. Consequently, the 10-point displacement experiments for LH-RH receptors were performed twice for the control group and only once for the treated groups. ¹²⁵I-labeled [D-Trp⁶]LH-RH was bound to one class of specific binding sites with high affinity ($K_d = 7.19 \pm 0.94$ nM) and low capacity ($B_{max} = 373.6 \pm 30.8$ fmol/mg of membrane protein). Treatment with SB-75 slightly increased the binding affinity of LH-RH receptors ($K_d = 4.38$ nM), and decreased the concentration of LH-RH receptors (234.5 fmol/mg of membrane protein), while treatment with [D-Trp⁶]LH-RH did not change the binding affinity and maximal binding capacity of LH-RH receptors.

In order to investigate whether the decrease in the number of EGF binding sites observed by radioreceptor assay was correlated with the suppression of EGFR mRNA levels, Northern blot analysis was performed. Gene transcripts were

Table I. Characteristics of EGF receptors in membranes of OV-1063 human epithelial ovarian cancer xenografts in nude mice after treatment with LH-RH agonist [D-Trp⁶]LH-RH, LH-RH antagonist SB-75, or bombesin/GRP antagonist RC-3095.

	K_d (nM)	B_{max} (fmol/mg protein)
Control	0.78 ± 0.04	289.4 ± 11.3
[D-Trp ⁶]LH-RH	0.77 ± 0.23	260.0 ± 20.3
SB-75	$0.37 \pm 0.11^*$	$66.1 \pm 7.7^{**}$
RC-3095	0.75 ± 0.05	278.1 ± 8.7

The results were obtained from 10-point displacement experiments in triplicate tubes. The results are shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. control by Student's t-test.

observed at approximately 10 and 4 kb, as shown in Fig. 2a. Ethidium bromide staining of the agarose gels indicated the presence of approximately equal amounts of RNAs (Fig. 2b). The EGFR mRNA was quantitated by densitometric analysis of the 10 kb band. The results, shown in Fig. 3, demonstrate about 75% decrease in the level of mRNA for EGFR after the treatment with SB-75. No changes were seen in the 4 kb mRNA species.

Discussion

The present study shows that the inhibition of growth of OV-1063 human epithelial ovarian cancer xenografts in nude mice by administration of LH-RH antagonist SB-75 is accompanied by a marked decrease in number of EGF binding sites and the levels of mRNA for EGFR.

Treatment with LH-RH agonists or antagonists leads to selective medical hypophysectomy and chemical castration and provides an efficacious approach for the treatment of some sex-hormone dependent tumors such as prostate and breast cancers (5). High levels of gonadotropins in women after the menopause have been implicated in ovarian carcinogenesis (5,6). The reduction in blood levels of gonadotropins and sex-steroids, induced by agonists and antagonists of LH-RH, may be useful for the treatment of ovarian cancer. LH-RH agonists including [D-Trp⁶]LH-RH have been tried clinically for the treatment of women with epithelial ovarian cancer, and the response rate vary from 10 to 50% (5). However, this work and our previous study (14) show that growth of ovarian OV-1063 cancers in nude mice was not inhibited by [D-Trp⁶]LH-RH, despite a significant suppression of serum LH levels (14). Thus, it is unlikely that the gonadotropins alone can be responsible for stimulating the growth of OV-1063 cancer.

Previously, we showed that SB-75 can suppress the growth of OV-1063 cells *in vitro* at concentrations 10 times lower than those of [D-Trp⁶]LH-RH most likely through high

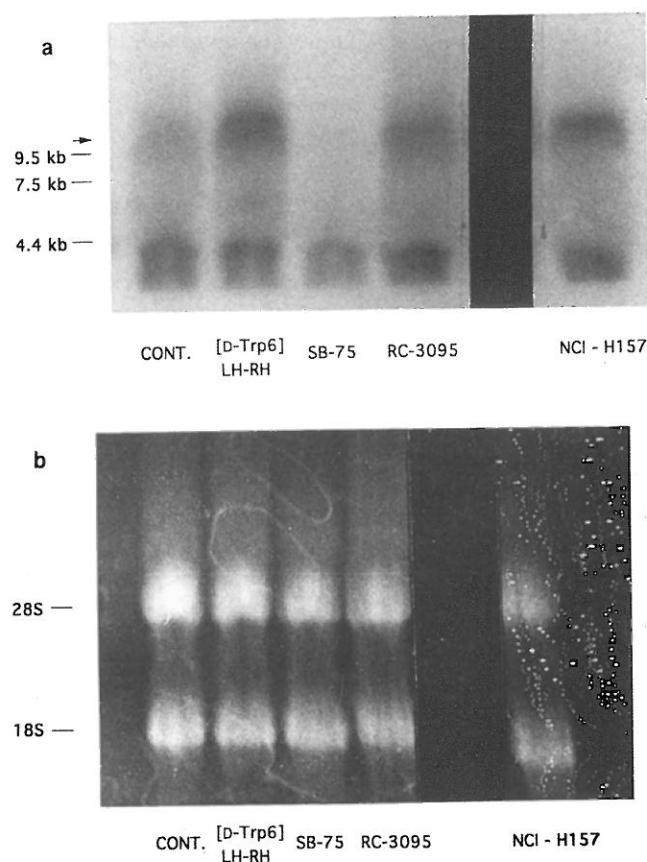


Figure 2. a: Northern blot analysis of EGF receptor mRNA. Total RNA, isolated by acid guanidinium thiocyanate-phenol-chloroform method, was fractionated on 1.2% agarose gels and transferred onto a nylon membrane. The blots were hybridized to the EGF receptor complementary DNA probe. The arrow indicates 10 kb mRNA band for EGFR. b: rRNAs loaded on the gel are shown after staining with ethidium bromide. Tumor tissue of NCI-H157 human non-small cell lung cancer xenograft in nude mice was used as a positive control. Representative data are shown. The same results were obtained from three independent experiments.

affinity LH-RH receptors (13). Emons *et al* (17) demonstrated the presence of high affinity LH-RH binding sites in EFO 21 and EFO 27 human epithelial ovarian cancer cell lines and showed that both the agonist [D-Trp⁶] LH-RH and antagonist SB-75 inhibited the proliferation of these cell lines *in vitro*. Collectively, these findings support the view that the antitumor effect of SB-75 in OV-1063 cancer cells *in vivo* might be exerted, at least in part, through LH-RH receptors. Antagonist SB-75, but not agonist [D-Trp⁶] LH-RH decreased the concentration of EGF receptors and the levels of mRNA for EGFR.

We identified two hybridizable bands of mRNA for EGFR, 10 kb and 4 kb. Previous studies examining the expression of EGFR mRNA from ovarian cancer tissue have varied in terms of the members and sizes of detectable mRNA. While the band approximately 10 kb is consistently reported (3,4,16), additional 5.6 (3,16) and 2.9 kb bands (3) have also been detected. The reason for the presence of the additional size mRNA may be related to intrinsic properties of the particular tumor from which the RNA was isolated. In our study in OV-1063 tumors, only the 10 kb mRNA was altered by treatment with SB-75. Previously, we have shown that inhibition of growth of estrogen-dependent MXT

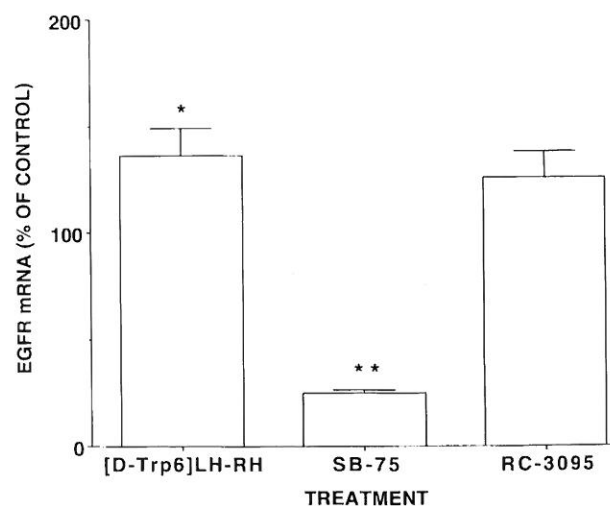


Figure 3. Densitometric analysis of EGF receptor mRNA. The levels of EGF receptor mRNA in the treated groups were divided by the level in the control tumor and expressed as a percentage of control value. Results are the mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control by Student's t-test.

mammary cancer in mice by SB-75 and [D-Trp⁶] LH-RH was associated with a significant decrease in the number of EGFRs in tumor membranes (11). This down-regulation of EGFRs by LH-RH agonist and antagonist might be responsible for the inhibitory effect of this class of analogs on *in vivo* proliferation of some cancers. Bombesin/GRP antagonist RC-3095 was used in our study because previously we have shown that RC-3095 inhibits the growth of mammary, prostatic, colorectal and other cancers (12). The inhibition of tumor growth by RC-3095 was associated with a major decrease in EGFR levels in membranes of these cancers. In our study, RC-3095 failed to inhibit the growth of OV-1063 tumors in nude mice. This might be due to the lack of endogenous autocrine bombesin/GRP stimulating these cells or the absence of bombesin/GRP receptors in the membranes of this tumor.

The exact molecular mechanism of action of LH-RH antagonist SB-75 on the level of EGFR mRNA and the concentration of EGFR remains to be elucidated. Certain growth factors, in addition to affecting their respective receptors, can induce transmodulation of other receptors, particularly those for EGF. There are several mechanisms by which different agents can influence EGFR (for review, see ref. 12): i) modulation of receptor binding or kinase activity through phosphorylation, ii) down regulation of receptors through internalization and iii) modulation of receptor synthesis at transcriptional level. LH-RH is known to initiate a series of intracellular signals in gonads, which cause the stimulation of phospholipase A₂, C, and D⁹. The stimulation of phospholipase A₂ results in the production of arachidonic acid, which acts as a substrate for the generation of lipooxygenase metabolites. The stimulation of phospholipase C activity leads to the hydrolysis of polyphosphoinositides, generating inositol 1, 4, 5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ is responsible for the release of Ca²⁺ from intracellular stores, and DAG stimulates protein kinase C activity. In rat luteal cells in primary culture, LH-RH agonists activates both phospholipase A₂ and phospholipase C,

but LH-RH antagonists activate only phospholipase A2 (18). Lack of stimulatory effect on phospholipase C by LH-RH antagonists might result in the failure to activate protein kinase C. It has been shown by run-on transcription in isolated nuclei derived from epithelioid tumor lines, that activation of protein kinase C results in a 5-fold induction of EGFR gene transcription (19). In view of these findings, our observation that LH-RH antagonist SB-75 greatly reduced the levels of EGFR mRNA might result from lack of stimulation of pathways that activate protein kinase C, which could increase *de novo* transcription of the EGFR gene. LH-RH antagonists could also negatively regulate EGFR mRNA in a direct manner, as shown for dexamethasone in fetal rat lung cells (20). Other mechanisms might also be involved in modifying pools of EGFR mRNA, such as alterations in their rate of degradation.

In MIA PaCa-2 human pancreatic cancer cells, LH-RH analogues significantly inhibited the EGF-induced phosphorylation of the 170-kDa EGFR (21). Thus SB-75 may inhibit cancer cell growth by down-regulation of the EGFR through suppression of its phosphorylation on a tyrosine residue. Transmodulation of the EGFR may also result from threonine phosphorylation of the EGFR, catalyzed by protein kinase C (22). Involvement of SB-75 in some of these multiple pathways cannot be excluded.

In conclusion, our findings reported herein and in another study (14) suggest that the growth of OV-1063 human epithelial ovarian cancer xenografts in nude mice can be inhibited by the modern LH-RH antagonist SB-75 (Cetorelix). This effect was associated with a reduction in the number of EGFR on membranes of this tumor. SB-75 also decreased the levels of mRNA for EGFR. The exact molecular mechanism of action of LH-RH antagonists on the EGFR and its mRNA remains to be elucidated.

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