GnRH-II Antagonists Induce Apoptosis in Human Endometrial, Ovarian, and Breast Cancer Cells via Activation of Stress-Induced MAPKs p38 and JNK and Proapoptotic Protein Bax

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Abstract

Recently, we could show that gonadotropin-releasing hormone (GnRH)-II antagonists induce apoptosis in human endometrial, ovarian, and breast cancer cells in vitro and in vivo. In the present study, we have ascertained receptor binding and effects of GnRH-II antagonists on mitogenic signal transduction and on activation of proapoptotic protein Bax. The GnRH-II antagonists tested showed EC50 values for GnRH-I receptor binding in the range of 1 to 2 nmol/L. The GnRH-II agonist [d-Lys6]GnRH-II showed an EC50 value for GnRH-I receptor binding of ~1,000 nmol/L. Agonistic activity on GnRH-I receptor function with an EC50 of 13 nmol/L has been determined for [d-Lys6]GnRH-II. Antagonistic activities with EC50 values in the range of 1 nmol/L were determined for the GnRH-II antagonists. Treatment of human endometrial, ovarian, and breast cancer cells with GnRH-II antagonists resulted in time-dependent activation of stress-induced mitogen-activated protein kinases p38 and c-Jun NH2-terminal kinase. In addition, treatment with GnRH-II antagonists induced time-dependent activation of proapoptotic protein Bax. GnRH-II antagonists are not involved in activation of protein kinase B/Akt or extracellular signal-regulated kinase 1/2. The GnRH-II antagonists tested had similar binding affinities to the GnRH-I receptor comparable with that of GnRH-I antagonist Cetrorelix. Referring to the cAMP response element reporter gene activation assay, the GnRH-II agonist [d-Lys6]GnRH-II has to be classified as an agonist at the GnRH-I receptor, whereas the GnRH-II antagonists tested are clear antagonists at the GnRH-I receptor. GnRH-II antagonists induce apoptotic cell death in human endometrial, ovarian, and breast cancer cells via activation of stress-induced mitogen-activated protein kinases p38 and c-Jun NH2-terminal kinase followed by activation of proapoptotic protein Bax. [Cancer Res 2009;69(16):6473–81]

Introduction

Expression of gonadotropin-releasing hormone (GnRH)-I and its receptor as part of a negative autocrine/paracrine regulatory mechanism of cell proliferation has been shown in several malignant tumors, including cancers of endometrium, ovary, and breast (1–5). Besides GnRH-I, a second structural variant of GnRH exists in mammals. GnRH-II has antiproliferative effects on human endometrial, ovarian, and breast cancer cells, which are significantly greater than those of GnRH-I agonist Triptorelin (6). Induction of apoptosis is not involved in down-regulation of cancer cell proliferation induced by agonists of GnRH-I or GnRH-II (1). GnRH-I and GnRH-II agonists rather inhibit mitogenic signal transduction of growth factor receptors via activation of a phosphatidylinositol 3-kinase pathway (7–9).

Recently, we could show that antagonistic analogues of GnRH-II induce apoptotic cell death in human endometrial (10), ovarian (10), and breast cancer cells in vitro and in vivo. Treatment of human endometrial, ovarian, and breast cancer cells with GnRH-II antagonists resulted in apoptotic cell death via dose-dependent loss of mitochondrial membrane potential and activation of caspase-3 (10). These antitumor effects could be confirmed in nude mice. GnRH-II antagonists significantly inhibited the growth of xenotransplants of human endometrial, ovarian, and breast cancers in nude mice, without any apparent side effects (10).

GnRH-II antagonists appear to be suitable drugs for an efficacious and less toxic endocrine therapy for endometrial, ovarian, and breast cancers. To be able to further improve this therapy as well as to expand the therapeutic concept on further active agents and cancers, it is required to understand the underlying mechanisms. Therefore, we now have ascertained GnRH-I receptor binding of GnRH-II antagonists and assessed their effects on GnRH-I receptor function. In addition, we have analyzed the effects of GnRH-II antagonists on mitogenic signal transduction pathways involved in induction of apoptosis in human endometrial, ovarian, and breast cancer cells.

Materials and Methods

Cell lines and culture conditions. cDNA encoding human GnRH-I receptor was isolated from human pituitary cDNA library (MPI für Biophysik) and expressed in LTK mouse fibroblast cells. A single-cell clone with stable and high expression of human GnRH-I receptor was selected. In radioligand saturation binding experiments, GnRH antagonist Cetrorelix showed high-affinity binding to heterologously expressed human GnRH-I receptor with a Kd of 0.1 nmol/L. The cell line generated is well suited for determination of competitive binding characteristics of agonists and antagonists of human GnRH-I receptor.

To assess functional implications of receptor binding by GnRH-I competitive agents, a recombinant mouse fibroblast LTK-based cell line was established, which stably expressed human GnRH-I receptor and...
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luciferase reporter gene under control of a triple cyclic AMP response element (CRE) sequence linked to cytomegalovirus minimal promoter. GnRH-I agonist Triptorelin induced luciferase expression as monitored by ATP bioluminescence detection in nanomolar range. The established cell line is suitable to detect agonistic as well as antagonistic activities of human GnRH-I receptor binding agents.

Human endometrial cancer cell lines HeC-1A, HeC-1B, and Ishikawa, human ovarian cancer cell lines OVCAR-3 and EFO-21, and human breast cancer cell lines MCF-7 and T47-D were obtained from the American Type Culture Collection or the sources detailed previously (2, 5).

The cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in air as described previously (2–4).


Receptor binding assay. For receptor binding studies, iodinated Cetrorelix was used as tracer with ~80% peptide capable of specific receptor association. Receptor binding assays were done with intact cells under physiologic conditions (11). For displacement binding assays, cells were incubated with ~200 pmol/L [3H]Cetrorelix and different concentrations of unlabeled test compounds as competitor. Cell suspension in binding medium was layered on top of silicon/paraffin oil, incubated for 60 min at 37°C, and separated by centrifugation. The tips of the tubes containing cell pellet were cut off; subsequently, cell pellet and supernatant were analyzed by γ-ray radiation analysis. Amount of unspecific binding was determined by including unlabeled Cetrorelix at 1 pmol/L, final concentration and was typically ≤10% of total binding. EC50 values are calculated by using Graphpad Prism (GraphPad Software).

Functional CRE reporter gene activation assay. Cells were seeded 24 h before the experiment in medium supplemented with 1% FCS in white clear-bottomed 96-well MTPs. Cells were incubated for 6 h with 1 nmol/L Triptorelin and phosphodiesterase inhibitor Rolipram (agonist mode) or Rolipram alone (agonist mode) in the presence of different concentrations of the test compound. Subsequently, cells were lysed and ATP bioluminescence was measured. Calculation of EC50 values was done by using Graphpad Prism (GraphPad Software).

Western blot analysis. Effects of GnRH-II antagonists on activation of protein kinase B (PKB)/Akt, mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) 1/2, and stress-activated MAPKs protein kinase B (PKB)/Akt, mitogen-activated protein kinase (MAPK) activating enzyme. C-terminal residues of endogenous Bax were analyzed by immunoprecipitation followed by Western blot. Protein G Sepharose was washed twice with water, equilibrated with CelLytic buffer for 15 min, incubated with anti-Bax antibody 2D2 for 1 h, and then washed twice again with Laemmli buffer. Equal amounts of protein (80 μg) were added to each tube and further incubated for additional 1 h to facilitate immunoprecipitation. Proteins bound to protein G Sepharose were washed three times with CelLytic buffer to remove nonspecifically bound proteins. After final centrifugation, proteins bound to protein G Sepharose were dissolved in Laemmli buffer, separated on SDS-PAGE as described above, and subjected to Western blot analysis as described above using anti-Bax antibody 6A7 (1:500).

Results


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Basal expression of PTEN, basal activity of PKB/Akt, and effects of GnRH-II antagonist \([\text{Ac-D-2Nal}^1,\text{D-4Cpa}^2,\text{D-3Pal}^3,\text{Leu}^8,\text{D-Ala}^{10}]\)GnRH-II treatment on PKB/Akt and on ERK1/2 activity. To investigate whether the PTEN pathway or the Akt pathway is active in human endometrial cancer cell lines Hec-1A, Hec-1B, and Ishikawa, human ovarian cancer cell lines OVCAR-3 and EFO-21, and human breast cancer cell line MCF-7 and T47-D, we have analyzed the basal amount of PTEN and phospho-Akt (Fig. 2A). Hec-1A and Hec-1B endometrial cancer cell lines expressed PTEN but showed no activation of Akt. Ishikawa endometrial cancer cell line expressed phospho-Akt but not PTEN. Ovarian cancer cell line OVCAR-3 expressed PTEN but showed no activation of Akt, whereas ovarian cancer cell line EFO-21 was positive for phospho-Akt but negative for PTEN expression. Both tested breast cancer cell lines (MCF-7 and T47-D) expressed PTEN but showed no activation of Akt.

To examine whether GnRH-II antagonist treatment has effects on Akt activation, we have treated human endometrial cancer cell line Ishikawa (Fig. 2B) and ovarian cancer cell line EFO-21 (data not shown) with the GnRH-II antagonist \((10^{-7} \text{ mol/L})\) in a time-dependent manner and analyzed the amount of unphosphorylated Akt and phospho-Akt (Fig. 2B). In both cell lines, no effect of GnRH-II antagonist treatment on Akt expression and activation was detectable. The Akt pathway was not affected by the GnRH-II antagonist.

To examine whether the MAPK ERK1/2 signaling pathway is activated by GnRH-II antagonist treatment, phosphorylation of ERK1/2 and expression of c-Fos were analyzed. MCF-7 breast cancer cells (Fig. 2C, top), OVCAR-3 ovarian cancer cells (Fig. 2C, bottom), and EFO-21 ovarian cancer cells (Fig. 2C, bottom) were treated with the GnRH-II antagonist \((10^{-7} \text{ mol/L})\) for 3 h, and phosphorylation of ERK1/2 and expression of c-Fos were analyzed by Western blotting and immunohistochemistry, respectively. In all cell lines, no effect of GnRH-II antagonist treatment on ERK1/2 phosphorylation and c-Fos expression was detectable. The ERK1/2 pathway was not affected by the GnRH-II antagonist.
middle), and Ishikawa endometrial cancer cells (Fig. 2C, bottom) were treated with the GnRH-II antagonist (10⁻⁷ mol/L) in a time-dependent manner and the amount of phosho-ERK1/2 and c-Fos was analyzed. In MCF-7 breast cancer cells (Fig. 2C, top), OVCAR-3 ovarian cancer cells (Fig. 2C, middle), and Ishikawa endometrial cancer cells (Fig. 2C, bottom), no effects of GnRH-II antagonist treatment on ERK1/2 activation and c-Fos expression were detectable. The MAPK ERK1/2 signaling pathway was not affected by the GnRH-II antagonist.

Effects of GnRH-II antagonist [Ac-α-2Nal¹,α-4Cpa₂,α-3Pal₃,ᵬ,Leu⁸,α-Ala¹₀]GnRH-II treatment on stress-activated MAPKs JNK and p38 activity. To determine activation of JNK and p38 by GnRH-II antagonists, we treated MCF-7 human breast cancer cells, OVCAR-3 human ovarian cancer cells, and Ishikawa human endometrial cancer cells with the GnRH-II antagonist (10⁻⁷ mol/L) in a time-dependent manner and analyzed the amount of phospho-JNK (Fig. 3) and phospho-p38 (Fig. 3B). After GnRH-II antagonist treatment, phospho-JNK was increased in MCF-7 human breast cancer cells (Fig. 3A, top), OVCAR-3 human ovarian cancer cells (Fig. 3A, middle), and Ishikawa human endometrial cancer cells (Fig. 3A, bottom). Maximal JNK activation was observed 30 min (MCF-7, Fig. 3A, top) or 45 min (OVCAR-3, Fig. 3A, middle; Ishikawa, Fig. 3A, bottom) after GnRH-II antagonist treatment and decreased after that again. In some cases, a second maximum 90 min after treatment was detectable in the OVCAR-3 cell line (Fig. 3A, middle). GnRH-II antagonist treatment resulted in an increase of p38 phosphorylation in MCF-7 human breast cancer cells (Fig. 3B, top), OVCAR-3 human ovarian cancer cells (Fig. 3B, middle), and Ishikawa human endometrial cancer cells (Fig. 3B, bottom). In all three cell lines, maximal p38 activation was reached 45 min after GnRH-II antagonist treatment and remained constant after that (Fig. 3B).

Effects of GnRH-II antagonist [Ac-α-2Nal¹,α-4Cpa₂,α-3Pal₃,ᵬ,Leu⁸,α-Ala¹₀]GnRH-II treatment on MAPK ERK1/2 and c-Fos and stress-activated MAPKs JNK and p38 activity after pretreatment with p38 inhibitor SB203580, JNK inhibitor SP600125, or G protein αi/o inhibitor PTX. To check GnRH-II antagonist-induced activation of p38 and JNK, the tumor cells were pretreated with p38 inhibitor SB203580 or JNK inhibitor SP600125. GnRH-II antagonist (10⁻⁷ mol/L) treatment resulted in an increase of phospho-p38. Pretreatment with p38 inhibitor SB203580 but not JNK inhibitor SP600125 prevented the GnRH-II antagonist-induced increase of activated p38 (Fig. 4). Treatment with the GnRH-II antagonist (10⁻⁷ mol/L) resulted in an increase of phospho-JNK. Pretreatment with JNK inhibitor SP600125 but not p38 inhibitor SB203580 prevented the GnRH-II antagonist-induced increase of activated JNK (Fig. 4). ERK1/2 and c-Fos were not affected by GnRH-II antagonist treatment with or without inhibitor pretreatment (Fig. 4).

To analyze whether the effects of GnRH-II antagonist are mediated through PTX-sensitive G protein αi/o, the cells were...
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pre-treated with PTX. Neither GnRH-II antagonist-induced activation of p38 nor GnRH-II antagonist-induced activation of JNK were inhibited by pretreatment with PTX, indicating that these effects of the GnRH-II antagonist are not mediated through PTX-sensitive G protein αi/o (Fig. 4).

Effects of GnRH-II antagonist [Ac-α2Nal1,ω-4Cpa2,ω-3Pal3,6-Leu6,ω-Ala10]GnRH-II treatment on activation of Bax. To determine activation of proapoptotic protein Bax by GnRH-II antagonists, we treated MCF-7 human breast cancer cells (Fig. 5A), OVCAR-3 human ovarian cancer cells (data not shown), and Ishikawa human endometrial cancer cells (data not shown) with the GnRH-II antagonist (10⁻⁷ mol/L) in a time-dependent manner and analyzed the amount of activated Bax monomers and homodimers. After GnRH-II antagonist treatment, the amount of Bax homodimers was time-dependently increased, whereas the amount of Bax monomers was decreased in all three cell lines. Maximal Bax homodimerization was observed 240 min after GnRH-II antagonist treatment and remained constant after that.

Discussion

Previous work showed that already nanomolar concentrations of GnRH-II antagonists induce apoptotic cell death in human endometrial, ovarian, and breast cancer cells in vitro and in vivo via dose-dependent loss of mitochondrial membrane potential and activation of caspase-3 (10). To improve the GnRH-II antagonist therapy as well as to be able to expand the therapeutic concept on further active agents and other cancers, we tried to learn more about the underlying mechanisms. Now we have ascertained the effects of GnRH-II antagonists on mitogenic signal transduction and activation of proapoptotic protein Bax in human endometrial, ovarian, and breast cancer cells. In addition, we have assessed GnRH-I receptor binding and functional implications of GnRH-I receptor binding by GnRH-II antagonists.

We could show that in human endometrial, ovarian, and breast cancer cells GnRH-II antagonists induce apoptosis through activation of the intrinsic apoptotic pathway. GnRH-II antagonists interact with the signaling pathway of MAPKs by time-dependent activation of the stress-induced MAPKs p38 and JNK. Pretreatment with p38 inhibitor SB203580 prevented the GnRH-II antagonist-induced increase of activated p38 and pretreatment with JNK inhibitor SP600125 prevented the GnRH-II antagonist-induced increase of activated JNK. It is known that activation of p38 is associated with induction of apoptosis, p38 can cause phosphorylation of Bax and thus induces translocation of activated Bax to the mitochondrial membrane (12, 13). This mechanism could also be shown for JNK in hepatocytes (12, 14). Harris and Johnson could show JNK-dependent induction of apoptosis via phosphorylation of Bax in neurons (15). In the present study, we could show that these mechanisms are also involved in GnRH-II antagonist-induced apoptosis. After GnRH-II antagonist treatment, the amount of activated Bax homodimers was time-dependently increased. Gross and colleagues have shown that the conformational change in Bax after activation manifests in its homodimerization, translocation, and mitochondrial membrane insertion (16). Activation of the stress-induced MAPKs JNK and p38 followed by activation and homodimerization of the proapoptotic protein Bax seem to play an important role in induction of apoptosis by GnRH-II antagonists (Fig. 5B). By moving the balance from antiapoptotic to proapoptotic signals (Bad, Bax), the mitochondrial membrane is permeabilized and cytochrome c is released. Cytochrome c forms with ATP inhibitor caspase-9 and Apaf-1 the apoptosome, which leads to the

Figure 3. Time-dependent effects of GnRH-II antagonist [Ac-α2Nal1,ω-4Cpa2,ω-3Pal3,6-Leu6,ω-Ala10]GnRH-II (10⁻⁷ mol/L) treatment on activation of stress-activated MAPKs JNK (A) and p38 (B) in MCF-7 human breast cancer cells (top), OVCAR-3 human ovarian cancer cells (middle), and Ishikawa human endometrial cancer cells (bottom). Controls were treated with PBS. β-actin was used as loading control marker. After GnRH-II antagonist (10⁻⁷ mol/L) treatment, phosphorylated JNK was increased in all 3 cell lines (A). Maximal JNK activation was observed 30 min (MCF-7, A, top) or 45 min (OVCAR-3, A, middle; Ishikawa, A, bottom) after treatment and decreased after that again. In some cases, a second maximum 90 min after treatment was detectable in the OVCAR-3 cell line (A, middle). GnRH-II antagonist (10⁻⁷ mol/L) treatment resulted in an increase of p38 phosphorylation in all three cell lines (B). Maximal p38 activation was reached 45 min after treatment and remained constant after that. These are representative data obtained from three independent experiments in three different passages of each cell line.
activation of the effector caspase-3, which in turn activates the effector caspase-7 (17). Antiapoptotic proteins such as Bcl-1 and Bcl-xL are located on the outer membrane of the mitochondria and inhibit the release of cytochrome c. Proapoptotic proteins such as Bad and Bax are located within the cytosol and are recruited to the mitochondria after induction of death signaling where they induce cytochrome c release by channel formation within the mitochondrial membrane (18).

Several groups including ours have reported in the past that antiproliferative actions of GnRH-I analogues were inhibited by pretreatment with PTX, indicating that these effects are mediated through PTX-sensitive G protein \( \alpha_i/o \) (1, 8, 19–21). It is different with GnRH-II antagonists. Here, we found that neither GnRH-II antagonist-induced activation of p38 nor GnRH-II antagonist-induced activation of JNK was inhibited by pretreatment with PTX, indicating that these effects of GnRH-II antagonists are not mediated through PTX-sensitive G protein \( \alpha_i/o \). Recently, we had reported that GnRH-II antagonist-induced caspase-3 activity was slightly reduced by PTX pretreatment (10). Therefore, we had speculated that these effects of GnRH-II antagonists might be mediated through PTX-sensitive G protein \( \alpha_i/o \). However, the inhibiting effects of PTX pretreatment on GnRH-II antagonist-induced caspase-3 activity were very small and not significant (10).

White and colleagues have recently reported that coupling of the GnRH-I receptor to G protein \( \alpha_q/11 \), but not to G protein \( \alpha_i/o \) or G protein \( \alpha_s \), and consequent activation of ERK plays a crucial role in GnRH-mediated growth inhibition (22). In contrast, Maudsley and colleagues have shown that GnRH-I antagonists promote proapoptotic signaling in peripheral reproductive tumor cells by activating a G protein \( \alpha_i \)-coupling state of GnRH-I receptor (19).

Morgan and colleagues have recently reported that differences in levels of GnRH-I receptor and signaling differentially affect the

![Figure 4](image-url)
apoptotic machinery within cell lines and contribute to the cell type-specific effects of GnRH analogues on growth (23). In our cell lines, GnRH-I agonist Triptorelin and GnRH-I antagonist Cetrorelix induce antiproliferative effects by activating a G protein-coupling state of GnRH-I receptor (8), whereas induction of apoptosis by GnRH-II antagonists is G protein-coupled independent. The differences regarding G protein coupling seem to depend on ligand and cell context.

PKB/Akt plays an important role in the balance between surviving and death (apoptosis) of a cell (24). It could already be shown that PKB/Akt can inhibit induction of apoptosis by phosphorylation of the proapoptotic protein Bad on Ser136 (25). Therapy of leiomyoma with GnRH-I agonist Leuprorelin leads to a reduction of volume (26). It could be shown that the amount of phospho-Akt was reduced resulting in an increase of phospho-Bad. It is suspected that these effects lead to induction of apoptosis because the antiapoptotic proteins FLIP and PED/PEA15 are also inhibited (27). Treatment of prostate cancer cell lines DU-145 and LNCaP with GnRH-I agonist Triptorelin resulted in induction of apoptosis via inhibition of PKB/Akt (28). It must be taken into account, however, that in both cell lines the basal amounts of activated PKB/Akt are very high (29). The reduction of PKB/Akt activity seems to be linked to mitogenic signaling because the stress-activated MAPK JNK is activated after treatment with GnRH-I agonist Triptorelin. PKB/Akt is able to regulate JNK signaling by different mechanisms (30). In human endometrial cancer cell line Ishikawa and human ovarian cancer cell line EFO-21, we found overactivation of PKB/Akt due to inactivating PTEN mutations. In both cell lines, no effect of GnRH-II antagonist treatment on PKB/Akt expression and activation was detectable. The PKB/Akt pathway was not affected by GnRH-II antagonists. Apoptosis also could be induced in the other cell lines used in which basal activity of PKB/Akt is not increased. In αT3-1 pituitary cells, it could be shown that the GnRH-I agonist-induced reduction of insulin-like growth factor–induced activation of PKB/Akt could be restored by GnRH-I antagonists (31). PKB/Akt does not play a relevant role in induction of apoptosis by GnRH-II antagonists. Although GnRH analogues interact with phosphatidylinositol 3-kinase-PKB/Akt signaling in gynecologic cancer cells, this seems only to apply to agonists and not to antagonists.

Activation of ERK1/2 by GnRH-I and GnRH-II agonists was shown in ovarian cancer cells (32–34). GnRH-I antagonist Antide was shown to inhibit these GnRH-I and GnRH-II agonist-induced ERK1/2 activation (33). Our group found that epidermal growth factor–induced ERK1/2 activation was inhibited by GnRH-I agonist Triptorelin, GnRH-II agonist [d-Lys6]GnRH-II, and GnRH-I antagonist Cetrorelix (9, 35, 36). In the present study, we could show that GnRH-II antagonists are not involved in

Figure 5. A, time-dependent effects of GnRH-II antagonist [Ac-c-2Na1,c-4Cpa2,c-3Pal3,6,Leu8,c-Ala15]GnRH-II (10⁻⁷ mol/L) treatment on homodimerization of proapoptotic protein Bax in MCF-7 human breast cancer cells. Upper bands represent Bax homodimers (42 kDa) and lower bands represent Bax monomers (21 kDa). After GnRH-II antagonist treatment, the amount of Bax homodimers (42 kDa) was time-dependently increased whereas the amount of Bax monomers (21 kDa) was decreased. After 30 min of GnRH-II antagonist treatment, Bax monomers were not detectable anymore. These are representative data obtained from three independent experiments in three different passages of the cell line. Experiments using OVCAR-3 human ovarian cancer cells or Ishikawa human endometrial cancer cells gave identical results. B, schematic representation of GnRH-II antagonist signaling in human ovarian, endometrial, and breast cancer cells. GnRH-II antagonists induce apoptosis through the intrinsic apoptotic pathway via stress-induced MAPKs p38- and JNK-induced activation of pro-apoptotic protein Bax, loss of mitochondrial membrane potential, release of cytochrome C, and activation of caspase-3.
ERK1/2 activation. The involvement of the ERK1/2 pathway in apoptotic signaling is also a very rare process because activation of ERK1/2 normally leads to proliferation (37).

In the present study, we could show that all three GnRH-II antagonists tested have a high binding affinity to the GnRH-I receptor comparable with that of the GnRH-I antagonist Cetrorelix. The GnRH-II agonist [D-Lys6]GnRH-II has a considerably lower binding affinity to the GnRH-I receptor. It is possible that [D-Lys6]GnRH-II has a higher affinity to the putative GnRH-II receptor. However, GnRH-II agonist-induced activation of the GnRH-I receptor could already be confirmed (33). Referring to the CRE reporter gene activation assay, the GnRH-II agonist [D-Lys6]GnRH-II has to be classified as an agonist at the GnRH-I receptor, whereas the GnRH-II antagonists tested are clear antagonists at the GnRH-I receptor. These results may indicate that GnRH-II analogues act via the GnRH-I receptor. However, after knockdown of GnRH-I receptor expression, the GnRH-II antagonist-induced activation of p38 was only slightly reduced, indicating that the antitumor effects of GnRH-II antagonists are not exclusively mediated through the GnRH-I receptor. An additional pathway such as the putative GnRH-II receptor may be responsible for GnRH-II antagonist-induced apoptosis. The GnRH-I receptor binding assays were carried out using pituitary cells and fibroblasts. Therefore, we cannot confirm a complete antagonism in tumor cells because the GnRH signal transduction is cell context dependent (38). However, it cannot be ruled out that GnRH-II antagonists-induced apoptosis is mediated through the GnRH-I receptor. It would be very interesting to know whether GnRH-II antagonist-induced activation of p38 and subsequent apoptosis would be abrogated after knockdown of expression of the putative additional receptor for GnRH. Different knockdown experiments, however, using GnRH-II receptor antisense fragments resulted in apoptotic cell death (39). In addition, binding assays and functional assays for the putative GnRH-II receptor are not available at the moment. For additional studies, human tumor cells transfected with the well-known marmoset monkey GnRH-II receptor could be used, although the human putative GnRH-II receptor is different to the marmoset monkey GnRH-II receptor. It is supposed that, if the putative human GnRH-II receptor exists, it is shortened with only five transmembrane domains, whereas the marmoset monkey GnRH-II receptor is seven-transmembrane domain receptor (39, 40). At present, therefore, this question cannot be answered.

In conclusion, we could show that GnRH-II antagonists induce apoptotic cell death in human endometrial, ovarian, and breast cancer cells already in nanomolar doses. Apoptosis induced by GnRH-II antagonists is mediated through the intrinsic apoptotic pathway via stress-induced MAPKs p38- and JNK-induced activation of the proapoptotic protein Bax, loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspase-3. In addition we could show that GnRH-II antagonists couple to the GnRH-I receptor and are clear antagonists at the GnRH-I receptor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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