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

Stefanie Fister,1 Andreas R. Günthert,1 Babette Aicher,2 Klaus W. Paulini,2 Günter Emons,3 and Carsten Gründker1

1Department of Gynecology and Obstetrics, Georg-August-University, Göttingen, Germany and 2Drug Discovery and Preclinical Development, Æterna Zentaris GmbH, Frankfurt, Germany

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-Lys⁸]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 cyclic AMP 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.

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 phosphotyrosine phosphatase resulting in down-regulation of cancer cell proliferation (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 murine 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 Kᵢ 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 murine fibroblast LTK-based cell line was established, which stably expressed human GnRH-I receptor and...
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 OVCA-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, 3).

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% of 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 [125I]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-radiation analysis. Amount of specific binding was determined by including unlabeled Cetrorelix at 1 μmol/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 p38, JNK, and pERK (12–14) were analyzed by Western blot. Monoclonal mouse anti-human phospho-Akt, polyclonal rabbit anti-human p90 ribosomal S6 kinase (4EBP1), polyclonal rabbit anti-human p38, polyclonal rabbit anti-human phospho-p38, polyclonal rabbit anti-human p44/p42 MAPK, polyclonal rabbit anti-human phospho-p44/p42, polyclonal rabbit anti-human phospho-stress-activated protein kinase/JNK, and polyclonal rabbit anti-human stress-activated protein kinase/JNK antibodies were obtained from Cell Signaling. Polyclonal rabbit anti-human c-Fos antibody was purchased from Abcam and polyclonal anti-human β-actin antibody was from Sigma–Aldrich.

For determination of GnRH-II antagonist-induced MAPK activity, cells were treated without or with GnRH-II antagonists (10−7 mol/L) in a time-dependent manner. To inhibit GnRH-II antagonist-induced MAPK activation, cells were pretreated (30 min) with 3 μmol/L SB203580 (1 μmol/L; Promega), JNK inhibitor II SP600125 (1 μmol/L; CelLytic), or pertussis toxin (PTX; 20 μg/mL; Sigma–Aldrich) and then treated for 45 min with GnRH-II antagonist.

After incubation, cells were detached immediately with 0.5 g trypsin (Biochrom) and 0.1 mmol EDTA in 1 liter PBS/bovine serum albumin. Pellets were washed twice with PBS and resuspended with CelLytic buffer (Sigma) containing protease inhibitors (Sigma). Equal amounts of protein per sample (40 μg) were used and diluted to equal volumes with Laemmli buffer. Cell lysates were separated on SDS-PAGE (10%, ProSieve 50 Gel Solution; Cambrex) under reducing conditions and transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare). The nitrocellulose membranes were blocked with 5% instant skimmed milk powder, spray-dried (Naturaflex; Töpperl) in TBST [137 mmol/L NaCl, 2.7 mmol/L KCl, 0.1% Tween 20, 25 mmol/L Tris-HCl (pH 7.4)] for 1 h at room temperature, washed with TBST, and incubated at 4°C overnight with the appropriate antibody in an 1:500 (anti-β-actin) or an 1:1,000 dilution in TBST and then, following washings, incubated at room temperature with horseradish peroxidase–conjugated anti-mouse IgG or anti-rabbit IgG (GE Healthcare Europe). After rinsing, membranes were washed twice with water, equilibrated with Laemmli buffer for 15 min, incubated with anti-Bax antibody 2D2 (1:500) 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 Laemmli 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


Effects of GnRH-II antagonist [d-Lys6]GnRH-II and antagonist [Ac-d-2Nal1,D-4Cpa2,D-3Pal3,6,Leu8,D-Ala10]GnRH-II on GnRH-I receptor function. Agonistic activity with an EC50 of 13 nmol/L has been determined for GnRH-II agonist [d-Lys6]GnRH-II (Fig. 1B). GnRH-II antagonist [Ac-d-2Nal1,D-4Cpa2,D-3Pal3,6,Leu8,D-Ala10]GnRH-II showed no activity with respect to functional CRE activation by the human GnRH-I receptor. Antagonistic activity with an EC50 of 1.5 nmol/L was determined for GnRH-II antagonist [Ac-d-2Nal1,D-4Cpa2,D-3Pal3,6,Leu8,D-Ala10]GnRH-II (Fig. 1C). No antagonistic activity was observed for GnRH-II agonist [d-Lys6]GnRH-II (Fig. 1C).

Basal expression of PTEN, basal activity of PKB/Akt, and effects of GnRH-II antagonist \([\text{Ac-D-2Nal}^1,\text{D-4Cpa}^2,\text{D-3Pal}^3,\text{D-Ala}^8,\text{D-Ala}^N] \text{GnRH-II}\) 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) showed no activation of ERK1/2.
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 phospho-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-d-2NaI,d-4Cpa2,d-3Pal3,6,Leu8,d-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, no effects of GnRH-II antagonist treatment on ERK1/2 activation and c-Fos expression were detectable. These are representative data obtained from three independent experiments in three different passages of each cell line.

**Figure 2.** Effects of GnRH-II antagonist [Ac-d-2NaI,d-4Cpa2,d-3Pal3,6,Leu8,d-Ala10]GnRH-II (10^{-7} mol/L) treatment on PKB/Akt (A, B) and ERK1/2 (C) signaling. A, basal amounts of PTEN and activated PKB/Akt in human endometrial cancer cell lines Hec-1A, Hec-1B and Ishikawa, in human breast cancer cell lines MCF-7 and T47-D, and in human ovarian cancer cell lines OVCAR-3 and EFO-21. Due to inactivating PTEN mutations the levels of active PKB/Akt were very high in the endometrial cancer cell line Ishikawa and the ovarian cancer cell line EFO-21. B, time-dependent effects of GnRH-II antagonist (10^{-7} mol/L) treatment on expression and phosphorylation of Akt in Ishikawa human endometrial cells. No effects of GnRH-II antagonist treatment on Akt expression and activation were detectable. These are representative data obtained from three independent experiments in three different passages of each cell line. Experiments using EFO-21 human ovarian cancer cells gave identical results. C, time-dependent effects of GnRH-II antagonist (10^{-7} mol/L) treatment on ERK1/2 activity and downstream transcription factor c-Fos in MCF-7 human breast cancer cells (top), OVCAR-3 human ovarian cancer cells (middle), and Ishikawa human endometrial cancer cells (bottom). In all three cell lines, no effects of GnRH-II antagonist treatment on ERK1/2 activation and c-Fos expression were detectable. These are representative data obtained from three independent experiments in three different passages of each cell line.
pretreated 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-o-2Nal1,o-4Cpa2,o-3Pal3,6,Leu6,o-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 further active agents and other cancers, we tried to learn more about the underlying mechanisms. 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 activation of caspase-3 (10). To improve the 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.
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 relocated 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 αi/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 αi/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 αi/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 αq/11, but not to G protein αi/o or G protein αs, 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 αi-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. Effects of GnRH-II antagonist [Ac-o-2Nal1, o-4Cpa2,o-3Pal3,h-Leu6,o-Ala19]GnRH-II (10⁻⁷ mol/L) treatment on activation of stress-activated MAPKs p38 and JNK, and MAPK ERK1/2 and c-Fos after pretreatment without or with p38 inhibitor SB203580, JNK inhibitor SP600125, or G protein αi/o inhibitor pertussis toxin (PTX) in MCF-7 human breast cancer cells. Controls were treated with PBS or DMSO. β-actin was used as loading control marker. GnRH-II antagonist (10⁻⁷ mol/L) treatment resulted in an increase of phosphorylated p38. Pretreatment with p38 inhibitor SB203580 but not JNK inhibitor SP600125 prevented the GnRH-II antagonist-induced increase of activated p38. Treatment with GnRH-II antagonist (10⁻⁷ mol/L) resulted in an increase of phosphorylated JNK. Pretreatment with JNK inhibitor SP600125 but not p38 inhibitor SB203580 prevented the GnRH-II antagonist-induced increase of activated JNK. ERK1/2 and c-Fos were not affected by GnRH-II antagonist treatment with or without inhibitor pretreatment. Neither GnRH-II antagonist-induced activation of p38 nor GnRH-II antagonist-induced activation of JNK were inhibited by pretreatment with PTX. 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.
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-coupling 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-2-Nal⁴,D-4-Cpa⁵,D-3Pal⁶,Leu⁸,D-Ala¹⁰]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 of 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. 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.

Acknowledgments

Received 12/8/08; revised 3/27/09; accepted 6/2/09; published OnlineFirst 7/28/09.

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We thank Renate Dietrich, Matthias Läsche, and Hiltrud Schulz for excellent technical assistance.

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Stefanie Fister, Andreas R. Günthert, Babette Aicher, et al.


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