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Gonadotropin releasing hormone typ II antagonist induces apoptosis in MCF-7 and triple-negative MDA-MB-231 human breast cancer cells \textit{in vitro} and \textit{in vivo}

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Abstract

Introduction: Triple-negative breast cancer does not express estrogen and progesterone receptors and there is no overexpression/amplification of the HER2-neu gene. Therefore this subtype of breast cancer lacks the benefits of specific therapies which target these receptors. Today chemotherapy is the only systematic therapy for patients with triple-negative breast cancer. About 50–64% of human breast cancers express receptors for gonadotropin releasing hormone (GnRH), which might be used as a target. New targeted therapies are warranted. Recently we could show that antagonists of gonadotropin releasing hormone typ II (GnRH-II) induce apoptosis in human endometrial and ovarian cancer cells \textit{in vitro} and \textit{in vivo}. This was mediated through activation of stress-induced mitogen activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK) followed by activation of pro-apoptotic protein Bax, loss of mitochondrial membrane potential and activation of caspase-3. In the present study we have analyzed whether GnRH-II antagonists induce apoptosis in MCF-7 and triple-negative MDA-MB-231 human breast cancer cells that express GnRH receptors. In addition we have ascertained whether knock-down of GnRH-I receptor expression affects GnRH-II antagonist-induced apoptosis and apoptotic signaling.

Methods: Induction of apoptosis was analyzed by measurement of loss of mitochondrial membrane potential. Apoptotic signaling was measured by quantification of activated MAPK p38 and caspase-3 using the western blot technique. GnRH-I receptor protein expression was inhibited using the antisense knock-down technique. \textit{In vivo} experiments were performed using nude mice bearing xenografted human breast tumors.

Results: We could show that treatment of MCF-7 and triple-negative MDA-MB-231 human breast cancer cells with a GnRH-II antagonist resulted in apoptotic cell death \textit{in vitro} via activation of stress activated MAPK p38 and loss of mitochondrial membrane potential. In
addition, we could show GnRH-II antagonist-induced activation of caspase-3 in MDA-MB-231 human breast cancer cells. After knock-down of GnRH-I receptor expression, GnRH-II antagonist-induced apoptosis and apoptotic signaling was only slightly reduced, indicating that an additional pathway mediating the effects of GnRH-II antagonists may exist. The GnRH-I receptor seems not to be the only target of GnRH-II antagonists. The antitumor effects of the GnRH-II antagonist could be confirmed in nude mice. The GnRH-II antagonist inhibited the growth of xenotransplants of human breast cancers in nude mice completely, without any apparent side effects.

**Conclusions:** GnRH-II antagonists seem to be suitable drugs for an efficacious and less toxic endocrine therapy for breast cancers including triple-negative breast cancers.

**Introduction**

Breast cancer is the most frequent malignant disease in women, with more than 1,000,000 new cases and 370,000 deaths yearly worldwide [1]. About 75-80% of breast cancers are hormone receptor-positive and express estrogen and progesterone receptors [2, 3]. Approximately 15-20% of breast cancers overexpress/amplify the HER2-neu gene, with around half of these co-expressing steroid hormone receptors. For patients with hormone-receptor-positive or HER2-neu-positive tumors effective targeted therapies have been developed. About 10-15% of breast cancers do not express either estrogen and progesterone receptor and also do not overexpress/amplify the HER2-neu gene [4-6]. These so called triple-negative breast cancers lack the benefits of specific therapies which target these receptors. Triple-negative breast cancer is relatively chemo-sensitive to conventional cytotoxic agents such as cisplatin but the effectiveness is only for short duration. Therefore, the development of new therapies is of great interest.
The expression of gonadotropin releasing hormone (GnRH-I) and its receptor as a part of a negative autocrine/paracrine regulatory mechanism of cell proliferation has been demonstrated in a number of malignant tumors, including cancers of endometrium, ovary and breast [7]. In these cancers the \textit{in vitro} proliferation can be inhibited by agonistic analogs of GnRH-I in a dose- and time dependent manner [7-11]. GnRH-I antagonists also have marked antiproliferative activity in most endometrial, ovarian and breast cancer cell lines tested \textit{in vitro}, indicating that the dichotomy of GnRH agonists and antagonists might not apply to the GnRH system in cancer cells [7-11].

Besides GnRH-I a second structural variant of GnRH exists in mammals. GnRH-II is totally conserved in structure from fish to mammals. It differs from GnRH-I in 3 amino acids. GnRH-II receptor was found in different species including non-human primates. Its existence in the human is controversial. There are several lines of evidence, however, for a functional GnRH-II receptor [12]. GnRH-II has antiproliferative effects on human endometrial, ovarian and breast cancer cells which are significantly greater than those of the superactive GnRH-I agonist Triptorelin [13]. Induction of apoptosis is not involved in the downregulation of cancer cell proliferation induced by agonists of GnRH-I or GnRH-II [7]. GnRH-I and GnRH-II agonists rather inhibit mitogenic signal transduction of growth factor receptors via activation of a phosphotyrosine phosphatase resulting in downregulation of cancer cell proliferation [14-16].

Recently, we could show that antagonistic analogs of GnRH-II induced apoptotic cell death in human endometrial and ovarian cancer cells \textit{in vitro}, via dose-dependent loss of mitochondrial membrane potential and activation of caspase-3 [17]. These anti-tumor effects could be confirmed in nude mice. GnRH-II antagonists significantly inhibited the growth of xenotransplants of human endometrial and ovarian cancers in nude mice, without any apparent side effects [17]. Apoptosis induced by GnRH-II antagonists is mediated through the intrinsic apoptotic pathway via stress-induced MAPKs p38- and JNK-induced activation of
the pro-apoptotic protein Bax, loss of mitochondrial membrane potential, release of cytochrome C and activation of caspase-3 [17, 18]. In addition we could demonstrate that GnRH-II antagonists couple to the GnRH-I receptor and have similar binding affinities to the GnRH-I receptor as the GnRH-I antagonist Cetrorelix [18]. Furthermore, we could show that [D-Lys$^6$]-GnRH-II is an agonist at the GnRH-I receptor whereas GnRH-II antagonists are clear antagonists at the GnRH-I receptor [18].

About 50–64% of human breast cancers express GnRH-I receptors [19-22]. Since GnRH-II antagonists appear to be suitable drugs for an efficacious and less toxic endocrine therapy for endometrial and ovarian cancers, the question arises whether GnRH-II antagonist treatment could also be a new therapeutic option for breast cancers. Especially for triple-negative breast cancers, defined by the lack of both estrogen and progesterone receptor as well as a lack of overexpression/amplification of the HER2-neu gene, the therapeutic options today are very limited.

In the present study we have analyzed whether a GnRH-II antagonist induces apoptosis in estrogen receptor/progesterone receptor positive MCF-7 human breast cancer cells, which have a normal expression of HER2-neu and triple-negative MDA-MB-231 human breast cancer cells in vitro and in vivo. In addition we have ascertained whether knock-down of GnRH-I receptor expression affects GnRH-II antagonist-induced apoptosis measured by loss of mitochondrial membrane potential and GnRH-II antagonist-induced apoptotic signaling analyzed by activation of stress activated MAPK p38 and caspase-3.

**Materials and methods**

**Cell lines and culture conditions**

The human breast cancer cell lines MCF-7 and MDA-MB-231 (triple-negative) were obtained from American Type Culture Collection (ATCC, Manassas, Virginia). In order to guarantee the identity of the cell lines over the years, the cells were expanded after purchase and
aliquots were stored in liquid nitrogen. Every half year a new frozen stock was opened and expanded to carry out the experiments. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air as previously described [8-10].

**Animals**

Female athymic (nude) mice (CD1 nu/nu), 6 to 8 weeks old on arrival, were obtained from Charles River (Sulzfeld, Germany). The mice were housed in sterile cages in a temperature-controlled room with 12-hour light/12-hour dark schedule and were fed autoclaved chow and water ad libitum. All experiments were done according to the German ethical guidelines and the German laws for protection of animals and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety, Department for Animal Protection, Oldenburg, Germany.

**GnRH-Analogs**

The GnRH-II antagonist [Ac-D2Nal¹, D-4Cpa², D-3Pal³, Leu⁸, D-Ala¹⁰]GnRH-II was developed by us and synthesized by Peptide Specialty Laboratories GmbH (Heidelberg, Germany) [17]. The GnRH-I agonist [D-Trp⁶]GnRH (Triptorelin) was kindly provided by Ferring Pharmaceuticals (Copenhagen, Denmark).

**GnRH-I receptor knock-down**

GnRH-I receptor knock-down was conducted as described previously [23]. A 43 bp fragment of the human GnRH-I receptor cDNA was cloned in antisense orientation (5’-CT AGA ACC ATG GAC TGT CCG ACT TTG CTG TTG CTT TTC AAA GC-3’) into the NheI/SalI sites of the eukaryotic expression vector, pIRES (Clontech, Palo Alto, California, USA), to produce the pGnRH-IR-antisense vector. Cells were grown to approximately 50% confluence on Nunc two-well chamber slides (immune cytochemistry) or in Nunc 100mm dishes
Transfections were done using Superfect liposome reagents and following the manufacturer’s instructions (Qiagen). After 12 h, transfected cells and non-transfected control cells were treated with the GnRH-I agonist Triptorelin (100nmol/l) to induce GnRH-I receptor protein internalization. Twelve hours later, the medium was changed and 20 ml PBS-BSA or an appropriate dilution of the GnRH-II antagonist [Ac-D2Nal\(^1\), D-4Cpa\(^2\), D-3Pal\(^3,6\), Leu\(^8\), D-Ala\(^10\)]GnRH-II was added, to a final concentration of \(10^{-7}\) M. Every 12 h, fresh PBS-BSA or GnRH-II antagonist was added. After 24 h of incubation, the medium was changed.

Knock-down of the GnRH-I receptor protein was evaluated by immune cytochemistry using a monoclonal mouse anti-human GnRH-I receptor antibody (clone A9E4; Research Diagnostics, Flanders, New Jersey, USA) and immunoblotting using a polyclonal rabbit antihuman GnRH-I receptor antiserum (Peptide Research Laboratories, Heidelberg, Germany) as described previously [23].

**Mitochondrial membrane potential**

For determination of GnRH-II antagonist-induced loss of mitochondrial membrane potential, cells with and without GnRH-I receptor knock-down were treated without or with the GnRH-II antagonist (\(10^{-9}\) and \(10^{-7}\) M) for 72 h. After incubation the cells were washed with PBS once and the mitochondrial membrane potential was detected using the JC-1 mitochondrial membrane potential detection kit according the instructions of the manufacturer (Biotium, Hayward, CA).

**Western blot analysis**

GnRH-II antagonist-induced activation of stress activated MAPK p38 and of caspase-3 was analyzed by western blot.

Polyclonal rabbit anti human phospho-p38 and polyclonal rabbit anti human p38 antibodies were obtained from Cell Signaling (Frankfurt/Main, Germany). Polyclonal rabbit anti-human
active caspase-3 antibody was purchased from from BD Pharmingen (Heidelberg, Germany) and polyclonal anti human β-actin antibody was from Sigma Aldrich (Deisenhofen, Germany).

For determination of GnRH-II antagonist-induced p38 activity, cells with (wild-type; WT) and without GnRH-I receptor expression (GnRH-I receptor knock-down, KD) were treated without or with the GnRH-II antagonist (10^{-7}M) for 45 minutes. For determination of GnRH-II antagonist-induced caspase-3 activity, cells with (wild-type; WT) and without GnRH-I receptor expression (GnRH-I receptor knock-down; KD) were treated without or with the GnRH-II antagonist (10^{-9}M and 10^{-7}M) for 48 h.

After incubation cells were detached immediately with 0.5 g trypsin (Biochrom, Berlin, Germany) and 5 mmol EDTA in 1 l PBS/BSA. The 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 Laemmlli-buffer. The cell lysates were separated on SDS-PAGE (10%, ProSieve® 50 Gel Solution, Cambrex, Verviers, Belgium) under reducing conditions and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare Europe, Munich, Germany). The nitrocellulose membranes were blocked with 5% instant skimmed milk powder, spray-dried (Naturaflor, Töpfer GmbH, Dietmannsried, Germany) in TBST (137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 25 mM Tris/Cl, pH 7.4) for 1 h at RT, washed with TBST and then 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 RT with horseradish peroxidase-conjugated anti mouse IgG or anti rabbit IgG (GE Healthcare Europe) at an 1:10,000 dilution in TBST for 1 h. After washings, specifically bound antibody was detected using the enhanced chemiluminescence kit (ECL; Millipore, Schwalbach, Germany). The bands were analyzed using the Kodak 1D image system (Kodak, New Haven, CT, USA).
In vivo studies
Tumors were initiated by subcutaneous injection of $1 \times 10^7$ cancer cells into the right flank of female athymic (nude) mice (CD1 nu/nu). After 2 (MDA-MB-231) or 3 (MCF-7) weeks all animals had developed solid tumors of about 200 mm$^3$ and treatment was initiated. The \textit{in vivo} experiments were done as follows: vehicle solution (control 1), 25 nmol of GnRH-I agonist Triptorelin (control 2) or 25 nmol of GnRH-II antagonist per mouse (5 mice per group) were injected intraperitoneally. Treatment was repeated every 2 days. Tumor volumes were measured on day 7, 11, 16, 21 of treatment (MCF-7) or on day 4, 8, 12, 16 of treatment (MDA-MB-231). The mice were killed after 21 (MCF-7) or 16 (MDA-MB-231) days.

Statistical Analysis
All experiments were repeated three times with different passages of the respective cell lines. The data were tested for significant differences by one-way analysis of variance followed by Student-Newman-Keuls' test for comparison of individual groups, after a Bartlett test had shown that variances were homogenous.

Results
Effects of GnRH-II antagonist treatment on induction of apoptosis in MCF-7 and triple-negative MDA-MB-231 human breast cancer cells \textit{in vitro}
To analyze the effects of GnRH-II antagonist treatment on induction of apoptosis in estrogen receptor/progesterone receptor positive MCF-7 human breast cancer cells, which have a normal expression of HER1-neu and triple-negative MDA-MB-231 human breast cancer cells, GnRH-II antagonist-induced loss of mitochondrial membrane potential ($\Delta \Psi$) was measured (Figure 1a). Treatment of MCF-7 and triple-negative MDA-MB-231 breast cancer cells with cytotoxic agent Doxorubicin ($10^{-9}$ M; positive control) or with the GnRH-II antagonist ($10^{-9}$ and $10^{-7}$ M) for 72 h resulted in a reduction of mitochondrial membrane
potential. After treatment with $10^{-7}$M of the cytotoxic agent Doxorubicin (DOX; positive control) mitochondrial membrane potential was decreased to $72.28 \pm 3.98$ % of control (MCF-7; $P<0.01$ vs. control; Figure 1b) or to $76.49 \pm 4.15$ % of control (MDA-MB-231; $P<0.01$ vs. control; not shown). Treatment with $10^{-9}$M of the GnRH-II antagonist resulted in a decrease of mitochondrial membrane potential to $64.51 \pm 6.95$ % of control (MCF-7; $P<0.001$; Figure 1a) or to $67.23 \pm 4.32$ % of control (MDA-MB-231; $P<0.001$ vs. control; not shown). After treatment with $10^{-7}$M of the GnRH-II antagonist mitochondrial membrane potential was reduced to $47.94 \pm 6.14$ % of control (MCF-7; $P<0.001$ vs. control; Figure 1a) or to $52.46 \pm 3.37$ % of control (MDA-MB-231; $P<0.001$ vs. control; not shown).

**Effects of GnRH-II antagonist treatment on induction of apoptosis in MCF-7 and MDA-MB-231 human breast cancer cells *in vitro* after knock-down of GnRH-I receptor expression**

To analyze whether the effects of the GnRH-II antagonist are mediated through the GnRH-I receptor, the expression of GnRH-I receptor protein was inhibited using the antisense knock-down technique.

A 43 bp cDNA encoding a fragment of the human GnRH-I receptor was cloned in antisense orientation into the NheI/SalI sites of the eucaryotic expression vector pIRES, to produce the pGnRH-I-R-antisense vector. Knock-down of the GnRH-I receptor protein was demonstrated by immune cytochemistry using a monoclonal mouse anti-human GnRH-I receptor antibody (Figure 1b-d) and immunoblotting using a polyclonal rabbit antihuman GnRH-I receptor antiserum [not shown; c.f. [23]]. After internalization of the GnRH-I receptor induced by GnRH-I agonist Triptorelin, a high density of novel synthesis of GnRH-I receptor protein, seen as GnRH-I receptor antigenicity, could be observed in non-transfected cells (Figure 1c), whereas transfected cells showed only slight GnRH-I receptor antigenicity (Figure 1d). As early as 1 day after knock-down of GnRH-I receptor expression, GnRH-I receptor protein
antigenicity was significantly reduced in comparison with the control. After 2, no more GnRH-I receptor protein could be detected; however, 4 days later a slight GnRH-I receptor protein antigenicity could be observed, indicating that the effectiveness of GnRH-I receptor knock-down begins to decrease after 6 days [not shown; c.f. [23]].

To analyze the effects of GnRH-II antagonist treatment on induction of apoptosis in GnRH-I receptor-negative (GnRH-I receptor knock-down) human breast cancer cells in comparison to the GnRH-I receptor-positive (wild-type) human breast cancer cells, GnRH-II antagonist-induced loss of mitochondrial membrane potential (ΔΨ) was measured (Figure 1a). After knock-down of GnRH-I receptor expression, GnRH-II antagonist-induced decrease of mitochondrial membrane potential was slightly reduced as compared to the effect of GnRH-II antagonist treatment in non-transfected cells. Treatment of the GnRH-I receptor knock-down cell lines with 10⁻⁹M of the GnRH-II antagonist resulted in a decrease of mitochondrial membrane potential to 75.35 ± 4.64 % of control (MCF-7; P<0.01 vs. control; not significant vs. WT; Figure 1a) or to 78.26 ± 3.47 % of control (MDA-MB-231; P<0.01 vs. control; not significant vs. WT; not shown). After treatment of the GnRH-I receptor knock-down cell lines with 10⁻⁷M of the GnRH-II antagonist mitochondrial membrane potential was reduced to 61.92 ± 5.27 % of control (MCF-7; P<0.001 vs. control; not significant vs. WT; Figure 1a) or to 64.93 ± 4.53 % of control (MDA-MB-231; P<0.01 vs. control; not significant vs. WT; not shown).

**Effects of GnRH-II antagonist treatment on apoptotic signaling in MCF-7 and MDA-MB-231 human breast cancer cells in vitro**

To analyze the effects of GnRH-II antagonist treatment on apoptotic signaling, GnRH-II antagonist-induced activation of stress activated MAPK p38 (Figure 2a) and caspase-3 (Figure 2b) in MCF-7 and MDA-MB-231 human breast cancer cells was measured.

Activation of p38 was considerably increased after GnRH-II antagonist treatment (Figure 2a,
third band) as compared to the untreated control (Figure 2a, first band). The effects of the GnRH-II antagonist on induction of apoptotic cell death could further be confirmed by measurement of caspase-3 activity. Treatment of MDA-MB-231 human breast cancer cells with cytotoxic agent Doxorubicin ($10^{-9}$ M; positive control) or with of the GnRH-II antagonist ($10^{-9}$ and $10^{-7}$ M) for 48 h resulted in increased caspase-3 activity (Figure 2b). After treatment with $10^{-9}$M of the cytotoxic agent Doxorubicin (positive control) caspase-3 activity was increased to 168.8 ± 17.54 % of control (MDA-MB-231; $P<0.05$; Figure 2b). Treatment with $10^{-9}$M of the GnRH-II antagonist resulted in an increase of caspase-3 activity to 195.8 ± 12.32 % of control (MDA-MB-231; $P<0.01$). After treatment with $10^{-7}$M of the GnRH-II antagonist caspase-3 activity was increased to 279.8 ± 25.38 % of control (MDA-MB-231; $P<0.001$). MCF-7 human breast cancer cells are caspase-3 deficient. Therefore, no caspase-3 activation was detectable.

**Effects of GnRH-II antagonist treatment on apoptotic signaling in MCF-7 and MDA-MB-231 human breast cancer cells in vitro after knock-down of GnRH-I receptor expression**

To analyze the effects of GnRH-II antagonist treatment on apoptotic signaling in GnRH-I receptor-negative (GnRH-I receptor knock-down) human breast cancer cells in comparison to the GnRH-I receptor-positive (wild-type) human breast cancer cells, GnRH-II antagonist-induced activation of stress activated MAPK p38 (Figure 2a) in GnRH-I receptor-negative (GnRH-I receptor knock-down) MCF-7 and MDA-MB-231 human breast cancer cells was measured (Figure 2a). Effects of GnRH-I receptor knock-down on caspase-3 activation were analyzed in GnRH-I receptor-negative (GnRH-I receptor knock-down) MDA-MB-231 human breast cancer cells (Figure 2b). After knock-down of GnRH-I receptor expression, GnRH-II antagonist-induced activation of p38 was slightly reduced (Figure 2a, fourth band) as compared to the effect of GnRH-II antagonist treatment in non-transfected cells (Figure 2a,
third band). The basal amount of phosphorylated p38 after knock-down of GnRH-I receptor expression alone (Figure 2a, second band) was only slightly decreased or nearly the same as compared to non-transfected cells without GnRH-II antagonist treatment (Figure 2a, first band). After knock-down of GnRH-I receptor expression, GnRH-II antagonist-induced caspase-3 activation was slightly reduced as compared to the effect of GnRH-II antagonist treatment in non-transfected cells. Treatment of the GnRH-I receptor knock-down cell lines with $10^{-9}$M of the GnRH-II antagonist resulted in an increase of caspase-3 activity to $170.6 \pm 11.71 \%$ of control (MDA-MB-231; $P<0.05$ vs. control; not significant vs. WT; Figure 2b). After treatment of the GnRH-I receptor knock-down cell lines with $10^{-7}$M of the GnRH-II antagonist caspase-3 activity was increased to $254.5 \pm 26.8 \%$ of control (MDA-MB-231; $P<0.01$ vs. control; not significant vs. WT; Figure 2b).

**Effects of GnRH-II antagonist treatment on tumor growth in vivo**

To show the proof-of-principle of an anti-tumor therapy using GnRH-II antagonists, nude mice bearing xenografted human breast tumors s.c. were treated with the GnRH-II antagonist $[\text{Ac-D2Nal}^1, \text{D-4Cpa}^2, \text{D-3Pal}^{3,6}, \text{Leu}^8, \text{D-Ala}^{10}]\text{GnRH-II}$.

Female nude mice bearing MCF-7 (Figure 3a) or triple-negative MDA-MB-231 (Figure 3b) tumors s.c. were treated without (control 1) or with 25 nmol per injection of GnRH-I agonist Triptorelin (control 2) or with 25 nmol per injection of the GnRH-II antagonist. The treatments were repeated every 2 days. The mice were killed after 16 (MDA-MB-231) or 21 (MCF-7) days of treatment. The increase of the tumor volume of the mice receiving therapy with GnRH-II antagonists was lower than with the control animals. After 7 days of treatment the differences on MCF-7 tumor growth (Figure 3a) became highly significant ($P<0.001$) and remained highly significant ($P<0.001$). The differences on MDA-MB-231 tumor growth (Figure 3b) became significant ($P<0.5$) after 4 days of treatment. On day 8 the differences became highly significant ($P<0.001$) and remained highly significant ($P<0.001$). Side effects
were not observed. To exclude that the anti-tumor effects of the GnRH-II antagonist is due to an interaction with the pituitary GnRH receptors, a second control group (control 2) was treated with 25 nmol per injection of the GnRH-I agonist Triptorelin. The increase of the tumor volume of the mice receiving therapy with GnRH-I agonist Triptorelin was nearly the same than with the animals of control 1. No anti-tumor effects using Triptorelin were observed.

**Discussion**

Previous work showed that nanomolar concentrations of GnRH-II antagonists induce apoptotic cell death in human endometrial and ovarian cancer cells *in vitro* and *in vivo* [17]. In addition we could show that apoptosis induced by GnRH-II antagonists is mediated through the intrinsic apoptotic pathway via stress-induced MAPKs p38- and JNK-induced activation of the pro-apoptotic protein Bax, loss of mitochondrial membrane potential, release of cytochrome C and activation of caspase-3 [17, 18]. Furthermore we could demonstrate that GnRH-II antagonists bind to the GnRH-I receptor and are clear antagonists at the GnRH-I receptor [18].

In the present study we could show that treatment of estrogen receptor/progesterone receptor positive MCF-7 and triple-negative MDA-MB-231 human breast cancer cells with the GnRH-II antagonist [Ac-D2Nal<sup>1</sup>, D-4Cpa<sup>2</sup>, D-3Pal<sup>3,6</sup>, Leu<sup>8</sup>, D-Ala<sup>10</sup>]GnRH-II resulted in apoptotic cell death via activation of stress activated MAPK p38 and loss of mitochondrial membrane potential. In addition, GnRH-II antagonist-induced activation of caspase-3 could be observed in MDA-MB-231 human breast cancer cells. MCF-7 human breast cancer cells are caspase-3 deficient [24]. Therefore, no caspase-3 activation was detectable. Apoptotic signaling in MCF-7 cells differs from caspase-3-positive breast cancer cell lines, involving activation of different caspases. In MCF-7 cells reduction of the mitochondrial membrane potential is caspase-independent [25].
Triple-negative breast cancer refers to a specific subtype of breast cancer that is characterized by lack of expression of both, estrogen and progesterone receptor as well as HER2-neu. Clinically triple-negative breast cancer is more aggressive and less responsive to standard therapies and associated poorer overall patient prognosis [26, 27]. To date, the therapeutic options are very limited leaving chemotherapy the only possible therapy. Treatment with a GnRH-II antagonist could be a new option for the therapy of triple-negative breast cancer. Recently we could show that GnRH-II antagonists bind to the GnRH-I receptor and are clear antagonists at the GnRH-I receptor [18]. However, after knock-down of GnRH-I receptor expression GnRH-II antagonist-induced apoptosis and apoptotic signaling 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 [18]. Therefore, we cannot confirm a complete antagonism in tumor cells since the GnRH signal transduction is cell context dependent [28]. 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 or not GnRH-II antagonist-induced activation of apoptosis and apoptotic signaling would be abrogated after knock-down of expression of the putative additional receptor for GnRH-II antagonists. Different knock-down experiments, however, using GnRH-II receptor antisense fragments resulted in apoptotic cell death (unpublished results). In addition, binding assays and functional assays for the putative GnRH-II receptor are not available at the moment. At present, therefore, this question cannot be answered.

We could show the proof-of-principle of an antitumor therapy using the GnRH-II antagonist [Ac-D2Nal\(^1\), D-4Cpa\(^2\), D-3Pal\(^3,6\), Leu\(^8\), D-Ala\(^10\)]GnRH-II \textit{in vivo} in nude mice bearing s.c. xenografts of human breast cancers. Nude mice bearing MCF-7 human breast cancers or MDA-MB-231 triple-negative human breast cancers were treated with the GnRH-II
antagonists [Ac-D2Nal\(^1\), D-4Cpa\(^2\), D-3Pal\(^3,6\), Leu\(^8\), D-Ala\(^{10}\)]GnRH-II. The increase of the tumor volume of the mice receiving therapy with the GnRH-II antagonist was significantly lower than with the control animals. Toxic side effects were not observed. 

Our findings could be the basis for a further evaluation in clinical trials. GnRH-I agonists and GnRH-I antagonists have been widely used in the therapy of cancer [29-33] and endometriosis [34-36] as well as in reproductive medicine [37-41]. Their effects are mainly due to the down-regulation of the hypothalamic-ovarian axis and the resulting medical castration. To exclude that the antitumor effects of the GnRH-II antagonist are mainly due to an interaction with the pituitary GnRH receptors and a subsequent reduction of ovarian estrogen production, control groups were treated with GnRH-I agonist Triptorelin. Because we could not observe antitumor effects in vivo after down-regulation of the hypothalamic-ovarian axis using the GnRH-I agonist Triptorelin, the down-regulation of the hypothalamic-ovarian axis alone cannot be responsible for the antitumor effects of GnRH-II antagonists. In addition, triple-negative MDA-MB-231 breast cancer cells are insensitive to estrogen deprivation. The GnRH-II antagonist seems to affect the tumor cells directly by inducing apoptosis.

**Conclusions**

We could show that the GnRH-II antagonist [Ac-D2Nal\(^1\), D-4Cpa\(^2\), D-3Pal\(^3,6\), Leu\(^8\), D-Ala\(^{10}\)]GnRH-II induces apoptotic cell death in MCF-7 and triple-negative MDA-MB-231 human breast cancer cells in vitro and in vivo. Apoptosis induced by the GnRH-II antagonist is mediated through the intrinsic apoptotic pathway via activation of stress-induced MAPK p38 and loss of mitochondrial membrane potential. GnRH-II antagonist-induced activation of caspase-3 could be observed in MDA-MB-231 human breast cancer cells. In addition, we could show that knock-down of GnRH-I receptor expression only slightly inhibited GnRH-II antagonist-induced apoptosis and apoptotic signaling indicating an additional pathway
mediating the effects of GnRH-II antagonists.

Thus, the GnRH-II antagonist [Ac-D2Nal\textsuperscript{1}, D-4Cpa\textsuperscript{2}, D-3Pal\textsuperscript{3,6}, Leu\textsuperscript{8}, D-Ala\textsuperscript{10}]GnRH-II seems to be a suitable drug for an efficacious and less toxic endocrine therapy for breast cancers including triple-negative breast cancers.

**Abbreviations**

Ac-D2Nal: N-acetyl-D-2-naphthylalanine; BSA: bovine serum albumin; cDNA: complementary deoxyribonucleic acid; D-4Cpa: D-4-cyclopropylalanine; D-3Pal: D-3-(3-pyridyl)-alanine; D-Ala: D-alanine; \( \Delta \psi \): mitochondrial membrane potential; DOX: doxorubicin; EDTA: ethylenediaminetetraacetic acid; GnRH: gonadotropin-releasing hormone; HER2-neu: human epidermal growth factor receptor 2; IgG: immunoglobulin G; JNK: c-Jun N-terminal kinase; KD: knock-down; Leu: leucine; MAPK: mitogen activated protein kinase; PBS: phosphate buffered saline; pIRES: plasmid containing the encephalomyocarditis virus internal ribosome entry site flanked by two multiple cloning sites; RT: room temperature; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST: tris buffered saline tween-20; WT: wild type.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CG designed the experiments, performed data analysis and statistics, and drafted the manuscript. CF, SF, and NN participated in the design of the experiments, carried out the *in vitro* and *in vivo* experiments. ARG participated in the study design and helped with data
analysis. GE helped with data analysis and statistics and helped to draft the manuscript. All authors read and approved the final manuscript.

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References


Figure legends

Figure 1
Effects of GnRH-II antagonist treatment on induction of apoptosis in GnRH-I receptor-positive (wild-type; WT) and GnRH-I receptor-negative (GnRH-I receptor knock-down; KD) MCF-7 human breast cancer cells in vitro. (a) Percentage of mitochondrial membrane potential (ΔΨ) after 72 h of treatment of GnRH-I receptor-positive (wild-type; WT) and GnRH-I receptor-negative (GnRH-I receptor knock-down; KD) MCF-7 breast cancer cells without (control = 100%) or with cytotoxic agent Doxorubicin (10^9 M; positive control) or with GnRH-II antagonist [Ac-D2Nal\textsuperscript{1}, D-4Cpa\textsuperscript{2}, D-3Pal\textsuperscript{3}, D-Lys\textsuperscript{6}, D-Ala\textsuperscript{10}]GnRH-II (10^{-7} M and 10^{-9} M). Columns represent means ± SEM of data obtained from three independent experiments in three different passages of the cell line. a, $P<0.001$ vs. control; b, $P<0.01$ vs. control. Experiments using MDA-MB-231 human breast cancer cells gave identical results. (b-d) Immune histochemical detection of GnRH-I receptor protein using a monoclonal mouse anti-human GnRH-I receptor antibody. (b) Control performed by omission of the primary antibody. (c) Non-transfected cells. (d) Cells transfected with pGnRH-I-R antisense expression vector.

Figure 2
Effects of GnRH-II antagonist treatment on apoptotic signaling in GnRH-I receptor-positive (wild-type; WT) and GnRH-I receptor-negative (GnRH-I receptor knock-down; KD) human breast cancer cells in vitro. (a) Amount of phosphorylated p38 after treatment without or with GnRH-II antagonist Ac-D2Nal\textsuperscript{1}, D-4Cpa\textsuperscript{2}, D-3Pal\textsuperscript{3,6}, Leu\textsuperscript{8}, D-Ala\textsuperscript{10}]GnRH-II (10^{-7}M) in non-transfected MCF-7 breast cancer cells and in MCF-7 breast cancer cells after knock-down of GnRH-I receptor expression. These are representative data obtained from three independent experiments in three different passages of the cell line. Experiments using MDA-MB-231
human breast cancer cells gave identical results. (b) Percentage of caspase-3 activity after 48 h of treatment of GnRH-I receptor-positive (wild-type; WT) and GnRH-I receptor-negative (GnRH-I receptor knock-down; KD) MDA-MB-231 breast cancer cells without (control = 100%) or with cytotoxic agent Doxorubicin ($10^{-9}$ M; positive control) or with GnRH-II antagonist [Ac-D2Nal$^1$, D-4Cpa$^2$, D-3Pal$^3$, D-Lys$^6$, D-Ala$^{10}$]GnRH-II ($10^{-7}$ M and $10^{-9}$ M). Columns represent means ± SEM of data obtained from three independent experiments in three different passages of the cell line. a, $P< 0.001$ vs. control; b, $P< 0.01$ vs. control; c, $P< 0.05$ vs. control.

**Figure 3**

Tumor volume of MCF-7 (a) and triple-negative MDA-MB-231 (b) human breast cancers xenografted into nude mice. The mice were treated without (control 1), with 25 nmol of GnRH-I agonist Triptorelin (control 2) or with 25 nmol of GnRH-II antagonist [Ac-D2Nal$^1$, D-4Cpa$^2$, D-3Pal$^{3,6}$, Leu$^8$, D-Ala$^{10}$]GnRH-II. Treatment was repeated every 2 days. Tumor volumes were measured on day 7, 11, 16, 21 of treatment (MCF-7; (a)) or on day 4, 8, 12, 16 of treatment (MDA-MB-231; (b)). The mice were killed after 21 (MCF-7; (a)) or 16 (MDA-MB-231; (b)) days. All experimental groups consisted of 5 animals. Vertical bars represent SEM. a, $P< 0.001$ vs. control; b, $P< 0.05$ vs. control.
Figure 1
Figure 2

A) Western blot analysis showing expression of p-p38, p38, and β-Actin. The table below shows the expression levels under different conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>p-p38</th>
<th>p38</th>
<th>β-Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GnRH-II agonist</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Knock-down</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

B) Graph showing Caspase-3 activity as a percentage of control. The bars represent different concentrations of GnRH-II antagonist:

Control: 100%
Positive Control: 100%
10⁻⁹ M (WT): 150%
10⁻⁷ M (WT): 250%
10⁻⁹ M (KD): 100%
10⁻⁷ M (KD): 150%

Significant differences are indicated by different letters (a, b, c) above the bars.
Figure 3

A

B

tumor volume [%]

tumor volume [%]

days after initiation of therapy

Control

Triptorelin

GnRH-II Antagonist

Figure 3