GnRH antagonists in the treatment of gynecological and breast cancers

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Abstract
Approximately 80% of human ovarian and endometrial cancers and 50% of breast cancers express GnRH and its receptor as part of an autocrine regulatory system. After binding of its ligand the tumor GnRH receptor couples to G-protein $\alpha_i$ and activates a variety of intracellular signaling mechanisms. (1) Through activation of a protein tyrosine phosphatase, autophosphorylation of growth factor receptors is reverted leading to an inhibition of mitogenic signaling and reduced cell proliferation. (2) Through activation of nuclear factor kappa B antiapoptotic mechanisms are induced protecting tumor cells from apoptosis induced, for example, by doxorubicin. (3) Through activation of the Jun kinase pathway AP-1 is induced, leading to cell cycle arrest in the G0/G1 phase. It seems reasonable to speculate that this system enables the tumor cell to reduce proliferation and to activate repair mechanisms while being protected simultaneously from apoptosis. Interestingly, GnRH antagonists show the same activity in this system as agonists, indicating that the dichotomy GnRH agonist–GnRH antagonist defined in the pituitary gonadotrope is not valid for the tumor GnRH system. Recently, a second type of GnRH receptor, specific for GnRH-II, has been identified in ovarian and endometrial cancers, which transmits significantly stronger antiproliferative effects than the GnRH-I receptor. GnRH antagonists have agonistic effects on this type II receptor. In animal models of human cancers, GnRH antagonists had stronger antitumor effects than GnRH agonists. Therefore, we performed a phase II clinical trial with the GnRH antagonist, cetrorelix (10 mg/day), in patients with ovarian or mullerian carcinoma refractory to platinum chemotherapy. Of 17 evaluable patients treated with cetrorelix, 3 obtained a partial remission (18%) which lasted for 2 to 6 months. Furthermore, 6 patients experienced disease stabilization (35%) for up to 1 year. In this very refractory patient population (median number of prior chemotherapies = 3) these results are quite remarkable when compared with palliative chemotherapy. In addition, cytotoxic GnRH analogs have been developed, where for example doxorubicin was covalently coupled to GnRH analogs. These compounds have superior antitumor effects in cancers expressing GnRH receptors as compared with native doxorubicin and allow for a targeted cytotoxic chemotherapy of gynecologic and breast cancers.

Introduction
The hypothalamic decapeptide gonadotropin hormone releasing hormone (GnRH), also called luteinizing hormone-releasing hormone (LHRH), functions as a key hormone in the regulation of mammalian reproduction (Schally 1994, Stojilkovic & Catt 1995, Stanislaus et al. 1998). In addition to its classic hypophysiotropic action, GnRH might function as a modulator of the activity of diverse systems in the brain and many peripheral organs (reviewed in Gründker et al. 2002a). It has been suggested that an autocrine/paracrine function of GnRH exists for example in the placenta, granulosa cells, myometrium, and lymphoid cells (reviewed in Emons et al. 1997, Gründker et al. 2002a).
**GnRH systems in human cancers**

Since 1985 the expression of GnRH and its receptor as well as direct antiproliferative effects of GnRH and its analogs have been demonstrated in a number of malignant human tumors, including cancers of the breast (Blankenstein et al. 1985, Miller et al. 1985, Eidne et al. 1987, Fekete et al. 1989, Baumann et al. 1993), ovary (Emons et al. 1989, Palewa et al. 1989, Emons et al. 1993a, Ohno et al. 1993, Thompson et al. 1991, Yano et al. 1994a,b, Kakar et al. 1994, Irmer et al. 1995), and endometrium (Srlakovic et al. 1990, Palewa et al. 1991, Emons et al. 1993b, Imai et al. 1994a,b, Irmer et al. 1994, Chatzaki et al. 1996). About 50% of breast, 70% of ovarian and 80% of endometrial cancers express GnRH and its receptor (Emons et al. 1997, Gründker et al. 2002a, Völker et al. 2002) (Table 1). These findings suggested the presence of an autocrine regulatory system in these cancers based on GnRH. Studies performed in our laboratory have demonstrated that the proliferation of ovarian cancer cells was significantly increased after treatment with an antiserum to GnRH, suggesting that GnRH produced by the tumor cells acts as a negative autocrine regulator of proliferation (Emons et al. 2000a). Native GnRH and GnRH agonists were found to inhibit in a dose- and time-dependent manner the proliferation of human breast, ovarian and endometrial cancer cell lines (reviewed in Emons et al. 1997, Gründker et al. 2002a). In most cancer cell lines tested, GnRH antagonists also induced a time- and dose-dependent inhibition of proliferation indicating that the dichotomy of GnRH agonist–GnRH antagonist as defined in pituitary gonadotropes does not apply to the GnRH system in human cancers. Here, antagonists act like agonists (Emons et al. 1997, Gründker et al. 2002a) (Fig. 1). Some investigators failed to detect direct antitumor effects of GnRH analogs in human cancer cell lines or observed them only at high concentrations of GnRH analog (reviewed in Gründker et al. 2002a). This phenomenon might be explained by the fact that the majority of the cell lines used by these authors did not express high affinity GnRH receptors (reviewed in Gründker et al. 2002a, Völker et al. 2002). In one ovarian cancer cell line (ES-2) stimulatory effects of a GnRH agonist were observed after 48 h when a low concentration (10 ng/ml) was used. After 72 h and in concentrations of 1 µg/ml only the inhibition of this cell line was observed. In this cell line, a GnRH antibody inhibited cell proliferation in a time- and concentration-dependent manner, suggesting that GnRH may function as a growth factor in this specific cell line (Arenciba & Schally 2000).

In a recent systematic study using well-established human ovarian and endometrial cancer cell lines, we found that 4 out of 6 ovarian and 5 out of 6 endometrial cancer cell lines expressed high affinity GnRH receptors. The proliferation of all these GnRH receptor-positive cell lines was reduced in a dose- and time-dependent manner by agonistic and antagonistic GnRH analogs (Völker et al. 2002). At a 10 pM agonist concentration, only a slight decrease in cell number to 85%–96% of control was observed. At a 1 nM concentration of the analog, the reduction in cell number was significant in all GnRH receptor-positive cell lines (71%–87% of control; P<0.01). The inhibitory effects were maximal at a 10 µM concentration of GnRH analogs and corresponded to 56%–71% of control. Stimulatory effects on proliferation were never observed even when low concentrations of GnRH analogs or short-term incubation (24 or 48 h) were used (Emons et al. 2000a, Völker et al. 2002). Thus our data and most reports in the literature suggest that in the majority of ovarian and endometrial cancers, GnRH and its receptor are part of a negative autocrine system which might be used therapeutically to inhibit cell proliferation by the application of GnRH analogs.

**Signal transduction of the GnRH system in human breast, ovarian and endometrial cancers**

During the last decade, the signal transduction mechanism mediating the antiproliferative effects of GnRH analogs in

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Expression of GnRH-I</th>
<th>Expression of GnRH-I receptor</th>
<th>Inhibitory effects of GnRH-I analogs on</th>
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<tbody>
<tr>
<td></td>
<td>Immuno/ mRNA</td>
<td>Radioreceptor mRNA</td>
<td>Proliferation Mitogenic signaling</td>
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<tr>
<td>Breast cancer</td>
<td>+ (+50%)</td>
<td>+ (+80%)</td>
<td>+ (+80%)</td>
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<tr>
<td>Ovarian cancer</td>
<td>+ (+80%)</td>
<td>+ (+80%)</td>
<td>+ (+80%)</td>
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<tr>
<td>Endometrial cancer</td>
<td>+ (+80%)</td>
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GnRH receptor which might explain this phenomenon. Also, could not find mutations or splice variants in the tumor

The finding that GnRH antagonists act like agonists in human cancers cannot be explained by mutations or splice variants of the GnRH receptor (Gründker et al. 2001a) and awaits further clarification.

Recently, it became evident that it is not only mitogenic signaling of growth factor receptors that is modulated by GnRH in human cancers. We could show that, in addition, nuclear factor κB is activated by GnRH in human ovarian and endometrial cancer cells (Gründker et al. 2000b). This effect is also mediated through G protein αi and leads to an inhibition of tumor cell apoptosis induced by for example the cytotoxic agent doxorubicin (Gründker et al. 2000b) (Fig. 3). Finally, we could show that binding of GnRH analogs to their receptors in cancers induces c-jun mRNA expression, c-Jun phosphorylation and AP-1 activation, again mediated through G protein αi (Gründker et al. 2001b). GnRH agonists induce Jun D-DNA binding in cancer cells, reduce DNA synthesis and lead to accumulation of cells in the G0/G1 phase of the cell cycle (Gründker et al. 2000b) (Fig. 3). Thus, at least three different mechanisms are activated by the GnRH receptor in tumor cells. (1) Stimulation of a protein tyrosine phosphatase, counter-acting autophosphorylation of growth factor receptors, leading to an inhibition of mitogenic signaling and reduced cell proliferation. (2) Stimulation of nuclear factor κB, leading to a protection of tumor cells from apoptosis. (3) Induction of AP-1 through activation of the Jun kinase pathway leading to cell cycle arrest in the G0/G1 phase. It seems

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passages of each cell line. In independent experiments run in duplicate, four different c-fos antagonists (Cetrorelix) induced inhibition of EGF-induced c-fos expression. c-fos expression of quiescent MCF-7 breast cancer cells (A = control = quiescent cells) and after treatment with 100 nM EGF (10 min) without (B) or with (C) previous treatment (15 min) with the GnRH antagonist Cetrorelix (CETRO; 100 nM) or with previous treatment with the GnRH antagonist Cetrorelix and with (2 ng/mL) PTX (D). After treatment with EGF, a significant increase in c-fos expression was observed (P < 0.001) (B). After treatment with Cetrorelix followed by EGF, no increase in c-fos expression was observed (C). After treatment with PTX, Cetrorelix-induced inhibition of EGF-induced c-fos expression was blocked indicating mediation through G-protein α1 (P < 0.001) (D). Columns represent means ± S.E.M. of data obtained from four independent experiments run in duplicate in four different passages of each cell line. a, P < 0.001 vs. control (A); b, P < 0.001 vs. EGF (B); c, P < 0.001 vs. EGF/CETRO (C).

Figure 2 Effects of pertussis toxin (PTX) on the GnRH antagonist Cetrorelix-induced inhibition of EGF-induced c-fos expression. c-fos expression of quiescent MCF-7 breast cancer cells (A = control = quiescent cells) and after treatment with 100 nM EGF (10 min) without (B) or with (C) previous treatment (15 min) with the GnRH antagonist Cetrorelix (CETRO; 100 nM) or with previous treatment with the GnRH antagonist Cetrorelix and with (2 ng/mL) PTX (D). After treatment with EGF, a significant increase in c-fos expression was observed (P < 0.001) (B). After treatment with Cetrorelix followed by EGF, no increase in c-fos expression was observed (C). After treatment with PTX, Cetrorelix-induced inhibition of EGF-induced c-fos expression was blocked indicating mediation through G-protein α1 (P < 0.001) (D). Columns represent means ± S.E.M. of data obtained from four independent experiments run in duplicate in four different passages of each cell line. a, P < 0.001 vs. control (A); b, P < 0.001 vs. EGF (B); c, P < 0.001 vs. EGF/CETRO (C).

reasonable to speculate that this GnRH system enables the tumor cell to reduce proliferation and activate repair mechanisms while being protected simultaneously from apoptosis.

Clinical applications of GnRH agonists in breast, ovarian and endometrial cancers

GnRH agonists have become a cornerstone in the systemic treatment of premenopausal women with estrogen-dependent breast cancer, both in the adjuvant as well as in the metastatic situation (Jonat 2001). Here, the essential mode of action of GnRH agonists is down-regulation of pituitary gonadotropin secretion, leading to a suppression of ovarian estrogen production, a state called reversible medical castration (Emons & Schally 1994). It remains unclear whether direct effects of GnRH analogs on breast cancer cells contribute to their marked efficacy mediated through estrogen withdrawal (reviewed in Emons & Schally 1994). In ovarian cancer, several phase II clinical trials have been performed in patients with relapsed, mostly platinum-resistant disease. Of 245 published patients, 23 (9%) had an objective remission and 64 (26%) had disease stabilization (Emons & Schulz 2000). In a large study by the European Organisation for Research and Treatment of Cancer (EORTC), only eleven (16%) out of 68 evaluable patients with progressive ovarian cancer treated with a GnRH agonist had disease stabilization, while no objective responses were observed. Median survival for patients with stable disease was 17 months, whereas for all patients it was 4 months (Duffaud et al. 2001).

It might be argued that the efficacy of an endocrine therapy like GnRH agonists might be reduced in patients with relapsed ovarian cancer refractory to standard chemotherapy. In an attempt to use GnRH agonist as a first-line systemic therapy, a prospective double-blind randomized trial was performed in which 135 patients with stage III or IV epithelial ovarian carcinoma received either monthly injections of the GnRH agonist [D-Trp6]GnRH (Triptorelin, 3.75 mg) or placebo until their deaths or termination of the trial respectively. All patients had standard cytoreductive surgery and were treated with a standard platinum-based chemotherapy, and, if necessary, with second- or third-line cytotoxic regimens. Progression-free and overall survival in patients receiving the GnRH agonist were not significantly different from that of patients receiving placebo (Emons et al. 1996b). At the time this study was designed and performed, the direct anti-apoptotic effects of GnRH agonists in tumor cells were not yet known (Gründker et al. 2000b). In view of our present knowledge, the lack of activity of the GnRH agonist in combination with chemotherapy might be interpreted as a neutralization of its direct anti-proliferative effects by its anti-apoptotic activity, protecting tumor cells, at least in part, from apoptosis induced by chemotherapy.

Also, in patients with relapsed or metastatic endometrial cancer GnRH agonists have been used. Up to now, the treatment of 144 women suffering from disseminated endometrial cancer with GnRH agonists has been reported. Objective remissions have been seen in 12% and stable diseases have been observed in 31% of these women (reviewed in Emons et al. 2000b, Noci et al. 2001).

Thus, single agent therapy with GnRH agonists in doses used for suppression of pituitary gonadotropin secretion has a modest activity in patients with relapsed or disseminated ovarian or endometrial cancer leading, in some cases, to long-lasting remissions or disease stabilization. As this treatment is virtually devoid of side effects, it remains a valid option in patients who will not tolerate or accept further chemotherapy. A combination of GnRH agonists with chemotherapy is probably not efficacious as the direct anti-proliferative effects of GnRH agonists are neutralized by their anti-apoptotic activity.

Clinical applications of GnRH antagonists

Using the human ovarian cancer cell line OV-1063 xenografted into nude mice, Yano et al. (1994b) demonstrated a significant inhibition of tumor growth by chronic treatment with the GnRH antagonist Cetrorelix but not with the GnRH
agonist Triptorelin. As both GnRH analogs induced a comparable suppression of the pituitary–gonadal axis, the authors speculated that antitumor effects of cetrorelix were exerted directly on GnRH receptors in tumors (Yano et al. 1994a,b). Assuming that the direct antitumor effects of GnRH antagonists might be superior to those of GnRH agonists, we designed a clinical trial with the GnRH antagonist cetrorelix in patients with ovarian or mullerian carcinoma refractory to platinum chemotherapy. In former trials with GnRH agonists, doses of the analogs (3–8 mg/month) had been used that were sufficient to suppress pituitary gonadotropin secretion. Tissue concentrations of LHRH agonists obtained with these conventional depot preparations of GnRH agonists (nM range, Emons & Schally 1994) might have been only marginally active at the tumor cell level. Therefore, in this trial, a cetrorelix dose of 10 mg/day was chosen in an effort to increase analog concentration at the tumor cells to a more efficacious level. In addition, during the first week of the trial, a pharmacological study was performed injecting increasing doses (1 mg/day to 10 mg/day) before the maintenance dose of 10 mg/day of cetrorelix was administered to all patients throughout the study. In this trial, performed at the MD Anderson Cancer Center, Houston, Texas, USA and at the University of Marburg, Germany, seventeen evaluable patients were treated. All patients had relapsed after at least one standard chemotherapy, 4 had two preceding chemotherapies, 5 had three prior chemotherapies and 8 had more than three chemotherapies before entering into the trial. Five patients had prior immuno- and three a prior hormonal therapy. Even in this group of heavily pretreated patients with refractory ovarian cancer, three patients (18%) experienced a partial remission with cetrorelix treatment lasting 2, 6 and 7 months, while 6 women (35%) had disease stabilization for 1–12 months. Median survival was 17 months. Toxicities included 1 anaphylactoid reaction (grade 4) controlled by corticoids and cimetidine, grade 2 histamine reaction (2 patients), grade 2 arthralgia (1 patient), minor hot flushes, headache, and local skin reaction at the injection site. With more experience on possible side effects, anaphylactoid and histamine reactions could be safely prevented by oral diphenhydramine (25 mg) and, if needed, cimetidine (300 mg/day) orally. With an injected dose of 10 mg/day, cetrorelix plasma concentrations remained constant and ranged between 30 and 60 ng/ml (20–40 nM). At this concentration significant antitumor effects of cetrorelix had been observed in vitro (Emons & Schally 1994). Thus, cetrorelix has some activity against ovarian cancer and, with appropriate co-medication, is well tolerated. In this very refractory patient population these
GnRH-II and its receptor

In non-mammalian vertebrates, it became evident that three structural variants of GnRH were present in individual species. A similar situation seems to exist in mammals. One of these GnRH variants is GnRH-II (also called chicken GnRH-II) which is entirely conserved in structure from fish to mammals (White et al. 1998, Urbanski et al. 1999). Recently, Millar et al. cloned a type II GnRH receptor from the marmoset monkey which is highly selective for GnRH-II (Millar et al. 2001). At the same time Neill et al. cloned the GnRH-II receptor from the rhesus monkey (Neill et al. 2001). Using RT-PCR and Southern blot analysis we could demonstrate the expression of GnRH-II receptor mRNA in human endometrial and ovarian cancer cell lines (Gründker et al. 2002c). The proliferation of these cell lines was reduced in a dose- and time-dependent manner by native GnRH-II (Fig. 5). These effects were significantly more potent than the antiproliferative effects of equimolar doses of the GnRH-I agonist triptorelin. In the GnRH-II receptor-positive but GnRH-I receptor-negative ovarian cancer cell line SK-OV-3 native GnRH-II but not the GnRH-I agonist triptorelin had antiproliferative effects (Gründker et al. 2002c). These findings open a new field of research on the role of GnRH-II in human cancers. As the anti-proliferative activity of native GnRH-II is significantly superior to that of the GnRH-I superagonists, superactive analogs of GnRH-II might become efficacious drugs for the therapy of human cancers expressing GnRH-II receptors.

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Figure 4 (A and B) Tumor volume of NIH: OVCAR-3 human ovarian cancers (A; GnRH-I receptor-positive) and SK-OV-3 human ovarian cancers (B; GnRH-I receptor-negative) xenografted into nude mice. Saline (control), doxorubicin (300 nmol/20 g) and AN-152 (300 nmol/20 g) were administered intravenously once on day 0. The numbers in brackets represent the numbers of animals alive. All experimental groups consisted of 5 animals. (C) Body weight of nude mice bearing HEC-1B human endometrial tumors. Saline (control), doxorubicin (300 nmol/20 g) and AN-152 (300 nmol/20 g) were administered intravenously once on day 0. All experimental groups consisted of 5 animals. Experiments in nude mice bearing NIH: OVCAR-3 or SK-OV-3 human ovarian cancers gave comparable results. Results are means with vertical bars representing S.E.M. *P < 0.05 versus control, **P < 0.01 versus control, ***P < 0.05 versus day 0 for AN-152; ns, not significant. From Grundker et al. 2002, with permission. © 2002, American Journal of Obstetrics and Gynecology.

Figure 5 Time-course experiments on cell proliferation of human endometrial cancer cell lines HEC-1A (A) and Ishikawa (B) and human ovarian cancer cell lines EFO-21 (C) and SK-OV-3 (D). Cells were incubated without (control) or with 10 µM native GnRH-II or with 10 µM GnRH-I agonist triptorelin. Cell number is given as a percentage of controls (100%). Each point represents the mean ± S.E.M. of three independent experiments, performed using different passages of the respective cell line in quadruple determinations. a, P < 0.001 versus control; b, P < 0.05 versus control; c, P < 0.05 versus triptorelin treatment; d, P < 0.001 versus triptorelin treatment. From Grundker et al. 2002c, with permission. © 2002, The Endocrine Society.

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