EXPERIMENTAL STUDY

Analogs of GnRH-I and GnRH-II inhibit epidermal growth factor-induced signal transduction and resensitize resistant human breast cancer cells to 4OH-tamoxifen

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Abstract

About 50–64% of human breast cancers express receptors for GnRH-I. Direct antiproliferative effects of analogs of GnRH-I on human breast cancer cell lines have been shown. They are at least in part mediated by antagonizing growth promoting effects of estradiol, epidermal growth factor (EGF) or insulin-like growth factor. Recently, expression of a putative receptor for GnRH-II in human tissues was demonstrated. Antiproliferative effects of GnRH-II in human endometrial and ovarian cancer cells were shown not to be mediated through the GnRH-I receptor. Now we demonstrate direct antiproliferative effects of the GnRH-I analog Triptorelin and the GnRH-II analog [D-Lys⁶]GnRH-II in MCF-7 and T47D human breast cancer cells expressing GnRH-I receptors and putative GnRH-II receptors. Pretreatment with Triptorelin or [D-Lys⁶]GnRH-II blocked EGF-induced autophosphorylation of EGF receptor and activation of mitogen-activated protein kinase (extracellular-signal-regulated kinase 1/2 (ERK1/2)) in these cells. In sublines of MCF-7 and T47D cells, which were developed to be resistant to 4OH-tamoxifen, HER-2/p185 was overexpressed. Pretreatment of these cell lines with Triptorelin or [D-Lys⁶]GnRH-II completely abolished resistance to 4OH-tamoxifen, assessed by 4OH-tamoxifen-induced apoptosis. Analogs of GnRH-I and GnRH-II counteract EGF-dependent signal transduction in human breast cancer cells with expression of receptors for GnRH-I and GnRH-II. Through this mechanism, they probably reverse acquired resistance to 4OH-tamoxifen mediated through overexpression or activation of receptors of the c-erbB family.

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Introduction

About 50–64% of human breast cancers express receptors for gonadotropin-releasing hormone I (GnRH-I), also called luteinizing hormone-releasing hormone (LHRH) (1–4). Direct antiproliferative effects of GnRH-I analogs have been described in GnRH-I receptor (GnRH-I-R)-positive breast cancer cell lines *in vitro* (5–8). Various studies demonstrated that direct inhibitory effects of GnRH-I analogs in human breast cancer cells are mediated at least in part by an antagonism of growth-promoting factors, such as estradiol, epidermal growth factor (EGF) or insulin-like growth factor (IGF) (9–11). In addition, Szepeshazy *et al.* (12) demonstrated direct GnRH-I-mediated growth inhibition and significant decrease of expression of receptors for EGF and IGF-I in estrogen-independent MXT mouse mammary carcinoma inoculated in BDF1 mice, which had undergone bilateral surgical ovariectomy. GnRH-I inhibits the expression of 60S acidic ribosomal

phosphoproteins P1 and P2 in MCF-7 cells, and thus affects the rate of protein translation as one mechanism of direct antiproliferative effect of GnRH-I in human breast cancer cells (13). Previously we showed that GnRH-I analogs significantly reduced EGF-induced expression of the immediate early response gene *c-fos* in MCF-7 cells (14).

The expression of GnRH-I and its receptor, GnRH-I-R, as a part of a negative autocrine regulatory system and direct antiproliferative effects of GnRH-I analogs have been demonstrated in a number of human malignant tumors, including ovarian, endometrial and prostate cancers. These studies also indicated that the dichotomy of GnRH antagonists and agonists might not apply to the GnRH system in cancer cells (reviewed in (15)). The GnRH-I-R-mediated signal transduction in human ovarian and endometrial cancers couples to G-protein α_i and activates a phosphotyrosine phosphatase counteracting EGF-induced tyrosine phosphorylation of EGF receptor (EGF-R), resulting in down-regulation of

mitogen-activated protein kinase (MAPK; extracellular-signalregulated kinase 1/2 (ERK1/2)), down-regulated *c-fos* expression and inhibition of estrogen-responsive elements and cell proliferation (15–18). In addition, GnRH-I activates nucleus factor κ B (NF- κ B) in ovarian cancer cells and protects these cells from doxorubicin-induced apoptosis (19). GnRH-I induces activator protein 1 (AP-1) and JunD DNA binding and reduces proliferation by cell-cycle arrest in G_{0/1} phase in human ovarian and endometrial cancer cells (20). It is reasonable to speculate that comparable GnRH-induced mechanisms of action exist in human breast cancer cells. In non-mammalian vertebrates three structural variants of GnRH were described in individual species, all decapeptides with almost similar amino acid composition but different functions in the regulation of reproduction. One of these GnRH variants is GnRH-II (also called chicken GnRH-II), which was totally conserved in structure in evolution from fish to mammals (21–23). The existence of three forms of GnRH suggests that three cognate receptor types would also be present in vertebrates (21, 23). Recently we showed that GnRH-II has antiproliferative effects on human endometrial and ovarian cancer cells, in which mRNA for a putative GnRH-II-R was detected (24). The effects of GnRH-II were greater than those of the superactive GnRH-I agonist Triptorelin. Although attempts to sequence the GnRH-II-R in human tissues have failed until now, we demonstrated that growth-inhibitory effects of GnRH-II are not mediated through the GnRH-I-R (25). It might be speculated that in addition to the autocrine growth regulatory system based on the expression of GnRH-I a similar system exists involving the putative GnRH-II-R.

The development of acquired resistance to antiestrogens in breast cancer is a major therapeutic problem. In human breast cancer, lack of response to endocrine therapy is often associated with decreased expression of the estrogen receptor α (ER α) and increased expression of the members of the *c-erbB* receptor family, including EGF receptor (EGF-R) and/or HER-2 oncogene product p185. HER-2/p185 overexpression results in hyperactivity of MAPK and tamoxifen resistance in MCF-7 cells (26). Inhibition of EGF-R/HER-2 kinase by specific inhibitor AG1478 reduced MAPK activity and enhanced tamoxifen action in resistant MCF-7 xenografts in athymic mice (26). In addition, treatment of MCF-7 cells resistant to tamoxifen with the inhibitor of EGF-R phosphotyrosine kinase (PTK), Iressa (ZD1839/gefitinib), or a monoclonal antibody to p185, trastuzumab (Herceptin®), reduced MAPK (ERK1/2) activity and inhibited cell growth (27).

The aim of this study was to show direct antiproliferative effects of the GnRH-I analog [D-Trp⁶]GnRH (Triptorelin) and the GnRH-II analog [D-Lys⁶]GnRH-II in human breast cancer cell lines MCF-7 and T47D. Then we demonstrated inhibitory effects of Triptorelin and [D-Lys⁶]GnRH-II on EGF-induced autophosphorylation of EGF-R and on MAPK (ERK1/2) activity. We

developed sublines of MCF-7 and T47D cells with secondary resistance to antiproliferative effects of 4OH-tamoxifen. These sublines were characterized and compared with parental cells for their expression of receptors for ER α and ER β , for expression of HER-2 oncogene product p185 and of EGF-R, and for expression for GnRH-I-R and GnRH-II-R. Finally, effects of Triptorelin, [D-Lys⁶]GnRH-II and Iressa on apoptosis induced by 4OH-tamoxifen in parental cells and 4OH-tamoxifen-resistant sublines were analysed.

Material and methods

Cell lines and culture conditions

The human breast cancer cell lines MCF-7 and T47D were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 and T47D cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. The medium used was based on Earle's salts and contained 2-fold concentrations of Earle's minimal essential medium, vitamins, essential and nonessential amino acids (Biochrom, Berlin, Germany). Other components were 2.2 g/l NaHCO₃ (Biochrom), 2.5 mg/l transferrin (Sigma, Deisenhofen, Germany) and 67 mg/l gentamycin sulfate (Biochrom). The medium was supplemented with 40 i.u./l insulin (Aventis, Frankfurt, Germany) and 10% fetal calf serum (FCS; Roche, Mannheim, Germany). 24 h prior to each experiment cell monolayers were washed with PBS and medium was replaced by Phenol Red- and FCS-free medium.

Establishment of MCF-7-TR and T47D-TR

To develop sublines resistant to 4OH-tamoxifen, MCF-7 and T47D cells were kept in culture as described above plus 4OH-tamoxifen (Sigma) at 125 nM. After 6 months of continuous exposure to 4OH-tamoxifen these cells showed proliferation in medium containing 4OH-tamoxifen comparable to that of parental cells without 4OH-tamoxifen, indicating the development of cell lines resistant to the growth-inhibitory properties of 4OH-tamoxifen. The resistant cell lines were named MCF-7-TR and T47D-TR, and were cultured for a further 3 months in medium containing 4OH-tamoxifen before characterization studies.

[³H]Thymidine incorporation

Prior to [³H]thymidine-incorporation assays we performed proliferation assays with increasing concentrations of Triptorelin and [D-Lys⁶]GnRH-II (1 nM–1 μ M), which showed dose-dependent inhibition of proliferation in MCF-7 and T47D cells. After 72 h Triptorelin and [D-Lys⁶]GnRH-II showed comparable growth-inhibitory effects on MCF-7 and

T47D human breast cancer cells (1 nM, less than 10% growth inhibition; 100 nM, about 50% growth inhibition; 1 μ M, about 80% growth inhibition). We focused further experiments on the dose of 100 nM of each analog. 10^7 cells were plated in 96-well dishes (Nunc, Roskilde, Denmark). Confluent cultures were washed twice with PBS and incubated for 72 h in 200 μ l medium containing [3 H]thymidine (85 Ci/mmol; 0.2 μ Ci per well; Amersham Biosciences, Little Chalfont, Bucks, UK) and the GnRH-I analog Triptorelin (100 nM; Ferring Arzneimittel, Kiel, Germany), the GnRH-II analog [D-Lys 6]GnRH-II (100 nM; Peptide Specialty Laboratories GmbH, Heidelberg, Germany) or 4OH-tamoxifen (0.25 μ M). Because of the very short half-life of human GnRH-II in serum we used the super-agonistic analog [D-Lys 6]GnRH-II. The cells were then washed with ice-cold PBS twice, treated with 10% trichloroacetic acid followed by 95% ethanol, and lysed with 400 μ l 0.25 M NaOH per well. Incorporated radioactivity was measured in a liquid scintillation counter (Beckman Coulter, Unterschleissheim, Germany).

Quantification of phosphorylated EGF-R and MAPK (ERK1/2) activity

Basal levels of phosphorylated EGF-R and activated MAPK (ERK1/2) were very low in all cell lines. Thus it was not possible to investigate any significant inhibitory effects of Triptorelin and [D-Lys 6]GnRH-I without previous EGF-induced autophosphorylation of EGF-R or activation of MAPK. To induce autophosphorylation of EGF-R and activation of MAPK, cells were treated with human recombinant EGF (Sigma) at 100 nM for 30 min. However, exposure to EGF (100 nM) for 5 and 15 min had no effect on autophosphorylation of EGF-R and activation of MAPK in our system with MCF-7 and T47D cells, so we focused further experiments on an activation time of 30 min with EGF. Triptorelin (100 nM), [D-Lys 6]GnRH-II (100 nM) or specific inhibitor Iressa (28) (ZD1839, 4.5 μ M; AstraZeneca, Wedel, Germany) was added 30 min prior to exposure to EGF. The cells were scraped from dishes with a rubber policeman, washed twice with PBS and then lysed on ice using a buffer (pH 6.8) containing 519 mM dithiothreitol (Sigma), 69 mM SDS, 0.1% Triton X-100 (Sigma), 62.5 mM Tris/HCl and 75 nM Bromphenol Blue. The cell lysates (10^4 cells per μ l lysate) were electrophoresed using SDS/PAGE (7.5% acrylamide/ N,N' -bis-methylene-acrylamide; AppliChem GmbH, Darmstadt, Germany) and then transferred to nitrocellulose membranes according to the instructions of the suppliers (Hybond $^{\text{TM}}$ -ECL $^{\text{TM}}$ nitrocellulose membrane; Amersham Biosciences). The nitrocellulose membranes were blocked in 5% instant skimmed-milk powder, spray-dried (Naturaflo; Töpfer GmbH, Dietmannsried, Germany) in TBST buffer (137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20 and

25 mM Tris/Cl, pH 7.4) for 1 h at room temperature, washed with TBST, and then incubated overnight by 4 °C with polyclonal rabbit anti-human phosphotyrosine antibody (Promega, Mannheim, Germany) or polyclonal rabbit anti-human/rat ACTIVE $^{\text{TM}}$ MAPK antibody pTEpY (anti-ERK1/2 polyclonal antibody; Promega) in a 1:200 or a 1:5000 dilution in TBST (Amersham Biosciences), respectively. After washing three times with TBST, specifically bound antibody was detected using the ECL $^{\text{TM}}$ Western-blotting analysis system (Amersham Biosciences). The bands were imaged by lightening and developing a radiographic film (Kodak BioMax MR film). Corresponding bands for phosphorylated EGF-R at 170 kDa and MAPK (ERK1/2) at 42 and 44 kDa were analysed using the Kodak 1D image system (Kodak, New Haven, CT, USA).

Isolation of mRNA, cDNA synthesis and PCR amplification

Polyadenylated RNA (poly(A) mRNA) was isolated from cells grown in monolayer using the Oligotex direct mRNA kit (Qiagen, Hilden, Germany). The concentration of poly(A) mRNA in each sample was determined by photospectrometry. First-strand cDNA was generated by reverse transcription of 1 μ g poly(A) mRNA using p(dT) $_{15}$ primers (Roche Diagnostics) with Moloney murine leukaemia virus reverse transcriptase according to the instructions of the suppliers (Life Technologies, Karlsruhe, Germany). The cDNAs (2 ng) were amplified in a 50 μ l reaction volume. The reaction mixture contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl $_2$, 200 μ M each dNTP, 1 μ M primers specific for human GnRH-I-R mRNA (29) (forward, 5'-CTG GCT GTG GAC ATC GCA TGT-3' reverse, 5'-ATG GCA GTC AGT GGC AGC AGA-3'), 1 μ M primers specific for the putative human GnRH-II-R mRNA (24) (forward, 5'-AGT CCA ATG GTA TGC TGG AGA-3'; reverse, 5'-ACC CGT GTC AGG GTG AAG AT-3'), 1 μ M primers specific for human ER α (forward, 5'-AAT CTA GAT AAT CGA CGC CAG-3'; reverse, 5'-GTG TTT CAA CAT TCT CCC TCC TC-3'), 1 μ M primers specific for human ER β (forward, 5'-TAG TGG TCC ATC GCC AGT TAT-3'; reverse, 5'-GGG AGC CAC ACT TCA CCA T-3'), 1 μ M primers specific for human EGF-R (forward, 5'-TGA TGG CTA GTG TGG ACA ACC-3'; reverse, 5'-CAT GAT ATT CTT TCT CTT CAG CA-3') or 1 μ M primers specific for human HER-2 oncogene product p185 (forward, 5'-GGC TGC TGG ACA TGG ACG AG-3'; reverse, 5'-GGG GCT GGG GCA GCC GCT C-3'), and 1.25 U AmpliTaq Gold $^{\text{®}}$ polymerase (Applied Biosystems, Weiterstadt, Germany). The reaction was performed in an Applied Biosystems DNA thermal cycler 9600. 35 cycles of amplification of PCR were carried out: denaturation at 94 °C for 30 s, annealing for 60 s at individual temperature (GnRH-I-R, 61 °C; GnRH-II-R, 60 °C; HER-2 and EGF-R, 58 °C; ER α and ER β , 56 °C), followed by extension at 72 °C for 60 s.

The PCR products were separated by gel electrophoresis in 1.5% agarose and visualized by ethidium bromide staining on a UV transilluminator. Corresponding bands were analyzed using the Kodak 1D image system.

Comparative flow-cytometry analysis

To analyse expression of GnRH-I-R and putative GnRH-II-R, HER-2 gene product p185-expression, and expression of EGF-R in parental and resistance-developed sublines of MCF-7 and T47D cells, 10^6 cells were resuspended in 50 μ l Hank's balanced salt solution (HBSS) containing 0.1% sodium azide, 1.5% Hepes, 2% FCS and 50 μ l diluted primary antibody polyclonal rabbit anti-human GnRH-R-I or polyclonal rabbit anti-human GnRH-R-II (both 1:400; Peptide Research Laboratories), mouse anti-HER2 (1:10; TAB250; Zymed Laboratories, San Francisco, CA, USA) or monoclonal anti-EGF-R antibody produced in mouse (1:10; clone 29.1; Sigma) were added and incubated on ice for 1 h. After washing, FITC-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (both Sigma), respective to the first antibody used, was added at 1:20 dilution. The cell suspension was incubated on ice for 1 h. Cells were washed in HBSS and immediately analysed by flow cytometry on FACScalibur equipment using Cellquest software (Becton Dickinson, Mountain View, CA, USA). Negative controls were performed by omission of primary antibodies. In each experiment 10^5 cells were counted.

Proliferation assays

The time-course and dose-dependent proliferation experiments were performed as described in detail previously (24, 30). Briefly, 20 000 cells of each cell line were plated in four-well cluster dishes (Nunc) and allowed to attach to the wells. After 24 h the medium was changed and 20 ml PBS/BSA or appropriate dilutions of 4OH-tamoxifen were added, resulting in final concentrations of 62.5–250 nM. Depletion of FCS resulted in complete de-adhesion and cell death in all cell lines on day 4, so we supplemented the medium with 10% stripped hormone-free FCS (Allgaeu BioTech Service, Goerisried, Germany). After 24 h of incubation the medium was changed. After 1, 2, 3 and 4 days the cells were counted in a Neubauer hemocytometer (24, 30).

Apoptosis assays and cell-cycle analysis

To quantify apoptosis and cell-cycle arrest we used a procedure similar to that described by Nicoletti *et al.* (31) based on detecting advanced DNA degradation. Briefly, a pellet containing 1×10^6 cells was gently resuspended in 500 μ l hypotonic fluorochrome solution containing 0.1% Triton X-100, 0.1% sodium

citrate and 50 μ g/ml propidium iodide (Sigma). The cell suspensions were kept at 4°C in the dark overnight before flow-cytometry analysis of cellular DNA content was performed using FACScalibur equipment with Cellquest software. In each experiment 10^5 cells were counted. To analyse apoptosis induced by 4OH-tamoxifen (250 nM), Triptorelin (100 nM), [D-Lys⁶]GnRH-II (100 nM) or Iressa (4.5 μ M), drugs or vehicle were added to the medium as described above. Cells were harvested after 72 h and prepared as described for further analysis.

Statistical analysis

All experiments were performed at least three times in different passages of the respective cell lines. Unless otherwise indicated, results are expressed as percentages of the respective controls and were pooled before being tested for statistically significant differences by one-way analysis of variance, followed by a Newman–Keuls' test for the comparison of individual groups.

Results

Direct antiproliferative effects of GnRH-I analog Triptorelin and GnRH-II analog [D-Lys⁶]GnRH-II

To show direct antiproliferative effects we compared [³H]thymidine incorporation after 72 h of exposure to GnRH-I analog Triptorelin and GnRH-II analog [D-Lys⁶]GnRH-II, both at the well-established concentration of 100 nM, with exposure to 4OH-tamoxifen (250 nM) in MCF-7 and T47D cells. In MCF-7 cells 4OH-tamoxifen reduced [³H]thymidine incorporation from $100 \pm 15.4\%$ in untreated control cells to $53.3 \pm 13.2\%$ ($P < 0.001$ compared with the control). Triptorelin reduced [³H]thymidine-incorporation in these cells to $64.9 \pm 12.8\%$ ($P < 0.001$) and [D-Lys⁶]GnRH-II to $55.9 \pm 9.7\%$ ($P < 0.001$). In T47D cells 4OH-tamoxifen reduced [³H]thymidine incorporation from $100 \pm 14.1\%$ in untreated control cells to $34.7 \pm 18.9\%$ ($P < 0.001$). Triptorelin reduced [³H]thymidine incorporation in these cells to $45.9 \pm 16.7\%$ and [D-Lys⁶]GnRH-II to $49.7 \pm 13\%$ (both $P < 0.001$ compared with the control; Fig. 1).

Effects of Triptorelin and [D-Lys⁶]GnRH-II on EGF-induced autophosphorylation of EGF-R

We compared effects of Triptorelin and [D-Lys⁶]GnRH-II on EGF-induced EGF-R tyrosine autophosphorylation to the effects of the specific PTK inhibitor Iressa in MCF-7 and T47D cells. The amount of detectable phosphorylated EGF-Rs in quiescent cells was very low in our assay. Treatment with human recombinant EGF

Figure 1 [^3H]Thymidine incorporation into human breast cancer MCF-7 and T47D cells after 72 h of exposure to 4OH-tamoxifen (250 nM), Triptorelin or [D-Lys 6]GnRH-II (both 100 nM). Boxes and whiskers represent median, s.e. and maximum plus minimum of data obtained from four independent experiments in four different passages of the cell lines. Results were tested by one-way analysis of variance, followed by a Newman–Keuls' test for the comparison of individual groups. MCF-7, $^aP < 0.001$ compared with all other groups; $^bP < 0.01$ compared with 4OH-tamoxifen and [D-Lys 6]GnRH-II; T47D, $^aP < 0.001$ compared with all other groups; $^bP < 0.01$ compared with Triptorelin and [D-Lys 6]GnRH-II.

(100 nM) for 30 min resulted in a significant increase of phosphorylated EGF-Rs in MCF-7 cells to $180 \pm 33\%$ and in T47D cells to $226.3 \pm 47.4\%$, as compared with quiescent cells (100%). Pretreatment with Iressa ($4.5 \mu\text{M}$) for 30 min, used as a positive control, completely abolished the effects of EGF on EGF-R autophosphorylation in MCF-7 and T47D cells. In MCF-7 and T47D cells pretreatment with Triptorelin or [D-Lys 6]GnRH-II (both 100 nM) for 30 min showed almost similar effects to Iressa (MCF-7/EGF + Triptorelin, $110.7 \pm 27.6\%$, $P < 0.05$ compared with EGF; MCF-7/EGF + [D-Lys 6]GnRH-II, $111.7 \pm 18.6\%$, $P < 0.01$; T47D/EGF + Triptorelin, $73 \pm 44.8\%$, $P < 0.01$; T47D/EGF + [D-Lys 6]GnRH-II, $56 \pm 43.1\%$, $P < 0.01$; Fig. 2). Experiments in the developed sublines MCF-7-TR and T47D-TR yielded comparable results (data not shown).

Effects of Triptorelin and [D-Lys 6]GnRH-II on EGF-induced MAPK (ERK1/2) activation

We then compared effects of Triptorelin and [D-Lys 6]GnRH-II on EGF-induced MAPK (ERK1/2) activity to the effects of Iressa. Treatment with EGF (100 nM) for 30 min resulted in a significant increase of MAPK activity in MCF-7 ($164.7 \pm 9.5\%$) and T47D ($222.3 \pm 33.6\%$) cells, as compared with quiescent cells. Pretreatment with Iressa ($4.5 \mu\text{M}$) for 30 min completely abolished the effects of EGF on MAPK activity in MCF-7 and T47D cells. In MCF-7 and T47D cells pretreatment with Triptorelin or [D-Lys 6]GnRH-II (both

100 nM) for 30 min showed comparable effects to Iressa (MCF-7/EGF + Triptorelin, $98.3 \pm 4\%$, $P < 0.01$ compared with EGF; MCF-7/EGF + [D-Lys 6]GnRH-II, $97.3 \pm 19.9\%$, $P < 0.01$; T47D/EGF + Triptorelin, $69.3 \pm 33.1\%$, $P < 0.001$; T47D/EGF + [D-Lys 6]GnRH-II, $65.3 \pm 48.8\%$, $P < 0.01$; Fig. 3). Experiments in the developed sublines MCF-7-TR and T47D-TR yielded comparable results. These cells did not show significant increased basal levels of MAPK activity compared with parental cells (data not shown).

Comparative characterization of parental MCF-7 and T47D cells and developed 4OH-tamoxifen-resistant sublines MCF-7-TR and T47D-TR

The developed sublines with secondary resistance to the growth-inhibitory effect of 4OH-tamoxifen were analysed for their expression of ER α and ER β , HER-2 gene product p185, EGF-R, GnRH-I-R and GnRH-II-R using reverse transcriptase (RT)-PCR. The cell-surface expression of p185, EGF-R, GnRH-I-R and GnRH-II-R was analysed by flow cytometry. In MCF-7-TR cells using RT-PCR we found a 3-fold up-regulation of mRNA expression of ER α , HER-2/p185 and EGF-R compared with parental cells. Expression of ER β was also increased slightly, but not significantly. There was no change in expression of mRNA for GnRH-I-R and GnRH-II-R (Fig. 4). Cell-surface expression of p185 and EGF-R were increased slightly in MCF-7-TR cells, whereas expression of GnRH-I-R and GnRH-II-R was

Figure 2 EGF-induced autophosphorylation of EGF-R in MCF-7 and T47D cells. Treatment with EGF (100 nM) for 30 min induced a significant increase of EGF-R autophosphorylation in all cell lines as compared with quiescent control cells (100%). Pretreatment with Iressa (4.5 μ M), Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) for 30 min completely blocked EGF-induced autophosphorylation of EGF-R in MCF-7 and T47D cells. Representative Western-blot analyses show effects on phosphorylated EGF-R in MCF-7 and T47D cells according to bands at 170 kDa. Columns represent means $\pm$ S.E. obtained from three independent experiments in three different passages of the cell lines. Statistical tests were as for Fig. 1. MCF-7, ^a $P < 0.05$ compared with EGF + Iressa and EGF + Triptorelin; ^b $P < 0.01$ compared with EGF + [D-Lys⁶]GnRH-II; T47D, ^a $P < 0.01$ compared with all other groups.

decreased slightly compared with parental MCF-7 cells (Fig. 5). In T47D-TR cells using RT-PCR we found a 2-fold up-regulation of mRNA-expression of HER-2 and a slightly but not significantly increased expression of EGF-R compared with parental T47D cells. Expression of mRNA for ER α , ER β and GnRH-II-R was clearly down-regulated in T47D-TR cells. There was no significant difference in expression of mRNA for GnRH-I-R in T47D cells and the developed subline (Fig. 4). Cell-surface expression of p185 was clearly increased in T47D-TR cells, whereas expression of EGF-R, GnRH-I-R and GnRH-II-R was decreased, compared with parental T47D cells (Fig. 5).

We then tested the effects of Triptorelin and [D-Lys⁶]GnRH-II on EGF-induced autophosphorylation of EGF-R and MAPK (ERK1/2) activity in the developed sublines.

Effects of 4OH-tamoxifen on proliferation in parental and resistant MCF-7 and T47D cells

In parental MCF-7 and T47D cells 4OH-tamoxifen showed time- and dose-dependent antiproliferative effects (Fig. 6). In MCF-7-TR cells 4OH-tamoxifen

showed strong proliferative effects with a maximum on day 3 compared with control cells without 4OH-tamoxifen. This effect may be explained by a lower proliferation rate in control cells due to depletion of 4OH-tamoxifen, which is regularly supplemented to the medium when MCF-7-TR cells are kept in culture. The proliferative effect of 4OH-tamoxifen in MCF-7-TR cells disappeared on day 4 (Fig. 6). In T47D-TR cells 4OH-tamoxifen had no significant effect on proliferation up to day 3 compared with control cells. However, depletion of 4OH-tamoxifen resulted in complete de-adhesion of the control T47D-TR cells on day 4 and cells showed morphological criteria of apoptosis, so cells treated with 4OH-tamoxifen cannot be quantified as percentages of untreated control cells on day 4 (Fig. 6).

Effects of Triptorelin, [D-Lys⁶]GnRH-II and Iressa on apoptosis induced by 4OH-tamoxifen-induced in parental and resistance-developed MCF-7 and T47D cells

In MCF-7 cells treatment with 4OH-tamoxifen (250 nM) for 72 h increased apoptosis from

Figure 3 MAPK (ERK1/2) activity in MCF-7 and T47D cells. Treatment with EGF (100 nM) for 30 min significantly increased MAPK activity in all cell lines as compared with quiescent control cells (100%). Pre-treatment with Iressa (4.5 μ M), Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) for 30 min completely blocked EGF-induced MAPK activity in all cell lines. Representative Western-blot analyses show effects on activated MAPK in MCF-7 and T47D cells according to bands at 42 and 44 kDa. Columns represent means $\pm$ s.e. obtained from three independent experiments in three different passages of the cell lines. Statistical tests were as for Fig. 1. MCF-7, ^a $P < 0.01$ compared with EGF + Triptorelin and EGF + [D-Lys⁶]GnRH-II, ^b $P < 0.001$ compared with EGF + Iressa; T47D, ^a $P < 0.01$ compared with EGF + Iressa and EGF + [D-Lys⁶]GnRH-II, ^b $P < 0.001$ compared with EGF + Triptorelin.

15.7 $\pm$ 2.5% (spontaneous apoptosis rate in untreated control cells) to 25.7 $\pm$ 1.5%. Treatment with Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) for 72 h did not significantly increase apoptosis rate in these cells. Addition of Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) to 4OH-tamoxifen was without significant effect on apoptosis induced by 4OH-tamoxifen. Comparable results were obtained in experiments with T47D cells (Fig. 7). In the sublines resistant to antiproliferative effects of 4OH-tamoxifen, MCF-7-TR and T47D-TR, 4OH-tamoxifen (250 nM) had no significant effect on apoptosis rate. Treatment with Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) for 72 h did not significantly increase apoptosis compared with spontaneous apoptosis rate of untreated control cells. In MCF-7-TR cells simultaneous treatment with 4OH-tamoxifen (250 nM) and Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM) significantly increased apoptosis from 12.3 $\pm$ 4.0% (spontaneous rate in untreated control cells) to 23.3 $\pm$ 6.7 and 25 $\pm$ 5.3%, respectively. In T47D-TR cells simultaneous treatment with 4OH-tamoxifen (250 nM) and Triptorelin or [D-Lys⁶]GnRH-

II (both 100 nM) significantly increased apoptosis from 10.7 $\pm$ 3.1% (spontaneous rate in untreated control cells) to 22.0 $\pm$ 4.4 or 22.0 $\pm$ 3.6%, respectively (Fig. 7). Exposure to Iressa (4.5 μ M) for 72 h did not increase apoptosis in parental MCF-7 and T47D cells or in the developed sublines. Simultaneous treatment with Iressa had no effect on apoptosis induced by 4OH-tamoxifen in parental cells and in the developed sublines (data not shown). We then analysed effects of Iressa on cell-cycle arrest in MCF-7 and T47D cells and in the developed sublines MCF-7-TR and T47D-TR. In all cell lines we found a tendency for cell-cycle arrest in the G₁ phase when cells were treated with Iressa (4.5 μ M) alone or in combination with 4OH-tamoxifen (0.25 μ M) for 72 h, but these effects were not statistically significant (data not shown).

Discussion

Previous work and our present data demonstrate direct antiproliferative effects of GnRH in GnRH-R-

Figure 4 RT-PCR analysis showing comparative semi-quantitative expression of mRNA of ER α , ER β , HER-2 oncogene product p185, EGF-R, GnRH-I-R and GnRH-II-R in parental MCF-7 and T47D cells and in the developed sublines MCF-7-TR and T47D-TR. Columns represent means $\pm$ s.e. obtained from three independent experiments in three different passages of the cell lines. Statistical tests were as for Fig. 1; n.s., not significant.

positive human breast cancer cells (5–8, 12). The antiproliferative effect of the GnRH-I analog Triptorelin and the GnRH-II analog [D-Lys⁶]GnRH-II in MCF-7 and T47D cells was not due to increased apoptosis. These data are in good accordance with previous results in human ovarian cancer cells, in which GnRH analogs induced JunD DNA binding and an extended cell cycle, but did not induce apoptosis (20). In our system Triptorelin and [D-Lys⁶]GnRH-II interfered with EGF-induced signal transduction and completely inhibited EGF-induced autophosphorylation of EGF-R and induction of MAPK (ERK1/2) activity in MCF-7 and T47D cells. In the sublines of MCF-7 and T47D cells that were developed to be resistant to the antiproliferative effects of 4OH-tamoxifen (MCF-7-TR and T47D-TR) treatment with Triptorelin and [D-Lys⁶]GnRH-II reinstated 4OH-tamoxifen sensitivity, as assessed by induction of apoptosis. In MCF-7-TR cells exposition to 4OH-tamoxifen showed proliferative effects compared with cells with depletion of 4OH-tamoxifen, whereas in T47D cells depletion or

exposure to increased concentrations of 4OH-tamoxifen had no effect on proliferation. In the developed sublines we found a slight down-regulated expression of GnRH-I-R and GnRH-II-R, whereas expression of the HER-2 oncogene product p185 was increased markedly in both cell lines. The expression of mRNA for EGF-R was also increased in MCF-7-TR and T47D-TR, but the cell-surface expression of EGF-R was hardly altered compared with parental cells. Although MCF-7-TR and T47D-TR showed slightly decreased cell-surface expression of GnRH-I-R and GnRH-II-R, exposure to Triptorelin and [D-Lys⁶]GnRH-II showed comparable effects on EGF-induced autophosphorylation of EGF-R and MAPK (ERK1/2) activation as in parental cells. Iressa (gefitinib/ZD1839) strongly inhibited EGF-induced autophosphorylation of EGF-R and MAPK (ERK1/2) activation in all cell lines but had no effect on apoptosis induced by 4OH-tamoxifen. In our system treatment with Iressa resulted in marginally increased cell-cycle arrest in the G₁ phase.

Figure 5 Comparative flow-cytometry analysis showing surface expression of HER-2 oncogene product p185, EGF-R, GnRH-I-R and GnRH-II-R in parental MCF-7 and T47D cells (thin lines) and in the developed sublines MCF-7-TR and T47D-TR (thick lines). Values for mean fluorescence of surface expression (negative control, < 10) were as follows: HER-2 oncogene product p185, 60.2 (MCF-7) versus 92.8 (MCF-7-TR), 187.1 (T47D) versus 427.7 (T47D-TR); EGF-R, 10.1 (MCF-7) versus 12.8 (MCF-7-TR), 61.3 (T47D) versus 44.0 (T47D-TR); GnRH I-R, 23.0 (MCF-7) versus 15.7 (MCF-7-TR), 50.3 (T47D) versus 32.0 (T47D-TR); GnRH II-R, 46.5 (MCF-7) versus 22.6 (MCF-7-TR), 157.6 (T47D) versus 69.0 (T47D-TR). Histograms show representative data obtained from three independent flow cytometry analysis experiments in three different passages of the cell lines.

Figure 6 Proliferation assays in parental and resistant MCF-7 and T47D cells with exposure to 4OH-tamoxifen in concentrations of 62.5–250 nM. The effects of 4OH-tamoxifen on cell proliferation are expressed as percentages of untreated control cells (100%). Graphs represent means $\pm$ S.E. obtained from three independent experiments in three different passages of the cell lines.

Figure 7 Quantification of apoptosis in parental and 4OH-tamoxifen-resistant MCF-7 and T47D cells after 72 h exposure to 4OH-tamoxifen (250 nM), Triptorelin or [D-Lys⁶]GnRH-II (both 100 nM), or GnRH analogs in combination with 4OH-tamoxifen. Columns represent means $\pm$ S.E. obtained from three independent experiments in three different passages of the cell lines. Statistical tests were as for Fig. 1. MCF-7, ^a $P < 0.001$ compared with control and Triptorelin, ^b $P < 0.01$ compared with [D-Lys⁶]GnRH-II; T47D, ^a $P < 0.01$ compared with control and Triptorelin, ^b $P < 0.05$ compared with [D-Lys⁶]GnRH-II; MCF-7-TR, ^a $P < 0.05$ compared with control, 4OH-tamoxifen, Triptorelin and [D-Lys⁶]GnRH-II; T47D-TR, ^a $P < 0.01$ compared with control, 4OH-tamoxifen, Triptorelin and [D-Lys⁶]GnRH-II.

Receptors for GnRH I are frequently expressed by breast cancers (1–4). Direct antiproliferative effects of GnRH-I analogs in human breast cancer cells *in vitro* or xenotransplanted into nude mice are mediated at least in part by an antagonism of growth-promoting factors, such as estradiol, EGF and IGF (8–12), and inhibit estradiol-induced serum-response element (SRE) activation and *c-fos* expression (18). We now demonstrate that the previously described GnRH-I-mediated inhibition of EGF-induced tyrosine phosphorylation of EGF-R and down-regulation of MAPK (ERK-1/2) activity in human ovarian and endometrial cancers (17, 30) also occur in human breast cancers.

Expression of mRNA for GnRH-II-R has been described in non-human primates and in human tissues. Until now, however, attempts to clone or sequence

a functional full-length receptor have not yet been successful (24, 32–35). In mammals, GnRH-II-Rs have been found to be more widely expressed in the body than GnRH-I-Rs, suggesting that GnRH-II may have various functions (36). The functional GnRH-II-R is expressed in various splice variants (37), but the human type II receptor gene homolog carries a frame-shift mutation and a premature stop codon, findings suggesting that a full-length type II receptor does not exist in humans (38). The antiproliferative effects of GnRH-II in human endometrial and ovarian cancer cells persist in cells after knockout of the GnRH-I-R, whereas the antiproliferative effect of GnRH-I agonist Triptorelin was completely abrogated in these cells, implying that the antiproliferative effects of GnRH-II are not mediated through the GnRH-I-R (25). Enomoto *et al.* (39) confirmed effects of GnRH-II in HHUA,

DU145, TSU-Pr1 and Jurkat cells with knocked-down GnRH-I-R and suggested functionality of a human type II GnRH-R splice variant. We recently identified a 43 kDa cell-membrane protein with GnRH-II-R antigenicity in human placenta, and endometrial, ovarian and prostate cancer cells by a polyclonal rabbit anti-human GnRH-II-R antiserum, which was generated using a peptide corresponding to the third extracellular domain of the GnRH-II-R. The 43 kDa band detected in Western-blot analysis was identified as a specific GnRH-II-binding site by photoaffinity labeling using ^{125}I -labeled (4-azidobenzoyl)-N-hydroxysuccinimide-[D-Lys⁶]GnRH-II (40). Using this polyclonal rabbit anti-human GnRH-II-R antiserum, we detected GnRH-II-R antigenicity at the cell surface of human MCF-7 and T47D breast cancer cells. In these cells the GnRH-II analog [D-Lys⁶]GnRH-II had antiproliferative effects, inhibited EGF-induced autophosphorylation of EGF-R and activation of MAPK (ERK1/2). Kim *et al.* (41) showed antiproliferative effects of GnRH-II in human ovarian cancer cells at the same concentration (100 nM), although they described activation of MAPK (ERK1/2) by GnRH-II. We now confirm the described interference of GnRH-II with the ERK1/2 pathway by attenuating EGF-stimulated MAPK activation.

Estrogen-dependent breast cancers can progress from an antiestrogen-sensitive to an antiestrogen-resistant state. HER-2/p185 or EGF-R overexpression and heterodimerization of members of the c-erbB family in human breast cancers results in activation of MAPK (ERK1/2), which phosphorylates Ser-118 in the ER, leading to ligand-independent ER activation with loss of the inhibitory effect of tamoxifen on ER-mediated transcription (26, 27, 42, 43). Tamoxifen recruits transcriptional corepressors to the AF-2 region in the hormone-binding domain of the ER to block ER-mediated transcription (44). HER-2/p185-induced hyperactivity of MAPK inhibits tamoxifen-mediated association of ER with nuclear receptor corepressor (N-CoR) in antiestrogen-resistant cells. Selective inhibition of MAPK by U0126 or AG1478 restores this association and abrogates antiestrogen-resistance in human breast cancers (26). HER-2 signaling activates the same signaling pathways activated by EGF and both receptors contribute to transformation in a cooperative manner (reviewed in (45)). Expression of EGF-R is found in more than 60% of human breast cancers and is inversely related to expression of ER and is a negative predictor for response to endocrine therapy *in vitro* and *in vivo* (27, 46, 47). Knowlden *et al.* (27) demonstrated reduced MAPK (ERK1/2) activity in developed tamoxifen-resistant MCF-7 cells, which generated a range of EGF-R specific ligands and transforming growth factor, after exposure to Iressa, trastuzumab (Herceptin) or MAPK-inhibitor PD098059, leading to growth inhibition in these cells. This indicates an autocrine release and action of EGF-R specific ligands inducing preferential EGF-R/HER-2

dimerization and downstream activation of the ERK pathway in tamoxifen-resistant cells. Gee *et al.* (48) showed that co-treatment with tamoxifen and Iressa or fulvestrant and Iressa is superior to treatment with tamoxifen alone in MCF-7 cells, and the combined treatment probably prevents secondary resistance to antiestrogens. In these cells increased EGF-R expression and MAPK activity induced by long-term administration of tamoxifen was prevented by co-treatment with Iressa, whereas Iressa alone was ineffective on growth inhibition (48). The aim of our study was to evaluate the effects of Iressa in comparison to Triptorelin and [D-Lys⁶]GnRH-II on apoptosis induced by 4 OH-tamoxifen up to 72 h, which was not increased by Iressa. We did not quantify growth inhibition by Iressa, which occurs significantly after 15 days in culture (48) and probably would occur in our cells after long-term administration too, indicated by the marginal effects of cell-cycle arrest in the G₁ phase. It is likely that the downstream activation of the ERK pathway in our system of 4OH-tamoxifen-resistant cells is not mediated preferentially by EGF-R/HER-2 dimerization, which can be blocked by Iressa or trastuzumab (27). The mechanism of tamoxifen resistance in our cell lines is not yet clear, since we cannot explain the up-regulation of mRNA expression of ER α and ER β in MCF-7-TR cells, whereas in T47D-TR cells mRNA expression of ER α and ER β was down-regulated. Although we could not describe hyperactivity of MAPK in our developed sublines, it is likely that Triptorelin and [D-Lys⁶]GnRH-II shows comparable activity to the specific MAPK inhibitor AG1478, which enhanced tamoxifen action in resistant MCF-7 cells (26). It is probable that increased expression of p185 and downstream activation of the ERK pathway are not the only mechanisms of tamoxifen resistance in our cell lines. Recently Jordan *et al.* (49) showed increased levels of phosphorylated phosphoinositide 3-kinase signaling component Akt1 in MCF-7 cells with developed tamoxifen resistance, which was activated by the ligands of the EGF-R, EGF and transforming growth factor α (TGF- α); in addition, Akt1 activation could be inhibited by Iressa in these cells (49). Activation of Akt/protein kinase B (PKB) is discussed to predict a worse outcome in patients with ER-positive breast cancer receiving antiestrogens and contributes to an aggressive phenotype of antiestrogen-resistant ER-positive breast cancers (49, 50). Kraus *et al.* (51) described inhibition of the phosphoinositide 3-kinase Akt/PKB pathway by GnRH analogs in human prostate cancer cells and these effects were amplified by specific phosphoinositide 3-kinase/PKB inhibitors. Further investigations on the effects of GnRH-I and GnRH-II on dimerization of members of the c-erbB family and interference with downstream activation of the ERK and Akt pathway in parental MCF-7 and T47D cells and the resistance-developed sublines are in progress.

In conclusion, the present study demonstrates antiproliferative effects of GnRH-I and GnRH-II analogs in human breast cancer cells with expression of GnRH-I-R and expression of putative GnRH-II-R. In these cells, analogs of GnRH-I and GnRH-II abrogated effects of EGF-stimulated ERK1/2 activation. The interference of GnRH-I and GnRH-II analogs with the ERK1/2 pathway was accompanied by restored sensitivity to 4OH-tamoxifen in sublines with developed resistance, which showed increased expression of the HER-2 gene product p185. These results might provide new therapeutic options in the combination treatment of breast cancer with secondary resistance to antiestrogens.

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