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Differential Effects of Aromatase Inhibitors and Antiestrogens on Estrogen Receptor Expression in Breast Cancer Cells

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Abstract. *Background: Estrogen receptors (ER) α and β play an important role in breast cancer. Recently, systemic adjuvant endocrine therapy with selective estrogen receptor modulator (SERM) tamoxifen has been challenged by aromatase inhibitors. Compared to antiestrogens, third-generation aromatase inhibitors (anastrozole and letrozole) exhibit an improved efficacy and tolerability. Materials and Methods: Using real-time PCR analysis, 21 breast cancer tissue samples were analysed for a change of the ERα/ERβ ratio during malignant progression. In stimulation experiments, differential effects of SERMs, ER antagonists and aromatase inhibitors have been investigated. Results: Transition from normal breast to grade 1 tumors was characterized by down-regulation of ERβ (relative quantification [RQ]=0.83, p=0.019), while transition from grade 1 to grade 3 tumors was associated with the decrease of ERα expression (RQ=1.14 vs. RQ=0.65, p<0.001). In stimulation assays, tamoxifen and fulvestrant increased ERα expression to RQ=1.51 (p=0.01) and RQ=1.42 (p<0.001), respectively, and left ERβ unchanged. In contrast, aromatase inhibitors up-regulated ERβ to RQ=1.23 (anastrozole, p=0.029) and RQ=1.38 (letrozole, p=0.048). Conclusion: Taken together, data indicate that SERMs/antiestrogens and aromatase inhibitors exhibit opposed effects on the ER expression of breast cancer cells: tamoxifen and fulvestrant up-regulate ERα expression, while aromatase inhibitors increase ERβ expression, which may contribute to the aromatase inhibitors' therapeutic superiority over antiestrogens.*

The effects of 17β-estradiol, mediated *via* estrogen receptors (ER) α and β, are crucial for breast cancer formation and tumor progression. ERα mediates transcriptional regulation of a variety of genes associated with angiogenesis, proliferation and invasion in breast carcinomas. With regard to the clinical therapy of breast cancer, ERα has been proven to be the most important target over the last decades, and its overexpression in breast carcinomas is routinely used as a predictor for endocrine therapy (1, 2). Expression of ERβ has also been demonstrated in various malignomas, including breast cancer (3). Several studies demonstrated that ERβ expression is a favorable prognostic factor, correlating with low histological grading, longer disease-free survival and response to tamoxifen (4, 5). When both ERs are co-expressed, ERβ exhibits an inhibitory effect on ERα-mediated gene expression (6-8). Moreover, it has been suggested that a change of ERα/ERβ ratio during tumorigenesis is more relevant than the absolute levels of ERα or ERβ (9). This hypothesis is supported by the finding that in ER-positive breast cancers the mean ratio ERα/ERβ is higher than in normal tissue (10).

Since most breast carcinomas are, at least initially, hormone responsive, systemic endocrine therapy using selective estrogen receptor modulators (SERMs) or aromatase inhibitors (AIs) is an established strategy for adjuvant breast cancer treatment. For many years, tamoxifen has been the gold standard for the treatment of hormone-dependent breast cancer (11). Recently, clinical trials demonstrated improved antitumoral efficacy and a favorable toxicity of third-generation AIs (letrozole, anastrozole and exemestane) as compared to tamoxifen, leading to a reassessment of the optimal adjuvant endocrine therapy for postmenopausal patients with breast cancer (12-16). The molecular basis underlying the superior efficacy of AIs is still unclear.

In this study, the impact of SERMs and antiestrogens (tamoxifen and fulvestrant) as well as aromatase inhibitors (anastrozole and letrozole) on the expression of ERα and ERβ in MCF-7 breast cancer cells was analyzed. *In vitro* data suggest that differential effects on the ERα/ERβ ratio may contribute to the therapeutic superiority of aromatase inhibitors.

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Materials and Methods

Compounds. Tamoxifen and fulvestrant (Faslodex™) were purchased from Sigma (Taufkirchen, Germany) and AstraZeneca (London, UK), respectively. The non-steroidal third-generation aromatase inhibitors anastrozole (Arimidex™) and letrozole (Femara™) were obtained from AstraZeneca (London, UK) and Novartis (Basel, Switzerland). Compounds were dissolved in ethanol or PBS, where appropriate, and diluted to the required concentration. 17β-estradiol (E2) and Δ^4-androstendion were purchased from Sigma.

Tissue samples. For total RNA isolation and purification, 21 liquid nitrogen snap frozen breast tissue samples (30 - 40 mg) were homogenized (Diax 100, Heidolph Instruments, Schwabach, Germany), passed through QIA-Shredder, and processed with RNeasy reagents (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Additionally, a DNase digestion step was performed on the extracted RNA. To adjust concentrations of the RNA preparations, samples were measured at wavelengths 260 nm and 280 nm (BioPhotometer, Eppendorf). RNA was stored at –80˚C for subsequent quantitative PCR analysis.

Real-time PCR. cDNA was prepared applying the Advantage RT-for-PCR-Kit (Clontech, Heidelberg, Germany). Real-time PCR was carried out utilizing the 7300 Real-time PCR System (Applied Biosystems, Foster City, USA). For all real-time PCR reactions, standard concentration of assays and Universal TaqMan™ PCR Mastermix (Applied Biosystems) were used. After an initial activation step of 95˚C for 10 minutes, 40 PCR cycles were performed using the following conditions: Denaturation at 95˚C for 15 seconds and annealing/extension at 60˚C for 1 minute. For either gene analyzed, the respective TaqMan™ Gene Expression Assay was used (for ERα: TaqMan™ #Hs00174860_m1; for ERβ: TaqMan™ #Hs00230957_m1; for β-Actin: TaqMan™ #Hs99999903_m1). Quantification of gene expression was accomplished by measuring the fractional cycle number at which the amount of expression reached the fixed threshold (cycle threshold, Ct). Relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method after normalisation to β-actin and is expressed as relative quantification (RQ value).

Cell culture. The well-established human breast cancer cell line MCF-7 has been used for all *in vitro* experiments. MCF-7 cells were maintained in phenol-red free RPMI 1640 supplemented with charcoal-stripped 10% fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin. Cell culture media and FCS were obtained from Gibco (Karlsruhe, Germany). Cells were kept in a humidified atmosphere at 37˚C of 5% CO_2.

Incubation. For stimulation experiments with tamoxifen and fulvestrant, cells were incubated with 100 nM of the respective compound for 48 hours, applying the cell culture conditions described above. Every sample contained 10 nM 17β-estradiol either alone (control) or in combination with the compound. For experiments with aromatase inhibitors, cells were incubated with 100 nM anastrozole or 100 nM letrozole for 48 hours. Δ^4-androstendion (100 nM) as substrate for the enzyme aromatase was added to approximate physiological conditions. For stimulation experiments with estradiol, cells were incubated for 48 hours with concentrations ranging from 0.1-100 nM. Following incubation, total RNA was extracted using RNeasy-Protect Mini (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified prior to quantitative real-time PCR analysis.

Data analysis. After evaluation of mRNA expression levels, statistical analysis was performed using the SPSS 13.0 software. Student's *t*-test was used to test differences in mRNA expression. $p<0.05$ was considered statistically significant.

Results

Altered expression of ERα and ERβ during carcinogenesis. Human breast tissue samples (n=21) were analyzed for ERα and ERβ mRNA expression. Of these, 7 derived from normal breast, 8 from histological grade 1 (G1) and 6 from grade 3 (G3) carcinomas. Neither of the patients had received endocrine therapy prior to biopsy. In all breast carcinomas irrespective of their histological grade expression of ERα and ERβ was observed. Compared to mean relative ERβ mRNA expression in normal breast tissue, lower ERβ expression in breast carcinomas was found. Normal breast tissue had RQ=1, ERβ expression was at RQ=0.83 (p=0.019) in G1 tumors and at RQ=0.88 (p=0.057) at G3 tumors (Figure 1). In contrast, with respect to ERα mRNA expression no significant change was observed comparing normal breast to G1 tumors, but the reduction of ERα expression during progression from G1 to G3 tumors was significant. Compared to normal breast, ERα expression remained unchanged in G1 tumors (RQ=1.14, n.s.), but significantly declined to RQ=0.65 ($p<0.001$) in G3 tumors (Figure 1).

Short-term estradiol stimulation of MCF-7 cells does not change ER expression. Figure 2 depicts results of the quantitative PCR analysis of ERα and ERβ expression in MCF-7 cells due to incubation with increasing concentrations of estradiol. For both ERα and ERβ, 48 hours of incubation was not found to change mRNA expression to a significant extent.

ERα/ERβ ratio is increased by antiestrogens but decreased by aromatase inhibitors. In MCF-7 breast cancer cells, both tamoxifen and fulvestrant significantly increased ERα mRNA expression within 48 hours of incubation (Figure 3). Tamoxifen elevated expression of ERα mRNA to RQ=1.51 (p=0.01) and fulvestrant to RQ=1.42 ($p<0.001$). In contrast, both compounds did not significantly change ERβ mRNA expression. Due to this, treatment with tamoxifen and fulvestrant led to a relevant increase of the ERα/ERβ ratio. In contrast, treatment of breast cancer cells with non-steroidal aromatase inhibitors increased ERα mRNA expression only marginally (anastrozole) or not at all (letrozole). However, both aromatase inhibitors were found to significantly up-regulate ERβ mRNA expression with RQ=1.23 (anastrozole; p=0.029) and RQ=1.38 (letrozole; p=0.048), respectively, thus decreasing the ERα/ERβ ratio.

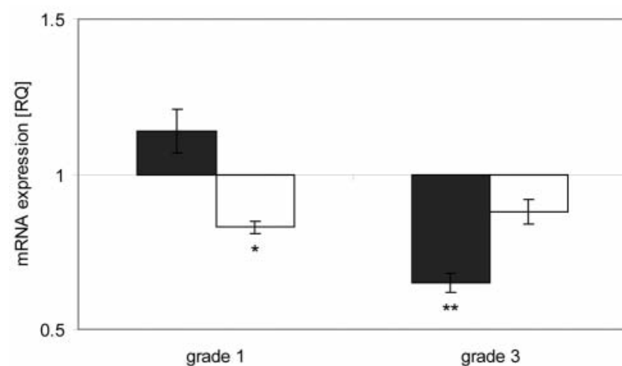

Figure 1. *Quantitative PCR analysis of ERα (■) and ERβ (□) expression in human breast tissue samples. G1, grade 1 tumor; G3, grade 3 tumor. Bars, SEM; *p<0.05; **p<0.001.*

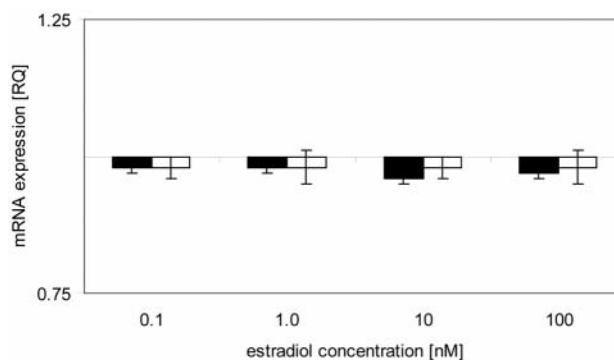

Figure 2. *Quantitative PCR analysis of ERα (■) and ERβ (□) expression in MCF-7 breast cancer cells after 48 hours of incubation with estradiol, applying a concentration range 0.1-100 nM. Columns, mean of three independent experiments; bars, SEM; *p<0.05.*

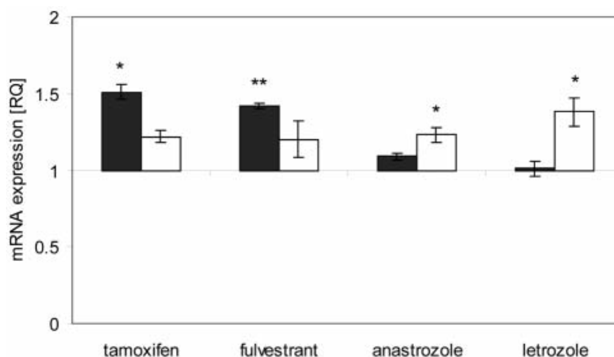

Figure 3. *Effect of selective estrogen receptor modulator tamoxifen, antiestrogen fulvestrant and aromatase inhibitors anastrozole and letrozole (all 100 nM) on ERα and ERβ mRNA expression in MCF-7 breast cancer cells, after 48 hours of incubation. (■), ERα; (□), ERβ. Columns, mean of three independent experiments; bars, SEM. *p<0.05; **p<0.001.*

Discussion

Both estrogen receptors ERα and ERβ play an important role in carcinogenesis, progression and treatment of breast cancer (10, 17, 18). While tumor promoting processes including proliferation, invasion and anti-apoptosis are mediated *via* ERα, activation of ERβ is associated with more beneficial effects (6-8). In this study, the differential ER expression in the course of malignant progression was analyzed. Furthermore, whether treatment with antiestrogens/SERMs or aromatase inhibitors would differentially modulate expression levels of both ERα and ERβ was also investigated.

Analyzing expression levels of ERα and ERβ in human breast tissue samples, the ratio ERα/ERβ was found to be significantly changed during malignant progression. Compared to mean ERβ expression in normal breast, expression levels were found to be decreased in G1 and G3 tumors (Figure 1). The difference in ERβ expression between G1 and G3 tumors was not significant. With respect to ERα expression, no significant change was observed during transition from normal breast tissue to G1 tumors. However, during progression from G1 to G3 tumors a highly significant decrease of ERα expression was demonstrated. These data are consistent with the literature, since there is evidence of a change of the ERα/ERβ ratio during breast cancer progression (19). It has been hypothesized that substantial changes of ER expression may be the major cause of a reduced estrogen responsiveness of breast cancer cells, thus leading to resistance to endocrine therapy (1). Also, loss of ERα expression and an inverse relation between ERβ expression and tumor grade have been described previously (20). The presented data indicate that the decrease of ER expression and change of ERα/ERβ ratio during malignant progression may occur in two distinct phases, namely a decline of ERβ expression, followed by the loss of ERα expression. According to these results, early steps of malignant progression (transition from normal breast to well-differentiated G1 tumors) are characterized by a decline of ERβ expression that during further progression remains constant. In contrast, loss of ERα expression occurs not until advanced de-differentiation, marked by the transition from well-differentiated G1 to poorly differentiated G3 tumors.

Prior to evaluating the impact of antiestrogens and aromatase inhibitors on the ERα/ERβ ratio, the short-term effect of estradiol on ERα and ERβ mRNA expression in MCF-7 breast cancer cells was investigated. As depicted in Figure 2, 48 hours of exposition to 0.1-100 nM estradiol did not change expression of ERβ. Concordantly, no effect on ER expression could be observed due to short-term (48 hours) estradiol deprivation (data not shown). In contrast, long-term effects of estradiol stimulation/deprivation on ER expression in breast cancer cells have been described previously (21). However, the lack of short-term estradiol effects on ER expression as presented here is relevant for subsequent stimulation of MCF-7 cells with antiestrogens and aromatase inhibitors, since these

compounds were capable of significantly changing ER expression in that very period of time.

As depicted in Figure 3, both tamoxifen and fulvestrant were found to significantly increase ERα mRNA expression within 48 hours. The expression level of ERβ remained stable, thus leading to a significantly increased ERα/ERβ ratio. Contrary to antiestrogens, both aromatase inhibitors anastrozole and letrozole decreased the ERα/ERβ ratio by significantly up-regulating ERβ expression (Figure 3). In the literature, controversial data on the effect of tamoxifen on ERα and ERβ expression have been reported; both increased and decreased ERα expression after tamoxifen stimulation have been described (22, 23). Conflicting data have been published for fulvestrant, too; some groups reported decreased ERα expression in breast cancer patients treated with fulvestrant (24, 22), while others observed elevated expression of ERα (25). The effects of aromatase inhibitors on ER expression have been previously investigated in breast cancer *ex vivo*, immunohistochemically comparing pretreatment tissue expression levels with those after treatment with aromatase inhibitors. In these studies, no consistent effects of anastrozole and letrozole on ER protein expression have been observed (26-28).

This study is the first to focus on the biologically relevant ratio ERα/ERβ rather than on the investigation of the antiestrogens' and aromatase inhibitors' effects on a single estrogen receptor but to focus on the biologically relevant ratio (Figure 4). Due to the tumor promoting effects of ERα-activation and antitumoral properties of ERβ, the finding that the ratio ERα/ERβ is increased by tamoxifen and fulvestrant is associated with prognostical and therapeutical unfavorable relevance. Furthermore, up-regulation of ERα expression in response to treatment with antiestrogens has been hypothesized to be a relevant cause of acquired resistance to endocrine treatment of breast cancer (29). In contrast, increased expression of ERβ caused by aromatase inhibitors may contribute to the therapeutical superiority of aromatase inhibitors, since ERβ inhibits ERα-mediated tumor promoting gene expression (6-8) and represents a favorable prognostic factor (4, 5). These results are the first to describe such *in vitro* effects of anastrozole and letrozole. Interestingly, in the present study the aromatase inhibitors exhibited their ERβ up-regulating effect in MCF-7 cells, which do not over-express aromatase but show expression only at low but detectable levels. This experimental setting resembles the situation in vivo and therefore further underlines the clinical relevance of these findings since it is well-known that less than 50% of all breast carcinomas over-express aromatase (30). In adjuvant systemic treatment of postmenopausal breast cancer patients, aromatase inhibitors are administered without consideration of the patients' tumoral aromatase expression status to prevent estrogen synthesis from androgen precursors by the enzyme aromatase localized predominantly in fat tissue. Due to the data presented in this study, patients with estrogen dependent breast carcinomas may benefit from aromatase inhibitor treatment not solely because of systemic reduction of estradiol but also because of up-regulation of ERβ expression within the tumor tissue itself.

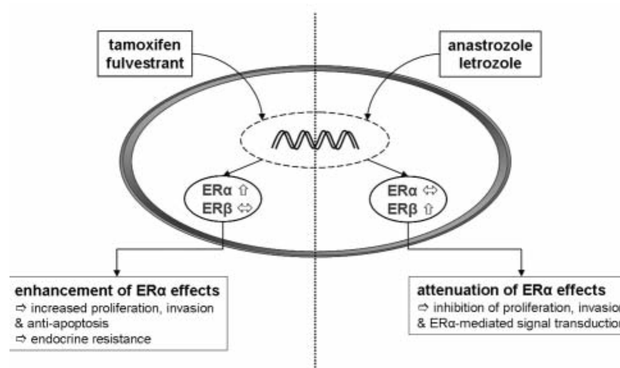

Figure 4. *Differential effects of antiestrogens/SERMs (LEFT) and aromatase inhibitors (RIGHT) on the ratio ERα/ERβ in breast cancer cells. Tamoxifen and fulvestrant enhance ERα effects by increasing the ratio ERα/ERβ; anastrozole and letrozole attenuate ERα effects by decreasing the ratio ERα/ERβ.*

Taken together, these results implicate that decrease of ER expression and change of the ERα/ERβ ratio during malignant progression occurs in two steps. With transition from normal breast tissue to grade 1 carcinomas, expression of ERβ decreases. Further de-differentiation from grade 1 to grade 3 carcinomas is then characterized by loss of ERα expression. Tamoxifen and fulvestrant were found to up-regulate ERα expression and to leave ERβ expression unchanged, while effects of anastrozole and letrozole were exactly the opposite. The fact that tamoxifen and fulvestrant increase the ratio ERα/ERβ whereas aromatase inhibitors decrease it may contribute to the superior efficacy of aromatase inhibitors in breast cancer therapy.

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