Effects of the Antiestrogens Tamoxifen and Raloxifene on the Estrogen Receptor Transactivation Machinery

MICHAEL GLAESER[1], DIETER NIEDERACHER[1], SIRUS DJAHANSOUZI[1], BETTINA HANSTEIN[1], RALF DITTRICH[2], MATTHIAS W. BECKMANN[2], PETER A. FASCHING[2] and SVEN ACKERMANN[2]

[1]*Department of Gynecology and Obstetrics, Heinrich-Heine-University, Duesseldorf;*
[2]*Department of Gynecology and Obstetrics, University Hospital Erlangen, Erlangen, Germany*

Abstract. *The influence of 17β-estradiol (E2), tamoxifen (TAM) and raloxifen (RLX) on the proliferation of breast (BC) and endometrial carcinoma cell lines (EC) and the expression of different compounds of the estrogen receptor (ER)-transactivation machinery were studied. E2 stimulated the proliferation of BC, but had no effect on the EC. TAM showed a biphasic effect on ER-positive cell lines. RLX showed an antagonistic suppression or no effect. The expression of ERα was down-regulated by E2, but not affected by selective estrogen receptor modulators.* ERβ *and progesterone receptor expressions were up-regulated by E2, TAM and OHT. This supports the hypothesis that* ERβ *expression is also regulated via the ERα-pathway. The steroid receptor coactivator (SRC) AIB1 expression was slightly decreased by E2 but not by antiestrogens (antiE). TIF2 expression was increased by E2, TAM and RLX, but SRC-1 expression was not. In comparison, the expressions of* ERβ *and progesterone receptor were strongly influenced by antiE, while the expression of SRCs was only slightly affected.*

The sexual steroid hormones estrogen and progesterone play an important role in the development, differentiation and function of normal breast and endometrial cells. Steroid hormones have certain effects in the development and growth of breast (BC) and endometrial carcinomas (EC), but the basis of these effects remains unclear (1). These effects may be indirect *via* a mitogenic stimulus or direct *via* activation of oncogenic pathways. The effects of estradiol are mediated by specific receptors, estrogen receptor α (ERα) and β (ERβ). The classical pathway of ER-mediated transactivation includes a ligand-dependent formation of homo- or heterodimers, binding to specific DNA elements (estrogen responsive element = ERE) in the promoter region of estrogen-responsive genes, recruitment of coactivators and transactivation of target gene expression (*e.g.,* progesterone receptor [PR] expression) (2). Alternative pathways of estrogenic action are *via* the AP-1 signal cascade (3) or protein-protein-interaction (4).

The selective estrogen receptor modulators (SERMs) tamoxifen (TAM) and raloxifene (RLX), and their derivatives 4-hydroxy-tamoxifen (OHT) and raloxifene-hydrochloride (RLX-HCl), inhibit ER-mediated transcriptional activation by competitive blocking of the ER ligand binding domain (5). SERMs are mixed antiestrogens with partial agonistic and antagonistic potential upon ERα and ERβ (6, 7). The tissue-specific differences of SERM action are believed to be due to diverse effects on the activation functions of ERs (5). The agonistic potential of TAM is reported to be mediated by the activation function-1 (AF-1) (8), while the antagonistic effect is caused by repression of the activation function-2 of ERα (9). Occasionally, the different responses of ERα and ERβ to antiestrogens are caused by the existence of an AF-1 in ERα, but not ERβ (6). Alterations in the components of the ER transactivation complex, concerning the ER-subtype (hetero- or homodimer) and the recruitment of coactivators, caused by the binding of a different ligand, may result in the different estrogenic potential of the complex. As major compounds of the ER transactivation complex, steroid receptor coactivators (SRCs) SRC1 (10), TIF2 (also GRIP1) (11, 12) and AIB1 (also RAC3, TRAM-1, ACTR, p/CIP) interact with ERs and enhance ER-mediated transcriptional activation (13-17). Overexpression of AIB1 or SRC1 increased ER-mediated transactivation (10, 13) and exhibited carcinogenic potential by stimulating cell proliferation, as shown for BC cell lines (18). Receptor-ligand complexes with different conformations have

Correspondence to: Dr. R. Dittrich, Department of Gynecology and Obstetrics, University Hospital Erlangen, Universitätsstraße 21-23, 91054 Erlangen, Germany. Fax: 0049-9131-853-3552, e-mail: ralf.dittrich@gyn.imed.uni-erlangen.de

Key Words: 17β-Estradiol (E2), SERM, breast, endometrium, tamoxifen, raloxifene.

Table I. *Steroid receptor status of breast and endometrial carcinoma cell lines determined by qdPCR and immunohistology.*

		ER-α		ER-β		PR	
		qdPCR	IH	qdPCR	IH	qdPCR	IH
EC cell lines	Ishikawa	+	+	+	n.c.	+	+
	KLE	–	–	–	n.c.	–	–
	RL-95-2	+	(+)	+	n.c.	+	+
BC cell lines	MCF-7	+	+	+	n.c.	+	+
	T47D	+	+	+	n.c.	+	+
	BT-20	–	–	+	n.c.	–	–

+ = positive, (+) = slightly positive, – = negative.

different affinities to SRCs, leading to altered transactivation enhancement by these complexes. SRCs are limiting factors to ER-mediated transactivation since ERs compete with other steroid receptors and transcription activators for coactivator binding (10, 19). This limitation leads to mutual squelching of steroid receptor-mediated transactivation. Increased expression of SRCs, caused by antiestrogenic treatment and increased availabillity for incorporation in the transactivation complex, would, therefore, lead to an enhanced ER-mediated transactivation, resulting in an increased cell proliferation, thus providing a mechanism with carcinogenic potential.

At present TAM treatment is the most frequently applied endocrine therapy against BC. As a negative side-effect, TAM increases the risk of EC (1). Clinical trials on RLX reported positive estrogenic effects on bone fractures, the cardiovascular system and lipoprotein concentration (20, 21). Therefore, RLX is indicated for osteoporosis prevention and the positive side-effect of reducing the BC risk. In contrast to TAM, no estrogenic effects in the endometrium were observed under RLX treatment (20, 22).

In this study, the question of whether these effects are caused by a different expressions of the steroid receptors and steroid receptor coactivators was examined. The effects of TAM and its derivative OHT, and RLX and RLX-HCl on the expression of steroid receptors and SRC in the BC cell line MCF7, which is responsive to hormonal treatment (23), were compared. PR expression was used as a reporter gene to show possible response to the estrogenic effects of these antiestrogens. Further, the effects of the SERMs on the cell proliferation of ER-positive and -negative BC and EC cell lines were analyzed to determine their estrogenic and antiestrogenic potential.

Materials and Methods

Cell lines. EC cell lines (Ishikawa, RL-95-2, KLE) and BC cell lines (MCF7, T47D and BT-20) were obtained from ATCC (Rockville, MD, USA). The culture conditions were applied, as recommended by ATCC. The ERα, ERβ and PR status of the cell lines indicated by ATCC was determined by immunohistochemical staining, as described previously (24), as well as quantitative RT-PCR (Table I).

Cell proliferation of all cell lines.
Kinetics: For analysis of cell proliferation, the BC cell lines MCF7, T47D and BT-20 and EC cell lines Ishikawa, KLE and RL-95-2 were grown in 96-well plates ($1x10^5$ cells per well) on phenol red-free media containing 10% fetal calf serum treated with dextran-coated charcoal. The cell lines were incubated with 17β-estradiol (10^{-8} M) (E2), TAM, OHT, RLX and RLX-HCl (10^{-6} M each) for 2, 4 and 6 days. The media containing (anti-) estrogens were renewed every 2 days. 0.1% ethanol (solvent) was used as the control for normal cell proliferation.
Determination of cell proliferation: After incubation with (anti)estrogens, cell proliferation was analyzed by the Cell Proliferation Kit I (MTT, Roche Diagnostics, Mannheim, Germany) and measured in a multiwell ELISA reader. The relative degree of cell proliferation was determined by the ratio: $n = A_H / A_C$ with A_H = absorbance by hormonal-incubated cells and A_C = absorbance by control cells incubated with 0.1% ethanol. Values were determined 2-4 times in 3 independent experiments.

Expression kinetics under hormonal stimulation in breast cancer cells. The kinetics of steroid receptor and steroid receptor coactivator expressions during incubation with (anti-)estrogens were examined in the ERα- and β-positive and PR-positive BC cell line MCF7, which is responsive to hormonal treatment (23). Cells ($2.5x10^5$) were plated in 6-well plates and maintained on phenol red-free media containing 10% dextran-coated charcoal-stripped fetal calf serum (CCS-FCS) for 24 h. For stimulation kinetics, each cell line was incubated with physiological concentrations of E2 (10^{-8} M), TAM (10^{-6} M), OHT (10^{-6} M), RLX (10^{-6} M) and RLX-HCl (10^{-6} M) for 4, 8, 12, 24 and 48 hours. For RNA extraction, the cells were washed with 5 ml phosphate-buffered saline (PBS) and directly suspended in 1 ml Trizol (Gibco BRL, Gaithersburg, MD, USA).

RNA extraction. The suspension was mixed with 200 μl chloroform, incubated for 2-3 min at room temperature (RT) and centrifuged for 15 min at 12000 xg and 4˚C. The supernatant was incubated with 500 μl isopropanol for 10 min at RT and centrifuged for 10 min at 12000 xg and 4˚C. The precipitated RNA was washed with 70% ethanol, dried at 55˚C and dissolved in diethylpyrocarbonate (DEPC)-treated distilled water.

Table II. *Optimized PCR conditions for quantification of expression of steroid receptor and steroid receptor coactivators.*

gene	primer (5'->3')	c(dNTPs) [M]	m(primer target) [pmol]	m(primer-reference) [pmol]	cycle numbers (target/ reference)	single step duration (denaturing/ annealing/elongation)
ERa	Cy5-GGAGACA TGAGAGCTGCCAAC CCAGCAGCATGTCGAAGATC	150	30	25	35/27	45 sec / 1 min / 1 min
PR	AAAGTGCTGTCAGGCTGGC Cy5-A TCACA TCTGGTTCAA TGCTC	150	30	30	32/27	45 sec / 1 min / 1 min
AIB1	Cy5- T ACTCTGTCA TCACCAGGCC GTCACTGAGGTGATCTCTGC	150	30	20	32/32	1 min / 1min / 1min
SRC1	Cy5-CTCTCA TCCACTGACCTTCTC TTGTTATTCAGTCAGTAGCTG	150	35	30	34/27	1 min / 1 min / 1 min 30 sec
TIF2	Cy5-GT A TTCAGAAGTTCCA TGCGC GTTTGTCCAGTCAGATCCGG	150	30	30	29/29	45 sec / 1 min / 1 min 15 sec
ER	TGCTTTGGTTTGGGTGATTGC Cy5- TTTGCTTTT ACTGTCCTCTGC	150	35	20	40 /27	1 min /1 min /1 min

cDNA synthesis. For cDNA synthesis, 5 μg of cell line RNA were denaturized at 90˚C for 5 min and cooled on ice. The cDNA synthesis mix contained 1x *Taq* polymerase reaction buffer (Pharmacia, Freiburg, Germany), 1 μl random-primer (Pharmacia), 125 mM $MgCl_2$, 20 mM of dATP, dCTP, dGTP and dTTP each, 0.4 M DTT, 2.91 mg/ml BSA, 0.5 μl RNasin (Promega, Madison, USA) and 0.5 μl Reverse Transcriptase (Pharmacia). The cDNA synthesis conditions were 10 min at 25˚C, 45 min at 42˚C and 5 min at 95˚C. The cDNA was diluted 1:5 in DEPC-treated distilled water. Two μl of diluted cDNA were subjected to PCR.

PCR conditions. The expressions of the steroid receptors ERα, ERβ and PR and steroid receptor coactivators SRC1, AIB1 and TIF2 in the BC cell line MCF7 were determined in comparison to the reference gene *β-Actin* as an internal standard. Primers for detection of *β-Actin* cDNA were 5'-F-ATTTGCGGTGGA CGATGGAG-3' (F=fluorescence [Cy5-] labelled) and 5'-AGAGATGGCCACGGCTGCTT-3', resulting in a 442 bp PCR product. Target and reference genes were co-amplified in multiplex PCR reactions (25) using different numbers of cycles for the reference and the target gene according to the "primer-dropping" method (26). *β-Actin* primers were "dropped-in" to be amplified in 27 cycles. For each target gene, the PCR conditions were optimized in terms of dNTP (150 μM in all reactions) and primer concentrations as well as duration of denaturation, annealing and elongation and cycle numbers for target and reference gene amplification. The primers were designed with an optimal annealing temperature of 58˚C (Table II). The PCR reactions contained 2 μl of 1:5-diluted cDNA, 150 μM dNTPs, 1xPCR buffer, 2.5 U *Taq* DNA polymerase (Pharmacia) and optimized amounts of target and reference gene primers in 50 μl reaction volume. The PCR reactions were started with a 10-min denaturation at 94˚C, prior to cycles of the optimized PCR conditions, followed by a final extension for 8 min at 72˚C.

PCR product analysis. Fluorescence-labelled products of multiplex RT-PCRs were analyzed on an automated fluorescent DNA sequencer (A.L.F., Pharmacia). The PCR products were measured in dilutions of 1:3, 1:8 and 1:15. 5.5 μl of the dilutions were mixed with 5.5 μl of stop solution (90% formamide, 10 mM EDTA, 0.3 % bromphenol blue). The mixture was heated for 5 min at 95˚C, cooled for 5 min on ice and loaded on a denaturing 6% polyacrylamide gel. The gels were run for 5 h at 38 W. Fluorescence-labelled DNA fragments were detected by laser activation. The data, automatically collected during the electrophoresis, were calculated using Allele links™ software (Pharmacia), which yields quantification of results in terms of peak size, height and area under the curve (25). Target gene expression (E_T) was determined in comparison to *β-Actin* expression in the equation: $E_T = P_T / P_\beta$ with P_T = peak area of the target gene signal and P_β = peak area of the *β-Actin* signal. Relative expression values (REV) were determined comparing the target gene expression in hormone-incubated cells with the expression in unstimulated control cells: REV = E_T (incubated) / E_T (control). The expression values were determined 3-4 times in 3 independent experiments.

Results

Effects of (anti-)estrogens on the cell proliferation of BC and EC cell lines. The proliferation of the BC cell lines MCF7, BT-20 and T47D was analyzed under treatment with estrogen and antiestrogens. Estradiol stimulated the cell proliferation of all 3 BC cell lines tested. Stimulation of the cell proliferation of the ER- and PR-positive cell line MCF7 was used as a control for responsiveness to this hormone, as described previously (23, 27). On incubation with E2, the MCF7 cell number increased up to 170% compared with untreated cells (after 6 days). In the ER-positive BC cell line, T47D cell proliferation was stimulated to nearly the same extent. The ER-/PR-negative cell line BT-20 showed the highest increase of cell number after 2 and 4 days of incubation, but the cell number dropped to the level of untreated control cells after 6 days of treatment (Figure 1a).

The antiestrogen TAM inhibited the cell proliferation of all 3 BC cell lines tested. While the number of BT-20 cells

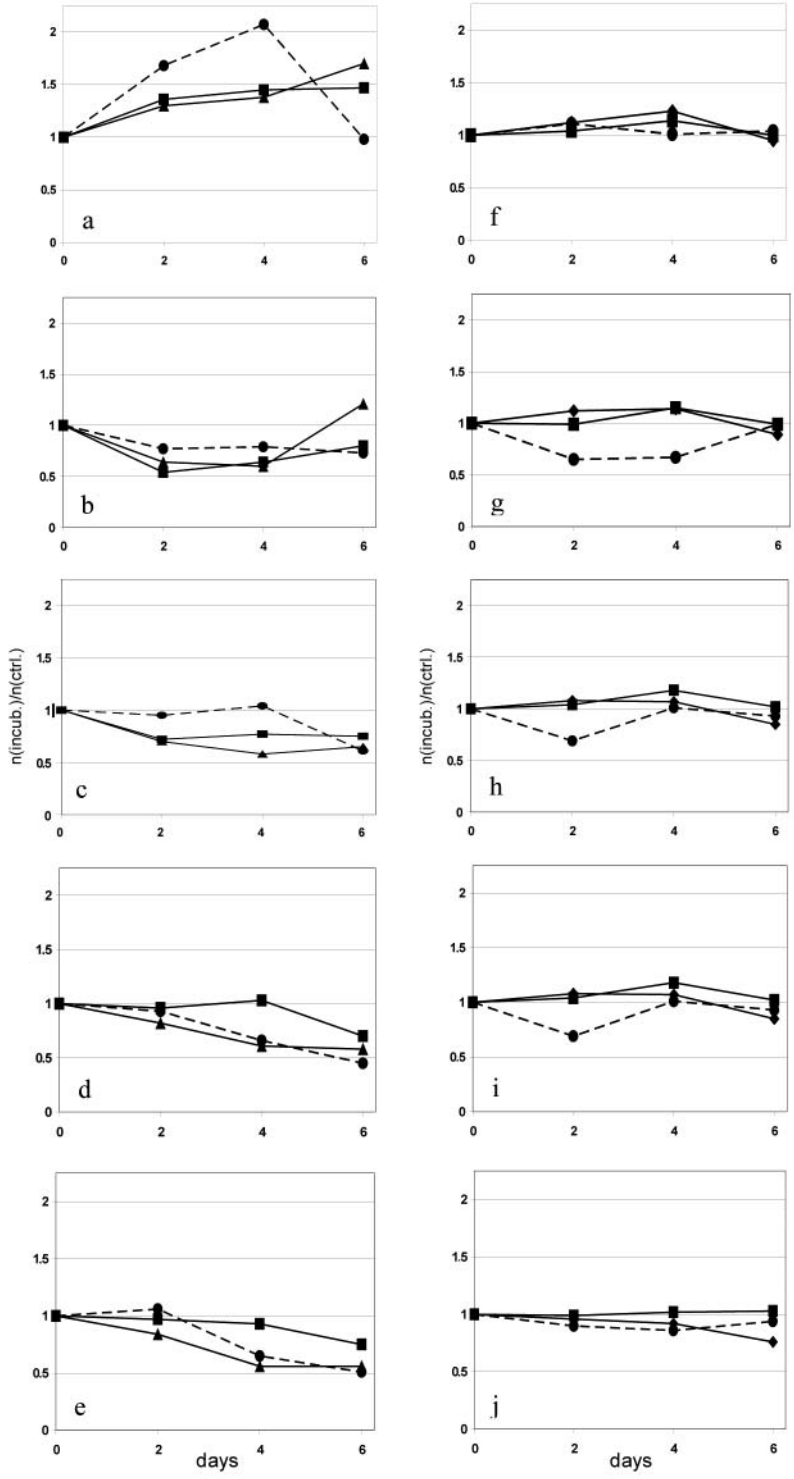

Figure 1. *Proliferation kinetics of breast (left, ● = BT-20, ■ = T47D, ▲ = MCF7) and endometrial (right, ● = Ishikawa, ■ = RL-95-2, ▲ = KLE) carcinoma cell lines. Cell lines were incubated with 10^{-8} M 17b-estradiol (a,f), 10^{-6} M tamoxifen (b,g), 10^{-6} M 4-OH-tamoxifen (c,h), 10^{-6} M raloxifene (d,i) and 10^{-6} M raloxifene-hydrochloride (e,j) for 2, 4 and 6 days. Cell proliferation was determined using the MTT cell proliferation kit (Roche).*

remained at a decreased level (73% of number of non-incubated control cells after 6 days), the MCF7 cell number increased after 2 days (60%) of incubation with TAM up to 121% compared to untreated cells after 6 days. This stimulatory effect was also found in T47D cells, but to a lesser extent. After a maximum decrease of cell number on the second day of incubation (64%), a slight increase was observed at the following time-points (up to 80%), but it

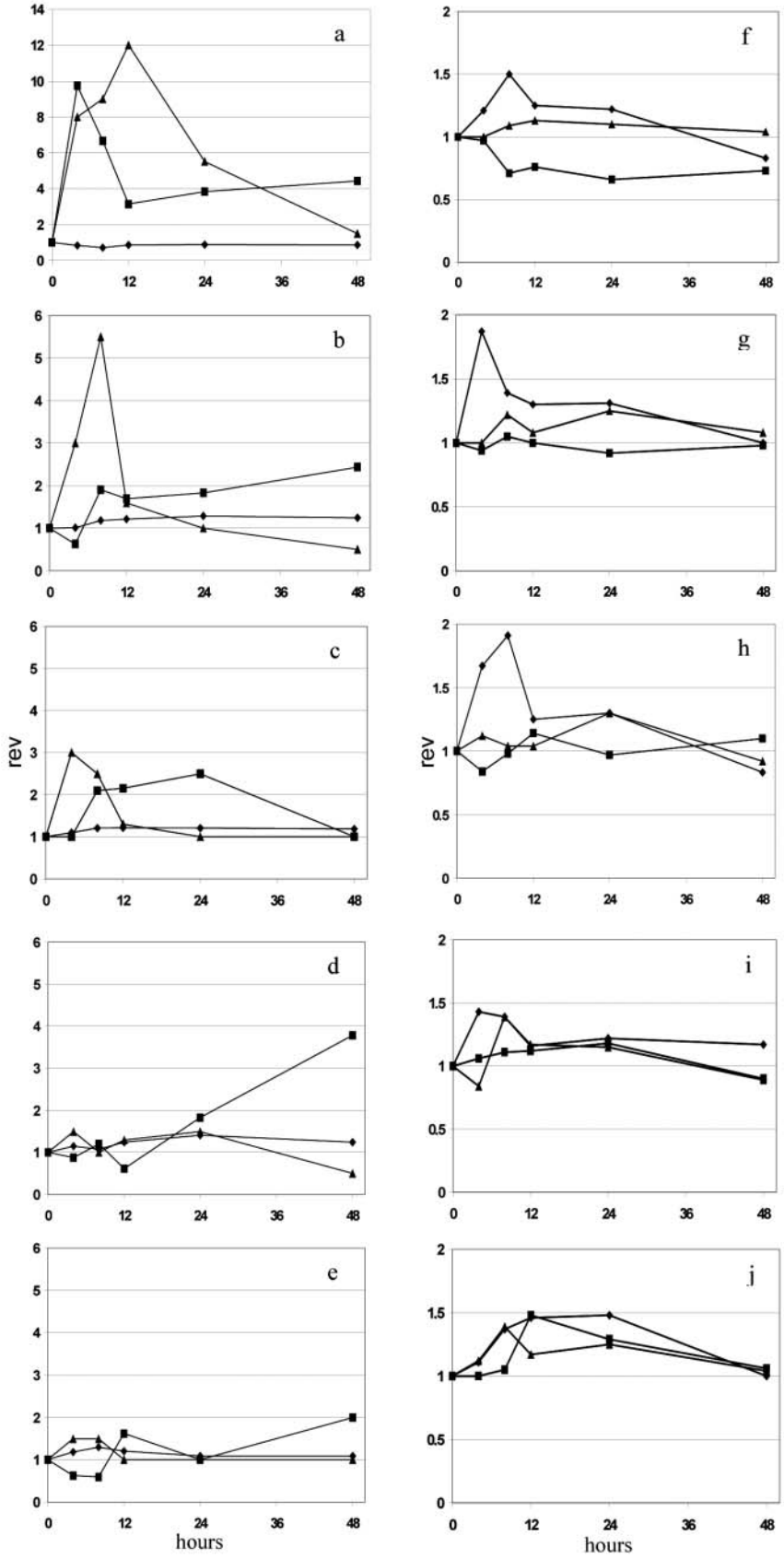

Figure 2. *Kinetics of steroid receptor (left, ◆ = ERa, ■ = ERb, ▲ = PR) and steroid receptor coactivator (right, (◆ = TIF2, ■ = AIB1, ▲ = SRC) expression in the breast cancer cell line MCF7 is demonstrated by the relative expression values (REV, y-axis) after 4, 8, 12, 24 and 48 hours of treatment with (a,f) E2, (b,g) TAM, (c,h) OHT, (d,i) RLX, (e,j) RLX-HCl.*

remained below control level (Figure 1b). With OHT incubation, the cell proliferation of MCF7 and T47D was suppressed (down to 58% and 72%) and remained at this decreased level. Proliferation of BT-20 cells was inhibited after 4 days (61% after 6 days) (Figure 1c).

RLX and RLX-HCl decreased the cell number of all 3 BC cell lines. Here, early suppression was much weaker compared to TAM- and OHT-treated cell lines. Late suppressive effects were distinct (MCF 7: 61% [RLX] and 56% [RLX-HCl] after 4 days; T47D: 70% and 75% after 6 days; 66% and 65% after 4 days) and remained at a reduced level (Figure 1 d,e).

In contrast to the increased cell proliferation observed during treatment with estrogen, incubation with the antiestrogens OHT, RLX and RLX-HCl caused a decrease in the cell proliferation rate of the BC cell lines MCF7, T47D and BT-20. A suppressive effect of TAM was apparent after 2 days of incubation, which changed to a relative stimulation of proliferation in the ER-positive cell lines MCF7 and T47D.

The cell proliferation of the EC cell lines Ishikawa, KLE and RL-95-2 was analyzed under the same conditions as for the BC cell lines. The ER-/PR-positive cell lines RL-95-2 and Ishikawa and the ER-/PR-negative cell line KLE were not influenced by incubation with estradiol showing only a slight stimulation (Figure 1f). While TMX and OHT did not affect the growth of RL-95-2 and KLE cells, both compounds had a suppressive effect on the proliferation of Ishikawa cells after 2 days, followed by an increase, similar to that seen with the BC cell lines MCF7 and T47D (Figure 1 g,h). In comparison, RLX and RLX-HCl did not alter the cell proliferation of the 3 EC cell lines tested (Figure 1 i,j).

While the proliferation of the BC cell lines was regulated by E2, TAM, OHT, RLX and RLX-HCl, these agents had no effect on the proliferation of the EC cell lines except the Ishikawa cell line.

Effects of (anti-)estrogens on the expression of steroid receptors. For analysis of the effects of (anti-)estrogens on the expressions of steroid receptors and steroid receptor coactivators, their expression levels in the estrogen-responsive BC cell line MCF7 were determined. The expression values were measured after 4, 8, 12, 24 and 48 hours of incubation with E2, TAM, OHT, RLX and RLX-HCl.

E2 had only a minor effect on ERα expression, decreasing the REV down to 71% of normal ERα expression (REV[ERα/E2/8h]=0.71) (Figure 2a). The expression of the PR was clearly up-regulated (up to 12-fold) by E2, demonstrating an estrogen-dependent enhancement of gene expression. The expression levels of ERβ were elevated by E2 to the same extent (~10-fold) as the PR. This result indicates that the ERβ expression is stimulated by E2 treatment in MCF7 cells.

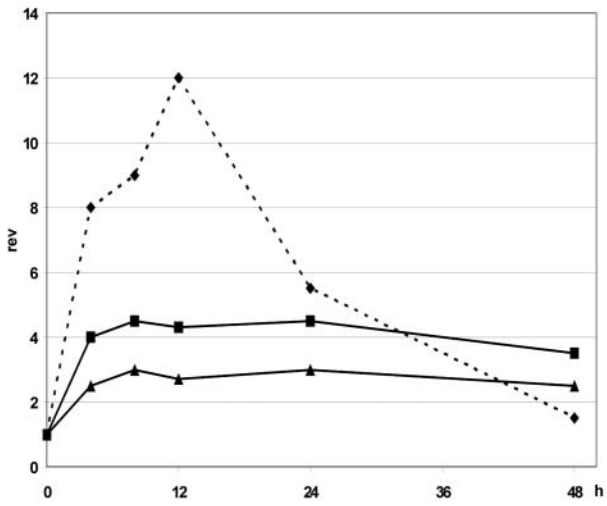

Figure 3. *Kinetic of progesterone receptor expression under incubation with E2 (◆) and co-incubations with E2 + TAM (■) and E2 + RLX (▲).*

TAM and OHT showed no significant effect on ERα expression. The REVs measured were all >1 (up to 1.41). The ERβ mRNA expression level was increased by TAM (up to 2-fold) and OHT treatment (up to 2.5-fold). The PR expression level showed a 5.5-fold rise by TAM and a 3-fold rise by OHT (Figure 2 b,c).

RLX treatment had a late suppressive effect on the expression of the PR, reducing its level down to 50% compared to untreated MCF7 cells. The ERα expression was not altered under RLX treatment, while the ERβ expression showed a response to RLX treatment. After 12 hours of treatment, the ERβ expression level was down-regulated to 62% of normal expression, but this suppression reversed into an increase of expression after 24 and 48 hours (3.9-fold) (Figure 2d). These two phases are possibly due to diverse direct or indirect mechanisms. This also indicates two different regulation mechanisms by which RLX influences ERβ and PR expression levels. Treatment with RLX-HCl showed the same suppression (60% of expression without hormonal treatment), followed by an increase of ERβ expression (2-fold after 48 hours) (Figure 2e).

To analyze the antiestrogenic potential of the SERMs, MCF7 cells were coincubated with E2 (10^{-8} M) and TAM (10^{-6} M) or RLX (10^{-6} M) (Figure 3). Coincubation with SERMs led to a suppression of the primary estrogenic stimulation (~12-fold) of PR expression (TAM: 37.5%; RLX: 25% of E2-stimulated PR mRNA level). Although TAM reduced the stimulative effect of E2, the PR expression was still increased compared to basic levels, reaching the same maximal level as observed with TAM treatment only. After 48 hours of coincubation with E2 and TAM, the PR expression was still elevated, obviously due the stimulative

effects of E2. RLX cotreatment did not block E2 stimulation of PR expression to its full extent, but lowered the E2-stimulated expression to only a 3-fold increase. Even this 3-fold stimulated expression remained at this level during the 48 hours of the experiment.

Effects of (anti-)estrogens on steroid receptor coactivator expression. The expression levels of the steroid receptor coactivators SRC-1, TIF2 and AIB1 were analyzed under the same conditions as described for the steroid receptors. E2 treatment of MCF7 cells had no effect on the expression level of SRC-1. TIF2 expression showed a slight increase (1.5-fold) after 8 hours of hormonal treatment. AIB1 expression was slightly down-regulated (66% of basic expression level after 24 hours) (Figure 2f).

Treatment of MCF7 cells with TAM and OHT did not alter the mRNA levels of SRC-1 and AIB1. In contrast, TIF2 expression was slightly increased under treatment with TAM (1.9-fold after 4 hours) and OHT (1.9-fold after 8 hours) (Figure 2 g,h).

In contrast to these effects observed under TAM and OHT treatment, incubation with RLX and RLX-HCl showed no effect on the expressions of the steroid receptor coactivators. The expression levels of SRC-1, TIF2 and AIB1 remained in the range of basic expression levels during RLX and RLX-HCl treatment (Figure 2 i,j).

Discussion

In hormone-sensitive tissues like breast and endometrium epithelium, estrogens play an important role in the development and progression of tumors. In breast tumors, cell proliferation stimulated by estrogens can be blocked by antiestrogenic therapy. Only tumors with positive steroid receptor status are responsive to this kind of therapy. For ER-positive breast tumors, TAM is the most common endocrine therapy (28). Although RLX has the same antiestrogenic potential on estrogen-dependent breast tumors, it is restricted as a preventive therapy against osteoporosis with the positive side-effect of not stimulating cell proliferation in the normal endometrium (20).

To elucidate the regulation mechanisms of SERMs, we analyzed their effects on the expression of the ER transactivation complex, steroid receptors and steroid receptor coactivators. Up-regulation of an estrogen-responsive gene by E2 was confirmed for the expression of the PR as previously reported (29). On the other hand, ERα expression was slightly down-regulated by E2 and confirmed previous reports with down-regulation of ERα expression by E2 in MCF7 (29-33). The effect may be due to auto-regulation by ERα binding to a specific inhibitory element in the promoter region of its own gene (33). This inhibition is mediated by all three promoters (34) and even the ERα protein level is decreased by this down-regulation of ERα mRNA levels (35). The low level of decrease found in our study might have been caused by differences in cell culture experiments (*e.g.,* hormone concentration, number of cell line passages before the experiment, serum-used).

ERβ expression was up-regulated by E2, probably mediated by an ERα-dependent mechanism. As ERβ was found to be a regulator of ERα transactivation (36), up-regulation of ERβ by ERα would represent a mechanism to alter and maybe limit ERα-mediated transactivation in response to estrogenic treatment. Formation of ERα-ERβ-heterodimers instead of ERα-ERα-homodimers is the crucial step in this scenario. ERβ would, therefore, play an important role in the control of estrogenic responses and the carcinogenic potential of (anti-)estrogenic agonism. Up-regulation of ERβ levels by estrogens might also lead to a responsiveness to estrogenic treatment despite a putative irresponsiveness due to a negative ERα status. This would underline the importance of a routinely analyzed ERβ status/expression in pathological diagnostics before and during hormonal therapy. Leygue *et al.* (37) reported, that in ER-positive breast tumors, the ERα:ERβ ratio was significantly higher than in normal breast tissue, while the expression of ERα and ERβ did not correlate in human breast tumors (38). In ovary cancer tissue, ERβ was also found to be decreased compared to normal ovaries (39). In hormone-independent tumors, a putative ERα-ERβ-control mechanism can be repressed supporting estrogen-stimulated carcinogenesis. Taken together, the up-regulation of ERβ and auto-regulation of ERα in response to E2 seem to be the mechanisms that control the extent of ERα-mediated transactivation. In further studies, the mechanism of ERβ expression regulation should be clarified, analyzing the promoter region in order to find putative regulative DNA elements (ERE, AP-1 elements).

TAM and its active metabolite OHT did not differ in their effect on steroid receptor expression, showing no effect on ERα expression. This was in accordance with observations by Saceda *et al.* (30, 31), but contradictory to data published by Davis *et al.* (32). The agonistic potential of TAM and OHT was demonstrated by its up-regulation of PR expression. The estrogenic effects of TAM/OHT were found to be mediated by the ERα-ERE-pathway depending on the cell type and ERE-promoter context (40). Levels of up-regulation by TAM/OHT in our experiments were lower than under E2 treatment. This is supposed to be caused by the fact that TAM/OHT agonist functions *via* the constitutively active AF-1 of ERα (41). AF-1 is weaker than AF-2, which is blocked by TAM/OHT (42). Compared to PR expression up-regulation, TAM/OHT showed only weak effects on ERβ expression, possibly indicating 2 different mechanisms of expression regulation. Coincubation of SERMs with E2 demonstrated the antagonistic potential of the agents suppressing E2-stimulated expression of the progesterone receptor.

In contrast to TAM, RLX and RLX-HCl had no effect on steroid receptor expressions. A low agonistic effect of RLX observed in reporter gene assays by Watanabe *et al.* (40) seems not to be sufficient to activate the target gene (*e.g.,* ERβ, PR) under physiological conditions.

As the balance between intracellular ERs and steroid receptor coactivators is important for transcriptional activation in response to (anti-)estrogens, we analyzed the impact of (anti-) estrogens on the expression of SRCs. Despite the fact that the structures of SRCs are very similar (43), they have different physiological roles (44, 45). SRCs are a limiting factor necessary for efficient ER transactivation (10). Squelching of nuclear receptors is reported to be caused by titration of coactivators (46). Squelching is a mechanism of regulation for steroid receptor action depending on a limited availability of SRCs, since SRC overexpression is supposed to enhance steroid receptor transactivation (13, 18). Influences on the regulation of coactivator expression, therefore, would alter the response to (anti-) estrogens and may have carcinogenic potential, *e.g.,* by stimulating cell proliferation. Another regulation mechanism depending on SRC expression levels is the balance between the levels of coactivators and levels of corepressors, as reported for a direct competition between the corepressors RIP140 and SRC-1 (47). The value of SRC-1 in the response of tumors to TAM was reported by Berns *et al.* (48), suggesting that high levels of SRC-1 indicate a favorable response to TAM of patients with recurrent BC. Our findings that SRC-1 expression is not influenced by (anti-)estrogens supports the importance of a constant expression level of this coactivator in these regulation mechanisms. In contrast to SRC-1, AIB1 was slightly down-regulated by E2. It has to be ascertained whether this minor effect is due to a direct inhibition of *AIB1* gene expression or to a indirect secondary effect. Another minor effect observed was that TIF2 expression was slightly increased under TAM and OHT treatment, while it was not affected by E2 or RLX treatment. The fact that this effect was observed with TAM/OHT treatment, but not under E2 treatment, may be a hint that TIF2 is especially important for AF-1 function effected by TAM/OHT and might be due to cell proliferation enhanced by TAM/OHT treatment.

In contrast to TAM/OHT, RLX and RLX-HCL treatment had no effect on the expression of coactivators SRC-1, TIF2 and AIB1, again showing no influence on the mechanisms regulating estrogen-dependent actions.

In this study, we showed that cell proliferation in BC cell lines is affected by TAM and RLX. While antagonistic effects by RLX were consistent, TAM treatment finally resulted in an increase of cell proliferation. This biphasic effect can be explained by its mechanisms of agonistic/antagonistic action. It may be due to the same mechanism causing resistance to TAM during BC therapy. As this effect was observed in the ER-positive cell lines MCF7 and T47D, but not in the ER-negative BT-20 cells, it is supposed to be mediated by the ER-pathway. TAM has been reported to exhibit its estrogenic potential by transactivation through the AF-1 of ERα (8). The decrease is probably caused by blockage of the AF-2, followed by a weaker agonistic action *via* AF-1. Although RLX also clearly showed antiestrogenic potential, TAM seems to be more potent. This may be due to a higher affinity to the estrogen receptors. The functions of antiestrogens seem not to be limited to the ERα-pathway as the ERα/β-negative cell line BT-20 showed the same responsiveness to (anti-)estrogenic treatment as the ERα-positive cell lines MCF7 and T47D. As this regulation is not mediated by an ERα- or ERβ-pathway, the estrogenic response of BT-20 must be mediated by other regulation mechanisms independent from estrogen receptors.

The antiestrogens TAM and RLX differed in their effects on the proliferation of BC cell lines. Since under RLX treatment no secondary effect of increased cell proliferation was found, although its antiestrogenic effectiveness was not as potent as that of TAM, it seems to be a more potent antiestrogen with regard to the development of resistance during BC treatment. However, clinical trials supporting these *in vitro* findings are pending (1). In BC therapy the decrease of cell proliferation is the dominant effect of TAM, as no agonistic potential was noted during the primary endocrine treatment of breast tumors. Negative estrogenic side-effects were observed in normal endometrium leading to induction of EC (1). Comparing these effects, there is an evident tissue-specific difference between BC and EC cell lines. The EC cell lines KLE, Ishikawa and RL-95-2 were insensitive to treatment with RLX. This is in accordance with the study of Hibner *et al.,* who also found no inhibition of growth of EC cells by RLX (49). KLE- and RL-95-2 cells were also unaffected by TAM, while Ishikawa cells showed the same biphasic effect as the BC cell lines MCF7 and T47D. This is probably due to the fact that Ishikawa cells express high levels of ERα and would support mediation by the ERα signal pathway. ERα expression alone is not sufficient to exert this effect, as shown by ERα-positive RL-95-2 cells not responding to TAM treatment. Possibly the availability of steroid receptor coactivators plays an important role in this mechanism. The antiestrogenic potential of TAM is due to inhibition of the AF-2 of ERs by blocking the binding of required coactivators. Considering tissue-specific differences between endometrial and breast cells, this effect of TAM might be one part of the mechanism leading towards the development of tumor cells in the endometrium under long-term treatment during BC therapy, as shown in clinical studies. As RLX does not exhibit this effect, it may be the better choice for endocrine therapy of BC. As EC cell lines were not affected by either TAM or RLX, an antiestrogen treatment of EC may be not indicated. However, depending on the ER-expression of EC, RLX may have inhibitory potential.

In summary, our experiments demonstrated that RLX and its derivate RLX-HCl exert no estrogenic properties on the cell proliferation of BC and EC cell lines, and on the expression of steroid receptors and steroid receptor coactivators. Together with its positive effects on bone mineral density (20) and the negative side-effects of TAM treatment on normal endometrial tissue and the development of resistance against TAM treatment during BC therapy, it may be considered as a more secure antiestrogen for endocrine BC therapy. Comparing, TAM and OHT showed no differences in (anti-)estrogenic potentials, although OHT has a higher affinity to both estrogen receptors than E2 and TAM (50).

Stimulative effects on BC cell proliferation were established by regulation of steroid receptor expression, while levels of SRCs were not influenced. SRC unresponsiveness to estrogenic effects seems to be a regulative mechanism that keeps estrogenic action in a certain range. Disturbance of this regulative balance may lead to enhanced estrogen-dependent transactivation, supporting tumor development in responsive target tissues such as breast and endometrium.

Acknowledgements

This work was supported by the Lilly Research Laboratories, Indianopolis, USA, represented by Lilly Deutschland GmbH, Bad Homburg, Germany. The results are part of the doctoral thesis of Dr. Michael Glaeser at the Heinrich-Heine-University, Duesseldorf, Germany.

References

1 Beckmann MW, Jap D, Kuschel B, Dall P, Lux MP, Hanstein B and Bender HG: Ovarian steroid hormones and anti-estrogens: risks for and prevention of cancers of the breast and endometrium in the menopause. Ger J Obstet Gynecol (Geburtshilfe Frauenheilkd) *60*: 77-85, 2000.

2 Flototto T, Djahansouzi S, Glaser M, Hanstein B, Niederacher D, Brumm C and Beckmann MW: Hormones and hormone antagonists: mechanisms of action in carcinogenesis of endometrial and breast cancer. Horm Metab Res *33*: 451-457, 2001.

3 Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ and Scanlan TS: Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science *277*: 1508-1510, 1997.

4 Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M and Auricchio F: Activation of the Src/p21ras/Erk pathway by progesterone receptor *via* cross-talk with estrogen receptor. EMBO J *17*: 2008-2018, 1998.

5 Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM and William L: McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. Breast Cancer Res Treat *44*: 23-38, 1997.

6 Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J and Nilsson S: Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Mol Pharmacol *54*: 105-112, 1998.

7 Zhang H, McElrath T, Tong W and Pollard JW: The molecular basis of tamoxifen induction of mouse uterine epithelial cell proliferation. J Endocrinol *184*: 129-140, 2005.

8 McInerney EM and Katzenellenbogen BS: Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. J Biol Chem *271*: 24172-24178, 1996.

9 Berry M, Metzger D and Chambon P: Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. EMBO J *9*: 2811-2818, 1990.

10 Onate SA, Tsai SY, Tsai MJ and O'Malley BW: Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science *270*: 1354-1357, 1995.

11 Voegel JJ, Heine MJ, Zechel C, Chambon P and Gronemeyer H: TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J *15*: 3667-3675, 1996.

12 Hong H, Kohli K, Trivedi A, Johnson DL and Stallcup MR: GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc Natl Acad Sci *93*: 4948-4952, 1996.

13 Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM and Meltzer PS: AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*: 965-968, 1997.

14 Li H, Gomes PJ and Chen JD: RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc Natl Acad Sci *94*: 8479-8484, 1997.

15 Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK and Rosenfeld MG: The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*: 677-684, 1997.

16 Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y and Evans RM: Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell *90*: 569-580,1997.

17 Takeshita A, Cardona GR, Koibuchi N, Suen CS and Chin WW: TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J Biol Chem *272*: 27629-27634, 1997.

18 Tai H, Kubota N and Kato S: Involvement of nuclear receptor coactivator SRC-1 in estrogen-dependent cell growth of MCF-7 cells. Biochem Biophys Res Commun *267*: 311-316, 2000.

19 Newman SP, Bates NP, Vernimmen D, Parker MG and Hurst HC: Cofactor competition between the ligand-bound oestrogen receptor and an intron 1 enhancer leads to oestrogen repression of ERBB2 expression in breast cancer. Oncogene *19*: 490-497, 2000.

20 Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, Draper M and Christiansen C: Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N Engl J Med *337*: 1641-1647, 1997.

21 Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A and Jordan VC: The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. JAMA *281*: 2189-2197, 1999.

22 Gustafsson JA: Raloxifene: magic bullet for heart and bone? Nat Med *4*: 152-153, 1998.
23 Levenson AS and Jordan VC: CF-7: the first hormone-responsive breast cancer cell line. Cancer Res *57*: 3071-3078, 1997.
24 Beckmann MW, Tutschek B, Gohring UJ, Engels K, Picard FK, Scharl A, Niederacher D and Schnurch HG: Immunohistochemical detection of epidermal growth factor receptor (EGF-R) in paraffin sections of breast carcinoma tissue: correlation and clinical significance. Geburtshilfe Frauenheilkd *55*: 258-265, 1995.
25 Glaeser M, Floetotto T, Hanstein B, Beckmann MW and Niederacher D: Gene amplification and expression of the steroid receptor coactivator SRC3 (AIB1) in sporadic breast and endometrial carcinomas. Horm Metab Res *33*: 121-126, 2001.
26 Wong H, Anderson WD, Cheng T and Riabowol KT: Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. Anal Biochem *223*: 251-258, 1994.
27 Otto AM: A one minute pulse of estradiol to MCF-7 breast cancer cells changes estrogen receptor binding properties and commits cells to induce estrogenic responses. J Steroid Biochem Mol Biol *54*: 39-46, 1995.
28 Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: overview of the randomised trials. Lancet *351*: 1451-1467, 1998.
29 Ree AH, Landmark BF, Eskild W, Levy FO, Lahooti H, Jahnsen T, Aakvaag A and Hansson V: Autologous down-regulation of messenger ribonucleic acid and protein levels for estrogen receptors in MCF-7 cells: an inverse correlation to progesterone receptor levels. Endocrinology *124*: 2577-2583, 1989.
30 Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M and Martin MB: Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol *2*: 1157-1162, 1988.
31 Saceda M, Lippman ME, Lindsey RK, Puente M and Martin MB: Role of an estrogen receptor-dependent mechanism in the regulation of estrogen receptor mRNA in MCF-7 cells. Mol Endocrinol *3*: 1782-1787, 1989.
32 Davis MD, VanderKuur JA and Brooks SC: Ligand structure influences autologous down-regulation of estrogen receptor-alpha messenger RNA. J Steroid Biochem Mol Biol *70*: 27-37, 1999.
33 Santagati S, Gianazza E, Agrati P, Vegeto E, Patrone C, Pollio G and Maggi A: Oligonucleotide squelching reveals the mechanism of estrogen receptor autologous down-regulation. Mol Endocrinol *11*: 938-949, 1997.
34 Donaghue C, Westley BR and May FE: Selective promoter usage of the human estrogen receptor-alpha gene and its regulation by estrogen. Mol Endocrinol *13*: 1934-1950, 1999.
35 Berthois Y, Dong XF, Roux-Dossetto M and Martin PM: Expression of estrogen receptor and its messenger ribonucleic acid in the MCF-7 cell line: multiparametric analysis of its processing and regulation by estrogen. Mol Cell Endocrinol *74*: 11-20, 1990.
36 Hall JM and McDonnell DP: The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinol *140*: 5566-5578, 1999.
37 Leygue E, Dotzlaw H, Watson PH and Murphy LC: Altered estrogen receptor alpha and beta messenger RNA expression during human breast tumorigenesis. Cancer Res *58*: 3197-31201, 1999.
38 Dotzlaw H, Leygue E, Watson PH and Murphy LC: Expression of estrogen receptor-beta in human breast tumors. J Clin Endocrinol Metab *82*: 2371-2374, 1997.
39 Brandenberger AW, Tee MK and Jaffe RB: Estrogen receptor alpha (ER-alpha) and beta (ER-beta) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-beta in neoplastic tissues. J Clin Endocrinol Metab *83*: 1025-1028, 1998.
40 Watanabe T, Inoue S, Ogawa S, Ishii Y, Hiroi H, Ikeda K, Orimo A and Muramatsu M: Agonistic effect of tamoxifen is dependent on cell type, ERE-promoter context, and estrogen receptor subtype: functional difference between estrogen receptors alpha and beta. Biochem Biophys Res Commun *236*: 140-145, 1997.
41 Webb P, Lopez GN, Uht RM and Kushner PJ: Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol *9*: 443-456, 1995.
42 Fawell SE, White R, Hoare S, Sydenham M, Page M and Parker MG: Inhibition of estrogen receptor-DNA binding by the "pure" antiestrogen ICI 164,384 appears to be mediated by impaired receptor dimerization. Proc Natl Acad Sci *87*: 6883-6887, 1990.
43 Leo C and Chen JD: The SRC family of nuclear receptor coactivators. Gene *245*: 1-11, 2000.
44 Lee SK, Kim HJ, Kim JW and Lee JW: Steroid receptor coactivator-1 and its family members differentially regulate transactivation by the tumor suppressor protein p53. Mol Endocrinol *13*: 1924-1933, 1999.
45 Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C and O'Malley BW: The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci *97*: 6379-6384, 2000.
46 Lopez GN, Webb P, Shinsako JH, Baxter JD, Greene GL and Kushner PJ: Titration by estrogen receptor activation function-2 of targets that are downstream from coactivators. Mol Endocrinol *13*: 897-909, 1999.
47 Treuter E, Albrektsen T, Johansson L, Leers J and Gustafsson JA: A regulatory role for RIP140 in nuclear receptor activation. Mol Endocrinol *12*: 864-881, 1998.
48 Berns EM, van Staveren IL, Klijn JG and Foekens JA: Predictive value of SRC-1 for tamoxifen response of recurrent breast cancer. Breast Cancer Res Treat *48*: 87-92, 1998.
49 Hibner M, Magrina JF, Lefler SR, Cornella JL, Pizarro AR and Loftus JC: Effects of raloxifene hydrochloride on endometrial cancer cells *in vitro*. Gynecol Oncol *93*: 642-646, 2004.
50 Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustafsson JA: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology *138*: 863-870, 1997.

Received September 14, 2005
Revised November 1, 2005
Accepted December 6, 2005