Single cell biochemistry to visualize antigen presentation and drug resistance

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A FRET profile of modifications in Estrogen Receptor α associated with resistance to anti-estrogens

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Anti-estrogen resistance is a major clinical problem in the treatment of breast cancer. In this study, Fluorescence Resonance Energy Transfer (FRET) analysis, a rapid and direct way to monitor conformational changes of ERα after anti-estrogen binding, was used to study the mechanism of anti-estrogen resistance. Ten different anti-estrogens all induce a rapid FRET response within minutes after the compounds have touched ERα in live cells. Phosphorylation of Serine 305 and/or Serine 236 of ERα by protein Kinase A and of Serine 118 by MAP kinase activity, which are both associated with resistance to anti-estrogens in breast cancer, influenced the FRET response differently for the various anti-estrogens. This resulted in seven different combinations of phospho-modifications in ERα, each of which induces resistance to (a) particular anti-estrogens(s), thereby providing a molecular selection criterion for resistance to the different anti-estrogens. The FRET response preceded activation/inactivation of ERα as measured in a traditional reporter assay. Most importantly, the FRET responses were indicative for resistance to anti-estrogens as measured by proliferation of hormone responsive T47D cells in the presence of anti-estrogens and under conditions of elevated PKA. Tamoxifen and EM-652 were the most sensitive to kinase activities, whereas ICI-182,784 (Fulvestrant), ICI-164,384 and resveratrol were the most resistant. The different responses of anti-estrogens to the different combinations of phospho-modifications in ERα elucidate why certain anti-estrogens are more prone than others to develop resistance, which is highly relevant in studying the mechanism of action of hormones and for endocrine therapy of human cancer.

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Significance

Estrogen receptor α (ERα)-positive breast cancer patients are commonly treated with anti-estrogen tamoxifen. However, only half of the recurrences in ER+ breast tumors respond to tamoxifen. We used Fluorescence Resonance Energy Transfer (FRET) to study resistance to anti-estrogens and established a profile for resistance to anti-estrogens that is based on accumulation of modifications in ERα by Protein Kinase A and MAP-kinase. These modifications convert the particular anti-estrogen from an antagonist into an agonist, stimulating then ERα activation and cell growth, a situation reminiscent to that in clinical breast cancers. This anti-hormone profile provides an explanation for the patterns of anti-estrogen resistance and may predict the regimen of successive endocrine treatment in breast cancer on the basis of modifications in ERα.

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Introduction

Each year approximately 250,000 women are diagnosed with breast cancer (BC) in Europe alone (International Agency for Research on Cancer, www-dep.iarc.fr). Three quarter of these patients have Estrogen Receptor (ER) positive disease and are commonly treated with tamoxifen. Despite a successful drug, almost 50% reduction in recurrence during 10 years of follow-up of ER-positive patients, and a reduction in mortality by a third, still a substantial proportion of BC patients who are treated with tamoxifen develop a relapse (1-3). In metastatic disease, first line endocrine therapy is beneficial in approximately 50% of ER-positive patients (4), whereas second line endocrine treatment results in a clinical benefit of 25-50% for treatment with tamoxifen after initial treatment with an aromatase inhibitor (5). Treatment with an aromatase inhibitor after tamoxifen treatment gives similar clinical benefits (6, 7). Such an improvement of treatment is also observed for the pure anti-estrogenic drug ICI-182,780 (Fulvestrant) after prior treatment with tamoxifen (8-10). Early diagnosis of anti-estrogen resistance could therefore lead to a proper patient selection for adequate therapy, which is of particular importance in the adjuvant setting.

A lead to early diagnosis of resistance to anti-estrogens is provided by the molecular mechanism of resistance to anti-estrogens. Anti-estrogens that bind the receptor inhibit its activity by modulating transactivation capacities of either the N-terminally located AF-1 and/or AF-2 at the C-terminus of ERα (11). The most carboxyl-terminal α-helix (H12) of the ER-LBD (Ligand Binding Domain) acts as a molecular switch for transactivation to occur. Its orientation determines the transcriptional read-out of the receptor. Binding of the different anti-estrogens to the LBD reorients H12 and conceals the coactivator-binding groove that consists of a pocket formed by α-helices 3, 4, 5 and 12 (12, 13). This distortion of H12 is not fixed, but occurs to various extents, depending on the side chain and polarity of the anti-estrogen applied (14). The extent of distortion can be measured using biophysical methods such as fluorescence resonance energy transfer (FRET) (15). Using FRET, we have demonstrated that anti-estrogens induce a conformational change that is overridden by phosphorylation of particular target sites on ERα, resulting in resistance to that anti-estrogen (16). For instance, resistance to tamoxifen is caused by phosphorylation of serine-305 of ERα by Protein Kinase A (PKA). Tamoxifen binds but then fails to induce the inactive conformation, invoking ERα-dependent transactivation instead. PKA activity thus induces a switch from antagonistic to agonistic effects of tamoxifen on ERα. In a retrospective clinical study, we confirmed that an elevated PKA level is associated with tamoxifen resistance in ER positive breast cancer (16). Serine-305 is also the target of p21-activated kinase, PAK-1 (17), and overexpression of PAK-1 is in a similar way associated with resistance to tamoxifen (18). In addition, resistance to anti-estrogens is also associated with modification of ERα by MAPK (19-21) and by the expression levels and/or phosphorylation status of cofactors such as SRC-1 (22) and SRC-3 (21, 23). Aberrant activation of other signaling pathways in ER-positive breast cancer cells will result in many downstream effects and the consequences for resistance to anti-estrogens are directly related to modification(s) of the estrogen receptor. This provides the possibility for immediate read-out of anti-estrogen resistance in these tumors. In particular using FRET, the response of ERα to tamoxifen is measured within 15 minutes and has been shown to correlate with consecutive events like transcription of a hormone responsive reporter gene and proliferation under conditions of anti-estrogens (16). Moreover, resistance to two different anti-estrogens used in the clinic, tamoxifen and ICI-182,780 (Fulvestrant), was distinguishable: resistance to tamoxifen was due to PKA-mediated phosphorylation of serine-305, whereas resistance to ICI-182,780 (Fulvestrant) required additional overexpression of cofactors cyclin D1 and SRC-1. Different structural requirements for anti-estrogen resistance are also foreseen by a different binding profile of randomly generated peptides to ERα in the presence of various anti-estrogens (24-26). Moreover, the 3D structures of the ligand binding domain of ERα bound to different anti-estrogens indicate anti-estrogen specific distortions of ERα (27).

In the present study we investigated the requirements for resistance to anti-estrogens using a FRET approach, and related these to consecutive ER transactivation events. This led to a distinctive profile of modification(s) in ERα that are associated with resistance to the various anti-estrogens. This anti-estrogen specific profile may be of use in providing a better prediction of anti-estrogen therapy and in optimal drug application and design.

Experimental Procedures

Cell culture and transfection

Human osteosarcoma U2OS cells were cultured in DMEM medium in the presence of 10% FCS and standard antibiotics. U2OS cells containing ERα constructs were cultured in phenol red-free DMEM medium containing 5% charcoal treated serum (CTS, Hyclone) 48 hours prior to analysis. For the FRET experiments, cells were cultured overnight on 2 cm round glass coverslips. 24 hours prior to analysis, cells were transfected with pcDNA3-YFP-ERα-CFP or mutants using PEI (Polyethylenimine, Mw 25kDa, Polysciences) (47). Estradiol (Sigma), 4-OH-tamoxifen (Sigma), raloxifene (Sigma), EM-652 (kindly provided by Dr. C. Labrie, University of Quebec, Canada), toremifene (Schering), resveratrol (trans-3,4',5-tridydroxystilbene) (Sigma), arzoxifene, lasofoxifene, ICI-164,384 (the last three kindly provided by Organon), GW7604, the active form of GW5638 (48) (kindly provided by GlaxoSmithKline), or ICI-182,780 (Tocris) were added at the concentrations indicated. Forskolin (Sigma) was added 15 min prior to measurements at a final concentration of 10^{-5} M.

YFP-ERα-CFP constructs

YFP-ERα-CFP constructs were generated as described previously (16). Site-directed mutagenesis of serine-118, 236 and 305 to alanine or glutamate was performed with the YFP-ERα-CFP construct as a template using the appropriate modified oligonucleotides. All constructs were verified by sequence analysis using 4Peaks (mekentosj.com). Protein expression was verified by Western blotting using antibodies against ERα (Stressgen Biotechnologies Corp) and GFP (49) and detected using an ECL detection kit (Amersham). The pcDNA3-YFP-ERα-CFP construct was transfected in U2OS cells that were inspected by confocal microscopy for YFP emission at 500–565 nm after 48 hours.

Fluorescence Resonance Energy Transfer (FRET)

Prior to FRET experiments, cells on coverslips were mounted in bicarbonate-buffered saline (containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 23 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES at pH 7.2) in a heated tissue culture chamber at 37°C under 5% CO2. Cells were analyzed on an inverted Zeiss Axiovert 135 microscope equipped with a dry Achroplan 63x objective. FRET equipment was as described previously (50). CFP was excited at 432 ± 5 nm and emission of YFP was detected at 527 nm and CFP at 478 nm. FRET was expressed as the ratio of YFP to CFP signals. The ratio was arbitrarily set as 1.0 at the onset of the experiment. Changes are expressed as percent deviation from this initial value of 1.0. For data acquisition, Felix software (PTI Inc.) was used. Data were plotted using proFit (QuantumSoft).

ERE-luciferase reporter assays

Luciferase assays were performed as described previously (51). In short, $8x10^4$ U2OS cells were plated in a 24-well plate culture dish and cultured overnight in CTS, after which cells were transfected with 10 ng of pcDNA3-YFP-ERα-CFP or mutants, 0.2 μg ERE-tk-Firefly luciferase (51) and 1 ng of SV40 Renilla luciferase construct using PEI. Directly after tranfection, 10^{-8} M estrogen, 10^{-7} M anti-estrogen or 10^{-6}M resveratrol was added to the cells that were cultured for 48 hr before harvesting. Membrane-permeable 8-Br-cAMP (32) was present during the last 16 hours at a final concentration of 0.1 mM.

Protein stability assay and western blotting of phospho-variants of ERα

For measuring ERα stability, $4x10^5$ of stably transfected YFP-ERα-CFP containing U2OS cells (16) were plated in a six well plate culture dish and cultured for two days in hormone-free CTS medium. Subsequently, 10^{-8} M estrogen, 10^{-7} M anti-estrogen or 10^{-6} M resveratrol was added to the medium, and cells were cultured for 48 hr before harvesting. Membrane-permeable 8-Br-cAMP (32) was added overnight when indicated at a final concentration of 0.1 mM. ERα stability was examined by Western blotting using antibodies against GFP (49) and anti-α tubulin (Sigma) as loading control and detected using an ECL detection kit (Amersham).
For characterization of the phospho-variants of ERα, U2OS cells were transfected with the YFP-ERα-CFP construct or S118E or S305A variants thereof, treated with 8-Br-cAMP and analyzed by Western blotting as described above using antibodies against GFP (49) or against phospho-S118-ERα(#2515, Cell Signaling, USA) or against phospho-S305-ERα (Upstate, USA).

Cumulative Growth Assay

The RNAi cassette from the previously described pSUPER-PKA-RIα vector (16) was recloned into the pRetroSuper (pRS) vector. pRS-GFP was generated by replacing the puromycin resistance gene by a cDNA encoding enhanced GFP (Clonetech). Ecotropic retroviruses were generated by transient transfection of the relevant constructs into EcoPack2 cells (Clonetech) as described (52). Virus containing supernatant was harvested 48-72 hr later and frozen in aliquots.

The cumulative growth advantage assay was performed as described previously (37). In short, T47D cells stably expressing the mouse ecotropic receptor were infected overnight with pRS-GFP-siRNA retroviruses, medium was refreshed the next day, and the cells were plated in different dilutions in 12-well plates in medium containing CTS alone, or CTS plus 10^{-8}M estrogen or 10^{-7}M anti-estrogen or 10^{-6}M resveratrol. Medium was replaced every 3 to 4 days and each week one set of 12 well plates was harvested and analyzed by FACS. The increase of GFP-positive cells over each period declines with higher percentages of initial GFP-positive cells (%GFP(t_0)) although the relative growth advantage is constant. This is represented by the formula: %GFP(t_2) = k x %GFP(t_1)/ [k x %GFP(t_1) + (100 - %GFP(t_1))], where k is the relative growth advantage of the GFP-positive cells over the negative cells. To obtain a measurement independent of the initial percentage of GFP-positive cells, we calculated k for every growth period (k = [(100 x %GFP(t_2)) - (%GFP(t_2) x %GFP(t_1))] / [(100 x %GFP(t_1)) - (%GFP(t_2) x %GFP(t_1))] and depicted cumulative growth over time.

Results

Characterization of the phospho-variants of ERα, and the effect of PKA activation on ERα protein stability in the presence of anti-estrogens

Anti-estrogens can be distinguished in selective estrogen receptor modulators (SERMs), such as tamoxifen, and full anti-estrogens or selective estrogen receptor downregulators (SERDs), such as ICI compounds 182,780 (Fulvestrant) and 164,384 (28), whereas anti-estrogen GW5638 has mixed SERM/SERD properties (29). Activation of ERα by estradiol is associated with degradation of ERα (30), whereas in case of binding to SERMs, ERα protein is stable. Binding to SERDs, such as Fulvestrant, results in proteasome-mediated degradation of the protein (31). Phosphorylation by PKA might also influence protein stability of ERα under conditions of anti-estrogens. We investigated this in U2OS cells stably transfected with wt YFP-ERα-CFP that were cultured in the presence of various anti-estrogens for 48 hours. During the last 16 hours half of the cells were cultured in the presence of 8-Br-cAMP (32). Subsequently, protein samples were isolated and analyzed by Western blotting for the relative levels of ERα protein (**Figure 1A**). The results confirmed the SERM nature of compounds tamoxifen, raloxifene, toremifene, EM-652, arzoxifene and lasofoxifene, for which levels of ERα proteins were maintained, whereas ERα levels in the presence of estradiol, and SERDs ICI-182,784 (Fulvestrant), GW5638 and ICI-164,384 were reduced. Treatment with the phyto-estrogen resveratrol had no apparent effect on stability of ERα. Addition of PKA stimulator 8-Br-cAMP resulted in enhanced levels of ERα protein with all anti-estrogens, except for ICI-182,780 (Fulvestrant).

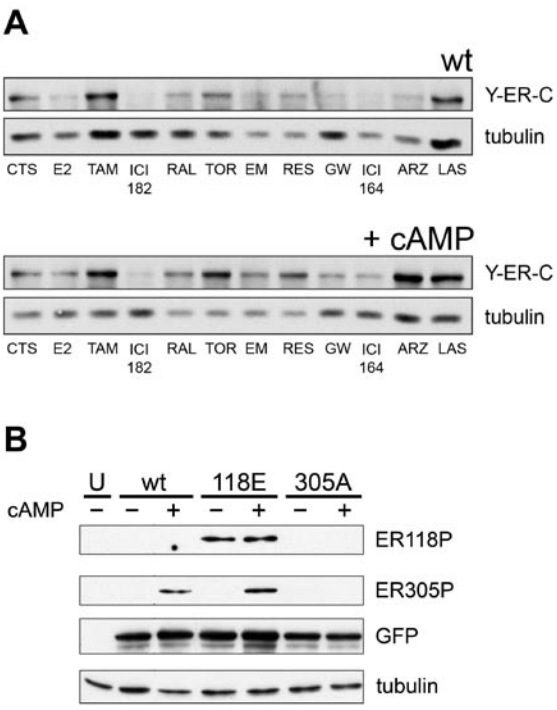

Figure 1. (**A**) Stability of ERα -protein in the presence of anti-estrogens and elevated PKA activity. Stably transfected YFP-ERα-CFP containing U2OS cells were cultured in the presence of CTS, E2 or the indicated anti-estrogens for two days, with the final 16 hours in the presence or absence of PKA-stimulator 8-Br-cAMP. The samples were analyzed for ERα protein expression by Western blotting, as described in Experimental Procedures. Anti-tubulin staining was used as a loading control.
(**B**) Characterization of the phospho-ERα variants. U2OS cells were transfected with the wild-type YFP-ERα-CFP construct or S118E or S305A mutants thereof, cultured in the presence or absence of 8-Br-cAMP and analyzed for the expression of wild-type ERα or the phospho-mutants of ERα. Anti-tubulin staining was used as a loading control. Absence of the phosphorylated S305-ERα protein in the cells transfected with S305A, but its presence in the PKA treated cells transfected with wild-type or S118E-ERα indicates the inability to phosphorylate mutant S305A-ERα by PKA.

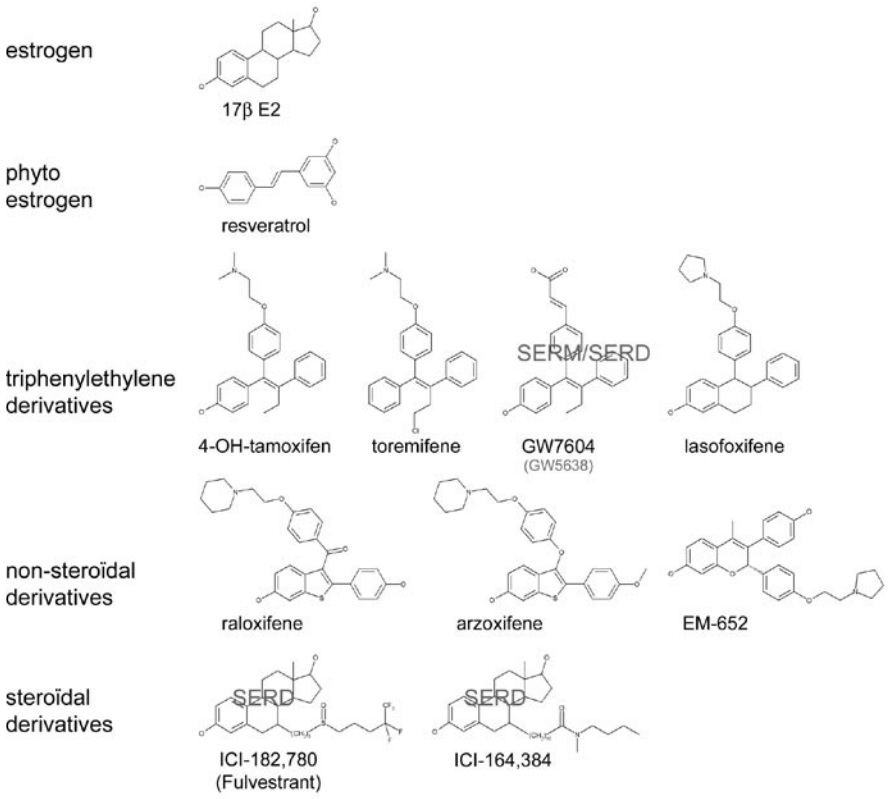

Table 1. Structure of estrogen-like and anti-estrogen-like SERMs and SERDs used in this study.

In this study we will use phospho-mutants of ERα that were characterized by Western blotting using phospho-ERα-specific antibodies (**Figure 1B**). U2OS cells containing either wild-type ERα, a S118E mutant that mimics phosphorylation by MAPK at that site or a 305A mutant that cannot be phosphorylated by PKA at that site, all showed equal levels of ERα, which was detected with an antibody recognizing the GFP tags at both sides of the protein. These ERα-GFP bands were also visible with an antibody detecting ERα (data not shown). The S118E-ERα containing cells showed expression of this protein using an antiserum that specifically detects phosphorylated S118-ERα, whereas treatment with PKA activator 8-Br-cAMP and the use of an antiserum that detects phospho-S305-ERα revealed the phospho-S305-ERα in the cells transfected with wild-type and S118E-ERα, but not in cells transfected with S305A-ERα.

Characteristics of anti-estrogens and FRET

Compounds with anti-estrogenic activity are either triphenylethylene derivatives such as tamoxifen, toremifene and GW5638, benzothiophenes such as raloxifene and arzoxifene, the chromane derivative acolbifene (EM-652), the tetrahydronaphtalene lasofoxifene, phyto-estrogens such as resveratrol, or steroidal derivatives, such as ICI compounds 182,780 (Fulvestrant) and 164,384. The compounds used in this study, and their structure are described in **Table 1**. They differ widely in biological effects *in vitro* and *in vivo* (33, 34).

Anti-estrogens can form hydrogen bonds with the amino acid residues in ERα. Agonist estradiol binds to glu353, arg394 and his524, whereas the anti-estrogens bind to additional amino acid residues, which together with the respective nature of the side chain of the anti-estrogens, results in different distortions of the LBD of ERα (14, 27). We measured such distortions by FRET, where we apply the various anti-estrogens to ER-negative U2OS cells, now transfected with a recombinant ERα with Yellow Fluorescent Protein (YFP) at the N- and Cyan Fluorescent protein (CFP) at the C-terminus. Application of anti-estrogens to these cells resulted in an altered position/

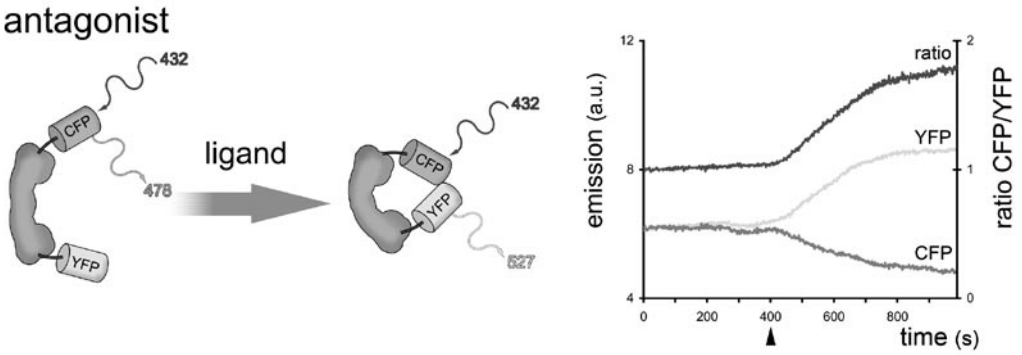

Figure 2. Inactivation of ERα by anti-estrogens measured by FRET. (**A**) Principle of FRET. Exciting CFP at 432nm results in emission at 478nm unless energy is transferred to YFP. Energy transfer depends on the orientation and distance between the two fluorophores and is highly sensitive to conformational changes. An increased YFP (at 527nm) at the expense of CFP emission can occur as the result of a conformational change of ERα. (**B**) FRET change induced by tamoxifen. Time course of emission of YFP (yellow) and CFP (blue) and corresponding ratio of YFP/CFP emission (red) of one YFP-wtERα-CFP containing U2OS cell after addition of 10^{-6} M 4-OH-tamoxifen (TAM).

orientation that induced a change in energy transfer between the two fluorophores. Using this approach, we are able to measure intramolecular changes of ERα as a consequence of exposure to anti-estrogens, which occurred within 15 minutes after administration of the anti-estrogens. The recombinant YFP-ERα-CFP construct retained the properties of wild-type ERα, and an optimal amount of YFP-ERα-CFP for FRET detection (0.5μg per 10^5 cells) in combination with an excess of anti-estrogens (10^{-6} M) was used in our FRET experiments (16). The principle of FRET and a representative experiment where FRET is detected in the form of the ratio between YFP and CFP following tamoxifen addition at 400 seconds, are presented in **Figure 2**.

Characterization of PKA-mediated resistance to anti-estrogens by FRET

The FRET changes induced by the various anti-estrogens in wt YFP-ERα-CFP containing U2OS cells are presented in box plots in **Figure 3A**, with the median value indicated. The box plots present data of at least three consistent, independent measurements. When the FRET changes showed variation, we included at least 10 additional measurement points. The data in **Figure 3A** demonstrated that anti-estrogens tamoxifen, EM-652, lasofoxifene, raloxifene, toremifene and GW5638 all showed a change in FRET (i.e. induced a conformational change in ERα) that was abolished by pretreatment of the cells with PKA activator forskolin as we had previously demonstrated for tamoxifen (16). The differences between the control and forskolin treated cells using these anti-estrogens were statistically significant ($p<0.05$). SERDs ICI-182,780 and ICI-164,384 showed a FRET change that was not affected by forskolin, whereas that of arzoxifene was reduced, but did not reach statistical significance. Phyto-estrogen resveratrol, which under the conditions used acts as anti-estrogen (35), showed no reduction in FRET change upon forskolin treatment.

Does PKA activation affect FRET changes for the sensitive anti-estrogens by phosphorylation of S305 of ERα, as we have demonstrated to be the case for tamoxifen (16)? To study this, we repeated the experiments using an YFP-ERα-S305A-CFP mutant, where serine-305 is replaced with alanine to prevent phosphorylation at this site, **Figure 3B**. Now PKA did not affect tamoxifen- and EM-652-induced FRET, indicating that PKA-associated resistance to these two anti-estrogens is dependent on PKA-mediated phosphorylation of S305. In case of the other SERMs, lasofoxifene, raloxifene, toremifene and GW5638 the FRET change was still abolished upon pretreatment with forskolin, suggesting that additional PKA driven events were responsible for FRET-predicted resistance to these anti-estrogens. The two other SERDs, ICI-182,780 (Fulvestrant) and ICI-164,384, were again insensitive to pretreatment with forskolin, as was resveratrol. Arzoxifene now showed a significant, but no absolute loss of FRET change upon forskolin pretreatment, suggesting that the effect of this com-

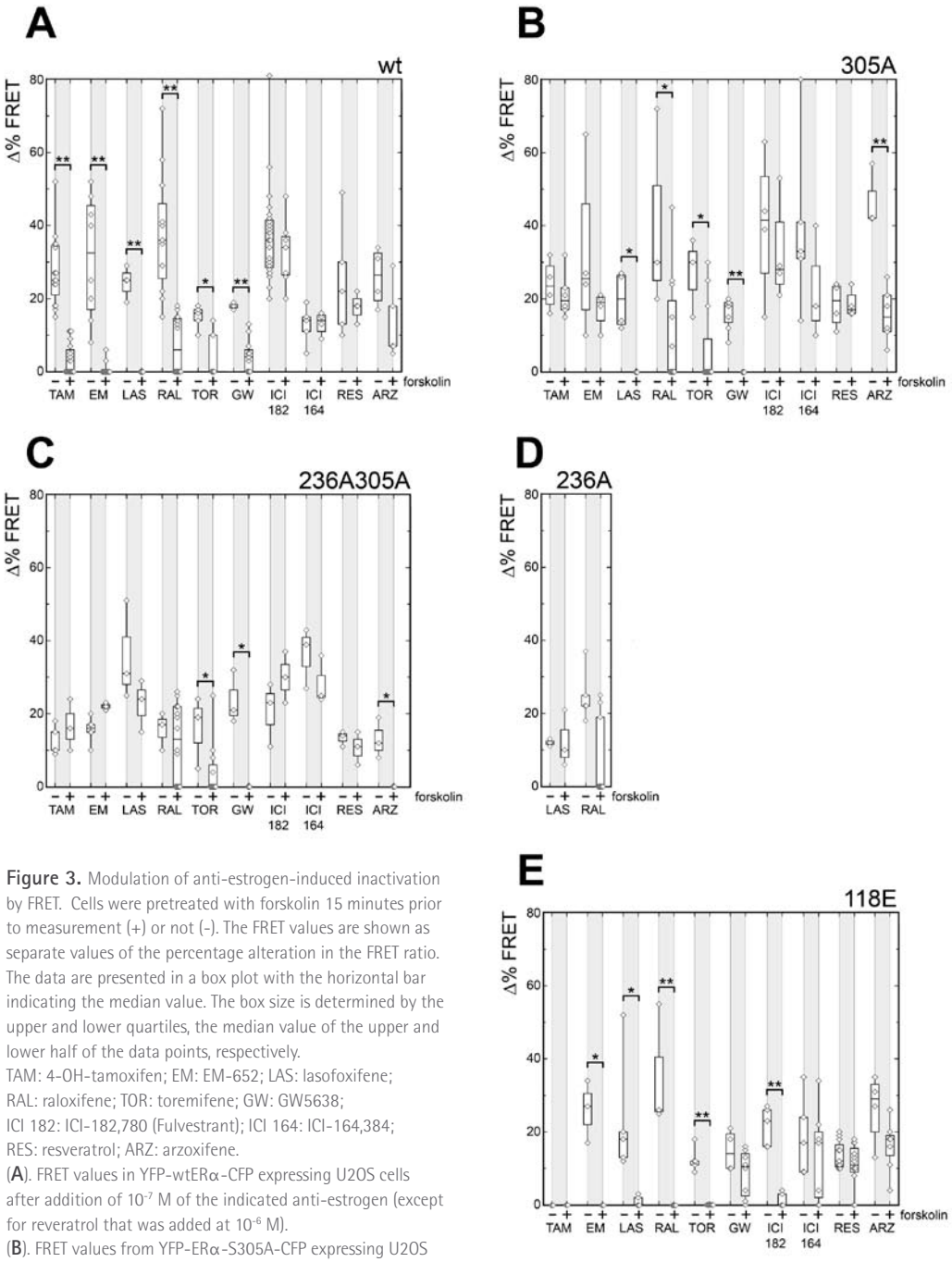

Figure 3. Modulation of anti-estrogen-induced inactivation by FRET. Cells were pretreated with forskolin 15 minutes prior to measurement (+) or not (-). The FRET values are shown as separate values of the percentage alteration in the FRET ratio. The data are presented in a box plot with the horizontal bar indicating the median value. The box size is determined by the upper and lower quartiles, the median value of the upper and lower half of the data points, respectively.
TAM: 4-OH-tamoxifen; EM: EM-652; LAS: lasofoxifene; RAL: raloxifene; TOR: toremifene; GW: GW5638; ICI 182: ICI-182,780 (Fulvestrant); ICI 164: ICI-164,384; RES: resveratrol; ARZ: arzoxifene.
(**A**). FRET values in YFP-wtERα-CFP expressing U2OS cells after addition of 10^{-7} M of the indicated anti-estrogen (except for reveratrol that was added at 10^{-6} M).
(**B**). FRET values from YFP-ERα-S305A-CFP expressing U2OS cells after addition of the anti-estrogens indicated in A.
(**C**). FRET values in YFP-ERα-S236A:S305A-CFP expressing U2OS cells after addition of the anti-estrogens indicated in A.
(**D**). FRET values in YFP-ERα-S236A-CFP expressing U2OS cells after addition of lasofoxifene or raloxifene.
(**E**). FRET values in YFP-ERα-S118E-CFP expressing U2OS cells after addition of the anti-estrogens indicated in A.
* indicates a statistically significant FRET reduction between forskolin treated and untreated samples, p value < 0.05.
** indicates a statistically significant FRET reduction between forskolin treated and untreated samples, p value < 0.01

pound was influenced by PKA-modifications of ERα at other sites than S305. In order to investigate the participation of other PKA target sites in ERα (36), we performed the FRET experiments with the YFP-ERα-S236A:S305A-CFP double mutant construct, where both PKA targets in ERα, serine 236 and 305, were replaced with alanine (**Figure 3C**). PKA pretreatment did not influence the conformational changes of YFP-ERα-S236A:S305A-CFP in response to tamoxifen and EM-652, as to be expected. Importantly, no FRET change was observed for lasofoxifene and raloxifene when pretreated with forskolin, whereas they were recorded with the single S305A mutant. This indicated that resistance to lasofoxifene and raloxifene was due to PKA-mediated phosphorylation of either S236 alone or to a combination of serine sites at positions 236 and 305. Using this double mutant, the reduction in FRET change for toremifene, GW5638 and arzoxifene upon 8-Br-cAMP treatment was still observed, indicating that PKA-mediated resistance of wild-type ERα to these anti-estrogens required other PKA-associated events outside ERα. In order to determine whether resistance to lasofoxifene and raloxifene required PKA-associated phosphorylation of S236 alone or of a combination of S236 and S305, we investigated YFP-ERα-S236A-CFP transfected U2OS cells (**Figure 3D**). The FRET change induced by lasofoxifene was completely abrogated upon pretreatment with forskolin, whereas FRET change induced by raloxifene was only partially affected. This indicated that FRET-predicted resistance to lasofoxifene required PKA-mediated phosphorylation of S236, whereas resistance to raloxifene was generated by PKA-mediated phosphorylation of either S236 or S305, or of a combination of both sites.

With respect to PKA-mediated resistance, five groups of anti-estrogens are to be distinguished:

A	Tamoxifen and EM-652, where resistance is associated with PKA-mediated phosphorylation of ERα at S305.
B	Lasofoxifene, where resistance is associated with PKA-mediated phosphorylation at S236 of ERα.
C	Raloxifene, where resistance is associated with PKA-mediated phosphorylation of ERα at either S236 or S305, or a combination of both.
D	Toremifene, GW5638 and arzoxifene, where resistance is associated with additional PKA-mediated events outside ERα.
E	SERDs ICI-182,780 (Fulvestrant) and ICI-164,384 and phyto-estrogen resveratrol that are PKA-insensitive with respect to resistance.

Characterization of MAPK/PKA associated resistance to anti-estrogens by FRET

In addition to the PKA pathway, activation of the MAPK pathway also influences activation of ERα (20) and may well be related to anti-estrogen resistance. We investigated this using an YFP-ERα-S118E-CFP construct in our FRET experiments, where serine-118 was replaced by glutamate, mimicking phosphorylation at that site by the activation of the MAPK pathway. A combination of this mutant with PKA activation by forskolin reflected the synergy between MAPK and PKA pathways in resistance to anti-estrogens. The ERα-S118E mutant did not show any conformational changes upon tamoxifen addition in the absence of PKA activation nor after forskolin pretreatment (**Figure 3E**), which supports previous reports that MAPK-mediated phosphorylation of S118 suffices to induce tamoxifen resistance of ERα (19). In contrast, the S118A mutant that cannot be phosphorylated by MAPK at this site behaved as wt ERα (data not shown). The other anti-estrogens still induced a conformational change of the ERα-S118E mutant, which was prevented by forskolin treatment for EM-652, lasofoxifene, raloxifene and toremifene, but not SERM/SERD GW5638 and SERD ICI-164,384. Importantly, PKA activation in cells expressing the ERα-S118E mutant did prevent a conformational change in response to SERD ICI-182,780 (Fulvestrant), suggesting that combined PKA and MAPK activity resulted in resistance to this compound on the basis of FRET measurements.

The results from the FRET experiments are summarized in **Figure 4** and provide a profile of modifications in ERα where the combination of effects of PKA and MAPK on resistance to anti-estrogens can be divided in seven categories:

A	MAPK-mediated phosphorylation of S118 that is associated with resistance to tamoxifen.
B	PKA-mediated phosphorylation of S305 that is associated with resistance to tamoxifen and EM-652.
C	PKA-mediated phosphorylation of S236 that is associated with resistance to lasofoxifene.
D	PKA-mediated phosphorylation of either S236 or S305, or a combination of both that is associated with resistance to raloxifene.
E	PKA effects outside ERα that affect resistance to toremifene, GW5638 and arzoxifene.

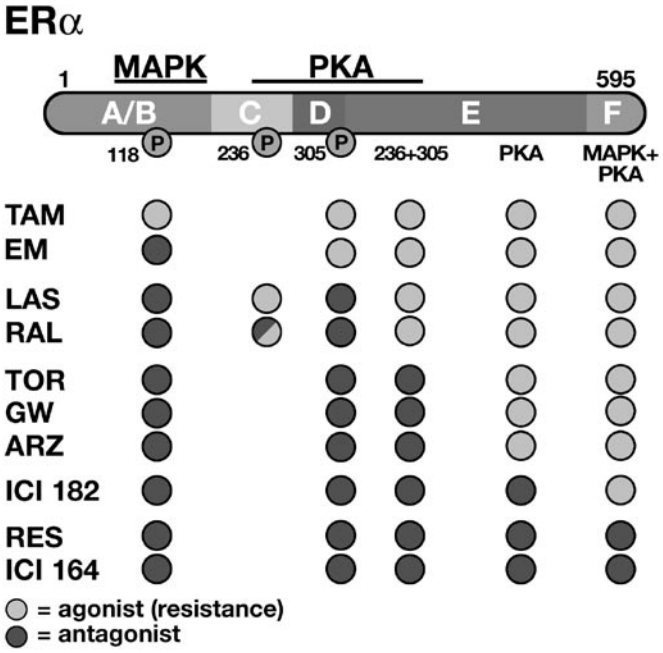

Figure 4. Summary of modifications in ERα that are associated with FRET-predicted resistance to anti-estrogens. The modification sites in ERα by MAPK (S118) and PKA (S236 and S305) are indicated. When no conformational change in wild-type ERα occurs in response to the various anti-estrogens, this is marked in green, indicating that ERα is insensitive towards the anti-estrogen (thus transcriptionally active) for a given modification status. For instance, PKA-mediated phosphorylation of YFP-wt-ERα-CFP showed no conformational change upon addition of tamoxifen and is therefore indicated in green. A conformational change in ERα in response to the various anti-estrogens is marked in red, indicating that ERα is sensitive towards the anti-estrogen (thus transcriptionally inactive) for a given modification status. A-F represent the various ER domains.protein was used as control.

F	A combined effect of MAPK and PKA on ERα that is associated with resistance to ICI-182,780 (Fulvestrant).
G	Insensitivity to MAPK and PKA of compounds ICI-164,384 and resveratrol.

Stimulation of anti-estrogen mediated transcriptional activation of ERα by PKA and MAPK

The compound-induced conformational changes of ERα indicated that phosphorylation of ERα by PKA and/or MAPK affected activation of ERα and might turn an antagonist into an agonist. We therefore investigated the ability of wildtype and mutant ERα to activate an ERE-containing reporter gene in the presence of these anti-estrogens with or without PKA activator 8-Br-cAMP in U2OS cells. These U2OS cells, devoid of endogenous ER expression, were transfected with constructs of ERα (variant), an ER-responsive luciferase reporter and an ER-insensitive Renilla luciferase as control for transfection efficiency (**Figure 5**). The expression level of ERα in these transiently transfected U2OS cells was similar to endogenous expression of ERα in T47D breast cancer cells as detected by Western blotting (data not shown). The results are presented in a range of 0% (read-out in cells without exogenous ER) to 100% luciferase activity (read-out of exogenous ER in the presence of 10^{-8} M estradiol, the optimal concentration used). In the absence of exogenous ER only slight variation in the background activation (set at 1 for hormone-free medium, CTS) of the reporter construct was observed (**Figure 5A**). The various anti-estrogens did not influence the reporter read-out in the absence of ERα, while addition of 8-Br-cAMP, which stimulated PKA activity, enhanced the read-out approximately three fold irrespective of the anti-estrogen (**Figure 5A**). Addition of wt YFP-ERα-CFP suppressed the read-out of the reporter assay in the absence of hormones (CTS), but stimulated it in the presence of E2 (**Figure 5B**). Treatment with 8-Br-cAMP enhanced the read-out in the presence of E2 even further, as has been reported before (36). 8-Br-cAMP also enhanced transcriptional read-out in the presence of anti-estrogens tamoxifen, EM-652, lasofoxifene, raloxifene, toremifene, GW5638 and arzoxifene, but less for ICI-182,780, ICI-164,384 and phyto-estrogen resveratrol (**Figure 5B**). This effect of PKA on transcriptional activation of ERα in the presence of anti-estrogens corresponded to the effect of PKA in the FRET experiments (**Figure 3**), indicating that an immediate measurement of the conformational change of ERα upon interaction with anti-estrogens preceded the transactivation of ER that we measured 48 hours after addition of the anti-estrogens.

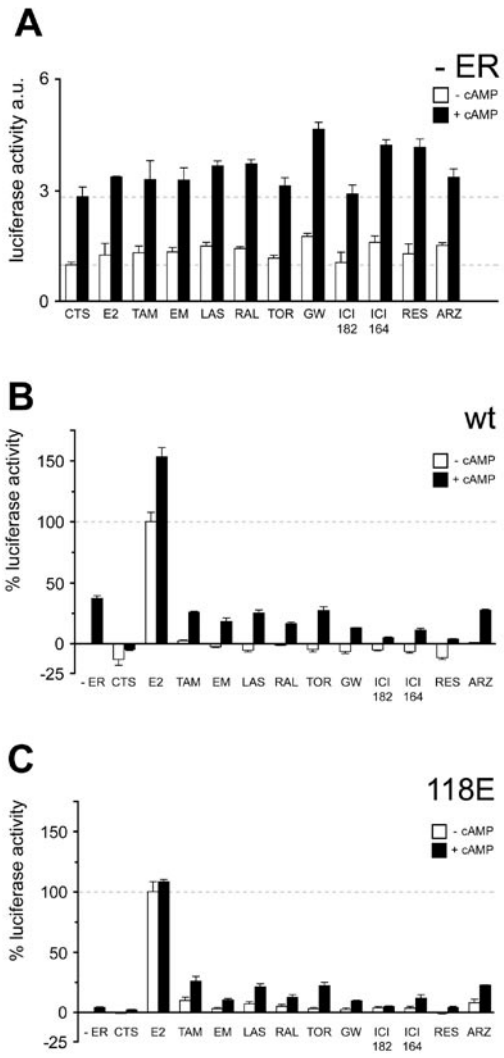

Figure 5. ER transcriptional activity measured by ERE-dependent luciferase assay. U2OS cells were transfected and cultured for two days in the presence of either CTS medium, 10^{-8} M Estradiol (E2), 10^{-7} M of the indicated anti-estrogens, or 10^{-6} M resveratrol and subsequently assayed by an ERE-luciferase assay. Bars indicate standard error in triplicate experiments. For abbreviations of the anti-estrogens, see legend Figure 3.
(**A**). ER dependent transcriptional activity in control U2OS cells (-ER)
(**B**). ER dependent transcriptional activity in YFP-wt-ERα-CFP transfected U2OS cells. The ratio of luciferase activity under conditions of E2 versus CTS was 25, which is set at 100%.
(**C**). ER dependent transcriptional activity in YFP-ERα-S118E-CFP transfected U2OS cells. The ratio of luciferase activity under conditions of E2 versus CTS was 54, which is set at 100%.

Using the YFP-ERα-S118E CFP mutant, which mimics phosphorylation by MAPK at this site, we observed that ERα occupancy of the promoter in the reporter construct enhanced the reporter read-out in the presence of tamoxifen, which was further stimulated by PKA activator 8-Br-cAMP (**Figure 5C**). Here again, the pattern of PKA mediated activation of ERα in the presence of anti-estrogens reflected the PKA-mediated effects that we observed in the FRET experiments (**Figure 3E**) with exception of SERD ICI-182,780 (Fulvestrant). No conformational change in the S118E mutant was detected in response to PKA activation in the presence of ICI-182,780, indicating an activated ERα (**Figure 3E**), whereas no enhanced transactivation by PKA was observed in the reporter assay. This may well be due to destruction of ERα in the presence of ICI-182,780 (Fulvestrant), as shown in **Figure 1A**.

Stimulation of anti-estrogen dependent proliferation by PKA

PKA-mediated resistance to anti-estrogens might result in proliferation of ER-dependent cells in the presence of anti-estrogens under conditions of PKA stimulation. To investigate this, we retrovirally transduced T47D breast cancer cells, which depend on estrogens for their growth, with a vector that allowed co-expression of the RNAi targeting PKA-RIα and a marker GFP gene. PKA-RIα is a negative regulator of PKA, knock down of PKA-RIα by RNAi leads to increased PKA activity, as has been demonstrated previously (16). In this assay, the GFP protein functions as a marker of transduced cells to determine the relative propagation of retrovirally transduced cells over controls, as determined by FACS. Relating the ratios of cells cultured in conditions of various anti-estrogens to those cultured in CTS, a cumulative growth advantage was determined as described before (37) (**Figure 6**). Elevated PKA activity resulted in a small relative growth disadvantage for T47D cells cultured in the presence of E2, which was likely due to PKA/E2-related apoptosis (38). However, increased PKA activity yielded a growth stimulatory effect on T47D cells cultured in the presence of, in particular, lasofoxifene, arzoxifene, tamoxifen and EM-652, and less for ICI-182,780. No or little growth stimulation was observed in the presence of ICI-164,384, GW5638, toremifene and resveratrol. PKA activity influenced anti-estrogen dependent growth of T47D cells in a similar way as it affected FRET in the presence of these anti-estrogens, with exception of

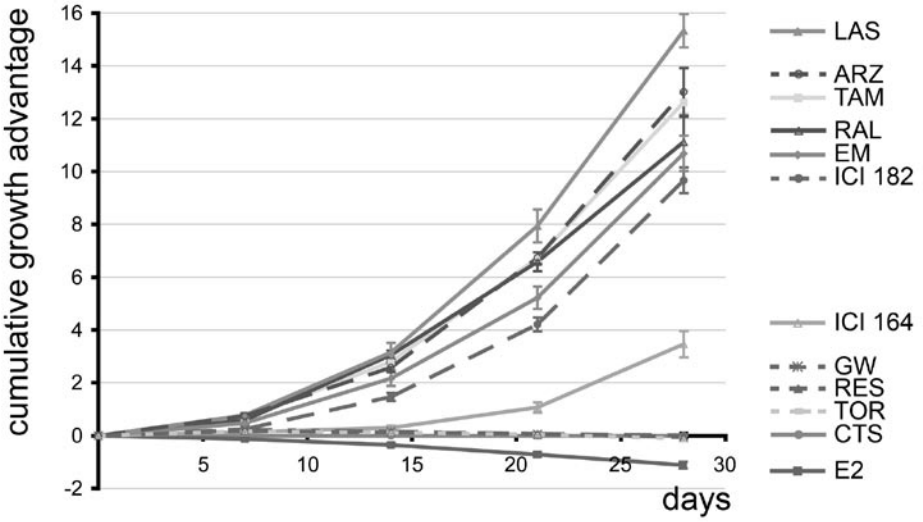

Figure 6. Cumulative growth advantage of T47D breast tumor cells with elevated PKA activity cultured in the presence of anti-estrogens. T47D cells co-expressing RNAi targeting PKA-RIα and GFP by retroviral transduction as described in Experimental Procedures were cultured in medium containing CTS alone, or CTS plus 10^{-8} M Estradiol (E2) or 10^{-7} M anti-estrogen (except for resveratrol that was added at 10^{-6} M). Medium was replaced every 3 to 4 days, and each week one 12 well plate was harvested and analyzed by FACS. The increase of GFP-positive cells in time was determined as described in Experimental Procedures. For abbreviations of the anti-estrogens, see legend Figure 3.

anti-estrogens GW5638 and torimefene. PKA activation affected FRET with these anti-estrogens (**Figure 3**), but elevated PKA did not result in growth stimulation. The reversed applied to ICI-182,780. The FRET change observed with GW5638 and toremifene was, however, influenced by PKA-associated events outside ERα, and may thus be cell type specific. For anti-estrogens such as tamoxifen, EM-652, raloxifene and lasofoxifene where resistance was associated with PKA-mediated modification(s) of targets in ERα, PKA activation did contribute to an increased anti-estrogen depended proliferation. For these anti-estrogens, our results show that conformational changes in ERα, as read by FRET, are coupled to transcriptional activation and cell proliferation.

Discussion

In endocrine treatment of breast cancer, early diagnosis of sensitivity for anti-estrogens may contribute to proper selection of adequate anti-estrogens for individual patients. This is especially relevant since patients benefit from consecutive treatment with different types of anti-estrogens (8, 9), which thus far is taking place on empirical basis. A profile of the modifications in ERα that are associated resistance to anti-estrogens as presented here in **Figure 4** may well contribute to rational matching of patients and compounds. This profile of ERα modifications is based on the immediate interaction between ERα and anti-estrogenic compounds, which takes place within 15 minutes after administration and is measured by FRET. The downstream effects of this interaction determine the applicability of these compounds and include stability and transactivation of the ERα and, ultimately, proliferation under anti-estrogenic conditions. Essential for a predictive profile is that the early conformational change in ERα by anti-estrogens as measured by FRET, is indicative of the following steps in the (inhibition of) anti-hormonal response. In the final outcome, our experiments clearly demonstrated the effect of elevated PKA activity on proliferation of ER-positive breast cancer cells under various anti-estrogen conditions (**Figure 6**). Phosphorylation of direct target sites in ERα leads to resistance predicted by FRET for the anti-estrogens tamoxifen, EM-652, raloxifene and lasofoxifene (**Figure 3A**) and moreover, conferred anti-estrogen-depended proliferation of T47D cells for the same anti-estrogens upon elevated PKA activity (**Figure 6**). Of the anti-estrogens for which resistance was due to PKA-associated targets outside ERα, i.e. GW5638, toremifene and arzoxi-

fene, only arzoxifene stimulated proliferation of T47D under activated elevated PKA conditions. The PKA-associated targets outside ERα may well include SRCs, and the effect of PKA-mediated phosphorylation of these cofactors in T47D cells, in which we measured the proliferation, might well differ from that in U2OS cells used for FRET experiments, due to different levels of SRCs and/or anti-estrogen specific effects that were not studied here. In case of GW5638, degradation of ERα is overriding resistance of ERα to the anti-estrogen as invoked by conformational changes. The first interaction between ERα and anti-estrogens, as measured by FRET, is therefore indicative of a conformational change in ERα that affects the later steps of activation of ERα, unless this conformational change leads to degradation of ERα.

In the more stringent group of antagonistic compounds, (ICI-182,780, ICI-164,384 and resveratrol), PKA activation did not, or only marginally affect proliferation for ICI-164,384 and resveratrol. In contrast, proliferation was stimulated by ICI-182,780. Remarkably, our FRET results also indicated that ICI-182,780 was the least stringent antagonist of this group, and was affected by activation of PKA in combination with MAPK (**Figure 3E**), or by elevated PKA in the presence of overexpression of cofactors SRC-1 and cyclin D1 (16). T47D cells moreover, also express ERβ which could be affected by anti-estrogens as well (39). In contrast to ERα, ICI-182,780 does not act as a SERD on ERβ (40), and even stimulates transactivation of ERβ in a fos:jun complex (41). These, as well as other events may influence the effect of PKA on anti-estrogen dependent proliferation of ER-positive breast tumor cells.

Our results from the reporter assay demonstrated that PKA activation enhances transactivation of ERα in the presence of E2, as has been reported before (36) (**Figure 5**), and in the presence of anti-estrogens that are responsive to PKA in the FRET assay (**Figure 3**). Here, the information obtained from the immediate interaction between anti-estrogens and ERα measured by FRET is indicative of the first transcriptional read-out from a hormone responsive reporter gene.

Phosphorylation by PKA is able to modulate the response to anti-estrogens, but does so differently for the various anti-estrogens, as is depicted in **Figure 4**. This can be explained by an anti-estrogen specific re-orientation of the LBD of ERα, which is counteracted by a specific set of PKA and/or MAPK associated phosphorylations in ERα, thereby converting the action of the antagonist into that of an agonist. The PKA- and MAPK-mediated modifications that are associated with FRET-predicted resistance result in a ranking of anti-estrogens that largely agrees with previous biological findings (34) and with structural differences between comparable compounds (**Table 1** and **Figure 4**). For the anti-estrogens in the triphenylethylene group (**Table 1**), the polarity of the side chain (COO in GW5638 versus $-N-C_2H_6$ in tamoxifen) correlated with their final effect in FRET analysis: resistance to anti-estrogen GW5638 required more stringent conditions than resistance to tamoxifen (**Figure 4**). The same applies to arzoxifene and raloxifene, and for ICI-164,384 in comparison with ICI-182,784 (Fulvestrant), where the former steroidal compound contains a more extended side chain and additional conditions appear to be required for resistance to these anti-estrogens.

The additional PKA-mediated events outside the ERα that are associated with resistance to toremifene, GW5638 and arzoxifene (**Figure 4**) may well involve phosphorylation of cofactors for which it has been demonstrated that the expression levels and/or phosphorylation status affect the extent of E2-mediated transactivation of ERα and its sensitivity to tamoxifen (23). The stringency of these modifications may well be different for the different anti-estrogens within this group, which may explain the increased proliferation in the presence of arzoxifene compared to GW5638 and toremifene that showed no PKA-mediated increase in proliferation (**Figure 6**). Our results do, however, show that PKA-mediated phosphorylation of particular sites of ERα, possibly in synergy with phosphorylation of SRCs, acts to confer resistance to anti-estrogens.

Although the results from our FRET and transactivation experiments do agree in general, loss of a change in FRET in the presence of anti-estrogens due to PKA activity does not lead to a full transactivation of ERα, as is observed in the presence of E2. Also proliferation under those conditions is only a fraction of that under E2 conditions (16). This suggests that, although the inactive state of ERα is abrogated by PKA pretreatment, the transcriptional active state of the ERα differs between activation by E2 and activation by PKA in the presence of anti-estrogens (W. Zwart *et al.*, in preparation). As for the reporter assay, these differences could be explained by a different promoter preference between ERα activated by E2 and ERα ac-

tivated by PKA in the presence of anti-estrogens (42). This difference in target preference is also obvious from different expression profiles of breast cancer cells (43), and more relevant to the present study, also of U2OS cells transfected with ERα (44), when E2 conditions are compared with conditions of different anti-estrogens. These data cast doubt on whether reporter assays that are used to measure E2-mediated transactivation of ERα are optimal to measure resistance to anti-estrogens under specific conditions.

Not only does the profile of modifications required for resistance provide a means for ranking anti-estrogens, it also describes conditions in specimens of breast cancer patients where resistance to a particular anti-estrogen can be anticipated. We (16) and others (18, 45) have demonstrated that elevated PKA and PAK-1 levels, as well as activation of PKA and PAK-1 in primary breast cancer is associated with resistance to tamoxifen, whereas resistance to tamoxifen treatment due to activation of the MAPK pathway has also been demonstrated before (46). The FRET profile based on ERα-modifications involved in resistance to tamoxifen, as presented in this study, provides predictive value for this form of anti-estrogen resistance, as well as a rationale for the selection of patients for adequate treatment with other anti-estrogens. When translated to the clinic the profile may predict the regimen of successive endocrine treatment modalities of breast cancer on the basis of modifications in ERα rather than by empirics.

Acknowledgements

We thank Lennert Janssen for assistance in generating the phospho-mutants of ERα, Anita Pfauth and Frank van Diepen for help with flow cytometry, Mathijs Voorhoeve for the pRetroSuper vectors and help with the growth assays, and Dr. K. Jalink for initial help with the FRET equipment. We thank Dr. F. Dijcks and Dr. G. Veeneman (Organon, Oss, the Netherlands) for the kind supply of ICI-164,384, lasofoxifene and arzoxifene, Dr. C. Labrie (University of Quebec, Canada) for the kind supply of EM-652, and Mrs. L. Bray (GlaxoSmithKline, Herthfordshire, UK) for kindly providing us with GW7604.

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