Classification of anti-estrogens according to intramolecular FRET effects on phospho-mutants of estrogen receptor α

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Abstract

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 estrogen receptor α (ERα) upon anti-estrogen binding, was used to characterize resistance to anti-estrogens. Nine different anti-estrogens all induced a rapid FRET response within minutes after the compounds have liganded to ERα in live cells, corresponding to an inactive conformation of the ERα. Phosphorylation of Ser^{305} and/or Ser^{236} of ERα by protein kinase A (PKA) and of Ser^{118} by mitogen-activated protein kinase (MAPK) influenced the FRET response differently for the various anti-estrogens. PKA and MAPK are both associated with resistance to anti-estrogens in breast cancer patients. Their respective actions can result in seven different combinations of phospho-modifications in ERα where the FRET effects of particular anti-estrogen(s) are nullified. The FRET response provided information on the activity of ERα under the various anti-estrogen conditions as measured in a traditional reporter assay. Tamoxifen and EM-652 were the most sensitive to kinase activities, whereas ICI-182,780 (Fulvestrant) and ICI-164,384 were the most stringent. The different responses of anti-estrogens to the various combinations of phospho-modifications in ERα elucidate why certain anti-estrogens are more prone than others to develop resistance. These data provide new insights into the mechanism of action of anti-hormones and are critical for selection of the correct individual patient-based endocrine therapy in breast cancer. [Mol Cancer Ther 2007;6(5):1526–33]

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Introduction

Three quarter of breast cancer patients have estrogen receptor (ER)–positive disease and are commonly treated with anti-estrogen tamoxifen. Despite being 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 breast cancer patients who are treated with tamoxifen develop a relapse and are to be treated with different anti-estrogens and/or aromatase inhibitors (1–3). Early diagnosis of anti-estrogen resistance could therefore lead to a proper patient selection for adequate therapy.

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 NH_2-terminally located AF-1 and/or AF-2 at the COOH terminus of ERα (4). The most carboxyl-terminal α-helix (H12) of the ER-ligand binding domain (ER-LBD) acts as a molecular switch for transactivation to occur. Its orientation determines the transcriptional readout 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 (5, 6). This distortion of H12 is not fixed, but occurs to various extents, depending on the side chain and polarity of the anti-estrogen applied (7). The conformational state of ERα can be measured using biophysical methods such as fluorescence resonance energy transfer (FRET; ref. 8). Using FRET, we have shown 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 (9). For instance, resistance to tamoxifen is caused by phosphorylation of Ser^{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 (9). Ser^{305} is also the target of p21-activated kinase, PAK-1 (10), and overexpression of PAK-1 is in a similar way associated with resistance to tamoxifen (11). In addition, resistance to anti-estrogens is also associated with modification of ERα by mitogen-activated protein kinase (MAPK; refs. 12–14) and by the expression levels and/or phosphorylation status of cofactors such as SRC-1 (15) and SRC-3 (14, 16). Aberrant activation of other signaling pathways in ER-positive breast cancer cells will result in post-translational modification(s) on the ER that affect resistance to anti-estrogens. In this way, and of clinical relevance, 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 Ser^{305}, whereas resistance to ICI-182,780 (Fulvestrant) required additional overexpression of cofactors cyclin D1 and SRC-1. Anti-estrogen characteristic requirements for resistance are also foreseen by a different binding profile of randomly generated peptides to ERα in the presence of various anti-estrogens (17–19). Moreover, the three-dimensional structures of the LBD of ERα bound to different anti-estrogens indicate anti-estrogen–specific distortions of ERα (20).

In the present study, we investigated the requirements for resistance to nine different anti-estrogens using a FRET approach and related these to consecutive ER transactivation events. This led to seven different combinations of phospho-modifications in ERα, each of which is associated with a FRET-related resistance to particular anti-estrogen(s). This anti-estrogen–specific profile reveals a mechanism for anti-estrogen resistance and provides a molecular explanation for the outcome of anti-estrogen therapy.

Experimental Procedures

Cell Culture and Transfection

Human osteosarcoma U2OS cells were cultured in DMEM in the presence of 10% FCS and standard antibiotics. U2OS cells containing ERα constructs were cultured in phenol red–free DMEM containing 5% charcoal-treated serum (CTS, Hyclone) 48 h before analysis. For the FRET experiments, cells were cultured overnight on 2-cm round glass coverslips. Twenty-four hours before analysis, cells were transfected with pcDNA3-YFP-ERα-CFP or mutants using polyethylenimine (PEI, M_w 25 kDa, Polysciences; ref. 21). Estradiol (Sigma), 4-OH-tamoxifen (Sigma), raloxifene (Sigma), EM-652 (kindly provided by Dr. C. Labrie, University of Quebec, Canada), toremifene (Schering), arzoxifene, lasofoxifene, ICI-164,384 (the last three kindly provided by Organon, Oss, the Netherlands), GW7604, the active form of GW5638 (ref. 22; kindly provided by GlaxoSmithKline), or ICI-182,780 (Tocris) were added at the concentrations indicated. Forskolin (Sigma) was added 15 min before measurements at a final concentration of 10^{-5} mol/L.

YFP-ERα-CFP Constructs

YFP-ER-CFP constructs were generated as described previously (9). Site-directed mutagenesis of Ser^{118}, Ser^{236}, and Ser^{305} to alanine or glutamate was done with the YFP-ERα-CFP construct as a template using the appropriate modified oligonucleotides. All constructs were verified by sequence analysis. Protein expression was verified by Western blotting using antibodies against ERα (Stressgen Biotechnologies Corp.) and green fluorescent protein (GFP; ref. 23) and detected using an enhanced chemiluminescence detection kit (Amersham). The pcDNA3-YFP-ERα-CFP construct was transfected in U2OS cells that were inspected by confocal microscopy for yellow fluorescent protein (YFP) emission at 500–565 nm after 48 h.

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-cyclic AMP (cAMP) and analyzed by Western blotting using antibodies against GFP (23) or against phospho-S118-ERα (Cell Signaling Technology) or against phospho-S305-ERα (Upstate).

Fluorescence Resonance Energy Transfer

Before FRET experiments, cells on coverslips were mounted in bicarbonate-buffered saline [containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 23 mmol/L $NaHCO_3$, 10 mmol/L glucose, and 10 mmol/L HEPES (pH, 7.2)] in a heated tissue culture chamber at 37°C under 5% CO_2. Cells were analyzed on an inverted Zeiss Axiovert 135 microscope equipped with a dry Achroplan 63× objective. FRET equipment was as described previously (24). Cyan fluorescent protein (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 done as described previously (25). In short, 8×10^4 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-ER-CFP or mutants, 0.2 μg ERE-tk-Firefly luciferase (25) and 1 ng of SV40 *Renilla* luciferase construct using PEI. Directly after transfection, 10^{-8} mol/L estrogen or 10^{-7} mol/L anti-estrogen was added to the cells that were cultured for 48 h before harvesting. Membrane-permeable 8-Br-cAMP (30) was present during the last 16 h at a final concentration of 0.1 mmol/L.

Results

Characterization of the Phospho-Variants of ERα

In this study we used phospho-mutants of ERα that were characterized by Western blotting using phospho-ERα–specific antibodies (Fig. 1B). U2OS cells containing either wild-type ERα, a S118E mutant that mimics phosphorylation by MAPK at that site, or a S305A 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 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

Anti-estrogens can be distinguished in selective ER modulators (SERM), such as tamoxifen, and full anti-estrogens or selective ER down-regulators (SERD), such as

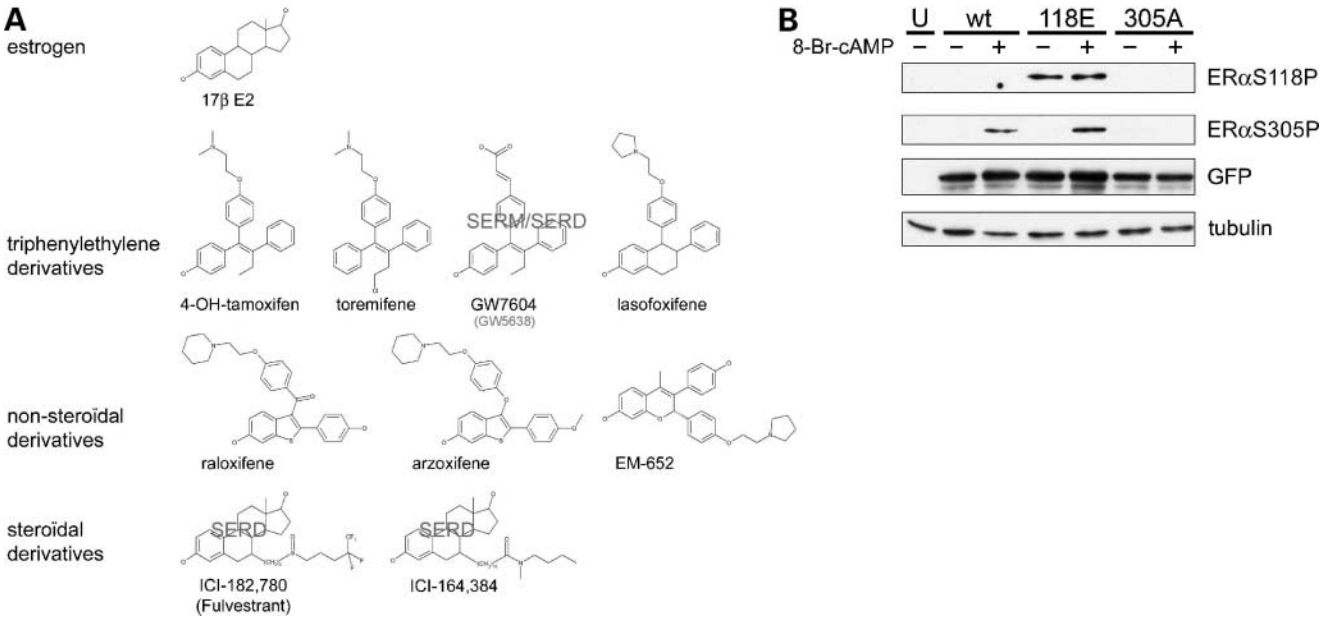

Figure 1. **A,** structure of estrogen-like and anti-estrogen-like SERMs and SERDs used in this study. **B,** characterization of the phospho-ERα variants. U2OS cells were transfected with the wild-type YFP-ERα-CFP construct or S118E or S305A mutants thereof, cultured in the presence or absence of 8-Br-cAMP 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.

ICI compounds 182,780 (Fulvestrant) and 164,384 (26), whereas anti-estrogen GW5638 has mixed SERM/SERD properties (27). The compounds used in this study and their structure are described in Fig. 1A. They differ widely in biological effects *in vitro* and *in vivo* (28, 29).

Anti-estrogens can form hydrogen bonds with the amino acid residues in ERα. Agonist estradiol binds to Glu^{353}, Arg^{394}, and His^{524}, 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 (7). We measured such distortions by FRET, where we applied 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 COOH terminus. Application of anti-estrogens to these cells resulted in an altered position/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 min 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 (0.5 μg per 10^5 cells) in combination with an excess of anti-estrogens (10^{-6} mol/L) was used in our FRET experiments (9). 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 s are presented in Fig. 2.

Characterization of PKA-Mediated Resistance to Anti-estrogens by FRET

The FRET changes induced by the various anti-estrogens in wild type (wt) YFP-ERα-CFP containing U2OS cells are presented in box plots in Fig. 3A, with the median value indicated. The box plots present data of at least three independent measurements. When the FRET changes showed variation, we included at least 10 additional measurement points. The data in Fig. 3A showed 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 an immediate PKA activator forskolin (30), as we had previously shown for tamoxifen (9). 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.

Does PKA activation affect FRET changes for the sensitive anti-estrogens by phosphorylation of S305 of ERα, as we have shown to be the case for tamoxifen (9)? To study this, we repeated the experiments using an YFP-ERα-S305A-CFP mutant, where Ser^{305} is replaced with alanine to prevent phosphorylation at this site (Fig. 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 Ser^{305}. In the 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. Arzoxifene now showed a significant, but no absolute loss of FRET change upon forskolin pretreatment, suggesting that the effect of this compound was influenced by PKA modifications of ERα at other sites than Ser^{305}. To investigate the participation of other PKA target sites in ERα (31), we did the FRET experiments with the YFP-ERα-S236A:S305A-CFP double mutant construct, where both PKA targets in ERα, Ser^{236} and Ser^{305}, were replaced with alanine (Fig. 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 Ser^{236} 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 forskolin treatment was still observed, indicating that PKA-mediated resistance of wild-type ERα to these anti-estrogens required other PKA-associated events outside ERα. To determine whether resistance to lasofoxifene and raloxifene required PKA-associated phosphorylation of Ser^{236} alone or of a combination of Ser^{236} and Ser^{305}, we investigated YFP-ERα-S236A-CFP–transfected U2OS cells (Fig. 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 Ser^{236}, whereas resistance to raloxifene was generated by PKA-mediated phosphorylation of either Ser^{236} or Ser^{305} or of a combination of both sites.

With respect to PKA-mediated resistance, five groups of anti-estrogens can be distinguished: (*a*) tamoxifen and EM-652, where resistance is associated with PKA-mediated phosphorylation of ERα at Ser^{305}; (*b*) lasofoxifene, where resistance is associated with PKA-mediated phosphorylation at Ser^{236} of ERα; (*c*) raloxifene, where resistance is associated with PKA-mediated phosphorylation of ERα at either Ser^{236} or Ser^{305}, 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 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 (12) and may well be related to anti-estrogen resistance. We investigated this using an YFP-ERα-S118E-CFP construct in our FRET experiments, where Ser^{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 (Fig. 3E), which supports previous reports that MAPK-mediated phosphorylation of Ser^{118} suffices to induce tamoxifen resistance of ERα (13). 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

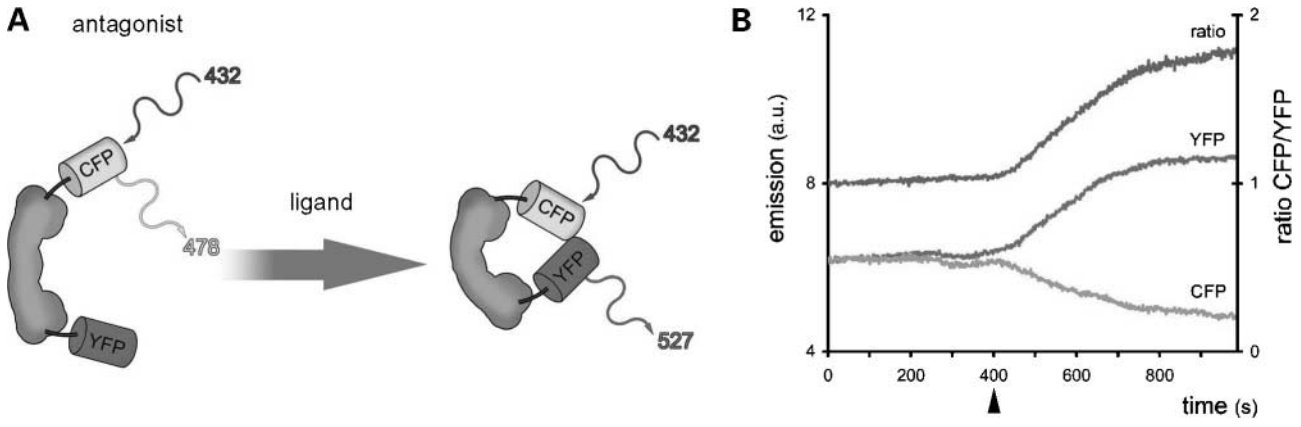

Figure 2. Inactivation of ERα by anti-estrogens measured by FRET. **A,** principle of FRET. Exciting CFP at 432 nm results in emission at 478 nm, unless energy is transferred to YFP. Energy transfer depends on the orientation and distance between the two fluorophores and is highly sensitive to conformational changes. An increased YFP (at 527 nm) 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 and CFP and corresponding ratio of YFP/CFP emission of one YFP-wtERα-CFP–containing U2OS cell after addition of 10^{-6} mol/L 4-OH-tamoxifen (TAM) added at 400 s (▲).

treatment for EM-652, lasofoxifene, raloxifene, and toremifene, but not for 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 Fig. 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 into seven categories: (*a*) MAPK-mediated phosphorylation of Ser^{118} that is associated with resistance to tamoxifen; (*b*) PKA-mediated phosphorylation of Ser^{305} that is associated with resistance to tamoxifen and EM-652; (*c*) PKA-mediated phosphorylation of Ser^{236} that is associated with resistance to lasofoxifene; (*d*) PKA-mediated phosphorylation of either Ser^{236} or Ser^{305}, 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; (*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 ICI-164,384.

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 conformation of ERα and might turn an antagonist into an agonist, as it did for tamoxifen. We therefore investigated the ability of wt and mutant ERα to activate an ERE-containing reporter gene in the presence of these anti-estrogens with or without PKA activator

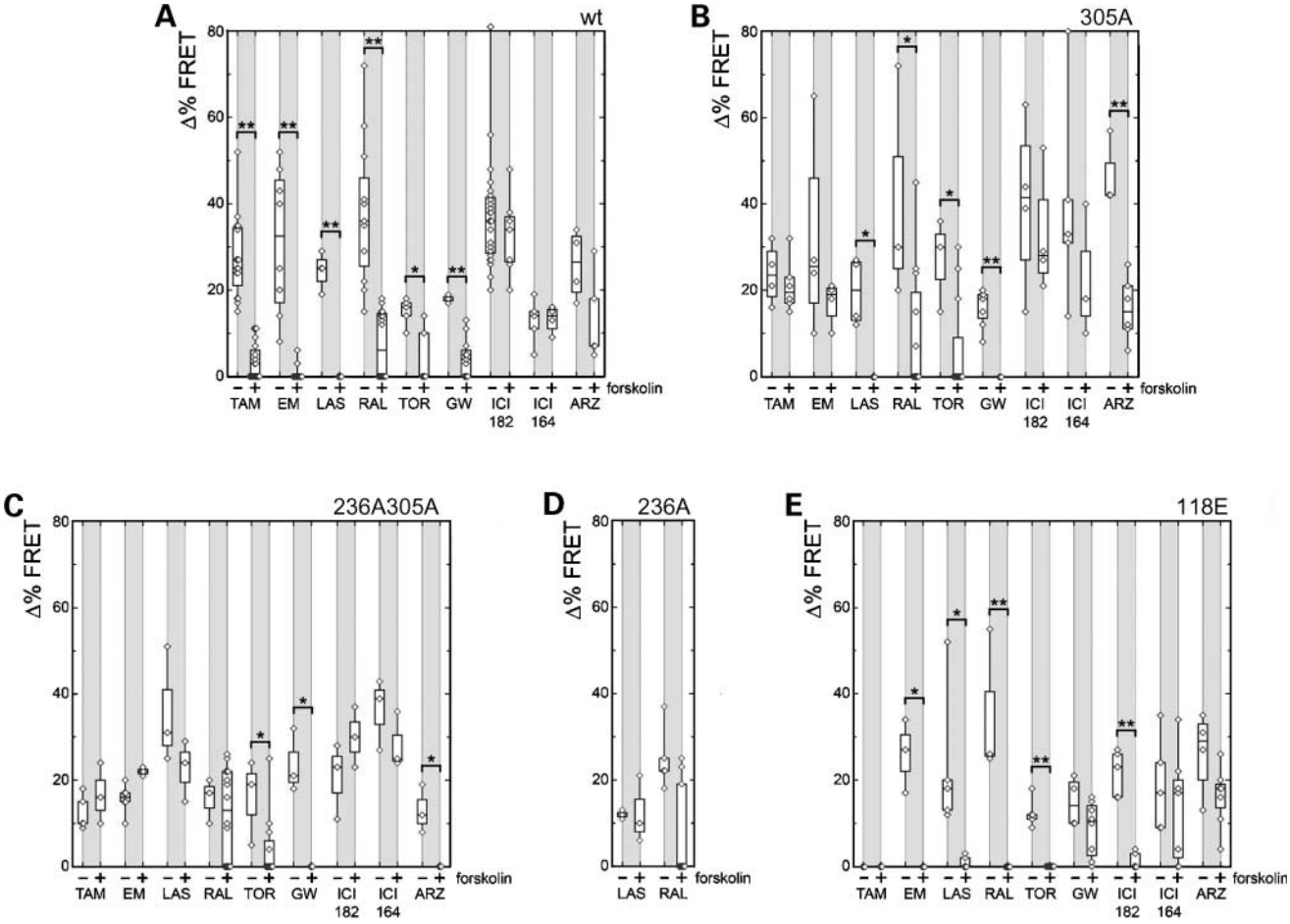

Figure 3. Modulation of anti-estrogen – induced inactivation by FRET. **A,** FRET values in YFP-wtERα-CFP – expressing U2OS cells after addition of 10^{-7} mol/L of the indicated anti-estrogen. Cells were pretreated with forskolin 15 min before measurement (+) or not (−). The FRET values are shown as 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; *ARZ,* arzoxifene. **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**). *, $P < 0.05$, statistically significant FRET reduction between forskolin-treated and untreated samples. **, $P < 0.01$, statistically significant FRET reduction between forskolin-treated and untreated samples.

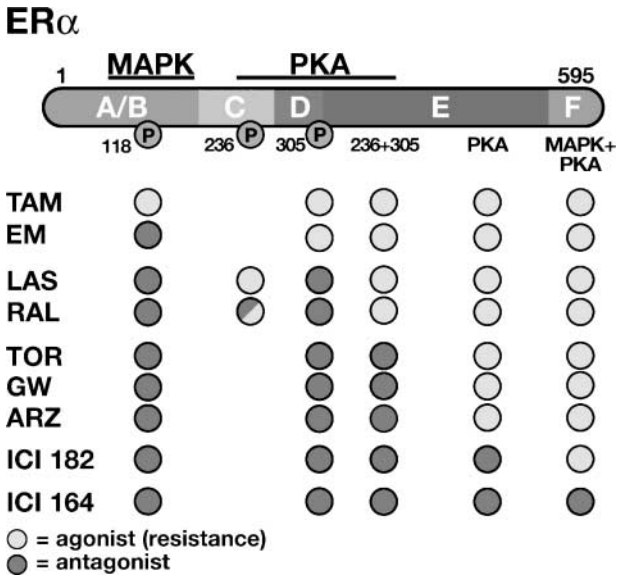

Figure 4. Summary of modifications in ERα that are associated with FRET-predicted resistance to anti-estrogens. The modification sites in ERα by MAPK (S118) and PKA (S236 and S305) are indicated. *White,* when no conformational change in wild-type ERα occurs in response to the various anti-estrogens, indicating that ERα is insensitive toward 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 white. *Gray,* a conformational change in ERα in response to the various anti-estrogens, indicating that ERα is sensitive toward the anti-estrogen (thus, transcriptionally inactive) for a given modification status. *A–F,* various ER domains.

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 (Fig. 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 related to CTS, which was set at 1. Without expression of ER, there was only a slight variation in the background readout of the ERE reporter assay irrespective of the various anti-estrogens, which was elevated approximately 3-fold by PKA (data not shown). In the presence of ERα, treatment with 8-Br-cAMP enhanced the readout of E2 (Fig. 5A), as has been reported before (31). 8-Br-cAMP also enhanced transcriptional activity of ERα in the presence of SERMs tamoxifen, EM-652, lasofoxifene, raloxifene, toremifene, GW5638, and arzoxifene significantly as compared with the respective SERMs under CTS conditions, but this was not significant for the SERDs ICI-182,780 and ICI-164,384 (Fig. 5A and Supplementary Data).[1] 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 (Fig. 3), indicating that an immediate measurement of the conformational change of ERα upon interaction with anti-estrogens provided information on the transactivation of ER that we measured 48 h after addition of the anti-estrogens. Using the YFP-ERα-S118E-CFP mutant, which mimics phosphorylation by MAPK at this site, we observed that the transcriptional activity of ERα-S118E in the presence of tamoxifen, but not for the other tested anti-estrogens, was significantly higher than for wt ERα (Fig. 5B and Supplementary Data),[1] corresponding to the FRET results. The transcriptional readout for the S118E

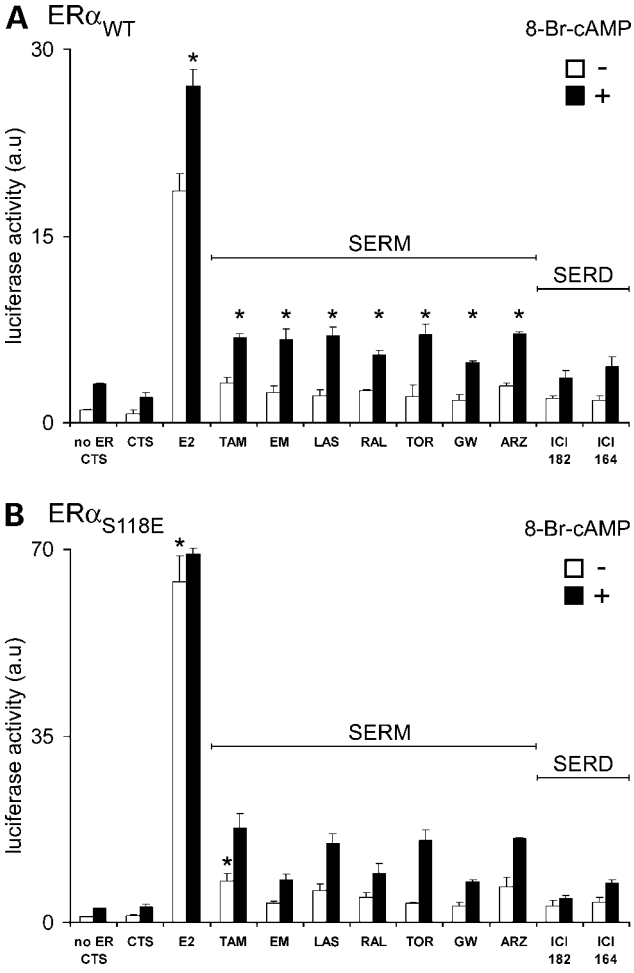

Figure 5. ER transcriptional activity measured by ERE-dependent luciferase assay. U2OS cells were transfected and cultured for 2 d in the presence of either CTS medium, 10^{-8} mol/L estradiol (*E2*) or 10^{-7} mol/L of the indicated anti-estrogens, and subsequently assayed by an ERE-luciferase assay. *Columns,* mean of triplicate experiments; *bars,* SD. For abbreviations of the anti-estrogens, see legend of Fig. 3. **A,** ER-dependent transcriptional activity in YFP-wt-ERα-CFP–transfected U2OS cells. The luciferase activity under conditions of CTS in the absence of ERα was set on 1. *, $P < 0.05$, statistically significant ER-dependent reporter readout between forskolin-treated and untreated samples. **B,** ER-dependent transcriptional activity in YFP-ERα-S118E-CFP–transfected U2OS cells. The luciferase activity under conditions of CTS in the absence of ERα was set on 1. *, $P < 0.05$, statistically significant ER-dependent reporter readout between the S118E mutant and wild-type ERα values.

[1] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

mutant in the presence of PKA stimulator 8-Br-cAMP differed significantly from control wild-type values for all tested anti-estrogens, including the two SERDs (Fig. 5B and Supplementary Data).[1] This was in line with the absence of a conformational change in ERα of the S118E mutant in response to PKA activation in the presence of ICI-182,780, indicating an activated ERα (Fig. 3E), but is in contrast to the conformational change observed under these conditions with ICI-164,384. The latter may well be due to differences in secondary effects between the two SERDs, which take place after the first interaction (that we measured by FRET), but before the transactivation that we measured in the ERE-luc assay (32, 33).

Discussion

In the endocrine treatment of breast cancer, early diagnosis of sensitivity for anti-estrogens will contribute to proper selection of adequate anti-estrogens for individual patients. This is especially relevant because patients benefit from consecutive treatment with different types of anti-estrogens (34, 35), which thus far is taking place on an empirical basis. A profile of the modifications in ERα that are associated to the resistance to anti-estrogens as presented here in Fig. 4 may well contribute to the 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 min after administration and is measured by FRET. Phosphorylation of direct target sites in ERα by PKA leads to resistance predicted by FRET for the anti-estrogens tamoxifen, EM-652, raloxifene, and lasofoxifene (Fig. 4), whereas indirect effects were measured for anti-estrogens GW5638, toremifene, and arzoxifene. The PKA-associated targets outside ERα may well include SRCs, and the effect of PKA-mediated phosphorylation of these cofactors in other cell types might well differ from that in U2OS cells used for FRET experiments, due to different levels of SRCs (15, 36).

Phosphorylation of ERα by PKA and/or MAPK is able to modulate the response to anti-estrogens, but does so differently for the various anti-estrogens, as is depicted in Fig. 4. This can be explained by an anti-estrogen–specific reorientation 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 the transcriptional activation of ERα, in particular for the SERMs tested in this study, and with previous biological findings (29) and structural differences between comparable compounds (Figs. 1A and 4). For the anti-estrogens in the triphenylethylene group (Fig. 1A), the polarity of the side chain (COOH in GW5638 versus -N-C_2H_6 in tamoxifen) correlated with the effect in FRET analysis: resistance to anti-estrogen GW5638 required more stringent conditions than resistance to tamoxifen (Fig. 4). The same applies to arzoxifene and raloxifene, and for ICI-164,384 in comparison with ICI-182,780 (Fulvestrant), where the former steroidal compound contains a more extended side chain and additional conditions seem 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 (Fig. 4) may well involve phosphorylation of cofactors for which it has been shown that the expression levels and/or phosphorylation status affect the extent of E2-mediated transactivation of ERα and its sensitivity to tamoxifen (16). Our results do, however, show that PKA- and MAPK-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 (9). 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.[2] As for the reporter assay, these differences could be explained by a different promoter preference between ERα activated by E2 versus ERα activated by PKA in the presence of anti-estrogens (37). This difference in target preference is also obvious from different RNA expression profiles of breast cancer cells (38) and, more relevant to the present study, also of U2OS cells transfected with ERα (39), when E2 conditions are compared with conditions of different anti-estrogens.

Not only does the profile of modifications required for resistance provide a means for ranking anti-estrogens, it also suggests conditions in specimens of breast cancer patients where resistance to a particular anti-estrogen can be anticipated. We (9) and others (11, 40) have shown that elevated PKA and PAK-1 levels, as well as activation of PKA and PAK-1 in primary breast cancer, are associated with resistance to tamoxifen. Resistance to tamoxifen treatment due to activation of the MAPK pathway has also been shown before (41). The FRET profile based on ERα modifications involved in resistance to tamoxifen, as presented in this study, may explain 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.

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[2] W. Zwart et al., in preparation.

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