



The Dysregulated Pharmacology of Clinically Relevant *ESR1* Mutants is Normalized by Ligand-activated WT Receptor

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ABSTRACT

The estrogen receptor (ER/*ESR1*) is expressed in a majority of breast cancers and drugs that inhibit ER signaling are the cornerstone of breast cancer pharmacotherapy. Currently, aromatase inhibitors are the frontline endocrine interventions of choice although their durability in metastatic disease is limited by activating point mutations within the ligand-binding domain of *ESR1* that permit ligand-independent activation of the receptor. It has been suggested that the most commonly occurring *ESR1* mutations would likely compromise the clinical activity of selective estrogen receptor downregulators and selective estrogen receptor modulators (SERMs) when used as second-line therapies. It was unclear, however, how these mutations, which are likely coexpressed in cells with ER^{WT}, may impact response to ER ligands in a clinically

meaningful manner. To address this issue, we dissected the molecular mechanism(s) underlying *ESR1*-mutant pharmacology in models relevant to metastatic disease. These studies revealed that the response of *ESR1* mutations to ligands was dictated primarily by the relative coexpression of ER^{WT} in cells. Specifically, dysregulated pharmacology was only evident in cells in which the mutants were overexpressed relative to ligand-activated ER^{WT}; a finding that highlights the role of allelism in determining ER-mutant pharmacology. Importantly, we demonstrated that the antagonist activity of the SERM, lasofoxifene, was not impacted by mutant status; a finding that has led to its clinical evaluation as a treatment for patients with advanced ER-positive breast cancer whose tumors harbor *ESR1* mutations.

Introduction

The estrogen receptor (ER/*ESR1*) is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and is expressed in the majority of luminal breast cancers (1, 2). Upon binding an estrogenic ligand, this transcription factor regulates the expression of genes required for cancer cell proliferation and survival. Not surprisingly, drugs that inhibit estrogen actions are the cornerstone of pharmacotherapy of breast cancers that express ER. Among the interventions most commonly used are the selective estrogen receptor modulator (SERM) tamoxifen, a drug which functions as an ER antagonist in breast cancer cells, and aromatase inhibitors (letrozole, anastrozole, or exemestane), competitive inhibitors of CYP19 (aromatase), the enzyme that converts androgens into estrogens (3, 4). Whereas both classes of drug effectively inhibit ER

signaling in breast cancer, it is now standard practice to use aromatase inhibitors in the adjuvant setting as frontline endocrine therapy in postmenopausal patients or in high-risk premenopausal patients when combined with ovarian suppression (4). Tamoxifen is primarily reserved for the adjuvant treatment of premenopausal patients with breast cancer at low risk for recurrence with or without interventions to achieve ovarian suppression (3, 5). These endocrine therapies have had a very significant impact on disease-free and overall survival in patients with breast cancer, although *de novo* and acquired resistance to either type of drug remains a significant clinical issue (6–9). However, the observation that ER remains engaged in the regulation of processes of importance in cancers that have escaped frontline endocrine interventions have led to the continued exploitation of this receptor as a therapeutic target (10).

Fulvestrant, a selective estrogen receptor downregulator (SERD), is used in patients who progress on frontline endocrine therapies and is given as monotherapy or in combination with targeted therapies (11). Drugs of this class function primarily as competitive inhibitors of agonist binding to ER, but their inhibitory activity is reinforced by a drug-induced conformational change that targets the receptor for proteasomal degradation (11, 12). Currently, fulvestrant is the only clinically approved SERD. Whereas this drug is a very effective inhibitor and downregulator of ER expression in cellular and animal models of breast cancer, its clinical utility is limited by its poor pharmaceutical properties and by the need to administer it as a large bolus intramuscularly (13, 14). Furthermore, it is not clear to what extent ER within tumors is occupied by fulvestrant at the maximum doses that can be delivered to patients (15). This has driven the search for oral SERDs (or SERMs) that are as effective as fulvestrant in inhibiting ER activity but which have tissue exposure levels sufficient to saturate the receptor. From these efforts emerged the first-generation oral SERDs GW5638, AZD9496 (NCT02248090, NCT03236874), and

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GDC-0810 (NCT01823835), all of which demonstrated efficacy in late stage disease but whose development has been discontinued (16–21). Other oral SERDs, like RAD1901 (NCT02338349), are currently in clinical development (22, 23).

Whereas the mechanisms underlying resistance to endocrine therapies are varied and complex, it is now clear that gain-of-function point mutations within the ligand-binding domain (LBD) of *ESR1* that permit it to exhibit constitutive transcriptional activity can confer resistance to aromatase inhibitors (24–26). Although rare in primary breast tumors, mutations in the *ESR1* LBD (ER^{mut}) occur in up to 40% of metastatic lesions, a finding that is consistent with their selection by conditions of extreme estrogen deprivation (24, 25, 27–31). Two of the most common mutations, Y537S and D538G (ER^{Y537S} and ER^{D538G}), account for roughly 70% of all *ESR1* mutations identified in patients with metastatic breast cancer (24, 25, 27–31). In addition to constitutively activating transcription, these mutations also exhibit distinct neomorphic activities that likely contribute to disease progression (32, 33). Notwithstanding these important differences, most attention has been focused on how these disease-associated mutations reduce the ER-binding affinity of some clinically important antagonists, an activity that may limit their therapeutic utility (24, 25, 27–31). The development of most SERDs was initiated before the prevalence of ER^{mut} s was fully appreciated, and it is now apparent that, as with fulvestrant, the affinity of ER^{mut} s for even the most contemporary SERDs is substantially reduced (~one order of magnitude; refs. 18, 23, 34). Thus, in addition to addressing whether inhibition of ER with these drugs is a viable approach to inhibit ER-positive, endocrine therapy-refractive disease, there remains an open question as to their efficacy in cancers expressing the ER^{mut} s (24–26). Thus, the primary goal of this study was to define the impact of ER^{mut} s on the pharmacology of ER ligands with a view to prioritizing existing drugs for clinical evaluation in patients. In addition, elucidation of the molecular mechanisms underlying the dysregulated pharmacology of ER^{mut} s was also undertaken with the goal of informing the identification of the next generation of ER modulators for use in the treatment of advanced breast cancer.

Materials and Methods

Cell lines and reagents

Fulvestrant (1047) and Raloxifene (2280) were purchased from Tocris. Estradiol (E8875) and 4-hydroxytamoxifen (H7904) were purchased from Sigma. Bazedoxifene (S2128) was purchased from Selleckchem. Lasofoxifene (HYA0038K), RAD1901 (HY19822A), GDC-0810 (HY12864), and AZD9496 (HY12870) were purchased from MedChem Express. SKBR3 and MCF7 cells used to generate the MCF7^l lines were purchased from ATCC which employs short tandem repeat analysis. MCF7^B and T47D cell lines were published previously (32, 33). The McDonnell laboratory routinely completes PCR-based *Mycoplasma* testing on all cell lines. All cell lines were used within 10–15 passages of thawing.

SKBR3 luciferase transcriptional reporter assay

SKBR3 cells were cotransfected with the 3X-ERE-TATA luciferase reporter gene (35) and expression constructs for either wild-type (WT) or mutant receptors using Eugene transfection reagent (Promega). pCMV- β -gal was used as a control for transfection efficiency. Ligands (dose titration of antagonists in the presence of 1 nmol/L E2) were added 5 hours posttransfection. Cells were lysed 24 hours later and the luciferase and β -gal assays were performed as described previously (36).

MCF7 luciferase transcriptional reporter assay

Cells were cultured in DMEM/F12 supplemented with 8% charcoal dextran-treated FBS. For siRNA transfection experiments, cells were plated over aliquoted siRNAs targeting the 3' UTR of ER (to knock-down endogenous ER) using Lipofectamine RNAiMax (Thermo Fisher Scientific) as per the manufacturer's protocol. After 48 hours, cells were cotransfected with the 7X-ERE-TATA luciferase reporter gene (35) and pCMV- β -gal. Assays were performed as described above for SKBR3, with the exception of 0.1 nmol/L E2 being used as a competitor. A detailed description of the derivation of MCF7 cells and the siRNA sequences utilized in these studies is described in Supplemental Methods.

Cofactor profiling

HepG2 cells were maintained in Basal Medium Eagles containing 8% FBS. For mammalian two-hybrid-based ER cofactor assay, cells were seeded in 96-well plates and transfected with VP16-ER, 5XGal4-Luc3, Gal4DBD-peptide fusion constructs (pM-peptides), and pCMV β -gal using Lipofectin as described previously (21, 37–39). Detailed description of peptide sequences and generation can be found in Supplemental Methods. Cells were then treated with saturating concentrations of ligands (10 μ mol/L for ER antagonists) for 48 hours. Assays were performed as described above. The data were standardized to avoid bias due to signal strength and clustered with the Ward hierarchical clustering method using JMP Pro 13 (SAS). The hierarchical cluster dendrogram was ordered by the first principal component.

Statistical analysis

Two-way ANOVA was utilized, comparing the logIC₅₀ of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. Significant differences ($P < 0.05$) were appropriately noted.

Results

The expression of clinically relevant ER mutants (ER^{mut} s) does not alter the pharmacology of ER ligands in cells expressing ER^{WT}

Prior studies that informed our current understanding of the pharmacology of ER^{mut} s in breast cancer cells were performed in model systems in which the mutants were expressed absent the WT receptor (ER^{WT} ; refs. 24–26, 34, 40). Whereas this may be an appropriate way to model the pharmacology of compounds in cells homozygous for the mutants, this approach does not take into account the heterogeneity of ER^{WT}/ER^{mut} expression in advanced ER-positive breast tumor cells that result from the selective pressure of endocrine therapy (26). To address this issue, we performed a comprehensive analysis of ER ligand pharmacology in cellular models in which ER^{WT} is expressed alone or in combination with ER^{mut} , the latter a scenario that is likely to represent what occurs within the majority of tumor cells in patients with metastatic disease.

To enable the evaluation of ER^{mut} pharmacology, we created MCF7 cell derivatives that express ER^{WT} alone (MCF7^{B-WT}) or both ER^{WT} and individual ER mutants (MCF7^{B-Y537S} and MCF7^{B-D538G}; ref. 32). The structures of the antagonists evaluated in this study are shown in Supplementary Fig. S1 and include most of the clinically relevant SERMs and SERDs that are available (16, 17, 22, 41). Interestingly, there was no change in cellular proliferation of cells expressing ER^{mut} s when compared with the MCF7^{B-WT} which expresses ER^{WT} (Fig. 1A–G). Importantly, a similar result was observed when ER transcription, as

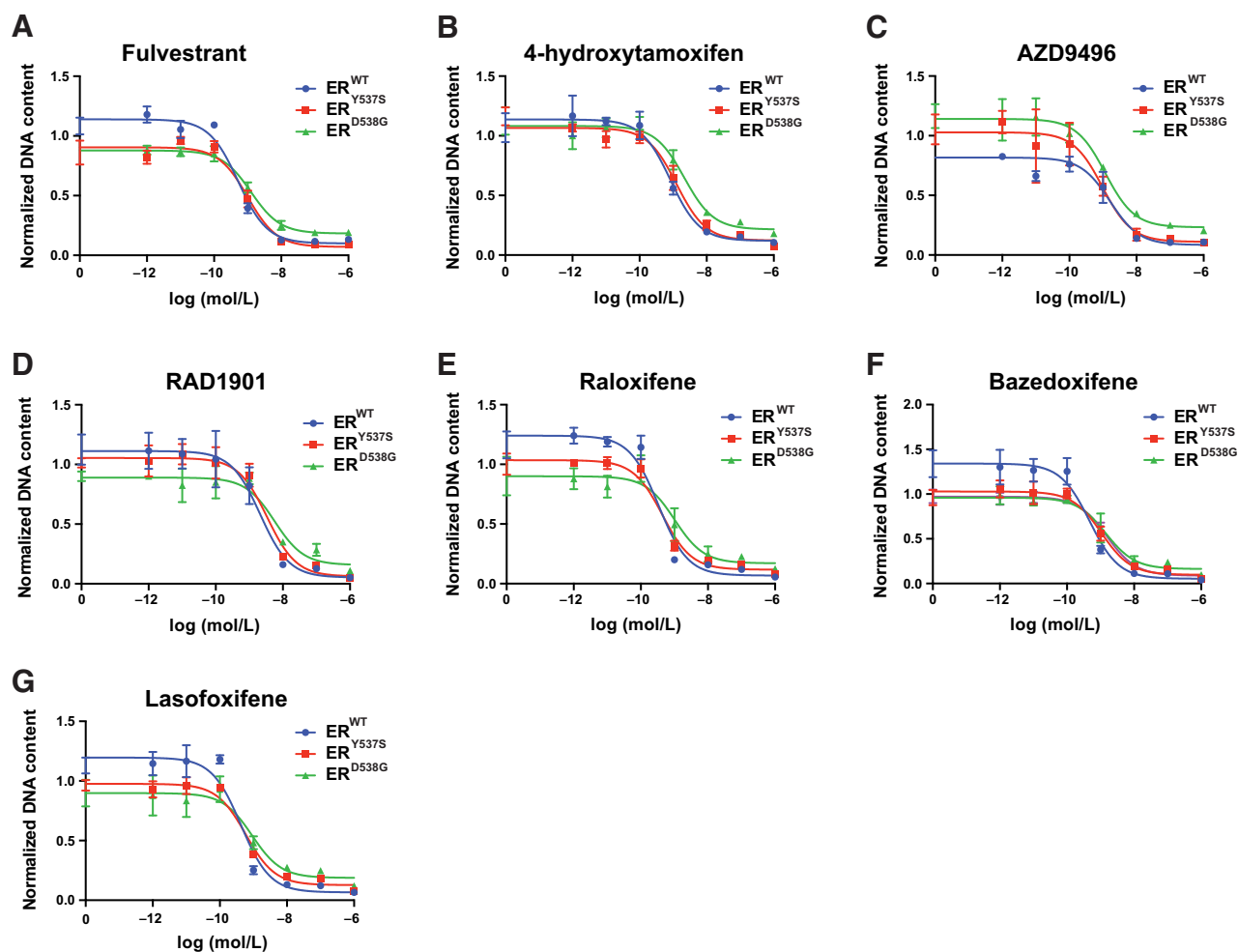


Figure 1.

Cells expressing both the ER^{WT} and ER^{mut}s have similar pharmacologic responses to antiestrogens when compared with cells only expressing ER^{WT}. **A–G**, MCF7^B cells were grown in DMEM-F12 media containing 2% FBS for 7 days while being treated with ER antagonists (10^{-12} – 10^{-6} mol/L). Cellular proliferation was assessed by measuring DNA content (Hoechst stain) and DNA content is normalized to vehicle. Data points are the mean of three technical replicates, and error bars are the SD of these replicates. Data presented are a representative of three independent experiments. Two-way ANOVA was utilized, comparing the logIC₅₀ of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. No significant differences ($P < 0.05$) were determined.

opposed to proliferation, was used to monitor ER activity (Supplementary Fig. S2). Specifically, as expected, basal (ligand-independent) ER transcriptional activity, assessed using an ERE-luciferase reporter, was higher in both MCF7^{B-Y537S} and MCF7^{B-D538G} cells when compared with the isogenic MCF7^{B-WT} cells. Furthermore, as observed in MCF7^{B-WT} cells, treatment with 17 β -estradiol (E2) increased ER-dependent transcriptional activity in both MCF7^{B-Y537S} and MCF7^{B-D538G} cell models (Supplementary Fig. S2A). Notably, however, no significant shift in potency or efficacy was observed for any of the ER ligands tested in this assay when comparing either MCF7^{B-Y537S} or MCF7^{B-D538G} with MCF7^{B-WT} (Supplementary Fig. S2B–S2I). Previous studies which demonstrated shifts in ligand potency in similar models were performed in hormone-stripped media where the activity of ER^{WT} is minimally active (32, 42).

We, and others, have reported extensively on the role of cell context in regulating ER pharmacology, a likely consequence of differences in coregulator expression (43, 44). Thus, we extended our studies to evaluate ER pharmacology in a second model, in which ER^{WT}-

expressing T47D cells were engineered to express ER^{Y537S} or ER^{D538G} in addition to endogenous ER^{WT} (Supplementary Fig. S3A and S3B; ref. 33). Interestingly, of the five mutant clones tested, only ER^{Y537SA} displayed resistance to any of the compounds analyzed (Supplementary Fig. S3A). Furthermore, using In-Cell Western assays in the MCF7^B cells (and derivatives), it was demonstrated that the potency of SERDs as assessed by receptor turnover was not affected by mutation status (Supplementary Fig. S4). We also confirmed that the expression of ER^{WT} and ER^{mut} did not change over time and were maintained under the conditions of our *in vitro* assays (Supplementary Fig. S5; ref. 45). Interestingly, the ER^{Y537SA} clone that displays partial resistance has a higher allelic frequency of ER^{Y537S} compared with the ER^{Y537SB} clone that does not show resistance (Supplementary Fig. S3A and Supplementary Fig. S5). However, our results appear to conflict with *in vitro* studies from others in which it was determined that ER^{Y537S} and ER^{D538G} display an altered response to clinically relevant SERMs and SERDs (24–26, 33, 34, 40).

The antagonist potency of SERDs and SERMs is reduced in cells expressing ER^{mut}s alone

To reconcile the discrepancies between our results presented here and those reported by others, we employed an overexpression model comparable with those that had been used previously to evaluate ER^{mut} pharmacology (24–26, 34, 40). Vectors expressing ER^{WT}, ER^{Y537S}, or ER^{D538G}, together with an ERE-luciferase reporter, were cotransfected into ER-negative SKBR3 breast cancer cells. Western immunoblot analysis was used to confirm that ER^{WT}, ER^{Y537S}, or ER^{D538G} are expressed at comparable levels (Supplementary Fig. S6). Using this model system, we demonstrated, as was observed in MCF7^B cells, that both ER^{Y537S} and ER^{D538G} exhibited constitutive transcriptional activity (Fig. 2A). However, while the efficacy of fulvestrant and 4-hydroxytamoxifen were comparable for all three receptors, the antagonist potency of these two clinically important compounds in cells expressing ER^{Y537S} or ER^{D538G} was reduced by approximately one order of magnitude when compared with ER^{WT} (Fig. 2B and C). The acidic SERDs, AZD9496 and GDC-0810, were found to be inactive as

antagonists on ER^{Y537S}, and indeed the latter compound functioned as a partial agonist in this assay (Fig. 2D and F). This is similar to our previous finding demonstrating that GW7604, a structurally distinct acidic SERD that is the 4-hydroxylated analog of GW5638, had reduced efficacy when assayed in ER^{Y537S}-expressing ovarian cancer cells (46). In addition, the potency of RAD1901, raloxifene, and bazedoxifene were reduced with subtle differences in the pharmacology noted when assayed on either ER^{Y537S} or ER^{D538G} (Fig. 2E and G–H). One of the most interesting findings in this study was that lasofoxifene, a SERM originally developed for the treatment/prevention of osteoporosis, was the only compound found to be as potent an antagonist when evaluated in cells expressing ER^{Y537S} or ER^{D538G} when compared with ER^{WT} (Fig. 2I). This latter observation is in agreement with the findings of a recent study from our group showing that lasofoxifene was as effective an inhibitor of ER^{mut}s as ER^{WT} in cellular models of gynecologic cancers (46). These findings have important clinical implications that could inform the optimal selection of ER antagonists for the treatment of patients with ER^{mut}s in advanced disease.

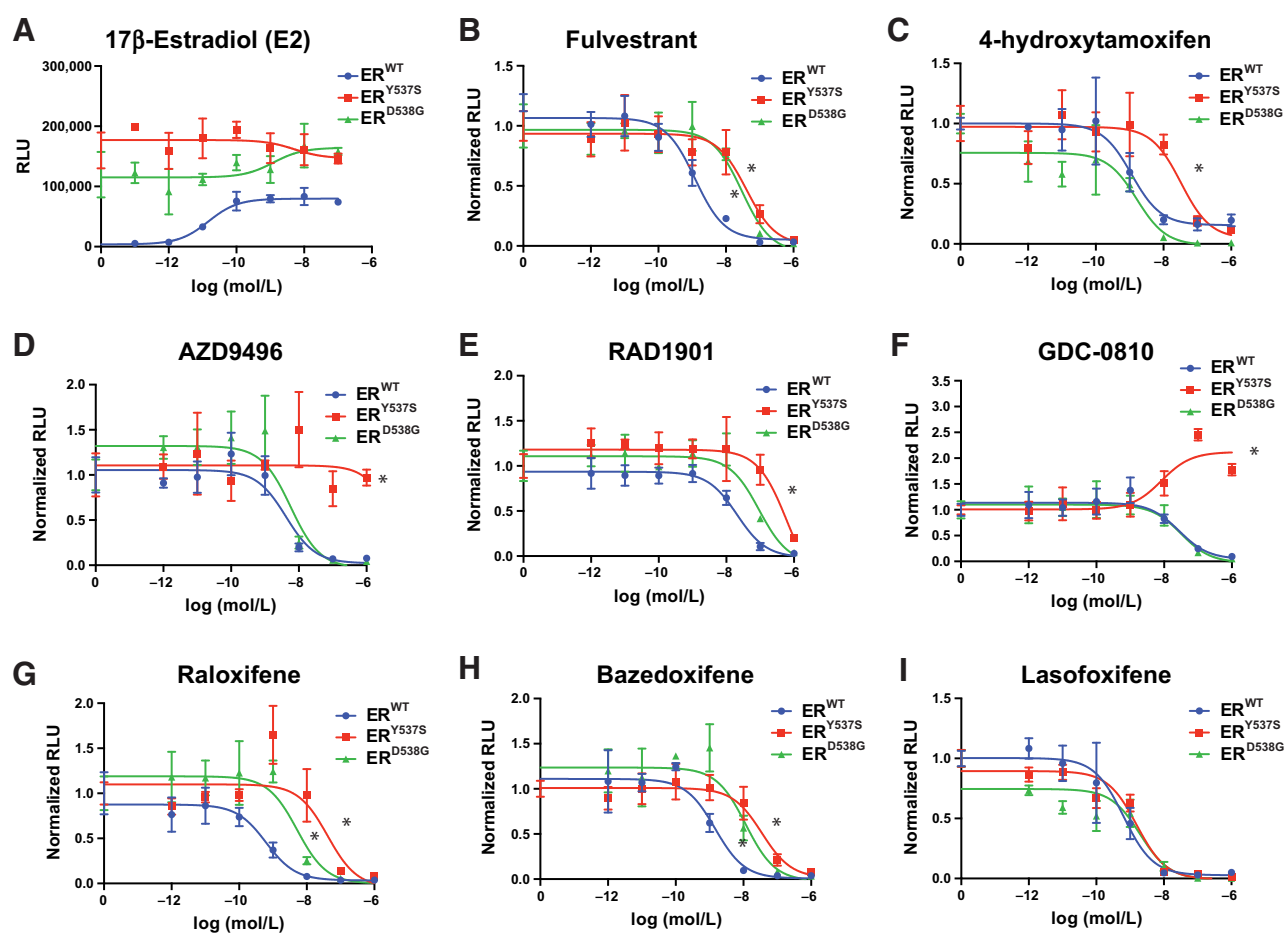


Figure 2.

ER^{mut}s confer antiestrogen resistance when expressed alone. SKBR3 (ER-negative breast cancer) cells were plated in phenol red-free media and transfected with an estrogen-responsive reporter gene (3X-ERE-tata-Luc) in the presence of ER^{WT}, ER^{Y537S}, or ER^{D538G}. After 5 hours, cells were treated with 17 β -estradiol (1 nmol/L; **A**) and ER antagonists (10⁻¹² to 10⁻⁶ mol/L; **B-I**). Firefly luciferase activity was assessed and normalized to β -galactosidase transfection control (Y-axis). Data points are the mean of three technical replicates, and error bars are the SD of these replicates. Data presented are a representative of three independent experiments. Two-way ANOVA was utilized, comparing the logIC₅₀s of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. Significant differences ($P < 0.05$) of the mutant IC₅₀s when compared with that of the WT that were determined by this analysis are represented with a star. For GDC-0810 and AZD9496 on ER^{Y537S}, the highest dose tested (10⁻⁶ mol/L) was used as a surrogate, as the IC₅₀ is greater than this value. The only compound that did not reach a significant difference for either mutant isoform was lasofoxifene.

ER ligands exhibit subtle differences in their ability to facilitate the interaction of ER^{mutts} with coregulators

We next embarked on studies to define the molecular basis of the differences in the pharmacology of ER^{WT}, ER^{Y537S}, or ER^{D538G}. Resolution of this issue, we anticipated, would allow for the optimal use of existing endocrine therapies, and inform the development of the next generation of ER modulators for breast cancer. Receptor conformation has emerged as the primary mechanism by which information flows from a ligand through the receptor to the transcriptional machinery (44, 47, 48). Similarly conformed ER ligand complexes can exhibit diverse activities in different cell contexts as a consequence of the cell-selective expression and differential recruitment of functionally distinct coregulators. Furthermore, subtle changes in ER structure, induced by structurally similar ligands, can result in different transcriptional outputs on individual target genes (37, 49, 50). Thus, it is possible that differences in the pharmacology of the ER^{mutts} noted in cellular models of ER-positive (MCF7^B and T47D cells) or ER-negative (SKBR3 cells) breast cancer could result from differences in cofactor expression and their differential recruitment by ER^{WT}, ER^{Y537S}, or ER^{D538G} upon ligand activation.

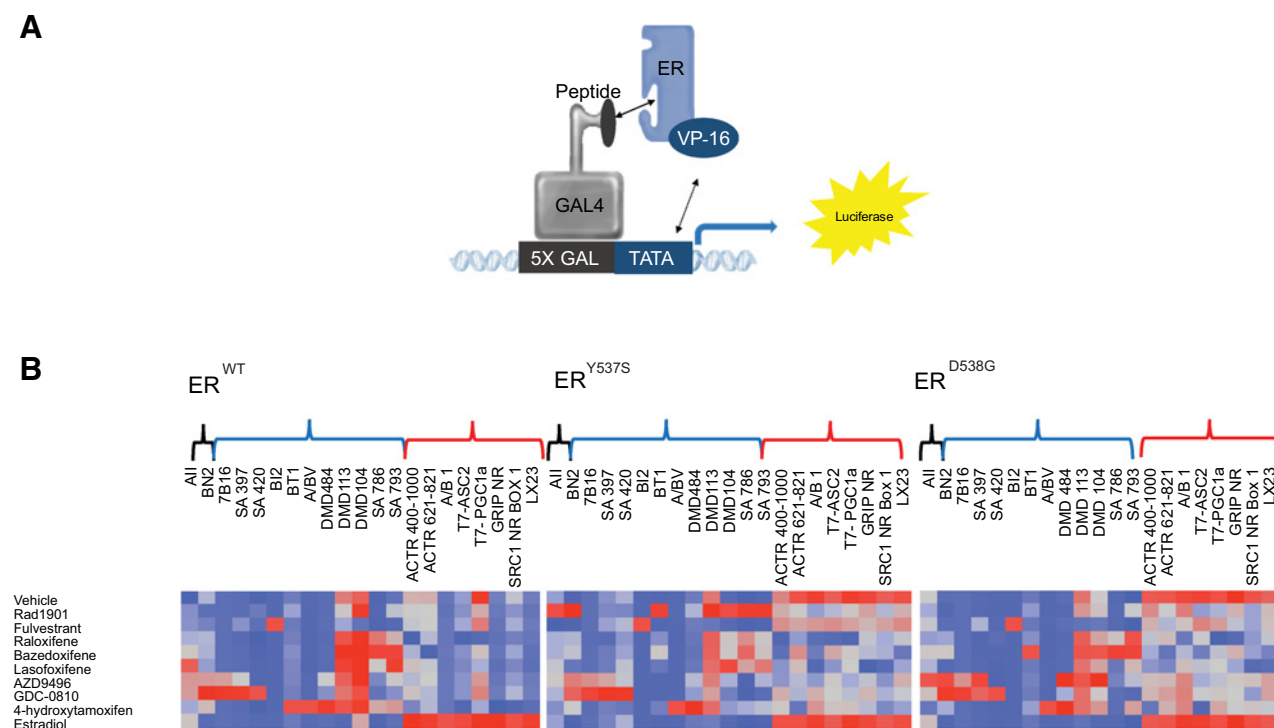
Given the primacy of receptor structure in determining pharmacologic output on ER, we evaluated the impact of SERMs and SERDs on the conformation of different receptor-ligand complexes using a cofactor peptide-binding assay, the utility of which we have described previously (21, 37–39). In this assay, short peptides identified using combinatorial peptide phage display, and peptides derived from the receptor interaction domains of validated coactivators (CoA) and corepressors (CoR), are expressed as GAL4-DBD peptide fusions (Fig. 3A). In addition, a control peptide that interacts with ER in the presence of any ligand (α II) was also utilized. Sequences and detailed information on all of the peptides used are included in the Supplemental Methods. ER^{WT}, ER^{Y537S}, or ER^{D538G} were modified to contain a VP-16 acidic activation domain at their amino termini (Fig. 3A). The interaction of the VP-16-ER proteins with the GAL4-peptide fusions in the presence of each ligand was assessed by measuring transcriptional activity on a GAL4-responsive luciferase reporter.

As expected given their constitutive activity, both mutant receptors interact in a ligand-independent manner, albeit to different degrees, with CoA-like peptides (designated with red brackets), and these interactions are further elevated upon the addition of E2 (Fig. 3B). The CoA interaction profiles of E2-activated ER^{WT}, ER^{Y537S}, or ER^{D538G} are surprisingly indistinguishable. Importantly, the constitutive interaction of the mutants with CoA peptides is only partially attenuated upon the addition of SERMs/SERDs (Fig. 3B). Notably, raloxifene and lasofoxifene appear to be the two most effective inhibitors of CoA-peptide binding to ER^{Y537S} or ER^{D538G}. Subtle quantitative differences in the binding of CoR-peptides (blue brackets) to the receptors in the presence of different ligands were also noted but there were no obvious differences in peptide binding preferences. One exception is the robust interaction of the RAD1901/ER^{Y537S} complex with a subset of the CoR peptides. We infer this to mean that this particular ligand-receptor complex may have an increased ability to recruit corepressors to ER^{mutt}. However, when taken together, it appears that the ER^{WT} can adopt different conformational states upon binding different ligands and these interactions are substantially similar in each of the mutant receptors. Taking into account the limitations of this study (i.e., surfaces on ER not probed with our current technology), we concluded that it is unlikely that the differences in the pharmacology of the ER^{mutts} observed in different cells can be attributed to differential coregulator binding alone.

The altered pharmacology of ER^{mutts} is only evident when their expression in cells exceeds that of the WT receptor

One of the key differences between MCF7^B (and T47D cells) and SKBR3 models is that in the latter cell line, ER^{Y537S} or ER^{D538G} are expressed in the absence of ER^{WT}. We considered it possible that in the MCF7^B (and T47D) cell background, ER^{WT} pharmacology dominates and normalizes the transcriptional activity of the mutants. We considered it likely, therefore, that by overexpressing the mutants relative to ER^{WT} in MCF7 cells, that the altered mutant pharmacology apparent in SKBR3 cells would emerge. To test this hypothesis, we generated MCF7 cells in which ER^{WT}, ER^{Y537S}, or ER^{D538G} expression was regulated in a doxycycline-inducible manner, allowing titratable expression of these proteins (MCF7^I) over endogenous ER^{WT}. Western immunoblot analysis confirmed that the increasing protein expression levels with increasing doses of doxycycline were comparable in each cell line (Supplementary Fig. S7). Considering the pharmacology noted in SKBR3 cells, we selected fulvestrant (potency shift observed with both mutants), AZD9496 (loss of efficacy as an inhibitor of ER^{Y537S}) and lasofoxifene (potency and efficacy unaffected by mutation status) for analysis in these model systems. The transcriptional activity and pharmacology of receptor combinations were assessed using a transfected ERE-luciferase reporter gene (Fig. 4). Relative to its activity on ER^{WT}, it was noted that increased expression of ER^{D538G} resulted in a reduction in fulvestrant potency, and a trend toward reduced potency was also noted with ER^{Y537S} (Fig. 4A). Likewise, the potency of AZD9496 on ER^{Y537S} was considerably reduced upon its overexpression in MCF7^I cells (Fig. 4B). The pharmacology of lasofoxifene was unaffected by receptor expression levels (Fig. 4C). No changes were noted in the activity of any ligand in cells overexpressing ER^{WT} alone (Supplementary Fig. S8). These data suggest that the altered pharmacology of ER^{mutts} may only be manifest when they are expressed at a higher level than ER^{WT}.

A series of experiments were designed to examine whether the altered pharmacology of select ER^{mutts} was solely an artifact of their overexpression or whether overexpression was required to outcompete a normalizing effect of ER^{WT}. To this end, the impact of altering the expression of ER^{WT} relative to ER^{mutts} was evaluated initially in SKBR3 cells. Western blots were used to confirm that the desired changes in receptor/mutant expression were accomplished (Supplementary Fig. S9). Consistent with the results presented in Fig. 2, the potency of fulvestrant and AZD9496 was reduced in SKBR3 cells expressing ER^{Y537S} or ER^{D538G} alone (Fig. 5A and B). However, as the expression of the ER^{WT} was increased to comparable levels with ER^{mutt}, the pharmacology of fulvestrant and AZD9496 was normalized to that which mirrored their activity on ER^{WT} (Fig. 5A and B). Lasofoxifene antagonist efficacy remained unchanged as the expression levels of ER^{WT} and ER^{mutt} were altered (Fig. 5C). It is important to note that in the absence of ligand, the constitutive activity of the mutant receptors is observed even when ER^{WT} is present (Supplementary Fig. S10). Thus, in the absence of hormone, the mutant is functionally in excess indicating that ER^{WT} activity, and not its expression alone, is required to achieve the normalization of ER pharmacology noted. This finding supports previous data in the literature that demonstrate that under conditions of extreme hormone deprivation, the resistance of ER^{mutts} to ER ligands is not affected by the presence of ER^{WT} (32, 42). To support these findings, we performed an analogous experiment in MCF7^I cells (Fig. 5D–F). In this context, we expressed ER^{mutts} in cells expressing endogenous ER^{WT} and consistent with our prior observations, the pharmacology of cells expressing ER^{WT} or ER^{mutts} were found to be indistinguishable. However, when the expression of the endogenous receptor was reduced using a siRNA directed against the 3' UTR of

**Figure 3.**

Differential cofactor recruitment reveals modest changes in overall receptor conformation between the WT and mutant receptors. **A**, A mammalian two-hybrid assay was used to evaluate ligand-dependent recruitment of peptides that mimic ER coregulators. **B**, Hep-G2 cells were cotransfected with VP-16-tagged WT or mutant ER, Gal4DBD-tagged peptides and a Gal4-responsive reporter gene and pCMV β -gal. Twenty-four hours later, cells were treated with saturating concentrations of ligands (10 μ M/L) and incubated for 48 hours. Normalized response, which was obtained by normalizing luciferase activity to β -galactosidase activity, was used as input for Ward hierarchical clustering. Heatmaps of mutant ERs are reordered to match the WT receptor. Results demonstrated a change in receptor conformation in response to ER-activating mutations. At the top of the graph, there are three classes of peptides: ligand indiscriminate (black), peptides associated with receptor inhibition (blue), and receptor activation (red). Data presented are a representative of two independent experiments.

the ER mRNA, the mutant pharmacology emerged. Doxycycline-induced expression of ER^{WT} and ER^{mut} and the effectiveness of the siRNA-mediated knockdown of endogenous ER protein levels were confirmed by In-Cell Western (Supplementary Fig. S11). As observed in SKBR3 cells, the constitutive activity of the mutant receptors was not diminished by coexpression of ER^{WT} (Supplementary Fig. S12). Importantly, the impact of ER^{WT}/ER^{mut} status on ligand pharmacology, established using a synthetic reporter assay, was also seen when the activity of ligands were assessed using endogenous target gene transcription on *PgR* and *GREB1* (Supplementary Fig. S13). Together, these results indicate that activated ER^{WT} can normalize the pharmacology of ER^{Y537S} and ER^{D538G}, and that tumor response to ER ligands following aromatase inhibitor therapy will depend on the relative coexpression of ER^{mut} and ER^{WT} in breast cancer cells. For reasons yet to be determined, the pharmacology of the SERM lasofloxifene is not affected by mutant status.

Discussion

The goal of this study was to define the molecular basis for the altered pharmacology exhibited by the most clinically relevant ER^{mut}s, information we anticipate could inform the selection of existing drugs for use in patients with advanced ER-positive breast cancer whose tumors harbor these mutations. In cell-based models of breast cancer, we made the important observation that when compared with ER^{WT}, the pharmacology, most notably antagonist potency, of these mutants

was significantly impacted by the relative coexpression of ER^{WT} and ER^{mut}. Previously, we and others have observed that the potency of existing ER antagonists was reduced in cells expressing either of the two most frequently occurring ER^{mut}s (ER^{D538G} and ER^{Y537S}; refs. 24, 25, 34, 40, 46). In this study, we have demonstrated that such differences are dependent on the relative expression level of both the ER^{WT} and ER^{mut}s and are only apparent under conditions where ER^{mut}s are substantially overexpressed relative to ER^{WT}. Given the previously reported neomorphic activities of the ER^{mut}s, it is possible that the differences in response to ER ligands may only be manifest on select endogenous target genes (32, 33). However, in our system, the activity of the mutants in the transcriptional reporter assays mirror their activities, in the presence of various ligands, when cell proliferation or endogenous target gene transcription is used as the readout. These findings are significant as prior studies that have informed our current understanding of the importance of ER^{mut}s in the pharmacotherapy of breast cancer were performed in cells expressing only ER^{mut}s in the absence of the ER^{WT} (24–26, 34, 40).

Whereas we have been able to confirm using several experimental models that ER^{WT} normalizes the activity of coexpressed ER^{mut}s, the mechanism(s) by which this activity occurs is elusive. One possibility is that ER^{WT} preferentially dimerizes with ER^{mut}s and simply outcompetes ER^{mut} homodimers. However, such a simple mechanism would require that the ER^{mut}s would exhibit reduced homodimerization/heterodimerization activity. It is more likely that in cells where ER^{WT} and ER^{mut} are present, and assuming no differences in dimerization

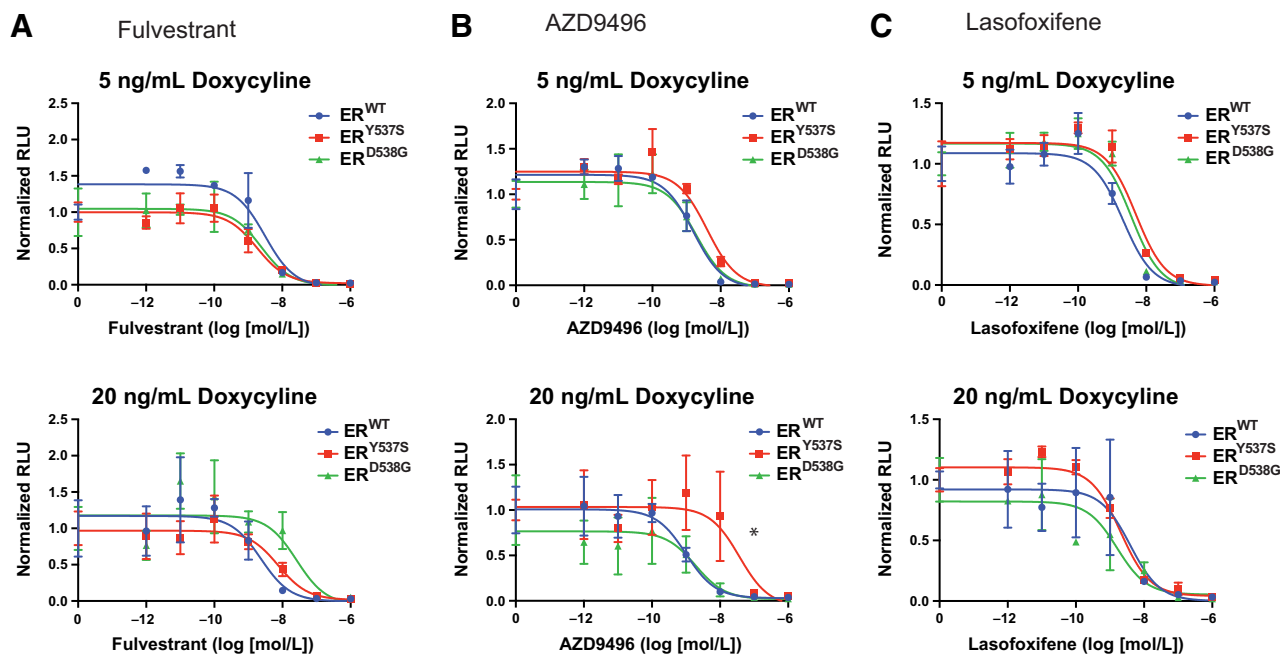


Figure 4.

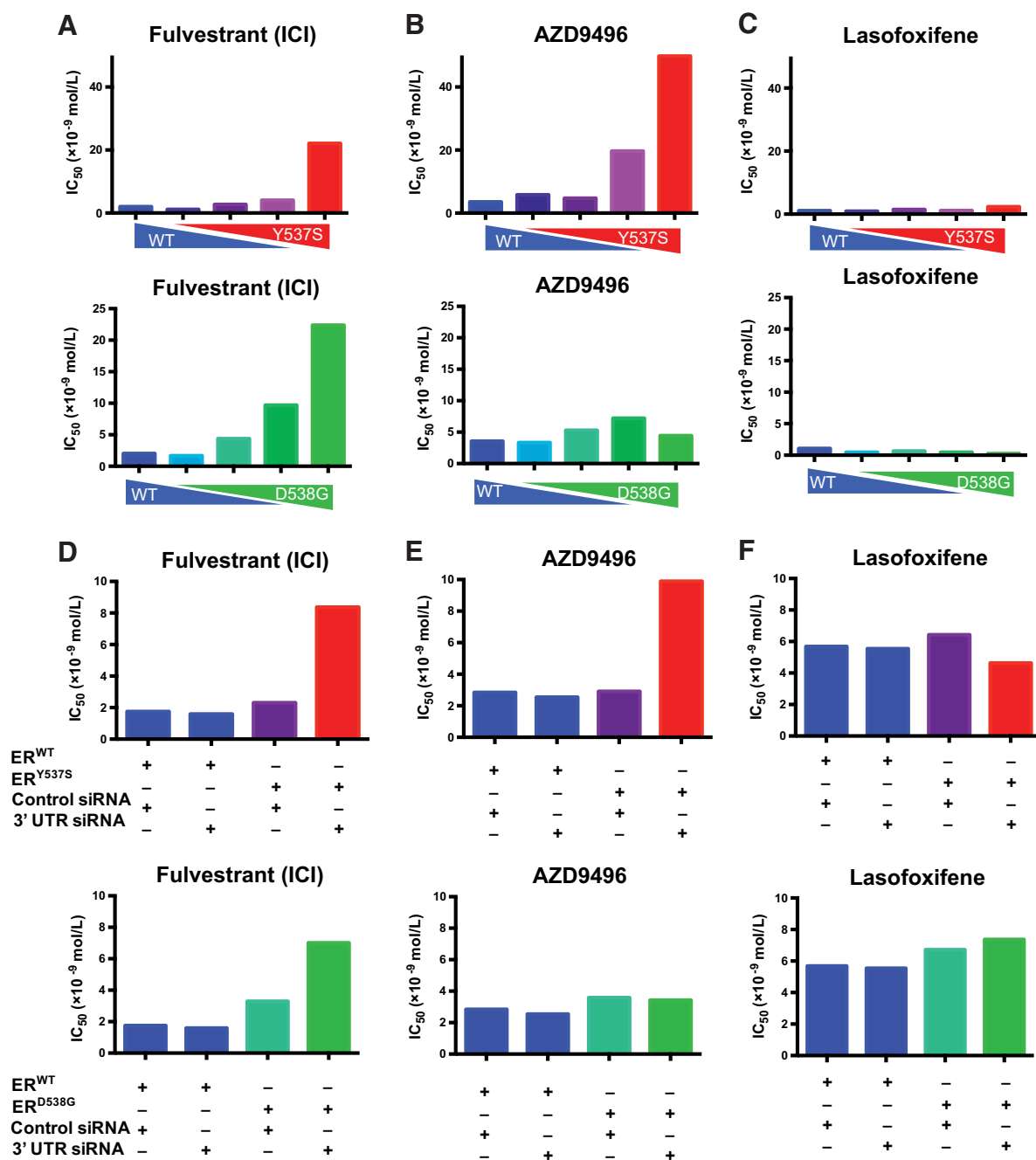
The altered pharmacology of ER^{mut}s can be manipulated by their expression level. **A–C**, MCF7 (ER-positive breast cancer) cells were engineered to express the WT or mutant receptors in a dose-dependent manner in response to doxycycline treatment over the endogenous ER^{WT}. Cells were plated in phenol red-free media for 48 hours with doxycycline 5 or 20 ng/mL as indicated and then transfected with an estrogen-responsive reporter gene (7X-ERE-tata-Luc). After 5 hours, cells were treated with 17 β -estradiol (0.1 nmol/L) and ER antagonists (10^{-12} to 10^{-6} mol/L). Firefly luciferase activity was assessed and normalized to β -galactosidase transfection control (Y-axis). Data points are the mean of three technical replicates, and error bars are the SD of these replicates. Data presented is a representative of three independent experiments. Two-way ANOVA was utilized, comparing the logIC₅₀ of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. Significant differences ($P < 0.05$) of the mutant IC₅₀s when compared with that of the WT that were determined by this analysis are represented with a star.

ability, that the majority of the receptor (75%) would exist in an ER^{WT}/ER^{WT} or ER^{WT}/ER^{mut} complex and that the presence of the WT receptor normalizes the response (potency) to ligands. The recent cryo-EM structure of the ER coregulator complex is informative as to how ER^{WT} may normalize the activity of the mutant (51). Specifically, it was observed that the establishment of a productive transcription complex requires each monomer in an ER dimer to engage a p160 coregulator (i.e., SRC-3) to establish a platform upon which p300 can be recruited. Thus, in an ER^{WT}/ER^{WT} or ER^{WT}/ER^{mut} complex, the conformational change(s) induced in ER^{WT} by antagonists would result in the expulsion of one or two SRC proteins from the complex and a productive transcriptional complex could not form. Using peptide-binding/cofactor-binding studies, we have demonstrated that the interaction of ER^{mut}s with CoAs is substantially inhibited upon the addition of saturating concentrations of most antagonists, explaining why the efficacy of existing inhibitors is not affected by the most commonly occurring mutations.

There are several immediate clinical implications of this work. It is clear that selection for ER^{mut}s by estrogen deprivation (aromatase inhibitor) manifests as resistance. However, given that most mutants would be expected to be coexpressed in breast cancer cells with ER^{WT}, it was unclear how they would impact the pharmacology of fulvestrant and other clinically important SERDs and SERMs. Our findings suggest that in ER^{WT}-expressing cells, the presence of a mutant receptor is unlikely to have any significant impact on response to existing antagonists unless its expression vastly exceeds that of ER^{WT} (or in cells in which it is solely expressed). Some clinical data supports

that assertion (29). Specifically, baseline and on treatment evaluation of ER^{mut}s in circulating tumor DNA was evaluated and correlated to fulvestrant response (alone or in combination with PI3K inhibition) as a part of the Phase II FERGI study (NCT01437566). The findings of this study demonstrated that median *ESR1* allele frequency was low at 0.45% and as such progression-free survival was not different in patients with ER^{mut}s compared with patients with ER^{WT} (29). Conversely, in the PALOMA-3 study (NCT01942135), there were observed differences in fulvestrant progression-free survival in response to mutation status (52, 53). Interestingly, these studies report a higher whole tumor allele frequency of ER^{mut}s, with a reported expression fraction of 0.10 (or 10%). This study also suggested that ER^{mut} containing clones were a small fraction of the whole tumor and as such the low allele frequency estimate was not representative of each individual cell. It is clear that cells expressing ER^{Y537S} emerged in the fulvestrant only arm of the PALOMA-3 arm and this has been taken as definitive evidence that this mutation reduces the potency of fulvestrant. We propose the alternative hypothesis that fulvestrant exposure is not sufficient to efficiently occupy ER^{WT}, or the ER^{mut}s, and that cells expressing the constitutively active mutants have a fitness advantage.

In our study, dysregulated ER^{mut} pharmacology is only manifest when the expression of the mutant receptor(s) exceeds that of its ER^{WT} counterpart. It is not clear how often this occurs in individual tumor cells and further research is needed to adequately assess allelism at the cellular level. Mutations in *ESR1* can be detected in clinical tumor samples and circulating tumor DNA using next-generation sequencing and droplet digital PCR (24, 25, 27–31, 52, 53). However, these

**Figure 5.**

The altered pharmacology of ER^{mut}s is only evident when expressed at a level higher than the WT receptor. **A-C**, SKBR3 (ER-negative breast cancer) cells plated in phenol red-free media supplemented with charcoal-stripped serum and transfected with an estrogen-responsive reporter gene (3X-ERE-tata-Luc) in the presence of different WT to mutant ER (Y537S or D538G) construct ratios. After 5 hours, cells were treated with 17 β -estradiol (1 nmol/L) and ER antagonists (10⁻¹² to 10⁻⁶ mol/L). Firefly luciferase activity was assessed and normalized to β -galactosidase transfection control. The IC₅₀s of each dose-response curve are plotted. Two-way ANOVA was utilized, comparing the logIC₅₀s of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. Significant differences ($P < 0.05$) of the mutant IC₅₀s when compared with that of the WT that were determined by this analysis are represented with a star. **D-F**, MCF7 (ER-positive breast cancer) cells were engineered to express the WT or mutant receptors in response to doxycycline treatment. Cells were plated in phenol red-free media for 48 hours with doxycycline and siRNA (control or targeting 3' UTR to knockdown the endogenous WT receptor) and then transfected with an estrogen-responsive reporter gene (7X-ERE-tata-Luc). After 5 hours, cells were treated with 17 β -estradiol (0.1 nmol/L) and ER antagonists (10⁻¹² to 10⁻⁶ mol/L). Firefly luciferase activity was assessed and normalized to β -galactosidase transfection control. The IC₅₀s of each dose-response curve are plotted. Two-way ANOVA was utilized, comparing the logIC₅₀s of all three independent experiments, to determine whether there were significant differences between the WT and mutant receptors. Significant differences ($P < 0.05$) of the mutant IC₅₀s when compared with that of the WT that were determined by this analysis are represented with a star. Data presented are a representative of three independent experiments.

assays are not designed to establish allelic frequency (homozygous vs. heterozygous *ESR1* alleles) on a single cell basis. The likely importance of ER^{mut} allelism was suggested in a recent study that revealed a propensity for a loss of heterozygosity of ER^{WT} when an ER^{mut} is also present in the tumors of patients on endocrine therapies (54). Specifically, in patients with breast cancer that harbored *ESR1* mutants, LOH of the WT allele drove 78% of *ESR1* mutant-specific allele balance, while background loss of alleles for nonmutant containing tumors also on endocrine therapy was only 30%. These data suggest that the ER^{WT} is important in determining ER^{mut} response to therapy and that tumors having a lower expression ER^{WT} have a survival advantage. The inability to assess ER^{WT}/ER^{mut} allelism in a facile manner reinforces the need to understand the relationship between ER expression level and ligand potency/efficacy as a means to select/develop pharmaceuticals for use in the treatment of patients whose tumors harbor ER^{mut}s.

It is likely that even in situations where the ER^{mut}s is expressed at a higher level compared with ER^{WT}, it is only of significance when potency is a limiting property of a drug (i.e., fulvestrant). However, our work suggests that most of the liabilities of the mutants can be mitigated by increasing the dose (assuming dose-proportional exposure and tolerable side-effect profile) of individual drugs as antagonist efficacy is not compromised by the expression of the most commonly occurring ER^{mut}s. This highlights the importance of drug exposure when considering new/existing drugs for use in the treatments of patients with mutant receptors. One approach that has been developed to address the reduced affinity of the mutants is to develop selective estrogen receptor covalent antagonists (SERCA; refs. 55, 56). The first of this new class of drugs, currently in clinical development, essentially converts tamoxifen into a covalent ER binder, thus mitigating the impact of the mutation on binding affinity. One SERCA is currently in clinical trials for patients with metastatic breast cancer progressing on endocrine therapy (NCT03250676; ref. 56). However, it is likely that locking the receptor in a “tamoxifen-induced conformation” is going to result in the selection of cancer cells which support the partial agonist activity of tamoxifen, an activity that is associated with acquired resistance (21, 57, 58).

The SERM lasofoxifene appears to have attributes that would make it particularly useful in patients where there is concern as to the contribution of ER^{mut}s to drug response. In this study, and an earlier study in gynecologic cancers, we demonstrated that this drug is an efficient antagonist whose actions are not influenced by mutant status (46). Lasofoxifene was initially developed for the treatment of climacteric symptoms and osteoporosis associated with menopause. It is currently under evaluation in the ELAINE trial (NCT03781063) to assess its efficacy compared with fulvestrant, post aromatase and CDK4/6 inhibitor therapy, as a treatment for patients whose tumors harbor ER^{mut}s. We also noted that the acidic SERDs [represented by GDC-0810, AZD9496, and previously GW7604 (46)] are ineffective

inhibitors of ER^{Y537S}. Thus, it is likely that the efficacy of this class of drugs will be diminished as the allelic frequency of ER^{Y537S} increases in patients. Currently, there are numerous new ER modulators, including LSZ102 (NCT202734615), AZD9833 (NCT03616586), GDC-9545 (NCT03332797), SAR429859 (NCT03284957), G1T48 (NCT03455270), and Zn-C5 (NCT03560531) under evaluation in the clinic (59). Notwithstanding the potential impact of ER^{Y537S} on the response to acidic SERDs, it appears as if the mutant status of tumors may not be a significant issue for drugs that achieve significant exposure to offset the decreased potency noted (for all but lasofoxifene). We believe the studies presented herein should emphasize approaches to achieve maximal drug exposure in tumors as opposed to developing new molecules that demonstrate increased affinity for the mutant receptors.

Disclosure of Potential Conflicts of Interest

K.J. Andreano has ownership interest (including patents) in Sermonix Pharmaceuticals. R. Jeselsohn reports receiving a commercial research grant from Lilly. M. Brown is a consultant at H3 Biomedicine, GTx, and Aleta Biotherapeutics; SAB member at Kronos Bio and GV20 Therapeutics; and reports receiving a commercial research grant from Novartis. S. Sammons is an advisor at Sermonix Pharmaceuticals and reports receiving a commercial research grant from Eli Lilly and Company. S.E. Wardell is a consultant at Zentalis Pharmaceuticals and has ownership interest (including patents) in Radius Health. D.P. McDonnell has ownership interest (including patents) in Sermonix and Radius Health. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.J. Andreano, J.G. Baker, S. Artham, S. Oesterreich, S. Sammons, S.E. Wardell, J.D. Norris, D.P. McDonnell

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The Dysregulated Pharmacology of Clinically Relevant *ESR1* Mutants is Normalized by Ligand-activated WT Receptor

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