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Bazedoxifene exhibits antiestrogenic activity in animal models of tamoxifen resistant breast cancer; implications for treatment of advanced disease

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Abstract

Purpose—There is compelling evidence to suggest that drugs that function as pure estrogen receptor (ER α) antagonists, or that down regulate the expression of ER α , would have clinical utility in the treatment of advanced tamoxifen and aromatase resistant breast cancer. Whereas such compounds are currently in development, we reasoned, based on our understanding of ER α pharmacology, that there may already exist among the most recently developed Selective Estrogen Receptor Modulators (SERMs) compounds that would have utility as breast cancer therapeutics. Thus, our objective was to identify among available SERMs those with unique pharmacological activities and to evaluate their potential clinical utility using predictive models of advanced breast cancer.

Experimental Design—A validated molecular profiling technology was used to classify clinically relevant SERMs based on their impact on ER α conformation. The functional consequences of these observed mechanistic differences on (a) gene expression, (b) receptor stability, and (c) activity in cellular and animal models of advanced endocrine resistant breast cancer was assessed.

Results—The high affinity SERM bazedoxifene was shown to function as a pure ER α antagonist in cellular models of breast cancer, and effectively inhibited the growth of both tamoxifen sensitive and resistant breast tumor xenografts. Interestingly, bazedoxifene induced a unique conformational change in ER α that resulted in its proteasomal degradation, although the latter activity was dispensable for its antagonist efficacy.

Conclusion—Bazedoxifene was recently approved for use in the European Union for the treatment of osteoporosis and thus may represent a near-term therapeutic option for patients with advanced breast cancer.

Keywords

estrogen receptor; SERM; bazedoxifene; SERD

Introduction

The primary goal of endocrine therapies in breast cancer is to block the transcriptional activity of estrogen receptor (ESR1, ER α) by either (a) inhibiting CYP19A1 (aromatase), the enzyme responsible for the conversion of androgens to estrogens, or (b) directly

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interfering with the transcriptional activity of the receptor. When used as primary interventions both approaches are similarly efficacious and improve outcome to the same degree. Notable, however, is the observation that a significant number of patients that exhibit *de novo* or acquired resistance to the antiestrogen tamoxifen subsequently respond to aromatase inhibitors (1–3). This finding highlights the continued dependence on ER α signaling within tumors in advanced disease, raising the possibility that even in tumors that are resistant to tamoxifen and aromatase inhibitors, the ER α signaling axis may remain a viable target.

The primary mechanisms underlying resistance to tamoxifen and aromatase inhibitors are somewhat different. However, it now appears that a common feature in either case involves the reprogramming of ER signaling pathways within tumor cells, allowing them to continue to capitalize on progrowth and survival pathways downstream of ER α . The currently available aromatase inhibitors effectively reduce the production of both peripherally and intratumorally generated estrogens, and resistance to these agents is not associated with an inability to effectively suppress estrogen production (4, 5). Rather, there is accumulating evidence that exposure of ER α -positive breast cancer cells to aromatase inhibitors renders them hypersensitive to either residual amounts of steroidal estrogens, dietary/environmental compounds with estrogenic activity, or to endogenously produced molecules that exhibit estrogenic activity but which do not require aromatization (6–8). With respect to the latter, we and others have shown that the oxysterol 27-hydroxycholesterol, a primary metabolite of cholesterol, exhibits robust estrogenic activity and is produced at levels that are likely to promote substantial tumor growth [(9) and unpublished data]. Thus, it is anticipated that high affinity ER α antagonists, or compounds that ablate ER α expression, would have clinical activity in aromatase resistant disease.

The mechanisms underlying resistance to tamoxifen are complex. When originally developed, tamoxifen was classified as an “antiestrogen”, a compound that competitively inhibited the binding of estrogens to ER α , thus freezing it in an apo-conformation. However, this simple model failed to explain how tamoxifen could manifest agonist activity in bone and in the endometrium, and did not explain the withdrawal responses noted in some breast cancer patients who progressed while on tamoxifen (10–13). It is now known that tamoxifen is not an “antiestrogen” *per se*, but rather a Selective Estrogen Receptor Modulator (SERM), a compound whose relative agonist/antagonist activity can differ between cells. Thus, while it is capable of functioning as an antagonist in the breast, tamoxifen can also manifest agonist activity in other tissues. This observation was significant in light of the early studies that suggested that resistance to tamoxifen represented a “switch” within breast cancer cells that enabled them to recognize tamoxifen as an agonist as opposed to an antagonist (14). Supported by a wealth of more recent preclinical and clinical data, it now appears that it is the ability of breast cancer cells to support the agonist activity of tamoxifen that is the primary driver of resistance. The mechanisms underlying the molecular pharmacology of tamoxifen in breast cancer are now well understood and have been informative with respect to the development of resistance. Specifically, it has been determined that tamoxifen does not freeze ER α in an apo-state but rather it induces a conformational change that enables the presentation of unique protein-protein interaction surfaces on the receptor that dictate its transcriptional coregulator binding preferences (15). Thus, the agonist activity of tamoxifen depends on the relative expression and/or the activity of functionally distinct coregulators in target tissues (16, 17). Whereas the specific coregulators that enable the agonist activity of tamoxifen remain elusive, there is considerable additional data to support this hypothesis. Most notably, by screening for compounds that induced a conformational change in ER α that did not present the protein-protein interaction surfaces required for tamoxifen action, we identified DPC974/GW5638 (18, 19). This compound was shown to be an effective inhibitor of ER action in xenograft

models of both tamoxifen sensitive and tamoxifen resistant breast cancers and yielded positive results in a heavily treated population of breast cancer patients with endocrine treatment resistant disease (20). Similarly, it has been shown that fulvestrant (ICI 182,780) also induces a unique conformational change in ER α , likewise functions as an antagonist in tamoxifen resistant xenograft models, and demonstrates efficacy in patients with advanced disease (15, 21, 22). Together these data suggest that by manipulating ER α structure and influencing coregulator engagement it is possible to develop compounds with useful activities in breast cancer.

Clearly, there is a very strong rationale to support the targeting of ER α in the setting of tamoxifen and aromatase resistant disease. Considering the current state of the art in this field, it is likely that drugs with utility in these disease states can be identified by screening for agents that (a) bind ER α with an affinity high enough to outcompete both endogenous and exogenous estrogens, (b) exhibit minimal agonist activity on those genes on which tamoxifen agonist activity is manifest in resistant breast cancer cells and (c) induce a structural change in ER α that disables the protein-protein interaction surfaces required for tamoxifen agonist activity. With these criteria in mind, we performed a comparative functional analysis of clinically relevant ER ligands, a study that revealed that bazedoxifene, a recently developed high affinity orally active SERM, inhibits ER α action in both tamoxifen sensitive and tamoxifen resistant xenograft models. This drug was recently approved for use in the EU for the treatment of osteoporosis. The findings presented herein should inform near term clinical studies of this drug in patients with advanced breast cancer.

Materials and Methods

Reagents

ER ligands included 17 β -estradiol (E2 – Sigma), ICI 182,780 (ICI – Tocris), 4-hydroxytamoxifen (4OHT – Sigma), raloxifene (Ral – Sigma) and endoxifen (endox – Sigma). Lasofoxifene (Laso) and bazedoxifene (BZA) were gifts from Wyeth, Inc (now Pfizer). Ligands were dissolved in ethanol or DMSO. Cycloheximide and MG132 were purchased from Sigma.

Cell culture

Cell lines were maintained in DMEM/F12 (MCF7) or RPMI (BT483, rBT474, and SKBR3) media (Invitrogen) supplemented with 8% FBS (Gemini), non-essential amino acids (Invitrogen), and sodium pyruvate (Invitrogen). Parental cell lines were obtained from ATCC, which authenticates cell lines by short tandem repeat profiling. Lapatinib resistant rBT474 cells and LTED MCF7 cells were maintained as described (23, 24). Cells were plated for experiments in media lacking phenol red and supplemented with 8% charcoal stripped FBS (Gemini). After 48 hours cells were treated with ER ligands and/or inhibitors as indicated, and were harvested for immunoblot or real time quantitative PCR analysis 24 hours after treatment.

Immunoblot analysis

Protein expression was analyzed as described (25) using antibodies sc-6259 (cytokeratin 18), sc-20680 (lamin A), sc-5546 (α -tubulin) and sc-8005 (ER α) (Santa Cruz Biotechnology).

RNA isolation and real time quantitative PCR

RNA isolation and analysis was performed as described (26). mRNA abundance was calculated using the $\Delta\Delta C_T$ method (27). Primer sequences are available upon request.

Mammalian 2 hybrid analysis

Transfections and analysis were performed as previously described (26).

Proliferation assays

3×10^3 MCF7 cells were plated per well in 96 well culture plates. One plate was decanted and frozen on day 1. Remaining plates were treated as indicated on days 1, 4, and 6, with identically treated plates harvested on days 1, 6, and 8. DNA content was detected using a FluoReporter assay (Invitrogen) per manufacturer's instructions.

Adenovirus production

Creation, production, and purification of an adenovirus expressing human ER α were previously described (28). 48 hours after plating, cells were infected with ER α adenovirus using multiplicity of infection (MOI) 0 or 100. Cells were treated with ER ligands as indicated immediately following infection and were harvested for immunoblot or real time quantitative PCR analysis 24 hours later. For proliferation assays, cells were infected and treated immediately and 2 or 5 days later.

Xenograft tumor analyses

All procedures were approved by the Duke University Institute for Animal Care and Use Committee. E2 dependent MCF7 or tamoxifen stimulated TamR tumors were initiated in the axial mammary gland of female NU/NU mice (~6 weeks age) by serial transfer. Briefly, ovariectomized recipient mice received hormone treatment via a timed release pellet (0.72 mg E2 or 5 mg tamoxifen/60 days – Innovative Research of America) implanted sc. 2 days later, an MCF7 or TamR tumor ~0.8–1 cm³ volume was excised under sterile conditions from a euthanized donor mouse, diced to ~2mm³ sections and implanted into the axial mammary gland under anesthesia (10g trochar). Tumor growth was measured 3 \times weekly by caliper (tumor volume = (A² \times B)/2, where A is the longer axis). When tumor volume reached ~0.2cm³ mice were randomized to continued E2 or tamoxifen treatment with placebo or BZA (5 mg/60 days) pellets implanted sc, or weekly injection with 5mg/mouse ICI 182,780 suspended in corn oil. For the BZA treatment only group E2 pellets were surgically removed simultaneous with BZA pellet insertion.

Analysis of tumor tissue

Tumors were excised upon reaching ~1cm³ and cryopreserved, and frozen tissues were pulverized. Protein expression was analyzed essentially as above. RNA was extracted using Trizol (Invitrogen) per manufacturer's instructions, and mRNA expression was detected as above.

Results

BZA induces a unique conformational change in ER α and inhibits its transcriptional activity in cellular models of breast cancer

We and others have demonstrated that tamoxifen partial agonist activity, and by inference tamoxifen resistance, can be attributed to specific ligand-induced conformational changes in ER α that presents a unique protein-protein interaction surface(s) that facilitates coregulator recruitment (15). This hypothesis was confirmed in studies which demonstrated that compounds that do not present the “tamoxifen surface” are effective in inhibiting the growth of tamoxifen resistant xenograft models of breast cancer (20). In recent years, several new high affinity SERMs have been developed for use in the treatment and prevention of osteoporosis. The goal of this study was to evaluate whether these clinically relevant

SERMs could have activity as breast cancer treatments and explore the potential of their near term use in this disease.

The impact of a series of chemically distinct SERMs on ER α conformation was evaluated using a previously described peptide-profiling technology that reads on the differential availability of protein-protein interactions presented on the receptor following ligand binding (15, 20). Specifically, peptide probes were selected that could identify those surfaces on ER α that were required for the agonist activity of estradiol and or tamoxifen. Using an in-cell two-hybrid assay, we determined that the SERMs bazedoxifene (BZA), lasofoxifene (Laso), and raloxifene (Ral) facilitate conformational changes in ER α that are distinct from those observed in the presence of 4-hydroxytamoxifen (4OHT) or the agonist 17 β -estradiol (E2). Furthermore, neither BZA, Laso or Ral occupied ER α interacted with probes that identified surfaces that were uniquely presented on the receptor upon binding ICI 182,780 (ICI) and GW7604 (Figure 1A). These results demonstrate the conformational flexibility of ER α and highlight a potential opportunity to identify compounds whose impact on receptor structure results in a favorable activity in breast cancer. These findings are in agreement with a prior study in which hydrogen/deuterium exchange (HDX) mass spectrometry was used to interrogate the impact of ligands on ER α conformation; studies which highlighted the structural uniqueness of the ER α -BZA complex (29).

Previously, we performed a broad survey of the transcriptional responses that occur within breast cancer cells when treated with different SERMs and identified a) genes regulated similarly by all SERMs tested, b) genes regulated only by 4OHT, and c) genes whose response differentiated SERMs (26). Using the most informative representative genes from this study we evaluated how differences in ligand-induced changes in ER α conformation translate into differences in gene expression. Several genes, ARHGGEF28 (RGNEF) for example, respond in a graded manner to different SERMs, with 4OHT exhibiting the greatest agonist activity, Ral and BZA being significantly less active, and ICI functioning as an inverse agonist (Figure 1B). Most notable, however, was the observation that the basal expression and estrogen dependent induction of AGR2, a gene associated with breast cancer progression during tamoxifen therapy, is repressed by ICI and BZA, but induced by Ral, Laso, and 4OHT (Figure 1C). Considering these data, a more comprehensive survey of the activity of different SERMs on the expression of a large number of genes whose expression was induced or repressed by tamoxifen was performed. Analysis of the data revealed that among the SERMs studied, the pharmacological profile of BZA was most comparable to the pure antagonist ICI, and thus it was brought forward for a more complete analysis in relevant models of breast cancer (Figure 1D). Importantly, it was shown that BZA inhibited estrogen dependent proliferation of MCF7 cells with efficacy similar to that of ICI and 4OHT (Figure 1E). As a more stringent test of activity, we assessed MCF7 cell proliferation in growth factor replete FBS media. Under these conditions 4OHT manifests significant partial agonist activity whereas ICI and BZA were effective antagonists (Figure 1F). It was further demonstrated that 4OHT was unable to inhibit insulin-stimulated proliferation of MCF7 cells whereas both ICI and BZA effectively inhibited this activity (Figure 1G). It was further demonstrated that ICI and BZA effectively inhibited the proliferation of lapatinib resistant rBT474 cells, tamoxifen sensitive subline of the HER2+ BT474 cells in which reactivation of ER α signaling is associated with the development of lapatinib resistance (24) (Figure 1H). Finally, BZA, 4OHT, and ICI similarly inhibited the proliferation of LTED MCF7 cells (Figure 1I), a cellular model of resistance to aromatase inhibitors. When taken together these data suggest that the conformational changes induced in ER upon binding ICI or BZA enable these agents to exhibit favorable activities in relevant models of breast cancer. These findings encouraged us to perform a comparative analysis of these drugs in *in vivo* models of breast cancer.

BZA attenuates estrogen dependent growth of MCF7-cell derived tumor xenografts

As an initial test of the therapeutic potential of BZA, its ability to inhibit the growth of E2 dependent MCF7 derived tumors was compared to ICI. For these experiments, MCF7 derived tumors were harvested from estrogen treated donor mice and similar sized tumor fragments were implanted into ovariectomized athymic nu/nu mice. All mice received estrogen supplementation until tumors reached 0.2cm³ at which time the tumor bearing mice were randomized to either of four groups. In three groups the estrogen supplementation was continued (1) alone or together with (2) BZA or with (3) ICI. In a fourth group of animals, the estradiol pellet was removed and the animals were administered BZA, a regimen intended to reveal any agonist activity of BZA not evident from the studies performed *in vitro*. The results of this analysis clearly indicate that both BZA and ICI significantly inhibit tumor growth rate with a delay in both tumor progression (Figure 2A–B) and tumor doubling time noted (Figure 2C). Furthermore, when administered alone, BZA did not exhibit agonist activity in this model (Figure 2B). It is important to note that ICI was administered at a dose of 5 mg/mouse injected subcutaneously once weekly, which translates to a dose ~1000 fold greater than that currently achievable in breast cancer patients. This dose of ICI greatly exceeds the dose of BZA used (5 mg/mouse in a 60 day continuous release pellet = ~83ug/kg/day), yet these drugs inhibited tumor growth and reduced tumor doubling time in a similar manner despite being administered at a dose only 7-fold greater than the daily dose of estradiol. Previously, it has been shown that ICI induces turnover of ER α in breast tumors and accordingly this drug, and others that exhibit similar activities, are now classified as Selective Estrogen Receptor Degraders (SERDs). Given the established SERD activity of ICI, the expression of ER α was measured in a subset of tumors harvested at the end of the study. Notwithstanding minor differences between tumors in each group, it was noted that the dosing regimen used to deliver ICI effected a substantial, although not absolute, turnover of the receptor. Interestingly, we also observed that ER α expression was similarly reduced in BZA treated tumors (Figure 2D). This was not totally unexpected as previously we and others have shown that BZA exhibits some SERD activity *in vitro* (30).

BZA attenuates ER α dependent growth of a tamoxifen resistant xenograft tumor model

The observation that BZA and ICI functioned similarly as inhibitors of either tamoxifen or estradiol induced gene expression in MCF7 cells *in vitro* led us to examine whether BZA would exhibit efficacy in an *in vivo* model of tamoxifen resistance (Figure 2E). Previously, we reported on the development and characterization of a xenograft model (TamR) in which resistance to tamoxifen is manifest (20). In brief, in this very stable tumor model, tamoxifen functions as an agonist whereas both of the SERDs, ICI and DPC974/GW5638, effectively inhibit growth (20). In contrast to the parental MCF7 tumors, which are unable to grow without E2 stimulation (data not shown), TamR tumors grow, albeit slowly, in the absence of estradiol supplementation, and their growth is dramatically stimulated upon administration of tamoxifen (5mg/60 day release pellet = ~83 μ g/kg/day) (Figure 3A). Analysis of tumor volume over time (~30 days) indicated that BZA significantly reversed tamoxifen stimulation of these tumors (Figure 3B), resulting in a growth rate that was equivalent to that observed when tumors were grown in the absence of tamoxifen (Figure 3C–D). Immunoblot analysis of whole cell extracts prepared from tumors harvested at the end of the study indicated that ER levels were significantly reduced in those treated with BZA whereas expectedly tamoxifen had a minimal effect on receptor expression (Figure 3E). To further differentiate the actions of BZA and Tam and to confirm adequate drug exposure, we assessed the expression of several ER target genes in tumor mRNA, selected for their ability to read on the agonist activity of tamoxifen. As expected from our previously published work (26), it was determined that the mRNAs encoding AGR2, AGR3 and KRT13 were significantly induced in tumors exposed to tamoxifen and that this activity

is completely inhibited by BZA. The inhibitory activity of tamoxifen on the basal expression of the estrogen responsive gene C1QTN6 was unaffected by BZA, indicating, not surprisingly, that these drugs are likely to similarly impact some target genes (26, 31, 32) (Figure 3F). These data indicate that BZA does not facilitate tumor growth in a validated model of tamoxifen resistance but rather inhibits tamoxifen stimulated growth of these tumors. These findings highlight the potential clinical utility of BZA as a breast cancer therapeutic.

BZA exhibits SERD activity in cellular models of ER α -positive breast cancer

BZA and ICI exhibited similar activities on gene regulation in MCF7 cell derived tumors and in their TamR variants, and both compounds resulted in a similar downregulation of ER α expression in these tumors. Of specific interest was the observation that treatment of MCF7 cell derived tumors with either BZA or ICI resulted in a similar downregulation of ER α expression. The pharmacology of ICI has been probed in detail and it has been concluded from these studies that this compound induces a conformational change in the receptor that targets it for degradation. Whereas the conformational change in ER α induced by BZA is distinct from that observed upon binding ICI [Figure 1A and (29)], we were interested in determining if the down regulation of ER α observed in BZA treated tumors reflects SERD activity, as is the case for ICI, or occurs in an indirect manner (i.e. decreased ER α mRNA expression). Notably, the impact of ligands on ER α stability/expression observed in tumors was recapitulated in cellular models of breast cancer. As shown, treatment of MCF7 (Figure 4A) or BT483 (Figure 4B) cells for 8 or 24 hours with BZA or ICI resulted in a loss of ER α expression, while 4OHT, endoxifen, Tam, Ral and Laso had little effect on the level of the receptor. For comparative purposes, and to validate the cell models, we demonstrated that treatment of cells with 17 β -estradiol resulted in a quantitative down regulation of ER α expression.

We next explored the mechanisms underlying the apparent SERD activity of BZA. Firstly, it was demonstrated that the effects of BZA, ICI or 17 β -estradiol on ER α expression occurred in a post-transcriptional manner as no differences in ER α mRNA expression were observed following drug treatment (Figure 4C). It was further demonstrated that ligand dependent downregulation of ER α expression was not affected by cotreatment with cycloheximide suggesting that receptor stability was an intrinsic property of the receptor ligand complex and did not require the *de novo* synthesis of ancillary proteins required for receptor turnover. Furthermore, 17 β -estradiol, ICI and BZA-mediated turnover of the receptor could be blocked by cotreatment of cells with the proteasome inhibitor MG132 (Figure 4D). Together these results suggest that, similar to ICI, BZA exhibits proteasome dependent SERD activity.

Inhibition of ER α turnover does not compromise the antagonist activity of BZA

Having demonstrated that BZA binding induces ER α turnover, we next performed a comparative analysis of this compound with ICI and GW7604, another SERD that has been evaluated previously in patients with breast cancer. When evaluated for their ability to inhibit 17 β -estradiol mediated induction of the transcription of GREB1 mRNA in MCF7 cells, it was observed that all three compounds were equally effective as antagonists with potencies that reflected their affinity for the receptor (Figure 5A); similar results were observed on other ER-responsive genes (26). However at saturating, maximally efficacious doses of each compound, it was noticed that, unlike what occurs in ICI or GW7604 treated cells, ER α expression was not completely down regulated in cells treated with BZA (Figure 5B). This raised the interesting question as to the requirement of receptor turnover for maximal antagonist activity, an important issue to address as it speaks to the utility of ER α measurements in tumors as a surrogate for the antiestrogenic actions of BZA.

Since proteasomal degradation of ER α , when occupied by different ligands, requires it to engage a specific E3 ligase(s), it stands to reason that if the expression of these latter enzymes are limiting that it should be possible to saturate the turnover process by overexpression of the receptor. Previously, we demonstrated that the ubiquitination pattern of 17 β -estradiol and ICI occupied ER α in cells were different (33, 34), suggesting that they may be targeted by different E3 ligases. In follow-up experiments, it was demonstrated that ICI mediated turnover of ER α was reduced upon receptor overexpression (35). Under the same conditions, however, a quantitative down regulation of 17 β -estradiol occupied receptor was observed. Thus, the ability to saturate the degradation process by overexpressing ER α afforded us the opportunity to assess whether turnover of the receptor was required for the antagonist activity of BZA. As shown in Figure 5C, the anticipated effects of the various SERMs/SERDs on ER α stability were observed in uninfected cells (MOI=0). However, in cells infected with a virus expressing ER α , up to 10-fold overexpression of the receptor was achieved, and under these conditions the SERD activity of ICI, BZA and GW7604 was saturated. We have previously demonstrated that receptor overexpressed in this manner is active and able to bind ligand (35). As observed previously, 17 β -estradiol mediated turnover of ER α was not impacted by any of these manipulations, ruling out non-specific effects of this viral overexpression protocol on the activity of the proteasome. The most important finding, however, is that overexpression of ER α had no significant effects on the antagonist efficacy of the SERMs/SERDs evaluated in this study. As expected overexpression of ER α did lead to an increase in ligand independent activation of transcription by the receptor; however, this activity was attenuated by all of the SERMs and SERDs tested (Figure 5D–E). Furthermore, the inhibition of ER α function by SERMs and SERDs, as measured by comparing the proliferation of infected and uninfected cells, was unaffected as well despite sustained overexpression of ER α (Figure 5F–J). When taken together these results suggest that although the removal of ER α by an ER ligand is a desirable attribute of antagonists, it is not required for the antagonist activity of the existing SERDs.

Discussion

In recent years there has been a resurgence of interest in ER α as a therapeutic in cancer. In part, this has been fueled by recent advances that have been made in the pharmaceutical exploitation of the androgen receptor (AR) in prostate cancer. Until relatively recently it was considered that the AR was not a viable target in castrate resistant prostate cancer. However, the spectacular clinical responses to Cyp17 inhibitors, like abiraterone, and to the new high affinity AR antagonist MDV3100 in late stage disease, have encouraged a reappraisal of ER as a therapeutic target in those breast cancers that have failed both tamoxifen and/or aromatase inhibitors (36, 37). As with AR, our understanding of the molecular pharmacology of ER has advanced tremendously in recent years, enabling the development of highly predictive mechanism-based screens for ER-modulators. Indeed, Laso and BZA emerged from *in vitro* screens that selected for compounds which (a) bound ER α and competitively displaced 17 β -estradiol (b) inhibited estrogen action in cellular models of breast cancer and (c) did not manifest ER α agonist activity in contexts where tamoxifen functioned as an agonist (38–40). In clinical trials, it was determined that both of these compounds exhibited robust estrogenic activity in bone and reduced the incidence of vertebral fractures by ~40% in osteoporotic post-menopausal patients (41, 42). In addition, as a secondary endpoint, Laso was shown to significantly decrease the risk of ER-positive breast cancer (43). However, Laso was subsequently found to have a less favorable clinical profile than BZA in the reproductive tract, in that its use was associated with significantly increased endometrial thickness and increased incidence of endometrial polyps, uterine leiomyoma, and vaginal bleeding (44). Laso has been approved in the EU for the treatment and prevention of osteoporosis but has not yet been registered. Recently, BZA has been approved and marketed in the EU for the same use. Our studies of the molecular

pharmacology of BZA and Laso indicate that the former most closely profiled with Fulvestrant (ICI) an approved SERD. Importantly, as demonstrated in this study BZA effectively inhibited the growth of tamoxifen sensitive and tamoxifen resistant breast cancer xenografts. Given these data, and the fact that this drug is currently available for use in humans, we believe that a near-term evaluation of its efficacy in patients with advanced disease is justified.

One of the unexpected findings made in this study is that BZA induces ER α turnover both *in vitro* and *in vivo* absent any impact on the expression of ER α mRNA. The results observed *in vivo* are of particular note as they indicate that a sustained knockdown of ER α protein expression can be accomplished by BZA over time and suggest the absence of feedback mechanism(s) to restore receptor levels. When considering the mechanisms by which resistance to endocrine therapies can arise, it is clear that it would be advantageous to chronically suppress ER α expression. In this manner, ER α , the target for coactivators and signaling pathways that impinge upon the ER-coregulator complex(s), is removed and signaling is attenuated. Importantly, we have shown that although BZA manifests SERD activity, receptor degradation is not required for its antagonist activity. This is an important finding as receptor overexpression has been considered as a possible explanation for resistance to tamoxifen in breast cancer.

Our studies have revealed that ICI and BZA exhibit very similar pharmacology and function similarly in relevant animal models of breast cancer, findings which provide rationale for its clinical evaluation. However, there has been significant concern of late as to the utility of ICI in breast cancer. Although this drug is approved for the treatment of tamoxifen/AI refractory tumors, its response rate as originally determined in the EFECT (Evaluation of Faslodex versus Exemestane) trial was ~10%, similar to intervention with exemestane a second line, steroidal AI (21). This low response rate was unexpected and was initially considered to indicate that SERD intervention would have limited use in the treatment of advanced breast cancer. However, a considerable amount of additional data has emerged of late indicating that the pharmacokinetic properties of fulvestrant, administered by IM injection, and not its mechanism of action, is likely that which limits its efficacy (45, 46). Indeed, despite the use of a loading dose three times higher than that evaluated in the EFECT trial, drug concentrations measured in the vicinity of the tumor were found to be insufficient to saturate the receptor (47). A compelling series of PET imaging studies confirmed poor delivery of fulvestrant to the tumor (43). Regardless, the results of the CONFIRM (Comparison of Fulvestrant in Recurrent or Metastatic Breast Cancer) trial suggested that this SERD may have improved biologic activity and clinical efficacy at higher doses than that originally approved (48). Specifically, it was noted that in the high-dose arm of this study, fulvestrant significantly delays time to cancer progression compared with standard-dose regimens. However, despite these advances in the delivery of fulvestrant, it is likely that the full therapeutic potential of SERD intervention has not been realized with this drug. Being a high affinity, orally bioavailable drug, BZA would not be limited by poor tumor access. Thus, its bio-availability presents the opportunity to evaluate the true potential of SERD activity in late stage disease.

Our studies introduce an apparent paradox in that BZA, originally developed as a SERM for use in the treatment and prevention of osteoporosis, actually profiles as a pure antagonist/SERD in the breast. The latter implies that in the context of bone, BZA exhibits ER α agonist activity in bone, a finding that on first glance is incompatible with it being a SERD. Indeed, DPC974/GW5638, an earlier molecule we developed that exhibited substantial SERD activity, is also bone protective (19). This raises the possibility that within the appropriate cells in bone that ER α stability is not impacted in the same manner as in breast cancer cells, a hypothesis that we are currently testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

Breast cancer remains the most commonly diagnosed cancer among women and the leading cause of cancer mortality in women worldwide. While targeted therapies such as tamoxifen and aromatase inhibitors are effective in treating estrogen receptor (ER) positive tumors, *de novo* and acquired resistance remain an impediment to durable clinical responses. However, ER α remains a therapeutic target in breast cancers that are resistant to both first and second line endocrine interventions. Evaluation of the molecular pharmacology of clinically relevant ER modulators revealed that the third generation selective estrogen receptor modulator (SERM) bazedoxifene had a unique mechanism of action and inhibited the growth of both tamoxifen sensitive and resistant ER α positive breast cancer xenografts. These findings provide strong support for the clinical evaluation of bazedoxifene in breast cancer patients who relapse while undergoing treatment with tamoxifen and/or an aromatase inhibitor.

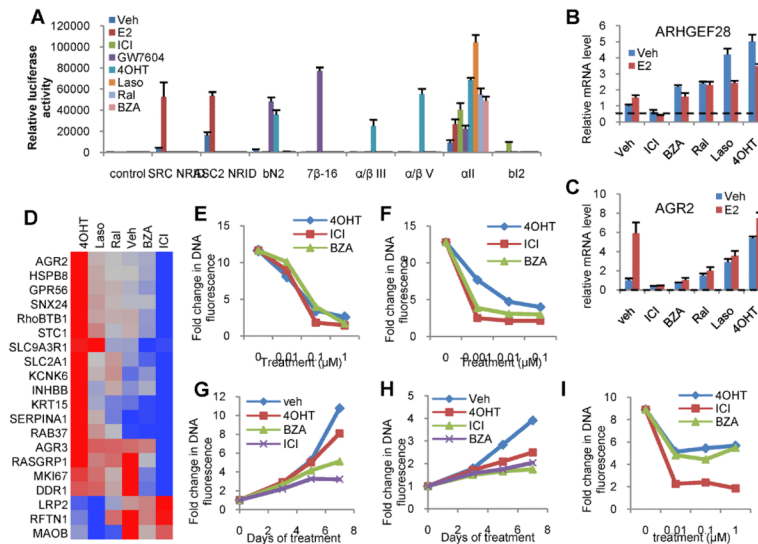


Figure 1. BZA induces a unique conformational change in ER α and inhibits ER α activity in cellular models of breast cancer

A) Interaction between ER α and conformation-specific peptides in a mammalian two-hybrid system. Triplicate wells of SKBR3 cells were transfected with plasmids expressing ER α fused to VP16 together with Gal4DBD alone (control) or Gal4DBD fused to ER interacting peptides noted on the horizontal axis. Cells were then treated with the indicated ER ligands (100 nM). Interaction of ER α with the Gal4DBD peptide constructs was detected through activation of a Gal4 responsive luciferase reporter construct and was normalized to detected β -galactosidase activity expressed in a constitutive manner using a second vector. Normalized response is expressed as fold increase over the detected level of interaction between Gal4DBD alone and ER-VP16 in the absence of ligand (Veh). B–D) MCF7 cells were treated for 24 hours with vehicle or 100nM ligand - ICI 182,780 (ICI), Bazedoxifene (BZA), Raloxifene (Ral), Lasofoxifene (Laso), or 4-hydroxytamoxifen (4OHT) - prior to RNA isolation and RT-qPCR analysis of the expression of mRNAs shown previously to be responsive to SERMs. mRNA expression was normalized to the expression of the 36B4 housekeeping gene. D) Fold changes as compared to vehicle of mRNAs of interest were transformed and normalized as previously described and are presented as a dendrogram constructed in JMP 9.0. E–F) MCF7 cells were plated in phenol red free media supplemented with charcoal stripped FBS (E) or in complete media supplemented with FBS (F) and were treated with the indicated ligands (100nM) on days 1, 4, and 6 of an 8 day proliferation assay. Cells in E were simultaneously treated with 1nM E2. DNA content as assessed by fluorescence serves as an indicator of cell proliferation. G) MCF7 cells were plated and treated as in E, but were treated with 20nM insulin instead of E2. H) Lapatinib resistant BT474 cells (rBT474) were plated in complete media supplemented with FBS and with 1 μ M GW2974 (EGFR inhibitor), and were then treated as in F. I) LTED MCF7 cells were plated in media supplemented with FBS that was stripped of growth factors twice using charcoal. Cells were treated with 0.01–1 μ M ligands on days 1, 4, and 6 of an 8 day proliferation assay and analyzed as in E. Values (relative increase in DNA fluorescence) in E–I were normalized to values detected in a duplicate plate of cells that was harvested on day 1 prior to the initial treatment. Data are representative of at least 3 independent experiments.

E,F – 756 1nM E2 and complete media

703 – H RBT474 in complete media

G – 875 GF prolif

Data from 647

H – proliferating in complete media
F/G – gene expression and degradation coordinated

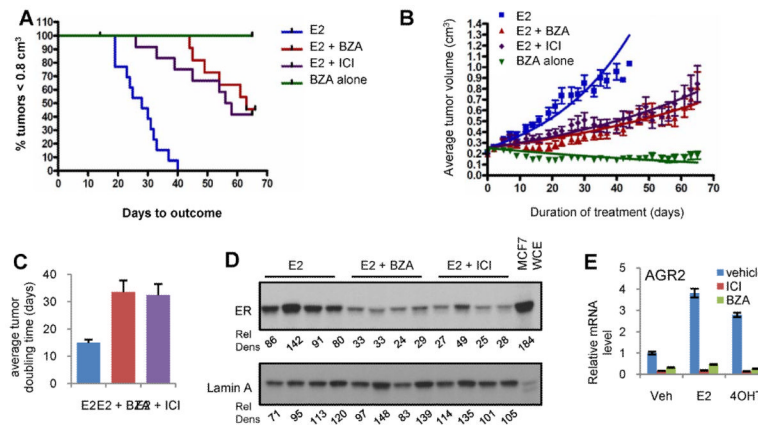


Figure 2. BZA attenuates ER and estrogen dependent growth of MCF7-cell derived tumor xenografts

Mice bearing MCF7 xenograft tumors were randomized (11–12 mice per group) at $0.2 \pm 0.025 \text{ cm}^3$ tumor volume to receive continued E2 stimulation (E2 alone), treatment with E2 together with either BZA (sc pellet) or ICI (weekly injection) or E2 withdrawal (pellet removed) together with BZA treatment (BZA alone). A) Days required for tumors to reach 0.8 cm^3 by Kaplan Meier analysis. Logrank test indicated significance ($p < 0.0001$) in comparison of all treatments to the estrogen control. Differences observed between the E2+ICI and E2+BZA groups were not significant. B) Tumor growth for each group is presented as the average tumor volume for each treatment group \pm SEM at each day of treatment, with the initial day of treatment at randomization considered to be day 0. Non-linear regression analysis for each growth curve is presented. C) Average doubling time for tumors within each group. Data for each replicate was fitted to an exponential growth regression model [$Y = \text{Start} \cdot \exp(K \cdot X)$, where we constrained the Start to being shared between all groups]. Doubling time was estimated from these curves and plotted as mean \pm SEM. Non-parametric Kruskal-Wallis analysis followed by the Dunn's test indicated significant differences ($P < 0.0001$) between both groups and E2. D) ER expression detected by immunoblot of whole cell extracts from tumors harvested from four representative mice from each group. Relative density (Rel Dens) of detected bands were calculated by mean density \cdot pixels and normalized to, and expressed as a percentage of, the average density detected for the vehicle treated samples. E) MCF7 cells were treated for 24 hours with vehicle, 17β -estradiol (1nM) or 4OHT (10nM) in the presence or absence of ICI or BZA (100nM). AGR2 mRNA expression was detected and normalized as in Figure 1.

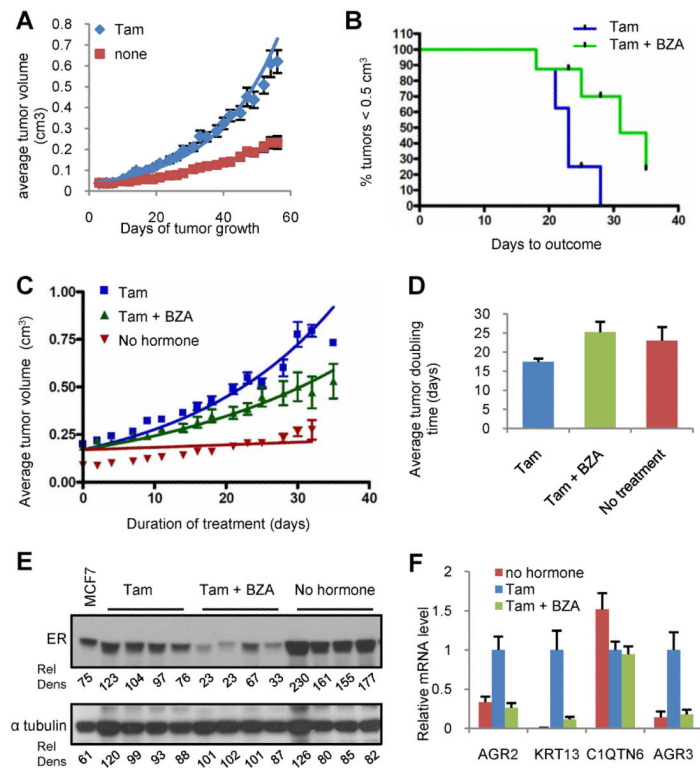


Figure 3. BZA attenuates ER dependent growth of tamoxifen resistant breast tumor xenografts
 A) Average tumor volume of tamoxifen resistant xenograft tumors implanted with or without tamoxifen stimulation (5 mg sc pellet). B–D) When Tam stimulated tumors attained $0.2 \pm 0.025 \text{ cm}^3$ tumor volume, animals were randomized (8–10 mice per group) to receive continued tamoxifen stimulation (tam alone) or treatment with Tam together with BZA (5mg sc pellet). B) Days required for tumors in each group to reach 0.5 cm^3 volume (Kaplan Meier analysis). Logrank test indicated significant difference ($p=0.0175$). C) Tumor growth for each group is presented as average tumor volume \pm SEM per study arm at each day of treatment, with the initial day of treatment at randomization considered to be day 0. Tumor volume for the unstimulated (no hormone) group is plotted corresponding to the first day of treatment for the \pm BZA groups. Non-linear regression analysis for each growth curve is presented D) Average doubling time for tumors in each group. Data for each replicate was fitted to an exponential growth regression model [$Y = \text{Start} * \exp(K * X)$, where we constrained the Start to being shared between all groups]. Doubling time was estimated from these curves and plotted as mean \pm SEM. Non-parametric Mann Whitney test indicated a significant difference ($P=0.0207$) between Tam and Tam+BZA. E) ER expression detected by immunoblot of whole cell extracts of tumors harvested from four representative mice from each group. Relative density (Rel Dens) was calculated as in Figure 2. F) Average expression level (\pm SEM) of tamoxifen regulated genes in tumors ($n=8-10$) from each treatment group. mRNA levels were detected and normalized to human 36B4 as in Figure 1. Data are plotted relative to the tamoxifen treated group.
 Graph – exp 558

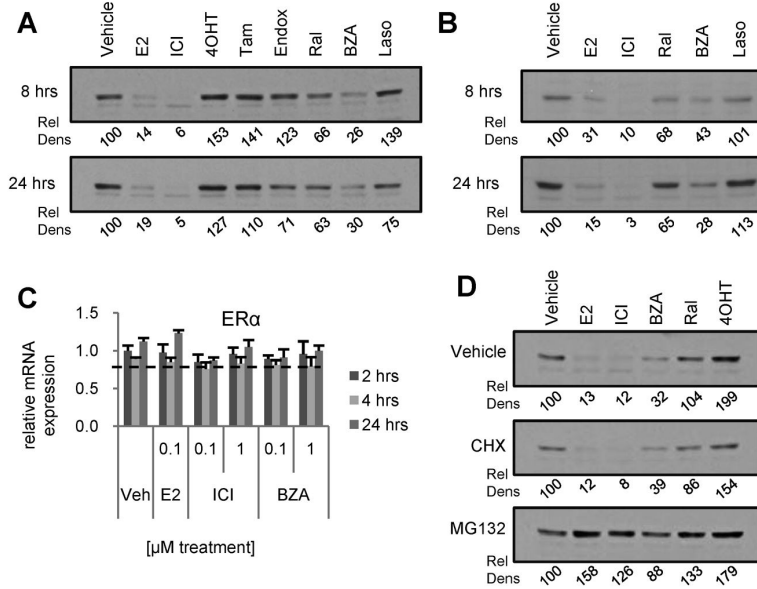


Figure 4. BZA reduces ERα expression in ERα-positive breast cancer cells

MCF7 (A) and BT483 (B) breast cancer cells were treated for 8 or 24 hours with E2 (100 nM) or ER antagonists (1 μM). ERα levels in these whole cell extracts were analyzed by immunoblot. C) MCF7 cells were pretreated 2 hours with MG132 (30 μM) or cycloheximide (CHX – 10 μg/ml) prior to 4 hours treatment with ER ligands (100 nM E2, 1 μM others). ER expression was analyzed by Western immunoblot analysis of whole cell extracts. D) MCF7 cells were treated for 2, 4 or 24 hours with 0.1 or 1 μM E2, BZA, or ICI prior to RNA isolation and detection of ERα mRNA by RT-qPCR. ER mRNA levels were normalized to the housekeeping gene 36B4. E) MCF7 cells were treated for 24 hours with E2 (100 nM) or SERDs (1 μM) and ERα levels were analyzed as in (A). Loading controls for panels A–B are illustrated in Supplementary Figure S1. Relative density (Rel Dens) of blot images was calculated as in Figure 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle treated control.

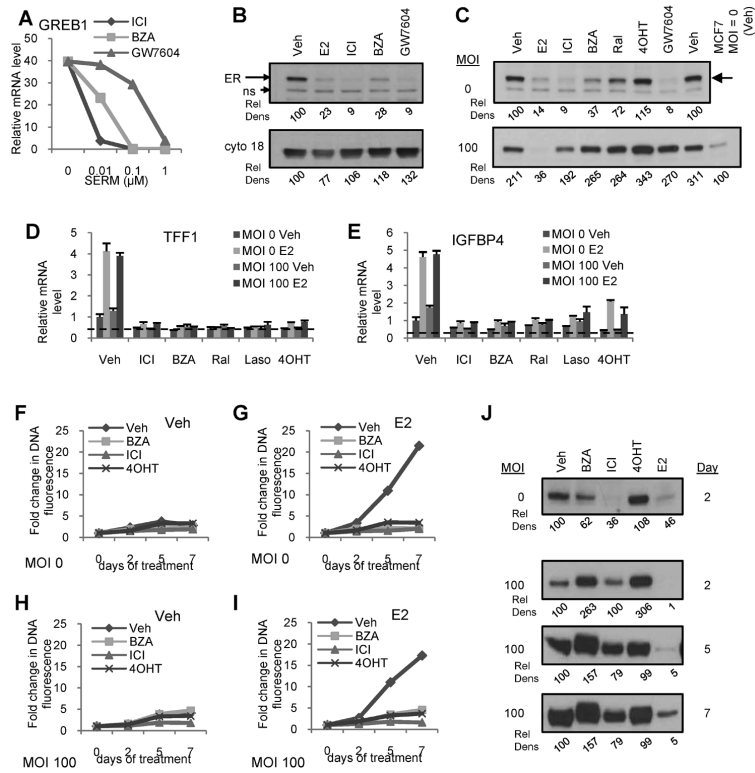


Figure 5. BZA dependent inhibition of ER-mediated transcriptional activity is not impacted by loss of ER turnover

A) MCF7 cells were treated for 24 hours with or without E2 (1nM) in the presence of the indicated SERDs (0.01–1 μ M). mRNA expression of target gene GREB1 was detected as in Figure 1. B) MCF7 cells were treated for 24 hours with E2 (100nM) or SERDs (1 μ M) and ER α levels were analyzed by immunoblot. C–E) MCF7 cells were infected with ER α adenovirus (MOI 100) or mock infected prior to 24 hours treatment with E2 (100 nM) or other ligands (1 μ M). C) ER α levels were analyzed by immunoblotting of whole cell extracts. D–E) Expression of ER target genes in cells infected in parallel and treated 24 hours with E2 (10 nM) in the presence or absence of SERMs or ICI (1 μ M) was analyzed by RT-qPCR as in Figure 1. F–G) MCF7 cells were infected as in C and treated with ligands (1 μ M) in the presence or absence of E2 (1nM) immediately, and 2 and 5 days following infection, with one replicate plate harvested prior to infection and 2, 5, and 7 days after infection. Cell proliferation in the absence (left) or presence (right) of estrogen was analyzed as in Figure 1. H) MCF7 cells were infected, treated with ligands, and harvested in parallel with F and G. ER α expression was analyzed as in C. Data are representative of at least 3 independent experiments. Relative density (Rel Dens) of blot images was calculated as in Figure 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle treated control. For 5C, values are normalized to, and expressed as a percentage of, the vehicle treated uninfected sample. In 5H, values are normalized to, and expressed as a percentage of, the vehicle control for each MOI and time.