Abstract

**Purpose:** There is compelling evidence to suggest that drugs that function as pure estrogen receptor (ER-α) antagonists, or that downregulate the expression of ER-α, would have clinical use in the treatment of advanced tamoxifen- and aromatase-resistant breast cancer. Although 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 (SERM) compounds that would have usage as breast cancer therapeutics. Thus, our objective was to identify among available SERMs those with unique pharmacologic activities and to evaluate their potential clinical use with 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 (i) gene expression, (ii) receptor stability, and (iii) activity in cellular and animal models of advanced endocrine-resistant breast cancer were 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. *Clin Cancer Res;* 19(9); 2420–31. ©2013 AACR.

Introduction

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

Breast cancer remains the most commonly diagnosed cancer among women and the leading cause of cancer mortality in women worldwide. Although 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 patients with breast cancer who relapse while undergoing treatment with tamoxifen and/or an aromatase inhibitor.

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 (ref. 9; 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 patients with breast cancer 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, although 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 seems 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). Although the specific coregulators that enable the agonist activity of tamoxifen remain elusive, there are 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 -resistant breast cancers and yielded positive results in a heavily treated population of patients with breast cancer with endocrine treatment–resistant disease (20). Similarly, it has been shown that fulvestrant (ICI 182,780; ICI) also induces a unique conformational change in ER-α, likewise functions as an antagonist in tamoxifen-resistant xenograft models and shows 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 usage in these disease states can be identified by screening for agents that (i) bind ER-α with an affinity high enough to outcompete both endogenous and exogenous estrogens, (ii) exhibit minimal agonist activity on those genes on which tamoxifen agonist activity is manifest in resistant breast cancer cells, and (iii) 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 conducted 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 -resistant xenograft models. This drug was recently approved for use in the European Union 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 17β-estradiol (E2; Sigma), ICI (Tocris), 4-hydroxytamoxifen (4OHT; Sigma), taloxifen (Sigma), and endoxifen (Sigma). Lasofoxifene and bazedoxifene were gifts from Wyeth, Inc. (now Pfizer). Ligands were dissolved in ethanol or dimethyl sulfoxide (DMSO). Cycloheximide and MG132 were purchased from Sigma.
Cell culture

Cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (MC7) or RPMI (BT483, rT474, and SKBR3) media (Invitrogen) supplemented with 8% FBS (Gemini), nonessential amino acids (Invitrogen), and sodium pyruvate (Invitrogen). Parental cell lines were obtained from American Type Culture Collection, which authenticates cell lines by short tandem repeat profiling. Lapatinib-resistant rT474 cells and LTED MCF7 cells were maintained as described previously (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 quantitative real-time PCR analysis 24 hours after treatment.

Immunoblot analysis

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

RNA isolation and quantitative real-time PCR

RNA isolation and analysis were conducted as described previously (26). mRNA abundance was calculated using the ΔΔCt method (27). Primer sequences are available upon request.

Mammalian two-hybrid analysis

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

Proliferation assays

Of note, 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 4, 6, and 8. DNA content was detected using a FluoReporter assay (Invitrogen) as per manufacturer’s instructions.

Adenovirus production

Creation, production, and purification of an adenovirus expressing human ER-α were previously described (28). Forty-eight 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 qRT-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. 17β-Estradiol-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 17β-estradiol or 5 mg tamoxifen/60 days; Innovative Research of America) implanted s.c. Two days later, an MCF7 or TamR tumor approximately of 0.8 to 1 cm³ in volume was excised under sterile conditions from a euthanized donor mouse, diced to approximately 2-mm³ sections, and implanted into the axillary mammary gland under anesthesia (10 g trochar). Tumor growth was measured 3 times weekly by caliper [tumor volume = (A² × B)/2, where A is the longer axis]. When tumor volume reached approximately 0.2 cm³ mice were randomized to continued 17β-estradiol or tamoxifen treatment with placebo or bazedoxifene (5 mg/60 days) pellets implanted s.c., or weekly injection with 5 mg/mouse ICI suspended in corn oil. For the bazedoxifene treatment, only group 17β-estradiol pellets were surgically removed simultaneously with bazedoxifene pellet insertion.

Analysis of tumor tissue

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

Results

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

We and others have shown that tamoxifen partial agonist activity, and by inference tamoxifen resistance, can be attributed to specific ligand-induced conformational changes in ER-α that present a unique protein–protein interaction surface(s) that facilitates coregulator recruitment (15). This hypothesis was confirmed in studies showing 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, lasofoxifene, and raloxifene facilitate conformational changes in ER-α that are distinct from those observed in the presence of 4OHT or the agonist 17β-estradiol.

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Furthermore, neither bazedoxifene, lasofoxifene, nor raloxifene occupied ER-α, and studies that highlighted the structural uniqueness of the ER-α–BZA complex (29).

Previously, we conducted a broad survey of the transcriptional responses that occur within breast cancer cells when treated with different SERMs and identified (i) genes regulated similarly by all SERMs tested, (ii) genes regulated only by 4OHT, and (iii) 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, ARHGEF28 (RGNEF) for example, respond in a graded manner to different SERMs, with 4OHT exhibiting the greatest agonist activity, raloxifene and bazedoxifene being significantly less active, and ICI functioning as an inverse agonist (Fig. 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 bazedoxifene but induced by raloxifene, lasofoxifene, and 4OHT (Fig. 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 conducted. Analysis of the data revealed that among the SERMs studied, the pharmacologic profile of bazedoxifene was most comparable with the pure antagonist ICI, and thus it was brought forward for a more complete analysis in relevant models of breast cancer (Fig. 1D). Importantly, it was shown that bazedoxifene inhibited estrogen-dependent proliferation of MCF7 cells with efficacy similar to that of ICI and 4OHT (Fig. 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 bazedoxifene were effective antagonists (Fig. 1F). It was further shown that 4OHT was unable to inhibit insulin-stimulated proliferation of MCF7 cells, whereas both ICI and bazedoxifene effectively inhibited this activity (Fig. 1G). It was further shown that ICI and bazedoxifene effectively inhibited the proliferation of lapatinib-resistant rBT474 cells, a tamoxifen-sensitive subline of the HER2+ BT474 cells in which reactivation of ER-α signaling is associated with the development of lapatinib resistance (ref. 24; Fig. 1H). Finally, bazedoxifene, 4OHT, and ICI similarly inhibited the proliferation of LTED MCF7 cells (Fig. 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 bazedoxifene enable these agents to exhibit favorable activities in relevant models of breast cancer. These findings encouraged us to conduct a comparative analysis of these drugs in in vivo models of breast cancer.

### Bazedoxifene attenuates estrogen-dependent growth of MCF7-cell–derived tumor xenografts

As an initial test of the therapeutic potential of bazedoxifene, its ability to inhibit the growth of 17β-estradiol–dependent MCF7-derived tumors was compared with that of 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 0.2 cm³ at which time the tumor-bearing mice were randomized to either of 4 groups. In 3 groups, the estrogen supplementation was continued (i) alone or together with (ii) bazedoxifene or with (iii) ICI. In a fourth group of animals, the estradiol pellet was removed and the animals were administered bazedoxifene, a regimen intended to reveal any agonist activity of bazedoxifene not evident from the studies conducted in vitro. The results of this analysis clearly indicate that both bazedoxifene and ICI significantly inhibit tumor growth rate with a delay in both tumor progression (Fig. 2A and B) and tumor doubling time noted (Fig. 2C). Furthermore, when administered alone, bazedoxifene did not exhibit agonist activity in this model (Fig. 2B). It is important to note that ICI was administered at a dose of 5 mg/mouse injected s.c. once weekly, which translates to a dose approximately 1,000-fold greater than that currently achievable in patients with breast cancer. This dose of ICI greatly exceeds the dose of bazedoxifene used (5 mg/mouse in a 60-day continuous release pellet = ~83 μg/kg/d), 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 (SERD). 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 affected a substantial, although not absolute, turnover of the receptor. Interestingly, we also observed that ER-α expression was similarly reduced in bazedoxifene-treated tumors (Fig. 2D). This finding was not totally unexpected as previously we and others have shown that bazedoxifene exhibits some SERD activity in vitro (30).

### Bazedoxifene attenuates ER-α–dependent growth of a tamoxifen-resistant xenograft tumor model

The observation that bazedoxifene and ICI functioned similarly as inhibitors of either tamoxifen- or estradiol-induced gene expression in MCF7 cells in vitro led us to examine whether bazedoxifene would exhibit efficacy in an in vivo model of tamoxifen resistance (Fig. 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 with the parental MCF7 tumors, which are unable to grow without 17β-estradiol 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 (5 mg/60-day release pellet = ~83 μg/kg/d; Fig. 3A). Analysis of tumor volume over time (≈30 days) indicated that bazedoxifene significantly reversed tamoxifen stimulation of these tumors (Fig. 3B), resulting in a growth rate that was equivalent to that observed when tumors were grown in the absence of tamoxifen (Fig. 3C and D). Immunoblot analysis of whole-cell extracts prepared from tumors harvested at the
Bazedoxifene inhibits ER-dependent tumor growth

Bazedoxifene 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 ± 0.025 cm³ tumor volume to receive continued 17β-estradiol (E2) stimulation (17β-estradiol alone), treatment with 17β-estradiol together with either bazedoxifene (s.c. pellet) or ICI (weekly injection) or 17β-estradiol withdrawal (pellet removed) together with bazedoxifene treatment (bazedoxifene alone). A, days required for tumors to reach 0.8 cm³ by Kaplan–Meier analysis. Log-rank 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 ± SEM at each day of treatment, with the initial day of treatment at randomization considered to be day 0. Nonlinear regression analysis for each growth curve is presented. C, average doubling time for tumors within each group. Data for each replicate were fitted to an exponential growth regression model [\(y = \frac{\text{Start}}{\text{exp}(k \times x)}\)], where we constrained the Start to being shared between all groups. Doubling time was estimated from these curves and plotted as mean ± SEM. Nonparametric Kruskal–Wallis analysis followed by the Dunn test indicated significant differences (P < 0.0001) between both groups and 17β-estradiol alone. D, ER expression detected by immunoblot of whole-cell extracts from tumors harvested from 4 representative mice from each group. Relative density (Rel Dens) of detected bands was calculated by mean density × pixels and normalized to, and expressed as a percentage of, the average density detected for the vehicle-treated group. E, MCF7 cells were treated for 24 hours with vehicle, 17β-estradiol (1 nmol/L) or 4OHT (10 nmol/L) in the presence or absence of ICI or bazedoxifene (100 nmol/L). AGR2 mRNA expression was detected and normalized as in Fig. 1.

The end of the study indicated that ER levels were significantly reduced in those treated with bazedoxifene, whereas, expectedly, tamoxifen had a minimal effect on receptor expression (Fig. 3E). To further differentiate the actions of bazedoxifene and tamoxifen 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 was completely inhibited by bazedoxifene. The inhibitory activity of tamoxifen on the basal expression of the estrogen responsive gene C1QTN6 was unaffected by bazedoxifene, indicating, not surprisingly, that these drugs are likely to similarly affect some target genes (refs. 26, 31, 32; Fig. 3F). These data indicate that bazedoxifene 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 use of bazedoxifene as a breast cancer therapeutic.

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

Bazedoxifene 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 bazedoxifene 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. Although the conformational change in ER-α induced by bazedoxifene is distinct from that observed upon binding ICI (Fig. 1A; ref. 29), we were interested in...
determining if the downregulation of ER-α observed in bazedoxifene-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 (Fig. 4A) or BT483 (Fig. 4B) cells for 8 or 24 hours with bazedoxifene or ICI resulted in a loss of ER-α expression, whereas 4OHT, endoxifen, tamoxifen, raloxifene, and lasofoxifene had little effect on the level of the receptor. For comparative purposes, and to validate the cell models, we showed that treatment of cells with 17β-estradiol resulted in a quantitative downregulation of ER-α expression.

We next explored the mechanisms underlying the apparent SERD activity of bazedoxifene. First, it was shown that the effects of bazedoxifene, ICI, or 17β-estradiol on ER-α expression occurred in a posttranscriptional manner as no differences in ER-α mRNA expression were observed following drug treatment (Fig. 4C). It was further shown that...
Cells were pretreated 2 hours with MG132 (30 μmol/L) and observed on other ER-responsive genes (26). However, at their affinity for the receptor (Fig. 5A); similar results were equally effective as antagonists with potencies that reflected MCF7 cells, it was observed that all 3 compounds were mediated induction of the transcription of GREB1 mRNA in when evaluated for their ability to inhibit 17β-estradiol, bazedoxifene, or ICI before RNA isolation and detection of ER-α mRNA by qRT-PCR. ER mRNA levels were normalized to the housekeeping gene 36B4. Loading controls for panels A and B are shown in Supplementary Fig. S1. Relative density (Rel Dens) of blot images was calculated as in Fig. 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle-treated control.

Having shown that bazedoxifene binding induces ER-α turnover, we next conducted a comparative analysis of this compound with ICI and GW7604, another SERD that has been evaluated previously in patients with breast cancer. Because 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 is limiting that it should be possible to saturate the turnover process by overexpression of the receptor. Previously, we showed that the ubiquitination patterns of 17β-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 shown that ICI-mediated turnover of ER-α was reduced upon receptor overexpression (35). Under the same conditions, however, a quantitative downregulation of 17β-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 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, 17β-estradiol-, ICI-, and bazedoxifene-mediated turnover of the receptor could be blocked by cotreatment of cells with the proteasome inhibitor MG132 (Fig. 4D). Together, these results suggest that, similar to ICI, bazedoxifene exhibits proteasome-dependent SERD activity.

Inhibition of ER-α turnover does not compromise the antagonist activity of bazedoxifene

Having shown that bazedoxifene binding induces ER-α turnover, we next conducted 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 17β-estradiol–mediated induction of the transcription of GREB1 mRNA in MCF7 cells, it was observed that all 3 compounds were equally effective as antagonists with potencies that reflected their affinity for the receptor (Fig. 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 downregulated in cells treated with bazedoxifene (Fig. 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 use of ER-α measurements in tumors as a surrogate for the antiestrogenic actions of bazedoxifene.

Figure 4. Bazedoxifene reduces ER-α expression in ER-α–positive breast cancer cells. MCF7 (A) and BT483 (B) breast cancer cells were treated for 8 or 24 hours with 17β-estradiol (100 nmol/L) or ER antagonists (1 μmol/L). ER-α levels in these whole-cell extracts were analyzed by immunoblot. C, MCF7 cells were pretreated 2 hours with MG132 (30 μmol/L) or cycloheximide (CHX; 10 μg/mL) before 4 hours treatment with ER ligands (100 nmol/L 17β-estradiol, 1 μmol/L 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 μmol/L 17β-estradiol, bazedoxifene, or ICI before RNA isolation and detection of ER-α mRNA by qRT-PCR. ER mRNA levels were normalized to the housekeeping gene 36B4. Loading controls for panels A and B are shown in Supplementary Fig. S1. Relative density (Rel Dens) of blot images was calculated as in Fig. 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle-treated control.

Table 1. Relative mRNA expression

| [μmol/L treatment] | Vehicle | E2 | ICI | 4OHt | Tam | Endox | BZA | Laso | 17β-estradiol | 100 nmol/L 17β-estradiol, bazedoxifene, or ICI | Before RNA isolation and detection of ER-α mRNA by qRT-PCR. ER mRNA levels were normalized to the housekeeping gene 36B4. Loading controls for panels A and B are shown in Supplementary Fig. S1. Relative density (Rel Dens) of blot images was calculated as in Fig. 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle-treated control. |
Figure 5. Bazedoxifene-dependent inhibition of ER-mediated transcriptional activity is not affected by loss of ER turnover. A, MCF7 cells were treated for 24 hours with or without 17\beta-estradiol (E2; 1 nmol/L) in the presence of the indicated SERDs (0.01–1 \mu mol/L). mRNA expression of target gene GREB1 was detected as in Fig. 1. B, MCF7 cells were treated for 24 hours with 17\beta-estradiol (100 nmol/L) or SERDs (1 \mu mol/L), and ER-\alpha levels were analyzed by immunoblot. C–E, MCF7 cells were infected with ER-\alpha adenovirus (MOI 100) or mock infected before 24 hours treatment with 17\beta-estradiol (100 nmol/L) or other ligands (1 \mu mol/L). C, ER-\alpha levels were analyzed by immunoblotting of whole-cell extracts. D and E, expression of ER target genes in cells infected in parallel and treated 24 hours with 17\beta-estradiol (10 nmol/L) in the presence or absence of SERMs or ICI (1 \mu mol/L) was analyzed by qRT-PCR as in Fig. 1. F–I, MCF7 cells were infected as in C, with 0 (F and G) or 100 (H and I) MOI of adenovirus expressing ER-\alpha and treated with ligands (1 \mu mol/L) in the presence or absence of 17\beta-estradiol (1 nmol/L) immediately, and 2 and 5 days following infection, with one replicate plate harvested before infection and 2, 5, and 7 days after infection. Cell proliferation in the absence (F and H) or presence (G and I) of estrogen was analyzed as in Fig. 1. J, MCF7 cells were infected, treated with ligands, and harvested in parallel with F through I. ER-\alpha 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 Fig. 2, but normalized to, and expressed as a percentage of, the density detected for the vehicle treated control. In H, values are normalized to, and expressed as a percentage of, the vehicle-treated uninfected sample.
the receptor was required for the antagonist activity of bazedoxifene. As shown in Fig. 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, bazedoxifene, and GW7604 was saturated. We have previously shown that receptor overexpressed in this manner is active and able to bind ligand (35). As observed previously, 17β-estradiol-mediated turnover of ER-α was not affected by any of these manipulations, ruling out nonspecific 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 (Fig. 5D and 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 (Fig. 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 pharmacological 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, such as 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 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, lasofoxifene and bazedoxifene emerged from in vitro screens that selected for compounds that (i) bound ER-α and competitively displaced 17β-estradiol, (ii) inhibited estrogen action in cellular models of breast cancer, and (iii) 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 approximately 40% in osteoporotic postmenopausal patients (41, 42). In addition, as a secondary endpoint, lasofoxifene was shown to significantly decrease the risk of ER-positive breast cancer (43). However, lasofoxifene was subsequently found to have a less favorable clinical profile than bazedoxifene 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). Lasofoxifene has been approved in the European Union for the treatment and prevention of osteoporosis but has not yet been registered. Recently, bazedoxifene was approved and marketed in the European Union for the same use. Our studies of the molecular pharmacology of bazedoxifene and lasofoxifene indicate that the former most closely profiled with fulvestrant (ICI), an approved SERD. Importantly, as shown in this study, bazedoxifene effectively inhibited the growth of tamoxifen-sensitive and -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 bazedoxifene induces ER-α turnover both in vitro and in vivo absent any impact on the expression of ER-α mRNA. The results observed in vitro are of particular note as they indicate that a sustained knockdown of ER-α protein expression can be accomplished by bazedoxifene 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 bazedoxifene 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 bazedoxifene exhibit very similar pharmacology and function similarly in relevant animal models of breast cancer, findings that provide a rationale for its clinical evaluation. However, there has been significant concern of late as to the use of ICI in breast cancer. Although this drug is approved for the treatment of tamoxifen/aromatase-inhibitor–refractory tumors, its response rate as originally determined in the EFECT (Evaluation of Faslodex vs. Exemestane) trial was approximately 10%, similar to intervention with exemestane as a second line, steroidal aromatase inhibitor (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 intramuscular injection, and not its mechanism of action, is likely that which limits its efficacy (45, 46). Indeed, despite the use of a loading dose 3 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 positron emission tomography 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 delayed 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, bazedoxifene would not be limited by poor tumor access. Thus, its bioavailability presents the opportunity to evaluate the true potential of SERD activity in late-stage disease.

Our studies introduce an apparent paradox in that bazedoxifene, 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, bazedoxifene exhibits ER-α agonist activity in bone, a finding that at 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 ER-α stability is not affected in the same manner as in breast cancer cells, a hypothesis that we are currently testing.

Disclosure of Potential Conflicts of Interest
S.E. Wardell is a consultant/advisory board member of Pfizer and Novo-lipid, Inc. E.R. Nelson is a consultant/advisory board member of NovoLipid, Inc. D.P. McDonnell has a commercial research grant from Pfizer and is a consultant/advisory board member of the same. No potential conflicts of interest were disclosed by the other author.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Wardell, E.R. Nelson, D.P. McDonnell
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