Disruption of estrogen receptor DNA-binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer

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Summary

A serious obstacle to successful treatment of estrogen receptor (ER)-positive human breast cancer is cell resistance to tamoxifen (TAM) therapy. Here we show that the electrophile disulfide benzamide (DIBA), an ER zinc finger inhibitor, blocks ligand-dependent and -independent cell growth of TAM-resistant breast cancer in vitro and in vivo. Such inhibition depends on targeting disruption of the ER DNA-binding domain and its communication with neighboring functional domains, facilitating ERα dissociation from its coactivator AIB1 and concomitant association with its corepressor NCoR bound to chromatin. DIBA does not affect phosphorylation of HER2, MAPK, AKT, and AIB1, suggesting that DIBA-modified ERα may induce a switch from agonistic to antagonistic effects of TAM on resistant breast cancer cells.

Introduction

The selective estrogen receptor modulator (SERM) tamoxifen (TAM), which binds to the estrogen receptor α (ERα) and partially inhibits its activity, is the most prolific therapeutic drug for the treatment of ER-positive breast cancer (Osborne, 1998). Adjuvant therapy studies of TAM show a 40%–50% reduction in the odds of recurrence and reduced mortality. Unfortunately, advanced breast cancers that initially respond well to TAM eventually become refractory to this compound (McDonnell and Norris, 2002; Jordan, 2004; Osborne et al., 2003; Shou et al., 2004).

ER functions in the nucleus as a transcriptional regulator of specific genes (Tsai and O'Malley, 1994). The structural organization of ERα consists of a ligand-independent transcription-activation domain (AF-1 domain), a DNA-binding domain (DBD), a ligand-binding domain (LBD), and a ligand-dependent transactivation domain (AF-2 domain) (Kumar et al., 1987; Ruff et al., 2000). Estrogen binding to ER alters its conformation, triggers receptor dimerization, and directly facilitates binding of the receptor complex to promoter regions of target genes, including sites known as estrogen-responsive elements (ERE), or indirectly through transcription factors such as AP-1 (Kushner et al., 2000). The recruitment of coactivators such as AIB1 and other proteins with acetyltransferase activity helps to unwind the chromatin, allowing transcription to occur (Brzozowski et al., 1997; Glass and Rosenfeld, 2000; Shang et al., 2000; Shiau et al., 1998; Smith et al., 1997). In contrast, the ER conformation induced by the binding of SERMs like TAM favors the recruitment of corepressors NCoR/SMRT and deacetylases that inhibit transcriptional activity in TAM-sensitive breast cancer cells (Keeton and Brown, 2005; Kurebayashi et al., 2000; Mak et al., 1999; Osborne et al., 2003; Shou et al., 2004). However, acquired resistance can be caused by alterations in the ER signal transduction pathway, converting the inhibitory SERM-ERα complex to a growth stimulatory signal (Jordan, 2004).

SIGNIFICANCE

Acquired resistance to antiestrogens is a major challenge to the clinical management of initially endocrine-responsive metastatic breast cancer. We have previously found that electrophilic DIBA and benzisothiazolone derivatives inhibited TAM-sensitive breast cancer cells by preferentially disrupting the vulnerable zinc fingers within the ER DNA-binding domain. Here we describe how DIBA restores the antagonistic action of TAM in resistant breast cancer cells through targeted disruption of the ER DNA-binding domain and its interaction with the proximal N-terminal domain to suppress ligand-dependent and -independent ER transcription and influence the recruitment of cofactor to the ER. These results show that small-molecule modification of the ER zinc finger may alter coactivator/corepressor functions, which are particularly relevant to TAM resistance.

Growing evidence indicates that crosstalk between ER and growth factor receptor signaling pathways (Brockdorff et al., 2003; Ibrahim and Yee, 2005; Osborne et al., 2005), especially the insulin-like growth factor receptor (IGFR) family and the epidermal growth factor receptor (EGFR) family (such as cErbB2 [HER2]), is one of the mechanisms for resistance to endocrine therapy in breast cancer (Schiff et al., 2004). In tumors with abundant ER, AIB1, and HER2, TAM behaves as an ER agonist and stimulates tumor growth (Osborne et al., 2005). High levels of activated AIB1 could reduce the antagonist effects of TAM, especially in tumors that also overexpress the HER2 receptor that activates MAPKs. TAM resistance may also be produced by decreased levels of the corepressor NCoR (Fujita et al., 2003; Lavinsky et al., 1998; Osborne, 1998).

The ER-DBD contains two nonequivalent Cys_4 zinc fingers (Laity et al., 2001; Ruff et al., 2000; Schoenmakers et al., 1999; Wikstrom et al., 1999), which function cooperatively in ER dimerization and DNA binding by stabilizing the secondary and tertiary structure of the ER-DNA complex (Maynard and Covell, 2001; Predki and Sarkar, 1992; Schwabe et al., 1993), leading to ligand-dependent ER transactivation and ER-mediated breast cancer cell and tumor growth. Moreover, interdomain communication between the N-terminal AF-1 domain and DBD of the nuclear receptors helps modulate structure- and ligand-independent functions of receptors (Brodie and McEwan, 2005; Kumar and Thompson, 2003; Shao et al., 1998; Takimoto et al., 2003). We have previously found that electrophilic DIBA and benzisothiazolone derivatives produced anticancer activity in TAM-sensitive human breast cancer cells by preferentially disrupting the vulnerable ER zinc fingers, thus blocking ER DNA binding and transactivation (Wang et al., 2004). Since this anti-breast-cancer strategy targeted ER at the level of its DNA binding, rather than the classical antagonism of estrogen binding, it is relevant to explore whether DIBA has the capacity to inhibit the growth of TAM-resistant breast cancer cells.

In this report, we investigated how DIBA restored the antagonist action of TAM on breast cancer, which was dependent on targeting disruption of the ER DNA-binding domain and its communication with neighboring transcription domains. Moreover, DIBA reduced ER association with coactivator AIB1 and enhanced ER association with corepressor NCoR. These findings provided the proof of principle for a potential for DIBA applicable to TAM-resistant breast cancer.

Results

DIBA suppresses TAM-resistant breast cancer cell growth

First we explored whether DIBA affects estrogen-mediated growth of TAM-resistant breast cancer cells. MCF-7/LCC2 is a selective ER-positive, TAM-resistant cell line (Brunner et al., 1993; Lilling et al., 2000). The specific ER ligand 17β-estradiol (E2) stimulated [^{3}H]thymidine incorporation in MCF-7/LCC2 and its parent MCF-7 cells, but the degree of stimulation in MCF-7/LCC2 is significantly less than that observed in E2-treated MCF-7 cells (Figures 1A and 1B). 4-Hydroxytamoxifen (4-OH-TAM) significantly inhibited MCF-7 cells, with an ED_{50} of 0.1 μM. A low dosage of DIBA enhanced TAM sensitivity, the ED_{50} deceasing 2-fold (0.05 μM) (Figure 1A). The TAM-resistant cell line MCF-7/LCC2 validated with relative resistance; however, a small dosage of DIBA (5 μM) restored 4-OH-TAM sensitivity, achieving over 90% inhibition of E2-driven proliferation at the lowest dosage tested of 4-OH-TAM (0.05 μM). Similarly, DIBA inhibited cell proliferation of MCF-7/HER2-18 (Figure 1C), another TAM-resistant MCF-7 derivative engineered to overexpress HER2 (Benz et al., 1993), and different types of ER-positive and TAM-resistant breast carcinoma cell lines including BT474 (Figure 1D), which expresses ER and is naturally gene amplified for HER2 and AIB1 (Lin et al., 1990; Anzick et al., 1997), and epithelial ZR-75 cells (Figure 1E) (Hoffmann et al., 2004) in a dose-dependent manner. These observations suggested that DIBA effectively restored the antagonist action of TAM on growth of TAM-resistant breast cancer cells.

In TAM-resistant cells, peptide growth factor signaling pathways appear to be important in modifying cell behavior, growth, and survival (Brockdorff et al., 2003; Ibrahim and Yee, 2005). Therefore, we examined whether DIBA impacted TAM-resistant cell growth mediated by stimulation of exogenous peptide growth factors. MCF-7/LCC2 cells (Figure 1F) were stimulated by IGF-1 alone or IGF-1 plus 4-OH-TAM. TAM did not block IGF-1-driven cell proliferation. However, adding DIBA at even 1 μM was sufficient to restore TAM inhibitory functions. These data demonstrated that DIBA also suppressed TAM-resistant cell growth mediated by growth factors.

Efficacy of DIBA on TAM-resistant breast cancer tumor growth in vivo

The in vivo efficacy of the DIBA was tested using nude mice bearing human MCF-7/LCC2 breast carcinoma xenografts. 4-OH-TAM alone did not significantly affect tumor growth. DIBA alone resulted in a dose-dependent inhibition of tumor growth, and a high dose (30 mg/kg) of DIBA reduced tumor volume to almost 50%. Moreover, treatment with 4-OH-TAM plus DIBA diminished tumor to undetectable levels (Figure 2A). Histopathological analysis (Figure 2B) showed a typical hypercellular solid carcinoma invading the dermis and subcutaneum, and the tumor cells had a high nuclear grade with frequent mitosis in the control vehicle (upper panel) or 4-OH-TAM alone-treated mice (middle panel). In contrast, marked reduction in tumor volume, partial encapsulation by fibrous connective tissue, and no significant invasion into surrounding skin tissue were observed in the mice treated with DIBA plus 4-OH-TAM (lower panel). These tumor cells with a low nuclear grade, focal glandular differentiation, and no frequent mitosis or necrosis were seen under higher magnification. No apparent toxicity was observed in liver or kidney in DIBA-treated mice, nor were there any significant changes in body weight gain compared with control mice (data not shown). Therefore, the data demonstrate that DIBA effectively reduces the growth of MCF-7/LCC2 TAM-resistant tumors in mice.

Synergism between DIBA and TAM on cell-cycle progression

Using propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis, we further evaluated TAM-treated cells within the cell cycle in the presence of DIBA (Figure 2C). E2-treated MCF-7/LCC2 cells showed decreased cells in the G0/G1 phase and an increased percentage of cells in the S and G2/M phases. Cells treated with TAM had a weak inhibitory effect on E2, increasing the percentage of cells in S/G2/M. By

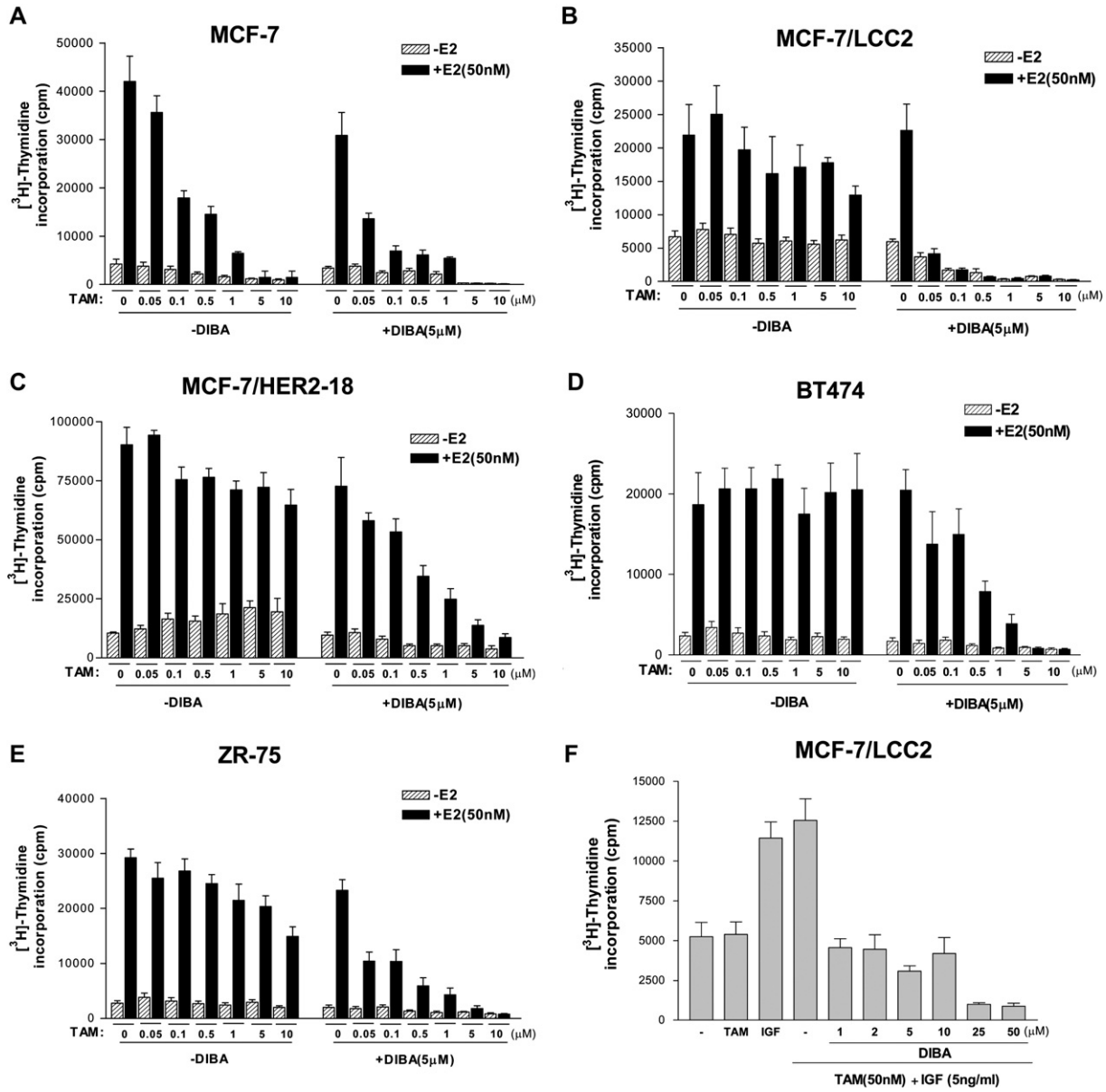

Figure 1. DIBA is a potent inhibitor of TAM-resistant breast cancer cell proliferation

A–E: Proliferation of MCF-7 (**A**), MCF-7/LCC2 (**B**), MCF-7/HER2-18 (**C**), BT474 (**D**), or ZR-75 (**E**) cells was examined by [^{3}H]thymidine incorporation assay. Starved cells were treated with DIBA for 2 hr, stimulated with (filled bars) or without (hatched bars) 50 nM E2, incubated with increasing concentrations of 4-OH-TAM, and analyzed 48 hr later. Data shown represent mean ± SEM.

F: Proliferation of starved MCF-7/LCC2 cells induced by 50 nM 4-OH-TAM or 5 ng/ml IGF-1 was also examined after treatment with increasing concentrations of DIBA. Data shown represent mean ± SEM.

contrast, in cells cotreated with E2 plus ICI 182780, the changes in cell-cycle status and growth induced by E2 were significantly inhibited. In the presence of DIBA combined with TAM, cell-cycle phase distribution induced by E2 shows a significant increase (from 60.7% to 81.7%) of cells in the G0/G1 phase, a decrease (from 30.1% to 6.4%) in the S phase, a decrease (from 7.9% to 5.4%) in the G2/M phase, and an increase (from 1.2% to 6.5%) in the sub-G1 phase. Also, DIBA enhanced the inhibitory effect of ICI 182780 on E2-stimulated cell growth. The FACS data further confirmed that DIBA restored the antagonist action of TAM on cell proliferation of TAM-resistant breast cancer cells analyzed by the [^{3}H]thymidine incorporation assay.

ER is necessary for synergism between DIBA and TAM

To determine whether targeted disruption of ER is necessary for DIBA to suppress cell growth of TAM-resistant breast cancer cells, we used BT474, an ER-positive but TAM-resistant breast cancer cell line, as a model system to examine the effect of depletion of ER on DIBA inhibition of cell growth of TAM-resistant cells (Figure 3A). The ER expressed in these cells was knocked down by using ERα-siRNA. The decreased level of ER was confirmed by western blot (Figure 3A, inset). Under identical conditions, DIBA rendered TAM inhibition on parent ER-positive cells, but was not able to sensitize TAM's suppression of growth of ER-depleted breast cancer cells. These data suggest that

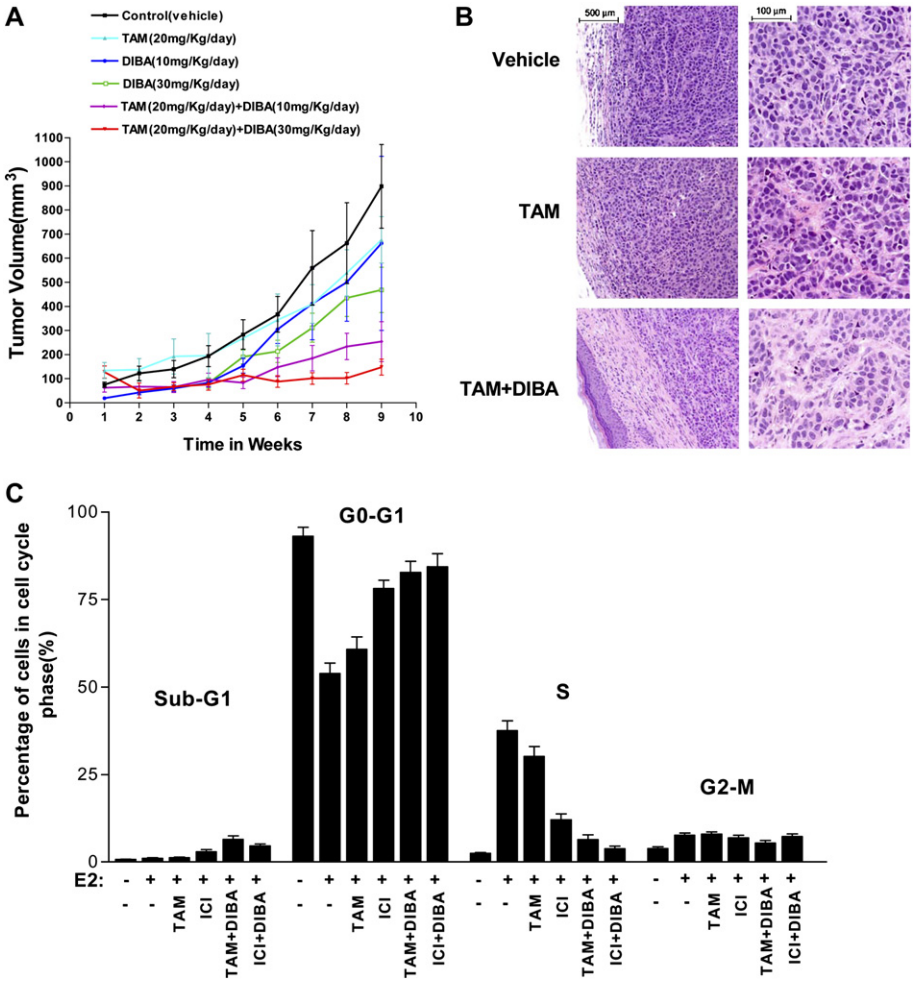

Figure 2. Synergism inhibition between DIBA and TAM on in vivo tumor growth and cell-cycle progression

A: Dose-dependent effect of DIBA and 4-OH-TAM on growth of MCF-7/LCC2 tumor in mice. Data shown represent mean ± SEM (n = 10 mice per group).

B: Morphology of MCF-7/LCC2 tumors treated with vehicle (upper panel), 4-OH-TAM at 20 mg/kg/day (middle panel), or 4-OH-TAM at 20 mg/kg/day plus DIBA at 30 mg/kg (lower panel).

C: MCF-7/LCC2 cells were synchronized by serum starvation, pretreated with 5 μM DIBA, and then stimulated with 50 nM E2, 50 nM TAM, or 1 μM ICI 182780. Cell-cycle distribution was examined by PI staining and FACS analysis. The results represent three independent experiments (mean ± SEM).

inhibition of DIBA on growth of TAM-resistant breast cancer cells depends on ER.

DIBA inhibits ER binding to DNA

To clarify whether the DIBA alters estrogen- or TAM-bound ER's ability to bind to its cognate ERE in TAM-resistant breast cancer cells, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts obtained from MCF-7/LCC2 cells (Figure 3B). E2- or 4-OH-TAM-treated cells displayed considerable ERE DNA-binding complexes, which could be partially supershifted with anti-ER, but not normal rabbit serum, confirming the specificity of these binding complexes. DIBA significantly decreased (80%) the E2- or TAM-induced ERE DNA-binding activity. In a similar experiment on androgen receptor (AR) in MCF-7/LCC2 (Figure 3C), DIBA did not inhibit AR DNA-binding activity.

Next, we examined whether DIBA affects ER binding to probes containing the AP-1, a nontypical repeat element. Estrogen or TAM induced substantial AP-1 binding activity (Figure 3D). The complexes were mostly supershifted with anti-Jun or anti-Fos antibodies. Anti-ER antibody just marginally decreased such complexes, suggesting that a low amount of ER may be bound to AP-1 sites under these conditions/cells. Moreover, DIBA did not display an inhibitory effect on E2- or TAM-stimulated AP-1-binding activity, possibly because ER binding to DNA is not required for its activity through the nonclassical AP-1 pathway (Jakacka et al., 2001; Webb et al., 1999). These data further support the specificity of DIBA influencing ER binding to DNA.

DIBA blocks occupancy of estrogen target gene promoters by ERα

We further used chromatin immunoprecipitations (ChIP) to directly assess whether DIBA impacts ERα binding to promoters of estrogen target genes. The presence of the specific promoters in the chromatin immunoprecipitates was analyzed by semiquantitative PCR by using specific pairs of primers spanning the estrogen-responsive regions in the pS2, c-Myc, and cathepsin D (CATD) gene promoters (Figure 3E). Stimulation with E2 and TAM dramatically increased ERα's occupancy of the above three promoters. DIBA remarkably decreased such occupancy of ERα to the target gene DNA sequences in chromatin. By contrast, ERα did not show any interaction with the distal promoter region (−3351 to −3551) of pS2 promoter. These results suggested DIBA directly influences the ability of ERα to bind to ERE in the promoter of target genes.

We also used ChIPs to examine whether DIBA affects ER binding to AP-1 site in a nontypical manner (Figure 3F). Stimulation with E2 and TAM induced a dramatic increase in the occupancy by c-*fos* or ERα of the AP-1 site, but not in the

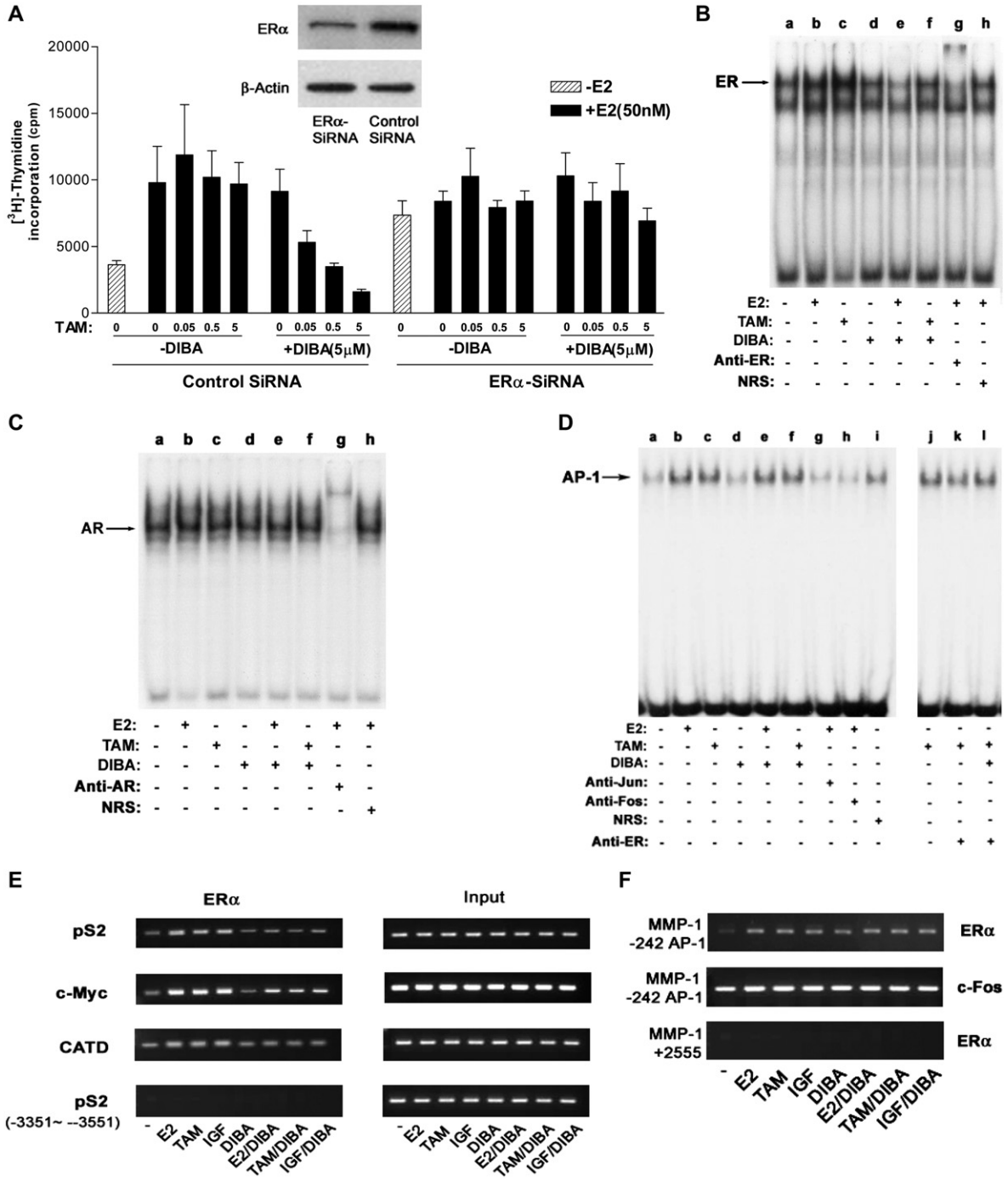

Figure 3. ER is necessary for DIBA to sensitize TAM inhibition

A: siRNA-mediated knockdown of ERα alters DIBA-mediated TAM inhibition of resistant cell growth. BT474 cells were transduced with ERα-siRNA or control vector and incubated for 96 hr. Levels of ERα expression were examined by western blotting (inset). Proliferation of the above transfected cells treated with DIBA and 4-OH-TAM in the presence of E2 was assayed by [^{3}H]thymidine incorporation. Data shown represent mean ± SEM.

B–D: DIBA inhibits E2-induced ERE (**B**), but not ARE (**C**) or AP-1 (**D**), DNA binding. MCF-7/LCC2 cells were treated with or without 5 μM DIBA for 2 hr, then stimulated with medium (−), 50 nM E2, or 50 nM 4-OH-TAM (+) for 20 min. Nuclear extracts were incubated in the absence of antibody, αER, αAR, αJun, αFos, or normal rabbit serum (NRS) in combination with ^{32}P-labeled oligonucleotide probes. Arrows indicate migrational location of each nonsupershifted ER, AR or AP-1 DNA complex.

E: The recruitment of ERα to the promoters of estrogen-responsive genes. MCF-7/LCC2 cells were treated with or without 5 μM DIBA for 2 hr, then stimulated with E2, 4-OH-TAM, or IGF-1 for 40 min. Soluble chromatin was prepared and immunoprecipitated with anti-ERα. The final DNA extractions were amplified using pairs of primers that cover the regions of pS2, CATD, and c-Myc gene promoters, as indicated. The distal region (approximately −3351 to −3551) of the pS2 gene promoter was examined for the presence of ERα (bottom row).

F: The recruitment of ERα to the promoter of an estrogen-induced AP-1-dependent gene MMP-1. Soluble chromatin was immunoprecipitated with antibodies against ERα or c-Fos. The final DNA extractions were amplified using pairs of primers that cover the AP-1 site as indicated or the non-AP-1-specific site (approximately +2555) of the MMP-1 gene promoter.

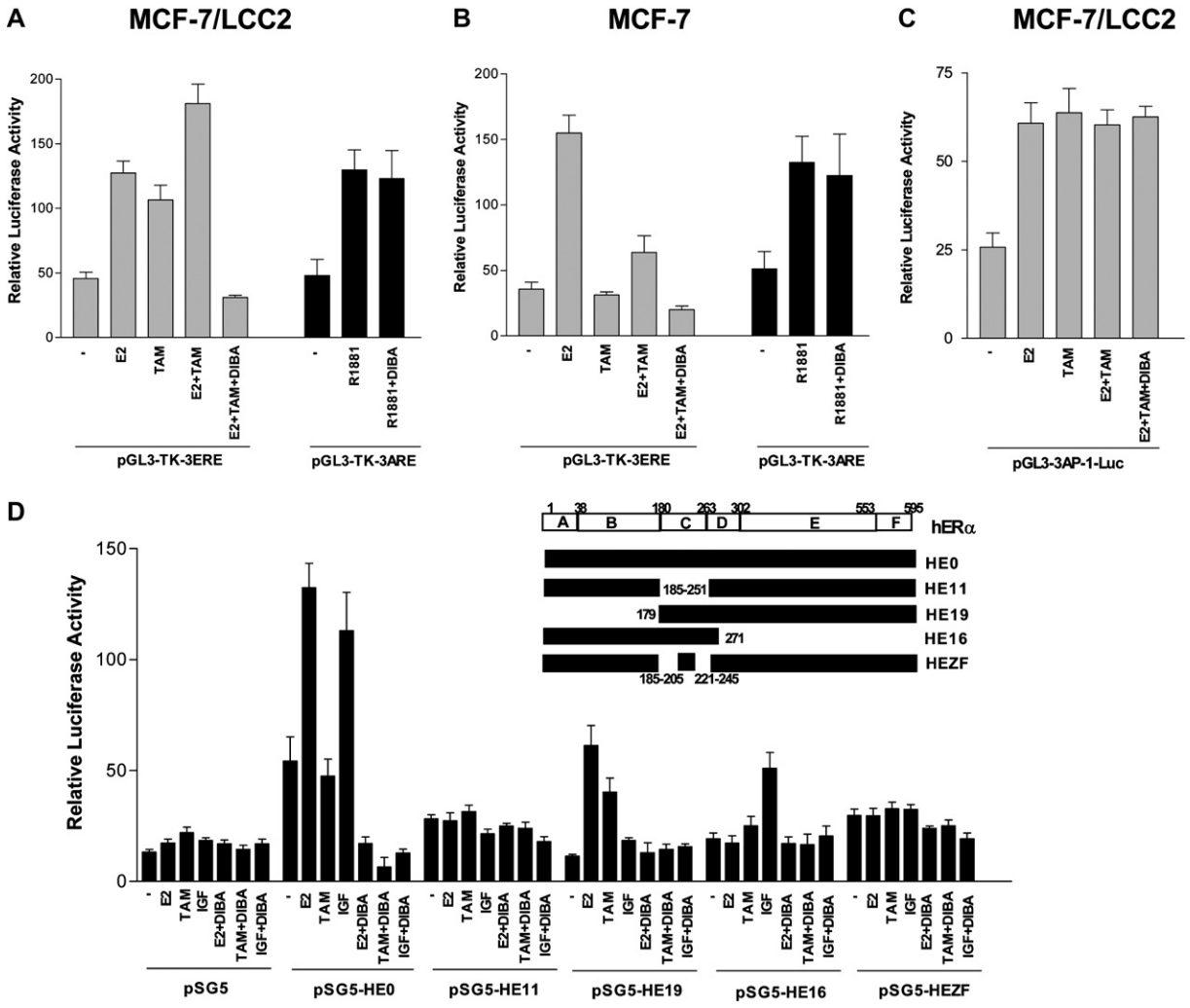

Figure 4. DIBA inhibits ERE transactivation

A–C: MCF-7/LCC2 or MCF-7 cells were transfected with a pGL3-TK-ERE luciferase, pGL3-TK-ARE luciferase, or pGL3-AP-1 luciferase construct, respectively. After addition of 4-OH-TAM (50 nM) and/or DIBA (5 μM) for 2 hr, cells were stimulated with or without 50 nM E2 or 100 nM R1881 for 16 hr. Luciferase activity of lysed cells was measured and normalized. Data shown represent mean ± SEM.

D: MDA-MB-468 cells were transfected with a wild-type ER (pSG5-HE0), a series of human ER deletion mutants including pSG5-HE11, pSG5-HE19, pSG5-HE16, pSG5-HEZF, or pSG5 control plasmids and a pGL3-TK-ERE luciferase reporter. After 24 hr, the transfected cells were treated with DIBA, E2, 4-OH-TAM, and IGF-1 for an additional 24 hr. Luciferase activity of lysed cells was measured and normalized. Data shown represent mean ± SEM.

non-AP-1-specific site in promoter of matrix metalloproteinase 1 (MMP-1), an estrogen-induced/AP-1-dependent gene promoter containing AP-1 sites but no ERE sequences (DeNardo et al., 2005). DIBA did not affect such occupancy of c-*fos* or ERα, consistent with the observation by EMSA.

DIBA inactivates ligand-dependent ERE transactivation

To determine whether DIBA might affect TAM-mediated ER transcription in TAM-resistant breast cancer cells, we tested transactivation of MCF-7/LCC2 (Figure 4A) and MCF-7 (Figure 4B) cells transfected with the ERE-luciferase reporter gene. E2 activated ERE transactivation in both cell lines. TAM alone suppressed E2-induced ERE transactivation in MCF-7 cells, whereas it increased ERE transactivation in MCF-7/LCC2 cells. DIBA significantly reduced ERE transactivation stimulated by 4-OH-TAM and E2 in MCF-7/LCC2 cells. By contrast, DIBA did not affect androgen-responsive element (ARE) transactivation mediated by R1881 in both MCF-7 and MCF-7/LCC2 cells (Figures 4A and 4B). Furthermore, DIBA did not inhibit transactivation of AP-1-luc (Figure 4C). These data indicate that DIBA selectively suppresses TAM-stimulated ER DNA binding and subsequent ERE transactivation.

To further validate the target specificity of DIBA on ligand-dependent ERE transcription in TAM-resistant breast cancer cells, we cotransfected the wild-type human ERα (HE0), a series of human ER deletion mutants (Kumar et al., 1987) including HEZF (ER depleted of zinc finger domains, ER-ΔZF), HE11 (ER depleted of DBD, ER-ΔDBD), HE19 (ER depleted of A/B regions but containing DBD and AF-2 domain, ER-ΔA/B), HE16 (ER depleted of D/E/F regions, ER-ΔD/E/F), or pSG5 control expression plasmid and the ERE-luciferase reporter gene into the ER-negative MDA-MB-468 cells. As shown in Figure 4D, overexpression of ERα, compared to pSG5 control, remarkably resulted in ERE transactivation. E2 strongly activated ERE

transactivation, whereas TAM did not show significant inhibition on such transactivation. Also, induction of ERE transactivation by E2 was observed in the cells overexpressed by ERα mutant HE19 containing completed AF-2 domain and DBD. Deletion of zinc finger domains (HEZF) or the entire DBD (HE11) resulted in decreasing ERE transactivation stimulated by E2, suggesting that zinc fingers in DBD are required for ligand-dependent ERE transactivation. DIBA significantly enhanced TAM inhibition in the wild-type ERα- or a mutant HE19 (ER-ΔA/B)-overexpressing cells, but not in HE11 (ER-ΔDBD)- or HEZF (ER-ΔZF)-overexpressing or control cells. Since DIBA, as a zinc finger inhibitor, has been demonstrated to preferentially disrupt the ER DNA-binding domain, the inhibitory effect of DIBA on ligand-induced ERE transcription in TAM-resistant breast cancer cells may be related to interruption of zinc finger domains within ER-DBD.

DIBA reduces ligand-independent ERE transactivation

The ligand-independent ERE transcription was also measured in the above wild-type ER or mutant-transfected MDA-MB-468 cells. As shown in Figure 4D, in the case of the wild-type ER (HE0)-overexpressing cells, IGF-1 strongly induced ERE transactivation. Deleting zinc finger domains or the entire DBD decreased ERE transactivation stimulated by either E2 or IGF-1, even though this mutant contains completed AF-1 and AF-2 domains. However, induction of ERE transactivation by IGF-1 was observed in the cells overexpressed by the ER mutant HE16 containing a completed N-terminal A/B domain and DBD, suggesting that DBD is required for both ligand-dependent and -independent ERE transactivation. IGF-1's activation of ERE transcription is not only dependent on the AF-1 domain itself, but is also mediated through the interaction between DBD and AF-1 domains, consistent with previous observations that long-range allosteric communication occurs in two separated domains of the androgen receptor (Brodie and McEwan, 2005), glucocorticoid receptor (Kumar et al., 1999), and progesterone receptor (Bain et al., 2000).

Moreover, DIBA blocked ERE transactivation stimulated by IGF-1 in the cells overexpressed by a wild-type ER or the ER mutant (HE16) containing A/B/C domains. Such an inhibitory effect of DIBA was not observed in the ER mutants HE11 (ER-ΔDBD)- and HEZF (ER-ΔZF)-transfected cells, indicating that inhibition of DIBA on the "steroid-independent activation" of ER by growth factor signals was related to DBD-mediated intramolecular communication with the AF-1 domain, which may also be involved in DIBA functionally suppressing TAM-resistant breast cancer cells.

DIBA decreases the TAM-bound ER association with AIB1

Activated AIB1 probably translocates to nucleus (Schiff et al., 2004), where it can interact with ER; therefore, we utilized a coimmunoprecipitation experiment to analyze whether DIBA impacts the ERα interaction with AIB1. Cell extracts were immunoprecipitated with an anti-ERα-specific antibody; immunoprecipitates were developed on western blots with anti-AIB1 (upper panel) or anti-ERα (lower panel). In MCF-7/LCC2 cells (Figure 5A), the AIB1 can be coprecipitated with ERα in cells treated with E2, 4-OH-TAM, or IGF-1, indicating that a direct protein-protein interaction occurs between nuclear receptor ERα and its coactivator AIB1 upon addition of E2, 4-OH-TAM, and IGF-1. Notably, DIBA significantly decreased such ER interaction with AIB1. In contrast, E2, but not TAM, induced this association between ERα and AIB1 in MCF-7 cells. These data support that the effect of DIBA on TAM-resistant MCF-7/LCC2 cells may be through dissociation of the coactivator AIB1 complexes from TAM-bound ERα.

DIBA increases association of TAM-bound ERα with NCoR

Several lines of evidence indicate that the nuclear receptor corepressor (NCoR) complex mediates the inhibitory effects of TAM (Keeton and Brown, 2005). Thus, we examined whether DIBA affects NCoR modulation of the response of ERα to TAM by using a coimmunoprecipitation experiment. Cell extracts were immunoprecipitated with an anti-NCoR specific antibody; immunoprecipitates were developed on western blots with anti-ERα (Figure 5B). In control MCF-7 cells, TAM induced the association between ERα and NCoR while E2 did not affect it, which may mediate the antagonistic effect of TAM on its sensitive cells. In MCF-7/LCC2 cells, a little ERα can be coprecipitated with NCoR, suggesting that a weak constitutive interaction occurs between nuclear receptor ERα and NCoR, which is consistent with the previous observations that interactions of ERα with NCoR in vitro appear to occur regardless of the ligand state of the receptor (Smith et al., 1997; Voss et al., 2005). Although E2 and IGF-1 significantly decreased such interaction, TAM alone did not increase it. DIBA remarkably increased NCoR association with ERα in the presence of E2, IGF-1, and TAM, suggesting that effect of DIBA on TAM-resistant MCF-7/LCC2 cells may also occur through association of the corepressor NCoR complexes with ERα.

DIBA mediates chromatin-associated recruitment of ERα and cofactors

To examine whether interaction between ERα and AIB1 or ERα and NCoR is chromatin associated, we performed ChIP assays of ER followed by the Re-ChIP analysis of either AIB1 or NCoR, analyzing the assembly of ERα-cofactor complex components on a well-characterized estrogen-responsive pS2 promoter (Figure 5C). The soluble chromatin derived from MCF-7/LCC2 cells was subjected to ChIP with ERα antibodies; subsequently, the released immune complexes were divided into two aliquots for the Re-ChIP using AIB1 antibodies or NCoR antibodies. The same Re-ChIP was also performed on the unbound supernatant fractions from the primary immunoprecipitation. The ChIP assay of ERα antibodies showed that strong binding of ERα to the pS2 promoter was induced by E2 or TAM. DIBA significantly decreased E2- or TAM-occupied ERα binding to the estrogen-responsive DNA sequences in the pS2 promoter. The Re-ChIP assay using AIB1 antibodies illustrated that E2 or TAM induced occupancy of the pS2 promoter by ER and the coactivator AIB1. However, the Re-ChIP assay using NCoR antibodies showed that a marginal recruitment of the NCoR occurred in the absence of ligand, while stimulating E2 or TAM abolished such promoter occupancy by ERα-NCoR complexes, indicating that interactions between ERα-AIB1 and between ERα-NCoR are chromatin associated. After DIBA treatment, there were very low levels of E2- or TAM-induced recruitment of ERα-AIB1 and ERα-NcoR complexes to chromatin. Combined with the data obtained from coimmunoprecipitation experiments (Figures 5A and 5B), these results suggested that DIBA-induced changes in ERα association with cofactors led to inhibition of ERα binding to DNA, in

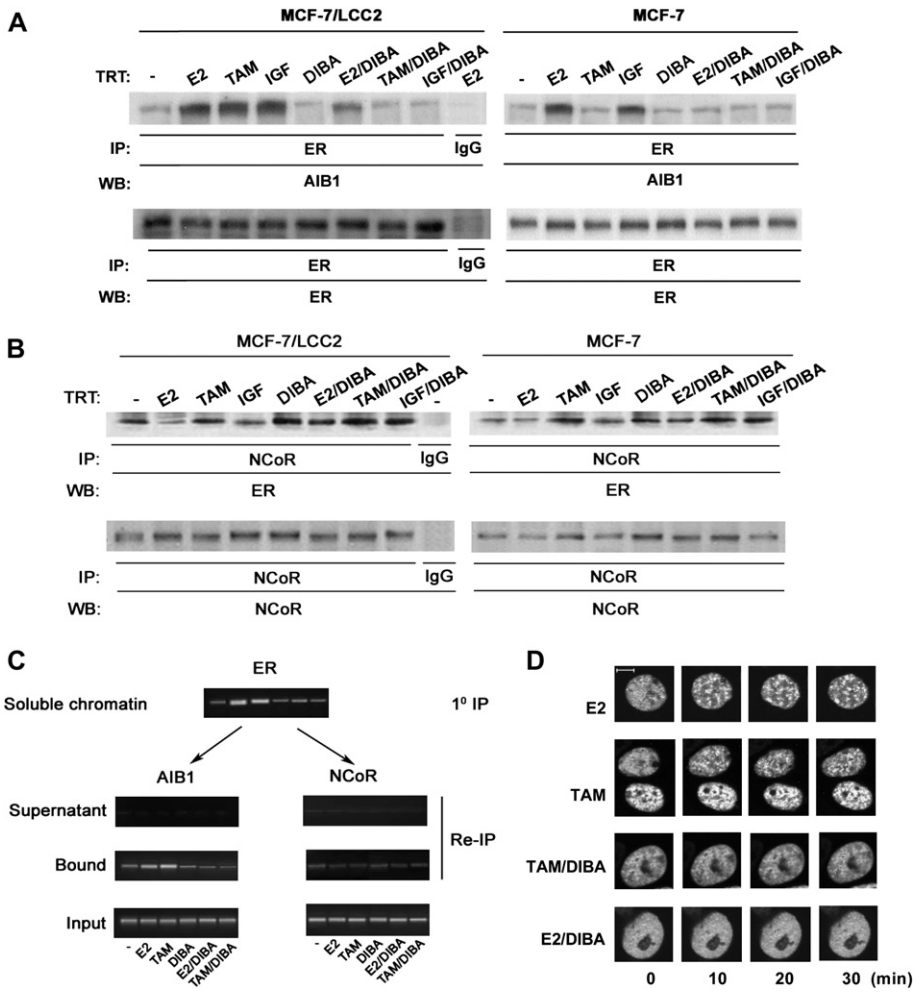

Figure 5. Effect of DIBA on ERα association with AIB1 or NCoR

A and B: ERα association with cofactors assayed by coimmunoprecipitation. MCF-7/LCC2 or MCF-7 cells were treated with or without DIBA for 2 hr, and then stimulated with E2, 4-OH-TAM, or IGF-1 for 24 hr before lyses. **A:** Western blotting analysis with anti-AIB1 (upper panel) or anti-ERα (lower panel) was performed on anti-ERα immunoprecipitates. **B:** Western blotting analysis was performed with anti-ERα (upper panel) or anti-NCoR (lower panel) on anti-NCoR immunoprecipitates.

C: Recruitment of ERα and cofactors assayed by ChIP-Re-ChIP. Soluble chromatin was immunoprecipitated with antibodies against ERα (1° IP). The supernatant was collected and reimmunoprecipitated with antibodies against AIB1 or NCoR (Supernatant Re-IP). Similar reciprocal Re-IPs were also performed on complexes eluted from the 1° IPs (Bound Re-IP).

D: Time course of GFP-ERα redistribution. MCF-7/LCC2 cells were transiently transfected with pEGFP-C2-hERα. Live cells expressing GFP-ERα were pretreated with vehicle or DIBA for 2 hr, followed by stimulation with 50 nM E2 or 50 nM TAM. Time courses of GFP-ER distribution were analyzed at 10 min intervals. Scale bar, 5 μm.

turn blocking transcription of target genes, which aided the synergism between DIBA and TAM.

Since cofactor association can influence ERα cellular localization, we used a transcriptionally active green fluorescent protein-ERα chimera (GFP-ERα) to examine whether DIBA affects ERα cellular distribution. MCF-7/LCC2 cells were transiently transfected with pEGFP-C2-hERα, and live cells expressing GFP-ERα were analyzed at 10 min intervals under confocal laser scanning microscopy. Without ligand, GFP-ERα was observed only in the nucleus, excluding the nucleolus, with a diffuse distribution. Upon adding E2, GFP-ERα was dramatically redistributed from a reticular to punctate pattern within the nucleus (Figure 5D). A similar reorganization occurred with TAM. In the cells pretreated with DIBA, neither E2 nor TAM produced the above apparent subnuclear redistribution patterns. These results demonstrated that DIBA inhibited E2- or TAM-induced ERα nuclear distribution.

DIBA dephosphorylates ERα at serine-167, but not serine-118

The human ERα AF-1 function is potentiated by the phosphorylation of serine residues of human ERα A/B domain after stimulation with its ligands and nonsteroidal growth factors (EGF and IGF-1) (Lannigan, 2003; Yamashita et al., 2005). We thus investigated whether DIBA may modulate ERα phosphorylation by using site-specific antiphosphoserine antibodies against ERα at Ser-118 or Ser-167 (Figure 6A). E2, 4-OH-TAM, and IGF-1 stimulated Ser-167 phosphorylation, whereas there was no significant difference in the level of phosphorylation of ERα at Ser-118 in MCF-7/LCC2 cells with the above treatments. While DIBA inhibited phosphorylation of ERα at Ser-167 induced by all stimuli, it affected neither Ser-118 phosphorylation nor the expression of ERα. It has been demonstrated that ERα phosphorylation at Ser-167, but not at Ser-118, conferred DNA binding and transcriptional activation (Joel et al., 1998) as well as TAM resistance (Campbell et al., 2001). Since the structure of the N-terminal AF-1 domain appears to be influenced by the DBD (Graham et al., 2000), and DIBA selectively reacts with zinc finger of ER-DBD, it seems likely that DIBA may interfere with phosphorylation of Ser-167 in AF-1 proximal to the DBD site through intramolecular communication, concurring with the above observation on the effect of DIBA in ligand-independent ERE transactivation (Figure 4D), and may also contribute to DIBA sensitizing the resistant cells to TAM.

DIBA does not affect expression and phosphorylation of AIB1 and MAPK

Since ER coactivator AIB1, like ER itself, is phosphorylated and activated by different signaling kinases, including the p42/44 MAPK, which can be activated by HER2 (Font de Mora and

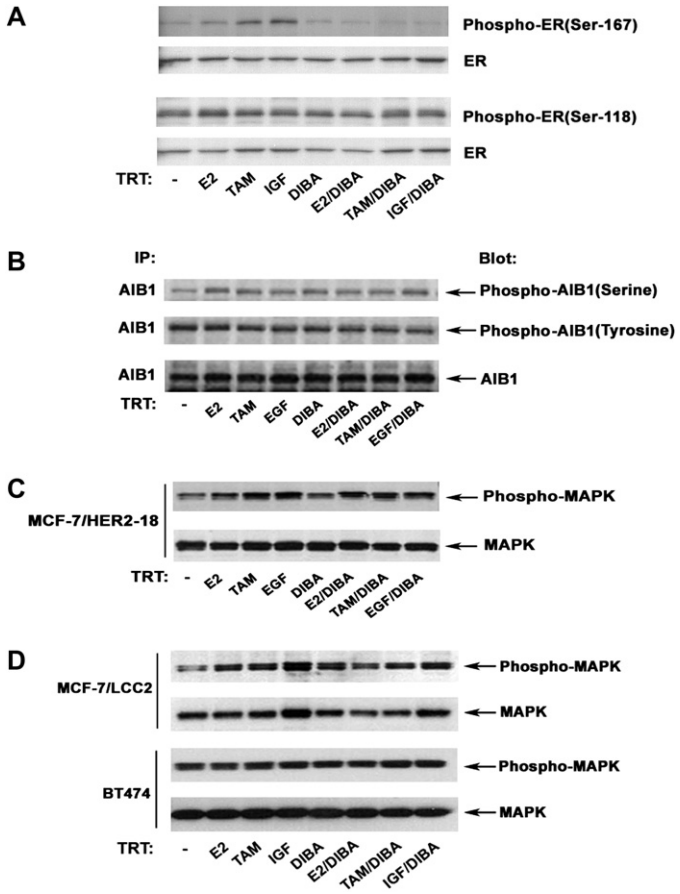

Figure 6. Effect of DIBA on expression and phosphorylation of ERα, AIB1, and MAPK

A: MCF-7/LCC2 cells were treated with or without DIBA for 2 hr, then simulated with E2, 4-OH-TAM, or IGF-1 for 20 min before lyses. Western blotting analysis was performed with anti-phospho-ER (Ser-167 or Ser-118) or anti-ER.
B: MCF-7/HER2-18 cell lysates were immunoprecipitated with anti-AIB1. Immunoprecipitates were blotted with anti-phosphoserine (upper panel), anti-phosphotyrosine (middle panel), or anti-AIB1 (lower panel).
C and D: Cell lysates of MCF-7/HER2-18 (**C**), MCF-7/LCC2, and BT474 (**D**) were analyzed with anti-phospho-MAPK for blot (upper panel) or anti-MAPK (lower panel) for re-blot.

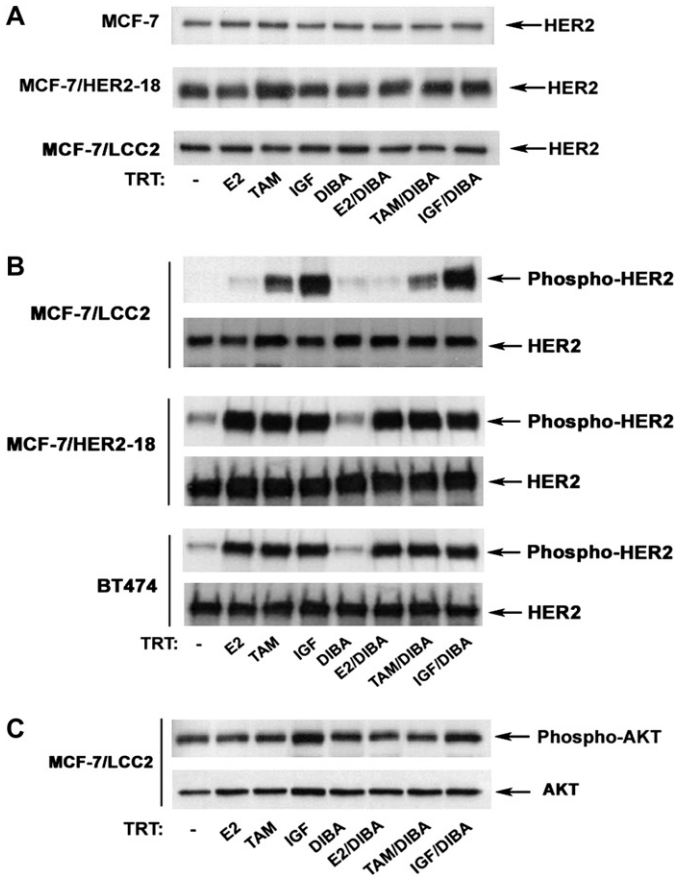

Figure 7. Effect of DIBA on expression and phosphorylation of HER2 and AKT

A: MCF-7, MCF-7/LCC2, and MCF-7/HER2-18 cells were treated with DIBA for 2 hr and then stimulated with E2, 4-OH-TAM, or IGF-1 for 20 min. HER2 expression was analyzed by western blotting with anti-HER2.
B: MCF-7/LCC2, MCF-7/HER2-18, and BT474 cells were treated as described in **A**, except that the antibodies were anti-phospho-HER2 for blot (upper panel) or anti-HER2 for re-blot (lower panel).
C: MCF-7/LCC2 cells were treated as described in **A**, except that the antibodies were anti-phospho-AKT for blot or anti-AKT for re-blot.

Brown, 2000), we examined whether DIBA attenuates phosphorylation of AIB1 in TAM-resistant cells (Figure 6B). MCF-7/HER2-18 cell extracts were immunoprecipitated with an anti-AIB1 specific antibody; the immunoprecipitates were developed on western blots with anti-phosphoserine (upper panel), anti-phosphotyrosine (middle panel), or anti-AIB1 (lower panel). The phosphorylation of serine, but not tyrosine, of AIB1 could be observed in cells stimulated with E2, 4-OH-TAM, and EGF. However, DIBA did not affect such phosphorylation, indicating that DIBA inactivates ER Ser-167 phosphorylation, but does not affect expression and phosphorylation of AIB1, possibly due to AIB1's lacking a zinc finger, although the signaling from the EGFR/HER2 family activates ER and AIB1 by the p42/44 MAPK.

We further examined whether DIBA disrupted phosphorylation of MAPK in different TAM-resistant breast cancer cell lines. Figure 6C shows the same pattern for the phosphorylation of MAPK as that for AIB1 in MCF-7/HER2-18 cells. Similar results were observed in MCF-7/LCC2 and BT474 cells (Figure 6D); the ratio of phospho-MAPK to total MAPK was not significantly changed after the treatment with DIBA.

DIBA does not influence expression and phosphorylation of HER2 and AKT

Since overexpression of HER2 and high levels of phosphorylated AKT may also contribute to TAM resistance (Gutierrez et al., 2005; Osborne et al., 2003), we examined whether DIBA disrupted expression and phosphorylation of HER2 and AKT in TAM-resistant breast cancer cell lines. Compared to MCF-7 cells, MCF7/HER2-18, MCF-7/LCC2, and BT474 cells expressed a considerable level of HER2 (Figures 7A and 7B). There are no significant changes in HER2 expression in DIBA-treated cells. Moreover, even though TAM and IGF-1 induced remarkable phosphorylation of HER2 (Stoica et al., 2000), DIBA did not affect it (Figure 7B), nor did DIBA significantly affect TAM- or IGF-1-stimulated phosphorylation of AKT (Figure 7C) in MCF-7/LCC2 cells. These results indicate the inhibitory effect of DIBA on TAM-resistant cell proliferation is not based on inactivation of HER2, MAPK, and PI3-K/AKT.

Discussion

ER plays a major role in many cases of breast cancer and apparently contributes to the growth of TAM-responsive, acquired TAM-resistant, and de novo ER-positive resistant models (Gee et al., 2005). By using siRNA to deplete ER of BT474, an ER-positive but TAM-resistant breast cancer cell line, we found that DIBA rendered TAM inhibition on parent ER-positive cells, but not on ER-depleted breast cancer cells (Figure 3A), suggesting that DIBA function on TAM-resistant breast cancer cells is ER dependent. We previously discovered that DIBA preferentially disrupted the vulnerable zinc fingers of the ERα DNA-binding domain, thus blocking ER DNA binding and transactivation (Wang et al., 2004). In this study, we used chromatin immunoprecipitations to directly assess ER binding to DNA on estrogen target genes. Stimulation with E2 and TAM dramatically increased ER's occupancy of the pS2, c-Myc, and CATD gene promoters. DIBA remarkably decreased such occupancy of ER to the target DNA sequences in chromatin (Figure 3E). These results suggested DIBA directly influences the ability of ER to bind to DNA, consistent with the data obtained from EMSA (Figure 3B). Moreover, DIBA resulted in inhibition of ligand-dependent and -independent ERE transactivation (Figure 4D). Therefore, targeted disruption of ER is necessary for DIBA, a zinc finger inhibitor, to sensitize TAM inhibition of resistant breast cancer cells through interfering with ER DNA binding and subsequent ERE transactivation.

Nuclear receptor function is modulated by transcriptional coregulators (Klinge et al., 2004; McKenna and O'Malley, 2002; Shang et al., 2000; Shang and Brown, 2002; Tikkanen et al., 2000). The relative level of these coactivators and corepressors might determine the balance of agonist and antagonist properties of TAM. Here, we used coimmunoprecipitation to clarify that DIBA decreased physical association of TAM-bound ER with its coactivator AIB1 (Figure 5A), whereas it increased ER interaction with its corepressor NCoR (Figure 5B). Moreover, ChIP experiments of ERα followed by either Re-ChIP of AIB1 or NCoR also showed that effect of DIBA on E2- or TAM-induced association between ER and AIB1 and dissociation between ERα and NCoR in the level of chromatin (Figure 5C), suggesting that DIBA-mediated changes in ERα interaction with cofactors resulted in blockage of TAM-bound ERα binding to targeted gene promoter and transcription. Notably, the ER-cofactor association caused by DIBA further influences TAM-bound ER nuclear distribution (Figure 5D), indicating other functional changes of ERα may have with chromatin on/off rates or shuttling. The above molecular mechanisms, by which the synergism between DIBA and TAM impacted ER activity, contributed to DIBA restoring TAM's antagonist action on TAM-resistant breast cancer cells (Figures 1 and 2). It may be important to note that in our previous report, the ED_{50} for DIBA ejection of zinc from recombinant ERα was 25 μM (Wang et al., 2004). Here we show effects on disrupting ERα/AIB1 or enhancing ERα/NCoR functions at 5-fold less, 5 μM, suggesting a range of molecular effects on ERα functions.

Several peptide growth factors and their intracellular signaling kinases, notably MAPK and AKT (Albanell and Baselga, 2001), have been shown to mediate cell proliferative responses and phosphorylate ERα on various AF-1 residues, promoting ERα transcriptional activity in a ligand-independent manner (Martin et al., 2000). In the case of TAM-resistant cells, we observed that exogenous IGF-1 stimulated phosphorylation of MAPK and AKT as well as ERα. Although DIBA did not affect phosphorylation of ERα at Ser-118, MAPK, or AKT, DIBA markedly inhibited phosphorylation of ERα at Ser-167 (Figure 6A), suggesting that inhibitory effects of DIBA on a powerful functional crosstalk engaged by the IGF-1 and ER pathways may occur through dephosphorylating ERα at Ser-167. Thus, DIBA disruption of ER zinc fingers resulted in not only perturbing DBD-dependent ERE transactivation (Figures 4A–4D), but also interfering with intramolecular communication between DBD and the N-terminal AF-1 domain (Figure 4D) to downregulate phosphorylation of Ser-167 (Figure 6A) induced by nonestrogenic stimulation in TAM-resistant breast carcinoma cells.

Overexpression of HER2 and high levels of phosphorylated AKT or ERK1/2 MAPK may also contribute to TAM resistance. MCF-7 cells stably transfected with HER2 (MCF-7/HER2-18) are de novo resistant to TAM, in contrast to their low-expressing, responsive MCF-7 counterparts (Benz et al., 1993; Konecny et al., 2003; Shou et al., 2004). Importantly, EGFR/HER2 signaling remains dependent on the ER in MCF-7/HER2-18 cells, as evidenced by their retained sensitivity to estrogen deprivation (Shou et al., 2004). DIBA did not inhibit phosphorylation of HER2, MAPK, and AKT (Figures 6 and 7) or of the coactivator AIB1 (Figure 6B). Moreover, DIBA did not display suppression of estrogen- or TAM-induced DNA binding (Figures 3D and 3F) and transactivation for AP-1 (Figures 3F and 4C), a nontypical ER-binding site. These results suggested that nongenomic actions of ERα may be not involved in the synergism between DIBA and TAM. However, this idea can not be totally excluded; most of the data suggest that DIBA blocks classical genomic sites of ERα.

In conclusion, DIBA resulted not only in inhibition of ligand-dependent ERα DNA binding and transcription, but also in effects on ligand-independent ERE transactivation. Of particular importance was the synergism between DIBA and TAM in regulating recruitment of cofactors to chromatin (decreasing the interaction of ERα with AIB1 and blocking dissociation between ERα and NCoR caused by E2 or TAM). Consequently, DIBA restores the antagonistic action of TAM in breast cancer cells that have acquired resistance, in turn quenching target gene expression and blocking cell growth of TAM-resistant breast cancer cells. These studies suggest a possible new approach in modifying TAM resistance and a potential role for small electrophilic compounds that can modify the particularly vulnerable zinc finger in ERα.

Experimental procedures

Cell and cell culture

The electrophilic compound DIBA (NSC654077) was from the Laboratory of Cell Biology, National Cancer Institute. The human breast carcinoma cell lines MCF-7, ZR-75, and MDA-MB-468 were obtained from ATCC (Manassas, VA). The MCF-7/LCC2 cell line was from Dr. R. Clarke. MCF-7/HER2-18 and BT474 cell lines were from Dr. K. Osborne. 4-OH-TAM and 17β-Estrodial were purchased from Sigma-Aldrich (St Louis, MO). ICI 182780 was from Tocris (Ellisville, MO). In experiments with estrogen or TAM, cells were cultured in phenol red-free and DMEM or RPMI 1640 supplemented with 5% charcoal-dextran-stripped fetal calf serum for at least 2 days.

Cell proliferation and cell-cycle analysis

Cell proliferation was examined by measuring DNA synthesis with [^{3}H]thymidine uptake (Wang et al., 2004). Cell cycle was analyzed by propidium iodide staining and FACS (Li et al., 2006).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (Wang et al., 2003). End-labeled [^{32}P] oligonucleotide probes correspond to the ERE consensus sequence: 5′-GATCCGTCAGGTCAC AGTGACCTGATGGATC-3′, ARE consensus: 5′-GAAGTCTGGTACAGG GTGTTCTTTTTG-3′, and AP-1 consensus: 5′-CGCTTGATGAGTCAGCCG GAA-3′, respectively.

Expression plasmids

pSG5-HE0, pSG5-HE11, pSG5-HE16, or pSG5-HE19 expression plasmids were kindly provided by Dr. P. Chambon, Universitė Louis Pasteur, France. pSG5-HEZF was created by site-directed mutagenesis of HE0 using the oligonuecleotide: 3305: 5′-CTCACTATAGGGCGAATTCCGGCCACGGACCAT GACCATGACCC-3′; 3306: 5′-CATATAGTCGTTATGTCCTTGAATACTTCTC TTGAAGAAGGCCTTGTAGCGAGTCTCCTTGGCAGATTCC-3′; 3307: 5′-GG CCTTCTTCAAGAGAAGTATTCAAGGACATAACGACTATATGTACGAAGTGG GAATGATGAAAGGTGGG-3′; 3308: 5′-TCAGACTGTGGCAGGGAAACCC TCTGCCTCCCCC-3′; 3309: 5′-AACTCGAGCTGGATCCTCAGACTGTGGC AGGGAAACCCTCTGCCTCCCCC-3′ resulting in deletion of amino acids 185–205 and 221–245.

Transfection of SiRNA for ERα

BT474 cells were transfected with an ERα-SiRNA construct or control vector for 96 hr according to the manufacturer's instructions (New England BioLabs, MA). Efficacy of the constructs was tested through western blot analysis of the respective target ERα in transfected cells.

Transfection of luciferase reporter plasmids

FuGene-6 was used for transfection of luciferase reporter plasmids or cotransfection of reporter gene plasmids with ER expression plasmids. Luciferase assays were performed according to the manufacturer's instructions (BD PharMingen, San Diego, CA).

Coimmunoprecipitation and western blot analysis

Coimmunoprecipitation and western blots were performed as previously described (Yang et al., 2000). Antibodies against ER, phospho-ER, AIB1, phospho-AIB1, NCoR, HER2, phospho-HER2, AKT, phospho-AKT, MAPK, phospho-MAPK, phosphotyrosine, and phosphoserine were from Upstate Biotechnology (Lake Placid, NY).

Chromatin immunoprecipitation

The ChIP assays were based on a protocol described by Shang et al. (2000). Cells were fixed by formaldehyde. Purified chromatin samples were immunoprecipitated with anti-ERα antibody. DNA, isolated from immunoprecipitated material following reversal of formaldehyde crosslinking, was amplified by PCR. Promoter-specific primers included: pS2, 5′-CCGGCCATCTCTCAC TAT-3′ (forward primer) and 5′-ATCTTGGCTGAGGGATCT-3′ (reverse primer); pS2 upstream primer pair for negative control, 5′-GAAGACTCCG CACCTCAGAC-3′ (forward primer) and 5′-CCCTTGTGGGGAATCTGG-3′ (reverse primer); c-Myc, 5′-CCGCCTGCGATGATTTATAC-3′ (forward primer) and 5′-AAGGTGGGGAGGAGACTCAG-3′ (reverse primer); Cathepsin D, 5′-TCCAGACATCCTCTCTGGAA-3′ (forward primer), 5′-GGAGCGG AGGGTCCATTC-3′ (reverse primer). MMP-1 promote, 5′-TTGCAACACCAA GTGATTCCA-3′ (forward primer) and 5′-CCCAGCCTCTTGCTACTCCA-3′ (reverse primer); MMP-1 non-AP-1 specific site, 5′-GAGTACAACTTACA TCGTGTTGCAG-3′ (forward primer) and 5′-ATATGGCTTGGATGCCATCA ATGTC-3′ (forward primer).

ChIP Re-ChIP

Complexes were eluted from the primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1]), followed by reimmunoprecipitation with the second antibodies (Shang et al., 2000). ChIP Re-ChIPs of supernatants were done essentially as the primary IPs.

Live microscopy

MCF-7/LCC2 cells were grown on 14 mm coverslips in 35 mm plates and transfected with a pEGFP-C2-hERα construct using FuGene-6. Before ligand addition, the starved cells were pretreated with DIBA for 2 hr. Images were acquired at 10 min intervals with a Zeiss LSM 510 confocal microscope using a 40×/1.3 NA oil immersion objective lens (Stenoien et al., 2000).

Human tumor xenografts

Human MCF-7/LCC2-derived tumor xenografts were established in female athymic Ncr-nu/nu nude mice (National Cancer Institute, Frederick, MD) as described previously (Brunner et al., 1993; Wang et al., 2004). Tumor volume is calculated as $a^2 \times b \times 0.5$, where "a" is the width and "b" is the length of the tumor. Formalin-fixed tissue sections were embedded in paraffin, stained with hematoxylin and eosin, and examined under a light microscope. Animal experimentation was reviewed and approved by NCI's Animal Research Committee.

Acknowledgments

We are very grateful to Dr. A.T. Maynard for helpful discussion in the initial stage, Dr. P. Chambon for kindly providing ER expression plasmids, Drs. J. Hartley and D. Esposito for help making pSG5-HEZF constructs, Drs. M. R. Anver, S. Lawrence and K. Rogers for help in pathology, Dr. O. M. Z. Howard for help in animal experiments, Drs. K. Noer and W. Li for help in analysis of cell cycles, and Mr. B. Harris and E. Cho for help in image analysis. This research was supported by the Intramural Research Program of the Center for Cancer Research, NCI/NIH, and also funded in part with federal funds from the NCI under contract # NO1-CO-12400.

Received: December 18, 2005
Revised: April 8, 2006
Accepted: September 28, 2006
Published: December 11, 2006

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