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Inhibition of Estrogen Receptor α-Mediated Transcription by Antiestrogenic 1,1-Dichloro-2,2,3-triarylcyclopropanes

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ABSTRACT

A novel class of pure antiestrogens, 1,1-dichloro-2,2,3-triarylcyclopropanes (DTACs), lack estrogenic activity in a mouse uterotrophic assay and inhibit the growth of estrogen-sensitive MCF-7 breast cancer cells (Day et al., 1991). Here, reporter assays were used to evaluate the effects of the DTACs on estrogen receptor α (ERα)-mediated transcription from either classic estrogen-response elements (EREs) or nonclassic AP-1 elements. Among the DTACs tested, only the compounds with smaller aromatic substituents, BDRM72 and BDRM81, displayed weak agonist activity on EREs. Their activity was less than that observed for the ER partial agonist, 4-hydroxytamoxifen (ZOHT). In competition experiments, the DTACs blocked estradiol-stimulated transcription from an ERE in a dose-dependent manner and were more effective inhibitors than ZOHT. Each of the DTACs was significantly less active than ZOHT or the pure antiestrogen ICI 182,780 (faslodex) in stimulating transcription from nonclassic AP-1 elements in the presence of ERα. DTACs did not modulate either basal or TPA (12-*O*-tetradecanoylphorbol-13-acetate)-stimulated transcription from an AP-1 element in the absence of ERα, indicating that they are not nonspecific inhibitors of transcription and that ERα is the drug target. Glutathione *S*-transferase pull-down assays were used to examine whether DTACs alter the interaction between ERα and the p160 coactivator, GRIP1. BDRM35, which has the same dimethylaminomethoxy and phenolic moieties as ZOHT, reduced binding by more than 50%. Thus, disruption of p160 coactivator recruitment by ERα may represent one mechanism by which DTACs function as antiestrogens. BDRM35 also suppresses estradiol induction of endogenous target genes c-myc and cyclin D1 in MCF-7 breast cancer cells.

Estrogen is critical in the development and differentiation of estrogen target tissues, including the breast and endometrium. Estrogen response is often retained and is sometimes exaggerated in neoplasms of these tissues. The cellular response to estrogens is mediated by the estrogen receptors (ER) α and β, which belong to the nuclear steroid hormone receptor superfamily (Tsai and O'Malley, 1994). These receptor proteins function as sequence-specific ligand-dependent transcription factors and regulate the expression of genes implicated in cell cycle control, differentiation, and cell survival (Tsai and O'Malley, 1994; Smith and O'Malley, 2004).

Because many neoplasms retain estrogen sensitivity and may even be estrogen-dependent, considerable effort has been placed on the development of compounds that block estrogen signaling. One goal is to suppress the growth of estrogen-sensitive and/or -responsive cancers, and in some situations to prevent their development. Compounds referred to as selective estrogen receptor modulators (SERMs) have shown considerable use in the treatment of women with ER-positive breast cancers (Tonetti and Jordan, 1996; Fisher et al., 1999; McDonnell, 1999). In addition, clinical trials have shown SERMs to be effective as chemopreventative agents in women at risk of developing breast cancer (Fisher et al., 1999). Tamoxifen has been one of the most widely used SERMs, and though it is antiestrogenic in breast tissue, it functions as an ER agonist in other tissues such as the endometrium (Gottardis et al., 1988; Berry et al., 1990). Tamoxifen has been observed to increase the risk of uterine neoplasms in postmenopausal women by 4-fold (Fisher et al., 1999). This adverse response to tamoxifen prompted the search for alternative agents that function as pure ER antagonists.

We previously reported the synthesis and biological evaluation of a novel class of antiestrogenic 1,1-dichloro-2,2,3-

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ABBREVIATIONS: ER, estrogen receptor; SERM, selective estrogen receptor modulator; DTAC, 1,1-dichloro-2,2,3-triarylcyclopropane; ERE, estrogen response element; ZOHT, 4-hydroxytamoxifen; E_2, estradiol; DES, diethylstilbesterol; DMSO, dimethyl sulfoxide; CMV, cytomegalovirus; GST, glutathione *S*-transferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ICI 182,780, faslodex.

triarylcyclopropanes (DTACs), finding that they lacked any demonstrable estrogenic activity in vivo (Day et al., 1991). Several of these compounds (BDRM23, BDRM36, BDRM64, and BDRM72) blocked estradiol stimulation of uterine weight gain in vivo and the proliferation of estrogen-sensitive MCF-7 breast cancer cells in vitro, demonstrating their antiestrogenic character. However, prior studies did not directly address whether DTACs function as antiestrogens by specifically altering ERα-mediated transcription.

There are multiple steps leading from estrogen exposure to a functional ER-dependent transcription response. An initial step involves ligand binding, followed by receptor conformational changes leading to release from an inhibitory complex with HSP90. ER then dimerizes and binds to estrogen response elements (EREs) located in the promoter of estrogen-regulated genes (Gronemeyer and Laudet, 1995). When bound to an agonist such as 17β-estradiol, the ER recruits proteins in the p160 class of transcription factors including SRC-1 (Oñate et al., 1995), GRIP1/TIF2 (Hong et al., 1996; Voegel et al., 1996; Norris et al., 1998) and AIB1 (Anzick et al., 1997) as well as the coactivators p300 (Yao et al., 1996) and cAMP response element-binding protein binding protein (Smith et al., 1998). Each of these possesses histone acetyltransferase activity. This sequence of steps results in increased transcription for estrogen-regulated genes. In addition to this classic model of gene regulation, the ER may also activate or repress transcription from alternative DNA sites including those of the transcription factors AP-1 (Paech et al., 1997; Kushner et al., 2000) and Sp1 (Saville et al., 2000). The outcome of ER regulation at DNA binding sites depends upon multiple factors including the cellular expression of coactivators and corepressors, the ER ligand, and the promoter context (Berry et al., 1990; Tzukerman et al., 1994; Katzenellenbogen et al., 1996). Because of the interesting activities previously found with the DTACs, we sought to increase our understanding of the molecular mechanism by which they function as antiestrogens. We therefore analyzed the effects of the DTACs on ERα-mediated transcription from EREs and AP-1 elements, as well as their effects on the ERα:GRIP1 coactivator interaction.

Materials and Methods

Cells. Estrogen receptor-negative CV-1 kidney cells were maintained in Dulbecco's modified Eagle's medium with 4.5 g/L-glucose (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 100 units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO_2 atmosphere. The human estrogen-dependent breast cancer cell line MCF-7 was purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Chemicals. 17β-Estradiol (E_2), diethylstilbestrol (DES), and 4-hydroxytamoxifen (ZOHT) were purchased from Sigma-Aldrich (St. Louis, MO). They were prepared as 1×10^{-2} M stocks in 100% ethanol and stored at −20°C. *Z*-1,1-Dichloro-2,3-diphenylcyclopropane (also known as Analog II) and the DTACs were synthesized as described previously (Magarian and Benjamin, 1975; Day et al., 1991; Singh et al., 1996; Overacre and Magarian, 1998), prepared as 1×10^{-3} M stocks in DMSO, and stored at −20°C. Before use in an experiment, each of the ligands was further diluted into the requisite amounts of DMSO and medium to keep vehicle concentrations constant.

Constructs. CMV-ERα contains the full-length coding sequence of human ERα (595 amino acids). ERE-tk-luciferase contains a single ERE cloned upstream of the thymidine kinase promoter and the luciferase gene (gift of Don DeFranco, Dept. of Pharmacology, University of Pittsburgh). Plasmid AP1-tk-luciferase was purchased from BD Biosciences Clontech (Palo Alto, CA). pGEX GST-GRIP1 563-1121 was described previously (Hong et al., 1996) and was a gift from Michael Stallcup (University of Southern California). CMV-SPORT-β-galactosidase was purchased from Invitrogen (Carlsbad, CA).

Transfections. For transient transfection assays, cells were plated in 6-well dishes at a density of 2×10^5 cells per well in phenol red-free Dulbecco's modified Eagle's medium (HyClone) containing 10% charcoal-dextran-stripped fetal bovine serum (HyClone). CV-1 cells were transfected using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Transfections contained 1.5 μg of reporter plasmid and 0.5 μg of ERα expression vector. After 16 h, the medium was replaced, and the cells were treated with vehicle or ligand for an additional 24 h. Cells were harvested and assayed for luciferase or β-galactosidase. Transfections were performed in duplicate, and each experiment was repeated at least three times.

Luciferase Assay. Cell monolayers were washed twice with ice-cold phosphate-buffered saline and incubated for 15 min in 250 μl of 1× cell culture lysis reagent (Promega, Madison, WI). Cell extracts were transferred to an Eppendorf tube and clarified by centrifugation for 2 min at 13,000 rpm at room temperature. The supernatants were transferred to a fresh tube and assayed using the luciferase assay system (Promega). For each assay, 10 μl of extract was diluted with 90 μl of 1× cell culture lysis reagent. Luminescence was read using an AutoLumat LB953 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Radiolabeled ER. [^{35}S]Methionine-labeled wild-type human ERα was prepared using the TNT Coupled reticulocyte lysate system (Promega) according to the manufacturer's protocol. The DNA template used in the in vitro transcription/translation reaction, plasmid T7 ER hormone binding domain, encodes the hormone binding half of human ERα (amino acids 275–595). Translation products were routinely analyzed by SDS gel electrophoresis to verify their integrity.

Preparation of GST-GRIP1. GST-GRIP1 synthesis was induced in XL-1 Blue cells transformed with the plasmid pGEX-GRIP1 563-1121 via the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.1 mM. After 2 h, the cells were collected and resuspended in 5.0 ml of ice-cold NETN buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The cell suspension was sonicated until clear, and Triton X-100 was added to a final concentration of 1%. The lysate was mixed for 30 min at 4°C and then clarified by centrifugation at 12,000*g* for 10 min at 4°C. Cell extracts were stored in 5.0-ml aliquots at −80°C until use. To prepare beads for the binding assay, glutathione-Sepharose beads (Amersham Biosciences Inc., Piscataway, NJ) were washed and equilibrated in NETN buffer. An aliquot (250 μl) of the washed beads was mixed with 5.0 ml of GST-GRIP1 cell extract for 1.5 h at 4°C. The beads were collected by centrifugation (3 min at 1000 rpm at 4°C) and washed three times with NETN buffer. The washed beads with bound GST-GRIP1 were resuspended in a final volume of 5.0 ml of binding buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8.0, containing 0.02% Tween 20, 100 μg/ml bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail) and used immediately.

GST Binding Assay. For each reaction, vehicle control or the appropriate ligand was diluted in 500 μl of binding buffer. In a separate tube, 2 μl of radiolabeled ERα was mixed with 500 μl of GST-GRIP1 beads. To measure the effect of each ligand on ERα binding to GRIP1, the contents of each tube were mixed and the reactions incubated for 4 h at 4°C on a rocking platform. The beads were collected by centrifugation and washed in 1.0 ml of wash buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, and 0.05% Tween 20). The wash was completely removed, and the beads were resuspended in 100 μl of fresh buffer. The suspension was trans-

ferred to a vial containing 3.0 ml of scintillation fluid, and the bound radioactivity was measured by scintillation spectrometry. For each assay, each reaction was performed in duplicate. Within each experiment, the amount of radioactivity bound in the presence of 10^{-9} M estradiol was set at 100% binding.

Preparation of Whole Cell Extracts. MCF-7 cells (4×10^5 cells per T25 flask) were cultured for 24 h in phenol red free medium containing 10% charcoal-stripped serum. DMSO, ZOHT, ICI 182,780, or BDRM35 were diluted in fresh medium and added to the cells 1 h before the addition of E_2. At the designated times, the medium was removed, and the adherent cell monolayers were washed twice in ice-cold phosphate-buffered saline. The cells were collected and lysed in TX-100/SDS buffer (1% TX-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl) supplemented with protease and phosphatase inhibitors. The extracts were clarified by centrifugation (10 min, 13,000*g*) at 4°C. Proteins were quantitated using the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Immunoblotting. Proteins (30 μg) were resolved by electrophoresis through precast 10% polyacrylamide Tris-HCl gels (Bio-Rad) and then transferred to polyvinylidene difluoride membrane. Membranes were probed using the monoclonal antibodies anti-c-myc (9E10) and anti-cyclin D1 (DCS-6), purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Complexes were detected using stabilized horseradish peroxidase-conjugated anti-mouse secondary antibody and SuperSignal reagent (Pierce). Actin was detected using the Ab-1 kit (Calbiochem, San Diego, CA).

Results

The compounds analyzed in this study are shown in Fig. 1. The parent cyclopropane, Analog II, is known to have antiproliferative activity in animal models of breast cancer and to be antiuterotrophic. It is interesting that the actions of Analog II seem to be mediated by inhibition of tubulin assembly (Ter Haar et al., 1997). The further DTAC triaryl analogs (1,1-dichloro-2,2,3-triarylcyclopropanes) do not have microtubule perturbing actions (R. Balachandran and B. W. Day, unpublished observations).

When bound by an agonist, the ER binds to EREs and adopts a conformation that facilitates recruitment of transcription coactivators resulting in increased gene expression. To determine whether the DTACs displayed estrogenic effects on transcription, ER-deficient CV-1 cells were cotransfected with an expression vector encoding human wild-type ERα (CMV-ERα) and an ERE-luciferase reporter construct (ERE-tk-luc). Transiently transfected cells were treated with the ER agonists E_2 or DES, the ER partial agonist (ZOHT), Analog II, or DTACs, and reporter gene activity measured after 24 h. Analog II and DTACs were used at a final concentration 1000× greater than E_2 to account for lower ER binding affinities (Day et al., 1991). At the concentrations used, none of the treatments had discernible effects on cell growth or morphology of the CV-1 cells (data not shown).

E_2 and the synthetic ER agonist, DES, increased ERE reporter gene expression (luciferase) as expected (Fig. 2). Luciferase activity was also increased by the partial agonist ZOHT, consistent with previously published reports (Berry et al., 1990). Analog II, BDRM72, and BDRM81 showed weak estrogenic activity as expected for small aromatic compounds, though to a lesser extent than did ZOHT. The remaining DTAC derivatives had no positive effect on transcription. Similar results were obtained in transiently transfected HeLa cells (data not shown), so the effects of the DTACs on ERα-mediated transcription from EREs were not cell-type specific.

To determine whether the DTACs negatively regulate transcription through the ER, competition experiments were conducted (Fig. 3). CV-1 cells were transiently transfected

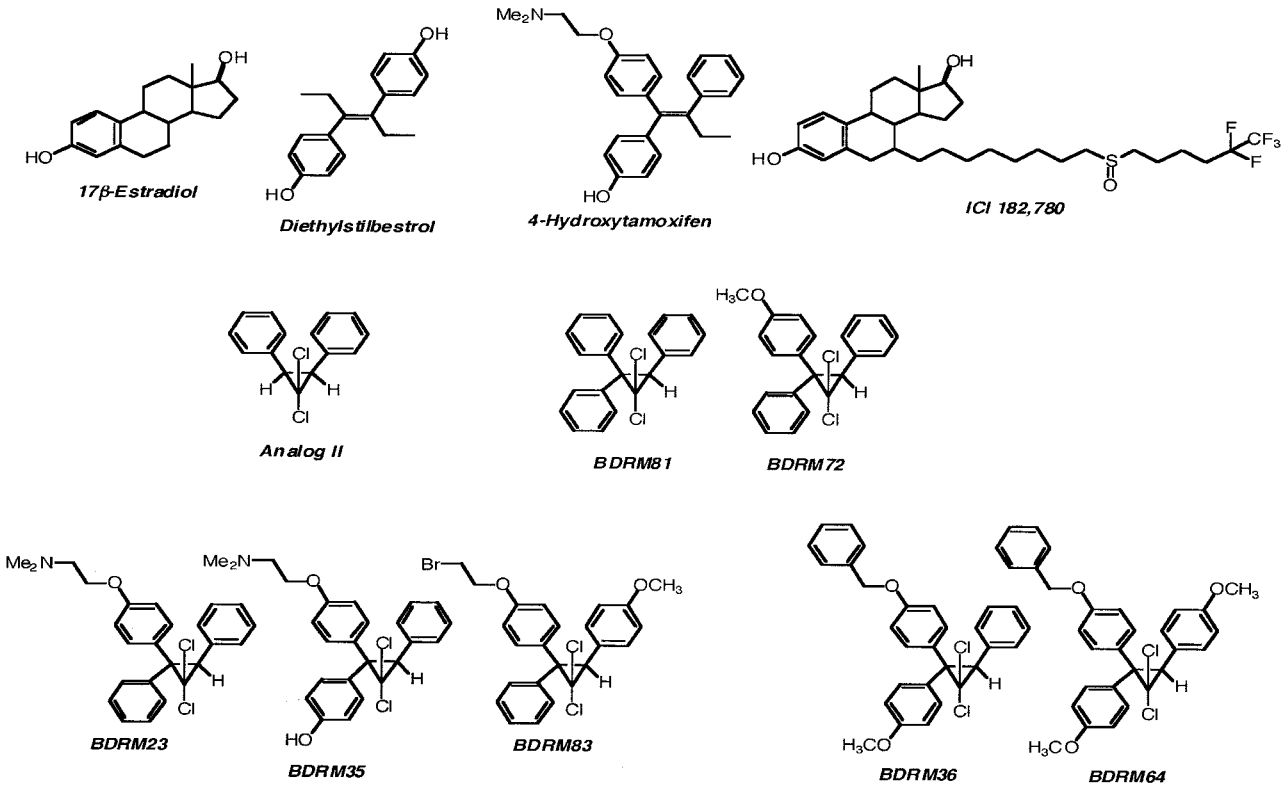

Fig. 1. Structures of the compounds examined in this study.

with CMV-ERα and ERE-tk-luc and then were treated with 10^{-8} M E_2 alone, test compound alone (10^{-5} M), or E_2 in combination with varying concentrations (10^{-7} to 10^{-5} M) of test compound. Reporter gene expression was measured after 24 h. The ER agonist DES itself increased reporter gene expression and did not display antiestrogenic character. In contrast, the ability of E_2 to stimulate reporter gene activity was reduced in a concentration-dependent manner by ZOHT, Analog II, and each of the DTACs. When Analog II and DTACs were used at a final concentration of 1×10^{-5} M, the level of reporter gene activity in cells treated with compound in the presence of E_2 approached that of cells treated with compound alone.

The results observed could have been caused by the ability of the test agents to block E_2-mediated transcriptional activation by specifically disrupting ER signaling. On the other hand, the compounds might function as general and nonspecific inhibitors of transcription. To address the specificity of DTACs, their ability to modulate transcription from an AP-1 DNA binding element was analyzed. CV-1 cells, which have no endogenous ERα, were transfected with an AP-1 reporter construct (AP-1-tk-luciferase) and then treated with ER agonists, ER antagonists, Analog II, or DTACs in the presence or absence of TPA, which was added to stimulate endogenous AP-1 activity (Fisch et al., 1989). In the absence of TPA, none of the known ER ligands or DTAC derivatives had any effect on transcription (Fig. 4). As expected, TPA treatment greatly increased AP-1 reporter gene activity ~8-fold over the DMSO control. TPA-stimulated AP-1 activity was not significantly affected by the addition of any of the estrogenic or antiestrogenic test agents, indicating that DTAC compounds are not general inhibitors of transcription (Fig. 4).

In addition to acting through conventional EREs to increase transcription, ERα can also regulate transcription through non-ERE sequences, including AP-1 sites (Paech et al., 1997; Kushner et al., 2000). To further assess the specificity of DTACs for modulating transcription in an ER-dependent manner, we tested whether they could alter the ability of the ERα to modulate transcription through an AP-1 element. CV-1 cells were cotransfected with CMV-ERα in addition to the AP-1-tk-luciferase reporter and treated with TPA (positive control), ZOHT, ICI 182,780, or a DTAC derivative (Fig. 5). Consistent with results obtained in HeLa cells (Paech et al., 1997), ZOHT and ICI 182,780 bound to ERα and stimulated transcription from the AP-1 reporter construct. Analog II and the DTAC derivatives also increased AP-1 transcription over DMSO alone but were consistently found to be weaker agonists than either ZOHT or ICI 182,780.

Upon binding an agonist, the ERα undergoes a conformational change that creates a hydrophobic surface favorable for interaction with p160 coactivator proteins, including GRIP1 (Hong et al., 1996; Voegel et al., 1996; Norris et al., 1998). ZOHT binding induces an alternative conformation in ERα, resulting in occlusion of the p160 coactivator binding site (Brzozowski et al., 1997; Shiau et al., 1998). To determine whether the DTACs disrupt p160 coactivator recruitment in a manner analogous to ZOHT, the ability of DTAC derivatives to bind and fold ERα for interaction with GRIP1 was measured using a GST pull-down assay. Neither ZOHT nor the DTAC compounds alone had a positive effect on inducing GRIP1-ERα interaction (Fig. 6A). In competition

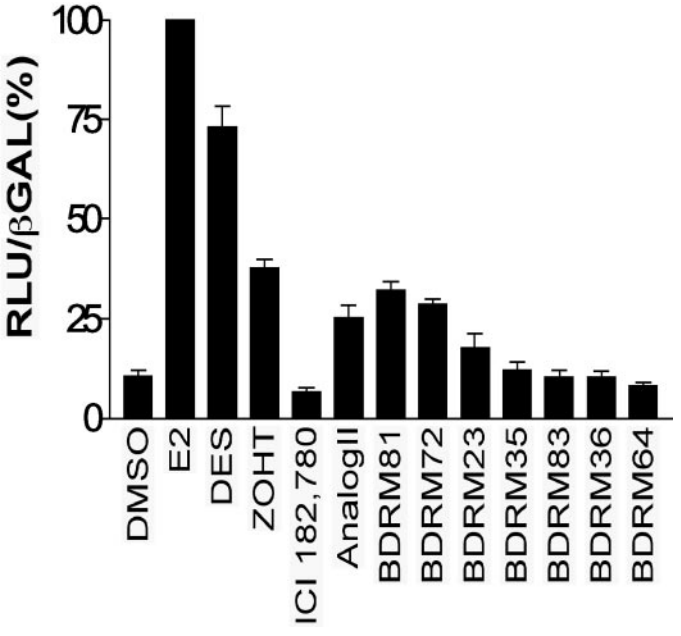

Fig. 2. High molecular weight DTACs do not stimulate ERα transcription. CV-1 cells were cotransfected with CMV-ERα, CMV-β-galactosidase, and ERE-tk-luciferase. After 16 h, E_2, DES, ZOHT, or the DTAC compounds were added. The final concentrations of each compound were 1×10^{-8} M E_2, DES, ZOHT, and ICI 182,780 and 1×10^{-5} M Analog II and DTACs. After an additional 24 h, the cells were harvested, and luciferase and β-galactosidase activity was measured. To control for transfection efficiency, luciferase activity was normalized to β-galactosidase activity. Data are expressed relative to the activity of E_2, which was set at 100%. Bars represent the mean ± S.D. of at least three independent experiments.

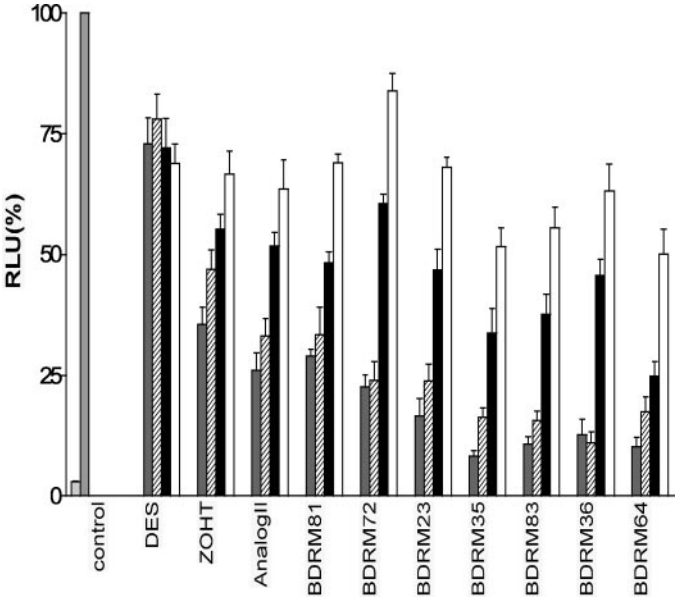

Fig. 3. DTACs block estradiol-stimulated ERα transcription in a dose-dependent manner. CV-1 cells were cotransfected with CMV-ERα and ERE-tk-luciferase. After 16 h, cells were treated with DMSO alone (control), 1×10^{-8} M E_2 (control), or 1×10^{-8} M E_2 in the presence of increasing concentrations of the indicated compound. After an additional 24 h, the cells were harvested, and luciferase activity was measured. Data are expressed relative to the activity of E_2 alone, which was set at 100%. Bars represent the mean ± S.D. of at least three independent experiments. White bars, 1×10^{-7} M compound plus E_2; black bars, 1×10^{-6} M compound plus E_2; hatched bars, 1×10^{-5} M compound plus E_2; gray bars, 1×10^{-5} M compound without E_2.

experiments, where E_2 was added simultaneously with a test compound, ZOHT and BDRM35 (both have the β-dimethylaminomethoxy side chain in the central ring and 4-phenolic moiety in the geminal phenyl ring of their respective triaryl systems) reduced the ability of E_2 to induce ERα binding to GRIP1 by more than 50% (Fig. 6B). A known pure antiestrogen, ICI 182,780 also effectively blocked the ability of ERα to bind GRIP1 in the presence of 10^{-9} M E_2. Analog II, BDRM23, and BDRM64 inhibited the GRIP1-ERα interaction to a lesser degree, approximately 30% (Fig. 6B). These data indicate that the DTACs do not induce binding of ERα to GRIP1, and some are able to inhibit p160 coactivator recruitment in the presence of E_2.

The data presented in Fig. 3 demonstrate that DTACs block E_2-mediated stimulation of an exogenous reporter gene. To test whether they similarly regulate the expression of endogenous genes, MCF-7 cells were pretreated with DMSO (solvent control) or an antiestrogenic compound for 1 h before E_2 addition. Effects of treatment on the expression of the well characterized E_2 target genes c-myc (Dubik and Shiu, 1988) and cyclin D1 (Frasor et al., 2003) were analyzed by Western blot (Fig. 7). A 1-h treatment with E_2 resulted in a substantial increase in c-myc expression. The magnitude of this induction was reduced to a similar extent by ICI 182,780, ZOHT, and BDRM35. The antiestrogenic compounds were also effective in blocking E_2 induction of cyclin D1 in MCF-7 cells, an effect that was observed 1 and 4 h post-E_2 addition.

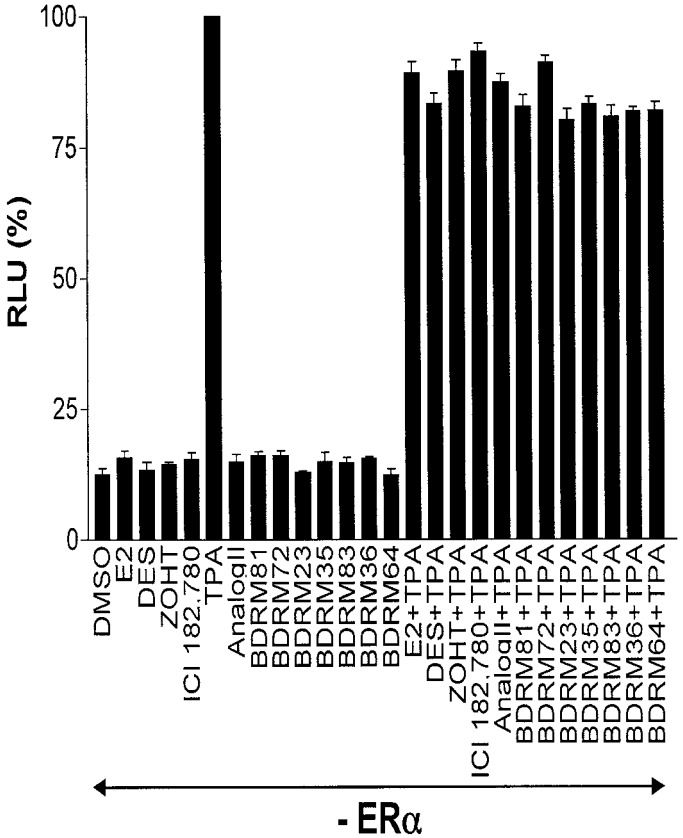

Fig. 4. DTACs are not general transcription inhibitors. CV-1 cells, which are ERα-deficient, were transfected with AP-1-tk-luciferase. The following day, cells were treated as indicated. DTAC compounds were used at a final concentration of 1×10^{-5} M. E_2, DES, ZOHT, and ICI 182,780 were used at a final concentration of 1×10^{-7} M. Cells were harvested, and luciferase activity was measured after 24 h of treatment. Data are expressed relative to the activity obtained for TPA alone, which was set at 100%. Bars represent the mean ± S.D. of at least three independent experiments.

Discussion

The DTACs represent a novel class of SERMs that function as apparent antiestrogens in vivo. To further characterize the mechanism of action of these compounds, we explored their effects on ERα-mediated transcription from classic EREs and nonclassic AP-1 elements. Effects were compared with those obtained with the well characterized SERM, tamoxifen and the pure ERα antagonist ICI 182,780. The DTACs are known to compete with estradiol for the rat uterine cytosol high-affinity low-capacity binding sites (presumably ERα) (Day et al., 1991) and inhibit estradiol-induced stimulation of transcription from EREs in a dose-dependent manner (Fig. 3). Taken together, these data indicate that the DTACs function as antiestrogens at the level of transcriptional regulation, effectively blocking the stimulatory effects of estradiol.

The DTACs displayed less estrogenic character in ERE-based mammalian reporter assays than did ZOHT. The DTACs also demonstrated less estrogenic character in AP-1-based mammalian reporter assays than did either ZOHT or ICI 182,780. Tamoxifen stimulation of ERα-mediated transcription at nonclassic EREs is observed in endometrial cells but not in breast cancer cells and parallels its effects on the in vivo response of these cell types (Tonetti and Jordan, 1996; Fisher et al., 1999; Shang and Brown, 2002). The diminished

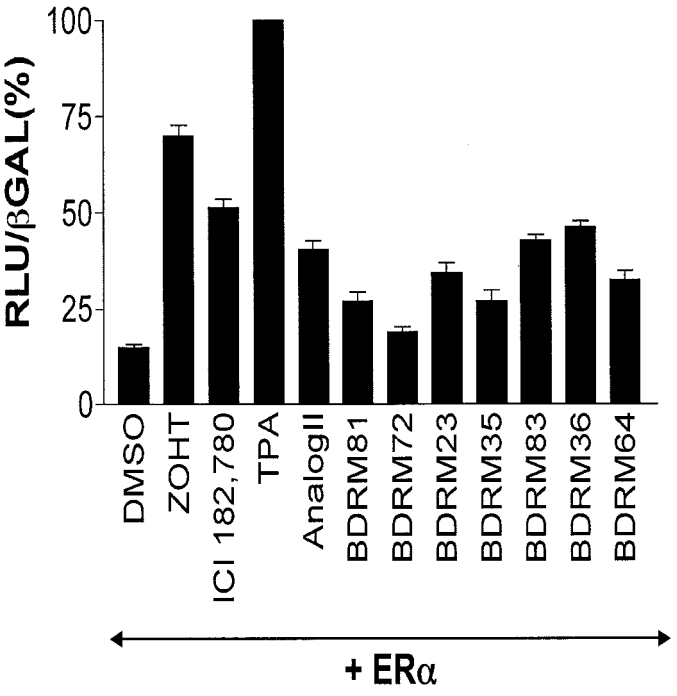

Fig. 5. DTACs target ERα to modulate transcription at AP-1 elements. CV-1 cells were cotransfected with CMV-ERα, CMV-β-galactosidase, and AP-1-tk-luciferase. After 16 h, E_2, DES, ZOHT, ICI 182,780, TPA, or the DTAC compounds were added. The final concentrations of each compound were 1×10^{-8} M E_2, DES, ZOHT, and ICI 182,780; 1×10^{-5} M Analog II and DTACs; and 1×10^{-7} M TPA. After an additional 24 h, the cells were harvested, and luciferase and β-galactosidase activity was measured. To control for transfection efficiency, luciferase activity was normalized to β-galactosidase activity. Data are expressed relative to the activity of TPA, which was set at 100%. Bars represent the mean ± S.D. of at least three independent experiments.

capacity of the DTACs to stimulate ERα-mediated transcription at AP-1 sites is consistent with the lack of uterine growth stimulation in vivo by these compounds (Day et al., 1991). These preclinical data suggest that, compared with tamoxifen, DTACs will be less likely to have the undesired effect of stimulating endometrial growth. The potential use of this novel class of pure antiestrogens therefore warrants further compound development and evaluation.

DTAC derivatives with smaller aromatic substituents, BDRM81 and BDRM72, having either no modification or only a methoxy group at the *para* position of the central phenyl ring, were the most active at eliciting transcription at EREs (Fig. 2) but were among the least active compounds at AP-1 sites (Fig. 5). These data support the idea that the requirements for DTAC stimulation of ERα-mediated transcription are different at EREs and AP-1 sites.

When bound by an agonist, the ER undergoes a conformational change that results in the formation of a hydrophobic groove on the surface of the ligand binding domain to which the nuclear receptor box (LXXLL motif) of p160 coactivators bind (Darimont et al., 1998; Eng et al., 1998; Feng et al., 1998; McInerney et al., 1998; Hong et al., 1999). The recruitment of p160 coactivators is sufficient for both gene activation and the ability of estrogens to stimulate the growth of breast cancer cells (Shang et al., 2000). ZOHT induces an alternative receptor conformation in which helix 12 of the ER occludes the coactivator binding site (Shiau et al., 1998). In this altered conformation, ZOHT stimulates the recruitment of corepressors rather than coactivators to endogenous estrogen-responsive promoters and represses transcription (Shang et al., 2000).

Based on the central role of p160 coactivators in transcriptional regulation by the ER, we examined whether the DTACs disrupt ER signaling by altering recruitment of the p160 coactivator GRIP1 in vitro. None of the DTACs induced

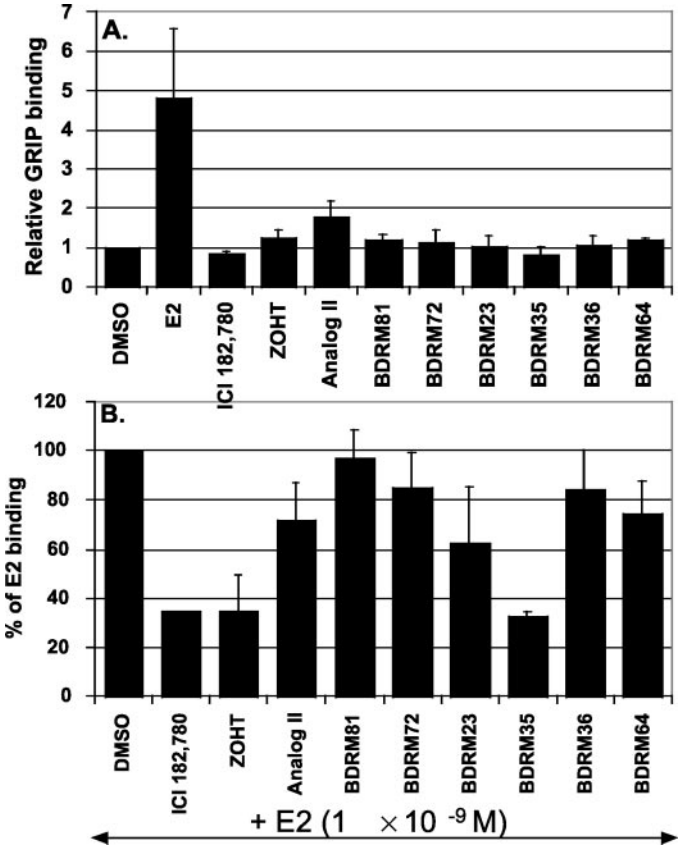

Fig. 6. Some DTACs interfere with the GRIP1-ERα interaction. The ability of DTAC compounds to modulate the interaction between ER and the p160 coactivator GRIP1 was measured using GST pull-down assays. GST-GRIP1 was bound to glutathione-Sepharose beads and then mixed with [^{35}S]methionine-labeled wild-type ERα as described under *Materials and Methods*. Immediately after mixing, the indicated ligands were added either alone (A) or in the presence (B) of E_2, and complexes were allowed to form for 4 h at 4°C. GST-GRIP1/ER complexes were subsequently collected by centrifugation, washed, and the bound radioactivity quantified. The concentration of each ligand was as follows: 1×10^{-9} M E_2; 1×10^{-7} M ICI; and 1×10^{-5} M ZOHT, Analog II, and DTACs. Bars represent the mean of at least two independent experiments. Data are expressed relative to DMSO solvent control (A) or the binding for E_2 alone (B), which was set at 100%.

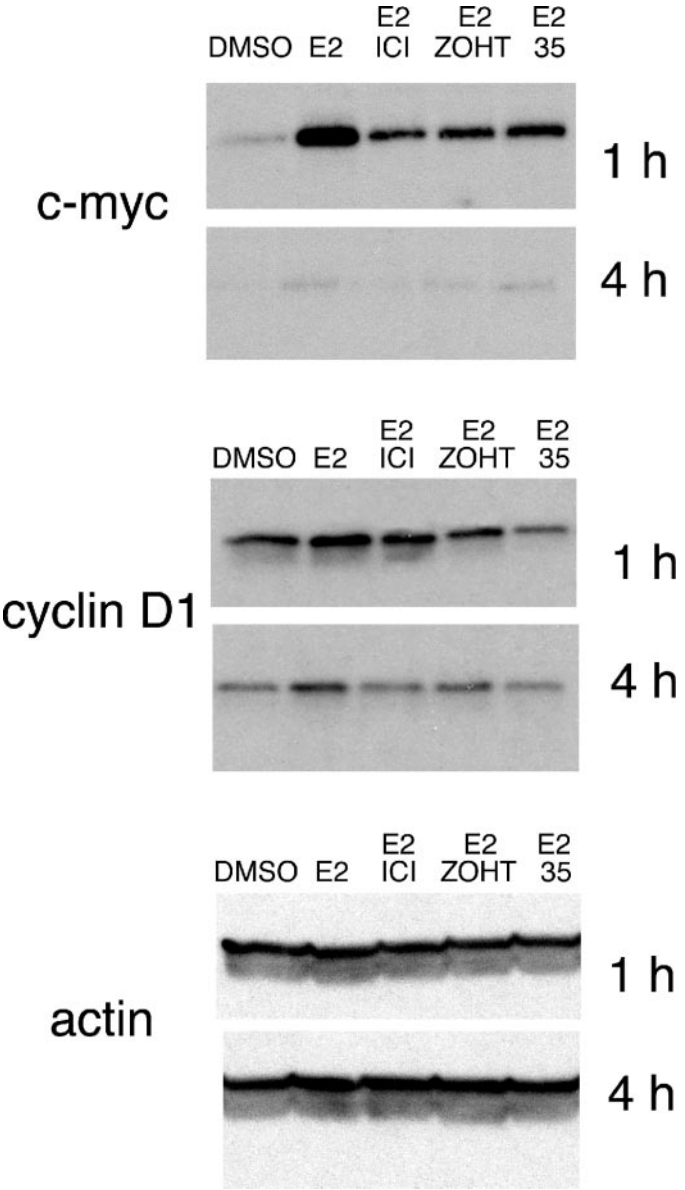

Fig. 7. BDRM35 blocks E_2 induction of endogenous target genes. MCF-7 cells were pretreated for 1 h with DMSO (solvent control), 1×10^{-7} M ICI 182,780, 1×10^{-7} M ZOHT, or 1×10^{-5} M BDRM35. At the conclusion of the pretreatment, E_2 was added to a final concentration of 1×10^{-9} M. Protein extracts were prepared 1 or 4 h post-E_2 addition. Protein expression was evaluated by Western blot using anti-c-myc or anti-cyclin D1 antibody at dilutions of 1:250 and 1:200, respectively. Blots were reprobed for actin as a control for protein quantitation and loading.

[^{35}S]methionine-labeled ERα to bind to GST-GRIP1 (Fig. 6A), which is consistent with their nonstimulatory transcriptional effects (Fig. 3); however, only BDRM35 was able to strongly inhibit E_2 stimulation of GRIP1-ERα binding as effectively as ZOHT and ICI 182,780 (Fig. 6B). BDRM35 and ZOHT share the same aryl substituents, suggesting that these features are important for preventing GRIP1 binding. Of note is the observation that BDRM23, which is the same as BDRM35 except that it lacks the phenolic substitution of the geminal aryl ring (and is therefore the dichlorocyclopropyl analog of tamoxifen rather than 4-hydroxytamoxifen), did not effectively disrupt the E_2-stimulated ERα-GRIP1 interaction. The phenolic moiety of 4-hydroxytamoxifen makes it a much better antiestrogen than tamoxifen, because it anchors the ligand within the hormone binding pocket of the ERα by forming hydrogen bonds with Glu353 and Arg394 (Brzozowski et al., 1997; Shiau et al., 1998). The absence of the phenolic moiety in BDRM23 may increase the mobility and decrease its affinity within the ligand binding pocket of ER, thereby reducing its inhibition of E_2-stimulated ERα-GRIP1 binding.

Analog II, BDRM23, and BDRM64 inhibited the GRIP1-ER interaction to a lesser degree, approximately 30% (Fig. 6B). The smallest DTAC derivatives, BDRM81 and BDRM72, did not significantly block E_2-induced GRIP1 binding in vitro. Therefore of the DTACs and known SERMs (ZOHT and ICI 182,780) tested in this study (Fig. 1), only those with the 4-position phenolic moiety were able to effectively prevent ERα-GRIP1 interaction. This correlates with the key phenolic position on the "A ring" of the natural and high-affinity ERα ligand, 17β-estradiol, and emphasizes the need to include it in future compound design.

It is also likely that more than one mechanism is involved in the DTAC inhibitory effects on estrogen signaling. It is possible that DTACs effectively recruit corepressors to EREs in vivo or reduce the binding of a different coactivator such as SRC-1 or AIB-1, depending on the cell type. The DTACs may also function similarly to the pure antiestrogen ICI 182,780, which disrupts E_2 signaling by stimulating ER degradation (Dauvois et al., 1992, 1993). The ability of DTACs to differentially modulate ER stability is under investigation.

Cyclin D1 and c-myc are well characterized E_2 target genes, the regulation of which seems to be central to the ability of E_2 to enhance the proliferation of MCF-7 breast cancer cells. We therefore studied the effects of BDRM35 on E_2 regulation of these genes as a way to assess its activity against endogenous targets of estrogen. We found that BDRM35 was as effective as ZOHT and ICI 182,780 in blocking E_2 stimulation of both c-myc and cyclin D1. Thus, BDRM35 functions as an antiestrogen and effectively blocks E_2-mediated stimulation of endogenous genes critical to the proliferation of breast cancer cells.

Estrogens signal physiologically through two distinct receptors, ERα and ERβ, which show differential tissue distribution, affinity for coactivators, and regulation by ER ligands (Suen et al., 1998; Wärnmark et al., 2001; Wong et al., 2001). We are cognizant of the fact that the precise outcome of DTAC administration in vivo will depend on effects on each of these receptors, and we are currently investigating the impact of the DTACs on ERβ signaling.

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