

A New Small Molecule Inhibitor of Estrogen Receptor α Binding to Estrogen Response Elements Blocks Estrogen-dependent Growth of Cancer Cells*

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Estrogen receptor α (ER α) plays an important role in several human cancers. Most current ER α antagonists bind in the receptor ligand binding pocket and compete for binding with estrogenic ligands. Instead of the traditional approach of targeting estrogen binding to ER, we describe a strategy using a high throughput fluorescence anisotropy microplate assay to identify small molecule inhibitors of ER α binding to consensus estrogen response element (cERE) DNA. We identified small molecule inhibitors of ER α binding to the fluorescein-labeled (fl)cERE and evaluated their specificity, potency, and efficacy. One small molecule, theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl) (TPBM), inhibited ER α binding to the flcERE (IC₅₀ ~ 3 μ M) and inhibited ER α -mediated transcription of a stably transfected ERE-containing reporter gene. Inhibition by TPBM was ER-specific, because progesterone and glucocorticoid receptor transcriptional activity were not significantly inhibited. In tamoxifen-resistant breast cancer cells that overexpress ER α , TPBM inhibited 17 β -estradiol (E₂)-ER α (IC₅₀ 9 μ M) and 4-hydroxytamoxifen-ER α -mediated gene expression. Chromatin immunoprecipitation showed TPBM reduced E₂-ER α recruitment to an endogenous estrogen-responsive gene. TPBM inhibited E₂-dependent growth of ER α -positive cancer cells (IC₅₀ of 5 μ M). TPBM is not toxic to cells and does not affect estrogen-independent cell growth. TPBM acts outside of the ER ligand binding pocket, does not act by chelating the zinc in ER zinc fingers, and differs from known ER α inhibitors. Using a simple high throughput screen for inhibitors of ER α binding to the cERE, a small molecule inhibitor has been identified that selectively inhibits ER α -mediated gene expression and estrogen-dependent growth of cancer cells.

Estrogen receptor α (ER α)³ is a member of the steroid/nuclear receptor family of transcription regulators and mediates cell growth and metastasis and resistance to apoptosis and immunosurveillance (1–5). ER α is activated by binding of 17 β -estradiol (E₂), or by the epidermal growth factor-activated extracellular signal-regulated kinase pathway and other signal transduction pathways (6). ER α -mediated gene transcription contributes to the development and spread of breast, uterine, and liver cancer (5, 7, 8). A role for ER action in ovarian cancer is supported by the recent finding that endocrine therapy is effective against relapsed ER-containing ovarian cancers (9, 10). Aromatase inhibitors that inhibit estrogen production and tamoxifen (Tam) and other selective estrogen receptor modulators (SERMs) are mainstays in treatment of estrogen-dependent cancers and have played an important role in developing our understanding of ER action (5, 7, 11, 12). Tam and other SERMs work by competing with estrogens for binding in the ligand binding pocket of ER. Over time, tumors usually become resistant to tamoxifen and other SERMs (13–15), requiring new strategies to inhibit ER α action.

In the best characterized model for ER action, ER α activates gene transcription by binding to palindromic estrogen response element (ERE) DNA and ERE half sites (4, 16, 17). Thus, an alternative to current approaches that primarily target ER action at the level of ligand binding is to target ER α at the level of its interaction with ERE DNA. Although targeting protein binding to DNA is attractive, until recently this approach was questioned, because small molecules may not disrupt the large interaction surfaces of protein-DNA and protein-protein complexes (18). However, several recent studies support the feasibility

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³ The abbreviations used are: ER α , estrogen receptor α ; E₂, 17 β -estradiol; cERE, consensus estrogen response element; flcERE, fluorescein-labeled cERE; FAMA, fluorescence anisotropy microplate assay; TPBM, theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl) (also known as 8-benzylsulfanylmethyl-1,3-dimethyl-3,7-dihydropurine-2,6-dione); Tam, tamoxifen; OHT, 4-hydroxytamoxifen; SERM, selective estrogen receptor modulator; PR, progesterone receptor; AR, androgen receptor; GR, glucocorticoid receptor; ARE, androgen response element; HRE, hormone response element; GRE/PRE, glucocorticoid/progesterone response element; Dox, doxycycline; ERU, estrogen responsive unit; HTS, high throughput screening; DIBA, disulfide benzamide; ChIP, chromatin immunoprecipitation; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PI-9, proteinase inhibitor 9.

HTS-derived Inhibitor of ER α Action

bility of using a high throughput screening (HTS) approach to identify small molecules that act directly at the binding interface, or allosterically by inducing a conformational change in the protein that alters the formation of a functioning macromolecular interface (19–24). Although it was not identified by HTS, disulfide benzamide (DIBA), an ER α zinc finger inhibitor (25), enhances the antagonist activity of Tam (26), providing support for our approach of identifying small molecule inhibitors targeting novel sites in ER action.

To inhibit ER α binding to the ERE, we developed and implemented an HTS fluorescence anisotropy microplate assay (FAMA) (27). We recently used FAMA to demonstrate active displacement in the binding of full-length SRC1 to ERE \cdot ER complexes (28). To use the FAMA as an HTS assay, a fluorescein-labeled consensus ERE (flcERE) is synthesized (28, 29). When polarized light excites the flcERE, the relatively small flcERE usually undergoes rotational diffusion more rapidly than the time required for light emission. Therefore, the position of the flcERE at the time of light emission is largely randomized, resulting in depolarization of most of the emitted light. When full-length ER α binds to the flcERE, the larger size of the flcERE \cdot ER α complex causes slower rotation, increasing the likelihood that the flcERE \cdot ER α complex will be in the same plane at the time of light emission as it was at the time of excitation. Therefore, the emitted light remains highly polarized. A receptor-DNA interaction increases fluorescence polarization and fluorescence anisotropy. Although fluorescence anisotropy assays based on using a labeled DNA binding site for the protein of interest represent an attractive approach, a study using this *in vitro* strategy to identify small molecule inhibitors of the b-zip DNA binding transcription factors failed to identify specific inhibitors that function in cells (30).

Here we used FAMA to conduct HTS and identified a small molecule, theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl) (TPBM, an 8-alkylthiothiated theophylline) (31, 32), that specifically inhibits E₂-induced, ER α -mediated, gene expression in intact cells, without significantly inhibiting PR- and GR-mediated gene expression. TPBM also inhibits E₂ and 4-hydroxytamoxifen (OHT, the active metabolite of Tam) induction of an endogenous gene in Tam-resistant breast cancer cells expressing elevated levels of ER α . ChIP demonstrates that TPBM decreases binding of E₂ \cdot ER α to a responsive gene. TPBM is not toxic to ER α -negative cells and exhibits dose-dependent inhibition of the estrogen-dependent growth of ER α -positive cancer cells. Our data show that an *in vitro* assay, using a protein-free consensus ERE and purified ER α , can identify small molecule inhibitors that block ER-mediated gene expression and estrogen-dependent growth of cancer cells.

EXPERIMENTAL PROCEDURES

Proteins—Full-length FLAG-tagged human ER α was expressed and purified as we described previously (27). Human FLAG-PR-B (33) and full-length, wild-type human FLAG-AR were purified as described (34).

Oligonucleotides—A 30-bp oligonucleotide containing the cERE was synthesized with fluorescein at its 5'-end using phosphoramidite chemistry and PolyPak II (Glen Research Corp, Sterling, VA) purified by the Biotechnology Center (Uni-

versity of Illinois, Urbana, IL). This flcERE was used in our earlier work describing FAMA (27, 28). The sequence of the fluorescein-labeled sense strand, with the cERE half sites underlined, is: 5'-fl-CTAGATTACAGGTCACAGTGACCT-TACTCA-3'. The flcARE is 5'-fl-CTAGATTACGGTACATGATG TTCTTACTCA-3'. The flcPRE is 5'-fl-CTAGATTACAGAACAAATCTGTTCTTACTCA-3'. The flcARE and flcPRE were synthesized and characterized as described for flcERE. To remove traces of free fluorescein present in some oligonucleotides (29), they were passed over a Centri-Sep column (usually used to remove free fluorescent dyes in DNA sequencing) following the supplier's directions (Princeton Separation, Princeton, NJ). Oligonucleotides were prepared at 10 μ M, and 50–100 μ l was loaded onto each column. To calculate oligonucleotide concentration, A_{260} values were measured. The method of Ozers *et al.* (35) was used to determine the degree of fluorescein incorporation, which was ~60% for the flcERE and slightly lower for flcARE and flcPRE. After column purification, the double-stranded probes were produced by annealing the fluorescein-labeled sense strand with an equimolar amount (both at 1 μ M) of the unlabeled antisense strand oligonucleotide in TE buffer (10 mM, Tris, pH 7.5, 1 mM EDTA) containing 100 mM NaCl at 100 °C for 5 min, followed by slow cooling in a water bath to form double-stranded probe.

High Throughput Screening Using FAMA—Previous microplate-based fluorescence polarization/anisotropy assays used 20- to 30- μ l volumes (19, 27, 30). To minimize protein use and to identify the appropriate concentrations of ER α to use in HTS, we carried out ER α binding studies in 10 and 20 μ l. As we recently reported for RNA-binding proteins (29), the use of small volumes results in only a slight decline in FA signal with no change in K_d or loss of reproducibility (Fig. 1). The only modification required for the 10- μ l assays was brief centrifugation of the 384-well plates to ensure that the sample volume was uniformly distributed across the bottom of the wells.

Two libraries of small molecules were screened. A library developed at the University of Illinois by K. Putt and P. Hergenrother contained ~9700 small molecules (22, 36, 37), and the NCI, National Institutes of Health Diversity Set contained 1990 small molecules. Prior to screening, the 10 mM library stocks were diluted to produce replica libraries in 384-well plates containing each small molecule at 0.25 mM (in DMSO). In our initial studies we were concerned that forming the flcERE \cdot ER α complex first and then adding the candidate small molecule inhibitors would miss small molecules that bind at the interface. We therefore screened using the “sequential” method in which the candidate small molecules were first incubated with ER α and followed by addition of the flcERE. The assay for binding of ER α to the flcERE is a modification of our earlier assay (27). Assays were carried out at room temperature in black wall 384-well microplates (Greiner/Bio-One) in a total volume of 10 μ l in buffer containing 20 mM Tris, pH 7.5, 10% glycerol, 0.2 mM EDTA, 2 mM dithiothreitol, 100 mM KCl, 0.5 ng/ μ l poly(dI:dC), 250 ng/ μ l bovine serum albumin, and 100 nM 17 β -estradiol (E₂). For high throughput screening, a master mix without ER α and probe was prepared at 4 °C. The master mix was divided into two parts. ER α was added to one part to 5 nM in the final assays. 7 μ l of the ER α -containing mix was then dispensed into

each well of a 384-well plate on ice. 100 nl of the compounds being tested was then added using a pin-transporter (V & P Scientific, Inc.) to a final concentration of 2.5 μM . The samples were mixed using the pin-transporter and sedimented by centrifugation for 2 min at 4 $^{\circ}\text{C}$ and incubated on ice for 10 min. The flcERE probe was added to the other aliquot of the mix to 1 nM. 3 μl of the mix containing the flcERE probe was added to each well containing ER α and the test compound. The samples were mixed using the pin-transporter, the plates were briefly centrifuged and incubated at room temperature for 10 min, and fluorescence anisotropy was measured using a BMG PheraStar (BMG Labtech) microplate reader (module: FP 485 520 520) with excitation at 485 nm and emission at 520 nm. To identify small molecules that were highly fluorescent, or quench fluorescence, fluorescence intensity was also measured.

Although there is no universally accepted standard of what change in signal constitutes a "hit" suitable for further evaluation, some researchers consider that any small molecule that results in a change of more than three standard deviations from the mean is appropriate for further study. Under the conditions of the HTS, the average change in anisotropy over the entire 384-well plate was 31.6 ± 2.7 S.D. The S.D. is 8.5%, and $3 \times$ S.D. is $\sim 25\%$. We therefore carried out further analysis of small molecules that, when present at 2.5 μM , altered the average change in anisotropy for binding of ER α to the flcERE by at least 25%. Re-screening the same plates demonstrated that the screen was reproducible. 76% of the primary hits scored as hits on re-screening a set of the initial plates (data not shown).

Dose-response curves for selected compounds were carried out as described for the HTS screen except that each well contained the indicated concentration of test compound. PR assays were carried out in the same buffer used for ER α assays and contained 1 nM flcPRE and 11 nM progesterone receptor B and 100 nM progesterone. The buffer used for AR was similar but also contained 5 μM ZnCl₂, 5 mM NaF, 0.6 μM CHAPS, and 100 nM dihydrotestosterone. AR assays contained 1 nM flcARE and 50 nM full-length wild-type AR. The concentrations of ER α , PR, and AR chosen for use produce 70–80% of maximum binding.

Reporter Gene Assays—The T47D-KBluc cells stably express an (ERE)₃-luciferase reporter gene (38). Cells were maintained in phenol red-free RPMI 1640 with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 10 mM Hepes, pH 7.5, 1 mM sodium pyruvate, 10% fetal bovine serum (Atlanta Biological, Atlanta, GA) and antibiotics. Four days before E₂ induction, the cells were switched to the above medium, with 10% 2 \times charcoal-dextran-treated calf serum instead of fetal bovine serum. 200,000 cells/well were transferred to each well of a 24-well plate. After 24 h the indicated concentrations of the test compounds were added in DMSO, and E₂ was added to 20 pM. After 24 h cells were washed once in phosphate-buffered saline, and 150 μl of 1 \times Passive Lysis Buffer (Promega, Madison, WI) was used to lyse the cells. Luciferase activity was determined using firefly luciferase reagents from Promega. T47D cells stably transfected to express GR and a mouse mammary tumor virus luciferase reporter that responds to liganded GR and PR were maintained and assayed in medium containing 5 nM progesterone for PR assays, or 2.5 nM dexamethasone for GR assays, essentially as described (39).

Evaluating Endogenous Gene Expression in Tam-resistant Breast Cancer Cells—A model for Tam-resistant breast cancers that overexpress ER α is MCF7ER α HA cells, which is a tetracycline-inducible MCF-7 cell line in which doxycycline (Dox) induces overexpression of ER α (40, 41). In contrast to MCF-7 cells, in these cells Tam and OHT are potent agonists (2, 42), and OHT, which stabilizes ER α , induced proteinase inhibitor 9 (PI-9) mRNA and protein more effectively than E₂ (2, 43). MCF7ER α HA cells were maintained in 10% 6 \times charcoal-dextran-treated fetal bovine serum (40, 41). All cells were in 0.1% DMSO vehicle and contained the indicated concentrations of TPBM added at the same time as the E₂ or OHT. To induce PI-9 mRNA, the cells were treated with 0.5 $\mu\text{g}/\text{ml}$ Dox to induce ER α and ethanol vehicle, 100 pM E₂, or 500 pM OHT for 24 h, mRNA was extracted and PI-9 mRNA levels were determined by quantitative reverse transcription-PCR as we recently described (2).

ChIP Assays—MCF7ER α HA cells were maintained as described above for studies evaluating endogenous gene expression. The cells were maintained for 24 h in medium containing 100 pM E₂ with or without 20 μM TPBM. To increase signals on the weak PI-9 promoter, in one experiment the E₂ concentration was raised to 10 nM for 45 min prior to cross-linking. The MCF7ER α HA were cross-linked with 1% formaldehyde and processed essentially as described (44). ER α -DNA complexes were immunoprecipitated with ER α -specific antibody (sc-8002 Santa Cruz Biotechnologies, Santa Cruz CA). PCR primers for PI-9 were: Forward 5'-CCT GAC CTG ACC CTG CTC-3'; Reverse 5'-CGC CTC CCA CGC TTT CTG-3'. Standard curves were produced using 1,000, 5,000, 10,000, 50,000, and 100,000 copies of each gene and primer and subject to real-time PCR using SYBR[®] Green PCR Master Mix (Applied Biosystems, Warrington UK) and the iCycler PCR thermocycler (Bio-Rad Laboratories).

Cell Growth and Toxicity Assays—ER α -positive BG-1 ovarian cancer cells (45) were provided by Prof. K. Korach. ER α -negative MDA-MB-231 human breast cancer cell lines were provided by Prof. A. Nardulli. The cells are maintained in phenol red-free minimal essential medium with 5% calf serum and antibiotics. 4 days before hormone induction, cells are switched to phenol red-free minimal essential medium containing 5% 2 \times charcoal-dextran-treated calf serum for BG-1 cells. For BG-1 cell growth assays, 250 cells in 100 μl of phenol-red-free medium were added to wells of a 96-well plate. After 24 h, the indicated concentrations of the test compounds and 10 pM E₂ or ethanol vehicle were added to each well. Compounds in DMSO were diluted in medium so that the DMSO concentration was not $>0.5\%$. Cell viability assays were carried out 5–6 days later using the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (MTS) (Promega).

ER α -negative MDA-MB-231 human breast cancer cells were used to test for generalized toxicity of the test compounds. To parallel the reporter gene assays in stably transfected T47D breast cancer cells, 5000 MDA-MB-231 cells per well were plated in a 96-well plate. The cells were maintained in the medium described above for the T47D cells. One day after plating, the same concentration of test compound used in the reporter gene assay (up to 30 μM) was added. After 24 h the cell

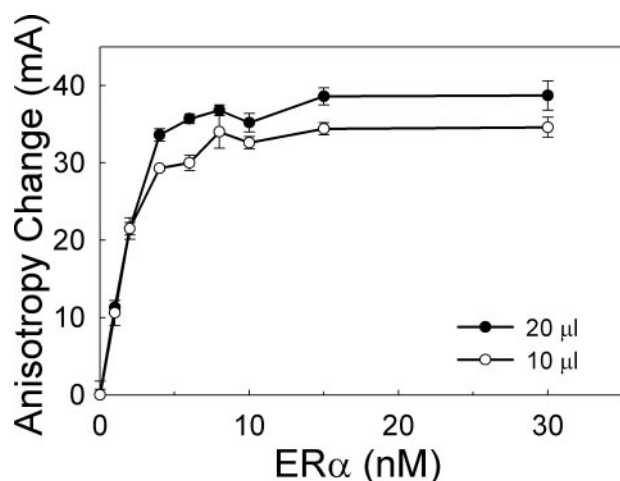


FIGURE 1. **A 384-well plate FAMA for 10- μ l volumes.** FAMA was carried out in samples containing 100 nM E₂ as described under "Experimental Procedures" in 384-well black wall microplates using either the standard 20- μ l volume (filled circles) or the 10- μ l volume used in the final HTS screen (open circles). Data represent the average increase in anisotropy observed after E₂:ER α binding to the flcERE. Data represent the mean \pm S.E. for four separate experiments.

proliferation assay was carried out as described above. A more stringent toxicity assay parallels the assay for inhibition of estrogen-dependent growth of BG-1 cells. 250 MDA-MB-231 cells were plated per well and maintained and assayed as described for the BG-1 cells. Several compounds without detectable toxicity in the 24-h assay inhibited MDA-MB-231 cell growth in the 5- to 6-day assay.

Western Blots—Western blots were performed as we recently described (43) with minor modifications. ER α was detected using a 1:2,000 dilution of ER α antibody ER6F11 (Bio Care Medical, Concord, CA). The blot was stripped for 15 min prior to reprobing with a 1:10,000 dilution of actin antibody.

RESULTS

The High Throughput Screen for Inhibitors of ER α —Development of the 10- μ l primary screen (Fig. 1) is detailed under "Experimental Procedures." The sequence of assays used to identify the lead inhibitor of ER α action in ER-dependent cancer cells is summarized in the flow chart in Fig. 2. In the initial high throughput screen, FAMA was used to assay binding of purified hER α to the flcERE in 384-well microplates. The validated hits were evaluated using FAMA for potency, efficacy, and specificity. Promising compounds were further tested in breast cancer cell lines stably transfected with reporter genes, in cell-based assays for toxicity, and for their ability to block estrogen-dependent cancer cell growth. The lead compound, TPBM, was then tested for its ability to inhibit E₂ and OHT induction of the endogenous PI-9 gene in Tam-resistant MCF7ER α HA cells. PI-9 is a granzyme B inhibitor that inhibits cytotoxic T lymphocyte (CTL) and natural killer (NK)-mediated apoptosis of target cells (2, 3). We used PI-9 as a test endogenous gene, because elevated expression of PI-9 is associated with a poor prognosis and reduced survival in several human cancers (46–48). PI-9 is a primary estrogen-regulated gene (49, 50). We recently showed that E₂ and OHT elicit robust >100-fold inductions of PI-9 mRNA in MCF7ER α HA cells (2). To begin to evaluate its site of action, we showed that TPBM

Identifying small molecules that block ER action in ER-dependent cancer

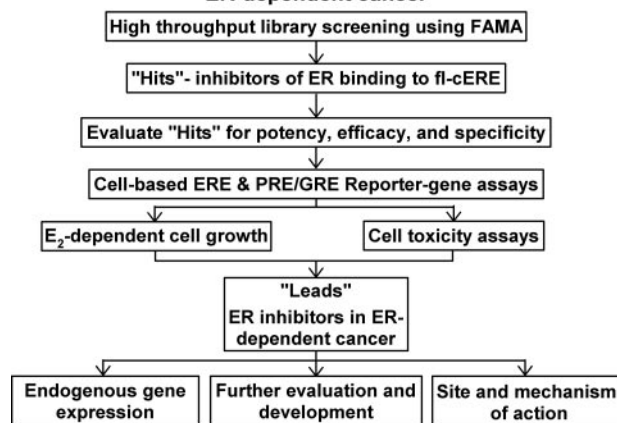


FIGURE 2. **Scheme for identification and characterization of small molecule inhibitors of ER α action in ER-dependent cancer cells.** Our strategy for identification of ER α antagonists included the following assays. (i) *In vitro* FAMA assays using purified proteins and DNA to carry out the HTS screen and to further characterize the verified hits for potency, efficacy, and specificity. (ii) Cell-based gene expression assays to determine potency and efficacy and to evaluate specificity using assays for PR- and GR-regulated gene expression. (iii) Cell growth assays for evaluating ability of the final candidates to block estrogen-dependent growth of cancer cells and for their generalized toxicity in ER α -negative cancer cells. (iv) Testing inhibitor potency and efficacy against an endogenous gene in Tam-resistant breast cancer cells. (v) Early studies to test known sites of ER α inhibitor action.

does not bind in the ligand binding pocket of ER α and that zinc does not block its inhibitory effect, indicating it is not an electrophile acting by chelating the zinc in the zinc fingers of ER.

Out of ~12,000 small molecules initially screened at 2.5 μ M, 262 reduced the anisotropy of the ER α -flcERE complex by >25% (see "Experimental Procedures"). After rescreening and eliminating compounds that no longer reduced the anisotropy change by >25%, displayed intrinsic fluorescence, were quenchers, or reduced the signal of the free flcERE probe, 56 structurally diverse compounds were selected for further testing. Most of the small molecules excluded from further analysis either displayed intrinsic fluorescence, or reduced the signal by a little over 25% in the initial assay and slightly less than 25% on re-testing.

Analysis of Hits for ER α Specificity, Potency, and Efficacy—Detailed potency and efficacy studies established IC₅₀ values required to block ER α binding to flcERE. Specificity was evaluated in dose-response studies by quantitative FAMA using purified full-length human PR binding to a fluorescein-labeled progesterone/glucocorticoid response element and full-length human AR binding to a fluorescein-labeled androgen response element. IC₅₀ values for inhibition of HRE binding by ER α , PR, and AR were determined for each of 56 small molecules identified in the primary screen and subsequent verification assays (Table 1). Most of the compounds inhibited more than one steroid receptor.

Structures (Fig. 3A) and dose-response curves (Fig. 3B) are presented for the four compounds subject to the most extensive analysis in cell-based studies (see below) and for two molecules representative of the diverse outcomes we observed. Compound 9568 (Fig. 3A) exhibited high potency and was the most specific ER inhibitor of the ~12,000 molecules tested (Fig. 3B, 9568). However, it is relatively large (M_r ~ 1,300) (Fig. 3A,

TABLE 1
IC₅₀ values for small molecule inhibitors of binding of steroid receptors to their HREs

IC₅₀ values were determined from dose-response curves (see Fig. 3B). For each small molecule binding of the indicated steroid receptor (ER α , AR, or PR) was determined at five concentrations (0.5, 1, 2.5, 5, and 10 μ M). The data represent the average of four independent sets of samples at each concentration. For ER, AR, and PR, FAMA was performed using the sequential method as described under "Experimental Procedures." The small molecules shown in Fig. 3 are in boldface.

Compound	IC ₅₀		
	ER α	AR	PR
		μ M	
92	2.5	0.5	5
340	>10	5.5	8
1387	4.5	4.8	6.5
1445	0.6	0.7	1.2
1529	4	5.2	7.5
1826	7	8	6
2067	5	3	10
2287	2.1	1.7	2.7
2674	5.5	5.8	6.2
3706	0.8	1	1.5
3710	2.5	5	2.9
3713	6	5.2	7
3813	2	4.8	6.5
3879	3.7	7.5	6.5
4456	7.5	>10	>10
4695	2.5	4.8	4
4700	2	2	2.7
4792	>10	7.5	>10
4864	8	6.5	8.8
5648	6.5	4.5	7.3
5649	9	6.7	8.5
5650	8	5.5	7.5
6119	9	7.4	9
6122	5.8	3.7	5.3
6454	5.8	>10	10
7107	5	10	>10
7122	5	7.5	10
7450	9.8	>10	>10
7473	9	>10	>10
7484	9.5	>10	>10
7487	9.5	>10	>10
8216	9	>10	>10
9064	>10	>10	>10
9503	2.6	>10	>10
9545	0.7	3	3.8
9548	1	4	5
9568	2	>10	>10
9671	>10	>10	>10
343040	>10	>10	>10
7810	>10	>10	>10
371847	>10	>10	>10
90737	10	>10	>10
95910	3	7.6	9.5
15596	5	>10	>10
130796	6	10	>10
360494	>10	>>10	>10
170008	9	9	10
125908	3.8	>10	>10
638432	2.5	5	7.4
34238	1.8	10	>10
91767	8	>10	>10
112257	10	>10	>10
109268	8	9	>10
43628	>10	>10	>10
306711	6.1	4	>10
146443	<0.5	0.5	0.4

9568) and had poor bioavailability in cell culture. Compound 9545 is representative of several small molecules that displayed good potency and efficacy but lacked specificity, having similar ability to inhibit binding of ER α , PR, and AR to their respective hormone response elements (HREs). Four structurally diverse molecules selected for further testing in cell-based assays exhibited good potency, with preferential inhibition of ER α binding to the flcERE relative to PR and AR

binding to their HREs (Fig. 3B, compounds 130796, 1529, 638432, and TPBM/95910).

Small Molecule Hits Inhibit ER-mediated Transcription in Intact Cells—The ability of each small molecule to inhibit ER-mediated gene expression in intact cells was tested in the ER α -positive T47D-KBluc breast cancer cell line that stably expresses an (ERE)₃-luciferase reporter gene (38). An E₂ dose-response curve showed that the cells exhibited strong E₂-dependent activation of the reporter gene with full induction at 50 pM E₂ (Fig. 4A). This is within the concentration range shown to induce PI-9 in MCF-7 cells (2) and several endogenous genes in HeLa cells stably transfected to express ER α (51).

Candidate small molecules were initially tested at 30 μ M in the T47D cell assay in medium containing 20 pM E₂ for 24 h prior to measuring luciferase activity (Fig. 4B). As expected a 100-fold molar excess of the antagonist ICI 182,780/Faslodex/Fulvestrant blocked activation of the reporter gene (Fig. 4B, +ICI). Small molecules that inhibited expression of the reporter by at least 50% and were not toxic in a short term 24-h toxicity test using MDA-MB-231 cells (see "Experimental Procedures," data not shown) were subjected to additional analysis. As shown in Fig. 4C, concentration-dependent inhibition of E₂-dependent ER α transactivation was observed with IC₅₀ values of 11.5 μ M 95910/TPBM, 22 μ M 1529, 3.5 μ M 130796, and 0.8 μ M 638432.

To establish specificity for ER α , we tested the small molecules for inhibition of GR and PR transactivation in T47D cells that express stably transfected GR and contain sufficient endogenous progesterone receptor B (but not AR, data not shown) to activate the stably expressed murine mammary tumor virus-luciferase reporter (39). Using T47D cells for the ER α , GR, and PR transactivation experiments minimized effects due to cell context. In preliminary experiments we found that 2.5 nM dexamethasone and 5 nM progesterone each elicited ~80% of maximum induction, the same relative level of transactivation used in our studies with E₂. These hormone concentrations resulted in transactivation that was specific for the receptor being tested (data not shown). In dose-response studies, higher concentrations of 638432 and 130796 were required to inhibit GR transactivation than ER α , and TPBM/95910 did not inhibit GR transactivation up to 20 μ M, with ~35% inhibition at 30 μ M (Fig. 5A). Compound TPBM/95910 did not significantly inhibit PR transactivation. Compounds 1529 and 130796 inhibited PR transactivation between 20 and 30 μ M (Fig. 5B).

Inhibition of the Estrogen-dependent Growth of Cancer Cells—A key goal of our studies was to determine whether small molecules selected for inhibition of binding of ER α to the cERE could block E₂-dependent growth of cancer cells. Consistent with earlier studies (45), we found that BG-1 cells exhibited a stronger and more reproducible E₂ stimulation of cell growth than MCF-7 cells (data not shown). Although the small molecules also inhibited estrogen-dependent growth of MCF-7 cells (data not shown), we focused most of our work on BG-1 cells. Data are shown for the most ER α -specific inhibitors, TPBM/95910 and 1529. Compound 1529 potently inhibited E₂-dependent growth of the BG-1 cells (IC₅₀ ~ 5 μ M). However, >5 μ M 1529 inhibited growth of the cells in the absence of E₂, suggesting a nonspecific effect at the higher inhibitor concen-

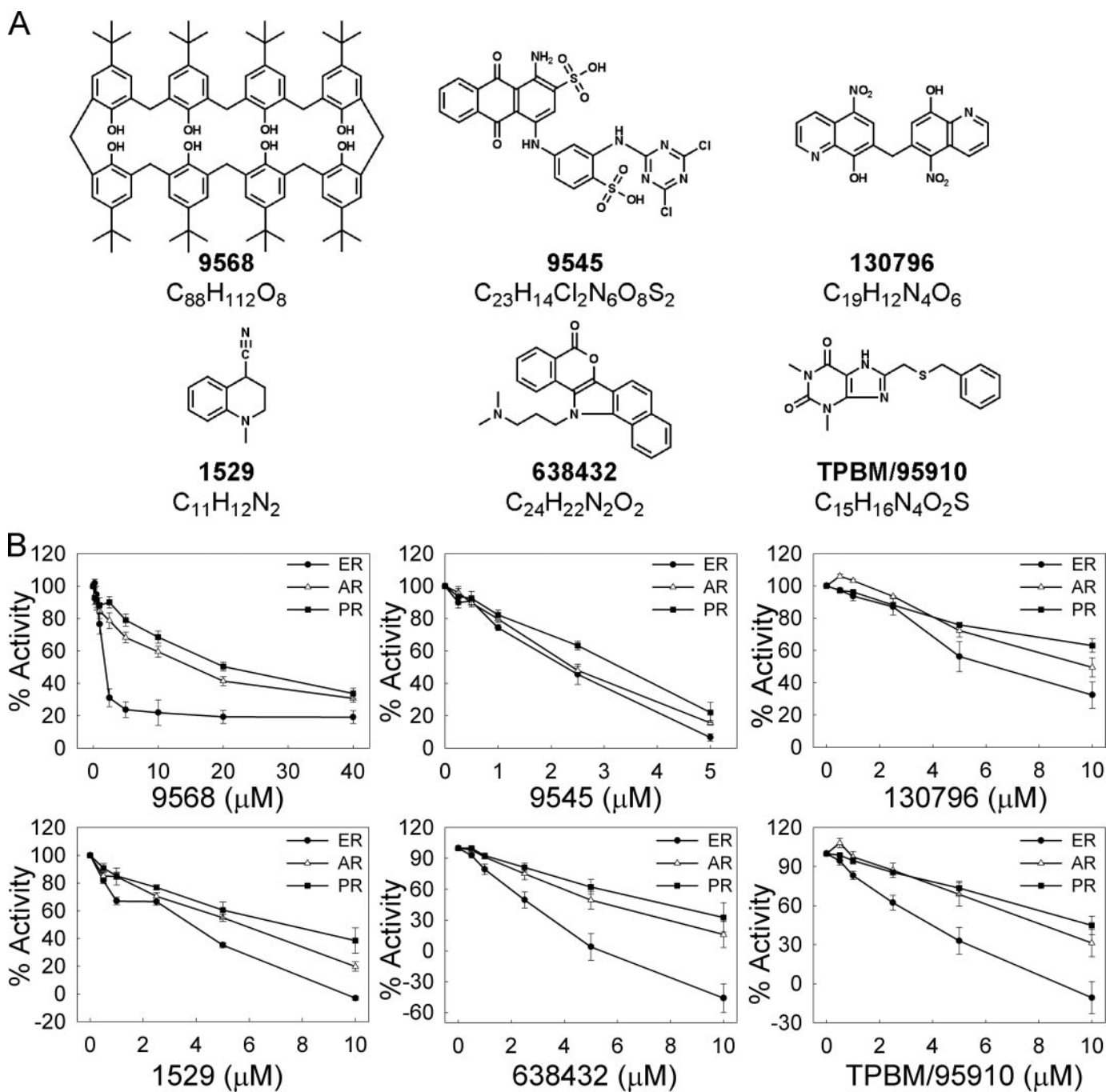


FIGURE 3. Dose-response curves for inhibition of ER α , PR, and AR binding to their HREs. A, the structures of the six compounds whose binding curves are shown in B. B, dose-response curves for ER α -selective and non-selective inhibitors identified in the primary HTS screen. The indicated concentrations of each small molecule were incubated with ER α (filled circles), AR (open triangles), and PR (filled squares) using the sequential method described under "Experimental Procedures." The anisotropy change on binding of each receptor to its respective response element was set equal to 100%. These anisotropy changes were: ER α \sim 35 mA units, PR \sim 90 mA units, and AR \sim 60 mA units. Because AR and PR are larger than ER, their binding to their HREs results in larger anisotropy changes. The data for compound TPBM/95910 represent a separate set of experiments from the data used to compile Table 1. The data represent the mean \pm S.E. for four separate experiments at each concentration.

tration (Fig. 6A, 1529). TPBM/95910 exhibited concentration-dependent inhibition of E₂-dependent growth of the BG-1 cells, with an IC₅₀ of 5 μM . At 30 μM , TPBM was as effective as a 100-fold excess of OHT in blocking E₂-dependent growth of BG-1 cells (Fig. 6A, TPBM).

To test for general cell toxicity, the ER α -negative MDA-MB-231 cell line was used. Growth of the MDA-MB-231 cells was unaffected by 1–20 μM 1529, but was reduced by 50–60% at 30

μM 1529 (Fig. 6B, 1529). The data suggest that, although 1529 elicits some E₂-dependent inhibition of cell growth, at higher concentrations it is toxic to cells. TPBM had no effect on E₂-independent growth of BG-1 cells (Fig. 6A, gray bars) or MDA-MB-231 cells (Fig. 6B). Studies of TPBM/95910, theophylline, 8-[(benzylthio)methyl]-(7Cl,8Cl) in the NCI, NIH Developmental Therapeutics Program testing program confirmed a lack of toxicity with 60 cancer cell lines over a wide range of

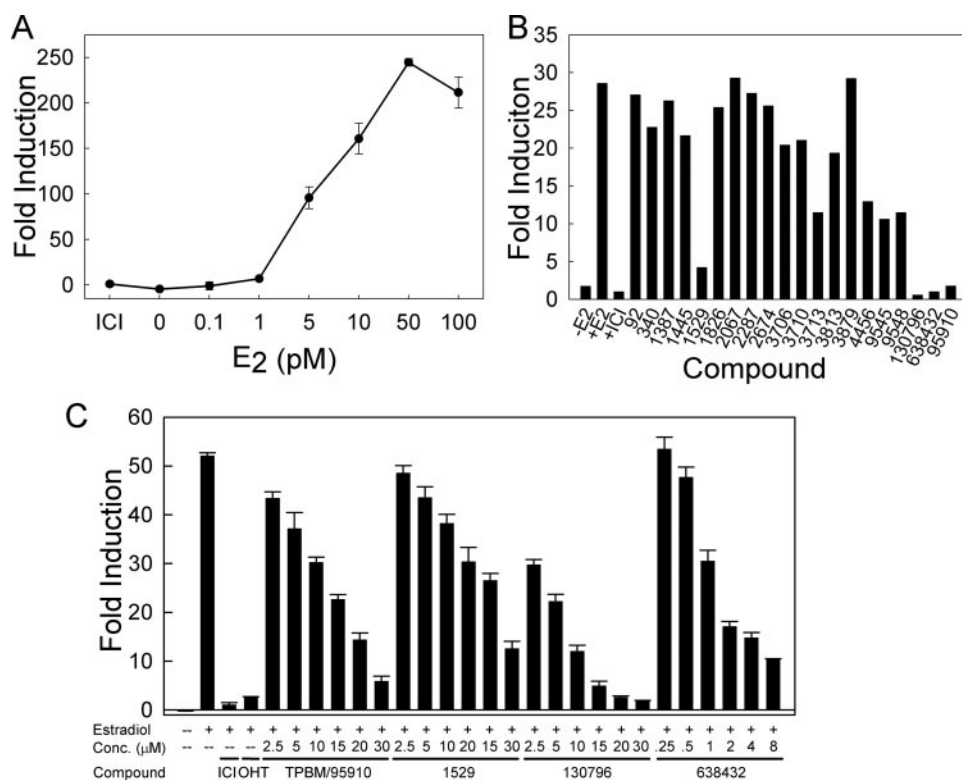


FIGURE 4. Effect of small molecules on ER-mediated gene expression in T47DKBluc cells. *A*, E₂ dose-response curve. The cells were maintained in medium containing either 1 nM ICI 182,780 (to test for traces of estrogens in the medium), or the indicated concentrations of E₂ and reporter gene expression was assayed after 24 h. The data represent the average of three independent experiments \pm S.E. *B*, inhibition of ER α -mediated gene expression by small molecules that inhibit binding of ER α to the ERE. Small molecules identified in the FAMA HTS screen, verified and further characterized for potency and specificity, were tested. Cells were incubated in medium containing 30 μ M inhibitor for 30 min, then 20 pM E₂ (*A*) was added, and the cells were incubated for an additional 24 h. Control experiments demonstrated that the DMSO used to dissolve the small molecules and the ethanol used to dissolve the E₂, separately and in combination, did not alter gene expression or reduce cell viability (data not shown). Data in *B* represent single experiments. *C*, dose-response curves for small molecules that inhibit ER-mediated gene expression. Assays were as described in *B*. In control experiments the cells were maintained for 24 h in medium containing 20 pM E₂, with or without 1 nM of ICI 182,780 or OHT. The indicated concentrations of each small molecule were incubated with the cells and E₂-ER-mediated gene expression assayed. The data represent the mean \pm S.E. for four separate experiments at each concentration. IC₅₀ values were obtained by curve-fitting using Sigma plot and had a high R² value.

concentrations up to 100 μ M TPBM. Of the 60 cell lines tested at 100 μ M TPBM/95910, only a few lung cancer cell lines showed >50% reduction in cell growth. Even at 100 μ M, TPBM/95910 did not inhibit growth of any of the 12 tested lines of breast and ovarian cancer cells by 50%.⁴ Thus, TPBM exhibits low toxicity to cells, and there is a large concentration difference between the 5 μ M IC₅₀ for TPBM inhibition of E₂-ER α -dependent growth of BG-1 cells and the >100 μ M TPBM required for inhibition of E₂-ER α -independent breast and ovarian cell growth.

TPBM Inhibits E₂ and OHT Induction of an Endogenous Gene in Tam-resistant Breast Cancer Cells—Development of resistance to Tam and other SERMs represents a major problem in endocrine therapy (7, 53–55). Thus, an important goal in the development of new inhibitors is to block ER α transcriptional activity in Tam-resistant breast cancer cells. Because TPBM targets binding of ER to DNA and does not compete with estrogens for binding as SERMs do, we explored whether TPBM is effective in Tam-resistant

MCF7ER α HA breast cancer cells. MCF7ER α HA cells are a tetracycline-inducible MCF-7 model for Tam-resistant breast cancer in which Dox induces overexpression of ER α (40, 41). In these cells Tam and OHT are potent agonists (2, 41). Because OHT stabilizes ER α , whereas E₂ down-regulates ER α , in MCF7ER α HA cells OHT is more effective than E₂ in inducing PI-9 (2).

Saturating E₂ (100 pM, Fig. 7A) and OHT (500 pM, Fig. 7B) induced PI-9 mRNA by 150- and 500-fold, respectively. TPBM (95910) elicited a concentration-dependent inhibition of E₂-ER α induction of PI-9 mRNA with an IC₅₀ of 8.5 μ M (Fig. 7A). 30 μ M TPBM (95910) was required to inhibit OHT-ER α induction of PI-9 mRNA by 48% (Fig. 7B). This is a stringent test, because Western blotting followed by PhosphorImager quantitation of band intensities shows that MCF7ER α HA cells, treated with Dox to induce ER α , express 3- to 4-fold more ER α in the presence of E₂ or OHT than MCF7ER α HA cells not treated with Dox (Fig. 7C). The less complete inhibition of PI-9 induction in the OHT-treated cells likely results from the ~4-fold higher level of ER α after OHT treatment than after E₂ treatment (Fig. 7C). The PI-9 gene is representative

of the many genes that contain complex estrogen response elements, including ERE half sites. These data show that TPBM inhibits ER α -mediated gene expression in Tam-resistant breast cancer cells that overexpress ER α .

ChIP Shows That TPBM Inhibits Binding of E₂-ER α to an Estrogen-regulated Gene—TPBM blocks binding of E₂-ER α to the flcERE in FAMA (Fig. 3). We used ChIP to test whether the ability of TPBMs to inhibit E₂ induction of PI-9 mRNA in MCF7ER α HA cells results from inhibition of E₂-ER α binding to the PI-9 estrogen-responsive region. MCF7ER α HA cells were maintained under the same conditions used to test the effect of TPBM on E₂ induction of PI-9 mRNA (Fig. 7A), and semi-quantitative ChIP (44) was performed. Although the signal in ChIP was low, TPBM inhibited binding of E₂-ER α to the PI-9 estrogen responsive unit (ERU) by 55% (Fig. 8). To evaluate the influence of TPBM on binding of E₂-ER α to the PI-9 ERU under conditions in which a stronger ChIP signal could be obtained, we exploited the observation that E₂-ER α binds to ERE-containing genes in an oscillatory fashion with time-dependent cycles of binding and release (56, 57). Although 100 pM E₂ produces a near maximal ~150-fold induction of PI-9 mRNA, after

⁴ Developmental Therapeutics Program, NCI/National Institutes of Health.

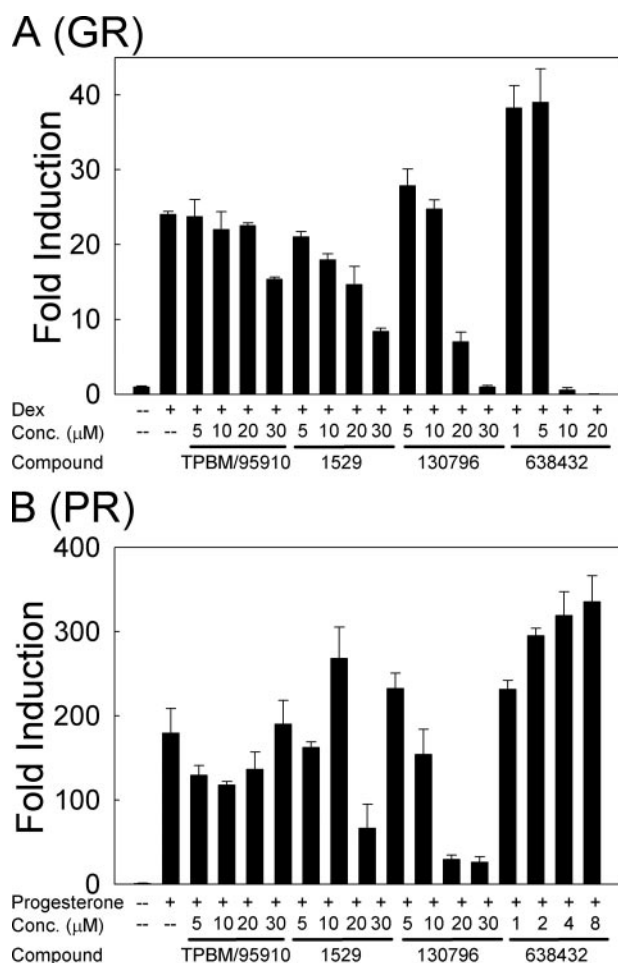


FIGURE 5. Effect of ER α inhibitors on GR- and PR-mediated gene expression in T47D cells. Assays were performed essentially as described for ER in the legend to Fig. 4 (A and B). The indicated concentration of each small molecule was incubated with the cells for 30 min followed by addition of 2.5 nM dexamethasone to assay GR transactivation (A) or 5 nM progesterone to assay PR transactivation (B). After 24 h, luciferase activity was measured. The data represent the mean \pm S.E. for four separate experiments at each concentration.

24 h in 100 pM E₂, binding of ER α to PI-9 was likely largely randomized. To enhance the ChIP signal we synchronized E₂·ER α binding by adding a pulse of 10 nM E₂ 45 min before cross-linking the cells. This resulted in a more robust binding of E₂·ER α to the PI-9 ERU and an increase of \sim 7.5-fold in ChIP occupancy units (Fig. 8). Under these conditions, binding of E₂·ER α to the PI-9 estrogen-responsive unit was decreased 72% (Fig. 8) in the presence of 20 μ M TPBM. At 20 μ M TPBM, induction of PI-9 mRNA was inhibited by 71% (Fig. 8). Thus, there was a good correlation between the ability of TPBM to inhibit induction of PI-9 mRNA and its ability to inhibit binding of E₂·ER α to the PI-9 gene. These data demonstrate that TPBM inhibits ER action in intact cells by decreasing binding of ER to EREs.

TPBM Does Not Bind in the Ligand Binding Pocket of ER α and Is Not a Zinc Chelator—We performed experiments to test the possibility that TPBM inhibits E₂·ER α binding to the flcERE by binding in the ER α ligand binding pocket or as an electrophile that complexes zinc in the zinc fingers of ER α . We found that increasing the concentration of E₂ to 10 μ M had no effect

on the ability of TPBM to inhibit binding of ER α to the flcERE (Fig. 9).

The only other known small molecule ER α inhibitor that acts outside the ER α ligand binding pocket is the electrophile DIBA, which chelates the zinc in the zinc fingers of the ER DNA binding domain (25, 26). Wang and coworkers showed that preincubating with zinc largely blocks inhibition of ER α by DIBA (25). At 5 μ M TPBM, binding of ER α to the flcERE was inhibited 76 \pm 7% (n = 3) in the absence of zinc and 68 \pm 6% (n = 3) in the presence of 50 μ M zinc. Under the same conditions, preincubating with zinc prevented the zinc chelator *ortho*-phenanthroline from inhibiting ER α binding to the flcERE (data not shown) and (27). Therefore, TPBM does not act by chelating the zinc in the zinc fingers of ER α and is a novel ER α inhibitor that acts outside of the ERs ligand binding pocket.

DISCUSSION

In this work, we describe a broadly applicable HTS system for identifying small molecules that inhibit interaction of DNA-binding proteins with their recognition sequences, demonstrate that a small molecule identified using this simple *in vitro* assay with isolated components also acts by reducing DNA binding in intact cells, and show that the candidate small molecule specifically and effectively blocks estrogen-dependent growth of cancer cells. TPBM is effective in Tam-resistant breast cancer cells making it a strong candidate for further therapeutic testing and development.

The HTS Screen—Usually, to identify small molecule inhibitors of macromolecular interactions in HTS screening, a mixture containing all of the components is assembled and then incubated with each compound in the library (30). We were concerned that pre-forming the E₂·ER α ·flcERE complex might eliminate those small molecules that bound at the protein·DNA interface. We therefore used the more complex approach of first incubating each test compound with E₂·ER α and then adding the flcERE. We compared this “sequential” screening method to the “mixture” method. Only a few compounds showed somewhat different potency as inhibitors of E₂·ER α binding to the flcERE when assayed by the sequential and mixture methods. Although both the mixture and sequential methods are robust screens (Z' > 0.5 (58)), the mixture method is preferred because it is easier to implement in large scale HTS for steroid receptors.

Because we were primarily searching for inhibitors, we screened the libraries at a concentration of E₂·ER α that results in \sim 80% of maximal binding. This reduced the chances of identifying activators that reduce the concentration of E₂·ER α required for maximal binding. A brief examination of 37 small molecules that resulted in increased anisotropy and did not display intrinsic fluorescence, showed that all 37 small molecules altered the anisotropy of the free flcERE probe and were therefore not genuine activators (data not shown). Screening the libraries at a receptor concentration that results in approximately half-maximal binding to the HRE is one way to determine the relative frequency of inhibitors and activators. However, screening at half-maximal binding results in smaller anisotropy changes and is better suited to HTS using the AR

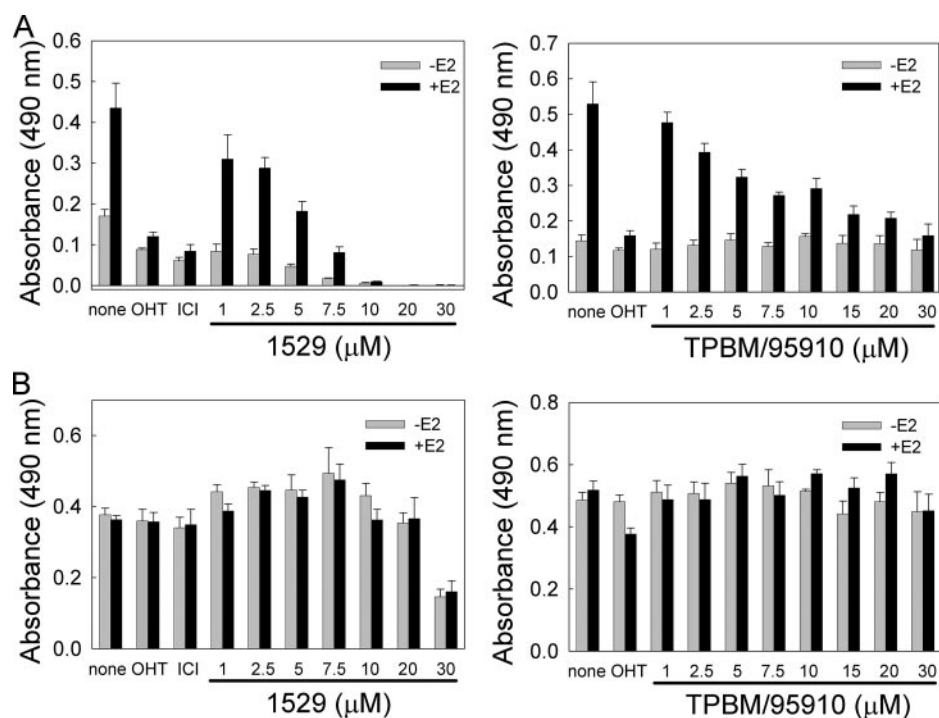


FIGURE 6. Small molecule inhibitors of ER-mediated gene expression block estrogen-dependent growth of cancer cells. A, BG-1 ovarian cancer cells were maintained in medium lacking E₂ (gray bars), or containing 10 pM E₂ (black bars). OHT and ICI were at 1 nM. The cells were maintained for 5 days in the presence of the indicated concentrations of TPBM/95910 or 1529 as described under "Experimental Procedures," and viable cells were determined using the cell titer Aqueous one solution cell proliferation assay. B, ER α -negative MDA-MB-231 cells were maintained in medium containing no E₂ (gray bars) or 10 pM E₂ (black bars). OHT and ICI were at 1 nM. The cells were maintained for 5 days in the indicated concentrations of TPBM/95910 and 1529, and viability was assayed as described using the cell titer Aqueous one solution cell proliferation assay. Cell plating and assays are described under "Experimental Procedures." The data represent the mean \pm S.E. for four separate experiments at each concentration. The IC₅₀ for TPBM/95910 was obtained by curve-fitting using Sigma plot and had a high R² value.

and PR, which are larger than ER α and produce much larger anisotropy increases when they bind to their HREs.

Because the composition of the two libraries we screened was not random, we cannot generalize about the chemical structures likely to be associated with ER α inhibitors. Although the structures of the inhibitors were highly diverse, a substantial percentage of the inhibitors, including the lead compound, TPBM, contained multiple rings that were joined by some sort of a flexible linker. Whereas some small molecules that were detected using FAMA did not function in the cell-based transfection assay, a high percentage of the molecules identified in the initial screen function in intact cells.

Identification and Characterization of an Inhibitor of ER α Action in Cancer Cells—The initial *in vitro* HTS screen employs a simple system containing only two pure components, a cERE and purified ER α . To be useful, inhibitors identified by this screening must inhibit ER α -mediated transcription in intact cells. Using a stably transfected breast cancer cell line that expresses endogenous ER α , we showed that TPBM elicits a concentration-dependent inhibition of reporter gene expression. The question of whether small molecules screened for the very different property of blocking binding of ER α to a cERE would also inhibit estrogen-dependent cancer cell growth was unresolved. At 30 μ M, TPBM and OHT both nearly completely inhibited estrogen-dependent growth of BG-1 cells. Interestingly, the IC₅₀ of 3.5 μ M for inhibition of binding of ER α to the

flcERE in FAMA is similar to the 5 μ M IC₅₀ for inhibiting the estrogen-stimulated component of BG-1 cell growth, suggesting an association between inhibition of ER α binding to EREs and inhibition of cell growth.

To be useful in antagonizing estrogen action in cancer cells, a small molecule should exhibit good specificity for ER α and low overall toxicity. TPBM inhibited E₂-ER α -dependent cell growth with an IC₅₀ of 5 μ M with no inhibition of the growth of ER α -negative MDA MB-231 cells up to 30 μ M. Independent testing of this compound against a panel of 60 cancer cell lines at the NCI, NIH Developmental Therapeutics Program showed that TPBM did not inhibit breast and ovarian cell growth up to 100 μ M. Thus, TPBM shows >10-fold greater potency for inhibiting E₂-ER α -dependent cell growth relative to nonspecific toxicity. In contrast, for several other ER α inhibitors (1529, 638432, and 130796) the concentrations required to inhibit estrogen-dependent growth of BG-1 cells was at most a few fold lower than the concentration that

was toxic to ER-negative MDA MD-231 cells. These compounds are unlikely to be useful as antagonists of ER action in cells.

We compared the ability of TPBM to inhibit reporter gene transcription mediated by ER α , PR, and GR in the same cell line expressing different reporter genes. Even at 30 μ M, TPBM has little effect on reporter gene transcription by PR and GR. Because we tested the specificity of TPBM against closely related steroid hormone receptors and because TPBM has little or no toxicity to cells, it is unlikely to significantly inhibit a broad range of DNA binding transcription regulators.

Another important aspect of our study was to identify an ER α inhibitor that is active in Tam-resistant breast cancer cells. Estrogen-dependent cancers undergo natural selection to Tam-resistant tumors through a variety of mechanisms, often maintaining expression of a functional ER α that is important for tumor growth (55). Recent studies show that an important feature of Tam-resistant breast cancer cells that retain dependence on ER α for growth is loss of dependence on SRC3 and other p160 coactivators for E₂-ER α -mediated gene transcription (41, 59). ER α in these tumors must still bind DNA to activate transcription. Thus, our screening strategy that targets DNA binding may have advantages compared with a screening strategy that targets binding of p160 coactivators to ER α .

TPBM effectively blocked E₂-dependent induction of PI-9 mRNA in Tam-resistant MCF7ER α HA cells (IC₅₀ 8.5 μ M). It is

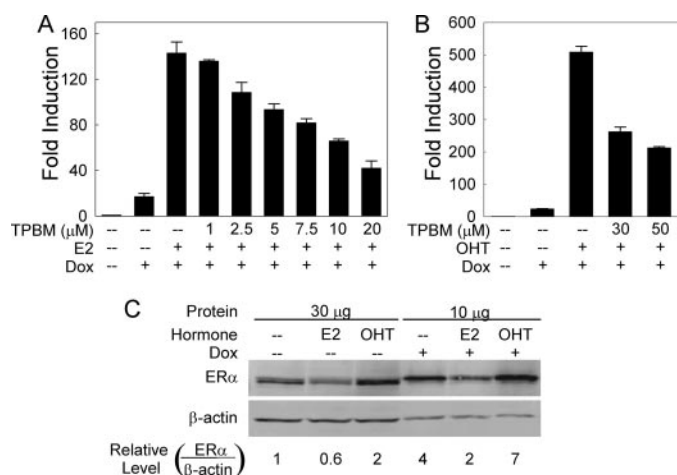


FIGURE 7. TPBM inhibits E₂- and OHT-mediated gene expression in a Tam-resistant cell line. MCF7ER α HA cells were maintained in 10% charcoal-stripped fetal bovine serum, treated with 0.5 μ g/ml Dox to induce ER α and 100 pM E₂ (A) or 500 pM OHT (B) and the indicated concentrations of 95910 for 24 h. The cells were harvested, and PI-9 mRNA levels were determined by quantitative reverse transcription-PCR as described under "Experimental Procedures." The high level of ER α in Dox-treated cells (E₂- and Dox+) results in some ligand-independent transactivation of PI-9 by ER α . The data represent the mean \pm S.E. for three separate experiments each assayed in triplicate. The IC₅₀ of 8.5 μ M for TPBM/95910 inhibition of E₂ induction of PI-9 was obtained by curve-fitting using Sigma plot and had a high R² value. C, Western blot analysis of ER α levels in MCF7ER α HA cells in the presence and absence of Dox. MCF7ER α HA cells were maintained in medium containing or lacking 0.5 μ g/ml Dox and no ligand, 100 pM E₂, or 500 pM OHT. The cells were harvested after 24 h, and total cell extracts were prepared and analyzed for ER α content by Western blot as described under "Experimental Procedures." To better visualize the differences in ER α levels in the uninduced and Dox-induced MCF7ER α HA cells 30 μ g (3 \times more protein) was run for each uninduced sample, and 10 μ g of protein was run for each sample from MCF7ER α HA cells in which ER α was induced with Dox. ER α antibody was used at a dilution of 1:2,000. Relative levels of ER α were calculated by PhosphorImager quantitation of band intensity and normalization to actin (actin antibody was a 1:10,000 dilution). The ratio of unliganded (-E₂ and -OHT) ER α to actin in the MCF7ER α HA cells not treated with Dox to induce ER α was set equal to 1. The ratios of ER α levels in the Dox-treated and uninduced (-Dox) MCF7ER α HA cells were 3.7, 3.1, and 4.0 for cells maintained in medium with no ligand, E₂, and OHT, respectively. The data in C are representative of other Western blots.

probably unusually difficult to inhibit ER α binding to the endogenous PI-9 ERU in the MCF7ER α HA cells and to the (ERE)₃ reporter in the T47D reporter gene cell line. In the MCF7ER α HA cells, the high level of ER α , 3–4 times higher than the already substantial level in MCF-7 cells (Fig. 7C) (40), coupled with use of near saturating E₂, likely makes it difficult to achieve effective inhibition. Furthermore, the (cERE)₃-luciferase reporter stably transfected in the T47D cells will exhibit strong cooperative binding of E₂·ER α to the three cEREs (60), making it difficult for an inhibitor to block ER α binding to the EREs. Interestingly, the IC₅₀ values of 10.5 and 8.5 μ M for inhibition of E₂·ER α -mediated gene expression from the (cERE)₃-luciferase reporter in T47D cells and from the endogenous PI-9 gene in MCF7ER α HA cells are somewhat higher than the IC₅₀ value of 5 μ M for inhibiting E₂-dependent cancer cell growth.

Using quantitative reverse transcription-PCR to measure the PI-9 mRNA level and semi-quantitative ChIP to measure occupancy of the PI-9 estrogen-responsive region, we observed a good correlation between the extent to which TPBM inhibits induction of PI-9 mRNA and the extent to which TPBM reduces E₂·ER α occupancy at the PI-9 gene. Thus, the primary mechanism by which TPBM exerts its intracellular action is by

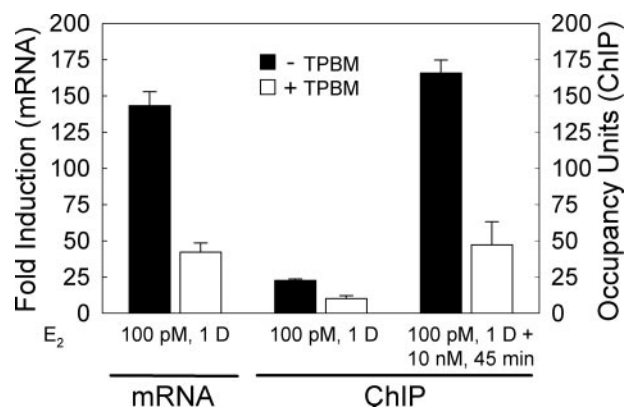


FIGURE 8. ChIP demonstrates that TPBM decreases binding of E₂·ER α to an estrogen-regulated gene. MCF7ER α HA cells were maintained for 24 h as described in the legend to Fig. 7A and used either for determination of PI-9 mRNA levels as described in "Experimental Procedures," or for ChIP (44) and as described in "Experimental Procedures." The MCF7ER α HA cells were maintained in medium containing 100 pM E₂ for 24 h in the absence (black bars) or presence (open bars) of 20 μ M TPBM. In one ChIP, additional 10 nM E₂ was added 45 min. before cross-linking the cells. The mRNA data is presented as -fold induction by E₂, with the level of PI-9 mRNA in control cells not treated with E₂ or Dox set equal to 1. The mRNA data represent the mean \pm S.E. for three separate experiments. The extent of association of E₂·ER α with the PI-9 ERU in the presence of E₂ or E₂ plus 20 μ M TPBM is presented in ChIP occupancy units normalized to 36B4 as a non-regulated gene (44). The ChIP data represent the average \pm S.E. of three assays. Decreased E₂·ER α occupancy of the PI-9 in cells maintained in E₂ plus 20 μ M TPBM was observed in multiple ChIP experiments. The difference between samples treated with E₂ and samples treated with E₂ plus 20 μ M TPBM was highly significant ($p < 0.01$ using the one-tailed Student's *t* test) for the mRNA induction and for both of the ChIPs. The robust nature of both ChIP experiments is demonstrated by the >25-fold increase in ChIP occupancy units in E₂-treated cells compared with control (-E₂ and -Dox) cells.

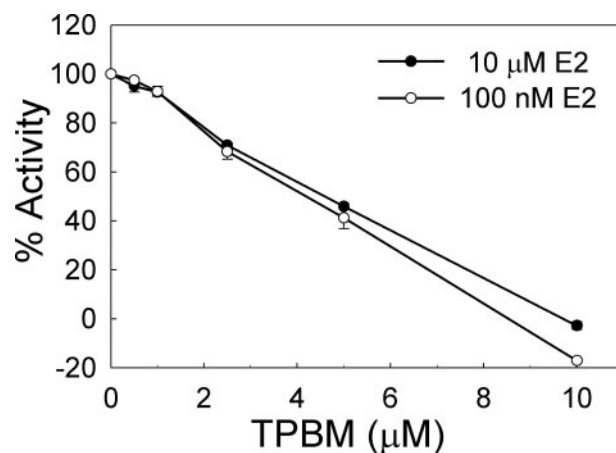


FIGURE 9. High concentrations of E₂ do not reduce the ability of TPBM to inhibit binding of E₂·ER α to the flcERE. Assays were carried out essentially as described in the legend to Fig. 3 and under "Experimental Procedures." E₂ was present at the standard concentration of 100 nM (open circles) or at 10 μ M (closed circles). The anisotropy change in the absence of inhibitor was set equal to 100%. The data represent the mean \pm S.E. for four separate experiments. Error bars that are not visible are smaller than the symbols.

decreasing interaction of ER α with regulatory regions of estrogen-responsive genes. It is likely that interaction of TPBM with ER α induces a conformational change in the receptor, and that one result of this conformational change is decreased association of E₂·ER α with the PI-9 gene. It is difficult to determine whether the conformational change that likely results from binding of TPBM to ER α in Tam-resistant breast cancer cells interferes not only with ERE binding but also with coactivator

binding. TPBM decreases association of E₂·ER α with the PI-9 gene making coactivator studies difficult. Also, in Tam-resistant MCF7ER α HA cells, ChIP fails to detect SRC3 at ER-regulated promoters, and the coactivators important for transactivation by E₂·ER α are presently unknown (41).

TPBM is structurally unrelated to the small molecules known to inhibit nuclear receptor function. β -Aminoketones were identified using HTS as inhibitors that covalently react with the thyroid hormone receptor and inhibit coactivator binding (19). Their specificity for the thyroid hormone receptor compared with other nuclear receptors has not been reported. DIBA is an electrophile originally identified as an inhibitor of binding of zinc finger proteins in retroviruses to their DNA binding sites and subsequently shown to inhibit ER action (25). Perhaps the most interesting property of DIBA is that it induces an ER α conformation that enhances the antagonist activity of Tam in Tam-resistant breast cancer cell lines (26). The utility of small molecules as probes for steroid receptor action was recently demonstrated by identification of a new coactivator binding surface on AR using small molecules selected by HTS as inhibitors of the binding of a coactivator peptide (52). These moderate potency (IC₅₀ ~ 50 μ M) small molecule inhibitors are structurally distinct from TPBM. Because TPBM does not act by binding in the ligand binding pocket of ERs, or by chelating the zinc in ER zinc fingers, and differs from known inhibitors, it represents a new class of ER inhibitor.

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