THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 19, pp. 12819–12830, May 9, 2008

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

Received for publication, December 5, 2007, and in revised form, March 12, 2008 Published, JBC Papers in Press, March 12, 2008, DOI 10.1074/jbc.M709936200

Chengjian Mao[‡], Nicole M. Patterson[‡], Milu T. Cherian[§], Irene O. Aninye[§1], Chen Zhang[‡], Jamie Bonéy Montoya[§], Jingwei Cheng[‡], Karson S. Putt[¶], Paul J. Hergenrother[¶], Elizabeth M. Wilson[‖], Ann M. Nardulli[§], Steven K. Nordeen[**], and David J. Shapiro[‡2]

*From the Departments of [‡]Biochemistry, [§]Molecular and Integrative Physiology, and [¶]Chemistry, University of Illinois, Urbana, Illinois 61810-3602, the [‖]Laboratories for Reproductive Biology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599-7500, and the **Department of Pathology, University of Colorado and Health Sciences Center, Denver, Colorado 80045*

Estrogen receptor α (ERα) plays an important role in several human cancers. Most current ERα 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 ERα binding to consensus estrogen response element (cERE) DNA. We identified small molecule inhibitors of ERα binding to the fluorescein-labeled (fl)cERE and evaluated their specificity, potency, and efficacy. One small molecule, theophylline, 8-[(benzylthio)methyl]-(7CI,8CI) (TPBM), inhibited ERα binding to the flcERE (IC_{50} ~ 3 μM) and inhibited ERα-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 ERα, TPBM inhibited 17β-estradiol (E_2)-ERα (IC_{50} 9 μM) and 4-hydroxytamoxifen-ERα-mediated gene expression. Chromatin immunoprecipitation showed TPBM reduced E_2·ERα recruitment to an endogenous estrogen-responsive gene. TPBM inhibited E_2-dependent growth of ERα-positive cancer cells (IC_{50} of 5 μM). 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 ERα inhibitors. Using a simple high throughput screen for inhibitors of ERα binding to the cERE, a small molecule inhibitor has been identified that selectively inhibits ERα-mediated gene expression and estrogen-dependent growth of cancer cells.

Estrogen receptor α (ERα)[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). ERα is activated by binding of 17β-estradiol (E_2), or by the epidermal growth factor-activated extracellular signal-regulated kinase pathway and other signal transduction pathways (6). ERα-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 ERα action.

In the best characterized model for ER action, ERα 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 ERα 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 feasi-

* This work was supported, in whole or in part, by National Institutes of Health Grants RO1 DK-071909 (to D. J. S.), RO1 DK 53884 (to A. N. M.) and by NICHD, NIH Grant HD16910 (to E. M. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] An NIH predoctoral trainee in cell and molecular biology.

[2] To whom correspondence should be addressed: Dept. of Biochemistry, University of Illinois, 600 S. Mathews Ave. Urbana, IL 61801. Tel.: 217-333-1788; Fax: 217-244-5858; E-mail: djshapir@uiuc.edu.

[3] The abbreviations used are: ERα, estrogen receptor α; E_2, 17β-estradiol; cERE, consensus estrogen response element; flcERE, fluorescein-labeled cERE; FAMA, fluorescence anisotropy microplate assay; TPBM, theophylline, 8-[(benzylthio)methyl]-(7CI,8CI) (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.

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 ERα 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 ERα 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·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 ERα binds to the flcERE, the larger size of the flcERE·ERα complex causes slower rotation, increasing the likelihood that the flcERE·ERα 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]-(7CI,8CI) (TPBM, an 8-alkylthiothiated theophylline) (31, 32), that specifically inhibits E_2-induced, ERα-mediated, gene expression in intact cells, without significantly inhibiting PR- and GR-mediated gene expression. TPBM also inhibits E_2 and 4-hydroxytamoxifen (OHT, the active metabolite of Tam) induction of an endogenous gene in Tam-resistant breast cancer cells expressing elevated levels of ERα. ChIP demonstrates that TPBM decreases binding of E_2·ERα to a responsive gene. TPBM is not toxic to ERα-negative cells and exhibits dose-dependent inhibition of the estrogen-dependent growth of ERα-positive cancer cells. Our data show that an *in vitro* assay, using a protein-free consensus ERE and purified ERα, 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 ERα 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 (University 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-CTAGATTAC<u>AGGTCA</u>CAG<u>TGACCT</u>-TACTCA-3′. The flcARE is 5′-fl-CTAGATTAC<u>GGTACA</u>T-GA<u>TG TTCT</u>TACTCA-3′. The flcPRE is 5′-fl-CTAGATTA-C<u>AGAACA</u>ATC<u>TGTTCT</u>TACTCA-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 μM, and 50–100 μl 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 μM) 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-μl volumes (19, 27, 30). To minimize protein use and to identify the appropriate concentrations of ERα to use in HTS, we carried out ERα binding studies in 10 and 20 μl. 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-μl 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·ERα 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 ERα and followed by addition of the flcERE. The assay for binding of ERα 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 μl in buffer containing 20 mM Tris, pH 7.5, 10% glycerol, 0.2 mM EDTA, 2 mM dithiothreitol, 100 mM KCl, 0.5 ng/μl poly(dI:dC), 250 ng/μl bovine serum albumin, and 100 nM 17β-estradiol (E_2). For high throughput screening, a master mix without ERα and probe was prepared at 4 °C. The master mix was divided into two parts. ERα was added to one part to 5 nM in the final assays. 7 μl of the ERα-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 °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 ERα 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× S.D. is ~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 ERα 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 ERα 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 ZnCl, 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 ERα, 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 E_2 induction, the cells were switched to the above medium, with 10% 2× 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 E_2 was added to 20 pM. After 24 h cells were washed once in phosphate-buffered saline, and 150 μl of 1× 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 ERα is MCF7ERαHA cells, which is a tetracycline-inducible MCF-7 cell line in which doxycycline (Dox) induces overexpression of ERα (40, 41). In contrast to MCF-7 cells, in these cells Tam and OHT are potent agonists (2, 42), and OHT, which stabilizes ERα, induced proteinase inhibitor 9 (PI-9) mRNA and protein more effectively than E_2 (2, 43). MCF7ERαHA cells were maintained in 10% 6× 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 E_2 or OHT. To induce PI-9 mRNA, the cells were treated with 0.5 μg/ml Dox to induce ERα and ethanol vehicle, 100 pM E_2, 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—MCF7ERαHA cells were maintained as described above for studies evaluating endogenous gene expression. The cells were maintained for 24 h in medium containing 100 pM E_2 with or without 20 μM TPBM. To increase signals on the weak PI-9 promoter, in one experiment the E_2 concentration was raised to 10 nM for 45 min prior to cross-linking. The MCF7ERαHA were cross-linked with 1% formaldehyde and processed essentially as described (44). ERα·DNA complexes were immunoprecipitated with ERα-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, 50,0000, 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—ERα-positive BG-1 ovarian cancer cells (45) were provided by Prof. K. Korach. ERα-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× 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 E_2 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).

ERα-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

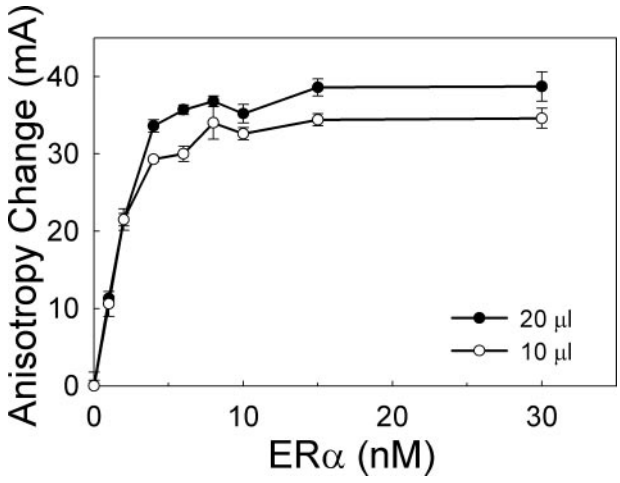

FIGURE 1. **A 384-well plate FAMA for 10-μl volumes.** FAMA was carried out in samples containing 100 nM E_2 as described under "Experimental Procedures" in 384-well black wall microplates using either the standard 20-μl volume (*filled circles*) or the 10-μl volume used in the final HTS screen (*open circles*). Data represent the average increase in anisotropy observed after E_2·ERα binding to the flcERE. Data represent the mean ± S.E. for four separate experiments.

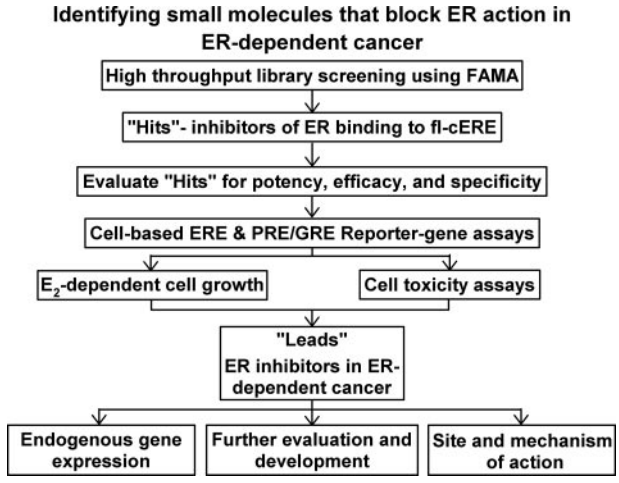

FIGURE 2. **Scheme for identification and characterization of small molecule inhibitors of ERα action in ER-dependent cancer cells.** Our strategy for identification of ERα antagonists included the following assays. (i) *In vitro* FAMA assays using purified proteins and DNA to carry out the HTS screen 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 ERα-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 ERα inhibitor action.

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. ERα was detected using a 1:2,000 dilution of ERα 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 ERα—Development of the 10-μl primary screen (Fig. 1) is detailed under "Experimental Procedures." The sequence of assays used to identify the lead inhibitor of ERα action in ER-dependent cancer cells is summarized in the flow chart in Fig. 2. In the initial high throughout screen, FAMA was used to assay binding of purified hERα 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 E_2 and OHT induction of the endogenous PI-9 gene in Tam-resistant MCF7ERαHA 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 E_2 and OHT elicit robust >100-fold inductions of PI-9 mRNA in MCF7ERαHA cells (2). To begin to evaluate its site of action, we showed that TPBM does not bind in the ligand binding pocket of ERα 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 μM, 262 reduced the anisotropy of the ERα-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 ERα Specificity, Potency, and Efficacy—Detailed potency and efficacy studies established IC_{50} values required to block ERα 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. IC_{50} values for inhibition of HRE binding by ERα, 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. 3*A*) and dose-response curves (Fig. 3*B*) 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. 3*A*) exhibited high potency and was the most specific ER inhibitor of the ~12,0000 molecules tested (Fig. 3*B*, *9568*). However, it is relatively large (M_r ~ 1,300) (Fig. 3*A*,

TABLE 1

IC_{50} values for small molecule inhibitors of binding of steroid receptors to their HREs

IC_{50} values were determined from dose-response curves (see Fig. 3*B*). For each small molecule binding of the indicated steroid receptor (ERα, AR, or PR) was determined at five concentrations (0.5, 1, 2.5, 5, and 10 μM). 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}		
	ERα	AR	PR
		μM	
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 ERα, 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 ERα binding to the flcERE relative to PR and AR binding to their HREs (Fig. 3*B*, 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 ERα-positive T47D-KBluc breast cancer cell line that stably expresses an $(ERE)_3$-luciferase reporter gene (38). An E_2 dose-response curve showed that the cells exhibited strong E_2-dependent activation of the reporter gene with full induction at 50 pM E_2 (Fig. 4*A*). 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 ERα (51).

Candidate small molecules were initially tested at 30 μM in the T47D cell assay in medium containing 20 pM E_2 for 24 h prior to measuring luciferase activity (Fig. 4*B*). As expected a 100-fold molar excess of the antagonist ICI 182,780/Faslodex/Fulvestrant blocked activation of the reporter gene (Fig. 4*B*, *+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. 4*C*, concentration-dependent inhibition of E_2-dependent ERα transactivation was observed with IC_{50} values of 11.5 μM 95910/TPBM, 22 μM 1529, 3.5 μM 130796, and 0.8 μM 638432.

To establish specificity for ERα, 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 ERα, 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 E_2. 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 ERα, and TPBM/95910 did not inhibit GR transactivation up to 20 μM, with ~35% inhibition at 30 μM (Fig. 5*A*). Compound TPBM/95910 did not significantly inhibit PR transactivation. Compounds 1529 and 130796 inhibited PR transactivation between 20 and 30 μM (Fig. 5*B*).

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 ERα to the cERE could block E_2-dependent growth of cancer cells. Consistent with earlier studies (45), we found that BG-1 cells exhibited a stronger and more reproducible E_2 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 ERα-specific inhibitors, TPBM/95910 and 1529. Compound 1529 potently inhibited E_2-dependent growth of the BG-1 cells (IC_{50} ~ 5 μM). However, >5 μM 1529 inhibited growth of the cells in the absence of E_2, suggesting a nonspecific effect at the higher inhibitor concen-

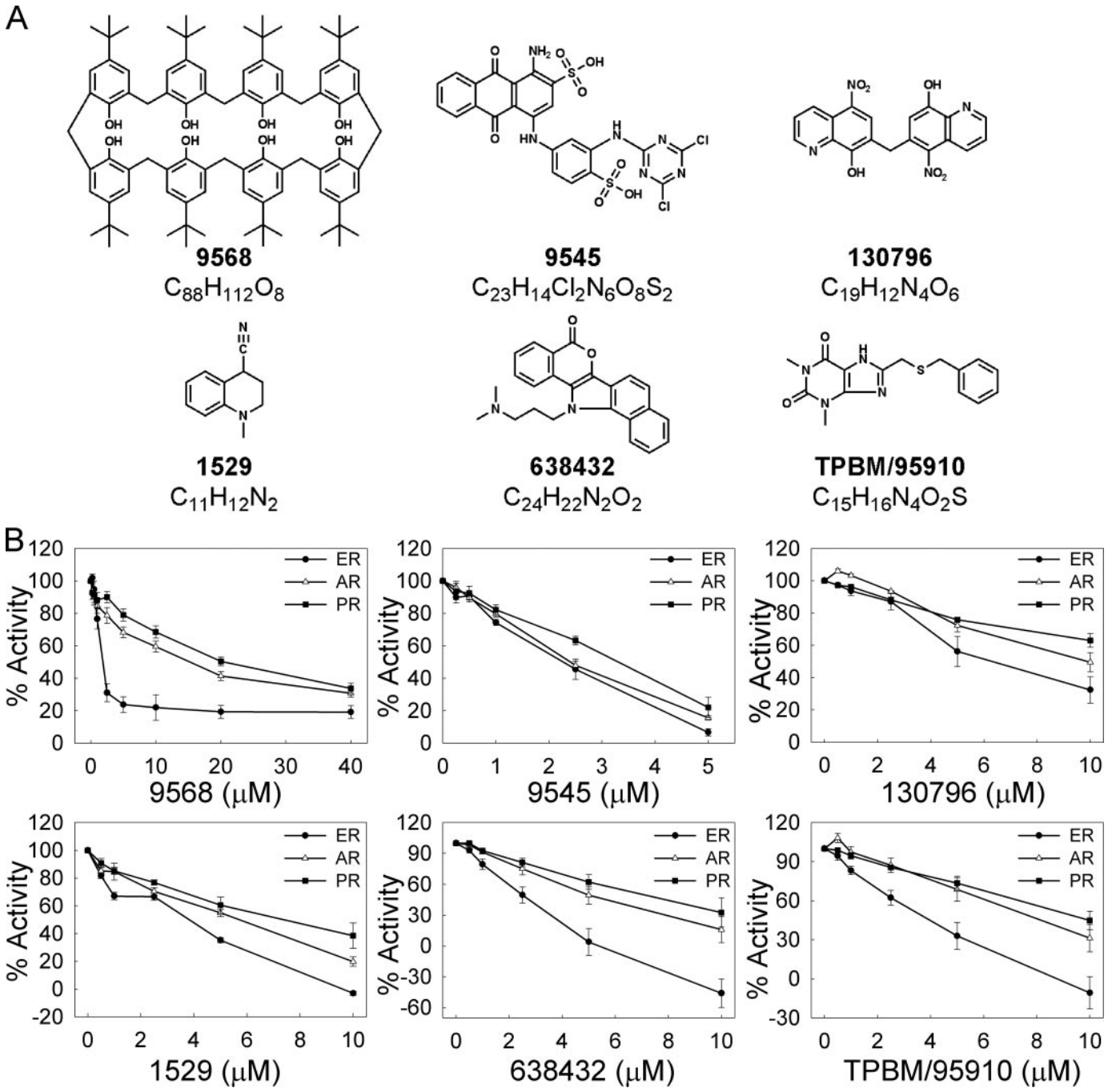

FIGURE 3. **Dose-response curves for inhibition of ERα, PR, and AR binding to their HREs.** *A*, the structures of the six compounds whose binding curves are shown in *B*. *B*, dose-response curves for ERα-selective and non-selective inhibitors identified in the primary HTS screen. The indicated concentrations of each small molecule were incubated with ERα (*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: ERα ~35 mA units, PR ~90 mA units, and AR ~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 ± S.E. for four separate experiments at each concentration.

tration (Fig. 6*A*, *1529*). TPBM/95910 exhibited concentration-dependent inhibition of E_2-dependent growth of the BG-1 cells, with an IC_{50} of 5 μM. At 30 μM, TPBM was as effective as a 100-fold excess of OHT in blocking E_2-dependent growth of BG-1 cells (Fig. 6*A*, *TPBM*).

To test for general cell toxicity, the ERα-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. 6*B*, *1529*). The data suggest that, although 1529 elicits some E_2-dependent inhibition of cell growth, at higher concentrations it is toxic to cells. TPBM had no effect on E_2-independent growth of BG-1 cells (Fig. 6*A*, *gray bars*) or MDA-MB-231 cells (Fig. 6*B*). Studies of TPBM/95910, theophylline, 8-[(benzylthio)methyl]-(7CI,8CI) in the NCI, NIH Developmental Therapeutics Program testing program confirmed a lack of toxicity with 60 cancer cell lines over a wide range of

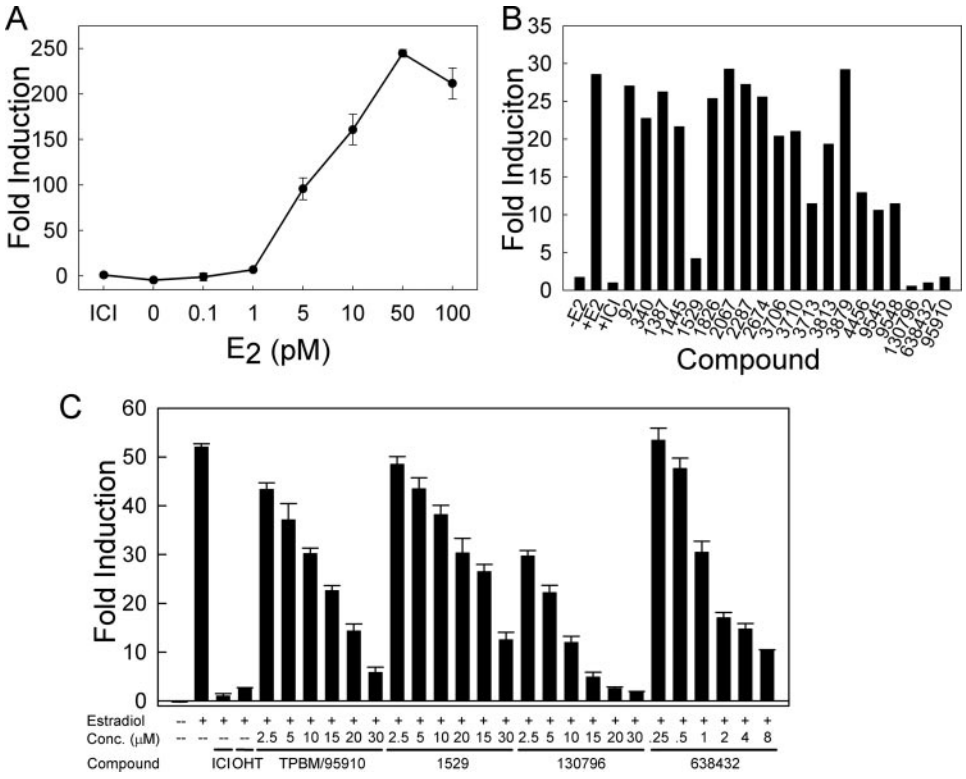

FIGURE 4. **Effect of small molecules on ER-mediated gene expression in T47DKBluc cells.** *A*, E_2 dose-response curve. The cells were maintained in medium containing either 1 nM ICI 182,780 (to test for traces of estrogens in the medium), or the indicated concentrations of E_2 and reporter gene expression was assayed after 24 h. The data represent the average of three independent experiments ± S.E. *B*, inhibition of ERα-mediated gene expression by small molecules that inhibit binding of ERα 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 μM inhibitor for 30 min, then 20 pM E_2 (*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 E_2, 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 E_2, with or without 1 nM of ICI 182,780 or OHT. The indicated concentrations of each small molecule were incubated with the cells and E_2·ER-mediated gene expression assayed. The data represent the mean ± S.E. for four separate experiments at each concentration. IC_{50} values were obtained by curve-fitting using Sigma plot and had a high R^2 value.

concentrations up to 100 μM TPBM. Of the 60 cell lines tested at 100 μM TPBM/95910, only a few lung cancer cell lines showed >50% reduction in cell growth. Even at 100 μM, 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 μM IC_{50} for TPBM inhibition of E_2·ERα-dependent growth of BG-1 cells and the >100 μM TPBM required for inhibition of E_2·ERα-independent breast and ovarian cell growth.

TPBM Inhibits E_2 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 ERα 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 MCF7ERαHA breast cancer cells. MCF7ERαHA cells are a tetracycline-inducible MCF-7 model for Tam-resistant breast cancer in which Dox induces overexpression of ERα (40, 41). In these cells Tam and OHT are potent agonists (2, 41). Because OHT stabilizes ERα, whereas E_2 down-regulates ERα, in MCF7ERαHA cells OHT is more effective than E_2 in inducing PI-9 (2).

Saturating E_2 (100 pM, Fig. 7*A*) and OHT (500 pM, Fig. 7*B*) induced PI-9 mRNA by 150- and 500-fold, respectively. TPBM (95910) elicited a concentration-dependent inhibition of E_2·ERα induction of PI-9 mRNA with an IC_{50} of 8.5 μM (Fig. 7*A*). 30 μM TPBM (95910) was required to inhibit OHT-ERα induction of PI-9 mRNA by 48% (Fig. 7*B*). This is a stringent test, because Western blotting followed by PhosphorImager quantitation of band intensities shows that MCF7ERαHA cells, treated with Dox to induce ERα, express 3- to 4-fold more ERα in the presence of E_2 or OHT than MCF7ERαHA cells not treated with Dox (Fig. 7*C*). The less complete inhibition of PI-9 induction in the OHT-treated cells likely results from the ~4-fold higher level of ERα after OHT treatment than after E_2 treatment (Fig. 7*C*). 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 ERα-mediated gene expression in Tam-resistant breast cancer cells that overexpress ERα.

ChIP Shows That TPBM Inhibits Binding of E_2·ERα to an Estrogen-regulated Gene—TPBM blocks binding of E_2·ERα to the flcERE in FAMA (Fig. 3). We used ChIP to test whether the ability of TPBMs to inhibit E_2 induction of PI-9 mRNA in MCF7ERαHA cells results from inhibition of E_2·ERα binding to the PI-9 estrogen-responsive region. MCF7ERαHA cells were maintained under the same conditions used to test the effect of TPBM on E_2 induction of PI-9 mRNA (Fig. 7*A*), and semi-quantitative ChIP (44) was performed. Although the signal in ChIP was low, TPBM inhibited binding of E_2·ERα to the PI-9 estrogen responsive unit (ERU) by 55% (Fig. 8). To evaluate the influence of TPBM on binding of E_2·ERα to the PI-9 ERU under conditions in which a stronger ChIP signal could be obtained, we exploited the observation that E_2·ERα binds to ERE-containing genes in an oscillatory fashion with time-dependent cycles of binding and release (56, 57). Although 100 pM E_2 produces a near maximal ~150-fold induction of PI-9 mRNA, after

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

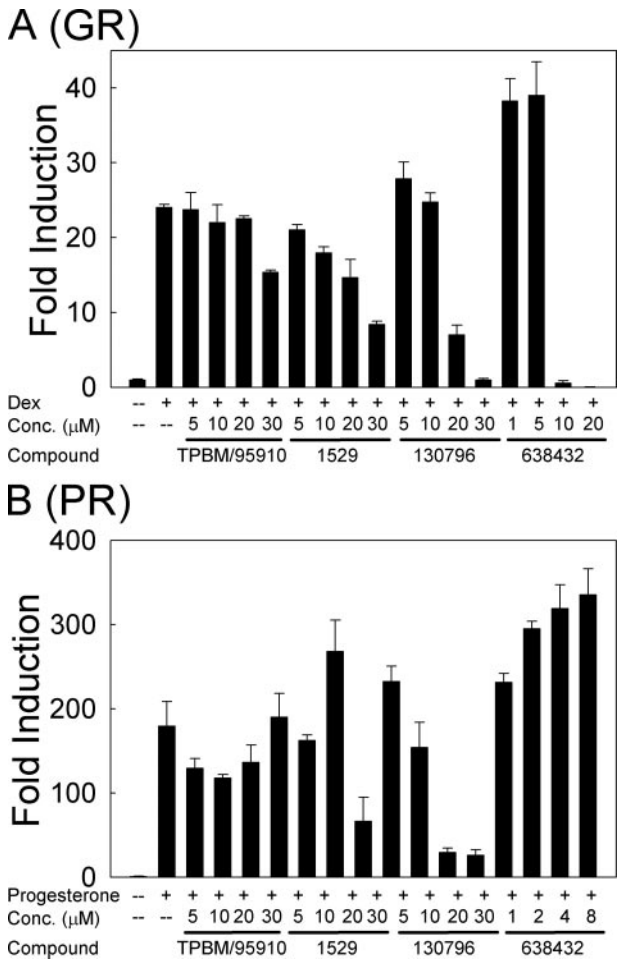

FIGURE 5. **Effect of ERα inhibitors on GR- and PR-mediated gene expression in T47D cells.** Assays were performed essentially as described for ER in the legend to Fig. 4 (*A* and *B*). The indicated concentration of each small molecule was incubated with the cells for 30 min followed by addition of 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 ± S.E. for four separate experiments at each concentration.

24 h in 100 pM E_2, binding of ERα to PI-9 was likely largely randomized. To enhance the ChIP signal we synchronized E_2·ERα binding by adding a pulse of 10 nM E_2 45 min before cross-linking the cells. This resulted in a more robust binding of E_2·ERα to the PI-9 ERU and an increase of ~7.5-fold in ChIP occupancy units (Fig. 8). Under these conditions, binding of E_2·ERα to the PI-9 estrogen-responsive unit was decreased 72% (Fig. 8) in the presence of 20 μM TPBM. At 20 μM 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 E_2·ERα 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 ERα and Is Not a Zinc Chelator—We performed experiments to test the possibility that TPBM inhibits E_2·ERα binding to the flcERE by binding in the ERα ligand binding pocket or as an electrophile that complexes zinc in the zinc fingers of ERα. We found that increasing the concentration of E_2 to 10 μM had no effect on the ability of TPBM to inhibit binding of ERα to the flcERE (Fig. 9).

The only other known small molecule ERα inhibitor that acts outside the ERα 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 ERα by DIBA (25). At 5 μM TPBM, binding of ERα to the flcERE was inhibited 76 ± 7% ($n = 3$) in the absence of zinc and 68 ± 6% ($n = 3$) in the presence of 50 μM zinc. Under the same conditions, preincubating with zinc prevented the zinc chelator *ortho*-phenanthroline from inhibiting ERα binding to the flcERE (data not shown) and (27). Therefore, TPBM does not act by chelating the zinc in the zinc fingers of ERα and is a novel ERα 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 E_2·ERα·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 E_2·ERα 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 E_2·ERα 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 E_2·ERα that results in ~80% of maximal binding. This reduced the chances of identifying activators that reduce the concentration of E_2·ERα 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

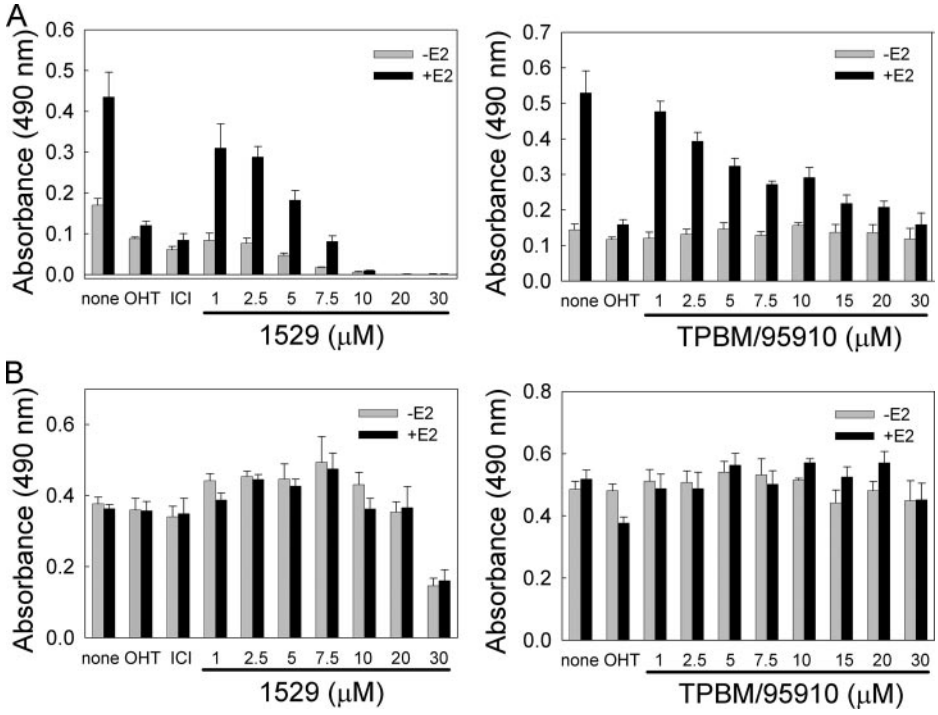

FIGURE 6. **Small molecule inhibitors of ER-mediated gene expression block estrogen-dependent growth of cancer cells.** *A*, BG-1 ovarian cancer cells were maintained in medium lacking E_2 (*gray bars*), or containing 10 pM E_2 (*black bars*). OHT and ICI were at 1 nM. The cells were maintained for 5 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*, ERα-negative MDA-MB-231 cells were maintained in medium containing no E_2 (*gray bars*) or 10 pM E_2 (*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 ± S.E. for four separate experiments at each concentration. The IC_{50} for TPBM/95910 was obtained by curve-fitting using Sigma plot and had a high R^2 value.

and PR, which are larger than ERα 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 ERα 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 ERα Action in Cancer Cells—The initial *in vitro* HTS screen employs a simple system containing only two pure components, a cERE and purified ERα. To be useful, inhibitors identified by this screening must inhibit ERα-mediated transcription in intact cells. Using a stably transfected breast cancer cell line that expresses endogenous ERα, 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 ERα to a cERE would also inhibit estrogen-dependent cancer cell growth was unresolved. At 30 μM, TPBM and OHT both nearly completely inhibited estrogen-dependent growth of BG-1 cells. Interestingly, the IC_{50} of 3.5 μM for inhibition of binding of ERα to the flcERE in FAMA is similar to the 5 μM IC_{50} for inhibiting the estrogen-stimulated component of BG-1 cell growth, suggesting an association between inhibition of ERα 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 ERα and low overall toxicity. TPBM inhibited E_2·ERα-dependent cell growth with an IC_{50} of 5 μM with no inhibition of the growth of ERα-negative MDA MB-231 cells up to 30 μM. 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 μM. Thus, TPBM shows >10-fold greater potency for inhibiting E_2·ERα-dependent cell growth relative to nonspecific toxicity. In contrast, for several other ERα 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 ERα, PR, and GR in the same cell line expressing different reporter genes. Even at 30 μM, 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 ERα 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 ERα that is important for tumor growth (55). Recent studies show that an important feature of Tam-resistant breast cancer cells that retain dependence on ERα for growth is loss of dependence on SRC3 and other p160 coactivators for E_2·ERα-mediated gene transcription (41, 59). ERα 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 ERα.

TPBM effectively blocked E_2-dependent induction of PI-9 mRNA in Tam-resistant MCF7ERαHA cells (IC_{50} 8.5 μM). It is

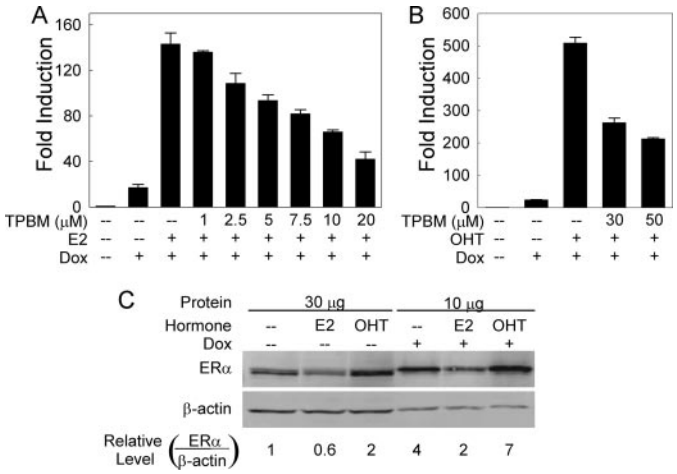

FIGURE 7. **TPBM inhibits E_2- and OHT-mediated gene expression in a Tam-resistant cell line.** MCF7ERαHA cells were maintained in 10% 6× charcoal-stripped fetal bovine serum, treated with 0.5 μg/ml Dox to induce ERα and 100 pM E_2 (*A*) or 500 pM OHT (*B*) and the indicated concentrations of 95910 for 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 ERα in Dox-treated cells (E_2- and *Dox*+) results in some ligand-independent transactivation of PI-9 by ERα. The data represent the mean ± S.E. for three separate experiments each assayed in triplicate. The IC_{50} of 8.5 μM for TPBM/95910 inhibition of E_2 induction of PI-9 was obtained by curve-fitting using Sigma plot and had a high R^2 value. *C*, Western blot analysis of ERα levels in MCF7ERαHA cells in the presence and absence of Dox. MCF7ERαHA cells were maintained in medium containing or lacking 0.5 μg/ml Dox and no ligand, 100 pM E_2, or 500 pM OHT. The cells were harvested after 24 h, and total cell extracts were prepared and analyzed for ERα content by Western blot as described under "Experimental Procedures." To better visualize the differences in ERα levels in the uninduced and Dox-induced MCF7ERαHA cells 30 μg (3× more protein) was run for each uninduced sample, and 10 μg of protein was run for each sample from MCF7ERαHA cells in which ERα was induced with Dox. ERα antibody was used at a dilution of 1:2,000. Relative levels of ERα were calculated by PhosphorImager quantitation of band intensity and normalization to actin (actin antibody was a 1:10,000 dilution). The ratio of unliganded ($-E_2$ and $-OHT$) ERα to actin in the MCF7ERαHA cells not treated with Dox to induce ERα was set equal to 1. The ratios of ERα levels in the Dox-treated and uninduced (*−Dox*) MCF7ERαHA cells were 3.7, 3.1, and 4.0 for cells maintained in medium with no ligand, E_2, and OHT, respectively. The data in *C* are representative of other Western blots.

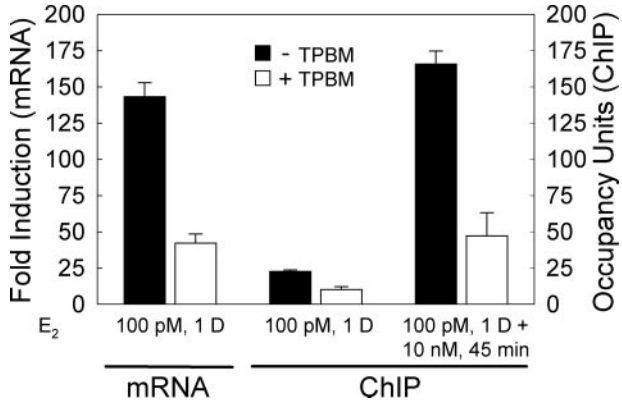

FIGURE 8. **ChIP demonstrates that TPBM decreases binding of E_2·ERα to an estrogen-regulated gene.** MCF7ERαHA cells were maintained for 24 h as described in the legend to Fig. 7*A* and used either for determination of PI-9 mRNA levels as described in "Experimental Procedures," or for ChIP (44) and as described in "Experimental Procedures." The MCF7ERαHA cells were maintained in medium containing 100 pM E_2 for 24 h in the absence (*black bars*) or presence (*open bars*) of 20 μM TPBM. In one ChIP, additional 10 nM E_2 was added 45 min. before cross-linking the cells. The mRNA data is presented as -fold induction by E_2, with the level of PI-9 mRNA in control cells not treated with E_2 or Dox set equal to 1. The mRNA data represent the mean ± S.E. for three separate experiments. The extent of association of E_2·ERα with the PI-9 ERU in the presence of E_2 or E_2 plus 20 μM TPBM is presented in ChIP occupancy units normalized to *36B4* as a non-regulated gene (44). The ChIP data represent the average ± S.E. of three assays. Decreased E_2·ERα occupancy of the PI-9 in cells maintained in E_2 plus 20 μM TPBM was observed in multiple ChIP experiments. The difference between samples treated with E_2 and samples treated with E_2 plus 20 μM 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 E_2-treated cells compared with control (*−E2* and *−Dox*) cells.

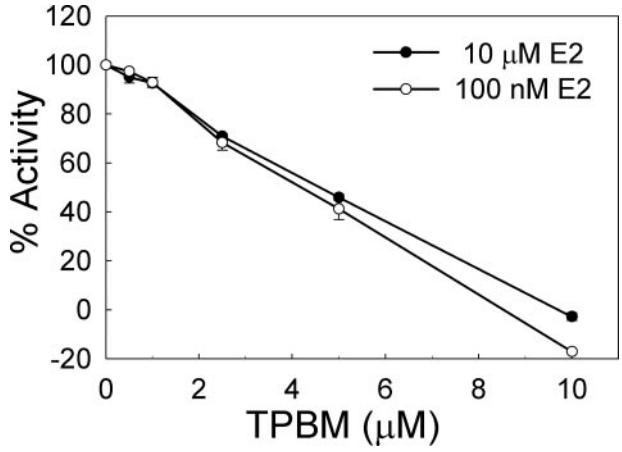

FIGURE 9. **High concentrations of E_2 do not reduce the ability of TPBM to inhibit binding of E_2·ERα to the flcERE.** Assays were carried out essentially as described in the legend to Fig. 3 and under "Experimental Procedures." E_2 was present at the standard concentration of 100 nM (*open circles*) or at 10 μM (*closed circles*). The anisotropy change in the absence of inhibitor was set equal to 100%. The data represent the mean ± S.E. for four separate experiments. *Error bars* that are not visible are *smaller than the symbols*.

probably unusually difficult to inhibit ERα binding to the endogenous PI-9 ERU in the MCF7ERαHA cells and to the $(ERE)_3$ reporter in the T47D reporter gene cell line. In the MCF7ERαHA cells, the high level of ERα, 3–4 times higher than the already substantial level in MCF-7 cells (Fig. 7*C*) (40), coupled with use of near saturating E_2, 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 E_2·ERα to the three cEREs (60), making it difficult for an inhibitor to block ERα binding to the EREs. Interestingly, the IC_{50} values of 10.5 and 8.5 μM for inhibition of E_2·ERα-mediated gene expression from the $(cERE)_3$-luciferase reporter in T47D cells and from the endogenous PI-9 gene in MCF7ERαHA cells are somewhat higher than the IC_{50} value of 5 μM for inhibiting E_2-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 E_2·ERα occupancy at the PI-9 gene. Thus, the primary mechanism by which TPBM exerts its intracellular action is by decreasing interaction of ERα with regulatory regions of estrogen-responsive genes. It is likely that interaction of TPBM with ERα induces a conformational change in the receptor, and that one result of this conformational change is decreased association of E_2·ERα with the PI-9 gene. It is difficult to determine whether the conformational change that likely results from binding of TBPM to ERα in Tam-resistant breast cancer cells interferes not only with ERE binding but also with coactivator

binding. TPBM decreases association of E_2·ERα with the PI-9 gene making coactivator studies difficult. Also, in Tam-resistant MCF7ERαHA cells, ChIP fails to detect SRC3 at ER-regulated promoters, and the coactivators important for transactivation by E_2·ERα 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 ERα 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 ($IC_{50} \sim 50\ \mu M$) 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.

Acknowledgments—We are grateful to Dr. K. Korach and Dr. E. Alarid, who provided the BG-1 ovarian cancer cells, and the MCF7ERαHA cells, respectively, to J. Johnson of the NCI Developmental Therapeutics Program for assistance in obtaining compounds for testing, and to Dr. S. McMasters of the Cell/Media facility for serum stripping and media preparation.

REFERENCES

1. Clarke, R., Liu, M. C., Bouker, K. B., Gu, Z., Lee, R. Y., Zhu, Y., Skaar, T. C., Gomez, B., O'Brien, K., Wang, Y., and Hilakivi-Clarke, L. A. (2003) *Oncogene* **22,** 7316–7339
2. Jiang, X., Ellison, S. J., Alarid, E. T., and Shapiro, D. J. (2007) *Oncogene* **26,** 4106–4114
3. Jiang, X., Orr, B. A., Kranz, D. M., and Shapiro, D. J. (2006) *Endocrinology* **147,** 1419–1426
4. O'Lone, R., Frith, M. C., Karlsson, E. K., and Hansen, U. (2004) *Mol. Endocrinol.* **18,** 1859–1875
5. Gradishar, W. J., and Cella, D. (2006) *JAMA* **295,** 2784–2786
6. Boonyaratanakornkit, V., and Edwards, D. P. (2004) *Essays Biochem.* **40,** 105–120
7. Deroo, B. J., and Korach, K. S. (2006) *J. Clin. Invest.* **116,** 561–570
8. Yager, J. D., and Davidson, N. E. (2006) *N. Engl. J. Med.* **354,** 270–282
9. Pandey, K. R. (2007) *BMJ.* **334,** 925
10. Smyth, J. F., Gourley, C., Walker, G., MacKean, M. J., Stevenson, A., Williams, A. R., Nafussi, A. A., Rye, T., Rye, R., Stewart, M., McCurdy, J., Mano, M., Reed, N., McMahon, T., Vasey, P., Gabra, H., and Langdon, S. P. (2007) *Clin. Cancer Res.* **13,** 3617–3622
11. Smith, I. E., and Dowsett, M. (2003) *N. Engl. J. Med.* **348,** 2431–2442
12. Winer, E. P. (2005) *J. Clin. Oncol.* **23,** 1609–1610
13. Boccardo, F. (2004) *Clin. Breast Cancer* **5,** Suppl. 1, S13–S17
14. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. (1997) *Breast Cancer Res. Treat.* **44,** 23–38
15. Lewis, J. S., and Jordan, V. C. (2005) *Mutat. Res.* **591,** 247–263
16. Carroll, J. S., and Brown, M. (2006) *Mol. Endocrinol.* **20,** 1707–1714
17. Klinge, C. M. (2001) *Nucleic Acids Res.* **29,** 2905–2919
18. Arkin, M. R., and Wells, J. A. (2004) *Nat. Rev. Drug. Discov.* **3,** 301–317
19. Arnold, L. A., Estebanez-Perpina, E., Togashi, M., Jouravel, N., Shelat, A., McReynolds, A. C., Mar, E., Nguyen, P., Baxter, J. D., Fletterick, R. J., Webb, P., and Guy, R. K. (2005) *J. Biol. Chem.* **280,** 43048–43055
20. Kung, A. L., Zabludoff, S. D., France, D. S., Freedman, S. J., Tanner, E. A., Vieira, A., Cornell-Kennon, S., Lee, J., Wang, B., Wang, J., Memmert, K., Naegeli, H. U., Petersen, F., Eck, M. J., Bair, K. W., Wood, A. W., and Livingston, D. M. (2004) *Cancer Cell* **6,** 33–43
21. Li, L., Thomas, R. M., Suzuki, H., De Brabander, J. K., Wang, X., and Harran, P. G. (2004) *Science* **305,** 1471–1474
22. Putt, K. S., Chen, G. W., Pearson, J. M., Sandhorst, J. S., Hoagland, M. S., Kwon, J. T., Hwang, S. K., Jin, H., Churchwell, M. I., Cho, M. H., Doerge, D. R., Helferich, W. G., and Hergenrother, P. J. (2006) *Nat. Chem. Biol.* **2,** 543–550
23. Verma, R., Peters, N. R., D'Onofrio, M., Tochtrop, G. P., Sakamoto, K. M., Varadan, R., Zhang, M., Coffino, P., Fushman, D., Deshaies, R. J., and King, R. W. (2004) *Science* **306,** 117–120
24. Moerke, N. J., Aktas, H., Chen, H., Cantel, S., Reibarkh, M. Y., Fahmy, A., Gross, J. D., Degterev, A., Yuan, J., Chorev, M., Halperin, J. A., and Wagner, G. (2007) *Cell* **128,** 257–267
25. Wang, L. H., Yang, X. Y., Zhang, X., Mihalic, K., Fan, Y. X., Xiao, W., Howard, O. M., Appella, E., Maynard, A. T., and Farrar, W. L. (2004) *Nat. Med.* **10,** 40–47
26. Wang, L. H., Yang, X. Y., Zhang, X., An, P., Kim, H. J., Huang, J., Clarke, R., Osborne, C. K., Inman, J. K., Appella, E., and Farrar, W. L. (2006) *Cancer Cell* **10,** 487–499
27. Wang, S. Y., Ahn, B. S., Harris, R., Nordeen, S. K., and Shapiro, D. J. (2004) *BioTechniques* **37,** 807–808, 810–807
28. Wang, S., Zhang, C., Nordeen, S. K., and Shapiro, D. J. (2007) *J. Biol. Chem.* **282,** 2765–2775
29. Mao, C., Flavin, K. G., Wang, S., Dodson, R., Ross, J., and Shapiro, D. J. (2006) *Anal. Biochem.* **350,** 222–232
30. Rishi, V., Potter, T., Laudeman, J., Reinhart, R., Silvers, T., Selby, M., Stevenson, T., Krosky, P., Stephen, A. G., Acharya, A., Moll, J., Oh, W. J., Scudiero, D., Shoemaker, R. H., and Vinson, C. (2005) *Anal. Biochem.* **340,** 259–271
31. Dietz, A. J., Jr., and Burgison, R. M. (1966) *J. Med. Chem.* **9,** 500–506
32. Dietz, A. J., Jr., and Burgison, R. M. (1966) *J. Med. Chem.* **9,** 160
33. Melvin, V. S., and Edwards, D. P. (2001) *Methods Mol. Biol.* **176,** 39–54
34. Askew, E. B., Gampe, R. T., Jr., Stanley, T. B., Faggart, J. L., and Wilson, E. M. (2007) *J. Biol. Chem.* **282,** 25801–25816
35. Ozers, M. S., Hill, J. J., Ervin, K., Wood, J. R., Nardulli, A. M., Royer, C. A., and Gorski, J. (1997) *J. Biol. Chem.* **272,** 30405–30411
36. Putt, K. S., and Hergenrother, P. J. (2004) *Anal. Biochem.* **326,** 78–86
37. Hergenrother, P. J. (2006) *Curr. Opin. Chem. Biol.* **10,** 213–218
38. Wilson, V. S., Bobseine, K., and Gray, L. E., Jr. (2004) *Toxicol. Sci.* **81,** 69–77
39. Nordeen, S. K., Kuhnel, B., Lawler-Heavner, J., Barber, D. A., and Edwards, D. P. (1989) *Mol. Endocrinol.* **3,** 1270–1278
40. Fowler, A. M., Solodin, N., Preisler-Mashek, M. T., Zhang, P., Lee, A. V., and Alarid, E. T. (2004) *FASEB J.* **18,** 81–93
41. Fowler, A. M., Solodin, N. M., Valley, C. C., and Alarid, E. T. (2006) *Mol. Endocrinol.* **20,** 291–301
42. Frasor, J., Chang, E. C., Komm, B., Lin, C. Y., Vega, V. B., Liu, E. T., Miller, L. D., Smeds, J., Bergh, J., and Katzenellenbogen, B. S. (2006) *Cancer Res.* **66,** 7334–7340
43. Cunningham, T. D., Jiang, X., and Shapiro, D. J. (2007) *Cell. Immunol.* **245,** 32–41
44. Schultz-Norton, J. R., McDonald, W. H., Yates, J. R., and Nardulli, A. M. (2006) *Mol. Endocrinol.* **20,** 1982–1995
45. Baldwin, W. S., Curtis, S. W., Cauthen, C. A., Risinger, J. I., Korach, K. S., and Barrett, J. C. (1998) *In Vitro Cell Dev. Biol. Anim.* **34,** 649–654
46. Medema, J. P., de Jong, J., van Hall, T., Melief, C. J., and Offringa, R. (1999) *J. Exp. Med.* **190,** 1033–1038
47. ten Berge, R. L., Meijer, C. J., Dukers, D. F., Kummer, J. A., Bladergroen, B. A., Vos, W., Hack, C. E., Ossenkoppele, G. J., and Oudejans, J. J. (2002) *Blood* **99,** 4540–4546

48. van Houdt, I. S., Oudejans, J. J., van den Eertwegh, A. J., Baars, A., Vos, W., Bladergroen, B. A., Rimoldi, D., Muris, J. J., Hooijberg, E., Gundy, C. M., Meijer, C. J., and Kummer, J. A. (2005) *Clin. Cancer Res.* **11,** 6400–6407
49. Krieg, A. J., Krieg, S. A., Ahn, B. S., and Shapiro, D. J. (2004) *J. Biol. Chem.* **279,** 5025–5034
50. Krieg, S. A., Krieg, A. J., and Shapiro, D. J. (2001) *Mol. Endocrinol.* **15,** 1971–1982
51. Cheng, J., Yu, D. V., Zhou, J. H., and Shapiro, D. J. (2007) *J. Biol. Chem.* **282,** 30535–30543
52. Estebanez-Perpina, E., Arnold, A. A., Nguyen, P., Rodrigues, E. D., Mar, E., Bateman, R., Pallai, P., Shokat, K. M., Baxter, J. D., Guy, R. K., Webb, P., and Fletterick, R. J. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 16074–16079
53. Jordan, V. C. (2001) *Ann. N. Y. Acad. Sci.* **949,** 72–79
54. Katzenellenbogen, B. S. (2000) *J. Soc. Gynecol. Investig.* **7,** Suppl. 1, S33–S37
55. Osborne, C. K., Shou, J., Massarweh, S., and Schiff, R. (2005) *Clin. Cancer Res.* **11,** 865s–870s
56. Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000) *Cell* **103,** 843–852
57. Schultz-Norton, J. R., Walt, K. A., Ziegler, Y. S., McLeod, I. X., Yates, J. R., Raetzman, L. T., and Nardulli, A. M. (2007) *Mol. Endocrinol.* **21,** 1569–1580
58. Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) *J. Biomol. Screen.* **4,** 67–73
59. Naughton, C., MacLeod, K., Kuske, B., Clarke, R., Cameron, D. A., and Langdon, S. P. (2007) *Mol. Endocrinol.* **21,** 2615–2626
60. Mattick, S., Glenn, K., de Haan, G., and Shapiro, D. J. (1997) *J. Steroid Biochem. Mol. Biol.* **60,** 285–294