Screening and characterization of estrogenic activity from a hydroxystilbene library

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Background: Compounds that either inhibit or induce an estrogen response *in vivo* are important as potential drugs and biochemical tools. Non-steroidal stilbene analogs such as tamoxifen are known to function as both estrogen agonists and antagonists depending upon the analog structure. This family of compounds is amenable to parallel-manifold synthesis because stilbene analogs are easily synthesized using a single-step olefination reaction.

Results: We have prepared a small 23-component hydroxystilbene library using a solid phase synthesis approach. The library was screened for estrogenic and antiestrogenic activity using a cell-based bioassay that measures estrogen receptor-mediated transcription of a reporter gene. Three of the analogs proved to have dose-dependent estrogenic activity with EC_{50} values between 5 μM and 15 μM. Further characterization of the hydroxystilbene-mediated estrogenic activity suggests that the agonist activity results from direct binding to the steroid site on the estrogen receptor with IC_{50} values of 1–10 μM.

Conclusions: The results of this study show that classic olefination chemistry can be adapted to a solid-phase format for parallel synthesis of analog libraries. Although yields varied for the individual analogs, sufficient quantity of pure material was obtained directly from the resin for structural characterization and biological evaluation. This study further validates solid-phase organic synthesis as a useful approach for rapid parallel-manifold library synthesis to augment both lead compound discovery and optimization.

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Introduction

Estrogen (17β-estradiol, Fig. 1a) is an important hormone that mediates a wide variety of cellular responses [1–4]. The biological responses triggered by estrogen are brought about by binding of the hormone to a specific nuclear estrogen receptor (ER). The hormone-bound ER forms an active dimer that functions as a transcription factor which mediates biological response by binding to specific promoter elements of DNA to initiate gene transcription (Fig. 1b). Compounds that either induce or inhibit cellular estrogen responses are important biochemical tools and potential candidates for drug development. Estrogens are used in hormone replacement therapy for postmenopausal women, and the estrogen antagonist tamoxifen (Fig. 1a) is currently used for the treatment of breast cancer. In addition to having antagonist properties, tamoxifen also behaves as a partial ER agonist and this property has been suggested to be responsible for the drug resistance and endometrial cancer that some patients develop after prolonged tamoxifen treatment [5–7].

A growing number of non-steroidal natural and synthetic aromatic compounds have been shown to possess estrogenic activity [8–12]. Plant flavonoids including genistein and coumestrol, and synthetic compounds such as phenolphthalein, alkylphenols, and dihydroxystilbenes, have been shown to be moderate to strong agonists of the ER. There is currently much debate over the health risks associated with estrogenic activity of compounds that are either present in the environment, or used as pharmaceuticals, pesticides, and herbicides [13,14].

We have initiated a research effort aimed at studying the activity of a series of non-steroidal estrogenic compounds to gain insight into the various molecular mechanisms of the estrogen response. The hydroxystilbene structure (Fig. 1a) was attractive as a starting point because similar hydroxylated aromatics are known to possess estrogenic activity [8], and because the synthesis of this structure is amenable to a manifold solid-phase approach allowing for rapid, parallel synthesis of a collection of analogs. This technique has enjoyed great success in the synthesis of large peptide libraries [15], and recent published work from the laboratories of Ellman [16,17], Hobbs-DeWitt [18], Kurth [19,20], and Zuckerman [21] suggests that solid-phase organic synthesis will be useful for creating non-peptide libraries as well.

Results

Solid-phase library synthesis

A 6 x 4 combinatorial matrix strategy was used to synthesize a small library of hydroxystilbenes on a polystyrene solid support using Horner–Emmons

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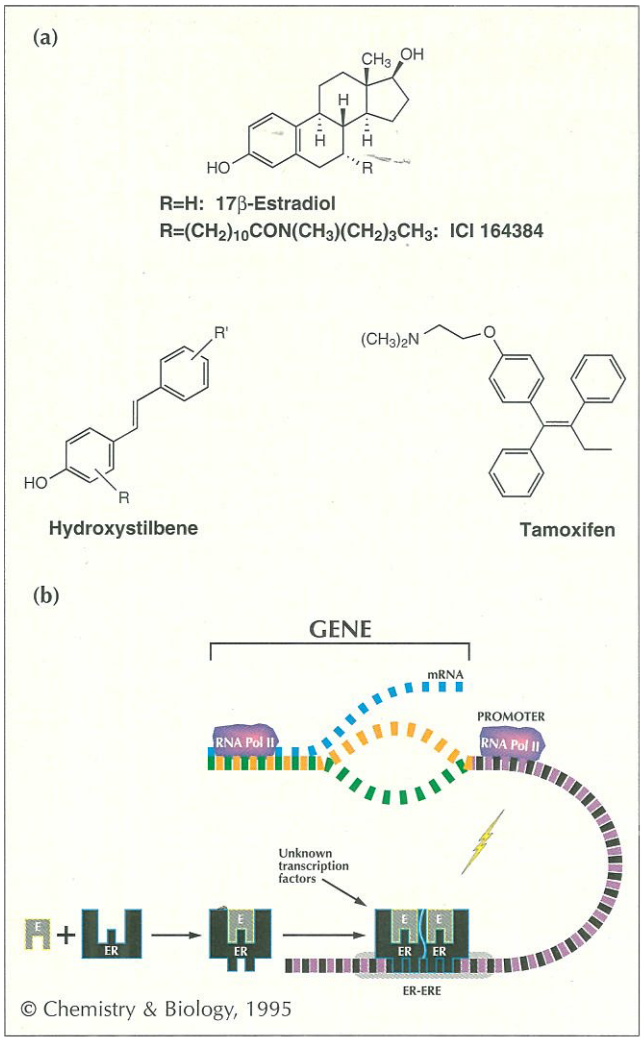


Fig. 1. Activation and inhibition of estrogen receptor signaling. **(a)** Compounds that bind to and mediate the activity of the estrogen receptor. Estradiol is the natural ligand, ICI 164384 is a potent antagonist, and tamoxifen has both agonist and antagonist activities. The agonist effects of hydroxystilbene derivatives are examined in this study. **(b)** Schematic of estrogen receptor activation. When estrogen (E) binds to the estrogen receptor (ER), the receptor can dimerize and bind to the estrogen response element (ERE), activating transcription of an estrogen-responsive gene.

olefination chemistry (Fig. 2). Four different hydroxybenzaldehydes were esterified with the acid-cleavable [4-(hydroxymethyl)phenoxy]acetic acid linker and attached to polystyreneaminomethyl (PAM) resin following the strategy developed by Ellman and coworkers [16]. At this stage, each hydroxybenzaldehyde-derivatized resin sample was split into six aliquots, and each aliquot was treated in parallel with a solution of one of six different benzylphosphonate anions (Fig. 2). After washing away the unreacted phosphonate and phosphate biproduct, the hydroxystilbenes were cleaved from the resin by treatment with trifluoroacetic acid (TFA) and filtered through silica. Only one of the reactions failed to produce any detectable hydroxystilbene product (4-fluorobenzyl phosphonate with resin bound 3-hydroxy-4 methoxybenzaldehyde) so that twenty-three different hydroxystilbene derivatives were generated by this procedure (Table 1). Further purification of the compounds proved to be unnecessary as judged by thin layer chromatography (TLC), NMR spectroscopy, and mass spectrometry. The chemical yields for these solid-phase syntheses ranged from excellent (85 %) to poor (7 %) and no optimization of the individual yields was attempted. We did not observe unreacted hydroxybenzaldehyde starting material in any of the resin-cleaved hydroxystilbene products. Polar baseline material was observed in the TLC of the low yielding reactions and was presumed to be intractable polymer which was removed in the silica gel filtration. It was not determined whether this material was formed in the olefination reaction or in the subsequent acid-catalyzed linker cleavage reaction. However, sufficient quantity of each compound (≥ 5 mg) was obtained by this method for structural characterization, screening, and further biological evaluation.

Estrogenic activity screens

The library of hydroxystilbene derivatives was screened for estrogenic activity in a cell-culture assay that relies on the ability of ligand-activated ER to bind an

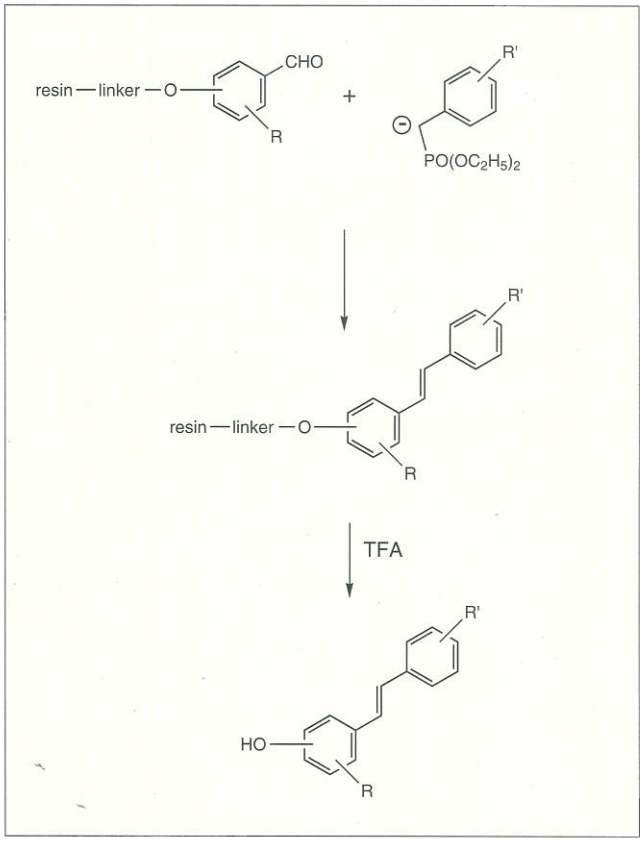
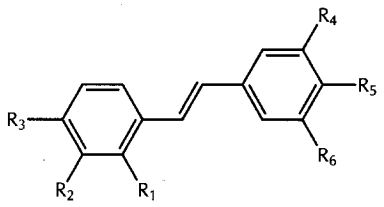


Fig. 2. Synthetic scheme for the solid phase synthesis of hydroxystilbene analogs. Four different hydroxybenzaldehydes were attached to resin following the strategy developed by Ellman and coworkers [16], then treated with a solution of one of six different benzylphosphonate anions. The resulting hydroxystilbenes were cleaved from the resin by treatment with trifluoroacetic acid (TFA) and filtered through silica.

Table 1. Hydroxystilbene analog library.



Compound	R_1	R_2	R_3	R_4	R_5	R_6	% yield*
1A	H	OH	H	H	H	H	44
1B	H	OH	H	H	NO_2	H	85
1C	H	OH	H	H	Br	H	45
1D	H	OH	H	OCH_3	H	OCH_3	41
1E	H	OH	H	H	F	H	40
1F	H	OH	H	F	H	H	35
2A	H	OH	OCH_3	H	H	H	8
2B	H	OH	OCH_3	H	NO_2	H	28
2C	H	OH	OCH_3	H	Br	H	20
2D	H	OH	OCH_3	OCH_3	H	OCH_3	25
2F	H	OH	OCH_3	F	H	H	16
3A	H	NO_2	OH	H	H	H	12
3B	H	NO_2	OH	H	NO_2	H	8
3C	H	NO_2	OH	H	Br	H	7
3D	H	NO_2	OH	OCH_3	H	OCH_3	7
3E	H	NO_2	OH	H	F	H	10
3F	H	NO_2	OH	F	H	H	14
4A	Cl	H	OH	H	H	H	55
4B	Cl	H	OH	H	NO_2	H	67
4C	Cl	H	OH	H	Br	H	57
4D	Cl	H	OH	OCH_3	H	OCH_3	46
4E	Cl	H	OH	H	F	H	40
4F	Cl	H	OH	F	H	H	62

*Yields based on resin-bound hydroxybenzaldehyde starting material as the limiting reagent.

estrogen response element and drive the production of chloramphenicol acetyl transferase (CAT) from a reporter gene [22]. After transient transfection with reporter genes, each hydroxystilbene was added to cultured ERC1 cells [22,23], a derivative of Chinese hamster ovary (CHO) cells that express human estrogen receptors, at a concentration of 50 μM. ER-regulated response was compared either to treatment with 17β-estradiol as a calibration standard or to treatment with an ethanolic vehicle. Hydroxystilbene series **1**, **2**, and **3** showed no measurable estrogenic activity (data not shown), whereas series **4** compounds showed weak estrogenic activity (Fig. 3). Of the series **4** compounds, **4A**, **4E**, and **4F** were found to provide the highest levels of estrogenic activity relative to 17β-estradiol.

To examine whether the series **4** compounds induce estrogenic activity through the ER, we tested the ability of the potent antiestrogen ICI 164384 (Fig. 1a) to inhibit the series **4** estrogenic activity. The ICI 164384 compound was found to inhibit the estrogenic activity of all the series **4** compounds (Fig. 3). As a negative control, CHO cells, which lack functioning ER, were transfected with the same estrogen-responsive reporter constructs and treated with 17β-estradiol and the series **4** hydroxystilbenes. As expected, no estrogenic activity was seen with these cells.

Dose response and ER binding of series 4

Dose response experiments were performed on the series **4** compounds over a concentration range of 0–100 μM (Fig. 4). For the most active compounds, **4A**, **4E**, and **4F**, saturation is observed at 50 μM. The effective concentration that provides 50 % maximum activity (EC_{50}) ranges from ~5 μM to ~15 μM for these three compounds. *In vitro* ER binding assays were performed on the three most active series **4** compounds to confirm that the estrogenic activity measured in the bioassay correlated with binding affinity for the ER. The inactive hydroxystilbene analog **3D** was included in the binding assay as a negative control. The ER-binding results for compounds **4A**, **4E**, and **4F** are consistent with the estrogenic bioassay, as each of the compounds show IC_{50} values of 1–10 μM for ER binding (Fig. 5). The two most active compounds in the bioassay, **4A** and **4F**, also show the highest affinity (1 μM) for the ER, although this affinity is approximately four orders of magnitude lower than that of 17β-estradiol. The analog **3D** which showed no activity in the bioassay also shows no binding affinity for the ER.

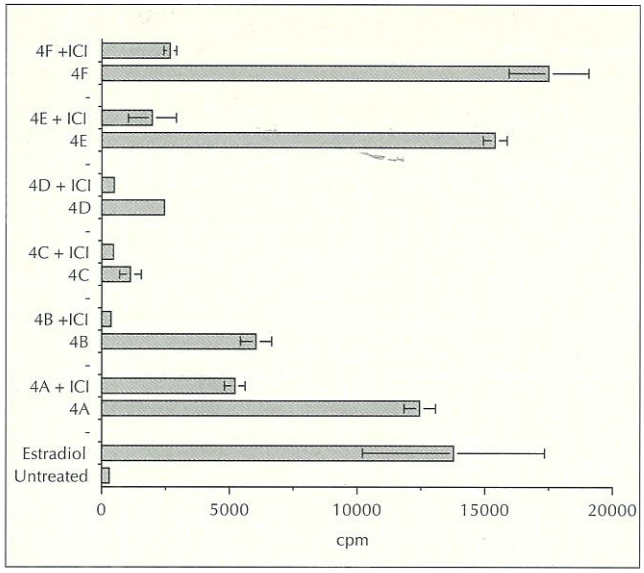


Fig. 3. Weak estrogenic activity of series **4** compounds and inhibition by treatment with antiestrogen. Induction of CAT enzyme activity was measured after transfection of ERC1 cells with a reporter gene followed by treatment with series 4 compound alone (50 μM) or series **4** compound plus the antiestrogen, ICI 164384 (1 μM). Induction with 10^{-9} M estradiol and ethanolic vehicle are shown as controls. Compounds **4A**, **4E**, and **4F** appear most active although they are approximately four orders of magnitude less potent than estradiol.

Discussion

The new technology of combinatorial organic synthesis has the potential to dramatically expedite the discovery and optimization of lead compounds for drug discovery [24]. Because the central feature of this technique involves organic synthesis on a solid support, the scope of combinatorial organic synthesis will hinge on the chemist's ability to adapt classic solution-phase organic reactions to the solid phase. Examples of non-peptide organic compounds that have been prepared by solid-phase synthesis include benzodiazepines [16,18], hydantoins [18], (N-substituted)glycine oligomers [21], and β-mercaptoketones [20]. From the results presented here, hydroxystilbenes can now be added to this rapidly increasing list. For the hydroxystilbene solid-phase synthesis the final chemical yields of the resin-cleaved compounds ranged from excellent (85 %) to poor (7 %). However, the purity of the resin-cleaved compounds was uniformly high and the time required to produce the twenty-three compound library was significantly decreased because the resin-bound aldehydes could be sub-divided and taken through the olefination step in a parallel-manifold fashion, and because byproducts and excess reagents could be removed in a simple washing/filtration step.

A fundamental difference between compounds like the hydroxystilbenes and biopolymers such as peptides (which are more easily adapted to solid-phase synthesis and combinatorial library generation) is that non-peptide compounds tend to have greater stability *in vivo*, greater bioavailability, and greater cell-permeability. The assay used to screen estrogenic activity of the hydroxystilbene library is a cell-based bioassay where activity is predicated on the ability of the compound to accumulate in the nucleus of the cultured cells. The fact that several of the series **4** compounds showed estrogenic activity in this assay demonstrates that the stilbene core structure has the membrane permeability required for a non-steroidal estrogen. Moreover, the observation that activity was only seen for series **4** shows that this bioassay is able to discriminate active compounds from inactive compounds in the context of a lead compound screen.

Additional experiments were performed to characterize further the estrogenic activity of the series **4** compounds identified in the initial screen. Four of the compounds (**4A**, **4B**, **4E**, and **4F**) showed clear dose response profiles over a concentration range of 0–100 μM (Fig. 4). The three most active compounds, **4A**, **4E**, and **4F**, show maximum activity at 50 μM and have EC_{50} values for estrogen response in the range of 5–15 μM. Three lines of evidence suggest that the series **4** estrogenic response is mediated by direct binding of the hydroxystilbene to the estrogen binding site of the ER. First, the fact that no estrogenic activity was observed in reporter gene-transfected CHO cells which lack a functional ER provides evidence that the response to the series **4** compounds was ER-mediated. Second, the observation that the response initiated by all the series **4** compounds could be inhibited by ICI 164384, a potent steroidal antiestrogen that competes with 17β-estradiol for binding to the ER, provides evidence that the hydroxystilbenes act through direct binding to the steroid binding site on the ER. Third, the binding affinity of the series **4** compounds to

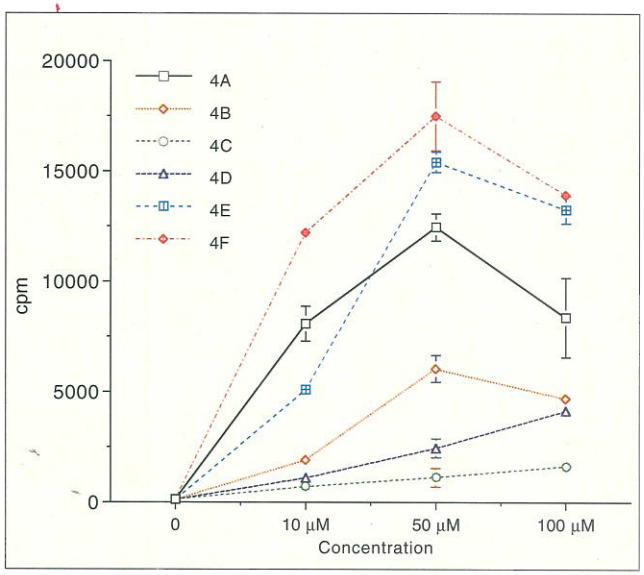


Fig. 4. Dose response curves for series **4**. Dose response experiments were performed on the series **4** compounds over a concentration range of 0–100 μM. For the most active compounds, **4A**, **4E**, and **4F**, saturation is observed at 50 μM. The effective concentration that provides 50 % maximum activity (EC_{50}) ranges from ~5 μM to ~15 μM for these three compounds.

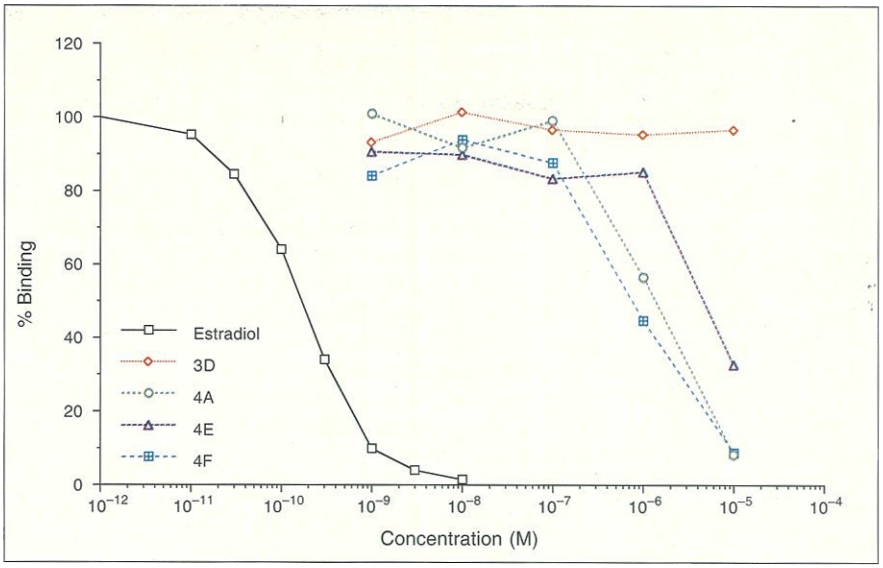


Fig. 5. ER binding/competition assay for compounds **4A**, **4E**, **4F**, and **3D**. *In vitro* ER binding assays were performed to confirm that the estrogenic activity measured in the bioassay correlated with binding affinity for the ER. The series **4** compounds show IC_{50} values of 1–10 μM for ER binding. Compound **3D**, which does not show biological activity, does not bind ER.

the ER was directly measured in a competition binding assay with 17β-estradiol and the measured IC_{50} values for the series **4** hydroxystilbenes correlate approximately with the EC_{50} values measured from the dose response bioassay.

Structure-activity relationships (SAR) in both the hydroxy-substituted and distal aromatic rings of the hydroxystilbenes are evident from the varying estrogenic activities of the library. Based on the observation that activity was only seen in the series **4** compounds, it appears that a *para* orientation between the hydroxyl substituent and the stilbene olefin is a requirement for ER-binding and activation. The *para* orientation is sensitive to additional substitution as evidenced by the fact that no estrogenic activity was observed for the series **3** (4-hydroxy-3-nitro) compounds; it is unclear whether steric or electronic factors are responsible for the lack of activity in series **3**. For the series **4** compounds, the three most active hydroxystilbenes bear either small fluorine substituents (**4E**, **4F**) or no substitution (**4A**) in the distal aromatic ring. This distal ring SAR is somewhat subtle; based on the dose-response data (Fig. 4), the 4'-Br substituted compound (**4C**) shows almost no activity, whereas the 4'-F substituted compound (**4F**) is the second most active member of the series. Here again, it is unclear whether steric effects, electronic effects, or a combination of both are responsible for the variations in estrogenic activity.

Significance

Advances in the field of combinatorial organic synthesis will depend on the adaptation of classic organic reactions to solid-phase synthesis. We have shown that the Horner–Emmons olefination process, a classic C–C bond-forming reaction, can be adapted to a solid-phase format for the preparation of hydroxystilbenes. Although substituent-dependent variation in chemical yield was observed in the preparation of a 23-component library, sufficient quantities of pure compounds were obtained without a purification step for structural characterization. Moreover, enough material was produced to screen each compound in a cell-based estrogenic assay, and to obtain more detailed characterization of the estrogenic activity of the positives identified from the screen.

This functional characterization shows that three of the hydroxystilbene analogs permeate cell membranes and trigger a dose-dependent estrogenic response with EC_{50} values in the range of 5–15 μM. Results from competition-response and ER-binding experiments with the antiestrogen ICI 164384 and 17β-estradiol provide evidence that the non-steroidal hydroxystilbene analogs elicit the estrogenic response through direct interaction with the steroid binding site of the estrogen receptor. In addition, structure-activity relationships for the hydroxystilbene pharmacophore are evident from the activity profile of the library. Such information could prove useful for predicting potential estrogenic activity of environmental pollutants and pharmaceuticals.

Materials and methods

Synthesis

The [4-(hydroxymethyl)phenoxy]acetic acid linker was attached to the hydroxybenzaldehydes and the resulting linker-derivatized aldehydes were attached to PAM resin according to the procedures of Ellman and colleagues [16]. The hydroxybenzaldehyde-derivatized resin was prepared on a 0.3–1.0 mmol scale and subdivided into six equal portions by weight for parallel treatment with the six different benzylphosphonate anions. The anions were generated in separate flasks by treating 1.3 mmol of the benzylphosphonate (10-fold molar excess over resin bound aldehyde) with 2.0 mmol sodium methoxide in 5 ml dimethyl formamide (DMF). After stirring the anion solution for 0.5 h at room temperature, a 0.13 mmol portion of resin aldehyde was added and the

mixture was stirred at room temperature overnight. Six such reactions corresponding to the number of benzylphosphonates were carried out in parallel. The reaction mixture was filtered and the resin was washed with (5 x 20 ml) of water, (5 x 20 ml) DMF, (5 x 20 ml) 1:1 DMF:water, and (5 x 20 ml) methylene chloride. The resin was then dried *in vacuo*.

The hydroxystilbene product was cleaved from the resin by treatment with 10 ml of a 95:5 solution of trifluoroacetic acid in water. The resulting slurry was stirred for 1–2 h at room temperature, then concentrated *in vacuo*. The crude residue was dissolved in 10 ml of a 1:2 solution of methanol in methylene chloride, filtered through a plug of silica gel, and concentrated *in vacuo* to provide the hydroxystilbene product in homogenous form as judged by TLC, NMR spectroscopy, and mass spectrometry. The chemical yields for the hydroxystilbenes ranged from 7 to 85 % based on the amount of resin-bound hydroxyaldehyde starting material as the limiting reagent.

Cell culture

ERC1 cells [22, 23] and CHO cells were cultured in Coon's F-12/Dulbecco's Modified Eagle's Medium (1:1) without phenol red (UCSF Cell Culture Facility) supplemented with 10 % iron-supplemented low estrogen calf serum (Sigma, St. Louis, MO; batch 19F-0156). ERC1 cells were supplemented with 50 μM zinc sulfate (Sigma, St. Louis, MO) and 40 μM cadmium sulfate (Sigma, St. Louis, MO) to maintain expression and selection of the ER gene.

Electroporation and CAT assays

Transfections and CAT assays were performed as described previously [22,25]. The expression vector $(GL45)_2$-CAT was made by ligating an oligonucleotide with *Hin*dIII ends into the *Hin*dIII site of pBL8. (pBL8 was a kind gift of G. Ryffel and contains a tk-CAT/SV40 fusion gene cloned into pEMBL8+ [26].) The GL45 oligo consisted of sequences –333/–288 of the vitellogenin A2 gene and was inserted in tandem with the second GL45 oligo. ERC1 cells (CHO cells that stably express human ER at supraphysiologic levels) were transiently transfected with the reporter construct by electroporation. Transfection efficiency and treatment toxicity were monitored by co-transfecting 2 μg per cuvette of β-human chorionic gonadotrophin (hCG) reporter plasmid [27] which was assayed with a standard kit using iodinated antibody (Tandem-R β-hCG Kit, Hybritech Inc., San Diego, CA).

Cells from multiple transfections were pooled and plated into six well dishes (Co-Star) and ERC1 cells were supplemented with 50 μM zinc sulfate. Cells were treated with stilbene derivative with or without ICI 164384 (1 μM) or estradiol at the concentrations indicated within 2 h, then assayed for CAT enzyme activity 24 to 48 h later using chloramphenicol and 8 mCi ml^{-1} tritiated acetyl Coenzyme-A (DuPont, Wilmington, DE) as previously described [22,25]. CAT enzyme activity was corrected to background and β-hCG counts were used as a measure of treatment toxicity. A single experiment representative of several independent experiments is presented. The data shown indicate the mean and standard deviation of duplicate estimations.

Estradiol competition ER-binding assay

MCF7 cells were grown as monolayer cultures in phenol red-free RPMI-1640 medium with 5 % charcoal-treated fetal bovine serum fortified with 0.2 ng ml^{-1} insulin (CT-FR). For experiments, cells were removed from their growth chamber with trypsin/EDTA, diluted in 1 % CT-FR to 1.75 (± 0.5) x 10^6 cells ml^{-1} and 100 μl were incubated with 50 μl of 0.4 nM ^{125}I-estradiol (2200 Ci $mmol^{-1}$, NEN Dupont) in 1 % CT-FR and 50 μl of the hydroxystilbenes in 1 % CT-FR (prepared in ethanol as 1000x stock solutions and diluted 250-fold in 1 % CT-FR). Control samples contained 1 % CT-FR with 0.4 % ethanol. Cells were incubated at 37 °C with 5 % CO_2 for 45 min with gentle mixing every 15 min. Following incubation, nuclei were prepared by lysing cells with 1 ml of ice-cold TPSG (0.2 % Triton X-100 in PBS containing 0.1 M sucrose and 10 % glycerol) as previously described [28]. The ^{125}I-estradiol in the nuclear pellet was measured in a gamma counter and the results are expressed as per cent ^{125}I-estradiol binding to ER in the nucleus in the absence (control = 100 %) and presence of increasing concentrations of the hydroxystilbene test compounds.

Supplementary material available

Physicochemical data for the linker-derivatized hydroxybenzaldehydes and hydroxystilbene compounds.

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