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# New antiestrogens from a library screen of homoallylic amides, allylic amides, and *C*-cyclopropylalkylamides

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**Abstract**—A new structural scaffold for antiestrogens was identified from the cell-based screening of transcriptional regulation properties of a 67-member library of homoallylic amides, allylic amides, and *C*-cyclopropylalkylamides. *C*-Cyclopropylalkylamide **3a** (*O*-ethyl-*N*-{2-[(1*S**,2*R**)-2-{(*R**)-[(diphenylphosphinoyl)amino](phenyl)methyl}cyclopropyl]ethyl}-*N*-[(4-methylphenyl)sulfonyl]carbamate) had antagonistic activity similar to that of tamoxifen and was further evaluated. Compound **3a** inhibited estradiol-induced proliferation of the ER-positive MCF-7 cells but had no effect on ER-negative MDA-MB231 human breast cancer cells. Furthermore, high micromolar concentrations of **3a** exhibited minimal cytotoxicity to the ER-negative line. The biological activities of the enantiomers of **3a** did not differ from one another nor from that of racemic **3a**.
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## 1. Introduction

The first clinically successful antiestrogen, tamoxifen (TAM), was developed in the 1970s and has since revolutionalized the therapy of breast cancer. The concept of developing selective estrogen receptor (ER) modulators (SERMs) arose after the discovery that TAM has mixed effects in humans, that is, it is antiestrogenic in the breast but estrogenic in the uterus.[1,2] The estrogen receptors, ERα and ERβ, belong to the type I nuclear hormone receptor family and are the products of different genes on separate chromosomes.[1–3] Both proteins have similar functional domains: an N-terminal domain, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). ERα has two activation functions (AFs) that contribute to transcriptional activity. AF1 is in the N-terminal region and is constitutive and mostly ligand independent. AF2 is ligand dependent and is located at the C-terminal region of the receptor.[1] Upon ligand binding, the ER dissociates from heat shock protein 90, dimerizes, and the ligand-bound homodimer binds to specific estrogen response elements (EREs) on DNA and stimulates transcription of specific genes. The LBD is the most studied region of the ER for drug discovery. This domain is multifunctional, mediating ligand binding, receptor dimerization, interaction with chaperone and coregulator proteins, nuclear translocation, and transcriptional activation. Upon agonist binding to the LBD, helix H12 of the ER moves to cap the ligand-binding cavity, leading to revelation of a region on the ER that is crucial for AF2 recruitment of transcription coactivators.[4] Helices H12, H3, H4, and H5 form a shallow hydrophobic pocket that recognizes LXXLL motifs (NR-boxes) on the p160 family of nuclear receptor coactivator proteins (e.g., steroid receptor co-activator 1 or SRC-1) as well as on the nuclear receptor corepressors, such as SMRT and N-CoR.[4] This region of the ER has been named the coregulator-binding domain (CBD). Different positioning of H12 upon binding by ligands to the LBD of the ER is widely believed to account for different mechanisms of action of pure antiestrogens, SERMs, and estrogen agonists.[5] The ERα is the target of interest in this study.

*Keywords*: Estrogen receptor α; Cell-based screen; Fluorescent estrogen; Transcription; Estrogen response element.
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ERα is a ligand-dependent transcription factor.[6] A critical event in the ERα regulation of a target gene expression is the binding of the DBD to EREs. It has been shown that the sequence of specific EREs in different target gene promoters alters receptor conformation.[7] The nature of specific genes targeted and the extent of their transcription depends on the subtle interplay between ligand, ER, coregulator recruitment, and EREs within the target gene promoter.[4] SERMs show mixed pharmacological effects, estrogenic or antiestrogenic, in different tissues.[8] This interplay has provided a potential mechanism for the tissue-specific effect of SERMs. Recently, a new type of antiestrogen has emerged targeting the interaction of ERα and SRC-3/pCIP/ACTR/RAC3.[9] SRC3 is overexpressed in 60% of breast cancers and also known to be involved in the development of TAM resistance.[10]

In the present work, a new structural class of ERα modulating agents was identified from a library screen of 67 homoallylic amides, allylic amides, and *C*-cyclopropylalkylamides utilizing constructs containing CMV promoter-driven ERα and the estrogen-regulated element of the *Xenopus* vitellogenin A2 gene inserted into the tk-luciferase plasmid transiently transfected into mammalian cells.[11] ERα antagonists were identified by their ability to antagonize 17β-estradiol (E2)-induced transcription in the transfected cells. One compound, *C*-cyclopropylalkylamide **3a**, was shown to inhibit E2-induced proliferation of ER-positive human breast cancer cell lines while having no effect on ER-negative cells.

## 2. Results and discussion

### 2.1. Library screen for potential ERα agonists and antagonists

Wipf et al.[12–14] recently reported a new method for the synthesis of homoallylic amides, allylic amides, and *C*-cyclopropylalkylamides. Many of the library components prepared through this method contained a combination of a di- or triaryl ring system with a central electron-rich (e.g., olefin) moiety. This is a structural feature common for some estrogens and many antiestrogens. The purpose of the initial screen was to determine if library members shown in Table 1 held potential for agonism or antagonism of ERα at a classical ERE. All library members were tested for ligand-dependent transcriptional activity of ERα using a transient transfection assay.[15] ER naïve HEK293 cells were co-transfected with three plasmids containing genes for CMV promoter-driven ERα, the reporter ERE-tk-Luc, and standard CMV-β-galactosidase (β-Gal) for transfection efficiency control using DOTAP liposomes transfection reagent. In this system, ERα and β-Gal are constitutively expressed, and compounds that cause the ERα to adopt an agonist-bound conformation cause binding of the receptor to the ERE and transcription of luciferase, providing a readout of ligand-dependent ERα transcriptional activation that can be standardized against β-Gal expression. Library members and TAM were screened at 10 μM. E2 at 10 nM was used as the positive control. Figure 1 shows the compound-induced fold of induction of luciferase activity compared to the activity in cells treated only with vehicle (DMSO, 0.1%). As expected, TAM showed modest estrogenic activity in this assay. In contrast, none of the library compounds promoted ER transcription (Fig. 1).

To search for ERα antagonists, the same experimental set up was used, but the compounds were tested for antagonism of E2-induced luciferase transcription. From the 67-member library, 13 compounds, structurally representative of all three subgroups (three homoallylic amides, three allylic amides, and seven *C*-cyclopropylalkylamides), were tested as potential antagonists. The transiently transfected HEK293 cells were co-incubated with 10 nM E2 and with the candidate antagonists at both 10 and 1 μM concentrations. TAM and cyclopropane **4**[16] (Fig. 3), known ERα antagonists, were used at 10 and 1 μM as positive controls (Fig. 2).

Two compounds, the allylic amide **2b** (Table 1) and the *C*-cyclopropylalkylamide **3a**, *O*-ethyl-*N*-{2-[(1*S**,2*R**)-2-{(*R**)-[(diphenylphosphinoyl)amino](phenyl)methyl}-cyclopropyl]ethyl}-*N*-[(4-methylphenyl)sulfonyl]-carbamate (Fig. 3), significantly inhibited E2-induced transcription at the ERE at 1 and 10 μM (Fig. 2). Preliminary tests for inhibition of E2-induced proliferation of MCF-7 cells showed compound **2b** to be inactive at the concentrations used (Table 2), whereas *C*-cyclopropylalkylamide **3a**, the second most active antagonist in the transcriptional screen, inhibited MCF-7 cell proliferation. Therefore, **3a** was evaluated in more detail for antagonistic activity in cell-based and protein–ligand displacement assays.

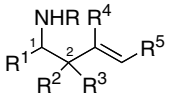
### 2.2. Compound 3a inhibits the E2-induced transcriptional activation of the reporter gene in a concentration-dependent manner

The *C*-cyclopropylalkylamide **3a** was further examined for concentration-dependent inhibition of the E2-induced transcriptional activation of ERα. Transfected HEK293 cells were stimulated with 1 nM E2 in the presence of a range of concentrations (3.2 nM to 50 μM) of compound **3a** or TAM (Table 2). $IC_{50}$ values were estimated from dose-dependence curves that best fit the data obtained as an average from at least two transfections done in triplicate (Fig. 4). Compound **3a** antagonized the effects of E2 in a concentration-dependent manner, yielding an $IC_{50}$ of $11 \pm 2$ μM. In this assay, TAM gave an $IC_{50}$ of $4.9 \pm 2.0$ μM.[20–22] Compound **3a** failed to induce transcription at ERE as compared to TAM and E2 (Fig. 1). These findings indicated that **3a** is likely to be full or partial antagonist.

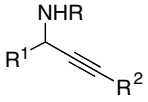
### 2.3. Compound 3a inhibits the E2-induced proliferation of ER-positive MCF-7 cells but does not effect ER-negative MDA-MB231 cell growth

Compound **3a** was further evaluated for antiestrogenic action in an E2-stimulated MCF-7 cell proliferation assay. It inhibited the E2 stimulated growth of MCF-7

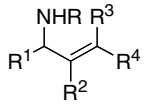
**Table 1.** Structures of the 67-member library



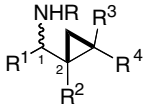

| Homoallylic amides[a] | $C_1$–$C_2$ | R | R1 | R2 | R3 | R4 | R5 |
|---|---|---|---|---|---|---|---|
| **1a** | *anti* | $P(O)Ph_2$ | Ph | $CH_2CH_2OSi(t\text{-Bu})Ph_2$ | H | H | H |
| **1b** | | $P(O)Ph_2$ | Ph | Me | Me | H | Me |
| **1c** | *anti* | $P(O)Ph_2$ | 4-$CO_2$Me–Ph | $CH_2CH_2OSi(t\text{-Bu})\ Ph_2$ | H | H | H |
| **1d** | *syn* | $P(O)Ph_2$ | 4-OMe–Ph | $C_4H_9$ | H | H | H |
| **1e** | *syn* | $P(O)Ph_2$ | Ph | $C_4H_9$ | H | H | H |
| **1f** | *syn* | Ts | Ph | $C_4H_9$ | H | H | H |
| **1g** | *syn* | Ts | Ph | $CH_2CH_2OSi(t\text{-Bu})Ph_2$ | H | H | H |
| **1h** | *syn* | $P(O)Ph_2$ | Ph | Et | H | Et | H |
| **1I** | *anti* | $P(O)Ph_2$ | Ph | Et | H | Et | H |
| **1j** | | $P(O)Ph_2$ | Ph | H | H | Me | H |
| **1k** | *syn* | 3,5-$(NO_2)_2$–PhC(O) | Ph | $C_4H_9$ | H | H | H |




| Propargylic amides | R | R1 | R2 |
|---|---|---|---|
| **1–1a** | $P(O)Ph_2$ | Ph | $C_4H_9$ |




| Allylic amides | R | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| **2a** | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2N(Ts)CO_2Et$ |
| **2b** | $P(O)Ph_2$ | (*E*)-PhCH=$C(CH_3)$ | H | H | $C_4H_9$ |
| **2c** | $P(O)Ph_2$ | Ph | H | H | $C_4H_9$ |
| **2d** | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2OSi(t\text{-Bu})Ph_2$ |
| **2e** | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2CO_2Si(i\text{-Pr})_3$ |
| **2f** | $P(O)Ph_2$ | (*E*)-PhCH=CH | H | H | $C_4H_9$ |
| **2g** | $P(O)Ph_2$ | 4-$CO_2$Me–Ph | H | H | $C_4H_9$ |
| **2h** | $P(O)Ph_2$ | 3-OMe–Ph | H | H | $C_4H_9$ |
| **2i** | $P(O)Ph_2$ | 2-OMe–Ph | H | H | $C_4H_9$ |
| **2j** | $P(O)Ph_2$ | 4-$NO_2$–Ph | H | H | $C_4H_9$ |
| **2k** | $P(O)Ph_2$ | 3-$NO_2$–Ph | H | H | $C_4H_9$ |
| **2l** | $P(O)Ph_2$ | 4-Cl–Ph | H | H | $C_4H_9$ |
| **2m** | $P(O)Ph_2$ | PhCC | H | H | $C_4H_9$ |
| **2n** | Ts | Ph | H | H | $C_4H_9$ |
| **2o** | Ts | $PhCH_2CH_2$ | H | H | $C_4H_9$ |
| **2p** | $P(O)Ph_2$ | Ph | H | Me | $C_4H_9$ |
| **2q** | $P(O)Ph_2$ | Ph | Me | H | H |
| **2r** | $P(O)Ph_2$ | Ph | Et | H | Et |
| **2s** | $P(O)Ph_2$ | 4-$CO_2$Me–Ph | $Si(CH_3)_3$ | H | (*E*)-CH=$CHC_6H_{13}$ |
| **2a** | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2N(Ts)CO_2Et$ |

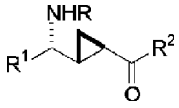



| *C*-Cyclopropylalkylamides[a] | $C_1$–$C_2$ | R | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|---|
| **3a** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2N(Ts)CO_2Et$ |
| **3b** | *anti* | $P(O)Ph_2$ | 3-OMe–Ph | H | H | $C_4H_9$ |
| **3c** | *anti* | $P(O)Ph_2$ | 2-OMe–Ph | H | H | $C_4H_9$ |
| **3d** | *anti* | $P(O)Ph_2$ | 4-Cl–Ph | H | H | $C_4H_9$ |
| **3e** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2OH$ |
| **3f** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2CO_2Si(i\text{-Pr})_3$ |
| **3g** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CO_2Me$ |
| **3h** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CH{=}CH_2$ |
| **3i** | *anti* | $P(O)Ph_2$ | Ph | H | H | $CH_2CH_2OSi(t\text{-Bu})Ph_2$ |

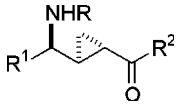
(*continued on next page*)

**Table 1** (*continued*)

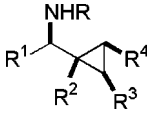
| *C*-Cyclopropylalkylamides[a] | $C_1$–$C_2$ | R | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|---|
| **3j** | *anti* | $P(O)Ph_2$ | Ph | H | H | $C_4H_9$ |
| **3k** | *syn* | $P(O)Ph_2$ | Ph | H | H | $C_4H_9$ |
| **3l** | *anti* | $P(O)Ph_2$ | PhCC | H | H | $C_4H_9$ |
| **3m** | *anti* | $CO_2CH_2Ph$ | Ph | H | H | $CO_2Me$ |
| **3n** | *anti* | $CO_2CH_2Ph$ | Ph | H | H | C(O)NH*i*-Pr |
| **3o** | *syn* | $CO_2CH_2Ph$ | Ph | H | Me | C(O)NH*i*-Pr |
| **3p** | *syn* | $P(O)Ph_2$ | Ph | H | Me | $C_4H_9$ |
| **3q** | *anti* | $P(O)Ph_2$ | Ph | Et | H | Et |
| **3r** | *anti* | $P(O)Ph_2$ | Ph | Me | H | $CH{=}CH_2$ |
| **3s** | *anti* | Ts | Ph | H | H | $C_4H_9$ |
| **3t** | *syn* | Ts | Ph | H | H | $C_4H_9$ |
| **3u** | *anti* | $CO_2CH_2Ph$ | Ph | Me | H | C(O)NH*i*-Pr |
| **3v** | *anti* | Ts | $PhCH_2CH_2$ | H | H | $C_4H_9$ |
| **3w** | *anti* | C(O)Ph | Ph | H | H | $C_4H_9$ |
| **3x** | *anti* | C(O)Ph-4-$NO_2$ | Ph | H | H | $C_4H_9$ |



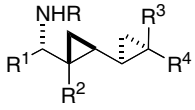

| *C*-Cyclopropylalkylamino acids[b] | R | R1 | R2 |
|---|---|---|---|
| **3–1a** | $CO_2CH_2Ph$ | Ph | (*S*)-NHCH(Me)Ph |
| **3–1b** | $CO_2CH_2Ph$ | Ph | NHPh-4-Br |
| **3–1c** | $CO_2CH_2Ph$ | Ph | L-Phe–OMe |
| **3–1d** | C(O)Ph-4-Br | Ph | OMe |




| *C*-Cyclopropylalkylamino acids[b] | R | R1 | R2 |
|---|---|---|---|
| **3–2a** | $CO_2CH_2Ph$ | Ph | L-Phe–OMe |




| *C*-Cyclopropylalkylamides | R | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| **3–3a** | $P(O)Ph_2$ | Ph | H | Me | $C_4H_9$ |
| **3–3b** | C(O)Ph-3,5-di$NO_2$ | Ph | H | Me | $C_4H_9$ |
| **3–3c** | C(O)Ph | Ph | H | Me | $C_4H_9$ |
| **3–3d** | $P(O)Ph_2$ | Ph | Me | H | H |




| *C*-Cyclopropylalkylamides | R | R1 | R2 | R3 | R4 |
|---|---|---|---|---|---|
| **3–4a** | C(O)Ph-3,5-$(NO_2)_2$ | Ph-4-$CO_2Me$ | H | Me | $C_6H_{13}$ |
| **3–4b** | $P(O)Ph_2$ | Ph | H | Me | $CH_2CH_2OH$ |
| **3–4c** | $P(O)Ph_2$ | Ph-4-$CO_2Me$ | H | Me | $C_6H_{13}$ |

[a] Diastereomerically pure.
[b] Enantiomerically pure.

cells in a concentration-dependent manner. Data shown in Figure 5 represents the percent of growth inhibition, where E2-stimulated growth of MCF-7 cells at day 6 was set to 100% growth (0% inhibition). *C*-Cyclopropylalkylamide **3a** was, as in the transcriptional antagonism assay, approximately 3-fold less potent then TAM against MCF-7 cells (Table 2). The $IC_{50}$ value obtained for TAM in this assay closely corresponds to previously reported values.[20–22]

The unusual structure of **3a** as compared to a variety of known antiestrogens[1,2] stimulated further evaluation of the specificity of **3a** in ER-negative MDA-MB231 human breast cancer cells. The growth inhibitory properties of **3a** were ER-dependent, as it had no effect on the proliferation of MDA-MB231 cells. *C*-Cyclopropylalkylamide **3a** did not demonstrate significant toxicity to these cells even at high micromolar concentrations (Table 2).

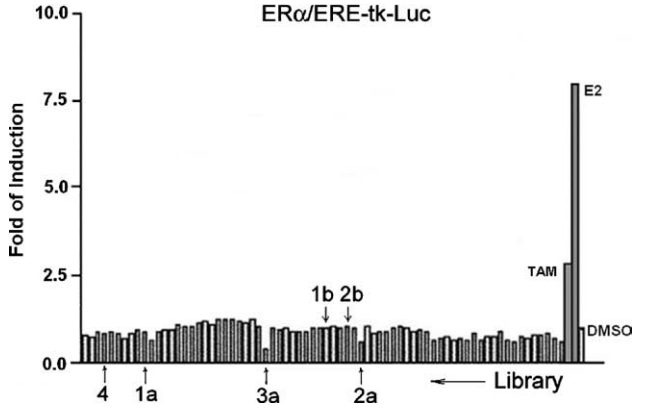



**Figure 1.** Screen of a 67-member library of homoallylic amides, allylic amides, and *C*-cyclopropylalkylamides for potential ERα agonism. ER naïve HEK293 cells were transfected with CMV promoter-driven human ERα, an ERE-tk-Luc reporter and a CMV-β-gal transfection control using DOTAP liposomes. Estradiol (E2) and tamoxifen (TAM) were used as positive controls. Library compounds and TAM were tested at 10 μM and E2 at 10 nM. Data represent fold of induction (mean ± SD, $N = 3$).

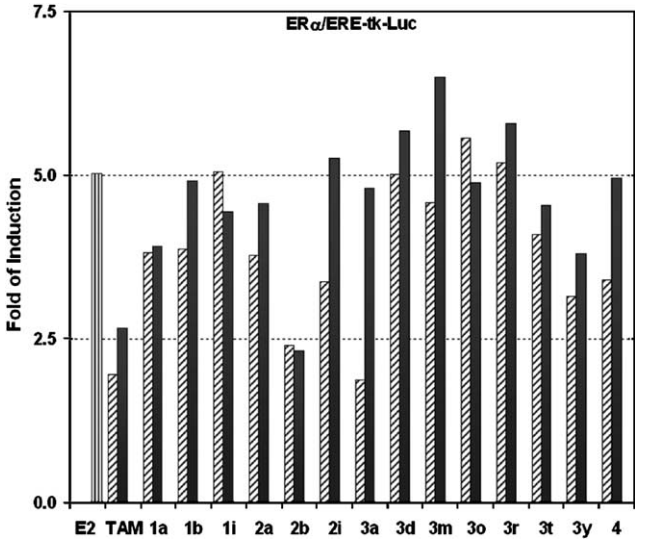



**Figure 2.** Screen of three homoallylic amides (**1a**, **1b**, **1i**), three allylic amides (**2a**, **2b**, **2i**), and seven *C*-cyclopropylalkylamides (**3a**, **3d**, **3m**, **3o**, **3r**, **3t**, **3y**) for potential ERα antagonism in the transcriptional assay. Library compounds, TAM, and **4** were tested at 10 μM (hatched bars) and 1 μM (black bars) in the presence of E2 at 10 nM. Data represents fold of induction (mean ± SD, $N = 3$) and stimulation control E2 ($N = 9$).

## 2.4. SAR of cell-based assays

To investigate the importance of the sulfonylcarbamate moiety of **3a**, a synthetic precursor of this compound, alkyne **5**[14] (Fig. 3), was tested in the MCF-7 antiproliferative assay and the transcriptional assays. Alkyne **5** contains the sulfonylcarbamate portion of **3a**. Interest in the effect of the sulfonylcarbamate moiety of these compounds was stimulated by a recent report on ER targeting agents that contain sulfonamide moieties[17] and the results of the antagonist screen (Fig. 2) where

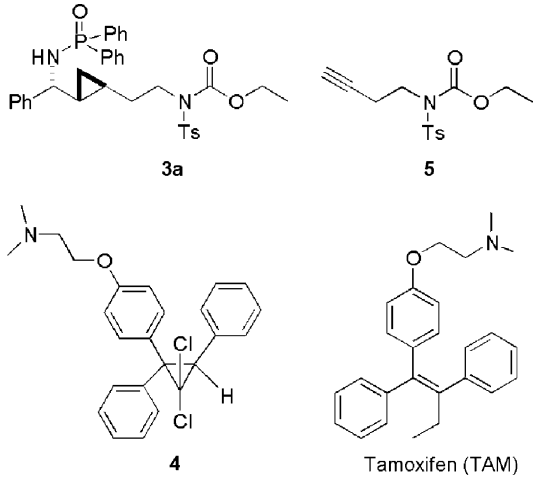



**Figure 3.** Structures of *C*-cyclopropylalkylamide **3a**, the synthetic precursor **5**, cyclopropane **4**, and TAM.

**Table 2.** Fifty percent inhibitory concentrations of compounds examined for E2-induced luciferase activity and MCF-7 cell proliferation, and for E2-independent MDA-MB231 cell proliferation

| Compound | ERα/ERE-tk-Luc $IC_{50}$ (μM) | MCF-7 $GI_{50}$ (μM) | MDA-MB231 $GI_{50}$ (μM) (e) |
|---|---|---|---|
| TAM | 4.9 ± 2.0 (a) | 3.9 ± 2.3 (b) | >50 (a) |
| **3a** | 11 ± 2 (a) | 12 ± 4 (c) | >50 (e) |
| **3a**-*ent1* | 13 ± 4 (a) | 8.8 ± 2.1 (b) | >50 (e) |
| **3a**-*ent2* | 23 ± 7 (a) | 13 ± 0 (b) | >50 (e) |
| **4** | — | 12 ± 0 (d) | — |
| **5** | — | >20 (d) | — |
| **2a** | — | 15 ± 0 (d) | — |
| **2b** | — | >20 (d) | — |
| **1a** | — | 13 ± 0 (d) | — |
| **1b** | — | 16 ± 1 (d) | — |
| Colchicine | — | — | 0.082 ± 0.009 (e) |

Values given are means ± SD. The range of concentrations used was 3.2 nM to 20 or 50 μM.
(a) $N = 6$, (b) $N = 8$, (c) $N = 20$, (d) $N = 4$, (e) $N = 8$.

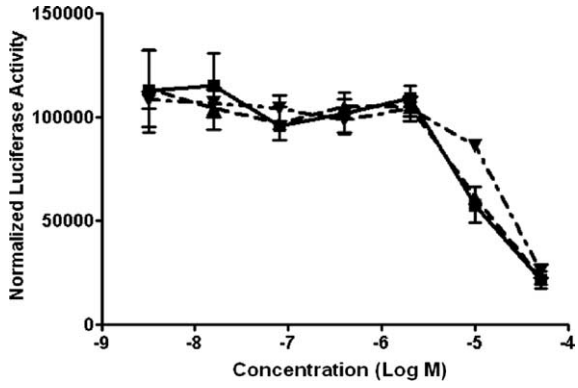



**Figure 4.** Compound **3a** inhibits E2-induced transcription in a concentration-dependent manner. The pure enantiomers, **3a**-*ent1* (▲) and **3a**-*ent2* (▼), and the racemate (■) of *C*-cyclopropylalkylamide **3a** show indistinguishable activities. Data represent normalized luciferase counts (means ± SD, $N = 3$).

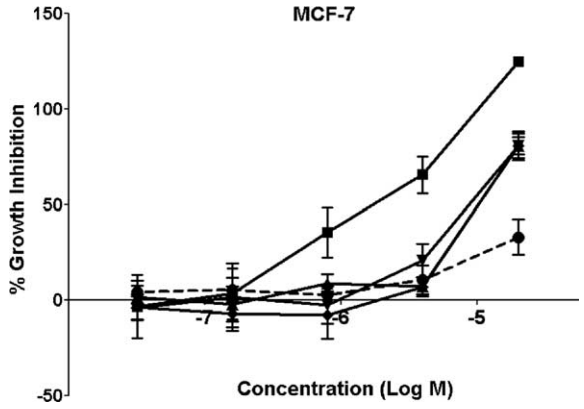



**Figure 5.** Compound **3a** (▲) and its enantiomers, **3a**-*ent1* (▼) and **3a**-*ent2* (♦) inhibit E2 (1 nM) induced proliferation of MCF-7 cells in a concentration-dependent manner. TAM (■) and alkyne **5** (●) were used as positive and negative controls, respectively. Data represent % growth inhibition (mean ± SD, $N = 4$).

structural analogs of **3a** lacking the sulfonylcarbamate had no antagonistic activity. The alkyne **5** demonstrated poor to moderate MCF-7 growth inhibition (Fig. 4), suggesting that the **3a** structure as a whole was necessary for biological activity. A similar lack of activity for **5** was observed in the transcriptional assay (data not shown). Based on these observations, we conclude that the lipophilic region containing the phosphinoylamide moiety is also necessary for the activity of compound **3a**.

### 2.5. Racemic 3a and its enantiomers have similar ERα antagonistic activity

Compound **3a** has three stereocenters. It is diastereomerically pure, but racemic.[13] Since biological activity is often due to a single enantiomer in a racemate,[18] the enantiomers of **3a** (**3a**-*ent1* and **3a**-*ent2*) were separated by chiral HPLC and tested side-by-side with the racemic mixture in the transcriptional assay (Fig. 4, Table 2). Interestingly, no difference was observed between the activities of the enantiomers of **3a**, both being equipotent to the racemate. The activities of the enantiomers and the racemate were also compared in the MCF-7 antiproliferative assay. Again, the enantiomers and the racemate were not statistically different in their inhibition of E2-induced growth of this ER-positive cell line (Fig. 5, Table 2).

### 2.6. Racemic 3a is a modest competitor at the ERα

Compound **3a** was tested for its ability to displace a fluorescent E2 derivative from the ERα. Recombinant human ERα was complexed with fluorescently labeled estradiol (ES2) and then treated with test agents. After 2 h, the fluorescence polarization was measured. ES2 bound to ERα protein gave high fluorescence polarization. In this assay the presence of a displacing ligand causes a decrease in the fluorescence polarization. Concentration dependence curves were constructed and $IC_{50}$'s were calculated from the best-fit curves for the controls E2 (5 ± 4 nM) and TAM (28 ± 20 nM). Although compound **3a** showed a concentration-dependent displacement of ES2 in this in vitro assay, its $IC_{50}$ value was above the highest concentration tested (>10 μM), leading to the conclusion that **3a** is only a modest E2 competitor at the ERα.

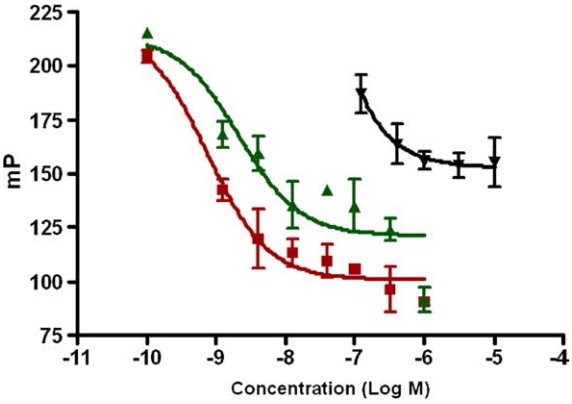



**Figure 6.** Compound **3a** (▼) ($IC_{50}$ > 10 μM) shows low potency in an in vitro ERα competition assay (Panvera) as compared to E2 (■) ($IC_{50}$ 5 ± 4 nM) and TAM (▲) ($IC_{50}$ 28 ± 20 nM). Data represent fluorescence polarization (mean ± SD, $N = 3$). The one site competition method in GraphPad Prism 4 software was used for constructing dose–response curves and calculating $IC_{50}$'s.

## 3. Discussion and conclusions

In this study, *C*-cyclopropylalkylamide **3a** was found to be a new ERα antagonist. A variety of in vitro assays have been developed to test for new antiestrogens.[19] Candidate antagonists for ERα should have specificity for the ERα LBD, be cell-permeable, stable under physiological conditions (e.g., cell culture medium) and, most importantly, prevent E2-induced ER transcriptional activation of target genes and inhibit E2-dependent cell proliferation. All of these benchmarks can be addressed in cell-based assays when physiological endpoints for antiestrogenic actions, inhibition of E2-induced breast cancer cell proliferation or E2-induced transcription, are examined. *C*-Cyclopropylalkylamide **3a** inhibited E2-induced MCF-7 cell proliferation and ERE-tk-Luc transcription, fulfilling these requirements in a concentration-dependent manner. In addition, compound **2b**, an allylic amide that showed significant antagonist activity in the transcriptional assay, failed to inhibit E2-induced MCF-7 cell proliferation at concentrations tested (Table 2). This finding demonstrated that at least two functionally distinct assays should be used in the determination of new potential antiestrogens. As a counter screen for selectivity, antiproliferative activity against the ER-negative MDA-MB231 human breast cell line was examined, where **3a** was found to be inactive. The biological activities of the individual enantiomers of compound **3a** were not statistically significantly different. Moreover, in vitro evaluation results demonstrated that **3a** only weakly displaced fluorescently labeled estradiol in an ERα competitor assay.

Overall, the unusual structure of **3a**, its ability to antagonize E2 in the cell-based assays, and its modest ability

to displace E2 in the binding pocket suggests new avenues for SERM design. The lack of differential activities of enantiomers in the biological assays was surprising. Molecular docking studies using the CAChe suite of algorithms with a model of the ERα LBD were conducted to compare the binding mode of both enantiomers of **3a**, as well as raloxifene (data not shown). These studies indicated that the phosphinoyl group of **3a** may serve as a cap to the binding pocket, interfering with helix 12 movement, while the remainder of **3a**'s structure binds deeper into the raloxifene binding cleft and may interact with residues at helices H3, H6, and H11, suggesting a partial or full antagonist binding mode. The fact that the phosphinoyl system lies essentially outside of the ligand binding pocket in the molecular docking exercises helps to explain the absence of enantioselectivity for this compound. In fact, both enantiomers of **3a** gave essentially identical binding scores. Crystallographic analyses of the ERα LBD soaked or co-crystallized with **3a**, should help to support or refute these hypotheses.

## 4. Experimental

### 4.1. Chemicals and cell culture

E2 and TAM citrate were purchased from Sigma (St. Louis, MO). All test compounds were prepared as 10 mM stock solutions in DMSO and stored at −30 °C until diluted. Cyclopropane **4** was prepared as described,[16] as were the *C*-cyclopropylalkylamides, homoallylic amides, allylic amides, and alkyne **5**.[12–14] The enantiomers of compound **3a** were resolved on a 0.46× 25 cm Chiralcel® OD column using 1 mL/min 7.5% isopropanol in hexanes as the mobile phase. The retention time for **3a**-*ent1* was 11.3 min, while that for **3a**-*ent2* was 17.8 min. The HEK293 cells were obtained from the American Type Culture Collection. ER positive MCF-7 and ER negative MDA-MB231 breast cancer cells were gifts from Dr. Marc Lippman. Prior to use in experiments, each ligand was further diluted into the requisite amounts of phenol red-free RPMI (Hyclone) medium containing 10% fetal bovine serum (FBS) (Hyclone) stripped of steroids with charcoal-coated dextran.

### 4.2. Plasmids and transient transfection assays

The CMV-ER ERα, ERE-tk-Luc, and CMV-β-gal plasmids were provided by Dr. Ron Evans at the Salk Institute. HEK293 cells were plated in 48-well plates with DMEM medium containing 10% FBS and allowed to attach and grow for 48 h. They were then transfected with the plasmids entrained in *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP) (Roche) at a density of 10,000 cells per well.[15,23] The liposomes contained 1.0 μg of reporter plasmid (ERE-tk-Luc), 0.5 μg of CMV-ERα, and 0.5 μg of the transfection control (CMV-β-gal) per well. Plasmids were premixed in PBS and mixed with DOTAP. Cells were treated with transfection mixture in serum- and phenol red-free DMEM. After 4 h, the medium was replaced with DMEM containing 10% FBS. Twenty-four hours later, the medium was changed to DMEM containing 10% dextran-coated charcoal-stripped FBS and test chemicals were added. After 24 h in the presence of test agent, cells were lysed and assayed for luciferase and β-galactosidase activities. Transfections were performed in triplicate, and each experiment was repeated at least twice.

**4.2.1. Luciferase assay.** The medium was removed and cell monolayers were frozen at −80 °C. After 30 min, 150 μL of cell culture lysis reagent was added to each well and incubated at 4 °C for an additional 30 min. Luciferase assay reagents were prepared as described previously.[15,23] Cell extracts were clarified by centrifugation for 3 min at 3000 rpm at room temperature and supernatants were transferred to 96-well assay plates. For each assay, 50 μL of supernatant was mixed with 50 μL of luciferase assay buffer. Luminescence was read using a Victor luminometer. Luciferase activity was normalized to-galactosidase activity and data was calculated as fold of induction as compared to vehicle control (DMSO, 0.1% final volume).

**4.2.2. Antagonism.** Antagonism in the luciferase assay was calculated using the following formula

$$\%\text{Inhibition} = [100 - (\text{L} + \text{E2})/(\text{E2})] \times 100,$$

where L + E2 represents normalized luciferase activity (using β-Gal as the internal control) in cells treated simultaneously with E2 and the test compounds, and E2 the normalized luciferase activity in cells treated with E2 alone. GraphPad Prism 4 software was used for constructing dose–response curves and calculating $IC_{50}$'s. $IC_{50}$'s were estimated from dose-dependence curves that best fit the data obtained as an average from at least two transfections performed in triplicate.

### 4.3. Antiproliferative assays

MCF-7 ERα positive breast cancer cells were plated in 96-well plates at 4000 cells/well in phenol red-free RPMI-1640 containing 10% dextran-coated charcoal-stripped FBS and 1 nM E2, and allowed to attach for 24 h. The cells were incubated in the presence of test compounds (at concentrations ranging from 25 pM to 50 μM) and 1 nM E2 for 6 days. Cell density was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye reduction assay using phenazine methanesulfonate as the electron acceptor as described previously.[24] Absorbance was measured at 490 nm minus that at 630 nm 2 h after incubation with the reagents. Data represents the average of at least two independent experiments done in quadruplicate. E2-stimulated growth of MCF-7 cells at day 6 was set to 100% growth.

MDA-MB231 ERα negative cells were plated in 96-well plates at 1000 cells/well and allowed to attach to the plastic for 72 h in phenol red-free RPMI-1640 containing 10% FBS. Test agents and control compounds (colchicine and TAM) were added over the range of 3.2 nM–50 μM and cells were incubated for 72 h. Cell density was

determined with the MTS assay. GraphPad Prism 4 software was used for constructing dose–response curves and calculating $GI_{50}$ values. $GI_{50}$ values were estimated from dose-dependence curves that best fit the data.

### 4.4. ERα competitor assay

The ERα competitor assay (Panvera) was performed according to manufacturer's recommendations with some modifications. Recombinant human ERα used in this homogenous assay was used at the recommended concentration of 15 nM and fluorescently labeled estradiol, Fluormone™ ES2, was used at 1 nM in the final mixture. Recombinant human ERα complexed with ES2 was distributed to all wells and then serial dilutions of test compounds were added. DMSO content was kept at 1%. The needed volume of 2× concentration of ERα complexed with ES2 (ER/ES2) was prepared on ice and distributed 20 μL/well in 384-well, square and black-bottom plates (Costar). Test compound dilutions, prepared in the screening buffer, were added 20 μL/well. E2 was used as a standard. After 2 h, the fluorescence polarization was measured using an Analyst™ AD & HT Assay Detection Systems reader (Molecular Devices) equipped with 485 nm excitation and 530 nm emission interference filters with the appropriate FL505 dichroic mirror. The instrumental set up was validated using serial dilutions (100 nM to 1 pM) of methylfluorescein (Sigma) in the screening buffer. Data were analyzed using GraphPad Prism's one site competition method (Fig. 6).

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