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# Antiproliferative activity of a series of novel cyclopropyl antiestrogens on MCF-7 human breast cancer cells in culture

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**The potential antitumor activity of a series of novel cyclopropyl compounds, which lack estrogen agonist activity, was evaluated in estogen receptor positive human breast cancer cells (MCF-7) in culture. The compounds were evaluated to determine their antiproliferative activity at a concentration of 1 μM at 2, 4 and 6 days of treatment by hemocytometer using the Trypan Blue exclusion method to count viable cells. Estradiol-induced reversibility of the antiproliferative activity of these compounds was also evaluated. The activity of a series of 19 diaryl- and triarylcyclopropyl compounds was examined. Thirteen compounds inhibited the growth of MCF-7 cells while six were inactive. Five of the 13 active compounds produced antiproliferative activity which was reversed by 0.1 μM estradiol. Thus, several of these novel cyclopropyl compounds may be useful in the treatment of hormone-dependent breast cancer and other estrogen-dependent tumors.**

*Key words:* Anticancer drugs, cyclopropyl antiestrogens, estrogen receptors, human breast cancer MCF-7 cells.

## Introduction

Breast cancer is one of the most common types of cancer in women today. Hormone-dependent breast tumors containing estrogen and progesterone receptors respond most favorably to antiestrogen treatment.[1] Tamoxifen, a non-steroidal antiestrogen, is used clinically for the treatment of advanced breast cancer in post-menopausal women, and as adjunct therapy in pre-menopausal women.[2] Tamoxifen binds to the estrogen receptor (ER) and blocks the effect of endogenous estradiol;[3] however, it produces both estrogenic and antiestrogenic activity. This partial estrogen agonist activity causes several undesirable effects in breast cancer patients, such as stimulation of ovarian estrogen production and an increased incidence of endometrial carcinoma.[4,5] Thus, there is a need for the development of pure estrogen antagonists, which would be better suited for the treatment of estrogen-dependent breast cancers.[6] In our search for a pure antiestrogen, we have synthesized several cyclopropyl compounds[7,8] and evaluated their estrogenic and antiestrogenic activity using the utreotropic and antiutreotropic assay in mice.[8,9] Compound **VI** (Analog II), one of these cyclopropyl derivatives, was found to be a pure estrogen antagonist in the mouse[9] and produced significant tumor regression in the rat DMBA-tumor model.[10–12] In order to evaluate the potential therapeutic effectiveness of these compounds in the treatment of human breast cancer, the activity of these compounds was examined on MCF-7 human breast cancer cells in culture.

## Materials and methods

### MCF-7 cell culture

MCF-7, estrogen receptor positive human breast cancer cells, were obtained from the Michigan Cancer Foundation. MCF-7 cells were grown in T-75 tissue culture flasks, as monolayer cultures in RPMI 1640 medium (without Phenol Red) supplemented with 2 mM L-glutamine, gentamicin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 5% calf serum. Medium without

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Phenol Red was used because Phenol Red is reported to be estrogenic in nature and thus can affect the assessment of antiestrogenic responses.[13,14] Cultures were grown at 37°C in a humid atmosphere containing 5% $CO_2$. When cultures reached confluency (usually at 7 days), they were subcultured using a 1:2 splitting ratio. Culture medium was changed on alternate days until confluency was reached. Viable cell counts were performed using the Trypan Blue exclusion method.[15]

### Cell proliferation study

In each experiment, the exponentially growing cells were trypsinized, counted and plated in multiwell plates at a density of $7.5 \times 10^4$ cells per well in 3 ml of media. After 2 days of incubation, when the cells were in an exponential growth phase, the test compounds were added. The test compounds, tamoxifen or estradiol, were dissolved in an absolute ethanol:polyethylene glycol 400 (45:55) mixture and added to the cultures following dilutions in culture medium. The final concentration of vehicle was 0.1% of the growth medium. Control wells received the same amount of vehicle alone. Exponentially growing cells were counted on the second, fourth and sixth days following addition of the experimental compounds using the Trypan Blue exclusion method.[15]

### Estrogen receptor determination

The affinity and binding capacity of the ER for estradiol was determined by Scatchard analysis using the whole-cell assay method.[16,17] MCF-7 cells were incubated with [$^3$H]estradiol (New England Nuclear, sp. act. 92.5 Ci/mmol) over a concentration range of 0.1–10 nM with or without a 200-fold excess of diethylstilbestrol (DES) in 0.4 ml of RPMI 1640 medium containing 0.1% bovine serum albumin for 60 min at 37°C. The bound [$^3$H]estradiol was extracted from the cells using 1 ml of absolute ethanol. The cells were incubated with ethanol for 30 min at 22°C. A 0.2 ml aliquot of the ethanol cell extract was transferred to 4 ml of liquid scintillation cocktail (Ready-Solv, Beckman) and was counted in a liquid scintillation counter (Beckman, model LS 1801). Specific bound [$^3$H]estradiol was determined by subtracting non-specific bound [$^3$H]estradiol (obtained in the presence of DES) from the total bound [$^3$H]estradiol.

### Statistics

Multiple group comparisons were made by using two-way ANOVA (Clear Lake Research Incorporated ANOVA program) on a Macintosh Plus computer. Individual groups were compared using Duncan's new multiple range test. Group comparisons resulting in $p$ values of less than 0.05 were considered to be statistically different. Compounds which caused a statistically significant reduction in viable cell number compared to the control group were considered to be active antiproliferative agents.

## Results

### Characterization of the MCF-7 cells

Estradiol (10 nM) stimulated the growth of MCF-7 cells while the standard antiestrogen tamoxifen (1 $\mu$M) inhibited the growth of MCF-7 cells on the fourth and sixth days (Figure 1).

The ER binding affinity for estradiol and the density of ER in MCF-7 cells as determined by Scatchard analysis using the whole-cell assay is presented in Figure 2. The specific bound [$^3$H]estradiol in MCF-7 cells was significantly higher than non-specific binding. The results of the whole-cell assay indicate that the binding capacity ($N$) of ER was 96 fM per million cells, while the binding affinity ($K_d$) was 0.53 nM/$\mu$g of DNA.

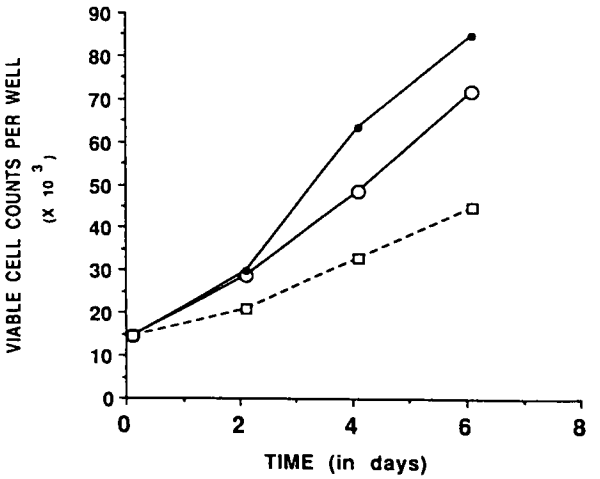



**Figure 1.** Effect of estradiol (10 nM), and tamoxifen (1 $\mu$M) on the growth of MCF-7 cells, after 2, 4 and 6 days of treatment. Each point represents the mean of duplicate samples and the values on days 4 and 6 of treatment were statistically significantly different from control. ○, Control; ●, estradiol; □, tamoxifen.

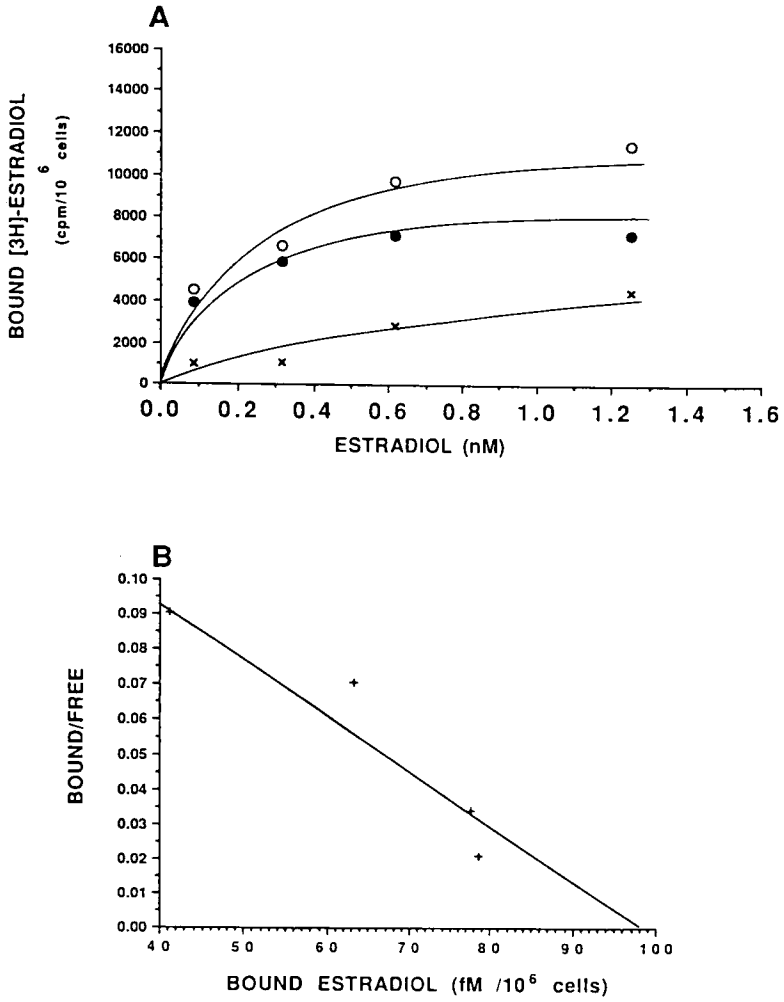



**Figure 2.** (A) Uptake of [$^3$H]estradiol by MCF-7 human breast cancer cells in culture after incubation for 1 h at 37°C with various concentrations of [$^3$H]estradiol in the presence of 200-fold higher concentration of diethylstilbestrol. Each point on the graph is the mean of triplicate samples. ○, Total binding; ●, specific binding; ×, non-specific binding. (B) Scatchard analysis of estrogen receptor in MCF-7 cells. Each point represents the mean of triplicate samples.

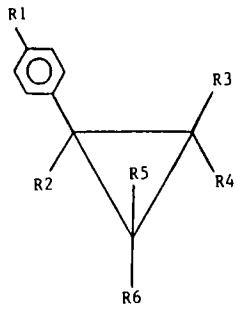
These results are consistent with the values reported by other investigators for MCF-7 cells[16–20] and clearly demonstrate the estrogen-dependent nature of the MCF-7 cells used in this study.

## Antiproliferative activity of the cyclopropyl compounds

Table 1 shows a list of compounds examined in this study. Compounds **I** to **XIII** inhibited the proliferation of MCF-7 cells at $10^{-6}$ M, on day 4 (Tables 2 and 3), whereas compounds **XIV–XIX** did not inhibit proliferation (Table 4). Of the 13 active compounds, eight (**I**, **II**, **IV**, **V**, **VII**, **VIII**, **XII** and **XIII**) inhibited cell growth on both days 4 and 6. Two of the active compounds (**II** and **IX**) reduced cell growth on day 2.

The criteria for estradiol reversibility was a significant reversal of compound-induced antiproliferative activity by estradiol on either day 2, 4 or 6. Accordingly, the antiproliferative activity of five of the active compounds (**I–V**) was reversed by a 10-fold lower concentration of estradiol (0.1 $\mu$M), as shown in Table 2. The activity of compounds **II–V** was reversed by estradiol, on day 4. The activity of compounds **IV** and **V** was reversed by estradiol on both days 4 and 6. The activity of compound **I** was partially

**Table 1.** Chemical structure of compounds examined in this study




| Compound | Isomer | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|
| **I** | *cis* | $OCH_3$ | H | $C_6H_4(p)OCH_3$ | H | Cl | Cl |
| **II** | – | OH | $C_6H_4(p)OH$ | $C_5H_{10}$ | | Cl | Cl |
| **III** | *trans* | H | H | H | $C_6H_5$ | H | H |
| **IV** | *trans* | OH | $C_2H_5$ | $C_2H_5$ | $C_6H_4(p)OH$ | H | H |
| **V** | *trans* | $OCH_3$ | $C_2H_5$ | $C_2H_5$ | $C_6H_4(p)OCH_3$ | H | H |
| **VI** | *cis* | H | H | $C_6H_5$ | H | Cl | Cl |
| **VII** | *cis* | $OCH_3$ | H | $C_6H_5$ | H | Cl | Cl |
| **VIII** | *cis* | $OCOCH_3$ | H | $C_6H_5$ | H | Cl | Cl |
| **IX** | *trans* | OH | $C_2H_5$ | $C_2H_5$ | $C_6H_4(p)OH$ | Cl | Cl |
| **X** | – | OH | $C_6H_4(p)OH$ | H | H | Cl | Cl |
| **XI** | – | $OCH_3$ | $C_6H_4(p)OH$ | H | H | Cl | Cl |
| **XII** | – | $OCH_3$ | $C_6H_4(p)OCH_3$ | H | H | Cl | Cl |
| **XIII** | – | $OCH_3$ | $C_6H_4(p)OCH_3$ | $C_5H_{10}$ | | Cl | Cl |
| **XIV** | *cis* | H | H | $C_6H_5$ | H | H | H |
| **XV** | *trans* | H | H | H | $C_6H_5$ | Cl | Cl |
| **XVI** | *trans* | $OCH_3$ | $C_2H_5$ | $C_2H_5$ | $C_6H_4(p)OCH_3$ | Cl | Cl |
| **XVII** | – | $OCH_3$ | $C_6H_4(p)OCH_3$ | $C_6H_5$ | H | Cl | Cl |
| **XVIII** | – | $OCOCH_3$ | $C_6H_4(p)OCOCH_3$ | $C_6H_5$ | H | Cl | Cl |
| **XIX** | *trans* | $OCOCH_3$ | H | H | $C_6H_5$ | Cl | Cl |

(*p*), At *para* position.

reversed by estradiol on day 6 and the activity of compound **II** was reversed by estradiol on day 2.

## Discussion

The growth of ER-positive MCF-7 cells used in this study was stimulated by estradiol and inhibited by tamoxifen (Figure 1). These compounds are widely used to evaluate the estrogen-responsive nature of MCF-7 cells,[21,22] since it is known that certain strains of MCF-7 cells have lost their estrogen responsiveness.[22] Finally, it is clear that the MCF-7 cells used in this study were estrogen-dependent, since the ER binding affinity ($K_d$) and ER binding capacity ($N$) as estimated using Scatchard analysis (Figure 2) is similar to that obtained by other investigators.[16-20]

The specific antiestrogenic action of tamoxifen on MCF-7 cell growth has been demonstrated at a concentration of 1 $\mu$M or lower; above this concentration the antiproliferative effects of tamoxifen on MCF-7 cell growth are believed to be due to a cytotoxic action.[23,24] Therefore, all the test compounds in the present study were evaluated at a concentration of 1 $\mu$M to examine their specific antiestrogenic properties.

The structure of the 19 novel cyclopropyl compounds, which were tested on the cultured human breast cancer cells in this study, are presented in Table 1. We have demonstrated, in a previous study, that some of these compounds are antiestrogenic; however, none of the compounds produced any estrogenic agonist activity and all of these compounds displayed weak ER binding affinity.[8,9] Thirteen compounds inhibited the growth of MCF-7 cells, as shown in Tables 2 and 3. It has been shown that the phenolic hydroxyl group serves as an anchoring point for initial binding with ER.[25] Therefore, these compounds may inhibit MCF-7 cell growth by binding to ER

**Table 2.** Influence of (estradiol-reversible) cyclopropyl compounds ($10^{-6}$ M) on the growth of MCF-7 cells

| Treatment[a] +Estradiol ($10^{-7}$M) | Viable MCF-7 cells per well (×1000) ± SEM | | | | | |
|---|---|---|---|---|---|---|
| | Day 2 | | Day 4 | | Day 6 | |
| | (−) | (+) | (−) | (+) | (−) | (+) |
| Control | 3.7 ± 0.8 | 3.82 ± 1.2 | 13.1 ± 0.3 | 17.2 ± 1.5 | 24.5 ± 6.5 | 29.1 ± 4.1 |
| **I** | 3.1 ± 0.0 | 4.7 ± 0.0 | 5.0 ± 1.2[b] | 5.0 ± 0.9[b] | 3.6 ± 0.0[b] | 10.1 ± 0.0[b,c] |
| **II** | 2.0 ± 0.04[b] | 3.5 ± 0.3[c] | 8.2 ± 0.6[b] | 12.4 ± 1.4[c] | 18.6 ± 11.6 | 27.0 ± 0.6 |
| **III** | 5.7 ± 2.6 | 10.6 ± 2.3 | 1.2 ± 0.2[b] | 14.5 ± 7.4[c] | 22.3 ± 0.1 | 27.1 ± 0.9 |
| **IV** | 3.0 ± 0.5 | 2.9 ± 0.7 | 6.8 ± 1.0[b] | 13.7 ± 1.5[c] | 14.1 ± 0.4[b] | 25.4 ± 1.0[c] |
| **V** | 11.8 ± 1.0 | 10.8 ± 1.7 | 7.6 ± 0.4[b] | 16.7 ± 0.8[c] | 15.2 ± 0.8[b] | 26.7 ± 3.5[c] |

[a] Compounds were tested at $10^{-6}$ M in the absence (−) or presence (+) of estradiol ($10^{-7}$ M).
[b] Statistically significant ($p < 0.05$) inhibition of growth of MCF-7 cells compared to non-estradiol stimulated control.
[c] Statistically significant ($p < 0.05$) reversal of growth inhibition in the presence of estradiol ($10^{-7}$ M).

**Table 3.** Influence of (estradiol-irreversible) cyclopropyl compounds ($10^{-6}$ M) on the growth of MCF-7 cells

| Treatment[a] +Estradiol ($10^{-7}$M) | Viable MCF-7 cells per well (×1000) ± SEM | | | | | |
|---|---|---|---|---|---|---|
| | Day 2 | | Day 4 | | Day 6 | |
| | (−) | (+) | (−) | (+) | (−) | (+) |
| Control | 3.7 ± 0.8 | 3.82 ± 1.2 | 13.1 ± 0.3 | 17.2 ± 1.5 | 24.5 ± 6.5 | 29.1 ± 4.1 |
| **VI** | 8.0 ± 1.0 | 5.5 ± 0.6 | 4.6 ± 1.0[b] | 5.7 ± 0.6[b] | 25.5 ± 4.0 | 34.6 ± 0.3 |
| **VII** | 2.5 ± 0.7 | 2.8 ± 0.4 | 5.0 ± 0.3[b] | 4.7 ± 0.3[b] | 14.2 ± 3.0[b] | 9.8 ± 2.0[b] |
| **VIII** | 3.8 ± 0.7 | 3.2 ± 0.1 | 7.2 ± 0.6[b] | 4.6 ± 1.7[b] | 6.7 ± 0.7[b] | 5.9 ± 2.2 |
| **IX** | 1.25 ± 0.04[b] | 1.7 ± 0.3[b] | 8.0 ± 0.04[b] | 7.2 ± 0.9[b] | 23.6 ± 2.6 | 17.5 ± 5.1 |
| **X** | 2.9 ± 0.3 | 5.1 ± 1.2 | 1.5 ± 0.5[b] | 7.9 ± 2.1[b] | 35.9 ± 7.2 | 10.4 ± 1.0 |
| **XI** | 3.4 ± 1.2 | 5.4 ± 1.4 | 4.5 ± 1.1[b] | 5.5 ± 1.5[b] | 20.8 ± 4.1 | 40.9 ± 6.5 |
| **XII** | 3.4 ± 0.6 | 3.2 ± 0.1 | 5.5 ± 0.2[b] | 6.4 ± 0.6[b] | 7.7 ± 0.6[b] | 7.6 ± 1.3[b] |
| **XIII** | 3.3 ± 0.8 | 5.4 ± 0.9 | 3.6 ± 1.7[b] | 4.3 ± 2.1[b] | 15.4 ± 1.7[b] | 12.2 ± 0.6[b] |

[a] Compounds were tested at $10^{-6}$ M in the absence (−) or presence (+) of estradiol ($10^{-7}$ M).
[b] Statistically significant ($p < 0.05$) inhibition of growth of MCF-7 cells compared to non-estradiol stimulated control.

**Table 4.** Influence of non-inhibitory cyclopropyl compounds ($10^{-6}$ M) on the growth of MCF-7 cells

| Treatment[a] +Estradiol ($10^{-7}$M) | Viable MCF-7 cells per well (×1000) ± SEM | | | | | |
|---|---|---|---|---|---|---|
| | Day 2 | | Day 4 | | Day 6 | |
| | (−) | (+) | (−) | (+) | (−) | (+) |
| Control | 3.7 ± 0.8 | 3.82 ± 1.2 | 13.1 ± 0.3 | 17.2 ± 1.5 | 24.5 ± 6.5 | 29.1 ± 4.1 |
| **XIV** | 9.1 ± 0.5 | 9.0 ± 0.3 | 13.0 ± 2.2 | 12.3 ± 0.8 | 18.8 ± 0.8 | 25.3 ± 0.2 |
| **XV** | 7.8 ± 0.4 | 8.5 ± 1.3 | 16.2 ± 1.1 | 15.5 ± 0.07 | 22.9 ± 7.8 | 17.4 ± 3.0 |
| **XVI** | 5.7 ± 1.5 | 9.1 ± 0.2 | 8.1 ± 0.1 | 12.5 ± 1.4 | 38.2 ± 3.3 | 19.9 ± 4.8 |
| **XVII** | 1.9 ± 0.5 | 1.6 ± 0.3 | 11.9 ± 2.3 | 7.2 ± 0.9 | 32.0 ± 2.7 | 28.1 ± 2.9 |
| **XVIII** | 2.9 ± 0.6 | 2.8 ± 0.2 | 25.8 ± 2.7 | 19.2 ± 8.4 | 21.9 ± 0.4 | 27.9 ± 0.4 |
| **XIX** | 6.9 ± 0.6 | 6.2 ± 0.3 | 15.6 ± 2.5 | 5.8 ± 2.9 | 16.9 ± 1.7 | 11.6 ± 0.8 |

[a] Compounds were tested at $10^{-6}$ M in the absence (−) or presence (+) of estradiol ($10^{-7}$ M).

at the phenolic hydroxyl group. All the compounds containing a phenolic hydroxyl group (**II, IV** and **IX–XI**) were found to be active on the basis of antiproliferative activity on MCF-7 cells. However, compounds not possessing a phenolic hydroxyl group also were found to produce antiproliferative activity. MCF-7 cells in culture are reported to cause estradiol metabolism.[26] Thus it is possible that the compounds which do not contain a phenolic hydroxyl group may be metabolized (i.e. metabolic demethylation of compounds **I, V, VII, XII, XIII** and hydroxylation of compounds **III, VI** and esterase hydrolysis of compound **VIII**), which would result in formation of a phenolic hydroxyl group, which in turn may be responsible for their activity.

An evaluation of the antiestrogenic nature of these compounds was carried out by testing their antiproliferative activity in the presence and absence of a 10-fold lower concentration of estradiol (0.1 $\mu$M). The activity of five active compounds (**I–V**) was reversed by estradiol and thus they may be considered to be antiestrogenic (Table 2). The presence of aromatic hydroxyl and methoxy groups in the *trans* configuration along with a hydrophobic region (H at R5 and R6) seems to be a sufficient requirement for the action of cyclopropyl compounds at the ER. This is evident from the complete estradiol-reversible antiproliferative activity of compounds **IV** and **V**, both on days 4 and 6 of treatment. Introduction of chloride into this hydrophobic region abolishes the antiproliferative action (i.e. compound **IX** caused estrogen-irreversible activity while compound **XVI** produced no antiproliferative activity). However, the hydrophobic region and *trans* configurations do not seem to be an absolute requirement for action of cyclopropyl compounds, as compounds **I–III**, which are devoid of these characteristics, also seem to act in an estrogen-reversible manner. The antiproliferative activity of compounds **I–III** cannot be solely attributed to their classical antiestrogenic action, since there was a lack of complete estradiol reversibility on both days 4 and 6. A non-antiestrogenic component, in addition to ER-mediated antiestrogenic effects, may contribute to the antiproliferative activity of these compounds, similar to that reported for tamoxifen.[24] Compounds **VI–XIII** may not be acting at the ER as indicated by their estradiol-irreversible activity. It is possible that these compounds might be acting as calmodulin antagonists[27] or as ligands for the antiestrogenic binding sites, similar to that reported for the triarylethylene series of antiestrogen.[24]

In conclusion, the antiproliferative response of the estrogen-dependent MCF-7 cells to compounds **I–V** supports the antiestrogenic nature of these five compounds. Thus these compounds have the potential to be useful in the treatment of and/or prevention of ER-positive human breast cancer.

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