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A comparison of the antitumor activity of two triarylcyclopropyl antiestrogens (compounds 4d and 5c) on human breast cancer cells in culture

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Compound 4d ((*E*)- and (*Z*)-1,1-Dichloro-2-[4-(benzyloxy)-phenyl]2,3-bis(4-methoxyphenyl) cyclopropane) and compound 5c ((*Z*)-1,1-Dichloro-2-[4-(benzyloxy)-phenyl]-2-(4-methoxyphenyl)-3-phenylcyclopropane) are two members of a novel series of triarylcyclopropyl compounds which have been shown to be pure antiestrogens. In the present study, the antiproliferative activity of 4d and 5c was examined on estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cells and A-549 human lung cancer cells. Compound 4d inhibited the growth of MCF-7 cells in a dose-related manner over a concentration range of 10^{-13} to 10^{-6} M while compound 5c inhibited MCF-7 cell growth in a dose-related manner over a concentration range of 10^{-9} to 10^{-5} M. Further, neither compound altered the growth of MDA-MB-231 or A-549 cells. Co-administration of estradiol reversed the antiproliferative activity of 4d but not 5c on MCF-7 cells. Both compounds bound specifically to ER in MCF-7 cells; however, the relative binding activity of 4d was five times greater than estradiol and 5000 times greater than 5c. The influence of 4d and 5c on the cell surface morphology of MCF-7 and MDA-MB-231 cells was studied using scanning electron microscopy. Both compounds, at a concentration of 10^{-6} M, reduced the density of microvilli on MCF-7 cells, which was reversed by co-administration of estradiol (10^{-9} M). These compounds did not alter the cell surface morphology of ER-negative MDA-MB-231 cells. In conclusion, the results of this study indicate that compound 4d is more potent than 5c as an inhibitor of breast cancer cell proliferation and suggest that a polar methoxy group on the β phenyl ring of compound 4d contributes to ER binding and ER-mediated antitumor activity. Further, these results suggest that one or both of these compounds may be highly effective in the treatment of estrogen-dependent breast cancer.

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Key words: Anticancer drugs, cyclopropyl antiestrogen, estrogen receptors, human breast cancer, MCF-7 cells, MDA-MB-231 cells.

Introduction

Antiestrogens are effective in controlling the growth of estrogen-dependent breast tumors.[1] The therapeutic efficacy of the antiestrogen tamoxifen in the treatment of advanced breast cancer in post-menopausal women and as adjunct therapy in pre-menopausal women is well established.[2] Although clearly of clinical value, tamoxifen is a partial estrogen agonist both *in vivo*[3] and *in vitro*.[4] The estrogen agonist activity of tamoxifen may be of clinical value in the prevention of bone loss and lowering low density lipoprotein cholesterol levels in post-menopausal women on long-term tamoxifen therapy.[5] However, the estrogen agonist activity of tamoxifen causes several undesirable effects in breast cancer patients such as stimulation of ovarian estrogen production and an increased incidence of endometrial carcinoma.[6,7] The estrogenic effects of tamoxifen have also been reported to increase the incidence of rat hepatocellular carcinoma at high dose[8] and to cause a possible increased risk of endometrial carcinoma.[9] Although tamoxifen has been reported to be tumoristatic to MCF-7 cells in nude mice,[10] its prolonged exposure can lead to tamoxifen-resistant[11] and tamoxifen-stimulable[12] tumors. Accordingly, prolonged tamoxifen treatment is known to ultimately fail.[13] Thus, antiestrogens devoid of estrogen agonist activity should be more effective in the treatment and/or prevention of breast cancer.

The search for a pure antiestrogen first led to the development of MER-25 but its clinical side-effects and lack of potency precludes its use in humans.[14]

During the past three decades several pure antiestrogenic compounds have been reported.[15–18] Our work with diaryl- and triarylcyclopropyl analogs indicates that these compounds are devoid of estrogenic activity in mouse uterine tissue.[15,16] The *p*-benzyloxy substituent on the α′ phenyl ring of the triarylcyclopropanes (**4d** and **5c**; see Figure 1) has been reported to be relatively non-polar and electron-rich at its distal region similar to the important dialkylaminoethoxy side-chain of tamoxifen. It has been suggested that these side-chains play an important role in mediating estrogen antagonist activity.[15] The polar group on the β phenyl ring (Figure 1) is reported to contribute to the estrogen receptor (ER) binding of cyclopropanes in the mouse uterine tissue.[16] Thus, it was of interest to examine the antitumor influence of *o*-benzyl protected (α′ ring) triarylcyclopropyl antiestrogens by substituting a polar methoxy group on either the α phenyl ring (compound **5c**) or both the α and β rings (compound **4d**).

In the present study, the relative importance of the polar methoxy group was evaluated by comparing the antitumor activity of **4d** and **5c**. Further, the antitumor activity of the cyclopropyl compounds was compared with tamoxifen in order to determine the potential therapeutic effectiveness of these compounds in the treatment of human breast cancer. Antitumor activity of these compounds was evaluated by measuring antiproliferative activity on ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cells in the presence and absence of estradiol. In addition, ER binding and cell surface morphology was determined in MCF-7 cells as well as the antiproliferative activity on A-549 human lung cancer cells to assess antitumor specificity for breast cancer.

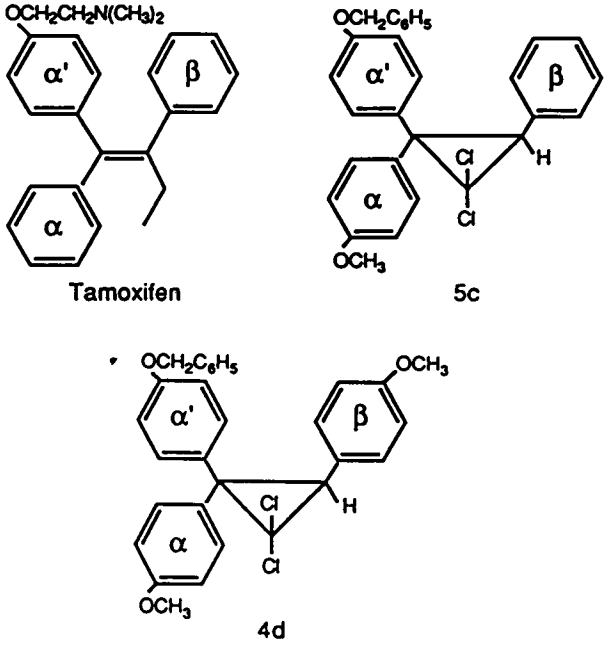

Figure 1. Chemical structures of compounds **4d**, **5c** and tamoxifen.

Materials and methods

Cell culture methods

The ER-positive MCF-7 human breast cancer cell line (passage #209) was obtained from the Michigan Cancer Foundation (Detroit, MI). 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 units/ml), streptomycin (100 μg/ml) and 5% calf serum containing a low level of estradiol as described previously.[19] Cultures were grown at 37°C in a humid 5% CO_2 atmosphere and fed on alternate days. When cultures reached confluence (usually at 7 days), they were subcultured using a 1:2 splitting ratio. Culture medium was changed on alternate days until the cells were confluent. In addition, the estrogen-dependent nature of these cells was characterized by their responsiveness to estradiol and tamoxifen and their estrogen receptor content as described earlier.[20]

The ER-negative MDA-MB-231 human breast cancer cell line (passage #37) and the A-549 human lung cancer cell line (passage #85) were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB-231 cells were grown under cell culture conditions which were similar to the MCF-7 cells except that Leibovitz L-15 medium supplemented with glutathione (16 mg/l) was used as the culture medium. The A-549 cell line was grown the same as MCF-7 cells, except for subculturing using a 1:3 splitting ratio every fifth day.

Cell proliferation studies

In each experiment the exponentially growing cells were trypsinized, counted and plated in multiwell plates at a density of 7.5×10^4 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 were dissolved in an absolute ethanol:polyethylene glycol

400 (45:55) mixture and added to the cell cultures following dilutions in culture medium. The final concentration of vehicle was 0.1% of the growth medium and shown not to alter cell growth.[21] Control wells received the same amounts of vehicle alone. The test medium was changed on alternate days for MCF-7 and MDA-MB-231 cell cultures, while it was changed daily for A-549 cell culture experiments. Exponentially growing cells were counted by hemocytometry on the scheduled days following addition of the experimental compounds using the Trypan blue exclusion method to determine cell viability as previously reported.[19,20] The antiproliferative activity of the test compounds was expressed as percent inhibition of control, which was calculated as follows:

$$\text{Antiproliferative activity} = \left(\frac{\text{viable cells}_{\text{control}} - \text{viable cells}_{\text{treated}}}{\text{viable cells}_{\text{control}}}\right) \times 100$$

ER relative binding activity (RBA) in MCF-7 cells

The MCF-7 cells were plated as described for the cell proliferation studies above and grown for 6 days in the growth medium and washed with Hanks' balanced salt solution. In order to determine the fraction of [^{3}H]estradiol specifically bound to ER, the cells, in triplicate wells, were incubated with 0.6 nM [^{3}H]estradiol (New England Nuclear, specific activity 92.5 Ci/mmol) with or without a 200-fold excess of DES in 0.4 ml of RPMI 1640 medium containing 0.1% bovine serum albumin for 60 min at 37°C. Parallel sets of wells were incubated with non-radioactive estradiol, tamoxifen, **4d** or **5c** at various concentrations over a range of 10^{-5} to 10^{-10} M. The bound [^{3}H]estradiol was extracted by incubating the cells with 1 ml of ethanol for 30 min at 22°C as described.[19,20] A 0.2 ml aliquot of the ethanol extract was transferred to 5 ml of liquid scintillation cocktail (Ready-Solv, Beckman) and counted on a liquid scintillation counter (Model LS 1801, Beckman). 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. The estradiol concentration which displaced 50% of [^{3}H]estradiol [$IC_{(50\ \text{estradiol})}$] served as the standard for estimation of RBA values, which were calculated as follows:

$$\text{RBA} = \frac{IC_{(50\ \text{estradiol})} \times 100}{IC_{(50\ \text{antiestrogen})}}$$

The $IC_{(50\ \text{antiestrogen})}$ is the concentration of antiestrogen (tamoxifen, **4d** or **5c**) that displaced 50% of the ER bound [^{3}H]estradiol.

Scanning electron microscopy (SEM)

Either MCF-7 or MDA-MB-231 cells were grown on coverslips placed in the bottom of six-well plates containing growth media. The cells were fixed with 2% glutaraldehyde in a phosphate buffer at pH 7.3, for 15 min at room temperature and then for 45 min at 4°C.[26] The coverslips were washed in media without calf serum. If required, the coverslips were stored at 4°C in 0.2 M sodium cacodylate buffer at pH 7.4. The coverslips were dehydrated through a graded series of ethanol (10–100%) before critical point drying. The coverslips were dried in carbon dioxide, mounted, grounded with silver colloids and shadowed with gold. The samples were then examined and photographed on a JEOL Model JSM-880 scanning electron microscope at 15 kV.

The density of microvilli (MV) on the cell surface was quantitated by counting the number of MV in five separate 1 μm^2 grids on the SEM photomicrographs as described previously.[19]

Statistics

Multiple group comparisons of the cell culture experiments were made by using either a one-way or two-way analysis of variance using the CLR ANOVA program (Clear Lake Research Incorporated Inc.) on a Macintosh Plus computer. Individual groups were compared using Duncan's new multiple range test. Group differences resulting in p values of less than 0.05 were considered to be significantly different.

Results

Antiproliferative activity

Compounds **4d**, **5c** and tamoxifen at a concentration of 10^{-6} M inhibited ($p < 0.05$) the growth of MCF-7 cells after 4 and 6 days of treatment, while estradiol (10^{-8} M) stimulated ($p < 0.05$) the growth of MCF-7 cells (Figure 2). In the dose-dependent study of antiproliferative activity, both compound **4d** and tamoxifen inhibited ($p < 0.05$) the growth of MCF-7 cells over the concentration range of 10^{-13} to 10^{-5} M (Figure 3). Compound **5c** inhibited MCF-7 cell proliferation ($p < 0.05$) over the concentration-range of 10^{-9} to 10^{-5} M.

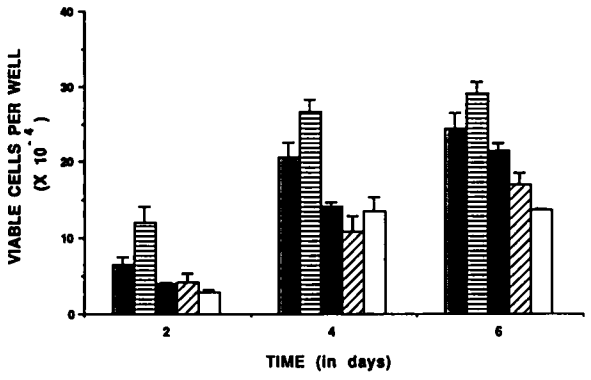

Figure 2. Effect of estradiol (10^{-8} M), **4d**, **5c** and tamoxifen (10^{-6} M) on the growth of MCF-7 cells, after 2, 4 and 6 days of treatment. Each points represents mean of duplicate samples ± SEM. ▨, Control; □, estradiol; ■, tamoxifen; ■, **4d**; □, **5c**.

In the study of estradiol-reversibility of antiestrogen antiproliferative activity on MCF-7 cells, compounds **4d**, **5c** or tamoxifen were administered at the concentration of 10^{-7} M for 4 days in the presence or absence of a 10-fold lower concentration of estradiol (10^{-8} M). As shown in Figure 4, estradiol (10^{-8} M) almost completely reversed the antiproliferative activity of tamoxifen. However, the same amount of estradiol caused a small, non-significant reversal of the anti-proliferative activity of compound **4d** and essentially no reversal of the activity of compound **5c**. Complete reversal of MCF-7 growth inhibition caused by compound **4d** was observed in the presence of an equimolar concentration of estradiol (10^{-7} M), while equimolar estradiol did not reverse the antiproliferative activity of compound **5c**.

Finally, neither compounds **4d**, **5c**, tamoxifen nor estradiol influenced the proliferation of ER-negative

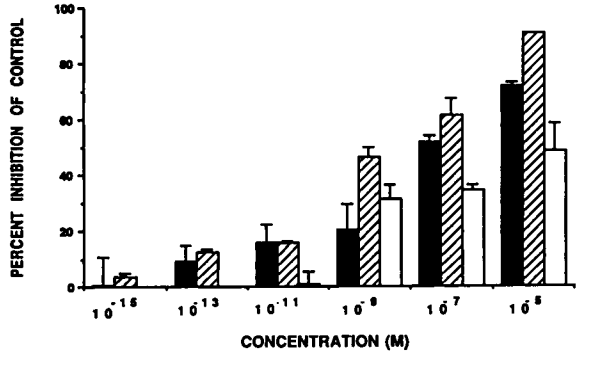

Figure 3. Dose-dependent antiproliferative activity of **4d**, **5c** and tamoxifen on MCF-7 cells. Each bar represents the mean of triplicate samples ± SEM. ▨, Tamoxifen; ■, **4d**; □, **5c**.

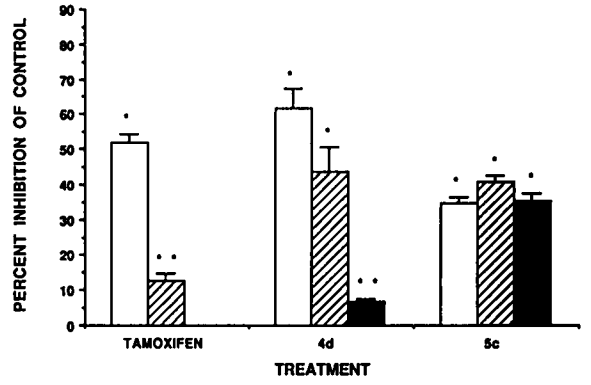

Figure 4. Antiproliferative activity of **4d**, **5c** and tamoxifen (10^{-7} M) in the presence or absence of estradiol (10^{-8} M) on MCF-7 cells. Each bar represent the mean of triplicate samples ± SEM. *Represents a significant ($p < 0.05$) antiproliferative treatment effect. **Represents a significant ($p < 0.05$) reversal of the antiproliferative treatment effect in the presence of estradiol. □, Compound; ■, compound + estradiol (10 nM); ■, compound + estradiol (100 nM).

MDA-MB-231 human breast cancer cells or human lung cancer A-549 cells (Table 1).

Cell surface morphology

The cell surface of control MCF-7 cells observed by SEM after 4 days of treatment was found to contain MV which were short, sparse and uniformly distributed (Figure 5A). Estradiol (10^{-8} M) increased the length and caused a 3-fold increase in the density of MV on the MCF-7 cell surface (Figure 5B). Compound **4d** (10^{-6} M) produced a marked reduction ($p < 0.05$) in MV (Figure 5C), while compound **5c** (10^{-6} M) caused only a small (20%) but significant ($p < 0.05$) decrease in the density of MV on the

Table 1. Influence of estradiol, **4d** and **5c** and tamoxifen on the growth of MDA-MB-231 ER-negative human breast cancer cells and A-549 human lung cancer cells

Treatment	Viable cells per well ± SEM	
	MDB-MB-231 cells[a] (×10⁴)	A-549 cells[b] (×10³)
Control	70.7 ± 2.7	98.0 ± 3.5
Estradiol (10^{-7} M)	68.7 ± 4.4	105.6 ± 2.8
Tamoxifen (10^{-6} M)	65.3 ± 6.1	120.0 ± 5.0
4a (10^{-6} M)	72.5 ± 3.6	102.0 ± 6.5
5c (10^{-6} M)	73.6 ± 5.2	106.0 ± 3.6

[a]MDA-MB-231 cells grown in six-well plates were counted on the fourth day of treatment.
[b]A-549 cells grown in 12-well plates were counted on the second day of treatment.

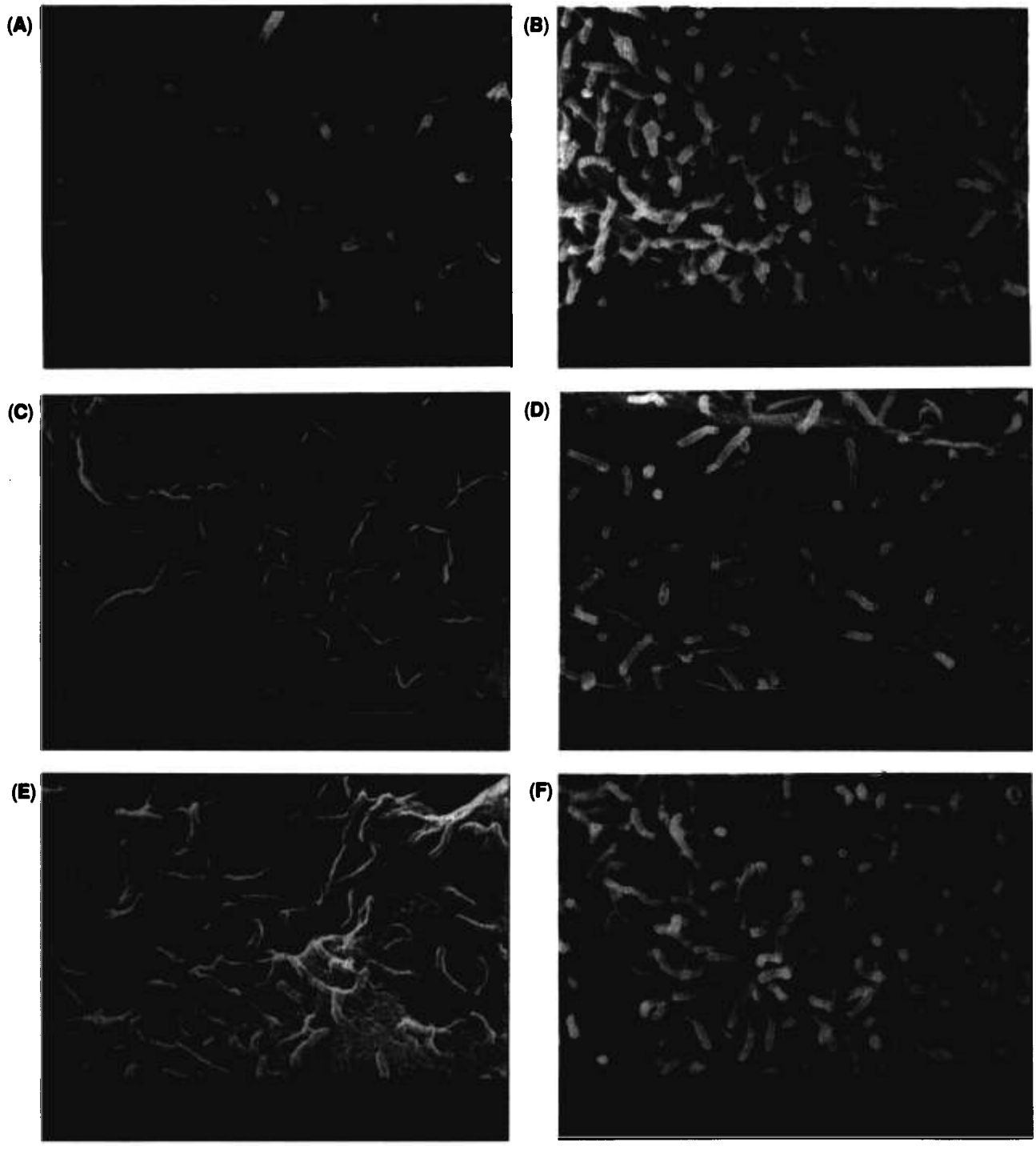

Figure 5. Scanning electron micrographs of MCF-7 cells (×24 000). (A) Control-treated with vehicle. (B) Treated with 10^{-8} M estradiol. (C) Treated with 10^{-6} M compound **4d**. (D) Treated with 10^{-6} M compound **5c**. (E) Treated with 10^{-6} M **4d** and 10^{-8} M estradiol. (F) Treated with 10^{-6} M **5c** and 10^{-8} M estradiol.

MCF-7 cell-surface, particularly in the central region, leading to a non-uniform distribution (Figure 5D).

Co-administration of estradiol (10^{-8} M) partially reversed the effects of compounds **4d** and **5c** on the density of cell surface MV (Figure 5E and F, respectively). However, the density, length and uniformity of MV in cells treated with **4d** and **5c** in the presence of estradiol was less ($p < 0.05$) than that observed with estradiol alone.

The MDA-MB-231 cells contained sparse, short and uniformly distributed MV. In this study, neither estradiol, **4d** nor **5c** altered the cell surface morphology of ER-negative MDA-MB-231 cells (data not shown).

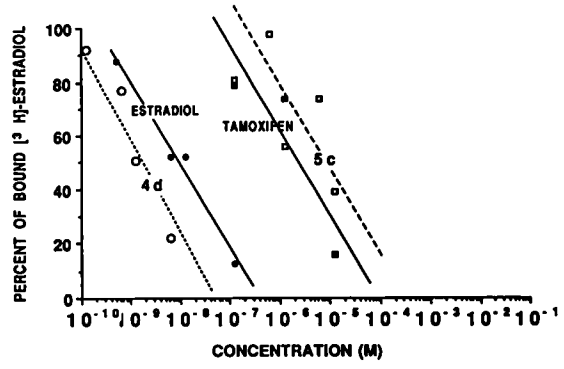

Figure 6. [^{3}H]Estradiol displacement curves for estradiol, **4d**, **5c** and tamoxifen from ER in MCF-7 cells. [^{3}H]Estradiol displacement was measured using the whole cell assay method. Each point represents the mean of triplicate samples. ●, Estradiol; ■, tamoxifen; ○, **4d**; □, **5c**.

ER RBA

The RBA and IC_{50} values for **4d**, **5c** and tamoxifen, presented in Table 2, were derived from the displacement curves (Figure 6). The results indicate an IC_{50} of 2.0 nM, 10.0 μM and 2.60 μM and an RBA of 500, 0.10 and 0.38 for **4d**, **5c** and tamoxifen, respectively. Thus, **4d** was found to have ~1300- to 5000-fold greater binding affinity for the ER of MCF-7 cells than tamoxifen and **5c**, respectively, in this study.

Discussion

Compounds **4d** and **5c** are members of a distinct chemical class of triarylcyclopropyl compounds which have been shown to be antiestrogens without estrogen agonist activity.[15,16] Therefore, the purpose of the present study was (i) to determine the potential therapeutic use of **4d** and **5c** in the treatment of breast cancer, and (ii) to establish the influence of a polar methoxy group substitution on the α and β rings in this triarylcyclopropyl series by comparing the antiproliferative activities of these two compounds. Tamoxifen and estradiol were used as standard antiestrogen and estrogen, respectively, in the evaluation of MCF-7 cell proliferation responsiveness since other investigators have reported a loss of ER and estrogen-mediated responsiveness in MCF-7 cells.[11,12] Accordingly, estradiol (10^{-8} M) stimulated and tamoxifen (10^{-6} M) inhibited the growth of MCF-7 cells as previously reported.[12,15]

Table 2. ER binding affinity

Treatment	IC_{50} (10^{-6} M)[a]	RBA
Estradiol	0.010	100.00
Tamoxifen	2.600	0.38
4d	0.002	500.00
5c	10.000	0.10

[a]The concentration of non-radiolabeled compound which displaced 50% [^{3}H]estradiol from ERs.

Antiproliferative experiments done with tamoxifen, **4d** and **5c** on MCF-7 cells indicate that all three antiestrogens inhibit cell growth; although **4d** was found to be the most potent inhibitor. Interestingly, in the estradiol reversibility experiments the antiproliferative activity caused by 10^{-7} M **5c** was not reversed; the activity of 10^{-7} M **4d** was only slightly reversed and the activity of 10^{-7} tamoxifen was completely reversed by 10^{-8} M estradiol. The exact reason for this lack of estradiol reversal with **5c** and **4d** is not completely clear; however, the extremely high binding affinity of **4d** for MCF-7 ER observed in this study would indicate that a greater concentration of estradiol would be required to cause reversal of the antiproliferative effects of this compound. Accordingly, 10^{-7} M estradiol caused a significant inhibition of **4d** antiproliferative activity. Further, since **4d** and **5c** bound specifically to the ER in MCF-7 cells and did not influence the growth of ER-negative MDA-MB-231 cells, it appears that the antiproliferative effects of these compounds on MCF-7 cells is largely ER mediated.

Alternatively, it is possible that one or both of these compounds are acting on the MCF-7 cells by an ER-independent mechanism. For example, it has been demonstrated that tamoxifen and other antiestrogens may interfere with cell proliferation by mechanisms other than direct ER binding, such as nuclear events including ER replenishment,[22] bind-

ing to a separate antiestrogen binding site (AEBS),[23] inhibition of the activity of calmodulin and protein kinase C,[24,25] and inhibition of growth factor activity in breast cancer cells.[26,27] Accordingly, future studies will examine other possible mechanisms of antiproliferative activity for these cyclopropyl compounds on breast cancer cells.

It has been shown that estradiol increases the length and density of MV on the surface of MCF-7 cells while tamoxifen decreases the estradiol-induced effects on MV.[28,29] In the present study, **4d** treatment decreased the length and density of MV on the surface of the MCF-7 cells while neither compound altered the MV on MDA-MB-231 cells. As in the antiproliferative experiments, **4d** was found to be more potent than **5c** in producing these ultrastructural changes. The influence of **4d** on the MV of MCF-7 cells was significantly reversed upon co-administration of estradiol. Thus, the cell-surface activity of these compounds appears to be ER dependent.

Considering the chemical structures of **4d** and **5c** as shown in Figure 1, it is clear that both compounds have a non-polar *p*-benzyloxy substituent on the α′ phenyl ring which is electron-rich at its distal position[15] and thus similar to the dialkylaminoethoxy side-chain on tamoxifen.[30] Likewise, the *p*-benzyloxy substituent on the α′ phenyl ring of the triarylcyclopropanes has been reported to be necessary for antiestrogenic activity.[15]

In a previous study, the antiestrogenic activities seen with **4d** and **5c** support the finding that a dialkylaminoethoxy side-chain is not always required for antagonism of estradiol-induced uterine weight gain.[15] A non-polar substituent at the *para* position of the α′ ring may still elicit antagonism as evidenced by the antiestrogenic activity of the α′ ring ethylated triarylethylene broparestral.[31] Removal of the benzyl groups from **4d** and **5c** to yield the corresponding phenols resulted in a complete loss of antiestrogenic activity.[15]

The antiproliferative activity observed in the present study clearly supports the previously reported hypothesis that the *p*-benzyloxy side-chain on the α′ phenyl ring, like the dialkylaminoethoxy side-chain of tamoxifen, is important for antiestrogenic activity and that a polar methoxy group on the α phenyl ring acts as an ER anchoring group, similar to the C3 phenolic hydroxyl group of estradiol.[15] Further, the polar group substitution at the β phenyl ring of the cyclopropyl compounds is reported to contribute to ER binding in rat uterine tissue.[16] Consistent with this finding, **4d** with a *p*-methoxy polar substituent on the β phenyl ring was found to have a much higher ER binding affinity than **5c** which has an unsubstituted β phenyl ring. Accordingly, compound **4d** was much more potent than compound **5c** in reducing MCF-7 cell proliferation and cell-surface morphology, which is consistent with the notion that these antitumor effects are ER mediated. In addition, these findings are consistent with the concept that ER binding affinity of an antiestrogen is related to its antiproliferative effect on breast cancer cells.[32]

In conclusion, the results of the present study demonstrate potent antitumor activity of the cyclopropyl compounds which is specific to ER-positive human breast cancer MCF-7 cells. Further, this study clearly indicates that the introduction of a polar methoxy group at the β phenyl ring of the triarylcyclopropane antiestrogens leads to an increase in antitumor activity on hormone-dependent cells. Moreover, the results suggest that this structure-related activity is a consequence of higher ER-binding affinity due to the presence of a *p*-benzyloxy side chain on the α′ ring and the polar methoxy group in the β phenyl ring. Finally, since both **4d** and **5c** have been shown to be pure antiestrogens and potent inhibitors of breast cancer cell growth, these compounds may be very effective in the treatment of breast cancer and/or the prevention of breast cancer in patients with a high risk of estrogen-dependent tumor development.

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