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Antitumor activity of a novel antiestrogen (Analog II) on human breast cancer cells

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Analog II (1,1-dichloro-*cis*-2,3-diarylcyclopropane), previously shown to be a pure antiestrogen in mice, was examined for potential antitumor activity on human breast cancer cells in culture. In this study, Analog II produced a dose-related antiproliferative effect on the growth of estrogen receptor (ER)-positive MCF-7 human breast cancer cells over a concentration range of 10^{-11} to 10^{-5}M. Analog II increased the fraction of MCF-7 cells in the G_2/M phase of the cell cycle. Further, this compound inhibited the growth of ER-negative MDA-MB-231 human breast cancer cells over a concentration range of 10^{-9} to 10^{-6}M. Using scanning electron microscopy to evaluate drug-induced changes in cellular morphology, it was observed that Analog II decreased the length and density of microvilli on both MCF-7 and MDA-MB-231 cells. The effects of Analog II on MCF-7 and MDA-MB-231 cell proliferation and morphology were not reversed in the presence of estradiol. In addition, the induction of estrogen-dependent genes in MCF-7 cells was not reversed by Analog II. It was observed that non-specific cytotoxicity may be responsible for part of the Analog II-induced inhibition on MCF-7 and MDA-MB-231 cell proliferation. However, the antitumor activity of this compound was found to be specific to human breast cancer cells since it did not alter the proliferation or viability of non-breast A-549 human lung cancer cells. In conclusion, these results indicate that Analog II is a potent antitumor agent, has a unique antitumor mechanism in breast cancer cells and may be effective in the treatment of breast cancer.

Key words: Analog II, anticancer drug, breast cancer, cyclopropyl antiestrogens, MCF-7, MDA-MB-231.

Introduction

Tamoxifen is a non-steroidal antiestrogen which is used in the treatment of hormone-dependent breast cancer in postmenopausal woman and as an adjunct therapy in premenopausal woman.[1] Although this compound is clinically effective and relatively safe, tamoxifen is known to produce partial estrogen agonist activity both *in vivo* and *in vitro*.[2] The estrogen agonist activity of tamoxifen is of clinical value in the prevention of bone loss and lowering low-density lipoprotein cholesterol levels;[1] however, there are also important disadvantages. For example, in MCF-7 human breast cancer cells tamoxifen produces partial estrogen agonist activity by stimulating the expression of progesterone receptor mRNA and other estrogen-dependent genes.[3-5] The estrogen agonist activity of tamoxifen is reported to be associated with undesirable side effects in breast cancer patients such as stimulation of ovarian estrogen production and an increased incidence of endometrial carcinoma.[6,7] Generally, tamoxifen is tumoristatic to MCF-7 cells in nude mice.[8] However, prolonged tamoxifen exposure can lead to tamoxifen-resistance[9] and tamoxifen-stimulated tumors.[10] Prolonged tamoxifen treatment in breast cancer patients is known to ultimately fail, since breast cancer is a polyclonal disease and while estrogen receptor (ER)-positive cell growth is inhibited by tamoxifen, the estrogen-independent cancer cells continue to grow.[1] Thus, antiestrogens devoid of estrogen agonist activity should provide an effective

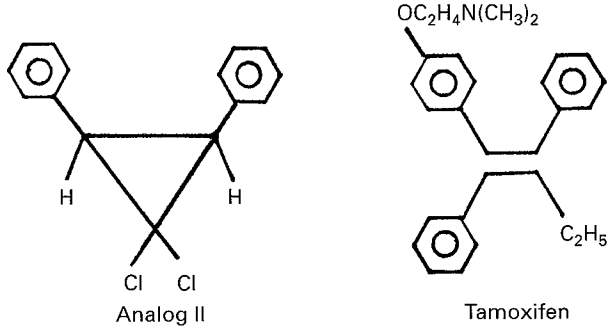

Figure 1. Chemical structure of tamoxifen and Analog II.

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strategy in the treatment and/or prevention of breast cancer.

Search for a pure antiestrogen led to the development of a novel series of cyclopropyl antiestrogens. Analog II (Figure 1; 1,1-dichloro-*cis*-2,3-diarylcyclopropane) was found to produce no detectable estrogen agonist activity in mouse uterine tissue and bound specifically to the rat uterine cytosolic ER.[11] Biodistribution studies with [^{3}H]Analog II in the mouse and rat indicate that this compound is taken up specifically and concentrated in estrogen-dependent tissue such as the uterus.[12]

Studies with Analog II in DMBA-induced mammary tumors in the rat demonstrated an inhibition of tumor growth to approximately the same extent as ovariectomy.[13] Further, Analog II was observed to be more effective than tamoxifen in protecting animals against the development of new DMBA-induced breast tumors during an 8 week treatment period.[13] In a separate study, Analog II was more effective than tamoxifen in reducing the incidence of animals with mammary tumors and the total tumor burden when administered 1 week before and 1 week after DMBA.[14] In a study using the DMBA-4 transplantable metastatic rat mammary tumor model, Analog II prevented metastatic tumor development for up to 1 month and tamoxifen for 3 weeks, following cessation of treatment.[15]

Because our previous studies have demonstrated that Analog II is a pure antiestrogen in the mouse[11] and inhibited the growth of DMBA-induced tumors in the rat,[13-15] the purpose of the present study was to examine the action of Analog II at the cellular level in human breast cancer cells and to evaluate its potential therapeutic usefulness. Thus, the antitumor action of Analog II was examined in this study using both the ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cell lines.

Materials and methods

Test compounds

Estradiol was obtained from Sigma (St Louis, MO). The antiestrogen tamoxifen was obtained from ICI America (Wilmington, DE), and Analog II from Wako Pure Chemicals (Richmond, VA). The test compounds were dissolved in an absolute ethanol:polyethylene glycol 400 (45:55) mixture and added to the cell cultures following dilutions in the culture medium. The final concentration of vehicle used was 0.1% of the growth medium as this concentration did not alter cell growth.[16] Control treatments received the same amounts of vehicle alone.

Cell culture methods

The ER-positive MCF-7 and MCF-7/E-3 human breast cancer cell lines were obtained from the Michigan Cancer Foundation (Detroit, MD). The ER-negative MDA-MB-231 human breast cancer cell line and A-549 human lung cancer cell line were obtained from the ATCC (Rockville, MD). MCF-7 and A-549 cells were grown 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 (5.4 pg/ml). Cultures were grown at 37°C in a humid 5% CO_2 atmosphere and fed on alternate days. These culture conditions were found to be optimal for the evaluation of antiestrogenic compounds in MCF-7 cells.[17]

MDA-MB-231 cells were grown under the same culture conditions except that Leibovit'z L-15 medium supplemented with glutathione (16 mg/l) was used instead of RPMI 1640.

Cell proliferation studies

Exponentially growing cells were trypsinized, counted and plated in multiwell plates at a density of 7.5×10^4 cells/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. 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 hemocytometer at the time indicated following addition of the experimental compounds using the Trypan blue exclusion method to quantify cell viability. The antiproliferative activity of the test compounds was expressed as percent inhibition of control, which was calculated as follows:

$$\text{Antiproliferative activity} = \frac{(\text{Viable cells}_{\text{control}} - \text{Viable cells}_{\text{treated}})}{\text{Viable cells}_{\text{controls}}} \times 100$$

The cytotoxicity of each compound was determined on the basis of treatment-induced cell viability and calculated as follows:

$$\text{Cytotoxicity} = \frac{(\%\ \text{cell viability}_{\text{control}} - \%\ \text{cell viability}_{\text{treated}})}{\%\ \text{cell viability}_{\text{control}}} \times 100$$

In the calculation of cytotoxicity the percent cell viability is the viable cell count divided by the total cell count (sum of viable cells and dead cells) in each well.

ER relative binding activity (RBA)

The MCF-7 cells were plated as described for the cell proliferation studies above. The cells were 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, Boston, MA; 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 triplicate wells were incubated with non-radioactive estradiol, tamoxifen or Analog II at various concentrations. The bound [^{3}H]estradiol was extracted by incubating the cells with 1 ml of ethanol for 30 min at 22°C as described.[18] A 0.2 ml aliquot of the ethanol cell extract was transferred to 4 ml of liquid scintillation cocktail and counted by liquid scintillation spectrometry. Specific bound [^{3}H]estradiol was determined by subtracting non-specific bound [^{3}H]estradiol (obtained in presence of DES) from the total bound [^{3}H]estradiol. The RBA values were calculated as follows:

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

$\text{IC}_{(50\ \text{estradiol})}$ is the concentration of estradiol which displaced 50% of [^{3}H]estradiol. $\text{IC}_{(50\ \text{antiestrogen})}$ is the concentration of antiestrogen (tamoxifen or Analog II) 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 six-well plates containing growth media. The cells were treated with antiestrogen or vehicle control for 4 days. Then the cells were fixed with 2% glutaraldehyde in a phosphate buffer at pH 7.3, as previously described.[17] 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 in CO_2. The coverslips were next 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 on the cell surface was quantitated by counting the number of microvilli in five separate 1 μm^2 grids on the SEM photomicrographs at $\times 30\ 000$.

Flow cytometry

Approximately 75 000 MCF-7 cells/well were seeded in 12-well plates and allowed to attach for 2 days. The growth medium was replaced with the media containing 10^{-6} M test compounds or diluent. Following a 2 day treatment period, a single cell suspension was obtained by trypsinizing the cells and passing the cells through a 22 gauge needle. The cells were fixed in 70% ethanol for 30 min at room temperature and then stored at 4°C until assayed by flow cytometry. The cells were pelleted and resuspended in 200 μl of cold HBSS. Following addition of 5 μl of RNase stock solution (150 400 units or 1.69 mg/ml of HBSS) the cells were incubated for 30 min at 37°C. Finally, the cells were stained with 10 μl of propidium iodide (1.8 mg/ml of HBSS) and DNA histograms were obtained on a Coulter, Epic IV flow cytometer as previously described.[19]

Northern analysis

Total RNA isolation. Highly estrogen responsive MCF-7/E-3 cells were used in these experiments. After a 24 h treatment of the cells with estradiol (10^{-9} M) or antiestrogens (10^{-6} M), total cellular RNA was isolated using RNAzol (Biotex, Houston, TX), according to the manufacturer's protocol. The amount of RNA was quantified by absorbance at 260 nm. Approximately 30-40 μg of total RNA was separated on a 1.2% agarose-6% formaldehyde denaturing gel and transferred to a GeneScreen Plus nylon membrane (New England Nuclear, Boston, MA) by capillary action. Transferred RNA was fixed to the membrane by baking at 80°C under continuous vacuum for 2 h.

Preparation of DNA Probes for Northern analysis. The cDNA for pS2 was obtained from ATCC and the cDNA for cath-D has been described.[20] Complementary DNA inserts were isolated from respective vector sequences by restriction enzyme digestion (*Pst*I for pS2; *Xba*I and *Sal*I for cath-D), following which the fragments were purified by agarose gel electrophoresis and recovered using the GeneClean II Kit (Bio 101, LaJolla, CA).

pS2, cath-D and β-actin (1.1 kb human cDNA) cDNA

probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL), using a random primer labeling kit (United States Biochemical, Cleveland, OH). Radiolabeled probes were purified using G-50 Sephadex spin columns (Boehringer Mannheim, Indianapolis, IN) and the probe used at a specific activity of $2\text{-}8 \times 10^8$ c.p.m./μg DNA.

Northern blot hybridization. The membrane was prehybridized at 65°C overnight in prehybridization/hybridization buffer [1% bovine serum albumin, 0.55 M sodium phosphate buffer, pH 7.2, 1 mM (EDTA), 7% SDS and 100 μg/ml denatured salmon sperm DNA]. Hybridization was performed at 65°C overnight in the aforementioned buffer, containing $2\text{-}8 \times 10^8$ c.p.m./μg probe. Following hybridization, the blots were washed twice at room temperature for 15 min each in wash I (1% BSA, 40 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA and 2% SDS). They were then washed at 55°C for 15 min in wash II (0.05% BSA, 40 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA and 0.4% SDS). The washed membranes were exposed to XAR film (Eastman Kodak, Rochester, NY). The resultant autoradiographs were analyzed on a laser densitometer (Ultrascan XL; LKB Instruments, Bromma, Sweden). Message size was determined by comparison to a RNA ladder (0.24-9.6 kb; Bethesda Research Laboratories, Gaithersburg, MD). Cath-D and pS2 mRNA levels were determined on the same blot which was subsequently stripped of the probe and reprobed with β-actin. Cath-D and pS2 were normalized relative to levels of the constitutively expressed β-actin mRNA in each lane.

Data analysis

Multiple group comparisons of the cell culture experiments were made using either a one-way or two-way ANOVA. 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 on MCF-7 cells

Analog II and the experimental antiestrogen standard, tamoxifen, at a concentration of 10^{-6} M inhibited the growth of MCF-7 cells on treatment days 4 and 6, while estrogen standard, estradiol, at a concentration of 10^{-8} M, stimulated the growth of MCF-7 cells (Figure 2). Tamoxifen and Analog II were tested over a concentration range of 10^{-11} to 10^{-5} M to compare their dose-related antiproliferative activity on MCF-7 cells, on the fourth day of treatment. Both compounds produced a dose-dependent inhibition of the proliferation of MCF-7 cells. In these experiments, Analog II was found to be a more potent inhibitor of cell proliferation than tamoxifen (Figure 3).

In a separate study with MCF-7 cells, co-administration of estradiol (10^{-8} M) reversed the antiproliferative activity produced by 10^{-7}M tamoxifen; however, estradiol (either 10^{-8} or 10^{-7} M) did not significantly alter the antiproliferative effect produced by 10^{-7} M Analog II (Table 1).

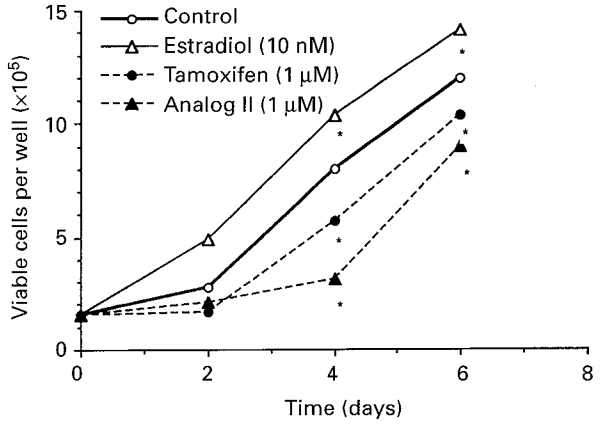

Figure 2. Influence of estradiol, tamoxifen and Analog II on the proliferation of MCF-7 cells at 2, 4 and 6 days of treatment. Each point represents the mean of duplicated samples ± SEM. *Indicates that the group mean was significantly different ($p < 0.05$) from the control group.

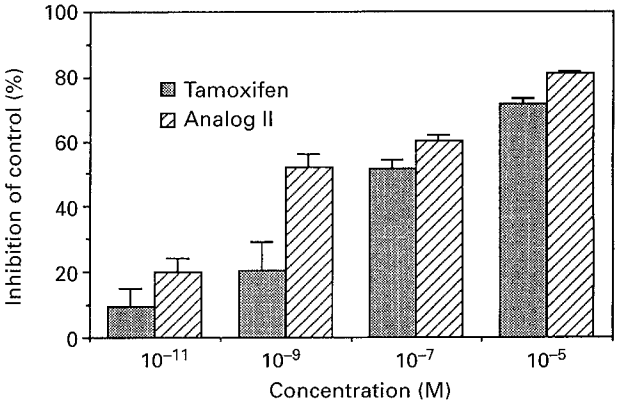

Figure 3. Dose–antiproliferative response (percent inhibition of control) of tamoxifen and Analog II on the proliferation of MCF-7 cells at 4 days of treatment. Each bar represents mean of triplicate samples ± SEM. *Indicates that the group mean is significantly different ($p < 0.05$) from the control group.

Table 1. The influence of estradiol co-administration on tamoxifen- and Analog II-induced antiproliferative activity of MCF-7 cells

Treatment	Inhibition of control[a] (%)
Tamoxifen (10^{-7} M)	51.80 ± 2.40[b]
Tamoxifen (10^{-7} M) + estradiol (10^{-8} M)	12.70 ± 2.00[b,c]
Analog II (10^{-7} M)	59.64 ± 4.55[b]
Analog II (10^{-7} M) + estradiol (10^{-8} M)	48.97 ± 5.65[b]
Analog II (10^{-7} M) + estradiol (10^{-7} M)	55.20 ± 7.50[b]

[a]Mean of triplicate viable cell-counts per well expressed as percent inhibition of control ± SEM.
[b]Statistically different ($p < 0.05$) from the control group.
[c]Statistically different ($p < 0.05$) from the tamoxifen-treated group.

Antiproliferative activity on MDA-MB-231 cells

Analog II (10^{-6} M) inhibited the proliferation of MDA-MB-231 cells on days 2, 4 and 6 of treatment (Figure 4). As expected, neither estradiol (10^{-7} M) nor tamoxifen (10^{-6} M) altered the growth of these ER-negative cells. In a dose-response study, with observations made on the fourth day of treatment, Analog II inhibited the growth of MDA-MB-231 cells over a concentration range of 10^{-9} to 10^{-6} M (Figure 5).

Cytotoxic activity on breast and non-breast cancer cell lines

Tamoxifen (10^{-7} M) inhibited the proliferation of MCF-7 cells but was not cytotoxic (Figure 6). Further, tamoxifen produced neither antiproliferative activity nor cytotoxicity, on either MDA-MB-231 human breast or A-549 human lung cancer cells. However, Analog II (10^{-7} M) produced a cytotoxic effect on both MCF-7 and MDA-MB-231 cells. The degree of antiproliferative activity and cytotoxicity produced by Analog II on the MDA-MB-231 cells was approximately the same, while the degree of antiproliferative activity of Analog II on MCF-7 cells was much greater than the cytotoxic effect. However, on the A-549 human lung cancer cell line, Analog II produced neither antiproliferative activity nor cytotoxicity.

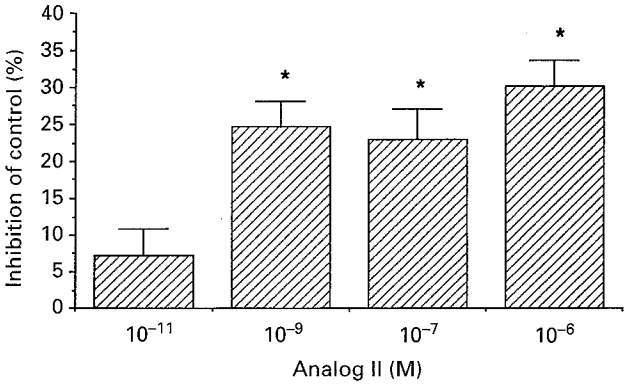

Figure 5. Dose–antiproliferative response (percent inhibition of control) of Analog II on the proliferation of MDA-MB-231 cells at 4 days of treatment. Each hatched bar represents the mean of triplicate samples ± SEM. *Indicates that the group mean is significantly different ($p < 0.05$) from the control group.

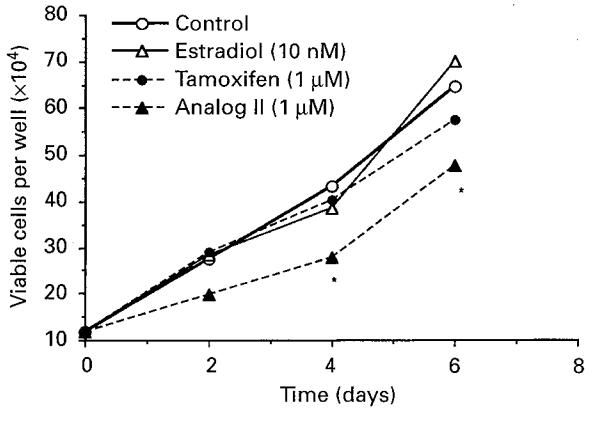

Figure 4. Influence of estradiol, tamoxifen and Analog II on the proliferation of MDA-MB-231 cells at 2, 4 and 6 days of treatment. Each points represents mean of triplicate samples ± SEM. *Indicates that values were significantly different ($p < 0.05$) from the control group.

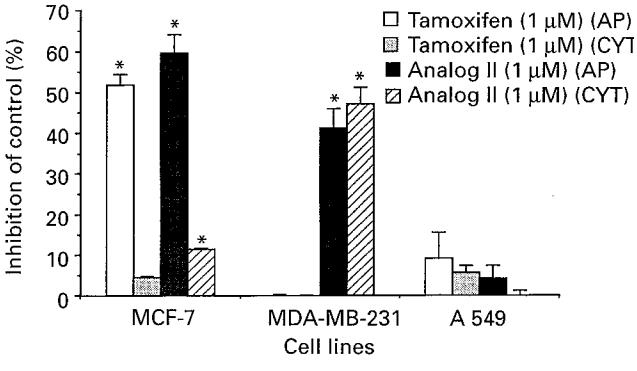

Figure 6. Comparison of antiproliferative activity (AP) and cytotoxicity (CYT) of tamoxifen and Analog II on MCF-7, MDA-MB-231 (human breast) and A-549 (human lung) cancer cell lines. Each bar represents the mean of triplicate samples ± SEM. *Indicates that the group mean is significantly different ($p < 0.05$) from the control group.

Effects on the ultrastructure of MCF-7 and MDA-MB-231 cells

The cell surface of MCF-7 human breast cancer cells was examined for ultrastructural alterations associated with a 4 day treatment of either estradiol (10^{-8} M), tamoxifen (10^{-6} M) or Analog II (10^{-6} M). In the control MCF-7 cells the cell surface microvilli (MV) were found to be short and uniformly distributed (Figure 7A). The Analog II-treated MCF-7 cells contained very few MV on the cell surface; however, some MV were observed on the peripheral surfaces of the cells (Figure 7B). In contrast, tamoxifen treatment did not alter the density of MV on MCF-7 cells (data not shown). However, the length and density of MV on MCF-7 cells was increased by estradiol (Figure 7C), as previously reported.[21,22] Co-administration of estradiol did not alter Analog II-induced changes in MV (Figure 7D).

In the control MDA-MB-231 cells, the cell surface MV were also found to be short and uniformly distributed (Figure 8A). In MDA-MB-231 cells, neither estradiol nor tamoxifen treatment altered either the length or density of cell surface MV (data not shown). However, Analog II treatment caused a significant reduction in the density of MV on MDA-MB-231 cells (Figure 8B) which was not altered by estradiol co-administration (data not shown).

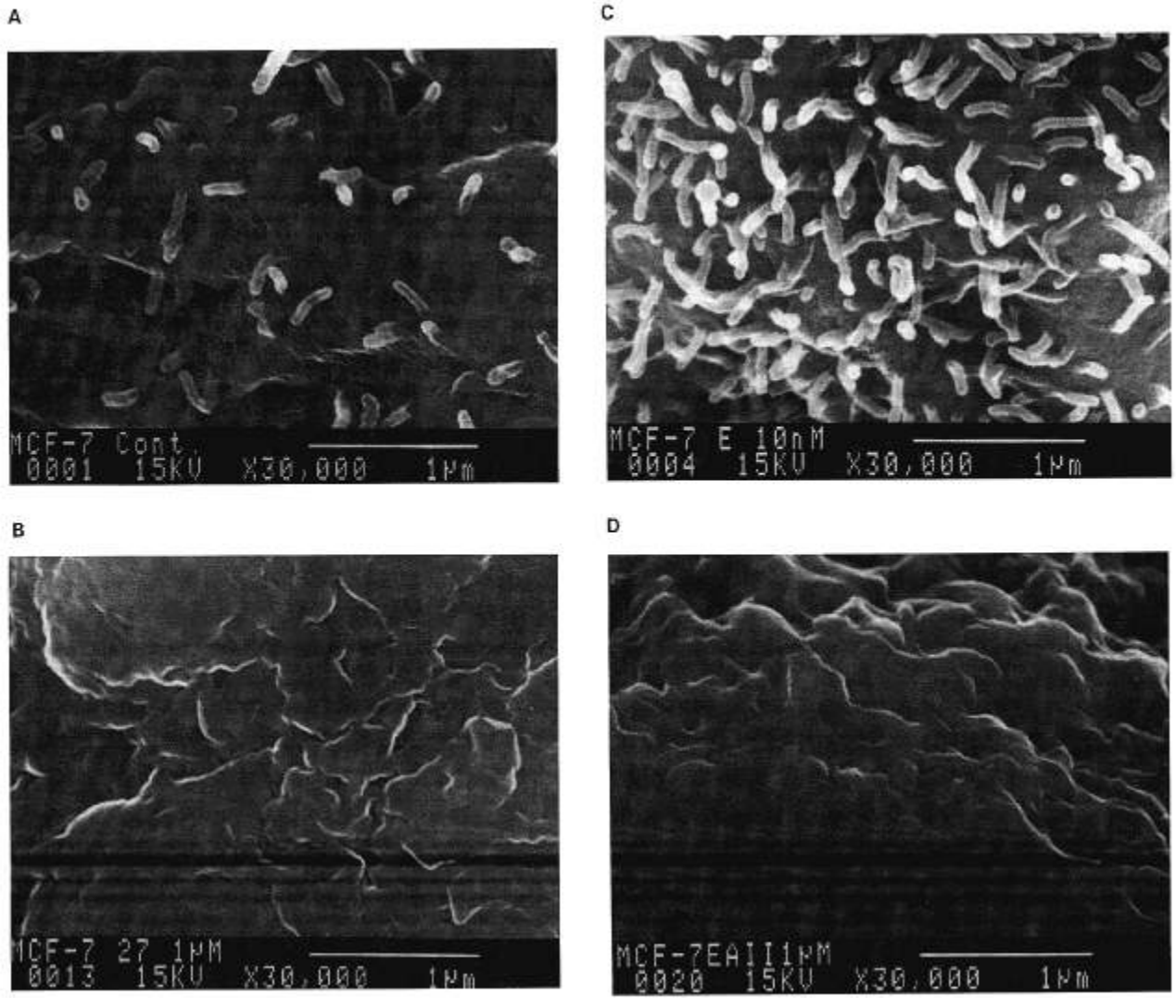

Figure 7. Scanning electron micrographs of MCF-7 cells at × 30 000. (A) Control—treated with vehicle; (B) Analog II (10^{-6} M) treated; (C) estradiol (10^{-8} M) treated; (D) Analog II (10^{-6} M) + estradiol (10^{-8} M) treated.

RBA

Figure 9 illustrates the [^{3}H]estradiol displacement curves produced by estradiol, tamoxifen and Analog II. In this study, the RBA values for tamoxifen and Analog II, derived from the displacement curves, were 0.5 and 0.04, respectively. These results indicate that Analog II has an ER binding affinity which is approximately 1/10 that of tamoxifen.

Cell cylce kinetic effects

The influence of Analog II on cell cycle kinetics of MCF-7 cells is illustrated in Figure 10. In these experiments Analog II treatment (10^{-6} M) decreased the fraction of cell in G_1 and S phases, and increased the percentage of cells in the G_2/M phase of the cell cycle ($p<0.05$), while tamoxifen increased the fraction of cell in the G_1 phase.[19]

Effects on pS2 and Cath-D gene expression

The effects of Analog II and estradiol (4 day treatment) on total cellular pS2 and cath-D mRNA in MCF-7 cells is shown in Figure 11. Estradiol (10^{-9} M) produced a 1.5-fold increase in pS2 and a 1.6-fold increase in cath-D mRNA. Analog II (10^{-6} M) caused a sight increase in pS2 and cath-D mRNA, but did not alter estradiol-induced increase in the mRNA levels of these genes. In control experiments with ER-deficient Chinese hamster lung cells, pS2 mRNA expression was undetect-

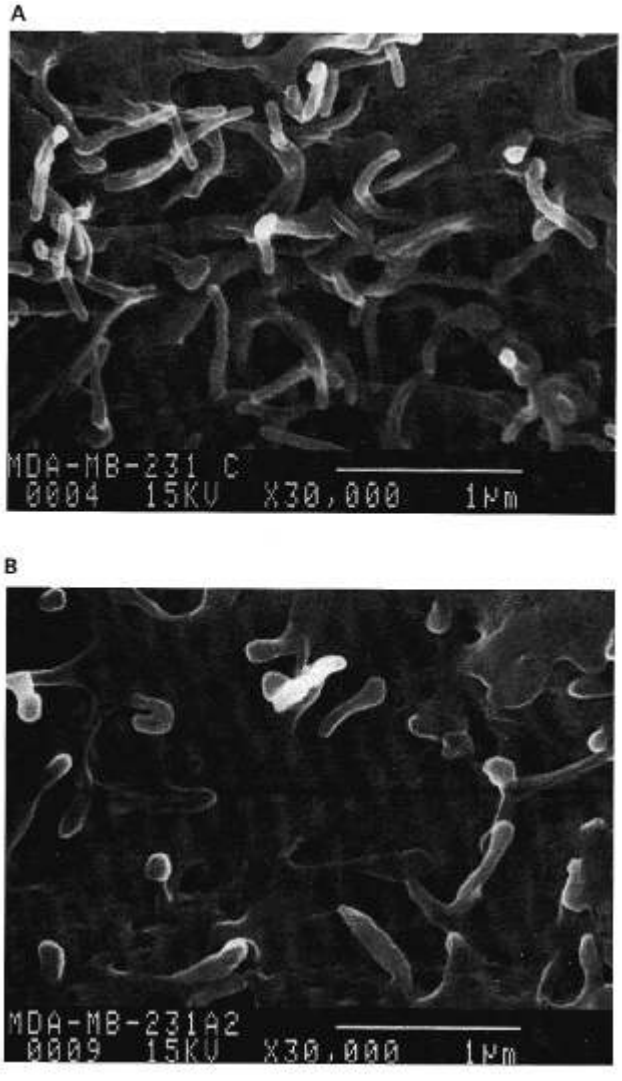

Figure 8. Scanning electron micrographs of MDA-MB-231 cells at ×30 000. (A) Control - treated with vehicle; (B) Analog II (10^{-6} M) treated.

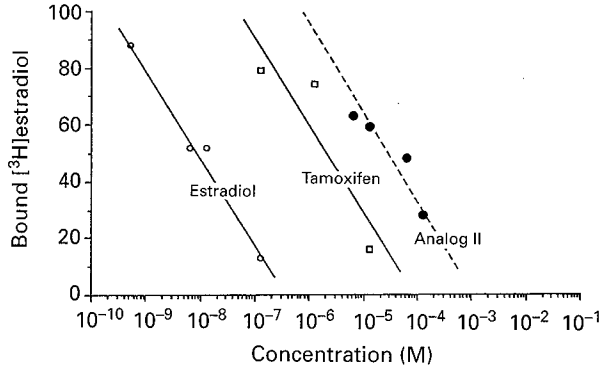

Figure 9. RBA of estradiol, tamoxifen and Analog II for ER in MCF-7 cells. Each point represents the mean of triplicate samples.

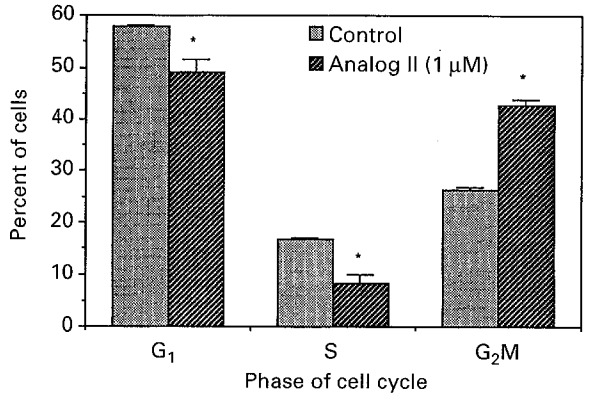

Figure 10. Influence of Analog II on the cell cycle distribution of MCF-7 cells. Each point represents the mean of triplicate samples ± SEM. *Indicates that the group mean is significantly different ($p<0.05$) from the control group.

able in both control and estradiol-treated cultures (data not shown).

Discussion

In previous studies, we have demonstrated that Analog II is an antiestrogen which binds specifically to the rat uterine cytosolic ER[11] and concentrates in estrogen-dependent tissues.[12] Analog II was found to be as effective as tamoxifen in inhibiting the growth of estrogen-dependent DMBA-induced tumors and more effective in preventing the development of new tumors during the treatment period.[13,14] Therefore, the major objective of the present study was to characterize the activity and the mechanism of action of Analog II in ER-positive MCF-7, human breast cancer, cells in culture. Based on the result of our previous studies, it was our hypothesis that Analog II would inhibit the proliferation of breast cancer cells by an ER-mediated mechanism.

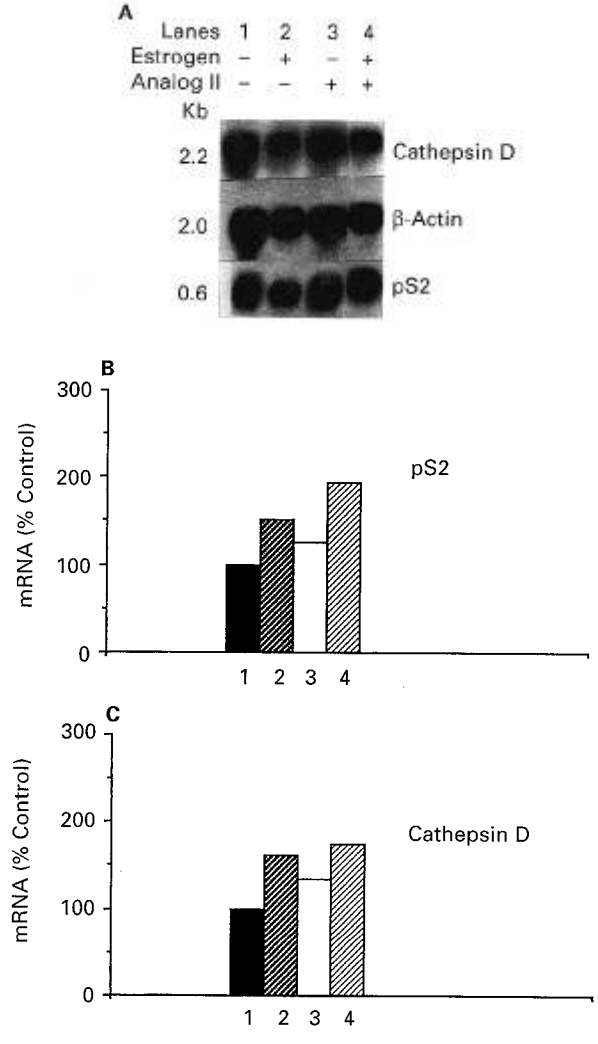

Figure 11. Influence of estradiol and Analog II on the expression of pS2 and cath-D mRNA in MCF-7 cells. This data is from a single experiment which is representative of two experiments.

In the present study, Analog II was found to be a potent inhibitor of breast cancer cell proliferation. Accordingly, Analog II was more potent than tamoxifen in inhibiting the growth of MCF-7 cells. However, the results of this study indicate that the Analog II-induced inhibition of MCF-7 cell growth may not occur as a result of a classic ER-mediated mechanism for the following reasons. (i) The ER-binding affinity of Analog II in MCF-7 cells did not correlate directly with the antiproliferative action of this compound on MCF-7 cells (Figure 9). (ii) The antiproliferative effect of Analog II on MCF-7 cells was not reversed in the presence of estradiol (Table 1). (iii) Analog II inhibited the growth of ER-negative MDA-MB-231 breast cancer cells (Figures 5 and 6). (iv) The effect of Analog II on the cell surface morphology of MCF-7 cells, was not reversed by estradiol. Moreover, Analog II reduced the density of MV on MDA-MB-231 cells (Figures 7 and 8). (v) Analog II did not inhibit estradiol-induced expression of the pS2 and cath-D genes which are known to be estrogen regulated (Figure 11). However, Analog II did bind specifically, although very weakly, to the ER and produced a much greater effect on the proliferation and cell surface morphology of MCF-7 than MDA-MB-231 cells.

This differential activity between ER-positive and ER-negative cell lines suggests that this compound acts to some extent by an estrogen-associated mechanism which is different than tamoxifen. This notion is also supported by the observation that the influence of Analog II on the cell cycle kinetics of MCF-7 cells is different than the established effect of tamoxifen to arrest cells in the G_1 phase.[22] We have previously observed that other cyclopropyl antiestrogens also arrest MCF-7 cells in the G_2/M phase.[19] Thus, Analog II may produce antiproliferative activity by delaying cell progression through the cell cycle. Accordingly, other antitumor agents such as vincristine and taxol inhibit mitotic activity at the G_2/M phase of the cell cycle.

Other possible mechanisms of antitumor activity of antiestrogens which have been proposed include inhibition of growth factor activity,[23] inhibition or down-regulation of intracellular calmodulin and protein kinase C activity.[24,25] In addition, tamoxifen and other triphenylethylene derivatives have also been shown to inhibit human breast cancer cell growth, in part by means of a non-estrogenic, perhaps cytotoxic mechanism.[26] Therefore, we examined the cytotoxicity of Analog II on breast cancer cells. The results

indicate that approximately 10% of the antiproliferative activity of Analog II on MCF-7 cells is related to cytotoxicity. However, in MDA-MB-231 cells, the majority of the antiproliferative activity of Analog II was found to be due to cellular cytotoxicity. In the A-549 human lung cancer cells, Analog II induced neither antiproliferative activity nor cytotoxicity. These results suggest that Analog II has a unique profile of antitumor activity and that its cytotoxic action appears to be breast cancer specific. Recently, Haar and Day[27] reported similar results on the cytotoxicity of MCF-7 and MDA-MB-231 cells and suggested that the cytotoxic effect of Analog II is associated with a major metabolite.

Alternatively, Analog II may have a different mechanism of action in ER-positive and ER-negative breast cancer cell lines. It is possible that the cytotoxicity, and thus part of the antiproliferative activity, of Analog II on both MDA-MB-231 and MCF-7 cells may be mediated by antiestrogen binding sites. These sites are known to be present and related to the cytotoxic effects of tamoxifen and triphenylethylene analogs in both cell lines.[28,29] Accordingly, the action of Analog II on other breast and non-breast tumor cell lines must be examined in order to determine conclusively that this compound is effective on a wide variety of human breast cancer cell types and specific for breast cancer.

The action of Analog II on the ultrastructure of breast cancer cells does not appear to be ER mediated since these effects were observed on both MCF-7 and MDA-MB-231 cells. Pathways unrelated to ER binding have been suggested for the morphological changes on the cell surface of MCF-7 cells.[21] Since drug-induced alterations in cell surface MV have been suggested to indicate a change in differentiation and transformation,[30,31] which may be related to the invasiveness of cancer cells, it is possible that these Analog II-induced changes in cell morphology may also be related to a reduction in the metastatic potential of breast cancer cells *in vivo*. Accordingly, Analog II has been reported to reduce the metastatic development of DMBA-4 tumor cells in the rat[15] and to reduce the onset of new tumor development in the DMBA-induced breast cancer model.[13,14] Further, this compound has been shown to reduce the invasiveness and motility of breast cancer cells.[32-34] In addition, the release of proteolytic enzymes, known to be involved in tumor cell invasion, is diminished by Analog II treatment of MCF-7 cells.[35]

In conclusion, Analog II clearly produces specific and potent antitumor activity on human breast cancer cells which appears to be unrelated to a specific ER-dependent mechanism and different than the action of tamoxifen. This unique antitumor spectrum of activity suggests that this compound may be therapeutically effective in the treatment of both ER-positive and ER-negative human breast tumors. As such it would have a distinct advantage over exisiting antiestrogens which are primarily effective against ER-positive breast tumors.[1] In addition, since the progression of primary breast tumors leads to metastatic tumors which are mainly ER-negative, agents such as Analog II may be useful in slowing or arresting metastatic development which is responsible, in large part, for breast cancer mortality.

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