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ORIGINAL ARTICLE

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Antitumor mechanism of action of a cyclopropyl antiestrogen (compound 7b) on human breast cancer cells in culture

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Abstract Cyclopropyl compound 7b [(*Z*)-1,1-dichloro-2-[4-[2-(dimethylamino)ethoxy] phenyl]-2-(4-methoxyphenyl)-3-cyclopropane] has been shown to be a pure antiestrogen in mouse uterine tissue. Antitumor activity was examined by evaluating the influence of 7b on the proliferation, estrogen receptor (ER) affinity and cell-surface morphology of ER-positive and ER-negative human breast cancer cells in culture. The antiproliferative potency of 7b was found to be equal to tamoxifen in ER-positive MCF-7 human breast cancer cells. Further, the antiproliferative activities of 7b and tamoxifen were reversed by coadministration of estradiol. Accordingly, the antiproliferative activity of compound 7b appears to be estrogen-mediated since it did not influence the growth of either ER-negative MDA-MB-231 human breast cells or A-549 human lung cancer cells in culture. An ER-dependent mechanism of action is also supported by the specific binding affinity of 7b for ER in MCF-7 cells. Further, a study of cell surface morphology using scanning electron microscopy (SEM) revealed that 7b reduced the density and distribution of microvilli (MV) on MCF-7 cells, which was reversed by coadministration of estradiol. Compound 7b did not alter the cell surface morphology of ER-negative MDA-MB-231 cells. In conclusion, 7b inhibited the growth of ER-positive MCF-7 cells in an estradiol-reversible manner, and had no effect on either ER-negative MDA-MB-231 cells or A-549 lung cancer cells. The results of this study confirm an antiestrogenic mechanism of action for 7b as previously observed in vivo and suggest that 7b would be effective in the treatment of estrogen-dependent breast cancer or as a prophylactic treatment for women with a high risk of breast cancer development.

Key words Cyclopropyl antiestrogen · Anticancer drug · Human breast cancer

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Introduction

Tamoxifen is a synthetic nonsteroidal antiestrogen which is commonly used for treatment of breast cancer [21, 30]. This compound antagonizes the action of estradiol at the cellular level by an estrogen receptor (ER)-associated mechanism [33]. Tamoxifen inhibits the growth of ER-positive breast cancer cells in culture [6, 31, 37] and DMBA-induced mammary tumors in the rat [25, 34]. The antitumor activity of tamoxifen is due to its estrogen antagonist activity. However, tamoxifen has estrogen agonist properties both in vitro and in vivo. For example, stimulation of the progesterone receptor [10, 14, 29], and expression of other ER-dependent mRNAs [7, 30, 32, 40] in human breast cancer cells in vitro and uterotropic stimulation in vivo [3, 22, 28] are attributed to its estrogen agonist activity. Accordingly, the estrogen agonist property of tamoxifen may be responsible for undesirable clinical side effects such as an increase in the incidence of endometrial carcinoma, and stimulation of ovarian estrogen production [4, 12]. Further, prolonged tamoxifen treatment is known to ultimately fail in most breast cancer patients [21]. Similarly, prolonged tamoxifen exposure can lead to tamoxifen-resistant [33] and tamoxifen-stimulable [13] tumors in animal models. Thus, antiestrogens devoid of estrogen agonist activity should be much more effective in the treatment of in situ and metastatic breast cancer.

Search for a pure antiestrogen led us to the development of a novel series of cyclopropyl antiestrogens. Compound 7b, a triarylcyclopropyl derivative (Fig. 1), has been found to be a pure antiestrogen without

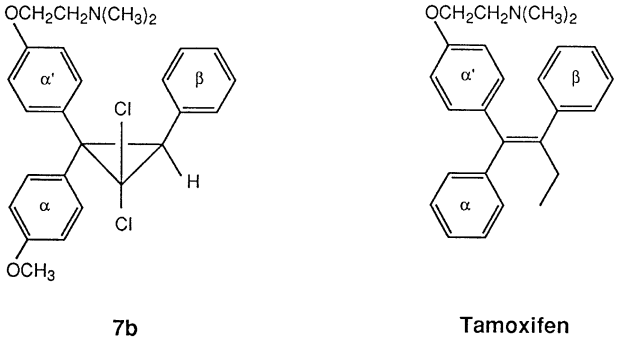

Fig. 1 Chemical structure of compound 7b and tamoxifen

estrogen agonist activity in the mouse uterotropic assay, ER binding specificity and antiproliferative activity on MCF-7 cells [8, 18]. Since compound 7b was found to be a pure estrogen antagonist, it was evaluated for antitumor activity on human breast cancer cells in the present study. In order to determine the therapeutic potential of this compound in the treatment of human breast cancer, the antitumor activity of 7b was compared with that of tamoxifen. The antitumor activity of 7b 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. Also ER binding and cell surface morphology of 7b was determined in MCF-7 cells. Further, in order to make an assessment of the breast cancer antitumor specificity, the antiproliferative activity of this compound on A-549 human lung cells was also examined in this study.

Materials and methods

Cell culture methods

The ER-positive MCF-7 human breast cancer cell line was obtained from the Michigan Cancer Foundation (Detroit, Mich.). MCF-7 cells were grown in T-75 tissue culture flasks, as monolayer cultures in RPMI-1640 medium (without phenol red) supplemented with 2 m*M* L-glutamine, 50 μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin and 5% calf serum containing a low level of estradiol [17]. Cultures were grown at 37 °C in a humid atmosphere containing 5% CO_2, and fed on alternate days. When cultures reached confluence (usually at 7 days), they were subcultured using a 1 : 2 splitting ratio. The culture medium was changed on alternate days until the cells were confluent. The estrogen-dependent nature of these cells was characterized by their responsiveness to estradiol and tamoxifen and their ER content [18].

The ER-negative MDA-MB-231 human breast cancer cell line and the A-549 human lung cancer cell line were obtained from the American Type Culture Collection (Rockville, Md.). The culture conditions used for MDA-MB-231 cells were the same as described above for MCF-7 cells except that Leibovitz' L-15 medium supplemented with 16 mg/l glutathione was used in place of RPMI-1640 medium. The A-549 cells were grown using the same conditions as MCF-7 cells, except for subculturing using a 1 : 3 splitting ratio every 5th 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 medium as previously described [18]. 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 had been shown not to alter cell growth [16]. 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, and daily for A-549 cell culture experiments. Exponentially growing cells were counted by hemocytometer on the scheduled days following the addition of the experimental compounds using the trypan blue exclusion method to determine cell viability as previously reported [19]. The antiproliferative activity of the test compounds was expressed as the percentage inhibition of control, calculated as follows:

Antiproliferative activity

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

ER relative binding activity in MCF-7 cells

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. The IC_{50} and realtive binding activity (RBA) values for estradiol and the antiestrogens were determined as previously described [20, 36]. Briefly, triplicate wells, were incubated with 0.6 n*M* [^{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 RPMI-1640 medium containing 0.1% bovine serum albumin for 60 min at 37 °C. Parallel sets of wells were incubated with nonradioactive estradiol, tamoxifen, and 7b at various concentrations. The bound [^{3}H]-estradiol was extracted by incubating the cells with 1 ml ethanol for 30 min at 22°C. A 0.2-ml aliquot of the ethanol extract was transferred to 4 ml liquid scintillation cocktail (Ready-Solv, Beckman) and counted in a liquid scintillation counter (Model LS 1801, Beckman). Specific bound [^{3}H]-estradiol was determined by subtracting nonspecific 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, and was 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 or 7b) that displaced 50% of the ER-bound [^{3}H]-estradiol.

Scanning electron microscopy

Either MCF-7 or MDA-MB-231 cells were grown on coverslips placed in the bottom of six-well plates containing growth medium. The cells were treated with estradiol (10^{-9} *M*), antiestrogen (10^{-6} *M*) or vehicle in the control group for 4 days. The cells were then 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. The coverslips were washed in medium 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% to 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 (SEM) at 15 kV [20, 39].

The density of microvilli (MV) on the cell surface was quantitated by counting the number of MV in five separate l-μm^2 grids on the SEM photomicrographs at $\times 30\,000$ magnification [20].

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, Austin, Tx.) on a Macintosh 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 statistically significant.

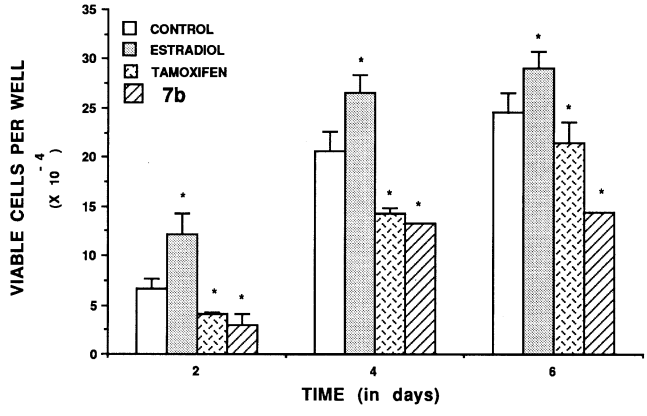

Fig. 2 Time-course of estradiol (10^{-8} *M*), tamoxifen (10^{-6} *M*) and 7b (10^{-6} *M*) treatment on the proliferation of MCF-7 cells. Each bar represents the mean of duplicate samples ± SEM (*significantly different from control)

Results

Antiproliferative activity

The influence of compound 7b, estradiol and tamoxifen were examined on the proliferation of MCF-7 cells, in exponential growth, at days 2, 4 and 6 of treatment. Estradiol (10^{-8} *M*) stimulated, while 7b and tamoxifen (10^{-6} *M*) inhibited, MCF-7 cell proliferation (Fig. 2). At 4 days of treatment, both tamoxifen and 7b decreased the growth of MCF-7 cells in a dose-dependent manner over a concentration range of 10^{-12} to 10^{-5} *M* (Fig. 3).

The antiproliferative activity of tamoxifen (10^{-7} *M*) was almost completely reversed by coadministration of estradiol (10^{-8} *M*) (Fig. 4). The antiproliferative activity of 7b (10^{-7} *M*) was completely reversed in the presence of equimolar estradiol (10^{-7} *M*), but only partially reversed by 10^{-8} *M* estradiol (Fig. 4).

The growth of ER-negative MDA-MB-231 cells at 4 days of treatment and the proliferation of A-549 human lung cancer cells at 2 days of treatment were not altered by estradiol, tamoxifen or 7b (Table 1).

Relative binding affinity

The IC_{50} and RBA for 7b and tamoxifen, presented in Table 2, were derived from the [^{3}H]-estradiol displacement curves (Fig. 5). The results revealed IC_{50} values of 95 μM and 2.10 μM and RBA values of 0.01 and 0.48 for 7b and tamoxifen, respectively. Thus, 7b was found to have an ER binding affinity that was approximately 48 times lower than tamoxifen in MCF-7 cells.

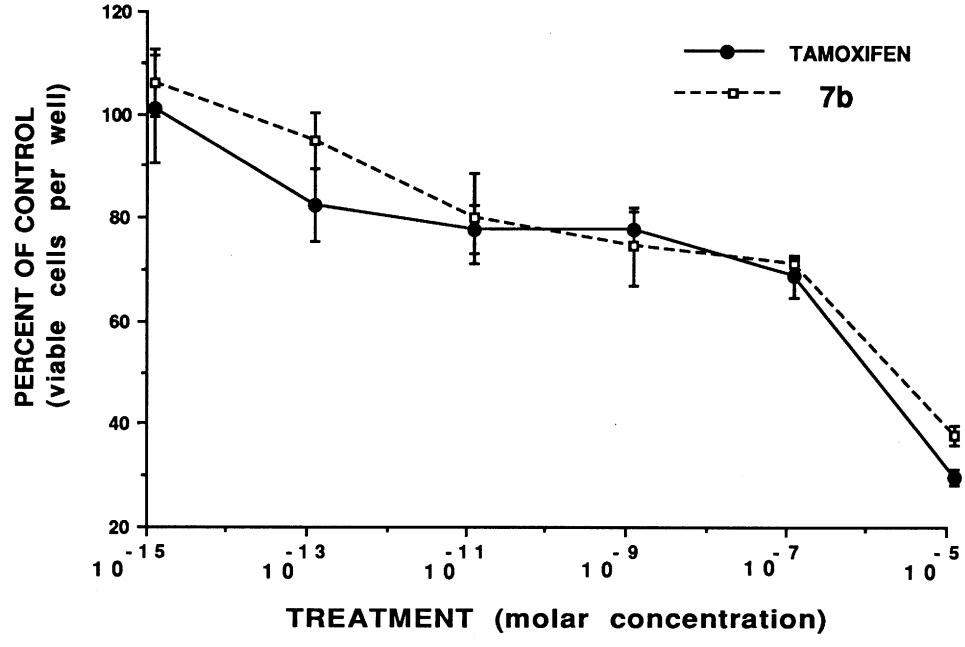

Fig. 3 Dose-response antiproliferative activity of 7b and tamoxifen on MCF-7 cells on day 4 of treatment. Each point represents the mean of triplicate samples ± SEM

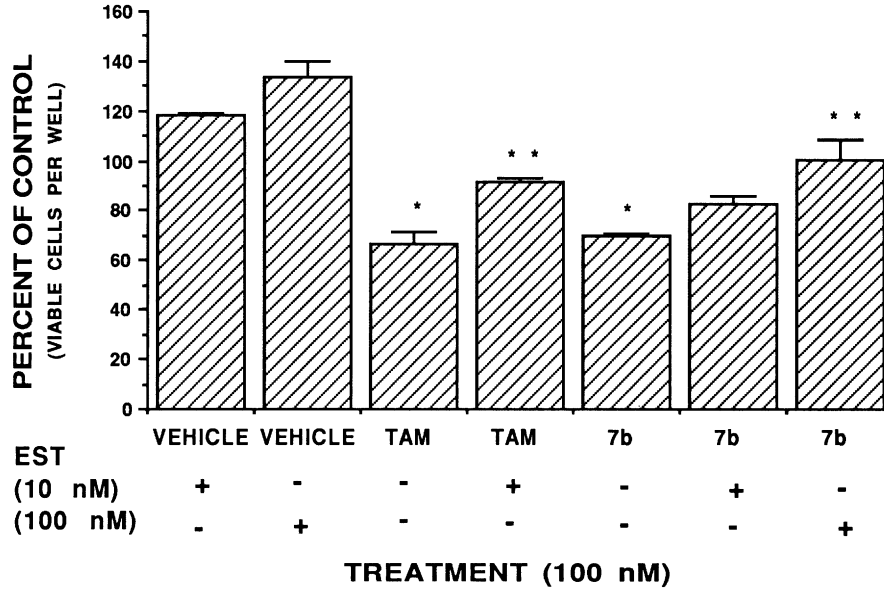

Fig. 4 Influence of estradiol on the antiproliferative activity of 7b and tamoxifen in MCF-7 cells on day 4 of treatment. Each bar represents the mean of triplicate samples ± SEM. (*$P < 0.05$, antiproliferative treatment effect, antiestrogen alone vs vehicle control; **$P < 0.05$, reversal of the antiproliferative treatment effect in the presence of estradiol, antiestrogen alone vs antiestrogen + estradiol)

Table 1 Influence of estradiol, tamoxifen and compound 7b 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	
	MDA-MB-231 cells[a] ($\times 10^{-4}$)	A-549 cells[b] ($\times 10^{-3}$)
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
7b (10^{-6} *M*)	79.7 ± 10.6	102.0 ± 6.5

[a]MDA-MB-231 cells grown in a 6-well plate were counted on the 4th day of treatment
[b]A-549 cells grown in a 12-well plate were counted on the 2nd day of treatment

Table 2 Estrogen receptor binding affinity

Treatment	IC_{50} (10^{-6} *M*)[a]	RBA
Estradiol	0.01	100.00
Tamoxifen	2.10	0.48
Compound 7b	95.00	0.01

[a]The concentration of non-radiolabeled compound responsible for displacing 50% [^{3}H]-estradiol from estrogen receptors

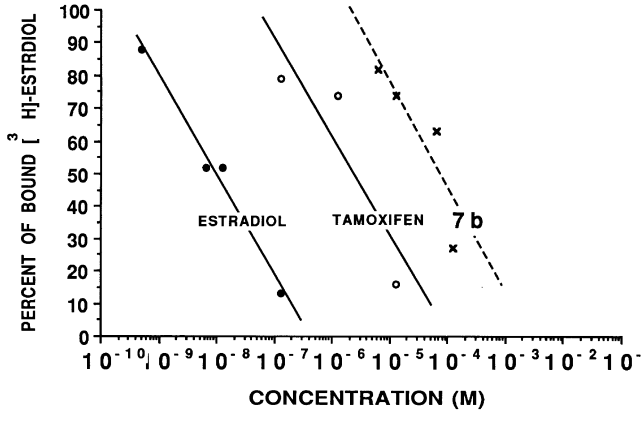

Fig. 5 [^{3}H]-estradiol displacement curves for estradiol, 7b and tamoxifen from ER in MCF-7 cells. [^{3}H]-estradiol displacement was measured using the whole-cell assay method. Each point represents the mean of triplicate samples

Cell surface morphology

The surface morphology of MCF-7 and MDA-MB-231 cells treated with either estradiol (10^{-8} *M*), or 7b (10^{-6} M) for 4 days was examined using SEM. No sign of cytotoxicity was observed in either the control or treated cells. The surface of control MCF-7 contained short, rare, uniformly distributed MV (Fig. 6A). In estradiol-treated MCF-7 cells there was an increase in length and density of MV on the cell surface (Fig. 6B) as previously reported by Vic et al. [39]. In 7b-treated MCF-7 cells a unique morphological feature was observed. The MV tended to aggregate in bunches of three or four, and formed tuft-like structures across the complete cell surface (Fig. 6C). Coadministration of estradiol (10^{-8} *M*) reversed the effect of tamoxifen and 7b on the cell surface morphology (Fig. 6D). Neither 7b nor estradiol treatment altered the morphology of MDA-MB-231 cells.

Discussion

Tamoxifen has been shown to inhibit the proliferation of MCF-7 cells in culture. However, specific antiestrogenic effects of tamoxifen on the growth of MCF-7 cells have been demonstrated at a concentration of

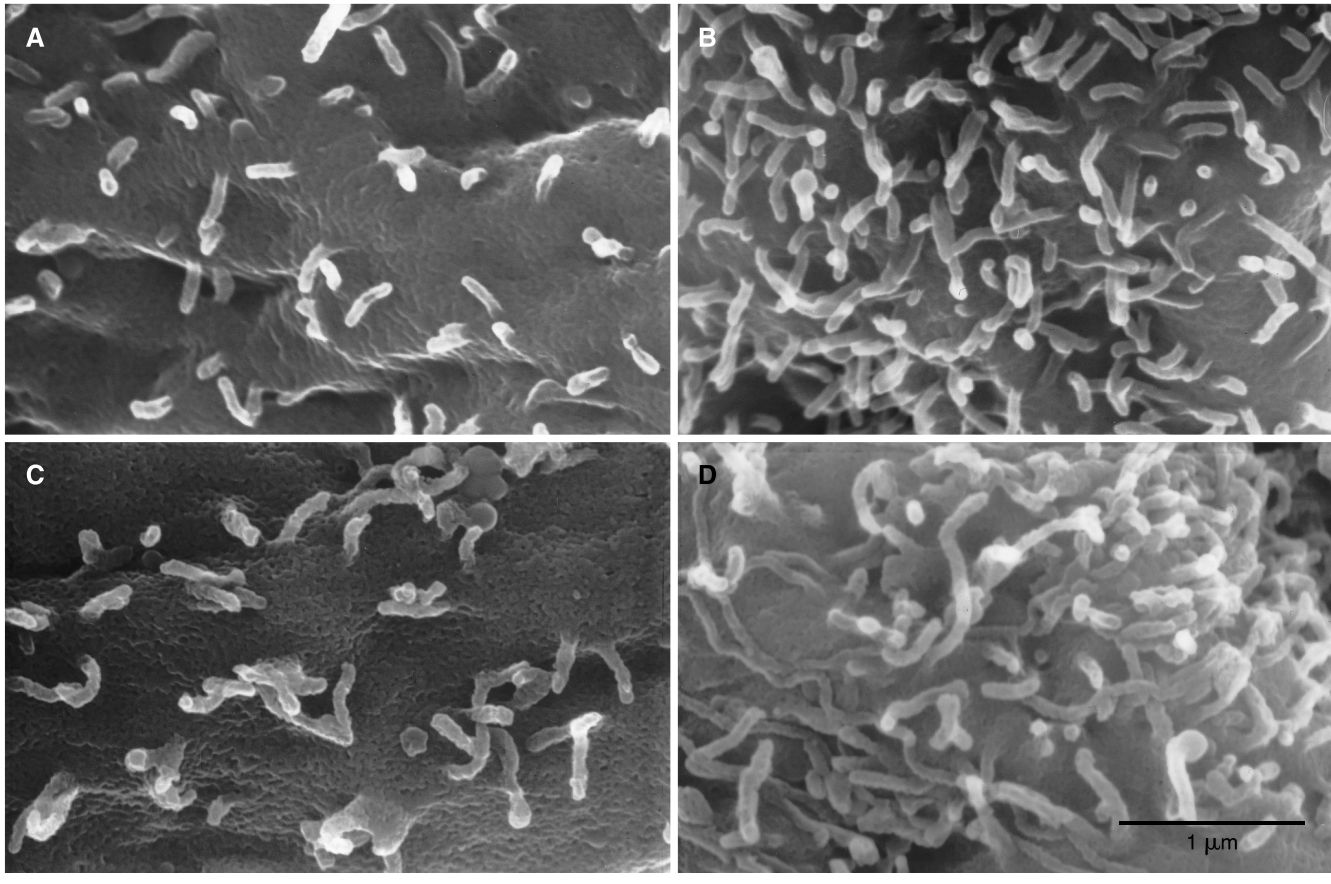

Fig. 6A–D Scanning electron micrographs of MCF-7 cells (×30 000). **A** Vehicle-treated control; **B** treated with 10^{-8} *M* estradiol; **C** treated with 10^{-6} *M* 7b; **D** treated with 10^{-6} *M* 7b and 10^{-8} *M* estradiol

10^{-6} *M* or lower; above this concentration the antiproliferative effect of tamoxifen on MCF-7 cell growth is believed to be due to a cytotoxic action [2]. Therefore, compound 7b was initially examind at a concentration of 10^{-6} *M* to evaluate its specific antiestrogenic properties. Tamoxifen and estradiol were used as standard antiestrogen and estrogen, respectively, in the evaluation of breast cancer cell proliferation responsiveness since a loss of ER and estrogen-mediated responsiveness in MCF-7 cells has been reported [5, 11]. As previously observed, estradiol (10^{-8} *M*) stimulated, and tamoxifen (10^{-6} *M*) inhibited, the growth of MCF-7 cells [18, 31]. In addition, the antiproliferative effect of 7b (10^{-6} *M*) on MCF-7 cells was also demonstrated in this study (Fig. 2). Further, the antiproliferative activity of 7b and tamoxifen in MCF-7 cells was found to be dose-related and approximately equipotent over a concentration range of 10^{-12} to 10^{-5} *M*, as shown in Fig. 3.

The antiproliferative activity of 7b appears to be ER-mediated. This notion is supported by several separate lines of evidence from the present study: (a) similar dose-related spectrum of antiproliferative activity of 7b and tamoxifen on ER-positive MCF-7 cells; (b) complete estradiol-induced reversibility of 7b antiproliferative activity in MCF-7 cells; (c) specific affinity of 7b for the ER of MCF-7 cells; and (d) absence of a 7b antiproliferative effect on ER-negative MDA-MB-231 breast cancer and A-549 lung cancer cells.

It is possible that the antiproliferative effects of 7b may also be mediated by an ER-associated mechanism which may or may not be initiated by ER binding. For example, antiestrogen-induced alteration of ER translocation to the nucleus [24], inhibition of genetic expression of cytoplasmic ER [23], and inhibition of the activity of calmodulin and/or protein kinase C to modulate the activation of ER in MCF-7 cells have been reported [27, 38]. In addition, inhibition of ER-dependent growth factor or growth factor receptor activity in breast cancer cells may be involved [9, 15, 26].

In the SEM experiments both tamoxifen and 7b reduced the density of MV on the surface of MCF-7 cells, although 7b caused a characteristic aggregation of MV on these cells. While the appearance of MV on the surface of 7b- and tamoxifen-treated MCF-7 cells differed, coadministration of estradiol reversed the morphological effects of both compounds. Further, neither tamoxifen nor 7b influenced the morphology of ER-negative MDA-MB-231 human breast cancer cells.

This evidence further supports an ER-mediated mechanism of antiestrogenic action for 7b in MCF-7 cells. It is possible that these specific drug-induced morphological changes may represent changes in differentiation, transformation or invasiveness of these cells [1, 35, 40, 41], but the exact significance of 7b action on cell morphology remains to be determined.

In conclusion, the results of the present study demonstrate that 7b produces specific antitumor activity on estrogen-dependent human breast cancer cells in culture. Further, the results indicate that the antitumor activity of 7b may be mediated by an ER-dependent mechanism of action and that this activity is comparable to that of tamoxifen. The similarity in chemical structure between 7b and tamoxifen suggest a similar pharmacological mechanism. Indeed the influence of these compounds on MCF-7 cell proliferation, cell surface morphology and estradiol-mediated reversal of these effects support this concept. The difference in ER binding affinity between tamoxifen and 7b may suggest a different ER-mediated mechanism. However, since 7b has been found to be a pure antiestrogen, without estrogen agonist activity in mouse uteri [8] and a potent inhibitor of breast cancer cell growth, this compound may be more effective than tamoxifen in the treatment of breast cancer and/or the prevention of breast cancer in patients with a high genetic risk.

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