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TAMOXIFEN AND ITS ACTIVE METABOLITE INHIBIT GROWTH OF ESTROGEN RECEPTOR-NEGATIVE MDA-MB-435 CELLS

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Abstract—Tamoxifen (TAM), the non-steroidal anti-estrogen most widely administered to breast cancer patients, acts, at least in part, by competing with estrogen receptors (ER). However, the existence of an alternative mechanism of action for this drug is supported by the clinical observations that: (a) 30% of patients with ER-negative cancer cells respond to TAM, and (b) 30% of patients with ER-positive cancer cells are not sensitive to this anti-estrogen. In this study, we observed that growth of the human ER-negative breast cancer cell line MDA-MB-435 was inhibited by TAM and 4-hydroxytamoxifen (4OH-TAM) in a concentration-dependent fashion. Both monoclonal enzymoimmunoassay and Dextran Charcoal Coated Scatchard radioimmunoassay analysis demonstrated that this MDA-MB-435 cell line does not express ER. The absence of ER in MDA-MB-435 cells was also demonstrated at the mRNA level by both northern blot hybridization and reverse transcription-polymerase chain reaction techniques. MDA-MB-435 cell proliferation was not affected by 17β-estradiol or by the pure anti-estrogen ICI 164384, further demonstrating that the observed effects of TAM and its active metabolite on the proliferation of MDA-MB-435 cells were due to an ER-independent mechanism, yet to be identified. MDA-MB-435 thus appears to be a promising original model for the study of the alternative ER-independent mechanisms of action of TAM.

Key words: tamoxifen; breast cancer; estrogen receptor negative; proliferation

Breast cancer is the second leading cause of death by cancer (after lung cancer) among women living in the United States and in affluent Western countries. Furthermore, it is the leading cause of death among American women 40–55 years of age [1] and has become increasingly prevalent in the United States over the past several decades [2–4].

Therefore, a major challenge facing today's breast cancer research is to develop and test more effective chemoprevention systems, mainly through pharmacological means. TAM||, a synthetic non-steroidal anti-estrogen, certainly bears investigation as a potential breast cancer chemopreventive drug. Indeed, TAM is currently used not only in the treatment of breast cancer but also in large-scale randomized trials to evaluate its potential in the prevention of the disease [5, 6]. The administration of TAM to patients with node negative breast cancer induces, in most cases, a substantial regression of the tumor, while significantly increasing the disease-free survival of the patients. The potential breast cancer chemopreventive action of TAM was suggested by the observation that patients receiving TAM as an adjuvant after the treatment of primary breast cancer showed a significant reduction in the incidence of contralateral breast carcinomas [7].

While TAM is used widely in both adjuvant breast cancer therapy and prospective clinical trials, the exact mechanisms of its action have not been completely elucidated. TAM treatment was first restricted to women with ER-positive tumors, based on the original observation that TAM competes with 17β-estradiol for its binding to ER and thereby inhibits steroid mitogenic effects. Such a mechanism of action was further supported by the observation that increasing concentrations of estrogens reversed the anti-proliferative action of TAM. The existence of alternate, ER-independent mechanisms of action of TAM was suggested by two clinical observations [8]: (a) 30% of women bearing ER-negative breast tumors respond to TAM treatment, and (b) 30% of patients with ER-positive tumors do not respond to this anti-estrogen.

TAM is known to affect many biological parameters that could be directly or indirectly involved in the control of its anti-proliferative action. It inhibits protein kinase C activity and modulates the cellular levels of growth stimulatory and inhibitory factors [9–11]. TAM reduces calcium

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|| Abbreviations: TAM, tamoxifen; 4OH-TAM, 4-hydroxytamoxifen; ER, estrogen receptor; TCA, trichloroacetic acid; DMEM, Dulbecco's Modified Eagle's Medium; RT-PCR, reverse transcription-polymerase chain reaction; cDNA, complementary DNA; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

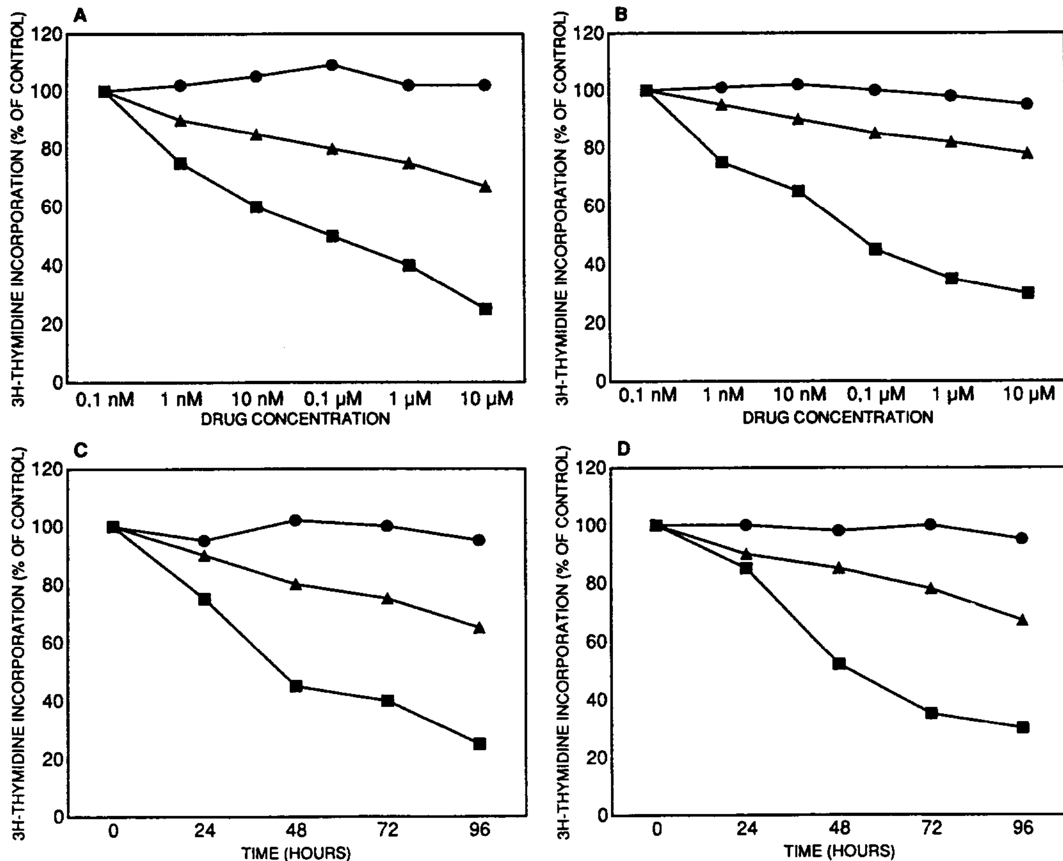

Fig. 1. Effects of TAM and 4OH-TAM on ER-positive and ER-negative breast cancer cell thymidine incorporation. MCF7 A/Z (■) MDA-MB-435 (▲) and MDA-MB-231 (●) were treated for 48 hr with increasing amounts of TAM (A) or 4OH-TAM (B). Alternatively, these cell lines were incubated for various time periods with TAM (C) or 4OH-TAM (D). Each point is the average of triplicate experiments and represents the incorporation of [^{3}H]thymidine (dpm/µg DNA) under the different experimental conditions, expressed as a percentage of control (vehicle only: MCF7 A/Z, 65,700 dpm/µg DNA; MDA-MB-435, 82,110 dpm/µg DNA; MDA-MB-231, 74,860 dpm/µg DNA). Intra-assay variation never exceeded 10% of the mean value.

influx by affecting its membrane channels. It inhibits lipid peroxidation, an effect that has been correlated with the cardiovascular benefits reported in women treated for breast cancer [12]. High concentrations of TAM are generally required to produce these inhibitory effects, leaving their biological significance open to discussion.

The description of an anti-estrogen binding protein that binds non-steroidal molecules, such as TAM, with high affinity has focused attention on the potential existence of a receptor, different from the ER, that would mediate their biological effects [13]. Estrogens do not displace TAM from this binding protein but, surprisingly, unsaturated fatty acids inhibit its binding. The physiological role of this protein and its natural ligand is still unknown.

In this study, we investigated the effects of TAM and its active metabolite, 4OH-TAM, on the proliferation of an ER-positive (MCF7 A/Z) and two ER-negative (MDA-MB-231, MDA-MB-435) human breast cancer cell lines. Unexpectedly, MDA-MB-435 proliferation was inhibited severely by TAM and 4OH-TAM. Moreover, ICI 164384, a synthetic anti-estrogen that strongly inhibited the proliferation of the ER-positive cell line MCF7 A/Z, did not affect MDA-MB-435 cell growth. This ER-negative breast cancer derived cell line constitutes an original model to identify the ER-independent mechanisms of TAM's anti-proliferative effects.

MATERIALS AND METHODS

Cell culture and treatments

MCF7 A/Z cells were provided by Dr. M. Mareel (University of Gent, Belgium). MDA-MB-231 cells were purchased from the American Type Culture Collection (U.S.A.). MDA-MB-435 cells were obtained from Dr. J. Price (University of Texas, Houston, TX, U.S.A.). MCF7 A/Z were grown in DMEM (GIBCO) and the other two cell lines in RPMI. Both media contained 10% fetal bovine serum, penicillin (100 µg/mL) and streptomycin

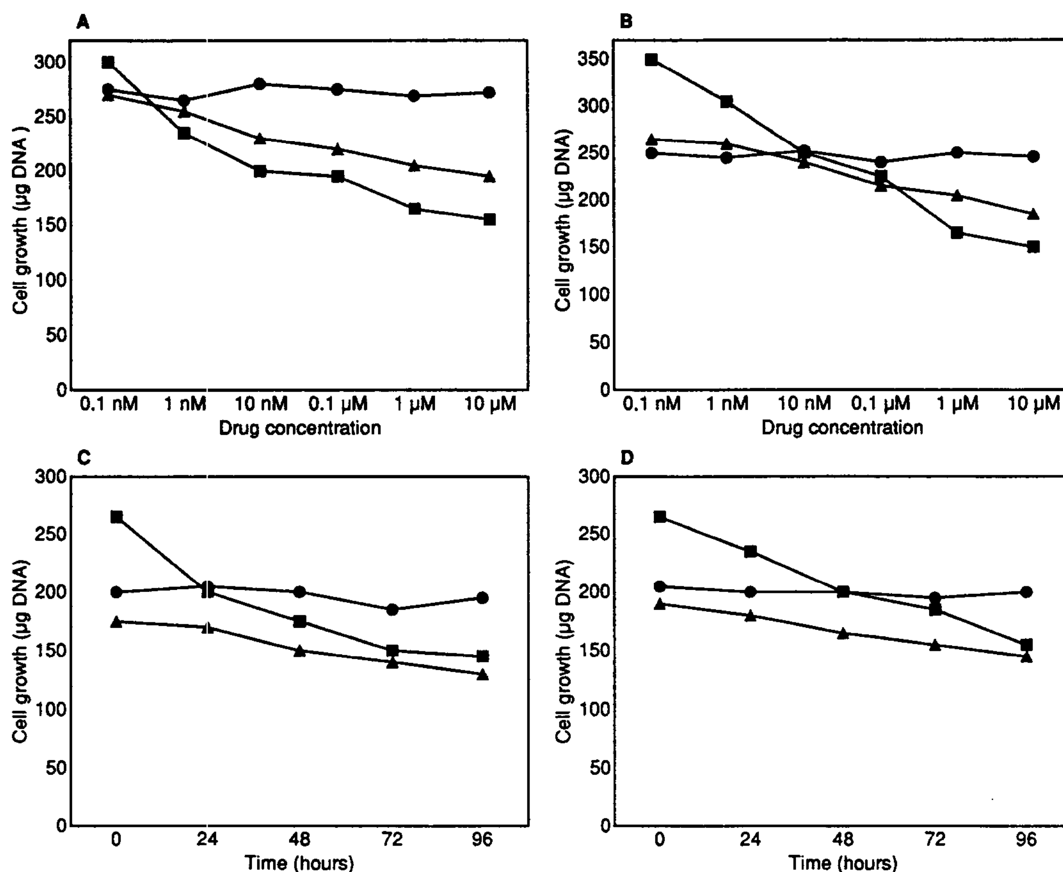

Fig. 2. Effects of TAM and 4OH-TAM on ER-positive and ER-negative breast cancer cell lines expressed as total DNA content per dish. MCF7 A/Z (■), MDA-MB-435 (▲) and MDA-MB-231 (●) were treated for 48 hr with increasing amounts of TAM (A) or 4OH-TAM (B). Alternatively, these cell lines were incubated for various time periods with TAM (C) or 4OH-TAM (D). Each point is the average of triplicate experiments. Intra-assay variation never exceeded 10% of the mean value.

(100 µg/mL). The cell lines were incubated routinely at 37° under 95% air:5% CO_2. Before each experiment, cells were grown for 2 days in phenol red free DMEM/Ham F-12 supplemented with 10% dextran charcoal-treated fetal bovine serum to eliminate contaminating estrogens.

TAM and 4OH-TAM were provided by Besins Iscovesco (Paris, France). 17β-Estradiol was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and ICI 164384 from ICI (Macclesfield, U.K.). All the chemicals were added to the cultures as concentrated stock solutions in pure ethanol (0.1% of the total volume).

Cell proliferation experiments

Cell growth was evaluated by [^{3}H]thymidine incorporation and DNA quantification as described [9]. Briefly, [*methyl*-^{3}H]thymidine (NEN, Boston, MA, U.S.A.) was added to the culture medium 3, 6, 12 and 18 hr before measurement, at a concentration of 5 µCi/mL medium. Since incorporation was still in the linear range after 12 hr of incubation, we decided to choose this incubation time for our experiments. Cells were then washed with PBS, treated for 10 min with TCA (5%), washed again with distilled water, and finally incubated for 2 hr in the presence of 3 mL of NaOH (0.5 M). The cell suspension was removed, and its radioactivity was measured in duplicate by liquid scintillation. Total DNA was quantified on the cell suspension by a fluorometric method [14]. The viability of the cells was determined routinely by a trypan blue exclusion assay. Less than 10% of the cells were killed after 48 hr of treatment with any of the tested compounds. The significance of the effects observed was evaluated by Student's *t*-test.

ER detection

ER content was determined in the different cell lines by two methods: an enzymoimmunoassay that measures the amount of receptor regardless of its biological activity, using a kit (Abbott Laboratories) [15], and a Dextran Charcoal Coated Scatchard method that evaluates the amount of biologically active receptor [16]. The ER determinations were performed on aliquots of cytosols prepared from the different cell lines described above.

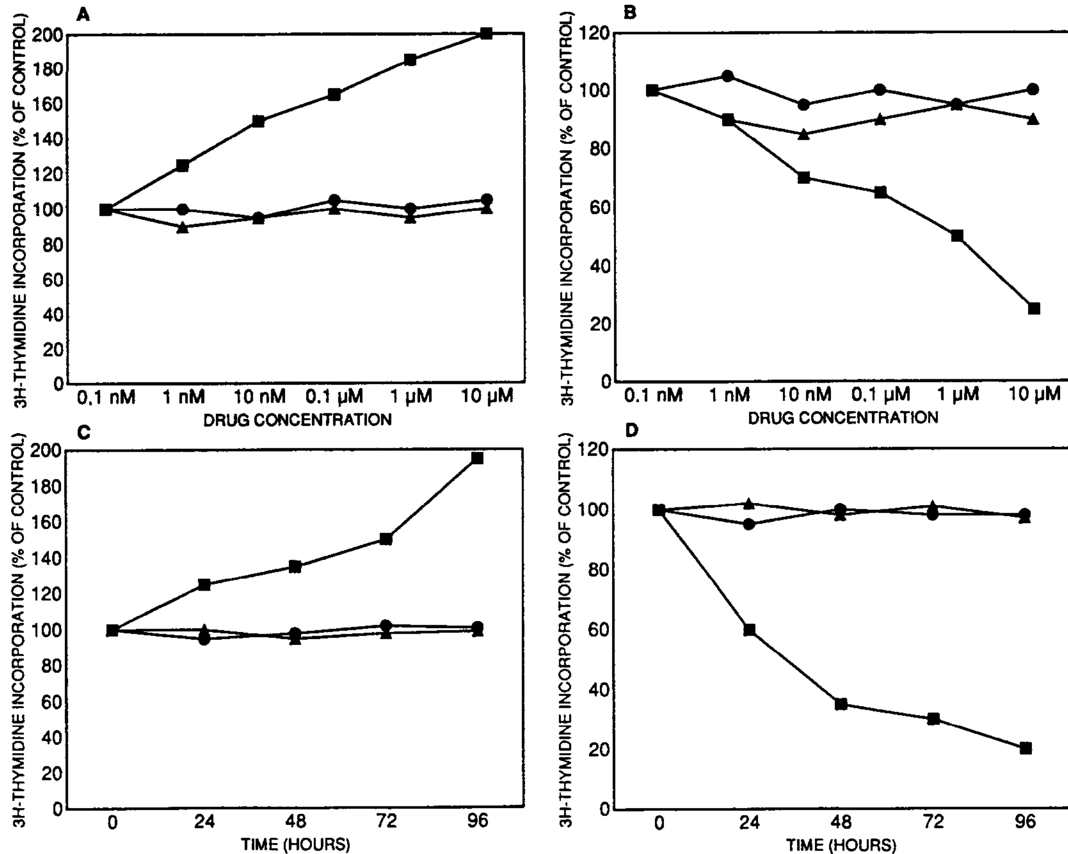

Fig. 3. Effects of 17β-estradiol and ICI 164384 on ER-positive and ER-negative breast cancer cell line thymidine incorporation. MCF7 A/Z (■), MDA-MB-435 (▲) and MDA-MB-231 (●) were incubated with increasing amounts of 17β-estradiol (A) or ICI 164384 (B). Alternatively, these cell lines were incubated for various time periods with 17β-estradiol (C) or ICI 164384 (D). Each point is the average of triplicate experiments and represents the incorporation of [^{3}H]thymidine (dpm/μg DNA) under the different experimental conditions, expressed as a percentage of control (vehicle only: MCF7 A/Z, 65,700 dpm/μg DNA; MDA-MB-435, 82,110 dpm/μg DNA; MDA-MB-231, 74,860 dpm/μg DNA). Intra-assay variation never exceeded 10% of the mean value.

Detection of ER mRNA transcript in MDA-MB-435 cells

Northern blotting. Total cellular RNA was extracted by a guanidium isothiocyanate-phenol-chloroform method (Biotecx, Houston, TX, U.S.A.) [17]. The extraction was performed by adding the reagent directly to the culture dishes and by aspirating the cell lysate several times into a Pasteur pipet. RNA (15 μg) was loaded onto 1.2% (w/v) agarose-formaldehyde gels and, after electrophoresis, transferred to Qiabrane membrane (Qiagen, Chatsworth, CA, U.S.A.). The RNA blot was hybridized at 42° to a [^{32}P]ER-specific 1.8 kb cDNA probe, according to the protocol previously described by Wever *et al.* [18]. After washing under stringent conditions (0.25 × SSC; 1% SDS at 37°), the membranes were exposed for 3 days at −80° to Fuji Medical X-ray film in an intensifying screen-equipped cassette. Total cellular RNA obtained from T47D, an ER-positive human breast cancer cell line, was added to the blot and used as a positive control [19].

RT-PCR. We used the RT-PCR technique to demonstrate further the absence of mRNA coding for the ER in MDA-MB-435 cells, using a protocol described previously [20]. Briefly, 1 μg of total RNA isolated from MDA-MB-435 cells, or from MCF7 A/Z cells as the positive control, was used as a template to synthesize single-stranded cDNA, using Superscript II reverse transcriptase (BRL, Gaithersburg, MD, U.S.A.) and an oligonucleotide deduced from the ER nucleotide sequence (from base 1287 to 1307). PCR was then performed using 5′ and 3′ primers, which correspond to nucleotides 850–870 and 1270–1289, respectively. The 483 bp putative cDNA amplified fragment was analyzed on an agarose electrophoresis gel. For each sample, we used a negative control omitting the reverse transcriptase enzyme and another control with no cDNA template. In addition, the RNA sample was incubated with RNase-free DNase (Boehringer Mannheim GmbH, Mannheim, Germany) before the PCR step to eliminate potentially contaminating genomic DNA, as described [20]. To control the integrity of the RNA template used in the

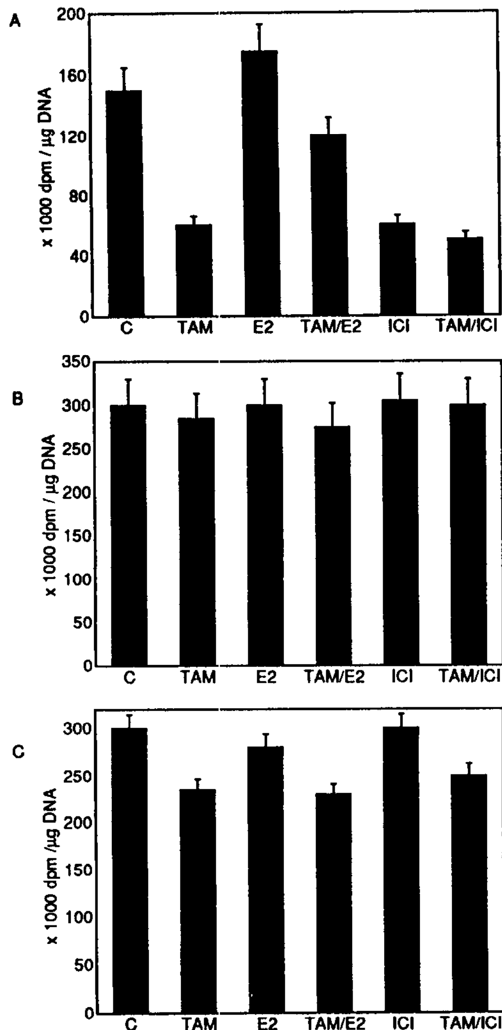

Fig. 4. Effects of treatments with TAM, 17β-estradiol (E2), TAM/17β-estradiol (TAM/E2), ICI 164384 (ICI) and TAM/ICI 164384 (TAM/ICI) on the growth of MCF7 A/Z (A), MDA-MB-231 (B) or MDA-MB-435 (C), treated as described in Materials and Methods (control = C). Each column represents the average of triplicate experiments. Intra-assay variation (represented as standard deviation) never exceeded 10% of the mean value.

Table 1. ER detection in MCF7 A/Z, MDA-MB-231 and MDA-MB-435 human breast cancer cells using radioimmunoassay (RIA) (DCC Scatchard) and enzymo-immunoassay (EIA) techniques

Cell lines	ER determination (fmol/mg protein)	
	RIA	EIA
MCF7 A/Z	600	650
MDA-MB-231	—	—
MDA-MB-435	—	—

experiments, we included a positive control in which we amplified a 581 bp actin cDNA fragment using an actin-specific downstream primer for the cDNA synthesis (1213–1230) and two oligonucleotides (from base 8 to 25 and from base 1160 to 1141) for the PCR amplification step [21].

RESULTS

Effect of estrogen and anti-estrogens on cell growth

To study the molecular mechanism of action of TAM, an ER-positive and two ER-negative human breast cancer cell lines were treated with increasing amounts of TAM and 4OH-TAM for 24–96 hr. After 2 days of treatment, TAM and 4OH-TAM were found to inhibit the growth of MCF7 A/Z(ER+) in a concentration-dependent manner (Fig. 1, A and B). The effects were similar whether expressed in dpm [^{3}H]thymidine/μg DNA (Fig. 1, A and B) or total DNA content/dish (Fig. 2, A and B). Unexpectedly, a similar inhibitory effect was observed when the ER-negative MDA-MB-435 cells were treated under the same conditions (Fig. 1, A and B). The TAM EC_{50} values (approximately 100 nM) were similar for the two cell lines. The growth inhibition time curves were equivalent regardless of the chemical or the cell line used (Fig. 1, C and D). Growth inhibition was stronger during the first 48 hr of treatment and diminished thereafter. However, the inhibition recorded after 48 hr of 4OH-TAM treatment was less pronounced with MDA-MB-435 (25%) than with MCF7 A/Z (50%). Neither TAM nor 4OH-TAM affected the growth of the MDA-MB-231 ER-negative cell line. The determination of total DNA content/dish showed similar results (Fig. 2, C and D), suggesting that thymidine incorporation was not influenced by factors other than cell growth.

The observation that the ER-negative MDA-MB-435 cell line was sensitive to TAM and 4OH-TAM led us to test the effects of 17β-estradiol and ICI 164384, a pure anti-estrogen with no estrogenic activity. 17β-Estradiol had no effect on MDA-MB-231 or MDA-MB-435 growth but significantly stimulated MCF7 A/Z proliferation (Fig. 3A). As expected, the pure synthetic anti-estrogen ICI 164384 inhibited the growth of MCF7 A/Z concentration dependently but did not affect that of the other two cell lines (Fig. 3B). Time curves for 17β-estradiol and ICI 164384 gave a different result. Growth stimulation of MCF7 A/Z by 17β-estradiol first showed a lag period during the first 24 hr of treatment and was then maximal through the following 24 hr (Fig. 3C). The influence of ICI 164384 is summarized in Fig. 3D.

The inhibitory effects of TAM on cell growth were tested in the presence of 17β-estradiol and ICI 164384. In MCF7 A/Z, 17β-estradiol, used at 10^{-8} M, partly reversed the growth inhibition of TAM. Conversely, the growth inhibitory effect of TAM on these cells was increased in the presence of 10^{-7} M ICI 164384 (Fig. 4A). Interestingly, the effects of TAM on MDA-MB-435 growth were not modified significantly by the simultaneous addition of either 17β-estradiol or ICI 164384 (Fig. 4C). Finally, the growth of MDA-MB-231 cells was not affected by

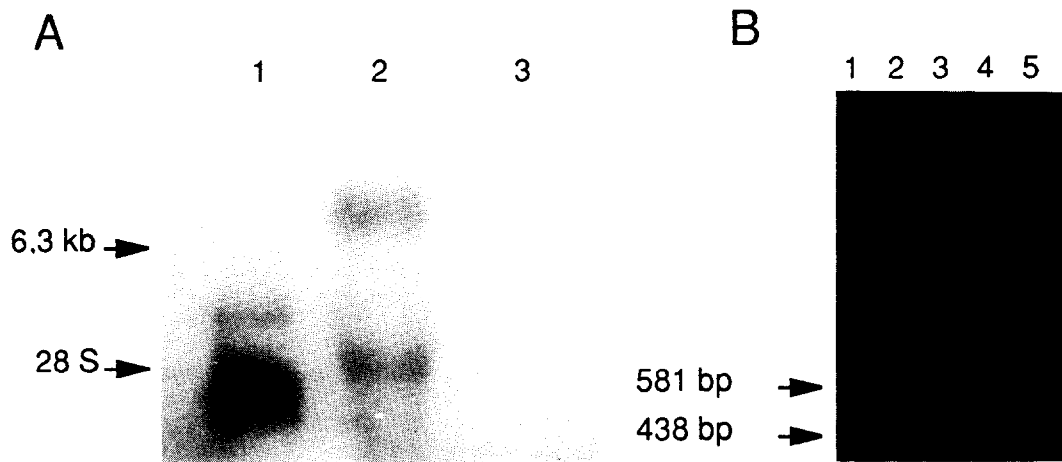

Fig. 5. Detection of ER mRNA in MDA-MB-435 cells. (A) Northern blot hybridization performed on MDA-MB-435 or T47D total cellular RNA, as described in Materials and Methods, showed a 6.3 kb transcript in T47D cells (lane 2) but not in MDA-MB-435 (lane 3). Lane 1 shows the molecular weight marker. (B) RT-PCR, performed as described in Materials and Methods, showed the presence of ER mRNA in MCF7 A/Z cells (lane 1) but not in MDA-MB-435 cells (lane 2). Control experiments included detection of ER mRNA in MDA-MB-435 by PCR without RT treatment (lane 3) and detection of actin mRNA with (lane 4) or without (lane 5) RT. The amplified cDNA actin fragment was of the size expected (581 bp).

the different mixtures of substances tested on the cell lines (Fig. 4B).

Estrogen receptor determination

The ER content in the three cell lines was determined by two methods. As shown in Table 1, MCF7 A/Z cells were the only ones to contain significant amounts of the receptor. The other two cell lines did not express any detectable amount of ER. These data were further confirmed by analysis of ER mRNA content. Northern blot analysis showed the absence of mRNA transcript in MDA-MB-435, while ER mRNA was detected in T47D total cellular RNA (Fig. 5A). The absence of ER mRNA in MDA-MB-435 cells was demonstrated further by RT-PCR performed on the total RNA of MDA-MB-435 cells. Using this technique, we were able to amplify the expected 438 bp DNA fragment of the ER cDNA in the positive control MCF7 A/Z cells, while no DNA fragment was detected in MDA-MB-435 cells (Fig. 5B). The ability to amplify a fragment of a control gene such as β-actin using the same total MDA-MB-435 RNA and the same RT-PCR protocol demonstrated the integrity of our RNA preparation (Fig. 5B).

DISCUSSION

The epidemiological evidence suggesting that TAM is a potential chemopreventive agent that could efficiently reduce the incidence of human breast cancer has encouraged studies aiming to identify the exact molecular mechanisms of action of this drug. The identification of alternate ER-independent mechanisms of action for this non-steroidal anti-estrogen has been hampered by the lack of appropriate models. In this study, we report the identification and characterization of the ER-negative human breast cancer cell line MDA-MB-435, which is sensitive to the growth inhibitory activities of TAM and its active metabolite. While inhibition was only 30% compared with the control, this effect was significant ($P < 0.01$) according to Student's t-test. Furthermore, the growth of another ER-negative cell line, MDA-MB-231, was not affected by these anti-estrogens, even when used at high concentrations (10 μM). For MCF7 A/Z and MDA-MB-435 cells, the inhibitory effect observed was obtained at a concentration of 10^{-6} M TAM, the usual pharmacological concentration at which TAM has been reported previously to be effective [22–24]. Moreover, ICI 164384, a synthetic anti-estrogenic compound, did not inhibit the growth of either of the two ER-negative cell lines, whereas it very significantly decreased the proliferation of the ER-positive MCF7 A/Z cell line. These data strongly suggest that MDA-MB-435 cells are sensitive to TAM through a pathway that does not require the ER. To our knowledge, these cells are the first ER-negative human breast cancer cells sensitive to pharmacological doses of TAM. While a previous report has shown that TAM inhibits the growth of the ER-negative breast cell line MDA-MB-330 [25], the experimental conditions used therein were very different from those used in our study. Indeed, MDA-MB-330 cells were treated with much higher concentrations of TAM (EC_{50} 10 μM), and the culture media were enriched with 10% fetal bovine serum, thus exposing the cells to an estrogenic stimulation. The authors concluded that the growth inhibition observed was most likely due to a cytotoxic effect of the drug [25].

More recently, Croxtall *et al.* [26] reported that TAM inhibits the growth of ER-negative A549 cells,

a human lung adenocarcinoma-derived cell line. 4OH-TAM did not modify the proliferation of these cells, even at a concentration of 1 μM. Used at 100-fold excess, neither estradiol nor ICI 164384 had any significant effect on TAM inhibitory action. Although the TAM effects were similar to those observed on the MDA-MB-435 cells, the 4OH-TAM data were drastically different and suggested that the parent compound and its metabolite acted through different mechanisms of action. Conversely, our results would indicate that the mode of action of these substances might follow the same pathway in our cell model. In this respect, our observation would support the possibility that the so-called anti-estrogen binding protein, which binds both TAM and 4OH-TAM [27, 28] with high affinity, mediates their inhibitory effects on cell growth.

The major problem in understanding the mechanism of action of TAM is certainly that the cellular response to the administration of the drug varies as a function of the organ studied, the animal species, or the origin of the cell line [29]. Moreover, the various effects of TAM are affected differently by 17β-estradiol, which reverses some but does not modify others. Finally, the TAM concentrations needed to observe the different effects span from the nanomolar to the micromolar range.

It is most likely that TAM and its metabolites have multiple sites of action. Many reports in the literature have demonstrated the participation of the ER in the TAM inhibition of breast tumor cell growth and of cell line proliferation [30, 31]. While not proven, the contribution of the anti-estrogen binding protein in TAM-induced inhibition, has been postulated and a role in the resistance of tumors to therapy has also been proposed [32, 33]. The growth-inhibitory action of TAM may also result from a direct inhibition of different enzyme activities: protein kinase C [34], Ca^{2+}-Mg^{2+}-ATPase [35] and calmodulin-dependent cAMP phosphodiesterase [36]. Such effects have only been observed *in vitro* at very high TAM concentrations (100 μM), making it unlikely that they occur *in vivo*. However, it should be kept in mind that *in vivo* 99% of the administered drug accumulated in the peripheral compartments, suggesting a strong tissue binding [37]. The presence of TAM in the phospholipid bilayer of the cellular membrane inducing fluidity changes could account for some of its effects on the activity of membrane bound proteins.

In vitro cell line models displaying different and specific answers to treatment by estrogens and anti-estrogens would be most helpful for the study of the biochemical mechanisms by which these drugs affect cell growth. As such, MDA-MB-435 cells appear to be an interesting model for the identification of the ER-independent mechanisms of action of TAM. The elucidation of all of these mechanisms of action will certainly lead to a safer use of TAM, in the treatment and prevention of cancer.

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