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DIFFERENTIAL REGULATION OF c-myc BY PROGESTINS AND ANTIESTROGENS IN T-47D HUMAN BREAST CANCER CELLS

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Summary—In order to investigate further the mechanisms associated with growth inhibition of human breast cancer cells by progestins and nonsteroidal antiestrogens, their effect on c-myc gene expression in T-47D-5 and T-47D cells has been investigated. The c-myc mRNA levels were differentially regulated by the synthetic progestin, medroxyprogesterone acetate and the nonsteroidal antiestrogen, monohydroxytamoxifen, in both cell lines. Antiestrogen treatment caused a persistent decrease in c-myc mRNA levels while the progestin caused a more complex response. Initially c-myc mRNA levels increased approx. 2-fold, this was followed by a decrease and then partial recovery. The end result, however, of each of these treatments is decreased cell number.

INTRODUCTION

In the presence of estrogens both progestins and nonsteroidal antiestrogens are growth inhibitory agents to receptor positive human breast cancer cells in culture [1, 2]. Since these agents interact with different receptors the mechanisms whereby they exert this growth inhibitory effect may differ. We have found that under these conditions progestins and antiestrogens differentially regulate the expression of EGF, TGF-α and TGF-β [1, 3] and possibly the EGF-receptor [4] gene in T-47D human breast cancer cells. While progestins inhibit TGF-β mRNA expression in T-47D cells, tamoxifen has no effect [3]. Furthermore, tamoxifen inhibits TGF-α expression [3] which is increased by progestins. EGF mRNA is increased in T-47D cells by progestins but unaffected by tamoxifen [1, 2]. These results suggest that the effects of antiestrogens and progestins on putative autocrine/paracrine growth factor gene expression in T-47D cells are different and cannot simply explain the growth inhibitory effects of these agents.

The c-myc gene is generally associated with cell proliferation, especially movement in and out of the cell cycle, in many cell types [5, 6]. Progestins and antiestrogens have been shown to have marked cell cycle effects on responsive human breast cancer cells [7, 8]. To explore further the mechanisms associated with the growth inhibitory effect of progestins and antiestrogens, the effect of these agents on c-myc expression was examined in T-47D and T-47D-5, human breast cancer cell lines.

EXPERIMENTAL

Cell culture

T-47D and T-47D-5, a subline which is more sensitive to the antiproliferative effects of progestins and antiestrogens [2], human breast cancer cells were obtained from sources as previously listed [2] and were grown in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum as previously described [2]. For the experiments, cells were plated at 1×10^6 in 150 mm diameter dishes, 2–3 days later the medium was replaced with fresh medium and MPA or monohydroxytamoxifen was added directly from 1000 × stock solutions in ethanol to achieve the concentrations indicated, and for the indicated periods of time. Cells were harvested by scraping off the monolayer with a rubber policeman. After centrifugation the cell pellet was frozen and stored at below −70°C until RNA preparation.

RNA extraction and Northern blot analysis

RNA was isolated by the guanidinium thiocyanate–cesium chloride method [9]. Total

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RNA was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, size-separated by electrophoresis on 1% (w/v) agarose gels containing 2.2 M formaldehyde and then blotted onto nitrocellulose. Filters were baked for 2 h at 80°C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized with an exon 2 human c-myc probe [10] labeled with [^{32}P] by nick-translation (Amersham, Oakville, Ontario) to a specific activity of 10^8 cpm/μg. Hybridizations, usually for 48 h, were performed at 42°C in the presence of 50% (v/v) deionized formamide, 5 × Denhardt's solution (1 × Denhardt's = 0.02% w/v each of BSA, Ficoll and polyvinylpyrrolidine), 5 × SSPE (1 × SSPE = 1.15 M NaCl, 0.01 M NaH_2PO_4 and 1 mM EDTA), 250 μg/ml denatured salmon sperm DNA and 0.1% SDS. At the end of the hybridization period the blots were washed twice in

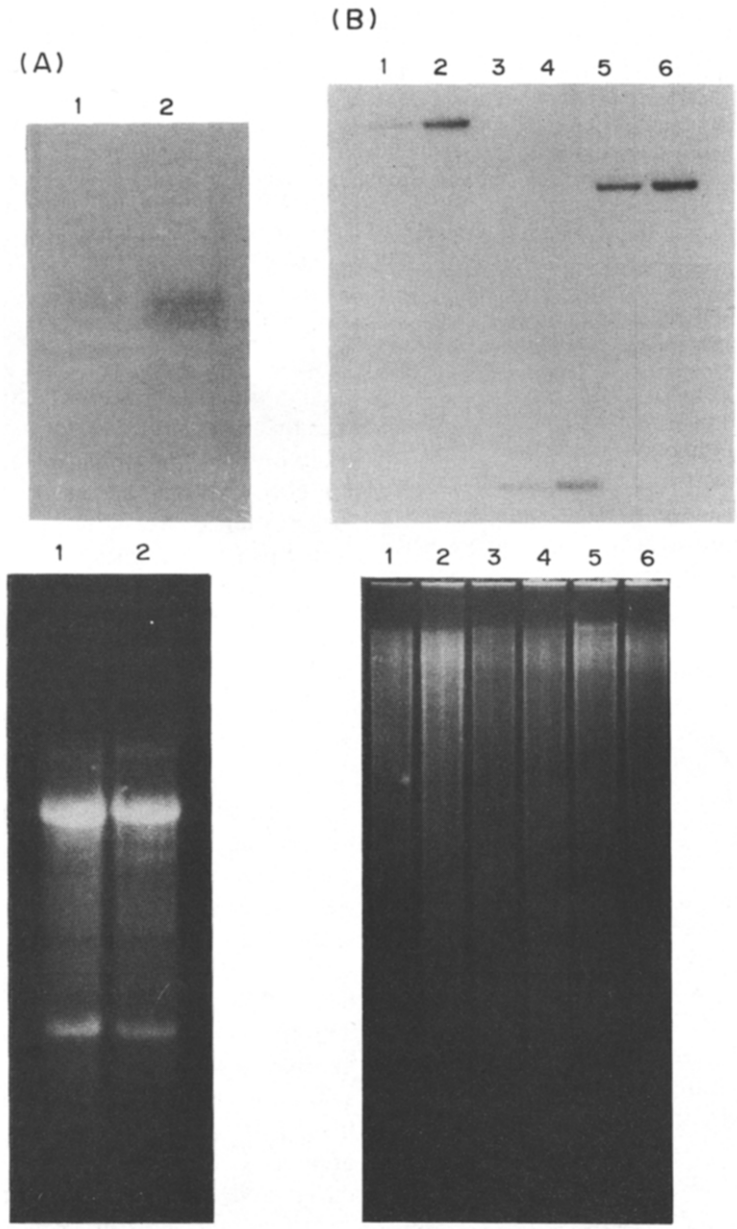

Fig. 1. (A): Top panel, Northern blot analysis of c-myc mRNA levels in exponentially growing T-47D (lane 1) and T-47D-5 (lane 2) cells. 30 μg of total RNA per cell line was used for the analysis; bottom panel, ethidium bromide stain of the above gel before transfer to nitrocellulose. (B): top panel, Southern blot analysis of the c-myc gene in DNA isolated from T-47D (lane 1, 3 and 5) and T-47D-5 (lane 2, 4 and 6) cells. 12.5 μg of DNA was subjected to restriction enzyme digestion with *Eco*RI (lane 1, 2), *Pvu*II (lane 3, 4) and *Bgl*II (lane 5, 6). The digested DNA was subjected to electrophoresis followed by transfer to nitrocellulose; bottom panel, ethidium bromide stain of above gel before transfer to nitrocellulose.

2 × SSC, 0.1% SDS 1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) for 15–30 min at room temperature, followed by three 20 min washes in 0.1 × SSC, 0.1% SDS at 65°C. Filters were exposed to Kodak XAR film at −70°C with an intensifying screen. Quantitation was achieved by densitometric scanning of various exposures of the autoradiograms (Biorad 1D analyst program) and was expressed relative to the control, arbitrarily assigned a value of 1.

RESULTS

The human breast cancer cell lines, T-47D and T-47D-5 contain detectable levels of an approx. 2.6 kb mRNA for the c-myc protooncogene [Fig. 1(A)]. Under conditions of logarithmic growth a comparison of the levels of c-myc mRNA between the two cell lines indicated that the T-47D-5 cells express approximately twice the level of that found in the T-47D cell line [Fig. 1(A)]. Southern blot analysis of DNA isolated from both cell lines indicates that the c-myc gene is present in T-47D-5 cells at approx. 5 times the copy number found in the T-47D cells [Fig. 1(B)].

The effect of two growth inhibitory agents, the antiestrogen monohydroxytamoxifen and the synthetic progestin medroxyprogesterone acetate (MPA) on the steady state level of c-myc mRNA was examined in both T-47D and T-47D-5 cells. At short times, 30–90 min after a single treatment a marked differential effect between the two compounds was observed in both cell lines (Fig. 2). Monohydroxytamoxifen decreased the level of c-myc mRNA as early as 30 min after treatment (Fig. 2, lane 2) with a maximal effect being evident at 90 min (Fig. 2, lane 6). Quantitation by densitometry and correction for RNA loading using the signal obtained by hybridizing the same filters with a ^{32}P-labeled 28S ribosomal cDNA probe, showed that c-myc RNA levels were decreased to 58 and 18% of control levels in T-47D and T-47D-5 cells, respectively. However, MPA caused a slight but reproducible increase in c-myc mRNA (using the 30 and 60 min time points; T-47D, 186 ± 30%, mean ± SEM, $n = 3$; T-47D-5, 180 ± 30%, $n = 3$) over this time period (Fig. 2 lanes 3, 5 and 7). The maximal effect was seen between 30 and 60 min after MPA treatment. The decreased level of c-myc mRNA due to monohydroxytamoxifen

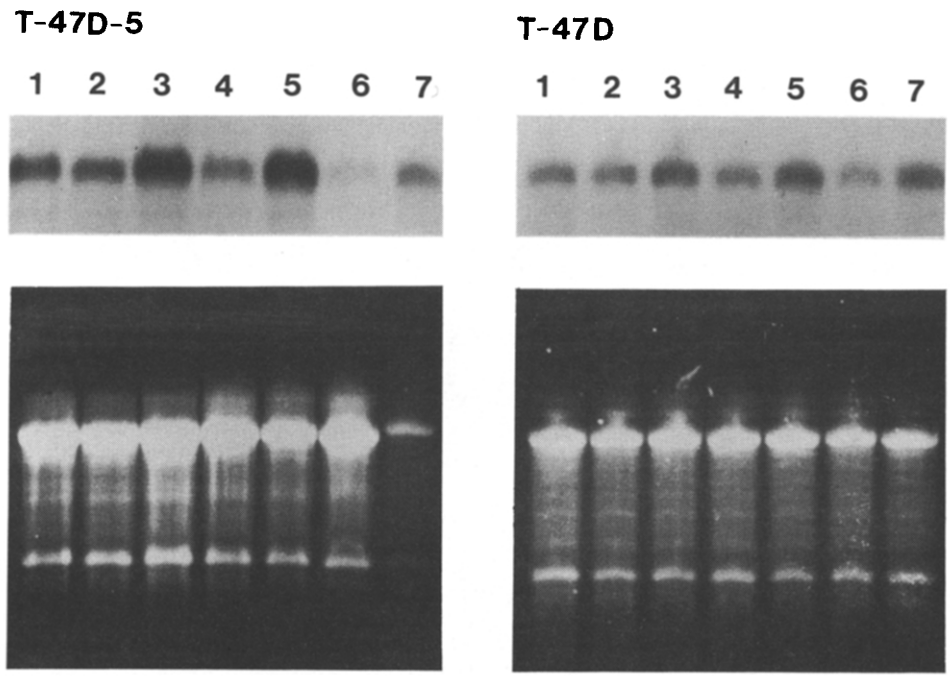

Fig. 2. The c-myc mRNA levels in T-47D-5 and T-47D cells which were untreated (lane 1) or had been treated with 1 μM MPA (lanes 3, 5 and 7) or with 1 μM monohydroxytamoxifen (lanes 2, 4 and 6) for 30 min (lanes 2 and 3), 60 min (lanes 4 and 5) and 90 min (lanes 6 and 7). 25–35 μg of total RNA for each group was subjected to Northern blot analysis. The bottom panel shows the ethidium bromide stained gels before transfer to nitrocellulose. The T-47D-5 autoradiogram was obtained by exposure to X-ray film for 20 h with an intensifying screen while that for T-47D cells was obtained by exposure for 48 h with an intensifying screen.

was maintained in both cell lines for at least 72 h which was the longest time point that we have examined [Fig. 3(A)]. However, the effect of MPA when examined over a longer time course [Fig. 3(B)] appeared to be biphasic with a decrease in c-myc mRNA levels occurring between 6 and 12 h after treatment. This decrease was maximal at 24 h. The level of c-myc RNA then was found to increase at 48 h in the case of the T-47D-5 cells and 72 h in the case of the T-47D cells.

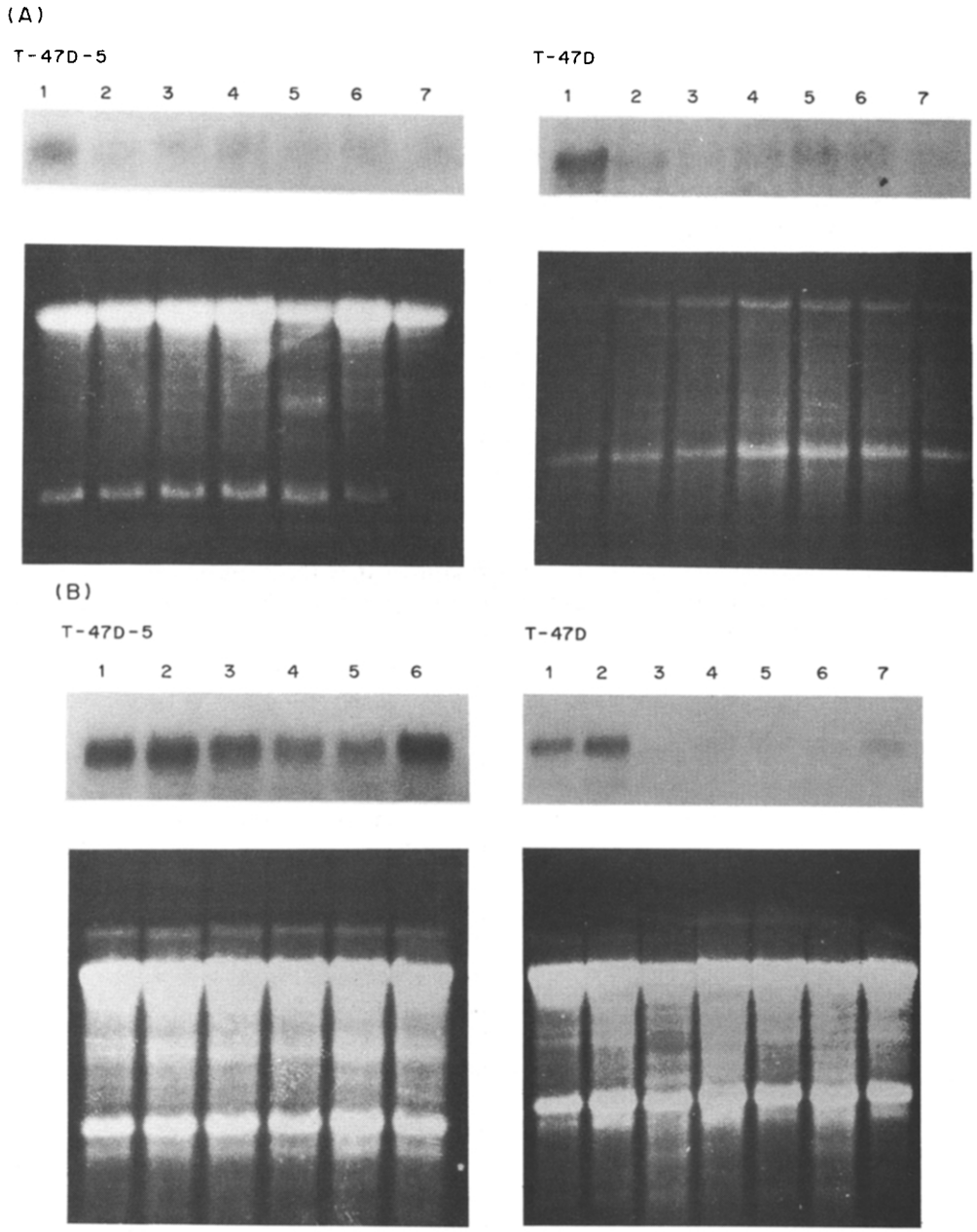

Fig. 3. (A): top panel, c-myc mRNA levels in T-47D-5 and T-47D cells which were untreated (lane 1) or treated with monohydroxytamoxifen (1 μM) for 1.5 h (lane 2), 6 h (lane 3), 12 h (lane 4), 24 h (lane 5), 48 h (lane 6) and 72 h (lane 7). 30 μg of total RNA per group was subjected to Northern blot analysis; bottom panel, ethidium bromide stained gels before transfer to nitrocellulose. The T-47D-5 autoradiogram was obtained by exposure to X-ray film for 3 days with an intensifying screen while that for T-47D cells was obtained by exposure for 7 days with an intensifying screen. (B): top panel, c-myc mRNA levels in T-47D-5 and T-47D cells which were untreated (lane 1) or treated with MPA (1 μM) for 1 h (lane 2), 6 h (lane 3), 12 h (lane 4), 24 h (lane 5), 48 h (lane 6) and 72 h (lane 7). 25 μg of total RNA per group was subjected to Northern blot analysis; bottom panel, ethidium bromide stained gels before transfer to nitrocellulose. The T-47D-5 autoradiogram was obtained by exposure to X-ray film for 11 h with an intensifying screen while that for T-47D cells was obtained by exposure for 20 h with an intensifying screen.

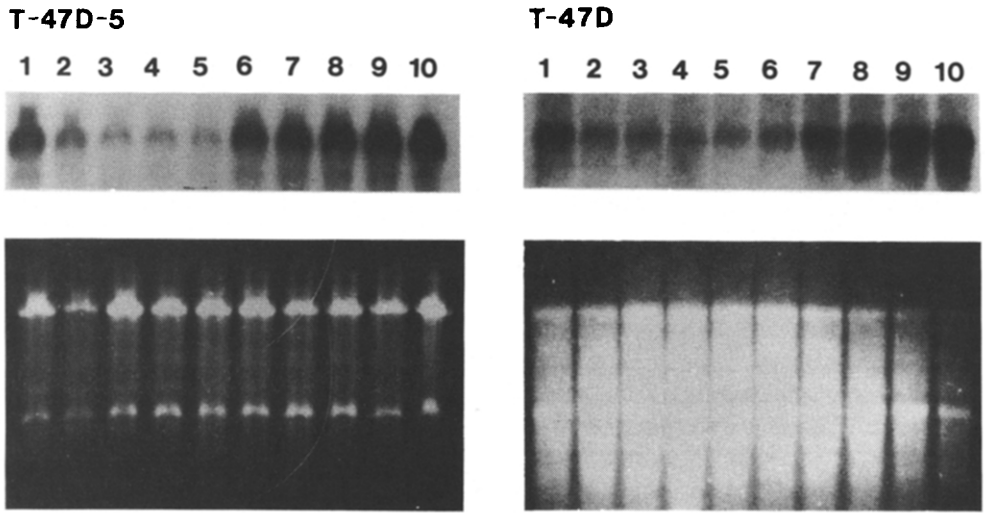

Fig. 4. Top panel, c-myc mRNA levels in T-47D-5 and T-47D cells which had been treated with vehicle alone (lanes 1 and 6), monohydroxytamoxifen (lanes 2, 3, 4 and 5) or MPA (lanes 7, 8, 9 and 10). The concentration of the two compounds were 10^{-9} M (lanes 2 and 7), 10^{-8} M (lanes 3 and 8), 10^{-7} M (lanes 4 and 9) and 10^{-6} M (lanes 5 and 10); bottom panel, ethidium bromide stains of the above gels before transfer to nitrocellulose. The T-47D-5 autoradiogram was obtained by exposure to X-ray film for 20 h with an intensifying screen while that for T-47D cells was obtained by exposure for 7 days with an intensifying screen.

Both monohydroxytamoxifen and MPA were used at a concentration of 1 μM in the above experiments. This is a high but noncytotoxic dose of the compounds [1, 2]. To ascertain the concentration range over which the effects described above are seen, a dose–response analysis was carried out. The results are presented in Fig. 4. As little as 10^{-9} M monohydroxytamoxifen decreased c-myc mRNA in both T-47D and T-47D-5 cells with a maximal effect occurring between 10^{-8} and 10^{-7} M. The initial increase in c-myc mRNA was seen in T-47D cells at 10^{-9} M MPA and above but in T-47D-5 cells the increase was only observed at 10^{-8} M MPA and above.

DISCUSSION

Expression of the c-myc gene has been found to be intimately associated with cell proliferation. The T-47D-5 cells have a higher steady state level of c-myc mRNA than T-47D cells. The increased c-myc expression in T-47D-5 cells can be explained, in part, by the presence of more c-myc gene copies per cell. T-47D-5 cells have been found to be hypertetraploid whereas T-47D cells are hyperdiploid [11]. However, this cannot explain the increased c-myc signal on Southern blot analysis of T-47D-5 DNA vs T-47D DNA since similar quantities of DNA were loaded. The c-myc gene is therefore amplified in T-47D-5 cells compared to the T-47D cells. Quantitation of the signal by densitometry indicated that there are at least 5 times more c-myc gene copies in T-47D-5 than in T-47D cells.

Stimulation of quiescent cells with growth factors [6] results in the movement of the cells into the cell cycle which is associated with a marked increase in c-myc expression. It was therefore not surprising that growth inhibition by the nonsteroidal antiestrogen, monohydroxytamoxifen, in both T-47D and T-47D-5 cells was accompanied by a rapid and sustained decrease in c-myc mRNA levels. Since tamoxifen has been shown previously to decrease the percentage of S phase cells and to concommitently increase the accumulation of cells in the G_0/G_1 phase of the cell cycle [7], the observed decrease in c-myc expression would be consistent with movement of cells out of the cell cycle. The c-myc mRNA levels have been previously shown not to vary substantially throughout the cell cycle [12, 13]. Altered expression reflects movement in and out of the cell cycle.

The effect of antiestrogen on c-myc mRNA levels probably occurs at the transcriptional level since it has been shown that estrogen stimulated growth in MCF-7 cells is accompanied by an increase in c-myc mRNA levels due to an increase in the transcription rate of the gene [14]. However, the initial stimulatory, inhibitory and partial recovery effect of MPA on c-myc mRNA levels was surprising since

this compound inhibits the growth of both the cell lines studied [1, 2]. The data obtained may however be explained in the light of the report from Clarke and Sutherland [15]. This group has studied the cell cycle kinetic effects of progestins on T-47D cells. Cells grown in phenol red-free, chemically defined medium were stimulated to grow by insulin. Treatment of these cells with the synthetic progestin ORG 2058 resulted in the inhibition of cell growth although initially there was a depletion of G_0/G_1 phase cells and a transient increase in the proportion of S phase cells peaking at 12 h after treatment. After 12 h the proportion of S phase cells fell rapidly as expected [8]. Although our growth conditions are different to those used by Clarke and Sutherland [15], the time course of the c-myc changes due to MPA treatment would be consistent with the phenomenon described by them. Following cell cycle arrest by MPA in T-47D cells a dose-dependent resumption of cell cycle progression has been reported [8]. The partial recovery of c-myc mRNA levels that we have observed after 72 h of treatment may reflect escape from G_0/G_1 arrest and re-entry into the cell cycle. It must however be remembered that the end-point of progestin and antiestrogen treatment is decreased cell number in T-47D and T-47D-5 cells [1, 2, 15]. The differential effect that we have observed between monohydroxytamoxifen and MPA might be expected to be associated with a difference in potency of the two compounds within each cell line. However, our previous data suggest that there is no difference in potency between the two compounds [2]. Furthermore, although the level of c-myc mRNA in T-47D-5 cells is higher than that in the T-47D cells, the modulation of c-myc expression by either monohydroxytamoxifen or MPA does not explain the differences in the sensitivity between the two cell lines to the growth inhibitory activity of these compounds [2].

The data presented in this report together with those previously published [1–3, 15–17] indicate that the mechanism(s) by which nonsteroidal antiestrogens and progestins cause growth inhibition of human breast cancer cells is different.

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