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Effects of Tamoxifen on Growth and Apoptosis of Estrogen-Dependent and -Independent Human Breast Cancer Cells

Roger R. Perry, MD, FACS, Yuan Kang, MD, PhD, and Bridget Greaves, BS

Background: Apoptosis ("programmed cell death") is an active process characterized by prominent nuclear changes and DNA cleavage, which distinguishes it from cellular necrosis. In this study we investigated whether tamoxifen (TAM) treatment of estrogen receptor ER(+) MCF-7 and ER(−) MDA-231 human breast cancer cells resulted in cytotoxicity and cellular changes typical of apoptosis.

Methods: Cytotoxicity was measured using a tetrazolium assay. Cellular morphologic changes were observed using transmission electron microscopy. DNA cleavage was assessed using 1.6% agarose gel electrophoresis and was also quantitated biochemically.

Results: Exposure of cells to TAM for 24 h resulted in dose- dependent cytotoxicity, and MCF-7 cells were somewhat more sensitive to TAM. TAM induced chromatin condensation around the nuclear periphery in both cell lines, changes typical of apoptosis. TAM-induced cytotoxicity correlated with dose-dependent DNA cleavage, which showed the characteristic "internucleosomal ladder." DNA cleavage occurred at a slightly lower TAM dose and occurred somewhat sooner in MCF-7 cells. TAM-induced DNA cleavage in MCF-7 cells was inhibited by the protein synthesis inhibitor cycloheximide, the RNA synthesis inhibitor actinomycin D, and by 17β-estradiol. However, in MDA-231 cells, DNA cleavage was inhibited by cycloheximide, partially but not significantly inhibited by actinomycin D, and not inhibited by 17β-estradiol.

Conclusions: TAM induces typical apoptosis in ER(+) or ER(−) human breast cancer cells. TAM induction of apoptosis in MCF-7 cells involves the estrogen receptor, and requires the synthesis of new protein and mRNA. TAM induction of apoptosis in MDA-231 cells depends primarily on protein synthesis. TAM-induced cytotoxicity and DNA damage appear to be explained in part by the induction of apoptosis.

Key Words: Apoptosis—Tamoxifen—Breast cancer—MCF-7 cells—MDA-231 cells.

The antiestrogen drug tamoxifen (TAM) is widely used for the treatment of breast cancer. Various studies suggest that TAM is useful for the treatment of women with early-stage breast cancer, regardless of whether or not their tumors express the estrogen receptor (ER) (1). TAM also has significant cytoxic and cytostatic effects (2,3), and cell cycle kinetic effects (4,5) toward both ER(+) and ER(−) breast cancer cell lines in vitro. The effects of TAM appear to be ER dependent at lower doses and ER independent at higher doses (5). Thus, TAM may have both ER-mediated and non-ER-mediated activity. The mechanism(s) of action of TAM, including its apparent activity against cells that lack ER, is poorly understood.

Apoptosis (programmed cell death) is a process that regulates cell number or eliminates damaged cells, and that differs morphologically and biochemically from cellular necrosis (6). Apoptosis occurs in both normal and neoplastic tissues and is an active

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Division of Surgical Oncology, Eastern Virginia Medical School, Norfolk, VA, 23507-1912
Address correspondence and reprint requests to Dr. R. R. Perry, Eastern Virginia Medical School, 825 Fairfax Avenue, Suite 610, Norfolk, VA 23507-1912, U.S.A.
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process characterized by condensation of nuclear chromatin, compaction of cytoplasmic organelles, reduction of cell volume, and internucleosomal DNA fragmentation, with eventual splitting of the cell into a series of membrane fragments that then undergo phagocytosis (7). Apoptosis may occur under normal physiologic circumstances including during development, in response to hormonal stimulation, during metamorphosis, and in the targets of T- and natural killer-cells (7).

The precise mechanism(s) whereby apoptosis is induced remains unknown. In most cases, apoptosis appears to be linked to the synthesis of new protein and the activation of specific genes (8). Evidence suggests that apoptosis is involved in cell death induced by a variety of chemotherapeutic drugs including doxorubicin (9), cisplatinum (10), nitrogen mustard (11), topoisomerase I inhibitors including camptothecin (12), topoisomerase II inhibitors including etoposide (12), cytokines including transforming growth factor-β_1 (TGF-β_1) (13), and tumor necrosis factor (TNF-α) and interferon gamma (IFN-γ) (14), glucocorticoids (15), androgens (16), and gonadotropin-releasing hormone analogues and somatostatin (17).

This report describes the cytotoxic effects of TAM toward ER(+) MCF-7 and ER(−) MDA-231 human breast cancer cells, and provides evidence that TAM-induced cytotoxicity correlates with morphologic changes and internucleosomal DNA cleavage typical of apoptosis. The effects of an RNA synthesis inhibitor, a protein synthesis inhibitor, and estrogen on TAM-induced DNA cleavage are also presented, and demonstrate that TAM-induced DNA cleavage is an active process. The results of this study suggest that TAM cytotoxicity appears to be explained, at least in part, by the induction of apoptosis.

MATERIALS AND METHODS

Cell lines

The MCF-7 cell line was originally obtained from the Michigan Cancer Foundation, and the MDA-231 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). These cell lines have been extensively studied and characterized. MCF-7 cells are ER(+) (18) and MDA-231 cells are ER(−) (19). Cells were maintained in α-minimal essential medium (α-MEM) supplemented with 5% fetal calf serum, glutamine, penicillin, and streptomycin. The cells were incubated at 37°C in a 95% air/5% CO_2 environment. Forty-eight hours before each experiment, the cells were transferred to phenol-red free medium with charcoal dextran stripped fetal calf serum to remove exogenous estrogens (20).

Drugs

Tamoxifen (trans 1-[4-β-dimethylaminoethoxyphenyl]-1,2-diphenylbut-1-ene, TAM) was provided by Zeneca Pharmaceuticals Group (Wilmington, DE, USA). A stock solution was prepared in 2% ethanol and was stored at −20°C. All other chemicals used were reagent grade and were obtained commercially.

Cytotoxicity assay

Cytotoxicity was measured by using a microculture tetrazolium assay as described by Carmichael et al. (21) with modifications (22). Briefly, 2×10^3 cells were incubated in each well of 96-well plates (Corning 25860, Corning, NY, USA) for 24 h before drug addition. Cells were then treated with various doses of TAM for up to 48 h. Controls consisted of untreated cells. After treatment, the cells were grown in drug-free medium for 48 h and then 100 μg of MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well for 4 h. The medium was removed by aspiration, and 150 μL of mineral oil was added to each well to solubilize the MTT formazan crystals. The spectrophotometric absorbance at 570 nm was determined using a scanning multiwell spectrophotometer (EL340, Bio-Tek Instruments, Inc., Winooski, VT,), and cell surviving fraction and IC_{50} values were calculated.

Electron microscopy

Breast cancer cells treated with TAM and untreated control cells were recovered from cultures and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde. The fixed cells were embedded in polybed 812 and sectioned, stained with uranyl acetate and lead citrate, and observed in a JEOL 1200 II transmission electron microscope (Japan Electronic Optical Ltd., Tokyo, Japan), operating at an accelerating voltage of 60 KV.

DNA isolation and gel electrophoresis

Breast cancer cells (4×10^6) were seeded into 75-cm^2 flasks (Falcon, Becton Dickinson). After 24 h the cells were treated at 37°C with different doses of TAM for varying periods of time. In some experiments, different inhibitors were added. Cellular DNA from each treatment group was isolated from

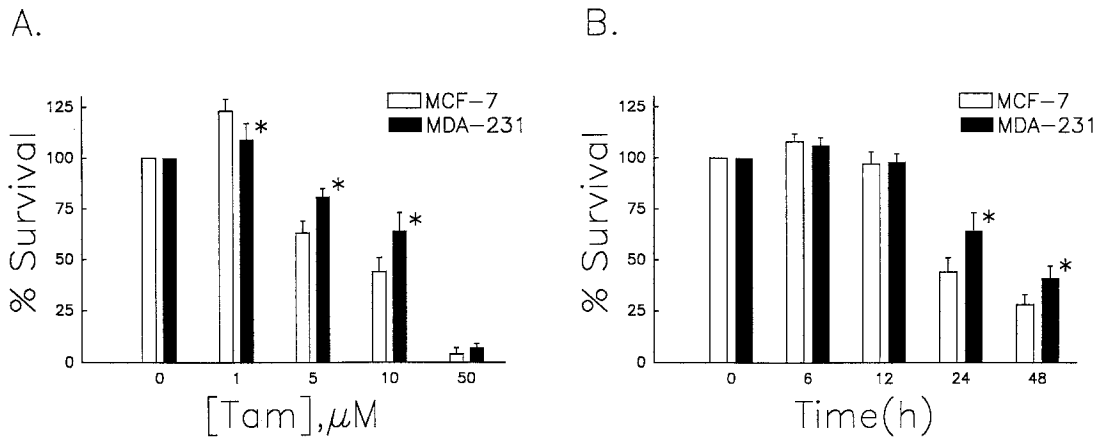

FIG. 1. Tamoxifen (TAM) cytotoxicity in ER(+) MCF-7 and ER(−) MDA-231 cells. A: Cells treated with different doses of TAM for 24h. B: Cells treated with 10 μ*M* TAM for up to 48 h. Cell survival was measured using the MTT assay. Each bar is plotted as the mean ± SE of four independent experiments. (*$p < 0.05$ compared with MCF-7 cells.)

5×10^6 cells. The cells were washed in phosphate-buffered saline, and the cell pellet was resuspended in 1.0 ml of 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, 10 m*M* EDTA (pH 7.0), containing 1% (wt/vol) sodium lauryl sarkosinate, and 0.5 mg/ml proteinase K. Proteolytic digestion was allowed to proceed at 50°C for 2 h. The DNA was precipitated with 2 vol of absolute ethanol, resuspended in 10 m*M* Tris-HCl, 1 m*M* EDTA buffer (pH 8.0), heated to 70°C, and loaded onto a 1.6% agarose gel. Pulse field electrophoresis was carried out in 40 m*M* Tris-acetate, 1 m*M* EDTA (pH 8.0), until the marker dye had migrated 4–5 cm. The gels were stained with ethidium bromide and DNA was visualized under UV light.

Measurement of DNA cleavage

DNA cleavage was measured as described by McConkey (23), with minor modifications. Briefly, breast cancer cells treated with TAM or untreated control cells were gently scraped from cultures and centrifuged at 500 *g*. The pellet was lysed with cold lysis buffer (20 m*M* EDTA, 0.5% Triton X-100, and 5 m*M* Tris-HCl, pH 8.3). After 30 min, the cell samples were centrifuged at 27,000 *g* to separate intact high-molecular-weight chromatin (pellet) from cleaved DNA fragments (supernatant). Diphenylamine reagent was added to the pellets and supernatants, and DNA content was determined by measuring the absorbance at 260 and 280 nm using a Hitachi U-2000 spectrophotometer (Hitachi Inc., Danbury, CT, USA). The results are expressed as the percentage of cleaved DNA, determined from the ratio of the DNA contents of the supernatants and pellets.

Statistics

All results are expressed as the mean ± SE. Differences between the means of different groups were determined using the Wilcoxon rank-sum test.

RESULTS

Cytotoxicity of TAM towards MCF-7 and MDA-231 cells

Cell survival curves were determined after treatment of MCF-7 and MDA-231 cells with different concentrations of TAM for 24 h (Fig. 1A). TAM induced dose-dependent cytotoxicity in both cell lines. The TAM IC_{50} values were 8.0 ± 0.7 μ*M* in MCF-7 cells and 15.2 ± 2.3 μ*M* in MDA-231 cells ($p < 0.05$). MCF-7 cells were somewhat more sensitive to stimulation at low TAM dose (1 μ*M*), and cytotoxicity at higher TAM doses (≥5 μ*M*) compared with MDA-231 cells (Fig. 1A). TAM also induced time-dependent cytotoxicity in both cell lines, and MCF-7 cells were slightly more sensitive to TAM (Fig. 1B).

Morphologic changes Induced by TAM

Cells were treated with TAM for 24 h and then studied using electron microscopy to see if the observed morphologic changes were typical of apoptosis. Compared with untreated cells, MCF-7 and MDA-231 cells treated with TAM exhibited chromatin condensation at the nuclear periphery, and reduction in nuclear size (Fig. 2). Other changes noted included shrinkage of total cell volume, compaction of cytoplasmic organelles, and dilatation and vacuolization of the endoplasmic reticulum.

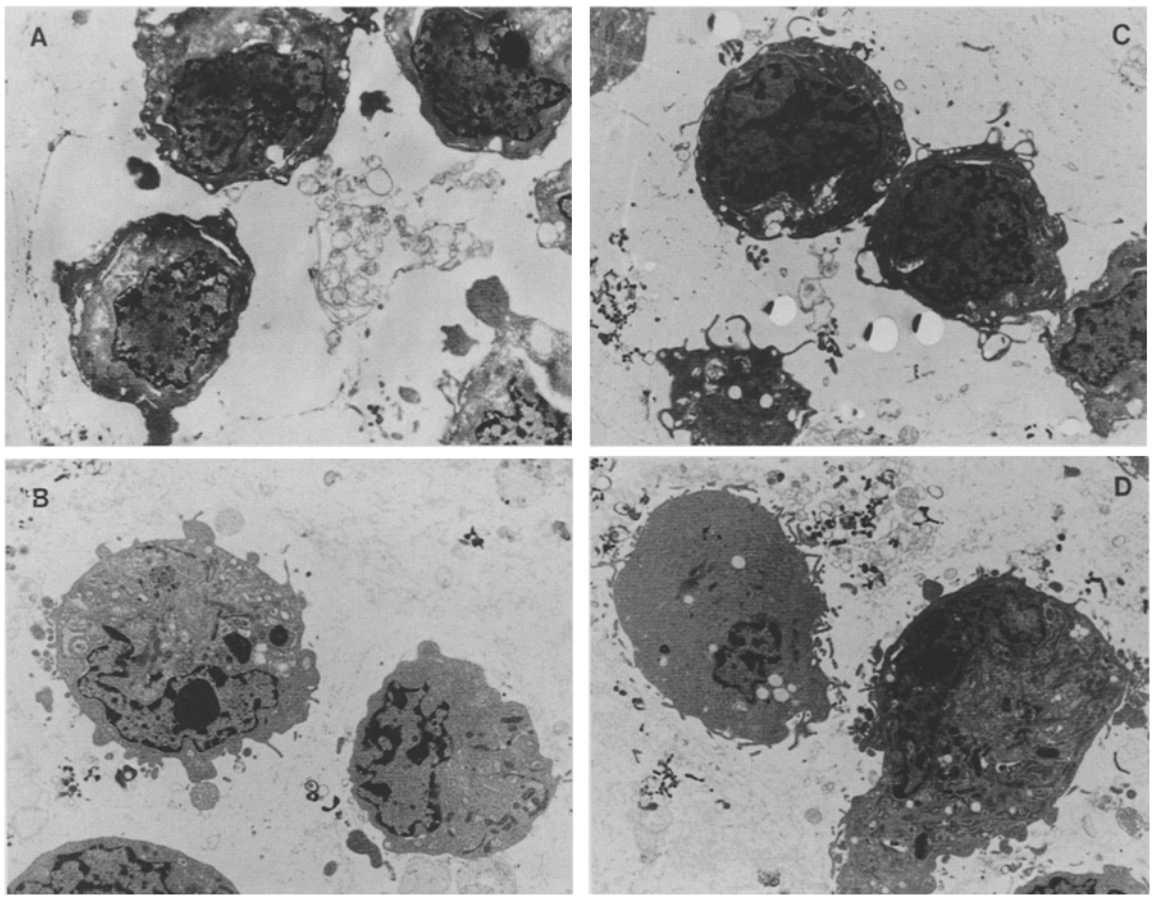

FIG. 2. Morphologic changes in MCF-7 and MDA-231 cells induced by tamoxifen (TAM) for 24 h. The electron micrographs shown are representative of the changes observed in four independent experiments. A: MCF-7 cells, control. B: MCF-7 cells treated with 10 μM TAM for 24 h. C: MDA-231 cells, control. D: MDA-231 cells treated with 10 μM TAM for 24 h. (Sections stained with uranyl acetate and lead citrate, ×6,000.)

These morphologic changes are consistent with apoptosis.

Effects of TAM on DNA cleavage

DNA was isolated from TAM-treated cells and subjected to agarose gel electrophoresis to determine whether DNA cleavage typical of apoptosis was observed. TAM induced dose-dependent DNA cleavage in both cell lines (Fig. 3). This cleavage exhibited the characteristic "internucleosomal ladder." Densitometry measurements of the gels (data not shown) and quantitative measurement of DNA cleavage (Table 1) demonstrated that cleavage occurred at a lower TAM dose in MCF-7 cells (5 μM) compared with MDA-231 cells (10 μM). The dose dependence of the TAM-induced DNA cleavage appeared to correlate with the observed cytotoxicity (Fig. 1A). Time course studies also demonstrated that TAM-induced DNA cleavage was time dependent (Fig. 4). Densitometry measurements of the gels (data not shown) and quantitative measurement of DNA cleavage (Table 2) demonstrated that 10 μM of TAM induced small but significant amounts of DNA cleavage sooner in MCF-7 cells (12 h) compared with MDA-231 cells (24 h). There was no increase in DNA cleavage in control cells in culture for up to 48 h.

Effects of inhibition of protein synthesis, inhibition of RNA synthesis, and of estrogen on DNA cleavage

In most instances, apoptosis is an active process, requiring synthesis of proteins and/or new RNA (8). Therefore, the ability of 10 μM TAM to induce DNA cleavage was assessed after the addition of the protein synthesis inhibitor cycloheximide (0.35 μM) or the RNA synthesis inhibitor actinomycin D (0.2 μM). The ability of TAM to induce DNA cleav-

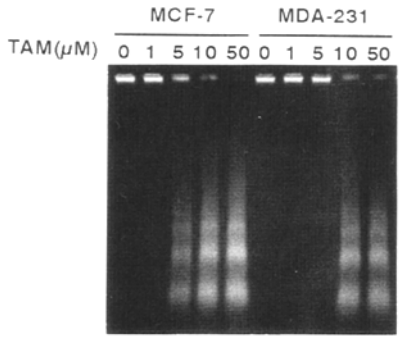

FIG. 3. Dose dependence of tamoxifen (TAM)-induced DNA cleavage. MCF-7 and MDA-231 cells were treated with various doses of TAM for 24 h. DNA was isolated and cleavage was determined using agarose gel electrophoresis. The results shown are typical of the four independent experiments performed.

age was also assessed after the addition of 17β-estradiol (10 μ*M*). These experiments demonstrated that TAM-induced DNA cleavage in MCF-7 cells was inhibited by cycloheximide, actinomycin D, and 17β-estradiol (Fig. 5, Table 3). However, in MDA-231 cells, TAM-induced DNA cleavage was inhibited by cycloheximide, and was partially, but not significantly, inhibited by actinomycin D. DNA cleavage in MDA-231 cells was not inhibited by 17β-estradiol.

DISCUSSION

TAM induces typical apoptosis in breast cancer cells, regardless of the cells' ER status. TAM-induced apoptosis is both time and dose dependent, occurring earlier and at lower doses in ER(+) cells. The results of inhibition experiments show that the mechanism is somewhat different in ER(+) cells compared with ER(−) cells. TAM induction of apoptosis in ER(+) cells occurs via an ER-mediated

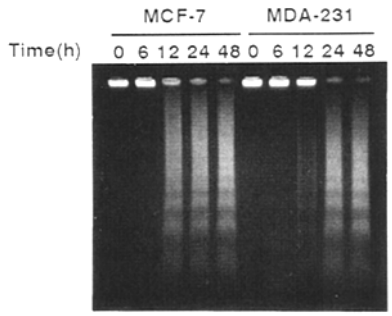

FIG. 4. Time dependence of TAM-induced DNA cleavage. Cells were exposed to 10 μ*M* TAM for 6–48 h. DNA was isolated and cleavage was determined using agarose gel electrophoresis. The results shown are typical of the four independent experiments performed.

TABLE 1. *Dose dependence of tamoxifen (TAM)-induced DNA cleavage (%) in MCF-7 and MDA-231 cells*[a]

TAM (μ*M*)	MCF-7	MDA-231
0	4.8 ± 0.7[b]	4.5 ± 0.5
1	5.4 ± 0.6	4.4 ± 0.6
5	10.5 ± 1.3[c]	5.6 ± 1.3
10	21.6 ± 1.6[c]	18.6 ± 1.8[c]
50	26.8 ± 2.3[c]	20.6 ± 2.2[c]

[a] Cells exposed to TAM for 24 h.
[b] $\bar{x}$ ± SE of six independent experiments.
[c] $p < 0.01$ as compared with control.

process and requires the synthesis of new protein and mRNA. On the other hand, TAM induction of apoptosis in ER(−) cells appears to depend primarily on protein synthesis. Apoptosis may not be the only mechanism of TAM- induced cytotoxicity, because 5 μ*M* of TAM caused some cytotoxicity in MDA-231 cells (Fig. 1A), yet DNA fragmentation was not observed.

Chemotherapeutic drugs and hormones with widely diverse mechanisms of action are capable of inducing apoptosis (9–12,15–17). Growth factors and cytokines including TGF-β_1 (13), and TNF-α and IFN-γ (14) have also been shown to trigger apoptosis. Other cytokines including epidermal growth factor, transforming growth factor-α (TGF-α), and basic fibroblast growth factor apparently inhibit apoptosis (24). The variety of substances with differing mechanisms of action that can trigger or inhibit morphologic and biochemical changes characteristic of apoptosis suggests the presence of a final common apoptoic pathway (25). Cells appear to possess a damage assessment mechanism, a cell cycle progression regulatory mechanism, and a trigger of apoptosis (26). However, it is unclear precisely how drug–tumor interactions result in cell death, nor is it known precisely how cells "sense" damage (27).

TABLE 2. *Time dependence of tamoxifen (TAM)-induced DNA cleavage (%) in MCF-7 and MDA-231 cells*[a]

Time (h)	MCF-7	MDA-231
0	4.8 ± 0.7[b]	4.5 ± 0.5
6	5.1 ± 0.7	5.5 ± 0.8
12	8.6 ± 1.1[c]	4.8 ± 0.9
24	21.6 ± 1.6[d]	18.6 ± 1.8[d]
48	36.9 ± 2.2[d]	30.6 ± 2.0[d]

[a] Cells exposed to 10 μ*M* TAM.
[b] $\bar{x}$ ± SE of six independent experiments.
[c] $p < 0.05$ as compared with control.
[d] $p < 0.01$ as compared with control.

Several genetic changes have been associated with apoptosis. Cells with increased expression of *c-myc* are more prone to undergo apoptosis when cell cycle progression is blocked (28). Cells with increased expression of wild type *p53* are also prone to undergo apoptosis (29). On the other hand, cells with increased expression of *bcl*-2 appear to be resistant to undergoing apoptosis (30,31). However, in no instance has an essential role for any gene in apoptosis in mammals been identified (25).

Cells that are inhibited from undergoing apoptosis are less sensitive to chemotherapeutic drugs, suggesting that prevention of apoptosis may be an important drug-resistance mechanism (31). Cells that are multidrug resistant (MDR) appear to maintain their sensitivity to TAM (32), and TAM may be useful in reversing MDR (22, 32). Whether TAM induces apoptosis in MDR cells is currently being studied by our laboratory.

The mechanism of action of TAM is poorly understood, but it appears TAM has both ER- and non-ER-mediated activity (1–5). Based on this study, TAM cytotoxicity in ER(+) and ER(−) cells is mediated, at least in part, via the induction of apoptosis. Type II estrogen binding sites have been identified, and may be a pathway through which some of the effects of TAM are exerted in ER(−) cells (33). However, in our study the addition of estrogen did not inhibit TAM-induced apoptosis in ER(−) cells, suggesting that apoptosis in these cells is triggered by another mechanism.

There are little data in the literature on the apoptotic effects of antiestrogens. One study examined the effects of 4-hydroxytamoxifen, a TAM metabolite, on apoptosis in breast cancer cells (34). At the dosage used, cytotoxicity and morphologic changes characteristic of apoptosis were observed in ER(+) cells, but neither cytotoxicity nor apoptosis occurred in ER(−) cells. We observed cytotoxicity and apoptosis with TAM treatment in ER(−) cells, but slightly higher dose and/or longer treatment duration were required compared with ER(+) cells. A recent study showed the antiestrogen drug toremifene also induced cytotoxicity and the morphologic changes of apoptosis in ER(+) cells, but a DNA ladder was not observed (35). Using somewhat different methodology, we observed a typical DNA ladder in cells treated with TAM.

It remains unclear precisely how TAM triggers apoptosis in the breast cancer cell lines examined in this study. However, because TAM causes a G_1/G_0 block (4,5,22), it may be that these cells already contain genetic alterations, such as abnormal expression of *c-myc,* which make them prone to undergo apoptosis in the presence of a cell cycle block (28). Alternatively, TAM may cause the release of growth factors such as TGF-β_1 (36,37), which may trigger apoptosis by activation of signal transduction pathways and/or by effects on *c-myc* expression (13,38). These are areas that are currently being investigated by our laboratory.

The levels of TAM used in this study are generally higher than those measured in the serum of patients receiving standard-dose TAM therapy. Patients taking 20–80 mg of TAM daily have mean serum levels of 0.2– 0.3 μ*M* (39). Patients treated with higher doses of TAM, up to 720 mg per day, have serum levels of up to 3.5 μ*M* (40). However, antiestrogen drugs accumulate in tissues at levels 16–30 times higher than in serum. For example, levels up to 6.6 μ*M* have been measured in brain metastases in patients taking 20–80 mg of TAM per day

TABLE 3. *Effect of inhibitors on tamoxifen (TAM)-induced DNA cleavage (%)*

Treatment group[a]	MCF-7	MDA-231
Control	4.6 ± 0.5[b]	4.2 ± 0.5
TAM (10 μ*M*)	20.3 ± 1.4	17.3 ± 0.9
TAM + estradiol (10 μ*M*)	6.2 ± 0.4[c]	19.4 ± 1.3
TAM + actinomycin D (0.2 μ*M*)	6.4 ± 0.6[c]	14.9 ± 1.2
TAM + cycloheximide (0.35 μ*M*)	6.5 ± 0.7[c]	7.4 ± 0.5[c]

[a] All groups treated for 24 h.
[b] $\bar{x}$ ± SE of six independent experiments.
[c] $p < 0.01$ as compared with TAM alone.

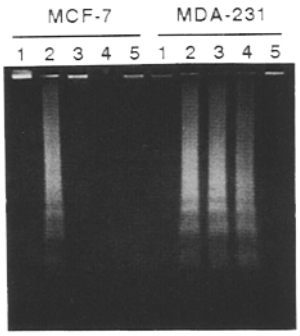

FIG. 5. Effect of inhibitors on tamoxifen (TAM)-induced DNA cleavage. The ability of 10 μ*M* TAM to induce DNA cleavage was assessed after the addition of various inhibitors. DNA was isolated and cleavage was determined using agarose gel electrophoresis. The results shown are typical of the four independent experiments performed. (Lane 1: saline control, Lane 2: 10 μ*M* TAM, Lane 3: TAM plus 17β-estradiol (10 μ*M*), Lane 4: TAM plus the RNA synthesis inhibitor actinomycin D (0.2 μ*M*), and Lane 5: TAM plus the protein synthesis inhibitor cycloheximide (0.35 μ*M*)).

(39). Thus, the concentrations of TAM used in this in vitro study may occur in clinical tumors treated with TAM.

Apoptosis may represent a common mechanism whereby a variety of antitumor treatments result in cell death. The particular cellular target may not be so important, but rather activation of the apoptotic pathway via direct induction, release of inhibition, or by signal transduction (25) may be critical for cytotoxicity to occur. Strategies and substances directed toward triggering apoptosis may thus represent a novel and potentially useful approach to treating cancer.

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