Pharmacodynamics of Tamoxifen and Its 4-Hydroxy and N-Desmethyl Metabolites: Activation of Caspases and Induction of Apoptosis in Rat Mammary Tumors and in Human Breast Cancer Cell Lines

Sandhya Mandlekar, Vidya Hebar, Konstantin Christov, and A-N. Tony Kong

ABSTRACT

The antiestrogen tamoxifen (TAM) is extensively metabolized by cytochrome P-450 in humans and rodents. The active, estrogen receptor-binding metabolites, 4-hydroxy TAM (OHT) and N-desmethyl TAM (DMT) have been well characterized. We showed that the s.c. injection of 1 mg/kg TAM in adult female Sprague Dawley rats bearing carcinoma-induced mammary tumors resulted in rapid serum decline of parent TAM but higher exposure of the metabolites, OHT and DMT. We found for the first time that the administration of TAM for a short time resulted in a delayed induction of caspase activity and apoptosis within the mammary tumors. When TAM, OHT, or DMT was added to human breast cancer cell lines in culture, each elicited a time- and dose-dependent induction of caspase activity, preceding apoptosis. Importantly, pretreatment of the cells with a pharmacological inhibitor of caspases [benzoxly Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk)] blocked apoptosis induced by all three of the compounds, implicating a critical role of caspases in TAM-, OHT-, or DMT-induced apoptosis. The results obtained from these studies suggest that one possible mechanism of inhibition of mammary carcinogenesis and tumor growth in vivo may be the induction of caspase-dependent apoptosis, and that the metabolites OHT and DMT may contribute to the antitumor effect of TAM.

INTRODUCTION

The antiestrogen TAM is widely used in the treatment of breast cancer and recently has been approved as a cancer chemopreventive agent in high-risk women (1). It is metabolized by the CYP subunits leading to the formation of OHT and DMT (Fig. 1), which have been extensively studied (2–7). The biotransformation in humans is primarily attributable to the action of CYP 3A4 (8) and CYP 2D6 (9). In humans as well as in rodents, OHT and DMT are shown to be the major metabolites, present in high levels in both the plasma and the tumor tissues (2, 4, 10, 11). It has been reported that the metabolites OHT and DMT possess several hundred times more or equal affinity, respectively, toward the ER as compared with the parent TAM (12, 13). Thus, OHT and DMT are considered to be more active than or equally active as TAM in retarding the estrogen-dependent cell growth and proliferation of ER-positive mammary tumors.

Recently, we showed that TAM induces apoptosis in both ER-positive and ER-negative human breast cancer cells by activating the caspase pathway (14). The caspases are intracellular cysteine-containing proteases that cleave various cytoplasmic, structural, as well as nuclear proteins in a tetrapeptide-sequence-specific manner after an aspartate residue (15, 16). The process of apoptosis, which has been shown to be responsible for anticancer drug-induced cell death and tumor regression, is dependent on activation of the caspase cascade (17). The 15 members of the caspase family have been classified into three subfamilies (18). The most upstream caspases in the cascade are caspases 8 and 9. The former is associated with the cell membrane death receptors through its death effector domain (19), whereas the latter is activated from its cytoplasmic precursor in an ATP-dependent manner on the binding of cytochrome c, released from the mitochondria in conjunction with Apaf-1 (20). The downstream or effector caspases comprise the caspases 3, 6, and 7. On proteolytic activation by upstream caspases, the effector caspases cleave a variety of protein substrates (21), including poly(ADP-ribose) polymerase [or PARP (22)], DNA fragmentation factor (23), DNA-dependent protein kinase (24), protein kinase C θ (25), and others. The cleavage of the various substrates contributes to the typical morphological and biochemical features observed in the apoptosis of cells.

Induction of apoptosis can be considered as one of the pharmacodynamic end points in the measurement of the efficacy of chemotherapeutic agents (26). It has been shown that daily administration of TAM (1 mg/kg) resulted in the regression of NMU-induced rat mammary tumors, and that this has been associated with the inhibition of cell proliferation and induction of apoptosis (27, 28). NMU-induced mammary tumors are ER-positive and have been used as a preclinical in vivo model for most of the breast cancer chemoprevention and therapy studies, because they are very similar in morphology and origin to human breast cancer (29). Because the antitumor mechanism of TAM action is yet to be completely understood and its metabolites have been shown to possess growth inhibitory activity, and because caspase-dependent apoptosis may be a determinant of the cytotoxicity of anticancer agents both in vitro and in vivo, we evaluated the role of TAM metabolism in terms of caspase activation and induction of apoptosis in rat mammary tumors as well as in human breast cancer cells. In contrast to most previous studies, here we assessed the early (1–4 days) cellular responses of mammary tumors to TAM and its metabolites.

MATERIALS AND METHODS

Cell Culture, Chemicals, and Treatment. Human breast cancer cell lines MCF-7 and BT-20 were obtained from American Type Culture Collection. The human breast cancer cell line MDA MB 231 was provided by Dr. Carol Westbrook (Department of Hematology and Oncology, College of Medicine, University of Illinois at Chicago). The cells were maintained in MEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. NMU, TAM, OHT, V-E (α-tocopherol), DAPI were all obtained from Sigma (St. Louis, MO). The hydrochloride of DMT was generously provided by Dr. M. Matilde Marques (Instituto Superior Tecnico, Lisbon, Portugal). The fluorogenic tetrapeptide substrates of caspase-3 (Ac-DEVD-MCA), and caspase-1 (Ac-YVAD-MCA) were obtained from Peptides International (Louisiana).
CAPESE INDUCTION BY TAM METABOLITES

isville, KY), and substrates for caspase-8 (Ac-IETD-MCA), caspase-9 (Ac-LEHD-FCA), and the pan-caspase inhibitor, z-VAD-fmk were purchased from Calbiochem (San Diego, CA). For treatment with chemicals, the medium was removed when cells were about 80% confluent and was replaced with serum-free MEM. For pretreatment, the cells were incubated with the respective inhibitors for 1 h followed by treatment with chemicals.

Animals and Treatment. The protocol for the animal experiment was approved by the Animal Care Committee of University of Illinois. Adult female Sprague Dawley rats (average weight, 250 g) were housed three per cage and fed 4% Purina diet ad libitum. The rats received two i.p. injections of NMU (Sigma; 50 mg/kg of body weight in acidified saline) at the age of 50 and 57 days. Three weeks after NMU injection, the animals were palpated weekly to monitor the appearance of mammary tumors. When the palpable tumors reached 5 mm in diameter, TAM (emulsified in corn oil at 1 mg/ml; injection volume, ~0.25 ml) treatment was initiated. All of the animals except the controls received two s.c. injections of TAM, 1 mg/kg of body weight, 12 h apart (8 p.m. and 8 a.m.). After several repeats of this schedule, the rat mammary tumors showed significant regression (30). Therefore, we sought to investigate the early signaling pathway leading to this response, i.e., induction of caspase-dependent apoptosis in mammary tumors. The rats were divided into groups of five animals each; one group was left untreated (control) and others were killed at 1.5, 3.5, 5, 7, 18, 24, 48, 72, and 96 h after the second TAM administration (no samples were taken in between the two TAM doses; and for the sake of convenience, the data are reported by considering the time of the second TAM administration, which was 12 h after the first dose as zero time point). After light ether anesthesia, the animals were killed by exsanguination via the abdominal aorta. Blood was collected, allowed to clot for 30 min at 4°C, and centrifuged at 12,000 rpm to separate the serum. The serum was stored at −70°C for subsequent HPLC analyses of TAM and metabolites concentration as described below. Mammary tumors were dissected out and cut into small portions, a portion of the sample was stored in formalin for histochimical analysis, and others were frozen at −70°C for subsequent determination of caspase activity.

HPLC Assay for TAM and Metabolites. The parent drug TAM and its metabolites (OHT, DMT, OHT), together with the internal standard TPP (which was metabolized by CYP 3A1 (rat), was formed rapidly after TAM administration and attained a Cmax of 385 ± 132 ng/ml at 5 h, and subsequently declined to BDL after 7 h (Fig. 2B; Table 1)). A second metabolite, DMT, presumably biotransformed by CYP 2D1 (rat) and also 3A1, appeared relatively late as compared with OHT (Fig. 2C). It reached a Cmax of 2136 ± 1516 ng/ml at 48 h, subsequently declined slowly, and was detected even at 96 h. The total exposure in rat serum of OHT and DMT was considerably higher than that of the parent TAM with regard to the area under the serum concentration versus time curve (AUC). The estimated AUC were 0.57, 2.66, and 14.9 µg/ml for TAM, OHT, and DMT, respectively (Table 1). Thus, after TAM administration, high levels of metabolites were found in the plasma of female rats.

Caspase Activity Assay. The tumors (about 100 mg) were first homogenized in lysis buffer [50 mM Tris (pH 7.4), 50 mM β-glycerophosphate, 15 mM MgCl2, 15 mM EDTA, 100 µM phenylmethylsulfonyl fluoride, 1 mM DTT, and 150 µM DTT digitoxin] by grinding with a pestle in a Potter-Elvehjem tissue homogenizer (Wheaton). The lysates were further sonicated twice for 15 s at 50 W using a tissue dismembrator (Fisher Scientific) and were kept on ice for 30 min. The lysates were clarified by centrifugation at 12,500 RPM for 20 min at 4°C. Caspase activity in the supernatant was determined as described previously (31). Briefly, 100 µg of total protein, as determined by the Bio-Rad protein assay (Bio-Rad, Richmond, VA), was incubated with 200 µM fluorogenic peptide substrates Ac-YVAD-MCA (caspase-1), Ac-DEVD-MCA (caspase-3), Ac-VEID-MCA (caspase-6), Ac-IETD-MCA (caspase-8) or Ac-LEHD-MCA (caspase-9) in 50 µl of caspase assay buffer [100 nM HEPES (pH 7.5), 10% sucrose, 10 mM DTT, and 0.1% 3-[3-(chloromadypropyl)dimethylamino]-1-propanesulfonate]. After incubation at 37°C for 2 h, the released AMC was measured with a spectrofluorometer (Perceptive Biosystems, Inc., Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The release of AMC was monitored at an emission wavelength of 460 nm. The release of AMC was monitored at an emission wavelength of 460 nm.
tumors in a time-dependent manner (Fig. 3A). The elevation in caspase activity was first seen at 18 h after the second TAM dose and reached the peak value at 24 h. The high caspase activity was sustained until 48 h, gradually decreasing at 72 h and returning to basal level at 96 h. Caspase-8 and -9 were also potently activated, although no induction in caspase-1 activity was detected under the experimental conditions. Apoptosis in mammary tumors was determined by TUNEL staining, apoptotic cells being recognized by brown staining of the nucleus (Fig. 3B). In control tumors, apoptotic cells were rare in peripheral proliferating areas but frequent in the areas close to necrosis (30, 32–35). In TAM-treated animals, the number of apoptotic cells started to increase 18 h after the second TAM administration (Fig. 3C), and the number of apoptotic cells continued to rise at subsequent sampling points, up to 96 h. The highest number of apoptotic cells were found in the animals killed at 48–72 h after the second TAM administration. Thus, kinetically, the activation of caspases (peak at 24 h) preceded the induction of apoptosis (peak at 48 h) in the rat mammary tumors. Because the plasma concentrations of TAM and OHT preceded, and those of DMT paralleled, the caspase kinetics, we next determined whether these metabolites of TAM were able to induce caspase-dependent apoptosis.

TAM Metabolites Are Active in Inducing Caspase Activation and Apoptosis in Human Breast Cancer Cell Lines. It has previously been reported that OHT was 100 times more potent than, and DMT was as active as, TAM in binding to the ER (12, 13). Also, the above data (Fig. 2), as well as previous findings (2, 4), suggest that high levels of TAM metabolites are formed after the administration of TAM to rats. Therefore, we wanted to determine whether these metabolites of TAM were capable of inducing caspase activity and/or apoptosis in human breast cancer cell lines. We recently reported that TAM (5 μM) induces the activity of caspase-3-, -8-, and -9-like proteases prior to inducing apoptosis in both ER-positive and ER-negative breast cancer cells (14). In the presence study, 5 μM of OHT or DMT was added to MCF-7 (ER-positive) or BT-20 and MDA-MB 231 (both ER-negative) human breast cancer cell lines, and induction of caspase activities and apoptosis were investigated over a period of 48 h. As shown in Fig. 4A, both metabolites caused a time-dependent increase in the activity of caspase-3-like protease in the ER-negative MDA MB-231 cells. The caspase-3 activity appeared at 15 h and peaked at 24 h after treatment. Whereas OHT also potently activated caspase-9, DMT did so to a lesser extent. Both of the metabolites also increased the activity of caspase-8, but neither of them activated caspase-1. Similar results were obtained in ER-negative BT-20 cells. In contrast, no activation of caspase-3 activity was observed in MCF-7 cells (data not shown), consistent with what was reported in the literature (36). The effect of the metabolites on cell death was studied using two methods as described previously (31, 37): (a) DAPI staining to detect nuclear changes such as fragmentation and condensation; and (b) trypan blue exclusion to detect cell death attributable to membrane damage. Induction of cell death by both compounds occurred at least after 24 h of exposure. The increase in condensed nuclei paralleled that of trypan blue–positively stained cells, which suggested that the cytotoxicity induced by the metabolites was attributable to both necrotic and apoptotic death (Fig. 4B). To further define the role of caspases in cell death induced by TAM metabolites, we pretreated the breast cancer cell lines with the broad-specificity caspase inhibitor, z-VAD-fmk, as described previously (31, 37). Pretreatment of the cells with z-VAD-fmk completely inhibited both OHT- and DMT-induced cell death (Fig. 4C), as detected by both DAPI and trypan blue staining. This suggests that the cell death induced by the metabolites of TAM was caspase-dependent. We had also reported that the lipid-soluble antioxidant V-E inhibited TAM-induced caspase activity and apoptosis in breast cancer cells (14). Similarly, V-E also inhibited OHT-induced, but not DMT-induced, caspase activity and cell death.

![Graph](image)

**Fig. 2. Concentrations of TAM (A) and its metabolites OHT (B) and DMT (C) in rat serum. TAM (1 mg/kg) was administered s.c. (8 p.m. and 8 a.m.) to female Sprague Dawley rats, and animals were killed at various time intervals as shown after the second dose. The serum concentration of TAM and metabolites was determined by HPLC. The individual data points were obtained from five animals. The mean serum concentrations and the pharmacokinetic parameter (AUC) are reported in Table 1.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>TAM (ng/ml)</th>
<th>OHT (ng/ml)</th>
<th>DMT (ng/ml)</th>
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<td>1.5</td>
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**Table 1** Rat serum concentrations of TAM and its metabolites (OHT, DMT) and the pharmacokinetic parameter

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a The data points shown are mean SE (n = 5).

b The AUC were estimated using the Noncompartmental analysis using WinNonlin software (Statistical Consultant, Apex, NC).
caspases were activated in NMU-induced rat mammary tumors after TAM administration. More than 95% of NMU-induced mammary tumors are ER positive and respond after treatment with antiestrogens with an increase in apoptotic cell death and a decrease in cell proliferation. In a recent study (33), TAM-induced cell death in mammary tumors has been evaluated at much later time points (5, 10, 20, and 28 days) as compared with the present study, and a consistent increase in apoptotic cells has been observed starting 5 days after the initiation of TAM treatment. In this study, after s.c. injection of 1.0 mg/kg TAM, we observed an increase in apoptotic cell death much earlier than previously reported, i.e., 24–48 h after TAM administration, and this was preceded by a sharp increase in various caspase activities including caspases-3, -8, and -9.

The HPLC analysis of rat serum after s.c. TAM administration indicated that the parent compound was metabolized to OHT and DMT. OHT was formed rapidly and disappeared rapidly, whereas peak levels of DMT did not occur until 48 h and were detected at 96 h. This observation is consistent with a previous report (4) in which rats were given 200 mg/kg TAM p.o. This pharmacokinetic difference between OHT and DMT suggests differential substrate specificity and reaction rate constants for the two cytochrome P-450 isoforms, presumably CYP 2D1 and 3A1 (CYP 2D6 and CYP 3A4 in humans in the formation of these metabolites from TAM. It is possible that the early conversion of TAM to DMT and a prolonged elimination phase for the parent as well as for the metabolites may have been undetected because of the limited sensitivity of UV-detection in our HPLC assay as compared with fluorescence or mass-spectrometric methods used by others. It remains to be determined whether CYP inhibition led to decreased clearance and, therefore, the accumulation of DMT. Importantly, both OHT and DMT were found in concentrations much higher than that of the parent drug. In addition, previous reports have shown that the concentration of TAM and its metabolites in mammary tumors are much higher than their plasma levels (2, 11, 38, 39). This, together with the facts that both metabolites are active ER-binding moieties and effective in inducing growth arrest of breast cancer cells, prompted us to investigate whether the metabolites themselves can induce caspase-dependent apoptosis. Whereas treatment with 1 mg/kg TAM resulted in high circulating levels of metabolites in rat serum (Fig. 2), no metabolites were detected in culture on treatment of human breast cancer cell lines with TAM at 5 μM for up to 48 h (data not shown), presumably because of low CYP activity. Therefore, to determine their individual activity, each of the TAM metabolites was added to human breast cancer cell lines. Both OHT and DMT induced caspase activation with similar kinetics comparable with that induced by TAM (Fig. 4A, Ref. 14), although OHT was somewhat more potent. Importantly, the general inhibitor of caspases, z-VAD-fmk, completely blocked cell death induced by all three of the compounds, which suggested that caspases may play a pivotal role in cell death (Fig. 4C) induced by these compounds. Interestingly, the lipid-soluble antioxidant V-E also completely inhibited TAM- and OHT-induced cell death but had little effect on DMT-induced cell death (Fig. 4C). The reasons for these differences are not clear but may be related to oxidative stress or cell membrane partitioning. Thus, it is conceivable that the delayed and sustained caspases activation and apoptosis in rat mammary tumors after TAM administration may be attributable to the conversion of TAM to its active metabolites OHT and DMT—because both metabolites are found in high concentrations in the rat mammary tumors (2, 38)—and/or could be attributable to the indirect effect of TAM that requires signaling intermediates. A confirmatory experiment to demonstrate the in vivo activity of the metabolites of TAM will be to administer the metabolites directly to mammary tumor-bearing rats and measure caspase activation and apoptosis in the tumors.
Cells were pretreated with 10 μM DMT. After treatment of MDA-MB 231 cells with 5 μM DMT (right panel) and apoptosis in human breast cancer cell lines by TAM metabolites, OHT (left panel) and V-E on cell death induced by TAM metabolites, OHT (left panel) and DMT (right panel). MDA-MB 231 cells were treated with 5 μM either OHT or DMT for various time intervals as indicated and enzymatic activity of caspases was determined by a fluorogenic assay as described in “Materials and Methods.” Data, means of three independent experiments (± SE). B, induction of apoptosis in human breast cancer cell lines by TAM metabolites. OHT (left panel) and DMT (right panel). After treatment of MDA-MB 231 cells with 5 μM of either metabolite for various time intervals as indicated, percentage of cell death was assessed by both DAPI and trypan blue staining as described in “Materials and Methods.” Data, means of three independent experiments (± SE). C, effect of caspase inhibitor and V-E on cell death induced by TAM metabolites, OHT (left panel) and DMT (right panel). MDA-MB 231 cells were pretreated with 10 μM Z-VAD-fmk or 0.3 mM V-E for 1 h followed by treatment with either 5 μM OHT or 5 μM DMT for 24 h, and the percentage of cell death was assessed by both DAPI and trypan blue staining as described in “Materials and Methods.” Data, means of three independent experiments (± SE).

Caspase-3 is the downstream effector caspase that cleaves various cytoplasmic and nuclear substrates, eventually giving rise to the typical morphological characteristics of apoptosis. A time-dependent induction in caspase-3 activity by TAM metabolites prior to the induction of apoptosis suggests that the caspase cascade may be responsible for the metabolite-induced cell death in human breast cancer cell lines and possibly also in rat mammary tumors. The more upstream caspases, caspase-8 and -9, were also activated by the metabolites. Caspase-8 belongs to the DED family, and is activated on upstream caspases, caspase-8 and -9, were also activated by the metabolites. Caspase-8 belongs to the DED family, and is activated on upstream caspases, caspase-8 and -9, were also activated by the second messengers and mediators of TAM- and metabolite-induced signaling events leading to caspase activation and apoptosis.

In summary, we have provided the first evidence that caspases are activated in rat mammary tumors early, within 18–24 h after TAM administration, and that the OHT and DMT metabolites of TAM can induce caspase-dependent apoptosis in various human breast cancer cells. This caspase-dependent apoptosis may contribute to the regression of rat mammary tumors after TAM treatment and may add to the effectiveness of TAM in breast cancer patients.

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