In vitro cytotoxicity of 4'-OH-tamoxifen and estradiol in human endometrial adenocarcinoma cells HEC-1A and HEC-1B

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Abstract. Tamoxifen (TAM), used to treat estrogen receptor (ER)-positive breast cancer, is a well known estrogen antagonist in the breast, but a partial estrogen agonist in the endometrium. In addition, TAM metabolites, such as 4'-hydroxy-tamoxifen (4-OH-TAM), have been shown to be more potent than the parent compound. The objective of this study was to determine the effects of 4-OH-TAM and estradiol (E₂) on two human endometrial adenocarcinoma cell lines, HEC-1B and HEC-1A. When HEC-1B cells were treated with lower concentrations (10-1,000 nM) of 4-OH-TAM or E₂ for 1-3 days, no significant difference in the percentage of cell survival was observed among the varying concentrations. At higher 4-OH-TAM or E₂ concentrations (1-100 µM), HEC-1B and HEC-1A cells responded similarly with an obvious decrease in cell growth noted at 10 and 100 µM 4-OH-TAM and 100 µM E₂. In order to address the observed cell death, DNA laddering was performed at various time intervals with 4-OH-TAM (10 µM) or E₂ (10 or 50 µM). DNA gel electrophoresis failed to show the typical laddering pattern (180-200 bp) observed in apoptosis. Furthermore, western blot analysis of caspase-8 and -3 failed to demonstrate caspase activity. These results suggest that apoptosis was not the underlying cellular mechanism of cell death. Due to the lack of apoptotic markers, a cytotoxic (cell death) effect was differentiated from a cytostatic (growth inhibition) effect confirming that cell death had occurred. In summary, micromolar concentrations of 4-OH-TAM induced a non-apoptotic cytotoxic effect in the endometrium; however further studies are needed to elucidate the cytotoxic pathway being utilized.

Introduction

Tamoxifen (TAM), a selective estrogen receptor modulator (SERM), has been studied for over 20 years as an anti-estrogenic drug used to treat estrogen receptor (ER)-positive breast cancer. By competing for binding at the ER, TAM has been proven to reduce the risk of developing breast cancer by 49% through its antagonistic properties (1). Binding of TAM results in decreased expression of genes that affect cell proliferation leading to diminished cell divisions in breast cancer (2). However, in the endometrium, TAM has been shown to act as a partial estrogen agonist, thus leading to the inappropriate proliferation of endometrial tissues. Consequently, the risk of developing endometrial cancer is increased by 3-fold in TAM-treated women (3). In addition, the agonistic effect of TAM has been shown to exhibit high variability depending upon cell type, promoter context, ambient estradiol concentration and ER subtype (α or β) (4,5). Furthermore, TAM has been shown to have significant in vitro cytotoxic and cytostatic effects on ER (+) and ER (-) breast cancer cells (6,7). A study by Reddel et al indicated that the effects of TAM are dose- and ER status-dependent (8). Specifically, their study showed that at low doses TAM effects are ER-mediated, whereas at higher doses TAM effects appeared to be ER-independent.

The antagonistic and agonistic properties inherent to TAM are also present in its numerous metabolites, specifically 4'-hydroxy-tamoxifen (4-OH-TAM). With the addition of a hydroxyl group, 4-OH-TAM has been shown to have a higher potency than TAM both in vitro and in vivo corresponding to a higher affinity for the ER (9). Of interest is the effect of 4-OH-TAM in ER-negative tissues, such as the rat (10) and mouse livers (11), where TAM-DNA adducts and 4-OH-TAM-induced DNA adducts have been demonstrated. Moreover, a study by Shibutani et al demonstrated TAM-DNA adduct formation in ER-positive tissues, such as the human endometrium (12). These results suggest the possibility of an ER independent pathway for TAM and its metabolites to induce cell proliferation.

A study by Castro-Rivera and Safe determined that ER-positive HEC-1A human adenocarcinoma cells treated with TAM exhibited both agonistic and antagonistic tendencies with respect to cell proliferation depending on the concentration used (13). Another study by Perry et al demonstrated the apoptotic effects of TAM in breast cancer cells regardless of ER status (14). The ER status of HEC-1A and HEC-1B cells has been debated in the literature. A recent study by Acconcia et al unequivocally demonstrated the presence of ER-α in both cell lines (15). However, their immunofluorescence data showed differences in the ER-α subcellular distribution, where HEC-1A ER-α was observed in both the cytosol and the...
nucleus, whereas, in HEC-1B cells ER-α was mostly located in the cytosol. The aim of the present study was to investigate the in vitro cytotoxicity of 4-OH-TAM and E₂ in human endometrial adenocarcinoma HEC-1A and HEC-1B cancer cell lines. We observed an obvious decrease in HEC-1B and HEC-1A cell growth noted at 10 and 100 µM 4-OH-TAM and 100 µM E₂. Furthermore, these micromolar concentrations of 4-OH-TAM induced a non-apoptotic cytotoxic effect.

Materials and methods

Cell lines and tissue culture conditions. HEC-1B and HEC-1A human endometrial adenocarcinoma cell lines were a generous gift from Dr. Cheryl Walker at M.D. Anderson (Smithville, TX, USA). HEC-1B and HEC-1A cells were harvested and protein extracts were prepared from Life Technologies (Grand Island, NY, USA) respectively supplemented with 10% fetal bovine serum (FBS, cat. #SH3039603) or charcoal stripped FBS (cat. #SH3006803; lacks endogenous steroid hormones, CSFBS) (both purchased from Hyclone Laboratories, Logan, UT, USA), 2% L-glutamine (cat. #G-7513) and 1% sodium pyruvate (cat. #S8636) (both obtained from Sigma, St. Louis, MO, USA). Cells were grown in 25 and 75 cm² culture flasks in a humidified atmosphere of 5% CO₂ at 37°C.

Compounds. The compounds 4-OH-TAM (cat. #H-7904) and E₂ (cat. #E-2758) were purchased from Sigma and dissolved in 100% ethanol, stored and protected from light in stock solutions of 1 mM at -20°C. The final concentration of ethanol in the culture media was consistently <0.1% (v/v).

Exposure to compounds and determination of cell growth. The media corresponding to HEC-1B and HEC-1A cells were replaced using 5% CSFBS 24 h prior to plating. Both cell lines were seeded (10,000 cells/well) in triplicates into 96-well plates. After 24 h, cells were incubated with 200 µl of various 4-OH-TAM or E₂ concentrations for 1-3 days at the conditions described above. Cells grown in the absence of 4-OH-TAM and E₂ were used as a control. Media were changed on day 2 to ensure proper nutrient content and effective drug concentration.

Cell viability was assessed using CellTiter 96® AQueous One Solution Cell Proliferation Assay (cat. #G3580; Promega, Madison, WI, USA) every 24 h. Briefly, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt; MTS) and an electron-coupling reagent (phenazine ethosulfate; PES) were added to each well. Cells were incubated for 1 h with 5% milk in PBS and probed with 0.25 µg/ml mouse anti-caspase-8 monoclonal antibody (cat. #551242; BD Biosciences, San Diego, CA, USA) or with 2 µg/ml mouse anti-caspase-3 monoclonal antibody (cat. #35-1600Z; Life Technologies) for 1 h at room temperature with constant agitation followed by a series of washes and incubated for 1 h with goat anti-mouse HRP conjugate (1:3,000 dilution; cat. #170-6516; BioRad). A chemiluminescent HRP signal detection system Amersham ECL™ Prime Western Blotting Detection Reagent (cat. #RPN2232; GE Healthcare, Buckinghamshire, UK) was used to detect the signal.

Determination of cell growth after exposure to the compounds. HEC-1B and HEC-1A cells were seeded in 25 cm² culture flasks and allowed to grow to 85% confluency. Cells were harvested and protein extracts were prepared from cells (adherent and detached cells) grown in the absence (control) or presence of 1, 10 or 100 µM 4-OH-TAM or E₂ (in 5% CSFBS media) for 24 h at the incubating conditions previously described. DNA fragments were then separated on a 2% Agarose E-Gel® (cat. #G5018-02; Life Technologies) and visualized with ethidium bromide under ultraviolet light.

Western blot analysis
Caspases. HEC-1A and HEC-1B cells were seeded in 25 cm² culture flasks and allowed to grow to 85% confluency. Cells were harvested and protein extracts were prepared from cells (adherent and detached cells) grown in the absence (control) or presence of 1, 10 or 100 µM 4-OH-TAM or E₂ (in 5% CSFBS media) for 24 h at the incubating conditions previously described followed by western blot analysis. In brief, whole cell lysates were prepared from log phase cells with 4X sample buffer (40% v/v glycerol, 4% SDS, 0.5% w/v bromophenol blue and 0.16 M Tris pH 7.0) plus 10% β-mercaptoethanol, subjected to gel electrophoresis on precast 12% SDS-polyacrylamide gels (cat. #456-1043; BioRad) and transferred to a polyvinylidene fluoride (PVDF) membrane (cat. #IPVH304F0; Merck Millipore Ltd., Co. Cork, Ireland). The membrane was blocked for 1 h with 5% milk in PBS and probed with 0.25 µg/ml mouse anti-caspase-8 monoclonal antibody (cat. #551242; BD Biosciences, San Diego, CA, USA) or with 2 µg/ml mouse anti-caspase-3 monoclonal antibody (cat. #35-1600Z; Life Technologies) for 1 h at room temperature with constant agitation followed by a series of washes and incubated for 1 h with goat anti-mouse HRP conjugate (1:3,000 dilution; cat. #170-6516; BioRad). A chemiluminescent HRP signal detection system Amersham ECL™ Prime Western Blotting Detection Reagent (cat. #RPN2232; GE Healthcare, Buckinghamshire, UK) was used to detect the signal.

Results

Determination of cell growth after exposure to the compounds. HEC-1B and HEC-1A cells were exposed to varying concentrations of 4-OH-TAM and E₂ to determine the effects of these compounds on cell survival. When comparing the different 4-OH-TAM or E₂ concentrations (10-1000 nM) used to treat the HEC-1B cells, no significant difference in the percent survival was observed (p≤0.260; p≤0.755) (Fig. 1A and B). However, as shown in
Fig. 2A, HEC-1B cells exposed to higher concentrations (1-100 µM) of 4-OH-TAM showed a significant difference in the percent cell survival between concentrations ($p \leq 0.0001$). Furthermore, at the concentrations of 10 and 100 µM, a definitive decrease in percent survival was noted when compared to the untreated cells (percent survival indicated by solid line).
and set to 100%). In contrast, as shown in Fig. 2B, HEC-1B cells treated with the same concentrations of E₂ underwent an initial proliferative response observed after 24 h at 1 and 10 µM, followed by an apparent decrease in cell survival that did not appear to be different from the non-treated cells. Treatment with 100 µM E₂ resulted in complete cell death. Furthermore, a significant difference in percent survival was noted between E₂ concentrations (p≤0.0001).

When HEC-1A cells were treated with the same high concentrations (1-100 µM) as HEC-1B cells of 4-OH-TAM or E₂, we observed a significant difference in the percentage of cell survival between concentrations (p≤0.0001, similar to HEC-1B cells. When comparing the percent survival of treated cells with the control, 4-OH-TAM appeared to decrease cell survival at 10 µM and complete cell death was observed at 100 µM (Fig. 3A). Similarly, as shown in Fig. 3B, E₂ caused complete cell death at 100 µM and there were significant differences between concentrations.

Absence of DNA laddering. To determine whether apoptosis was the underlying cellular mechanism responsible for the decreased percent cell survival observed in both cell lines exposed to 10 µM or 100 µM 4-OH-TAM or 100 µM E₂ (when compared to the control); DNA was extracted from adherent and floating HEC-1B and HEC-1A cells after 24 h of exposure with 10 µM 4-OH-TAM or E₂ and subjected to electrophoresis on a 2% agarose gel. No DNA laddering was observed in any of the samples (data not shown). Due to concern with the possibility of having extracted the DNA past a time-point where DNA fragmentation could be observed, HEC-1B and HEC-1A cells were incubated with 10 µM 4-OH-TAM or 50 µM E₂ and DNA was extracted at 6 and 12 h after exposure. Cells were exposed to a higher E₂ concentration (50 µM), since we thought that 10 µM E₂ was not high enough to induce apoptosis (refer to Fig. 2B and 3B). Again, no DNA laddering was observed in any of the samples suggesting that apoptosis was not the mechanism involved (Fig. 4).

Absence of caspase-3 and -8 activation as determined by immunoanalysis. To determine whether activation of caspase-8 and -3 had occurred, western blot analysis of the protein extracts collected from adherent and floating HEC-1B and HEC-1A cells grown in the absence (control) or presence of 1 or 10 µM 4-OH-TAM or E₂ for 10 and 24 h was performed. We did not observed the expected lower molecular weight bands for caspase-8 or -3 with any of the concentrations used or time-points (data not shown). Caspase-3 exists in cells as an inactive 32-kDa proenzyme and during apoptosis procaspase-3 is cleaved into 17- and 12-kDa active subunits by upstream proteases such as caspase-8. Similarly, during apop-
tosis, the proform of caspase-8a 55-kDa protein, is cleaved into smaller active subunits of 40/36 kDa (doublet) and 23 kDa.

Cytotoxic effect of 4-OH-TAM and estradiol. Due to the absence of apoptotic markers, we wanted to differentiate between the cytostatic and cytotoxic effects of 4-OH-TAM and E2. In order to do that, cells were incubated in the presence or absence of 10 µM 4-OH-TAM or 100 µM E2 for 24 h in 5% CSFBS. Trypan blue dye exclusion assay was used to assess the cytotoxic effect of 4-OH-TAM or E2 (Fig. 5). Cell viability of HEC-1B and HEC-1A cells treated with 10 µM 4-OH-TAM was determined to be <25% in comparison to the number of living cells at the time of exposure. When subjecting HEC-1B and HEC-1A cells to 1 µM 4-OH-TAM for 1-3 days, the growth patterns did not differ from the untreated cells, while cells treated with 10 and 100 µM 4-OH-TAM underwent a significant decrease in cell proliferation when compared to the control. These results differ from a study by Castro-Rivera and Safe (13) which determined that HEC-1A human endometrial adenocarcinoma cells treated with 1 µM TAM underwent a significant decrease in cell proliferation.

Discussion

The present study examined the effects of 4-OH-TAM and E2 on the proliferation of HEC-1B and HEC-1A human endometrial adenocarcinoma cells. Since endometrial cell proliferation is the most sensitive marker for differentiating agonistic vs. antagonistic activity (16), endometrial cell lines were subjected to hormonal treatments of 4-OH-TAM and E2 at varying concentrations (nM to µM) for 1-3 days. At concentrations ranging from 10-1000 nM of 4-OH-TAM or E2, HEC-1B cell proliferation did not differ from that of the untreated cells. Due to the apparent lack of effect of 4-OH-TAM on the growth patterns between treated and untreated HEC-1B cells, we decided to increase the concentrations of 4-OH-TAM and E2 to the micromolar range. When using 4-OH-TAM, a decreased proliferation of HEC-1A cells was not observed until the 4-OH-TAM concentration was increased to 10 µM, thus suggesting a difference in the effects of TAM and its metabolite 4-OH-TAM. This is an interesting result considering 4-OH-TAM has been shown to possess a higher potency than TAM (9). While micromolar concentrations of 4-OH-TAM and E2 appear to be clinically irrelevant, the plasma concentration range of TAM measured in chemotherapy patients has been recorded as 0.1-10 µM (17). Furthermore, patients treated with higher doses of TAM (720 mg per day) have serum levels of up to 3.5 µM with accumulated levels in tissues reaching 16-30 times higher than that of the serum (14). Therefore, the concentrations of 4-OH-TAM utilized in this study mirror actual concentrations of TAM recorded in patients undergoing chemotherapy.

Figure 4. DNA fragmentation analysis after 6 and 12 h in HEC-1B (A) and HEC-1A cells (B) after treatment with 4-hydroxy-tamoxifen (4-OH-TAM) or E2. DNA extracts were prepared as described in Materials and methods from cells treated with 10 µM of 4-OH-TAM or 50 µM E2. The DNA was visualized on a 2% agarose gel with ethidium bromide under UV light. A typical DNA laddering pattern (180-200 bp) was not observed.

Figure 5. Growth inhibition vs. cell death of HEC-1B and HEC-1A endometrial cancer cells. HEC-1B and HEC-1A cells were seeded into 6-well plates and allowed to attach for 24 h. Prior to exposure, cells were counted to determine the initial number of living cells (determined by trypan blue dye exclusion assay) at the time of exposure (represented by the line). HEC-1B and HEC-1A cells were then grown in the absence (control) or presence of 10 µM 4-OH-TAM or 100 µM E2 for 24 h. The percentage of living cells for each treatment was determined by dividing the number of living cells after the 24-h hormonal treatment exposure by the number of living cells counted prior to the start of the exposure. The number of living cells in either cell line treated with 100 µM E2 was not detectable (represented by ND).
Following observation of the HEC-1B and HEC-1A cells exposed to 1 or 10 µM E2 for 1-3 days, the growth patterns did not appear to be different from the untreated cells, whereas 100 µM E2 caused an obvious decrease in cell proliferation in both cell lines. Since HEC-1A and HEC-1B cells contain ER-α (15), estrogen treatment was hypothesized to induce an increase in cell growth in comparison to the untreated cells. However, the concentrations of estadiol used in this study were higher than physiological plasma concentrations of estrogen (~1 nM) (18) and thus may have led to the cytotoxic effect.

In order to determine the mechanism behind the observed decrease in cell proliferation, we looked for DNA laddering 24 h after exposure of the cells to 10 µM 4-OH-TAM or E2. DNA gel electrophoresis failed to show DNA-laddering in any of the samples. We were concerned with the possibility of having extracted the DNA at a time-point when it was too late to observe DNA fragmentation. Therefore, we extracted DNA at 6 and 12 h after exposure to 10 µM 4-OH-TAM or 50 µM E2. Another concern was that the E2 concentration (10 µM) may not have been high enough to induce apoptosis. Therefore, we increased the E2 concentration to 50 µM for the 6 and 12 h exposures. Neither of these measures resulted in the evidence of apoptosis through DNA laddering. Similarly, Dietze et al. found that 1 µM TAM, but not an equimolar concentration of 4-OH-TAM, induced apoptosis in ER-positive normal human mammary epithelial cells (HMECs) (19). This suggests that 4-OH-TAM induces cell death through a mechanism other than apoptosis.

Furthermore, western blot analysis of caspase-8 and -3 expression failed to unequivocally show caspase activation. The inactive form of caspase-8, a 55-kDa protein, is cleaved into smaller subunits of 40/36 kDa (doublet) and 23 kDa upon activation, whereas procaspase 3 (32 kDa) is cleaved into active 17- and 12-kDa subunits. Our data excluded the death receptor pathway of the apoptotic mechanism (which utilizes caspase-8) as the underlying cellular mechanism of the observed cell death. Therefore, the results of the present study and the lack of DNA ladder suggest that apoptosis was not the underlying mechanism used to cause cell death in the 4-OH-TAM and E2-treated HEC-1B and HEC-1A cells.

Although apoptosis is the most common form of programmed cell death there are alternative non-apoptotic mechanisms that can lead to regulated cell death such as autophagy, necroptosis or the non-regulated pathway of necrosis. In fact, mammalian cells have been shown to express cell death proteases even when they are not undergoing apoptosis (20). Furthermore, characteristics assumed to be unique to apoptosis, such as chromatin condensation and even DNA fragmentation, may not be strictly indicative of apoptosis (21). On a kinetic scale, morphological changes in cell structure may occur over a wide time range depending on cell type as noted in hepatocytes (2-3 h) and lymphocytes (36-48 h) (22). Considering all the data from these studies, a form of cross-talk appears to exist between apoptosis and other cytotoxic mechanisms ending in necrosis in which DNA laddering and caspase-8 activation may not be strictly characterized as apoptotic events. Several in vitro studies have shown that treatment of breast cancer cells with various antioestrogens or aromatase inhibitors induces cell death via unknown mechanisms (23-26). Therefore, although DNA laddering and western blot analysis of caspase-8 and -3 failed to demonstrate the activation of an apoptotic mechanism as a means of cell death, a cytotoxic effect mediated strictly by mechanisms resulting in necrosis may not be accurate. As a result, subsequent studies may focus on determining whether 4-OH-TAM-induced DNA adducts contribute to the observed cytotoxic effect or whether autophagy or necroptosis are at play. Furthermore, future aims may also include using endometrial cancer cells to replicate studies conducted with breast cancer cells. When analyzing TAM- and 4-OH-TAM-treated normal HMECs, apoptosis was found in TAM-treated, but not 4-OH-TAM-treated HMECs (19). It would be of interest to investigate whether similar results are noted with endometrial cancer cells, thus elucidating the differences between TAM and 4-OH-TAM cytotoxic effects in the endometrium.

Due to the obvious decrease in cell survival observed in both HEC-1B and HEC-1A cells exposed to 10 µM 4-OH-TAM or 100 µM E2 for 1-3 days in comparison to the untreated cells and the lack of apoptotic markers, we wanted to distinguish whether the decline observed was due to a cytostatic (growth inhibition) or cytotoxic (cell death) effect. After exposure to the above concentrations of 4-OH-TAM and E2, a cytotoxic effect was observed as determined by trypan blue dye exclusion assay. Indeed, the number of living cells decreased to <25 and 0% for HEC-1B and HEC-1A cells treated with 4-OH-TAM and E2, respectively. Therefore, the micromolar concentrations of 4-OH-TAM and E2 induced a cytotoxic effect resulting in extensive to complete cell death.

Overall, this study suggests that micromolar concentrations of 4-OH-TAM induce a non-apoptotic cytotoxic effect in the endometrium. However, subsequent studies are needed to elucidate the underlying mechanism involved in the cytotoxic effect of 4-OH-TAM and E2.

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