5-androstene 3β,17α diol induces autophagic cell death of human breast cancer cells and enhances radiation cytotoxicity

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Abstract
Herein, we demonstrate that the steroid, 5-androstene 3β,17α diol (17α-AED) induced cell death in human breast cancer cells MCF-7, MDA-MB231, T47D and TTU-1 at clinically relevant levels. 17α-AED treatment resulted in autophagic cell death without evidence of apoptosis in breast cancer cells revealed by increased cleavage of microtubule-associated protein-light chain 3 and the presence of acidic vesicular organelles in the absence of caspase 3, 8 or 9 activation and PARP processing. Increased phosphorylation of eukaryotic translational initiation factor 2a (eIF2α) was detected in treated MCF-7 cells and disruption of eIF2α signaling reduced autophagic cell death in 17α-AED treated MCF-7 cells. In breast cancer cell survival studies, 17α-AED synergistically potentiated the cytotoxicity of radiation treatment. Collectively, 17α-AED induces autophagic cell death in human breast tumors which is mediated, at least in part, by eIF2α signaling and may have potential therapeutic value for the treatment of breast tumors.

Keywords: autophagy; androstene steroids; breast cancer; radio-sensitizer

Introduction
One out of eight women is reported to develop breast cancer and, despite recent therapeutic advances, it is the second leading cause of cancer deaths in the United States [1]. Invasive ductal cancer is the most common form of breast cancer and comprises ~75% of all cases [1]. Surgery followed by local-regional radiation therapy is considered the standard primary treatment and can be combined with adjuvant hormonal and/or chemotherapy. Tamoxifen is commonly used for breast tumors that are positive for the expression of the estrogen receptor (ER) or progesterone receptor which may be used in conjunction with aromatase inhibitors to increase efficacy or with chemotherapeutic agents such as cyclophosphamide, methotrexate or fluorouracil [2]. A recent advance in the treatment of this disease involves the use of trastuzumab (herceptin). Trastuzumab is a mAb specific for the human epidermal growth factor receptor 2 (Her-2/neu) which is expressed on ~25% of breast cancer tumors and is used in combination with chemotherapy or radiation to specifically target Her-2/neu positive breast cancer cells [3,4]. Unfortunately, more than half of Her-2/neu positive breast tumors fail to respond or become resistant to trastuzumab-based chemotherapy [5]. Therefore, there is a need to investigate novel therapeutic agents for the treatment of this cancer which may be used as a single agent or as adjuvant to enhance standard therapeutic modalities such as radiation.

Using an experimental mammary adenocarcinoma model, Schwartz et al. [6] demonstrated that the naturally occurring adrenal steroid dehydroepiandrosterone (DHEA) inhibited the spontaneous development of breast tumors. Additional studies showed that in vivo administration of DHEA suppresses the growth of human ZR75-1 breast tumor xenografts [7]. The steroid, 5-androstene 3β,17α diol (17α-AED), is a metabolite of DHEA and possesses significantly greater anti-tumor activity than DHEA [8]. We have shown that pharmacologically achievable concentrations of 17α-AED inhibit the proliferation of human ER+ ZR75-1 and ER- MDA-MB231 human breast cancer cells, but cellular toxicity and associated cell death pathways were not analyzed [9].

Autophagy is generally considered a temporary cell survival response to starvation or a toxic environment in which cytosolic proteins are recycled; however, autophagy can also lead to type II programmed cell death [10-13]. For the initiation of autophagy, beclin-1 binds with class III phosphoinositide-3-kinase class III/ p150 lipase [14,15]. Subsequently, an ubiquitin-like conjugation system involving Atg12-Atg5 and microtubule-associated
protein 1 light chain 3 (LC3) - phosphatidylethanolamine mediates the expansion of the pre-autophagosome membrane and the engulfment of organelles and protein constituents [10,14,15]. During this process LC3-I (18 kD) is converted to LC3-II (16 kD) and is incorporated into the membrane. At a later stage, autophagosome fuses with a lysosome to form an autolysosomes/acidic vesicular organelle (AVO) and the contents of the vesicle are degraded and recycled [10,14,15]. Previous investigations with 17α-AED showed that tumor cell cytotoxicity can be mediated by the induction of either apoptotic or autophagic programmed cell death pathways depending on the etiological origin of the neoplasm. In this regard, 17α-AED induces apoptosis in leukemias and autophagy malignant gliomas [16-18]. In this report, we demonstrate that 17α-AED induces autophagic cell death in human breast tumors as evident by LC3 processing and the presence of AVOS, both biomarkers for autophagy, without caspase activation or poly (ADP-ribose) polymerase (PARP) cleavage. The neuro-steroid, 5-androstene 3β,17α diol; induces caspase activation or poly (ADP-ribose) polymerase (PARP) cleavage. The neuro-steroid, 5-androstene 3β,17α diol; induces caspase activation or poly (ADP-ribose) polymerase (PARP) cleavage. The neuro-steroid, 5-androstene 3β,17α diol; induces caspase activation or poly (ADP-ribose) polymerase (PARP) cleavage. The neuro-steroid, 5-androstene 3β,17α diol; induces caspase activation or poly (ADP-ribose) polymerase (PARP) cleavage.

Materials and methods
Cell lines and culture
Cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum as monolayers at 37°C, 5% CO2 and 100% humidity and passed biweekly with trypsin in the absence of antibiotics. All culture reagents and supplements were obtained from Invitrogen Life Sciences (Carlsbad, CA). The MCF-7 breast adenocarcinoma cell line expresses ERα and a low level of ERβ and the MDA-MB231 breast adenocarcinoma cell line is ERα negative, expresses a non-functional, truncated form of ERβ devoid of the steroid binding domain [20,21]. The MCF-7 and MDA-MB231 cell lines were obtained from the NCI Frederick Cancer Research Facility. The T47D mammary ductal carcinoma cell line is ERα positive and expresses a moderate level of ERβ. The TTU-1 mammary ductal carcinoma cell line is ERα negative and the ERβ status is unknown [22]. T47D and TTU-1 cell lines were a gift from Dr. Shawn Holt (Department of Pathology, Virginia Commonwealth University, Richmond, VA).

Cytotoxicity assays
Tumor cells (1x10⁴/well) were cultured in 96-well plates in the presence of 17α-AED (3 – 60µM), Custom Steroid Synthesis, Voorhees, NJ or vehicle (PEG100:ethanol v/v) for 48 h and the level of released lactate dehydrogenase (LDH) was quantified using a LDH-Cytotoxicity Assay Kit II (BioVision, Mountain View, CA). The percentage of cytotoxicity was measured according to the supplied protocol. All culture conditions were performed in triplicate.

Clonogenic assays
Cells were seeded into 6-well cluster plates (250 – 1000 cells/well) and were allowed to adhere for 8 h. 17α-AED or vehicle was added in triplicate at the indicated doses (1- 20µM final). After 48 h of treatment, medium was replaced with drug-free culture medium. In combination experiments, cells were treated with 17α-AED for 24 h; then exposed to radiation (0.5 – 2.0 Gy) at a rate of 3.90 Gy/min; and cultured for an additional 24h after which media was replaced with drug-free medium. Cells were cultured for an additional 8 – 12 d; rinsed with PBS; fixed with methanol; and colonies were stained with 0.5 % crystal violet. Colonies containing > 50 cells were enumerated; the surviving fraction was calculated; and data was normalized to vehicle treated controls.

Transient transfection and 17α-AED treatment
MCF-7 cells were seeded into 60 mm dishes and incubated overnight followed by transfection with 4 µg of plasmid DNA expressing dominant-negative eIF2αS51A (DN-eIF2α) or wild type eIF2α (wt-eIF2α, gifts from Dr. Constantinos Koumenis, University of Pennsylvania) using Metafectene Pro (Biontex, Martinsried, Germany). After 48h, cells were treated with 17α-AED (10 µM) or vehicle for 15 - 48h and then assessed for LC3-cleavage; levels of CHOP or cell death by trypan blued exclusion as indicated.

Immunoblotting
Immunoblotting was performed using whole cell lysates as previously described [18]. Briefly, cells were incubated with 17α-AED (0 – 20 µM, final) or vehicle for 15 - 48h, after which lysates were loaded on SDS/PAGE gels and transferred to nitrocellulose membranes. The membranes were incubated overnight with primary antibodies followed by incubation with a horseradish peroxidase conjugated secondary antibody (Rockland Immunochemicals, Gilbertsville, PA). Immunoreactive bands were visualized using chemiluminescence (SuperSignal, Pierce Chemicals, Rockford, IL). The membranes were re-blotted with a mouse anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO) and used as a loading control. The following primary Abs were used to detect their respective proteins and cleavage products: caspase -3, -8, -9 and PARP were from Santa Cruz Biotechnology (Santa Cruz, CA); LC3 (Novus Biologicals, Littleton, CO); total eIF2α or phospho-specific (Ser51) eIF2α (Cell Signaling Technology, Beverly, MA); and CCAAT/enhancer-binding protein homologous protein (CHOP, BioLegend, San Diego, CA).

Detection of acidic vesicular organelles
Acidic vesicular organelles were detected in cells by staining with acridine orange as previously described [18]. Cells were seeded into 6-well plates and cultured in the presence of vehicle or 10 - 20 µM of 17α-AED for 72h. Select cultures were supplemented with 3-methyladenine (10 mM, 3-MA, Sigma-Aldrich), an inhibitor of class III phosphoinositol-3-kinase.
Acridine orange (1.0 µg/ml, Sigma-Aldrich) was added to the cultures for the last 15 min. Total cells were collected and analyzed by flow cytometry using a FACSCanto II (BD Biosciences, San Jose, CA) and cells containing AVOs were identified as double positive cells in the Q2 quadrant of FL1, FL3 histograms.

Statistics
Statistical analysis was performed using a one way analysis of variance test and mean values ± the standard error of the mean are shown. Differences were considered to be significant when the calculated p value was less than 0.05. SigmaPlot, version 11 (Systat Software, Inc., San Jose, CA) was used for regression analysis of the clonogenic data. The Chou-Talalay method was used to determine the nature of the interaction between 17α-AED and radiation by calculating combination index (CI) values using CalcuSyn software (Biosoft, Cambridge, UK) [23].

Results
Treatment with 17α-AED induces cell death of human breast cancer cells
The cytotoxic effects of 17α-AED were evaluated on MCF-7, MDA-MB231, T47D and TTU-1 human breast tumor cells which were either ERα positive or negative and varied on the level and functional status of ERβ. Cells were treated with titrated concentrations of 17α-AED (3 – 60 µM) and cell viability was assessed in short-term (48 h) LDH release assays. The results shown in Figure 1 indicate that the LD50 for 17α-AED on the cells tested is between 8 - 15µM, with the MCF-7 cells being the most sensitive (LD50 ~ 8.0 µM). Similar results were obtained using short-term, trypan blue exclusion viability assays (data not shown). Next, we assessed the irreversible anti-tumor effects on breast tumor cell survival after a brief exposure to 17α-AED using the more sensitive clonogenic assay. MCF-7, MDA-MB231, T47D and TTU-1 cells were treated with various concentrations of the steroid for 48 h and then cultured in drug-free medium for >1 week and the surviving fractions were calculated. The results in Figure 2 show increased cell sensitivity (LD50 = 3–10 µM), indicating that 17α-AED induced additional damage manifested over a longer period of time. These results demonstrate that 17α-AED induces a significant level of cell death in a concentration dependent fashion in the breast tumor cell lines tested regardless of ERα/β steroid signaling status, suggesting that anti-tumor activity is independent of the ER.
17α-AED induces autophagy in breast cancer cells in the absence of apoptosis

We assessed the activation of type I and type II programmed cell death pathways in breast tumor cells treated with 17α-AED. MCF-7 cells were exposed to increasing concentrations (0–10 µM) of 17α-AED for 48 h and were analyzed for biochemical markers for the induction of apoptosis and autophagy. Proteolytic processing of initiator caspases-8 and -9; and effector caspase-3 was used to assess the induction of apoptosis [24,25]. Increased conversion of LC3-I (18 kDa) to LC3-II (16 kDa) was used as a marker for autophagy [26]. The results shown in Figure 3a indicate that LC3 processing was augmented in correlation with increasing concentrations of 17α-AED, as indicated by an increased level of LC3-II and a corresponding reduction of LC3-I in treated MCF-7 cells. In contrast, there was no apparent activation of caspases -3, -8, or -9 with the concentrations tested. Similar experiments were conducted with T47D, TTU-1 and MDA-MB231 breast tumor cells in which apoptosis was assessed by caspase-3 cleavage and PARP processing and autophagy was detected by LC3 conversion. As shown in Figure 3b, treatment of these cells with 20 µM (~ LD-50 as determined in 48 h cytotoxicity assays) of 17α-AED for 48h induced autophagy as evident by LC3 conversion without the activation of apoptosis as indicated by the absence of caspase-3 and PARP cleavage. As a positive control for the induction of apoptosis, U937 myeloma cells treated with 17α-AED were included [17].

To provide further evidence for 17α-AED induced autophagy in breast tumor cells, we analyzed steroid treated MCF-7 and MDA-MB231 cells for the presence of AVOs which is a late event in autophagy [27]. A pilot time course study demonstrated that MCF-7 cells treated for 72 h with 17α-AED(10 µM) was optimal for the detection of intracellular AVOs (data not shown). A significant level of AVO formation of 21.6% was readily detected in treated MCF-7 and MDA-MB231 cells (10µM and 20µM 17α-AED, respectively) but not in vehicle treated cells.

Figure 2. Exposure of breast tumor cells to 17α-AED reduces cell survival.
In clonogenic assays, MCF-7, MDA-MB231, TTU-1 and T47D cells were exposed to titrated concentrations of 17α-AED or vehicle for 48 h; then cultured in drug-free medium; and surviving colonies were enumerated. Each experiment was conducted two additional times and yielded similar results.
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The addition of the class III phosphoinositol-3-kinase inhibitor, 3-MA (1mM), to MCF-7 or and MDA-MB231 cell cultures treated with 17α-AED reduced the presence of AVOs to baseline levels (Figure 4a). Representative histograms used for the detection of AVOs in MCF-7 cells are shown in Figure 4b. These findings strongly indicate that 17α-AED treatment induces autophagic cell death in human breast cancer cells.

eIF2α signaling in human breast cancer cells treated with 17α-AED

eIF2α activation results in the transient repression of translation with the exception of specific endoplasmic reticulum-stress associated mRNAs such as CHOP and can induce autophagy in cells [28]. We investigated the activation status of eIF2α in MCF-7 and MDA- MB231 cells treated with 17α-AED (10 and 20µM, respectively). As shown in Figure 5a, there is an increased level of eIF2α phosphorylation in these cells after 24 h of 17α-AED exposure while there were no noticeable changes in the total level of eIF2α. These results suggest eIF2α activation may be involved with 17α-AED induced autophagic cell death in breast cancer cells.

To establish a connection between eIF2α phosphorylation and the anti-tumor effects of 17α-AED on breast tumor cells, we disrupted eIF2α signaling in MCF-7 cells by transient transfection with a DN-eIF2α construct [29]. As shown in Figure 5b, cells transfected with DN- eIF2α have reduced protein levels of CHOP after 17α-AED treatment as compared to treated, transfection control cells, confirming down-modulation of eIF2α signaling. Moreover, 17α-AED induced LC3 cleavage and cell death was attenuated in MCF-7 cells with reduced eIF2α activity as compared to transfection control cells exposed to the drug (Figure 5b and 5c). These results implicate the involvement of eIF2α signaling in 17α-AED induced autophagic death in human breast cancer cells.

17α-AED potentiates the cytotoxic effects of radiation on human breast cancer cells

We assessed the combined effects of 17α-AED treatment and
Figure 4. AVO formation in breast cancer cells treated with 17α-AED.
a) MCF-7 and MDA-MB231 cells were treated with 17α-AED (10 μM and 20 mM, respectively); 17α-AED and 3-MA (10 mM); or vehicle for 3 days and the percentage of cells containing AVOs was evaluated. Mean values of 3 independent experiments are shown. P<0.005.
b) Representative histograms of MCF-7 cells treated with vehicle (left); 10μM of 17α-AED (middle); or 10 μM of 17α-AED in the presence of 10 mM 3-MA (right) are shown. Annotated numbers indicate the percentage of cells positive for AVOs (quadrant Q2).

Figure 5. eIF2α signaling in 17α-AED treated breast tumor cells.
a) MCF-7 and MDA-MB231 cells were exposed to 17α-AED (17α, 10 μM and 20 μM, respectively) or vehicle (veh) for 24 h. Immunoblotting was performed to detect the phosphorylated form of eIF2α (p-eIF2α) or total eIF2α (t-eIF2α). b) MCF-7 cells were transfected with a DN-eIF2α or wild-type eIF2α (wt-eIF2α, control) plasmid construct and 48 h later cells were treated with 17α-AED (17α, 10 μM) or vehicle (veh). After 15 h of treatment, immunoblotting for CHOP was performed and, after 48 h of drug treatment, LC3 conversion was assessed. c) A fraction of the transfected MCF-7 cells were exposed to vehicle or 17α-AED (17α, 10 μM) for 48 h and cell viability was evaluated. The results indicate that disruption of eIF2α signaling reduces 17α-AED induced cell death. (p<0.01). Representative results of 2 independent experiments are shown.
radiation on breast tumor cell survival to determine if the steroid can enhance the anti-tumor activity of a conventional form of breast cancer therapy. MCF-7 and MDA-MB231 cells were exposed to escalating concentrations of 17α-AED for 48 h and, midway through drug treatment, cells were subjected to increasing doses of radiation. The results shown in Figure 6 indicate that the presence of 17α- AED significantly enhances the cytotoxic activity of radiation on the tumor cells. To establish the nature of the combined effects, CI values were calculated based upon normalized colony formation where a CI value <1 indicates synergy. As shown in Table 1, there is synergy between the two treatments in the fixed dose ratios tested which is readily appreciable in the MCF-7 cells and MDA-MB231 cells exposed to the higher combinations, as compared to the single treatment groups.

Discussion
We have previously demonstrated that 17α-AED inhibits the proliferation of human breast cancer cells; however, the cytotoxic activity of 17α-AED and the mechanisms involved in the anti-tumor activity have not been investigated [9]. Initial studies evaluated both short-term and long-term cytotoxicity of 17α-AED on several human breast cancer cells. The results of these studies were similar and revealed that the LC50 of 17α-AED was less than 15.0µM on the tumor cell lines tested with MCF-7 cell being the most sensitive. Moreover, responsiveness to 17α-AED is most likely not mediated through
ER signaling since the steroid induced cell death in both ERα+, ERα- breast tumors and MBA-MDA231 cells which lack steroid induced, ERβ signaling [19,21]. This is of importance because a majority of treatment approaches for breast cancer involve targeting this hormone receptor. Likewise, the role of the tumor suppressor protein p53 can be considered negligible since both p53 wild-type (MCF-7) and p53 mutant (MDA-MB231 and T47D) cell lines were sensitive to 17α-AED treatment [30]. Aspinall et al. [31] recently reported that 5-androstene 3β,17α diol (the epimer of 17α-AED) stimulates the proliferation of human breast tumor cells through an ER mediated mechanism. This is noteworthy since the 5-androstenediols epimers are chemically identical, only differing in the orientation of the hydroxyl group on C-17; however this results in major different biological effects and underscores the strict structure-activity relationship of these steroids [32].

Subsequent studies investigated the activation of type I (apoptosis) and type II (autophagy) programmed cell death pathways in breast tumor cells in response to 17α-AED treatment. MCF-7 cells exposed to escalating concentrations of 17α-AED were positive for LC3 cleavage, a biochemical marker for autophagy [26]. In addition, a significant percentage of breast cancer cells contained acidic autolysosomes after androstene treatment. In contrast, no increase in the degree of the activation of initiator caspases-8 and 9 or of the effector caspase-3 was detected in 17α-AED treated MCF-7 cells. Similar results were obtained when MDA- MB231, TTTU-1 and T47D breast cancer cells were treated with the steroid. Collectively, these results indicate that 17α-AED, when used at pharmacologically relevant concentrations, induces autophagic cell death in human breast tumor cells, regardless of ERα status.

Phosphorylation of eIF2αresults in the general inhibition of protein synthesis under a variety of stressful conditions such as nutrient starvation or the accumulation of unfolded proteins in the endoplasmic reticulum and may lead to apoptosis or autophagy [28,33]. 17α- AED treatment of MCF-7 and MDA-MB231 cells resulted in increased levels of eIF2α phosphorylation, suggesting an involvement in the biological effects of the steroid. Down modulation of eIF2α signaling resulted in reduced autophagy and cell death in MCF-7 breast cancer cells exposed to 17α-AED. This indicates that eIF2α activity may play a role in the anti-tumor activity of 17α-AED on breast tumors. It has been estimated that the risk of breast cancer recurrence is >20% after conservative surgery and loco-regional radiotherapy is routinely used in an attempt to eradicate residual disease [34]. However, in addition to the discomfort there may be harmful consequences that accompany radiation treatment; therefore, a therapy that would increase the efficacy of radiation and allow for a significant reduction to exposure levels would be beneficial. In the last series of experiments, we demonstrated that treatment of breast tumor cells with 17α-AED synergistically enhances the anti-tumor effects of radiation. Exposure of MCF-7 cells to radiation has been reported to promote autophagy which is generally considered a cytoprotective response [35]. Kim et al. [36] recently reported that radiation induces autophagy in MCF-7 cells by the activation of the endoplasmic reticulum response and protein kinase-like endoplasmic reticulum kinase/ eIF2α signaling and that the treatment with tunicamycin, a well known inducer of endoplasmic reticulum stress, greatly enhances the radiosensitivity of the MCF-7 cells. It is tempting to speculate, that in our studies of combination 17α-AED and radiation treatment of breast tumor cells, the co-activation of endoplasmic reticulum stress and eIF2α signaling overrides the cytoprotective aspect of autophagy to potentiate cancer cell death.

**Conclusions**

Our findings demonstrate that in vitro treatment with 17α-AED induces autophagic cell death in human breast tumor cells which appears to be independent of ERα/β. Additionally, the cytotoxic activity of this steroid on breast cancer cells is dependent, at least in part, to eIF2α signaling. Finally, we have shown that 17α-AED either enhance or synergistically interacts with radiation to potentiate breast tumor cell death. It is possible that 17α-AED may have therapeutic value in the treatment of breast tumors as a single agent or an adjuvant.

**List of abbreviations**

17α-AED, 5-androstene 3β,17α diol, AVOs: acidic vesicular organelles, CHOP, CCAAT: enhancer-binding protein homologous protein, CI: combination index, DHEA: dehydroepiandrosterone, DN: dominant-negative, eIF2 α: eukaryotic translation initiation factor 2, ER: estrogen receptor, Her-2/neu: human epidermal growth factor receptor 2, LC3: microtubule-associated protein-light chain 3, 3-MA: 3-methyladenine PARP: poly (ADP-ribose) polymerase, wt: wild type.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

MRG designed the study; interpreted the experimental results; and critically reviewed and approved the manuscript. WJ and TP executed the immunoblotting; eIF2a signaling disruption experiments; cytotoxicity and clonogenic assays; and were involved with data and image acquisition, RML conceptualized and co-designed the study; interpreted the experimental results; and critically reviewed and approved the manuscript.

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