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Autophagy and the functional roles of Atg5 and beclin-1 in the anti-tumor effects of 3β androstene 17α diol neuro-steroid on malignant glioma cells

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ABSTRACT

In this study, we demonstrate that the anti-tumor activity of the neuro-steroid, 3β androstene 17α diol (17α-AED) on malignant glioma cells is mediated by the induction of autophagy. 17α-AED can inhibit the proliferation an induce cell death of multiple, unrelated gliomas with an IC_{50} between 8 and 25 μM. 17α-AED treatment induced the formation of autophagosomes and acidic vesicular organelles in human malignant gliomas which was blocked by bafilomycin A1 or 3-methyladenine. Cleavage of microtubule-associated protein-light chain 3 (LC3), an essential step in autophagosome formation, was detected in human malignant glioma cells exposed to 17α-AED. In 17α-AED treated T98G glioma cells there was an increase in the autophagy related proteins Atg5 and beclin-1. Silencing of *ATG5* or *beclin-1* with small interfering RNA significantly reduced the incidence of autophagy in 17α-AED treated malignant gliomas and attenuated the cytotoxic effects of the neuro-steroid indicating that the induction of autophagy mediates the anti-glioma activity of 17α-AED rather than serving as a cyto-protective response. These results demonstrate that 17α-AED possesses significant anti-glioma activity when used at pharmacologically relevant concentrations in vitro and the cytotoxic effects are resultant from the induction of autophagy.

1. Introduction

Glioblastomas are grade IV astrocytic neoplasms derived from the glial lineage; are the most frequent of primary central nervous system tumors, accounting for 69% of the glial tumors; and are the most malignant [1]. Traditional treatment for this disease is surgical resection accompanied by radiation and is generally considered palliative with the 5-year survival rate of 2% [2]. A recent advance in the treatment of this brain cancer is the use of the alkylating agent, temozolomide, in combination with radiation therapy which has shown to extend the median survival of malignant glioma patients an additional 2.5 months [3,4].

Androstene steroids are produced in neuro-ectodermal tissue by the metabolism of dehydroepiandrosterone [5]. The androstene steroids exist in α- and β-epimeric forms and, although chemically identically, the biological actions of the two epimers are distinctly different. 3β androstene 17β diol (17β-AED) is an enhancer of immune regulation [6–10]. In contrast, 3β androstene 17α diol (17α-AED) possesses potent anti-tumor activity but no known immune function. 17α-AED can inhibit the proliferation and induce cell death of human MDA-MB231 and ZR75-1 breast adenocarcinomas; U937 and HL-60 lymphomas; and T98G and U251MG malignant gliomas [11–13]. However, the mechanism of the anti-tumor effects of 17α-AED is different in malignant gliomas as compared to that of the other tumors. In this regard, 17α-AED induces apoptotic type I programmed cell death in the breast tumors and the lymphomas but not in malignant gliomas [11–13]. Human malignant glioma cells exposed to 17α-AED do not display hallmark features of apoptosis such as DNA fragmentation and caspase activation, suggesting that an alternate cell death pathway is utilized in these cells [13].

Autophagy is generally associated with a cell survival response to starvation or a toxic environment and is a ubiquitous process in which cells degrade cytosolic materials such as proteins and organelles [14–17]. In this process, cytoplasmic constituents are sequestered within the developing membrane of a nascent

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autophagosomal vesicle which then fuses with a lysosome to form an autolysosome. Here, the contents of the vesicle are degraded and recycled. Autophagosome formation involves two functional groups of Atg proteins. The first group is a lipase signaling complex. In mammalian cells, the class III phosphatidylinositol-3 kinase (PI3K) and p150 form a complex with beclin-1 (orthologue of yeast Atg6) to initiate the nucleation of the pre-autophagosome [18,19]. The second group consists of an ubiquitin-like conjugation system which is required for pre-autophagosome expansion and completion. It is composed of the Atg12-Atg5 conjugation system and microtubule-associated protein 1 light chain 3 (LC3) which is the mammalian orthologue of yeast Atg8. LC3 is shuttled to the autophagosomal membrane by the Atg12-Atg5 complex. LC3-I (18 kDa) is converted to LC3-II (16 kDa) which is integrated into the autophagosome membrane [16,18]. Both conjugation systems are interdependent and autophagy does not proceed if there is a defect in one of the systems.

Although autophagy is generally viewed as a cell survival mechanism, in some cases it can be a form of type II programmed cell death [17,20,21]. The role of autophagy in cancers such as malignant glioma is controversial. In this regard, the induction of autophagy may be a cyto-protective mechanism in response to cytotoxic agents or the nutrient-poor tumor micro-environment. On the other hand, autophagy may act as a tumor suppressor mechanism and induce cell death which has been such as observed when apoptosis is blocked [14,20–22].

Here, we show that 17α-AED can inhibit the proliferation and induce cell death of multiple, unrelated glioma cell lines. Based upon our recent findings, we hypothesized that, in human malignant gliomas, the in vitro anti-tumor effects of 17α-AED may be mediated by autophagic type II programmed cell death. To test this, we analyzed 17α-AED treated malignant glioma cells for the presence of autophagosomes; elevated levels of autophagy associated proteins and the conversion of LC3-I to LC3-II. Our findings revealed that 17α-AED induced autophagic cell death in malignant glioma cells. Furthermore, the disruption of the autophagic process in malignant glioma cells with small interfering RNA (siRNA) targeting *ATG5* or beclin-1 attenuated the biological effects of 17α-AED.

2. Materials and methods

2.1. Cell lines and culture

Glioma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and non-essential amino acids as adherent monolayers at 37 °C, passed biweekly with trypsin in the absence of antibiotics. All tissue culture reagents and supplements were obtained from Invitrogen (Carlsbad, CA). The human glioblastoma cell lines LN-18, LN-229, LN-Z308, T98G, U251MG, U87MG were maintained by the Neuro-Oncology Research Group at Virginia Commonwealth Medical Center, Richmond, VA. The human LN-18, LN-229, LN-Z308, U87MG, T98G, U87MG and rat F98 cells were acquired from the American Type Culture Collection (Manasas, VA) and the U251MG cell line was obtained from the European Collection of Cell Cultures (Wiltshire, UK). GBM6 is a primary human malignant glioma and was a gift from the laboratory of C. David James (University of California – San Francisco). GBM6 was serially passaged in nude mice and for in vitro experiments, short-term (5–14 days) primary cultures were generated from a tumor mince as originally described by Sarkaria et al. [23]. The murine GL261 glioma was a gift from Dr. Gregory Plautz, Cleveland Clinic Foundation, Cleveland, OH. All cell lines were tested to be negative for mycoplasma contamination (MycoTect, Invitrogen).

2.2. Proliferation assays

The proliferation of tumor cells was assessed by the incorporation of ^{3}H-TdR (Amersham Biosciences, Piscataway, NJ) as described by Graf et al. [13]. Briefly, glioma cells (1×10^4/well) were cultured in a 96-well, tissue culture plate in the presence of 17α-AED (3–200 μM, provided by Dr. Loria) for 3 days. Control wells were treated with vehicle (50% PEG400/50% ethanol). During the last 15 h of culture, cells were pulsed with 1 μCi of ^{3}H-TdR and stored at −80 °C. Incorporation of ^{3}H-TdR was analyzed using a 96-well plate harvester and a beta-plate reader (Packard, Meridien, CT). Data are expressed as percent reduction in proliferation of the mean of triplicate experimental cultures as compared to the mean of cultures treated with vehicle.

2.3. Cytotoxicity assays

Lactate dehydrogenase (LDH) released into the culture medium from cells with damaged plasma membranes was used to determine the level of cell death. Glioma cells were prepared in triplicate as in the proliferation assay and after 3 days of culture the level of released LDH was quantified using a LDH-Cytotoxicity Assay Kit II (BioVision, Mountain View, CA) according to the manufactures directions. Percentage of cytotoxicity was measured by the following formula: (experimental release − spontaneous release/total release − spontaneous release) × 100 where spontaneous release is from comparable cultures treated with vehicle.

2.4. Detection of acidic vesicular organelles

Acidic vesicular organelles can be detected in cells by staining with acridine orange [24,25]. Tumors cells were seeded into 6-well cluster plates and cultured in the presence of vehicle or 10–20 μM of 17α-AED for 3 days as described above. In some cases, 20 nM of bafilomycin A1 or 1–10 mM of 3-methyladenine (3-MA, both from Sigma, St. Louis, MO) was added to the cultures for the last 48 h as indicated. At the end of the culture period, acridine orange (1.0 μg/ml, Sigma) was added to each culture and cells were incubated for 15 min. Total floating and adherent cells were collected; washed with PBS and analyzed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA). When excited with a 488 nm laser, the nucleolus of acridine orange stained cells fluoresces bright green which can be detected in the FL1 channel and acidic vesicles emit a bright red fluorescence which can be detected in the FL3 channel [25,26]. Forward scatter threshold was adjusted to omit cellular debris and 10,000 ungated events were analyzed. Tumor cells containing AVOs were identified as double positive cells in the Q2 quadrant of the FL1, FL3 histogram.

2.5. Transmission electron microscopy

T98G malignant glioma cells were grown on plastic coverslips in 24-well cluster plate in the presence of vehicle or 17α-AED (10 μM) for 3 days. T98G cells were then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h; rinsed with 0.1 M cacodylate buffer; post-fixed in 1% OsO_4; and dehydrated with graded ethanol to PolyBed 812 (Polysciences, Warrington, PA) embedding media. T98G cells were flat embedded with cell side down on BEEM embedding capsules (Electron Microscopy Sciences, Fort Washington, PA) filled with fresh resin and polymerized overnight in a 60 °C oven. The embedded coverslips were peeled off to expose the monolayer; thin sections (70–60 nm) were generated on an ultramicrotome; placed on 200 mesh copper grids; and stained with uranyl acetate and lead citrate for contrast. The grids were viewed in the JEOL 1210 transmission electron microscope at 80 kV.

2.6. Western blotting

Tumor cells were seeded in a 6-well cluster plate and incubated with 17α-AED (10–20 μM, final) or vehicle as described above. When 3-MA was used, cells were pre-incubated for 1 h with the inhibitor before 17α-AED treatment. After 6–72 h, non-adherent and adherent cells were collected and cell lysates were prepared and immunoblotting was conducted as previously described [13]. Briefly, total protein was resolved on a 4–12% NuPAGE MES gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were incubated with rabbit polyclonal Abs specific for beclin-1, Atg5 (both from Santa Cruz Biotechnology, Santa Cruz, CA); LC3 (a generous gift from Dr. Seiji Kondo, University of Texas M.D. Anderson Cancer Center, Houston, TX and Novus Biologicals, Littleton, CO); phosphorylated-Akt (Ser473); total Akt; phosphorylated mammalian target of rapamycin (mTOR, Ser2448); and total mTOR (Cell Signaling Technology, Beverly, MA) followed by an incubation with a goat anti-rabbit Ab conjugated to horseradish peroxidase (Rockland Immunochemicals, Gilbertsville, PA). Immuno-reactive bands were visualized using chemiluminescence.

2.7. siRNA transfection

For siRNA transfection, 2×10^5 malignant glioma cells were seeded into 6-well plates overnight. Next day, the culture media was replaced with serum-free Optimem (Invitrogen) and glioma cells were transfected with 100 nM of *ATG5*, *beclin-1* or control siRNA (Santa Cruz Biotechnology) using oligofectamine (Invitrogen). Over the course of 6 h, cultures were supplemented with fetal bovine serum in a step-wise fashion to a final 10% concentration. Two days after transfection, a fraction of the cells was subjected to Western blotting for Atg5 or beclin-1. The remaining glioma cells were treated with 17α-AED and analyzed for AVO formation; used in proliferation and cytotoxicity assays or lysed and used for LC3 immunoblotting.

2.8. Statistics

Statistical analysis was performed using the Student's *t*-test. Mean value of at least three independent experiments are shown ± the standard error of the mean (S.E.). Differences were considered to be significant when the calculated *p* value was less than 0.05.

3. Results

3.1. 17α-AED inhibits the proliferation of human and rodent glioma cell lines and induces cell death

Several human and rodent glioma cell lines were treated with titrated concentrations of 17α-AED to determine the dose of 17α-AED that is required to inhibit 50% of cell proliferation as compared to cells treated with vehicle (IC_{50}). The results, shown in Table 1, indicate that the IC_{50} for 17α-AED on the gliomas tested is between 8 and 30 μM. The median IC_{50} for the gliomas tested is 16.47 μM ± 2.01.

Next, the cytotoxic effects of 17α-AED were evaluated in malignant cells. Human GBM6, T98G, U87MG, LN-18 and LN-Z308 malignant glioma cells were treated with titrated doses of 17α-AED for 3 days and cell viability was assessed by measuring the release of LDH. The results are shown in Fig. 1 and demonstrate that the primary GBM6 and the T98G glioma cells are the most sensitive to 17α-AED with a LD_{50} of <10 μM, consistent with the proliferation data. The other human glioma cell lines were also killed by 17α-AED with a LD_{50} ~20 μM. These results demonstrate that 17α-AED can induce a significant level of cell death in a dose dependent fashion in these five human malignant glioma cell lines.

Table 1
IC_{50} of 17α-AED on several different human and rodent glioma cell lines.[a].

Glioma	Species	IC_{50} (μM ± S.E.)
GBM6	Human	8.52 ± 1.50
LN-18	Human	10.65 ± 0.45
LN-229	Human	17.60 ± 0.90
LN-Z308	Human	10.75 ± 0.05
T98G	Human	8.50 ± 1.30
U251MG	Human	15.85 ± 0.75
U87MG	Human	22.35 ± 1.77
F98	Rat	13.65 ± 1.55
RT-2	Rat	18.05 ± 0.25
GL261	Murine	17.25 ± 1.05

[a] Mean percentages of at least two independent experiments are shown, ±S.E.

3.2. 17α-AED treatment of human malignant glioma cells induces the development of acidic vesicular organelles

The development of AVOs is a late event in the autophagy and the formation of these vesicles can be detected with the lysosomal tropic agent acridine orange [25]. In a time course study, T98G glioma cells were exposed to 17α-AED (10 μM) and analyzed for the generation of AVOs by acridine orange staining and flow cytometry. There were no significant differences in the presences of AVOs in T98G glioma cells exposed to 17α-AED for 24 and 48 h as compared to sham treated cells (data not shown). However, after 72 h, a significant level of AVO formation could be readily detected in the 17α-AED treated T98G cells but not in T98G cells treated with vehicle (Fig. 2A and B). Moreover, the addition of 3-MA, an inhibitor of class III PI3K which blocks autophagic sequestration, to T98G glioma cultures exposed to 17α-AED for 72 h significantly decreased the level of AVOs from 33.83% ± 0.89 S.E. to 20.23% ± 0.59 ($p < 0.001$, Fig. 2C) [19,27]. Bafilomycin A1 is an inhibitor of vacuolar H^+-ATPase and inhibits the fusion between autophagosomes and lysosomes [15,28,29]. The presence of bafilomycin A1 in 17α-AED treated T98G cultures reduced the generation of AVOs to levels that were close to control treated T98G cells (Fig. 2D and Table 2).

These investigations were expanded to determine if 17α-AED could induce AVO formation in other human malignant gliomas. LN-18, LN-229, LN-Z308, U87MG, and U251MG malignant glioma cells were exposed to 20 μM of 17α-AED or vehicle in the presence or absence of bafilomycin A1 (20 nM). After 3 days, the development of AVOs was assessed by acridine orange staining and flow cytometry.

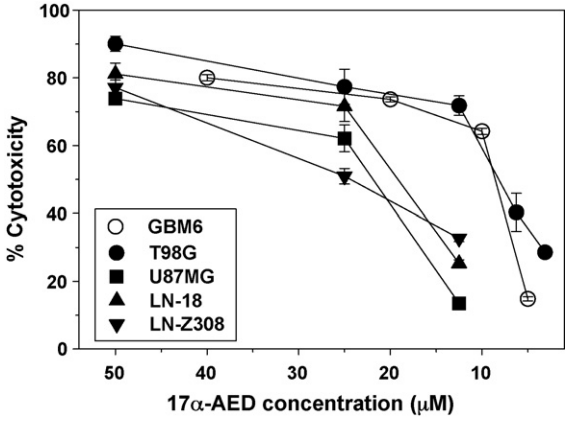

Fig. 1. 17α-AED is cytotoxic to human malignant glioma cells. After exposure to titrated doses of 17α-AED for 3 days the degree of cell death was determined based upon the measurement of LDH activity released from the cytosol of damaged cells. This experiment was conducted three times and yielded similar results.

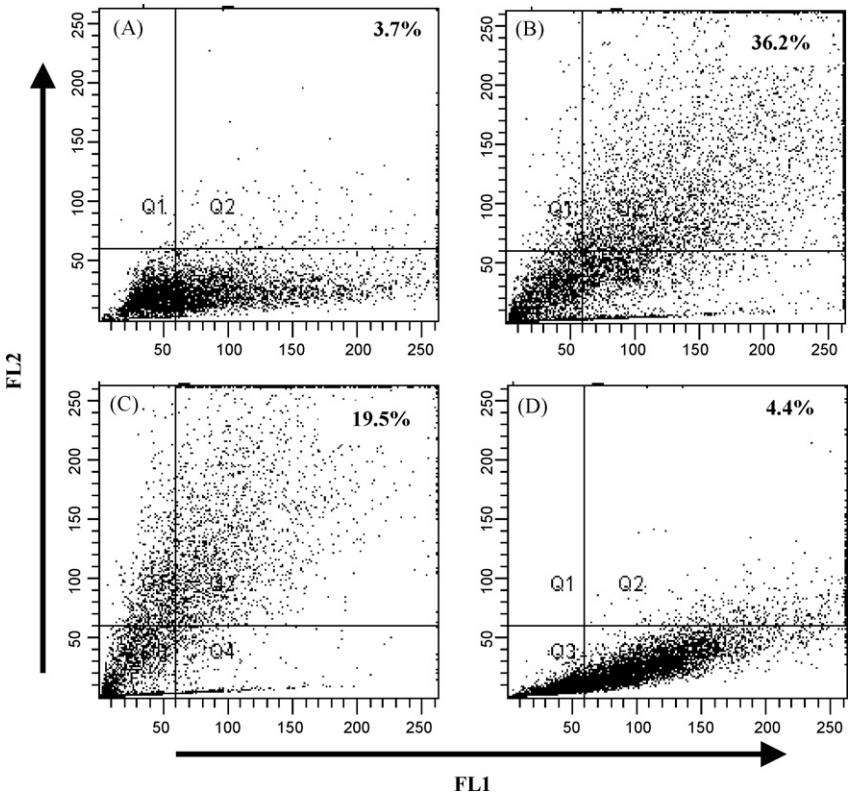

Fig. 2. Acidic vesicular organelle formation in human T98G malignant glioma cells treated with 17α-AED. T98G glioma cells treated with vehicle (A) or 10 μM 17α-AED (B) and stained with acridine orange after 72 h of treatment. Annotated numbers indicate the percentage of cells positive for AVOs in quadrant Q2. T98G cells were exposed to 17α-AED (10 μM) for 72 h and inhibitors were added for the last 48 h. (C) The presence of 3-MA (10 mM) significantly reduced the degree of AVO formation and (D) the addition of bafilomycin A1 (20 nM) inhibited the generation of AVOs in treated T98G cells. Representative results of three independent experiments are shown.

The results are shown in Table 2 and indicate that 17α-AED treatment induced the formation of AVOs in a significant percentage of cells in each of the gliomas tested as compared to vehicle treated cells ($p < 0.005$). Of the gliomas treated with 17α-AED, the overall mean percentage containing AVOs was 23.14 ± 8.38 which represents >300% increase over the mean value of controls (5.18 ± 0.05). Moreover, the addition of bafilomycin A1 effectively blocked 17α-AED induced AVO formation in the malignant glioma cells.

3.3. Presence of autolysosomes in T98G malignant glioma cells treated with 17α-AED

Autolysosomes arise from the fusion of autophagosomes and lysosomes which is a late stage event in the autophagic process. Autolysosomes are ultra-structures that can be detected by electron microscopy and are characterized by a single membrane and contain degraded proteins and organelles [17,30]. Based upon the results form the AVO formation studies, T98G glioma cells were exposed to vehicle or 10 μM of 17α-AED for 72 h and then processed for transmission electron microscopy. Analysis of cellular ultra-structures revealed the presence of multiple autolysosome-like bodies in the cytoplasm of 17α-AED treated cells (Fig. 3). These vesicles tended to be located adjacent to the nucleus and appeared as dark vesicular bodies with a single membrane containing debris suggesting that they were late stage autophagosomes or autolysosomes [17,30]. The vesicles were ~500 nm in size and were rarely present in control treated T98G glioma cells.

Table 2
Percentage of malignant glioma cells with acidic vesicular organelles after 3 days of exposure to 17α-AED ± the presence of bafilomycin A1.[a]

Glioma	Vehicle	17α-AED[b]	+baf[c]
LN-18	5.35 ± 1.20	17.25 ± 1.85	5.35 ± 2.25
LN-229	5.20 ± 0.59	22.52 ± 2.54	5.85 ± 0.95
LN-Z308	5.20 ± 0.40	30.70 ± 2.90	4.05 ± 2.05
T98G	5.10 ± 0.73	35.73 ± 0.93	3.97 ± 1.54
U251MG	5.02 ± 0.47	14.40 ± 1.64	5.50 ± 1.70
U87MG	5.22 ± 0.18	18.26 ± 1.15	4.95 ± 1.25
Overall means	5.18 ± 0.05	23.14 ± 8.38	4.94 ± 0.32

[a] Mean percentages ± S.E. are shown.
[b] 20 μM of 17α-AED, 10 μM for T98G cells.
[c] Bafilomycin A1 (baf) 20 nM.

3.4. 17α-AED treatment induces increased levels of beclin-1 and Atg5 in T98G glioma cells and increased LC3 conversion in several different human malignant gliomas

T98G malignant glioma cells were cultured in the presence of 10 μM of 17α-AED or vehicle and the levels of beclin-1 and Atg5 as well as the conversion of LC3-I to LC3-II was determined by Western blotting after 72 h of treatment. Beclin-1 is a member of the lipase signaling complex which is essential in the induction of autophagy [16,18,19]. An elevated level of beclin-1 can be detected in T98G cells exposed to 17α-AED (Fig. 4A). In contrast, only a low level of beclin-1 can be detected in vehicle treated T98G cells. Atg5 and LC3 are constituents of the conjugation system and play a role in the extension of the autophagosomal membrane [16,18]. Fig. 4A shows an increase in the level of Atg5 protein in T98G cells treated with 17α-AED over the basal level of Atg5 in vehicle treated T98G cells. The conversion of LC3-I to LC3-II is a strong biochemical marker for the induction autophagy [31]. As shown in Fig. 4A, there is a significant level of the processing of LC3-I to LC3-II in T98G glioma cells treated with 17α-AED. A low background level of LC3-II can be seen in T98G glioma cells treated with vehicle. In order to

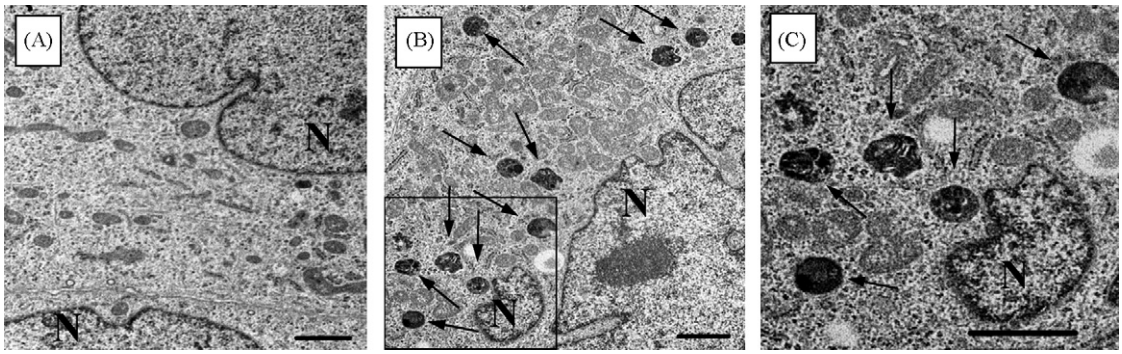

Fig. 3. Presence of autolysosomes in T98G glioma cells exposed to 17α-AED. Transmission electron microscopic analysis of the ultra-structure of human T98G malignant glioma cells treated for 3 days with (A) vehicle or (B) 17α-AED (10 μM). 17α-AED treated T98G glioma cells with multiple autolysosome-like vesicles in the cytoplasm (arrows). Note that the nucleus (N) in control and treated T98G cells was intact with no evidence of chromatin condensation or fragmentation. (C) Boxed region of 17α-AED treated T98G cells shown in (B) at a higher magnification. Bar = 1 μm.

obtain information as to the rate of the induction of autophagy in T98G glioma cells treated with 17α-AED, we performed a time course study in which the level of LC3 cleavage was assessed. The results are shown in Fig. 4B and clearly indicate LC3 processing after 12 h of 17α-AED (10 μM) exposure which proceeded over time. This suggests that the induction of autophagy by 17α-AED treatment in T98G cells, as measured by LC3 processing, is rapid as compared to the generation of AVOs.

Petiot et al. [19] demonstrated that 3-MA blocks autophagic sequestration by the inhibition of class III PI3K and we observed that 3-MA treatment attenuated 17α-AED induced formation of AVOs in T98G malignant glioma cells. In a continuation of these experiments, we determined the effect that this inhibitor would have on Atg5 protein levels and LC3 conversion. T98G glioma cells were treated with vehicle or 17α-AED (10 μM) in the presence or absence of 3-MA. As shown in Fig. 4C, there was a reduction in the conversion of LC3-I to LC3-II and a decrease in Atg5 protein in T98G glioma cells that were treated with 17α-AED plus inhibitor as compared to T98G glioma cells treated solely with 17α-AED or inhibitor.

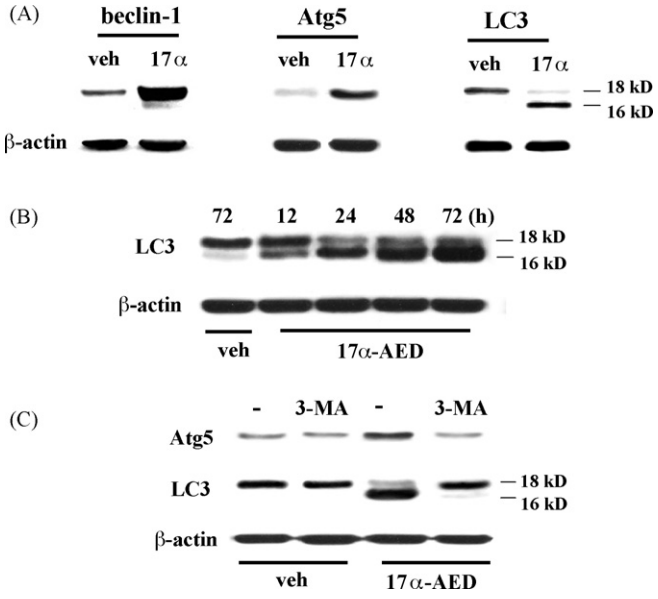

Fig. 4. Increased protein levels of beclin-1, Atg5 and LC3 conversion in T98G glioma cells exposed to 17α-AED. (A) T98G glioma cells were treated with vehicle (veh) or 10 μM 17α-AED (17α) for 72 h. T98G cells were lysed and 5–25 μg of total protein were subjected to Western blot analysis and probed with antibodies against beclin-1, Atg5 or LC3. Increased levels of beclin-1 and Atg5 as well as increased conversion of LC3-I (18 kDa) to LC3-II (16 kDa) can be detected in the treated T98G cells as compared to controls. Representative results from three experiments are shown. (B) T98G cells were exposed to 17α-AED (10 μM) for the indicated times and immunoblotting was performed to determine the degree of LC3 conversion over time. Representative results from two time course experiments are shown. (C) Same as in (A); however, when 3-MA (1 mM) is included in the cultures, 17α-AED treated T98G glioma cells have reduced levels of LC3-II and Atg5. This experiment was conducted twice and yielded similar results. An anti-β-actin mAb was used to show equal protein loading.

The induction of autophagy, as detected by LC3 processing, was also assessed in several different human malignant gliomas cells after exposure to 17α-AED or vehicle. The results in Fig. 5 show that in vehicle treated glioma cells there is little or no conversion of LC3-I to LC3-II. In contrast, all of the malignant glioma cells treated with 17α-AED have a robust level of LC3-II protein and an associated decrease in the level of LC3-I. The results from these findings indicate that 17α-AED can induce autophagy in human malignant glioma cells.

3.5. Transfection of human T98G malignant glioma cells with beclin-1 or ATG5 siRNA reduces the biological effects of 17α-AED

The Atg12-Atg5 and the Atg8 (LC3)-PE conjugation systems are interdependent and a disruption in one system has a direct negative effect on the autophagic process [18]. Beclin-1 is required for the induction of autophagy. Therefore, perturbation of the levels of Atg5 or beclin-1 should result in reduced autophagy and the attenuation of the biological effects of 17α-AED on human malignant glioma cells. To test this, T98G, U251MG and LN-229 glioma cells were transiently transfected with siRNA targeting *ATG5* or *beclin-1* to specifically knock down the intracellular levels of these proteins. As shown in Fig. 6A, 48 h after transfection with *ATG5* or *beclin-1* siRNA (100 nM) there was a significant, although not complete, reduction in the levels of these proteins in malignant glioma cells as compared to glioma cells transfected with non-targeting, scrambled siRNA (control, 100 nM). Furthermore, as shown in Fig. 6B, transfection with *ATG5* or *beclin-1* siRNA resulted in a significant decrease in the percentage of malignant glioma cells containing AVOs and the degree of LC-3 processing (Fig. 6C) in glioma cells after 17α-AED treatment as compared to glioma cells transfected with control siRNA.

To further advance our understanding of the role of autophagy in malignant gliomas treated with 17α-AED, we evaluated the consequences of the disruption of autophagy by treatment with *ATG5* or *beclin-1* siRNA on the anti-glioma effects of 17α-AED. Malignant glioma cells were transfected with scrambled, *ATG5* or *beclin-1* siRNA and after 48 h the glioma cells were exposed to 17α-AED in cytotoxicity and proliferation assays. The results indicate that the inhibition of the autophagic process with *ATG5* or *beclin-1* siRNA resulted in the significant attenuation of the

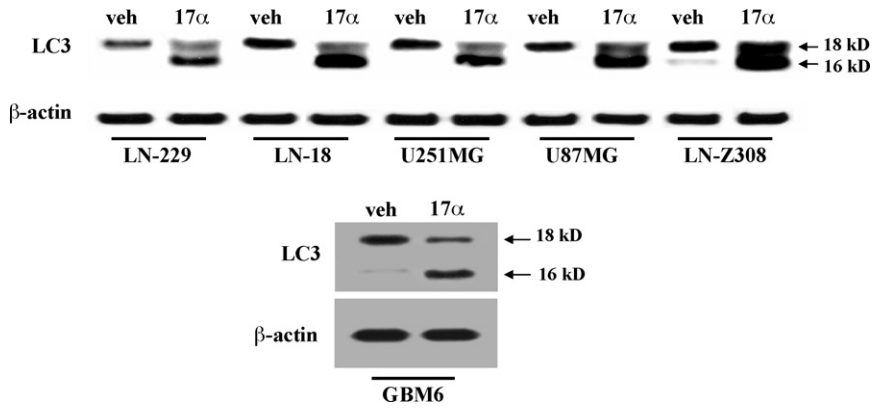

Fig. 5. 17α-AED treatment induces LC3 cleavage in several human malignant gliomas. Cell lysates were obtained from human LN-229; LN-18; U251MG; U87MG; and LN-Z308 cells treated with 20 μM 17α-AED; primary GBM6 glioma cells exposed to 8.5 μM 17α-AED or vehicle for 3 days. Twenty-five microgram of total protein were used for Western blot analysis using anti-LC3 antibody. LC3-II protein is readily detected in all of the 17α-AED treated glioma cell lines and is absent or barely detectable in gliomas treated with vehicle. Data are from one representative ($n = 2$). An anti-β-actin mAb was used to show equal protein loading.

anti-proliferative effects and cytotoxic activity of 17α-AED as compared to control cells transfected with scrambled siRNA (Fig. 6D and E). These results argue that the induction of autophagy by 17α-AED in malignant glioma cells is a means by which this neuro-steroid exerts its anti-tumor activity rather than functioning as a cyto-protective response.

3.6. Reduced Akt/mTOR signaling in T98G malignant glioma cells treated with 17α-AED

It has been shown that the inhibition of the PI3K/Akt/mTOR pathway induces autophagy in human malignant glioma cells [32,33]. Therefore, we investigated the affects of 17α-AED treatment on this signaling pathway in T98G glioma cells by analyzing the activation status of Akt and mTOR signaling proteins. In a time course study, decreased phosphorylation of Akt and mTOR could be detected in T98G cells after 12 h of exposure to 17α-AED as compared to T98G cells treated with vehicle (Fig. 7). The levels of phosphorylated Akt and mTOR in treated T98G cells continued to decrease over time while the levels of total Akt and mTOR did not change. These results suggest that modulation of PI3K/Akt/mTOR signaling may be at least one of the pathways by which 17α-AED exerts its effects on human T98G glioma cells.

4. Discussion

In our previous studies with the androstenediol neuro-steroids, we demonstrated that 17α-AED but not its epimer 17β-AED can induce apoptosis in human MDA-MB231 and ZR75-1 breast adenocarcinomas; as well as U937 and HL-60 lymphomas [11,12]. In contrast, 17α-AED treatment did not cause T98G and U251MG malignant gliomas to undergo apoptosis although the neuro-steroid did have an anti-tumor effect on these two glioma cell lines [13]. These findings indicated the functional specificity of the anti-tumor effects of the adrostenediol molecules in terms of the orientation of the hydroxyl group on C-17 (α- or β-orientation) and that a different cell death pathway may be utilized in tumors of glial origins as compared to other tumors exposed to 17α-AED. In this investigation, we demonstrated the neuro-steroid 17α-AED, when used at clinically relevant concentrations in vitro, can inhibit the proliferation and induce cell death of several malignant glioma cell lines by the induction of autophagy.

Paglin et al. [25] has recently demonstrated that the formation of AVOs in cancer cells in response to radiation is a morphological characteristic of the autophagic process and the generation of AVOs can be blocked by bafilomycin A1 and 3-MA. A significant level of AVOs could be detected in human malignant glioma cells after 72 h of treatment with 17α-AED, but not at shorter exposure times of 24 or 48 h. The formation of AVOs could be inhibited by the presence of bafilomycin A1 or 3-MA. Moreover, electron microscopy of T98G glioma cells treated with 17α-AED for 72 h revealed the presence of numerous autolysosomes or late stage autophagosomes with single membranes in the cytoplasm of the glioma cells, providing strong evidence for the induction of autophagy.

In the next series of experiments, we analyzed 17α-AED treated T98G glioma cells for the presence of biochemical markers associated with autophagy. Beclin-1 is the homologue of yeast Atg6 and is involved with the initiation of autophagic vesicle nucleation through a lipid kinase signaling complex [16]. Atg5 and LC3 proteins are from two separate, but interdependent, ubiquitin-like conjugation pathways that are required for the expansion and completion of the autophagosome. [16,18] We show that beclin-1 and Atg5 levels are increased in T98G malignant glioma cells after exposure to 17α-AED. In addition, there is a dramatic increase of conversion of LC3 I to LC3 II in T98G glioma cells treated with 17α-AED relative to control cells. The addition of 3-MA to T98G glioma cultures treated with 17α-AED resulted in a decrease in Atg5 protein and LC3 conversion.

A time course experiment revealed detectable levels of LC3 processing after 12 h of 17α-AED exposure. In contrast, AVO formation in 17α-AED treated T98G glioma cells could not be detected until after 72 h of exposure. This suggests that the induction of early autophagic events, i.e. the formation and expansion of the pre-autophagosome membrane as indicated by LC3 processing, is very rapid as compared to late stage events of autophagy such as the formation of AVOs and the fusion of autophagosomes with lysosomes to generate autolysosomes. Double membrane autophagosomes would most likely be present in 17α-AED treated T98G cells at a time after the induction of autophagy and before the generation of autolysosomes.

Using several additional human malignant glioma cells lines, we have demonstrated that 17α-AED can induce the formation of AVOs, which can be blocked with bafilomycin A1. Furthermore, LC3-I is converted to LC3-II in these human glioma cells after exposure to the neuro-steroid. These results indicate that the induction of autophagy by 17α-AED is not exclusive to T98G glioma cells but perhaps a common death pathway utilized in tumors of glial origin in response to treatment with this neuro-steroid. Exposure of T98G cells to 17β-AED (5–40 μM) failed to induce LC3 conversion (Jia, Loria and Graf, unpublished observation).

To compliment the morphological and biochemical evidence of 17α-AED induced-autophagy, we used siRNA gene silencing

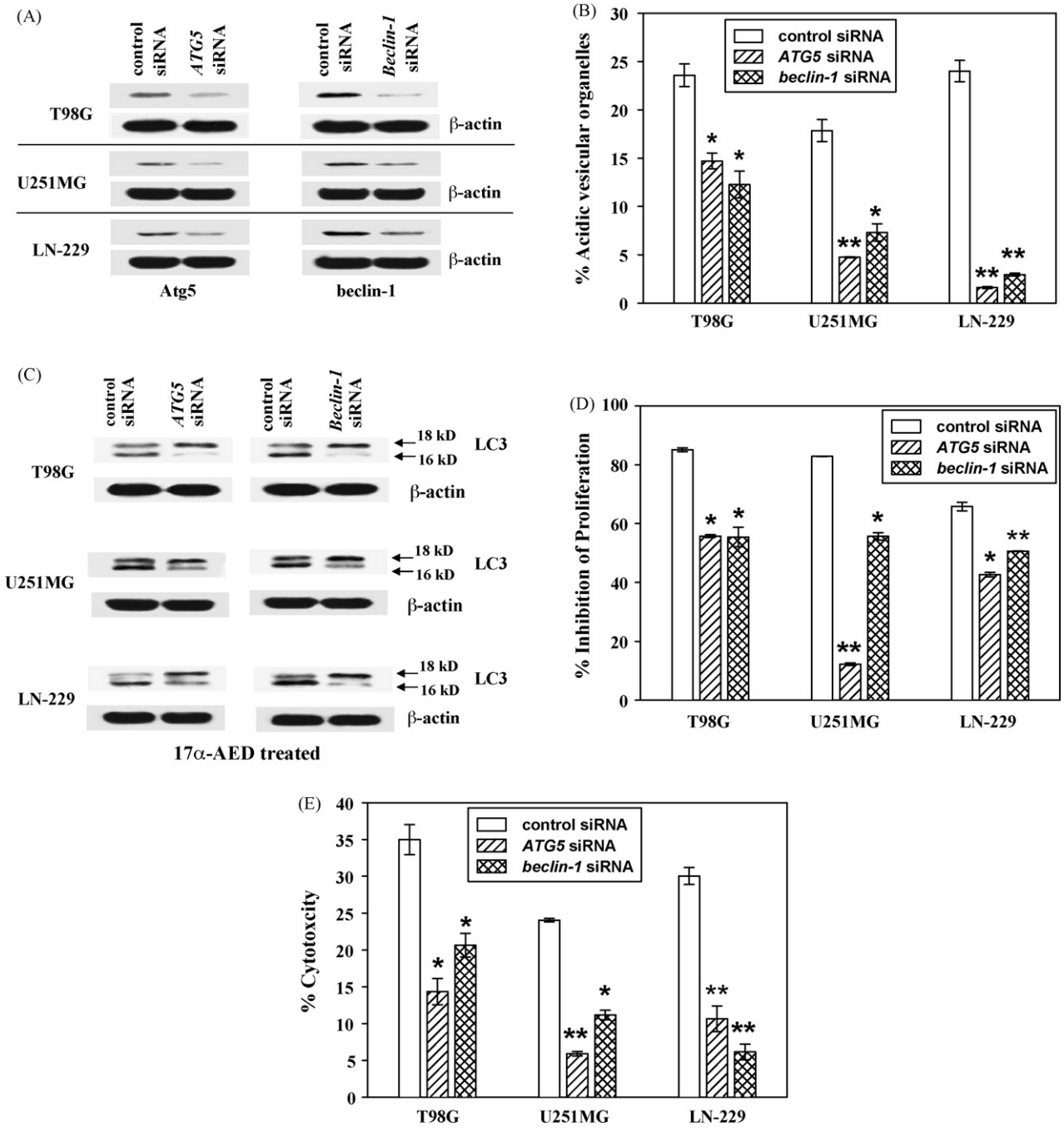

Fig. 6. *ATG5* or *beclin-1* siRNA treatment of human malignant glioma cells attenuates the anti-tumor effects of 17α-AED. T98G, U251MG or LN-229 glioma cells were transfected with 100 nM of *ATG5*, *beclin-1* or scrambled siRNA. (A) After 48 h, a fraction of the cells were subjected to Western blotting which revealed a reduction in Atg5 or beclin-1 protein levels in *ATG5* or *beclin-1* siRNA treated cells as compared to controls. The remaining malignant glioma cells were treated with 17α-AED (10 μM for T98G cells and 20 μM for U251MG and LN-229 cells) for 3 days and the biological effects of 17α-AED on the transfected cells was assessed. Knock down of Atg5 or beclin-1 protein levels resulted in a significant reduction in the (B) generation AVOs and (C) LC3 conversion in malignant glioma cells exposed to 17α-AED as compared to glioma cells transfected with scrambled siRNA. The anti-glioma effects of 17α-AED were significantly attenuated in *ATG5* or *beclin-1* siRNA transfected malignant glioma as compared to glioma cells transfected with scrambled siRNA in terms of (D) inhibition of proliferation as well as (E) cytotoxic activity ($^{*}p < 0.01$; $^{**}p < 0.005$). Representative results from two independent siRNA experiments are shown.

to disrupt the autophagic process by specifically knocking down Atg5 or beclin-1 protein levels in T98G, U251MG and LN-229 glioma cells. Although this approach did not completely eliminate the presence of these proteins, the degree to which their levels were reduced significantly attenuated the biological effects of 17α-AED on glioma cells. The incidence of autophagy, as measured by the formation of AVOs, was significantly lowered in malignant glioma cells treated with *ATG5* or *beclin-1* siRNA in response to 17α-AED treatment as compared to glioma cells transfected with control siRNA. These results are in accordance with the findings of Boya et al. [34] in which targeted reduction of Atg5 or beclin-1 by siRNA interrupted the induction of autophagy by nutrient starvation in HeLa cells. In a different study involving the modulation of Atg5 protein, Yousefi et al. [35] showed that enforced over expression of *ATG5* in HeLa cells promoted autophagy and sensitized the cells to chemotherapeutic agents where as treatment of HeLa or MDA-MB231 breast cancer cells with *ATG5* siRNA reduced the cytotoxic effects of these agents. Iwamaru et al. [32]

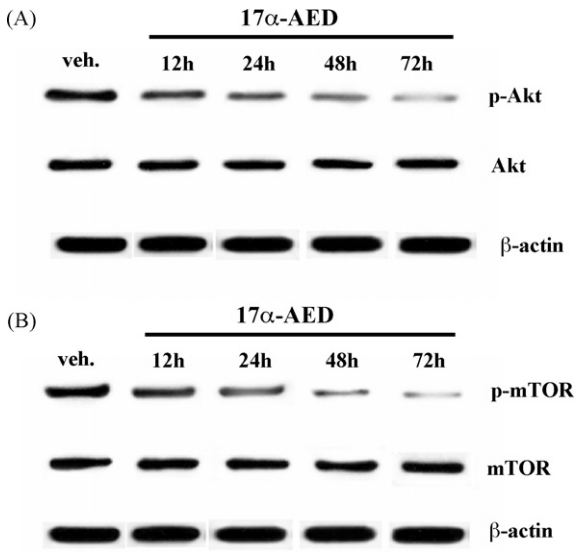

Fig. 7. Decreased phosphorylation of Akt and mTOR proteins in 17α-AED treated T98G glioma cells. T98G cells were exposed to 10 μM 17α-AED for the indicated times and lysed. Ten microgram of total protein was subjected to Western blot analysis using antibodies specific for the phosphorylated forms of (A) Akt (p-Akt-ser473); total Akt; (B) phosphorylated mTOR (p-mTOR-ser2448) or total mTOR. Data are from one representative ($n = 2$). An antibody for β-actin was used to show equal protein loading.

recently used siRNA directed against beclin-1 to attenuate the cytotoxic effects of rapamycin on malignant glioma cells, and by this means, demonstrated that the induction of autophagy was a primary mediator of the anti-tumor effects of rapamycin on malignant glioma cells. Our findings are similar, in that disruption of the autophagic process by siRNA mediated reduction of Atg5 or beclin-1 resulted in a decrease in the anti-glioma effects of 17α-AED on malignant glioma cells. These results indicate that the induction of autophagy is a mechanism by which 17α-AED exerts its biological effects of malignant glioma cells. This is an important point to consider. A recent review by Kroemer and Levine [17], pointed out that features of autophagy may be present during cell death and referred to this as 'cell death with autophagy' as compared to 'cell death by autophagy' in which autophagy is the mechanism of cell death. Our results from the *ATG5* or *beclin-1* siRNA experiments argue strongly for the latter case in that glioma cell death, as a result of 17α-AED treatment, is mediated by autophagy.

It is commonly accepted that the extreme resistance of malignant gliomas to cancer therapies is due to their rapid proliferation and intense resistance to apoptosis. Their aggressive growth can be attributed partially to constitutive activation of the PI3K/Akt/mTOR pathway [1,36]. Where as their resistance to apoptosis has been accredited in part to signaling abnormalities in this cell death pathway such as upregulation of bcl2 family members (including the recently identified molecule Bcl2-like-12) and phosphoprotein enriched in astrocytes-15 and p53 mutations [14,37,38]. Therefore, in tumor cells such as malignant gliomas, where the apoptotic pathway is commonly dysfunctional, it is understandable that an alternate death pathway, such as autophagy may be utilized in response to cytotoxic agents as compared to tumor cells possessing a more intact apoptotic pathway. However, it has been demonstrated that there is cross-talk between apoptosis and autophagy and if one pathway is blocked, cell death can progress by the alternate route. In this regard, Kanzawa et al. [26,39] has shown that blocking temozolomide- or arsenic trioxide-induced autophagy in malignant glioma cells with bafilomycin A1 results in apoptotic cell death. It is possible that in our experiments in which the autophagic process was disrupted by *beclin-1* or *ATG5* siRNA that the apoptotic pathway may be activated in glioma cells treated with 17α-AED; however, the finding that toxicity was decreased in the Atg5 and beclin-1 knock down cells treated with the neuro-steroid as compared to control argues against this. On the other hand, Shimizu et al. [40] demonstrated that treatment of Bax/Bak double knockout mouse embryonic fibroblasts with the pro-apoptotic agents etoposide or staurosporine induced autophagy.

Disruption of the PI3K/Akt/mTOR pathway with siRNAs or pharmacological inhibitors can induce autophagy in human glioma cells [32,33]. In a time course study, we demonstrated decreased activation of both Akt and mTOR in T98G glioma cells exposed to 17α-AED which was detectable after 12 h of treatment. This time frame corresponds to the induction of LC3 cleavage in 17α-AED treated T98G cells. It is possible that modulation of Akt/mTOR signaling may be involved with 17α-AED induced autophagy in human glioma cells.

In summary, our morphological, biochemical and genetic data supports our hypothesis that in vitro treatment with 17α-AED neuro-steroid induces type II programmed cell death, autophagy, in human malignant glioma cells. Moreover, the anti-tumor effects of 17α-AED are not unique to T98G glioma cells. In this regard, we have shown that 17α-AED possesses anti-tumor activity towards several different human and rodent gliomas and the neuro-steroid can induce autophagy in several, unrelated human malignant gliomas. The utilization of this alternate cell death pathway in response to 17α-AED treatment may be exclusive to tumors of glial origin since 17α-AED exposure results in apoptotic cell death in breast adenocarcinomas and lymphomas.

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