The neuro-steroid, 3β androstene 17α diol exhibits potent cytotoxic effects on human malignant glioma and lymphoma cells through different programmed cell death pathways

MR Graf*, W Jia¹ and RM Loria²
¹Department of Neurosurgery and the Massey Cancer Center, Virginia Commonwealth University Medical Center, PO Box 980631, Richmond, VA 23298-0631, USA; ²Departments of Microbiology and Immunology and Pathology, Virginia Commonwealth University Medical Center, PO Box 980631, Richmond, VA 23298-0631, USA

The neuro-steroids 3β-androstene-17α-diol (17α-AED), 3β-androstene-17β-diol (17β-AED), 3β-androstene-7α,17β-triol (7α-AET) and 3β-androstene-7β,17β-triol (7β-AET) are metabolites of dehydroepiandrosterone and are produced in neuro-ectodermal tissue. Both epimers of androstenediols (17α-AED and 17β-AED) and androstene triols (7α-AET and 7β-AET) have markedly different biological functions of their chemical analogue. We investigated the cytotoxic activity of these neuro-steroids on human T98G and U251MG glioblastoma and U937 lymphoma cells. Proliferation studies showed that 17α-AED is the most potent inhibitor, with an IC₅₀ of 15 μM. For T98G glioma, 90% inhibition was achieved with 25 μM of 17α-AED. Other neuro-steroids tested only marginally suppressed cell proliferation. Reduced cell adherence and viability could be detected after 18 h of 17α-AED exposure. Treatment with 17α-AED induced a significant level of apoptosis in U937 lymphoma cells, but not in the glioma cells. Cytopathology of 17α-AED-treated T98G cells revealed the presence of multiple cytoplasmic vacuoles. Acridine orange staining demonstrated the formation of acidic vesicular organelles in 17α-AED-treated T98G and U251MG, which was inhibited by bafilomycin A1. These findings indicate that 17α-AED bears the most potent cytotoxic activity of the neuro-steroids tested, and the effectiveness may depend on the number of hydroxyls and their position on the androstene molecule. These cytotoxic effects may utilize a non-apoptotic pathway in malignant glioma cells.

British Journal of Cancer (2007) 97, 619 – 627 doi:10.1038/sj.bjc.6603894 www.bjcancer.com
Published online: 17 July 2007 © 2007 Cancer Research UK

Keywords: androstene neuro-steroids, glioblastoma, lymphoma, malignant glioma, programmed cell death

Glioblastomas are grade IV astrocytic neoplasms derived from the glial lineage and represent ~50% of primary central nervous system (CNS) tumours. They are essentially incurable with a median survival of approximately 18 months after primary diagnosis, regardless of treatment. Traditional treatment for this disease is surgical resection accompanied by radiation and is generally considered palliative with the 5-year survival rate of 2% (Surawicz et al, 1998).

Nitrosourea chemotherapeutic agents are often used as adjuvant with radiation; however, the therapeutic benefit of the inclusion of these agents is controversial (Walker et al, 1980; Stupp and Weber, 2005a,b). The reduced efficacy of these compounds may be attributed to their limited penetration through the blood brain barrier, and the resistance of migrating glioblastoma cells to apoptosis (Kemper et al, 2004; Lefranc et al, 2005). Temozolomide is an alkylating agent that can access the CNS, and recent clinical studies have shown that temozolomide administration combined with radiation therapy can improve the overall survival of malignant glioma patients (Stupp et al, 2001; Stupp and Weber, 2005a,b). Stupp and Weber (2005a,b) demonstrated that temozolomide combined with radiation therapy prolonged the survival of glioblastoma patients with a median increase in survival of 2.5 months. Despite the recent advancement in the use of temozolomide in the treatment of glioblastomas, the prognosis of these patients remains poor and there continues to be a need for the development of effective therapies for the treatment of this disease.

Androstene steroids are produced in neuro-ectodermal tissue by the metabolism of the common steroid dehydroepiandrosterone (DHEA) (Labbe, 2002). The androstenes are neuro-steroids that exist in α- and β-epimeric forms (Figure 1) and, although chemically identical, the biological actions of the two epimers are distinctly different. In this regard, researchers have demonstrated that 3β androstene 17α diol (17α-AED) can inhibit the proliferation and induce apoptosis of human U937 and HL60 lymphoma cells (Huynh and Loria, 1997; Loria, 2002). In addition, human MDA-MB231 and ZR75-1 breast cancer cells also undergo apoptotic cell death when treated with 17α-AED by a mechanism that is independent of oestrogen and androgen receptors (Huynh et al, 2000). 17α-AED does not have inhibitory or apoptotic effects on normal cells (Loria, 2002). In contrast, the epimer, 3β/
Antitumour effects of androstene neuro-steroids  

MATERIALS AND METHODS

Cell lines and culture

Glioma cells were cultured in DMEM supplemented with 10% FBS and non-essential amino acids as adherent monolayers at 37°C, passed biweekly with trypsin in the absence of antibiotics. Cell lines were routinely screened for mycoplasma contamination (Mycotect, Invitrogen, Carlsbad, CA, USA). All tissue culture reagents and supplements were obtained from Invitrogen. The human glioblastoma cell lines T98G and U251MG were maintained by the Neuro-Oncology Research Group at Virginia Commonwealth Medical Center (Richmond, VA, USA). The T98G and was originally acquired from the American Type Culture Collection (ATCC, Manasas, VA, USA), and the U251MG cell line was obtained from the European Collection of Cell Cultures (Wiltshire, UK). The human U937 histiocytic lymphoma cell line (Sundstrom and Nilsson, 1976) was obtained from the ATCC and grows in suspension.

Proliferation assay

The proliferation of tumour cells was assessed by the incorporation of 3H-TdR (Amersham Biosciences, Piscataway, NJ, USA) in a 96-well, flat-bottom tissue culture plate. T98G and U251MG glioma cells (1 × 10^4 well^-1) and U937 lymphoma cells (2 × 10^4 well^-1) were cultured in the presence of 17β-AED, 17β-AET, 7α-AET or 7α-AED (provided by Dr Loria) at concentrations ranging from 200–3 μM in a final volume of 200 μl well^-1 for 3 days. Control wells were treated with an equivalent amount of vehicle (50% PEG400/50% ethanol). During the last 15 h of culture, cells were pulsed with 1 μCi of 3H-TdR and stored at −80°C. Incorporation of 3H-TdR was used as a measure of proliferation and was analysed using a 96-well plate harvester and a beta-plate reader (Packard, Meriden, CT, USA). Data are expressed as percent reduction in proliferation of the mean of triplicate experimental cultures as compared to the mean of sham-treated cultures.

Cell viability assay

A total of 5 × 10^3 T98G or U251MG glioma cells were seeded in a six-well cluster plate and incubated for 6–8 h, after which medium was removed and wells were rinsed with Hank’s Balanced Salt Solution (HBSS, Invitrogen). Fresh medium supplemented with 17β-AED or equivalent amount of vehicle was added to the cultures at a concentration of 10 μM for T98G cells and 15 μM for U251MG cells. After 18 h of exposure to 17β-AED, cultures were viewed with a phase-contrast microscope and photographed. Floating cells were collected. Adherent cells were trypsinized, pooled with the non-adherent cells, centrifuged and the pellet was resuspended in phosphate-buffered saline (PBS). U937 cells were seeded at a concentration of 4 × 10^5 cells ml^-1 and after 6–8 h, 17β-AED (10 μM final) or vehicle was added to the cultures. After 18 h, cells were collected, pelleted and resuspended in PBS. Over 200 cells in total were counted in each sample on a hemacytometer, and viable live cells were distinguished from dead cells by trypan blue exclusion. Mean values s.e. are shown.

Assay for DNA fragmentation

Tumour cells were seeded at a concentration of 1 × 10^5 cells well^-1 in a six-well cluster plate and incubated for 6–8 h before addition of 17β-AED (10 μM for T98G and U937; 15 μM for U251MG) or equivalent amount of vehicle. After 4 days, detached and adherent cells were collected as previously described and washed twice with PBS. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) was used for the detections of DNA cleavage in the treated cells (APO-DIRECT, BD Biosciences, San Diego, CA, USA). Cells were analysed on a FACSCanto flow cytometer (BD Biosciences). Forward scatter threshold was adjusted to omit cellular debris; 10,000 events were counted and positive TUNEL staining was read in the FL1 channel (FITC).

Western blotting

Tumour cells were seeded in a six-well cluster plate and incubated with 17β-AED (10 μM, final) or vehicle as described above. After 3 days, spent medium was collected, centrifuged and the pellet was added back to the original cell cultures. Cells were lysed with RIPA buffer, lysates were collected, pelleted and resuspended in PBS. Over 200 cells in total were counted in each sample on a hemacytometer, and viable live cells were distinguished from dead cells by trypan blue exclusion. Mean values s.e. are shown.
USA). A 25 μg weight of total protein was run on a 4–12% NuPAGE MES gel (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk + 0.1% Tween-20 in PBS for 1 h. The membranes were incubated overnight with rabbit polyclonal Ab specific for poly (ADP-ribose) polymerase (PARP) or caspase 3 (both from Cell Signaling Technology, Beverly, MA, USA) at 4°C, washed with 0.1% Tween-20 in PBS and incubated with goat anti-rabbit Ab conjugated to horseradish peroxidase (Rockland Immunocchemicals, Gilbertsville, PA, USA) for 1 h. Immunoreactive bands were visualized by chemiluminescence (SuperSignal, Pierce Chemicals). The same membranes were reblotted with a mouse anti-β-actin mAb (Sigma, St Louis, MO, USA), as described above, and used as a loading control.

**Cytopathology**

For microscopic analysis, 5 × 10^4 T98G glioma cells were added to each chamber of a four-chamber glass slide and incubated for 15 h. Medium was then removed and wells were gently washed with HBSS. Fresh medium was added and supplemented with 17α-AED for a final concentration of 10 μM. After 30 h, medium was removed, wells were gently rinsed with HBSS, cultures were fixed with 5% buffered formalin in DMEM. Chamber slides were then subjected to routine haematoxylin and eosin staining. Cells were viewed with a Nikon Eclipse E800 microscope and images obtained with a Diagnostic Instruments Spot RT CCD camera.

**Detection of acidic vesicular organelles**

Acidic vesicular organelles (AVO) can be detected in cells by staining with acridine orange (Traganos and Darzynkiewicz, 1994; Paglin et al, 2001). Tumours cells were seeded into six-well cluster plates and cultured in the presence of vehicle or 10–15 μM 17α-AED as described above, for 3 days. In some cases, bafilomycin A1 (50 nM, Sigma) was added to the cultures for the last 48 h. At the end of the culture period, acridine orange (Sigma) was added to each culture (1.0 μg ml⁻¹ final) and cells were incubated for 15 min. Total floating and adherent cells were collected; washed with PBS and analysed on a FACSCanto flow cytometer. 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 (Paglin et al, 2001; Kanzawa et al, 2003b). Forward scatter threshold was adjusted to omit cellular debris and 10 000 ungated events were analysed. Tumour cells containing AVO were identified as double positive cells in the Q2 quadrant of the FL1, FL3 histogram.

**Statistics**

Statistical analysis was performed using the Student’s t-test. Differences were considered to be significant when the calculated P-value was less than 0.05.

**RESULTS**

**Androstene steroids inhibit tumour cell growth and induce cell death**

In order to determine the antiproliferative potential of the androstene neuro-steroids, human T98G glioma, U251MG glioma and U937 lymphoma cells were cultured in the presence of 17α-AED, 17β-AED, 7α-AET and 7β-AET at titrated doses ranging from 3–200 μM, for 3 days. The results are shown in Figure 2, and demonstrate that 17α-AED is the most potent inhibitor of these neuro-steroids. In this regard, 17α-AED at a dose of 25 μM inhibited the proliferation of T98G glioma and U937 lymphoma cells by 90% or greater and U251MG glioma cell proliferation was inhibited by 80%. For each tumour, there was a significant dose response to 17α-AED when used between 3 to 25 μM. The approximate concentration of 17α-AED to inhibit 50% of cell proliferation (IC₅₀) was 10 μM for T98G and U937 and 15 μM for U251MG. In contrast, 17β-AED, 7α-AET and 7β-AET could only suppress 30% or less of tumour cell proliferation at the 50 μM dose, and these antiproliferative effects modestly increased at higher concentrations as compared to the effects of 17α-AED. Therefore, 17α-AED is significantly more effective in inhibiting malignant...
glioma and lymphoma cell proliferation than its chemically identical β-epimer (17β-AED); consequentially, nonspecificity does not appear to be an issue. Additionally, 17α-AED is also markedly more effective than either 7α-AET or 7β-AED, and the inhibitory effect of 17α-AED is dose dependent. Subsequent studies focused on the cytotoxic effects of 17α-AED when used at the approximate IC50 concentration.

In the proliferation studies above, it may be possible that 17α-AED interferes with the ability of the glioma cells to efficiently adhere to the tissue culture plate and form a healthy monolayer. To address this, we incubated the glioma cells for 6–8 h before adding 17α-AED to the cultures. This time period allowed the glioma cells to firmly attach to the tissue culture plate. Vehicle or 17α-AED (10 μM) was then added to T98G cultures. The morphological effects of the neuro-steroid on the glioma culture could be detected as early as 8 h post-treatment, and included reduced cellular adherence and de-attachment of cells from the tissue culture plate. Figure 3A–D show phase-contrast images of a representative T98G culture after 18 h of exposure to vehicle or 17α-AED. The effect of 17α-AED (15 μM) on U251MG glioma cultures was similar (data not shown). Immediately following photography, the viability of the cultures was assessed by trypan blue exclusion. The results, shown in Figure 3E, indicate that 17α-AED-treated T98G, U251MG and U937 cultures contain a significant percentage (33.2, 17.4 and 28.4%, respectively, P<0.01) of non-viable cells as compared to sham-treated cultures which was ≤10%. Cell death in 17α-AED-treated T98G glioma and U937 lymphoma was approximately three times greater than of vehicle-treated cells.

17α-AED induces apoptosis of human U937 lymphoma cells, but not T98G and U251MG glioma cells

Several standard assays for apoptosis were conducted on U937 lymphoma and the malignant glioma T98G and U251MG cells treated with 17α-AED or vehicle for 3–4 days. Nuclear DNA fragmentation is a morphological change that occurs in apoptotic cells.
cells and is a classical feature of apoptosis (Nagata et al., 2003). A TUNEL assay was performed on the tumour cells treated with 17α-AED to detect DNA cleavage, which was quantified by flow cytometry. The results are shown in Figure 4. In the U937 lymphoma cultures treated with 17α-AED, 39.4% of the cells were TUNEL positive, indicating that these cells were apoptotic. Furthermore, induction of apoptosis by 17α-AED treatment of U937 lymphoma cells was dose dependent, as revealed by annexin V staining, with an LD₅₀ between 6.25 and 12.5 μM (data not shown). In contrast to U937 cells, only a low level, 7.7% of T98G glioma cells treated with 17α-AED were positive for TUNEL, and the percentage of TUNEL-positive U251MG cells exposed to 17α-AED remained at the level of sham-treated U251MG cells. Proteolytic processing of the effector caspase 3 and PARP are biochemical events of apoptosis and the presence of their cleavage products are markers of this cell death pathway (Nunez et al., 1998; Oliver et al., 1998). Western blotting was conducted using whole-cell lysates from human U937, T98G and U251MG cells treated with 17α-AED, to determine the extent of caspase 3 and PARP cleavage. The results, shown in Figure 5, demonstrate that U937 lymphoma cells treated with 17α-AED have an increased level of cleaved caspase 3 and PARP as compared to cells treated with vehicle. In contrast, no cleavage of caspase 3 was detected in treated T98G and U251MG malignant glioma cells, and there was no change in background levels of PARP cleavage noted in the 17α-AED-treated glioma cells as compared to glioma cells exposed to vehicle. Caspase 3 cleavage and PARP processing was not detected in U937, T98G and U251MG tumour cells exposed to 17α-AED for

Figure 4 17α-AED treatment induces DNA fragmentation of U937 lymphoma cells. (A) U937 lymphoma cells; (B) T98G glioma cells and (C) U251MG glioma cells were treated with vehicle (left panels) or 17α-AED (right panels). After 4 days, the degree of DNA cleavage was assessed by TUNEL staining and used as a measure of apoptosis. Annotated numbers reflect the percentage of cells staining positive for apoptosis (x-axis, FITC-A). This experiment was performed twice and yielded similar results.
24 or 48h, thereby excluding the possibility of early apoptotic cell death in these experiments (data not shown). These results demonstrate that 17α-AED treatment induces U937 lymphoma cells to undergo apoptosis, which is in accordance with our previous report (Huynh and Loria, 1997). However, the major cytotoxic effects of 17α-AED on malignant glioma cells appear to be mediated by a non-apoptotic pathway.

Cytopathic effects of 17α-AED on T98G glioma cells

Human T98G glioma cells were cultured for 30 h in the presence of vehicle or 17α-AED at a final concentration of 10 μM. Analysis of haematoxylin and eosin-stained cultures revealed significant morphological alterations as a result of 17α-AED treatment. In the sham-treated T98G culture (Figure 6A), glioma cells displayed a large, pale staining nucleus containing prominent nucleoli, and were epithelioid in appearance. Also in the control culture, numerous mitotic figures were apparent and, in a small percentage of cells, marginal cytoplasmic vacuolization could be noted. In the 17α-AED-treated cultures (Figures 6B and C), very few mitotic figures were present, and a substantial number of T98G cells contained multiple, cytoplasmic vacuoles which, in some cases, appeared to displace the nucleus. Vacuolization was often accompanied by the presence of small, basophilic bodies. The presence of these vacuoles in the 17α-AED-treated T98G cells was also detected by flow cytometry as an increase of cellular side scatter (data not shown).

17α-AED induces autophagosome formation in malignant glioma cells

Cytoplasmic vacuolization is a feature of certain death pathways such as necrosis and autophagy, but not apoptosis, and the presence of acidic vacuoles or AVO is a characteristic of autophagic cell death (Paglin et al, 2001; Lefranc and Kiss, 2006). Vital staining with acridine orange was used to detect the presence of AVO in tumour cells treated with 17α-AED. The results shown in Figure 7A indicate that more than 40% of T98G glioma cells and only 4.0% of U937 lymphoma cells (Figure 7B) treated with the neuro-steroid contained AVO. A high percentage of AVO was also detected in U251 malignant glioma cells treated with 17α-AED, as compared to sham-treated cells (data not shown). Bafilomycin A1 specifically inhibits AVO formation by blocking the fusion of autophagosomes and lysosomes (Yamamoto et al, 1998). The addition of bafilomycin A1 prevented the generation of AVO in 17α-AED-treated T98G glioma cells (Figure 7A) and U251MG glioma cells (data not shown). These results suggest that 17α-AED induces autophagic cell death in human malignant glioma cells, but not in lymphoma cells.

DISCUSSION

The anticancer effects of DHEA have been widely investigated and researchers have shown that treatment with DHEA can inhibit the growth of different tumours such as colon adenocarcinoma (Schulz et al, 1992), pancreatic carcinoma (Melvin et al, 1997) and breast cancer (Rocuzzi et al, 1993). Additionally, DHEA treatment has been shown to retard the onset of chemically induced cancers such as mammary, liver and prostate carcinogenesis in experimental rat models (Simile et al, 1995; Lubet et al, 1998; Rao et al, 1999). More recently, Yoshida et al (2003) reported that DHEA and its metabolites have antiproliferative effects on human hepatoma and human colonic adenocarcinoma cell lines. The androsterone steroid molecules used in this study are naturally synthesized in neuro-ectodermal tissue by the metabolism of DHEA, and represent a true class of neuro-steroids. In this investigation, we examined the cytotoxic effects of chemically identical or similar androsterone derivatives on two human malignant glioma cell lines and a human lymphoma cell line. Our findings demonstrate that the androsterone neuro-steroids can inhibit the proliferation of these tumour cells, induce cell death,
and, in the case of glioma cells, evoke cytopathological changes consistent with autophagy.

The results of the proliferation studies showed that there is a significant difference in the potency of the four androstene neuro-steroids. The percentage of growth inhibition for 17α-AED at 50 μM dose was well above 80% for all of the tumours, clearly indicating that 17α-AED is the most effective inhibitor of tumour cell proliferation of the androstene steroids tested. In contrast, 17β-AED, which is chemically identical, only marginally inhibited proliferation at this concentration; therefore nonspecificity is not an issue. The IC₅₀ for 17α-AED is < 20 μM for the glioblastoma and the lymphoma cells. This is particularly noteworthy when one considers that the IC₅₀ of temozolomide on malignant glioma cell cultures has been reported to be 100 μM or greater (Sankar et al., 1999; Kanzawa et al., 2003a, 2004). In addition, investigations by Yoshida et al. (2003) on the antiproliferative action of DHEA on human cancer cell lines demonstrated that DHEA at 100 μM could inhibit ~ 50% of Hep G2 hepatoma cell proliferation and 30% of Caco-2 colonic adenocarcinoma cell proliferation; however, there was no significant reduction in tumour cell viability when DHEA was used at this dose. Also in this study, the investigators showed that etiocholanolone, a metabolite of DHEA; had the most potent cytotoxic activity, with an IC₅₀ of ~ 80 μM on Hep G2 cells. This is approximately 10 times less potent than that of the cytotoxic effects of 17α-AED on human U937 lymphoma and T98G malignant glioma cells.

When used at high concentrations, 17β-AED, 7α-AET and 7β-AET were able to inhibit proliferation of the tumour cells, although the degree of inhibition was marginal as compared to that of 17α-AED. In terms of chemical structure, these results are interesting because 17α-AED and 17β-AED are chemically identical but differ in the placement, that is, above or below the plane of the androstene molecule, of a hydroxyl on the seventeenth carbon and yet exhibit an extreme difference their capacity to inhibit tumour cell proliferation. Furthermore, the addition of a hydroxyl group at the seventh position in either α or β orientation on the 17β-AED molecule does not increase the antiproliferative effects of 17β-AED. Overall, the results of the proliferation studies suggest that the potency of the antiproliferative effects of the androstenes may be dependent upon the placement and the number of the hydroxyl groups on the tetra-cyclic, androstene molecule.

Subsequent studies demonstrated that morphological effects could be noted on glioblastoma cultures after 18 h of exposure to 17α-AED, which included disruption of the cell monolayer and decreased adherence to the tissue culture plate. Also at this time point, reduced cell viability could be detected in the treated tumour cell cultures. The induction of cell death by 17α-AED were not as pronounced on U251MG cells, as compared with the T98G and U937 cells, suggesting that the U251MG cells may be more resistant to this neuro-steroid. Based on our previous work showing the effects of 17α-AED in human myeloid leukaemic cell lines, it is apparent that the effectiveness of this molecule is target cell dependent (Huynh and Loria, 1997). This is confirmed in this experiment.

Previous studies from our group have shown that 17α-AED treatment induces apoptosis in human breast and leukaemic
cancer cells, as demonstrated by TUNEL and electron microscopy (Huynh and Loria, 1997; Huynh et al., 2000). However, it does not appear that apoptosis plays a major role in the death of malignant glioma cells treated with 17α-AED as shown by the nominal degree of DNA fragmentation; lack of caspase 3 cleavage and PARP cleavage as compared to sham-treated glioma cells. Additionally, preliminary electron microscopic analysis of the ultrastructure of T98G glioblastoma cells treated 3 days with 17α-AED did not reveal classical apoptotic features such as blebbing of the plasma membrane and DNA condensation (Graf et al., unpublished results). In some CNS diseases involving neuronal cell death, such as amyotrophic lateral sclerosis and Huntington's disease, traditional apoptotic features are not present (Dal Canto and Gurney, 1994; Turmaine et al., 2000).

Cytopathology revealed novel morphological changes in T98G cells treated for 30 h with 17α-AED. Most notable was the presence of numerous, large vacuoles in the cytoplasm of the treated cells, which was often accompanied with small, basophilic bodies. Acridine orange staining of 17α-AED-treated cells demonstrated that a large percentage of T98G glioma cells, but not U937 lymphoma cells, contain acidic vacuoles, and that their formation could be inhibited with bafilomycin A1.

Two forms of programmed cell death have been recently proposed (Bursch et al., 2000). Common features of type I programmed cell death, also referred to as apoptosis, include caspase activation, chromatin condensation and DNA cleavage (Jaattela, 2002). In contrast, autophagy is a form of type II programmed cell death, which is caspase independent, includes the formation of AVOs and may proceed in the absence of DNA cleavage (Lefranc and Kiss, 2006). Recent studies of human malignant glioma cells treated with temozolomide or arsenic trioxide have revealed that cell death occurs not by apoptosis, but rather by the induction of autophagy (Kanzawa et al., 2004, 2004). In our studies, human malignant glioma cells exposed to 17α-AED displayed features characteristic of autophagy, the presence of AVO and the ability of bafilomycin A1 to inhibit AVO formation, but not of apoptosis. Therefore, it is possible that the antiangioma effects of this neuro-steroid may be mediated by autophagic, type II programmed cell death. Biochemical and genetic studies are in progress to identify the extent to which 17α-AED induces autophagic cell death in the glioma cells, and to determine the molecular mechanisms involved. However, this is the first report of the antiangioma effects of 17α-AED.

To summarize, we have demonstrated that, of the androstene neuro-steroids tested, 17α-AED is the most potent inhibitor of human malignant glioma cells and lymphoma cells, with an IC50 in the rage of 10 to 20 μM. In fact, 17α-AED used at a concentration of 25 μM, can inhibit tumour cell proliferation by 80% or greater. These findings indicate that the cytotoxic effects of the neuro-steroids are dependent on the number and position of the hydroxyl groups on the androstene molecules as well as the aetiology of the tumour. It is apparent that the cytotoxic activity of 17α-AED on lymphoma cells is mediated by apoptosis; however, in malignant gliomas, it is likely that a nonapoptotic pathway of programmed cell death is utilized, such as autophagy. 17α-AED may have clinical relevancy in the treatment of brain tumour patients, because it is a neuro-steroid that can readily cross the blood brain barrier and possesses potent antiangioma activity.

ACKNOWLEDGEMENTS

We are highly grateful for the expert assistance of Dr John Bigbee in the analysis of the morphological effects of the neuro-steroids on T98G cells. We would also like to thank the James and Martha Betts Foundation for their support. This work was supported in part by a grant from the MCV Medical Center AD Williams Trust Funds (M.R.G.) and the SCDR Cancer Research Fund (R.M.L.). Microscopy was performed at the VCU – Dept. of Neurobiology & Anatomy Microscopy Facility, supported, in part, with funding from NIH-NINDS Center core Grant SP30NS047463.

REFERENCES

Ben Nathan D, Padgett DA, Loria RM (1999) Androstenediol and dehydroepiandrosterone protect mice against lethal bacterial infections and lipopolysaccharide toxicity. J Med Microbiol 48: 425 – 431
Buccuzzi G, Di Monaco M, Brignardello E, Leonardi L, Gatto V, Pizzini A, Gallo M (1993) Dehydroepiandrosterone antiestrogenic action through androgen receptor in MCF-7 human breast cancer cell line. Anticancer Res 13: 2267 – 2272
Bursch W, Ellinger A, Gerner C, Frohwein U, Schulte-Hermann R (2000) Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann N Y Acad Sci 926: 1 – 12
Dal Canto MC, Gurney ME (1994) Development of central nervous system pathology in a murine transgenic model of human amyotrophic lateral sclerosis. Am J Pathol 145: 1271 – 1279
Huynh PN, Carter Jr WH, Loria RM (2000) 17 Alpha androstenediol on oncogenic myeloid cell lines. Cancer Detect Prev 24: 435 – 444
Huynh PN, Loria RM (1997) Contrasting effects of alpha- and beta-androstenediol on oncogenic myloid cell lines in vitro. J Leukoc Biol 62: 258 – 267
Jaattela M (2002) Programmed cell death: many ways for cells to die decently. Ann Med 34: 480 – 488
Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S (2000) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 7: 448 – 457
Kanzawa T, Germano IM, Kondo Y, Ito H, Kyo S, Kondo S (2003a) Inhibition of telomerase activity in malignant glioma cells correlates with their sensitivity to temozolomide. Br J Cancer 89: 922 – 929
Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I (2003b) Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res 63: 2103 – 2108
Kemper EM, Booger W, Thuis I, Beijnen JH, van Tellingen O (2004) Modulation of the blood-brain barrier in oncology: therapeutic opportunities for the treatment of brain tumours? Cancer Treat Rev 30: 415 – 423
Lathe R (2002) Steroid and steroid 7-hydroxylation: ancient pathways. Steroids 67: 967 – 977
LeFranc F, Brotchi J, Kiss R (2005) Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. J Clin Oncol 23: 2411 – 2422
LeFranc F, Kiss R (2006) Autophagy, the Trojan horse to combat glioblastomas. Neurosurg Focus 20: E7
Loria RM (1997) Antiglucocorticoid function of androstenediol. Psycho-neuroendocrinology 22(Suppl 1): S103 – S108
Loria RM (2002) Immune up-regulation and tumor apoptosis by androstenedione. Steroids 67: 953 – 966
Loria RM, Conrad DH, Huff T, Carter H, Ben Nathan D (2000) Androstenediol and androstenedione. Protection against lethal radiation and restoration of immunity after radiation injury. Ann N Y Acad Sci 917: 860 – 867
Lubet RA, Gordon GB, Prough RA, Lei XD, You M, Wang Y, Grubbs CJ, Steele VE, Kelloff GJ, Thomas CF, Moon RD (1998) Modulation of methylNitrosourea-induced breast cancer in Sprague Dawley rats by dehydroepiandrosterone: dose-dependent inhibition, effects of limited exposure, effects on peroxisomal enzymes, and lack of effects on levels of Ha-Ras mutations. Cancer Res 58: 921 – 926
Melvin WS, Boros LG, Muscarella P, Brandes JL, Johnson JA, Fisher WE, Schirmer WJ, Ellison EC (1997) Dehydroepiandrosterone-sulfate inhibits pancreatic carcinoma cell proliferation in vitro and in vivo. Surgery 121: 392 – 397
Nagata S, Nagase H, Kawane K, Mukae N, Fukuyama H (2003) Degradation of chromosomal DNA during apoptosis. *Cell Death Differ* 10: 108 – 116

Nunez G, Benedict MA, Hu Y, Inohara N (1998) Caspases: the proteases of the apoptotic pathway. *Oncogene* 17: 3237 – 3245

Oliver Fj, de la RG, Rolli V, Ruiz-Ruiz MC, de Murcia G, Murcia JM (1998) Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. *J Biol Chem* 273: 33533 – 33539

Padgett DA, Loria RM (1994) *In vitro* potentiation of lymphocyte activation by dehydroepiandrosterone, androstenediol, and androstenetriol. *J Immunol* 153: 1544 – 1552

Padgett DA, Loria RM, Sheridan JF (2000) Steroid hormone regulation of antiviral immunity. *Ann N Y Acad Sci* 917: 935 – 943

Paglin S, Hollister T, Delohery T, Hackett N, McMullin M, Sphicas E, Domingo D, Yahalom J (2001) A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 61: 439 – 444

Schulz S, Klann RC, Schonfeld S, Nyce JW (1992) Mechanisms of cell growth inhibition and cell cycle arrest in human colonic adenocarcinoma cells by dehydroepiandrosterone: role of isoprenoid biosynthesis. *Cancer Res* 52: 1372 – 1376

Simile M, Pascale RM, De Miglio MR, Nufris A, Daino L, Seddaiu MA, Muroni MR, Rao KN, Feo F (1995) Inhibition by dehydroepiandrosterone of growth and progression of persistent liver nodules in experimental rat liver carcinogenesis. *Int J Cancer* 62: 210 – 215

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO (2005a) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987 – 996

Stupp R, Weber DC (2005b) The role of radio- and chemotherapy in glioblastoma. *Oxidat. Med* 20: 184 – 187

Surawicz TS, Davis F, Freels S, Laws Jr ER, Menck HR (1998) Brain tumor survival: results from the National Cancer Data Base. *J Neurooncol* 40: 151 – 160

Traganos F, Darzynkiewicz Z (1994) Lysosomal proton pump activity: supravital cell staining with acridine orange differentiates leukocyte subpopulations. *Methods Cell Biol* 41: 185 – 194

Turmaine M, Raza A, Mahal A, Mangiarini L, Bates GP, Davies SW (2000) Nonapoptotic neurodegeneration in a transgenic mouse model of Huntington’s disease. *Proc Natl Acad Sci USA* 97: 8093 – 8097

Walker MD, Green SB, Byar DP, Alexander Jr E, Batzdorf U, Brooks WH, Hunt WE, MacCarty CS, Mahaley Jr MS, Mealey Jr J, Owens G, Ransohoff J, Robertson JT, Shapiro WR, Smith Jr KR, Wilson CB, Strike TA (1980) Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. *N Engl J Med* 303: 1323 – 1329

Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y (1998) Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct Funct* 23: 33 – 42

Yoshida S, Honda A, Matsuzaki Y, Fukuhama S, Tanaka N, Takagiwa A, Fujimoto Y, Miyazaki H, Salen G (2003) Anti-proliferative action of endogenous dehydroepiandrosterone metabolites on human cancer cell lines. *Steroids* 68: 73 – 83