Effects, in an in-vivo model system, of 1,2,3,4-tetrahydroisoquinoline on glioma

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The effects of 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) on the growth of glioma was tested *in vitro* and *in vivo*. Normal cultured rat astrocytes and C6 rat glioma were used as a differential screen to test the effects of EDL-155. The compound was preferentially cytotoxic for C6 glioma (EC50 = 1.5 μmol/l) relative to cultured neonatal astrocytes (EC50 = 27.4 μmol/l). When compared with a standard chemotherapeutic agent, carmustine (1,3-bis[2–chloroethyl]-1-nitrosourea), or temozolomide, EDL-155 was more selective and more potent in our differential tissue culture assay. The effect of EDL-155 was also tested in an animal model in which C6 glioma was transplanted into the brains of Sprague–Dawley rats. EDL-155 was delivered directly onto the tumor by an osmotic minipump directly into the brain or by intraperitoneal injection. Animals treated with EDL-155 had significantly smaller tumors than did control animals treated with carrier solution. We observed anatomical changes in cultured glioma cells treated with EDL-155 that were consistent with selective destruction of mitochondria and the induction of autophagy. These changes were not observed in normal astrocytes cultured from rat pups. The selective killing of glioma in tissue culture and in the rat brain models indicates that EDL-155 has potential therapeutic value in treating this type of brain cancer.
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Introduction

Gliomas are the most common form of primary brain cancer. High-grade tumors such as glioblastoma multiforma (WHO grade IV) are relatively frequent and have a devastating prognosis [1,2]. In the United States, approximately 18 000 patients are diagnosed with high-grade glioblastoma every year. The 80% mortality rate among these patients at 12 months after diagnosis is due, in part, to the infiltrating nature of the tumors [3], which makes surgical excision difficult. However, without complete surgical resection of these tumors, there is little chance of a long-term cure [4]. Patients rarely respond to standard chemotherapeutic protocols [5]. Even with this aggressive treatment, patients with glioblastoma multiforma survive, on average, less than 1 year after diagnosis [6,7].

One obvious goal of basic and clinical research efforts is to find a therapy that will significantly extend the survival of patients with high-grade brain tumors. Wide ranges of therapeutic agents and strategies have been and are currently being applied against glioblastomas. These include toxins directed at extracellular protein domains [8], gene-based therapy using viral delivery systems [9–11], natural products such as curcumin [12], inorganic compounds such as selenite [13], and any of a number of others [14–18]. Many of these efforts were frustrated by the infiltrating nature of glioblastomas and the isolation of the brain by the physical and biological nature of the blood–brain barrier. To date, one of the more promising areas remains the development of small molecules that may provide the therapeutic advantage needed to treat these high-grade tumors.

We began our current efforts to identify a small molecule to treat brain cancer by examining the effects of a library of compounds on the growth of rat C6 glioma cells in culture. We found that 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) had selective cytotoxic activity against rat C6 glioma cells relative to its action against primary cultures of rat neonatal astrocytes [19]. The EC50 values for EDL-155 were approximately 1.5 μmol/l in C6 glioma as compared with a value of 27.4 μmol/l in primary astrocytes. The dose–response curves of the typical brain cancer chemotherapeutic drugs 1,3-bis[2–chloroethyl]-1-nitrosourea (BCNU), 5-fluorouracil, and melphalan had higher EC50 than EDL-155 values, as

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well as less selectivity. These initial studies suggested that EDL-155 might be of considerable efficacy in treating cancer of the brain.

In this study, we have examined the effects of EDL-155 on cultured cells and in an animal model commonly used in glioma research. Using an animal model to approximate the consequences of human disease has both strengths and weaknesses [20–24]. The most common model is the injection of tumor cells into the brain. This model allows us to deliver a specific number of tumor cells into the brain and then treat the animals with potentially therapeutic agents. This type of system is accepted and extensively used in glioma research [25–28]. We present in-vitro data comparing the effect of EDL-155 to the response of C6 glioma to the commonly used therapeutic agent BCNU and temozolomide, as well as in-vivo data demonstrating the effectiveness of EDL-155 in killing C6 glioma within the environment of the brain.

Method

Cell cultures

Glial cells were cultured from the cerebral cortex of Sprague–Dawley rat pups using a protocol described by McCarthy and de Vellis [29] and modified by Geisert and Stewart [30]. The animals were anesthetized by cold and decapitated, after which their brains were immediately removed. We placed the cortices in a Petri dish containing 10 ml of Hank's balanced salt solution (HBSS). The tissue was placed in 20 ml of 0.1% trypsin in HBSS for 10 min. The astrocytes were plated at a density of 5×10^3 cells/cm^2 into T-75 culture flasks. The cells were allowed to grow to confluence in Eagle's basal medium (BME) with 10% fetal calf serum. The C6 glioma cell line was purchased from American Type Culture Collection (Manassas, Virginia, USA). For studies in which C6 glioma were transplanted into the brain, we used a C6 cell line produced in our laboratory that caries the β-galactosidase marker. The cell line was transfected with the pCMVβ expression vector (Clontech, Palo Alto, California, USA); stable transfectants were selected using G418 (Invitrogen, Carlsbad, California, USA). Three glioma cell lines, U87, T98G, and A172, were purchased from American Type Culture Collection.

Screening and dose–response assays

EDL-155 was synthesized in the laboratory of Dr Duane D. Miller [19]. BCNU was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Temozolomide was also purchased from Sigma-Aldrich. Screening assays were performed according to the method of Wagner *et al.* [31]. Briefly, the primary cultures of astrocytes and cultures of C6 glioma cell lines were handled identically with respect to treatment concentrations and the manipulations of cells for screening assays. The cells were treated with trypsin and transferred to 96-well plates at a cell density of 10^3 cells/mm^2. The cells were grown overnight in 100 µl of 10% fetal calf serum (FCS) BME in a 37°C incubator containing a humid 5% CO_2 atmosphere. All samples were dissolved completely to make a 100 µmol/l stock solution and diluted to produce a series of concentrations. A 10 µl aliquot of each of these initial solutions was added to 190 µl of 2% FCS BME to produce the test concentration. The vehicle solution, 10% dimethyl sulfoxide in saline, was tested as a control. Dilutions were performed so that cosolvent concentrations did not vary for any particular experiment. Immediately before treatment, the 10% FCS BME was suctioned off the cells and replaced with the 190 µl of 2% FCS BME. The cultured cells were incubated with test compound for 4 days. The cells were fixed with 4% paraformaldehyde, stained with 0.1% cresyl violet stain. The cell number was determined in a manner similar to that described by Wagner *et al.* [31]. The screening data were collected as four wells for each dose per compound (screening) or concentration (dose–response curve). In addition, the average growth of cells in eight wells given no treatment was used as a negative control for each plate. The cells for dose–response curves were grown in the same medium and handled in a manner similar to that used in the screening assays. This same assay system was used to define the dose–response curves for all other cell lines and compounds.

Data analysis

The cytotoxic character of each compound was reported as the percentage of cell survival, calculated as the average A_{560} for treated cells divided by A_{560} of untreated (negative control) cells, and expressed as a percentage. Values less than or equal to 100% indicate a cytostatic or cytotoxic effect. Dose–response curves and EC50 values were attained via plots of percent survival versus concentration.

Electron microscopy

For electron microscopic examination, cultured cells were rinsed in phosphate-buffered saline (PBS) and placed in 2% glutaraldehyde in phosphate buffer (pH 7.3). The cells were post fixed in 1% osmium tetroxide for 1 h at room temperature. The cells were rinsed in PBS, dehydrated, and infiltrated with Spurr embedding medium. Silver to gold sections were cut on a microtome (Ultracut E; Reichert Histostat, Reichert Microscope Services, Austria). The sections were examined using a 2000EX JEOL (Tokyo, Japan) electron microscope.

Mito Tracker Green staining

To identify mitochondrial membranes at the light microscopic level we used Mito Tracker Green FM, a green-fluorescence mitochondrial stain (Molecular Probes, Eugene, Oregon, USA). After fixation in 4% paraformaldehyde, the cells were stained with 100 nmol/l Mito Tracker Green at room temperature for 20 min. The cells

were rinsed in PBS and examined using a Nikon confocal microscope C1 (Nikon, Japan) with excitation wavelength of 485 nm and emission 520 nm filter.

In-vivo model with intracranial delivery

Male Sprague–Dawley rats ($n = 12$, 250–350 g) were deeply anesthetized with 13 mg/kg of rompun and 87 mg/kg of ketalar in preparation for surgery. The rats were monitored during surgery to ensure that they remained deeply anesthetized and unresponsive to pain. The skull was surgically exposed, a burr hole was placed in the skull, and an indwelling cannula was lowered 2.5 mm below the cerebral cortex into the hippocampus. The cannula was fixed to the surface of the skull with dental cement. For in-vivo experiments, we used C6 gliomas that had been stably transfected in our laboratory with a *β-Gal* reporter gene. One day before surgery, the cultures of C6 glioma were extensively rinsed in HBSS and placed in BME with 10% rat serum (Equitech-Bio, Kerrville, Texas, USA). The next day, the cells were treated with trypsin and placed in HBSS. Approximately 5×10^5 C6 glioma cells were delivered into the brain over 20 min. At this point, the cannula was connected to an Alzet miniosmotic pump (model 2001, Alzet Osmotic Pumps, Cupertino, California, USA) that delivered 1 µl/h of treatment solution for 7 days. The pump was then placed under the skin and the incision was closed with surgical staples. Rats were allowed to recover and returned to the animal care facility. The animals were then assigned to one of two treatment groups: six rats received carrier solution (HBSS) only; the other six rats received 7 µmol/l of EDL-155 in HBSS. During the 7-day treatment period, the rats were monitored daily.

After a survival period of 8 days, the rats were deeply anesthetized with 26 mg/kg of rompun and 174 mg/kg of ketalar and perfused through the heart with saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were sectioned at 50 µm with a freezing microtome. One set of sections was rinsed in PBS and reacted with X-gal. The remaining sections were stored in borate-buffered saline (pH 8.4) at 4°C. One 1-in-5 series of sections was mounted on glass slides and stained by the Nissl method, using cresyl violet stain.

The size of tumors in each animal was measured. The section from each rat that displayed the maximum extent of the tumor was identified and photographed using a digital camera on a dissecting microscope. The digital images were coded and the codes kept by one investigator (E.E.G.). The digital images were analyzed to define the area of the tumor using the program NIH image. This work was conducted in a blinded manner (W.E.O.). The codes were released after which the data were compiled and analyzed using a Student's *t*-test.

In-vivo model with intraperitoneal administration

Male Sprague–Dawley rats ($n = 21$, 250–350 g) were deeply anesthetized with (13 mg/kg, rompun and 87 mg/kg, ketalar). The skull was surgically exposed, a hole was drilled in the skull, and 5×10^5 C6 glioma cells were delivered into the caudate nucleus. The incision was closed with surgical staples and the animal was allowed to recover and returned to the animal care facility.

Approximately 24 h after tumor cell implantation, the first intraperitoneal administration of EDL-155 (20 mg/kg) was begun and continued twice daily for the next 7 days. Eleven rats received vehicle only (10% dimethyl sulfoxide in HBSS) and 10 rats received EDL-155. After a survival period of 8 days, each rat was deeply anesthetized with 26 mg/kg rompun and 174 mg/kg ketalar and perfused through the heart with saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The brains were sectioned at 50 µm and a 1-in-5 series of sections were processed as described above. In the later experiments the serial section allowed us to define tumor volume. The digital images were taken from each section and coded. The codes were kept by one investigator (E.E.G) and the digital images were analyzed to define the volume of the tumor using the program NIH image. This work was conducted in a blinded manner (X.W.). The codes were released and the data compiled and analyzed using a Student's *t*-test.

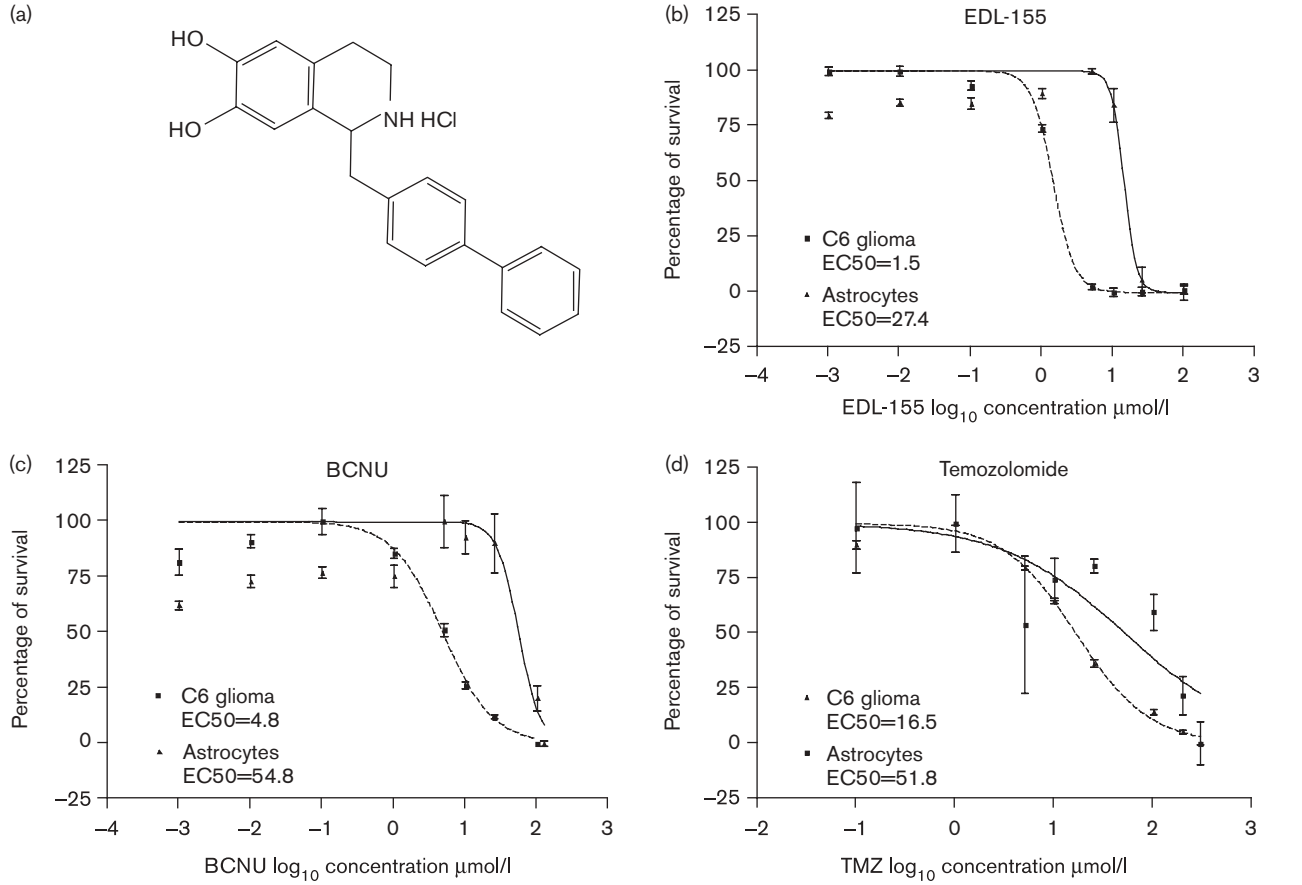
Results

Earlier studies have demonstrated that EDL-155 at concentrations that do not affect the growth of cultured rat neonatal astrocytes is cytotoxic against C6 glioma. To expand these initial findings, we compared the effects of EDL-155 with that of BCNU in tissue culture. In a direct comparison, we examined the effects of EDL-155 on cultured C6 glioma relative to normal cultured astrocytes and in an animal model of brain cancer.

In-vitro comparison of EDL-155 to BCNU and temozolomide

As an initial test of the efficacy of EDL-155 in selectively killing glioma, we compared the ability of EDL-155 to kill rat C6 glioma to that of BCNU and temozolomide, alkylating agents used to treat brain cancer (Fig. 1). In addition, we evaluated the relative toxicity of each compound on normal brain astroglial cells cultured from neonatal rat pups. When cultured cells were treated with different concentrations of BCNU, there was a dramatic decrease in the viability of cells as the concentration of BCNU approached 20 µmol/l. The BCNU was more effective at killing the C6 glioma than normal astrocytes. With BCNU, the EC50 for astrocytes was 54.8 µmol/l, whereas that for C6 glioma was 4.8 µmol/l (Fig. 1c). When temozolomide was used to treat cultured cells, the EC50 for normal astrocytes was 51.8 and for the C6 glioma it was 16.5 (Fig. 1d). When we examined the effects of

Fig. 1



(a) Shows the structure of 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155). (b) (c), and (d) illustrate the dose–response curves for EDL-155 (b), 1,3-bis[2–chloroethyl]-1-nitrosourea (BCNU) (c), and temozolomide (TMZ) (d) for normal rat brain astrocytes and C6 glioma. In (b), (c), and (d), the percentages of surviving cells are plotted against the concentration of the drug. In (b), the percentage of cells surviving after 4 days of treatment with different concentrations of EDL-155 is shown. Note that the C6 glioma (EC50 = 1.5 μmol/l) is more sensitive to EDL-155 than are astrocytes (EC50 = 27.4 μmol/l). For cultures treated with BCNU (c), the EC50 for C6 glioma is 4.8 μmol/l and for normal astrocytes the EC50 is 54.8 μmol/l. When cultures are treated with TMZ the EC50 for glioma is 16.5 μmol/l and for astrocytes it is 51.8 μmol/l.

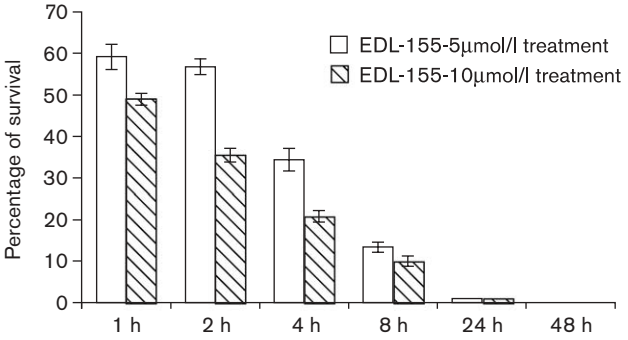
EDL-155 on these cultured cell types, there was a dramatic difference in the response of C6 glioma relative to that of normal brain astrocytes. Specifically, C6 gliomas were more sensitive to the effects of the EDL-155 than were the normal brain astrocytes. The normal astrocytes had an EC50 of 27.4 μmol/l; the C6 glioma had an EC50 of 1.5 μmol/l. Thus, there is a 18-fold difference in the effective concentration of EDL-155 needed to kill C6 glioma cells relative to the concentration needed to kill normal brain astrocytes.

To obtain an estimate of the exposure necessary to kill glioma, we varied the duration of exposure of C6 glioma to EDL-155 (Fig. 2). In this experiment, C6 glioma cells were treated with either 5 or 10 μmol/l EDL-155 and the treatment duration varied from 1 to 48 h. The cells were kept in culture for a total of 4 days, and then fixed with paraformaldehyde. The number of surviving cells was determined. Exposure for only 4 h was sufficient to kill more than 50% of the glioma cells in culture. Exposure for 24 or 48 h completely killed the cultured C6 glioma cells. Thus, it takes a relatively brief exposure of 4 h to have a significant inhibitory effect on the growth of C6 glioma and after a 24-h exposure in culture to EDL-155 the C6 glioma is eliminated.

Human glioblastoma

To examine the effects of EDL-155 on human glioma, we conducted a series of dose–response studies using three human cell lines, U87, A172, and T98G (Fig. 3). Cells were treated with concentrations of EDL-155 ranging from 0.001 to 100 μmol/l. The EC50 for the U87 cell line was 10.8 μmol/l. Slightly more EDL-155 was needed to kill the A172 cell line, which thus had an EC50 of 17.2 μmol/l. The T98G cell line required a concentration of EDL-155 with an EC50 of 16.9 μmol/l. These results

Fig. 2



The duration of treatment with 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) in C6 glioma is shown. The cells were exposed to either 5 or 10 μmol/l EDL-155 for different periods: 1, 2, 4, 8, 24, or 48 h. The cells were then allowed to grow for a total of 4 days, at which time we assessed the number of surviving cells. With 24-h treatment, almost all of the cells were killed; with a 48-h exposure, no living cells were observed. A 4-h exposure to 10 μmol/l EDL-155 resulted in 80% killing of the C6 glioma.

demonstrated not only that the isoquinoline EDL-155 can kill human glioma, but that there is a considerable range in the EC50 values for the cell lines tested.

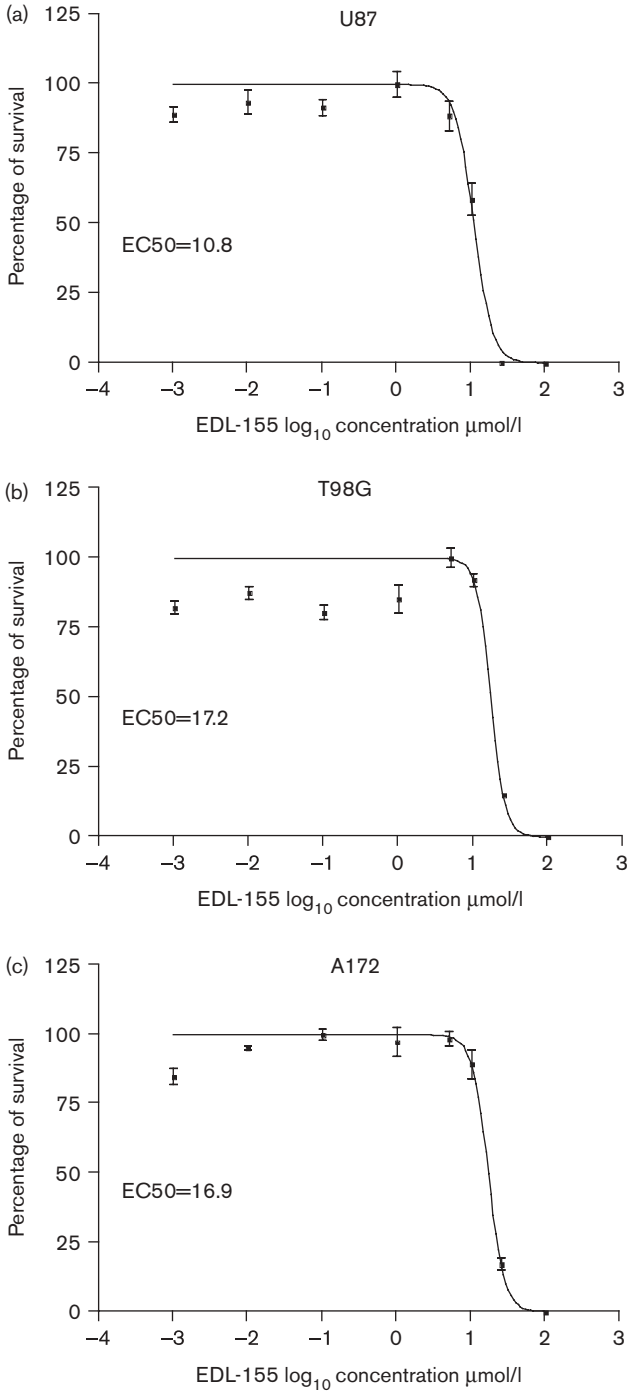
During this dose–response experiment, we noted that all of the cells treated with higher concentrations of drug developed a series of large vacuoles that were visible under phase microscopy (data not shown). We examined this finding further by culturing U87 cells and staining them with toluidine blue (data not shown). When we examined the cells, we were surprised to find that the nuclear morphology of the EDL-155-treated cells was similar to that of the control cells. The one obvious difference between the treated cells and the control cells was the presence of large vacuoles in cytoplasm of the treated cells. These structures were not observed in the control cells. To stain the cells for DNA and potentially mycoplasma, an additional set of cultures was stained for DNA using the Hoechst stain (data not shown). The results were similar, with no discernible difference in the nuclear morphology and the presence of large vacuoles in the EDL-155-treated cells that were negative for DNA.

Ultrastructure of EDL-155-treated cells

To aid in defining early changes occurring after EDL-155 treatment, we examined cultured cells at the electron microscopic level, defining the two morphological features associated with the death of the glioma cells. For these experiments we used normal rat astrocytes, C6 rat glioma cells, U87 human glioma cells, and T98G human glioma cells. Cells were cultured; one set of cultures was then treated with carrier solution, whereas the second set was treated with EDL-155. We used a treatment of 7 μmol/l EDL-155 for 16 h to provide a relatively effective dose with a shortened duration to capture early changes occurring in the cells. At this dose there were no visible differences between the ultrastructure of control cells (data not shown). In addition, the normal brain astrocytes treated with EDL-155 (Fig. 4a) showed exactly the same ultrastructure as the control astrocytes. The nuclei appeared similar in both sets of cultures, as did all other morphological features, including the mitochondria and the endoplasmic reticulum. In the C6 glioma cells, however, there was a significant difference between the control cells and the treated cultures. In the EDL-155-treated cultures, the C6 glioma cells had numerous large vacuoles; no such vacuoles were observed in the control cells (Fig. 4b). Moreover, very few mitochondria were present in the treated glioma and the ones we could identify were severely disrupted. In some cases vacuoles surrounded by double membranes were observed (arrowhead in Fig. 4b). Similar ultrastructural features were observed in the human glioma. In the U87 glioma cells, there were many large vacuoles throughout the cytoplasm and some of these vacuoles appeared to contain fragments of mitochondria. On careful examination, we were not able to find many intact mitochondria in the EDL-155 U87 cells (Fig. 4c), whereas in the cells treated with the control solution, mitochondria were easily seen in the cytoplasm. This was also the case for the T98G cells treated with EDL-155 (Fig. 4d). In the U87 glioma, T98G glioma, and C6 glioma cells treated with EDL-155, the mitochondria were severely disrupted or missing. Many fragments of what appeared to be mitochondria were observed in large vacuoles and some of the structures were surrounded by double membranes.

These findings provide the first indication of a selective mechanism for the effects of EDL-155. The selective disrupted mitochondria in cultured glioma cells are one of the key features of the EDL-155 treatment. This does not occur in cultures of normal rat brain astrocytes treated with EDL-155 or in the control cells. The second observation relevant to the mechanism of action of

Fig. 3



The dose–response curves for 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) on three human gliomas is illustrated: (a) U87 cells, (b) T98G cells, and (c) A172 cells. All three human cell lines have similar EC50s: U86 = 10.8, T98G = 17.2, and A172 = 16.9.

EDL-155 is that we did not observe nuclear fragmentation or condensation, which typically are associated with apoptosis. Finally, there are many large vacuoles in the glioma treated with EDL-155 and many of these are surrounded by double membranes. These morphological features are always observed in cells that are sensitive to EDL-155 treatment.

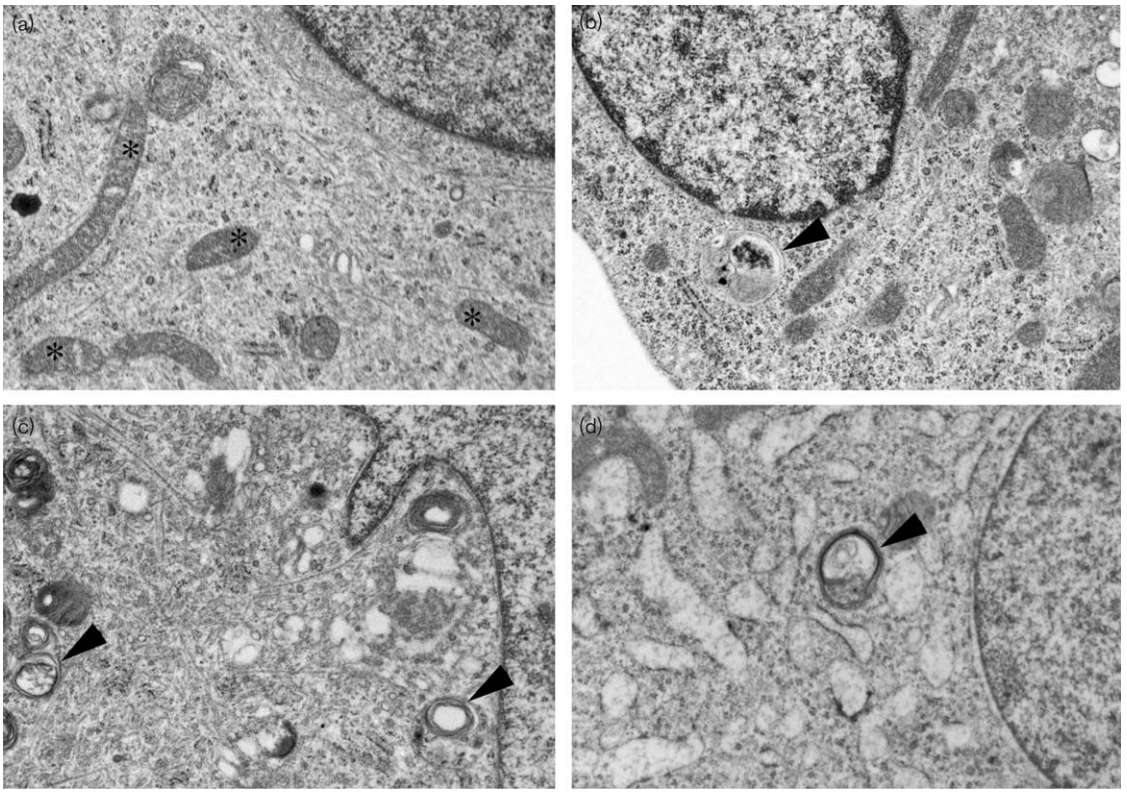
To provide an independent examination of the effects of EDL-155 on mitochondria, we ran a similar experiment staining the mitochondria with Mito Tracker Green and examining the cultures with the confocal microscope (Fig. 5). In these experiments we observed a distinct change in the staining pattern within all of the glioma cells treated with EDL-155. In the control cells there was a fine stippling pattern associated with normal mitochondria (Fig. 5a and b). After treatment with EDL-155 the cells no longer had the fine stippling pattern, instead the glioma contained large round (vacuole-like) structures that were positive for the Mito Tracker Green marker (Fig. 5c and d). The lack of mitochondria, the large vacuoles, the vacuoles with double membranes, and the intact nuclear morphology are all consistent with the hypothesis that EDL-155 is sending the glioma into autophagy or programed cell death type II. This possibility will be considered further in the Discussion section.

In-vivo glioma models with intracranial delivery of EDL-155

To determine whether EDL-155 has any potential therapeutic value, we used a rat model. A cannula was implanted in the hippocampus of each rat, after which cultured C6 glioma cells carrying the β-glactosidase marker gene were delivered through the cannula, which was then connected to an Alzet miniosmotic pump. The pump was implanted and began delivering treatment to the transplanted glioma within 24 h of surgery.

In control animals, which were treated with the carrier solution (HBSS), relatively large tumors were observed in the brains 8 days after implantation. The C6 glioma formed a large mass out of which cells extended, infiltrating the surrounding tissues attached to local blood vessels. Indeed, cells marked blood vessels a considerable distance away from the bulk of the tumor (Fig. 6a). A dramatic difference was observed in the size of the tumors in rats treated with 7 µmol/l of EDL-155. After 7 days of EDL-155 treatment, the tumors were considerably smaller than those in control rats. In addition, there did not seem to be the same degree of infiltration as that observed in control rats. Most of the tumor appeared to be associated with the region immediately surrounding the body of the treatment cannula, as if it had grown between the metal sides of the cannula used to deliver the drug or into the neural tissue surrounding the cannula (Fig. 6b). Surprisingly, EDL-155 treatment did not seem to be detrimental to the surrounding neural tissue. Examining sections near the treatment sites revealed that all of the cells were present

Fig. 4



The effects of 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) on normal rat astrocytes (a), rat C6 glioma (b), human U87 glioma (c), and human T98G glioma (d) are shown in four electron micrographs. All of the cells were treated with 7 μmol/ of EDL-155 for 16 h. In normal rat astrocytes treated with EDL-155, the ultrastructure appears to be normal. The nuclear membrane and mitochondria (asterisk) are intact, and there are no obvious autophagosomes in the cytoplasm. In the C6 glioma, U87 cells, and T98G glioma, the nuclei are intact. Very few mitochondria are, however, observed and some mitochondria are severely disrupted. Large membrane-bound vacuoles throughout the cytoplasm in all glioma cell lines are also observed, and many of these structures are bounded by double membranes (arrowhead), the hallmark of autophagosomes. All micrographs are taken at a magnification of $\times$ 20 000.

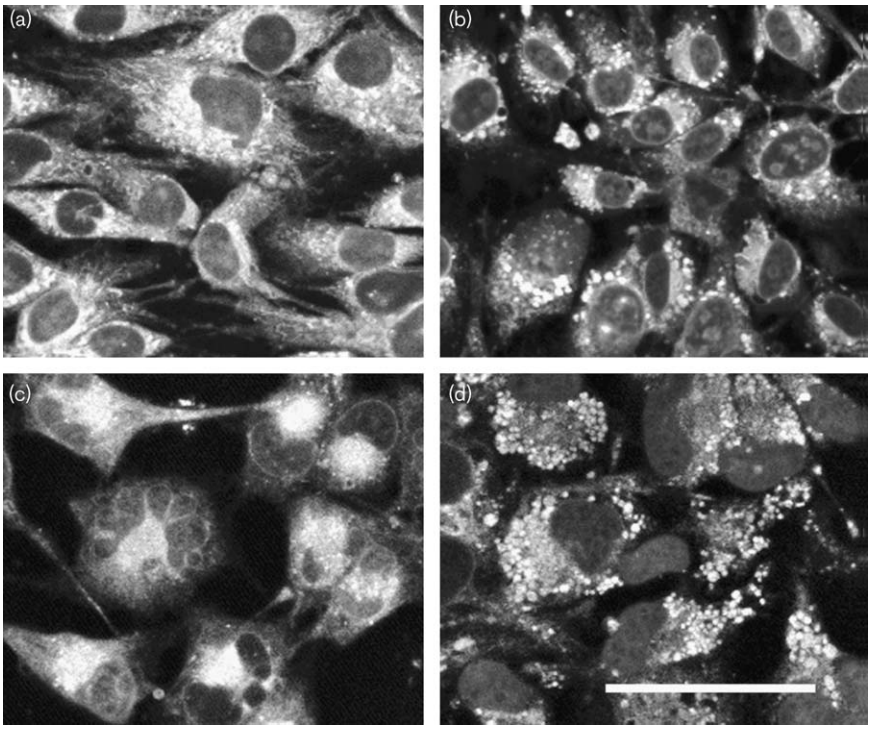
within the brain and that there were no signs of extensive reactive gliosis (Fig. 6c). A few glioma cells could be observed in association with the vascular supply in these distal sections, but no major collections of tumor cells could be found.

To obtain a measure of the size of tumors, we identified the section within each animal that displayed the greatest extent of the tumor. That section was photographed and the area of the tumor was measured using the program NIH image. The data for all rats are shown in Fig. 7. For the rats treated with the carrier solution only ($n = 6$ control animals) the tumors ranged in size from 7.5 to 20.6 mm^2. In rats treated with EDL-155 ($n = 6$), the tumors were generally smaller than those in the other groups. One animal was an outlier, with a tumor measuring 12 mm^2. This tumor had grown up into the cortex; the tip of the treatment cannula appeared to be in the lateral ventricle. All of the other animals in the EDL-155 treatment group had smaller tumors than those observed in the control treatment group. The size of the tumors in the EDL-155-treated rats ranged from 1.6 to 5.1 mm^2. The mean size of tumor in the EDL-155-treated group was $5 \pm 3.7\ \mu m^2$, and in the control group it was $13 \pm 5.8\ \mu m^2$. This difference was significant at the $P < 0.02$ (Student's t-test).

In-vivo glioma models with intraperitoneal delivery of EDL-155

For the second set of in-vivo experiments, the C6 glioma was implanted in the caudate nucleus and the animals were allowed to rest for 1 day before the intraperitoneal treatment began. After the 7 days of treatment the experiment was ended and the brains examined to determine the size of the tumors. In control animals, relatively large tumors were observed in the caudate nucleus. The C6 glioma could be observed as a large mass and out of this mass, cells were observed infiltrating the surrounding tissues attached to local blood vessels. After 7 days of intraperitoneal treatments with EDL-155 (20 mg/kg), the tumors appeared to be smaller compared with those observed in the vehicle control animals. The

Fig. 5



The effects of 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) on mitochondrial membranes are shown. Two cell lines are shown: rat C6 glioma (a and b) and human U87 glioma (c and d). (a and c) are controls that received no compound. The cells in (b and d) were treated with 25 μmol/l EDL-155 for 16 h. The cultures were stained with Mito Tracker Green. Notice that in the control cells the mitochondrial staining is a fine stippled pattern throughout the cytoplasm. After 16 h of EDL-155 treatment, the mitochondrial membranes have coalesced into large vacuoles within the cells. These data confirm our findings at the electron microscopic level with a decrease in mitochondria and an increase in large debris containing vacuoles. The scale bar in (d) represents 40 μm.

tumors were reconstructed from serial sections to define the total tumor volume in each animal. The data for all animals are shown in Fig. 8. The mean size of the EDL-155 treatment tumors was significantly smaller than the tumor size in vehicle control animals (13.2 ± 5.3 vs. $18.7 \pm 6.3\,mm^2$; Student's *t*-test $P < 0.03$). These data indicate that the intraperitoneal EDL-155 treatment was capable of inhibiting the growth of the C6 glioma tumors within this rat model of brain cancer.

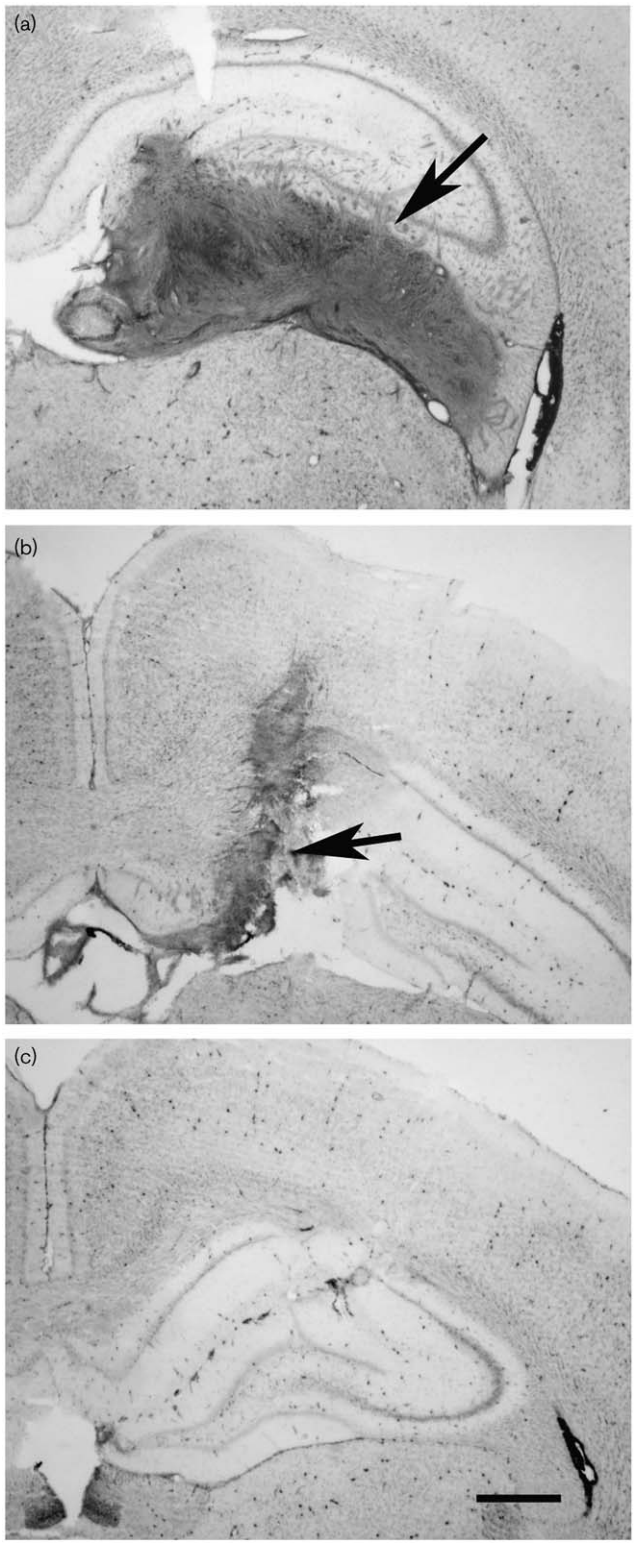
Discussion

In this study we have tested the ability of EDL-155 to selectively kill C6 glioma in culture and in an animal model of brain cancer. We identified EDL-155 as an antiglioma agent in a screen of a library of 500 compounds [19]. On the basis of these initial findings, we wished to compare the efficacy of EDL-155 in killing glioma cells relative to that of a common chemotherapeutic agent BCNU. In a tissue culture assay, we generated dose–response curves to determine the effectiveness of EDL-155 in killing C6 glioma and normal neonatal rat astrocytes. Our in-vitro results demonstrate that EDL-155 is more selective in killing C6 glioma than in killing normal brain astrocytes. Furthermore, a comparison of the dose–response curves of EDL-155 to that of the commonly used anticancer agent BCNU reveals that the EDL compound is considerably more selective in its killing of glioma than is BCNU. In addition, it is more selective than temozolomide [32,33].

The effectiveness of EDL-155 in killing C6 glioma was extended by testing the compound in a rat model of brain cancer. C6 glioma was implanted into the brains of Sprague–Dawley rats and an osmotic minipump delivered drug directly onto the tumor. After 7 days of treatment, there was a prominent tumor in all of the animals that received carrier solution only. When we examined the animals treated with EDL-155, we found that the tumors were smaller (61.6%) than those of the animals treated with carrier solution only. We also tested EDL-155 in a model where the drug was delivered by intraperitoneal injection. In this latter model, there was a significant decrease (29.4%) in tumor size but not to the same extent as that observed with intracranial delivery of the

compound. This type of model is well characterized [24,26,27,34] and allows direct testing of the effectiveness of compounds in treating transplanted glioma [28,35].

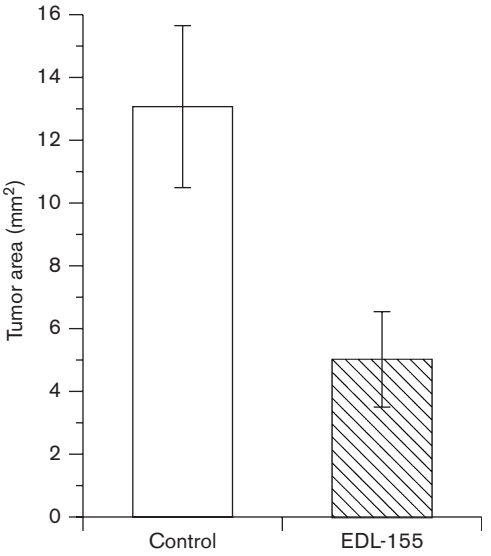
Fig. 6



The growth of C6 glioma in the brains of rats treated with vehicle only (a), and 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) (b) is shown. Approximately 50 000 labeled C6 gliomas were transplanted and immediately treated for 7 days. Regions of the control treatment are shown at a higher magnification to illustrate the invasiveness of the tumor (a, arrow). EDL-155 (5 μmol/l) was delivered at a rate of 1 ul/h treatment for 7 days and this resulted in considerably less tumor growth (b, arrow). Notice that transplanted glioma is found mainly around the tract of the cannula implanted to deliver the treatment. In a section 250 mm form the tumor in the EDL-155-treated animals the tissue is relatively intact (c). There is no significant neuronal loss and no indication of reactive gliosis. The scale bar in (c) represents 1 mm.

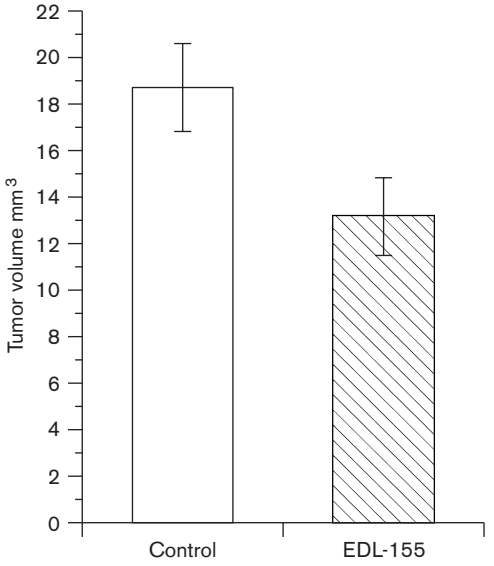
In our early in-vivo experiments, we began intracranial treatment immediately after the transplantation of the cells. Although this approach was technically the most efficient means of testing the effects of EDL-155 on the growth of glioma in the environment of the brain, it did not properly mimic the situation that would occur in a human diagnosed with brain cancer. In these cases, the glioma is well established before any treatment begins. Nonetheless, these initial experiments showed that EDL-155 could decrease tumor size relative to carrier solution only. In the second set of in-vivo experiments, the C6 glioma was implanted into the caudate nucleus and the EDL-155 was delivered by intraperitoneal injection. These treatments began 1 day after the implantation. The results of the intraperitoneal delivery

Fig. 7



The area of the tumor was measured in each animal. Animals were treated with carrier solution only (control, $n=6$), or with 7 μmol/l of 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) ($n=6$). The mean tumor size is shown in mm^2 with standard error bars. The animals treated with EDL-155 had significantly smaller tumors than those observed in the control rats (Student's *t*-test, $P<0.02$).

Fig. 8



The volume of the tumor for each animal treated by intraperitoneal administration of carrier solution only (control, $n=11$), or with 7 μmol/l 1-(biphenyl-4-ylmethyl)-1,2,3,4-tetrahydroisoquinoline-6,7-diol (EDL-155) ($n=10$) is shown. The volume of each tumor was measured and the data is displayed as mean tumor volume and standard error in mm^3. The animals treated with EDL-155 had significantly smaller tumors than those observed in the control rats (Student's *t*-test, $P<0.03$).

experiment were not nearly as favorable as those that delivered EDL-155 directly into the brain. In this clinically more relevant model, there are complications imposed by the blood–brain barrier. In a related study of EDL-155, we have found that EDL-155 does not readily cross the blood–brain barrier [36].

We focused on the potential ability of EDL-155 to selectively kill C6 glioma. In tissue culture, EDL-155 was cytotoxic to glioma cells at a considerably lower concentration than that used for normal astrocytes cultured from the neonatal rat. In this assay, we treated normal brain cells (neonatal astrocytes) that were mitotically active. Within the environment of the adult rat brain, very few normal cells are in the cell cycle. Most of them are terminally differentiated or resting in G0. In this environment, a 7 μmol/l solution of EDL-155 seemed not to be detrimental to the tissues surrounding the treatment cannula. The fact that there was little to no degeneration or reactive gliosis at the end of the cannula is favorable for the potential use of EDL-155 in treating cancers within the normal environment of the brain.

One intriguing aspect of EDL-155 treatment that occurred in the C6 glioma and human glioma cell lines was the induction of large vacuoles, and the nuclei appeared relatively normal. When the cells were examined at the electron microscopic level, a decreased number of mitochondria was evident in the treated glioma. These changes, which were not observed in primary cultures of astrocytes treated with EDL-155, are suggestive of the mechanism whereby EDL-155 selectively kills glioma cells. The lack of nuclear condensation or fragmentation strongly indicates that the cells do not die by apoptosis, a conclusion that is supported by the apparent absence of DNA laddering in cells treated with EDL-155 (unpublished observation). Rather, the intact nuclei and the presence of large vacuoles suggest that the cells are dying of programed cell death type II or autophagy (for a review see [37]). Gliomas seem to be particularly sensitive to autophagy [38–40], which has been reported to occur in these tumors after treatment with temozolomide [41], volatile anesthetics [42], inhibitors of PI3 kinase [43], or the natural product curcumin [12]. After treatment of gliomas with sodium selenite, cells also die of autophagy [13] and the primary target seems to be mitochondrial. Kim *et al.* [13] have provided evidence that selenite preferentially damages mitochondria in gliomas, thus sending the cells into autophagy.

The potential importance of mitochondria in the progression and development of cancer was recognized relatively early [44] and is again receiving considerable attention (for reviews see [45,46]). The earliest anatomical effect observed in glioma treated with EDL-155 is the disruption of mitochondria, suggesting that these organelles are the initial target of EDL-155. It is possible that the selectivity we observe with EDL-155 is related to differences in mitochondrial proteins. For example, the deamination of Bcl-X_L could produce a molecular target in the mitochondria of cancer cells that is not present in normal tissues [47]. This type of selective vulnerability in mitochondria could make them a major target for cancer therapies. Recently, several studies have identified compounds that target mitochondria in cancer cells [48–52]. The results of this study suggest that EDL-155, like other anticancer compounds, has a primary molecular target in the mitochondria. Alternatively, the selectivity of the drug may be because of the susceptibility of gliomas to autophagy. We are currently conducting experiments to determine which of these hypotheses is most tenable.

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7 241 774 and is owned by The University of Tennessee Research Foundation.

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