EDL-291, a novel isoquinoline, presents antiglioblastoma effects *in vitro* and *in vivo*

Xiang-Di Wang[a], Natalie E. Freeman[a], Renukadevi Patil[b], Shivaputra A. Patil[b], Suchareeta Mitra[a], William E. Orr[a], Clint W. Abner[a], Charles Ryan Yates[b], Duane D. Miller[b] and Eldon E. Geisert[a]

To investigate the effectiveness of EDL-291, a 6,7-dimethoxy-1-[4-(4-methoxypyridin-3-yl)benzyl]-1,2,3,4-tetrahydroisoquinoline dihydrochloride compound, in inhibiting the survival of glioblastoma *in vitro* and *in vivo*. Dose–response curves were generated to determine the EC_{50} in rat and human glioblastoma cell lines by treatment with different dilutions of EDL-291. To evaluate the architecture of the glioblastoma cells after treatment with EDL-291, the rat and human glioblastoma cells were stained with Mito Tracker Green FM. To determine whether autophagy was induced in EDL-291-treated glioblastoma cells, both rat and human glioblastoma cell lines were stained with acridine orange and light chain-3 immunoblots were performed. The efficacy of EDL-291 was monitored *in vivo* using a rat glioblastoma model. Rat glioblastoma cells were transplanted into an intracranial rat model, followed by infusions of saline, a low dose of EDL-291 (20 mg/kg for the first half hour, followed by 40 mg/kg EDL-291 in saline for 4 h), or a high dose of EDL-291 (60 mg/kg for the first half hour, followed by 90 mg/kg EDL-291 for 4 h). EDL-291 inhibits glioblastoma *in vitro* by destroying the mitochondria as shown with Mito Tracker Green FM. Acridine orange staining and light chain-3 immunoblots suggest that autophagy is induced when glioblastoma cells are treated with EDL-291. *In vivo*, a low dosage of EDL-291 is sufficient and effective in reducing glioblastoma tumor size. EDL-291 selectively induces cell death in rat and human glioblastoma cell lines by the induction of autophagy. EDL-291 exhibits antiglioblastoma effects both *in vitro* and *in vivo*.

Anti-Cancer Drugs 2012, **23**:494–504

Keywords: autophagy, brain cancer, glioblastoma, isoquinoline

[a]Department of Ophthalmology, Hamilton Eye Institute, College of Medicine and [b]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, USA

Correspondence to Xiang-Di Wang, MD, PhD, Department of Ophthalmology, Hamilton Eye Institute, College of Medicine, University of Tennessee Health Science Center, Memphis, TN 38163, USA
Tel: +1 901 448 7823; fax: +1 901 448 5028;
e-mail: xwang17@uthsc.edu

Received 30 November 2011 **Revised** 25 January 2012
Revised form accepted 29 January 2012

Introduction

Malignant glioblastoma is the most frequent and aggressive brain neoplasm originating in the central nervous system. In conjunction with the biological and clinical aggressiveness of glioblastoma, patients diagnosed with glioblastoma have a high mortality rate. The median survival time of the majority of glioblastoma patients is generally less than 1 year from the time of diagnosis and, unfortunately, most patients die within 2 years even under the most favorable circumstances [1–3]. According to WHO, glioblastoma is ranked as a grade IV tumor and possesses characteristics such as abnormal cells that reproduce rapidly, formation of new blood vessels to maintain the rapid growth of the tumor, and within the center of the tumor, there are necrotic areas [4].

The current standard of care for glioblastoma patients is surgical resection to remove as much of the tumor as possible; however, due to the invasive nature of glioblastoma, surgery is often followed by concomitant and adjuvant radiation therapy and chemotherapy. Remarkable advances have been made in the areas of (a) surgical techniques, (b) treatment options such as cytotoxic chemotherapy, targeted systemic therapy, vaccines, and gene therapy [5–7], and (c) advances in delivery of treatment such as intra-arterial drug administration [8,9], direct intratumoral injection [10], blood–brain barrier disruption [11], and convection-enhanced delivery [12]. Even though these remarkable advances have been set forth, the prognosis of glioblastoma remains poor, with more than 90% of the tumors reoccurring within a couple of centimeters of the primary tumor. It is surprising that this cancer is so intrusive, even though therapies have been created to specifically target angiogenesis, mechanisms associated with cell growth [13–17], and the individual genes that are responsible for the growth of glioma such as the isocitrate dehydrogenase 1 gene [18].

Glioblastoma research has traditionally been conducted using cell lines derived from malignant gliomas such as U87, U251, and A-172. These studies are usually followed by or paralleled with an in-vivo model of glioblastoma. One of the major setbacks in the development of brain cancer drugs is the inability of the molecule to cross the blood–brain barrier and the blood–tumor barrier. Several

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328351ee4f

strategies have been proposed to resolve this problem such as (a) intra-arterial drug administration and gene therapy [8–10], (b) direct intratumoral injection [10], and (c) designing anticancer drugs that are lipid soluble with a neutral pH, not highly bound to plasma proteins, and have a molecular weight less than 200 Da [19–21].

The current goal for our laboratory is to develop compound(s) with a low molecular weight, good penetration of the blood–brain barrier, and a relatively safe profile to treat high-grade glioblastoma. Over the past 9 years, we have identified a variety of 1,2,3,4-tetrahydroisoquinoline derivatives that have inhibited the growth and survival of glioblastoma both *in vitro* and *in vivo* [22–24]. The current study evaluates the efficacy of EDL-291 in rat and human glioblastoma cell lines as well as a rat in-vivo model in order to better understand the biology of glioblastoma treatment and attempt to maintain quality of life by delaying disease progression.

Methods

Cell culture

Astroglial cells were cultured from the cerebral cortex of Sprague–Dawley rat pups using a protocol described in McCarthy and de Vellis [25] and modified by Geisert and colleagues [26,27]. The animals were anesthetized by cold (placed in ice), decapitated, and their brains were immediately removed. The cortices were placed in a Petri dish containing 10 ml of Hank's balanced salt solution (HBSS; Mediatech Inc., Manassas, Virginia, USA). The tissue was placed in 20 ml of 0.1% trypsin (Invitrogen Life Technologies, Carlsbad, California, USA) in HBSS for 10 min. The astrocytes were plated at a density of 5×10^3 cells/cm^2 in a T-75 culture flask (Sigma Aldrich, St Louis, Missouri, USA). The cells were grown to confluence in Eagle's basal medium (BME) (Mediatech Inc.) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Hyclone, Rockford, Illinois, USA). The C6 rat glioblastoma cell line and human glioma cell lines (U87, T98G, and A-172) were purchased from the American Type Culture Collection (ATTC, Manassas, Virginia, USA) and were maintained in BME (Mediatech Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.), 1% Hepes (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies), and 1% L-glutamine (Invitrogen Life Technologies). The human glioblastoma cell line, U251, was a kind gift from Dr Wei Zhang (National Cancer Institute, NIH Bethesda, Maryland, USA) and the cells were maintained in RPMI 1640 (Thermo Fisher Scientific Inc.) supplemented with 10% FBS (Thermo Fisher Scientific Inc.), 1% Hepes (Invitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies), and 1% L-glutamine (Invitrogen Life Technologies).

In-vitro EDL-291 cytotoxicity assay

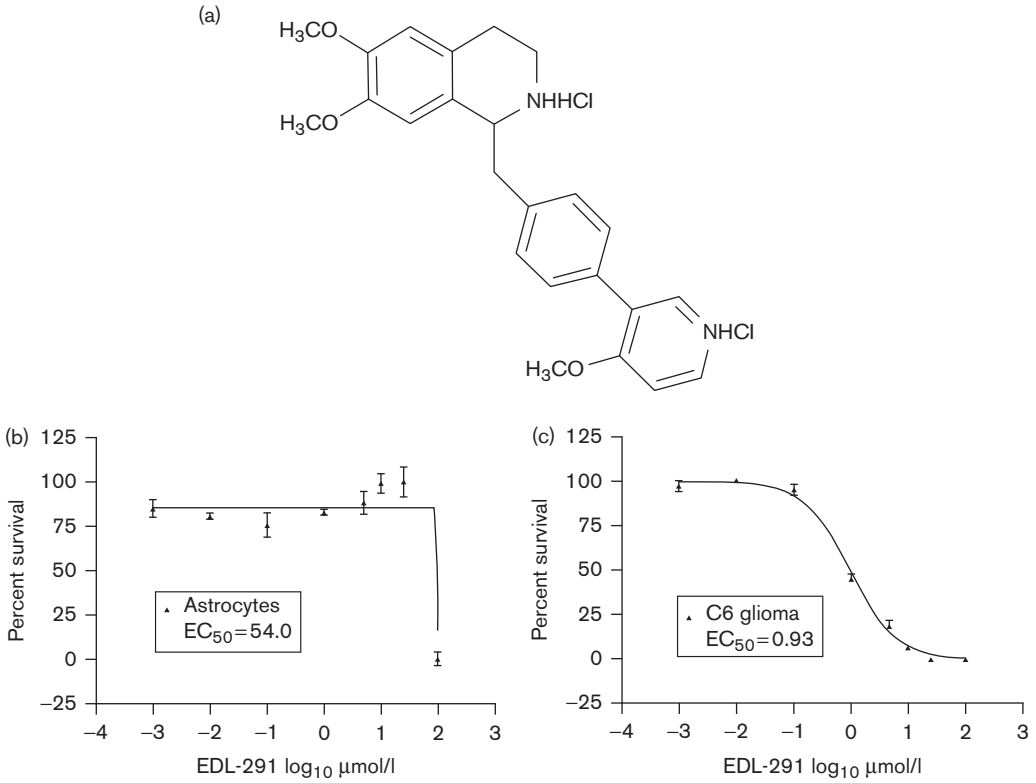
EDL-291 was synthesized in the laboratory of Dr Duane D. Miller and the structure of EDL-291 is presented in Fig. 1a. Rat and human glioblastoma cells (C6, U87, U251 T98G, and A-172) were transferred into 96-well plates at a cell density of 1×10^3 cells/well. The cells were cultured overnight in 200 µl of culture medium (described above) in a 37°C incubator containing a humid 5% CO_2 atmosphere. Before treatment, the culture medium was replaced with 2% FBS culture medium, which contained either vehicle [dimethyl sulfoxide (DMSO); Sigma Aldrich] or different concentrations of EDL-291 (0.001, 0.01, 0.1, 1.0, 5.0, 10.0, 25.0, and 100.0 µmol/l). The DMSO concentrations remained uniform in all wells at 0.01% (v/v). The cells were incubated for 96 h and fixed with 4% paraformaldehyde, stained with 0.1% cresylecth violet (Sigma Aldrich), and the absorption was monitored on a BioTek µQuant Spectrophotometer at 490 nm (BIO-TEK Instruments Inc., Winooski, Vermont, USA). The cell number was determined by quantifying the intensity of the blue stain, which is directly proportional to the number of intact nuclei in the well [22].

The effect of the EDL-291 on cell growth or cell death is presented as percent survival. This is calculated by taking the average absorbance at 590 nm for treated cells, divided by absorbance of the untreated cells (negative control), and expressed as a percentage. Values less than 100% indicate a diminished growth or cytotoxicity of EDL-291. The raw data were entered into GraphPad Prism (GraphPad Software, San Diego, California, USA) and nonlinear regression was performed to calculate the best-fit curve as well as the EC_{50}. Different concentrations of EDL-291 (0.001, 0.01, 0.1, 1.0, 5.0, 10.0, 25.0, and 100.0 µmol/l) were used for the calculation of the EC_{50} values.

Western blot of light chain-3

C6, U87, and U251 glioma cells were treated with 0, 5, 20, and 40 µmol/l of EDL-291 and the cells were collected at 4 and 16 h after treatment. The glioma cells (5×10^6 cells) were washed with PBS (Invitrogen Life Technologies), lysed with 2× Laemmli's buffer (Sigma Aldrich), and the total cell lysates were homogenized. Equal amounts of the cell lysates were loaded (40 µg) on 4–12% SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, California, USA), transferred onto a PVDF membrane (Sigma Aldrich), and blocked with 4% nonfat dry milk. The membrane was incubated with the light chain-3 (LC3; Cell Signaling Technology, Danvers, Massachusetts, USA) primary antibody at a concentration of 1 : 2000 (4°C) overnight, washed with Tris-buffered saline (TBST; Sigma Aldrich), incubated in goat anti-rabbit IgG (H&L), HRP conjugate secondary antibody (Promega, Madison, Wisconsin, USA) for an hour at room temperature, washed in TBST (Sigma Aldrich), and visualized using an ECL detection kit (Thermo Fisher Scientific Inc.) and Kodak 4000MM image station. To assess equal protein loading, GAPDH antibody (Cell

Fig. 1



EDL-291 dose–response curves for normal rat astrocytes and C6 rat glioblastoma. The structure of 6,7-dimethoxy-1-[4-(4-methoxypyridin-3-yl)benzyl]-1,2,3,4-tetrahydroisoquinoline dihydrochloride, EDL-291 (a). The dose–response curves for normal rat astrocytes (b) and C6 rat glioma after EDL-291 treatment for 4 days (c). The concentrations of EDL-291 ($\log_{10}$ μmol/l) are presented on the *x*-axis, whereas the percent survival of the cells is presented on the *y*-axis. Each concentration is represented by a mean and SD of the mean. The rat astrocytes display an EC_{50} of 54.0 μmol/l and the C6 glioma cells have an EC_{50} of 0.93 μmol/l.

Signaling Technology) was used at a concentration of 1 : 1000 and incubated overnight (4°C) washed with TSBT (Sigma Aldrich), incubated in goat anti-rabbit IgG (H&L), HRP conjugate secondary antibody (Promega) for an hour at room temperature, washed in TBST (Sigma Aldrich), and visualized using an ECL detection kit (Thermo Fisher Scientific Inc.) and Kodak 4000MM image station.

Mitochondria staining with Mito Tracker Green FM

C6, U87, and U251 cells were cultured on 24-well glass-bottom collagen-coated culture plates (MatTek Corporation, Ashland, Massachusetts, USA). The cells were washed with PBS and fresh culture media and incubated at 37°C. After a 24-h incubation, the cells were treated with either vehicle (DMSO) or EDL 291 (5 and 25 μmol/l) for 16 h. Sixteen hours after treatment, the cells were fixed with 4% paraformaldehyde (Sigma Aldrich) and stained with 100 nmol/l Mito Tracker Green FM (Molecular Probes, Eugene, Oregon, USA) for 20 min at room temperature. The cells were rinsed in PBS and examined at an excitation wavelength of 485 nm and emission 520 nm filter using a Nikon C1Si spectral confocal system with a Nikon TE2000-E2 inverted confocal microscope at ×60 objective lens magnification (Nikon, Tokyo, Japan).

Acridine orange staining for autophagy detection

C6, U87, and U251 cells were cultured on a glass-bottom collagen-coated 24-well cell culture plate (MatTek Corporation) and treated with 0, 5, and 25 μmol/l of EDL-291 for 4 and 16 h. The cells were stained with acridine orange (Molecular Probes–Invitrogen, Carlsbad, California, USA) at a final concentration of 1 μg/ml in culture media containing *2%* FBS. The cells were incubated at 37°C for 15 min, washed with PBS, and fresh media containing *2%* FBS were added to the cells. Stained vacuoles were visualized in a Nikon C1Si spectral confocal system with a Nikon TE2000-E2 inverted confocal microscope at ×60 objective lens magnification.

In-vivo glioblastoma model

Male Sprague–Dawley cannulated rats fitted with a vascular access harness ($n = 27$, 250–350 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA) in order to develop the C6 rat

glioma intracranial tumor model. The day before surgery, C6 glioma cultures were rinsed in HBSS and placed in BME with 10% rat serum (Equitech-Bio Inc., Kerrville, Texas, USA). On the day of the surgery, the cells were trypsinized and incubated in HBSS.

Before surgery, the animals were anesthetized with xylazine (13 mg/kg; Butler Schein, Dublin, Ohio, USA) and ketamine (87 mg/kg; Butler Schein). The rats were monitored throughout the surgery to ensure that they remained deeply anesthetized and unresponsive to painful stimuli. Approximately 5×10^5 C6 glioblastoma cells in 5 µl of HBSS were delivered through an infusion 2.5 mm below the cerebral cortex into the hippocampus over a 5-min period. The animals were allowed to recover and returned to the animal care facility. Four days after the tumor was transplanted, the animals were assigned to one of the three treatment groups: nine rats received carrier solution only (0.9% saline injection solution), nine rats received a low-dose infusion of EDL-291 (20 mg/kg for the first half hour, followed by 40 mg/kg EDL-291 in saline for 4 h), and nine rats received a high-dose infusion of EDL-291 (60 mg/kg for the first half hour, followed by 90 mg/kg EDL-291 for 4 h). The animals received infusions through the vascular access on the back. The animals were continuously monitored during the infusion (4.5 h) and throughout the 8-day treatment period.

Histology

After 8 days of treatment, the rats were anesthetized with xylazine (26 mg/kg) and ketamine (174 mg/kg). The animals were perfused through the heart with saline, followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). The animals were decapitated, the heads were soaked in 4% paraformaldehyde for 3 days, and the brains were removed from the skull. The brains were postfixed in 4% paraformaldehyde for 24 h and placed in a 30% sucrose solution. The brains were sectioned at 50 µm with a freezing microtome. One set of sections was rinsed in PBS, whereas the remaining sections were stored in borate-buffered saline (pH 8.4) at 4°C. One one-in-five series of sections were mounted on glass slides and stained by the 0.1% cresylecth violet. The digital images were taken from each section and coded (codes were kept by one investigator, Xiang Di-Wang). The digital images were analyzed to define the volume of the tumor (27 tumors) using the program NIH image.

Statistics

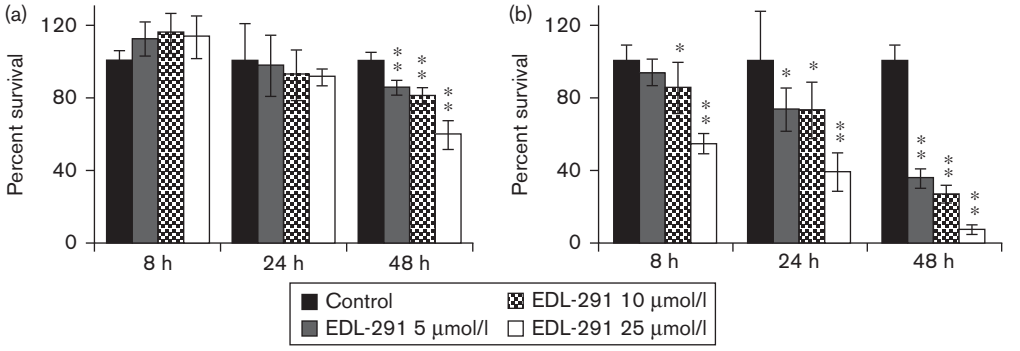
All in-vitro data are expressed as a mean of values and ± SD of the mean. Comparisons between treatment groups were made using Student's *t*-test. For the in-vivo studies, the data were compiled and analyzed using a Mann–Whitney *U* nonparametric test. This work was conducted in a blinded manner (William E. Orr).

Results

The effects of EDL-291 on glioblastoma cells *in vitro*

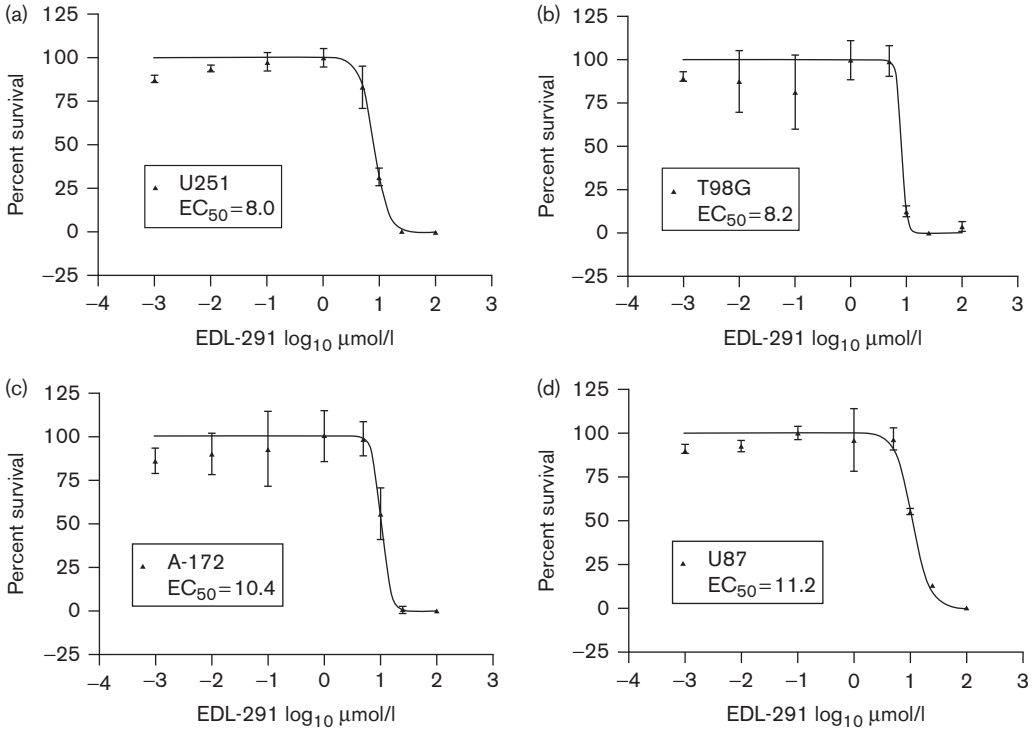
Dose–response curves were generated for normal rat astrocytes and C6 glioblastoma cells in order to test the efficacy of EDL-291 (Fig. 1b and c). The normal astrocytes (Fig. 1b) and C6 glioblastoma (Fig. 1c) cells were treated with different dilutions of EDL-291 (0.001, 0.01, 0.1, 1.0, 5.0, 10.0, 25.0, and 100.0 µmol/l) for 96 h. The cells were stained with cresylecth violet and the absorption was measured at 490 nm, which was directly proportional to the number of intact nuclei (viable cells). A marked difference was observed in the response of C6 glioblastoma cells relative to that of normal astrocytes. Specifically, the C6 glioblastoma cells are more sensitive to the effects of EDL-291 than the normal brain astrocytes. The normal astrocytes displayed an EC_{50}

Fig. 2



Dose–response and time–response studies for normal rat astrocytes and C6 glioblastoma cells exposed to EDL-291. Both the normal astrocytes (a) and the C6 glioma (b) cells were exposed to EDL-291 at concentrations of 0, 5, 10, and 25 µmol/l for 8, 24, or 48 h. The cells were incubated (37°C) for a total of 4 days, at which time the number of surviving cells was determined. Time is displayed on the *x*-axis (8, 24, and 48 h) and the percent survival of cells is presented on the *y*-axis. Each concentration is represented by a mean and SD of the mean. Within 8 h of treatment with EDL-291 (25 µmol/l), approximately 50% of the glioblastoma cells did not survive. After 48 h of EDL-291 exposure, the survival of the glioblastoma cells was inhibited at all concentrations tested. Significant decrease in growth is indicated by an $*P<0.05$ and $**P<0.01$ (Student's *t*-test).

Fig. 3



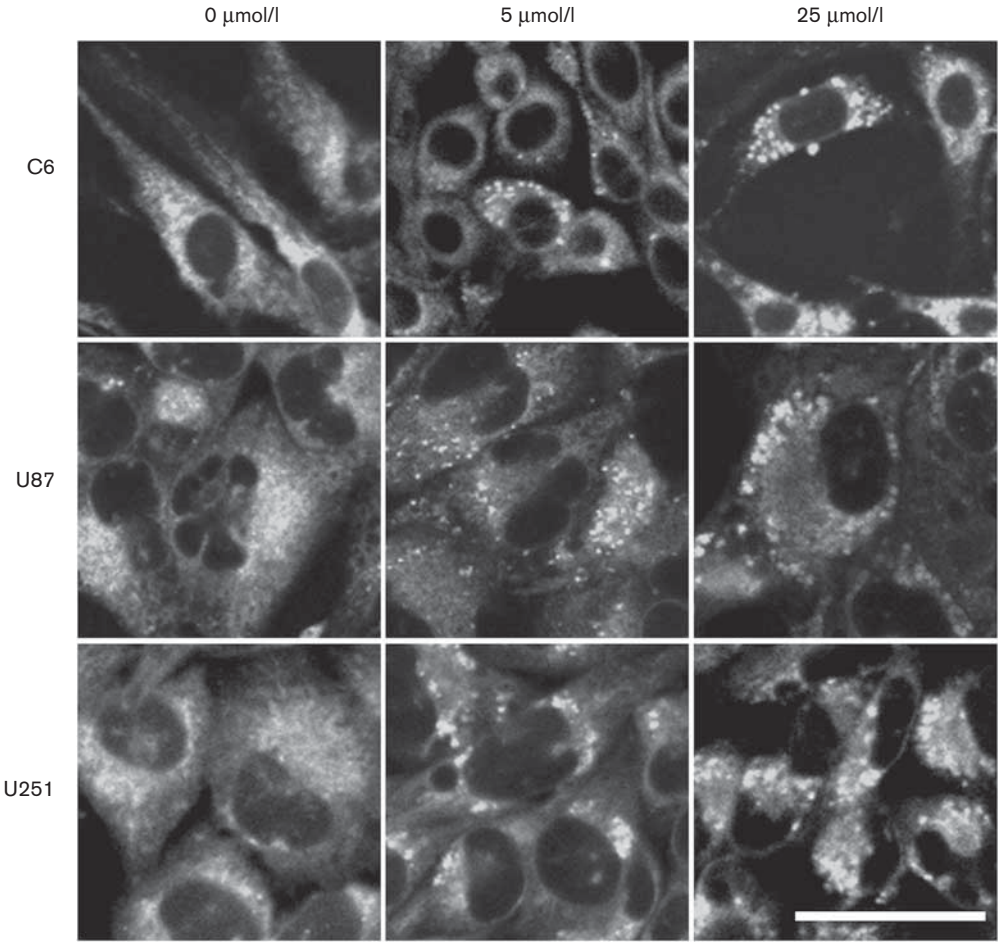
EDL-291 dose–response curves were generated for human glioblastoma cells in order to determine the EC_{50}. The concentrations of EDL-291 (log_{10} μmol/l) are presented on the *x*-axis, whereas the percent survival of the cells is presented on the *y*-axis. Each concentration is represented by a mean and SD of the mean. U251 human glioblastoma cells have an EC_{50} of 8.0 (a). T98G human glioblastoma cell lines have an EC_{50} of 8.2 (b). The A-172 human glioblastoma cell line has an EC_{50} of 10.4 (c). U87 human glioblastoma cells have an EC_{50} of 11.2 (d).

value of 54.0 μmol/l, whereas C6 glioblastoma displayed an EC_{50} value of 0.9 μmol/l when treated with EDL-291. A 58-fold difference in the effective concentration of EDL-291 is needed to inhibit C6 glioblastoma relative to the concentration needed to inhibit the growth of normal astrocytes, indicating that EDL-291 is selectively cytotoxic to the C6 glioblastoma cells.

To determine the optimal time and concentration necessary to inhibit C6 glioblastoma cells, the duration of exposure and concentration of EDL-291 were varied in normal astrocytes and C6 glioblastoma cells. Both the normal astrocytes and the glioblastoma cells were treated with increasing concentrations of EDL-291 (0, 5, 10, and 25 μmol/l) for 8, 24, and 48 h. The percentage of surviving cells was quantified as shown in Fig. 2. The normal astrocytes (Fig. 2a) were not as affected by EDL-291 treatment. There was no significant difference at any concentration for EDL-291 after treating the cells for 8 and 24 h. After 48 h of treatment, there was a significant decrease in the growth at all concentrations tested ($P < 0.01$, Student's *t*-test). For the C6 glioblastoma, there was a significant decrease ($P < 0.05$, Student's *t*-test) in the survival within 8 h with concentrations of EDL-291 above 10 μmol/l (Fig. 2b). Exposing the glioblastoma cells for 8 h at 25 μmol/l was sufficient to inhibit more than 50% of the glioblastoma cells ($P < 0.01$, Student's *t*-test). At 24 h of treatment, there was a decrease in cell survival of C6 glioblastoma treated with all concentrations of EDL-291 (5 μmol/l $P < 0.05$, 10 μmol/l $P < 0.05$, and 25 μmol/l $P < 0.01$, Student's *t*-test). At 48 h, there was a significant decrease in cell survival at all concentrations ($P < 0.01$, Student's *t*-test): a 64% decrease was observed when treated with 5 μmol/l EDL-291, a 73% decrease in cell survival at 10 μmol/l EDL-291, and a 93% decrease in cell survival with 25 μmol/l EDL-291. These results indicate that EDL-291 has the ability to inhibit the growth and survival of the rat glioblastoma cells, which seems to be dose and time dependent while having a minimal effect on the growth and survival of normal astrocytes. In addition, a brief exposure (8 h) of EDL-291 (25 μmol/l) has inhibitory effects on the rate of survival of the glioblastoma cells and a prolonged exposure (48 h) seems to almost completely inhibit glioblastoma survival, while having minimal effects on normal astrocytes.

As our overall goal is to develop chemotherapeutic agents for treating human glioblastoma, we examined the effects of EDL-291 on several human glioblastoma cell lines: U251, T98G, A-172, and U87. To create dose–response curves, the human glioblastoma cell lines were treated

Fig. 4



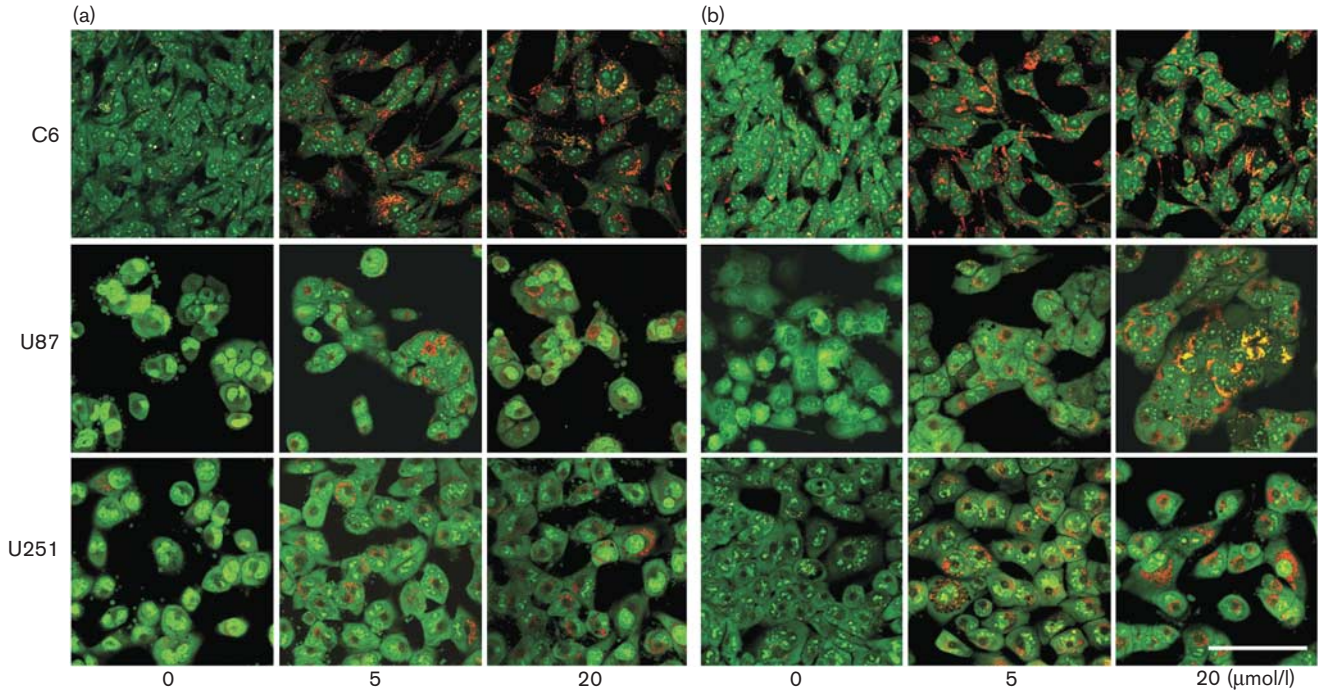
Photomicrographs displaying the effects of EDL-291 on mitochondrial membranes of rat and human glioblastoma cell lines. To examine the architecture of glioblastoma cells, C6 rat glioblastoma, U87 human glioblastoma, and the U251 human glioblastoma cells were treated with EDL-291 (0, 5, and 25 μmol/l) for 16 h. Mito Tracker Green FM staining was performed immediately following the 16-h treatment. The mitochondrial staining present within the control samples (C6, U87, and U251 treated with 0 μmol/l) was seen as a fine stippled pattern throughout the cytoplasm; however, after the EDL-291 treatment (5 and 25 μmol/l), the mitochondrial membranes had coalesced into large vacuoles within the cells. The scale bar represents 20 μm.

with increasing concentrations of EDL-291 for 96 h, ranging from 0.001 to 100 μmol/l (Fig. 3). From the dose–response curves, we calculated the EC_{50} as follows: the U251 and T98G glioblastoma cell lines had similar EC_{50} at 8.0 and 8.2 μmol/l, respectively (Fig. 3a and b). The A-172 glioma cell line had a higher EC_{50} of 10.4 μmol/l and the U87 required an even higher concentration of EDL-291 at 11.2 μmol/l (Fig. 3c and d). These results demonstrate that EDL-291 is capable of inhibiting the growth of several human glioblastoma cell lines and the required concentration between the four human cell lines ranges between 8 and 11.2 μmol/l (Fig. 3).

Induction of autophagy in EDL-291-treated glioblastoma cells

Our publications have shown that EDL-155, which is structurally similar to EDL-291, disrupts the mitochondrial structure and induces autophagy in glioblastoma and retinoblastoma cells [27,28]. To reveal the architecture of the glioblastoma cells, C6, U251, and U87 glioblastoma cells were treated with vehicle (DMSO) or EDL-291 (5 and 25 μmol/l) and the cells were stained with MitoTracker Green FM. In this study, confocal microscopy was used to visualize the effects of EDL-291 on the mitochondria (Fig. 4). In the control group, the glioblastoma nuclei were located in the middle of the cells, the cells appeared to have smooth plasma membranes, and there as a fine stippling pattern associated with normal mitochondria. With increasing concentrations of EDL-291, the nuclei remained intact; however, the mitochondria lost the fine stippling pattern and appeared to be severely disrupted with punctate staining. There did not appear to be any nuclear morphology changes associated with apoptosis such as fragmentation or condensation in either the control or the EDL-291-treated

Fig. 5



Photomicrographs of the confocal microscopy analysis of glioblastoma cells treated with EDL-291 and stained with acridine orange. C6 rat glioblastoma cells, U87 human glioblastoma cells, and U251 human glioblastoma cells treated with EDL-291 (0, 5, and 20 μmol/l) for 4 h (a) and 16 h (b), followed by acridine orange staining. The cell types (C6, U87, and U251) can be seen on the left side of the panels and the concentrations of EDL-291 (0, 5, and 20 μmol/l) are presented at the bottom of the panels. Note the formation of acridine orange-accumulating autophagic vacuoles (orange–red fluorescence) in the EDL-291 treated glioblastoma cells as the time and dosage of EDl-291 increase. Autophagic vacuoles were observed in all of the glioblastoma cell lines within 4 h of treatment with a low dose of EDL-291, 5 μmol/l. The scale bar represents 50 μm.

glioblastoma cells. In contrast, the morphological data seem to suggest that the mechanism for EDL-291-treated glioma involves the disruption of mitochondria.

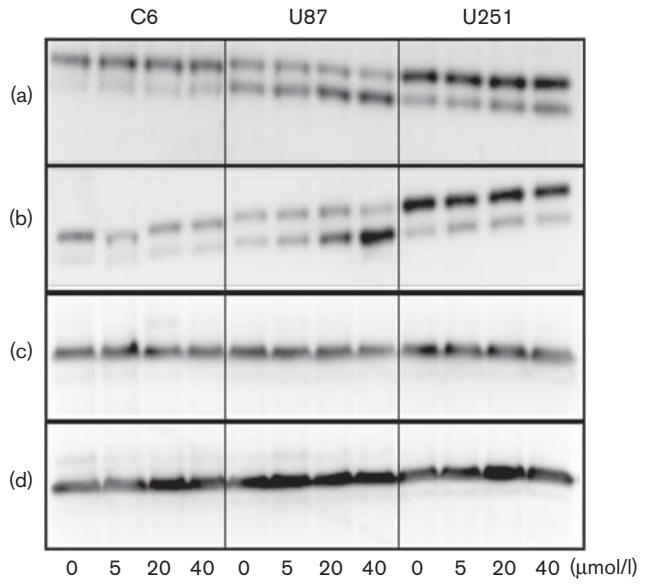
To determine whether EDL-291 induces autophagy in glioblastoma cells, C6, U87, and U251 glioma cells were stained with acridine orange (Fig. 5). This technique allows the characterization of acidic vesicular organelle formation, a hallmark of autophagy. The cells that are positive for acidic vesicular organelle formation display an enhanced red to orange–yellow fluorescence. Each of the glioblastoma cell lines was treated with EDL-291 (0, 5, and 20 μmol/l) for 4 and 16 h. The formation of the acidic vesicular organelles appeared to increase after 4 h of treatment even at the low dosage of EDL-291 (5 μmol/l) in C6, U87, and U251 glioblastoma cells. In addition, the effects appeared to be both dose and time dependent.

Another hallmark of autophagy is a shift in the molecular weight of microtubule-associated protein 1 LC3. If autophagy does occur in glioblastoma cells treated with EDL-291, then there should be a characteristic shift in the molecular weight of LC3. To determine whether EDL-291 induces autophagy in glioblastoma cell lines, C6, U87, and U251 cells were treated with EDL-291 (0, 5, 20, and 40 μmol/l) for 4 and 16 h. After treatment with EDL-291, the cell lysates were probed with an LC3 antibody. As shown in Fig. 6, probing with an LC3 antibody demonstrated a characteristic shift in the molecular weight of LC3, from LC3-I to LC3-II. The shift in the molecular weight of LC3 indicates that the glioblastoma cells treated with EDL-291 are undergoing autophagy (Fig. 6a and b). A slight shift in LC3 was observed in the C6 rat glioma cells at 4 h (Fig. 6a), indicating the formation of autophagosomes; however, at 16 h, the shift seems to decrease if not disappear (Fig. 6b). A prominent shift in LC3 was observed in the U87 human glioblastoma cells at 4 and 16 h (Fig. 6a and b). The U251 human glioblastoma cell line treated with EDL-291 displayed a shift in LC3 molecular weight after 4 h of treatment; however, the majority of the lysates reside in the LC3-I phase. A shift in LC3 molecular weight was observed in U251 cells after a 16-h treatment with either 5 or 20 μmol/l of EDL-291. Figure 6c and d displays the glioblastoma cell lysates probed with GAPDH, which suggests relatively equal protein loading.

The effects of EDL-291 *in vivo*, intracranial glioblastoma model

To determine whether EDL-291 may be effective as an in-vivo therapy, we used a rat intracranial model to inject

Fig. 6



LC3 and GAPDH immunoblots of the glioblastoma cells treated with EDL-291. (a, b) Immunoblots of LC-3 using lysates (40 μg) from rat and human glioblastoma cell lines (C6, U87, and U251) treated with EDL-291 (0, 5, 20, 40 μmol/l) for 4 h (a) and 16 h (b). A hallmark of autophagy is the shift in the molecular weight of LC3 from LC3-I to LC3-II shown on the right side of the immunoblots. (c, d) Glioblastoma lysates probed with GAPDH to assess equal protein loading.

the C6 glioma cell line [27] into cannulated rats fitted with a vascular harness. This study included three groups: (a) control (saline), (b) low dose of EDL-291 (20 mg/kg for the first half hour, followed by 40 mg/kg EDL-291 in saline for 4 h), and (c) high dose of EDL-291 (60 mg/kg for the first half hour, followed by 90 mg/kg EDL-291 in saline for 4 h). Representative sections from each treatment group are shown in Fig. 7a. In the control animals (received vehicle only), relatively large tumors were observed in the brains 8 days after implantation. The C6 glioblastoma cells formed a large mass occupying the majority of the cortex and the cells extended from the tumor, infiltrating into the surrounding tissues, and appeared to attach to local blood vessels. In comparison with the control animals, a marked difference was observed in the size of the tumors in the animals treated with EDL-291. After 8 days of EDL-291 treatment, the tumors were smaller than those in control rats. In addition, there did not appear to be the same degree of infiltration as that observed in control rats. Surprisingly, EDL-291 treatment did not appear to be detrimental to the surrounding neural tissue. Examining sections near the treatment sites revealed that there were no signs of extensive reactive gliosis. 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.

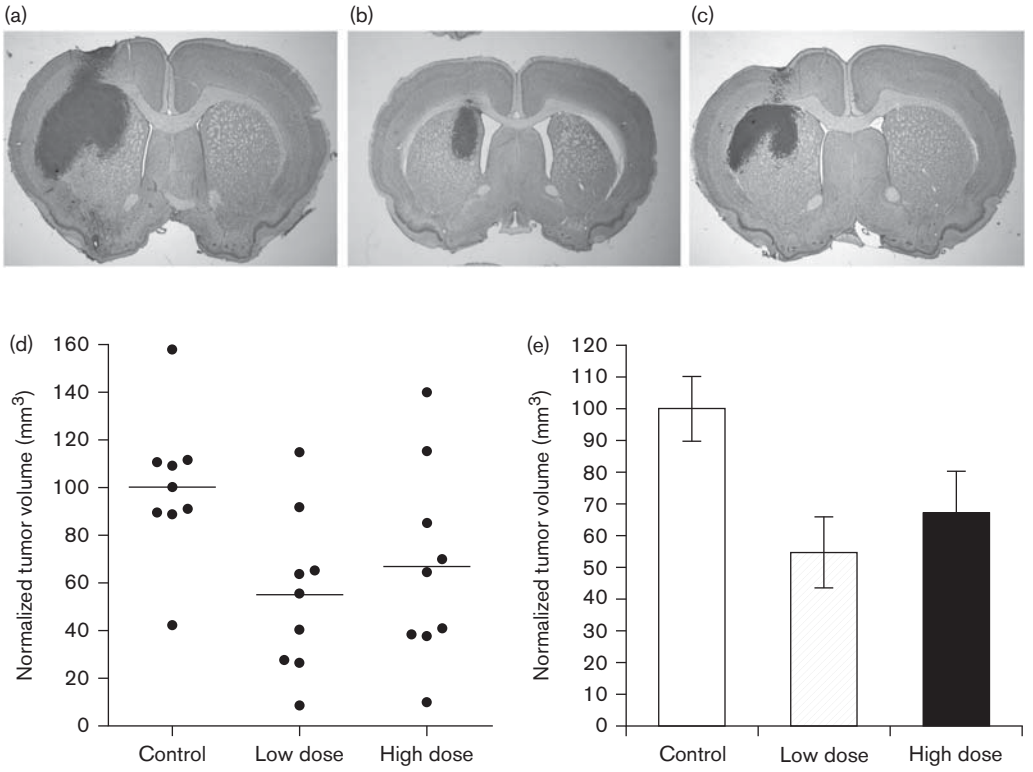
The compiled data for all the animals are displayed in Fig. 7d and e. The animals treated with vehicle had a mean tumor size of 22.5 mm^3. In the animals treated with the low dose of EDL-291, the tumors were smaller, with a mean equal to 13.2 mm^3. The mean tumor size in the high-dose EDL-291-treated rats was 17.3 mm^3. The difference between the tumor size of the low-dose EDL-291-treated group and the control group was significant, *P*-value less than 0.05 (Mann–Whitney *U*-test). The difference between the tumor size of the high-dose EDL-291-treated group and the control group was significant at a *P*-value of less than 0.05 level (Mann–Whitney *U*-test). The data indicate that there is no significant difference between the two EDL-291-treated groups with a *P*-value equal to 0.25. These results suggest that EDL-291 treatment is effective in reducing the glioblastoma tumor size and a low dose is sufficient in reducing tumor volume.

Discussion

The search for a potent and effective antiglioblastoma drug is a major challenge. Our laboratory has focused on the discovery and development of small-molecule technology to create life-changing therapeutics for brain cancer. One of our lead compounds, EDL-155, has been shown to be highly selective for inhibiting the growth of glioblastoma both *in vitro* and *in vivo* [22,27]. Anatomical changes were observed in cultured glioblastoma cells treated with EDL-155 in which the mitochondria were destroyed and autophagy was induced. Recent publications within our laboratory show that local delivery of EDL-155 is effective in killing retinoblastoma cells and EDL-155 appears to induce autophagic cell death [28]. We discovered a flaw in the EDL-155 compound, in which 90% of the drug was being metabolized by catechol-*O*-methyltransferase after a single pass through the liver [24]. The soft targets of EDL-155 were identified and the compound was modified to prevent its metabolism. The modified version of EDL-155 was named EDL-291 and the properties of EDL-291 were nearly optimal to treat central nervous system tumors. EDL-291 has (a) a molecular weight of 463 Da, (b) is stable and easy to work with, (c) has a respectable safety profile, and (d) does not appear to have effects that are detrimental to healthy cells.

In this study, the in-vitro effects of EDL-291 were examined on malignant rat and human glioblastoma cell lines. The in-vitro studies concluded that EDL-291 selectively inhibits C6 rat glioblastoma cells as well as human glioblastoma cell lines U87, U251, T98G, and A-172. EDL-291 exhibits a 58-fold difference in the EC_{50} between C6 rat glioblastoma cells and the normal rat astrocytes. EDL-291 was also effective in inhibiting a variety of human glioblastoma cells with concentrations less than 11.2 μmol/l (8.2–11.2 μmol/l). A significant finding is that EDL-291 appears to be protective on

Fig. 7



The effects of EDL-291 treatment on the intracranial glioblastoma model. The growth of C6 glioblastoma cells in the brains of rats treated only with vehicle (a), low-dose EDL-291 (20 mg/kg EDL-291 for the first half hour, followed by 40 mg/kg EDL-291 in saline for 4 h), (b) and a high dose of EDL-291 (60 mg/kg EDL-291 for the first half hour, followed by 90 mg/kg EDL-291 for 4 h) (c). The *n*-value is equal to nine animals per group. Approximately 50 000 C6 glioblastoma cells were transplanted. The dark area in the cortex represents the growth of the C6 glioblastoma cells. Regions of the control treatment illustrate the invasiveness of the tumor (a). EDL-291 was delivered for 8 days and this resulted in less tumor growth (b, c). There was no noticeable neuronal loss and no indication of reactive gliosis. The area of the tumor was measured in each animal. The mean tumor size is shown in mm^3 with SD bars. The animals treated with EDL-291 had smaller tumors than those observed in the control rats ($P < 0.05$, Mann–Whitney *U*-test). The individual dot graph shows normalized tumor volume by groups (d). Each horizontal line shows the average volume of the group. The bar graph displays the comparison between the control group and the treated groups (e). Using Student's *t*-test, the *P*-value between control and the low-dose the EDL-291 group was found to be less than 0.05 and the *P*-value between the control and the high-dose EDL-291 group was less than 0.05.

supporting cells while displaying detrimental effects on the survival of glioblastoma cells.

When the C6 rat and human glioblastoma cells were examined at the electron microscopy level, there was a decrease in the number of mitochondria in the EDL-291-treated glioblastoma. These changes suggest that EDL-291 is damaging to the glioblastoma cells. Furthermore, EDL-291 appears to induce autophagy in both rat and human glioblastoma as shown with acridine orange staining and a molecular weight shift in LC3 protein (a hallmark of autophagy).

In general, glioblastoma cells seem to be particularly sensitive to autophagy [29–32] and many chemotherapeutic agents have been shown to induce autophagy in a variety of cancer types [33–35]. Tamoxifen and rapamycin have been used in the treatment of breast and ovarian cancer and both treatments appear to activate autophagy at low doses [36–38]. A recent investigation showed that seven out of 12 tumors analyzed by transmission electron microscopy, including breast and lung cancer, had evidence of autophagy, suggesting that autophagy occurs within many tumor types [39,40]. With regard to glioblastoma, Kanzawa *et al.* [32] demonstrated that treatment of glioblastoma cell lines with temozolomide alone causes autophagic cell death.

The effectiveness of EDL-291 as an in-vivo therapy was monitored using a rat intracranial model fitted with a vascular access harness. The vascular access operating system is a reliable model that is well accepted because it is atraumatic and induces minimal stress for serial infusion of any pharmaceutical agents over a given period of time in conscious unrestrained rats [41]. C6 glioma cells were implanted into the brain and the animals were infused with saline, a low dose of EDL-291, or a high dose of EDL-291. After 8 days, a prominent tumor was observed in all of the animals that received only saline. The EDL-291-treated animals had tumors that were

significantly smaller than those observed in the control animals ($P < 0.05$, Mann–Whitney *U*-test). The results suggest that EDL-291 is effective at crossing the blood–brain barrier and there appeared to be no signs of toxic side effects such as fluctuations in body weight (gain/loss), abnormal clinical signs, or behavioral changes.

Novel therapies for treating glioblastoma are urgently needed and this study shows that EDL-291 could be developed for a potential antiglioma therapy. An intravenous injection of the compound appears to be absorbed *in vivo* and EDL-291 crosses through the blood–brain barrier to assert its effects without side effects in a rat glioblastoma model. Although EDL-291 is effective in reducing the tumor size, we believe that direct therapy and/or a combination therapy would result in a more effective glioblastoma treatment. Thus, the future direction of this study will be to determine an optimal combinational therapy to help treat glioblastoma as well as EDL-291 effects in direct tumor treatment.

Acknowledgements

This work was supported by the College of Pharmacy, the Center for Neurobiology of Brain Disorders, and the Van Vleet Foundation at the University of Tennessee Health Science Center; Unrestricted Grant from Research to Prevent Blindness; and ED Laboratories. This technology has a US patent number WO 2008064329 A1 20080529 and is owned by the University of Tennessee Research Foundation.

Conflicts of interest

No competing financial interests exist for authors Natalie E. Freeman, Suchareeta Mitra, and Clint W. Abner. Authors Xiang-Di Wang, Renukadevi Patil, Shivaputra A. Patil, William E. Orr, Charles Ryan Yates, Duane D. Miller, and Eldon E. Geisert are listed on the patent entitled 'Substituted tetrahydroisoquinoline compounds for cancer therapy' US Patent number WO 2008064329 A1 20080529. The compound is licensed to ED Laboratories, a wholly owned subsidiary of RX Bio Holding Company. Dr Eldon E. Geisert and Dr Duane D. Miller are founders and board members of ED Laboratories.

References

1 Surawicz TS, Davis F, Freels S, Laws ER Jr, Menck HR. Brain tumor survival: results from the National Cancer Data Base. *J Neurooncol* 1998; **40**: 151–160.
2 Laws ER, Parney IF, Huang W, Anderson F, Morris AM, Asher A, *et al.* Survival following surgery and prognostic factors for recently diagnosed malignant glioma: data from the Glioma Outcomes Project. *J Neurosurg* 2003; **99**:467–473.
3 Stupp R, Hegi ME, van den Bent MJ, Mason WP, Weller M, Mirimanoff RO, *et al.* Changing paradigms – an update on the multidisciplinary management of malignant glioma. *Oncologist* 2006; **11**:165–180.
4 Kleihues P, Sobin LH. World Health Organization classification of tumors. *Cancer* 2000; **88**:2887.
5 Lefranc F, Brotchi J, Kiss R. 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* 2005; **23**:2411–2422.
6 Lefranc F, Sadeghi N, Camby I, Metens T, Dewitte O, Kiss R. Present and potential future issues in glioblastoma treatment. *Expert Rev Anticancer Ther* 2006; **6**:719–732.
7 Stummer W, Reulen HJ, Meinel T, Pichlmeier U, Schumacher W, Tonn JC, *et al.* Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias. *Neurosurgery* 2008; **62**:564–576, discussion 564–576.
8 Assietti R, Olson JJ. Intra-arterial cisplatin in malignant brain tumors: incidence and severity of otic toxicity. *J Neurooncol* 1996; **27**:251–258.
9 Barnett FH, Scharer-Schuksz M, Wood M, Yu X, Wagner TE, Friedlander M. Intra-arterial delivery of endostatin gene to brain tumors prolongs survival and alters tumor vessel ultrastructure. *Gene Ther* 2004; **11**:1283–1289.
10 Hamstra DA, Moffat BA, Hall DE, Young JM, Desmond TJ, Carter J, *et al.* Intratumoral injection of BCNU in ethanol (DTI-015) results in enhanced delivery to tumor – a pharmacokinetic study. *J Neurooncol* 2005; **73**: 225–238.
11 Liu HL, Hua MY, Chen PY, Chu PC, Pan CH, Yang HW, *et al.* Blood–brain barrier disruption with focused ultrasound enhances delivery of chemotherapeutic drugs for glioblastoma treatment. *Radiology* 2010; **255**:415–425.
12 Lopez KA, Tannenbaum AM, Assanah M, Linskey K, Yun J, Kangarlu A, *et al.* Convection-enhanced delivery of topotecan into a PDGF-driven model of glioblastoma prolongs survival, ablates tumor initiating cells and recruited glial progenitors. *Cancer Res* 2011; **71**:3963–3971.
13 Di Tomaso E, Snuderl M, Kamoun WS, Duda DG, Auluck PK, Fazlollahi L, *et al.* Glioblastoma recurrence after cediranib therapy in patients: lack of ''rebound'' revascularization as mode of escape. *Cancer Res* 2011; **71**: 19–28.
14 Zuniga RM, Torcuator R, Jain R, Anderson J, Doyle T, Ellika S, *et al.* Efficacy, safety and patterns of response and recurrence in patients with recurrent high-grade gliomas treated with bevacizumab plus irinotecan. *J Neurooncol* 2009; **91**:329–336.
15 Ali SA, McHayleh WM, Ahmad A, Sehgal R, Braffet M, Rahman M, *et al.* Bevacizumab and irinotecan therapy in glioblastoma multiforme: a series of 13 cases. *J Neurosurg* 2008; **109**:268–272.
16 Galanis E, Buckner JC. Enzastaurin in the treatment of recurrent glioblastoma: a promise that did not materialize. *J Clin Oncol* 2010; **28**:1097–1098.
17 Butowski N, Chang SM, Lamborn KR, Polley MY, Parvataneni R, Hristova-Kazmierski M, *et al.* Enzastaurin plus temozolomide with radiation therapy in glioblastoma multiforme: a phase I study. *Neuro Oncol* 2010; **12**:608–613.
18 Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, *et al.* IDH1 and IDH2 mutations in gliomas. *N Engl J Med* 2009; **360**:765–773.
19 Misra A, Ganesh S, Shahiwala A, Shah SP. Drug delivery to the central nervous system: a review. *J Pharm Pharm Sci* 2003; **6**:252–273.
20 Pardridge WM. The blood–brain barrier: bottleneck in brain drug development. *NeuroRx* 2005; **2**:3–14.
21 Beduneau A, Saulnier P, Benoit JP. Active targeting of brain tumors using nanocarriers. *Biomaterials* 2007; **28**:4947–4967.
22 Mohler ML, Kang GS, Hong SS, Patil R, Kirichenko OV, Li W, *et al.* Discovery of antiglioma activity of biaryl 1,2,3,4-tetrahydroisoquinoline derivatives and conformationally flexible analogues. *J Med Chem* 2006; **49**:5845–5848.
23 Song P, Ma F, Wang F, Wang X, Patil R, Ramagiri S, *et al.* Plasma and cerebrospinal fluid pharmacokinetics of the novel tetrahydroisoquinoline EDL-155 in rats. *Cancer Chemother Pharmacol* 2008; **61**:1037–1044.
24 Ramagiri S, Ma F, Kosanam H, Wang X, Patil R, Miller DD, *et al.* Fast and sensitive liquid chromatography/electrospray mass spectrometry method to study ocular penetration of EDL-155, a novel antitumor agent for retinoblastoma in rats. *J Mass Spectrom* 2009; **44**:786–793.
25 McCarthy KD, de Vellis J. Alpha-adrenergic receptor modulation of beta-adrenergic, adenosine and prostaglandin E1 increased adenosine 3′:5′-cyclic monophosphate levels in primary cultures of glia. *J Cyclic Nucleotide Res* 1978; **4**:15–26.
26 Geisert EE Jr, Stewart AM. Changing interactions between astrocytes and neurons during CNS maturation. *Dev Biol* 1991; **143**:335–345.
27 Kang GS, Wang XD, Mohler ML, Kirichenko OV, Patil R, Orr WE, *et al.* Effects, in an in-vivo model system, of 1,2,3,4-tetrahydroisoquinoline on glioma. *Anticancer Drugs* 2008; **19**:859–870.
28 Nassr M, Wang X, Mitra S, Freeman-Anderson NE, Patil R, Yates CR, *et al.* Treating retinoblastoma in tissue culture and in a rat model with a novel isoquinoline derivative. *Invest Ophthalmol Vis Sci* 2010; **51**:3813–3819.
29 Hay N. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* 2005; **8**:179–183.
30 Megalizzi V, Mathieu V, Mijatovic T, Gailly P, Debeir O, De Neve N, *et al.* 4-IBP, a sigma1 receptor agonist, decreases the migration of human cancer

cells, including glioblastoma cells, in vitro and sensitizes them in vitro and in vivo to cytotoxic insults of proapoptotic and proautophagic drugs. *Neoplasia* 2007; **9**:358–369.
31 Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D, *et al*. A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 2006; **9**:341–349.
32 Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004; **11**:448–457.
33 Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, *et al*. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 2006; **10**:51–64.
34 Turcotte S, Chan DA, Sutphin PD, Hay MP, Denny WA, Giaccia AJ. A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. *Cancer Cell* 2008; **14**:90–102.
35 Yu L, Alva A, Su H, Dutt P, Freundt E, Welsh S, *et al*. Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* 2004; **304**:1500–1502.
36 Abedin MJ, Wang D, McDonnell MA, Lehmann U, Kelekar A. Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ* 2007; **14**:500–510.
37 Bursch W, Ellinger A, Kienzl H, Torok L, Pandey S, Sikorska M, *et al*. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. *Carcinogenesis* 1996; **17**:1595–1607.
38 Ogier-Denis E, Codogno P. Autophagy: a barrier or an adaptive response to cancer. *Biochim Biophys Acta* 2003; **1603**:113–128.
39 Aoki H, Kondo Y, Aldape K, Yamamoto A, Iwado E, Yokoyama T, *et al*. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. *Autophagy* 2008; **4**:467–475.
40 Alva AS, Gultekin SH, Baehrecke EH. Autophagy in human tumors: cell survival or death? *Cell Death Differ* 2004; **11**:1046–1048.
41 Blouin A, Molez S, Pham D, Ayach B, Dussault P, Escher E, *et al*. A novel procedure for daily measurements of hemodynamical, hematological, and biochemical parameters in conscious unrestrained rats. *J Pharmacol Toxicol Methods* 2000; **44**:489–505.