Treating Retinoblastoma in Tissue Culture and in a Rat Model with a Novel Isoquinoline Derivative

Mohamed Nassr,1 XiangDi Wang,1 Suchareeta Mitra,1 Natalie E. Freeman-Anderson,1 Renu Kadevi Patil,2 C. Ryan Yates,2 Duane D. Miller,2 and Eldon E. Geisert1

PURPOSE. To investigate the effectiveness of a novel isoquinoline derivative, EDL-155, in killing retinoblastoma in vitro and in vivo.

METHODS. Dose-response curves were generated in which Y79 retinoblastoma cells tagged with luciferase (Y79-Luc) were treated with serial concentrations of EDL-155. Electron microscopy was used to evaluate the ultrastructural morphology of EDL-155–treated Y79 cells. To determine whether autophagy was induced in EDL-155–treated Y79-Luc cells, staining with acridine orange and LC-3 immunoblot analysis was performed. To evaluate the efficacy of EDL-155 in vivo, Y79-Luc retinoblastoma cells were injected into the vitreous cavity of newborn rats, followed by periocular injections of EDL-155 (20 mg/kg/day) or an equivalent dosage of saline.

RESULTS. EDL-155 appeared to destroy the retinoblastoma cells in vitro with an EC50 of 9.1 μM. EDL-155–treated retinoblastoma cells displayed a lack of viable mitochondria and the presence of autophagosomes wrapped in the characteristic double membranes. Acridine orange staining of EDL-155–treated retinoblastoma cells demonstrated the accumulation of vacuoles, and the immunoblots displayed a shift in molecular weight of LC-3, indicative of incorporation into autophagosome vesicles. In the retinoblastoma animal model, four doses of EDL-155 were delivered over 4 days, which was sufficient to see a significant decrease (P = 0.01) in viable intraocular tumors. Seven of the 25 rats treated with EDL-155 had no detectable living tumor. No significant decrease in viable tumor was observed in control animals.

CONCLUSIONS. EDL-155 appears to eliminate retinoblastoma cells by disrupting mitochondria and inducing autophagy. Local delivery of EDL-155 may be an effective therapy for some types of ocular cancers. (Invest Ophthalmol Vis Sci. 2010;51:3813–3819) DOI:10.1167/iovs.09-5042

From the 1Department of Ophthalmology, College of Medicine, and the 2Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee. 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 funds from Research to Prevent Blindness; and ED Laboratories. Submitted for publication December 10, 2009; revised February 17, 2010; accepted February 19, 2010.

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MATERIALS AND METHODS

Luciferase Assay

Human Y79 retinoblastoma cells carrying the luciferase reporter gene (Y79-Luc) were a generous gift from Michael Dyer at St. Jude Children’s Research Hospital (Memphis, TN). The Y79-Luc cells were maintained in basal medium Eagle (BME; Mediatech, Herndon, VA) supplemented with 10% fetal calf serum (HyClone, Logan, UT). The cells were plated at a density of 10^5 cells/well in a 96-well plate and were cultured in a 37°C incubator containing a humid 5% CO2 atmosphere. Before treatment, the 10% FCS BME was replaced with 2% FCS BME containing either vehicle (dimethyl sulfoxide [DMSO] in normal saline) or serial concentrations of EDL-155 (0.001–100 μM). EDL-155 was synthesized in the laboratory of one of the authors (DDM). Dilutions of EDL-155 were made so that the DMSO concentrations remained at 0.01% (vol/vol). The Y79-Luc cells were incubated for 24, 48, and 96 hours. Luciferin (150 μg/mL) was added to the culture medium, and luminescence was monitored on a microplate reader spectrophotometer (FLX800; μQuant; BioTek, Winooski, VT).

Electron Microscopy

A human retinoblastoma cell line, Y79 (ATCC, Manassas, VA), was maintained as described. Y79 cells were treated with EDL-155 (5 μM) for 19 hours before fixation. Cultured Y79 cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in PBS (pH 7.3). The cells were centrifuged and postfixed in 1% osmium tetroxide for 1 hour at room temperature. Next, the Y79 cells were rinsed in PBS, dehydrated, and infiltrated with Spurr embedding medium. Silver to gold sections were cut on a microtome (Ultracut E; Histostat; Reichert, Morris Plains, NJ), and the sections were examined under an electron microscope (2000EX; JEOL Tokyo, Japan).

LC-3 Immunoblotting

Y79-Luc cells were treated with EDL-155 (0, 5, 20 μM) for 4 and 48 hours. After treatment, the cell lysates were run on an SDS-PAGE gel (8%–20%), and proteins were transferred to a nitrocellulose membrane and probed for Ponceau to ensure equal protein loading (data not shown). LC-3 antibody (Cell Signaling, Danvers, MA) was used at 1:1000 dilution, followed by the secondary antibody, peroxidase conjugated donkey anti-rabbit, at 1:1000 dilution. LC-3 was detected with an enhanced chemiluminescence detection kit (Thermo Scientific Pierce, Rockford, IL) and a digital imaging system (Image Station 4000 MM; Kodak, Rochester, NY).

Detection of Autophagic Vacuoles

Y79-Luc tumor cells were treated with EDL-155 (0, 5, 20 μM) for 4 and 48 hours. The cells were stained with 1 g/mL acridine orange (Molecular Probes-Invitrogen, Carlsbad, CA) for 15 minutes, washed with media, and examined under a confocal microscope (Nikon, Tokyo, Japan) at 60× objective lens magnification.

In Vivo Y79-Luc Model

Y79-Luc cells (100,000) were suspended in Hank’s buffered salt solution and injected into the right eye of 60 Sprague-Dawley rat pups within 24 hours of birth (day 0). On day 10, luciferin (150 mg/kg body weight; Xenogen, Cranbury, NJ) was injected into the peritoneal cavity of each rat, and 30 minutes later the animals were scanned using a digital imaging system (Image Station 4000 MM; Kodak). Of the 60 pups injected with Y79-Luc cells, 35 displayed luminescence in the eye. The animals that presented luciferase-positive eyes were randomly assigned to receive EDL-155 (n = 25) or control vehicle (n = 10). Rats in the treatment group received daily periocular injections of EDL-155 (20 mg/kg; 0.01% DMSO in normal saline) for 4 days. To monitor tumor viability, the animals were scanned on day 1 (before treatment), day 3 (after two treatments), and day 5 (after four treatments). Data were normalized to the initial luminescence coming from eyes on day 1,

FIGURE 1. (A) Structure of 1-biphenyl-1-ylmethyl-1,2,3,4-tetrahydroisoquinoline-6,7-diol hydrochloride (EDL-155). (B–D) Dose-response curves were generated using a luciferase assay at 24 hours (B), 48 hours (C), and 96 hours (D) after EDL-155 treatment.
setting the luminescence units to 100 and monitoring all subsequent changes relative to this starting value. The statistical significance of the difference between the means was determined using the one-tailed Student’s *t*-test.

**Histology**

The rats were anesthetized with 13 mg/kg rompum and 87 mg/kg ketalar perfused through the heart (with saline), followed by 4% paraformaldehyde in PBS (pH 7.3). Both eyes were removed and embedded in plastic and were sectioned at a thickness of 1 μm and stained with toluidine blue. All experimental animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RESULTS**

**Effects of EDL-155 on Y79 Cells In Vitro**

To test the efficacy of an isoquinoline derivative, EDL-155, (Fig. 1A) on retinoblastoma cells, dose-response curves were generated (Figs. 1B–D). Y79-Luc cells were treated with serial dilutions of EDL-155 (0.001–100 μM) for 24, 48, and 96 hours. Luminescence intensity was quantified, which enabled us to monitor the viability of the Y79-Luc retinoblastoma cells by directly observing the effects of the treatment over time. Twenty-four hours after treatment, we observed a decrease in luminescence intensity from the Y79-Luc cells, and the EC₅₀ of EDL-155 was 36.2 μM (Fig. 1B). The data also demonstrated that the luminescence and EC₅₀ concentrations continued to decrease over time with an EC₅₀ of 27.3 μM after 48 hours (Fig. 1C) and an EC₅₀ of 9.1 μM at 96 hours (Fig. 1D). These results suggest that the Y79-Luc cells are relatively sensitive to the cytotoxic effects of EDL-155. A gradual decrease in luminescence over time was observed in these studies, which suggests a decline in adenosine triphosphate production that may be the result of a lack of normal mitochondrial function in the EDL-155–treated Y79-Luc cells.

**Ultrastructural Changes with EDL-155 Treatment**

To evaluate the ultrastructural effects of EDL-155 on mitochondria and the general morphology of the retinoblastoma cells, Y79 cells were incubated with EDL-155 (5 μM) for 19 hours. When the morphology of the retinoblastoma cells was evaluated, the control cells appeared to be normal Y79 cells with intact mitochondria and normal nuclear morphology (Figs. 2A, 2B). In contrast, the Y79 cells treated with EDL-155 displayed distinct differences in the morphology that could be observed even at low magnification (Figs. 2C, 2D). The most prominent features and morphologic differences in the EDL-155–treated tumor cells was the presence of many large vacuoles throughout the cytoplasm and the appearance of severely disrupted mitochondria. When examined at high magnification, the EDL-155–treated Y79 cells appeared to have organelles with double membranes, unlike the control cells (Figs. 2C, 2D). The double-layered organelles appeared to be autophagosomes, a hallmark of autophagy (programmed cell death type 2).20–22 Similar to the control cells, the EDL-155–treated cells displayed relatively normal nuclear morphology. Evidence to support nuclear fragmentation or condensation, hallmarks of apoptosis (programmed cell death type 1),20–22 was not observed. These ultrastructural findings provide important implications when analyzing the mechanistic action of EDL-155 in retinoblastoma cells. Although EDL-155 appears to disrupt the mitochondria in retinoblastoma cells, the morphologic hallmarks observed do not appear related to apoptosis. Instead the results show that EDL-155 induces autophagy in retinoblastoma cells.

**Induction of Autophagy in EDL-155–Treated Y79 Cells**

The ultrastructural changes observed in the EDL-155–treated retinoblastoma cells led us to examine the potential role of autophagy. One of the hallmarks of autophagy is a shift in the molecular weight of microtubule-associated protein 1 light chain 3 (LC-3).23 LC-3 is critical for amino acid starvation-induced autophagy and is associated with the autophagosome membrane.23 If autophagy occurs within the retinoblastoma cells, there should be a characteristic shift in the molecular weight of LC-3 and, thus, the formation of autophagosomes.

To determine whether EDL-155 induced autophagy in retinoblastoma cells, Y79-Luc cells were treated with EDL-155 (0, 5, 20 μM) for 4 and 48 hours and were probed for LC-3. As shown in Figure 3, probing with LC-3 demonstrated a characteristic shift in molecular weight of LC-3 from LC-3 I to LC-3 II. The shift in molecular weight of LC-3 indicates that the cells treated with EDL-155 were undergoing autophagy.

To verify that autophagy was induced in the EDL-155–treated retinoblastoma cells, Y79 cells were stained with acridine orange. Acridine orange stains the cytoplasm and nucleolus bright green, whereas the acidic compartments (autophagosomes) fluoresce bright red or orange.24,25 In Figure 4, the photomicrographs display Y79-Luc tumor cells treated with EDL-155 (0, 5, 20 μM) for 4 and 48 hours. In general, these results displayed an increase in red/orange fluorescence when the Y79-Luc cells were treated with EDL-155, indicating the accumulation of acridine orange in the acidic compartments of the Y79-Luc tumor cells. Visualization of these acidic organelles confirmed the formation of autophagosomes in EDL-155–treated retinoblastoma cells and the induction of autophagy. Therefore, three independent approaches—
electron microscopy, shifting molecular weight of LC-3,
acridine orange staining—the results demonstrated that EDL-
155 induced autophagy in the Y79-Luc cells.

**Effect of EDL-155 In Vivo**

To test the effectiveness of EDL-155 as an in vivo therapy, we
used an animal model in which human retinoblastoma cells,
Y79-Luc, were injected into the eyes of newborn rats. The
luciferase tagged cells allowed us to monitor the growth of the
intraocular tumor by imaging luminescence from the eye.

Representative scans from a control animal and an EDL-
155-treated animal are shown in Figure 5. Intraocular lumines-
cence from the control rat increased from day 1 to day 3 (Figs.
5A, 5B) and remained elevated at day 5 (Fig. 5C), indicating
that there was an increase in the intraocular tumor size. In the
EDL-155-treated animal, little change in intraocular lumines-
cence was observed after two doses (day 3 scan; Fig. 5E).
However, after four doses (day 5 scan; Fig. 5F), there was a
significant decrease in intraocular luminescence. Of the 25 rats
treated with EDL-155, 23 had less luminescence coming from
the tumor-bearing eye after four treatments than was present
before treatment. Furthermore, seven of the 25 treated animals
had no detectable intraocular luminescence after four doses of
EDL-155.

These results were quantified and are presented in Figure 6.
The changes occurring in luminescence within the animals
were standardized, as described in Materials and Methods. On
day 1, both groups had a relative value of 100 with no error
bars. Because each animal was set to a value of 100, there was
no variability at this point. On day 3, the relative amount of
luminescence increased in both groups. There was, on aver-
age, less luminescence from the treated group, but at this early
time point this difference was not significant. By day 5, there
was a dramatic and significant difference between the treated
The mean luminescence of the control group appeared to decrease; however, there was not a significant difference from that observed on day 3. In the EDL-155-treated group, there was a significant decrease in luminescence. The mean luminescence of the EDL-155-treated group was significantly decreased on day 5 compared with day 3. Furthermore, there was a significant decrease relative to the values seen in the control group. Because of the rapid growth of the tumors in the control animals, the in vivo experiments were terminated on day 5. These data establish that EDL-155 caused a significant reduction in luminescence from the transplanted Y79 cells, and the data suggest a reduction in intraocular tumor size.

The animals appeared to tolerate the EDL-155 treatment. There was no significant difference in weight gain between treated animals and control animals. The control animals gained an average of 5.4 g over the treatment period, whereas treated animals gained an average of 4.6 g. These amounts represent a 24.2% weight gain for the control group and a 23.6% weight gain for the treated group. No differences were observed in the animals' motor skills or general appearance. In addition, we did not observe any local inflammation around the eyes of the EDL-155–treated animals compared with the eyes of the control animals.

To confirm that the intraocular tumor was affected by EDL-155 treatment, we examined the morphology of the tumor within the eye. Histologic sections demonstrated that the number of viable tumor cells was approximately equal to the luminescence observed at day 5 (Fig. 7). In the control animals, there was a considerable amount of tumor within the vitreous chamber, and in some cases the tumor was invading the retina (Fig. 7A). Occasionally, the tumor cells were observed invading the anterior and posterior chambers and penetrating the lens. When there was no luminescence, the Y79-Luc cells appeared to be dead (Fig. 7B). Many of the nonviable Y79-Luc cells were filled with large vacuoles or were in an advanced state of degradation. Thus, the morphology of the intraocular tumor treated with EDL-155 indicates that EDL-155 killed the retinoblastoma cells.

**DISCUSSION**

To study the effects of EDL-155 in vivo, we chose a retinoblastoma rat model developed by Michael Dyer’s group. The model is well characterized and very useful for short-term studies. Although this model does not perfectly recapitulate human retinoblastoma, it does allow us to directly test the effectiveness of treating transplanted retinoblastoma with EDL-155. The rat pups began receiving EDL-155 treatment in the second week of life, before the blood-retinal barrier was fully developed. In humans, the average age of detection is 18 months, well after the blood-retinal barrier is formed. Thus, in this model, EDL-155 would have greater access to tumor cells than would be expected in humans. Furthermore, the rat has a very small eye compared with the human eye, and the concentration of drug within the limited space of the rat vitreous may be considerably higher than in the human. In human eyes, the difference in pharmacokinetics may make delivery of the drug difficult, and the intraocular drug concentration may not be sufficient at the site of tumors.

The results of the retinoblastoma animal model indicated a significant decrease in the luminescence emitted from the eyes of the EDL-155–treated animals compared with that emitted from control animals. Of the 25 animals given EDL-155 treat-
ment, seven had no luminescence after four treatments over 4 days. This was not observed in the animals treated with vehicle alone. This retinoblastoma model provided information that may prove beneficial in treating human retinoblastoma or other ocular cancers.

In previous studies,16,17 we found that EDL-155 appeared to kill cultured glioma by sending the cells into autophagy. Similar to the EDL-155–treated glioma, the EDL-155–treated retinoblastoma displayed large vacuoles in the cytoplasm, and the nuclei appeared to be intact. When we examined the cells treated with EDL-155 at the electron microscopic level, normal mitochondria were absent while organelles wrapped in double membranes (autophagosomes) were present. These changes were not observed when we examined primary cultures of astrocytes treated with EDL-155.18 The lack of nuclear condensation or fragmentation indicated that the cells did not die by apoptosis. In addition, cytoplasmic DNA laddering was absent in cells treated with EDL-155 (unpublished observation, 2005). All these observations indicate that autophagy may be a potential mechanism of action of EDL-155. It is important to note that these results do not completely eliminate the possibility that apoptosis is not involved at some point; however, as discussed, results do conclude that autophagy is induced in retinoblastoma cells treated with EDL-155.

Support of autophagy as the mechanism of cell death comes from the presence of double membranes surrounding many of the vacuoles in the EDL-155–treated cells. These double membranes are a hallmark of autophagy.29 To provide additional evidence of autophagy as a mechanism of action, we examined two techniques to identify autophagy, LC-3 immunoblots and acridine orange staining. When cells enter autophagy, acidic autophagosomes are formed. In this process, LC-3 is modified, enters the autophagosomes, and undergoes a characteristic shift in molecular weight. In the present study, we observed a shift in LC-3 molecular weight only when cells were treated with EDL-155, providing further support that the cells were entering autophagy. The final evidence of autophagy comes from cells stained with acridine orange, which highlights newly formed acidic autophagosomes. We observed the red/orange staining of small organelles when the retinoblastoma cells were treated with EDL-155. Collectively, these data indicates that autophagy is a potential mechanism of action of EDL-155.

Autophagy has been implicated in cancer initiation and progression. Work is still being conducted to define the functional role of autophagy in cancer. Many treatments have the ability to induce autophagy (for a review see Ref. 30), including arsenic trioxide,31,32 imatinib mesylate,33 temozolomide,34,35 rapamycin,36-38 dopamine,39,41 temozolomide,34,35 Vitamin D analog,42,45 resveratrol,46 and the sphingolipid ceramide.49,50 Several studies have identified small molecules that induce autophagy in PC3 prostate cancer51 or in renal cell carcinoma.52 In the present study, we have demonstrated that EDL-155 induces autophagy in retinoblastoma cell lines. We are examining the molecular interactions of EDL-155 in vitro to define the precise mechanism of action and the molecular target of EDL-155.

The potential benefits of using EDL-155 to treat local cancers are its rapid clearance (342.5 § 49.9 ML/min/kg) and high volume of distribution (13,005 § 1187 ML/kg).18 After intravenous administration, EDL-155 undergoes extensive metabolism in the liver by cytochrome P-450 and in peripheral tissues by catechol-O-methyltransferase. Periocular administration increases the availability of the compound to the eye while decreasing systemic toxicity. We have tested only one concentration of EDL-155 (20 mg/kg).

In our animal model, EDL-155 is an effective treatment for retinoblastoma. Our data clearly indicate that this mono-therapy is capable of producing significant tumor reduction. Most clinical protocols and preclinical research studies in animal models of retinoblastoma have used combinations of drugs. For example, one therapy uses topotecan with carboplatin of retinoblastoma (transgenic and xenograft), which has been shown to be superior to the combination of vincristine, carboplatin, and etoposide.53 Recently, nutlin-3, an inhibitor of MDM-p53 interaction in cells, was proposed for use in treating retinoblastoma. It was shown to be effective when used in combination with topotecan in a murine model of retinoblas-
toma.54 Each of these compounds is associated with toxic side effects. Our initial studies have shown that EDL-155, used alone, provides a relatively effective therapy with no observable toxic side effects. Obviously the use of EDL-155 in combination with other chemotherapeutic agents may prove to be a more effective treatment, and future studies will examine the potential synergistic interactions of EDL-155.

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