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**Original**

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**Background:** Glioblastoma is a highly aggressive primary malignant brain tumor. New treatments, especially ones that can eradicate subpopulations with stem-cell-like properties, are currently being explored. In vitro, epirubicin has demonstrated toxicity towards such populations. However, epirubicin fails to readily cross the blood-brain barrier. Furthermore, its efficacy against stem-like tumor cells growing within the neural tissue has not been fully addressed. Polymeric micelles are an advanced system developed to enhance drug delivery into tumors.

**Methods:** We have investigated the effect of epirubicin micelles against murine glioma initiating cells (GICs) in vitro and ex vivo.

**Results:** In vitro, epirubicin micelles inhibited the sphere growth of GICs in a dose-dependent manner. Furthermore, they also inhibited the growth of GIC-based tumors in ex vivo cultured brain slices from the tumor-bearing mice. This effect was inversely proportional to the tumor size at the beginning of the treatment.

**Conclusions:** Our results suggest that epirubicin micelles are effective against murine glioma cells with stem cell properties and their use should be initiated at an early stage.

**Key Words**: epirubicin, epirubicin micelle, glioblastoma, glioma initiating cells, microglia

## Introduction

Glioblastoma (GBM) is the most aggressive primary malignant brain tumor in adults. Currently, even the best available therapy, which includes surgical resection, temozolomide administration, and adjuvant radiotherapy,[1] is not curative. In malignant tumors, one of the major causes of a poor prognosis is the existence of cells with stem-like properties. Such cells can arise from the malignant transformation of normal tissue stem cells and act as tumor-initiating cells. Furthermore, they can give rise to both pluripotent and differentiated daughter cells, thereby sustaining and propagating primary and recurrent tumors.[2-5] In addition, tumor cells with stem-like properties exhibit an inherent resistance to therapy and are thus considered the true targets for novel therapies.[5,6] In the case

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of malignant brain tumors, cells with stem-like properties are important in all stages, including tumor initiation, maintenance, and recurrence.[7-9] However, each of these three populations might genetically and/or phenotypically differ[10] and therefore warrant specific targeting analyses.

Anthracyclines are chemotherapeutic agents that are widely used in the treatment of solid tumors, such as ovarian and breast cancer.[11-14] Recently epirubicin, an anthracycline, has been identified as one of the FDA-approved drugs that displayed a strong toxic effect against primary tumor cells isolated from surgically-resected glioblastoma samples and maintained as stem-cell cultures in vitro.[15] However, previous studies have reported that epirubicin does not readily cross the blood-brain barrier (BBB).[16]

In recent years, several drug delivery systems have been developed to enhance drug penetration and accumulation into tumors. In brain malignancies, such systems, sometimes further modified, are also expected to overcome the obstacle of the BBB. This can be achieved either by formulations which enhance transport across the BBB, by combination with transient opening of the BBB using chemical or physical agents, or by taking advantage of the already disrupted BBB to ensure specific accumulation of the drugs.

Polymeric micelles exploit the phenomenon known as enhanced permeability and retention effect (EPR). The enhanced permeability of abnormal tumor vessels allows the micellar delivery into tumors, while poor drainage prevents them from being washed out.[17-19] Furthermore, micelles are designed to release their contents in response to low pH, facilitating drug release in the tumor cell lysosomes.[19-21] Epirubicin micelles have been developed and are currently undergoing a phase 1 clinical trial in advanced solid tumors and soft tissue sarcoma. However, their efficacy against glioma cells with stem cell properties has not yet been evaluated.

Here, we investigated the effect of epirubicin micelles in a murine glioblastoma model, with a focus on glioma initiating cells (GICs) and the early stage of tumor formation.

## Materials and Methods

### Cell culture

GICs were established from *Ink4a/Arf* $^{-/-}$ NSCs/NPCs as previously described.[22] The culture medium (NSM) consisted of serum-free DMEM/F12 (Wako, Osaka, Japan) supplemented with recombinant human epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA), basic fibroblast growth factor (PeproTech) each at 20 ng/ml, 200 ng/ml heparan sulfate (Sigma Aldrich, MS, USA), and B27 supplement without vitamin A (Invitrogen, Carlsbad, CA, USA). In all experiments, GICs were dissociated to obtain single-cell suspensions before use.

### Chemicals and reagents

Epirubicin micelles (NC-6300) were synthesized at NanoCarrier Co., Ltd (Kashiwa, Japan) as previously described.[21, 23] Epirubicin micelles were provided by Kowa (Aichi, Japan). Epirubicin was purchased from Cosmo Bio (Tokyo, Japan). Temozolomide was purchased from LKT Laboratories (St. Paul, MN, USA).

### Micelle preparation

Epirubicin micelles were first dissolved in ultrapure water to allow the spontaneous formation of micelles (5 μmol of epirubicin/L). The micelles were then passed through an ADVANTEC 0.20 μm filter (ADVANTEC, Tokyo, Japan) and frozen at −80°C until use.

### Sphere growth assay

Cells were plated in ultra-low binding 96-well plates (Corning, Corning, NY, USA) at a density of 500 cells per well, with or without the addition of drugs at indicated concentrations. The plates were incubated at 37°C and 5% $CO_2$/95% humidified air and cultured for 7 days. Image acquisition and analysis were performed with Biorevo BZ9000 (Keyence, Osaka, Japan) 7 days after plating, 6 wells for each group. Sphere images were obtained at the plane of maximum size.

### Cell survival assay

Cell viability was measured using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt] assay.

GICs were seeded in 96-well plates at a density of 5 × $10^3$ cells per well and incubated with or without drugs at indicated concentrations for 24-48 h. Subsequently, 10 μl of WST-8 solution was added to each well, and the plates were incubated for an additional 1 h at 37°C. A plate reader (Perkin Elmer, Waltham, MA, USA) was used to measure the absorbance of each well at 450 nm. The percentage of viable cells was calculated by comparison with the control wells. In each experiment, 5 wells were quantified for each group.

**Orthotopic implantation experiments**

All animal experiments were approved by the Animal Care and Use Committee of the Keio University School of Medicine, Tokyo, Japan (No. 11020). Intracranial implantations were performed as previously described.[22] Briefly, 6-week-old female C57BL/6J mice were anesthetized and placed in a stereotactic frame. GICs suspended in Hank's balanced solution were injected into the right hemisphere at the following coordinates: 2 mm lateral and 1 mm anterior to the bregma, 3 mm below the surface of the brain. The injection was performed over 2 min, with an additional 2-min pause before removing the syringe. The hole was sealed with bone wax and the scalp was closed using an autoclip applicator.

**Explant culture, drug treatment, and imaging**

Brains of C57BL/6 mice implanted with GICs were dissected and cut into 150-200 μm coronal slices using a Leica VS1200 vibratome (Leica, Wetzlar, Germany). Brain explants were cultured on Millicell-CM culture plate inserts (Merck Millipore, Billerica, MA, USA) in glass-bottom dishes (IWAKI, Shizuoka, Japan). The slices were maintained in NSM as previously described.[24] NSM with or without the indicated drugs was changed daily. Serial images were acquired with a Fluoview FV10i inverted confocal microscope (Olympus, Tokyo, Japan). All images in one series were acquired the same settings: laser excitation wavelength 473 nm, transmissivity 30% (for slices from mice n#1, n#2) and laser excitation wavelength 473 nm, transmissivity 50% (for slices from mice n#3, n#4). Images are presented as acquired at the initial settings, without corrections for the increase in background signal due to tissue autofluorescence or free epirubicin fluorescence.[25] Composite images of 4 adjacent fields, with a Z-stack of 5 images covering a depth of 40 μm, were automatically generated and the length and width of the tumor areas were measured with the Fluoview Software v4.2. At the end of the experiment, brain slices were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned to a thickness of 4 μm. Sections were stained with a rabbit polyclonal antibody to Iba-1 (013-27691, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan).

**Statistical analysis**

Quantitative data are presented as mean ± SD from each experiment, representative of at least three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A p value $< 0.05$ was considered statistically significant.

## Results

To analyze the effect of epirubicin and epirubicin micelles on GIC survival, proliferation, and invasion, we used an *Ink4a/Arf* $^{-/-}$, *GFP* and *Hras*$^{V12}$ - expressing GIC cell line with a medium proliferation- and high infiltrative-potential. Cell survival assays indicated that short-term treatment with 250 μM temozolomide reduced the number of viable GICs by approximately 70%, similar to a previous report in a more proliferative, but less invasive GIC line with the same genetic background.[24] In contrast, epirubicin and epirubicin micelles induced a concentration-dependent decrease in the number of viable cells at concentrations as low as 1-1,000 nM (**Figure 1**), with a 50% reduction in the number of viable cells at concentrations lower than 100 nM (**Figure 1**).

Next, we investigated the drugs in proliferating GICs grown in a 3D sphere culture, continuously exposed to the drugs for a period of one week. Both free epirubicin and epirubicin micelles inhibited sphere growth at concentrations as low as 1 nM (**Figure 2**). The effect of epirubicin was slightly stronger than that of micelles expected to release an equivalent concentration; however, the micelles exhibited a dose-dependent effect and reduced sphere size by approximately 90% at 100 nM (**Figure 2**). Temozolomide was less effective even at mi-

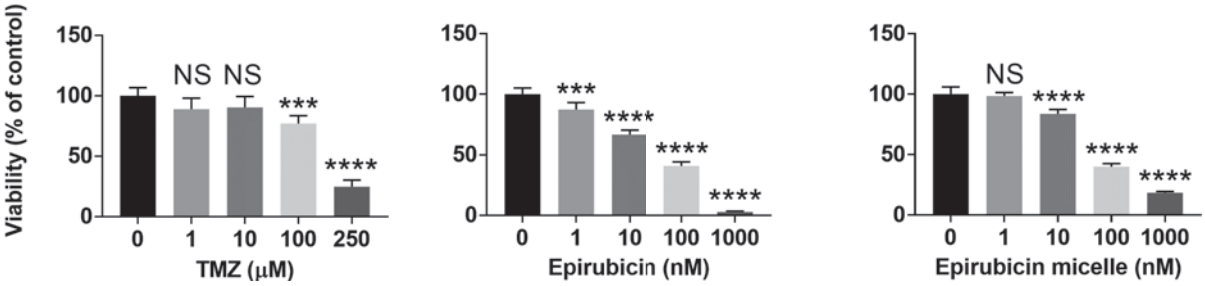


**Figure 1** GIC survival after treatment with temozolomide (TMZ) and epirubicin. A total of 5,000 cells were seeded in each well in 100 μl NSM and treated with drugs at the indicated concentrations. Quantification data from a representative experiment are shown. NS, not significant; ***p<0.001; ****p<0.0001.

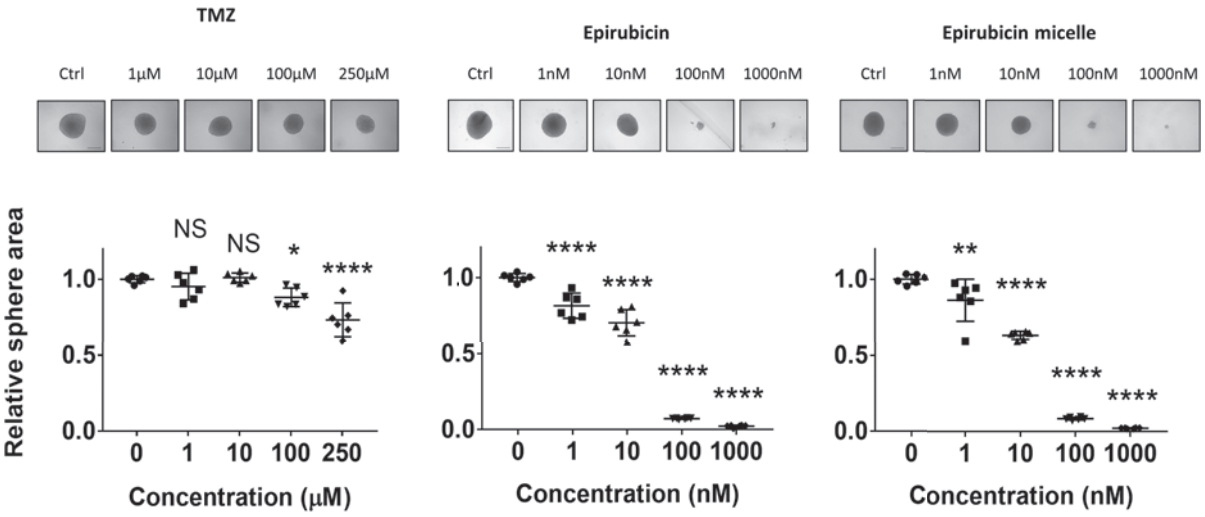


**Figure 2** Sphere growth of drug-treated GICs. A total of 500 cells were seeded in each well in 100 μl NSM and treated with drugs at the indicated concentrations. Images were acquired on day 7. Quantification of the sphere area relative to the control is shown for a representative experiment. NS, not significant; *p<0.05; **p<0.01; ****p<0.0001. Scale bar: 300 μm.

cromolar concentrations (**Figure 2**).

Finally, in order to assess the drugs on tumors in their native microenvironment, the treatment was performed using cultured coronal brain slices from mice injected with the GICs. Tumors formed in syngeneic mice and grown in slice culture exhibited cellular pleomorphism, mitoses, and giant cells (**Figure 3**, lower left panel) and were highly invasive (**Figure 3**, left upper and lower panels). They also displayed an accumulation of Iba-1-positive microglia around the tumor mass, as well as small invasive foci (**Figure 3**, right upper and lower panels). Epirubicin micelles (3 μM) significantly inhibited tumor growth in tumors with initial diameters of less than 1 mm (**Figure 4**). In contrast, the inhibitory effect was less pronounced in tumors which exceeded 1 mm (**Figure 5**). Notably, epirubicin micelles did not inhibit tumor cell invasion and were slightly less effective than free epirubicin, independent of tumor size (**Figure 4** and **Figure 5**).

## Discussion

Our results demonstrated that epirubicin and epirubicin micelles inhibited the growth of the genetically engineered murine GICs both in vitro and ex vivo.

The results showing the efficacy of epirubicin against GICs in vitro contributed to existing data and demonstrated the toxicity against cells with stem-cell-like properties.[15] Importantly, our results further showed that the toxic effect of epirubicin in GICs was preserved in the

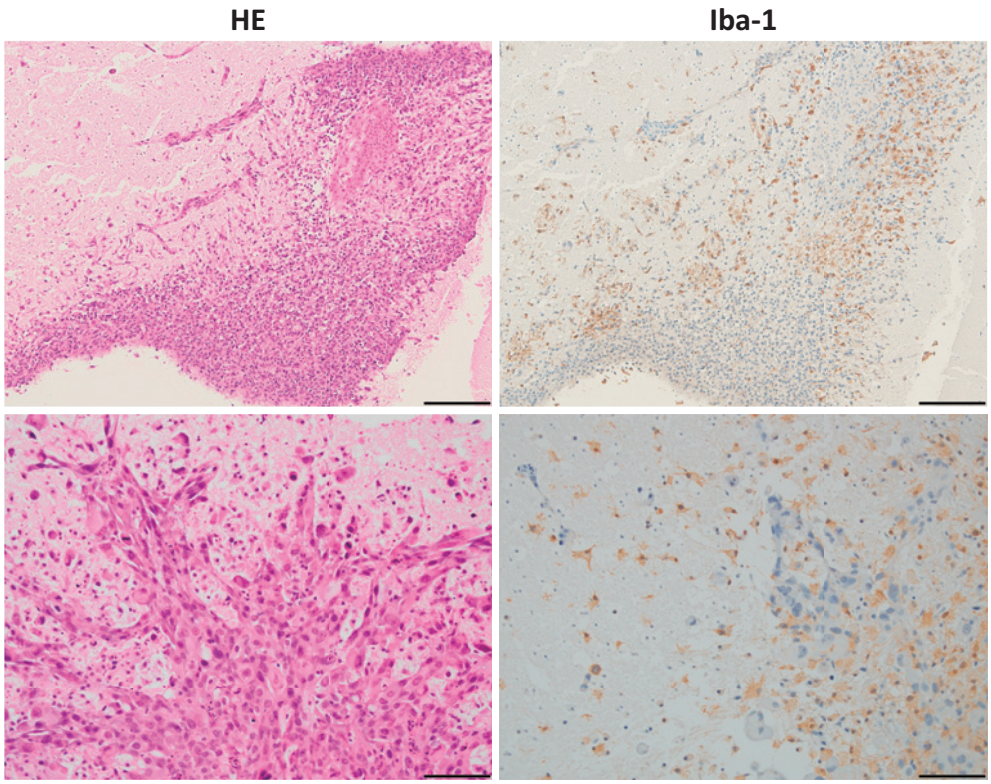


**Figure 3** Histopathological analysis of GIC-based tumors in cultured brain slices. Untreated slices cultured in NSM were stained with hematoxylin-eosin (HE; left panels, low and high magnification) and antibody to microglial marker Iba-1 (right panels, low and high magnification). Scale bar: upper panels, 200 μm; lower panels, 100 μm.

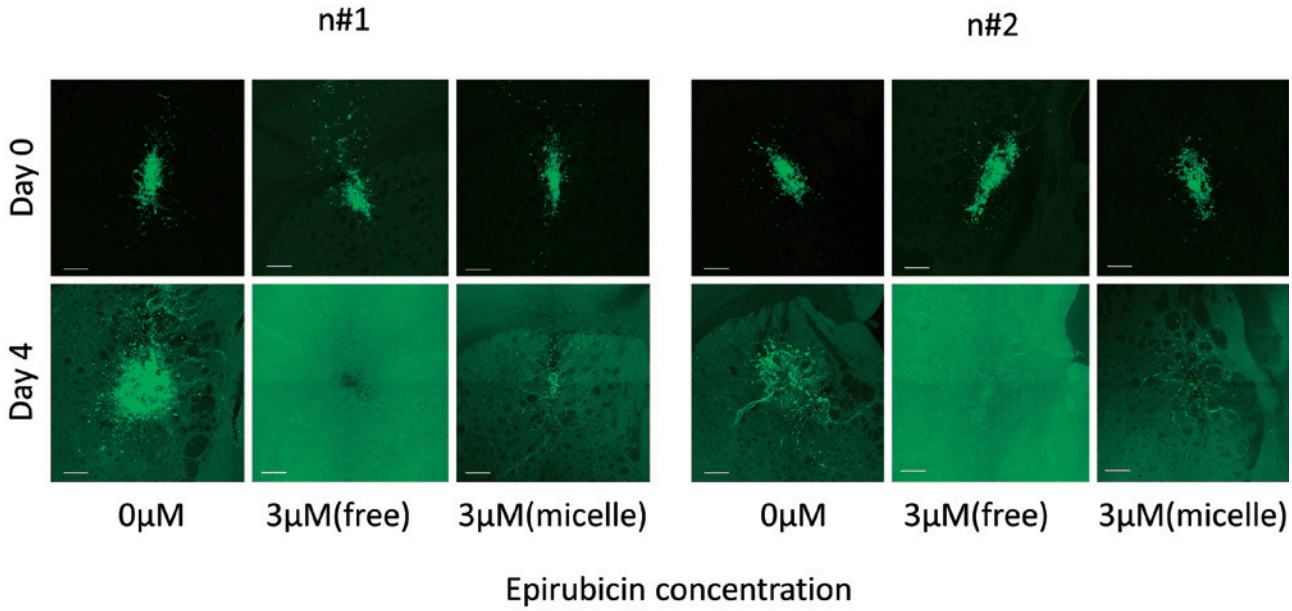


**Figure 4** The effect of epirubicin on cultured coronal brain slices. Slices were treated with the indicated drug concentrations for 4 days. Representative images for tumors with an initial maximum length of less than 1 mm. Scale bar: 300 μm.

micellar formulation. Since delivery in the form of micelles is expected to increase the intratumoral concentration and decrease systemic side-effects, these results highlight the potential of epirubicin micelles against most

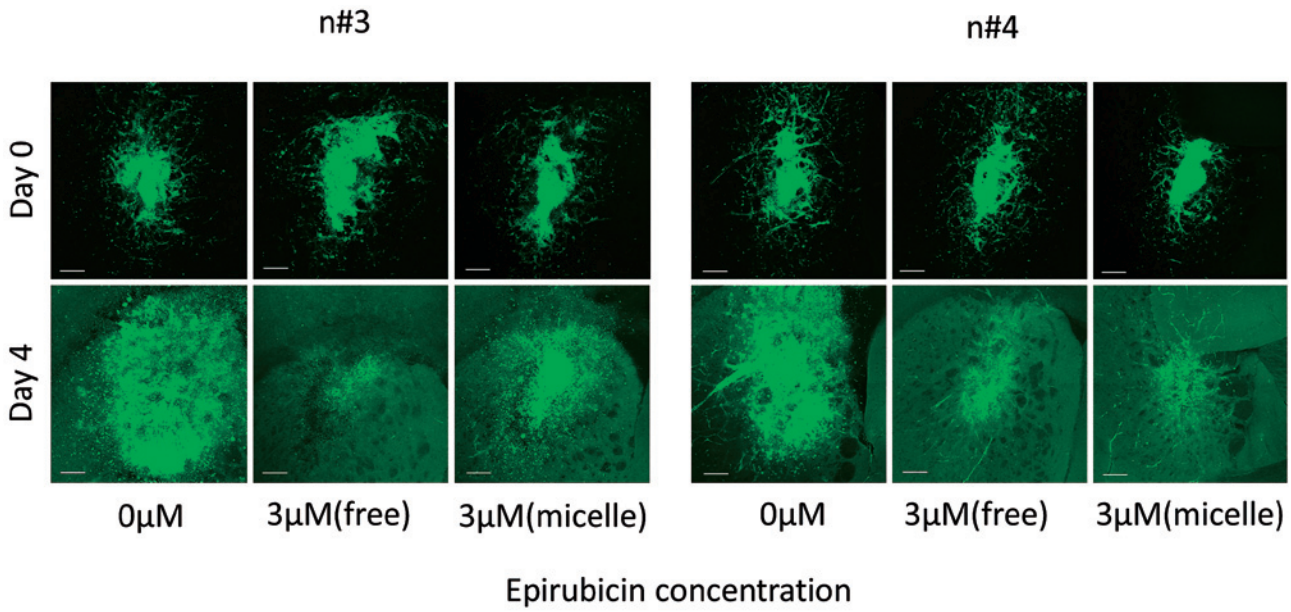


**Figure 5** The effect of epirubicin on cultured coronal brain slices. Slices were treated with the indicated drug concentrations for 4 days. Representative images for tumors with an initial maximum length of more than 1 mm. Scale bar: 300 μm.

malignant cells contributing to glioma initiation, the GICs.

We also observed that epirubicin micelles reduced the growth of GIC-based tumors in brain slice explants. The induced murine GIC model, based on the overexpression of H-Ras$^{V12}$ in NSCs with a deletion in the *Ink4/Arf* locus, exhibits pronounced cellular pleomorphism, endothelial proliferation, pseudopalisading, and infiltration of the tumor cells, resulting in pathological features closely resembling those associated with glioblastoma.[22] The GIC-based tumors, formed by implantation of GICs into immunocompetent mice, are thus highly aggressive. The efficacy of epirubicin and epirubicin micelles against GIC-based tumors warrants their further evaluation in this model.

Notably, our results also suggested that, in GIC-based tumors, the effect of micelles seemed to be inversely proportional to the size of the tumors at the start of treatment. This result needs to be validated in vivo, using animals with intracranially implanted-GICs. A careful assessment of the number and permeability of blood vessels, along with an analysis of the tight junctions and the BBB in tumors of different sizes will also be needed to find the best therapeutic window for micelle administration.

In our model, the efficacy of the epirubicin micelles was slightly weaker than that of free epirubicin. This finding is consistent with previous reports and has been attributed partially to a more rapid uptake of the free drug and a more gradual release of the compound from micelles.[26] In addition, we found a higher number of single cells which have invaded into the normal parenchyma in the micelle-treated slices. This could also be due primarily to the time lag before the release of the drug from micelles, time lag during which GICs continue to infiltrate into the normal brain. Studies exposing the explants to a single dose of drugs and with longer periods of observation are needed to further investigate these aspects.

Targeting infiltrative cells is also an important issue in vivo, where the micelles are expected to act mainly through EPR, rather than directly crossing the BBB, thus having the strongest effect against cells within the tumor mass. Newer formulations have cyclic Arg-Gly-Asp (cRGD) peptides on the surface of the micelles, peptides which allow binding to integrins overexpressed on endothelial cells and/or GBM cells. Such micelles have shown a higher intratumoral penetration in GBM spheroids and xenograft models[26] and might be further exploited to target tumor cells which have invaded along blood vessels.

Furthermore, the efficacy of epirubicin micelles could also be enhanced by combination therapies. For instance, in mouse models of colon and pancreatic cancer, epirubi-

cin micelles are part of a promising treatment strategy called sonodynamic therapy (SDT).[27] SDT involves the delivery of high-intensity, focused ultrasound, with epirubicin micelles as the sonosensitizer agent. This minimally invasive combination has shown better results than the delivery of epirubicin micelles alone, indicating a promising method of enhancing their effect.[27, 28]

## Conclusion

In conclusion, our results demonstrate that epirubicin micelles are effective against GICs in vitro and during the early stages of tumor formation in cultured brain slices. They also suggest that the use of epirubicin micelles should be initiated as early as possible.

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**Conflicts of Interest**: The authors have no conflicts of interest directly relevant to the content of this article.

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