Imidazole antifungal compounds exert their antipathogenic effects through inhibition of sterol biosynthesis. These drugs have also recently been identified as candidate anticancer agents for several solid tumors including glioblastoma. However, their effects on glioma-initiating cells (GICs), i.e., glioma cells with stemlike properties that are able to initiate tumors, remain unclear. Consequently, we examined the effects of the optically active imidazole compound luliconazole on mouse GICs and GIC-based tumors. Luliconazole impaired in a concentration-dependent manner the growth of spheres formed by GICs in vitro. In contrast to the inhibitory effects of ionizing radiation and temozolomide on sphere growth, that of luliconazole was attenuated by the addition of exogenous cholesterol. Exposure to luliconazole of brain slices derived from mice with orthotopic GIC implants for 4 days in culture resulted in a marked increase in the number of tumor cells positive for cleaved caspase-3, but without a similar effect on normal cells. Furthermore, in brain slices, luliconazole inhibited the expansion of GIC-based tumors and the parenchymal infiltration of tumor cells. Our findings therefore indicate that luliconazole effectively targets GICs, thereby providing further support for the antitumorigenic effects of imidazole antifungal compounds.

**Keywords:** luliconazole, glioma-initiating cell, glioma, metabolism

**Introduction**

Drug repositioning offers the possibility of reducing the cost and time required for the development of new therapeutic agents for types of cancer with few current treatment options. Antifungal drugs of the azole class, including imidazoles and triazoles, prevent synthesis of the fungal cell wall by inhibiting the activity of lanosterol 14α-demethylase (CYP51), which catalyzes the conversion of lanosterol to 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol and thereby ultimately gives rise to the cell wall component ergosterol.1,2 In humans, CYP51 is essential for cholesterol biosynthesis, but the higher affinity of antifungal azoles for the fungal ortholog3,4 has been exploited to determine doses of these agents that confer a maximal therapeutic benefit with minimal side effects. Antifungal azoles have also recently emerged as candidate anticancer agents. The imidazole compound clotrimazole has been found to inhibit the proliferation or viability of melanoma, Lewis lung carcinoma, and breast cancer cell lines.5–7 Ketoconazole has also manifested antitumor effects in hepatocellular carcinoma and prostate cancer and is currently under evaluation in clinical trials for prostate cancer.8–10 The effects of imidazole antifungal agents on glioblastoma, the most lethal primary brain malignancy, have also

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been investigated. Clotrimazole, ketoconazole, miconazole, and econazole were found to reduce the viability of multiple glioma cell lines in vitro. However, glioblastomas include fractions of cells with stemlike properties that contribute to the initiation or propagation (or both) of these tumors. To achieve an improvement in prognosis for glioblastoma, repositioned drugs would also need to be effective against such stemlike cells. Ketoconazole and miconazole reduce the number of glioblastoma stemlike cells in vitro. However, the activities of most imidazole antifungals against glioma-initiating cells (GICs) remain unclear. Consequently, we investigated the effects of luliconazole, an imidazole previously untested for anticancer properties, on mouse GICs in vitro and in brain explants.

Materials and Methods

Cell culture

Neural stem-progenitor cells isolated from Ink4a/Arf-null mice were genetically engineered to express the oncoprotein H-RasV12 and either dsRed or green fluorescent protein as a fluorescent marker (RasR and RasG cells, respectively), as previously described. The transformed cells, which function as GICs, were cultured in serum-free Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Wako, Osaka, Japan) supplemented with recombinant human epidermal growth factor and basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), heparan sulfate (Sigma-Aldrich, St. Louis, MO, USA), and B27 supplement without vitamin A (Invitrogen, Carlsbad, CA, USA).

Reagents

Luliconazole was provided by Nihon Nohyaku (Tokyo, Japan) and was dissolved in dimethyl sulfoxide (Wako). Synthetic cholesterol supplement (S5442) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and B27 supplement without vitamin A (Invitrogen, Carlsbad, CA, USA).

Sphere formation assay

GICs were seeded in low-binding 96-well plates (Corning, Corning, NY, USA) at a density of 100 cells per well, with or without the indicated drugs. Images of the resulting spheres were acquired with a Biorevo BZ9000 inverted microscope (Keyence, Osaka, Japan) at 7 days after plating. The sphere area was quantified using the Hybrid Cell Count function of the Keyence analysis software.

Cytotoxicity assay

Cells were plated at a density of 5000 per well in 96-well plates and cultured for 24 h before the addition of luliconazole at various concentrations. After culture for an additional 24 h, the number of live cells was determined using formazan-based Cell Count Reagent SF (Nacalai Tesque, Kyoto, Japan).

Explant culture and analysis

All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Animal Care and Use Committee of Keio University School of Medicine (No. 11020). RasR cells (5 × 10⁴) or RasG cells (1 × 10⁵) were injected stereotactically into the right forebrain of female C57BL/6 J mice at 6 weeks of age, as previously described. Within 7 days of cell injection, the brain was isolated and transferred to ice-cold DMEM-F12 containing 1% penicillin-streptomycin mix (Nacalai Tesque). Coronal slices (200 μm thick) of the brain were prepared as previously described and were cultured at the air–fluid interface on Millicell-CM culture inserts (Merck Millipore, Billerica, MA, USA) placed in 3.5-cm glass-bottom dishes (AGC Techno Glass, Shizuoka, Japan). For drug treatment, the brain explants were incubated in medium supplemented with luliconazole or vehicle, with the medium being refreshed daily. Given that the effect for equimolar drug concentrations is weaker in brain explants than in cultured cells, the final drug concentration was determined based on preliminary experiments starting at 100 µM luliconazole. Imaging of the tumors formed in the brain slices was performed on days 0, 2, and 4 with an FV10i inverted confocal microscope (Olympus, Tokyo, Japan). Multifield composites were generated and processed with Fluoview software. After the final imaging, the explants were immersed in 4% paraformaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm for immunohistochemical analysis with rabbit polyclonal antibodies to cleaved caspase-3 (9661S; Cell Signaling Technology, Danvers, MA, USA).

Statistical analysis

All experiments were performed at least three times. Quantitative data are presented as means ±SDs and were compared using one-way analysis of variance (ANOVA) followed by Dunnett’s or Tukey’s post hoc test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered statistically significant.
Results

Luliconazole inhibits sphere growth and viability of GICs in vitro

The ability to grow as spheres in floating culture is one of the characteristics of brain tumor-initiating cells and correlates with tumorigenic capacity. First, we investigated the effect of luliconazole on the ability of GICs to grow as spheres. At concentrations of 20 and 30 µM, luliconazole inhibited the expansion of spheres formed by GICs that express both H-RasV12 and dsRed (RasR cells) without affecting the shape of the sphere or the aggregation ability, whereas higher concentrations of the drug markedly inhibited sphere formation (Fig. 1A, B). Quantification of the sphere dimensions revealed an approximately 50% reduction in sphere area in the presence of luliconazole at 30 µM (Fig. 1B). The drug also reduced the number of viable GICs in adherent culture, but this effect was less pronounced than the inhibition of sphere growth (Fig. 1C).

Luliconazole inhibits tumor growth and parenchymal infiltration in brain explants

Cultured brain slices are an effective system for evaluating the response of tumors formed by GICs to both cytostatic and cytotoxic drugs. Sequential imaging of the tumors formed after implantation of RasR GICs into the right forebrain of mouse recipients showed that the tumor area and the infiltration of tumor cells into the normal tissue parenchyma of brain slices continued to increase during 4 days in culture (Fig. 2A). In slices treated with 200 µM luliconazole, however, tumor expansion was inhibited, and the fluorescent cell density was markedly reduced compared with control cultures (Fig. 2A). Given
that we previously found that certain compounds inhibit mass expansion without affecting invasion of the remaining viable GICs into the surrounding brain tissue, we examined the effects of luliconazole on brain slices implanted with a more infiltrative line of GICs expressing H-RasG12V and green fluorescent protein (RasG cells). Luliconazole markedly reduced the tumor size in such brain explants (Fig. 2B), and it also significantly reduced the distance over which surviving single tumor cells invaded into the normal parenchyma (Fig. 2B, C).

Luliconazole induces cell death in GICs in brain explants

Histopathologic analysis of brain explants at the end of study treatment can yield additional information on drug cytotoxicity for both tumor cells and normal cells. Tumors formed by RasR GICs and treated with luliconazole (200 µM) manifested cell shrinkage and nuclear fragmentation compared with control tumors (Fig. 3A, B). Consistent with these morphological changes and with reports that ketoconazole and posaconazole induce apoptosis in glioblastoma xenograft models, immunohistochemical
analysis revealed that the number of GICs positive for cleaved caspase-3, a marker of apoptosis, markedly increased in the luliconazole-treated tumors (Fig. 3C,D). In contrast, perivascular and periventricular regions of normal brain tissue, both of which are sensitive to drug toxicity, showed no substantial change in the number of cells positive for cleaved caspase-3 after exposure to luliconazole (Fig. 3E,F).

**Cholesterol rescues sphere growth in the presence of luliconazole**

Finally, we examined whether the effects of luliconazole on GICs might be attenuated by the presence of exogenous cholesterol. To test the rescue effect in conditions of maximal treatment-induced damage, spheres were treated with luliconazole at 50 μM (maximal inhibition of sphere growth, Fig. 1), temozolomide at 500 μM, or radiation at 5 Gy. Whereas synthetic cholesterol induced an approximately 1.5-fold increase in the area of control GIC spheres formed during culture for 7 days, it increased the area of spheres formed in the presence of 50 μM luliconazole by a factor of approximately 15 (Fig. 4A,B), representing a marked rescue effect. In contrast, cholesterol did not attenuate the inhibitory effects on sphere growth of either ionizing radiation (Fig. 4C) or temozolomide, the major chemotherapeutic drug used for the treatment of glioblastoma (Fig. 4D).

**Discussion**

We have herein shown that luliconazole, an imidazole antifungal agent, impairs the growth of spheres formed by GICs in vitro and also inhibits the growth and invasion of GIC-based tumors in cultured brain slices. We further found that the effect of luliconazole on GIC sphere growth in vitro was attenuated by the addition of exogenous cholesterol.
The evaluation of compounds for therapeutic indications beyond their initial use may lead to the identification of single repurposable drugs or of entire classes of agents that can be repurposed.\textsuperscript{17,18} In the latter case, the testing of several compounds in the same class is usually warranted to establish proof of concept. Clotrimazole and ketoconazole were previously found to have antiglioma effects.\textsuperscript{11,12} Our results now indicate such effects for a further drug, luliconazole, which was developed in Japan as an optically active imidazole with high potency against dermatophytes and other fungi.\textsuperscript{19–21}

Glioblastomas contain fractions of cells with stemlike properties, properties that also confer on these cells intrinsic drug resistance and high invasiveness.\textsuperscript{22–24} It is important that such fractions be eradicated for a lasting improvement in prognosis, and failure to inhibit the survival or infiltrative abilities of these cells by current treatments is thought to be at the root of the poor prognosis for malignant gliomas. Luliconazole inhibited sphere formation by our GIC cell lines \textit{in vitro}, such sphere formation being an indicator of stemlike properties and tumor aggressiveness.\textsuperscript{16} This drug also induced caspase-dependent tumor cell death and inhibited tumor cell invasion in brain explants, thereby manifesting efficacy with regard to two major characteristics of GICs.

These facts notwithstanding, the concentration of luliconazole required to induce a significant antitumor effect \textit{ex vivo} was five times that needed \textit{in vitro}. One possible reason for this difference is a low penetration of the drug into brain slices. Another factor might be the high concentration of cholesterol present in the brain,\textsuperscript{25} in contrast to the cholesterol-free culture medium used for assay of

![Fig. 4. Effect of cholesterol on the inhibitory action of luliconazole on sphere growth.](image_url)

(A) Representative phase-contrast images of RasR GIC spheres formed after culture in the absence or presence of 50 µM luliconazole and the absence or presence of synthetic cholesterol (Chol) for 7 days. Scale bars, 300 µm. (B) Relative sphere area for spheres as in (A). (C,D) Relative sphere area for RasR GIC spheres cultured for 7 days either in the absence or presence of synthetic cholesterol after exposure to 5 Gy of ionizing radiation (IR) (C) or in the absence or presence of 500 µM temozolomide (TMZ) (D). All quantitative data are means ±SDs for representative experiments (n = at least five spheres per treatment group). **P < 0.01, ****P < 0.0001; NS, not significant (one-way ANOVA followed by Tukey’s test).
sphere formation.

Lanosterol 14α-demethylase catalyzes a reaction in human cells similar to that which it mediates in fungi. Inhibition of this enzyme results in the accumulation of lanosterol and can also ultimately lead to depletion of downstream cholesterol. This effect is considered negligible in human cells at doses at which imidazole antifungals are used in clinical settings; however, this effect has been confirmed after the use of high-dose ketoconazole in prostate cancer.26 Ketocconazole was shown to significantly lower cholesterol and increase lanosterol levels in cultured normal human fibroblasts and in the serum of patients with advanced prostate cancer.26 However, the contribution of cholesterol inhibition to the antitumor action of imidazole antifungals remains relatively unexplored. The antiproliferative effect of clotrimazole in glioblastomas has been attributed to its interference with the downstream cholesterol. This effect is considered negligible in human cells similar to that which it mediates in fungi. Inhibition of this enzyme results in the accumulation of lanosterol 14α-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and Candida albicans CYP51 with azole antifungal agents. Yeast 1999; 15: 755–763. PMID:10398344, DOI:10.1002/(SICI)1097-0061(19990630)15:9<755::AID-YEA417>3.0.CO;2-8

In summary, our findings indicate that luliconazole effectively targets GICs in vitro and ex vivo, thereby providing further support for the antitumorigenic effects of imidazole antifungals compounds.

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Conflicts of Interest

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