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# Inhibition of tumor growth and prolonged survival of rats with intracranial gliomas following administration of clotrimazole

**M. Humayun Khalid, M.D., Ph.D., Yoshiharu Tokunaga, M. Div., M.D., Anthony J. Caputy, M.D., and Eric Walters, Ph.D.**

*Department of Biochemistry and Molecular Biology, Howard University College of Medicine, Washington, DC; Department of Neurosurgery, Nagasaki University School of Medicine, Nagasaki, Japan; and Department of Neurological Surgery, George Washington University, Washington, DC*

*Object.* Clotrimazole, an imidazole derivative and inhibitor of cytochrome P-450, inhibits the proliferation of cancer cells by downregulating the movement of intracellular $Ca^{++}$ and $K^+$ and by interfering with the translation initiation process. Clotrimazole inhibits the proliferation of human glioblastoma multiforme cells; it induces morphological changes toward differentiation and blocks the cell cycle in the $G_0/G_1$ phase. In vitro, clotrimazole enhances the antitumor effect of cisplatin by inducing wild-type p53–mediated apoptosis. The authors examined the effect of clotrimazole on tumor growth, sensitivity to cisplatin, and survival of rats with intracranial gliomas.

*Methods.* Cultured C6 and 9L glioma cells were exposed to clotrimazole, and cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide colorimetric assay. Clotrimazole produced a dose- and time-dependent inhibition of cell proliferation. The growth inhibitory effect of clotrimazole could not be overcome by exogenous stimulation with epidermal growth factor. Both C6 and 9L glioma cells were implanted into the rat brain and after 5 days, the animals were treated with a daily single dose of clotrimazole for 8 consecutive days. Clotrimazole treatment caused a significant inhibition of intracranial tumor growth. The survival of rats with 9L gliomas was analyzed after 10 days of treatment with clotrimazole, cisplatin, or a combination of clotrimazole and cisplatin. Rats treated with either drug displayed a significantly prolonged survival time; however, the combination treatment resulted only in an additional survival benefit.

*Conclusions.* Clotrimazole effectively inhibits cell proliferation and tumor growth, and prolongs survival of rats with intracranial gliomas. Clotrimazole may be considered a potential anticancer drug for treatment of intracranial gliomas.

**Key Words • glioma • clotrimazole • growth inhibition • cisplatin • survival • rat**

Ion channels and $[Ca^{++}]_i$ are thought to be involved in cell proliferation and may play a role in tumor development. Clotrimazole, an imidazole derivative, inhibits voltage- and ligand-stimulated mechanisms of $[Ca^{++}]_i$ influx in nucleated cells[30,45] and $[Ca^{++}]_i$-activated $K^+$ channels in human red blood cells.[2] Clotrimazole inhibits the proliferation of normal and several cancer cell lines including human GBM lines.[1,4,7,20,21,25] The growth inhibitory effects of clotrimazole are mediated by its ability to deplete $[Ca^{++}]_i$ stores and to prevent the rise in cytosolic $[Ca^{++}]_i$ by blocking the capacitative influx of $[Ca^{++}]_i$ through the plasma membrane that normally follows mitogenic stimulations.[7]

A number of mechanisms are involved in the cell-growth inhibitory action of clotrimazole. Clotrimazole activates protein kinase R, induces phosphorylation of eIF2alpha, inhibits protein synthesis at the level of translation initiation, decreases the expressions of cyclins, induces the expression of wild-type p53, decreases the level of c-myc, increases the mRNA of migration-inhibitory protein-8/14, blocks the cell cycle in the $G_0/G_1$ phases, and induces apoptosis.[1,21,23] Cancer cells are characterized by a high rate of glycolysis, and clotrimazole effectively decreases the levels of glucose 1,6-biphosphate and fructose 1,6-biphosphate, the two allosteric stimulatory signal molecules of glycolysis, by detaching them from the cell cytoskeleton. Clotimazole detaches hexokinase from the mitochondria and reduces the ATP content—actions that result in cell viability.[16,34] This drug also has been shown to inhibit angiogenesis in vitro and in vivo.[10,42]

Previously, we demonstrated that clotrimazole inhibits the growth of human GBM cells in vitro by inducing cellular differentiation and cell-cycle arrest at the $G_0/G_1$ phase, increasing the levels of cellular GFAP and wild-type p53, decreasing the levels of c-myc and c-fos proteins, and inducing apoptosis.[21] We also demonstrated that the growth inhibitory effects of clotrimazole on GBM cells could not be overcome by exogenous stimulation by EGF or c-myc peptide.

*Abbreviations used in this paper:* ATP = adenosine triphosphate; $[Ca^{++}]_i$ = intracellular calcium; DMEM = Dulbecco modified Eagle medium; DMSO = dimethyl sulfoxide; EGF = epidermal growth factor; FBS = fetal bovine serum; GBM = glioblastoma multiforme; GFAP = glial fibrillary acidic protein; MTT = 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide; SD= standard deviation.

The present study was designed to determine the effect of clotrimazole on two animal cell line models in vitro and in vivo. We observed that similar to human GBM cells,[21] the in vitro growth of C6 and 9L glioma cells was inhibited by clotrimazole in a dose- and time-dependent manner. Moreover, clotrimazole treatment decreased the size of rat intracranial C6 and 9L tumors. Previously, we demonstrated that the combination of clotrimazole and cisplatin synergistically enhances the cytotoxicity of cisplatin by inducing apoptosis in human GBM cells in vitro. In the present study we analyzed the survival of animals bearing intracranial 9L gliomas, which were treated with clotrimazole, cisplatin, or a combination of both drugs. We found that either drug alone significantly increases the survival of these rats.

## Materials and Methods

*Cell Lines and Culture*

Two rat glioma cell lines, C6 and 9L, were used in the experiments. The cells were grown in culture in DMEM supplemented with 10% heat-inactivated FBS, penicillin, and gentamycin at 37˚C in a humidified atmosphere of 95% air/5% $CO_2$.

*In Vitro Cell Proliferation*

Semiconfluent cells were trypsinized and replated onto 96-well flat-bottom culture plates ($5 \times 10^4$ cells/plate) in DMEM containing 10% FBS. The cells were allowed to attach to the wells for 24 hours. Subsequently, various concentrations of clotrimazole (dissolved in DMSO as a stock solution) or DMSO alone (used as vehicle) were added and the cells were allowed to grow for 72 additional hours. Cell growth was assessed by performing an MTT colorimetric assay. The time-dependent growth response of clotrimazole was examined by treating the C6 and 9L cells with a dose of clotrimazole (25 and 30 μM, respectively) for up to 96 hours. At desired time intervals, the effects of treatments on cell growth were determined by performing the MTT assay. In brief, 10 μl of MTT reagent was added to each well and mixed by tapping gently on the side of the wells; the cells were incubated at 37˚C for 4 hours in the $CO_2$ incubator until MTT formazan production. The MTT formazan was dissolved in 100 μl of isopropanol with 0.04 N HCl in each well. Finally, the absorbances were measured on a microplate reader with a test wavelength of 570 mm and a reference wavelength of 630 mm.

*Clotrimazole Treatment and EGF Stimulation of Glioma Cells*

The C6 cells were grown in serum-free DMEM for 24 hours, after which they were cultured either in routine culture medium (DMEM and FBS) or in $Ca^{++}$-free DMEM to which clotrimazole (25 μM) had been added in the presence or absence of exogenous recombinant human EGF (20 ng/ml) for 72 hours. Cell proliferation was quantified by performing an MTT assay.

*Animals and the Brain Tumor Model*

Male Fischer-344 rats, each weighing between 250 and 300 g, were housed in cages in an air-conditioned room and had free access to standard laboratory food and water. All animal experiments conformed strictly to the university guidelines for animal experimentation. The rats were anesthetized by Fluothane inhalation and then mounted on a stereotactic instrument. The skull of each rat was exposed and a small hole (0.8 mm inner diameter) was drilled through the cranium. A 10μl suspension of either C6 or 9L glioma cells ($5 \times 10^6$ viable cells) was injected into the caudate nucleus of the right hemisphere (coordinates: rostral + 1.5 mm, lateral + 2.5 mm, ventral − 6.5 mm, relative to bregma and the dural surface). The injection was administered by using a 25-gauge needle attached to a 10-μl chromatography syringe. The hole was immediately sealed with bone wax, the scalp incision was sutured, and povidone iodine was applied to prevent infection.

*Treatment of the Rats*

Three groups of rats (eight rats/group), inoculated with C6 or 9L glioma cells, were used for the analysis of tumor growth. The appropriate drug was given to the rat 5 days after tumor cell inoculation. In the first group the rats received no treatment, in the second group the animals received peanut oil (1 ml as vehicle), and in the third group the animals received clotrimazole (125 mg/kg body weight) in peanut oil. Each group of rats was housed in a separate cage and allowed free access to food and water. Drugs or vehicle were administered through intraperitoneal injections given once daily for a total of 8 days. During this period, all the rats were examined every day to assess their health and any evidence of drug toxicity. After 8 days, the animals were anesthesized, killed by decapitation, and their brains were removed. Immediately afterward, the brain tumors were separated macroscopically, weighed, fixed in 10% neutral formalin, and embedded in paraffin. Eight-micrometer tissue sections were stained with H & E for histological examination. In every tumor, a central and two periphery sections were examined to assess the degree of cellularity, the presence and number of mitotic figures, the presence of necrotic foci, and vascularity. For each tumor, cellularity was assessed as the total number of cells (cell density) in these three sections. This number was calculated by counting the number of cells in 30 randomly selected light microscopy HPFs (10 HPFs from each peripheral section and 10 HPFs from the central section) displayed on a video monitor and averaging this number to provide the mean number of cells (± SD). At the same time, the mean number of mitotic figures and the mean vascularity (determined by the number of blood vessels including microvascular endothelial proliferative components in these three sections) in a given tumor were assessed. Additionally, we thoroughly searched other areas of the brain, spinal cord, liver, spleen, kidney, stomach, and intestine for macroscopic evidence of local and distal metastasis and drug toxicity.

*Survival of Rats*

Seven days after of intracranial 9L tumor cell implantation, four groups of rats (seven rats/group) were treated in the following manner. In the first group the rats received no drug but instead 1 ml of normal saline (through intraperitoneal injection) as a control vehicle; in the second group the animals received a single intracarotid infusion of 1.4 mg/kg cisplatin only on Day 1 of treatment as we described previously;[45] in a third group the animals received one intraperitoneal injection of 125 mg/kg clotrimazole daily for 10 days; and in the fourth group the rats received a single intracarotid infusion of cisplatin on Day 1 of treatment, followed by daily treatment with clotrimazole, as described earlier, for 10 days. Each group of rats was housed in a separate cage and checked daily to determine their food intake status and general health. The survival of drug- and vehicle-treated rats was followed until all the rats had died; the survival time of each group was then calculated.

*Sources of Supplies*

The two cell lines, C6 and 9L, were obtained from the American Type Tissue Culture Collection (Rockville, MD). Clotrimazole, cisplatin, and recombinant EGF peptide were purchased from Sigma Chemical Co. (St. Louis, MO). The MTT Assay Kit was purchased from Chemicon Intl. Co. (Temecula, CA).

*Statistical Analysis*

Mean values and SDs were calculated and probability values were obtained using the unpaired Student t-test. A probability value less than 0.05 was considered significant. Kaplan–Meier survival curves and the log-rank test were used for survival analysis.

## Results

*Clotrimazole Causes Decreased Proliferation of Glioma Cells*

We examined the treatment effects of clotrimazole on the proliferation of C6 and 9L glioma cells. As shown in Fig.

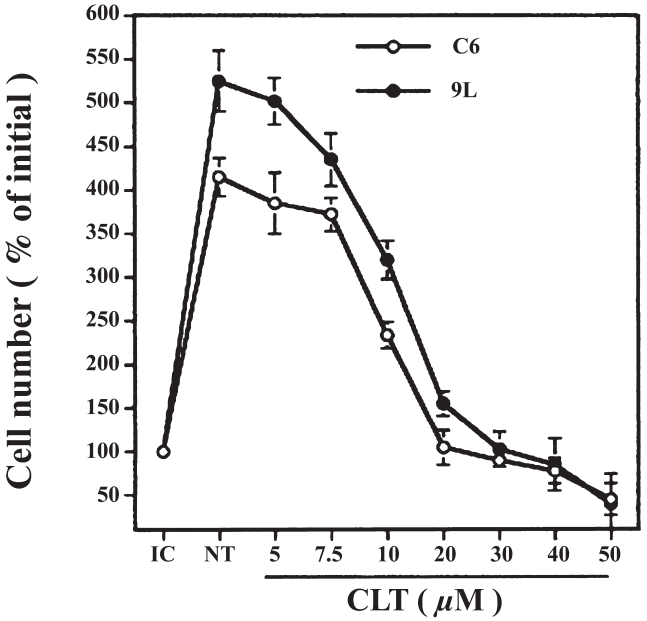



FIG. 1. Graph demonstrating dose-response curves of clotrimazole (CLT) with respect to cell proliferation in the C6 and 9L glioma cell lines. The cells were treated with clotrimazole at the indicated concentrations for 72 hours and cell viability was determined using the MTT colorimetric assay, as described in *Materials and Methods*. Values represent the means ± SDs of three separate experiments. IC = initial cells; NT = nontreated cells.

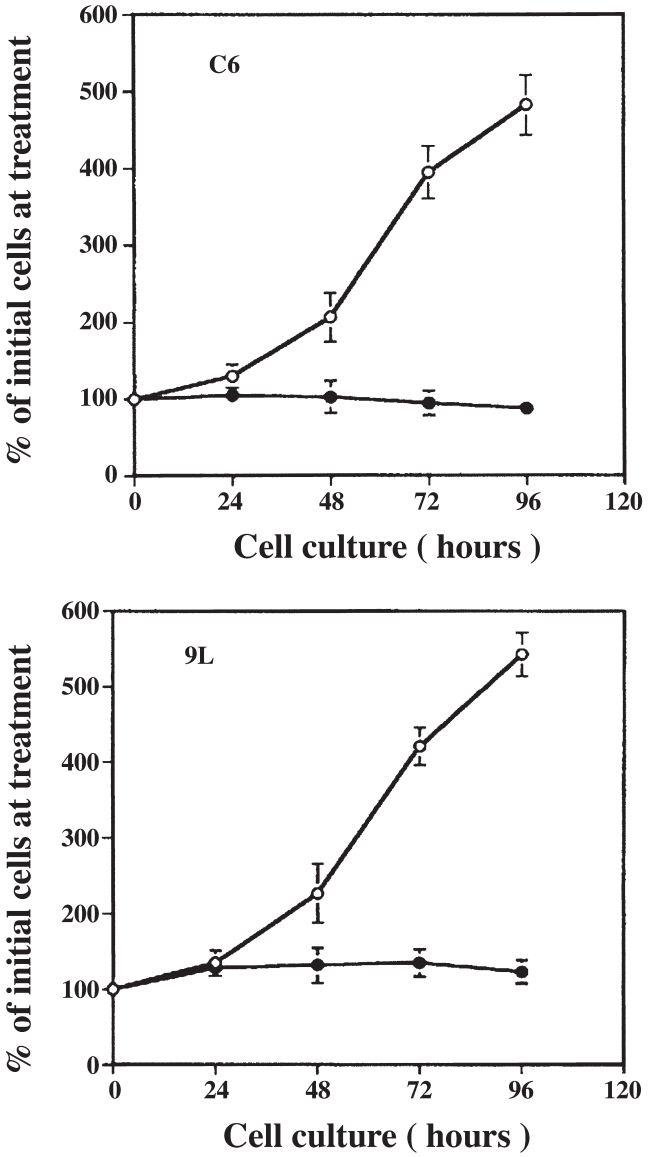



FIG. 2. Graphs showing the effects on cell growth of clotrimazole treatment at indicated time periods in C6 *(upper)* and 9L *(lower)* glioma cells. Untreated cells *(open circles)*, and clotrimazole-treated cells (25 μM for C6 cells and 30 μM for 9L cells; *closed circles*) were tested. The MTT assay was used. Values represent means ± SDs of three separate experiments.

1, clotrimazole inhibited the growth of glioma cells in a dose-dependent fashion. A near-complete inhibition of cell growth was achieved with clotrimazole concentrations of 25 and 30 μM in C6 and 9L gliomas, respectively. These concentrations of clotrimazole were less effective in blocking cell growth, however, when applied to gliomas with a high cell density ($10^5$ cells/plate) (data not shown).

We studied the influence of treatment time on the proliferation of C6 and 9L cells at concentrations of 25 μM and 30 μM clotrimazole, respectively. The growth of C6 cells was almost completely blocked by clotrimazole over a 96-hour treatment (Fig. 2A), whereas the growth of 9L cells remained active for the first 24 hours of treatment, but was almost completely blocked when examined at 48 hours and remained blocked up to 96 hours (Fig. 2B).

### *Failure of EGF Stimulation to Overcome the Growth-Inhibitory Effect of Clotrimazole*

Clotrimazole can block EGF-stimulated DNA synthesis and cell proliferation by preventing the rise in $[Ca^{++}]_i$ that normally follows after mitogenic stimulation to promote cell growth. We therefore examined the influence of recombinant human EGF on the growth inhibitory effect of clotrimazole on C6 cells that had previously been serum starved for 24 hours. The simultaneous addition of EGF in the presence of clotrimazol had no influence on the growth inhibitory effect of the drug (Fig. 3).

### *Clotrimazole Inhibition of Intracranial Tumor Formation*

During the 8 days of drug or vehicle treatments, all the rats appeared to be healthy and there was no sign or symptom of drug toxicity. With the exception of one rat in the untreated group, all animals in this experiment displayed a slight gain in body weight before they were killed. As shown in Figs. 4 and 5, clotrimazole inhibited the tumor formation of C6 and 9L gliomas in the rat. In rats with C6 gliomas, the tumor weight (mean ± SD) was 600.41 ± 60.67 mg in the untreated group, 586.02 ± 43.84 mg in the peanut oil–treated group, and 277.50 ± 67.54 mg in the clotrimazole-treated group (Fig. 4a). In the rats with 9L gliomas the tumor weight (mean ± SD) was 626.20 ± 56.47 mg in the untreated group, 615.11 ± 63.94 mg in the peanut oil–treated group, and 289.13 ± 48.31 mg in the clotrimazole-treated group. The treatment of rats with peanut oil had almost

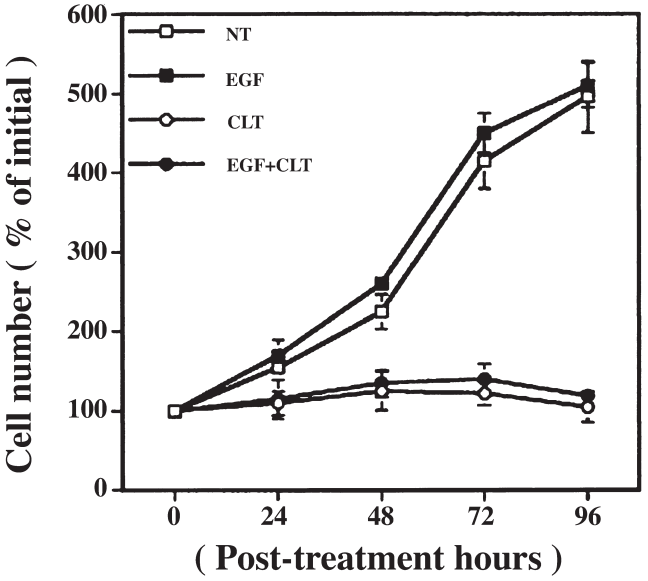



FIG. 3. Graph showing the effects of recombinant EGF on the cell growth inhibition by clotrimazole in C6 cells. Cells were serum starved for 24 hours and then incubated with serum plus DMEM containing clotrimazole (25 μM) alone or clotrimazole plus EGF (20 ng/ml) for 72 hours. Cell proliferation was determined using the MTT colorimetric assay. Values represent means ± SDs of three separate experiments. Stimulation with EGF had no or very little influence on clotrimazole-induced cell growth inhibition.

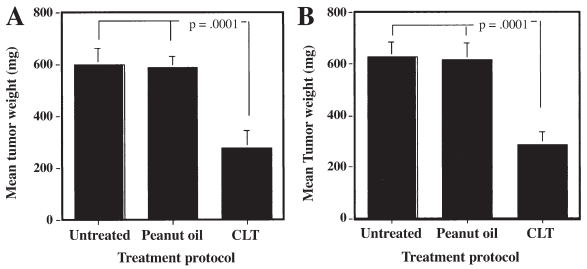



FIG. 4. Graphs showing the effects of clotrimazole treatments on intracranial tumor growth of C6 (A) and 9L (B) gliomas in rats. Viable glioma cells ($5 \times 10^6$ cells/rat) were injected into the caudate nucleus of the right hemisphere and drug treatment was initiated 5 days after tumor cell inoculation. Three groups composed of eight rats each were used; one group received no treatment, the second group received peanut oil (1 ml as vehicle), and the third group received clotrimazole (125 mg/kg body weight) in peanut oil. Values represent the means ± SDs of tumor weight in each group of rats and the probability value was obtained using an unpaired Student t-test.

no influence on tumor growth when compared with untreated rats, regardless of what glioma was harbored. Clotrimazole-treated rats had significantly smaller tumors compared with untreated rats ($p = 0.0001$ for animals with C6 gliomas and $p = 0.0001$ for those with 9L lesions). Histological examinations of these tumors were also performed. In C6 tumors the cell density (mean ± SD) was 2262.50 ± 362.53 cells/tumor in the untreated group, 2209.51 ± 294.09 cells/tumor in the peanut oil–treated group, and 1182.25 ± 152.89 cells/tumor in the clotrimazole-treated group (Fig. 5a). Among the 9L tumors these values were 2159.63 ± 169.41, 2140.88 ± 242.99, and 1490.50 ± 165.08 cells/tumor, respectively (Fig. 5b). Clotrimazole-treated tumors had significant decreased cell densities compared with tumors that were either untreated or treated with peanut oil ($p = 0.0001$ for C6 gliomas and $p = 0.0001$ for 9L lesions). The number of mitotic figures was also decreased in both tumor types in animals treated with clotrimazole compared with rats that were untreated or given peanut oil (data not shown). There was no evidence of tumor metastasis or abnormal changes in the spinal cord, liver, spleen, kidney, stomach, and intestine.

*Clotrimazole Prolonged Survival of Rats With Gliomas*

At the end of treatment period, some rats became slightly lethargic. We could not verify food intake of individual animals because the rats were housed in groups. At Day 9 of treatment, mild hemiparesis was experienced by four rats in the untreated group, two rats in each of the clotrimazole and cisplatin treatment groups, and one rat in the combined clotrimazole–cisplatin treatment group. Despite this, all the rats maintained adequate food and water intake. Five days after the end of the treatment period, all rats demonstrated a slight increase in body weight, and no further body weight measurement was taken during the remainder of the observation period. We concluded that all rats had died as a consequence of increased intracranial pressure caused by the tumor. Three rats in the untreated group and three in the clotrimazole-treated group became paraplegic, and nasogastric feeding was administered 2 days before their deaths.

The survival of rats with intracranial 9L gliomas was analyzed and the results were plotted using a Kaplan–Meier survival graph (Fig. 6). The survival times (means ± SDs) were as follows: in untreated control rats, 20.14 ± 3.67 days (range 15–25 days); in clotrimazole-treated rats, 27 ± 6.63 days (range 18–37 days); in cisplatin-treated rats, 28.14 ± 5.87 days (range 20–38 days); and in clotrimazole- and cisplatin-treated rats, 31 ± 7.12 days (range 19–39 days). In rats treatment with clotrimazole significantly prolonged the duration of survival compared with no treatment with clotrimazole ($p = 0.038$, log-rank test). Treatment with cisplatin also significantly prolonged the length of survival of rats in comparison to the untreated rats ($p = 0.006$, log-rank test). The combined clotrimazole–cisplatin treatment prolonged survival ($p = 0.0015$ for a comparison of untreated and clotrimazole–cisplatin treatment groups); however this difference in survival time was not significant compared with either clotrimazole or cisplatin treatment alone ($p = 0.4286$ for a comparison of clotrimazole-treated rats and rats treated with both clotrimazole and cisplatin; and $p = 0.2981$ for a comparison of cisplatin-treated rats and rats treated with both drugs).

## Discussion

In previous studies investigators have shown that clotrimazole is an effective antiproliferative agent against several normal and cancer cell lines both in vitro and in vivo.[1,4,20,21,23,47] In the present study, we demonstrated that clotrimazole inhibits the growth of rat C6 and 9L glioma cells in vitro and intracranial tumor formation in vivo. Moreover, treatment of rats with intracranial 9L gliomas by clotrimazole significantly prolongs their survival.

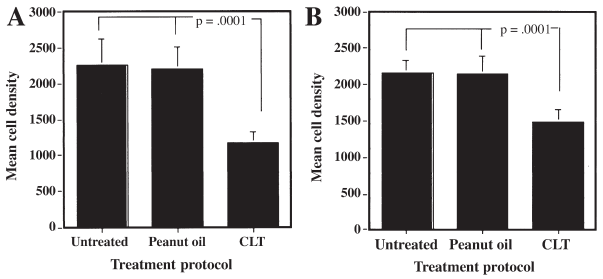



FIG. 5. Graph showing effects of clotrimazole on the cell density of rat C6 (A) and 9L (B) intracranial gliomas. The tumor sections were stained with H & E. In each tumor, a central and two periphery sections were examined to determine degrees of cellularity. Values represent means ± SDs.

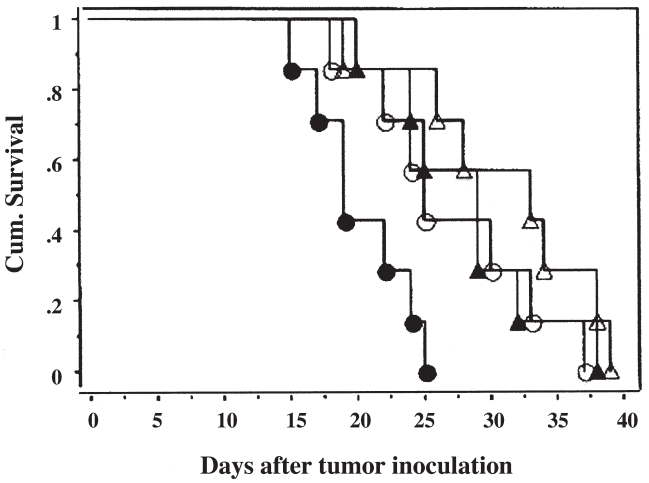



FIG. 6. Kaplan–Meier graph showing survival of rats with 9L gliomas given no drug treatment *(black circles)*, and comparable rats given clotrimazole alone *(white circles)*, cisplatin alone *(black triangles)*, or a combination of clotrimazole plus cisplatin *(white triangles)*. Seven days after the tumor cells were injected into the rat brains, the animals were treated by intraperitoneal injection with clotrimazole for 10 days or by intracarotid infusion with cisplatin (single infusion at Day 1 of treatment) or with a combination of both drugs. The significance of difference was determined using the log-rank test. Probability values are listed in *Results*. Cum = cumulative.

Also known as a cytochrome P-450 inhibitor, clotrimazole inhibits cell proliferation by altering the movement of $[Ca^{++}]_i$ and $K^+$.[7] A number of other mechanisms are involved in the growth inhibitory action of clotrimazole. It blocks growth factor signaling pathways, activates protein kinase R, phosphorylates eIF2alpha, interferes with protein synthesis at the level of translation initiation, and decreases expression of growth-promoting cyclins that subsequently inhibit cyclin-dependent kinase activity and blockage of the cell cycle in the $G_1$ phase.[1] Previously, we demonstrated that clotrimazole inhibits the growth of human GBM cells in vitro by inducing cellular differentiation, causing cell cycle arrest at the $G_0/G_1$ phase, increasing cellular GFAP and wild-type p53, decreasing levels of c-myc and c-fos proteins, and inducing apoptosis.[21] In the same study, we also demonstrated that a decrease in human GBM cell growth and viability produced by exposure to clotrimazole, is partially dependent on RNA and protein synthesis. The in vitro cell growth pattern of rat gliomas in the present study (Figs. 1 and 2) is quite similar to the results obtained in our human GBM study.[21]

Binding of growth factors to specific receptors in growth-arrested cells triggers an array of ionic signals. An increase in cytosolic $Ca^{++}$ released from intracellular stores, capacitative $Ca^{++}$ influx through the plasma membrane, and activation of $Ca^{++}$-dependent $K^+$ channels in the plasma membrane are critical events in the cascade of mitogenic stimulation that leads to DNA synthesis.[35,40,46] These mitogenic events are potential therapeutic targets for inhibition of proliferation of malignant cells. Clotrimazole inhibits cell proliferation by decreasing $[Ca^{++}]_i$ stores and the rising level of cytosolic $Ca^{++}$ by preventing its entry through the plasma membrane, which normally occurs after growth factor stimulation.[7] Clotrimazole blocks growth factor–stimulated DNA synthesis.[7,21] Previously, we demonstrated that clotrimazole treatment of human GBM cells in culture decreased DNA synthesis and increased the number of cells in the $G_0/G_1$ phase.[21] In the study of human GBM cells and in the present report on rat glioma cells, we found that the growth-inhibitory effect of clotrimazole on these cells could not be overcome by simultaneous treatment with EGF. Clotrimazole could compete with specific growth factor receptors in the plasma membrane and interfere with the mitogenic pathways located downstream of the growth factor–receptor interaction. Hence, the inhibition of cell proliferation warrants the need for more studies that are aimed at determining the underlying mechanisms that affect second messenger signaling within these cells.

The clotrimazole concentrations of 25 to 30 μM, which we used in previous[21] and present in vitro experiments, were very effective for inhibiting cell growth without apparent toxicity, whereas much higher concentrations were found to be toxic in both studies. Benzaquen and colleagues[7] have demonstrated that as little as 10 μM clotrimazole completely inhibits both DNA synthesis and cell growth in a number of normal and cancer cell lines, even in the presence of platelet-derived growth factor and basic fibroblast growth factor. In contrast, 10 μM clotrimazole minimally inhibits the growth of human and rat glioma cells in vitro, as we have observed previously[21] and in the present study. The dosage of clotrimazole that we used in the present in vivo experiments (125 mg/kg/day, given once daily by an intraperitoneal injection) provided adequate therapeutic efficacy and did not reveal any macroscopic evidence of tumor metastasis or abnormal changes in the spinal cord, liver, spleen, kidney, stomach, and intestine. The dosages of clotrimazole used by investigators in other in vivo studies ranged from 100 to 160 mg/kg/day in single or divided doses administered by oral gavage for variable time periods (range 5–28 days).[7,11,41,43] In most of these studies, no major side effect or significant change in blood or hepatic parameter was observed.

The mechanism of interaction between clotrimazole and cisplatin and their combined action(s) during treatment of intracranial tumors are unknown. Previously, we demonstrated that combined clotrimazole–cisplatin treatment significantly enhanced human GBM cell cytotoxicity in vitro compared with administration of either drug alone.[21] In the present study, we observed that when clotrimazole or cisplatin alone was administered to rats harboring intracrani-

al 9L gliomas, each drug conferred a significant survival benefit when compared with the fate of untreated rats. Although not statistically significant, the combined cisplatin–clotrimazole treatment of rats with 9L gliomas resulted in an additional survival benefit over the survival of rats treated with either drug alone. Failure to demonstrate a significant survival benefit with this combination regimen in vivo is a mater of debate. The results of in vitro experiments do not always correspond to results that are obtained in vivo. Multiple factors are involved in the survival of animals with tumors, one of which may be the timing of drug administration. Moreover, the combination of cisplatin and clotrimazole on liver metabolism and subsequent serum drug levels may influence the action of individual drugs in this group of animals. Clotrimazole induces greater expression of wild-type p53 in human GBM cells whose parent cells had wild-type or mutant-type p53, and it has been hypothesized that apoptosis mediated by this highly expressed wild-type p53 could be involved in the increased sensitivity to cisplatin.[21] A similar mechanism might be involved in the rat glioma in vivo after treatment with the combination of clotrimazole and cisplatin. Restoration of wild-type p53 function in GBM and other cells renders the cells more sensitive to irradiation or cisplatin-induced apoptosis.[13,14,21] It has been demonstrated that modification of tumor cells with wild-type p53, followed by treatment with cisplatin in vivo, significantly prolonged the survival of rats with intracranial 9L gliomas.[14]

The exact mechanism underlying the cytotoxicity of cisplatin, which involves changes in calcium channels and alterations in calcium hemostasis in tumor cells, is unclear. High-dose cisplatin chemotherapy for patients with cancer induces an acute decrease in the serum level of total calcium.[17] When released from intracellular stores, but not moved from extracellular space, $Ca^{++}$ plays a role in cisplatin-induced cell injury.[22] Both cis-diamminedichloroplatinum (cisplatin) and trans-diamine dichloroplatinum block the voltage-dependent $Ca^{++}$ current in a concentration-dependent manner.[24] In human GBM and transitional cell carcinoma cell lines, nifedipine, a calcium-channel blocker, enhances the antitumor effect of cisplatin in vitro and in vivo, and in the absence of normal extracellular $Ca^{++}$, nifedipine enhances the cytotoxicity of cisplatin by inducing apoptosis.[25,48] Calcium-channel blockers can easily cross the blood–brain barrier and their primary action is to reduce the number of open channels, thus restricting the influx of $Ca^{++}$ into the cell.[27] In the rat glioma model, administration of a $Ca^{++}$ antagonist causes a selective dose-dependent increase in blood–brain permeability within the tumor tissue and enhances drug delivery to malignant brain tumors without affecting normal parts of the brain.[28] It may be that clotrimazole, like other Ca antagonists, increases blood–brain and blood–tumor barrier permeability to facilitate increased cisplatin delivery to tumors. This area of research is the subject of future investigation.

The metabolism of cancers, including gliomas, is characterized by a high rate of aerobic glycolysis. The agents that inhibit glucose metabolism can block the proliferation of tumor cells by inhibiting the allosteric regulators of glycolysis bound at the cytoskeleton and mitochondria-bound hexokinase.[15,16,32,34] Clotrimazole causes the detachment of glycolytic enzymes, such as phosphofructokinase, aldolase, glucose 1,6-biphosphate, and fructose 1,6-biphosphate, from the cytoskeleton, which is followed by a reduction in ATP content and a decrease in cancer cell viability.[15,16] The mitochondria-bound hexokinase enhances uptake of $[Ca^{++}]_i$ by the mitochondria, resulting in increased intramitochondrial oxidative metabolism and ATP production.[12,26] Various factors such as $[Ca^{++}]_i$ and $[Ca^{++}]_i$-mobilizing hormone increase mitochondrially bound hexokinase, which is found in many tumor cells including melanoma and glioma.[3,6,19,29,32,34] Inhibitors of hexokinase inhibit cell proliferation and DNA synthesis, increase apoptosis in vitro, and inhibit tumor growth in vivo.[29] It has been reported that clotrimazole detaches hexokinase from the mitochondria and that this is followed by decreased cell viability.[34] Intracranial glioma contains as much as a 40-fold higher level of hexokinase and 300-fold level of glucose-6-phosphate deoxyglucose in tumors compared to the normal cortex.[19] Because the cytoskeleton and mitochondria are recognized as important modulators of cell function, proliferation, differentiation, and neoplasia, the detachment of glycolytic enzymes from the cytoskeleton and mitochondria induced by clotrimazole, as well as their inhibitory action on cell proliferation, warrants the use of clotrimazole as a promising treatment for gliomas in future studies.

In the present study, we used the 9L gliosarcoma–Fischer rat model for therapeutic approaches because of our familiarity with this model.[45] Although C6 cells are not syngeneic to the Fischer rat, we used the C6 glioma–Fischer rat model for a comparison with the 9L tumor model. We found that the tumor growth pattern and therapeutic efficacy of clotrimazole are almost identical in both the C6–Fischer rat and 9L–Fischer rat models. Although no currently available animal brain tumor model exactly simulates human high-grade brain tumors, the rat models that are currently available have provided a wealth of information on in vitro and in vivo biochemical and biological properties of brain tumors and their in vivo responses to various therapeutic modalities such as chemotherapy, radiotherapy, gene therapy, and immunotherapy. Ideally, valid brain tumor models should be derived from glial cells grown in vitro and in vivo with predictable and reproducible growth patterns that simulate human gliomas. In addition, they should possess weak or nonimmunogenic properties, and their response to therapy, or lack thereof, should resemble that of human brain tumors.[5] Two of the most popular rat glioma models include the C6 glioma and the 9L gliosarcoma. The C6 glioma, which was chemically induced in an outbred Wistar rat, is also syngeneic to the BDIX rat and the Sprague–Dawley rat,[18,38] but not syngeneic to any inbred strain. Nevertheless, the C6 rat glioma induces a vigorous immune reaction that may mimic a specific anti-tumor response in Wistar rats.[33] Although there have been some therapeutic and radioimaging studies[31,44] in which the C6–Fischer rat model was used, its potential to evoke an alloimmune response in nonsyngeneic systems can mimic therapeutic efficacy, and the model might have a serious limitation if used in survival studies. The 9L gliosarcoma, which was chemically induced in an inbred Fischer rat, has been one of the most widely used of all rat brain tumor models and has provided much useful information relating to brain tumor biology and therapy. Thus, a better option might have been to use human genograft cell lines in nude mice or rats, or an alternate rodent tumor model for in vivo therapeutic studies.

Although clinically clotrimazole is mostly used as an ex-

ternal treatment for fungal infections, systemic administration of clotrimazole has been reported for the treatment of patients with sickle cell disease, oral candidiasis, oral thrush in patients infected by the human immunodeficiency virus, and fungal infection in recipients of liver transplants.[8,9,36,37] In most of these studies, clotrimazole (10 mg in oral troche) was administered five times a day for variable time periods (range 7–120 days), and no major adverse reaction to the drug was reported. Brugnara, et al.,[8] have demonstrated that at the relatively low dosage of 20 mg/kg/day for 2 weeks, clotrimazole was well tolerated with side effects of mild dysuria and pyuria, likely due to urinary excretion of clotrimazole metabolites. An increase in hemoglobin and indirect bilirubin levels were noted; however, total bilirubin, lactate dehydrogenase, and alkaline phosphate levels were normal. These changes returned to normal when clotrimazole was discontinued. In the same study, at a higher dose (30 mg/kg/day for 2 weeks), some patients experienced significant nausea and vomiting, with increased plasma alanine transaminase and aspartate tramsaminase levels, which returned to baseline after clotrimazole was discontinued. It is absolutely necessary to measure serum levels of clotrimazole in rats during the period of treatment, although in the present in vivo study, we did not do this in any rats. It has been reported that the plasma level of clotrimazole in patients with sickle cell disease who received a dosage of 20 mg/kg/day for 2 weeks, varied between 0.03 and 0.44 μM.[8] These values are in accordance with the blood level of clotrimazole following oral administration in healthy volunteers and in patients with systemic mycoses.[39] Plasma levels of clotrimazole (given orally at a dosage of 30 mg/kg/day) in two patients were 0.41 μM and 0.74 μM, respectively. Micromolar levels of clotrimazole metabolites were also present in plasma following clotrimazole administration in five patients (range 2.8–12.5 μM).[8] In healthy volunteers, the clotrimazole concentration peaked within 6 hours of oral administration and returned nearly to baseline levels by 24 hours. High-density lipoproteins appear to be the main carriers of this drug in both normo- and hypertriglyceridemic plasma.[39] There is only one previous report of administration of clotrimazole in an animal tumor model,[7] and our present in vivo experiment is the second tumor model ever reported. Although both studies demonstrated significant benefit of clotrimazole administration in animal tumor models without apparent toxicity, more studies are needed before this drug may be used in patients.

## Conclusions

The findings of this study indicate that clotrimazole is an effective agent to inhibit cell proliferation in vitro and intracranial tumor growth in vivo. Treatment with clotrimazole prolongs the survival of rats with intracranial gliomas, and provides an additional survival benefit when administered in combination with cisplatin. Clotrimazole may be a potentially useful anticancer drug in treating malignant gliomas including human GBM. Its potential warrants additional study.

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*Address reprint requests to:* M. Humayun Khalid, M.D., Ph.D., Department of Biochemistry and Molecular Biology, Howard University College of Medicine, 520 West Street, Northwest, Washington, DC 20059. email: humayunkhalid@hotmail.com.