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Clotrimazole induces a late G₁ cell cycle arrest and sensitizes glioblastoma cells to radiation *in vitro*

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Abstract

Tumor cells are characterized by their high rate of glycolysis and clotrimazole has been shown to disrupt the glycolysis pathway thereby arresting the cells in the G₁ cell cycle phase. Herein, we present data to support our hypothesis that clotrimazole arrests tumor cells in a radiosensitizing, late G₁ phase. The effects of clotrimazole were studied using the glioblastoma cell line, U-87 MG. Flow cytometry was used to analyze cell cycle redistribution and induction of apoptosis. Immunoblots were probed to characterize a late G₁ cell cycle arrest. Nuclear and cytoplasmic fractions were collected to follow the clotrimazole-induced translocation of hexokinase II. Clonogenic assays were designed to determine the radiosensitizing effect by clotrimazole. Our studies have shown a dose-dependent and time-dependent clotrimazole arrest in a late G₁ cell cycle phase. Concurrent with the late G₁ arrest, we observed an overexpression of p27^{Kip} along with a decreased expression of p21^{Cip}, cyclin-dependent kinase 1, cyclin-dependent kinase 4, and cyclin D. Clotrimazole induced the translocation of mitochondrial-bound hexokinase II to the cytoplasm and the release of cytochrome c into the cytoplasm. Clotrimazole-induced apoptosis was enhanced when combined with radiation. Clotrimazole was shown to sensitize tumor cells to radiation when the cells were irradiated for 18 h post-clotrimazole treatment. The disruption of the glycolysis pathway by clotrimazole leads to cell cycle arrest of U-87 MG cells in the radiosensitizing late G₁ phase. The use of clotrimazole as a radiosensitizing agent for cancer treatment is novel and may have broad therapeutic applications.

Keywords

antitumor; clotrimazole; glioblastoma; hexokinase II; radiosensitizing

Introduction

Clotrimazole has been marketed as a topical treatment for fungal infections since 1973 and now is commonly available as an over-the-counter cream or by prescription. In the early

1980s an oral clotrimazole tablet was available for the treatment of oral thrush, skin infections, and for prophylaxis of oropharyngeal candidiasis in patients who were immunocompromised, such as HIV or cancer patients [1,2]. Besides its anti-fungal activity, clotrimazole has been studied for its antitumor effects on cancer cells.

The initial study, in 1984, by Mukhtar *et al.* [3] showed for the first time that the topical application of clotrimazole to the skin of mice dramatically reduced the 3-methylcholanthrene-induced formation of skin tumors by blocking the metabolic enzymes required to convert the precarcinogen, polycyclic aromatic hydrocarbons, to the active carcinogenic metabolites. Henceforth, additional evidence has shown that clotrimazole inhibited tumor cell growth in a variety of cancer cells [4–8]. The in-vitro studies by Khalid *et al.* [9] showed that clotrimazole inhibited glioblastoma cell growth, arrested cells in the G₁ cell cycle phase, and induced apoptosis. Subsequently, it was shown that clotrimazole reduced the synthesis and expression of G₁ cyclins such as cyclins D, E, and A at the level of translation, thereby inhibiting the associated cyclin-dependent kinase (cdk) activity required for progression into the S phase leading to a growth arrest in the G₁ cell cycle phase [9–12].

The two most radiation-sensitive phases of the cell cycle are the G₂/M phase and the late G₁ phase [13,14]. The limited studies with clotrimazole did not differentiate in which stage of the G₁ phase the arrest was located. However, a recent study with a clotrimazole analog, NC381, indicated that cell cycle arrest was in late G₁ at the G₁–S phase transition [15]. Therefore, we hypothesized that clotrimazole would arrest tumor cells in the late G₁ cell cycle phase, thereby sensitizing tumor cells to radiation treatment (RT). Herein, we studied the in-vitro effects of clotrimazole using the glioblastoma cell line, U-87 MG. Clotrimazole treatment resulted in the translocation of hexokinase II (HKII) from the mitochondria to the cytoplasm and the release of cytochrome c. This report describes a dose response by clotrimazole to arrest cells in the G₁ cell cycle phase with a concurrent decrease of cells in S phase and G₂/M phase. Using immunoblots to characterize the G₁ cell cycle arrest, we identified the arrest to be in the late G₁ phase. To support our hypothesis, clotrimazole-induced apoptosis was enhanced when treatment combined with radiation and clotrimazole showed a significant dose-dependent radiation-sensitizing effect on the U-87 MG cells.

Methods

Cells and cell culture

The human glioblastoma cell line, U-87 MG, was obtained from Dr Su-Huang (Ludwig Institute for Cancer, University of California, San Diego). The cells were maintained in Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum, and 2 mmol/l L-glutamine. Cell cultures were maintained in a humidified chamber at 37°C with 5% CO₂.

Analysis of cell cycle redistribution

The U-87 MG cells were plated in six-well tissue culture dishes and on reaching 50–60% confluence; the cells were treated with dimethyl sulfoxide (DMSO), 10, 20, or 40 µmol/l

clotrimazole (Sigma–Aldrich, St Louis, Missouri, USA), while maintaining equal concentrations of DMSO (0.5%) in the culture medium. The cells were collected at various time points and processed for cell cycle analyses as described earlier [16]. Briefly, all floating and adherent cells were collected and fixed in 70% ethanol. The fixed cells were incubated in PBS containing ribonuclease A (0.5 mg/ml) and propidium iodide (20 µg/ml). The data were collected at the UAB FACS Core Facility using a Becton Dickinson FACSCalibur system (Becton Dickinson, San Jose, California, USA) and analyzed using ModFit LT v2.0 software (Verity Software House, Inc., Topsham, Maine, USA). DNA histograms corresponding to G₁, S, and G₂/M populations were expressed as a percentage of the total area of the DNA.

Immunoblotting for the detection of cell cycle-related proteins

U-87 MG cells were exposed to 40 µmol/l clotrimazole for 18 h while maintaining the DMSO concentration in the culture medium at 0.5%. Cell lysates were collected as described earlier [17]. Equal concentrations of protein were separated by SDS–PAGE and transferred to Immobilon-P membrane (Millipore Corp., Bedford, Massachusetts, USA) by electroblotting. The immunoblots were blocked in 10% milk–Tris-buffered saline with 0.05% Tween 20 (TBS–T) for 1 h at room temperature. Primary antibodies; anti-p27^{Kip}, anti-p21^{Cip}, anti-p-reti-noblastoma protein (pRb) (Ser807/811), anti-p-pRb (ser780), anti-pRb, and anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA); anti-cyclin D and anti-cdk4 were purchased from Millipore Corp (Billerica, Massachusetts, USA); anti-cdk1 was purchased from Thermo Fisher Scientific (Rockford, Illinois, USA). All primary antibodies were incubated overnight at 4°C in 2% milk–TBS–T at the appropriate dilutions recommended by the manufacturer. The immunoblots were washed in TBS–T followed by incubation for 1–2 h at room temperature with horseradish peroxidase-tagged species-specific secondary antibodies. The immunoblots were washed with TBS–T with a final wash in TBS and developed using Amersham ECL reagents (GE Healthcare Bio-Sciences Corp, Piscataway, New Jersey, USA).

Isolation of mitochondrial and cytoplasmic fractions

U-87 MG cells were exposed to 40 µmol/l clotrimazole or an equal concentration of DMSO (0.5%) for 6 h; then the cells were collected and processed as described earlier [18,19]. Briefly, the cells were collected by centrifugation (500 × g for 10min), the supernatant removed and the cells suspended in three-cell volumes with an ice-cold isolation buffer (20mmol/l HEPES, pH 7.4, 10mmol/l KCl, 1.5 mmol/l MgCl₂, 0.2 mmol/l EDTA, 250 mmol/l sucrose, 1 mmol/l DTT, 1 mmol/l PMSF 1% protease inhibitor cocktail). The cells were mechanically disrupted by 20 strokes with a 26-gauge needle and the cell debris was removed by centrifugation at 1200 × g for 10 min. The mitochondrial-enriched supernatant was centrifuged at 15000 × g for 10 min. The enriched mitochondrial fraction (pellet) and cytosolic fraction (supernatant) were collected and quantitated using a BCA Protein assay (Thermo Fisher Scientific).

The fractions (20 µg per lane) were separated by SDS–PAGE and transferred to the Immobilon-P membrane. The immunoblots were processed as described above, using the primary antibodies; anti-HKII (Cell Signaling Technologies, Beverly, Massachusetts, USA),

anti-cytochrome c (BD Biosciences, San Jose, California, USA) or anti-actin (Santa Cruz Biotechnology), and the horseradish peroxidase-tagged species-specific secondary antibodies.

Densitometry

The intensity of the immunoblot bands was quantified using the Kodak Molecular Imaging software (Carestream Health, New Haven, Connecticut, USA). The results were expressed as a ratio of the intensity of the protein of interest to that of actin from the same source.

Detection of apoptosis

The induction of cell death from exposure to clotrimazole, radiation or the combination of the two agents was quantified by using the Annexin V-FITC apoptosis detection kit (BioVision Research Products, Mountain View, California, USA). U-87 MG cells were treated with 40 $\mu\text{mol/l}$ clotrimazole for 24 h before exposure to 3 Gy RT [X-Rad 320 (Precision X-Ray, Inc., N. Bedford, Connecticut, USA)]. The cells were collected according to the manufacturer's protocol at the following time points after clotrimazole treatment; 24 h (0 h after RT), 48 h (24 h after RT), and 72 h (48 h after RT). The data were generated using a Becton Dickinson FACScan system and analyzed using CellQuest v3.1 software (Becton Dickinson).

Cell survival assay to determine radiosensitization by clotrimazole

Standard cell survival assays were used to determine whether clotrimazole sensitizes tumor cells to ionizing RT. Log-phase U-87 MG cells were plated in 60 mm dishes in appropriate numbers to attain 20–200 colonies per plate. The cells were allowed to attach for less than 16 h, then exposed to clotrimazole (5, 10 or 20 $\mu\text{mol/l}$) or vehicle (DMSO) maintaining 0.5% DMSO in all the treatment groups. After an 18-h exposure to clotrimazole, the cells were mock-irradiated or irradiated with various doses of radiation (^{60}Co Picker Unit, Cleveland, Ohio, USA). After an incubation period of 12–15 days, the colonies were fixed with methanol and stained with crystal violet. Colonies of more than 50 cells were counted and analyzed as described by our group [17]. The surviving fraction (SF) was defined as the number of colonies counted divided by the number of cells plated multiplied by plating efficiency. Plating efficiency was defined as the number of colonies counted divided by the numbers of cells plated in the mock-irradiated group. From the survival data, we calculated the SF at 2 Gy ($\text{SF}_{2\text{ Gy}}$) and the radiation dose that resulted in a 37% survival fraction (D_{37}).

Statistical analysis

All experiments were the combined results of two to three independent experiments. The results were expressed as the average $\pm$ standard error. For the apoptosis assays, the statistical significance among treatment groups was determined using a two-tailed, Student's *t*-test [20]. The cell survival assays were evaluated by a linear regression model with the statistical procedure PROC GLM (SAS v9.1, SAS Institute Inc., Cary, North Carolina, USA). The model was built with clotrimazole doses, radiation doses, and the possible interaction between the clotrimazole and radiation doses. Treatment groups with *P* values less than or equal to 0.05 were considered significant.

Results

Effect of clotrimazole on cell cycle distribution

Clotrimazole has been shown to inhibit DNA synthesis after a 24-h exposure of the drug in the lung, colon, and melanoma cell lines [21]. To determine the effect of clotrimazole on cell cycle distribution, we exposed nonsynchronized U-87 MG cells to increasing doses of clotrimazole and collected cells at various time points for cell cycle analysis. The results shown in Fig. 1 indicate a dose-dependent cell cycle redistribution beginning as early as 12 h after exposure to clotrimazole treatments. Specifically, clotrimazole treatments accumulated cells in the G₁ phase of the cell cycle in a time-dependent and dose-dependent manner compared with vehicle-treated control cells (Fig. 1a). The fraction of cells in G₁ increased from 65% for the control cells to 70, 76, and 81% at 18 h after 10, 20, and 40 μ mol/l clotrimazole treatment, respectively. The cells showed a concomitant dose-dependent decrease of cells in the S phase beginning 12 h after exposure to clotrimazole and maintained to the 30-h time point (Fig. 1b). Less than 2% of the cells were entering the S phase after an 18-h exposure to clotrimazole, which was maintained for the duration of the observed time period. A dose-dependent decrease in the cells entering the G₂/M phase was evident after an 18-h exposure to clotrimazole, with a maximum decrease in cells entering G₂/M after exposure to 40 μ mol/l clotrimazole for 24–30 h (Fig. 1c).

Clotrimazole modulates the expression of cell cycle regulatory proteins

To determine whether the administration of clotrimazole has the potential to sensitize tumor cells to RT, we investigated whether the G₁ cell cycle arrest induced by clotrimazole was late in the G₁ phase, near the G₁–S transition. Immunoblots were conducted to analyze the cell cycle regulatory proteins; the cyclins and cdks, associated with a late G₁ arrest (Fig. 2).

The loss of cyclin D expression before the G₁/S restriction point has been shown to prevent cells from entering the S phase [22]. Corresponding to the G₁ growth arrest and the reduction of cells entering the S phase, we observed a four-fold decrease in cyclin D expression after exposure to clotrimazole for 18 h, which was maintained throughout the observed clotrimazole-induced G₁ cell cycle arrest (Fig. 2). Concurrent with the decrease in cyclin D, we observed a 1.7-fold decrease in cdk4 after exposure to clotrimazole for 18 h. As expected, the levels of cyclin D and cdk4 were maintained in the DMSO-treated control group.

In addition to its role in mitosis, cdk1 also facilitates the G₁–S transition by showing increased expression as the cells enter the S phase [23–25]. In Fig. 2, immunoblot analysis showed a 2.7-fold decrease of cdk1 expression at 24 h in clotrimazole-treated cells that corresponded to the increase in cells in the G₁ phase and decrease in cells in the S and G₂/M phases, whereas the DMSO-treated control cells resulted in a 1.8-fold increase in cdk1 expression over the same time period.

The cdk inhibitor, p27^{Kip}, has been found to have a major role in the progression of cells through the G₁–S transition. The overexpression of p27^{Kip} induces G₁ growth arrest and blocks cell cycle progression into the S phase [26–28]. Corresponding to the clotrimazole-treated G₁ arrested cells, our results indicated that exposure to clotrimazole for 24 h resulted

in a 1.8-fold increase in p27^{Kip} expression, whereas the DMSO-treated control cells showed a stable expression of p27^{Kip} (Fig. 2). Treatment with clotrimazole reduced the expression level of p21^{Cip} by 1.6-fold, beginning 24 h after exposure to the drug.

Another key regulatory protein important in the G₁–S phase transition is the pRb [25,29,30]. pRb remains in an inactive state, bound to the transcription factor E2F until phosphorylated by the activated cyclin D–cdk4/6 and cyclin E–cdk2 kinases [31]. Immunoblot analysis of the pRb showed a nearly two-fold decreased phosphorylation at serine 807/811 and a reduced phosphorylation at serine 780 after exposure to clotrimazole for 24–30 h, with the total pRb protein levels remaining constant (Fig. 3). The decreased phosphorylation of pRb in the clotrimazole-treated cells corresponds to the decrease in cyclin D and cdk4 expression along with the increased expression of p27^{Kip}, which is an inhibitor of cyclin E–cdk2 kinase activity. No decrease in phosphorylated or total pRb was observed in the DMSO-treated control cells.

Effect of clotrimazole on hexokinase II and induction of apoptosis

Located in the outer mitochondrial membrane, HKII is a critical enzyme in the glycolysis pathway. Clotrimazole has been shown to induce the detachment of HKII from the mitochondrial membrane and thereby reduce ATP levels in HeLa, B16 melanoma, CT-26 colon adenocarcinoma, and Lewis lung carcinoma cell lines [4,7,18]. We analyzed the cytoplasmic and mitochondrial fractions from the clotrimazole-treated U-87 MG cells and showed a translocation of HKII from the mitochondrial fraction to the cytoplasmic fraction after a 6-h exposure to 40 µmol/l clotrimazole, which was not observed in DMSO-treated control cells (Fig. 4). The translocation of HKII from the mitochondria to the cytoplasm was accompanied by the release of cytochrome c indicating that this may be one mechanism for clotrimazole-induced apoptosis.

To determine whether clotrimazole induced apoptosis in the U-87 MG cell lines, we exposed the cells to 40 µmol/l clotrimazole and observed an increase in apoptotic cells after exposure for 24 h, approximately 10% higher than background levels (Fig. 5). The increase in apoptotic cells was maintained at 48 h, then increased to 20% above background after 72-h exposure to clotrimazole. When the clotrimazole-treated U-87 MG cells were combined with a single 3 Gy dose of RT, a significant increase in apoptotic cells was detected at 24h ($P = 0.021$) and 72h ($P = 0.048$) compared with the clotrimazole-treated cells. The combined treatment of clotrimazole and radiation also induced apoptosis in 10–20% more cells than the radiation-only treated cells.

Clotrimazole as a radiosensitizing agent

We have shown by cell cycle and immunoblot analyses that clotrimazole induced cell cycle arrest in late G₁ phase after an 18-h exposure. Tumor cells arrested in late G₁ phase of the cell cycle have the potential to be sensitive to radiation-induced DNA damage. Therefore, we exposed the U-87 MG cells to clotrimazole for 18 h before increasing doses of RT. Cell survival curves showed significant sensitivity to RT after an 18-h exposure to clotrimazole (Fig. 6). When clotrimazole and radiation were administered concurrently, we observed no radiosensitizing effect (data not shown). For the U-87 MG cells, clotrimazole showed a

significant dose-dependent radiosensitizing effect at 5 $\mu\text{mol/l}$ ($P = 0.0455$), 10 $\mu\text{mol/l}$ ($P = 0.0040$), and 20 $\mu\text{mol/l}$ ($P = 0.0019$) compared with the DMSO control survival curve. The combined effect of 40 $\mu\text{mol/l}$ clotrimazole and radiation was too cytotoxic to produce meaningful survival curves (data not shown). From the cell survival data, we calculated the D_{37} and $SF_{2\text{ Gy}}$ for each treatment group. For the U-87 MG cells exposed to 0, 5, 10, and 20 $\mu\text{mol/l}$ clotrimazole, D_{37} was calculated to be 3.12, 2.70, 2.45, and 2.31 Gy, respectively, and the $SF_{2\text{ Gy}}$ was reduced from 59 to 53 to 48 to 44% after the cells were exposed to 0, 5, 10, and 20 $\mu\text{mol/l}$ clotrimazole, respectively.

Discussion

More than 50 years ago, Warburg [32] proposed that the high rate of glycolysis played a critical role in maintaining tumor cells. Subsequently, it has been shown that HKII is overexpressed in cancer cells and has a direct relationship with two major hallmarks of tumor cells, the increase in glycolysis and ATP production and the decreased ability to undergo apoptosis [33,34]. HKII catalyzes the conversion of glucose to glucose-6-phosphate in the first step of the glycolysis pathway. Clotrimazole has been shown to disrupt the glycolysis pathway by preventing HKII from binding to the voltage-dependent anion channel, thereby allowing for the binding of Bax to voltage-dependent anion channel followed by the release of cytochrome c and induction of apoptosis [4,18].

We hypothesized that the clotrimazole-induced translocation of HKII from the mitochondria to the cytoplasm and corresponding depletion of cellular ATP levels, would induce a late G_1 cell cycle arrest. This hypothesis is supported by a study treating non-small cell lung cancer cells with a clotrimazole analog (NC381), which showed that G_1 arrest was at the G_1 -S transition [15]. Our results showed that clotrimazole-treated U-87 MG cells resulted in a two-fold increase of p27^{Kip} protein expression corresponding to the increased arrest of cells in the G_1 phase and corresponding decrease in cells in the S phase and G_2 /M-phase. p27^{Kip} is a key regulatory protein for the progression of cells from G_0 through G_1 to the S phase. There is a marked increase in p27^{Kip} as cells enter G_1 , followed by a sharp decrease in p27^{Kip} as the cells enter the S phase [26–28, 35–37]. Excess levels of p27^{Kip} protein have been found in cell–cell contact and transforming growth factor- β G_1 -arrested cells, with p27^{Kip} bound to cyclin E–cdk2 complex thereby blocking the phosphorylation of pRb and progression into the S phase [35]. Our findings expand on earlier reports of clotrimazole-induced G_1 arrest by indicating that the arrest is in late G_1 , which corroborates the findings that the clotrimazole analog, NG381, arrested cells in the late G_1 phase [15,38,39].

Cell cycle redistribution by clotrimazole into a radiosensitive late G_1 phase along with the increase in apoptosis when combined with radiation lead us to hypothesize that clotrimazole would sensitize tumor cells to radiation. In this study we observed that clotrimazole significantly enhanced the sensitivity of human glioblastoma cells to RT. This enhancement of radiosensitivity was observed when cancer cells were exposed to clotrimazole for 18 h before RT, corresponding to the late G_1 arrest. Cell cycle redistribution into the late G_1 phase is likely to be an important mechanism by which clotrimazole enhanced radiosensitivity [13,14].

Interestingly, it has been described that the expression of p21^{Cip} is required for a G₂ arrest after DNA damage by radiation and blocks irradiated-induced apoptosis [40–42]. We have shown that clotrimazole reduced the expression of p21^{Cip} thereby potentially enhancing apoptosis and the cytotoxic effect of RT. Although clotrimazole has been widely studied for its growth-inhibitory effect on various tumor cells, this is the first report to show a radiosensitizing effect.

Clotrimazole has been shown to sensitize human glioblastoma cell lines to cisplatin by enhanced inhibition of tumor cell growth and induction of apoptosis [9]. We present a scheme for the radiosensitizing and chemosensitizing effects by clotrimazole in Fig. 7.

However, a detailed mechanism by which clotrimazole acts as a radiosensitizing agent needs to be further investigated. The increased dependence for glycolysis as an energy source for cancer cells has made this pathway an important target for anticancer therapy [33,34].

Clinically relevant concentrations of clotrimazole have been attained in earlier studies. It has been shown that the oral administration of clotrimazole at 35 mg/kg bodyweight for 28 days is tolerable in 92% of the patients [46]. Earlier studies have shown that plasma levels of 3.5–11 µmol/l drug concentration can be achieved at 9 h after taking an oral dose of 1 g clotrimazole tablet and if clotrimazole is in an oil solution, then plasma levels of 10–30 µmol/l drug concentration can be achieved [46,47]. These levels are in the range of an effective concentration for enhancing RT *in vitro*.

Studies have shown the ability of imidazoles to cross the blood–brain barrier. In one study, cryptococcal meningitis was prevented from occurring in HIV patients by fluconazole when administered as a maintenance therapy [48]. In another study, clotrimazole was shown to inhibit intracranial gliomas in a rat model and significantly prolong survival [10]. Therefore, clotrimazole or clotrimazole analogs should be evaluated as a radiosensitizing agent for the treatment of malignant gliomas in which the current standard treatment consists of surgical resection followed by radiation therapy.

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Fig. 1.

Redistribution of the cell cycle by clotrimazole (CLT). U-87 MG cells were treated with CLT for various time points and processed for cell cycle analysis. DNA histograms corresponding to (a) G₁ phase, (b) S phase, and (c) G₂/M phase populations were expressed as a percentage of the total area of the DNA. The data represents the average $\pm$ standard error from three independent experiments, with each independent experiment done in triplicate. DMSO, dimethyl sulfoxide.

Fig. 2.

The modulation of the expression of cell cycle regulatory proteins in U-87 MG cells after exposure to 40 μmol/l clotrimazole (CLT) or dimethyl sulfoxide (DMSO) treated control cells. Total cell lysates were collected and equal concentrations of protein (15 μg) was separated and electrophoretically blotted. Immunoblot analysis was used to follow the expression of cyclin D, cyclin-dependent kinase 1 (cdk1), cdk4, p27^{Kip}, and p21^{Cip} over a 30 h exposure time period. Actin was used as a loading control. Densitometry was used to quantitate the expression levels of each protein (data not shown). The representative result of one immunoblot is shown from three independent experiments.

Fig. 3.

The modulation of the retinoblastoma protein (pRb) and the phosphorylation of pRb in U-87 MG cells after exposure to 40 $\mu\text{mol/l}$ clotrimazole (CLT) or dimethyl sulfoxide (DMSO)-treated control cells. Total cell lysates were collected and equal concentrations of protein (15 μg) was separated and electrophoretically blotted. Phospho-Ser 807/811, phospho-Ser 780, and total pRb protein levels were detected by immunoblot during the treatment time period. Actin was used as a loading control. Densitometry was used to quantify the expression levels of each protein over the 30 h time period. The representative result of one immunoblot is shown from three independent experiments.

Fig. 4.

Immunoblot analysis for the translocation of hexokinase II (HKII) and the release of cytochrome c (Cyto C) from the mitochondria to the cytoplasm after U-87 MG cells were exposed to 40 $\mu\text{mol/l}$ clotrimazole (CLT) or dimethyl sulfoxide (DMSO) for 6 h. Mitochondrial and cytoplasmic fractions were isolated and equal protein concentrations (20 μg) were separated and electrophoretically blotted. The immunoblots were probed for HKII and Cyto C to observe the effect by CLT on the translocation of HKII and the release of Cyto C. Actin was used as a loading control. Densitometry was used to quantify the expression levels of each protein in the cytoplasmic and mitochondrial cell fractions. The representative result of one immunoblot is shown from two independent experiments.

Fig. 5.

Detection of apoptosis in U-87 MG cells treated with clotrimazole (CLT) for 24 h, followed by radiation treatment (RT) (3 Gy). Cells were collected and processed immediately after 3 Gy RT (24 h time point), or 48 and 72 h after treatment with CLT. Data points represent the average $\pm$ standard error of three independent experiments, with each independent experiment done in triplicate. A significant increase in the detection of apoptotic cells after RT as compared with CLT treated cells was observed at 24 h (* $P=0.021$) and 72 h (** $P=0.048$).

Fig. 6.

Cell survival curves for the detection of radiosensitization by clotrimazole (CLT). U-87 MG cells were treated with 5 $\mu\text{mol/l}$ CLT ($\diamond$), 10 $\mu\text{mol/l}$ CLT ($\triangle$), 20 $\mu\text{mol/l}$ CLT ($\circ$) or vehicle [dimethyl sulfoxide (DMSO), $\square$] for 18 h before the treatment with radiation. After an incubation period of 12–15 days, the colonies were fixed, stained and counted, analyzed, and graphed (surviving fraction vs. radiation dose). The average plating efficiencies were 43% (DMSO), 34% (5 $\mu\text{mol/l}$ CLT), 21% (10 $\mu\text{mol/l}$ CLT), and 5% (20 $\mu\text{mol/l}$ CLT). A significant radiosensitizing effect by CLT was observed at 5 $\mu\text{mol/l}$ (* P = 0.0455), 10 $\mu\text{mol/l}$ (** P = 0.0040), and 20 $\mu\text{mol/l}$ (** P = 0.0019) as compared with the DMSO control group. All data are the combined results from three independent experiments, with each independent experiment done in triplicate.

Fig. 7.

A schematic representation of the anticancer activity of clotrimazole using data presented herein and earlier published. The mitochondrial-bound hexokinase II enzyme converts glucose to glucose-6-phosphate in the first step of the glycolysis pathway. Clotrimazole disrupts the glycolysis pathway by the translocation of hexokinase II from the voltage-dependent anion channel on the outer mitochondrial membrane to the cytoplasm [4,43,44]. Bax is now capable of binding to the voltage-dependent anion channel and inducing the release of cytochrome c into the cytoplasm, thereby inducing apoptosis [18]. The translocation of hexokinase II interferes with the glycolysis pathway and reduces ATP production [45]. The cell responds by arresting in late G₁, thereby preventing the cells from progressing into the DNA synthesis phase [39]. The arrested cells are sensitive to radiation and cisplatin treatments, with an enhancement of apoptosis [9].