Analysis of the Antitumor Activity of Clotrimazole on A375 Human Melanoma Cells

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Abstract. *Aim: The current study was designed to characterize the anticancer effects of clotrimazole on human cutaneous melanoma cells. Materials and Methods: The v-raf murine sarcoma viral oncogene homolog B1 V600E mutant melanoma cell line A375 was used as an in vitro model. Characterization tools included analyses of cell viability, gene expression, cell-cycle progression, annexin V reactivity and internucleosomal DNA fragmentation. Results: Clotrimazole induced cytotoxicity in A375 human melanoma cells without significant changes of human keratinocyte cell viability. Clotrimazole, at a concentration that approximates the inhibitory concentration 50% (IC_{50}) value (i.e. 10 μM), reduced the expression of hexokinase type-II, induced cell-cycle arrest at G_1–S phase transition, altered annexin V reactivity and induced DNA fragmentation without evidence of necrosis. Conclusion: The current study provides evidence of a remarkable pro-apoptotic effect by clotrimazole against human melanoma cells, with a different mechanism of action and timeline of the apoptosis-related events when compared to cisplatin.*

Clotrimazole is an anti-fungal imidazole derivative which has been used in the clinic for more than 20 years. It is available for the treatment of mycoses, oral candidiasis in immuno-compromised patients, and for skin infections. Its anti-fungal effect is related to the inhibition of fungal sterol 14α-demethylase, a microsomal cytochrome *P450*-dependent enzyme (1). In addition to its anti-fungal properties, it has been reported that clotrimazole has therapeutic effects in sickle cell disease (2) and secretory diarrhea (3).

Clotrimazole also had growth-inhibitory effects on several human cancer cell lines, including lung (4), colorectal (5), breast (6, 7), and endometrial (8) cancer and inhibited tumor growth in a xenograft rat model of intracranial glioma, prolonging survival (9). Previous studies showed that clotrimazole, described as a calmodulin antagonist (7, 10), inhibited the proliferation of human cancer cells *via* disruption of cellular calcium homeostasis (11). It releases Ca^{2+} from intracellular stores while inhibiting Ca^{2+} influx and blocking intermediate-conductance Ca^{2+}-activated potassium channels (IK) (1, 11, 12). Further studies demonstrated that clotrimazole blocks the cell cycle in the G_1 phase, induces apoptosis and inhibits *in vivo* angiogenesis (13-15). Moreover, clotrimazole effectively reduces both glucose consumption (which represents the primary source of energy for tumor cells) and energy metabolism by inhibiting glycolysis and ATP production, leading to reduction of tumor cell viability (16, 17). This effect seems to be due to the interference of the drug with the activity of glycolytic enzymes, particularly by reducing hexokinase (HK) binding to the outer mitochondrial membrane and detaching phosphofructokinase-1 and aldolase from the cytoskeleton (4, 18, 19).

Cutaneous melanoma is one of the most aggressive forms of human cancer and the most aggressive type of skin cancer, with an increasing incidence worldwide. Cutaneous melanoma originates from neuroectodermal melanocytes, which normally do not proliferate in the adult skin, and it is characterized by invasive local growth and early formation of metastases (20). Metastatic melanoma is associated with

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Key Words: Clotrimazole, melanoma, A375 cells, apoptosis, cell cycle, hexokinase.

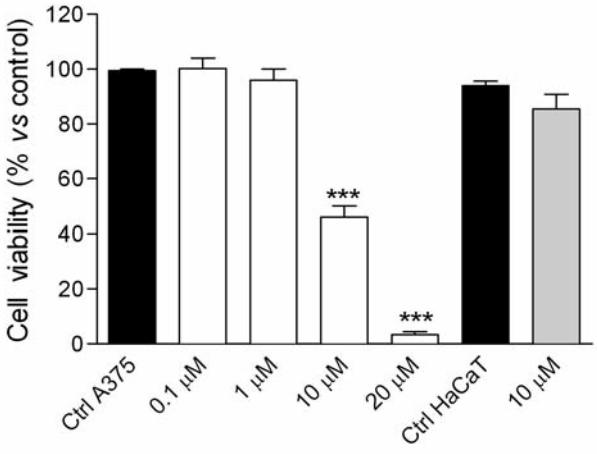

Figure 1. *Effect of clotrimazole on cell viability. The drug was tested at 0.1-20 μM for 48 h on A375 cells and 10 μM for 48 on HaCaT cells. Data were obtained from three independent experiments and expressed as mean±standard error of the mean. $^{***}p<0.001$ versus control (Ctrl).*

poor prognosis and limited therapeutic options. As a matter of fact, advanced melanoma is generally refractory to conventional chemotherapy (21) and novel therapeutic strategies are required.

The current study was designed to characterize the anticancer effects of clotrimazole on A375 cells (used as an *in vitro* model of human cutaneous melanoma) and to investigate the possible underlying mechanisms of its activity.

Materials and Methods

Chemicals. Clotrimazole was obtained from Tocris Bioscience (Bristol, UK) and cisplatin from Sigma-Aldrich (Milan, Italy). Compounds were dissolved in their specific solvents (dimethyl sulfoxide and milliQ water for clotrimazole and cisplatin, respectively) and further diluted in sterile culture medium immediately before their use. DMSO did not exceed 0.3% v/v in the culture medium.

Cell culture. The human cutaneous melanoma cancer cell line A375 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with L-glutamine (2 mM), 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Sigma-Aldrich, Milan, Italy). The human keratinocyte cell line, HaCaT (Lonza, Walkersville, MD, USA), was cultured in DMEM complemented with 10% FBS, 4.5 g/l glucose, and 2 mM L-glutamine in the presence of 50 IU/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Low cell passages (between 5 and 20) were used in the present study.

Cell viability assay. Cell viability was measured using a method based on the cleavage of 4-(3- (4-iodophenyl)-2- (4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzene disulfonate (WST-1) to formazan by mitochondrial dehydrogenase activity following the manufacturer's instructions (Cell proliferation reagent WST-1; Roche, Mannheim, Germany). Briefly, cells (5×10^3/well) were seeded in 96-well plates in medium with 10% FBS; after 24 h, the complete medium was replaced with clotrimazole-containing medium with 1% FBS. To this aim, clotrimazole was dissolved in DMSO and then diluted in low-serum medium at a concentration range of 0.1-20 μM. Fresh stock

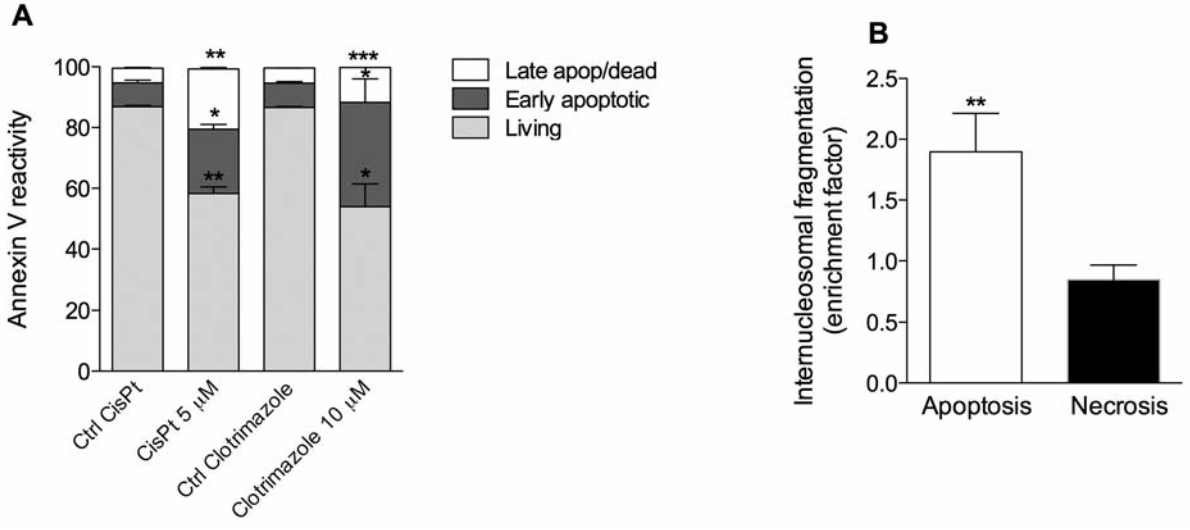

Figure 2. *Effect of clotrimazole on apoptosis. A: Percentage of apoptotic cells after clotrimazole at 10 μM for 48 h. Cisplatin (CisPt) at 5 μM for 48 h was used as reference compound. B: Internucleosomal DNA fragmentation expressed as enrichment factor of cytosolic nucleosomal fragments versus control (vehicle-treated cells). Data were obtained from three independent experiments and expressed as mean±standard error of the mean. $^{*}p<0.05$, $^{**}p<0.01$, $^{**}p<0.001$ versus control.*

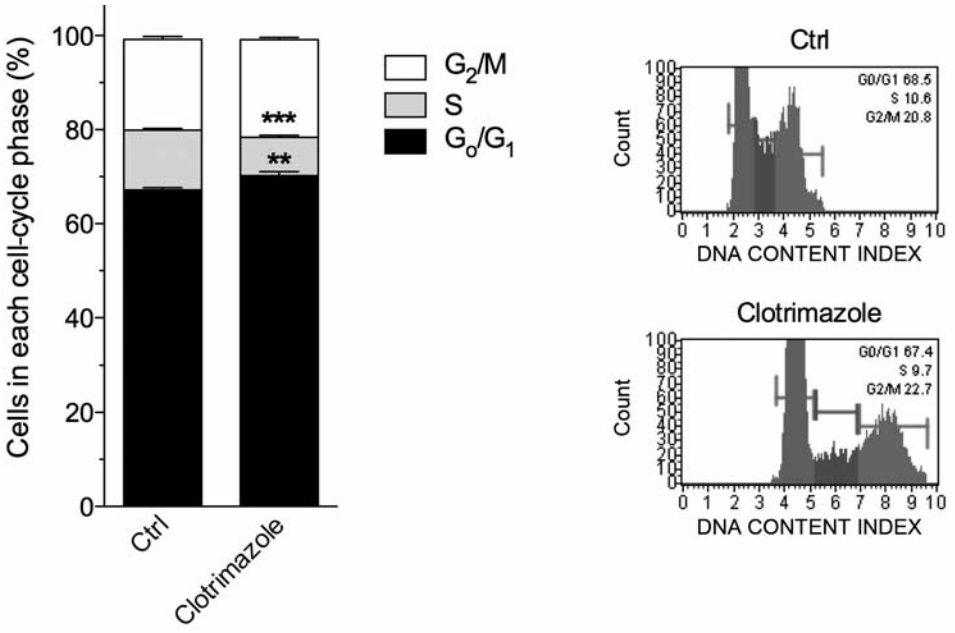

Figure 3. *Effect of clotrimazole on cell-cycle progression. A375 cells were treated with clotrimazole at 10 μM for 48 h. Results are presented as percentage of cells in the different phases (G_0/G_1, G_2 or S) of cell cycle versus total cell number. Representative cell-cycle histograms of treated and untreated cells are also reported. Data were obtained from three independent experiments and expressed as mean±standard error of the mean. $^{**}p<0.01$ versus the control cells at G_0/G_1; $^{***}p<0.001$ versus the control cells at S.*

solutions were prepared on the day of the experiment. Effects were evaluated after 48 h. Vehicle-treated cells were incubated as controls. The final concentration of DMSO in each well did not exceed 0.3% (v/v). This concentration did not affect the cell proliferation or apoptosis of the investigated cell line. Following drug exposure, WST-1 was added and the absorbance was measured at 450 nm using a microplate reader Victor2TM (Wallac, PerkinElmer, Waltham, MA, USA). Optical density values from vehicle-treated cells were considered to represent 100% of cell viability.

Cell death detection. The mechanism of cell death (apoptosis or necrosis) was quantitatively determined using the cell death detection ELISAPLUS assay (Roche Life Science, Indianapolis, IN, USA), as recommended by the manufacturer. A375 cells (1×10^4 cells/well) were plated in 96-well plate in medium with 10% FBS; after 24 h, the complete medium was replaced with 10 μM clotrimazole-containing medium with 1% FBS. The clotrimazole concentration was chosen on the basis of the inhibitory concentration 50% (IC_{50}) value obtained in cell viability experiments. After drug treatment for 24 h at 37˚C, cells were centrifuged and the supernatant maintained at 4˚C to assess necrosis (the DNA fragments released from the cells due to necrosis are present in the supernatant layer). The cell pellet containing the apoptotic bodies was suspended in lysis buffer and incubated for 30 min at room temperature. After centrifugation, supernatant and cell lysate solutions (20 μl) were placed in triplicate into wells of streptavidin-coated microplates, and 80 μl of the immunoreagent containing a mixture of anti-histone-biotin and anti-DNA mAb conjugated with peroxidase (POD) were added. The plate was covered with adhesive foil and incubated for 2 h at room temperature in a shaking incubator at 300 rpm. The unbound antibodies were washed with a specific buffer and the amount of nucleosomes retained by the POD in the immunocomplex was determined photometrically with 2,2'-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid as substrate, using a microplate reader at 405 nm.

Annexin V and 7-amino-actinomyosin (7-AAD) assay. Dual staining with annexin V conjugated to fluorescein-isothiocyanate (FITC) and 7-AAD was performed using the commercially available kit (Muse Annexin V and Dead Cell Kit; Merk KGaA, Darmstadt, Germany). A375 cells were treated with DMSO (control), 10 μM clotrimazole, or 5 μM cisplatin (used as positive control) for 48 h. Both floating and adherent cells were collected, centrifuged at 300 ×*g* for 5 min and suspended in cell culture medium. Then, a 100-μl aliquot of cell suspension (about 5×10^4 cells/ml) was added to 100 μl of fluorescent reagent and incubated for 10 min at room temperature. After incubation, the percentages of living, apoptotic and dead cells were acquired and analyzed by Muse™ Cell Analyzer (Merck KGaA) in accordance with the manufacturer's guidelines. Double staining was used to distinguish between viable, early apoptotic, necrotic, and late apoptotic cells. Annexin V conjugated to fluorescein (annexin V:FITC)-positive and 7-AAD-negative cells were identified as early apoptotic, while annexin V:FITC-positive and 7-AAD-positive cells were identified as in late apoptosis or necrotic.

Cell-cycle analysis. The distribution of A375 cells in the different phases of the cell cycle was performed using the MuseTM Cell Analyzer. Briefly, cells were treated with clotrimazole (10 μM) or vehicle for 48 h. Adherent cells were collected and centrifuged at 300 ×*g* for 5 min. The pellet was washed with phosphate-buffered saline (PBS) and suspended in 100 μl of PBS; finally cells were

slowly added to 1 ml of ice-cold 70% ethanol and maintained overnight at −20˚C. Cell suspension aliquot (containing at least 2×10^5 cells) was then centrifuged at 300 ×g for 5 min, washed once with PBS and suspended in the fluorescent reagent (MuseTM Cell Cycle reagent). After incubation for 30 min at room temperature in the dark, measurements of the percentage of cells in the different phases were acquired.

Real-Time PCR analyses of HK-II gene expression. The assessment of HK II mRNA levels was performed by real-time reverse transcription polymerase chain reaction (real-time RT PCR). In brief, total RNA was extracted from control cells as well as from cells treated with clotrimazole (10 μM) for 24 h, using Rneasy® Mini Kit (Qiagen, Milan, Italy) according to manufacturer's instructions. Purity of the RNA samples was determined by measuring the absorbance at 260:280 nm. cDNA synthesis was performed with 1 μg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. Primers used for RT-PCR were 5'-GATTTCACCAAGCGTGGACT-3' (forward) and 5'-CCACACCCACTGTCACTTTG-3' (reverse) for HK-II, and 5'-GTGAAGGTCGGAGTCAACG-3' (forward) and 5'-GGTGAA GACGGCCAGTGGACTC-3' (reverse) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-PCR reactions consisted of 10 μl SsoFast ™ Eva Green® Supermix (Bio-Rad, Hercules, CA, USA), 0.3 μl of both 25 μM forward and reverse primers, 20 ng of cDNA and milliQ RNase/DNase-free water up to 20 μl. All reactions were performed for 40 cycles using 58˚C and 59˚C as annealing temperature for HK-II and *GAPDH*, respectively. The HKII gene mRNA levels for each sample were normalized against *GAPDH* mRNA levels, and the relative expression was calculated by using the cycle threshold (Ct) value. PCR specificity was verified by both melting curve analysis and gel electrophoresis.

Effect of clotrimazole on HK activity. The ability of clotrimazole to modulate HK activity was evaluated by using the Hexokinase Colorimetric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Specifically, cells were treated with clotrimazole at 10 μM for 48 h and the effect was compared to that of control cells (*i.e.* cells treated with vehicle). HK activity was determined by a coupled enzyme assay, in which glucose is converted to glucose-6-phosphate by hexokinase, which is oxidized by glucose-6-phosphate dehydrogenase to form NADH. The resulting NADH reduces a colorless probe resulting in a colorimetric (450 nm) product proportional to the HK activity present. One unit of HK is the amount of enzyme that will generate 1 μmol of NADH per min at pH 8.0 at room temperature. The absorbance values at 450 nm were measured every 5 min for a total time of 40 min.

Statistical analysis. All experiments were performed in triplicate and the results were analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data are shown as mean values±standard error of the mean. Statistical analyses were performed by Student's *t*-test or one-way ANOVA followed by Bonferroni's multiple comparison test.

Results

Effect on cell viability. Clotrimazole, in the concentration range of 0.1-20 μM for 48 h, reduced A375 cell viability in a concentration-dependent manner, with a mean IC_{50} value of 9.88±0.36 μM (Figure 1). Noteworthy, clotrimazole tested at 10 μM for 48 h (that is, a concentration that approximates the IC_{50} mean value obtained for A375 cells) did not significantly affect HaCaT cell viability (Figure 1).

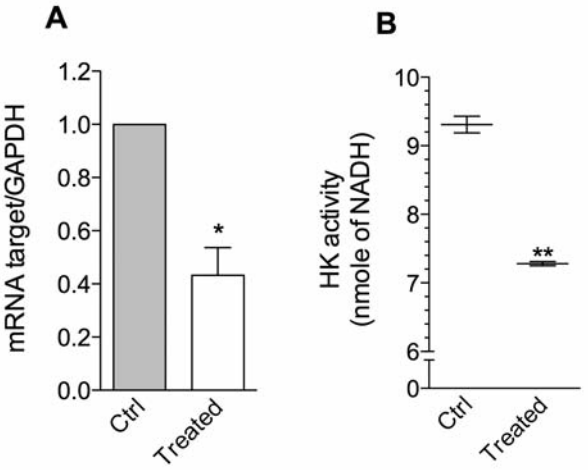

Figure 4. *Effects of clotrimazole on hexokinase expression and activity. A: Real-time PCR assessment of HK-II gene expression normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) after clotrimazole at 10 μM for 24 h. B: Colorimetric assay of hexokinase activity (expressed as nmole of NADH) after clotrimazole at 10 μM for 48 h. Data are obtained from three independent experiments and expressed as mean±standard error of the mean. $^*p<0.05$, $^{**}p<0.01$ versus the control (Ctrl).*

Effect on apoptosis. Experiments assessing annexin V reactivity/cell membrane permeabilization were performed to differentiate early apoptosis from late apoptosis and necrosis. Our findings clearly demonstrated that clotrimazole at 10 μM for 48 h induced apoptosis predominantly in the early stage, with only a modest contribution in the late stages of the process (Figure 2A). In contrast, cisplatin, used as reference compound, promoted cell death by inducing both apoptosis and necrosis of A375 cells (Figure 2A). Apoptosis induced by clotrimazole was more pronounced than that observed with cisplatin when tested at an equiactive concentration, *i.e.* 5 μM (Figure 2A). Clotrimazole at a concentration that approximates the IC_{50} value (*i.e.* 10 μM) induced internucleosomal DNA fragmentation and accumulation of histone-complexed DNA fragments in the cytoplasmic fraction of A375 cells, compared to vehicle-treated cells (Figure 2B). We did not observe an increased production of histone-complexed DNA fragments directly in the culture supernatant (Figure 2B), confirming that clotrimazole induced apoptotic cell death without any involvement of necrosis.

Effects on cell cycle. The analysis of cell-cycle phase distribution of asynchronous cells was assessed by flow cytometry. After treatment with clotrimazole at 10 μM for

48 h, the percentage of S-phase cells decreased compared to the control (11.8 and 20.0% total events, respectively). The parallel increase in the proportion of cells in G_0/G_1 phase in treated (44.6% total events) *versus* untreated (39.5% total events) cells suggest that clotrimazole induced a cell-cycle arrest at G_1 to S phase transition (Figure 3).

Effects on HK expression and activity. To provide insights into the mechanisms underlying the cytotoxic effect of clotrimazole observed on A375 melanoma cells, we analyzed the levels of expression of the HK-II by real-time PCR, in the presence or absence of clotrimazole at 10 μM for 24 h. Our findings demonstrated that treatment of A375 cells with clotrimazole induced a significant ($p<0.05$) decrease in HK-II expression (Figure 4A). We further assessed the effect on HK activity and found that clotrimazole, at 10 μM for 48 h, significantly ($p<0.01$) reduced enzyme activity, compared to control cells (Figure 4B).

Discussion

In the present study, we demonstrated that clotrimazole exerted a concentration-dependent reduction of the A375 melanoma cell viability with a remarkable cytotoxic effect, comparable to that observed for cisplatin. In order to mechanistically explain our observations, we investigated the ability of clotrimazole to induce cell-cycle perturbation and apoptosis. In our experimental conditions, clotrimazole was found to induce a block in cell-cycle progression at the G_1 to S phase transition. Such evidence is consistent with data obtained in human glioblastoma (22), lung cancer (3) and oral squamous carcinoma cells (11) investigating cell-cycle effects of clotrimazole. Interestingly, late G_1 phase is one of the most radiosensitizing phases of the cell cycle and experimental evidence has been provided on the ability of clotrimazole to sensitize glioblastoma cells to radiation *in vitro* (14).

Our results also demonstrated that clotrimazole induced apoptosis in A375 cells, as shown by the alteration of the annexin V reactivity and accumulation of histone-complexed DNA fragments in the cytoplasmic fraction of the cell lysate. Noteworthy, no evidence of necrosis was observed, suggesting that apoptosis was the major mechanism by which clotrimazole induced cell death in A375 cells. By comparing equiactive concentrations from concentration-response curves obtained in cell viability experiments, we demonstrated that clotrimazole primarily induced apoptosis in the early stage, while cisplatin induced apo-necrosis. Different timing of induction of apoptosis observed in the current study was in line with data showing how induction of different modes of cell death by cisplatin in cells may represent an interesting pharmacological strategy in the development of new combination schedules for glioblastoma (22) and gastric cancer (23).

HK-II 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 (24, 25). In the current study, we provided evidence that treatment with clotrimazole at the IC_{50} value induced a significant reduction of both expression and activity of HK in A375 cells. It is, therefore, conceivable that under our experimental conditions, cell-cycle arrest and apoptosis were correlated with the clotrimazole-induced inhibition of HK-II and the corresponding depletion of cellular ATP levels. Such a hypothesis has also been suggested by Liu and co-workers (14), who demonstrated that clotrimazole was able to translocate mitochondrial-bound HK-II to the cytoplasm inducing the release of cytochrome *c* into the cytoplasm on human glioblastoma cells. Interestingly, A375 cells harbor a v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) missense mutation (V600E) (26), which has been demonstrated to cause the up-regulation of genes involved in glycolysis (27). We demonstrated that clotrimazole, at a concentration that approximates the IC_{50} value in cell viability experiments, significantly reduced HK-II expression and induced apoptosis in A375 cells without affecting the viability of proliferating human keratinocytes, suggesting that the compound may exhibit some selectivity for cancer cells. These findings are in agreement with the Warburg effect (28), as well as with evidence showing that melanoma cells exhibit a higher rate of glycolysis, compared to their normal counterpart cells (27).

Overall, findings of the current study demonstrated a pro-apoptotic activity of clotrimazole against human melanoma cells, comparable to that observed for cisplatin. Furthermore, the ability of clotrimazole to alter the glycolysis pathway and induce cell cycle arrest in the G_1-S phase transition suggest that this drug could sensitize tumor cells to radiation and chemotherapeutic agents.

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