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Abstract

In this study, we demonstrated that MIC dose-dependently arrested various human cancer cells at the G0/G1 phase of the cell cycle. The protein levels of p53, p21/Cip1, and p27/Kip1 were significantly elevated by MIC treatment in COLO-205 cells. Electrophoretic mobility gel shift assays (EMSA) showed that the nuclear extracts of the MIC-treated COLO-205 cells exerted a significant binding between wild type p53 and its consensus-binding site present in the p21/Cip1 promoter. These results suggested that the p53-associated signaling pathway is involved in the regulation of MIC-induced cancer cell growth arrest. By immunoblot analysis, we demonstrated that cyclin D3 and cyclin-dependent kinase-4 (CDK4) protein levels were inhibited by MIC-treatment in the cancer cells. Significant therapeutic effect was further demonstrated in vivo by treating nude mice bearing COLO-205 tumor xenografts with MIC (50 mg/kg, i.p.). The protein expression of p53 was significantly increased in MIC-treated tumor tissues by immunohistochemical staining technique. DNA fragmentation and TUNEL assay were performed and demonstrated that apoptosis occurred in tumor tissues treated with MIC. Our study provides the novel mechanisms of antitumor effects of MIC and such results may have significant applications for cancer chemotherapy.

Keywords: Oral antifungal agents, apoptosis, cell cycle

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The discovery of antifungal activity of azole compounds represented an important therapeutic advance. Miconazole (MIC), ketoconazole (KT), itraconazole, and fluconazole are currently commercially available (Bodey, 1992). Among its disadvantage are limited absorption in the absence of gastric acid and its potential for drug-drug interactions; many clinicians believe that topical MIC is a relatively effective agent for the treatment of most mycotic infections (Diehl, 1996). Because of its limited activity and toxicity, the MIC has now been replaced by newer agents (such as terbinafine) (Leenutaphong *et al.*, 1999; McClellan *et al.*, 1999). MIC, KT, bifonazole, clotrimazole, econazole, isoconazole and tioconazole are known inhibitors of cytochrome p-450 dependent steroidogenic enzymes (Ayub and Levell, 1989). Another study indicated that KT and MIC inhibits cholesteryl ester formation in macrophages by blocking the intracellular transport of endocytosed cholesterol from lysosomes to the endoplasmic reticulum (Aikawa *et al.*,

1999). These antifungal imidazoles MIC and KT are known to inhibit synthesis of essential cell membrane components. Furthermore, MIC can exert direct physiochemical cell membrane damage at relatively high levels, but KT cannot (Beggs, 1984).

The antitumor effects of anti-fungal agents (such as KT) were investigated in several other laboratories (Blagosklonny *et al*., 2000; Bok and Small, 1999; Heyns *et al*., 1985; Mahler and Denis, 1992; Trachtenberg, 1984a; Trachtenberg, 1984b; Trachtenberg and Pont, 1984). In this study, we further demonstrated that MIC induced growth inhibition in various human cancer cells through G0/G1 cell cycle arrest. The therapeutic efficacy was further examined in vivo by treating athymic mice bearing COLO-205 tumor xenografts with MIC (50 mg/Kg, i.p.). This study provides further evidences that the antifungal agent, MIC, might also have significant applications for cancer chemotherapy.

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MIC Induces G0/G1 Cell Cycle Arrest in Various Human Cancer Cells with Different p53 Status

As shown in figure 1, MIC (10-50 μM) induced a dose-dependent inhibition of cell growth in various human cancer cells. As compared to human cancer cells, the MIC-induced growth arrest of human normal keratinocytes (#76 KhGH) was less profound (Figure 1). Figure 2A showed a representative fluorescence-activated cell sorter (FACS) analysis of DNA content at various times after release from quiescence by incubation in culture media supplemented with 10% FCS and 0.1% DMSO. Figure 2B showed that MIC (20 μM) induced a significant accumulation (>85 %) of cells in G0/G1 phase of the cell cycle, suggesting that the observed growth inhibitory effect of MIC in the figure 1 was due to an arrest of DNA replication in the cell cycle.

Dose-dependent Response of Cells to MIC-induced G0/G1 arrest

As shown in the figure 3, significant apoptosis was induced in cells treated with higher dose of MIC (> 40 ì M). However, G0/G1 arrest was observed in cells exposed to lower concentration of MIC (< 30 ì M). Our recent report indicated that p53 was involved in KT-induced G0/G1 arrest and apoptosis in COLO 205 cells (Chen *et al*., 2000; Ho *et al*., 1998). The present study further demonstrated that G0/G1 cell cycle arrest and apoptosis were easily induced in the cells with wild type p53 (COLO 205 and Hep G2) by MIC treatment. Such results suggesting that p53 might be involved in MIC-induced G0/G1 arrest and apoptosis.

MIC-Induced Cancer Cells Apoptosis through Caspase-3 Activation

Figure 3 shows that the sub-G1 peak was observed in cells treated with higher doses of MIC (> 40 ì M). Such results revealed that apoptotic cells were presented in MIC treated group. We further demonstrated that COLO 205 and HT 29 cells treated with MIC (20-50ì M) exhibited morphological changes were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Figure 4A). The apparent DNA ladder appeared at 24 hr after 30 ì M of MIC treatment in the COLO 205 cells (Figure 4A). Figure 4B shows that the caspase-3 was activated in COLO 205 cells at 24 hr after MIC (30ì M) exposure. Previous report demonstrated that the substrate of caspase-3 is the poly-ADP ribose polymerase (PARP) (Tewari *et al*., 1995). Western blotting analysis revealed that the Mr. 116,000 PARP molecule was degraded to a relatively stable Mr. ~85,000 fragment at 24 hr after MIC (10-35ì M) treatment (Figure 3C). Our study demonstrated that MIC-induced cancer cells apoptosis was at least through caspase-3 pahway.

The p53 and p21/CIP1 Were the Key Regulators in MIC-induced G0/G1 Arrest

Based on the FACS analysis presented in the figure 2A showed that 0, 15, 18 and 24 h represents the G0/G1, S, G2/M and 2nd G0/G1 phase. Accordingly, this time point (15 h) was selected for studying the dose-dependent effect of MIC and the changes of p53 proteins for induction of G0/G1 arrest was determined by western blotting analysis (Figure 5). Our data

demonstrated that the activated p53 was more significantly induced in the COLO 205 cells (with wild type p53) (Figure 5 A). As shown in the Figure 5 B, the MIC-treated cells showed that the up-regulation of p21/Cip1 protein expression was observed initially at 6 h after MIC treatment and persisted for at least 24 h (Figure 5B). In contrast, in the DMSO-treated control group, the expression of p21/Cip1 in the cell was up-regulated at 6 h after cells were challenged with 10% FCS and then rapidly down regulated at 15 h after treatment.

To further demonstrate the p53 protein in cells was activated by MIC treatment, Electrophoretic mobility gel shift assay (EMSA) was conducted in both of the COLO 205 and HT 29 cells. The EMSA results showed that the nuclear extracts of the MIC-treated COLO 205 cells exerted a significant binding between wild type p53 protein and its consensus-binding site in the p21/Cip1 promoter region (Figure 5C).

MIC Induces Elevation of p21/Cip1, p27/Kip1 and Inhibition of Cyclin D3 and CDK4 Protein Expression

As shown in figure 5 and 6, the protein levels of both p53 and p21/Cip1 were induced and the CDK4 protein expression was inhibited in the MIC-treated COLO 205 cells (with wild type p53). Interestingly, the other cell cycle negative regulator, p27/Kip1, protein expression were more significantly induced in the p53-null (HL 60), p53-deleted (Hep 3B) and the p53 His^{273} mutant (HT 29) cells. Such results implied that p27/Kip1 may be involved in the MIC-induced G0/G1 cell cycle arrest through a p53-independent pathway in these cells.

As shown in the figure 6, the protein levels of cyclin D3, and CDK4 in the MIC-treated cells were down-regulated after treatment with MIC while the cyclin D1 and PCNA were not changed significantly. In this study, the faster migration form of cyclin A2 (58 kDa) and cyclin B, which promote cells entry from G0/G1 into S and from S into G2/M phase respectively, were also down regulated dose-dependently in MIC-treated cancer cells (Figure 6). The protein level of CDK2 was not significantly changed in MIC-treated cells. We further determined the CDK2-associated protein, cyclin E, protein expression and demonstrated that the cyclin E protein was slightly inhibited in MIC-treated cells (Figure 6).

MIC-induced G0/G1 Arrest Was Through Inhibit of CDK4 Kinase Activity

Our results revealed that the decreased CDK4 kinase activity was concomitant with increased expression of p21/Cip1 and p27/Kip1 in cells treated with MIC (Figure 7). These results implied that the G0/G1 arrest induced by MIC was due to decrease the kinase activity of CDK2 and CDK4 mediated by an increase of p21/Cip1 (or p27/Kip1)-CDKs association.

MIC Causes Tumor Regression in vivo

We further examined the therapeutic efficacy of MIC in vivo by treating athymic mice bearing COLO-205 tumor xenografts, using concentrations of MIC (50 mg/Kg). After establishment of palpable tumors (mean tumor volume, 200 mm^3), animal received intraperitoneal injections of MIC three times per week, as well as DMSO for a negative control. After 6 weeks, tumor volume in MIC was significant inhibited in comparison with DMSO-treated controls (Figure 8 A and B). In mice receiving these treatment regimens, no gross signs of toxicity were observed (body weight, visible inspection of general appearance and microscopic examination of individual organs) (Figure 8 C). However, the tumor weight and the tumor/body weight ratio were strongly inhibited in the MIC-treated group (Figure 8 D and E). Our results provide further evidences that such observations may have significance of application for cancer chemotherapeutic purposes.

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