Antitumor Effects of Miconazole on Human Colon Carcinoma Xenografts in Nude Mice through Induction of Apoptosis and G0/G1 Cell Cycle Arrest

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Miconazole (MIC), a promising oral antifungal agent, has been used worldwide in the treatment of superficial mycosis. 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 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 ip). The protein expression of p53 was significantly increased in MIC-treated tumor tissues by immunohistochemical staining and Western blotting analysis. 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.

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Key Words: miconazole; G0; G1 arrest; apoptosis; p53; nude mice.

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2 Abbreviations used: CDK, cyclin-dependent kinase; CKIs, CDK inhibitors; EMUSA, electrophoretic mobility gel shift assays; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; IP, immunoprecipitation; KT, ketoconazole; MIC, miconazole; PARP, poly(ADP ribose) polymerase.
could inhibit hepatic metastasis from a human pancreatic adenocarcinoma (Tzanakakis et al., 1990) and reduce the incidence of pulmonary metastases in the nude mice melanoma model (Nardone et al., 1988). We have recently demonstrated that administration of KT induced apoptosis and G0/G1 cell cycle arrest in various types of human cancer cells (Ho et al., 1998; Chen et al., 2000). We also demonstrated that the oral antifungal agent griseofulvin induced apoptosis and G2/M arrest through abnormal microtubule polymerization (Ho et al., 2001). The therapeutic effects of griseofulvin were further examined in vivo by treating athymic mice bearing COLO 205 tumor xenographs, using either 50 mg/kg griseofulvin or 5 mg/kg nocodazole and combined treatment of both agents. Combined treatment of griseofulvin and nocodazole significantly enhanced the effect of nocodazole, leading to cessation of tumor growth. Such results implied that combined administration of chemotherapeutic agents (such as nocodazole) and griseofulvin might provide a novel therapy for colorectal cancer (Ho et al., 2001).

The current options for treating human cancer are limited to excision surgery, general chemotherapy, radiation therapy, and, in a minority of breast cancers that rely on estrogen for their growth, antiestrogen therapy. Obviously, there is an urgent need for new therapeutic strategies focusing on inhibition of cancer cell proliferation. As in our previous studies, the oral antifungal agents griseofulvin and KT have been demonstrated to repress tumor cell growth in vitro and in vivo (Ho et al., 1998, 2001; Chen et al., 2000). The antitumor effects of antifungal 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 and Pont, 1984; Trachtenberg, 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 xenographs with 50 mg/kg ip MIC. This study provides further evidences that the antifungal agent MIC might also have significant applications for cancer chemotherapy.

MATERIALS AND METHODS

Cell lines and cell culture. The HT 29 and COLO 205 cell lines were isolated from human colon adenocarcinoma (American Type Culture Collection HMIC-38 and CCL-222; Barchiesi et al., 2000). HEP 3B and HEP G2 cell lines were derived from human hepatocellular carcinoma (American Type Culture Collection HB-8064 and HB-8065; Knowles et al., 1980). HL 60 was developed from human myeloid leukemia cells. The cell line 76 Kgh (ATCC CRL 8858) was composed of keratinocytes derived from normal human epidermis. The p53 gene in the COLO 205 and HEP G2 cells were wild type (Ho et al., 1996; Bressac et al., 1990). In contrast, the p53 gene is mutated in codon 273 in HT 29 cells (Newkirk et al., 1995). The p53 gene was found to be partially deleted (5 kb) in HEP 3B cells (Bressac et al., 1990) and null in the HL 60 cells (Jui et al., 1998). Cell lines were grown at 37°C in a 5% carbon dioxide atmosphere in Eagle’s minimal essential medium (for HEP 3B and HEP G2) or RPMI 1640 (for COLO 205, HT 29, and HL 60 cells) supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycin, and 0.3 mg/ml glucose. A 3:1 mixture of Ham’s F-12 medium and DMEM medium (for 76 Kgh cells) supplemented with 10% FCS, 40 ng/ml hydrocortisone, 0.01 mg/ml cholaer toxin, 0.005 mg/ml insulin, and 10 ng/ml epidermal growth factor was used.

Determination of cell viability. Human cancer cells and keratinocytes were treated with MIC (10–50 μM). Cell viability was determined at indicated times based on the trypan blue exclusion method. The viability percentage was calculated based on the percentage of unstained cells as described previously (Ho et al., 1998).

Determination of cell growth curve. Human colon cancer (10 × 10⁶), hepatoma (12 × 10⁶), human leukemia (10 × 10⁴), and human normal keratinocytes (10 × 10⁴) cells were plated in 25-mm petri dishes. The next day, the medium was changed and MIC (10–50 μM) was added. Control cells were treated with dimethyl sulfoxide in a final concentration of 0.05% (v/v). The incubation medium was renewed every day during the experiment. At the end of incubation, cells were harvested for cell count with a hemocytometer.

Cell synchronization, drug treatment, and flow cytometry analysis. At 24 h after plating of cells, medium was removed. Cells were washed three times with phosphate-buffered saline and then incubated with medium containing 0.04% FCS for 24 h. Under such conditions, cells were arrested in G0/G1 as determined by flow cytometry analysis. After serum starvation, the low-serum (0.04% FCS) medium was removed and the cells were then stimulated by the addition of medium containing 10% FCS. MIC solutions were prepared by dissolving this compound in a final concentration of 0.05% DMSO. The cell cycle stages in MIC- and mock-treated groups were measured by flow cytometry analysis. Cells were incubated with 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), and DNA content was measured using a FACScan laser flow cytometer analysis system (Becton-Dickinson, San Jose, CA; 15,000 events were analyzed for each sample.

Western analysis. Western blotting analysis was performed by the method described previously (Ho et al., 2001). Briefly, cell lysates were prepared, electrophoresed, immunoblotted with antibodies, and then visualized by incubating with the colorogenic substrates (nitroblue tetrazolium, NBТ and 5-bromo-4-chloro-3-indolyl phosphate, BCIP (Sigma). The expression of GAPDH was used as the control for equal protein loading.

Immunoprecipitation, CDK2, and CDK4 kinase activity assay. The p21-associated CDK2-2 kinase activity was determined as described by Wu et al. (1990). Briefly, using 2 μg anti-CDK2 antibody and 20 μl protein A agarose beads, the protein complexes were precipitated from 200 μg of protein lysate per sample as described above. Beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 40 μl of “hot” kinase solution [0.25 μl (2.5 μg) of histone H1, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed using 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.

Similarly, the CDK4 kinase activity was determined as described by Wu et al. (1996) with some modifications. Briefly, MIC-treated cells were lysed in Rb lysis buffer (50 mM Hepes–KOH, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupetin and aprotinin) and immunoprecipitated with 2 μg anti-CDK4 antibody. The protein complexes in beads were washed twice with Rb lysis buffer and then once with Rb kinase assay buffer (50 mM Hepes–KOH, pH 7.5, containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM MgCl₂, and 1 mM DTT). Phosphorylation of Rb was measured by incubating the beads with 40 μl of hot Rb kinase solution [0.25 μl (2 μg) Rb–GST fusion protein, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of Rb kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed using 12% SDS–PAGE, and the gel was dried and subjected to autoradiography.
Electrophoretic mobility shift (EMSA) assay (Somasundaram et al., 1997). The double-stranded DNA probe used in the experiment contained the following sequence: the p21 promoter (5'-CAGGAACAGTCTCCAACATGGTAGC-3'). The radiolabeled DNA (4 ng, 100,000 cpm) was incubated with nuclear extract in 15 μl of binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 200 mM NaCl, and 1 μg probe DNA) on ice for 5 min. The samples were electrophoresed in a 5% polyacrylamide gel at room temperature for 3 h at 130 V. The gel was dried on Whatman 3M paper and exposed to Fuji X-ray film at −70°C.

Treatment of COLO 205-derived xenografts in vivo (Ho et al., 2001). COLO 205 cells were grown in RPMI 1640 supplemented with 10% FCS as described above. Cells were harvested through two consecutive trypsinizations, centrifuged at 300g for 5 min, washed twice, and resuspended in sterile PBS. Cells (5 × 10⁶) in 0.2 ml were injected subcutaneously between the scapulae of each nude mouse (purchased from National Science Council Animal Center, Taipei, Taiwan). After transplantation, tumor size was measured using calipers and the tumor volume was estimated according to the formula tumor volume (mm³) = L × W²/2, where L is the length and W is the width (Oki et al., 1998). Once tumors reached a mean size of 200 mm³, animals received ip injections of either 25 μl DMSO or 50 mg/kg MIC three times per week for 6 weeks.

DNA fragmentation analysis in tumor tissues treated with MIC. The tumor tissues were excised at the end of experiment. Part of the tumor was frozen in liquid nitrogen and the remainder was fixed and stained with TdT FragEL DNA fragmentation detection kit (Calbiochem Co., Cambridge, MA) for detection of apoptotic cells in tumor tissues. The DNA was isolated from frozen tumor tissues in the MIC-treated and control group by incubation overnight with nuclease-free proteinase K at 55°C in 10 mM Tris, pH 7.5, 150 mM EDTA, and 0.5% sodium dodecyl sulfate (Ho et al., 1995). The DNA was then used for detection of DNA laddering as described in our recent report (Ho et al., 1998).

Immunohistochemical staining. Paraffin-embedded blocks were sectioned at about 4-μm thickness, deparaffinized, and rehydrated. After microwave pretreatment in citrate buffer, pH 6.0, for antigen retrieval, slides were immersed in 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. After washing, slides were incubated overnight at 4°C with the p53-specific antibody (Santa Cruz Biotechnology) in a dilution of 1:50. After a second incubation with a biotinylated anti-mouse antibody, slides were incubated with peroxidase-conjugated streptavidin (DAKO LSAB + kit; Dako Corp., Carpinteria, CA). Reaction products were visualized by immersing slides in a diaminobenzidine tetrachloride and finally counterstained with malachite green. We performed control immunostaining using preabsorption of anti-p53 antibody with human synthetic p53 peptide (Santa Cruz Biotechnology) to determine the specificity of primary antibody.

RESULTS

MIC-Induced Cytotoxicity in Human Cancer Cells

In this study, normal human keratinocyte were treated with different concentrations (10–50 μM) of MIC and the viability of the cells was determined (Fig. 1A). As seen in Fig. 1A, no significant influence on the viability of the keratinocytes was observed when these cells were treated with lower doses of MIC (<40 μM) (Fig. 1A). Lower viability (66 ± 6.17%) was only detected in cells treated with a higher dose of MIC (50 μM). In Figs. 1B and 1C, different types of human cancer cells and normal keratinocytes were treated with either a lower dose (20 μM, Fig. 1A) or a higher dose (50 μM, Fig. 1B) of MIC at the indicated time points. Significant cytotoxicity was observed in all types of human cancer cells treated with 50 μM MIC (Fig. 1C). In contrast, influences of viability observed in cancer cells treated with the lower dose (20 μM) of MIC were not significant (Fig. 1B). Such results indicate that human cancer cells were more sensitive to MIC exposure than normal human keratinocytes.

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

As shown in Fig. 1, the lower dose of MIC (20 μM) did not affect the cell viability of human cancer cells. To investigate whether MIC arrested human cancer cell growth, the cell growth numbers were then determined (Fig. 2). MIC (10–50 μM) induced a dose-dependent inhibition of cell growth in various human cancer cells (Figs. 2A–2E). The cell growth inhibitory effects on keratinocytes were less profound in response to the lower dose (<30 μM) of MIC treatment. Signif-
icant cell growth arrest and cytotoxicity effects were only observed in keratinocytes treated with a higher dose of MIC (>40 μM) 48 h later (Fig. 2F). Such results indicated that normal keratinocytes were more resistant than cancer cells to MIC treatment.

In order to examine further the actions of MIC on the cellular mitotic cycle, the COLO 205 cells were switched to media with 0.04% FCS for 24 h to render them quiescent and to synchronize their mitotic activities at the G0/G1 phase. They were then returned to culture media supplemented with 10% FCS and, at various times thereafter, were harvested for flow cytometry analyses. Figure 3A shows 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 3B shows that MIC (20 μM) induced a significant accumulation (>85%) of cells in the G0/G1 phase of the cell cycle, suggesting that the observed growth inhibitory effect of MIC shown in Fig. 2 was due to an arrest of DNA replication in the cell cycle.

**Dose-Dependent Response of Cells to MIC-Induced G0/G1 Arrest**

As illustrated in Fig. 3, the biggest difference in the G0/G1 cell population between the MIC-treated and control groups was seen 15 h after replacement with complete medium. Accordingly, this time point (15 h) was selected for studying the dose-dependent effect of MIC and the minimal dose of KT required for induction of G0/G1 arrest determined by flow cytometry analysis (Fig. 4). As shown in Fig. 4, significant apoptosis was induced in cancer cells (COLO 205, Hep 3B, Hep G2, and HL 60 cells) treated with higher doses of MIC (>40 μM) (Figs. 4B–4E). However, G0/G1 arrest was observed in cells exposed to lower concentrations of MIC (<30 μM) (Figs. 4A and 4B). Our recent reports indicated that p53 was involved in KT-induced G0/G1 arrest and apoptosis in COLO 205 cells (Ho et al., 1998; Chen et al., 2000). The present study further demonstrated that G0/G1 cell cycle arrest and apoptosis were easily induced by MIC treatment in cells with wild-type p53 (COLO 205 and Hep G2). Such results...
suggest that p53 might be involved in MIC-induced G0/G1 arrest and apoptosis.

**MIC-Induced Cancer Cell Apoptosis through Caspase-3 Activation**

Figure 4 shows that the sub-G1 peak was observed in cells treated with higher doses of MIC (10–35 μM). Such results revealed that apoptotic cells were present in the MIC-treated group. We further demonstrated that COLO 205 and HT 29 cells treated with MIC (20–50 μM) exhibited morphological changes accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Fig. 5A). The apparent DNA ladder appeared at 24 h after 30 μM MIC treatment in the COLO 205 cells (with wild-type p53) (Fig. 5A). To further measure the caspase-3 activity in cell response to MIC treatment, human cancer cells (COLO 205 and HT 29) and normal human keratinocytes were treated with MIC in a dose-dependent manner (10–30 μM) and the caspase-3 and poly(ADP ribose) polymerase (PARP) protein levels were then detected. Figure 5B shows that the caspase-3 was more easily activated in COLO 205 cells at 24 h after MIC exposure. The activated caspase-3 was not detected in normal keratinocytes during 24 h of MIC treatment. A previous report demonstrated that the substrate of caspase-3 is PARP (Tewari et al., 1993). Western blotting analysis revealed that the M, 116,000 PARP molecule was degraded to a relatively stable M, ~85,000 fragment 24 h after MIC (10–35 μM) treatment (Fig. 5C). Our study demonstrated that MIC induced cancer cell apoptosis at least through the caspase-3 pathway.

**p53 and p21/CIP1 Were the Key Regulators in MIC-Induced G0/G1 Arrest**

Previous studies have demonstrated that the p53 protein is a potent transcription factor, which is activated and accumulated in the nucleus in response to different DNA-damaging agents (Haffner and Oren, 1995; Kastan et al., 1991), leading to cell cycle arrest or apoptosis occurrence (Ko and Prives, 1996; Levine, 1997). Our recent reports demonstrated that KT induced p53 protein expression and caused the occurrence of apoptosis and G0/G1 cell cycle arrest in various human cancer cells (Ho et al., 1998; Chen et al., 2000).

Based on the FACS analysis presented in Fig. 3A, 0, 15, 18, and 24 h represent the G0/G1, S, G2/M, and second G0/G1 phases. 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 (Fig. 6). Our data demonstrated that the activated p53 was more significantly induced in the COLO 205 cells (with wild-type p53) (Fig. 6A). As shown in Fig. 6B, the MIC-treated cells showed that the up-regulation of p21/Cip1 protein expression was observed initially at 6 h (Fig. 6B, lane 4) after MIC treatment and persisted for at least 24 h (Fig. 6B, lane 12). In contrast, in the DMSO-treated control group, the expression of p21/Cip1 in the cell was up-regulated at 6–15 h (Fig. 6B, lanes 3 and 5) after cells were challenged with 10% FCS and then rapidly down-regulated at 18 h (Fig. 6B, lane 7) after treatment.

As described previously, p53 was demonstrated to be a transcription factor that up-regulates the p21/Cip1 protein expression (el-Deiry et al., 1993). These results suggest that the increased expression of p53 and p21/Cip1 protein is critical for MIC-mediated G0/G1 arrest. To further demonstrate that p53 protein in cells was activated by MIC treatment, EMSA was conducted in both the COLO 205 and the 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 (Fig. 6C).

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

To further scrutinize the role p53 plays in G0/G1 arrest in cells treated with MIC, four types of human cancer cells with different p53 status were studied and the levels of cell-cycle-regulated protein expression were determined after MIC (10–50 μM) treatment (Fig. 7). As shown in Figs. 6 and 7, 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, in the other cell-cycle-negative regulator, p27/Kip1, protein expression was more significantly induced in the p53-null (HL 60), p53-deleted (Hep 3B), and p53 His^{273} mutant (HT 29) cells. These 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 Fig. 7, the protein levels of cyclin D1, D3, and CDK4 in the MIC-treated cells were down-regulated after treatment with MIC, while PCNA was induced in the Hep 3B and HL 60 cells. Previous studies have demonstrated that two types of cyclin A (A1, 66 kDa and A2, 58 kDa) exist in mammalian cells. Cyclin A1 differs from other cyclins in its highly restricted expression pattern. Besides its expression during spermatogenesis (Ravnik and Wolgemuth, 1999; Liu et al., 1998), cyclin A1 is expressed in hematopoietic progenitor cells and in acute myeloid leukemia (Muller et al., 1999). Cyclin A2 is the major regulator of the cell cycle progression and its synthesis is required for progression to S phase (Howe et al., 1995). In this study, the faster migration forms of cyclin A2 (58 kDa) and cyclin B, which promote cell 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 (Fig. 7). The protein level of CDK2 was not significantly changed in MIC-treated cells. We further determined the CDK2-associated protein, cyclin E, expression and demonstrated that the cyclin E protein was slightly inhibited in MIC-treated cells (Fig. 7).

**MIC-Induced G0/G1 Arrest Was through Inhibition of CDK4 Kinase Activity**

As described above, p21/Cip1 and p27/Kip1 were potent inhibitors of CDKs. We suggest that MIC treatment may result in the inhibition of CDK4 kinase, which is necessary for cells to overcome the restriction point in the G0/G1 phase of the cell cycle. Our results revealed that the decreased CDK4 kinase

**FIG. 4.** Dose-dependent induction of G0/G1 phase arrest and apoptosis in human cancer cells. (A) HT 29, (B) COLO 205, (C) Hep 3B, (D) Hep G2, (E) HL 60, and (F) keratinocytes cells were treated by MIC (10–80 μM). FACS analysis of DNA content after 15 h release from quiescence by incubation in culture media supplemented with 10% FCS or MIC. Three samples were analyzed in each group, and values represent the mean ± SE.
activity was concomitant with increased expression of p21/Cip1 and p27/Kip1 in cells treated with MIC (Fig. 8). These results implied that the G0/G1 arrest induced by MIC was due to decreased 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,

**Fig. 5.** MIC induced human cancer cell apoptosis through caspase-3 activation. (A) DNA fragmentation analysis in human colon cancer cells undergoing MIC-induced apoptosis. COLO 205 and HT 29 cells were treated with MIC (20–50 μM) and DNA fragmentation was examined 24 h later. Cells were mock treated with DMSO (0.05%) as controls. (B) Caspase-3 activation induced by MIC. The COLO 205, HT 29, and keratinocyte cells were treated with MIC (10–30 μM) and the caspase-3 level was detected by immunoblotting analysis. (C) The PARP protein was degraded by activated caspase-3. The COLO 205, HT 29, and keratinocyte cells were treated with MIC (10–35 μM) and the protein extracts were isolated for immunoblotting analysis.

**Fig. 6.** Effect of MIC on the p53 signaling regulatory proteins in human cancer cells with different p53 status. (A) Dose-dependent response of MIC-induced p53 protein expression in human cancer cells. The HT 29, COLO 205, and HL 60 cells were synchronized with 0.04% FCS for 24 h as described under Materials and Methods. After synchronization, cells were then released into complete medium (10% FCS) containing MIC (10–60 μM) for 15 h. The control group was treated with DMSO (0.05%, v/v). Protein extracts (100 μg/lane) were separated by SDS-PAGE, immunoblotted with p53-specific antibody, and detected using the NBT/BCIP system. (B) Time-dependent response of MIC-induced p21/Cip1 protein levels in COLO 205 cells. COLO 205 cells were synchronized with 0.04% FCS and 0.05% DMSO with or without MIC (30 μM), the cells were prepared for protein extraction and EMSA assays were performed.
using concentrations of MIC (50 mg/kg). After establishment of palpable tumors (mean tumor volume, 200 mm$^3$), animals 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 significantly inhibited in comparison with DMSO-treated controls (Figs. 9A and 9B). 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) (Fig. 9C). However, the tumor weight and the tumor/body weight ratio were strongly inhibited in the MIC-treated group (Figs. 9D and 9E). Our results provide further evidence that such observations may have significance for cancer chemotherapeutic purposes.

p53 Induction and Apoptosis Are the Major Mechanisms of MIC-Inhibited Tumor Growth in Vivo

In order to demonstrate that apoptosis was induced in MIC-treated tumor tissues, DNA was extracted from tumor tissues in the control and the MIC-treated groups. Our results demonstrated that significant DNA fragmentation occurred in MIC-treated tumor xenografts (Fig. 10A). The apoptotic cells in tumor tissues were further detected microscopically by

![FIG. 7.](image_url) Effect of MIC on the expression levels of G0/G1 phase regulatory proteins in human cancer cell lines with various p53 status. The cells were rendered quiescent by incubation for 24 h in the cultured media containing 0.04% FCS. After 15-h release from quiescence by incubation in culture media supplemented with 10% FCS and 0.05% DMSO with or without MIC (20–50 μM), the cells were harvested and protein extracts (100 μg per lane) were separated by SDS–PAGE. After electrophoresis, proteins were transferred onto Immobilon-P membranes, probed with proper dilutions of specific antibodies, and then detected by using the NBT/BCIP system. Membrane was also probed with anti-GAPDH antibody to correct for differences in protein loading.

![FIG. 8.](image_url) MIC-induced G0/G1 cell cycle arrest through p53-dependent and -independent signaling pathway. Human cancer cells with different p53 status (HT 29, COLO 205, or HL 60) were rendered quiescent by incubation for 24 h in the cultured media containing 0.04% FCS. After 15-h release from quiescence by incubation in culture media supplemented with 10% FCS and 0.05% DMSO with or without MIC (20 μM), the cells were harvested for protein extraction. Protein extracts (100 μg/lane) were separated by SDS–PAGE. After electrophoresis, proteins were transferred onto Immobilon-P membranes, probed with proper dilutions of specific antibodies, and then detected by using the NBT/BCIP system. The CDK2 and CDK4 kinase activity were detected as described under Materials and Methods.
The growth of COLO 205 tumor xenografts in nude mice was reduced by MIC treatment. COLO 205 cells were injected subcutaneously between the scapulas of athymic nude mice. Once tumor volume reached approximately 200 mm$^3$, the animal received an injection of 50 mg/kg ip MIC or DMSO three times per week for 6 weeks. (A) Gross appearance of subcutaneous tumors after treatment with MIC (left) or DMSO (right) for 6 weeks. Insets show the isolated tumors. (B) Average tumor volume of DMSO-treated (circles, $n=5$) versus MIC-treated (squares, $n=5$) nude mice. (C) Body weight, (D) tumor weight, and (E) tumor/body weight ratio were measured at the end of the experiment. Five samples were analyzed in each group, and values represent the mean ± SE. Comparisons were subjected to Student’s $t$ test. *Significantly different at $p < 0.05$.
FIG. 10. MIC induces apoptosis and increased expression of p53 protein in COLO 205 tumors. (A) Tumor tissues excised from nude mice with COLO 205 tumor xenografts were dissected and DNA fragmentation was examined. (B) The paraffin-embedded sections were stained with TUNEL assay for detection of apoptotic cells (arrowhead). (C) The protein extracts were isolated from the frozen tumor tissues and the p53, p21/Cip1, and p27/Kip1 proteins were detected by immunoblotting analysis. Microscopic view of the paraffin sections stained with H & E shown in (D) DMSO- (as a control) and (E) MIC-treated groups. Immunohistochemical stain analysis of the p53 protein expression in (F) DMSO (as a control) and (G) MIC-treated tumor sections. Magnification 400×
TUNEL staining (Fig. 10B, arrowhead). We further demonstrated that the p53, p21/Cip1, and the p27/Kip1 proteins were significantly induced in the tumor tissues by Western blotting analysis (Fig. 10C). Such results were consistent with the in vitro studies as described above. The morphology of the apoptotic cells in tumor tissues was also observed by the H & E staining technique (Figs. 10D and 10E). Our results demonstrated that apoptotic cells observed in the MIC-treated group were more significant in tumor tissues compared with the control group (Figs. 10D and 10E, arrowhead). To further investigate the localization of the induced p53 protein in tumor tissues treated with MIC, an immunohistochemical stain technique was performed. As shown in Fig. 10, the p53 protein was significantly induced and accumulated in neoplastic cells in MIC-treated tumor tissues (Fig. 10G, arrowhead) compared with the DMSO-treated group (Fig. 10F). Our results implied that inhibition of tumor cell growth through induction of the p53-signaling pathway appeared to contribute significantly to the inhibition of tumor growth.

DISCUSSION

MIC was demonstrated to be a nonselective inhibitor of cytochrome P450 by inhibition of hydroxylation and epoxidation and thereby has been widely used in different studies as a model compound (Chu et al., 2000; Oyekan, 2000). The inhibitory effects of MIC on conjugative metabolism using rabbit liver microsomes in vitro have been demonstrated (Sawamura et al., 2000). Studies have demonstrated that drug interactions occurred in patients when MIC was administered simultaneously with anticoagulant therapy (Ogard and Vestergaard, 2000; Silingardi et al., 2000; Thirion and Zanetti, 2000). Moreover, MIC has been found to attenuate the increase in cerebral blood flow elicited by glutamate (Bhardwaj et al., 2000) and to exert an effect on stimulation of dengue virus replication in culture cells (Lee et al., 2000). MIC has also been demonstrated as a plasma membrane H(+)ATPase blocker (Flaadt et al., 2000) and it affects the intracellular calcium levels in HL 60 cells (Harper and Daly, 2000). Human plasma concentrations of MIC were monitored either by high-performance liquid chromatographic analysis (Carrier and Parent, 2000; Kobylinska et al., 1996) or by gas chromatographic–mass spectrometry (Szathmary and Luhmann, 1988). A previous study in humans receiving a 600 mg MIC injection b.i.d. daily for 2 days showed that serum levels of MIC were 4.62 ± 2.15 µg/ml (Taguchi et al., 2000). MIC prolonged the anticoagulant (warfarin) actions in rabbits (D’Mello et al., 1992). Plasma concentration of MIC was 0.2–0.5 µg/ml during ip administration of MIC for 6 days (50 mg/kg/12 h). MIC has also been used in the treatment of mice infected with Coccidoides immitis (148 mg/kg sc or im single-dose injection of MIC). Plasma concentrations of MIC after 5 h of im and sc administration were determined to be 2.09 and 1.57 µg/ml, respectively (Levine et al., 1975). This study, although we do not have any data, reveals the plasma concentration of the MIC in drug-treated mice. The concentrations of MIC in human and animal plasma after administration of drug might be at the microgram per milliliter level. Our study demonstrated that the G0/G1 arrest in human COLO 205 cells was clearly induced by MIC at a concentration of 10 µM (4.79 µg/ml). Since a goal of this study was to develop a rational therapy for human cancer, we do not emphasize the effects of MIC at concentrations exceeding the clinically achievable dose (Taguchi et al., 2000). This study demonstrated absolutely the new mechanisms of antitumor effects of MIC, including apoptosis induction and G0/G1 cell cycle arrest in human cancer cells. These observations provide significant information for consideration of MIC in clinical cancer chemotherapy.

The tumor suppressor p53 has been implicated in a variety of cellular processes (Greenblatt et al., 1994; Bates and Vousden, 1996). However, p53 has undisputed roles in the induction of cell growth arrest and apoptosis (el-Deiry et al., 1994). Our studies provide evidence that lower doses of MIC (<30 µM) induce cell cycle arrest whereas higher doses of MIC (>40 µM) induce cell apoptosis, especially in COLO 205 (with wild-type p53) cells. Time-dependent experiments demonstrated that G0/G1 phase of the cell cycle in COLO 205 (with wild-type p53) cells was more easily induced by MIC and these arrested cells may eventually die by apoptosis. In contrast, in HT 29 cells (p53 His77 mutant, with functional wild type; Niewolik et al., 1995), MIC induced a G0/G1 cell cycle arrest dose dependently but less apoptotis was observed. Significant apoptosis was induced by MIC in the p53-null (HL 60) and the p53-deleted (Hep 3B) cells. Recently, several studies demonstrated that wild-type p53 plays a major role in G0/G1 cell cycle arrest and apoptosis (St. John et al., 2000; Chen et al., 2000), whereas mutated p53 could not sufficiently induce apoptosis (Rowan et al., 1996). Our results therefore suggest MIC induces p53 transcriptional activation in mediating cell cycle arrest in appropriate cell types but shows a less significant role in apoptotic function. We further demonstrated that the process of G0/G1 cell cycle arrest induced by MIC in COLO 205 cells was correlated with the induction of the p53-associated signaling pathway. Our results provided evidence of increased binding of p53 protein with the p53-specific consensus-binding sequence present in the p21/Cip1 promoter (Fig. 6C). These results support the hypothesis that the p53-signaling pathway may be involved in mediating MIC-induced apoptosis and growth arrest in human cancer cells.

The p21/Cip1 is induced by the p53 tumor suppressor upon DNA damage (Gartel et al., 1996). Previous studies demonstrated that the cell growth arrest was mediated by transcriptional activation of p21/Cip1, which binds and inactivates the CDKs required for cell cycle progression (el-Deiry et al., 1993,
Recently, overexpression of p27 has been shown to induce incidence of spontaneous pituitary tumors (Feroffi, 1996), and highly intensi
gous for p21/Cip1 and pRb are more tumor prone than those is thought to play a critical role in negative regulation of cell
nding also reinforces evidence that p21/Cip1 does not act
bition of CKI activity is one of the factors causing uncontrolled
The outcome of CKI induction in most cells is cessation of cell
The p23 was exclusively located in the cytosol and was a true
the inhibition of cancer cell proliferation would make it a very
Another CKI is p27/Kip1, which mediates growth arrest and is thought to play a critical role in negative regulation of cell
cells. A recent paper demonstrated that increased apoptosis
was accompanied by increased PCNA + cells in renal tissues of the acute puromycin aminonucleoside nephrosis animal (Fernandez et al., 2001). On the other hand, in agents (such as
bioflavonoid or gonadotropin-releasing hormone agonist) or
was studied in this paper revealing that significant p21/
Cip1 protein induction was observed only in the COLO 205
receptors were also observed in the EMSA. Recent studies suggest that p21/Cip1
does have tumor suppressor properties. P21/Cip1 mutations are
found at a low frequency in several human tumors (Malkowicz et al., 1996) and a p21/Cip1 mutation, which was demonstrated to
specifically abrogate its binding to CDKs, was identified in a
primary breast tumor (Balbin et al., 1996). The human papilloma virus E7 transforming protein interacts with p21/
Cip1, disrupting its interaction with CDKs (Funk et al., 1997; Jones et al., 1997). Keratinocytes from p21/Cip1-null mice are
cells by MIC treatment. Such results implied that MIC-induced
proliferation in a wide variety of tumor cells with different p53
status, and treatment of cells with MIC led to G0/G1 arrest
through the induction of p21/Cip1 and p27/Kip1. We also
provide evidence that MIC inhibited cell growth not only by
increasing CKIs (p21/Cip1 and p27/Kip1) levels but also by
decreasing cyclin D3/CDK4 kinase activity. The consequent reduction of CDK4 activity by CKIs is most likely responsible for
the MIC-induced G0/G1 arrest in tumor cells. The present
studies suggest that MIC may have chemotherapeutic properties by inducing the inhibitory activity of the negative regulators of the cell cycle. Therefore, it is quite pertinent to
investigate the direct mechanism by which MIC activates CKIs in
the cells. Our study provides the basis of molecular mechanisms for MIC in cancer treatment. The universality of MIC in the inhibition of cancer cell proliferation would make it a very attractive agent for cancer chemotherapy.

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REFERENCES

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