Abstract. Miconazole (MIC), an antifungal agent, displays anti-tumorigenic activity in various types of human cancers, including bladder cancer, yet its mechanism of antitumor action is not well understood. In the present study, we demonstrated that, in a cell viability assay, MIC had a cytotoxic effect on human T24, J82 and TSGH-8301 bladder cancer cells in a dose- and time-dependent manner, but did not exhibit significant toxicity toward human peripheral blood mononuclear cells. Cell cycle analysis revealed that MIC at concentrations of 25 and 50 µM significantly caused G0/G1 arrest in the TSGH-8301 and T24 cells, respectively. DNA fragmentation, mitochondrial membrane potential and western blot analyses showed that MIC inhibited the growth of these cells by both mitochondrial-mediated and death receptor (DR5) -mediated apoptosis pathways. Specifically, MIC increased the protein levels of p21 and p27, but decreased the expression of cyclin E1, CDK2 and CDK4. MIC augmented the expression of DR5, cleaved forms of caspase-3 -8 and -9, poly(ADP-ribose) polymerase and Bax, decreased the expression of Bcl-2 but increased cytosol levels of cytochrome c. Our results suggest that MIC inhibits the growth of bladder cancer cells through induction of G0/G1 arrest and apoptosis via activation of both the extrinsic and intrinsic apoptotic pathways. MIC is a potential chemotherapeutic agent for treating bladder cancer in humans.

Introduction

Triazoles, including miconazole (MIC), ketoconazole (KT) and fluconazole (FT), are used as fungicides in agriculture and as antifungal drugs in humans (1,2). Topical MIC is efficacious for the treatment of most mycoses (3-5). MIC buccal tablets have recently been approved by the US Food and Drug Administration for the treatment of oropharyngeal candidiasis in human immunodeficiency virus (HIV)-infected patients (6). MIC alters the fungal cell membrane, and exerts its therapeutic effect mainly from the endoplasmic reticulum, independent of phospholipase C activity and external Ca2+ influx (14).
Although these findings demonstrate the antitumor activity of MIC, the mechanism underlying the effect of MIC on bladder cancer remains unknown. In the present study, we evaluated the effect of MIC on bladder cancer cell apoptosis and characterized its underlying molecular mechanism.

Materials and methods

Cell culture and reagents. Human bladder cancer cell lines T24 (p53-mutant) and TSGH-8301 (wild-type p53) were purchased from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). The J82 (p53-mutant) cell line was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors. All of these cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA) and 1% antibiotic-antimycotic solution. Cells were incubated at 37°C with 5% CO₂. Miconazole nitrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). MIC was prepared in dimethyl sulfoxide (DMSO) to a final concentration of 0.2 mmol/l.

Cytotoxicity assay. The cytotoxicity of MIC in T24, and TSGH-8301 cells and PBMCs was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich), which was performed as previously described (14). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium bromide (MTS) assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions. Cells were seeded on 96-well plates for 24 h, and then treated with pifithrin-α (PFT-α), NAC or caspase inhibitors with and without MIC at 50 µM. After incubation for 24 h, 20 µl of CellTiter 96 Aqueous One Solution Reagent (Promega) was added to each well and incubated for 1-4 h followed by absorbance reading at 490 nm.

Flow cytometry. Cells (1x10⁶) were treated with MIC for 24 h. All cells were collected after trypsinization followed by fixation and permeabilization with 70% ethanol at -20°C overnight. After washing with ice-cold phosphate-buffered saline (PBS), the cells were incubated with propidium iodide solution (PI: 0.2 mg/ml, RNase: 20 µg/ml, 0.1% Triton X-100) for 30 min at 37°C. Flow cytometry was performed using WinMDI v2.9 (Scripps Research Institute, La Jolla, CA, USA). Ten thousand events/sample were counted for triplicate experiments.

DNA fragmentation analysis. Cells were treated with various concentrations of MIC. At indicated time points, the cells were collected by 0.25% (v/v) trypsin digestion, followed by centrifugation at 2,000 x g for 10 min. Cells were lysed in 800 µl of lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA and 0.3% Triton X-100), and treated with RNase (0.1 mg/ml) followed by proteinase K. The extracted DNA was electrophoresed on 2% agarose gels and stained with ethidium bromide (15).

Western blotting. Equal amounts of protein (measured by Bradford assay) were loaded on 10-15% sodium dodecyl sulfate polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with 5% non-fat milk in Tris-buffered saline and Tween-20 (TBST) buffer (20 mM Tris-HCl, 120 mM NaCl, 0.1% Tween-20). The membranes were incubated with antibodies against TNF-related apoptosis inducing ligand (TRAIL), death receptors (DR4 and DR5) (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), DR5 (1:10,000; Abcam, Cambridge, MA, USA), β-actin, Bcl-2, Bax, caspase-3, -8 and -9, poly(ADP-ribose) polymerase (PARP) (1:1,000; Cell Signaling Technology, Boston, MA, USA), cytochrome c (1 µg/ml; from BD Pharmingen, San Diego, CA, USA), Smac/DIABLO, cyclin D1, cyclin-dependent kinase 2 (CDK2), CDK4 (1:1,000; GeneTex, Hsinchu City, Taiwan), p53, p21, p27 and cyclin E1 (1:1,000; Epitomics, Burlingame, CA, USA) at 4°C overnight. After washing, the blots were incubated with horseradish peroxidase-labeled secondary antibodies for 1 h, and visualized using enhanced chemiluminescence.

Mitochondrial membrane potential assay. Cells were seeded onto 10-cm dishes and treated with various concentrations of MIC for 24 h, followed by staining with 5 µM JC-1 (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C. Fluorescence was monitored using a plate reader at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission). Mitochondrial membrane potential (ΔΨm) changes were indicated by the changes in the ratio of 590 nm (red) to 540 nm (green) fluorescence.

Reactive oxygen species detection. Intracellular reactive oxygen species (ROS) were determined using the fluorescent superoxide indicator dihydroethidium (DHE; Setareh Biotech, LLC, Eugene, OR, USA) (16-19). T24 and TSGH-8301 cells were treated with 50 µM MIC for 8, 16 or 24 h, and then incubated with 2 µM DHE in serum-free medium, at 37°C for 15 min, and then analyzed by flow cytometry.

Statistical analyses. Statistical comparisons were performed using unpaired, two-tailed Student's t-tests. p-values of <0.05 were considered to indicate a statistically significant result.

Results

MIC is cytotoxic to T24, J82 and TSGH-8301 cells. We determined the cytotoxic effect of various concentrations of MIC (Fig. 1A) on T24, J82 and TSGH-8301 cells using the MTT assay. The cell viability of both T24 (p53 mutant) and TSGH-8301 (p53 wild-type) cells was lower than that of the J82 bladder cancer cells after treatment with 50 µM MIC. We therefore chose T24 and TSGH-8301 cells for further assays. MIC exhibited cytotoxic effects in a concentration- and time-dependent manner (Fig. 1B and C). After MIC treatment for 24, 48 and 72 h, the IC₅₀ values were estimated to be 47.5±0.5, 42.0±1.8 and 37.3±0.3 µM, respectively, in the T24 cells; and 45.9±0.4, 32.2±0.8 and 29.4±0.2 µM, respectively, in the TSGH-8301 cells. Treatment of PBMCs with 100 µM MIC for 24 h showed a lower cytotoxic effect (85% cell-survival rate) than that noted in the bladder cancer cells (Fig. 1B). Thus, human bladder cancer cells were more sensitive than PBMCs to the cytotoxic effect of MIC. Furthermore, the morphological changes in MIC-treated cells at 24 h were examined using a
phase-contrast microscope. Cell proliferation in the T24 and TSGH-8301 cells was inhibited with 25 µM MIC, but the cells were mostly alive (Fig. 1D). At concentrations of ≥50 µM, MIC induced cell death with marked morphologic changes, such as shrinkage, rounding and floating of the cells, indicating that MIC at higher concentrations induced apoptotic cell death.

MIC induces cell cycle arrest and apoptosis in a p53-dependent manner in T24 and TSGH-8301 cells. To elucidate the mechanism underlying MIC-induced cell death, we analyzed the cell cycle phase distribution in the T24 and TSGH-8301 cells treated with various MIC concentrations for 24 h. The percentage of cells in the sub-G1 phase, indicating cell death, in the T24 and TSGH-8301 cells treated with 75 µM MIC (7.8±0.3 and 14.8±3.5%, respectively; Fig. 2A and B) was significantly higher than the percentages in the untreated (control) cells (0.9±0.1 and 1.1±0.8%, respectively). Additionally, the percentages of T24 cells in G0/G1 transition after treatment with 25 and 50 µM MIC (65.6±4.3 and 76.0±4.0%, respectively) were significantly higher than that in the untreated cells (56.8±4.6%; Table I). Similarly, the percentage (63.6±0.4%) of TSGH-8301 cells in G0/G1 transition after treatment with 25 µM MIC was significantly higher than that noted in the untreated cells (52.7±1.0%; Table I). Thus, MIC caused G0/G1 cell cycle arrest in both cell lines.

To further investigate the type of cell death caused by MIC, we assessed DNA fragmentation in both types of cells. MIC-treated cells exhibited significant internucleosomal DNA degradation, and elicited a DNA fragment ladder on 2% gels, which was significantly increased in the T24 and
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Figure 2. Cell cycle analysis of miconazole (MIC)-treated bladder cancer cells. (A) T24 and TSGH-8301 cells were treated with 25, 50 or 100 µM MIC for 24 h. Subsequently, the cells were stained with propidium iodide (PI), and then analyzed by flow cytometry. (B) The percentages of (a) T24 and (b) TSGH-8301 cells at each phase of the cell cycle were subjected to statistical analysis. Statistical significance: *p<0.05, and **p<0.01 as compared with the control.

Table I. Cell cycle phase distribution of T24 and TSGH-8301 cells following MIC treatment.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>MIC (µM)</th>
<th>Sub-G1 (%)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T24</td>
<td>0</td>
<td>0.9±0.1</td>
<td>56.8±4.6</td>
<td>16.2±5.0</td>
<td>24.5±0.0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>1.4±0.2</td>
<td>65.6±4.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.3±4.6</td>
<td>15.5±0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.4±0.3</td>
<td>76.0±4.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.5±3.5</td>
<td>12.6±0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.8±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.7±3.9</td>
<td>8.3±4.0</td>
<td>22.8±0.3</td>
</tr>
<tr>
<td>TSGH-8301</td>
<td>0</td>
<td>1.1±0.8</td>
<td>52.7±1.0</td>
<td>19.2±1.2</td>
<td>23.2±2.8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2.0±1.3</td>
<td>63.6±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.8±4.4</td>
<td>16.5±0.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.8±0.6</td>
<td>55.9±3.6</td>
<td>13.9±2.8</td>
<td>23.5±3.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>14.8±3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.1±7.0</td>
<td>23.7±0.7</td>
<td>21.1±0.7</td>
</tr>
</tbody>
</table>

T24 and TSGH-8301 cells were treated without or with MIC (25, 50 and 100 µM) for 24 h, and cell cycle distribution was determined using flow cytometry. Data are expressed as mean ± SD of experiments (n=3); *p<0.05 vs. control; **p<0.01 vs. control. MIC, miconazole.

TSGH-8301 cells treated with 75 µM MIC for 24 h (Fig. 3A) and 50 µM MIC for 24, 48 and 72 h (Fig. 3B) relative to the control. Importantly, TSGH-8301 cells exhibited more DNA fragmentation than T24 cells after 50 µM MIC.
treatment for 24 h (Fig. 3B). This indicated that the TSGH-8301 cells, expressing wild-type p53, were more sensitive to MIC-induced apoptosis than the T24 cells, which express p53 with an in-frame deletion of tyrosine 126.

**MIC increases p21 and p27 levels, and inhibits cyclin E1, CDK2 and CDK4 expression in T24 and TSGH-8301 cells.**

We then investigated the roles of cell cycle arrest and p53 in MIC-induced growth inhibition of bladder cancer cells, using western blot analysis. The levels of p21 and p27 were significantly increased, but those of cyclin E1, CDK2 and CDK4 were decreased after treatment with 25 or 50 µM MIC in the TSGH-8301 and T24 cells, as compared to these levels in the untreated cells (Fig. 4). Notably, MIC, at 25 and 50 µM, stimulated p53 expression in the TSGH-8301 (wild-type) cells, but not in the T24 (p53 mutant) cells as compared to the untreated cells. However, 25 or 50 µM MIC inhibited expression of cyclin D1, which is required for G1/S transition (20), in the TSGH-8301 cells, resulting in growth arrest. However, MIC at these concentrations stimulated cyclin D1 expression in the T24 cells. Thus, the TSGH-8301 cells were more sensitive to MIC-induced G0/G1 cell cycle arrest than the T24 cells, suggesting that cyclin D1 and possibly p53 are involved in cell cycle arrest. We hypothesized that, in p53-mutant T-24 cells, MIC-stimulated expression of cyclin D1 decreased the susceptibility of these cells to MIC-induced G0/G1 cell cycle arrest as compared with the TSGH-8301 cells.

**MIC induces caspase-dependent apoptosis in T24 and TSGH-8301 cells.**

Apoptotic pathways include the mitochondrial ‘intrinsic pathway’ and a DR-related ‘extrinsic pathway’, which involves activation of caspase-9, -8, and -3/-7, and cleavage of PARP (21). Thus, to clarify which apoptotic pathway is induced by MIC, levels of the cleaved/activated forms of caspase-8, -9 and -3, and PARP were determined by western blotting. Cleavage of PARP was induced in the TSGH-8301 and T24 cells treated with 50 and 75 µM MIC for 24 h (Fig. 5A). Therefore, both the mitochondrial and DR pathways were found to be involved in MIC-induced apoptosis in these bladder cancer cells. Moreover, MIC induced a higher level of apoptosis in the TSGH-8301 cells when compared to the T24 cells. Importantly, the level of the cleaved form of
caspase-8 (~41 kDa) was significantly higher in the TSGH-8301 cells than that in the T24 cells after treatment with ≥12 µM MIC. To determine the roles of caspases in MIC-induced apoptosis, the cells were treated with 50 µM MIC in the presence of vehicle only (control), or 10 µM caspase-3, -8 or -9 inhibitors (z-DEVD, z-IETD and z-LEHD, respectively) for 24 h. As shown in Fig. 5B, these caspase inhibitors partially reversed MIC-induced apoptosis in both the T24 and TSGH-8301 cell lines (*p<0.05; **p<0.01). Notably, in the T24 cells, there was still a moderate difference between cells treated with MIC plus caspase-8 inhibitor and those treated with MIC alone (*p<0.05).

**MIC induces DR-mediated apoptosis independent of p53 in T24 and TSGH-8301 cells.** To confirm that MIC induces extrinsic apoptosis, we determined the effect of MIC on the expression of proteins in the DR pathway in the T24 and TSGH-8301 cells. Treatment with 50 µM MIC increased expression of DR5 (Fig. 6A), but moderately decreased DR4 levels in both cell lines. However, treatment of these cells with 75 µM MIC almost completely abolished the expression of DR4, DR5 and β-actin (an internal control) (Fig. 6A). This suggests the non-specific cytotoxicity of MIC at 75 µM. To confirm that DR5 is involved in MIC-induced apoptosis in both cell types, we performed MTT assay. Pre-treatment of cells with anti-DR5 blocking antibody significantly attenuated MIC-induced apoptosis in both types of bladder cancer cell lines (Fig. 6B). To define the role of p53 in MIC-induced cell death, T24 and TSGH-8301 cells were treated with 50 µM MIC without or with 1 and 10 µM PFT-α (a p53 inhibitor) for 24 h. The cell survival was then determined using MTS assay. As shown in Fig. 6C, PFT-α at 1 µM, significantly reversed...
MIC-induced cytotoxicity in both cell types, particularly TSGH-8301 cells which express wild-type p53. PFT-α treatment attenuated MIC-increased expression of cleaved PARP in the T24 and TSGH-8301 cells (Fig. 6D). PFT-α treatment attenuated MIC-increased expression of cleaved PARP in the T24 and TSGH-8301 cells and cyclin D1 only in the TSGH-8301 cells (Fig. 6D). These results suggest that MIC induced apoptosis in T24 cells in a p53-independent, but cyclin D1-dependent manner.

**MIC induces apoptosis through the ROS-mediated mitochondrial pathway in T24 and TSGH-8301 cells.** To confirm that the intrinsic, mitochondrial pathway is involved in MIC-induced bladder cancer cell apoptosis, we measured Δψm in MIC-treated T24 and TSGH-8301 cells using the fluorescent cationic dye JC-1; loss of Δψm is an indicator of mitochondrial damage during apoptosis (22). JC-1 aggregates in normally polarized mitochondria, emitting red fluorescence, but in apoptotic depolarized cells, where Δψm is reduced, JC-1 is diffused as monomers throughout the cells and emits green fluorescence. A concentration-dependent decrease in red fluorescence was observed and the ratios of green/red fluorescence were estimated in the 75 µM MIC-treated T24 and TSGH-8301 cells (98.1/1.6 and 97.8/1.9%, respectively; Fig. 7A and B). This indicated that MIC treatment reduced Δψm in both cancer cell lines.

We then examined the expression of the proapoptotic protein Bax (23), which triggers cytochrome c release, and of Bcl-2, an anti-apoptotic protein, which inhibits cytochrome c release (24). Treatment of T24 and TSGH-8301 cells with >50 µM MIC for 24 h resulted in increased expression of Bax and/or cleaved Bax protein, and decreased expression of Bcl-2 protein in the T24 cells (Fig. 7C). Since loss of Δψm promotes the release of cytochrome c and Smac/DIABLO into the cytosol (21), we determined the levels of cytochrome c and Smac/DIABLO in the cytosolic fractions of
T24 and TSGH-8301 cells that were treated with higher concentrations of MIC (50 and 75 µM). As shown in Fig. 7D, MIC increased cytochrome c and Smac/DIABLO levels in the cytosol in a concentration-dependent manner in both T24 and TSGH-8301 cells. Taken together, these results indicated that the mitochondrial pathway was also involved in the MIC-induced apoptosis. Since ROS can alter the cellular redox state and Δψm (25,26), we investigated whether MIC-treated T24 and TSGH-8301 cells produce ROS. DHE staining yielded increased red fluorescence intensity (Fig. 8A) and ROS production (Fig. 8B) in both MIC-treated cell lines over time (8-24 h). Furthermore, JC-1 staining yielded increasing green fluorescence intensity in both cell lines during the same period (Fig. 8C and D), indicating a reduction in Δψm. Moreover, pre-treatment of these cells with the antioxidant N-acetyl-L-cysteine (NAC) increased cell survival rates (~10%) in both types of cells (Fig. 8E). Collectively, these results suggest that MIC-induced mitochondrial-mediated apoptosis is associated with ROS production in bladder cancer cells.

Discussion

In the present study, we demonstrated that MIC inhibits cell growth in human bladder cancer cells in vitro by inducing both mitochondrial- and DR5-mediated apoptosis. The cytotoxic effects of MIC in both bladder cancer cell lines were similar to that previously reported for colon cancer cells (COLO205; IC50 ~50 µM) (12). MIC caused cell cycle arrest at G0/G1 transition in both the T24 and TSGH-8301
The tumor-suppressor p53 plays a pivotal role in cell growth arrest and apoptosis (27,28). Western blotting revealed the elevation of p21 and p27 kinase inhibitors in both MIC-treated bladder cancer cells and a decrease in cyclin E1, CDK2 and CDK4 kinase. Given the differential expression of tumor-suppressor p53 and cyclin D1 kinase in the two bladder cancer cell lines used, we hypothesized that the effect of MIC on cyclin D1 expression in these cells is dependent on the p53 status. MIC induced downregulation of cyclin D1 in the TSGH-8301 cells (expressing wild-type p53), but not in the T24 cells (expressing p53 mutant). This was evidenced by the additive effect of MIC and PFT-α on the downregulation of cyclin D1 in the TSGH-8301 cells (Fig. 6D). The downstream protein of p53, p21/WAF1 (also known as cyclin-dependent kinase inhibitor), which binds to and inhibits the activity of cyclin-CDK2 and CDK4/6 complexes, functions as a regulator of cell cycle progression at the G1 and S phases (29). The expression of the corresponding gene is tightly controlled by...
p53, through which p21 mediates p53-dependent cell cycle
G1 phase arrest in response to a variety of stress stimuli (30).

Additionally, p27 belongs to the Cip/Kip family of CDK
inhibitors, which prevents activation of cyclin E-CDK2 or
cyclin D-CDK4 complexes, thus controlling cell cycle progres-
sion at G1 (31). Therefore, MIC may cause cell death in both
bladder cancer cell lines by arresting the G0/G1 phase of the
cell cycle. The magnitude of MIC-induced apoptosis in the two
bladder cancer cell lines was found to be associated with the
p53 status. These results also supported the hypothesis that
MIC-induced cell-growth inhibition in both bladder cancer cell
lines involves the p53 pathway. Furthermore, higher MIC doses
(50 and 75 μM) induced apoptosis in both bladder cancer cell
lines, as demonstrated by DNA fragmentation and increased
caspase-9, -8 and -3, and PARP levels. Caspases play pivotal
roles in the initiation and execution of the DR- and mitochon-
drial-mediated apoptotic pathways. Our data also revealed that
MIC-induced apoptosis in both bladder cancer cell lines was
associated with the p53 status, and that TSGH-8301 cells were
more susceptible than T24 cells to MIC-induced cell apoptosis.
Notably, a putative p53 inhibitor, PFT-α, at 1 and 10 μM,
effectively protected both TSGH-8301 and T24 cell types from
MIC (50 μM)-induced apoptosis or cell death as compared
to cells treated with MIC alone, regardless of the presence or
absence of p53 mutation in these cells (Fig. 6C). We reason that
PFT-α protects against MIC-induced apoptosis or cell death in a
p53-independent, but in a cyclin D1-dependent manner in
T24 cells. MIC (50 μM) increased the expression of cyclin D1
in the T24 cells, but attenuated cyclin D1 expression in the
TSGH-8301 cells as compared with the control cells treated
without MIC. PFT-α is known to protect cancer cells (HCT116)
due to DNA damage-induced apoptosis by a p53-independent
mechanism involving cyclin D1 (32). Notably, PFT-α did not
affect the expression of p53 and DR5, but protected against
apoptosis in both the T24 and TSGH-8301 cells treated with
MIC as evidenced by decreased levels of cleaved PARP in these
cells (Fig. 6D). The reason that PFT-α did not significantly
affect the expression of p53, or other transcriptional factors,
such as NF-κB (33,34), C/EBP homologous protein (35,36),
Elk 1 (2) and YY1 (37) is not clear. It may be under the influence
of DR5 expressed in these cells treated with MIC. Activation of
caspase-8 is associated with the DR signaling cascade and the
extrinsic pathway (38). Our results revealed that both bladder
cancer cell lines treated with ≥50 μM MIC for 24 h exhibited
upregulation of caspase-8 and DR5. However, apoptosis was
partially blocked with an anti-DR5 blocking antibody. Thus, we
speculate that MIC induces apoptosis through the DR pathway
by activation of caspase-8 and the downstream caspase-3 in
both bladder cancer cell lines.

ROS cause Δψm dysfunction and release of cytochrome c,
which activates caspase-3, leading to mitochondrial-mediated
apoptosis (39). MIC has been shown to induce actin cytoskel-
eton stabilization and ROS accumulation upon killing of yeast
cells (7). We demonstrated that MIC induced mitochondrial-
mediated apoptosis in the T24 and TSGH-8301 bladder cancer
cells, as determined by Δψm decrease, the release of cyto-
chrome c and Smac/DIABLO, activation of caspase-9 and -3,
and a change in the Bax/Bcl-2 ratio (23). Treatment with higher
doses of MIC enhanced ROS levels in both bladder cancer cell
lines, which parallelled the decrease of Δψm over the same
period, but this effect was blocked by the antioxidant NAC.
Thus, MIC-induced apoptosis in the T24 and TSGH-8301
bladder cancer cells involved the mitochondrial pathway and
was modulated by ROS generation.

In conclusion, we demonstrated that MIC caused G0/G1 cell cycle arrest in human bladder cancer cells, and
thus, induced apoptosis in these cells through both the extrinsic
DR5-dependent and intrinsic mitochondrial-mediated
pathways. MIC appeared to cause cell cycle arrest through
both p53-independent and ROS-dependent mechanisms, as
evidenced by the inability of NAC to influence the protec-
tive effect of PFT-α on MIC-induced cell cycle arrest (data
not shown). Since MIC has been shown to interact with lipid
rafts/caveolae in target cells (7), we hypothesized that MIC
causes cell cycle arrest and activates both apoptosis pathways,
at least in part, by interaction with lipid rafts/caveolae in the
plasma membrane, where DR5 transduces signaling (40). MIC
has been widely used as an antifungal agent without severe
side effects, even with prolonged use (5,41,42). Thus, the anti-
cancer effect of MIC may be of potential clinical significance
for the treatment of bladder cancer in humans.

Acknowledgements

The present study was supported by the Taichung Veterans
General Hospital, and the Hung Kung University (grant
no. TCVGH-HK1028007).

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