Ketoconazole-Induced Apoptosis through P53-Dependent Pathway in Human Colorectal and Hepatocellular Carcinoma Cell Lines

Yuan-Soon Ho,* Pei-Wen Tsai,* Cheng-Fei Yu,* Hsu-Ling Liu,* Rong-Jane Chen,* and Jen-Kun Lin†,‡

*School of Medical Technology, Taipei Medical College and †Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China

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The requirements for orally administered antifungal agents are (a) high efficacy against the infecting microorganism, (b) low toxicity for the host, and (c) high, or at least constant, bioavailability. Agents which meet these requirements include griseofulvin, flucytosine, some azole derivatives (ketoconazole [KT], itraconazole, and fluconazole) and the allylamine (terbinafine) (Monika, 1993). KT and fluconazole areazole antifungal agents, each with a broad spectrum of activity against both superficial and systemic mycoses. The target enzyme, fungal lanosterol demethylase, contains cytochrome P450 as a coenzyme (Rodriquez and Acosta, 1996). Although KT is very effective for this indication, its use is associated with adverse reactions, which can be sufficiently severe to require discontinuation of therapy. The most common side effect is hepatotoxicity; less common are adrenal crisis and interstitial pneumonitis (Sarver et al., 1997; Babovic-Vuksanovic et al., 1994). With worldwide usage of KT, numerous cases of KT-induced hepatitis have been documented (Bercoff et al., 1985; Stricker et al., 1986; Lake-Bakaar et al., 1987; Benson et al., 1988; Cauwenbergh, 1989; Brusko and Marten, 1991). In superficial infections, use of KT is limited to short-term therapy because of hepatotoxicity during long-term treatment of fungal infections such as onychomycosis (Lake-Bakaar et al., 1987; Chien et al., 1997). The rechallenge of KT in patients has resulted in enhanced hepatotoxicity (Lake-Bakaar et al., 1987; van Parys et al., 1987). Histologic analysis of KT-induced hepatotoxicity shows massive centriflobular necrosis in which the hepatotoxicity was primarily of the hepatocellular type.

Trachtenberg et al. (1983) and Eichenberger et al. (1989) first reported that KT has antitumor activity in prostate cancer and suggested a direct cytotoxic effect on androgen-independent prostate cancer (AI PCa). In another study, Avishay et al. demonstrated that the combination of KT and adriamycin has a 55% prostatic-specific antigen (PSA) response rate in patients with AI Pca and that this combination is worthy of additional study (Sella et al., 1994). Using different response criteria, several investigators reported 15% response rates of AI PCa to KT (Trump et al., 1989; Johnson et al., 1988; Gerber and Chodak, 1990).

Over the past few years, there has been increasing recognition of the important role of cell death in determining tissue cell number and how a lack of cell death under appropriate physiologic conditions can contribute to cellular transformation and malignant cell growth (Kerr, 1971; Kerr et al., 1972). Cell death permits the selective elimination of excess cells, such as in morphogenesis and the immune system, and also permits the maintenance of tissue homeostasis (Steller, 1995; White, 1996). The majority of these cell deaths share a common morphology, including a reduction in cell volume, blabbing of the plasma membrane, chromatin aggregation, and finally fragmentation of DNA. This morphologically distinct form of cell death is called apoptosis (Kerr, 1971).

The level of p53 accumulation of KT-induced apoptosis. It was found that KT induced nuclear liver cells. We further investigated the molecular mechanisms of various type of human cancer cells and in a primary culture of rat liver cells. We found that p53 protein in a dose- and time-dependent manner. The level of p53 protein was elevated approximately three times as much in treated cells 24 h after KT (5 μM) exposure as in cells receiving mock treatment. We found that cells containing wild-typep53 (COLO 205 and Hep G2) were more sensitive to KT exposure. The bax protein was induced and the bcl-2 protein was inhibited by KT in cells containing wild-typep53 (Hep G2, COLO 205) but not in cells without p53 (Hep 3B). The caspase-3 was activated 24 h after KT treatment. The Poly-(ADP ribose) polymerase (PARP) and the lamin A degradation was induced by KT, which promoted nuclear membrane disassembly and eventually caused apoptosis. Our results also indicated that none of the PKC gene family was involved in KT-induced apoptosis.© 1998 Academic Press

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1 To whom correspondence should be addressed. Fax: (011) 886 – 2-2391 – 8944.

2 Abbreviations used: AI PCa, androgen-independent prostate cancer; IRP, ICE-related protease; KT, ketoconazole; LNAC, [scap][r]-N-acetyl-cysteine; PARP, poly-(ADP ribose) polymerase; PKC, protein kinase C; prICE, protease resembling ICE; PSA, prostatic-specific antigen.
In this manuscript, we demonstrate KT-induced apoptosis in different human cancer cells and in a primary culture of rat liver cells. The aim of this study was to evaluate the molecular mechanisms of the apoptotic effects induced by KT so that further mechanistic investigations of cytotoxicity in human cancer cells could be performed. The major nuclear membrane protein (lamin A, and poly-[ADP ribose] polymerase [PARP]) degradation induced by KT was detected by Western blot analysis. The alterations of gene expression associated with the cell cycle (p53), signal transduction (protein kinase C [PKC]), and apoptosis (Caspase-3, bax, and bcl-2) in human cancer cells treated with KT was also investigated in this study.

MATERIALS AND METHODS

Chemicals. KT, fluconazole, amphotericin B, and griseofulvin were purchased from the Sigma Chemical Co. (St. Louis, MO). Flucytosine was purchased from the Merck Co. The protein assay kit was purchased from Bio-Rad Labs. (Hercules, CA).

Cell lines and cell culture. The cell line HT 29 (HTB-38; American Type Culture Collection, Rockville, MD) was isolated from a moderately well-differentiated grade II human colon adenocarcinoma (Semple et al., 1978). The cell line COLO 205 (CCL-222; American Type Culture Collection) was developed from a poorly differentiated human colon adenocarcinoma. Hep 3B cells (HB 8064; American Type Culture Collection) were derived from a human hepatocellular carcinoma line (Aden et al., 1979; Knowles et al., 1980; Darlington et al., 1987). The cell line Hep G2 (HB 8065; American Type Culture Collection) was derived from a human hepatocellular carcinoma (Aden et al., 1979; Knowles et al., 1980; Darlington et al., 1987) and contained wild-type p53 (Bressac et al., 1990). The cell line CCD-922SK (CRL 1828; American Type Culture Collection) was derived from normal human fibroblasts. The p53 gene in the COLO 205 and CCD-922SK cells was cloned into the TA cloning vector (Invitrogen, San Diego, CA) and sequenced. The p53 gene in COLO 205 and CCD-922SK cells was of the wild type (Ho et al., 1996, 1997). In HT-29 cells, p53 is mutated in codon 273 (Niewolt et al., 1995). The p53 gene has been found to be partially deleted (7 kb) in Hep 3B cells (Darlington et al., 1987). Cell lines were grown at 37°C in a 5% CO2 atmosphere in Eagle’s minimal essential medium (for Hep 3B, Hep G2, and CCD-922SK cells), RPMI 1640 (for COLO 205 and HT-29 cells) supplemented with 10% fetal calf serum, 50 [mu]g/ml gentamicin, and 0.3 mg/ml glutamine.

Rat hepatocyte culture procedure. The isolation and the culturing of the rat hepatocytes were done according to methods previously reported (Acosta et al., 1980; Davila et al., 1990). Briefly, the hepatocytes were obtained from postnatal Sprague–Dawley rats (i.e., 8 to 11 days of age). The liver was perfused by retrograde perfusion of the portal vein with a hypodermic syringe containing approximately 2 ml of warm Hanks’ balanced salt solution with albumin fraction V (3 mg/ml) and collagenase type II (80 U/ml). After mechanical dissociation and centrifugation of the tissue homogenates several times, the resulting pellets were resuspended in 4 ml of Williams’ medium E, which contained 0.001% (w/v) insulin, 0.005% (w/v) hydrocortisone, 10% (v/v) newborn bovine serum, 200 [mu]g/ml potassium penicillin G, 200 [mu]g/ml streptomycin sulfate, and 4 [mu]g/ml amphotericin B. The cell suspension was diluted to a volume of 0.5 x 106 cells/well in Falcon 24-well multiplates. Cultures were grown in a CO2 incubator with 95% (v/v) air and 5% (v/v) CO2 at 37°C and maintained at a pH of 7.2 to 7.4.

Chemical exposure. We initiated a study in which experiments were conducted on the third day after the initial plating of the hepatocytes. For the toxicity studies, stock solutions (50 mM) of each antifungal drug were prepared by dissolving the compounds in DMSO. The stock solutions of drugs were then diluted with serum- and antibiotic-free medium to yield final concentrations ranging from 0 to 50 [mu]M. The final treatment concentration of DMSO was 0.25% (v/v) DMSO/culture medium. After various periods of incubation, cells were sedimented for protein preparation or for DNA fragmentation analysis.

Indirect immunofluorescence staining. Cells were plated on glass cover slides in 12-well culture dishes. After treatment with various agents, the cells were fixed for 10 min in acetone–ethanol (1:1, v/v) at ~20°C and dried. For immunofluorescence, the slides were incubated for 45 min at 37°C in a humidified chamber with a primary antibody diluted 1:30 in PBS with 0.05% (v/v) Tween 20. The slides were washed three times at room temperature with PBS, incubated for another 30 min with biotinilated goat anti-mouse (or anti-rabbit) antibody (diluted 1:100 in PBS and 0.05% [v/v] Tween 20), and washed again. Finally, the slides were incubated for 10 min with fluorescein isothiocyanate-conjugated streptavidin, washed thoroughly, and mounted with Glycerol (Dako Corp., Carpinteria, CA).

Preparation of protein lysate. Treated and untreated cells were rinsed three times with ice-cold PBS, pelleted at 800g for 5 min and lysed in 500 [mu]l of freshly prepared extraction buffer (10 mM Tris–HCl, pH 7: 140 mM sodium chloride; 3 mM magnesium chloride; 0.5% [v/v] NP-40; 2 mM phenylmethylsulfonyl fluoride; 1% [w/v] aprotinin; and 5 mM dithiothreitol) for 20 min on ice. The extracts were cleared by centrifugation for 30 min at 10,000g.

SDS–PAGE and immunoblotting. Proteins were normalized to 50 [mu]g lane resolved on 12.5% (w/v) SDS–PAGE and blotted onto an immobilon P membrane with a semidry electroblotting apparatus (TE70; Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight at room temperature with blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% [v/v] Tween 20; 4% [w/v] nonfat dry milk, and 0.1% [w/v] sodium azide). Antibodies used for immunoblot assays including polyclonal rabbit antisera specific for human bax (Ab-1, CAN Bioscience Co.), were raised against synthetic peptides corresponding to amino acids 150 to 165 of human p21-bax. Mouse monoclonal antibodies included lamin A (JOL4, Se-rotec Co.); PARP (ABR Co.) (Lamarre et al., 1988), and the PKK gene family (Transduction Laboratory, Inc.).

RESULTS

Cytotoxic Effects of KT in Human Cancer Cells

As Fig. 1A shows, human hepatocarcinoma (Hep G2) cells were treated with various concentrations (0–50 [mu]M) of KT and the viability of the Hep G2 cells was determined. The viability of Hep G2 cells was less than 30% 72 h after exposure to KT (25 [mu]M). However, no influence on the viability of the Hep G2 cells was observed when these cells were treated with DMSO (0.25%,
v/v). In Figs. 1B and 1C, the cytotoxic effects on human cancer cell lines and on the primary culture of rat liver cells with exposure to KT (25 and 50 μM, respectively) are demonstrated. The human COLO 205 cells were the most sensitive to KT exposure. Similar results were also seen in Hep G2 cells.

**KT-Induced Apoptosis in Different Human Cancer Cell Lines**

The cells treated with KT (25 μM) exhibited morphological changes and were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Figs. 2A and 2B). These results further confirmed the occurrence of apoptotic cell death after KT exposure. As is shown in Fig. 2A, after exposure of Hep G2 cells to KT (0, 5, and 25 μM), both the attached and detached cells were harvested at 12, 24, and 48 h after drug treatment. In each case, nucleosomal DNA ladders typical of apoptosis were visible on agarose gel after staining with ethidium bromide (Fig. 2A). In the low-dose group (5 μM), the DNA ladder appeared at 24 h after drug treatment (Fig. 2A), whereas in cells exposed to a high dose of KT (25 μM) the DNA ladder appeared early, at 12 h (Fig. 2A). Several additional human cancer cell lines were also examined for KT-induced apoptosis. DNA ladders were clearly evident 24 h after all of the human cancer cell lines were exposed to KT (5 μM) (Fig. 2B).

**Nuclear Accumulation of P53 Protein in KT-Treated Cells**

Changes in the amount and localization of p53 protein in KT-treated Hep G2 cells were monitored by indirect immunofluorescence staining with a p53-specific monoclonal antibody (pAb 1620). Without KT exposure, positively stained cells were rarely observed except for a few cells with extended cytoplasm and large nuclei. When cells were exposed to KT (5 μM) 24 h later, the intensity of the fluorescence in the nuclei...
and the number of cells positively stained for wild-type p53 increased notably (Fig. 3A).

To determine whether the observed nuclear accumulation of p53 was due to increased amounts of p53, Western blotting analysis was performed using cell extracts prepared from KT-treated Hep G2 cells. Figure 3B shows that the amount of p53 increased with increasing doses of KT to 50 μM. At a lower dose (1 μM), the Hep G2 cells exhibited a one- to twofold
FIG. 4. Upregulation of bax by KT in human hepatocellular carcinoma cells. (A) Immunofluorescent staining of bax protein in Hep G2 cells. The cells on the right received mock treatment as controls. The cells on the left were treated with KT (5 μM). After 24 h, the cells were fixed and stained with bax-specific antibody using the biotin–streptavidin system. The bax protein induction in Hep G2 (B) and Hep 3B (C) cells was observed following exposure to KT (5 μM) for 12, 24, and 48 h. Untreated cells (Controls) were harvested at different times as shown on the right. Proteins were normalized to 50 μg/lane and the amount of bax and α-tubulin was visualized by video densitometry to compensate for unequal loading of protein in different lanes. The relative bax levels were normalized to α-tubulin levels. Each blot is representative of three similar experiments.
The levels and the kinetics of the \textit{bax} protein induction in Hep G2 (with wild-type \textit{p53}, Fig. 4B) and Hep 3B (with deleted \textit{p53}, Fig. 4C) cells were investigated using Western blot analysis. The results showed that a significant \textit{bax} induction was observable in Hep G2 but not in Hep 3B cells after exposure to KT (5 \textmu M) (Fig. 4B). On the other hand, the \textit{bcl-2} levels in cells of different \textit{p53} status were investigated for their sensitivity to KT-mediated inhibition of \textit{bcl-2} protein. Significant inhibition of \textit{bcl-2} occurred in Hep G2 and COLO 205 cells (with wild-type \textit{p53}) in a time-dependent manner after KT (5 \textmu M) treatment (Figs. 5A and 5C). In contrast, the \textit{bcl-2} levels in Hep 3B cells (with deleted \textit{p53}, respectively) was not inhibited significantly after KT treatment (Fig. 5D). It seemed that the inhibition of \textit{bcl-2} by KT was dependent on the competent function of the \textit{p53} gene in the treated cells.

### FIG. 6.

The Caspase-3 protein activation, \textit{PARP} degradation, and \textit{lamin A} cleavage coincided in Hep G2 cells undergoing apoptosis. (A) Total cell lysates were prepared from Hep G2 cells treated with KT (5 \textmu M) in a time-dependent manner. Protein extracts were separated by SDS–PAGE and were stained with Comassie blue to reveal the bulk of the cellular polypeptide. (B) Caspase-3 activation, (C) \textit{PARP} degradation, and (D) nuclear \textit{lamin A} cleavage were initially observed 12 h after treatment with KT (5 \textmu M).
**PARP and Lamin A Protein Degradation was Induced by KT**

The protein lysates of the Hep G2 cells treated with KT were separated by SDS–PAGE and stained with Comassie blue; the bulk of the cellular polypeptide remained intact (Fig. 6A). As Fig. 6B shows, the caspase-3 was activated in Hep G2 cells 12 to 24 h after KT exposure. According to a previous report, the substrate of caspase-3 is PARP (Tewari et al., 1995). We further explored the possibility that activation of caspase-3 may also induce nuclear lamina protein degradation. Western blotting analysis revealed that the $M_r$ 116,000 PARP molecule was degraded to a relatively stable $M_r$ ~85,000 fragment (Fig. 6C) at 12 h after KT treatment. Cleavage of lamin A was also observed 12 h later (Fig. 6D) with a time point that paralleled the DNA fragmentation (Fig. 2A) observed when cells were treated with KT (5 $\mu$M).

As described above, cleavage of lamin A protein occurred when the cells were treated with KT. To ascertain whether the cleavage of lamin A was unique to KT-induced apoptosis, Hep G2 cells were treated with a variety of antifungal agents. Results obtained with these agents are shown in Fig. 7. In this study, KT was highly effective in inducing lamin A degradation, whereas fluconazole was moderately effective. In contrast, griseofulvin and flucytosine could only weakly cause lamin A degradation (Fig. 7A). It is of interest to note that KT and fluconazole have some degree of structural similarity, as is illustrated in Fig. 7B.

**KT-Induced Apoptosis is not through PKC Signaling Pathway**

Although the factors regulating apoptosis remain obscure, the involvement of PKC has been implied by a great deal of evidence (Ferraris et al., 1977). The multigene PKC family encodes for multiple isoforms with possibly distinct biological effects. PKC isoenzymes are classified as conventional PKC ($\alpha$, $\beta$, and $\gamma$), novel PKC ($\sigma$, $\epsilon$, $\eta$, and $\theta$), and atypical isoenzyme ($\lambda$/$\pi$ and $\xi$). PKC activation has been shown to variably block or promote apoptosis depending on the cell type and cell state (Lucas and Sanchez-Margalet, 1995). In order to further scrutinize the critical role of PKC function involved in the KT-induced apoptosis, we screened PKC isoenzyme expression using Western blotting analysis and found that none of the PKC gene family described above was altered by KT in Hep G2 cells (data not shown).

**DISCUSSION**

KT is used worldwide as an antifungal agent with a broad spectrum of activity against both systemic and superficial mycosis. However, its use is also associated with adverse reactions, which can require discontinuation of therapy. The most common side effect is hepatotoxicity (Lake-Bakaar et al., 1987; Benson et al., 1988; Cauwenbergh, 1989; Brusko and Marten, 1991; Chien et al., 1997). A previous study (Rodriguez and Acosta, 1995) demonstrated that KT was more toxic than fluconazole in a primary rat liver culture system by measuring the leakage of the cytosolic enzyme, LDH, into the medium; by measuring the mitochondria reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT; by assessing lysosomal uptake of neutral red; and by gross morphology. We suggest that the results described above may have been due to apoptotic cell death induced by KT. In this study, an in vitro experimental model system was established to study the molecular mechanisms of hepatotoxicity induced by KT. Primary rat liver cells were cultured and demonstrated that apoptosis occurred with exposure to KT.

KT is also an anticancer drug used for the treatment of advanced prostate cancer (Trachtenberg et al., 1983; Eichenberger et al., 1989). According to previous studies, apoptosis induced by a wide variety of chemotherapeutic agents has been found to be dependent on a normal $p53$ status, which may be
a determinant of the chemosensitivity of tumor cells (Perego et al., 1996). Our investigations dealt with the cellular responses of different human cancer cells (with various p53 statuses) to KT exposure. In this study, Hep G2 and COLO 205 cells (with wild-type p53) were very sensitive to KT, whereas Hep 3B and HT-29 cells (with deleted and mutated p53, respectively) exhibited an intermediate sensitivity to KT. According to the previous reports described, p53 inhibits expression of bcl-2 and accelerates expression of bax (Miyashita et al., 1994; Miyashita and Reed, 1995). In our recent study (Jiang et al., 1996), apoptosis was induced by various stimuli (such as staurosporine, quinacrine, and cycloheximide) in Hep G2 cells. Most of these stimuli induced both p53 and myc protein expression. However, none of the stimuli altered the expression levels of bcl-2 or bax protein. Our other observations indicated that busulphan (a protein alkylating agent) and vincristine (which binds to tubulin and blocks the mitotic process) did not induce bax protein expression in Hep G2 cells (unpublished data). However, both our own studies and those of others demonstrated that agents that cause DNA damage (such as NO and γ-irradiation) might elevate p53 and bax levels and eventually cause apoptosis (Ho et al., 1996, 1997; Kitada et al., 1996). These results implied that the p53-regulated bax protein elevation was not induced by all of the apoptosis-inducing agents. The role of KT in induction of p53 and bax still required scrutiny.

Recently, numerous mammalian proteases have been identified whose expressions induce apoptosis (Kumar, 1995; Fraser and Evan, 1996; Earnshaw and Takahashi, 1996). Several studies have demonstrated that the death signal is transduced by a protease cascade (Martin and Green, 1995). For instance, activated TX (also called ICH-2 or ICE_{re}) cleaves proICE (Faucheu et al., 1995), and activated ICE cleaves pro-Caspase-3 (Lamarre et al., 1988). Pro-Caspase-3 has also been shown to be cleaved by nuclear granzyme B (Darmon et al., 1995). The known target of Caspase-3 is PARP (Lamarre et al., 1988; Tewari et al., 1995). Clearly, intracellular protease is an important component of the apoptotic machinery. In this study, Hep G2 cells were treated with KT for various lengths of time and subjected to SDS–PAGE. All of the protein lysates were present in amounts similar to those found in control cells. In contrast, Caspase-3 was activated and the major structural protein of the nuclear envelope (PARP and lamin A) had been degraded. The results presented in this study raise the possibility that KT might have activated an endogenous cellular protease that is normally present in an extranuclear compartment of the cell.

KT is an inhibitor of cytochrome p450 (Rodriguez and Acosta, 1996). As in previous studies (Duthie et al., 1994, 1995), Hep G2 cells were used for studying xenobiotic liver toxicity using the well-characterized hepatotoxic chemicals, bromobenzene, cyclosporin A, and tamoxifen. In these studies, KT was used as an inhibitor of cytochrome p450 to evaluate whether the toxicity required the activation of bromobenzene by p450. The studies demonstrated that KT did not affect on the toxicity of bromobenzene. However, viability of the cells treated by KT (50 μM) decreased as compared to that of the control cells (72.5% and 81%, respectively) after a 180-min period of incubation. These results were consistent with our observation and we further demonstrated that apoptosis was induced after long-term exposure to KT at this dosage (50 μM) (Fig. 1A).

In summary, our investigations demonstrated that wild-type p53 or p53-regulated gene products (such as bax and bcl-2) were altered (increased in bax and decreased in bcl-2) in human cancer and rat liver cells by KT exposure. Furthermore, other genes may also be involved in the cellular responses to KT exposure, an involvement that may eventually lead to the cells undergoing apoptosis.

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