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Ketoconazole Potentiates Terfenadine-Induced Apoptosis in Human Hep G2 Cells Through Inhibition of Cytochrome p450 3A4 Activity

Ying-Jan Wang,[1] Cheng-Fei Yu,[2] Li-Ching Chen,[3] Chien-Ho Chen,[2] Jen-Kun Lin,[4] Yu-Chih Liang,[5] Chien-Huang Lin,[2] Shyr-Yi Lin,[5] Chin-Fa Chen,[6] and Yuan-Soon Ho[2]*

[1]Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan, Taiwan
[2]Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan
[3]Department of Nursing, TzuChi University, Hualien, Taiwan
[4]Institute of Biochemistry, College of Medicine, National Taiwan University, Taiwan
[5]Department of Internal Medicine, Taipei Medical University Hospital, Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan
[6]Department of Laboratory Medicine, Taipei Medical University Hospital, Taipei, Taiwan

Abstract Terfenadine (TF) is a highly potent histamine H1 receptor antagonist that in clinically effective doses is free of significant central nervous system side effects. Ketoconazole (KT) is a worldwide used oral antifungal agent with a broad spectrum of activity against both superficial and systemic mycosis. Simultaneously administration of KT and TF has been reported to induce several potent symptoms including cardiotoxicity, excitotoxicity, inhibition of blood mononuclear cells proliferation, and cardiovascular toxicity. However, the intracellular molecular mechanisms of TF–KT interactions in cells were still uncertain. In this study, we first demonstrated that TF (5–30 μM) induced apoptosis in several types of human cancer cell lines including human hepatoma (Hep G2), colorectal cancer (COLO 205), and fibroblast (CCD 922SK) cells for 24 h. The cellular responses to TF-induced apoptosis were demonstrated to be associated with the *p53*-signaling pathway, including induction of *p53, p21/Cip1*, *p27/Kip1*, *bax* protein expression and inhibition of *bcl-2* protein expression. To realized the role of H1 receptor involved in TF-induced apoptosis, different H1 receptor antagonists including promethazine, mequitazine, and chlorpheniramin (50–100 μM) were administered and demonstrated that these chemicals cannot induced apoptosis through the H1 receptor signaling pathway. Interestingly, we found that the apoptotic effect of TF (2.5 μM) was significantly potentiated by KT (1 μM) treatment in Hep G2 cells through inhibition of the cytochrome p450 3A4 (CYP 3A4) activity. Such results were demonstrated by decreased of the TF activity with recombinant CYP 3A4, which prepared from baculovirus-infected insect cells.Our results provide the molecular basis of TF–KT interaction and this information should allow more rational forecasting of the risk for TF therapy during co-administration of KT. J. Cell. Biochem. 87: 147–159, 2002.

Key words: apoptosis; terfenadine; ketoconazole; *p53*; cytochrome p450 (3A4)

Terfenadine (TF), was first reported by Brandon et al. (1980), appears to be the first antihistamine act as a highly potent H1 histamine receptor antagonist that in clinically effective doses lack of side effects such as sedation, impaired psychomotor performance, and excessive mucosal drying [Brandon and Weiner, 1980; Rafferty and Holgate, 1987; Kaliner and Check, 1988]. Recently, TF is known to have direct effects on electrical activities in the

Abbreviations used: CYP 3A4, cytochrome p450 3A4; DMSO, dimethylsulfoxide; ITRA, itraconazole; KT, ketoconazole; *PARP*, Poly-(ADP ribose) polymerase; PBS, phosphate buffer saline; PMSF, phenylmethyl sulfonyl fluoride; SDS–PAGE, sodium dodesyl sulfate–polyacrylamide gel electrophoresis; TF, terfenadine.

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*Correspondence to: Yuan-Soon Ho, PhD, Graduate Institute of Biomedical Technology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan.
E-mail: hoyuansn@tmu.edu.tw

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heart [Liu et al., 1997; Lu and Wang, 1999]. The mechanisms were demonstrated by block sodium current [Lu and Wang, 1999] and L-type Ca^{2+} channel current [Liu et al., 1997] in myocytes isolated from experimental animal. Other studies demonstrated that TF potentiated the excitotoxic response to *N*-methyl-D-aspartate (NMDA) receptor agonists in cerebellar neurons [Diaz-Trelles et al., 1999, 2000]. In addition, antigen/mitogen-induced peripheral blood mononuclear cells proliferation was inhibited by TF in a concentration-dependent manner [Holen et al., 1995]. The metabolisms of the TF to its desalkyl and hydroxy metabolites were demonstrated to be mediated by human liver microsomal enzymes cytochrome p450-3A (CYP 3A4) isoforms [Ling et al., 1995; Rodrigues et al., 1995]. The testosterone 6 beta-hydroxylation has been shown to be the principal enzyme involved in the first step in TF's biotransformation (formation of azacyclonol and TF alcohol) [Ling et al., 1995].

Ketoconazole (KT) is an azole antifungal agent with a broad spectrum of activity against both superficial and systemic mycosis [Bisschop et al., 1979]. The target enzyme of the KT were identified as the fungal lanosterol demethylase which contains cytochrome P450 as a coenzyme [Gupta et al., 1986]. As described above, the metabolism of TF was through CYP 3A4 [Ling et al., 1995; Rodrigues et al., 1995], whereas the KT is a potent and selective inhibitor of this enzyme [Boxenbaum, 1999a; Parker et al., 2000]. Several studies demonstrated that clinical adverse interactions of TF with azole antifungals agent (KT) may be related to inhibition of TF biotransformation [Jurima-Romet et al., 1994; Rodrigues et al., 1995; Hey et al., 1996a]. Simultaneously administration of KT and TF may predict a large and potentially hazardous impairment of TF clearance by KT [Haaz et al., 1998; Baune et al., 1999; Boxenbaum, 1999b; Herman et al., 1999]. In addition, KT is now been demonstrated as a potent inhibitor of cytochrome p450(17) alpha (17alpha-hydroxylase/C(17,20)-lyase), which is required for androgen synthesis and is applicable in treatment of some types of prostate cancer [Nnane et al., 1998; Grigoryev et al., 1999]. Trachtenberg et al. [Trachtenberg and Pont, 1984] and Williams et al. [Williams, 1984] first reported that the KT has antitumor activity in prostate cancer and suggested a direct cytotoxic effect on androgen-independent prostate cancer. Recently, our studies demonstrated that apoptosis and G0/G1 phase of cell cycle arrest were induced by KT in different types of human cancer cell lines through the *p53*-signaling pathway [Ho et al., 1998; Chen et al., 2000]. Our results provide the direct evidences indicated that some additional mechanisms (such as apoptosis induction and cell growth inhibition) affect by KT might be applied in some other types of cancer chemotherapy.

Apoptosis is an active and gene-directed form of cell death with well characterized morphological and biochemical features [King and Cidlowski, 1995]. In this study, we first demonstrated that apoptosis was easily induced in human cancer cell lines by TF. Our data revealed that such apoptosis-inducing effects by TF were not correlated with the H1-receptor signaling pathway. We found that TF-induced apoptosis was significantly potentiated by KT in human Hep G2 cells through inhibition of CYP 3A4 activity. The specific aims of this study were to investigate the molecular events of apoptosis in cells induced by TF. The KT-inhibited CYP3A4 in Hep G2 cells in the potentiation of TF-induced apoptosis was also demonstrated. Our results provide more direct evidences of the toxic effects in clinical patients during co-administration of KT and TF.

MATERIALS AND METHODS

Chemicals

TF, KT, chlorpheniramine, and promethazine were purchased from Sigma Chemical Co. (St. Louis, MO). The mequitazine was purchased from Asahi Chemical Industry (Tokyo, Japan). The protein assay kit was purchased from Bio-Rad Co. (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]. HL 60 cells was developed from human myeloid leukemia cells. 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. [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, [Knowles et al., 1980; Darlington et al., 1987] and contains 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 cell line #76 KhGH (CRL 8858; ATCC) was composed of keratinocytes derived from normal human epidermis. 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]. In HT-29 cells, *p53* is mutated in codon 273. [Niewolik 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% carbon dioxide atmosphere in Eagle's minimal essential medium (for Hep 3B, Hep G2, and CCD-922SK cells), RPMI 1640 (for HL 60, COLO 205, and HT-29 cells) supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycin, and 0.3 mg/ml glutamine. A 3:1 mixture of Ham's F12 medium and DMEM medium (for #76 KhGH cells) supplemented with 10% FCS, 40 ng/ml hydrocortisone, 0.01 mg/ml cholera toxin, 0.005 mg/ml insulin, and 10 ng/ml epidermal growth factor [Ho et al., 2001].

Chemical Exposure and Determination of Cell Viability

For the toxicity studies, stock solutions (50 mM) of each drug were prepared by dissolving the compounds in dimethylsulfoxide (DMSO). Then the stock solutions of drugs were diluted with serum-free and antibiotics-free medium to yield final concentrations ranging from 0 to 15 μM. The final treatment concentration of DMSO was 0.05% (v/v) DMSO/culture medium. After various periods of incubation, cells were sedimented and the viability of the cells was determined immediately by the trypan blue exclusion assay. The viability percentage was calculated based on the percentage of unstained cells.

Analysis of DNA Fragmentation

Treated or mock-treated cells were grown in 9-cm Petri dishes. Both attached and detached cells were harvested, washed twice with ice-cold PBS, and the DNA was then isolated as described in our previous study [Ho et al., 1998]. Samples were electrophoresed in a 1.5% (w/v) agarose gel and DNA was visualized by ethidium bromide staining.

Preparation of Protein Lysate, SDS–Polyacrylamide Gel Electrophoresis (PAGE), and Immunoblotting

The protein preparation has been described previously [Ho et al., 1998; Chen et al., 2000]. Treated or untreated subconfluent monolayers of cells in 9-cm Petri dishes were rinsed three times with ice-cold phosphate-buffered saline (PBS), scraped off with a rubber policeman, pelted at 800*g* for 5 min and lysed in 500 μl 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; 5 mM dithiothreitol) for 20 min on ice. The extracts were cleared by centrifugation for 30 min at 10,000*g*.

Equal amounts of proteins (50 μg) (estimated using Bio-Rad protein assay kit, Bio-Rad Labs.) were separated by 12.5% (w/v) SDS–PAGE on 0.75 mm mini-gels (Migdet System; Pharmacia, Inc., Piscataway, NJ) and were transferred to an immobilon P membrane (Millipore Corp., Bedford, MA) with a semidry electroblotting apparatus (TE70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm^2 for 40 min in 25 mM Tris-HCl, pH 8.3; 192 mM glycine; and 20% (v/v) methanol. 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) non-fat dry milk; and 0.1% (w/v) sodium azide).

Antibodies and Immunoblot Analysis

Antibodies used for immunoblot assays included the polyclonal rabbit antisera (used at 0.1%, v/v) specific for human *bax* protein (Ab-1, CAN Bioscience Co., CA) were raised against synthetic peptide corresponding to amino acids 150–165 of human *bax* protein. Mouse monoclonal antibodies include *p53*, *p21/Cip1*, *p27/Kip1* (Transduction Laboratories, Lexington, KY), *PARP*, *bad*, *bcl-2*, caspase-3, and *PCNA* (Santa Cruz, Inc., CA). SDS–PAGE and electroblotting to nitrocellulose filters were performed as described above. Filters were incubated for 1 h with primary antibody, washed three times, and then incubated with alkaline phosphatase-conjugated secondary antibody (immunoglobulin G) in PBS and 0.5%

(v/v) Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were visualized by incubating with the colorigenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co.).

Metabolism of TF by Recombinant Human CYP 3A4

Microsomes prepared from baculovirus-infected insect cells expressing CYP 3A4 were obtained from Genetest (Woburn, MA) [Nakajima et al., 1999]. The recombinant CYP 3A4 was coexpressed with cytochrome b5 [Kobayashi et al., 2000]. Control microsomes were from insect cells infected with wild-type baculovirus. Microsomes from insect cells containing recombinant CYP 3A4 (50 pmol/ml) were incubated with TF (2.5 μM) at 37°C for 30 min. In this study, KT and itraconazole (ITRA) were used as a selective CYP 3A4 inhibitor as described in the previous study [Jurima-Romet et al., 1994; Baldwin et al., 1995; Newton et al., 1995].

RESULTS

TF-Induced Apoptosis in Human Cancer Cell Lines

In the Figure 1A, human hepatocellular carcinoma (Hep G2), colon cancer (COLO 205), normal human fibroblast (CCD 922SK), and normal human keratinocyte (#76 KhGH) cells were treated with various concentrations (5–15 μM) of TF and the viability of these cells were determined in a time dependent manner. As seen in Figure 1A,B, the viability of the Hep G2 and COLO 205 cells was less than 30% at 24 h after exposure to TF (15 μM). No significant cytotoxicity was observed in control fibroblast (CCD-922SK) and keratinocyte (#76 KhGH) cells treated with DMSO (0.05%, v/v). Our results indicated that human cancer cells including Hep G2 and COLO 205 were more sensitive

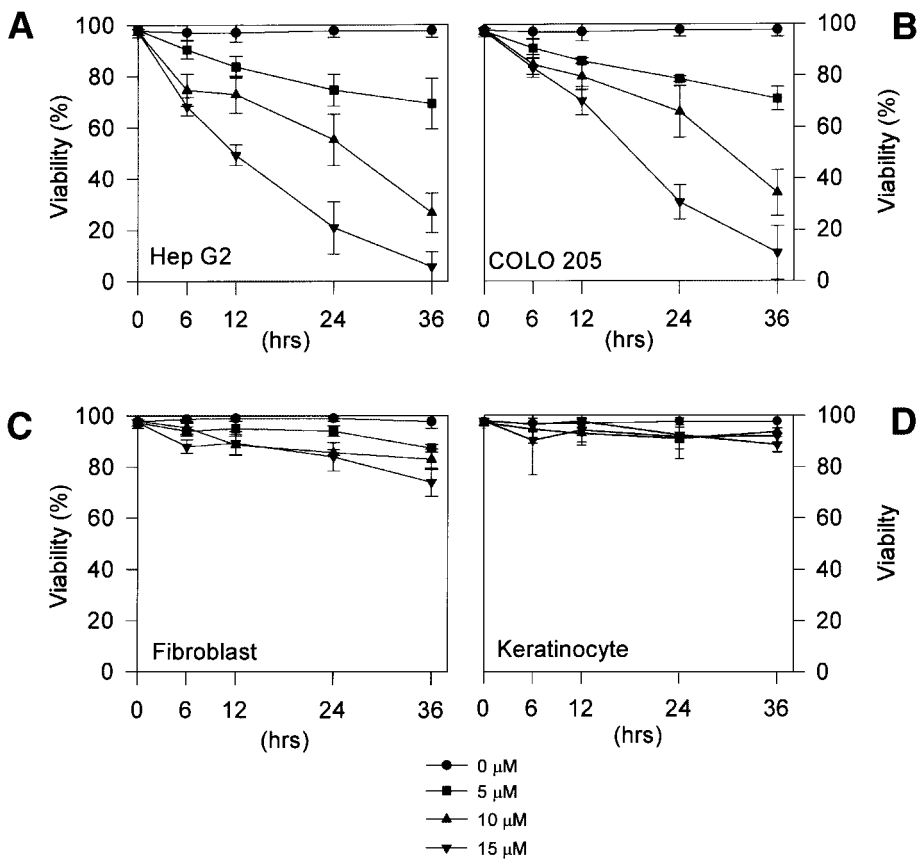

Fig. 1. Viability of the human cancer and fibroblast cells treated with TF. (**A**) Hep G2, (**B**) COLO 205, (**C**) CCD 922SK, and (**D**) #76 KhGH cells were treated with various concentrations of TF (5–15 μM) at the indicated time points. The viability was then determined by trypan blue exclusion assay as described in Materials and Methods. Results are the mean of three independent experiments.

to TF exposure than in normal cells, such as CCD-922SK and #76 KhGH cells (Fig. 1C,D).

To investigate whether the cytotoxic effects of TF observed in human cancer cells were due to the presence of apoptotic cell death. Cells were treated with TF (5–30 μM) for 24 h and DNA fragmentation analysis were performed. As shown in Figure 2A,B, significant DNA ladders were observed in both of the Hep G2 and COLO 205 cells after 10 μM of TF treatment for 24 h. Our results indicated that such apoptotic-induction effect of TF was not cell-type specific. We further demonstrated that normal human fibroblast (CCD 922SK) cells were more resistant to TF treatment when compared to cancer cells (Fig. 2C).

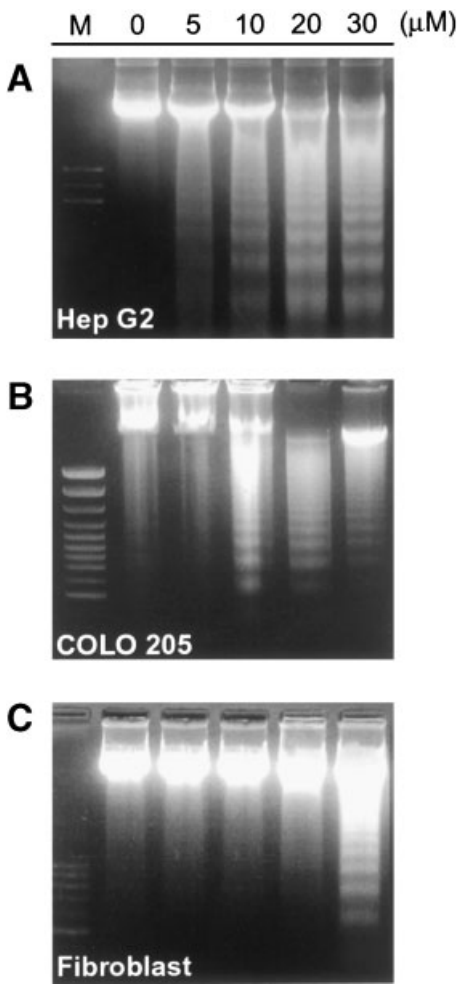

Fig. 2. DNA fragmentation analysis in human cancer cells undergoing TF-induced apoptosis. Human (**A**) Hep G2, (**B**) COLO 205, and (**C**) CCD 922SK cells were treated with TF in a dose dependent manner (5–30 μM) and DNA fragmentation was examined 24 h later. Cells in **lane 1** received mock treatment of DMSO (0.05%) as a negative control.

Elevation of the p53 Protein in TF-Treated Hep G2 and COLO 205 Cells

The tumor suppressor, p53, has been implicated in a variety of cellular processes [Greenblatt et al., 1994; Bates and Vousden, 1996]. However, the undisputed roles of p53 are the inducing of cell growth arrest and apoptosis [el-Deiry et al., 1994]. To further investigate the role of *p53* involved in TF-induced apoptosis, human COLO 205, and Hep G2 cells with wild type *p53* status were treated with TF (1–15 μM) for 24 h and the levels of cell cycle and apoptosis-regulated proteins expression were determined (Fig. 3). As shown in the Figure 3, the amount of *p53, p21/Cip1*, and *p27/Kip1* in both cells was increased with increasing doses of TF up to 15 μM. In addition, significant induction of *bax* and inhibition of *bcl-2* were observed in both cells exposed to TF (Fig. 3). The activated caspase-3 protein was detected in both cells during 24 h of TF (>10 μM) treatment. According to the previous report [Tewari et al., 1995], the substrate of caspase-3 is poly-ADP ribose polymerase (*PARP*). Western blotting analysis revealed that the *PARP* (116 KDa) molecule was degraded to a relatively stable (85 KDa) fragment at 24 h after TF (>10 μM) treatment (Fig. 3). The *bad* protein expression were also

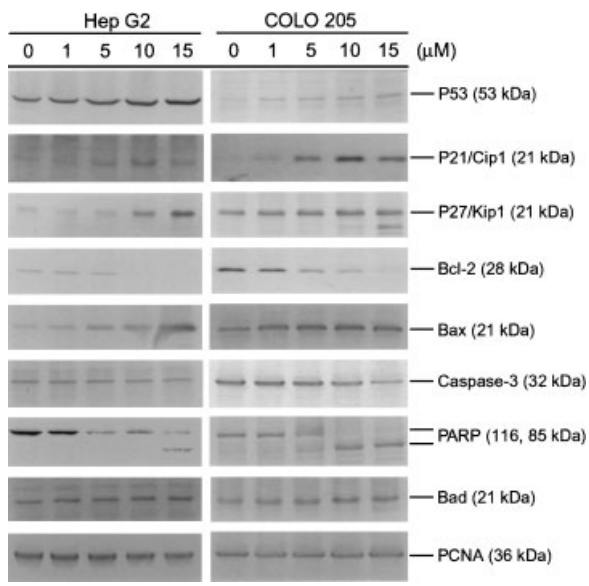

Fig. 3. TF-induced apoptosis and cell cycle regulated proteins expression in the Hep G2 and COLO 205 cells. The Hep G2 (**left panel**) and COLO 205 (**right panel**) cells were treated with TF (1–15 μM) for 24 h. The protein lysates of these cells were isolated and normalized to 50 μg/lane. Each blot is representative of at least three different experiments.

induced in cells treated with TF (>10 μM) whiles the *PCNA* expressionwas not changed.

Histamine H1-Receptor Antagonist Cannot Induce Human Cancer Cells Apoptosis

As described above, TF is a specific histamine H1 receptor antagonist [Rafferty and Holgate, 1987]. To further scrutinize whether antihistamine that against H1 receptor could induce apoptosis in human cancer cells, three additional clinical used antihistamine specific against H1 receptor namely promethazine [Nakamura et al., 1996], mequitazine [Nakamura et al., 1998], and chlorpheniramine [Yasuda et al., 1995] were used for determination of their apoptosis induction effects. As shown in the Figure 4A, all these types of antihistamine were added to the Hep G2 cells in a high concentration of 50–100 μM for 24 h exposure. Our results demonstrated that any types of cells death including apoptosis (Fig. 4A, lanes 2–7) and necrosis (Fig. 4B, lanes 2–7) were not observed in Hep G2 cells even in high concentration (100 μM) of H1 receptor antagonist treatment. However, DNA laddering was observed in lower dose (20 μM) of TF-treated Hep G2 cells. Such results implied that TF-induced apoptosis in Hep G2 cells might not be through the H1-receptor signaling pathway.

KT Potentiate the TF-Induced Apoptosis Effects in the Hep G2 Cells

The metabolic reactions of TF are presumed to be mediated by Cytochrome P450-3A isoforms. The azole antifungal agent KT was a highly potent inhibitor, having mean inhibition constants (Ki) of 0.037 and 0.34 μM for desalkyl- and hydroxy-terfenadine formation, respectively. The ITRA also was a potent inhibitor, with Ki values of 0.28 and 2.05 μM, respectively. [von Moltke et al., 1996]. Recently, several studies demonstrated that clinical adverse interactions of TF with azole antifungals agents (KT and ITRA) may be related to inhibition of TF biotransformation [Jurima-Romet et al., 1994; Rodrigues et al., 1995; Hey et al., 1996a]. To realize whether the drug-induced cellular apoptotic effects were involved in the clinical observed TF–KT and TF–ITRA interactions, the minimal concentrations of TF, ITRA, and KT that required for induction of apoptosis during combine treatment of both agents in Hep G2 cells were measured. The drug-induced apoptotic cells (appeared in the sub G1 phase) were

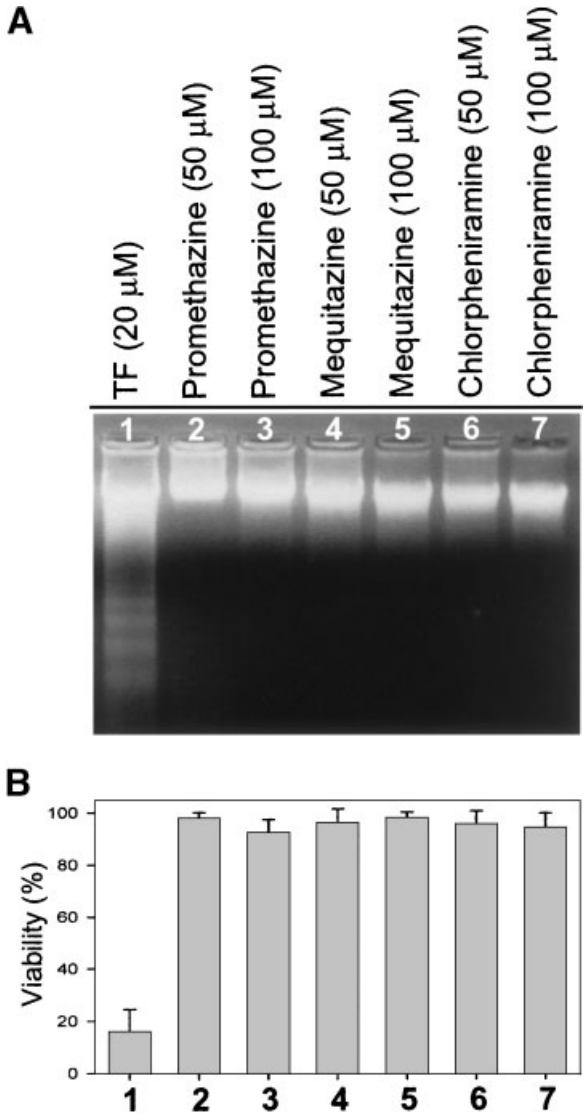

Fig. 4. Different antihistamines which specific against to the H1 receptor cannot induce apoptosis in human Hep G2 cells except for TF. Hep G2 cells were treated either with 50–100 μM of promethazine, mequitazine, or chlorpheniramine for 24 h. Cells in the **lane 1** were treated with 20 μM of TF for 24 h as a positive control. **A**: The DNA were isolated from these cells and detected for DNA fragmentation. **B**: The cell viability was determined by trypan blue exclusion assay as described in Materials and Methods.

detected by flow cytometry analysis. Interestingly, our results revealed that 1 μM of KT and ITRA were the minimal concentration that required for induction of significant apoptosis in the Hep G2 cells when treated with TF (2.5 μM) simultaneously (Fig. 5A,B). Accordingly, our data further demonstrated that the minimal concentration of TF required for induction of apoptosis in the presence of KT (1 μM) and ITRA (1 μM) were measured as 1 μM

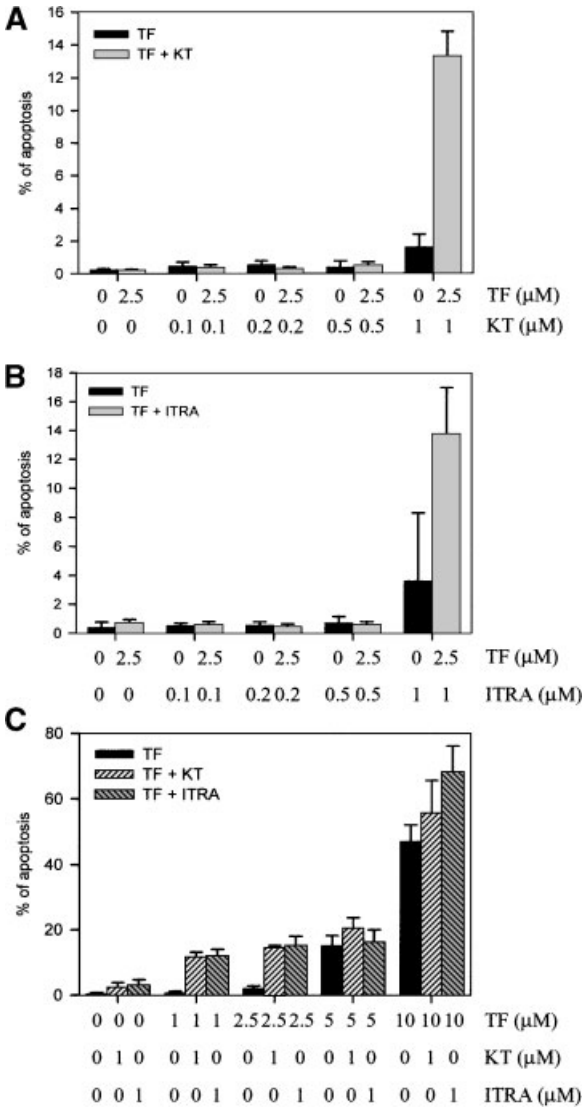

Fig. 5. Determination of the minimal concentrations of KT, ITRA, and TF that required for apoptosis induction in combine treatment experiments. Hep G2 cells were simultaneously treated with (**A**) TF (2.5 μM) plus KT (0.1–1 μM), (**B**) TF (2.5 μM) plus ITRA (0.1–1 μM), and (**C**) TF (1–10 μM) simultaneously with either KT (1 μM) or ITRA (1μM) for 24 h. FACS analysis of DNA content was conducted when Hep G2 cells were harvested after drug treatment. Percentage of cells in the Sub G1 phase of the cell cycle was determined using established CellFIT DNA analysis software. Three samples were analyzed in each group, and values represent the mean value.

(Fig. 5C). Such results implied that the CYP 3A4 might be play an important role in the TF-induced apoptosis in our system.

To further investigate whether these observations were cell-type specific, additional four types of human cancer cell lines including human hepatocellular carcinoma (Hep 3B), leukemia (HL 60), and colon cancer (COLO 205 and HT 29) cells were selected. Our data revealed that apoptosis induction by combine treatment of TF (2.5 μM) and KT (1 μM) were more profound in hepatocellular carcinoma (Hep G2 and Hep 3B) cells when compared to the colon cancer (COLO 205 and HT 29) cells (Fig. 6). The apoptotic cells were significantly observed by 1 μM of KT treatment in HL 60 cells (Fig. 6E; bar 4). However, no significant potentiation of the apoptotic effects were observed in the HL 60 cells when combine treatment with TF (2.5 μM) and KT (1 μM) (Fig. 6E; bar 5).

TF Induced Apoptosis was Potentiated by KT Through Inhibition of Intracellular Cytochrome p450 3A4 (CYP 3A4)

Previous studies demonstrated thatbiotransformation of the TF to its desalkyl and hydroxy metabolites were presumed to be mediated by human liver microsomal enzymes CYP 3A isoforms [Ling et al., 1995; Rodrigues et al., 1995]. The CYP 3A4 enzyme in the Hep G2 cells was significant inhibited by KT (1.0 μM), which is a potent and selective inhibitor [Boxenbaum, 1999a; Parker et al., 2000]. Substantially inhibited metabolism of TF by KT may lead to profound increases of TF exposure [Haaz et al., 1998; Baune et al., 1999; Boxenbaum, 1999b; Herman et al., 1999]. The apoptotic effects of TF in Hep G2 cells were first demonstrated in this study. Accordingly, to further clarify the roles of CYP 3A4 involved in TF-induced apoptosis, the Hep G2 cells were treated with TF (2.5 μM), KT (1 μM) or combine treatment of both agents for 24 h and the DNA fragmentation was then analyzed (Fig. 7A). As shown in the Figure 7A (lanes 2, 3), DNA fragmentation was not observed in the Hep G2 cells treated either with KT (1 μM) or TF (2.5 μM) alone. However, DNA fragmentation was only observed significantly in Hep G2 cells by combined treated with both agents (Fig. 7A, lane 6). Our results also demonstrated that the p53, p21/Cip1, and p27/Kip1 proteins expressions were significantly enhanced by combine treatment of KT (1 μM) plus TF (2.5 μM) when compare to TF- or KT-treated alone (Fig. 7B). Our results shown in the Figure 7A,B clearly demonstrated that KT (1 μM) potentiated the TF (2.5 μM)-induced DNA laddering effects in the Hep G2 cells and such apoptosis effects might be induced through the p53, p21/Cip1, and p27/Kip1 proteins activation.

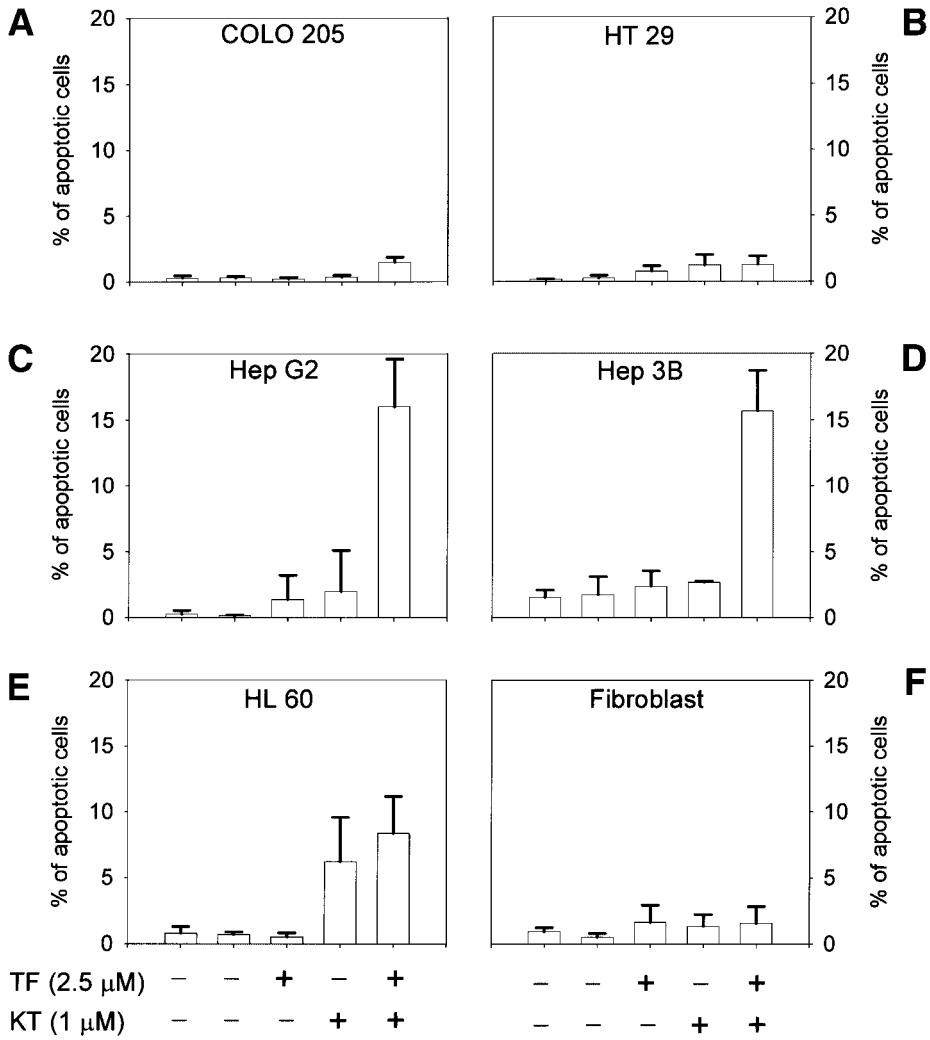

Fig. 6. Synergistic induction of apoptosis by TF and KT in different types of human cancer cells. Human (**A**) COLO 205, (**B**) HT 29, (**C**) Hep G2, (**D**) Hep 3B, (**E**) HL 60, and (**F**) CCD 922SK cells were treated with either TF (2.5 μM), KT (1 μM), or combined treatment for 24 h. Cells were treated with DMSO (0.05%) as a control group. FACS analysis of DNA content was conducted when cells were harvested after drug treatment. Percentage of cells in the Sub G1, G0/G1, S, and G2/M phases of the cell cycle were determined using established CellFIT DNA analysis software. Three samples were analyzed in each group, and values represent the mean value.

To further investigate whether the KT-potentiated DNA laddering effect that induced by TF was due to inhibition of CYP 3A4 activity in Hep G2 cells. Microsomes from baculovirus-infected insect cells expressing CYP 3A4 were prepared to examine the inhibitory effects of CYP 3A4 involved in metabolism of TF. The TF (2.5 μM) was preincubated with the recombinant CYP 3A4 (30 pmol of CYP/ml) at 37°C for 30 min [Kobayashi et al., 2000]. The CYP 3A4-treated TF (2.5 μM) was then added to the Hep G2 cells for DNA laddering analysis (Fig. 7A, lane 4). Our results demonstrated that DNA laddering effects were completely attenuated in Hep G2 cells when combine treatment of KT (1 μM) and CYP 3A4-inactivated TF (2.5 μM) (Fig. 7A, lane 5). Such results further implied that KT-inhibited CYP 3A4 activity may increase the concentrations of TF present in the cultured medium.

DISCUSSIONS

TF-Induced Human Cancer Cells Apoptosis Was Through the p53-Dependent and-Independent Signaling Pathway

In this study, Hep G2 and COLO 205 cells (with wild type *p53*) were very sensitive to TF.

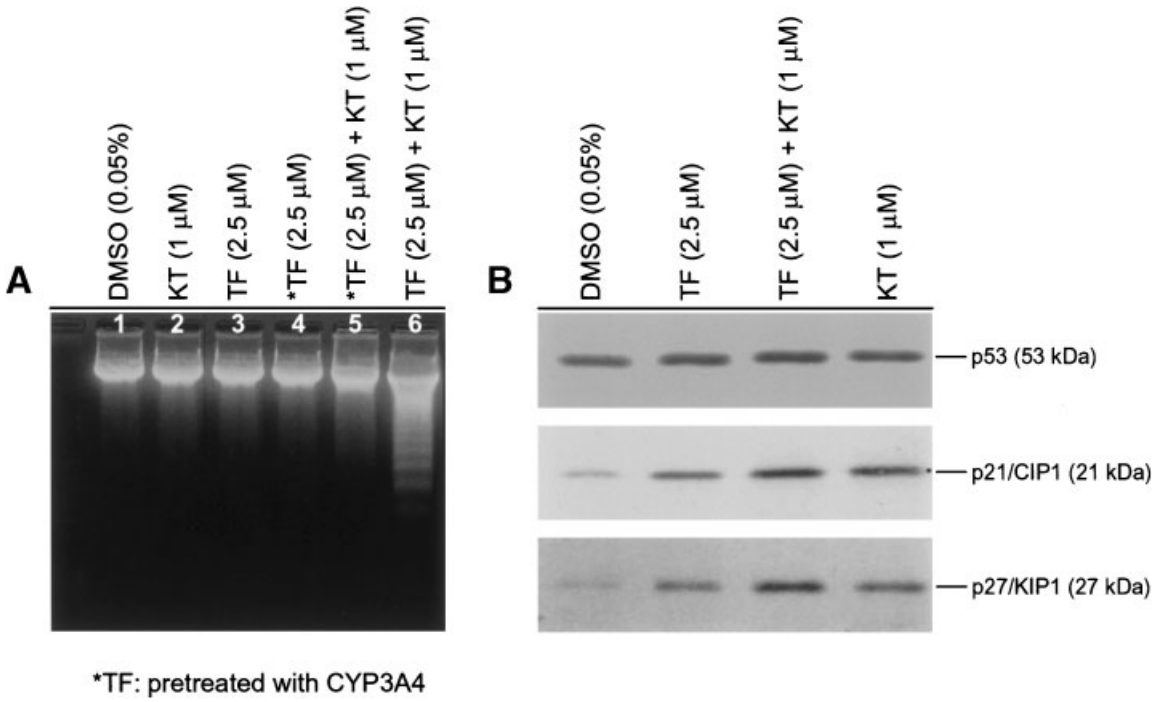

Fig. 7. TF-induced DNA fragmentation was potentiated by KT through induction of p53, p21/Cip1, and p27/Kip1. **A**: Hep G2 cells were treated with TF, KT, or both for 24 h in the following treatments: (**lane 1**) DMSO (0.05%), (**lane 2**) KT (1 μM), (**lane 3**) TF (2.5 μM), (**lane 4**) TF (2.5 μM) pre-incubated with microsomes prepared from insect cells (containing recombinant CYP 3A4) for 30 min, the mixtures were then added to cells, (**lane 5**) TF (2.5 μM) was pre-incubated with microsomes prepared from insect cells (containing recombinant CYP 3A4) for 30 min, KT (1 μM) was added to these mixtures then treated for Hep G2 cells immediately for additional 24 h, or (**lane 6**) combine treated with both TF (2.5 μM) and KT (1 μM). The DNA was isolated and analyzed for DNA laddering effects from these cells treated with drugs 24 h later. **B**: Hep G2 cells were treated with TF, KT, or both for 24 h in the following treatments: (**lane 1**) DMSO (0.05%), (**lane 2**) TF (2.5 μM), (**lane 3**) KT (1 μM), or (**lane 4**) combine treated with both TF (2.5 μM) and KT (1 μM). The p53, p21/Cip1, and p27/Kip1 proteins expression were analyzed by Western blotting analysis from these cells treated with drugs.

The *p53* protein expression was significantly induced in both cells as evidenced by immunoblotting analysis. According to the previous reports described, *p53* inhibits expression of *bcl-2* and induces 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 the *bcl-2* and *bax* protein. On the other hand, others' and ours studies demonstrated that agents which cause DNA damage (such as NO and γ-radiation) might elevate *p53* and *bax* levels, inhibit *bcl-2* expression and eventually cause apoptosis [Ho et al., 1996, 1997, 1999a,b; Kitada et al., 1996]. Such results implied that the *p53*-regulated *bax* and *bcl-2* protein expression was not changed by all of the apoptotic inducing agents. In this study, the *bcl-2* and *bax* protein expression were significantly changed by TF treatment in Hep G2 and COLO 205 cells. The direct evidence of *p53*-regulated TF-induced *bcl-2* and *bax* protein expression will be investigated in our further study by *p53*-specific antisense oligonucleotide as described recently [Chen et al., 2000]. On the other hand, we demonstrated that the Hep 3B cells (with deleted *p53*) were also exhibited a significant sensitivity to TF-induced apoptosis. Such results implied that the *p53*-signaling pathway was the one but not the only one way to induce apoptosis by TF.

KT Affects the Normal Functions of Liver and Intestine Through Inhibition of Microsomal CYP Enzymes

KT is a worldwide used antifungal agent with a broad spectrum of activity against both systemic and superficial mycosis. However, its

use was associated with adverse reactions and the most common side effects are hepatotoxicity [Lewis et al., 1984; Lake-Bakaar et al., 1987; Benson et al., 1988]. Many reports demonstrated that KT interfere the biotransformation of many hazardous compounds and the drug–drug interactions with KT in man are due to the effects of this drug on hepatic microsomal activity [Fleishaker et al., 1996; Floren et al., 1997; Khoo et al., 1998]. Recently, advanced studies demonstrated that hepatic microsomal enzyme CYP 3A4 was selectively inhibited by KT in vitro and in vivo [Boxenbaum, 1999a,b; Gibbs et al., 2000]. The roles of CYP 3A isoforms are responsible for the metabolism of a majority of therapeutic compounds [Meredith et al., 1985; Mosca et al., 1985], and they are abundant in the intestine and liver [Tsunoda et al., 1999]. KT has also been reported for CYP1A1 inhibition in the intestine in some individuals [Paine et al., 1999]. All these results revealed that the hepatotoxicity and intestinal toxicity induced by KT may be due to inhibition of microsomal enzymes in human tissues.

Inhibition of CYP 3A4 Activity in an Effective Concentration of KT Cannot Induced Apoptosis in Human Cancer Cells

The CYP 3A4 enzyme in cultured Hep G2 cells was nearly complete inhibited by KT in a concentration of 1 μM [Boxenbaum, 1999a; Parker et al., 2000]. However, in such a concentration of KT (1 μM) cannot induce apoptosis in Hep G2 cells (Fig. 6C). Our previous studies demonstrated that apoptosis and G0/G1 cell cycle arrest were induced by KT in a concentrations of more than 5 μM in different types of human cancer cells including human hepatocellular carcinoma (Hep G2 and Hep 3B), and colorectal cancer (COLO 205 and HT 29) cells [Ho et al., 1998; Chen et al., 2000]. CYP 3A4 was reported involved in the biotransformation of TF [Ling et al., 1995; Rodrigues et al., 1995], whereas the TF does not have the same inhibitory potential for cytochrome P-450 isozymes as KT [Back et al., 1989]. Another study demonstrated that KT strongly inhibited *N*-desmethyladinazolam demethylation (IC 50 = 0.14 μM) studied with human liver microsomes using substrate concentration in a range of 10–1,000 μM [Venkatakrishnan et al., 1998].The inhibition constants (Ki) of KT for the desalkyl and hydroxy pathway of TF metabolism were averaged of 0.024 and 0.237 μM, respectively [von Moltke et al., 1994]. These results indicated that 1.0 μM of KT could inhibit the intracellular CYP 3A4 activity in a significant level. However, our results demonstrated that inhibition of CYP 3A4 enzyme activity by KT in an effective concentration (1.0 μM) was not induced apoptosis in human Hep G2 cells (Figs. 6C and 7A). Our results revealed that KT-induced apoptosis was only observed in a concentration of more than 5 μM (Fig. 2A) and the apoptosis signaling pathway has been demonstrated to be associated with the p53-signaling pathway in our recent study [Ho et al., 1998; Chen et al., 2000].

Inhibition of CYP 3A4 Decreased the Biotransformation of TF and Increased Exposure of TF in Cultured Hep G2 Cells

As shown in the Figure 6, combine treatment of KT (1.0 μM) with TF (2.5 μM) significantly induced apoptosis in Hep G2 cells. However, this apoptotic effect induced by TF was completely attenuated when TF was pretreated with recombinant CYP 3A4. Our data suggested that inhibition of intracellular CYP 3A4 by KT in Hep G2 cells decreased the biotransformation of TF in cultured medium, such results may eventually increased and prolonged the TF exposure. A previous study used a mathematical model, based on the in vitro Ki values and the usual clinical range of plasma KT concentrations (1–5 μg/ml; 1.88–0.94 μM), predicted that plasma TF levels during coadministration of KT would increase by a factor ranging from 13- to 59-fold relative to the same dose of TF given without KT. Actual plasma TF levels during TF–KT coadministration in a clinical pharmacokinetic study were close to those predicted by the model [von Moltke et al., 1994]. These plasma levels were associated with prolongation of the corrected QT interval, thereby explaining the potentially life-threatening ventricular arrhythmias reportedly associated with TF–KT cotherapy [von Moltke et al., 1996].

Implications of the Drug–Drug Interaction of KT and TF in Clinical Therapeutic Purpose

TF was reported by Brandon et al. (1980), appears to be the first antihistamine do not block cholinergic or central H1 receptors and thus do not produce the side effects, such as sedation, impaired psychomotor performance, and excessive mucosal drying [Brandon and Weiner, 1980]. Recently, the adverse effects of TF were illustrated in several studies including

cardiotoxic and excitotoxic responses [Liu et al., 1997; Diaz-Trelles et al., 1999; Lu and Wang, 1999]. Several additional studies demonstrated that clinical adverse interactions of TF with azole antifungals agent (KT) may be related to inhibition of TF biotransformation [Jurima-Romet et al., 1994; Rodrigues et al., 1995; Hey et al., 1996a]. The mechanisms were now been investigated that simultaneously administration of KT and TF may predicted a potentially hazardous impairment of TF clearance by KT [Haaz et al., 1998; Baune et al., 1999; Boxenbaum, 1999b; Herman et al., 1999]. For example, human TF hepatic extraction goes from 95% in the absence of a competitive inhibitor to 35% in the presence of KT (200 mg oral intake every 12 h dosed to steady-state) [Boxenbaum, 1999a]. Clinical patients coadministration of KT and TF induced a polymorphic ventricular tachycardia that is associated with prolongation of the QT interval [Monahan et al., 1990]. The cardiotoxic effects were demonstrated in TF–KT cotherapy in animal experimental model [Hey et al., 1996a,b; Roberts and Llenas, 1996]. Although some adverse effects was observed by TF–KT cotherapy in patients, the molecular mechanisms of the drug–drug interactions involved in these adverse effects were not clearly investigated. Our results provide the molecular basis of TF–KT interactions in vitro and such results required further investigations in animal experiment.

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