Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food and Chemical Toxicology 43 (2005) 1483–1495
www.elsevier.com/locate/foodchemtox

Molecular mechanisms of econazole-induced toxicity on human colon cancer cells: G0/G1 cell cycle arrest and caspase 8-independent apoptotic signaling pathways

Yuan-Soon Ho [a], Chih-Hsiung Wu [b], Hsiao-Min Chou [c], Ying-Jan Wang [d], How Tseng [e], Chien-Ho Chen [f], Li-Ching Chen [g], Chia-Hwa Lee [h], Shyr-Yi Lin [i,*]

[a] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei 110, Taiwan*
[b] *Department of Surgery, School of Medicine, Taipei Medical University and Hospital, Taipei 110, Taiwan*
[c] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei 110, Taiwan*
[d] *Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan 701, Taiwan*
[e] *Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan*
[f] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei 110, Taiwan*
[g] *Department of Nursing, TzuChi University, Hualien 970, Taiwan*
[h] *Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei 110, Taiwan*
[i] *Department of Internal Medicine, Taipei Medical University Hospital, Department of Internal Medicine, School of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*

Received 9 September 2004; accepted 12 April 2005

Abstract

Econazole (Eco), a potent broad-spectrum anti-fungal agent, has been used in the treatment of superficial mycosis. Eco is a store-operated Ca^{2+} channel antagonist which induces cytotoxic cell death of leukemia. However, little is known about its cytotoxic effect upon solid tumor cells. The purpose of this study is to investigate both the in vitro and in vivo molecular mechanisms of Eco-induced toxicity on colon cancer cells. We used COLO 205 cell line and nude mice xenograft model to investigate the cytotoxic effect of Eco. We demonstrated that lower doses Eco (5–20 μM) arrested human colon cancer cells at the G0/G1 phase of the cell cycle. The protein levels of p53, p21/Cip1, and p27/Kip1 were significantly elevated while CDK2 and CDK4 kinase activity were significantly suppressed by Eco treatment in COLO 205 cells. At higher doses (40–60 μM), Eco induced COLO 205 cells apoptosis evidenced by ladder formation in DNA fragmentation assay and sub-G1 peak in flow cytometry analysis. Western blot analysis showed that caspases 3, 9 but not 8 were activated by high dose Eco treatment to the COLO 205 cells accompanied with cytochrome *c* and apoptosis-inducing factor (AIF) translocation. Significant anti-tumorigenesis effect was further demonstrated in vivo by treating nude mice bearing COLO 205 tumor xenografts with Eco 50 mg/kg intraperitoneally. Our findings highlight the molecular mechanisms underlying the Eco-induced toxicity on colon cancer cells.

Keywords: Econazle; Colon cancer; Apoptosis; Cytotoxicity; Cell cycle

Abbreviations: AIF, apoptosis-inducing factor; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DMSO, dimethylsulfoxide; Eco, Econazle; FCS, fetal calf serum; iNOS, nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NBT, nitroblue tetrazolium; PARP, poly-ADP-ribose polymerase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; VDAC, voltage-dependent anion channel.

* Corresponding author. Tel.: +886 2 27361661x3210; fax: +886 2 27393447.
E-mail address: sylin@tmu.edu.tw (S.-Y. Lin).

0278-6915/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2005.04.002

1. Introduction

Econazole (Eco), an anti-fungal imidazole compound, has been used in the treatment of vulvovaginal candidiasis (Dellenbach et al., 2000; Osser et al., 1991) and superficial fungal infection (Aron-Brunetiere et al., 1977; Prajna et al., 2003; Rigoni et al., 1989). Eco has been shown to inhibit lipopolysaccharide-inducible nitric oxide synthase (iNOS) activity in rat aortic rings and cultured J774 murine macrophage cells (Bogle and Vallance, 1996) suggesting that it exerted a potential anti-inflammatory effect. Furthermore, Eco at 50 μM reduced prostaglandin E2 production in rabbit platelets suggesting that it inhibited cyclooxygenase activity (Jiang et al., 1991). Eco at dose of 1 μg/ml was found to reduce cell numbers, viability and survival, and DNA synthesis in the NS1 myeloma line (Denyer et al., 1985). More importantly, Eco has been demonstrated to exert selective inhibition of leukemic and breast cancer cell lines than hemopoietic progenitors using clonogenicity assays. (Soboloff et al., 2002; Zhang et al., 2002). These findings suggested the potential anti-tumor effects of Eco.

The anti-fungal agents have been shown to exert anti-tumor effect. For example, ketoconazole has been used in the treatment of hormone-dependent prostate cancer (Blagosklonny et al., 2000; Mahler and Denis, 1992; Trachtenberg and Pont, 1984). Ketoconazole inhibited 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). As in our previous studies, the oral anti-fungal agents griseofulvin and ketoconazole have been demonstrated to suppress tumor cell growth in vitro and in vivo (Chen et al., 2000; Ho et al., 2001; Ho et al., 1998; Wang et al., 2002). Griseofulvin significantly potentiated the anti-cancer effect of nocodazole, leading to cessation of tumor growth. These results suggested that combined administration of anti-fungal agent and chemotherapeutic agents might provide a novel therapy for colorectal cancer (Ho et al., 2001).

Colorectal cancer is the second leading cause of cancer mortality in Western societies and one of the most common malignancies worldwide (Chinery et al., 1997). Although intensive research during the past few years has led to considerable improvement in the treatment and diagnosis at an early stage, yet for those patients with advanced stage or recurrent disease, the prognosis is still not satisfactory (Courtney et al., 2004; Gill et al., 2003; Lieberman and Atkin, 2004). Therefore, experimental and clinical investigators continue to search for new therapeutic strategies. One approach, as pursued in this study, seeks to identify medicinal agents capable of retarding the cell cycle and/or activating the cellular apoptotic response in the cancerous cells. We demonstrated that low dose Eco induced growth inhibition in colon cancer cells through G0/G1 cell cycle arrest. Furthermore, high dose Eco induced colon cancer cells apoptosis mediated via mitochondrial pathway which was not depended on caspase 8 activation. The anti-tumor effect was further examined in vivo by treating Eco to athymic mice bearing COLO 205 tumor xenografts. This study provides novel molecular mechanisms of Eco-induced cytotoxicity upon colon cancer which might have potential application for colon cancer treatment.

2. Materials and methods

2.1. Cell lines and cell culture

The COLO 205 cell line was isolated from human colon adenocarcinoma (American Type Culture Collection CCL-222). The cell line FCH, a homozygous familial hypercholesterolemia cell (CRL 1831; American Type Culture Collection), was derived from primary cultures of normal colon epithelial cells (Siddiqui and Chopra, 1984). The cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml), and 0.3 mg/ml glutamine for COLO 205; grown in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12, 1:1 with 2.5 mM L-glutamine, 1.2 g/l sodium bicarbonate, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 10% FCS, 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 100 ng/ml hydrocortisone and 10 mM HEPES for CRL 1831 in a humidified incubator (37 °C, 5% CO_2). Eco (Sigma Chemical Co., St. Louis, MO) was added at the indicated doses in 0.05% dimethylsulfoxide (DMSO). For control specimens, the same volume of DMSO was added in a final concentration of 0.05% (v/v) without Eco.

2.2. Determination of cell viability

COLO 205 and CRL 1831 cells were treated with Eco (10–30 μM). Cell viability was determined at the indicated times based on MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and allowed to adhere overnight. After removing the medium, 200 μl of fresh medium per well, containing 10 mmol/l Hepes (pH 7.4) was then added. Then, 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added to the wells and the plate was incubated for 2–4 h at 37 °C in the dark. The medium was removed and 200 μl DMSO and 25 μl Sorensens's glycine buffer was added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

2.3. Determination of cell growth curve

COLO 205 (1×10^5), CRL 1831 (1×10^5) cells were plated in 35 mm petri dishes. The next day, the medium was changed and Eco (5–60 μM) was added. Control cells were treated with DMSO 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.

2.4. Cell synchronization, drug treatment, and flow cytometry analysis

At 24 h after plating of cells, cells were washed three times with phosphate-buffered saline (PBS) and then incubated with medium containing 0.04% FCS for additional 24 h. Under such conditions, cells were arrested in G0/G1 as determined by flow cytometry analysis (Lee et al., 2003). After serum starvation, the low-serum (0.04% FCS) medium was removed and the cells were then challenged by addition of medium containing 10% FCS. Eco solutions were prepared in a final concentration of 0.05% (v/v) DMSO. The cell cycle stages in the Eco and DMSO-treated groups were measured by flow cytometry analysis. Cells were harvested and stained with propidium iodide (50 μg/ml) (Sigma Chemical Co., St. Louis, MO), and DNA content was measured using a FACScan laser flow cytometer analysis system (Becton–Dickinson, San Jose, CA); and 15,000 events were analyzed for each sample.

2.5. Western analysis

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

2.6. Immunoprecipitation, CDK2, and CDK4 kinase activity assay

The p21/Cip1-associated CDK-2 kinase activity was determined as described previously (Wu et al., 1996). Briefly, using 2 μg anti-CDK2 antibody and 20 μl protein A agarose beads, the protein complexes were precipitated from 200 μg of protein lysates per sample as described above. Beads were washed twice with lysis buffer and then once with kinase assay buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT). Phosphorylation of the 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-^{32}P]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 previous report (Wu et al., 1996) with some modifications. Briefly, Eco-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 $MgCl_2$, 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-^{32}P]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.

2.7. Caspase activity assay

Caspase activity was measured by using caspases 3 (Promega, Madison, WI) and 9 (Chemicon, Temecula CA) colorimetric activity assay kits as previous described (Ho et al., 2003; Lin et al., 2001). Briefly, COLO 205 cells were lysed by addition of cell lysis buffer and protein concentration was measured. Caspase activity was assayed at 37 °C in 100 μl of assay buffer containing 50 μg (for caspase 3) or 30 μg (for caspase 9) of the indicated colorimetric peptide. Caspase activity was measured by the release of *p*-nitroaniline (*p*NA) from the labeled substrates Ac-DEVD-*p*NA and Ac-LEHD-*p*NA for caspase 3 and 9, respectively, and the free *p*NA was quantified at 405 nm.

2.8. Treatment of COLO 205-derived xenografts in vivo

COLO 205 cells were grown in RPMI 1640 supplemented with 10% FCS as described above. Cells were harvested through two consecutive trypsinizations, centrifuged at 300*g* for 5 min, washed twice, and resuspended in sterile PBS. Cells (5×10^6) 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 (mm^3) = $L \times W^2/2$, where L is the length and W is the width (Ho et al., 2001). Once tumors reached a mean size of 200 mm^3, animals received intraperitoneal injections of either 25 μl DMSO or 50 mg/kg Eco three times per week for 6 weeks.

2.9. Analysis of DNA fragmentation

Analysis of DNA fragmentation was performed as previously described (Ho et al., 1996). Briefly, the Eco and DMSO-treated cells were seeded on 100 mm dishes. The DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamyl alcohol (24:1 v:v), then precipitated with 0.1 volume of sodium acetate, pH 4.8, and 2.5 volumes of ethanol at −20 °C overnight, and finally centrifuged at 13,000*g* for 1 h. Genomic DNA was quantitated, and equal amounts of DNA sample in each lane were electrophoresed in a 2% agarose gel. The DNA was visualized by ethidium bromide staining.

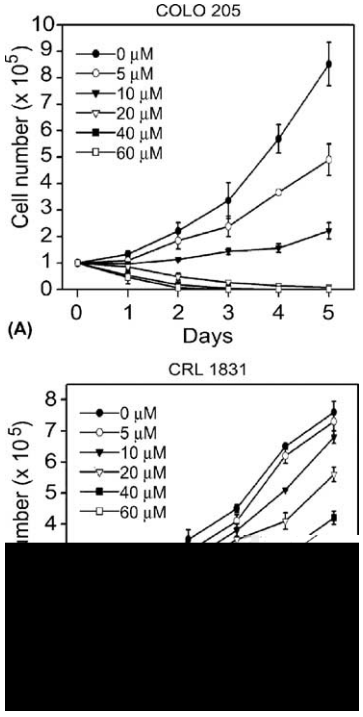

Fig. 1. Cell growth inhibitory effects of Eco on colon cancer cells and normal human colon epithelial cells. (A) Human colon cancer (COLO 205) cells and (B) Human normal colon epithelial cells (CRL 1831) were treated with various concentrations of Eco (5–60 μM) for 1–5 days. Dose-dependent suppression of cell growth was observed in COLO 205 cells and normal colon epithelial cells. Media with or without Eco were changed everyday until cell counting. Three samples were analyzed in each group and the results were presented as means ± SE.

3. Results

3.1. Eco-induced cytotoxicity in COLO 205 cells

To investigate whether Eco inhibited colon cancer cell growth, COLO 205 and human normal colon epithelial cells (CRL 1831) were treated with different doses (5–60 μM) of Eco for 1–5 days and the cell growth numbers were then determined (Fig. 1). Eco at lower doses (5–10 μM) suppressed COLO 205 cell proliferation while at higher dose (>20 μM) induced cell death (Fig. 1A). Interestingly, such effect was less profound on normal human colon epithelial cells treated with Eco (Fig. 1B). We further demonstrated that the viability of COLO 205 was significantly decreased when exposed to more than 10 μM Eco for 24 h (Fig. 2A). In contrast, less significant influence of viability was observed in normal colon epithelial cells treated with same dose of Eco (Fig. 2B). These results indicated that colon cancer cells were more susceptible to Eco treatment than normal human normal colon epithelial cells.

3.2. Eco induced G0/G1 cell cycle arrest and apoptosis in COLO 205 cells

In order to determine the action of Eco on a specific phase of cell cycle, the COLO 205 cells were synchronized by switching them to media with 0.04% FCS for

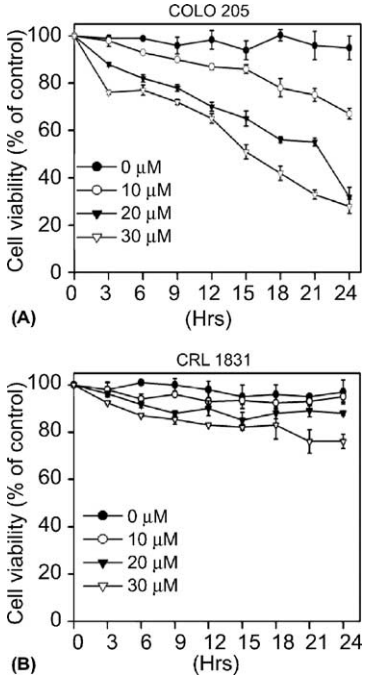

Fig. 2. Dose and time-dependent response of viability of human cells treated with Eco. (A) COLO 205 cell and (B) Normal colon epithelial cells viability was determined after exposure to various concentrations of Eco (10–30 μM) for the indicated time points. Three samples were analyzed in each group and the results were presented as means ± SE.

24 h to render them quiescent. Cells were then returned to culture media containing 10% FCS which stimulated cell proliferation with or without 20 μM Eco and, at various times thereafter, they were harvested for flow cytometry analysis. Fig. 3A (lower panel) showed that 20 μM Eco induced a significant accumulation (>85%) of cells in the G0/G1 phase of the cell cycle compared to 0.05% DMSO control (upper panel), suggesting that

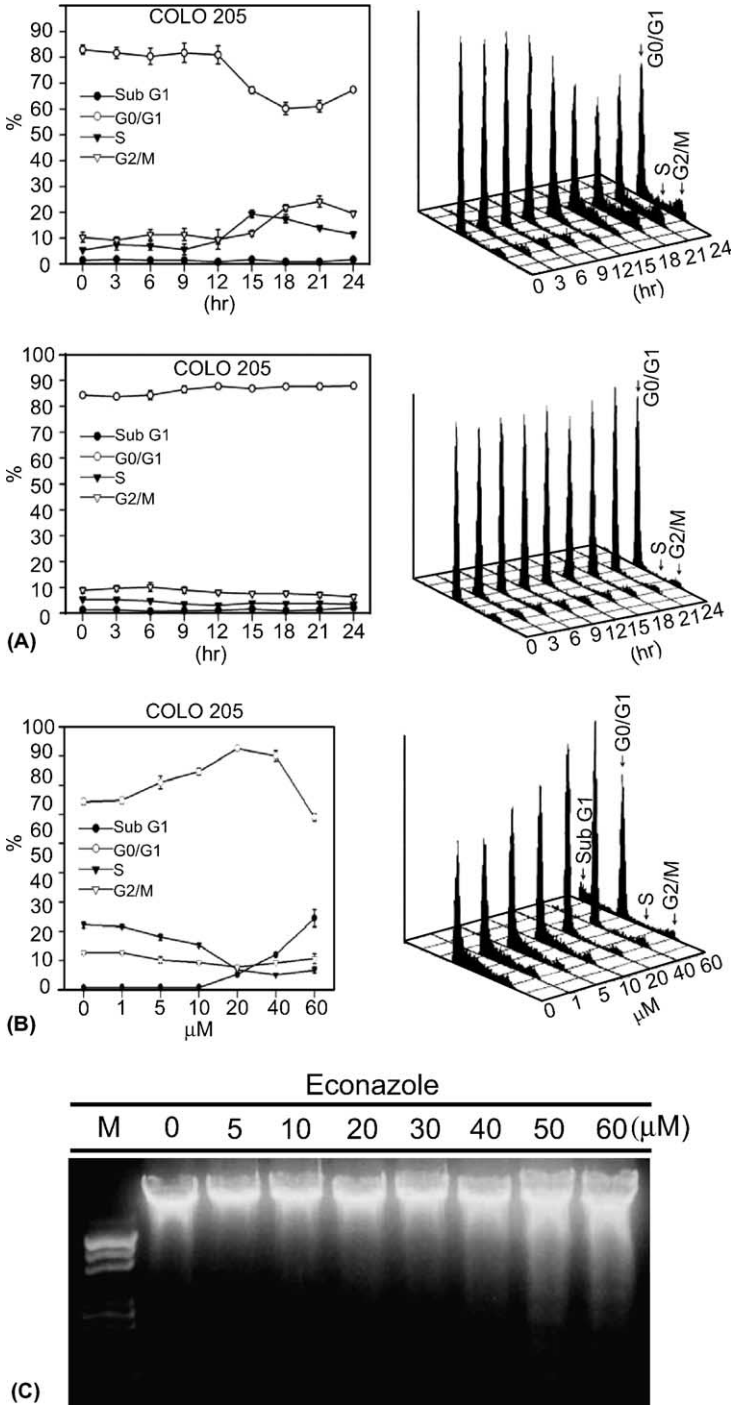

Fig. 3. Effect of Eco on cell cycle regulation of COLO 205 cells. Time-dependent effects of (A) FACS analysis of DNA content was conducted after COLO 205 cells were released for quiescence by incubation in culture media supplement with 10% FBS containing 0.05% DMSO (upper panel) or Eco (20 μM in 0.05% DMSO, lower panel). (B) Dose dependent response of Eco on cell cycle regulation. COLO 205 cells were quiescent and released by incubation in culture media supplement with 10% FBS and Eco (1–60 μM) treatment for 24 h. Percentage of cells in 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 were presented as means ± SE and (C) DNA fragmentation assay in COLO 205 cells. Cells were treated with Eco (5–60 μM) or DMSO (0.05%) as control and DNA fragmentation was examined 24 h later. M is the molecular weight marker.

the growth inhibitory effect of Eco was due to an arrest of DNA replication in the cell cycle. Fig. 3B demonstrated the dose-dependent effect of Eco on the cell cycle regulation. The results demonstrate that Eco (20 μM) induced an accumulation (up to 85%) of the COLO 205 cells at the G0/G1 phase of the cell cycle. However, higher dose (40–60 μM) of Eco induced COLO 205 cell death as evidenced by sub-G1 peak in the flow cytometry analysis. DNA fragmentation assay also demonstrated that DNA ladder formation was observed at 24 h after Eco (40–60 μM) treatment to colon cancer cells (Fig. 3C).

3.3. p53, p21/Cip1 and p27/Kip1 were the key regulators in the Eco-induced G0/G1 arrest in COLO 205 cells

As shown in Fig. 4, treatment of COLO 205 cells with lower doses (5–20 μM) Eco for 24 h induced increased of p53, p21/Cip1, p27/Kip1, cyclin E and decreased of phospho-CDK2 and phospho-Rb protein expression in a dose-dependent manner. In order to determine whether the changes of the levels of cyclins and CDKs were also involved in the Eco-induced G0/G1 arrest, we then examined cyclins and CDKs protein levels in response to Eco treatment. Eco at the concentrations of 5–20 μM did not induce any significant changes of the levels of cyclin A, D1, D3, CDK4, and proliferating cell nuclear antigen (PCNA). However, in the higher doses (40–60 μM)-treated groups, the phospho-CDK2, CDK4, cyclins A2, D1, and D3 protein levels were significantly down regulated in the COLO 205 cells. Such results might be due to cellular toxicity occurred in the COLO 205 cells treated with higher dose (40–60 μM) Eco (Fig. 4).

3.4. Eco-induced G0/G1 arrest was through inhibition of CDK2 and CDK4 kinase activity

It has been demonstrated that the progression of cell cycle activity is associated with coordinated successive activation of certain CDKs occurred late in the G1 phase and is instrumental in the transition from the G1 to the S phase (Hunter and Pines, 1994; Morgan, 1995). As described previously (Sherr and Roberts, 1995), the p21/Cip1 and p27/Kip1 were potent inhibitors of CDK2 and CDK4 which regulated the cells entering the restriction point in the G0/G1 phase of the cell cycle. Based on the flow cytometry analysis in the COLO 205 cells (Fig. 3A), the time points at the 0, 15, 18 and 24 h after released from quiescence represents the G0/G1, S, G2/M and the second G0/G1 phases of the cell cycle, respectively. Accordingly, these time points were selected for Western blot analysis to examine the effects of Eco on the expression of cell cycle regulatory proteins. We demonstrated that low dose Eco (20 μM) significantly suppressed CDK2 and CDK4 kinase activity

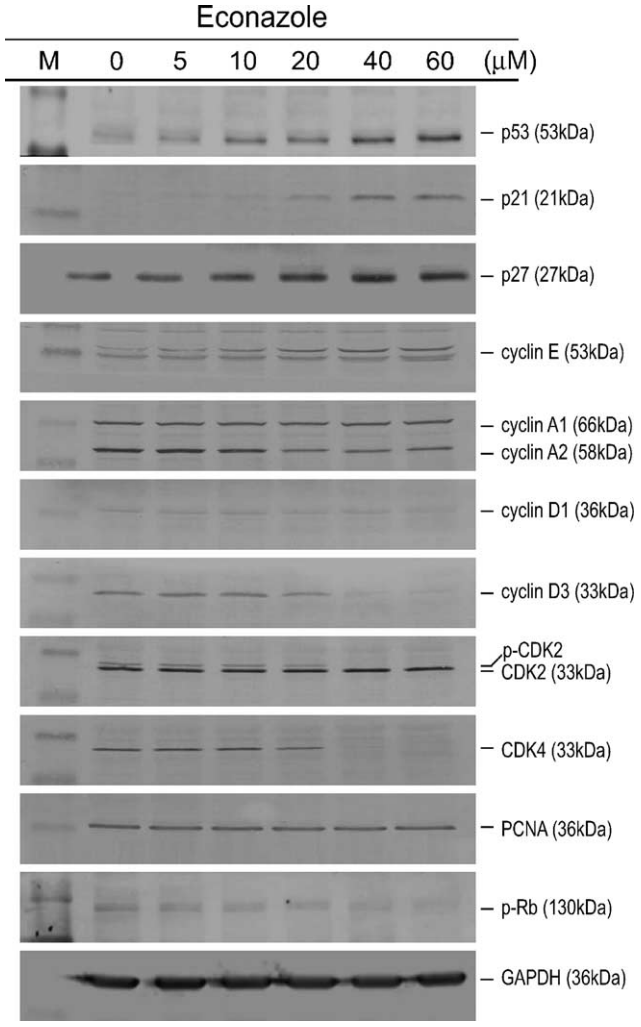

Fig. 4. Effect of Eco on the expression of G0/G1 phase regulatory proteins in COLO 205 cells. The cells were rendered quiescent by incubation for 24 h in the cultured media containing 0.04% FCS. After 15 h released from quiescence by incubation in culture media supplement with 10% FCS and 0.05% DMSO with or without Eco (5–60 μM), the cells were harvested and protein extracts (100 μg per lane) were separate by SDS-PAGE. After electrophoresis, proteins were transferred onto Immobilion-P membranes, probed with proper dilution of specific antibodies, and then detected by using the NBT/BCIP system. Membrane was also probed with anti-GADPH antibody to correct for difference in protein loading. M is the molecular weight marker.

(Fig. 5A) with concomitant increased p21/Cip1 and p27/Kip1 protein expression as compared to DMSO control groups (Fig. 5B). These results suggested that the G0/G1 arrest induced by Eco was due to decreased of the CDK2 and CDK4 kinase activity mediated by an induction of p21/Cip1 and p27/Kip1 expression.

3.5. Eco activated the caspase 3, 9 but not caspase 8

Since it has been demonstrated that the occurrence of apoptosis requires the activation of caspases (Thornberry and Lazebnik, 1998), we investigated the involvement of

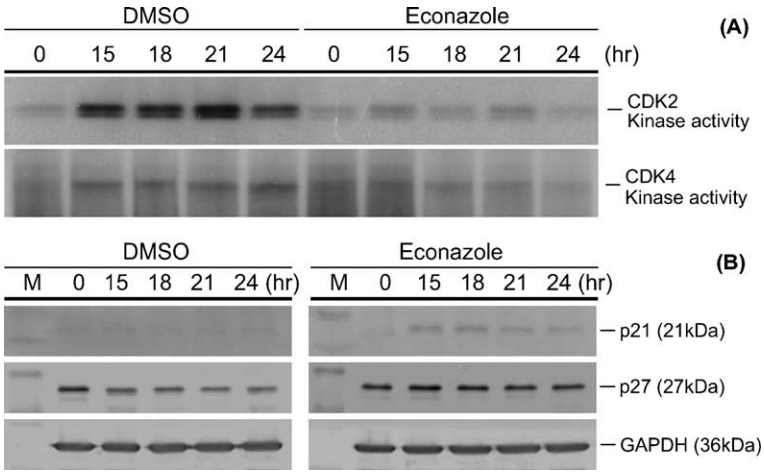

Fig. 5. (A) Time course study of Eco on the CDK2 and CDK4 kinase activity. The cells were rendered quiescent by incubation for 24 h in the cultured media containing 0.04% FCS. The cells then were released from quiescence by incubation in culture media supplement with 10% FCS and 0.05% DMSO with or without Eco (20 μM), the cells were harvested and 200 μg protein per lane was used for the CDK2 and CDK4 kinase activity assay as described in the materials and methods and (B) Time course study of Eco on the expression of G0/G1 phase regulatory proteins in COLO 205 cells. The cells were prepared as (A) and protein extracts (100 μg per lane) were separate by SDS-PAGE. After electrophoresis, proteins were transferred onto Immobilion-P membranes, probed with proper dilution of specific antibodies, and then detected by using the NBT/BCIP system. Membrane was also probed with anti-GADPH antibody to correct for difference in protein loading. M is the molecular weight marker.

caspase activation in the Eco-induced apoptosis in the COLO 205 cells by Western blot analyses. Eco at higher dose (40–60 μM) activated the caspase 3 evidenced by a decreased in the protein level of procaspase 3 and degradation of the poly-ADP-ribose polymerase (PARP), the substrate for caspase 3 in both dose-dependent and time course studies (Figs. 6A and B). To further elucidate the apoptotic pathways involved in the activation of caspase 3, we examined the changes of caspases 8 and 9 protein levels in the Eco-treated COLO 205 cells. Treatment of COLO205 cells with high dose (40–60 μM) Eco activated caspase 9 evidenced by degradation of the procaspases 9 as well as the appearance of its cleavage product but not caspase 8 (Figs. 6A and B). To further confirm the Western blot results, we performed caspase activity assays. As shown in Fig. 6C, treatment of COLO 205 cells with 60 μM Eco induced caspase 3 activity about 6 folds and 13.5 folds at 9 and 12 h compared to control, respectively, while induced caspase 9 activity about 2 folds and 5.5 folds at 9 and 12 h compared to control, respectively.

To further confirm that caspase 8 was truly not activated, Western blot analysis was performed in the COLO 205 cells treated with high dose (60 μM) Eco in a time dependent study (Fig. 6D). Consistent results were shown in Fig. 6E, high dose (60 μM) Eco induced COLO 205 cells apoptosis started after 6–12 h of treatment as evidenced by DNA laddering formation. However, under the same condition, caspase 8 activation was not detected even in a prolonged (48 h) Eco treatment in the COLO 205 cells detected by Western blot analysis (Fig. 6D). In contrast, TNFα (100 ng/ml)-treated COLO 205 cells which acted as a positive control demonstrated caspase 8 activation by 24 h as well as cleavage of its substrate, bid protein, suggesting that caspase 8 was not involved in Eco-induced apoptosis in the COLO 205 cells (Fig. 6D). We also tested whether Fas/FasL pathways were involved in the caspase 8 activation in COLO 205 cells. Treatment with 100 ng/ml FasL to COLO 205 cells for 24 h failed to activate caspase 8 and bid protein (Fig. 6D). These results were consistent with a previous report that COLO 205 cells were resistant to FasL-mediated apoptosis (Herbeuval et al., 2003).

3.6. Eco upregulated p53 and Bax protein and translocated cytochrome c from mitochondria to cytosol and AIF from mitochondrial to nucleus

Our study demonstrated that in the COLO 205 cells treated with higher doses (40–60 μM) of Eco, the Bax protein was induced at a significantly higher level than the control cells, whereas Bcl-2 and Bid expression remained the same as control (Fig. 7A). We further found that higher doses (40–60 μM) of Eco dose-dependently increased translocation of cytochrome *c* from mitochondria to cytosol in the COLO 205 cells (Fig. 7A). To examine whether cytochrome *c* release was biologically functioning in initiating apoptosome assembly, immunoprecipitation was performed with cytosolic preparations from Eco-treated cells using cytochrome *c* antibody. As shown in Fig. 7A, Western blotting detected a significantly increase of Apaf-1 in cytochrome *c* coprecipitates from COLO 205 cells.

In this study, extremely high dose (60 μM) of Eco induced significant apoptosis in COLO 205 cells as evidenced by DNA laddering occurred at 6–12 h (Fig. 6E). According to the previous study that the tumor

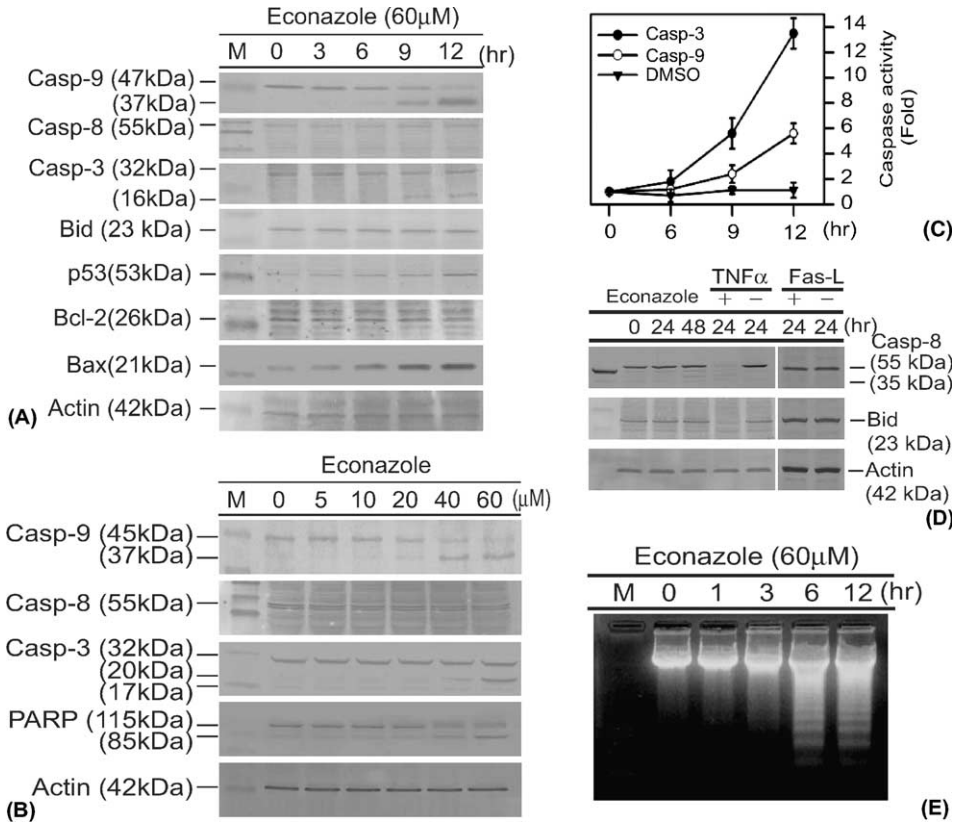

Fig. 6. Effect of Eco on the expression of apoptosis regulatory proteins in COLO 205 cells. The cells were rendered quiescent by incubation for 24 h in the cultured media containing 0.04% FCS. The cells then were released from quiescence by incubation in culture media supplement with 10% FCS and 0.05% DMSO with or without Eco. (A) Time course. (B) Dose-dependent study of Western blotting were performed. M is the molecular weight marker. Membrane was also probed with anti-actin antibody to correct for difference in protein loading. (C) Eco-induced caspase activities in COLO 205 cells. Cells were treated with 60 μM of Eco for various time periods. Caspase activities were measured as described in Materials and Methods. Data represent means ± SE for three determinations. (D) Caspase 8 is not involved in the Eco-induced apoptosis. Western blot analysis of caspase 8 and bid proteins time course study treated with high dose (60 μM) Eco, 100 ng/ml TNFα, and 100 ng/ml FasL to COLO 205 cells. Membrane was also probed with anti-actin antibody to correct for difference in protein loading and (E) High dose (60 μM) Eco caused DNA ladder formation starting after 6 h of treatment. M is the molecular weight marker.

suppressor p53 exerts its anti-neoplastic activity primarily through the induction of apoptosis (Schmitt et al., 2002). Recent study further demonstrated that the cytosolic p53 directly activated the proapoptotic Bax protein in the absence of other proteins to permeabilize mitochondria and engaged the apoptotic program (Chipuk et al., 2004). In our study, time-dependent experiment demonstrated that Eco induced p53 and Bax protein expression concurrently as early as 6–9 h (Fig. 6A). Similar responses of Eco-induced increased of the p53 (Fig. 4) and Bax (Fig. 7A) protein levels were found in the dose-dependent experiments.

Our data revealed that increased amounts of cytochrome *c* and Apaf-1 proteins were detected in the cytosolic fractions of the Eco-treated cells (Fig. 7B). The release of mitochondrial factors such as cytochrome *c*and Smac/DIABLO is considered a prerequisite for caspase 9 activation in apoptosis (Du et al., 2000; Li et al., 1997; Liu et al., 1996; Verhagen et al., 2000). In this study, presence of cytosolic cytochrome *c* was detected at 6–12 h after 60 μM Eco treatment leads to Apaf-1-mediated apoptosome assembly and rapid activation of caspase 9 (Fig. 6A). On the other hand, the Smac/DIABLO which translocated from mitochondria to cytosol was observed as early as 3–6 h (Fig. 7B) which then interacts with the XIAP (Du et al., 2000; Verhagen et al., 2000), and blocks its inhibitory effects on activated caspases 9 and 3. Translocation of AIF from mitochondrial to nucleus has been shown to activate apoptosis. Our results demonstrated that Eco at high dose induced AIF translocation from mitochondria to nucleus evidenced by Western blot analysis (Fig. 7B).

3.7. Eco inhibited colon tumor growth in vivo

We further examined the anti-tumor effect of Eco in vivo by treating athymic mice bearing COLO 205 tumor xenografts. After established palpable tumors (mean tumor volume, 200 mm^3), animals received Eco at dosage of 50 mg/kg or DMSO (control) three times a week. The tumor volume was significant smaller in the Eco-treated group than the DMSO-treated control started

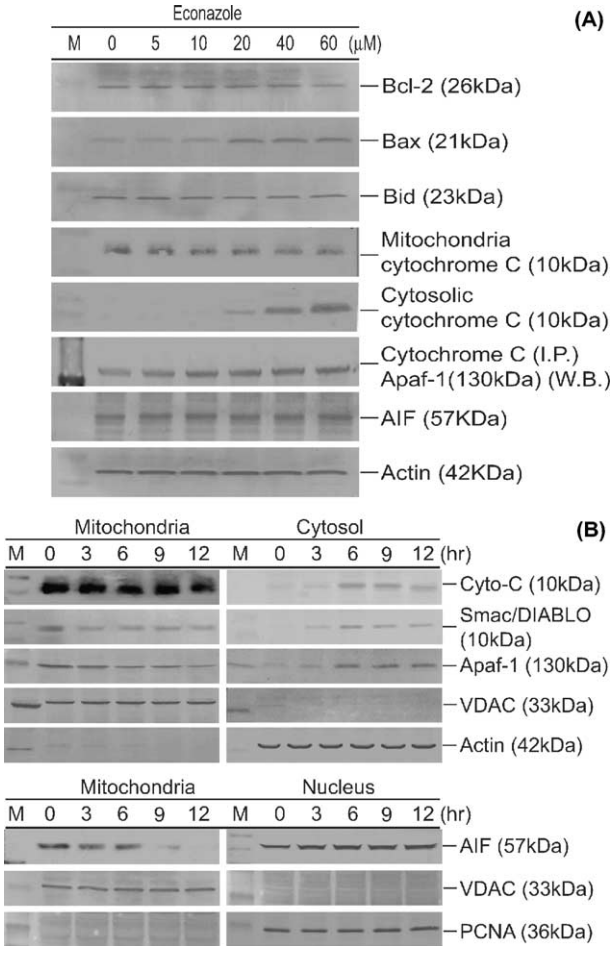

Fig. 7. Effect of Eco on the expression of apoptosis associated proteins. M is the molecular weight marker. (A) Dose-dependent study of Western blotting was performed.. Membrane was also probed with anti-actin antibody to correct for difference in protein loading. (B) Translocation of apoptosis associated factors in COLO 205 cells. Western blot analysis of time course study treated with high dose (60 μM) Eco. Upper panel, mitochondrial voltage-dependent anion channel (VDAC) and actin were used as loading controls for mitochondrial and cytosolic proteins, respectively. Lower panel, VDAC and PCNA were used as loading controls for mitochondrial and nuclear proteins, respectively. M is the molecular weight marker.

from the second to the sixth week (Fig. 8A). In mice receiving these treatment regimens, no gross signs of toxicity were observed including body weight (Fig. 8B) and visible inspection of general appearance, and microscopic examination of individual organs such as intestinal epithelium histology (data not shown). Additionally, the tumor weight and the tumor/body weight ratio were strongly inhibited in the Eco-treated group (Figs. 8C and D).

4. Discussion

Eco has been used to treat superficial fungal infections and vaginal candidiasis. Successful treatment using Eco in a case of middle aged women with opportunistic pulmonary aspergillosis had been reported (Hantschke et al., 1978). Toxicity and teratogenicity studies in guinea-pigs and rats indicated that Eco was well tolerated (Thienpont et al., 1975). However, little is known about its use in oral or intravenous applications (Heel et al., 1978; Midgley et al., 1981). The present study was undertaken to examine the cytotoxic effect of Eco on human colon cancer cells. Our in vitro studies demonstrated that Eco at concentrations of 5–20 μM inhibited growth rate of COLO 205 cells in a dose-dependent manner, but less significant in normal human colon epithelial cells. These results were due to an inhibitory effect of econzole on the mechanisms for cell division in the subcultured cancer cells. Furthermore, when Eco concentration was increased to 40–60 μM, apoptosis was observed in COLO 205 cells. In vivo studies show that intraperitoneal administration of Eco caused a significant suppression of the COLO 205 tumor mass. To our knowledge, this is the first demonstration that Eco induces toxicity of colon cancer cells both in vitro and in vivo.

By flow cytometry analysis, we demonstrated that Eco at lower concentrations (5–20 μM) induced G0/G1 cell cycle arrest of COLO 205. Cell cycle progression is regulated by successive, coordinated activation of CDKs (Hunter and Pines, 1994; Morgan, 1995), whose activity is controlled by their association with a series of regulatory subunits called cyclins and a group of CDK-inhibitory proteins designated CKIs (Sherr and Roberts, 1995). Among these CKIs are two known as p21/Cip1 and p27/Kip1. The p21/Cip1 is induced by p53 tumor suppressor gene on DNA damage (Gartel et al., 1996). Transcriptional activation of p21/Cip1 associated with and inactivated the CDKs which resulted in G0/G1 arrest (el-Deiry et al., 1994; el-Deiry et al., 1993). The human papilloma virus E7 oncoprotein interacted with p21/Cip1 protein and abrogated p21/Cip1-mediated inhibition of CDK2 activity (Funk et al., 1997). Keratinocyte from p21/Cip1-null mice are more tumorigenic than the wild type counterpart following expression of an activated ras allele (Missero et al., 1996) suggesting that p21/Cip1 is a candidate tumor suppressor gene. Our data demonstrated that p53 protein induction occurred in advance of p21/Cip1 suggesting that p53 might transcriptionally activate p21/Cip1 which in turn mediated Eco-induced G0/G1 arrest in COLO 205 cells.

p27/Kip1, a cell cycle regulatory protein, controls G1/S transition checkpoint by inhibiting the activity of different kinds of cyclin/CDK complexes in vitro and in vivo, including CDK2 and CDK4 complexes (Polyak et al., 1994). Homozygous deletion of p27/Kip1 alleles in mice results in enhanced growth, hyperplasia of various organs, and the spontaneous formation of pituitary tumors (Kiyokawa et al., 1996; Nakayama et al., 1996). Overexpression of p27/Kip1 leads to apoptotic cell death in mammalian cells suggesting a potential therapeutic

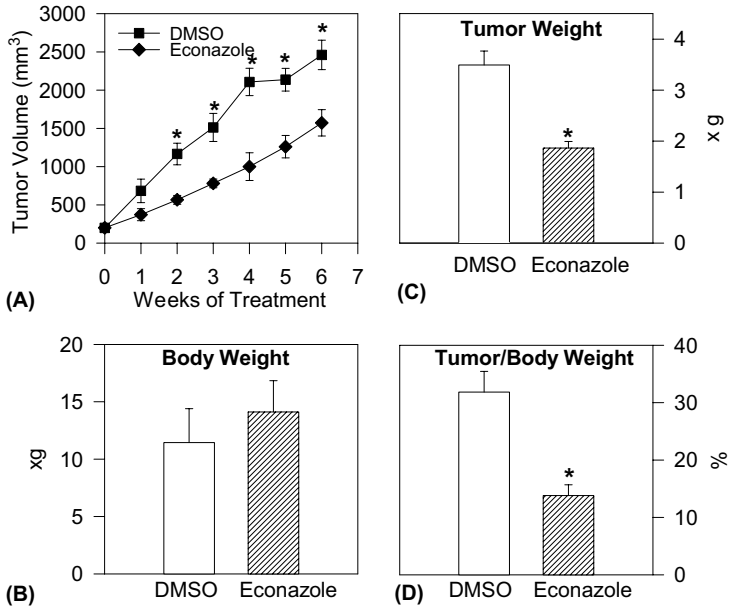

Fig. 8. The growth of COLO 205 tumor xenografts in nude mice was suppressed by Eco treatment. Athymic nude mice injected with COLO 205 cells into subcutaneous tissue of inter-scapular area. Once tumor volume reached approximately 200 mm^3, the animal received treatment of 50 mg/kg Eco or DMSO intraperitoneally three times per week for 6 weeks. (A) Average tumor volume of DMSO-treated (circle, $n = 5$) versus Eco-treated ($n = 5$) nude mice. (B) Body weight. (C) Tumor weight and (D) Tumor/body weight ratio were measured at the end of the experiment. Five samples were analyzed in each group, and values represent the means ± SE. Comparisons were subjected to Student's t test. *Significant difference at $p < 0.05$.

approach aimed at elevating p27/Kip1 expression for treatment of human cancers (Wang et al., 1997). In our study, low dose Eco induced p21/Cip1 and p27/Kip1 protein expression, suppressed the CDK2 and CDK4 kinase activity and induced G0/G1 arrest while there were no significant changes of cyclins or CDKs expression suggesting that p21/Cip1 and p27/Kip1 are the main regulators of Eco-induced cell cycle inhibition.

By flow cytometry and DNA fragmentation analyses, we also demonstrated that high dose Eco (40–60 μM) caused apoptosis in COLO 205 cells. The extrinsic, caspase 8/FADD pathway and intrinsic, mitochondrial pathway are the two major signal pathways regulated apoptosis process. Activaton of bid by caspase 8 links the extrinsic to the intrinsic apoptotic pathway through mitochondrial damages (Li et al., 1998). It has been shown that clinically applied anti-cancer drugs such as cisplatin, topotecan, and gemcitabine induced apoptosis of solid tumor cells through caspase 8 dependent pathways (Ferreira et al., 2000). Tumor resistance to cytotoxic drugs may occur through altered expression of caspase 8, upregulation of caspase 8 inhibitors or sequestration of caspase 8 (Kim et al., 2001). Furthermore, hypoxia is known to be associated with interference to chemotherapy or radiotherapy and hypoxia-induced apoptosis mainly relies mitochondrial pathways (Weinmann et al., 2004). Our results demonstrated that Eco-induced apoptosis in colon cancer cell death was primarily through mitochondrial pathway which was not depended on caspase 8 and bid activation. These findings suggested that Eco might be a useful salvage agent in the management of chemotherapy resistant colon cancer.

The bax gene promoter region contains four motifs with homology to consensus p53-binding sites suggesting that bax is a p53 primary-response gene, involved in a p53 transcriptionally regulated induction of apoptosis (Miyashita and Reed, 1995). However, recent study further demonstrated that the cytosolic p53 directly activated the proapoptotic Bax protein (Chipuk et al., 2004). Our study demonstrated that Eco induced p53 and Bax protein expression concurrently. Based on these findings, we propose that when p53 accumulates in the cytosol, it can function analogously to the BH3-only subset of proapoptotic Bcl-2 proteins to activate Bax and trigger the apoptosis mediated by mitochondria-signaling processes.

It has been discovered that in response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effector such as AIF (Lorenzo et al., 1999). AIF is a phylogenetically ancient mitochondrial intermembrane flavoprotein endowed with the unique capacity to induce caspase-independent peripheral chromatin condensation and large-scale DNA fragmentation when added to purified nuclei (Cande et al., 2002). In our study, high dose Eco treatment induced translocation of AIF from mitochondria to nucleus suggesting a peculiar molecular mechanism in the Eco-induced cytotoxicity toward colon cancer.

Based on the results of our present study, we proposed a model for the molecular mechanisms of

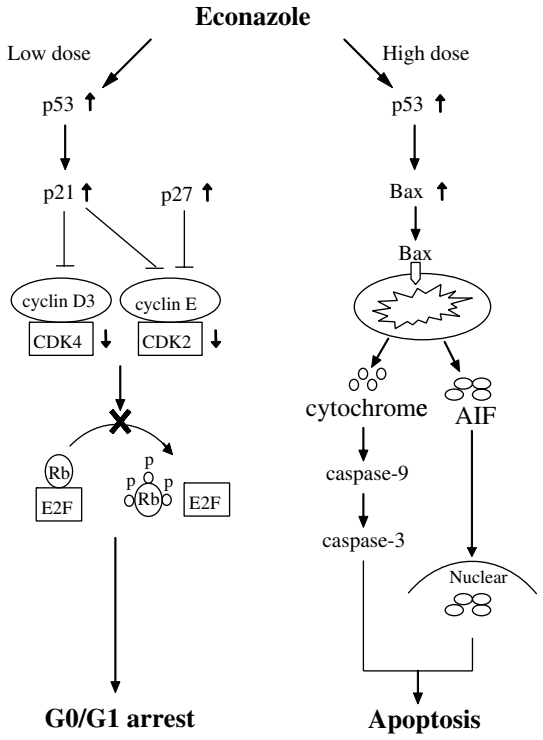

Fig. 9. Mode for Eco-induced cytotoxic effect on COLO 205 cells. In response to low dose Eco, p53, p21/Cip1 and p27/Kip1 proteins were elevated and resulted in suppression of CDK2 and CDK4 activities. Subsequently, hypophophorylation of Rb protein and induction of G0/G1 arrest occurred. In response to high dose Eco, p53 protein elevation and Bax translocation to mitochondria which induced cytochrome *c* translocation from mitochondria to cytosol. Caspase 9 and 3 were activated and resulted in apoptosis. High dose Eco also induced translocation of AIF from mitochondria to nucleus and participated in apoptotic process.

Eco-induced cytotoxic effect on COLO 205 cells, as shown in Fig. 9 (Ashkenazi and Dixit, 1998; Green and Reed, 1998). Our experimental findings introduce our basic observations that this anti-fugal agent-Eco can specifically inhibit cell proliferation and induce apoptosis in colon cancer cells both in cultures and also in colon cancer bearing nude mice. The results from the present in vitro and in vivo studies highlight the molecular mechanism of Eco-induced cytotoxicity upon colon cancer which might have potential application for the disease.

Acknowledgement

Supported by the National Science Council of Taiwan; grant NSC 92-2314-B-038-029 and NSC 92-2314-B-038-045.

References

Aron-Brunetiere, R., Dompmartin-Pernot, D., Drouhet, E., 1977. Treatment of pityriasis capitis (dandruff) with econazole nitrate. Acta Derm. Venereol. 57 (1), 77–80.

Ashkenazi, A., Dixit, V.M., 1998. Death receptors: signaling and modulation. Science 281 (5381), 1305–1308.

Blagosklonny, M.V., Dixon, S.C., Figg, W.D., 2000. Efficacy of microtubule-active drugs followed by ketoconazole in human metastatic prostate cancer cell lines. J. Urol. 163 (3), 1022–1026.

Bogle, R.G., Vallance, P., 1996. Functional effects of econazole on inducible nitric oxide synthase: production of a calmodulin-dependent enzyme. Br. J. Pharmacol. 117 (6), 1053–1058.

Cande, C., Cohen, I., Daugas, E., Ravagnan, L., Larochette, N., Zamzami, N., Kroemer, G., 2002. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84 (2–3), 215–222.

Chen, R.J., Lee, W.S., Liang, Y.C., Lin, J.K., Wang, Y.J., Lin, C.H., Hsieh, J.Y., Chaing, C.C., Ho, Y.S., 2000. Ketoconazole induces G0/G1 arrest in human colorectal and hepatocellular carcinoma cell lines. Toxicol. Appl. Pharmacol. 169 (2), 132–141.

Chinery, R., Brockman, J.A., Peeler, M.O., Shyr, Y., Beauchamp, R.D., Coffey, R.J., 1997. Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21WAF1/CIP1 via C/EBPbeta. Nat. Med. 3 (11), 1233–1241.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M., Green, D.R., 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303 (5660), 1010–1014.

Courtney, E.D., Melville, D.M., Leicester, R.J., 2004. Review article: chemoprevention of colorectal cancer. Aliment. Pharmacol. Ther. 19 (1), 1–24.

Dellenbach, P., Thomas, J.L., Guerin, V., Ochsenbein, E., Contet-Audonneau, N., 2000. Topical treatment of vaginal candidosis with sertaconazole and econazole sustained-release suppositories. Int. J. Gynaecol. Obstet. 71 (Suppl 1), S47–S52.

Denyer, M.D., Hubbard, R., Marks, V., 1985. The effects of econazole on the growth of murine myeloma cells and its use in hybridoma production. Dev. Biol. Stand. 60, 503–508.

Du, C., Fang, M., Li, Y., Li, L., Wang, X., 2000. Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell 102 (1), 33–42.

el-Deiry, W.S., Harper, J.W., O'Connor, P.M., Velculescu, V.E., Canman, C.E., Jackman, J., Pietenpol, J.A., Burrell, M., Hill, D.E., Wang, Y., et al., 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res. 54 (5), 1169–1174.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., Vogelstein, B., 1993. WAF1, a potential mediator of p53 tumor suppression. Cell 75 (4), 817–825.

Ferreira, C.G., Span, S.W., Peters, G.J., Kruyt, F.A., Giaccone, G., 2000. Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res. 60 (24), 7133–7141.

Funk, J.O., Waga, S., Harry, J.B., Espling, E., Stillman, B., Galloway, D.A., 1997. Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. Genes Dev. 11 (16), 2090–2100.

Gartel, A.L., Serfas, M.S., Tyner, A.L., 1996. p21—negative regulator of the cell cycle. Proc. Soc. Exp. Biol. Med. 213 (2), 138–149.

Gill, S., Thomas, R.R., Goldberg, R.M., 2003. Review article: colorectal cancer chemotherapy. Aliment. Pharmacol. Ther. 18 (7), 683–692.

Green, D.R., Reed, J.C., 1998. Mitochondria and apoptosis. Science 281 (5381), 1309–1312.

Hantschke, D., Schulte, R., Dabag, S., Greschuna, D., 1978. Treatment with econazole of a case of pulmonary aspergillosis. Mykosen Suppl. 1, 230–235.

Heel, R.C., Brogden, R.N., Speight, T.M., Avery, G.S., 1978. Econazole: a review of its antifungal activity and therapeutic efficacy. Drugs 16 (3), 177–201.

Herbeuval, J.P., Lambert, C., Sabido, O., Cottier, M., Fournel, P., Dy, M., Genin, C., 2003. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. J. Natl. Cancer Inst. 95 (8), 611–621.

Ho, Y.S., Duh, J.S., Jeng, J.H., Wang, Y.J., Liang, Y.C., Lin, C.H., Tseng, C.J., Yu, C.F., Chen, R.J., Lin, J.K., 2001. Griseofulvin potentiates antitumorigenesis effects of nocodazole through induction of apoptosis and G2/M cell cycle arrest in human colorectal cancer cells. Int. J. Cancer 91 (3), 393–401.

Ho, Y.S., Ma, H.Y., Chang, H.Y., Wei, B.L., Lee, C.C., Ho, S.Y., Guo, H.R., Wu, T.P., Chang, W.H., Wang, Y.J., 2003. Lipid peroxidation and cell death mechanisms in rats and human cells induced by chloral hydrate. Food Chem. Toxicol. 41 (5), 621–629.

Ho, Y.S., Tsai, P.W., Yu, C.F., Liu, H.L., Chen, R.J., Lin, J.K., 1998. Ketoconazole-induced apoptosis through P53-dependent pathway in human colorectal and hepatocellular carcinoma cell lines. Toxicol. Appl. Pharmacol. 153 (1), 39–47.

Ho, Y.S., Wang, Y.J., Lin, J.K., 1996. Induction of p53 and p21/WAF1/CIP1 expression by nitric oxide and their association with apoptosis in human cancer cells. Mol. Carcinog. 16 (1), 20–31.

Hunter, T., Pines, J., 1994. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 79 (4), 573–582.

Jiang, Y.Y., Zhang, B., Chen, X.S., Long, K., 1991. Effects of econazole and clotrimazole on TXB2 and PGE2 production in calcimycin-stimulated neutrophils and arachidonic acid-stimulated platelets. Zhongguo Yao Li Xue Bao 12 (3), 276–280.

Kim, P.K., Mahidhara, R., Seol, D.W., 2001. The role of caspase-8 in resistance to cancer chemotherapy. Drug Resist. Updat. 4 (5), 293–296.

Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A., Koff, A., 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). Cell 85 (5), 721–732.

Lee, W.S., Chen, R.J., Wang, Y.J., Tseng, H., Jeng, J.H., Lin, S.Y., Liang, Y.C., Chen, C.H., Lin, C.H., Lin, J.K., Ho, P.Y., Chu, J.S., Ho, W.L., Chen, L.C., Ho, Y.S., 2003. In vitro and in vivo studies of the anti-cancer action of terbinafine in human cancer cell lines: G0/G1 p53-associated cell cycle arrest. Int. J. Cancer 106 (1), 125–137.

Li, H., Zhu, H., Xu, C.J., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94 (4), 491–501.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., Wang, X., 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91 (4), 479–489.

Lieberman, D.A., Atkin, W., 2004. Review article: balancing the ideal versus the practical-considerations of colorectal cancer prevention and screening. Aliment. Pharmacol .Ther. 19 (Suppl 1), 71–76.

Lin, S.Y., Chang, Y.T., Liu, J.D., Yu, C.H., Ho, Y.S., Lee, Y.H., Lee, W.S., 2001. Molecular mechanisms of apoptosis induced by magnolol in colon and liver cancer cells. Mol. Carcinog. 32 (2), 73–83.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86 (1), 147–157.

Lorenzo, H.K., Susin, S.A., Penninger, J., Kroemer, G., 1999. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell death differ. 6 (6), 516–524.

Mahler, C., Denis, L., 1992. Management of relapsing disease in prostate cancer. Cancer 70 (1 Suppl), 329–334.

Midgley, I., van Bladeren, P.J., Hawkins, D.R., Chasseaud, L.F., Darragh, A., Brodie, R.R., Walmsley, L.M., 1981. The metabolic fate of 3[H]econazole in man. Xenobiotica 11 (9), 595–608.

Missero, C., Di Cunto, F., Kiyokawa, H., Koff, A., Dotto, G.P., 1996. The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. Genes Dev. 10 (23), 3065–3075.

Miyashita, T., Reed, J.C., 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80 (2), 293–299.

Morgan, D.O., 1995. Principles of CDK regulation. Nature 374 (6518), 131–134.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y., 1996. Mice lacking p27 (Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. Cell 85 (5), 707–720.

Nardone, P.A., Slotman, G.J., Vezeridis, M.P., 1988. Ketoconazole: a thromboxane synthetase and 5-lipoxygenase inhibitor with anti-metastatic activity in B16-F10 melanoma. J. Surg. Res. 44 (4), 425–429.

Osser, S., Haglund, A., Westrom, L., 1991. Treatment of candidal vaginitis. A prospective randomized investigator-blind multicenter study comparing topically applied econazole with oral fluconazole. Acta Obstet. Gynecol. Scand. 70 (1), 73–78.

Polyak, K., Lee, M.H., Erdjument-Bromage, H., Koff, A., Roberts, J.M., Tempst, P., Massague, J., 1994. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78 (1), 59–66.

Prajna, N.V., John, R.K., Nirmalan, P.K., Lalitha, P., Srinivasan, M., 2003. A randomised clinical trial comparing 2% econazole and 5% natamycin for the treatment of fungal keratitis. Br. J. Ophthalmol. 87 (10), 1235–1237.

Rigoni, C., Toffolo, P., Cantu, A., Beretta, D., Terenzio, C., 1989. 1% econazole hair-shampoo in the treatment of pityriasis capitis; a comparative study versus zinc-pyrithione shampoo. G. Ital. Dermatol. Venereol. 124 (11–12), LXVII–LXX.

Schmitt, C.A., Fridman, J.S., Yang, M., Lee, S., Baranov, E., Hoffman, R.M., Lowe, S.W., 2002. A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. Cell 109 (3), 335–346.

Sherr, C.J., Roberts, J.M., 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 9 (10), 1149–1163.

Siddiqui, K.M., Chopra, D.P., 1984. Primary and long term epithelial cell cultures from human fetal normal colonic mucosa. In vitro 20 (11), 859–868.

Soboloff, J., Zhang, Y., Minden, M., Berger, S.A., 2002. Sensitivity of myeloid leukemia cells to calcium influx blockade: application to bone marrow purging. Exp Hematol. 30 (10), 1219–1226.

Thienpont, D., Van Cutsem, J., Van Nueten, J.M., Niemegeers, C.J., Marsboom, R., 1975. Bilogical and toxicological properties of econazole, a broad-spectrum antimycotic. Arzneimittelforschung 25 (2), 224–230.

Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281 (5381), 1312–1316.

Trachtenberg, J., Pont, A., 1984. Ketoconazole therapy for advanced prostate cancer. Lancet 2 (8400), 433–435.

Tzanakakis, G.N., Agarwal, K.C., Vezeridis, M.P., 1990. Inhibition of hepatic metastasis from a human pancreatic adenocarcinoma (RWP-2) in the nude mouse by prostacyclin, forskolin, and ketoconazole. Cancer 65 (3), 446–451.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., Vaux, D.L., 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102 (1), 43–53.

Wang, X., Gorospe, M., Huang, Y., Holbrook, N.J., 1997. p27Kip1 overexpression causes apoptotic death of mammalian cells. Oncogene 15 (24), 2991–2997.

Wang, Y.J., Yu, C.F., Chen, L.C., Chen, C.H., Lin, J.K., Liang, Y.C., Lin, C.H., Lin, S.Y., Chen, C.F., Ho, Y.S., 2002. Ketoconazole potentiates terfenadine-induced apoptosis in human Hep G2 cells through inhibition of cytochrome p450 3A4 activity. J. Cell Biochem. 87 (2), 147–159.

Weinmann, M., Jendrossek, V., Handrick, R., Guner, D., Goecke, B., Belka, C., 2004. Molecular ordering of hypoxia-induced apoptosis: critical involvement of the mitochondrial death pathway in aFADD/caspase-8 independent manner. Oncogene.

Wu, X., Rubin, M., Fan, Z., DeBlasio, T., Soos, T., Koff, A., Mendelsohn, J., 1996. Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. Oncogene 12 (7), 1397–1403.

Zhang, Y., Crump, M., Berge, S.A., 2002. Purging of contaminating breast cancer cells from hematopoietic progenitor cell preparations using activation enhanced cell death. Breast Cancer Res. Treat. 72 (3), 265–278.