IN VITRO AND IN VIVO STUDIES OF THE ANTICANCER ACTION OF TERBINAFINE IN HUMAN CANCER CELL LINES: G0/G1 P53-ASSOCIATED CELL CYCLE ARREST

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Terbinafine (TB) (Lamisil©), a promising oral antifungal agent used worldwide, has been used in the treatment of superficial mycosis. In our study, we demonstrated that TB dose-dependently decreased cell number in various cultured human malignant cells. Flow cytometry analysis revealed that TB interrupts the cell cycle at the G0/G1 transition. The TB-induced cell cycle arrest in colon cancer cell line (COLO 205) occurred when the cyclin-dependent kinase (cdk) system was inhibited just as the levels of p53, p21/Cip1 and p27/Kip1 proteins were augmented. In the TB-treated COLO 205, the binding between p53 protein and p53 consensus binding site in p21/Cip1 promoter DNA probe was increased. Pretreatment of COLO 205 with p53-specific antisense oligodeoxynucleotide decreased the TB-induced elevations of p53 and p21/Cip1 proteins, which in turn led to arrest in the cell cycle at the G0/G1 phase. Moreover, in the p53 null cells, HL60, TB treatment did not induce cell cycle arrest. Taken together, these results suggest an involvement of the p53-associated signaling pathway in the TB-induced antiproliferation in COLO 205. We further examined whether administration of TB could affect the growth of tumor xenografts in athymic mice bearing COLO 205 tumors. The current options for treating human cancer are limited to excision surgery, general chemotheraphy, radiation therapy and, in a minority of breast cancers that rely on estrogen for their growth, antiestrogen therapy. Although there has been considerable improvement in the treatment of cancer, the overall prognosis remains not good. Therefore, investigators continue to search for new therapeutic strategies. One approach, as pursued in our study, seeks to identify medicinal agents capable of retarding the cell cycle and/or activating the cellular apoptotic response in the cancerous cells. Recently, we have shown that a number of antifungal agents exert antiproliferative and/or apoptotic activities in various malignant cells in vitro and in vivo. For instance, our previous studies showed that ketoconazole (Nizoral©) induced cell cycle arrest at the G0/G1 phase of the cell cycle and the occurrence of apoptosis in hepatoma and colon cancer cells,1,2 whereas griseofulvin (Gri-fulin®) induced apoptosis and cell cycle arrest at the G2/M phase through abnormal microtubule polymerization.3 We also showed that combined treatment of griseofulvin and nocodazole (ND) significantly enhanced the therapeutic efficacy in the treatment of cancerous cells in athymic mice bearing COLO 205 tumor xenografts.3 In the present study, we examined the antitumoral activity of terbinafine (TB) (Lamisil®).

TB is a newly synthesized oral antimycotic drug in the allylamines class: a fungicidal agent that inhibits ergosterol synthesis at the stage of squalene epoxidation.4 It shows a good safety profile and relatively few drug interactions.5 The cream form and oral tablet of TB have been approved for clinical uses in the United States.6 The oral formulation has been on the market in various countries for more than 8 years, and as of 1997, more than 7.5 million individuals had been treated with this drug.7 Here, we showed that TB inhibited the proliferation of tumor cells in vitro and in vivo. The experimental findings reported below highlight the molecular mechanisms of TB-induced antitumoral activity.

MATERIAL AND METHODS

Cell lines and cell culture
The HT 29 (p53 mut)8 and COLO 205 (p53 wild)9 cell lines were isolated from human colon adenocarcinoma (American Type

Key words: terbinafine (Lamisil®); anticaner; G0/G1 cell cycle arrest; nude mice; immunohistochemistry

Abbreviations: AS, antisense oligonucleotide; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; cdk, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; FACS, fluorescence-activated cell sorter; NBT, nitro blue tetrazolium; ND, nocodazole; TB, terbinafine.

Grant sponsor: National Science Council; Grant numbers: 89-2314-B-038-036, 91-2320-B-038-045, 90-2320-B-038-032.

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Received 21 November 2002; Revised 26 February 2003; Accepted 27 February 2003

DOI 10.1002/ijc.11194

Culture Collection (ATCC) HTB-38 and CCL-222). Hep 3B (p53 partially deleted)16 and Hep G2 (p53 wild)17 cell lines were derived from human hepatocellular carcinoma (ATCC HB-8064 and HB-8065).18 Human gingival fibroblasts were harvested by enzymatic dissociation. The HL 60 cell line (p53 null) was derived from human myeloid leukemia cells (59170; ATCC). The cell lines were grown in MEM (for Hep 3B, Hep G2 and human gingival fibroblasts) or RPMI-1640 (for COLO 205, HT 29 and HL 60 cells) supplemented with 10% FCS, 50 μg/ml gentamicin (Gentamicin®) and 0.3 mg/ml glutamine in a humidified incubator (37°C, 5% CO2). The p53-specific antisense (5'-CGGGCTTCTC-CATGCCTGA-3') and sense (5'-ACTGCCATGGAGGAGCCG-3') phosphothioates (S-oligos) were designed as described in our previous study.2 Synthesized and purified using high-performance liquid chromatography by Genset (Evry Cedex, France).

**Determination of cell growth curve**

Human colon cancer, hepatoma, leukemia and human normal fibroblast cells at a density of 1 x 104 were plated in 35 mm Petri dishes. TB was added at the indicated doses in 0.05% DMSO. For control specimens, the same volume of the 0.05% DMSO without TB was added. Media with and without TB were changed daily until cell counting.

**Flow cytometry**

The COLO 205 and HT 29 cells were synchronized as previously described.2 After the cells had grown to 70–80% confluency, they were rendered quiescent by incubation for 24 hr in RPMI-1640 containing 0.04% FCS and challenged with 10% FCS. Then, after release using trypsin-EDTA, they were harvested at 3000 RPM for 5 min, and then stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

**Immunoprecipitation and kinase activity assay**

As previously described,13 the TB-treated cells were lysed in Rb lysis buffer and immunoprecipitated with anti-cdk4 antibody (2 μg). The protein complexes in beads were washed twice with Rb lysis buffer and then once with Rb kinase assay buffer. The level of phosphorylated Rb (for pRb), histone H1 (for cdk2) and glutathione x-transferase-Rb fusion protein (for cdk4) were measured by incubating the beads with 40μl of hot Rb kinase solution (0.25 μl (2 μg) of Rb-GST fusion protein, 0.5μl of (γ-32P) ATP, 0.5μl of 0.1 mM ATP and 38.75 μl of Rb kinase buffer) at 37°C for 30 min, and then stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

**Electrophoretic mobility shift assay (EMSA)**

The double-stranded DNA probe used in the experiment contained the p21/Cip1 promoter (5'-CAGGAACAGTCCCAACAT-GTTGAGC-3') with p53 consensus binding site. The radiolabeled DNA (4 ng, 100,000 cpm) was incubated with nuclear extract in 15 μl of binding buffer (10 mM TRIS-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, 200 mM NaCl, and 1 μg probe of DNA) on ice for 5 min. The samples were electrophoresed in a 5% polyacrylamide gel, dried on Whatman 3M paper and then exposed to Fuji x-ray films at 70°C.

**Treatment of COLO 205-derived xenografts in vivo**

The COLO 205 cells (5 x 104) in 0.1 ml of RPMI-1640 were injected subcutaneously between the scapulae of each nude mouse (purchased from National Science Council animal center, Taipei, Taiwan). After transplantation, the tumor size was measured using calipers, and the tumor volume was estimated by the following formula: tumor volume (mm3) = 1/2 x L x W2,2,2 where L is the length and W is the width of the tumor.15 Once the tumor reached a volume of 200 mm3,16 animals received intraperitoneal injections of DMSO (25 μl), TB (50 mg/kg), ND (5 mg/kg) or TB plus ND 3 times per week for 6 weeks.

**DNA fragmentation analysis in tumor tissues isolated from the TB-treated mouse**

The tumor tissues were excised at the end of each experiment. One part of the tumor tissue was frozen in liquid nitrogen for DNA isolation; the remainder was fixed in 4% paraformaldehyde for detection of apoptotic cells using TdT FragEL™ DNA fragmentation detection kit (Calbiochem Co., Cambridge, MA). The DNA isolated from the frozen tumor tissues was used for detection of DNA laddering, a marker of apoptosis, as described previously.1

**Immunocytochemical staining analysis of the expressions of p53, p21/Cip1 and p27/Kip1 proteins in the COLO 205 tumor tissues**

As previously described,16,17 paraffin-embedded blocks were sectioned at 5–7 μm thickness. After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, slides were immersed in 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. After intensive washing with PBS, slides were incubated overnight at 4°C with the p53, p21/Cip1 and p27/Kip1 antibodies in a dilution of 1:50. After a second incubation with a biotinylated antimouse antibody, slides were incubated with peroxidase-conjugated streptavidin (DAKO LSAB+ kit; Dako Corp., Carpinteria, CA). Reaction products were visualized by immersing slides in a diaminobenzidine tetrahydrochloride and finally counterstained with hematoxylin.

**Statistics**

All data were expressed as the mean value ± SE. Comparisons were subjected to 1-way ANOVA followed by Fisher’s least significant difference test. Significance was accepted at p < 0.05.
RESULTS

Inhibition of cell proliferation in TB-treated human malignant cells

We examined the effect of TB on the growth of various human cancer cells. The cells were cultured for 5 days with or without TB (30–120 μM), and then the cells were harvested and counted. These data show that TB decreased cell number in cultured human cancer cells (COLO 205, HT 29, Hep G2, Hep 3B and HL 60) in a dose-dependent manner. When TB concentration was increased to 60 μM, cell growth arrest or cell death was observed in these cancer cells. In contrast, TB at concentrations of 30–60 μM did not inhibit the growth rate of the cultured human gingival fibroblasts (Fig. 1f). However, when TB concentration was increased to 120 μM, a 50% growth inhibition was observed.

Arrest of cell cycle at the G0/G1 phase by TB in human cancer cells

In order to demonstrate more sharply the actions of TB on a specific phase of the cell cycle, the cancer cells (COLO 205 and HT 29) were all synchronized by switching them to media with 0.04% FCS for 24 hr to render them quiescent. When they were returned to culture media containing 10% FCS and 0.05% DMSO (control) or 90 μM TB in 0.05% DMSO (which started them all on a new cell cycle), and at various times thereafter, they were harvested for flow cytometry analysis. Figure 2 shows the representative FACs analyses of DNA content of the DMSO-(left panel) and the 90 μM TB- (right panel) treated COLO 205 (Fig. 2a) and HT 29 (Fig. 2b) cells at various times after the cells' release from quiescence. The results demonstrate that TB induced an accumulation (>90%) of the COLO 205 and HT 29 cells at the G0/G1 phase of the cell cycle, suggesting that the observed growth inhibitory effect of TB on the COLO 205 and HT 29 cells was due to an arrest of G0/G1 phase in the cell cycle. Figure 3 demonstrated the dose effect of TB on the G0/G1 arrest. As illustrated in Figure 3a–c, TB induced G0/G1 arrest in COLO 205, HT 29 and Hep G2 cells in a dose-dependent manner. In Hep 3B and HL 60 cells (p53 null), however, TB (10–150 μM) did not induce G0/G1 arrest, but dose-dependently caused the occurrence of apoptosis as evidenced by the presence of the sub G1 (Fig. 3d and e). Importantly, treatment of human fibroblasts with TB did not induce cell cycle arrest or cell death (Fig. 3f).

TB-induced G0/G1 arrest is reversible

To test the reversibility of the TB-induced G0/G1 arrest, the COLO 205 cells were switched to media with 0.04% FCS to render them quiescent. They were then returned to culture media supplemented with 10% FCS and 0.05% DMSO or 90 μM TB in 0.05% DMSO. After 24 hr of treatment with TB, the cells were washed 3 times with PBS and then returned to media containing 10% FCS without TB. In response to TB treatment, the COLO 205 cells were arrested at the G0/G1 phase. Figure 4 demonstrated that the TB-induced G0/G1 cell cycle arrest was not reversed by TB removal, and this inhibition lasted for at least 7 days. We further tested
whether TB can induce cell cycle arrest at a lower concentration with longer exposure. As illustrated in Figure 5, treatment of COLO 205 with TB at a concentration as low as 1 μM for 4 days can induce significant G0/G1 cell cycle arrest.

**Effects of TB on the levels of cell cycle regulatory proteins**

To investigate the underlying molecular mechanisms of TB-induced G0/G1 arrest, the COLO 205 cells were switched to media with 0.04% FCS to render them quiescent at the G0/G1 phase. They were then returned to culture media supplemented with 10% FCS and 0.05% DMSO with or without TB (60 μM), and at various times thereafter, they were harvested for protein extraction and Western blot analysis. Based on the FACS analysis in the COLO 205 cells, 0, 15, 18 and 24 hr after release from quiescence represents the G0/G1, S, G2/M and 2nd G0/G1 phases of the cell cycle, respectively (Fig. 2a). Accordingly, these time points were selected for protein extraction and Western blot analysis to examine the effects of TB on the expression of cell cycle regulatory proteins. As shown in the Figure 6a (upper panel), the level of p21/Cip1 protein in the DMSO-treated COLO 205 cells was increased significantly at 3 hr after the cells were challenged with 10% FCS, and then rapidly declined to an undetectable level at 9 hr after treatment. This result was consistent with a previous report showing that transient induction of p21/Cip1 was required for the increased stability of cdk kinases activity. The TB-treated COLO 205 cells, on the other hand, showed a persistent increase in p21/Cip1 protein level after TB treatment (Fig. 6a, lower panel).

A previous study showed that the p27/Kip1 protein level was high in the quiescent cells, and then decreased rapidly after stimulated with serum. A similar finding was observed in Figure 6b, showing that the level of p27/Kip1 protein in the COLO 205 cells was high after 24 hr serum starvation (left panel, 0 hr), and then decreased after challenged with 10% FCS (left panel, 15 hr). In contrast, the increased p27/Kip1 protein levels in the TB-treated COLO 205 cells were maintained at high levels after 10% FCS treatment (right panel, 0–24 hr). The levels of cyclin D3, cdk2 and cdk4 proteins were downregulated in the TB (60 μM)-treated COLO 205 cells, while the levels of cyclin D1 and PCNA proteins were not changed (Fig. 6b, right panel). Cyclin A2 and cyclin B, which promoted the cell entrance into the S and G2/M phases, respectively, were also downregulated in the TB-treated COLO 205 cells (Fig. 6b, right panel). The levels of cyclin E protein, which is associated with cdk2, were slightly elevated in the TB-treated cells (Fig. 6b, right panel). Moreover, the levels of pRb were downregulated in the TB-treated COLO 205 cells.

The results shown in Figure 3 demonstrated that TB induced cell cycle arrest at the G0/G1 phase in human cancer cells with either wild-type p53 (COLO 205 and Hep G2) or p53 His273 mutant (HT 29). In contrast, TB induced apoptosis in HL 60 (p53 null) and Hep 3B (p53 partial deleted). These data suggest that TB induced the cancer cells to undergo G0/G1 cell cycle arrest or apoptosis dependent on the p53 status of the cells. To further test this hypothesis, the dose effects of TB on the levels of cell cycle regulatory protein were conducted in 4 different human cancer cell lines, COLO 205 (p53 wild type), HT 29 (p53 His273 mutant), Hep 3B (p53 partial deleted) and HL 60 (p53 null). As illustrated in Figure 7, TB increased the levels of p53, p21/Cip1 and p27/Kip1 proteins, and decreased cyclin D and cdk4 in COLO 205 and HT 29 cells. In HL 60 and Hep 3B cells, TB treatment did not change the levels of p53 and p21/Cip1 proteins, but significantly increased the levels of p27/Kip1 protein.
P53-activated signaling pathway was involved in TB-induced G0/G1 arrest

The p53 protein has been suggested to be a potent transcription factor involved in the regulation of cell cycle arrest and occurrence of apoptosis. As illustrated in Figure 7, the levels of p21/Cip1 and p53 proteins were dose-dependently increased in TB-treated COLO 205 and HT 29 cells, suggesting that upregulation of p53 and p21/Cip1 might be involved in the TB-mediated G0/G1 arrest in these cells. To further test this hypothesis, we examined the TB effects on the levels of p21/Cip1, p27/Kip1 and p53 proteins, and the cdk4 kinase activity in 3 human cancer cell lines (COLO 205, HT 29 and Hep 3B). As shown in Figure 8, TB at a concentration of 60 μM induced a strong decrease in the assayable cdk4 kinase activity in COLO 205 (p53 wild type) cells and a slight decrease in the TB-treated HT-29 (p53 His273 mutant) and Hep 3B (p53 partial deletion) cells. The electrophoretic mobility gel shift assay was conducted by using p21/Cip1 promoter DNA, which contains p53 consensus binding site, to demonstrate that the p53 binding activity was more significantly increased in the nuclear extracts of the TB-treated COLO 205 cells (Fig. 8b, lane 4) than in those of the TB-treated HT 29 cells (Fig. 8b, lane 2).

To further demonstrate that increased p53 expression correlated with G0/G1 arrest in the TB-treated cells, the experiment illustrated in Figure 8c was carried out. Thus, in the sample labeled TB (for 60 μM TB-treated alone), the p53 and p21 electrophoretogram bands were increased in intensity, while the G0/G1 population was increased by about 2.3-fold (Figure 8c, lane 2). Sample TB+AS was treated with a p53 antisense oligonucleotide (AS), which blocked the expression of p53. Consequently, in this sample, the levels of p53 and p21 proteins did not increase and the TB addition to sample TB+AS failed to induce the increased G0/G1 population, which was evident in the TB sample (Figure 8c, lane 3).

TB potentiates the apoptotic effects of ND

Combined treatment of the cells with drugs affecting different cell cycle checkpoints has been suggested to be 1 of the approaches to enhance the drug-induced apoptotic effect in human malignant cells. Accordingly, we cotreated the COLO 205 cells with TB, which causes G0/G1 arrest, and ND, which arrests the cells at the G2/M phase, and examined the degree of the occurrence of apoptosis. Genomic DNAs extracted from TB-treated COLO 205 were examined by gel electrophoresis. They were found to display the DNA ladder patterns characteristic of cells undergoing apoptosis when ND was at a concentration of 50 nM or higher (Fig. 9a, lane 5). In the presence of 10 μM TB, which does not induce DNA ladder patterns in COLO 205 cells (Fig. 9a, lane 9), ND induced the DNA ladder pattern in the COLO 205 cells at a concentration as low as 1 nM (Fig. 9a, lane 10). This finding indicates that TB enhanced the ND-induced apoptosis in the COLO 205 cells.

Given the enhancement by TB of the ND-induced apoptosis of COLO 205 cells in vitro, we next determined whether administration of TB could affect the ND-induced obvious decline in tumor size in an in vivo setting. A reduction in tumor volume between mice given TB, ND or TB plus ND vs. those given vehicle (DMSO...
plus peanut oil) was detected (Fig. 9b–e). Importantly, the tumor volume and tumor weight of the mice treated with TB plus ND were significantly reduced compared to those treated with TB or ND alone (p < 0.05), suggesting that TB enhanced the ND-induced reduction in tumor size.

Both cell cycle arrest and occurrence of apoptosis are involved in TB-inhibited tumor growth in vivo

Since, retardation of the cell cycle and activation of the cellular apoptotic response are 2 major mechanisms preventing tumor growth, we examined the TB effect on the cell cycle and apoptosis occurrence of the solid tumor derived from the implanted COLO 205. The particular evidence for the occurrence of apoptosis in the tumor isolated from the TB-treated animal includes DNA strand breaks caused by endonuclease, which can be detected in situ by nick end-labeling tissue sections with dUTP-biotin by terminal deoxynucleotidyl transferase (Fig. 10a), and fragmentation of DNA, which can be examined by gel electrophoresis (Fig. 10b).

The contents of p53 and p21/Cip1 were increased in the tumor isolated from the TB-treated mouse (Fig. 10c), suggesting that the inhibition of the progression of cell cycle activity was involved in the TB-induced decline in tumor size. The TB-induced upregulation of p21 and p53 in the COLO 205 tumor were further confirmed by immunocytochemical staining technique. The DMSO-treated animals (control) expressed very little, if any, p53, p21/Cip1 and p27/Kip1 activity in the COLO 205 tumor tissue (Fig. 11a–c). In contrast, p53, p21/Cip1 and p27/Kip1 immunoreactivities were strongly induced in the TB-treated tumor tissues (Fig. 11d–f). Interestingly, the p53 immunoreactive cells were observed over the whole tissue section. Among these cells, some expressed p21/Cip1 and others expressed p27/Kip1. The p21/Cip1 (Fig. 11e, blue square) and p27/Kip1 (Fig. 11f, red square) immunoreactive cells located in different areas of the COLO 205 tumor tissues. Figure 11g–i shows the percentage of cells expressing p53, p21/Cip and p27/Kip1 respectively.

DISCUSSION

The present study was undertaken to investigate the anticancer mechanisms of TB. Our in vitro studies demonstrated that TB inhibited proliferation and induced apoptosis in cultured human cancer cells. In vivo studies showed that intraperitoneal administration of TB at a dose of 50 mg/kg caused a substantial decline in tumor size of the COLO 205 tumor mass. An increased expression of p21/Cip1 and p53 proteins and the occurrence of apoptosis in the solid tumor isolated from the TB-treated mouse suggest that both cell cycle inhibition and apoptotic cell death contribute to the antitumor effects of TB. To our knowledge, this is the first demonstration that TB...
**FIGURE 6** – Time effect of TB on cyclin and cdk protein levels in COLO 205 cells. (a) In the DMSO-treated COLO 205, 10% FCS caused a transient increase in p21/Cip1 protein (upper panel). In contrast, TB caused a persistent increase in p21/Cip1 protein level (lower panel). (b) In response to TB treatment, the levels of cyclin A2, B and D3, and cdk2, cdk4 and pRb proteins were downregulated, while cyclin E and p27/Kip1 were slightly upregulated. The COLO 205 cells were synchronized with 0.04% FCS for 24 hr, and then released into complete medium (10% FCS) containing TB (60 μM) for the indicated time points. COLO 205 cells were also treated with DMSO (0.05%, v/v) as a control group. Protein extracts (100 μg/lane) were separated by SDS-PAGE, probed with specific antibodies and detected using the NBT/BCIP system.
FIGURE 7 – Dose effect of TB on the cell cycle regulatory protein levels. TB dose-dependently increased the levels of p53, p21/Cip1 and p27/Kip1 proteins, and decreased the levels of cyclin D3 and cdk4 proteins in COLO 205 and HT 29 cells. In HL 60 and Hep 3B cells, TB treatment did not change the levels of p53 and p21/Cip1 proteins, but significantly increased the levels of p27/Kip1 protein in a dose-dependent manner. The cells were rendered quiescent for 24 hr, and then challenged with 10% FCS and treated with various concentrations of TB (60–150 μM) for an additional 15 hr. Protein extracts (100 μg/lane) were separated by SDS-PAGE, probed with specific antibodies and detected using the NBT/BCIP system. Membranes were also probed with anti-GAPDH antibody to correct for differences in protein loading.
inhibits the growth of colon cancer cells both in vitro and in vivo through retardation of the cell cycle and activation of the cellular apoptotic response in the cancer cells.

The inhibitory effect of TB on cell growth does not appear to be limited to the COLO 205 cells, as similar inhibition has also been observed in other transformed cultured cells, such as HT 29, Hep G2, Hep 3B and HL 60 (Fig. 1). However, it seems that TB exerts its antitumor activity through retarding the cell cycle or activating the cellular apoptotic response dependent on the p53 status of the cancer cells. In our study, we observed that TB dose-dependently induced cell cycle arrest at the G0/G1 phase in the cancer cells with wild-type p53 (COLO 205 and Hep G2) and the occurrence of apoptosis in the cells with p53 null (HL 60) or partial deletion p53 (Hep 3B). A previous report demonstrated that HT-29 cells contain a point mutation at codon 273 (Arg→His) of the p53 gene. In the HT-29 cell, TB treatment caused cell cycle arrest instead of apoptosis (Fig. 3b). This result is consistent with a recent report showing that mutant p53 was not sufficient to induce apoptosis. The mutant-type p53 protein in HT-29 cells is recognized by pAb DO-1 (recognizes all p53 proteins) and pAb 1620 (recognizes wild-type conformation of p53), but not by pAb 240 (recognizes mutant conformation of p53). In our study, the mutant-type p53 protein in HT-29 cells (with wild-type p53 conformation) could be detected with pAb 1620. However, the degree of p53 induction by TB in HT-29 cells was much less than that observed in COLO 205 cells (Fig. 8a). These observations demonstrate that both the COLO 205 cells, which express wild-type p53, and the HT
29 cells with "conformationally" wild-type p53 were sensitive to TB-induced G0/G1 arrest. Such results imply that the p53 signaling pathway is involved in the G0/G1 cell cycle arrest.

Treatment of the COLO 205 cells with TB resulted in an increase in the levels of p21/Cip1, p27/Kip1 and p53 proteins and a decrease in the levels of cyclin A2, B and D3, and cdk2 and cdk4 proteins (Figs. 6 and 7). Among these changes, p53 seems to have a major contribution to the TB-induced G0/G1 arrest in the COLO 205 cells. P53, the tumor suppressor, has been implicated in a variety of cellular processes.25,26 However, the undisputed roles of p53 are the induction of cell growth arrest and apoptosis.27,28 In response to TB treatment, the expression of p53 in COLO 205 and HT 29 was significantly upregulated. The TB-induced increase of p53 did bind to the p21/Cip1 promoter DNA, which contains the p53 consensus binding site (Fig. 8b). In our study, we further demonstrated that the process of G0/G1 cell cycle arrest induced by TB in the COLO 205 cells is correlated with the activation of the p53-associated signaling pathway, as evidenced by the p53-specific antisense oligonucleotide experiment (Fig. 8c). Moreover, TB-induced G0/G1 arrest was not observed in the p53 null cells, HL 60 (Fig. 3e). These data further support the notion that p53 is involved in the TB-induced antiproliferation. Observation of an increased expression of p53 and p21/Cip1 proteins and the occurrence of apoptosis in the solid tumor isolated from the TB-treated mouse supports the hypothesis that the p53 signaling pathway is involved in TB-induced decline in tumor size of the COLO 205 tumor. An increased expression of p21/Cip1 protein and a decrease of the assayable cdk4 kinase activity (Fig. 8a) in the TB-treated COLO 205 cells suggest that TB treatment caused an increase in p53 protein level, which in turn upregulated the p21/Cip1 level, and finally induced a decrease in the cdk kinase activity. The consequent reduction of cdk4 activity by p21/Cip1 is most likely responsible for the TB-induced G0/G1 arrest in the COLO 205 cells.

In our study, we try to clarify the roles of the p21/Cip1 and p27/Kip1 protein expression, which involved G0/G1 arrest and/or apoptosis induced by TB. Previous studies have demonstrated that p21/Cip1 arrests the cell cycle through binding and inactivating the cdk's, which are required for cell cycle progression.27,28 A number of studies have suggested that p21/Cip1 does have tumor suppressor properties. P21/Cip1 mutations have been found in several human tumors,29 and a p21/Cip1 mutation, which was demonstrated to specifically abrogate its binding to cdk's, was identified in a primary breast tumor.30 P27/Kip1 also mediates growth arrest and is thought to play a critical role in negative regulation of cell division in vivo.31 In contrast to p21/Cip1, mice with the p27/Kip1 gene null showed an increased body size, female sterility and a high incidence of spontaneous pituitary tumors.32 In our study, we demonstrated that the p27/Kip1 was significantly induced by TB in both HL 60 (p53 null) and Hep 3B (p53 deleted) cells (Fig. 7). However, significant G0/G1 phase cell cycle arrests in both the Hep 3B and HL 60 cells were not observed (Fig. 3d and e). Recent study demonstrated that adenovirally mediated p27/Kip1 overexpression leads to apoptosis in human cancer cells. In sharp contrast, a similar overexpression of p21/Cip1 results in G1-S arrest, but minimum cytotoxicity was observed.33 Another study demon-
strated that p27/Kip1 expression was associated with spontaneous apoptosis and Bax protein expression in tumor sections from oral and orthopharyngeal carcinoma in vivo and in vitro.34,35 All these results imply that p27/Kip1 protein might play an important role in TB-induced apoptosis, but not in cell growth arrest.

The ability of chemotherapeutic agents to inhibit cancer cell growth and to initiate apoptosis is an important determinant of their therapeutic response. However, significant toxicity at high doses has precluded the use of chemotherapeutic agents as a monotherapy for cancers. Combination therapy is a potential method to help reduce a compound’s undesirable toxic effects but still maintain or enhance its antitumor efficacy. Recently, our study demonstrated that griseofulvin, an oral-antifungal agent, potentiates the anticancer activities of ND, a clinically used chemotherapeutic agent, in vivo.3 In our present study, we further demonstrated an enhancement of TB on the ND-induced apoptosis. Historically, TB has been used as an orally active broad-spectrum antifungal drug, especially active in patients with histoplasmosis or nonmeningeal cryptococcosis.36,37 A previous study has demonstrated that approximately 70% of TB is absorbed after an oral dose (250 mg).38 and the maximum plasma concentrations of 0.5–1.5 μg/ml are reached within 2 hr.39–41 Another report in a human study showed that the plasma level of TB after daily oral receiving of 250 mg doses of TB for 4 weeks was 1.7 ± 0.77 μg/ml (5.83 μM).40 TB is highly lipophilic and keratophilic. It extensively accumulates at the adipose tissues, keratin-rich tissues (such as dermis, epidermis and nail) and other organ tissues.42,43 The concentrations of the TB in the tissue levels exceeded that of plasma as early as 1 day after stop of medication, and this difference continued to increase until the last day of tissue sampling. Here, we showed that administration of TB at a concentration as low as 1 μM for 3 days arrested the COLO 205 cells at the G0/G1 phase of the cell cycle (Fig. 5). We further demonstrated that the TB-induced cell cycle arrest was irreversible (Fig. 4). Such results imply that continued administration of lower-dose TB could reach the therapeutic concentrations in plasma. Importantly, flow cytometry analysis showed that at the doses (10–150 μM) used in our in vitro studies, TB was not cytotoxic for the cultured untransformed human fibroblasts, nor did the TB have any effect on cell proliferation in this culture (Fig. 3). However, the
FIGURE 11 – Immunolocalization of p53, p21/Cip1 and p27/Kip1 proteins in COLO 205 tumor tissues. (a–c) Strong immunoreactivities of p53 (D, blue and red squares), p21/Cip1 (E, blue square) and p27/Kip1 (F, red square) proteins were detected in COLO 205 tumor tissues isolated from the TB-treated nude mice, but not from the DMSO-treated mice. The tumor tissues were cut into 5–7 μm thickness, and serial sections were stained with the specific antibodies against human p53 (a and d), p21/Cip1 (b and e) and p27/Kip1 (c and f) for determination of specific antigen in tumor tissues. Green arrows indicate the representative p53 (d), p21/Cip (e) or p27/Kip1 (f) immunoreactive cells (brown) (200×). The percentage of cells expressing p53 (g), p21/Cip (h) and p27/Kip1 (i) was calculated.
study of TB effect on the cell growth rate showed that treatment of TB at a concentration of 120 µM for 5 days reduced cell count by 50% in human fibroblasts (Fig. 1f). This might be explained by intracellular accumulation of TB due to a daily change of culture medium for 5 days. Additionally, the dose (50 mg/kg of body weight) used in the present study was not cytotoxic for the vital organs.

Although animal studies of TB-induced antitumour action are still ongoing, the findings from the present in vitro and in vivo studies strongly suggest the potential applications of TB in the treatment of human cancer. The universality of TB in the inhibition of cancer cell proliferation would make it a very attractive agent for cancer chemotherapy.

ACKNOWLEDGEMENTS

Our study was supported by National Science Council (NSC) grant NSC 89-2314-B-038-036 to Dr. Ho, and NSC 91-2320-B-038-045 and NSC 90-2320-B-038-032 to Dr. Lee.

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