Int. J. Cancer: **106,** 125–137 (2003)

Publication of the International Union Against Cancer

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

Wen-Sen LEE[1,2], Rong-Jane CHEN[3], Ying-Jan WANG[3], How TSENG[1], Jiiang-Huei JENG[4], Shyr-Yi LIN[5], Yu-Chih LIANG[5], Chien-Ho CHEN[10], Chien-Huang LIN[10], Jen-Kun LIN[6], Pei-Yin HO[7], Jan-Show CHU[8], Wei-Lu HO[8], Li-Ching CHEN[9] and Yuan-Soon HO[10]*

[1]*Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan*
[2]*Department of Physiology, School of Medicine, Taipei Medical University, Taipei, Taiwan*
[3]*Department of Environmental and Occupational Health, National Cheng Kung University Medical College, Tainan, Taiwan*
[4]*School of Dentistry, College of Medicine, National Taiwan University, Taipei, Taiwan*
[5]*Department of Internal Medicine, School of Medicine, Taipei Medical University, Taipei, Taiwan*
[6]*Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan*
[7]*Graduate Institute of Cellular and Molecular Biology, Taipei Medical University, Taipei, Taiwan*
[8]*Department of Pathology, School of Medicine, Taipei Medical University, Taipei, Taiwan*
[9]*Department of Nursing, TzuChi University, Hualien, Taiwan*
[10]*Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan*

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 tumors derived from human colon cancer cells in an *in vivo* setting. COLO 205 cells implanted subcutaneously in nude mice formed solid tumor; subsequent intraperitoneal injections of TB (50 mg/kg) led to obvious decline in tumor size, up to 50–60%. In these tumors, increases in the p21/Cip1, p27/Kip1 and p53 proteins and the occurrence of apoptosis were observed. Combined treatment with TB and nocodazole (ND), a clinically used anticancer agent, potentiated the apoptotic effect in COLO 205. These findings demonstrate for the first time that TB can inhibit the proliferation of tumor cells *in vitro* and *in vivo*.

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

The current options for treating human cancer are limited to excision surgery, general chemotherapy, 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 (Grifulvin®) 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* mutant)[8] and COLO 205 (*p53* wild)[9] cell lines were isolated from human colon adenocarcinoma (American Type

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.

*Correspondence to: Graduate Institute of Biomedical Technology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. Fax: +886-2-2739-3422. E-mail: hoyuansn@tmu.edu.tw

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)[10] and Hep G2 (*p53* wild)[10] cell lines were derived from human hepatocellular carcinoma (ATCC HB-8064 and HB-8065).[11] 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 gentamycin (Gentamicin®) and 0.3 mg/ml glutamine in a humidified incubator (37°C, 5% CO_2). The *p53*-specific antisense (5′-CGGCTCCTC-CATGGCAGT-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 1×10^4 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% confluence, 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 various times, washed twice with PBS/0.1% dextrose and fixed in 70% ethanol at 4°C. Nuclear DNA was stained with a reagent containing propidium iodine (50 μg/ml) and DNase-free RNase (2 U/ml) and measured using a fluorescence-activated cell sorter (FACS). The population of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickinson, San Jose, CA).

Protein extraction and Western blot analysis

As previously described,[2] the frozen tumor was pulverized in liquid N_2 and then mixed with lysis buffer (0.5 M TRIS-HCl, pH 6.8, 0.4% SDS). For cell cultures, the cells were seeded onto 150 mm dishes and grown in RPMI-1640 (for COLO 205, HT 29 and HL 60 cells) or MEM (for Hep 3B) supplemented with 10% FCS. After the cells had grown to subconfluence, they were rendered quiescent. The cells were released from quiescence with culture medium supplemented with 10% FCS. TB in 0.05% DMSO or 0.05% DMSO without TB was added to the cells at various concentrations, and the mixture was allowed to incubate for 17 hr. Western blot analysis was performed as previously described.[2,12] Immunodetection was carried by probing with proper dilutions of specific antibodies at room temperature for 2 hr. Anti-p21/Cip1, anti-p27/Kip1, anti-p53 and anti-GAPDH monoclonal antibodies (Santa Cruz, Inc., Santa Cruz, CA); anticyclin B1, D1 and D3, anti-cyclin-dependent kinase (cdk) 2 and cdk4, and proliferating cell nuclear antigen (PCNA) monclonal antibodies (Transduction Laboratories, Lexington, KY) were used at a concentration of 1:1,000 dilution. Anti-cyclin A and E polyclonal antibodies (Transduction, San Diego, CA) were used at a concentration of 1:250 dilution. The secondary antibodies, alkaline phosphatase-coupled anti-mouse or anti-rabbit antibody (Jackson, Westgrove, PA), were incubated at room temperature for 1 hr at a concentration of 1:5,000 or 1:1,000 dilution, respectively. The specific protein complexes were identified by incubating with the colorigenic substrates (nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP); Sigma Chemical Co., St. Louis, MO). In each experiment, membranes were also probed with anti-GAPDH antibody to correct for differences in protein loading.

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 s-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-^{32}P) 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)[14]

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[3]

The COLO 205 cells (5×10^6) 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 (mm^3) = $1/2 \times L \times W$,[2,2] where L is the length and W is the width of the tumor.[15] Once the tumor reached a volume of 200 mm,[3,3] 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 tetrachloride 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 $p < 0.05$.

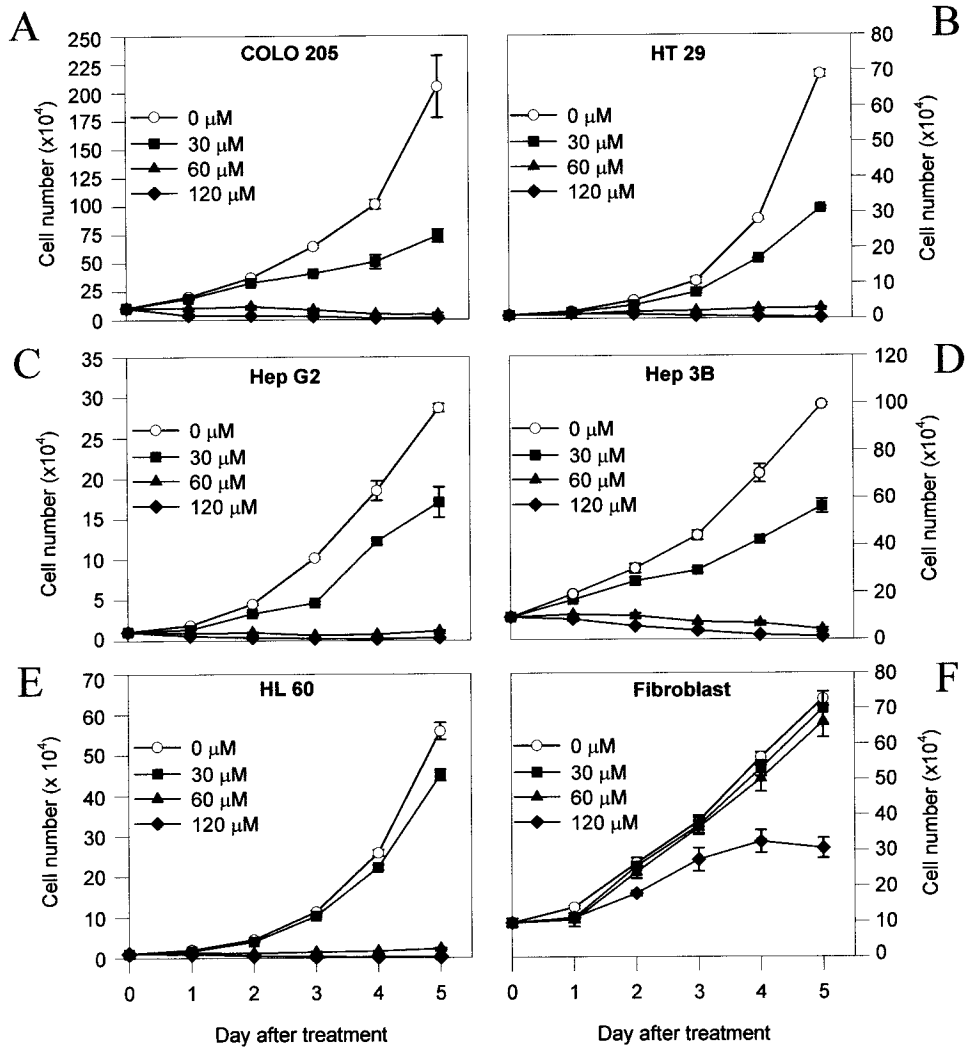

FIGURE 1 – Dose-dependent effects of TB on cell number in human malignant and normal cells. COLO 205 (*a*), HT 29 (*b*), Hep G2 (*c*), Hep 3B (*d*), HL 60 (*e*) and normal human fibroblast (*f*) cells were treated with various concentrations of TB (30–120 μM). Media with or without TB were renewed daily until cell counting. Three samples were analyzed in each group. Values represent mean ± SE.

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. 1*f*). 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. 2*a*) and HT 29 (Fig. 2*b*) 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 3*a–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. 3*d* and *e*). Importantly, treatment of human fibroblasts with TB did not induce cell cycle arrest or cell death (Fig. 3*f*).

TB-induced G0/G1 arrest is irreversible

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 removable, and this inhibition lasted for at least 7 days. We further tested

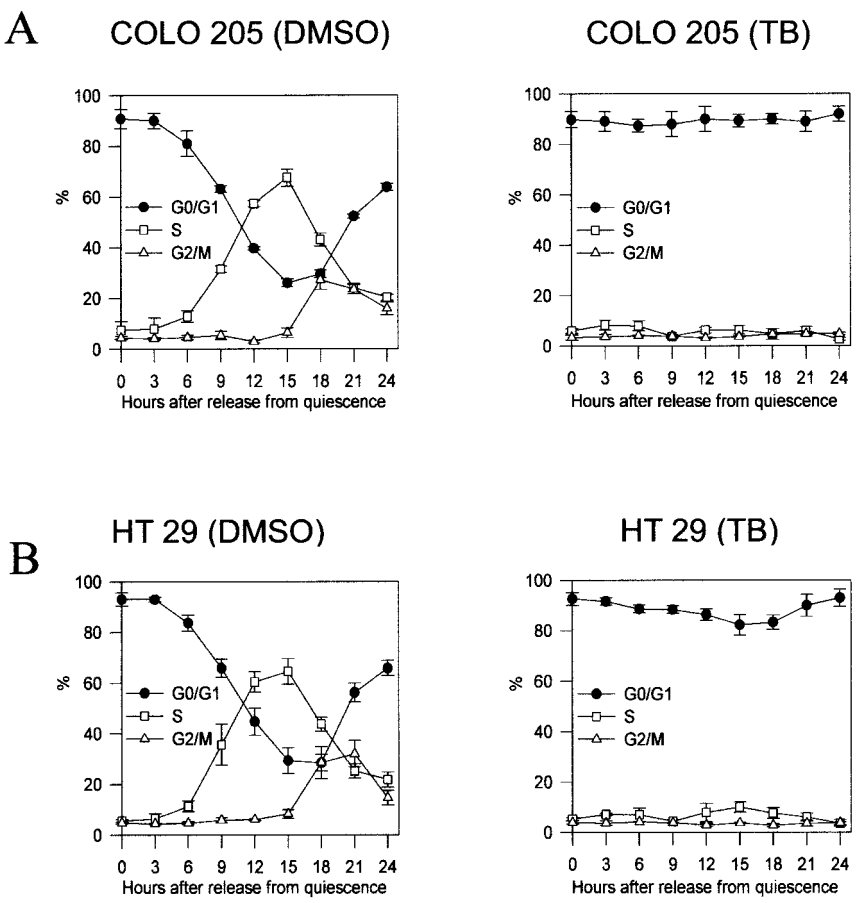

FIGURE 2 – Time-dependent response of TB-induced G0/G1 phase arrest in COLO 205 cells. COLO 205 (*a*) and HT 29 (*b*) cells were synchronized with 0.04% FCS for 24 hr as described in Material and Methods. After synchronization, the cells were then released into complete medium (10% FCS) containing 0.05% DMSO (left panel) or 90 μM TB in 0.05% DMSO (right panel). Percentage of cells in 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 ± SE.

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. 2*a*). 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 6*a* (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.[18] The TB-treated COLO 205 cells, on the other hand, showed a persistent increase in p21/Cip1 protein level after TB treatment (Fig. 6*a*, 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.[19] A similar finding was observed in Figure 6*b*, 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. 6*b*, 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. 6*b*, right panel). The levels of cyclin E protein, which is associated with cdk2, were slightly elevated in the TB-treated cells (Fig. 6*b*, 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* His^{273} 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 His^{273} 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 D3 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.

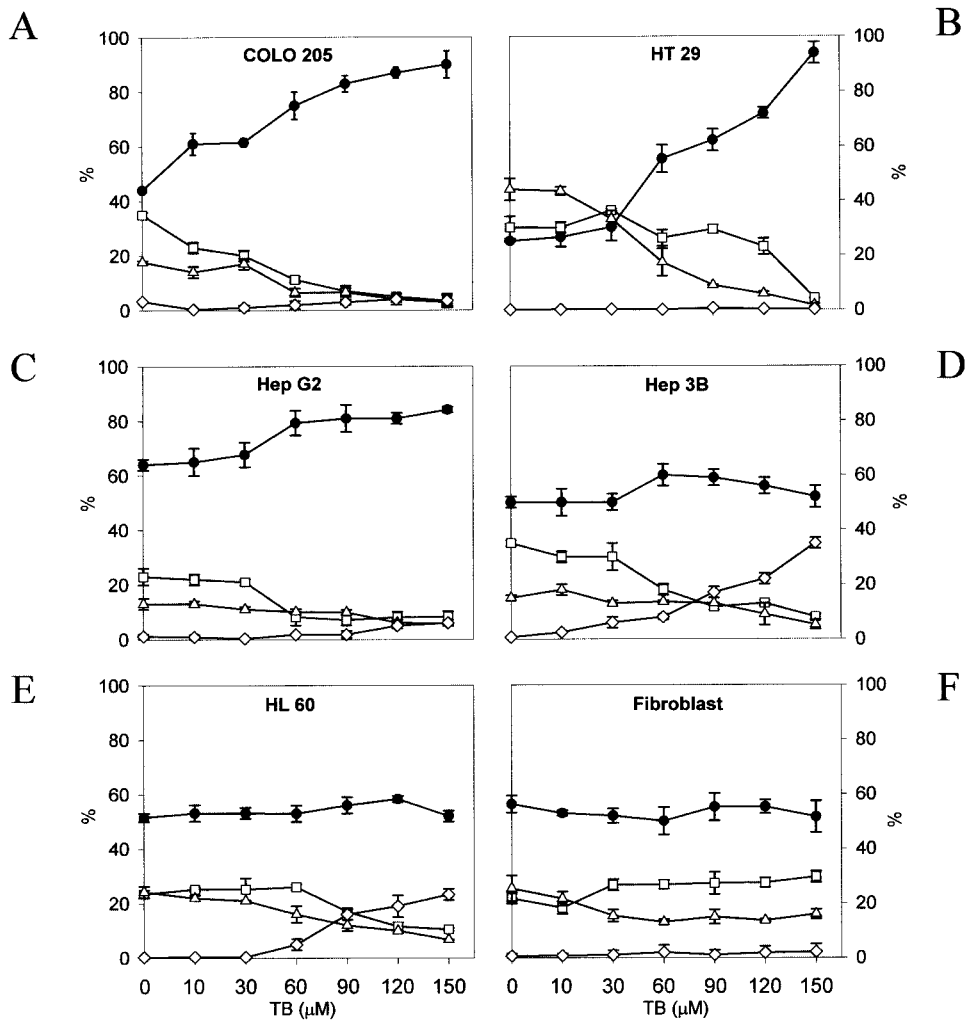

FIGURE 3 – Effects of TB on cell cycle and apoptosis in human cancer cells. TB dose-dependently induced cell cycle arrest at the G0/G1 phase in COLO 205 (*a*), HT 29 (*b*) and Hep G2 (*c*). In Hep 3B (*d*) and HL 60 (*e*), TB caused the occurrence of apoptosis in a dose-dependent manner. Treatment of human fibroblast for 15 hr did not cause cell cycle arrest or apoptosis (*f*). FACS analysis of DNA content 15 hr after release from quiescence by incubation in culture media supplemented with 10% FCS and various concentrations of TB in 0.05% DMSO. Percentage of cells in 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 ± SE.

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.[20,21] 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*a*, 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* His^{273} 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. 8*b*, lane 4) than in those of the TB-treated HT 29 cells (Fig. 8*b*, lane 2).

To further demonstrate that increased p53 expression correlated with G0/G1 arrest in the TB-treated cells, the experiment illustrated in Figure 8*c* 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 8*c*, 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 8*c*, 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.[22] 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. 9*a*, lane 5). In the presence of 10 μM TB, which does not induce DNA ladder patterns in COLO 205 cells (Fig. 9*a*, lane 9), ND induced the DNA ladder pattern in the COLO 205 cells at a concentration as low as 1 nM (Fig. 9*a* 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

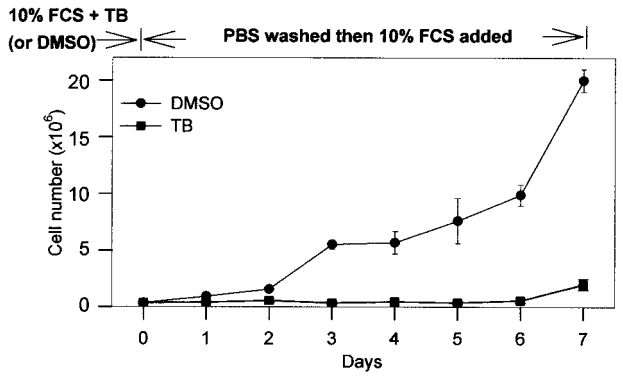

FIGURE 4 – Reversibility of the TB-induced inhibition of cell proliferation. TB-induced inhibition of cell proliferation was not reversed by removal of TB. The COLO 205 cells were released from quiescence by incubation in culture media supplemented with 10% FCS and 0.05% DMSO without or with 90 μM TB for 24 hr. After 24 hr of treatment with TB, the cells were washed twice with PBS, replaced with fresh 10% FCS without DMSO or TB. The TB-induced inhibition of cell proliferation was sustained for at least 7 days after removal of TB. The cultured cell numbers in DMSO- and TB-treated groups were counted every day. Three samples were analyzed in each group, and values represent the mean ± SE.

plus peanut oil) was detected (Fig. 9*b–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. 10*a*), and fragmentation of DNA, which can be examined by gel electrophoresis (Fig. 10*b*). The contents of p53 and p21/Cip1 were increased in the tumor isolated from the TB-treated mouse (Fig. 10*c*), suggesting that the inhibition of the progression of cell cycle activity was involved in the TB-induced decline in tumor size. The TB-induced upregulations 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. 11*a–c*). In contrast, p53, p21/Cip1 and p27/Kip1 immunoreactivities were strongly induced in the TB-treated tumor tissues (Fig. 11*d–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. 11*e*, blue square) and p27/Kip1 (Fig. 11*f*, red square) immunoreactive cells located in different areas of the COLO 205 tumor tissues. Figure 11*g–i* shows the percentage of cells expressing p53, p21/Cip and p27/Kip1 respectively.

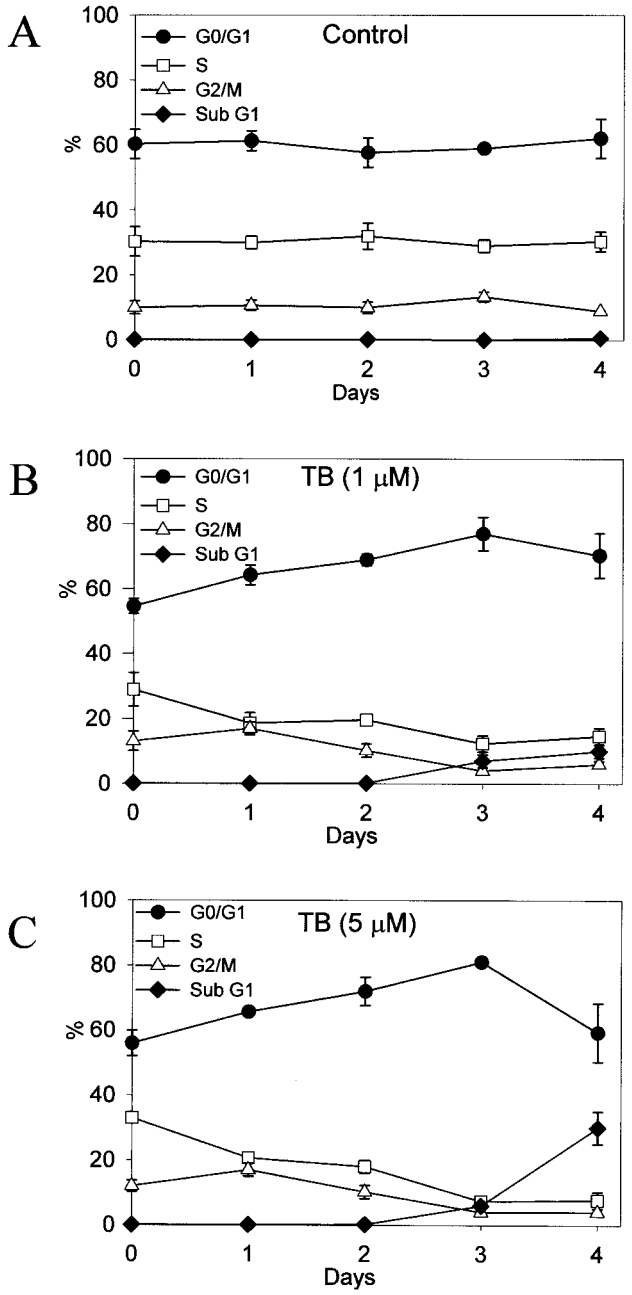

FIGURE 5 – Effects of lower doses of TB (1–5 μM) on cell cycle arrest in human COLO 205 cancer cells. TB dose- and time-dependently induced cell cycle arrest at the G0/G1 phase in COLO 205 cells. The COLO 205 cells were exposed to culture media supplemented with 10% FCS and various concentrations (1 and 5 μM) of TB in 0.05% DMSO for the indicated times. Media with and without TB were changed daily until flowcytometry analysis was performed. Percentage of cells in 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 ± SE.

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

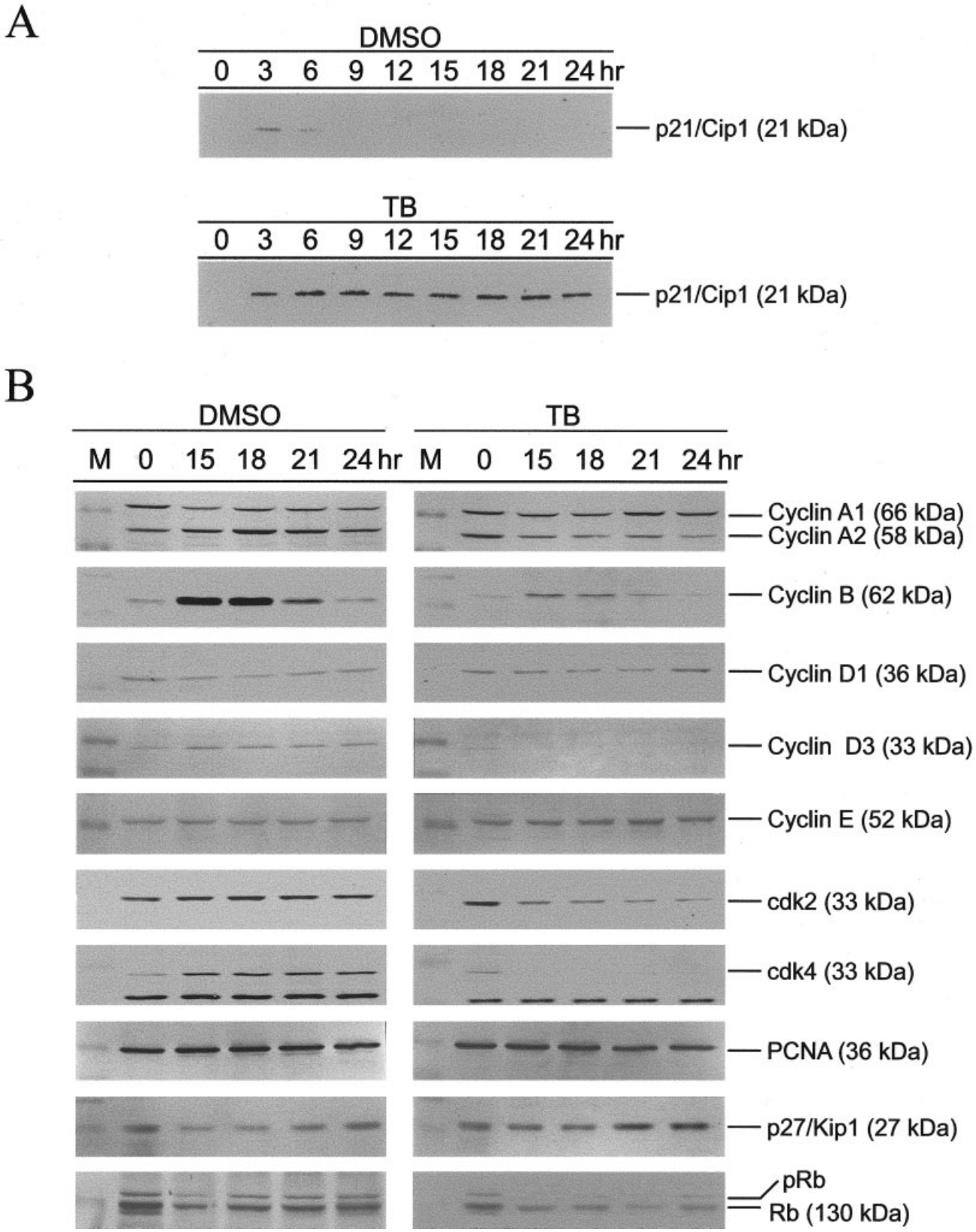

FIGURE 6 – Time effect of TB on cyclin and cdk protein levels in COLO 205 cells. (*a*) In the DMSO-treated COLO 205, 10% FCS caused a transit increase in p21/Cip1 protein (upper panel). In contrast, TB caused a persistent increase in p21/Cip1 protein level (lower panel). (*b*) In response to TB treatment, 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.

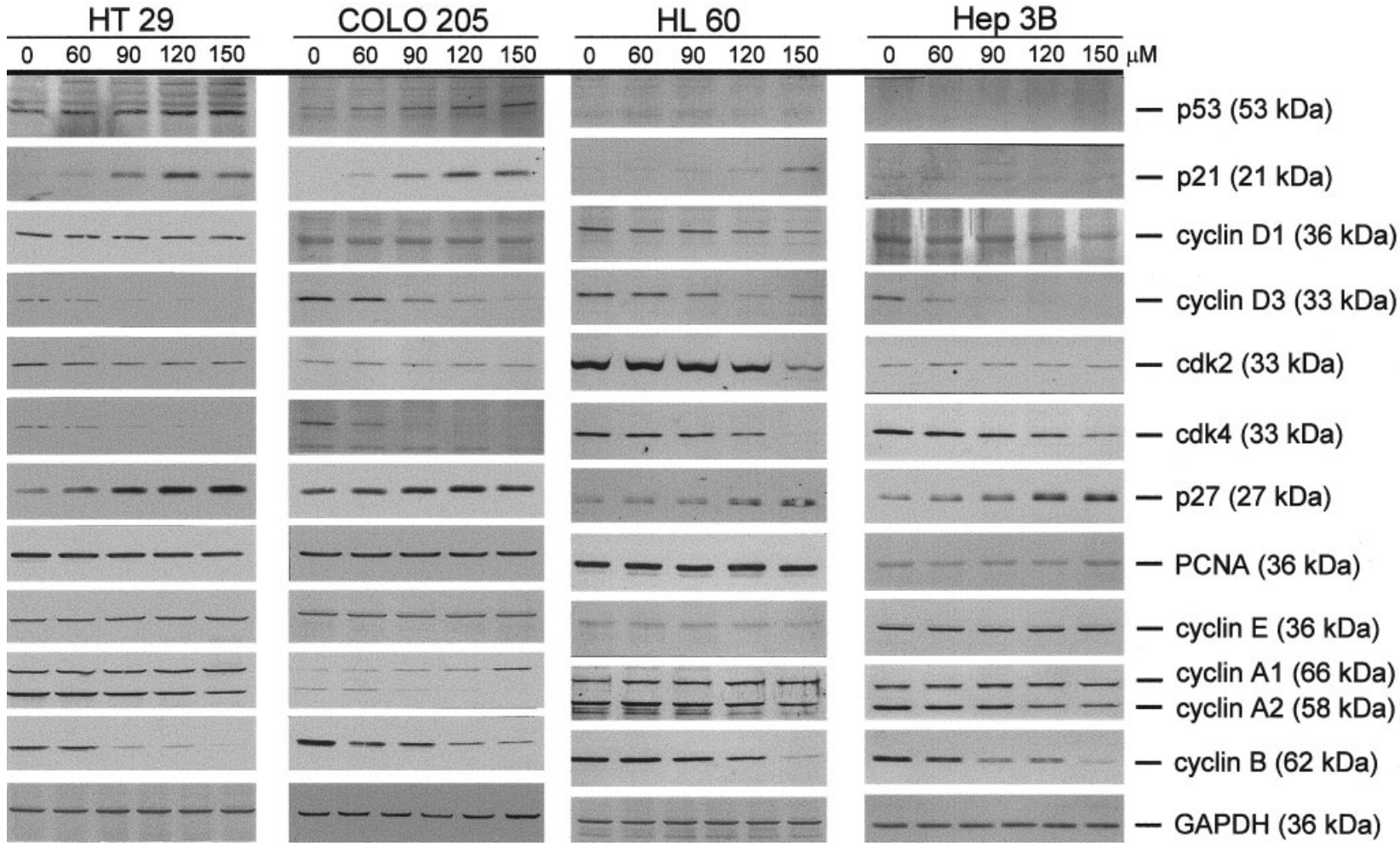

FIGURE 7 – Dose effect of TB on the cell cycle regulatory protein levels. TB dose-dependently increased the levels of p53, p21/Cip1 and p27/Kip1 proteins, and decreased the levels of cyclin D3 and cdk4 proteins in COLO 205 and HT 29 cells. In HL 60 and Hep 3B cells, TB treatment did not change the levels 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.

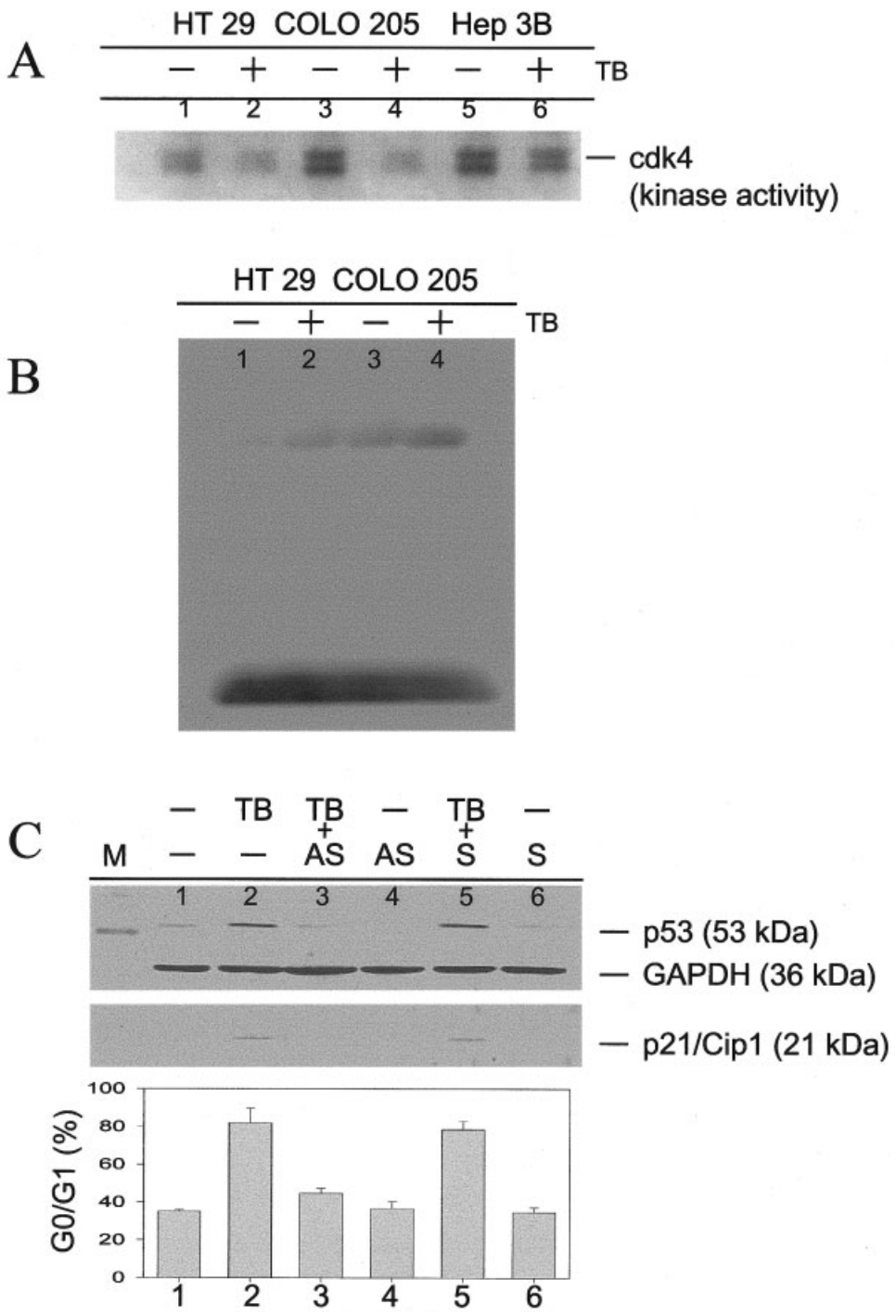

FIGURE 8 – Involvement of p53 signaling pathway in the TB-induced G0/G1 cell cycle arrest in COLO 205 cells. (*a*) TB strongly decreased the assayable cdk4 kinase activity in COLO 205 cells (p53 wild type). In the HT 29 (p53 His^{273} mutated) and Hep 3B (p53 partial deleted) cells, TB slightly decreased the cdk4 kinase activity. The cells were treated with 60 μM TB (+) or 0.05% DMSO (–) for 15 hr after release from quiescence. (*b*) Treatment of COLO 205 cells, but not HT 29 cells, with 60 μM TB increased the binding between p53 protein and the p53 consensus binding site in the p21/Cip1 promoter DNA probe. (*c*) Antisense p53 oligonucleotide abolished the TB-mediated increases of p53 and p21 protein levels and cell population at the G0/G1 phase. Antisense or sense p53 was added to COLO 205 at a final concentration of 20 μM 16 hr before the cell was challenged with 10% FCS and 60 μM TB treatment for an additional 15 hr. The percentage of cells in the G0/G1 phase of the cell cycle, determined using established CellFIT DNA analysis software, is shown in the bottom. M, size marker; AS, antisense p53 oligonucleotide; S, sense p53 oligonucleotide.

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.[8] In the HT-29 cell, TB treatment caused cell cycle arrest instead of apoptosis (Fig. 3*b*). This result is consistent with a recent report showing that mutant *p53* was not sufficient to induce apoptosis.[23] 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*).[24] 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. 8*a*). These observations demonstrate that both the COLO 205 cells, which express wild-type *p53*, and the HT

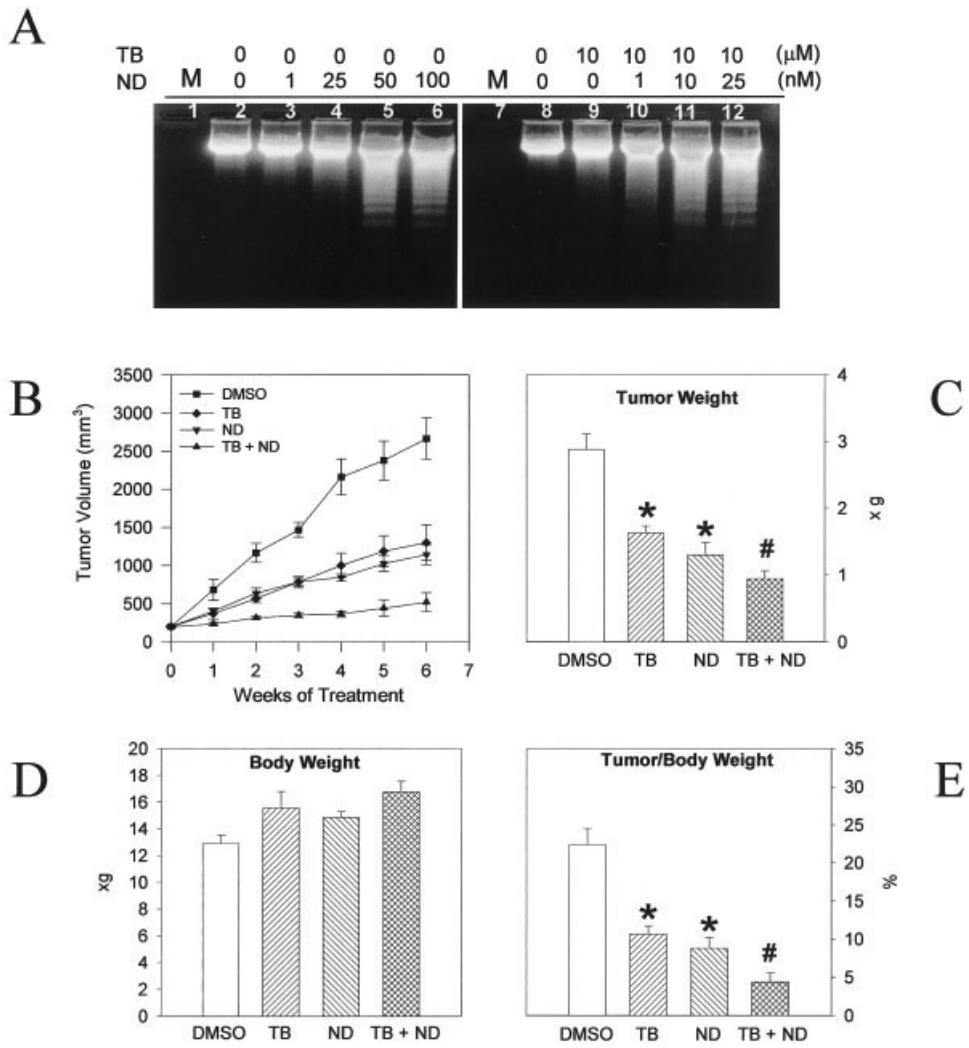

FIGURE 9 – TB reduces the growth rate of tumors and potentiates the antitumor activity of ND in nude mice. (*a*) Potentiation of ND-induced apoptosis by TB. Induction of apoptosis in COLO 205 cells was shown by DNA fragmentation using electrophoresis of genomic DNA. DNA fragmentation was examined at 24 hr after drug treatment. In lanes 2 and 8, cells received mock treatment as controls. M (lanes 1 and 7), DNA size marker. (*b*) Average tumor volume of DMSO-treated *vs.* drug-treated nude mice ($n = 5$). Tumor weight (*c*), animal body weight (*d*), and tumor/body weight ratio (*e*) were measured at the end of the experiment. Five samples were analyzed in each group, and values represent the mean ± SE. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $p < 0.05$. *, TB-, ND-, and TB+ND-treated group different from DMSO-treated group; #, TB+ND-treated group different from TB- or ND-treated group.

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. 8*b*). 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. 8*c*). Moreover, TB-induced G0/G1 arrest was not observed in the *p53* null cells, HL 60 (Fig. 3*e*). These data further support the notion that *p53* is involved in the TB-induced antiproliferaton. 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. 8*a*) 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 cdks, 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 cdks, 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. 3*d* 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-

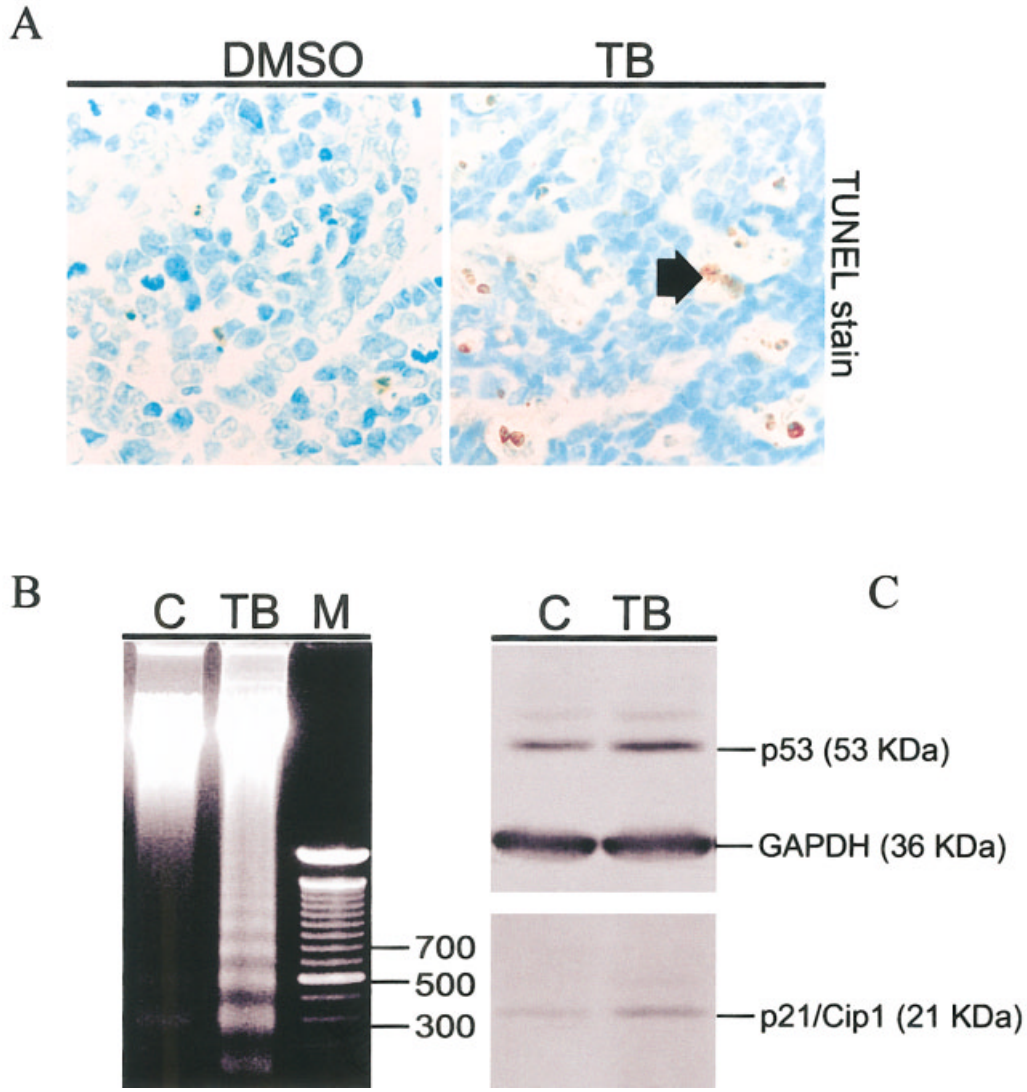

FIGURE 10 – TB causes the occurrence of apoptosis and increases the levels of p53 and p21/Cip1 proteins in the COLO 205-xenografted tumor. (*a*) Light micrographs of COLO 205 tumor tissues stained *in situ* by the TdT-mediated dUTP-biotin nick end-labeling method to detect the DNA breaks (400×). Arrow indicates representative apoptotic cells. (*b*) Induction of apoptosis by TB in COLO 205 tumor is also shown by DNA fragmentation using electrophoresis of genomic DNA. (*c*) Western blot analyses show the levels of p53 and p21/Cip1 proteins in COLO 205 tumor. COLO 205 tumors were isolated for protein extraction at 6 weeks after DMSO or TB treatment. Membranes were also probed with anti-GAPDH antibody to correct for differences in protein loading.

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 1 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*f*). However, the

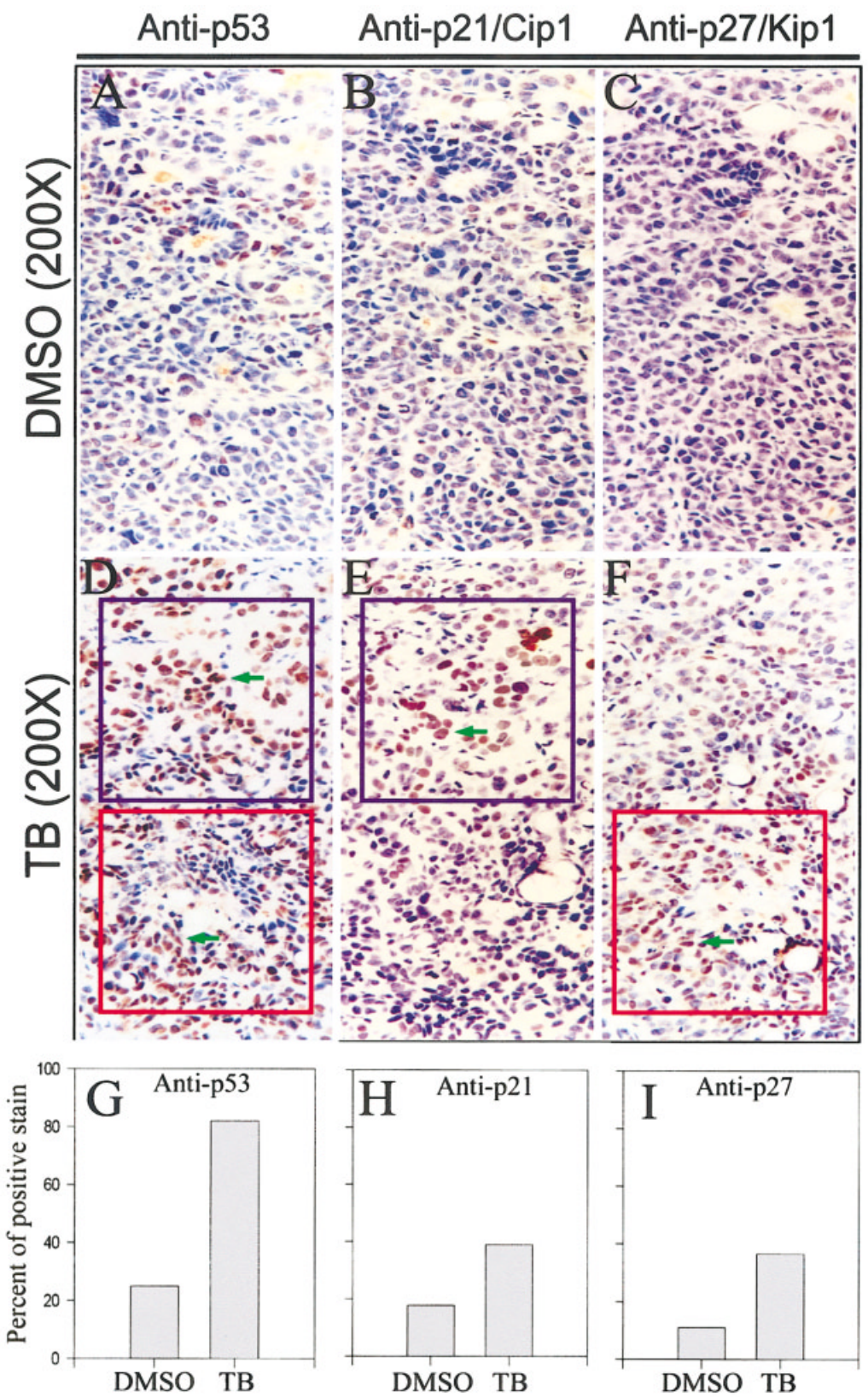

FIGURE 11 – Immunolocalization of p53, p21/Cip1 and p27/Kip1 proteins in COLO 205 tumor tissues. (*a–c*) Strong immunoreactivities of p53 (D, blue and red squares), p21/Cip1 (E, blue square) and p27/Kip1 (F, red square) proteins were detected in COLO 205 tumor tissues isolated from the TB-treated nude 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 caculated.

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. 1*f*). 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 *in vivo* studies was not cytotoxic for the vital organs.

Although animal studies of TB-induced antitumoral 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.

REFERENCES

1. Ho YS, Tsai PW, Yu CF, Liu HL, Chen RJ, Lin JK. Ketoconazole-induced apoptosis through P53-dependent pathway in human colorectal and hepatocellular carcinoma cell lines. Toxicol Appl Pharmacol 1998;153:39–47.
2. Chen RJ, Lee WS, Liang YC, Lin JK, Wang YJ, Lin CH, Hsieh JY, Chaing CC, Ho YS. Ketoconazole induces G0/G1 arrest in human colorectal and hepatocellular carcinoma cell lines. Toxicol Appl Pharmacol 2000;169:132–41.
3. Ho YS, Duh JS, Jeng JH, Wang YJ, Liang YC, Lin CH, Tseng CJ, Yu CF, Chen RJ, Lin JK. Griseofulvin potentiates antitumorigenesis effects of nocodazole through induction of apoptosis and G2/M cell cycle arrest in human colorectal cancer cells. Int J Cancer 2001;91:393–401.
4. Petranyi G, Ryder NS, Stutz A. Allylamine derivatives: new class of synthetic antifungal agents inhibiting fungal squalene epoxidase. Science 1984;224:1239–41.
5. Abdel-Rahman SM, Nahata MC. Oral terbinafine: a new antifungal agent. Ann Pharmacother 1997;31:445–56.
6. Gupta AK, Shear NH. Terbinafine: an update. J Am Acad Dermatol 1997;37:979–88.
7. Gupta AK, del Rosso JQ, Lynde CW, Brown GH, Shear NH. Hepatitis associated with terbinafine therapy: three case reports and a review of the literature. Clin Exp Dermatol 1998;23:64–7.
8. Niewolik D, Vojtesek B, Kovarik J. p53 derived from human tumour cell lines and containing distinct point mutations can be activated to bind its consensus target sequence. Oncogene 1995;10:881–90.
9. Ho YS, Wang YJ, Lin JK. Induction of p53 and p21/WAF1/CIP1 expression by nitric oxide and their association with apoptosis in human cancer cells. Mol Carcinog 1996;16:20–31.
10. Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR, Ozturk M. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc Natl Acad Sci U S A 1990;87:1973–7.
11. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980;209:497–9.
12. Lee WS, Jain MK, Arkonac BM, Zhang D, Shaw SY, Kashiki S, Maemura K, Lee SL, Hollenberg NK, Lee ME, Haber E. Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. Circ Res 1998;82:845–51.
13. Wu X, Rubin M, Fan Z, DeBlasio T, Soos T, Koff A, Mendelsohn J. Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. Oncogene 1996;12:1397–403.
14. Somasundaram K, Zhang H, Zeng YX, Houvras Y, Peng Y, Wu GS, Licht JD, Weber BL, El-Deiry WS. Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. Nature 1997;389:187–90.
15. Oki E, Sakaguchi Y, Toh Y, Oda S, Maehara Y, Yamamoto N, Sugimachi K. Induction of apoptosis in human tumour xenografts after oral administration of uracil and tegafur to nude mice bearing tumours. Br J Cancer 1998;78:625–30.
16. Lee WS, Smith MS, Hoffman GE. Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone. Proc Natl Acad Sci USA 1990;87:5163–7.
17. Lee WS, Harder JA, Yoshizumi M, Lee ME, Haber E. Progesterone inhibits arterial smooth muscle cell proliferation. Nat Med 1997;3:1005–8.
18. LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. New functional activities for the p21 family of CDK inhibitors. Genes Dev 1997;11:847–62.
19. Coats S, Flanagan WM, Nourse J, Roberts JM. Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science 1996;272:877–80.
20. Ko LJ, Prives C. p53: puzzle and paradigm. Genes Dev 1996;10:1054–72.
21. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323–31.
22. Li CJ, Li YZ, Pinto AV, Pardee AB. Potent inhibition of tumor survival in vivo by beta-lapachone plus taxol: combining drugs imposes different artificial checkpoints. Proc Natl Acad Sci USA 1999;96:13369–74.
23. Rowan S, Ludwig RL, Haupt Y, Bates S, Lu X, Oren M, Vousden KH. Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant. EMBO J 1996;15:827–38.
24. Webley KM, Shorthouse AJ, Royds JA. Effect of mutation and conformation on the function of p53 in colorectal cancer. J Pathol 2000;191:361–7.
25. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855–78.
26. Bates S, Vousden KH. p53 in signaling checkpoint arrest or apoptosis. Curr Opin Genet Dev 1996;6:12–8.
27. el-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994;54:1169–74.
28. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817–25.
29. Malkowicz SB, Tomaszewski JE, Linnenbach AJ, Cangiano TA, Maruta Y, McGarvey TW. Novel p21WAF1/CIP1 mutations in superficial and invasive transitional cell carcinomas. Oncogene 1996;13:1831–7.
30. Balbin M, Hannon GJ, Pendas AM, Ferrando AA, Vizoso F, Fueyo A, Lopez-Otin C. Functional analysis of a p21WAF1,CIP1,SDI1 mutant (Arg94 — Trp) identified in a human breast carcinoma. Evidence that the mutation impairs the ability of p21 to inhibit cyclin-dependent kinases. J Biol Chem 1996;271:15782–6.
31. Naumann U, Weit S, Rieger L, Meyermann R, Weller M. p27 modulates cell cycle progression and chemosensitivity in human malignant glioma. Biochem Biophys Res Commun 1999;261:890–6.
32. Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. Cell 1996;85:733–44.
33. Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res 1997;57:5441–5.
34. Fujieda S, Inuzuka M, Tanaka N, Sunaga H, Fan GK, Ito T, Sugimoto C, Tsuzuki H, Saito H. Expression of p27 is associated with Bax expression and spontaneous apoptosis in oral and oropharyngeal carcinoma. Int J Cancer 1999;84:315–20.
35. Wang X, Gorospe M, Huang Y, Holbrook NJ. p27Kip1 overexpression causes apoptotic death of mammalian cells. Oncogene 1997;15:2991–7.
36. Rademaker M, Havill S. Griseofulvin and terbinafine in the treatment of tinea capitis in children. N Z Med J 1998;111:55–7.
37. Caceres-Rios H, Rueda M, Ballona R, Bustamante B. Comparison of terbinafine and griseofulvin in the treatment of tinea capitis. J Am Acad Dermatol 2000;42:80–4.
38. Jensen JC. Clinical pharmacokinetics of terbinafine (Lamisil). Clin Exp Dermatol 1989;14:110–3.
39. Humbert H, Cabiac MD, Denouel J, Kirkesseli S. Pharmacokinetics of terbinafine and of its five main metabolites in plasma and urine, following a single oral dose in healthy subjects. Biopharm Drug Dispos 1995;16:685–94.
40. Kovarik JM, Mueller EA, Zehender H, Denouel J, Caplain H, Millerioux L. Multiple-dose pharmacokinetics and distribution in tissue of terbinafine and metabolites. Antimicrob Agents Chemother 1995;39:2738–41.
41. Kovarik JM, Kirkesseli S, Humbert H, Grass P, Kutz K. Dose-proportional pharmacokinetics of terbinafine and its N-demethylated metabolite in healthy volunteers. Br J Dermatol 1992;126(Suppl 39):8–13.
42. Faergemann J, Zehender H, Denouel J, Millerioux L. Levels of terbinafine in plasma, stratum corneum, dermis-epidermis (without stratum corneum), sebum, hair and nails during and after 250 mg terbinafine orally once per day for four weeks. Acta Derm Venereol 1993;73:305–9.
43. Hosseini-Yeganeh M, McLachlan AJ. Tissue distribution of terbinafine in rats. J Pharm Sci 2001;90:1817–28.