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Pretreatment with an Ethanolic Extract of *Taiwanofungus camphoratus* (*Antrodia camphorata*) Enhances the Cytotoxic Effects of Amphotericin B

Ling-Yi Chen,[†] Ming-Thau Sheu,[‡,§] Der-Zen Liu,[∥] Chuh-Kai Liao,[†] Hsiu-O Ho,[‡] Woei-Yao Kao,[⊥] Yaun-Soon Ho,[#] Wen-Sen Lee,[†] and Ching-Hua Su*[,†,▽]

[†]Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei 110, Taiwan, Republic of China

[‡]School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei 110, Taiwan, Republic of China

[§]Clinical Research Center and Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei 110, Taiwan, Republic of China

[∥]Graduate Institute of Biomedical Materials and Engineering, College of Oral Medicine, Taipei Medical University, Taipei 110, Taiwan, Republic of China

[⊥]Department of Hematology and Oncology, Tri-Service General Hospital National Defense Medical, Taipei 110, Taiwan, Republic of China

[#]School of Medical Laboratory Science & Biotechnology, College of Medicine, Taipei Medical University, Taipei 110, Taiwan, Republic of China

[▽]Department of Microbiology and Immunology, Taipei Medical University, Taipei 110, Taiwan, Republic of China

ABSTRACT: *Taiwanofungus camphoratus*, a well-known Chinese medicine used in Taiwan, possesses several pharmacological functions, including anticancer effects. In the present study, we aimed to investigate a novel anticancer effect by pretreating cancer cells with an ethanolic extract of *T. camphoratus* (TCEE) followed by the administration of an antifungal agent amphotericin B (AmB). Both TCEE and AmB showed significant dose-dependent cytotoxicity in HT29 cells. Pretreatment with a nontoxic dose of TCEE enhanced the cytotoxicity of AmB. Furthermore, significant apoptotic cell death was found in cells treated with TCEE and AmB. A combination treatment with AmB plus TCEE resulted in a significant repression of tumor growth in HT29 xenografts. Collectively, our results indicated that combined treatment with AmB and TCEE effectively induced apoptosis and inhibited tumor growth. In the future, TCEE may serve as a potential complementary and alternative medicine to treat patients suffering from colorectal cancer.

KEYWORDS: *Taiwanofungus camphoratus*, colon cancer, antifungal agents, *Antrodia camphorata*, apoptosis, amphotericin B

INTRODUCTION

Taiwanofungus camphoratus (syn. *Antrodia camphorata*), a rare Ganoderma-like fungus, has been used as a component of nutraceuticals and healthy foods to modulate the immune system or enhance liver function in Taiwan. In the past decade, it has been reported to have several pharmacological functions including anticancer,[1−5] antihepatotoxic,[6,7] antihypertensive,[8] anti-inflammatory,[5,9,10] antioxidant,[11,12] and neuroprotective[13] effects. To date, 78 components of *T. camphoratus* have been identified and structurally elucidated.[14] The bioactive constituents of TCEE include diterpenoids, triterpenoids, sesquiterpene lactone, benzenoids, and polysaccharides.[9,13,15−17] Among the compounds isolated from *T. camphoratus*, 22 belong to the triterpenoids category and have either ergostane or lanostane skeletons. Triterpenoids have known pharmacological activities and are considered to be potential anticancer agents.[18] Notably, they have been shown to have selective cytotoxicity against tumor cells rather than normal cells in various types of cancer.[19,20]

Cancer chemotherapy commonly employs a cocktail strategy in which multiple drugs are used. To be considered successful, a drug combination should have advantages such as increased therapeutic efficacy, reduced toxicity to the host, and minimal drug resistance. Amphotericin B (AmB) is a polyene antifungal agent that is widely used to treat deep-seated fungal infections.[21] It has recently received attention because of its potential for use as an anticancer agent; for example, it has been found to potentiate the cytotoxicity of cisplatin in various types of cancer.[22,23] AmB interacts specifically with ergosterol-containing membranes, forming micelles. The attachment of the drug moieties to the acyl side chains of ergosterol induces the formation of the membrane pore complex. These complexes alter cell membrane permeability, resulting in the efflux of certain important electrolytes, such as potassium and magnesium ions, from the cytosol. The disruption of membrane pumps and membrane potential leads to the apoptosis of the ergosterol-containing

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cells.[24] At high doses, essential cytoplasmic components, especially proteins, leak out, which leads to further apoptotic destruction of the cell. Upon cell shrinkage and decreases in the intracellular concentrations of electrolytes, particularly potassium ions, DNA fragmentation and caspase 3-like activity occur, leading to cellular apoptosis.[25]

This study aims to investigate a novel anticancer regimen in which the polyene antibiotic AmB and an ethanolic extract of *T. camphoratus* were selected and examined for potential effects. We demonstrated that combination treatment with TCEE and AmB in a sequential manner leads to a significant decrease in the viability of HT29 cells in a dose-dependent manner. This result indicated that the aforementioned dosing regimen exerts an anticancer effect on HT29 cells. Furthermore, an inhibitory effect of the combined medications was observed in tumor-bearing animals, whereas no significant tumor regression occurred when each drug was used separately. These results suggest that pretreatment of TCEE enhanced the sensitivity of tumor cells toward cell membrane destruction induced by AmB and that the combination may serve as an alternative therapy for colon cancer.

MATERIALS AND METHODS

AmB and TCEE Preparation. AmB was purchased from Sigma Aldrich Co. (St. Louis, MO). A pulverized crude extract of solid state cultivated *T. camphoratus* was provided by Well Shine Biotechnology Development (Taipei, Taiwan) and prepared as previously described;[11] the resulting compound contained 15−20% triterpenoids and 1−2% polysaccharides. Pulverized *T. camphoratus* was suspended in 95% alcohol (1:40 w/v), and the suspension was incubated overnight on a rotary shaker. The insoluble portion was removed by filtration. The filtrate was then lyophilized to obtain the TCEE. A stock solution of TCEE was prepared by dissolving TCEE powder in absolute EtOH to a final concentration of 100 mg/mL and stored at −20 °C.

Cell Culture. The human colon cancer cells line HT29 was purchased from the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan, Republic of China) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Gibco, Grand Island, NY) under standard culture conditions (37 °C, 95% humidified air and 5% CO_2).

MTT Assay. Cells were seeded in 12-well culture plates at a concentration of 5×10^5/well and incubated for 24 h prior to treatment. HT29 were treated with noncytotoxic doses of TCEE (50 μg/mL) and AmB (1.5−6.0 μM); they were first pretreated with TCEE for 24 h, followed by treatment with AmB for an additional 24 h. MTT solution (0.55 mg/mL) was then added to each well, and the plates were further incubated at 37 °C for 4 h in a humidified incubator with 5% CO_2. The culture medium was then removed and mixed with 500 μL of DMSO. Aliquots of 200 μL were transferred into 96-well plates. The optical density was measured at 550 nm with a Microplate Autoreader (Bio-Tek Instruments, VT). All experiments were performed in triplicate.

HT29-Derived Xenografts Animal Model. ICR nude mice (nu/nu, male) were obtained from the National Science Council animal center, Taipei. The study was approved and conducted in accordance with the guideline for the care and use of laboratory animals by the Animal Research Committee at Taipei Medical University. A suspension of 5×10^6 HT29 cells in 0.1 mL of RPMI 1640 was injected subcutaneously between the scapulae of each nude mouse. After implantation, the tumor size was measured using calipers, and the tumor volume was estimated based on the following formulas: tumor volume (mm^3) = $1/2 \times L \times W^2$, where L is the length and W is the width of the tumor.[26] In the first experiment, once the tumor had reached a volume of 200 mm^3, the animals were divided into three groups; they either received iv injections of 100 μg/kg AmB per day for 4 weeks, were fed 10 mg/kg TCEE per day for 4 weeks, or were fed 10 mg/kg TCEE per day for 2 weeks and then given iv injections of 100 μg/kg AmB per day for 2 weeks. Another set of animals given PBS alone was used as the control group (control). In the second experiment, once the tumor reached a volume of 200 mm^3, animals were divided into three groups; they either received iv injections of 100 μg/kg AmB per day for 30 days (AmB group), were fed 10 mg/kg TCEE per day for 30 days (TCEE group), or were fed 10 mg/kg TCEE per day for the first 2 days and then received iv injections of 100 μg/kg AmB on the third day for 10 cycles (for the same total period of 30 days; TCEE + AmB group). Another set of animals given PBS alone was used as the control group (control).

Annexin V−PI Staining. An Annexin V (FITC-conjugated) apoptosis kit (BioVision, Mountain View, CA) was used to analyze the number of apoptotic cells according to the manufacturer's instructions. Cells (5×10^5) were cultured in six-well plates and treated with TCEE (50 μg/mL) and AmB (1.5−6.0 μM) by pretreating them with TCEE for 24 h and then treating with AmB or vehicle for an additional 24 h. After trypsinization, the floating and adherent cells were collected and washed with ice-cold PBS and then resuspended in 0.5 mL of binding buffer, 5 μL of Annexin V−FITC, and 10 μL of propidium iodide (PI). After incubation for 15 min in the dark, the cells were imaged under a fluorescent microscope or resuspended for flow cytometric analysis. The green (530 nm) and red (>650 nm) fluorescence emission from 10000 cells illuminated with blue (488 nm) excitation light was measured with a FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ) and analyzed using CellQuest software (Beckton Dickinson).

Cell Cycle Analysis. The cellular DNA content was determined by flow cytometric analysis of PI-labeled cells. After treatment, cells were harvested, fixed in ice-cold 70% ethanol, stored at 4 °C, washed with phosphate-buffered saline (pH 7.2), treated with 25 mg/mL RNase A at 37 °C for 30 min, and stained with 50 μg/mL PI for 30 min. For flow cytometric analysis, a FACSCalibur flow cytometer (Becton Dickinson) equipped with a single argon ion laser was used. The DNA content of 10000 cells per analysis was monitored using the FACSCalibur system. A minimum of 10000 cells per sample was used for analysis performed using CellQuest software. Representative flow cytometry patterns are shown. The data were analyzed using the software ModFit LT 3.0 (Verity Software House, Topsham, ME).

Mitochondrial Membrane Potential Analysis. The loss of mitochondrial membrane potential was quantitatively determined by flow cytometry using the MitoProbe JC- 1 Assay Kit according to the manufacturer's instructions (Molecular Probes). Briefly, cells were trypsinized, washed twice with warm PBS, and resuspended in warm PBS to a concentration of 1×10^6 cells/mL. The cells were then stained with 10 μL of 200 μM JC-1 dye at 37 °C for 30 min in the dark and analyzed immediately by FACScan flow cytometry using CellQuest analysis software (Becton Dickinson).

Western Blot Analysis. Cells in 10 cm culture dishes were harvested at the indicated times following treatment with TCEE (50 μg/mL) and AmB (1.5−6.0 μM) (pretreatment with TCEE for 24 h followed by treatment with AmB for an additional 24 h) or vehicle alone and washed with ice-cold PBS. The cells were incubated in ice-cold RIPA buffer [1 M Tris (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% SDS, 10% DOS, and 10% NP40] with freshly added protease inhibitor cocktail tablets (Roche, United States) on ice for 30 min. The cells were scraped, and the lysates were collected and centrifuged at 13000 rpm for 30 min at 4 °C. Then, the supernatant was collected, aliquoted, and stored at −70 °C. The protein concentration of the lysates was measured using a BCA protein assay kit (Pierce, United States) according to manufacturer's protocol. Cellular proteins (40 μg) were separated on 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels and transferred onto PVDF (Amersham Biosciences, United States) at

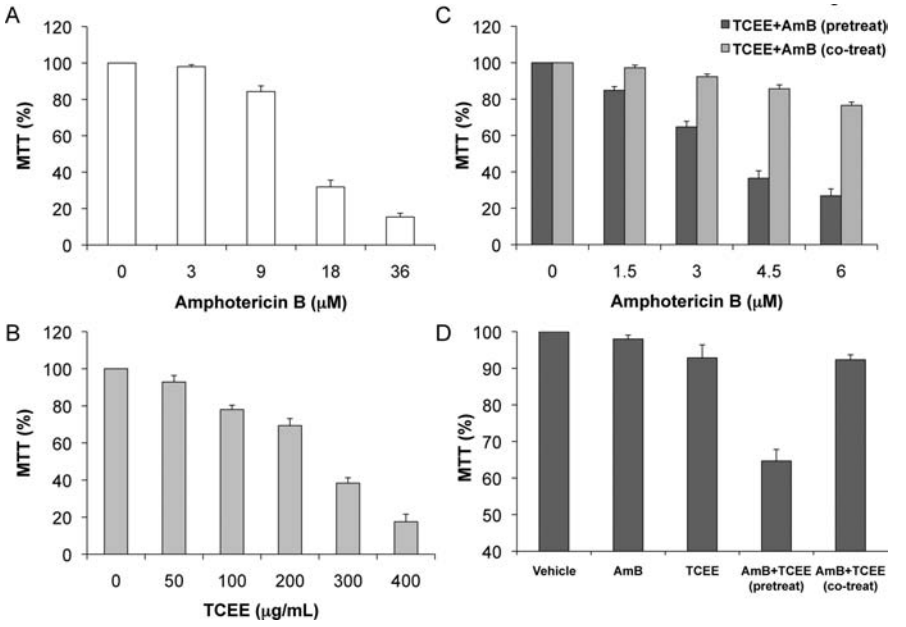

Figure 1. Susceptibility of HT29 cells to the cytotoxic effects of AmB and TCEE used alone or in combination. MTT assays were performed as described in the Materials and Methods to evaluate HT29 cell cytotoxicity treated with the indicated concentrations of (A) AmB, (B) TCEE, or (C) a combination of AmB and TCEE. (D) Pretreament of TCEE followed by AmB enhanced cytotoxicity in HT29 cells. The results of the MTT assay demonstrate that 3 μM AmB and 50 μg/mL TCEE exerted enhanced inhibitory effects on HT29 cells at 48 h following treatment. The data represent the mean ± SD of five independent experiments, normalized to the viability of the control cells.

30 V overnight. The blots were blocked by incubation in 10% skim milk in PBS for 1 h at room temperature or overnight at 4 °C. The blots were washed with TBST (TBS with 0.1 Tween 20) three times for 15 min and incubated with the appropriate primary antibody for 2 h at room temperature or overnight at 4 °C. The blots were washed with TBST three times for 5 min each time followed by incubation with the corresponding secondary antibody horseradish peroxidase conjugate for 1 h at room temperature. Membranes were incubated with ECL plus Western blotting detection reagents (Amersham Biosciences, United States) and analyzed using a Fuji LAS-3000 imaging system (Japan). The antibodies and dilutions used in this study included anti-Cyclin D1 mAb (1/3000; Upstate, United States), anti-Cdk2 Ab (1/4000; Calbiochem, Germany), anti-β actin mAb (1/4000; Chemicon, CA), antirabbit HRP-linked Ab (1/5000; Cell Signaling, United States), and antimouse HRP-linked Ab (1/5000; Cell Signaling). The band intensity was quantified by arithmetic analysis using the software ImageJ (NCBI). The protein levels were normalized to their internal β-actin and displayed as the relative expression.

Statistical Analysis. The data are presented as the means and standard deviations (means ± SDs). The results were analyzed using a two-tailed Student's t test to assess statistical significance. Values with $P < 0.05$ were considered to be statistically significant.

RESULTS

Cytotoxicity of AmB and TCEE in the HT29 Cells. First, we examined the cytotoxicity caused by treatment with both AmB and TCEE. As shown in Figure 1A,B, both AmB and TCEE inhibited HT29 proliferation in a dose-dependent manner. To investigate the combined effects of AmB and TCEE, we chose nontoxic doses of AmB (0, 1.5, 3, 4.5, and 6 μM) and TCEE (50 μg/mL). We examined two different treatment regimens (cotreatment and pretreatment) to evaluate the effects of AmB plus TCEE. Human HT29 cells were either cotreated or pretreated with TCEE 50 μg/mL in combination with serial doses of AmB at 0, 1.5, 3.0, 4.5, and 6.0 μM for 24 h. While cotreatment with TCEE with AmB had only a mild antiproliferative effect, pretreatment with TCEE dramatically enhanced cytotoxicity of AmB (Figure 1C,D). Therefore, treating TCEE and AmB in a sequential manner significantly inhibited the growth of HT29 cells as compared to treatment with either drug alone (Figure 1A,B).

Induction of Apoptosis in HT29 Cells by Combined Treatment with AmB and TCEE. It has been reported that various types of extracts of *T. camphoratus* can induce apoptosis in in vitro culture systems,[7] and the disruption of membrane pumps and membrane potential by AmB also leads to apoptosis of the ergosterol-containing cells.[24] Therefore, we examined whether combined treatment with AmB and TCEE also induced apoptosis. As shown in Figure 2A, HT29 cells were treated with the aforementioned combination of the two agents, and annexin V−PI double staining was performed to examine the apoptotic effect of combined treatment with AmB and TCEE in HT29 cells. While treatment with AmB alone failed to cause cell death or apoptosis (data not shown), the viability of cells treated with TCEE and AmB in a sequential manner significantly decreased, and the percentage of apoptotic cells significantly increased in a dose-dependent manner ($P < 0.01$; Figure 2B,C).

In addition to annexin V staining, mitochondrial membrane potential in cells pretreated with TCEE followed by AmB was also examined. The loss of mitochondrial membrane potential is one of the important mechanisms leading to apoptosis. As shown in Figure 3A, combined treatment with AmB and TCEE dramatically decreased the mitochondrial membrane potential ($P < 0.01$), which confirmed our previous results. Furthermore, we examined the expression of the apoptotic protein Bax and the antiapoptotic protein Bcl-2 (Figure 3B). Pretreatment of TCEE followed by AmB significantly reduced the level of Bcl-2 without affecting Bax. Collectively, AmB combined with TCEE pretreatment decreases cell viability by increasing apoptosis.

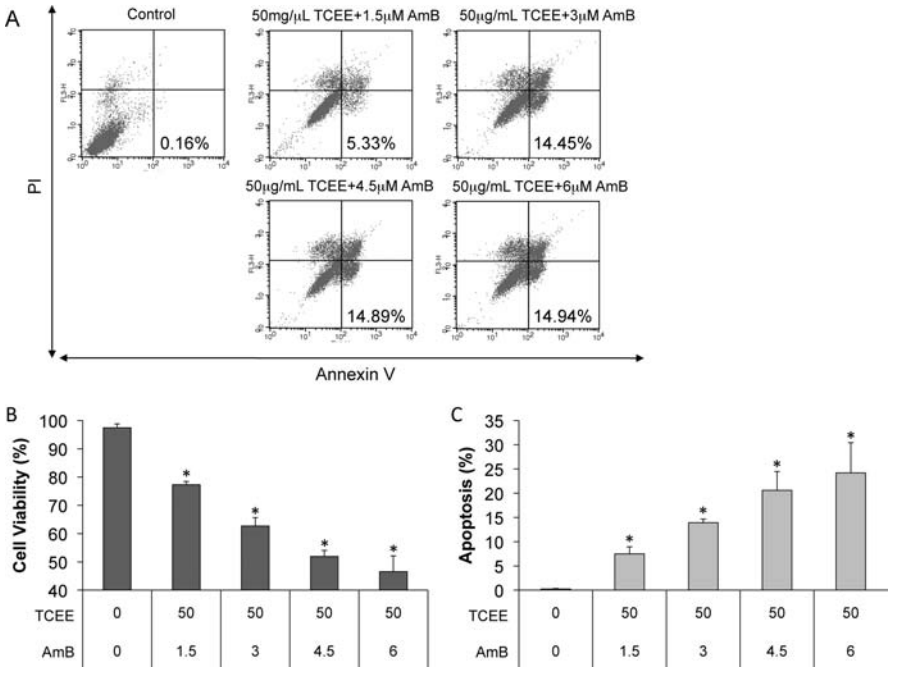

Figure 2. Apoptotic effects of TCEE and AmB in HT29 cells. Cells were treated with either TCEE or AmB or a combination at the indicated concentrations. Cells were then harvested and stained with Annexin V and PI for flow cytometric analysis. (A) The induction of apoptosis by combined treatment with AmB and TCEE. (B) Bars represent the percentage of viable cells (Annexin V- and PI-negative cells). (C) Bars represent the percentage of apoptotic cells (Annexin V-positive cells) evaluated by surface phosphatydylserine externalization. The data are the mean ± SD of four independent experiments. *P < 0.01 indicates a statistically significant change with respect to the control samples.

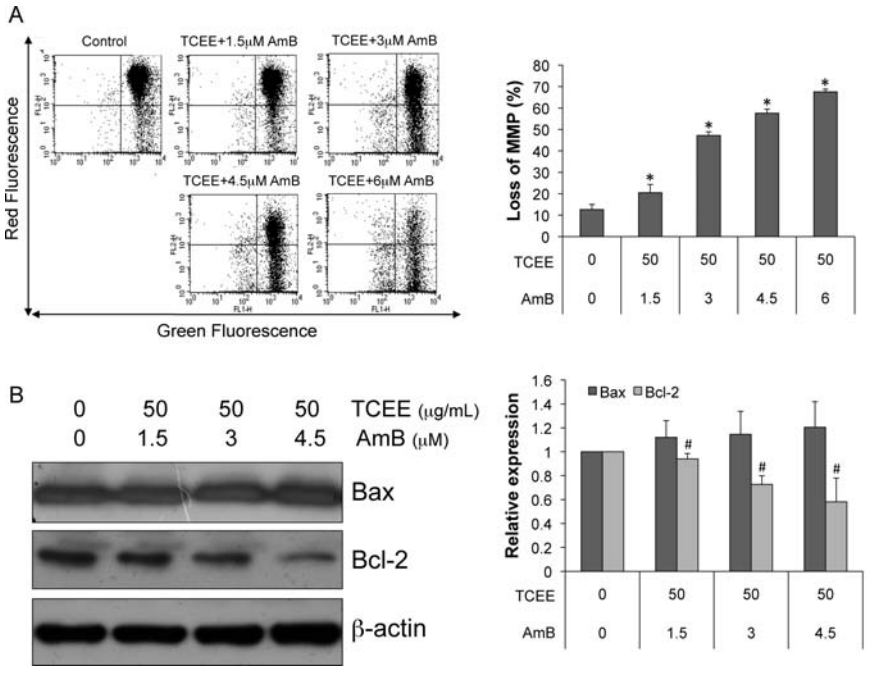

Figure 3. Loss of mitochondrial membrane potential and a decrease in the level of antiapoptotic protein in HT29 cells induced by combined treatment with TCEE and AmB. HT29 cells were treated with vehicle or a combination of TCEE and AmB at the indicated concentrations. (A) The mitochondrial membrane potential was determined using JC-1 staining and assessed via flow cytometry. Left: Representative results of four independent experiments. Right: Bars represent the percentage of cells that lose mitochondrial membrane potential. (B) The expression levels of Bax and Bcl-2 were detected using Western blotting, as detailed in the Materials and Methods. Left: Western blot analysis of Bax and Bcl-2 expression. Right: Quantification of Bax and Bcl-2 expression by densitometric analysis. The data presented are from at least three independent experiments and expressed as the mean ± SD; *P < 0.01 or #P < 0.05 indicates a statistically significant change with respect to the control samples.

Cell Cycle Arrest Induced by TCEE in HT29 Cells. To examine a mechanism other than apoptosis that might mediate the anticancer effect of the two drugs, we investigated the role of TCEE in regulating cell cycle progression using flow cytometry. HT29 cells pretreated with TCEE for 24 h and subsequently treated with AmB for 0, 6, 12, 18, or 24 h resulted in a significant increase in the percentage of cells in the G2/M phase (Figure 4A,B). The G2/M cell cycle arrest caused by TCEE occurred as early as 6 h post-treatment. Previous researchers have shown that cdc2 binds to cyclin B1 to form the M phase promoting factor (MPF), which induces mitosis. In our study, the treatment resulted in a reduction of cyclin B1 in HT29 cells as compared to the vehicle control (Figure 4C). Unexpectedly, the protein levels of CDK2 increased upon combined treatment with 4.5 μM AmB and 50 μg/mL of TCEE (Figure 4C). Despite the effects of AmB and TCEE on apoptotic pathway, the results indicated that combined treatment with AmB and TCEE also affects the expression of G2/M phase-related proteins.

Inhibitory Effect of TCEE and AmB on Xenograft Growth in Nude Mice. Because TCEE and AmB showed promising cytotoxic activity in HT29 cells in vitro, we investigated their therapeutic efficacy in a mouse xenograft model. The therapeutic effects of combined treatment with TCEE and AmB on HT29 xenograft growth in nude mice were evaluated. Two different combination regimens were applied. In the first method, mice were fed 10 mg/kg TCEE per day for 2 weeks and then injected with AmB (100 μg/kg, iv) per day for 2 weeks. Under these conditions, the toxicity of these agents as measured by a decrease in body weight (Figure 5A) was mild. HT29 xenografted mice treated with this combination of TCEE and AmB showed a significant reduction in tumor size as compared to the vehicle control, AmB, or TCEE alone (Figure 5B,C; $P < 0.01$). These results demonstrate that TCEE enhanced the antitumor effects of AmB in vivo.

Next, we investigated whether a short, more compact treatment regimen would further enhance the efficacy of the AmB/TCEE combination. The mice were fed 10 mg/kg TCEE per day for 2 days and then injected with AmB (100 μg/kg, iv) on the third day; this treatment was repeated for 10 cycles (a total period of 30 days), as detailed in the Materials and Methods. There were no significant alterations in body weight (Figure 6A), whereas a 93% reduction in tumor growth was observed in mice treated with the AmB and TCEE combination ($P < 0.01$; Figure 6B). The TCEE and AmB combination significantly suppressed the growth of HT29 cells in xenografted mice (Figure 6C). Remarkably, complete remission was achieved in two out of six mice (Figure 6D). The results indicated that the novel combination of TCEE and AmB possesses potent antitumor therapeutic efficacy with relatively mild toxicity.

DISCUSSION

In the present study, we demonstrated that both TCEE and AmB induce cytotoxicity in a dose-dependent manner. Pretreatment with TCEE followed by AmB dramatically inhibited cell proliferation in HT29 cells, whereas cotreatment with these two agents showed no significant effects. AmB's biological action is most intimately related to its ability to form hydrophilic pores in the hydrophobic plasma membrane, causing an increase in the permeability of the cells to ions and small molecules.[27,28] It has been proposed that the interaction between membrane sterols and AmB is responsible for AmB's selectivity. The selective toxicity of AmB to fungi results from its capacity to bind more strongly to ergosterol, the principal fungal sterol, than to cholesterol, the principal sterol of mammalian cells. However, the nature of the mechanism underlying its biological activity is still under dispute. Recently, polyene antibiotics have gained attention for their potential applications in cancer treatment.[29,30] Before their use in clinical practice, the insensitivity of cancer cells to polyene antibiotics needs to be addressed. It has been suggested that selective substitution of cholesterol with one of its biosynthetic precursors [e.g., 7-dehydrocholesterol (7-DHC)] in hepatoma or a combination of a high cholesterol diet and specific inhibitors of cholesterol biosynthesis may overcome this limitation.[31,32] In addition, the structure of ergosterol, notably the presence of a 5,7-dien system in the sterol molecule, is responsible for the high sensitivity of the fungal membrane to AmB.[12,13,29] Membranes containing any sterol with a 5,7-dien system, for example, ergosterol and 7-DHC, are at least 10 times more sensitive to AmB than a membranes without such sterols. Charbonneau et al. also found that the affinity of 7-DHC for AmB in a lipid matrix is higher than that of cholesterol.[33] Because the extract of *T. camphoratus* contains sterols with ergostane or lanostane skeletons, it is possible that treating cancer cells with the extract of *T. camphoratus* might increase the susceptibility of the plasma membrane of cancer cells to AmB by the insertion of ergostane triterpenoids or might influence the vital barrier functions of the membrane by the insertion of lanostane triterpenoids, leading to cell death.

Another possible mechanism for the effects observed in this study is the caspase pathway that drives the apoptotic events in various cell lines and is brought on by treatment with TCEE or AmB. Previous studies have shown that upon treatment with this drug combination, there is a substantial lag between activation of the intrinsic and extrinsic apoptotic pathways.[34−37] Flow cytometry analysis revealed that this drug combination resulted in G2/M arrest and an elevation in the number of cells undergoing apoptosis. The results indicated that the induction of cell cycle arrest and apoptosis are the main mechanisms underlying the antitumor effect of this drug combination.

To gain a better understanding of the mechanisms of the G2/M arrest and apoptosis caused by this drug combination, we proceeded to investigate the levels of key proteins regulating the G2/M phase transition and apoptosis. The cell cycle of eukaryotes is regulated by many proteins, such as cyclin-dependent protein kinases (CDKs), cyclins, and cyclin-dependent protein kinase inhibitors (CDKIs).[38,39] The cell cycle has three main checkpoints, including (notably for this study) the G2/M checkpoint. The main regulator of this checkpoint is the cyclinB1−Cdc2 protein complex, also known as the M phase promoting factor (MPF). The levels of the cyclin B1 and Bcl-2 proteins were decreased in HT29 cells exposed to either AmB or TCEE alone. The combination of the drugs resulted in a more significant change in the protein levels.

In the animal experiment, we chose TCEE oral doses that have not shown significant toxicity in mice. We evaluated the antitumor activity of AmB, TCEE, and the combination of the drugs on the growth of HT29 xenografts in nude mice. The results were similar to those of the in vitro experiments. Pretreatment with TCEE via the oral route followed by AmB iv administration significantly inhibited HT29 tumor growth further verifying the antitumor effects of these two drugs on HT29 cells. In addition to TCEE plus AmB-induced apoptosis, other reported biological effects of this combination, such as to reductions in

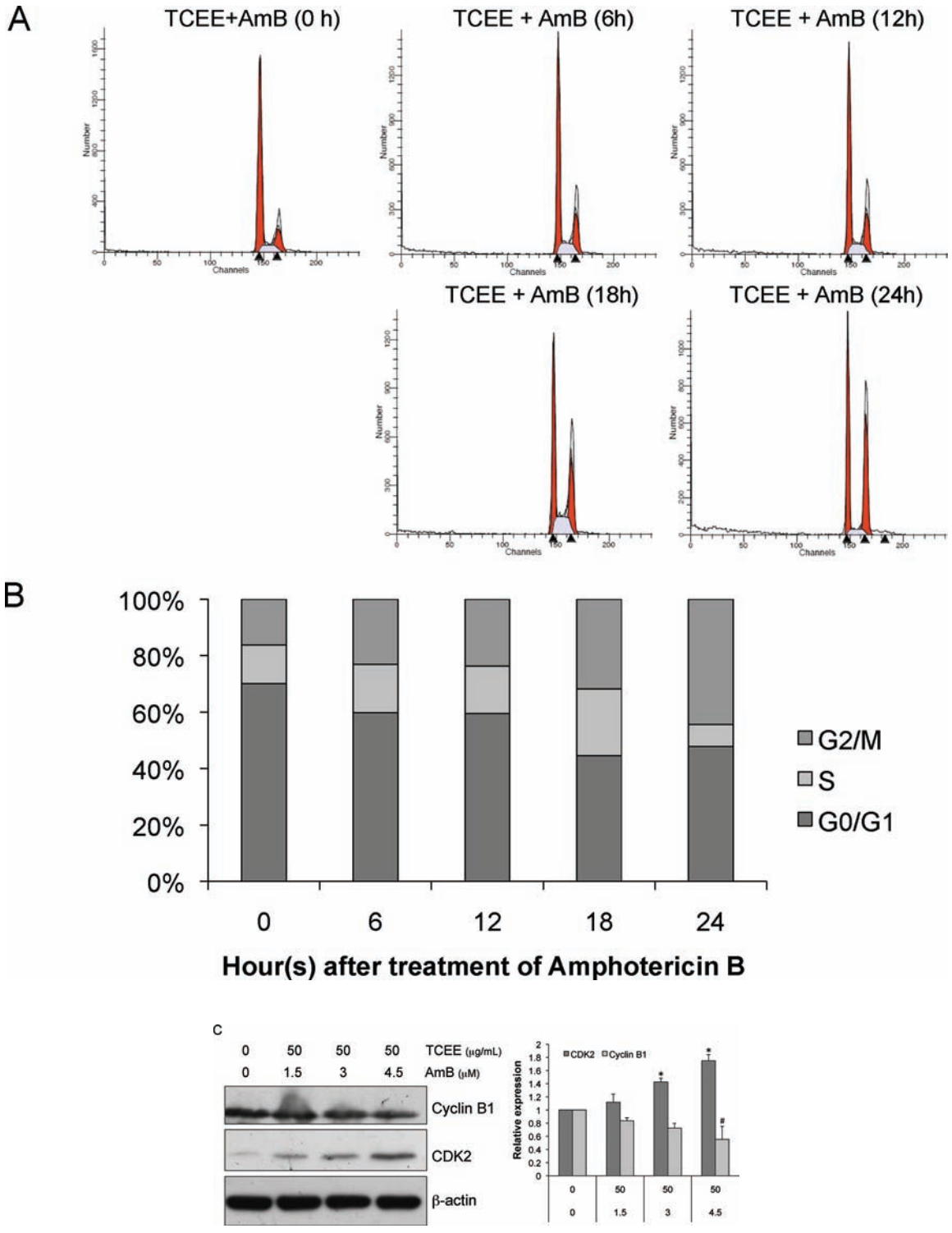

Figure 4. Induction of G2/M arrest in HT29 cells treated with TCEE and AmB. (A and B) HT29 cells were pretreated with 50 μg/mL TCEE for 24 h and followed by 3 μM AmB for the indicated time. The cells were stained with PI and analyzed for DNA content by flow cytometry. (A) The data were analyzed using the ModFit cell cycle analysis software. The profiles are representative histograms of triplicate assays. (B) The percentage of cells in each phase of the cell cycle is shown in the bar graphs. (C) The expression levels of cyclin B1 and CDK2 were detected using Western blotting. Left: Western blot analysis of CDK2 and cyclin B1 expression. Right: Quantification of CDK2 and cyclin B1 expression by densitometric analysis. The data presented are from at least three independent experiments and expressed as the mean ± SD; $^*P < 0.01$ or $\#P < 0.05$ indicates a statistically significant change with respect to the control samples.

PDGF-induced smooth muscle cell (SMC) proliferation and migration,[40] may contribute to its efficacy in this model.

In conclusion, the anticancer effect of the combination of AmB and TCEE is likely due to the induction of cell cycle arrest and

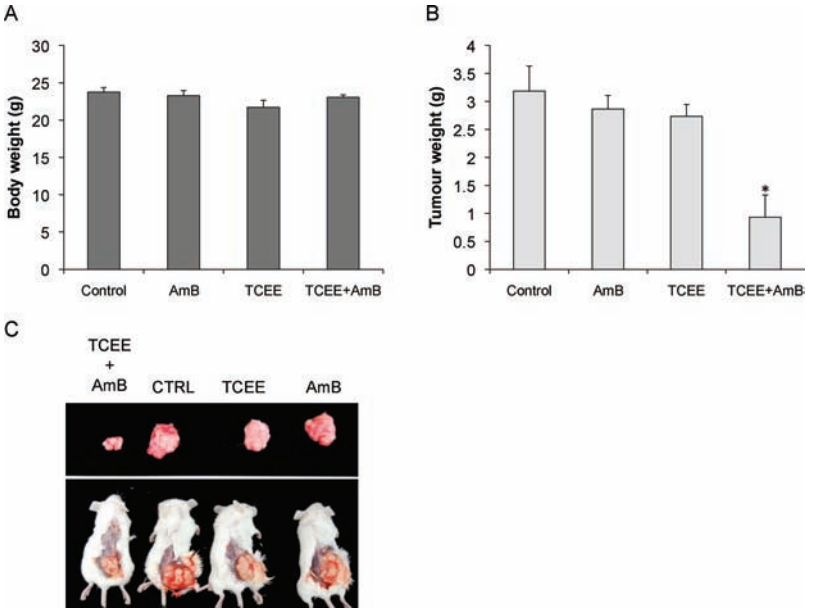

Figure 5. Therapeutic effects of TCEE and AmB in nude mice bearing human HT29 xenografts. After the tumor size reached 200 mm^3, the effects of two different combinations of AmB and TCEE were evaluated, as detailed in the Materials and Methods. Animals were divided into four groups including those given intravenous (iv) injections of AmB (0.1 mg/kg) per day for 4 weeks (AmB group), fed 10 mg/kg TCEE for 4 weeks (TCEE group), fed 10 mg/kg TCEE per day for 2 weeks and then given iv injections of AmB (0.1 mg/kg) per day for 2 weeks (TCEE+AmB group), and given PBS (control). (A) Average body weight. (B) Average tumor weight. (C) Representative subcutaneous tumors. $N = 6$ for all groups. The results are expressed as the mean ± SD; $^*P < 0.01$ indicates a statistically significant change with respect to the control samples.

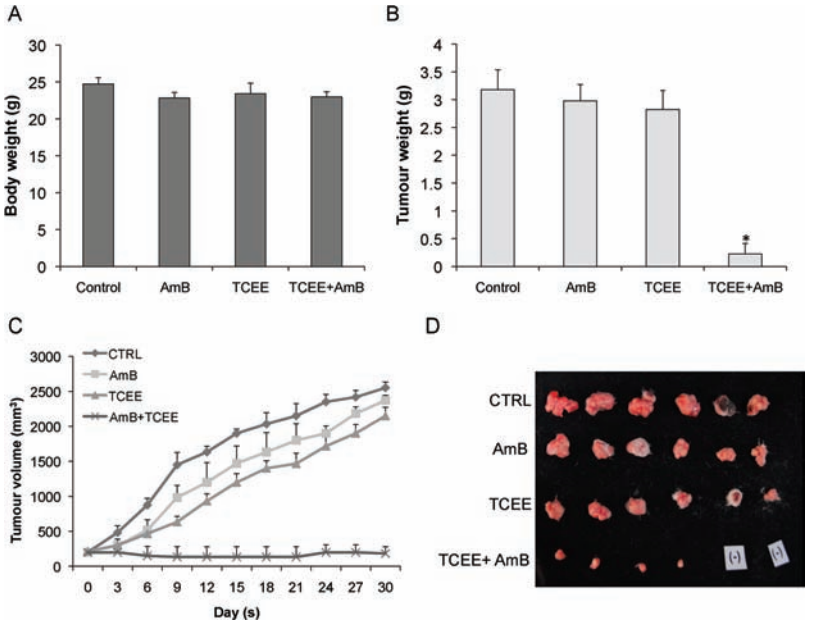

Figure 6. Inhibition of HT29 xenografts by a combination of TCEE and AmB. Animals were divided into four groups including those given intravenous (iv) injections of AmB (0.1 mg/kg) per day for 30 days (AmB group), fed 10 mg/kg TCEE per day for 30 days (TCEE group), fed 0.1 mg/kg TCEE per day for the first 2 days and then given iv injections of AmB (0.1 mg/kg) on the third day for 10 cycles (for the same total period of 30 days) (TCEE + AmB group), and given PBS (control). (A) Average body weight. (B) Average tumor weight. (C) Average tumor size changes. (D) Representative subcutaneous tumors. $N = 6$ for all groups. The results are expressed as the mean ± SD; $^*P < 0.01$ indicates a statistically significant change with respect to the control samples.

apoptosis. The mechanisms through which these effects occur are probably caused by alteration in the levels of certain key cell cycle and apoptotic regulatory proteins. Our results suggest that this drug combination has clinical potential for the treatment of patients suffering from colorectal cancer and may serve as an alternative medicine.

AUTHOR INFORMATION

Corresponding Author

*Tel: +886-2-27361661, ext. 2011. Fax: +886-2-23787795. E-mail: curator@tmu.edu.tw.

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