Terrein induces apoptosis in HeLa human cervical carcinoma cells through p53 and ERK regulation

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Abstract. Terrein, a fungal metabolite derived from *Aspergillus terreus*, has been shown to have a variety of biological activities in human cells including inhibition of melanogenesis, as well as anti-inflammatory, antioxidant and anticancer properties. In the present study, terrein was shown to have marked anticancer activity on HeLa human cervical carcinoma cells. Terrein exhibited inhibition of proliferation within the same ranges for other cancer cell types with an IC_{50} at 0.29 mM. The growth inhibition that induced cell death was via apoptosis mechanisms. Chromatin condensation was observed using the Hoechst 33342 stain, a DNA-specific dye. The increase of DNA fragmentation or the sub-G0 peak was also detected by flow cytometry. The signaling used by terrein to induce apoptosis was via the death-receptor and mitochondrial pathways; the cleavage of specific fluorogenic substrates by caspase-3, -8 and -9 activities are clearly demonstrated. The mitochondria were damaged as demonstrated by the decrease of the red/green ratio of the JC-1 staining and the increase of the *Bax*/*Bcl-2* expression ratio. Further analysis of the upstream signaling by the quantitative real-time polymerase chain reaction showed that *p53*, *p21* and *ERK* were upregulated which indicates the importance of their roles on terrein signaling. This study is the first to show that terrein has an effect on the anticancer properties in cervical cancer cells by inducing apoptosis through p53 and ERK regulation. Our data may help expand the function of the terrein compound and may also aid in the discovery of new anticancer agents.

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Introduction

Human cervical carcinoma is the second most common cancer among women worldwide, with about 500,000 new cases and 250,000 related deaths occurring every year, primarily in developing countries (1). Cervical cancer can be cured by radical surgery or radiotherapy for the patients diagnosed with cervical cancer in the early stages, while chemotherapy or neoadjuvant chemotherapy is the primary option for patients with advanced cervical cancer (2). However, the available chemotherapeutic agents are not completely effective in patients with advanced cervical cancer due to the lower chemosensitivity of the cervical cancer cells. Therefore, effective chemotherapeutic agents are required to improve the 5-year survival rate of these patients (3). Cancer is a disease of uncontrolled cell growth or proliferation and a lack of apoptosis; therefore, any agent that can block the cell proliferation or induce apoptosis in the cancer cells could prove to be a potent anticancer agent. To date, several anticancer drugs (such as, paclitaxel, doxorubicin, etoposide and cisplatin) already in use in the clinical setting have been proven to be apoptosis-inducing agents (4-7). Thus, apoptosis induction is a promising direction in the development of new anticancer agents.

Several sources from plants, marine organisms and microorganisms are used to produce anticancer agents. In microorganisms, it has been shown that both bacteria and fungi are valuable sources of bioactive compounds. However, most anticancer drugs developed from microorganisms currently used in the clinical setting are from bacteria (8). The anticancer properties of metabolites from fungi have yet to be fully elucidated. Terrein ($C_8H_{10}O_3$) is a bioactive, fungal, secondary metabolite which was first isolated from *Aspergillus terreus* in 1935 (9). The chemical structure of terrein contains free hydroxyl groups at positions 4 and 5 of the cyclopentenone ring (Fig. 1) (10,11). Terrein has been reported to have several biological activities. It has been shown that terrein functions as a melanogenesis inhibitor by reducing the tyrosinase production in the spontaneously immortalized mouse melanocyte cell line of Mel-Ab (11,12). In lipopolysaccharide (LPS)-induced inflammation of human

dental pulp cells, terrein has been shown to function as an anti-inflammatory agent (13). In MC3T3-E1 fibroblast cells grown on a titanium surface, biocompatible material, terrein was found to reduce the oxidative stress demonstrating anti-oxidant activity (14). Aside from the activities mentioned, terrein has also been shown to suppress the proliferation of human skin keratinocyte cells (15). Markedly, terrein has been shown to inhibit the growth of several types of cancer cells. In prostate cancer cells, terrein has been reported to work as an angiogenesis inhibitor (16). In lung cancer, terrein has been shown to function as a proteasome inhibitor that promotes cell death by apoptosis (10). Additionally, terrein has suppressive growth effects in ABCG2-expressing breast cancer cells by inducing the apoptosis mechanism (17). Thus, terrein is a promising compound, particularly for its anticancer properties; it may provide a new option in cancer therapeutics. In this study, we further examined the anticancer properties of terrein in cervical cancer cells (HeLa), as well as the signaling induced through ERK, p53 and caspase-3, -8 and -9, which have yet to be reported for terrein function.

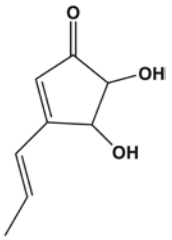

Figure 1. The structure of terrein.

Materials and methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM), Medium 199, fetal bovine serum (FBS), 0.25% Trypsin-EDTA, penicillin-streptomycin, TRIzol Reagent, Taq DNA Polymerase, SuperScript® VILO™ cDNA Synthesis kit and Hoechst 33342 were purchased from Gibco (Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO), RNase and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium citrate, dithiothreitol (DTT) and ethidium bromide were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Material 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from USB Corp., (Cleveland, OH, USA) and agarose from Vivantis (Oceanside CA, USA). The JC-1 mitochondrial membrane potential assay kit was purchased from Biotium, Inc. (Hayward, CA, USA). The caspase colorimetric assay kit was from Calbiochem Merck KGaA (Darmstadt, Germany). Power SyBR® Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA).

Preparation of terrein. Terrein was extracted from the culture broth of fungi *Aspergillus terreus* CRI301. The crude extract was carried out using ethyl acetate as a solvent. The EtOAc extract was concentrated *in vacuo*, and then the crude extract from the broth was fractionated and purified by use of the Sephadex LH-20 (2 cm inner diameter and 125 cm long), using MeOH as an eluent. Spectroscopic analysis was used for the compound characteristics.

Cell culture and maintenance. The human cervical carcinoma cell line (HeLa) was kindly provided by Dr Mathurose Ponglikitmongkol, Department of Biochemistry, Faculty of Science, Mahidol University, Thailand. The immortalized porcine epithelial glandular (PEG) cells were kindly provided by Dr Chatsri Deachapunya, Srinakharinwirot University. The HeLa cells were maintained in DMEM supplemented with 10% FBS and with 1% penicillin-streptomycin. The PEG cells were cultured in DMEM containing 5% FBS, 1% L-glutamine, 1% non-essential amino acid, 0.1% insulin and 1% penicillin-streptomycin. Both specimens were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cytotoxicity assay. The cytotoxicity assay was performed by the MTT method (18). The HeLa and PEG cells at $1x10^4$ cells/100 μl/well were seeded onto a 96-well plate and incubated overnight. The cells were then treated with terrein at 5, 0.5, 0.05, 0.005, 0.0005 and 0.00005 mM for 24 h. An untreated group was combined with 1% DMSO and used as a negative control. Following 24 h of cell treatment, the MTT dye [Thiazolyl Blue Tetrazolium Bromide: (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added at 0.5 mg/ml into each well and incubated for 3 h. The formazan crystal products formed were dissolved by the addition of 100 μl of DMSO. After 15 min, the amount of purple formazan was determined by measuring the optical density (OD) using the ELISA microplate reader at 595 nm. The experiment was performed in triplicate and the percentage of cell viability was calculated as: % Viability = [OD of treated cells/OD of control cells] x 100.

Nuclear morphological observation. The effect of terrein on the nuclear morphological changes was investigated by Hoechst 33342 staining (19). Briefly, the HeLa cells at $4x10^5$ cells/well were seeded onto a 12-well plate and treated with terrein at 0, 0.3, 0.6 and 1.5 mM for 24 h. At the end of the treatment, both the adherent and non-adherent cells were collected. Then, the cells were fixed with 3.7% (vol/vol) paraformaldehyde for 10 min at room temperature, permeated with 0.1% Triton X-100 for another 10 min at room temperature and stained with Hoechst 33342 (1 mg/ml of phosphate-buffered saline; PBS) at 37˚C for 15 min. The nuclear morphology was observed with a fluorescent microscope (Olympus, Tokyo, Japan).

Analysis of apoptotic sub-G0 population. The sub-G0 population was analyzed using flow cytometry as previously described (20). The HeLa cells at $1x10^6$ cells/well were plated on a 6-well plate and treated with terrein at 0, 0.3, 0.6 and 1.5 mM. After 24 h, the treated cells were trypsinized and washed twice with ice-cold PBS. The cell pellet was resuspended in 1 ml PBS and gently fixed (drop by drop) with 4 ml of absolute ethanol at -20˚C for 5-15 min. Following centrifugation, the ethanol was discarded and 5 ml of PBS was added to the cell pellet which was then allowed to rehydrate for 15 min. Subsequently, each sample was incubated with 500 μl of 100 μg/ml RNase for 20 min at 37˚C. After washing with PBS, the cell pellet was gently resuspended in 500 μl of PI solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton

Table I. Oligonucleotides used in real-time PCR.

Gene	Forward primer	Reverse primer
p53	5'-ACTAAGCGAGCACTGCCCAA-3'	5'-ATGGCGGGAGGTAGACTGAC-3'
p21	5'-TATGGGGCTGGGAGTAGTTG-3'	5'-AGCCGAGAGAAAACAGTCCA-3'
Bax	5'-GCGTCCACCAAGAAGCTGAG-3'	5'-ACCACCCTGGTCTTGGATCC-3'
Bcl-2	5'-TGTGGCCTTCTTTGAGTTCG-3'	5'-TCACTTGTGGCCCAGATAGG-3'
ERK2	5'-GCCTGGCCCGTGTTGCAGAT-3'	5'-CGCCCCTCCAAACGGCTCAA-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTCA-3'	5'-GACAAGCTTCCCGTTCTCAG-3'

X-100) at 4˚C in a darkened environment overnight. Each sample was measured using flow cytometry (BD FACSCanto, Becton-Dickinson, Lincoln Park, NJ, USA) using the Consort 30 program (Becton-Dickinson).

Caspase activity assay. Caspase-3, -8 and -9 activities were measured using fluorescent assay kit detection (Calbiochem Merck KGaA), according to the manufacturer's instructions. Briefly, HeLa cells at $1x10^6$ cells/well were placed on a 6-well plate, treated with terrein at 0, 0.3, 0.6 and 1.5 mM for 12 h. After the treatment, supernatants from cell lysates were incubated with fluorogenic substrates using DEVD-AFC (caspase-3-like), IETD-AFC (caspase-8-like) and LEHD-AFC (caspase-9-like), at 37˚C for 2 h prior to monitoring with a fluorescent microplate reader with excitation set at 400 nm, and emissions at 505 nm.

Analysis of mitochondrial transmembrane potential. The changes in mitochondrial membrane potential ($\Delta\Psi m$) were detected using a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1) dye (Biotium Inc.). In healthy cells, the JC-1 accumulates in the mitochondria as JC-1 aggregates (fluorescence is red) and also in the cytoplasm as JC-1 monomers (fluorescence is green). In early apoptosis, the $\Delta\Psi m$ collapses, making JC-1 aggregates unable to accumulate within the mitochondria and dissipate into the JC-1 monomers leading to a loss of the red fluorescence. Therefore, collapse of the $\Delta\Psi m$ is exhibited by a decrease in the ratio of red to green fluorescence (21). In accordance with the terrein treatment, HeLa cells at $1x10^4$ cells/well were placed on a 96-well plate, treated with terrein at 0, 0.3, 0.6 and 1.5 mM for 6 h. Following treatment, the cells were harvested and incubated with a JC-1 reagent solution at 37˚C for 20 min, then washed twice with PBS and suspended once more in the PBS. The samples were analyzed by a fluorescence microplate reader and measured with both the red fluorescence (excitation 550 nm, emission 600 nm) and the green fluorescence (excitation 485 nm, emission 535 nm). The ratio of red fluorescence intensity vs. green fluorescence intensity was calculated and presented as the means ± SD. This experiment was performed in triplicate.

Real-time polymerase chain reaction (real-time PCR). To analyze the effect of terrein on the expression of apoptosis-related genes (*p53*, *Bax*, *Bcl-2*, *p21* and *ERK2*), real-time PCR was used. HeLa cells at $1x10^6$ cells/well were plated on a 6-well plate, treated with terrein at 0, 0.3, 0.6 and 1.5 mM for 24 h. Following treatment, the cells were harvested and washed with 500 μl of PBS. The total RNA was extracted using a TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and quantified by use of OD measurement at 260 and 280 nm using a spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) (all RNA samples had an A260/A280 ratio >1.8).

The isolated total RNA (2.5 μg) was reverse-transcribed to cDNA with the SuperScript VILO cDNA Synthesis kit (Invitrogen). The reaction mixture was composed of 4 μl of 5X VILO reaction mix, 2 μl of 10X SuperScript enzyme mixture, and DEPC-treated water in a total volume of 25 μl. The reaction mixture was incubated at 25˚C for 10 min, at 42˚C for 60 min and the reaction was terminated by heating at 85˚C for 5 min. The resultant cDNA was stored at -20˚C until further use. The PCR primers were obtained from BioDesign Co., Ltd., Pathumthani, Thailand. PCR primers were designed by Primer 3.0 and BLAST search to check the specificity. The primer sequences used are listed in Table I.

Real-time quantitative-PCR was performed on an ABI StepOnePlus (Applied Biosystems), using 96-well microtiter plates. The reaction was carried out in a total volume of 20 μl, containing 2.5 μl of the cDNA sample (equivalent to 75 ng), 1 μl of 0.5 μM each of the primer and 10 μl of SYBR-Green Reaction Mix (Applied Biosystems). PCR amplification was performed in duplicated wells. The cycling conditions were: 10 min polymerase activation at 95˚C and 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec. In addition, the real-time reaction of the products was examined by analyzing the melting point after each reaction. A sample without cDNA was used as a negative control and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The baseline and the threshold were set automatically by the software. The crossing point of the amplification curve with the threshold represents the cycle threshold (Ct). The fluorescence threshold Ct values were calculated, and the ΔCt values were determined using the formula $\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$. The $\Delta\Delta$Ct values were then calculated based on the formula $\Delta\Delta Ct = \Delta Ct$ treated - ΔCt untreated. The expression level of the target gene in the treated cells was measured relative to the level observed in the untreated cells and was quantified using the formula $2^{-\Delta\Delta CT}$ (22). The PCR products were electrophoresed on a 2% agarose gel and stained by ethidium bromide under UV light.

Statistical analysis. The results of each experiment were expressed as the means ± standard deviation (SD, for each group n=3). The data were processed with the GraphPad Prism

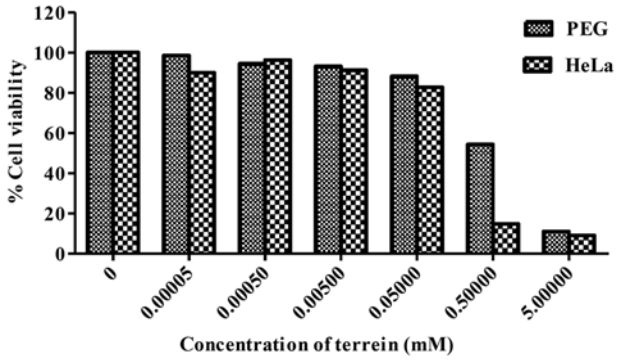

Figure 2. The effect of terrein on cell viability as determined by the MTT assay. HeLa and PEG cells were treated with various concentrations of terrein (mM) for 24 h. Control cell cultures were exposed to the vehicle, 1% DMSO. Data are expressed as the means ± SD, n=3.

5 software. Statistical significance was assessed by one-way ANOVA analysis of variance to evaluate the significance of differences between the groups.

Results

Effect of terrein on cell viability. Terrein was tested for its cytotoxicity against human cervical cancer cells (HeLa) and normal cells (PEG) by the MTT assay. As shown in Fig. 2, terrein significantly inhibited the growth of PEG and HeLa cells in relation to the concentration used. The IC_{50} values were at 0.53 mM for PEG and 0.29 mM for HeLa. It is noteworthy that the percentage of cell viability comparing the cancer and the normal cells differed significantly when using terrein at a concentration of 0.5 mM which was approximately 18 and 60%, respectively. The results indicate a considerable potential of the cytotoxicity effect on human cervical cancer HeLa cells with lower toxicity on normal PEG cells.

Terrein induces apoptosis in HeLa cells. To evaluate the mode of cell death induced by terrein in HeLa cells, the experiment was carried out by staining cells with the DNA specific dye, Hoechst 33342. The cell samples were compared between the terrein-treated cells and the untreated control HeLa cells. The concentrations of terrein were 0, 0.3, 0.6 and 1.5 mM and were treated for 24 h. As depicted in Fig. 3, the untreated control cells displayed normal, round nuclei (Fig. 3a), while the cells treated with terrein exhibited characteristics of apoptosis, such as cell shrinkage, nuclear condensation and fragmentation in a dose-dependent manner (Fig. 3b-d).

As the apoptotic cells with fragmented nuclei appear as cells with hypodiploid DNA content and could be detected at sub-G0 peak with flow cytometry, the numbers of the apoptotic sub-G0 population were quantified. The result demonstrated that the terrein-treated HeLa samples had significantly increased in the sub-G0 phase as compared to the untreated sample (Fig. 4Aa-d). The statistics of each phase of the cell cycle showed that the sub-G0 populations increased as the doses of terrein increased from 11.90 to 26.37 and 84.93%. (Fig. 4B). These results suggest that apoptosis is the mode of cell death used by terrein against HeLa cells.

Induction of apoptotic signaling is triggered by the death receptor and the mitochondrial pathway. Apoptosis is triggered by sequential activation of caspases, a group of cysteine proteases, and proceeds primarily through two pathways. The extrinsic or death receptor pathway involves activation of caspase-8 and is initiated by ligand interaction with death receptors. Second, the intrinsic or mitochondrial pathway is activated by an imbalance between pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family at the mitochondria and cytosol, resulting in the release of cytochrome *c* from the mitochondria, which in turn activates caspase-9. Both caspase-8 and caspase-9 activate caspase-3 which acts as a common downstream part of the two major apoptosis path-

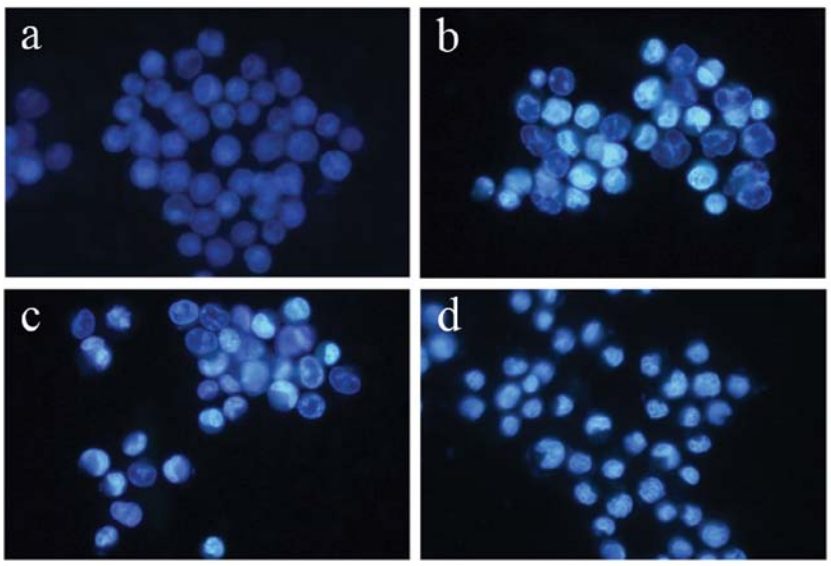

Figure 3. The changes of the nuclear morphology of cervical cancer cells upon treatment with terrein. HeLa cells treated with terrein at (a) 0, (b) 0.3, (c) 0.6 and (d) 1.5 mM for 24 h were fixed and stained with the DNA binding dye Hoechst 33342. Condensed and fragmented nuclei were observed under a fluorescent microscope. Condensed and fragmented nuclei were observed under a fluorescent microscope (x400).

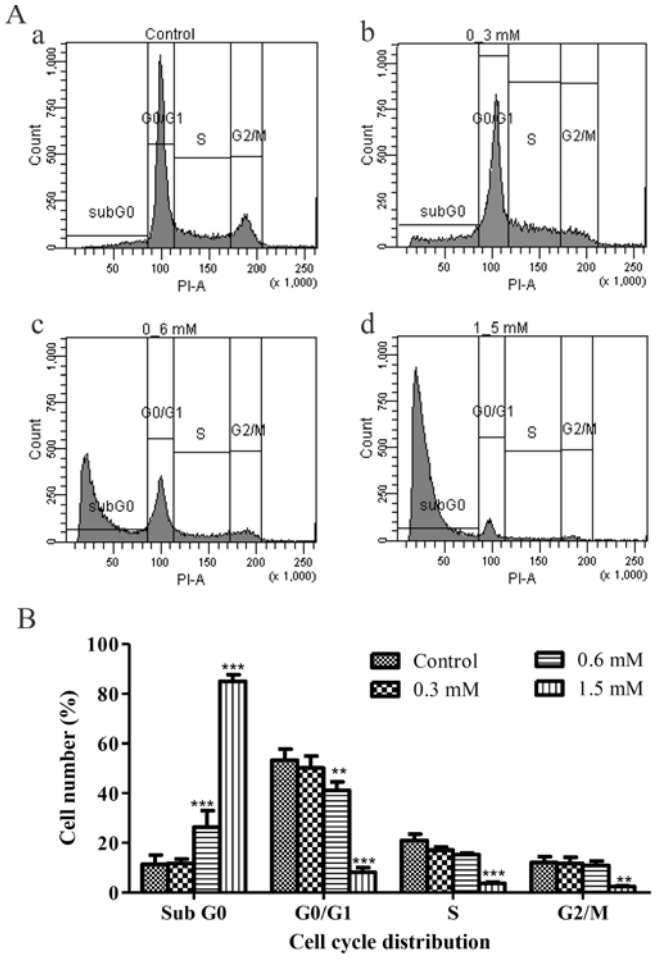

Figure 4. The effect of terrein on the cell cycle distribution of cervical cancer cells upon terrein treatment. (A) HeLa cells were treated with terrein at (a) 0, (b) 0.3, (c) 0.6 and (d) 1.5 mM for 24 h and analyzed by flow cytometry. Histograms show the number of cells per channel (vertical axis) vs. the DNA content (horizontal axis). The data shown are representative of three independent experiments with similar findings. (B) The values indicate the percentage of cells at the indicated phases of the cell cycle. The significant differences of the treated cells from the untreated control group are indicated by $^{**}P<0.01$, $^{***}P<0.001$.

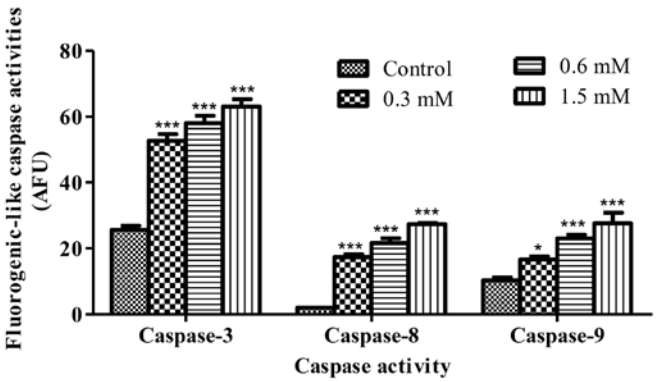

Figure 5. The activation of cellular caspase-3, -8 and -9 in cervical cancer cells after terrein treatment. Lysate from HeLa cells treated with terrein (0, 0.3, 0.6 and 1.5 mM) for 12 h were used to measure the catalytic activity of caspase by using fluorogenic substrates. The data shown are representative of three independent experiments. The significant differences from control are indicated by $^{*}P<0.05$, $^{***}P<0.001$.

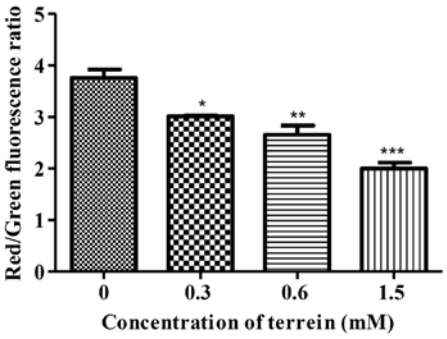

Figure 6. The dissipation of mitochondrial membrane potential of cervical cancer cells upon treatment with terrein. HeLa cells treated with different concentrations of terrein (0, 0.3, 0.6 and 1.5 mM) for 6 h were stained with JC-1 and the average ratio of red/green fluorescence intensity was analyzed by flow cytometry. The data shown are representative of three independent experiments. The significant differences from control are indicated by $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

ways resulting in apoptosis (23). To address the apoptotic pathway in the terrein-treated HeLa cells, measuring of the fluorogenic substrate cleavage was performed. The result of the fluorescence intensity showed that terrein significantly activated caspase-8, caspase-9 and caspase-3 function after 12 h of treatment. Caspase activity increased significantly when compared to the control group of untreated cells in a concentration-dependent manner (Fig. 5). In addition, the activity of each caspase was inhibited by their specific inhibitor provided by the kit (data not shown). These results suggest that terrein activates the signaling of both the death receptor and mitochondrial pathways.

To confirm the cascade, the damage to the mitochondria was analyzed using a specific dye for mitochondrial, JC-1, staining. Upon quantification by flow cytometry, the HeLa cells treated with terrein at 6 h presented with decreasing $\Delta\Psi m$ as compared to the control group of untreated cells in a dose-dependent manner (Fig. 6). Then, we investigated the expression of Bcl-2 family proteins and whether they were involved with the damage to the mitochondria. The expression of *Bax* (pro-apoptotic) and *Bcl-2* (anti-apoptotic) was selected for investigation. As a result, terrein increased the expression of *Bax* (Fig. 7a) and decreased the expression of *Bcl-2* (Fig. 7b) in a dose-dependent manner by real-time PCR. An increase in the *Bax*/*Bcl-2* ratio (Fig. 7c) indicates that upregulation of these Bcl-2 family proteins are upstream events causing damage to the mitochondria.

Apoptotic signaling is mediated by p53 and ERK activation. The tumor suppressor gene, *p53*, is known to be responsible for the inhibition of cell growth and/or the commitment to apoptosis. Meanwhile, p53 protein regulates the expression of the downstream effector p21, a potent inhibitor of cell cycle kinases, in which both are in response for DNA damage. In addition, p53 regulates apoptosis via upregulation of the expression of *Bax* and blocks the function of Bcl-2. Thus, it is possible that a substantial increase in the *Bax*/*Bcl-2* ratio may have resulted from p53 function. The level of the expression of *p53* and *p21* were then examined. As shown in Fig. 8a and b, the expression of mRNA from both *p53* and *p21* was upregulated suggesting

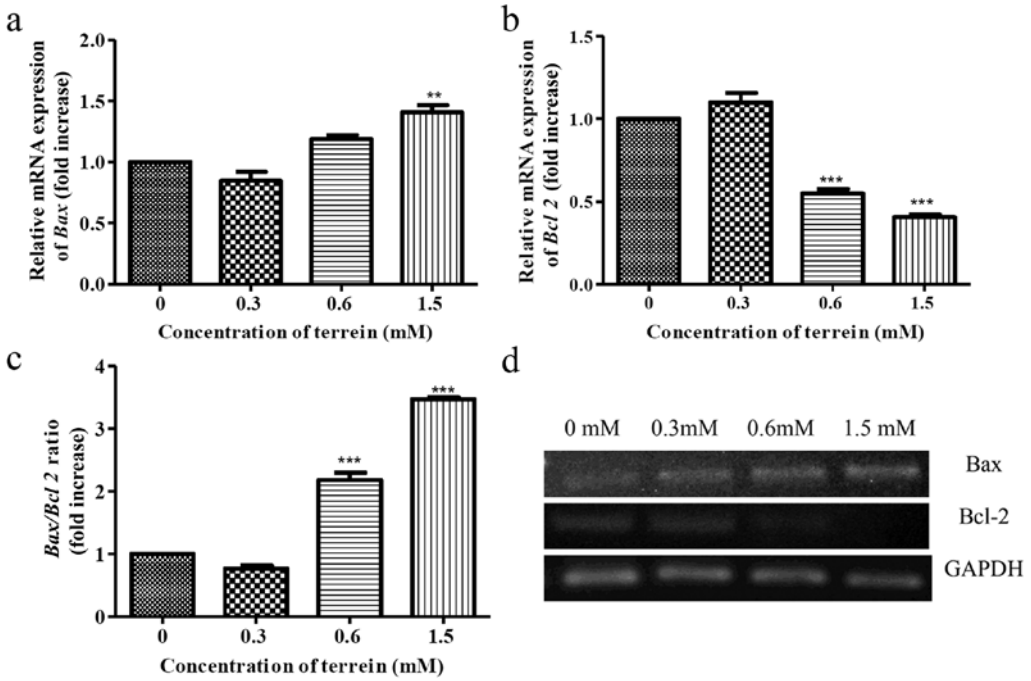

Figure 7. Dose-dependent effects of terrein on *Bcl2* and *Bax* mRNA expression levels in HeLa cells, treated with different concentrations of terrein (0, 0.3, 0.6 and 1.5 mM) for 24 h. mRNA expression levels were determined by quantitative real-time PCR. GAPDH mRNA was used as an internal control. (a) The relative gene expression level of *Bax*. (b) The relative gene expression level of *Bcl-2*. (c) Bar charts show the ratio of *Bax/Bcl-2*, analyzed from the quantitative real time-PCR results. (d) PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The data shown are representative of three independent experiments. The significant differences from control are indicated by $^{**}P<0.01$, $^{***}P<0.001$.

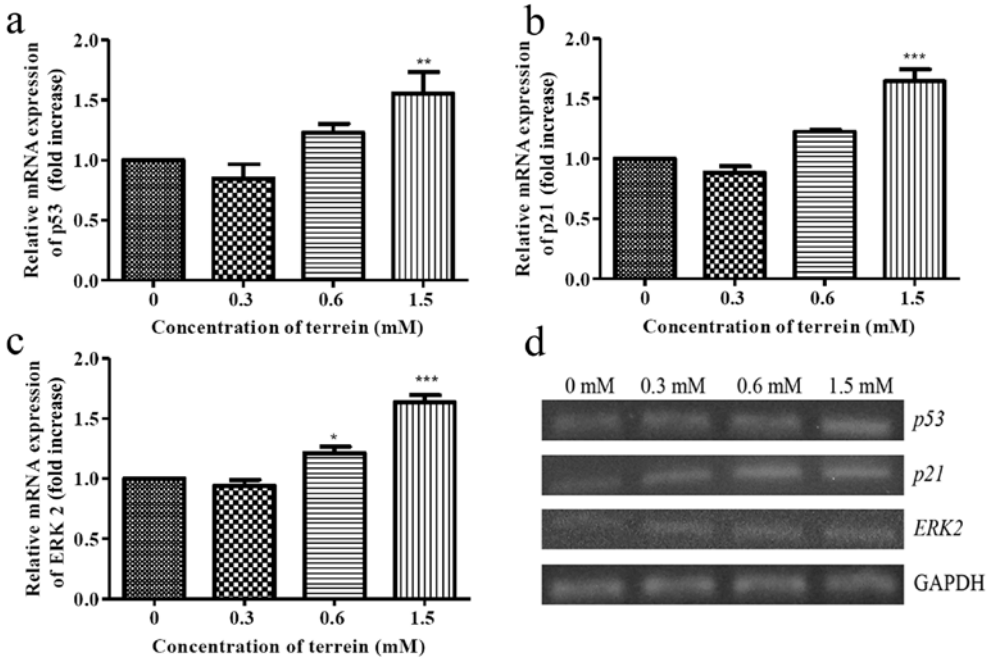

Figure 8. Dose-dependent effects of terrein on *p53*, *p21* and *ERK2* mRNA expression levels in HeLa cells, treated with different concentrations of terrein (0, 0.3, 0.6 and 1.5 mM) for 24 h. mRNA expression levels were determined by quantitative real-time PCR. GAPDH mRNA was used as an internal control. (a) The relative gene expression level of *p53*. (b) The relative gene expression level of *p21*. (c) The relative gene expression level of *ERK2*. (d) PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The significant differences from control are indicated by $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

that the upstream signaling of the mitochondrial pathway was induced by terrein. To investigate this signaling triggered by terrein, further upstream mediators of p53 were evaluated. As is well known, several protein kinases may function to activate p53 and ERK2 may be the kinase that is responsible for the DNA damage. Therefore, we selected ERK2 to study the level of expression in response to the terrein treatment. As depicted in Fig. 8c, the mRNA expression of *ERK2* increased following terrein treatment in a dose-dependent manner indicating the involvement of ERK signaling.

Discussion

The present study is the first to demonstrate that terrein, a fungal metabolite, induces apoptosis in cervical cancer cells via p53 and ERK signaling. As previously shown, terrein has a variety of effects including anti-inflammatory (13), anti-oxidant (14), anti-proliferative (15) and skin-whitening properties (11-12). The effects of terrein on cancer cells have also been reported. In androgen-dependent prostate cancer cells (LNCaP-CR), terrein demonstrates angiogenesis inhibition by blocking the secretion of angiogenin with an IC_{50} of 13 μM (16). In human lung tumoral cell lines (NCI-H292), terrein acts as proteasome inhibitor by suppressing the chymotrypsin- and trypsin-like activities with the IC_{50} of 0.3 mM. Also, in these lung tumor cells, terrein was able to induce apoptotic cell death at concentrations of 0.15 mM and 0.3 mM (10). In breast cancer cells (MCF-7), terrein markedly inhibited cell proliferation in IC_{50} of 1.1 nM (17). Meanwhile, for normal cells, it has been shown that terrein has of non-cytotoxic effects in human keratinocyte at the concentration of 1-50 μM (15). Comparing these data, our study found that the IC_{50} for cervical cancer cells was at 0.29 mM, while in normal porcine epithelial glandular (PEG) cells it was at 0.53 mM (Fig. 2). These data suggest that the dose of terrein to induce cancer cell death is cell type-dependent. The concentration appears to be high (mM range) but this effective dose has almost the same value exhibited in lung tumor cells. The inhibition concentration at 50% of terrein treatment in HeLa cells did not differ significantly from normal PEG cells. This indicates that terrein also exhibits cytotoxic action on normal cells. However, at approximately 0.5 mM of terrein, the percentage of cell viability of HeLa cells was approximately 18%, while in the normal PEG cells it was approximately 60% which was represented by the difference in the sensitivity.

The evaluation of the mechanism used by terrein to trigger cervical cancer cell death is implicated via apoptosis. As shown in Figs. 2-5, chromatin condensation, DNA fragmentation and caspase activation were clearly demonstrated. These are distinct characteristics of the apoptosis mechanism (24). To develop an anticancer agent, apoptosis is the preferable mechanism as it does not trigger the inflammation process observed in necrosis, another form of cell death. As previously shown, several anticancer drugs use apoptosis as their target mechanism, therefore, terrein is another promising compound for development as an anticancer agent (4-7).

The pathway to induce apoptosis is initiated by two major pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway integrates extracellular signals through the binding of external ligands to death receptors located at the plasma membrane such as the Fas/FasL interaction. Engagement of these receptors by their specific ligand induces their trimerization and leads to the assemblage of the death-inducing signaling complex (DISC). In this complex, procaspase-8 is activated and in turn cleaves and activates executioner caspases including caspase-3, caspase-6 or caspase-7 (25,26). The intrinsic pathway is triggered by the activation of the pro-apoptotic Bcl-2 family proteins known as Bax or Bak. These proteins have shown the ability to form pores in the mitochondrial outer membranes, thereby allowing permeabilization of cytochrome *c* release to cytosol. Cytochrome *c* binds to the adaptor apoptotic protease activating factor-1 (Apaf-1) forming a large multi-protein structure known as the apoptosome. The apoptosome then recruits and activates procaspase-9 into the active form which further activates the downstream effector caspases for the death receptor pathway, finally resulting in cell death (27,28).

However, several reports have shown that the cascade of the extrinsic and intrinsic pathway is not fully separated in some cases. Caspase-8 can initiate death via caspase-3 directly or it can trigger the mitochondrial pathway via Bid cleavage (25). Cells that perform directly to the cascade from caspase-8 to caspase-3 are called type I cells, while the cells relative to the cascade initiated from caspase-8 and that have death enhancement via the mitochondrial pathway are called type II cells (29). As shown in this study, terrein activates both caspase-8 and caspase-9 (Fig. 5). Also, the changes in the ratio of *Bax*/*Bcl-2* expression and the dissipation of the $\Delta\Psi m$ were detected (Figs. 6 and 7). These data suggested that terrein-induced apoptosis in cervical cancer cells may display as type II signaling, which is consistent with the reports that HeLa cells triggered by apoptosis-inducing agents usually perform as type II cells (30,31).

p53 plays an important role in several cellular processes. It controls the cell cycle, cell senescence and cell apoptosis. To regulate the apoptosis mechanism, p53 mediates the expression of several proteins that are involved in the release of cytochrome *c* from the mitochondria, and Bax, Noxa, Puma, AIP1 and APAF1 are also included (32). We also demonstrated that *Bax* is upregulated upon treatment with terrein, and this may be due to the function of the transcriptional activation by p53. As shown by our results, the level of *p53* expression increased upon treatment with terrein (Fig. 8a). In addition, the level of *p21*, the cyclin-dependent kinase 2 inhibitor that is transcriptionally activated by p53, was also upregulated (Fig. 8b). These data support the critical role of p53 in terrein-mediated cervical cancer cell death which correlates with previous studies of bioactive agents, such as capsaicin (33), eurycomanone (34), flavonoid quercetin (35), kaempferol-7-*O*-β-D-glucoside (36) and cisplatin (37).

Our study also analyzed the role of extracellular signal-regulated kinase (ERK) signaling and whether or not it is involved in terrein-induced apoptotic cell death. As previously described, ERK2 is involved in cell death by interaction with phosphorylated p53 (38). Thus, we determined the level of *ERK2* expression in response to terrein treatment. As depicted in Fig. 8c, the level of the expression of *ERK2* increased in a dose-dependent manner. These data suggest that ERK may act upstream of p53 and that consequently leads to cell death by apoptosis. In addition, it has been reported in HeLa cells that ERK activation is associated with the upregulation of p53 expression upon treatment with shikonin (39) and H_2O_2 (40). Otherwise, it is assumed that ERK may act upon the activation of caspase-8. As it has been shown, the prolonged activation of ERK1/2 induces FADD-independent caspase-8 activation and cell death (41,42). As is demonstrated by our study, the upregulation of *ERK2* is possibly an important mediator that activates p53, caspase-8 and caspase-9, leading to the destruction of the cancer cells.

In conclusion, our study demonstrated that terrein is a potential candidate as an anticancer agent as it was shown to induce cytotoxicity and apoptosis in cervical cancer cells. The apoptosis pathway may be type II signaling which mediates through ERK signaling. ERK acts as a mediator to regulate the activation of both caspase-8 and p53. The downstream effect of the p53, particularly Bax, was upregulated and significantly leads to the dissipation of the $\Delta\Psi m$. Consequently, caspase-9 and caspase-3 are activated finally initiating the cleavage of all cellular substrates and genetic materials.

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