A Bibenzyl from *Dendrobium ellipsophyllum* Inhibits Epithelial-to-Mesenchymal Transition and Sensitizes Lung Cancer Cells to Anoikis

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**Abstract.** Background: Anti-metastasis therapy may become the potential means of improving survival of cancer patients. As the ability of cancer cells to change phenotype from epithelial to mesenchymal has been recognized as an important hallmark of cancer metastasis, this study provides information regarding the effect of a bibenzyl, namely 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB), isolated from *Dendrobium ellipsophyllum*, in inhibiting epithelial-to-mesenchymal transition (EMT) and sensitization of lung cancer cells to anoikis. Materials and Methods: Human lung cancer H292 cells were treated with non-cytotoxic doses of TDB for 24 h prior to evaluation of anoikis and anchorage-independent growth. The proteins relevant to EMT and anoikis resistance were examined in TDB-treated H292 cells via western blot analysis. Results: A significant increase in apoptosis induced by cell detachment was found in TDB-treated H292 cells. The formation of tumor in anchorage-independent growth assay was found to be dramatically reduced in response to the compound. Furthermore, western blot analysis of proteins involved in EMT revealed that treatment with TDB resulted in the increase of E-cadherin and the decrease of vimentin and transcription factor SNAIL, indicating EMT suppression. Concomitantly with EMT inhibition, the activity of pro-survival pathways, including activated protein kinase B (pAKT) and activated extracellular signal-regulated kinase (pERK), were found to be significantly reduced. Conclusion: Because EMT, anoikis resistance and anchorage-independent growth are among important factors facilitating cancer metastasis, TDB shows potential to be developed as an anti-metastasis agent.

The process of epithelial–mesenchymal transition (EMT) was recently recognized as an important factor in the facilitation of metastasis (1, 2). Among various types of cancer, lung cancer has become one of the most life-threatening diseases because it has a very high mortality rate (3). Worldwide information shows that patients with lung cancer are frequently found with the signs of metastasis at the time of diagnosis (4-6). Like other types of cancer, metastasis of cancer cells results in difficulty in treatment, as well as relapse of the disease (7).

Indeed, the process of metastasis involves the ability of cancer cells to migrate away from primary tumors and invade in blood circulation (8). Importantly, *in vivo* and *in vitro* studies have demonstrated that the capability of the cells to resist death after detachment or anoikis is critical for successful metastasis (9-11). Anoikis is one type of apoptotic cell death triggered by the loss of contact with the extracellular matrix (12, 13). Even though the mechanisms of cancer in resisting anoikis are still largely unknown, up-to-date data point out that cancer cells escape from anoikis by several ways, including up-regulation of survival signals including protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) (14, 15). In addition, the mesenchymal phenotype of cancer cells induced by the EMT has been implicated in cancer cell metastasis by increasing anoikis resistance, as well as enhancing growth of secondary tumors (16-18).

In line with our work in identifying possible candidate compounds for use for anti-metastasis approaches, compounds isolated from the plants in the genus *Dendrobium* have garnered much scientific attention. Several compounds isolated from *Dendrobium* plants, including chrysotobilbenzyl, chrysotoxine, crepidatin, and moscatilin, have been shown to possess anti-metastasis activity (19, 20).
The present study aimed to investigate the potential activity of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB), isolated from *D. ellipsophyllum*, on the EMT of human lung cancer cells. This work also investigated the consequent effects of TDB-mediated EMT inhibition on anoikis sensitivity and anchorage-independent growth. The findings gained from this study may benefit the development of this compound for anti-metastasis therapy.

**Materials and Methods**

**Cell culture.** Human H292 lung cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml penicillin/ streptomycin (Gibco, Gaithersburg, MA, USA). Cells were maintained in a humidified incubator containing 5% CO2 at 37°C.

**Chemical reagents.** TDB with approximately 98% w/w was isolated from *D. ellipsophyllum*. Botanical identification was performed in comparison with herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment. A voucher specimen (BS-DE-052555) is on deposit at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Briefly, the whole plant of *D. ellipsophyllum* was dried and ground into powder. This dry powder (4.8 kg) was then extracted with methanol (3 x 10 l) at room temperature. The methanolic filtrate was filtered and evaporated under reduced pressure to give a viscous mass (400 g). This material (200 g) was subjected to vacuum–liquid chromatography (VLC) (Kieselgel 60, 70-320 mesh; Merck; Darmstadt, Germany) on silica gel (n-hexane–ethyl acetate gradient to give five fractions (A-E). Fraction D (63 g) was separated by VLC over silica gel, eluted with n-hexane–ethyl acetate gradient to give seven fractions (D1-D7). Fraction D5 (5.4 g) was subjected to medium pressure liquid chromatography (MPLC) over silica gel, eluted with n-hexane–ethyl acetate gradient to give 14 fractions (D5A-D5N). Fraction D5G (954.3 mg) was separated by column chromatography (Kieselgel 60, 230–400 mesh, Merck; Darmstadt, Germany) over silica gel (n-hexane–ethyl acetate, gradient), and further purified on Sephadex LH20 (25-100 μm; Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden) with acetone to obtain TDB. For structural confirmation, the compound was analyzed through mass spectrophotometer and nuclear magnetic resonance spectroscopy (NMR) as described. Mass spectra were recorded on a UPLC mass spectrometry (Waters 2996-2695, ESI-MS). NMR spectra were recorded on a Bruker Avance DPX-300 FT-NMR spectrometer or a Varian Unity INOVA-500 NMR spectrometer. Trypsin, Hoechst33342, 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethylsulfoxide (DMSO) and agarose were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Antibody to E-cadherin, vimentin, transcription factor SLUG, transcription factor SNAIL, ERK, pERK (Thr202/Thr204), AKT, pAKT (Ser 473), β-actin and specific horseradish peroxidase (HRP)-link secondary antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**Cell viability assay.** H292 lung cancer cells were treated with TDB at various concentrations (0.5-20 μM) for 24 h or left untreated as a control. After indicated time, cell culture medium was replaced by 100 μl/well of MTT solution (0.4 mg/ml) and left in the dark at 37°C for 4 h. After that, MTT solution was removed and 100 μl/well of DMSO was added to dissolve the formazan crystals. The intensity of formazan color was measured at 570 nm using microplate reader (Anthros, Durham, NC, USA). All analyses were performed in at least three independent replicate cultures. The cell viability was calculated from optical density (OD) ratio of treated to non-treated control cells and presented as a percentage to that of non-treated controls.

**Anoikis assay.** H292 cells that had been treated with non-toxic concentrations of TDB for 24 h were detached. A single-cell suspension in culture medium was seeded into an ultra-low attachment plate (Corning, Acton, MA, USA) at a density of 1.5x10⁵ cells/ml. The cells were harvested and incubated with 20 μM of XTT for detection of cell viability at 0, 6, 12, and 24 h. After light protection at 37°C for 4 h, the intensity of the formazan product from XTT was measured at 450 nm using a microplate reader.

**Nuclear staining assay.** Apoptotic and necrotic cell death was determined by the Hoechst33342/PI co-staining assay. Treated cells were incubated with 10 μM of the Hoechst33342 dye and 5 μg/ml of the PI dye for 30 min at 37°C in the dark. Modes of cell death were visualized under a fluorescence microscope (Olympus IX51 with DP70). Blue fluorescent Hoechst33342 was detected as apoptotic cells, while the red fluorescence of PI represented necrotic cells.

**Anchorage-independent growth assay.** Anchorage-independent cell growth was carried out in two-layer soft agar. Briefly, 1% agarose was melted and kept at 55°C in a water-bath. The bottom layer was a mixture of 1% agarose and RPMI culture medium at a ratio of 1:1. After adding 500 μl/well of the mixture into a 24 well-plate, the agar was solidified at 4°C for 5 min. The upper layer was prepared from 1% agarose and a single-cell suspension of TDB-treated H292 cells to give the final concentration of 0.33% agarose and provide 1,500 cells/well (250 μl). The upper layer was allowed to set in an incubator at 37°C for 4 h, then the culture medium was added on top. Fresh complete RPMI medium (250 μl/well) was added every three days. After two weeks, colonies were photographed in order to count and measure colony number and size, respectively.

**Western blot analysis.** After treatment with TDB at non-toxic concentrations for 24 h or left untreated as a control. H292 cells were harvested and lysed on ice for 60 min. The protein content of cell lysate was determined using BSA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein of each sample was separated by size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. The membrane was blocked in 5% skim milk in TBST (25 mmol/l Tris-HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature. Then the membrane was probed with primary antibody of E-cadherin, vimentin, SLUG, SNAIL, ERK, pERK (Thr202/Thr204), AKT, pAKT (Ser 473) and β-actin (Cell Signaling, Danvers, MA, USA) at 4°C overnight and washed three times with TBST for 8 min. After that, the membrane was incubated with HRP-conjugated secondary antibody (Cell Signaling, Danvers, MA, USA) for 2 h at room temperature. The
signal of immunoreactive proteins was detected by enhanced chemiluminescence (Supersignal West Pico, Pierce). The quantitative analysis was performed using analyst/PC densitometric software (Bio-Rad Laboratory, Hercules, CA, USA).

Statistical analysis. Mean data from at least three independent experiments were normalized to the non-treated controls. Statistical analysis was performed using one-way ANOVA. A p-value of less than 0.05 was considered as statistically significant.

Results

Effect of TDB on viability and proliferation of human H292 lung cancer cells. To investigate the possible anti-metastasis activity of TDB, its effect on cell viability of lung cancer cells was firstly clarified. H292 cells were incubated with different concentrations of TDB (0-20 μM) for 24 h and cell viability was determined by MTT assay. Figure 1A shows that TDB significantly reduced cell viability of H292 cells at concentrations greater than 10 μM. Therefore, the compound at 0-5 μM was considered as non-toxic concentration and selected for further experiments. For proliferation assay, cells were treated with 0-5 μM of TDB for 24, 48, and 72 h. Cell proliferation was analyzed by MTT at each time point. The results obtained from the MTT assay revealed that TDB at a concentration of 5 μM significantly suppressed cell proliferation at 48 h. Furthermore, at the detection time of 72 h, all concentrations used exhibited significant antiproliferative effects (Figure 1B).

To confirm the above results, the effect of TDB on apoptosis of cells was evaluated by Hoechst33342/propidium iodide (PI) co-staining assay. The nuclear morphological analysis indicated that treatment of TDB at concentrations of 0-5 μM for 24 h caused no significant apoptotic cell death, as indicated by the absence of condensed or fragmented nuclei (Figure 1C).
Effect of TDB on anoikis susceptibility of lung cancer cells. H292 cells were incubated with non-cytotoxic concentrations of TDB for 24 h. The cells were then cultured under detached conditions in a low attachment plate. Cell viability of detached cells was evaluated at 0, 6, 12 and 24 h through XTT assay. Figure 2A indicates that in the absence of TDB treatment, viability of the H292 cells gradually decreased over time and at 24 h after cell detachment, approximately 60% of the cells remained viable. Importantly, treatment of the cells with TDB at 1 and 5 μM significantly reduced viability of these detached cells, indicating that the compound possesses an anoikis-sensitizing effect.

To confirm the above findings, nuclear staining assay with Hoechst33342 and PI was performed. After detachment for 24 h, cells were subjected to the nuclear staining assay. The results from Hoechst/PI co-staining revealed that TDB significantly increased anoikis responses of the cells as indicated by the significant increase of cells containing condensed or fragmented nucleus stained with Hoechst33342 dye. Notably, PI-positive necrotic cells were barely detectable under such treatment conditions (Figure 2B).

TDB inhibits tumor cells growth under anchorage-independent conditions. As the growth of cancer cells in anchorage-independent growth reflects aggressiveness of cancer cells as well as metastasis potentials (21), the present study further investigated whether TDB could suppress growth of cancer cells such conditions. H292 cells were treated with TDB (0-5 μM) for 24 h before being subjected to the soft agar anchorage-independent growth assay as described in Materials and Methods. After 14 days, the colony size and number of each treatment were determined. Figure 3 indicates that treatment with TDB at non-toxic concentrations resulted in significant inhibition of tumor cell colony formation in a dose-
dependent manner. Both the number of colonies and size of the colony were significantly suppressed on treatment with TDB in comparison to those of the non-treated control group. These results confirmed the inhibitory effect of TDB on the metastasis potential of lung cancer cells.

**TDB suppresses survival pathways and inhibits EMT.** Having shown that TDB had the ability to sensitize cells anoikis, as well as suppress lung cancer cell growth in an anchorage-independent manner, the underlying mechanisms of anti-metastasis effects of TDB were further investigated. An increase in cellular survival mechanisms such as via AKT and ERK has been long known to render cells resistant to anoikis (14, 15). Cells were treated with TDB for 24 h, and then the levels of activated and total forms of AKT and ERK were evaluated by western blotting. Figure 4A shows that TDB significantly reduced levels of activated AKT (pAKT) and of activated ERK (pERK), while having only minimal effects on the total levels of both proteins. These data indicate that TDB may suppress the pro-survival signals of the cancer cells and then may, at least in part, be implicated in its anoikis sensitization.

Recent studies have shown the predominant role of EMT on metastasis of many types of human cancer, including lung cancer (22, 23). Since the induction of EMT was demonstrated to be a major cause of anoikis resistance in lung cancer, the effect of TDB on EMT was further investigated. The EMT markers E-cadherin, vimentin, SLUG, and SNAIL were evaluated in TDB-treated cells. Figure 4C and D show that treatment of H292 cells with TDB resulted in the increase of E-cadherin together with the decrease of vimentin and SNAIL in comparison to those of non-treated cells. These data indicate that EMT of these lung cancer cells was suppressed by the addition of TDB.
Discussion

The interaction of cells with their surrounding extracellular matrix through integrins has long been known to be critical for the survival of solid tumor cells. In detail, such interaction provides pro-survival signals, such as AKT and ERK, that help maintain viability and attenuate apoptotic response to death stimuli (24, 25). Evidence shows that certain cancer cells resist detachment-induced apoptosis by increasing their levels of cellular-activated AKT, as well as of ERK (14, 15).

Although the mechanism regarding augmentation of pro-survival signals in anoikis-resistant cancer cells remains unclear, many cancer experts consider the EMT of cancer cells an important player. During cancer cell dissemination, solid cancer cells are detached from their extracellular matrix and begin to change their phenotype from epithelial to mesenchymal. It is well-known that EMT is the cell physiological mechanism that allows cells to migrate, invade, and resist anoikis (26). The markers of EMT consist of the decrease of the epithelial protein E-cadherin, and the increase of mesenchymal proteins such as vimentin, SNAIL, and SLUG (27, 28). Indeed, the importance of E-cadherin loss in metastasis of several cancer cell types has been highlighted in a number of in vitro and in vivo studies (29, 30). The loss of E-cadherin has also been accepted as the hallmark of EMT. In the present study, we found that treatment of lung cancer cells with TDB significantly inhibited EMT by reducing expression of mesenchymal proteins such as vimentin and SNAIL. Even though the effect of TDB on SLUG was barely detected, expression of E-cadherin was significantly restored.
In addition to describing the effect of TDB on EMT, we provide information regarding the suppression of cellular pro-survival signals. Many studies indicate the significance of abnormal regulation of growth factor receptors that activates pro-survival signaling pathways, such as the PI3K/AKT and ERK pathways, leading to an inhibition of anoikis (14, 31). Our results indicated that if the cancer cells resist anoikis by such means, TDB is able to sensitize cells to anoikis by suppressing the levels of activated AKT and ERK.

In summary, our study has provided novel and significant data indicating that bibenzyl TDB is a promising candidate for drug development because of its inhibition of EMT, a hallmark of metastasis, attenuation of survival of cancer cells during detachment, and growth inhibition of the cells in anchorage-independent manner. As in many types of cancer, metastasis is an important factor negatively-influencing on success of therapy, as well as patient quality of life, such therapeutic strategies involving anti-metastasis approaches should not be ignored.

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References


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