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A bibenzyl from *Dendrobium ellipsophyllum* mediates apoptosis in human lung cancer cells via modulation on cellular reactive oxygen species

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Abstract

The modulation on cellular reactive oxygen species (ROS) affects various cell activities including survival and death. Apoptosis, a program cell death, is efficiently mediated by both ROS generators and anti-oxidant compounds. Recently, apoptosis-inducing effect of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum*, a Thai orchid has been reported in human lung cancer cells. This study aimed to evaluate ROS-modulating activity of TDB and its regulation on apoptosis in human lung cancer cells. After treatment with various concentrations of TDB (10-100 µM), MTT assay revealed the significant reduction of viability in human lung cancer H460 cells. Lower level of cellular ROS detected by 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) fluorescent probe was indicated in lung cancer H460 cells incubated with TDB (50 µM) for 3-6 h compared with non-treated control cells. Pretreatment with hydrogen peroxide (H_2O_2) at non-toxic concentration (100 µM) successfully preserved viability in lung cancer cells treated with 50 µM of TDB. Moreover, Hoechst33342 staining showed that apoptosis is significantly decreased in H460 cells incubated with 100 µM of H_2O_2 for 30 min prior exposure with TDB (50 µM) compared with the cells only treated with TDB. These results demonstrated that TDB induces apoptosis in human lung cancer cells via alteration on cellular ROS level. The novel information obtained from this study would clarify the apoptosis-regulating mechanisms of TDB for further development as an effective anti-cancer drug.

Introduction

Lung cancer is one of the most prevalent cancer in both male and female patients, worldwide.[1] It can be categorized into small cells (SCLC) and non-small cells (NSCLC) which account approximately 85% of all lung cancer cases.[2, 3] According to high successful rate and patient compliance, chemotherapeutic drugs have been recommended for initial and metastasis stage of lung cancer pathology.[4] However, the incidence of chemotherapeutic resistance and serious side-effects oppose the benefits of current chemotherapy.[5, 6] Natural extracts have been highlighted as a potential source for searching of novel anti-cancer drugs with human safety profile.[7, 8]

Various anti-cancer compounds mediate apoptotic cell death in cancer cells dependently on reactive oxygen species (ROS).[9, 10] ROS has been recognized as signaling molecules that regulate many cellular activity including survival, proliferation and cell death.[11, 12, 13] Oxidative stress induced by anti-cancer drugs cause DNA damage and activate apoptosis cascade.[14, 15] Nevertheless, induction of apoptosis is also reported in various cancer cells exposed with anti-oxidant extracts.[16, 17]

The compound, 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB), (fig. 1b) was extracted from *Dendrobium ellipsophyllum*, a Thai orchid (fig. 1a). Recently, anti-cancer activity of TDB has been reported in human lung cancer cells.[18, 19, 20] Selective activity of TDB is evidenced with higher IC50 in dermal papilla cells compared with various lung cancer cells.[21] However, the effect of TDB on cellular ROS level has not been investigated. This study aimed to evaluate ROS modulating activity of TDB and its regulation on apoptosis in human lung cancer cells.

A)

B)

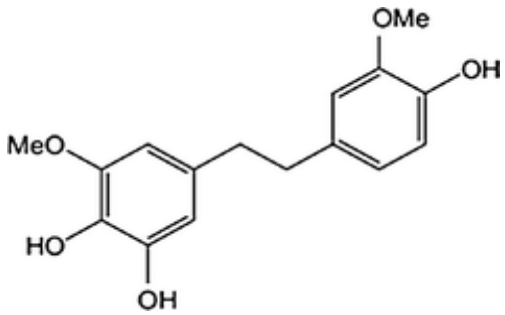

Figure 1. A) *Dendrobium Ellipsophyllum* or Euang Thong **B)** Chemical structure of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB)

Methodology:

Chemical reagents

All chemical reagents including MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), hoechst33342, propidium iodide (PI), DMSO (dimethysulfoxide), 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) fluorescent probe, N-acetyl cysteine (NAC) and 3% hydrogen peroxide solution were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA).

Preparation for 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB)

D.ellipsophyllum extract containing ≥ 98% of 4,5,4′-trihydroxy-3,3′-dimethoxybibenzyl (TDB) was provided by Assoc. Prof. Boonchoo Sritularak, Ph.D., Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Before adding into cultured cells, TDB was dissolved in DMSO and diluted to desired concentrations in optimum medium. The final concentration of DMSO in cell culture medium should be less than 0.5%.

Cell culture

Human lung cancer H460 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and 100

units/mL penicillin/streptomycin (Gibco, Gaithersburg, MA, USA). The cells were maintained under 5% CO_2 at 37 ºC until reach 70-80% confluence before using for further experiments.

Cytotoxicity assay

Cell viability was determined by MTT colorimetric assay. After indicated treatment, H460 cells at density of 1×10^4 cells/well in 96-well plates were further incubated with 0.4 mg/mL of MTT in dark place at 37 °C for 4 h. The MTT solution was replaced with 100 µl DMSO to dissolve the purple formazan crystal. The absorbance of formazan solution was measured at 570 nm using a microplate reader (Anthros, Durham, NC, USA). Cell viability was calculated from the absorbance value (OD) and presented as percentage related to with non-treated control cells.

$$\text{Cell viability}(\%) = \frac{\text{A570}_{\text{with sample}}}{\text{A570}_{\text{with control}}} \text{x } 100$$

Nuclear staining assay

Mode of cell death was detected via co-staining of Hoechst33342 and propidium iodide (PI). Human lung cancer cells were stained with 10 µM of Hoechst33342 and 5 µg/mL PI for 30 min at 37 °C. Then, mode of cell death was evaluated under a fluorescent microscope (Olympus IX51 with DP70). Apoptotic cells with condensed chromatin and/or fragmented nuclei were stained with bright blue fluorescence of Hoechst33342. Meanwhile PI-positive cells were presented necrosis cell death.

Detection of cellular reactive oxygen species

Cellular ROS level was determined by 2′,7′-dichlorofluorescein diacetate ($DCFH_2$-DA) fluorescent probe (Sigma, St. Louis, MO, USA). After seeding H460 cells in 96-well plates at density of $1x10^4$ cells/well for 12 h, the cells were incubated with 10 µM $DCFH_2$-DA at 4 °C for 15 min. Then pre-incubated cells were further cultured in RPMI containing TDB (0-50 µM), NAC (1 mM) or H_2O_2 (100 µM) for 0-6 h. The cellular ROS level at each time point was determined by measurement of $DCFH_2$-DA fluorescent intensity using microplate reader at the excitation and emission wavelengths of 488 and 538 nm, respectively. The relative cellular ROS level was calculated from the fluorescent intensity at indicated time point divided by the fluorescent intensity of non-treated control cells at 0 h.

Statistical analysis

Data representative of three independent experiments were presented as mean ± standard deviation (SD). The significant differences between multiple groups were compared via analysis of variance (ANOVA), followed by individual comparisons with Scheffe's post hoc test. Statistical significance was considered at $p \leq 0.05$.

Results and Discussion

TDB induced-apoptosis in human lung cancer cells

After incubation of human lung cancer H460 cells with various concentrations of TDB for 24 h, there was significant reduction of viability in the cells treated with 10-100 µM of TDB (fig. 2A). Cell viability was decreased approximately 50% in the cells cultured with 100 µM of TDB for 24 h. Co-staining with Hoechst33342 and PI notified condensed DNA and fragmented nuclease which were unique morphology of apoptosis cells in H460 cells after exposure with TDB at 50-100 µM for 24 h. Meanwhile red fluorescence of PI was rarely observed in all TDB-treated cells (fig 2B).

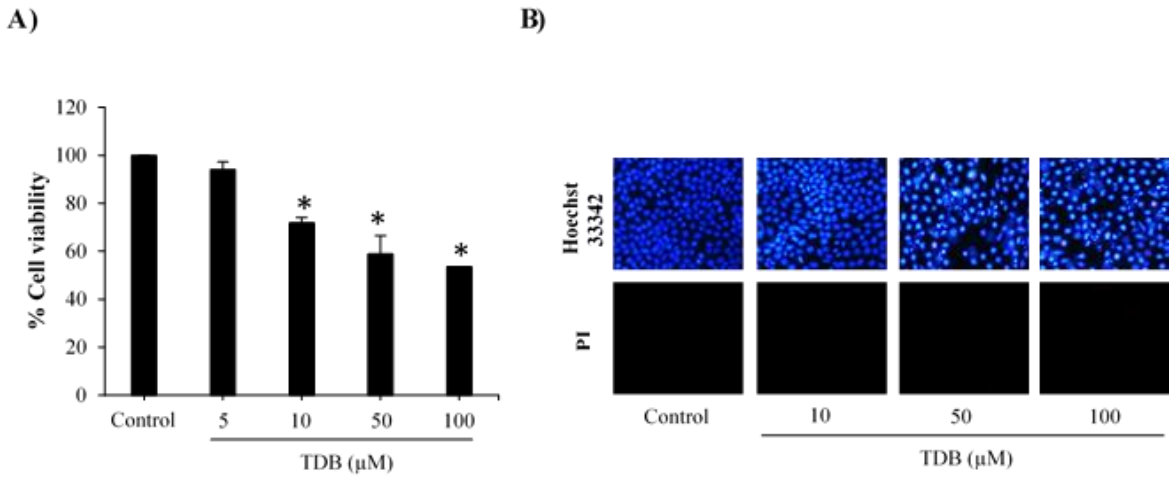

Figure 2. Cytotoxicity of TDB in human lung cancer cells **A)** MTT assay revealed the significant reduction of cell viability in lung cancer cells incubated with 10-100 µM of TDB for 24 h. **B)** Apoptosis presented with bright blue fluorescence of Hoechst33342 was observed after incubation of H460 cells with TDB at 50-100 µM for 24 h whereas there was no detection of necrosis cell. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

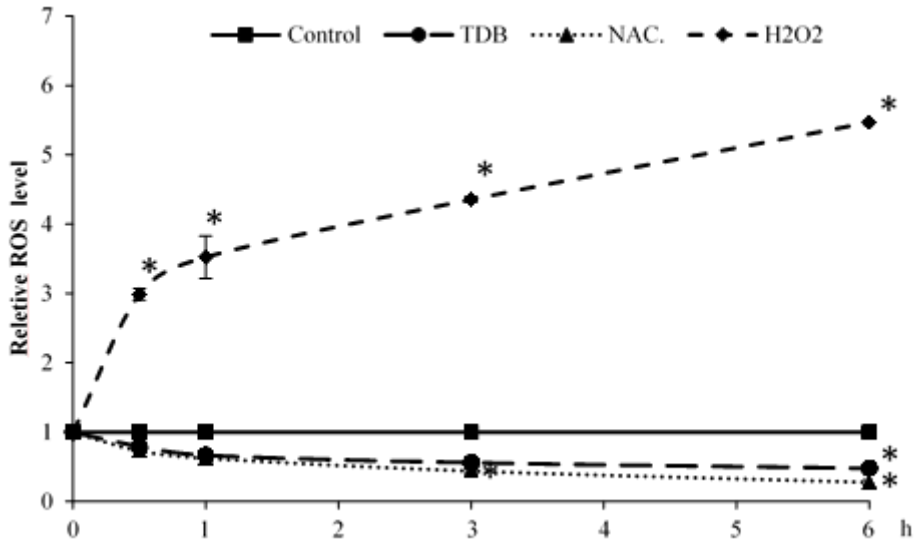

Figure 3. The relative of ROS level in human lung cancer H460 cells after treatment with TDB 50 µM for 0.5, 1, 3 and 6 h. The cells treated NAC (1 mM) and H_2O_2 (100 µM) were considered as negative and positive control, respectively. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells at the same time point.

TDB modulates cellular ROS level in human lung cancer cells

Figure 3 shows the lower level of cellular ROS in H460 lung cancer cells incubated with TDB at 50 µM for 3-6 h compare with non-treated control cells at same time point. The reduction of relative ROS level was also observed after treatment of H460 cells with NAC (1 µM), a well-known anti-oxidant compound. It was worth nothing that non-toxic concentration (100 µM) of H_2O_2 dramatically increased ROS in human lung cancer cells at early incubated time (30 min). These results demonstrated that TDB at 50 µM possessed anti-oxidant activity in human lung cancer cells.

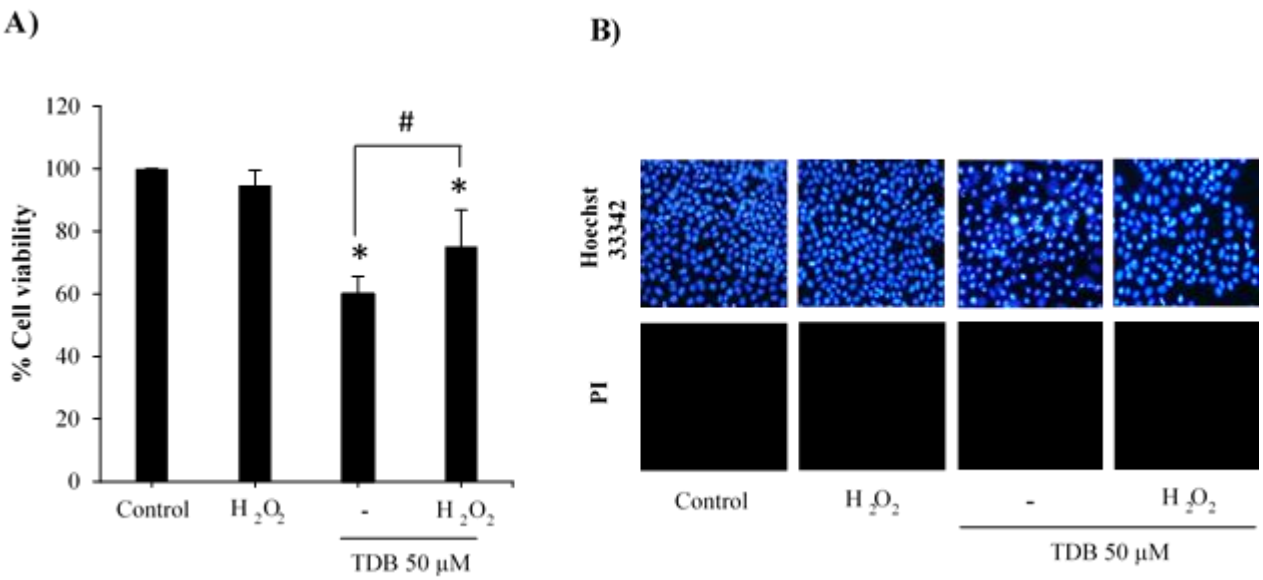

Figure 4. TDB mediated-apoptosis via ROS dependence **A)** Pre-treatment with 100 µM of H_2O_2 restrained cytotoxicity of TDB in lung cancer H460 cells **B)** The diminution of apoptosis cell death was notified in the cells pretreated with 100 µM of H_2O_2 for 30 min before further incubation with 50 µM of TDB for 24 h compared with H460 cells cultured with only TDB. Data are represented as mean ± SD from three independent experiments. $^{*}p \leq 0.05$ versus non-treated control cells. $^{\#}p \leq 0.05$ versus the cells treated with only TDB.

Role of anti-oxidant activity in TDB induced-apoptosis

According to anti-oxidant activity, the involvement between the alteration on cellular ROS and cytotoxicity of TDB was further investigated. Figure 4A indicates that pre-treatment with 100 µM of H_2O_2, a ROS generator for 30 min significantly preserved cell viability in lung cancer cells exposed with 50 µM of TDB. Interestingly, pre-incubation with H_2O_2 retrained apoptosis induced by TDB (50 µM) in human lung cancer cells (fig.4B). Notably, treatment with 100 µM of H_2O_2 for 24 h did not alter viability in human lung cancer cells. These results indicated that TDB mediated apoptosis in human lung cancer cells via ROS dependence.

Conclusion

TDB extracted from *D.ellipsophyllum* manifested apoptosis-inducing effect in human lung cancer cells through anti-oxidant activity.

References

1. Cheng TY, Cramb SM, Baade PD, Youlden DR, Nwogu C, Reid ME. J Thorac Oncol.2016; 11:1653-1671.
2. Zhang J, Gold KA, Lin HY, Swisher SG, Xing Y, Lee JJ, Kim ES, William WN Jr. J Thorac Oncol. 2015; 10:682-690.
3. Hanna JM, Onaitis MW. J Carcinog. 2013; 12:6.
4. Harrington SE, Smith TJ. JAMA. 2008; 299:2667-2678.
5. Shanker M, Willcutts D, Roth JA, Ramesh R. Lung Cancer (Auckl). 2010; 1:23-36.
6. Ihbe-Heffinger A, Paessens B, Berger K, Shlaen M, Bernard R, von Schilling C, Peschel C. Support Care Cancer. 2013; 21:1665-1675.
7. Greenwell M, Rahman PK. Int J Pharm Sci Res. 2015; 6:4103-4112.
8. Wang H, Khor TO, Shu L, Su ZY, Fuentes F, Lee JH, Kong AN. Anticancer Agents Med Chem. 2012; 12:1281-1305.

9. Wangpaichitr M, Wu C, Li YY, Nguyen DJM, Kandemir H, Shah S, Chen S, Feun LG, Prince JS, Kuo MT. Savaraj N. Oncotarget. 2017; 8:49275-49292.
10. Circu ML, Aw TY. Free Radic Biol Med. 2010 48:749-762.
11. Liou GY, Storz P. Free Radic Res. 2010; 44:479-496.
12. Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W. Oxid Med Cell Longev. 2016; 2016:4350965.
13. Orrenius S. Drug Metab Rev. 2017; 39:443-455.
14. Dai X, Guo G, Zou P, Cui R, Chen W, Chen X, Yin C, He W, Vinothkumar R, Yang F, Zhang X, Liang G. J Exp Clin Cancer Res. 2017; 36:120.
15. Wang JP, Hsieh CH, Liu CY, Lin KH, Wu PT, Chen KM, Fang K. Oncol Lett. 2017; 14:3503-3509.
16. Jeong CH, Joo SH. J Cancer Prev. 2016; 21:13-20.
17. Seo SU, Kim TH, Kim DE, Min KJ, Kwon TK. Redox Biol. 2017; 13:608-622.
18. Chaotham C, Chanvorachote P. J Nat Med. 2015 ;69:565–574
19. Chaotham C, Pongrakhananon V, Sritularak B, Chanvorachote P. Anticancer Res. 2014; 34:1931–1938.
20. Tanagornmeatar K, Chaotham C, Sritularak B, Likhitwitayawuid K, Chanvorachote P. Anticancer Res.2014; 34:6573–6579.
21. Hlosrichok A, Sumkhemthong S, Sritularak B, Chanvorachote P, Chaotham C. J Nat Med. 2018. [Epub ahead of print]

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