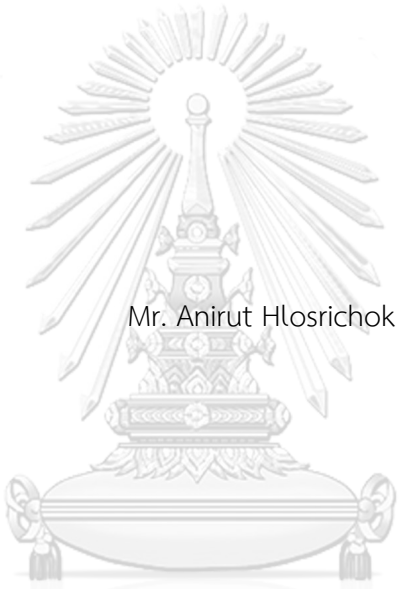
EFFECTS OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* ON INDUCTION OF APOPTOSIS AND INHIBITION OF CANCER STEM-LIKE CELLS IN HUMAN LUNG CANCER H460 CELLS

Mr. Anirut Hlosrichok



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
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ผลของ 4,5,4'-ไตรไฮดรอกซี-3,3'-ไดเมทอกซีไบเบนซิล จากเอื้องทองต่อการเหนี่ยวนำอะพอพโทซิสและการยับยั้งเซลล์มะเร็งต้นกำเนิดในเซลล์มะเร็งปอดของมนุษย์ชนิด เอช460

นายอนิรุจน์ เหล่าศรีโชค

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชเคมี ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Thesis Title	EFFECTS OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* ON INDUCTION OF APOPTOSIS AND INHIBITION OF CANCER STEM-LIKE CELLS IN HUMAN LUNG CANCER H460 CELLS
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Field of Study	Biomedicinal Chemistry
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ความล้มเหลวของยาเคมีบำบัดในปัจจุบัน นำมาสู่การกลับมาเป็นซ้ำของโรคมะเร็งและการเสียชีวิตที่เพิ่มขึ้นในผู้ป่วยโรคมะเร็งปอด การศึกษานี้ทดสอบสาร 4,5,4'-ไตรไฮดรอกซี-3,3'-ไดเมทอกซีไบเบนซิล สารกลุ่มไบเบนซิลที่สกัดจากเอื้องทองเพื่อพัฒนาเป็นสารต้านมะเร็งชนิดใหม่สำหรับโรคมะเร็งปอด ฤทธิ์จำเพาะในการต้านมะเร็งแสดงด้วยค่าความเข้มข้นการยับยั้งร้อยละ 50 มากกว่า 300 ไมโครโมลาร์ ในเซลล์รากผม ในขณะที่ค่าความเข้มข้นการยับยั้งร้อยละ 50 ในเซลล์มะเร็งปอดชนิด เอช460 มีค่าประมาณ 100 ± 5.18 ไมโครโมลาร์ จากผลการวิเคราะห์ด้วย Flow cytometry ยืนยันว่าเมื่อได้รับสารสกัด ที่ความเข้มข้น 50 ไมโครโมลาร์ เป็นเวลา 24 ชั่วโมง ทำให้เกิดการตายแบบอะพอพโทซิสทั้งช่วงต้นและช่วงท้ายอย่างมีนัยสำคัญ แต่ไม่พบการตายแบบนีโครซิสในเซลล์มะเร็งปอดชนิด เอช460 พบการเพิ่มขึ้นของโปรตีนชนิด p53 ซึ่งเป็นโปรตีนยับยั้งมะเร็งหลังจากทดสอบด้วยสารสกัดที่ความเข้มข้น 10-50 ไมโครโมลาร์ การกระตุ้นโปรตีน p53 นั้นเกี่ยวข้องกับการเพิ่มขึ้นของโปรตีนส่งเสริมอะพอพโทซิสชนิด Bax การลดลงของโปรตีนต้านอะพอพโทซิสชนิด Bcl-2 และ Mcl-1 และการยับยั้งวิถีรอดชีวิต Akt กลไกที่เป็นไปได้ที่เกี่ยวข้องกับการกระตุ้นอะพอพโทซิส คือการเปลี่ยนระดับของอนุพันธ์ออกซิเจนที่ว่องไว เมื่อตรวจสอบด้วย 2', 7'- dichlorofluorescein diacetate พบระดับของอนุพันธ์ออกซิเจนที่ว่องไว ภายในเซลล์ชนิด เอช460 ลดลงหลังทำการทดสอบด้วยสารสกัด (ความเข้มข้น 50 ไมโครโมลาร์) เป็นเวลา 3-6 ชั่วโมง นอกจากนี้การย้อมเซลล์ด้วยสี Hoechst33342 ยังแสดงให้เห็นการตายของเซลล์แบบอะพอพโทซิสลดลงอย่างมีนัยสำคัญในเซลล์ เอช460 ที่ได้รับไฮโดรเจน เปอร์ออกไซด์ 100 ไมโครโมลาร์ นาน 30 นาทีก่อนทดสอบด้วยสารสกัด (ความเข้มข้น 50 ไมโครโมลาร์) เมื่อเปรียบเทียบกับเซลล์ที่ได้รับสารสกัดเพียงอย่างเดียว นอกจากนี้สารสกัดยังยับยั้งลักษณะเซลล์ต้นกำเนิดในเซลล์มะเร็งปอดชนิด เอช460 ซึ่งยืนยันจากการทดลองด้วยเทคนิค spheroid assay และ flow cytometry พบว่า เซลล์มะเร็งต้นกำเนิดที่แสดงออกโปรตีนชนิด CD133 ลดลงหลังจากได้รับสารสกัด อีกทั้งระดับโปรตีนที่เกี่ยวข้องในการถอดรหัสการแสดงออกลักษณะเซลล์ต้นกำเนิด Nanog และ Sox2 นั้นลดลง ขณะที่ไม่มีการเปลี่ยนแปลงของโปรตีน Oct4 พบการลดของโปรตีนควบคุมชนิด β-catenin โดยสรุปสาร 4,5,4'-ไตรไฮดรอกซี-3,3'-ไดเมทอกซีไบเบนซิล สกัดจากเอื้องทองมีฤทธิ์ยับยั้งเซลล์มะเร็งต้นกำเนิดและการชักนำให้เกิดการตายแบบอะพอพโทซิสที่จำเพาะต่อเซลล์มะเร็งโดยการลดระดับอนุพันธ์ออกซิเจนที่ว่องไวในเซลล์มะเร็งปอดของมนุษย์ ข้อมูลที่ได้จากการศึกษานี้ยืนยันศักยภาพในการพัฒนาสาร 4,5,4'-ไตรไฮดรอกซี-3,3'-ไดเมทอกซีไบเบนซิล เป็นสารต้านมะเร็งที่มีความปลอดภัยต่อไป

สาขาวิชา	ชีวเวชเคมี	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Apoptosis, Bibenzyl, Dendrobium ellipsophyllum, ROS, Cancer Stem-like Cells

Anirut Hlosrichok : EFFECTS OF 4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL FROM *DENDROBIUM ELLIPSOPHYLLUM* ON INDUCTION OF APOPTOSIS AND INHIBITION OF CANCER STEM-LIKE CELLS IN HUMAN LUNG CANCER H460 CELLS. Advisor: Asst. Prof. Chatchai Chaotham, Ph.D.

Failure of current chemotherapeutic drugs leads to the recurrence of tumors and increased mortality in lung cancer patients. This study investigated 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB), a bibenzyl extracted from *Dendrobium ellipsophyllum* Tang and Wang as a novel anticancer agent for lung cancer. Selective anticancer activity of TDB against human lung cancer cells was demonstrated with a high half maximal inhibitory concentration (IC_{50}) approximately > 300 μM in dermal papilla cells, while IC_{50} in human lung cancer H460 cells was approximately 100 ± 5.18 μM. Flow cytometry confirmed that TDB (50 μM) treatment for 24 h, caused significant early and late apoptosis but not necrosis in H460 cells. The up-regulation of p53, a tumor-suppressor protein, was observed in human lung cancer cells treated with 10-50 μM of TDB. The mechanism for apoptosis was attributed to p53 activation, which was related to up-regulation of pro-apoptotic Bax, reduction of anti-apoptosis Bcl-2 and Mcl-1 proteins and suppression on protein kinase B (Akt) survival pathway. Another possible mechanism involved in TDB-induced apoptosis in H460 cells was the modulation of reactive oxygen species (ROS), as intracellular ROS level was decreased by TDB (50 μM) treatment for 3-6 h as detected by 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA) fluorescent probe. Moreover, Hoechst33342 staining showed that apoptosis was significantly decreased in H460 cells incubated with 100 μM of hydrogen peroxide (H_2O_2) for 30 min prior exposure to TDB (50 μM) compared with the cells only treated with TDB. Additionally, TDB inhibited cancer stem cell (CSC)-like characteristics in H460 cells as evidenced in spheroid assay and flow cytometry analysis, whereby CSCs, CD133-positive cells markedly decreased after TDB treatment. TDB caused a decrease in protein levels of the stemness-related transcription factors; Nanog and Sox2 while there was no alteration of Oct4. There was the reduction of related regulator proteins, β-catenin. In conclusion, TDB extracted from *D. ellipsophyllum* demonstrated stemness suppression and selective apoptosis induction via intracellular ROS reduction in human lung cancer cells. The results obtained from this study strengthen the potential development of TDB as an

Field of Study: Biomedicinal Chemistry Student's Signature .....

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LIST OF ABBREVIATIONS

%	=	Percentage
℃	=	Degree Celsius
μM	=	Micromolar
β-catenin	=	Beta-catenin
ANOVA	=	Analysis of variance
ALDH1A1	=	Aldehyde dehydrogenase 1 family member A1
Apaf-1	=	Apoptosis protease activating factor-1
Akt	=	Protein kinase B
pAkt	=	Phosphorylation Protein kinase B
Erk	=	Extracellular signal-regulated kinases
pErk	=	Phosphorylation extracellular signal-regulated kinases
Bak	=	BCL2 homologous antagonist/killer
Bcl-2	=	B-cell lymphoma 2
CSCs	=	Cancer stem-like cells
c-FLIP	=	Cellular-FLICE inhibitory protein
CO_2	=	Carbon dioxide
$DCFH_2$-DA	=	2, 7-dichlorofluorescein diacetete
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DR5	=	Death receptor 5
FBS	=	Fetal bovine serum
GSK3β	=	Glycogen synthase kinase-3 beta
pGSK3β		Phosphorylation glycogen synthase kinase-3 beta
h	=	Hour, Hours
H_2O_2	=	Hydrogen peroxide

IC_{50}	=	The half maximal inhibitory concentration
KCl	=	Potassium Chloride
Mcl-1	=	Myeloid cell leukemia sequence 1
Min	=	Minute (S)
mL	=	Milliliter
mM	=	Millmolar
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NAC	=	N-acetylcysteine
Nanog	=	Homeobox protein NANOG
NaCl	=	Sodium Chloride
$O_2^{\cdot-}$	=	Superoxide anion
Oct4	=	Octamer-binding transcription factor 4
$^{\cdot}OH$	=	Hydroxyl radical
PI	=	Propidium iodide
PUMA	=	P53—unregulated modulator of apoptosis
ROS	=	Reactive oxygen species
RPMI	=	Roswell Park Memorial Institute's medium
S.D.	=	Standard deviation
SOX2	=	Sex determining region Y-box 2
TNF	=	Tumor necrosis factor
TRAIL	=	TNF-Related Apoptosis Inducing Ligand
TDB	=	4,5,4'-TRIHYDROXY-3,3'-DIMETHOXYBIBENZYL

CHAPTER I

INTRODUCTION

Despite several decades of intensive studies, cancer especially lung cancer still remains a major health problem worldwide (1). The high prevalence and estimated death rate of lung cancer are indicated in both male and female patients (2). Due to aggressive features of active proliferation, instantaneous spreading and drug resistance, the 5-year survival rate in lung cancer patients has not improved (3, 4). Among various treatments, administrations of chemotherapeutic drugs are essentially recommended for lung cancer patients at both initial and advance stage (5, 6). However, the failure of available chemotherapies has been evidenced with the recurrence of tumor pathology after treatment with current anticancer drugs (7, 8). Moreover, toxicity to non-cancer cells leading to severe side effects such as hair loss, nephrotoxicity and myocardial toxicity restricts the achievement of modern anticancer drugs (9, 10).

Induction of apoptosis has been recognized as a major mechanism of novel chemotherapeutic agents (11). Apoptosis or process of programmed cell death is normally featured by distinct morphological changes and biochemical mechanisms. Apoptosis is regulated through the balance between anti- and pro-apoptosis signaling (12). Bcl-2 family proteins compose with various regulatory proteins that either accelerate or inactivate apoptosis cascade. The imbalance between pro-apoptosis and anti-apoptosis proteins initiates the releasing of cytochrome c from mitochondria following with the activation of caspase cascade and eventually apoptotic cell death. Cancer cells possess the capability to oppose apoptosis through the up-regulation of anti-apoptosis Bcl-2 family proteins (13, 14). The over-expression of Bcl-2 (B-cell lymphoma 2) and Mcl-1 (myeloid cell leukemia 1) has been revealed in lung cancer cells isolated from patients (15). Both Bcl-2 and Mcl-1 involve with aggressive behaviors of chemotherapeutic resistance in human lung cancer cells (16, 17). Deprivation of Bax (Bcl-2-associated X protein), a pro-apoptosis protein also reduces the susceptibility to drug-induced apoptosis in various cancer cells (18-20). Moreover, role of p53, a tumor suppressor protein has been elucidated to influence with apoptosis induction (21, 22).

Activation of p53 function directly increases the expression of pro-apoptosis proteins and consequently influence on down-regulation of Bcl-2 and Mcl-1 (23, 24). Moreover, pro-survival machinery of Akt (Protein kinase B) and Erk (Extracellular signal–regulated kinase) are also retrained through the trigger of p53 (25, 26). Therefore, stimulation and restoration of p53 function has been focused as a target molecule of novel anticancer drugs (27, 28).

Recently, cancer-stem like cells (CSCs) has been recognized as a small population that involves with initiation, metastasis and recurrence of tumor pathology (29). CSCs possess stemness properties of quiescence, self-renewal and pluripotency which leads to low susceptibility to current chemotherapeutic drugs (30, 31). The overexpression of stemness transcription factors and marker proteins including Nanog, Oct4, Sox2, CD133 and ALDH has been elucidated in CSCs of various cancer cells (32). Role of CSCs on aggressive behaviors especially chemotherapeutic resistance have been elucidated with the presence of CSCs isolated from both lung cancer specimens and cell lines (33). According to critical influence on cancer pathology, the investigation on novel anticancer drugs targets on suppression of CSCs sub-population (34, 35).

Role of reactive oxygen species (ROS) on aggressive behaviors of cancer cells has been well recognized. Cellular redox status in cancer cells is tightly controlled through the overexpression of antioxidant enzymes. Oxidative stress down-regulates the expression of anti-apoptosis proteins such as Bcl-2 and signaling molecules of Akt survival pathway (36-38). Moreover, apoptosis can be generated in ROS-activated p53 which results in up-regulation of Bax (39). In term of modulation on stemness phenotypes, overexpression of Oct4 and Sox2 has been revealed in CSCs at hypoxia condition in which cellular ROS are highly produced (40). Nevertheless, there are the reports about potential anticancer activity of anti-oxidant compounds. Increasing of H_2O_2 can suppress on Wnt/β-catenin pathway in CSCs (41). The effect of well-known antioxidant N-acetyl cysteine (NAC) on expression of p53 and apoptosis induction had been illustrated (42).

In searching for an effective chemotherapy, compounds from natural resource have been attracted for their human safety and various potential therapeutic profiles (43). Recently, cytotoxicity and anti-metastasis activity of 4,5,4'-trihydroxy-3,3'-

dimethoxybibenzyl (TDB), a bibenzyl extracted form Dendrobium ellipsophyllum in human lung cancer cells have been demonstrated (44-46). However, anticancer activity and related mechanisms of TDB have not been reported. This study aims to evaluate the apoptosis inducing effect of TDB in human lung cancer cells. The alteration of apoptosis-regulating molecules after treatment with TDB will be clarified. Furthermore, TDB suppressed-CSCs will be investigated in human lung cancer cells. The information obtained from this study will strengthen the potential development of TDB as a promising chemotherapeutic drug.

Research Questions

1. Does 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl induce apoptosis in human lung cancer cells?
2. Does 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl suppress stemness in human lung cancer cells?
3. Does reactive oxygen species involve with anticancer activities of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl in human lung cancer cells?

Objectives

1. To investigate apoptosis inducing activity and related mechanisms of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl in human lung cancer cells.
2. To investigate suppressive activity of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl in cancer stem-like cells of human lung cancer cells.

Hypothesis

A bibenzyl, 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl extracted from *Dendrobium ellipsophylum* induces apoptosis and represses stemness in human lung cancer cells via modulation on Bcl-2 family proteins and stemness-regulating transcriptional factors, respectively.

Expected benefits

The underlying mechanisms of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl in regulating apoptosis and cancer stem-like cells in human lung cancer cells.

CHAPTER II

LITERATURE REVIEWS

1. Lung cancer

Among various types of cancer, lung cancer accounts for major cause of cancer-related mortality worldwide (47). The cancer statistics in United State reveals that the highest death rate in both male and female cancer patients results from lung cancer. Moreover, the estimated incidence and death rate in lung cancer continuously increase (48). Risk factors of lung cancer include smoking, asbestos, gas toxicity and genetic variation (49).

Lung cancer cells can be categorized as small cell and non-small cell lung cancer depending on their morphology (50). Non-small cells which are a common type of lung cancer compose with 3 subtypes including adenocarcinoma, squamous carcinoma and large cell carcinoma. Adenocarcinoma found in the mucosal lining of lung accounts about 40% of all lung cancer patients. Squamous carcinoma or epidermoid carcinoma occurs in epithelium of the bronchi. Large cell carcinoma is high spreading lung cancer cells. Although small cell lung cancer is only 10 to 15% in all patients, they possess high growth and spreading phenotype (51).

1.1 Treatments for lung cancer

Despite intensive research for effective treatments, 5-years survival and recurrence rate in lung cancer patients does not improve (17). The current therapeutic approaches for lung cancer compose with surgery, radiation and chemotherapy (52). Surgery is the most effective treatment but it is not suitable for advanced stage with the spreading of lung cancer cells to various secondary organs (53). Radiation and chemotherapy are administrated in lung cancer patients with metastasis tumor pathology due to their systemic mechanism of actions (54). Recently, chemotherapeutic drugs have been recommended for both curing and preventive treatments of recurrence lung cancer (55). However, the achievement rate of current chemotherapeutic drugs is still low. Resistance to drugs-induced cell death and severe side effects restrain the effectiveness of chemotherapeutic drugs (56). About 50% of

cancer cells isolated from lung tumor had low susceptibility to available chemotherapy (57). Resistance to drug-induced apoptosis substantially involves with failure of current chemotherapies and mortality in various types of cancer especially lung cancer (58). One of the most limitations that restrict the benefits of chemotherapeutic drugs is the toxicity to non-cancerous tissue (9). Damage on dermal papilla cells consequence with hair loss is a common adverse effect found in patients administrated with current anticancer drugs (10). Although it is not a cause of life-threatening, hair loss can devastate quality of life which results in the withdrawal of chemotherapy in cancer patients (59-61).

1.2 Chemotherapeutic resistance in human lung cancer

Drug resistance is a well-known phenomenon that results when the disease becomes resistant to medication (62). This concept was first considered when the bacteria became resistant to antibiotics. Since then, similar mechanisms have been found in other diseases, such as cancer (58). Cancer drug resistance remains a major obstacle in medical oncology. Clinical resistance may occur before or after cancer treatment (63). Chemotherapeutic resistance may be due to the elimination of apoptotic pathways resulted from the unacceptable gene mutation or down-regulation in apoptotic protein coding such as p53, a tumor suppressor protein (64). Low expression of the Bcl-2 pro-apoptotic proteins induces drug resistance in various cancer cells (18, 65). Moreover, up-regulation of Bcl-2 anti-apoptotic protein such as Mcl-1 (myeloid cell leukemia 1) and Bcl-2 (B-cell lymphoma 2) strongly contribute as drug resistance factor in a clinical study (66). Recently, the presence of cancer stem-like cells (CSCs) has been illustrated to influence with chemotherapeutic resistance and recurrence of cancer pathology (30). Not only stemness transcriptional factors but anti-apoptotic Bcl-2 family proteins including Bcl-2, Mcl-1 and Bcl-XL (B-cell lymphoma-extra-large) are highly expressed in CSCs (67)

2. Apoptotic cell death

Apoptosis is a form of programmed cell death in multiple organisms. It involves with biochemical reactions that cause specific cell death including cell blabbing, cell membrane changes, chromatin condense and DNA breaks apart (68). The removal of apoptotic debris does not cause any damage to the neighboring tissue, unlike necrosis cell death (69). In addition, apoptosis is also important for the removal of unsuitable cells such as mutant cells, in which apoptosis is a process that prevents the growing of abnormal cells (70).

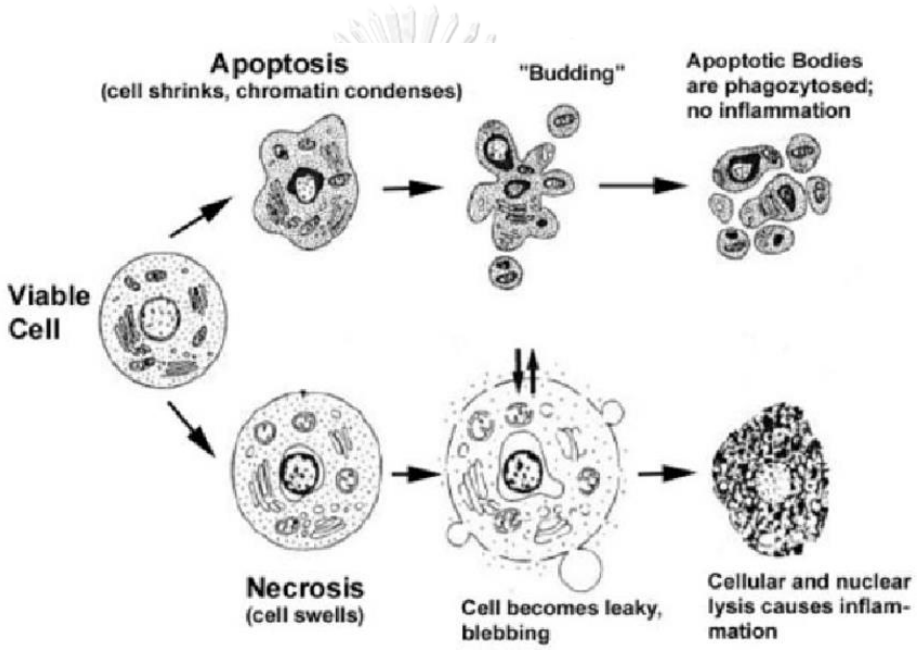


Figure 1 Various type of cell death. Apoptosis cells death have formation of apoptotic bodies is nuclear condensation, DNA damage. Apoptosis cell deaths have a controlled physiologic process and removing components of cells without destroy another cells. Necrosis is pathway of cell death together with membrane breakdown and release organelle to environment (71).

Apoptosis is stimulated via multi-signal pathways and imposed by multi-complex of extrinsic and intrinsic machinery (72). The process of apoptosis involve with an energy dependent cascade of molecular incidence. The extrinsic and intrinsic pathways make an effort on the identical execute pathway which starts by the cleavage of

caspase-3 and effect in DNA fragmentation, degradation for cytoskeletal and mature of apoptotic bodies (73). Apoptosis has been found to be involved with initiation of tumor pathology. The modification of apoptotic pathways presented in cancer cells authorizes unlimited cell proliferation and mechanism to drug-induced cell death (74). Therefore, stimulating apoptosis signal pathway is an attractive target for cancer medication (75).

2.1 Extrinsic pathway of apoptosis

The extrinsic pathway induces apoptosis through the activation of caspases cascade via signaling from cell-surface death receptors such as death receptor 5 (DR5) (76, 77). Previous research found that combination treatment of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and chemotherapeutic drugs can sensitize apoptosis in cancer cells through the expression of DR5 protein (78). Moreover, cellular FLICE-inhibitory protein (c-FLIP) acts as an inhibitor of extrinsic signal-induced apoptosis (79). The interaction between death receptors and specific ligand activates caspase8, a down-stream signaling of extrinsic pathway. c-FLIP restrains apoptosis through suppression on the conversion of inactive caspase8 (pro-caspase8) to activated caspase8 (80).

2.2 Intrinsic Pathway

The intrinsic signaling pathway involves with apoptosis process linked a various non-receptor-mediated triggers (81). Machinery of intrinsic apoptosis creates intracellular signals that alters mitochondrial membrane (82). Proteins related to apoptosis, which target the mitochondria, are effective in a variety of ways such as penetrating into mitochondrial membrane resulting increase of membrane permeability. The permeability of mitochondrial membrane leads to leaking of the apoptotic initiating molecule (68). There are many inducers that can initiate intrinsic pathway. Some growth factors, hormones and cytokines can conduct to defeat of repression of death programs, so triggering apoptosis. Other stimuli consisting with radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals also induce apoptosis via intrinsic pathway (83).

The control and regulation of this mitochondria mediated-apoptotic are influenced by Bcl-2 family of proteins. Down regulation of Bcl-2 resulted from overexpression of p53 generate intrinsic apoptosis (84). Main mechanism of Bcl-2 family proteins on inducing apoptosis is the regulation of cytochrome c releasing from the mitochondria (85). After leaking from mitochondrial membrane, cytochrome c interacts with APAF-1 (apoptotic protease activating factor 1) to form apoptosome, a proto-caspase9 enzyme. Cleavage of pro-caspase9 or activated caspase9 stimulates the execute caspase cascade of caspase3 and eventually proceed apoptotic bodies (86).

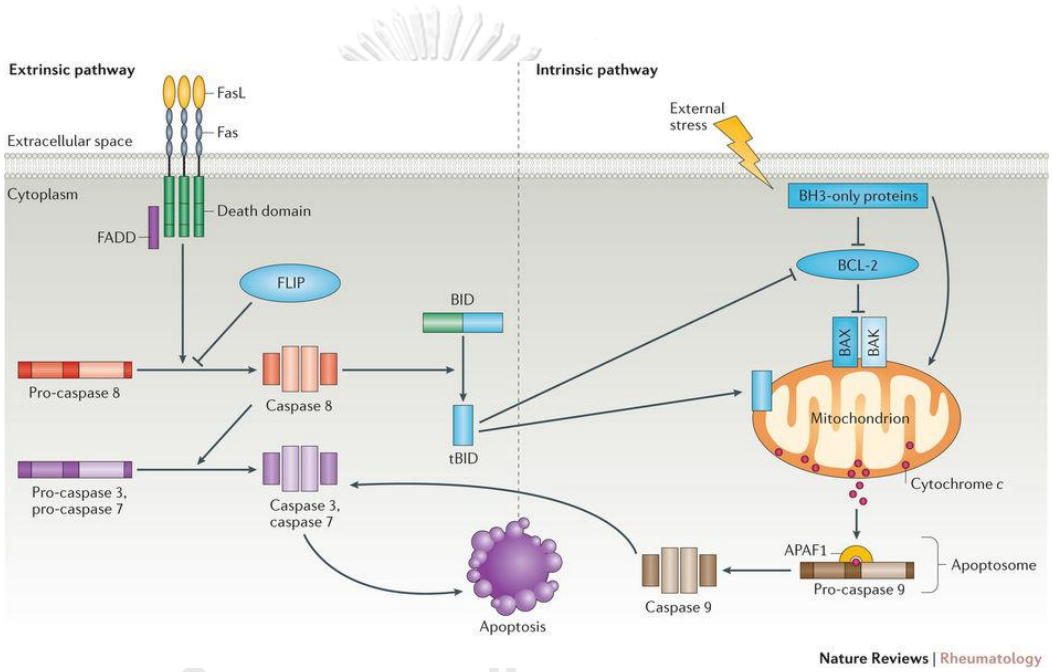


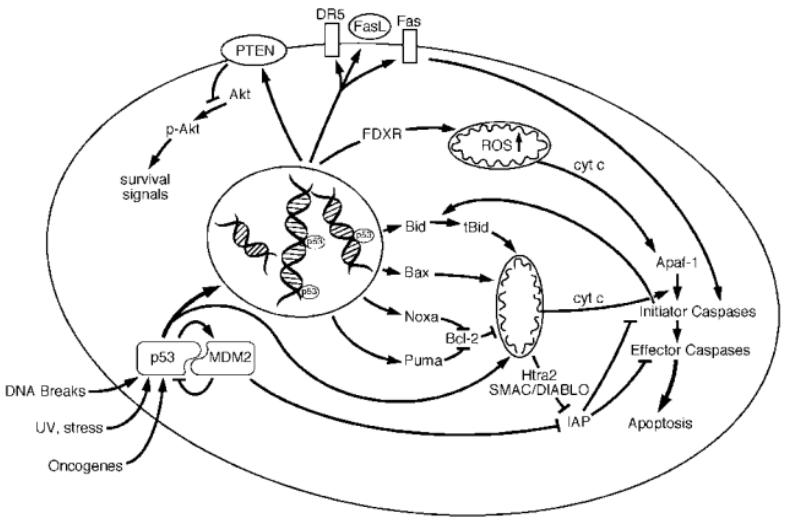
Figure 2 The extrinsic and intrinsic pathways of apoptosis (87).

Interestingly, many chemotherapeutic drugs and natural compounds trigger intrinsic apoptosis in various cancer cells. Current chemotherapy for lung cancer such as cisplatin, actinomycin D and afatinib mediate apoptosis through decreasing of anti-apoptosis Bcl-2 proteins including Bcl-2 and Mcl-1 (88, 89). Moreover, ouabain, a natural extract can down-regulate Mcl-1 protein and induce apoptosis in lung cancer cells (90). Meanwhile, treatment with some natural extracts promote the expression

of Bax, pro-apoptosis protein and successfully induce apoptosis in human lung cancer cells (91, 92).

2.3 p53-induced apoptosis pathway

The tumor suppressor protein, p53 is a genetic transcription factor that controls the expression of multiple genes involving in cell cycle, growth, senescence and death in response to cellular stress (93). p53 activates a wide signalling network that acts on both two major apoptotic pathways, external and internal pathways (94). p53-induced extrinsic pathway involves with the engagement of particular death receptors that belong to the tumor necrosis factor receptor (TNFR) family and formation of death-inducing signaling complex (DISC) (95). These lead to respective activation of caspase8 and caspase3, which in turn induces apoptosis. Most death receptors associating with external stimuli are FAS and DR5 receptor (96).



Figurer 3 A model for p53-induced apoptosis (97).

p53-induce intrinsic apoptotic pathway is dominated by the bcl2 family proteins, which controls the release of cytochrome c from the mitochondria (98). Bax is activated via by p53 in cellular response to stress condition (99). The BH3-only proteins, Noxa and Puma are encoded by p53-inducible genes. Anti-apoptosis role of Noxa and Puma is enhancing activity of Bax and Bak through inhibition of Mcl-1, Bcl-2 and Bcl-XL (99).

The activation of p53 not only involves with apoptosis role of Bcl-2 family proteins but also modulates the expression of survival molecules (100). Previous study has shown that the alteration of protein kinase B (Akt) signaling pathway is resulted from the inhibition on p53 in which is a general mechanism to uncontrollably proliferate and escape apoptosis in cancer cells. Akt also regulates the activity of p53 through the activation of murine double minute 2 (MDM2)-mediated targeting of p53 (101). Extracellular signal–regulated kinases (Erk) control survival and proliferation of cell through activation of mitogen-activated protein kinase (MAPK) cascade. Suppression on p53 can results the inhibition of Erk survival signal and eventually inhibition of cell proliferation and deterrence of cell cycle (26).

3. Cancer stem-like cells

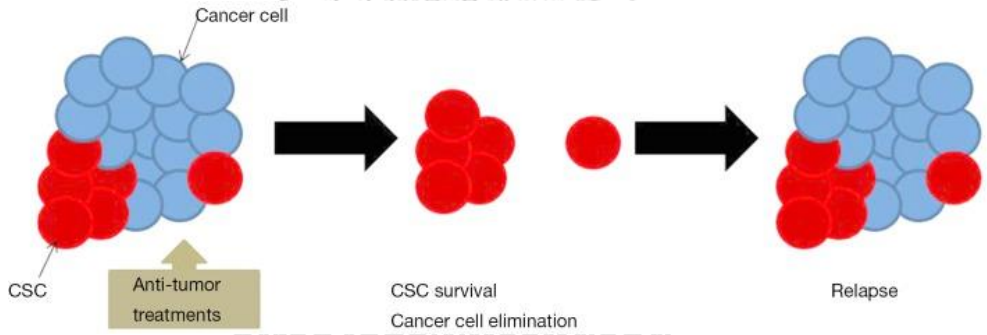


Figure 4 Role of cancer stem-like cells in recurrence of tumor pathology (102).

Stem cells (SCs) are unique cells possess capability to self-renewal and produce differentiated offspring in different types of cells. In spite of small population, SCs are important for replenishing and repair damage tissue (102). Cancer stem–like cells (CSCs), a small cell cluster within the tumor hold stemness characters which facilitate initiation, metastasis and recurrence of cancer pathology. Recently, CSCs are considered as a major factor for low achievement rate of current therapeutic treatments (103). Available chemotherapies successfully induce apoptosis cell death only in cancer cells but not in CSCs. The over-expression of anti-apoptotic Bcl-2 family

proteins have been indicated in persisting CSCs (104). Unresponsive CSCs play a critical role on generation of new and recurrence tumor (102). Due to aggressive features including cancer generator, high metastasis and chemotherapeutic resistance, the attempt to investigate and develop novel anti-cancer drugs emphasize on elimination of CSCs sub-population (105). In addition, there is a report about targeting cell death in CSCs induce by various natural compounds (106).

Table 1 Protein marker for CSCs in lung cancer (107).

Markers	Cellular/molecular function	CSCs
CD44	Hyaluronic acid receptor	NSCLC; breast; hematopoietic
uPAR/CD87	Urokinase plasminogen activator (uPA) receptor	SCLC
CD90	Tissue specific differential glycosylation	NSCLC
CD117	Growth factor receptor	NSCLC; neuroendocrine lung; hematopoietic
CD133	Unknown	Lung, brain, colon, pancreas
CD166	Activated leukocyte cell adhesion molecule (ALCAM)	NSCLC, SCLC
ALDH	Alcohol metabolism	Lung; hematopoietic, breast, prostate
BMI-1	Represses the tumor suppressor phosphatase and tensin homolog (PTEN)	NSCLC, SCLC
EpCAM	Cell-cell contact adhesion strength and tissue plasticity	NSCLC
FZD	Progression development, morphogenesis drug resistance	NSCLC
PODXL-1	Sodium-hydrogen exchange regulatory cofactor 2	SCLC
PTCH	Differentiation, branching morphogenesis	SCLC
SP	Drug resistance	Squamous lung carcinoma/NSCLC; hematopoietic, brain, breast
ALDH	Early stem cell differentiation	NSCLC

The presence of CSCs in lung cancer has been evidenced with detached spheroid formation and *in vivo* tumor initiation of sub-population isolated from both lung cancer specimens and cell lines (108, 109). Moreover, lung CSCs can be identified through the evaluation on SCs marker protein such as prominutesin-1 (CD133), CD44 and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) (107). CD133 and ALDH1A1 are recommended as marker for CSCs in various cancer (110-112). Table 1 show an overview of used lung CSC markers.

3.1 Regulation on stemness property in lung cancer stem-like cells

The regulation on stemness phenotypes including self-renewal and pluripotency in both normal SCs and CSCs is modulated by stemness transcriptional factors. Sex

determine region Y-box 2 (Sox2), Nanog and octamer-binding transcription factor 4 (Oct4) are transcriptional factors that mediate stemness property in various cancer cells including lung cancer (113, 114). Complex of Oct4, Sox2 and Nanog recruits essential transcriptional factors to produce stemness regulating proteins (115). Suppression on Oct4 expression not only inhibits stemness phenotypes but also metastasis feature in CSCs of lung cancer cells (116). Spheroid formation under detached condition, cell cycle arrest and apoptosis cell death in CSCs are regulated by Sox2 level (117, 118). Up-regulation of Sox2 transcriptional factor has been reported in various type of lung cancer (119, 120).

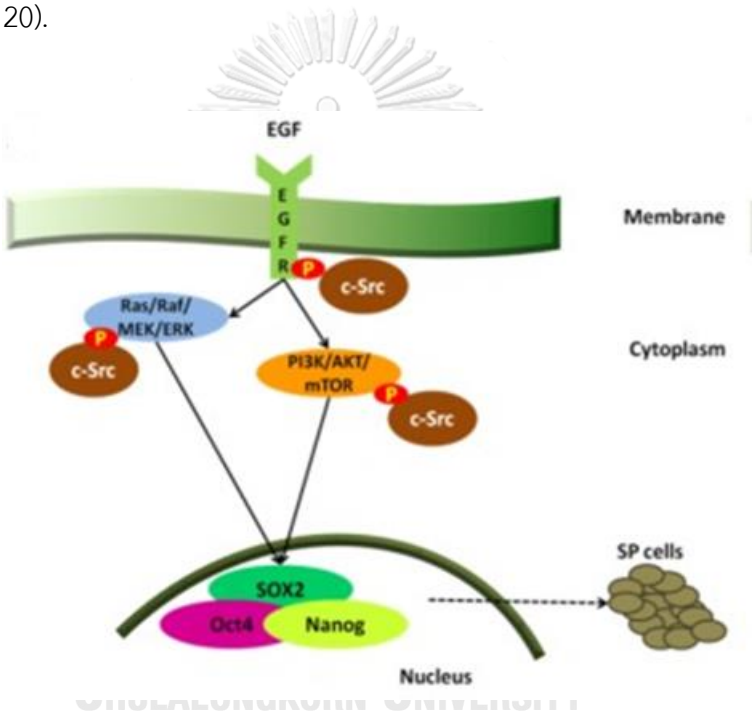


Figure 5 Role of epidermal growth factor (EGF) mediated-survival molecules on stemness transcriptional factor complex (120).

Nanog has been selected for prognostic marker for lung cancer (121, 122). Lung cancer patients with overexpression of Nanog and Oct4 demonstrated low survival rate (114). Aggressive features of chemotherapeutic resistance, epithelial to mesenchymal transition (EMT) and self-renewal in CSCs are regulate by Nanog (113). Additionally, survival signals of Erk and Akt mediate transcriptional function of Nanog/Oct4/Sox2 complex in lung cancer CSCs (120).

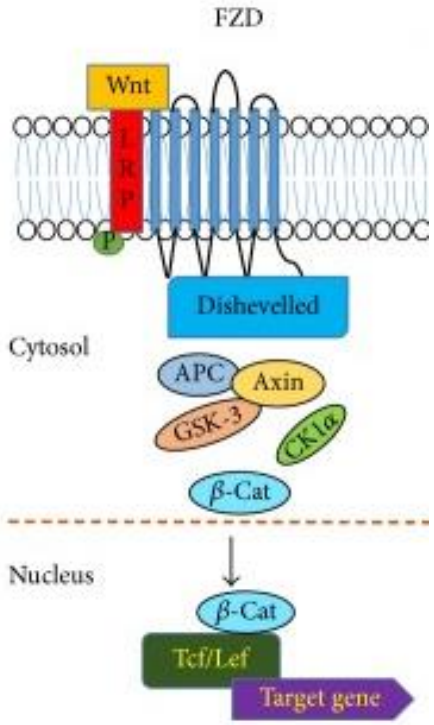


Figure 6 Wnt/β-catenin signaling for activation of gene transcription (123).

The expression of stemness transcriptional factor has been revealed to influence by Wnt/β-catenin pathway (124, 125). Activated Wnt receptors, FZD and LRP prevent degradation of β-catenin resulting in accumulation of β-catenin in nuclease. Dephosphorylate β-catenin is retrieved from destructive complex of axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), and casein kinase Iα (CKIα) (123). The interaction between β-catenin and T-cell factor (TCF) stimulate the transcription of Oct4, Sox2 and CD44 (126). Interestingly, p53 also regulates the function of GSK3β which in turn suppress CSCs via inhibition on Wnt/β-catenin pathway (127). Inactivation of p53 liberates stemness and EMT features in sub-population cancer cells (128). Stemness phenotypes and Oct4 expression of CSCs in lung cancer cells has been modulated via Wnt/β-catenin signal (129, 130). Suppression on Wnt/β-catenin effectively eliminates CSCs population in lung cancer cells (131).

4. Reactive oxygen species (ROS) and cancer

Reactive oxygen species or ROS are free radicals of oxygen derivatives that actively interact with other molecules. At physiological condition, cellular ROS are continuously produced via oxygen-dependent metabolisms. There are various types of cellular ROS however superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicle ($OH^{\cdot}$) are often found in metabolism process (132). The amount of cellular ROS is tightly controlled at the optimum level by cellular antioxidant mechanisms such as glutathione redox system, superoxide dismutase and catalase enzyme (133). At low level, ROS act as importance signaling molecules in essential survival pathways. However, the accumulation of ROS or oxidative stress cans affect normal cellular functions and result in dysregulation of survival and death (134).

The uncontrollable proliferation in cancer cells has been revealed to be involved with the high level of basal cellular ROS compared with non-cancerous cells. The increase of cellular ROS causes genetic mutation, dysregulation of survival and death and eventually tumor formation (135). According to the relevance to cancer pathology, various anticancer drugs possess the capability to modify cellular ROS balance in cancer cells. Although oxidative stress inducing is a potential characters of chemotherapy drugs, antioxidant compounds also has been proposed as candidate for anticancer drugs (136). Therefore, underlying mechanisms in alteration of ROS level, ROS-targeted molecules and affected cancer phenotypes need to be individually clarified in pro- and anti-oxidant chemotherapeutic drugs.

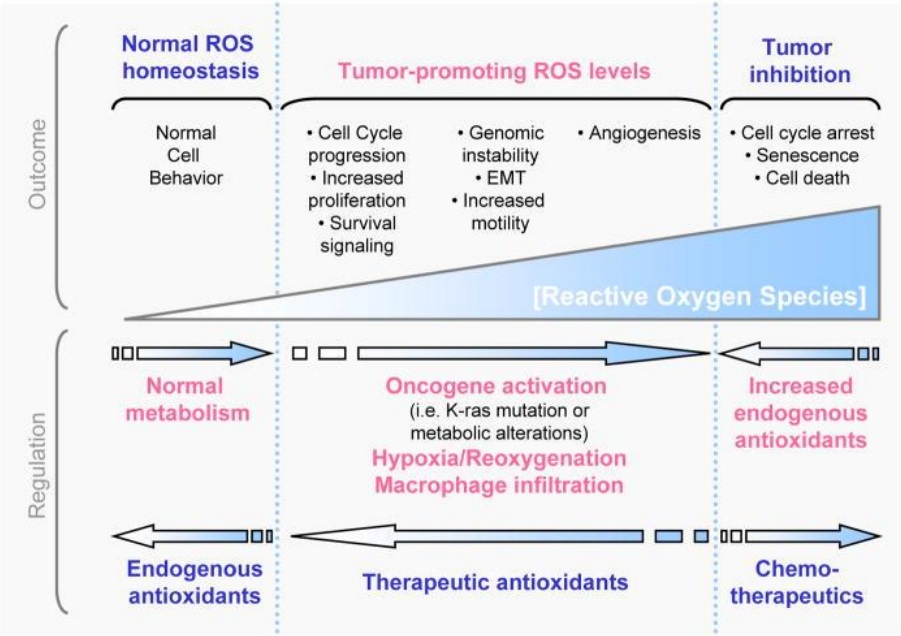


Figure 7 Cellular ROS level and effect on cancer cells (137).

4.1 ROS regulating apoptosis

Various anticancer compounds induce apoptosis via ROS dependence. Cisplatin, a current chemotherapeutic drug for lung cancer generate H2O2 that causes DNA damage and apoptosis cell death (138). Down-regulation on survival pathways and anti-apoptosis proteins have been reported after treatment ROS generating natural compounds in cancer cells (36-38). Meanwhile, antioxidants also show anticancer activity through initiation of apoptosis. Modulation on cellular redox status results in cytochrome c releasing form mitochondria (139). Selective anticancer activity of metformin is mediated by reduction of cellular ROS level in pancreatic cancer cells (140). Cellular redox status closely regulates function and expression of tumor suppressor protein, p53. Oxidative stress stimulates p53 activity consequence with expression Bax and PUMA and apoptosis (39). Moreover, p53 has been demonstrated to manipulate cellular redox status through up-regulation of antioxidant enzymes including glutathione peroxidase (141). The effect of well-known antioxidant N-acetyl cysteine (NAC) on expression of p53 and apoptosis induction had been illustrated (42).

4.2 Role of ROS on stemness in cancer cells

Cellular ROS level is a critical factor driving behaviors of stem cells. At low ROS level, stem cells are at quiescent stage. Meanwhile, differentiation and proliferation are activated in oxidative stress condition in stem cells (142). In cancer stem-like cells (CSCs), cellular ROS level is lower than both cancer and non-cancer cells. The overexpression on antioxidant enzymes has been reported in CSCs (142, 143). The accumulation of ROS can modulate stemneess-regulating pathways. Increasing of H_2O_2 suppresses on Wnt/β-catenin pathway in CSCs (41). Overexpression of Oct4 and Sox2 has been revealed in CSCs at hypoxia condition in which cellular ROS are highly produced (40). Therefore, dysregulation on cellular ROS level has been proposed as target mechanism to eradicate CSCs (142). Recently, natural compound-induced ROS effectively restrain self-renewal in CSCs (144).

5. *Dendrobium ellipsophyllum*

Natural compounds have gained an attention in anticancer research because of their human safety profile and various potential therapeutic effects (43, 145). Recently, a bibenzyl with the chemical name of 4, 5, 4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* Tang and Wang has been shown cytotoxicity in human lung cancer cells (45). Furthermore, potential anti-metastasis activity of TDB is evidenced with inhibition on migration and sensitization to detachment-induced cell death through suppression on EMT regulating molecules (44, 46). Nevertheless, anticancer activity on apoptosis induction and CSCs in human lung cancer cells as well as related mechanisms of TDB has not been reported. This study aimed to evaluate the apoptosis-inducing effect of TDB in human lung cancer cells and to investigate the alteration of apoptosis-regulating molecules caused by the extract. It is desired that the study findings may strengthen the potential for development of TDB as a chemotherapeutic drug.

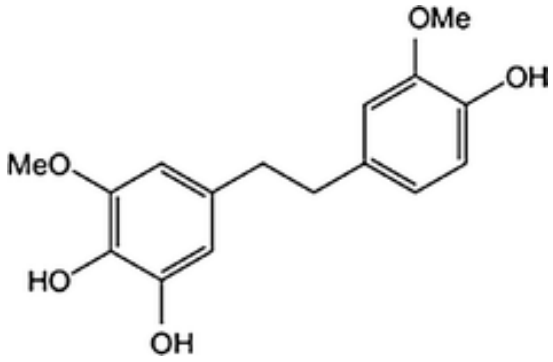


Figure 8 Molecular structure of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB) a bibenzyl extracted from *Dendrobium ellipsophyllum.*

CHAPTER III

METERIALS AND METHODS

Material

1. Chemical reagents

The brown viscous extract containing ≥98% of 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* was kindly provided by Assoc. Prof. Boonchoo Sritularak, Ph.D., Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. All chemical reagents including MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Hoechst33342, propidium iodide (PI), DMSO (dimethysulfoxide) were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Apoptosis detection kit of Alexa Fluor® 488 Annexin V was obtained from Thermo Fisher Scientific (Waltham, MA, USA.). Primary antibody of Bcl-2, Mcl-1, Bax, p53, Akt, pAkt (Ser 473), Erk, pErk (Thr 981), Nanog, Oct4, Sox2, β-catenin, GSK3β, pGSK3β (ser 9), β-actin and specific horseradish peroxidase (HRP)-link secondary antibody were bought from Cell Signaling Technology, Inc. (Danver, MA, USA). Specific antibody of CD133 for determination of stem cell protein markers were purchased from Cell-signaling Technology (Denver's, MA, USA).

2. Cell culture

Human lung cancer H460 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). They were maintained in Roswell Park Memorial Institute (RPMI) medium (Gibco, Gaithersburg, MA, USA). Meanwhile, human dermal papilla DPCs cells were purchased from Applied Biological Materials Inc. (Richmond, Canada) and maintained in Prigrow III medium (Applied Biological Materials Inc., Richmond, Canada). All cell culture mediums were 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 cultured under 5% CO_2 at 37 ºC until reached 70-80% confluence before using for next experiments.

Methods

1. Preparation for 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB)

Before adding into cultured cells, TDB were dissolved in DMSO and diluted to desired concentrations in optimum medium. The final concentration of DMSO in cell culture medium should less than 0.05%.

2. Cytotoxicity assay

Cell viability was determined by MTT colorimetric assay. After treatment with 0-100 µM of TDB for 24 h, the cells placed in 96-well plates at density of $1x10^4$ cells/well 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.

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

3. Nuclear staining assay

Apoptosis and necrosis cell death were detected via co-staining of Hoechst33342 and propidium iodide (PI). Cells that were treated or left untreated with 1-50 µM TBD for 24 h were stained with 10 µM of Hoechst33342 and 5 µg/ml PI for 30 min at 37 °C. The mode of cell death was examined and scored under a fluorescence 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.

4. Flow cytometry analysis

Mode of cell death was also evaluated by flow cytometry using an Annexin V-FITC apoptosis kit (Thermo Fisher Scientific, Waltham, MA, USA). Human lung cancer H460 cells were cultured at a density of 1×10^6cells/well in 6-well plate for 12h. Then, the cells were incubated with TDB at 0-50 μM for 24h. Treated-cells were collected and prepared as single cell suspension in phosphate buffer saline (PBS) pH 7.4. Annexin V-FITC/propidium staining **was** performed according to the manufacturer's instructions. Briefly, the cell suspensions were centrifuged and resuspended in 100 μl of 1× binding buffer. The cells were stained with 10 μl of Annexin V-FITC (1 μg/ml) and 5 μl of PI (2.5 μg/ml) for 15 min. Apoptosis and necrosis cells were analyzed via a FACScan flow cytometer using CellQuest software (Becton-Dickinson, Redlands, CA, USA).

To evaluation of cancer stem-like cell population, secondary spheroids treated with TDB (0-100 μM) were collected and prepared as single cell suspension in phosphate buffer saline (PBS) pH 7.4. The cell suspensions were centrifuged and remove supernatant and cell pallets were incubated overnight at 4 °C with rabbit anti-CD133 antibody (Choi D et al., 2009). The cells were washed with PBS pH 7.4 and incubated for 1 h with Alexa Flour 488-conjugated goat anti-rabbit secondary antibody (Life Technology, Eugene, OR, USA). Fluorescence intensity was determined by flow cytometry using a 488-nm excitation beam and a 519-nm band-pass filter (FACSort; Becton Dickinson, Rutherford, NJ, USA). The mean fluorescence intensity was quantified by CellQuest software (Becton-Dickinson, Redlands, CA, USA).

5. Enrichment of cancer-stem like cells

Spheroid formation assay was chosen for culture and enrichment of cancer stem-like cells from human lung cancer cells (146). Briefly, human lung cancer cells were prepared in RPMI containing 1% FBS in and cultured in a 24-well ultralow attachment plate at density of 2.5×10^3 cell/well. After formation of primary spheroids for 7 days, cancer colonies were dispersed into single cell suspension by using 1 mM EDTA. The cell suspension in RPMI containing 1% FBS were further culture under detachment

condition in a 24-well ultralow attachment plate at density of 2.5 x 10^3 cell/well for 14 days. Then, single secondary spheroid was isolated and maintained in a 24-well ultralow attachment plate at approximately 1 spheroid/well. The spheroid size and morphology were observed under microscope (Olympus IX51 with DP70) at day 0, 3, 5 and 7 after treatment with various concentrations (0-100 μM) of TDB. This protocol is adapted from other studies using the tracking of cancer stem cell markers including CD133 and ALDH1A1 to detect cancer-stem like cells (147, 148).

6. Limiting dilution assay (LDA)

Limiting dilution assay is a method to measure stem cell frequency in the population (149). LDA used in this study was slightly modified from previous described (150). Human lung cancer cells were plated in the gradually decreasing numbers from 200 cells/well to 1 cell/well in 200 μl on a 96-well ultralow attachment plate and cultured for 14 days, whereupon the number of wells containing spheroid for each cell plating density (number of positive culture) were recorded. Spheroid formation with TDB-treated cells were calculated in all wells compared to control. The number and morphology of colonies were observed under microscope (Olympus IX51 with DP70) at day 14. Using NIS-Elements D software, sixteen images were taken for each well to capture all colonies and finally collated to make one photo through the same software.

7. Detection of cellular reactive oxygen species

Cellular ROS is determined by 2,7-dichlorouorescein diacetate ($DCFH_2$-DA) fluorescent probe (Sigma, St. Louis, MO, USA) for general detection non-specific ROS. After placed 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 culture in RPMI containing TDB (0-50) μM for 0 - 3 h. The cellular ROS level at each time point was determined through the measurement of $DCFH_2$-DA fluorescence intensity using microplate reader at the excitation and emission wavelengths of 488 and 538 nm, respectively.

8. Western blot analysis

After treatment with TDB (0-50 μM) for 24 h, human lung cancer H460 cells were incubated with lysis buffer containing 20 mM TrisHCl (pH 7.5), 1 % Triton X-100, 150 mM sodium chloride, 10 % glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) for 30 min at 4 ºC. The cellular lysates were centrifuged at 10,000 rpm (4 ºC) for 10 min then supernatant was collected. Total protein content was determined using a Bicinchoninic acid assay (BCA assay) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). After subjection of 40 μg of protein from each treatment through SDS-PAGE, separated protein were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5 % non-fat dry milk in TBST (25 mM Tris-HCl ,pH 7.5, 125 mM NaCl and 0.05 % Tween 20)) at 25 ºC for 1 h. The optimum primary antibodies were added onto the membranes. After incubation at 4 ºC for 12 h, the membranes were washed with TBST for 7 min × three times. The membranes were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at 25 ºC. Finally, the signal form specific proteins were detected using a chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA). The analyst/PC densitometry software package (Bio-Rad Laboratories, Hercules, CA, USA) was used for quantification of protein signal.

9. Statistical analysis

Data were presented as mean ± standard deviation (SD) from three independent experiments. The significant differences between multiple groups were compared via analysis of variance (ANOVA), followed by individual comparisons with Turkey's post-hoc test. SPSS programs (IBM Inc., NY, USA) was used for statistical analysis. Statistical significance is considered at $p \leq 0.05$.

Conceptual framework

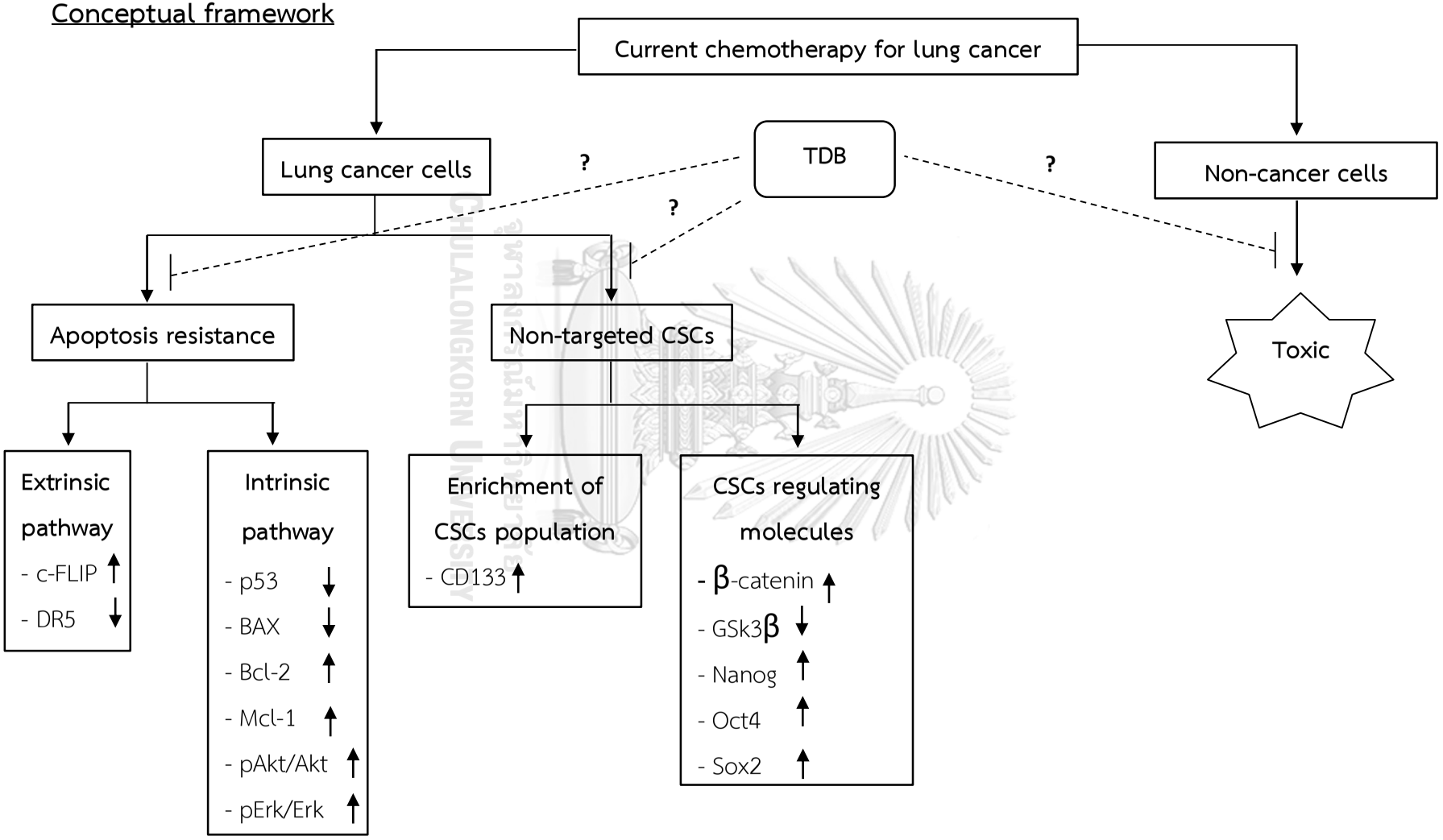


Figure 2.9 Conceptual framework

Experimental design

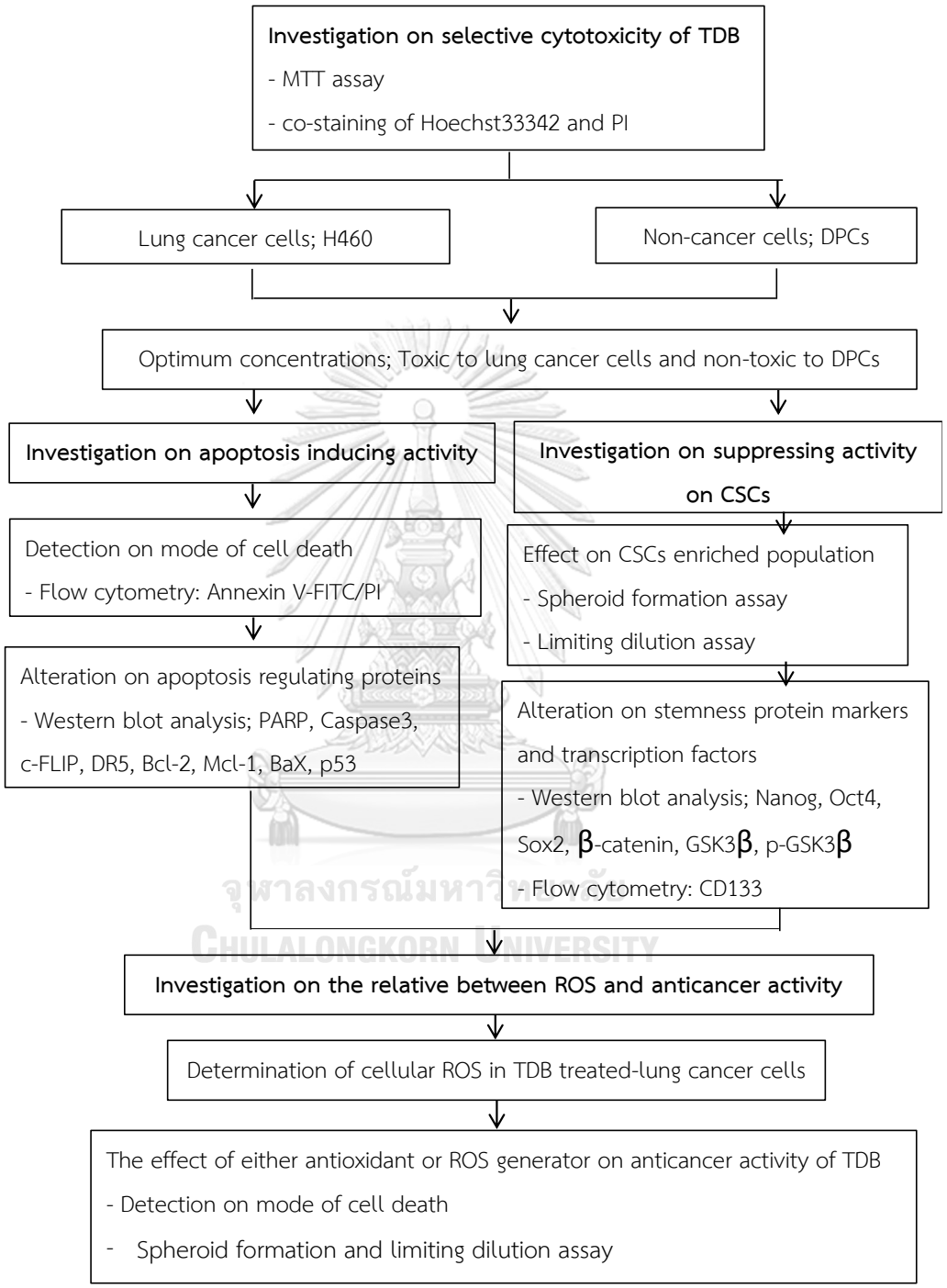


Figure 2.10 Experimental design

Experimental Design

1. **Investigation on selective cytotoxicity of TDB in various human lung cancer cells**

The anticancer activity of TDB was evaluated in H460 lung cancer cells. Lung cancer at $1X10^4$ cell/well in 96-well were treated with 0-300 μM of TDB at 37 °C for 24 hours then cell viability will be detected via MTT assay. For investigate the cytotoxicity of TDB in normal human cells, MTT viability assay was performed in DPCs treated with TDB (0-300 μM) for 24. The 50% inhibition concentration (IC_{50}) of TDB were calculated. Moreover, co-staining of Hoechst33342 and PI was also performed to confirm cell death in TDB-treated cells.

2. **Investigation on apoptosis induction and related mechanisms of TDB in human lung cancer cells**

Apoptosis cell death was investigated through flow cytometry in lung cancer H460 cells treated with 0-50 μM of TDB for 24 h. The alteration of caspase3 and PARP in TDB-treated H460 were analyzed via western blot analysis to confirm apoptosis. Western blot analysis was also used for determination the expression of Bcl-2, Mcl-1, Bax, c-FLIP, DR5 and p53 to investigate TDB-induced intrinsic and extrinsic apoptosis pathways.

3. **Investigation on suppressing effect of TDB on CSCs in human lung cancer cells**

Number and size of CSCs enriched population were evaluated through spheroid formation assay and limiting dilution assay (LDA) to reveal the effect of TDB (0-100 μM) on CSCs in lung cancer cells. The alteration of β-catenin, GSK3β and Nanog, Oct4, Sox2 transcription factor were analyzed in TDB-treated CSCs by western blot analysis. Flow cytometry was performed to determine alteration of CSCs markers, CD133 in CSCs of human lung cancer cells.

4. Investigation on the relation cellular ROS and anticancer activity in TDB treated-human lung cancer cells

The modulations on cellular ROS level were evaluated in lung cancer cells incubated with TDB for 0-3 h. To clarify the relation between TDB-modulating ROS and anticancer activity including apoptosis induction and CSCs suppression, human lung cancer cells were pretreatment with either antioxidant (N-acetyl cysteine) or ROS generator (H_2O_2) at non-toxic concentration for 30 min before expose with TDB. Then %cell viability, %apoptosis and relative number and size of CSCs spheroid formation were investigated and compared between pretreated and non-pretreated lung cancer cell.

CHAPTER IV

RESULTS

1. TDB induces apoptosis in human lung cancer cells

1.1 TDB treatment selectively affects cell viability in human lung cancer cells

The 4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl (TDB) extracted from *Dendrobium ellipsophyllum* has selective toxicity to human lung cancer cells. After incubation with various concentrations of TDB for 24 h, there was significant reduction of viability in human lung cancer H460 cells treated with 10-100 μM of TDB. This cytotoxic effect was found to be dose-dependent, and the IC_{50} was calculated using the equation (y = 0.1746x + 32.54) wherein 50% cell viability reduction is estimated to occur with 100 ± 5.18 μM TDB treatment for 24 h (figure 4.3). MTT results reflect the selective cytotoxicity of the compound towards H460 cells while Hoechst33342/PI co-staining of H460 cells treated with 50-100 μM of TDB for 24 h showed condensed DNA and fragmented nuclei, indicating TDB causes apoptosis (figure 4.1B).

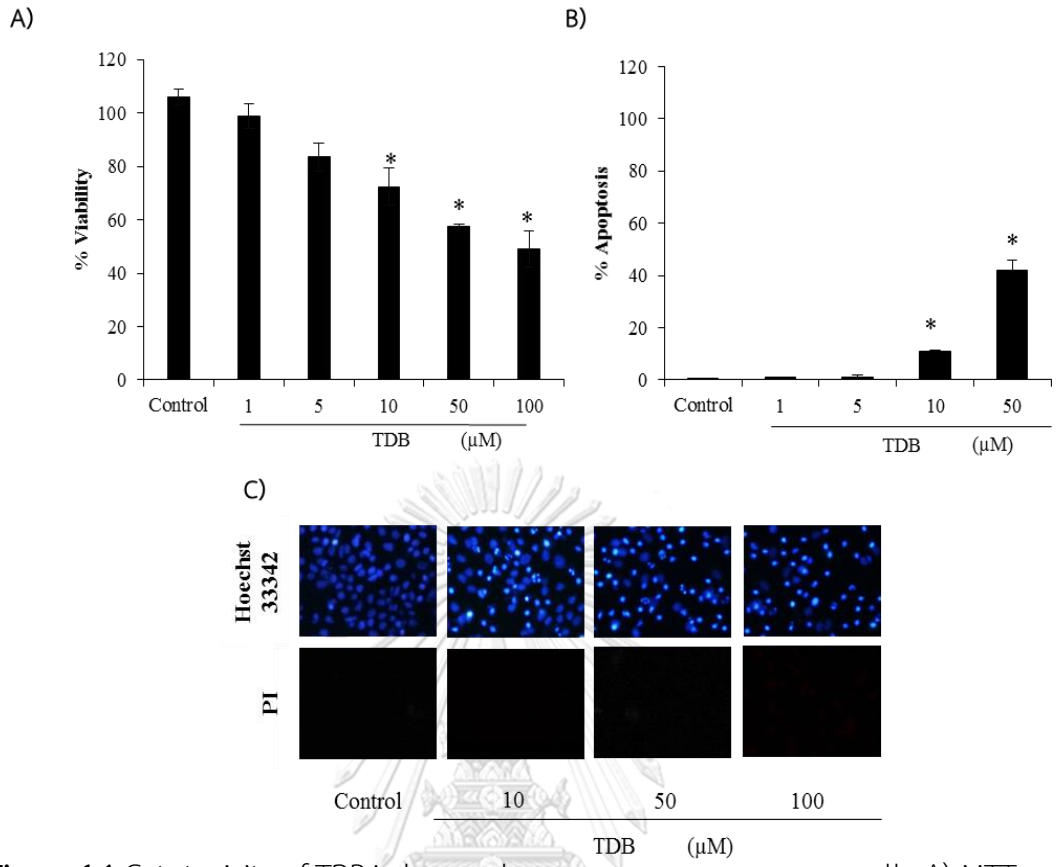


Figure 4.1 Cytotoxicity of TDB in human lung cancer cells A) MTT assay revealed the significant reduction of cell viability in lung cancer cells incubated with 10-100 μM of TDB for 24 h. B) Percent apoptosis induced by TDB 0-50 μM for 24 h in H460 lung cancer cells. C) Apoptosis presented as bright blue fluorescence of Hoechst33342 was observed after incubation of H460 cells with TDB at 50-100 μM for 24 h, whereas necrosis was undetected. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

Meanwhile, human dermal papilla cells (DPCs) incubated with TDB (0-100 μM) for 24 h showed no statistical significance of cell viability (figure 4.2A). Furthermore, co-staining with Hoechst33342 and PI did not reveal the characteristic morphology of apoptosis or necrosis in DPCs after exposure with TDB at 10-100 μM for 24 h (figure 4.2B), indicating that the toxic range in H460 cells is not enough to induce cell death in non-cancer DPCs.

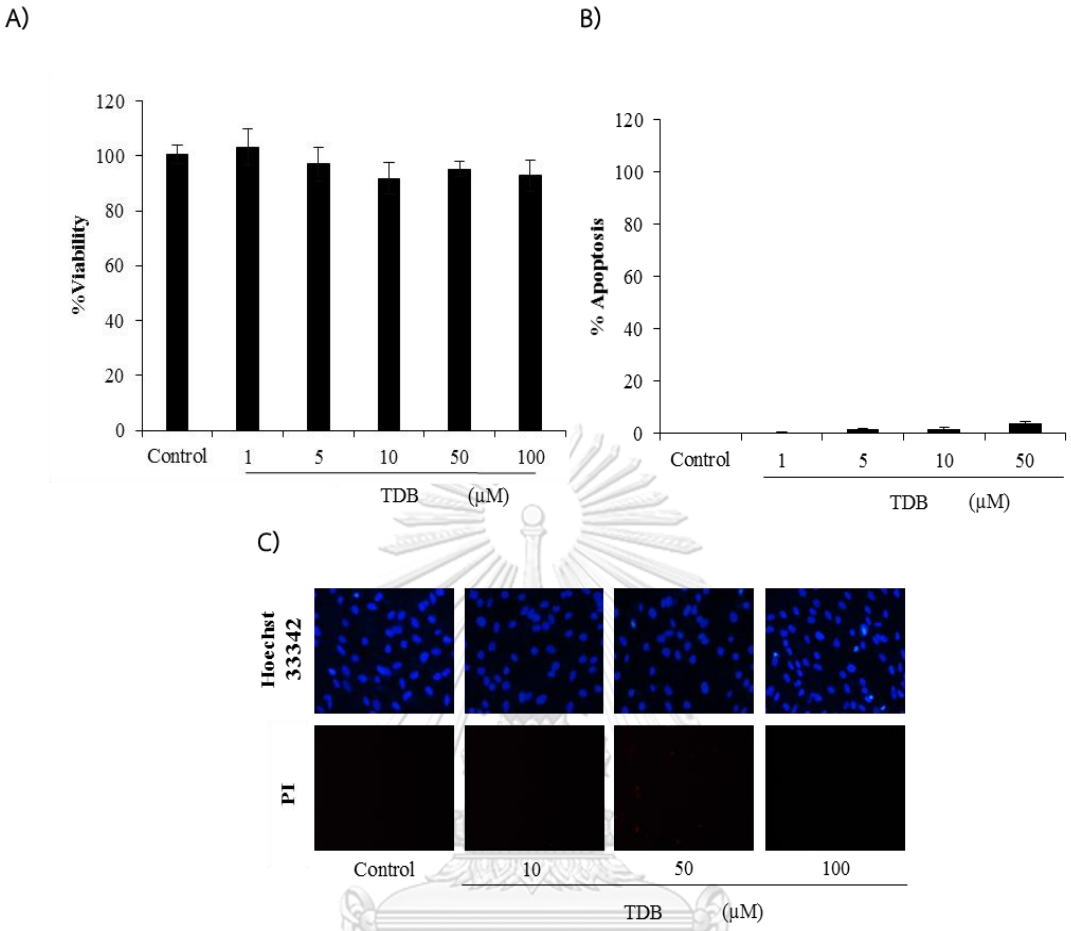


Figure 4.2 Cytotoxicity of TDB in human dermal papilla cells (DPCs) A) MTT assay revealed no significant reduction of cell viability in human dermal papilla cells incubated with 1-100 μM of TDB for 24 h. B) There was no significant change of percent apoptosis in DPCs cells after treatment with TDB 0-50 μM for 24 h. C) Hoechst33342/PI co-staining did not reveal apoptosis or necrosis in DPCs cells treated with TDB at 50-100 μM for 24 h. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

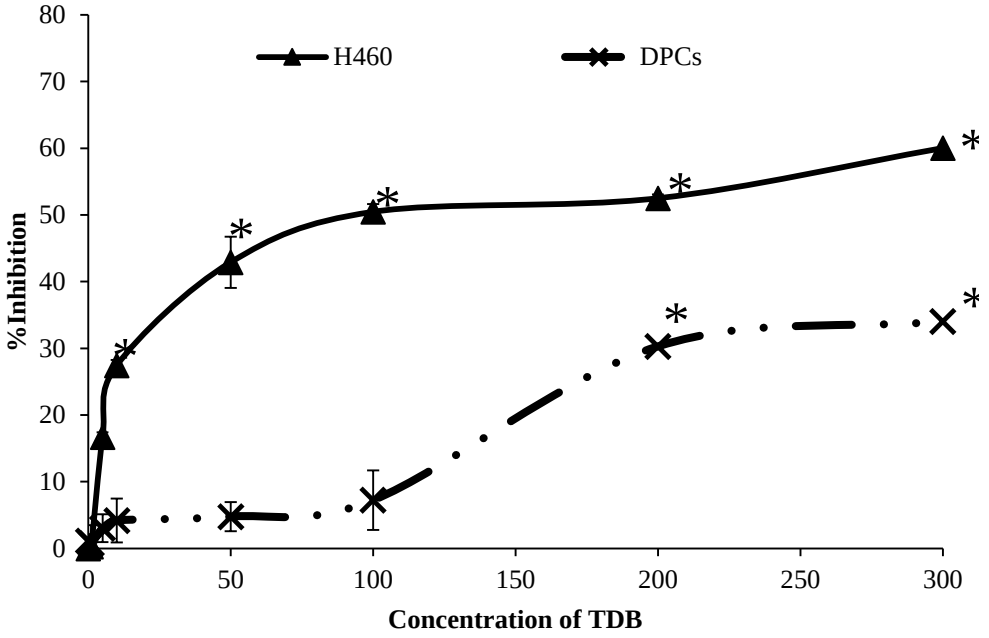


Figure 4.3 Selective anticancer activity of TDB in human lung cancer cells. The %inhibition on cell viability in human lung cancer H460 cells after treatment with TDB for 24 h was demonstrated in dose response curve of log scale. MTT assay revealed the significant reduction of cell viability in lung cancer cells incubated with 10-300 μM of TDB. This graph depicts %Inhibition = 100 - % cell viability for H460 and DP cells. Meanwhile, TDB showed no diminution (0-100 μM) and lower %inhibition (200-300 μM) on viability in DPCs cells compared with lung cancer cells treated with same concentrations. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

1.2 Flow cytometry detects the apoptosis-inducing effect of TDB

Apoptosis-inducing effect of TDB was further confirmed through annexin V-FITC/PI staining assay using flow cytometry. Annexin-V interacts specifically with phosphatidylserine which is characteristically presented on the surface of cell membrane during apoptosis while PI confirms cell death by staining cells that lose membrane integrity (151). Data analysis from flow cytometry revealed the increase of annexin V-stained human lung cancer H460 cells when incubated with TDB at 50 μM

for 24 h (figure 4.4 A). Figure 4B details the significant increase of both early (annexin V positive) and late (annexin V positive and PI positive) stage of apoptosis in TDB-treated cells compared with non-treated control cells. It was worth noting that PI-positive cells were not observed after exposure of H460 cells to TDB.

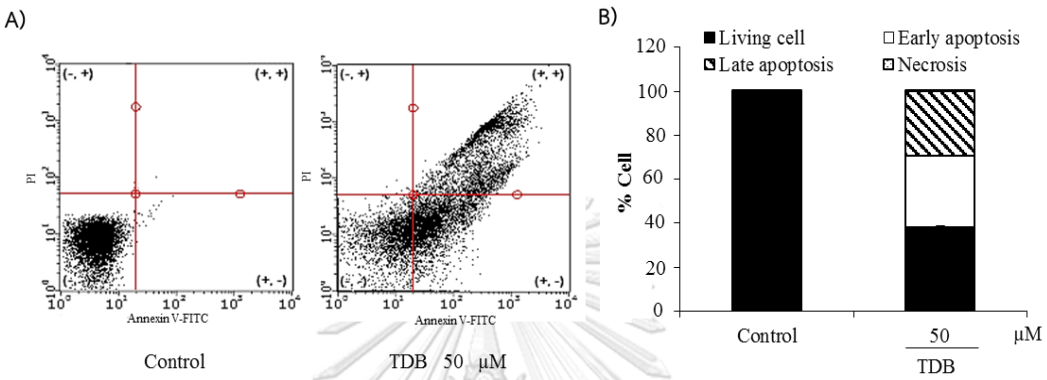


Figure 4.4 A) Chromatogram obtained from single cell analysis via flow cytometry indicated significant proportion of lung cancer H460 cells in apoptosis after treatment with 50 µM of TDB for 24 h. B) Flow cytometry also confirmed no necrosis detection in TDB-treated H460 cells. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

1.3 Alteration of apoptosis regulating proteins in TDB treated-lung cancer cells

Evaluation on apoptosis marker proteins was performed by western blot technique. The alteration on caspase cascade involving in apoptosis was first evaluated. Upon the incubation of human lung cancer H460 cells with 10-50 µM of TDB for 24 h, there was a significant increase of cleaved caspase-3 and cleaved PARP, both indicating the activation of caspase-3, an executor caspase in apoptosis cascade (figure 4.5 A). Because cleaved of PARP is a substrate of activated caspase-3 (152). Figure 4.5 B indicates the conversion from poly (ADP-ribose) polymerase (PARP) to

cleaved PARP correlated with augmentation of activated caspase-3 in TDB-treated H460 cells.

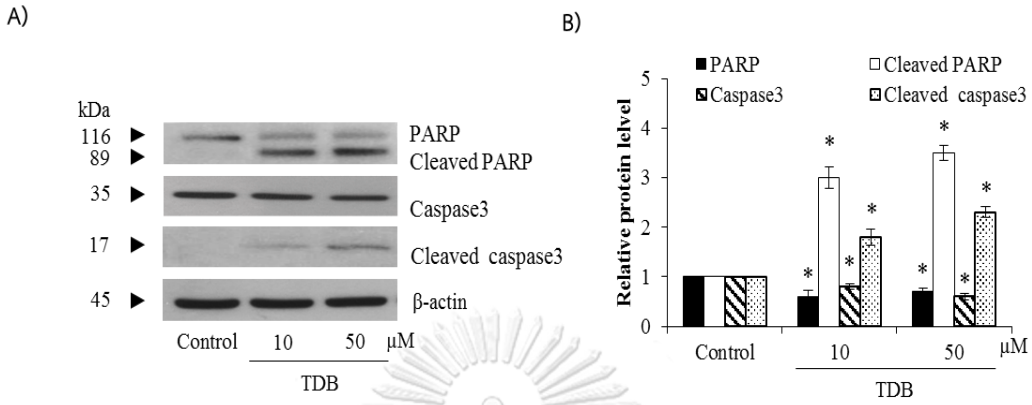


Figure 4.5 The activation of an apoptosis caspase cascade is demonstrated by A) the increase of cleaved caspase-3 and B) the conversion of PARP to cleaved PARP in human lung H460 cancer cells after treatment with 10–50 μM of TDB. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

1.4 TDB triggers intrinsic apoptosis pathway in human lung cancer cells

There are two major apoptosis pathways including intrinsic and extrinsic mechanism (153). The results showed that treatment for 12 h with TDB (10-50 μM) induced intrinsic pathway alteration of Bcl-2 family proteins in lung cancer cells. Anti-apoptosis Bcl-2 and Mcl-1 were decreased while the level of BAX, pro-apoptosis protein was increased by incubation of TDB in human lung cancer H460 cells (figure 4.6 A and B)

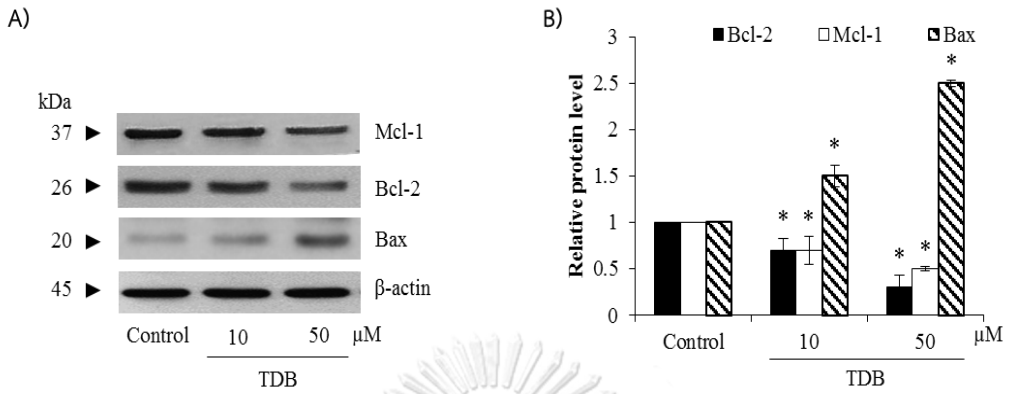


Figure 4.6 A) Western blot analysis elucidated the decline of anti-apoptosis proteins; Bcl-2 and Mcl-1 in human lung cancer cells incubated with 10–50 μM of TDB for 12 h. B) TDB also obviously up-regulated the expression level of Bax, a pro-apoptosis protein in H460 lung cancer cells in a dose-dependent manner. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

In order to clarify the involvement between TDB and extrinsic apoptosis, the alteration of death receptor (DR5) and caspase8 inhibitor (c-FLIP) was investigated. The results indicated that TDB did not induce apoptosis via extrinsic apoptosis pathway because DR5 and c-FLIP protein levels were unaltered after TDB treatment at 10-50 μM for 12 h (figure 4.7)

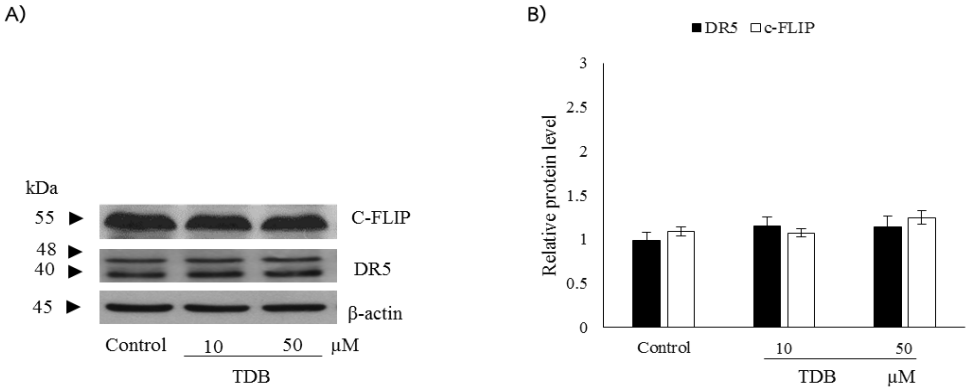


Figure 4.7 A) Protein levels of DR5 and C-FLIP in human lung cancer cells incubated with 10–50 μM of TDB for 12 h. B) TDB does not significantly alter levels of DR5, c-FLIP protein in H460 lung cancer cells. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

1.5 TDB activates p53 tumor suppressor pathway

Down-regulation of p53, a tumor suppressor protein is associated with uncontrolled cell division and malignancy (154). Chromatin damage and cellular stress are known to stimulate p53, resulting in cell cycle interruption and apoptosis induction (Shapiro GI et al, 1999). The p53 protein is also known to suppress Akt and Erk survival signaling pathways (155). Figure 4.8 A shows that p53 level was increased in human lung cancer cells after incubation with TDB at 10-50 μM for 12 h, while pro-survival Akt and p-Akt (phosphorylated-Akt) levels were also significantly reduced. However, no alteration of the Erk and p-Erk (phosphorylated-Erk) was noted (figure 4.8 B).

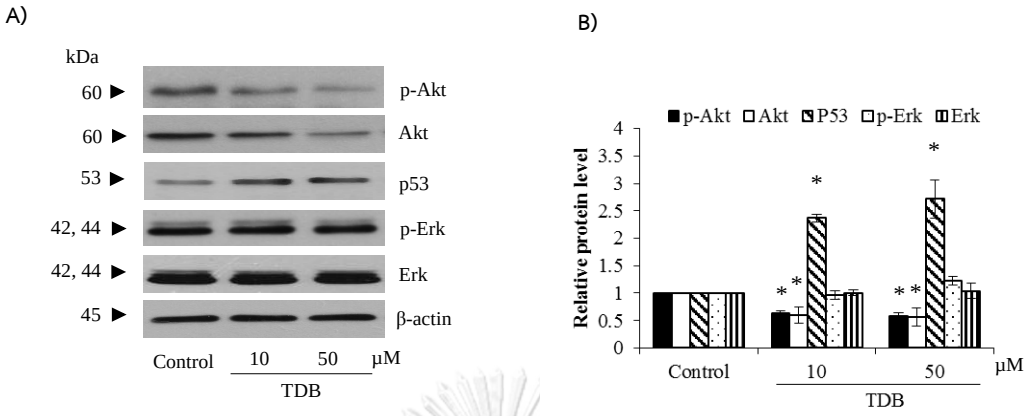


Figure 4.8 A) TDB modulated p53, Akt and p-Akt levels. B) Notably, the modification on survival pathway of Erk and p-Erk were not obviously detected in TDB-treated H460 cells. Data are represented as mean ± SD from three independent experiments. *$p \leq$ 0.05 versus non-treated control cells.

2. Investigation on relationship between ROS modulation and apoptosis induction in TDB-treated H460 lung cancer cells

2.1 TDB alters intracellular ROS level in human lung cancer cells

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 (156, 157). Thus, the alteration of cellular ROS level in H460 lung cancer cells was detected after treatment with TDB. Figure 4.8 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 1 mM of N-acetyl cysteine (NAC), a well-known anti-oxidant compound. It was worth nothing that non-toxic concentration (100 μM) of hydrogen peroxide (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.

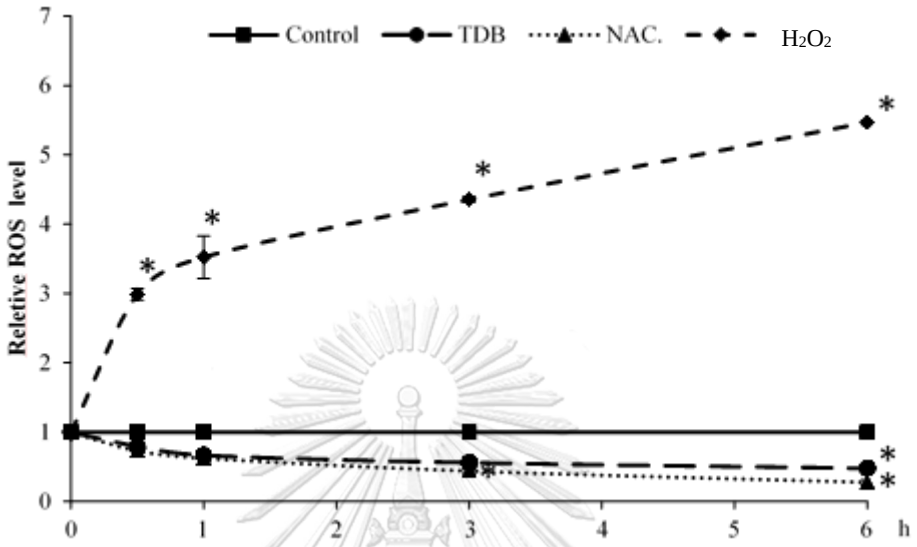


Figure 4.9 The relative of ROS level detected in human lung cancer H460 cells after treatment with TDB 50 μM for 0.5, 1, 3 and 6 h. NAC (1 mM) and H_2O_2 (100 μM) treatment were used as negative and positive control, respectively. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells at the same time point.

2.2 Apoptosis-inducing activity of TDB is related to its modulation on cellular ROS

Due to the observed anti-oxidant activity of TDB in H460 cells, the link between cellular ROS alteration and cytotoxicity was further investigated. Figure 4.9 A indicates that pretreatment of human lung cancer cells with 100 μM of H_2O_2, a ROS generator for 30 min significantly preserved cell viability in the cells exposed with 50 μM of TDB for 24 h. Interestingly, pre-incubation with H_2O_2 retrained apoptosis induced by TDB (50 μM) in human lung cancer cells (figure 4.10 B). These results indicate that TDB-mediated apoptosis in human lung cancer cells is ROS-dependent.

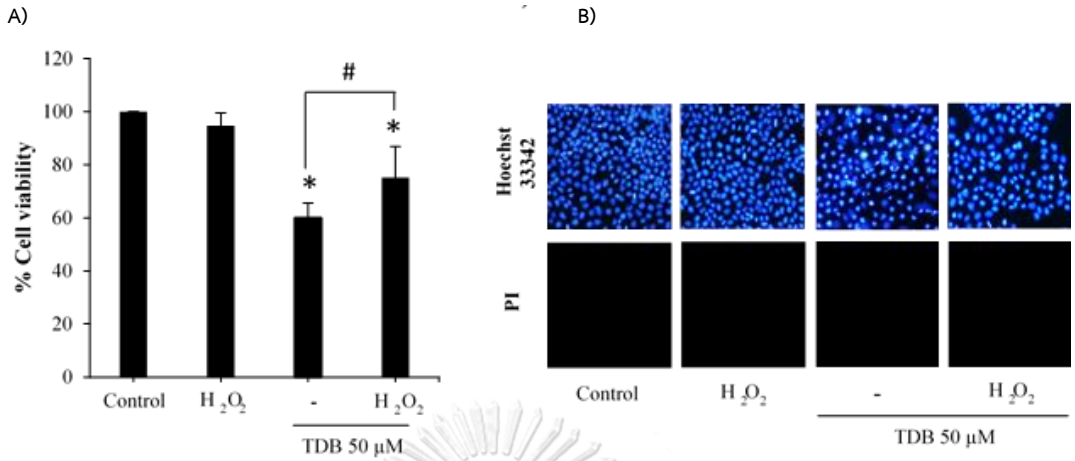


Figure 4.10 TDB mediates ROS-dependent apoptosis A) Pretreatment with 100 µM of H2O2 decreased TDB cytotoxicity in lung cancer H460 cells B) The diminution of apoptosis cell death was notified in the cells pretreated with 100 µM of H_2O_2 for 30 min before further incubation with 50 µM of TDB for 24 h. 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.

3. TDB suppresses CSCs in human lung cancer cells

3.1 Tumor initiating activity of CSCs enriched population evaluated through spheroid formation assay

Cancer stem-like cells (CSCs) enriched population is successfully selected through culture of human lung cancer cells in serum free medium under detachment condition for 21 days (158). The capability to generate new tumor colony of CSCs enriched population was investigated in spheroid formation assay. After further culture for 7 days in ultra-low attachment plate, there was significant reduction of relative size of tumor colony in CSCs enriched population treated with TDB at 50-100 μM (figure 4.10 A). Moreover, the inhibition on tumor initiating activity in CSCs enriched H460 population was early observed at day 3 of incubation with TDB (figure 4.11 B).

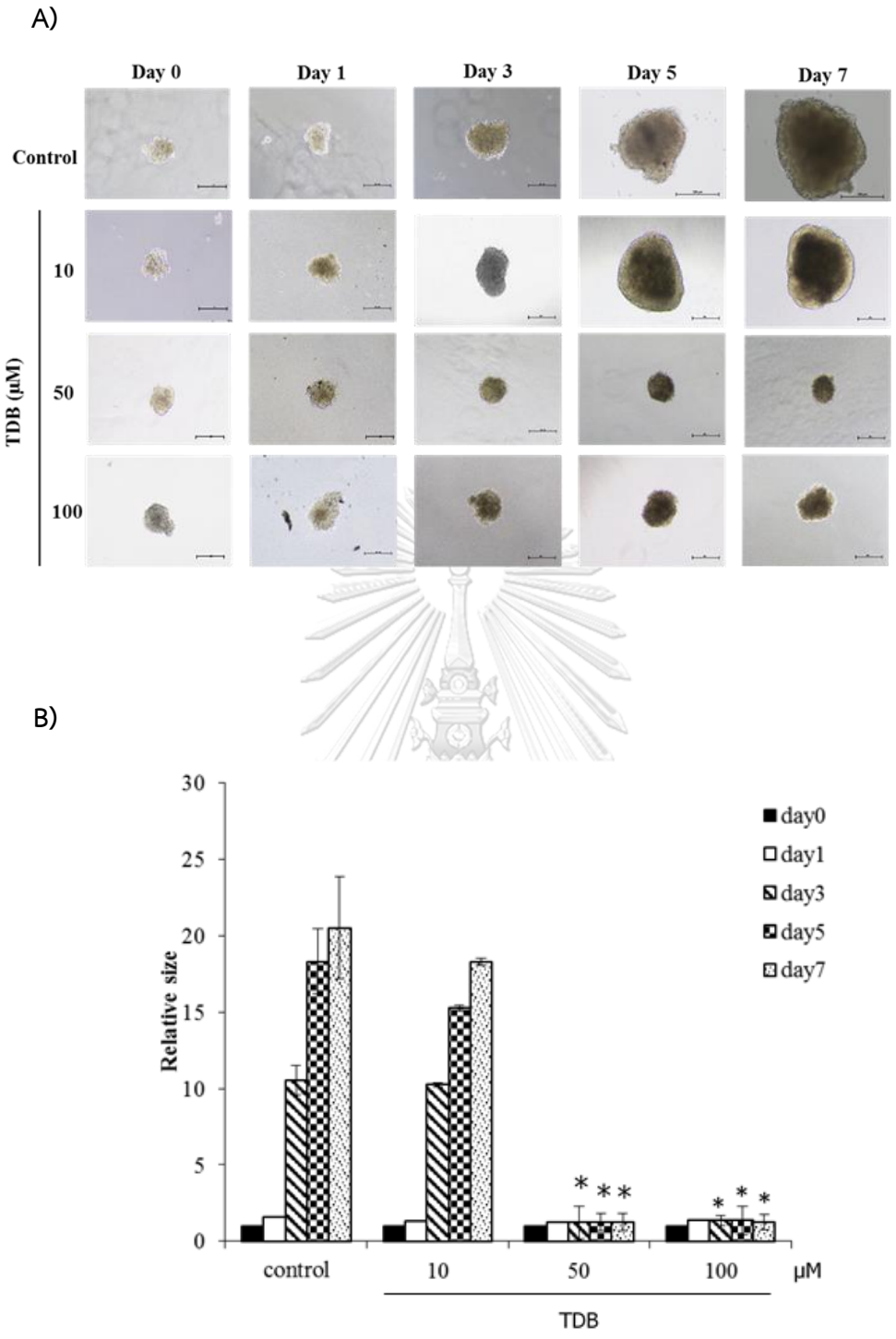


Figure 4.11 Effect of TDB on in vitro spheroid formation A) CSCs enriched H460 cells were cultured with or without TDB (10-100 μM) in ultra-low attachment plates for 7 days. **B)** There was the reduction of relative size of CSCs enriched colony of human lung cancer cells after incubation with 50-100 μM of TDB for 3 days. Data are

represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells at the same time point.

3.2 Limiting dilution assay (LDA) shows the decrease in CSCs population in TDB-treated lung cancer cells

The limiting dilution assay (LDA) is a method used to determine number of CSCs in a certain population (150). The LDA was performed to examine the effect of TDB on suppression of CSCs. The LDA results suggest that the CSC populations decreased with treatment of 10-100 μM TDB (figure 4.12A and B). The results remarkably show that while the CSCs in the non-treated control cells expectedly possess the ability to form tumor spheres, the CSCs in H460 cells treated with TDB were not able to generate colonies. This further provides evidence that TDB treatment reduces activity or the presence of CSC subpopulations.

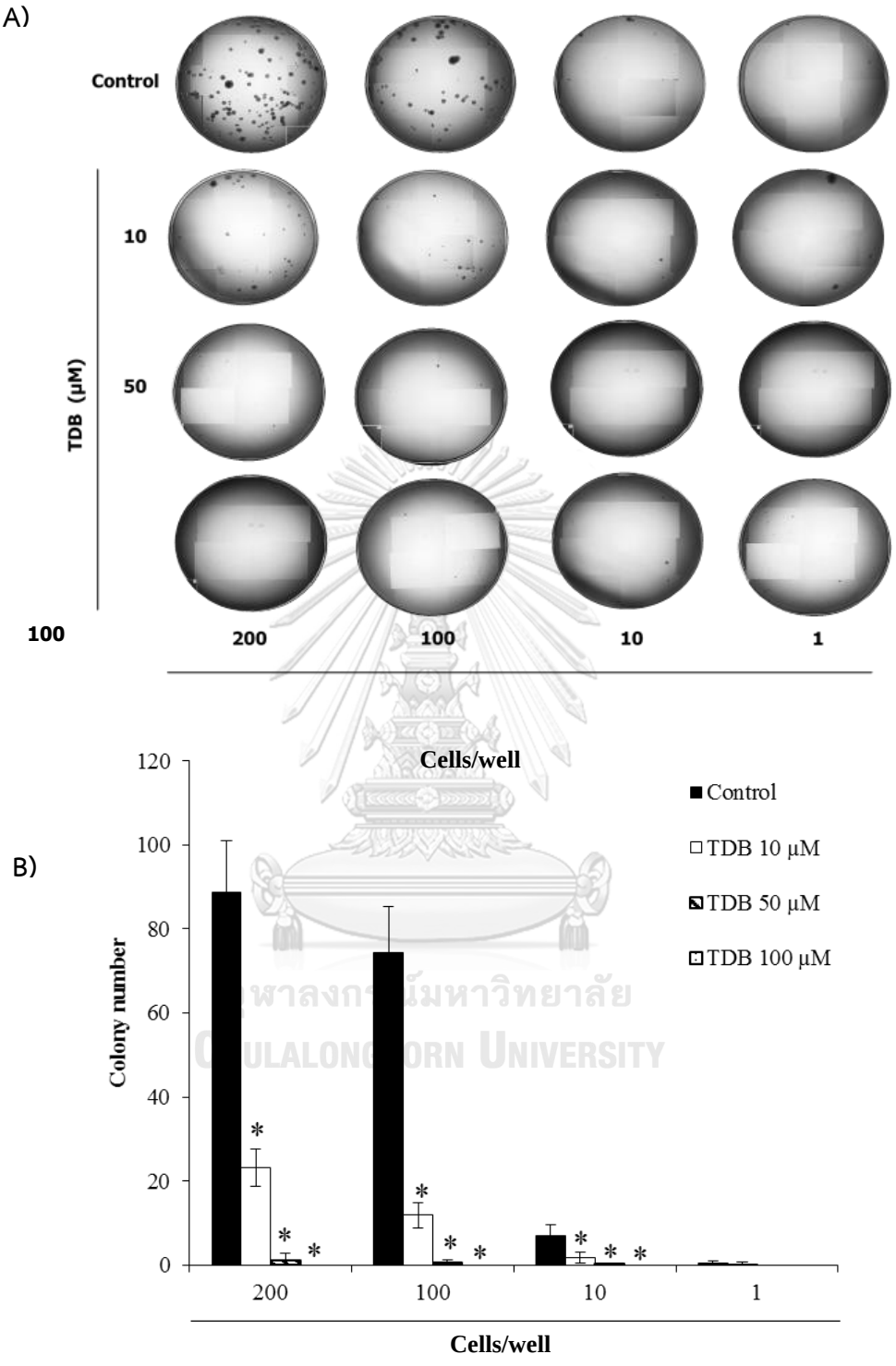


Figure 4.12 A) Representative photos of wells with spheroid growth at day 14 obtained by limited dilution assay treated with TDB 0-50 μM. **B)** Number of counted colony after treatment with TDB 0-50 μM after 14 days of incubation. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

3.3 TDB alteration of CSC markers in enriched CSCs population of H460 cells treated with TDB

To repair the damage induced by chemical, physical or mutational causes, CD133+ progenitor or stem cells are activated (108). Stem cells have the crucial ability of self-renewal which would be detrimental in the cancer context. To track the CSCs subpopulation, flow cytometry was used to detect CD133, a commonly used marker for CSCs in lung cancer. Flow cytometry of single cells obtained from enriched CSCs colony showed the significant reduction of CD133-positive cells in treatment with TDB at 50 µM for 3 days (figure 4.13).

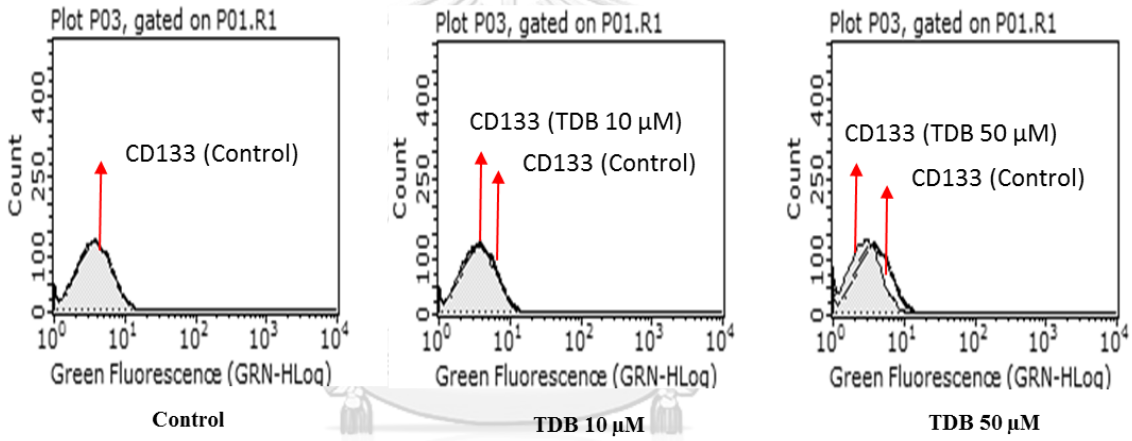


Figure 4.13 Chromatogram obtained from flow cytometry of CSCs enriched population revealed the reduction of CD133-positive H460 cells upon treatment with TDB at 10-50 µM.

3.4 TDB regulates the expression of stemness-related transcription factors

Up-regulation of the three proteins Oct4, Nanog and Sox2 are crucial in the development and maintenance of stemness feature in cancer cells. Because the obvious inhibition on growth of CSCs colonies was already observed, the enriched CSCs populations of H460 treated with TDB 0-50 µM for 3 days were analyzed for the expression of stemness regulating proteins through western blotting analysis. After culture with 50 µM of TDB, expression level of stemness transcription factors, Sox2 and

Nanog were significantly decreased. On the other hand, TDB did not alter Oct-4 protein level when compared to untreated control (figure 4.14 A and B).

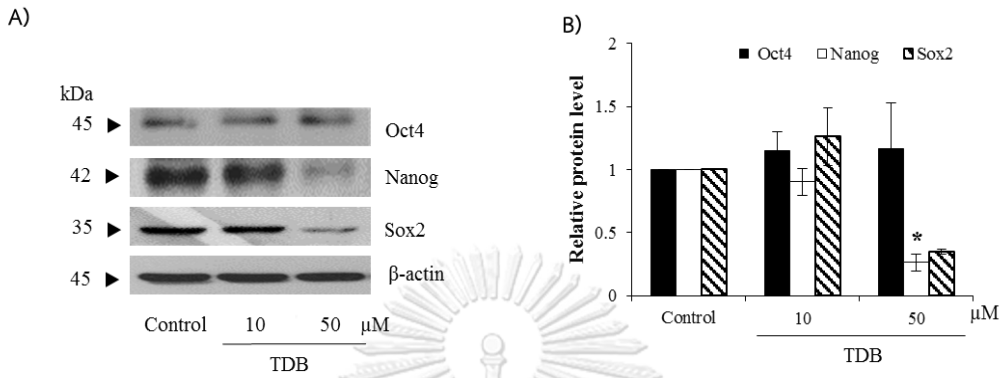


Figure 4.14 A) Western blot analysis revealed TDB effect on Sox2, Oct-4 and Nanog in CSCs-enriched population of human lung cancer cells B) The obviously alteration of Nanog and Sox2 expression level were indicated in treatment with 50 μM of TDB. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

Due to β-catenin's role as transcription factor promoting stem cell maintenance, its inhibition is a current therapeutic goal for cancer treatment (159). GSK3β causes phosphorylation of β-catenin which in turn activates its degradation (160). While, the levels of β-catenin were decrease, western blot analysis revealed no change in GSK3β and pGSK3β expression level in lung CSCs enriched population treated with 10-50 μM of TDB (figure 4.15 A and B).

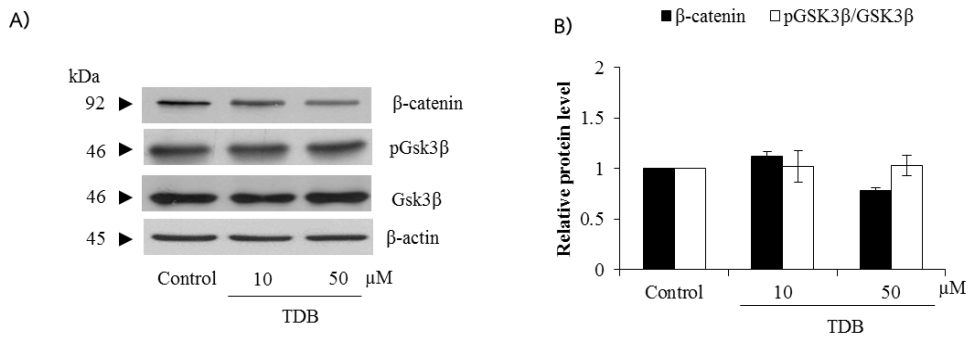


Figure 4.15 A) Western blot analysis revealed TDB effect on β-catenin and pGSK3β/GSK3β expression in CSCs enriched population of human lung cancer cells incubated with 0–50 μM of TDB for 3 days. B) The alteration of pGSK3β/GSK3β expression level was not changed while there were the decrease levels of β-catenin in TDB treated cells. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

3.5 Role of ROS on inhibitory effect of TDB on CSCs in human lung cancer cells

Investigation on ROS role in stemness is an important emerging field as previous research shows that the existence of CSCs is modulated by ROS. Spheroid formation assay measures the capacity of CSCs in a population to generate a new tumor colony. After 7 days of incubation in ultra-low attachment plate, relative size of CSCs-enriched spheroid colonies was significantly reduced by TDB treatment at 50 μM. Interestingly, There were no significant alteration of colony size between CSCs enriched population treated with TDB (50 μM) compared with combination treatment of H_2O_2 (10 μM) and TDB (figure 4.16). It was found that H_2O_2 did not affect the inhibitory activity of TDB on CSCs population.

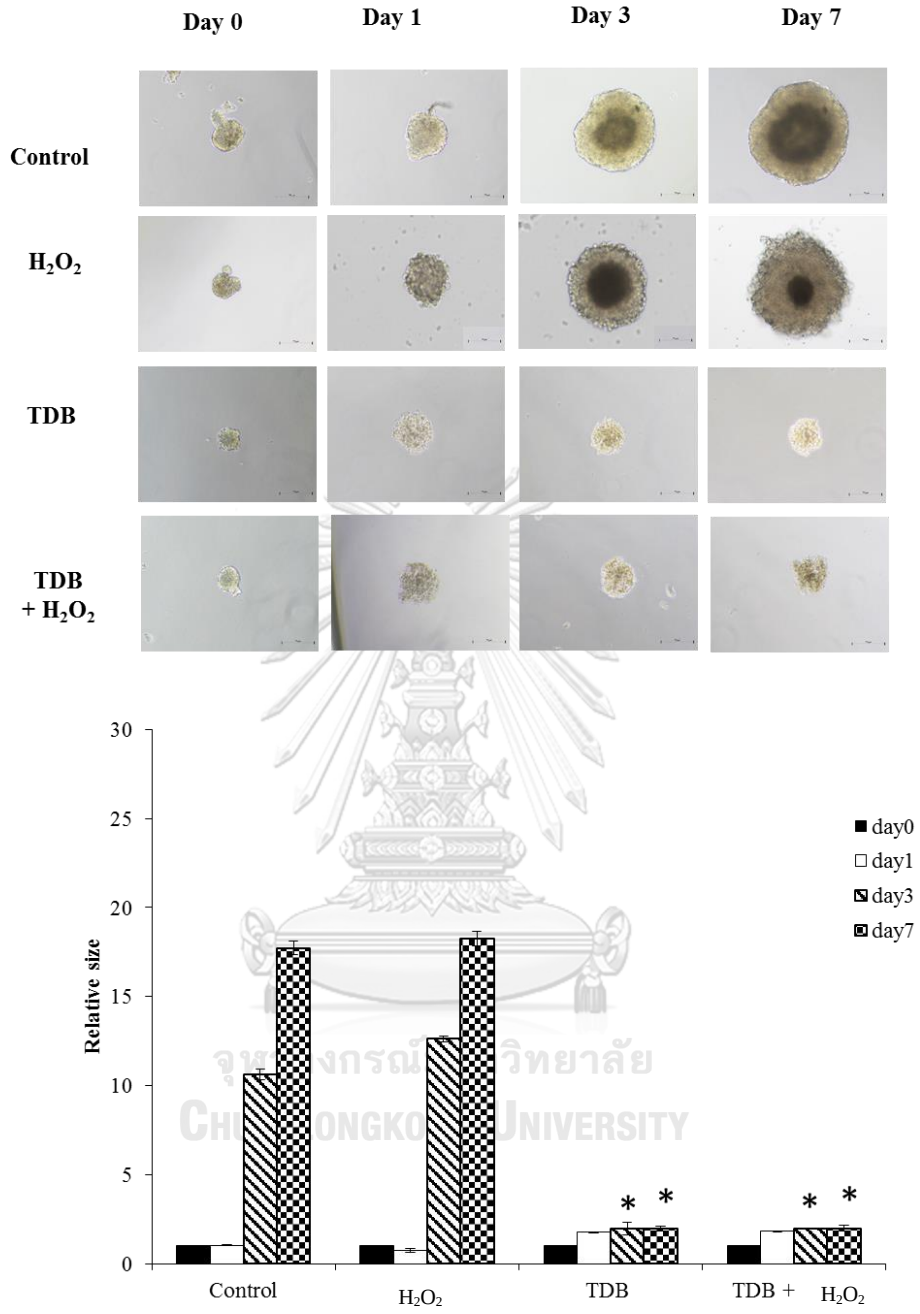


Figure 4.16 Effect of ROS in spheroid formation **A)** CSC-enriched H460 cells were cultured with treated with TDB (50 μM), H_2O_2 (10 μM) and TBD (50 μM) + H_2O_2 pre-treatment in ultra-low attachment plates for 7 days. **B)** Relative size of CSCs enriched colony of human lung cancer cells. Data are represented as mean ± SD from three

independent experiments. *$p \leq 0.05$ versus non-treated control cells at the same time point.

3.6 Role of ROS on inhibitory activity of TDB on CSCs

To study the specific role of ROS in CSCs was farther investigated via Limiting dilution assay (LDA). Figure 4.17 indicated no significant alteration of colony number between TDB (50 μM) treatment and pretreatment with TDB following with H_2O_2 (10 μM).

These results demonstrated that inhibitory effect of TDB on CSCs in human lung cancer cells does not involve with it's antioxidant activity.

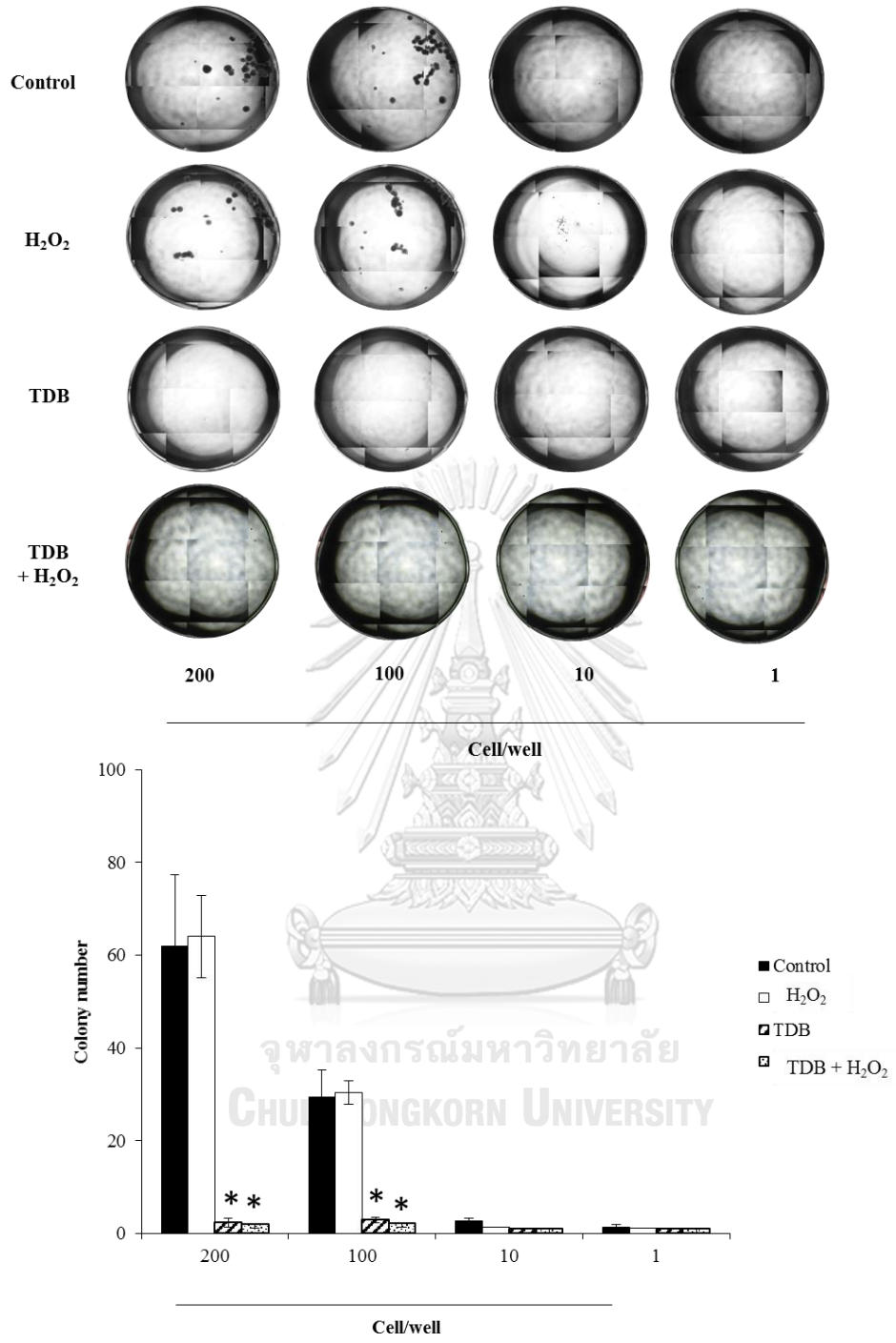


Figure 4.17 A) On day 14, photos were taken of spheroids obtained by limiting dilution assay with H_2O_2 10 μM treatment, TDB 50 μM treatment and TBD 50 μM + H_2O_2 10 μM treatment as compared to non-treated control. **B)** Relative colony count was determined. Data are represented as mean ± SD from three independent experiments. *$p \leq 0.05$ versus non-treated control cells.

CHAPTER V
DISCUSSION AND CONCLUSION

Lung cancer has a very high recurrence rate, and the highest mortality rate among various cancers (161, 162). There are many factors for the aggressive nature of the disease, among which are the up-regulation of pro-survival and anti-apoptotic proteins, and the presence of cancer stem cells in the population (163, 164). Natural compounds have garnered much research interest because of the ability to induce apoptosis in cancer cells while maintaining a good safety profile (43). Apoptosis induction is a primary therapeutic goal in cancer because apoptosis causes less damage to surrounding tissue, compared with the more destructive necrosis (165, 166). Extracts from *Dendrobium ellipsophyllum* have previously showed cytotoxic, anoikis sensitization and migration suppression effects in lung cancer cells (44-46). Consistent with such results, the IC_{50} of TDB (4,5,4'-trihydroxy-3,3'-dimethoxybibenzyl) in H460 cells as 100 ± 5.18 μM was reported in this study. Although it is already known that TDB can induce apoptosis, the apoptosis regulating pathways have never been revealed. This study provides more proof furthering the potential of the TDB as an anticancer agent.

This research provides good evidence for the further development of TDB as an anti-cancer drug because we show that it possesses selective cytotoxicity to cancer cells. The induction of apoptotic cell death in H460 lung cancer by TDB but not in dermal papilla cells indicates that the extract is more selective towards cancer cells (figure 4.1). Dermal papilla are cells forming the normal hair follicle, and as such are rapidly dividing normal cells (167, 168). This characteristic makes these cells a target of cancer drugs leading to the known side effect of chemotherapy-induced alopecia (CIA). The use of dermal papillae allows an approximation of selectivity of a cancer drug and helps address this common side effect in many chemotherapeutic regimens (169, 170). This is important because CIA decreases quality of life of patients (3). The safety profile of TDB at 0–100 μM was indicated with the selective cytotoxicity against human lung cancer cells with no alteration on cell viability of human DPCs (figure. 4.2).

Moreover, it is possible that TDB is not toxic to DPCs because of its antioxidant effect, whereas cisplatin usually induces reactive oxygen species (ROS) related to cell death in DPCs (170). Likewise, TDB might induce DNA damage in cancer cells because DNA repair mechanism is defective in cancer cells (171), while DP cells have intact repair mechanisms. Overall, these results indicate a good safety profile and selective anticancer activity of the TDB from *D. ellipsophyllum.*

The major novel finding accomplished through this study is the elucidation of the pathway involved in TDB effect of apoptosis induction. TDB induces apoptosis by the up-regulation of the p53 tumor suppressor protein, intrinsic pathway of apoptosis and the related attenuation of survival signaling (figure. 4.5-4.8). Altered function and down-regulation of p53 suppressor protein have been correlated to the aggressive nature of lung cancer (172, 173). Evidence showed that restored p53 function is enough to initiate apoptosis in lung cancer cells (174). The confirmation of apoptosis-inducing activity was evidenced by apoptosis features detected, including the localization of phosphatidylserine on the cell membrane outer surface, caspase-3 activation and related decrease of its substrate, PARP in TDB-treated lung cancer cells (figure. 4.5). Moreover, the up-regulation of p53 noted in TDB treatment is also consistent with other evidence showing other natural extracts inducing apoptosis via p53 activation (175, 176). As a transcription factor, p53 is known to regulate multiple signaling pathways. Cells overexpressing p53 exhibited down-regulation of anti-apoptosis proteins and pro-survival proteins (23, 25). Likewise, TDB treatment weakens Akt survival signaling in human lung cancer cells (figure. 4.8). Moreover, Akt also promotes drug resistance via the PI3K/Akt pathway (177). Up-regulation of Akt is also associated with chemotherapeutic failure in lung cancer (178, 179). The reduction of p53 and Akt signaling found in TDB-treated lung cancer cells supports the possibility to develop TDB as an anticancer compound.

Another point investigated in this study is TDB effect on the Bcl-2 family proteins, as these proteins are the key players in intrinsic pathway of apoptosis (131, 180). The TDB-induced alteration of Bcl-2 family proteins could result from p53 activation. The augmentation of Bax as well as the reduction of Mcl-1 and Bcl-2 corresponded with the expression of p53 in human lung cancer cells that were

exposed with TDB (figure. 5.1). The up-regulation of pro-apoptosis Bax and down-regulation of anti-apoptotic proteins, Mcl-1 and Bcl-2, are both known to be the effects of the transcriptional function of p53 (181, 182). Bax activity is a crucial step in mitochondria-mediated apoptosis, because it increases the permeabilization of mitochondrial membrane that results in the release of cytochrome c triggering the apoptosis cascade (183). Moreover, the Mcl-1 and Bcl-2 over-expression in lung cancer causes increased resistance to chemotherapeutic drugs and poorer prognosis for patients (66, 184). The attenuation of these anti-apoptosis proteins successfully induces apoptosis in cancer cells (185).

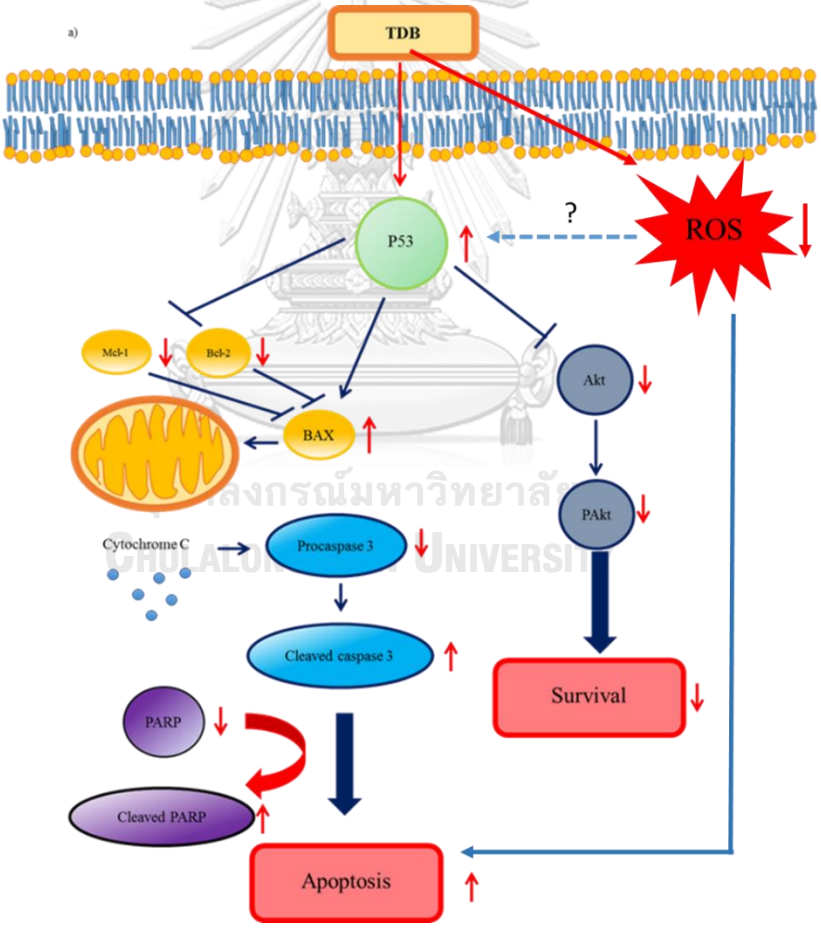


Figure 5.1 TDB induces ROS-dependent apoptosis by intrinsic pathway and decreases signaling by p53 activation (186).

Another interesting highlight of this research is that TDB cytotoxicity is linked to ROS modulation observed in cells treated with TDB. The apoptotic effect induced by many natural compounds is dependent on reactive oxygen species (ROS) modulation (187, 188). ROS are known to act as signaling molecules that regulate the survival or death of cells (137). While oxidative stress is a known effect of several anticancer drugs that cause DNA damage and subsequent apoptosis (158), some anti-oxidant extracts also have been shown to induce apoptosis in various cancer types (189). An example of a known anti-cancer compound that decreases ROS is curcumin (190, 191). Other phenolic compounds have antioxidant effect and can induce DNA damage in cancer cells. An example of which is resveratrol which can induce apoptosis in cancer cells by DNA damage (192). Similarly, we report that TDB cytotoxicity is linked to its antioxidant effect (figure 4.9-4.10). It is possible that antioxidant compounds can induce apoptosis by DNA damage through methylation on DNA bases (193).

Most importantly, the novel activity established herein is the inhibition of cancer stem cell characteristics in lung cancer cell population. It is already known that CSCs play an important role in tumorigenesis. CSCs retain the key stem-cell characteristics of pluripotency and self-renewal. Because of this, CSCs are theorized to originate from general or progenitor stem cells (194). It is important to study CSCs because they are considered to generate various types of malignant cells (195). CSCs contribute to tumor progression and other aggressive features such as resistance to chemotherapy and radiotherapy (103). Natural products are valuable pharmaceutical products because of their efficacy and relative safety. More importantly, such moieties have already shown great promise in the treatment of CSCs, which is a major problem in therapy (106, 196).

Spheroid culture of cancer cell lines is known to induce an enrichment in CSCs. The spheroid formation assay and limiting dilution assay (LDA) results suggested that TDB treatment reduced the size and number of CSCs population (figure 4.11-4.12). Furthermore, the tracking of the population of H460 stem cells marked by known stem cell markers CD133 by flow cytometry revealed the reduction of the stem cell population after a 3-day treatment with 50 μM TDB treatment (figure. 4.13). Although, the results did show that decreased alterations of β-catenin, the transcription factors

Sox2, Nanog protein were decreased (figure 4.14-4.15). The transcription factor, Sox2 contributes to the maintenance of cell renewal, differentiation of stem cells and also aids in stem cell reprogramming (197). Moreover, pluripotency and stem cell reprogramming are regulated by the Nanog master transcription factor (198). Significantly, poor prognosis in lung cancer patients may be correlated with upregulation of NANOG (122, 199). Other research shows that lung malignancy patients with overexpression for Nanog Furthermore Oct4 exhibited low survival rate (121, 125).

TDB treatment caused decreased levels of Sox2 and Nanog but Oct4 level is unaltered. The expression of these stemness transcriptional factors has been related to the Wnt/β-catenin pathway, which was investigated in this study (124, 125). The interaction between β-catenin and T-cell factor (TCF) is known to stimulate the transcription of Oct4, Sox2 and CD44 (126). Evidence shows that p53 also regulates the function of GSK3β which can suppress CSCs via Wnt/β-catenin pathway inhibition (127, 131). Because inactivation of p53 is known to induce stemness in cancer cell subpopulations (128), p53 down-regulation induced by TDB was very promising result. However, upon investigating this pathway, it was found that TDB does not significantly alter p-GSK3β/GSK3β levels. Such results suggest that other pathways may be involved in the decreased expression of the stemness transcription factors induced by TDB. Other pathways to be investigated may include the Notch signaling pathway and Hedgehog pathway among others (200, 201). More importantly, the PI3K/Akt pathway may be involved in CSC regulation. There is also evidence that down-regulation of Oct4 and Nanog transcription factors may be a consequence of phosphorylated Akt reduction (114, 196). TDB-induced p-Akt down-regulation may contribute to suppression of cancer stem cell-like phenotype (figure. 5.2).

Interestingly, the experiment results of the spheroid assay suggest that ROS does not seem to cause CSC inhibition induce by TDB. Treatment of non-toxic concentration of H_2O_2 did not alter the suppression effect of TDB on size of CSCs enriched population (figure 4.16). Non-dependence on ROS modulation of CSCs inhibitory effect of TDB was also supported by LDA assay (figure. 4.12).

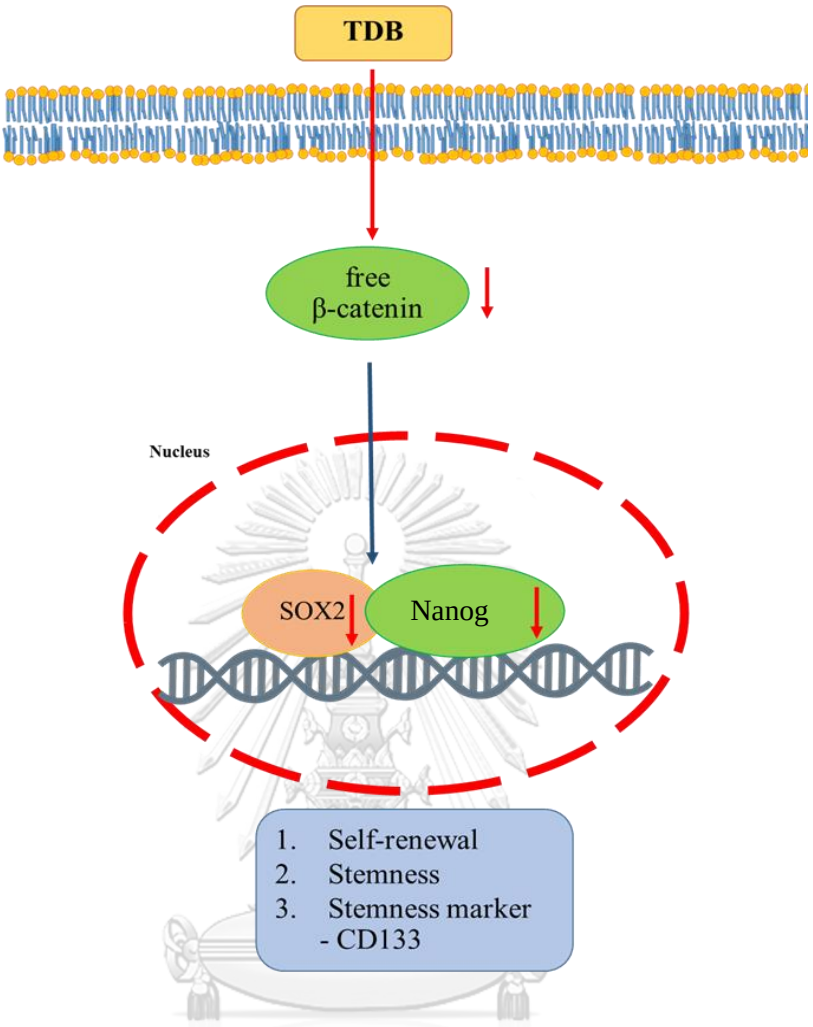


Figure 5.2 The scheme represents the effect of TDB on human lung cancer cells. The present study reveals that TDB has an ability to reduce the level of CD133, a known CSC marker, by suppressing the activation of self-renewal factors Nanog and Sox 2.

By conclusion, TDB extracted from *D. ellipsophyllum* demonstrated stemness suppression, selective apoptosis induction via intracellular ROS reduction in human lung cancer cells. The results obtained from this study strengthens the potential development of TDB as an anticancer compound with a favorable safety profile and high efficacy that could provide a novel solution to treatment failure due to malignant behavior and stemness.

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APPENDIX

APPENDIX

TABLES AND FIGURES OF EXPERIMENTAL RESULTS

Table 2 Cell viability of human lung cancer cells after treatment with TDB for 24 h

TDB extracts (μM)	Cell viability (%)
Control	100 ± 0.00
1	98.04 ± 3.24
5	83.84 ± 3.51
10	71.47 ± 5.25*
50	57.61 ± 1.01*
100	52.34 ± 5.98*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 3 percent apoptosis of human lung cancer cells after treatment with TDB for 24 h

TDB extracts (μM)	Apoptosis (%)
Control	0.00 ± 0.00
1	0.82 ± 0.25
5	1.14 ± 0.63
10	11.05 ± 0.93*
50	47.95 ± 4.04*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 4 Cell viability of human dermal papilla cells after treatment with TDB for 24 h.

TDB extracts (μM)	Cell viability (%)
Control	100 ± 0.00
1	103.90 ± 6.16
5	93.81 ± 5.66
10	96.53 ± 4.43
50	95.67 ± 2.94
100	93.81 ± 6.29

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 5 percent apoptosis of human dermal papilla cells after treatment with TDB for 24 h

TDB extracts (μM)	Apoptosis (%)
Control	0.00 ± 0.00
1	0.37 ± 0.18
5	1.65 ± 0.23
10	1.70 ± 0.64
50	5.02 ± 0.79*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 6 inhibition of human lung cancer cell and human dermal papilla cells after treatment with TDB for 24 h.

TDB extracts (μM)	H460 cells Inhibition (%)	DPs cells Inhibition (%)
Control	0.00 ± 0.00	0.00 ± 0.00
1	0.27 ± 0.67	0.71 ± 0.76
5	16.67 ± 2.14	3.01 ± 2.74
10	27.44 ± 0.59	4.18 ± 2.08
50	42.90 ± 0.72	4.76 ± 3.26
100	50.46 ± 0.81	7.24 ± 2.18
200	52.49 ±3.83	30.30 ± 4.46
300	60.01 ± 1.16	34.05 ± 0.24

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 7 Mode of cell death detected in TDB-treated lung cancer cells via flow cytometry

TDB extracts (μM)	Living cell (%)	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Control	99.27 ± 0.02	0.57 ± 0.42	0.16 ± 0.11	0 ± 0.00
50	37.98 ± 0.21	32.69 ± 0.32	29.22 ± 0.65	0.11 ± 0.62

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 8 Relative level of apoptosis marker proteins in lung cancer cells treated with TDB for 24 h

TDB extracts (μM)	Relative protein level			
	PARP	Cleaved-PARP	Caspase 3	Cleaved-caspase 3
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10	0.61 ± 0.12*	3.05 ± 0.23*	0.70 ± 0.09*	2.00 ± 0.16*
50	0.72 ± 0.07*	3.50 ± 0.15*	0.60 ± 0.15*	2.30 ± 0.11*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 9 Relative level of intrinsic apoptosis proteins in lung cancer cells treated with TDB for 12 h

TDB extracts (μM)	**Relative protein level**		
	Mcl-1	Bcl-2	BAX
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10	0.70 ± 0.15*	0.70 ± 0.13*	1.30 ± 0.11*
50	0.40 ± 0.13*	0.30 ± 0.03*	1.60 ± 0.05*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 10 Relative level of extrinsic apoptosis proteins in lung cancer cells treated with TDB for 12 h

TDB extracts (μM)	Relative protein level	
	DR5	c-FLIP
Control	1.00 ± 0.00	1.00 ± 0.00
10	1.15 ± 0.09	1.07 ± 0.04
50	1.13 ± 0.10	1.24 ± 0.05

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 11 Relative level of p53 regulating proteins in lung cancer cells treated with TDB for 12 h

TDB extracts (μM)	**Relative protein level**				
	Akt	p-Akt	Erk	p-Erk	p53
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10	0.60 ± 0.05*	0.63 ± 0.15*	1.00 ± 0.02	0.98 ± 0.01	2.37 ± 0.07*
50	0.56 ± 0.07*	0.58 ± 0.17*	1.03 ± 0.03	1.02 ± 0.14	2.71 ± 0.30*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 12 Relative ROS level in human lung cancer cells treated with TDB

Time (h)	Control	TDB 50 μM	NAC 1 mM	H_2O_2 100 μM
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0.5	1.00 ± 0.00	0.79 ± 0.01	0.72 ± 0.02	2.99 ± 0.08*
1	1.00 ± 0.00	0.66 ± 0.01	0.60 ± 0.07	3.52 ± 0.30*
3	1.00 ± 0.00	0.56 ± 0.02	0.52 ± 0.02*	4.35 ± 0.04*
6	1.00 ± 0.00	0.47 ± 0.03*	0.23 ± 0.03*	5.46 ± 0.03*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 13 Cell viability of human lung cancer cells after treatment with TDB for 24 h with or without pretreatment with H_2O_2

Treatment	Cell viability (%)
Control	100±0.00
H_2O_2 100 μM	94.82±4.61
TDB 50 μM	60.14±5.48*
TDB 50 μM and H_2O_2 100 μM	75.16±11.76*, #

Values are means of the independent triplicate experiment ± SD.

* $p < 0.05$ versus non-treated control.

# $p < 0.05$ versus the cells treated with only TDB.

Table 14 Relative size of CSCs enriched H460 lung cancer cells

Day	control	TDB 10 μM	TDB 50 μM	TDB 100 μM
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
1	1.59 ± 0.04	1.34 ± 0.05	1.25 ± 0.01	1.38 ± 0.07
3	10.51 ± 0.97	10.25 ± 0.13	1.23 ± 1.05*	1.35 ± 0.30*
5	14.29 ± 2.12	15.25 ± 0.19	1.24 ± 0.56*	4.69 ± 0.95*
7	16.50 ± 3.35	18.22 ± 0.22	1.25 ± 0.57*	3.50 ± 0.50*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control at the same time point.**

Table 15 Number of forming colony of human lung cancer cells evaluated through limiting dilution assay (LDA)

TDB (μM)	200 cells/well	100 cells/well	10 cells/well	1 cell/well
Control	88.63 ± 12.35	74.27 ± 11.06	7.00 ± 2.49	0.54 ± 0.52
10	23.27 ± 4.43*	11.90 ± 2.98*	1.81 ± 0.01*	0.20 ± 0.00
50	1.09 ± 0.00*	0.54 ± 0.00*	0.09 ± 1.33*	0.00 ± 0.42
100	0.00 ± 1.64*	0.00 ± 0.69*	0.00 ± 0.30*	0.00 ± 0.00

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 16 Relative level of stemness regulating proteins in CSCs enriched population of human lung cancer cells

TDB extracts (μM)	Relative protein level		
	Sox2	Nanog	Oct-4
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10	1.26 ± 0.23	0.90 ± 0.10	1.14 ± 0.14
50	0.35 ± 0.02*	0.26 ± 0.06*	1.16 ± 0.36

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 17 Relative protein level of β-catenin related pathways in CSCs enriched population of human lung cancer cells

TDB (μM)	Relative protein level	
	β-catenin	pGSK3β/GSK3β
Control	1.00 ± 0.00	1.00 ± 0.00
10	1.16 ± 0.21	1.02 ± 0.44
50	0.74 ± 0.03*	1.03 ± 0.23

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control.**

Table 18 Effect of ROS on **r**elative size of CSC-enriched H460 spheroid colonies

Day	control	TDB 50 μM	H_2O_2 10 μM	H_2O_2 10 μM + TDB 50 μM
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
1	1.03 ± 0.03	1.74 ± 0.04	0.71 ± 0.01	1.81 ± 0.02
3	10.62 ± 0.30	1.96 ± 0.36*	12.62 ± 0.14	1.95 ± 0.03*
7	17.72 ± 0.38	1.95 ± 0.14*	18.24 ± 0.42	1.98 ± 0.16*

Values are means of the independent triplicate experiment ± SD.

*** $p < 0.05$ versus non-treated control at the same time point.**

Table 19 Effect of ROS on CSCs evaluated through limiting dilution assay (LDA)

TDB (μM)	200 cells/well	100 cells/well	10 cells/well	1 cell/well
Control	62.00 ± 15.25	29.33 ± 10.11	2.67 ± 4.58	1.33 ± 0.57
TDB 50 μM	2.34 ± 0.97*	2.93 ± 0.56*	1.00 ± 0.01	1.00 ± 0.00
H_2O_2 10 μM	64.00 ± 8.88	30.33 ± 2.51	1.30 ± 0.00	1.20 ± 0.00
TDB 50 μM + H_2O_2 10 μM	2.10 ± 0.96*	2.30 ± 0.50*	1.00 ± 0.02	1.00 ± 0.00

Values are means of the independent triplicate experiment ± SD.

* $p < 0.05$ versus non-treated control.

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PUBLICATION	-
AWARD RECEIVED	-