ผลของสารเบนโซฟีโนน-3 และมอสคาทิลินต่อการแพร่กระจายของเซลล์มะเร็งปอดของมนุษย์ชนิดไม่ใช่เซลล์เล็ก

นางสาวเกศริน บุษรานนท์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECT OF BENZOPHENONE-3 AND MOSCATILIN ON METASTASIS IN HUMAN NON-SMALL CELL LUNG CANCER CELLS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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เกศริน บุษรานนท์ : ผลของสารเบนโซฟีโนน-3 และมอสคาทิลินต่อการแพร่กระจายของเซลล์มะเร็งปอดของมนุษย์ชนิดไม่ใช่เซลล์เล็ก (EFFECT OF BENZOPHENONE-3 AND MOSCATILIN ON METASTASIS IN HUMAN NON-SMALL CELL LUNG CANCER CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร.ปิติ จันทร์วรโชติ, 56 หน้า.

การสัมผัสสารที่มีผลส่งเสริมมะเร็งสามารถส่งผลทำให้โรคมะเร็งมีความรุนแรงมากขึ้น ในงานวิจัยนี้ ได้ศึกษาถึงผลของสารเบนโซฟีโนน-3 (Benzophenone-3) ซึ่งเป็นสารกันแดดที่พบได้ทั่วไปในผลิตภัณฑ์เครื่องสำอาง สามารถกระตุ้นให้เซลล์มะเร็งปอดเกิดการแพร่กระจายได้ สารเบนโซฟีโนน-3 ในระดับความเข้มข้นต่ำ สามารถกระตุ้นให้เกิดการตายในสภาวะไร้การยึดเกาะ (anoikis) ในเซลล์มะเร็งปอดได้อย่างมีนัยสำคัญ นอกจากนี้สารเบนโซฟีโนน-3 ยังสามารถเร่งอัตราการเจริญเติบโตของกลุ่มก้อนมะเร็งและจำนวนของกลุ่มก้อนมะเร็งในสภาวะไร้การยึดเกาะได้จาก anchorage-independent growth assay การศึกษานี้ได้ค้นพบว่ากลไกที่อยู่เบื้องหลังพฤติกรรมเหล่านี้ของเซลล์มะเร็งคือ กระบวนการการเปลี่ยนแปลงจากเซลล์ที่มีลักษณะเยื่อบุผิวไปเป็นเซลล์ที่มีลักษณะมีเซนไคม์ (epithelial to mesenchymal transition, EMT) ร่วมกับการเพิ่มระดับของคาวีโอลีน-1 (Caveolin-1) ซึ่งกลไกทั้งสองนี้สามารถช่วยให้เซลล์มีความดื้อต่อการตายแบบไร้สภาวะยึดเกาะ (anoikis resistance) โดยการเพิ่มระดับโปรตีนที่เกี่ยวข้องกับการมีชีวิตรอด ผลจากการศึกษานี้ พบว่าสารเบนโซฟีโนน-3 กระตุ้นให้เกิดการเพิ่มขึ้นของโปรตีน extracellular-signal-regulated kinase (ERK) สารเบนเซฟีโนน-3 ยังมีผลกระตุ้นการเพิ่มขึ้นของระดับของโปรตีนต้านอะพอพโทซิส (anti-apoptosis) เช่น Bcl-2 และ Mcl-1 ในงานวิจัยก่อนหน้านี้ได้มีการรายงานถึงระดับสารเบนโซฟีโนน-3 ที่สะสมในเลือดของผู้บริโภคที่สัมผัสโดนผลิตภัณฑ์ที่มีส่วนผสมของสารเบนโซฟีโนน-3 เพราะฉะนั้นการศึกษาเกี่ยวกับผลของ สารเบนโซฟีโนน-3 ต่อเซลล์มะเร็งน่าจะเป็นประโยชน์ต่อความปลอดภัยของการใช้สารชนิดนี้ในผู้ป่วยโรคมะเร็งด้วย ถึงแม้ว่าสารเบนโซฟีโนน-3 จะมีฤทธิ์ไปในทางส่งเสริมความรุนแรงของโรคมะเร็ง แต่ก็ยังมีสารสมุนไพรที่สกัดมาจากดอกกล้วยไม้ ชื่อว่า มอสคาทิลิน (Moscatilin) ที่มีรายงานว่ามีผลไปในทางยับยั้งความรุนแรงของโรคมะเร็งประกอบไปด้วยคุณสมบัติการต้านการเพิ่มจำนวนเซลล์ (anti-proliferation) และการต้านการเคลื่อนที่ของเซลล์ (anti-migration) ในงานวิจัยนี้พบว่ามอสคาทิลินมีความสามารถในการยับยั้งการแพร่กระจายของเซลล์มะเร็งโดยผ่านการยับยั้งการทำงานของโปรตีน Akt, ERK และ Mcl-1 ในงานวิจัยนี้ได้พบว่า มอสคาทิลินสามารถเพิ่มอัตราการตายของเซลล์มะเร็งในสภาวะไร้การยึดเกาะโดยผ่านกลไกที่กล่าวมาข้างต้นได้ ซึ่งการค้นพบนี้อาจนำไปสู่การพัฒนายาต้านโรคมะเร็งจากสารมอสคาทิลินได้ในอนาคต

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชศาสตร์ชีวภาพ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: BENZOPHENONE-3 / CANCER METASTASIS / NON-SMALL CELL LUNG CANCER / EPITHELIAL TO MESENCHYMAL TRANSITION / ANOIKIS RESISTANCE / MOSCATILIN

KESARIN BUSARANON: EFFECT OF BENZOPHENONE-3 AND MOSCATILIN ON METASTASIS IN HUMAN NON-SMALL CELL LUNG CANCER CELLS. ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., 56 pp.

Exposure to compounds with cancer-potentiating effect can contribute to the progression of cancer. Herein we have discovered for the first time that benzophenone-3 (BP-3), a chemical used as sunscreen in various cosmetic products, enhances the ability of lung cancer cells to undergo metastasis. The exposure of the lung cancer cells to BP-3 at non-toxic concentrations significantly increased the number of anoikis resistant cells in a dose-dependent manner. Also, BP-3 increased the growth rate as well as number of colony accessed by anchorage-independent growth assay. We found that the underlying mechanisms of such behaviors were the epithelial to mesenchymal transition (EMT) process of cancer cell, and the increase in caveolin-1 expression. As both mechanistic events have been shown to mediate anoikis resistance via augmentation of cellular survival signals, our results further revealed that the BP-3 treatment significantly up-regulated extracellular-signal-regulated kinase (ERK). Also, such compound increased the cellular levels of anti-apoptotic Bcl-2 and Mcl-1 proteins. As the presence of substantial level of BP-3 in plasma of the consumers has been reported, this finding may facilitate the further investigations which lead to the better understandings and evidences concerning the safety of use in cancer patients. Interestingly, a natural compound extracting from traditional orchids called moscatilin was previously reported with promising effects for cancer therapy including anti-proliferation and anti-migration properties. This study also found that moscatilin had the ability to attenuate metastasis process by suppressing the activity of Akt, ERK, and Mcl-1 resulting in a resistance in anoikis process. This study also found that moscatilin had the ability to attenuate metastasis process targeting the above-mentioned anoikis resistance pathways. This finding may indeed facilitate the development of moscatilin for the treatment of cancer.

Department:	Biochemistry and Microbiology	Student's Signature	.....
		Advisor's Signature	.....
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LIST OF ABBREVIATIONS

%	=	percentage
^{o}C	=	degree Celsius
µg/L	=	microgram/Liter
µM	=	micromolar
Akt	=	protein kinase B
ANOVA	=	analysis of variance
Bcl-2	=	B cell leukemia/lymphoma 2
BP-3	=	Benzophenone-3
Cav-1	=	caveolin-1
CO_2	=	carbon dioxide
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	dimethyl sulfoxide
E-Cad	=	E-cadherin
ECM	=	extracellular matrix
EMT	=	Epithelial to Mesenchymal Transition
ERK	=	extracellular-signal-regulated kinase
EtOH	=	ethanol
G	=	gram
h	=	hour, hours
H_2O_2	=	hydrogen peroxide
MAPK	=	mitogen activated protein kinase
Mcl-1	=	myeloid cell leukemia sequence 1
Min	=	minute (S)
Ml	=	milliliter
mM	=	millimolar

MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-Cad	=	N-cadherin
NSCLC	=	non-small cell lung cancer
PBS	=	phosphate buffer saline
PI	=	propidium iodide
RPMI	=	Roswell Park Memorial Institute's medium
SCLC	=	small cell lung cancer
S.D.	=	standard deviation
U	=	unit

CHAPTER I
INTRODUCTION

Cancer remains a leading public health problem as the number of death in patients has been shown to be increasing every year. Many factors including both endogenous and exogenous are contributed to the progression of cancer making cancer a highly complex disease (Floor et al., 2012; Siegel et al., 2014). A knowledge regarding the possible risk of chemical compositions in daily life products has garnered an increased attention and concern of the usage of such chemicals in cancer patients or the populations. Recently, there was reported the possible effects of triclosan, a widely used anti-microbial agent, in potentiating epithelial to mesenchymal transition that may further facilitate the metastasis process (Winitthana, Lawanprasert & Chanvorachote, 2014). Other reports also revealed that many chemical compounds presenting in daily products might confer the cancer cells toward more aggressive phenotypes (Whysner et al., 2004; Huff, 2013). Therefore, it is reasonable that novel knowledge on compound toxicology should be frequently updated to prevent any adverse outcomes from exposing to the compounds especially in cancer patients.

Indeed, cancer metastasis is a multistep process which has been shown to be a major cause of cancer-related death. Metastasis involves ability of tumor cell resisting to anoikis, migrating and invading the surrounding tissues, and forming secondary tumors (Valastyan & Weinberg, 2011). A sufficient number of investigations has pointed out that the behavior of cancer cell called “epithelial to mesenchymal transition (EMT)”, is a hallmark of high aggressiveness as EMT provides the mechanistic properties for anoikis resistance and cell motility (Onder et al., 2008; Wheelock et al., 2008; Klymkowsky & Savagner, 2009; Voulgari & Pintzas, 2009; Chanvorachote et al., 2012; Nurwidya et al., 2012; Shi et al,. 2013). The EMT process causes cancer cells to up-regulate their survival signals including Mcl-1, Bcl-2 and activated Akt and ERK levels

(Larue & Bellacosa, 2005; Song et al,. 2005; Youle & Strasser, 2008). Likewise, our previous findings are in coherent with other reports on the positive role of caveolin-1 (Cav-1) on cancer aggressiveness. Cav-1 was shown to potentiate lung cancer cells resistance to anoikis (Ravid et al., 2006; Rungtabnapa et al., 2011; Chunhacha & Chanvorachote, 2012.), and increase migration and invasion (Ho et al., 2010; Halim, Luanpitpong & Chanvorachote, 2012). Cav-1 was also shown to stabilize the level of anti-apoptotic Mcl-1 protein during cellular detachment (Chunhacha et al., 2012). Alterations in expressions of these proteins contribute to cancer metastatic behavior.

A benzophenone-3 (BP-3) (2-hydroxy-4 methoxybenzophenone or oxybenzone (INN name)) is a sunscreen agent that is frequently found in a number of cosmetic products. Commercially, it is claimed as a broadband UV filter and used in concentrations ranging up to 10% w/w alone or in combination with other UV filters (Calafat et al., 2008). As BP-3 is highly lipophilic, the compound can be absorbed through human skin. Interestingly, BP-3 was shown to accumulate in human body and was found in urine and breast milk (Gonzalez et al., 2006). 10% of BP-3 lotion exposure through dermal application resulted in average urine level of 140 μg/L and 60 μg/L in male and female, respectively (Janjua et al., 2004). Until now, BP-3 has only limited data regarding potentiating effects on cancer metastasis. The present study aimed to investigate the effect of this compound in human non-small cell lung cancer cells owning to its high incidence of metastasis (Chaffer & Weinberg, 2011; Chanvorachote et al., 2012). The knowledge gained from the present study may promote the concern of use of this compound in cancer patients.

Despite the potential of BP-3 to potentiate cancer aggressiveness, many natural compounds have been shown to possess the contradict result. Moscatilin, a bibenzyl derivative extracted from Thai orchid has been one of most interesting compound in cancer therapy as it was reported with anti-proliferation and anti-migration properties (Ho & Chen, 2003; Kowitdamrong et al., 2013; Klongkumnuankarn et al., 2015). In breast

cancer, it was evidenced that moscatilin was able to inhibit metastasis through Akt- and Twist-dependent mechanisms without causing any toxicity in the normal cells (Pai et al., 2012). In addition, moscatilin increased the population of esophageal cancer cells in the sub-G1 phase in a dose- and time-dependent manner. From the protein expression analysis, it was illustrated that moscatilin at 5 and 10 μM targeted polo-like kinase 1 (PLK-1) and cyclin B1 to inhibit cancer cells' growth and induce apoptosis (Chen et al., 2013). Kowitdamrong and his colleagues (2013) have reported that moscatilin inhibited the endogenous reactive oxygen species (ROS) leading to a decrease in migration and invasion of human lung adenocarcinoma H23 cells. In the present study, the anti-metastasis property of moscatitlin was investigated to reveal the novel mechanism of moscatilin as an anti-cancer compound, which may benefit in the discovery of new compound for cancer therapy.

Research Questions

1. Does benzophenone-3 affect H460 non-small cell lung cancer cells aggressiveness?
2. Is the molecular mechanism of benzophenone-3's effects on H460 non-small cell lung cancer cells related to pro-survival proteins and anoikis?
3. Does moscatilin affect H460 non-small cell lung cancer cells anoikis?
4. Is the molecular mechanism of moscatilin's effects on H460 non-small cell lung cancer cells related to pro-survival proteins and anoikis?

Objectives

1. To investigate the effects of benzophenone-3 and moscatilin on H460 non-small cell lung cancer cells including colony formation and anoikis assay.
2. To investigate the molecular mechanisms of benzophenone-3's and moscatilin's effects on H460 non-small cell lung cancer cells which are related to pro-survival proteins and anoikis.

Hypothesis

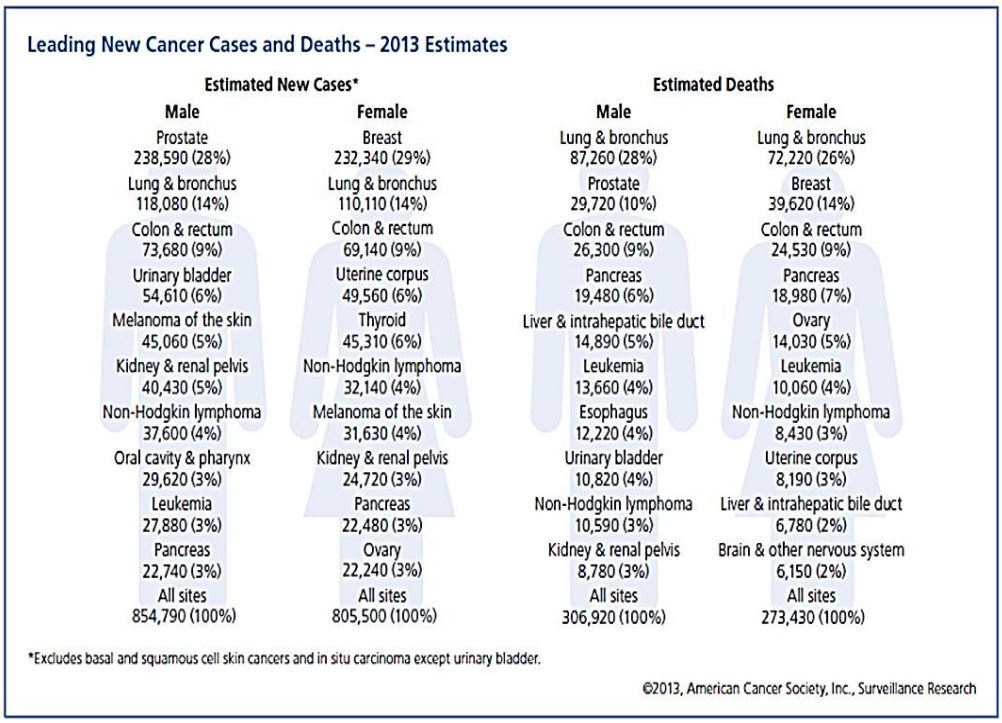
1. Benzophenone-3 promotes colony formation and inhibits anoikis of H460 non-small cell lung cancer cells.
2. The molecular mechanisms of benzophenone-3's effects on H460 non-small cell lung cancer cells are related to survival and EMT pathways.
3. Moscatilin inhibits colony formation and promotes anoikis of H460 non-small cell lung cancer cells.
4. The molecular mechanisms of moscatilin's effects on H460 non-small cell lung cancer cells are related to survival and EMT pathways.

CHAPTER II

LITERATURE REVIEW

Lung cancer

The most fundamental cause of death from cancer was reported to be lung cancer as lung cancer has been accused for about 1 in 4 of all cancer related deaths according to the American cancer society (Siegel et al., 2014). An estimation of 158,080 deaths was expected to occur in 2016 (Siegel et al., 2014). Moreover, lung cancer was found to be the second most diagnosed type of cancer in the United States ranking after prostate cancer in men and breast cancer in women (Fig. 1). 224,390 new patients diagnosed with lung cancer were expected in 2016 which accounts for approximately 14% of all cancer diagnoses (Siegel et al., 2014).



Leading New Cancer Cases and Deaths – 2013 Estimates

Estimated New Cases*		Estimated Deaths	
Male	**Female**	**Male**	**Female**
Prostate 238,590 (28%)	Breast 232,340 (29%)	Lung & bronchus 87,260 (28%)	Lung & bronchus 72,220 (26%)
Lung & bronchus 118,080 (14%)	Lung & bronchus 110,110 (14%)	Prostate 29,720 (10%)	Breast 39,620 (14%)
Colon & rectum 73,680 (9%)	Colon & rectum 69,140 (9%)	Colon & rectum 26,300 (9%)	Colon & rectum 24,530 (9%)
Urinary bladder 54,610 (6%)	Uterine corpus 49,560 (6%)	Pancreas 19,480 (6%)	Pancreas 18,980 (7%)
Melanoma of the skin 45,060 (5%)	Thyroid 45,310 (6%)	Liver & intrahepatic bile duct 14,890 (5%)	Ovary 14,030 (5%)
Kidney & renal pelvis 40,430 (5%)	Non-Hodgkin lymphoma 32,140 (4%)	Leukemia 13,660 (4%)	Leukemia 10,060 (4%)
Non-Hodgkin lymphoma 37,600 (4%)	Melanoma of the skin 31,630 (4%)	Esophagus 12,220 (4%)	Non-Hodgkin lymphoma 8,430 (3%)
Oral cavity & pharynx 29,620 (3%)	Kidney & renal pelvis 24,720 (3%)	Urinary bladder 10,820 (4%)	Uterine corpus 8,190 (3%)
Leukemia 27,880 (3%)	Pancreas 22,480 (3%)	Non-Hodgkin lymphoma 10,590 (3%)	Liver & intrahepatic bile duct 6,780 (2%)
Pancreas 22,740 (3%)	Ovary 22,240 (3%)	Kidney & renal pelvis 8,780 (3%)	Brain & other nervous system 6,150 (2%)
All sites 854,790 (100%)	All sites 805,500 (100%)	All sites 306,920 (100%)	All sites 273,430 (100%)

*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

Figure 1 Estimated new cancer and deaths worldwide for most cancer sites in 2013 diagnoses (Siegel et al., 2014)

The major cause of lung cancer was shown to be from exposing to carcinogenic compounds such as certain metals, organic chemical and especially cigarette smoking. In recent years, accumulating compounds were reported to potentiate a higher risk in cancer patients. More knowledge on compound toxicology is important to prevent patient deteriorating from the disease.

Types of lung cancer

According to cellular growth and metastasis pattern, lung cancer was classified into 2 major types; small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer (SCLC) was found at approximately 10-15 % of all lung cancer patients. SCLC has an irregular morphology which was known as oat cell cancer. The most common body site for SCLC was found to be at the bronchi. Despite the lower incident compared to the other type of cancer, SCLC tends to grow and spread at higher rate (Siegel et al., 2014).

Non-small cell lung cancer (NSCLC) was more frequently found with approximately 85-90 % incident rate. NSCLC can be further classified into squamous cell carcinoma, adenocarcinoma and large cell carcinoma according to the cellular morphology.

Squamous cell carcinoma is found at the earliest stage of NSCLC with approximately 25-30 % occurrence. This type of cancer usually colonizes along airways of the lungs. Patients diagnosed with squamous cell carcinoma often relate to smoking behavior and usually found the tumor located near a bronchus in the middle of the lungs (Siegel et al., 2014).

The most frequently found type of NSCLC was adenocarcinoma. It was claimed to be a slow growing type of lung cancer compared to the other types. It was reported that the adenocarcinoma was usually found on the outer part of the lungs. Patients with adenocarcinoma was observed to have a better prognosis than any other types

of lung cancer carcinoma (Siegel et al., 2014).

Large cell carcinoma is the minority type of NSCLC. However, it has a faster growth and spreading rate than other types of NSCLC, resulting in a more complication in the treatment of cancer (Siegel et al., 2014).

As it is observed that NSCLC is 4 times more common than SCLC is. Moreover, NSCLC is a common type related to chemical exposure; therefore, in this study we used NSCLC to observe the effect of BP-3 exposure.

Metastasis

Patients who are diagnosed with lung cancer usually have metastasized cancer cells (Siegel et al., 2014). Metastasis is a rapid biological process of cancer when cancer cell spread from primary site to form into secondary tumor (Chambers et al., 2002; Geiger & Peeper, 2009). Metastasis was found as the major cause of death in cancer patients as it was accounted for more than 90% of cancer death (Mehlen & Puisieux, 2006). After the metastasis is detected, less than 16% of the patients were able to survive the 5-year duration. Lung cancer cells are able to metastasize to other organs throughout the body such as bone, brain and lymph nodes. The attenuation of metastasis process is, therefore, the ultimate goal of cancer therapy and can result in significantly improved quality of life of lung cancer patients (Mehlen & Puisieux, 2006).

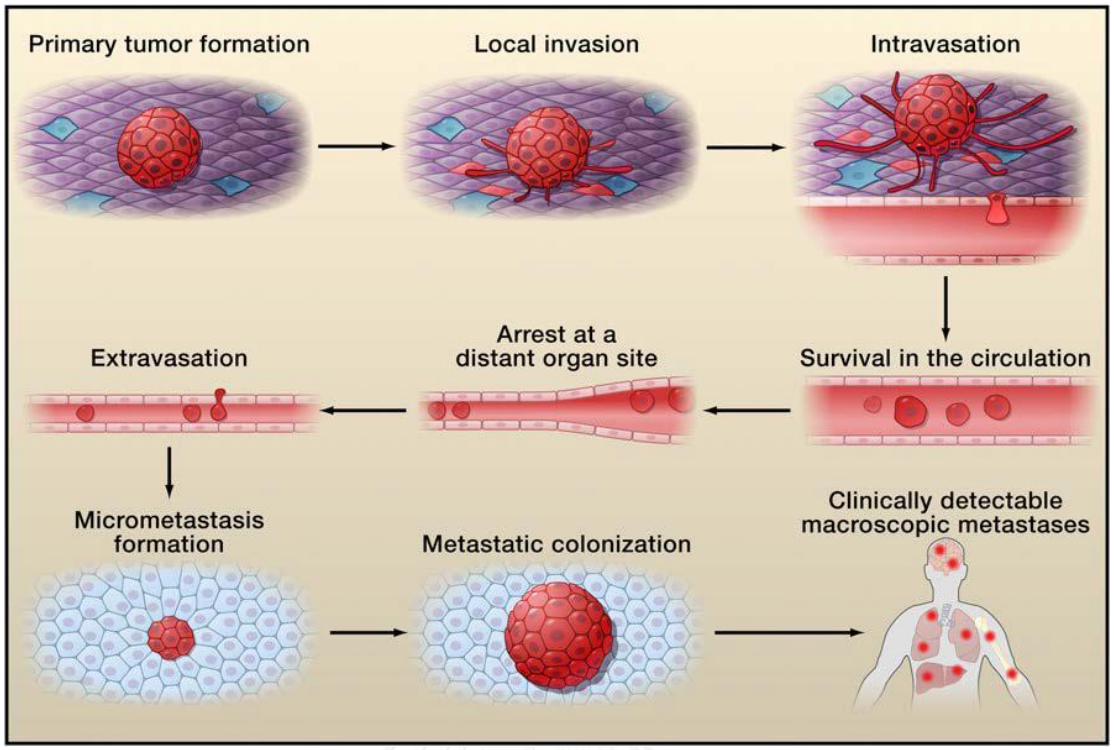


Figure 2 The metastatic cascade (Mehlen & Puisieux, 2006)

The sequences of metastasis are described as followed;

1) At the primary site, cancer cells detach from the basement membrane that the cells were attached to, then migrate and invade through the surrounding extracellular matrix (ECM).

2) When cancer cells reach the vessels, they intravasate into the blood and lymph vessels.

3) The detached cancer cells circulate in the circulatory or lymphatic system.

4) At the suitable environment, the detached cancer cells extravasate out of the vessels, then migrate and invade to the secondary site.

5) At the new secondary site, the proliferative programs are re-activated and cause the secondary tumor growth.

Anoikis

When cancer cells are detached from ECM at the primary site, the body has a protective mechanism to prevent cellular detachment and the formation of undesirable colony in the body. This mechanism is called anoikis which is a programmed cell death that activate when cells detach from the surrounding ECM causing cells to undergo apoptosis (Chiarugi & Giannoni, 2008). Tissue homeostasis is the result of anoikis process. A failure of cell to undergo anoikis or anoikis resistance was found to be the fundamental cause of disease development such as cancer, cardiovascular disease and diabetes (Taddei et al., 2011; Paoli, Giannoni & Chiarugi, 2013).

For the metastasis to occur, the cancer cells have to become resistant to anoikis and be able to survive after their detachment from the primary site and spread through the vascular system and then form into the secondary tumor at distal organs (Fig 3).

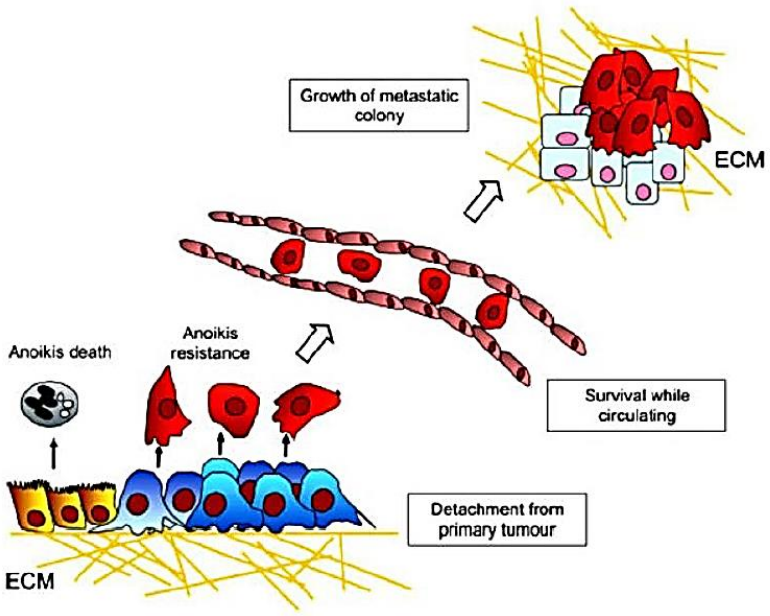


Figure 3 Anoikis resistance and cancer metastasis (Chiarugi & Giannoni, 2008)

Therefore, it can be said that anoikis resistance is the prerequisite mechanism for the cancer cells to undergo metastasis (Chiarugi & Giannoni, 2008). The molecular mechanisms underlying anoikis resistance are not fully explored. However many studies have proposed possible mechanism including epithelial to mesenchymal transition (EMT) which was found to intensify the survival pathways and attenuate the apoptotic pathways when the cancer cells are detached (Paoli, Giannoni & Chiarugi, 2013).

Epithelial to mesenchymal transition

The epithelial to mesenchymal transition (EMT) process was claimed as a hallmark of cancer aggressiveness. The transdifferentiation from epithelial cell to elongated mesenchymal phenotypes allow cancer cells to gain ability to become more adaptable (Yilmaz & Christofori, 2009). The process of EMT is well known to be related to cancer metastasis as the critical mechanism driving cells towards anoikis resistance (Kumar et al., 2011). The molecular proteins that were reported to mark EMT process include N-cadherin, Vimentin, Snail and Slug (Kumar et al., 2011). N-cadherin is an intercellular protein that link between mesenchymal cells. An increase in expression of N-cadherin makes cancer cells to come more into contact with stromal fibroblasts and less into contact with structural epithelial cells facilitating the invasion into the surrounding tissue. Vimentin is a type of cytoskeleton found in mesenchymal cells. Migrative mesenchymal cells require to have the high strength intermediate filament, vimentin, in order to tolerate the traction force during the movement (Heatley, Whiteside & Maxwell, 1993). Snail and Slug are transcription factors responsible for expression of EMT related proteins (Fenouille et al., 2012; Sánchez-Tilló et al., 2012). Increases in the expression of these proteins are the hallmark of EMT.

Survival signaling pathway

In normal condition, cell survival signaling pathways such as PI3K/Akt and MAPK/ERK pathways can be activated by growth factors-receptor interaction, cell-cell interactions and cell-ECM interactions as shown in Fig 4 (Enomoto et al., 2005; Lu & Xu, 2006).

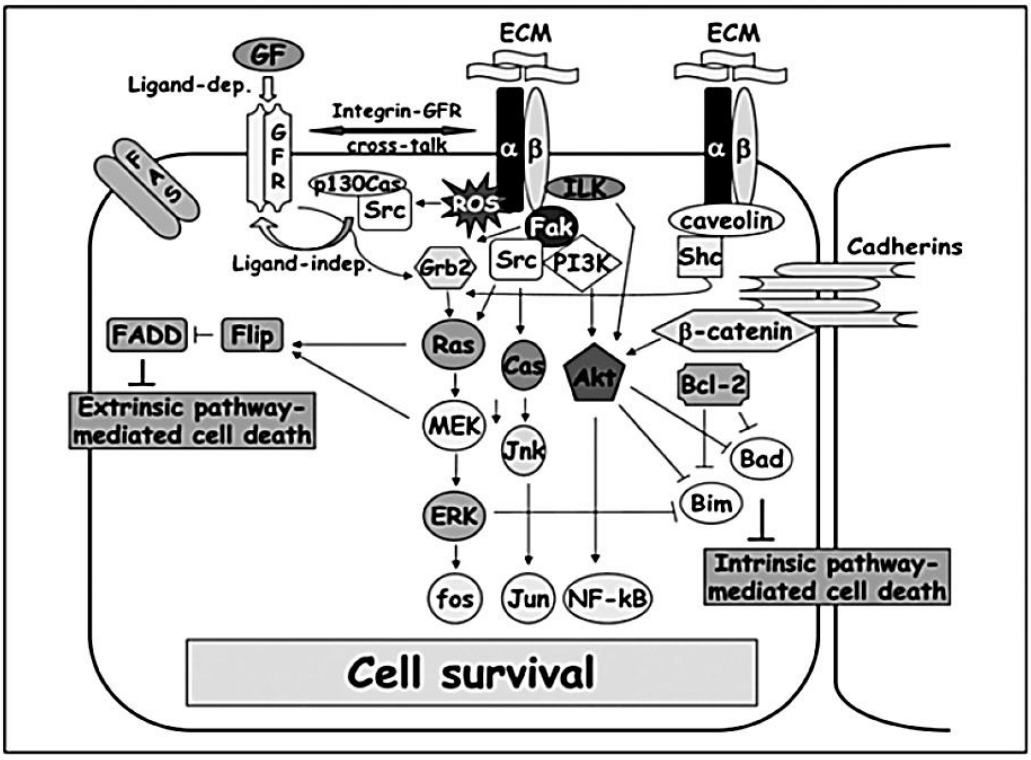


Figure 4 Survival signaling pathways include growth factor receptor activation, intergrin-ECM engagement and cadherin mediated cell-cell interaction (Lu & Xu, 2006).

The inhibition of apoptosis process is also essential for the cells to survive. These inhibition processes include the down regulation of the expression of pro-apoptotic factors as well as the up regulation of the expression of anti-apoptotic factors (Lu & Xu, 2006). The PI3K pathway which can be induced by many survival factors, leads to an activation of Akt which plays an important role in survival signaling.

Activated Akt phosphorylates and inhibits the pro-apoptotic Bcl-2 family members lead to cell survival. Many growth factors and cytokines induce anti-apoptotic Bcl-2 family members. These pro-survival Bcl-2 family members (e.g., Bcl-2 and Mcl-1) retain the integrity of mitochondria, protecting the cell from cytochrome c release which can lead to the activation of caspase-9 (Youle & Strasser, 2008; Song et al., 2014).

The MAPK/ERK (Mitogen-activated protein kinases, originally called ERK, Extracellular signal-regulated kinases) pathway transfers a signal from the receptor on the surface of the cell to the DNA inside the nucleus of the cell. The signal is induced by the molecular binding of the receptor on the cell surface and the appropriate growth factor. The pathway includes many proteins, including MAPK, which activated by adding phosphate groups. This can lead to an alteration of the DNA in the nucleus and the expression of proteins producing some changes in the cell, such as cell division and cell survival (Lu & Xu, 2006).

Benzophenone-3

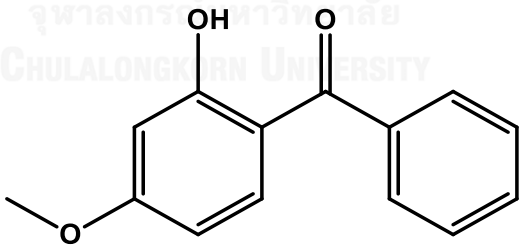


Figure 5 Chemical structure of benzophenone-3

Benzophenone-3 (BP-3) or oxybenzone (INN name) is used as a broadband UV filter at concentrations of up to 10 % weight/weight in sunscreen products alone or in combination with other type of UV filters. Other than the usage in sunscreens, BP-3 is also used commonly in other types of cosmetic products such as photo protection

skin care or mixed with flavoring ingredient at concentrations ranging between 0.05 - 0.5 %. It is also used in the manufacture of insecticides, agricultural chemicals, hypnotics, antihistamines, and other pharmaceuticals as an additive in plastics, coatings, and adhesive formulations. Due to the unavoidable daily exposure to the chemical and the lipophilicity of the compound, BP-3 was claimed to be able to get absorbed through human skin which might cause some effect that is still unknown.

Bioaccumulation of BP-3 was reported to occur in human as BP-3 has been detected in human urine and in breast milk (Janjua et al., 2004). BP-3 can be absorbed through dermal application then accumulated and excreted in the urine. According to the study, the topical application of a 4% lotion over the body daily for 5 days resulted in 1.2-8.7% of the BP-3 recovered in the urine (Gonzalez et al., 2006). In addition, 10% of BP-3 lotion exposure through dermal application resulted in average urine level of 140 µg/L and 60 µg/L in male and female respectively (Janjua et al., 2004).

The effects to human health from exposing to BP-3 at low environmental doses are still unknown. The clinical dermal application reported some cases of photo allergy and allergy to BP-3. Male reproductive toxicity has been reported after chronic high dose application in animal studies (Feigeal, 2009). BP-3 also reported with weak estrogenic activity and weak anti-androgenic activity. No human hormonal changes were observed during four days of application of 10 % BP-3 lotion (Calafat et al., 2008). In genetic and toxicology study, BP-3 showed that there was no mutagen in Salmonella typhimurium strains TA98, TA100, TA1535, or TA1537 (Whysner et al., 2009). The International Agency for Research on Cancer (IARC) has no ratings as to human carcinogenicity of BP-3.

Even though the effect to human health is still largely unknown, but the toxicology and carcinogenesis of BP-3 were reported in animal studies (Calafat et al., 2008). An in vivo study was done in rats and mice. It was found that the survival rate of the groups exposed to 1,250 ppm BP-3 was significantly less than that of controls.

Under the conditions of these 2-year studies, there was some incidence of carcinogenic activity of BP in male rats. Thus, evidences of carcinogenic activity of BP in rats and mice raise a question whether BP-3 can promote cancer metastasis in the carcinogenesis safety aspect of this compound.

Moscatilin

Moscatilin, 4,4′-dihydroxy-3,3′,5′-trimethoxybibenzyl or a bibenzyl derivative extracted from medicinal orchid. The isolation of moscatilin was done by chromatographic separation of a methanol extract prepared from the dried and powdered whole plate of *Dendrobium pulchellum* (Orchidaceae), which is known in Thai as "Ueang chang nao". The methanol extraction from the whole plant of dendrobium was illustrated with 80% cytotoxicity at concentration of 50 µg/mL in human small cell lung cancer H460 cells (Kowitdamrong et al., 2013; Klongkumnuankarn et al., 2015).

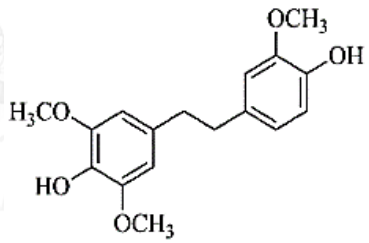


Figure 6 Chemical structure of moscatilin

Moscatilin was reported with pharmaceutical benefits including anti-oxidant and anti-inflammatory (Kowitdamrong et al., 2013). Also moscatilin was claimed for many promising effects as a cancer therapeutic agent such as anti-cancer and anti-migration activities (Ho & Chen, 2003; Pai et al., 2012; Chen et al., 2013; Klongkumnuankarn et al., 2015). According to Pai and his colleague (2012), moscatilin was able to reduce breast cancer metastasis in both *in vitro* and *in vivo*. The study

reported that moscatilin targeted the expression of Akt and Twist in the mRNA level leading to a significantly decreased in migration and invasion without causing any toxicity in the normal cells (Pai et al., 2012). Moreover, in the squamous cell carcinoma and adenocarcinoma esophageal cancer cell lines, moscatilin was able to induce apoptosis and mitotic catastrophe in a dose- and time-dependent manner (Chen et al., 2013). In addition, moscatilin possesses an anti-migration effect through inhibiting the endogenous reactive oxygen species (ROS) leading to a reduced in FAK and Akt activation in human lung adenocarcinoma H23 cells (Kowitdamrong et al., 2013). However, moscatilin's role in anoikis susceptibility as well as the process of EMT is still unknown. Knowledge regarding the anoikis-sensitizing effects of moscatilin and the mechanisms of action targeted the above-mentioned anoikis resistance pathways may facilitate the development of this promising compound for the treatment of cancer.

CHAPTER III
MATERIALS AND METHODS

Materials

NCI-H460 and A549 human lung carcinoma cells, human keratinocyte HaCat cells and HK-2 (human proximal tubular epithelial cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, phosphate buffer saline (PBS) and trypsin-EDTA were obtained from GIBCO (Grand Island, NY, USA). Benzophenone-3 (BP-3), dimethysulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Gibthai Company, Ltd. (Life technologies, MD, USA). Agarose, BCA protein assay kit, and nitrocellulose membranes were purchased from Bio-Rad Laboratories, Inc. (Biorad, USA). PD98059 and antibodies for Akt, pAkt, ERK, pERK, Bcl-2, Mcl-1, N-cadherin, E-cadherin, vimentin, slug, snail and β-actin, and peroxidase conjugated secondary antibodies were obtained from Cell Signaling (Denvers, MA, USA). Complete Mini cocktail protease inhibitor was obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA). Lysis buffer and chemiluminescent substrate were purchased from Thermo Scientific (Supersignal West Pico; Pierce, Rockfore, IL, USA).

Methods

Cell culture

The Attach cells H460 were cultured in RPMI-1640 medium. A549, HaCat and HK-2 cells were cultured in DMEM medium. Detached cells were grown in six-well plates pre-coated with polyHEMA (1.2%). Cells were cultured at 37 °C in a 5 % CO_2

humidified incubator. Both of these mediums were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/mL streptomycin. All cells were sub-cultured using trypsin-EDTA at 70-80 % confluence (Winitthana, Lawanprasert & Chanvorachote, 2014).

Treatment preparation

BP-3 was dissolved with DMSO to make a stock solution at concentration of 0.1 mg/µL. Moscatilin was dissolved with DMSO to make a stock solution at concentration of 100 µM. Small amount of all stock solutions were aliquot into separated eppendrops to reduce any unnecessary freeze-thaw cycles. Stock solutions were kept at -20 °C. The final concentration of DMSO in the treatment was less than 0.1 % to prevent any cytotoxicity due to the solvent (Winitthana, Lawanprasert & Chanvorachote, 2014).

Cell viability assay

Cell viability was analyzed using a colorimetric MTT assay. 10^4 cells in 100 µL of RPMI or DMEM medium were seeded into each well of a 96-well plate and incubated overnight for cell attachment. Then, the cells were treated with different concentrations of BP-3 (0–300 µg/L) for 24 hours for the cell viability assays. After the indicated times, the medium was removed and replaced with MTT solution for 4 hours at 37 °C. Formazan product was solubilized with 100 µL DMSO, and the intensity was measured at 570 nm using a microplate reader (Anthros, Durham, NC, USA). Viable cells were represented as the percentage of cell viability relative to control cells (Winitthana, Lawanprasert & Chanvorachote, 2014).

Apoptosis and necrosis evaluation assay

Nuclear co-staining with Hoechst 33342 and propidium iodide (PI) was used to determine apoptotic and necrotic cell death. After the treatment, cells were incubated with 10 µM Hoechst 33342 and 5 µM PI for 30 minutes at 37 °C in the dark. Cells were then visualized and imaged under a fluorescence microscope (Olympus 1X51 with

DP70). The blue fluorescent Hoechst dye detected apoptotic cells while the red fluorescent PI detected necrotic cells. The number of apoptotic and necrotic cells were represented as the percentage in each treatment group relative to the control (Winitthana, Lawanprasert & Chanvorachote, 2014).

Anoikis assay

To prevent cell adhesion, tissue culture six-well plates were coated with 200 µl (6 mg/ml in 95 % ethanol) of 2-hydroxyethylmethacrylate (poly-HEMA) and left to evaporate overnight in a laminar flow hood at room temperature. Adherent H460 or A549 cells in culture plates were trypsinized into a single-cell suspension in RPMI or DMEM medium and then seeded into poly-HEMA-coated plates at a density of 1×10^5 cells/ml. Suspended cells were treated and incubated at 37°C before harvested 0, 3, 6, 9, 12, and 24 hours for cell viability test using MTT assay (Winitthana, Lawanprasert & Chanvorachote, 2014).

Colony formation assay

Anchorage-independent growth was determined by colony formation assay in soft agar as described by Koleske et al. (1995) with minor modifications. In brief, the treated H460 and A549 cells from six-well plate monolayer cultures were prepared into a single-cell suspension by trypsin and 1.5 mM EDTA. 2×10^4 Cells were suspended in RPMI or DMEM containing 10 % FBS and 0.3 % agarose before seeding into a 24-well plate on top of a layer of solidified RPMI or DMEM containing 10% FBS and 1% agarose. Then the culture was left in 37 °C for the colony to form between the agarose layer. The cells were fed every 3 days by adding 200 µl of RPMI or DMEM with 10 % FBS. Colonies were assayed by photograph at x10 magnification after 2 weeks. Relative colony number and diameter were determined by dividing the values of the treated cells by those of the control cells (Winitthana, Lawanprasert & Chanvorachote, 2014).

Western blot analysis

Cells were seeded at a density of 5×10^5 cells/well into poly-HEMA-coated six-well plates overnight. After specific treatments, the cells were harvested and incubated with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 % Triton X-100, 150 mM sodium chloride (NaCl), 10 % glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail for 60 minutes on ice. Then, cell lysates were collected, and the protein content was determined by using the BCA protein assay kit. Equal amounts of protein from each sample (60 µg) were denatured by heating at 95 ℃ for 5 minutes in Laemmli loading buffer and subsequently loaded onto 10 % SDS-polyacrylamide gels. After separation, the proteins were transferred onto 0.45 µm nitrocellulose membranes, and the transferred membranes were then blocked in 5 % nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20) for 1 hour and subsequently incubated with a specific primary antibody overnight at 4 ℃. The membranes were washed three times with TBST for 10 minutes and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG for 2 hours at room temperature. After three washes with TBST, the immune complexes were detected by using chemiluminescence and quantified using the analyst/PC densitometry software (Bio-Rad) (Winitthana, Lawanprasert & Chanvorachote, 2014).

Statistical analysis

The data were obtained from at least four independent experiments and presented as the mean ± standard deviation (SD). Statistical analysis was used to assess the data by using one-way ANOVA and a post hoc test at a significance level of $p < 0.05$. SPSS version 17.0 was analyzed for significance of all statistical analysis.

Conceptual framework

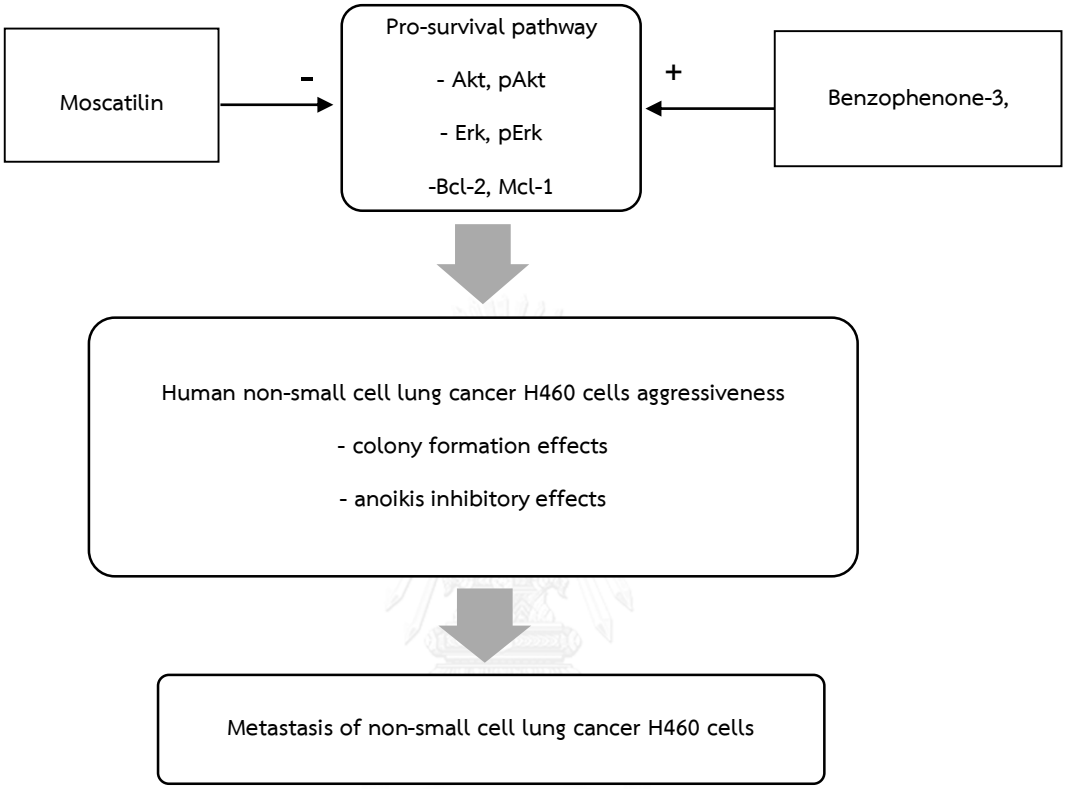


Figure 7 Conceptual framework

Experimental design

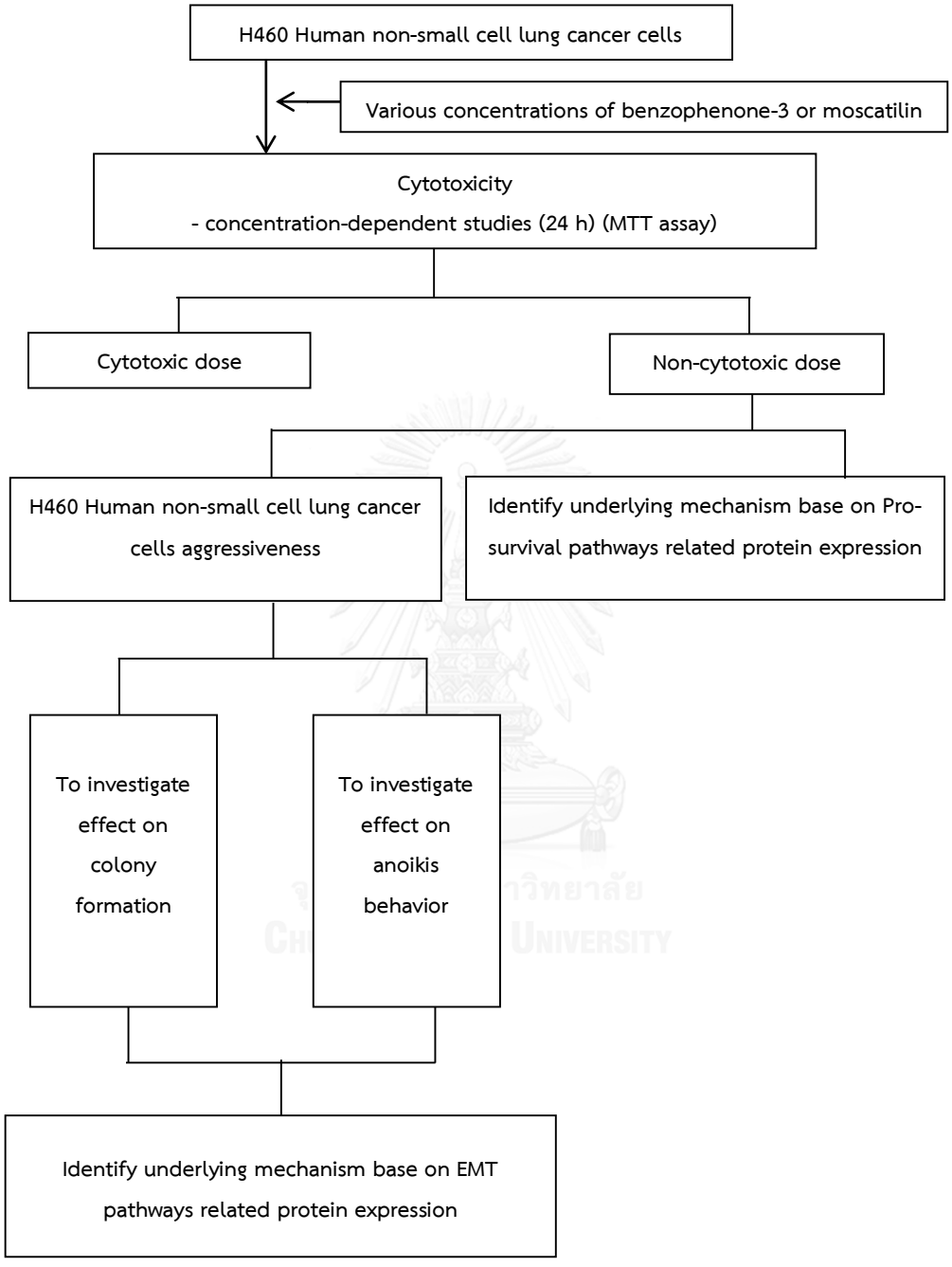


Figure 8 Experimental design

CHAPTER IV
RESULTS

Effect of BP-3 on cell viability

- BP-3 does not cause cytotoxicity in lung cancer cell lines

In order to clarify the possible cancer potentiating effects of BP-3 in lung cancer cells, the cytotoxic effect of BP-3 on human lung cancer cell lines, H460 and A549, was first characterized. Cells were treated with various concentrations of BP-3 (0-300 µg/L) for 24 h and cell viability was measured by MTT assay. Figure 9b shows that BP-3 has no significantly changed in viability of H460 and A549 cells at all concentrations

- BP-3 does not cause cytotoxicity in normal epithelial cells

In addition, the toxic effect of the compound was evaluated in the normal epithelial cells including human proximal tubular epithelial cell, HK-2, and human normal keratinocyte, HaCaT, cells. Figure 9c shows that BP-3 caused no significant toxic effect in both HK-2 and HaCaT cells at the indicated concentrations. Therefore, BP-3 at the concentrations of 0-300 µg/L was considered as non-toxic concentration and was used for further experiments.

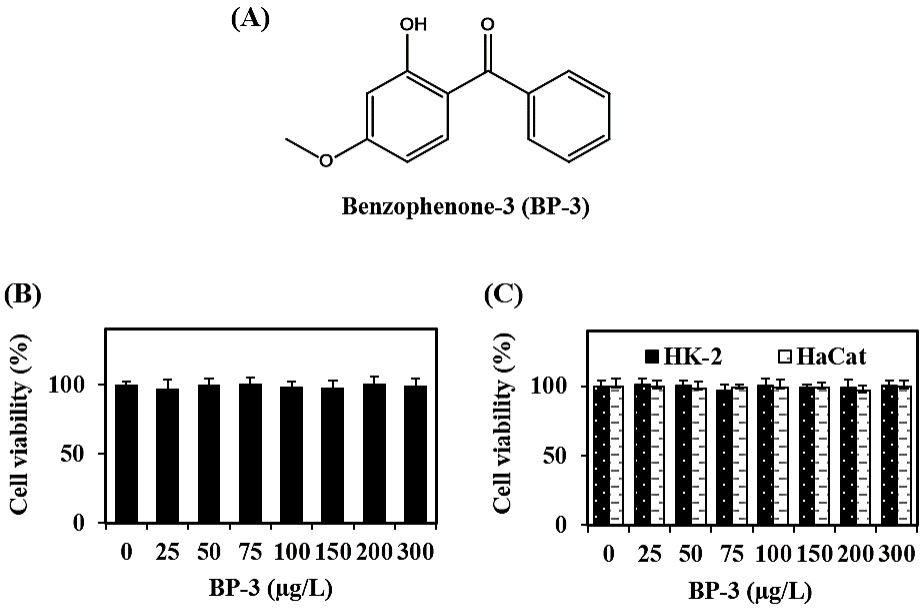


Figure 9 Cytotoxic effect of BP-3 on H460, HK-2 and HaCat cells. **a** The chemical structure of BP-3. **b** H460 Cells were treated with BP-3 (0–300µg/L) for 24 h. **c** HK-2 and HaCat cells were treated with BP-3 (0–300µg/L) for 24 h. The percentage of cell viability was determined by MTT assay. Values are means of the three independent triplicate samples ± SE. *P 0.05 versus non-treated control.

Effect of BP-3 on colony formation in lung cancer cell

- BP-3 promotes colony formation in H460 cell

As anchorage-independent growth has been recognized as an important characteristic of the highly metastasis cancer cells, we next investigated the effect of BP-3 on cancer cell growth in such condition. Briefly, H460 cells were seeded in agarose layer to prevent cell-cell interaction and extracellular matrix adherence. Then BP-3 at the concentrations of 0-150 µg/L was treated to the agarose-seeded cells. The colony number and colony size after 7 and 10 days were determined as indicated in Materials and Methods. Colony number and colony size of each treatment (Fig. 10a) were calculated as a percentage in comparison to those of control group and shown in Fig. 10b, c, respectively. The results revealed that BP-3 at the concentrations of 50, 100

and 150 µg/L significantly increased colony formation of H460 cells in both number and size. Such observations indicated that BP-3 have a cancer potentiating effect by enhancing anchorage-independent survival and growth of lung cancer cells.

- BP-3 promotes colony formation in H460 cell

In order to confirm the colony formation effect of BP-3 in another lung cancer cells, the effects of BP-3 on anchorage-independent growth of H460 cells was investigated. Fig. 10b shows that BP-3 at the doses of 50 – 150 µg/L significantly increased cell survival in the detached condition in consistent with the result from H460 cell line.

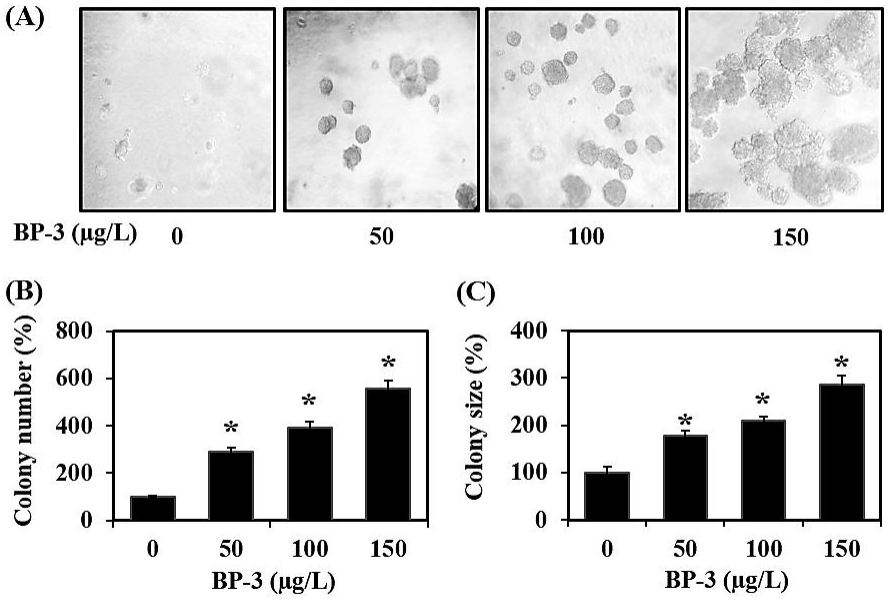


Figure 10 Effect of BP-3 on anchorage-independent growth of lung cancer H460 cells. **a** Cells were treated with BP-3 (0-150 µg/L) and subjected to soft agar colony formation assay, as described in ‘‘Materials and Methods’’. Representative fields from three independent experiments were photographed after the cells were cultured for 7 and 10 days. Scale bar is 1,000 mm. **b–c** Colony number and colony size was determined by image analyzer on the 10th day of culture. Values are means of the three independent triplicate samples ± SE. *P, 0.05 versus non- treated control.

Effect of BP-3 on anoikis resistance in lung cancer cell

- BP-3 induces anoikis resistance in H460 cell

Having shown that the BP-3 facilitates the growth of the cancer cells in detached condition, we next examined whether the compound could attenuate the detachment-induced apoptosis or anoikis in these cells. Anoikis is a critical inhibitory process for metastasis. To determine the effect of BP-3 on anoikis response of the H460 cells, the cells were detached, suspended in the serum free culture media containing BP-3 (0 – 150 µg/L) for 0-24 hours, and cell survival was determined by MTT assay at 0, 3, 6, 9, 12, and 24 hours. Fig. 11a shows that BP-3 treatment significantly increased cell survival at the dose of 50 – 150 µg/L with approximately 50-70 % of cells remaining viable. Hoechst33342/PI staining assay indicated that necrosis cell death was not detectable in the control and the BP-3-treated cells at all conditions. The apoptotic cells with fragmented and/or condensed nuclei were significantly decreased in the cells treated with BP-3 (Fig 11b, c). The results suggested that BP-3 treatment mediates anoikis resistance in the lung cancer H460 cells.

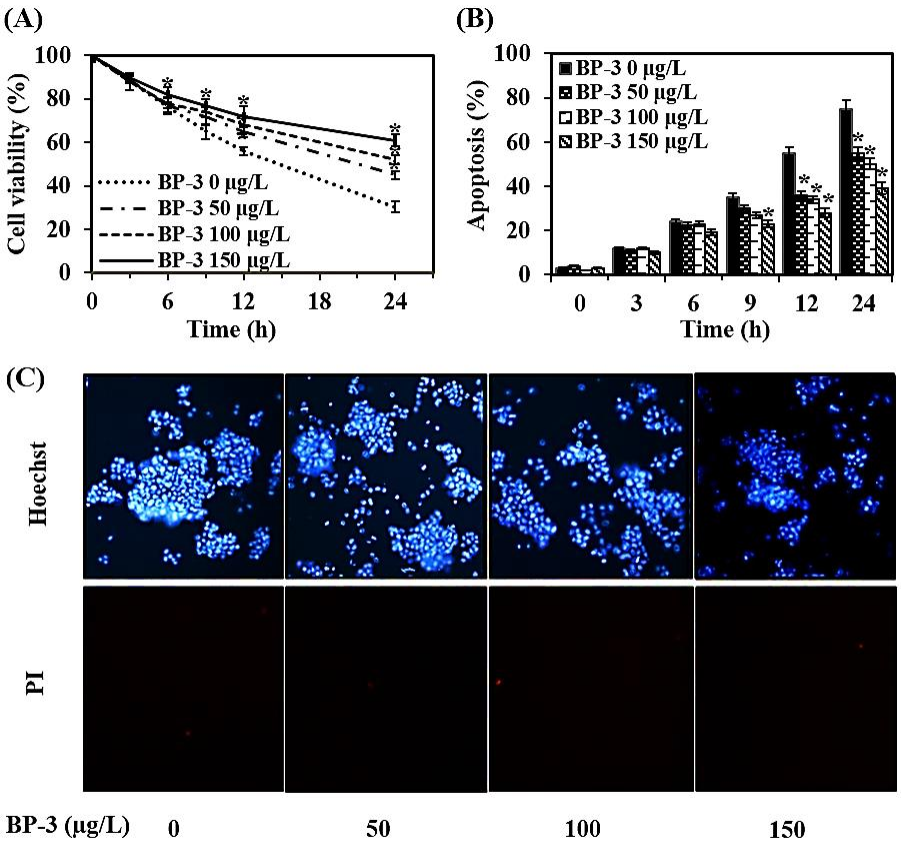


Figure 11 Effect of BP-3 on anoikis response. **a** Detached H460 cells were incubated with BP-3 (0-150 µg/L) in poly-HEMA coated plates. Cell viability was determined by MTT assay at indicated times. Values are means of three independent triplicate experiments ± SE. *P, 0.05 versus non-treated control at time 0. **b** Percentage of apoptotic cells was evaluated by Hoechst33342 staining. Values are means of three independent triplicate experiments ± SE. *P, 0.05 versus non-treated control at time **c** After treatment for 24 h, nuclear morphology of the cells was detected by Hoechst33342/PI co-staining assay and visualized under a fluorescence microscope.

- BP-3 induces anoikis resistance in A549 cell

In order to confirm the anoikis resistant effect of BP-3 in another lung cancer cells, the effects of BP-3 on anoikis process of A549 cells was investigated. Consistent to the results obtained from H460 cells, BP-3 significantly increased colony formation of A549 in both number and size of the colonies (Fig. 12c-e).

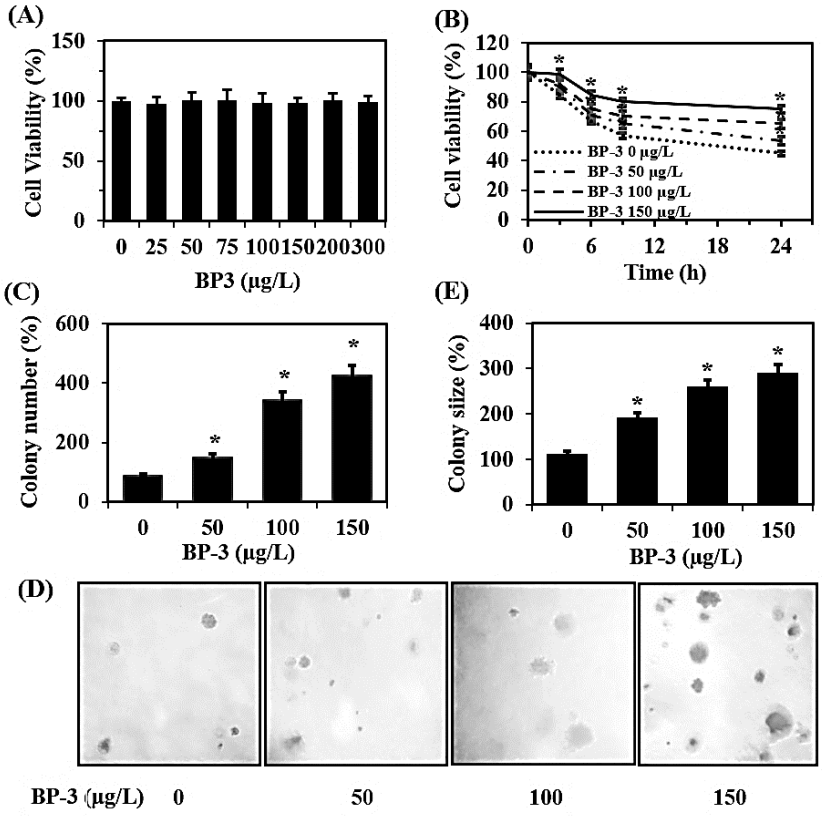


Figure 12 Effect of BP-3 on A549 cells. **a** Cytotoxic effect BP-3 on A549 cells was determined by MTT assay. Values are means of the three independent triplicate samples ± SE. *P,0.05 versus non-treated control. **b** Detached A549 cells were incubated with BP-3 (0-150 µg/L) in poly-HEMA coated plates and cell viability was determined by MTT assay at indicated times. Values are means of three independent triplicate experiments ± SE. *P,0.05 versus non-treated control at time 0. **c–e** Cells were treated with BP-3 (0-150 µg/L) and subjected to soft agar colony formation assay. Representative fields from three independent experiments were photographed after the cells were cultured for 7 and 10 days. Scale bar is 1,000 mm. Colony number and colony size were determined by image analyzer on the 10th day of culture. Values are means of the three independent triplicate samples ± SE. *P, 0.05 versus non- treated control.

Effect of BP-3 on epithelial to mesenchymal transition (EMT) in lung cancer cell

- BP-3 increases expression of EMT marker proteins in H460 cell

Collectively, our results showed that BP-3 could promote anoikis resistance as well as anchorage-independent growth of the lung cancer cells. A huge number of evidence has indicated that the cancer cells process of EMT is an important hallmark of aggressive cancer cells and such process was shown in several studies to potentiate anoikis resistance and metastasis (Eccles & Welch 2007; Geiger & Peeper 2009; Nurwidya et al., 2012). We next clarified whether BP-3 could induce EMT by evaluating the EMT markers using western blot analysis. The cells were treated with BP-3 at the concentration of 150 µg/L and EMT related proteins including N-cadherin, E-cadherin, Vimentin, Slug, and Snail were determined (Fig. 13a-f). Results indicated that the treatment of the cells with BP-3 significantly increased of N-cadherin (Fig. 13b) and Vimentin (Fig. 13c) in a time-dependent manner as those proteins were found significantly different from control as early as 9 hours after the treatment. Furthermore, the EMT major transcription factors Slug and Snail were found to significantly up-regulated at 12 and 24 hours (Fig. 13e, f). These results suggested that BP-3 can mediated EMT in the lung cancer cells and may responsible for facilitating the anoikis resistant as well as metastatic phenotypes of the lung cancer cells.

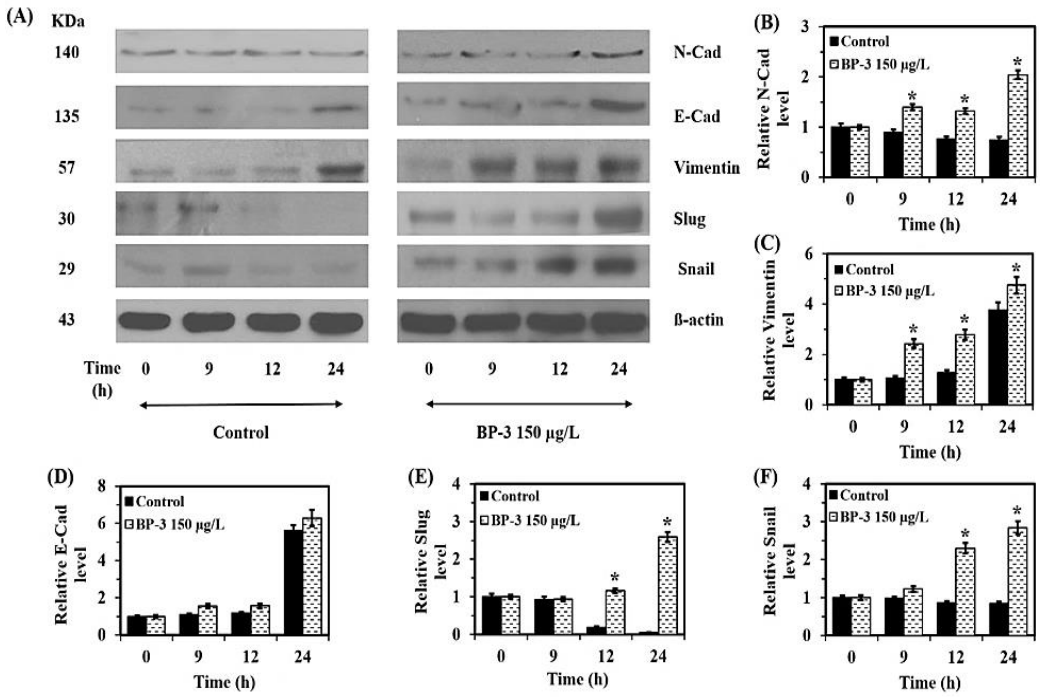


Figure 13 Effect of BP-3 on EMT markers. **a** Cells were treated with BP-3 (0-150 µg/L) for 0, 9, 12 and 24 h in detached condition. The level of N-cadherin, E-cadherin, vimentin, slug and snail were determined by western blot analysis. Blots were reprobed with β-actin to confirm equal loading. **b–f** The immunoblotting signals were quantified by densitometry and mean data from independent experiments were normalized to the results. The data present means of the three independent triplicate samples± SE. *P, 0.05 versus non-treated

Effect of BP-3 on survival signal in detached lung cancer cell

- BP-3 increases expression of pro-survival proteins in detached H460 cell

EMT has been shown to induce anoikis resistance by increasing survival pathways such as Akt and ERK (Song et al., 2005; Lu & Xu, 2006). We next investigated whether the anoikis resistance of the BP-3 treated cells was mediated through the increase of these survival signals. The H460 cells were detached in the presence or absence of BP-3 and the activated level of Akt and ERK were evaluated by western blotting. Our results aligned with the mentioned concept as the activated ERK significantly increased and slightly elevated activated Akt was detected in the BP-3-treated cells in comparison to those of non-treated control cells (Fig. 14b, c). The active Akt and ERK are key mediators for the induction of the downstream pro-survival proteins. We further found that the pro-survival Mcl-1 and Bcl-2 proteins were significantly increased in response to BP-3 treatment (Fig. 14d, e). Besides, we found that the level of Cav-1, a known mediator of anoikis resistance (Chunhacha & Chanvorachote, 2012; Chunhacha et al., 2012; Halim, Luanpitpong & Chanvorachote, 2012) was significantly up-regulated in the lung cancer cells treated with BP-3 (Fig. 14f). The increase of N-cadherin in the EMT cancer cells was shown to mediate survival of the cells through activation of Akt and ERK (Chiarugi & Giannoni, 2008; Paoli, Giannoni & Chiarugi, 2013). Besides, Cav-1 was reported to be able to increase the level of Mcl-1 and activated Akt in the detached lung cancer cells (Chunhacha et al., 2012). Cells were treated with BP-3 in a dose-dependent manner and the expression level of activated Akt, total Akt, activated ERK, total ERK, Mcl-1, Bcl-2 and Cav-1 were determined by Western blotting assay. Fig. 11g, h shows that BP-3 at the concentrations of 50-150 µg/L caused a significant increase of activated ERK, Mcl-1, Bcl-2 and Cav-1 expression over the control level. Together, these findings at least, in part, revealing the novel mechanism of BP-3 in regulation of anoikis resistance via EMT as well as Cav-1 machineries.

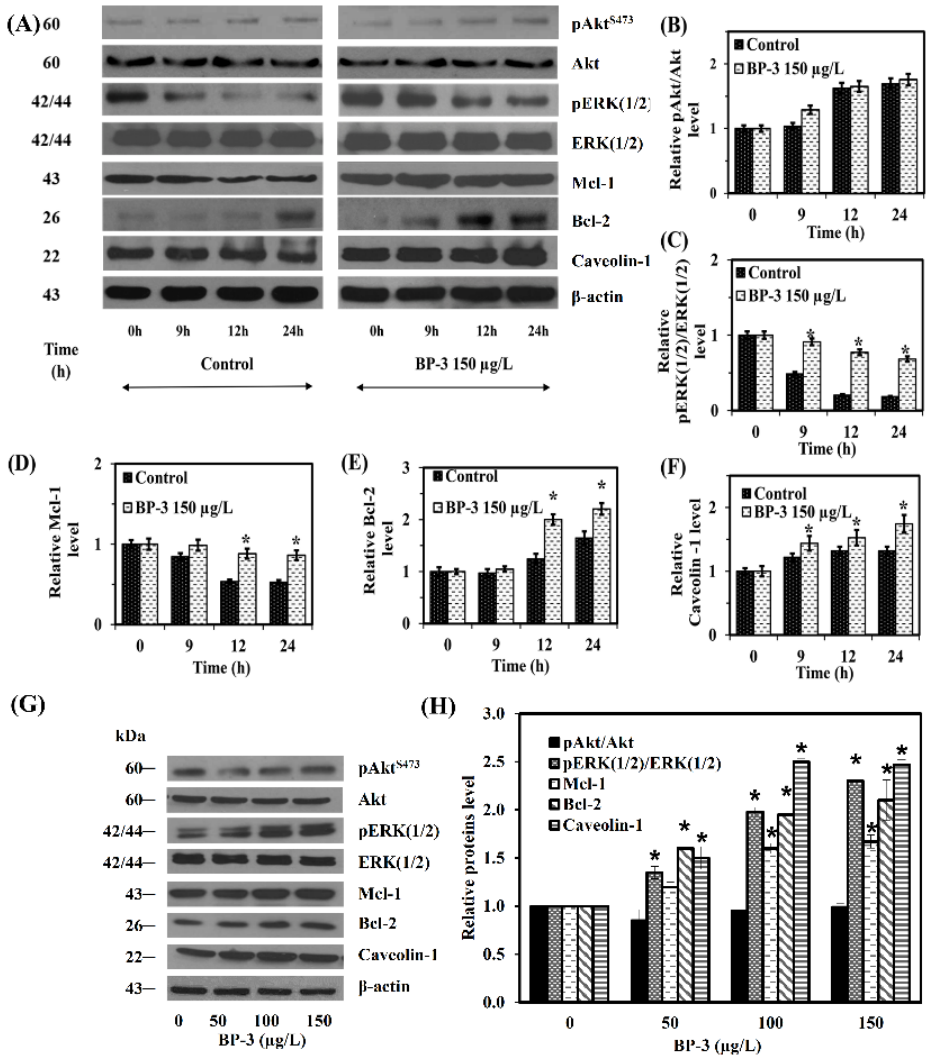


Figure 14 Effect of BP-3 on anoikis regulatory proteins. **a** H460 cells were treated with BP-3 (150 μg/L) for 0, 9, 12 and 24 h in detached condition. The levels of survival proteins, including activated Akt, total Akt, activated ERK (p42/44), total ERK, Mcl-1, Bcl-2, and Cav-1 were determined by western blotting. Blots were reprobed with beta-actin to confirm equal loading. **b–f** The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the results. The data present means of the three independent triplicate samples± SE. *P, 0.05 versus n on-treated. **g** Cells were treated with BP-3 (0-150 μg/L) for 12 h in detached condition. The levels of survival proteins, including activated Akt, total Akt, activated ERK (p42/44), total ERk, Mcl-1, Bcl-2, and Cav-1 were determined by western blotting. Blots were reprobed with beta-actin to confirm equal loading. **h** The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the results. The data present means of the three independent triplicate samples± SE. *P, 0.05 versus non-treated.

Effect of ERK inhibitor (PD98059) on cell viability and anoikis regulatory proteins.

To confirm whether ERK is the key regulator in BP-3-mediated anoikis resistance, PD98059 (ERK inhibitor) was used. H460 cells were detached, suspended in the serum free culture media containing non-cytotoxic concentrations of BP-3 (150 μg/L) in the presence or absence of PD98059 (15 μM) for 12 hours, and cell survival was determined by MTT assay. Fig. 12a shows that BP-3 treatment significantly increased cell survival after detachment with approximately 20% cell survival over non-treated control. Interestingly, addition of ERK inhibitor suppressed anoikis resistance in BP-treated cells back to the base-line level in non-treated cells. These results confirmed that BP-3 treatment mediates anoikis resistance via ERK-dependent mechanism.

The expression of downstream protein signals of ERK was further determined. Detached H460 cells were treated with BP-3 or BP-3 and PD98059 for 12 hours, and the protein expression were analyzed by western blot analysis. Fig. 15b, c show that treatment of the cells with BP-3 significantly increased phosphorylated ERK, whereas the expression of total ERK was not altered. Also, the downstream proteins Mcl-1 and Bcl-2 were found to increase as results of ERK activation. ERK inhibitor significantly abolished the inductive effect of BP-3 on p-ERK, Mcl-1 and Bcl-2 expressions. These results strongly supported the role of ERK signaling in BP-3- induced survival in lung cancer H460 cells.

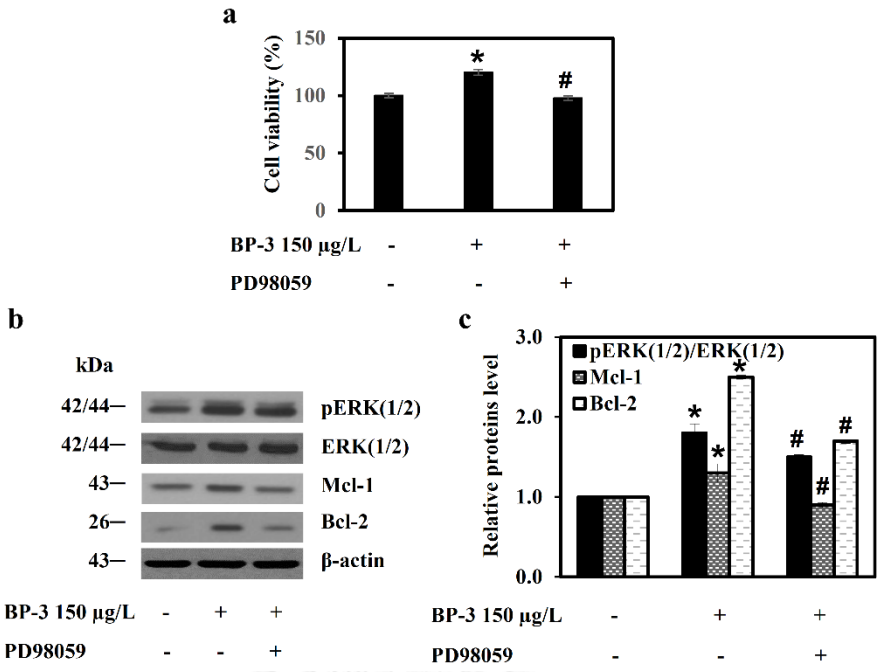


Figure 15 Effect of ERK inhibitor (PD98059) on cell viability and anoikis regulatory proteins. **a** H460 cells were treated with BP-3 (150 µg/L) in the presence or absence of ERK inhibitor (PD98059) for 12 h in detached condition. Cell viability was determined by MTT assay at indicated times. Values are means of three independent triplicate experiments ± SE. *P, 0.05 versus non-treated control. #P, 0.05 versus BP-3 treated alone. **b** The levels of proteins, including activated ERK (p42/44), total ERK, Mcl-1, Bcl-2 were determined by western blotting. Blots were reprobed with beta-actin to confirm equal loading. **c** The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to the results. The data present means of the three independent triplicate samples± SE. *P, 0.05 versus non-treated. #P, 0.05 versus BP-3 treated alone

Effect of natural compound (moscatilin) on inhibiting epithelial to mesenchymal transition (EMT) in lung cancer cell

- Moscatilin does not cause cytotoxicity in H460 cell

In order to determine the concentration of moscatilin used in the study, the cytotoxicity of moscatilin was evaluate in the lung cancer cells H460 by MTT assay and nuclear staining assay. Cells were treated with moscatilin with moscatilin 0-5 μM for 24 hours. Figure 13b-d shows that moscatilin at concentration of 0-1 μM has no cytotoxic effect on H460 cells. Moreover, the cytotoxic effect of moscatilin was confirmed by Hoechst 33342/PI co-staining assay. It can be been that the nuclear condensation of apoptotic cells was not observed at 0-1 μM of moscatilin.

- Moscatilin does not cause cytotoxicity in HaCat cell

In order to confirm the non-cytotoxicity of moscatilin used in the study, the cytotoxicity of moscatilin was evaluate in the human normal keratinocytes, HaCat, by MTT assay. HaCat cells were treated with moscatilin with moscatilin 0-5 μM for 24 hours. Figure 16b shows that moscatilin at concentration of 0-1 μM has no cytotoxic effect on HaCat cells. The cytotoxic effect of moscatilin was also confirmed by Hoechst 33342/PI co-staining assay. Figure shows that the nuclear condensation of apoptotic cells was not observed at 0-1 μM of moscatilin. Both HaCat cytotoxic results were in consistency to the results from H460 cells. Therefore moscatilin at concentration 0-1 μM was further used in this study.

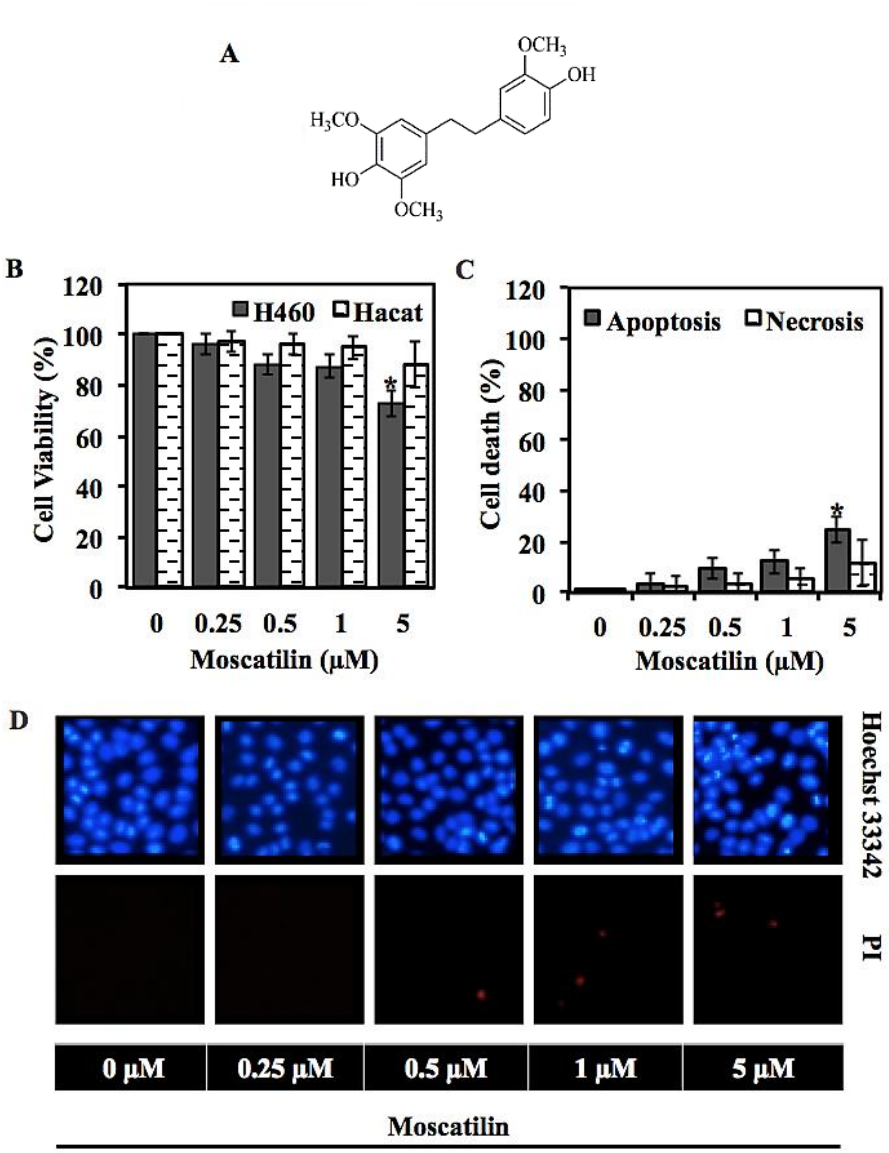


Figure 16 Cytotoxic effects of moscatilin **a** on human lung cancer H460 cells and keratinocyte HaCat cells. **b** H460 and HaCat cells were treated with moscatilin (0-5 μM) for 24 h. Percentage of cell viability was determined by MTT assay. The viability of untreated cells was represented as 100%. **(b and c)** H460 cells were treated with moscatilin (0-5 μM) for 24 h. Apoptotic and necrotic cell death were evaluated using Hoechst 33342/PI staining and calculated as a percentage compared with non-treated control cells. The data present means ± SD (n = 4). * $P < 0.05$ vs. non-treated cells.

- Moscatilin inhibits anoikis resistance in H460 cell

In order to determine the anoikis sensitization of moscatilin in H460 cell, the anoikis assay was performed. H460 cells were treated with 0-1 μM of moscatilin 24 hours before detached into a suspended condition in serum free culture medium. At 0, 3, 6, 9, 12, 24 hours the suspended cells were harvested for cell viability assays, MTT and Hoechst 33342/PI assay. Figure 17 shows that the survival rate of the treated cells decreased in a dose-dependent manner at each experimental time. Moscatilin at 0.25 μM significantly decreased the viability of detached cells at 24 hours compared to the control. At 3 hours after the detachment moscatilin at 1 μM significantly reduced the cell viability to below 80 %. The anoikis sensitizing effect of moscatilin was confirmed by Hoechst 33342/PI assay. The results from both assays were in consistency while there were no PI-positive necrotic cells observed in all experimental conditions. All in all, these outcomes has claimed that moscatilin at non-cytotoxic centration was able to inhibit the anoikis resistance behavior of lung cancer cells.

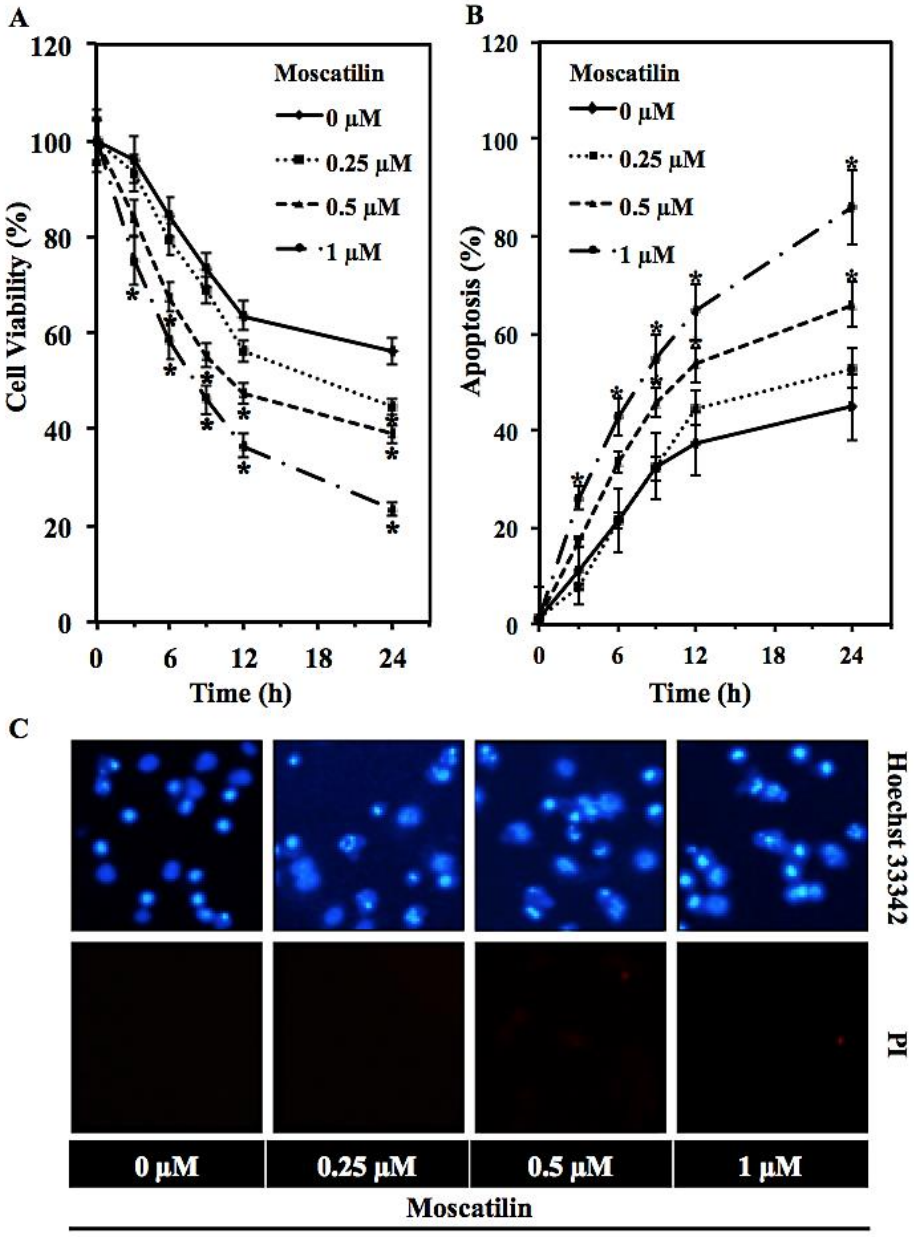


Figure 17 Anoikis sensitizing effect of moscatilin on human lung cancer H460 cells. (A) H460 cell were treated with non-cytotoxic concentrations of moscatilin (0-1 μM) for 24 h, and cell survival was determined by MTT assay. The viability of untreated cells was represented as 100%. (B and C) H460 cells were treated with moscatilin (0-5 μM) for 24 h. Apoptotic and necrotic cell death were determined by Hoechst 33342/PI staining and calculated as a percentage compared with non-treated control cells. All plots are means ± SD (n = 4). * $P < 0.05$ vs. non-treated cells.

- Moscatilin reduces colony formation in H460 cell

In order to investigate the reduction of anchorage-independent growth of moscatilin in H460 cell, the colony formation assay was carried out. After 24 hours of 0-1 μM moscatilin treatment, the treated cells were subjected to colony formation test between agarose layers. Figure 18 shows that at day 7 moscatilin treatment was able to reduce the growth of the cancer colonies in both number and size. 1 μM of moscatilin was able to reduce the number of colonies down to 43 % and the average diameter of the colonies was down to 35 % compared to the control. It is, therefore, reasonable to state that moscatilin has the ability to inhibit the anchorage-independent growth of lung cancer cells.

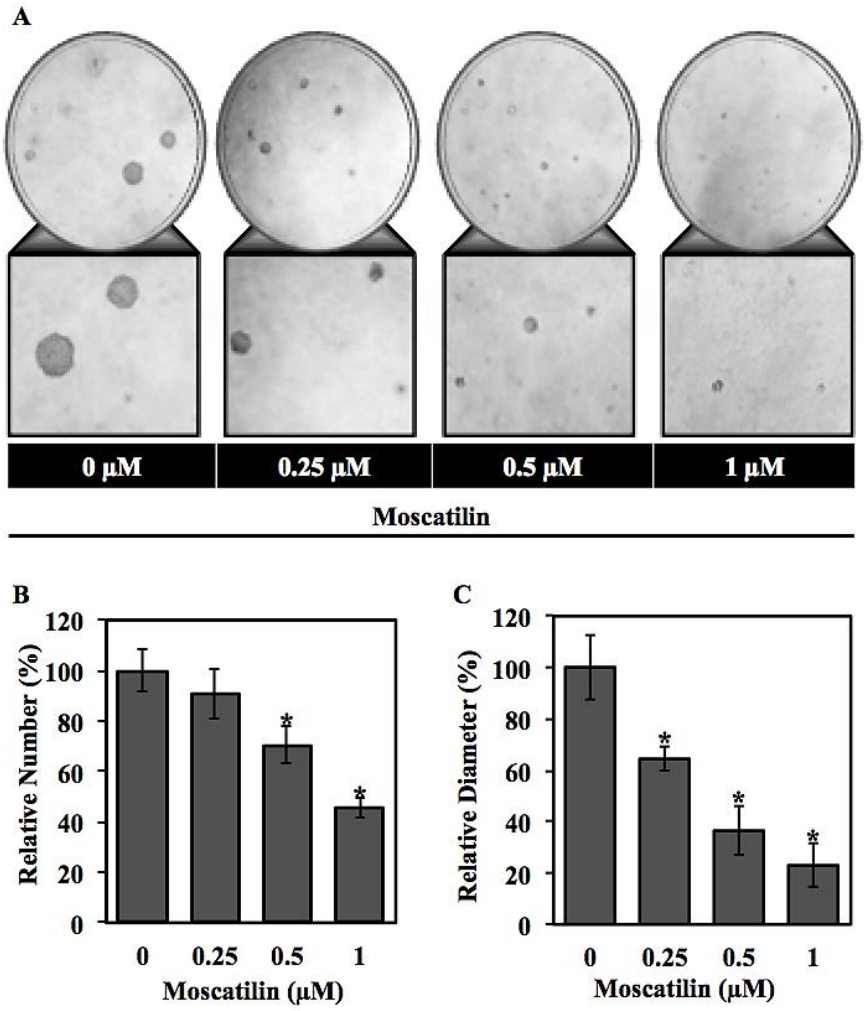


Figure 18 Moscatilin inhibits anchorage-independent growth of H460 cells. (A) After treatment with moscatilin (0-1 µM) for 24 h, H460 cells were suspended and subjected to colony-formation assay. Colony ×4 images were captured after day 7. (B) Colony number and (C) colony diameter were analyzed and calculated as relative to the non-treated control cells. The relative number and relative diameter of untreated cells was represented as 100%. The data present means ± SD (n = 4). * $P < 0.05$ vs. non-treated cells. Representative fields from four independent experiments were photographed.

- Moscatilin decreases expression of EMT marker proteins in H460 cell

As it was clearly reported that anoikis resistance behavior of cancer cell is the result of the EMT process, so we hypothesized that moscatilin may have the ability to decrease EMT process of cancer cells. In order to prove the statement, the expressions of EMT marker proteins were evaluated after moscatilin treatment. 0-1 μM moscatilin was treated to H460 cells for 24 hours before detaching the cells into suspended condition for 12 hours. After that H460 cells were harvested and subjected to protein measurement in Western blotting analysis. EMT marker proteins expressions including N-cadherin, E-cadherin, vimentin, Snail and Slug were evaluated. It can be seen from figure 19 that 1 μM caused the expression of N-cadherin, vimentin Snail and Slug to decrease whereas the expression of E-cadherin was increased, which correlated with the EMT inhibition process.

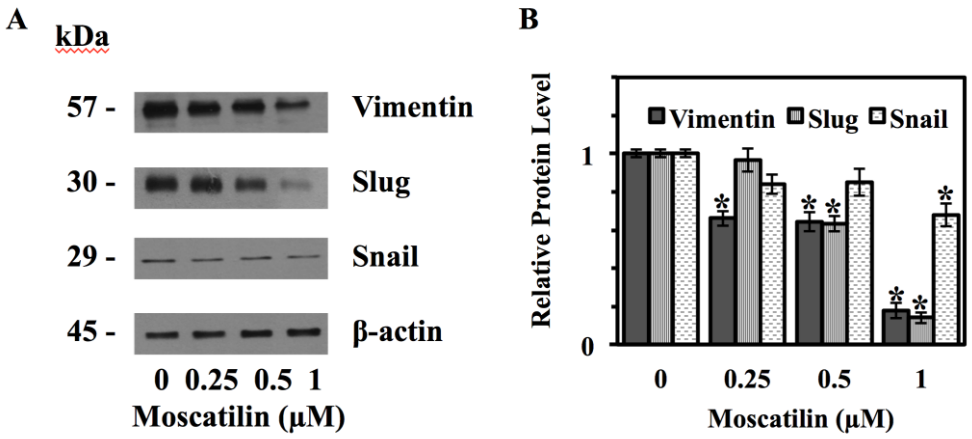


Figure 19 Effect of moscatilin on EMT markers. (A) After treatment with moscatilin (0-1 μM) for 24 h, H460 cells were treated with moscatilin (0-1 μM) for 12 h in detached condition. The levels of Vimentin, Slug and Snail were determined by western blot analysis. The blots were re-probed with β-actin to confirm equal loading. (B) The immunoblotting signals were quantified by densitometry and mean data from independent experiments were normalized to the untreated control. The data present means ± SD (n = 4). * $P < 0.05$ vs. non-treated cells

- *Moscatilin decreases expression of pro-survival proteins in detached H460 cell*

In order to clarify the mechanism of moscatilin in regulating anoikis sensitizing property, the expression of anoikis resistance-associated proteins including activated Akt (phosphorylated Akt at Ser 473), activated ERK (phosphorylated ERK (p44/42)), Mcl-1, and Cav-1, were determined. 0-1 μM moscatilin was treated to H460 cells for 24 hours before protein measurement in Western blotting analysis. The results indicated that treatment with moscatilin at 0.5 and 1 μM significantly reduced the active forms of Akt and ERK in comparison to the control group. Moreover, Mcl-1 protein, a downstream protein of Akt and ERK, was found to be significantly reduced in response moscatilin treatment. As the up-regulation of Cav-1 is associated with anoikis resistance in many cancers including lung cancer, the effect of moscatilin on Cav-1 expression was also evaluated. Figure 20 shows that the level of Cav-1 was significantly down regulated in response to 1 μM moscatilin treatment. In conclusion, this present study reveals that moscatilin possesses activity in enhancing the anoikis response of the cancer cells by suppressing survival Akt, ERK, anti-apoptotic Mcl-1, and Cav-1 proteins through EMT inhibition.

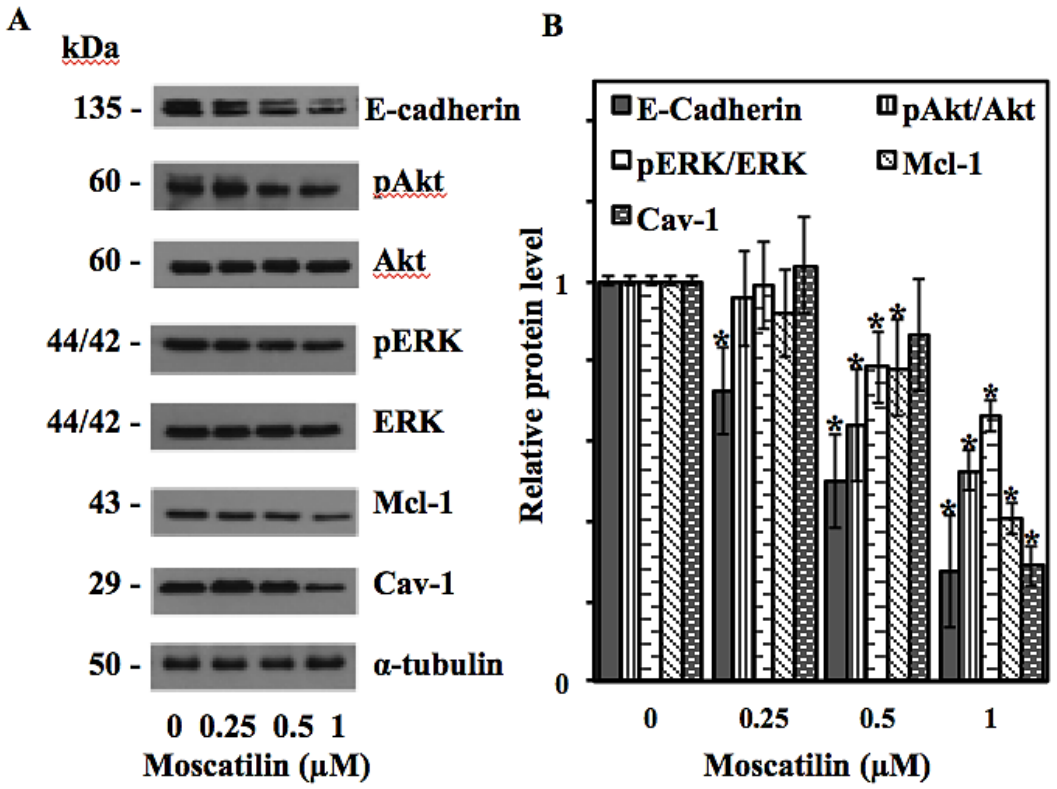


Figure 20 Effects of moscatilin on anoikis regulatory proteins. (A) H460 cells were treated with moscatilin (0-1 μM) for 24 h. Levels of E-cadherin, activated Akt, total Akt, activated ERK (p44/42), total ERK (p44/42), Mcl-1, and Cav-1 were determined by Western blotting. The blots were re-probed with α-tubulin to confirm equal loading. (B) The immunoblotting signals were quantified by densitometry and mean data from independent experiments were normalized to the untreated controls. The data present means ± SD (n = 4). * $P < 0.05$ vs. non-treated cells.

CHAPTER V
DISCUSSION AND CONCLUSION

Lung cancer is among the leading deadly diseases with the poorest clinical outcome. The metastasis in lung cancer has been accepted to be a critical factor accentuates the soaring death rate (Eccles & Welch, 2007; Geiger & Peeper, 2009; Nurwidya et al., 2012). In certain cases, adaptive mechanisms of cancer cells in response to some stimuli were shown to enhance the progression of the disease. Exposure to the anti-cancer agents like cisplatin, a widely used drug for the treatment of cancers has been shown to confer cell migration, invasion, and drug resistance (Chanvorachote et al., 2012). Therefore, a conception that tumor cells adapt themselves in order to survive in the altered conditions and sometimes result in more aggressive phenotypes has gained more interest in cancer research.

BP-3 has been used for several years as an active sunscreen in many types of cosmetic as well as daily used products such as flavoring agent or photo protective products. As sufficient evidences have indicated that BP-3 is able to absorbed through skin and accumulate in the human body (Janjua et al., 2004; Gonzalez et al., 2006), the concern of usage of this agent has been raised. Herein, we have showed for the first time that the exposure of the human lung cancer cells to the non-toxic concentrations of BP-3 could confer to the cells behavioral change toward mesenchymal phenotypes. The BP-3-treated cell exhibited the augmented abilities to survive and growth in an anchorage-independent condition.

EMT process has been shown in a number of studies as an important process facilitating metastasis. The alteration of cellular phenotypes form epithelial to mesenchymal drives the cancer cells to survive in detached condition, facilitates migration, and invasion (Yilmaz & Christofori, 2009; Sánchez-Tilló et al., 2012). Herein

we have revealed for the first time that exposure of the lung cancer cells to BP-3 cells increased the expression of EMT marker proteins. Interestingly, the aggressive behaviors of the cells including anoikis resistance and growth in anchorage-independent manner were found to be potentiated in the BP-3-treated cells.

EMT has been shown to induce anoikis resistance by increasing activated levels of survival pathways such as Akt and ERK (Song et al., 2005; Lu & Xu, 2006). Indeed, a key molecular feature up-regulating in the EMT cells, N-cadherin, was shown to augment cellular pro-survival signals such as Akt and ERK (Chiarugi & Giannoni, 2008; Paoli, Giannoni & Chiarugi, 2013). Our results aligned with the mentioned concept as the activated ERK were significantly up-regulated and slightly increased activated Akt were detected in the BP-3-treated cells in comparison to those of non-treated control cells (Fig. 14). Because the active Akt and ERK are key mediators for the induction of down-stream pro-survival proteins, we thus tested whether the active Akt and ERK could increase the pro-survival proteins. Mcl-1 and Bcl-2 are anti-apoptotic proteins that have been shown to be up-regulated by the function of active Akt and ERK. Accordingly, we found that treatment of the cells with BP-3 resulted in a significantly increase of Mcl-1 and Bcl-2. Furthermore, the Cav-1, a protein that has been shown to mediate anoikis resistance in lung cancer cells (Ravid et al., 2006; Chunhacha & Chanvorachote, 2012; Lloyd, 2012), was shown herein to be up-regulated in response to BP-3 treatment (Fig. 14).

The Cav-1 protein was shown to increase metastatic potential of several cancer cells including lung cancer cells by potentiating abilities of the cells to migrate, invade, and resist to anoikis (Chunhacha & Chanvorachote, 2012; Chunhacha et al., 2012; Halim, Luanpitpong & Chanvorachote, 2012). Cav-1 was shown to stabilize the level of anti-apoptotic Mcl-1 in the detached lung cancer cells (Chunhacha et al., 2012). In addition, the present of Cav-1 was shown to sustain the activated function of Akt in anoikis cancer cells (Li et al., 2003). Collectively, our resulted suggested that BP-3 may potentiate anoikis resistance as well as growth in anchorage-independent manner via EMT and Cav-1-dependent mechanisms (figure 21).

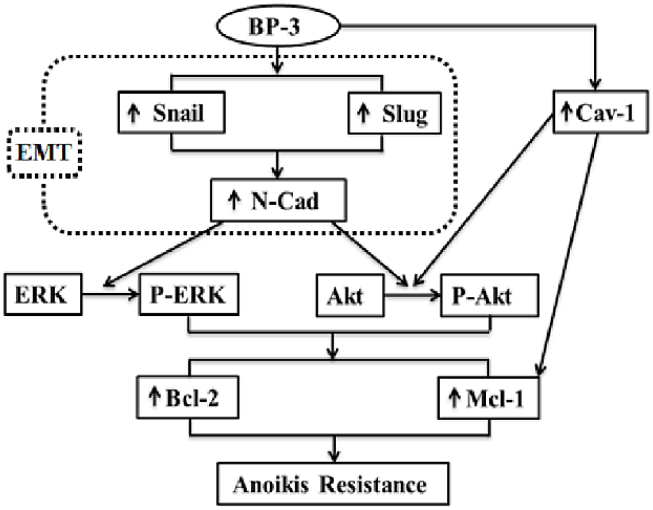


Figure 21 The scheme representing mechanism of BP-3 in regulation of cancer cell anoikis resistance through EMT and caveolin-1- dependent pathways.

Regarding angiogenesis, it is observed in lung cancer which is essential in the process of primary tumor growth, proliferation and metastasis (Eccles & Welch, 2007; Geiger & Peeper, 2009; Nurwidya et al., 2012). As angiogenesis is responsible for an increase in blood supply to tumor cells, it is possible that BP-3 in blood circulation

would have a better chance to expose to the cancer cells. Therefore, we agree that angiogenesis would enhance the effect of BP-3 by such a mean.

Despite toxicity of BP-3, moscatilin, bibenzyl derivative extracted from medicinal orchid which is widely found in agricultural countries like Thailand, has been revealed to have an anti-metastasis potential in lung cancer cells. Our study has provided more evidence supporting the anti-cancer activity of moscatilin. We have found that moscatilin was able to inhibit anoikis-resistant phenotypes of lung cancer cells by suppressing cell survival Akt, ERK pathways and Cav-1 through inhibition of EMT process (figure 22). This finding may be a strong evidence leading to a further investigation and development of moscatilin targeting anti-metastasis approach.

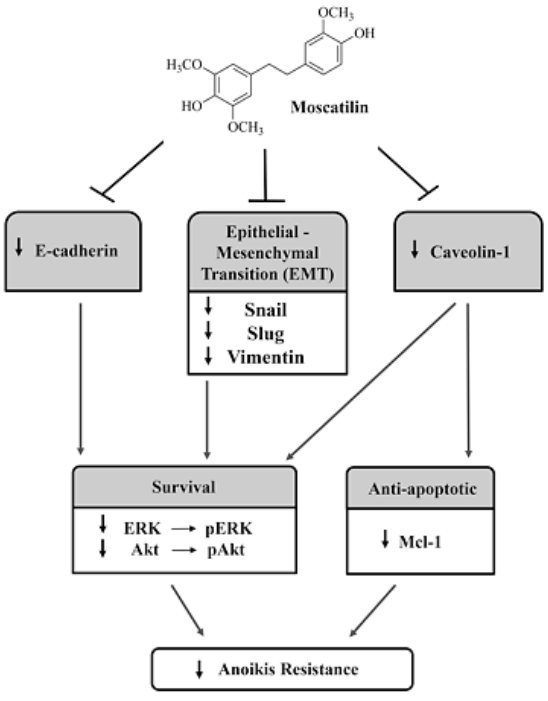


Figure 22 The scheme represents the effect of moscatilin on anoikis regulatory proteins. The present study reveals that moscatilin has ability to sensitize anoikis in the H460 lung cancer cells via the suppression of E-cadherin-mediated survival, EMT, and Cav-1.

In conclusions, our study showed that BP-3 may promote lung cancer metastasis by induces EMT and increase Cav-1 that confer anoikis resistance and growth in detached condition. Nevertheless, a natural compound, moscatilin has the ability to counteract the EMT behavior of aggressive lung cancer cells. This study provides novel information of BP-3, a chemical that is widely used in health care products and the novel cancer therapy potential of moscatilin. The findings may lead to further investigations that promote the caution of use of BP-3-containing products in cancer patients.

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REFERENCES

Calafat, A.M., Wong, L.Y., Ye, X., Reidy, J.A. & Needham, L.L., 2008. Concentrations of the sunscreen agent benzophenone-3 in residents of the United States: National Health and Nutrition Examination Survey 2003–2004. *Environmental Health Perspectives*, 116(7), pp.893–897.

Chaffer, C.L. & Weinberg, R.A., 2011. A perspective on cancer cell metastasis. *Science*, 331(6024), pp.1559–1564.

Chambers, A.F., Groom, A.C. & MacDonald, I.C., 2002. Metastasis: Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer*, 2(8), pp.563–572.

Chanvorachote, P., Luanpitpong, S., Chunhacha, P., Promden, W. & Sriuranpong, V., 2012. Expression of CA125 and cisplatin susceptibility of pleural effusion-derived human lung cancer cells from a Thai patient. *Oncology Letters*, 4(2), pp.252–256.

Chen, C.A., Chen, C.C., Shen, C.C., Chang, H.H. & Chen, Y.J., 2013. Moscatilin induces apoptosis and mitotic catastrophe in human esophageal cancer cells. *Journal of Medicinal Food,* 16(10), pp.869-7.

Chiarugi, P. & Giannoni, E., 2008. Anoikis: A necessary death program for anchorage-dependent cells. *Biochemical Pharmacology*, 76(11), pp.1352–1364.

Chunhacha, P. & Chanvorachote, P., 2012. Roles of caveolin-1 on anoikis resistance in non small cell lung cancer. *International Journal of Physiology, Pathophysiology and Pharmacology*, 4(3), pp.149–155.

Chunhacha, P., Pongrakhananon, V., Rojanasakul, Y. & Chanvorachote, P., 2012. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. *AJP: Cell Physiology*, 302(9), pp.C1284–C1292.

Chunhacha, P., Sriuranpong, V. & Chanvorachote, P., 2013. Epithelial-mesenchymal transition mediates anoikis resistance and enhances invasion in pleural effusion-derived human lung cancer cells. *Oncology Letters*, 5(3), pp.1043–1047.

Eccles, S.A. & Welch, D.R., 2007. Metastasis: recent discoveries and novel treatment strategies. *Lancet*, 369(9574), pp.1742–1757.

Enomoto, A., Murakami, H., Asai, N., Morone, N., Watanabe, T., Kawai, K., Murakumo, Y., Usukura, J., Kaibuchi, K. & Takahashi, M., 2005. Akt/PKB Regulates Actin Organization and Cell Motility via Girdin/APE. *Developmental Cell*, 9(3), pp.389–402.

Feigeal, A.I., 2009. Toxicological evaluation of benzophenone. *The EFSA Journal*, 1104, pp.1–30.

Fenouille, N., Tichet, M., Dufies, M., Pottier, A., Mogha, A., Soo, J.K., Rocchi, S., Mallavialle, A., Galibert, M.D., Khammari, A., Lacour, J.P., Ballotti, R., Deckert, M. & Tartare-Deckert, S., 2012. The Epithelial-Mesenchymal Transition (EMT) Regulatory Factor SLUG (SNAI2) Is a Downstream Target of SPARC and AKT in Promoting Melanoma Cell Invasion N. A. Hotchin, ed. *PLoS ONE*, 7(7), p.e40378.

Floor, S.L., Dumont, J.E., Maenhaut, C. & Raspe, E., 2012. Hallmarks of cancer: of all cancer cells, all the time? *Trends in Molecular Medicine*, 18(9), pp.509–515.

Geiger, T.R. & Peeper, D.S., 2009. Metastasis mechanisms. *BBA - Reviews on Cancer*, 1796(2), pp.293–308.

Gonzalez, H., Farbrot, A., Larkö, O. & Wennberg, A.M., 2006. Percutaneous absorption of the sunscreen benzophenone-3 after repeated whole-body applications, with and without ultraviolet irradiation. *British Journal of Dermatology*, 154(2),

pp.337–340.

Halim, H., Luanpitpong, S. & Chanvorachote, P., 2012. Acquisition of anoikis resistance up-regulates caveolin-1 expression in human non-small cell lung cancer cells. *Anticancer Research*, 32(5), pp.1649–1658.

Heatley, M., Whiteside, C. & Maxwell, P., 1993. Vimentin expression in benign and malignant breast epithelium. *Journal of Clinical Pathology,* 46(5), pp. 441–445.

Ho, C.-C., Huang, P.S., Huang, H.Y., Chen, Y.S., Yang, P.C. & Hsu, S.M., 2010. Up-Regulated Caveolin-1 Accentuates the Metastasis Capability of Lung Adenocarcinoma by Inducing Filopodia Formation. *The American Journal of Pathology*, 161(5), pp.1647–1656.

Ho, C.-K. & Chen, C.-C., 2003. Moscatilin from the Orchid *Dendrobrium loddigesiils* a Potential Anticancer Agent. *Cancer Investigation*, 21(5), pp.729–736.

Huff, J., 2013. Benzene-induced Cancers: Abridged History and Occupational Health Impact. *International Journal of Occupational and Environmental Health*, 13(2), pp.213–221.

Janjua, N.R., Mogensen, B., Andersson, A.M., Petersen, J.H., Henriksen, M., Skakkebaek, N.E. & Wulf, H.C., 2004. Systemic Absorption of the Sunscreens Benzophenone-3, Octyl-Methoxycinnamate, and 3-(4-Methyl-Benzylidene) Camphor After Whole-Body Topical Application and Reproductive Hormone Levels in Humans. *The Journal of Investigative Dermatology*, 123(1), pp.57–61.

Klongkumnuankarn, P., Busaranon, K., Chanvorachote, P., Sritularak, B., Jongbunprasert, V. & Likhitwitayawuid, K., 2015. Cytotoxic and Antimigratory Activities of Phenolic Compounds from *Dendrobium brymerianum. Evidence-Based Complementary*

and Alternative Medicine, pp.1–9.

Klymkowsky, M.W. & Savagner, P., 2009. Epithelial-Mesenchymal Transition. *The American Journal of Pathology*, 174(5), pp.1588–1593.

Kowitdamrong, A., Chanvorachote, P., Sritularak, B. & Pongrakhananon, V., 2013. Moscatilin Inhibits Lung Cancer Cell Motility and Invasion via Suppression of Endogenous Reactive Oxygen Species. *BioMed Research International*, 2013(1), pp.1–11.

Kumar, S., Park, S.H., Cieply, B., Schupp, J., Killiam, E., Zhang, F., Rimm, D.L. & Frisch, S.M., 2011. A Pathway for the Control of Anoikis Sensitivity by E-Cadherin and Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology*, 31(19), pp.4036–4051.

Larue, L. & Bellacosa, A., 2005. Epithelial–mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways. *Oncogene*, 24(50), pp.7443–7454.

Li, L., Cheng, H.R., Salahaldin, A.T., Chengzhen, R. & Timothy, C.T., 2003. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. *Molecular Cell Biology*. 23(24). pp. 9389–9404.

Lloyd, P.G., 2012. Caveolin-1, antiapoptosis signaling, and anchorage-independent cell growth. Focus on “Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells.” *AJP: Cell Physiology*, 302(9), pp.C1282–C1283.

Lu, Z. & Xu, S., 2006. ERK1/2 MAP kinases in cell survival and apoptosis. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)*, 58(11), pp.621–

631.

Mehlen, P. & Puisieux, A., 2006. Metastasis: a question of life or death. *Nature Reviews Cancer*, 6(6), pp.449–458.

Nurwidya, F., Takahashi, F., Murakami, A. & Takahashi, K., 2012. Epithelial Mesenchymal Transition in Drug Resistance and Metastasis of Lung Cancer. *Cancer Research and Treatment*, 44(3), pp.151–156.

Onder, T.T., Gupta, P.B., Mani, S.A., Yang, J., Lander, E.S. & Weinberg, R.A., 2008. Loss of E-Cadherin Promotes Metastasis via Multiple Downstream Transcriptional Pathways. *Cancer Research*, 68(10), pp.3645–3654.

Pai, H.-C., Chang, L.H., Peng, C.Y., Chang, Y.L., Chen, C.C., Shen, C.C., Teng, C.M. & Pan, S.L., 2012. Moscatilin inhibits migration and metastasis of human breast cancer MDA-MB-231 cells through inhibition of Akt and Twist signaling pathway. *Journal of Molecular Medicine*, 91(3), pp.347–356.

Paoli, P., Giannoni, E. & Chiarugi, P., 2013. Anoikis molecular pathways and its role in cancer progression. *BBA - Molecular Cell Research*, 1833(12), pp.3481–3498.

Ravid, D., Maor, S., Werner, H. & Liscovitch, M., 2006. Caveolin-1 inhibits anoikis and promotes survival signaling in cancer cells. *Advances in Enzyme Regulation*, 46(1), pp.163–175.

Rungtabnapa, P., Nimmannit, U., Halim, H., Rojanasakul, Y. & Chanvorachote, P., 2011. Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. *AJP: Cell Physiology*, 300(2), pp.C235–C245.

Sánchez-Tilló, E., Liu, Y., de Barrios, O., Siles, L., Fanlo, L., Cuatrecasas, M., Darling, D.S., Dean, D.C., Castells, A. & Postigo, A., 2012. EMT-activating transcription factors in cancer: beyond EMT and tumor invasiveness. *CMLS Cellular and Molecular Life Sciences*, 69(20), pp.3429–3456.

Shi, Y., Wu, H., Zhang, M., Ding, L., Meng, F. & Fan, X., 2013. Expression of the epithelial-mesenchymal transition-related proteins and their clinical significance in lung adenocarcinoma. *Diagnostic Pathology*, 8(1), pp.1–1.

Siegel, R., Ma, J., Zou, Z. &. Jemal, A., 2014. Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, 64(1), pp.9–29.

Song, G., Ouyang, G. & Bao, S., 2005. The activation of Akt/PKB signaling pathway and cell survival. *Journal of Cellular and Molecular Medicine*, 9(1), pp.59–71.

Song, L., Coppola, D., Livingston, S., Cress, D. & Haura, E.B., 2014. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biology & Therapy*, 4(3), pp.267–276.

Taddei, M.L., Giannoni, E., Fiaschi, T. & Chiarugi, P., 2011. Anoikis: an emerging hallmark in health and diseases. *The Journal of Pathology*, 226(2), pp.380–393.

Valastyan, S. & Weinberg, R.A., 2011. Tumor Metastasis: Molecular Insights and Evolving Paradigms. *Cell*, 147(2), pp.275–292.

Voulgari, A. & Pintzas, A., 2009. Epithelial–mesenchymal transition in cancer metastasis: Mechanisms, markers and strategies to overcome drug resistance in the clinic. *BBA - Reviews on Cancer*, 1796(2), pp.75–90.

Wheelock, M.J., Shintani, Y., Maeda, M., Fukumoto, Y. & Johnson, K.R., 2008. Cadherin switching. *Journal of Cell Science*, 121(6), pp.727–735.

Whysner, J., Reddy, M.V., Ross, P.M., Mohan, M. & Lax, E.A., 2004. Genotoxicity of benzene and its metabolites. *Mutation Research/Reviews in Mutation Research*, 566(2), pp.99–130.

Winitthana, T., Lawanprasert, S. & Chanvorachote, P., 2014. Triclosan Potentiates Epithelial-To-Mesenchymal Transition in Anoikis-Resistant Human Lung Cancer Cells. *PLoS ONE*, 9(10), p.e110851.

Yilmaz, M. & Christofori, G., 2009. EMT, the cytoskeleton, and cancer cell invasion. *Cancer and Metastasis Reviews*, 28(1-2), pp.15–33.

Youle, R.J. & Strasser, A., 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nature Reviews Molecular Cell Biology*, 9(1), pp.47–59.

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