ผลของมอสคาทิลินต่อการเคลื่อนที่ของเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก

นายอัครวุฒิ โกวิทย์ดำรงค์

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EFFECTS OF MOSCATILIN ON MIGRATION OF NON-SMALL CELL LUNG CANCER CELLS

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นายอัครวุฒิ โกวิทย์ดำรงค์: ผลของมอสคาทิลินต่อการเคลื่อนที่ของเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็ก. (EFFECTS OF MOSCATILIN ON MIGRATION OF NON-SMALL CELL LUNG CANCER CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ภญ. ดร.วริษา พงศ์เรขนานนท์ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ภก. ดร.ปิติ จันทร์วรโชติ , 102 หน้า.

โรคมะเร็งปอดเป็นสาเหตุของการเสียชีวิตในผู้ป่วยโรคมะเร็งทั่วโลกและส่วนใหญ่ของผู้ป่วยเกือบทั้งหมดเสียชีวิตจากการแพร่กระจายของเซลล์มะเร็ง การเคลื่อนที่และการรุกล้ำเป็นกระบวนการที่จำเป็นของการแพร่กระจายของเซลล์มะเร็งที่มีความสำคัญในโรคมะเร็ง ทศวรรษที่ผ่านมามีการพัฒนาสารจากสมุนไพรที่มีฤทธิ์ทางชีวภาพเพื่อเป็นตัวเลือกในการยับยั้งการแพร่กระจายของเซลล์มะเร็งโดยมอสคาทิลินเป็นสารหนึ่งที่น่าสนใจสกัดได้จากกล้วยไม้ไทย *Dendrobium pulchellum* พบว่ามีฤทธิ์ต้านมะเร็งหลายชนิด แต่อย่างไรก็ตามยังไม่มีข้อมูลผลของมอสคาทิลินต่อการเคลื่อนที่และรุกล้ำของเซลล์ ในการศึกษานี้แสดงให้เห็นถึงฤทธิ์ของมอสคาทิลินในความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ในการยับยั้งการเคลื่อนที่ของเซลล์มะเร็งปอดชนิดที่ไม่ใช่เซลล์เล็กชนิด เอช23 ผลการยับยั้งของมอสคาทิลินมีความสัมพันธ์กับการลดระดับของอนุพันธ์ออกซิเจนที่ว่องไวภายในเซลล์โดยพบว่าไฮดรอกซิลเป็นอนุพันธ์ที่สำคัญในการลดการเกิดฟิโลโพเดีย การวิเคราะห์ด้วยเวสเทิร์นบลอทพบว่ามอสคาทิลินสามารถลดระดับของ activated focal adhesion kinase (phosphorylated FAK, Tyr 397) และ activated ATP-dependent tyrosine kinase (phosphorylated Akt, Ser 473) ในขณะที่ FAK และ Akt รวมภายในเซลล์ไม่มีการเปลี่ยนแปลง จากการศึกษานี้แสดงให้เห็นถึงโมเลกุลพื้นฐานที่เกี่ยวข้องกับฤทธิ์ของมอสคาทิลินในการยับยั้งการเคลื่อนที่และรุกล้ำของเซลล์มะเร็งปอดและแสดงให้เห็นถึงศักยภาพของมอสคาทิลินในการยับยั้งการแพร่กระจายเพื่อใช้ในการรักษามะเร็งปอดต่อไป

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สาขาวิชา...เภสัชวิทยา..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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AKKARAWUT KOWITDAMRONG: EFFECTS OF MOSCATILIN ON MIGRATION OF NON-SMALL CELL LUNG CANCER CELLS. ADVISOR: VARISA PONGRAKHANANON, Ph.D., THESIS CO-ADVISOR: ASSIST. PROF. PITHI CHANVORACHOTE, Ph.D., 102 pp.

Lung cancer is the leading cause of death among cancer patients worldwide, and most of them have died from metastasis. Migration and invasion are prerequisite processes associated with high metastasis potential in cancers. During the past decade, efforts have been made to develop cancer treatment options based on the anti-metastasis mechanism. Researchers have identified some biological agents that are believed to have an ability against cancer metastasis. Moscatilin is a major constituent isolated from the Thai orchid *Dendrobium pulchellum*, has been shown to have anticancer effect against numerous cancer cell lines. However, little is known regarding the effect of moscatilin on cancer cell migration and invasion. The present study demonstrates that non-toxic concentrations of moscatilin were able to inhibit human non-small cell lung cancer H23 cell migration and invasion. The inhibitory effect of moscatilin was associated with an attenuation of endogenous reactive oxygen species (ROS), in which hydroxyl radical ($OH^{\bullet}$) was identified as a dominant species in the suppression of filopodia formation. Western blot analysis also revealed that moscatilin possed its ability to down-regulate activated focal adhesion kinase (phosphorylated FAK, Tyr 397) and activated ATP-dependent tyrosine kinase (phosphorylated Akt, Ser 473), whereas their parental counterparts were not detectable changed. In conclusion, these results indicate the novel molecular basis of moscalitin-inhibiting lung cancer cell motility and invasion and demonstrate a promising anti-metastatic potential of such an agent for lung cancer therapy.

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Field of Study:….Pharmacology…….... Advisor's Signature V. Pongr.

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LIST OF ABBERVIATIONS

%	= percentage
°C	= degree Celsius
µg	= microgram (s)
µl	= microlitre (s)
Akt	= adenosine triphosphate dependent tyrosine kinase
ANOVA	= analysis of variance
bFGF	= bovine fibroblast growth factor
BM	= basement membrane
Cdc42	= cell division cycle 42
CO_2	= carbon dioxide
$DCFH_2$-DA	= dichlorofluorescein diacetate
DHE	= dihydroethidium
DMNQ	= dimethoxynaphthoquinone
DMSO	= dimethyl sulfoxide
DNA	= deoxyribonucleic acid
ECM	= extracellular matrix
ED_{50}	= effective dose for 50% of the population exposed to the drug
EDTA	= ethylene diamine tetraacetic acid
EGF	= epidermal growth factor receptor
EMT	= mesenchymal transition

eNOS = endothelial nitric oxide synthase

ERK = extracellular signal-regulated kinase

ERM = ezrin-radixin-moesin

et al. = et alibi, and others

EtOH = ethanol

F-actin = filamentous actin

FAK = focal adhesion kinase

FAT = focal adhesion targeting

FBS = fetal bovine serum

$FeSO_4$ = ferrous sulphate

g = gram

h = hour, hours

H_2O_2 = hydrogen peroxide

H23 = non-small lung cancer cell line: adrenocarcinoma

HCT116 = colon cancer cell line

HK2 = human kidney 2: human renal proximal tubule cells

HPF = hydroxyphenyl fluorescein

HUVECs = human umbilical vein endothelial cells

IC_{50} = the half maximal inhibitory concentration

IGF = insulin-like growth factor

JNK = c-Jun N-terminal kinase

l = litre (s)

LCC	= large cell carcinoma
MAPK	= mitogen-activated protein kinases
MeOH	= methanol
min	= minute (s)
mg	= milligram (s)
ml	= milliliter
mM	= millimolar
MMP	= matrix metalloprotease
MTT	= 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	= sodium chloride
nM	= nanomolar
NSCLC	= non-small cell lung cancer
$O_2^{\bullet-}$	= superoxide anion radical
$OH^{\bullet}$	= hydroxyl radical
OD	= optical density
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate-buffered saline
PI	= propidium iodide
PI3K	= phosphatidylinositol-3 kinase
PKC	= protein kinase C
RAW 264.7	= mouse leukemic monocyte macrophage cell lines

ROS	= reactive oxygen species
RNA	= ribonucleic acid
RNase	= ribonuclease
RPMI	= Roswell Park memorial institue’s medium
SCC	= squamous cell carcinoma
SCLC	= small cell lung cancer
S.D.	= standard deviation
SDS	= sodium dodecyl sulfate
Ser	= serine
TBST	= tris-buffered saline, 0.1 % Tween 20
TNM	= tumor, node, and metastasis
Tyr	= tyrosine
VEGF	= vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Lung cancer has killed more than one million patients each year, making it the most lethal form of cancers (Parkin, 2001). Despite important improvements in diagnosis and treatment choices, the fatality rate among lung-cancer patients remain high because most patients are diagnosed at the time when their conditions have already reached the advanced stage or metastasis (Thomas *et al*., 2001). More than 90% of lung-cancer patients have died from metastasis. After the metastasis is established, less than 15 % of patients have survived for longer than 5 years (Thomas *et al*., 2001). Such statistics have indicated that the process of migration and invasion must have occurred at a much earlier stage in the process of tumor progression. Consequently, metastasis is a hallmark of malignancy and no other effective treatment option is now available for the patients with cancer metastasis. Many studies have been conducted in order to explore alternative medication against cancer metastasis (Ray and Jablons, 2009).

Metastasis process is a complicated series of biological steps where malignant cells spread from the primary tumor to other target sites via migration, intravasation, transportation, extravasations, and adhesion of the detached cells (Fidler, 2005). Prior to place at secondary site tumor must migrate and invade through basement membrane out of primary (Yamaguchi and Condeelis, 2007). Therefore, the knowledge of cell-movement mechanisms proves crucial in understanding tumor metastasis and exploring the effective ways for prevention.

Aberrant generation of cellular ROS was tightly associated with several metastasis cancers such as lung (Luanpitpong *et al*., 2010), prostate (Lim *et al*, 2005) and colon cancers (Lei *et al*, 2011). Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) served as important regulators of various physiological pathways during cancer metastasis including angiogenesis, cancer motility, and invasiveness (Luanpitpong *et al*., 2010; Hung *et al*., 2012). Scientific evidence also showed that cancer migration and invasion are obviously regulated by reactive oxygen species (ROS) (Luanpitpong *et al*., 2010; Hung *et al*., 2012). It was demonstrated that treatment with antioxidant such as ascorbic acid caused a reduction in cancer motility and invasion (Wei *et al*., 2003), and conversely the addition of exogenous ROS enhances these activities (Storz, 2005).

Several molecular mechanisms were showed that migration and invasion are mainly regulated by focal adhesion kinase (FAK) signaling pathways. FAK facilitates signaling and functions at sites of integrin adhesion, regulating the turnover of these adhesion sites (Schaller, 2010). FAK is a non-receptor protein tyrosine kinase localized prominently in focal adhesions (Schaller *et al*., 1992). It plays role in a positive regulator of both cell motility and survival. The ability of tumor cells to migrate has been found to be linked to increased FAK expression, phosphorylation and catalytic activity (Schaller, 2010). During cell motility, FAK stimulates Akt/ERK signaling, that is localized at the leading edge of motile cells in actin-rich protruding structures. It has been reported that down regulation of p-Akt and p-ERK expression was able to inhibit cell migration and invasion both *in vivo* and *vitro* study (Kennedy *et al*., 1999). Furthermore, FAK regulates GTPase family of proteins, cell division cycle 42 (Cdc42). This family plays an essential role in modulating actin reorganization associated with cell motility. Filopodia, meanwhile, has been found to play an essential role in cell motility and invasion by protrusion at the edge of motile

cells for attachment and gliding controlled by FAK (Sinha and Yang, 2008). Therefore, the suppression of these migration and invasion regulatory proteins would inhibit cancer metastasis.

Developed from the aforementioned findings, this study focuses on the potential of Moscatilin (4,4'-dihydroxy-3,3',5'-trimethoxybibenzyl) in inhibiting cancer metastasis. Moscatilin is a bibenzyl component derived from the Thai orchid, *Dendrobium pulchellum* or "Ueang chang nao" in Thai (Chanvorachote *et al*., 2012). Moscatilin was reported to have various pharmacological properties such as anti-inflammatory (Liu *et al*., 2009), antioxidant (Zhang *et al*., 2007) and anti-platelet aggregation (Chen *et al*., 1994). Recently, it has demonstrated anticancer activity against many kinds of cancer, for example induction of cell cycle G2-M arrest, suppression of tumor angiogenesis and induction of cell apoptosis (Miyazawa et al., 1999; Ho and Chen, 2003). However, the role of moscatilin on tumor metastasis has not been characterized. This study thus aims to examine the effect of moscatilin on lung cancer migration and invasion as well as the underlying mechanisms. The result of this study could be useful in the development of this compound for cancer therapy.

Research questions

1. Does moscatilin inhibit the H23 non-small cell lung cancer migration and invasion?

2. What is the underlying mechanism of moscatilin in inhibiting H23 non-small cell lung cancer migration and invasion?

Hypothesis

Moscatilin is able to inhibit migration and invasion through disruption of FAK signaling in H23 non-small cell lung cancer.

Objectives

1. To determine the effects of moscatilin on H23 non-small cell lung cancer migration and invasion.

2. To investigate the mechanism of moscatilin on the inhibition of H23 non-small cell lung cancer migration and invasion.

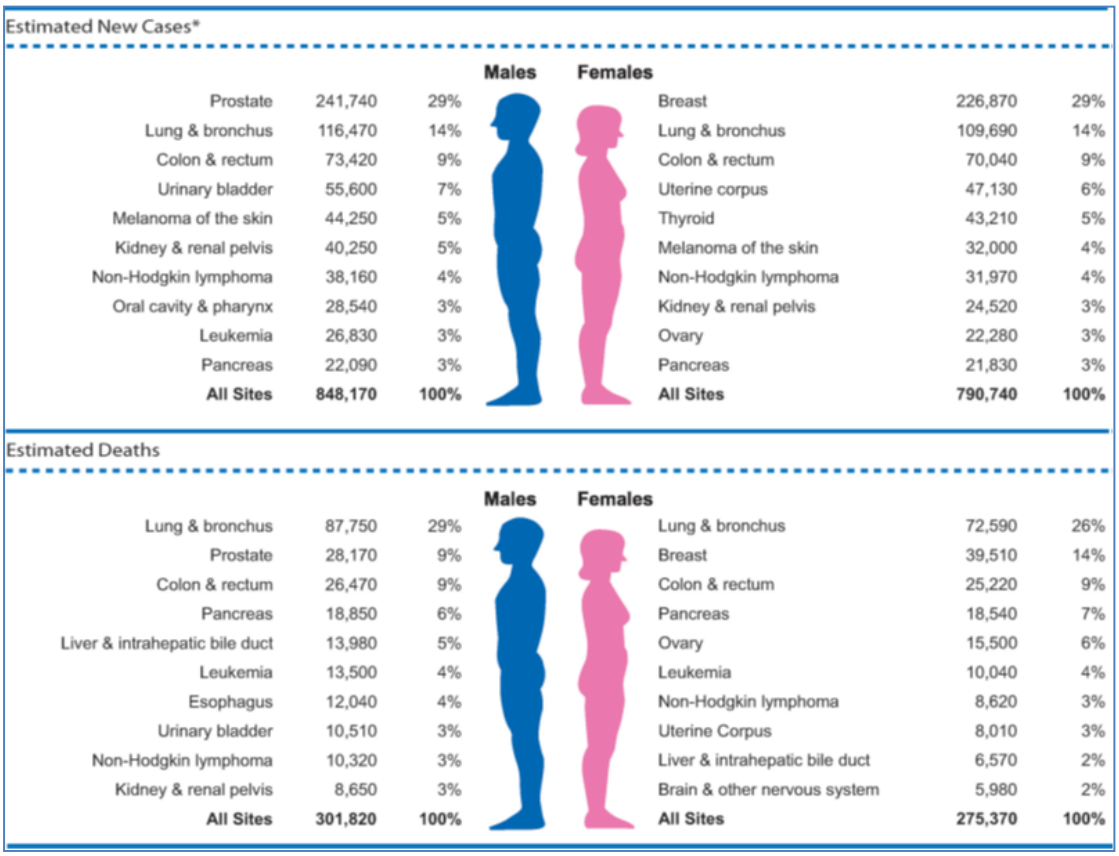
CHAPTER II

LITERATURE REVIEW

Lung cancer

Lung cancer is the deadliest and commonly-found form of cancer. In terms of prevalence, lung cancer is the second top killer among both male and female patients (Siegel *et al*., 2012). Lung cancer has a very poor prognosis, making it difficult in detection of the disease at an early stage. As a result, most patients have already reached an advanced stage of lung cancer when they started the treatment. The survival rate for lung-cancer patients is thus less than 15 % (Ray and Jablons, 2009).

If molecular techniques and biomarkers for identifying cancer risks and medical prognosis improve, early diagnosis of lung cancers may become common and prevent cancer progression. At present, over 70% of patients with lung cancer have developed symptoms of advanced local or metastatic disease that is not responsive to therapy (Spira and Ettinger, 2004; Visbal *et al*., 2005; Gridelli *et al*., 2007). Lung cancer-related deaths have also increased largely in response to the growing number of smokers. Cigarette smoking is a big risk factor, which is blamed for approximately 80% of lung-cancer cases. Other causes include idiopathic pulmonary fibrosis, asbestos exposure, and environmental radon (Parkin *et al*., 2005; Sun *et al*., 2007).



Estimated New Cases*

	Males			Females	
Prostate	241,740	29%		Breast	226,870	29%
Lung & bronchus	116,470	14%		Lung & bronchus	109,690	14%
Colon & rectum	73,420	9%		Colon & rectum	70,040	9%
Urinary bladder	55,600	7%		Uterine corpus	47,130	6%
Melanoma of the skin	44,250	5%		Thyroid	43,210	5%
Kidney & renal pelvis	40,250	5%		Melanoma of the skin	32,000	4%
Non-Hodgkin lymphoma	38,160	4%		Non-Hodgkin lymphoma	31,970	4%
Oral cavity & pharynx	28,540	3%		Kidney & renal pelvis	24,520	3%
Leukemia	26,830	3%		Ovary	22,280	3%
Pancreas	22,090	3%		Pancreas	21,830	3%
All Sites	**848,170**	**100%**		**All Sites**	**790,740**	**100%**

Estimated Deaths

	Males			Females	
Lung & bronchus	87,750	29%		Lung & bronchus	72,590	26%
Prostate	28,170	9%		Breast	39,510	14%
Colon & rectum	26,470	9%		Colon & rectum	25,220	9%
Pancreas	18,850	6%		Pancreas	18,540	7%
Liver & intrahepatic bile duct	13,980	5%		Ovary	15,500	6%
Leukemia	13,500	4%		Leukemia	10,040	4%
Esophagus	12,040	4%		Non-Hodgkin lymphoma	8,620	3%
Urinary bladder	10,510	3%		Uterine Corpus	8,010	3%
Non-Hodgkin lymphoma	10,320	3%		Liver & intrahepatic bile duct	6,570	2%
Kidney & renal pelvis	8,650	3%		Brain & other nervous system	5,980	2%
All Sites	**301,820**	**100%**		**All Sites**	**275,370**	**100%**

Figure 1 Estimated new cancer cases and deaths worldwide in 2012 (Siegel *et al*., 2012)

Lung cancers are classified by histology into 2 types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The NSCLC constitutes for 85% of the lung cancer cases while the SCLC accounts for the rest. During the last few decades, adenocarcinoma subtype of NSCLC has been the most commonly-found histological type of lung cancer (Mason, 2005). The incidences of SCLC, which is also a commonly-found histological subtype of lung cancer among smokers, have decreased over the same period (Mason, 2005).

Types of lung cancer

Lung cancers are divided into two main categories based on the disease's characteristics (Mason, 2005).

1. **Non-small-cell lung cancer** or NSCLC: Accounting for about 85% of all lung-cancer cases, it contains three subtypes as follows:

- **Adenocarcinoma**: It usually occurs to mucus-producing cells in the center or outer regions of the lung. As it develops slowly, it causes few or no noticeable symptom(s) at all until it is far advanced. By that time, it is extremely aggressive, spreads fast, and causes death. More than half of cases, it metastasizes to the brain (Alberg *et al*., 2005; Devesa *et al*., 2005; Ginsberg *et al*., 2005).

- **Squamous cell carcinoma**: It occurs to reserved cells, which are responsible for replacement of damaged or injured cells in the bronchial epithelium or major airways. The squamous cell carcinoma is usually found in the middle of the lung, either in a main lobe or in a bronchus branch. Over time, it will grow in size and causes activities in the lung. However, squamous cell carcinoma usually does not spread as rapidly as different types of lung cancer do. The direct cause of squamous cell carcinoma is smoking. This subtype accounts for about 25% of all lung-cancer incidences (Alberg *et al*., 2005; Devesa *et al*., 2005; Ginsberg *et al*., 2005).

- **Large cell carcinoma** or LCC: These tumorous cells develop in smaller bronchi or scarred tissue around the outer part of the lungs. They divide and replicate fast, forming tumors that threateningly spread from the lungs to other parts of the patients' body. Of all lung-cancer cases, 10% are related to LCC (Alberg *et al*., 2005; Devesa *et al*., 2005; Ginsberg *et al*., 2005).

2. **Small-cell-lung cancer** (SCLC): Similarity to squamous cells, SCLC may develop from reserved cells or other cells in the epithelium. It accounts for approximately 20% of all lung-cancer cases. Chemotherapy works well against SCLC, but if it is treated by chemotherapy, this subtype is very aggressive and usually turns lethal very fast (Alberg *et al*., 2005; Devesa *et al*., 2005; Ginsberg, *et al*., 2005).

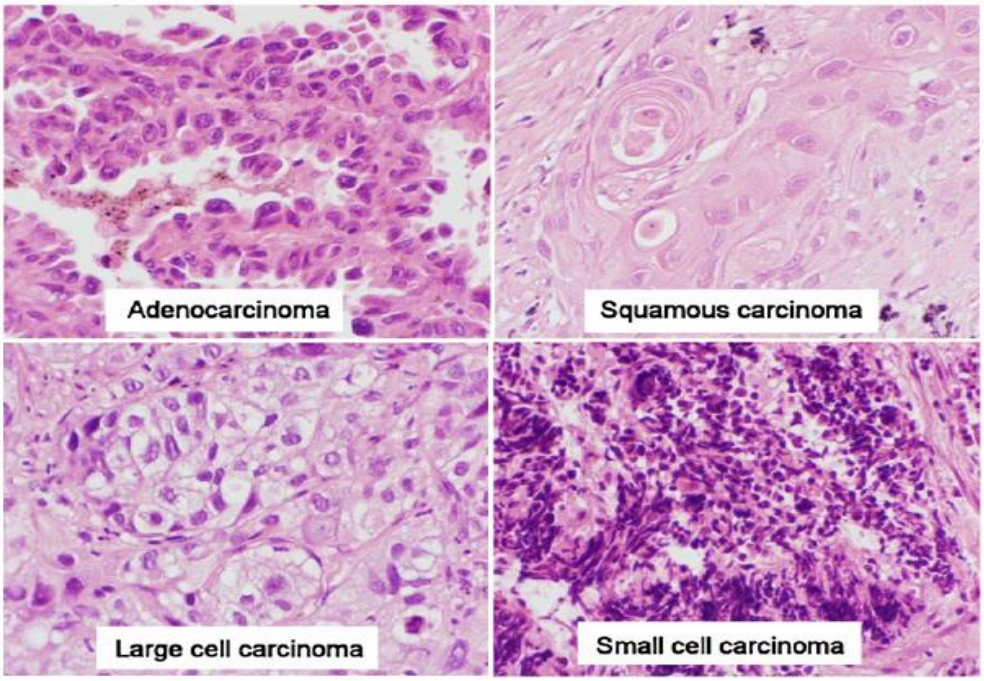


Figure 2 Histological types of lung cancer (Motadi *et al*., 2007)

Staging of non-small-cell lung cancer

Tumor, node, and metastasis (TNM) marks different staging system for NSCLC. Staging is subject to the size and location of the primary tumor, the presence of cancer cells in lymph nodes involvement, and the spread of the lung cancer to other parts of the body. Stage of cancer can be used to predict the survival of patients as well as to guide them towards the most appropriate treatment regimen or clinical trials. Therefore, it is necessary that tumor size, lymph-node status, and possible presence of metastases be determined before doctors start treating each cancer patient (Spira and Ettinger, 2004).

Stage	Tumor	Node	Metastasis	General Description	Survival Rate	
					1 Yr	5 Yr
Non–small-cell lung cancer						
Local						
IA	T1	N0	M0	T1 tumor: ≤3 cm, surrounded by lung or pleura; no tumor more proximal than lobe bronchus	94	67
IB	T2	N0	M0	T2 tumor: >3 cm, involving main bronchus ≥2 cm distal to carina, invading pleura; atelectasis or pneumonitis extending to hilum but not entire lung	87	57
IIA	T1	N1	M0	N1: involvement of ipsilateral peribronchial or hilar nodes and intra-pulmonary nodes by direct extension	89	55
Locally advanced						
IIB	T2	N1	M0		73	39
	T3	N0	M0	T3 tumor: invasion of chest wall, diaphragm, mediastinal pleura, pericardium, main bronchus <2 cm distal to carina; atelectasis or pneumonitis of entire lung		
IIIA	T1	N2	M0		64	23
	T2	N2	M0			
	T3	N1	M0			
	T3	N2	M0	N2: involvement of ipsilateral mediastinal or subcarinal nodes		
IIIB	Any T	N3	M0	N3: involvement of contralateral (lung) nodes or any supraclavicular node	32	3
Advanced						
IIIB	T4	Any N	M0	T4 tumor: invasion of mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; separate tumor nodules; malignant pleural effusion	37	7
IV	Any T	Any N	M1	Distant metastasis	20	1
Small-cell lung cancer						
Limited disease				Evidence of tumor confined to ipsilateral hemithorax; can be encompassed by a single radiation port		
Extensive disease				All other diseases, including metastatic disease		

Figure 3 Staging classifications for lung cancer (Mountain, 2000)

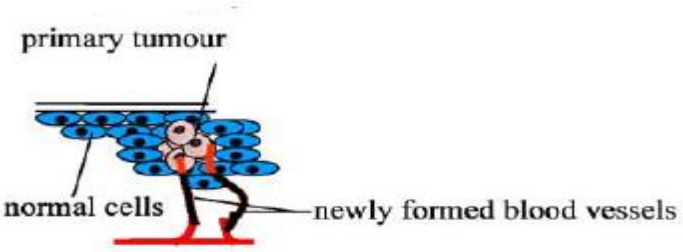
Metastasis

Metastasis cancer is a complex molecular phenomenon in which cancer cells move from the primary site to other parts of the body, forming secondary tumor (Fidler, 1991). Cancer metastasis is a major cause of deaths among cancer patients. Therefore, metastasis cancer poses a deeply serious clinical problem (Parkin, 2001). Cancer metastasis involves a series of biological steps. To produce clinically relevant lesions, metastatic cells must survive all the steps. If the disseminating tumor cells fail to complete just one of the steps, it wills not completely success a metastasis. The efficiency of the invasive and metastatic cells is usually poor. Only 0.01% of circulating cells successfully establish metastasis. Metastasis capability of cancers are depends largely on the interactions of their cell surface molecules with the microenvironment (Glaves *et al*., 1983).

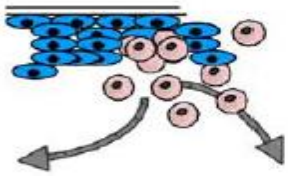
The major steps of metastasis are (Chambersetal, 2002; Fidler, 2003):

1. The creation of a new blood vessel to supply tumor mass called “angiogenesis”.
2. The migration of tumor cells from the primary tumor site.
3. The invasion penetrates the basement membrane and extracellular matrix surrounding the tumor.
4. Intravasation of the tumor cells into the blood stream or lymphatic circulation.
5. Adhesion of the circulating tumor cells to the endothelial cell lining at the target organ site. Small tumor cell aggregates are detached.
6. Invasion of the tumor cells through the endothelial cells layer, surrounding basement membrane and target organ tissue.
7. The growth of secondary tumor at the target organ.
8. Repetition of the process beginning at step 1.

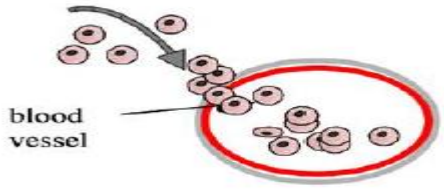
1.Angiogenesis



2.De-adhesion of tumor cells

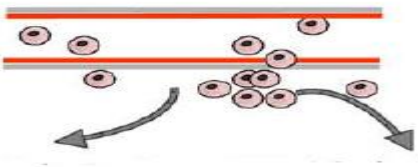


3.Invasion and migration through the basement membrane and extracellular matrix and subsequent invasion of basement membrane of endothelium



4.Intravasation

5.Adhesion of cancer cells to endothelial cell



6.Extravasation **7.Colonisation**

Figure 4 The metastatic cascade (Brooks *et al.*, 2009)

Migration

Cell migration is an integral part in biological phenomena, both in normal and pathological events (Von der Mark *et al.*, 1999; Cho and Klemke, 2000). In adults, cell migration is required in homeostatic processes for example, for skin to get new cells that continuously migrate from basal layer, and for leukocytes to outer part for bacteria consumption (Friedl and Weigelin, 2008). Cell migration also happens in pathological processes such as in events of tumor metastasis, chronic inflammations, and vascular diseases. Tumor growth always includes the formation of new blood vessels, which are possible via the proliferation and migration of their endothelium. In cancer metastasis, some cancer cells are able to move from the primary tumor to other organ where they create secondary or metastatic tumors (Laufenburger and Horwit, 1996). If researches provide greater understanding in the molecular mechanisms that controls metastatic progression, new treatment options related to adhesion molecules and cancer microenvironment may be developed and introduced for the benefits of patients. Future researches indeed should focus on chemokine, growth factor, cell adhesion molecules and extracellular proteases (Staff, 2001; Yamaguchi *et al*., 2005).

Invasion

Invasion involves the process of cancer cells moving through extracellular matrix barriers (Stylli *et al*., 2008). The invasive process is initiated by deregulation of genetic in tumor cells that paves way for imbalanced stimulatory and inhibitory physiology events. Biological molecules also play major roles in the invasion during physiological events such as angiogenesis, embryogenesis and morphogenesis (Nakada *et al*., 2007). Also, the extracellular matrix may contain stored proteinases that can be triggered by the pseudopodia of invading cell. One of the early events in the cancer cell invasion and metastasis is the proteolytic degradation of ECM component (Clark *et al*., 2008).

Focal adhesion kinase (FAK)

Nearly a decade ago, focal adhesion kinase (FAK) was first identified in chicken embryo cells from transformation of v-Src as a non-receptor tyrosine kinase that played a crucial role of integrins in mediating cell signalling and signaling pathways growth factor receptor at sites of integrin adhesion. Structurally, FAK is consisted of a tyrosine kinase domain sandwiched by a C-terminal domain with focal adhesion targeting (FAT) sequence and a N-terminal FERM domain. The FERM domain of FAK structure is similar to that of cytoskeletal organization proteins like ezrin, radixin, and moesin (ERM) family of proteins, talin and signaling molecules. Activation of FAK has a big role in the cell survival, proliferation, migration and invasion. All these processes are implicated in cancer progression and metastasis. FAK is activated as soon as an auto-inhibitory intra-molecular interaction between its amino terminal FERM domain and the tyrosine kinase domain is disrupted. The activated FAK forms a complex with Src family kinases, which initiates multiple downstream signaling pathways through phosphorylation of other proteins like Akt, Jnk and Erk pathway. Several studies reported the FAK phosphorylation selectively increased at Tyr-397 at the leading edge of motile cells. Such findings suggested that FAK was a key player in both cell migration and cell invasion (Ilic *et al*., 2001; MacPhee *et al*., 2001). The higher levels of FAK expression and activity were shown to link with malignant or metastatic diseases and poor prognosis (Miyazaki *et al*., 2003). The suppression of either FAK activity or expression would be benefit in cancer therapy (Golubovskaya, 2010).

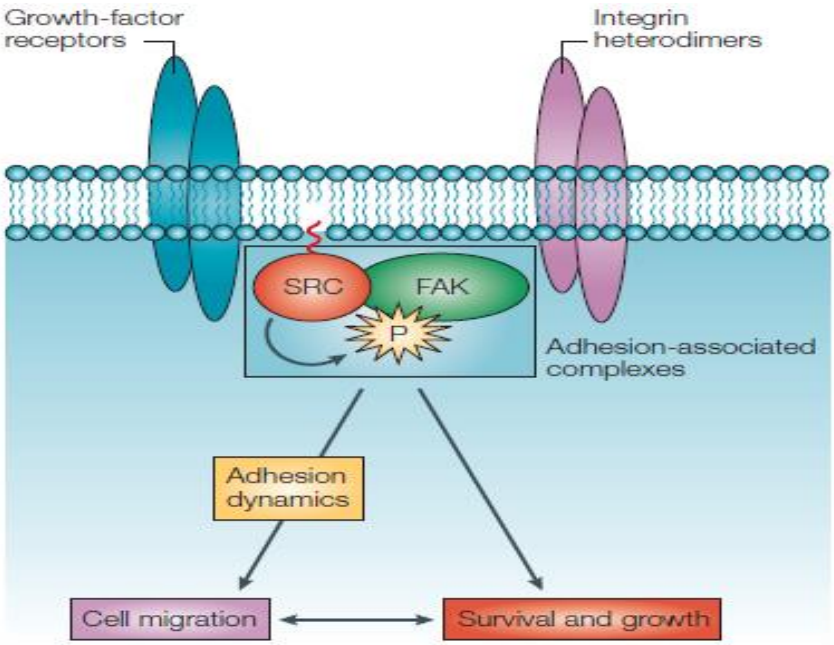


Figure 5 Focal adhesion kinase (FAK) as a signaling (McLean *et al*., 2005)

FAK in lung cancer

Tissue specimens in lung cancer, phosphorylated FAK is the major component among 100-130 kDa phosphorylated protein associating with poor patient prognosis (Nishimura *et al*., 1996). In addition, increased phosphorylation of FAK is found in lung cancer sample whereas it absent in normal tissues (Imaizumi *et al*., 1997). The increased phosphorylation of FAK was closely associated with the involvement of cancer and disease-free survival time. Stimulation of small-cell lung cancer cells with hepatocyte growth factor (HGF) activated c-Met and increased phosphorylation FAK at Y397, are therapeutic target in lung tumorigenesis (Maulik *et al*., 2012). Together, FAK signaling is found to be a significant factor in the early stages of human lung adenocarcinoma metastasis, indicating FAK involvement in tumorigenesis (Carelli *et al*., 2006).

FAK in migration and invasion

FAK-mediated migration and invasion signaling pathways, inhibition of FAK can prevent cell migration and invasion processes (Benlimame *et al*., 2005). Researches show that FAK is activated in invading fibrosarcoma and regulates metastasis (Hauck *et al*., 2001). In breast cancers, high FAK expression is related to tumor aggressive phenotype (Lark *et al*., 2005). Consequently, FAK expression in preinvasive ductal carcinoma in situ (DCIS) tumors suggests that the survival function of FAK occurs as an early event in tumorigenesis (Lightfoot *et al.*, 2004). Several FAK downstream signaling pathways have already been blamed for enhanced cell migration and invasion, such as PI3K signalling. The activated PI3K facilitates cell migration via its effector Cdc42, which is a main regulator of actin cytoskeleton and filopodia in cell motility (Cain and Ridley, 2009). FAK also controls cell migration through the Rho subfamily of small GTPases and the assembly and disassembly of

actin filaments (Kaibuchi *et al*, 1999). Therefore, the expression and activity of FAK is linked to numerous migration and invasion pathways (Zhao and Guan, 2009).

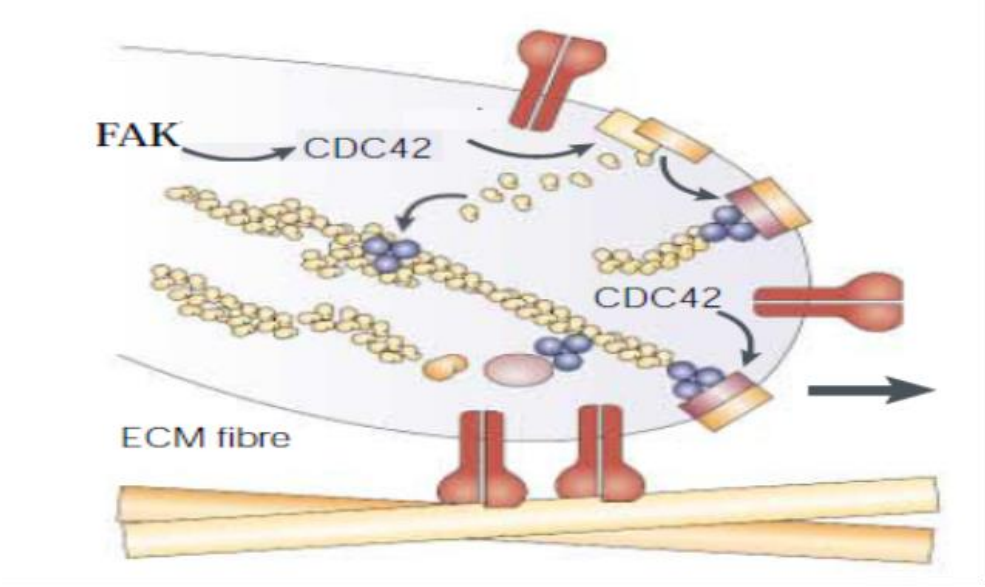


Figure 6 Focal adhesion kinase (FAK) signaling in cell migration (Friedl and Wolf, 2003)

Adenosine triphosphate dependent tyrosine kinase (Akt)

As Akt is a main effector of PI3K (phosphoinositide 3-kinase), PI3K/Akt pathway can regulate many cellular processess associated with cancer. Akt has significant impacts on cell survival, proliferation, migration, invasion and angiogenesis (Song *et al*., 2005). In mammals, there are three distinct genes encoded for Akt namely Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). Their isoforms are activated by similar mechanisms via PI3K signaling. Ligand stimulation triggers PI3-kinase, the upstream activator of Akt, by directly binding PI3-kinase subunits with either the activated, phosphorylated receptor or adaptor proteins phosphorylated by receptor kinase activity (Myers *et al*., 1993).

Akt is a key regulator of various processes through its ability to control cell invasion and cell migration. It can control cell migration, actin organization, cell-to-cell adhesion and extracellular matrix degradation (Brader and Eccles, 2004). Several studies have concluded that Akt1 is able to increase fibroblast motility via phosphorylating girdin, an actin-binding protein that promotes stress fiber formation and lamellapodia (Cenni *et al.*, 2003). In squamous carcinoma cells, the expression of a constitutively active Akt1 allele pushes epithelial to mesenchymal transition (EMT) or motility (Grille *et al.*, 2003). Therefore, Akt signaling pathway is commonly linked to increased cell migration and invasion in many cancer cell types (Vivanco and Sawyers, 2002).

Cell division cycle 42 (Cdc42)

Cdc42 is a member of the Rho family of small GTPases. It functions as a molecular switch, allowing the molecule to change from an active GDP-bound form to an inactive GTP-bound form and vice versa in response to diverse signals (Sinha and Yang, 2008). RhoGTPases regulates microtubule and intermediate filament dynamics during cell migration, which begins upon the stimulation of cell surface molecules like integrin, receptor tyrosine kinases, cytokine receptors and G-protein coupled receptors (Sinha and Yang, 2008). Cdc42 activation interacts with and activates various downstream effectors, often through the induction of conformational changes within the effector molecules. Many studies have confirmed the role of Cdc42 in the regulation of the multistep cell-migration process (Cerione, 2008; Aznar, 2001).

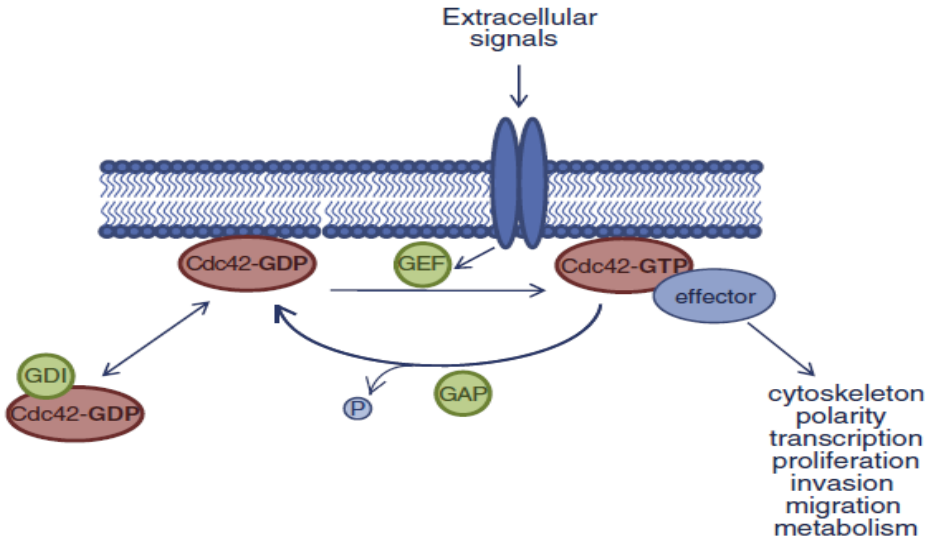


Figure 7 Biochemical regulation of Cell division cycle 42 **(**Cdc42) activity (Stengel and Zheng, 2011)

Filopodia

Filopodia, actin-containing spikes, has facilitated cell migration. However, they are under the control of proteins like Mena/VASP, diaphanous and fascin. Fascin is an actin bundling protein, having the ability to bind filaments in lamellipodial networks to promote filopodia formation (Svitkina *et al*., 2003). Phosphorylation, the small GTPases Rac and Cdc42 have controlled its actin bundling activity (Hashimoto *et al*., 2007). Comprising three isoforms, murine diaphanous is implicated in different cytoskeletal activities such as the interaction with actin filament and microtubules (Faix and Grosse, 2006). However, its action in filopodia is believed to mainly rely on its facility to act as an activation of actin filament nucleation and elongation factor. Mena/VASP proteins also have the capability to promote the elongation of actin in filopodia formation. In addition to enhancing cell motility, filopodia can facilitate many other cellular processes. Among them are wound healing, adhesion to the extracellular matrix and guidance towards chemoattractants (Gupton and Gertler, 2007; Faix and Rottner, 2006). As these proteins are implicated in the promotion of cancer metastasis, both and filopodial actin dynamics are identified as important players in this malignant process (Applewhite, 2007).

Small GTPases and filopodia formation

Small GTPases of the Rho superfamily have certainly related to the regulation of cell morphology and actin cytoskeleton. RhoGTPases are Rac1, Cdc42 and RhoA. According to some studies, RhoA contributes to the formation of stress fibres and focal adhesions. Rac1 encourages lamellipodium formation. Cdc42, meanwhile, plays a role on filopodia formation by interacting with multiple proteins (Nobes and Hall, 1995). In migration signaling pathway, Cdc42 is inducted the ARP2/3 complex-dependent actin filament nucleation through activation of WASP and N-WASP. TheARP2/3 complex is considered a main nucleation of the branched actin network.

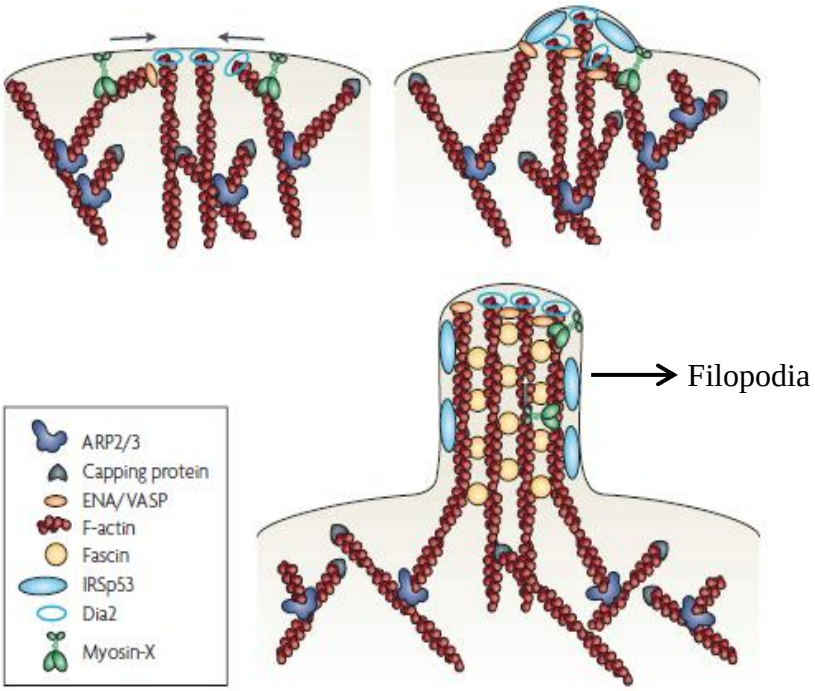


Figure 8 Filopodia formation (Mattila and Lappalainen, 2008)

The extracellular signal-regulated kinase (ERK)

The extracellular signal-regulated kinase (ERK), also named the 42-/44-kDa mitogen-activated protein kinase (MAPK) pathway, is involved in a variety of cell types by diverse intracellular or extracellular stimuli such as Raf-1. Raf-1 actions as a MAPK kinase kinase (MAP3K) and activates MAPK/ERK kinase 1 and 2 (MEK1/2; also called MAPK kinase1/2) by serine phosphorylation. MEK1/2, dual-speciicity protein kinases, then catalyze the phosphorylation of ERK1 and ERK2 (p44 MAPK and p42 MAPK) on tyrosine and threonine residues. Phosphorylated ERK1/2 involved the regulation of a large diversity of processes such as survival, motility, differentiation and proliferation (Pages *et al*., 1993; Seger and Krebs, 1995). In this respect, the ERK signaling pathway is found to have played a role in the regulation of cell motility. Several studies indicate that ERK activity at the membrane periphery is a prerequisite to focal adhesion disassembly, favouring cell spreading and motility (Klemke et al., 1997; Welch, et al. 2000). This notion corresponds to the findings that ERK is a target of Src-FAK signalling (Viala and Pouyssegur, 2004). Several studies provide downstream of ERK regulate the activity of the Rho family of small GTPases in colon cancer migration and invasion (Vial *et al*., 2003). Furthermore, the importance of activation of ERK pathway in the cells motility mechanism has been informed, in which ERK1/2 phosphorylates enhances the myosin light-chain kinase (MLCK) activity leading to increase MLC phosphorylation and enhance cell migration (Shen *et al*., 2010). Consequently, the regulation of cell migration and invasion most probable characterizes a major mechanism by which the ERK signaling cascade promotes cancer migration and metastasis.

Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) are broadly defined as highly reactive molecules containing oxygen which normally produced by cellular metabolic process. Despite ROS are essential for cellular biological functions, depletive production of ROS or excessing of cellular antioxidant molecules and/or enzymes leading to oxidative stress and consequently alteration in physiological and pathological such as regulation of cell cycle progression and apoptosis. ROS has been widely studied to be elevated in several cancers such as lung cancer, prostate, colon and others (Zhou *et al*, 2003). ROS are highly reactive because they contain unpaired electron as well as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) and (Aslan and Ozben, 2003; Poli *et al*., 2004).

The first ROS that superoxide anion ($O_2^{\bullet-}$) are produced from the mitochondrial respiratory chain by NADPH oxidases as in the equation below (Gina *et al*, 2009):

$$2O_2 + NAD(P)H \longrightarrow 2O_2^{\bullet-} + NADP^- + H^+ \text{ oxidase}$$

Superoxide anion mainly undergoes dismutation to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (Gina *et al*, 2009):

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

H_2O_2 is converted to H_2O by catalase and glutathione peroxidase:

$$2H_2O_2 \xrightarrow{catalase} 2\,H_2O + O_2$$

$$2GSH + H_2O_2 \xrightarrow{glutathione\ peroxidase} GS\text{-}SG + 2H2O$$

Hydroxyl radical ($OH^{\bullet}$) is formed by the reaction of H_2O_2 and $O_2^{\bullet-}$ with through two main reactions which are Haber-Weiss reaction and Fenton reaction.

These reactions are catalyzed by transition metal such as iron and copper (Gina *et al*, 2009):

Haber-Weiss reaction

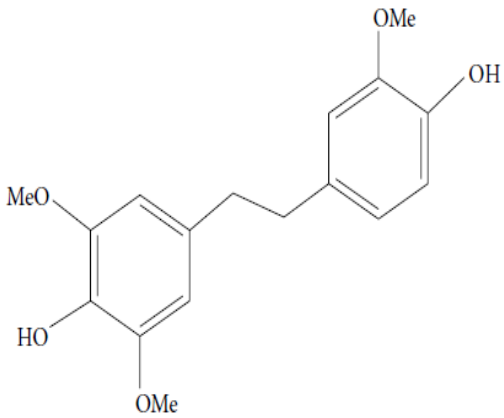
$$H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe^{2+}} OH^{\bullet} + OH^{-} + O_2$$

Fenton reaction

$$H_2O_2 + Fe^{2+} \longrightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$$

The biological functions of ROS and its important roles in cancer development and disease progression have been investigated for several decades. Cancer cells are known to be metabolically active and under increased oxidative stress, presumably in association with dysfunction of metabolic regulation and uncontrolled cell proliferation (Boonstra and Post, 2004). Recently, the regulation of ROS signaling in cancer metastasis was promoted. Several major signal transduction pathways such as FAK, PI3K and Rho-GTPase pathways, that are known to associates with regulation transcriptional of metastasis-related genes such as MMPs and integrins (Brinckerhoff and Matrisian, 2002; Nelson and Melendez, 2004; Cully *et al*., 2006), were regulated by ROS (Bokoch and Diebold, 2002; Matsuzawa and Ichijo, 2005; Hordijk, 2006). Several studies reports involved the effects of ROS on cell migration such as cytoskeleton remodeling. For example, a TGF-beta plays an important role in actin cytoskeletal change of endothelial cells which was associated by ROS (Hu *et al*. 2005). Therefore, the role of ROS in cell migration is linked to both its critical impact on the signaling pathway and its oxidative activity to modify structural protein.

Moscatilin



(A) (B)

Figure 9 Moscatilin (A) Structure of moscatilin (B) *Dendrobium pulchellum*

Moscatilin (4,4'-dihydroxy-3,3',5'-trimethoxybibenzyl) is a bibenzyl component derived from the Thai Dendrobium pulchellum orchid (Chanvorachote *et al*., 2012) . This substance can also be extracted from another species of genus Dendrobium such as *Dendrobium moscatum* (Majumder and Sen, 1987), *Dendrobrium loddigesii* (Ho and Chen, 2003), *Dendrobium aphyllum* and *Dendrobium secundum*. Moscatilin was reported to used as a traditional medicine for fever, maintaining a healthy stomach replenishing body fluid, and serving as a natural anti-platelet agent (Chen *et al*., 2008). Recently, several studies show that moscatilin has antimutagenic and anti-angiogenesis activities, as well as inhibits platelet aggregation (Chen *et al*., 2008; Hwang *et al*., 2010; Tsai *et al*., 2010).

Anti-mutagenic activity of moscatilin

Moscatilin has anti-cancer effects in many cell lines including colon, lung and other organs but not uniformly across cell lines (Chen *et al*., 2008). For example, while the substance works well against lung cancer and stomach cancer, it proves ineffective against liver cancer. Recently, moscatilin has also been informed to induced cell cycle arrest by increasing the proportion of sub-G1 populations in HCT-116 cells (Chen *et al*., 2008). It has the ability to induce the phosphorylation of JNK1/2 caused by inhibited tubulin polymerization. Moreover, it can activate caspase9 and caspase3 to induce apoptosis. Furthermore, it induces DNA damage, H2AX phosphorylation and up-regulation of p21 and p53 leading to cell death (Chen *et al*., 2008).

Anti-angiogenesis activity of moscatilin

Moscatilin was shown to against the formation of new blood vessels in human umbilical vein endothelial cells (HUVECs) and lung cancer cells (Tsai *et al*., 2010). Recently, moscatilin from *Dendrobium loddigesii* has been reported to inhibited A549 lung cancer cell growth and their migration, and attenuate tube formation in HUVECs cells by suppressing bovine fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Moscatilin, morevoer, has showed the inhibitory effects on Akt, ERK1/2 and eNOS signaling pathways in HUVEC (Tsai *et al*., 2010).

Anti-platelet aggregation of moscatilin

Moscatilin has demonstrated antiplatelet aggregation activity *in vitro*, it suppressed the aggregation of collagen and arachidonic acid-induced platelets (Chen *et al*., 1994). According to many studies, MeOH extract of the stem of *Dendrobium loddigesii* including moscatilin inhibited platelet aggregation in rabbit platelet with arachidonic acid and collagen. The findings have reflected that several different

compounds from different Dendrobium species have the ability to inhibit the platelet aggregation. Therefore, such compounds can be developed for the treatment of cardiovascular diseases. Moreover, compounds isolated from the MeOH of *Dendrobium nobilestems* has weakened lipopolysaccharide-induced nitric oxide generation in mouse macrophage RAW 264.7 cells (Hwang *et al*., 2010).

While moscatilin is reported to have potent antitumor activity, it remains unclear how it has played a role in cancer metastasis. This study is thus investigated the effect of moscatilin on non-small cell lung cancer migration and invasion in and identified the mechanism focusing on the proteins mediating cancer migration and invasion.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

Moscatilin was obtained from Dr. Boonchoo Sritularak (Chulalongkorn University, Bangkok, Thailand); fetal bovine serum, L-glutamine, penicillin, and streptomycin were obtained from Gibco, Corp. (Gaithersburg, MA, USA); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Amplex red, dichlorofluorescein diacetate ($DCFH_2$-DA), dihydroethidium (DHE), dimethysulfoxide (DMSO), dimethoxy-naphthoquinone (DMNQ), ferrous sulphate ($FeSO_4$), Hoechst 33342, hydrogen peroxide (H_2O_2), hydroxyphenyl fluorescein (HPF), propidium iodide (PI) and phalloidin-tetramethylrhodamine B isothiocyanate conjugate were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA); RNase A was obtained from Applichem, Inc. (Cheshire, CT, USA); Matrigel was obtained from BD Biosciences, Inc. (Woburn, MA, USA); protease inhibitor cocktail was obtained from Roche Molecular Biochemicals, Inc. (Indianapolis, IN, USA); nitrocellulose membranes was obtained from Bio-rad Laboratories, Inc. (Hercules, CA, USA); BCA protein assay kit and Supersignal west pico chemiluminescent was obtained from Thermo Scientific, Inc. (Rockford, IL, USA); Akt rabbit antibody, p-Akt rabbit antibody, FAK rabbit antibody, p-FAK rabbit antibody, Erk1/2 rabbit antibody, p-Erk1/2 rabbit antibody, Cdc42 rabbit antibody, β-actin rabbit antibody and peroxidase conjugated secondary anti-rabbit IgG antibody were obtained from Cell Signaling Technology, Inc. (Denvers, MA, USA); and Immobilon Western chemiluminescent HRP substrate was obtained from Millipore, Corp. (Billerica, MA, USA).

2. Equipment

Automated cell counter (TC20, Bio-rad Laboratories Inc., Hercules, CA, USA), auto pipette: 2-10 µl, 10-100 µl, 20-200 µl and 20-1,000 µl, balance, bottle: 100 ml, 250 ml, 500 ml and 1,000 ml (Schott, Elmsford, NY, USA), carbon dioxide incubator, centrifuge, conical tube: 15 ml and 50 ml (Corning Inc., Tewksbury, MA, USA), disposable pipette: 5 ml and 10 ml, flow cytometer (FACSort, Becton Dickinson, Rutherford, NJ, USA), fluorescence microscope (Olympus IX51 with DP70, Olympus America Inc., Center valley, PA, USA), fluorescence microplate reader (Perkin Elmer Inc., Waltham, MA, USA), imaging densitometer (Bio-Rad Laboratories Inc., Hercules, CA, USA), laminar flow cabinet, microplates: 6-well, 24-well and 96-well (Costar Tewksbury, MA, USA), pipette tips for 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl, pH meter, transwell plate: 24-well (Costar), vertex mixer.

Methods

1. Sample preparation

Moscatilin was dissolved in DMSO and distilled deionized water to achieve indicated concentrations containing less than 0.1% DMSO, which show no cytotoxic in H23 and HK2 cells.

2. Cell culture

Human lung adenocarcinoma H23 cells and Human renal proximal tubule HK2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H23 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and HK2 were cultured in Dulbecco's Modified Eagle's Medium (DMEM). All media were supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum in 5% CO_2 at 37°C

with humidified incubator. All experiments were designed when cells reached 80-90% confluence.

3. Cell viability assay

Cell viability was determined by MTT assay, which measured the ability of the cells to reduce MTT (yellow) to purple formazan product by using mitochondrial reductase enzyme. Briefly, H23 cells were seeded $1x10^4$ cells per well onto 96-well plates for 24 h. After the treatment, cells were incubated with 100 µl of 500 µg/ml MTT solution for 4 h at 37°C. Then, MTT solution was removed and 100 µl of DMSO was added to dissolve the formazan product. The intensity of formazan product was read by a microplate reader at 570 nm. All analyses were performed in at least three independent experiments. Cell viability was expressed as the percentage that was calculated from absorbance of MTT-treated cells relative to controlled cells, as shown in the following equation.

$$\% \text{ Relative cell viability} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

4. Apoptosis assay

Mode of cell death were identified by Hoechst 33342 and PI co-staining and DNA content assay.

For Hoechst 33342 and PI co-staining assay. Briefly, H23 cells were seeded $1x10^4$ cells onto 96-well plates for 24 h. After specific treatments, cells were incubated with 10 µg/mL of the Hoechst and 5 µg/mL PI dye for 30 min at 37°C. The apoptotic cells were shown by having condensed chromatin and/or DNA fragmentation whereas necrotic cells showed loss of membrane integrity. Hoechst 33342 is permeable dye, which has ability to stain apoptotic and necrotic cells while PI is impermeable dye, which stained only necrotic cells. Cells were visualized using

a fluorescence microscope. The number of apoptosis and necrosis were counted and represented as percentage relative to controlled cells.

For DNA content analysis assay. Briefly, H23 cells were seeded $4x10^5$ cells onto 6-well plate. After specific treatments, cells were trypsinized and centrifuged at 4500 rpm for 5 min at 4 °C. Then, the cell pellets were resuspended with cold 99% absolute ethanol and kept at -20°C for 24 h. After an incubation at -20 °C for 24 h, the cells were centrifuged to collects the cell pellets. And then, cell pellets were resuspended in 0.5 ml of cell-cycle assay PI buffer containing10% PBS 10x, 10% RNaseA 1 mg/ml, 0.1% Triton-X100, 0.1% 0.5M EDTA, 2% PI 10 mg/ml at a dark room for 30 min. DNA content analysis was carried out by a flow cytometer (10,000 gated events/treatment). Apoptotic DNA fragmentation were determined by sub-G_0/G_1 phase of cell cycle analysis.

5. Migration determination

Migration determination was performed by wound healing assay and transwell migration assay.

Wound healing migration assay: briefly, H23 cells were cultured 2×10^5 cells onto 24-well plate containing with 10 % FBS in RPMI. After cell monolayer was scratched a minor wound on the cells in each well by a sterile 200 µl tip. Next, cells were washed with PBS, replaced with RPMI serum-free medium containing specific treatment and photographed at the start of incubation, and also at 12, 24 and 48 h incubation time. Cells migration to wound surface was monitored by microscope. The width of the wound at different intervals was then compared to its size at the start of the incubation.

Transwell migration assay: Briefly, H23 cells were seeded 3×10^4 cells per well and treated with specific treatment containing RPMI serum-free medium before

being added to the upper compartment of 24-transwell plate. The lower compartment was filled with conditioned 10 % FBS in RPMI that acted as a chemoattractant. After incubation of the chamber for 12, 24 and 48 h, non-migrated cells were removed from the upper surface of the chamber cotton swab and rinsed in PBS. Cells were fixed with cold methanol for 10 min, and stained with Hoechst 33342 for 30 min. The number of cells migrating to the lower chamber was counted under fluorescence microscope in 12 fields per treatment.

6. Invasion determination

Invasion determination was made in a transwell invasion assay. Briefly, H23 cells were seeded 3×10^4 cells per well onto upper chamber of 24-transwell plate, in which the membrane was coated with matrigel, and treated with specific treatment containing RPMI serum-free medium. The lower compartment contained 10 % FBS in RPMI as chemotractant. After the incubation for 12, 24 and 48 h, non-invaded cells were removed from the upper surface of the chamber by cotton swab and rinsed with PBS. Cells were fixed with cold methanol for 10 min, and stained with Hoechst 33342 for 30 min. The number of invaded cells to the lower chamber was determined by fluorescence microscope in 12 fields per treatment.

7. Cell morphology characteristics

Morphology of cells was characterized by phalloidin staining. Phalloidin were used to stain filamentous actin. Briefly, cells were seeded 3×10^3 cells per well onto 96-well plates for 24 h. After the treatment, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Next, cells were permeabilized in 0.1% Triton-X100 in PBS for 4 min and blocked with 0.2% bovine serum albumin fraction V (BSA) for 30 min. At the indicated times, cells were incubated with rhodamine-phalloidin diluted 1:100 in PBS for 15 min, and washed

three times by PBS. Cells were imaged by fluorescence microscope. Characteristic of filopodia was represented in relations to controlled cells.

8. Reactive Oxygen Species Detection

Intracellular ROS were determined using specific ROS detection probe including dichlorofluorescein diacetate ($DCFH_2$-DA; ROS probe), hydroxyphenyl fluorescein (HPF; specific $OH^{\bullet}$ probe), dihydroethidium (DHE; specific $O_2^{\bullet -}$ probe) And Amplex red (specific H_2O_2 probe). Briefly, H23 cells were seeded at density of $1x10^4$ cells onto 96-well plates for 24 h. After specific treatments, cells were incubated with either 100 μM of $DCFH_2$-DA, 100 μM of HPF, 100 μM of DHE or 10 mM of Amplex red for 30 min at 37◦C after which they were washed and immediately analyzed for fluorescence intensity using a fluorescence microplate reader using a 480-nm excitation beam and a 530-nm bandpass filter for detecting DCF fluorescence, a 490-nm excitation beam and a 515 nm band-pass filter for HPF, a 530-nm excitation beam and a 590 nm band-pass filter for Amplex red or a 488-nm excitation beam and a 610-nm band-pass filter for DHE.

9. Western blot analysis

To investigate protein expressions, H23 cells were seeded at 4×10^5 cells per well onto 6-well plate. Cells were then treated with various concentrations of moscatilin for 24 h. After a specific treatment, cells were washed twice with cold-PBS and incubated at 4 °C with lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail for 30 min. After the centrifugation of lysates at 12000 rpm for 15 min at 4 °C, supernatants were collected to determine protein content by using the BCA protein assay. Each sample was denaturized by heating at 95◦C for 5 min with Laemmli loading buffer (225 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 9% 2-

mercaptoethanol, and 0.009% bromphenol blue). Subsequently, proteins (60 μg protein) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the separation, proteins were transferred onto 0.45 μm nitrocellulose membranes. The transferred membranes were blocked for 1 h in 5% skim milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20). Membranes were washed three times with TBST for 7 min and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed three times with TBST for 7 min, incubated with horseradish peroxidase-conjugated specific secondary antibodies for 2 h at room temperature. Following the incubation, the immune complexes were detected by chemiluminescence and quantified by imaging densitometry using analyst/PC densitometry software. Mean densitometric data from independent experiments were normalized with β-Actin protein.

10. Statistical analysis

Normalized to results in the non-treated control, data from at least three independent experiments were presented as mean ± S.D. An analysis of variance (ANOVA) and post hoc test (Tukey's test) were used for statistical analysis and multiple comparisons. Values of $P < 0.05$ were regarded to be statistically significant.

11. Experimental design

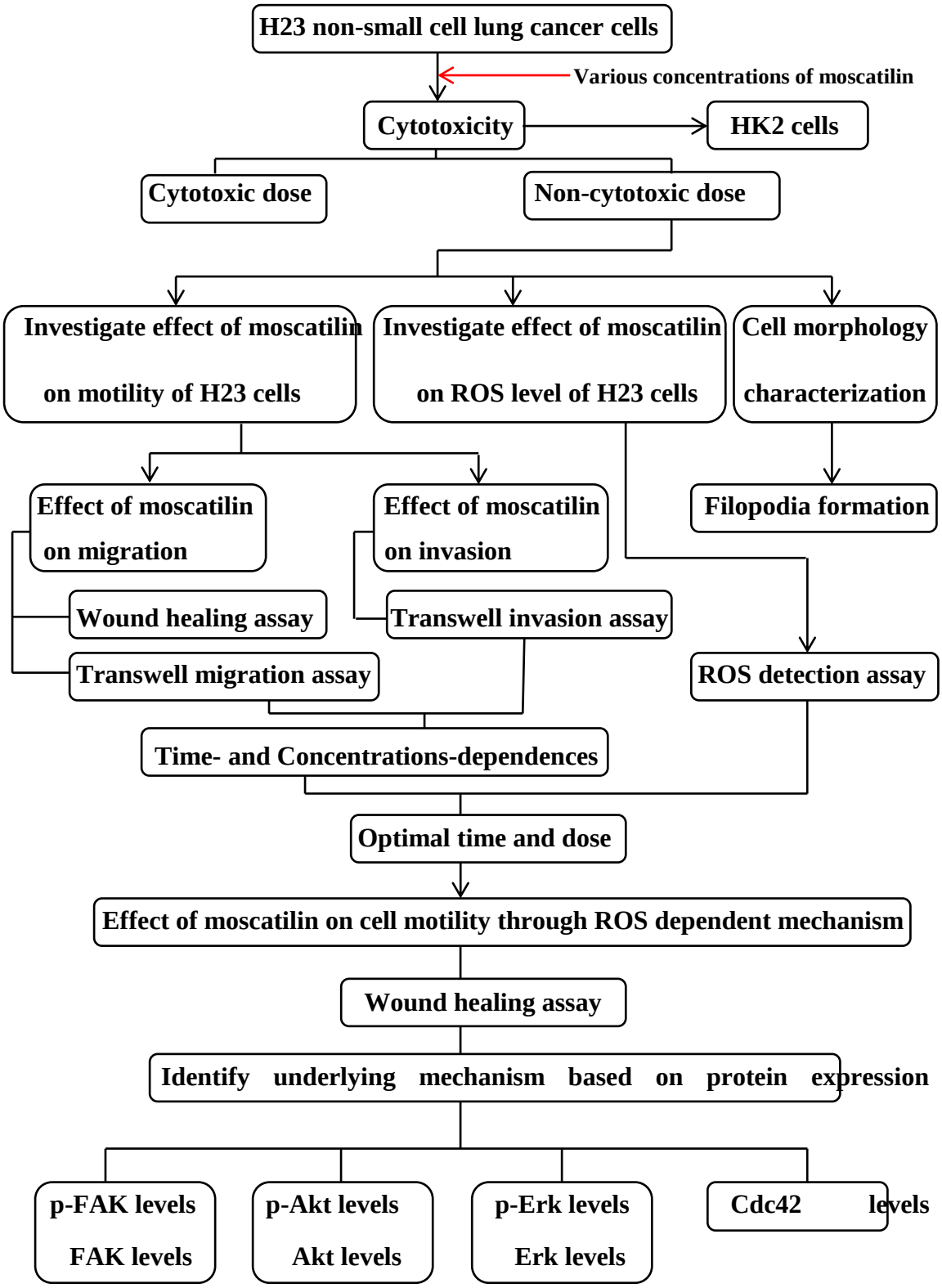


Figure 10 Experimental design of this study

11.1. Conceptual framework

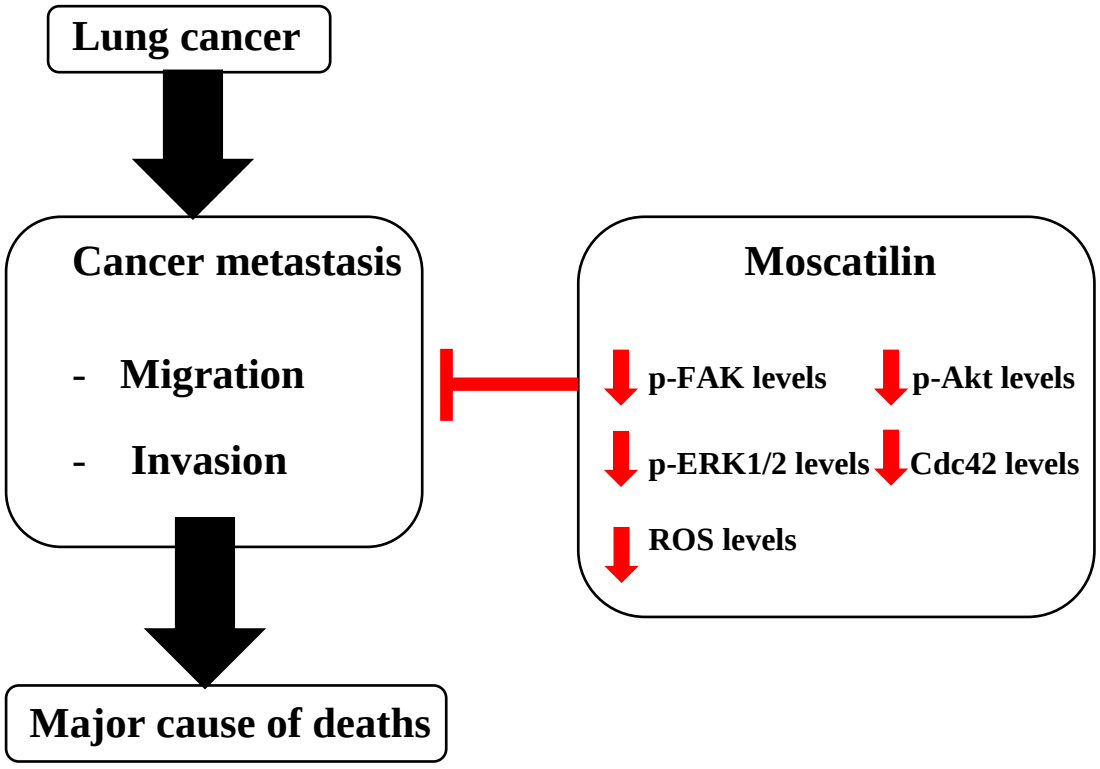


Figure 11 Conceptual framework of this study

11.2. Investigation on cytotoxic effect of moscatilin in non-small cell lung cancer H23 cells

To investigate the cytotoxic effect of moscatilin, the cell viability of H23 cells on cytotoxic effect was evaluated by MTT assay.

11.3. Investigation on mode of cell death in response to moscatilin treatment in non-small lung cancer H23 cells

To investigate the effect of moscatilin on mode of cell death, apoptotic and necrotic cells were determined by Hoechst 33342 and PI co-staining assay. Furthermore, subG_0/G_1 phase was evaluated by DNA content analysis assay.

11.4. Investigation the effect of moscatilin on cell migration

To investigate the effect of moscatilin on cancer migration, the ability of H23 cell migration in response to various concentrations of moscatilin (0-1 μM) was evaluated by wound healing assay and transwell migration assay.

11.5. Investigation the effect of moscatilin on cell invasion

To investigate the effect of moscatilin on cancer invasion, the ability of H23 cell invasion in response to various concentrations of moscatilin (0-1 μM) was evaluated by using transwell invasion assay.

11.6. Investigation the effect of moscatilin on filopodia formation

To investigate the effects of moscatilin on filopodia formation, the effect of moscatilin on filopodia formation was evaluated by using phalloidin staining in H23 cells.

11.7. Investigation the mechanism of moscatilin-inhibiting migration

To investigate the mechanism of moscatilin-inhibited migration pathway, the mechanism of moscatilin on attenuates migration was evaluated by western blot analysis, using specific antibody to p-FAK, FAK, p-Akt, Akt, p-ERK1/2, ERK1/2 and Cdc42.

11.8. Investigation the effect of moscatilin on endogenous reactive oxygen species level

To investigate effect of moscatilin on endogenous reactive oxygen species level after indicated treatment, endogenous reactive oxygen species level were determined using ROS probe as dichlorofluorescein diacetate ($DCFH_2$-DA) by ROS detection assay.

11.9. Identification of specific reactive oxygen species generation in response to moscatilin-inhibiting migration

To investigate specific reactive oxygen species generation in response to moscatilin, specific reactive oxygen species were determined using specific probe, namely HPF (hydroxyl radical probe), amplex red (hydrogen peroxide probe),or DHE (superoxide anion probe) by ROS detection assay. Furthermore, antioxidant effect of moscatilin on cancer migration was evaluated by wound healing assay.

11.10. Investigation the effect of moscatilin on HK2 cell death

To investigate the effect of moscatilin on HK2 cell death, the cell viability of HK2 cells on cytotoxic effect was evaluated by MTT assay.

CHAPTER IV

RESULTS

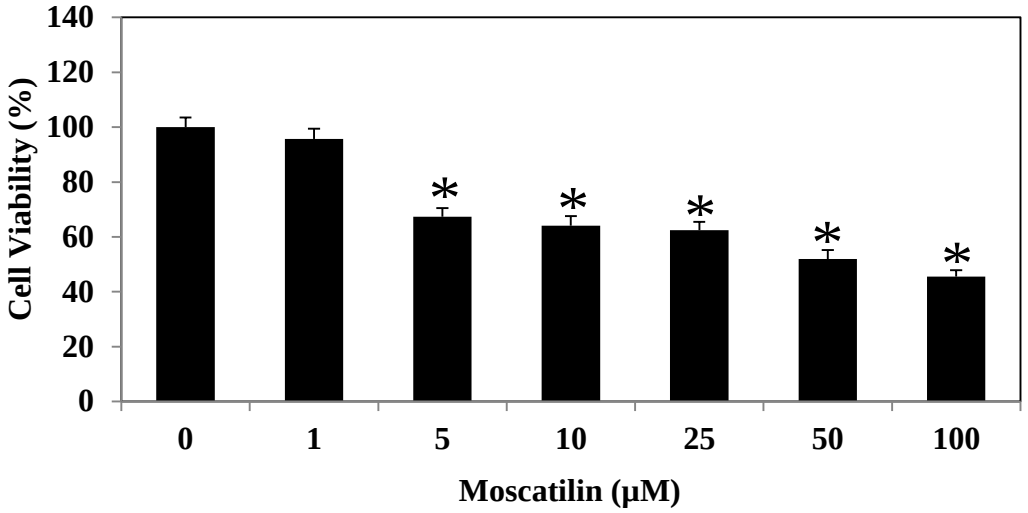
1. Investigation on cytotoxic effect of moscatilin in non-small cell lung cancer H23 cell

To investigate the inhibitory effect of moscatilin on cancer migration and invasion, prerequisite information regarding its cytotoxicity is crucial. MTT assay was used to determine cytotoxic effects. The investigation involved the treatment of H23 cell lines with different concentrations of moscatilin (0-100 μM) for 24 h.

Figure 12A showed that treatment with moscatilin at the concentration ranging from 0.25 to 1 μM had no significant effect on H23 cell viability, while moscatilin at the higher concentrations than 1 μM caused significantly decrease in cell viability. At the concentration of 50 μM decreased cell viable with approximately to 50%. In addition, non-toxic dose of moscatilin (0-1 μM) did not affect relative cell proliferation both 12, 24, 48 and 72 h (Figure 12B).

This study indicated that the lower doses of moscatilin (0-1 μM) caused neither toxic nor proliferative effects on lung cancer H23 cells.

A



B

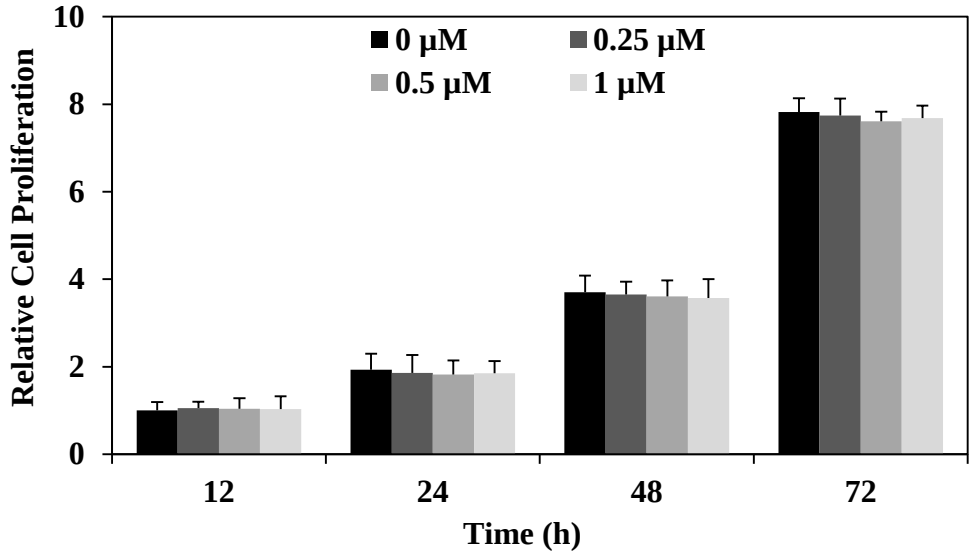


Figure 12 Cytotoxicity of moscatilin on non-small cell lung cancer H23 cells. (A) Cells were treated with various concentrations of moscatilin (0-100 μM) for 24 h. (B) Cells were treated with various concentrations of moscatilin (0-1 μM) for various times (0-72 h). Cytotoxicity was determined by MTT assay. Data represented the means ± SD (n=3). *P < 0.05 vs. non-treated control cells.

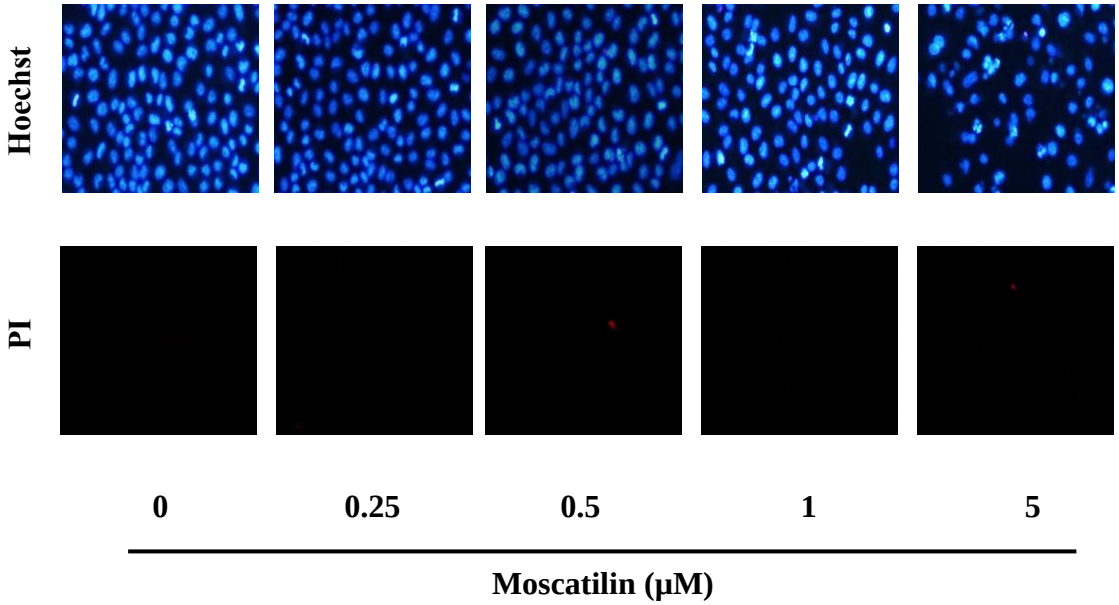
2. Investigation on mode of cell death in response to moscatilin treatment in non-small lung cancer H23 cells

To confirm the acquisition of non-toxic doses of moscatilin by MTT assay, Hoechst 33342 and PI co-staining assay, as well as DNA content analysis assay were performed. H23 cell lines were treated with various non-toxic doses of moscatilin (0-5 μM).

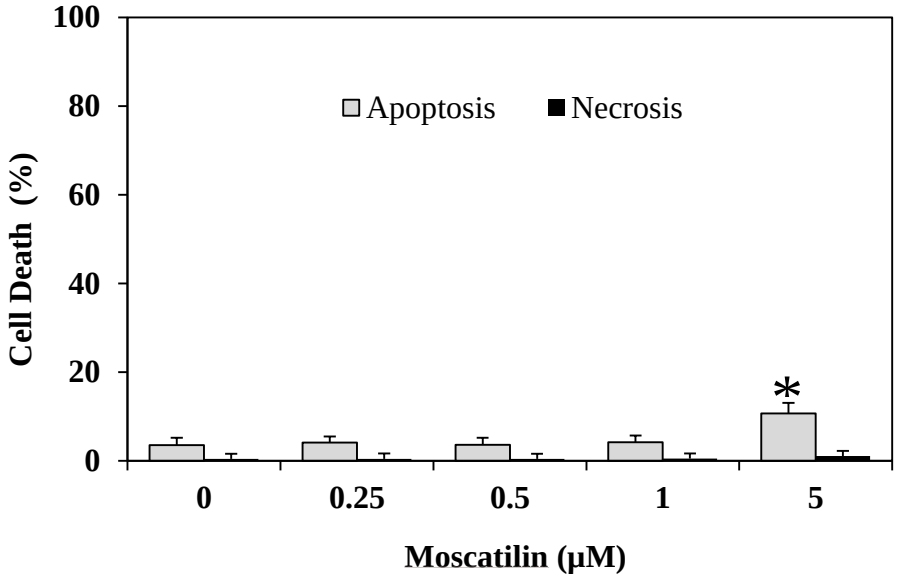
Hoechst and propidium iodide co-staining assay revealed that non-toxic dose of moscatilin (0-1 μM) caused neither apoptosis nor necrosis while moscatilin at concentrations more than 5 μM showed significantly increase apoptotic cells (Figure 13A and B). In addition, DNA content analysis demonstrated that non-toxic dose of moscatilin did not enhance cell proliferation whereas the increase of cell in sub-G_0/G_1 phase was observed in response to 5 μM of moscatilin, indicating suppression of cell growth (Figure 13C and D).

This result confirmed previous finding that non-toxic dose of moscatilin (0-1 μM) had no cytotoxicity and proliferative effect, and these concentrations were used in further study.

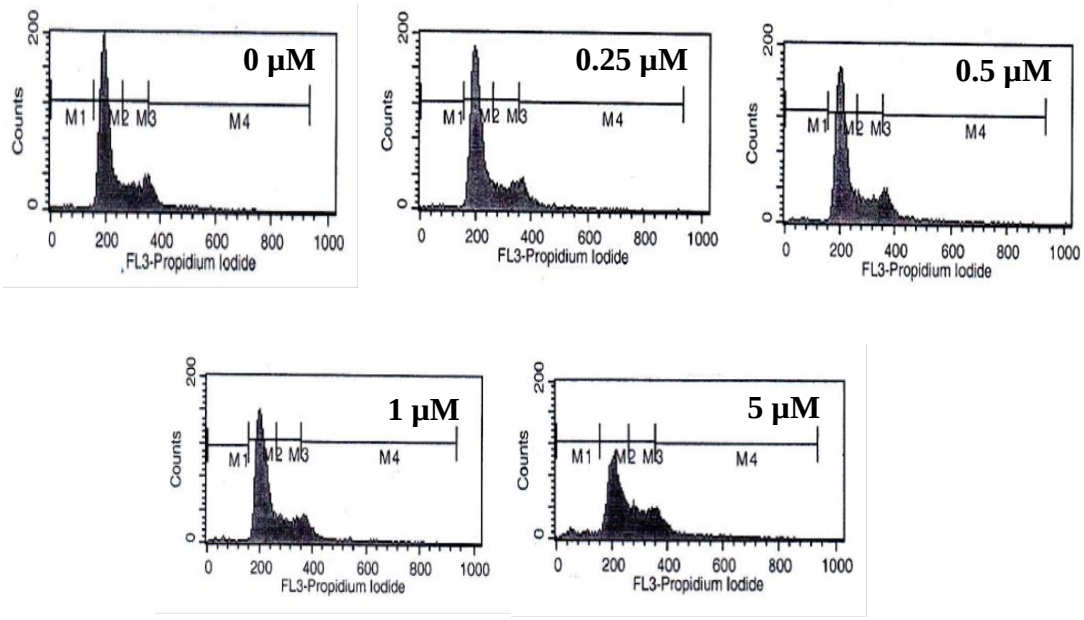
A


Hoechst
PI
0
0.25
0.5
1
5
Moscatilin (μM)

B


100
80
60
40
20
0
Cell Death (%)
Apoptosis
Necrosis
*
0
0.25
0.5
1
5
Moscatilin (μM)

C



D

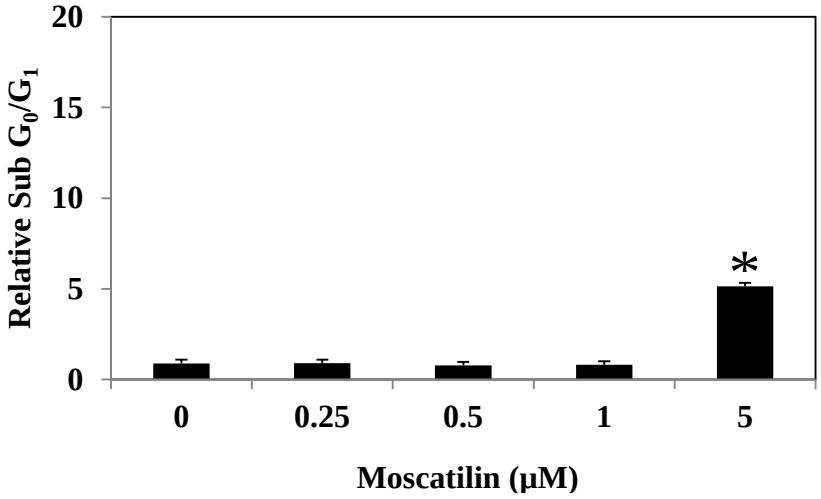


Figure 13 Mode of cell deaths in response to moscatilin treatment in non-small lung cancer H23 cells. Cells were treated with various concentrations of moscatilin (0-5 µM) for 24 h. (A) Nuclear morphology of Hoechst33342/PI-positive cells was visualized under fluorescence microscopy. (B) Percentage of apoptotic and necrotic

cells were obtained from Hoechst 33342/PI co-staining assays. (C) Cellular apoptosis was determined by DNA content analysis using flow cytometry. (D) Sub-G_0/G_1 of the cell-cycle was calculated and represented as a relative value. Data represented the means ± SD (n=3). *$P < 0.05$ vs. non-treated control cells.

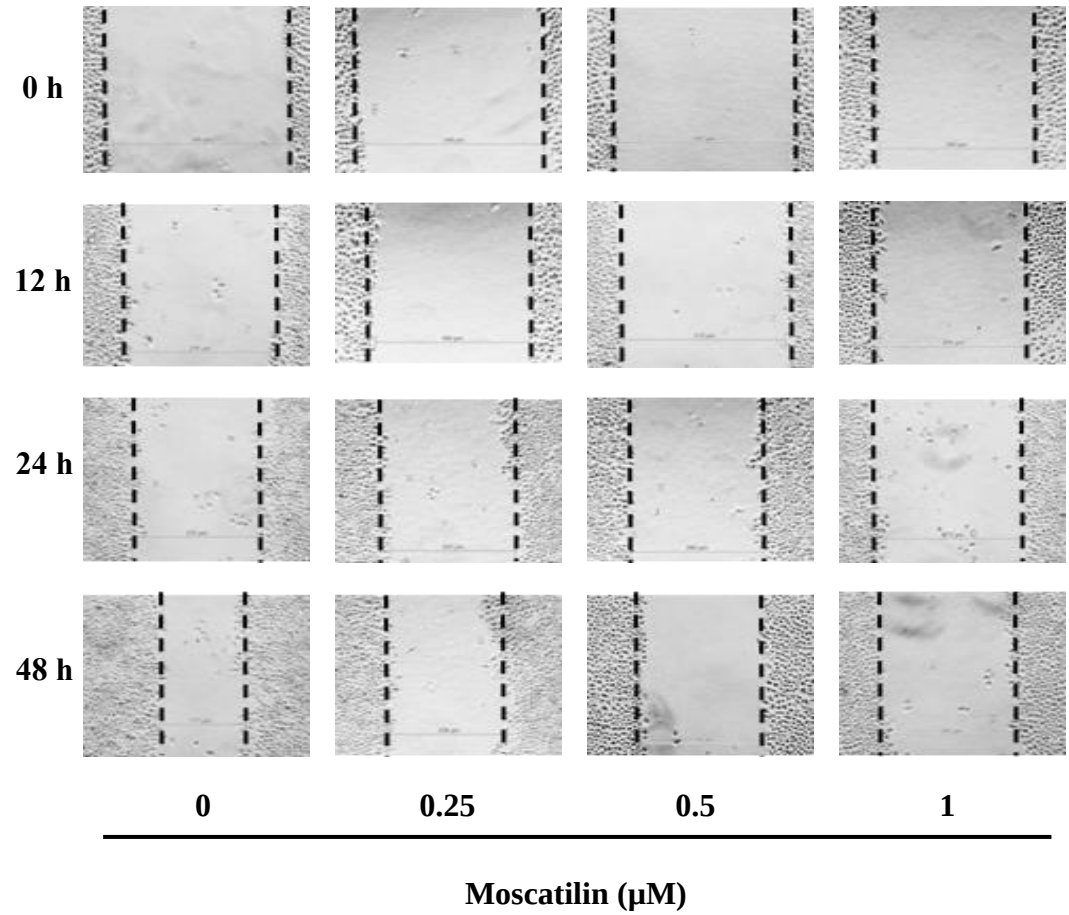
3. Investigation the effect of moscatilin on cell migration

Migration has been indicated as a one of aggressive characteristic of metastasis cancer (Brooks *et al.*, 2009). The negative regulatory role of moscatilin on lung cancer migration was investigated by wound healing and Boyden chamber assays.

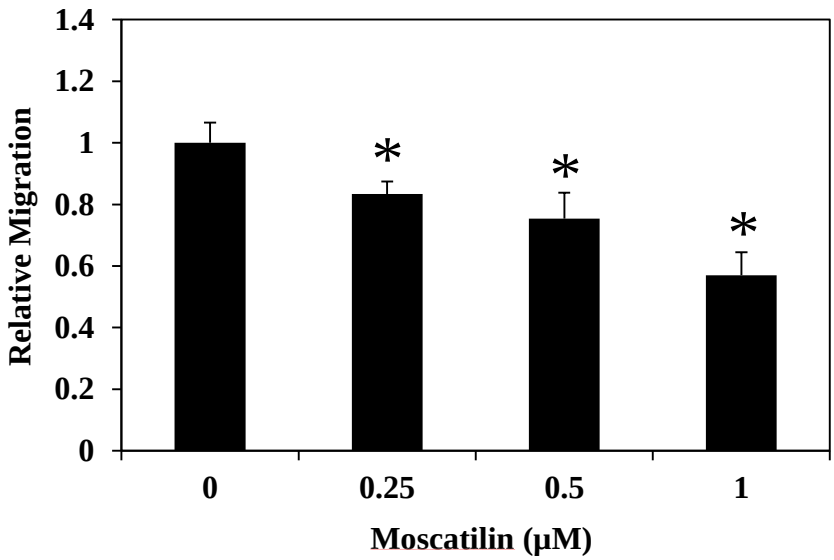
Wound healing assay showed that treatment with non-toxic dose of moscatilin (0-1 μM) inhibited the H23 cell migration in a dose- and time-dependent manner. Approximately 0.80-, 0.75- and 0.55-fold of relative migration level were found in cells treated with 0.25, 0.5 and 1 μM at 24 h, respectively, comparing with non-treated control cells (Figure 14). In addition, transwell migration assay supported above finding that the migrating cells on lower side of membrane were decreased gradually in dose- and time-dependent manners (Figure 15).

These results suggest the promising role of moscatilin on regulation of lung cancer migration.

A


0 h
12 h
24 h
48 h
0
0.25
0.5
1
Moscatilin (μM)

B


Relative Migration
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
0.25
0.5
1
Moscatilin (μM)

C

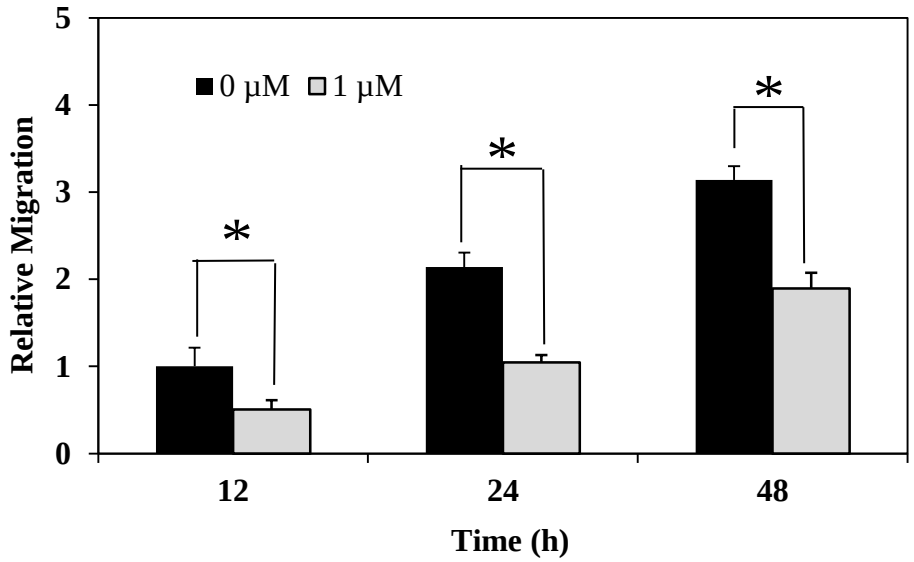
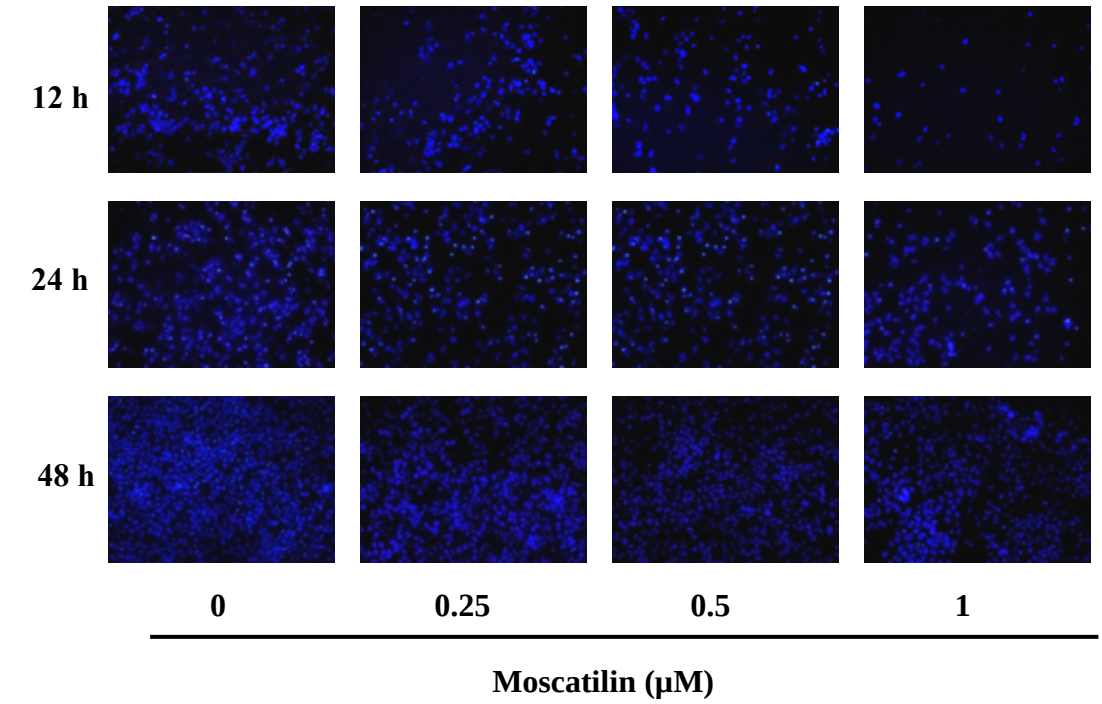
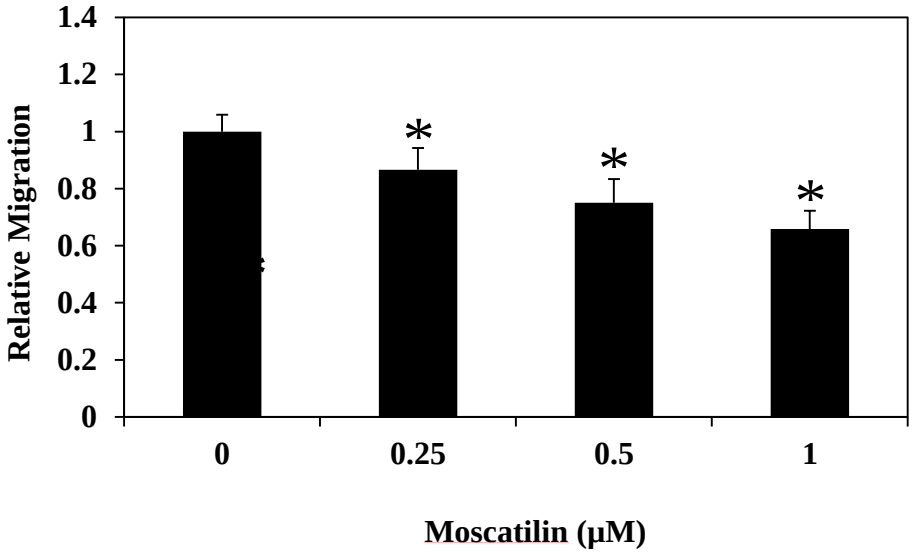


Figure 14 Effects of moscatilin on H23 cell migration (wound healing assay). (A) Confluent monolayer of H23 cells were wounded using a 1-mm width tip and incubated with non-toxic dose of moscatilin for various times. After indicated treatment, wound space was captured under microscope. (B) Confluent monolayer of H23 cells were wounded using a 1-mm width tip and incubated with non-toxic dose moscatilin (1 μM) or without for 24 h. (C) Confluent monolayer of H23 cells were wounded using a 1-mm width tip and incubated with 1 μM moscatilin or without for various times (0-48 h). Wound space was analyzed and represented as migration level relatively to the change of those in non-treated cells. Data represent the means ± SD (n=3). *P < 0.05 vs. non-treated control cells.

A


12 h
24 h
48 h
0
0.25
0.5
1
Moscatilin (μM)

B


Relative Migration
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
0.25
0.5
1
Moscatilin (μM)

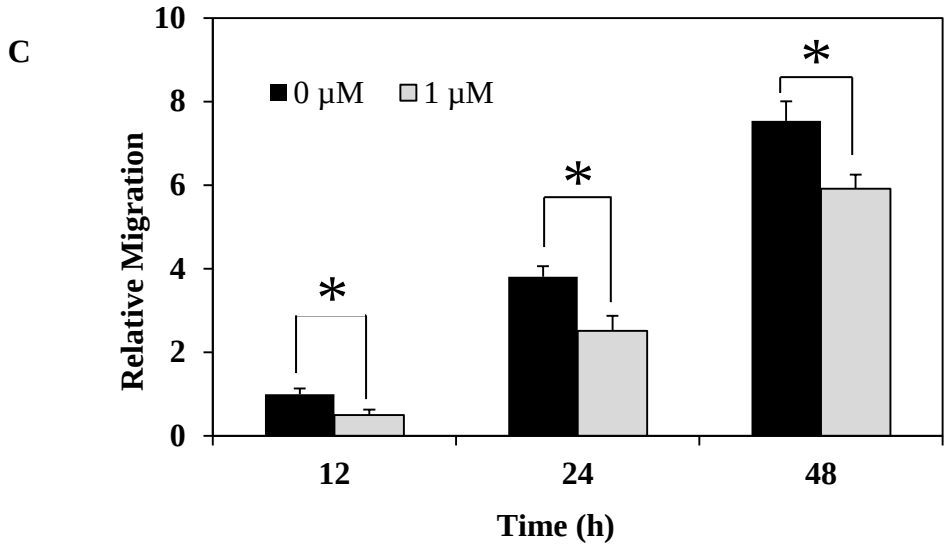


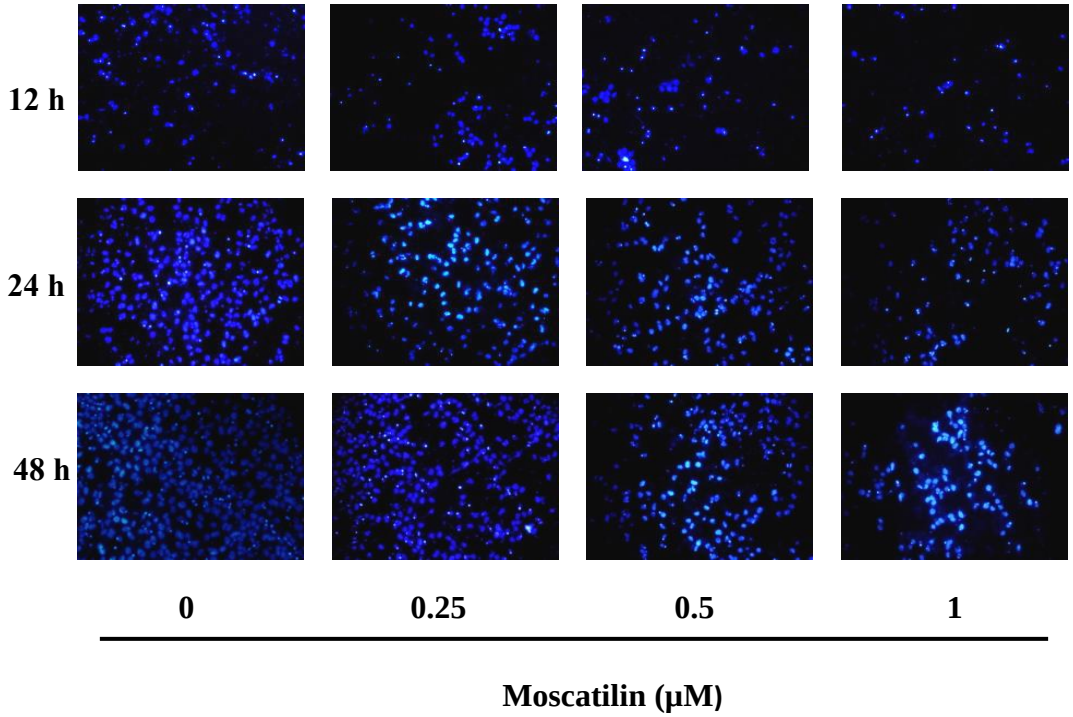
Figure 15 Effects of moscatilin on H23 cell migration (transwell migration assay). (A) H23 cell invasion was examined by transwell migration assay after being incubated with a non-toxic dose of moscatilin for various times. After indicated, migrating cells were stained with Hoechst 33342 and visualized under fluorescence microscopy (B) H23 cells were treated with non-toxic doses moscatilin (0-1 µM) for 24 h. (C) H23 cells were treated with 1 µM moscatilin for various times (0-48 h). The relative of the cell migration was calculated and expressed. Data represented the means ± SD (n=3). *P < 0.05 vs. non-treated control cells.

4. Investigation the effect of moscatilin on cell invasion

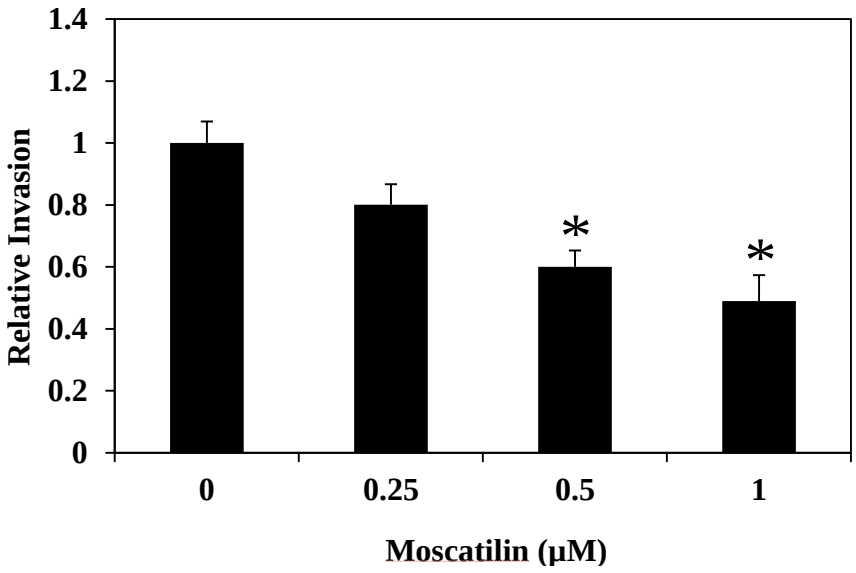
To further investigate the effect of moscatilin on lung cancer cell invasion, H23 cells were treated with non-toxic dose of moscatilin (0-1 μM) for various times (0-48 h), and invaded cells were examined by transwell invasion assay. Figure 16 show that non-toxic concentrations of moscatilin decreased a number of invaded cells through the lower side of chamber in a dose- and time-dependent fashion, which approximately 0.63- and 0.50-fold of relative invaded cells were observed in response to 0.5 and 1 μM of moscatilin at 24 h, respectively.

These results revealed the promising role of moscatilin on the regulation of lung cancer invasion.

A


12 h
24 h
48 h
0
0.25
0.5
1
Moscatilin (μM)

B


Relative Invasion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
0.25
0.5
1
Moscatilin (μM)

C

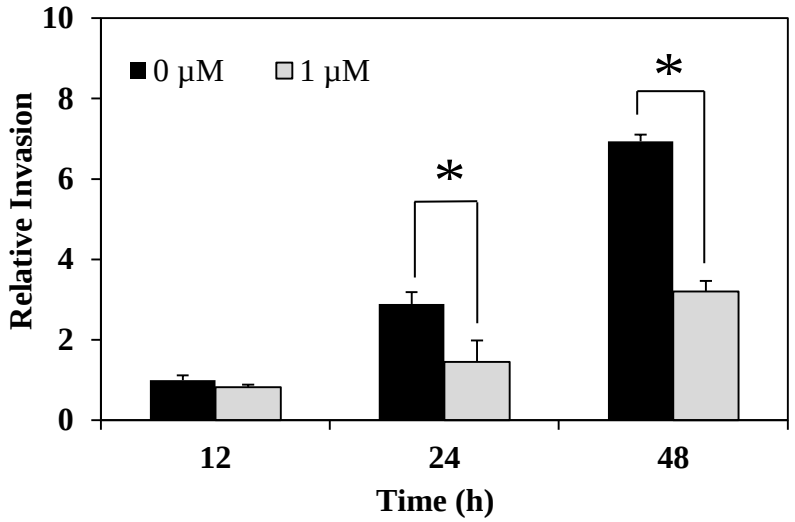


Figure 16 Effects of moscatilin on H23 cell invasion. (A) H23 cell invasion was examined by transwell invasion assay after being incubated with a non-toxic dose of moscatilin for various times. After indicated treatment, invade cells were stained with Hoechst 33342 and visualized under fluorescence microscopy (B) H23 cells were treated with non-toxic doses moscatilin (0-1 μM) for 24 h. (C) H23 cells were treated with 1 μM moscatilin for various times (0-48 h). The relative of the cell invasion was calculated and expressed as relative value. Data represented the means ± SD (n=3). *P < 0.05 vs. non-treated control cells (time = 12).

5. Investigation the effect of moscatilin on filopodia formation

The characteristic of filopodia or the filamentous actin elongation has been indicated to promoted cell migration (Mattila and Lappalainen, 2008). To examine cytoskeleton alignment, H23 cell lines were treated with various non-toxic doses of moscatilin (0-1 μM) for 24 h.

The results showed that the treatment of moscatilin at the concentration ranging from 0.25 to 1 μM decreased a portion of filopodia formation as compared with the untreated control cells in dose-dependent manner (Figure 17).

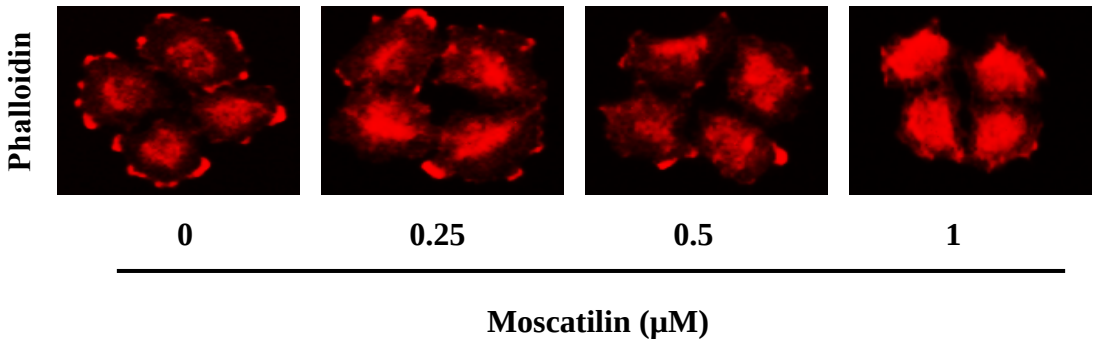


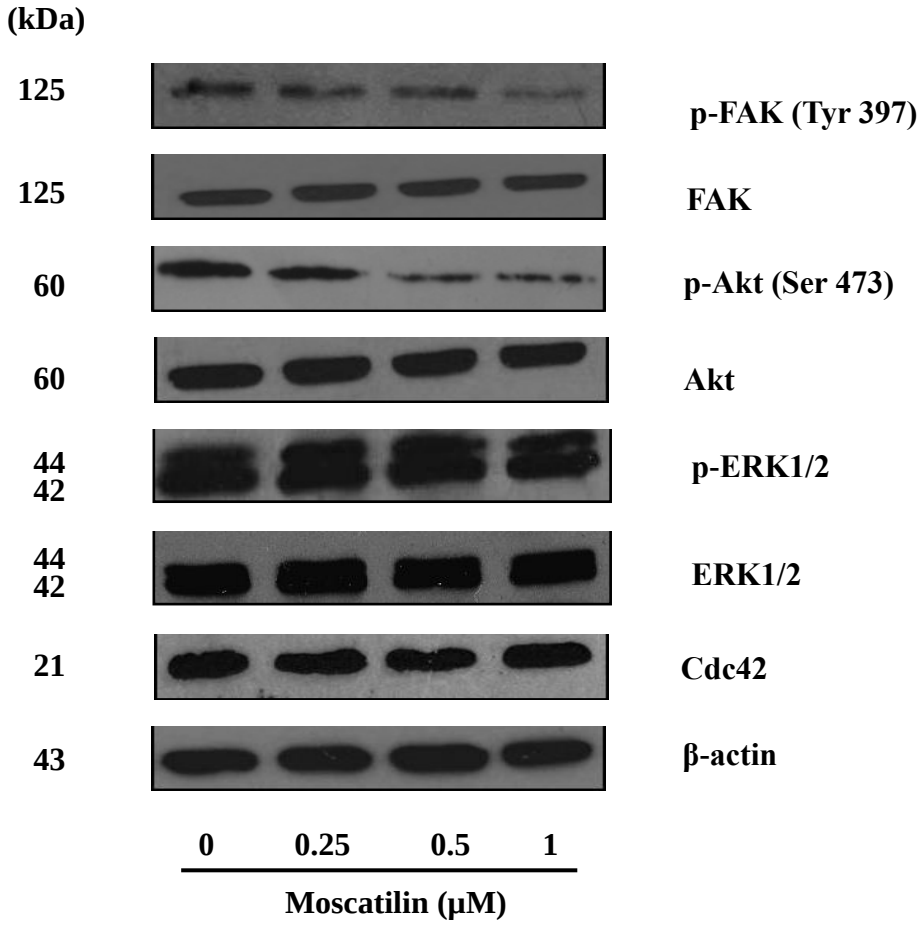
Figure 17 Effects of moscatilin on filopodia formation. After being treated with a non-toxic dose of moscatilin for 24 h, cells were stained with phalloidin, and examined under fluorescent microscope.

5. Investigation the mechanism of moscatilin-inhibiting migration

In order to clarify the inhibitory mechanism of moscatilin in migration pathway, the study focused on key indicators namely p-FAK, p-Akt, p-ERK1/2 and Cdc42, which reported to have implicated in cell migration in several studies (Schmitz *et al*., 2000; Schlaepfer and Mitra, 2004; Shukla *et al*., 2007). In this study, the protein level was evaluated by western blot analysis**.**

The results showed that treatment of moscatilin at non-toxic concentrations (0-1 μM) for 24 h significantly decreased the phosphorylation of FAK at Tyr 397 and phosphorylation of Akt at Ser 473 as compared with non-treated control cells, whereas their preserved parental counterparts were not changed (Figure 18). Futhermore, the level of p-ERK1/2 and Cdc42 were not affected by moscatilin. These results suggested that moscatilin attenuated cell migration through FAK- and Akt-dependent pathway.

A



B

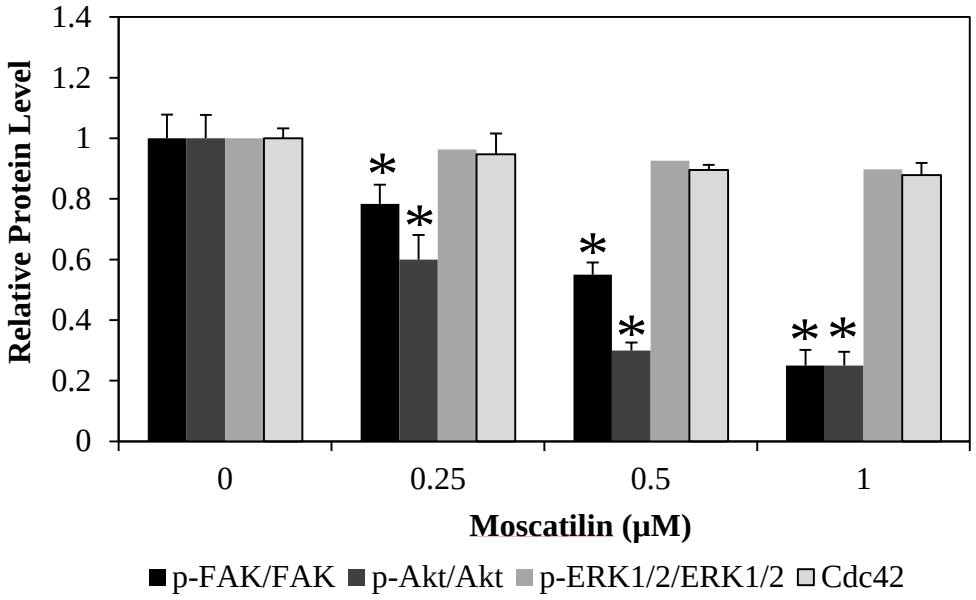


Figure 18 Effects of moscatilin on migratory-related proteins. (A) H23 Cells were

treated with various non-toxic doses of moscatilin (0-1 μM) for 24 h and analyzed for protein expression using western blot analysis as described under Materials and Methods. Cells were collected and analyzed for phosphorylated-FAK (Tyr 397), FAK proteins, phosphorylated-Akt (Ser 473), Akt, phosphorylated-Erk1/2, Erk1/2 and Cdc42 proteins. Blots were reprobed with β-actin to confirm equal loading. (B) The immunoblot signals were quantified by densitometry and mean data from four independent experiments were presented. Values are means of samples ± SD. *P < 0.05 vs. non-treated control cells.

6. Investigation the effect of moscatilin on endogenous reactive oxygen species level

It has been well documented that endogenous ROS, namely, hydrogen peroxide, superoxide anion and hydroxyl radical are continuously produced inside the living cells (Lee *et al*., 2000). Substantial studies have indicated the regulatory role of such specific ROS in cell behaviors including migration and invasion (Luanpitpong *et al*., 2010; Hung *et al*., 2012), and most evidence indicated that these specific ROS play distinguishable roles in cell motility. In order to provide the precise mechanism of moscatilin in the regulation of cell migration, cells were treated with non-toxic concentrations of moscatilin (0-1 μM), and cellular ROS were investigated by $DCFH_2$-DA, ROS detection probe and analyzed by fluorescence microplate reader from 0-3 h. The result showed that moscatilin caused a significantly decrease of endogenous ROS level in concentration- and time-dependent manners (Figure 19).

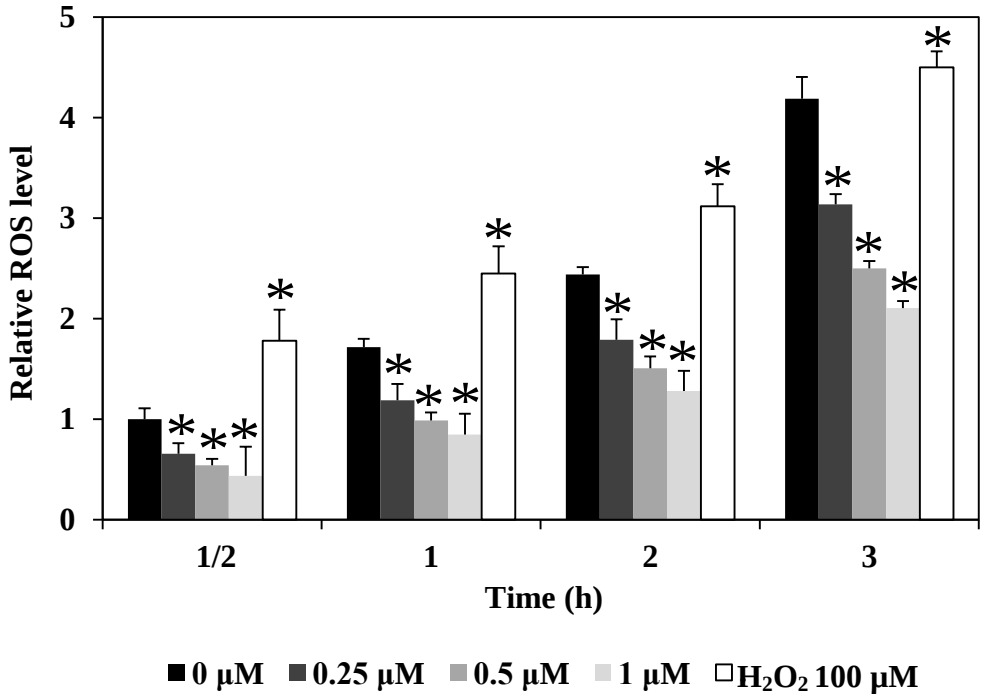


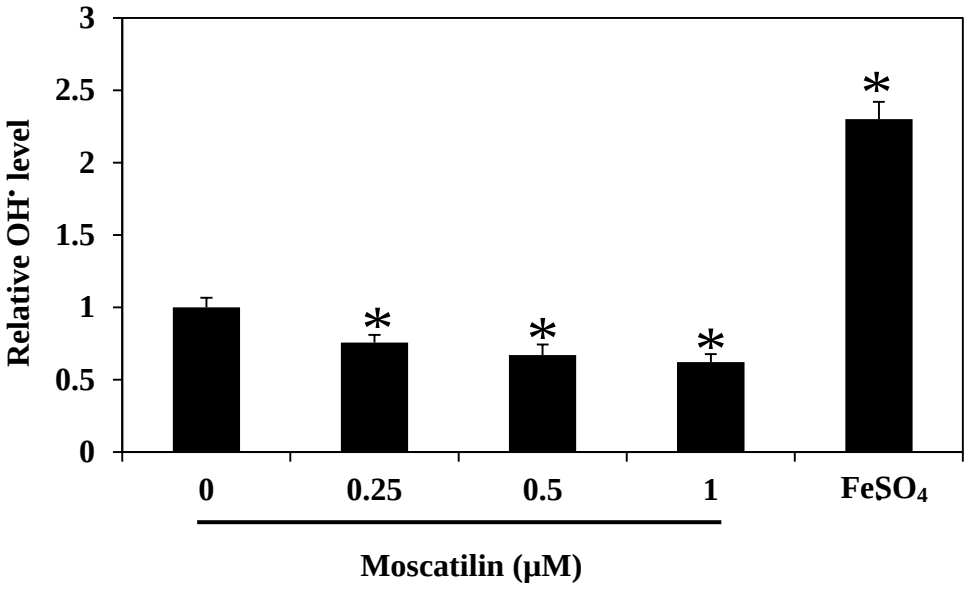
Figure 19 Effects of moscatilin on endogenous reactive oxygen species level. H23 cells were treated with various nontoxic doses of moscatilin (0-1 μM) for various times (0-3 h), and treated with H_2O_2 as positive control. Endogenous cellular ROS levels were determined by $DCFH_2$-DA probe. Data represented the means ± SD (n=3). *P < 0.05 vs. non-treated control cells of each time point.

7. Identification of specific reactive oxygen species generation in response to moscatilin-inhibiting migration

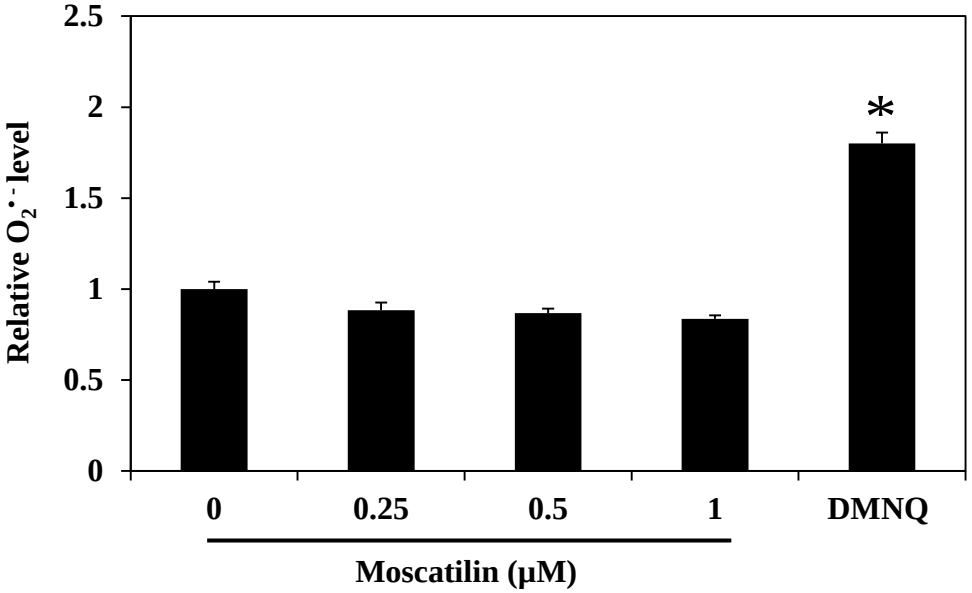
In order to identify the specific ROS involved in our tested conditions, cells were treated with moscatilin (0-1 μM) for 3 h and incubated with specific ROS detection probes: HPF, Amplex red and DHE. Interestingly, moscatilin shows an antioxidant effect, by which the level of $OH^{\bullet}$ is substantially decreased in response to moscatilin treatment (Figure 20A). While no change was observed regarding the level of $O_2^{\bullet-}$ and H_2O_2 in comparison with non-treated control cells (Figures 20B and C), suggesting that endogenous $OH^{\bullet}$ is a targeted species eliminated by moscatilin. To confirm the anti-$OH^{\bullet}$ effect of moscatilin, cells were treated with specific $OH^{\bullet}$ generator (ferrous sulfate) in the presence of moscatilin for 3 h, and ROS levels were identified using $DCFH_2$-DA-specific ROS detection probes. Figure 21A clearly demonstrates that an extensive increase in ROS level mediated by ferrous sulfate was in turn suppressed gradually by moscatilin in a dose-dependent fashion. This novel finding indicated that moscatilin shows a potent antioxidant against endogenous ROS, and $OH^{\bullet}$ is the most affected species.

Parallel study was conducted to investigate the relevance of antioxidant effect of moscatilin on cancer migration, cells were pre-incubated with $OH^{\bullet}$ generator in the presence or absence of moscatilin treatment. Wound healing assay show that ferrous sulfate treatment significantly enhanced the migration of cells, and the addition of moscatilin was able to abolish such an effect (Figure 21B). These findings suggest that anti-migrative effect of moscatilin was associated with its ability to suppress endogenous $OH^{\bullet}$.

A


Relative OH˙ level
3
2.5
2
1.5
1
0.5
0
*
*
*
*
0
0.25
0.5
1
FeSO4
Moscatilin (μM)

B


Relative O2˙- level
2.5
2
1.5
1
0.5
0
*
0
0.25
0.5
1
DMNQ
Moscatilin (μM)

C

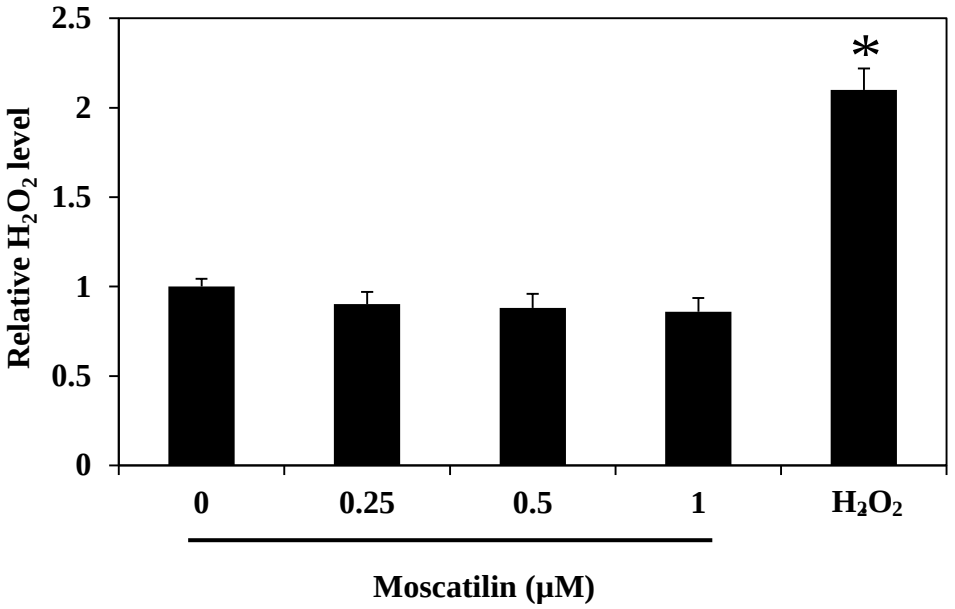
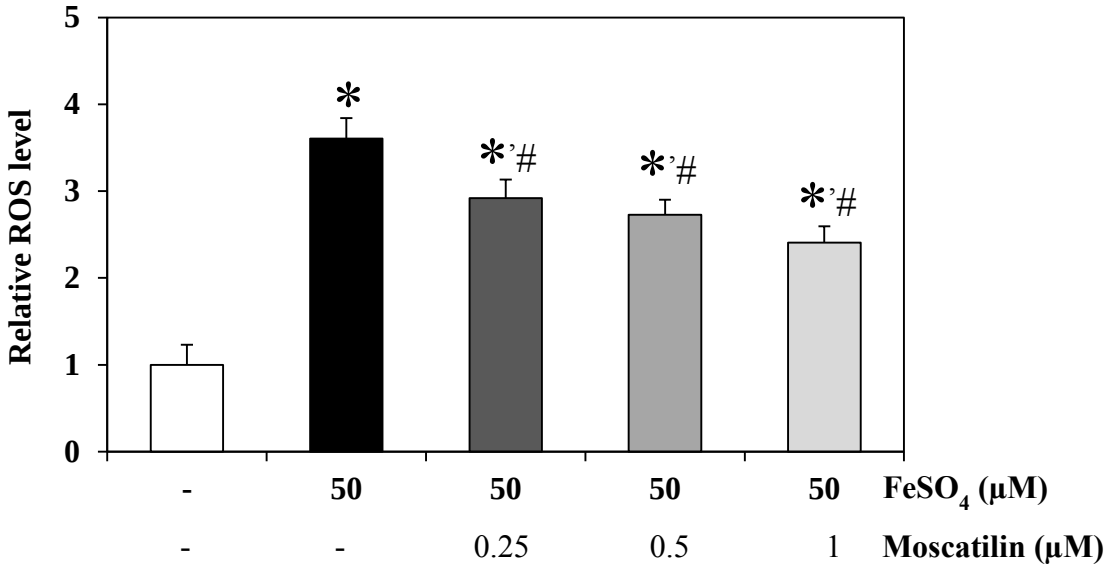


Figure 20 Specific reactive oxygen species generation in response to moscatilin. H23 cells were treated with various non-toxic doses of moscatilin (0-1 μM). (A) After the indicated treatment for 3 h, cells were incubated with HPF probe. Hydroxyl radical level was detected using fluorescence microplate reader. *P < 0.05 versus non-treated control cells. $FeSO_4$ was used as positive control (B) Superoxide anion level was detected by DHE probe. *P < 0.05 versus non-treated control cells. DMNQ was used as positive control. (C) Hydrogen peroxide level was examined using Amplex red probe. *P < 0.05 versus non-treated control cells. H_2O_2 was used as positive control.

A



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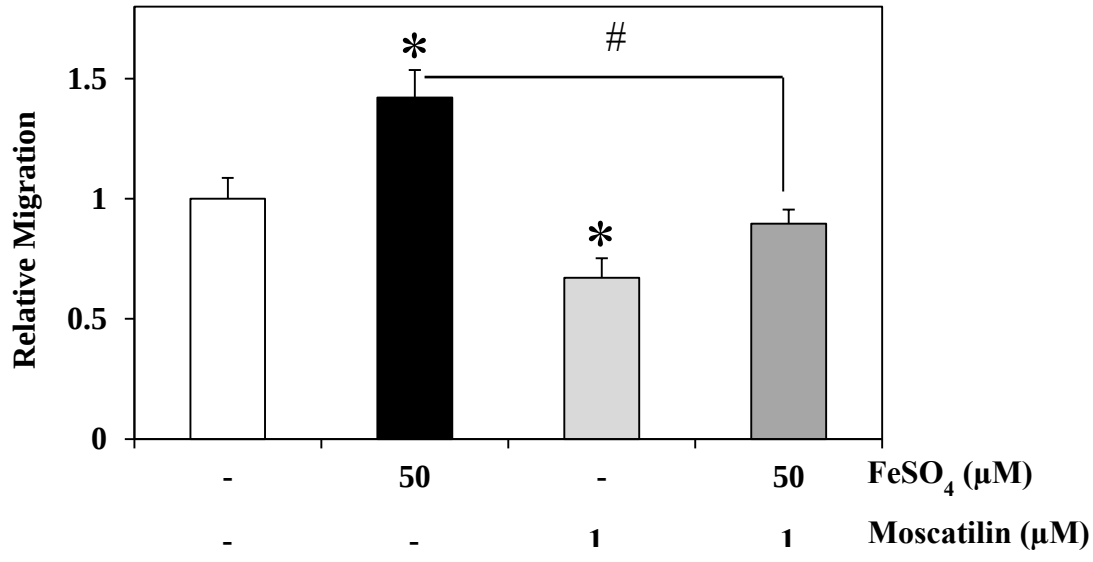

Figure 21 Effects of moscatilin on cell motility through ROS mechanism. (A) Cells were pre-treated with 50 μM of $FeSO_4$ for 30min prior to moscatilin treatments (0-1 μM) for 3 h. Endogenous ROS level were determined by using $DCFH_2$-DA probe. Values are mean ± SD (n = 3). *P < 0.05 versus nontreated control cells. #P < 0.05 versus ferrous sulfate treated cells. (B) Confluent monolayer of H23 cells was

wounded using a 200 µl width tip and treated with moscatilin (1 µM) in the presence or absence of 50 µM $FeSO_4$ for 24 h. Wound space was analysed and represented as migration level relatively to the change of those in non-treated cells. *$P < 0.05$ versus non-treated control cells. #$P < 0.05$ versus ferrous sulphate treated cells.

8. Investigation the effect of moscatilin on HK2 cell death

Because major concern for anti-cancer drugs is cytotoxic to normal cells, the study was test whether moscatiin at mentioned concentrations caused toxic to the human renal HK2 cells. MTT assay was used to determine cytotoxic effects. The investigation involved the treatment of HK2 cell lines with non-toxic concentrations of moscatilin (0-1 μM).

Figure 22 showed that the treatment with moscatilin at the concentration ranging from 0.25 to 1 μM had no significant effect on HK2 cell viability. This study indicated that the lower doses of moscatilin (0-1 μM) exhibited no cytotoxic effect on HK2 cells.

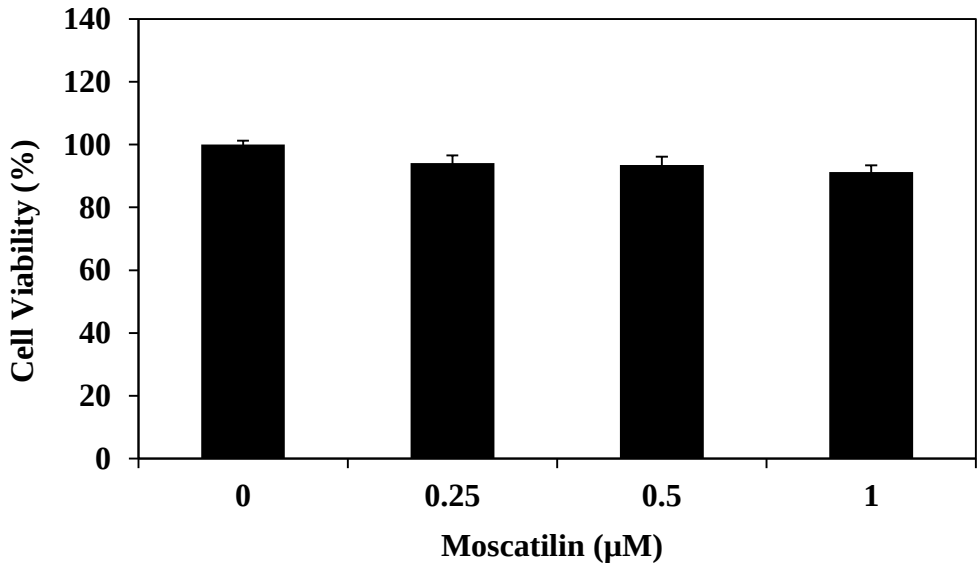

Figure 22 Effects of moscatilin on HK2 cell death. HK2 cells were treated with non-toxic concentrations of moscatilin (0-1 μM) for 24 h. Cytotoxicity was determined by MTT assay. Data represented the means ± SD (n=3). *P < 0.05 vs. non-treated control cells.

CHAPTER V

DISCUSSION AND CONCLUSION

Lung cancer is one of the most frequent cancers, due to cancer metastasis (Ray and Jablons, 2009). The early detection of cancer, prevention of metastasis, and investigation of the mechanism of metastatic activity are all vitally important because they can prolong survival rate (Leber and Efferth, 2009).

Cancer metastasis is a complex multistep process whereby cancer cell migration and invasion are crucial in determining the capability of cancer to metastasize. Cancer migration is characterized by the movement of cancer to other places which initiates by the dynamic change of cytoskeleton including protrusion of cell membrane and actin-myosin contraction (Fidler, 2002). Even though the invasion of the cancer cells was shown to share certain molecular mechanisms with cell migration, invasion is more focused on the ability of cancer to disrupt basement membrane and extracellular matrix by secreting the proteolytic enzyme to destruct the meshwork of basement membrane, prior to migration through surrounding tissue (Fidler, 2002). Most of metastasis cancer cells exhibit these aggressive behaviors which limit the effectiveness of cancer therapy and result in high mortality rate of lung cancer patients (Ray and Jablons, 2009).

Many studies have been conducted in the past decade to explore biological agents that have an ability to inhibit cancer metastasis. According to numerous researches, moscatilin, a major constituent of *Dendrobium pulchellum*, is one such interested in its antimutagenic activity against several cancer types and inhibition of platelet aggregation (Chen *et al*., 2008; Hwang *et al*.; Tsai *et al*., 2010). Moscatilin was also reported to have various pharmacological properties such as

antiinflammation and antioxidant (Hwang *et al*. 2010; Tsai *et al*., 2010). Previous studies indicated that bibenzyl derivative from this genus possess cytotoxic effect against many cancer cell lines such as moscatilin, gigantol and dengraol, and exhibited antioxidant activity (Zhang *et al*., 2007; Sritularak *et al*., 2011). This also provided further evidence supporting the promising role of this natural compound for treatment of metastasis cancers. This findings show that nontoxic doses of moscatilin were able to inhibit lung cancer cell migration and invasion (Figures 14, 15 and 16). This work also reported herein for the first time that such an inhibitory effect was involved with the potential of moscatilin to attenuate endogenous ROS of which $OH^{\bullet}$ was identified to be an affected species.

The role of ROS in cancer behavior has been well described including the regulation of cell motility and invasiveness (Storz, 2005; Luanpitpong *et al*., 2010). Recently, specific ROS, $O_2^{\bullet-}$, and H_2O_2 were shown to exhibit a negative regulatory effect on cell migration and invasion, whereas OH· encourages such activities (Luanpitpong *et al*., 2010). Previously, moscatilin was reported to have antioxidant effect (Zhang *et al*, 2007), and this further found that this substance reduced endogenous OH· and thus inhibited migratory action of the cells (Figure 19, 20 and 21). Consistent with previous report, this study found that the addition of ferrous sulfate promoted cancer cell motility, which can be conversed by treatment with moscatilin.

Emerging evidence showed that several signalling molecules such as focal adhesion kinase (FAK), Akt/phosphatidylinositol-3-kinase (PI3K), and p44/42 Mitogen-activated protein kinases (ERK1/2) play enhancing roles in motility of cells (Shukla *et al*., 2007; Teranishi *et al*., 2009). Recently, focal adhesion kinase (FAK) pathway has gained increasing attention as migratory-related proteins (Teranishi *et al*., 2009). During cell motility, FAK accumulated at the site of integrin and the

phosphorylated form of FAK was shown to serve as binding site for Src (Mitra and Schlaepfer, 2006). FAK-Src complexes enhance actin polymerization and filopodia formation through Cdc42-dependent mechanism (Mitra and Schlaepfer, 2006; Mattila and Lappalainen, 2008). In addition, Akt and ERK signaling were implicated in cancer migration and invasion, of which the suppression of either their expressions or activity by silencing plasmid or specific inhibitor was able to attenuate these activities (Si *et al*., 2012). Accumulative studies have demonstrated that these mentioned proteins function independently from each other (Peng *et al*., 2005), and some evidence showed the linkage of them on cell motility (Hayashi *et al*., 2008). FAK activation was shown to mediate Akt phosphorylation which resulted in cell movement. According to this report, the reduction of Akt activation found in this study might be a consequent event as downstream effector in response to moscatilin-attenuating FAK phosphorylation. Even ERK and Cdc42 were indicated to potentiate cells to migrate and invade (Mattila and Lappalainen, 2008; Teranishi *et al*., 2009), this study demonstrated that moscatilin impeded migratory activity of H23 cells via ERK and Cdc42-independent mechanisms. These results provide a mechanistic insight into the mechanism of moscatilin on cancer cell migration and invasion through the suppression of endogenous ROS and FAK and Akt activation.

In conclusion, this study reported a novel finding on moscatilin suppressing migratory behaviour of lung cancer cells and its molecular mechanism. The migrative-inhibitory effect of moscatilin was through an attenuation of endogenous $OH^{\bullet}$ (Figure 18). In addition, moscatilin reduced FAK and Akt activation, which, at least in part, is responsible for its antimigratory effects. Since cell motility and invasion were critical implicated in cancer metastasis, this study thus provides information and highlights potential of this natural-based compound for clinical use to overcome cancer metastasis.

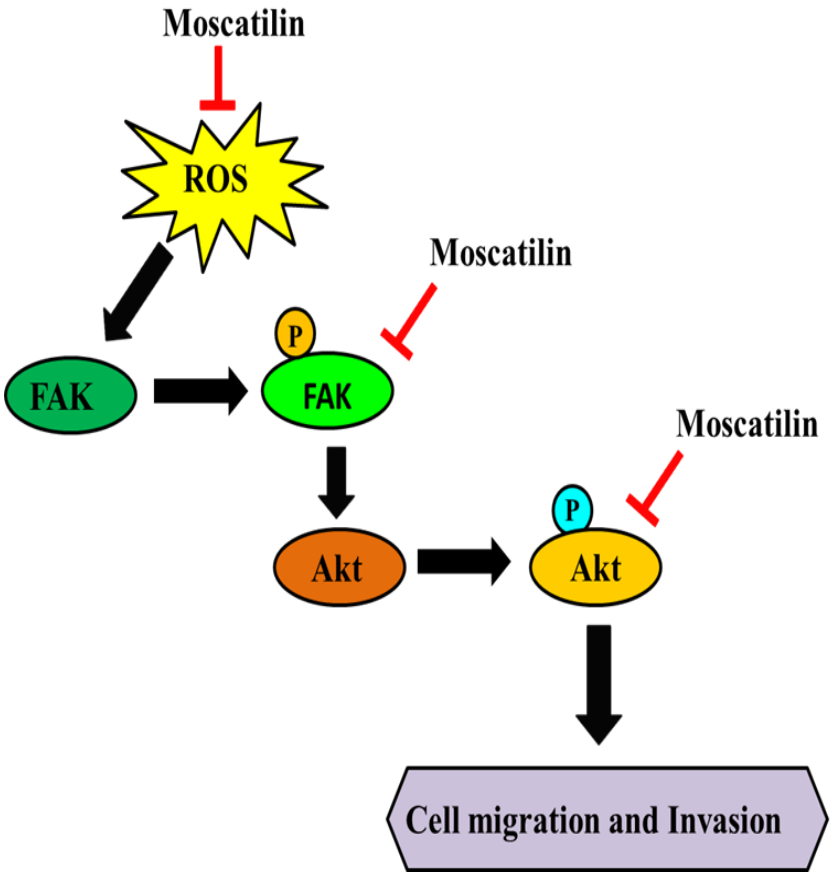

Figure 23 A schematic diagram summarizes the inhibitory effect of moscatilin on lung cancer cell migration and invasion.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The percentage of H23 cell viability was determined by MTT assay after treatment with various concentrations of moscatilin for 24 h.

Moscatilin (μM)	Cell viability (%)
0	100.00 ± 3.48
1	95.69 ± 3.77
5	67.35 ± 3.11*
10	64.06 ± 3.54*
25	62.49 ± 2.94*
50	52.96 ± 3.20*
100	46.48 ± 3.27*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA.

Table 2 The relative of H23 cell proliferation was determined by MTT assay after treatment with non-toxic concentrations of moscatilin (0-1 μM) at various time points.

Time (h)	Relative cell proliferation			
	0	0.25	0.5	1
12	1.00 ± 0.19	1.93 ± 0.37	3.7 ± 0.38	7.82 ± 0.32
24	1.05 ± 0.15	1.86 ± 0.41	3.65 ± 0.29	7.74 ± 0.39
48	1.04 ± 0.24	1.82 ± 0.32	3.61 ± 0.36	7.61 ± 0.22
72	1.03 ± 0.29	1.85 ± 0.28	3.57 ± 0.43	7.68 ± 0.29

Each value represents mean ± S.D. of three independent experiments.

Table 3 The percentages of apoptotic and necrotic cells were determined by Hoechst 33342 and propidium iodide co-staining assay after treatment with moscatilin (0-5 μM) for 24 h.

Moscatilin (μM)	Apoptotic cells (%)	Necrotic cells (%)
0	3.53 ± 1.66	0.45 ± 0.17
0.25	4.11 ± 1.40	0.48 ± 0.21
0.5	3.59 ± 1.62	0.44 ± 0.15
1	4.23 ± 1.49	0.51 ± 0.19
5	10.7 ± 1.37*	1.11 ± 0.30

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA.

Table 4 The percentage of sub-G_0/G_1 was determined by propidium iodide staining and flow cytometry after treatment with non-toxic concentrations of moscatilin (0-5 μM) for 24 h.

Moscatilin (μM)	Sub-G_0/G_1 (%)
0	0.89 ± 0.11
0.25	0.90 ± 0.20
0.5	0.78 ± 0.17
1	0.81 ± 0.19
5	5.14 ± 0.37*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA.

Table 5 Relative cell migration (wound closure) of H23 cells was determined by wound healing assay after treatment with non-toxic concentrations of moscatilin (0-1 μM) for 24 h.

Time (h)	Relative cell migration (wound closure)			
	0	0.25	0.5	1
24	1.00 ± 0.15	0.83 ± 0.12*	0.65 ± 0.21*	0.48 ± 0.12*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA.

Table 6 Relative cell migration (wound closure) of H23 cells was determined by wound healing assay after treatment with moscatilin (1 μM) or without for various times.

Time (h)	Relative cell migration (wound-healing)	
	0	1
12	1.00 ± 0.11	0.50 ± 0.16*
24	2.14 ± 0.15	1.04 ± 0.12*
48	3.11 ± 0.20	1.89 ± 0.13*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells at 12 h ($P < 0.05$) determined by One-way ANOVA.

Table 7 Relative cell migration (transwell) of H23 cells was determined by transwell migration assay after treatment with non-toxic concentrations of moscatilin (0-1 μM) for 24 h.

Time (h)	Relative cell migration (transwell)			
	0	0.25	0.5	1
24	1.00 ± 0.28	0.80 ± 0.17*	0.75 ± 0.24*	0.66 ± 0.23*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells at 12 h ($P < 0.05$) determined by One-way ANOVA.

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Table 8 Relative cell migration (transwell) of H23 cells was determined by wound healing assay after treatment with moscatilin (1 μM) or without for various times.

Time	Relative cell migration (wound-healing)	
(h)	0	1
12	1.00 ± 0.15	0.54 ± 0.22*
24	3.81 ± 0.28	2.51 ± 0.23*
48	7.51 ± 0.31	5.46 ± 0.31*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells at 12 h ($P < 0.05$) determined by One-way ANOVA.

Table 9 Relative cell invasion of H23 cells was determined by transwell invasion assay after treatment with non-toxic concentrations of moscatilin (0-1 μM) for 24 h.

Time (h)	Relative cell invasion			
	0	0.25	0.5	1
24	2.89 ± 0.27	0.86 ± 0.17	0.76 ± 0.28*	0.50 ± 0.24*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells at 12 h ($P < 0.05$) determined by One-way ANOVA.

Table 10 Relative cell invasion of H23 cells was determined by transwell invasion assay after treatment with moscatilin (1 μM) or without for various times.

Time	Relative cell invasion	
(h)	0	1
12	1.00 ± 0.12	0.82 ± 0.16*
24	2.89 ± 0.27	1.45 ± 0.24*
48	6.93 ± 0.35	3.20 ± 0.27*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells at 12 h ($P < 0.05$) determined by One-way ANOVA.

Table 11. The relative protein levels of p-FAK/FAK, p-Akt/Akt, p-Erk1/2/Erk1/2 and Cdc42 were determined by Western blot analysis after treatment with non-toxic concentrations of moscatilin (0-1 μM) for 24 h.

Moscatilin (μM) / Protein	Relative protein level			
	0	0.25	0.5	1
p-FAK/FAK	1.00 ± 0.07	0.78 ± 0.17*	0.55 ± 0.14*	0.23 ± 0.13*
p-Akt/Akt	1.00 ± 0.06	0.65 ± 0.08*	0.36 ± 0.18*	0.28 ± 0.18*
p-ERK1/2/ERK1/2	1.00 ± 0.07	0.96 ± 0.06	0.92 ± 0.14	0.89 ± 0.17
Cdc42	1.00 ± 0.05	0.94 ± 0.14	0.89 ± 0.11	0.87 ± 0.14

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA.

Table 12 Relative ROS level of H23 cells was determined by using $DCFH_2$-DA probe after treatment with non-toxic concentrations of moscatilin (0-1 μM) for various times (0-3 h).

Time (h)	Relative ROS level				
	0	0.25	0.5	1	$H_2O_2$100 μM
1/2	1.00 ± 0.19	0.65 ± 0.16*	0.54 ± 0.11*	0.43 ± 0.29*	1.78 ± 0.31*
1	1.71 ± 0.18	1.18 ± 0.16*	0.98 ± 0.17*	0.84 ± 0.11*	2.45 ± 0.27*
2	2.44 ± 0.27	1.79 ± 0.17*	1.51 ± 0.21*	1.28 ± 0.19*	3.12 ± 0.22*
3	4.19 ± 0.21	3.13 ± 0.19*	2.50 ± 0.20*	2.16 ± 0.17*	4.50 ± 0.16*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA. H_2O_2 was used as positive control.

Table 13 Relative $OH^{\bullet}$ level of H23 cells was determined by using HPF probe after treatment with non-toxic concentrations of moscatilin (0-1 µM) for 3 h.

Treatment	Relative $OH^{\bullet}$ level
Control	1.00 ± 0.16
Moscatilin 0.25 µM	0.75 ± 0.15*
Moscatilin 0.5 µM	0.67 ± 0.21*
Moscatilin 1 µM	0.62 ± 0.18*
$FeSO_4$ 50 µM	2.30 ± 0.25*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA. $FeSO_4$ was used as positive control.

Table 14 Relative $O_2^{\bullet-}$ level of H23 cells was determined by using DHE probe after treatment with non-toxic concentrations of moscatilin (0-1 µM) for 3 h.

Treatment	Relative $O_2^{\bullet-}$ level
Control	1.00 ± 0.21
Moscatilin 0.25 µM	0.88 ± 0.19
Moscatilin 0.5 µM	0.86 ± 0.24
Moscatilin 1 µM	0.83 ± 0.18
DMNQ 5 µM	1.80 ± 0.32*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA. DMNQ was used as positive control.

Table 15 Relative H_2O_2 level of H23 cells was determined by using Amplex red probe after treatment with non-toxic concentrations of moscatilin (0-1 µM) for 3 h.

Treatment	Relative ROS level
Control	1.00 ± 0.14
Moscatilin 0.25 µM	0.90 ± 0.16
Moscatilin 0.5 µM	0.87 ± 0.18
Moscatilin 1 µM	0.85 ±0.17
H_2O_2 100 µM	2.12 ± 0.21*

Each value represents mean ± S.D. of three independent experiments. * refers significant difference from non-treated control cells ($P < 0.05$) determined by One-way ANOVA. H_2O_2 was used as positive control.

Table 16 Relative ROS level of H23 cells was determined by using $DCFH_2$-DA probe after pre-treatment with non-toxic concentrations of moscatilin (0-1 µM) prior to $FeSO_4$ for 3 h.

Treatment	Relative ROS level
Control	1.00 ± 0.23
$FeSO_4$ 50 µM	3.60 ± 0.21*
$FeSO_4$ 50 µM+Moscatilin 0.25 µM	2.91 ± 0.23*,#
$FeSO_4$ 50 µM+Moscatilin 0.5 µM	2.72 ± 0.17*,#
$FeSO_4$ 50 µM+Moscatilin 1 µM	2.40 ± 0.18*,#

Each value represents means ± S.D. of three independent experiments. * and # refers significant difference from non-treated control cells and positive control, respectively, ($P < 0.05$) determined by One-way ANOVA. $FeSO_4$ was used as positive control.

Table 17 Relative cell migration of H23 cells was determined by wound healing assay after treatment with moscatilin (1 µM) in the presence or absence of 50 μM of $FeSO_4$ for 24 h.

Treatment	Relative cell migration
Control	1.00 ± 0.18
$FeSO_4$	1.45 ± 0.13*
Moscatilin 1 µM	0.67 ± 0.08*
$FeSO_4$ 50 µM + Moscatilin 1 µM	0.59 ± 0.09#

Each value represents means ± S.D. of three independent experiments. * and # refers significant difference from non-treated control cells and positive control, respectively, ($P < 0.05$) determined by One-way ANOVA. $FeSO_4$ was used as positive control.

Table 18 The percentage of HK2 cell viability was determined by MTT assay after treatment with non-toxic concentrations of moscatilin (0-1 μM) for 24 h.

Moscatilin (μM)	Cell viability (%)
0	100.00 ± 1.25
0.25	94.14 ± 2.41
0.5	93.52 ± 2.59
1	91.30 ± 2.13

Each value represents means ± S.D. of three-independent experiments.

VITA

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