ผลของอวาเบนต่อการหลุดของเซลล์มะเร็งปอดชนิด เอช 23

นางสาวธิดารัตน์ เรืองหิรัญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

EFFECT OF OUABAIN ON THE DETACHMENT OF H23 LUNG CANCER CELL

Miss Thidarat Ruanghirun

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2014

Thesis Title	EFFECT OF OUABAIN ON THE DETACHMENT OF H23 LUNG CANCER CELL
By	Miss Thidarat Ruanghirun
Field of Study	Pharmacology
Thesis Advisor	Associate Professor Pithi Chanvorachote, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Pharmaceutical Sciences
(Assistant Professor Rungpetch Sakulbumrungsil, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Pornpimol Kijsanayotin, Ph.D.)

.....Thesis Advisor
(Associate Professor Pithi Chanvorachote, Ph.D.)

.....Examiner
(Assistant Professor Suree Jianmongkol, Ph.D.)

.....External Examiner
(Associate Professor Chatchai Chinpaisal, Ph.D.)

ธิดารัตน์ เรืองหิรัญ : ผลของอวาเบนต่อการหลุดของเซลล์มะเร็งปอดชนิด เอช 23 (EFFECT OF OUABAIN ON THE DETACHMENT OF H23 LUNG CANCER CELL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร.ปิติ จันทร์วรโชติ{, 77 หน้า.

อวาจัดเป็นสเตียรอยด์ฮอร์โมนชนิดหนึ่ง ที่สามารถสังเคราะห์ขึ้นได้ภายในร่างกายของมนุษย์ และพบว่ามีผลต่อเซลล์มะเร็งหลายชนิด อย่างไรก็ตามผลของมันที่มีต่อการแพร่กระจายของเซลล์มะเร็งยังไม่เป็นที่ทราบแน่ชัด ในการศึกษานี้แสดงให้เห็นว่าอวาเบนในขนาดที่ไม่เป็นพิษต่อเซลล์มะเร็งปอด สามารถกระตุ้นให้เซลล์มะเร็งปอดหลุดออกจากเนื้อเยื่อฐานได้ อวาเบนที่ความเข้มข้น 0-10 พิโคโมลาร์ สามารถกระตุ้นให้เซลล์มะเร็งปอดหลุดออกจากเนื้อเยื่อฐาน โดยขึ้นกับความเข้มข้นและเวลาที่ใช้ ในขณะที่ส่งผลเพียงเล็กน้อยต่อการมีชีวิตรอดของเซลล์ โดยเกิดผ่านการกระตุ้น focal-adhesion kinase (FAK) และ ATP-dependent tyrosine kinase (Akt) นอกจากนี้ยังพบว่าอวาเบนมีผลลดระดับโปรตีน Alpha-5 และ beta-1 integrins ซึ่งการหลุดของเซลล์มะเร็งนั้นนับว่าเป็นขั้นตอนเริ่มต้นที่มีความจำเป็นในการแพร่กระจายของมะเร็ง โดยประโยชน์ในการศึกษาครั้งนี้ทำให้เราได้มีความเข้าใจถึงพื้นฐานชีววิทยาระดับโมเลกุลของมะเร็งเพิ่มมากขึ้น

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5476237833 : MAJOR PHARMACOLOGY

KEYWORDS: OUABAIN / CANCER METASTASIS / CELL DETACHMENT / LUNG CANCER

THIDARAT RUANGHIRUN: EFFECT OF OUABAIN ON THE DETACHMENT OF H23 LUNG CANCER CELL. ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D.{, 77 pp.

A human steroid hormone, ouabain, has been shown to play a role in several types of cancer cell behavior; however, its effects on cancer metastasis are largely unknown. Here, we demonstrate in human lung cancer cells that sub-toxic concentrations of ouabain facilitate cancer cell detachment from the extracellular matrix. Ouabain at concentrations of 0-10 pM significantly enhances cell detachment in dose- and time- dependent manners, while having minimal effect on cell viability. The detachment-inducing effect of ouabain was found to be mediated through focal-adhesion kinase and ATP-dependent tyrosine kinase pathways. Alpha-5 and Beta-1 integrins were found to be down-regulated in response to ouabain treatment. Since detachment of cancer cells is a prerequisite process for metastasis to begin, these insights benefit our understanding of the molecular basis of cancer biology.

Department:	Pharmacology and Physiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmac□logy	
Academic Year:	2014	

ACKNOWLEDGEMENTS

Firstly, this research project would not be accomplished without the help of my great advisor, Associate Professor Pithi Chanvorachote. I cannot thank enough for his valuable insight and input in helping me complete this research project.

I would like to sincerely thank my colleagues in this research unit, Dr. Chatchai Choatham, Miss Thitita Unahabnokha, Mrs. Piyaparisorn Wongvaranon, Miss Nuttida Yongsanguanchai and Miss Sopanya Charoenrungruang for their kind supports and assistances. I also deeply appreciate Dr. Preedakorn Chunhacha and Miss Kanuengnit Choochuay for their kind advices and great encouragement. Another indispensable person I wish to thank is Dr. Varisa Pongrakhananon for her assistance in checking my grammars and thesis writing.

In addition, I would like to express my sincerely thank to all the professors in the Department of Pharmacology and Physiology, Chulalongkorn University for their knowledgeable advices and suggestions.

This study was supported by The 90th Aniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University. I am thankful that I was selected to receive this fund to carry out this research study.

Finally, I am very grateful to my parents, Mr. Surapol and Mrs. Suda Ruanghirun, for all of their understanding and for their supports throughout my life. I am forever indebted to them.

CONTENTS

Page

Page

LIST OF TABLE

LIST OF FIGURES

LIST OF ABBERVIATIONS

%	=	percentage
℃	=	degree Celsius
µg	=	microgram
µl	=	microliter
µM	=	micromolar
α	=	alpha
β	=	beta
Akt	=	adenosine triphosphate dependent tyrosine kinase
ANOVA	=	analysis of variance
BCA	=	bicinchoninic acid
CMA	=	concanamycin A
DMEM	=	Dulbecco's Minimal Essential Medium
ECM	=	Extracellular matrix
et al.	=	et alii, and other people
FAK	=	focal adhesion kinase
FAs	=	focal adhesion
FAT	=	focal adhesion targeting
FBS	=	fetal bovine serum

FERM	=	four-point-one, ezrin, radixin, moesin
Grb2	=	growth factor receptor-bound protein 2
h	=	hour
min	=	minute
ml	=	milliliter
mM	=	milimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTs	=	microtubule
nM	=	nanomolar
NSCLC	=	non-small cell lung cancer
OB	=	Ouabain
p-AKT	=	phosphorylated AKT
PBS	=	phosphate buffer saline
PDGF	=	platelet-derived growth factor
p-FAK	=	phosphorylated FAK
pH	=	potential of hydrogen ion
PI	=	propidium iodide
PI3-kinases	=	phosphatidyl-inositol 3-kinases

pM	=	picomolar
RPMI	=	Roswell Park Memorial Institute
SCLC	=	small cell lung cancer
S.D.	=	standard deviation
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	=	serine
TBS-T	=	tris-buffered saline, tween 20
Tyr	=	tyrosine

CHAPTER I

INTRODUCTION

Despite advances in chemotherapy, as well as novel strategies to overcome lung cancer, this type of cancer remains a leading cause of death (1). The majority of deaths of patients with lung cancer are due to metastasis, a process by which cancer cells spread from their origin to other parts of the patient's body (2-5). In order to elucidate molecular targets for the development of novel therapies, insights into lung cancer cells biology regarding metastasis are very important. Cancer metastasis consists of several key components (6, 7). While other steps of metastasis such as anoikis, migration, invasion, and cancer cell adhesion to the endothelial surface have been intensively studied, the molecular basis of cancer cell detachment from the original tumor is largely unknown.

This early dissemination of cancer cells from their extracellular matrix (ECM) was shown to be critical to successful metastasis. and such a process occurs *via* the cascades of focal adhesion dissociation (8-11). Even though the mechanisms of cancer cell detachment are not yet defined, evidence indicates that focal adhesion kinase (FAK) and ATP-dependent tyrosine kinase (Akt) play important roles in both cell detachment as well as cell motility (12, 13). Activation of FAK or Akt was shown to induce focal adhesion disassembly in many cell models (14, 15). Furthermore, integrins, proteins linking focal adhesion complex with components of the ECM were

shown to be critical for proper cell adhesion. The focal adhesion sites are major regulated by FAK resulting in integrin internalization and FAs turn over. Among various types of integrins, α5 and β1 integrins have garnered predominant attention in cancer research since their activity was shown to be important for adhesion in several types of cancer (16-19). Moreover, several evidences indicate that E-cadherin, the major protein plays a key role on cell-cell contact usually lose or down-regulated during cell dissemination processes (20-22).

Ouabain, an endogenous substance that has been identified as a human hormone, has gained increasing attention in cancer research (23-26). Ouabain was reported to found in plasma in concentrations ranging from 2-770 pM (27) and was shown to sensitize cancer cells to death induced by TNF-related apoptosis-inducing ligand (TRAIL) (28). Because of cancer cells in the patient's body are likely exposed to ouabain and there is no report indicating effects of ouabain in regulation of cancer cells detachment, the present study aimed to elucidate the possible regulatory role of the substance. The knowledge gained from this study may lead to a better understanding of cancer biology and may help in the search for new molecular targets for novel anticancer strategies.

Research Questions

1. Does ouabain at physiological levels has an effect on H23 non-small cell lung cancer cells detachment?
2. What is the underlying cellular mechanism on the effect of ouabain at physiological levels on H23 non-small cell lung cancer cells detachment?

Objectives

1. To investigate the effect of ouabain on H23 non-small cell lung cancer cells detachment.
2. To study the underlying mechanism of ouabain on H23 non-small cell lung cancer cells detachment.

Hypothesis

Ouabain can trigger H23 non-small cell lung cancer cells detachment by inducing alteration in adhesion proteins.

CHAPTER II

LITERATURE REVIEW

Lung cancer

Lung cancer is one of the most aggressive cancer, with approximately more than 1.6 million new cases diagnosed each year and the major death of this cancer is due to colonization of cancer cells in distant organs usually in brain, lymph nodes and bones (1-5). The most common cause of lung cancer is long term cigarette smoking which accounts for 90% of the patients. The other causes are due to several factors including genetic factors, radon gas, asbestos, and air pollution (29, 30). The main types of lung cancer are divided into two main type small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which are classified by their biological and treatment methods. Moreover, NSCLS also subdivided into squamous cell (epidermoid) carcinoma, adenocarcinoma and large cell (undifferentiated) carcinoma (31).

Small cell lung cancer (SCLC) is found in approximately 10-15% of the patients. The major cause of this type is correlated strongly with cigarette smoking. In This type, cancer cell grows rapidly and usually metastasize when diagnosed. Most of the patients die within only 2 to 4 months from diagnosis if they do not receive proper treatment (32). The treatment is not a surgical procedure but this type of

cancer responds very well to chemotherapy when compared to the other type, Non-small cell lung cancer (NSCLC) (33).

Non-small cell lung cancer (NSCLC) is found in 85-90% of all lung cancers and is commonly diagnosed at an advanced stage. Surgery may be the treatment of choice for an early stage of NSCLC (34). However, even with the surgical treatment the recurrence of this type of cancer still found in some patients. It was found that most of the relapsed patients died from metastasis at distant organs. The relapse may happen before surgery or after surgery indicating that NSCLC is often found as a systemic disease at the time of diagnosis (35).

Metastasis

Cancer metastasis is the characteristic that cancer cells break away from their primary sites and travel to other parts of the body and establish new tumor at new sites (9, 36). Lung cancer is more likely to spread than other types of cancer. The sites that lung cancer cells usually metastasis is brain, lymph node and bone (37). The metastasis of cancer is considering the leading cause of death in patients. Lung cancer patients whose have metastatic lesion have an average 5-year survival rate less than 15% (38).

The metastasis cascades can be divided into two main processes. The first process is involved in the physical translocation of cancer cells from the original sites to other sites. The second process is encompassed with an ability of cancer cells to develop and adapt into the new environment (6).

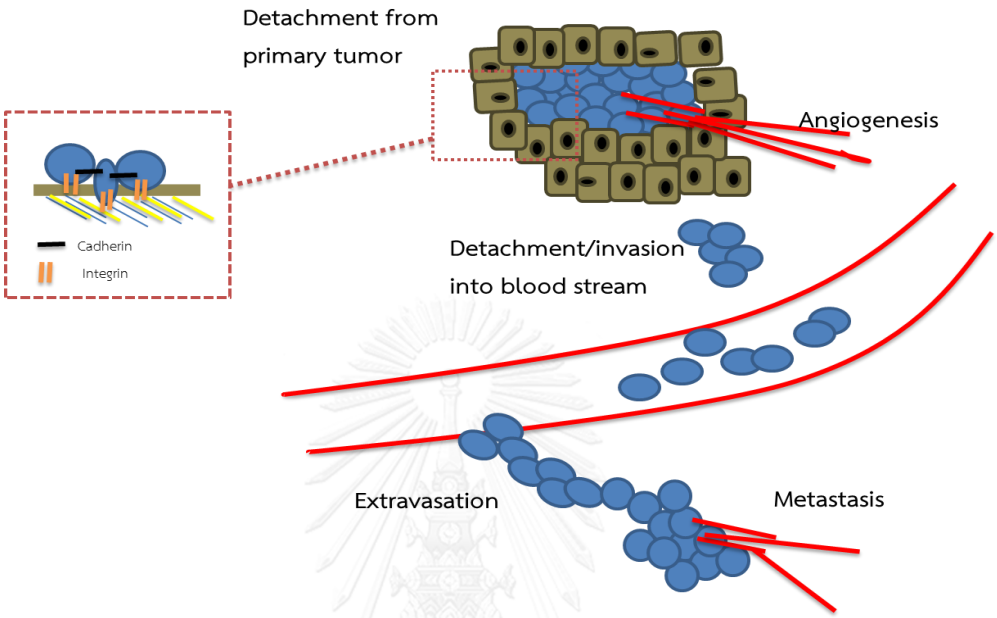


Figure 1 The metastasis cascade

Cancer cells metastasis usually involves the following steps:

- Detachment: cancer cells must detach from their original tumor site (7).
- Local invasion: cancer cells can invade the tissue around the initial lesion and penetrate through their basement membrane (7).
- Intravasation: cancer cells must entry into the blood or lymph vessel (7).
- Circulation: cancer cells must survive in the circulation or lymphatic system and move through other parts of the body (7).
- Arrest and extravasation: the adhesion of cancer cells to capillary wall happen at

the target site. Then, cancer cells can invade through the capillary wall and migrate into the surrounding tissue (7).

- Proliferation: cancer cells colonize and divide at the target site to form new tumor (7).
- Angiogenesis: the formation of new blood vessels to supply oxygen and nutrients that necessary for growth and survival for new tumor (7).

Detachment of cancer cell

In the metastasis cascades, it was indicated that the detachment of cancer cells from their parent tumor is an initial step in metastasis (6, 7, 9). The dissemination of cancer cells from their extracellular matrix (ECM) was shown to be critical to a successful metastasis (30, 39). An adhesion molecule named integrin plays a key role in cell-ECM adhesion. Integrin is a family of transmembrane receptor that links the intracellular actin-cytoskeleton to their ECM. The area of cell-ECM adhesion is known as focal adhesion (FAs) contacts (40). When cancer cells detach for metastasis, these FAs must be destabilized (41). E-cadherin is a protein that plays a critical role in cell adhesion contact and many studies indicate that during metastasis, cancer cells always loss of E-cadherin expression (20, 21).

Because of the detachment of cancer cells from their ECM is a prerequisite to a successful metastasis. Therefore, it is necessary to study proteins that regulate the detachment processes. In this review we focus on four proteins that play pivotal

roles in cancer cell detachment including integrin, focal adhesion kinase (FAK), ATP dependent tyrosine kinase (Akt) and E-cadherin.

Integrin

Integrin is major cell surface adhesion receptors for ECM. These cell-ECM adhesion areas are known as focal adhesion (FAs) contacts (40). Integrin plays crucial roles in cell survival and tissue development. Integrin activation leads to critical cellular responses including gene expression, regulation of cell survival processes including differentiation and proliferation (40, 42). Apart from their physiological roles, integrin is also involved in many pathological conditions such as inflammation and tumor progression (43).

Integrin structure is a large heterodimer glycoprotein, consisting of two alpha –α and beta –β subunits that link together with non-covalent bonding (40). In mammals, 18 α-subunits and 8 β-subunits have been characterized to combine into 24 cell surface integrin receptors. Each integrin comprises of three domains, cytoplasmic portion, single transmembrane portion and extracellular portion (40). The adhesion to ECM in each type of integrin is defined by α-subunits while the cytoplasmic tail of β-subunits binds to adaptor proteins that connect to the actin filaments inside the cell (40).

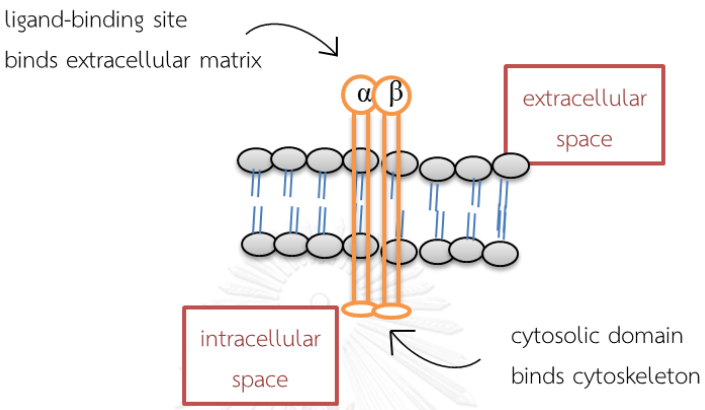


Figure 2 The structure of integrin

Among various types of integrin, α5 and β1 integrins have garnered predominant attentions in cancer research area because of their important roles in cancer progression in multiple types of cancer including in lung cancer (44, 45). Moreover, α5 and β1 integrins demonstrate the predominant role in cell-ECM adhesion (16-19). Studies in several cancers including lung cancer indicate that loss of β1 integrins expression weakens the ability to adhere to ECM and promote cancer cell motility (46).

Integrin and focal adhesion turnover

The invasive property of cancer cells is known to involve with the loss of cell adhesion to their original sites, the expression of motile phenotypes and also the detachment into blood circulation to travel to other sites of the body (7, 8). Many literatures show that before cancer cells detach from their ECM, the focal contacts must be disrupted in termed of focal adhesion (FAs) turnover (10, 11).

It was found that internalization of integrin can facilitates the focal adhesion turnover (47). The internalization of integrin is major regulated by focal adhesion protein (FAK) (14). FAK plays a major role in FAs turnover which will be discussed in detail later.

The process of integrin internalization occurs after FAK activation. Then integrin is internalized into plasma membrane and rapidly trafficked to early endosome. After that, they are sorted to recycle or degrade in lysosome, but most of the internalized integrin is recycled back to plasma membrane. The result of this event leads to cancer cells detaching from their ECM and travelling into blood circulation (48, 49).

Focal adhesion kinase (FAK)

Focal adhesion kinase is an intracellular protein tyrosine kinase which is recruited at FAs where integrin binds to ECM. The structure of FAK is composed of three domains; a focal adhesion targeting (FAT), a kinase domain and a FERM domain. FAT domain mediates colocalozation of FAK to FAs (50). FAK is an important protein that regulates diverse cellular processes such as survival, proliferation and migration. Numerous studies indicated that deregulation of FAK is the key event in the development and progression of cancer. It has been reported that an increase in expression and/or activation of FAK in cancer are strongly associated with invasive and aggressive phenotypes (51, 52).

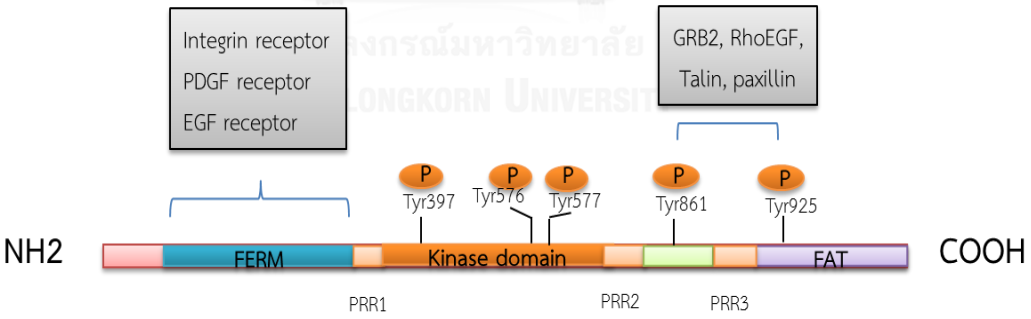


Figure 3 The structure of FAK

FAK and focal adhesion (FAs) turnover

Cumulative evidence indicates that an activation of FAK is the crucial step for FAs turnover. FAK was autophosphorylated at Tyr397 and subsequently phosphorylated at multiple tyrosine residues within FAK. Phosphorylation at Tyr925 is essential for the interaction of FAK with Grb2. the binding of Grb2 to FAK recruits dynamin (microtubules binding protein) to FAs. The extension of microtubules to the FAs is a key process that facilitates integrin internalization. Thus, the activation of FAK at tyr397 is the key to regulate integrin internalization and subsequently FAs turnover (10).

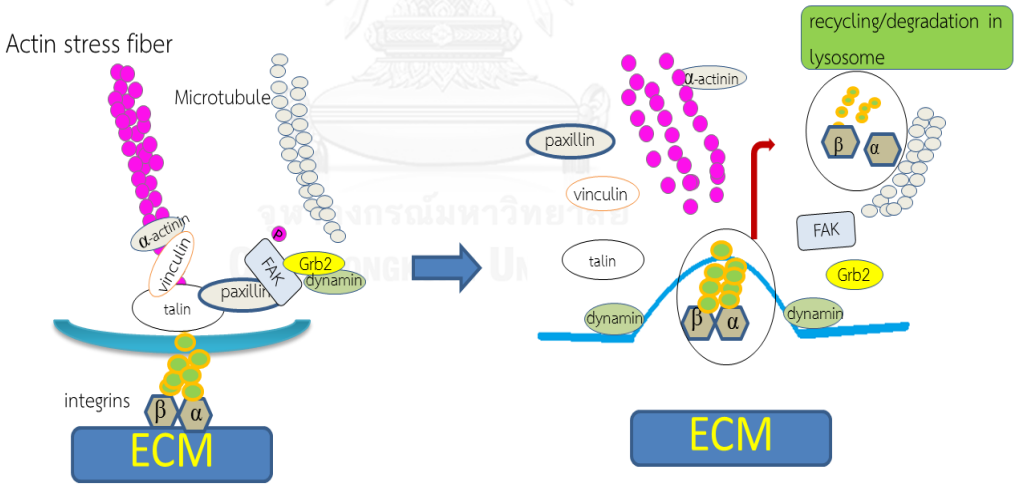


Figure 4 FAK regulates focal adhesion turnover

ATP dependent tyrosine kinase (Akt)

ATP dependent tyrosine kinase or Akt is a serine/threonine-specific protein kinase which is activated by signaling through phosphatidyl-inositol 3-kinases (PI3-kinases) (53). The activation of Akt by phosphorylation at serine/threonine residues regulates multiple cellular responses such as cell survival, gene expression, proliferation and differentiation (54). In cancers, Akt plays crucial roles to promote tumor growth, survival, migration and invasion (55). Moreover, Akt has been shown to activate transcription factor, Snail, which is known to repress of the E-cadherin gene. This repression of E-cadherin can cause cell-cell dissociation (13). Thus, an increase in activation level of Akt is strongly correlated with motile phenotype and invasiveness of cancer cells. A recent study of Higuchi and colleagues in 2013 demonstrate an overexpression of active Akt in fibroblasts increased autophosphorylation of FAK at Tyr397 and resulting in FAs turnover. In addition, this study found that Akt knockdown resulting in prevention of FAs turnover (15).

E-cadherin

E-cadherin is expressed by most of normal epithelial tissues and plays a key role in cell-cell adhesion. The structure of E-cadherin is composed of a large extracellular domain, a transmembrane domain and a cytoplasmic domain. The extracellular domain mediates cell–cell contact in the presence of calcium while the cytoplasmic domain interacts with the actin cytoskeleton within the cell (56).

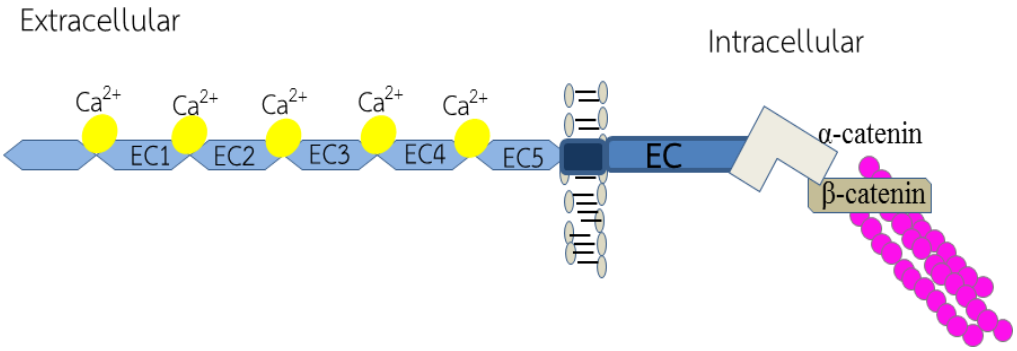


Figure 5 The structure of E-cadherin

E-cadherin has pivotal roles in epithelial cell behavior, tissue formation, and suppression of cancer (57). In cancer cells, loss of E-cadherin promotes metastasis (58). Importantly, clinical studies have shown that patients who lost of E-cadherin expression had the poor overall survival (59-61).

Ouabain

Ouabain, is a member of cardiac glycoside isolated from the ripe seeds of African plants *Strophanthus gratus* and the bark of *Acokanthera ouabaio* (62). In 1991 endogenous ouabain-like compound which was identical to the plant-derived ouabain was found in the human plasma (63). There are compelling evidences indicate that ouabain is synthesized in the adrenal gland (64) and hypothalamus (65). It is still unclear about physiological role of endogenous ouabain, but it was found that the endogenous ouabain is upregulated in the plasma in response to sodium depletion. Moreover, many studies indicate that endogenous ouabain may play a

role in causing hypertension and cardiac hypertrophy (66). Plasma concentrations of endogenous ouabain seem to vary in the range of 2-770 pM whereas, the average concentration of endogenous ouabain in normal condition was found at 30 pM (27, 67). An elevated concentration of endogenous ouabain was found under different conditions such as congestive heart failure, essential hypertension and renal failure (68).

At the present, ouabain and its related cardiotonic steroids have garnered an increasing attention in cancer research. A growing body of evidence indicates that ouabain has an anti-cancer activity against many cancers. For example treatment of ouabain results in cell cycle inhibitor and growth arrest in breast cancer cell (69). Furthermore, the study of Huang and colleagues indicated that ouabain induced a significant loss of mitochondrial membrane potential and subsequently caused a sustained reactive oxygen species production and a severe apoptosis in human prostate cancer cell (24).

Accumulative evidence indicates that treatment with ouabain at high concentration resulting in cell detachment and death (70, 71). In the recent study in 2014 of Heredia and colleagues demonstrate that ouabain treatment in epithelial renal canine cells (MDCK) at 300 nM induced tight junction (TJs) disassembly through internalization and degradation of the TJs protein (72). These data leads to a question whether ouabain at physiological concentrations can regulate the

detachment of cancer cells from their sites of adhesion because of the detachment of cancer cells is the first requirement in cancer metastasis. Therefore, this study aims to investigate the effect of ouabain at its physiological concentrations in the regulation of cancer cells detachment and hopefully the results from this study may fulfill the understanding of endogenous ouabain in cancer biology.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and Reagents

Ouabain, concanamycin A (CMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342 and propidium iodide (PI) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Matrigel was obtained from Becton Dickinson, Inc. (Mississauga, Ontario, Canada). Rabbit monoclonal antibody to integrin, p-FAK, FAK, p-Akt and Akt were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2. Equipment

Automated cell counter (TC ^{10TM} ***Bio-Rad Laboratories***, Inc., Hercules, CA, USA), autopipette: 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl and pH meter CG 842 (Schott, Elmsford, NY,USA), cell culture plate: 6-well and 96-well, carbon dioxide incubator, conical tube: 15 ml and 50 ml and pipette tips for 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl (Corning Inc., MA, USA), Centrifuge (Z 383, Hermle labortechnik Gmbh, Weihngen, Germany), fluorescence microscope (Olympus IX51 with DP70, Olypus America Inc., Center valley, PA, USA), microplate reader (Wallac 1420, Perki Elmer Inc., MA, USA), Laminar flow cabinet (Bosstech scientific instruments, USA).

Methods

1. Cell culture

Human lung adenocarcinoma H23 cell was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H23 cell was cultured in RPMI-1640 medium. The medium was supplement with 10% fetal bovine serum, 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco, MD, USA) in 37 °C with 5% CO_2. H23 cells were subculture when they reach 80-90% confluence.

2. Cell viability assay

Cell viability was evaluated using MTT assay. Briefly, H23 cells were seeded at a density of 10^4 cells/well in 96-well plate, incubated for 12 h. After that, they were treated with different concentrations of ouabain for 12 h. Cell viability was determined by incubation with MTT solution (5.0 mg/ml in **phosphate-buffered** saline (PBS)) and incubated at 37°C for 4 h. The optical density was then determined by a microplate reader (Anthros, Durham, NC, USA) at 570 nM, and the percentage of viable cells was calculated relative to control cells as follow equation.

$$\text{Cell viability (\%)} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

3. Nuclear staining assay

Apoptotic and necrotic cell death were determined by Hoechst 33342 and PI co-staining. After treated H23 cells with different concentrations of ouabain, cells were incubated with 10 µM of Hoechst 33342 and 5 µg/ml of PI for 30 min at 37°C.

Apoptotic cells with condensed chromatin and/or fragmented nuclei and necrotic cells with PI-positive were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70). The Percentage of apoptotic and necrotic cells were calculated as follow.

$$\text{Apoptotic cell (\%)} = \frac{\text{number of apoptotic cells in treatment}}{\text{number of apoptotic cells in control}} \times 100$$

$$\text{Necrotic cells (\%)} = \frac{\text{number of necrotic cells in treatment}}{\text{number of necrotic cells in control}} \times 100$$

4. Cell-ECM adhesion assay

Cell-ECM adhesion assay was performed as follows. Briefly, matrigel was thawed at 4°C for 12 h, diluted to final concentration at 0.2 μg/ml, spread evenly on the surface of 96-well plates, and incubated at 37°C for 12 h. Cells were treated with non-toxic concentrations of ouabain, then trypsinized to produce single cells and immediately seeded onto matrigel-coated plate. Cells were allowed to adhere to the matrigel for 30 min at 37°C, then the cells were washed three times with PBS. Adherent cells were evaluated by MTT assay and scoring under an inverted microscope (Olympus IX51 with DP70). Percentage of adherent cells was calculated as follow.

$$\text{Cell adhesion (\%)} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

$$\text{Cell count (\%)} = \frac{\text{number of adherent cells to matrigel in treatment}}{\text{number of adherent cells to matrigel in control}} \times 100$$

5. Cell-ECM detachment assay

Suspended cells (5×10^3 cells/well) were seeded on matrigel-coated 96-well plates for 12 h. Cells were then treated with non-toxic concentrations of ouabain for 6 h. After that, cells were dissociated from matrigel by incubated with 0.1% trypsin at 37°C for 8 min. The remaining cells were determined using MTT assay and scored under an inverted microscope (Olympus IX51 with DP70). Percentage of remaining cells was calculated as follow.

$$\text{Remaining cells (\%)} = \frac{OD_{570} \text{ of treatment}}{OD_{570} \text{ of control}} \times 100$$

$$\text{Cell count (\%)} = \frac{\text{number of remaining cells adhesion to matrigel in treatment}}{\text{number of remaining cells adhesion to matrigel in control}} \times 100$$

6. Western blot analysis

After specific treatments, cells were washed twice with cold PBS and incubated with lysis buffer containing 20 mM TrisHCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 100 mM phenylmethyl sulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 40 min on ice, collected whole cell lysates and centrifuged at 12,000 rpm at 4°C for 15 min. The supernatants of the whole cell lysates were collected and assayed for protein

content using the BCA protein assay. Loading dye was added to equal amounts of protein from each sample then, denatured by heating at 95℃ for 5 min. The proteins containing loading dye were separated 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After separation, the proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and then, blocked the nonspecific proteins with 5% nonfat dry milk in TBS-T (25 mM TrisHCl pH 7.5, 125 mM sodium chloride and 0.05% Tween 20) for 1 h at room temperature. The membranes were then washed three times with TBS-T for 8 min and incubated with primary antibody overnight. The membranes were then wash three times with TBS-T and then, incubated with secondary anti-rabbit antibody for 2 h. Following the incubation, the proteins were then visualized using an enhanced chemiluminescence detection kit.

For analysis of western blot, β-actin was loaded to confirm equal amount of protein in each sample. Then, the data obtained from measuring intensity of western blot band were dividing by the value of its non-treated control. Therefore, the control in each experiment would have the value equal to 1. The relative protein level was calculated as follow.

$$\text{The relative protein level} = \frac{\text{intensity of band in each treatment}}{\text{intensity of its non-treated control}}$$

7. Statistical analysis

Data were presented as the means ± S.D. from three independent experiments. Statistical analysis was carried out using one-way ANOVA following *post hoc* test for comparing more than two groups and independent sample t-test for comparing between two groups. Determined the statistical significant at $p<0.05$. SPSS 18.0 (SPSS Inc., Chicago, **IL**, USA) was used for all statistical analyses.

Experimental design

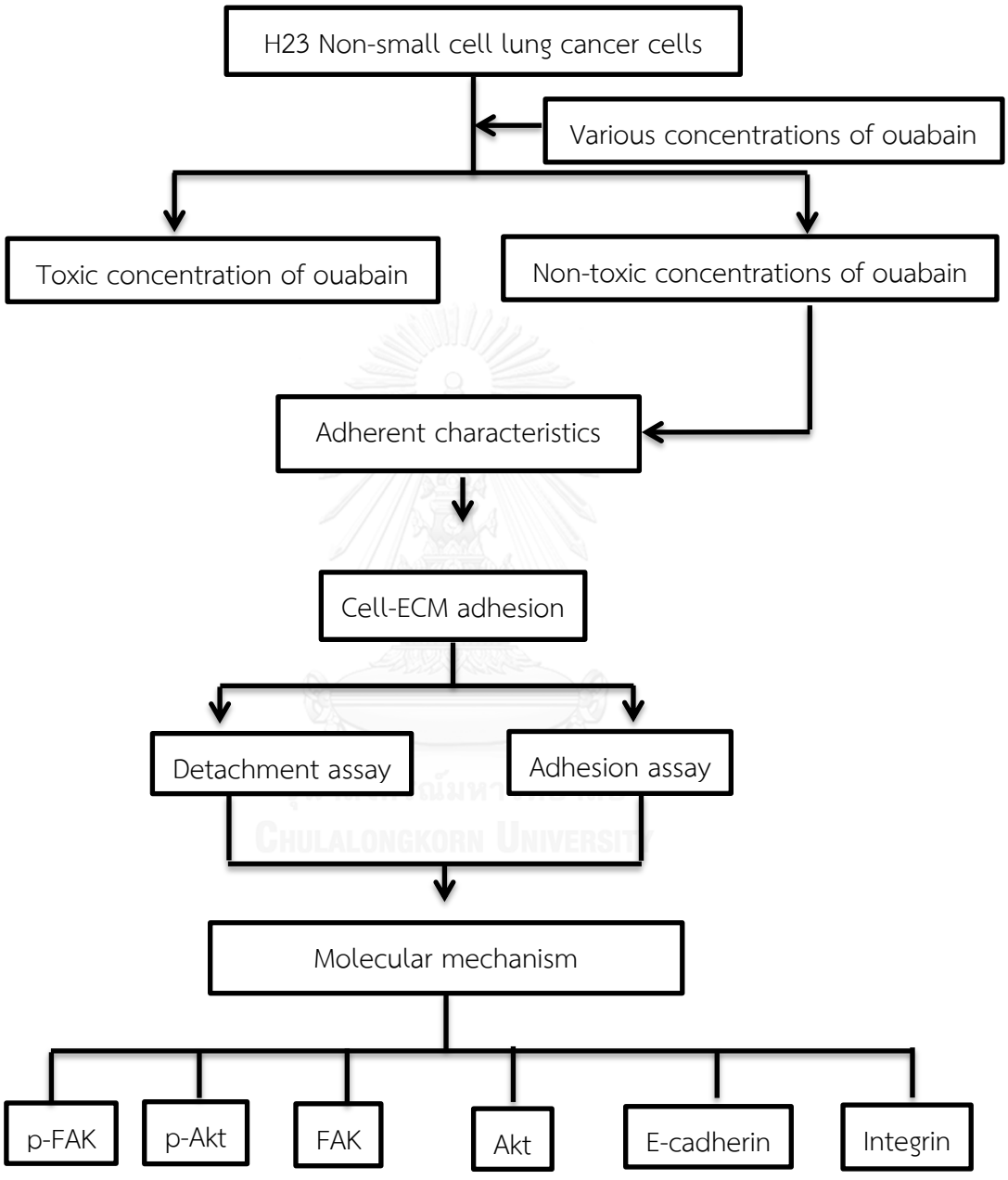


Figure 6 Experimental design of this study

Conceptual framework

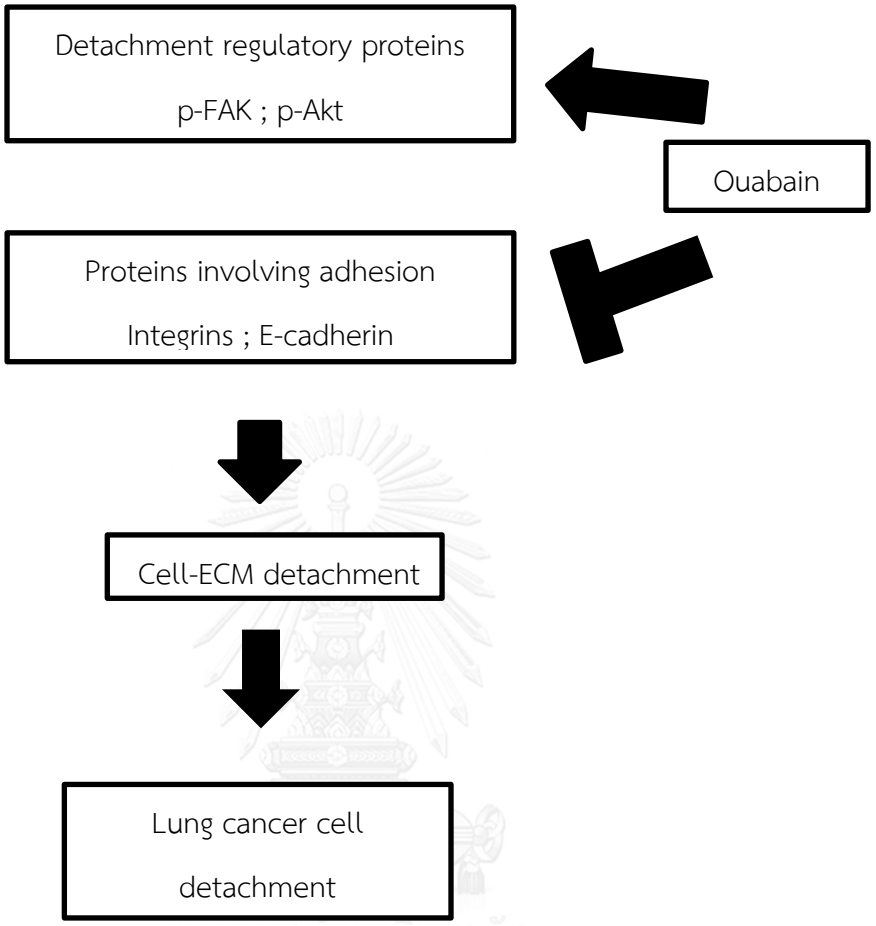


Figure 7 Conceptual framework of this study

Investigation on the cytotoxic effect of ouabain in H23 and HaCat cells.

To evaluate an effect of ouabain on H23 and HaCat cell viability. The cell viability was determined by MTT assay. Non-toxic concentrations of ouabain on H23 cells were used in further experiments.

Investigation the mode of cell death in response to ouabain treatment in H23 cells.

To determine whether of non-toxic concentrations of ouabain cause apoptosis and necrosis on H23 cells. Mode of cell death was evaluated by Hoechst/PI co-staining assay. Then, determined the mode of cell death by observed under fluorescence microscope.

Investigation the effect of ouabain on cell-extracellular matrix (ECM) detachment.

To elucidate the effect of ouabain on lung cancer cell-ECM detachment, H23 cells were treated with non-toxic concentrations of ouabain. The effect of ouabain on cell-ECM detachment was determined by cell-ECM adhesion and cell-ECM detachment assays.

Investigation the effect of ouabain on cell-cell dissociation.

To elucidate the effect of ouabain on cell-cell dissociation. H23 cells were treated with non-toxic concentrations of ouabain for 6 h. Cells were then photograph by inverted microscope to determine cell-cell dissociation.

Investigation on molecular mechanisms that induced by ouabain in H23 non-small cell lung cancer.

To identify the underlying mechanism of ouabain on lung cancer cells detachment. Western blot analysis is performed to evaluate the level of following proteins consist of p-FAK, FAK, p-Akt, Akt, integrins and e-cadherin.

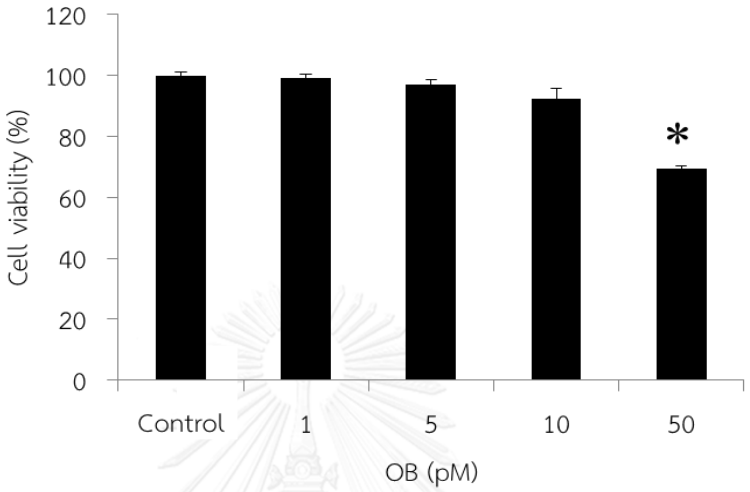
CHAPTER IV

RESULTS

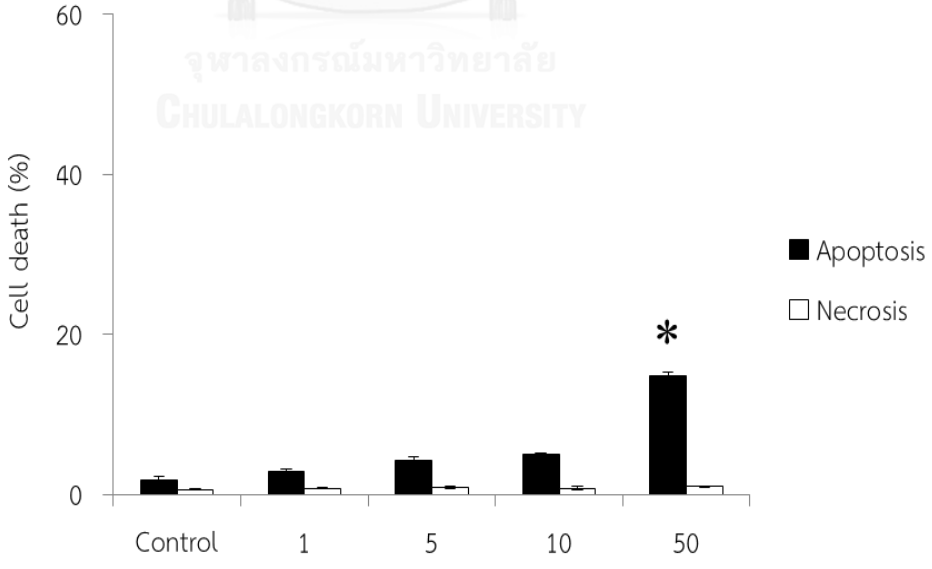
1. Effect of ouabain on the viability of human lung cancer H23 cells.

Firstly, I characterized the effects of ouabain on the survival of human lung cancer H23 cells. Because previous studies have reported the concentrations of ouabain and ouabain-like substances in human plasma to range from 2-770 pM, the concentration range of 0-50 pM of ouabain was selected for use in the present study. H23 cells were cultured in the presence and absence of ouabain (0-50 pM) for 12 h, and cell viability was determined by MTT assay. Figure 7A shows that when H23 cells were treated with ouabain, at concentrations ranging from 0-10 pM, neither cytotoxicity nor proliferative effects were observed. A significant decline in cell viability was detected in the cells treated with 50 pM ouabain, with approximately 70% of the cells remaining viable. Accordingly, the Hoechst33342 and PI staining assay indicated that ouabain at concentrations 0-10 pM did not cause apoptosis or necrosis (Figure 8B and C).

A



B



C

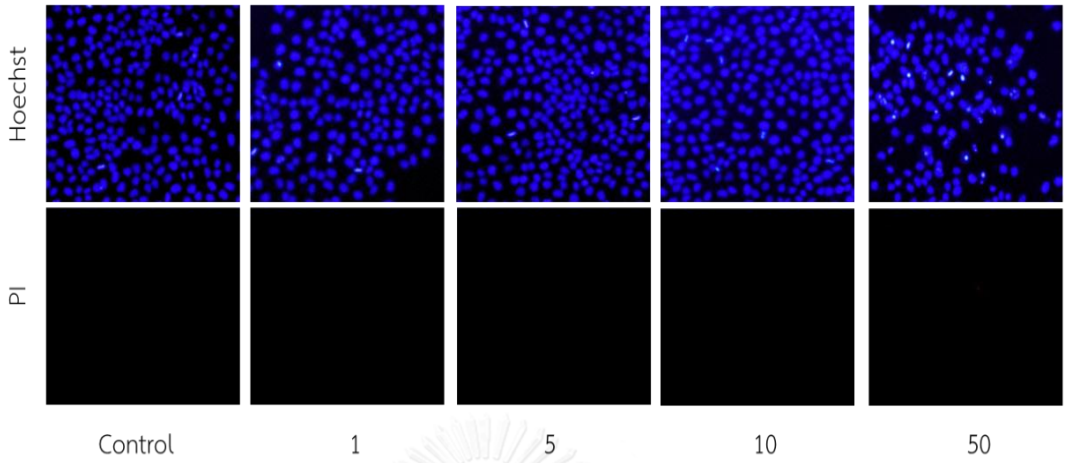


Figure 8 Effect of ouabain on cytotoxicity in lung cancer H23 cells.

A: Effect of ouabain on H23 cell viability. Cells were treated with different concentrations (0-50 pM) of ouabain for 12 h. The cell viability was analyzed using the MTT assay. B: Apoptotic and necrotic cell count from Hoechst33342/PI staining of ouabain-treated in H23 cells for 12 h. C: The pictures of Hoechst33342/PI staining of ouabain-treated in H23 cells for 12 h. The data are the mean ± S.D. (n = 3). * $p<0.05$ *versus* the non-treated control.

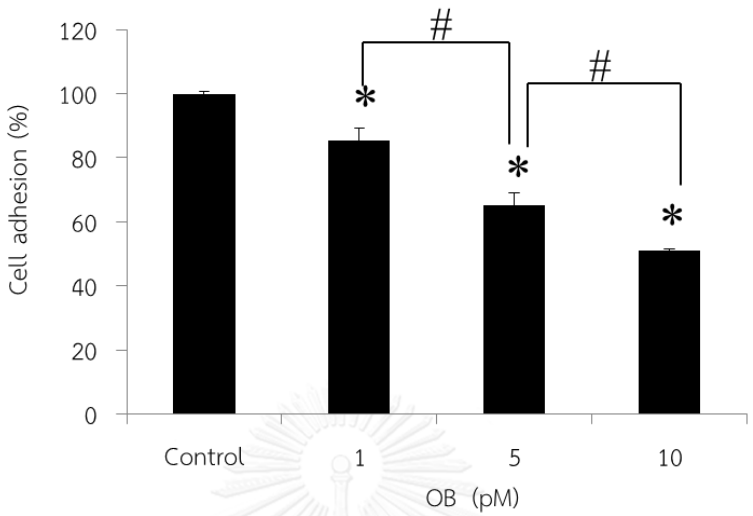
2. Ouabain enhanced lung cancer H23 cells detachment.

To elucidate the effect of ouabain on lung cancer cell detachment, I performed cell-ECM adhesion and detachment assays. For cell-ECM adhesion assay, cells were incubated with ouabain at non-toxic concentration (0-10 pM) for 0-6 h and were subjected to the cell-ECM adhesion assay. After trypsinization, cells were allowed to adhere to the matrigel surface for 30 min and the adhered cells were

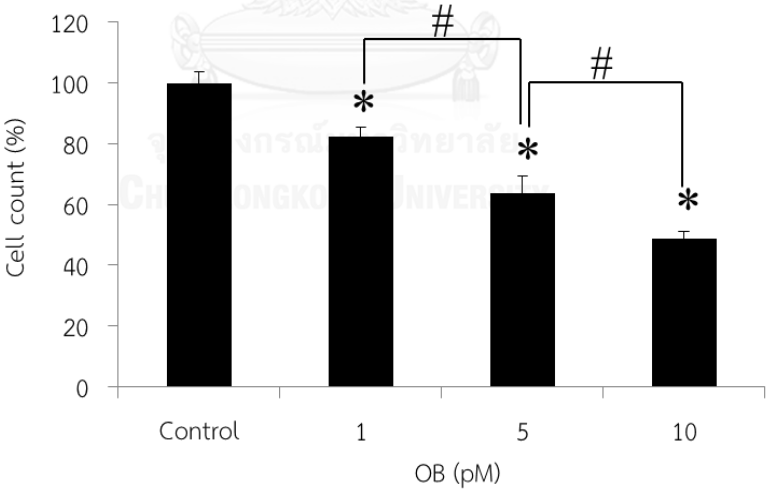
determined by MTT assay and cell counting. Figure 9 shows that treatment with ouabain significantly reduced the number of adhered cells in dose- (Figure 9 A-C) and time-dependent manners (Figure 9 D-F). Treatment with ouabain at 5 and 10 pM reduced the adhesion of cells to approximately 65% and 50%, respectively (Figure 9 A and B).

Furthermore, I elucidated the effect of ouabain on cancer cell detachment using cell detachment assay. Cells were sparsely seeded in order to avoid cell-cell contact. After 12 h incubation, the cells were incubated with 0-10 pM ouabain for 6 h and were dissociated from matrigel surfaces by incubating with 0.1% trypsin at 37°C for 5-10 min. The percentage of remaining cells to total cells was assessed by MTT assay and cells scored under an inverted microscope. Figure 10 shows that treatment with ouabain significantly enhanced the detachment of the cells in dose-dependent manner. Together, these results suggest that ouabain at physiological concentration weakened lung cancer cell adhesion to ECM surface and facilitate detachment.

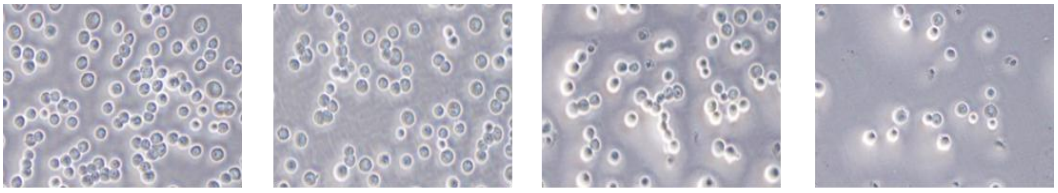
A


Cell adhesion (%)
120
100
80
60
40
20
0
#
#
*
*
*
Control
1
5
10
OB (pM)

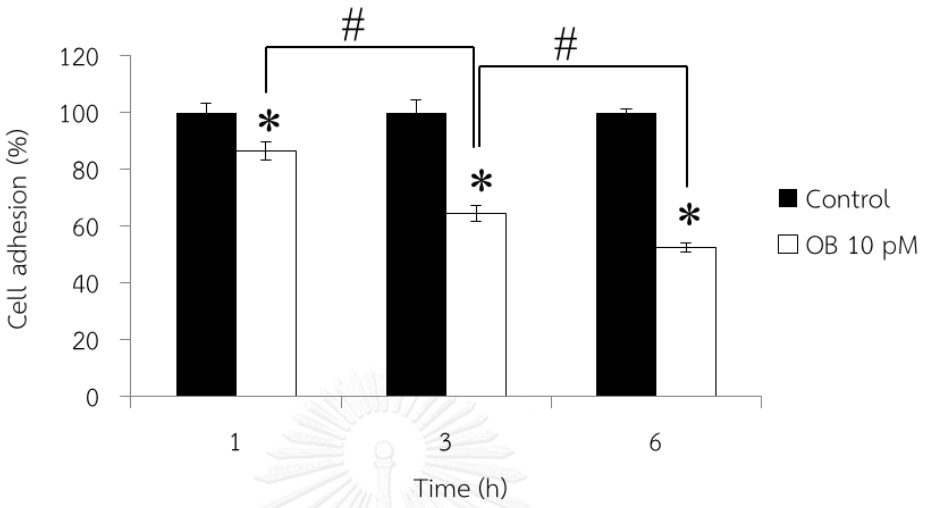
B


Cell count (%)
120
100
80
60
40
20
0
#
#
*
*
*
Control
1
5
10
OB (pM)

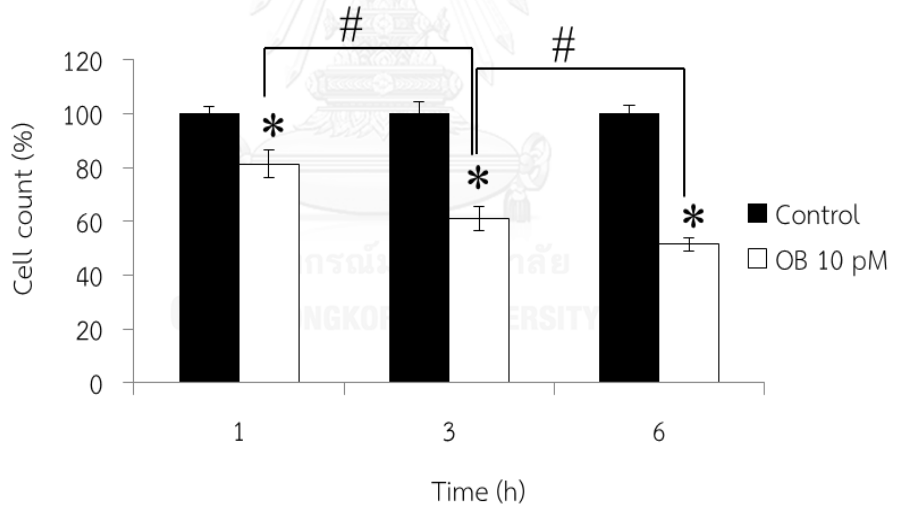
C


Control
OB 1 pM
OB 5 pM
OB 10 pM

D


#
#
120
100
80
60
40
20
0
Cell adhesion (%)
*
*
*
Control
OB 10 pM
1
3
6
Time (h)

E


#
#
120
100
80
60
40
20
0
Cell count (%)
*
*
*
Control
OB 10 pM
1
3
6
Time (h)

F

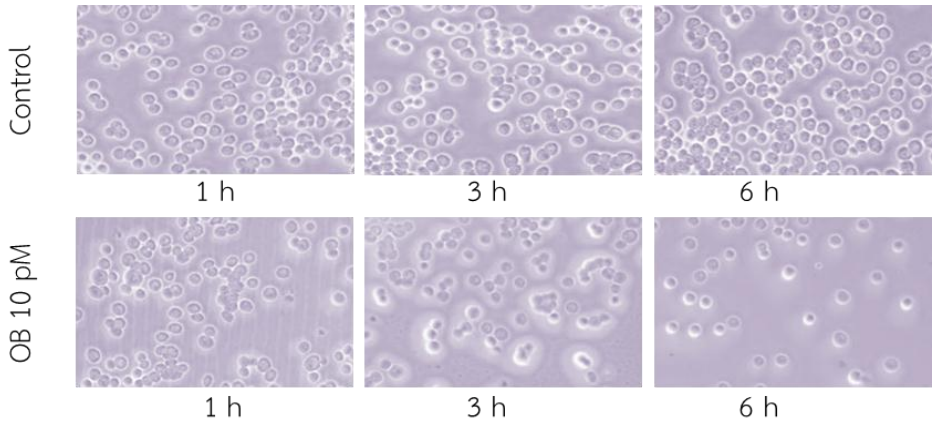
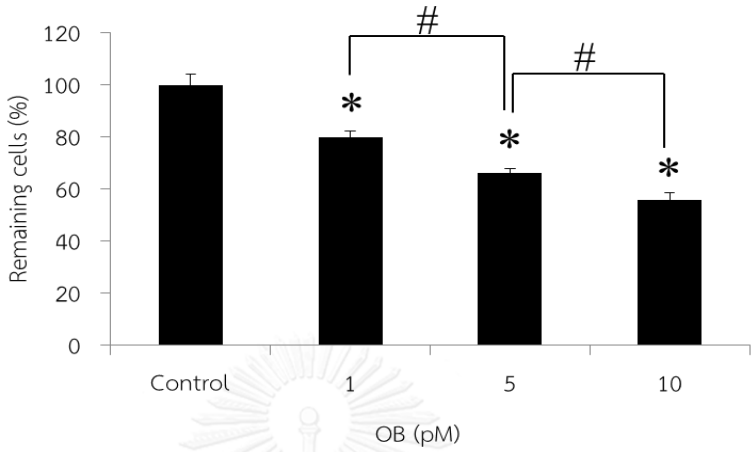

Control
OB 10 pM
1 h
3 h
6 h
1 h
3 h
6 h

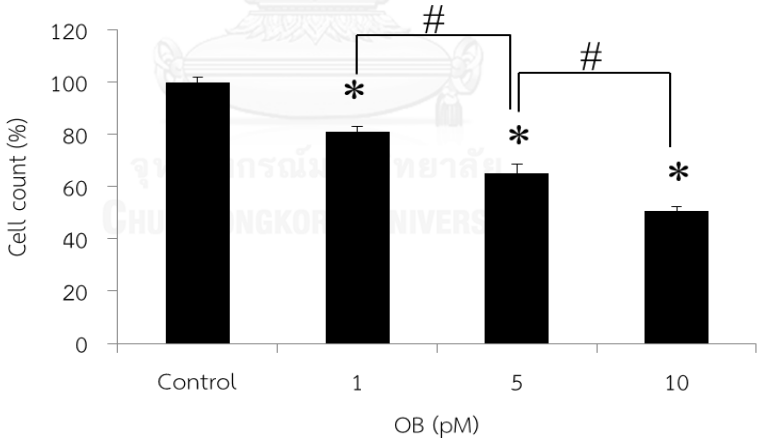
Figure 9 Effect of ouabain on H23 cell adhesion.

H23 cells were exposed to ouabain at different concentrations for 6 h and subjected to cell-extracellular matrix (ECM) adhesion assay. Cells were allowed to adhere to the matrigel surface for 30 min and the adhered cells were determined by MTT assay (A) and cell counting (B). C: Phase-contrast images of adhered cells. For time-dependent experiments, cells were treated with 10 pM ouabain for 1-6 h and subjected to the assay. Cell adhesion was evaluated by MTT assay (D) and cell counting (E). F: Phase-contrast images of adhered cells. The data are the mean ± S.D. (n = 3). (*) Represent the significant different from the non-treated control , (#) represent the significant different from each treatment. Determined the statistical significant at $p<0.05$.

A


Remaining cells (%)
120
100
80
60
40
20
0
#
#
*
*
*
Control
1
5
10
OB (pM)

B


Cell count (%)
120
100
80
60
40
20
0
#
#
*
*
*
Control
1
5
10
OB (pM)

C

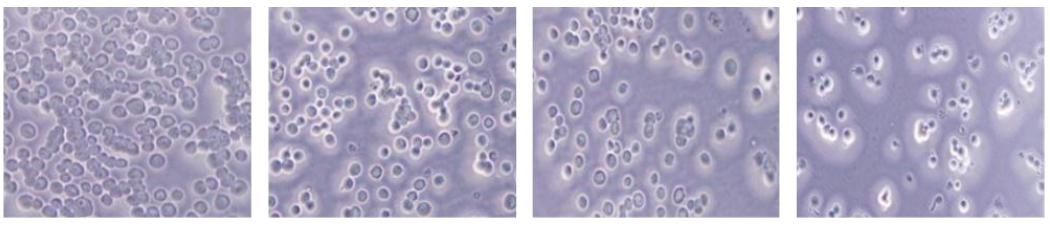

Control
OB 1 pM
OB 5 pM
OB 10 pM

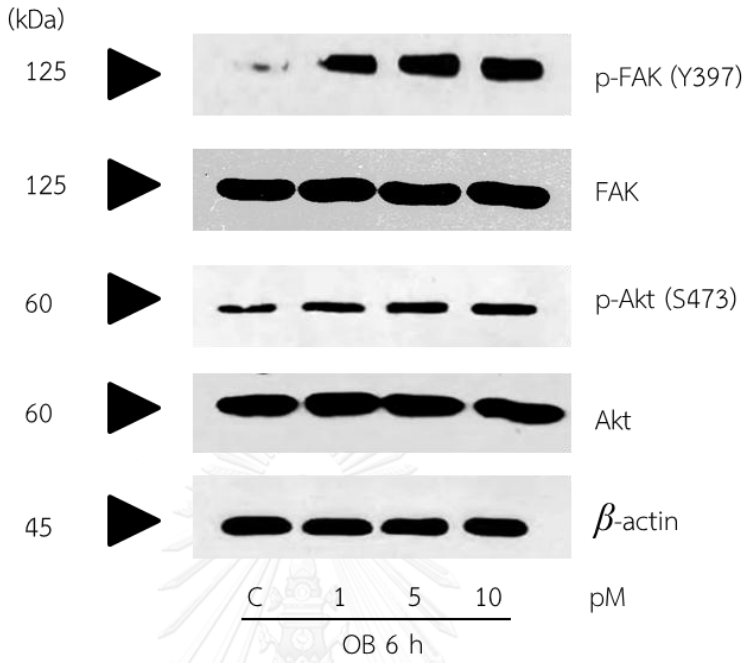
Figure 10 Effect of ouabain on H23 cell detachment.

H23 cells were incubated with 0-10 pM of ouabain (OB) for 6 h and were dissociated from matrigel surfaces by incubated with 0.1% trypsin at 37°C for 5-10 min. The percentage of remained cells to total cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (A) and cell scoring (B) under an inverted microscope. C: Phase-contrast images of remaining cells. The data are the mean ± S.D. (n = 3). (*) Represent the significant different from the non-treated control , (#) represent the significant different from each treatment. Determined the statistical significant at $p<0.05$.

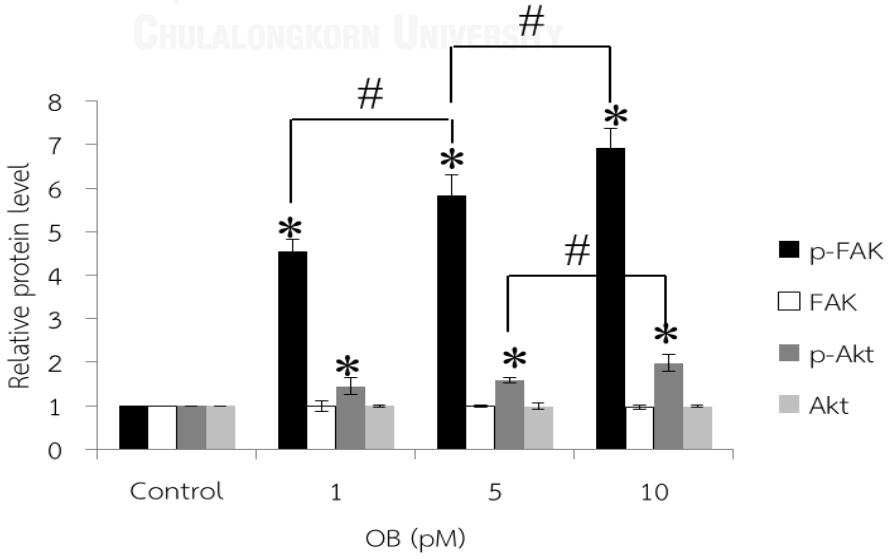
3. Ouabain induces FAK and Akt activation.

Having demonstrated the potentiating effect of ouabain on lung cancer cell detachment, I next examined the underlying mechanism, focusing on the expression level and activation of the proteins known to play roles in cancer cell-ECM detachment. H23 cells were treated with non-toxic concentrations of ouabain and expression of FAK, activated FAK (phosphorylation at Tyr 397), Akt and activated Akt (phosphorylation at Ser 473) were evaluated by western blot analysis. Figure 11 indicates that treatment with ouabain significantly increased the levels of phosphorylated FAK (Tyr 397) and phosphorylated Akt (Ser 473), whereas ouabain exposure had no significant effect on the levels of total FAK and total Akt. For dose-dependent assessment, cells were treated with 0, 1, 5 and 10 pM of ouabain for 6 h and expressions level of these proteins was evaluated. Figure 11 A and B indicates that treatment with ouabain increased the activation of FAK and Akt when compared to the non-treated control. Moreover, this result shows that the activation of FAK was dose-dependent manner. For time-dependent assessment, cells were treated with 10 pM of ouabain for 0-6 h, and the level of proteins was detected by western blotting. Figure 11 C and D show that an increase of ouabain exposure caused an increase in activation of FAK and Akt in time-dependent manner. These results indicate the involvement of FAK and Akt in facilitating the detachment of H23 lung cancer cells.

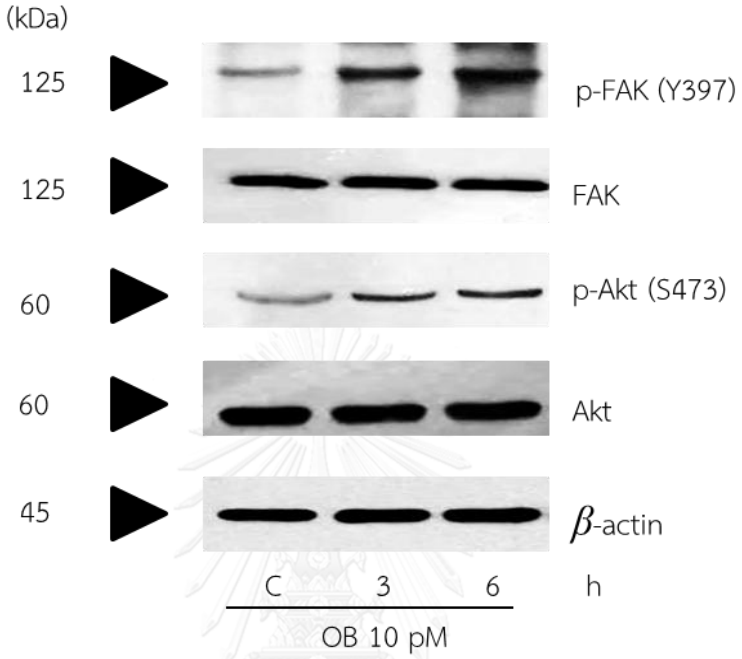
A



B



C



D

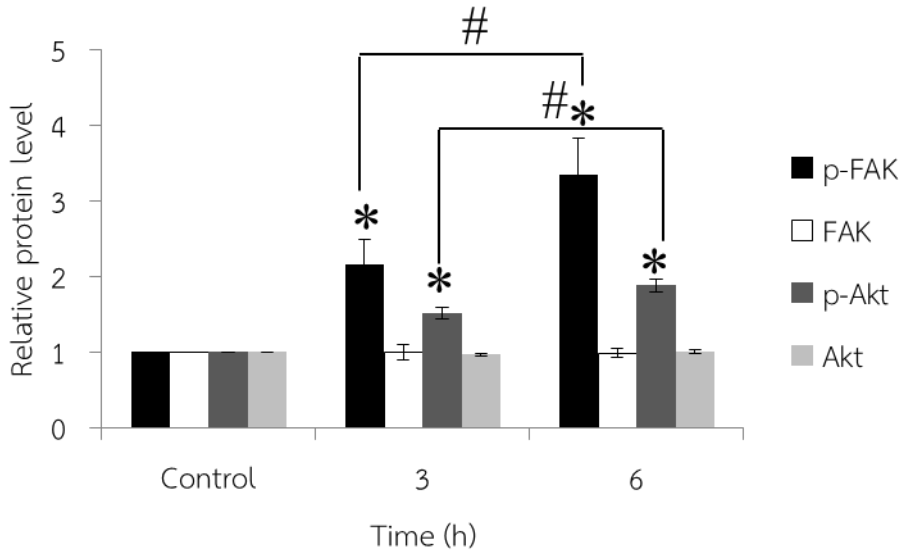


Figure 11 Ouabain activates the focal-adhesion kinase (FAK)-ATP-dependent tyrosine kinase (Akt) pathways.

A: H23 cells were treated with 0-10 pM of ouabain for 6 h, and the expression of phosphorylated FAK, total FAK, phosphorylated Akt, and total Akt were determined by western blotting. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. C: Cells were treated with 10 pM ouabain for 0-6 h and proteins were evaluated. B and D: The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. (n = 3). (*) Represent the significant different from the non-treated control , (#) represent the significant different from each treatment. Determined the statistical significant at $p<0.05$.

4. Ouabain induces the reduction of α5, β1 integrins.

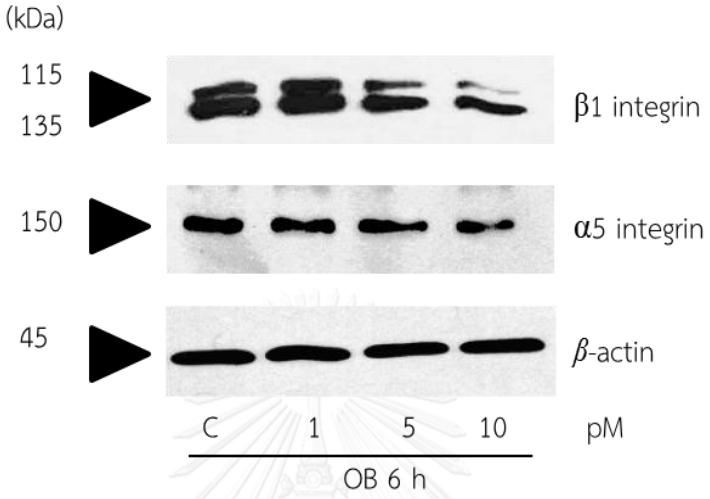
As α5 and β1 integrins have been implicated in cancer cell adhesion to the ECM surface (19), I tested whether the detachment of H23 cells in the present study was associated with the decrease of these integrins. H23 cells were treated with non-toxic concentrations of ouabain and the results were showed in figure 12. I found that treatment with ouabain at 1, 5 and 10 pM for 6 h significantly decreased the cellular level of α5 and β1 integrins. Moreover, treatment with ouabain at 10 pM for 3 and 6 h significantly decreased the cellular level of α5 and β1 integrins in time-dependent manner. From these results, I next examined effect of ouabain on the alteration level of other integrins subunits. H23 cells were treated with ouabain 10 pM for 3 and 6 h and the level of αv, β3 and β5 integrins was evaluated by western blot analysis. Interestingly, I found that treatment with ouabain at 10 pM for 3 and 6 h caused no effect on the alteration level of αv, β3 and β5 integrins (Figure 13).

Because cellular integrins were previously shown to be tightly regulated *via* degradation through the lysosomal pathway, I further tested whether treatment with ouabain could facilitate this degradation of integrins. H23 cells were treated with ouabain in the presence or absence of concanamycin A (CMA, a specific inhibitor that blocks lysosomal degradation of proteins) for 6 h and level of integrins was determined. Figure 12 C and D show that treatment with ouabain down-regulated α5 and β1 integrins in a time-dependent manner and the addition of CMA significantly

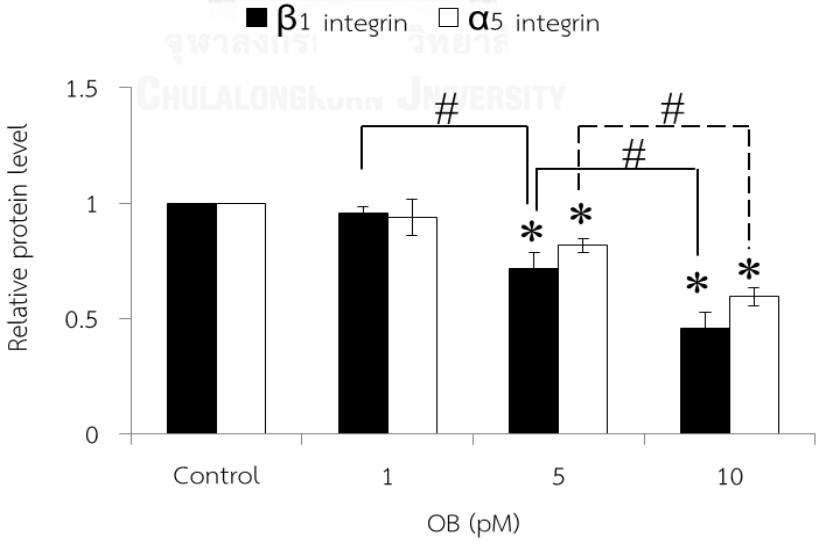
inhibited such effects of ouabain. These results indicate that ouabain facilitates cancer cell detachment by mediating α5 and β1 integrins degradation through a lysosomal mechanism.

Taken together with these observations, it is likely that this endogenous substance at biological concentrations could enhance cancer cell detachment *via* FAK and Akt activation, as well as facilitating of integrin α5 and β1 degradation.

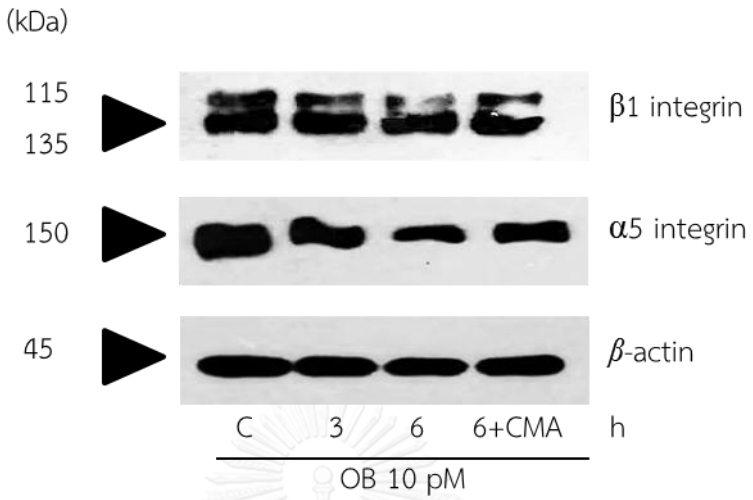
A


(kDa)
115
135
β1 integrin
150
α5 integrin
45
β-actin
C
1
5
10
pM
OB 6 h

B


β1 integrin
α5 integrin
Relative protein level
1.5
1
0.5
0
#
#
#
*
*
*
*
Control
1
5
10
OB (pM)

C



D

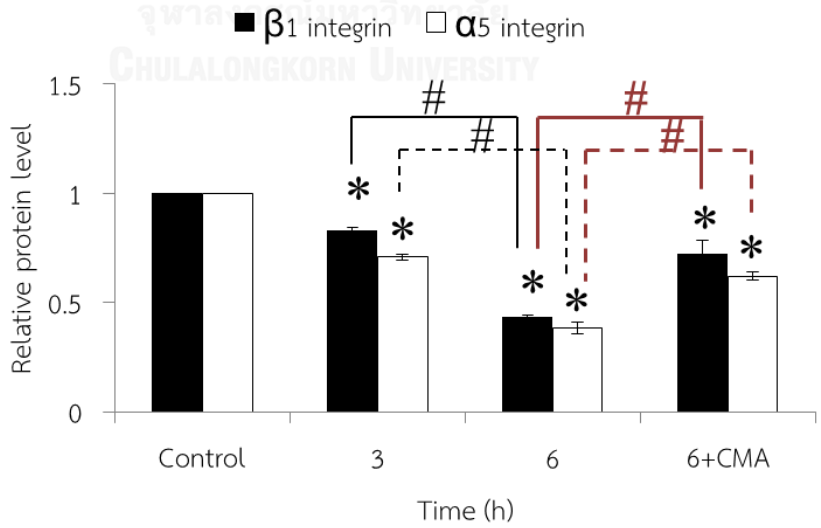
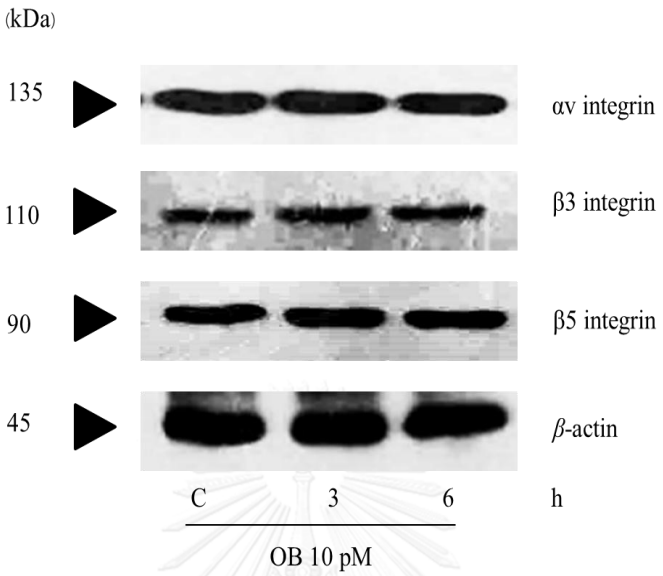


Figure 12 Ouabain down-regulates α5 and β1 integrins via lysosomal pathway.

A: H23 cells were treated with 0-10 pM of ouabain for 6 h, and the expression of α5 and β1 integrins were determined by western blotting. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. C: Cells were treated with 10 pM ouabain for 0-6 h in the presence or absence of lysosomal inhibitor concanamycin A (CMA) and proteins were evaluated. B and D: The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. (n = 3). (*) Represent the significant different from the non-treated control , (#) represent the significant different from each treatment. Determined the statistical significant at $p<0.05$.

A



B

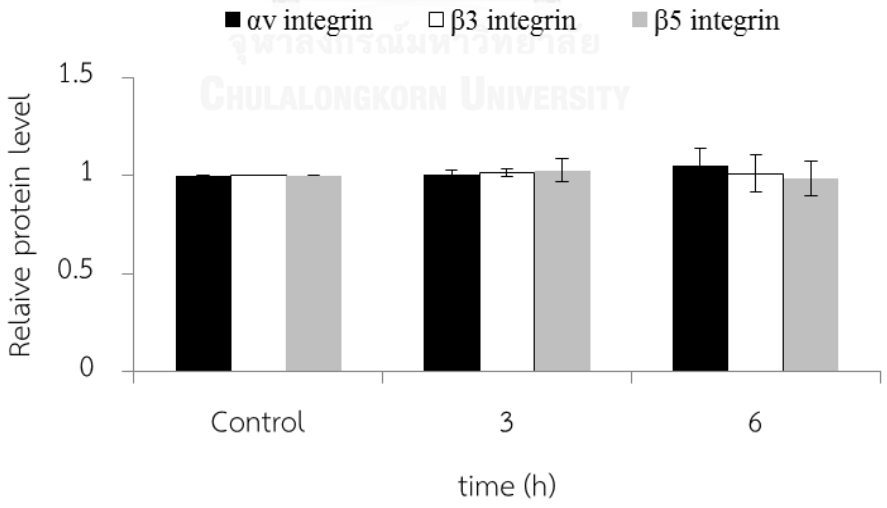


Figure 13 Ouabain had no effect on the level of αv, β3 and β5 integrins.

A: H23 cells were treated with 10 pM of ouabain for 3 and 6 h, and the expression of αv, β3 and β5 integrins were determined by western blotting. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. B : The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. (n = 3). Determined the statistical significant at $p<0.05$.

5. Ouabain had no effect on E-cadherin protein level.

Loss of E-cadherin expression always observed in highly invasive cancer cells and promotes metastasis by disrupting intercellular contacts, an early step in metastatic dissemination (21). From this reason, I next investigated effect of ouabain on the alteration level of E-cadherin. Cells were treated with 0-10 pM ouabain for 6 h, and the expression of E-cadherin was determined by western blotting. Figure 14 shows that E-cadherin protein level in ouabain-treated H23 cells was not significantly different from the control cells. The result Indicate that detachment of H23 cells that trigger by ouabain at physiological level was not related to the reduction of E-cadherin protein level.

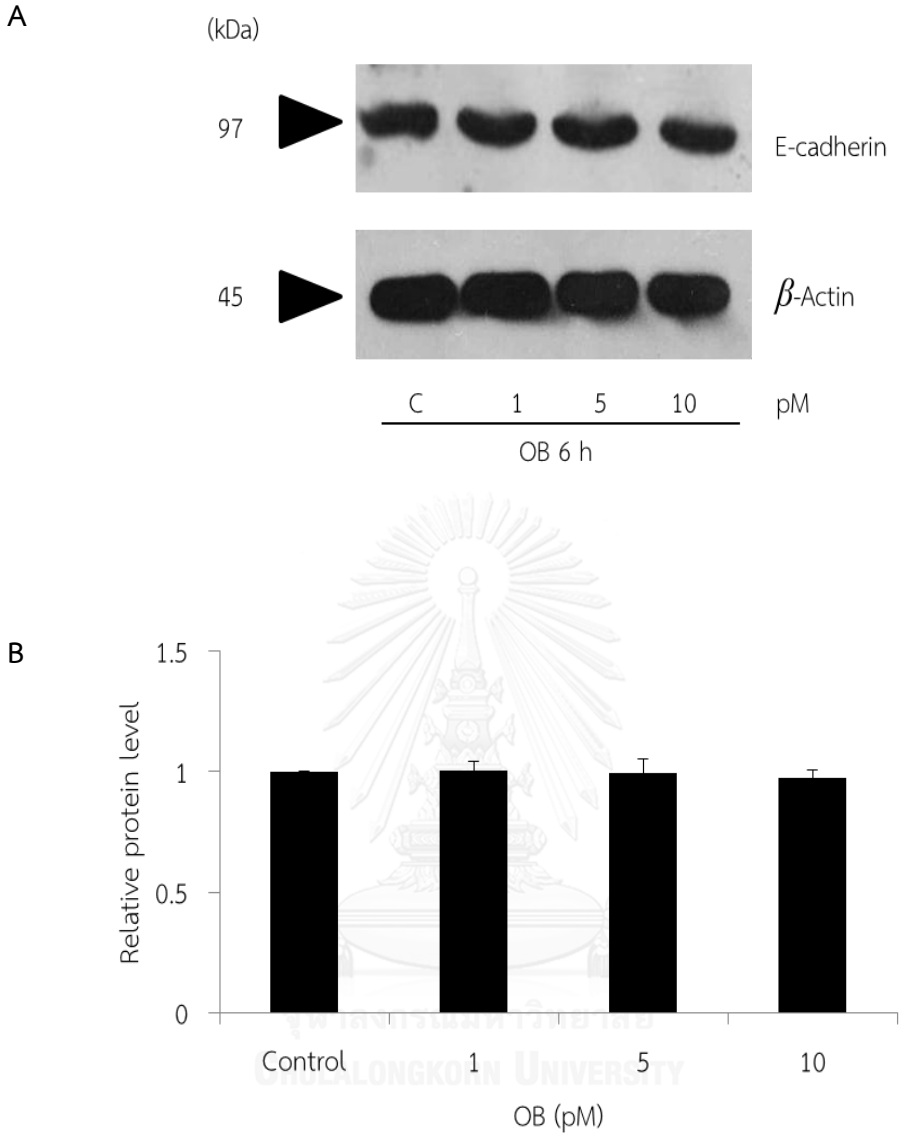


Figure 14 Ouabain had no effect on E-cadherin protein level.

A: Cells were treated with 0-10 pM ouabain for 6 h, and the expression of e-cadherin was determined by western blotting. To confirm equal loading of the samples, the blots were reprobed with β-actin antibody. B: The immunoblot signals were quantified by densitometry. The data are the mean ± S.D. (n = 3). Determined the statistical significant at $p<0.05$.

CHAPTER V

DISCUSSION AND CONCLUSION

Ouabain, a cardiac glycoside member isolated from plants, was found to be endogenously produced in humans and classified as a human hormone (63). It has garnered increasing attention in cancer research. Here, I demonstrate for the first time that ouabain at physiological concentrations (0-10 pM) facilitates lung cancer cell detachment. Even though the detachment of cancer cells from their original tumor is accepted as an important initial process of cancer metastasis, there are only few studies elucidating the cellular mechanisms of cell detachment.

Interestingly, ouabain and its related compounds are found in human plasma at different concentrations depending on the status of individuals as well as methods of assessment. In most cases, plasma ouabain concentrations are reported to be between 2-770 pM (27). The present study aimed to investigate the effect of this endogenous substance at its physiological levels because of cancer cells in the patient's body are likely exposed to ouabain. I therefore selected concentrations of 0-50 pM; however, ouabain at concentrations of more than 50 pM caused significant cytotoxic towards human lung cancer cells. This result is consistent with the previous experiments indicating that ouabain possesses activity against many types of cancers (24-26, 69). Besides its direct cytotoxicity, the previous study in my laboratory found that ouabain at non-toxic concentrations sensitized TRAIL-mediated death by down-

regulating anti-apoptotic myeloid cell leukemia sequence 1 (MCL-1) protein in H292 and H460 cells (28).

Cell-ECM adhesion plays an important part for survival of the cancer cells by activating the survival signal through integrin-Akt-dependent mechanisms (73). Although the detachment of cells will disrupt such survival and trigger detachment-mediated apoptosis (anoikis), certain cancer cells have an ability to resist anoikis and further spread (74, 75). In order for cell detachment, integrins binding to extracellular compartments must be disrupted and previous studies show that such dissociation of integrin from their ECM resulted from the activation of FAK (10, 11). In certain work, FAK was shown to be activated by active Akt. Therefore, activation of Akt could be regulate FAs turn over by stimulation through FAK and leads to FAs disassembly (15). The internalization of integrins, under certain conditions, leads to the degradation of cellular integrins *via* the lysosomal pathway (48, 49). Consistent with this finding, our results indicate that ouabain-mediated cell detachment involves FAK and Akt activation, and down-regulation of cellular α5 and β1 integrins but the level αv, β3 and β5 integrins were not affected by ouabain treatment. From this observation, it was noteworthy why other integrins proteins were not affected by ouabain treatment. In order to fulfill this observation, it is necessary to further study of the mechanism in the reduction of such proteins in detail later.

The reduction of cellular α5 and β1 integrins may be due to an increase in degradation process rather than the decrease in protein synthesis. Because of protein

synthesis involves in the transcription and translation of gene in DNA which would require a much longer time to be able to detect the difference in protein expression (76). This assumption was confirmed by using specific inhibitor of lysosomal degradation (CMA). I found that the cellular level of α5 and β1 integrins was significantly increased. From these observations indicate that ouabain reduced cellular α5 and β1 integrins through the lysosomal pathway.

Moreover, a growing body of evidence indicates that cancer cells that loss of E-cadherin expression is likely to separate form their origin tumors and spread to other parts of the body (61). In this work, our results demonstrate that ouabain at its physiological levels was no effect on the alteration of E-cadherin level.

In summary, this study demonstrated the possible role of ouabain at its physiological level on the detachment of lung cancer cells. The detachment of cancer cells by ouabain involves the activation of FAK and Akt signaling pathway. Moreover, ouabain exposure reduced the level of α5 and β1 integrins through lysosomal degradation resulting in cell-ECM detachment. The knowledge gained from this study may benefit our understanding of cancer biology and cancer metastasis.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: a cancer journal for clinicians. 2010;60(5):277-300.
2. Yokoi K, Kamiya N, Matsuguma H, Machida S, Hirose T, Mori K, et al. Detection of brain metastasis in potentially operable non-small cell lung cancer: a comparison of CT and MRI. Chest. 1999;115(3):714-9.
3. Coleman RE. Metastatic bone disease: clinical features, pathophysiology and treatment strategies. Cancer treatment reviews. 2001;27(3):165-76.
4. Park HY, Kim YH, Kim H, Koh WJ, Suh GY, Chung MP, et al. Routine screening by brain magnetic resonance imaging decreased the brain metastasis rate following surgery for lung adenocarcinoma. Lung cancer (Amsterdam, Netherlands). 2007;58(1):68-72.
5. Iordanidou L, Trivizaki E, Saranti S, Georgakopoulos A, Bolanos N, Baltagiannis N, et al. Is there a role of whole body bone scan in early stages of non small cell lung cancer patients. Journal of BUON : official journal of the Balkan Union of Oncology. 2006;11(4):491-7.
6. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nature reviews Cancer. 2003;3(6):453-8.
7. Coghlin C, Murray GI. Current and emerging concepts in tumour metastasis. The Journal of pathology. 2010;222(1):1-15.

8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

9. Weiss L, Ward PM. Cell detachment and metastasis. Cancer metastasis reviews. 1983;2(2):111-27.

10. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M. Turnover of Focal Adhesions and Cancer Cell Migration. International Journal of Cell Biology. 2012;2012.

11. Broussard JA, Webb DJ, Kaverina I. Asymmetric focal adhesion disassembly in motile cells. Current opinion in cell biology. 2008;20(1):85-90.

12. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature. 1995;377(6549):539-44.

13. Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, Lee-Kwon W, et al. The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer research. 2003;63(9):2172-8.

14. Hamadi A, Bouali M, Dontenwill M, Stoeckel H, Takeda K, Ronde P. Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. J Cell Sci. 2005;118(19):4415-25.

15. Higuchi M, Kihara R, Okazaki T, Aoki I, Suetsugu S, Gotoh Y. Akt1 promotes focal adhesion disassembly and cell motility through phosphorylation of FAK in growth factor-stimulated cells. J Cell Sci. 2013;126(Pt 3):745-55.

16. Truong H, Danen EH. Integrin switching modulates adhesion dynamics and cell migration. Cell adhesion & migration. 2009;3(2):179-81.

17. Schaffner F, Ray AM, Dontenwill M. Integrin $\alpha 5\beta 1$, the Fibronectin Receptor, as a Pertinent Therapeutic Target in Solid Tumors. Cancers. 2013;5(1):27-47.

18. Hirasawa M, Shijubo N, Uede T, Abe S. Integrin expression and ability to adhere to extracellular matrix proteins and endothelial cells in human lung cancer lines. British Journal of Cancer. 1994;70(3):466-73.

19. Roca-Cusachs P, Gauthier NC, Del Rio A, Sheetz MP. Clustering of alpha(5)beta(1) integrins determines adhesion strength whereas alpha(v)beta(3) and talin enable mechanotransduction. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(38):16245-50.

20. Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochimica et biophysica acta. 1994;1198(1):11-26.

21. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer research. 2008;68(10):3645-54.

22. Canel M, Serrels A, Frame MC, Brunton VG. E-cadherin–integrin crosstalk in cancer invasion and metastasis. Journal of Cell Science. 2013;126(2):393-401.

23. Blaustein MP. Physiological effects of endogenous ouabain: control of intracellular Ca2+ stores and cell responsiveness. The American journal of physiology. 1993;264(6 Pt 1):C1367-87.

24. Huang YT, Chueh SC, Teng CM, Guh JH. Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer PC-3 cells. Biochemical pharmacology. 2004;67(4):727-33.

25. Ozdemir T, Nar R, Kilinc V, Alacam H, Salis O, Duzgun A, et al. Ouabain targets the unfolded protein response for selective killing of HepG2 cells during glucose deprivation. Cancer biotherapy & radiopharmaceuticals. 2012;27(8):457-63.

26. Gasper R, Vandenbussche G, Goormaghtigh E. Ouabain-induced modifications of prostate cancer cell lipidome investigated with mass spectrometry and FTIR spectroscopy. Biochimica et biophysica acta. 2011;1808(3):597-605.

27. Gottlieb SS, Rogowski AC, Weinberg M, Krichten CM, Hamilton BP, Hamlyn JM. Elevated concentrations of endogenous ouabain in patients with congestive heart failure. Circulation. 1992;86(2):420-5.

28. Chanvorachote P, Pongrakhananon V. Ouabain downregulates Mcl-1 and sensitizes lung cancer cells to TRAIL-induced apoptosis. American journal of physiology Cell physiology. 2013;304(3):C263-72.

29. Landi MT, Dracheva T, Rotunno M, Figueroa JD, Liu H, Dasgupta A, et al. Gene Expression Signature of Cigarette Smoking and Its Role in Lung Adenocarcinoma Development and Survival. PLoS ONE. 2008;3(2):e1651.

30. Bryant A, Cerfolio RJ. Differences in epidemiology, histology, and survival between cigarette smokers and never-smokers who develop non-small cell lung cancer. Chest. 2007;132(1):185-92.

31. Martin N, Hahn S, McKenna WG. Molecular Biology and Genetics of Lung Cancer. In: Jeremić B, editor. Advances in Radiation Oncology in Lung Cancer. Medical Radiology Radiation Oncology: Springer Berlin Heidelberg; 2005. p. 3-11.

32. Jackman DM, Johnson BE. Small-cell lung cancer. The Lancet.366(9494):1385-96.

33. Simon GR, Wagner H. Small cell lung cancer. Chest. 2003;123(1 Suppl):259s-71s.

34. Ettinger DS, Akerley W, Bepler G, Blum MG, Chang A, Cheney RT, et al. Non-small cell lung cancer. Journal of the National Comprehensive Cancer Network : JNCCN. 2010;8(7):740-801.

35. Zaric B, Stojsic V, Tepavac A, Sarcev T, Zarogoulidis P, Darwiche K, et al. Adjuvant chemotherapy and radiotherapy in the treatment of non-small cell lung cancer (NSCLC). Journal of Thoracic Disease. 2013;5(Suppl 4):S371-S7.

36. Bacac M, Stamenkovic I. Metastatic cancer cell. Annual review of pathology. 2008;3:221-47.

37. Tsuboi M, Ohira T, Saji H, Miyajima K, Kajiwara N, Uchida O, et al. The present status of postoperative adjuvant chemotherapy for completely resected non-

small cell lung cancer. Annals of thoracic and cardiovascular surgery : official journal of the Association of Thoracic and Cardiovascular Surgeons of Asia. 2007;13(2):73-7.

38. Richards MA, Stockton D, Babb P, Coleman MP. How many deaths have been avoided through improvements in cancer survival? BMJ : British Medical Journal. 2000;320(7239):895-8.
39. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nature reviews Cancer. 2002;2(8):563-72.
40. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110(6):673-87.
41. Petit V, Thiery JP. Focal adhesions: structure and dynamics. Biology of the cell / under the auspices of the European Cell Biology Organization. 2000;92(7):477-94.
42. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. J Cell Sci. 2006;119(Pt 19):3901-3.
43. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY). 1999;222(2):124-38.
44. Roman J, Ritzenthaler JD, Roser-Page S, Sun X, Han S. alpha5beta1-integrin expression is essential for tumor progression in experimental lung cancer. American journal of respiratory cell and molecular biology. 2010;43(6):684-91.

45. Mierke CT, Frey B, Fellner M, Herrmann M, Fabry B. Integrin alpha5beta1 facilitates cancer cell invasion through enhanced contractile forces. J Cell Sci. 2011;124(Pt 3):369-83.

46. Rintoul RC, Sethi T. The role of extracellular matrix in small-cell lung cancer. The Lancet Oncology. 2001;2(7):437-42.

47. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG. Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. The Journal of Cell Biology. 2009;187(5):733-47.

48. Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. Traffic (Copenhagen, Denmark). 2006;7(1):14-21.

49. Bridgewater RE, Norman JC, Caswell PT. Integrin trafficking at a glance. Journal of Cell Science. 2012;125(16):3695-701.

50. Hall JE, Fu W, Schaller MD. Focal adhesion kinase: exploring Fak structure to gain insight into function. International review of cell and molecular biology. 2011;288:185-225.

51. Golubovskaya VM, Kweh FA, Cance WG. Focal adhesion kinase and cancer. Histology and histopathology. 2009;24(4):503-10.

52. Schaller MD. Cellular functions of FAK kinases: insight into molecular mechanisms and novel functions. J Cell Sci. 2010;123(Pt 7):1007-13.

53. Stambolic V, Woodgett JR. Functional distinctions of protein kinase B/Akt isoforms defined by their influence on cell migration. Trends in Cell Biology.16(9):461-6.

54. Shtilbans V, Wu M, Burstein DE. Current overview of the role of Akt in cancer studies via applied immunohistochemistry. Annals of diagnostic pathology. 2008;12(2):153-60.

55. Cicenas J. The potential role of Akt phosphorylation in human cancers. The International journal of biological markers. 2008;23(1):1-9.

56. van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cellular and molecular life sciences : CMLS. 2008;65(23):3756-88.

57. Takeichi M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development (Cambridge, England). 1988;102(4):639-55.

58. Sulzer MA, Leers MP, van Noord JA, Bollen EC, Theunissen PH. Reduced E-cadherin expression is associated with increased lymph node metastasis and unfavorable prognosis in non-small cell lung cancer. American journal of respiratory and critical care medicine. 1998;157(4 Pt 1):1319-23.

59. Kang H, Min B, Lee K, Kim N, Kim S, Choi J, et al. Loss of E-cadherin and MUC2 Expressions Correlated with Poor Survival in Patients with Stages II and III Colorectal Carcinoma. Ann Surg Oncol. 2011;18(3):711-9.

60. Umbas R, Isaacs WB, Bringuier PP, Schaafsma HE, Karthaus HF, Oosterhof GO, et al. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer research. 1994;54(14):3929-33.

61. Byers SW, Sommers CL, Hoxter B, Mercurio AM, Tozeren A. Role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: measurement of cell-cell adhesion strength. J Cell Sci. 1995;108 (Pt 5):2053-64.

62. Ogawa H, Shinoda T, Cornelius F, Toyoshima C. Crystal structure of the sodium-potassium pump (Na+,K+-ATPase) with bound potassium and ouabain. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(33):13742-7.

63. Hamlyn JM, Blaustein MP, Bova S, DuCharme DW, Harris DW, Mandel F, et al. Identification and characterization of a ouabain-like compound from human plasma. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(14):6259-63.

64. Laredo J, Shah JR, Lu ZR, Hamilton BP, Hamlyn JM. Angiotensin II stimulates secretion of endogenous ouabain from bovine adrenocortical cells via angiotensin type 2 receptors. Hypertension. 1997;29(1 Pt 2):401-7.

65. Tymiak AA, Norman JA, Bolgar M, DiDonato GC, Lee H, Parker WL, et al. Physicochemical characterization of a ouabain isomer isolated from bovine hypothalamus. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(17):8189-93.

66. Manunta P, Messaggio E, Ballabeni C, Sciarrone MT, Lanzani C, Ferrandi M, et al. Plasma ouabain-like factor during acute and chronic changes in sodium balance in essential hypertension. Hypertension. 2001;38(2):198-203.

67. Balzan S, Neglia D, Ghione S, D'Urso G, Baldacchino MC, Montali U, et al. Increased circulating levels of ouabain-like factor in patients with asymptomatic left ventricular dysfunction. European journal of heart failure. 2001;3(2):165-71.

68. Bagrov AY, Shapiro JI, Fedorova OV. Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. Pharmacological reviews. 2009;61(1):9-38.

69. Kometiani P, Liu L, Askari A. Digitalis-induced signaling by Na+/K+-ATPase in human breast cancer cells. Molecular pharmacology. 2005;67(3):929-36.

70. Contreras RG, Shoshani L, Flores-Maldonado C, Lazaro A, Cereijido M. Relationship between Na(+),K(+)-ATPase and cell attachment. J Cell Sci. 1999;112 (Pt 23):4223-32.

71. Akimova OA, Poirier M, Kotelevtsev SV, Hamet P, Orlov SN. The death of ouabain-treated renal epithelial cells: evidence against anoikis occurrence. Apoptosis : an international journal on programmed cell death. 2008;13(5):670-80.

72. Rincon-Heredia R, Flores-Benitez D, Flores-Maldonado C, Bonilla-Delgado J, Garcia-Hernandez V, Verdejo-Torres O, et al. Ouabain induces endocytosis and

degradation of tight junction proteins through ERK1/2-dependent pathways. Experimental cell research. 2014;320(1):108-18.

73. Bolos V, Gasent JM, Lopez-Tarruella S, Grande E. The dual kinase complex FAK-Src as a promising therapeutic target in cancer. OncoTargets and therapy. 2010;3:83-97.

74. Frisch SM, Screaton RA. Anoikis mechanisms. Current opinion in cell biology. 2001;13(5):555-62.

75. Rennebeck G, Martelli M, Kyprianou N. Anoikis and survival connections in the tumor microenvironment: is there a role in prostate cancer metastasis? Cancer research. 2005;65(24):11230-5.

76. Sonenberg N. Protein Synthesis in Eukaryotes: IRES-mediated Internal Initiation. [Internet]. 2006 [cited 19 December 2014]. Available from: http://onlinelibrary.wiley.com/doi/10.1038/npg.els.0000544/abstract

APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The percentage of H23 cell viability after treatment with various concentrations of ouabain (0-50 pM) for 12 h was determined by MTT assay.

Ouabain (pM)	Cell viability (%)
Control	100.00 ± 0.96
1	99.24 ± 1.07
5	97.06 ± 1.66
10	92.18 ± 3.50
50	69.56 ± 0.80*

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 2 The percentage of apoptotic and necrotic cell count from Hoechst33342/PI co-staining assay after treatment with various concentrations of ouabain (0-50 pM) for 12 h.

Ouabain (pM)	Apoptosis (%)	Necrosis (%)
Control	1.77 ± 0.42	0.61 ± 0.06
1	2.77 ± 0.39	0.73 ± 0.09
5	4.23 ± 0.41	0.79 ± 0.14
10	5.03 ± 0.12	0.76 ± 0.26
50	14.74 ± 0.58*	0.94 ± 0.07

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 3 The percentage of cell adhesion to ECM after treatment with various concentrations of ouabain (0-10 pM) for 6 h was determined by MTT assay.

Ouabain (pM)	Cell adhesion (%)
Control	100.00 ± 0.88
1	85.39 ± 3.76*,#
5	65.27 ± 3.78*,#
10	50.91 ± 0.49*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 4 The percentage of cell adhesion to ECM after treatment with various concentrations of ouabain (0-10 pM) for 6 h was determined by cell counting.

Ouabain (pM)	Cell count (%)
Control	100.00 ± 3.73
1	82.18 ± 3.11*,#
5	63.73 ± 5.62*,#
10	48.84 ± 2.40*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 5 The percentage of cell adhesion to ECM after treatment with ouabain 10 pM for 1, 3 and 6 h was determined by MTT assay.

Time (h)	Cell adhesion (%)	
	Control	OB 10 pM
1	100.00 ± 3.46	86.52 ± 3.15*,#
3	100.00 ± 4.58	64.55 ± 2.91*,#
6	100.00 ± 1.36	52.53 ± 1.64*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by independent-sample t-test at $p<0.05$.

Table 6 The percentage of cell adhesion to ECM after treatment with ouabain 10 pM for 1, 3 and 6 h was determined by cell counting.

Time (h)	Cell count (%)	
	Control	OB 10 pM
1	100.00 ± 2.59	81.23 ± 5.18*,#
3	100.00 ± 4.56	61.10 ± 4.41*,#
6	100.00 ± 3.26	51.52 ± 2.55*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by independent-sample t-test at $p<0.05$.

Table 7 The percentage of remained cell that adhesion to ECM after detachment was determined by MTT assay.

Ouabain (pM)	Remaining cell (%)
Control	100.00 ± 4.22
1	79.74 ± 2.40*,#
5	66.33 ± 1.40*,#
10	55.78 ± 2.89*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 8 The percentage of remained cell that adhesion to ECM after detachment was determined by cell counting.

Ouabain (pM)	Cell count (%)
Control	100.00 ± 1.77
1	81.18 ± 1.74*,#
5	65.10 ± 3.48*,#
10	50.87 ± 1.52*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 9 The Relative protein level of p-FAK, FAK, p-Akt and Akt was determined by western blot analysis after treatment with ouabain (0-10 pM) for 6 h.

Ouabain (pM) / Protein	Relative protein level			
	Control	1	5	10
p-FAK	1.00	4.54 ± 0.30*,#	5.84 ± 0.47*,#	6.94 ± 0.44*,#
FAK	1.00	0.99 ± 0.11	0.98 ± 0.02	0.97 ± 0.05
p-Akt	1.00	1.45 ± 0.20*	1.59 ± 0.06*,#	1.98 ± 0.20*,#
Akt	1.00	1.00 ± 0.02	0.99 ± 0.05	0.98 ± 0.03

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 10 The Relative protein level of p-FAK, FAK, p-Akt and Akt was determined by western blot analysis after treatment with ouabain 10 pM for 0, 3 and 6 h.

Time (h) / Protein	Relative protein level		
	Control	3	6
p-FAK	1.00	2.16 ± 0.23*,#	3.36 ± 0.34*,#
FAK	1.00	1.00 ± 0.10	0.99 ± 0.06
p-Akt	1.00	1.56 ± 0.18*,#	1.93 ± 0.17*,#
Akt	1.00	0.97 ± 0.06	1.01 ± 0.09

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 11 The Relative protein level of β1 and α5 integrins were determined by western blot analysis after treatment with ouabain (0-10 pM) for 6 h.

Ouabain (pM) / Protein	Relative protein level			
	Control	1	5	10
β1 integrin	1.00	0.95 ± 0.02#	0.72 ± 0.07*,#	0.46 ± 0.06*,#
α5 integrin	1.00	0.94 ± 0.09	0.82 ± 0.06*,#	0.60 ± 0.06*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 12 The Relative protein level of β1 and α5 integrins were determined by western blot analysis after treatment with ouabain 10 pM for 0, 3 and 6 h and after pretreatment with CMA 1 uM for 6 h.

Time (h) / Protein	Relative protein level			
	Control	3	6	6 + CMA
β1 integrin	1.00	0.83 ± 0.01*,#	0.43 ± 0.01*,#	0.73 ± 0.06*,#
α5 integrin	1.00	0.71 ± 0.01*,#	0.38 ± 0.03*,#	0.62 ± 0.02*,#

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Asterisks (*) represent the significant different from the non-treated control and (#) represent the significant different from each treatment. Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 13 The Relative protein level of αv, β3 and β5 integrins were determined by western blot analysis after treatment with ouabain 10 pM for 0, 3 and 6 h.

Time (h) / Protein	Relative protein level		
	Control	3	6
αv integrin	1.00	1.00±0.02	1.05±0.09
β3 integrin	1.00	1.02±0.02	1.01±0.09
β5 integrin	1.00	1.03±0.06	0.99±0.09

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

Table 14 The Relative protein level of E-cadherin was determined by western blot analysis after treatment with ouabain (0-10 pM) for 6 h.

Ouabain (pM) / Protein	Relative protein level			
	Control	1	5	10
E-cadherin	1.00	1.01±0.04	0.99±0.06	0.97±0.03

The data are the mean ± S.D. for triplicate independent experiments (n = 3). Statistical significant was determined by One-way ANOVA following post hoc test at $p<0.05$.

VITA

Miss Thidarat Ruanghirun was born in December 20, 1987 in Samutprakarn. She received Bachelor's degree of Pharmacy from faculty of Pharmacy, Huachiew Chalermprakiet University in 2012.