ผลของกรดอัลฟาไลโปอิกต่อการตายที่เหนี่ยวนำด้วยเคมีบำบัดและสภาวะไร้การยึดเกาะของเซลล์มะเร็งปอดของมนุษย์ชนิดไม่ใช่เซลล์เล็กเอช 460

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EFFECTS OF ALPHA-LIPOIC ACID ON CHEMOTHERAPY AND DETACHMENT-INDUCED CELL DEATH IN HUMAN NON-SMALL CELL LUNG CANCER H460 CELLS

Miss Punyawee Puchsaka

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology and Toxicology
Department of Pharmacology and Physiology
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Thesis Title	EFFECTS OF ALPHA-LIPOIC ACID ON CHEMOTHERAPY AND DETACHMENT-INDUCED CELL DEATH IN HUMAN NON-SMALL CELL LUNG CANCER H460 CELLS
By	Miss Punyawee Puchsaka
Field of Study	Pharmacology and Toxicology
Thesis Advisor	Associate Professor Pithi Chanvorachote, Ph.D.
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ปุณยวีร์ พืชสะกะ : ผลของกรดอัลฟาไลโปอิกต่อการตายที่เหนี่ยวนำด้วยเคมีบำบัดและสภาวะไร้การยึดเกาะของเซลล์มะเร็งปอดของมนุษย์ชนิดไม่ใช่เซลล์เล็กเอช 460 (EFFECTS OF ALPHA-LIPOIC ACID ON CHEMOTHERAPY AND DETACHMENT-INDUCED CELL DEATH IN HUMAN NON-SMALL CELL LUNG CANCER H460 CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร.ปิติ จันทร์วรโชติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ภก. ดร. ฉัตรชัย เชาว์ธรรม, 87 หน้า.

ความล้มเหลวของการรักษาด้วยยาเคมีบำบัดและการแพร่กระจายของเซลล์มะเร็งเป็นสาเหตุสำคัญของอัตราการเสียชีวิตที่เพิ่มขึ้นในโรคมะเร็งปอด การเปลี่ยนแปลงสภาวะรีดอกซ์ภายในเซลล์เนื่องมาจากสิ่งกระตุ้นจากภายในเซลล์หรือสารจากภายนอกเซลล์ส่งผลอย่างมากต่อวิถีสัญญาณและการแสดงออกของเซลล์ การศึกษานี้พบว่าเซลล์มะเร็งปอดที่ได้รับกรดอัลฟาไลโปอิกมีปริมาณของไฮโดรเจนเปอร์ออกไซด์ และซุปเปอร์ออกไซด์แอนไอออนภายในเซลล์เพิ่มสูงขึ้น และการเพิ่มขึ้นของอนุพันธ์ออกซิเจนที่ว่องไวนี้ส่งผลต่อการลดของโปรตีนอินทิกรินชนิดเบต้า-1 และเบต้า-3 ตามลำดับ ซึ่งโปรตีนอินทิกรินมีความเกี่ยวข้องกับลักษณะความรุนแรงและการแพร่กระจายของเซลล์มะเร็ง เซลล์มะเร็งปอดที่ได้รับกรดอัลฟาไลโปอิกมีความสามารถในการรอดชีวิตจากการตายแบบอะนอยคิสและการเจริญเติบโตในสภาวะไร้การยึดเกาะได้ลดลง นอกจากนี้กรดอัลฟาไลโปอิกยังเพิ่มความไวต่อการตายแบบอะพอพโทซิสที่เหนี่ยวนำด้วยยาเคมีบำบัดคือ ซิสพลาติน อีโทโปไซด์และพาคลิแทคเซล พบการลดลงของอินทิกรินชนิดเบต้า-1 และเบต้า-3 โดยไม่พบการเปลี่ยนแปลงของอินทิกรินชนิดแอลฟา-5 และแอลฟา-วี ในเซลล์มะเร็งปอดที่ได้รับกรดอัลฟาไลโปอิก นอกจากนี้พบการลดลงของโปรตีนในวิถีสัญญาณรอดชีวิตชนิด p-AKT และโปรตีนที่ต้านการตายแบบอะพอพโทซิสชนิด Bcl-2 สอดคล้องกับการเปลี่ยนแปลงของโปรตีนอินทิกริน ผลการศึกษาด้วยการตรวจวัดปริมาณอนุพันธ์ออกซิเจนที่ว่องไวและสารต้านอนุมูลอิสระที่จำเพาะแสดงให้เห็นว่า ไฮโดรเจนเปอร์ออกไซด์ และซุปเปอร์ออกไซด์แอนไอออนที่เหนี่ยวนำด้วยกรดอัลฟาไลโปอิกมีบทบาทสำคัญต่อการลดของอินทิกรินชนิดเบต้า-1 และเบต้า-3 ตามลำดับ การศึกษานี้ได้แสดงถึงผลของกรดอัลฟาไลโปอิกต่อลักษณะความรุนแรงของเซลล์มะเร็งปอดและบทบาทของซุปเปอร์ออกไซด์แอนไอออนและไฮโดรเจนเปอร์ออกไซด์ ต่อการควบคุมการแสดงออกของอินทิกริน การตายแบบอะนอยคิสและการเพิ่มความไวต่อการตายแบบอะพอพโทซิสที่เหนี่ยวนำด้วยยาเคมีบำบัด

ภาควิชา	เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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PUNYAWEE PUCHSAKA: EFFECTS OF ALPHA-LIPOIC ACID ON CHEMOTHERAPY AND DETACHMENT-INDUCED CELL DEATH IN HUMAN NON-SMALL CELL LUNG CANCER H460 CELLS. ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., CO-ADVISOR: CHATCHAI CHAOTHAM, Ph.D., 87 pp.

Chemotherapeutic failure and metastasis are the main causes of high mortality rate in lung cancer. Alteration of cellular redox status in response to endogenous stimuli or exogenous compounds has a significant impact on cell signaling and behavior. Herein we divulge for the first time that lung cancer cells exposed to alpha-lipoic acid (LA) resulted in a higher level of cellular hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$) , and such an increase of the specific reactive oxygen species (ROS) downregulated integrin β1 and β3, the integrins known for potentiating aggressive behaviors and metastasis. The LA-treated cells exhibited significant decrease in their abilities to survive in detached condition or anoikis resistance and grow in anchorage-independent soft agar assay. Furthermore, LA sensitized the cells to cisplatin, etoposide and paclitaxel-induced apoptosis. For underlying mechanism, we found that the treatment of the cells with LA significantly decreased integrin β1 and β3, while had no effect on integrin α5 and αv. Interestingly, survival protein p-AKT and anti-apoptotic protein Bcl-2 were reduced in an association to such integrin modulations. Using ROS probes and selective anti-oxidants, we have proved that H_2O_2 and $O_2^{\cdot-}$ induced by LA are key players for the decrease of β1 and β3 integrins, respectively. These findings indicate a novel effect of LA as well as specific ROS, $O_2^{\cdot-}$ and H_2O_2 in integrin regulation, anoikis and chemotherapeutic sensitizations.

Department:	Pharmacology and Physiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmacology and Toxicology	Co-Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	=	percentage
ºC	=	degree Celsius
µM	=	micromolar
AKT	=	protein kinase B
ANOVA	=	analysis of variance
Apaf-1	=	apoptosis protease activating factor-1
Bad	=	BCL2-associated death promoter
Bak	=	BCL2 homologous antagonist/killer
Bax	=	BCL2-associated X
Bcl-2	=	B-cell lymphoma 2
Bcl-XL	=	B-cell lymphoma-extra large
Bid	=	BH3 interacting domain death agonist
Bim	=	BCL2-like protein 11
Cis	=	cisplatin
CO_2	=	carbon dioxide
$DCFH_2$-DA	=	2, 7-dichlorofluorescein diacetate
DHE	=	dihydroethidium
DMSO	=	dimethyl sulfoxide
ECM	=	extracellular matrix
EMT	=	epithelial-mesenchymal transition
ERK	=	extracellular signal regulated kinase
EtOH	=	ethanol
Eto	=	etoposide
FAK	=	focal adhesion kinase
GSH	=	glutathione

h	=	hour, hours
H_2O_2	=	hydrogen peroxide
HPF	=	3'–(p-hydroxyphenyl) fluorescein
LA	=	alpha-lipoic acid
Mcl-1	=	myeloid cell leukemia sequence 1
min	=	minute (S)
ml	=	milliliter
mM	=	millimolar
MnTBAP	=	Mn(III)tetrakis (4-benzoic acid) porphyrin chloride
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	=	N-acetylcysteine
NSCLC	=	non-small cell lung cancer
$O_2^{\bullet-}$	=	superoxide anion radical
$^{\bullet}OH$	=	hydroxyl radical
Pac	=	paclitaxel
PBS	=	phosphate buffer saline
PI	=	propidium iodide
ROS	=	reactive oxygen species
RPMI	=	Roswell Park Memorial Institute's medium
S.D.	=	standard deviation
TNF	=	tumor necrosis factor
U	=	unit

CHAPTER I
INTRODUCTION

Lung cancer is the most prevalent cause of mortality in cancer patients worldwide. Approximately 85% of lung cancer patients are characterized as non-small cell lung cancer (NSCLC) [1]. Although, surgical resection is the most successful treatment but chemotherapeutic drugs are recommended in many cases for palliation and prolong life of lung cancer patients [2]. Recently, chemotherapeutic resistance in lung cancer cell is continuously reported. There are many cancer patients experiencing the relapse and ultimately death because of treatment failure. However, not only resistance to chemotherapy-induced cell death but also highly metastasis can lead to failure of cancer treatment and aggressive behavior of cancer [3].

Cancer metastasis is a complicated process. It begins with the detachment from the primary tumor, migration and invasion through surrounding tissues, extravasation and survival in blood or lymphatic circulation. Then, metastatic cancer cells are carried to the distant target organs and proliferate at the secondary site [4]. For non-cancerous cells, detachment from extracellular matrix (ECM) usually induces a specific type of apoptosis, called anoikis. Anoikis is a self-defensive mechanism for extermination of inappropriate cells. However, aggressive cancer cells have developed several strategies to overcome anoikis such as increase of survival proteins, down-regulation of pro-apoptotic proteins and integrin switch [5].

Integrins are a large family of cell surface receptors mediating adhesion of the cell to the surrounding ECM. Heterodimeric structure of integrins composed of α and β subunit. The interaction between integrins and ECM is essentially requires for initiation of survival signal in both normal and cancer cells. Complete adhesion of integrins and ECM can activate diverse signals which regulates survival, proliferation,

migration and differentiation. Integrin-ECM interaction mediate survival signals through pathway of protein kinase B (AKT) and extracellular-signal-regulated kinases (ERKs) and also stimulate the expression level of anti-apoptotic Bcl-2 family proteins, Mcl-1 and Bcl-2. Meanwhile, pro-apoptotic proteins such as Bax, Bad, Bid and Bim are suppressed via integrin-ECM interaction [6]. Interestingly, the alteration of integrin subunit or integrin switch implicates in aggressive behaviors of cancer cells. Previous studies have demonstrated that integrin subunit of $\alpha 4$, αv, $\alpha 5$, $\beta 1$, and $\beta 3$ associate with cell invasion and metastasis [7, 8]. Furthermore, overexpression of integrin $\beta 1$ subunit involves with chemotherapeutic resistance in many types of cancer cells such as lung cancer, myeloma, myeloid leukemia, breast cancer and glioma [9].

In the past decade, considerable attention has been focused on the potential of natural compounds to enhance chemotherapeutic treatment and prevent cancer metastasis. Among them, alpha-lipoic acid (LA) has gained a lot of attention since it has been shown to possess anti-cancer activity against various cancer cells, predominantly through facilitating apoptosis [10-14]. The study in lung cancer demonstrated that LA has a pro-oxidant activity to generate reactive oxygen species (ROS) specifically superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and concurrently increase apoptosis. LA regulates anti-apoptotic Bcl-2 protein via H_2O_2-dependent proteasome degradation [11]. Additionally, the pro-oxidant effect of LA to generate ROS especially $O_2^{\cdot-}$ and enhance colon cancer cell apoptosis was reported. LA-induced apoptosis can be attenuated by pre-treatment with anti-oxidant [10]. Moreover, LA also demonstrate anti-metastatic effect on inhibition of migration and invasion in bladder cancer cells [15].

However, the sensitizing effect of LA on anoikis and chemotherapeutic susceptibility remains largely unknown. Therefore, this study aims to investigate the effect of LA and its related-mechanisms on detachment-induced cell death and apoptotic activity of conventional chemotherapy drugs in human non-small lung cancer cells. These might stimulate the development of this natural compound as synergistic therapy for treatment of aggressive lung cancer cells.

Research Questions

1. Does LA enhance apoptotic activity of conventional chemotherapy?
2. Does LA sensitize detachment-induced cell death?
3. Does LA affect the alteration of integrin expression pattern?
4. What are the possible mechanisms of LA in sensitization of chemotherapeutic activity and detachment-induced cell death?

Objectives

1. To evaluate the effect of LA on sensitization of chemotherapeutic drug-induced apoptosis in human non-small lung cancer cells.
2. To evaluate the effect of LA on anoikis sensitization
3. To evaluate the effect of LA in alteration of integrin expression.
4. To investigate the underlying molecular mechanisms of LA in sensitization of chemotherapeutic activity and anoikis in human non-small lung cancer cells.

Hypothesis

LA can sensitize anoikis and enhance anti-cancer effect of conventional chemotherapy in non-small lung cancer cells via down regulation of $\beta 1$, $\beta 3$, $\alpha 5$ and αV integrin subunits.

Expected benefits

The finding from this study, including the effect and underlying mechanisms of LA in potentiating anoikis and chemotherapeutic response may benefit the development of this natural compound as the combination therapy with conventional chemotherapeutic drugs in order to enhance treatment effectiveness. Furthermore, this study would initiate the development of LA as anti-metastatic drugs.

CHAPTER II
LITERATURE REVIEWS

1. Lung cancer

Lung cancer is the fundamental cause of death from cancer worldwide. This is due to the highly migrative and rapidly metastatic ability of lung cancer. There are two main types of lung cancer which grow and spread differently, including small-cell lung carcinomas or SCLCs (about 15% of all lung cancer cases) and non-small cell lung carcinomas or NSCLCs (about 85%). Although, SCLC tends to grow and spread much faster than NSCLC but the most common type of lung cancer is NSCLC [1, 16]. Depending on histology and staging of tumor pathology, recommended treatments for NSCLC are surgery, radiotherapy, chemotherapy, and targeted therapy. In early stage (stage I and II), the primary treatment is tumor resection followed by chemotherapeutic treatments. The first-line drug for treatment of lung cancer is a platinum-based (cisplatin or carboplatin). In addition to surgical resection, chemotherapy and radiotherapy are treatment strategies for advanced or metastatic tumor disease. It was claimed that chemotherapy significantly improved patient's overall survival rate in the clinical studies. Combination regimens between platinum-based with taxanes (paclitaxel, docetaxel, or vinorelbine), antimetabolites (gemcitabine or pemetrexed) or vinca alkaloids (vinblastine) are adviced for tumor pathology at stage IV [16]. However, successful rate of lung cancer treatment is very low. About 50% of patients face with the recurrence of cancer pathology mostly result from chemotherapeutic failure. Aggressive cancer cells can mediate intrinsic or acquired resistance mechanisms under chemotherapy [17].

2. Mechanisms of chemotherapy resistance

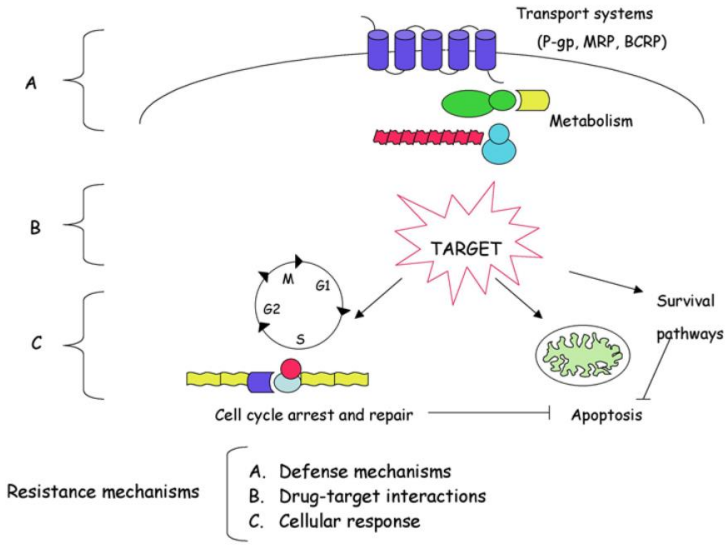

Figure 2.1 The main resistance mechanisms [18]

Resistance of chemotherapy drugs is commonly found in advanced metastatic cancer cells. There are two major pathway of chemotherapeutic resistance which are acquired and intrinsic mechanisms. Certainly, both manifestation resistant cells can develop simultaneous resistance of structurally and functionally diverse antitumor agents. The relevant mechanisms that contribute to cellular resistance including reduction of intracellular drug concentration via defensive factor, alteration of drug–target interaction, and changes in cellular response as shown in Figure 2.1. In particularly, the increase of cellular ability to repair DNA damage associated with resistance to chemotherapeutic drugs including alkylating agents, platinum compounds, and topoisomerase inhibitors [18].

Because anticancer drugs primarily induce apoptotic cell death, tumor cells can mediate chemotherapeutic resistance via up regulation of anti-apoptotic proteins or down regulation of pro-apoptotic proteins. In some types of tumors, a high level of

Bcl-2 or Mcl-1, anti-apoptotic Bcl-2 family proteins, result in poor response to chemotherapy. On the other hand, down-regulation of BAX expression associates with low apoptosis induced by chemotherapeutic drugs. Furthermore, some studies have shown that AKT, the serine/threonine kinase, survival signal is overexpressed in several metastatic tumor [19].

3. Apoptosis

Generally, cells have self-destruction programmed called apoptosis. There are two different pathways initiating apoptosis. According to Figure 2.2, the extrinsic pathway can be mediated by death receptors on the surface of the cell. Meanwhile, the intrinsic pathway in Figure 2.3 is triggered by cellular stress such as DNA damage, oxidative stress and microtubule disruption resulting in alteration of mitochondrial membrane. Activation of cysteine aspartyl-specific proteases (caspases) is a latter step of programed cell death from both pathways. Active caspases degrade cellular organelles leading to specific biochemical and morphological changes. Apoptotic induction is tightly regulated by a number of factors, including Bcl-2 family members, apoptotic inhibitor proteins, and several protein kinases [19].

(1) Extrinsic apoptosis pathway

The extrinsic signaling pathways associated with tumor necrosis factor (TNF) receptor including TNF, CD95 (Fas) and TNF-related apoptosis including ligand (TRAIL), play a crucial role in the cell death signal from the surface of the cell to the intracellular signaling pathways. This signal starts with the activation of CD95 or TNF receptor following with receptor recruitment of FADD and RIP into a death-inducing signaling complex (DISC). After that, procaspase8, an initiator caspase, which is activated by DISC complex, can lead to the cleavage of the downstream effectors including caspase 3, 6 and 7, resulting in apoptotic morphology [20, 21].

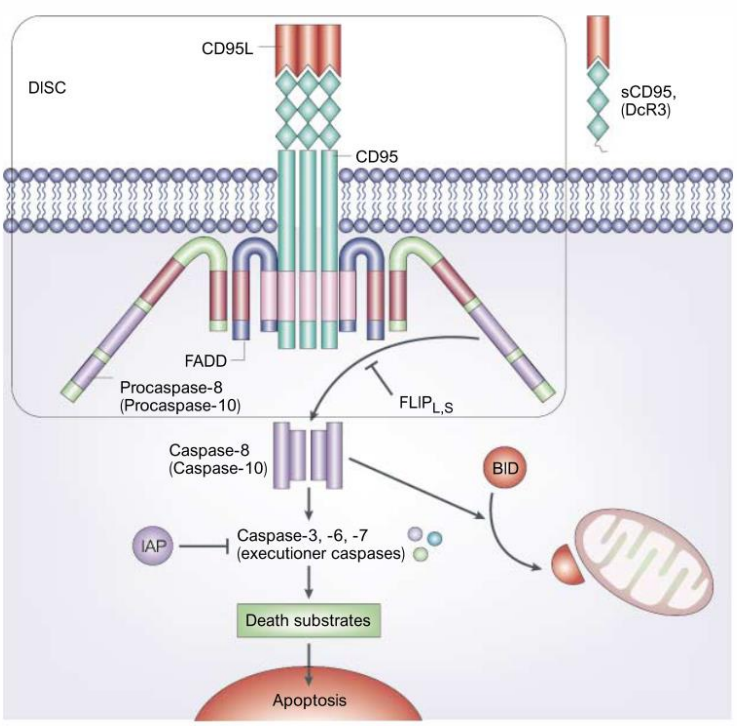

Figure 2.2 Apoptosis signaling through death receptors [19]

(2) Intrinsic or mitochondrial apoptosis pathway

The intrinsic pathway is associated with mitochondria that is triggered by various stimuli. Absence of growth factors, DNA damage and cellular oxidative stress cause a leakage on the mitochondria membrane leading to the imbalance of the mitochondrial transmembrane potential and the release of cytochrome c from mitochondrial intermembrane space into cytoplasm. The binding of cytochrome c to APAF-1 induces apoptosome forming which mediates the activation of caspase-9 execution caspase cascade. Intrinsic apoptotic pathways are regulated by the Bcl-2 family proteins. Anti-apoptotic proteins including Mcl-1, Bcl-2, and Bcl-XL prevent the formation of mitochondrial pores, protecting membrane integrity, and inhibiting the release of cytochrome c. While other pro-apoptotic proteins including Bax and Bak can translocate from the cytosol into mitochondria and can homodimerize or

heterodimerize with Bak or Bid resulting in forming mitochondrial pores, increasing membrane permeability, and releasing of cytochrome c [21, 22].

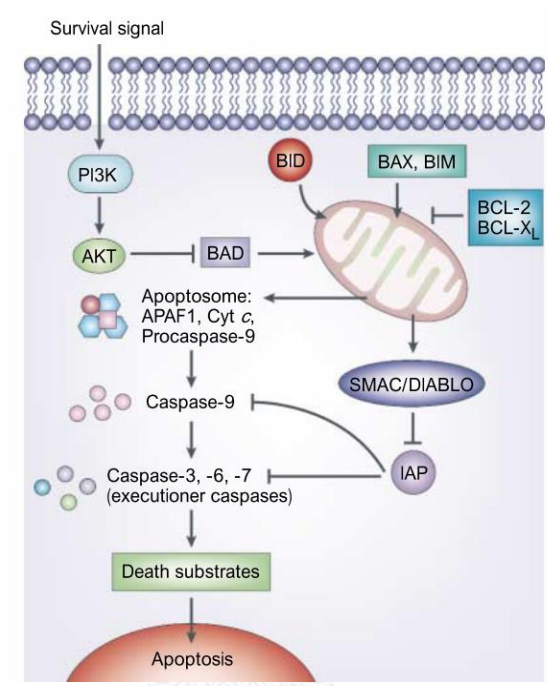

Figure 2.3 Apoptosis signaling through mitochondria [19]

4. Anoikis

Anoikis is a subset of apoptosis that is triggered by upon cell detachment from ECM. Anoikis acts as a regulating mechanism in prevention of anchorage-independent cell growth and attachment to an inappropriate environment, following with avoiding the colonization of secondary tumors or metastasis stage of cancer disease. The induction of anoikis program mediated by the interplay of two apoptotic pathways which intrinsic pathway or extrinsic pathway as shown in Figure 2.4. The Bcl-2 family proteins are the major proteins of both processes as same as apoptosis pathway [5, 6].

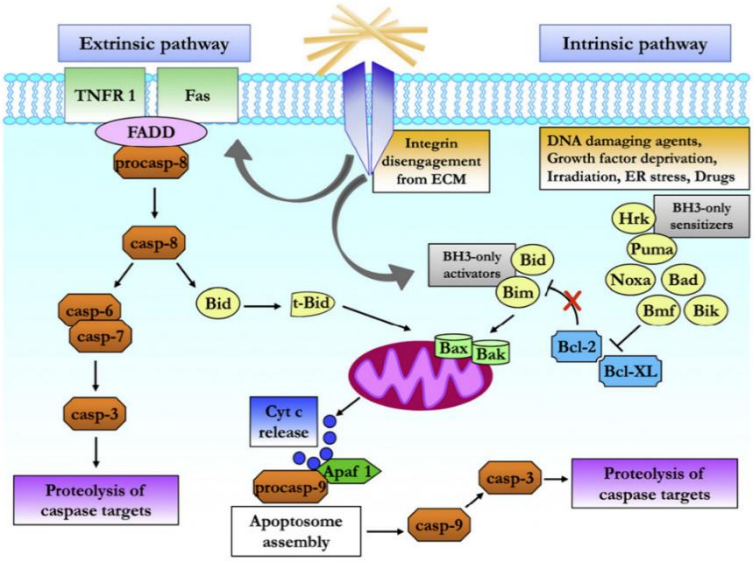

Figure 2.4 Molecular pathway of anoikis [5]

However, cancer cells can rapidly adapt several mechanisms to resist anoikis by activation of survival signaling or inhibition of apoptotic pathway. Anoikis resistance is a critical step that caused the detached cancer cells from primary tumor still survive in the absence of adhesion, which facilitates cancer cells ability to metastasize to distant organs from the tissue that cancer cell originated in as shown in Figure 2.5.

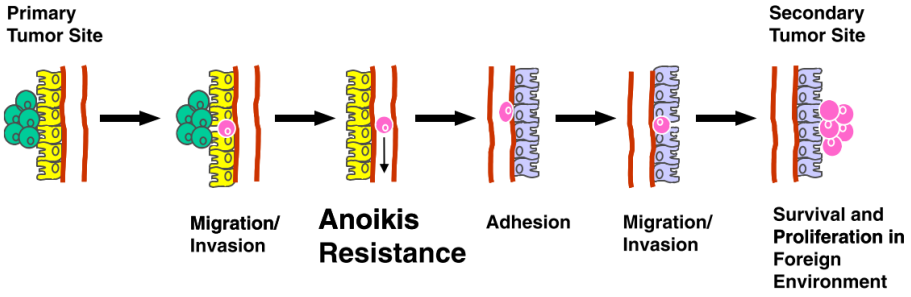

Figure 2.5 Step in metastasis [23]

Anoikis resistance mechanism

When the cell detach from ECM, cancer cells can evade anoikis by altering expression of adhesion receptor or integrin switch, promoting an epithelial–mesenchymal transition (EMT)-like phenotype in Figure 2.6. Moreover, tumor cells can upregulate downstream pro-survival signals, such as phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), NF-κB and Rho GTPase. These prosurvival signals result in survival, proliferation and migration pathways. The overexpression of receptor tyrosine kinases (RTKs) also leads to the suppression of anoikis. Alternatively, the other mechanisms including autophagy and entosis deregulate and adapt their metabolism to escape apoptosis and promote survival [5, 24].

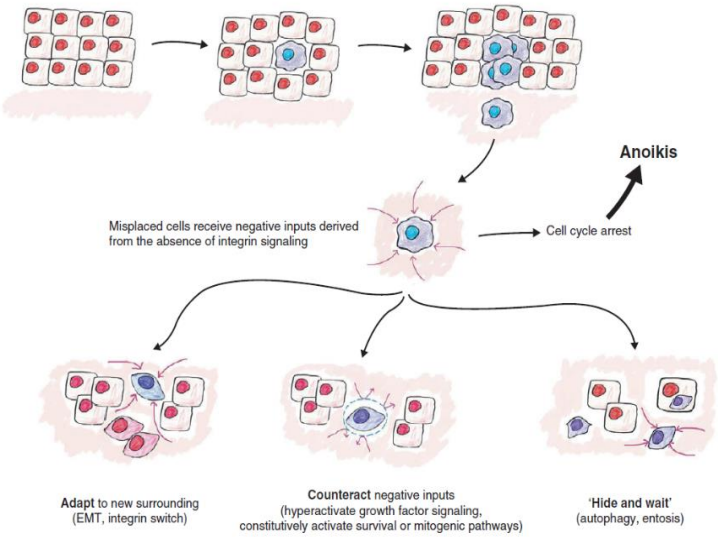

Figure 2.6 Mechanisms of overcome anoikis [24]

5. Integrins

Integrins are glycoproteins transmembrane receptors consisting of 18α and 8β subunits which non-covalently bind to form 24 different transmembrane heterodimers. Basic structure of integrin is shown in Figure 2.7. Each specific heterodimerize integrin differently bind to ECM proteins [25, 26].

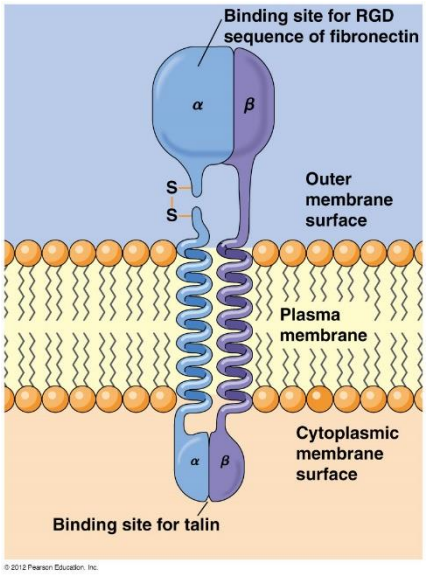

Figure 2.7 Basic structure of integrin

Integrins can be classified into three groups by main functional including vascular integrins, cell-cell adhesion integrins, and cell-ECM adhesion integrins [27]. Thus, they play important roles in many biological processes including migration, invasion, survival, and proliferation. Activated integrins can be induced by cytoplasmic event called 'inside-out activation' and integrin-ligand interaction called 'outside-in activation'. Regulation of integrin function associated with conformational changes, integrin clustering, and integrin trafficking. Endocytosis plays an crucial step of integrin turnover and integrin redistribution in anchorage dependent cells [28].

5.1 Integrin signaling in normal cells survival

Integrins bind to ECM result in signaling that necessary for cells survival. There are four heterodimers of integrins including $\alpha5\beta1$, $\alpha v\beta3$, $\alpha1\beta1$ and $\alpha6\beta1$ recognized as the essential signal transducer for cell survival. Integrins also communicates signals from the outside of the cell directly into the cell through the recruitment of non-receptor tyrosine kinases such as focal adhesion kinase protein (FAK) and Src families. These activations lead to the activation of many cellular signaling pathways especially MAPK and PI3K pathways as shown in Figure 2.8. In addition, integrins regulate the expression and activity of the Bcl-2 protein family. For example, ligation of integrins $\alpha5\beta1$ or $\alpha v\beta3$ result in increased expression of Bcl-2. In the other way, ligation of integrin $\alpha v\beta3$ also inhibit expression of Bax, by downregulation of transcriptional activity of p53 [29].

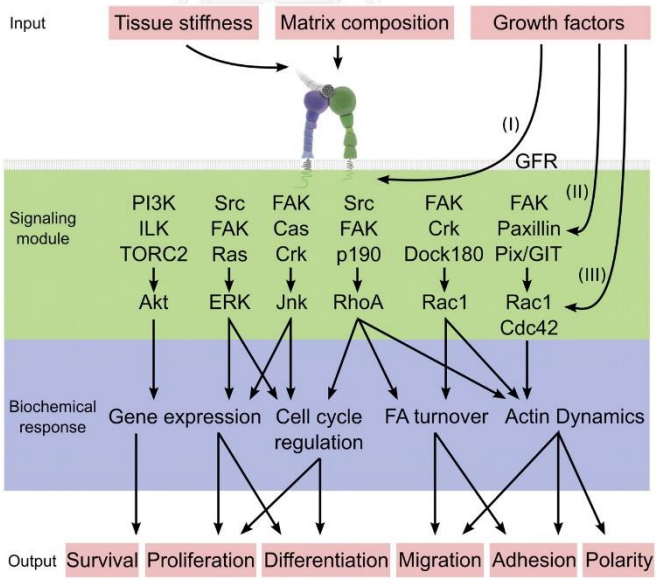

Figure 2.8 Integrin signaling [30]

5.2 Integrins in cancer

The integrin family of cell adhesion receptors play an importance role in cellular functions in both normal and cancer cells. Binding between integrins and ECM provide signaling necessary for cell migration, invasion, proliferation, survival, angiogenesis and trigger to tumor progression. In addition, integrin can crosstalk with growth factor receptors. Integrin dimerization mediates cell survival via several downstream pathways including activation of PI3K-AKT pathway, MAPK pathway and NF-κB signaling leading to an up regulation of Bcl-2 or FLIP, and a down regulation of p53. Each heterodimer integrin receptor regulates different pathways. For example, αvβ3 can crosstalk with fibroblast growth factor receptor (FGFR) resulting in prevention of apoptosis through inhibit apoptosis pathway. Moreover, αvβ3 extremely increases anchorage-independent tumor cell survival [26].

5.3 Role of integrin in chemotherapeutic resistance

Interestingly, several studies have shown that integrin is obviously associated with chemotherapeutic resistance. Overexpression of integrin β_1 was found to enhance chemotherapy resistance in small cell lung cancer due to the activation of the PI3K-AKT pathway [31, 32]. In multiple myeloma, α4β1 and α5β1 bind to fibronectin contributed to resistance to doxorubicin and melphalan [33]. In breast cancer cells, α2β1 and α5β1 have been shown that protect from vincristine and paclitaxel [34]. Glioma cells are protected from the topotecan when adhered to vitronectin through αvβ3 and αvβ5 [35]. Some integrins subtypes including α2, α4, α5, and β1 interacted with the CXCR4 chemokine receptor induces chemoresistance [36]. Furthermore, silencing of β1 integrin bring about sensitization chemotherapeutic effect of cisplatin and gefitinib and leading to impaired migration and invasion ability of the non-small cell lung cancer A549 cells [37].

5.4 Role of integrin in anoikis resistance and cancer metastasis

In non-cancerous cells, the interaction of integrins and ECM contributes to cell-adhesion. Adhesion of cell to ECM promote pro-survival signals, while detachment from the ECM triggers normal cell to undergo ‘‘anoikis’’. Conversely, cancer cells have an unusual character that allows them to escape anoikis as they can survive and growth in anchorage-independent condition. Therefore, cancer cells may metastasize to other organs. The mechanisms of anoikis resistance not only integrin switch, but also crosstalk with other kinases and adaptor proteins in focal complexes. Result in, generation of downstream pro-survival signals such as the PI3K/AKT and MEK/ERK pathways. It was known that the switching in integrin expression can lead to different downstream signaling pathways. In melanoma cells $\alpha v\beta 3$ integrin has a positive role in induction of anoikis resistance. High expression levels of $\alpha v\beta 5$ and $\alpha v\beta 6$ contribute to anoikis resistance phenotype. In addition, overexpression of $\beta 4$ integrin causes an activation of PI3K and inducing anoikis resistance. Activation of PI3K/AKT pathway can regulate transcription factors activities that control the level of expression of apoptotic genes or phosphorylation of pro-apoptotic proteins and inhibit their functions [5]. Moreover, integrin plays a role in cancer cell migration and invasion. Several studies have shown increased expression of $\alpha v\beta 3$, $\alpha 6\beta 4$, $\alpha 5\beta 1$ enhance tumor cell invasiveness and metastasis [8, 38].

6. Reactive oxygen species (ROS)

ROS are highly reactive oxygen-containing molecules that have a single unpaired electron. There are two major types of ROS, free oxygen radicals such as hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet -}$), nitric oxide ($NO^{\bullet}$), organic radicals ($R^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and non-radical ROS including hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), organic hydroperoxides (ROOH), hypochloride (HOCl). Among them, $O_2^{\bullet -}$, H_2O_2,

$^{\bullet}OH$ and $NO^{\bullet}$ are the most studied ROS in cancer. ROS are essential for biological functions. They play an important role in interaction and modification the structure of proteins, transcription factors and genes. Additionally, ROS are associated with cell survival, growth, differentiation and inflammation.

ROS is major generated in the mitochondria. In general, electron leakage from the respiratory complex of mitochondrial can react with oxygen, leading to formation of $O_2^{\bullet-}$, which can be transformed to other ROS [39].

In cancer cells, increased of ROS generation have been reported. Role of $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$ and $NO^{\bullet}$ are wildly investigated in various cancer cells. High levels of ROS in cancer cells can result from augmented metabolic activity, increased cellular receptor signaling, oncogene activity, increased activity of inflammatory enzymes such as cyclooxygenases, lipoxygenases, or through crosstalk with immune cells. Increasing of ROS generation in cancer cells lead to raise of antioxidant capacity and maintain the ROS levels under the toxic threshold, whereas excessive amounts of ROS above the toxic threshold level, resulting in cell death as shown in Figure 2.9 [39, 40].

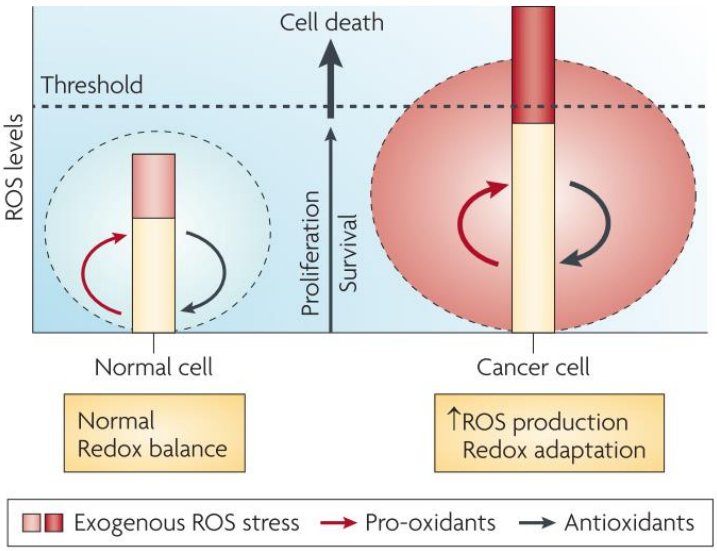

Figure 2.9 Cancer redox biology: biological basis for therapeutic selectivity [40]

Mounting evidence reported that ROS are the key mediator of Bcl-2 down-regulation and induced cancer cells apoptosis [11, 41-43]. On the other hand, some study reported that H_2O_2 inhibit cancer cells anoikis and mediated chemotherapeutic resistance [44, 45]. Moreover, the function of integrin strongly relate with cellular ROS level. The interaction between integrin and ECM stimulate the production of cellular ROS which in turn is key mediator for activation of various integrin-signaling pathways [46, 47]. Integrin activation is associated with increased ROS production by NADPH-oxidases, 5-lipoxygenase, and release from mitochondria [48]. However, ultimate alteration of cellular redox status might abolish the function of integrin. Induction of cellular H_2O_2 has been demonstrated to suppress adhesive function of integrin β1 [49].

7. Alpha-lipoic acid (LA)

LA is a natural substance synthesized in small amounts by plants, animals and human body. It is essential for several mitochondrial enzyme complexes as a co-factor such as pyruvate, α-ketoglutarate, and branched chain α-keto acids [50]. Its structure is an octanoic acid bridged with two sulfurs as shown in Figure 2.10. LA can rapidly converted to reduced form, dihydrolipoic acid (DHLA) in many tissues by mitochondrial dihydrolipoamide dehydrogenase and cytosolic glutathione reductase. The major of LA metabolism is mitochondrial β-oxidation. For soluble ability, LA can soluble in methanol, ethanol, diethyl ether and chloroform but not in water (XLogP3 = 1.7) [51].

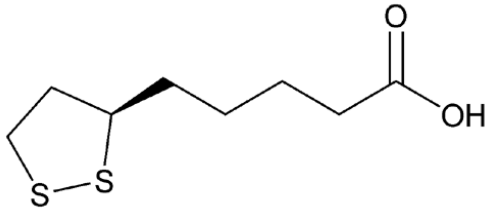

Figure 2.10 The structure of LA

In pharmacological properties, LA has been shown to act as a pro-oxidant as well as an anti-oxidant depending on cell type and cellular redox status. Some studies reported that LA and DHLA act as a free radical scavenger, namely hydroxyl radical, hypochlorous acid, and singlet oxygen. In addition, LA can assist in regenerate endogenous antioxidants, including vitamins C and E and glutathione [51-53]. LA has been reported that can increase the amount of intracellular glutathione (GSH) in melanoma cell lines (*in vitro*) and in murine neuroblastoma (*in vivo*) [54]. On the other hand, LA was reported to exert prooxidant properties *in vitro*. LA and DHLA were shown to promote mitochondrial permeability transition (MPT) in hepatocytes and isolated rat liver mitochondria and stimulate superoxide anion production. Moreover, LA and DHLA have been shown to induced mitochondrial Ca^{2+} release [51].

LA is proved to be effective against diabetic peripheral neuropathy [55], atherosclerosis [56], inflammatory skin diseases [57], reperfusion arrhythmias [58], and Parkinson's disease [59] because it's antioxidant properties. Conversely, several studies have reported that LA has anti-cancer activities. It facilitates cancer cells apoptosis in various cancer cells such as breast, hepatic, leukemic, colon, and lung cancers via down regulation of AKT survival pathway, inhibition of anti-apoptotic Bcl-2 proteins and/or activation of pro-apoptotic proteins [10-14, 60]. LA It was reported that LA act as pro-oxidant by increasing mitochondrial $O_2^{\cdot-}$production in human colon cancer HT29 cells [10]. The colon cancer cells were also shown to have a lower antioxidant capacity and were more susceptible to LA-induced apoptosis than the normal non transformed cells. LA has been shown ability to increase ROS generation lead to p53 activation, induction of Bax and induced apoptosis in hepatoma cells [12]. Moreover, pro-oxidant activity of LA has reported in human lung epithelial H460 cells. Rapid generation of H_2O_2 induced by LA triggered to increase antiapoptotic Bcl-2 degradation and, eventually cell death [11]. In addition to anti-cancer activity, LA also has an anti-metastasis to inhibit cell migration and invasion in bladder cancer via down regulation

of β1 integrin [15] and anti-proliferative in NSCLC [61]. While there are many studies report that LA can protect chemotherapy toxicity such as intestinal toxicity from methotrexate [62], hyperlipidemic cardiomyopathy from cyclophosphamide [63], ototoxicity and nephrotoxicity from cisplatin [64, 65], and cardiotoxicity from doxorubicin [66].

Although, LA has several pharmacological properties, however, whether LA sensitizes anoikis and enhance the effect of chemotherapy in human non-small cell lung cancer remains largely unknown. This study, therefore aims to investigate the effects of LA on anoikis induction and enhance chemosensitivity and its related-mechanisms.

CHAPTER III
MATERIALS AND METHODS

Materials

1. Cell culture

Human lung cancer epithelial H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). H460 cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 100 U/ml of penicillin/ streptomycin in a 5% CO_2 environment at 37°C. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM EDTA. Cells were cultured until reach the 100% confluence before using.

2. Chemicals and Reagents

RPMI 1640 medium, FBS, L-glutamine, penicillin/ streptomycin, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from GIBCO (Grand Island, NY, USA). LA, cisplatin, catalase, Hoechst33342, N-acetylcysteine (NAC), propidium iodide (PI), dimethysulfoxide (DMSO), absolute ethanol, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA), dihydroethidium (DHE) and hydroxyphenyl fluorescein (HPF) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Paclitaxel, etoposide, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) were obtained from Calbiochem (San Diego, CA, USA). WST-1 was obtained from Roche Applied Science (Mannheim, Germany). Agarose was obtained from Bio-Rad (Hercules, CA, USA). Antibodies for Bcl-2, Mcl-1, Bax, caspase-3, FAK, phosphorylated FAK (Y397), AKT, phosphorylated AKT (S473), integrin α5, integrin αv, integrin β1, integrin β3, β-actin and peroxidase-labeled specific

secondary antibodies were obtained from Cell Signaling Technology, Inc. (Denver, MA, USA).

3. Equipments

Laminar flow cabinet (Bosstech, Bangkok, Thailand), carbon dioxide incubator (Thermo fisher scientific, Waltham, MA, USA), autopipette: 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl (pipetman, Gilson, Ohio, USA), pipette tips for 2-10 µl, 10-100 µl, 20-200 µl and 200-1,000 µl (Corning, NY, USA), cell culture plate: 96-well, 24-well, 6-well, and ultra-low attachment plate (Nunc, Corning, NY, USA), conical tube: 15 ml and 50 ml (Neptune, Corning, NY, USA) , bottle: 100 ml, 250ml, 500 ml and 1,000 ml (Duran, Mainz, Gernany), Automated cell counter (TC20, Bio-Rad, Singapore), pH meter (SevenCompactS220, Mettler-toledo, Bangkok, Thailand), vertex mixer (Scientific industries, NY, USA), centrifuge (Z383K, HERMEL Laboratechnik, Germany), ELISA reader (Anthros, Durham, NC, USA) and fluorescence microscope (Olympus IX51 with DP70, Japan).

Methods

1. Sample preparation

Various concentrations of LA were prepared by dissolved with absolute ethanol and diluted by completed RPMI 1640 media. The final concentration of absolute ethanol used in all of the experiments was 0.1%. The results from the treated cells were compared with the non-treated cell exposed to the 0.1% final concentration of absolute ethanol.

2. Assessment of cell viability assay

Cell viability was determined by MTT colorimetric assay which, measures the cellular capacity to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide or MTT (yellow) to the purple formazan crystal by mitochondria dehydrogenase enzymes. Briefly, cells in 96-well plate were incubated with 0.4 mg/ml of MTT for 4 h at 37°C. The supernatant was then removed and 100 µl DMSO was added to dissolve the formazan product. The intensity was measured at 570 nm using an ELISA reader (Anthros, Durham, NC, USA). The absorbance of crystal formazan is referred to amount of living cells. All analyses were performed at least three independent replicate cultures.

Cell viability is calculated as follow:

$$Cell\ viability\ (\%) = \frac{A570\ with\ treatment}{A570\ with\ control} \times 100$$

3. Proliferation assay

Human lung cancer cells were seeded at a density of 2×10^3 cells/well in 96-well plates. Cell proliferation was determined by MTT assay at 0, 24, 48, and 72 h after exposure to LA at indicated concentrations (0-10 µM) for 48 h. The absorbance of formazan product which was dissolved by DMSO was measured by spectrophotometry at 570 nm using a microplate reader.

4. Anoikis assay

Cell viability in detachment condition was determined by WST-1, tetrazolium salt. WST-1 is cleaved to a soluble formazan by the succinate-tetrazolium reductase, which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. Briefly, cells were treated with non-toxic concentrations of LA for 48 h and then detached into a single-cell suspension in the RPMI serum free medium. After that cells were seeded into ultra-low attachment plate and incubated at 37°C, 5% CO_2 for various times up to 24 h. After specific times of incubation,

detached-cell suspension were seeded in 96-well plate. Then, WST-1 reagent was added and incubated for 2 h. The intensity of formazan dye was measured at 450 nm using an ELISA reader (Anthros, Durham, NC, USA).

Cell viability is calculated as follow:

$$Cell\ viability\ (\%) = \frac{A450\ with\ treatment - A450\ blank}{A450\ with\ control - A450\ blank} \times 100$$

5. Nuclear staining assay

Apoptotic and necrotic cells death were detected by Hoechst33342 and propidium iodide (PI) co-staining. After specific treatments, cells were stained with 10 μM of the Hoechst and 5 μg/ml PI dyes for 30 min at 37°C. The apoptotic cells having condensed chromatin and/or fragmented nuclei stained with Hoechst33342 and PI-positive necrotic cells were visualized and scored under a fluorescence microscope (Olympus IX51 with DP70).

6. Colony formation assay

Anchorage-independent growth was determined by the colony formation assay in soft agar. Briefly, H460 cells were treated with non-toxic concentrations of LA for 48 h and prepared into a single-cell suspension in RPMI containing 10% FBS and 0.33% low melting temperature agarose. After that, 250 μl of these single-cell suspension were plated into a 24-well plate over a 250 μl layer of solidified RPMI supplemented with 10% FBS and 0.5% agarose. Completed RPMI media was add about 250 μl every three days. The survival colonies were visualized and scored under a microscope after 14 days.

7. Western blot analysis

After specific treatments, cells were incubated in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 % Triton X-100, 150 mM sodium chloride, 10 % glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN, USA) at 4°C for 60 min on ice. Cell lysates were collected and determined for protein content using the BSA assay (Bio-Rad, Hercules, CA, USA). Proteins (60µg) were resolved under denaturing conditions by 10 % SDS-PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h in 5 % non-fat dry milk in TBST (25 mM Tris–HCl, pH 7.4, 125 mM sodium chloride, 0.05 % Tween 20) and incubated with appropriate primary antibodies at 4 °C for 12 h. Membranes were washed three times with TBST for 5 min and incubated with horseradish peroxidase-labeled isotype specific secondary antibodies for 1 h at room temperature. The immune complexes were then detected by an enhanced chemiluminescence detection system (Supersignal West Pico; Pierce; Rockford, IL, USA) and quantified using Analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA, USA). Protein expression level of Bcl-2, Mcl-1, Bax, caspase-3, FAK, p-FAK, AKT, p-AKT, α5 integrin, αv integrin, β1 integrin, β3 integrin, β-actin) were investigated and β-actin was used as a loading of control in each treatment.

8. ROS detection

Intracellular H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ were determined by flow cytometry using $DCFH_2$-DA, DHE and HPF as fluorescent probes, respectively. Cells at 1.5 x 10^5 cells/well were seeded overnight into 6-well plates. Before LA treatment, the cells were incubated either with 10 µM $DCFH_2$-DA, 10 µM DHE or 10 µM HPF for 30 min at 4°C, after which they were washed with PBS and treated with 10 µM of LA for 1-6 h. After indicated time, cells were washed, resuspended in PBS, and immediately analyzed for

fluorescence intensity using FACSCaliber (Beckton Dickinson, Rutheford, NJ, USA) at the excitation and emission wavelengths of 488 and 538 nm, respectively, for detecting DCF fluorescence, at 488 and 610 nm for DHE and 490 and 515 nm for HPF. Mean fluorescence intensity was quantified by CellQuest software analysis of the recorded histograms. Relative fluorescence was calculated as a ratio of the treated to the non-treated control fluorescence intensity.

9. Statistical Analysis

All data were expressed as the means ± SD from three or more independent experiments. Multiple comparisons were examined for significant differences of multiple groups, using analysis of variance (ANOVA), followed by individual comparisons with Scheffe's post-hoc test. Statistical significance is set at $P<0.05$

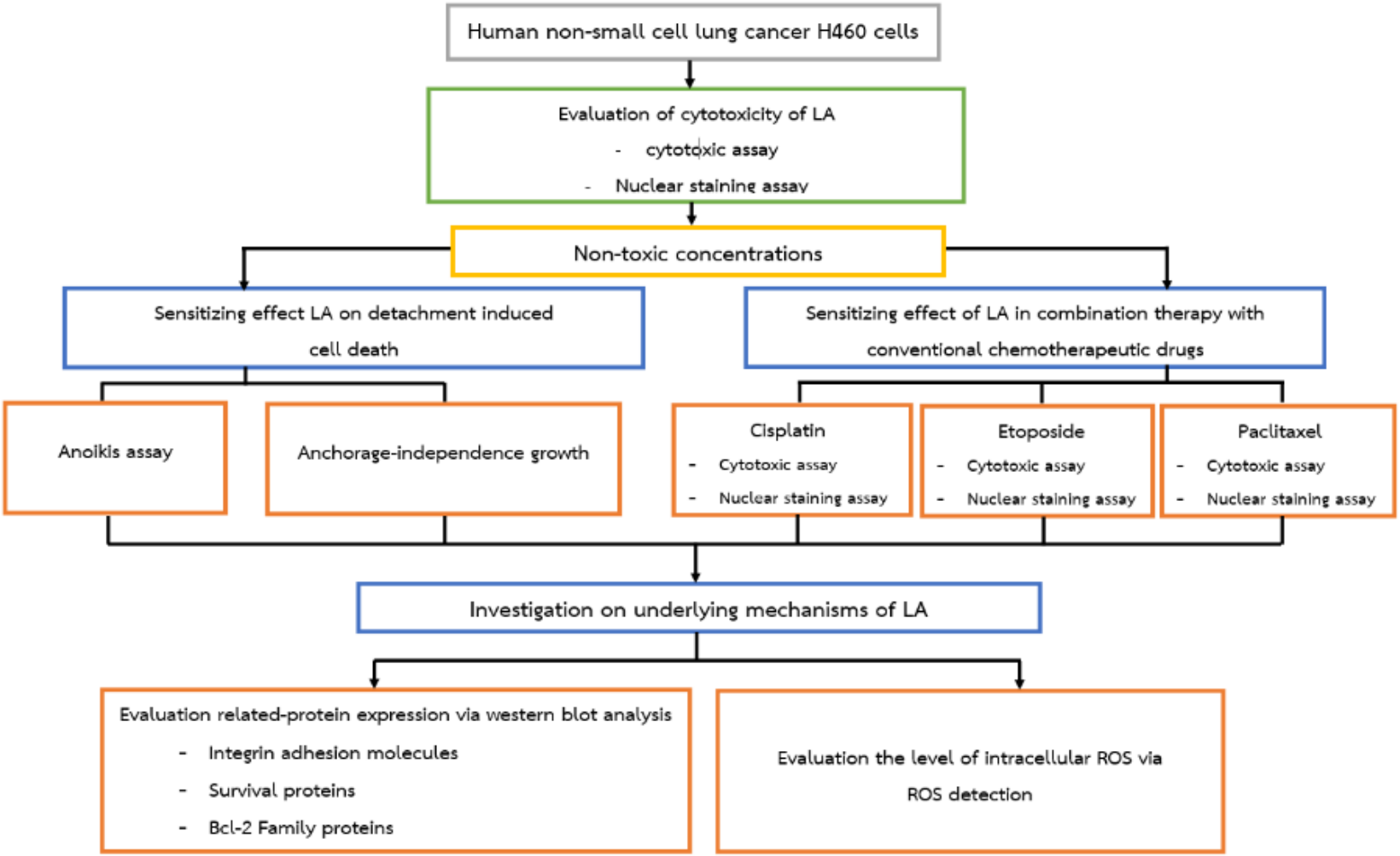

Figure 3.1 Experimental designs

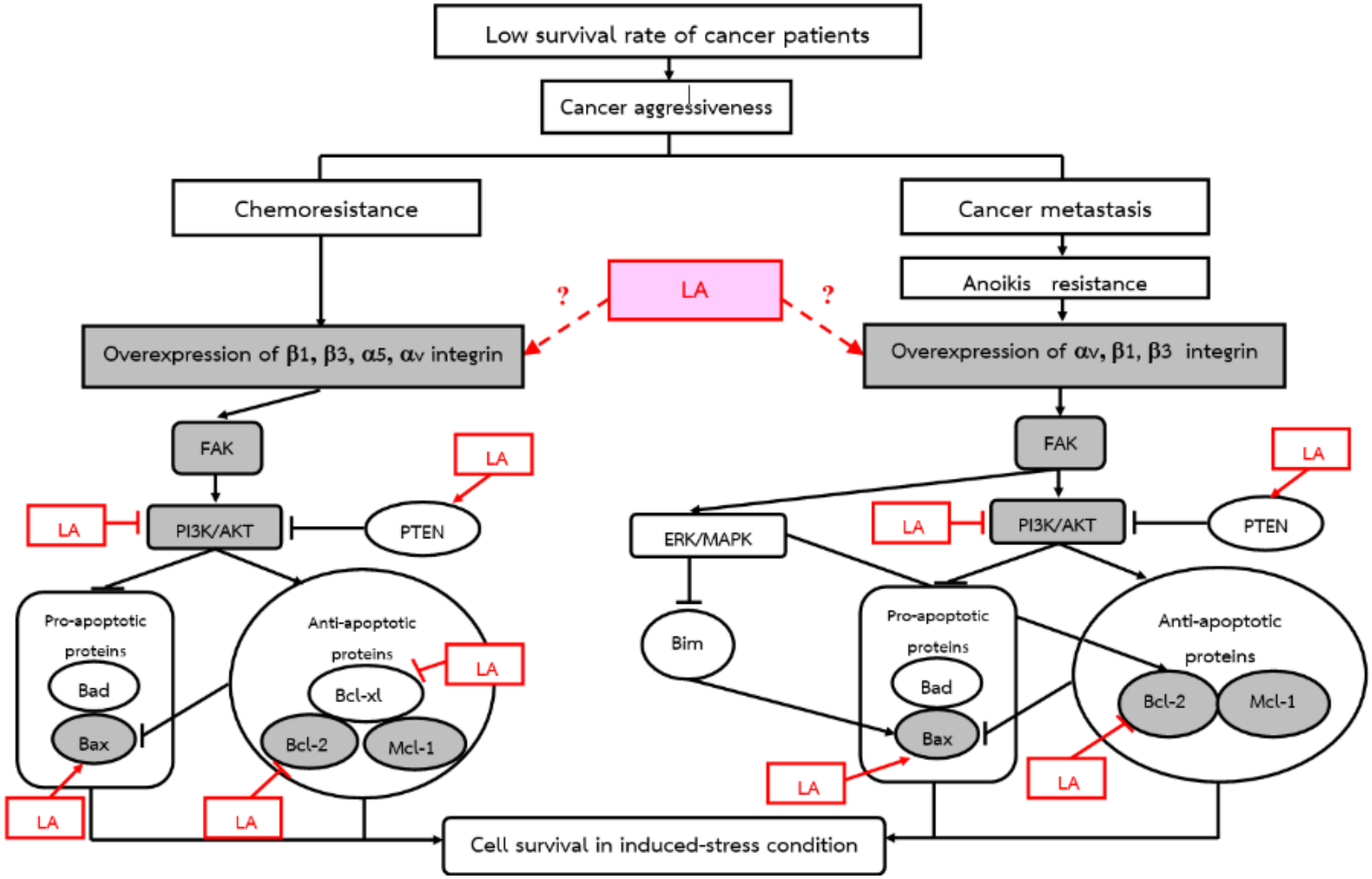

Figure 3.2 Conceptual framework of this study

Part I : Investigation on the cytotoxic effects of LA in human lung cancer cell H460 cells.

1. Effect of LA on cell viability in H460 cells

To eradicate the direct cytotoxic effect of LA, which overwhelm the sensitizing effect. The cytotoxic effects of LA in human lung cancer H460 cells in attachment condition was first characterized. Cells were seeded at $5x10^3$ cells/well in 96-well and culture at 37 C 5% CO_2 for 12 h. Then, they were treated with various concentrations of LA (0-100 μM) and incubated at 37°C for 48 h. Cell viability and mode of cell death were determined by MTT and nuclear staining assay (Hoechst - PI staining assay). Non-toxic concentrations of LA which cause no significant effect and not less than 90% on cell viability at 48 h were used in further experiments.

2. Effect of LA on proliferation in lung cancer H460 cells

To study the effect of LA on cell proliferation of human lung cancer cells, H460 cells. Cells were seeded at 1×10^3 cells/well in a 96-well plate for 12 h. The cells were then pretreated with non-toxic concentrations of LA for 48 h and further cultured for various times (at 24, 48, and 72 h). Then, cell viability was examined by MTT assay.

Part II : Investigation on the sensitizing effect of LA on anoikis in human lung cancer H460 cells.

1. Effect of LA on anoikis in human lung cancer H460 cells

To study the effect of LA on detachment-induced cell death, H460 cells were seeded at $1.5x10^5$ cells/well in a 6-well plate. Then, the cells were pretreated with various non-toxic concentrations of LA for 48 h. Pretreated cells were then detached into a single-cell suspension in RPMI serum free medium and seeded into ultra-low attachment plate at a density of 1×10^5 cells/well. After 0-24 h of incubation

time, cell survival was determined by WST-1 assay and modes of cell death were determined using nuclear staining assay (Hoechst - PI staining assay).

2. Effect of LA on anchorage-independent growth of human lung cancer H460 cells.

An ability to growth in anchorage-independent condition has been shown to be tightly related to the anoikis resistance potential that allows cancer cells to survive in the absence of adhesion and travel to the secondary site (5, 6). This study, therefore investigates the effect of LA on anchorage-independent growth through colony formation or soft agar assay. H460 cells were cultured layer of agarose in order to mimic detachment condition. Briefly, 250 μl of the bottom layer which is a mixture of 1% agarose and completed RPMI 1640 media at a ratio 1:1 was prepared in 24 well-plate. The upper layer was prepared from 1% Agarose and a single cell suspension of pretreated-H460 cells at non-toxic concentration for 48 h. Finally, 250 μl upper layer contains 0.335 agarose with the cells at 1,000 cell/well. Then completed RPMI medium was added on top. Fresh completed RPMI medium (250 μl/well) was added every 3 days. The colony formation was visualized and scored under a microscope after 7 and 14 days.

Part III : Investigation on the effect of LA on susceptibility of human lung cancer H460 cells to chemotherapeutic agents.

Human lung cancer cells were pretreated in the present and absent of various non-toxic concentrations of LA for 48 h. After incubation, cells were exposed to anticancer agents, including low dose cisplatin, etoposide or paclitaxel for 24 h and cell viability was determined by MTT assay**.** Apoptosis and necrosis were determined using nuclear staining assay (Hoechst - PI staining assay).

Part IV : Investigation on the underlying mechanisms of LA in human lung cancer H460 cells.

1. **Investigation on the involve mechanisms of LA in human lung cancer H460 cells.**

Anoikis is triggered by loss of cell anchorage which associated with change in integrin level and related-survival downstream signaling. To investigate effect of LA on regulation of proteins in anoikis mechanism, H460 cells were pretreated with various non-toxic concentrations of LA. After that the levels of anoikis regulating-proteins, including α5, αv, β1, β3 integrin, p-FAK, FAK, p-AKT, AKT were determined by western blot analysis with specific antibody of each protein.

2. **Investigation on the involve mechanisms of LA on susceptibility of human lung cancer H460 cells to chemotherapeutic agents.**

Several studies have been shown that β1 integrin enhance chemotherapy resistance in various cancers. In order to investigate the possible mechanisms of LA on the susceptibility of chemotherapy drugs. H460 cells at $1x10^5$ cells/well in 6-well plate were treated with various sub-toxic concentrations of LA for 48 h. After that the levels of proteins such as p-FAK, FAK, p-AKT, AKT, Mcl-1, Bcl-2, and Bax were determined by western blot analysis with specific antibody of each protein. Additionally, the level of interested-proteins in H460 cells incubated with low dose cisplatin, etoposide or paclitaxel in presence or absence of LA were also evaluated.

Part V : Investigation on the underlying mechanism of LA in regulating integrin

1. **Investigation on the level of intracellular ROS induced by LA in H460 cells**

Previous studies have indicated that the LA possesses the pro-oxidant activity to generate ROS in attached condition [10, 11]. To prove the ability of LA to generate ROS in human lung cancer cells, H460 cells. Cells were seeded at a density of 1×10^5 cells/ml. The cells were pretreated with ROS probe before treated with various sub-toxic concentrations of LA and incubated at 37°C for 0-6 h. After incubation, intracellular ROS detection was then determined by flow cytometry as mentioned in methods using $DCFH_2$-DA as a fluorescent probe for H_2O_2, DHE as a probe for $O_2^{\cdot-}$ as well as HPF as a probe for $O_2^{\cdot-}$.

2. **Investigation on the effect of ROS induced by LA on integrin expression, anoikis induction and chemosensitization in H460 cells.**

To determine the effect of ROS induced by LA on integrin expression and sensitizing effect of anoikis and chemotherapeutic induced-apoptosis cell death. Cells were pretreated with pan- or specific ROS scavengers, treated with LA, and subjected to anchorage-independent growth or drug sensitization assays as previously described.

CHAPTER IV
RESULTS

1. Effects of LA on viability of human lung cancer H460 cells

To investigate the cytotoxic effect of LA, the non-toxic concentrations of LA were firstly determined. Human lung cancer H460 cells were cultured in RPMI medium in the absence or presence of LA (0-100 μM) for 48 h, and cell viability was determined by MTT viability assay.

The results indicated that treatment with 0-10 μM of LA caused no significant difference of % cell viability in H460 lung cancer cells compared with non-treated control cells (Fig. 4.1A). The cytotoxicity effect of LA was early observed in the presence of 50 μM LA with approximately 85% viable cells. To confirm the effect of LA on cell toxicity, mode of cell death was evaluated by Hoechst33342 and PI co-staining assay. Figure 4.1B,C demonstrate that apoptotic cells containing condensed and/or fragmented nuclei were not detectable in response to LA treatment at the concentrations of 0-10 μM. Treatment doses of 50 and 100 μM caused a significant increase in cell apoptosis over the control, while necrosis was barely detected in all concentrations.

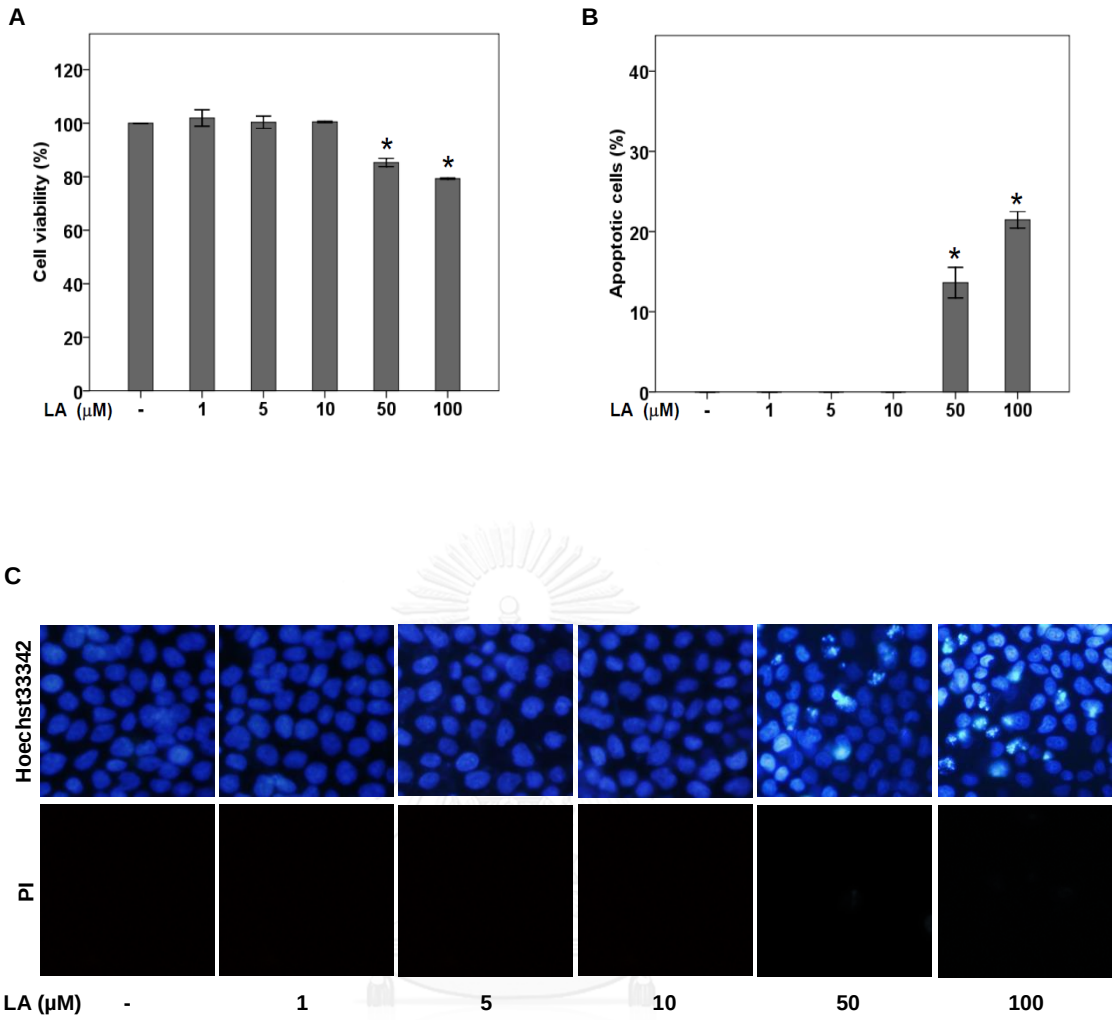

Figure 4.1 Effect of LA on cell viability of human lung cancer H460 cells. After incubation of the H460 cells with various concentrations of LA (0-100 μM) for 48 h, **A** cell viability was determined by MTT assay. Mode of cell death was evaluated by Hoechst33342 and PI co-staining. **B** % Cell apoptosis was calculated from the cells presenting condensed and fragmented DNA. **C** Apoptosis and necrosis cells were captured under fluorescent microscope. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

2. LA inhibits cell proliferation in human lung cancer H460 cells

To determine the effect of LA on cell proliferation. Cells were pretreated with non-toxic concentrations (0-10 μM) for 48 h. Then, pretreated cells were subjected to plate in 96-well plates. Cell proliferation was determined by MTT assay at 0, 24, 48, and 72 h.

Figure 4.2 showed that LA at 10 μM significantly decreased the relative cell proliferation at 24 h of incubation time when compared with non-treated control. For 48 and 72 h of incubation, relative proliferation obviously decreased in all LA-treated group in dose dependent manner. Taken together, 10 μM of LA obviously suppressed proliferation of human lung cancer cells.

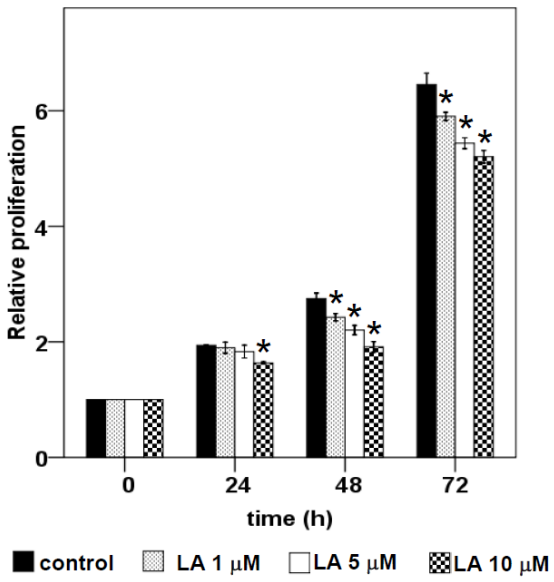

Figure 4.2 Effect of LA on cell proliferation. H460 cells were pretreated with non-toxic concentrations (0-10 μM) of LA for 48 h and subjected to cell proliferation assays. Effect of LA on cell proliferation was evaluated by MTT assay after culturing in the normal condition for 0-72 h. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

3. LA sensitizes anoikis and inhibits anchorage-independent growth

Anoikis, a critical mechanism in prevention of adherent-independent cell growth and attachment to an inappropriate matrix, following with avoiding colonizing of distant organs or metastasis stage of cancer disease. The ability to grow independently of cell adhesion of tumor cells has been shown to be an important hallmark of aggressive metastatic cells [5, 6]. Next, the effect of LA on anchorage-independent survival and growth was investigated by soft agar colony formation assay. The cells were pretreated with LA at non-toxic concentrations (0-10 μM) for 48 h prior to subject in the layer of agarose containing RPMI medium as described in Materials and Methods. Figure 4.3 show that LA treatment significantly inhibited survival and growth of the lung cancer cells in a dose-dependent manner. Both the number and size of colonies were significantly suppressed in LA-treated cells in comparison to those of non-treated control. Our results revealed that LA treatment sensitizes anoikis and inhibits growth of these cells in detached condition as indicated by the significant decrease in the number and size of colony, respectively.

It was noted that there was no significant difference of cell viability between LA-pretreated H460 cells and non-treated control group in anoikis assay. At detachment condition, %cell viability of human lung cancer cells was gradually decreased in time dependent manner. However, pretreatment with LA (1-10 μM) had no effect in alteration of cell viability after culture at detachment condition for 24 h. Data was shown in appendix.

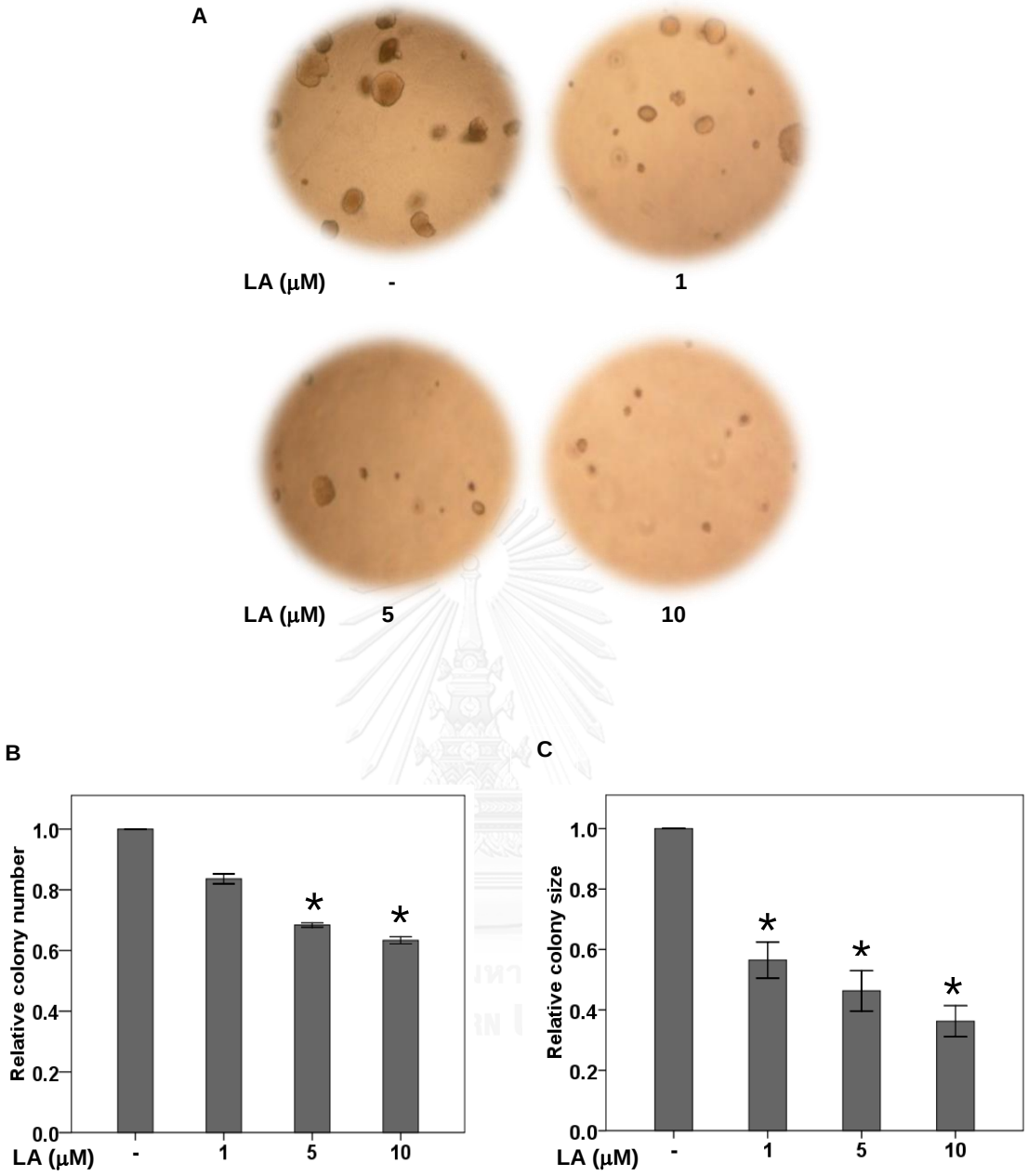

Figure 4.3 Effect of LA on anchorage-independent growth. H460 cells were pretreated with non-toxic concentrations (0-10 μM) of LA for 48 h and subjected to soft agar and cell proliferation assays. **A** Colony was captured after 14 days. **B** Numbers and **C** size of cell colony were determined and presented as relative ratio to those of the untreated control cells. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

4. LA depletes integrin β1, and β3 and downstream signaling.

As recent evidences have reported the role of certain integrins like integrin α5, αv, β1, and β3 in potentiating lung cancer anoikis resistance, migration, and metastasis [5, 67] the expression of integrins in response to LA treatment was analyzed. The lung cancer cells were exposure to non-toxic concentrations of LA as previously described and the level of integrin α5, αv, β1, and β3 were examined by western blotting. Figure 4.4A, B illustrate the dramatic reduction of integrin β1, and β3 in response to LA treatment. In terms of integrin α5 and αv, we found no alteration.

Integrins was shown to mediate anoikis resistance in several cancer cells by increasing cellular survival signals such as FAK and PI3K/AKT pathways [5, 24]. We further tested such downstream molecular targets of integrins and found that in correlation to integrin β1 and β3 depletion, p-FAK and p-AKT were strongly downregulated in the presence of LA at the concentrations of 0-10 μM. Meanwhile, there was no difference in the level of total FAK and AKT (Fig. 4.4C, D). These data suggested the possible mechanism of LA in attenuation of anoikis resistance and growth via the suppression of integrins and their related downstream pro-survival pathway.

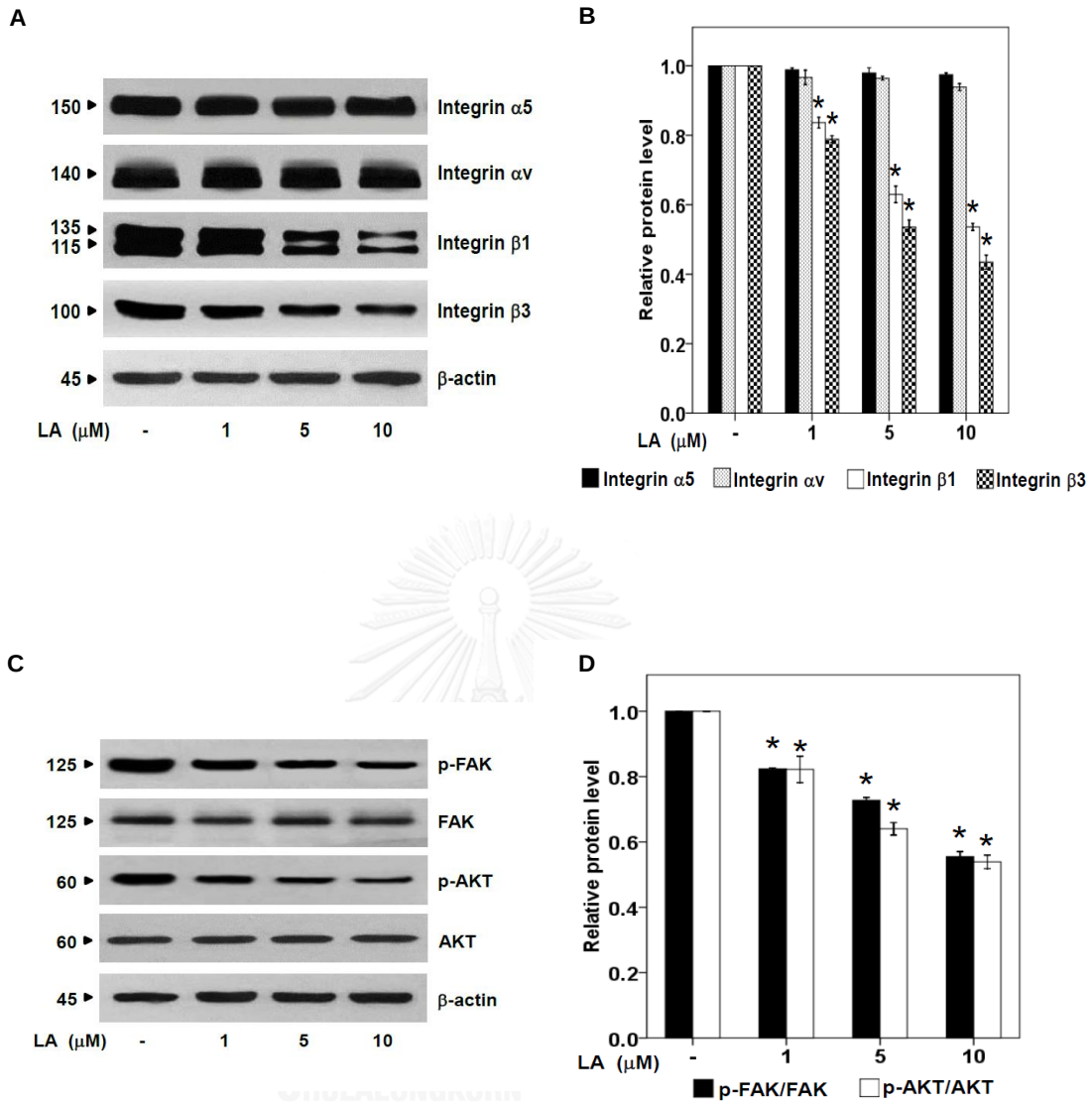

Figure 4.4 LA downregulates integrin β1 and β3 and their downstream signalings. H460 cells were treated with LA at non-toxic concentrations (0-10 μM) for 48 h. **A** The expression levels of integrin α5, integrin αv, integrin β1 and integrin β3 were analyzed by western blot analysis. **B** The immunoblot signals were quantified by densitometry. (**C**, **D**) Downstream signaling of integrin: FAK, p-FAK (Y397), AKT, p-AKT (S473) were evaluated. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control.

5. LA sensitizes chemotherapy-induced apoptosis in human lung cancer cells

A number of evidence has indicated the role of integrins in regulation of chemotherapy resistance [9, 31, 68, 69]. Therefore, we evaluated the effect of LA treatment on the susceptibility to current chemotherapeutic drugs used for lung cancer treatment. H460 lung cancer cells were culture in completed RPMI medium with or without non-toxic concentrations (0-10 μM) of LA for 48 h. Cisplatin, etoposide or paclitaxel was then added into pretreated cells for 24 h, before viable cells were detected by MTT assay.

As presented in Figure 4.5A, 4.6A and 4.7A, the incubation of H460 cells with either 25 μM cisplatin, 25 μM etoposide, or 0.1 μM paclitaxel for 24 h significantly reduced cell viability to 88.89%, 81.62%, and 77.17%, respectively. Importantly, pretreatment of the cells with non-toxic concentration of LA (10 μM) for 48 h prior to drug treatment remarkably sensitized the lung cancer cells to drug-induced apoptosis (Fig. 4.5-4.7). It is worthy noted herein that the sensitization to cisplatin- and etoposide-induced apoptosis was also notified in lung cancer cells pretreatment with of LA at low dose (5 μM) (Fig. 4.7). These findings add up the information that the observed depletion of integrin $\beta 1$ and $\beta 3$ and their survival counterparts mediated by LA influents drug susceptibility in these lung cancer cells.

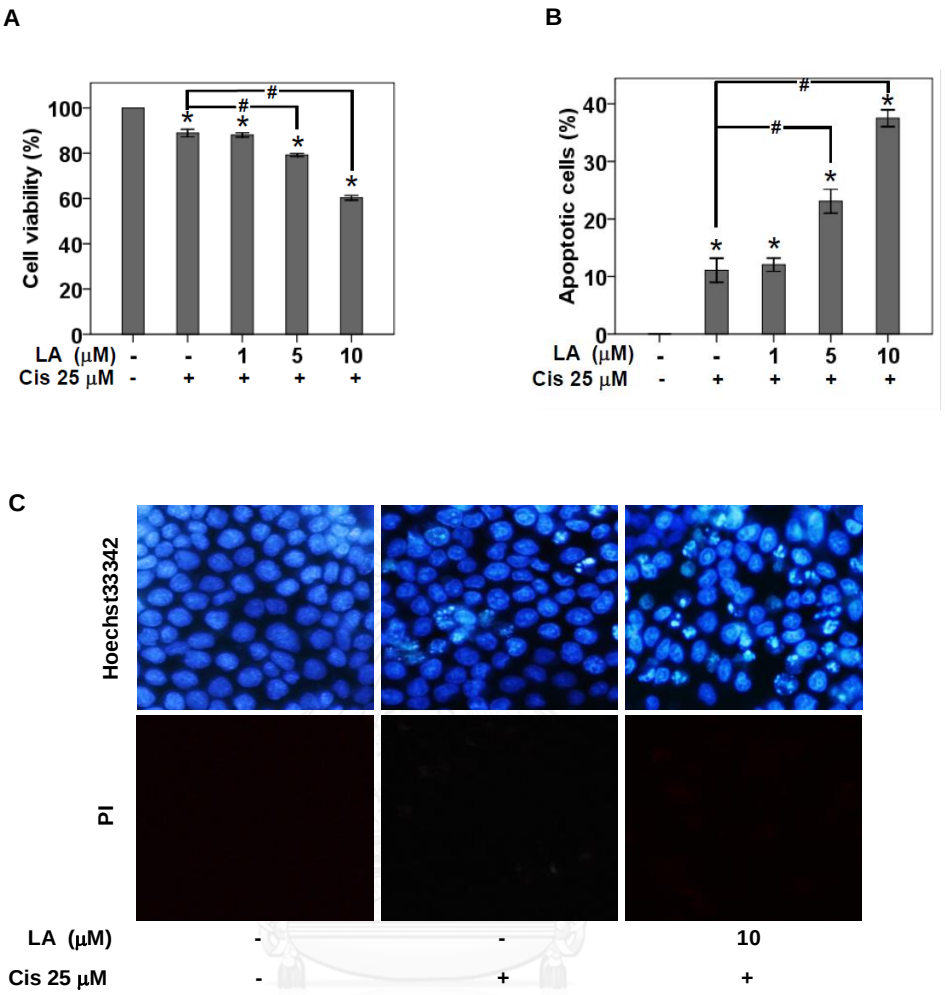

Figure 4.5 LA enhances apoptotic response of the cells to cisplatin drugs. H460 cells were pretreated with LA (0-10 μM) for 48 h and exposed to cisplatin for 24 h. **A** Cell viability of was evaluated by MTT assay. **B and C** The apoptosis and necrosis were determined under fluorescent microscope using Hoechst33342 and PI co-staining assay. Values are means of independent triplicate experiments ± SD. * $p < 0.05$ versus untreated control. # $p < 0.05$ versus cisplatin-treated control.

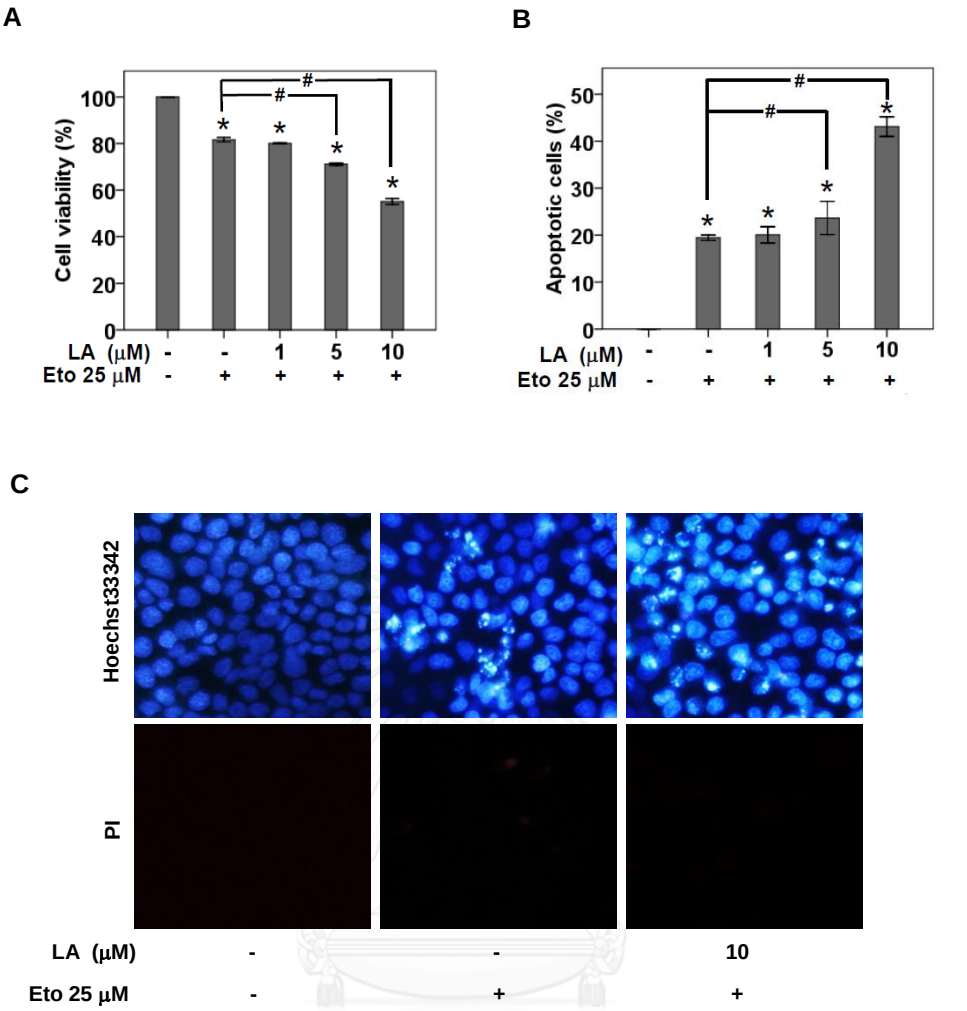

Figure 4.6 LA enhances apoptotic response of the cells to etoposide drugs. H460 cells were pretreated with LA (0-10 μM) for 48 h and exposed to etoposide for 24 h. **A** Cell viability was evaluated by MTT assay. **B and C** The apoptosis and necrosis were determined under fluorescent microscope using Hoechst33342 and PI co-staining assay. Values are means of independent triplicate experiments ± SD. * $p < 0.05$ versus untreated control. # $p < 0.05$ versus etoposide-treated control.

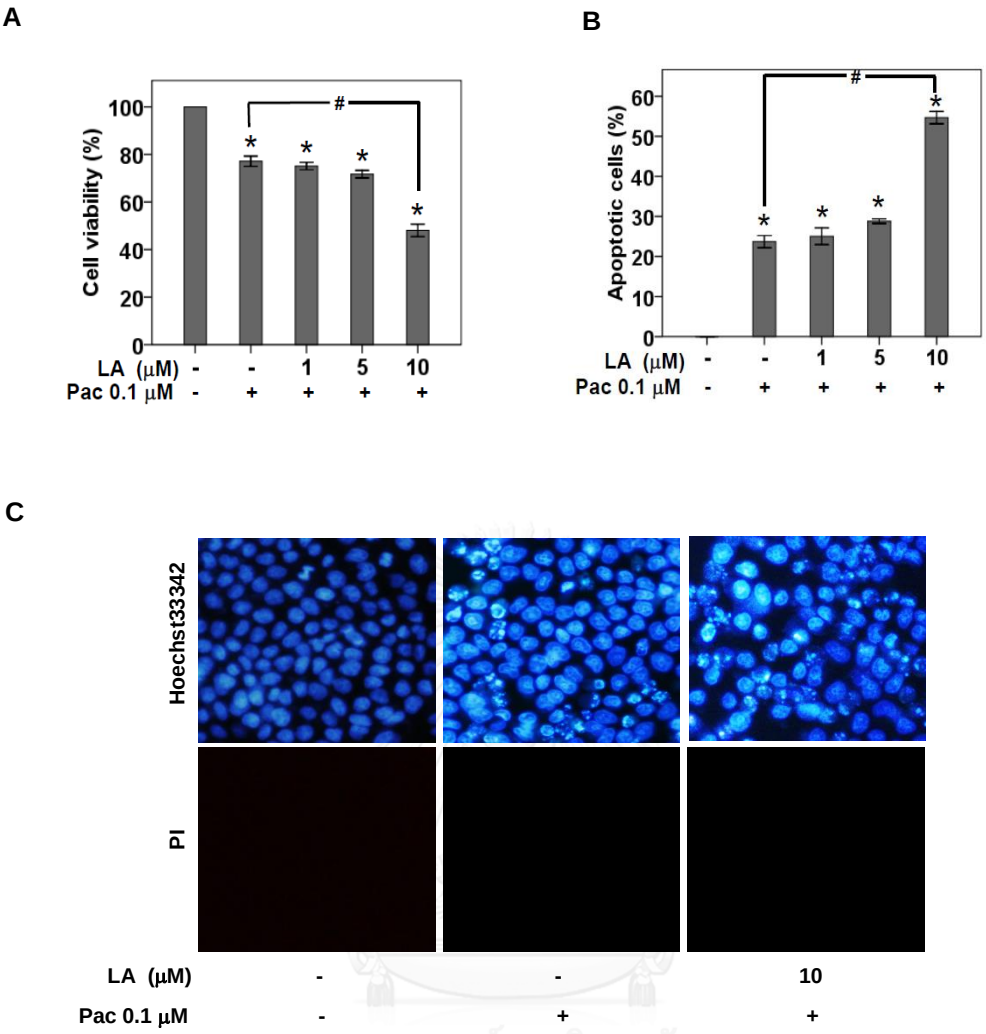

Figure 4.7 LA enhances apoptotic response of the cells to paclitaxel drugs. H460 cells were pretreated with LA (0-10 μM) for 48 h and exposed to paclitaxel for 24 h. **A** Cell viability was evaluated by MTT assay. **B and C** The apoptosis and necrosis were determined under fluorescent microscope using Hoechst33342 and PI co-staining assay. Values are means of independent triplicate experiments ± SD. * $p < 0.05$ versus untreated control. # $p < 0.05$ versus paclitaxel-treated control.

6. Investigation on mechanism of LA sensitizes anoikis and chemotherapeutics induce apoptosis

To evaluate the effect of LA on survival and apoptosis regulatory proteins namely p-FAK, FAK, p-AKT, AKT, Mcl-1, Bcl-2, Bax, and caspase-3. The cells were pretreated with 10 μM of LA for 48 h, treated with cisplatin, etoposide, or paclitaxel for 24 h, and the proteins were determined by western blotting. As expected treatment the cells with either LA or drug alone significantly reduced p-FAK and p-AKT. The combination of LA and drug caused further reduction of such survival proteins (Fig. 4.8, 4.9, and 4.10).

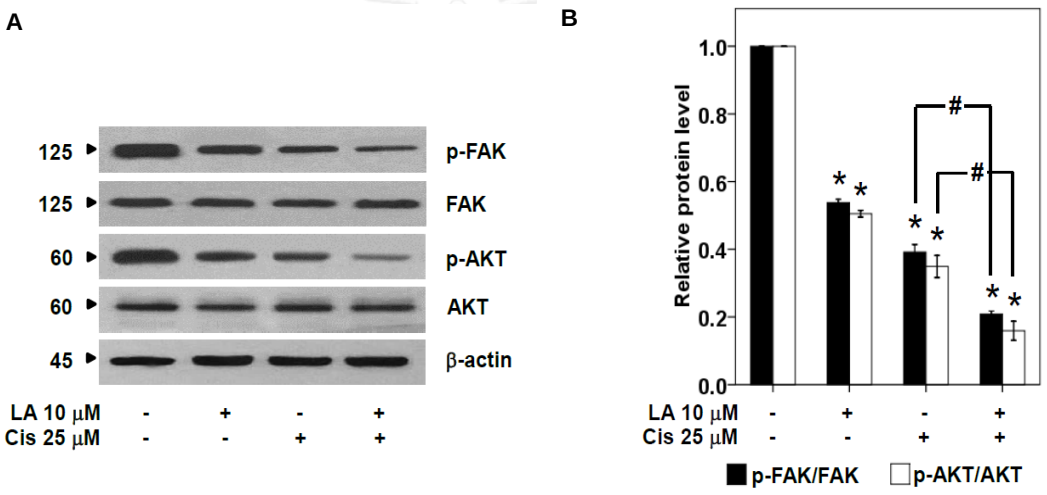

Figure 4.8 LA decreases activated FAK and AKT. The cells were pretreated with LA (0-10 μM) for 48 h and treated with cisplatin for 24 h. Western blot analysis shows the level of FAK, p-FAK (Y397), AKT, p-AKT (S473). The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus cisplatin-treated control.

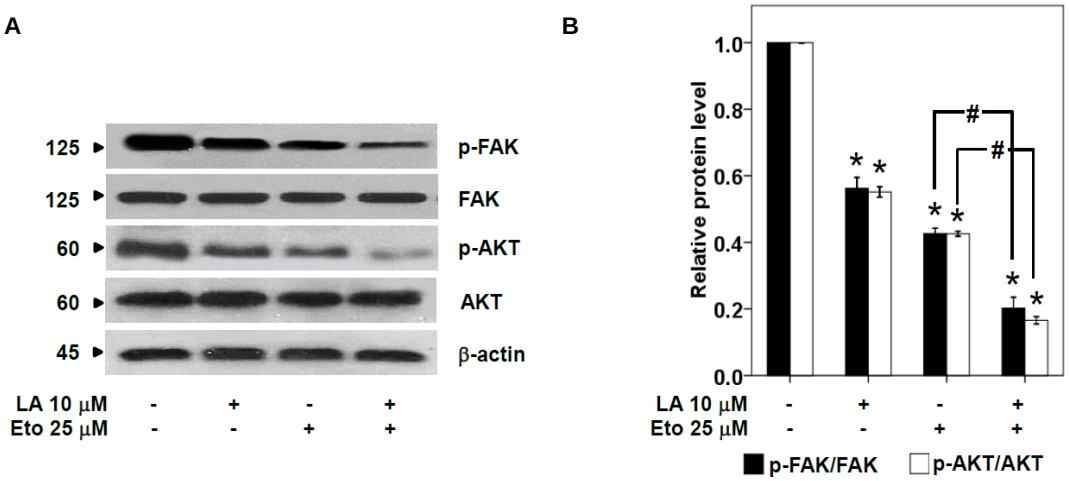

Figure 4.9 LA decreases activated FAK and AKT. The cells were pretreated with LA (0-10 μM) for 48 h and treated with etoposide for 24 h. Western blot analysis shows the level of FAK, p-FAK (Y397), AKT, p-AKT (S473). The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus etoposide-treated control.

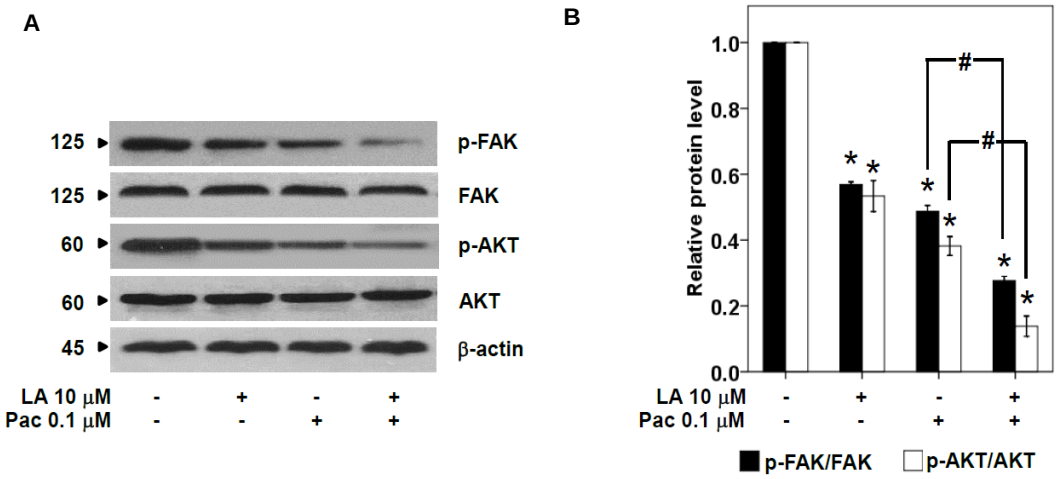

Figure 4.10 LA decreases activated FAK and AKT. The cells were pretreated with LA (0-10 μM) for 48 h and treated with paclitaxel for 24 h. Western blot analysis shows the level of FAK, p-FAK (Y397), AKT, p-AKT (S473). The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus paclitaxel-treated control.

Moreover, the downstream anti-and pro-apoptotic members of Bcl-2 family protein were evaluated. We found that the key anti-apoptotic proteins Bcl-2 and Mcl-1 significantly depleted in response to LA and drug treatment, while the pro-apoptotic Bax significantly increased (Fig. 4.11-4.13). Also, the cleavage form of caspase-3 significantly increased in response to the combination treatment. Taken together, we provide supportive information that LA sensitizes the cell response to chemotherapeutic drugs via FAK and AKT-dependent mechanism.

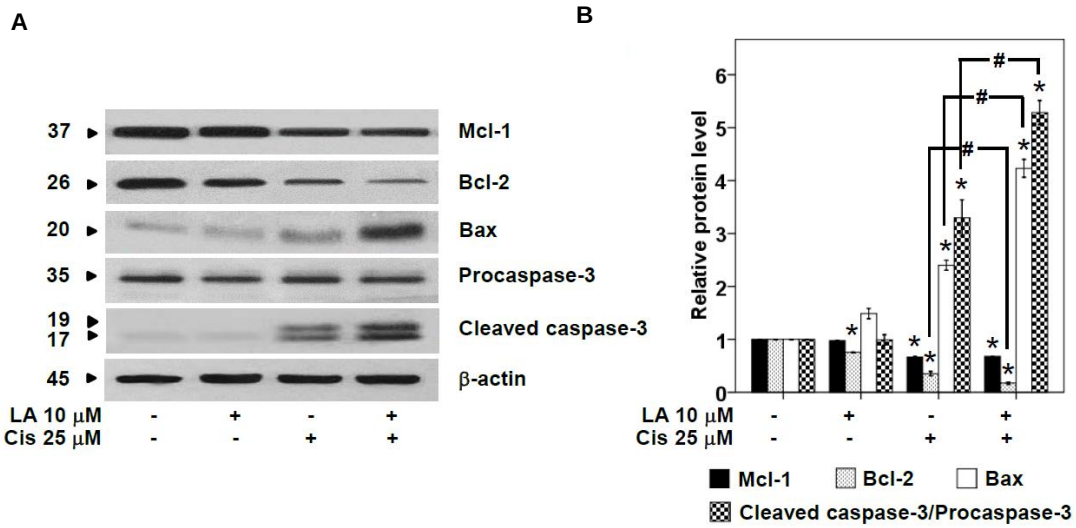

Figure 4.11 The alteration of Bcl-2 family proteins in combination treatment of LA in combination with cisplatin in H460 lung cancer cells. H460 cells were pretreated with LA at non-toxic concentrations (0-10 μM) for 48 h and then, were incubated with cisplatin for 24 h. The alteration of anti-apoptotic proteins, Mcl-1 and Bcl-2, pro-apoptotic protein, Bax, and caspase-3 were examined by western blot analysis. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus cisplatin-treated control.

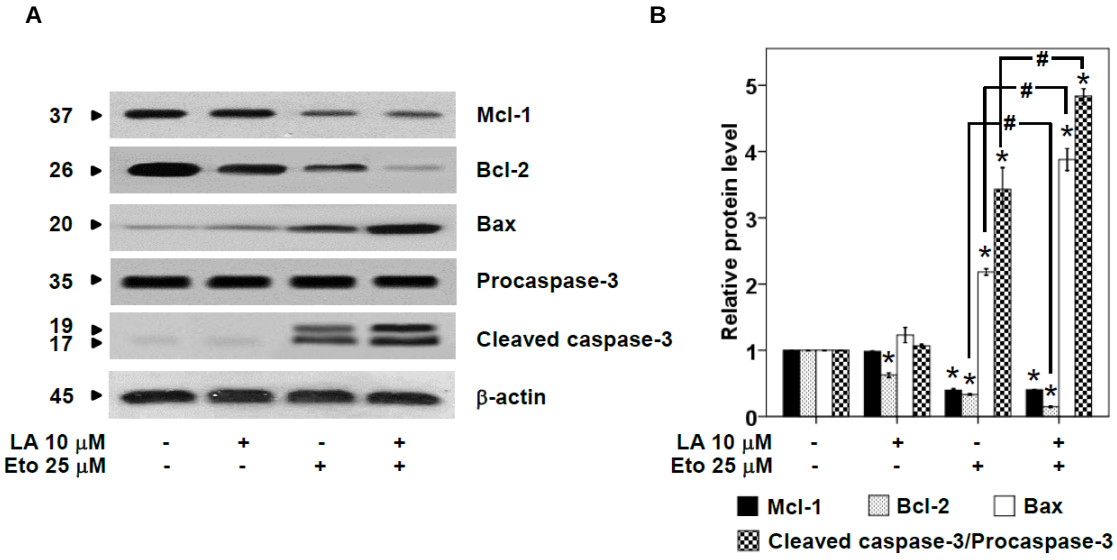

Figure 4.12 The alteration of Bcl-2 family proteins in combination treatment of LA in combination with etoposide in H460 lung cancer cells. H460 cells were pretreated with LA at non-toxic concentrations (0-10 μM) for 48 h and then, were incubated with etoposide for 24 h. The alteration of anti-apoptotic proteins, Mcl-1 and Bcl-2, pro-apoptotic protein, Bax, and caspase-3 were examined by western blot analysis. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. $^{\#}$ $p < 0.05$ versus etoposide-treated control.

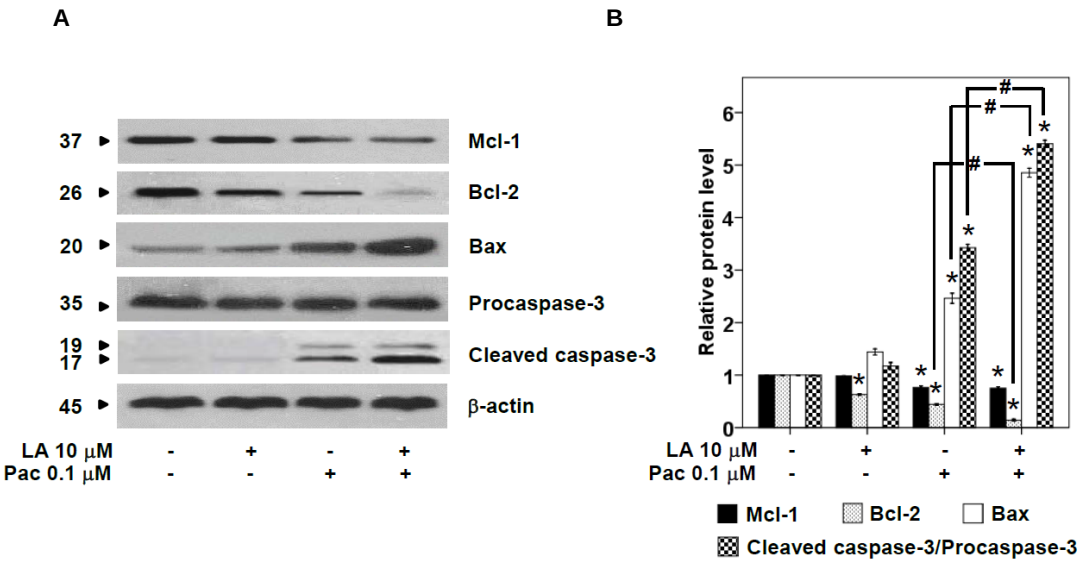

Figure 4.13 The alteration of Bcl-2 family proteins in combination treatment of LA in combination with paclitaxel in H460 lung cancer cells. H460 cells were pretreated with LA at non-toxic concentrations (0-10 μM) for 48 h and then, were incubated with paclitaxel for 24 h. The alteration of anti-apoptotic proteins, Mcl-1 and Bcl-2, pro-apoptotic protein, Bax, and caspase-3 were examined by western blot analysis. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Values are means of the independent triplicate experiments ± SD. * $p < 0.05$ versus non-treated control. # $p < 0.05$ versus paclitaxel-treated control.

7. Effect of LA induced cellular ROS generation

ROS are crucial signaling molecules in cell biology. Previous studies have indicated that the LA possesses the pro-oxidant activity to generate ROS in attached condition [10, 11]. To prove the ability of LA to generate ROS in H460 cells. Cells were seeded at a density of 1×10^5 cells/ml. Cells were pre-incubated with a specific ROS probe ($DCFH_2$-DA as a fluorescent probe for H_2O_2, DHE as a probe for $O_2^{\cdot-}$ as well as HPF as a probe for $OH^{\cdot}$) for 30 min prior to non-toxic concentrations of LA and incubated at 37°C for 0-6 h. Accumulation of intracellular ROS is then determined by flow cytometry as mentioned in methods. The result as shown in Figure 4.14A indicate that treatment of the cells with 10 μM LA significantly increased of intracellular $O_2^{\cdot-}$ and H_2O_2. However, there was no alteration of $OH^{\cdot}$ level in the cell treated with LA (Fig 4.14).

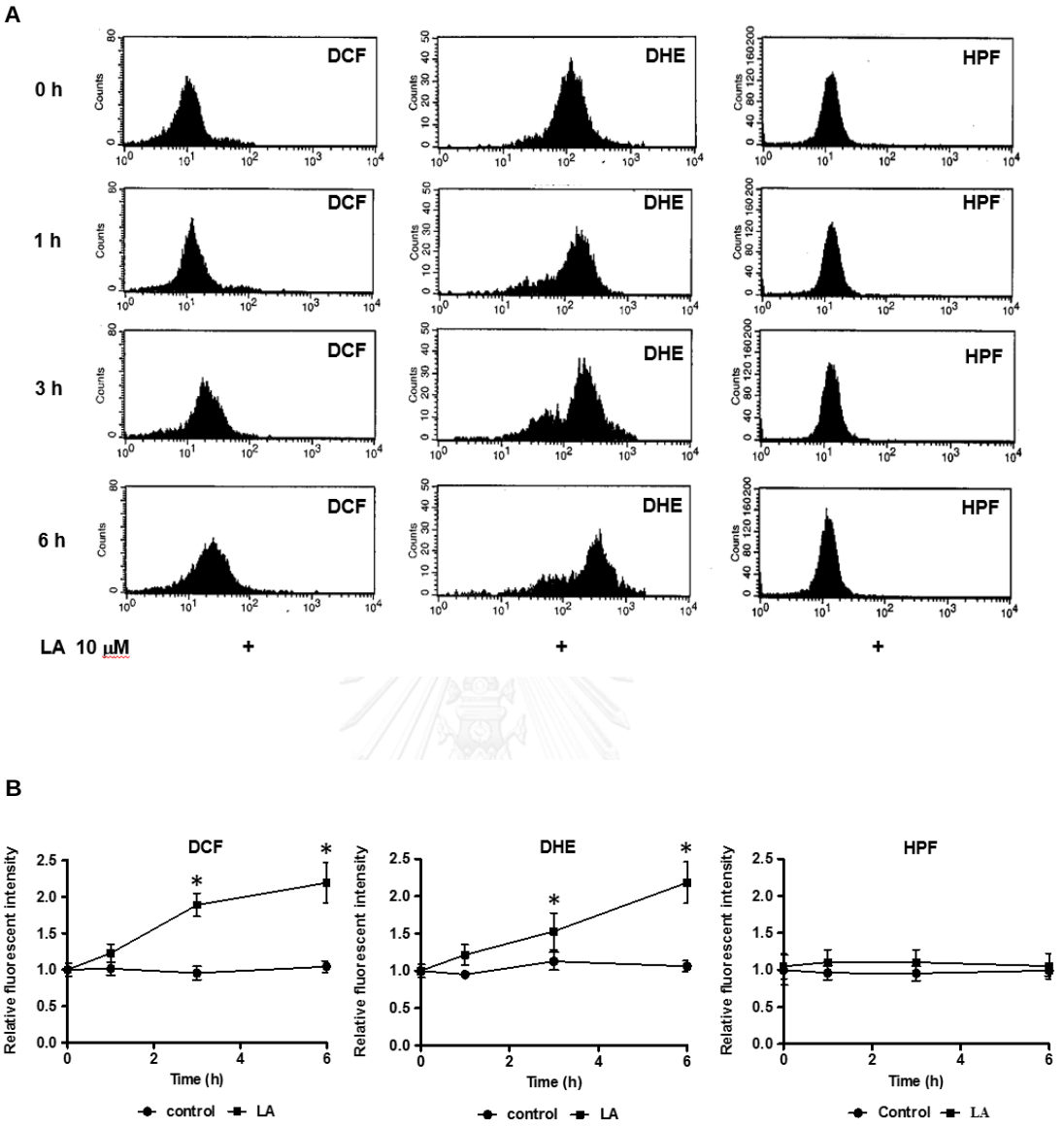

Figure 4.14 LA generates ROS in human lung cancer cells. Subconfluent (70-80%) monolayers of H460 cells were treated with 10 μM of LA for 1-6 h. **A** The time-point specific alteration of H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ after 10 μM LA treatment. **B** Intracellular levels of H_2O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ were detected by flow cytometric analysis using specific ROS fluorescent probes, DCF, DHE and HPF, respectively.* $p < 0.05$ versus untreated control cells.

8. $O_2^{\cdot-}$ and H_2O_2 regulate integrin expression in LA treated-lung cancer cells

ROS have been shown to regulate protein expression in many steps of protein processing including transcription, translation, and degradation [11, 41, 42, 44, 70, 71]. In order to clarify the underlying mechanism of LA in downregulation of integrins, broad and specific ROS scavengers were added prior to LA treatment, and the level of integrin β1 and β3 was determined by western blot analysis. Figure 4.15 show that treatment of the cells with NAC, a broad ROS scavenger successfully restored the expression of integrin β1 and β3. Attractively, H_2O_2 scavenger, catalase specifically prevented the reduction of integrin β1 but not β3 in LA treated H460 cells (Fig. 4.16). In the other way, integrin β3 was preserved by the pretreatment with $O_2^{\cdot-}$ scavenger (MnTBAP), but such scavenger has no effect on integrin β1 (Fig. 4.17). In conclusion, our results suggested that LA decreases integrin β1 via H_2O_2 induction, while reduces integrin β3 via $O_2^{\cdot-}$.

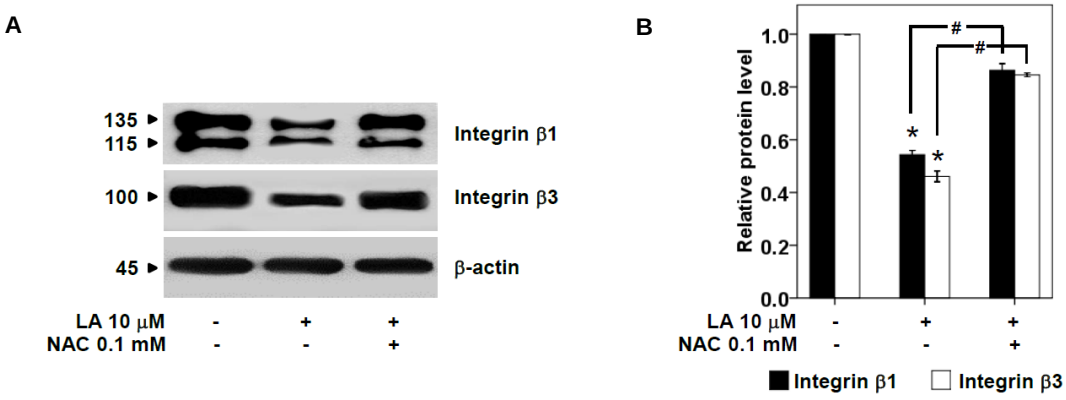

Figure 4.15 LA modulates integrin expression via ROS. For broad ROS scavengers, H460 lung cancer cells were pretreated with NAC for 1 h and treated with 10 μM of LA for 48 h. Western blot results indicate the expression of integrin β1 and β3 in the cell pretreated with 0.1 mM NAC. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Data represent as the means ± SD. * $p < 0.05$ versus untreated control cells. # $p < 0.05$ versus LA-treated control.

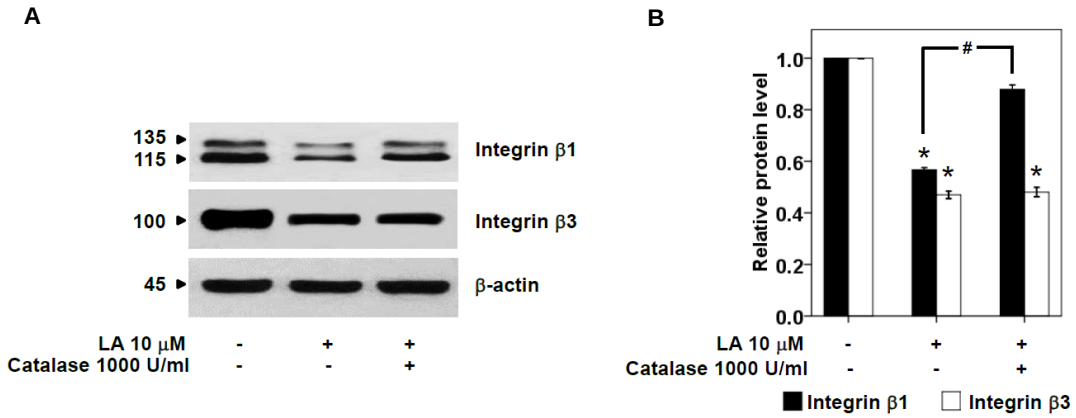

Figure 4.16 LA modulates integrin expression via ROS. For H_2O_2 scavengers, H460 lung cancer cells were pretreated with catalase for 1 h and treated with 10 μM of LA for 48 h. Western blot results indicate the expression of integrin β1 and β3 in the cell pretreated with 1000 U/ml catalase. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Data represent as the means ± SD. * $p < 0.05$ versus untreated control cells. # $p < 0.05$ versus LA-treated control.

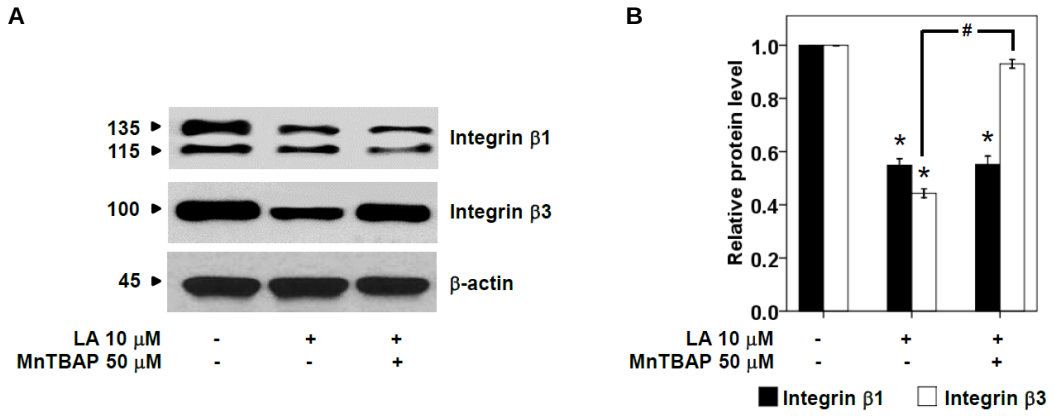

Figure 4.17 LA modulates integrin expression via ROS. For $O_2^{\cdot-}$ scavengers, H460 lung cancer cells were pretreated with MnTBAP for 1 h and treated with 10 μM of LA for 48 h. Western blot results indicate the expression of integrin β1 and β3 in the cell pretreated with 50 μM MnTBAP. The blots were re-probed with β-actin to confirm equal loading. The immunoblot signals were quantified by densitometry. Data represent as the means ± SD. * $p < 0.05$ versus untreated control cells. # $p < 0.05$ versus LA-treated control.

To provide the supportive information regarding the role of specific ROS-mediating effect of LA on integrins alteration, anoikis, and drug responses, the cells were pretreated with specific ROS scavengers, treated with LA, and subjected to anchorage-independent growth or drug sensitization assays as previously described. Results indicated that the pan ROS scavenger NAC and H_2O_2 scavenger, catalase abolished effects of LA on anoikis as well as drug-mediated apoptosis. Meanwhile, MnTBAP, $O_2^{\cdot-}$ scavenger, inhibited anoikis induction effect but had only slightly effect on chemosensitization (Fig.4.18A, B). These results have confirmed the mechanistic roles of specific ROS on LA sensitization of lung cancer cells to anoikis and chemotherapeutic agents.

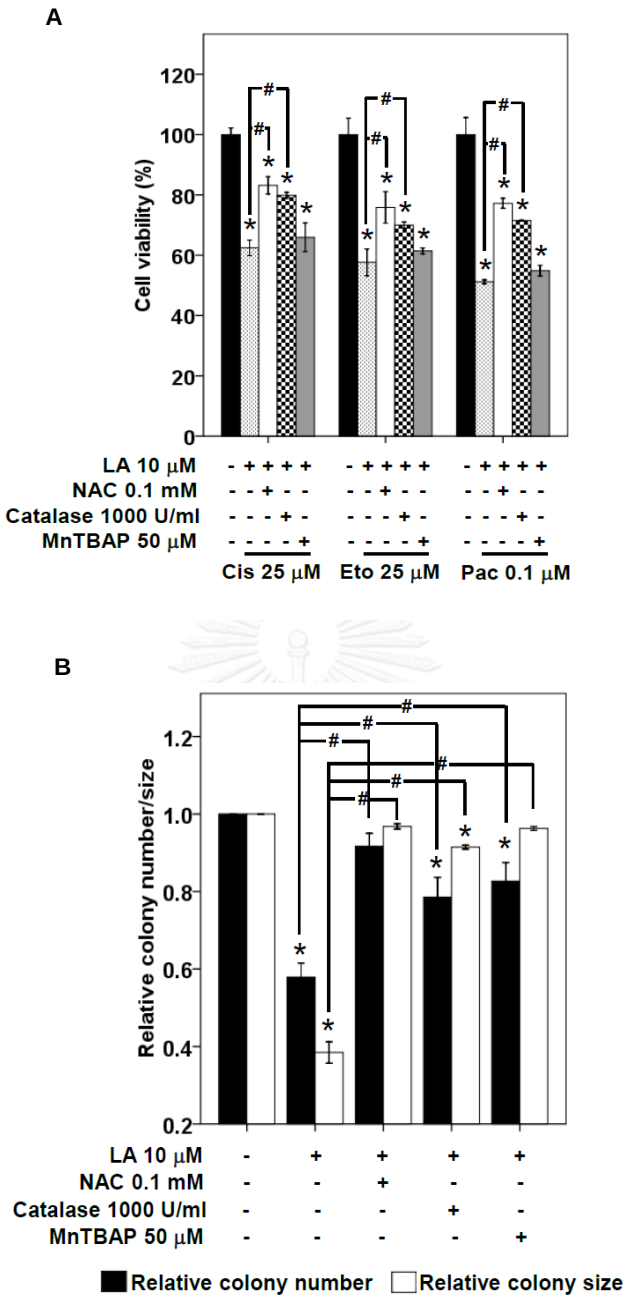

Figure 4.18 LA modulates drug and anoikis sensitization via ROS. For anti-oxidant experiments, H460 lung cancer cells were pretreated with ROS scavengers for 1 h and treated with 10 μM of LA for 48 h. The effect of LA on **A** chemotherapeutic sensitization and **B** anchorage-independent growth analysis of ROS scavengers-pretreated H460 cells. Data represent as the means ± SD. * $p < 0.05$ versus untreated control cells. # $p < 0.05$ versus chemotherapy with LA-treated control cells.

CHAPTER V
DISCUSSION AND CONCLUSION

According to safety profile and anti-cancer activity, LA has garnered interests as a potential compound for cancer therapy. LA was shown to sensitize cancer cells to apoptosis by modulating cellular redox status resulting in the downregulation of anti-apoptotic and pro-survival proteins [10, 11]. Herein we have provided novel information regarding regulatory effect of LA on intergrin pattern through specific ROS induction. The H_2O_2 and $O_2^{\cdot-}$ induced by LA treatment caused the decrease of integrin $\beta 1$ and $\beta 3$, respectively. As integrins are the key initiators for AKT through the interaction with ECM proteins [72], and AKT provides major cell survival signals [73], the decline of such integrins are likely to weakening the cancer cells.

Many evidence points out that the abilities of cells to resist anoikis as well as chemotherapeutic drug-induced apoptosis are major obstacles for the positive clinical outcome in lung cancer patients [17]. In general, resistance to chemotherapeutic agents in cancers is caused by many possible ways including active drug efflux, high level of survival proteins, modification of drug targets, and mutation in cellular checkpoint signals [74]. In lung cancer, the role of AKT on drug resistance has been highlighted as it was shown to constitutively active in NSCLC cells and such an activation status of the protein strongly promotes cellular survival and resistance to chemotherapy and radiation [75]. As generator of cellular AKT signal, integrins, transmembrane receptors, have garnered increasing attention in the cancer research field. In particular, the increase of certain integrins in the cancer cells was shown to be tightly associated with the augmented metastasis and drug resistance. Integrin $\beta 1$ causes chemotherapy resistance through the activation of PI3K/AKT pathway, and the depletion of such an integrin regained apoptosis response to cisplatin and gefitinib in

lung cancer cells [31, 32, 37, 67]. Besides, overexpression of integrin β1 is required for the anoikis resistance and anchorage-independent growth [76], suggesting the positive role of this integrin on cancer metastasis. Likewise, previous study demonstrated that integrin β3 positively affects anchorage-independent growth of lung cancer cells [77]. Consistence with these findings, we found that downregulation of integrins β1 and β3 mediated by LA reversed drug and anoikis resistance (Fig. 4.4-4.5).

Two different models were used to evaluate survival in detachment condition. For anoikis assay, the sensitizing effect of LA on detachment-induced cell death was not presented. It is noted that anoikis is the investigation on cell viability only after detachment for 24 h, whereas survival in detachment condition for longer time (14 days) is indicated in soft agar assay. Cell-cell adhesion seems to play a major role in cell survival at the beginning of detachment [5]. In order to escape cell death and grow in detached condition for longer period of time, cancer cells require epithelial-mesenchymal transition (EMT) [24]. Integrin β1 was reported that the centrally implicated in EMT induction, possibly protecting the cells from anoikis [78]. Moreover, integrin β3 was found that promote oncogenic TGF-β signaling and its stimulation of EMT and metastasis in breast cancer cells [79]. Although, LA did not alter cell death at the beginning of detachment but the reduction of anchorage-independent growth was obviously demonstrated in LA-treated lung cancer cells. These might result from the down-regulation of integrin in lung cancer cells treated with LA.

Roles of ROS in cancer pathology are well established especially in terms of survival and death [43, 80, 81], and aggressive behaviors such as chemotherapeutic resistance, survival in detachment condition, migration and invasion [41, 45, 70]. LA has been shown to act as a pro-oxidant [82, 83] as well as an anti-oxidant [52, 53] depending on cell type and cellular redox status. Various studies have reported about ROS induction activity of LA in cancer cells [11, 12]. Our results showed that treatment of the lung cancer cells with LA resulted in the significant increase of intracellular $O_2^{\cdot-}$

and H_2O_2 (Fig. 4.8). Using specific anti-oxidants, broad ROS scavenger NAC, $O_2^{\cdot-}$ scavenger MnTBAP, and H_2O_2 scavenger catalase, successfully reversed the effects of LA on integrin β1 and β3 expression (Fig. 4.9), drug sensitization (Fig. 4.10A), anchorage-independent colony formation (Fig. 4.10B). Nevertheless, $O_2^{\cdot-}$ and H_2O_2 are reactive molecules produced in the stressed cells and/or in the crosstalk between cells and inflammation within the tumor microenvironment [84]. The knowledge gained from the present study may fulfill the better understanding in the molecular basis of ROS on integrins in lung cancer.

In summary, our data provide evidence that LA regulates integrin expression pattern and sensitizes human lung cancer cells to anoikis and chemotherapy. LA decreases integrin β1 and β3 via specific ROS induction as shown in schematic diagram (Fig. 5.1). These data provide the novel anti-cancer approaches that may support the development of LA to benefit anti-cancer therapy.

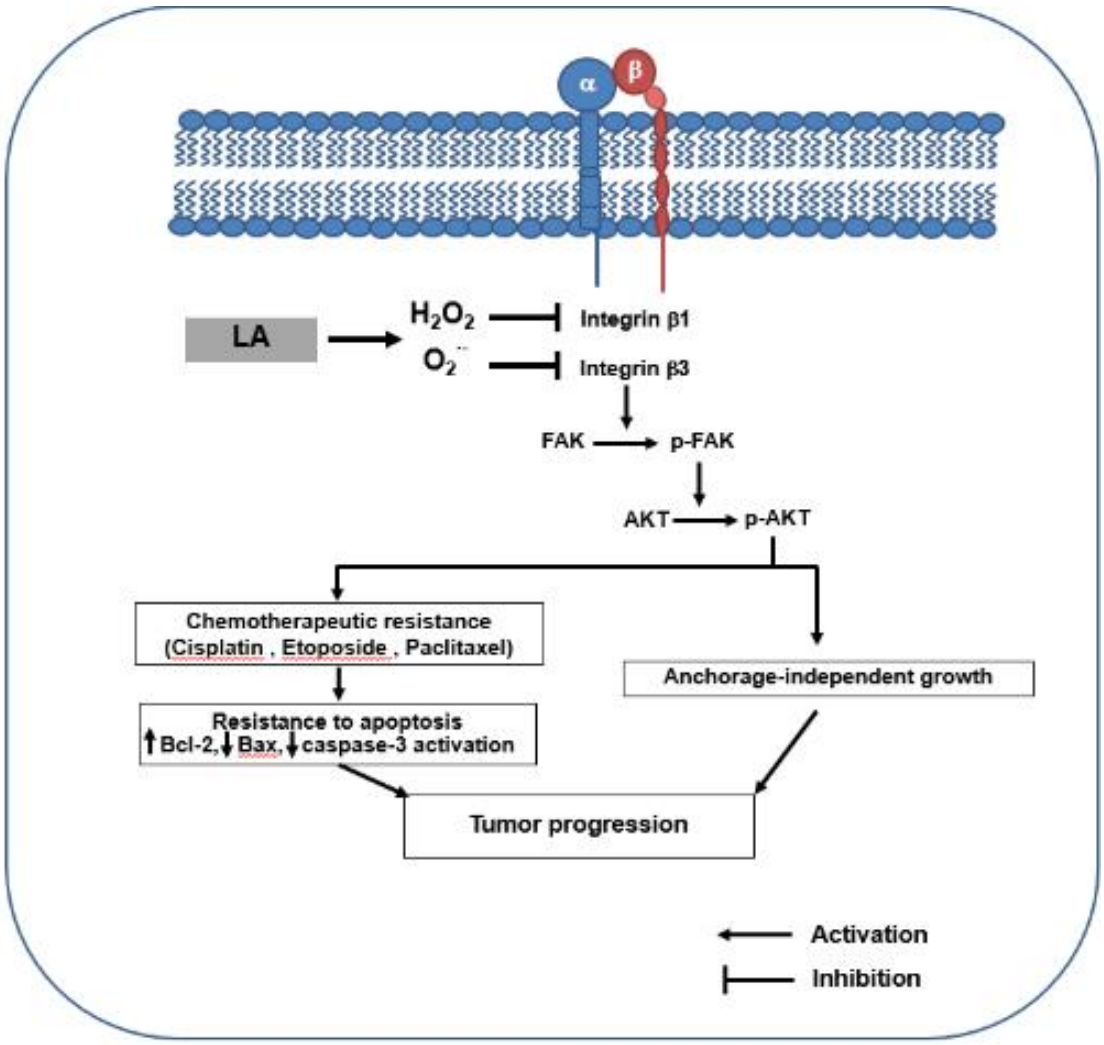

Figure 5.1 Proposed mechanistic scheme of LA sensitization. LA sensitizes human lung cancer cells to anoikis and chemotherapeutic drugs induced apoptosis via the induction of H_2O_2 and $O_2^{\cdot-}$, which reduce integrin β1 and β3 expression, respectively. The reduction of such integrins then causes the decrease of p-FAK and its downstream survival signal AKT.

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APPENDIX

APPENDIX

TABLES AND FIGURES OF EXPERIMENTAL RESULTS

Table 1 The percentage of H460 viability by MTT cytotoxicity assay after treatment with various concentrations of LA (0-500 μM)

LA (μM)	Cell viability (%)
Control	100.00 ± 0.00
1	101.93 ± 3.08
5	100.33 ± 2.30
10	100.48 ± 0.32
50	85.30 ± 1.53*
100	79.29 ± 0.25*
500	33.43 ± 2.40*

Values are mean ± SD (n=3); * *P* < 0.05 versus non-treated control

Table 2 The relative proliferation of H460 cells after pretreatment with non-toxic concentrations of LA (0-10 μM)

LA (μM)	Relative proliferation			
	Time 0	Time 24	Time 48	Time 72
Control	1.00 ± 0.00	1.94 ± 0.01	2.75 ± 0.09	6.45 ± 0.20
1	1.00 ± 0.00	1.90 ± 0.10	2.43 ± 0.06*	5.90 ± 0.07*
5	1.00 ± 0.00	1.83 ± 0.11	2.20 ± 0.08*	5.44 ± 0.09*
10	1.00 ± 0.00	1.64 ± 0.02*	1.91 ± 0.09*	5.20 ± 0.11*

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control

Table 3 The relative colony number and size of H460 cells after pretreatment with non-toxic concentrations of LA (0-10 μM) by soft agar assay

LA (μM)	Relative colony number	Relative colony size
Control	1.00 ± 0.00	1.00 ± 0.00
1	0.95 ± 0.03	0.56 ± 0.08*
5	0.68 ± 0.01*	0.46 ± 0.09*
10	0.63 ± 0.02*	0.36 ± 0.07*

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control

Figure 6.1 Effect of LA on anoikis induction by anoikis assay

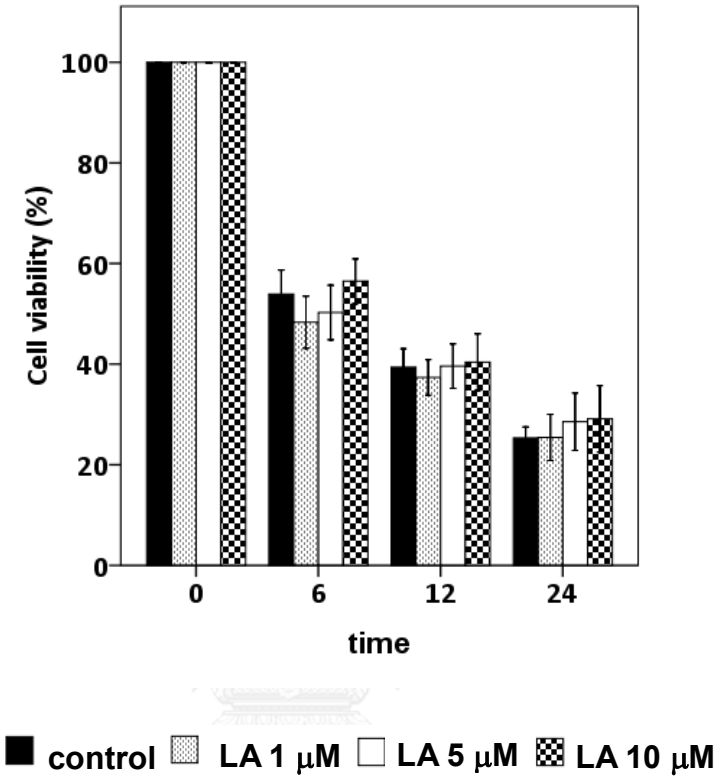

Table 4 The percentage of H460 viability by anoikis assay

LA (μM)	Cell viability (%)			
	Time 0	**Time 6**	**Time 12**	**Time 24**
Control	100.00 ± 0.00	53.94 ± 4.77	39.39 ± 3.67	25.33 ± 2.19
1	100.00 ± 0.00	48.32 ± 5.17	37.34 ± 3.55	25.45 ± 4.59
5	100.00 ± 0.00	50.26 ± 5.43	39.60 ± 4.42	28.56 ± 5.71
10	100.00 ± 0.00	56.49 ± 4.42	40.41 ± 5.63	29.14 ± 6.61

Table 5 The relative protein level values of integrins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) in attachment condition

LA (μM)	Relative protein levels			
	Integrin α5	Integrin αV	Integrin β1	Integrin β3
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
1	0.98 ± 0.01	0.97 ± 0.02	0.84 ± 0.02*	0.79 ± 0.03*
5	0.98 ± 0.02	0.96 ± 0.02	0.63 ± 0.02*	0.54 ± 0.02*
10	0.97 ± 0.04	0.94 ± 0.03	0.54 ± 0.04*	0.44 ± 0.02*

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control

Table 6 The relative protein level values of downstream-signaling proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) in attachment condition

LA (μM)	Relative protein levels	
	p-FAK/FAK	p-AKT/AKT
Control	1.00 ± 0.00	1.00 ± 0.00
1	0.82 ± 0.01*	0.82 ± 0.04*
5	0.73 ± 0.01*	0.64 ± 0.02*
10	0.56 ± 0.02*	0.54 ± 0.02*

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control

Table 7 The percentage of H460 viability by MTT cytotoxicity assay after pretreatment with various concentrations of LA and then treated with cisplatin

Groups	Cell viability (%)	Apoptotic cells (%)
Control	100.00 ± 0.00	0.00 ± 0.00
Cis 25 μM	88.89 ± 1.65*	11.09 ± 2.08*
LA 1 μM + Cis 25 μM	88.06 ± 0.94*	12.05 ± 1.15*
LA 5 μM + Cis 25 μM	79.22 ± 0.67*,#	23.09 ± 2.08*,#
LA 10 μM + Cis 25 μM	60.29 ± 1.04*,#	37.51 ± 1.47*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 8 The percentage of H460 viability by MTT cytotoxicity assay after pretreatment with various concentrations of LA and then treated with etoposide

Groups	Cell viability (%)	Apoptotic cells (%)
Control	100.00 ± 0.00	0.00 ± 0.00
Eto 25 μM	81.63 ± 0.96*	19.44 ± 0.58*
LA 1 μM + Eto 25 μM	80.03 ± 0.30*	20.06 ± 1.73*
LA 5 μM + Eto 25 μM	71.17 ± 0.55*,#	23.64 ± 3.51*,#
LA 10 μM + Eto 25 μM	55.13 ± 1.24*,#	43.10 ± 2.08*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 9 The percentage of H460 viability by MTT cytotoxicity assay after pretreatment with various concentrations of LA and then treated with paclitaxel

Groups	Cell viability (%)	Apoptotic cells (%)
Control	100.00 ± 0.00	0.00 ± 0.00
Pac 0.1 μM	77.18 ± 2.15*	23.72 ± 1.53*
LA 1 μM + Pac 0.1 μM	75.13 ± 1.55*	25.04 ± 2.08*
LA 5 μM + Pac 0.1 μM	71.71 ± 1.55*	28.80 ± 0.58 *
LA 10 μM + Pac 0.1 μM	48.08 ± 2.61*,#	54.67 ± 1.53*,#

Values are mean ± SD (n=3); * *P* < 0.05 versus non-treated control and # *P* < 0.05 versus only chemotherapy-treated group

Table 10 The relative protein level values of downstream-signaling proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and cisplatin

Groups	Relative protein levels	
	p-FAK/FAK	p-AKT/AKT
Control	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.54 ± 0.01*	0.50 ± 0.01*
Cis 25 μM	0.39 ± 0.02*	0.35 ± 0.03*
LA 10 μM + Cis 25 μM	0.21 ± 0.01*,#	0.16 ± 0.03*,#

Values are mean ± SD (n=3); * *P* < 0.05 versus non-treated control and # *P* < 0.05 versus only chemotherapy-treated group

Table 11 The relative protein level values of downstream-signaling proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and etoposide

Groups	Relative protein levels	
	p-FAK/FAK	p-AKT/AKT
Control	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.57 ± 0.01*	0.54 ± 0.05*
Eto 25 μM	0.49 ± 0.02*	0.38 ± 0.03*
LA 10 μM + Eto 25 μM	0.28 ± 0.02 *,#	0.14 ± 0.03*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 12 The relative protein level values of downstream-signaling proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and paclitaxel

Groups	Relative protein levels	
	p-FAK/FAK	p-AKT/AKT
Control	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.56 ± 0.03*	0.55 ± 0.02*
Pac 0.1 μM	0.42 ± 0.02*	0.43 ± 0.01*
LA 10 μM + Pac 0.1 μM	0.20 ± 0.04*,#	0.17 ± 0.02*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 13 The relative protein level values of apoptotic proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and cisplatin

LA (μM)	Relative protein levels			
	Mcl-1	Bcl-2	Bax	Cleaved caspase-3/procaspase-3
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.98 ± 0.01	0.63 ± 0.03*	1.23 ± 0.12*	1.07 ± 0.02
Cis 25 μM	0.40 ± 0.02*	0.33 ± 0.01*	2.18 ± 0.05*	3.43 ± 0.33*
LA 10 μM + Cis 25 μM	0.40 ± 0.01*	0.15 ± 0.01*,#	3.88 ± 0.17*,#	4.84 ± 0.11*,#

Values are mean ± SD (n=3); * *P* < 0.05 versus non-treated control and # *P* < 0.05 versus only chemotherapy-treated group

Table 14 The relative protein level values of apoptotic proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and etoposide

LA (μM)	Relative protein levels			
	Mcl-1	Bcl-2	Bax	Cleaved caspase-3/procaspase-3
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.98 ± 0.01	0.76 ± 0.01*	1.49 ± 0.10*	0.99 ± 0.10
Eto 25 μM	0.67 ± 0.02*	0.35 ± 0.04*	2.40 ± 0.09*	3.30 ± 0.34*
LA 10 μM + Eto 25 μM	0.68 ± 0.02*	0.17 ± 0.02*,#	4.23 ± 0.17*,#	5.29 ± 0.23*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 15 The relative protein level values of apoptotic proteins over controls after pretreatment with non-toxic concentrations of LA (0-10 μM) and paclitaxel

LA (μM)	Relative protein levels			
	Mcl-1	Bcl-2	Bax	Cleaved caspase-3/procaspase-3
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
LA 10 μM	0.98 ± 0.01	0.63 ± 0.01*	1.44 ±0.06*	1.17 ± 0.07
Pac 0.1 μM	0.76 ± 0.03*	0.44 ± 0.02*	2.46 ± 0.10*	3.43 ± 0.06*
LA 10 μM + Pac 0.1 μM	0.75 ± 0.02*	0.14 ± 0.02*,#	4.85 ± 0.09*,#	5.41 ± 0.06*,#

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control and # $P < 0.05$ versus only chemotherapy-treated group

Table 16 The relative fluorescent intensity in H460 cells by flow cytometry in response to non-toxic concentrations of LA

Time (h)	Relative fluorescent intensity					
	DCF		DHE		HPF	
	control	LA	control	LA	control	LA
0	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.09	1.00 ± 0.21	1.00 ± 0.21
1	1.00 ± 0.07	1.23 ± 0.11	0.98 ± 0.06	1.21 ± 0.14	0.96 ± 0.10	1.06 ± 0.21
3	0.98 ± 0.08	1.89 ± 0.12*	1.04 ± 0.12	1.53 ± 0.25*	0.95 ± 0.1	1.06 ± 0.21
6	1.00 ± 0.08	2.19 ± 0.21*	1.09 ± 0.07	2.18 ± 0.28*	0.99 ± 0.07	1.00 ± 0.21

Values are mean ± SD (n=3); * $P < 0.05$ versus non-treated control

VITA

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