EFFECT OF SUB-TOXIC CONCENTRATIONS OF CISPLATIN ON CAVEOLIN-1 EXPRESSION AND ANOIKIS IN LUNG CANCER CELLS

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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 2011
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ผลของซิสพลาตินที่ความเข้มข้นต่ำกว่าระดับความเป็นพิษต่อ

การแสดงออกของ คาวิโอลิน-1 และการตายแบบอะนอยคิสในเซลล์มะเร็งปอด

นางสาวฐิติพร ส่งเสริม

Thesis Title   EFFECT OF SUB-TOXIC CONCENTRATIONS OF
                        CISPLATIN ON CAVEOLIN-1 EXPRESSION AND
                        ANOIKIS IN LUNG CANCER CELLS
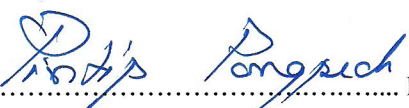By   Miss Thitiporn Songserm
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ฐิติพร ส่งเสริม: ผลของซิสพลาตินที่ความเข้มข้นต่ำกว่าระดับความเป็นพิษต่อ
การแสดงออกของ คาวิโอลิน-1 และการตายแบบอะนอยคิสในเซลล์มะเร็งปอด.
(EFFECT OF SUB-TOXIC CONCENTRATIONS OF CISPLATIN ON
CAVEOLIN-1 EXPRESSION AND ANOIKIS IN LUNG CANCER CELLS)
อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภก. ดร.ปิติ จันทร์วรโชติ, 77 หน้า.

ซิสพลาตินเป็นยาเคมีบำบัด ซึ่งในบางกรณีการใช้ยานี้ในการรักษาโรคมะเร็งก่อให้เกิดการเพิ่ม
การดำเนินไปและความรุนแรงของโรคได้ เนื่องจากสาเหตุของการเกิดเหตุการณ์เหล่านี้ยังไม่มีข้อมูลด้าน
พื้นฐานโมเลกุลที่แน่ชัด งานวิจัยนี้จึงทำการศึกษาผลของการได้รับซิสพลาตินในความเข้มข้นที่ต่ำกว่า
ระดับความเป็นพิษต่อลักษณะการตายแบบอะนอยคิสในเซลล์มะเร็งปอดชนิด H460 โดยการศึกษาให้
ซิสพลาตินในความเข้มข้นต่ำกว่าระดับความเป็นพิษแก่เซลล์เป็นเวลา 24 ชั่วโมง หลังจากนั้นจะทำให้
เซลล์หลุดจากการยึดเกาะและทำการวัดการรอดชีวิตของเซลล์ จากการศึกษาพบว่าเมื่อเซลล์ได้รับซิส
พลาตินในความเข้มข้นต่ำกว่าระดับความเป็นพิษมีผลทำให้เกิดการดื้อต่อการตายแบบอะนอยคิสเกิดขึ้น
ในลักษณะที่ขึ้นกับความเข้มข้นของซิสพลาติน ซึ่งการเพิ่มขึ้นของการรอดชีวิตของเซลล์ที่หลุดจากการยึด
เกาะเหล่านี้พบว่ามีความเกี่ยวข้องกับการเพิ่มขึ้นของระดับโปรตีนคาวิโอลิน-1 โดยเซลล์ที่ถูกเพิ่มการ
แสดงออกของคาวิโอลิน-1 พบว่ามีการเพิ่มขึ้นของการดื้อต่อการตายแบบ อะนอยคิส ในขณะที่เซลล์ที่ถูก
ยับยั้งการแสดงออกของคาวิโอลิน-1 พบผลในลักษณะที่ตรงกันข้ามคือมีการตายแบบอะนอยคิสเพิ่มขึ้น
ยิ่งกว่านั้นการศึกษานี้ยังพบว่าอนุพันธ์ออกซิเจนที่ว่องไวซึ่งถูกกระตุ้นโดยซิสพลาตินยังเป็นสาเหตุหลัก
ของการทำให้เกิดการเพิ่มขึ้นของคาวิโอลิน-1 ในกลไกนี้ด้วย โดยมีการเพิ่มขึ้นของซุปเปอร์ออกไซด์ แอน
ไอออนและไฮโดรเจนเปอร์ออกไซด์ภายในเซลล์ หลังจากเซลล์ตอบสนองต่อการ ได้รับซิสพลาติน แต่
อย่างไรก็ตามมีเฉพาะไฮโดรเจนเปอร์ออกไซด์เท่านั้นที่พบว่ามีการกระตุ้นทำให้เกิดการเพิ่มขึ้นของคาวิโอ
ลิน-1 หลังจากได้รับซิสพลาตินในความเข้มข้นที่ต่ำกว่าระดับความเป็นพิษการศึกษานี้ให้ข้อมูลเกี่ยวกับ
ผลของการ ได้รับซิสพลาตินในความเข้มข้นต่ำกว่าระดับความเป็นพิษต่อเซลล์มะเร็ง ซึ่งทำให้เพิ่มความ
เข้าใจต่อลักษณะทางชีวภาพของเซลล์มะเร็ง และเป็นประโยชน์ต่อการพัฒนาการรักษาโรคมะเร็งด้วยยา
เคมีบำบัด

ภาควิชา <u>เภสัชวิทยาและสรีรวิทยา</u> ลายมือชื่อนิสิต <u>ฐิติพร ส่งเสริม</u>
สาขาวิชา <u>เภสัชวิทยา</u> ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก <u> </u>
ปีการศึกษา <u>2544</u>

5376586733: MAJOR   PHARMACOLOGY

KEYWORDS : CISPLATIN / CAVEOLIN-1 / HYDROGEN PEROXIDE / ANOIKIS / LUNG CANCER CELLS

THITIPORN SONGSERM: EFFECT OF SUB-TOXIC CONCENTRATIONS OF CISPLATIN ON CAVEOLIN-1 EXPRESSION AND ANOIKIS IN LUNG CANCER CELLS. ADVISOR: ASST PROF. PITHI CHANVORACHOTE, Ph.D., 77 pp.

Cisplatin-based chemotherapy, in certain cases, is followed by enhanced progression or increased aggressiveness of cancers. Because the molecular basis of such phenomenon is largely unclear, this study thus investigated the possible impact of cisplatin exposure on anoikis characteristics of human lung carcinoma H460 cells. Cells were treated with sub-toxic concentrations of cisplatin for 24 h, followed by detachment, and examined for viable cells. We found that cisplatin exposure caused cells to resist anoikis in a dose-dependent manner. The attenuation of cell anoikis was found to be tightly associated with the elevation of caveolin-1 (Cav-1) level in these cells. Cav-1 transfected (H460/Cav-1) cells were demonstrated in the present study to exhibit high anoikis resistance whereas ShRNA Cav-1 transfected (H460/shCav-1) cells expressed opposite behavior. Further, This study found that reactive oxygen species (ROS)-induced by cisplatin played a key role on such Cav-1 up-regulation. Cellular superoxide anion and hydrogen peroxide were up-regulated in response to cisplatin treatment; however, only hydrogen peroxide was found to induce Cav-1 elevation. Together, This findings demonstrate the novel effect of sub-toxic cisplatin treatment on cancer cells which may lead to a better understanding of cancer biology and the improvement of chemotherapy.

Department :   Pharmacology and Physiology   Student's Signature *Thitiporn   Songserm*

Field of Study : Pharmacology   Advisor's Signature *Pithi Chan*

Academic Year : 2011

ACKNOWLEDGEMENTS

This thesis could not successfully complete without the kindness of my advisor, Assistant Professor Pithi Chanvorachote, who gave good advice and be guidance of this thesis since start until successfull. Moreover, he gave appreciate suggestion, checked and corrected the fault of this thesis.

Next, I would like to express my thanks to the lab members, Dr. Varisa Pongrakhananon, Mr. Chatchai choatham, Mr. Preedakorn Chunhacha, Mr. Wongsakorn Suchaoin, Miss Kanittha Pongjit, Miss Chuanpit Ninsontia, Mr. Hasseri Halim, Miss Apiriya Dhumrongvaraporn, Miss Nuttida Yongsanguanchai and other members for supporting, experimental teaching, and commenting on everything in this project.

I would like to thank Department of Pharmacology and Physiology, Faculty of Pharmaceutical sciences, at Chulalongkorn University, for giving me an incredible opportunity to study in Master degree. Furthermore, this research was partly supported by Thailand Research Fund (to P. Chanvorachote) and the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

Besides, I would like to thank my thesis committees, Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D., Assistant Professor Rataya Luechapudiporn, Ph.D. and Assistant Professor Patamawan Phuagphong, Ph.D. for valuable comments and suggestions.

Lastly, my graduation would not be acheived without best wish from my parents, Mr.Worrayut and Mrs.Supattra Songserm, who help me for everything and always gives me greatest love throughout my life. I would like to special thanks to my close friends for their help, support and encouragement. Thank you so much.

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LIST OF ABBREVIATIONS

%	=	percentage
°C	=	degree Celsius
μM	=	micromolar
ANOVA	=	analysis of variance
Cav-1	=	caveolin-1
CAT	=	catalase
CO_2	=	carbon dioxide
$DCFH_2$-DA	=	2,7-dichlorofluorescein diacetate
DFO	=	deferoxamine
DHE	=	dihydroethidium
DMSO	=	dimethyl sulfoxide
et al.	=	et alibi, and others
$FeSO_4$	=	ferrous sulfate
g	=	gram
GSH	=	glutathione
h	=	hour, hours
H_2O_2	=	hydrogen peroxide
HPF	=	3'-(p-hydroxyphenyl) fluorescein
min	=	minute (s)
ml	=	milliliter
mM	=	millimolar
MnTBAP	=	Mn(III)tetrakis (4-benzoic acid) porphyrin chloride
NAC	=	*N*-acetylcysteine
NPV	=	Sodium pyruvate
$O_2^{\bullet-}$	=	superoxide anion radical
$^{\bullet}OH$	=	hydroxyl radical
PBS	=	phosphate buffer saline
PI	=	propidium iodide
ROS	=	reactive oxygen species
RPMI	=	Roswell Park Memorial Institute's medium

S.D.   = standard deviation

U   = unit

CHAPTER I

INTRODUCTION

Among various chemotherapeutic agents, cisplatin or *cis*-diamminechloro-platinum (II) has been long used and recognized as a potent effective drug for treatment of various solid tumors including ovarian, bladder, cervical, and lung cancer (Loehrer and Einhorn, 1984; Sleijfer, Meijer, and Mulder, 1985; Ozols, 1992; Wong and Giandomenico, 1999). Unfortunately, in some patients, the cancer relapses (Gerl *et al.*, 1997; Cognetti *et al.*, 2012), and acquires resistance to chemotherapy (Parez, 1998; Kelland, 2007; Li *et al.*, 2010; Oliver *et al.*, 2010), or metastasis occurs (Baselga, 2005). An effort has been made in determining the possible mechanisms in controlling such tumor relapse and drug resistance. Accumulative studies reported that cancer resists cisplatin through the induction of intracellular antioxidant activity, the attenuation of drug uptake, the alteration of drug detoxification and an increases of drug efflux (Godwin *et al.*, 1922; Kelly *et al.*, 1998; Hall *et al.*, 2008; Peklak-Scott *et al.*, 2008). However, the mechanisms facilitating cancer metastasis are still largely unknown.

Cancer metastasis is a multi-step process involving with cell invasion, dissemination and migration, by which the survival of detached cells in circulation is an important step determining the fate of metastatic cancer. Since anoikis is a cellular mechanism of detachment-induced apoptosis, inhibiting cancer cells from successful metastasis; the tolerance to this mechanism thus facilitates cancer cell survival during

systemic circulation and the formation of secondary tumor at distance sites (Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossmann, 2002; Mehlen and Puisieux, 2006). Several molecular signaling molecules were found to regulate anoikis mechanism including Caveolin-1 (Cav-1) (Rungtabnapa *et al*., 2011). Cav-1, a 21-24 kDa structural protein in plasma membrane, has been shown to be involved in cancer progression as a tumor promoter protein (Terence and Michael, 2005; Di *et al*.; 2007). The elevation of Cav-1 in several types of cancer, including lung, breast, prostate, and pancreatic cancer, was strongly associated with the metastatic potential characteristic of cancer (Nasu, Timme, and Yang, 1988; Yang *et al*., 1988; Thompson, 1999; Ho *et al*., 2002; Suzuoki, Miyamoto, and Kato, 2002). It was reported that Cav-1 enhances anchorage-independent growth of non-small cell lung cancer cells, which is a crucial mechanism providing survival of metastatic cancer cells (Rungtabnapa *et al*., 2011).

Reactive oxygen species (ROS), namely superoxide anion, hydrogen peroxide, and hydroxyl radical, have been long shown to regulate several cellular behaviors (Sies, 1997; Polytarchou, Hatziapostolou, and Papadimitriou, 2005; Halliwell, 2007; Klaunig, Kamendulis, and Hocevar, 2010). The aggressiveness of cancer, such as invasion, migration and resistance to detachment-induced apoptosis, was reported to be tightly associate with the oxidative status of cancer cells (Brown and Bicknell, 2001; Ishikawa *et al*., 2008; Nishikawa, Hashida, and Takakura, 2009). Recently, we showed that hydrogen peroxide produced during cell detachment plays a role in rendering lung carcinoma cell resistant to anoikis (Rungtabnapa *et al*., 2011). Interestingly, the mechanism of hydrogen peroxide-mediated anoikis resistance was found to involve the up-regulation of the cellular protein Cav-1.

Cisplatin treatment resulted in an induction of cellular oxidative stress leading to cell apoptosis (Miyajima *et al*.,1997; Schweyer *et al*., 2004; Seve and Dumontet, 2005; Berndtsson *et al*., 2006). However, whether or not sub-toxic concentrations of this drug affect cellular ROS production and cell behaviors are largely unknown. It remains to be further investigated whether sub-toxic cisplatin treatment could have an effect in facilitating cancer cell metastasis by making cells resistant to anoikis. Using pharmacological approaches, we investigated the effect of cisplatin exposure on anoikis resistance in non-small cell lung cancer H460 cells. The anoikis resistance in our system has been found to be involved with Cav-1 induction. The present study provides novel information regarding the cancer cell biology which has a potential to facilitate the improvement of chemotherapeutic outcome.

Research questions

1. Are sub-toxic concentrations of cisplatin able to alter Cav-1 expression and anoikis resistance in lung cancer cells?

2. What is the mechanism of sub-toxic doses of cisplatin in mediations of Cav-1 expression and anoikis resistance?

Hypothesis

Sub-toxic cisplatin treatment facilitates lung cancer cells anoikis resistance by the increase of Cav-1 protein via ROS-dependent mechanism.

Objectives

1. To study the effect of sub-toxic concentrations of cisplatin on Cav-1 protein exprssion and anoikis characteristic in lung cancer cells.

2. To investigate the mechanism of sub-toxic concentrations of cisplatin in

   regulation of Cav-1 expression and anoikis characteristic in lung cancer cells.

CHAPTER II

LITERATURE REVIEWS

Cisplatin

Cis–diamminedichloroplatinum (II) (Cisplatin) is an inorganic platinum compound, which has a structure as shown in figure 2.1. It has been long used and recognized as a potent and effective chemotherapeutic agent against many solid tumors including ovarian, bladder, cervical, and lung cancer (Loehrer and Einhorn, 1984; Sleijfer, Meijer, and Mulder, 1985; Ozols, 1992; Wong and Giandomenico, 1999); given alone or in combination with other drugs, such as cyclophosphamide, bleomycin and etoposide. Cisplatin became the first FDA-approved platinum compound for cancer treatment in the year 1978 (Kelland, 2007).

$$H_3N \diagdown \diagup Cl$$
$$Pt$$
$$H_3N \diagup \diagdown Cl$$
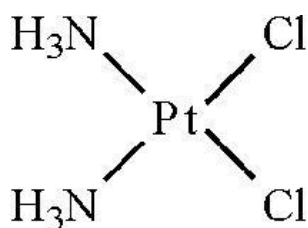
Figure 2.1 Structure of cisplatin (Eckstein, 2011)

Cisplatin is an alkylating agent. DNA-adduct is its major pathway for antitumor properties. Water molecules that through hydrolysis or aquation would displaced the chloride atom of cisplatin (electrophile) and formed 1,2-intrastrand cross-links within the DNA between the N7 atoms of guanines of the DNA

(nucleophilic molecules) in dividing cells. Resulting in bulky adducts that impair replication and transcription, which leads to cell-cycle arrest, DNA repair and cell death (Wang and Lippard, 2005) as demonstrated in figure 2.2.
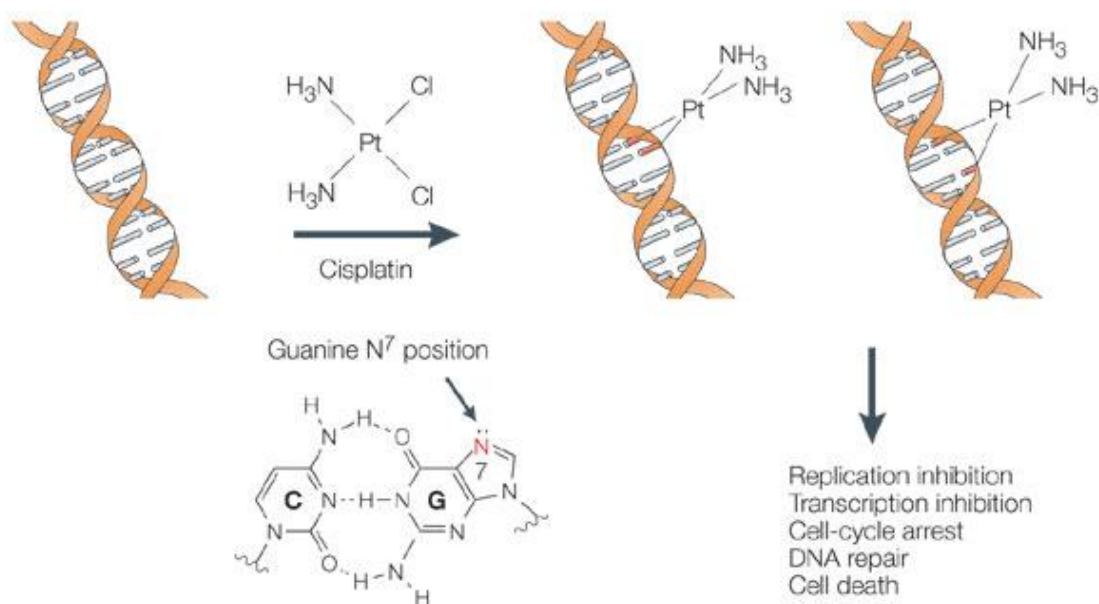
Figure 2.2 Mechanism of action of cisplatin (Wang and Lippard, 2005)

Cisplatin induced cellular oxidative stress has been recognized as an important cytotoxic mode of action (Miyajima *et al.*, 1997; Wang *et al.*, 2008). Cisplatin had been proven to elevate the intracellular reactive oxygen species (ROS) level in many cells (Bragado *et al.*, 2007; Stewart *et al.*, 2007). Cellular oxidative stress induced by cisplatin has been shown to contribute to its cytotoxicity and the increased antioxidant mechanisms of cancer cells attenuate cisplatin-induced apoptosis (Wong and Giandomenico,1999; Seve and Dumontet, 2005). Reactive form of cisplatin is able to interact with other cellular targets such as plasma membrane, lipids, proteins,

especially the negatively charged of thiol-containing species including glutathione (GSH). Depletion or inactivation of GSH and related cellular antioxidants by cisplatin are expected to shift the cellular redox status which leads to ROS production and oxidative stress within the cells (Istvan and Robert, 2003; Rebillard *et al*., 2008). Furthermore, cisplatin can also causes damage to the mitochondrial function, resulting in the increase of ROS via the disrupted respiratory chain (Kruidering *et al*., 1996). Previous study had shown that cisplatin mediated reactive oxygen species (ROS) formation though the induction of the proteasomal degradation of Bcl-2 protein, in which believed to be the key mechanism of cisplatin inducing cell apoptosis in lung cancer cells. (Chanvorachote *et al*., 2006)

Although cisplatin had been considered as a highly effective cancer chemotherapeutic drug, it still has major barriers limiting its uses and efficacy, such as, its toxicity including nephrotoxicity, neurotoxicity, ototoxicity (Kelland, 2007) and drug resistance (Stewart, 2007). The efficacy of cisplatin is frequently attenuated due to de novo drug resistance in the advanced stage of cancer or in cancer cells acquiring cisplatin resistance during therapy (Wong and Giandomenico, 1999). Multiple mechanisms that mediate intrinsic or acquired resistance to cisplatin have been identified. Studies have reported that cancer resistance to cisplatin can be through the induction of antioxidant activity within cell, the attenuation of drug uptake, the alteration of drug detoxification and the increasing in drug efflux etc. (Godwin *et al*., 1922; Kelly *et al*., 1998; Hall *et al*., 2008; Peklak-Scott *et al*.,2008).

Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. The oxygen are highly reactive due to the presence of unpaired valence shell electrons. In normal physiologic condition, ROS are generated in metabolic pathway of cell especially through the electron transport chain within mitochondria, and the phagocytosis in the immune system (Salganik, 2001). However, ROS levels can increase during environmental stress (e.g., UV or heat exposure) (Devasagayam *et al*., 2004), resulting in cell structures damaged. Normally, cells are protected from these ROS by enzymes such as superoxide dismutases (SOD), catalases, lactoperoxidases, glutathione peroxidases (GPx), glutathione reductase (GR), glutathione-*S*-transferase (GST) and peroxiredoxins. Also, small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, lipoic acid, ubiquinone and glutathione (GSH) play important roles as cellular antioxidants (Salganik, 2001; Chirino and Pedraza-Chaverri, 2009).

ROS are produced from cellular metabolism in bodies; especially the electrons from the mitochondrial electron transport chain that is considered as a major source of ROS production. The first ROS that are produced from the mitochondrial electron transport chain is superoxide anion ($O_2^{\bullet-}$). Then, the superoxide anion would be changed into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 would then be converted to water by catalase (CAT) or glutathione peroxidase. However, in the presence of reduced transition metals such as ferrous ion (Fe^{2+}) or copper ion (Cu^+), hydrogen peroxide will be transformed to hydroxyl radical ($\bullet OH$), which is very toxic to cells, called Fenton reaction (Halliwell and Gutteridge, 1984; Salganik,

2001). The Haber-Weiss reaction is the combination between Fenton reaction and the reduction of metal ions by superoxide anion, it can generate hydroxyl radical as well (Nordberg and Arner, 2001; Valko *et al*., 2007). ROS generation as demonstrated in figure 2.3.

$$H_2O_2 + Fe^{2+} / Cu^+ \longrightarrow OH^\bullet + OH^- + Fe^{3+} / Cu^{2+} \qquad \text{Fenton reaction}$$

$$O_2^{\bullet-} + Fe^{3+} / Cu^{2+} \longrightarrow O_2 + Fe^{2+} / Cu^+ \qquad \text{Reduction of metal ions}$$

$$H_2O_2 + O_2^{\bullet-} \longrightarrow OH^\bullet + OH^- + O_2 \qquad \text{Haber-Weiss reaction}$$
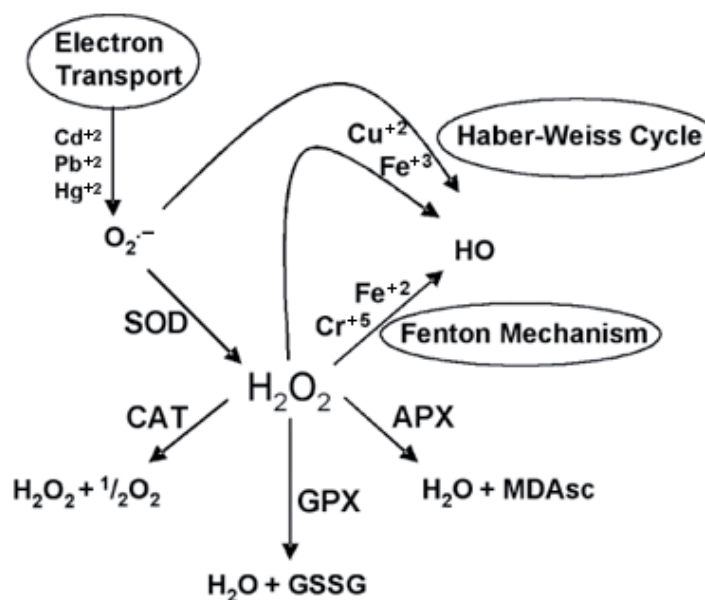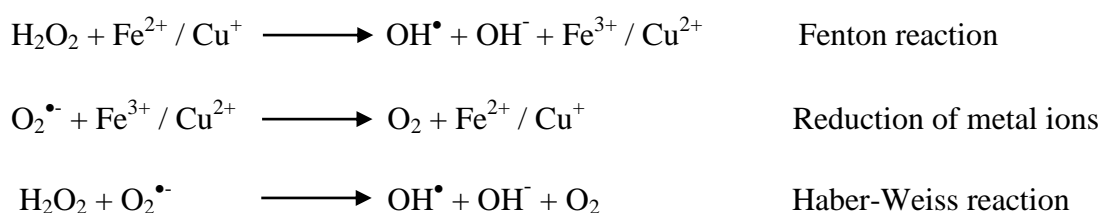
Figure 2.3 ROS generation (Benavides, Gallego, and Tomaro, 2005)

In general, the most common harmful effects of these reactive oxygen species on the cell are DNA damage (Klaunig, Kamendulis, and Hocevar, 2010), oxidations of polyunsaturated fatty acids in lipids (Sorg, 2004) and oxidations of amino acids in proteins (Berlett and Stadtman, 1997). However, reactive oxygen species can be

beneficial when they are used attack and kill unwanted cells by the immune system (Segal, 2005).

Oxidative stress is caused by the disturbance in the balance between production and elimination of ROS within cells. This may be originated by an overproduction of substances of ROS or by the depletion of antioxidant defenses (Sies, 1997). In human, oxidative stress is involved in many diseases including, atherosclerosis, Parkinson's disease, heart failure, myocardial infraction, Alzheimer's disease, and diabetes mellitus (Sorg, 2004; Valko *et al.*, 2007).

In cancer biology, some cancer aggressive activities like cell invasion, migration, metastasis and anoikis resistance were reported to tightly associate with the oxidative status of the cancer cells. In metastasis of cancer cell, ROS can act as promoting (Brown and Bicknell, 2001; Ishikawa *et al.*, 2008; Nishikawa, Hashida, and Takakura, 2009) or inhibitory (Maeda *et al.*, 2001) effects depending on the type of ROS, the molecular background of cells and tissues, the location of ROS production, and the amount and persistence of individual ROS. Increased in ROS generation and oxidative stress has been shown to developed many human metastatic cancers especially lung cancer (Chung-man, 2001). Moreover, previous studies have shown that hydrogen peroxide produced during cell detachment plays a role in rendering lung carcinoma cell resistance to anoikis (Rungtabnapa *et al.*, 2011).

Anoikis

Anoikis or detachment-induced apoptosis is subset of apoptosis that occurring when epithelial cells are detached from surrounding extracellular matrix (ECM)

(Frisch and Screaton, 2001) as show in figure 2.4. This detachment causes a loss of cell-matrix interaction that could restrict inappropriate cell survival by inducing cell cycle arrest and apoptotic cell death. Since cell-matrix adhesion have major effects to cytoskeletal structure, gene regulation, differentiation and cell growth control. In attach cells, cell-specific activation of integrins and their downstream signaling mediators, including the non-receptor tyrosine kinase Src, focal adhesion kinase (FAK) and integrin-linked kinase (ILK), protect cells from anoikis (Frisch and Francis, 1994). Anoikis is a self-defense mechanism that organisms use to eliminate cells in an inappropriate environment. Anoikis resistance that cells are able to adapt to new location allows tumor cells to expand and invade adjacent, giving rise to metastasis (Guadamillas *et al*., 2011).
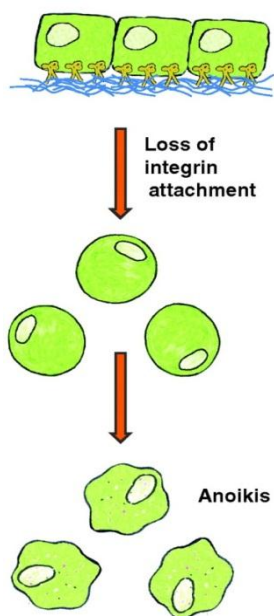
Figure 2.4 Anoikis or detachment-induced apoptosis (Guadamillas *et al*., 2011)

Cancer metastasis, a spread of cancer cells to secondary sites, is a multi-step process involving cell invasion, dissemination and migration. By which the survival rate of detached cells in the circulation is an important factor in determining the fate of metastatic cancer. Since anoikis is a cellular mechanism of detachment-induced apoptosis inhibiting cancer cells, it has been shown to be a principal mechanism of inhibition of tumor metastasis. The tolerant to this mechanism thus facilitates cancer cell survival during systemic circulation and formation of secondary tumor at distance sites. Anoikis resistance is depended with the tumor metastasis level and cancer metastasis state (Frisch and Francis, 1994; Grossmann, 2002; Mehlen and Puisieux, 2006). Anoikis can be regulates by many signaling pathways. Previous studies indicated that Cav-1 inhibits anoikis of cancer cell and promotes metastasis (Fiucci *et al*., 2002; Ravid *et al*., 2005; Ravid *et al*., 2006). Moreover, metabolic and oxidative stress can be activated by loss of cell adhesion. Production of reactive oxygen species (ROS) that is generated by detachment of cells correlates with anokis (Li *et al*., 1999). Therefore understanding the anoikis mechanisms of cancer cells could lead to the novel therapeutic strategies for metastatic cancers.

Caveolin-1 (Cav-1)

Caveolins are a member of a protein that includ three members in vertebrates, such as caveolin-1-3, all of which encode 20-24 kDa proteins. They are found the most in plasma membrane but also are in the golgi, endoplasmic reticulum (ER), cytoplasm. Expressions of caveolins different in each tissue. Caveolin-1 (Cav-1) is the most finding member that is found highest levels in endothelial cells, adipocytes, smooth muscle cells, and type I pneumocytes. Caveolin-2 (Cav-2) is coexpressed with

Cav-1 that requires Cav-1 in function, while the Cav-3 expresses mainly in muscle cells. Caveolin is localized to caveolae "little caves" that is plasma membrane specializations as shown in figure 2.5. Which function of cavolin within caveolae are scaffolding protein and signaling molecules. Caveolins associated with many important cellular processes, such as vesicular transport, signal transduction, cholesterol homeostasis, and tumor suppression. (Williams and Lisanti, 2004). Recently, the role of caveolin-1 in the regulation of cancer progression and metastasis gains increasing attention.
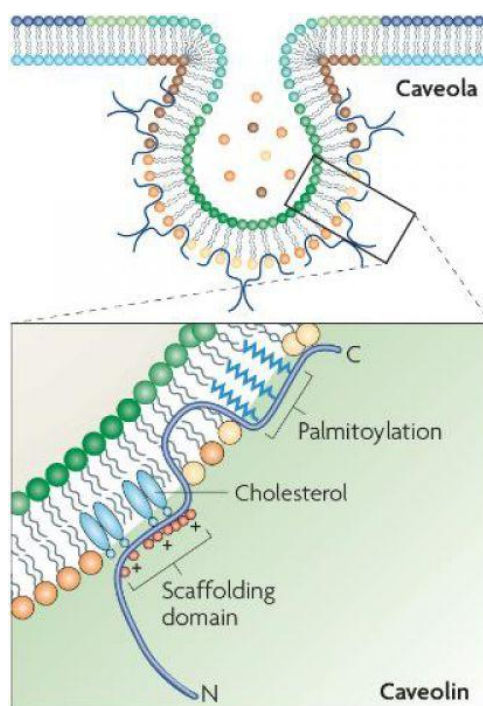
Figure 2.5 Caveolae and component of caveolae (Parton and Simons, 2007)

Caveolin-1 shows a long putative hairpin intramembrane domain of caveolae which N and C termini of Cav-1 localizes in the cytoplasm. Cav-1 binds with 1-2 cholesterol molecules and is palmitoylated in C-temini. Two caveolin-1 monomers will form to dimer. The domains present in Cav-1 including Carboxy-terminal

membrane attachment domain, Transmembrane domain, Amino-terminal membrane attachment domain and Oligomerization domain as shown in figure 2.6. Cav-1 is synthesized in endoplasmic reticulum (ER) and is transported from ER to Golgi complex, and then and is transported directly to the plasma membrane (Parton and Simons, 2007). Cav-1 have two isoforms including α and β that different in number of amino terminus. The β isoform has a shorter amino terminus than the α isoform (Williams and Lisanti, 2004).
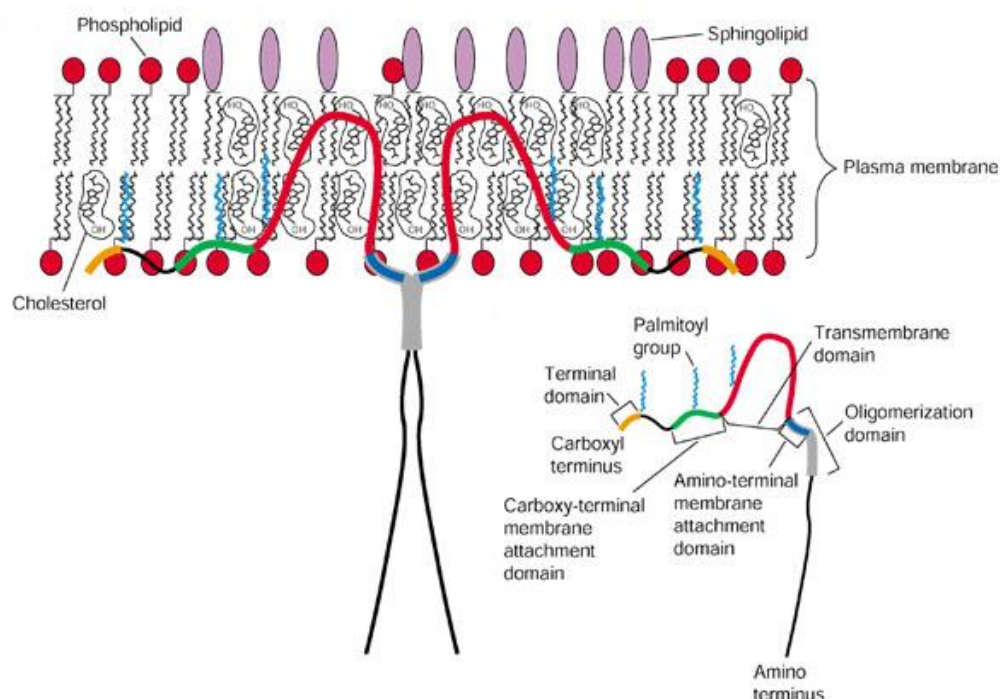
Figure 2.6 Membrane topology and structure of caveolin-1

(Williams and Lisanti, 2004)

Caveolin-1 is a key protein involved in tumor metastasis. Moreover, it has been shown to be involved with cancer progression, as a tumor promoter protein (Terence and Michael, 2005; Dana *et al*., 2006; Di *et al*.; 2007). Recently, genetic

knockout of caveolin-1 is shown to result in elevated sensitivity to viral-induced mammary tumorigenesis and to carcinogen-induced skin tumorigenesis (Capozza *et al*., 2003; Williams *et al*., 2003). Several investigators have reported that the elevation of Cav-1 in several cancers including lung, breast, prostate, and pancreas cancers was strongly associated with the cancer metastasis (Nasu, Timme, and Yang, 1988; Yang *et al*., 1988; Thompson, 1999; Ho *et al*., 2002; Suzuoki, Miyamoto, and Kato, 2002). Previous studies suggested that Cav-1 expression correlated with several aggressive behaviors of lung cancer cells including multidrug-resistance (Lavie and Liscovitch, 1997; Lavie, 1998; Shatz and Liscovitch, 2004) and anoikis resistance (Chanvorachote *et al*., 2009; Rungtabnapa *et al*.2011).

Lung cancer

Lung cancer is the leading cause of cancer-related death worldwide especially in men. In the year 2008, up to 1.4 million deaths was from lung cancer (Jemal *et al*., 2010). The main causes of lung cancer are exposure to ionizing radiation, carcinogens (such as tobacco smoking) and viral infection which causes damaged lungs bronchial epithelium. As the damages increases, tissues become cancer (Vaporciyan *et al*., 2000). The main types of lung cancer are small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Small cell lung carcinoma (SCLC) is less common that arise in the larger airways (primary and secondary bronchi) and grow rapidly (Collins *et al*., 2007). SCLC are associated with endocrine and paraneoplastic syndrome because these cancer cells contain dense neurosecretory granules that vesicles containing neuroendocrine hormones. Cigarette smoking is main cause of this lung cancer type (Barbone *et al*.,1997).

Another type of lung cancer is non-small cell lung carcinomas (NSCLC) , the major type of lung cancer that have three main sub-types including squamous cell lung carcinoma, adenocarcinoma, and large-cell lung carcinoma. Moreover, there are several other types that occur less frequently and are grouped together because their prognosis and management are similar.

1. **Squamous cell lung carcinoma** (SCC) is more common in men than in women. It is closely associated with a history of tobacco smoking, more than other types of lung cancer (Kenfield, 2008). SCC arises centrally in larger bronchi, and while it often metastasizes to lymph nodes.

2. **Large-cell lung carcinoma** (LCLC) is originated from transformed epithelial cells in the lung. This non-small cell lung carcinomas is heterogeneous group of undifferentiated malignant neoplasms. LCLC is differentiated from small cell lung carcinoma primarily by the larger size of the anaplastic cells which have high cytoplasmic-to-nuclear size ratio.

3. **Lung adenocarcinoma** is the most common type of lung cancer in current that occur with non smokers. (Subramanian and Govindan, 2007). Adenocarcinomas account for approximately 40% of lung cancers. It is more often seen in peripheral of lung than small cell lung cancer and squamous cell lung cancer. (Travis *et al*.,1995)

Treatment for lung cancer depends on the specific cell type of cancer. SCLCs are sensitive to chemotherapy more than non-small cell lung carcinoma. The majority

advanced non-small-cell lung cancer patients are treated with cisplatin or carboplatin, in combination with gemcitabine, docetaxel, paclitaxel, etoposide, or vinorelbine. However, only; 30% of these patients respond to treatments (Socinski, 2004). Among patients that initially respond, most patients develop resistant cancer. Both inherent and acquired drug resistance are major barriers to successful chemotherapy of lung cancer. The main causes of cancer-related death which frequently occurs in lung cancer patients are metastasis and chemotherapeutic resistance (Hanahan and Weinberg, 2000; Hoffman, Mauer, and Vokes, 2000). Moreover study reported that up-regulation of caveolin-1 has been observed in lung cancer cells (Burgermeister *et al*., 2008) especially lung cancer cell in non-small cell lung carcinoma type (Sunaga *et al*., 2004). Various studies shown that expression of Cav-1 associated with poor prognosis and drug resistance in lung cancer patients (Yoo *et al*., 2003; Ho *et al*., 2008). Since NSCLC is the most diagnosed type of lung cancer and being at an advanced stage at the time of diagnosis. Therefore strategy of chemotherapeutic sensitization may improve the therapy and benefit to clinical outcome.

CHAPTER III
MATERIALS AND METHODS

Materials

1. Chemicals and Reagents

Cisplatin, *N*-acetylcysteine (NAC), reduced glutathione (GSH), sodium pyruvate, catalase, Mn(III)tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), deferoxamine (DFO), hydrogen peroxide (H_2O_2), ferrous sulfate ($FeSO_4$), 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA), propidium iodide (PI) and Hoechst 33342 were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Dihydroethidium (DHE) and 3'-(*p*-hydroxyphenyl) fluorescein (HPF) were from Molecular Probes, Inc. (Eugene, OR, USA). Resazurin was purchased from Invitrogen (Carlsbad, CA, USA). Specific antibody for Cav-1 and peroxidase-conjugated secondary antibody were obtained from Abcam (Cambridge, MA, USA), and specific antibody for β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Equipments

Carbon dioxide incubator, Laminar flow cabinet, Micropipette: 2-10 μl, 10-100 μl, 20-200 μl and 200-1,000 μl, Pipette tips for 2-10 μl, 10-100 μl, 20-200 μl, and 200-1,000 μl, Cell culture plate: 6-well and 96-well normal (Nunc) and Ultra low-attached plate, bottle: 100 ml, 250ml, 500 ml, and 1,000 ml (Duran), Conical tube: 15 ml and 50 ml (Neptune), Disposable pipette: 1ml and 5ml, pH meter, Vertex mixer, Hemocytometer, Fluorescence microplate reader (Beckton Dickinson, Rutherford,

Nj), Fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-U), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Power pac^{TM} Basic)

Methods

1. Sample preparation

Cisplatin are prepared by diluting with deionized water to obtain the desired concentrations.

2. Cell culture

Human lung carcinoma H460 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells are cultured in a 5% CO_2 environment at 37°C using RPMI 1640 medium. The RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml of penicillin/streptomycin.

3. Plasmid and transfection

Cav-1 plasmid was obtained from the American Type Culture Collection (Manassas, VA, USA), and Cav-1 knockdown shRNACav-1 plasmids were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cav-1 overexpressing (H460/Cav-1) and Cav-1 knockdown (H460/shCav-1) cells were established by transfection of the H460 cells with Cav-1 overexpressing or Cav-1 knockdown shRNACav-1, respectively, according to the manufacturer's instruction. Briefly, H460 cells at 60% confluence were incubated with 15 µl of Lipofectamine 2000 reagent and 2 µg of Cav-1 or shRNA-Cav-1 plasmids. After 16 h, the medium was replaced with culture

medium containing 10% fetal bovine serum. Approximately two days after the beginning of transfection, the single-cell suspensions were plated onto 75-ml culture flasks and cultured for 60 days with antibiotic selection. The expression of Cav-1 protein in the transfectants was quantified by western blot analysis.

4. Resazurin cytotoxicity assay

Cell viability was determined by Resazurin cytotoxicity assay which change resazurin to resorufin (resazurin product) by using the reducing power of living cells to quantitatively measure the proliferation of cells. Briefly, H460 cells were seeded in 96-well plates and allowed to attach for 24 h. After specific treatments, cells were incubated with 1:50 resazurin for 1 h at 37°C. Fluorescence intensity of resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and 590 nm (emission wavelength) using a microplate reader. Cell viability was calculated as a percentage relative to that of non-treated cells. All analyses were performed for at least three independent replicate experiments.

Cell viability was calculated as follow:

$$\text{Percentage of cell viability} = \frac{\text{Fluorescence intensity of treatment}}{\text{Fluorescence intensity of control}} \times 100$$

5. Apoptosis and necrosis assay

Apoptotic and necrotic cell death was evaluated by Hoechst 33342 and propidium iodide (PI) co-staining. After specific treatments, cells were incubated with 10 μM of Hoechst 33342 and 5 μg/ml PI dye for 30 min at 37°C. Apoptotic cells having condensed chromatin, fragmented nuclei and PI-positive necrotic cells were

visualized and scored under a fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-U).

6. Anoikis assay

H460 cells were seeded in 6-well plates and treated with various concentrations of cisplatin for 24 h. Adherent cells were then detached and seeded in 6-well ultra low-attached plates in RPMI medium at a density of 40,000 cells/well. Suspended cells were incubated under normal culturing condition at 37°C for various times up to 24 h. Cells were harvested, washed and incubated with 1:50 resazurin for 1 h at 37°C, and the fluorescence intensity of resazurin product (resorufin) was measured at 530 nm (excitation wavelength) and 590 nm (emission wavelength) using a microplate reader. Relative cell viability was calculated as a percentage relative to that of non-treated cells. All analyses were performed for at least three independent replicate cultures.

7. ROS detection

Intracellular ROS were determined using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH$_2$-DA), superoxide anion was determined by dihydroethidium (DHE), and hydroxyl radical was determined by 3'-(p-hydroxyphenyl) fluorescein (HPF). Cells were incubated with 10 μM of DCFH$_2$-DA, HPF, or DHE for 30 min at 4ºC, after which they were immediately analyzed for fluorescence intensity by fluorescence microplate reader (SpectraMax M5; Molecular Devices Crop., Sunnyvale, CA, USA) using a 480-nm excitation beam and a 530-nm bandpass filter for detecting DCF fluorescence, a 490-nm excitation beam and a 515-

nm band-pass filter for HPF, or a 488-nm excitation beam and a 610-nm band-pass filter for DHE, and were visualized under fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-U).

8. Western blot analysis

After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris·HCl (pH 7.5), 1% TritonX-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min on ice. Cell lysates were collected and determined for protein content using the Bradford method (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins for each sample (40 μg) were denatured by heating at 95°C for 5 min with Laemmli loading buffer and were subsequently loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel for electrophoresis (PAGE). After separation, proteins were transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST [25 mM Tris·HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20] and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and quantified using analyst/PC densitometry software (Bio-Rad).

9. Statistical analysis

Mean data from at least three independent experiments were normalized to the results of the non-treated controls. Statistical differences between means were determined using an analysis of variance (ANOVA) and post hoc (Tukey's test) at a significance level of $p<0.05$, and are presented as the mean $\pm$ SD.
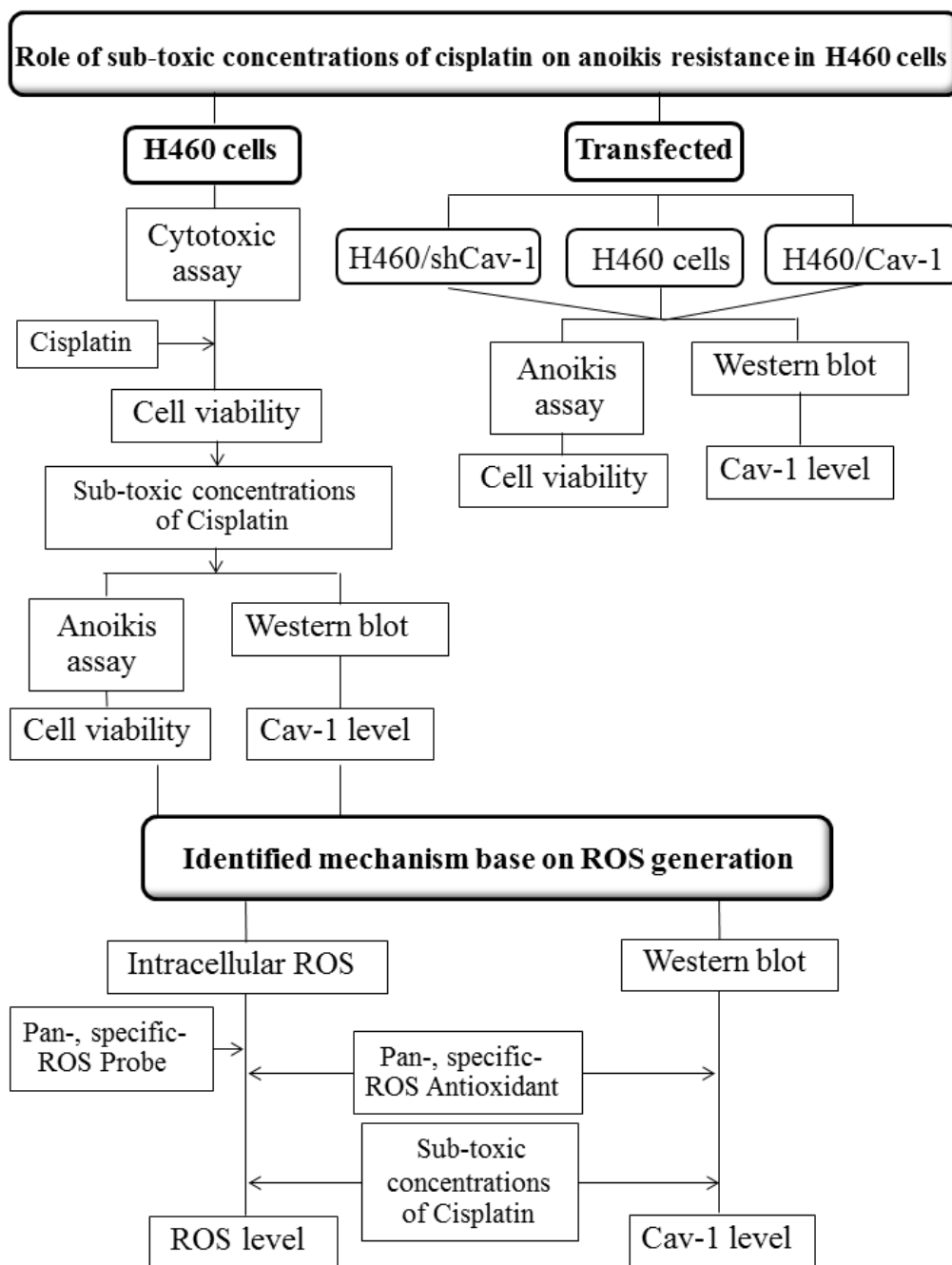
10. Experimental design

10.1 Experimental design

Figure 3.1 Experimental design of this study

10.2 Conceptual framework of this study
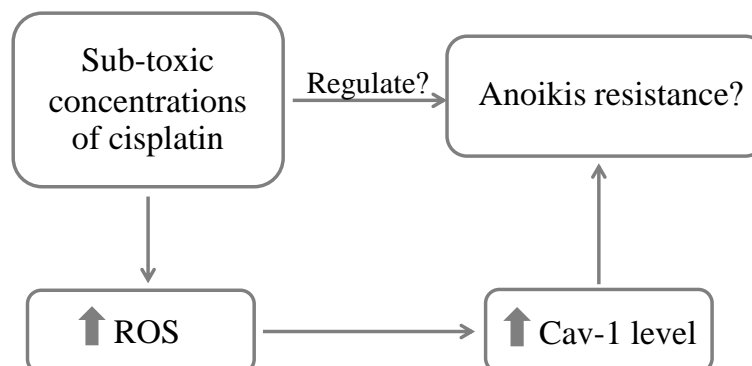
Figure 3.2 Conceptual framework of this study

10.3 Investigation on the cytotoxic effects of cisplatin in lung carcinoma cells

The effect on cell viability of sub-toxic concentrations were determined by resazurin cytotoxicity assay. H460 cells were seeded in 96-well plate, treated with various concentrations of cisplatin (0-100 μM) for 24 h, and then cell viability was analyzed. In addition, cells were similarly treated with cisplatin as above mentioned, apoptotic and necrotic cell deaths were determined by Hoechst 33342 and propidium iodide co-staining. Hoechst-positive cells containing condensed or fragmented nuclear fluorescence and necrotic PI-positive cells were visualized and scored under a fluorescence microscope (Nikon Inverted Microscope Eclipse Ti-U).

10.4 To determine the effect of sub-toxic concentration of cisplatin on anoikis susceptibility of H460 cells

H460 cells were seeded in 6-well plate, and treated with various sub-toxic doses of cisplatin for 24 h. Cells were then detached and incubated in ultra low-attached plates for various times (0-24 h). After that they were determined for cell

viability by the Resazurin cytotoxicity assay. To determine characteristic of detachment-induced apoptosis and further confirmed the effect of cisplatin exposure in alteration of H460 cells anoikis was by staining with Hoechst 33342 and propidium iodide.

10.5 Investigation on the effect of Cav-1 on anoikis characteristic of H460 cells

Cells were stably transfected with Cav-1 overexpressing, shRNA Cav-1, or control plasmids. After selection and propagation, Cav-1 overexpressing, shCav-1, and control cells were subjected to Western blot analysis as described in the Materials and Methods section. To confirm the correlation of Cav-1 level and anoikis resistance, after detachment the cell viability and its apoptotic, necrotic cell deaths was evaluated by the anoikis assay and Hoechst 33342 and propidium iodide co-staining , respectively.

10.6 Investigation on Sub-toxic concentrations of cisplatin mediated Cav-1 level

Having shown that Cav-1 played a role in attenuating anoikis response of H460 cells. To determined the effect of sub-toxic cisplatin exposure on cellular Cav-1 level. Cells were treated with various concentrations of cisplatin for 24 h, and its effect on Cav-1 expression was evaluated by Western blot analysis.

10.7 To determine the effect of sub-toxic concentrations of cisplatin induced cellular ROS generation

Cisplatin-induced cellular ROS generation has been widely accepted as one mechanism in the mode of action of cisplatin. It is very interesting that whether

cisplatin in low concentrations causing no cytotoxic effect could modulate cellular ROS and affect cell behaviors or not. Cells were incubated with sub-toxic dose of cisplatin and observed intracellular ROS over times. These cells were pre-incubated with specific ROS probe (DCFH$_2$-DA) for 30 min prior to cisplatin treatment. Accumulation of intracellular ROS inside these cells was evaluated by fluorescence microplate reader. To confirm the results of ROS induction ability of sub-toxic doses of cisplatin, known antioxidants N-acetylcysteine (NAC) and reduced glutathione (GSH) were added to the cells before cisplatin treatment.

In addition, Specific ROS-induced by sub-toxic concentrations of cisplatin were determined using specific ROS scavengers, namely sodium pyruvate (hydrogen peroxide scavenger), MnTBAP (superoxide anion inhibitor), and deferoxamine (hydroxyl radical inhibitor), and specific ROS probes, namely DHE for superoxide detection and HPF for hydroxyl radical detection. Although DCFH$_2$-DA probe was not specific for hydrogen peroxide detection, evidence had shown that sodium pyruvate could abolish cisplatin-mediated hydrogen peroxide induction. The fluorescence signals were evaluated by fluorescence microscope and fluorescence microplate reader.

10.8 ROS induced by sub-toxic doses of cisplatin up-regulate Cav-1 and anoikis resistance in lung cancer cells

ROS inductions were concomitant with Cav-1 up-regulation. To test the role of ROS on cisplatin-mediated Cav-1 induction, cells were treated with sub-toxic concentrations of cisplatin in the presence or absence of pan-ROS scavengers, reduced glutathione and N-acetylcysteine for 24 h, and Cav-1 expression was determined by

Western blotting. Furthermore, to identify which ROS was involved in this process, H460 cells were pre-treated with specific ROS antioxidants including deferoxamine, MnTBAP, sodium pyruvate or catalase, prior to cisplatin treatment, and Cav-1 expression level was analyzed by Western blotting.

If the results suggest toward that which ROS induced by cisplatin was able to regulate Cav-1 expression in these cells, the effect of that exogenous ROS treatment on Cav-1 level was evaluated to confirm the findings. Cav-1 level was analyzed by Western blotting.
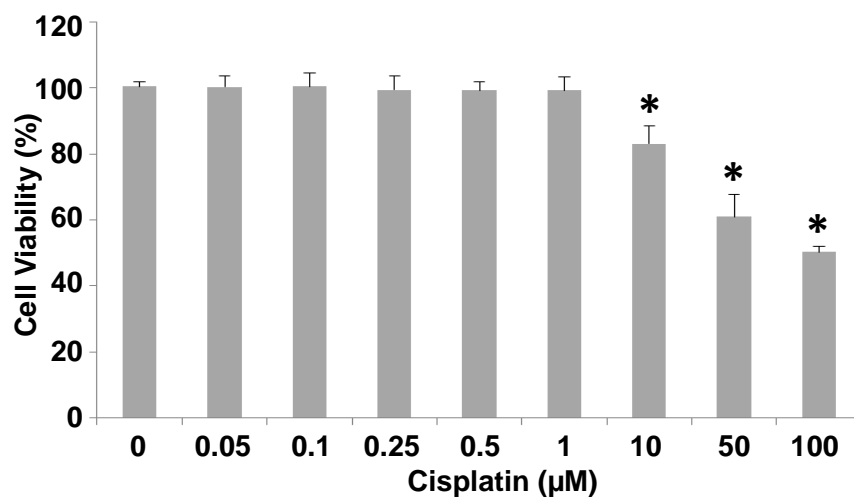
CHAPTER IV

RESULTS

1. Cisplatin mediated cytotoxicity in lung carcinoma H460 cells.

To investigate the cytotoxic effect of sub-toxic concentrations of cisplatin, this study first examined cytotoxic effect of cisplatin on H460 cells by cytotoxicity, apoptosis, and necrosis assays. Cells were left untreated or treated with cisplatin at the concentrations of 0.05, 0.1, 0.25, 0.5, 1, 10, 50 and 100 µM. After 24 h, cell viability and cell death were determined.

The result showed that the treatment of cisplatin at the concentration range from 0.05 to 1 µM had no significant effect on H460 cell viability, while cisplatin at the concentrations of 10, 50 and 100 µM caused significant decrease in viable cells, with approximately 83, 62 and 57%, respectively (As shown in figure 4.1A). In addition, the condensed or fragmented nuclear fluorescence study supported that no apoptotic were detected in response to cisplatin at concentrations of 0.05-1µM. As the dose of cisplatin increased to 10, 50, and 100 µM, cisplatin-mediated apoptosis was increased to 15, 37, and 52%, respectively (As shown in figure 4.1A). While necrotic PI-positive cells were barely detected at all concentrations of cisplatin (As shown in figure 4.1B, C). These results suggested that concentrations of cisplatin at 0.05-1µM had no significant cytotoxic effect on H460 cells.

A

B

C

Hoechst

PI

Control **Cisplatin 0.05 μM** **Cisplatin 0.1 μM** **Cisplatin 0.25 μM** **Cisplatin 0.5 μM**

Hoechst

PI

Cisplatin 1 μM **Cisplatin 10 μM** **Cisplatin 50 μM** **Cisplatin 100 μM**
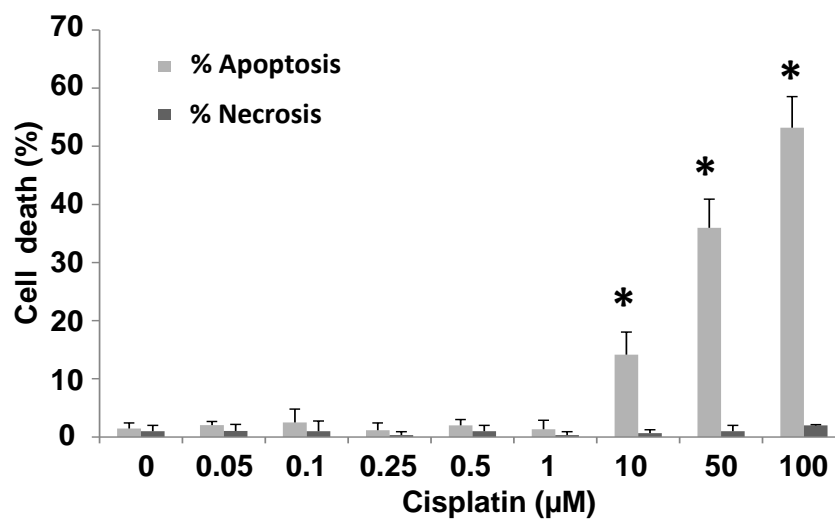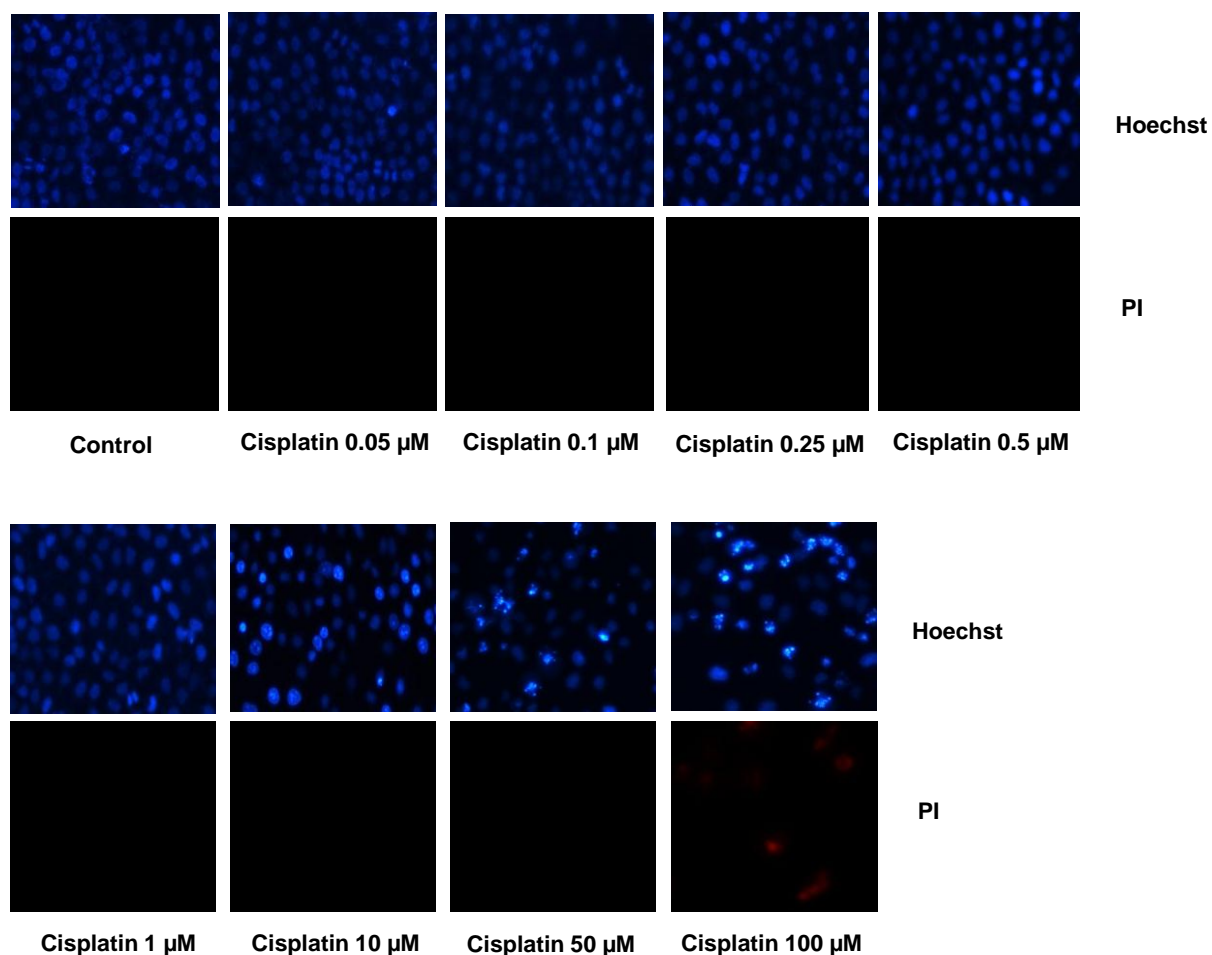
Figure 4.1 Cisplatin mediated cytotoxicity in lung carcinoma H460 cells. **A)** H460 cells were treated with cisplatin (0, 0.05, 0.1, 0.25, 0.5, 1, 10, 50, and 100 μM) for 24 h, and cell viability was determined by resazurin cytotoxicity assay. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control. **B)** Nuclear morphology of apoptosis and necrosis wasdetermined by Hoechst 33342/propidium iodide staining at 24 h after cisplatin treatment. . Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control. **C)** Morphology of apoptotic and necrotic cells were visualized under fluorescence microscope.

2. Effect of sub-toxic concentrations of cisplatin on anoikis susceptibility of H460 cells

To determine the effect of sub-toxic concentrations of cisplatin on anoikis susceptibility of the cells, H460 cells were seeded in 6-well plates, and treated with sub-toxic concentrations of cisplatin (0, 0.05, 0.1, 0.25, 0.5 and 1 µM) for 24 h. Cells were then detached and incubated in ultra low-attached plates for various times (0, 3, 6, 12 and 24 h) and cell viability determined by the Resazurin cytotoxicity assay and cell death detection by Hoechst 33342 and Propidium Iodide (PI) co-staining assay.

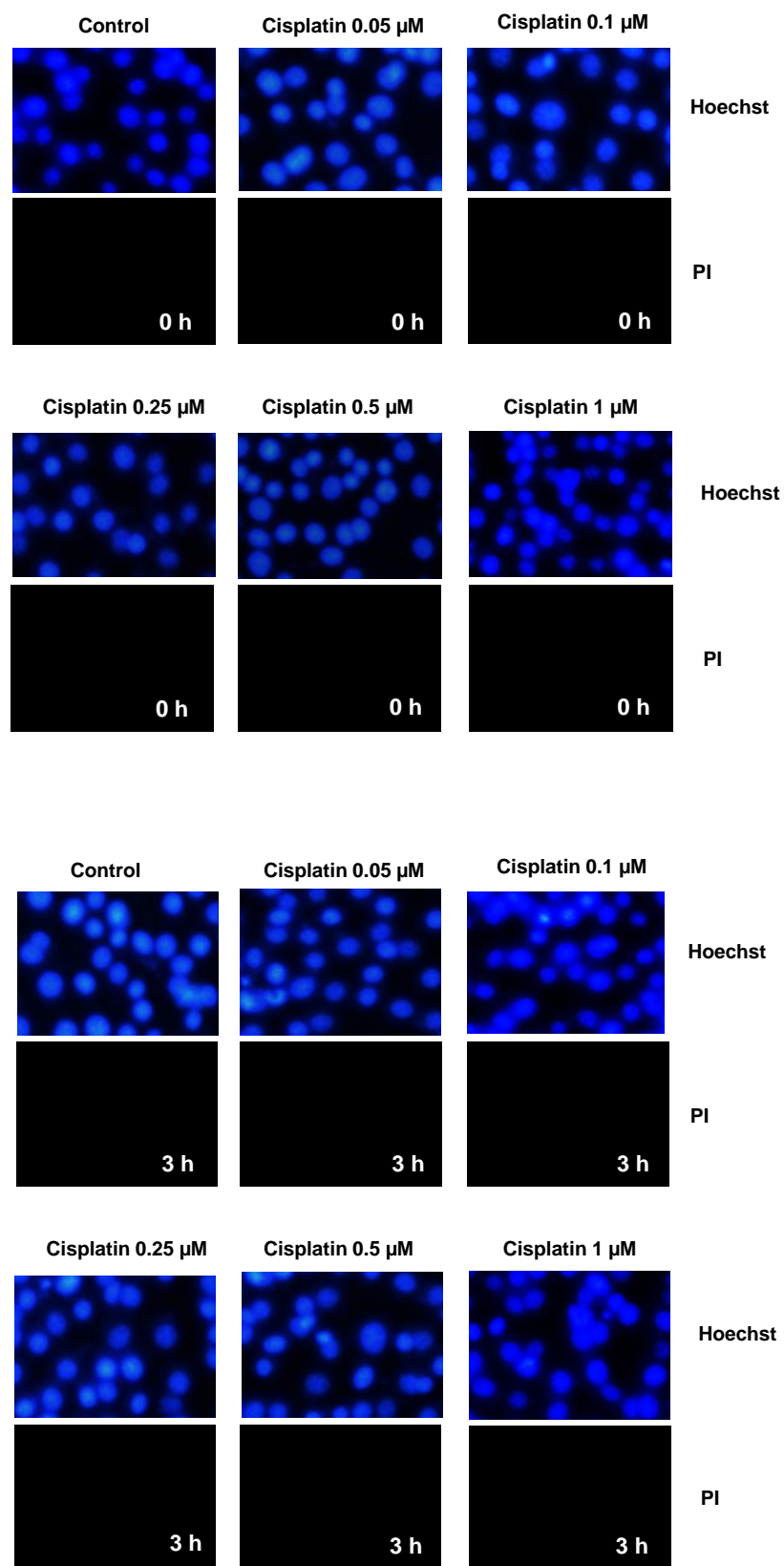The result showed that cisplatin treatment prior to cell detachment significantly increased anoikis resistance in a dose-dependent manner. At 6 h after detachment, anoikis sensitivity alteration could be observed with approximately 89 and 91% of viable cells in cells treated with 0.5 and 1 µM of cisplatin respectively, in comparison 81% survival of non-treated control cells (As shown in figure 4.2A). In addition, Figure 4.2B shows the characteristic morphology of detachment-induced apoptosis and further confirmed the effect of cisplatin exposure on inhibition of H460 cells anoikis. It is worth notting that nuclear PI fluorescence was not detectable in this experiment. These results suggested that sub-toxic concentrations of cisplatin regulate anoikis resistance on H460 cells.

A

B

Control	Cisplatin 0.05 µM	Cisplatin 0.1 µM	

			Hoechst
			PI
6 h	6 h	6 h	

Cisplatin 0.25 µM	Cisplatin 0.5 µM	Cisplatin 1 µM	

			Hoechst
			PI
6 h	6 h	6 h	

Control	Cisplatin 0.05 µM	Cisplatin 0.1 µM	

			Hoechst
			PI
12 h	12 h	12 h	

Cisplatin 0.25 µM	Cisplatin 0.5 µM	Cisplatin 1 µM	

			Hoechst
			PI
12 h	12 h	12 h	
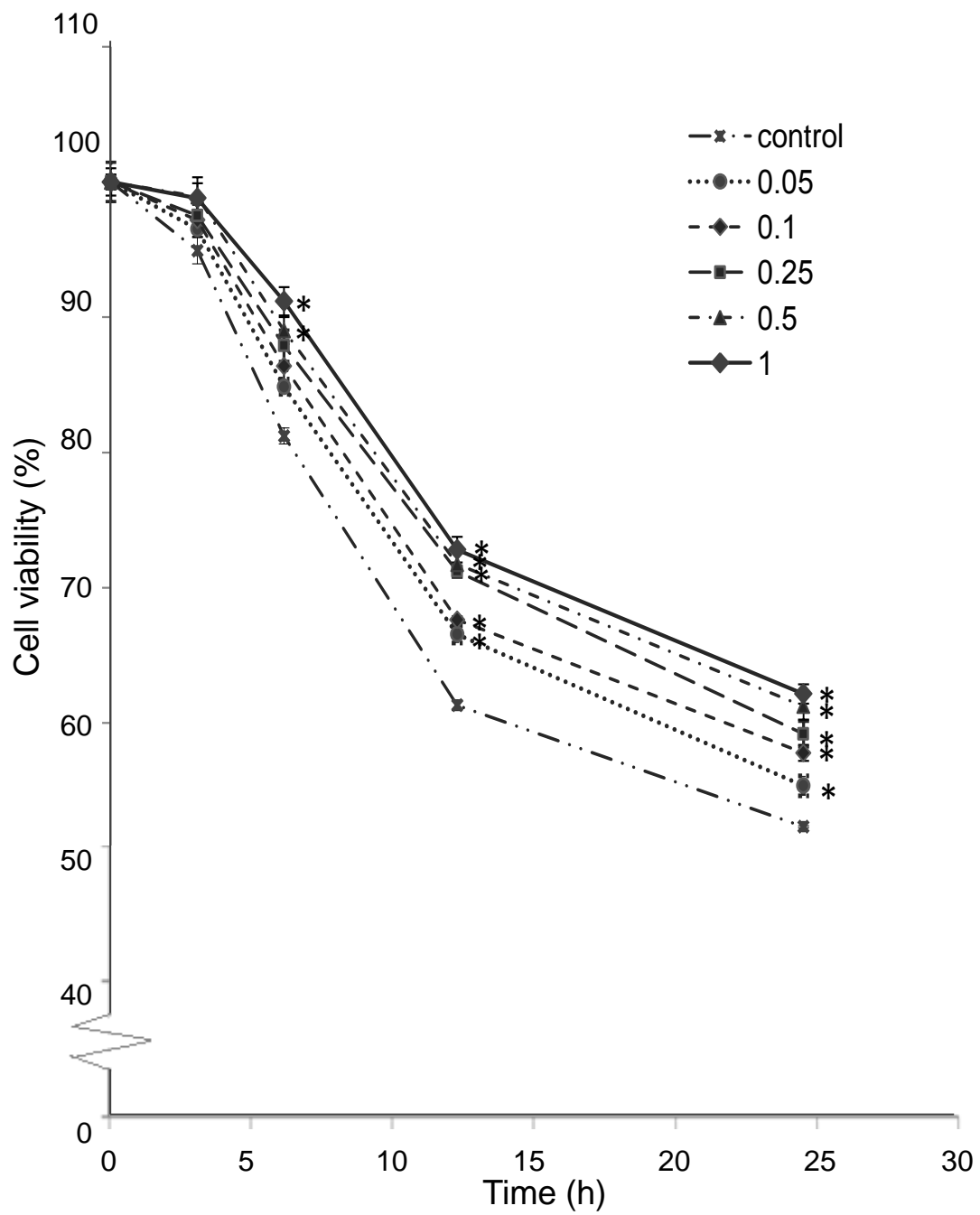
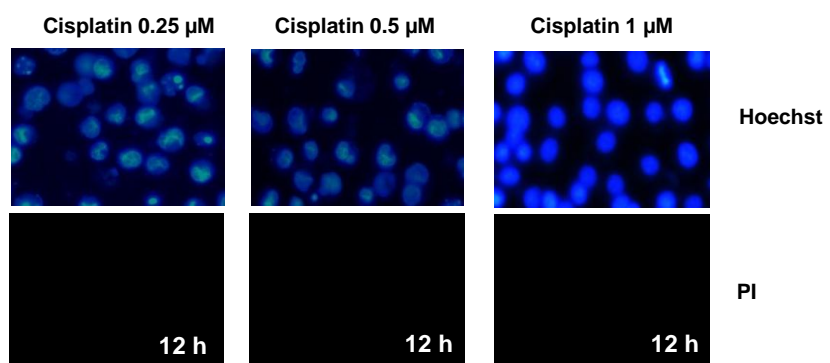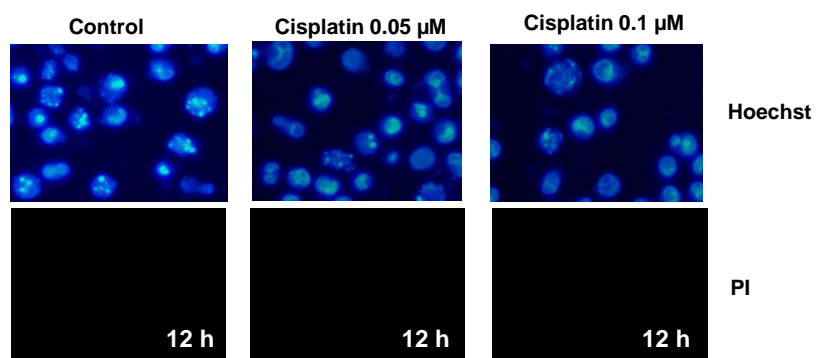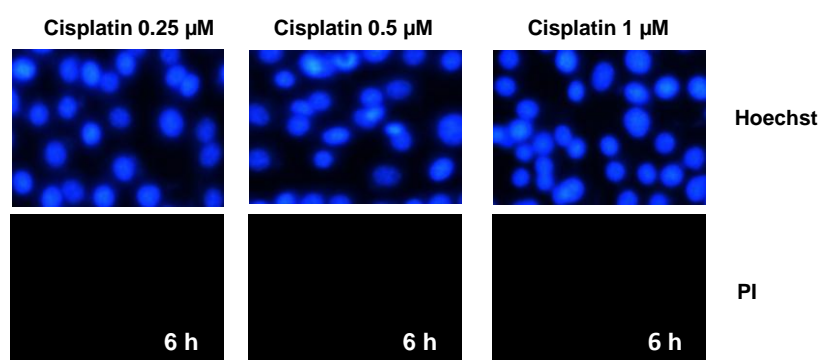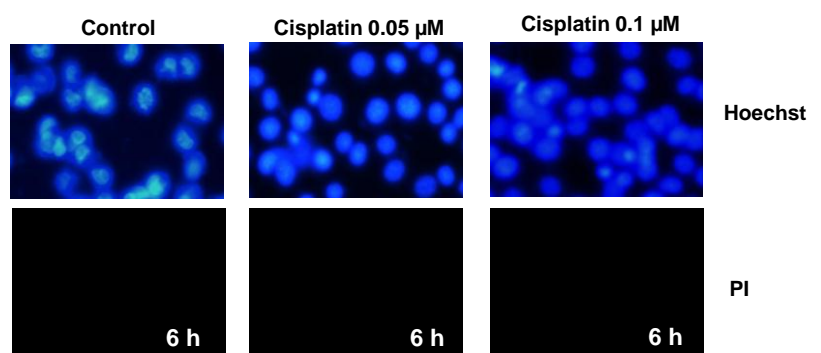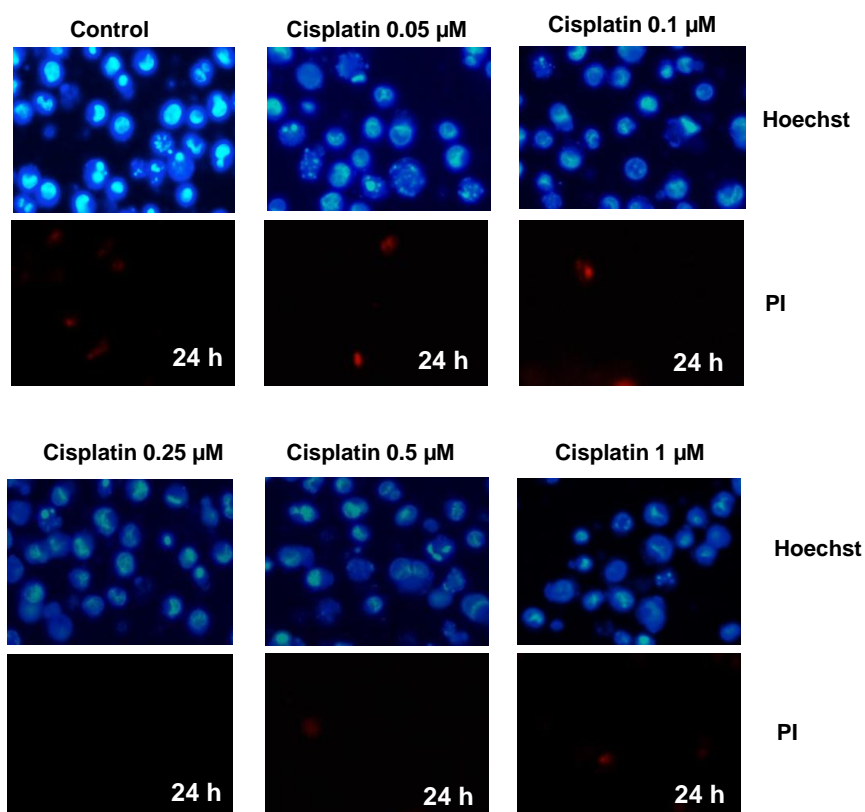**Figure 4.2** Effect of sub-toxic concentrations of cisplatin on anoikis susceptibility of H460 cells. **A)** H460 cells were treated with (0, 0.05, 0.1, 0.25, 0.5 and 1 µM) of cisplatin for 24 h, and the cells were suspended in ultra low-attached plate for various times (0, 3, 6, 12 and 24 h). Cell survival was determined at the indicated times by resazurin cytotoxicity assay. The viability of cells is presented as cell viability relative to that of non-detachment controls. Values are means±S.D; (n=3); * *P*<0.05 *versus* non-treated control. **B)** Anoikis nuclei stained with Hoechst 33342 and propidium iodide was visualized under fluorescence microscope

3. To determine the effect of Cav-1 on anoikis characteristic of H460 cells

Cav-1 has been demonstrated to regulate cancer cell aggressiveness and metastasis in various types of cancer (Terence and Michael, 2005; Di Vizio *et al*., 2007). In particular, our previous study indicated the role of Cav-1 in inhibition of cell anoikis (Rungtabnapa *et al*., 2011). Cells were stably transfected with Cav-1, shRNACav-1, or control plasmids. After selection and propagation, Cav-1- and shCav-1-transfected cells and control cells were subjected to anoikis assay and western blot analysis as described in the Materials and Methods. Western blotting detected with anti-Cav-1 antibodies showed the substantial elevation of Cav-1 level in the cells stably transfected with Cav-1 plasmid, while a significantly reduced Cav-1 level was detected in shCav-1-transfected cells (As shown in figure 4.3A).

To confirm the correlation of Cav-1 level and anoikis resistance, cell viability after detachment was evaluated. Figure 4.3B shows that Cav-1-transfected cells exhibited higher (~80%) of cell survival at 0-24 h after detachment compared with control cells (~40%), whereas cell viability of shRNACav-1-transfected cells was extensively reduced to ~20%. Hoechst33342 assay also showed an increase in nuclear fluorescence and chromatin condensation of the detached cells over time in all these cells, with the highest proportion of anoikis being observed in shRNACav-1-transfected cells (As shown in figure 4.3C). In contrast, no detectable change in nuclear PI fluorescence was identified during the detachment period of time.

A
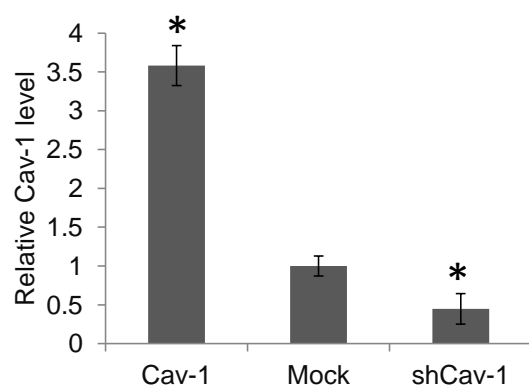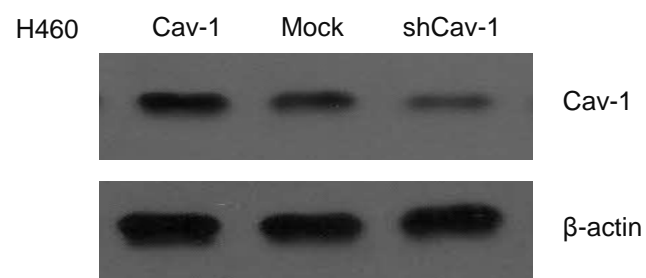
B
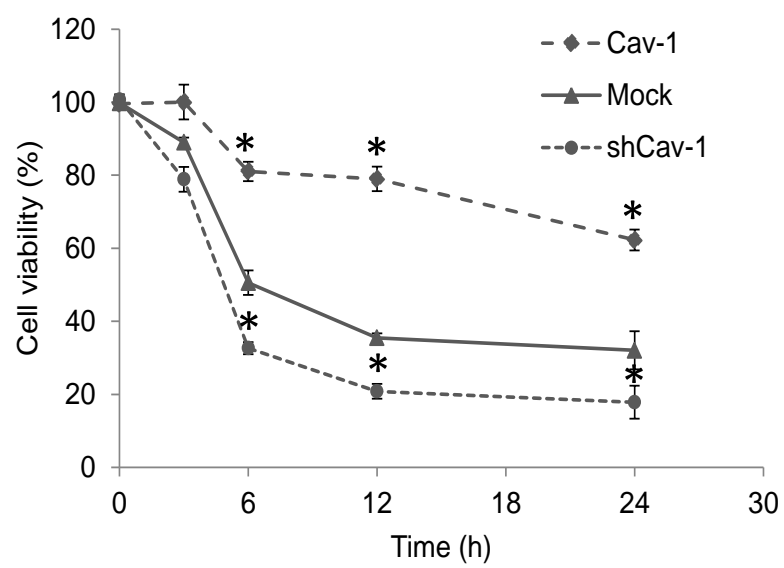
C
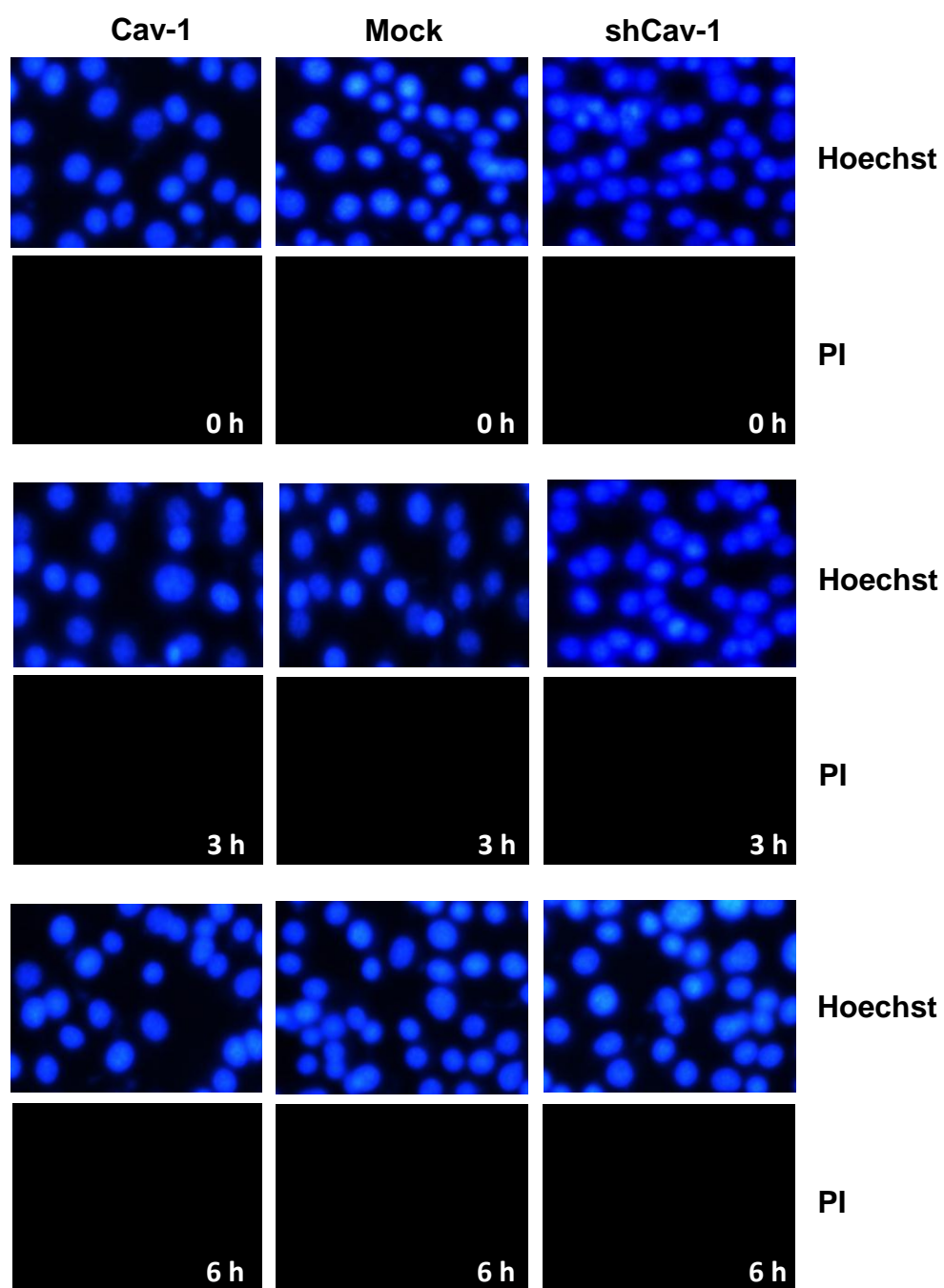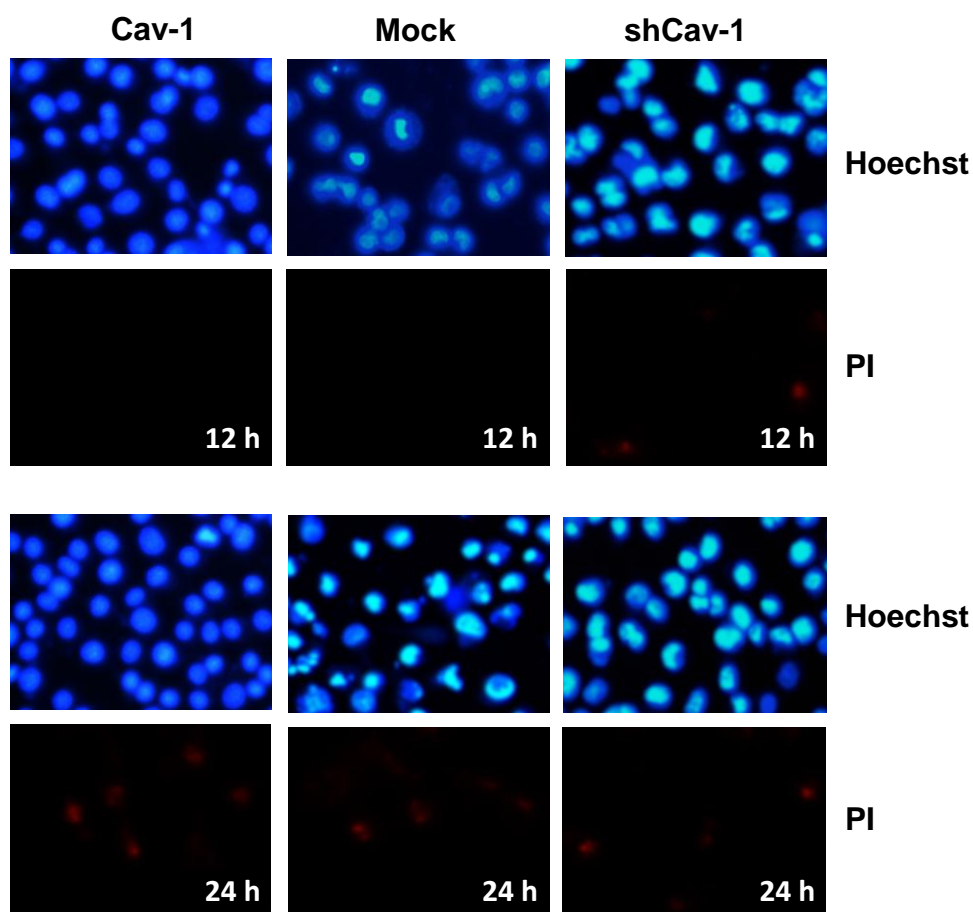
Figure 4.3 Effect of Cav-1 on anoikis characteristic of H460 cells. **A)** Cells were stably transfected with mock , Cav-1, or shRNA-Cav-1 plasmids, and analyzed for Cav-1 expression levels by western blotting. Values are means±S.D. (n=3); * $P<0.05$ *versus* control-transfected cells. **B)** Cells were detached and suspended in ultra low-attached plates for various times (0, 3, 6, 12, 24 h). After indicated times, cell survival was evaluated by resazurin cytotoxicity assay. Values are means±S.D. (n=3); * $P<0.05$ *versus* control-transfected cells. **C)** Apoptosis and necrosis cells were determined by Hoechst 33342 and propidium iodide, and visualized under fluorescence microscope.

4. Investigation on Sub-toxic concentrations of cisplatin mediated Cav-1 level

To determined the effect of sub-toxic concentrations of cisplatin on Cav-1 level of H460 cells. Cells were treated with sub-toxic concentrations of cisplatin (0, 0.05, 0.1, 0.25, 0.5 and 1 µM) for 24 h, and Cav-1 level was evaluated by western blot analysis.

The results clearly showed that the Cav-1 level of cells was increased in response to sub-toxic concentrations of cisplatin treatment in a dose-dependent manners. Which these results suggested that sub-toxic cisplatin exposure was able to elevate Cav-1 level (As shown in figure 4.4).
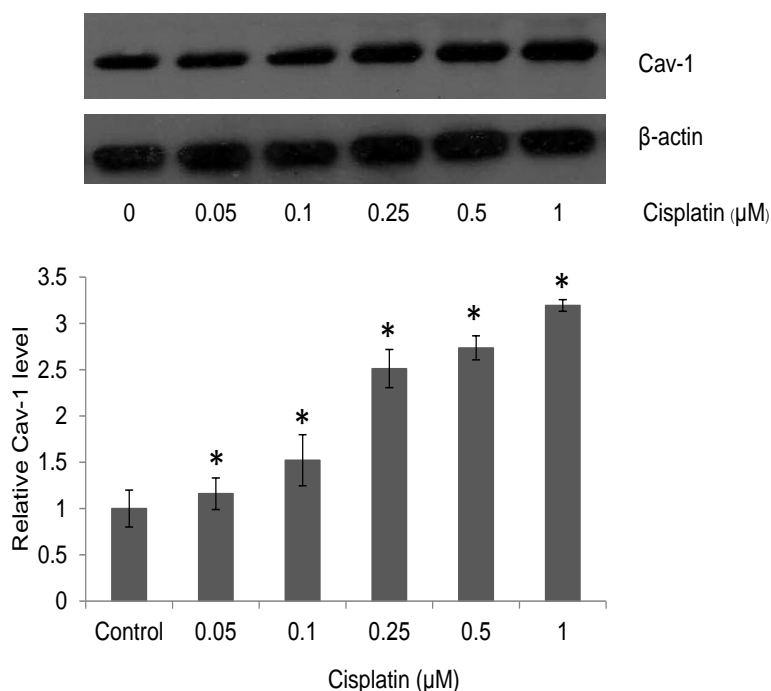
Figure 4.4 Sub-toxic concentrations of cisplatin increased cellular Cav-1 level. H460 cells were treated with cisplatin (0, 0.05, 0.1, 0.25, 0.5 and 1 µM) for 24 h, and Cav-1 expression levels were then determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. Immunoblot signals were

quantified by densitometry and mean data from independent experiments were normalized to non-treated cells. Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control cells.

5. Effect of sub-toxic concentrations of cisplatin induced cellular ROS generation

Cisplatin-induced cellular ROS generation has been widely accepted as one mechanism in the mode of action of cisplatin. It is very interesting to consider whether cisplatin low concentrations causing no cytotoxic effect could modulate cellular ROS and affect cell behaviors. H460 cells were incubated with a sub-toxic dose of cisplatin (1 μM) and determined intracellular ROS at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 h. Cells were pre-incubated with a specific ROS probe (DCFH$_2$-DA) for 30 min prior to cisplatin treatment. Accumulation of intracellular ROS inside these cells was evaluated by fluorescence microplate reader and the results indicated that cisplatin did induced cause a significant increase in ROS production of the cells as early as 1.5 h compared to non-treated control (As shown in figure 4.5.1A).

In order to confirm the results of ROS induction by cisplatin, known antioxidants 1 mM NAC and 1 mM GSH were added to the cells before cisplatin treatment. Administration of such antioxidants dramatically inhibited both base-line ROS production and cisplatin-mediated ROS up-regulation in these cells, confirming the effect of cisplatin on the cellular ROS profile (As shown in figure 4.5.1A). Next, To confirmed the effect of sub-toxic concentration of cisplatin on cellular ROS profile, these cells visualized under fluorescence microscope. The results shown that it is in the same direction (As shown in figure 4.5.1B).

Furthermore, To identified specific species of ROS induced by sub-toxic concentrations of cisplatin using specific ROS probes and scavengers, and evaluated the fluorescence signals by fluorescence microscopy and fluorescence microplate reader. The fluorescence intensity were detected by fluorescence microscope reader at 0, 0.25, 0.5, 0.75, 1, 2 and 3 h. First, cells were similarly treated with cisplatin in the presence of specific ROS probes, namely, $DCFH_2$-DA for hydrogen peroxide detection and specific ROS scavengers, namely sodium pyruvate (hydrogen peroxide scavenger). The fluorescence result indicated that treatment with 1 μM cisplatin induced hydrogen peroxide production as indicated by significantly increased cellular fluorescence intensity was detected at 0.75, 1, 2 and 3 h (As shown in figure 4.5.2A). Although $DCFH_2$-DA probe was not specific for hydrogen peroxide detection, evidence that sodium pyruvate abolished cisplatin-mediated ROS induction (As shown in figure 4.5.2A, B), suggesting that hydrogen peroxide was the main ROS induced by sub-toxic concentration of cisplatin.

Second, DHE phobe and MnTBAP (superoxide anion inhibitor) were used for superoxide anion detection. The result indicated that the fluorescence intensity of superoxide anion was markedly increased after 0.75 h of cisplatin treatment (As shown in figure 4.5.3A). Moreover, the addition of MnTBAP dramatically decreased the fluorescence intensity of superoxide anion originated from cisplatin treatment (As shown in figure 4.5.3A, B), suggesting that superoxide anion radical were produced in response to the treatment of cisplatin.

Finally, Intracellular hydroxyl radical induction was evaluated by response to sub-toxic concentration of cisplatin in H460 cells using HPF as a fluorescent probe and deferoxamine as a hydroxyl radical inhibitor. The fluorescence signals were

evaluated by fluorescence microscope and fluorescence microplate reader. The result showed that sub-toxic concentration of cisplatin treatment caused no induction in HPF signal compared to the non-treated control (As shown in figure 4.5.4A). Also, addition of deferoxamine could not alter the HPF signal compared to the cisplatin- and non-treated cells (As shown in figure 4.5.4A, B). Hydroxyl radical was not significantly induced in this sub-toxic cisplatin mediated ROS induction.

A

B

DCFH$_2$-DA

control   Cisplatin 1 µM   +GSH   +NAC
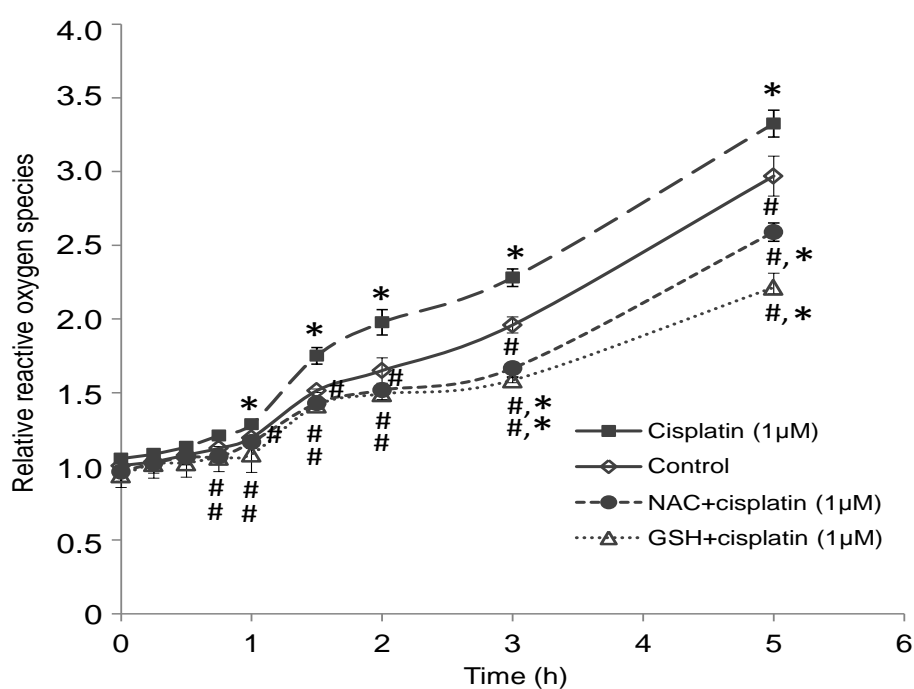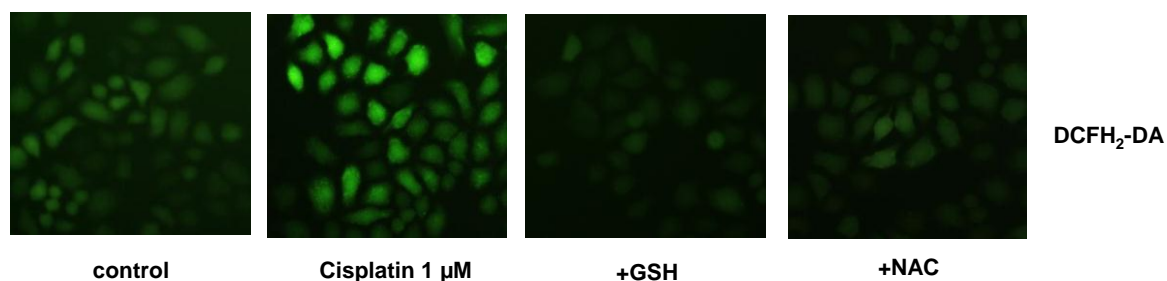
Figure 4.5.1 Sub-toxic concentrations of cisplatin induced ROS generation. **A)** H460 cells were pre-incubated with DCFH$_2$-DA prior to incubated with sub-toxic concentrations of cisplatin (1 µM) and observed intracellular ROS at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 h. ROS generation were determined by fluorescence microplate reader. To confirm the results of ROS induction ability of sub-toxic doses of cisplatin, known antioxidants 1 mM N-acetylcysteine (NAC) and 1 mM reduced glutathione (GSH) were added to the cells before cisplatin treatment. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control; [#] $P<0.05$ *versus* cisplatin-treated cells. **B)** Cellular ROS signal at 3h after cisplatin treatment was visualized under fluorescence microscope.

A

B
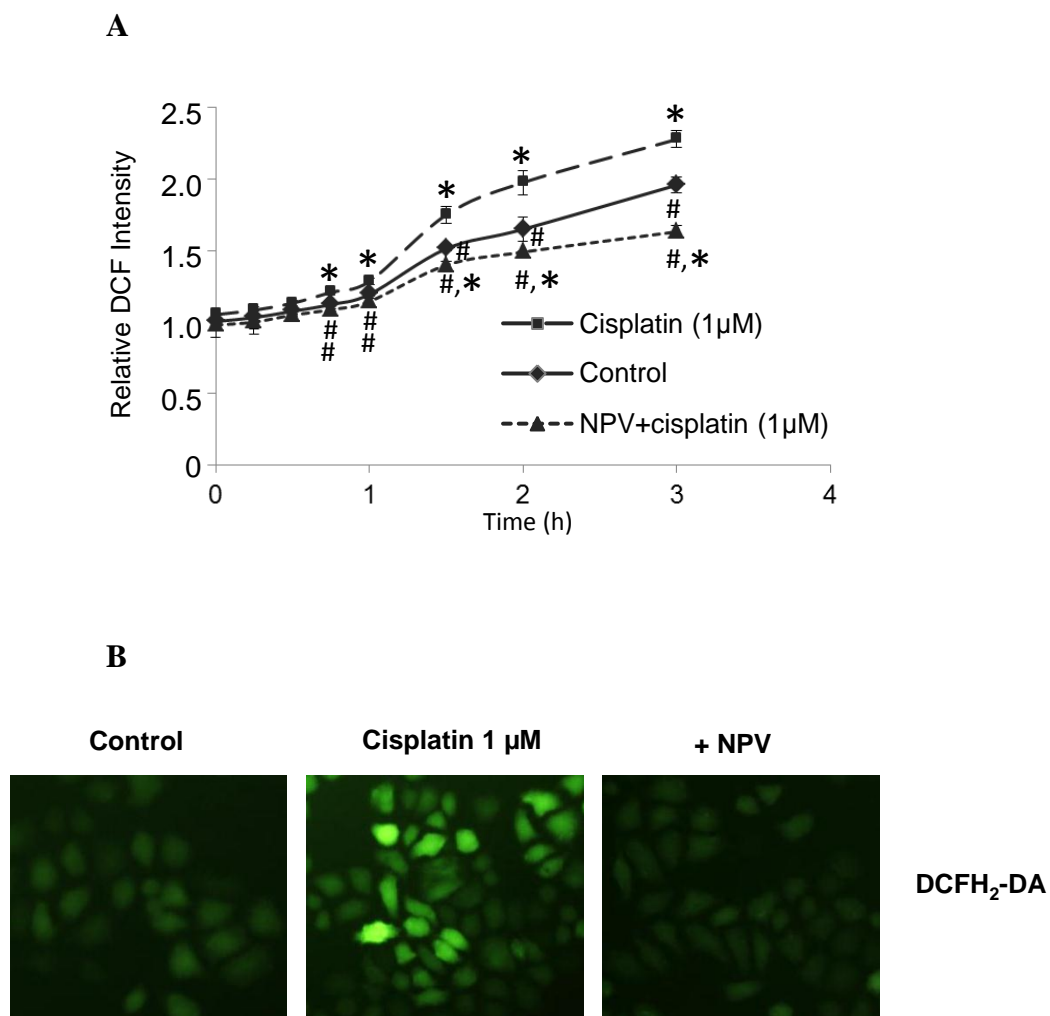
Figure 4.5.2 To identified specific species of ROS (Hydrogen peroxide) induced by sub-toxic concentrations of cisplatin. **A)** H460 cells were pre-incubated with $DCFH_2$-DA for 30 min prior to incubated with sub-toxic concentrations of cisplatin (1 μM) and observed intracellular ROS at 0, 0.25, 0.5, 0.75, 1, 2, 3 h. ROS generation were determined by fluorescence microplate reader. To confirm the results of Hydrogen peroxide induction ability of sub-toxic doses of cisplatin, specific antioxidant 1 μM sodium pyruvate (NPV) was added to the cells before cisplatin treatment. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control; [#] $P<0.05$ *versus* cisplatin-

treated cells. **B)** Hydrogen peroxide signals were determined by under fluorescence microplate reader and visualized under fluorescence microscope.

A

B
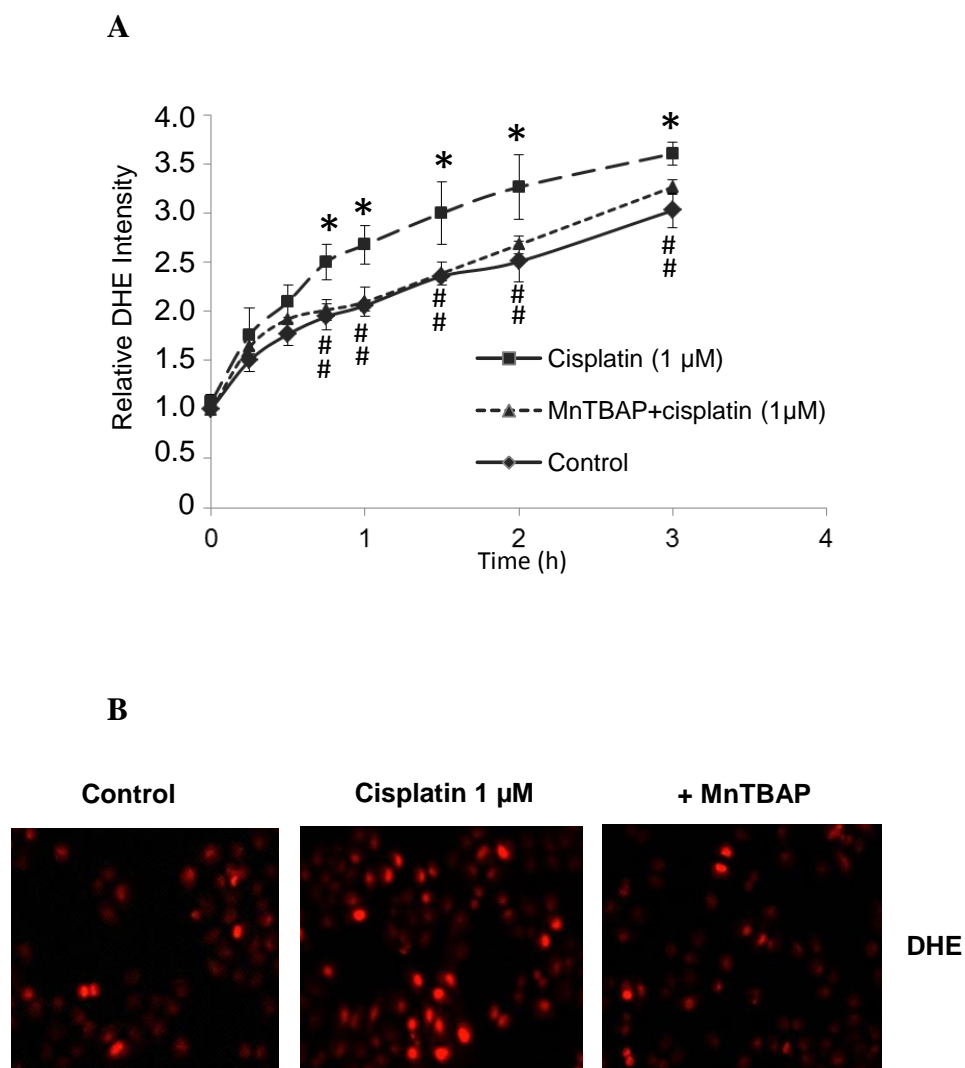
Figure 4.5.3 To identified specific species of ROS (Superoxide anion) induced by sub-toxic concentrations of cisplatin. **A)** H460 cells were pre-incubated with DHE for 30 min prior to incubated with sub-toxic concentrations of cisplatin (1 µM) and observed intracellular ROS at 0, 0.25, 0.5, 0.75, 1, 2, 3 h. ROS generation were determined by fluorescence microplate reader. To confirm the results of Superoxide

anion induction ability of sub-toxic doses of cisplatin, specific antioxidant 50 μM MnTBAP was added to the cells before cisplatin treatment. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control; [#] $P<0.05$ *versus* cisplatin-treated cells. B) Superoxide anion signals were determined by under fluorescence microplate reader and visualized under fluorescence microscope.

A

B

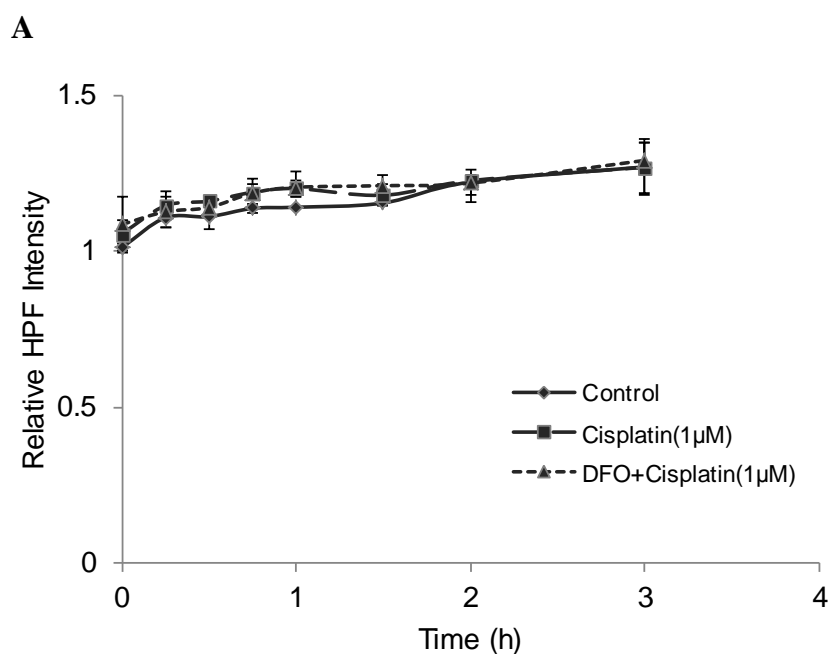**Figure 4.5.4** To identified specific species of ROS (Hydroxyl radical) induced by sub-toxic concentrations of cisplatin. **A)** H460 cells were pre-incubated with HPF for 30 min prior to incubated with sub-toxic concentrations of cisplatin (1 μM) and observed intracellular ROS at 0, 0.25, 0.5, 0.75, 1, 2, 3 h. ROS generation were determined by fluorescence microplate reader. To confirm the results of Hydroxyl radical induction ability of sub-toxic doses of cisplatin, specific antioxidant 1 μM Deferoxamine (DFO) was added to the cells before cisplatin treatment. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control; # $P<0.05$ *versus* cisplatin-treated cells. **B)** Hydroxyl radical signals were determined by under fluorescence microplate reader and visualized under fluorescence microscope.
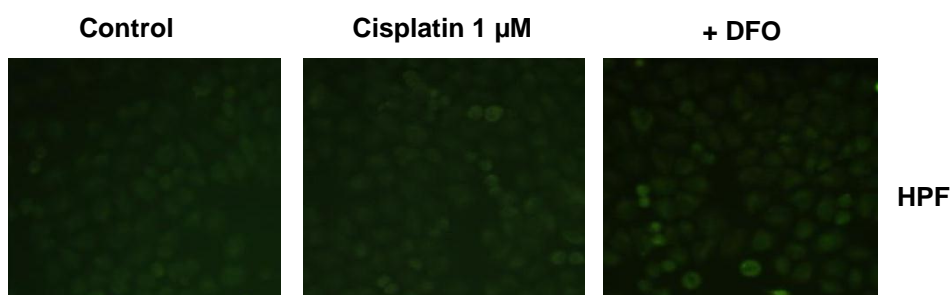
6. ROS induced by sub-toxic concentrations of cisplatin up-regulate Cav-1 and anoikis resistance in lung cancer cells

To determined that ROS inductions were the dominant factor which up-regulation of Cav-1. H460 cells were treated with cisplatin (0.25, 1 μM) in the presence or absence of pan-ROS scavengers, such as reduced glutathione (1 mM) and N-acetylcysteine (1 mM) for 24 h. The Cav-1 expression was determined by Western blotting. The result indicated that cisplatin treatment for 24 h caused a significant increase in Cav-1 level and the upregulating effect of sub-toxic concentrations of cisplatin on Cav-1 expression was inhibited by NAC and GSH (As shown in figure 4.6A).

Having shown that sub-toxic dose of cisplatin could increase cellular superoxide anion and hydrogen peroxide. Additional, to identified which ROS were involved in this process. H460 cells were pre-incubated with specific ROS

antioxidants, such as deferoxamine (1 mM), MnTBAP (50 μM), sodium pyruvate (1 mM) or catalase (5,000 U/ml) prior to 1 μM treatment of cisplatin; Cav-1 expression was determined by western blotting. The result indicated that sub-toxic concentration of cisplatin treatment increased the cellular Cav-1 level and addition of hydrogen peroxide scavengers, sodium pyruvate and catalase completely inhibited cisplatin-induced up-regulation. Whereas, pre-incubated with deferoxamine and MnTBAP had not decreased the Cav-1 level that were induced by sub-toxic concentration of cisplatin (As shown in figure 4.6B).

The results suggested that only hydrogen peroxide induced by cisplatin was able to regulate Cav-1 expression in H460 cells, the effect of that exogenous exogenous hydrogen peroxide treatment on the Cav-1 level is evaluated to confirm the findings. H460 cells were treated with hydrogen peroxide (100 μM) alone and the Cav-1 level was analyzed by western blotting. The results shows that hydrogen peroxide significantly elevated Cav-1 expression level as compared to non-treated controls (As shown in figure 4.6C). These results indicated that hydrogen peroxide is a major positive regulator of Cav-1 expression in response to cisplatin treatment.

A
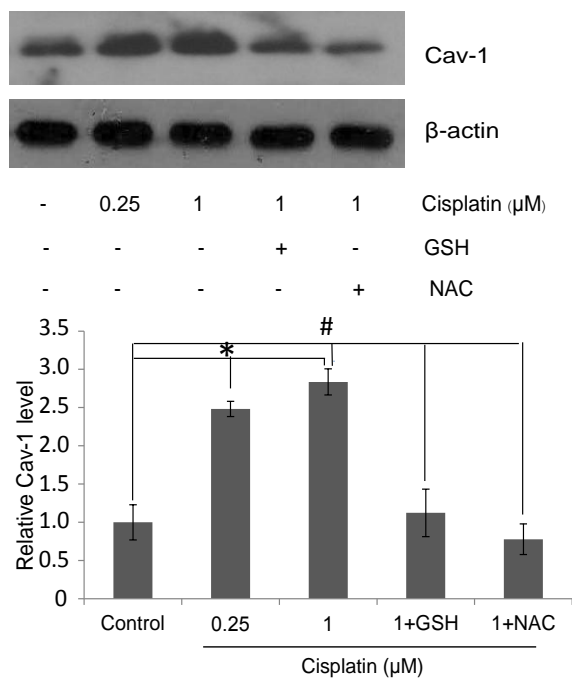
B
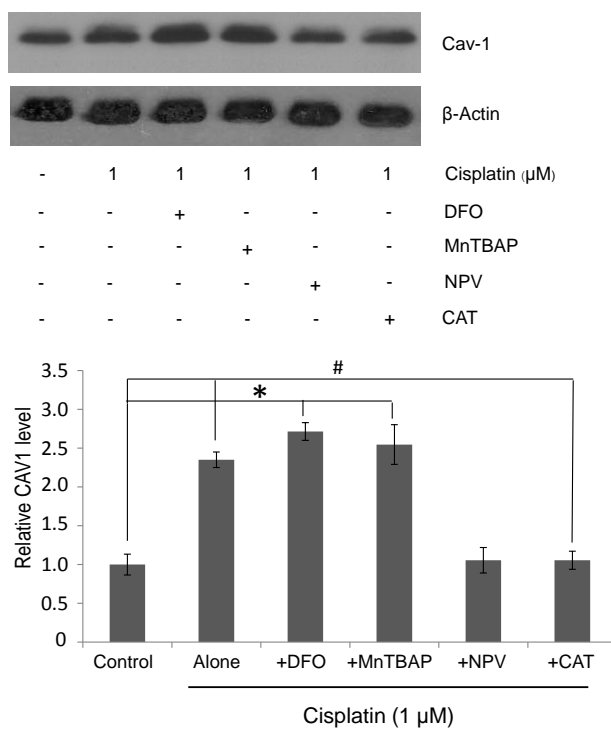
C
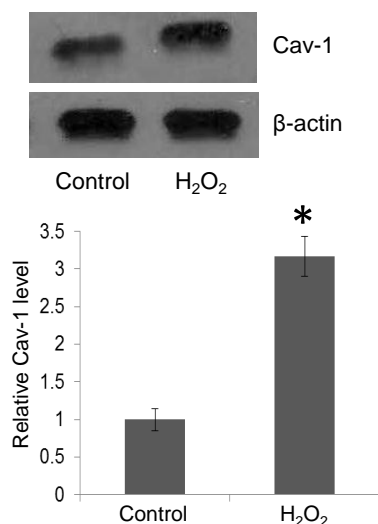
Figure 4.6 ROS induced by sub-toxic doses of cisplatin up-regulate Cav-1 level regulates. **A)** H460 cells were left untreated or pre-treated with 1 mM reduced glutathione (GSH) or 1 mM N-acetyl cysteine (NAC) for 30 min, and treated with 1 μM cisplatin for 24 h. Caveolin-1 (Cav-1) level was evaluated by western blot analysis. **B)** Cells were left untreated or pre-treated with 1 μM deferoxamine (DFO), 50 μM MnTBAP, 1 μM sodium pyruvate (NPV) or 5,000 U/ml catalase (CAT) for 30 min and the cells were treated with cisplatin for 24 h. **C)** H460 cells were treated with hydrogen peroxide (100 μM) for 24 h and Cav-1 levels were determined by western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. Immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized to non-treated cells. Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control cells; [#] $P<0.05$ *versus* cisplatin-treated cells.

CHAPTER V

DISCUSSION AND CONCLUSION

The acquisition of cisplatin resistance, frequently found in human lung cancer, has been considered as an important but complex obstacle to effective chemotherapy (Nishio *et al*., 1999; Hoffman, Mauer and Vokes, 2000; Socinski, 2004). Although the exact mechanism by which lung cancer cells tolerate cytotoxic drug remains elusive, higher aggressiveness of surviving cancer cells after cisplatin-based regimens, existing as the principal problem in the cancer treatment, has continuously been reported. The present study demonstrated for the first time that sub-lethal concentrations of cisplatin renders human lung carcinoma cells resistant to detachment-induced apoptosis. Furthermore, This study found that cisplatin-generated ROS were responsible for Cav-1 up-regulation and, subsequently, anoikis resistance.

It is widely known that cisplatin treatment causes an induction of several ROS, namely superoxide anion, hydrogen peroxide, and hydroxyl radical, and such an increase of ROS causes cytotoxic effects on cells (Wang *et al*., 2008; Chanvorachote *et al*., 2009; Pongjit and Chanvorachote, 2011). However, less is known regarding the ROS generated by sub-toxic concentrations of cisplatin. This study report herein for the first time that a low concentration of cisplatin was able to increase production of specific ROS, namely hydrogen peroxide and superoxide anion (As shown in figure 4.5.2 and 4.5.3). Results further revealed the effect of hydrogen peroxide in rendering cells resistant to anoikis. Most metastatic cancer cells resist detachment-induced apoptosis (anoikis). Anoikis, plays a principal role in inhibition of cancer cell

spreading from the original site to others. Several studies suggested that Cav-1 expression correlated with several aggressive behaviors of lung cancer cells, including multidrug-resistance (Lavie *et al*., 1997; Lavie and Liscovitch, 1998; Shatz and Liscovitch, 2004) and anoikis resistance (Rungtabnapa *et al*., 2011). The present study demonstrated that after treatment with sub-toxic concentrations of cisplatin, Cav-1 was up-regulated in a dose-dependent manner (As shown in figure 4.4), and such up-regulation of Cav-1 had an inhibitory effect on cell anoikis (As shown in figure 4.2). To confirm these results, This study determined role of Cav-1 on anoikis resistance. Gene overexpression and knockdown experiments indicated that Cav-1 protein regulated anchorage independent growth (As shown in figure 4.3). Furthermore, we revealed that Cav-1 expression in cisplatin-treated H460 cells was dependent on the oxidative stress induced by cisplatin. Addition of antioxidant GSH and NAC was able to attenuate the ROS induction and, subsequently, Cav-1 up-regulation (As shown in figure 4.6A). Previous studies reported that cisplatin-mediated death was related to the induction of cellular hydrogen peroxide (Choi *et al*., 2004; Wang *et al*., 2008) and hydroxyl radical (Jiang *et al*., 2007) production. However, in this study, a low concentration of cisplatin up-regulated hydrogen peroxide but did not alter the hydroxyl radical level (As shown in figure 4.5B and 4.5D). Since induction of cellular ROS by cisplatin was previously shown to be dose-dependent (Chanvorachote *et al*., 2009), it is possible that the production of cellular hydroxyl radical may be attenuated on low dose cisplatin treatment and was overwhelmed by cellular antioxidants.

Various effects of specific ROS have been shown in many studies. This study thus identified the specific ROS involved in the mechanism of cisplatin-mediated Cav-1 up-regulation and anoikis resistance. Specific ROS scavengers as well as

specific ROS probes were used and results indicated that super oxide anion and hydrogen peroxide are two key ROS present in cisplatin-treated cells. The increase of Cav-1 in response to cisplatin exposure was mainly due to hydrogen peroxide but not superoxide anion (As shown in figure 4.6B). These finding indicated an appreciable ROS generated by cisplatin in anoikis resistance. To potentiate these findings, our results demonstrated treatment of cells with exogenous hydrogen peroxide promoted the up-regulation of Cav-1 (As shown in figure 4.6C). In accordance with a previous study reporting the crucial role of hydrogen peroxide on Cav-1 expression and cell anoikis (Rungtabnapa *et al*., 2011), the present study indicates that hydrogen peroxide induced by sub-toxic concentrations of cisplatin can help cancer cells resist to detachment-induced apoptosis and may facilitate the metastatic ability of cancer cells. In summary, this study reported a novel effect and an underlying mechanism of sub-toxic concentrations of cisplatin in regulating anoikis resistance in human lung carcinoma H460 cells. Exposure to cisplatin at the sub-toxic concentrations induced ROS generation (mainly superoxide and hydrogen peroxide). Hydrogen peroxide induced by such cisplatin exposure mediated Cav-1 up-regulation and anoikis resistance in these cells. Since the ability to up-regulate cellular ROS production, especially of hydrogen peroxide, is found in a number of chemotherapeutic agents and other drugs, this finding might at least lead to further investigations that facilitate a better understanding regarding cancer cell biology and benefit the design of more effective treatment strategies for chemotherapy.

This study could be beneficial to the design chemotherapy treatment aimed at overcoming anoikis resistance and cancer metastasis. Since this study found that have the correlation between sub-toxic concentrations of cisplatin with anoikis resistance,

especially anoikis resistance that generated from ROS induction by cisplatin. This may be more prudent in the use of chemotherapy at inappropriate concentrations. As well the result of this study also provide careful about factors that influence the concentration of chemotherapy in the blood such as elimination or distribution of drug, multi-drug resistance receptor and drug detoxification, which can cause cancer metastasis. Moreover treatment with ROS-base cancer therapy in present must also be considered both advantage and disadvantage. Since there are many factors such as level of ROS, type of ROS, type of cancer and state of cancer that affect to the action of this drug.

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APPENDIX

APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The percentage of H460 viability was determined by rezasurin cytotoxicity assay after treatment with various concentration of cisplatin (dose dependency).

Cisplatin (μM)	Cell viability (%)
Control	100.00 ± 0.00
0.05	96.28 ± 7.97
0.1	95.55 ± 3.30
0.25	94.65 ± 7.48
0.5	94.18 ±3.96
1	92.63 ± 4.09
10	83.04 ± 2.83
50	62.39 ± 5.47
100	57.89 ± 2.35

Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control

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Table 2 The percentage apoptotic and necrotic cells were determined by Hoechst

33342/propidium iodide staining.

Cisplatin (µM)	Apoptotic cells (%)	Necrotic cells (%)
Control	1.67 ± 3.43	1.14 ± 4.63
0.05	2.48 ± 2.59	1.07 ± 5.76
0.1	3.42 ± 6.47	1.03 ± 7.02
0.25	1.56 ± 3.97	0.24 ± 2.65
0.5	2.65 ±3.25	1.23 ± 3.84
1	1.67 ± 6.86	0.48 ± 2.24
10	14.32 ± 4.78	1.05± 2.68
50	35.47 ± 5.33	1.42 ± 3.44
100	53.01 ± 5.36	3.19 ± 1.58

Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control

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Table 3 The percentage of H460 viability was determined by anoikis assay after treatment with sub-toxic concentrations of cisplatin (μM) at various time points (time dependency).

Time (h)	Cell viability (%)					
	Control	0.05	0.1	0.25	0.5	1
0	100±1.61	100±1.40	100±4.33	100±0.82	100±1.36	100±0.48
3	94.92±4.37	96.51±4.82	97.18±2.42	97.51±3.66	98.91±0.38	98.80±2.89
6	81.22±2.55	84.87±2.27	86.40±3.01	87.90±4.78	89.02±1.54	91.18±1.61
12	61.32±1.30	66.56±1.42	67.64±1.92	71.21±1.27	71.75±1.59	72.82±0.17
24	52.34±0.23	55.36±0.60	57.80±1.48	59.20±0.40	61.25±0.79	62.15±0.30

Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control

Table 4 The percentage of Cav-1 overexpressing, shCav-1, and control cells viability were determined by anoikis assay at various time points (time dependency).

Time (h)	Cell viability (%)		
	Mock	Cav-1 overexpressing	shCav-1
0	100±1.47	100±0.80	100±1.38
3	88.99±4.76	100±1.33	78.88±3.39
6	50.54±2.62	81.03±3.35	32.67±1.65
12	35.49±3.37	78.98±1.20	20.83±2.03
24	32.04±2.87	62.28±5.23	17.85±4.53

Values are means±S.D. (n=3); * $P<0.05$ *versus* control-transfected cells

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Table 5 The relative reactive oxygen species in H460 cells was quantified by fluorescence microplate reader in response to sub-toxic concentration of cisplatin (1µM) in the present and absent of ROS scavenger.

Time (h)	Relative reactive oxygen species			
	Control	Cisplatin	NAC+Cisplatin	GSH+Cisplatin
0	1.00±0.00	1.05±0.55	0.96±0.66	0.94±0.85
0.25	1.03±0.00	1.08±0.77	1.02±0.02	1.02±0.10
0.5	1.08±0.02	1.13±0.02	1.06±0.05	1.02±0.12
0.75	1.12±0.00	1.21±0.12	1.07±0.02	1.06±0.01
1	1.19±0.02	1.28±0.02	1.16±0.02	1.08±0.04
1.5	1.51±0.06	1.75±0.01	1.43±0.04	1.41±0.02
2	1.65±0.09	1.98±0.09	1.51±0.03	1.50±0.01
3	1.96±0.06	2.28±0.05	1.67±0.02	1.59±0.02
5	2.97±0.09	3.33±0.14	2.59±0.06	2.21±0.10

Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control

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Table 6 The relative DCF intensity in H460 cells was quantified by fluorescence microplate reader in response to sub-toxic concentration of cisplatin (1μM) in the present and absent of ROS scavenger.

Time (h)	Relative DCF intensity		
	Control	Cisplatin	NPV+Cisplatin
0	1.00±0.00	1.05±0.55	0.98±0.08
0.25	1.03±0.00	1.08±0.77	1.00±0.09
0.5	1.08±0.02	1.13±0.02	1.05±0.01
0.75	1.12±0.00	1.21±0.12	1.09±0.01
1	1.19±0.02	1.28±0.02	1.14±0.02
1.5	1.51±0.06	1.75±0.01	1.40±0.03
2	1.65±0.09	1.98±0.09	1.49±0.01
3	1.96±0.06	2.28±0.05	1.63±0.04

Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control

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Table 7 The relative DHE intensity in H460 cells was quantified by fluorescence microplate reader in response to sub-toxic concentration of cisplatin (1µM) in the present and absent of ROS scavenger.

Time (h)	Relative DHE intensity		
	Control	**Cisplatin**	**MnTBAP+Cisplatin**
0	1.00±0.03	1.08±0.08	0.99±0.01
0.25	1.50±0.11	1.76±0.28	1.64±0.07
0.5	1.77±0.18	2.10±0.17	1.92±0.12
0.75	1.95±0.13	2.50±0.10	2.01±0.10
1	2.06±0.05	2.68±0.20	2.10±0.15
1.5	2.36±0.05	3.00±0.31	2.39±0.17
2	2.51±0.21	3.26±0.33	2.68±0.09
3	3.03±0.18	3.60±0.12	3.27±0.08

Values are means±S.D. (n=3); * $P<0.05$ *versus* non-treated control

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Table 8 The relative HPF intensity in H460 cells was quantified by fluorescence microplate reader in response to sub-toxic concentration of cisplatin (1μM) in the present and absent of ROS scavenger.

Time (h)	Relative HPF intensity		
	Control	Cisplatin	DFO+Cisplatin
0	1.01±0.02	1.06±0.04	1.09±0.09
0.25	1.11±0.03	1.15±0.05	1.13±0.05
0.5	1.11±0.04	1.16±0.02	1.14±0.02
0.75	1.14±0.01	1.19±0.05	1.19±0.03
1	1.14±0.00	1.20±0.03	1.21±0.05
1.5	1.16±0.00	1.18±0.03	1.21±0.03
2	1.22±0.04	1.23±0.02	1.22±0.01
3	1.27±0.09	1.27±0.08	1.29±0.06

Values are means±S.D. (n=3); * *P*<0.05 *versus* non-treated control

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VITA

Miss. Thitiporn Songserm was born on September 16, 1986 in Bangkok. She received her B.Pharm from the faculty of Pharmacy, Silapakorn university in 2010.