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3,4-dihydroxy-5,4'-dimethoxybibenzyl sensitizes non-small cell lung cancer cells to cisplatin-induced apoptosis via p53 upregulation

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3,4-DIHYDROXY-5,4'-DIMETHOXYBIBENZYL SENSITIZES NON-SMALL CELL LUNG CANCER CELLS TO CISPLATIN-INDUCED APOPTOSIS VIA P53 UPREGULATION

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3,4-ไดไฮดรอกซี-5,4'-ไดเมทอกซีไบเบนซิลทำให้เซลล์มะเร็งปอดชนิดไม่ใช่เซลล์เล็กไวต่อการตายแบบอะพอพโทซิสจากซิสพลาทินผ่านการปรับเพิ่ม พี 53

น.ส.ฮาร์ดยันติ เอกะ ปูตริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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By	Miss Hardyanti Eka Putri
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ฮาร์ดยันติ เอกะ ปูตริ : 3,4-ไดไฮดรอกซี-5,4'-ไดเมทอกซีไบเบนซิลทำให้เซลล์มะเร็งปอดชนิดไม่ใช่เซลล์เล็กไวต่อการตายแบบอะพอพโทซิสจากซิสพลาทินผ่านการปรับเพิ่ม พี 53. (3,4-DIHYDROXY-5,4'-DIMETHOXYBIBENZYL SENSITIZES NON-SMALL CELL LUNG CANCER CELLS TO CISPLATIN-INDUCED APOPTOSIS VIA P53 UPREGULATION) อ.ที่ปรึกษาหลัก : ศ. ภก. ดร.ปิติ จันทร์วรโชติ

มะเร็งปอดนั้นเป็นโรคร้ายแรงที่มีอันตรายต่อชีวิตลำดับต้นๆ ซึ่งมีตัวเลขการเสียชีวิตมากที่สุดในกลุ่มโรคมะเร็งทั้งหลาย การตั้งเป้าหมายค้นหาโปรตีนพิเศษที่สามารถควบคุมการเจริญเติบโตของมะเร็งและการแพร่กระจายของเนื้อร้ายนั้นถือว่าเป็นงานที่น่าสนใจเป็นอย่างมากในกระบวนการรักษาโรคมะเร็ง ด้วยเหตุนี้ งานวิจัยชิ้นนี้มีวัตถุประสงค์ในการตรวจสอบผลของสาร 3,4-ไดไฮดรอกซี-5 4'-ไดเมท็อกซีเบนซิล (DS-1) ในการมุ่งหวังให้เกิดกระบวนการลดทอนโปรตีน P53 ด้วยยีน MDM2 และการยับยั้งการเปลี่ยนสภาพเซลล์ (EMT) ในเซลล์มะเร็งปอด ประสิทธิผลของสาร DS-1 หรือสารที่ผสมกับยาต้านซิสพลาตินในเซลล์มะเร็งปอดนั้นได้รับการทดสอบโดยกระบวนการ MTT, การย้อมนิวเคลียส, และการทดสอบวิธี Annexin V/PI การแสดงผลของโปรตีนที่เกี่ยวเนื่องกับอะพอบโทซิสและการเปลี่ยนสภาพเซลล์นั้นได้รับการทดสอบโดยการวิเคราะห์ด้วยเทคนิคเวสเทิร์นบล็อต อาการแพร่กระจายของเนื้อร้ายนั้นได้รับการประเมินโดยการกำหนดลักษณะรูปร่างเซลล์ การแพร่กระจาย การรุกรานเซลล์อื่น และการทดสอบการเจริญเติบโตที่ไม่ต้องอาศัยเซลล์ยึดเกาะ (anchorage-independent cell growth) ในการตรวจสอบบทบาทของสาร DS-1 ต่อการสร้างภาวะเสถียรและลดโปรตีน P53 นั้น ผู้วิจัยได้ดำเนินการทดสอบไซโคลเฮกซิไมด์ และ การทดสอบการตกตะกอนโปรตีน (Immunoprecipitation) และโปรตีน P53 สภาพดีได้รับการทดสอบโดยวิธีอิมมูโนฟลูออเรสเซนต์ เพื่อที่จะยืนยันและแสดงให้เห็นถึงปฏิสัมพันธ์ต่อกันระหว่างสาร DS-1 และโปรตีน MDM2 ผู้วิจัยได้ดำเนินกระบวนการวิเคราะห์โดยคอมพิวเตอร์ (*in silico*) ผลลัพธ์ที่ได้แสดงให้เห็นว่า สาร DS-1 ให้สภาวะเป็นพิษต่อเซลล์ และทำให้เซลล์มะเร็งปอดรับเอากระบวนการอะพอบโทซิสของยาต้านมะเร็งซิสพลาติน สาร DS-1 ก่อให้เกิดการเพิ่มระดับของโปรตีน P53 ในระดับเซลล์อย่างมีนัยสำคัญ ในขณะที่ โปรตีน P53 สภาพดีนั้นมีการเปลี่ยนแปลงในระดับเล็กน้อยมาก สาร DS-1 ที่รวมกับยาต้านมะเร็งซิสพลาตินอาจจะยกระดับโปรตีน p-p53 (Ser15) และการส่งสัญญาณผ่านเซลล์ของโปรตีน P53 (Bax, Bcl-2 และ Akt) ซึ่งจะนำไปสู่กระบวนการอะพอบโทซิสในระดับที่สูงกว่าได้ การวิเคราะห์การตกตะกอนโปรตีนเผยให้เห็นว่า สาร DS-1 ลดจำนวนโปรตีนเชิงซ้อน P53-ยูบิควิตินลง ซึ่งเป็นขั้นตอนที่เกิดก่อนการสลายโปรตีน P53 โปรแกรมการจำลองการจับกันยังพิสูจน์ได้ว่า สาร DS-1 มีปฏิสัมพันธ์ต่อโปรตีน MDM2 ภายใต้ขอบเขตพันธะของโปรตีน P53 โดยปฏิกิริยาพันธะเคมีระหว่างคาร์บอนกับไฮโดเจน ที่สาร Lys27 ปฏิกิริยา Pi-Alkyl ที่ lle37 และ Leu30 และปฏิกิริยาแวนเดอร์วาลล์ (Van der Waals) ที่ lle75, Val51, Val69, Phe67, Met38, Tyr43, Gly34, Phe31, และ Lys27 กระบวนการจัดการสาร DS-1 และยาซิสพลาตินในเซลล์มะเร็งปอดส่วนหลักที่ค่อยๆ แพร่สู่ร่างกายแสดงให้เห็นถึงผลลัพธ์ที่สอดคล้องกันของการกระตุ้นการรับยาต้านมะเร็งซิสพลาตินที่เพิ่มมากขึ้น สำหรับการแพร่กระจายของมะเร็งนั้น สาร DS-1 มีฤทธิ์ยับยั้งการเพิ่มตัวของเซลล์มะเร็งปอดอย่างได้ผลเมื่อเปรียบเทียบกับกลุ่มเป้าหมาย พฤติกรรมรุนแรงของเซลล์มะเร็ง เช่น ความสามารถในการแพร่กระจายและความสามารถในการรุกรานเซลล์อื่นได้ถูกลดระดับลงอย่างมากในเซลล์ที่ได้รับสาร DS-1 นอกจากนี้ การวิเคราะห์การเจริญเติบโตที่ไม่ต้องอาศัยเซลล์ยึดเกาะได้พิสูจน์ว่า สาร DS-1 สามารถระงับการเจริญเติบโตของเซลล์มะเร็งที่เกิดจากการยึดเกาะ โดยเห็นผลจากการลดจำนวนลงอย่างมีนัยของขนาดและจำนวนเซลล์ ในกระบวนการทำงาน ผู้วิจัยพบว่าสาร DS-1 สามารถสยบการเปลี่ยนสภาพเซลล์ โดยเห็นได้จากการลดจำนวนลงของตัววัดการเปลี่ยนสภาพเซลล์ที่เรียกว่าเอ็น-แคดเฮริน, รูปแบบ Snail และ Slug ในขณะที่ตัววัดค่า อี-แคดเฮรินมีค่ามากขึ้น ทั้งนี้ สาร DS-1 ยังแสดงให้เห็นถึงการลดลงของระดับโมเลกุล integrin B1 ด้วยหลักฐานที่พบนั้นทำให้พิสูจน์ได้ว่า สาร DS-1 สามารถเป็นตัวยับยั้งโปรตีน MDM2 และการป้องกันการแพร่กระจายของเนื้อร้ายโดยผ่านทางการระงับโมเลกุล integrin และ โปรตีน FAK ซึ่งอาจจะเป็นประโยชน์ต่อการพัฒนาส่วนผสมนี้เพื่อใช้ในการรักษาโรคมะเร็งปอดได้

สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อนิสิต .....
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KEYWORD: Non-Small Cell Lung Cancer DS-1 bibenzyl MDM2 p53 antimigration and chemosensitizer.
Hardyanti Eka Putri : 3,4-DIHYDROXY-5,4'-DIMETHOXYBIBENZYL SENSITIZES NON-SMALL CELL LUNG CANCER CELLS TO CISPLATIN-INDUCED APOPTOSIS VIA P53 UPREGULATION. Advisor: Prof. PITHI CHANVORACHOTE, Ph.D.

Lung cancer is a leading fatal malignancy with the highest number of cancer deaths. Targeting a specific protein regulating cancer progression and metastasis has been attracting much attention for the development of cancer therapy. Thus, this study was aimed to investigate the effect of 3,4-dihydroxy-5,4′-dimethoxybibenzyl (DS-1) in targeting MDM2-attenuating p53 function and inhibition EMT in lung cancer cells. The efficacy of DS-1 or combined with cisplatin in lung cancer cells was determined by MTT, nuclear staining, and Annexin V/PI assays. The expression of apoptosis- and EMT-related proteins was determined by western blot analysis. Metastatic behaviors were evaluated by cell shape characterization, migration, invasion, and anchorage-independent cell growth assay. To investigate the role of DS-1 on stabilization and degradation of p53, cycloheximide chasing assay and immunoprecipitation were conducted, and the active form of p53 was investigated by immunofluorescent staining assay. To confirm and demonstrate the site interaction between DS-1 and MDM2 protein, the *in silico* computational analysis was performed. The results showed that DS-1 exhibited a cytotoxic effect and sensitized lung cancer cells to cisplatin-induced apoptosis. DS-1 caused a significant increase in cellular level of p53 protein, while the active form of p53 (phosphorylation at Ser15) was insignificant change. DS-1 combined with cisplatin could enhance p-p53 (Ser15) and p53 downstream signaling (Bax, Bcl-2, and Akt), leading to higher level of apoptosis. Immunoprecipitation analysis revealed that DS-1 decreased p53-ubiquitin complex, a prerequisite step of p53 proteasomal degradation. Molecular docking simulation further evidenced that DS-1 interacts with MDM2 within p53-binding domain by carbon-hydrogen bond interaction at Lys27, π-Alkyl interactions at Ile37, and Leu30, and Van der Waals interactions at Ile75, Val51, Val69, Phe67, Met38, Tyr43, Gly34, Phe31, and Lys27. Treatment of DS-1 and cisplatin in patient-derived primary lung cancer cells showed the consistent effects of increasing cisplatin sensitivity. For cancer metastasis, DS-1 significantly inhibited the proliferation of lung cancer cells compared to the control group. The aggressive behavior of cancer cells including migration and invasion ability was significantly reduced in the DS-1-treated cells. Besides, anchorage-independent growth analysis provides evidence that DS-1 could suppress the growth of the cancer cell in detached condition indicated by the significant reduction in cell colony size and number. For mechanisms, we found that DS-1 suppressed EMT indicated by the reduction of EMT markers namely N-cadherin, Snail, and Slug, while increasing epithelial maker of E-cadherin. Also, DS-1 was shown to decrease the cellular levels of integrin β1. Our findings provide evidence of DS-1 as an MDM2 inhibitor and metastasis prevention through integrin and FAK suppression, which may benefit the development of this compound for lung cancer treatment.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....

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%	=	Percentage
μM	=	Micrometer
Akt	=	protein kinase B
ANOVA	=	Analysis of variance
Bax	=	Bcl-2-associated X-protein
Bcl2	=	B-cell lymphoma 2
BSA	=	bovine serum albumin
Cadherin	=	Calcium-dependent adhesion
CHX	=	Cycloheximide
CI	=	Combination Index
DDR	=	DNA damage response
DMSO	=	Dimethyl sulfoxide
E1	=	Ub-activation enzyme
E-cadherin	=	Epithelial cadherin
ECM	=	Extra Cellular Matrix
EMT	=	Epithelial to Mesenchymal Transition
Et al.	=	Et alibi, and others
FA	=	Fraction affected
FAK	=	Focal Adhesion Kinase
FBS	=	Fetal Bovine Serum
MDM2	=	Mouse-double minute 2
mL	=	milliliter
MOMP	=	Mitochondrial outer membrane permeabilization
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	=	Sodium chloride
N-cadherin	=	Neural cadherin
NSCLC	=	Non–Small Cell Lung Carcinoma
°C	=	Degree celsius

p-Akt	=	phospho-Akt
PBS	=	phosphate-buffered saline
p-FAK	=	phospho-Focal Adhesion Kinase
PI	=	propidium iodide
p-p53	=	phospho-p53
RhoA	=	Ras Homolog Gene Family Member A
RPMI	=	Roswell Park Memorial Institute
TP53	=	tumor protein p53
Ub	=	ubiquitin
Ub-p53	=	Ubiquitinated p53
wt-p53	=	wild-type p53

CHAPTER I

INTRODUCTION

Background and Rationale

Lung cancer remains the leading cause of cancer-related death worldwide, which is accounted for 18.4% of the total cancer deaths in 2018 (Bray et al., 2018). Of note, Non–small cell lung carcinoma (NSCLC) is the most commonly diagnosed type of lung cancer (Molina et al., 2008). Moreover, studies reported 20%-40% of advanced lung cancer patients related to metastasis, which is a serious sigh undergoing several symptoms leading to decrease quality of life (Cho et al., 2019; Lee et al., 2019). This because the obligate of cancer metastasis acquires malignant tumor motility to migrate and invade from the origin site of the primary tumor to a secondary site (Huysentruyt and Seyfried, 2010). Chemotherapy remains the mainstay treatment for NSCLC. Unfortunately, for long term therapy patients are reported to develop resistance (Zappa and Mousa, 2016). Recently, drug targeting specific proteins involving in cancer progression and aggressiveness has shown a promising efficacy for clinical application. Thus, an investigation of new potent compounds to target the cancer-associated protein has gained increasing attention in cancer and molecular pharmacological researches.

One potential target of drug action for lung cancer therapy is the Mouse-double minute 2 (MDM2) protein (Chène, 2003; Wang et al., 2012). MDM2 protein is encoded by the MDM2 gene functioning as a key negative regulator of p53, a tumor suppressor protein (Shi and Gu, 2012). The p53 protein secures stability of genome by commanding cell fate to cell cycle arrest or apoptosis in response to cellular stress and DNA damage (Kumari et al., 2014). On activation, p53 directly regulates the transcriptional function of apoptosis inducers. Following this, the cellular responses to p53 encourage the induction of pro-apoptosis proteins such as Bcl-2-associated X-protein (Bax) and depletion of anti-apoptosis proteins including B-cell lymphoma 2 (Bcl-2) (Fischer, 2017; Aubrey et al., 2018). Therefore, the deletion of p53 networks facilitates the formation of serious diseases, particularly the impact on cancer initiation and rapid tumor progression (Royds and Iacopetta, 2006). In case of tumor protein p53 (TP53) mutation,

deletion in TP53 copy number, or aberrant activation of MDM2 protein, it have been defined as the main regulators of the perturbation of p53 activation (Jackson and Lozano, 2013; Liu et al., 2016; Kastenhuber and Lowe, 2017). For lung cancer, p53 inactivation or elimination resulted in tumor progression and poor response to chemotherapy including cisplatin (Mitsudomi et al., 2000). It was shown that the function of p53 depends on the cellular available level (Lavin and Gueven, 2006).

MDM2 was found to be substantially overexpressed in malignancies (Jones et al., 1998), and such an increase in MDM2 associates with poor prognosis and chemoresistance (Hou et al., 2019). MDM2 interacts with p53 and ligases the p53 to ubiquitin molecule, which intern targets p53 for proteasomal degradation (Moll and Petrenko, 2003). p53-binding domain or hydrophobic pocket domain at the NH2 terminus (residues 25-109) of MDM2 binds to N-terminal domain (residues 18-26) of p53. Once they became complex, MDM2 as E3 ligase specific for p53 required E1-E2 ubiquitins that later is recognized by proteasome to accomplish protein degradation (Moll and Petrenko, 2003). Several factors have been reported to disturb this MDM2-p53 complex including cellular stress (Hu et al., 2012), DNA damage, phosphorylation of p53, and MDM2 inhibitor (Shangary and Wang, 2009). Interestingly, reactivation of p53 by hampering the interaction between p53 and MDM2 has shown beneficial effects in cancer cells (Yue et al., 2017).

Besides reactivation of p53 as tumor suppressor, p53 has shown a contol in primary phases or metastastic progression (Shiota et al., 2008) In order to regulate EMT, p53 retains the transcriptional protein to attenuate EMT, whereas lack of p53 protein contributes to EMT phenotypes like increasing of Slug expression correlates with loss of E-cadherin in p53-null tumors (Smit and Peeper, 2008). Moreover, the ability of p53 in control of mesencymal like cell morphology involvel in the Rho family of small GTPases. The activity of RhoA govers to elongated morphology characteristic of cancer cells (Sahai and Marshall, 2003). In p53-null cells, most subsequent studies have reported the increased activity of RhoA after loss of p53. This indicate that p53 loss can activate a more mesenchymal phenotype in human cancer. Moreover, considering that targeting MDM2 is the key factor to restore p53 protein level and has been associated with angiogenesis (Helei Huo et al., 2019), MDM2-targeted therapy thus may

be a promising strategy for treating advanced malignancies.

In addition to cancer metastasis, the change of cancer cell phenotype of epithelial to mesenchymal transition (EMT) is essential for cell migration and invasion progression (van Zijl et al., 2011; Fares et al., 2020). The major hallmarks of this process are identified as loss of cell polarity and dissociation of cell-cell junction integrated with extracellular matrix (ECM) (Dongre and Weinberg, 2019). EMT resulted in the alteration of motility and adhesion regulatory proteins including the switch of E-cadherin to N-cadherin with the induction of mesenchymal proteins such as Snail and Slug (Nurwidya et al., 2012; Sosa Iglesias et al., 2018). Toward cell metastasis, N-cadherin, a mesenchymal phenotype, facilitates the interaction of cancer and stromal cells conferring cancer cell migration and invasion (Asnaghi et al., 2010) (Chen et al., 2015; Dongre and Weinberg, 2019). Both E-cadherin and N-cadherin are adherents junction molecules (Araki et al., 2011). However, E-cadherin expression is only exhibited in epithelial tissue maintaining cell integrity and homeostasis, which has been correlated with a greater prognosis and long term total survival time in lung cancer (Asnaghi et al., 2010) whereas N-cadherin is mostly expressed in mesenchymal cells that responsible for cell motility (Yu et al., 2019). Thus, cells highly expressing N-cadherin acquire the capacity to migrate and invade surrounding stroma and subsequently spread through the blood and lymphatic vessels to distant sites (Mrozik et al., 2018). Equally important, the cadherin alteration was found to be associated with zink-finger-family transcriptional activity encompassing Snail, and Slug (Wheelock et al., 2008). It has been demonstrated that Snail-transfected cells promoted the induction of N-cadherin and suppression of E-cadherin in which accomplished the progress of EMT (Takkunen et al., 2006). Thus, dysregulation of N-cadherin, as well as the transcriptional factor related to cancer behavior, has been investigated as intention therapy to combat cancer metastasis (Mrozik et al., 2018; Song et al., 2018).

Also, another factor involved in successful metastasis is that cells ought to against anoikis, apoptosis induction during detachment (Buchheit et al., 2014). To allow this plasticity, integrin serves as a heterodimeric transmembrane receptor for cellular adhesion to ECM (Alanko et al., 2015; Seguin et al., 2015). On activation, the interface of specific extracellular ligands and outer domain of integrin cause dimerization of **α**

and β glycoprotein subunits of integrin to convey the ligand cues, extracellular signal to intracellular transduction (Cooper and Giancotti, 2019). As a result, it required adaptor protein-mediated kinase activation including the FAK signaling pathway. It has been confirmed that the integrin-mediated FAK apparatus contribute to control cell adhesion, mobility, phenotype synthesis, and cell survival and to restrict the anoikis induction. (Silginer et al., 2014; Alanko et al., 2015). The common integrins in epithelial cells such as αvβ3 and α5β1 are found overexpressed in many cancer (Desgrosellier and Cheresh, 2010). Although the functional activity of these integrins implies in biological activity, integrins have been indicated to facilitate the cancer cell survival in response to any cellular stress within the circulation, and cell adhesion to blood vessel endothelium at the secondary site (Alanko et al., 2015). Moreover, the expression of integrin β1 has been correlated to poor prognosis in NSCLC (Dingemans et al., 2010). Along with development of antimetastasis, previous studies have evidenced the correlation between integrin expression and metastasis by which down-regulation of integrin suppressed metastatic behavior (Li et al., 2012; Petpiroon et al., 2017) suggesting that this protein is deemed as a target of cancer therapy.

3,4-dihydroxy-5,4′-dimethoxybibenzyl (DS-1), identified in *Dendrobium signatum*, has shown cytotoxic activity in several cancer types including breast cancer, liver cancer, and colon adenocarcinoma cells (Mittraphab et al., 2016). This suggests that DS-1 may exhibit anticancer potential. Moreover, other natural products isolated from *Dendrobium signatum* including Gigantol has been demonstrated that it potentially suppressed cancer cell metastasis (Charoenrungruang et al., 2014, Unahabhokha et al., 2016) and cancer stem cell phenotypes in lung cancer cells (Bhummaphan and Chanvorachote, 2015). Structurally, DS-1 consists of a phenyl group or phenyl ring which is related to one benzene ring. Importantly, a previous study reported that several MDM2 inhibitors in human clinal trials for cancer treatments with phenyl ring showed the binding affinity to the MDM2 domain (Yujun Zhao, et al., 2014). This indicates that DS-1 may exibit the potential to occupy MDM2 pocket. As its activity in sensitized chemotherapy as well as targeting MDM2 or p53 mechanisms are not known, this study aimed at investigating the effect of DS-1 or combined with cisplatin in p53 level and interaction with MDM2 protein. In addition, Along with anti-metastasis

approaches for Non-Small Cell Lung Cancer, the present study also aimed to investigate the potential effect of DS-1 on EMT process. By conducting pharmacological, experimental, and computational modeling assessment, we demonstrated how DS-1 interruption MDM2-p53 complex resulted in the up-regulation of p53 protein level and DS-1-inhibited the mesenchymal phenotypes. Furthermore, our study provides intriguing data of a potent compound that can be developed as anti-metastasis particularly for NSCLC cells.

Research questions

1. Does DS-1 has an effect on apoptosis induction in lung cancer cells?
2. Does DS-1 has an effect on MDM2-p53 complex in H460 cancer cells?
3. Does DS-1 sensitize lung cancer cells to cisplatin-triggered apoptosis?
4. Does DS-1 has an effect on EMT characteristics in Non-Small Lung Cancer cells?

Objectives

1. To identify the effect of DS-1 on apoptosis induction in lung cancer cells
2. To identify the effect of DS-1 on MDM2-p53 complex in H460 lung cancer cells
3. To identify the sensitization effect of DS-1 in lung cancer cells to cisplatin exposure
4. To determine the mechanisms involved in DS-1 sensitizing H460 lung cancer cells to cisplatin-induced apoptosis
5. To investigate the effect of DS-1 on EMT characteristics in Non-Small Lung Cancer cells

Hypothesis

1. DS-1 interacts with the p53-binding domain of MDM2 that mediates the accumulation of p53 and sensitizes lung cancer cells to cisplatin-induced apoptosis.
2. DS-1 affects EMT in Non-Small Lung Cancer cells through integrin β1 and p-FAK suppression

CHAPTER II

LITERATURE REVIEW

Lung cancer

Above a decade lung cancer becomes the most incident for all cancer cases with the highest mortality rate both in men and women (de Groot et al., 2018). As reported, the lung cancer case in 2008 was identified about 1.6 million cases with 1.4 million mortalities and lamentably increased up to 1.8 million cases and 1.6 million lung cancer death in 2012. Among the cases, two histological types, Non-small cell lung cancer (NSCLC) and Small cell lung cancer (SCLC), are found that NSCLC is the common one around 80-85% of all lung cancer cases. Moreover, the newly diagnosed patients are in the late stage of lung cancer that well-known to be associated with extremely poor progress. Many therapy options have been developed in order to offer a cure for all cancer patients. The available treatments unfortunately nowadays for patients mainly are limited by several reasons: the required test for individual pharmacotherapies, the patient's capability of response, chemotherapy side effects, and expansion of chemoresistance (Molina et al., 2008; Zappa and Mousa, 2016).

Development of lung cancer starts once the lung cells changing or turning into mutation. The occurrence of this is because of various initiation risk factors, the extrinsic factor including toxic substances or intrinsic factors like genetics of family history. American Lung Association revealed that the major factor recognized in more than 80% of lung cancer patients is tobacco smoke: the active or even passive smoking is exposed by toxic chemicals of tobacco. Understanding the lung cancer risk factors beneficially inspires a strategy to prevent the incident. The expanding evidence showed that controlling tobacco use in the US has taken participation in lung cancer prevalence (Warren and Cummings, 2013; Balogh et al., 2014).

Treatment preference for lung cancer patients in terms to determine the precise treatment depends on the tumor type; NSCLC or SCLC, and stage; early stage or late stage. Staging helps confirm the tumor cell prognosis: the higher stage patient is, the more tumor cells have spread. Standard treatments for patients with Non-Small Lung cancer include surgery, radiotherapy, chemotherapy, targeted therapy, or

combination therapy. based on lung cancer stage, patients diagnosed in early-stage (I-II) are considered for surgical treatment and or combined with chemotherapy as neoadjuvant or adjuvant therapy. However, some patients may not capable of the surgery, then they receive radiation therapy or a combination of radiation therapy and chemotherapy. Moreover, patients diagnosed with late-stage lung cancer can be treated with chemotherapy. That also has a different response to all patients. Alternatively, palliative therapy can be a strategy to enhance the quality of life to other patients who cannot respond to chemotherapy (Chan and Hughes, 2015; Lemjabbar-Alaoui et al., 2015; Zappa and Mousa, 2016).

Cisplatin

Cisplatin, cis-diamminedichloridoplatinum(II), is most often used as initial chemotherapy Non-Small Lung Cancer patients with advanced-stage; cisplatin, first introduced by M. Peyrone in 1844 and admitted by FDA (Food and Drug Administration) in 1978 for use of testicular and bladder cancer treatment, is now widely used for several solid malignancies. The most important, the use of cisplatin in lung cancer patient, indeed, depends on the tumor cell type or stage, and individual comorbidities (Cosaert and Quoix, 2002; Kelland, 2007; Amarasena et al., 2008). According to the characteristics of tumor cells, studies have reported that the advanced-stage lung cancer patients, who possess unrespectable cancer or metastatic NSCLC, respond to the cisplatin regiment with 10% survival rate at one year (Baxevanos and Mountzios, 2018). In combination treatment, cisplatin plus pemetrexed was effectively used in nonsquamous NSCLC (Scagliotti et al., 2008). However, in accordance with long term therapy, side effects of cisplatin become a concern and also might not be tolerated for some patients. Collectively, the sensitive tumor cells to cisplatin no longer enable to develop chemoresistance leading to therapy failure (Shen et al., 2012; Barr et al., 2013).

The role of cisplatin as anticancer drug mainly causes DNA damage owing to the contained platin stating on target guanines, thus eliciting transcriptional inhibition, cell cycle arrest, DNA repair, and cell death. In term of molecular mechanisms, studies reported that the mode of action of cisplatin is recognized in the different cellular components. Fundamentally, the action of cisplatin in cell starts when it gets into the

cells. The evidence showed that in cytoplasm, cisplatin cause ROS induction. This facilitates cisplatin to take an action on the mitochondrial outer membrane permeabilization (MOMP), thereby leading to apoptosis induction via the intrinsic pathway. In the nucleus, the aquated cisplatin attaches to DNA bases. This modulates the DNA damage by initiating DNA damage response (DDR), thus resulting in cell death (Galluzzi et al., 2011; Shen et al., 2012).

The molecular mechanism of cisplatin, as mentioned above, involves several intracellular activities. The fact that the cell adaptation after cisplatin exposure act in several pathways by which reduce the uptake and accumulation of cisplatin within the cells, these mechanisms are divided into four part including Mechanisms of pre-target resistance where prevention to cisplatin before reaching the target of action. That occurs by which 1) reduce cisplatin update by depletion of uptake transporter, CTR1; 2) increase the elimination of cisplatin by eliciting efflux transporter, ATP7A/ATP7B and MRP2; 3) inactivate cisplatin by increasing glutathione (GHS).

Cisplatin-induced oxidative stress

Reactive oxidative stress (ROS) normally exhibits during cellular activity, but it can be controlled by the production of antioxidants such as GSH, glutathione; SOD, superoxide dismutase; and CAT, catalase, thus can maintain homeostasis of cellular component. Once cell under stress condition, it promotes the high level of ROS leading to cellular protein damage, and fatal DNA lesions. In terms of the mode of action of cisplatin, an accumulating study reported that by upregulating excessive ROS, cisplatin enables to cause depletion of mitochondrial membrane potential. Cells undergo apoptosis. This because induction of ROS has shown its role in generating extrinsic and intrinsic apoptosis cascade.

Cisplatin-induced apoptosis

The main effects of cisplatin as anticancer drugs cause cell death by apoptosis induction. The mechanism involves, Initially, genotoxic stress induced by cisplatin modulates a defect in survival signaling pathway that contributes to apoptotic-programmed cell death. These have been confirmed that biochemical activities including regulation of cytochrome-c out from mitochondria, activation of caspase-9,

reduction of Bcl-2, induction of DNA damage mediates apoptotic cell death after cisplatin exposure.

The molecular mechanisms of cisplatin, as mentioned above, involve several intracellular activities. The cell adaptation after cisplatin exposure act in several pathways by which reduce the uptake and accumulation of cisplatin within the cells, these mechanisms are divided into four parts: mechanisms of pre-target resistance, mechanisms of on-target resistance, mechanisms of post-target resistance, and mechanisms of off-target resistance (Galluzzi et al., 2011). Mechanisms of pre-target resistance are prevention to cisplatin before reaching the target of action. That occurs by which 1) reduce cisplatin update by lowering of uptake transporter, CTR1; 2) increase the elimination of cisplatin by eliciting efflux transporter, ATP7A/ATP7B, and MRP2; 3) inactivate cisplatin by increasing glutathione (GHS). The mechanism of on-target resistance is that DNA lesions by cisplatin require capability on repairing system that gives a chance to repair the damage and also cells could allow the existence of unrepaired DNA. Thus, by this process cells are able to tolerate cisplatin exposure (Lindahl and Barnes, 2000, Wood et al., 2000). Mechanism of post-target resistance involves the alteration of genetic and signaling cascade resulting in cells escape from the apoptosis pathway. For instance, studies reported that cell resistance to cisplatin has shown the deficiency of Bax triggering apoptosis but mostly enhanced survival signaling cascade including MAPK and JNK pathway (Brozovic et al., 2004, Biagosch et al., 2010).

p53 protein

Cells continuously proliferate in proper control and maintenance. During living cells, however, they can be exposed to several cellular stresses. In turn, cellular stresses initiate the genomic instability that can be resulted in gene mutation or delegation. Consequently, expansion of the genomic instability can develop transformation cells. Thus, cells need a proper system to maintain itself during cellular stresses and prevent the initiation stage of cancer development.

p53 or TP53 (tumor protein 53) is recognized as an important tumor suppressor protein that limits the cell division. Studies revealed the function of p53 is associated with involvement in cancer development (Vousden and Ryan, 2009). It is

not only induction of DNA damage but also protecting cells to expand cancer initiation. As its dubbed, p53 is “the guardian of the genome” by which modulate apoptotic induction, gene amplification, and senescence (Sujan et al., 2017). Hence, the functional change of this protein leads to cells growing abnormally. It has been identified that the mutation of p53 protein has been found in various of cancer for 38%-50% including ovarian, esophageal, colorectal, head and neck, larynx, and lung cancer (Olivier et al., 2010).

Structural features of p53

Structurally, p53 composes of three main domains (Figure 2.1) that can be activated on the N-terminal or C-terminal domain with phosphorylation of serine/T in each (Yogosawa and Yoshida, 2018). The activation of p53 is mainly in response of cellular environment or under stress condition within three steps: stabilization of p53, action on DNA binding, and transcriptional fuction (Zilfou and Lowe, 2009). The function of p53 in cellular growth arrest pathway is mediated through a dual role, transcriptional-dependent in the nucleus, and transcriptional-independent in the mitochondria (Figure 2.2) (Chi, 2014).

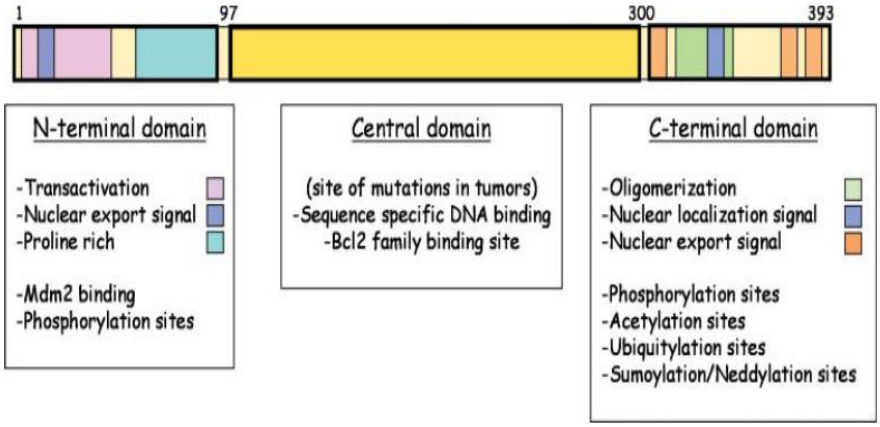


Figure 2.1 Structure of p53 (Yee and Vousden, 2005)

p53 plays as a transcriptional factor that upregulates several pro-apoptosis gene expression including Noxa, Bax, and Puma within nucleus mediated by directly interacting between p53 transactivation domain at 15-29 residues (p53TAD) and transcriptional machineries such as TBP, p300/CBP, and hTAFII31 (Wang et al., 2010). On the other hand, the interaction of p53 and anti-apoptosis proteins, Bcl-2, and Bcl-

XL leads to lack of mitochondrial membrane permeability and thus promotes transcription-independent apoptosis (Moulder et al., 2018).

In normal cells, the amount of p53 is maintained at low level (Liu and Kulesz-Martin, 2000) due to degradation protein by Mdm2 and ubiquitin-protein binging on N-terminal domain. This complex also causes the co-location of p53 from nucleus to cytoplasm (Dai and Gu, 2010). As a result, p53 is accumulated in the cytoplasm in an inactive state (Cory and Adams, 2002).

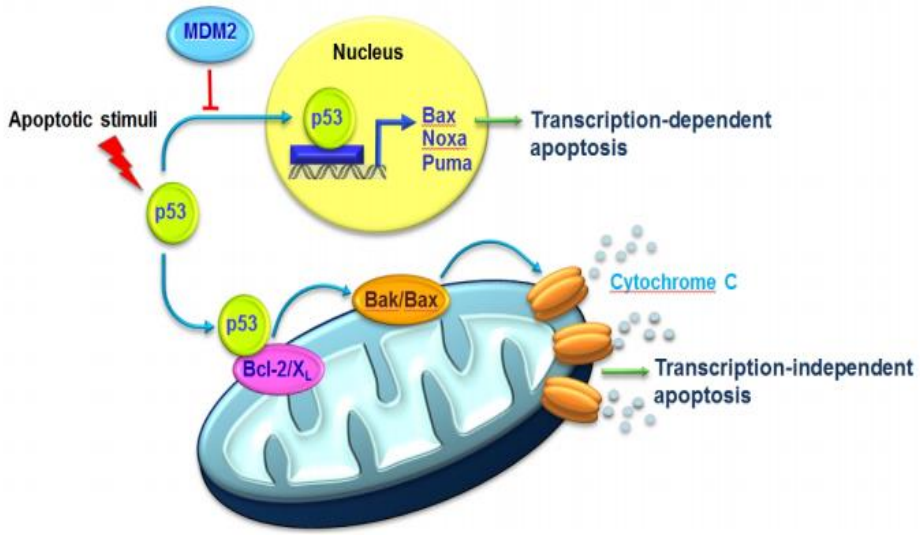


Figure 2.2 Activation of p53 in a dual different cellular pathway (Chi, 2014)

The increase of p53 expression levels within cells is the critical factor for its activation. Accumulation of p53 is regulated by Mouse Double Minute 2 (MDM2) which is E3 ubiquitin ligase protein, a specific p53 inhibitor, and responsible for p53 degradation via proteasome pathway (Figure 2.3). Structurally, MDM2 is a 491 amino acid, consists of several domains: 1) N-terminal p53 interaction domain, 1) central acidic domain, 2) zinc finger domain, and 3) C-terminal ring domain. Whereas p53 possesses 393 amino acids: N-terminal transactivation domains (TAD1 and TAD2, residues ~1–40 and ~40–61, respectively), the proline-rich domain (PR, ~ 64–92), the central DNA-binding domain (DBD, residues ~ 100–300), the oligomerization domain (OD, residues ~ 323–355), and the unstructured C-terminal domain (CTD, residues 364–393). It has been confirmed that the binding site of the mdm2-p53 complex is at hydrophobic pocket domain at the NH2 terminus (residues 25-109) of MDM2 and amphipathic peptide at the NH2 terminus (residues 18-26) of p53. Moreover, the mutation at the mdm2-

binding site p53 (p53 residues Leu14, Phe19, Leu22, Trp23, and Leu26, and Leu26) have shown resistance to degradation by mdm2. Likewise, mutations of MDM2 at residues Gly58, Glu68, Val75, or Cys77 result in lack of p53 binding.

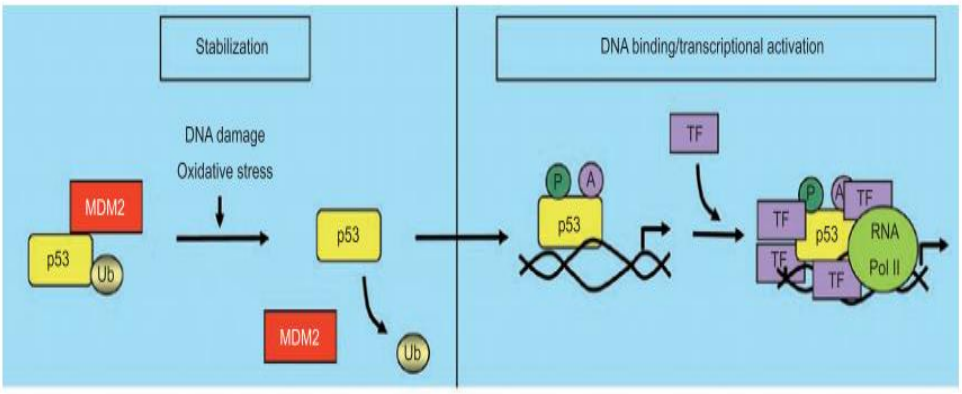


Figure 2.3 mode of p53 activation (Brooks and Gu, 2010)

Apoptosis-programmed cell death

Program cell death is a crucial occurrence within biological systems. Two major types of program cell death, well-commonly known as necrotic and apoptotic type, are distinguished on the mechanism by which leads the cell to undergo mortality (Kim et al., 2006). Morphologically, necrotic cells are indicated by cell swelling and disfigurement of organelles mainly caused by environmentally determined, thus resulting in launch of intracellular component and inflammatory whereas apoptotic cells are indicated by cell shrinkage, condensation, and also DNA fragmentation mainly relevance to cell suicide, thus the dying cells are abolished by phagocytosis (Häcker, 2000; Ziegler and Groscurth, 2004; Rello et al., 2005).

The molecular mechanisms of apoptotic programmed cell death involve vast activity of several biochemical. Two categories of the mechanism which is disposed of different apoptotic-related protein in each have been identified (Dorn, 2013). Category I called extrinsic pathway involves death receptor known as member of the tumor necrosis factor (TNF) receptor that relay death signal from extracellular through activation of caspase 8 within cytoplasm triggering apoptosis. Whereas Category II called intrinsic pathway involves the mitochondrial pathway. The intracellular signaling stimuli initiate loss of membrane potential of mitochondria. As a result, the cytochrome-c enables to enter the cytoplasm then generating signal activates caspase-

9 and caspase-3. Once the cell decides to suicide, it continuously suppresses DNA-repairing system and regulates the function of PARP protein to fragment the DNA and finally, the dying cells are removed by phagocytosis process (Kim et al., 2006).

B-cell lymphoma-2 (Bcl-2) family including pro- and anti-apoptosis which contributes to the process of apoptosis recognized has relevance with the activation of p53. High level of p53 is not only regulating the transcription of target gene but also collectively increasing pro-apoptosis protein such as Bax, and Bad, suppressing the anti-apoptotic protein (Prochazkova et al., 2004). Understanding the apoptotic-modulating protein can help better to decide the effective strategy for chemosensitizer.

MDM2 protein

Murine double minute 2 (MDM2) is protein highly regulated because of role in modulating p53. MDM2 protein, also called as human double minute 2 (HDM2), was firstly identified in Balb/c3T3 fibroblast cell line (3T3DM) of tumor-bearing mice and located at chromosome. It has been reported by Bioportal for Cancer Genomics that MDM2 overexpression in prominent in lung cancer patients, breast cancer, liver cancer, and colorectal cancer. The amplification of MDM2 is a contributing factor of tumorigenesis in human by involving in genomic instability (M. Reza, 2017) The negative regulatory factor for p53 is MDM2. In biological condition, autoregulatory loop of MDM2–p53 leads to p53 protein as in normal condition (Marine & G Lozano., 2010). At this condition, the expressions of both MDM2 and p53 are a very low. At the mechanistic level, MDM2 binds to p53 via its N-terminal domain and forms the complex of MDM2-p53. The binding mechanism initiate the p53 transcription activation region and decreases p53 transcription activity. The MDM2 ring finger domain will then act as an E3 ligase and ubiquitinate p53. Proteasome interaction contributes to the degradation of the p53 protein. MDM2 will also specifically eliminate p53 from cells to reduce the p53 protein (Helei Hou, 2019) Several studies reported the potential correlation between MDM2 and chemoresistance in several cancers. One study proposed that MDM2 inhibit apoptosis mediated by cisplatin, which contribute to cisplatin resistance. In addition, the dysregulation of MDM2 and p53 loop epidermoid carcinoma affects to cisplatin tolerance. Cisplatin can induce both phosphorylation of p53 and mdm2 up-regulation which subsequently affects to minimize cisplatin

resistance and induce cisplatin resistance, respectively. Thus, the up-regulation of MDM2 plays a role in cisplatin tolerance and cisplatin sensitivity (Helei Hou, 2019).

Drug sensitizer

Chemosensitization is one of the strategies for cancer treatment. Simply put, one drug is administered to augment cancer cell sensitivity to another chemotherapy agent (Gupta et al., 2011). The changing of cancer cells to more sensitive involves the alteration of its physiology and metabolism. And thus increasing the development of program cell death when getting the second exposure of the treatment. Foremost of this mode is an enhancement of efficacy, delaying chemoresistance, and lowering dosages and toxicity of conventional chemotherapy agent. Therefore, chemosensitization is a somewhat promising approach for cancer therapy ahead (Cree et al., 2002; Campbell et al., 2012).

Several strategies to achieve those goals of chemosensitization have been described by Shabbits (2003). Most of the focus targets involve modulating program cell death and preventing the mechanism of resistance of cancer cells to chemotherapy. Current scientific evidence reports have been revealed the promising outcome of chemosensitization approach (Shabbits et al., 2003). Whereas, in lung cancer therapy the strategies for chemosensitization have been found several targets in order to enhance the effect of chemotherapy agents such as Bcl-2, RAS-MAP pathway, and cell cycle regulation (Chanvorachote et al., 2009; Aung et al., 2017).

Epithelial-mesenchymal transition (EMT)

The alteration from epithelial cells to mesenchymal cells provides a chance for cell metastasis and invasion. This process is indicated by loss of epithelial phenotypes whereas upregulation of mesenchymal phenotypes during tissues or organ development, also best known as epithelial-mesenchymal transition (EMT) (Nistico et al., 2012; Garg, 2017).

Like normal cells, cancer cells also have the ability to move or migrate from the original cancer cells (primary area) to another tissue or organ (secondary area). Primary lung cancer cells metastasize through the blood vessels to various distant organs such as liver, peritoneum, and bone. In patients with cardia cancer, the secondary cancer cells have been found in lung, nervous system, and bone. Whereas

In patients with non-cardia cancer and adenocarcinoma mostly metastasize to the peritoneum and, bone and ovaries respectively (Riihimaki et al., 2016).

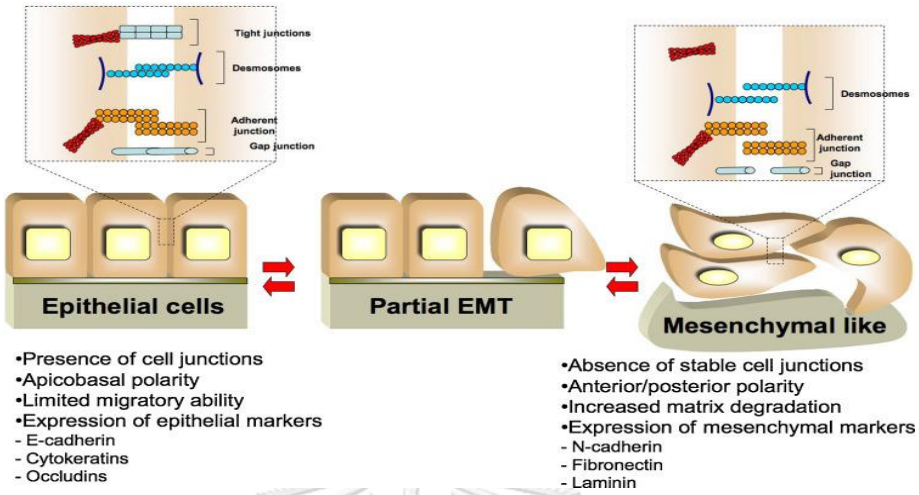


Figure 2.3 epithelial-mesenchymal transition (Vergara et al., 2016)

Metastasis has been associated with the failure of chemotherapy because of the improvement of mesenchymal phenotypes during cell metastasis influence cell response to chemotherapy. For example, Snail, one of the mesenchymal markers, is transcription factor that initiates changing phenotypes toward EMT process. Hence, it increases cell motility, migration, and invasion. In addition, snail also is an important factor that causes chemoresistance due to the inhibition of apoptosis process by activating the survival signaling pathway like PI3K or p21 for cell proliferation. The evidence showed that suppression of snail by antisense oligonucleotides in the mouse model with colonic neoplasia provides the increasing number of cell death (Roy et al., 2004). Furthermore, the expression of snail in cell survival is resistant to apoptotic stimuli and DNA damage (Barrallo-Gimeno and Nieto, 2005) (Siddik, 2009) (Smith and Bhowmick, 2016). Moreover, another mesenchymal phenotype like N-cadherin increases whereas epithelial characteristic, E-cadherin, is downregulated in cisplatin-resistant lung cancer cells (Huang et al., 2016).

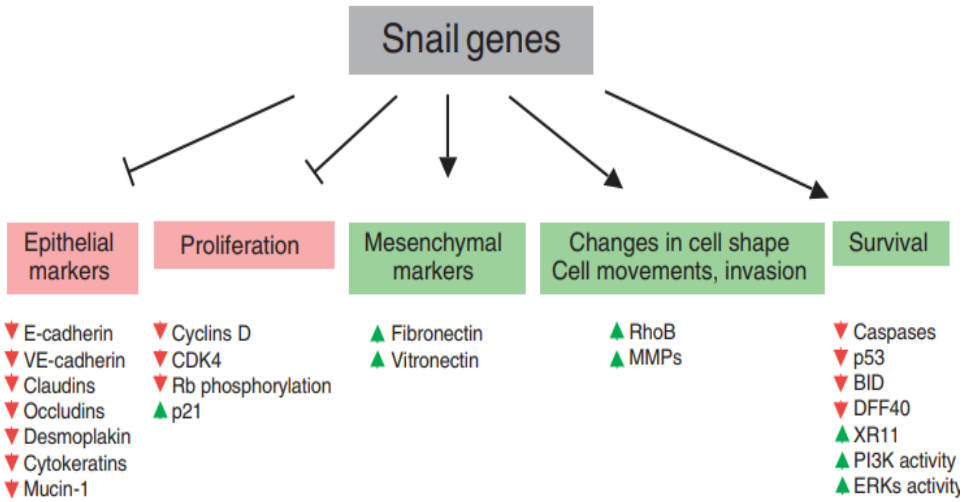


Figure 2.4 Snail targets (Barrallo-Gimeno and Nieto, 2005)

The progression of EMT is regulated by several signaling cascade including SMAD, PI3K-AKT, ERK-MAPK, and JNK signaling. For example, the interaction between ligan and TGF-beta type 1 receptor generates the downstream signaling like PI3K-AKT then translocates to the nucleus in order to enhance the EMT related phenotypes (Gonzalez and Medici, 2014).

Integrin

Epithelial cells are surrounded by cell walls called extracellular matrix which is essential for living cells. This mediated the transmembrane cell-surface receptor, integrin. Nowadays, 24 types of integrin heterodimers have been identified as a form of combination 18 α- and 8 β-subunits. the way of cells sense and response to microenvironment is facilitated by the binding of integrin expression and EMC. Thus, integrin could mediate cell adhesion and intracellular signaling (Lambert et al., 2012). In addition, integrin also serves extracellular communication, cell stability in tissue, and cells reposition toward migration and wound repair during cell development. The activation of integrin described as a transduction signal through integrin, which it is divided into multiple processes: 1) a signal within cells can promote the activation of integrin via the binding of talin to the β-integrin, in turn, 2) the active form of the integrin allows intracellular signal transduction and activate the downstream signaling including focal adhesion formation and actin cytoskeletal reorganization, 3) growth

factor also involved in activation of integrin. It generates a specific signal that enables to cross-tall with the integrin and elicits signal in the same pathway (Figure 2.5) In the same process of cancer, integrin-mediated cancer progression, and metastasis (Hamidi et al., 2016).

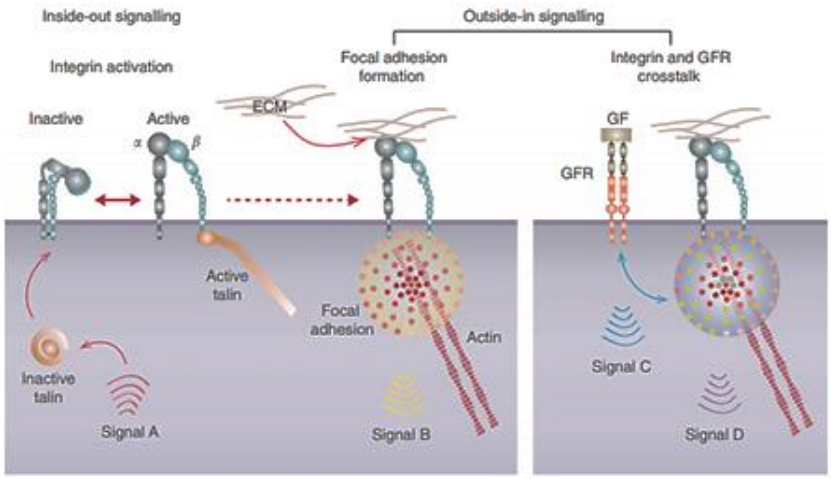


Figure 2.5 Activation of integrin (Hamidi et al., 2016)

Several studies have demonstrated the complexity function and diversity of integrin in cancer cells integrin-mediated cancer progression diversity of cancer cells (Table 2.1).

Table 1. Integrin in cancer progression

Tumor type	Integrins expressed	Associated phenotypes
Melanoma	αvβ3 and α5β1	Vertical growth phase and lymph node
Breast	α6β4 and αvβ3	Increased tumor size and grade, and decreased survival (α6β4). Increased bone metastasis (αvβb3)
Prostate	αvβ3	Increased bone metastasis
Pancreatic	αvβ3	Lymph node metastasis
Ovarian	α4β1 and αvβ3	Increased peritoneal metastasis (α4β1) and tumor proliferation (αvβ3)
Cervical	αvβ3 and αvβ6	Decreased patient survival
Glioblastoma	αvβ3 and αvβ5	Both are expressed at the tumor-normal tissue margin and have a possible role in invasion
Non-small-cell lung carcinoma	α5β1	Decreased survival in patients with lymph node-negative tumors

(Desgrosellier and Cheresh, 2010)

In addition, interrupting integrin function to treat cancer is a comprehensive and ongoing area of cancer research. Integrins are functionally based on the present of extracellular ligand, the focal adhesion formation, and in contact of cytoskeletal (Desgrosellier and Cheresh, 2010). Any Inhibition of these events prevents integrin functions and thus resulting in pharmacological effect. Based on a review of the existing anti-integrin therapeutics and preclinical literature, an integrin antagonism is widely used as target (Millard et al., 2011). However, the activation and downstream signaling of integrin indicates that alternative approaches are feasible. Integrin downstream Molecules downstream would also be beneficial therapeutic approach (Hamidi et al., 2016). for example, Focal adhesion kinase (FAK), a non-receptor tyrosine kinase acting in b-integrin signaling, is increased in focal adhesions and together with another kinase for adhesion turnover, actin cytoskeleton dynamics, and cell changed morphology and thus impacts cancer cell migration and cell invasion (Parsons et al., 2010). Several

clinical trials with FAK (GSK2256098, PF04554878, VS-4718, and so on) and Src (dasatinib) inhibitors are currently underway (Golubovskaya, 2014).

3,4-dihydroxy-5 4'-dimethoxybenzyl (DS-1)

Dendrobium is well-known as the major orchid found in tropical forests in North and Northeast Thailand. Previously, the therapeutic use of *Dendrobium* has been purposed in Traditional Chinese Medicine (TMC) as a universal remedy. Since then, accumulation of studies investigated the feature effects of *Dendrobium* isolates in different kinds of species including alkaloid, flavanoid and sesquiterpenoid have shown pharmacological activity such as anti-diabetic, anti-cancer, anti-metastasis, and anti-pyretic (Cakova et al., 2017; Khwanduean Rattana, 2017). 3,4-dihydroxy-5 4'-dimethoxybenzyl (DS-1) (Figure 2.6) was identified in both *Dendrobium signatum* and *Dendrobium candidum*. DS-1 isolated in a whole *Dendrobium signatum* plant has been shown cytotoxic effect in several cancer cells (Li et al., 2008; Mittraphab et al., 2016). The anticancer effect of DS-1 in Non-Small Cell Lung Cancer is not yet investigated.

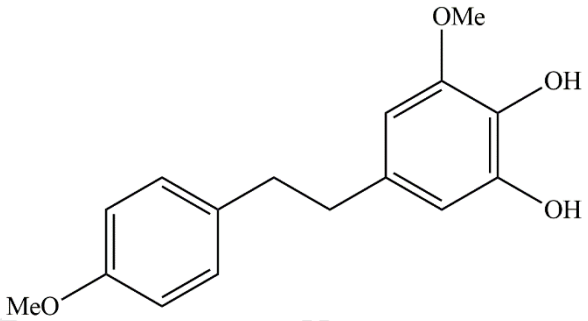


Figure 2.6 Chemical structure of 3,4-dihydroxy-5 4'-dimethoxybenzyl (DS-1)

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

3,4-dihydroxy-5 4'-dimethoxybenzyl (DS-1), 3)-4,5-dimethylthiazol-2-yl-(2, 5-diphenyltetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), Hoechst 33342, Propidium Iodide (PI), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St . Louis, MO). Roswell Park Memorial Institute (RPMI) 1640, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and Immunoprecipitation kit were purchased from Thermo Fisher Scientific Inc (Waltham, MA). Antibodies for Bax, Bcl-2, p53, p-p53 (Ser15), Akt, p-Akt, E-cadherin, N-cadherin, snail, slug, vimentin, integrin-β1, integrin-β3, integrin-α5, RhoA, FAK, p-FAK (Try397), ubiquitin (P4D1) and GAPDH were purchased from Cell Signaling Technology (Beverly, MA) and chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA)

2. Equipment

Laminar flow cabinet (BossTech), microscopy (Nikon Eclipse TS100, Japan), carbon dioxide incubator, Autopipette: 2-10 µL, 10-100 µL, 20-100 µL, and 200-1.000 µL, pipette tips: 2-10 µL, 10-100 µL, 20-100 µL, and 200-1.000 µL, cell culture plate: 96-well, 24-well, and 6-well (Costar), conical tube: 15 mL and 50 mL (Neptune), bottle: 250 mL, 500 mL, and 1000 mL (Duran), disposable pipette: 5 mL, Automated cell counter (TC20 Bio-Rad), pH meter, vortex mixer, balance, microplate reader, flow cytometer (Millipore Sigma) and fluorescence microscope (Nikon eclipse Ts2)

Methods

1. DS-1 preparation

DS-1 was isolated from *Dendrobium signatum* (Figure 2.5). The whole plant of *Dendrobium signatum* was purchased from Jatujak market, Bangkok, in October 2012, as previously described (Mittraphab et al., 2016). The extraction process of the dried plant (3.5 kg) was using MeOH at room temperature. The obtained extract (200 g) was partitioned with EtOAc and *n*-BuOH to give EtOAc extract (41 g), a n-BuOH extract (130 g), and an aqueous extract (28 g). Then, The EtOAc extract was further separated by vacuum-liquid chromatography on silica gel (acetone: *n*-hexane, gradient) to yield 8 fractions (A-H). Fraction E was again fractioned by CC (acetone: *n*-hexane, gradient). DS-1 (184 mg) was obtained after the purification on Sephadex LH-20 (acetone). NMR spectroscopy was performed to determine its purity. DS-1 was dissolved in dimethylsulfoxide (DMSO) as a stock solution. Different concentration of DS-1 for each experimental group was prepared and diluted in cell culture media. The concentration of DMSO (< 0.2%) in the tested compound solution was no cytotoxic effect on H460 cells.

2. Cell culture

The H460 and H292 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO_2 environment at 37^0C.

3. Preparation of patient-derived primary lung cancer cells from malignant pleural effusion

Preparation of patient-derived primary lung cancer cells was conducted following the approved protocol by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 365/62). This study was carried out in accordance with the principles of World Medical Association Declaration of Helsinki. All participants had been diagnosed with recurrent or advanced stage non-small cell lung cancer at the King Chulalongkorn Memorial Hospital. Primary cells in pleural effusion (500-1.000 mL) were collected through thoracentesis. The samples were then

centrifuged at 300 g for 10 min, at 4°C. The cells were re-suspended in RPMI medium with 10% FBS, 2 mM L-glutamine, and 100 units/ml of each of penicillin and streptomycin and cultured for 10-15 passages. They were characterized as the patient-derived primary cancer cells (ELC12, ELC16, ELC17, and ELC20).

4. MTT assay

Cell viability and cytotoxic effect of DS-1 alone or combined with cisplatin were determined by MTT colorimetric assay. Briefly, cells were plated in 96-well culture plates at density 1 x 10^4 cells/well in culture medium and allowed to attach overnight. Media was then substituted by new media containing the indicated compound and cells were incubated for 24 hours. After the treatment, media containing compound was replaced by MTT solution to each well for 4 hours incubation in dark room. An intensity reading of the formazan product is measured at 570 nm using a microplate reader, and the percentage of viable cells was calculated in relation to control cells. All analyses were performed in 3 independent replicate cell cultures. Cell viability was calculated as follow:

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

Anti-proliferation effect of DS-1 is also analyzed by MTT assay. Briefly, cells (2 x 10^3 cell/well) were pretreated with DS-1 (0-100 μM) for 24 hours. cells then incubated up to 48 hours. Cell viability was measured at different time point 0, 24, and 48 hours. Cell proliferation rate was calculated as follows: OD at 24 h or 48 h divided by OD at 0 h, and the relative cell proliferation was determined by comparing the cell proliferation rate of treated cells and untreated cells.

5. Apoptosis and Necrosis assay

Apoptotic cells and necrotic cells induced by DS-1 were determined by visualizing the cell morphology. Firstly, cells in 96-well plate were treated with DS-1 (0-200 μM) for 24 hours. After that cells were rinsed with PBS then incubated with 5 μM PI and 10 μM Hoechst 33342 in PBS. Cells were analyzed and captured under fluorescence microscopy (Nikon Eclipse Ts2). Red- and blue-fluorescent nuclear represent necrotic and apoptotic/viable cells respectively.

6. Annexin-V/PI binding assay

Apoptotic cell death was analyzed by Annexin-V/PI binding assay. After the indicated treatment, cells were harvested by trypsinization, then washed with PBS and collected by centrifugation, then cells were incubated in 100 µL of binding buffer containing 5 µL of FITC-Annexin V and 1 µL of PI solution at room temperature. After 15 minutes of incubation adding 500 µL of binding buffer, the stained cells were immediately measured by flow cytometry.

7. Cycloheximide chasing assay

Cycloheximide (CHX) chasing assay was performed to identified p53 half-life in response to DS-1 treatment. Briefly, cells were treated with DS-1 (50 µM And 100 µM) with or without 50 µg/mL CHX, for 0, 1, 3, and 6 hours. The cells were then collected and lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). The p53 protein level at each time point was determined by western blot as aforementioned.

8. Immunoprecipitation

Ubiquitination of p53 protein was determined by immunoprecipitation assay. Cells were pretreated with proteasome inhibitor MG132 (10 µmol/L) for 1 hour and then treated with DS-1 for 6 hours. After that cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer at 4°C for 20 minutes. The supernatant was separated by centrifugation at 14.000 × g for 10 min at 4°C, measured for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA). 200 µg proteins with anti-p53 antibodies were incubated with the magnetic beat for overnight at 4^0C. Then western blot assay was conducting and using the ubiquitin (P4D1) Mouse mAb to detect the expression of Ub-p53 complex.

9. Western Blot Analysis

The protein expression indicating the molecular mechanism of DS-1 in lung cancer cells was identified by western blotting analysis. The cells were placed onto 60 mm2 plates overnight. After treatments, the cells were washed with cold PBS and incubated with a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM sodium

chloride (NaCl), 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min on ice. Cell lysates are collected, and the protein content was determined by using the BCA protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The amount of protein from each loaded onto 7.5-10% SDS-polyacrylamide gels. After separation, the proteins are transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad), and the transferred membranes were blocked in 5% nonfat dry milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween 20) for 1 h and subsequently incubated with a specific antibody overnight at 40C. Then, the membranes were washed three times in 5 minutes with TBST and incubated with horseradish peroxidase (HRP)–conjugated anti-rabbit or anti-mouse IgG isotype-specific secondary antibodies in 5% nonfat dry milk for 2 hours at room temperature. After three-time washed with TBST, the immune complexes were detected using chemiluminescence and the appearance band will be quantified using Image-J software.

10. Immunofluorescence staining assay

The expression of p-p53 was analyzed by immunofluorescence staining assay. Firstly, cells were treated with the indicated treatment. After that, cells were washed twice with PBS and fixed by 4% paraformaldehyde at 4^0C for 10 minutes. Permeabilization of the cells was performed by incubating the cells with 0.1% Triton x and 1% fetal bovine serum in PBS at 4^0C for 10 min. The cells were blocked with 3% BSA at room temperature for 30 minutes and incubated with p-p53, primary antibody, at 4^0C overnight (0.1% saponin and 1% fetal bovine serum in PBS). After that cells were washed again with PBS, then incubated with secondary antibody for 1 hour at room temperature. After staining, cells were visualized and captured using fluorescence microscopy.

11. Structural Modeling

In order to identify the binding modes and binding interactions of the DS-1 molecule bound to MDM2 protein, the molecular docking technique on the basis of CHARMm-based DOCKER (CDCOKER) will be utilized to identify binding orientation, the

position of compound (ligand) on protein targets, as well as interaction energy between them.

CDOCKER is based on a molecular dynamics simulated-annealing-based algorithm, which generates the various conformations of ligand before putting into the protein binding site. In the present study, 100 independent docking runs will be performed to obtain the complex formation between DS1 and MDM2 protein. Then, conformer with the lowest CDOCKER interaction energy as referred to the best pose will be selected and further analyzed.

The acquired results will be determined in terms of interaction energy and binding orientation between DS-1 and p53-MDM2 complex.

11.1 Preparation of DS-1 structure

The 3D structure of DS-1 is built and optimized at the HF/6-31G(d) level of theory using the Gaussian09 software.

11.2 Preparation of p53-MDM2 complex

The X-ray structure of p53-MDM2 complex is obtained from Protein Data Bank (PDB ID: 1YCR). The binding pocket of p53-MDM2 complex is firstly identified before testing its interaction with DS-1. This has been previously studied that the binding site of the p53-MDM2 complex is at hydrophobic pocket domain at the NH2 terminus (residues 25-109) of MDM2 and amphipathic peptide at the NH2 terminus (residues 18-26) of p53. Thus, the p53-binding pocket can be defined as the potential active site of the MDM2 protein to bind the ligand.

11.3 Molecular docking

Molecular docking of DS-1 with MDM2 will be carried out using the CDOCKER program in the Accelrys Discovery Studio 2.5Accelrys Inc.. CDOCKER will be used to dock DS-1 into the ligand-binding site of MDM2 and the interaction energy of each pose will be obtained. The process is generated based on the stimulation of annealing approach (i.e. heating and cooling steps). 100 poses will be ranked from its CDOCKER interaction energy values as following equation. Binding energy calculation:

Ebinding = energy of complex (Ecomplex) – (energy of protein (Eprotein) – energy of ligand (Eligand))

11.4 Molecular dynamics (MD) simulation

MD simulation of the best conformer of DS-1-MDM2 complex taken from molecular docking will be performed using AMBER 16 software package. The additional results in terms of structural analyses and binding affinity of DS1-MDM2 complex can be revealed by MD simulation. In the present study, MD simulation will be conducted to confirm the stability and recognition of DS-1 toward MDM2. The basic principle of MD simulation is to calculate forces (bonded and non-bonded interaction between atom in the system) based on Newton's second law of motion. Note that bonded interaction includes bond length, bond angle, and torsion, whereas non-bonded interaction includes electrostatic interaction and van der walls interactions. These interactions are commonly called “forces field”.

12. Filopodia formation assay

Cells at a density of 5 x 10^4 cells/well in 96 well-plate were treated with DS-1 (0-100 μM) for 24 hours. The cells were continuously fixed with 4% paraformaldehyde for 10 min. After permeabilized with 0.1% Triton-X in PBS for 5 min, the cells were rinsed with PBS, blocked for unspecific binding with 0.2% BSA in PBS for 30 min, then incubated with a 1:100 dilution of phalloidin-rhodamine in PBS for 30 min, rinsed in PBS three times and mounted in 50% glycerol in PBS. Cell morphology of untreated and treated groups was capture captured using a fluorescence microscope (Nikon Eclipse Ts2, Japan; 40x magnification).

13. Migration Assay

Cell migration was determined by wound healing assays. A monolayer of cells was cultured in 96-well plates. The wound space was created by 1-mm-wide tips. After that, media was removed and washed with PBS. The cell monolayers were incubated with non-toxic concentration of DS-1 and permitted to migrate for 24 and 48 hours under a phase-contrast microscope. The percentage of the migration level was calculated as previously described (Yue et al., 2010), the different of wound area measured at 0 hours and wound area measured at 24 or 48 hours is divided by wound area measured at 0 hours, then multiplied by 100%.

14. Invasion Assay

An invasion assay was performed using a 24-well transwell unit with polycarbonate (PVDF) filters (8 mm pore size). Each membrane was coated with 0.5% matrigel (BD Biosciences, Bedford, MA, USA) on the upper surface of the chamber overnight at 37°C in the incubator. The cells were plated in the upper chamber at the density of 2×10^4 cells/100 μL in serum-free medium and were incubated with non-toxic concentrations of DS-1 and a medium containing 10% FBS was added to the lower chamber of the unit. After 24 hours, the top medium and matrigel were removed and the cells on the upper side of the membrane were removed with a cotton swab while the bottom side was stained with Hoechst 33342. The cells were visualized and scored under a fluorescence microscope.

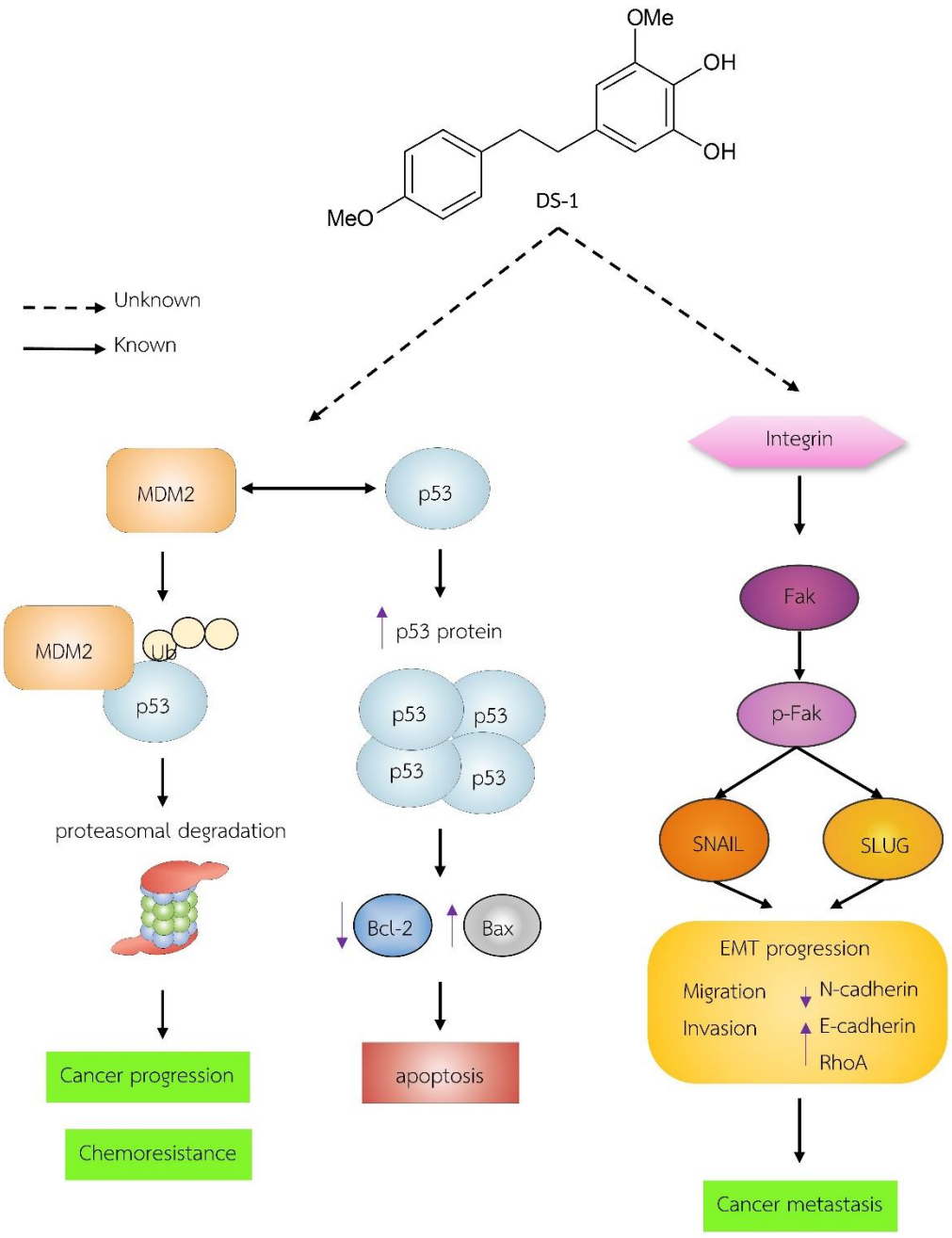
15. Anchorage-independent cell growth assay

Anchorage-independent cell growth was design in two-layer of soft agar. Briefly, the first layer was made by preparing 1% agarose and RPMI culture medium at ratio 1:1. The mixture was added into 24-well plate (500 μL/well) and placed at 4^0C. The second layer contained a mixture of the treated cell suspension in culture media and 0.33% agarose gel. After the first 3 h incubation and followed by every 3 days, culture medium with 10% FBS was added. Then 14 days later, colonies were photographed under microscopy (Nikon Eclipse Ts2, Japan; 10x and 20x magnification) and measured the colony number and size using ImageJ software.

16. Statistical analysis

All numeric values are represented as the mean ± S.D from three independent experiments. Multiple comparisons were examined by analysis of variance (ANOVAs), followed by the Tukey posthoc test using Prisma 8 (Graphpad Software, San Diego, CA, USA). Statistical significance was set at $*p<0.05$.

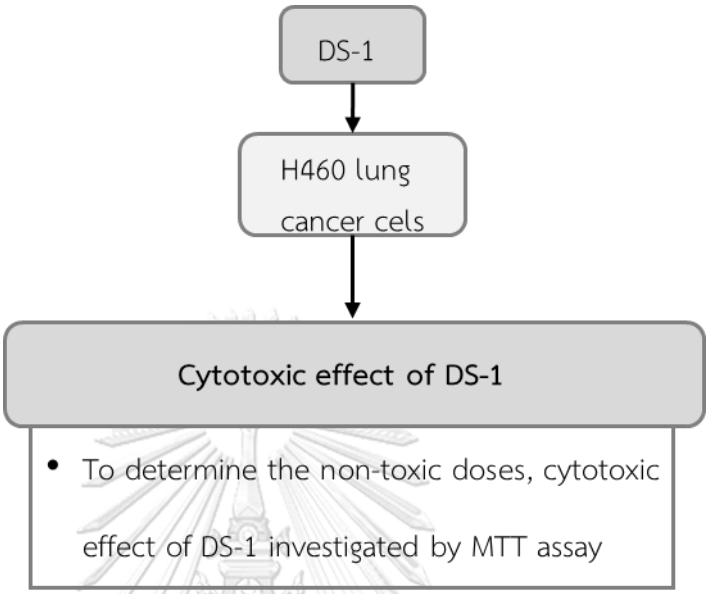
17. Conceptual framework



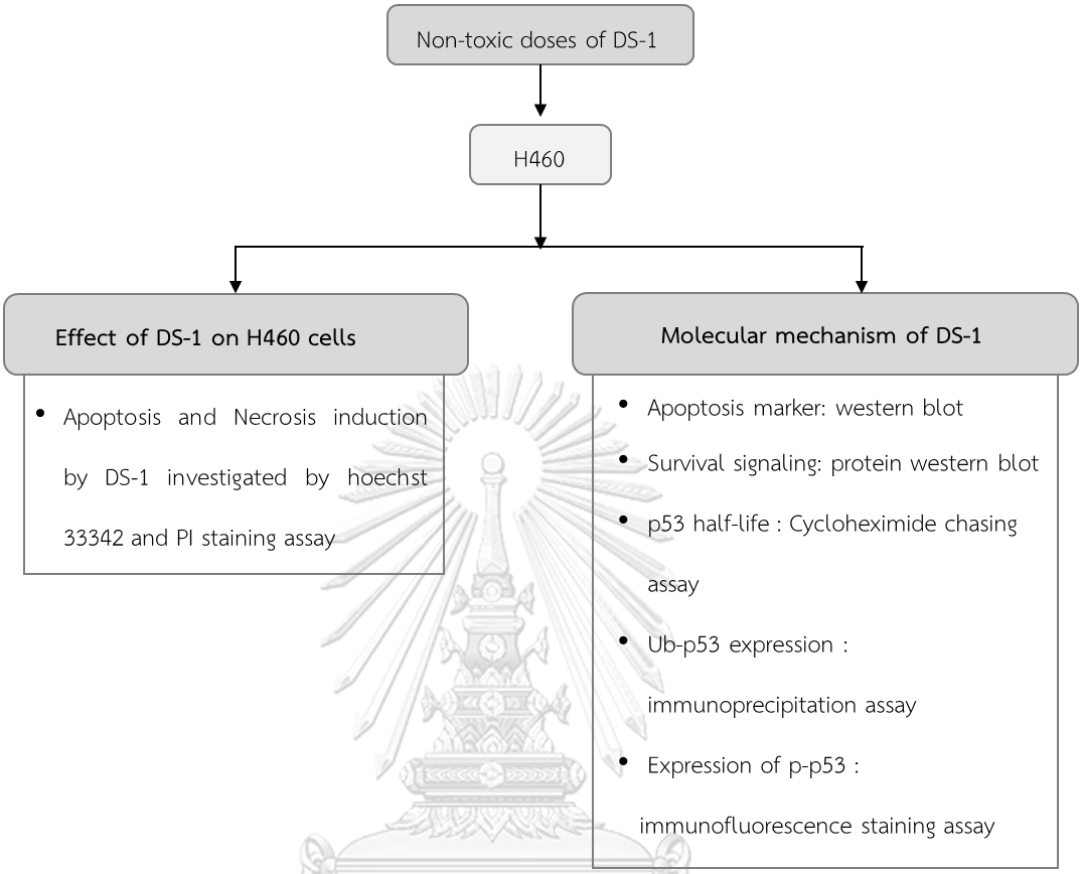
18. Experimental Design

This study was designed as following;

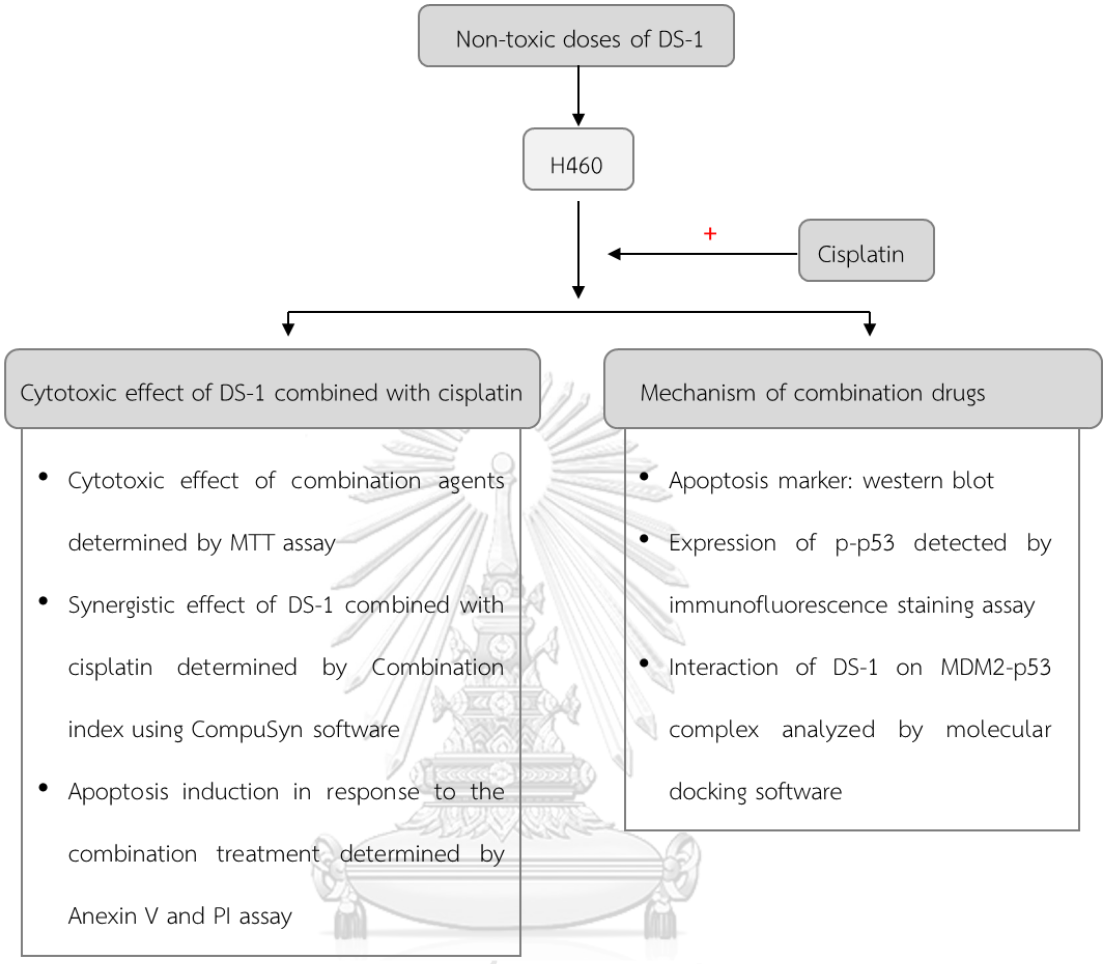
Part I Cytotoxic Effect of DS-1 on H460 cells



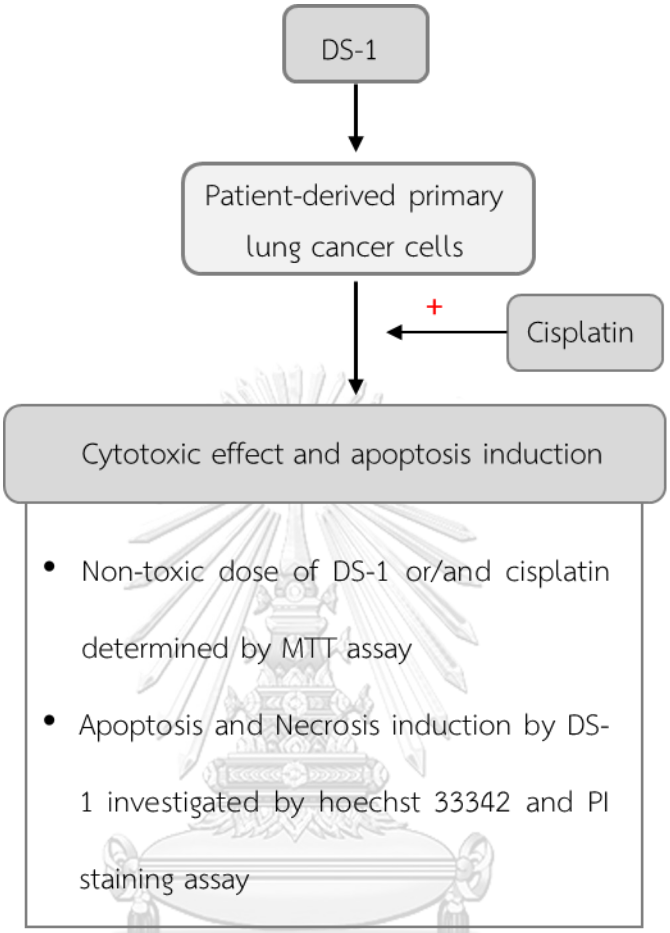
Part II Effect of DS-1 on p53 protein level



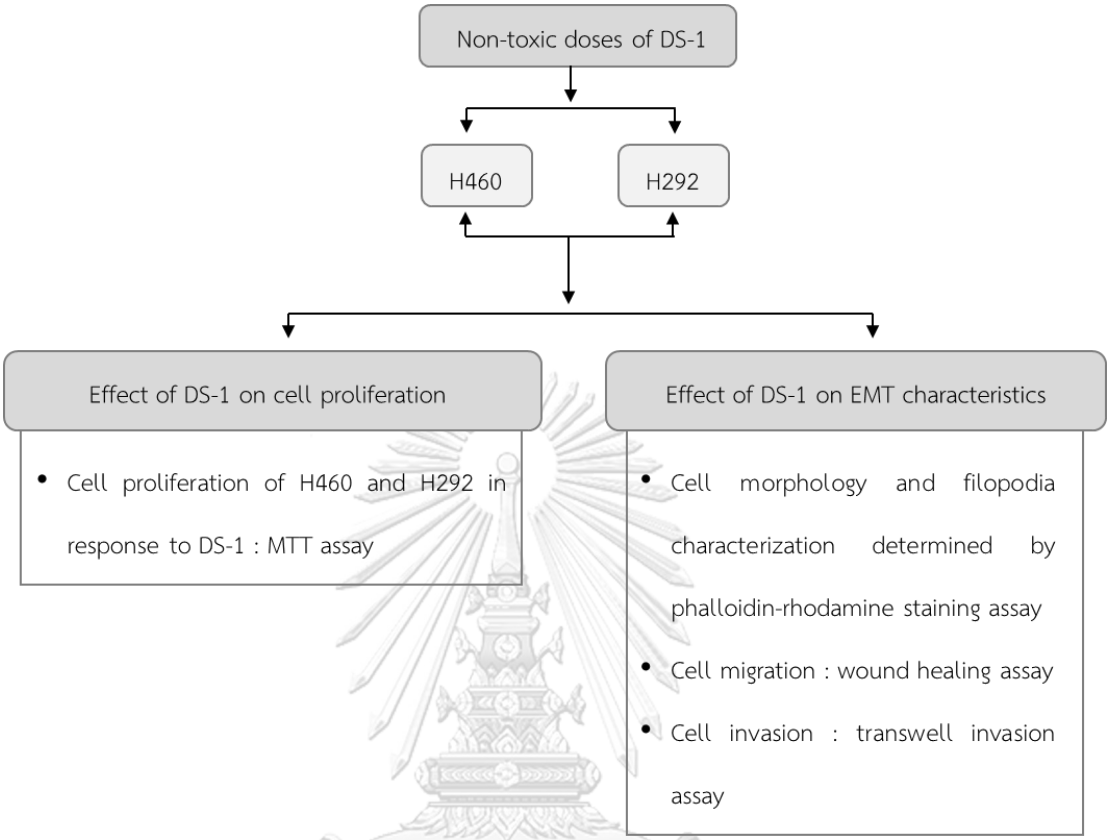
Part III Effect of DS-1 combined with cisplatin in H460 cells



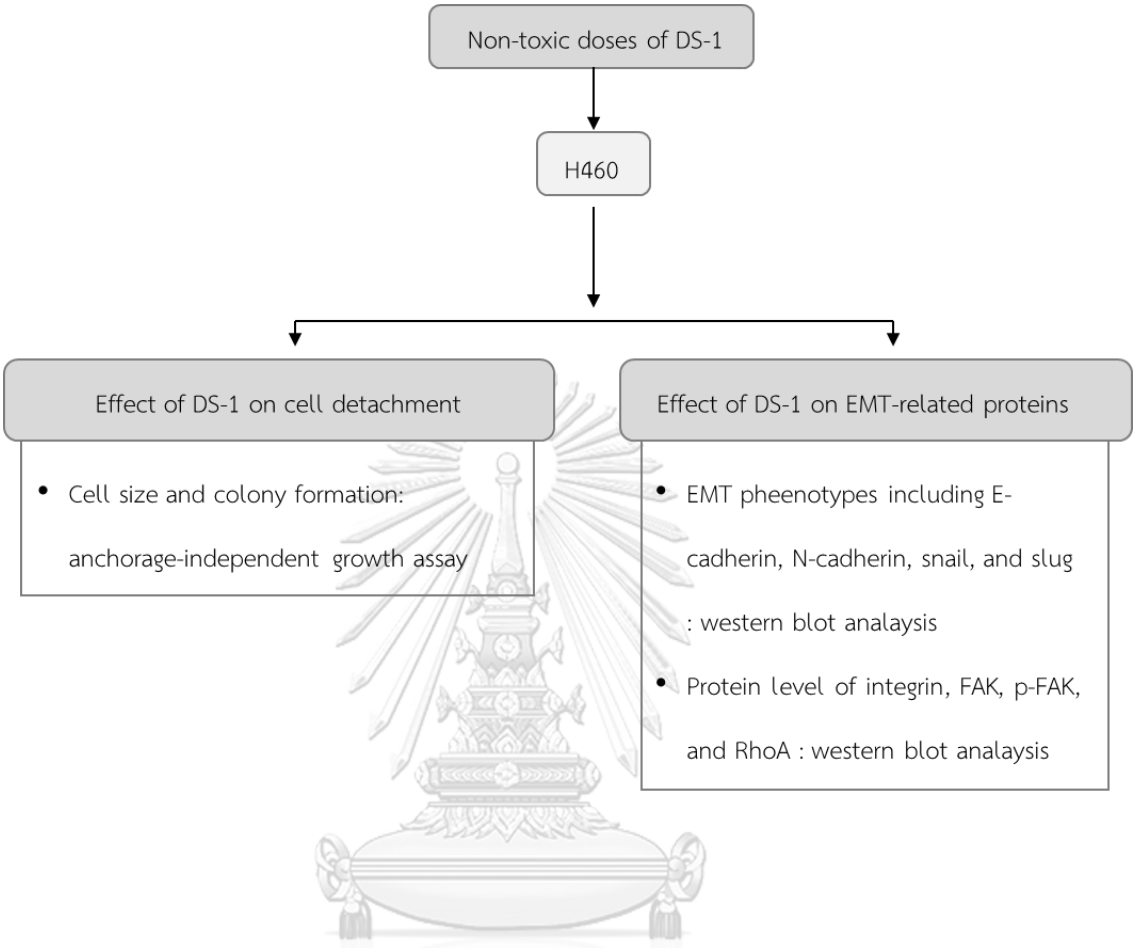
Part IV Effect of DS-1 and/or cisplatin in patient-derived primary lung cancer cells



Part V Effect of DS-1 on EMT characteristics



Part VI Effect of DS-1 on EMT-related protein



19. Cytotoxic effect and apoptosis induction of DS-1 on NSCLC cell

19.1 Cytotoxic effect of DS-1

Evidence has been revealed that DS-1 has the capability to suppress cell viability in several cancer cell (Mittraphab et al., 2016), thus indicating that DS-1 may benefit to be developed as an anticancer agent. An attempt to inspect the effect of DS-1 on NSCLC cells, finding the toxicity, and specifying the non-toxic doses was the first investigation.

Cells in 96-well plate were treated with DS-1 in several dose concentrations (0-200μM) and let them in incubator at 37^0C for 24 h. Enzyme activity indicating the viable cells was measured by MTT assay. The non-toxic dose of DS-1 was determined by which concentration has no significant effect compared with control group and reduces cell viability no lower than 70%. Altogether, we could reveal IC50 concentration of DS-1.

19.2 Effect of DS-1 in apoptosis induction

To confirm the cytotoxic effect of DS-1 that may affect apoptosis or necrosis induction on NSCLC cells. Apoptosis and Necrosis staining assay was performed. The cells were treated with DS-1 (0-150μM) for 24 h. Then cell nucleus will be stained with Hoechst and PI. Morphological change for all treatment and the non-treatment group was observed under fluorescent microscopy.

20. Mode of action of DS-1 in H460 cells

As a prior test to investigate DS-1 toxicity on NSCLC, this study further extends more evidence by identifying the molecular mechanism. Cancer cells keep growing in the supports of apoptosis stimuli inhibition and survival signaling system (Kalimuthu and Se-Kwon, 2013). Thereby, apoptosis and survival signaling are therapeutic target to develop an anticancer agent. To aim so, the related proteins including Bcl-2, Bax, Akt, p-Akt, and p53 were investigated.

Subconfluence Cells 80-90% in 6-well plate are treated with non-toxic doses of DS-1 (0-100 μM) and incubated for 24 h. The protein expressions of apoptosis and survival signaling pathways were determined by western blot analysis.

21. Synergistic effect of DS-1 combined with cisplatin

The standard therapy for NSCLC still tends to use platinum-based drug including cisplatin. The mechanism of cisplatin triggering DNA damage is categorized as a non-specific anti-cancer drug. it is well known that cisplatin cause nephrotoxicity and neurotoxicity as the advertising effect and the inherent resistance also enable to limits therapy achievement. In comparison in the use of cisplatin as double therapy versus monotherapy, studies demonstrated that the median survival of combination therapy with cisplatin is significantly higher than cisplatin alone. Increasing the efficacy, optimizing the side effect, and delaying resistance evolution are an expectation of the combination therapy by meaning that to enhance the curative outcome (Cosaert and Quoix, 2002; Cheong et al., 2005; Amarasena et al., 2008). Here, we tested the response of H460 cells to a combination of DS-1 and cisplatin treatment.

Cells were seeded onto a 96-well plate. After overnight, the media was replaced by new media containing DS-1 (0-100 μM). 24 h later cells incubate again with cisplatin (0-50μM) for 24 h and 48 h. After that, the viable cells were measured by MTT assay. In an effort to determine whether this combination treatment has a synergistic effect, the combination index (CI) was calculated using compuSyn software.

22. Mechanism underlines DS-1 sensitizing H460 cells to cisplatin treatment

The natural product has been widely used for many diseases. In the development of anti-cancer agent, natural product with the specific molecular mechanism has beneficially shown the effectivity to enhance chemotherapy potency (Demain and Vaishnav, 2011).

In order to delve the mechanism by which DS-1 sensitizes H460 cells to cisplatin, immunoprecipitation assay was performed. Cells within 6-well plate were treated with MG132 for 3 h prior to DS-1 exposure for 6 h. Up to 250 μg protein into Eppendorf with magnetic bead and add anti-p53 for incubation overnight at 4^0C. Protein complex then detected by western blot analysis

23. Molecular mechanism of combination of DS-1 and cisplatin

To confirm the effect of DS-1 combined with cisplatin in apoptosis induction, cells were incubated with DS-1 prior to 24 h then treated with cisplatin for 24 h. The

expression of apoptosis-related proteins such as Bcl-2, Bax, p53 is investigated by western blot assay as mentioned above.

24. Effect of DS-1 combined with cisplatin on p-p53 expression

To identify the expression of p-p53 after combination treatment, cells were incubated with DS-1 for 24 hours and then incubated again for 24 hours with cisplatin. The p-p53 expression was visualized by immunofluorescence assay. The treated cells were then incubated with p-p53 antibody overnight and fluorochrome-conjugated secondary antibody (Alexa Fluor® 488 Conjugate) for 1 hour at room temperature. After staining, cells were visualized and captured using fluorescence microscopy.

25. Cytotoxic effect of DS-1 combined with cisplatin in patient-derived primary lung cancer cells

To provide more evidence of the beneficial effect of DS-1 and combined with cisplatin, we further investigated the cytotoxic and synergistic effect in patient-derived primary lung cancer cells. Cells were treated with DS-1 with or without cisplatin for 24 h. Then, viable cells were measured by MTT assay.

26. Effect of DS-1 on cell migration and invasion

26.1 Effect of DS-1 on cell morfology

Cancer cells originally grow within tissue. As they continuously proliferate, cancer spread in late-stage enables to metastasis through breaking out the normal tissue and reaches new region of the body (Bacac and Stamenkovic, 2008). During cell metastasis, the process follows several steps by which initiated by the change of cellular morphology. For instance, epithelial to mesenchymal transformation (EMT) is characterized by a prolonged cell spindle of the cytoskeleton. This is indicated as a higher metastatic potential than non-metastatic cells (Leber and Efferth, 2009; Lyons et al., 2016).

To determine whether DS-1 inhibits the initiation stage of EMT progress. We previously observed the different cell morphology between control and treatment groups as mentioned above. The cell size was measured by ImageJ software.

26.2 Effect of DS-1 on cell migration and invasion

Furthermore, as the morphological modification, it supports the cell behavior yielded in cell migration and invasion through the tissue to enter circulation where cells are facilitated to other organs or tissue. The ability of cell migration after DS-1 treatment was analyzed by measuring the area of wound healing model. Whereas the ability of cell invasion after DS-1 treatment was evaluated the number of cells that invade through transwell filter compared to the control group.

27. Effect of DS-1 on cell growth in an anchorage-independent model

It has been well known that most of epithelial cells able to survive and proliferate require the proper conditions: cells attach within tissue and generate the activation of many survival signaling pathways (Guadamillas et al., 2011). Alike normal cells, cancer cells potentially defense themselves to survive within a detach condition called anchorage-independent. During EMT, cells travel from primary site to secondary site by detaching form. To determine whether DS-1 suppresses cell growth during anchorage-independent, cells pretreated with non-toxic doses of DS-1 for 24 hours were placed in adhesion model using anchorage-independent growth assay as mentioned in materials and methods.

28. Effect of DS-1 on EMT phenotypes

Cell metastasis evolvement is initiated by which the transformation of cells from epithelial cells with their cell-cell adhesion and cell polarity to mesenchymal cells with migratory and invasion ability (Mittal 2016). To investigate the effect of DS-1 on EMT-regulated protein. Western blot assay was performed to detect the protein expression of N-cadherin, E-cadherin, snail, and slug. Moreover, integrin expression has been also associated with EMT progression (Bai et al. 2010; Larzabal et al. 2014). Thus integrin β1, integrin β3, integrin α5 as well as the downstream pathway including FAK protein level were determined by western blot assay.

29. Expected benefit and application

This finding demonstrated the novel activity of a pure compound DS-1 including a specific molecular mechanism on regulating MDM2-p53 complex with sensitizing

effect in NSCLC cells toward cisplatin-induced apoptosis and the potential effect as EMT inhibitor. This information is beneficial for developing DS-1 as a new potent anticancer agent for NSCLC.

CHAPTER IV

RESULTS

1. Cytotoxic effect of DS-1 on lung cancer cells and normal lung cells

a. Cytotoxic effect of DS-1

The cytotoxic effect of DS-1 has been addressed in several cancer cells including breast cancer cells, liver cancer cells, and colon adenocarcinoma cancer cells (Mittraphab et al., 2016). In the present study, we examined the effect of DS-1 on H460 lung cancer cells. To determine the non-cytotoxic and cytotoxic concentration of DS-1, cells were first treated with DS-1 for 24 hours. After that, the cells were incubated with MTT reagent in dark room for 4 hours. The formazan product was diluted by DMSO, then measured by microplate reader at 570 nm. The result showed that DS-1 reduced cell viability in a dose-dependent manner and significantly reduced at 150 μM and 200 μM that indicated as toxic doses (Figure 4.1)

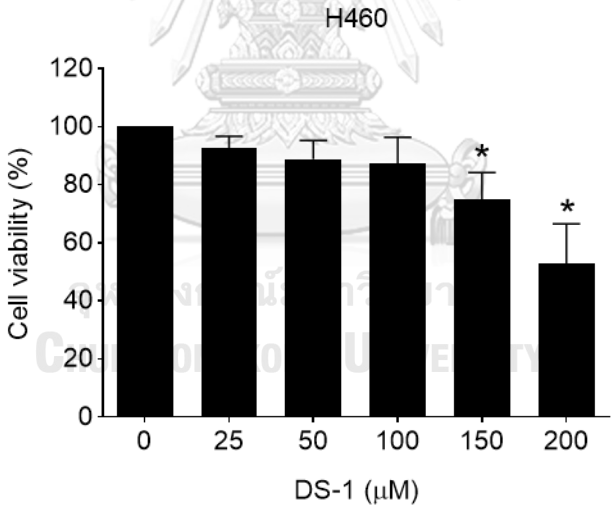


Figure 4.1 Cytotoxic effect of DS-1 on H460 lung cancer cells. Cells were treated with several doses of DS-1 (0-200 μM) for 24 hours. Viable cells were identified by MTT assay. The percentage of the cell viability was calculated relative to control cells (taken as 100% viability). Values are means ± SD of three independent experiments, *P < 0.05 versus non-treated cells.

b. Effect of DS-1 on apoptosis and necrosis induction

To confirm the cytotoxic effect of DS-1, we further investigated the apoptosis and necrosis cell morphology. H460 cells were similarly treated with DS-1 and analyzed by Hoechst 33342 and PI staining assay. As shown in figure 4.2, the apoptotic morphology indicated by intense nuclear fluorescence, chromatin condensation, and fragmentation was seen along with the increasing dose concentration of DS-1. This revealed that DS-1 positively has potential on apoptosis induction in H460 cells after reaching the hight dose.

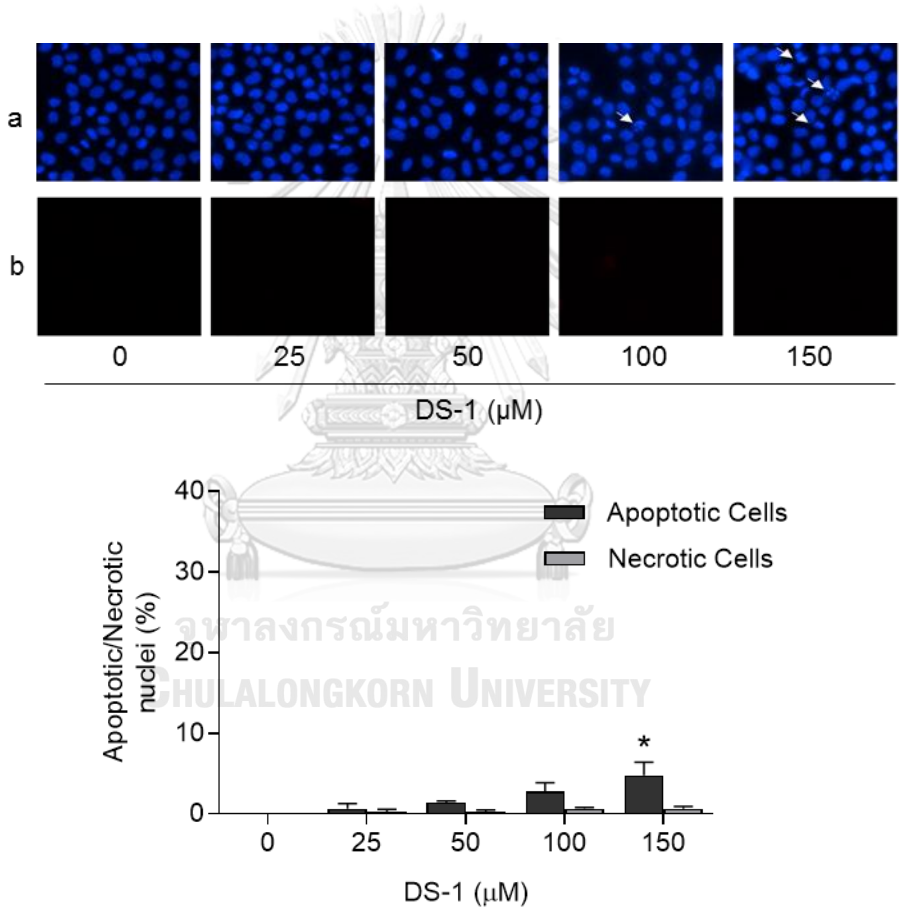


Figure 4.2 The apoptotic and necrotic cells were stained by (a) Hoechst 33342 and (b) propidium iodide. Scale bar represents 25 μm. The percentage of apoptotic and necrotic nuclei was calculated as the total apoptotic/necrotic cell of the treatment group divided by the total apoptotic/necrotic cell of the control group and multiplied by 100%. The data is presented as mean ± SD (n=3). *p<0.05 versus control group.

2. DS-1 sensitizes cisplatin-induced apoptosis in H460 cells

a. Cytotoxic effect of DS-1 combined with Cisplatin

To determine the sensitizing effect of DS-1 on H460 cells to cisplatin treatment, we further investigated the cytotoxic effect of DS-1 combined with cisplatin on H460 cells. Cells were pretreated with DS-1 for 24 hours prior then once again treated with cisplatin for 24 or 48 hours. The cell viability was analyzed by MTT assay. The results indicated that the living cells were reduced more at combination treatment than cisplatin alone in a dose- and time-dependent manner (Figure 4.3).

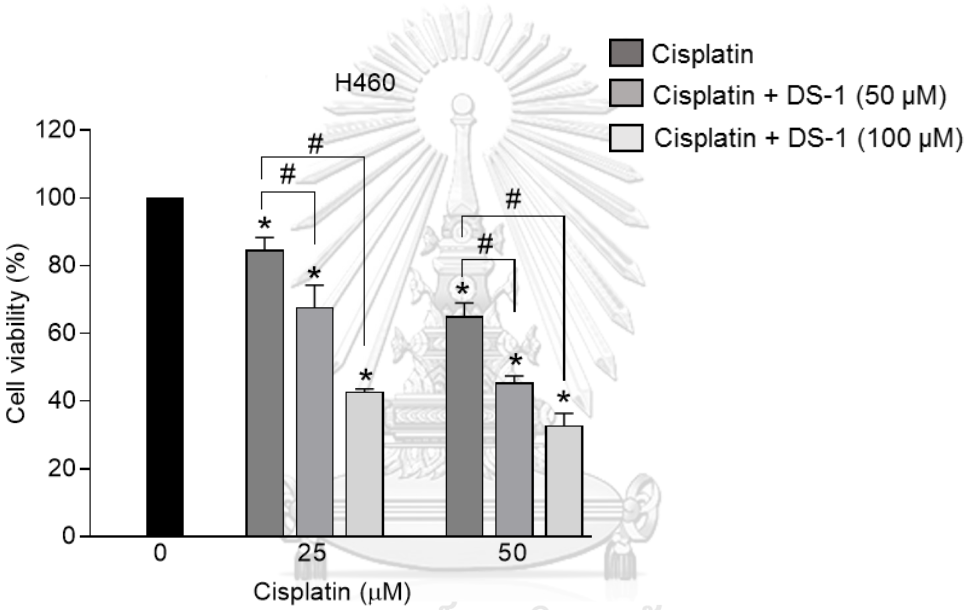


Figure 4.3 DS-1 enhances the effect of cisplatin-induced cytotoxicity on H460 lung cancer cells. Cells were treated with DS-1 (50 and 100 μM) for 24 hours before 24 hours cisplatin (25 and 50 μM) exposure. Viable cells were measured by MTT assay. The percentage of cell viability was calculated and compared to untreated cells. The data is presented as mean ± SD (n=3). *$p<0.05$ versus control group, $^{\#}p<0.05$ versus cisplatin-treated cells.

b. Effect of DS-1 combined with cisplatin on apoptosis induction

Since having shown that DS-1 could sensitize cells to cisplatin-induced cytotoxicity, to identify the effect of DS-1 in cisplatin-induced apoptosis, we then performed annexin V conjugated PI using Flow cytometry to measure the increasing number of apoptotic cells. Cells were pre-treated with DS-1 for 24 hours, then

incubated with cisplatin for 24 hours. The cells were stained with annexin v and PI. Consistently, the results showed that in combination treatment of DS-1 and cisplatin has a high percentage of apoptotic cells (Figure 4.4). Accordingly, it could be assumed that DS-1 has the capability to sensitize the cells to cisplatin-induced apoptosis.

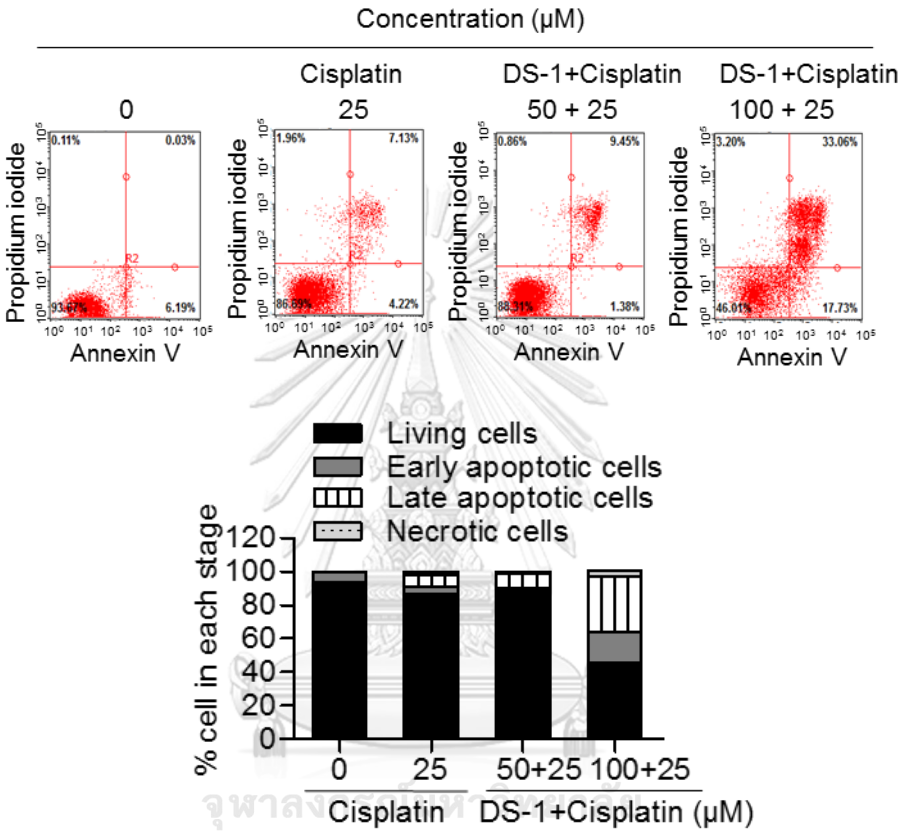


Figure 4.4 Density maps of annexin V apoptosis detection results. After the treatment of DS-1 and cisplatin, apoptotic cells were measured by flow cytometry. Rates of living, early apoptosis, late apoptosis, and necrosis cells were obtained after the annexin V/PI analysis. .

c. Combination index of DS-1 plus cisplatin

The combination index (CI) was also calculated by CompuSyn software. CI < 1, CI = 1, and CI > 1 represented synergism, additive, and antagonism effect, respectively. DS-1 plus cisplatin showed CI lower than 1 implying that this combination has a synergistic effect (Figure 4.5).

DS-1 (µM)	Cisplatin (µM)	Fa	CI
50	25	0.35	0.35542
50	50	0.54	0.06605
100	25	0.57	0.03346
100	50	0.67	0.02076

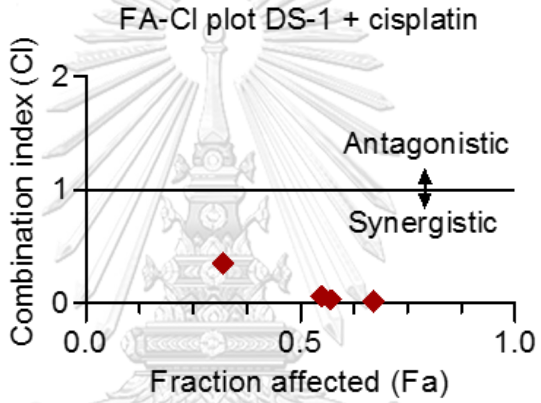


Figure 4.5 Combination index (CI)-fraction affected (Fa) plot of DS-1 and cisplatin combination in H460 cells. Fraction affected; the range is 0-1, where Fa= 0 and 1 represented 100% viability *and 0% viability, respectively, and input into CompuSyn Software (Biosoft, Ferguson, MO, USA). CI < 1, CI = 1, and CI > 1 represented synergism, additive, and antagonism effect, respectively.*

3. DS-1 increases the expression of p53 on H460 cells

a. Effect of DS-1 on protein-regulated apoptosis

To investigate the mechanism of action of DS-1, the non-toxic concentrations of DS-1 (25-100 µM) were continuously evaluated on apoptotic-related proteins including p53, Bax, and Bcl-2 and survival signaling cascade proteins including Akt and p-Akt. H460 cells were treated with DS-1 (0-100 µM) for 24 hours. The protein expression was detected by western blot analysis. Interestingly, the results showed the increasing level of p53 protein expression in the DS-1 treated cells up to 2.24-6.00-fold higher than that of the control group. Moreover, DS-1 also affected the induction

of Bax to 1.99-fold and inhibition of Bcl-2 0.26-fold protein expression to at 100 μM of DS-1, but slightly suppressed the expression of Akt/p-Akt protein compared to the untreated group (Figure 4.6A,B), given that DS-1 might regulate the accumulation of p53 protein, which in turn generates the cellular response including up-regulation of Bax and down-regulation of Bcl-2.

b. Effect of DS-1 on p53 half-life

p53 functions as an important regulatory for apoptosis induction in response to cellular stress stimulus including aberration of oncogene activation and DNA damage (Fridman and Lowe, 2003). Thereupon, rapid degradation, short half-life, and low intracellular level of p53 promote cancer cells thrive (Ozaki and Nakagawara, 2011). Accordingly, for the therapeutic approach, several studies have purposed the restoration of p53 function as a potential drug mechanism of action (Bossi and Sacchi, 2007; Martinez, 2010; Hientz et al., 2017). To evaluate whether DS-1 up-regulates p53 level through increasing p53 stability, the CHX chasing assay was performed. CHX has been reported as a protein synthesis inhibitor and is widely used to determine half-life of a protein (Schneider-Poetsch et al., 2010). Cells were subjected to 50 μg/mL CHX with or without DS-1 (50 μM and 100 μM) for 0, 1, 3, and 6 hours followed by western blot analysis. In the present of CHX, the expression of p53 protein was inhibited and the reduction of the protein is dominantly dependent on the rate degradation. As shown in figure 4.6 C and D, the result showed that DS-1 could increase the p53 half-life in CHX-treated cells from ~ 0.5 hours to ~ 1.10 and 1.84 hours at 50 and 100 μM of DS-1, respectively. The half-life of p53 within cells is limited by its negative regulator MDM2 protein. This because the MDM2 targets p53 and ligases the protein with ubiquitin molecules for proteasomal degradation (Marine and Lozano, 2010).

c. Effect of DS-1 on Ub-p53 level

Since DS-1 increased the level of p53 expression in H460 cells at the non-toxic dose, we presumed that DS-1 might act on the preventive degradation of p53 protein that leads to increasing the accumulation of p53 protein in the nucleus. To confirm the effect of DS-1 on the prevention of p53 degradation, immunoprecipitation and western blot analysis were performed to detect the formation of Ub-p53. H460 cells

were pretreated with MG132 (10 μM), a specific proteasome inhibitor, for 1 hour, and treated with DS-1 (50-100 μM) for 6 hours. As shown in figure 4.6E, the results indicated that the Ub-p53 complex was dramatically suppressed after DS-1 treatment, suggesting that DS-1 increases p53 protein by delaying the ubiquitin-proteasome degradation process.

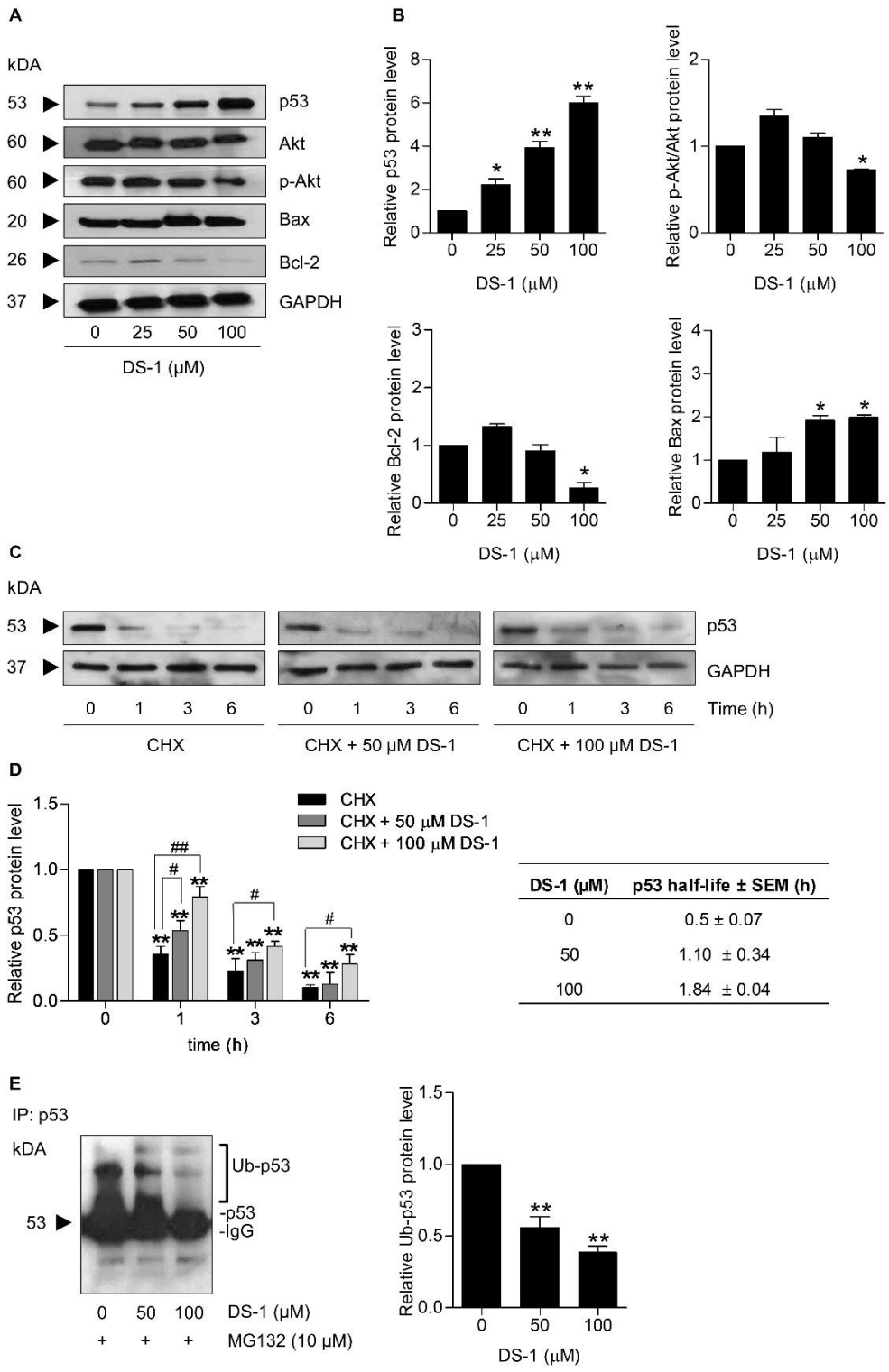


Figure 4.6 The effect of DS-1 on apoptosis-related protein. (A) Cells were treated with DS-1 (0-100 μM) for 24 hours. The protein expression was detected by western blot assay. (B) The intensity was qualified by densitometry. (C) Cycloheximide was carried out to determined the half-life of p53 protein. Cells were subjected to DS-1 (50 and

100 μM) with or without 50 μg/mL cycloheximide (CHX) for 0,1, 3, and 6 hours. The p53 protein expression was detected by western blot. (D) The relative protein levels were calculated by densitometry. The half-life of p53 protein was calculated using GraphPad Prism's one-phase decay algorithm. The data is presented as mean ± SD (n=3). **p<0.01 versus untreated cells at 0 hour, $^{\#}$p<0.05, $^{\#\#}$p<0.01 versus CHX-treated cells (E) Cells were pretreated with proteasome inhibitor MG132 (10 μmol/L) for 1 hour and then treated with DS-1 (50-100 μM) for 6 hours. Cell lysates were immunoprecipitated with anti-p53 antibody, and the ubiquitinated protein level (ubiquitin (P4D1) mouse mAb) was analyzed by western blotting assay. The intensity of Ub-p53 protein level was qualified by densitometry and calculated relative to control. The values of all experiments are means of independent triplicate experiments ± SD *p<0.05, **p<0.01 versus control group.

4. The combination of DS-1 and cisplatin enhanced the expression of p-p53 on H460 cells

a. Effect of DS-1 combined with cisplatin on protein-related apoptosis

Having shown that DS-1 sensitizes lung cancer cells to cisplatin-induced apoptosis, we further identified the mechanistic pathways involving in apoptosis induction including expression apoptosis-related protein, p53, Bax, Bcl-2, Akt/p-Akt, and activation of p53 in combination treatment. Cells were pretreated with DS-1 (50-100 μM) for 24 hours and treated with cisplatin (25-50 μM) for 24 hours. The indicated proteins were investigated by western blot. As shown in figure. 4.7A-C, DS-1 could enhance the effect of cisplatin in regulating the p53-dependent apoptosis cascade including induction of Bax, suppression of Bcl-2, and attenuation of Akt/p-Akt. As the p53 is known to be stabilized and activated in response to DNA damage stimuli via phosphorylation at Ser-15 and the activated p53 then accumulated in the nucleus for its transcriptional activities.

b. Effect of DS-1 combined with cisplatin on p-p53 level

The p-p53 expression was evaluated by immunofluorescent assay and visualized under fluorescence microscopy. We investigated the activate status and nuclear localization of the protein in response to DS-1, cisplatin, and combination treatments. Figure. 4.7 D and E indicate that treatment of the cells with DS-1 slightly

increased the p-p53 level concentrated at the nucleus of cells, whereas cisplatin treatment could also enhance the intense signal of p-p53. Interestingly, the combination treatment dramatically increased the activated p53 and nuclear localization, suggesting that DS-1 may, at least in part, induce the accumulation of naive p53 prior to cisplatin treatment and such accumulation of the protein intern promoted the activation of p53. These indicated that the rule of DS-1 on cisplatin-induced apoptosis acts on upregulation p53 to sensitize H460 lung cancer cells. Then the increasing p53 protein is activated after exposure to cisplatin.

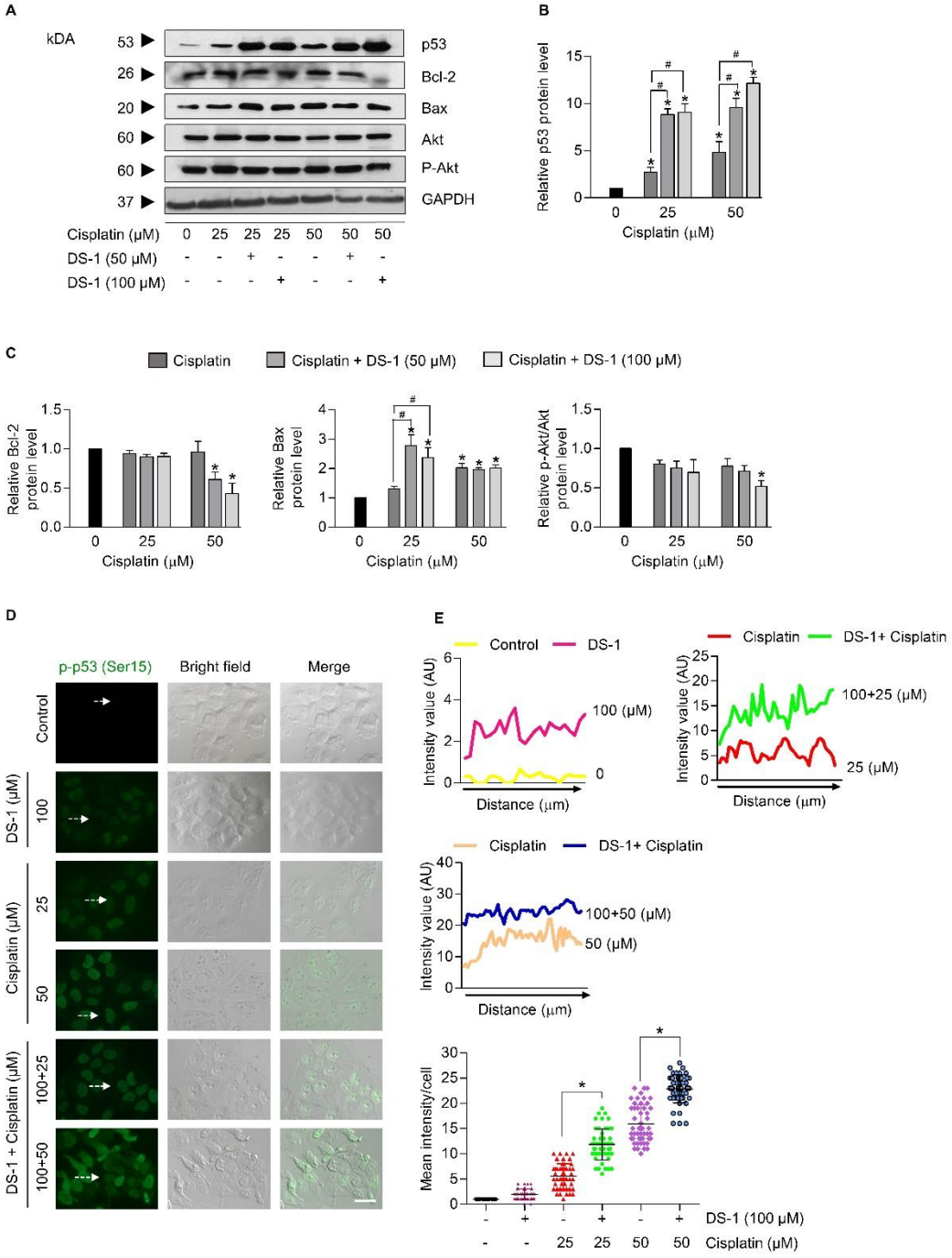


Figure 4.7 The effect of DS-1 combined with cisplatin on apoptosis-related protein. (A) After 24 hours of DS-1 (50-100 μM) treatment, cells were exposed to cisplatin (25 and 50 μM) for 24 hours. The protein expression was detected by western blot assay. (B-C) The intensity was qualified by densitometry. The relative protein level was calculated.

The membrane blots were re-probed with GAPDH to confirm equal loading of the sample. The data is presented mean ± SD (n=3) *p<0.05 versus control group, #p<0.05 versus cisplatin-treated cells. (D) The representative images show phosphorylation of p53 (green) investigated by Immunofluorescence. Scale bar represents 25 μm. (E) The fluorescence intensity of p-p53 was analyzed using ImageJ software. The diagram illustrated signal intensity along the dotted line. The mean intensity/cell from at least 50 cells was calculated relative to control group. The data is presented as mean ± SD (n=3) *p<0.05 versus cisplatin-treated cells.

5. DS-1 interacts with the binding pocket of MDM2 protein

A study reported that intrusion on the interaction of MDM2 and p53 complex has been revealed as a way to prevent p53 degradation (Klein and Vassilev, 2004), giving a thought that DS-1 may inhibit ubiquitination of p53 by targeting MDM2 protein. To predict the binding mode of DS-I into MDM2, molecular docking was performed using CHARMm-based DOCKER (CDOCKER) program in the Accelrys Discovery Studio 2.5Accelrys Inc. The 3D structure of DS-1 was built and optimized at the HF/6-31G(d) level of theory using the Gaussian09 software while the X-ray structure of MDM2-p53 complex was retrieved from Protein Data Bank (PDB ID: 1YCR). The results of the study showed that DS-1 could bind with the binding pocket of MDM2 (Figure 4.8). Accordingly, Molecular Dynamic (MD) simulation was also carried out to define its structural analyses and binding affinity. As observed, DS-1 with MDM2 showed the binding affinity. DS-1 was found to contact with the binding pocket of MDM2 by carbon-hydrogen bond at Lys27, π-Alkyl interactions at Ile37 and Leu30, and Van der Waals interactions at Ile75, Val51, Val69, Phe67, Met38, Tyr43, Gly34, Phe31, and Lys27 (Figure 4.8C). Hence, this molecular docking study provides a prediction of the DS-1 ability to reside with numerous active pockets of MDM2. Moreover, MD simulation also showed the structural stability and interaction mechanism of the protein-ligand complex, which verified that the Root-mean-square deviation (RMSD) plot of MDM2-DS-1 complex reached a plateau and continuously stable after 50 ns (Figure 4.8D-G). Moreover, hydrogen bond was analyzed by AMBER, which was computed with 100 ns simulation. The length and angle cutoffs of hydrogen bonds used was ≤ 3.5 Å and ≥ 120o, respectively. DS-1 bound with MDM2 pocket with approximately ~2 ± 1 hydrogen

bonds. The number of atom contact in intermolecular interaction of MDM2-DS-1 complex was also analyzed in time course simulation. The results showed that ~21 ± 7 intermolecular atom contacts were found in the simulation which indicated our simulation model is stable.

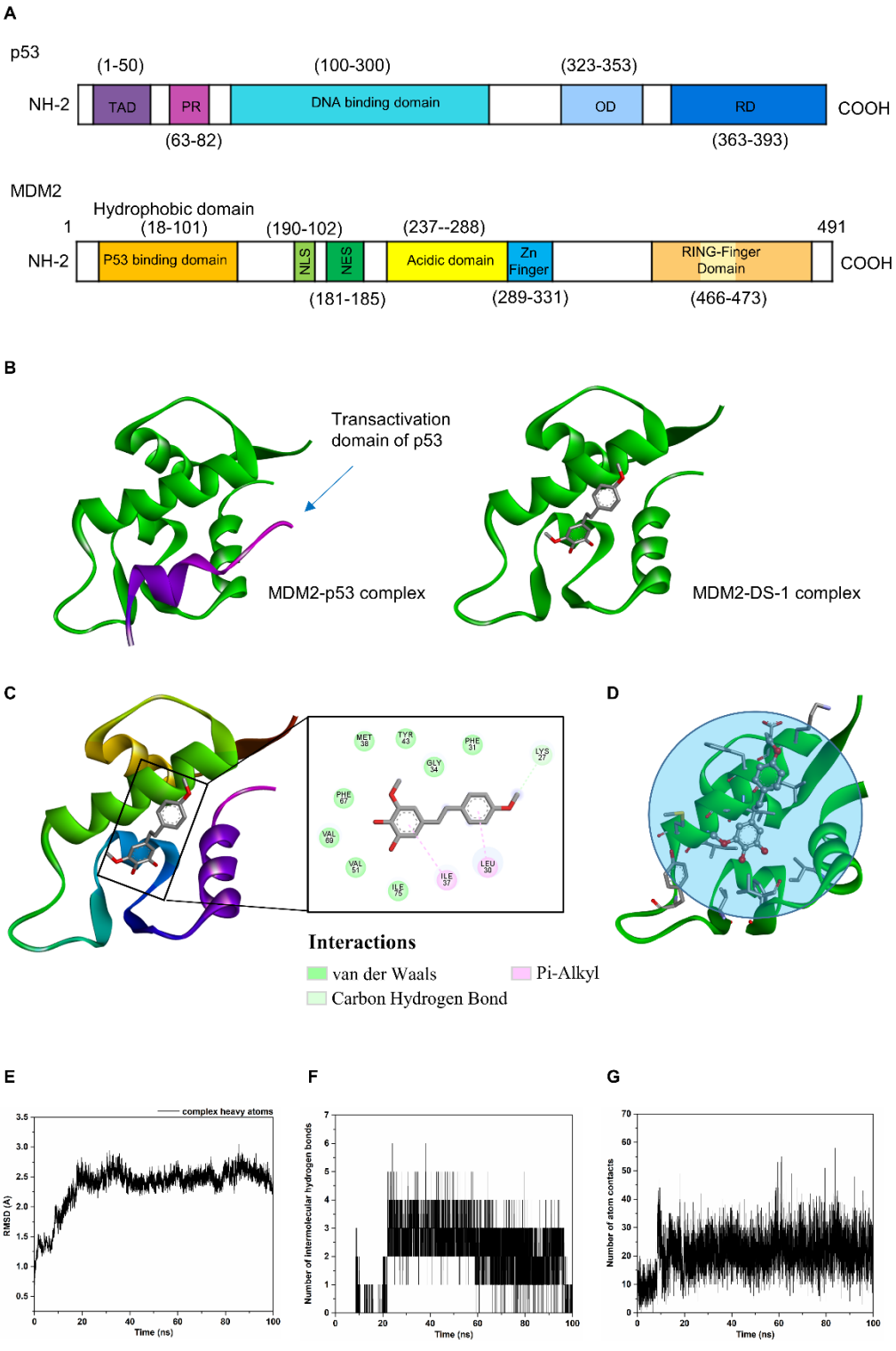


Figure 4.8 Molecular docking model of DS-1 and MDM2. (A) Domain structure of p53 (upper) and MDM2 (lower). p53 possesses 393 amino acids: N-terminal transactivation domains (TAD1 and TAD2, residues ~1–40 and ~ 40–50, respectively), the proline-rich domain (PR, ~ 64–82), the central DNA-binding domain (DBD, residues ~ 100–300), the oligomerization domain (OD, residues ~ 323–353), and the unstructured C-terminal domain (CTD, residues ~ 363–393). MDM2 is a 491 amino acid, consists of several domains, N-terminal p53 interaction domain (Hydrophobic domain, residues ~ 18-101), Nuclear localization signal (NLS, residues ~ 18-101), Nuclear export signal (NSE, residues ~ 181-185), central acidic domain (residues ~ 237-285), zinc finger domain (residues ~ 289-331), and Ring-finger domain (residues ~ 466-473). (B) Comparison of the 2D structure complex of MDM2 (green), p53 (magenta), and DS-1. (C) The binding site of DS-1 on MDM2 protein. The interaction between them is represented in different colors (carbon-hydrogen bond interaction at Lys27, π-Alkyl interactions at Ile37 and Leu30, and Van der Waals interactions at Ile75, Val51, Val69, Phe67, Met38, Tyr43, Gly34, Phe31, and Lys27). (D) An illustration of The number of intermolecular atom contacts between DS-1 and MDM2. (E) Structure stability of DS-1-conjugated MDM2 was evaluated by Root-mean-square deviation (RMSD) analysis. (F) The number of intermolecular hydrogen bonds between protein and ligand was computed for simulation time. (G) The number of intermolecular atom contacts between protein and ligand (calculated from 4 Å sphere of ligand) was determined for simulation time.

6. Effect of DS-1 on patient-derived primary lung cancer cells

We next investigated the DS-1 sensitizing effect on primary lung cancer cells. The patient-derived primary lung cancer cells were generally characterized. The cells were treated with various concentrations of DS-1 (0-150 μM) and/or cisplatin (0-50 μM) for 24 hours. Cell viability was measured by MTT assay. The result indicated that DS-1 or cisplatin alone decreased the viable cells significantly. Importantly, the combination showed that DS-1 could enhance the cells to cisplatin-induced cytotoxicity (Figure 4.9A, B). To confirm, apoptosis and necrosis were evaluated by co-staining of Hoechst 33342 and Propidium Iodide (PI). After the 24 hours incubation of DS-1, cisplatin, or combination, cells were incubated with Hoechst 33342 and PI and visualized under

fluorescent microscopy. The results show the consistent data inducting that DS-1 could sensitize cisplatin-induced apoptosis and the combination treatment caused the highest apoptosis response compared to cisplatin or DS-1 treatment (Figure. 4.9C, D).

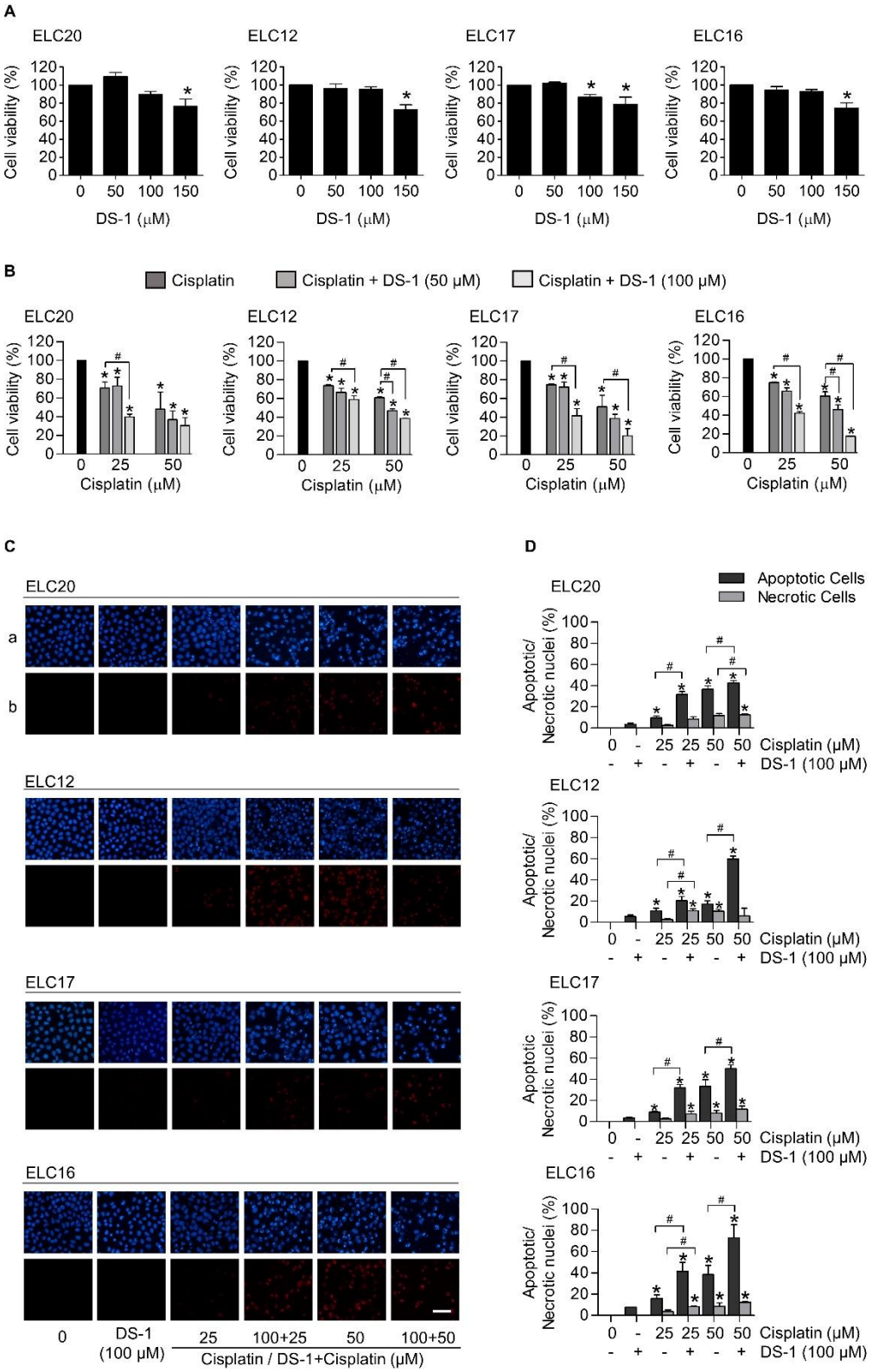

A
ELC20
ELC12
ELC17
ELC16
Cell viability (%)
DS-1 (μM)
B
Cisplatin
Cisplatin + DS-1 (50 μM)
Cisplatin + DS-1 (100 μM)
Cisplatin (μM)
C
a
b
0
DS-1 (100 μM)
25
100+25
50
100+50
Cisplatin / DS-1+Cisplatin (μM)
D
Apoptotic Cells
Necrotic Cells
Apoptotic/ Necrotic nuclei (%)
Cisplatin (μM)
DS-1 (100 μM)

Figure 4.9 DS-1 sensitizes patient-derived primary lung cancer cells. The cells were treated with DS-1 (0 -150 μM) for 24 hours or combined with cisplatin (25 and 50 μM). (A-B) Living cells were measured by MTT assay. The percentage of cell viability was calculated and compared to untreated cells. (C) The apoptotic and necrotic cell morphology stained by (a) Hoechst 33342 and (b) propidium iodide were visualized under fluorescent microscopy. Scale bar represents 50 μm. (D) The percentage of apoptotic and necrotic nuclei was calculated relative to control group. The data is presented mean ± SD (n=3) *p<0.05 versus control group, #p<0.05 versus cisplatin-treated cells.

7. Effect of DS-1 on proliferation of H460 and H292 cells

A previous study showed that multiple cascades control the proliferation and metastasis of cancer cells in a different way. However, the unregulated proliferation of cancer cells could facilitate metastasis progression (Endo et al., 2019). Therefore, we first looked at cell proliferation of H460 and H292 cells in the presence of DS-1. Briefly, cells were incubated the cells in 96-well plate with various doses of DS-1 (0-100 μM) for 0, 24, and 48 h. The increasing number of cells for each time point was measured by MTT assay. The results showed that relative cell proliferation at 24 h was fairly attenuated at DS-1 treatment, while at 48 h of cell incubation was gradually suppressed on both the lung cancer cells (Figure 4.10).

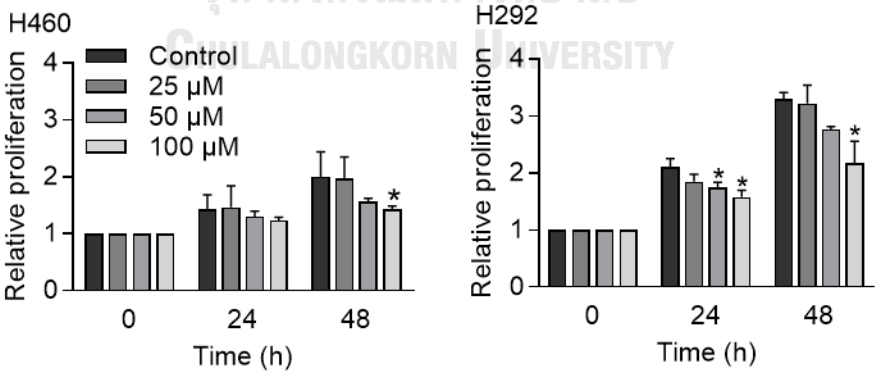


Figure 4.10 DS-1 inhibits cell proliferation of H460 cells. Cells were treated with DS-1 (0-100 μM). Cell viability was analyzed using MTT assay at 0, 24, and 48 h. Relative proliferation was calculated by comparing with the control group. The data is presented at mean ± SD (n=3), *p<0.05 versus control group.

8. DS-1 inhibited migration and invasion of H460 and H292 cells

a. Effect of DS-1 on cell morphology

To examine the effect of DS-1 on cell behavior, we further assessed the EMT-related morphological change. Normally, epithelial cell morphology is characterized as round, elongated, or spherical. On development of cancer migration and invasion, cells tend to exhibit morphological transformation of epithelial cells to a spindle mesenchymal cell shape which is characterized by the form of filopodia or plasma membrane protrusions (Elaskalani et al. 2017). Cells were treated with DS-1 for 24 h. After that, the phalloidin-rhodamine staining assay was conducted. As shown in Figure 4.11, relative filopodia per cells of DS-1 treatment group (100 μM) reduced the formation of filopodia of both cells suggesting that DS-1 might exhibit prevention on mesenchymal-like morphology.

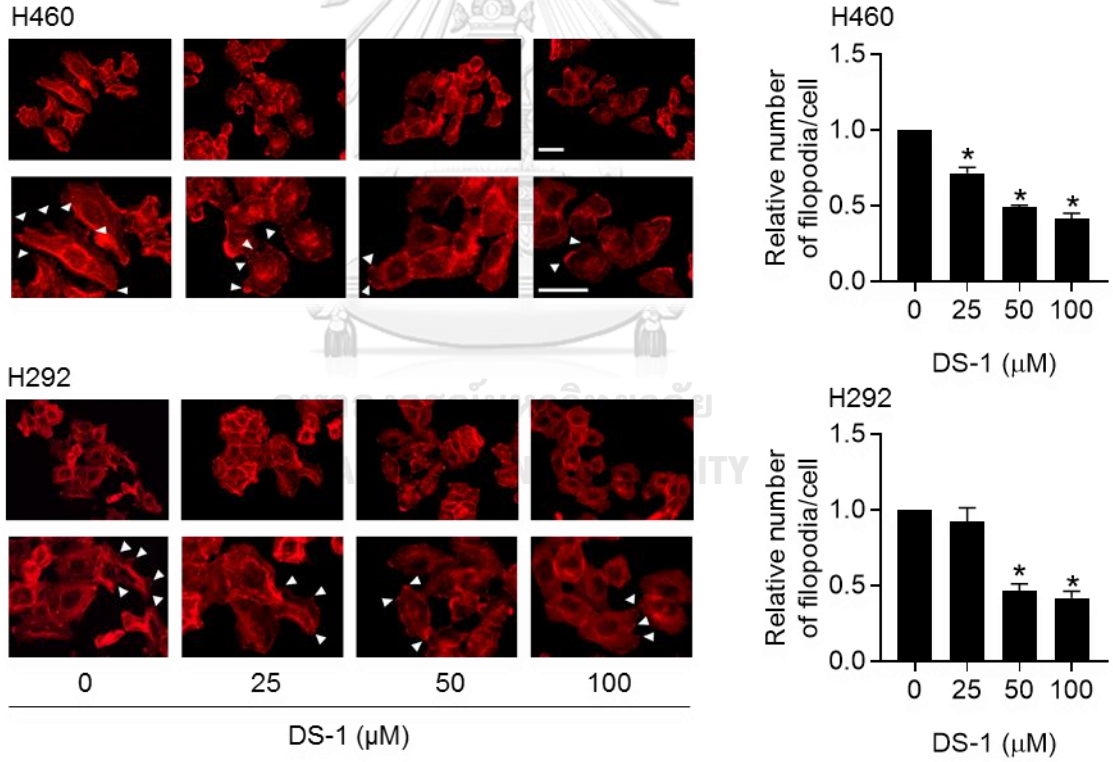


Figure 4.11. Effect of DS-1 on filopodia formation of H460 and H292 cells. After treatment with nontoxic concentrations of DS-1, cells were stained with phalloidin-rhodamine and examined using fluorescent microscopy. Filopodia characteristics are indicated by arrowheads. Scale bar represents 100 μm. Relative number of filopodia

per cell compared with the control. Data are shown as the mean ± SD (n=3). *p<0.05 versus non-treated control.

b. Effect of DS-1 on cell migration and invasion

Next, we tested DS-1 on regulating migration and invasion of H460 and H292 cells. Wound healing assay and transwell invasion assay were performed. Cell movement was captured under phase bright microscopy at 0, 24, and 48 h. The results showed that DS-1 prevented cell migration of H460 and H292 cells in a dose-dependent manner. Similarly, transwell invasion results showed a reduced number of H460 and H292 cell invasion at 25, 50, and 100 μM of DS-1 compared to control group (Figure 4.12). These evidenced that DS-1 potentially inhibited NSCLC migration and invasion.

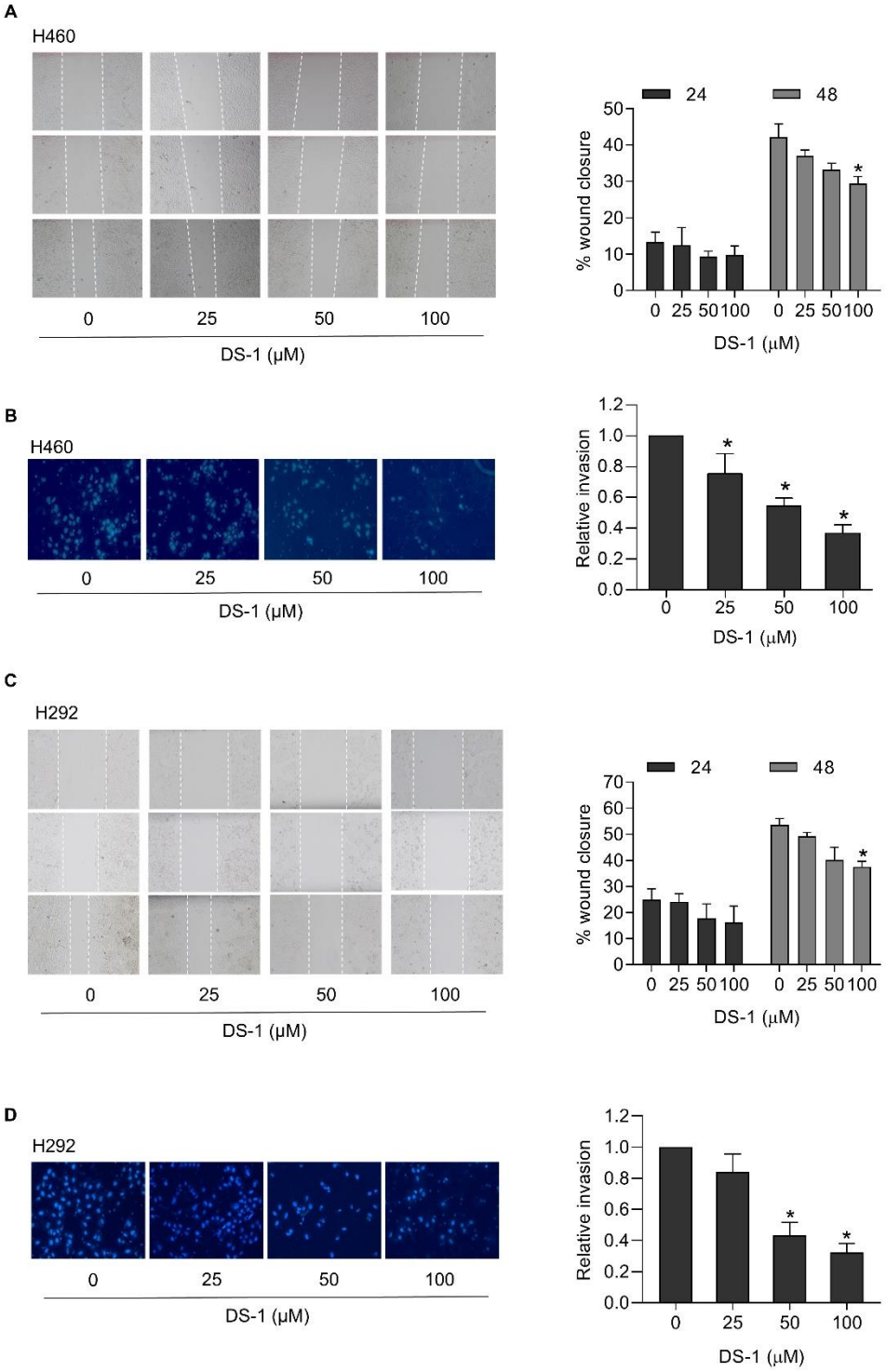


Figure 4.12 DS-1 inhibits migration and invasion of H460 cells. Cells were pretreated with DS-1 (0-100 μM) for 24 h. Cell migration was evaluated by wound healing assay. The percentage of wound closure migration level at 24 and 48 h was determined by comparing it with the wound area at 0 h. Cell invasion was investigated by transwell

migration assay (pore size: 8 μm). The treated cells with DS-1 were inserted onto the upper chamber containing the set of matrigel and incubated for 48 h. The invaded cells were stained with Hoechst 33342 and visualized by fluorescence microscopy (20X magnification). The relative invasion was calculated as total invaded cells of the treatment group divided by total invaded cells of control group. The data is presented as mean ± SD (n=3). *$p<0.05$ versus control group.

c. Effect of DS-1 on Anchorage-independent growth

Cancer metastases are related to aggressiveness in which cells enable to survive in detached condition (Buchheit et al., 2014). As they were resistant to anoikis, the invaded cells get into systemic circulation form metastatic colonies and reach distant tissue (Nguyen et al., 2009). It has been clarified that such interaction of tumor cells and platelets mediate the survival cascade and elude tumor cells from shear stress (Yu et al., 2014). Thus, we further questioned whether DS-1 could inhibit H460 cell growth in the anchorage-independent model. Cells were pre-treated with DS-1 (0-100 μM) for 24 h, then subjected to soft agar anchorage-independent growth assay as illustrated in materials and methods. Our result exhibited that DS-1 significantly reduced the number as well as the size of colonies (Figure 4.13).

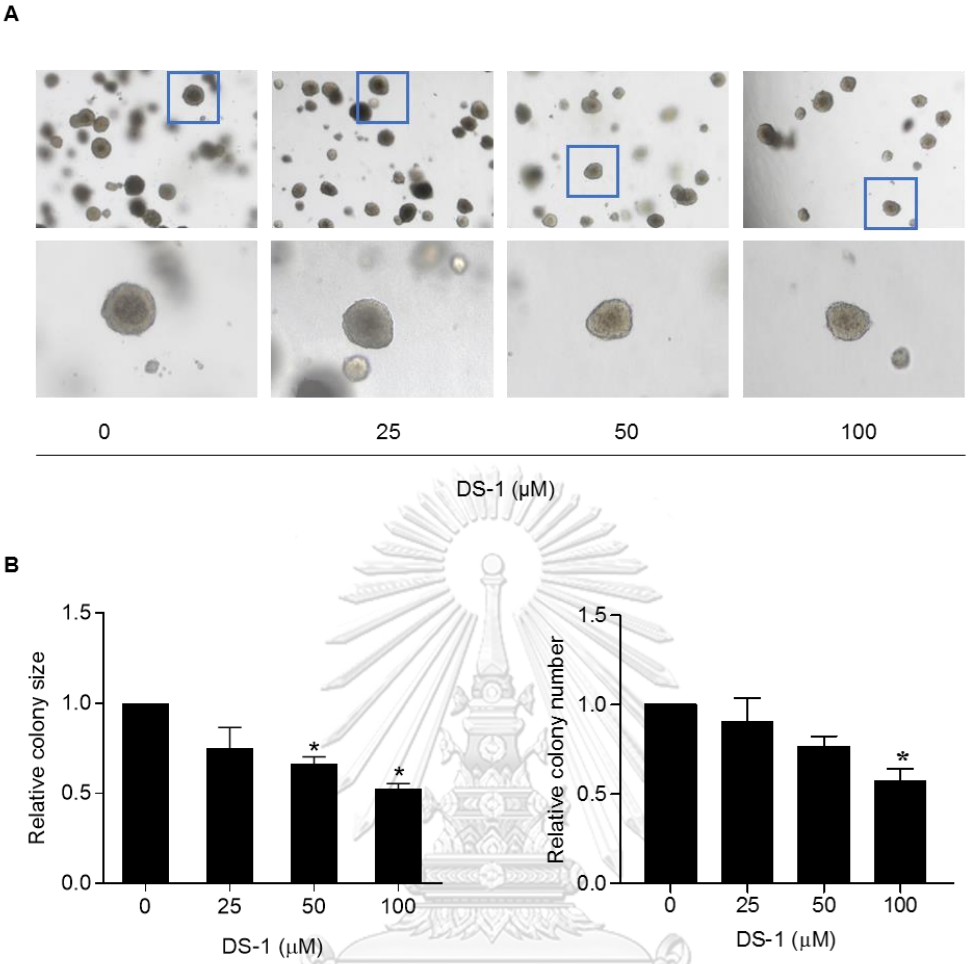


Figure 4.13 DS-1 inhibits cell growth of H460 cells in the anchorage-independent condition. (A-B) Cells were pretreated with DS-1 (0-100 μM) for 24 hours and subjected to anchorage-independent assay. After 14 days of incubation, cells were captured under phase bright 10X (upper) 20X (lower) magnification. The results were represented in relative number and size of the colonies. The data is present mean ± SD (n=3). *p<0.05 versus control group.

9. DS-1 suppressed EMT properties

Cell metastasis evolvement is initiated by which the transformation of cells from epithelial cells with their cell-cell adhesion and cell polarity to mesenchymal cells with migratory and invasion ability (Mittal, 2016). Following our findings, DS-1 exhibited an inhibitory effect on NSCLC cell migration and invasion. We suspected that DS-1 might regulate EMT properties. Western blot assay was performed to detect EMT-related protein expression. As shown in Figure 4.14A DS-1 downregulated the

expression of mesenchymal markers such as N-cadherin, snail, and slug but increased epithelial marker such as E-cadherin. These indicated that DS-1 inhibits the initiation of cell metastasis by regulating the cadherin switching. Moreover, integrin expression has been also associated with the EMT progression (Bai et al., 2010; Larzabal et al., 2014). We next investigated the expression of integrin including α5, β1, and β3. After 24 h of the DS-1 treatment, integrin β1 expression was significantly decreased at 100 μM concentration of DS-1. However, the moderate change was shown on integrin α5 and β3 expression in response to DS-1 exposure (Figure 4.14B). Furthermore, total FAK protein and p-FAK (Try397) were also determined. We found that DS-1 reduced the active form of FAK compare to the control group. As FAK/p-FAK is one of the downstream pathways of integrin that contributes to cancer metastasis, these results indicated the mechanism of action of DS-1 in which might diminish integrin β1-mediated FAK activation to cell migration.

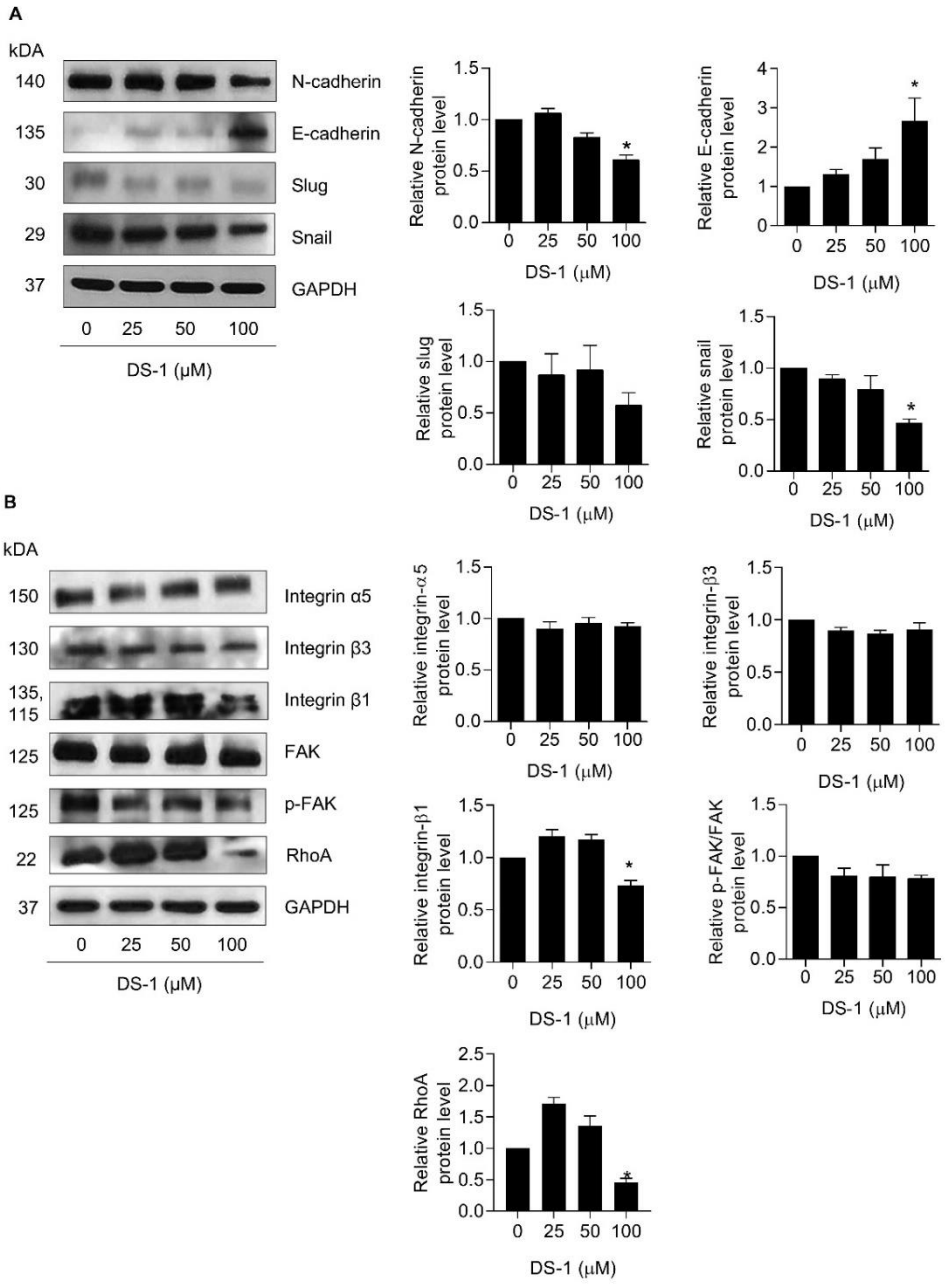


Figure 4.14 Effect of DS-1 on EMT regulatory proteins in H460 cells. (A-J) Cells were treated with 25, 50, 100 μM of DS-1. The protein expression was detected by Western blot and quantified using ImageJ software. Relative protein level was calculated and compared with untreated group. The data is present mean ± SD (n=3). *p<0.05 versus control group.

CHAPTER V

DISCUSSION AND CONCLUSION

The gene encoding p53 (TP53) in early year of the discovery was initially recognized as an oncogene but after ten years it was correctly recognized as a tumor suppressor, which gain much attention in p53 research (Levine and Oren, 2009). p53 protein has feature function not only as a tumor suppressor but also as transcriptional factor (Zilfou and Lowe, 2009). The dimer form between DNA-binding domine (DBD) of p53 (residues 90-300) (Figure 2.3) and response element (RE) of DNA is recognized to initiate p53 acting on transcriptional alteration that regulates several cellular responses including cell cycle arrest, apoptosis cell death, senescence and DNA repair system (Wang et al., 2010). The fundamental of p53 activation is mediated by multiple cellular stress or a genotoxic exposure on the cells which causes the translocation of p53 from the cytoplasm to nucleus and stabilization of p53, turns to its active form (Liang and Clarke, 2001; Sansome et al., 2001; Kumari et al., 2014).

These functions maintain genome stability and preventing survival and propagation of harmful cancer cells (Laptenko and Prives, 2006). Moreover, the employment of p53 activation for cancer has shown a positive effect on cell sensitivity to chemotherapy (Trepel et al., 1997; Hernandez-Valencia et al., 2018). However, it has been classified as the p53 status in many cancer types as inactivated or loss of function that is related to cancer progression (Soussi and Wiman, 2015). Moreover, tumor protein p53 (TP53) mutation, deletion in TP53 copy number, and aberrant activation of MDM2 protein have been defined as the main regulators of the perturbation of p53 activation (Jackson and Lozano, 2013; Kastenhuber and Lowe, 2017), 2013; Liu et al., 2016; Kastenhuber and Lowe, 2017).

MDM2 continuously depletes p53 through its ubiquitin E3 ligase activity. The ubiquitination of p53 is an important step for the degradation of the protein via proteasome function. An approach to inhibit p53 degradation by interfering MDM2-p53 interaction has garnered increasing attention recently as p53 suppression or inactivation is well-defined to potentiate oncogenic potentials. As the concept of targeting MDM2 and restoring normal p53 function has offered traction for novel anti-

cancer therapeutics, the present study demonstrates how a bibenzyl-phenanthrene, isolated from *Dendrobium Signatum*, interacts with MDM2 and inhibits MDM2-dependent p53 degradation in human lung cancer cells. We also found that the increase of p53 level in lung cancer cells could enhance cancer cell response to cisplatin in lung cancer cells and pleural effusion-derived primary lung cancer cells (Figure. 4.9).

Considering MDM2-p53 interaction, the functional domain of MDM2 provides the fundamental principle for the oncogene properties (Figure 4.8). The N-terminal p53-binding domain is required for MDM2-p53 interaction. The acidic domain and an adjacent zinc finger connect the interaction of MDM2 with many ribosomal proteins (RPs). And the RING finger domain is where the E3 ubiquitin ligase activity of MDM2 and mediates the ubiquitin-conjugating E2 enzyme to promote the target protein ubiquitination (Canon et al., 2015). In association with p53 inactivation, MDM2-p53 interaction causes the suppression of p53 transcriptional function via exporting of p53 protein into the cytoplasm or promoting the degradation (Hayashi et al., 2006; Oliner et al., 2016). MDM2 blocks the N-terminal trans-activation domain of p53 and facilitates p53 degradation through the ubiquitin-proteasome pathway (Liang and Clarke, 2001; Asher et al., 2002). Therefore, the half-life of p53 could be prolonged by protecting p53 from the ubiquitination process (Pant and Lozano, 2014). Many studies have highlighted the impact of the high level of MDM2 in cancers.

Studies have demonstrated that cellular level of MDM2 protein was responsible for the inactivation of p53 and the high level of MDM2 associated with tumor progression in sarcoma (Turc-Carel et al., 1986; Meltzer et al., 1991; Oliner et al., 2016). Recently, about forty types of malignancies, including solid tumors, sarcomas, and leukemia were reported to have elevated MDM2 expression that associate with poor clinical prognosis and decreased response to therapeutic agents (Rayburn et al., 2005; Oliner et al., 2016). Specifically, a study in NSCLC patients found that 21% of 116 patients had overexpressed of MDM2. The overexpression of MDM2 protein mediated by MDM2 gene amplification has been linked with metastasis, radioresistance, as well as chemoresistance (Rayburn et al., 2005). Also, it suppresses the accumulation and

activation of p53 apoptotic pathway (Zhao et al., 2014). These findings have supported the concept that targeting MDM2 offers a promising means for cancer treatment.

In the present study, we have found that non-toxic doses of DS-1 increased p53 protein by preventing p53-ubiquitination. Consequently, DS-1 caused the induction of apoptosis-related proteins including Bax and suppression of Bcl2, and Akt/p-Akt (Figure. 4.6A,B), and apoptosis induction was found at high concentration of DS-1 (150 μM) (Figure. 4.2). Interestingly, DS-1 at non-toxic concentration (100 μM) barely increases the activated p53 in comparison to cisplatin (Figure. 4.7D,E). It is possible that the low concentrations of DS-1 accumulated p53 protein but did not affect the activation of p53. Since aberrant activation of MDM2 protein was frequently found in cancer cells (Higashiyama et al., 1997; Wade et al., 2013). Studies targeting the interaction of p53 and MDM2 have shown a beneficial effect on the reactivation of p53 protein that mediates apoptosis induction in cancer cells (Moll and Petrenko, 2003; Yue et al., 2017; Moulder et al., 2018). Mechanistically, the Hydrophobic domain (residues 18-101) of MDM2 binds p53 tumor-suppressor at N-terminal domain of p53 (Phe19, Leu22, Trp23, and Leu26) (Shangary and Wang, 2008) which have been found as the basis for the therapeutic design to develop an inhibitor of MDM2-p53 interaction. Moreover, It has been previously described that compounds that act as MDM2 inhibitor and interact with the hydrophobic domain of MDM2 like Nutlin, the first MDM2 inhibitor to enter phase I clinical trials showed the interaction with MDM2 at Trp23, Leu26 and Phe19 could interfere its interaction with p53 (Zhao et al., 2015). Interestingly, the result of the present study showed that DS-1 interacts with the binding pocket of MDM2 at Lys27, Leu30, Ile37, Ile75, Val51, Val69, Phe67, Met38, Tyr43, Gly34, and Phe31 (Figure 4.8B,C). In turn, the presence of DS-1 probably inhibits the interaction of p53 and MDM2 leading to stabilize and restore the intracellular p53 protein. In line with this study, several compounds targeting activity MDM2 also induced p53-mediated apoptosis in cancer cells (Sun et al., 2008; Ohnstad et al., 2011; Yu et al., 2019). A study in Xenograft models of non-small lung cancer cells showed that the inhibition of MDM2 and p53 interaction could enhance apoptotic response and cell cycle arrest (Hai et al., 2015). Additionally, of clinical relevance, drugs targeting MDM2 have been extensively studied in many cancer types and some of which are being tested in clinical trials such

as MK-8242, SAR405838, RO5045337, RO5503781, CGM097, and DS-3032b (Zhao et al., 2015). Notably, p53 driven by MDM2 plays a crucial role in apoptosis by regulating the activation of p53.

Moreover, chemotherapy including cisplatin remains the first treatment option because it has shown the substantial benefits for NSCLC patients (Cosaert and Quoix, 2002; Horita et al., 2017). Albeit initial treatment for lung cancer is responding well to cisplatin, the long-term treatment proves to be ineffective and unsatisfactory due to the development of drug resistance (Galluzzi et al., 2012; Barr et al., 2013). In one study, cisplatin-caused DNA damage triggers the elevating of MDM2-p53 loop in tumor cells. Simultaneously, this has been linked to the ability of a protein called Mushashi-2 (MSI-2) governing the E3 ligase activity of MDM2 to accelerate p53 degradation (Hou et al., 2019). Moreover, a study confirmed that MDM2–p53 autoregulatory loop contributes to cell resistance, that upregulation of MDM2 expression and non-phosphorylated p53 were exhibited in cisplatin-resistant tumor cells (Sheng et al., 2017). Therefore, previous studies have reported that MDM2 inhibitor combined with cisplatin was expected to be a promising strategy to enhance treatment outcomes (Ambrosini et al., 2007; Canon et al., 2015; Deben et al., 2015). Moreover, in cultured cells of non-small cell lung cancer, treatment of an MDM2 inhibitor, and cisplatin synergistically induced apoptosis cell death compared to Nutlins-3a or cisplatin alone (Deben et al., 2015). Small molecules that inhibit MDM2 including Nutlin-3a, induced p53 activation and showed to be effective in combination with known chemotherapy (Ohnstad et al., 2011). In a xenograft model of NSCLC, mice subcutaneously injected with NCI-H460 cells, AMG 232 (MDM2 inhibitor) combined with cisplatin in mice showed synergistic antitumor activity, at the same time the activity of p53 was increased (Canon et al., 2015). Consistent with our study, we also reported that the synergistic effect of DS-1-combined with cisplatin induces programmed cell death by apoptosis via enhancing the activation of p53 and its downstream apoptotic pathway. Collectively, we provided feature information of a new compound together with the mode of action of DS-1 by which enhances the expression of p53 protein gives a chance to cisplatin upregulate the phosphorylation of p53 and thus increased the cytotoxic effect and apoptotic cells in NSCLC.

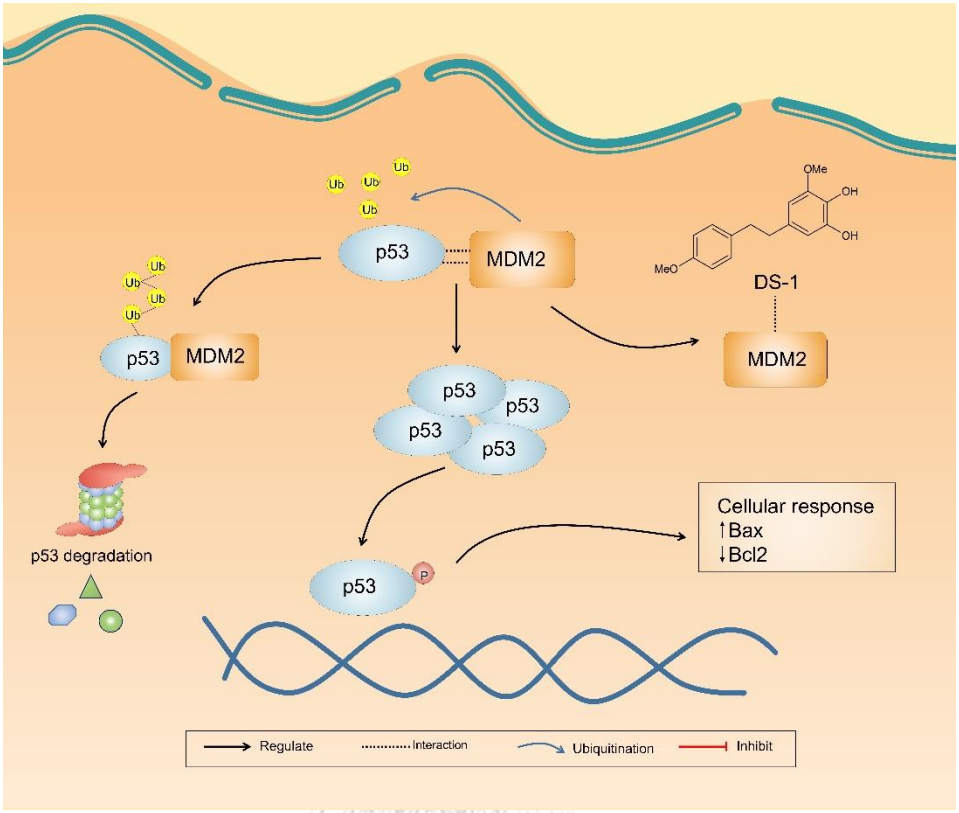


Figure 5.1 Systematic mechanism of action of DS-1. The correlation between p53 and MDM2 is an autoregulatory feedback loop. p53 promotes the expression of MDM2. Successively, MDM2 inhibits p53 activity and mediates p53 degradation. The mechanisms of DS-1 on NSCLC; DS-1 interacts with MDM2 resulting in accumulating p53 intracellular protein prevent p53 ubiquitination, as observer DS-1 reduces ubiquitin-p53 complex.

For cancer metastasis, despite EMT phenomenon in such cellular biology taking place in many processes during the development of living cells, aberration EMT has been associated with metastasis and resistance evolvement in many types of cancer cells (Savagner 2010; Xiao and He 2010). A study has conclusively demonstrated EMT as cancer hallmark is purposed for the initiation of cancer cell migration and invasion, which is related to the risk of lung cancer in treatment failure (Messaritakis et al., 2017). Suppressing molecular mechanisms governing EMT has been considered an effective target for anticancer strategies in many preclinical and early-clinical study (Yang et al., 2019). Certain integrins are emerging as pivotal factors of cancer cell

refraining from apoptosis during metastasis. The integrin function to respond to the extracellular signal transduction and generate the coordinate signaling pathway activation of focal adhesion kinase (FAK) (Alanko et al., 2015). FAK signaling pathway activation supporting cell proliferation and survival has been reported also to regulate the transcriptional factor of EMT (Zouq et al., 2009; Sulzmaier et al., 2014). Therefore, EMT-regulating property is a considerable strategy to impede cancer metastasis.

Previously, studies interested in the active compounds of Dendrobium species have revealed the anti-metastasis effect in Non-small cell lung cancer cells (Chaotham et al. 2014; Charoenrungruang et al. 2014). In the present study, an expanded investigation of another isolate of Dendrobium signatum, 3,4-dihydroxy-5,4′-dimethoxybibenzyl (DS-1), aimed to determine the anti-metastasis potency in NSCLC cells. For cancer metastasis, it has been revealed that most cancer cells could generate survival signals once in contact with the basement membrane (Nguyen et al. 2009). Whereas, in the condition of the loss of cell-cell adhesion toward cell dissemination, invaded cancer cells defense to survive in circulation, termed anoikis resistance, and thus succor to reach the secondary distance(Simpson et al. 2008). Interestingly, we found that DS-1 could inhibit cell growth in the anchorage-independent condition (Figure 4.13). Moreover, the occurrence of EMT, typically characterized by reduction of epithelial markers such as E-cadherin, and enhancement of mesenchymal markers including N-cadherin is controlled by activation such transcriptional factors including snail, and slug (Dongre and Weinberg 2019). Studies demonstrated the correlation between snail protein and N-cadherin, where the induction of slug/snail in lung cancer cells caused increasing of N-cadherin as well as cancer cell movement and invasion (Wang et al., 2013; Yang et al., 2017). Moreover, as has been studied, snail showed the ability to inhibit the expression of E-cadherin by blocking the binding of its promoter (CDH1) (Herranz et al. 2008), which plays important role in chemoresistance as well as radioresistance (Kurrey et al. 2009). Thereupon, small molecules that showed inhibition of these proteins could prevent metastasis advancement (Otsuki et al., 2018). In agreement with the present study, DS-1 caused migration and invasion prevention of lung cancer cells (Figure 4.12) together with accentuation of mesenchymal properties (N-cadherin, snail, and slug) and enhancement of E-cadherin (Wheelock et al. 2008).

Importantly, the protein expression of integrin β1 and p-FAK was also reduced in response to DS-1 treatment (Figure 4.14). This might possible that DS-1 could inhibit migration and invasion through suppression on integrin β1-mediated FAK pathway.

In conclusion, our study revealed the potential effect of DS-1 on suppression of integrin and activation of downstream pathway of FAK, which showed a promising purpose to block EMT step cancer cells toward metastasis. Taken together, our study provided the basic data to support the further investigation of DS-1 as anticancer therapy.

REFERENCES

Alanko J, Mai A, Jacquemet G, Schauer K, Kaukonen R, Saari M, Goud B and Ivaska J (2015). "Integrin endosomal signalling suppresses anoikis." Nature Cell Biology 17(11): 1412-1421.

Amarasena I U, Walters J A E, Wood-Baker R and Fong K (2008). "Platinum versus non-platinum chemotherapy regimens for small cell lung cancer." Cochrane Database of Systematic Reviews(4).

Ambrosini G, Sambol E B, Carvajal D, Vassilev L T, Singer S and Schwartz G K (2007). "Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1." Oncogene 26(24): 3473-3481.

Araki K, Shimura T, Suzuki H, Tsutsumi S, Wada W, Yajima T, Kobayahi T, Kubo N and Kuwano H (2011). "E/N-cadherin switch mediates cancer progression via TGF-β-induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma." British Journal of Cancer 105(12): 1885-1893.

Asher G, Lotem J, Sachs L, Kahana C and Shaul Y (2002). "Mdm-2 and ubiquitin-independent p53 proteasomal degradation regulated by NQO1." Proc Natl Acad Sci U S A 99(20): 13125-13130.

Asnaghi L, Vass W C, Quadri R, Day P M, Qian X, Braverman R, Papageorge A G and Lowy D R (2010). "E-cadherin negatively regulates neoplastic growth in non-small cell lung cancer: role of Rho GTPases." Oncogene 29(19): 2760-2771.

Aubrey B J, Kelly G L, Janic A, Herold M J and Strasser A (2018). "How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression?" Cell Death & Differentiation 25(1): 104-113.

Aung T N, Qu Z, Kortschak R D and Adelson D L (2017). "Understanding the Effectiveness of Natural Compound Mixtures in Cancer through Their Molecular Mode of Action." Int J Mol Sci 18(3).

Bacac M and Stamenkovic I (2008). "Metastatic cancer cell." Annu Rev Pathol 3: 221-247.

Bai J, Zhang J, Wu J, Shen L, Zeng J, Ding J, Wu Y, Gong Z, Li A, Xu S, Zhou J and Li G (2010). "JWA regulates melanoma metastasis by integrin alphaVbeta3 signaling." Oncogene 29(8): 1227-1237.

Balogh E P, Dresler C, Fleury M E, Gritz E R, Kean T J, Myers M L, Nass S J, Nevidjon B, Toll B A, Warren G W and Herbst R S (2014). "Reducing tobacco-related cancer incidence and mortality: summary of an institute of medicine workshop." Oncologist 19(1): 21-31.

Barr M P, Gray S G, Hoffmann A C, Hilger R A, Thomale J, O'Flaherty J D, Fennell D A, Richard D, O'Leary J J and O'Byrne K J (2013). "Generation and characterisation of cisplatin-resistant non-small cell lung cancer cell lines displaying a stem-like signature." PloS one 8(1): e54193-e54193.

Barrallo-Gimeno A and Nieto M A (2005). "The Snail genes as inducers of cell movement and survival: implications in development and cancer." Development 132(14): 3151-3161.

Baxevanos P and Mountzios G (2018). "Novel chemotherapy regimens for advanced lung cancer: have we reached a plateau?" Ann Transl Med 6(8): 139.

Bhummaphan, N. and P. Chanvorachote (2015). "Gigantol Suppresses Cancer Stem Cell-Like Phenotypes in Lung Cancer Cells." Evid Based Complement Alternat Med 2015: 836564.

Bossi G and Sacchi A (2007). "Restoration of wild-type p53 function in human cancer: relevance for tumor therapy." Head Neck 29(3): 272-284.

Bray F, Ferlay J, Soerjomataram I, Siegel R L, Torre L A and Jemal A (2018). "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." CA Cancer J Clin 68(6): 394-424.

Brooks C L and Gu W (2010). "New insights into p53 activation." Cell Res 20(6): 614-621.

Buchheit C L, Weigel K J and Schafer Z T (2014). "Cancer cell survival during detachment from the ECM: multiple barriers to tumour progression." Nature Reviews Cancer 14(9): 632-641.

Cakova V, Bonte F and Lobstein A (2017). "Dendrobium: Sources of Active Ingredients to Treat Age-Related Pathologies." Aging Dis 8(6): 827-849.

Campbell B C, Chan K L and Kim J H (2012). "Chemosensitization as a means to augment commercial antifungal agents." Front Microbiol 3: 79.

Canon J, Osgood T, Olson S H, Saiki A Y, Robertson R, Yu D, Eksterowicz J, Ye Q, Jin L, Chen A, Zhou J, Cordover D, Kaufman S, Kendall R, Oliner J D, Coxon A and

Radinsky R (2015). "The MDM2 Inhibitor AMG 232 Demonstrates Robust Antitumor Efficacy and Potentiates the Activity of p53-Inducing Cytotoxic Agents." Mol Cancer Ther 14(3): 649-658.

Chan B A and Hughes B G M (2015). "Targeted therapy for non-small cell lung cancer: current standards and the promise of the future." Translational lung cancer research 4(1): 36-54.

Chanvorachote P, Pongrakhananon V, Wannachaiyasit S, Luanpitpong S, Rojanasakul Y and Nimmannit U (2009). "Curcumin sensitizes lung cancer cells to cisplatin-induced apoptosis through superoxide anion-mediated Bcl-2 degradation." Cancer Invest 27(6): 624-635.

Chaotham C, Pongrakhananon V, Sritularak B and Chanvorachote P (2014). "A Bibenzyl from Dendrobium ellipsophyllum inhibits epithelial-to-mesenchymal transition and sensitizes lung cancer cells to anoikis." Anticancer Res 34(4): 1931-1938.

Charoenrungruang S, Chanvorachote P, Sritularak B and Pongrakhananon V (2014). "Gigantol, a bibenzyl from Dendrobium draconis, inhibits the migratory behavior of non-small cell lung cancer cells." J Nat Prod 77(6): 1359-1366.

Chen S, Zhao S, Wang X, Zhang L, Jiang E, Gu Y, Shangguan A J, Zhao H, Lv T and Yu Z (2015). "Crocin inhibits cell proliferation and enhances cisplatin and pemetrexed chemosensitivity in lung cancer cells." Transl Lung Cancer Res 4(6): 775-783.

Chène P (2003). "Inhibiting the p53–MDM2 interaction: an important target for cancer therapy." Nature Reviews Cancer 3(2): 102-109.

Cheong K, Spicer J, Chowdhury S and Harper P (2005). "Combination therapy versus single agent chemotherapy in non-small cell lung cancer." Expert Opin Pharmacother 6(10): 1693-1700.

Chi S-W (2014). "Structural insights into the transcription-independent apoptotic pathway of p53." BMB reports 47(3): 167-172.

Cho Y J, Cho Y M, Kim S H, Shin K-H, Jung S-T and Kim H S (2019). "Clinical analysis of patients with skeletal metastasis of lung cancer." BMC Cancer 19(1): 303.

Cooper J and Giancotti F G (2019). "Integrin Signaling in Cancer: Mechanotransduction, Stemness, Epithelial Plasticity, and Therapeutic Resistance." Cancer Cell 35(3): 347-367.

Cory S and Adams J M (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer 2(9): 647-656.

Cosaert J and Quoix E (2002). "Platinum drugs in the treatment of non-small-cell lung cancer." British journal of cancer 87(8): 825-833.

Cree I A, Knight L, Di Nicolantonio F, Sharma S and Gulliford T (2002). "Chemosensitization of solid tumor cells by alteration of their susceptibility to apoptosis." Curr Opin Investig Drugs 3(4): 641-647.

Dai C and Gu W (2010). "p53 post-translational modification: deregulated in tumorigenesis." Trends Mol Med 16(11): 528-536.

de Groot P M, Wu C C, Carter B W and Munden R F (2018). "The epidemiology of lung cancer." Translational lung cancer research 7(3): 220-233.

Deben C, Wouters A, Op de Beeck K, van Den Bossche J, Jacobs J, Zwaenepoel K, Peeters M, Van Meerbeeck J, Lardon F, Rolfo C, Deschoolmeester V and Pauwels P (2015). "The MDM2-inhibitor Nutlin-3 synergizes with cisplatin to induce p53 dependent tumor cell apoptosis in non-small cell lung cancer." Oncotarget 6(26): 22666-22679.

Demain A L and Vaishnav P (2011). "Natural products for cancer chemotherapy." Microbial biotechnology 4(6): 687-699.

Desgrosellier J S and Cheresh D A (2010). "Integrins in cancer: biological implications and therapeutic opportunities." Nature Reviews Cancer 10(1): 9-22.

Dingemans A-M C, van den Boogaart V, Vosse B A, van Suylen R-J, Griffioen A W and Thijssen V L (2010). "Integrin expression profiling identifies integrin alpha5 and beta1 as prognostic factors in early stage non-small cell lung cancer." Molecular Cancer 9(1): 152.

Donehower L A, Harvey M, Slagle B L, McArthur M J, Montgomery C A, Jr., Butel J S and Bradley A (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." Nature 356(6366): 215-221.

Dongre A and Weinberg R A (2019). "New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer." Nature Reviews Molecular Cell Biology 20(2): 69-84.

Dorn G W, 2nd (2013). "Molecular mechanisms that differentiate apoptosis from programmed necrosis." Toxicol Pathol 41(2): 227-234.

Elaskalani O, Razak NBA, Falasca M, Metharom P. 2017. Epithelial-mesenchymal transition as a therapeutic target for overcoming chemoresistance in pancreatic cancer. World J Gastrointest Oncol. 9(1):37-41

Endo Y, Lyon S, Shen Y, Mohan N and Wu W J (2019). "Cell proliferation and invasion are regulated differently by EGFR and MRP1 in T-DM1-resistant breast cancer cells." Scientific Reports 9(1): 16383.

Fares J, Fares M Y, Khachfe H H, Salhab H A and Fares Y (2020). "Molecular principles of metastasis: a hallmark of cancer revisited." Signal Transduction and Targeted Therapy 5(1): 28.

Fischer M (2017). "Census and evaluation of p53 target genes." Oncogene 36(28): 3943-3956.

Galluzzi L, Senovilla L, Vitale I, Michels J, Martins I, Kepp O, Castedo M and Kroemer G (2011). "Molecular mechanisms of cisplatin resistance." Oncogene 31: 1869.

Garg M (2017). "Epithelial, mesenchymal and hybrid epithelial/mesenchymal phenotypes and their clinical relevance in cancer metastasis." Expert Rev Mol Med 19: e3.

Golubovskaya V M (2014). "Targeting FAK in human cancer: from finding to first clinical trials." Front Biosci (Landmark Ed) 19: 687-706.

Gonzalez D M and Medici D (2014). "Signaling mechanisms of the epithelial-mesenchymal transition." Sci Signal 7(344): re8.

Guadamillas M C, Cerezo A and del Pozo M A (2011). "Overcoming anoikis – pathways to anchorage-independent growth in cancer." Journal of Cell Science 124(19): 3189-3197.

Gupta S C, Kannappan R, Reuter S, Kim J H and Aggarwal B B (2011). "Chemosensitization of tumors by resveratrol." Ann N Y Acad Sci 1215: 150-160.

Häcker G (2000). "The morphology of apoptosis." Cell and Tissue Research 301(1): 5-17.

Hai J, Sakashita S, Allo G, Ludkovski O, Ng C, Shepherd F A and Tsao M-S (2015). "Inhibiting MDM2-p53 Interaction Suppresses Tumor Growth in Patient-Derived

Non–Small Cell Lung Cancer Xenograft Models." Journal of Thoracic Oncology 10(8): 1172-1180.

Hamidi H, Pietila M and Ivaska J (2016). "The complexity of integrins in cancer and new scopes for therapeutic targeting." Br J Cancer 115(9): 1017-1023.

Hayashi S, Ozaki T, Yoshida K, Hosoda M, Todo S, Akiyama S and Nakagawara A (2006). "p73 and MDM2 confer the resistance of epidermoid carcinoma to cisplatin by blocking p53." Biochem Biophys Res Commun 347(1): 60-66.

Hernandez-Valencia J, Garcia-Villa E, Arenas-Hernandez A, Garcia-Mena J, Diaz-Chavez J and Gariglio P (2018). "Induction of p53 Phosphorylation at Serine 20 by Resveratrol Is Required to Activate p53 Target Genes, Restoring Apoptosis in MCF-7 Cells Resistant to Cisplatin." Nutrients 10(9).

Hientz K, Mohr A, Bhakta-Guha D and Efferth T (2017). "The role of p53 in cancer drug resistance and targeted chemotherapy." Oncotarget 8(5): 8921-8946.

Higashiyama M, Doi O, Kodama K, Yokouchi H, Kasugai T, Ishiguro S, Takami K, Nakayama T and Nishisho I (1997). "MDM2 gene amplification and expression in non-small-cell lung cancer: immunohistochemical expression of its protein is a favourable prognostic marker in patients without p53 protein accumulation." British Journal of Cancer 75(9): 1302-1308.

Horita N, Nagashima A, Nakashima K, Shibata Y, Ito K, Goto A, Yamanaka T and Kaneko T (2017). "The best platinum regimens for chemo-naive incurable non-small cell lung cancer: network meta-analysis." Scientific Reports 7(1): 13185.

Hou H, Sun D and Zhang X (2019). "The role of MDM2 amplification and overexpression in therapeutic resistance of malignant tumors." Cancer Cell Int 19: 216.

Huang D, Duan H, Huang H, Tong X, Han Y, Ru G, Qu L, Shou C and Zhao Z (2016). "Cisplatin resistance in gastric cancer cells is associated with HER2 upregulation-induced epithelial-mesenchymal transition." Scientific Reports 6: 20502.

Huysentruyt L C and Seyfried T N (2010). "Perspectives on the mesenchymal origin of metastatic cancer." Cancer Metastasis Rev 29(4): 695-707.

Jackson J G and Lozano G (2013). "The mutant p53 mouse as a pre-clinical model." Oncogene 32(37): 4325-4330.

Jones S N, Hancock A R, Vogel H, Donehower L A and Bradley A (1998). "Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis." Proc Natl Acad Sci U S A 95(26): 15608-15612.

Kalimuthu S and Se-Kwon K (2013). "Cell survival and apoptosis signaling as therapeutic target for cancer: marine bioactive compounds." International journal of molecular sciences 14(2): 2334-2354.

Kastenhuber E R and Lowe S W (2017). "Putting p53 in Context." Cell 170(6): 1062-1078.

Kelland L (2007). "The resurgence of platinum-based cancer chemotherapy." Nat Rev Cancer 7(8): 573-584.

Khwanduean Rattana S S (2017). "Micropropagation of Dendrobium signatum Rchb.f." Pertanika J. Trop. Agric. Sci.

Kim R, Emi M and Tanabe K (2006). "The role of apoptosis in cancer cell survival and therapeutic outcome." Cancer Biology & Therapy 5(11): 1429-1442.

Kumari R, Kohli S and Das S (2014). "p53 regulation upon genotoxic stress: intricacies and complexities." Mol Cell Oncol 1(3): e969653.

Kurrey N K, Jalgaonkar S P, Joglekar A V, Ghanate A D, Chaskar P D, Doiphode R Y and Bapat S A (2009). "Snail and slug mediate radioresistance and chemoresistance by antagonizing p53-mediated apoptosis and acquiring a stem-like phenotype in ovarian cancer cells." Stem Cells 27(9): 2059-2068.

Lambert A W, Ozturk S and Thiagalingam S (2012). "Integrin signaling in mammary epithelial cells and breast cancer." ISRN Oncol 2012: 493283.

Laptenko O and Prives C (2006). "Transcriptional regulation by p53: one protein, many possibilities." Cell Death Differ 13(6): 951-961.

Larzabal L, de Aberasturi A L, Redrado M, Rueda P, Rodriguez M J, Bodegas M E, Montuenga L M and Calvo A (2014). "TMPRSS4 regulates levels of integrin alpha5 in NSCLC through miR-205 activity to promote metastasis." Br J Cancer 110(3): 764-774.

Lavin M F and Gueven N (2006). "The complexity of p53 stabilization and activation." Cell Death & Differentiation 13(6): 941-950.

Leber M F and Efferth T (2009). "Molecular principles of cancer invasion and metastasis (review)." Int J Oncol 34(4): 881-895.

Lee J S, Hong J H, Sun D S, Won H S, Kim Y H, Ahn M S, Kang S Y, Lee H W and Ko Y H (2019). "The impact of systemic treatment on brain metastasis in patients with non-small-cell lung cancer: A retrospective nationwide population-based cohort study." Scientific Reports 9(1): 18689.

Lemjabbar-Alaoui H, Hassan O U, Yang Y-W and Buchanan P (2015). "Lung cancer: Biology and treatment options." Biochimica et biophysica acta 1856(2): 189-210.

Levine A J and Oren M (2009). "The first 30 years of p53: growing ever more complex." Nature Reviews Cancer 9(10): 749-758.

Li N, Zhang J P, Guo S, Min J, Liu L L, Su H C, Feng Y M and Zhang H L (2012). "Down-regulation of β3-integrin inhibits bone metastasis of small cell lung cancer." Mol Biol Rep 39(3): 3029-3035.

Li Y, Wang C L, Guo S X, Yang J S and Xiao P G (2008). "Two new compounds from Dendrobium candidum." Chem Pharm Bull (Tokyo) 56(10): 1477-1479.

Liang S H and Clarke M F (2001). "Regulation of p53 localization." Eur J Biochem 268(10): 2779-2783.

Liu C, Cai Z, Jin G, Peng D, Pan B-S, Zhang X, Han F, Xu X and Lin H-K (2018). "Abnormal gametogenesis induced by p53 deficiency promotes tumor progression and drug resistance." Cell Discovery 4(1): 54.

Liu Y and Kulesz-Martin M (2000). "P53 regulation and function in normal cells and tumors." Medicina (B Aires) 60 Suppl 2: 9-11.

Liu Y, Chen C, Xu Z, Scuoppo C, Rillahan C D, Gao J, Spitzer B, Bosbach B, Kastenhuber E R, Baslan T, Ackermann S, Cheng L, Wang Q, Niu T, Schultz N, Levine R L, Mills A A and Lowe S W (2016). "Deletions linked to TP53 loss drive cancer through p53-independent mechanisms." Nature 531(7595): 471-475.

Lyons S M, Alizadeh E, Mannheimer J, Schuamberg K, Castle J, Schroder B, Turk P, Thamm D and Prasad A (2016). "Changes in cell shape are correlated with metastatic potential in murine and human osteosarcomas." Biology open 5(3): 289-299.

Marine J C and Lozano G (2010). "Mdm2-mediated ubiquitylation: p53 and beyond." Cell Death & Differentiation 17(1): 93-102.

Martinez J D (2010). "Restoring p53 tumor suppressor activity as an anticancer therapeutic strategy." Future oncology (London, England) 6(12): 1857-1862.

Meltzer P S, Jankowski S A, Dal Cin P, Sandberg A A, Paz I B and Coccia M A (1991). "Identification and cloning of a novel amplified DNA sequence in human malignant fibrous histiocytoma derived from a region of chromosome 12 frequently rearranged in soft tissue tumors." Cell Growth Differ 2(10): 495-501.

Messaritakis I, Kotsakis A and Georgoulias V (2017). "Association of epithelial-to-mesenchymal transition circulating tumor cells in non-small cell lung cancer (NSCLC) molecular subgroups." Journal of thoracic disease 9(12): 4817-4820.

Millard M, Odde S and Neamati N (2011). "Integrin targeted therapeutics." Theranostics 1: 154-188.

Mitsudomi T, Hamajima N, Ogawa M and Takahashi T (2000). "Prognostic significance of p53 alterations in patients with non-small cell lung cancer: a meta-analysis." Clin Cancer Res 6(10): 4055-4063.

Mittal V (2016). "Epithelial Mesenchymal Transition in Aggressive Lung Cancers." Adv Exp Med Biol 890: 37-56.

Mittraphab A, Muangnoi C, Likhitwitayawuid K, Rojsitthisak P and Sritularak B (2016). "A New Bibenzyl-phenanthrene Derivative from Dendrobium signatum and its Cytotoxic Activity." Nat Prod Commun 11(5): 657-659.

Molina J R, Yang P, Cassivi S D, Schild S E and Adjei A A (2008). "Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship." Mayo Clinic proceedings 83(5): 584-594.

Moll U M and Petrenko O (2003). "The MDM2-p53 interaction." Mol Cancer Res 1(14): 1001-1008.

Moulder D E, Hatoum D, Tay E, Lin Y and McGowan E M (2018). "The Roles of p53 in Mitochondrial Dynamics and Cancer Metabolism: The Pendulum between Survival and Death in Breast Cancer?" Cancers 10(6): 189.

Mrozik K M, Blaschuk O W, Cheong C M, Zannettino A C W and Vandyke K (2018). "N-cadherin in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer." BMC Cancer 18(1): 939.

Nguyen D X, Bos P D and Massague J (2009). "Metastasis: from dissemination to organ-specific colonization." Nat Rev Cancer 9(4): 274-284.

Nistico P, Bissell M J and Radisky D C (2012). "Epithelial-mesenchymal transition: general principles and pathological relevance with special emphasis on the role of matrix metalloproteinases." Cold Spring Harb Perspect Biol 4(2).

Nurwidya F, Takahashi F, Murakami A and Takahashi K (2012). "Epithelial mesenchymal transition in drug resistance and metastasis of lung cancer." Cancer research and treatment : official journal of Korean Cancer Association 44(3): 151-156.

Ohnstad H O, Paulsen E B, Noordhuis P, Berg M, Lothe R A, Vassilev L T and Myklebost O (2011). "MDM2 antagonist Nutlin-3a potentiates antitumour activity of cytotoxic drugs in sarcoma cell lines." BMC cancer 11: 1-11.

Oliner J D, Saiki A Y and Caenepeel S (2016). "The Role of MDM2 Amplification and Overexpression in Tumorigenesis." Cold Spring Harb Perspect Med 6(6).

Olivier M, Hollstein M and Hainaut P (2010). "TP53 mutations in human cancers: origins, consequences, and clinical use." Cold Spring Harb Perspect Biol 2(1): a001008.

Otsuki Y, Saya H and Arima Y (2018). "Prospects for new lung cancer treatments that target EMT signaling." Developmental Dynamics 247(3): 462-472.

Ozaki T and Nakagawara A (2011). "Role of p53 in Cell Death and Human Cancers." Cancers 3(1): 994-1013.

Pant V and Lozano G (2014). "Limiting the power of p53 through the ubiquitin proteasome pathway." Genes Dev 28(16): 1739-1751.

Parsons J T, Horwitz A R and Schwartz M A (2010). "Cell adhesion: integrating cytoskeletal dynamics and cellular tension." Nat Rev Mol Cell Biol 11(9): 633-643.

Petpiroon N, Sritularak B and Chanvorachote P (2017). "Phoyunnanin E inhibits migration of non-small cell lung cancer cells via suppression of epithelial-to-mesenchymal transition and integrin αv and integrin β3." BMC Complementary and Alternative Medicine 17(1): 553.

Prochazkova J, Lichnovsky V, Kylarova D, Erdosova B and Vranka P (2004). "Involvement of p53 and Bcl-2 family proteins in regulating programmed cell death and proliferation in human embryogenesis." Gen Physiol Biophys 23(2): 209-229.

Rayburn E, Zhang R, He J and Wang H (2005). "MDM2 and human malignancies: expression, clinical pathology, prognostic markers, and implications for chemotherapy." Curr Cancer Drug Targets 5(1): 27-41.

Rello S, Stockert J C, Moreno V, Gamez A, Pacheco M, Juarranz A, Canete M and Villanueva A (2005). "Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments." Apoptosis 10(1): 201-208.

Riihimaki M, Hemminki A, Sundquist K, Sundquist J and Hemminki K (2016). "Metastatic spread in patients with gastric cancer." Oncotarget 7(32): 52307-52316.

Roy H K, Iversen P, Hart J, Liu Y, Koetsier J L, Kim Y, Kunte D P, Madugula M, Backman V and Wali R K (2004). "Down-regulation of SNAIL suppresses MIN mouse tumorigenesis: modulation of apoptosis, proliferation, and fractal dimension." Mol Cancer Ther 3(9): 1159-1165.

Royds J A and Iacopetta B (2006). "p53 and disease: when the guardian angel fails." Cell Death & Differentiation 13(6): 1017-1026.

Sansome C, Zaika A, Marchenko N D and Moll U M (2001). "Hypoxia death stimulus induces translocation of p53 protein to mitochondria. Detection by immunofluorescence on whole cells." FEBS Lett 488(3): 110-115.

Savagner P (2010). "The epithelial-mesenchymal transition (EMT) phenomenon." Ann Oncol 21 Suppl 7: vii89-92.

Scagliotti G V, Parikh P, von Pawel J, Biesma B, Vansteenkiste J, Manegold C, Serwatowski P, Gatzemeier U, Digumarti R, Zukin M, Lee J S, Mellemgaard A, Park K, Patil S, Rolski J, Goksel T, de Marinis F, Simms L, Sugarman K P and Gandara D (2008). "Phase III study comparing cisplatin plus gemcitabine with cisplatin plus pemetrexed in chemotherapy-naive patients with advanced-stage non-small-cell lung cancer." J Clin Oncol 26(21): 3543-3551.

Schneider-Poetsch T, Ju J, Eyler D E, Dang Y, Bhat S, Merrick W C, Green R, Shen B and Liu J O (2010). "Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin." Nature chemical biology 6(3): 209-217.

Seguin L, Desgrosellier J S, Weis S M and Cheresh D A (2015). "Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance." Trends Cell Biol 25(4): 234-240.

Shabbits J A, Hu Y and Mayer L D (2003). "Tumor Chemosensitization Strategies Based on Apoptosis Manipulations." Molecular Cancer Therapeutics 2(8): 805-813.

Shangary S and Wang S (2008). "Targeting the MDM2-p53 interaction for cancer therapy." Clin Cancer Res 14(17): 5318-5324.

Shangary S and Wang S (2009). "Small-molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy." Annual review of pharmacology and toxicology 49: 223-241.

Shen D-W, Pouliot L M, Hall M D and Gottesman M M (2012). "Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes." Pharmacological reviews 64(3): 706-721.

Sheng W, Dong M, Chen C, Wang Z, Li Y, Wang K, Li Y and Zhou J (2017). "Cooperation of Musashi-2, Numb, MDM2, and P53 in drug resistance and malignant biology of pancreatic cancer." FASEB J 31(6): 2429-2438.

Shi D and Gu W (2012). "Dual Roles of MDM2 in the Regulation of p53: Ubiquitination Dependent and Ubiquitination Independent Mechanisms of MDM2 Repression of p53 Activity." Genes Cancer 3(3-4): 240-248.

Siddik Z H M, Kapil (2009). Drug Resistance in Cancer Cells, Springer Science.

Silginer M, Weller M, Ziegler U and Roth P (2014). "Integrin inhibition promotes atypical anoikis in glioma cells." Cell Death & Disease 5(1): e1012-e1012.

Simpson C D, Anyiwe K and Schimmer A D (2008). "Anoikis resistance and tumor metastasis." Cancer Lett 272(2): 177-185.

Smith B N and Bhowmick N A (2016). "Role of EMT in Metastasis and Therapy Resistance." J Clin Med 5(2).

Song D, Zhao J, Su C, Jiang Y and Hou J (2018). "Etoposide induced NMI promotes cell apoptosis by activating the ARF-p53 signaling pathway in lung carcinoma." Biochem Biophys Res Commun 495(1): 368-374.

Sosa Iglesias V, Giuranno L, Dubois L J, Theys J and Vooijs M (2018) "Drug Resistance in Non-Small Cell Lung Cancer: A Potential for NOTCH Targeting?" Frontiers in Oncology 8, 267 DOI: 10.3389/fonc.2018.00267.

Soussi T and Wiman K G (2015). "TP53: an oncogene in disguise." Cell Death & Differentiation 22(8): 1239-1249.

Sujan D, Dhruv K, Meshram S, Agrawal S and Smt) Sunita Meshram D (2017). p53: the guardian of genome, apoptosis, and its role in carcinogenesis.

Sulzmaier F J, Jean C and Schlaepfer D D (2014). "FAK in cancer: mechanistic findings and clinical applications." Nature reviews. Cancer 14(9): 598-610.

Sun S H, Zheng M, Ding K, Ding K, Wang S and Sun Y (2008). "A small molecule that disrupts Mdm2-p53 binding activates p53, induces apoptosis and sensitizes lung cancer cells to chemotherapy." Cancer biology & therapy 7(6): 845-852.

Takkunen M, Grenman R, Hukkanen M, Korhonen M, Garcia de Herreros A and Virtanen I (2006). "Snail-dependent and -independent epithelial-mesenchymal transition in oral squamous carcinoma cells." J Histochem Cytochem 54(11): 1263-1275.

Trepel M, Scheding S, Groscurth P, Horny H P, Malipiero U, Brugger W, Dichgans J and Weller M (1997). "A new look at the role of p53 in leukemia cell sensitivity to chemotherapy." Leukemia 11(11): 1842-1849.

Turc-Carel C, Limon J, Dal Cin P, Rao U, Karakousis C and Sandberg A A (1986). "Cytogenetic studies of adipose tissue tumors. II. Recurrent reciprocal translocation t(12;16)(q13;p11) in myxoid liposarcomas." Cancer Genet Cytogenet 23(4): 291-299.

Unahabhokha, T., P. Chanvorachote, B. Sritularak, J. Kitsongsermthon and V. Pongrakhananon (2016). "Gigantol Inhibits Epithelial to Mesenchymal Process in Human Lung Cancer Cells." Evid Based Complement Alternat Med 2016: 4561674.

van Zijl F, Krupitza G and Mikulits W (2011). "Initial steps of metastasis: cell invasion and endothelial transmigration." Mutation research 728(1-2): 23-34.

Vergara D, Simeone P, Franck J, Trerotola M, Giudetti A, Capobianco L, Tinelli A, Bellomo C, Fournier I, Gaballo A, Alberti S, Salzet M and Maffia M (2016). "Translating epithelial mesenchymal transition markers into the clinic: Novel insights from proteomics." EuPA Open Proteomics 10: 31-41.

Vousden K H and Ryan K M (2009). "p53 and metabolism." Nature Reviews Cancer 9: 691.

Wade M, Li Y-C and Wahl G M (2013). "MDM2, MDMX and p53 in oncogenesis and cancer therapy." Nature Reviews Cancer 13(2): 83-96.

Wang B, Fang L, Zhao H, Xiang T and Wang D (2012). "MDM2 inhibitor Nutlin-3a suppresses proliferation and promotes apoptosis in osteosarcoma cells." Acta Biochim Biophys Sin (Shanghai) 44(8): 685-691.

Wang B, Xiao Z, Ko H L and Ren E C (2010). "The p53 response element and transcriptional repression." Cell Cycle 9(5): 870-879.

Wang Y, Shi J, Chai K, Ying X and Zhou B P (2013). "The Role of Snail in EMT and Tumorigenesis." Current cancer drug targets 13(9): 963-972.

Warren G W and Cummings K M (2013). "Tobacco and lung cancer: risks, trends, and outcomes in patients with cancer." Am Soc Clin Oncol Educ Book: 359-364.

Wheelock M J, Shintani Y, Maeda M, Fukumoto Y and Johnson K R (2008). "Cadherin switching." J Cell Sci 121(Pt 6): 727-735.

Wheelock M J, Shintani Y, Maeda M, Fukumoto Y and Johnson K R (2008). "Cadherin switching." J Cell Sci 121(Pt 6): 727-735.

Xiao D and He J (2010). "Epithelial mesenchymal transition and lung cancer." Journal of thoracic disease 2(3): 154-159.

Yang X, Han M, Han H, Wang B, Li S, Zhang Z and Zhao W (2017). "Silencing Snail suppresses tumor cell proliferation and invasion by reversing epithelial-to-mesenchymal transition and arresting G2/M phase in non-small cell lung cancer." Int J Oncol 50(4): 1251-1260.

Yang X-G, Zhu L-C, Wang Y-J, Li Y-Y and Wang D (2019). "Current Advance of Therapeutic Agents in Clinical Trials Potentially Targeting Tumor Plasticity." Frontiers in oncology 9: 887-887.

Yee K S and Vousden K H (2005). "Complicating the complexity of p53." Carcinogenesis 26(8): 1317-1322.

Yogosawa S and Yoshida K (2018). "Tumor suppressive role for kinases phosphorylating p53 in DNA damage-induced apoptosis." Cancer Sci 109(11): 3376-3382.

Yu J-G, Ji C-H and Shi M-H (2019). "Nitroxoline induces cell apoptosis by inducing MDM2 degradation in small-cell lung cancer." The Kaohsiung Journal of Medical Sciences 35(4): 202-208.

Yu L X, Yan L, Yang W, Wu F Q, Ling Y, Chen S Z, Tang L, Tan Y X, Cao D, Wu M C, Yan H X and Wang H Y (2014). "Platelets promote tumour metastasis via interaction

between TLR4 and tumour cell-released high-mobility group box1 protein." Nat Commun 5: 5256.

Yu W, Yang L, Li T and Zhang Y (2019). "Cadherin Signaling in Cancer: Its Functions and Role as a Therapeutic Target." Frontiers in oncology 9: 989-989.

Yue P Y K, Leung E P Y, Mak N K and Wong R N S (2010). "A Simplified Method for Quantifying Cell Migration/Wound Healing in 96-Well Plates." Journal of Biomolecular Screening 15(4): 427-433.

Yue X, Zhao Y, Xu Y, Zheng M, Feng Z and Hu W (2017). "Mutant p53 in Cancer: Accumulation, Gain-of-Function, and Therapy." Journal of Molecular Biology 429(11): 1595-1606.

Zappa C and Mousa S A (2016). "Non-small cell lung cancer: current treatment and future advances." Translational lung cancer research 5(3): 288-300.

Zhao Y, Aguilar A, Bernard D and Wang S (2015). "Small-molecule inhibitors of the MDM2-p53 protein-protein interaction (MDM2 Inhibitors) in clinical trials for cancer treatment." J Med Chem 58(3): 1038-1052.

Ziegler U and Groscurth P (2004). "Morphological Features of Cell Death." Physiology 19(3): 124-128.

Zilfou J T and Lowe S W (2009). "Tumor suppressive functions of p53." Cold Spring Harb Perspect Biol 1(5): a001883

Zouq N K, Keeble J A, Lindsay J, Valentijn A J, Zhang L, Mills D, Turner C E, Streuli C H and Gilmore A P (2009). "FAK engages multiple pathways to maintain survival of fibroblasts and epithelia: differential roles for paxillin and p130Cas." Journal of cell science 122(Pt 3): 357-367.

APPENDIX

Table of Experimental Results

Table 2. The percentage of cell viability was determined by MTT assay after the treatment of DS-1 (0-200 μM) for 24 hours.

DS-1 (μM)	Cell Viability (%)
0	100.00 ± 0.00
25	92.38 ± 3.20
50	88.92 ± 3.67
100	87.42 ± 5.20
150	75.13 ± 5.27*
200	52.71 ± 8.00*

Value represents means ± SD of three independent experiments, *p<0.05 versus control group.

Table 3. The percentage of apoptotic and necrotic H460 cells was investigated by Nucley staining assay using Hoechst 33342

DS-1 (μM)	% Apoptosis	% Necrosis
0	0.00 ± 0.00	0.00 ± 0.00
25	0.66 ± 0.62	0.26 ± 0.30
50	1.40 ± 0.20	0.24 ± 0.24
100	2.79 ± 1.08	0.65 ± 0.18
150	4.82 ± 1.63*	0.67 ± 0.28

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group.

Table 4. The percentage of cell viability was investigated by MTT assay after the treatment of DS-1 combined with cisplatin

DS-1 (μM)	Cisplatin (μM)	Cell Viability (%)
0	0	100.00 ± 0.00
-	25	84.65 ± 3.69*
50	25	67.75 ± 6.50*#
100	25	42.83 ± 0.76*#
-	50	65.07 ± 3.87*
50	50	45.53 ± 1.92*#
100	50	32.77 ± 3.59*#

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group, #p<0.05 versus cisplatin-treated cells.

Table 5. Number of apoptotic H460 cells treated with DS-1 and cisplatin was investigated by Annexin and PI staining assay using flow cytometry

DS-1 (μM)	Cisplatin (μM)	% cell in each stage			
		a	b	c	d
0	0	93.67	6.19	0.03	0.11
0	25	86.69	4.22	7.13	1.96
50	25	88.31	1.38	9.45	0.86
100	25	46.01	17.73	33.16	3.20

a: Living cells, b: Early apoptotic cells, c: Late apoptotic cells, d: Necrotic cells.

Table 6. Combination index of DS-1 plus cisplatin

DS-1 (μM)	Cisplatin (μM)	Fa	CI
50	25	0.32	0.35542
50	50	0.54	0.06605
100	25	0.57	0.03346
100	50	0.67	0.02076

Fa: Fraction affected, CI: Combination index

Table 7. Relative apoptosis-related protein of H460 cells was detected by western blot analysis

DS-1 (μM)	Relative protein level			
	p53	p-Akt/Akt	Bcl-2	Bax
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25	2.23 ± 0.44*	1.34 ± 0.14	1.32 ± 0.08	1.19 ± 0.59
50	3.93 ± 0.50**	1.10 ± 0.09	0.91 ± 0.18	1.91 ± 0.20*
100	6.00 ± 0.54**	0.721 ± 0.03*	0.26 ± 0.15*	1.98 ± 0.10*

Value represents means ± SD of three-independent experiments, *p<0.05, **p<0.01 versus control group.

Table 8. Relative p53 protein after the treatment of DS-1 and cycloheximide (CHX) was detected by western blot analysis

Time (h)	Relative p53 protein level		
	CHX	CHX + DS-1 (50 μM)	CHX + DS-1 (100 μM)
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
1	0.36 ± 0.06*	0.53 ± 0.08*#	0.79 ± 0.08*#
3	0.23 ± 0.10*	0.31 ± 0.06*	0.42 ± 0.04*
6	0.10 ± 0.03*	0.13 ± 0.09*#	0.28 ± 0.07*#

Value represents means ± SD of three-independent experiments, *p<0.01 versus control group, #p<0.01 versus CHX-treated cell group.

Table 9. Relative Ub-p53 level of H460 cells after the treatment of DS-1 and MG132 was detected by immunoprecipitation and western blot analysis

DS-1 (μM)	Relative Ub-p53 protein level
0	1.00 ± 0.00
50	0.59 ± 0.19*
100	0.38 ± 0.08*

Value represents means ± SD of three-independent experiments, *p<0.01 versus control group

Table 10. Relative apoptosis-regulated proten level of H460 cells treated with DS-1 and cisplatin was determined by western blot assay

DS-1 (μM)	Cisplatin (μM)	Relative protein level			
		p53	p-Akt/Akt	Bcl2	Bax
0	0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
0	25	2.70 ± 0.90*	0.80 ± 0.08	0.94 ± 0.07	1.31 ± 0.14
50	25	8.82 ± 1.02*#	0.75 ± 0.15	0.90 ± 0.05	2.78 ± 0.64*
100	25	9.05 ± 1.59*#	0.70 ± 0.16	0.90 ± 0.07	2.36 ± 0.34*
0	50	4.81 ± 1.13*	0.78 ± 0.17	0.96 ± 0.13	2.02 ± 0.25*
50	50	9.53 ± 1.75*#	0.71 ± 0.12	0.61 ± 0.17*	1.96 ± 0.10*
100	50	12.12 ± 1.12*#	0.51 ± 0.14*	0.42 ± 0.23*	2.02 ± 0.18*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group and #p<0.05 versus cisplatin-treated cells.

Table 11. Mean of p-p53 intensity in response to DS-1 and/or cisplatin treatment

DS-1 (μM)	Cisplatin (μM)	Mean of intensity
0	0	1.00 ± 0.00
100	0	4.78 ± 16.13
0	25	5.57 ± 2.46
100	25	11.10 ± 3.03*
0	50	16.28 ± 4.33
100	50	22.89 ± 2.86*

Value represents means ± SD of three-independent experiments, *p<0.05 versus cisplatin-treated cells

Table 12. The percentage of cell viability of patient-derivated lung cancer cells was determined by MTT assay after the treatment of DS-1 and/or cisplatin

DS-1 (μM)	% Cell viability			
	E20	E12	E17	E16
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
25	109.70 ± 3.64	96.19 ± 4.28	102.21 ± 0.92	94.42 ± 3.17
50	89.74 ± 2.86	95.12 ± 2.38	86.55 ± 2.55*	92.95 ± 1.81
100	76.62 ± 6.57*	72.59 ± 4.56*	78.95 ± 6.37*	74.59 ± 4.62*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group

DS-1 (μM)	Cisplatin (μM)	% Cell viability	
		E20	E12
0	0	100.00 ± 0.00	100.00 ± 0.00
-	25	70.51 ± 6.18*	73.44 ± 1.86*
50	25	72.81 ± 9.05*	66.51 ± 7.54*
100	25	39.69 ± 2.84*#	58.92 ± 6.62*#
-	50	47.93 ± 1.82*	60.66 ± 1.05*
50	50	36.77 ± 9.30*	46.85 ± 3.39*#
100	50	30.32 ± 8.38*	38.44 ± 0.54*#

DS-1 (μM)	Cisplatin (μM)	% Cell viability	
		E17	E16
0	0	100.00 ± 0.00	100.00 ± 0.00
-	25	74.51 ± 0.81*	74.68 ± 1.08*
50	25	72.19 ± 5.25*	65.51 ± 6.32*
100	25	41.66 ± 7.55*#	42.19 ± 3.13*#
-	50	51.23 ± 1.21*	60.27 ± 4.77*
50	50	38.49 ± 4.79*	46.04 ± 8.88*#
100	50	20.23 ± 7.79*#	17.53 ± 0.70*#

Value represents means ± SD of three-independent experiments, *$p<0.05$ versus control group and #$p<0.05$ versus cisplatin-treated cells.

Table 13. The percentage of apoptotic and necrotic patient-derived lung cancer cells was investigated by Nucley staining assay using Hoechst 33342

DS-1 (μM)	Cisplatin (μM)	Primary-derived patient E20 cells	
		% Apoptosis	% Necrosis
0	0	0.00 ± 0.00	0.00 ± 0.00
100	-	3.60 ± 1.00	0.00 ± 0.00
-	25	9.57 ± 1.90*	2.40 ± 1.00
100	25	31.71 ± 2.88*#	8.33 ± 2.35
-	50	36.56 ± 3.24*	11.65 ± 1.98
100	50	42.67 ± 2.20*#	12.59 ± 0.92*#

DS-1 (μM)	Cisplatin (μM)	Primary-derived patient E12 cells	
		% Apoptosis	% Necrosis
0	0	0.00 ± 0.00	0.00 ± 0.00
100	-	5.45 ± 1.42	0.00 ± 0.00
-	25	10.77 ± 2.58*	2.41 ± 1.02
100	25	20.36 ± 3.82*#	10.79 ± 2,00*#
-	50	17.12 ± 3.05*	10.29 ± 1.97*
100	50	59.81 ± 2.71*#	5.97 ± 7.35

DS-1 (μM)	Cisplatin (μM)	Primary-derived patient E16 cells	
		% Apoptosis	% Necrosis
0	0	0.00 ± 0.00	0.00 ± 0.00
100	-	7.86 ± 0,00	0.00 ± 0.00
-	25	15.84 ± 3.41*	3.41 ± 1.65
100	25	41.41 ± 8.40*#	8.40 ± 0.68*#
-	50	38.28 ± 8.55*	8.55 ± 3.19*
100	50	72.87 ± 12.31*#	12.31 ± 0.68*

DS-1 (μM)	Cisplatin (μM)	Primary-derived patient E17 cells	
		% Apoptosis	% Necrosis
0	0	0.00 ± 0.00	0.00 ± 0.00
100	-	3.40 ± 0.73	0.00 ± 0.00
-	25	8.95 ± 0.78*	2.47 ± 1.08
100	25	31.95 ± 2.99*#	7.21 ± 2.58*
-	50	33.27 ± 6.30*	7.99 ± 2.59*
100	50	50.14 ± 3.52*#	11.54 ± 3.29*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group and #p<0.05 versus cisplatin treated cells.

Table 14. Relative cell proliferation of H460 and H292 was determined by MTT assay after incubation with DS-1 for 24 hours and 48 hours

DS-1	Relative cell proliferation of H460		
(μM)	0h	24h	48h
0	1.00 ± 0.00	1.43 ± 0.25	2,00 ± 0.43
25	1.00 ± 0.00	1.46 ± 0.37	1.96 ± 0.37
50	1.00 ± 0.00	1.30 ± 0.10	1.56 ± 0.05
100	1.00 ± 0.00	1.23 ± 0.05	1.43 ± 0.06*

DS-1	Relative cell proliferation of H292		
(μM)	0h	24h	48h
0	1.00 ± 0.00	2.10 ± 0.14	3.29 ± 0.12
25	1.00 ± 0.00	1.84 ± 0.09	3.21 ± 0.32
50	1.00 ± 0.00	1.74 ± 0.13*	2.76 ± 0.04
100	1.00 ± 0.00	1.57 ± 0.12*	2.16 ± 0.39*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group

Table 15. Relative filopodia formation of H460 and H292 was determined by phalloidin-rhodamine staining assay

DS-1 (μM)	Relative number of filopodia/cell	
	H460	H292
0	1.00 ± 0.00	1.00 ± 0.00
25	0.72 ± 0.07*	0.92 ± 0.16
50	0.49 ± 0.03*	0.47 ± 0.08*
100	0.41 ± 0.07*	0.42 ± 0.07*

Table 16. The percentage of migration level of H460 and H292 cells treated with DS-1 was investigated by wound healing migration assay

DS-1	% migration level of H460 cells	
(μM)	24h	48h
0	13.39 ± 2.61	42.16 ± 6.33
25	12.51 ± 4.75	36.95 ± 2.90
50	9.30 ± 1.53	33.22 ± 3.01
100	9.83 ± 2.40	29.39 ± 3.38*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group

DS-1	% migration level of H292 cells	
(μM)	24h	48h
0	24.88 ± 4.23	53.57 ± 4.19
25	24.07 ± 3.14	49.22 ± 2.61
50	17.70 ± 5.54	40.11 ± 8.58
100	16.24 ± 6.13	37.51 ± 3.77*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group

Table 17. Relative invasion of H460 and H292 cells treated with DS-1 was investigated by transwell invasion assay

DS-1	Relative invasion	
(μM)	H460	H292
0	1.00 ± 0.00	1.00 ± 0.00
25	0.69 ± 0.04*	0.84 ± 0.11
50	0.54 ± 0.05*	0.43 ± 0.08*
100	0.36 ± 0.05*	0.33 ± 0.05*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group

Table 18. Relative colony size and number of H460 cells treated with DS-1 was determined by anchorage-independent growth

DS-1 (μM)	Relative colony number	Relative colony size
0	1.00 ± 0.00	1.00 ± 0.00
25	0.90 ± 0.24	0.75 ± 0.20
50	0.76 ± 0.10	0.66 ± 0.07*
100	0.57 ± 0.13*	0.52 ± 0.05*

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group.

Table 19. Relative protein level of EMT-regulated protein of H460 was investigated by western blot assay

DS-1 (μM)	Relative protein level		
	N-cadherin	E-cadherin	Snail
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25	1.06 ± 0.08	1.32 ± 0.20	0.90 ± 0.07
50	0.83 ± 0.10	1.70 ± 0.48	0.79 ± 0.24
100	0.60 ± 0.09*	2.66 ± 1.01*	0.46 ± 0.08*

DS-1 (μM)	Relative protein level		
	Integrin β1	Integrin β3	Integrin α5
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25	1.20 ± 0.10	0.90 ± 0.06	0.90 ± 0.12
50	1.17 ± 0.09	0.87 ± 0.05	0.95 ± 0.10
100	0.74 ± 0.07*	0.90 ± 0.11	0.92 ± 0.07

DS-1 (μM)	Relative protein level		
	Slug	RhoA	p-FAK/FAK
0	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
25	0.86 ± 0.36	1.71 ± 0.18	0.80 ± 0.14
50	0.92 ± 0.42	1.53 ± 0.06	0.80 ± 0.20
100	0.57 ± 0.22	0.46 ± 0.10*	0.78 ± 0.06

Value represents means ± SD of three-independent experiments, *p<0.05 versus control group.

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