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Effect of hydroquinone 5-o-cinnamoyl ester of renieramycin m on the suppression of lung cancer stem cells

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EFFECT OF HYDROQUINONE 5-*O*-CINNAMOYL ESTER OF RENIERAMYCIN M ON THE SUPPRESSION OF LUNG CANCER STEM CELLS

Miss Nattamon Hongwiangchan

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ผลของไฮโดรควิโนน 5-*โอ*-ซินนาโมอิล เอสเทอร์ของเรนิอีราไมซินเอ็มในการยับยั้งเซลล์ต้นกำเนิดมะเร็งปอด

น.ส.ณัฐมล หงษ์เวียงจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	EFFECT OF HYDROQUINONE 5-*O*-CINNAMOYL ESTER OF RENIERAMYCIN M ON THE SUPPRESSION OF LUNG CANCER STEM CELLS
By	Miss Nattamon Hongwiangchan
Field of Study	Pharmaceutical Sciences and Technology
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ณัฐมล หงษ์เวียงจันทร์ : ผลของไฮโดรควิโนน 5-*โอ*-ซินนาโมอิล เอสเทอร์ของเรนิอีราไมซินเอ็มในการยับยั้งเซลล์ต้นกำเนิดมะเร็งปอด. (EFFECT OF HYDROQUINONE 5-*O*-CINNAMOYL ESTER OF RENIERAMYCIN M ON THE SUPPRESSION OF LUNG CANCER STEM CELLS) อ.ที่ปรึกษาหลัก : ศ. ภก. ดร.ปิติ จันทร์วรโชติ

เซลล์ต้นกำเนิดมะเร็งเป็นประชากรมะเร็งที่มีจำนวนน้อยที่มีความสามารถในการก่อเนื้องอกและสามารถแบ่งเซลล์ทดแทนตนเองได้ เซลล์ต้นกำเนิดมะเร็งเป็นตัวขับเคลื่อนของการทำให้เกิดการสร้างมะเร็งขึ้นใหม่ การพัฒนาของมะเร็ง ความล้มเหลวในการรักษาและการกลับมาเป็นซ้ำของโรค ด้วยเหตุผลนี้สารประกอบใหม่ซึ่งมุ่งเป้าไปที่เซลล์ต้นกำเนิดมะเร็งจึงเป็นวิธีที่มีแนวโน้มในการควบคุมมะเร็ง ในการศึกษานี้ไฮโดรควิโนน 5-*โอ*-ซินนาโมอิล เอสเทอร์ของเรนิอีราไมซินเอ็ม (ซิน-อาเอ็ม) แสดงให้เห็นว่าสามารถยับยั้งเซลล์ต้นกำเนิดมะเร็งปอด ซิน-อาเอ็มเป็นพิษต่อเซลล์มะเร็งปอดที่มีค่าความเข้มข้นการยับยั้งร้อยละ 50 ประมาณ 15 ไมโครโมลาร์ ซิน-อาเอ็มยับยั้งเซลล์ต้นกำเนิดมะเร็งโดยการยับยั้งการสร้างโคโลนี (colony) และยับยั้งการก่อตัวของเนื้องอกลักษณะทรงกลม (tumor spheroid formation) นอกจากนี้ประชากรเซลล์ต้นกำเนิดมะเร็งถูกแยกและบำบัดด้วยซิน-อาเอ็ม เซลล์ต้นกำเนิดมะเร็งที่ถูกบำบัดด้วยซิน-อาเอ็มเกิดการตายภายใน 24 ชั่วโมง ซิน-อาเอ็มแสดงการทำลายซี-มิก (c-Myc) โปรตีนควบคุมการรอดชีวิตและเซลล์ต้นกำเนิดโดยลดเครื่องหมายเซลล์ต้นกำเนิดมะเร็ง (CSC markers) และปัจจัยการถอดรหัสเซลล์ต้นกำเนิด (stem cell transcription factors) เช่น ALDH1A1, Oct4, Nanog และ Sox2 สำหรับการควบคุมชั้นบน (up-stream regulation) เราพบว่าซิน-อาเอ็มยับยั้งเอเคที (Akt) อย่างมีนัยสำคัญและทำให้ปัจจัยการถอดรหัสต้นแบบ (pluripotent transcription factors) ลดลง ซิน-อาเอ็มยังยับยั้งเอ็มทอ (mTOR) ในขณะที่ p-GSK3β (Ser9) ลดลงเล็กน้อย การยับยั้ง Akt/mTOR ทำให้เกิดการยูบิควิตินของ c-Myc และส่งเสริมการย่อยสลาย กลไกของ Akt ที่ควบคุมความเสถียรของ c-Myc ได้รับการตรวจสอบด้วย wortmannin ซึ่งเป็นตัวยับยั้ง Akt เราใช้การทดลองระดับโมเลกุลและฟีโนไทป์ของเซลล์ต้นกำเนิดมะเร็งเพื่อแสดงการออกฤทธิ์การยับยั้งแบบใหม่ของสารประกอบนี้ในเซลล์ต้นกำเนิดมะเร็งเพื่อเป็นประโยชน์ในการรักษาแบบมุ่งเป้าไปที่เซลล์ต้นกำเนิดมะเร็งสำหรับการรักษามะเร็งปอด

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Nattamon Hongwiangchan : EFFECT OF HYDROQUINONE 5-*O*-CINNAMOYL ESTER OF RENIERAMYCIN M ON THE SUPPRESSION OF LUNG CANCER STEM CELLS. Advisor: Prof. PITHI CHANVORACHOTE, Ph.D.

Cancer stem cells (CSCs) are distinct cancer populations with tumorigenic and self-renewal abilities. CSCs are drivers of cancer initiation, progression, therapeutic failure, and disease recurrence. Thereby, novel compounds targeting CSCs offer a promising way to control cancer. In this study, the hydroquinone 5-*O*-cinnamoyl ester of renieramycin M (CIN-RM) was demonstrated to suppress lung cancer CSCs. CIN-RM was toxic to lung cancer cells with a half-maximal inhibitory concentration around 15 μM. CIN-RM suppressed CSCs by inhibiting colony and tumor spheroid formation. In addition, the CSC population was isolated and treated and the CSCs were dispatched in response to CIN-RM within 24 h. CIN-RM was shown to abolish cellular c-Myc, a central survival and stem cell regulatory protein, with the depletion of CSC markers and stem cell transcription factors ALDH1A1, Oct4, Nanog, and Sox2. For up-stream regulation, we found that CIN-RM significantly inhibited Akt and consequently decreased the pluripotent transcription factors. CIN-RM also inhibited mTOR, while slightly decreasing p-GSK3β (Ser9). Inhibiting Akt/mTOR induced ubiquitination of c-Myc and promoted degradation. The mechanism of how Akt regulates the stability of c-Myc was validated with the Akt inhibitor wortmannin. Taken together, we utilized molecular experiments and the CSC phenotype to reveal the novel suppressing the activity of this compound on CSCs to benefit CSC-targeted therapy for lung cancer treatment.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
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In drawing things to a close, I would like to express my deep appreciation to my dearest parents for their love, understand and encouragement which give me to accomplish my goal.

Nattamon Hongwiangchan

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CHAPTER I

INTRODUCTION

1.1 Background and rational

Cancer stem cells (CSCs) are a leading cause of cancer aggressiveness that enhance the ability of cancer to disseminate. They are the small section that carrying asymmetric division to remain the constant proportion and self-renewal properties. This unique population has been identified as a tumor initiator that progresses cancer (1, 2). Recent studies have highlighted the CSC population as a critical regulator of disease relapse, as CSCs have very high detoxification ability and augmented drug resistance pathways; therefore, targeting CSCs is recognized as a promising way to control cancer (3).

CSCs exhibit biological activities and stem cell properties through several pluripotent transcription factors, such as Oct4, Nanog, and Sox2 (4). Among the prominent regulators of pluripotency, the protein kinase B (Akt)/c-Myc axis has garnered increasing interest. Akt plays an essential role in various aspects of tumor growth, survival and therapeutic resistance in numerous types of cancer (5, 6). c-Myc is a proto-oncogene transcription factor that regulates several downstream signaling pathways. The functions of c-Myc include cell cycle progression, survival metabolism and stem cell activity (7, 8). Overexpression of the c-Myc protein results in aggressiveness and therapeutic resistance of cancers because of over-activated stemness properties (8, 9). The cellular availability of c-Myc is dependent on the stability of the protein which is controlled by Akt. Akt promotes the stability of c-Myc by inhibiting phosphorylation at threonine 58 (Thr58), which prevents protein degradation (10, 11). In addition, mTOR regulates c-Myc-driven tumorigenesis (12) and controls c-Myc stability (13).

A series of studies have demonstrated the cooperation between c-Myc and other self-renewal transcription factors, including Nanog, Sox2 and Oct4. These three self-renewal transcription factors possess predominant stemness activity (14, 15). c-Myc cooperates with other factors to regulate self-renewal. However, recent studies have demonstrated that c-Myc plays a role as a regulator of other self-renewal transcription factors by inducing the transcription and activity of the proteins (14). Hence, inhibiting Akt which is upstream of various CSC related regulators, is a promising strategy.

Marine-derived compounds are interesting biologically active compounds in cancer therapy. These compounds have complex structures that interact with various biomolecular targets to suppress or promote biological functions for treatment purposes (16). Natural marine compounds and their synthetic derivatives have been investigated in clinical trials (17). Marine-derived anticancer drugs, such as trabectedin and cytarabine, have been approved by the United States Food and Drug Administration (18). According to recent studies, the tetrahydroisoquinoline family and marine/microbial alkaloids such as renieramycins, ecteinascidins, saframycins, safracins and naphthyridinomycins, exhibit anticancer properties against cancer cells (19).

Renieramycin M is a marine alkaloid in the bistetrahydroisoquinolinequinone family that has been isolated from the Thai blue sponge, *Xestospongia* sp. renieramycin M exhibits anticancer activities in several cancer cells, such as lung, breast and colon (20). Interestingly, this compound not only suppresses non-stem cancer cells but also suppresses CSCs in lung carcinoma (21). Hydroquinone 5-*O*-cinnamoyl ester of renieramycin M (CIN-RM), which contains an additional cinnamoyl ester on C-5 and a hydroxyl moiety at C-8 of ring A, displays better apoptosis-inducing potency in H292 lung cancer cells (22). CIN-RM was synthesized by a two-

step chemical modification of renieramycin M involving palladium catalyzed hydrogenation and Steglich esterification (23). However, no study has reported on the suppression of CSCs by CIN-RM. This research aims to investigate the potential effect of CIN-RM on CSC of lung cancer and elucidated the underlying mechanism, which involved Akt regulating the c-Myc pathway. However, no study has reported on the suppression of CSCs by CIN-RM. This research aims to investigate the potential effect of CIN-RM on CSC of lung cancer and elucidated the underlying mechanism, which involved Akt regulating the c-Myc pathway.

1.2 Objectives of the study

1.2.1 To investigate the effect of hydroquinone 5-*O*-cinnamoyl ester of renieramycin M (CIN-RM) on lung cancer stem cell suppression.

1.2.2 To evaluate the underlying mechanism(s) of CIN-RM against CSC property in lung cancer effects caused by CIN-RM on lung cancer.

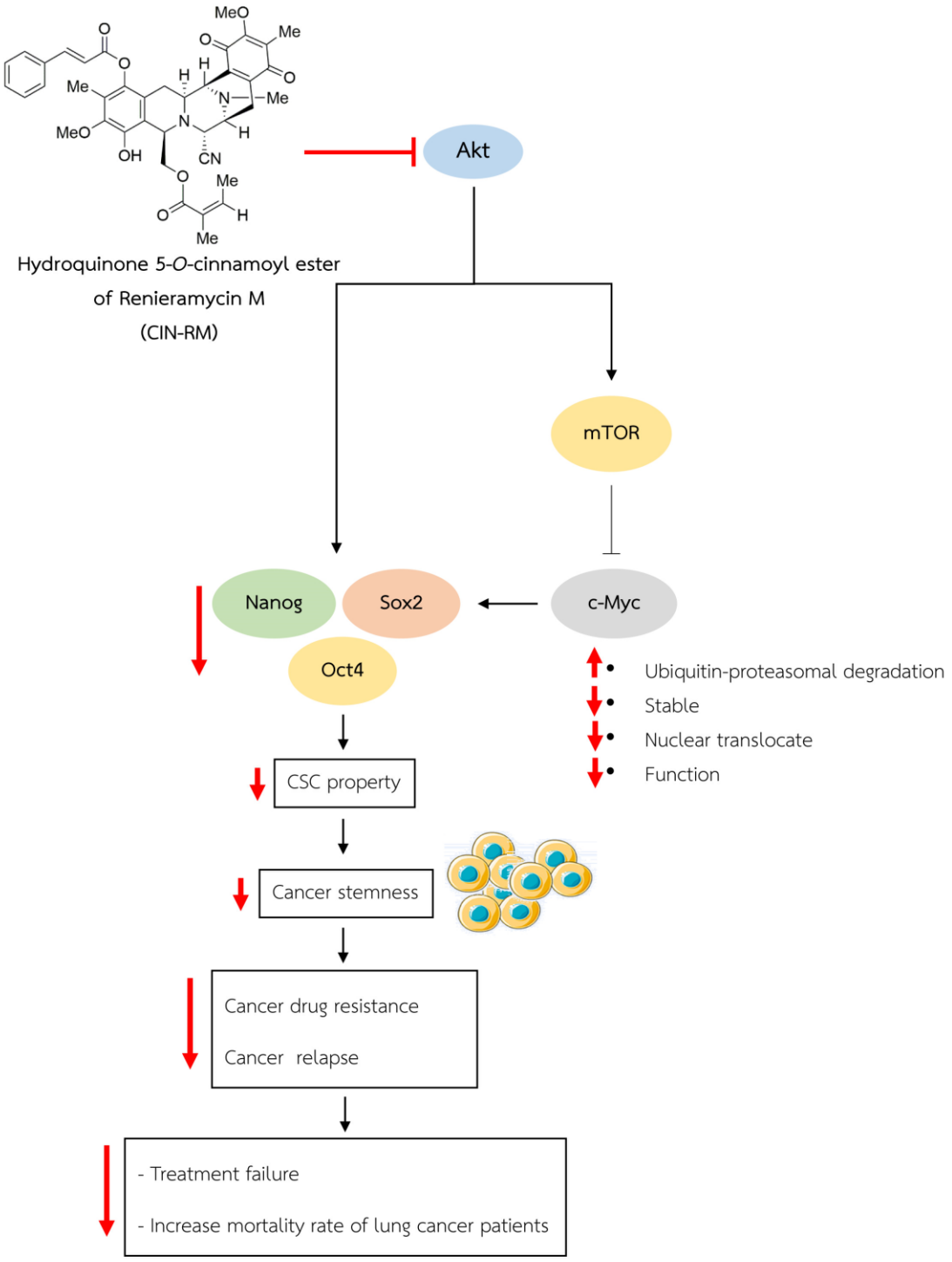
1.3 Hypothesis of the study

1.3.1 CIN-RM can suppress CSC by inhibiting AKT/c-Myc signaling pathway in lung cancer

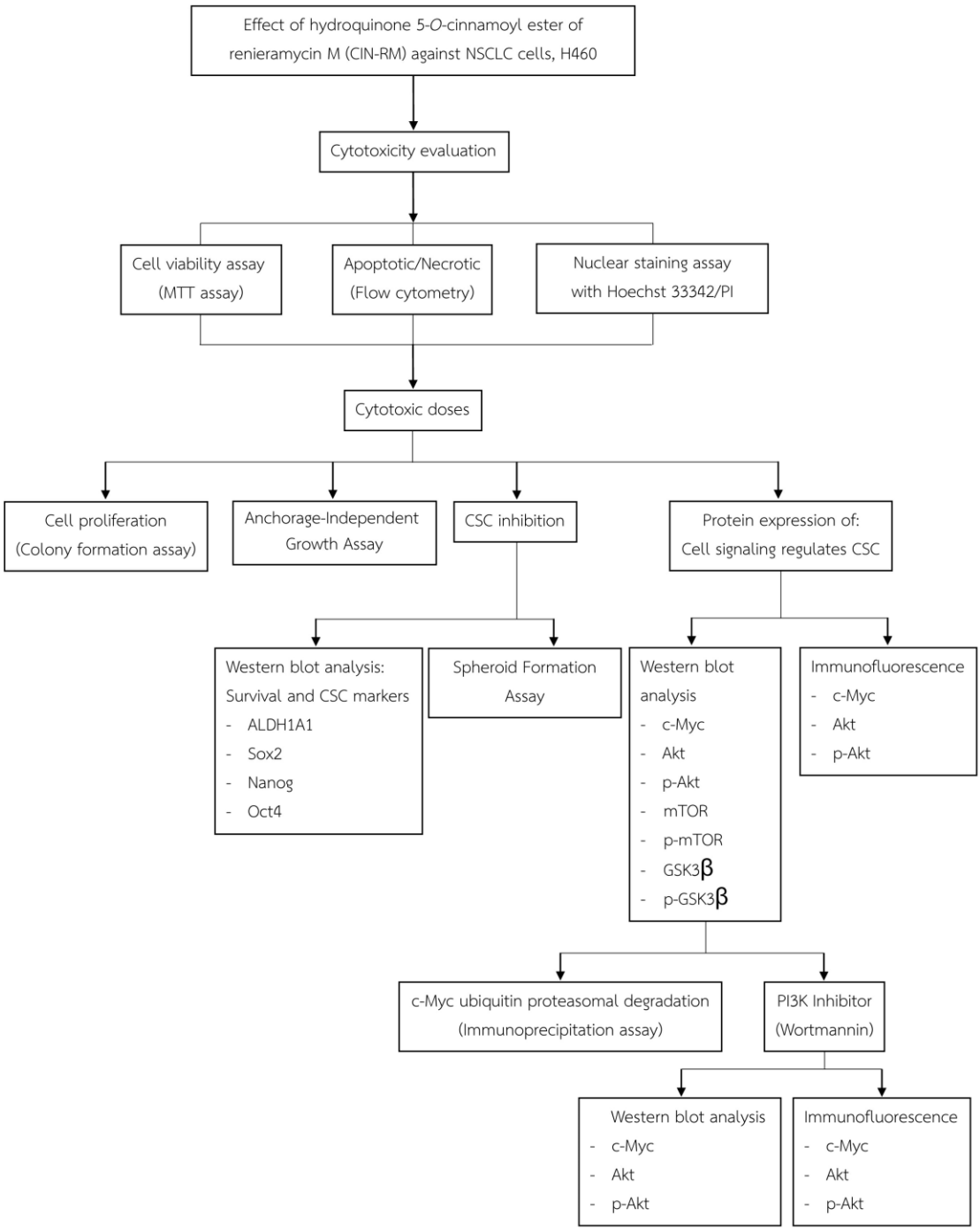
1.4 Benefits of the study

This study will provide the preliminary data of hydroquinone 5-*O*-cinnamoyl ester of renieramycin M including suppression of stem cell mechanism in non-small cell lung cancer and maintenance stem cell. The study can be used to further develop CIN-RM as an alternative treatment.

1.5 Conceptual framework



1.6 Research design



CHAPTER II

LITERATURE REVIEW

2.1 Lung cancer

Lung cancer is a cancer that initiates in the lungs and causes a death worldwide (24). Every year, over 1.8 million of lung cancer patients have been diagnosed (25). It is caused by bronchial mucosa cells that have been irritated for a long time, so it may be called a bronchogenic carcinoma. In 2018, 2.09 million of new cases and also 1.76 million of deaths have been reported. The cancer incidence and death rates have increased considerably compared to 2012. Smoking is still the major risk factor of this cancer (26).

2.1.1 Symptoms

Most lung cancer has not showed an obvious symptom in the initial stages. There are signs indicating the occurrence of the disease when more cancer growths. It can be observed from chronic cough, chest pain, fatigue, shortness of breath, pain while breathing or coughing, and weight loss. Some of rare symptoms may be occurred such as wheezing, changes of fingertip and nail shape, high fever, difficulty swallowing, hoarseness, and swelling of face and neck (27).

2.1.2 Risk factor

There are various risk factors for lung cancer including air pollution, genetic factor, and tobacco smoking. These factors are the causes of cancer development (27).

- Air pollution

Air pollution, both indoor and outdoor, is one of the main risk factor for lung cancer. This factor acquires more than 30% of the causes of lung cancer (28). The releasing of chemicals (SO_2, NOx, CO, and heavy metals) to the air and dust (PM) are considered important to cause air pollution and directly affect the respiratory system (29). In Taiwan, PM 2.5 was found to affect lung adenocarcinoma (AdLC) and the survival of patients (30).

- Genetic factor

Factors that cause cancer may come from external factors, but sometimes internal factors can also affect cancer, such as genetic factor. Family history is a genetic risk. If the family has a history of lung cancer, it will enhance the risk of disease as well (27). Genetic variation in the region $5_p15.33$ TERT-CLPTM1Ll affect lung cancer in non-smokers (31). Mutation of the epidermal growth factor receptor (EGFR) can induce cancer and the EGFR mutation in NSCLC patients is more common in female (Over 40%) than male (less than 15%) (32).

- Tobacco Smoking

Many researches have reported that tobacco smoking may be the major cause of this cancer, with a risk of 90% and 60% in male and female, respectively (32). In the smoking patients, the predicted prognosis is poorer than non-smoking (33). Tobacco smoke contains many carcinogenic (polyaromatic hydrocarbons, N-nitrosamines, acetaldehyde), these chemicals can cause lung cancer development in smokers and also secondhand smokers (34).

2.1.3 Type of Lung cancer

There are 2 subtypes, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) accounting for approximately 15% and 85% of all lung cancer respectively (35). NSCLC, the main type of lung cancer, is categorized into 3 subtypes including squamous cell lung carcinoma (25–30%) which has a strong relationship with smoking, large-cell lung carcinoma (5–10%) which starts in the middle of the lungs and can spread into nearby lymph nodes, and lung adenocarcinoma (40%) which is a key type of lung cancer. Lung adenocarcinoma has a slow growth rate and can be detected before it spreads outside the lungs compared to other types of lung cancer (36) (Figure 1).

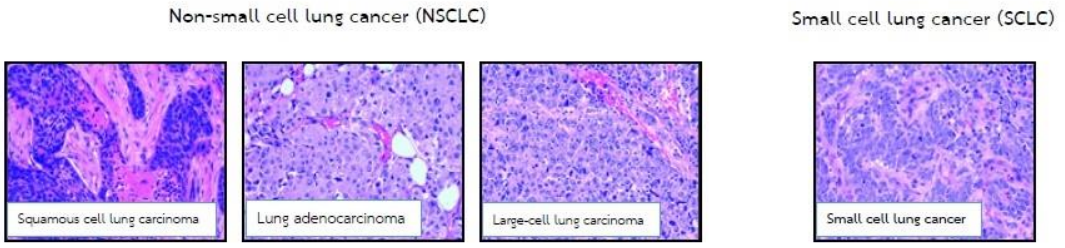


Figure 1 Histological classification of lung cancer cell (37)

2.1.4 Treatments for lung cancer

There are many options to cure lung cancer base on the type, the stage, and the patient's condition. The first treatment is surgical treatment, patients with stage I and II that have not lymph node spread, are suitable for surgical treatment (38). The second treatments are targeted therapy, chemotherapy, and immunotherapy. For NSCLC, the driver mutations are an important part of diagnostic of NSCLC. From

previous research, 50% of NSCLC patients had oncogenic drivers, and targeted therapy could improve overall survival (OS). This is the reason why FDA approved tyrosine kinase inhibitors (TKI), a one type of targeted therapeutic drug, for NSCLC treatment. However, the targeted therapy of SCLC has not been approved by the FDA yet. For immunotherapy, immune checkpoint inhibitors (ICIs) can be used to combine with chemotherapy (35). The last treatment is radiotherapy which is very essential in lung cancer treatment and it is used in palliative care. It can improve therapeutic response via a combination of immunotherapeutic agents and radiatio (39).

2.2 Cancer Stem cell (CSC)

Stem cells (SCs) are specialized cells which have the ability to self-renew, develop differentiate offspring in several different types of cells. Even with a small population, SCs are essential for replenishing aging cell and repairing damaged tissues (40). Cancer stem cells (CSCs), a small population inside the tumor hold stemness characters which promote tumor initiation, cell metastasis and tumor recurrence. Currently, CSCs are key factor contributing to low rate of successful treatments (41). Available chemotherapeutic drugs induce programmed cell death in normal cancer cells but not in CSCs (3). Other than that, CSCs show several characteristics like normal pluripotent stem cells including self-renewal, specific gene expression and protein markers, and the signaling pathways (42). These characteristics point out that CSCs are associated with tumor development and progression. In addition, previous research reported that the intrinsic and the extrinsic alterations in the SC tumor microenvironment, together with the mutations and the epigenetic regulations are involved and responsible for CSCs development (Figure. 2) (43).

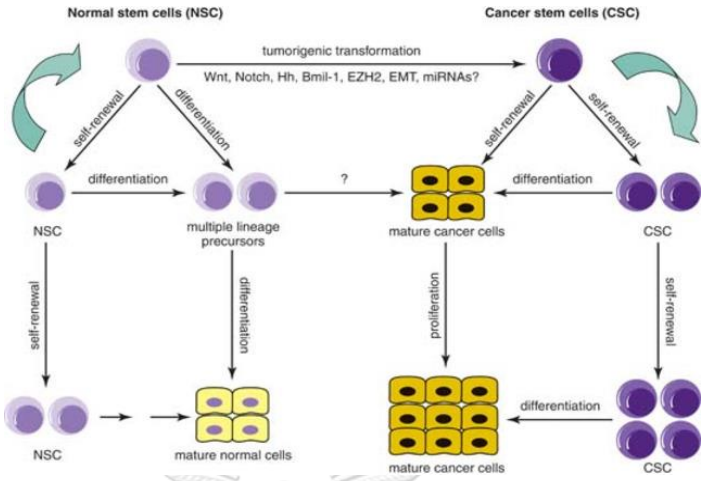


Figure 2 The implication of SCs in term of development and progression of tumors

(44)

CSCs is a small sub-population of cells within tumor and its characteristics are the same with NSCs. CSCs have the ability to initiate tumor formation, widespread proliferation, and cancer chemotherapeutic drug resistant (42). As shown in figure 3, CSCs (red) can self-renew and developed in cancer to form tumor bulk (yellow). When the tumor grows, cells can go limited benign growth or all cells may form disseminated malignancies. Therefore, cells are resistance to drug and leading to cancer recurrence (45).

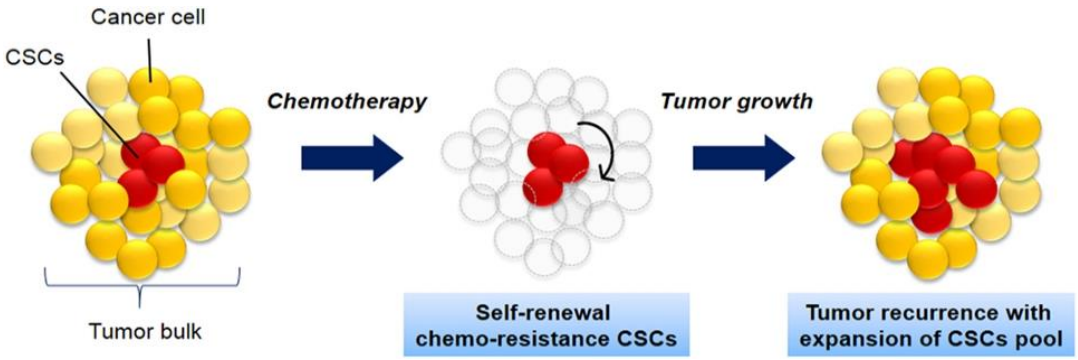


Figure 3 Cancer stem cells biology

(45)

In the United States, 2012 there are 226,160 of lung cancer patients were diagnosed and also 160,340 of deaths were reported (46). Although the diagnosis and treatment have been advance over the past decade, the prognosis remains poor due to its resistance in therapy, rapid tumor growth, and its ability to spread (47). CSCs are responsible for the aggressive phenotypes of lung carcinoma. CSCs expresses stem cell markers in lung cancer including CD133, CD44, ALDH, Oct4, and Nanog (48). Moreover, it important to be able to distinguish NSCLC and SCLC on the basis of CSC characteristics because it could effect on treatment strategies and prognosis (49).

2.2.1 Cancer stem cell marker

CSCs is a potential driving force of initiating new tumors and progression of cancer because of the self-renewal, drug resistance, metastasis and cancer recurrence (50). Up-regulation of specific stem cell protein markers including ALDH1A1 which relate to tumorigenesis and chemoresistance properties of CSCs (51).

ALDH

Another marker that use to identify and isolate the CSCs is aldehyde dehydrogenase. ALDH is an enzymes which can regulate the cell differentiation of NSCs (52). ALDH, intracellular enzymes, is involve in detoxification process and drug resistance in SCs (53). One of the members of the ALDH family is ALDH1. It is a cytosolic isoenzyme. The expression of ALDH1 of lung cancer cells have indicated highly tumorigenic and cancer cell cloning properties (54). Moreover, expression of ALDH1A1 on CSCs displayed chemotherapy resistance and EGFR-TKI (epidermal growth factor receptor tyrosine kinase inhibitors) resistance (55).

2.2.2 Cancer stem cell transcription factor

CSC can increase the expression of transcription factors which can regulate pluripotency property and self-renewal such as Nanog, Sox2, and Oct4, these factors making CSC different from NSCs. The overexpression of these proteins marker were determine in CSCs than ESC or normal cell that affects proliferation property and self-renewal (4). Transcription factors of stemness can modulated the stem phenotype such as self-renewal and pluripotency in NSCs and CSCs. Sox2, Nanog and Oct4 are commonly transcriptional factors that mediate CSCs properties in various cancer such as lung cancer (56),(57). Oct4 /Sox2 /Nanog complex recruits important transcriptional factors to induce stemness regulating proteins (58).

Nanog

Nanog, transcription factor, shows a critical factor in maintain the capacity of self-renewal of ESCs in the development of embryonic (59). In lung cancer patients with overexpression of this protein, the prognosis is worsened. Therefore, in the lung

cancer, Nanog protein is used as an key predictor in the prognosis (60). Nanog can control the self-renewal and cell potency in ESCs. In addition, the combination of Nanog with other protein including Oct4, Sox2 and Lin28 may be used to induce effective reprogramming process in fibroblasts to provide the induced pluripotent stem cells (iPSCs) (61). Nanog is not only regulates self-renewal and induced pluripotent stem cells but also displays in regulating ESCs cell cycle. Previous research has indicated that ESC clones with overexpression of Nanog protein have expedited S-phase entry. For the Nanog physiological conditions, the C-terminal was bind to CDK6 and CDC2 5 A at regulatory region, which mediate S-phase entry (62). Moreover, NanogP8 is more expressed in T-cell acute lymphoblastic leukemia and when knockdown of NanogP8 can suppressed proliferation of cells and self-renewal, induced apoptosis and cell cycle arrest via a p53 pathway (63). In addition, downregulation of Nanog cause G0/G1 cell cycle arrest, reduced cyclin E expression and STAT3 in breast cancer cell line (64). Nanog is important for control ESCs cell cycle. Therefore, the investigated Nanog in term of LCSC may be useful in the development of new therapies.

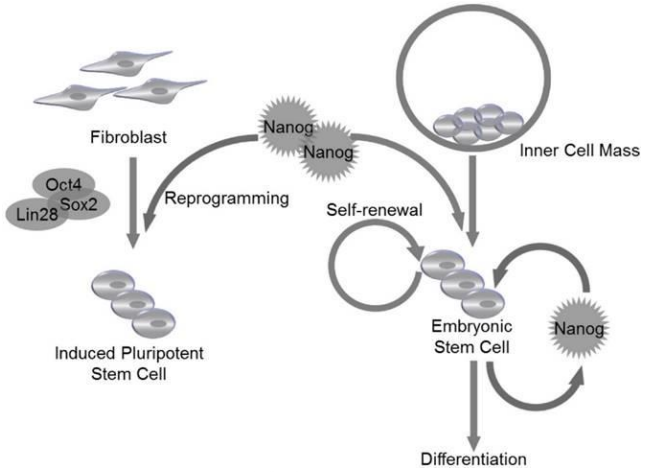


Figure 4 Nanog controls self-renewal and pluripotency of SCs (61)

Sox2

SOX2 (SEX determining region (SRY) homology box 2) is o the stem cell transcription factor. Up-regulation of Sox2 is currently reported in various type of lung carcinoma such as SCLC, squamous cell lung carcinoma and lung adenocarcinoma (65). Spheroid formation and CSCs apoptosis are regulated by Sox2 protein level (66). Previous research found that Sox2 has functionally in the repair the pluripotent of iPS and ES. In addition, Sox2 has been accepted as a powerful oncogene in numerous cancer, where it controls CSCs and functionally relates to hallmarks rule as shown in Figure 5 (67).

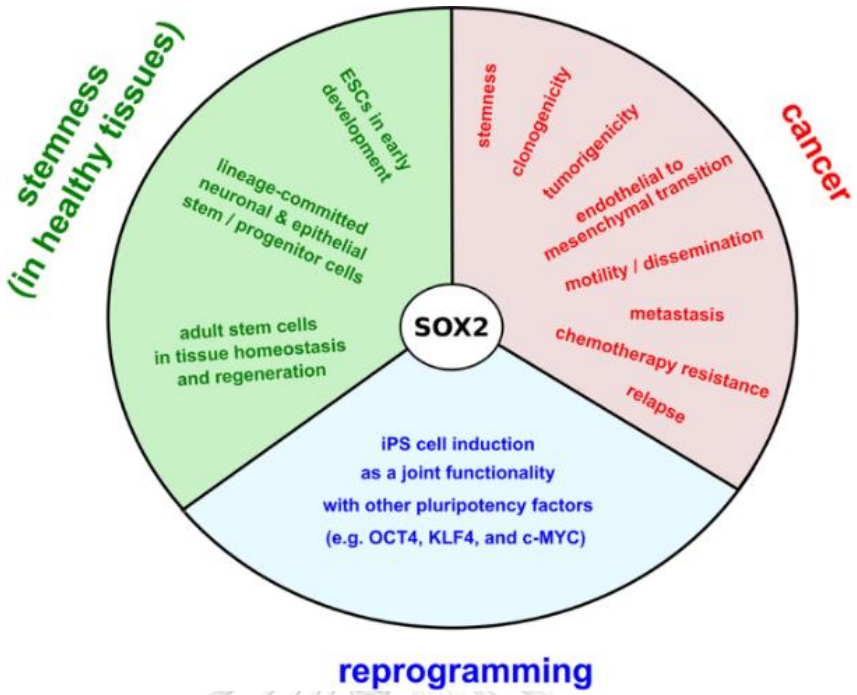


Figure 5 The overview of SOX2 functionality (67)

Oct4

Oct4, Octamer-binding transcription factor 4, is the one of CSCs transcription factor. It is an essential regulator of ESC fate. Cancer cells are similar in appearance to early embryonic cells, with common characteristics including deathless, undifferentiated, and invasive (68). So, dysregulation of overexpressed proteins during the embryonic stage can cause development of cancer. Oct4 is especially expressed in ESCs and a large amount is needed to maintain self-renewal of ESCs, which mean Oct4 is a main regulator of pluripotency in mammalian development (69). A previous study has showed that the expression of Oct4 shows a critical factor in keeping CSC characters in lung cancer with $CD133^{+}$ cells and suppression on Oct4 expression inhibits stemness phenotypes and metastasis feature in CSCs of lung cancer cells (70).

2.2.3 Cancer stem cell signaling pathways

From currently research reported that CSCs have various similar characters to NSCs including self-renewal and differentiation. Both CSCs and NSCs share many major pathway to remain its survival (71). CSCs have been reported to show various properties of embryonic or SCs tissue and cancer development pathways including Akt signaling pathway which plays an essential role in various aspects of tumor growth, survival and therapeutic resistance in numerous types of cancer (5, 6). The critical driving pathways of CSCs including the AKT signaling pathway was increased in cancers with high CSC properties (72). So, activation of this signaling may give a significant role in extension of CSCs and resistance to drug.

Akt regulate cancer stem cells

The AKT also known as a protein kinase B (PKB). It is an oncogenic protein which control survival, proliferation, apoptosis mechanism. The critical phosphorylation sites of Akt are Thr308 and Ser473 and it phosphorylates a several of downstream such as FOXO1, GSK3β, and mTOR (73). Activation of Akt/mTOR pathway enhanced CSC phenotypes in various cancer such as prostate cancer, breast cancer, and colorectal cancer (6).

Oct4/Sox2/Nanog complex recruits important transcriptional factors to induce stemness regulating proteins (58). These transcription factors were shown to be activated via several pathways including Akt pathway (65). The previous study showed that Akt directly regulates Oct4 and Sox2 activity (74-77). Akt increases the stability of the Oct4 protein by phosphorylating Oct4 at threonine 235. Phosphorylated Oct4 enters to the nucleus and interacts with Sox2, which in turn activate the transcription of Nanog (78).

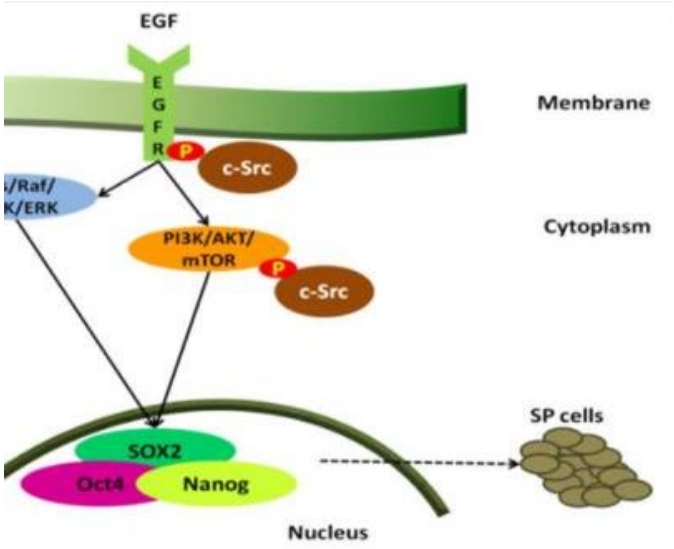


Figure 6 Role of Akt regulate stemness transcriptional factor complex (65)

The mammalian target of rapamycin (mTOR)

mTOR is a serine/threonine kinase. mTOR signaling pathway is critical in regulating growth, survival, metabolism of cell. The function of it acts through two structurally protein complexes which are mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is regulated by many pathways including the Akt signaling pathway. mTORC1 regulates several proteins, including protein phosphatase 2A (PP2A). Many previous research reported that mTOR signaling pathway is accumulated in various types of cancers. In the solid tumors, mTOR is dysregulated in approximatetl 30% of cancers. In NSCLC, PI3K signaling pathway activation is found about 50–70% of cancer patients with Akt phosphorylation (79). mTOR can also control the stability of c-Myc through PP2A inhibition (80).

c-Myc regulates cancer stem cells

c-Myc, proto-oncogene, plays a role as stem cell transcription factor that regulates several down-stream pathway. There are many functions of c-Myc such as cell cycle progression, cell survival, and stem cell activity (7, 8). Overexpression of c-Myc leading to over-activation of stemness properties (8, 9). c-Myc can cooperate with self-renewal transcription factors including Nanog, Sox-2 and Oct-4 to regulate the self-renewal property. (14). It is also a co-factor of Oct4/Sox2/KLF4 during pluripotent stem cell reprogramming process (81).

c-Myc gene is targeted in Wnt pathway. It is a critical role in switch. After suppression of β-catenin/TCF-4 activity of colorectal cancer cells, the decreasing of Myc gene leading to the transcription of p21, that results in differentiation and triggers G1 arrest (82).

Notch signaling can activate PI3K/AKT signaling pathway (83), and c-Myc. A recent research reported that in CRC cell lines, the activation of the Notch signaling could transcriptionally bind to c-Myc and cyclin D1 (84). Myc and cyclin D1 are related to the progression of the cell cycle. So, suppressing the Notch pathway for anti-cancer effect may involve in inhibition of the cell cycle progression (85-87).

Moreover, self-renewal in colorectal CSCs is depend on the Hedgehog-GLI via connect with the Wnt pathway (88). GLI-1 can reduces the expression of c-Myc, and can suppress the cell proliferation (89). It can indicate that c-Myc transcription factor is important in CSC therapy.

c-Myc maintains the chemoresistance

c-Myc protein is significantly role in drug resistance. The overexpression of c-Myc trigger downstream genes which related to cell cycle regulation and genomic instability (90-92). Several chromosomal abnormalities occur when c-Myc inducing genomic instability such as elevation of elements, centromere and telomere fusions, DNA double strand breaks, and genetic mutation. (92-95). In addition, the aggregation of genomic instability takes the susceptibility of tumor cells to DNA-damaging agents (Figure 7) (96, 97).

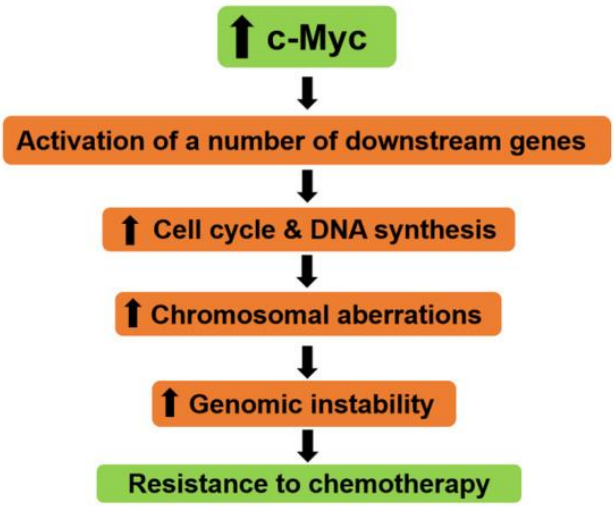


Figure 7 Roles of c-Myc in chemoresistance (8)

Akt/c-Myc signaling pathway in cancer stem cell

PI3K/Akt signaling pathways are important to cancer on physiological and pathological conditions including differentiation, proliferation, and cell survival (98). Previously researches showed that activation of the PI3K/Akt signaling pathway leading to EMT and enhanced CSC phenotypes on prostate cancer radioresistance

(99). PI3K/Akt pathway is significant for prostate CSCs maintenance and that targeting PI3K signaling may be beneficial in PC treatment by eliminating prostate CSCs (100). Myc is characterized as a regulator genes and proto-oncogenes which encode important nuclear transcription factors. As c-Myc has been recognized as an essential regulator of CSCs (7). Swords et al. demonstrated that when Akt pathway was disrupted, c-Myc was found to be rapidly decreased as a results of destabilization and enhanced degradation (11). c-Myc, a major downstream target of Akt, accompany Oct4, Nanog and Sox2 to promote self-renewal in CSCs (14). The stability of c-Myc is controlled by the Akt/mTOR pathway. mTOR inhibits Ser62 de-phosphorylation on c-Myc by hindering PP2A activity (80) leading to the stabilization of c-Myc.

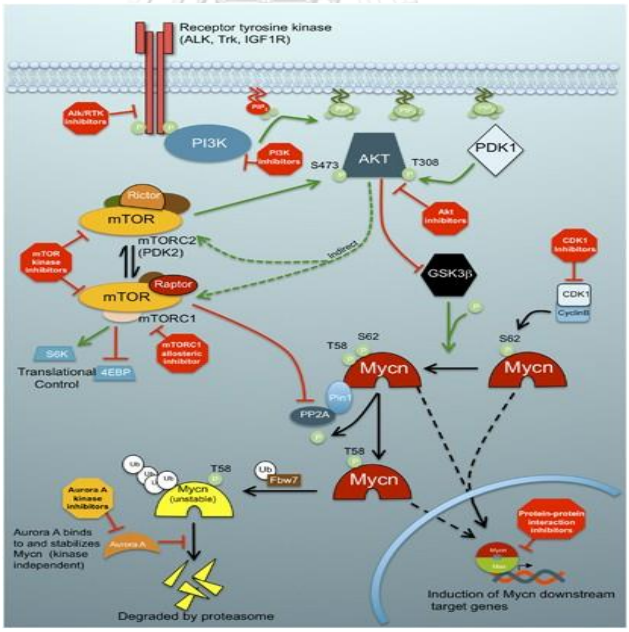


Figure 8 The stability of c-Myc is controlled by the Akt/mTOR pathway (80)

Not only stability of the protein requires Akt/mTOR activity, but c-Myc cellular function was shown to depend on Akt activity (101). The present research indicated that Thr308 and Ser473 are the 2 main Akt phosphorylation sites; activation of Thr308 phosphorylation may increase the Akt enzymatic activity (12). The phosphorylation at Threonine 58 (Thr 58) and serine 62 (Ser 62) of c-Myc is significant for c-Myc ubiquitin-proteasomal degradation (Figure 9) (10).

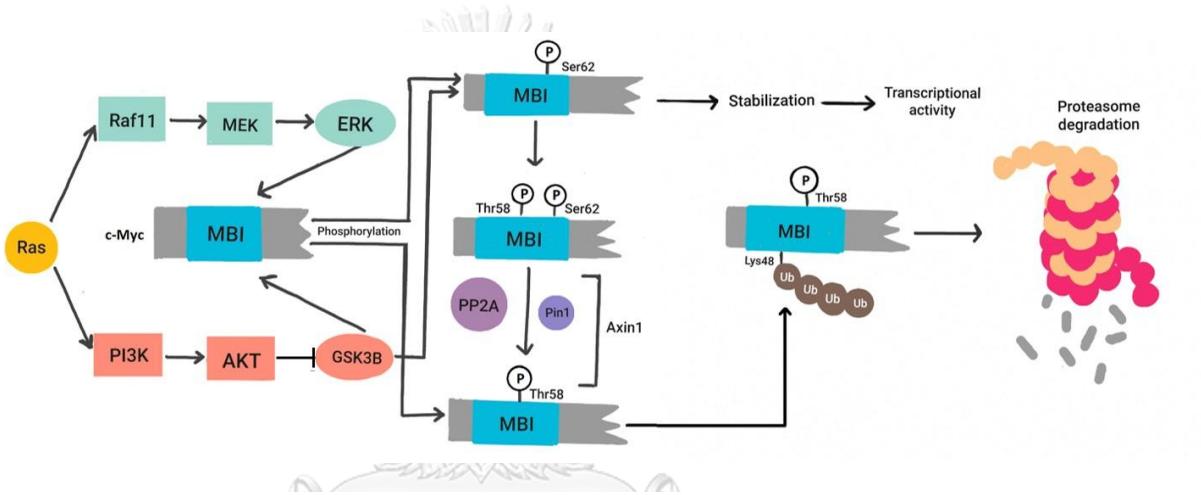


Figure 9 c-Myc ubiquitin-proteasomal degradation mechanism (10)

Taken together, inhibition of CSCs, whether it be alteration of CSC markers, or inhibition of CSC signaling pathways, may be a possible alternative for lung cancer treatment under such circumstances. At present, several studies indicate that marine compounds from various sources can inhibit CSCs. For example, 5-*O*-acetyl-renieramycin T induced apoptosis and decrease the CSC markers expression (CD44 and CD133) and decrease Nanog stem cell transcription factor via Akt signal pathway (102). *Crambe crambe* (CR), marine sponge extract, strongly reduced pancreatic and prostate CSCs (103). renieramycin M (RM) suppress CSC-like phenotypes in H460 lung

cancer cells (21). CIN-RM has more potent cytotoxicity than RM in H292 lung cancer cells. CIN-RM induced apoptosis through a p53-dependent mechanism (22). Therefore, in this research is interested in inhibits lung CSCs by using CIN-RM, which are marine compounds.

2.3 Phytochemicals and anti-cancer activities

Renieramycins, marine alkaloids classified into the tetrahydroisoquinoline family (19). 1,2,3,4-tetrahydroisoquinoline analogs show potential anti-cancer activity in many type of cancer such as lung cancer, and colon cancer (104). The 1,2,3,4-tetrahydroisoquinoline motif is the one of important as a minor groove DNA alkylator which can covalently bonds at N2-amine of guanine. This specifically functionalization result in DNA bending and leading to DNA damage in cancer cells (105). This result has indicate the significant of 1,2,3,4-tetrahydroisoquinoline scaffold as a model for anti-cancer candidates. Renieramycin M, bis-1,2,3,4-tetrahydroisoquinolinequinone alkaloid, is isolated from the Thai blue sponge *Xestospongia* sp. (106). Moreover, renieramycin M and its derivatives which modify ester side chains at C-22, showed a potential cytotoxicity in cancer cell lines including colon cancer , lung cancer , and breast cancer (107). From previously study of structure activity of RM and its derivatives with linear and aromatic ester side chains that focus on C-22 and C-5 have been evaluated for cytotoxic in NSCLC cell lines (23), which has been reported to be the world's leading cause of death (108). From present findings, the chemical modification of ester side chains at position C-22 and C-5 play a major relationship between structure and its cytotoxicity.

Hydroquinone 5-*O*-cinnamoyl ester of renieramycin M (CIN-RM) (Figure 10) was semi-synthesized from renieramycin M which is isolated from the Thai blue sponge *Xestospongia* sp. CIN-RM was obtained from two-step semi-synthesis of RM involving hydrogenation with 20% Pd(OH)2/C in EtOAc to obtain the

bishydroquinonerenieramycin M (HQ-RM) followed by esterification with cinnamoyl chloride and the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI.HCl) and 4-dimethylaminopyridine (DMAP) resulted in cinnamoyl substituent at C-5 position (23). Previous studies have revealed that renieramycin analogs have antimicrobial and anti-proliferation effects (109). For instance, renieramycin M induced apoptosis via p53-dependent signaling pathway and also inhibit progression and metastasis in lung cancer cell (110). Renieramycin M is an anti-metastatic agent by sensitizing anoikis-resistant in H460 lung cancer cells to anoikis via suppress anoikis-resistance mechanisms (111). Bishydroquinone renieramycin M (HQ-RM) induces apoptosis of H292 lung cancer cells through a mitochondria pathway (112). CIN-RM induced apoptosis effect on H292 lung cancer cells (22). From the previous research, it has not been reported about the suppression of CSCs activity on lung cancer of CIN-RM. Therefore, the researcher is interested in study the suppression of lung CSCs activity, which should lead to the discovery of important anticancer compound which may play a role in the development of a new type of anticancer drugs that effective treatment.

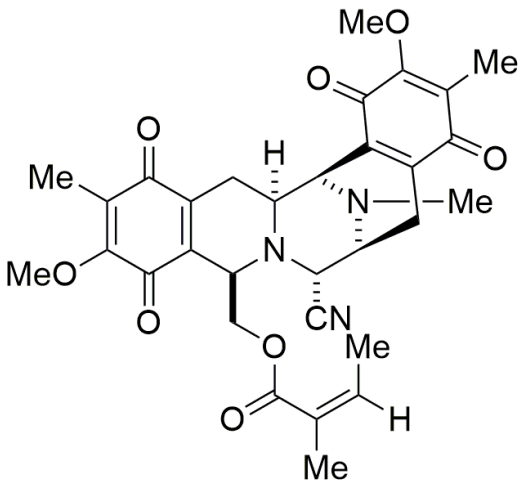


Figure 10 Structure of Hydroquinone 5-*O*-cinnamoyl ester of renieramycin M (CIN-RM)

CHAPTER III

METHODOLOGY

3.1 Material and Instruments

3.1.1 Non-small Cell Lung Cancer Cell Lines and Cultures

Non-small cell lung cancer cells used in the experiment was H460. H460 was obtained from the American Type Culture Collection (Manassas, VA, USA). H460 were grown in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 units/mL of each of penicillin and streptomycin under 5% carbon dioxide (CO_2) at 37 °C condition in an incubator.

3.1.2 Hydroquinone 5-O-cinnamoyl ester of renieramycin M (CIN-RM) preparation

CIN-RM is dissolved with dimethyl sulfoxide (DMSO) and diluted with Roswell Park Memorial Institute (RPMI) 1640 Medium cell culture in 10% fetal bovine serum (FBS) to obtain the desired concentration. The final concentration of DMSO will be less than 0.5% solution, which shows no signs of cytotoxicity.

3.1.3 Chemical and Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin, fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine, and trypsin-EDTA were acquired from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), propidium iodide (PI), Hoechst 33342, Triton X-100 ,bovine serum albumin (BSA), MG132, and paraformal-dehyde were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). Agarose was obtain from Bio-Rad Laboratories (Hercules, CA, USA). RIPA buffer were acquired from Cell Signaling Technology, Inc. (Danvers, MA). The primary antibodies

were used in the experiments including β-Actin (#4970), GSK3β (#9323), p-GSK3β (#9832), mTOR (#2983), p-mTOR (#5536), Akt (#9272), phosphorylated Akt or p-Akt (#4060), c-Myc (#5605), Nanog (#4903), Oct4 (#2840), Sox2 (#3579), and ALDH1A1 (#36671) were acquired from Cell Signaling Technology (Danvers, MA, USA). The primary antibody ubiquitin (ab7780) was purchased from Abcam (Cambridge, UK). The respective secondary antibodies, anti-rabbit IgG (#7074) and anti-mouse (#7076) were also obtained from Cell Signaling Technology (Danvers, MA, USA).

3.1.4 Equipment

- CO_2 incubator (Thermo forma)
- Oven (United instrument Co., Ltd., Thailand)
- Water bath (Memmert, Chatcharee Holding Co., Ltd., Thailand)
- Fume hood FH120 (BossTech)
- Nikon Eclipse Ts2 microscope
- Microplate reader Perkin Elmer VICTOR3 (Anthros, Durham, USA)
- Guava flow cytometer (Merck Millipore)
- SDS-PAGE (Bio-rad)
- Chemiluminescent ImageQuant LAS4000
- 60- and 100-mm dish culture (Corning Inc., USA)
- 6, 24 and 96 well plate (Corning Inc., USA)
- 1.5 ml microcentrifuge tube (Corning Inc., USA)
- 0.2-2 µl, 2-20 µl, 10-200 µl and 200-1000 µl micropipettes (Corning Inc., USA)
- 2 µl, 20 µl, 200 µl and 1000 µl micropipette tips (Corning Inc., USA)

3.2 Methods

3.2.1 Cytotoxicity Assay

To study the cytotoxicity of CIN-RM on NSCLC cell lines (H460), MTT colorimetric assay was used. H460 $1.5x10^4$ cells/well were cultured in 96-well tissue culture plate with 100 µL/well RPMI in 10% FBS at 37 °C under 5% CO2 in incubator overnight. Cells in 96-well plates were treated with various concentrations of CIN-RM (0, 0.1, 0.5, 1, 5, 10, 20 µM) for 24 h. After that, cells were incubated with 0.4 mg/mL MTT for 3 h at 37°C. Then, 100% DMSO was added to dissolve the formazan crystals. The intensity of the MTT product was measured at 570 nm by a microplate reader (Anthros, Durham, NC, USA). The percentage of cell survival (% Cell viability) and IC50 were calculated as described in the manufacturer's protocol (7sea Biotech, Shanghai, China). Cell viability (%) = (ODexperi-ment – ODblank)/(ODcontrol – ODblank) × 100%.

3.2.2 Nuclear Staining Assay

Apoptosis and necrosis cells death were analyzed with Hoechst 33342 and PI fluorescent DNA co-staining assay. The cells were seeded 1×10^4 cells/well in 96 well plate and incubated overnight. Then, the cells were treated with CIN-RM at various concentrations (0-20 µM) for 24 h. After that, cells were incubated with 10 µg/mL Hoechst 33342 and 5 µg/mL PI for 30 min at 37°C. Then, cells were visualized by fluorescence microscopy (Nikon ECLIPSE Ts2) and the analysis was evaluated by ImageJ software.

3.2.3 Apoptosis Assay

Apoptotic and necrotic cells were investigated using Annexin V-FITC apoptosis kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were treated with various concentrations (0-20 µM) of CIN-RM, incubated at 37 °C for 24 h. Treated-cells were

trysinized and suspended in 70 µL of 1X binding buffer. Then, incubated with Annexin V/FITC in the dark at room temperature for 15 min. Then, binding buffer was added up to 400 µL and the cells were stained with PI before performed flow cytometry assay. The cells were analyzed with BD FACSDiva 8.0.2 flow cytometry systems.

3.2.4 Colony Formation Assay

H460 cells were pretreated with various concentrations (0-20 µM) of CIN-RM for 24 h. Next, CIN-RM-treated cells were detached and seeded approximately 300 cells/well onto a 6-well plate and let them formed colonies at 37 ℃ for 7days. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature, followed by staining with crystal violet solution at room temperature for 30 min and washed with tap water. The colony number and size were investigated by OpenCFU software.

3.2.5 Anchorage-Independent Growth Assay

Soft agar colony formation assay was used to determined anchorage-independent growth cell growth. The cells were pretreated with various concentrations (0-20 µM) of CIN-RM for 24 h. For the preparation of the agar, 1:1 ratio mixture of RPMI medium containing 10% FBS and 1% agarose in a 24-well plate to form a bottom layer. For an upper layer contain 8×10^3 living cells/mL in the agarose gel with 10% FBS and 0.3% agarose. When the upper layer was solidified, RPMI medium containing 10% FBS was added and then incubated at 37°C. Phase-contrast images of colony formation were taken at day 7, 14, and 21 of treatment using a phase-contrast microscope (Olympus IX51 with DP70, Melville, NY, USA). The colony number and size were investigated by ImageJ software.

3.2.6 Spheroid Formation Assay

H460 cells were pretreated with various concentrations (0-20 μM) of CIN-RM for 24 h. The cells were detached and seeded approximately 2.5 × 10^3 cells/well onto a 6-well ultralow attachment plate with in serum free RPMI medium and incubated. Spheroid for-mation was determined after 7 days using a phase-contrast microscope (Nikon ECLIPSE Ts2). The analysis was evaluated by ImageJ software.

Meanwhile, H460 cells were seeded approximately 2.5 × 10^3 cells/well onto a 6-well ultralow attachment plate with serum free medium and incubated for 7 days to form primary spheroids. Then, the primary spheroids were suspended into single cells and seeded onto a 96-well ultralow attachment plate with serum free medium for 14 days to form secondary single spheroids. After that, the spheres were treated with various concentrations of CIN-RM (0–20 μM) and incubated for 24 h in an environment of 37 °C with 5% CO_2. At 24 h after treatment, apoptosis cell death was analyzed with Hoechst 33342 and imaged using a phase-contrast microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan).

3.2.7 Immunofluorescence

H460 cells were seeded in 96-well plates at the density of 8 × 10^3 cells/well and incubated overnight. Then, H460 cells were treated with various concentrations (0-20 μM) of CIN-RM and 2.5 and 5 μM of wortmannin. Next, cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilized by 0.5% of Triton X-100 in PBS for 5 min, and then blocking with 10% of FBS in 0.1% of Triton X-100 for 1 h at room temperature. Primary antibody of c-Myc and p-Akt at proportional 1:200 in 10% of FBS were applied before incubation overnight at 4 °C. After that, Alexa Fluor 488 IgG secondary antibody was added and incubated for 1 h in dark at room temperature. Hoechst 33342 was used to stain cell nucleuses and then visualized

under fluorescent microscope (Nikon ECLIPSE Ts2, Tokyo, Japan) and the analysis was evaluated by ImageJ software.

3.2.8 Immunoprecipitation Assay

H460 cell lines were pretreated with 10 μM of MG132 for 1 h followed by 10 μM of CIN-RM for 3 h. The treated cells were obtained and lysed with RIPA buffer. The magnetic beads from Dynabeads™ Protein G Immunoprecipitation Kit were washed with washing buffer and incubated with c-Myc primary antibody (Ab) in binding buffer about 10 min. Then, adding the protein lysate to the bead-Ab complex suspension overnight at 4℃. Then, the complex was washed 3 times with 100 μL washing buffer. Supernatant was discorded and then the elution buffer was added for detaching the Ab-Ag complex from the beads. Finally, western blot analysis was used to evaluate the ubiquitinated c-Myc protein.

3.2.9 Western blot analysis

Western blotting was a technique for detecting specific proteins in a sample. Cells were seeded at density 4 x 10^5 cells/well in 6 well plates overnight. Cells were treated with CIN-RM at various concentrations (0-20 μM) for 24 h and 2.5 and 5 μM of wortmannin for 12h . Then, cells were washed with PBS (on ice) and incubated on ice for 30 min with 1X RIPPA 60 μL containing 10x RIPA buffer 100 μL, protease inhibitors (PI) 100 μL, PMSF 10 μL, and Triton X 10 μL. Protein content was analyzed using BCA protein assay. The extracted proteins were separated with gel electrophoresis using 7.5%-15% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel). After that, the proteins were transferred from the gel to the polyvinylidene difluoride (PVDF) membrane, blocked with milk medium (Tris-HCl (pH 7.5) 25 mM, NaCl 125mM, and 0.05% Tween20 (TBST) 0.05%) and 5% nonfat dry milk powder for 2 h, and incubated overnight with primary antibodies that specific to the proteins

(ALDH1A1, Nanog, Oct4, Sox2, GSK3β, p-GSK3β, mTOR, p-mTOR, Akt, p-Akt, c-Myc, and beta-actin). Then, the membranes were washed with TBST 3 times and then incubated with the secondary antibodies for 2 h at room temperature. Immunoreactive proteins were detected with the chemiluminescent evaluation system and subsequently exposed by Chemiluminescent ImageQuant LAS4000. Protein bands were analyzed using the ImageJ software.

3.2.10 Statistical analysis

The results from three independent experiments (n = 3) were presented as means ± standard deviation for each group. Statistical differences between groups were analyzed using an analysis of variance (ANOVA), followed by individual comparisons with Schefft's post-hoc test. For two-group comparison, t-test analysis was calculated. The statistic was calculated by SPSS software program version 16 (SPSS Inc., Chicago, IL, USA). The p-value of less than 0.05 was considered as statistically significant. *p < 0.05, **p < 0.01, and ***p < 0.001. GraphPad prism 5 was used to created graphs in this experiment (GraphPad Software, San Diego, CA, USA).

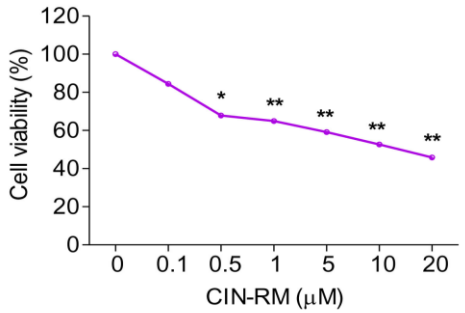
CHAPTER IV

RESULTS

4.1 Selective cytotoxicity of CIN-RM in human lung cancer cells

To elucidate the anticancer potential of CIN-RM (Figure 10), we determined the cytotoxic profile of CIN-RM in lung cancer H460 cells. The cells were incubated with CIN-RM (0–20 μM) for 24 h. The results showed that CIN-RM significantly reduced the viability of H460 cells (Figure 11A) with a half maximal inhibitory concentration (IC_{50}) value of 14.64 ± 7.09 μM (Figure 11B).

A



B

Cell	IC_{50} ± SEM (μM)
H460	14.64 ± 7.09

Figure 11 Effect of CIN-RM on cell viability of lung cancer H460 cells.

(A) The significant reduction of % cell viability in human lung cancer H460 cells with CIN-RM at 0–20 μM for 24 h. (B) The half maximal inhibitory concentration (IC_{50}) of CIN-RM in H460 cells was 14.64 ± 7.09 μM. All data are presented as the mean ± SEM (n = 3). * $p < 0.05$, and ** $p < 0.01$ compared with untreated cells.

Further investigations of CSC-targeting activity of the compound were performed in H460 cells treated with 0–20 μM CIN-RM. Apoptosis is characterized by condensation and fragmentation of DNA. Therefore, we examined whether the majority of the cytotoxic effects caused by CIN-RM was related to apoptosis. Hoechst 33342 staining was used to evaluate the nuclear morphology of the CIN-RM-treated cells. H460 cells were treated with 0–20 μM CIN-RM for 24 h. In addition, propidium iodide (PI), fluorescence dye was used to detect for necrosis; however, no PI-positive cells were detected at the tested CIN-RM concentrations. These results reveal that 1-20 μM CIN-RM induced apoptosis cell death indicated by the clear presence of DNA condensation and/or fragmentation (Figure 12). The reduction in cell viability detected by MTT correlated well with the induction of apoptosis at the same concentrations.

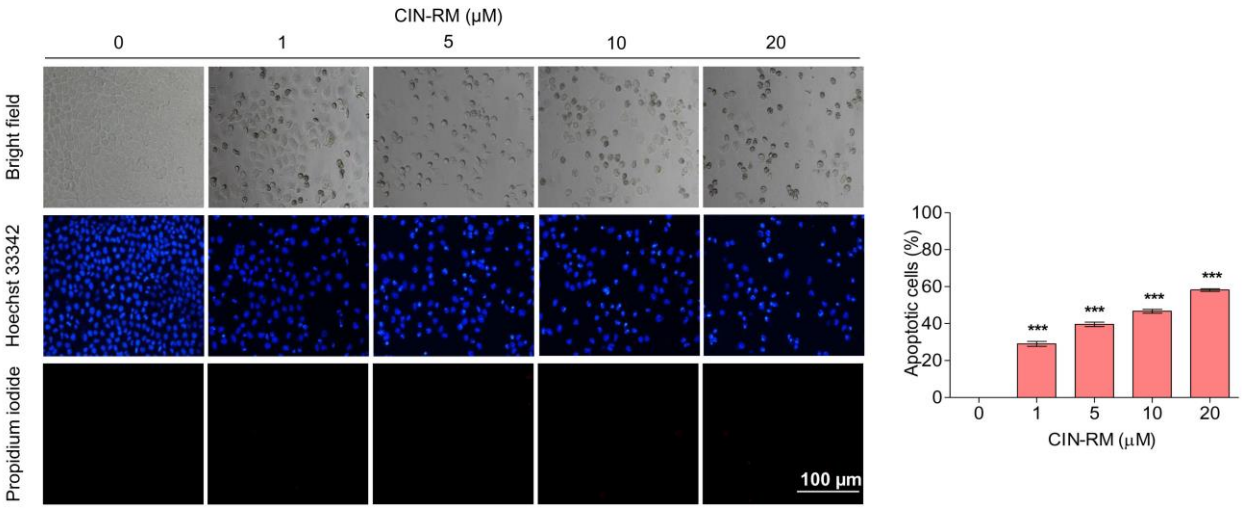


Figure 12 Apoptosis effect of CIN-RM on H460 cells.

Apoptotic H460 cells were detected by Hoechst 33342/PI staining and visualized by fluorescence microscopy. The percentage of apoptotic cells in CIN-RM-treated cells was analyzed. All data are presented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

The apoptosis inducing activity of CIN-RM was confirmed by annexin-V-FITC/PI staining with flow cytometry analysis. Similar to the nuclear staining assay, CIN-RM significantly induced apoptotic cell death in concentration of 1-20 μM (Figure 13). Our results suggest that apoptosis was the main mode of cell death of the CIN-RM treated cells.

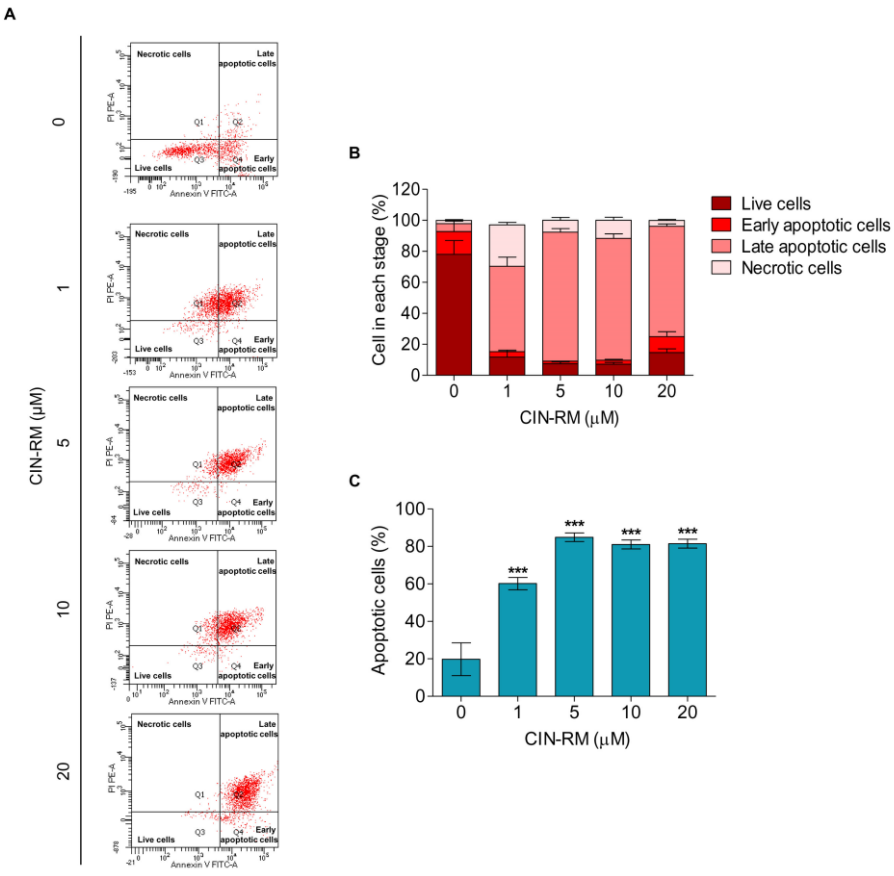


Figure 13 Effect of CIN-RM on apoptosis by flow cytometry on H460 cells.

(A) To confirm apoptosis inducing activity in H460 cells, the cells were treated with various concentrations of CIN-RM (0–20 μM) for 24 h, and apoptosis was evaluated by annexin V-FITC/PI staining. (B-C) The percentage of cells in each stage and the percentage of apoptotic cells were calculated. All data are presented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

It is widely accepted that CSCs can escape apoptosis in response to chemotherapy. Next, we tested whether CIN-RM had this effect on a resistant cell population in the modified colony formation assay. Surviving H460 cells after a 24-h CIN-RM (1, 5, 10, and 20 μM) treatment were subjected to a clonogenic assay without further treatment. Crystal violet-stained colonies represent the capability to reproduce a new cancer colony from a single cell was shown in Figure 14. It was shown that the resistant cells receiving CIN-RM at 1-20 μM could not form the colonies

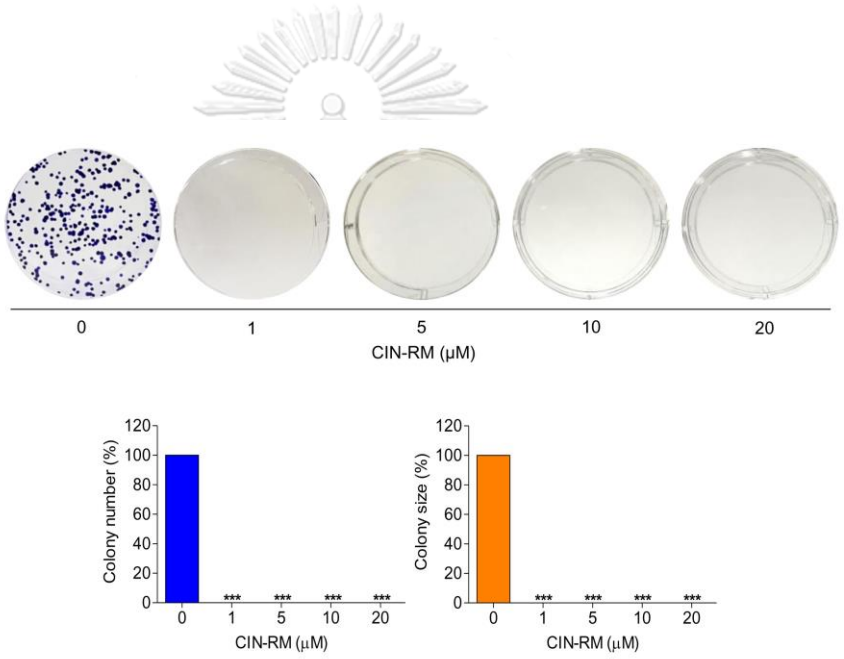


Figure 14 Effect of CIN-RM on cell proliferation by colony formation assay on H460 cells.

Cells were treated with various concentrations of CIN-RN (0–20 μM) for 24 h before being subjected to forming colonies for 7 days, then the colonies were stained with crystal violet. All data are presented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

4.2 CIN-RM Attenuates Anchorage-Independent Growth and Suppresses CSC Spheroid Formation

It was previously reported that the process of anchorage-independent growth of cancer cells reflects the anoikis-resistant capability of malignant tumor cells (113). To test whether CIN-RM could suppresses such cancer cell survival and the ability to grow under detached conditions, H460 cells were treated with CIN-RM for 24 h. The surviving cells were collected and grown for 7, 14, and 21 days in soft agar for the anchorage-independent growth assay. The number and size of the growing cancer colonies were determined and calculated relative to those of the untreated control. The results indicated that the CIN-RM-pretreated cells exhibited decreased anchorage-independent growth compared with the untreated control (Figure 15). As illustrated in Figure 15 , the numbers of H460 cell colonies decreased significantly in response to the 1, 5, 10 and 20 μM CIN-RM treatment and the percentage of the colony size in response to 1, 5, 10 and 20 μM CIN-RM were 100%. These results suggest that the CIN-RM treatment may affect the signaling pathways influencing the growth of cancer cells in the detached condition.

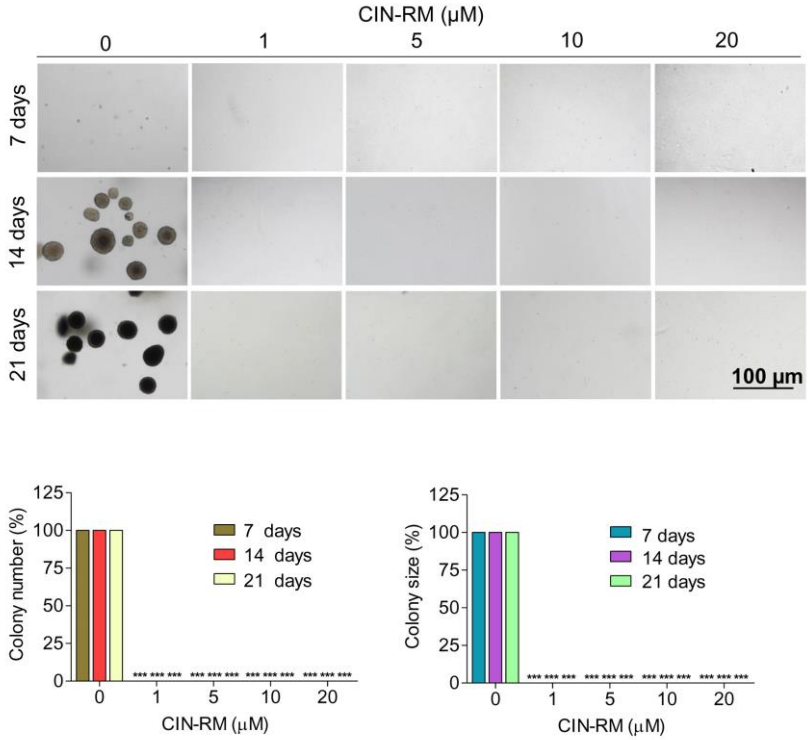


Figure 15 CIN-RM suppresses anchorage-independent growth of H460 cells.

Cells were pretreated with CIN-RM for 24 h, and the surviving cells were subjected to an anchorage-independent growth assay. Data are represented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

As the ability of cancer cells to form tumor spheroids has been used to reflect the CSC phenotype, we studied the effect of CIN-RM on spheroid formation. H460 cells were treated with various concentrations of CIN-RM (0–20 μM) for 24 h, and the cells were subjected to the spheroid formation assay. The primary spheroids were captured under a microscope after 7 days. The results show that the untreated control cells had a high ability to form primary tumor spheroids, whereas the cells treated with CIN-RM exhibited a completely abolished of tumor spheroids at any dose (Figure 16).

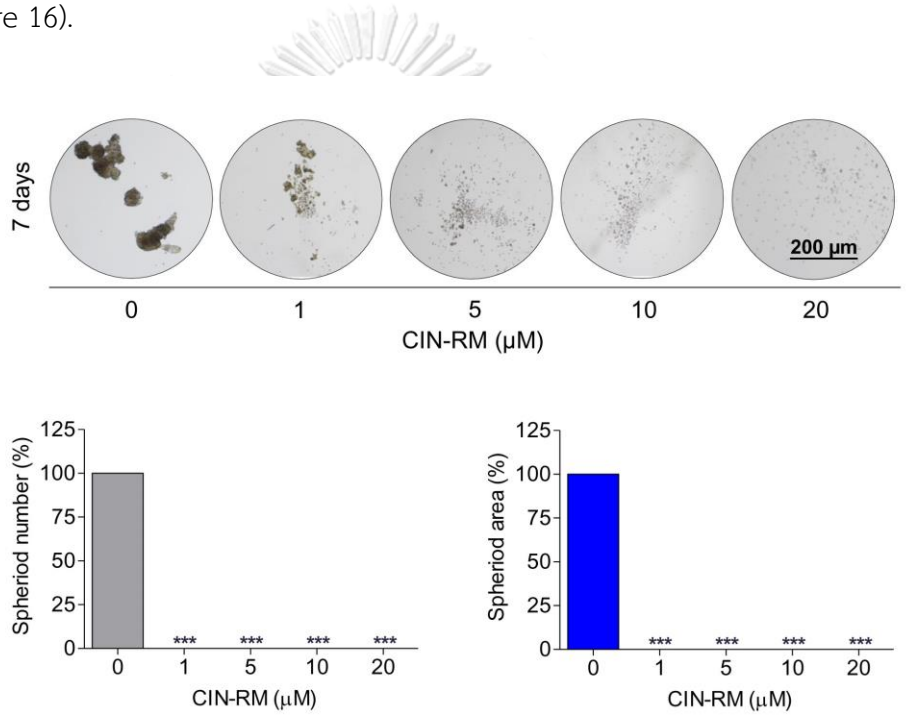


Figure 16 Effect of CIN-RM on spheroid formation on H460 cells.

Cells were pretreated with CIN-RM for 24 h and allowed to form primary spheroids for 7 days, and the spheroid of the CSC population was determined. Data are represented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

To further confirm this CSC suppressing activity, a CSC-rich population was established form secondary spheroid of control cells. The CSC spheroids were seeded in ultralow attach 96-well plates at a density of one spheroid per well. The spheroids were treated with CIN-RM (0-20 μM) for 24 h. The results showed that the untreated control spheroid survived and maintained the integrity of tumor spheroid, whereas the CIN-RM-treated cells revealed a dissociated pattern of spheroids (Figure 17A). Hoechst 33342 staining further revealed apoptosis character of DNA fragmentation and/or DNA condensation in the CIN-RM treated cells (Figure 17B).

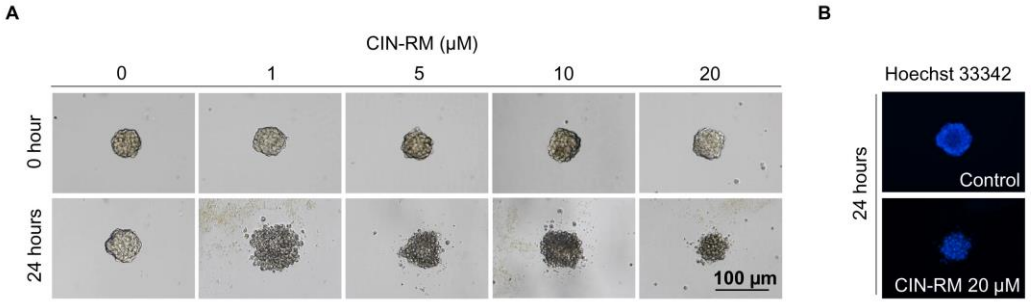


Figure 17 Effect of CIN-RM on CSC secondary spheroid on H460 cells.

(A) The CSC single spheroid was treated with toxic concentrations of CIN-RM for 24 h. (B) The apoptosis cell death was analyzed with Hoechst 33342.

After showing that CIN-RM suppressed CSC properties, we next confirmed this result by evaluating the CSC markers in CIN-RM-treated cells. CSCs highly express self-renewal transcription factors and detoxifying enzymes, such as ALDH1A1, Oct4, Nanog, and Sox2 (4). The H460 cells were treated with various concentrations of CIN-RM (0-20 μM) for 24 h and expression of the ALDH1A1, Oct4, Nanog, and Sox2 proteins was measured (Figure 18A). The results showed that Oct4 and Sox2 were dramatically decreased at 1 μM of CIN-RM, while Nanog decreased significantly at 5 μM. ALDH1A1 decreased significantly at 10 μM (Figure 18B) when compared with the untreated control.

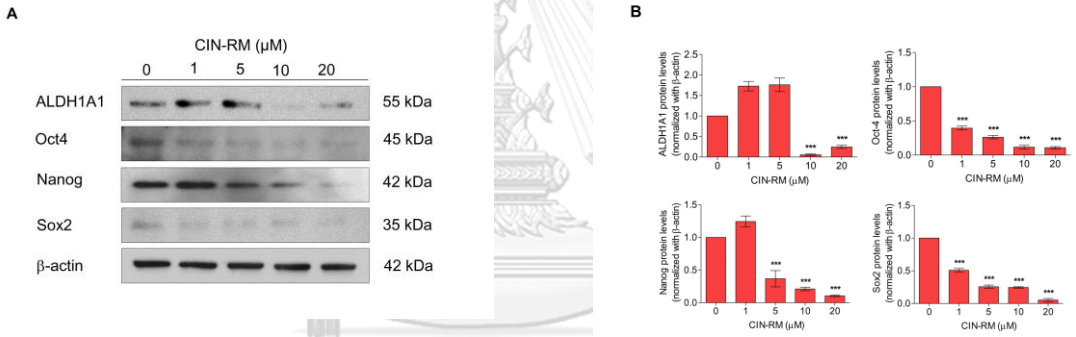


Figure 18 CIN-RM suppresses cancer stem cell (CSC)-like phenotype of human lung cancer cells.

(A) H460 cells were treated with various concentrations (0–20 μM) of CIN-RM for 24 h. The expression of ALDH1A1, Oct4, Nanog, and Sox2 were determined by Western blotting. β-actin was determined to confirm equal loading of the samples. (B) Densitometry of each protein was calculated and the results were presented as relative protein levels when compared with untreated control. Data are represented as the mean ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

4.3 CIN-RM Suppression of CSC Is Mediated Via Akt Inhibition

c-Myc and Akt play major roles in cell survival, proliferation and stem cell properties. It has known that Akt controls the degradation of c-Myc by ubiquitin proteasomal degradation (11). To investigate whether CIN-RM suppresses CSCs through the Akt/c-Myc signaling pathway, H460 cells were treated with various concentrations (0–20 μM) of CIN-RM and investigated by western blot analysis (Figure 19A). The results indicated that the expression of p-Akt, p-mTOR, and c-Myc decreased significantly, while the expression of p-GSK3β (Ser9) decreased slightly compared to those of non-treated control cells (Figure 19B), suggesting that the CSC-suppressive activity of the compound may, at least in part, via Akt/c-Myc inhibition.

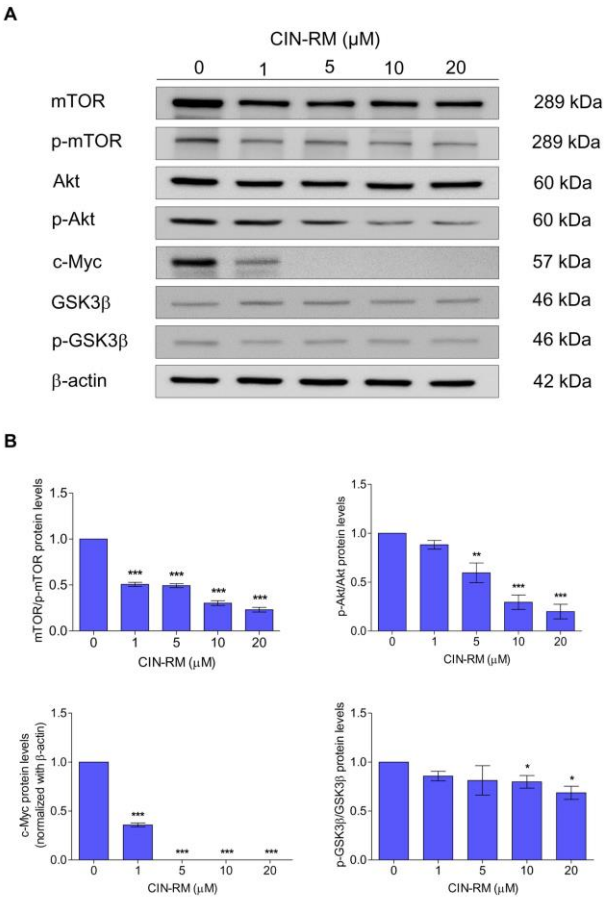


Figure 19 CIN-RM suppresses CSCs through the Akt/c-Myc signaling pathway.

(A) H460 cells were treated with various concentrations (0–20 μM) of CIN-RM for 24 h and the expression levels of mTOR, p-mTOR, GSK3β, p-GSK3β, Akt, p-Akt, and c-Myc protein were investigated by Western blotting. (B) Blots were reprobed with β-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry. Values are presented as means ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with untreated cells.

We further confirmed the inhibitory effect of CIN-RM on the Akt/c-Myc signaling pathway using an immunofluorescence staining assay. H460 cells were treated with 0–20 μM of CIN-RM for 12 h before incubated with p-Akt and c-Myc primary antibodies. Overall, p-Akt and c-Myc fluorescence intensity decreased significantly in the cytoplasm and nucleus (Figure 20,21). Interestingly, while the level of p-Akt was evenly distributed in both cell compartments (Figure 20B), c-Myc was predominantly located in the nucleus of un-treated control cells (Figure 21B).

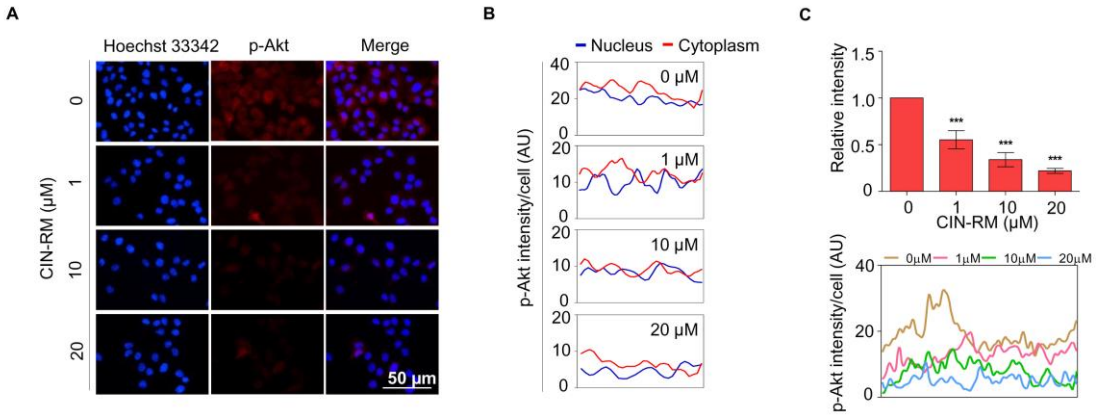


Figure 20 CIN-RM suppresses p-Akt on H460 cell.

(A) H460 cells were treated with CIN-RN at toxic concentrations for 12 h. The cellular levels of p-Akt were determined by immunofluorescence analysis. (B) The fluorescence intensity of the nucleus and cytoplasm were analyzed by ImageJ software. (C) The fluorescence intensity was analyzed by ImageJ software. Values are presented as means ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

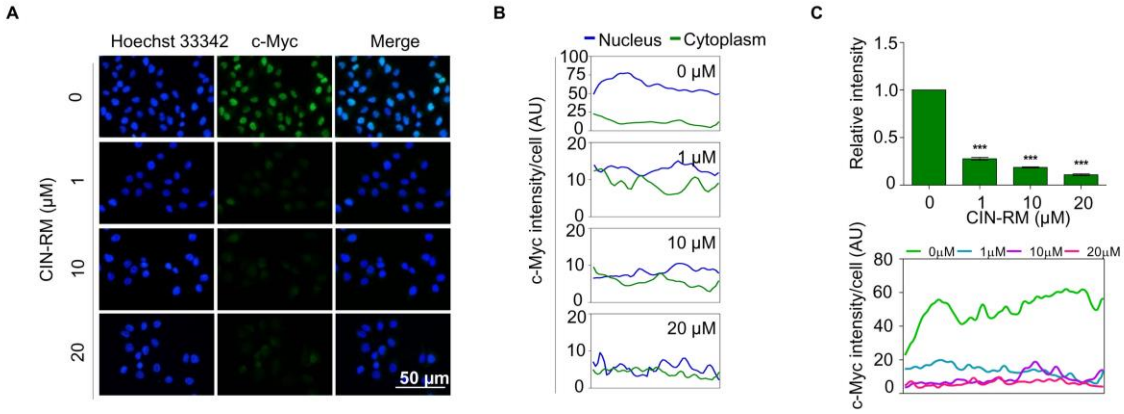


Figure 21 CIN-RM suppresses c-Myc on H460 cell.

(A) H460 cells were treated with CIN-RN at toxic concentrations for 12 h. The cellular levels of c-Myc were determined by immunofluorescence analysis. (B) The fluorescence intensity of the nucleus and cytoplasm were analyzed by ImageJ software. (C) The fluorescence intensity was analyzed by ImageJ software. Values are presented as means ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

Emerging research has shown that Akt affects degradation of c-Myc via the ubiquitin proteasomal pathway (114). Enhanced c-Myc degradation has also been linked to a reduction of CSC transcription factors, including Sox2, Oct4 and Nanog (115). This study further investigated whether downregulation of Akt by CIN-RM resulted in degradation of the c-Myc ubiquitin proteasomal. H460 cells were pretreated with 10 μM of the proteasomal inhibitor MG132 for 1 h followed by 10 μM of CIN-RM for 3 h. The c-Myc-ubiquitin complex was evaluated by immunoprecipitation assay (Figure 22A). Figure 22B indicates that the level of the c-Myc-ubiquitin complex increased approximately two-fold in CIN-RM-treated cells. These results demonstrate that suppressing CSCs with CIN-RM occurred through Akt-dependent c-Myc destabilization.

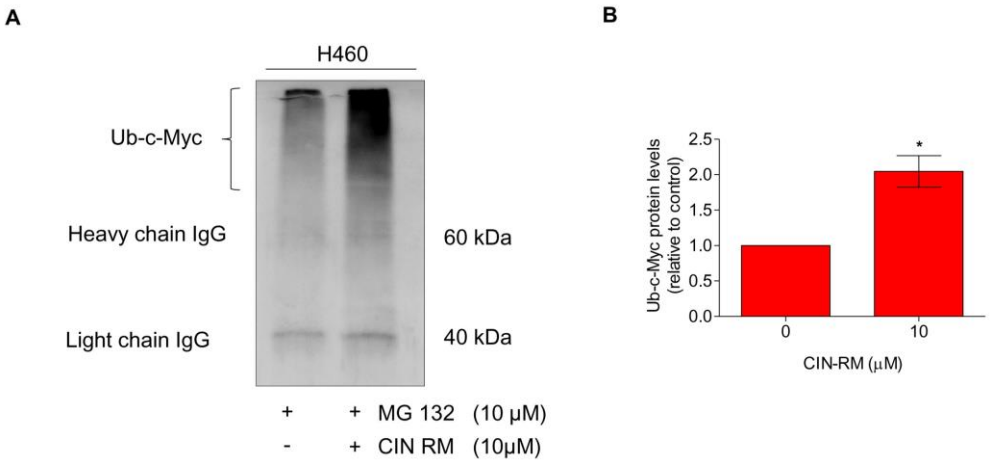


Figure 22 Effect of CIN-RM on degradation of the c-Myc ubiquitin proteasomal. (A) H460 cells were treated with 10 μM MG132 for 1 h followed by 10 μM CIN-RM for 3 h. The specific c-Myc protein was immunoprecipitated using an antibody against c-Myc. The immunocomplex was evaluated by immunoblotting using ubiquitin antibodies. (B) Densitometry of Ub-c-Myc protein complex level was calculated. Values are presented as means ± SEM (n = 3). * $p < 0.05$ compared with untreated cells.

The PI3K inhibitor wortmannin was used to validate the regulation in H460 cells to bolster the finding that Akt regulates the stability of c-Myc. Cells were treated with wortmannin at 2.5 and 5 μM for 12 h and the Akt, p-Akt and c-Myc protein levels were determined. Wortmannin significantly decreased the p-Akt and c-Myc levels, while the Akt protein levels remained unchanged (Figure 23). The immunofluorescence staining assay confirmed suppression of Akt by the PI3K inhibitor and further revealed that c-Myc was consequently suppressed (Figure 24,25).

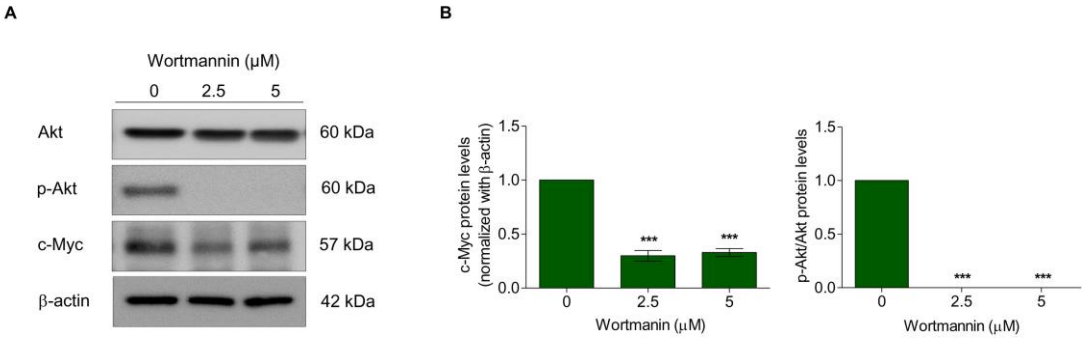


Figure 23 Wortmannin suppresses Akt/c-Myc signaling pathway.

(A) H460 cells were treated with 2.5 and 5 μM of wortmannin for 12 h and the expression levels of Akt, p-Akt, and c-Myc were analyzed by Western blotting. (B) Blots were reprobed with β-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry. Values are presented as means ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

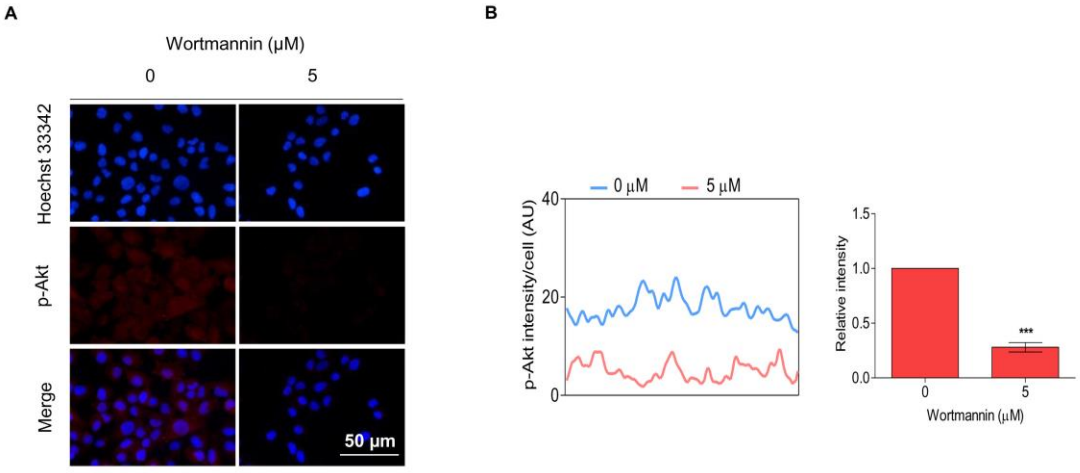


Figure 24 Wortmannin suppresses p-Akt on H460 cell.

(A) H460 cells were treated with wortmannin at 2.5 and 5 μM for 12 h. The cellular levels of p-Akt were determined by immunofluorescence analysis. (B) The fluorescence intensity was analyzed by ImageJ software. Values are presented as means ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

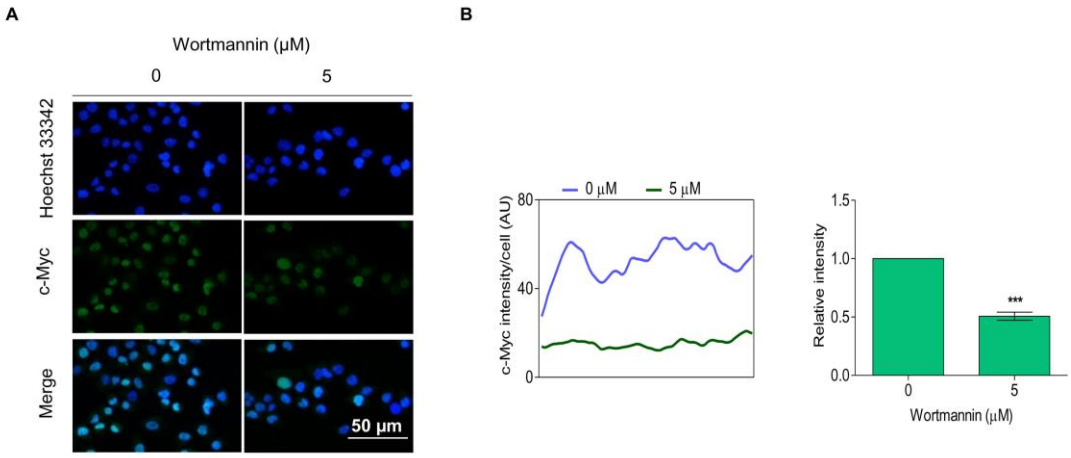


Figure 25 Wortmannin suppresses c-Myc on H460 cell.

(A) H460 cells were treated with wortmannin at 2.5 and 5 μM for 12 h. The cellular levels of c-Myc were determined by immunofluorescence analysis. (B) The fluorescence intensity was analyzed by ImageJ software. Values are presented as means ± SEM (n = 3). *** $p < 0.001$ compared with untreated cells.

CHAPTER V

DISCUSSION AND CONCLUSION

CSCs are a unique sub-population within tumors that are linked to low-rated successful treatments. CSCs have several cellular defensive mechanisms to escape conventional treatments (116). The remaining CSCs that are not eliminated by chemotherapy initiate and promote cancer relapse (42). Therefore, the molecules targeting CSCs as well as the mechanism maintaining cancer stemness should offer a novel promising treatment for cancer (117). CSCs commonly overexpress specific CSC markers, such as ALDH1A1, and possess a high level of pluripotent transcription factors, including Nanog, Sox2 and Oct4. The CSCs in many cancers are enhanced by Akt and its downstream regulator, c-Myc (4, 5). As the stem cell rich population of cancers has a highly active Akt signaling mechanism and elevated c-Myc (5, 118), these two proteins have been recognized as important drug targets for cancer treatment (119). In this study, we demonstrated the activity of CIN-RM in suppressing lung cancer CSCs with a possible underlying compound action mechanism.

Marine-derived compounds from various sources have been demonstrated to inhibit CSCs. For example, 5-*O*-acetyl-renieramycin T induces apoptosis and decreases expression of the CSC markers (CD44 and CD133) and decreases the Nanog stem cell transcription factor via the Akt signaling pathway (102). An extract of the marine sponge *Crambe crambe* (CR) strongly reduces pancreatic and prostate CSCs (103). RM suppressed CSC-like phenotypes in H460 lung cancer cells (21). CIN-RM (Figure 10) was semi-synthesized from renieramycin M which is isolated from the Thai blue sponge *Xestospongia* sp. (23). A previous study demonstrated that CIN-RM shows potential as an anticancer agent by triggering apoptosis-inducing factors (AIF) and a caspase cascade leading to apoptotic cell death in lung cancer (22). Our study revealed that the CIN-RM treatment resulted in a significant induction of apoptotic

cell death and inhibited cell proliferation (Figure 12-14). We have added up the novel information that CIN-RM significantly obstructed anchorage independent cell growth and inhibited the ability to form tumor spheroids (Figure 15,16) and eradicated the formed spheres (Figure 17).

ALDH1A1, Nanog, Oct4 and Sox2 are reported stem cell markers in lung cancer (4, 51). Overexpression of ALDH represents highly tumorigenic, cancer cell cloning properties (54) and reveals chemo-resistance (55). Moreover, Nanog, Sox2 and Oct4 are pluripotent transcription factors regulating self-renewal capacity. In our study, we discovered that CSCs suppressed the activity of CIN-RM by inhibiting ALDH1A1 and the pluripotency transcription factors (Figure 18). For the up-stream regulatory mechanism, CSC transcription factors were shown to be activated via several pathways including Akt. It was previously shown that Akt directly regulates Oct4 and Sox2 activity (74-77). Akt increases the stability of the Oct4 protein by phosphorylating Oct4 at threonine 235. Phosphorylated Oct4 enters to the nucleus and interacts with Sox2, which in turn activate the transcription of Nanog (78).

In addition, c-Myc, a major downstream target of Akt, accompany Oct4, Nanog and Sox2 to promote self-renewal in CSCs (14). c-Myc is a co-factor of Oct4/Sox2/KLF4 during pluripotent stem cell reprogramming (81). Akt regulated the stability of c-Myc via a GSK3β-dependent mechanism (10) Similarly, the stability of c-Myc is controlled by the Akt/mTOR pathway. mTOR inhibits Ser62 de-phosphorylation on c-Myc by hindering PP2A activity (80) leading to the stabilization of c-Myc. The expression levels of the p-mTOR, p-Akt and c-Myc proteins significantly decreased in response to CIN-RM (Figure 19). The results of the immunofluorescence assay demonstrated in the same manner that CIN-RM significantly decreased the intensity of p-Akt and c-Myc in cytoplasm and nucleus, respectively, of H460 cells (Figure 20,21). The stability of c-Myc is dependent on the control by Akt in

protecting c-Myc proteasomal degradation by inhibiting GSK3β (11). Therefore, disrupting the Akt signal could result in indirect suppression of CSCs by destabilizing c-Myc. From these results, CIN-RM only slightly affected p-GSK3β (Ser9), but strongly decreased p-mTOR expression (Figure 19), suggesting that CIN-RM regulated c-Myc degradation through the Akt/mTOR pathway.

CIN-RM was obtained from two-step semi-synthesis of RM involving hydrogenation at C-5 and C-8 position followed by esterification with cinnamoyl at C-5 position. Previous studies reported that RM was toxic to H460 cell with IC_{50} around 40 μM (110), while in this study founded that CIN-RM was toxic to H460 cell with IC_{50} around 15 μM. Another research also reported that CIN-RM at 100 μM reduced viability of H292 cells more than RM at the same concentration (22). From previous studies, the key structure–activity relationship studies of RM and its derivatives featuring ester side chains at C-5 have been investigated for the cytotoxicity on NSCLC (120) (23). From the current findings, the modified ester side chains at C-5 showed a critical structure-cytotoxicity relationship. According to the cytotoxicity on NSCLC, a small acyl ester at C-5 is importance for the high potencies of RM derivatives compound**.** The additional ester side chain at C-5 involves an increase in hydrophobic interaction and the hydrogen bonding in the DNA alkylation process to improve the cytotoxicity (20). Therefore, RM modification can be assumed that CIN-RM is more toxic than RM because of the ester side chains at C-5 position.

CIN-RM exhibited a potential CSC-targeting activity by inhibiting Akt. As CSCs have been shown to drive cancer progression, drug resistance, metastasis and relapse, this compound may offer novel approaches for the improvement of highly resistant and relapsing cancers. In addition, the Akt inhibitory effect of the compound may be useful for many human cancers in which Akt is over-activated with highly drug resistant characteristics. Furthermore, this novel model could be developed as a new molecular targeting Akt for cancer treatment.

In conclusion, this study demonstrated that CIN-RM suppressed CSCs in H460 cells by inhibiting the AKT/c-Myc signaling pathway, resulting in the downregulation of the stem cell transcription factors, Nanog, Oct4 and Sox2 (Figure 26). This study will be useful to further develop CIN-RM as an alternative treatment for CSCs in lung cancer.

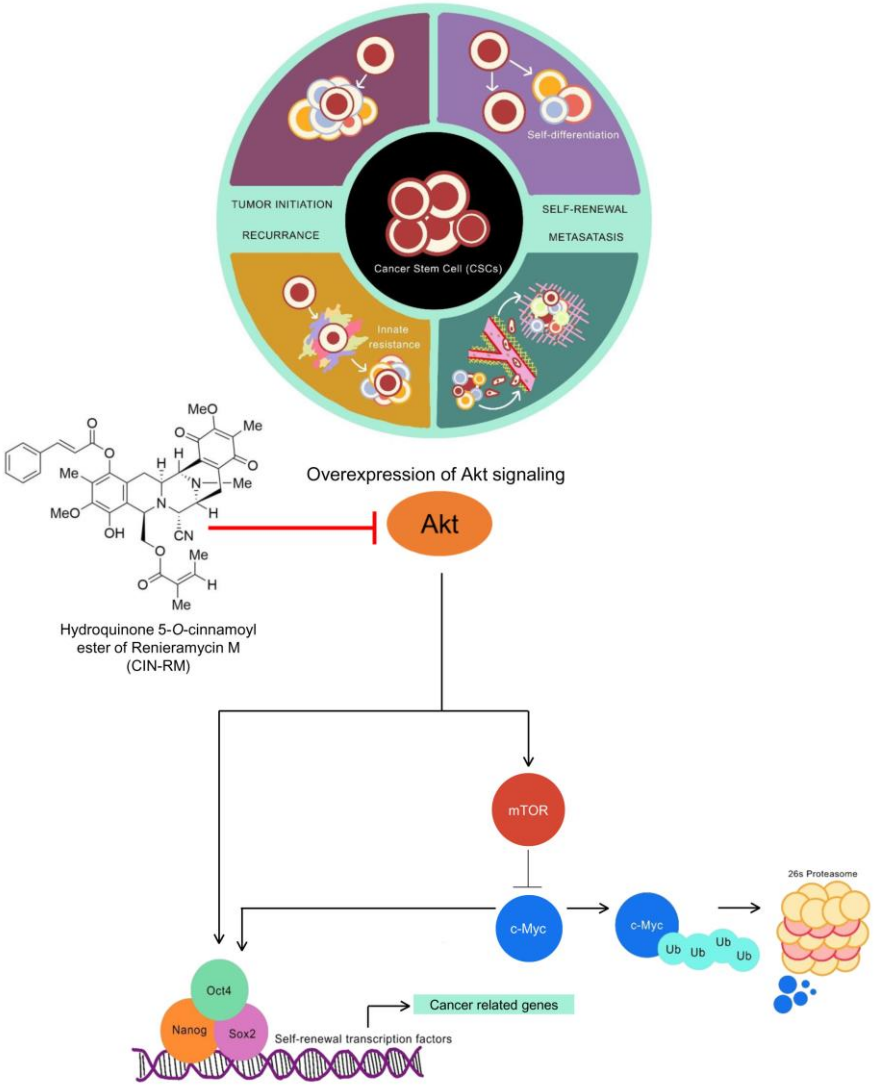


Figure 26 The proposed regulatory pathway involving in CSC suppression of CIN-RM.

CSCs are the major cause of therapeutic failure due to their ability of self-renewal and tumor initiation. They take part in cancer recurrence and metastasis. Akt signaling

and related pathways are upregulated in CSCs leading to cancer aggressiveness. CIN-RM could directly interact and inhibit Akt function resulted in the reduction of stem cell transcription factors. In addition, the inhibition of Akt triggers c-Myc proteasomal degradation. In the absence of upstream pluripotency factors Nanog, Oct4 and Sox2 and the protein co-factor c-Myc, the CSCs were depleted.

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