EFFECT OF 5-*O*-(*N*-BOC-L-ALANINE)-RENIERAMYCIN T IN THE SUPPRESSION OF LUNG CANCER STEM CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ผลของ 5-*โอ*-(*เอ็น*-บ็อค-แอล-อะลานีน)-เรนิอีราไมซินที่ในการยับยั้งเซลล์ต้นกำเนิดมะเร็งปอด

น.ส.ดารินทิพย์ สุขสมัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	EFFECT OF 5-*O*-(*N*-BOC-L-ALANINE)-RENIERAMYCIN T IN THE SUPPRESSION OF LUNG CANCER STEM CELLS
By	Miss Darinthip Suksamai
Field of Study	Pharmaceutical Sciences and Technology
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ดารินทิพย์ สุขสมัย : ผลของ 5-*โอ*-(*เอ็น*-บ็อค-แอล-อะลานีน)-เรนิอีราไมซินทีในการยับยั้งเซลล์ต้นกำเนิดมะเร็งปอด. (EFFECT OF 5-*O*-(*N*-BOC-L-ALANINE)-RENIERAMYCIN T IN THE SUPPRESSION OF LUNG CANCER STEM CELLS) อ.ที่ปรึกษาหลัก : ศ. ภก. ดร.ปิติ จันทร์วรโชติ

เซลล์ต้นกำเนิดมะเร็งเป็นตัวขับเคลื่อนที่กระตุ้นความก้าวร้าวและการแพร่กระจายโดยการใช้สัญญาณที่เกี่ยวข้องกับเซลล์ต้นกำเนิด ในการศึกษานี้ 5-*โอ*-(*เอ็น*-บอค-แอล-อะลานีน)-เรนิอีราไมซิน ที (โอบีเอ-อาที) แสดงให้เห็นว่าสามารถยับยั้งสัญญาณเซลล์ต้นกำเนิดมะเร็งและกระตุ้นให้เกิดการตายของเซลล์แบบอะโพโทซิส โอบีเอ-อาทีแสดงความเป็นพิษต่อเซลล์ที่มีค่าความเข้มข้นการยับยั้งร้อยละ 50 ประมาณ 7 ไมโครโมลาร์และการตายของเซลล์แบบสื่อกลางถูกตรวจโดย annexin V/PI และวิธีการย้อมติดสีของนิวเคลียร์ ในทางกลไก โอบีเอ-อาทีมีความสามารถในการกระตุ้นให้เกิดการตายของเซลล์แบบอะโพโทซิสผ่านการส่งสัญญาณที่ขึ้นกับพี 53 และยังสามารถยับยั้งสัญญาณของเซลล์ต้นกำเนิดมะเร็งได้ด้วย วิถีที่ขึ้นกับพี 53 ถูกบ่งชี้โดยการเหนี่ยวนำของโปรตีนพี 53 และการลดของโปรตีนต้านการเกิดอะโพโทซิส เอ็มซีแอล-วันและบีซีแอล-ทู และ cleaved PARP ถูกตรวจพบในเซลล์ที่บำบัดด้วยโอบีเอ-อาที ที่น่าสนใจคือโอบีเอ-อาทีมีความสามารถในการยับยั้งเซลล์ต้นกำเนิดมะเร็งได้ เนื่องจากมันสามารถลดการสร้างเนื้องอกทรงกลมได้ นอกจากนี้ พบว่าโอบีเอ-อาทีสามารถกระตุ้นให้เกิดการตายของเซลล์แบบอะโพโทซิสของประชากรที่มีเซลล์ต้นกำเนิดมะเร็งและการยุบตัวของเนื้องอกทรงกลม เครื่องหมายเซลล์ต้นกำเนิดมะเร็ง CD133, Oct4 และ Nanog ลดลงอย่างเห็นได้ชัดหลังบำบัดด้วยโอบีเอ-อาที สำหรับการควบคุมเซลล์ต้นกำเนิดมะเร็งชั้นบนเอเคทีและ ซีมิก นั้นพบว่าลดลงอย่างมีนัยสำคัญ ชี้ให้เห็นว่าเอเคทีอาจเป็นเป้าหมายของการดำเนินการที่อาจเกิดขึ้น การสร้างแบบจำลองโมเลกุลทางคอมพิวเตอร์แสดงให้เห็นว่าโอบีเอ-อาทีมีปฏิสัมพันธ์กับโมเลกุลเอเคที การศึกษานี้แสดงให้เห็นว่าผลของการยับยั้งเซลล์ต้นกำเนิดมะเร็งแบบใหม่ของโอบีเอ-อาทีโดยผ่านการยับยั้งเอเคทีนี้อาจเป็นประโยชน์ต่อการรักษาโรคมะเร็งได้

สาขาวิชา เภสัชศาสตร์และเทคโนโลยี ลายมือชื่อนิสิต .....

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Darinthip Suksamai : EFFECT OF 5-*O*-(*N*-BOC-L-ALANINE)-RENIERAMYCIN T IN THE SUPPRESSION OF LUNG CANCER STEM CELLS. Advisor: Prof. PITHI CHANVORACHOTE, Ph.D.

Cancer stem cells (CSCs) drive aggressiveness and metastasis by utilizing stem cell-related signals. In this study, 5-*O*-(*N*-Boc-L-alanine)-renieramycin T (OBA-RT) was demonstrated to suppress CSC signals and induce apoptosis. OBA-RT exerted cytotoxic effects with a half-maximal inhibitory concentration of approximately 7 μM and mediated apoptosis as detected by annexin V/PI and nuclear staining assays. Mechanistically, OBA-RT exerted dual roles, activating p53-dependent apoptosis and concomitantly suppressing CSC signals. A p53-dependent pathway was indicated by the induction of p53 and the depletion of anti-apoptotic Mcl-1 and Bcl-2 proteins. Cleaved PARP was detected in OBA-RT-treated cells. Interestingly, OBA-RT exerted strong CSC-suppressing activity, reducing the ability to form tumor spheroids. In addition, OBA-RT could induce apoptosis in CSC-rich populations and tumor spheroid collapse. CSC markers, including CD133, Oct4, and Nanog, were notably decreased after OBA-RT treatment. Upstream CSCs regulating active Akt and c-Myc were significantly decreased; indicating that Akt may be a potential target of action. Computational molecular modeling revealed that OBA-RT could interact with an Akt molecule with high affinity. This study has revealed a novel CSC inhibitory effect of OBA-RT via Akt inhibition, which may improve cancer therapy.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
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Special thanks to Assistant Professor Supakarn Chamni, Ph.D. for providing 5-O-(N-Boc-L-Alanine)-Renieramycin T for use in this study.

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.

Darinthip Suksamai

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.1 Background and rational

Lung cancer is an important human cancer. At present, several strategies are used for lung cancer treatment, including surgery, chemotherapy, radiotherapy, and targeted therapy; however, drug resistance and the spread of the cells to form metastases frequently result in poor prognosis and treatment failure. Advances in molecular and clinical research have highlighted the role of a cancer cell population, namely cancer stem cells (CSCs), and the concept of CSCs has dramatically altered the understanding view of cancer cell biology, pathogenesis, and the clinical response (1). Therefore, the current drug discovery theme has focused on the undifferentiated cancer cell population, as the available therapy primarily eradicates the non-CSC population in the tumor, thereby sparing drug-resistant CSCs (2). High tumorigenic potentials augment cellular survival and drug-resistant mechanisms, and the metastatic abilities of lung CSCs depend on the specific stem cell-related signaling pathways (3).

Myc is a family of proto-oncoproteins that regulate cell growth, survival, and proliferation (4). In lung cancer, c-Myc is recognized as a key factor facilitating cell growth, drug resistance, and dissemination. In addition, its dominant role in controlling CSC properties supports the concept that targeting c-Myc could be a potential method for lung cancer therapy (5). A number of studies and observations show the co-incidence of c-Myc and activated PI3K/Akt in transformed cells. Moreover, the PI3K/Akt/c-Myc signaling axis could promote CSC properties in cancers (6).

The deregulation of Akt is associated with several features of cancers, and Akt-targeting compounds can improve cancer therapies. In addition, a number of Akt

inhibitors have been investigated for lung cancer treatment (7). Natural tetrahydroisoquinoline of marine origin and their analogs, such as ecteinascidins from tunicates, exhibit potent cytotoxicity against several types of cancer cells, and they have been approved for clinical use in the treatment of cancers, including advanced soft-tissue sarcoma and ovarian cancer in the case of ecteinascidin 743 (trabectedin) (8) and metastatic small cell lung cancer in the case of the semisynthetic analog, namely lurbinectedin (9).

However, the mechanism of action of ecteinascidins is not fully understood. Ecteinascidins can exert anticancer activities via binding with DNA and DNA-binding proteins and mediating cell apoptosis (10). Ecteinascidins target Akt as it can dramatically decrease phosphorylated Akt (s473-AKT or p-Akt (11) and reduce the expression level of several anti-apoptotic proteins, such as Bcl-2 and Mcl-1 (12). A recent pre-clinical study in the xenograft mice model of uterine cervical cancer revealed that lurbinectedin effectively eliminates CSCs (9). Renieramycins, which are bis(tetrahydroisoquinoline)quinone alkaloids found in sea sponges and nudibranchs, are members of the same soframycin family as tris(tetrahydroisoquinoline) ecteinascidins (13), have also demonstrated potent anti-cancer activities (14), particularly renieramycin M (RM), which is the major bis(tetrahydroisoquinolinequinone) constituent isolated from potassium cyanide-pretreated Xestospongia collected in Thailand (15) and the Philippines (16). RM can sensitize resistance to anoikis via decreasing cellular levels of survival and apoptotic proteins (including p-Akt, p-ERK, Bcl-2, and Mcl-1) (15) and attenuate CSC-like phenotypes (17) in H460 cells. Similar to derivatizations of ecteinascidins (18), late-stage modifications on either A- or E-ring quinone of RM have been found to alter the mode of action and cellular targeting pattern which can lead to enhanced selectivity and activity. A series of 5-*O*-Boc-amino ester derivatives of RM is synthesized and successfully used in a structural cytotoxicity relationship study (19), indicating that Boc-protected amino acid moieties serve as empirical groups in the introduction of additional compound–target intermolecular interaction networks and modifying their physicochemical properties. The cinnamoyl ester derivative of RM has shown superior cytotoxicity compared with the parent compound (20) and it can

suppress CSCs potentially by inhibiting Akt (21). Renieramycin T (RT), a hybrid renieramycin-ecteinascidin analog derived from RM with a methylhydroxybenzodioxole unit resembling ecteinascidin (22), could promote p53-dependent apoptosis via near-to-complete depletion of Mcl-1 and partly decrease the cellular level of Bcl-2, whereas RT could not affect Akt (23). Trabectedin-mimic derivative of RT, 5-*O*-acetyl-renieramycin T (5-*O*-acetyl RT), could significantly deplete Akt and reverse CSC-associating cisplatin resistance in non-small-cell lung carcinoma (NSCLC) (24). Protections at the phenolic alcohol at C-5 can enhance the cytotoxicity of RT derivatives (25). Based on previous developments, we synthesized the 5-*O*-(*N*-Boc-L-alanine)-renieramycin T (OBA-RT) from RM and investigated the induction of cancer cell apoptosis and CSC-suppressing effect. Using molecular pharmacological and computational modeling approaches, we reported the potential CSC-targeting activities of this new compound, which could improve anticancer therapy.

1.2 Objective

1.2.1. To investigate suppressive activity of OBA-RT on CSC-associated phenotypes in lung cancer cells.

1.2.2. To evaluate the underlying mechanism in suppression lung cancer stem cells by OBA-RT

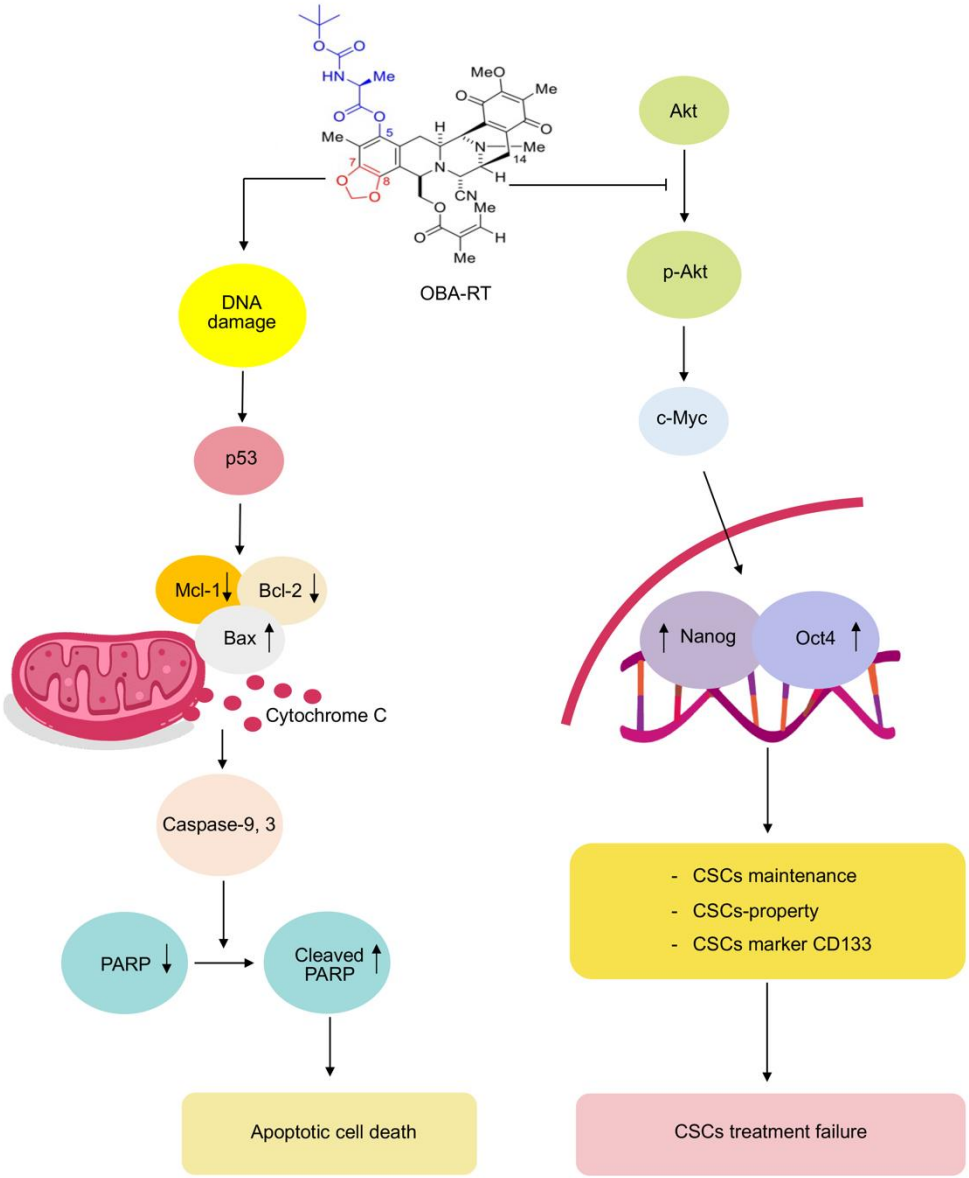
1.3 Hypothesis

OBA-RT could induce lung cancer cell apoptosis through p53-dependent pathway by reduction of anti-apoptotic MCl-1, BCl-2 and increased expression of pro-apoptotic Bax and cleaved-PARP and were able to suppress CSC-associated phenotypes in lung cancer cells by inhibiting AKT signaling pathway, resulting in decreased of Nanog and Oct4.

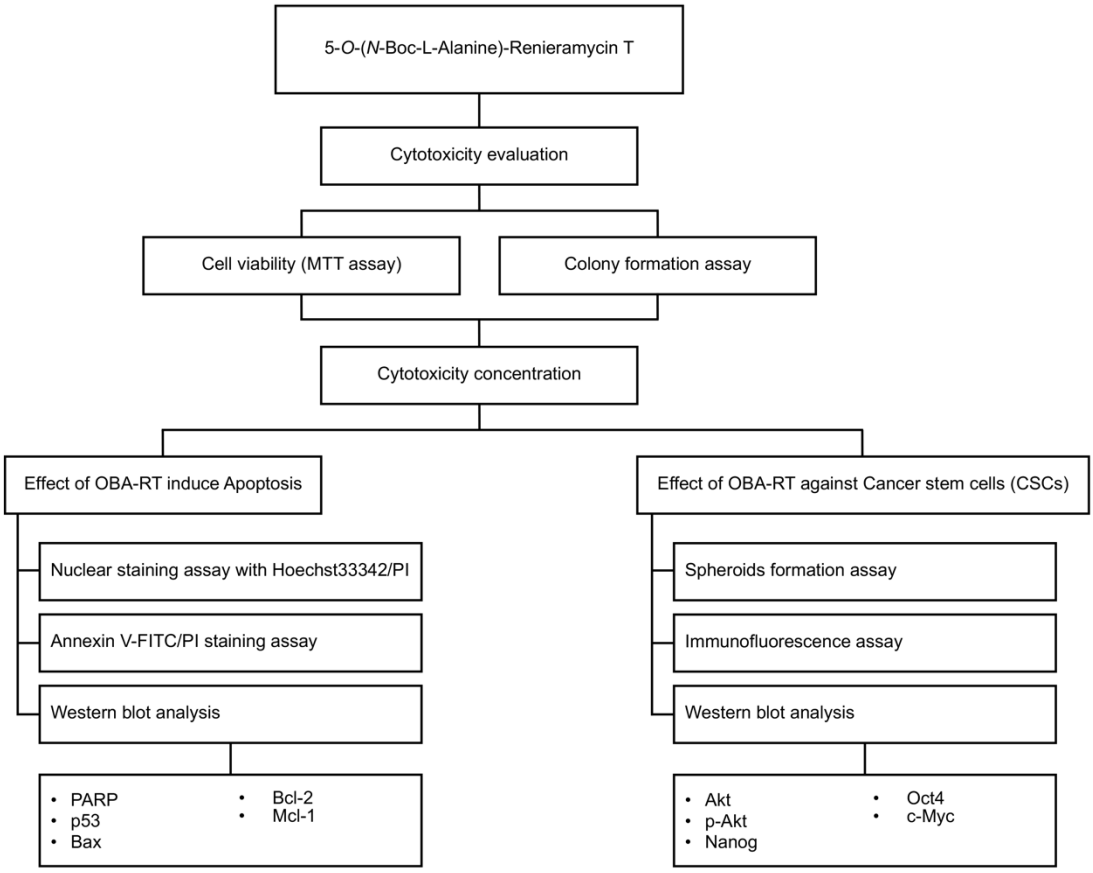
1.4 Expected benefic of the study

The result from this study may elucidate the preliminary data of OBA-RT in suppression of CSC-associated phenotypes in lung cancer cells and suppression of stem cell mechanism. Thus, this study may benefit the development of OBA-RT for anti-cancer treatment.

1.5 Concepture framework



1.6 Experimental design



CHAPTER II

LITERATURE REVIEW

2.1 Lung Cancer

Lung cancer is the most frequent diagnosed malignancy and the leading cause of cancer-associated death in both Thailand and worldwide. It initiates from normal lung cells that grow irregularly out of control and from a mass called tumor. A tumor interferes the function of the lung, that provides oxygen to the body through the blood vessels and lastly cause the patient death. Despite of advances in early diagnosis and various standard treatments, lung cancer still has a low prognosis. The 5-year relative survival rate is approximately 19% which is lower than other types of cancers (26).

Epidemiology

According to the American Cancer Society's publication, cancer Facts & Figures 2021 reported that lung cancer is the second most regular cancer and the most important cause of cancer death in both male and female despite the incidence rates have declined since 2000. From 2007 to 2016, lung cancer incidence rates decreased by 3% per year in male and by 1.5% per year in female. Likewise, the death rate decreased by 4% per year in male and by 3% per year in female. However, an estimated 131,880 deaths from lung cancer (69,410 in men and 62,470 in women) will be occurred and 235,760 new cases of lung cancer (119,100 in men and 116,660 in women) will be evaluated in 2020 by The American Cancer Society's estimates for lung cancer in the United States. The 5-year survival rate are 19% depended on various factors, such as the subtype of lung cancer, and the stage of disease (26). In Thailand, lung is the third leading site of cancer that has 8.86% incidence rate (males 5.46% and female 3.40%) of all cancer in 2017. The age-adjusted incidence rate in 2017 for lung cancer of males is higher than females (27).

Estimated New Cases

Males				Females		
Prostate	248,530	26%		Breast	281,550	30%
Lung & bronchus	119,100	12%		Lung & bronchus	116,660	13%
Colon & rectum	79,520	8%		Colon & rectum	69,980	8%
Urinary bladder	64,280	7%		Uterine corpus	66,570	7%
Melanoma of the skin	62,260	6%		Melanoma of the skin	43,850	5%
Kidney & renal pelvis	48,780	5%		Non-Hodgkin lymphoma	35,930	4%
Non-Hodgkin lymphoma	45,630	5%		Thyroid	32,130	3%
Oral cavity & pharynx	38,800	4%		Pancreas	28,480	3%
Leukemia	35,530	4%		Kidney & renal pelvis	27,300	3%
Pancreas	31,950	3%		Leukemia	25,560	3%
All Sites	**970,250**	**100%**		**All Sites**	**927,910**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	69,410	22%		Lung & bronchus	62,470	22%
Prostate	34,130	11%		Breast	43,600	15%
Colon & rectum	28,520	9%		Colon & rectum	24,460	8%
Pancreas	25,270	8%		Pancreas	22,950	8%
Liver & intrahepatic bile duct	20,300	6%		Ovary	22,950	5%
Leukemia	13,900	4%		Uterine corpus	12,940	4%
Esophagus	12,410	4%		Liver & intrahepatic bile duct	9,930	3%
Urinary bladder	12,260	4%		Leukemia	9,760	3%
Non-Hodgkin lymphoma	12,170	4%		Non-Hodgkin lymphoma	8,550	3%
Brain & other nervous system	10,500	3%		Brain & other nervous system	8,100	3%
All Sites	**319,420**	**100%**		**All Sites**	**289,150**	**100%**

Figure 1 Estimated new cancer cases and deaths worldwide in 2020

Rick factor (28)

1. Tobacco and smoking including secondhand smoke

Smoking is the most important risk factor in lung cancer. About 80% to 90% lung cancer death involved with smoking.

2. Asbestos

People who work with asbestos such as in mills, mines, and shipyards are risked to lung cancer.

3. Radon

Radon is a naturally occurring radioactive gas that comes from the breakdown of radioactive elements. Therefore, the US Environmental Protection Agency (EPA), radon is the second dominant cause of lung cancer.

4. Exposure to other cancer-causing agents in the workplace such as arsenic, beryllium, cadmium, coal products, diesel exhaust, and radiation.

5. Occupational exposures such as painting, paving, rubber manufacturing, roofing and chimney sweeping

6. Air pollution

7. Personal or family history of lung cancer

8. Tuberculosis

Symptoms (29, 30)

Symptoms of localized lung cancer

1. Persistent coughing
2. Chest pain, shoulder, or back not related to pain from coughing
3. Change in color or volume of phlegm
4. Shortness of breath or difficult breathing
5. Hoarseness
6. Harsh sounds with each breath (stridor)
7. Recurrent lung problems, such as bronchitis or pneumonia
8. Coughing up sputum or mucus or blood (hemoptysis)

Symptoms of lung cancer that can occur elsewhere in the body:

1. Loss of appetite or unexplained weight loss
2. Muscle wastage (cachexia)
3. Stagnancy
4. Headaches, bone or joint pain

5. Bone fractures unrelated to accidental injury
6. Neurological symptoms, such as unsteady gait or memory loss
7. Swelling of neck or facial
8. General weakness
9. Bleeding and blood clots

Types of lung cancer (27, 31)

There are comprising into two types such as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC).

1. Non-small cell lung cancer (NSCLC) is the most frequent type of lung cancer, about 85% of lung cancers. There are 3 main kinds including;

- **Adenocarcinoma**: Adenocarcinoma is the most frequently found of lung cancer among entire male and female.
- **Squamous cell carcinoma**: This type is found 25% of all lung cancers.
- **Large cell carcinoma**: This type acquires 10% to 15 % of NSCLC.

2. Small cell lung cancer (SCLC) is found in about 15% of lung cancers. They have a tendency to grow faster than NSCLC.

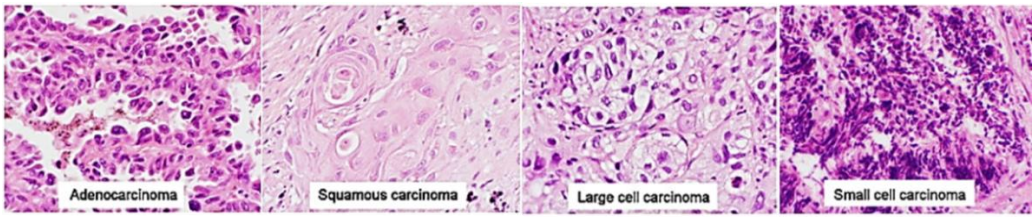


Figure 2 Histological types of lung cancer

Staging of lung cancer (33)

- Stage 0: This stage is called *in situ*. The cancer is significantly “in place” and no metastasis.

- Stage I: Tumors are 3 cm or less in size but no metastasis to any lymph nodes.

- Stage II: Tumor size larger than 3 cm but 7 cm or less in size with metastasis to nearby lymph nodes.

- Stage III: Tumor of any size with metastasis to lymph nodes but Stage III cancers has not metastasis to other distant parts of the body.

- Stage IV: This stage is the most advanced stage of lung cancer. Tumor of any size with or without metastasis to nearby lymph nodes but has metastasis to other distant parts of the body.

Treatments of lung cancer (31)

Treatments for lung cancer patient depend on the type of cancer, tumor characteristics, stages of cancer, and metastasis to other distant parts of the organs. The treatments of lung cancer can be including;

1. Surgery

The most stage I and stage II will be treated with surgical to remove the lobe or section of the lung that involving the tumor.

2. Chemotherapy

Chemotherapy is used in early and advanced stage of lung cancer or tumor that cannot be removed surgically. This treatment may be used in combination with radiation and targeted therapy. Chemotherapeutic drugs are usually given in oral form or intravenous form or sometimes both. The drugs most used in lung cancer are cisplatin, carboplatin, and pemetrexed etc.

3. Radiation therapy

This treatment can be used in combination with chemotherapy for preventing the disease relapse after surgery. It uses high-energy rays to eliminate the cancer cells. In stage IV patients, radiation therapy is used for symptom alleviation.

4. Targeted therapy

Targeted therapy drugs are designed to attack specific molecules that are important for cancer growth and metastasis. Targeted therapy of advanced-stage lung cancer with certain molecular biomarkers may be used in combination with chemotherapy, radiation therapy or interventional pulmonology. The targeted therapeutic drugs used in lung cancer are including erlotinib, afatinib, ceritinib and bevacizumab etc.

5. Immunotherapy

Immunotherapy is a new treatment option for cancer as it's designed to activate immune system to attack and kill cancer cells. Immunotherapy is divided into 4 main types; monoclonal antibodies, therapeutic vaccines, checkpoint inhibitors and adoptive T-cell transfer.

Currently, there are many different types of treatment to treat lung cancer. And a number of chemotherapy drugs and drugs targeting programmed cell death have been used, where apoptosis programmed cell death is the primary mechanism used in cancer treatment. Therefore, apoptotic cell death is further discussed.

2.2 Apoptotic cell death

The processes of apoptosis, programmed cell death, generally have different morphology and energy-dependent biochemical mechanisms. Cell death is an essential component of many processes such as hormone-dependent atrophy, normal cell turnover, embryo development, proper development and function of

the immune system, and apoptosis induced. Chemical Improper cell death (Whether too little or too much) is the factor in various human conditions, such as neurological disease, autoimmune disorders, ischemic damage, and many cancers. The ability to alter cell life or death is recognized as having great therapeutic potential (34).

Cytology of apoptotic cell death

The different stages of apoptotic cell death begin with the contraction of cells and the condensation of chromatin along with the formation of cell membranes. Organelles and nucleus and scales fragments begin to form of the apoptosis body, which is eventually swallowed up by macrophages or neighboring cells by endocytosis /phagocytosis. Cell components to extracellular fluid result in cells being neatly eliminated without causing an inflammatory response (35).

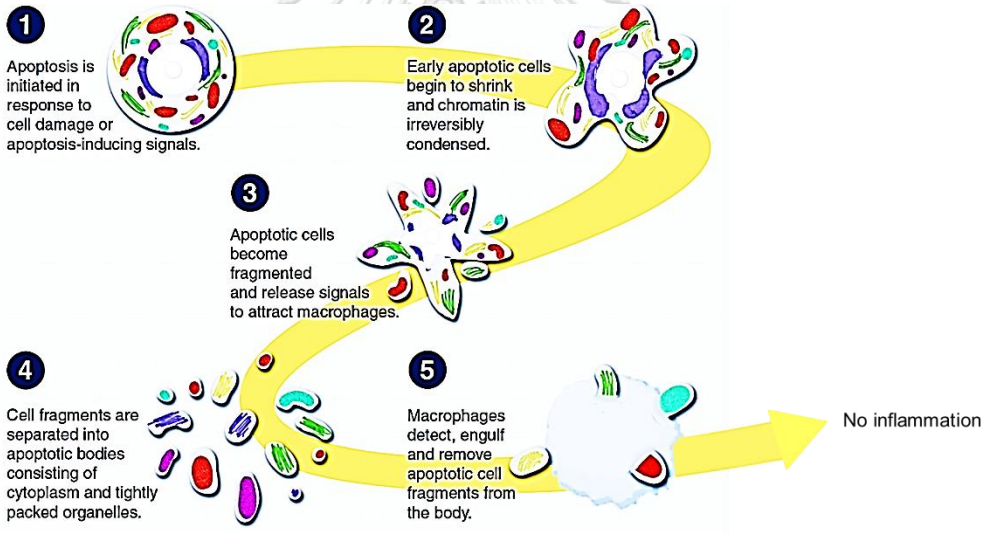


Figure 3 Cytology of apoptosis cell death

(36)

Mechanism of apoptosis

Cell death is regulated by activation of the Caspase family of proteins, which are central to the cell death mechanism, as it is both the initiator and the executioner. There are two pathways that can activate caspases. It's called the intrinsin and extrinsin pathway. Eventually, both pathways lead to activation of an action pathway, resulting in apoptosis (37, 38).

Intrinsic pathway is an apoptosis program that starts inside the cell, usually in response to injury, UV rays, low oxygen levels (hypoxia), DNA damage, tumor formation, oxidative stress, or anticancer drugs. The intracellular pathways of cell death are tightly regulated by members of the Bcl-2 family of proteins, which can be divided into anti-apoptotic and pro-apoptotic proteins. The pro-apoptotic proteins are made up of 2 sub-families: BH123. Such as Bax and Bak and BH3- proteins such as Bid, Bad, Bim, PUMA and NOXA promote apoptosis cell death by increasing the release of cytochrome C. On the other hand, proteins are anti-apoptosis including Bcl-2, Bcl-xl and Mcl-1 inhibits apoptosis cell death with blocking the release of cytochrome C. Usually, the most anti-death proteins (Bcl-2 and Bcl-xl) are on the cytosolic surface of the outer mitochondrial membranes to maintain the integrity of the mitochondria. Prevents the released of cytochrome C from inappropriate mitochondria. These proteins bind to and inhibit pro-apoptotic proteins including Bax and Bak, resulting in apoptosis cell death inhibition. After an apoptotic stimulant, the pro-apoptotic BH3 protein binds to and inhibits the anti-apoptotic protein, leading to the activation of the pro-apoptotic BH123, Bax, and Bak. Activating Bak and Bax promotes the release of cytochrome C into the cytosol, then the cytochrome C binds to the apoptotic protease-activating factor 1 (APAF1), forming an apoptosome to recruit and motivate the caspase-9. Once activated, caspase-9 can activate the executioner caspase-3, leading to apoptosis cell death (38-40).

Another initiation pathway, extrinsic pathway, involves membrane receptor mediated interactions. Extrinsic pathways can be initiated by binding to extracellular signaling proteins such as tumor necrosis factor (TNF), Fas ligand, and TNF-related apoptosis-induces ligand (TRAIL) to the cell surface cell-surface death receptors, a protein transmembrane belonging to tumor necrosis factor (TNF) receptor. This leads to intracellular recruitment of adapter protein including Fas-related death domain (FADD) and initiator caspase-8. Which produces a lethal induced signaling complex (DISC). The formation of DISC results in activation of the initiator caspase, activating downstream executioner caspase such as caspase-3, which leading to cell death (38-40).

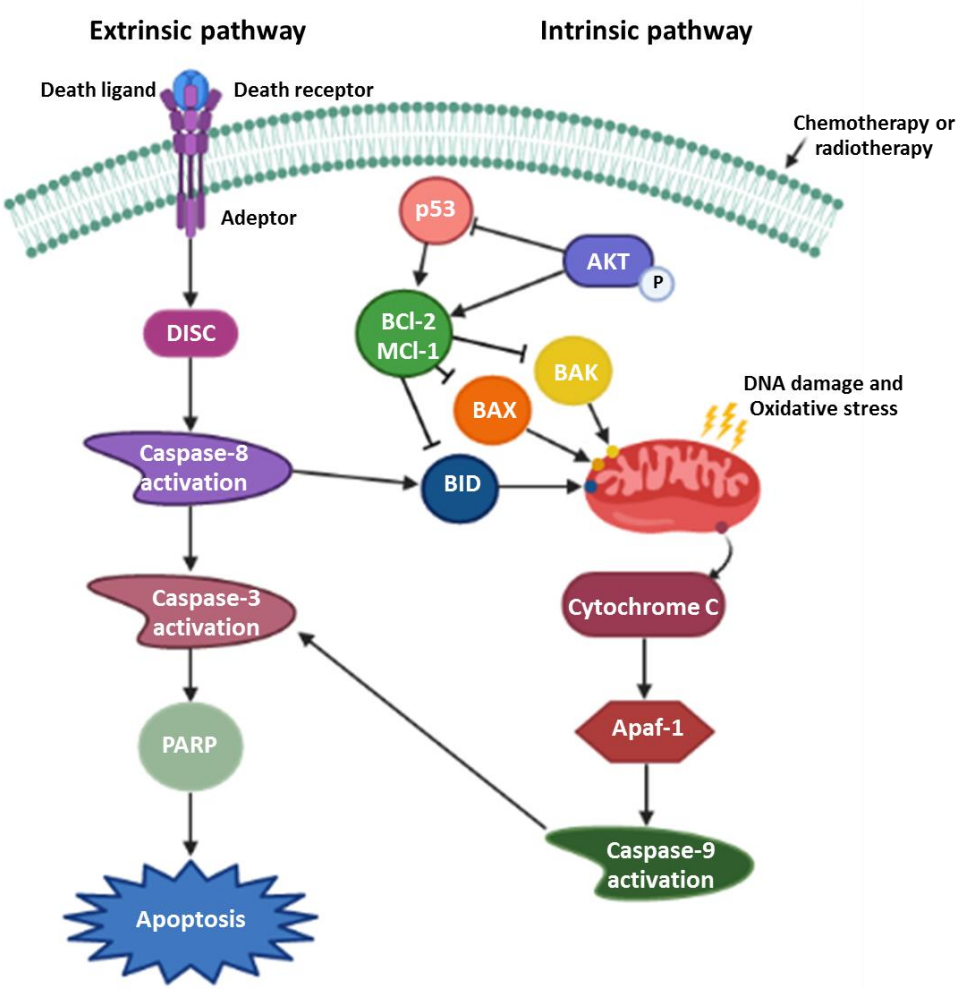


Figure 4 The intrinsic and extrinsic apoptotic pathways

p53-induced apoptosis pathway

The tumor suppressor protein p53 is a genetic transcription factor that regulates the expression of multiple genes involved in the cell cycle, senescence, growth, and death in response to cellular stress (41). p53 activates signaling network acting on two major of apoptotic pathways, external and internal pathways (42). The p53-induced extrinsic pathway is associated with involvement of specific mortality receptors belonging to the family of necrotic factor receptor (TNFR) and the formation of a signaling complex (DISC) (43). The activated caspase-8 and caspase-3 respectively, which induce apoptosis. The most common death receptors linked to external stimuli are FAS and DR5 receptor (44).

p53-induce intrinsic apoptotic pathway is regulated by proteins of the Bcl2 family, which regulate cytochrome c release from mitochondria (45). Bax is activated via p53 in the cellular response to stress conditions. The BH3-only proteins, Noxa and Puma are encoded by p53-inducible genes. The anti-apoptosis role of Noxa and Puma improves Bax activity by inhibiting Mcl-1, Bcl-2 and Bcl-XL (46).

The activation of p53 not only involves with apoptosis role of Bcl-2 family proteins but also modulates the expression of survival proteins (47). Previous study has shown that the altering of protein kinase B (Akt) signaling pathway is resulted from the inhibition on p53 in which is a general mechanism to uncontrollably proliferate and escape apoptosis in cancer cells.

c-Myc, signalling apoptosis, is an important for induces apoptosis due to the inability of efficiently up-regulate p53 as a result of the MDM2 that increase levels. Thus, treatment with the MDM2 inhibitor Nutlin will be re-established the stabilization of p53 and promoted apoptosis cell death (48).

However, there is a treatment for lung cancer by inducing apoptosis. The survival rate remains low due to a number of issues such as chemotherapeutic resistance. Therefore, a better treatment strategy is needed. At present, inhibition of cancer stem cell (CSC) is used in cancer treatment because they are involved in cancer metastasis

2.3 Cancer stem cell (CSC)

Stem cells (SCs) are specialized cells that have the ability to self-renewal and produce different offspring in different types of cells. Even with a small population, SCs are important for replenishing and repairing damaged tissue (49). Cancer stem cells (CSCs) are small clusters within tumors hold stemness characters which facilitate initiation, metastasis and recurrence of cancer pathologies. CSCs are the main factors contributing to the low achievement rate of present therapeutic treatments (50). At present, chemotherapies drugs can induce of apoptosis in cancer

cells only, but not in CSCs due to the expression of anti-apoptosis Bcl-2 family proteins has been identified persistent in CSCs (51). Unresponsive CSC plays a key role in tumor regeneration and its recurrence (49) due to its aggressive properties, including cancer builders, high metastasis, and resistance to chemotherapy. Examinations and developing new anti-cancer drugs focus on the elimination of CSCs Subpopulation (52). There are also reports on targeting cell death in CSC caused by a wide variety of marine natural compounds.

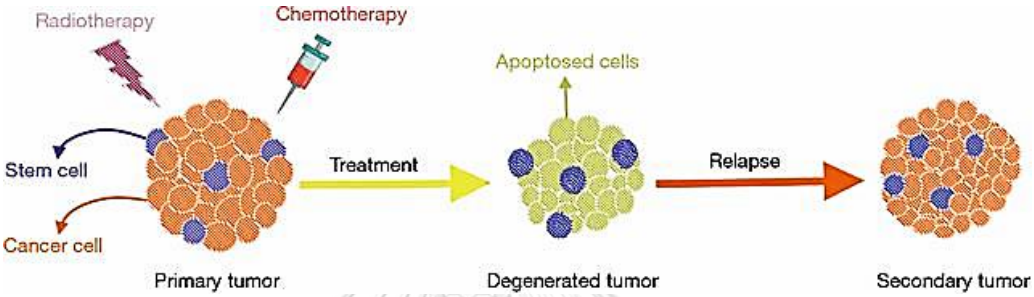


Figure 5 Role of cancer stem cells in recurrence of tumor pathology (53)

The presence of CSC in lung cancer has been demonstrated with the formation of a separate spheroid and in vivo tumor initiation of Subpopulation isolated from both lung cancer specimens and cell lines. Lung CSCs was also identified by evaluation of SCs marker proteins such as prominutesin-1 (CD133) and ALDH (Aldehyde Dehydrogenase). CD133, a pentaspan membrane glycoprotein, is one of the most prominent biomarkers used to isolate of cancer stem cells (CSCs) (54). CD133 is a marker that indicates the characteristics of cells with high tumorigenicity and high ability to form spheroids (55). CD133 expression was positively associated with poor outcomes for cancer patients (56). Thus, the expression of CD133 might be responsible for metastasis, CSCs tumourigenesis, and chemo-resistance by interacts with the Wnt/β-catenin and PI3K-Akt signalling pathways (57). ALDH is cancer prognostic marker in CSCs that is an enzyme that participates in important cellular mechanisms as detoxification and drug resistance (58). In currently, CD133 and ALDH are recommends as markers for CSC in various cancers. Table 1 provides an overview of used lung CSC markers (59).

Table 1 Protein marker for CSCs in lung cancer (59)

Markers	Cellular/molecular function	CSCs
CD44	Hyaluronic acid receptor	NSCLC; breast; hematopoietic
uPAR CD87	Urokinase plasminogen activator (uPA) receptor	SCLC
CD90	Tissue specific differential glycosylation	NSCLC
CD117	Growth factor receptor	NSCLC; neuroendocrine lung; hematopoietic
CD133	Unknown	Lung, brain, colon, pancreas
CD166	Activated leukocyte cell adhesion molecule (ALCAM)	NSCLC, SCLC
ALDH	Alcohol metabolism	Lung; hematopoietic, breast, prostate
BMI-1	Represses the tumor suppressor phosphatase and tensin homolog (PTEN)	NSCLC, SCLC
EpCAM	Cell-cell contact adhesion strength and tissue plasticity	NSCLC
FZD	Progression development, morphogenesis drug resistance	NSCLC
PODXL-1	Sodium-hydrogen exchange regulatory cofactor 2	SCLC
PTCH	Differentiation, branching morphogenesis	SCLC
SP	Drug resistance	Squamous lung carcinoma NSCLC; hematopoietic, brain, breast
ALDH	Early stem cell differentiation	NSCLC

Regulation on the progenitor properties in lung cancer stem cells

The regulation on stemness phenotypes including self-renewing and possibility in both normal SCs and CSCs were adjusted by the stemness transcriptional factors such as Nanog and octamer-binding transcription factor 4 (Oct4). It is a transcription factor that mediates the production of various cancer cells, including lung cancer (60, 61). Oct4 expression suppression not only suppresses the stemness phenotype but also metastatic characteristics in the CSC of lung cancer cells (62). Nanog was selected as a prognostic marker for lung cancer (63, 64), lung cancer patients with overexpression of Nanog and Oct4 showed a low survival rate (65), the aggressive properties of chemical drug resistance. Therapy and self-renewal in CSC were regulated by Nanog (66).

c-Myc regulates cancer stem cells

Myc is a nuclear transcription factor that mainly regulates cell cycle, cell growth, metabolism, and survival. Myc family proteins contain c-Myc, l-Myc, and n-Myc. Among them, c-Myc can become a promising therapeutic target molecule in cancer (67). c-Myc, one of the stem cells markers, has recently been considered to serve as a link between malignancy and ‘stemness’ (68). The overexpression of c-Myc leading to over-activation of stemness properties (67). c-Myc can cooperate with self-renewal transcription factors (Nanog and Oct4) to regulate the self-renewal

property (69). Thus, it can indicate that c-Myc transcription factor is important in CSC therapy.

Cancer stem cell signalling pathway Akt regulate cancer stem cells

Current research reports that CSCs are similar to various NSCs, including self-renewal and differentiation. Both CSCs and NSCs have many pathway to remain its survival (70). The anti-CSC strategies have been assessed by focusing on inhibition of many intracellular signaling pathways, such as Wnt/TCF, Signal transducer and activator of transcription 3 (STAT3), NF-**K**B and Akt. Akt signaling can be considered as a key regulator for cancers and CSC phenotypes. It is well known that Akt signaling plays a critical role in regulating CSC maintenance and properties (71).

Akt regulate cancer stem cells

Akt, also known protein kinase B (PKB), is an overactive proto-oncogene in most cancers. Activation of Akt requires phosphorylation at Thr308; phosphorylation at Ser473 enhances catalyst activity (72). Activation of Akt pathway enhanced CSC phenotypes in various cancer including prostate cancer, breast cancer, non-small-cell lung cancer, and colorectal cancer (73).

Oct4/Sox2/Nanog function in combinatorial complex recruits important transcriptional factors to induce stemness regulating proteins (74). These transcription factors have been shown to be activated through several pathways, including the Akt pathway (75). Previous studies have shown that Akt directly regulates Oct4 and Sox2 activities (76-78). Akt increases the stability of 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.

Interestingly, chemotherapy drugs and a number of marine natural compounds induce apoptosis in cancer cells. Current chemotherapy for lung cancer, such as cisplatin, afatinib and actinomycin D, causes apoptosis by a decrease in anti-apoptosis protein including Bcl-2 and Mcl-1. Moreover, Renieramycin T, a marine

natural extract, can regulate Mcl-1 protein and induce apoptosis of lung cancer cells. So, treatment with marine natural extracts promotes the expression of Bax, a protein pro-apoptosis, and has successfully induced apoptosis and suppressed cancer stem cell (CSC) of lung cancer cells (23, 24).

2.4 Marine derived products

The marine environment is an innumerable and diverse resource of potent bioactive compounds. Thus, the marine derived product is an extraordinary resource that used for the discovery and developments of new anticancer drugs. Recently antitumor, antimicrobial, and anti-inflammatory effects were reported by marine natural products. According to numerous research, the marine natural environment will be produced large amounts of potent substances that can inhibit survival, proliferation in cancer cells. The structural and chemical properties of substances from marine organisms were not found in terrestrial natural products due to their structures are becoming more complex and diverse. Therefore, these marine-derived molecules have the ability to interact with a number of biomolecular targets to suppress or induce specific biological functions against different types of cancer cells (23, 79).

In currently, there are five drugs isolated from marine organisms that approved by FDA for use as pharmaceutical drugs in cancer treatment including brentuximab vedotin, cytarabine, eribulin mesylate, and trabectidine (80). In addition, there are lots of marine drugs still in clinical trials such as gemcitabine from marine sponge and plitidepsin from tunicate (81). For this reason, it can be concluded that marine environment has abundant source of drugs that could be potential candidates for cancer treatment.

Renieramycin

Renieramycins, marine natural products, are alkaloids in tetrahydroisoquinoline family. Renieramycins are derived from marine natural products that found in diverse marine organisms such as sponge the genera *Reniera, Cribrochalina, Xestospongia, and Neopetrosia* (80). However, the extraction and isolation of reniramycins were also found to be unstable and decomposed as very unstable amino alcohol functionality at Carbon-21 in their structure will be converted into stable aminonitrile compounds by pretreatment with KCN (potassium cyanide) in methanolic buffer solution (80, 82)

The derivatives of renieramycin (renieramycins A-Y) are extracted from many species of sponges and contain a chemical structure associated to other isoquinoline natural products, which include quinocarcins, naphthyridinomycins, ecteinascidins, and saframycins, as well as become a target molecule for the study, synthesis, and research in biology for the treatment of cancer.

5-*O*-(*N*-Boc-Alanine)-Renieramycin T (OBA-RT)

Figure 6 the blue sponge *Xestospongia sp.*

The most prominent compound in reniemycins family is renieramycin T that extracted from KCN pretreated with methanolic extract of the blue sponge *Xestospongia sp.* that found in Thailand and the Philippines. As the chemical structure of renieramycin T, a renieramycin–ecteinascidin hybrid marine natural product in the tetrahydroisoquinoline alkaloid family, is the first example of a hybrid pentacyclic core. Recently, renieramycin T has become an attractive target for

synthetic and biological studies of highly substituted phenol and a condensed 1,3-dioxole ring, similar to the left carbon framework of ecteinascidins (83). The addition of an amino acid (alanine) by *N*-Boc-alanine esterification of the phenol moiety of renieramycin T to furnish the 5-O-(*N*-Boc-Alanine)-Renieramycin T (OBA-RT) (Figure 7) (19), having an identical structure to the anti-cancer drug Ecteinascidin 743 (Yondelis®) that approved by U.S. Food and Drug Administration (FDA). It has been reported that renieramycins family, including renieramycin M and renieramycin T, and derivative have a strong cytotoxicity, with IC_{50} (inhibitory concentration) values in the range of concentrations at nanomolar (nm) in many human cancer cell lines, including lung carcinoma (QC56 cells), pancreatic cancer (AsPC cells), colon cancer (DLD and HCT116 cells), breast cancer (MDA-MB-435 cells), and non-small-cell lung cancer cells (22, 84). In 2019, Korrakod reported that the renieramycin T can be inducing apoptosis in H460 cells through p53-dependent pathway which down-regulated of anti-apoptotic Mcl-1 and Bcl-2 proteins. The compound mediates Mcl-1 depletion by increasing the ubiquitin-proteasomal degradation of the protein and Mcl-1 was shown to control for survival and progression of cancer (85). In 2019, Wipa reported that the 5-*O*-acetyl renieramycin T (*O*-acetyl RT) can be inducing apoptosis and suppressing the expression of cancer stem cell (CSC) markers in H292 cells. We also found that the *O*-acetyl RT has the ability to increase cisplatin-induced apoptosis and reduce the number of cisplatin-induced $CD133^+$ cells. Therefore, *O*-acetyl RT may be a promising option as an allergen in cancer chemotherapy to reduce resistance and inhibit cancer progression (24). In 2009, Kornvika found that renieramycin M (RM) and their derivatives had anti-proliferative activities against colon (HCT116), and breast (MDA-MB-435) cancer cells with a very low IC_{50} (86). In 2016, bishydroquinone renieramycin M (HQ-RM), a modificated form of renieramycin M, had highly potent anticancer activity, greater than its parental RM. From this study, it had cytotoxicity and apoptosis induction in NSCLCs by an increase in the pro-apoptotic Bax protein and a decrease in the anti-apoptosis protein Bcl-2 and Mcl-1 (87) However, the mechanisms of OBA-RT that inhibit cancer stem cell and metastasis in NSCLC cells have not been elucidated yet especially. Thus, the effects of OBA-RT will be studied in this research.

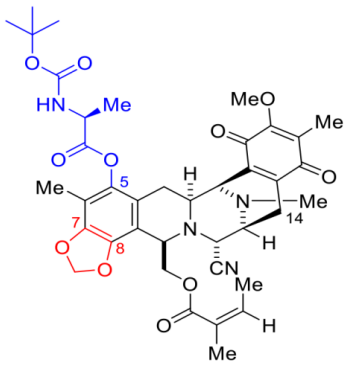


Figure 7 The synthesis of 5-*O*-(*N*-Boc-Alanine)-Renieramycin T (OBA-RT).

CHAPTER III

METHODOLOGY

3.1 Material and Instruments

3.1.1. Cell Lines and Culture

Human non-small cell lung cancer (NSCLC) cell lines, A549 (ATCC® CCL-185™, RRID: CVCL_0023) cells were obtained from the American Type Culture Collection (Manassas, VA, USA). A549 cells were cultured in DMEM medium. The medium was supplemented with 10% FBS, 2 mM L-glutamine and 100 units/ml of each penicillin and streptomycin at 37 ℃ with 5% CO_2 in a humidified incubator.

3.1.2. Preparation of the OBA-RT Stock Solution

OBA-RT was prepared by dissolving it in dimethyl sulfoxide (DMSO) solution and then stored at -20 ℃. It was freshly diluted with medium to the desired concentrations before using. The final concentration of DMSO was less than 0.5% solution, which shown no signs of cytotoxicity.

3.1.3. Reagents and Antibodies

Dulbecco's Modified Eagle's Medium (DMEM) medium, fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, phosphate-buffered saline (PBS) and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Hoechst 33342, propidium iodide (PI), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA). The following primary antibodies, PARP (#9532), p53 (#9282), Mcl-1 (#94296), Bcl-2 (#4223), BAX (#5023), Akt (#9272), phosphorylated Akt (#4060), Nanog (#4903), Oct4 (#2840), c-Myc (#5605) and GADPH (#5174), were obtained from Cell Signaling Technology (Danvers, MA, USA).

CD133 (#CA1217) was obtained from Cell Applications (San Diego, CA, USA). The respective secondary antibodies, anti-rabbit IgG (#7074) and anti-mouse (#7076) were 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)
- Nikon Eclipse Ts2 microscope
- 0.2-2 µl, 2-20 µl, 10-200 µl and 200-1000 µl micropipettes (Corning Inc., USA)
- Microplate reader Perkin Elmer VICTOR3 (Anthros, Durham, USA)
- Guava flow cytometer (Merck Millipore)
- SDS-PAGE (Bio-rad)
- Chemiluminescent ImageQuant LAS4000
- 60 mm dish culture (Corning Inc., USA)
- 6, 24 and 96 well plate (Corning Inc., USA)
- 15 and 50 ml conical tube (Corning Inc., USA)

3.2 Methods

3.2.1. Cell viability

A549 cells were cultured in 96-well plates at density 1×10^4 cells/well and incubated overnight at environment 5% CO2. Cells were treated with OBA-RT at concentrations 0 to 25 µM for 24 h. After treatments, 100 µL of MTT reagent (0.4 mg/mL) was added to each well and incubated for 3 h. The formazan crystals were dissolved in DMSO and measured using microplate reader (Anthros, Durham, NC, USA) at wavelength 570 nm.

3.2.2. Colony formation assay

The survival ability to colonize single cancer cells was investigated by colony formation assay. After treatments, cells were cultured into 6-well plates at density of 300 cells/well and incubated for 7 days. The cells were washed with 1X PBS, fixed with 4% paraformaldehyde (Sigma Chemical, St. Louis, MO, USA) for 30 min, and 0.5% crystal violet solution was used for staining. Cells were washed with 1X PBS three times, numbers and sizes of colonies were counted.

3.2.3. Apoptotic assay

A549 cells were seeded in 96-well plates at density 1×10^4 cells/well and allowed to attach overnight. Cells were incubated with various concentrations of OBA-RT at 0 to 25 μM for 24 h, and then the cells were co-stained with 10 μM of Hoechst 33342 (Sigma, St. Louis, MO, USA) and propidium iodide (PI) (Sigma, St. Louis, MO, USA) for 30 min in darkness. A fluorescence microscopy (Olympus DP70, Melville, NY, USA) was performed to image the apoptotic cells.

In addition, annexin V-FITC apoptosis Kit (Thermo Fisher Sciencetific, Waltham, MA, USA) was assessed to investigate the apoptosis and necrosis cells. A549 cells were seeded into 24-well plates at a density of 1.5×10^4 cells/well and incubated overnight. Cells were treated as indicated concentrations of OBA-RT (0-25 μM) for 24 h, then cells were harvested and suspended in the binding buffer followed by incubated with Annexin V and PI for 15 min in darkness. Apoptotic and necrotic cells were assessed by Guava easyCyteTM flow cytometry (Merk, DA, Germany).

3.2.4. Spheroid formation assay

A549 cells were pre-treated with concentrations of OBA-RT (0–25 μM) for 24 h. The treated cells were seeded onto ultralow-attachment plate at a density 2.5×10^3 cells/well in DMEM medium containing 1% FBS (v/v) (Merck, DA, Germany) for 7 days to form spheroids. At days 3 and 7, the numbers and sizes of spheroids

were photographed using a phase-contrast microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan).

3.2.5. CSC-rich population

The enrichment of the CSC subpopulation in cancer cells was successfully performed through three-dimensional (3D) spheroid-formation assay. A549 cells were seeded onto 24-well ultralow-attachment plates approximately 2.5×10^3 cells/well with serum-free medium to form primary spheroids for 7 days. After that, primary spheroids were resuspended into single cells were seeded onto 96-well ultralow-attachment plates for 14 days to form CSC-rich spheroids. After 14 days, CSC-rich spheroids were treated with concentrations of OBA-RT (0-25 μM) for 24 h. After treatment, apoptosis cell death was analyzed with Hoechst 33342 and size of single spheroid was captured using a phase-contrast microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan).

3.2.6. Western blot analysis

A549 cells were seeded at a density of 4×10^5 cells/well in 6 well plates overnight. Cells were treated with OBA-RT (0–25 μM) for 24 h. Then, cells were washed with cold 1X PBS and incubated in RIPA buffer, 1% Triton x-100, 100 mM PMSF, and a protease inhibitor for 30 min on ice. Protein concentrations were quantified using BCA protein assay kit from Pierce Biotechnology (Rockford, IL). Cells lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) (Bio-Rad Laboratories Inc., CA, USA). The membrane was blocked with 5% (w/v) non-fat dry milk power (Merck, DA, Germany) at room temperature for 2 h and each membrane was incubated with the specific primary antibodies for overnight at 4 °C, as well as incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA) for 2 h at room temperature. The protein expressions were observed using chem-iluminescence (Supersignal West Pico;

Pierce, Rockford, IL, USA) and were quantified using ImageJ software (NIH, Bethesda, MD, USA).

3.2.7. Immunofluorescence Assay

A549 cells were seeded into 96-well plates at a density 1×10^4 cells/well and incubated overnight. After treatments with OBA-RT for 24 h, the cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton-x for 5 min, and blocked with 4% BSA for 1 h at room temperature. The cells were incubated with an anti-CD133 antibodies overnight at 4 °C. After that, incubated with secondary antibodies for 1 h, and Hoechst 33342 (Sigma, St. Louis, MO, USA) for 30 min at room temperature in darkness, and mounted with 50% glycerol (Merck, DA, Germany). Confocal images were assessed under fluorescence microscope (Nikon ECLIPSE Ts2, Tokyo, Japan) and analyzed by ImageJ software.

3.2.8. Statistical analysis

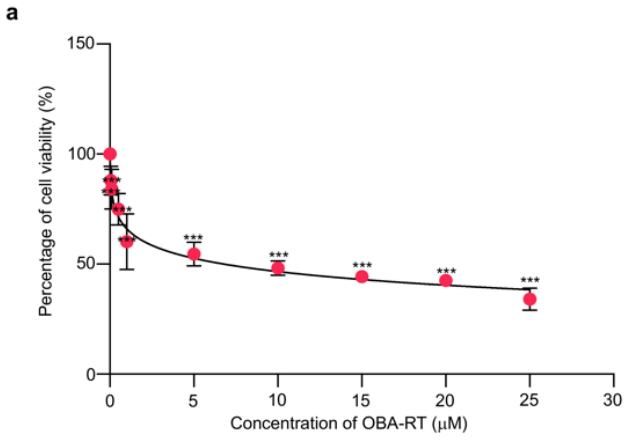
All results were compared and expressed as mean ± standard error of the mean (SEM) from at least triplicate independent experimental. Statistical analyses were evaluated using an analysis of variance (ANOVA) followed by Tukey HSD post hoc test. The statistic was calculated by using SPSS version 28 (IBM Corp., Armonk, NY, USA). Statistically significant of difference was indicated as *p-values less than 0.05. GraphPad prism 9 was used for creating graphs in all experiments (GraphPad Soft-ware, San Diego, CA, USA).

CHAPTER IV

RESULTS

4.1. Selective cytotoxicity of OBA-RT in non-small human lung cancer cells

We determined the cytotoxic profile of OBA-RT in NSCLC A549 cells to elucidate the anti-cancer potential of OBA-RT. After treating the cells with various concentrations of OBA-RT (0–25 μM) for 24 h, cell viability was evaluated by using the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay. The results showed that OBA-RT significantly reduced cell viability of A549 cells (Figure 8a) with the half-maximal inhibitory concentration (IC_{50}) value of 7.30 ± 0.04 μM (Figure 8b). The cytotoxic effects of OBA-RT were considered non-toxic at concentrations ≤ 0.05 μM in A549 cells.



b

Cell lines	IC_{50} ± SEM (μM)
A549	7.30 ± 0.04

Figure 8 OBA-RT reduces cell viability of non-small-cell lung cancer (NSCLC) cells.

(a) A549 cells were treated with various concentrations of OBA-RT (0–25 μM) for 24 h. Cell viability was determined by MTT assay. (b) The half-maximal inhibitory concentration (IC_{50}) at 24 h was calculated. Data represent as mean ± SEM (n=3). ***$p < 0.0001$ compared with untreated control cells.

We confirmed the effect of OBA-RT in decreasing cell survival by investigating the surviving cell after treatment by colony formation assay. Surviving A549 cells after treatment with OBA-RT (5, 10, and 25 μM) for 24 h were counted and seeded to colony formation assay without further treatment. Crystal violet-stained colonies, which could reproduce a new cancer colony from a single cell, are shown in Figures 9. The results showed that the resistant cells receiving OBA-RT at 5 to 25 μM could not form colonies. (Figure 9).

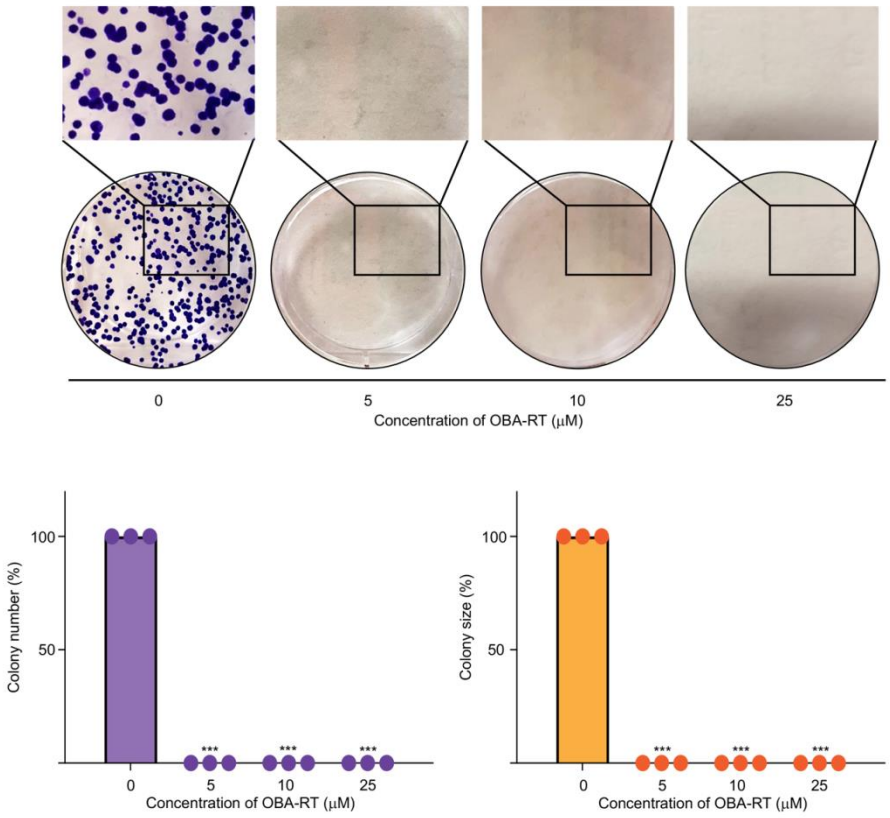


Figure 9 Effect of OBA-RT on cell proliferation by colony formation assay on A549 cells.

A549 cells were treated with various concentrations of OBA-RT (0–25 μM) for 24 h before being subjected to forming colonies for 7 days. Colony was stained by crystal violet. Data represent as mean ± SEM (n=3). ***$p < 0.0001$ compared with untreated control cells.

4.2. OBA-RT Induced Apoptosis through p53 Activation

In determining the mode of cell death induced by OBA-RT, the A549 cells were treated with OBA-RT (0–25 μM) for 24 h, and the apoptosis and necrosis cells were quantified the using the Hoechst 33342/ propidium iodide (PI) double staining assay. Hoechst 33342 staining was used to evaluate the nuclear morphology of apoptotic cells showing condensed or fragmented nuclei, whereas the PI (PI) stained the nucleus of necrotic cells. The results indicated that OBA-RT could increase apoptosis in a dose-dependent manner, whereas necrotic cells were minimally detected in response to all treatments. Therefore, OBA-RT primarily induced apoptotic cell death in our experimental setting (Figure 10a-b).

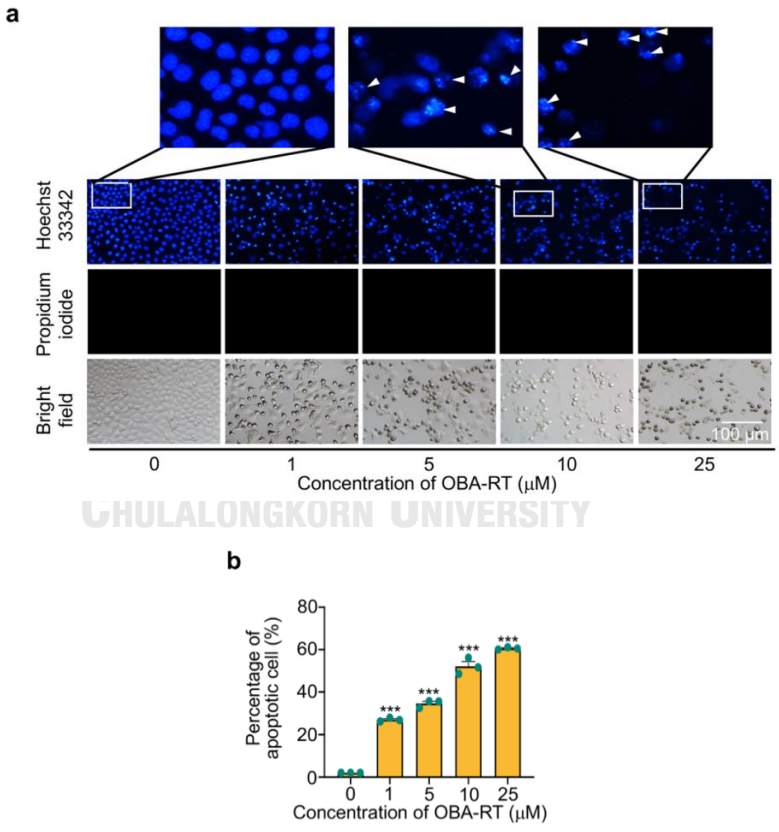


Figure 10 Apoptosis effect of OBA-RT on A549 cells.

(a, b) The nuclei of A549 cells treated with OBA-RT were stained with Hoechst 33342/ propidium iodide (PI) and calculated as a percentage compared with untreated control cells. The fragmented nuclei of apoptotic cells were indicted by arrowhead.

Data represent as mean ± SEM (n=3). ***p < 0.0001 compared with untreated control cells.

Other apoptotic cell feature of the presence of extracellular phosphatidylserine was determined to confirm the apoptosis-inducing effect of OBA-RT. Flow cytometric analysis of Annexin V/PI staining of the OBA-RT-treated cells showed that OBA-RT could increase the number of Annexin V-positive apoptotic cells (Figure 11a). As shown in Figure 11b, the percentage of early apoptotic cells was 27.28%, 43.72%, and 59.66% in A549 cells treated with OBA-RT at concentrations of 5, 10, and 25 μM, respectively.

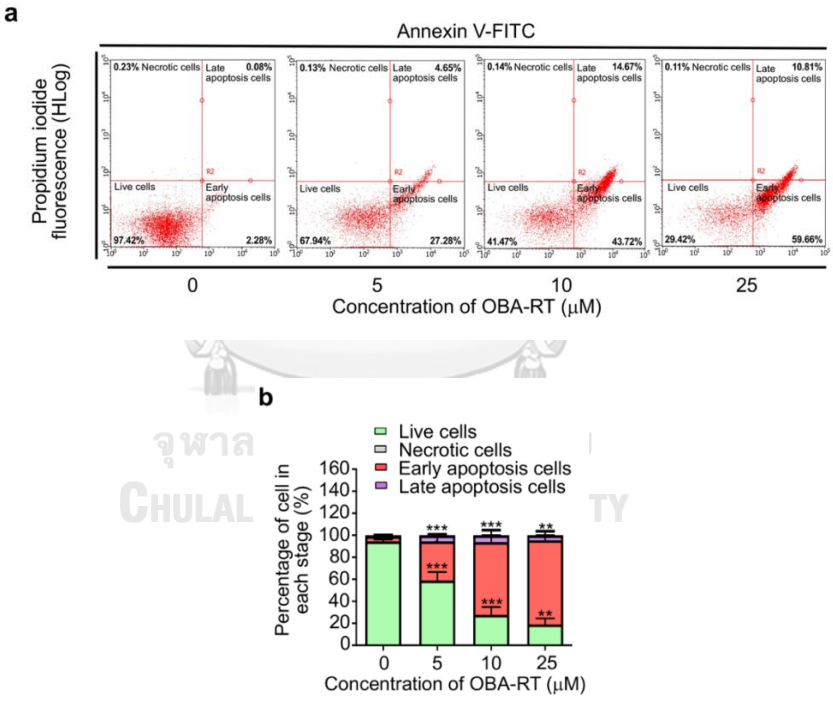


Figure 11 Effect of OBA-RT on apoptosis by flow cytometry on A549 cells.
(a) To confirm the apoptosis-inducing effect of OBA-RT, the A549 cells were treated with OBA-RT (0–25 μM) for 24 h. Apoptotic and necrotic cell death were determined using the Annexin V/PI staining assay. (b) Percentages of cells at each stage were calculated. Data represent as mean ± SEM (n=3). **p < 0.01 and ***p < 0.0001 compared with untreated control cells

In addition, the specific apoptotic marker protein, namely, poly (ADP-ribose) polymerase (PARP), and its cleaved form were detected in the treated cells. For mechanistic analysis, we monitored the alteration of apoptosis regulatory proteins, which belong to the Bcl-2 family and its upstream regulator p53 proteins. Protein determination was performed by western blotting. Lung cancer cells were treated with OBA-RT (0–25 μM) for 24 h. Western blotting revealed that in response to OBA-RT treatment, the cleaved form of PARP was significantly increased compared with the untreated control cells as shown in Figure 12a, b.

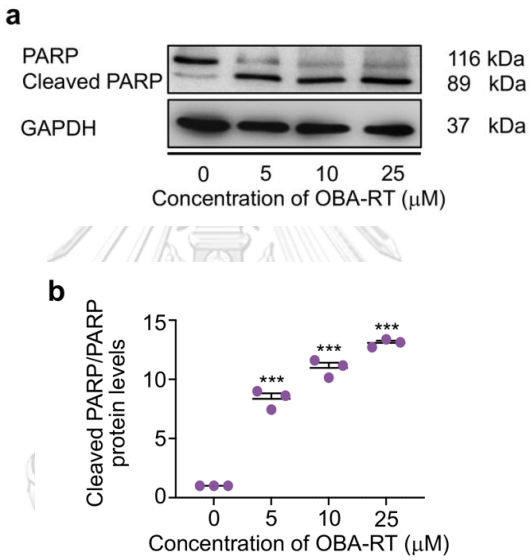


Figure 12 Effect of OBA-RT on apoptosis by Western blot analysis on A549 cells. (a) A549 cells were treated with OBA-RT (0-25 μM) for 24 h. The expression of cleavage of PARP and PARP were examined by Western blot analysis. GAPDH was determined to confirm equal loading of the samples. (b) Relative protein levels were quantified by densitometry. Data represent as mean ± SEM (n=3). ***$p < 0.0001$ compared with untreated control cells.

For apoptosis induction, the major regulators of p53-dependent apoptosis such as p53, anti-apoptotic proteins (Mcl-1 and Bcl-2), and pro-apoptotic proteins (Bax) were investigated in OBA-RT-treated cells. The results revealed that p53 was dramatically increased in response to compound treatment. Moreover, anti-apoptotic Bcl-2 and Mcl-1 were decreased, whereas pro-apoptotic Bax were found to be slightly altered. (Figure 13a, b).

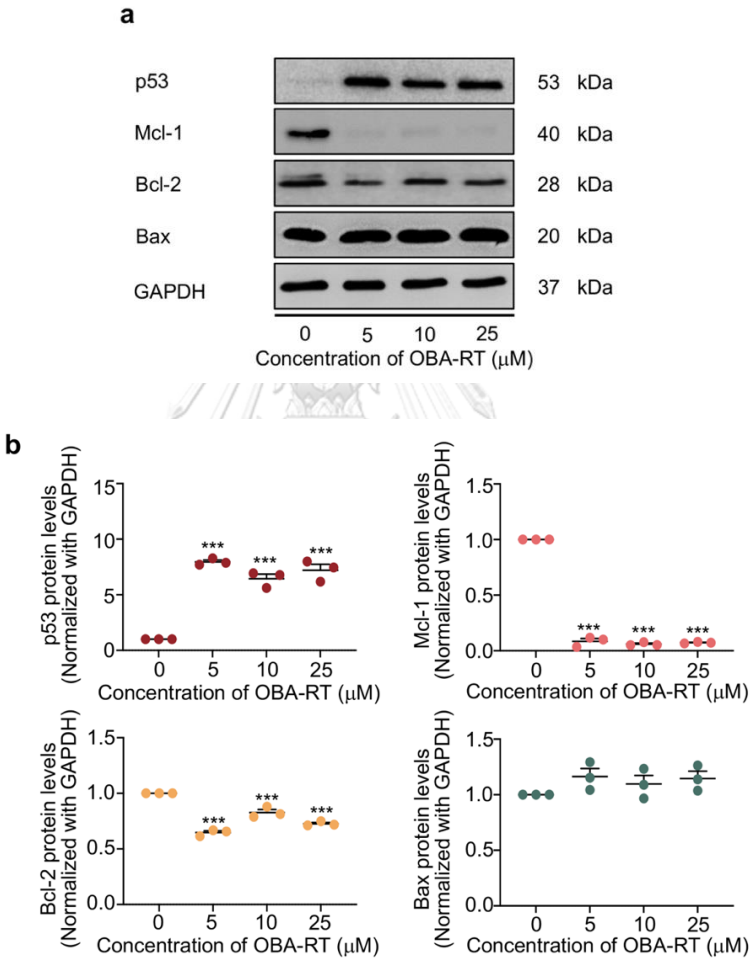


Figure 13 Effect of OBA-RT induces apoptosis via p53-dependent manner

(a) A549 cells were treated with OBA-RT (0-25 μM) for 24 h. The expression levels of apoptosis-associated proteins Bcl-2, Mcl-1, Bax and p53 proteins in A549 cells treated with OBA-RT (0-25 μM) for 24 h were examined by Western blot analysis. To confirm equal loading of the protein samples, the blots were reprobed with the GAPDH

antibody. (b) Relative protein levels were quantified by densitometry. Data represent as mean ± SEM (n=3). ***$p < 0.0001$ compared with untreated control cells.

4.3. OBA-RT Suppresses CSC Spheroid Formation

CSCs become an important target for the determination of novel anti-cancer drugs. The ability of cancer cells to form tumor spheroids has been refer to augmented CSC potentials. Next, we tested whether OBA-RT possessed CSC-suppressing activity. A549 cells were treated with OBA-RT at concentrations of 0-25 μM for 24 h, and the cells were subjected to a spheroid formation assay. The results showed that the cells treated with OBA-RT (5-25 μM) exhibited a reduced ability to form tumor spheroids in a concentration-dependent manner (Figure 14a-c).

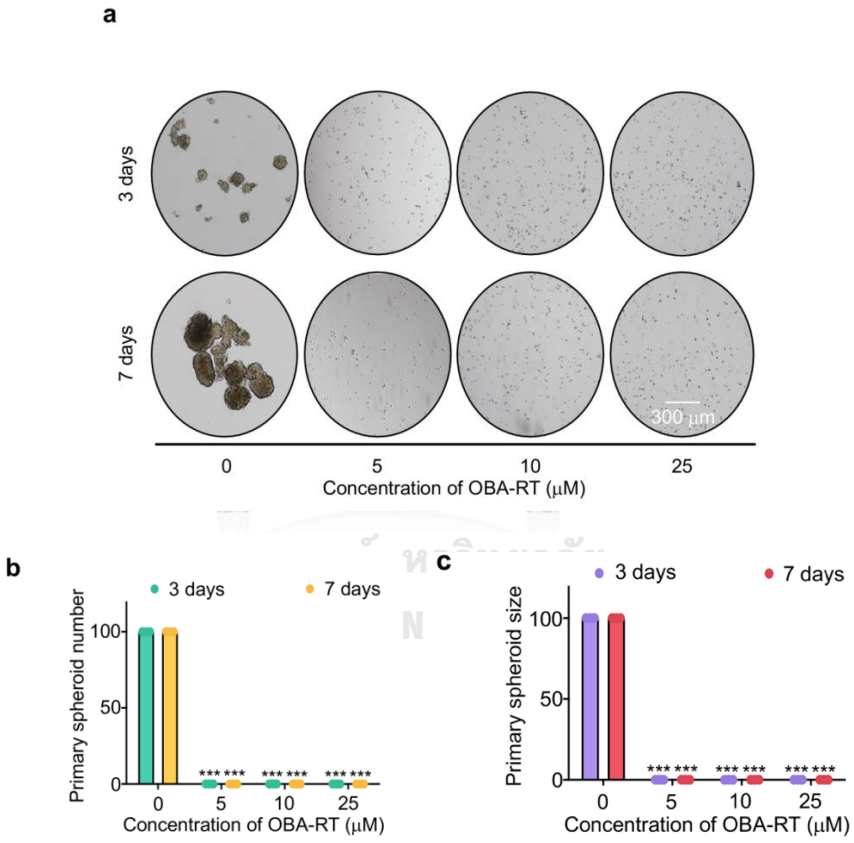


Figure 14 Effect of OBA-RT suppresses cancer stem cell (CSC) phenotypes of human non-small cell lung cancer A549 cells.

To assess the spheroid formation activity (a) Cells were pre-treated with OBA-RT for 24 h and allowed for 7 days to form primary spheroids. The numbers (b) and sizes (c) of primary spheroids were calculated. Data represent as mean ± SEM (n=3). ***$p < 0.0001$ compared with untreated control cells.

To further confirm CSC-killing population, the apoptotic induction of OBA-RT in CSC population of A549 cells was elucidated. A CSC-rich population of the lung cancer cells was established. The CSC spheroids were seeded in 96-well plates by ultralow attachment at a density of one spheroid per well. The spheroids were treated with OBA-RT (0-25 μM) for 24 h. In addition, the non-treated spheroid exhibited a normal survived feature, and the OBA-RT-treated spheroids detached and dissociated (Figure 15a). Hoechst 33342 staining of the treated spheroid further revealed the apoptotic character of DNA fragmentation and/or DNA condensation in the OBA-RT treated spheroid (Figure 15a-c). Collectively, OBA-RT possessed anti-CSC phenotypes, which can induce CSC apoptosis.

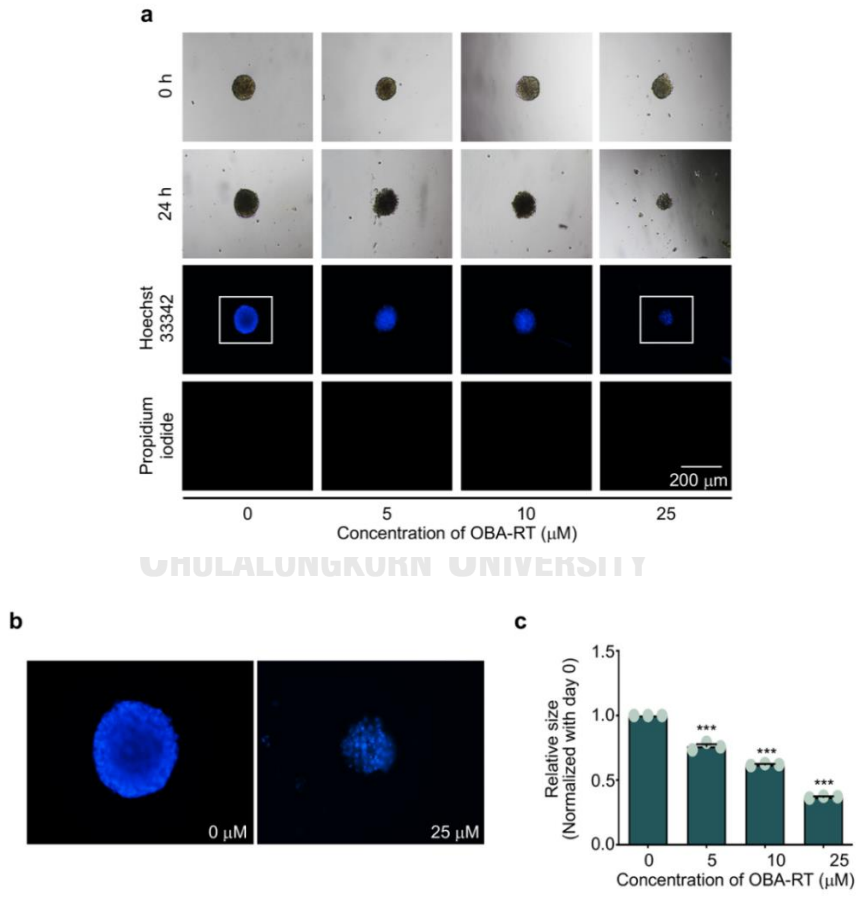


Figure 15 Effect of OBA-RT on CSC-rich populations on A549 cells.

(a) To further confirm the CSC-killing activity of OBA-RT, the CSC-rich populations in 3D culture were established by forming the primary spheroids for 7 days. The primary spheroids were suspended into single cells to form CSC-rich spheroids for 14 days in

ultralow attachment 96-well plates. The CSC-rich spheroids were then treated with OBA-RT at concentrations of 0-25 μM for 24 h. (b) The apoptosis cells were determined by Hoechst 33342 staining. (c) Relative size of CSC spheroids was quantified. Data represent as mean ± SEM (n=3). ***p < 0.0001 compared with untreated control cells.

4.4. OBA-RT Suppresses CSC Signals in A549 Cells

We determined CD133, a well-known CSC marker in response to compound treatment, to confirm the CSC-suppressing effect of OBA-RT. The cells were similarly treated with 0-25 μM OBA-RT for 24 h. The level of CD133 was then analyzed by immunofluorescence detected by a specific CD133 antibody. Figure 16a-b show that CD133 fluorescence intensity at concentrations of 5–25 μM significantly decreased when compared with the non-treatment control.

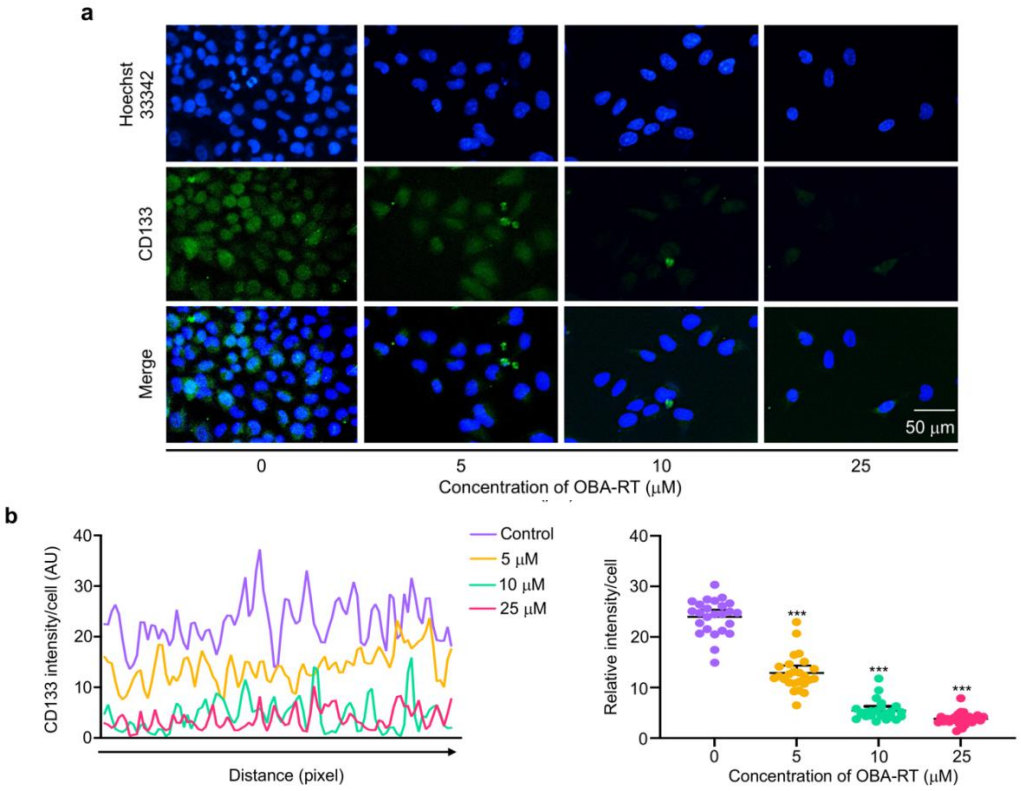


Figure 16 Effect of OBA-RT Suppresses Cancer Stem Cell Signals in A549 Cells

(a) OBA-RT inhibits Akt and suppresses CSCs. A549 cells were treated with OBA-RT for 24 h. The cells were co-stained with CD133 antibodies and Hoechst 33342. The expression of CD133 was examined by immunofluorescence (IF). (b) The fluorescence intensity was analyzed by ImageJ software. Data represent as mean ± SEM (n=3). *** $p < 0.0001$ compared with untreated control cells.

Inhibiting CSC-maintaining cellular signals is a potential way to reduce and improve clinical outcome in CSC-driving cancers, including lung cancer. The stemness properties of cancer are regulated by several pathways, and the Akt pathway can regulate pluripotent transcription factors, namely, Nanog and Oct4. Considering that OBA-RT could suppress the CSC phenotypes in lung cancer cells, we further tested whether this compound could effectively inhibit the CSC upstream signals via Akt inhibition and deplete the transcription factors of stem cells. The A549 cells were treated with various concentrations of OBA-RT (0-25 μM) for 24 h. In addition, CSC transcription factors, namely, Oct4 and Nanog, and CSC regulatory proteins, namely, Akt, p-Akt, and c-Myc proteins, were analyzed by Western blot analysis. The results revealed that Nanog, Oct4, and c-Myc were significantly decreased after OBA-RT treatment at concentrations of 5 and 25 μM. Akt signaling was highlighted as a therapeutic target for CSC-driven and malignant cancers; thus, the protein expression of phosphorylated Akt/Akt ratio was evaluated. After treatment of OBA-RT (5–25 μM) for 24 h, the p-Akt/Akt ratio was dramatically diminished when compared with the non-treatment control (Figure 17a, b).

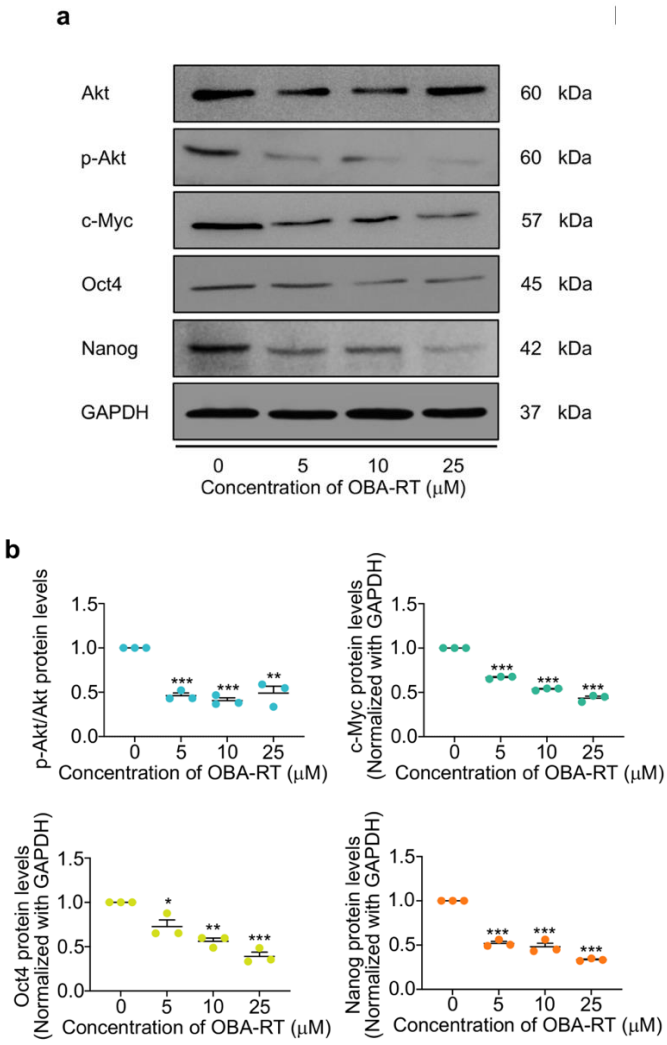


Figure 17 OBA-RT suppresses cancer stem cell (CSC)-like phenotype of human lung cancer cells.

(a) A549 cells were treated with various concentrations (0–20 μM) of OBA-RT for 24 h. The expression of activated Akt (p-Akt), total Akt and the expression levels of stemness-related proteins, Oct4, Nanog, and c-Myc in A549 cells treated with OBA-RT (0–25 μM) for 24 h were examined by Western blot analysis. To confirm equal loading of the protein samples, the blots were re-probed with the GAPDH antibody. (b) Relative protein levels were quantified by densitometry. Data represent as mean ± SEM (n=3). * $0.01 \leq p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared with untreated control cells.

4.5. Molecular docking simulations indicated the OBA-RT interactions with the allosteric pocket of Akt-1 protein

We performed a molecular docking simulation of OBA-RT with Akt (PDB code: 5KCV) to evaluate the possibility of a direct interaction between OBA-RT and Akt. In verifying the docking protocol, we redocked miransertib into its original binding site of Akt using Autodock Vina. The root mean square deviation (RMSD) of the redocked ligand was a small RMSD value (0.484 Å). The results (Figure 18) indicated that the docking protocol was correct (RMSD < 2 Å) *(88)*. The binding energies of OBA-RT and co-crystal ligand miransertib have been reported in Table 2. OBA-RT could bind with Akt-1 with binding energy of −8.1 kcal/mol. As shown in Figure 6, OBA-RT forms two hydrogen bonds with Thr82 and Glu203 and forms hydrophobic interactions with Asn53, Asn54, Ser56, Ala58, Gln79, Trp80, Leu202, Ser205, Leu264, Lys268, Val270, and Asp292.

Table 2 Binding energy in kcal/mol of OBA-RT compared to co-crystal ligand miransertib.

Compounds	Binding Energy (kcal/mol)	Hydrogen Bond Interactions	Hydrophobic Interactions
Miransertib (Co-crystal ligand)	-12.8	Thr211, Tyr272	Asn53, Gln79, Trp80, Thr82, Leu210, Leu264, Val270, Tyr272, Asp274, Ile290
OBA-RT	-8.1	Thr82, Glu203	Asn53, Asn54, Ser56, Ala58, Gln79, Trp80, Leu202, Ser205, Leu264, Lys268, Val270, Asp292

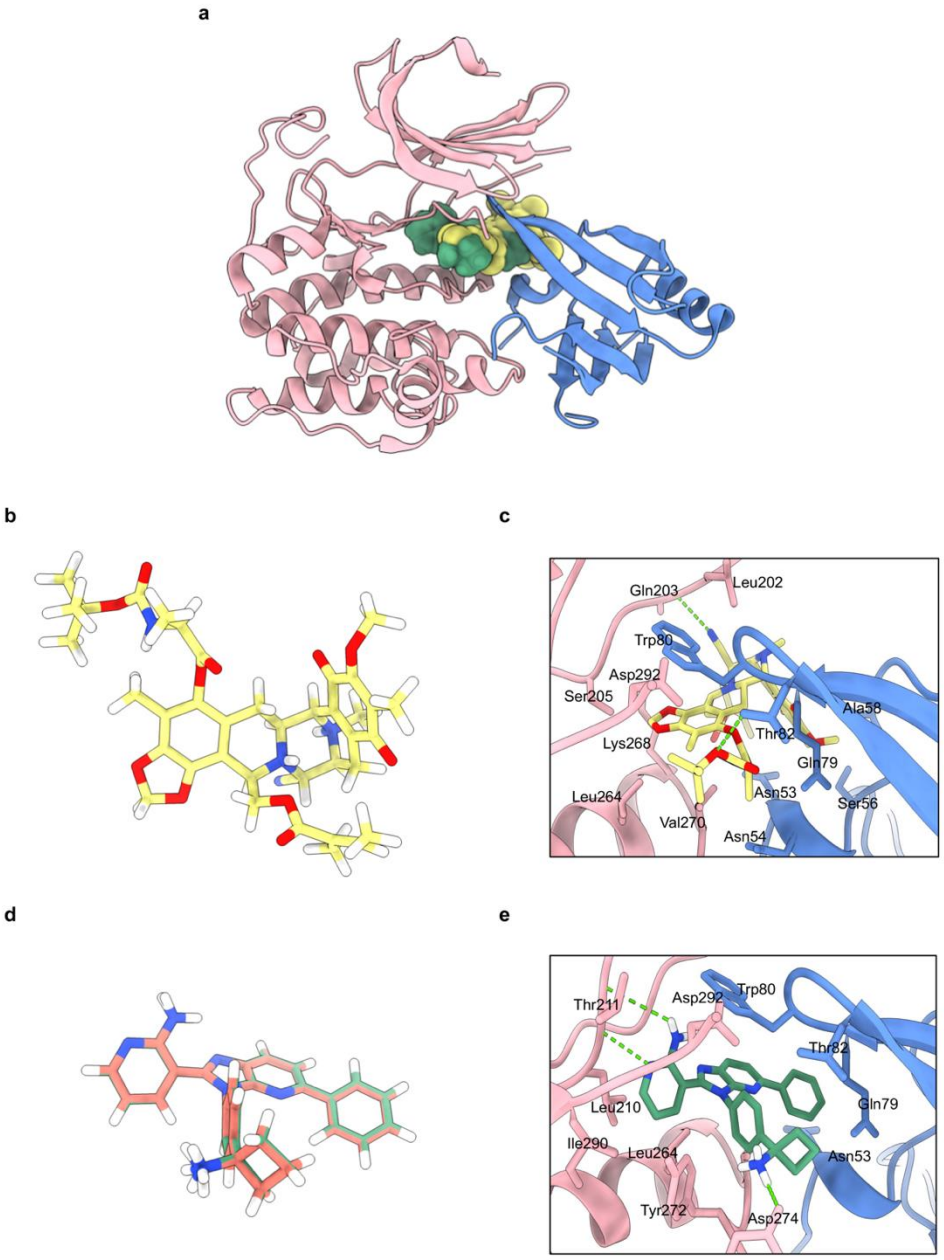


Figure 18 Molecular docking simulations indicated the OBA-RT interactions with the allosteric pocket of Akt-1 protein

(a) Co-crystal structure of Akt-1 in complex with OBA-RT and miransertib (PDB code: 5KCV). The kinase domain is shown in pink, PH domain in blue, OBA-RT in yellow, and miransertib in green. (b) 3D chemical structure of OBA-RT. (c) The binding mode of OBA-RT to the allosteric pocket of Akt-1. One of two major hydrogen bondings

was formed between the carbamate ether oxygen of Boc-L-alanine moiety and Thr82 while the butyl group was contributed to hydrophobic interactions with Leu264, Leu268, and Val270. (d) Redocking of miransertib in Akt-1 (PDB code: 5KCV); overlap of the co-crystal ligand miransertib (red) and redocking (green). (e) The binding mode of miransertib to the allosteric pocket of Akt-1. Hydrogen bonds are displayed as the green dashed lines.

CHAPTER V

DISCUSSION AND CONCLUSION

We reported facile and concise semi-synthesis of OBA-RT from RM isolated from the blue sponge Xestospongia sp. using benzoquinone/naphthoquinone-type photoredox chemistry (89) and Stiglich esterification. In addition, photo-transformation might account for the abiotic formation of hydroxybenzodioxole in naturally occurring saframycin-type tetrahydroi-soquinolin-equinone as an alternative enzymatic oxidative cyclization to the biosynthesis (89). This photosynthetic approach for C–H activation at C-5 would be useful for other tet-rahydroisoquinolinequinones, such as jorunnamycins, although the extent of utility and compatibility with other substitutions require further investigation. Compared with the three-step hydrogenation/esterification/oxidation scheme (19), this synthesis strategy should be amenable for any 5-*O*-conjugation of RT and related compounds. For example, the synthesis of a series of amino acid RT conjugates allowing the study of structure–activity relationship could be performed using this two-step scheme. Moreover, it could enable creative functionalization such as antibody–drug conjugations, fluorescence dye ligation for microscopy, and activity-based or photoaffinity probes for a target engagement study based on proteomics.

A549 cells are human non-small cell lung cancer cell line and are responsible for the diffusion of water and electrolytes across the alveoli (90, 91). A549 is a mutated KRAS cell. This mutation gene plays a large role in the aggressiveness of lung cancer and chemotherapy resistance. Constitutively active KRAS has been implicated in numerous human cancers, such as colon, breast, melanoma and including lung cancer, due to its ability to activate downstream RAF/MEK/ERK and PI3K/AKT. Cellular stemness is in part of AKT dependent and inhibited through AKT targeted inhibitor (92, 93). Therefore, A549 is appropriated as lung cancer stem cell model for AKT signaling inhibitor.

In this study, our data indicated that OBA-RT has a cytotoxic effect on human A549 cells with (IC_{50}) value of 7.30 ± 0.04 μM and, it displays molecular pharmacological properties in cancer cells similar to previously reported structurally related compounds (Figure 8, a-b). Our study revealed that the OBA-RT treatment could significantly inhibit cell viability (Figure 9, a–b) by inducing apoptotic cell death. We have added up the novel information that OBA-RT significantly inhibited the ability to form colonies (Figure 9) and eradicated the formed spheres. Due to OBA-RT can induce DNA damage and cell cycle arrest at the G0/G1 checkpoint but also triggering the apoptosis through caspase-3 activation (94) starting at concentration 5 μM.

Apoptosis mechanism is one of the important processes that regulate cancer cell survival. Several cancer researchs has aimed to study in this signaling pathway. Our study demonstrated that the OBA-RT treatment resulted in a significant induced of apoptotic cell death (Figure 10–11). This tumor suppressor p53 protein plays an important role in regulating DNA repair, cell cycle arrest, and apoptotic cell death. DNA double stranded breaks (DSBs) is the phenomena for DNA damage response (DDR) which evaluated by gamma-H2AX activation (γ-H2AX). γ-H2AX is a marker protein for DNA damage repair in DSBs resulting in activating Ataxia telangiectasia-mutated (ATM) kinases (95). In response to DNA damage, p53 were activated via ATM kinases (96). The activation of p53 resulted in the alteration of the cellular balance of Bcl-2 family proteins, thereby increasing pro-apoptotic members and decreasing anti-apoptotic proteins. This alteration creates the release of mitochondrial contents to the cytoplasm, and such contents motivate the function of caspases leading to apoptotic cell death. However, inducing apoptosis is not sufficient to eliminate cancer. In this research, the results show that OBA-RT has a mechanism of action similar to that of RT by inducing the p53-dependent signaling pathway and suppressing Mcl-1, which is an anti-apoptotic marker (Figure 13, a-b). Interestingly, the protein analysis shows a predominant effect of the cellular protein levels of Mcl-1. Mcl-1 is an anti-apoptotic protein that has gained increasing interest in lung cancer

cell biology because it is highly expressed in lung cancer (97). Furthermore, Mcl-1 is important for the survival of lung cancer cells.

Particular populations of cancer cells, namely, CSCs, have been reported as key driving factors for malignancy in several cancers. The conventional cancer therapy can only eliminate cancer cells but not CSCs. The CSCs can escape and obscure resulting in relapse of the disease in the future (2). Therefore, study of anti-cancer stem cells will be the highest promising approach. Indeed, different anti-CSC strategies have been assessed by inhibiting many intracellular signaling pathways, such as Wnt/TCF, signal transducer, and activator of transcription 3, namely, NF-**K**B and Akt. Akt signaling can be considered as a key regulator for cancers and CSC phenotypes. Notably, Akt signaling plays a critical role in regulating CSC maintenance and properties. Akt has been implicated in many aspects of cancer, such as cell proliferation, differentiation, and survival (71). Previous studies have revealed that Akt is directly linked to the master pluripotency factor Oct4 (98) and regulating transcription factor Nanog, Sox2, and reversed therapy resistance (99). A series of reports has exhibited that Akt inhibition may lead to CSC suppression. For example, Rhodes revealed that GSK690693 is a novel Akt kinase inhibitor that has recently entered phase I clinical trials. GSK690693 inhibited proliferation and induced apoptosis in a subset of tumor cells with potency consistent with intracellular inhibition of Akt kinase activity that showed reductions in phosphorylated Akt substrates in vivo (100). In 2019, Chantarawong reported that *O*-acetyl RT can suppress CSCs in lung cancer by depleting the AKT signal (24). Interestingly, *O*-acetyl RT has a chemical structure similar to OBA-RT. Hongwiangchan also reported that the CIN-RM suppressed CSCs by inhibiting the AKT signaling pathway, resulting in the downregulation of stem cell transcription factors, including Nanog, Oct4, and Sox2 (21). Nanog and Oct4 are the key transcription factor that controls self-renewal and pluripotency of CSCs, which is a prognostic biomarker in lung CSC under regulation by the Akt signaling pathway (101). Thus, it has been demonstrated that the inhibition of the Akt pathway could suppress CSCs. Moreover, spheroids formation that imitates in vivo conditions and contains key tumor features, especially drug

resistance and stem-like phenotype, serve as a more robust and valuable model for in vitro screening in lung cancer treatments. In this research, this compound demonstrated a potential in killing CSC properties as shown in Figure 15, a-c. Interestingly, our protein of interest, i.e., p-Akt, c-Myc, were dramatically abolished by OBA-RT treatment (Figure 17, a-b). Since the master regulators of CSCs can be controlled by Akt signaling. Therefore, inhibition of Akt will affect in reducing downstream factors that relate to CSC domination. Our results indicated that OBA-RT could suppress CSCs by decreasing of CD133, Nanog, and Oct4 expression (Figure 16-17) via an Akt-dependent pathway.

The inhibition of Akt at an essential binding site for protein activity is a powerful strategy. At present, several critical binding sites have been focused. Consequently, the allosteric Akt inhibitors have been highly emphasized because of their role in blocking the kinase activity of Akt and interfering a pleckstrin homology (PH)-domain membrane-mediated recruitment (102). This inhibition prevents Akt kinase activation and phosphorylation. In this study, considering the allosteric mechanism of OBA-RT, we performed molecular docking simulations using the binding interaction pattern of OBA-RT with the allosteric pocket of Akt-1. The molecular docking result revealed that OBA-RT could bind with Akt-1 with a binding energy of −8.1 kcal/mol, which is suitable for a potential interaction with Akt. The binding interaction pattern of OBA-RT with the allosteric pocket of Akt-1 is illustrated in Figure 18. The allosteric pocket of Akt-1 was located between the kinase domain and N-terminal PH domain (103). OBA-RT forms a hydrogen bond with Gln203 and hydrophobic interaction with Leu202, Ser205, Leu264, Lys268, Val270, and Asp292 of the kinase domain. Moreover, it forms a hydrogen bond with Thr82 and hydrophobic interaction with Asn53, Asn54, Ser56, Ala58, Gln79, and Trp80 in the PH domain. Trp80 has been reported as an important residue for the allosteric Akt-1 inhibitor (104). The Boc-L-alanine extension contributed significantly to the overall affinity of OBA-RT to Akt, that is, the hydrogen bond formed between the carbamate ether oxygen and the hydroxyl group of Thr82 and the hydrophobic interaction formed between the terminal tert-butyl group and Leu264, Lys268, and Val270. In addition,

OBA-RT showed a similar binding pattern compared with miransertib, an oral allosteric Akt-1 inhibitor, by hydrophobic interaction with Trp80. Thus, the analyses suggest that OBA-RT could interact with Akt-1 via an allosteric mechanism, which demonstrates the ability of OBA-RT to inhibit Akt-1, following the previous experimental results. This result could support the conclusion that OBA-RT could be a potential anti-cancer agent by targeting Akt activation through an allosteric mechanism. Based on our computational analysis, OBA-RT-resistant cell lines with Akt variant harboring mutations in a key residue predicted to directly bind to OBA-RT can be generated for experimental validation. In vitro biophysical analyses for the determination of binding parameters between Akt and OBA-RT such as isothermal titration calorimetry and thermal shift assay might be conducted to verify the target engagement. By functionalizing OBA-RT with biotin or a bio-orthogonal group to be used in pull-down assays, chemo proteomics could be used to identify the complete set of cellular targets beyond Akt.

The cellular target profiling provided in this work would contribute to a new perspective of the tetrahydroisoquinoline antitumor antibiotics and may inform further systematic medicinal chemistry development of compounds in this class with defined molecular pharmacology details for next-generation therapy for intractable cancers. Currently, there are many ways to cure cancers, which include combination therapy. Combination therapy, a treatment modality that combines two or more therapeutic agents, is a cornerstone of cancer therapy. Interestingly, the combination of anti-cancer drugs enhance efficacy by synergistic effect. Combination therapy may target the same key pathways (105). Therefore, the combination between OBA-RT and other standard chemotherapy such as cisplatin, doxorubicin or etoposide might be the focusing point. Since the conventional drugs can only eliminate normal cancer cells but not cancer stem cell. Thus, adding OBA-RT would be raising up the successive rate for lung cancer therapy.

The results provide novel and significant data of the new derivative of RT (OBA-RT) to be considered as a potential therapy for lung CSCs. The compound has a potent apoptotic and CSC-suppressing activity in lung cancer cells (Figure 19, a-b). In

addition, the OBA-RT molecule could exert allosteric inhibition on Akt protein. As Akt is critical for cancer cell survival and stemness phenotypes, our results might be used in emphasizing OBA-RT as a potential therapy for CSC and Akt-driven cancers.

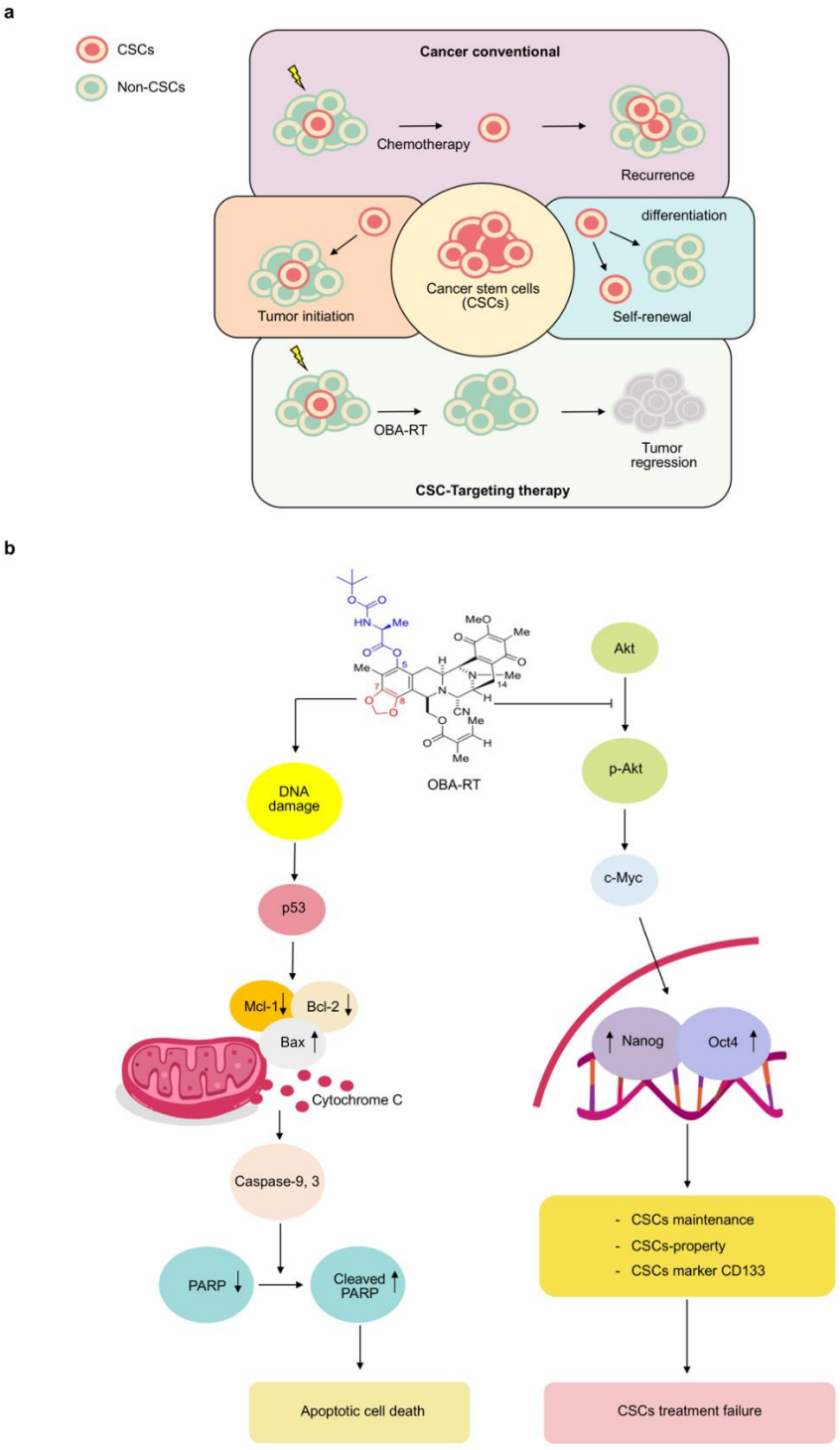


Figure 19 The proposed regulatory pathway of OBA-RT in inhibition of CSC and induction of apoptosis.

(a) CSCs drive cancer initiation, progression, and therapeutic failure due to their abilities to initiate cancer, induce self-renewal and tumorigenicity, and augmented pluripotent signals. CSCs are known to be highly resistant to chemotherapy and cause cancer relapse. Specific treatment to CSCs may induce cancer collapse and prevent the relapse of the disease. (b) Akt signaling pathways are critical for CSC properties and apoptotic cell death leading to cancer aggressive-ness. OBA-RT could inhibit Akt function, resulting in the apoptotic inducing and cancer stem cell suppression activity in lung cancer cells.

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