ANTI-MIGRATORY AND ANTI-INVASIVE ACTIVITIES OF 22-*O*-(*N*-BOC-L-GLYCINE) ESTER OF RENIERAMYCIN M AGAINST HUMAN LUNG CANCER H460 CELLS

Miss Yamin Oo

A Thesis Submitted in Partial Fulfillment of the Requirements
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ฤทธิ์ยับยั้งการย้ายถิ่นและยับยั้งการรุกรานของ 22-*โอ*-(*เอ็น*-บ็อค-แอล-ไกลซีน) เอสเทอร์ของเรนิอีรา ไมซินเอ็มต่อเซลล์มะเร็งปอดมนุษย์ชนิด เอช460

น.ส.ยามิน อู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Miss Yamin Oo
Field of Study	Pharmaceutical Sciences and Technology
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ยามิน อู : ฤทธิ์ยับยั้งการย้ายถิ่นและยับยั้งการรุกรานของ 22-*โอ*-(*เอ็น*-บ็อค-แอล-ไกลซีน) เอสเทอร์ของเรนิอีราไมซินเอ็มต่อเซลล์มะเร็งปอดมนุษย์ชนิด เอช460 . (ANTI-MIGRATORY AND ANTI-INVASIVE ACTIVITIES OF 22-*O*-(*N*-BOC-L-GLYCINE) ESTER OF RENIERAMYCIN M AGAINST HUMAN LUNG CANCER H460 CELLS) อ.ที่ปรึกษาหลัก : ผศ. ภก. ดร.ฉัตรชัย เชาว์ธรรม

อุบัติการณ์ของมะเร็งปอดในระยะแพร่กระจายมีผลอย่างมากต่อการกลับมาเป็นซ้ำและอัตราการเสียชีวิต ปัจจุบันยังไม่มีวิธีการรักษาที่สามารถยับยั้งการย้ายถิ่นของเซลล์ ซึ่งเป็นกระบวนการสำคัญในการแพร่กระจายของมะเร็งปอด ในการศึกษานี้ได้ศึกษาผลของ 22-*โอ*-(*เอ็น*-บ็อค-แอล-ไกลซีน) เอสเทอร์ของเรนิอีราไมซินเอ็ม (22-Boc-Gly-RM) ซึ่งเป็นอนุพันธ์ของอะมิโนเอสเทอร์สังเคราะห์ของ bistetrahydroisoquinolinequinone alkaloid ที่สกัดได้จาก *Xestospongia* sp., ต่อพฤติกรรมการย้ายถิ่นของเซลล์มะเร็งปอดมนุษย์ หลังจากทดสอบด้วย 22-Boc-Gly-RM ที่ความเข้มข้นไม่เป็นพิษ (0.5-1 ไมโครโมลาร์) เป็นเวลา 24 ชั่วโมง พบว่า 22-Boc-Gly-RM สามารถยับยั้งการเคลื่อนที่ของเซลล์มะเร็งปอดมนุษย์ชนิด เอช460 ได้อย่างมีประสิทธิภาพโดยประเมินจากการปิดรอยแผล อัตราการเคลื่อนที่ของเซลล์ (Transwell migration) และการเคลื่อนที่จากก้อนมะเร็ง ความสามารถในการรุกรานผ่านส่วนประกอบของเมททริกซ์ของเซลล์มะเร็งปอดเอช460 ถูกยับยั้งเมื่อเลี้ยงในอาหารเลี้ยงเซลล์ที่ประกอบด้วย 22-Boc-Gly-RM ที่ความเข้มข้น 0.1-1 ไมโครโมลาร์ การลดลงของโครงสร้างแอคตินที่ย้อมด้วย phalloidin สอดคล้องกับการลดลงของโปรตีน Rac1-GTP ที่วิเคราะห์ด้วย western blot เมื่อได้รับ 22-Boc-Gly-RM ความเข้มข้น 0.1-1 ไมโครโมลาร์ พบการยับยั้งสัญญาณ p-FAK/p-Akt และยับยั้งกระบวนการเปลี่ยนไปเป็นเซลล์มีเซนไคม์ ซึ่งแสดงได้จากเพิ่มขึ้นของโปรตีน E-cadherin และการแสดงออกของโปรตีน N-cadherin ที่ลดลง พบการเปลี่ยนแปลงของโปรตีนที่เกี่ยวข้องกับการรุกราน ได้แก่ การลดลงของเมทริกซ์เมทัลโลโปรตีเนส ชนิด MT1-MMP, MMP-2, MMP-7 และ MMP-9 รวมถึงการเพิ่มขึ้นของตัวยับยั้งเมทัลโลโปรตีเนส ชนิด TIMP2 และ TIMP3 ในเซลล์ เอช460 ที่ได้รับ 22-Boc-Gly-RM ดังนั้น 22-Boc-Gly-RM จึงเป็นสารที่มีแนวโน้มที่ดีสำหรับใช้ยับยั้งการแพร่กระจายของมะเร็งปอดผ่านการยับยั้งพฤติกรรมการย้ายถิ่นและยับยั้งการเปลี่ยนแปลงไปเป็นเซลล์มีเซนไคม์

สาขาวิชา	เภสัชศาสตร์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Yamin Oo : ANTI-MIGRATORY AND ANTI-INVASIVE ACTIVITIES OF 22-*O*-(*N*-BOC-L-GLYCINE) ESTER OF RENIERAMYCIN M AGAINST HUMAN LUNG CANCER H460 CELLS. Advisor: Asst. Prof. CHATCHAI CHAOTHAM, Ph.D.

The incidence of metastasis stage crucially contributes to high recurrence and mortality rate in lung cancer patients. Unfortunately, no available treatment inhibits migration, a key metastasis process in lung cancer. In this study, the effect of 22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M (22-Boc-Gly-RM), a semi-synthetic amino ester derivative of bistetrahydroisoquinolinequinone alkaloid isolated from *Xestospongia* sp., on migratory behavior of human lung cancer cells was investigated. Following 24 h of treatment, 22-Boc-Gly-RM at non-toxic concentrations (0.5-1 μM) effectively restrained motility of human lung cancer H460 cells assessed through wound healing, transwell migration and multicellular spheroid models. The capability to invade through matrix component was also repressed in H460 cells cultured with 0.1-1 μM 22-Boc-Gly-RM. The dose-dependent reduction of phalloidin-stained actin stress fibers corresponded with the down-regulated Rac1-GTP level presented via western blot analysis in 22-Boc-Gly-RM-treated cells. Treatment with 0.1-1 μM of 22-Boc-Gly-RM obviously caused suppression of p-FAK/p-Akt signal and consequent inhibition of epithelial to mesenchymal transition (EMT), which was evidenced with augmented level of E-cadherin and reduction of N-cadherin expression. The alteration of invasion-related proteins in 22-Boc-Gly-RM-treated H460 cells was indicated by the diminution of matrix metalloproteinases (MT1-MMP, MMP-2, MMP-7 and MMP-9) as well as the up-regulation of tissue inhibitors of metalloproteinases (TIMP), TIMP2 and TIMP3. Thus, 22-Boc-Gly-RM is a promising candidate for anti-metastasis treatment in lung cancer through inhibition of migratory features associated with suppression on EMT.

Field of Study:	Pharmaceutical Sciences and Technology	Student's Signature .....
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LIST OF ABBREVIATIONS

%	=	percentage
ºC	=	degree Celsius
µg/mL	=	microgram per milliliter
µM	=	micromolar
22-Boc-Gly-RM	=	22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M
Akt	=	protein kinase B
ANOVA	=	analysis of variance
BSA	=	Bovine serum albumin
CO_2	=	carbon dioxide
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	dimethyl sulfoxide
E-cad	=	E-cadherin
ECM	=	Extracellular matrix
EDTA	=	Ethylenediaminetetraacetic acid
EMT	=	Epithelial to mesenchymal transition
FAK	=	focal adhesion kinase
FBS	=	fetal bovine serum
h	=	hour, hours
kDa	=	kilodalton
min	=	minute, minutes

mL	=	milliliter
MMPs	=	matrix metalloproteinases
MT1-MMP	=	membrane-type matrix metalloproteinase
MTT	=	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NaCl	=	sodium chloride
N-cad	=	N-cadherin
NSCLC	=	non-small cell lung cancer
p-Akt	=	phosphorylated protein kinase
PBS	=	phosphate buffer saline
p-FAK	=	phosphorylated focal adhesion kinase
PI	=	propidium iodide
Rac1-GTP	=	Ras-related C3 botulinum toxic substrate 1
RPMI	=	Roswell Park Memorial Institute's medium
SD	=	Standard deviation
TIMP	=	tissue inhibitor of matrix metalloproteinase
µl	=	microliter

CHAPTER I

INTRODUCTION

Incidence and mortality rate of lung cancer have been increasing in both male and female patients all over the world [1]. The unusually high mortality rate is linked to cancer metastasis, which is the formation of a secondary tumor far from the original cancer pathology [2]. Approximately 70% of lung cancer patients have been reported to have advanced or metastatic disease that leads to poor prognosis and lowered 5-year survival rates [3]. Tumor metastasis occurs via a series of steps including detachment from the primary tumor, migration and invasion into surrounding tissue, intravasation to blood circulation and finally extravasation to form new tumor colonies at secondary sites [4]. Among these, migration and invasion are recognized as cancer hallmarks, while also serving as critical parameters of the metastasis cascade [5]. A growing body of evidence suggests that combating cancer metastasis by inhibiting migration and invasion is the key to successful cancer treatment [6, 7]. However, no available therapy provides satisfactory outcome in addressing cancer migration and invasion, highlighting the need for the development of a novel anti-metastasis drug.

The motility of migrating cancer cells involves the coordination of spatio-temporal changes of cellular actin filaments mediated by ras-related C3 botulinum toxin substrate 1 (Rac1), a protein featuring prominently in cancer cell migration and invasion [8]. Activated Rac1 (Rac1-GTP) facilitates cytoskeletal reorganization,

promoting cell migration via extracellular matrix (ECM) adhesion [9, 10]. Additionally, focal adhesion kinase (FAK) has been reported to modulate ECM adhesion activity of cancer cells through serving as an upstream regulator of Rac1 [4, 11]. The role of epithelial to mesenchymal transition (EMT) in enabling effective metastasis has been widely noted [12]. EMT occurs when epithelial cancer cells abolish adherence proteins, lose cell-cell contact and change to a mesenchymal phenotype thus gaining the ability to cross ECM by initiating migration and invasion [13]. Lung cancer cells in the advanced stages reveal alterations in transmembrane adhesion molecules, particularly the upregulation of N-cadherin and diminution of E-cadherin, which are features that characterize EMT [14, 15]. These cadherin alterations are dependent upon upstream sequence of FAK/Akt (Protein kinase B) signaling [16, 17]. By inhibiting the phosphorylation of both FAK (p-FAK) and Akt (p-Akt), a significant upregulation of E-cadherin expression and a simultaneous downregulation of N-cadherin expression occurs in various cancer cells [18, 19]. Moreover, the down-regulation of p-FAK and p-Akt in both *in-vitro* and *in-vivo* models resulted in suppressed cancer metastasis [18, 19], urging our further interest.

The invasion process involves a family of proteases called matrix metalloproteinases (MMPs), zinc-dependent enzymes capable of digesting ECM components [20]. Among the MMP subtypes, evidence suggests that matrix metalloproteinase 2 (MMP-2), 7 (MMP-7) and 9 (MMP9) as well as membrane type1-MMP (MT1-MMP) are responsible for promoting invasion and metastasis, especially in

lung cancer [20-22]. Wherein, ECM degradation, which enables metastasis, is inhibited by MMP inhibitors called tissue inhibitor of metalloproteinases (TIMPs) [23]. In fact, several reports have shown that up-regulation of TIMP2 and TIMP3 effectively suppresses invasion in various cancers including lung [24-26], pancreatic [27],thyroid [28] and colorectal cancer [29]. Moreover, inhibition of p-Akt and p-FAK suppresses cancer cell invasion by down-regulating MMP-2, MMP-7 and MT1-MMP expression [16, 30].

The marine bistetrahydroisoquinolinequinone alkaloids isolated from Thai blue sponge *Xestospongia* sp. (Figure 1) such as renieramycin M (**1**), renieramycin T (**2**) and jorunnamycin A (**3**) were reported as potential anti-metastasis natural compounds for lung cancer. Renieramycin M sensitized anoikis-resistant lung cancer cells by suppressing Akt survival protein level [31]. Meanwhile jorunnamycin A demonstrated anti-metastasis potential in human lung cancer cells via the inhibition of pro-survival FAK/Akt signal and suppression on EMT [32]. In addition, anticancer activity of renieramycin T was evidenced with apoptosis-inducing effect [33]. Recently, the new series of hydroquinone monoester derivatives of renieramycin M have been reported as the potential cytotoxic agents against human lung cancer cells [34, 35]. The hydroquinone analogues of renieramycin M such as hydroquinone 5-*O*-acetyl ester (**4**) and hydroquinone 5-*O*-cinnamoyl ester (**5**) significantly induced apoptosis by increasing expression of apoptosis-inducing factor [35, 36]. Among various semi-synthetic derivatives, 22-*O*-amino ester derivative of renieramycin M namely 22-*O*-(*N*-Boc-L-

glycine) ester of renieramycin M (**6**) exhibits potent anticancer activity against human lung cancer, which was indicated with lower half-maximal inhibitory concentration (IC_{50}) compared to renieramycin M [34]. However, the underlying mechanisms relating to migration and invasion of these marine alkaloids are not thoroughly elucidated. In this study, 22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M (22-Boc-Gly-RM) is elaborated its anti-metastasis target focusing on migration and invasion activity of human lung cancer cells. The results would further elucidate the potential of these marine-derived alkaloids, especially the promising semi-synthetic derivative (22-Boc-Gly-RM), to become novel anti-metastasis agents.

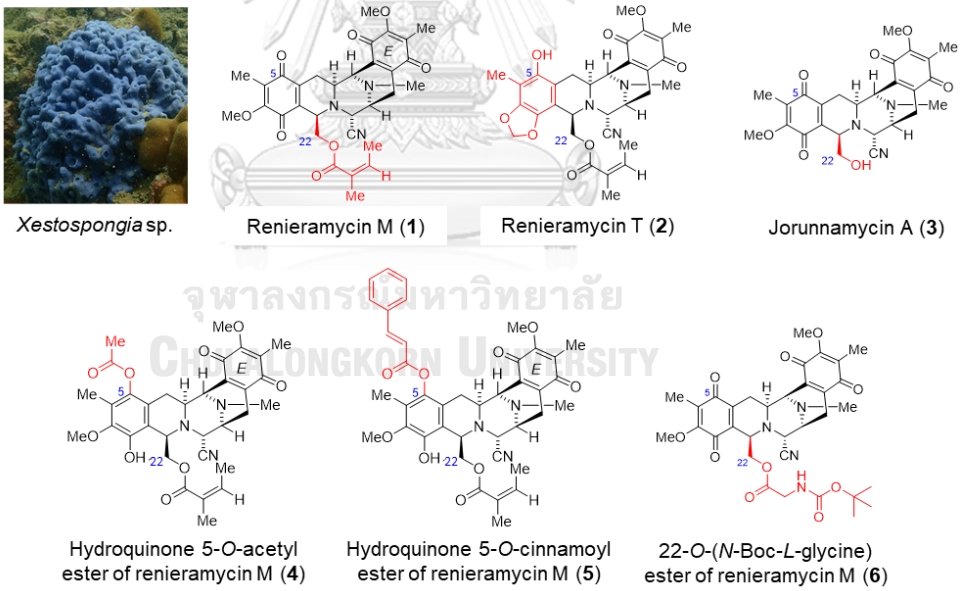


Figure 1 Thai blue sponge Xestospongia sp., renieramycin M (1),renieramycin T (**2**) and jorunnamycin A (**3**) and the new series of hydroquinone monoester derivatives of renieramycin M **(4-6)** wherein **(6)** represents chemical structure of 22-O-(N-Boc-L-glycine) ester of renieramycin M.

Research questions

1. Does 22-Boc-Gly-RM effectively inhibit migration and invasion in human lung cancer cells in vitro?
2. Which mechanism pathways of 22-Boc-Gly-RM inhibit migration and invasion of human lung cancer cells?

Objectives

1. To investigate the inhibitory effect of 22-Boc-Gly-RM on migration and invasion in human lung cancer H460 cells
2. To investigate the underlying mechanisms of 22-Boc-Gly-RM related in the inhibiting migration and invasion in human lung cancer H460 cells

Hypothesis

1. 22-Boc-Gly-RM could inhibit migration and invasion in human lung cancer H460 cells
2. 22-Boc-Gly-RM could modulate FAK regulating migratory signals in human lung cancer H460 cells

Expected benefits

The information of anti-migratory and anti-invasive activity and related mechanisms of 22-Boc-Gly-RM, which is the first anti-lung cancer mechanistic study of 22-*O*-acyl ester derivatives of renieramycin M and further development of 22-Boc-Gly-RM as a potential candidate for an anti-metastasis therapeutic agent.

CHAPTER II

LITERATURE REVIEWS

1. Lung cancer

1.1 Lung cancer metastasis

According to statistics of the International Agency Research for Cancer, there were an estimated 1.8 million cancer death and 2.2 million new cancer cases from 36 countries worldwide were predicted in 2020 [37]. Almost one-quarter of the global population are associated with lung cancer which still caused more deaths than the four leading causes of cancer (brain, prostate, colorectal, and brain cancers). Among all types of cancer, lung cancer is the most diagnosed cancer (11.7% of total cases) and seconding for mortality (18% total cancer deaths) attributed to both male and female patients [37, 38]. Approximately 90% of lung cancer deaths are primarily due to its high incidence of metastasis pathology in which the tumor has spread beyond the primary site [39]. Lung cancer is divided into two broad histologic classes, non-small cell lung cancer (NSCLC) and small cell lung cancer (~15%), each of which having separate biological behavior. Based on histological types, NSCLC is further subdivided into adenocarcinoma, large cell carcinoma, and squamous cell carcinoma which grows and spread in different ways. Non-small cell lung cancer (NSCLC) accounts for >80% of all cases of lung cancer, particularly from a patient with advanced or metastasis disease which is associated with a poor

prognosis [40]. Unfortunately, approximately 57% proportion of lung cancer patients are diagnosed at late or metastasis (stage IV disease) with the survival of patients at 5 year after diagnosis is only 5% [38]. The metastasis of NSCLC has been influenced by the histological subtype at diagnosis as well as age and sex of the patient [41]. Lung cancer metastasis spread readily into most frequent sites such as bone, brain, liver, and adrenal gland [42]. The evidence shows that lung cancer cells can survive under hypoxia, inactivated immune cells, and can migrate from the primary site and potentially lead to metastasis [43]. Despite the development of an advanced new treatment regimen, there is no curative treatment for advanced-stage metastasis lung cancer.

1.2 Therapy for lung cancer and advanced-stage NSCLC

The management and treatment of clinical lung cancer cases generally comprising surgery, radiotherapy, chemotherapy, and targeted therapy which can be treated alone or in combination with each other. The choice of treatment options is depending on cell types, stages of cancer, and patient performance status [44]. The primary treatment for most cancers in the early-stage disease (stage I and II) is surgery, which is only curative therapy and 25% of the patient are respectable at diagnosis [45]. For patients who contain unresectable tumors, radiotherapy is a treatment of choice. While surgery may be curative in the early stage, but most patients need following chemotherapy

treatment even who has been taken out obvious cancerous tissue during surgery. Although the standard option of the early stage of NSCLC is surgery and radiotherapy however the therapeutic efficacy of radiotherapy is limited for the patient whose cancer spread distantly. So far, one out of every three patients of NSCLC have an advanced tumor (stage IV of the disease) that is surgically ineradicable for whom systemic chemotherapy remains the basis of a palliative treatment to prolong to lengthen life and improve quality of life [46, 47]. Chemotherapy is the critical foundation for the patient with all stages of NSCLC cancer starting from an early resectable tumor into an advanced metastasis stage whose only treatment is chemotherapy. The current first-line treatment for stage IV NSCLC patients includes platinum-based chemotherapy used as a single cytotoxic agent with a median survival of 6.5 months. The combination of doublets of cisplatin or carboplatin with taxanes or vinca alkaloids expands the survival times to 10 months [48]. Also, combination chemotherapy with targeted therapy (monoclonal VEGF) or pemetrexed has an overall survival advantage up to 12 months and is first-lined licensed therapy for stage IIIB and IV non-squamous NSCLC [49, 50]. Moreover, approved second-line therapy such as docetaxel (Taxotere), pemetrexed, erlotinib, and gefitinib are licensed for the treatment of all non-small cell lung cancers. The combination of one of these agents found better tolerability in phase II and randomized clinical trials in metastasis cancer, but the development of

acquired or innate resistance limits therapeutic success in cancer patients [51, 52]. Also, the effect of radiation during radiotherapy can even induce distant metastasis, tumor migration, and invasive potential of cancer cells [53, 54]. On the other hand, NSCLC cells are less sensitive to radiation therapy and less affected by current therapies when compared with SCLC cells. Consequently, pretreatment of chemotherapy makes the cancer cells more sensitive to radiotherapy while there seems to be no significant difference in the median survival rate of radiotherapy alone. The series of chemotherapeutic drugs against NSCLC do not adequately establish the activity of cytotoxic agents as migration and invasion suppression in metastasis [55]. Despite the development of advanced new treatment but appear to have different side effect and overall survival rate not impressive and the responses to current standard treatment are poor except for localized tumor. Hence, these challenges might urgently need to develop a more efficacious and better-tolerated chemotherapeutic treatment to counteract this issue.

2. Cancer metastasis

Metastasis has been shown to have aggressive behavior of cancer cells which is the primary cause of death responsible for 90% of all cancer death and chemotherapeutic failure among cancer patients [56]. Limited success has been occurred in metastatic disease due to the reason that cancer therapy are mainly focuses on the term of inhibition of cancer growth, with few emphases

on treating cancer metastasis. It is the single most challenging obstacle in cancer management and its prevention might be the key goal in cancer research [57]. It is well established that metastasis is complex, the multistep process whereby cancer cells spread throughout the body, growing new colonies at a distance site away from originating tumor. Briefly, the metastasis cells begin with detachment from the existing tumor mass, undergo migration and invasion into basement membrane (BM) and extracellular matrix (ECM), enter to blood vessels and lymphatic systems (intravasation), leave from bloodstream (extravasation), finally adhere and colonize at the distant target organ. Not all the cells are led to initiate a new tumor after extravasation but rather go through cell death and the remaining alive cell are undergo proliferate or dormancy to give rise to metastases (Figure 2) [58]. Recently, the interest in cancer research has primarily shifted to the method which can detect on the early stage before the metastasis late-stage incurable and life-threatening disease. The rapid advance of molecular and cellular level associated with metastasis and identifying biochemical signaling pathways may lead to the understanding of better potential targets against metastasis cancer.

The metastasis process involved multiple sequential steps and biochemical events which are contributed by four vital steps such as detachment, migration, invasion, and adhesion, each of these steps are orchestrated and interrelated to each other [57]. For instance, cell migration

includes detachment, invasion, adhesion whereas cell invasion contains migration and adhesion. All these functions are regulated by molecular biochemical events and are affected by the surrounding extracellular matrix which together contributes to metastatic cascade [59]. Additionally, the cancer cell adhesiveness between cell-cell and cell to extracellular matrix interaction which are a distinctive characteristic of metastasis cells. During the cell migration and invasion, the disruption of cell-cell adhesion allows the cancer cell to detach from the initial tumor and alteration of cell-matrix interaction provides the cell to invade into the secondary site [57].

Among the multistage process, the tumor cell migration and invasion are an important circumstances for tumor progression, these critical steps may impact the delivery of cancer cells into the blood circulation and subsequently migrate and invade through the basement membrane, the extracellular matrix of surrounding tumor and endothelial cells of the target organ [60]. Most lung cancer patients are diagnosed at the late stage which already beyond local migration and invasion stages [3]. Hence, cancer cell migration and invasion are two fundamentals earlier steps for metastasis and hallmark of malignancy [61]. These dynamic changes of cells are accomplished by remodeling of cell-cell adhesion and cell-matrix interaction to address destination sites. When the migratory cells reach the circulation, it invades into the basement membrane to form secondary tumor [62]. The consequences of cancer cell migration

facilitate tumor progression in vitro and in vivo, moreover, concomitant with clinically poor prognosis in cancer patients [57]. The current advances are highlighted in understanding the molecular mechanism by which the cancer cells migration and invasion counteract tumor metastasis, including experimental methods and clinical observations. A better understanding of tumor cell migration and invasion is pivotal not only for perceiving the mechanism underlying cancer progression but also for better strategies for cancer treatment and patient prognosis.

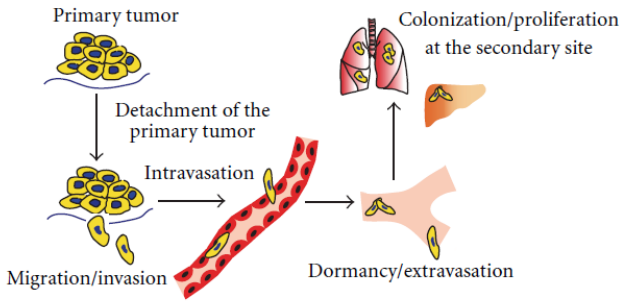


Figure 2 The steps of the metastatic cascade [58]

2.1 Migration

In multicellular animals, cell migration is a critical event throughout life not only in physiological embryonic development, wound healing, and immunosurveillance process but also in pathological diseases such as cancer,

inflammation, and rheumatoid arthritis [63]. Cell migration is a coordinated process of cytoskeleton dynamic and reorganization, cell adhesion [64]. To enable movement, the cells use a multistep process by formation of membrane protrusion at the leading edge to sense either soluble chemoattractant or repellent and move along with gradient, a process called chemotaxis [65]. The key requirement for cell migration is polymerization of G-actin monomer into filamentous F-actin which pushes the protrusion at leading-edge forward [66]. The changes in cytoskeleton rearrangement and cell adhesion determine cellular shape changes induced by actin polymerization. This temporal and spatial reorganization of cell structure allows the cells to respond to chemotactic signals such as growth factor or soluble factor from local or distance site of neighboring cells or extracellular matrix [67]. Moreover, cell motility be leading role in cell adhesion, detachment, migration, and invasion [68]. The key driver of cell migration starts with extension of protrusion at the leading edge and attribute of new adhesion at the front, translocate cell body, and dissociate adhesion at the rear. All of these steps undergo reorganization or assembly and disassembly of the actin cytoskeleton [68]. All the processes are contributed to maintaining normal conditions as well as in cancer progression.

In disease conditions, such as cancer cells use unregulated protrusive growth, for example during tumor metastasis, the cancer cells penetrate

extracellular or neighboring cells through two different mechanisms which can interchangeable between individual and collective cell movement [2, 69]. Malignant cells extensively use both types of modes in the healing of wound surface, tissue rearrangement as well as in the process of invasion and metastasis. In the 2D environment, most of the cells are moved by protrusion at the leading edge by cause of actin polymerization. The movement in the 3D matrix environment is more complex as a result of switching two modes [69, 70]. Single cell detaches from the original tumor undergo two migration patterns. For instance, Friedl showed that cells move in a ‘‘mesenchymal’’ fashion motion that had localized proteolysis on ECM surface [69, 71]. This motility is utilized by not only cancer cells but also normal fibroblast cells. Similarly, Sahai and Marshall mentioned ‘‘elongated’’ and ‘‘rounded associated motility’’ mediated by Rac or Rho/ROCK signaling [72]. Single-cell motility was observed in lymphoma, melanoma, breast cancer, and lung cancer. Most motile epithelial cells typically move with mesenchymal motion term as ‘‘epithelial-mesenchymal transition’’ – EMT – is the conversion of the epithelial cell to the motile and common event during carcinogenesis in vitro [73]. In contrast to the individual movement of cells, tumor cell can also migrate in groups, one is chain-like migration is displayed by melanomas and the second movement prefers as collective migration and invasion which mimics during development process [4]. In the epithelial cells, the cell

migration occurred after the dissociation of attachment from the cell, and majority of cancer are originated from epithelial cells. They are usually associated with the extension of dynamic F-actin mediated cellular protrusion (lamellipodia, filopodia) which is critical for motile cells with the help of mechanical force or protease activities as shown in Figure 3 [2, 57]. Membrane protrusion is the functional aspect of most of the motile cells for stabilization of directional movement and study of target proteins involved in the transduction of migratory signals are center of attention for cancer cell researchers [74]. Numerous cell surface receptor such as tyrosine kinase is stimulated by several external stimuli and induce response in the cell via downstream signaling molecules [75].

Several intracellular molecules might seem to interplay in migration, however, small GTPase-dependent cell signaling is essential for malignant motility and migration. Rho-GTPase family belongs to the superfamily of small GTPase, critical molecules for cell migration by sending a signal from the receptors to the cytoskeleton and has been implicated in many cellular processes including actin organization, cell morphogenesis, cell polarity, and progression of metastasis different tumors [74]. The activation of Rho-GTPase (GTP bound form) by extracellular cues are important to initiate signaling cascade and control the actin cytoskeleton [76]. The function of Rho-GTP involved in the stimulation of the first step of actin polymerization such as

nucleation which stabilizes multimer of actin monomer for elongation of the new actin filament [74, 77]. The classical Rho-GTPase such as RAC, Ras-related C3 botulinum toxin substrate-1 especially (Rac1), is required for the organization of the cytoskeleton, cell migration, and invasion through localized polymerization of actin [78]. Active Rac1 (Rac1-GTP) generates protrusion at the front of the cell and regulate dynamic F-actin assembly during chemotaxis which is directed cell movement toward chemoattract agent [79]. Dysregulation of Rac1 signaling is a prerequisite of cell migration and invasion, two fundamental steps in the metastatic cascade [80]. Rac1 overexpression has been found in non-small cell lung cancer, oral squamous cancer, breast cancer and gastric cancer [81]. Furthermore, Rac1 can mediate in vitro migration, invasion, whereas in vivo, it initiates lung colonization in xenograft mice [10]. Active form Rac1-GTP enhanced in the population of human NSCLC. Taken together, the observed effect of overexpressed Rac1 was detected in primary lung cancer tissue than normal lung specimens [81]. The significant association of Rac1 suppression reduced lung metastasis in mouse colorectal adenoma model [82]. Emerging evidence has shown that Rac1 has also been considered as an aggressive phenotype of lung cancer cells and lymph node metastasis in NSCLC patients [83, 84]. In addition, one studied mention that Rac1 inhibitor (NSC23766) could inhibit lung cancer migration, invasion, and rearrangement of cytoskeleton. The interesting point is that disruption of Rac1 function can

enhance chemotherapy sensitivity in lung cancer patients [81]. Interference of cell motility becomes an appealing approach in developing agents for metastasis cancer. The interference of Rac1 that is involved in cell migration could be potential targets for antimetastatic therapy.

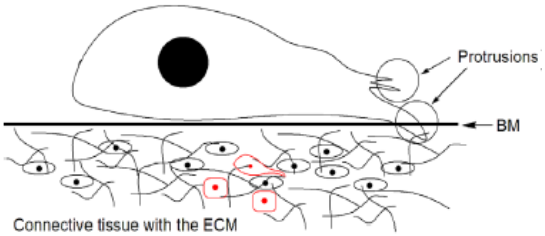


Figure 3 Invasive migration of metastasis cancer cells [2]

2.2 Invasion

The mounting evidence indicated that cancer cells need tumor environments not only to proliferate but also require to interact with their microenvironment to allow the disseminated cell to colonize at the distant organ for successfully metastasized [85]. In addition, the tumor microenvironment serves as a premetastatic niche whereas the key components of such niche are extracellular matrix (ECM) which play a significant role in cancer metastasis [86]. The extracellular matrix is characterized by its high dynamic structure and undergoes balanced remodeling [67]. The process of remodeling is primarily achieved through cleavage by proteases matrix metalloproteinase (MMPs) specific enzyme for ECM degradation [87]. ECM is the scaffold of the proteins to anchor and ligand

reservoir of growth factors. When MMPs degrade ECM, it subsequently releases this growth factor which in turn promotes metastasis spread [67, 88]. The tumor-associated proteolytic activity could be implemented for degradation of cell-basement membrane and tumor cell invasion by MMPs which is the essential process of transition carcinoma to a malignant invasive tumor [67]. This process helps the invasive cell to create a locally specific region by which tumor cells through the matrix barrier and spread into the surrounding tissue [57].

MMPs are zinc-dependent endopeptidase which is the main group of enzymes for cancer cell invasion and capable of degrading basement membrane and ECM components [87]. MMPs are synthesized as inactive zymogen and activated by other MMPs, and their proteolytic activity is controlled by protease inhibitors such as tissue inhibitor metalloproteinase (TIMPs) [89]. Both the expression and the activity of MMPs is increased in tissue and blood of cancer patient [90]. MMPs and their inhibitors have been contributed in all stages of cancer events, start from early stages into clinically metastasis progression. Over 23 members of proteinase have been identified, alternatively classified as soluble MMPs and membrane-type MMPs (MT-MMPs). Based on complex structure and substrate specificity and degradation of extracellular components, they are generally subdivided into six groups (collagenase, gelatinase, stromelysins, matrilysins, membrane-type MMP, and

other non-classified MMPs) [87]. In the previous observation, invasion of single amoeboid cells was observed in lung [20], breast, ovarian, prostate cancer [91, 92]. The follow-up studies demonstrate that increased MMPs expression in the blood of NSCLC patients is correlated with poor lung cancer patient's survival [20]. Furthermore, the catalytic activity of MMP can be regulated by endogenous inhibitors TIMP (tissue inhibitor of membrane proteinase), TIMP2, and TIMP3, but not TIMP1 to control the excessive breakdown of ECM [93]. In general, TIMP overexpression by cancer cells represent less invasive metastasis capacity, nevertheless, the individual TIMP overexpression might be diverse with a different type of tumor cells [23, 94].

The hallmark of cancer invasion is the up regulation of proteolytic enzymes (MMPs). The inhibition of ECM degradation by MMPs is effective for the inhibition of mesenchymal cell migration [88]. It is well-known that invasive malignant tumor cells are expressed several MMPs and involve in several tumor signaling pathways. Several studies have been published that MMP-2, MMP-9 (gelatinase A) which is very strongly correlated with NSCLC metastasis, resistance to chemotherapy, and low survival rate [19, 95]. The MMP-9 expression level is higher in NSCLC tissue and serum and tends to increase tumor size. Further, they destroyed structural components of lung tissue by breaking down the collagen type IV basement membrane [96, 97].

Moreover, membrane-type MT1-MMPs expression levels are significantly higher in human lung cancer in comparison with normal lung tissue. MMP-14 are frequently expressed in both epithelial tumor cells of mouse and human NSCLC [98]. As they localized on the surface of the cells and inhibit invasion of lung cancer cells by degradation of collagen and numerous ECM components in vitro. Furthermore, MT-MMP is a cell motility enhancer and specific activator for MMP-2 which promotes the secondary growth at the metastasis site [99]. In addition, active MMP-2 are found in the tumor sample, concomitantly believed the MT1-MMP is also increased [100]. In NSCLC, the expression MMP-2 has been shown to correlate with invasive phenotype of tumor cell and chemotherapy resistance [101]. Interestingly, in the study of the three-dimensional experiment, MT1-MMP induces the activation of the tumor by enhancing the invasion of Matrigel [102].

In addition, initial studies suggested that tumor-expressed MMP-7 are highly correlated with the aggressive phenotype of malignant tumors including lung cancer by proteolysis of ECM components leading to invasion and metastasis [103]. MMP-7 overexpression has been detected in malignant tumors including lung [103], colorectal [104, 105], gastric [106] and pancreatic cancers [107]. The previous finding revealed MMP-7 controls epithelial cell migration by controlling cell-matrix interaction. The poor survival rate of lung cancer patients was correlated with increase MMP-7 expression in the tumor [108, 109].

To take a step forward, a hindrance to the activity of matrix metalloproteinases on tumor growth and metastasis was achieved by overexpression of tissue inhibitor metalloproteinase (TIMP). TIMPs contain four types of proteins (TIMP1,2,3 and 4) that play a role in the remodeling of ECM in both physiological and pathological conditions [93]. Unbalanced between the level of MMPs and TIMPs during carcinogenesis could be critical for invasive potential and worsen the patient outcome [23]. Despite the complex role of TIMPs in cancer, the expression of TIMP-2 and TIMP-3 inhibits cell migration and invasion in vitro and in vivo [24, 28]. The burden of metastasis tumor showing decreased in the lung of TIMP-2 treatment mice [110]. TIMP-3 belongs tumor suppressor gene in most types of cancer and the loss of TIMP-3 has been linked to increasing tumor size and advanced stage metastasis [111]. Based on previous evidence in studying inhibition of invasion by MMPs could be potentially targeted for NSCLC.

3. Epithelial to mesenchymal transition

EMT can occur in both physiological development processes and pathological conditions. During developmental EMT, (a) cell morphology is rearrangement by coordination of cell-cell, cell-ECM, and soluble signal (b) epithelial cells are release through proteolytic disruption of basement membrane and (c) fully detached cells undergo mesenchymal phenotype

[112]. In the case of disease, the EMT process is uncoordinated, disorganized, and cell-autonomous fashion. EMT program has been shown to enhance chemoresistance, metastasis, and great therapeutic interest in the cancer patient [113]. Furthermore, EMT is the characteristic feature of metastatic cancer cells, by which epithelial cells are transformed into mesenchymal characteristics exploited via dissociated of cell-cell junctions [114]. The breakage of adhesion between cell-cell junction to loss epithelial integrity and cadherin are involved in the regulation of cell-cell adhesion which are mainly controlled by the type 1 classical cadherin members of E-cadherin and N-cadherin [115].

Metastasis starts when polarized epithelial cancer cells detach from localized tumors and mesenchymal cells invade into adjacent tissues by EMT. General features of EMT include the cells change morphology, lose polarity and downregulated epithelial markers (E-cadherin), up-regulate mesenchymal markers (N-cadherin) which increase cell motility [73]. E-cadherin is important for maintaining epithelial phenotype and decrease their expression is the hallmark of EMT and subsequently induce migration and invasion in vitro and in vivo [116]. E-cadherin has been reported as a potent tumor suppressor because their downregulation is always associated with more aggressive, metastasis dissemination in malignant tumors. Furthermore, the suppression of E-cadherin is associated with poor prognosis in NSCLC patients [117]. Most

cancer cells with mesenchymal phenotype are found to correlate with drug resistance and poor prognosis [118]. On the contrary of this loss, N-cadherin is expressed non-epithelial and stromal cell and one of mesenchymal cadherin involved in adhesion of cell into the stroma [119]. This cadherin switch seems a clinical indicator for poor prognosis in metastasis [116]. N-cadherin upregulation are indicator of EMT and correlated with promote in cell-adhesion and migration regardless of expression and function of E-cadherin [120]. The critical role of cadherin switch makes protein as attractive target for cancer therapy.

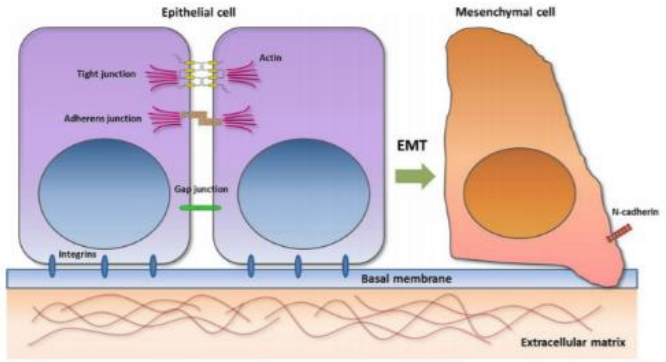


Figure 4 Schematic representation of epithelial to mesenchymal transition [121]

4. FAK signal-regulating migratory behaviors

4.1 Downstream signaling pathway of FAK: Targeting Rac1-inhibited cell motility

The key signaling pathway controlling cell motility in cancer is FAK which is a non-receptor cytoplasmic tyrosine kinase localized in the focal adhesion of the cells. Elevated FAK level in different tumor cell types was reported to induce tumor progression and metastasis [122, 123] . FAK activation has been

linked to modify signaling molecules involving PI3K/Akt that in turn promote actin cytoskeleton rearrangement cell spreading and migration[124]. Akt signaling stimulates actin remodeling and protrusion of leading-edge through Rac1-GTP protein modulate capabilities of migration and invasion of cancer cells [30]. Also, activated FAK has been implicated in the activation of downstream Rac-induced lamellipodia protrusion to enhance the movement of cancer cells [125]. The previous studies have identified a cell migration pathway triggered by PI3K/Akt and Rac1-GTP activation of FAK downstream in both epithelial and non-epithelial cell lines [126, 127]. Consistent results revealed that natural compound (*Dendrobium ellipsophyllum*, α-tomatine)-treated cells exhibit migration and invasion suppression effect by mediating FAK, Akt signaling in lung cancer cells [16, 128].

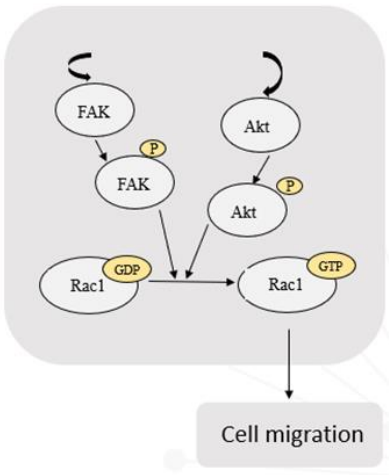


Figure 5 Rac signaling downstream migration through FAK, Akt mediated pathway [129]

4.2 Downstream signaling pathway of FAK: Targeting MMP-inhibited cell invasion

FAK has been shown to promote invasion by modifying actin dynamic and cell-matrix interaction [130]. Protein kinase B (Akt) which is localized at the leading edge of actin-rich moving cells and their downregulated expression inhibits cancer invasion [131]. Additionally, the involvement of Akt in reduced expression and activity of MMP has been studied in migration and invasion in vitro and in vivo [132]. Most published studies revealed that activation of FAK and downstream PI3K, Akt signaling pathway associated with cell invasiveness process. This is consistent with the prior report, MMP-mediated matrix degradation is achieved through activation of FAK which subsequently stimulated PI3K/Akt signaling [133, 134]. The compounds Nobiletin and Sinulariolide3,4-Dihydroxybenzalactone demonstrated that observed inhibitory of MMP expression on the invasion of cancer cells is due to FAK, PI3K, AKT pathway [135-137].

4.3 Downstream signaling pathway of FAK: Targeting EMT-inhibited cell motility

Meanwhile, phosphorylated FAK is an upstream modulator of cadherin proteins and FAK overexpression leads to induction of the EMT pathway [138]. PI3K/Akt signaling pathways activated various molecules that involve in EMT, cell-ECM interaction, and ECM proteolysis [139]. The researcher found that the

Akt signaling pathway is involved in EMT induced-MMPs downregulation in lung epithelial cells [140]. In another study, PI3K/Akt signaling is required for induction of EMT through upregulation of cadherin molecules [139]. The number of pieces of evidence suggests that several natural compounds such as Kaempferol and Batastasin III inhibit EMT in human lung cancer cells via downregulation of FAK/Akt signaling pathways [17, 141].

This concept makes sense knowledge to hypothesize dramatic inhibition of these activated molecules could be minimized the impact of tumor cell invasion and migration. The upstream FAK-Akt mediates migratory downstream effectors signaling to play a vital role in cell migration, invasion, and metastasis of lung cancer.

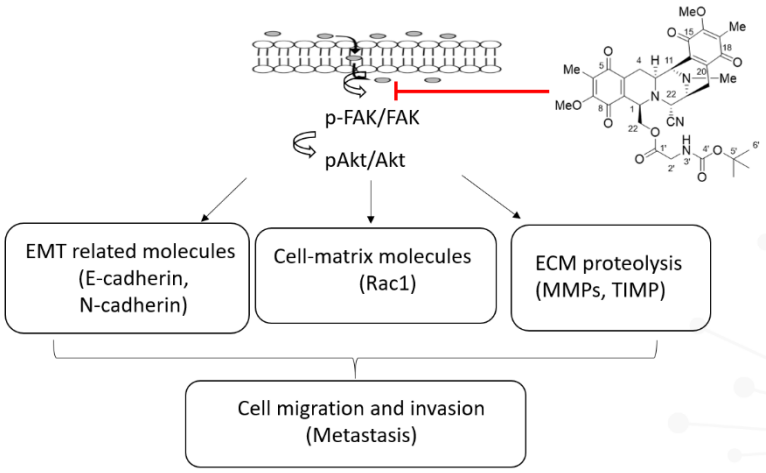


Figure 6 Signaling pathways of downstream that related with EMT and cell-matrix molecule and proteolysis involved in cell migration and invasion [139]

5. Anticancer activity of tetrahydroisoquinoline alkaloid from marine source

At the present, most active compounds used in cancer chemotherapy are derived from natural products. As early as 1995, the national cancer institute (NCI) was reported that 4% of marine natural products isolated from different organisms were found to contain the antitumor compound [142]. As a result of their complex structure, marine molecules can interact with various biological targets to either inhibit or enhance biological activity in the living cells. Most marine natural alkaloids have novel chemical structures belong to the tetrahydroisoquinoline family such as ecteinascidins, renieramycins, saframycins and jorunnamycins have been reported as potential anticancer candidates against several cancer cells lines including lung cancer [143].

Ecteinascidin 743 (ET-743), the first marine anticancer agent has been approved as a lead compound under the trade name Yondelis (ET-743) for the treatment of soft tissue sarcoma by the European Union in 2007 [144]. ET-743, tetrahydroisoquinoline alkaloid isolated from tunicate extract *Ecteinascidia turbinata* found in Caribbean ocean [145]. Their potent anti-proliferation activity of ecteinascidin in a variety of tumor cells and human tumor xenografts including melanoma, breast, ovarian as well as non-small cell lung cancer by unique mechanisms such as DNA minor groove binding activity and block cell cycle progression at the late phase [142, 146]. The presence of low amounts in their natural sources face obstacles in the isolation process and researchers

have been focusing on preparing synthetic processes to get efficient quantity [147].

Another member of natural marine bistetrahydroisoquionoline alkaloid, renieramycins which were isolated from different marine organism. The renieramycins are structurally and biologically related to ecteinescidin 743 (ET 743) [148] and exhibited promising cytotoxicity profiles against a wide range of cancer cell lines such as lung, breast, and colon cancer [149]. Renieramycin M possessed potential antimetastatic activity via apoptosis cell death mechanism and sensitized anoikis resistance against human H460 lung cancer cells [31]. Besides, another marine alkaloid containing an identical core structure, jorunnamycin A was recently reported as the antimetastasis agent by inhibition of epithelial to mesenchymal transition and sensitized anoikis in human lung cancer cells [32]. Until recently, the new generation of hydroquinone monoester derivatives of reneiramycin M was evaluated as potential cytotoxic agents in metastasis non-small cell lung cancer H23 and H460 cell lines [34, 150]. These facts have emphasized the essential role of naturally occurring renieramycins as the promising synthetic target for anticancer agents [151]. Surprisingly, the structural modification of renieramycins at C-22 and C-5 position showed strong antiproliferative activity and less unwanted toxic necrotic effect on the cells [148]. Based on the semi-synthesis approach, a new series of renieramycins such as 22-*O*-amino ester and 5-*O*-amino ester has been

continuously developed. Among the two new series, the cytotoxicity potency of 22-*O*-amino ester derivative against non-small cell lung cancer cell lines greater than the 5-*O* series [34].

5.1 22-*O*-(*N*-Boc-L-Glycine) ester of renieramycin M as a potential anticancer agent

Among the marine renieramycins, the most prominent renieramycin M, which was isolated from Thai blue sponge *Xestospongia* sp. found in Si-Chang Island, Gulf of Thailand [152]. The new semi-synthetic 22-*O*-amino ester derivative of renieramycin M was prepared by Chamni's laboratory through the transformation of renieramycin M to journamycin A with three steps of hydrogenation, hydride reduction and air-oxidation followed by treated with commercially available *N*-tert-butyloxycarbonyl amino acid such as *N*-Boc-L-glycine, which has been listed as excellent yield (93%) with the most cytotoxic potency among the 22-*O*-ester series [34]. In recent years, the structure-cytotoxicity relationship of renieramycin and its semi-synthetic derivative of ester side chains at C-22 and C-5 have been explored against the non-small cell lung cancer cell lines [150, 151]. Interestingly, additional ester motif substituent by replacing angelate ester at C-22 showed moderate to high cytotoxic potency with nano-molar concentration in human lung cancer cell lines [149].

Based on the structural analysis of 22-Boc-Gly-RM, both ester motif and aminoacyl moiety on ring B of tetrahydroisoquinoline core are essential for hydrophobic interaction with hydrogen bonding through interaction with other macromolecules during DNA alkylation process, which is similar with ecteinascidin mechanism. The cytotoxicities of newly synthesized 22-*O*-ester derivatives were significantly more potent than the parent compound and control drugs cisplatin and doxorubicin [34]. In the ongoing research toward the development of the derivative, 22-*O*-(N-Boc-L-glycine) ester of renieramycin M by putting amino ester-containing carbamate as protecting abbreviation group (Boc) being a model to further study as a prodrug [153, 154] and antibody-drug conjugate providing amide and carbamate linkage to connect cytotoxicity of the molecule [34, 155]. Herein, we reported *in vitro* cancer cell-based studies of 22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M and to identify cellular mechanism corresponding to cancer cell migration and invasive mechanism against H460 NSCLC cell lines. This is the first study of 22-*O*-ester derivative of renieramycin M which adds to the interest in the new series as a potential anti-lung cancer candidate (Figure 1).

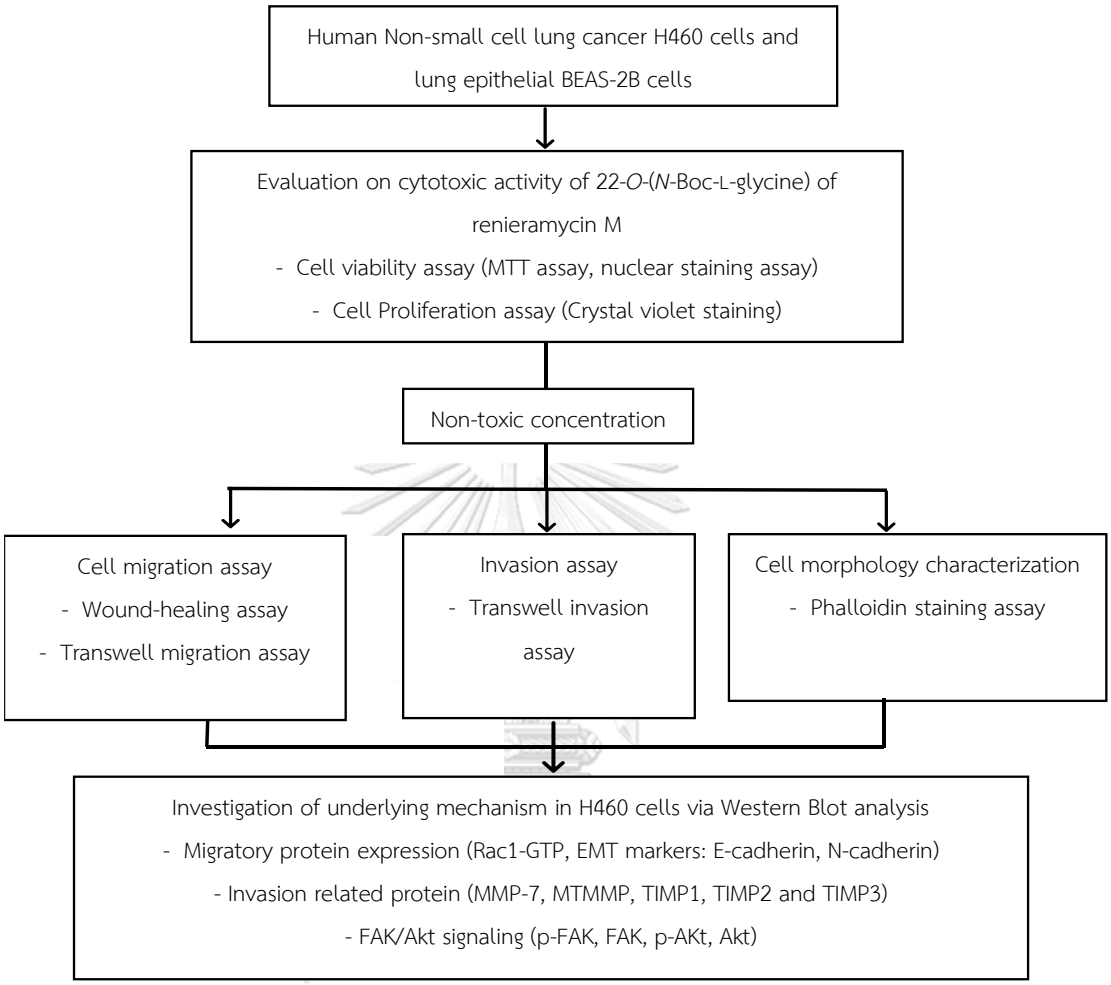


Figure 7 Experimental design

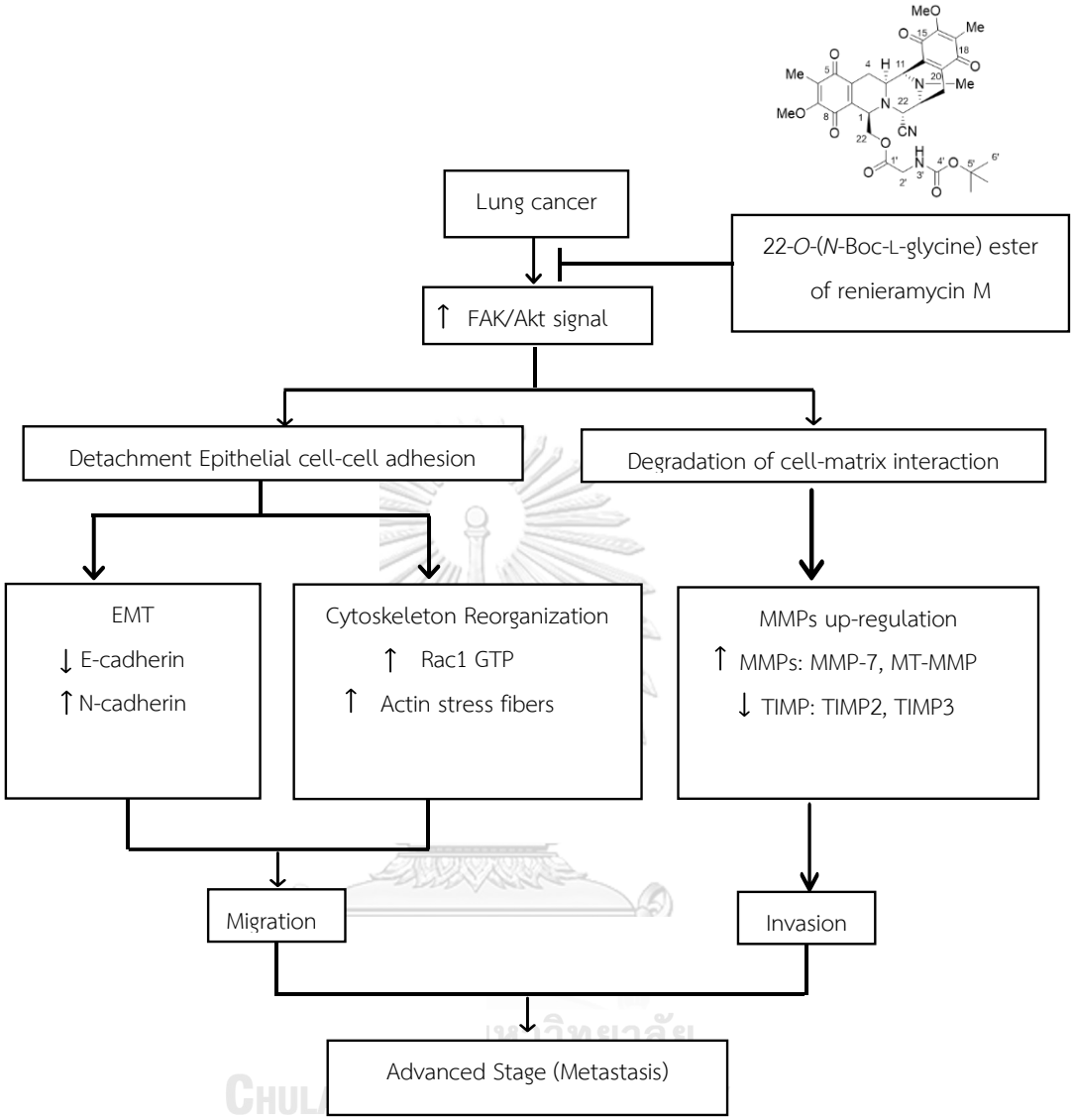


Figure 8 Conceptual framework

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemical and reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, phosphate buffer solution (PBS, pH 7.4), fetal bovine serum (FBS), 200 mM L-glutamine solution, 0.25% Trypsin/EDTA, and 10,000 units/ml penicillin/streptomycin solution were procured from Gibco (Gaithersburg, MA, USA). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst33342, propidium iodide (PI), 1% (w/v) crystal violet solution, paraformaldehyde powder, 37% (w/v) formaldehyde solution, Actinomycin D, 30% (w/w) hydrogen peroxide solution and methanol solution were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from EMD Millipore Corporation (Billerica, MA, USA). Alexa Fluor 568 phalloidin and Matrigel matrix were ordered from Invitrogen (Carlsbad, CA, USA) and Corning (Cambridge, MA, USA), respectively. Bicinchoninic acid (BCA) protein assay kit and SuperSignal West Pico PLUS chemiluminescent substrate were sourced from Thermo Scientific (Rockford, IL, USA). Primary antibodies specific for Akt, p-Akt (Ser473), FAK, p-FAK (Tyr397), E-cadherin, N-cadherin, MT1-MMP, MMP-2, MMP-7, MMP-9, TIMP2, TIMP3, β-actin and horseradish peroxidase (HRP) conjugated specific secondary antibodies were

obtained from Cell Signaling Technology (Danvers, MA, USA). Specific antibody for Rac1-GTP was purchased from NewEast Biosciences (Malvern, PA, USA).

2. Preparation of 22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M

22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M (22-Boc-Gly-RM) was semi-synthesized from jorunnamycin A by esterification as described in the previous studies [34, 150]. The obtained yellow powder of 22-Boc-Gly-RM was kept at room temperature avoid from light and moisture until use. The stock solution of 22-Boc-Gly-RM was prepared in DMSO and kept at -20°C until used in cell experiments. The DMSO stock solution of 22-Boc-Gly-RM was diluted with culture medium to obtain the desired concentration containing final concentration of DMSO less than 0.5% (v/v).

3. Cell culture

Human lung cancer H460 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute (RPMI 1640) medium. While normal human bronchial epithelial BEAS-2B cell (American Type Culture Collection, Manassas, VA, USA) and human keratinocyte HaCaT cell line (CLS, Heidelberg, Germany) were cultured in Dulbecco's modified eagle medium (DMEM). All the medium was supplemented with solution of 10% FBS, 2 mmol/L L-glutamine and 100 units/ml penicillin/streptomycin (Gaithersburg, MA, USA) under 5% CO_2 at 37°C until 70-80% confluence before using in further experiments.

Methods

1. Cell viability assay

The viable cells were assessed by using colorimetric MTT assay to determine non-toxic concentrations of 22-Boc-Gly-RM. Human lung cancer H460 cells were seeded at density of 1×10^4 cells/well in 100 µL RPMI medium containing 10% FBS into 96-well plates. After overnight incubation for cell attachment, the cells were treated with 22-Boc-Gly-RM (0-20 µM) for 24 h then further incubated with 0.4% (w/v) MTT solution at 37°C for 4 h protected from light. The intensity of purple formazan crystals solubilized in DMSO was measured at 570 nm via microplate reader (PerkinElmer Inc, Waltham, MA, USA). The percent cell viability was calculated as optical density (OD) ratio between treated to non-treated control cells.

2. Nuclease staining assay

Mode of cell death was examined through nuclear costaining of Hoechst33342 and PI. After indicated treatment, human lung cancer cells at 1×10^4 cells/well in 100 µL RPMI medium containing 10% FBS in 96-well plates were stained with 10 µM Hoechst33342 and 5 µg/mL PI in PBS (pH 7.4) for 30 min protected from light. Assessment of the morphology of fluorescent cells after costaining was done via a fluorescence microscope (Olympus IX51 Inverted Microscope, Olympus Corporation, Tokyo, Japan). Apoptosis cells presenting the

bright blue fluorescence of Hoechst33342 and necrosis cells stained with red PI fluorescence [156] were observed and reported as %cell death.

3. Determination of cell proliferation

To access the effect on cell proliferation, human lung cancer H460 cells at a density of 2×10^3 cells/well in 96-well plates were incubated with 10% FBS in RPMI medium (100 µL) with or without non-toxic concentrations of 22-Boc-Gly-RM. After culture for 12-72 h, the density of attached cells was estimated through crystal violet staining. The detached dead cells were removed after twice washing with 100 µL de-ionized water. After fixation with 1% (w/v) formaldehyde for 30 min, the cells were further incubated with 0.05% (w/v) crystal violet for 30 min. Excess dye was washed 2 times with 100 µL de-ionized water. After air-drying overnight, the crystal violet-stained cells were solubilized in methanol for measurement of OD at 570 nm by a microplate reader (PerkinElmer Inc, Waltham, MA, USA) [157]. Relative proliferation was a calculated as a ratio between OD of cells with respective treatments to OD of untreated control cells at 12 h.

4. Wound healing assay

The two-dimensional motility of lung cancer cells treated with 22-Boc-Gly-RM was investigated through wound healing or scratch wound assay. The monolayer of lung cancer H460 cells was prepared by seeding with 100 µL of single cell suspension prepared in RPMI medium containing 10% FBS at density of 4×10^4 cells/well in 96-well plates for overnight. Then, a scratch wound was

generated by creating a straight line on the cell monolayer with 20 µL pipette tip. The cells were washed with PBS to remove cell debris and further cultured with serum-free RPMI medium containing non-toxic concentrations of 22-Boc-Gly-RM. The width of the scratch wound was captured by using an inverted microscope (Nikon Ts2 Inverted microscope, Japan Optical Industries Co., Ltd., Tokyo, Japan) at 0, 6, 12, and 24 h of the incubation time. The relative migration level was calculated as the following formula.

$$\text{Relative migration}_{\text{at T}} = \frac{\text{Width of wound}_{\text{treatment at 0 h}} - \text{Width of wound}_{\text{treatment at T h}}}{\text{Width of wound}_{\text{control at 0 h}} - \text{Width of wound}_{\text{control at 6 h}}}$$

5. Three-dimensional migration and invasion assay

Boyden chamber 24-well plate (Corning Costar, State, MA, USA) was used to evaluate three-dimensional migration and invasion. Single-cell suspension of human lung cancer H460 cells in 100 µL serum-free RPMI medium was seeded at density of 5×10^4 cells/well on filter membrane of the transwell insert. The ability of human lung cancer cells to migrate from the upper chamber containing non-toxic concentration of 22-Boc-Gly-RM in serum-free culture medium was examined after placing the transwell inserts on a 24-well plate filled with 500 µL of 10% FBS in RPMI medium for 24 h. The number of migrated cells under the transwell membrane (pore size at 8 µm) was visualized under a fluorescence microscope (Olympus IX51 Inverted Microscope, Olympus Corporation, Shinjuku, Tokyo, Japan) after staining with 10 µM Hoechst33342 for 30 min. For transwell invasion assay,

the upper chamber was pre-coated with 0.5% Matrigel to mimic ECM before seeding of 100 µL of H460 cells suspension in serum-free culture medium at density of 5×10^4 cells/well. After induction by 10% FBS in RPMI medium (500 µL) as a chemoattractant in the lower chamber, the invasive cells under the transwell insert were stained with Hoechst33342 (10 µM) and examined under a fluorescence microscope. The ratio of migrated/invaded cells between treated and untreated group was presented as relative migration/invasion level.

6. Morphological characterization

In fixed conditions, cell morphology can be visualized by using phalloidin dye, which stains cytoskeleton actin filaments [158]. Briefly, human lung cancer cells (5×10^3 cells/well) were cultured with or without 22-Boc-Gly-RM at non-toxic concentrations in an 8-well chamber slide for 24 h. After removing culture medium, the cells were fixed with 4% paraformaldehyde at room temperature for 20 min. Then, the cells were washed with PBS, permeabilized with 0.1% Triton X for 5 min, and further blocked with 3% BSA in PBS for 30 min. The cells were then incubated with Alexa Fluor 568 phalloidin (1:100 in PBS) for 2 h at room temperature. After washing 3 times with PBS, Hoechst33342 solution (10 µg/mL) was added and placed for 15 min in a dark place to stain nuclei. Morphological changes were examined and photographed under a fluorescence microscope (Olympus IX51 Inverted Microscope, Olympus Corporation, Tokyo, Japan). The

staining intensity of stress fibers was analyzed by Image J software and presented as a value relative to untreated control cells.

7. Cancer spheroid-based migration and invasion assays.

Three-dimensional cancer spheroids were derived after culture of lung cancer H460 cells in 200 µl of RPMI containing 10% FBS at density of 1×10^4 cells/well in 96-well round bottom ultra-low attachment plate (Corning, Tewksbury, MA, USA) for 4 days [159]. To evaluate cell motility, single cancer spheroids were then transferred onto 96-well flat bottom plate and further incubated with non-toxic concentration of 22-Boc-Gly-RM in 100 µL serum-free RPMI medium for 24 h [160]. For cancer spheroid-based invasion assay, the obtained multicellular spheroids in 96-well round bottom ultra-low attachment plate were embedded into 100 µL 0.5% Matrigel. After solidification for 1 h under 37°C, the spheroids were cultured in 100 µL serum-free RPMI medium with or without 22-Boc-Gly-RM for 24 h [161]. The migratory and invasive capacities of cancer cells from multicellular spheroids were observed under an inverted microscope (Nikon Ts2 Inverted microscope, Japan Optical Industries Co., Ltd., Tokyo, Japan) at 0, 6, 12, and 24 h of the incubation time. The relative migrated/invaded area was calculated as the following formula.

$$\text{Relative migrated/invaded}_{\text{at T h}} = \frac{\text{Spheroid area}_{\text{treatment at T h}} - \text{Spheroid area}_{\text{treatment at 0 h}}}{\text{Spheroid area}_{\text{control at 6 h}} - \text{Spheroid area}_{\text{control at 0 h}}}$$

8. Western blot analysis.

After the indicated treatment, the protein lysate was extracted from human lung cancer cells incubated with lysis buffer solution (Merck, DM, Germany) composed of 20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM disodium EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/mL leupeptin and protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) at 4ºC for 30 min. The clear supernatant was collected and determined for total protein content by the BCA protein assay kit. Equal amount of denatured (95ºC) protein sample was loaded onto SDS-PAGE. The separated proteins were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), which was followed by blocking with 5% skim milk in TBST (25 mM Tris-HCl pH 7.5, 125 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. Then, the membrane was probed with specific primary antibodies at 4ºC overnight with gentle agitation. The membranes were washed with TBST for 5 min × 3 times and probed with HRP-conjugated specific secondary antibodies for 2 h at room temperature. The results were analyzed with help of chemiluminescence detection reagent and protein intensity was measured with analyst/PC densitometric software (Bio-Rad, Hercules, CA, USA) using β-actin as loading standard in each experiment.

9. Statistical Analysis

Data were represented as means ± standard derivation (SD) from three independent experiments. For statistical analysis, one-way ANOVA followed by Tukey HSD's post hoc test was carried out with SPSS program (SPSS Statistics Version 22.0, IBM, Armonk, NY, USA). The statistical significance was considered at $p < 0.05$.

CHAPTER IV

RESULTS

1. Cytotoxicity profile of 22-Boc-Gly-RM in human lung cancer cells

To investigate anti-metastasis activity, the cytotoxic profile of 22-Boc-Gly-RM was primarily evaluated in human lung cancer H460 cells. After culture for 24 h, the significant reduction of %cell viability determined by MTT assay

was revealed in the cells treated with 22-Boc-Gly-RM at 5-20 μM compared with untreated control (Figure 9 a). To confirm MTT assay, costaining of Hoechst33342/PI was performed to trace mode of cell death. Treatment with 50 μM Actinomycin D and 500 μM hydrogen peroxide for 24 h served as positive controls for apoptosis and necrosis induction, respectively, in human lung cancer cells [162]. Corresponding with the viability results, (Figure 9 c) demonstrates no apoptosis in human lung cancer cells incubated with the lower concentrations (0.1-1 μM) of 22-Boc-Gly-RM. Increased %apoptosis (Figure 9 b) signified by bright blue fluorescence of condensed DNA/fragmented nuclei stained by Hoechst33342 was noted in the cells after culture with 22-Boc-Gly-RM at 5-20 μM. Notably, there was no detectable necrosis in all 22-Boc-Gly-RM-treated cells. According to these results, 22-Boc-Gly-RM at 0.1-1 μM were considered as the non-toxic concentration range and selected for further investigations.

The effect on cell proliferation was additionally evaluated in human lung cancer cells cultured with 22-Boc-Gly-RM (0-1 μM) for 12-72 h. Results from the crystal violet assay, which serves to indirectly quantify viable cells, indicate the anti-proliferative effect of 22-Boc-Gly-RM after 48-72 h of incubation time (Figure 9 d). The incremental increase of proliferation in human lung cancer H460 cells was clearly observed after culture for 48-72 h while there was no alteration of relative proliferation in H460 cells cultured with 0.1

μM of 22-Boc-Gly-RM. Interestingly, 22-Boc-Gly-RM at 0.5-1 μM remarkably suppressed proliferation promptly at 24 h and continued to lower proliferation levels in human lung cancer H460 cells compared with untreated control cells at 48-72 h. It should be noted, however, that culture with 0.1-1 μM 22-Boc-Gly-RM for 12 h did not alter proliferative activity in lung cancer H460 cells.

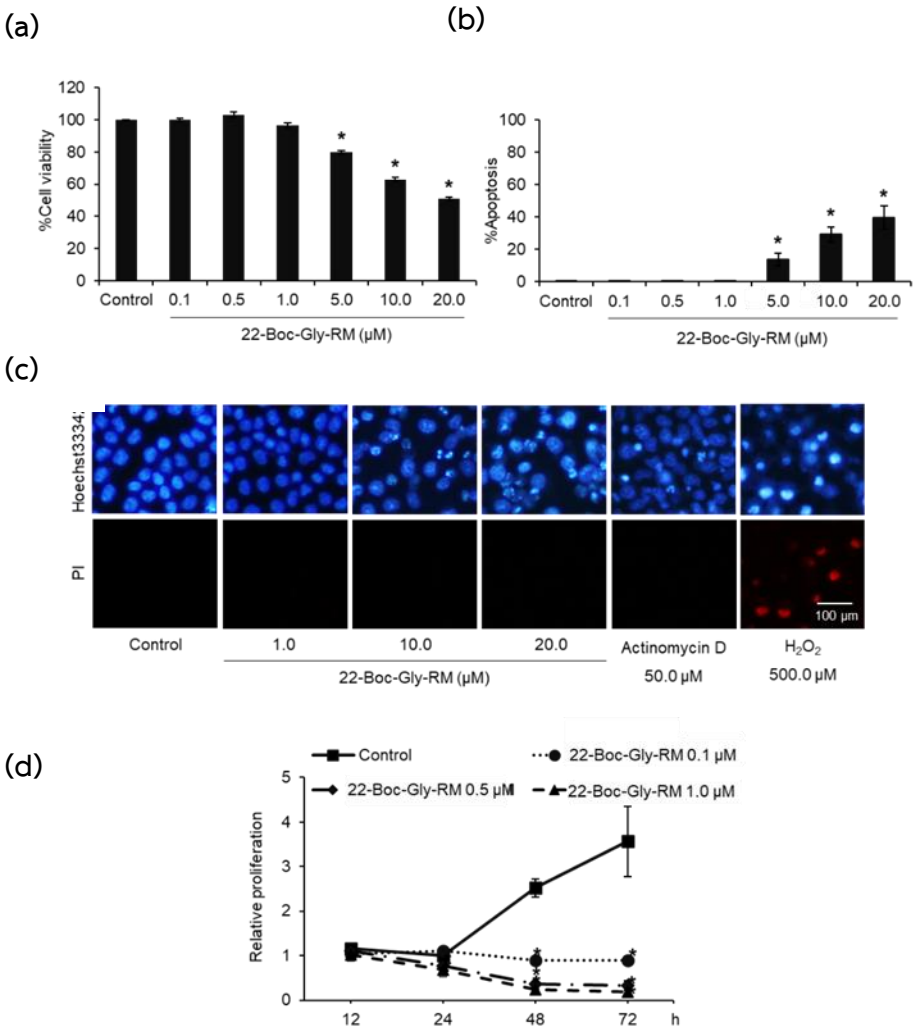


Figure 9 Cytotoxicity of 22-Boc-Gly-RM in human lung cancer cells.**(a)** Culture with 22-Boc-Gly-RM at 0.1-1 μM for 24 h causes no decrease on viability which was assessed via MTT assay in human lung cancer H460 cells. **(b)** The augmentation of %apoptosis was noted in H460 cells treated with 5-20 μM of 22-Boc-Gly-RM for 24 h compared with untreated control group. **(c)** Costaining with Hoechst33342/propidium iodide (PI) obviously depicted apoptosis stained with bright blue fluorescence of Hoechst33342 in H460 cells cultured with 10-20 μM 22-Boc-Gly-RM and Actinomycin D (50 μM), the positive control for apoptosis induction. Notably, necrosis cell death, which is indicated by red fluorescence from PI, was obviously observed in lung cancer H460 cells only after incubation with 500 μM hydrogen peroxide (H_2O_2) but not with 22-Boc-Gly-RM. **(d)** Crystal violet assay revealed the antiproliferative effect of 22-Boc-Gly-RM at non-

toxic concentrations (0.1-1 μM) in human lung cancer H460 cells after the incubation for 48-72 h while there was no alteration of proliferative activity observed early at 12 h. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point.

2. Diminish motility in lung cancer cells cultured with 22-Boc-Gly-RM

As motility and invasion are major determinants of metastasis cancer cells to disseminate and generate secondary tumor, the anti-metastasis potential of 22-Boc-Gly-RM was initially evaluated through wound healing assay. To minimize proliferative activity, the closure of scratch wound was evaluated in lung cancer H460 cell monolayer cultured in serum-free RPMI medium containing 0-1 μM 22-Boc-Gly-RM. The aggressive nature of human lung cancer cells was demonstrated with the dramatic increase of relative migration level after culture for 12-24 h (Figure 10 a). Intriguingly, the suppressive effect on cell motility was illustrated with wider wound space in the cell monolayers incubated with 0.5-1 μM of 22-Boc-Gly-RM compared with the untreated cells at 12-24 h (Figure 10 b). Although the modulation of wound closure might result from both anti-proliferation and anti-motility effect, diminished migratory activity was promptly noted in H460 cells after 12 h of the incubation with 22-Boc-Gly-RM (0.5-1 μM) which did not show alteration of cell proliferation (Figure 9 d).

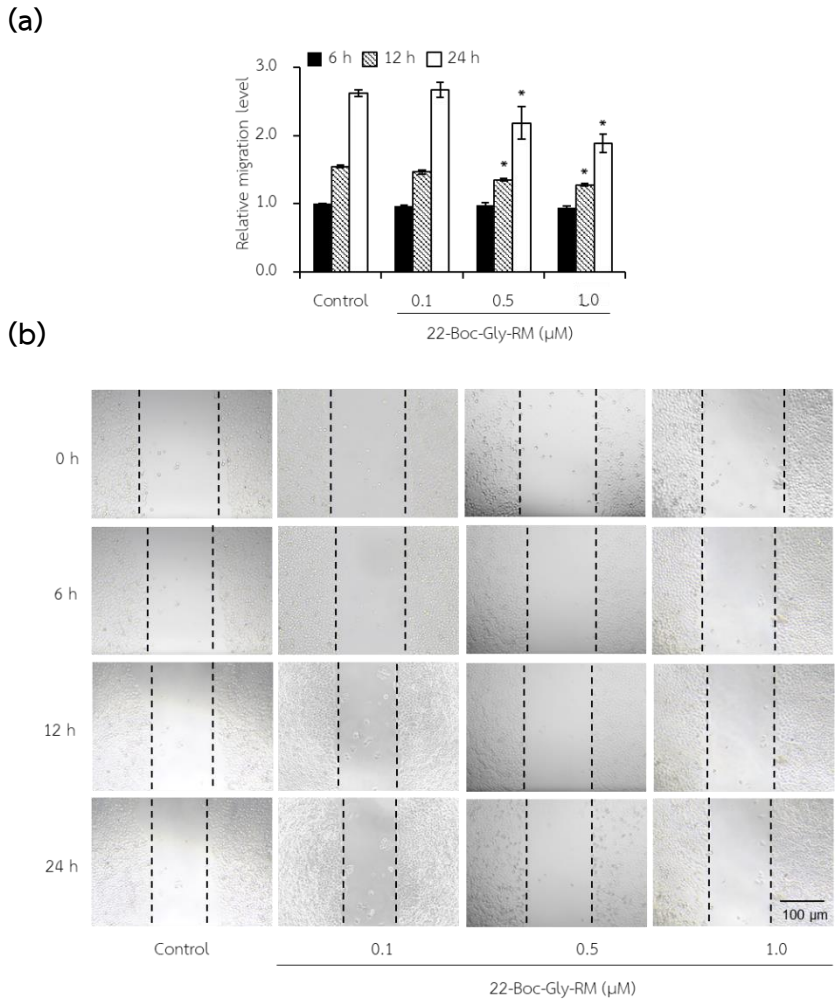


Figure 10 22-Boc-Gly-RM inhibits motility in human lung cancer cells. **(a)** Relative migration level indicated lower motility of lung cancer H460 cells cultured with 22-Boc-Gly-RM at 0.5-1 μM for 12-24 h compared with non-treated control cells. **(b)** Migratory activity of human lung cancer H460 cells depicted with the closure of scratch wound during 0-24 h of incubation time. Interestingly, treatment with 0.5-1 μM of 22-Boc-Gly-RM restrained motility in H460 cells as depicted with larger wound space at 12-24 h compared with control group. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point.

3. Three-dimensional migration and invasion behavior suppressed by 22-Boc-Gly-RM

The inhibitory activity of 22-Boc-Gly-RM on motility and invasion of single cells directly responding to chemoattractant was detected via transwell three-dimensional model. Human lung cancer H460 cells were cultured in serum-free RPMI medium with or without 22-Boc-Gly-RM (0.1-1 μM) on filter membrane of the transwell insert that was placed on 24-well plate containing 10% FBS in culture medium. Following 24 h of the incubation period, the migrated cells that passed through the filter membrane were stained with Hoechst33342 as presented in (Figure 11 a). Treatment with 0.1-1 μM of 22-Boc-Gly-RM dramatically decreased the amount of transwell migrated cells in a concentration-dependent manner when compared with untreated control group (Figure 11 b).

To imitate invasion process that is commonly found in tumor pathology [75], the transwell insert of Boyden chamber was pre-coated with thin layer of 0.5% Matrigel before on top with the single-cell suspension of human lung cancer cells in serum-free medium. Invasive capability of lung cancer H460 cells depicted with numerous cells passed through filter membrane covered with ECM components (Figure 11 c). Notably, the number of invaded H460 cells represented as relative invasion level was significantly decreased after culture with 22-Boc-Gly-RM (0.1-1 μM) for 24 h (Figure 11 d).

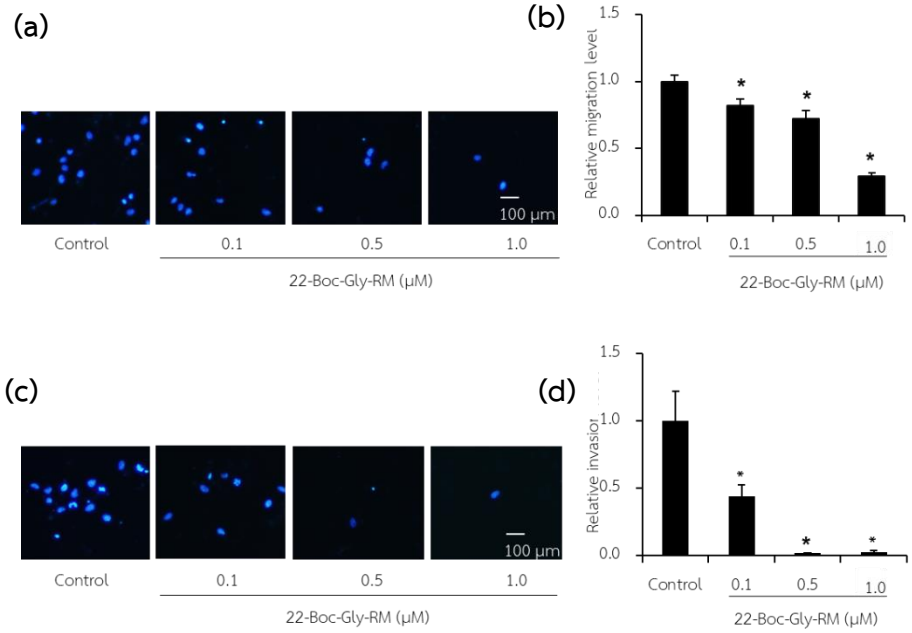


Figure 11 Transwell migration and invasion activity in human lung cancer cells suppressed by 22-Boc-Gly-RM. **(a)** Lung cancer H460 cells that passed through the filter membrane of transwell insert were stained with Hoechst33342 and observed under a fluorescence microscope. **(b)** The reduction of three-dimensional migrated cells was indicated with lower relative migration level in the cells cultured with 0.1-1 μM of 22-Boc-Gly-RM for 24 h compared with untreated control cells. **(c)** The invasive activity of human lung cancer H460 cells was depicted with Hoechst33342-stained cells located under the transwell inserts coated with 0.5% Matrigel. Culture for 24 h with 22-Boc-Gly-RM at 0.1-1 μM significantly decreased the invaded H460 cells. **(d)** Corresponding with Hoechst33342 staining results, the relative invasion level also indicated a dose-dependent suppressive effect of 22-Boc-Gly-RM in human lung cancer cells. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control

4. 22-Boc-Gly-RM rearranges cytoskeleton actin in human lung cancer cells

Migratory activity involves the reorganization of cytoskeleton. Elongated actin filaments (F-actin) or actin stress fibers, which enables cell motility are generated through polymerization of actin monomers [163]. The alteration of cell morphology and rearrangement of cellular actin filament was examined in 22-Boc-Gly-RM-treated H460 cells after staining with Alexa Fluor 568-phalloidin. The amount of actin stress fiber/cell was significantly reduced in the cells cultured with 22-Boc-Gly-RM (0.5-1 μM) compared to untreated control cells (Figure 12 a). Correspondingly, figure 12 b, which reveals results from immunofluorescence microscopy, clearly depicts the attenuation of F-actin formation in lung cancer H460 cells cultured with 0.5-1 μM 22-Boc-Gly-RM for 24 h. In contrast, well-oriented stress fibers were noted in the non-treated cells. It is worth noting that rounded morphology with less membrane protrusions was also observed in 22-Boc-Gly-RM-treated lung cancer cells.

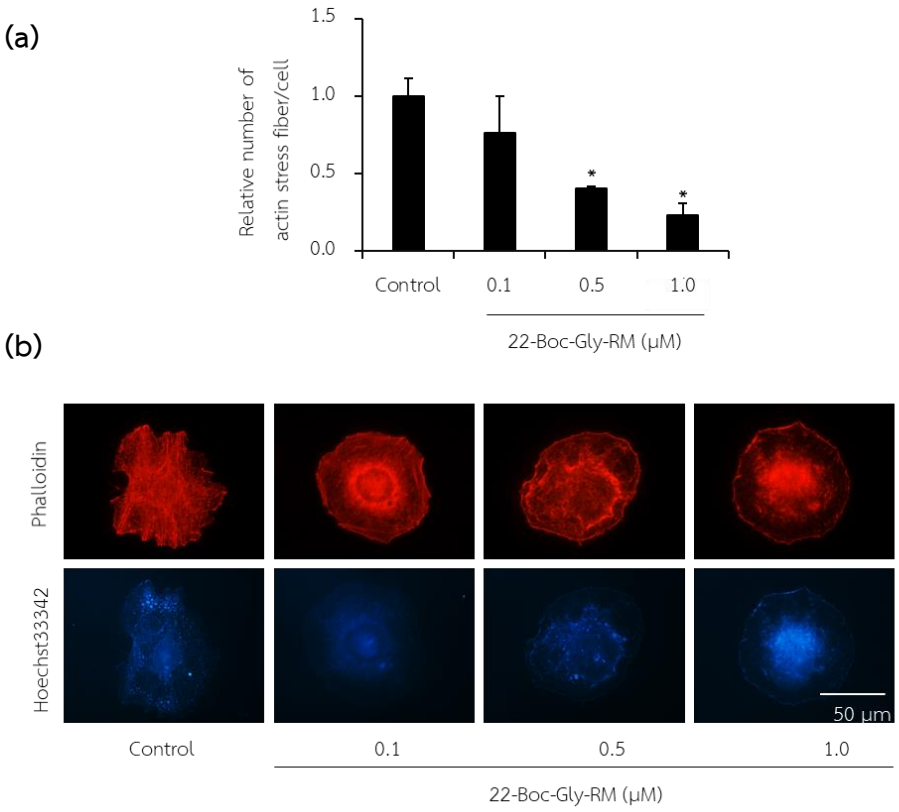


Figure 12 Cell morphology and actin filament formation altered by 22-Boc-Gly-RM. After incubation with 0-1 μM of 22-Boc-Gly-RM for 24 h, the alteration of morphology and actin filament formation was observed in lung cancer H460 cells stained with Alexa Fluor 568-tagged phalloidin under fluorescence microscope. **(a)** The number of formed actin filaments in the cells treated with 22-Boc-Gly-RM was relatively lower compared to non-treated control cells. **(b)** Not only reduced actin stress fibers but also poorly organized actin filament and non-polarized morphology was obviously demonstrated in lung cancer cells cultured with 0.5-1 μM of 22-Boc-Gly-RM. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control

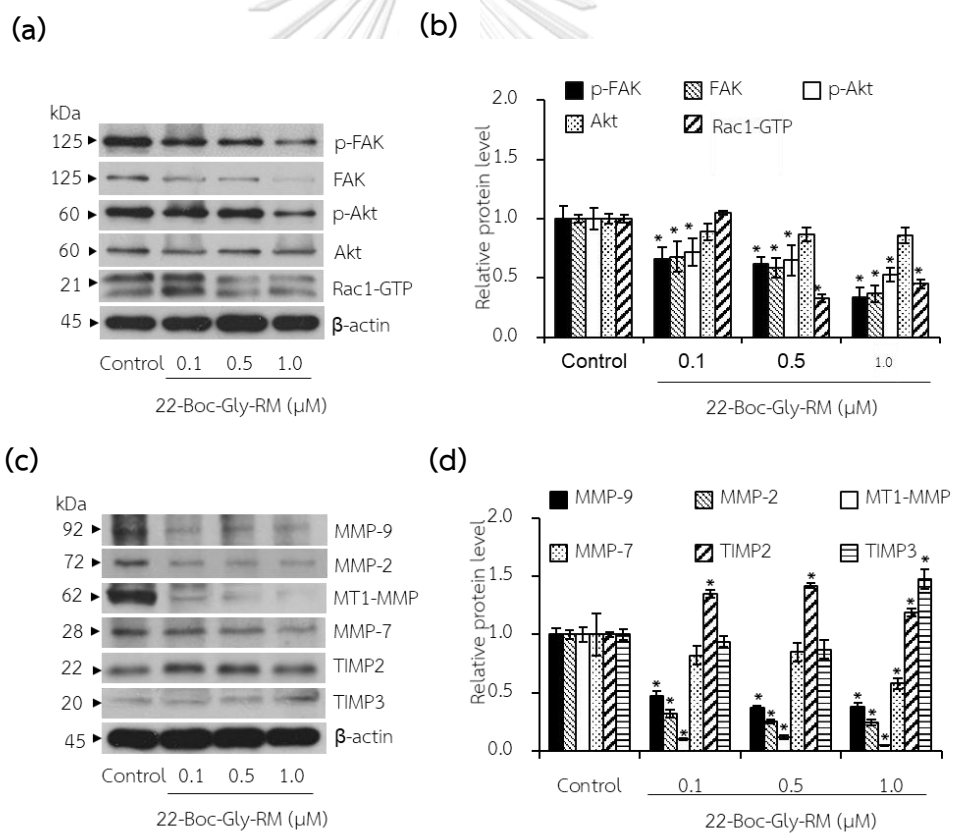
5. Modulation on FAK-mediating migratory and invasive signals and suppression of EMT by 22-Boc-Gly-RM

Due to its major role in actin polymerization [163], the alteration of Rac1-GTP expression level was investigated via western blot analysis. Figure 13 a illustrates the decreased protein level of Rac1-GTP in human lung cancer H460 cells treated with 0.5-1 μM of 22-Boc-Gly-RM for 12 h, which is positively correlated with the attenuating effect of 22-Boc-Gly-RM on F-actin formation (Figure 12) and migration activity (Figure 10). Interestingly, downregulation of upstream regulatory molecules including p-FAK, FAK and p-Akt was also indicated after culture H460 cells with 0.1-1 μM 22-Boc-Gly-RM (Figure 13 b).

Not only motility-mediating molecules but also MMPs and related proteins, which are involved with invasive capability, were modulated by 22-Boc-Gly-RM. Diminution of MT1-MMP, MMP-2, MMP-7, and MMP-9 as well as upregulation of MMP inhibitors, TIMP2 and TIPM3, was observed in lung cancer cells incubated with 1 μM 22-Boc-Gly-RM for 12 h (Figure 13 c). It is worth noting that 22-Boc-Gly-RM at lower concentrations (0.1-0.5 μM) also altered the expression level of MT1-MMP, MMP-2, MMP-9 and TIMP2 in lung cancer H460 cells.

Epithelial-to-mesenchymal transition (EMT), another downstream pathway affected by p-FAK/FAK signals, is a critical character of highly metastatic cancer cells, which was investigated in 22-Boc-Gly-RM-treated

human lung cancer cells. Corresponding to the less polarized cell morphology (Figure 12), culture with 0.1-1 μM of 22-Boc-Gly-RM repressed the expression of N-cadherin, a mesenchymal protein marker, in human lung cancer H460 cells (Figure 13 e). Moreover, E-cadherin, a protein presenting epithelial cell phenotypes, was overexpressed in the cells incubated with 22-Boc-Gly-RM (Figure 13 f). Taken together, these results strengthen the suppressive effect of 22-Boc-Gly-RM on EMT in human lung cancer cells.



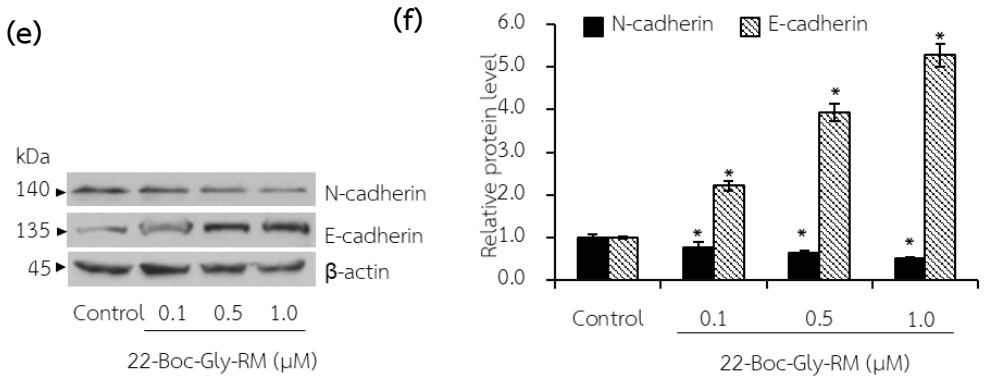


Figure 13 Modulatory effect of 22-Boc-Gly-RM on migration-related proteins was indicated with **(a)** down-regulation of FAK and p-FAK and consequent reduction of p-Akt in human lung cancer cells. **(b)** Correlated to the suppression on the upstream signals, treatment with 22-Boc-Gly-RM at 0.5-1 μM for 12 h significant diminished expression level of Rac1-GTP in lung cancer H460 cells. Not only **(c)** decreased expression of MT1-MMP, MMP-2, MMP-7 and MMP-9 but also **(d)** up-regulated TIMP2 and TIMP3, the inhibitors of MMPs, in lung cancer cells were mediated by 22-Boc-Gly-RM. Additionally, the alteration of EMT marker proteins including **(e)** N-cadherin and **(f)** E-cadherin indicated inhibitory role of 22-Boc-Gly-RM on EMT in lung cancer H460 cells. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control.

6. 22-Boc-Gly-RM restrains migratory and invasive activity in multicellular lung cancer spheroids

Since the spheroid model is a well-established in vitro model that mimics behaviors of in vivo tumor mass, various anticancer activities including suppressive effect on migration and invasion of therapeutic compounds have been suitably evaluated in multicellular cancer spheroids [160, 161, 164]. The aggressive features of multicellular cancer spheroids derived from lung cancer H460 cells were evidenced with the dramatic increase of relative migrating area (Figure 14 a) and invading area (Figure 15 a) expanding from cancer spheroids after culture for 12-24 h. Intriguingly, treatment with 0.5-1 μM obviously restrained migratory and invasive activity of lung cancer cells in multicellular spheroids promptly at 12 h as indicated in Figure 14 b and 15 b, respectively. Despite significant anti-migration activity after culture for 12-24 h, anti-invasive effect of 22-Boc-Gly-RM at lower concentration (0.1 μM) was clearly depicted in H460 spheroids after 24 h of treatment only. These suppressive effects on migration and invasion in multicellular cancer spheroids additionally support anti-metastasis potential of 22-Boc-Gly-RM in human lung cancer cells.

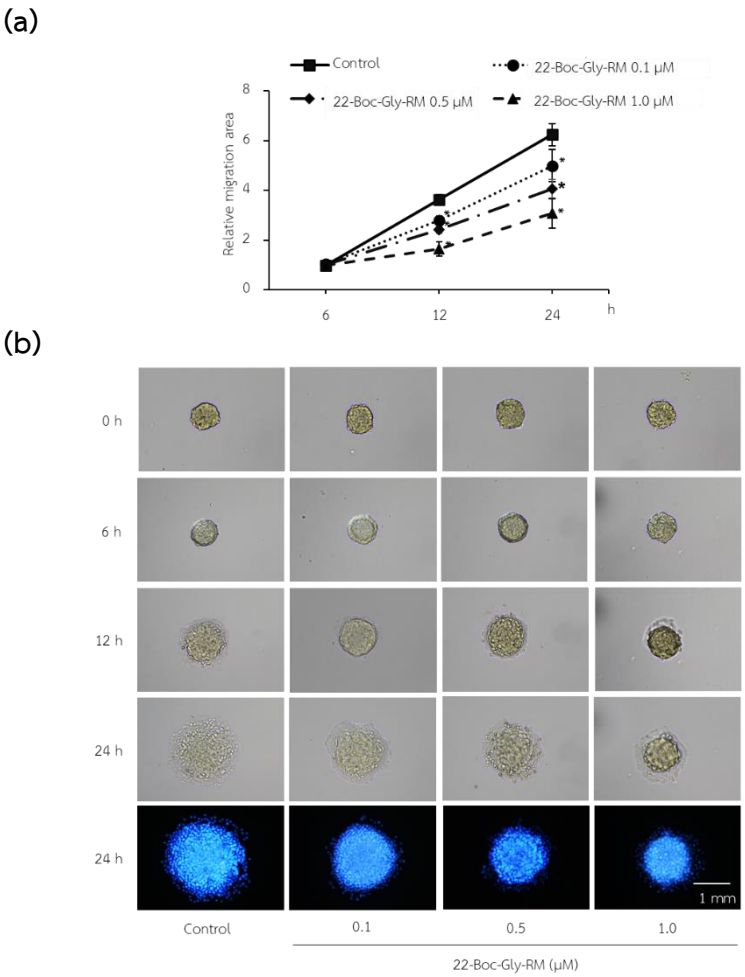


Figure 14 Anti-migratory effect of 22-Boc-Gly-RM in multicellular lung cancer spheroids. **(a)** Relative migrated area was evaluated in single cancer spheroids derived from lung cancer H460 cells after plating onto flat bottom 96-well plate for 0-24 h. **(b)** The expansion of the migratory area of lung cancer H460 spheroids detected under microscope was restrained after treatment with 0.1-1 μM of 22-Boc-Gly-RM for 12-24 h. Nuclear staining with Hoechst33342 was performed to clearly demonstrated migrated area expanding from multicellular H460 spheroids. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point

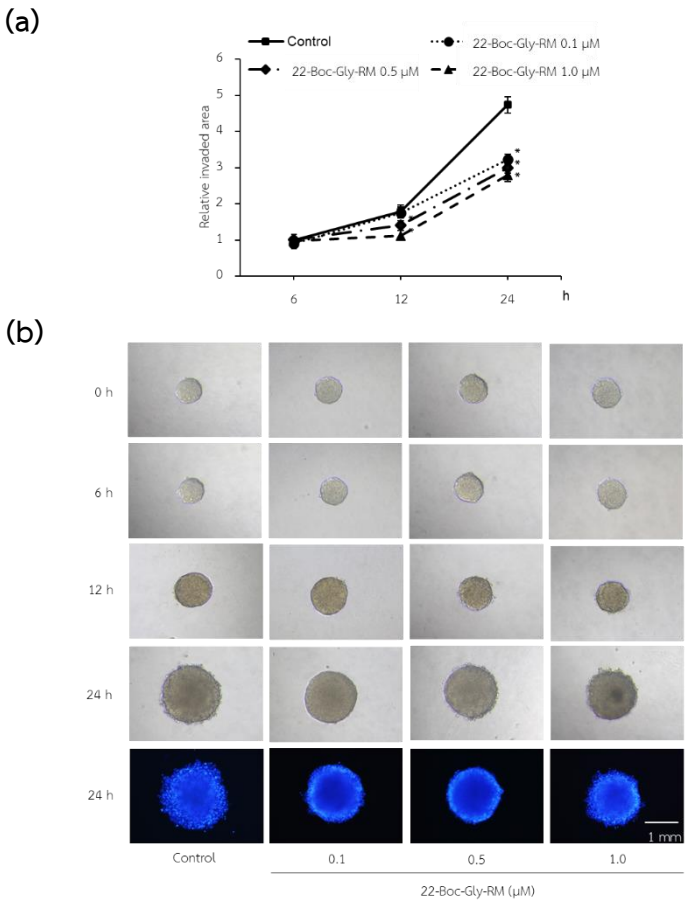


Figure 15 22-Boc-Gly-RM diminishes invasive capability in multicellular spheroids derived from human lung cancer cells. Single cancer spheroids obtained from lung cancer H460 cells were embedded in solidified 0.5% Matrigel before evaluating invasive capability under an inverted microscope. The dramatic increase of **(a)** relative invaded area corresponding with the enlargement of **(b)** spheroid area was depicted in three-dimensional (3D) H460 spheroids. Culture with 22-Boc-Gly-RM (0.1-1 μM) for 12-24 h significantly inhibited invasive activity of lung cancer cell in multicellular spheroids. The invaded area expanding from 3D cancer spheroids was also observed under fluorescence microscope after staining with Hoechst33342. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point

7. Cell viability analysis of 22-Boc-Gly-RM in normal human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells

To identify the cytotoxicity effect in normal cells, MTT and nuclease staining assay were performed after treatment of normal human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells with 0.1-1 μM of 22-Boc-Gly-RM for 24 h. The percentage of viable cells was presented in figure 16. After treatment with 0.1 to 1 μM, about 90 to 100% viable BEAS-2B cells were remained and there was no significant reduction of cell viability in both of BEAS-2B cells (Figure 16 a) and HaCaT cells (Figure 16 b). Notably, there was no detectable apoptosis and necrosis in 22-Boc-Gly-RM-treated BEAS-2B (Figure 16 c) and HaCaT cells (Figure 16 d). Hence, 22-Boc-Gly-RM at range between 0.1 to 1 μM were chosen for subsequent investigations on cell proliferation and wound-healing migration assay.

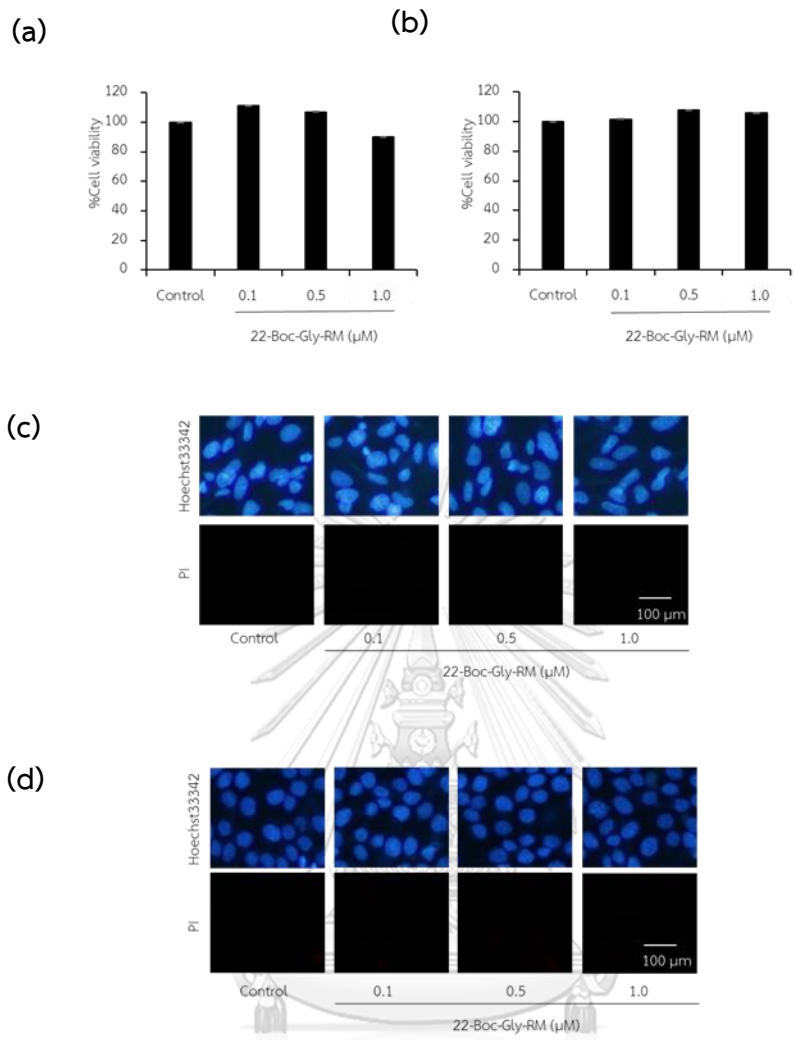
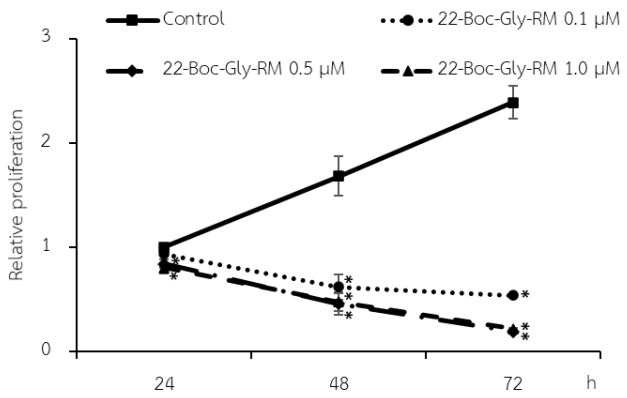


Figure 16 Effect of 22-Boc-Gly-RM on cell viability in normal cells. Treatment with 22-Boc-Gly-RM at 0.1-1 μM indicated no significant changes on viability assessed via MTT assay in **(a)** human bronchial epithelial BEAS-2B cells **(b)** human keratinocyte HaCaT cells. No apoptosis and necrosis cell death were detected in 22-Boc-Gly-RM-treated **(c)** BEAS-2B and **(d)** HaCaT cells after costaining with Hoechst33342 and propidium iodide (PI). Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control.

8. Anti-proliferation effect of 22-Boc-Gly-RM in normal human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells

Before performing cell migration assay, the effect on cell proliferation was additionally investigated in normal human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells incubated with non-toxic concentrations of 22-Boc-Gly-RM for 24-72 h. The results obtained from crystal violet assay which serves to indirectly quantify viable cells indicated anti-proliferation effect at 48-72 h by 22-Boc-Gly-RM (0.1-1 μM) treated BEAS-2B (Figure 17 a) and HaCaT cells (Figure 17 b). Compare to control cells at 24 h, 22-Boc-Gly-RM at 0.5-1 μM remarkably suppressed proliferation in BEAS-2B cells while only 22-Boc-Gly-RM at the highest concentration 1 μM obviously lowered relative proliferation levels in HaCaT cells.

(a)



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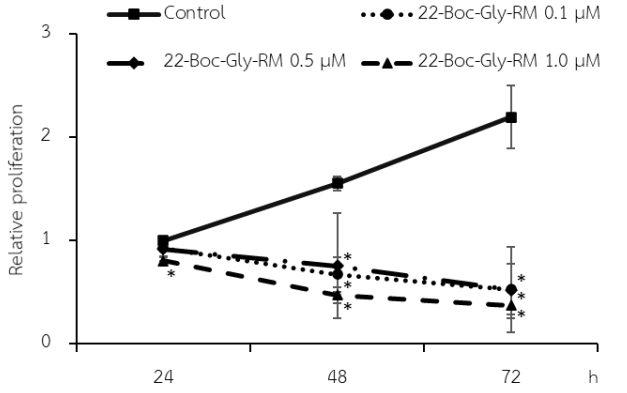
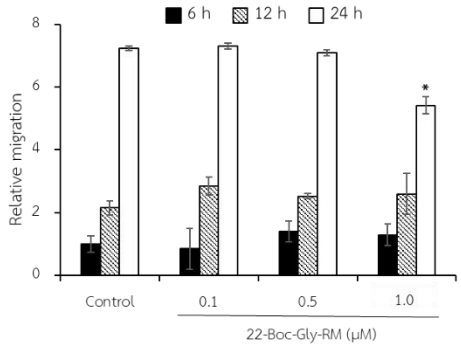


Figure 17 Effect of 22-Boc-Gly-RM on proliferation of normal cells. Cell proliferation of normal human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells was determined via crystal violet assay. The diminution of cell proliferation was observed after treatment with 0.1-1 μM 22-Boc-Gly-RM for 48-72 h in **(a)** BEAS-2B cells and **(b)** HaCaT cells. Notably, anti-proliferative effect of 22-Boc-Gly-RM at 1 μM was promptly observed at 24 h. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point.

9. Investigation of cell migration in 22-Boc-Gly-RM-treated human bronchial epithelial BEAS-2B cells and human keratinocyte HaCaT cells

The coordinated movement of the cell population in two-dimension is displayed by epithelial monolayer which occur in both pathological and physiological condition. The speed of motion of the cells is measured with wound healing assay, making cell-free area in the confluent monolayer by treating the cells with 0.1-1 μM 22-Boc-Gly-RM in serum free medium. Treatment with 0.1-0.5 μM 22-Boc-Gly-RM for 6-24 h did not alter cell motility in BEAS-2B cells (Figure 18a). Nevertheless, 24-h incubation with 22-Boc-Gly-RM at 1 μM inhibited closure of scratch wound in BEAS-2B cells (Figure 18b). Interestingly, no significant change of cell migration in human keratinocyte HaCat cells cultured with 0.1-0.5 μM 22-Boc-Gly-RM for 0-24 h compared with non-treated control cells (Figure19a and b). Taken together, these results suggest that 22-Boc-Gly-RM ranged between 0.1-0.5 μM does not moderate migratory behavior in normal human cells.

(a)



(b)

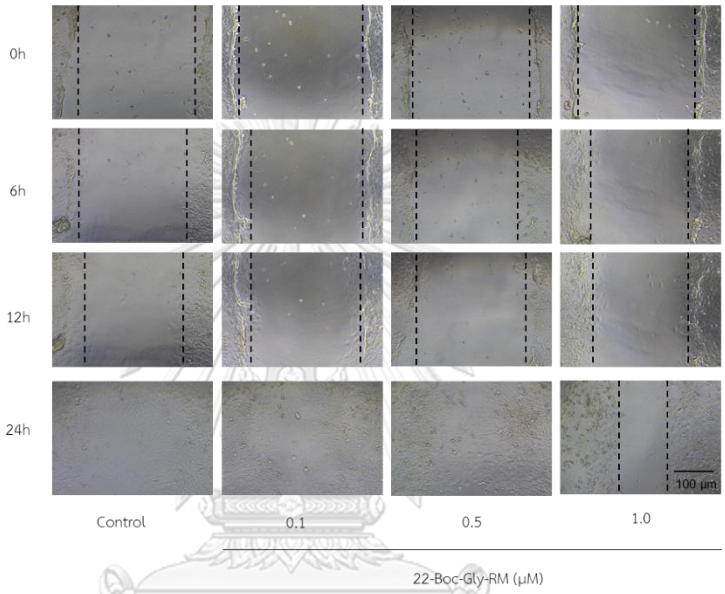


Figure 18 Effect of cell motility of 22-Boc-Gly-RM in BEAS-2B cells is observed through wound-healing assay. **(a)** Relative migration level of BEAS-2B cells cultured with 0.1-1 μM 22-Boc-Gly-RM for 6-24 h. **(b)** Treatment with 1 μM 22-Boc-Gly-RM for 24 h restrained cell motility in BEAS-2B cells. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point.

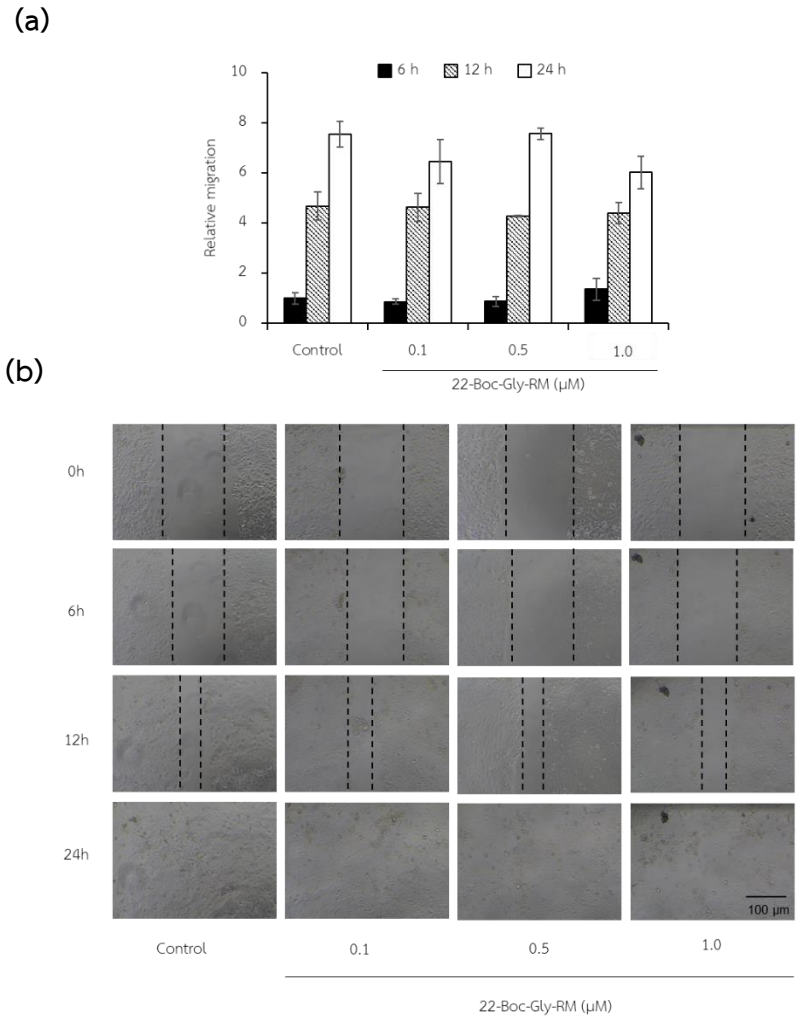


Figure 19 Effect of cell motility of 22-Boc-Gly-RM in HaCaT cells is observed through wound-healing assay. **(a)** Intriguingly, no reduction of relative migration level was observed in HaCaT cells after treated with 0.1-1 μM 22-Boc-Gly-RM for 6,12 and 24 h when compared with control cells. **(b)** The scratch wound was completely closed in 22-Boc-Gly-RM-treated HaCaT cells. Data represent means ± SD of three independent experiments. *$p < 0.05$ compared with non-treated control at the same time point.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Lung cancer has been reported to possess aggressive features of high proliferation and metastasis progression [165]. The capability of some cancer cells to migrate and invade from the original tissue to secondary sites fuels the metastatic process [166]. Studies indicate the emerging benefits of inhibiting cancer cell migration and invasion as a novel strategy for metastasis prevention [16, 26, 29, 167]. Recently, the promising anti-metastasis activity of marine bistetrahydroisoquinolinequinone alkaloids isolated from Thai blue sponge *Xestospongia* sp. including renieramycin M and jorunnamycin A was highlighted through reports indicating suppression on anchorage-independent growth and sensitization of detachment-induced cell death in human lung cancer cells [31]. To maintain cytotoxicity and improve cancer selectivity, a semi-synthesized version of renieramycin M containing a hydroquinone amino ester extension was prepared to gain insights into anticancer activity and specific protein targets [34]. Taken together with the attenuation on migration and invasion activity presented in this study, the semi-synthesized hydroquinone monoester derivative of renieramycin M, 22-Boc-Gly-RM, has demonstrated multi-targeted potential use against metastasis in human lung cancer cells.

It has previously been noted that FAK/PI3K/Akt signaling plays a crucial role in the regulation of metastasis in different cancers [16]. The overexpression and

phosphorylation of FAK serves as one of the hallmarks of cell motility and invasion while PI3K/Akt activation is identified as part of the downstream pathway of FAK. Accordingly, the interference of FAK expression by various compounds was reported to suppress metastasis [16, 17]. The results from western blotting show that the relative level of p-FAK and p-Akt is critical for 22-Boc-Gly-RM-inhibited tumor migration and invasion (Figure. 13a and b). Although both total and phosphorylated forms of FAK and Akt were down-regulated, whether FAK and/or Akt is targeted molecule of 22-Boc-Gly-RM should be further investigated. FAK/Akt signaling has also been described as the upstream regulator of Rac1-GTP, an intracellular transducer that regulates actin polymerization resulting in cytoskeleton reorganization and cell motility. Increased invasion and motility in cancer cells as well as increased formation of actin-rich protrusions at the cell margin of plasma membrane called lamellipodia were reported to be mediated by RAC1 in a PI3K-dependent manner [168]. In this study, formation of lamellipodia (Figure. 12), which is related with Rac1-GTP expression level is significantly reduced in 22-Boc-Gly-RM-treated lung cancer cells in comparison with untreated control cells (Figure. 13a and b). The decreased Rac1-GTP level directly correlates with the attenuating effect of 22-Boc-Gly-RM on F-actin formation (Figure. 12) and the moderation of migration activity (Figure. 10). The gain of motility and invasion capacity accompanied by restructuring of the actin cytoskeleton and the formation of invasive protrusions like lamellipodia are imbued when cancer cells become more mesenchymal [169].

In cancer, EMT occurs when epithelial cells acquire mesenchymal features that permit escape from the primary tumor. This temporary transition state allows the early stages of metastasis to occur by facilitating migration and invasion and enabling survival under detachment conditions [170]. Metastatic cancer cells have been reported to exhibit EMT features including increased N-cadherin and decreased E-cadherin expression [171]. Down-regulation of E-cadherin is a crucial event for EMT program that leads to the loss of epithelial cell phenotype particularly their adhesive feature and well-maintained cell-to-cell contacts. On the other hand, upregulation of N-cadherin promotes mesenchymal characteristics such as basement membrane degradation, cell motility and cell invasion, which are acquired for progression from carcinoma to metastasis [2]. When E-cadherin is upregulated, N-cadherin becomes down-regulated and metastasis is inhibited [171]. Interestingly, the current study found that the inhibitory effect of 22-Boc-Gly-RM in human lung cancer cells also involved with the reduction of N-cadherin as well as up-regulation of E-cadherin (Figure. 13e and f). Moreover, it has been reported that p-FAK is an upstream modulator of cadherin proteins and the overexpression of FAK can trigger EMT pathway [138]. Taken together, the present results reveal that 22-Boc-Gly-RM treatment caused the reduction of FAK level thereby mediating the reduction of downstream Akt, resulting in the upregulation of E-cadherin and downregulation of N-cadherin in human lung cancer cells. In addition to our previous study [32], this current finding provides evidence that bistetrahydroisoquinolinequinone alkaloids isolated from *Xestospongia* sp. and their

derivatives effectively inhibit metastasis in various lung cancer models via suppressing FAK/Akt/EMT pathway.

The excessive degradation of ECM is an essential step for cell migration and invasion [23]. The invasive ability associated with mesenchymal phenotype of EMT comes from the central role of MMPs in the breakdown of cell barriers and basement membrane [87]. MT1-MMP, an MMP commonly expressed in lung carcinoma, serves as a powerful enzyme capable of promoting invasion and metastasis through degradation of ECM macromolecules [172]. Some MMPs such as MMP-2 and MMP-7 have also been found to have prognostic value to predict tumor recurrence and clinical outcome [20, 58]. This study focuses on the effects of the compound on MMP-2, MMP-7, MMP-9 and MT1-MMP, which are notable drug targets of clinically tested inhibitors and natural compounds in development [20]. Among various MMPs, MT1-MMP and MMP-7 are found in tumors with high metastasis potential and aggressive features [20, 90, 103]. Low survival rate of lung cancer patients, along with increased metastasis risk, were shown to be correlated with increased expression of MMP-1, MMP-7 and MMP-9 [20, 103]. Fortunately, MMP activity can be restrained by their inhibitors, TIMPs [23]. Herein, the results indicate that 22-Boc-Gly-RM remarkably diminished expression of MMP-2, MMP-7, MMP-9 and MT1-MMP (Figure. 13c and d). In contrast, the levels of tissue inhibitor of MMPs, TIMP2 and TIMP3, were upregulated in response to 22-Boc-Gly-RM treatment (Figure. 13c and d). The upstream regulation of MMP activity is specifically regulated by FAK overexpression, which is mediated through Akt activation to enhance

the invasiveness of lung cancer cells [138, 156]. Thus, the results on MMP and TIMP regulation correlate with the p-Akt and p-FAK suppression (Figure. 13a and b) and EMT regulation (Figure. 13e and f) mediated by 22-Boc-Gly-RM. Overall, these findings reveal that the diminution of MMPs and accumulation of their inhibitors potentially suppresses invasive activity through ECM modulation in human lung cancer cells (Figure. 11c and d). Although the inhibitory effect of 22-Boc-Gly-RM on migratory and invasive activity of human lung cancer cells was also shown in cancer spheroid model which is known to mimic *in vivo* tumor features (Figure. 14 and 15), further animal studies would strengthen anti-metastasis potential of 22-Boc-Gly-RM, a semi-synthetic marine derivative compound.

The purpose of newly discovered natural molecules isolated from natural source that possess anticancer properties aim to destroy only cancerous cells without harmful to normal cells. 22-Boc-Gly-RM demonstrated cytotoxic effect on lung cancer H460 cells without exhibiting significant toxicity to human bronchial epithelial BEAS-2B cell and human keratinocyte HaCaT cells. Interestingly, due to owing motif of tetrahydroisoquinoline in 22-Boc-Gly-RM, exhibit inhibition on proliferation of cells at the indicated time. Herein, the study has found that 22-Boc-Gly-RM was not shown interference on normal cells motility regardless on anti-proliferation effect at 24 h has presented in figure 18, figure 19 respectively. However, based on the results of studies, concentration of highest non-toxic concentration of 1 μM 22-Boc-Gly-RM might interplay both of inhibition effect leading to contrive on BEAS-2B cells without damage

to keratinocyte HaCaT cells. Thus, the additional augment associated with choosing of optimum non-toxic concentration would be important concern and 0.5 μM 22-Boc-Gly-RM could be the choice for further tests by pre-clinical study to justify if it is effective for prevention of lung cancer migration, invasion and metastasis.

Conclusions

In summary, the present work describes the compound of 22-Boc-Gly-RM from semi synthesis origin and its mechanism of action such as inhibition of migration and invasion pathway have been tested on human lung cancer H460 cells and non-cancerous cells. Due to broad range of 22-Boc-Gly-RM's action, the mechanism underlying its ability to interfere in cancer cell migration and invasion are innumerable. These include, modulation of cell-cell and cell-matrix interaction, cytoskeleton reorganization, epithelial to mesenchymal transition, and extracellular matrix remodeling by matrix metalloproteinases. In this study, the mechanism by which 22-Boc-Gly-RM, a 22-*O*-acyl ester derivative of renieramycin M exhibit the inhibitory role on the migration and invasion in human lung cancer cells through suppression on FAK/Akt-mediating signal, which is evidenced by the modulation of cytoskeleton regulating protein (Rac1-GTP), MMP-related molecules (MT1-MMP, MMP-2, MMP-7, MMP-9, TIMP2 and TIMP3) and EMT markers (E-cadherin and N-cadherin). The proposed mechanism by which 22-Boc-Gly-RM suppress migration and invasion of lung cancer H460 cells through inactivation of FAK/Akt signaling pathway as well as downregulation

of Rac1-GTP protein and EMT marker and further suppress expression of MMPs protein as presented in figure 20. The obtained information would facilitate the development of 22-Boc-Gly-RM as an effectively anti-metastasis agent for lung cancer treatment.

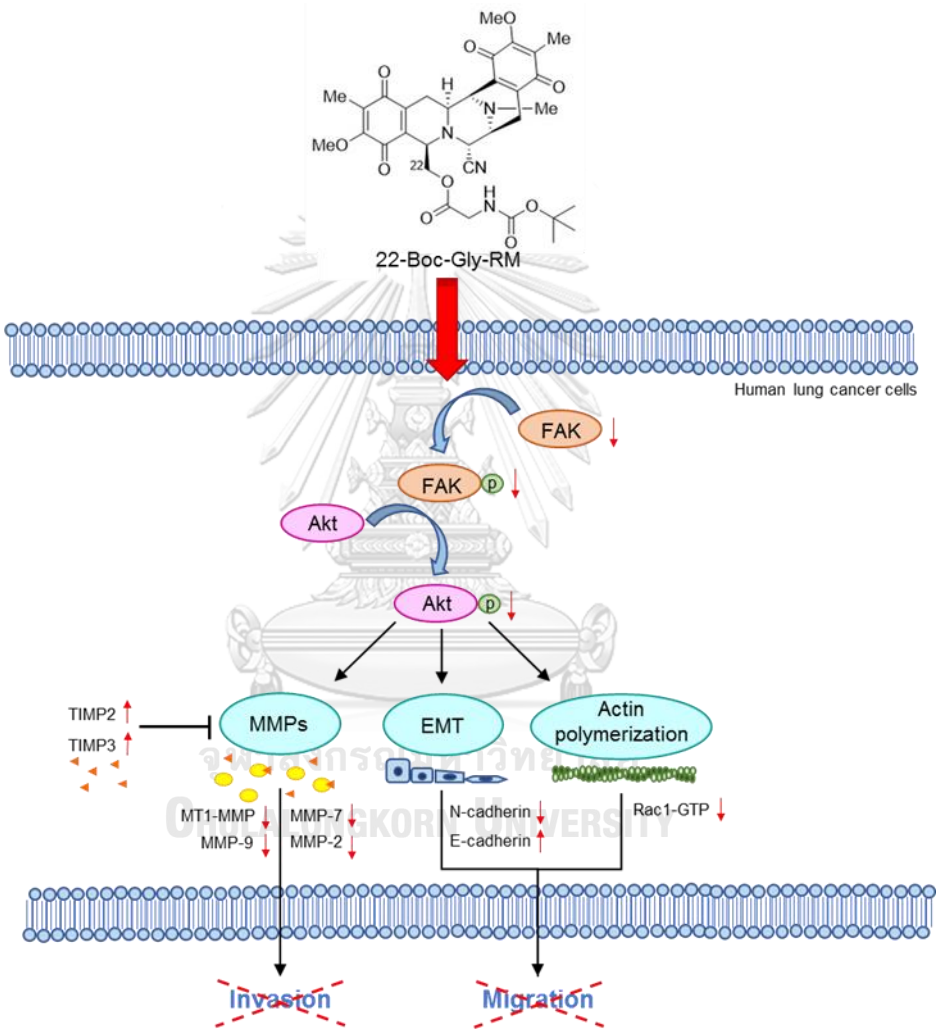


Figure 20 Proposed mechanistic scheme of suppressive activity of 22-O-(N-Boc-L-glycine) ester of renieramycin M (22-Boc-Gly-RM) on migration and invasion behaviors in human lung cancer cells. The symbols and arrows in red color indicates effects of the compound. It is seen that 22-Boc-Gly-RM down-regulates FAK-mediated migratory signals associatied with alteration of matrix metalloproteinase enzyme (MMPs), tissue

inhibitor of metalloproteinases (TIMPs) and Rac1-GTP as well as suppression on epithelial to mesenchymal transition (EMT) which is involved with migrating and invading capacity of human lung cancer cells.

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APPENDIX

TABLES OF EXPERIMENTAL RESULTS

Table 1 The percent cell viability (a) and the percent apoptosis (b) after treatment with 0-20 μM of 22-*O*-(*N*-Boc-L-glycine) ester of renieramycin M or (22-Boc-Gly-RM) on human lung cancer H460 cells.

(a)

22-Boc-Gly-RM (μM)	Cell viability (%)
Control	100±0.02
0.1	100.19±1.10
0.5	103.20±1.70
1	96.66±1.60
5	80.13±1.00*
10	62.92±1.19*
20	50.92±1.13*

(b)

22-Boc-Gly-RM (μM)	Apoptosis (%)
Control	0.26±0.06
0.1	0.26±0.05
0.5	0.12±0.08
1	0.29±0.07
5	13.74±3.86*
10	29.22±4.54*
20	39.65±7.11*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 2 The relative proliferation of H460 cells after treatment with 0-1 μM of 22-Boc-Gly-RM for 12 h, 24 h, 48 h and 72 h.

22-Boc-Gly-RM (μM)	Relative proliferation			
	12 h	24 h	48 h	72 h
Control	1.16±0.10	1.00±0.00	2.52±0.21	3.57±0.79
0.1	1.04±0.05	1.12±0.02	0.90±0.02*	0.89±0.09*
0.5	1.11±0.13	0.78±0.04*	0.36±0.03*	0.33±0.11*
1	1.03±0.15	0.68±0.16*	0.24±0.02*	0.18±0.09*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 3 The relative migration of H460 cells monolayer after treatment with 0-1 μM of 22-Boc-Gly-RM for 6 h, 12 h and 24 h.

22-Boc-Gly-RM (μM)	Relative migration		
	6 h	12 h	24 h
Control	1.00±0.00	1.54±0.02	2.63±0.05
0.1	0.97±0.01	1.46±0.03*	2.67±0.11
0.5	0.98±0.04	1.35±0.02*	2.19±0.23*
1	0.94±0.03*	1.28±0.02*	1.89±0.13*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 4 Relative migration level (a) and relative invasion level (b) of lung cancer H460 cells after cultured with 0-1 μM of 22-Boc-Gly-RM for 24 h.

(a)

22-Boc-Gly-RM (μM)	Relative migrated cells
Control	1.00±0.05
0.1	0.82±0.05*
0.5	0.72±0.07*
1	0.29±0.02*

(b)

22-Boc-Gly-RM (μM)	Relative invaded cells
Control	1.00±0.22
0.1	0.44±0.09*
0.5	0.02±0.00*
1	0.03±0.01*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 5 The relative number of actin stress fiber in lung cancer H460 cells after incubation with 0-1 μM of 22-Boc-Gly-RM for 24 h.

22-Boc-Gly-RM (μM)	Relative number of actin stress fiber per cell
Control	1.00±0.11
0.1	0.76±0.24
0.5	0.40±0.01*
1	0.23±0.08*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 6 The relative protein level of migration relating protein (a), invasion related protein (b) and epithelial to mesenchymal protein marker (c) after treatment with 0-1 μM of 22-Boc-Gly-RM for 24 h in lung cancer H460 cells.

(a)

22-Boc-Gly-RM (μM)	Relative protein level				
	p-FAK	FAK	p-Akt	Akt	Rac1-GTP
Control	1.00±0.10	1.00±0.03	1.00±0.09	1.00±0.04	1.00±0.04
0.1	0.66±0.10*	0.68±0.13*	0.72±0.12*	0.89±0.07	1.05±0.02
0.5	0.62±0.06*	0.59±0.08*	0.65±0.13*	0.87±0.06	0.33±0.03*
1	0.34±0.08*	0.37±0.07*	0.53±0.05*	0.86±0.07	0.45±0.03*

(b)

22-Boc-Gly-RM (μM)	Relative protein level					
	MMP-9	MMP-2	MT1-MMP	MMP-7	TIMP2	TIMP3
Control	1.00±0.05	1.00±0.04	1.00±0.06	1.00±0.18	1.00±0.02	1.00±0.05
0.1	0.47±0.04*	0.32±0.03*	0.10±0.01*	0.82±0.08	1.35±0.03*	0.94±0.05
0.5	0.37±0.02*	0.25±0.02*	0.12±0.02*	0.85±0.08	1.42±0.02*	0.87±0.08
1	0.38±0.03*	0.24±0.02*	0.05±0.00*	0.58±0.05*	1.19±0.03*	1.47±0.08*

(c)

22-Boc-Gly-RM(μM)	Relative protein level	
	N-cadherin	E-cadherin
Control	1.00±0.08	1.00±0.03
0.1	0.77±0.12*	2.22±0.11*
0.5	0.64±0.05*	3.93±0.20*
1	0.52±0.04*	5.28±0.28*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 7 The relative migrated area (a) and relative invaded area (b) of spheroid cancer H460 cells after treated with 0-1 μM of 22-Boc-Gly-RM for 6 h, 12 h and 24 h.

(a)

22-Boc-Gly-RM (μM)	Relative migration area		
	6 h	12 h	24 h
Control	1.00±0.13	3.63±0.22	6.24±0.45
0.1	1.04±0.15	2.79±0.17*	5.00±0.64*
0.5	1.01±0.16	2.42±0.12*	4.06±0.37*
1	0.98±0.20	1.65±0.29*	3.08±0.59*

(b)

22-Boc-Gly-RM (μM)	Relative invasive area		
	6 h	12 h	24 h
Control	1.00±0.16	1.79±0.17	4.74±0.23
0.1	0.91±0.13	1.76±0.15	3.23±0.15*
0.5	1.01±0.15	1.41±0.14*	3.00±0.15*
1	0.97±0.18	1.13±0.13*	2.80±0.17*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 8 The percent cell viability after incubated with 0-1 μM of 22-Boc-Gly-RM on (a) normal lung BEAS-2B cells and (b) human keratinocyte HaCaT cells.

(a)

22-Boc-Gly-RM (μM)	Cell viability (%)
Control	100.00±0.03
0.1	111.34±0.01
0.5	106.96±0.12
1	90.22±0.01

(b)

22-Boc-Gly-RM (μM)	Cell viability (%)
Control	100.00±0.02
0.1	101.74±0.03
0.5	107.97±0.02
1	105.82±0.04

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 9 The relative proliferation of (a) BEAS-2B cells and (b) HaCaT cells after treatment with 0-1 μM of 22-Boc-Gly-RM for 12 h, 24 h, 48 h and 72 h.

(a)

22-Boc-Gly-RM (μM)	Relative proliferation		
	24 h	48 h	72 h
Control	1.00±0.00	1.68±0.19	2.39±0.16
0.1	0.94±0.05	0.62±0.12*	0.54±0.02*
0.5	0.84±0.09*	0.46±0.10*	0.19±0.01*
1	0.80±0.01*	0.47±0.09*	0.22±0.01*

(b)

22-Boc-Gly-RM (μM)	Relative proliferation		
	24 h	48 h	72 h
Control	1.00±0.00	1.55±0.07	2.20±0.30
0.1	0.93±0.09	0.67±0.17*	0.53±0.24*
0.5	0.92±0.12	0.76±0.51*	0.53±0.41*
1	0.80±0.05	0.48±0.08*	0.38±0.13*

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

Table 10 The relative migration of (a) BEAS-2B cells and (b) HaCaT cells monolayer after treatment with 0-1 μM of 22-Boc-Gly-RM for 6 h, 12 h and 24 h.

(a)

22-Boc-Gly-RM (μM)	Relative migration		
	6 h	12 h	24 h
Control	1.00±0.25	2.15±0.22	7.23±0.06
0.1	0.85±0.65	2.85±0.28	7.30±0.09
0.5	1.41±0.33	2.52±0.09	7.09±0.10
1	1.29±0.35	2.60±0.65	5.42±0.28*

(b)

22-Boc-Gly-RM (μM)	Relative migration		
	6 h	12 h	24 h
Control	1.00±0.23	4.69±0.57	7.53±0.51
0.1	0.87±0.11	4.63±0.56	6.45±0.87
0.5	0.88±0.21	4.29±0.04	7.57±0.22
1	1.37±0.44	4.40±0.42	6.02±0.65

Values are means of the independent triplicate experiments ± SD.

*p < 0.05 versus non-treated control

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