ผลของสารเทอริอีนในการยับยั้งการแพร่กระจายและการสร้างหลอดเลือด ของเซลล์มะเร็งปอด

INHIBITORY EFFECTS OF TERREIN ON LUNG CANCER CELL METASTASIS AND ANGIOGENESIS

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Graduate School Srinakharinwirot University

2019

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ไพวัลย์ บัวจันทร์

ปริญญานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตร
ปรัชญาดุษฎีบัณฑิต สาขาวิชาชีวภาพการแพทย์
คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
ปีการศึกษา 2562

INHIBITORY EFFECTS OF TERREIN ON LUNG CANCER CELL METASTASIS AND ANGIOGENESIS

PAIWAN BUACHAN

A Dissertation Submitted in partial Fulfillment of Requirements

for DOCTOR OF PHILOSOPHY (Biomedical Sciences)

Faculty of Medicine Srinakharinwirot University

2019

THE DISSERTATION TITLED

INHIBITORY EFFECTS OF TERREIN ON LUNG CANCER CELL METASTASIS AND ANGIOGENESIS

BY

PAIWAN BUACHAN

HAS BEEN APPROVED BY THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES AT SRINAKHARINWIROT UNIVERSITY

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Title	INHIBITORY EFFECTS OF TERREIN ON LUNG CANCER CELL METASTASIS AND ANGIOGENESIS
Author	PAIWAN BUACHAN
Degree	DOCTOR OF PHILOSOPHY
Academic Year	2019
Thesis Advisor	Assistant Professor Doctor Wanlaya Tanechpongtamb , Ph.D.

Cancer metastasis is the leading cause of mortality for cancer patients and over seventy percent of lung cancer patients were diagnosed at advanced or metastatic stages resulting in increasing the incidence of mortality. Terrein is a secondary bioactive fungal metabolite that isolated from the *Aspergillus terreus.* Numerous studies have demonstrated that it has various biological activities including the inhibition of melanogenesis, anti-inflammatory and anticancer properties. In this study, we also analyzed on the anticancer effect of terrein but targeting through the mechanism of metastatic inhibition of lung cancer cells that has never been reported. Human lung cancer cells (A549) were used to determine the cytotoxic effect of terrein compared to normal cell lines Vero, L6 and H9C2 using MTT assay. The results indicated that terrein significantly inhibited cell viability of all of those cells tested with a different level of toxicity which IC_{50} were at 229 μM, 870 μM, 1,240 μM and 579 μM, respectively. A significant increased cytotoxic effect was clearly observed in A549 cells by LDH assay. The inhibitory effect of terrein on cell proliferation assessed by IncuCyte indicated significantly suppressed A549 cell proliferation. To further investigate the effect of terrein on the migration and invasion processes, both the normal and the aggressive phenotype of A549 grown in the hypoxic conditions were performed. The results revealed that terrein significantly inhibited cancer cell migration and invasion of both normoxia and hypoxia conditions. In terms invasive ability, qPCR demonstrated that terrein decreased MT1-MMP, MMP-2 and MMP-9 expression. This result was supported by a reduction of MMP-2 and MMP-9 activity evaluated by gelatin zymography. Angiogenesis processes of A549 including VEGF secretion, capillary-like tube formation as well as VEGF/VEGFR2 interaction were further determined by ELISA, tube formation assay and western blotting, respectively, which the suppression was indicated. These data were accompanied by a reduction of integrin and FAK protein and its downstream mediators (e.g. PI3K, AKT, mTORC1 and P70S6K). All these data indicate that terrein was able to inhibit all major processes of metastasis in human lung cancer cells, which is crucial for cancer treatment.

Keyword : Terrein, non-small cell lung cancer, migration, invasion, metastasis

ACKNOWLEDGEMENTS

For the success of the thesis, I would like to give this page to express my sincere gratitude to many people who have contribution during my study. First of all, I would like to express my sincere gratitude and deepest appreciation to my advisor, Asst. Prof. Wanlaya Tanechpongtamb, and co-advosor Asst. Prof. Maneekarn Namsa-aid and Asst. Prof. Foengchat Jarintanan, for their kindness, encouragement, guidance, valuable advise, constructive and intensive supervised throughout the laboratory works and preparation of this thesis.

I would like to thank Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University for providing all instruments and facilities on my research. I also express my appreciation to the members of advisory committee for their helpful guidance and valuable suggestions.

This work was supported by research grants from Faculty of Medicine, Srinakharinwirot University, Thailand (2560) and National Research Council of Thailand: NRCT (2562). In addition, I would like to thank Postgraduate Student Exchange (GRAD S-4-62) program for giving me the opportunity to study in Canada.

I am so grateful that I received the opportunity to study in Canada through the Canada-ASEAN Scholarships and Educational Exchanges for Development (SEED) program. I was able to complete research at York University, which was a great place to develop my skills and scientific thinking and to widen my vision. Especially, I would like to thank Professor Gary Sweeney, Hye Kyoung Sung, Vivian Vu and all my fellow lab members for sharing their knowledge and lab techniques with me. I will never forget their kindness, friendliness, and eagerness to help me.

Finally, I would like to give all my heart to my family and my friends for their love, caring, consulting, understanding and many encouragements throughout my study. Without their supports, my success would never come true.

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CHAPTER I
INTRODUCTION

Lung cancer or pulmonary carcinoma is the uncontrolled growth of abnormal cells in one or both of the lungs. These abnormal cells can spread to other organs of the body through lymphatic system or bloodstream which is called metastatic stage [1]. The causes of lung cancer are from several risk factors which the most important factor is long-term tobacco smoking. For people who have never smoked, the risk factors are exposure to second-hand smoke, radon gas, asbestos, air pollution as well as genetic factors [2, 3]. The epidemiology study reported that lung cancer has been the leading cause of cancer death among men since 1950s. In 2018, the GLOBOCAN database which studies cancer of 36 cancers in 185 countries in the world reported that about 2.1 million people or 11.6% of total cancer were diagnosed as lung cancer. Lung cancer deaths worldwide is estimated at 1.8 million death or 18.4% of the total, which was ranking as the first of total cancer. In Thailand, lung cancer are estimated at 23,957 (15.1%) for new lung cancer cases and 21,371 (20.1%) as lung cancer death in 2018 [4]. Over 70% of lung cancer patients were diagnosed at advanced stages or metastatic stages resulting in increasing the incidence of mortality [5].

The metastasis is a complex process which the cancer cells spread from a primary site of tumor to distant sites. It is composed of several processes including cell proliferation, adhesion, invasion, migration and angiogenesis [5]. The cancer cells in this stage or malignant cells have the ability of rapidly growing, attachment and degradation of proteins that composed of extracellular matrix (ECM). This leading to the movement of the malignant cells through the circulation and starting of new colony at the distant organs. A newly developing tumors therefore has to synthesize new blood vessels to supply oxygen and nutrients. However, this newly vessels mostly display of abnormalities and actually cannot supply enough of oxygen. This condition makes hypoxic environment to the malignant cells. To survive in this environment, cancer cells have to adapt themselves by expression more mediators that involved in the process of tumor proliferation and malignant progression [6]. Moreover, various genes of the

involving processes are upregulated including cellular proliferation, differentiation, tumor glycolysis, angiogenesis, metastasis and invasion [7-9]. Hence, tumor cells become more resistant to standard therapies such as chemotherapy and radiotherapy [9]. As the new ways for cancer treatment are not presently established , both of these treatments has to be the method of choices even have an unpleasant results, also the side effects to normal cells [5]. So, this is the reason to propose this work in order to develop new treatment or new anticancer agent that could specifically and directly targeting the cancer cells in the metastasis condition.

Currently, the world has turned attention to use natural substances for cancer treatment as natural substances have less in side effects. Therefore, in this research study, it is interesting to bring the substance from nature to detect the property of anticancer. Terrein, a fungal metabolite, is selected as a candidate in this work [10]. This compound has been shown to exhibit various biological activities including melanogenesis inhibition [11], an anti-inflammation [12] and anticancer [13]. Activity of terrein on human hepatoma demonstrated that terrein could inhibit Bel-7402 cells proliferation through the initiation of cell cycle arrest [14]. Terrein has also been shown to inhibit cell proliferation and angiogenesis of head and neck cancer through suppressing angiogenin production [15]. However, the effect of terrein on the inhibition of human lung cancer cell proliferation, metastasis and angiogenesis have not been fully clarified. Thus, this study would like to investigate the effect of terrein on the inhibition of human lung cancer cell proliferation, metastasis and angiogenesis, as well as observing the effect of terrein on the aggressive phenotype of lung cancer cell in hypoxic environment.

Objectives

1. To determine the effect of terrein against human lung cancer cell viability and proliferation.

2. To examine whether terrein could inhibit metastasis process both in the normal and hypoxic condition.

3. To examine whether terrein could inhibit angiogenesis process.

4. To investigate the effect of terrein on the expression of metastasis mediators such as FAK, PI3K, AKT, mTOR and P70S6K.

Hypothesis

Terrein, a fungal metabolite from *Aspergillus terreus*, may act as anticancer agent in lung cancer cells. The mechanisms are possibly via inhibiting cancer cells proliferation and metastasis processes including migration, adhesion and invasion. In addition, terrein may has these effects both in the normal and hypoxic condition.

CHAPTER II
REVIEW OF THE LITERATURE

This part will be divided into six topics as normal lung, lung cancer, metastasis, angiogenesis, hypoxia and terrein compound.

2.1 The normal lung

The lungs are a pair of soft, spongy, cone-shaped organs located either side of the chest cavity. Each lung is divided into lobes. The left lung has two lobes, upper and lower. The right lung is slightly larger than the left and has three lobes as upper, middle, and lower [16, 17]. The windpipe which is also known as the trachea divides into one left and one right breathing tube or called as bronchi. These breathing tubes continue to divide into many smaller tubes or bronchioles. The bronchioles end in tiny air sacs known as alveoli which look like clusters of grapes. There are more than 300 million alveoli in normal lungs as shown in Figure 1 [17, 18].

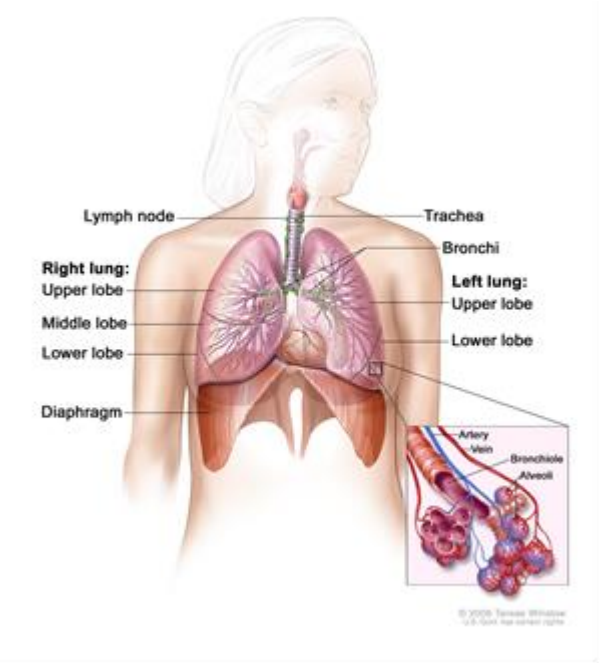


Figure 1 Anatomy of respiratory system

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

2.1.1 Function of the lungs

The lungs are the functional structures of the respiratory system. The primary function is to exchange gases; taking oxygen into the blood circulation and get rid of carbon dioxide out of the body. Other function is to warming, filtering, and humidifying the inhaled air. Also, the lungs help keeping balance of body pH to ensure that cells and enzymes in the body will have an optimal condition [16].

2.1.1.1 How the lungs function

When breathe in, air enters through the nose or mouth. Normally, the nose is the preferred route as it has a filter to decrease some irritants from the air. However, when large amount of air is needed (for example exercise), the mouth breathing is more suitable.

After entering the nose or mouth, the air then travels down to the larynx, pharynx and moves down to right and left bronchus. From bronchus, the air moves further into smaller bronchioles, through alveolar ducts and into the alveoli where the exchange of gases take place. Surrounding each alveoli are tiny blood vessels or capillaries which look like a net. This is where the place that oxygen diffuses from the wall of the alveoli into the enveloping capillaries and through the blood circulation. At the same time, carbon dioxide or "exhaust" gas from the body diffuses out of the blood and into the alveoli. After that, the carbon dioxide travels up through the bronchioles, the trachea and finally out of the body through the nose or mouth by breathe out. For the body to function well, oxygen must enter the blood and carbon dioxide must leave the blood at a regular rate [17].

2.1.2 Diseases of the lungs

Smoking, infections, air pollutants and genetics are responsible for most of lung diseases which can be occurred in any parts. Lung diseases that commonly found affecting the airways are asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, while affecting the air sacs (alveoli) are pneumonia, tuberculosis and lung cancer [19].

2.2 Lung cancer

Lung cancer is the disease that abnormal cells growth without controlled in one or both of the lungs. While normal cells reproduce and develop into healthy lung tissue, these abnormal cells reproduce faster and form tumors that interfering the normal lung functions. In the severe case, the cancer cells may spread from the tumor through the bloodstream or lymphatic system, and to other organs. This stage known as metastasis which is the major cause of death in patient [1].

2.2.1 Epidemiology of lung cancer

The worldwide statistics of cancer are presently based on the data from GLOBOCAN, a project of the International Agency for Research on Cancer (IARC) which

is the specialized cancer agency of the World Health Organization (WHO). Data from GLOBOCAN 2018, estimated global cancer rising to 18.1 million new cases, 9.6 million deaths, when compared with 14.1 million new cases, 8.2 million deaths, in 2012 (4).

For lung cancer, it is the most commonly diagnosed cancer in the world when both men and women are calculated. In 2018, 2.1 million of new lung cancer cases are estimated to occur, accounting for about 11.6% of total cancer diagnoses (Figure 2). When observing through each sex, the number of lung cancer was strongly marked in men than in women. The number of lung cancer cases in men was about 1.4 million or 14.5% (Figure 3). For women, the incidence of lung cancer was generally lower at about 725,352 or 8.4% (Figure 4) [4].

The mortality rate shows that lung cancer was the most leading cause of cancer death worldwide, estimated to be nearly one in five (1.8 million death or 18.4%) of the total. This data was supported from the statistics found in men and women as shown in Figure 2-4 [4]. Data from GLOBOCAN 2018 shows that both the incidence and mortality of lung cancer increased from 2012. The WHO estimates that lung cancer deaths worldwide will continue to rise, mostly attributable to the increase in global tobacco use, especially in Asia. If this current trends continue, it is estimated that new cancer cases would increase to 1.9 billion in 2025 [20].

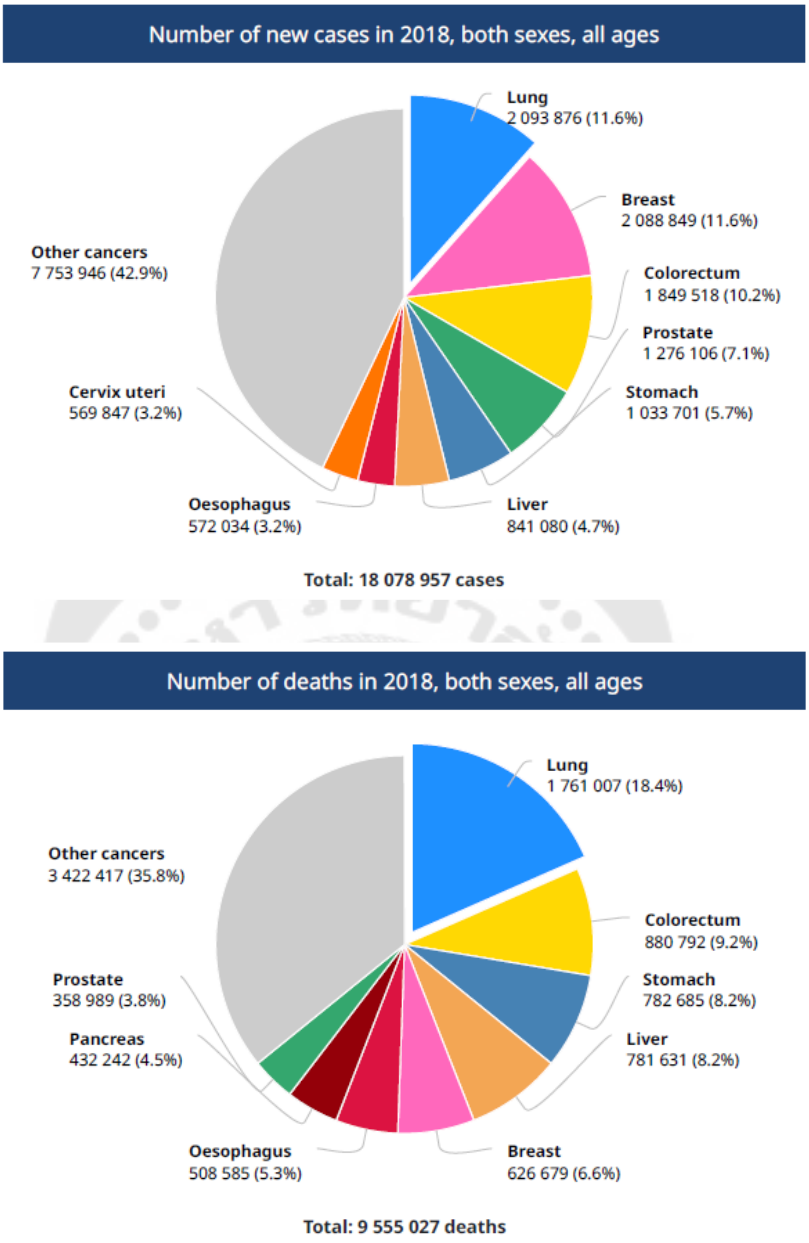


Figure 2 Estimated incidence and mortality of cancer: Both sexes

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

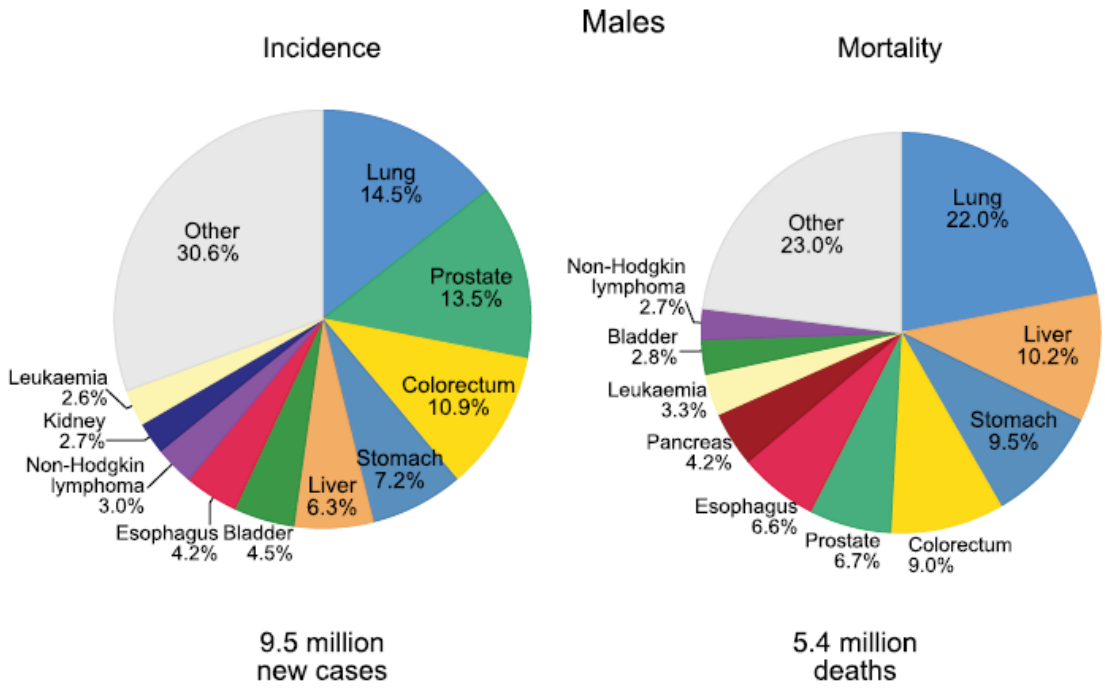


Figure 3 Estimated incidence and mortality of cancer in men

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

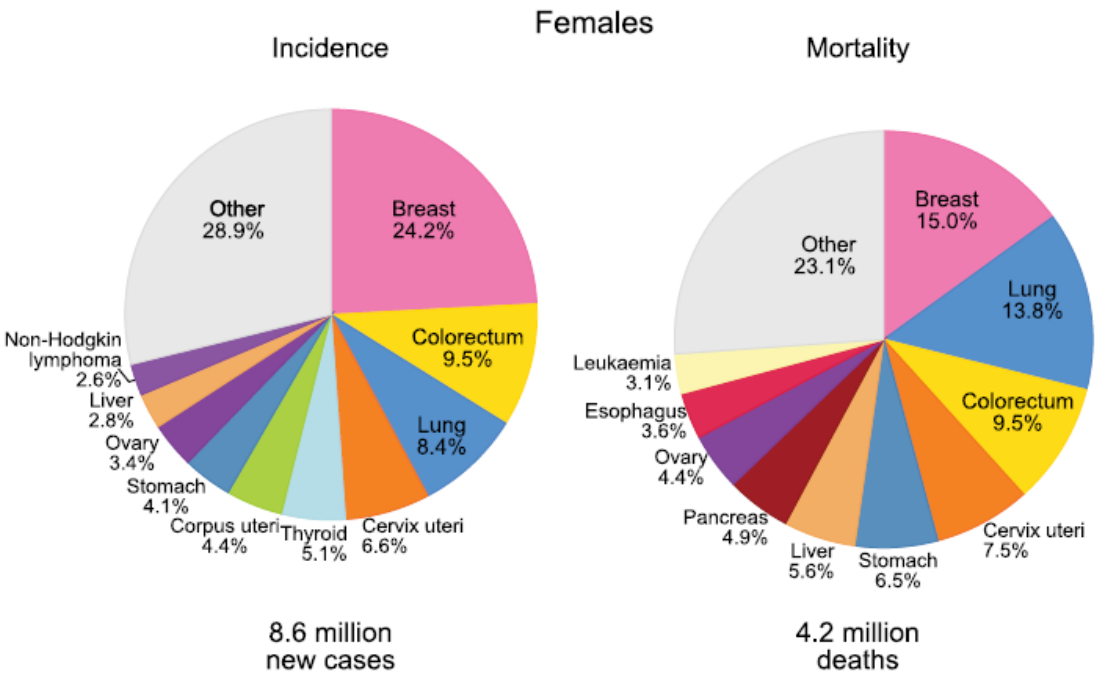


Figure 4 Estimated incidence and mortality of cancer in women

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

In Thailand, lung cancer is diagnosed in both men and women (Figure 5) [21]. An estimated of 23, 957 new lung cancer cases occurred in 2018, accounting for about 14.1% of total cancer diagnosed, which was ranking as the first of total cancer, while 5 year prevalent cancer cases were at 21,508. Lung cancer was also reported to be the leading cause of cancer death which accounting for about 21,371 death or 20.1%, which was ranking second after liver cancer as shown in Table 1 [21].

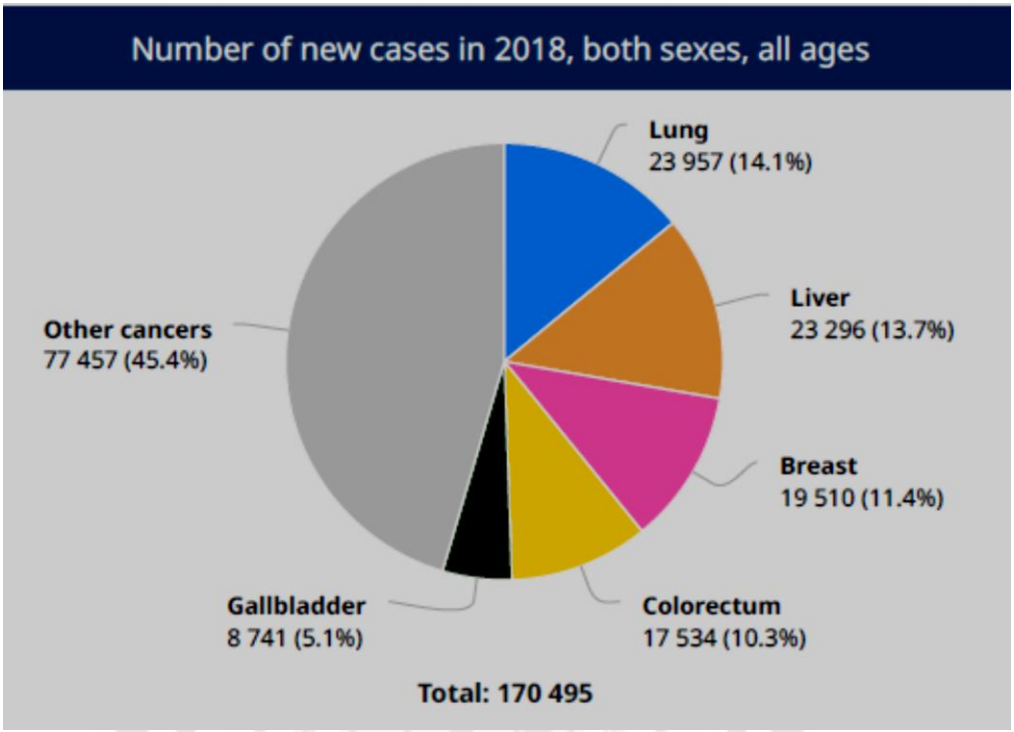


Figure 5 Estimated incidence of cancer in Thailand: Both sexes

Source: GLOBOCAN 2018: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2018. International Agency for Research on Cancer.Internet available from: http://gco.iarc.fr/today/data/factsheets/populations/764-thailand-fact-sheets.pdf.

Table 1 Estimated incidence, mortality and prevalence of all cancer sites in Thailand: Both sexes.

Incidence, Mortality and Prevalence by cancer site										
	New cases				Deaths				5-year prevalence (all ages)	
Cancer	Number	Rank	(%)	Cum.risk	Number	Rank	(%)	Cum.risk	Number	Prop.
Lung	23 957	1	15.08	2.32	21 371	2	20.10	2.22	21 508	31.09
Liver	23 296	2	14.67	2.45	23 154	1	21.77	2.43	17 116	24.74
Breast	19 510	3	12.28	3.95	5 902	4	5.55	1.17	59 457	167.63
Colon	9 097	4	5.73	0.94	5 152	5	4.84	0.52	21 096	30.49
Gallbladder	8 741	5	5.50	0.87	7 608	3	7.15	0.81	9 066	13.10
Cervix uteri	8 622	6	5.43	1.70	5 015	6	4.72	1.00	23 823	67.16
Rectum	8 110	7	5.11	0.87	4 144	7	3.90	0.43	20 512	29.65
Prostate	6 467	8	4.07	1.52	2 886	12	2.71	0.55	14 330	42.51
Non-Hodgkin lymphoma	6 049	9	3.81	0.65	3 139	10	2.95	0.33	15 811	22.85
Leukaemia	4 721	10	2.97	0.50	3 825	8	3.60	0.41	12 218	17.66
Lip, oral cavity	4 572	11	2.88	0.45	2 351	13	2.21	0.23	12 147	17.56
Stomach	4 004	12	2.52	0.40	3 370	9	3.17	0.35	5 341	7.72
Oesophagus	3 563	13	2.24	0.37	3 060	11	2.88	0.32	3 261	4.71
Thyroid	3 393	14	2.14	0.36	384	25	0.36	0.04	11 291	16.32
Ovary	3 254	15	2.05	0.64	1 940	15	1.82	0.39	8 427	23.76
Bladder	3 202	16	2.02	0.32	1 725	17	1.62	0.16	8 683	12.55
Corpus uteri	2 671	17	1.68	0.56	837	21	0.79	0.18	8 060	22.72
Pancreas	2 297	18	1.45	0.24	2 243	14	2.11	0.23	1 529	2.21
Nasopharynx	2 200	19	1.39	0.24	1 421	18	1.34	0.16	6 245	9.03
Brain, nervous system	2 164	20	1.36	0.24	1 865	16	1.75	0.21	5 664	8.19
Larynx	1 572	21	0.99	0.16	877	20	0.82	0.09	4 326	6.25
Kidney	1 417	22	0.89	0.16	818	22	0.77	0.09	3 273	4.73
Multiple myeloma	1 251	23	0.79	0.13	1 105	19	1.04	0.11	2 728	3.94
Oropharynx	886	24	0.56	0.09	435	23	0.41	0.05	2 560	3.70
Hypopharynx	794	25	0.50	0.09	428	24	0.40	0.05	1 168	1.69
Penis	643	26	0.40	0.15	252	27	0.24	0.05	1 801	5.34
Melanoma of skin	536	27	0.34	0.06	290	26	0.27	0.03	1 525	2.20
Salivary glands	526	28	0.33	0.06	198	28	0.19	0.02	1 237	1.79
Anus	327	29	0.21	0.03	166	29	0.16	0.02	809	1.17
Hodgkin lymphoma	305	30	0.19	0.03	137	30	0.13	0.01	1 037	1.50
Testis	254	31	0.16	0.05	51	33	0.05	0.01	892	2.65
Vulva	228	32	0.14	0.04	81	31	0.08	0.01	690	1.95
Vagina	130	33	0.08	0.02	66	32	0.06	0.01	343	0.97
Kaposi sarcoma	38	34	0.02	0.00	24	35	0.02	0.00	102	0.15
Mesothelioma	34	35	0.02	0.00	27	34	0.03	0.00	35	0.05
All cancer sites	170 495	-	-	16.32	114 199	-	-	11.28	330 716	478.03

2.2.2 Types of lung cancer

Lung cancer is divided into two main types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [(2)].

1. Small cell lung cancer (SCLC)

Small cell lung cancer (SCLC), which is previously known as “oat cell carcinoma”, has been found at 10% to 15% of lung cancers. It is an aggressive malignancy type that typically caused by tobacco smoking. The obvious characterized of SCLC is a rapid growth rate and short doubling time. In addition, SCLC has early establishment of widespread metastatic pathology. Patients are commonly diagnosed

with metastatic disease and chemotherapy is considered as the primary method of treatment.

2. Non-small cell lung cancer (NSCLC)

Non-small cell lung cancer (NSCLC) is the common type (80-85%) of lung cancers. NSCLC has different suptypes which different in the type of cells, the rate of growth and how it spreads to other sites. NSCLC can be divided into three subtypes;

Adenocarcinoma: Accounting for about 40% of lung cancers are adenocarcinomas. These cancers start in the cells that line the alveoli which are normally secrete substances such as mucus. Adenocarcinoma is also found in the outer parts of the lung. This type of lung cancer can occur in both of smokers and non-smokers. It is more common in women than in men, and it has a tendency to occur in younger people than other types of lung cancer.

Squamous cell carcinoma: This type is accounting for about 25% to 30% of all lung cancers and can also called epidermoid carcinoma. These cancers begin in squamous cells, which are thin, flat and look like fish scales. This cell type normally lines inside of the airways in the lungs. This cancer type occurs mainly in smokers and founds in the central part of the lungs, near a main airway (bronchus).

Large cell carcinoma: This type accounts for about 10% to 15% of lung cancers. It can appear in any parts of the lung. It is a fast growing and spreading, which can make it harder to treatment. This subtype also known as *large cell neuroendocrine carcinoma* which is grow quickly similar to SCLC [2, 18].

2.2.3 Stages of lung cancer

The stage of lung cancer describes the tumor size and whether it has spread. The stage is one of the most important factors in deciding how to treat the cancer and determining how successful treatment should be.

1. Stages of SCLC

The stage of SCLC can be divided into two stages, which are limited stage and extensive stage. The stage of SCLC is important for making decision of the way of treatment. Chemotherapy combined with radiation therapy may be used to try the cancer in limited stage, while chemotherapy alone may be a better alternative for extensive stage cancer [22].

- Limited stage

The cancer is found in only one lung that mean only one side of the chest and including nearby lymph nodes on the same side of the chest.

- Extensive stage

The cancer has spread widely throughout the lung, to the other lung, to lymph nodes on the other side of the chest, or to other parts of the body.

2. Stages of NSCLC

NSCLC staging uses the TNM system which T refers to the size of the primary tumor, N is the presenting of cancer in the lymph nodes and M is metastasis, how the cancer cell has spread to other parts of the body. Then, NSCLC can be divided into 6 stages as occult, 0, I, II, III and IV [18].

Occult stage

The occult stage or hidden stage, cancer cells are found in sputum (mucus coughed up from the lungs) or bronchial washing (a sample of cells taken from inside the airways that lead to the lung) but cancer cannot be seen in the lung by imaging tests or bronchoscopy. Cancer may have spread to other parts of the body [18].

Stage 0 (carcinoma in situ)

Stage 0 is also known as carcinoma in situ, which located in the place of starting. In stage 0, abnormal cells are found in the lining of the airways. These abnormal cells may become cancer and spread into nearby normal tissue [18].

Stage I

The cancer cells are formed. This stage is divided into stages IA and IB.

Stage IA: The tumor is in the lung only about 3 centimeters or smaller.

Stage IB: The tumor size about 3 - 5 centimeters. Cancer has not spread to the lymph nodes but spread to the main bronchus. Additionally, cancer has also spread to the innermost layer of the lung lining. Part of the lung has collapsed or become inflamed in the area where the trachea joins the bronchus as shown in Figure 6 [18].

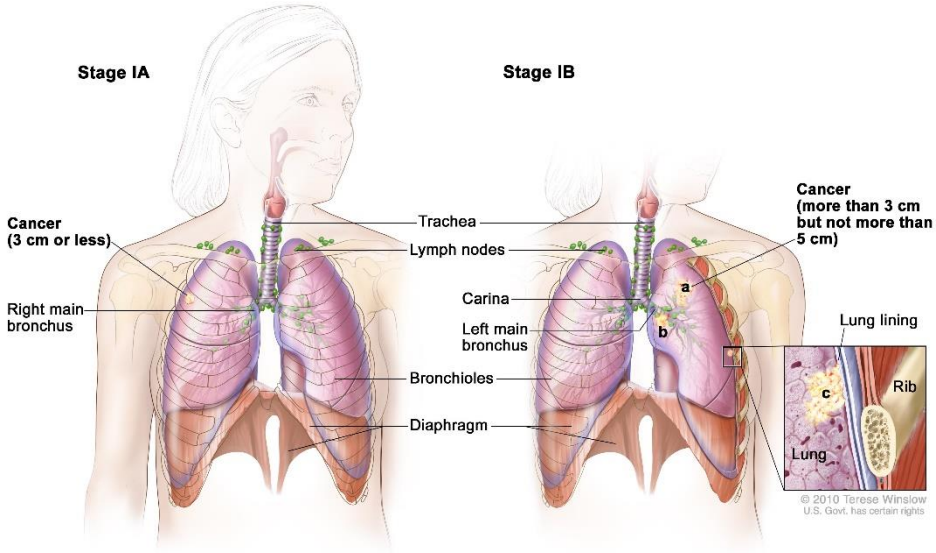


Figure 6 Display stage I of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

Stage II

Stage II is divided into stages IIA and IIB. Each stage IIA and IIB can be divided into two sections depending on the size of the primary tumor, where the tumor is found, and whether cancer cells found in the lymph nodes.

Stage IIA:

(1) The tumor is not larger than 5 centimeters. Cancer cells have spread to lymph nodes on the same side of the chest as the primary tumor, to the main bronchus and to the innermost layer of the lung lining. Part of the lung has collapsed or become inflamed in the area where the trachea joins the bronchus.

(2) The tumor is larger than 5 centimeters but not larger than 7 centimeters. Cancer cells have not spread to the lymph nodes but spread to the main bronchus. In addition, cancer cells have also spread to the innermost layer of the lung lining. Part of the lung has collapsed or become inflamed in the area where the trachea joins the bronchus. Two sections of stages IIA as shown in Figure 7 [18].

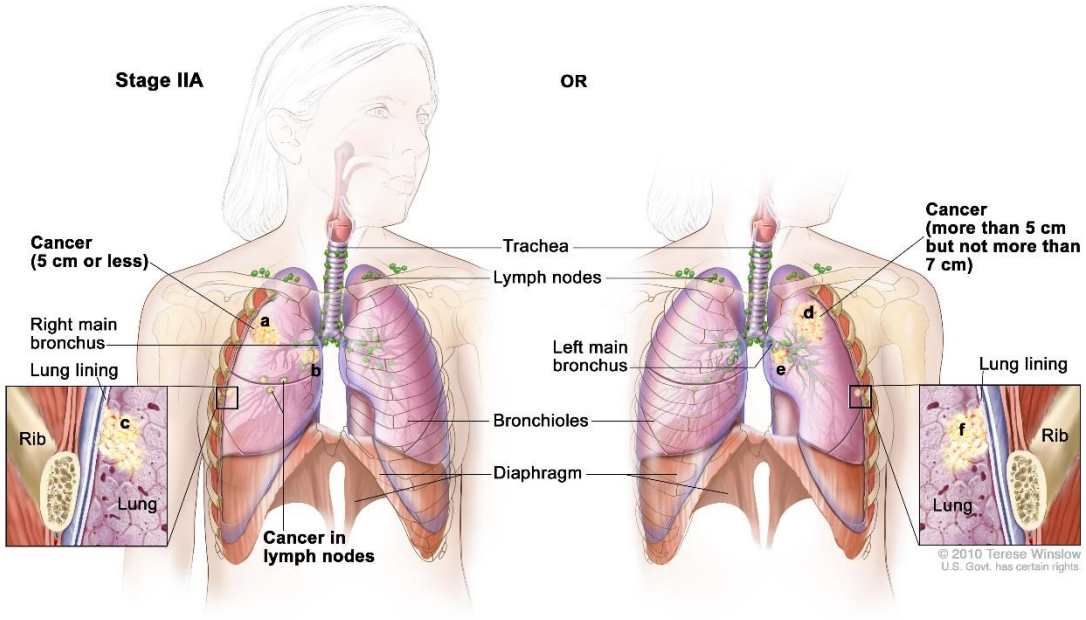


Figure 7 Display stages IIA of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

Stage IIB:

(1) The tumor is larger than 5 centimeters but not larger than 7 centimeters. Cancer cells have spread to nearby lymph nodes on the same side of the chest as the primary tumor, to the main bronchus and to the innermost layer of the lung lining. Part of the lung has collapsed or become inflamed in the area where the trachea joins the bronchus.

(2) The tumor is larger than 7 centimeters. Cancer cells have not spread to the lymph nodes but spread to the main bronchus, the diaphragm or the nerve that controls it, the chest wall or the lining of the chest wall and the membrane around the heart. The whole lung has collapsed or become inflamed. There are one or

more separate tumors in the same lobe of the lung. Two sections of stages IIB are shown in Figure 8 [18].

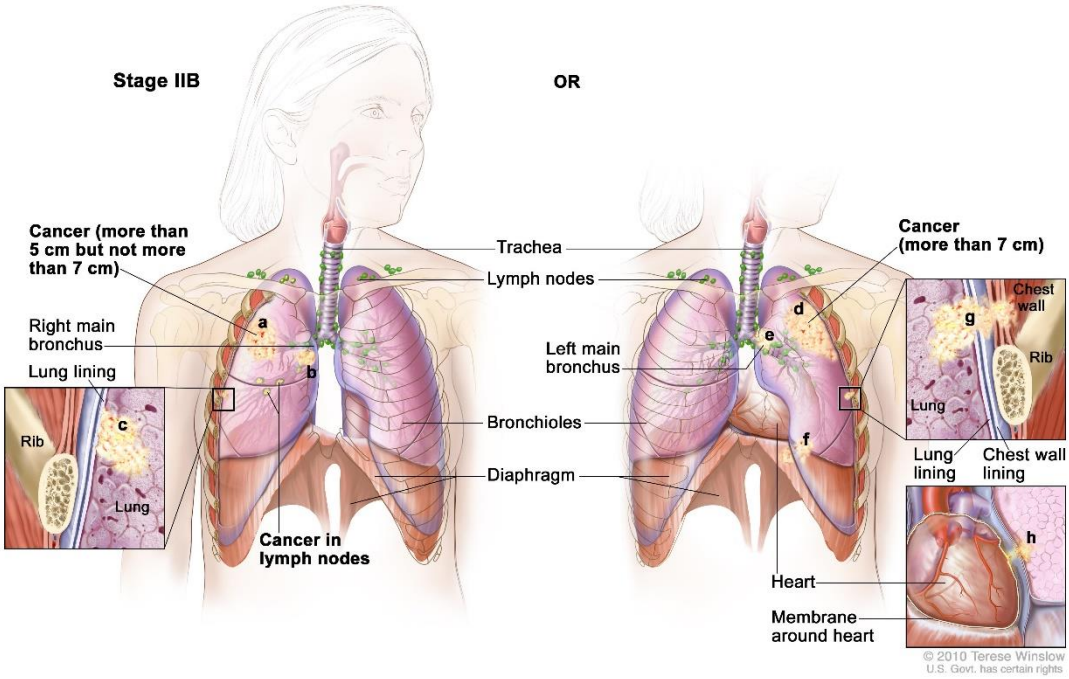


Figure 8 Display Stage IIB of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

Stage III

Stage IIIA can be divided into three sections based on the size of the primary tumor, where the tumor is found, and which lymph nodes have cancer (if any).

(1) The tumor may be any size. Cancer cells have spread to the lymph nodes on the same side of the chest as the primary tumor. Cancer cells may have spread to the main bronchus, membrane around the lung, chest wall lining or chest wall, diaphragm and the nerve that controls it, and membrane around the heart. Part of the

lung or the whole lung may have collapsed or become inflamed. There may be one or more separate tumors in the same lobe of the lung (Figure 9) [18].

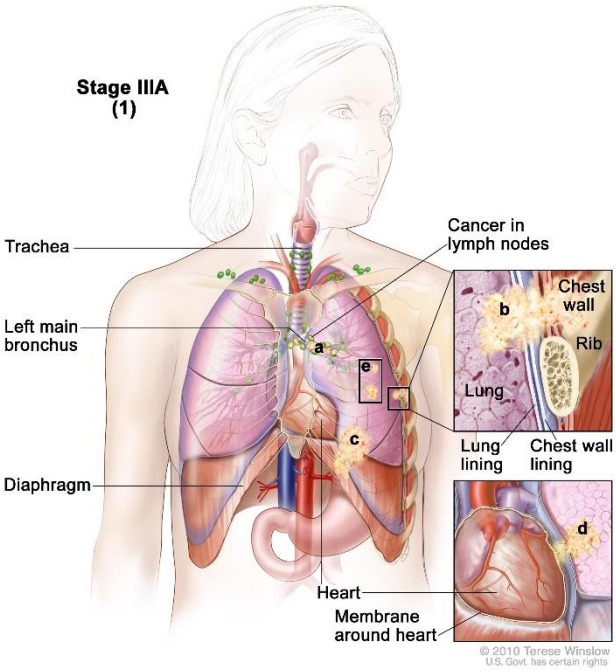


Figure 9 Display Stage IIIA (1) of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

(2) The tumor may be any size. Cancer cells have spread to lymph nodes on the same side of the chest as the primary tumor, to main bronchus, membrane around the lung, chest wall lining or chest wall, diaphragm and the nerve that controls it and heart or the membrane around it. In addition, cancer cells may have spread to major blood vessels that lead to or from the heart, trachea, esophagus, sternum and carina. The whole lung may have collapsed or become inflamed. There may be one or more separate tumors in any of the lobes of the lung with cancer (Figure 10) [18].

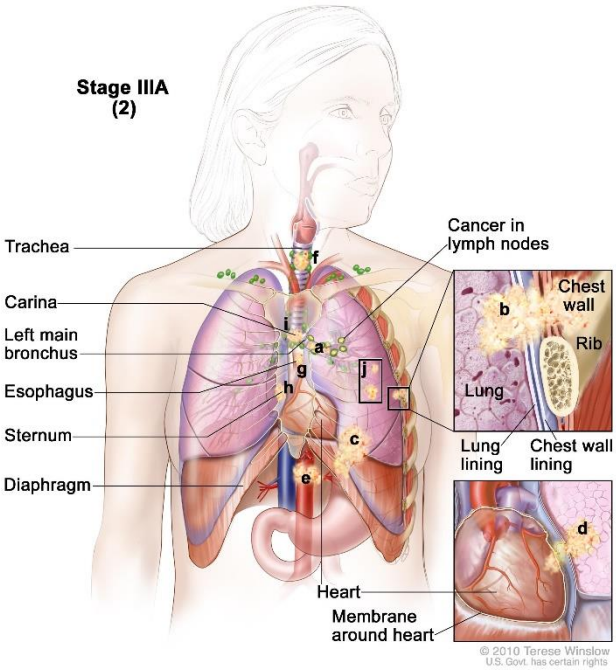


Figure 10 Display Stage IIIA (2) of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

(3) The tumor may be any size. Cancer cells have not spread to the lymph nodes but spread to the heart, major blood vessels that lead to or from the heart, trachea, esophagus, sternum and carina (Figure 11) [18].

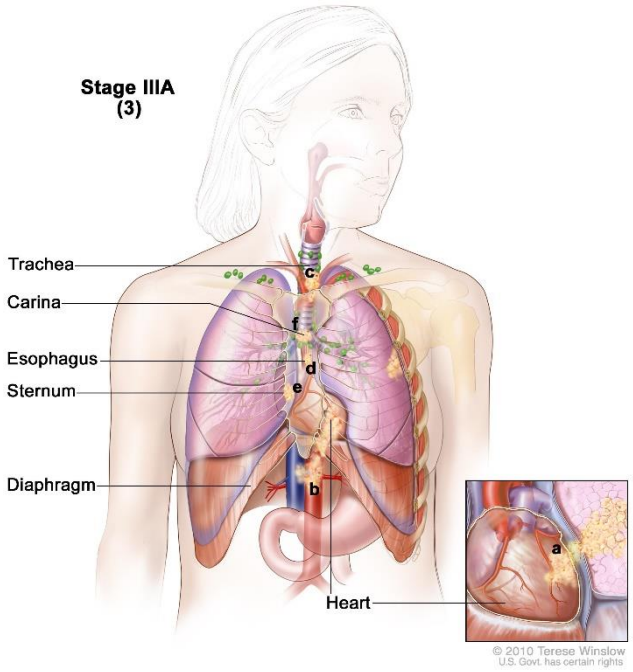


Figure 11 Display Stage IIIA (3) of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

Stage IIIB can be divided into two sections depended on the size of the tumor, where the tumor is found and which lymph nodes have cancer.

(1) The tumor may be any size. Cancer cells have spread to lymph nodes above the collarbone or on the opposite side of the chest as the primary tumor. Cancer may have spread to main bronchus, membrane around the lung, chest wall lining or chest wall, diaphragm and the nerve that controls it included heart or the membrane around it. In addition, cancer cells may have spread to major blood vessels that lead to or from the heart, trachea, esophagus, sternum and carina. Part or all of the lung may have collapsed or become inflamed. There may be one or more separate tumors in any of the lobes of the lung with cancer (Figure 12) [18].

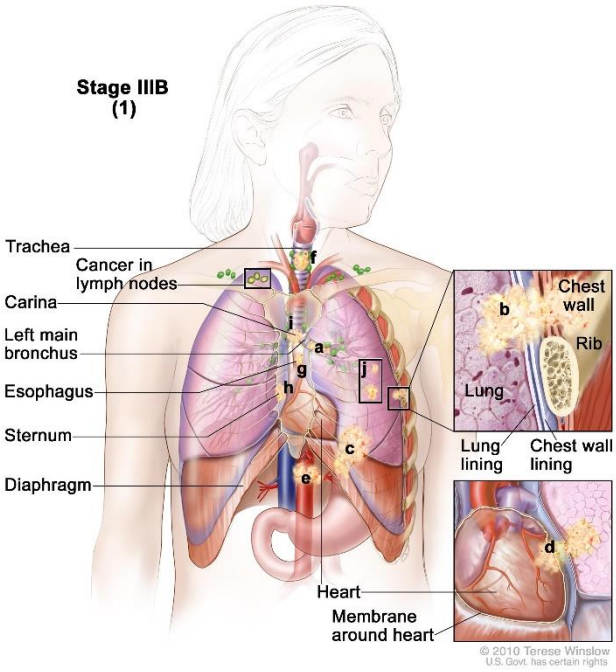


Figure 12 Display Stage IIIB (1) of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

(2) The tumor may be any size. Cancer cells have spread to lymph nodes on the same side of the chest as the primary tumor. Additionally, cancer cells have spread to heart, major blood vessels that lead to or from the heart, trachea, esophagus, nerve that controls the larynx, sternum and carina. There may be separate tumors in different lobes of the same lung (Figure 13) [18].

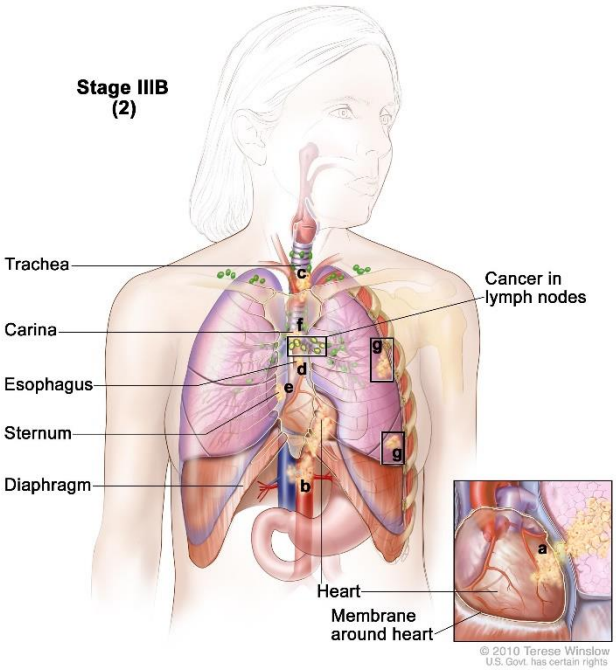


Figure 13 Display Stage IIIB (2) of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

Stage IV

In stage IV, the tumor may be any size and cancer cells may have spread to lymph nodes. There are one or more tumors in both lungs. Cancer can be seen in fluid around the lungs or the heart. Moreover, cancer cells have spread to other parts of the body such as the brain, liver, adrenal glands, kidneys or bone (Figure 14) [18].

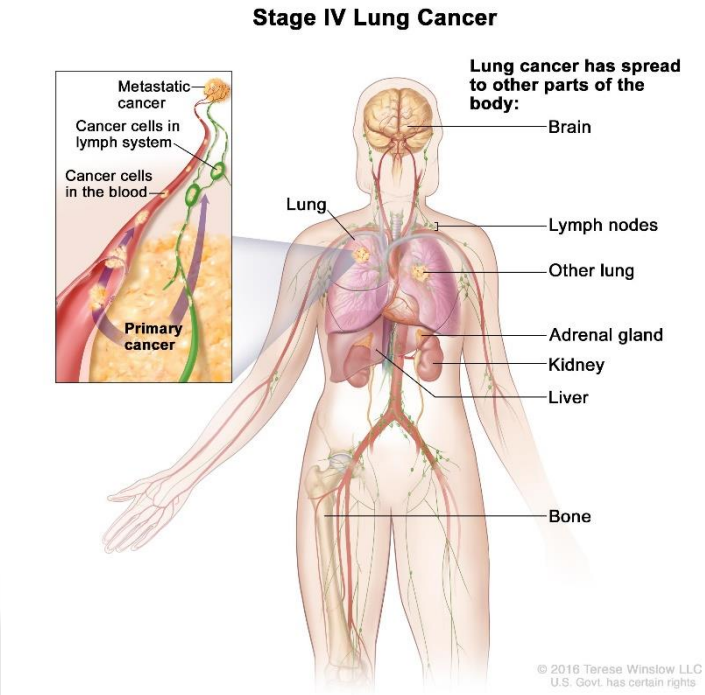


Figure 14 Display Stage IV of NSCLC

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

2.2.4 Signs and symptoms of lung cancer

The signs and symptoms are depending on the localization of the primary tumor and the metastasis condition. The early stage of the disease is few and vague symptoms. Usually, the symptoms of lung cancer not appear until the disease is already at the advanced stage. The most common symptoms of lung cancer are [2, 18]:

- Chest discomfort or pain.
- A cough that doesn't go away or gets worse over time.
- Trouble breathing.
- Wheezing.
- Blood in sputum (mucus coughed up from the lungs).
- Hoarseness.

- Loss of appetite.
- Weight loss for unknown reason.
- Feeling very tired.
- Trouble swallowing.
- Swelling in the face and/or veins in the neck.

Moreover, when lung cancer has spread to other parts of the body, the common symptoms are:

- Bone pain.
- Headache, weakness or numbness of an arm or leg, dizziness, balance problems, or seizures caused by cancer spread to the brain or spinal cord.
- Yellowing of the skin and eyes (jaundice) caused by cancer spread to the liver.

2.2.5 Lung cancer risk factors

Different type of lung cancers have different risk factors. Some risk factors can control such as exposure to chemicals or other substances and some behavior. But, some risk factors cannot control such as age and family history [2]. Several risk factors that potentially developed lung cancer are described below.

1. Tobacco smoke

Tobacco smoke is the most important risk factor for lung cancer development [2 3]. About 80% of lung cancer deaths are resulting from smoking [2]. Tobacco smoke is a complex aerosol composed of gaseous and particulate compounds. Many of them are obviously harming to the body. Some of them are addictive substances such as nicotine and tar [3]. Nicotine is not carcinogenic, but it is an extremely fast-acting drug because it can reaches the brain within 15 seconds of being inhaled, which is the reason of the highly addictive property. Tar is a major

component of lung cancer risk. It contains many chemicals that cause cancer. When a smoker inhales cigarette smoke, 70% of the tar remains in the lungs [24].

Tobacco smoke consists of chemical constituents more than 7,000, which more than 60 are known carcinogens. The carcinogens that particularly concern for lung carcinoma are the tobacco-specific *N*-nitrosamines (TSNAs). TSNAs, including 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), N′-nitrosonornicotine (NNN), N-nitrosoanabasine (NAB), and N-nitrosoanatabine (NAT) are crucial carcinogens specifically found in tobacco [25]. NNK is known to induce lung adenocarcinoma in experimental animals. NNK can bind to DNA and form DNA adducts that leads to permanent mutations [3].

2. Secondhand smoke

Secondhand smoke is the combination of smoke from the burning end of a cigarette and the smoke emitted from smokers [26], also called environmental tobacco smoke that can leads to an increased risk for lung cancer [2]. National committees and organizations have summarized that exposure to environmental tobacco smoke is one important cause of lung cancer [27]. The U.S. Environmental Protection Agency (EPA) has reported substances found in secondhand smoke and known to cause human cancer, they are arsenic, benzo(a)pyrene, cadmium, chromium, nickel and NNK [28]. Nonsmokers who live with a smoker and exposed to secondhand smoke are found an excess risk of developing lung cancer by 20-30% [29]. The risk of developing lung cancer can be increased with the longer of duration and the higher of level of exposure to secondhand smoke [30]. Moreover, involuntarily exposure in children can be affected by secondhand smoke more than adults [29, 30].

3. Exposure to radon

The U.S. Environmental Protection Agency has defined radon to be the second important cause of lung cancer after tobacco smoke [31]. Each year, it is estimated that 15,000 to 22,000 lung cancer deaths in the United States are related to

radon. Radon is an inert radioactive gas released from the naturally occurring decay of the elements uranium, thorium and radium that ubiquitous in rocks and soil. It is an invisible, odorless, tasteless gas that permeates the ground and diffuses into the air. Radon gas usually exists at very low levels outdoors. However, in areas without sufficient ventilation such as underground mines, people who inhale radon can accumulate to levels that increase risk of developing lung cancer [32].

The radon decays quickly and emits tiny radioactive particles, known as radon decay products and subsequent alpha particle emission. α-radiation is highly damaging to tissues including the respiratory epithelium. When inhaled, these radioactive particles can damage the cells and genetic material, so long-term exposure to radon can contribute to lung cancer [33, 34].

4. Exposure to asbestos

Asbestos is a group of minerals that naturally occurring in the environment as bundles of fibers, which used in commercial and industrial applications. Asbestos are divided into 2 main groups: serpentine asbestos (chrysotile) and amphibole asbestos that both groups of asbestos have been linked with cancer [3]. People can be exposed to asbestos from inhaling asbestos fibers in the air. Also, it can also enter the body through eating when the people consume contaminated food or liquids. When breathed in, asbestos fibers can be trapped in the lungs and remain there for a long time. Over time, these fibers can accumulate and result in inflammation, which can affect breathing and contribute to health problems [35].

5. Exposure to air pollution

The IARC has classified outdoor air pollution as a carcinogen and can cause lung cancer. Air pollution is a mixture of many different substances such as diesel engine exhaust, solvents, metals, dust and smoke [36]. Exposure in long-term and accumulating high level of outdoor air pollution increases the risk of lung cancer. Moreover, occupational exposures to diesel exhaust, especially in persons in the

trucking industry, they are increasing the risk of lung cancer at approximately 30% to 50% [3].

6. Genetic factors

Most cases of the lung cancer are not associated with inherited gene changes. These cancers are linked to somatic mutations, which occur only in some cells in the lung. The risk factors for lung cancer can occur when genetic mutations build up in important genes, specifically genes that control cell growth, cell division or the repair of damaged DNA. These changes can lead to abnormal cell growth and divide uncontrollably to form a tumor. The mutations in lung cells often cause from exposure to factors in the environment such as chemical carcinogens in tobacco smoke [37, 38].

Gene mutation that associated with lung cancer is epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog *(KRAS)* and anaplastic lymphoma kinase *(ALK)*, which are exclusive in patients with NSCLC.

The EGFR, a receptor-tyrosine kinase, which can be transactivated by G protein-coupled receptors and controls cell growth and proliferation via a transduction signaling pathway [39]. EGFR is expressed on the cell surface of NSCLC [40]. A mutation of this receptor can contribute to overexpression of the tyrosine kinase domain in the cell membrane. As a result, cells are unregulated growth and proliferation, leading to tumor formation [41-43]. The overexpression of EGFR in NSCLC is related to tumor development, poor prognosis and drug resistance [44]. Activating *EGFR* mutations are more common observed in patients with adenocarcinomas and nonsmokers women and 50% of Asians and 10% of non-Asians [45].

KRAS proto-oncogene is one of the most frequent mutations in NSCLC. *KRAS* mutations are found in adenocarcinomas subtype of NSCLC approximately 25%. These mutation most commonly found in smokers or smokers in the past. However, it can also found in patients who have never smoke [46, 47].

ALK rearrangements are receptor tyrosine kinases (RTKs), which fusion with the echinoderm microtubule-associated protein-like 4 (EML4) gene. This results in the abnormal expression and activation of tyrosine kinase in the cytoplasm of cancer

cells. These mutations found about 2-7% of patients with adenocarcinoma subtype of NSCLC who have never smoke or smokers in the past. ALK mutations occur as independently of EGFR and KRAS gene mutations (47, 48).

7. Other factors

Other factors for lung cancer include:

- Gender
- Age
- Race and Ethnicity
- Diet
- Infections
- Agents in the workplace
- Talc and talcum powder [2, 3]

2.2.6 Tests for diagnosing lung cancer

Tests and procedures to detect and diagnose lung cancer are often done at the same time. Some of the following tests and procedures may be used.

1. Physical examination and history

Physical examinations for checking general signs of health, including checking for signs of disease, such as lumps or other abnormalities. A history of the patient's health behaviors, including smoking and past works, illnesses and treatments will also be taken into account [18].

2. Laboratory tests

Medical procedures that are need to test samples of tissue, blood, urine, or other substances in the body. These tests help to diagnose disease, plan and check treatment, or monitor the disease over time [18].

3. Chest x-ray

An x-ray that is produces images of the organs and bones inside the chest. An x-ray is a type of energy beam that can go through the patient body and onto film, making a picture of areas inside the body without having to make an incision. This can help to diagnose, monitor and treat many medical conditions (Figure 15) [18].

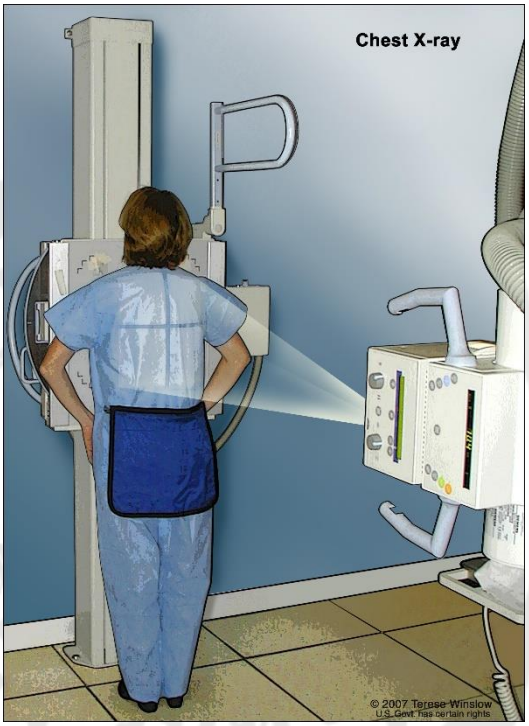


Figure 15 Chest x-ray

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

4. CT scan (computed tomography)

CT scan is a procedure that uses X-rays to makes a series of detail images of areas inside the body such as the chest, taken from different angles. The

images are made by a computer linked to an x-ray machine. A dye may be injected into a vein or swallowed to help the organs or tissues appear more clearly [18].

5. Sputum cytology

Sputum cytology is a procedure to examine a sample of sputum (mucus coughed up from the lungs and in the airways) under a microscope to determine whether cancer cells are present [18].

6. Fine-needle aspiration (FNA) biopsy of the lung

The tissue or fluid is removed from the lung by using a thin needle. The needle is inserted into the chest through the skin on the chest and into a tumor which can make sure the needle goes to the right part of the lung by watching it through ultrasound or a CT scanner. A sample is removed with the needle and sent to the laboratory. A pathologist then evaluates the sample under a microscope to look for cancer cells (Figure 16) [18].

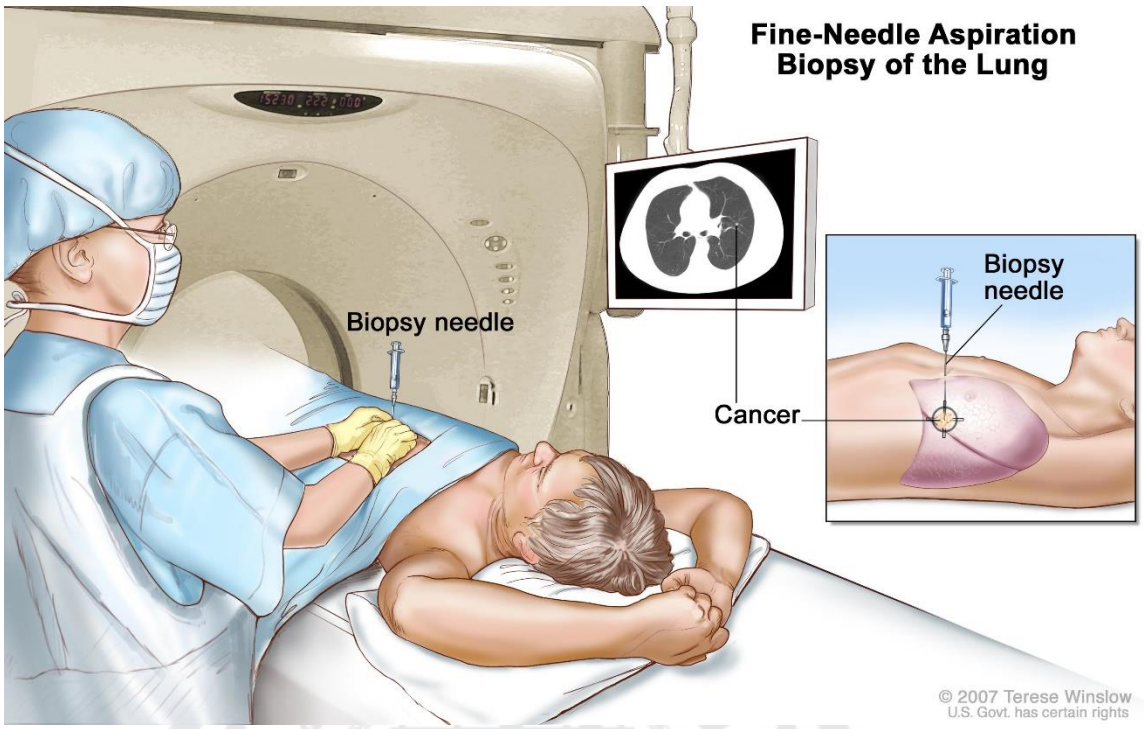


Figure 16 Fine-needle aspiration biopsy of the lung

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

7. Bronchoscopy

Bronchoscopy is a procedure to look inside the larynx, trachea and smaller airways in the lung for abnormal areas. A bronchoscope is inserted through the nose or mouth into the trachea and reach to lungs. A bronchoscope is a thin, tube-like instrument with a light source and a camera at the end for viewing. It may also have an equipment to remove tissue samples, which are examined under a microscope for signs of cancer (Figure 17) [18].

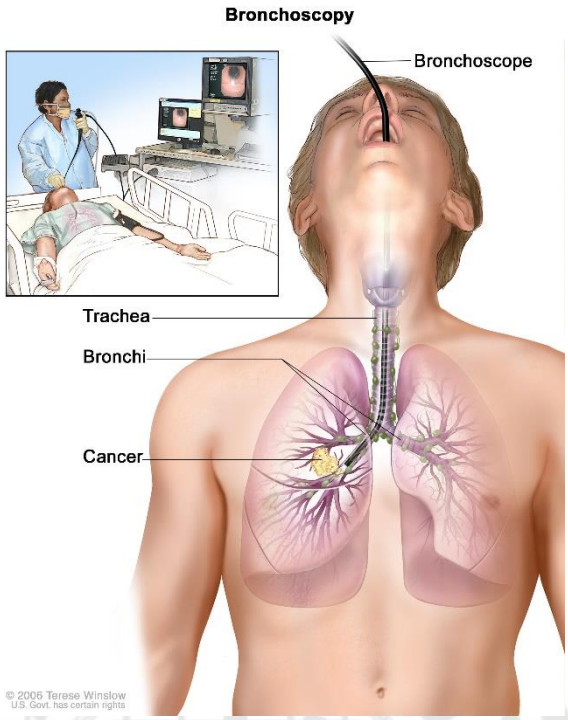


Figure 17 Bronchoscopy

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

8. Thoracoscopy

Thoracoscopy is a surgical procedure using to look at the organs inside the chest to check for abnormal areas. A thoracoscope is a thin, tube like instrument containing a tiny camera inserted through an incision to visually examine and also have an equipment to remove tissue or lymph node samples for checking under a microscope for signs of cancer. Thoracotomy is used in cases that if certain tissues, organs, or lymph nodes can't be reached. In this procedure, a larger incision is made between the ribs and the chest is opened [18].

9. Thoracentesis

The fluid is removed from the space between the lining of the chest and the lung by using a needle. A pathologist examines the fluid under a microscope for cancer cells [18].

10. Immunohistochemistry

Antibodies are used to check for certain antigens in a sample of tissue. The antibody is usually linked to a radioactive substance or a fluorescent dye, when the antibodies bind to the antigen in the tissue sample, the radioactive substance or a fluorescent dye is activated that causes the tissue to light up under a microscope [18].

2.2.7 Lung cancer treatment and therapy

The choice of treatment depends on many factor to select the correct and effective of treatment. Treatment options depend on the stage of the cancer whether the size of the tumor located in the lung only or has spread to other places in the body. In addition, signs and symptoms, and general health of patient.

1. Surgery

The surgery are used to treat lung cancer which can be divided into four types.

- Wedge resection: Surgery to remove part of the lung lobe containing cancer and some of the normal tissue around it. When a slightly larger amount of tissue is taken, it is called a segmental resection (Figure 18) [18].

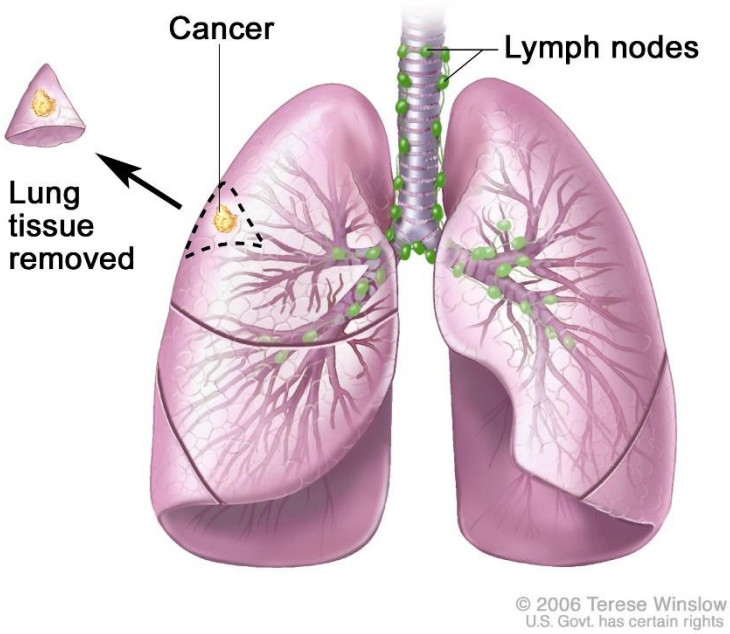


Figure 18 Wedge resection of the lung

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

- Lobectomy: surgery to remove a whole lobe of the lung that containing cancer (Figure 19) [18].

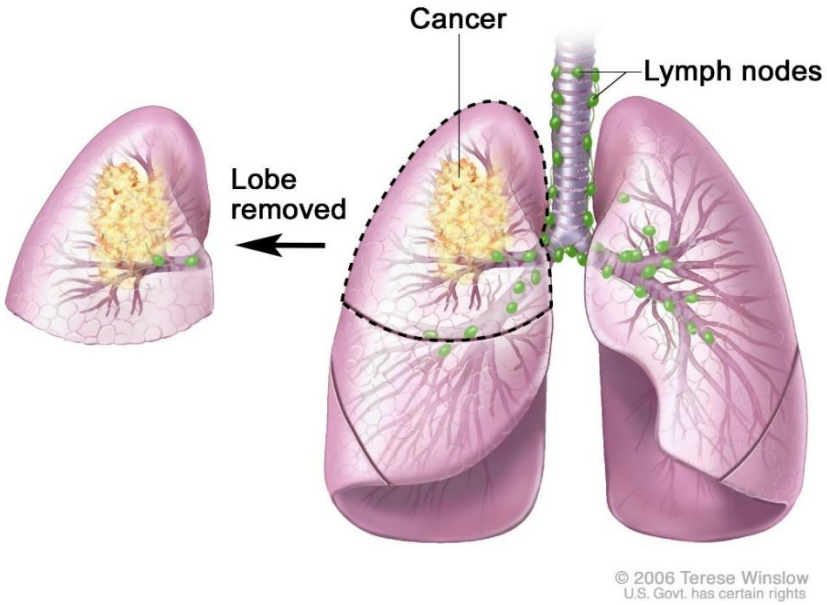


Figure 19 Lobectomy

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

- Pneumonectomy: surgery to remove one whole lung (Figure 20) [18].

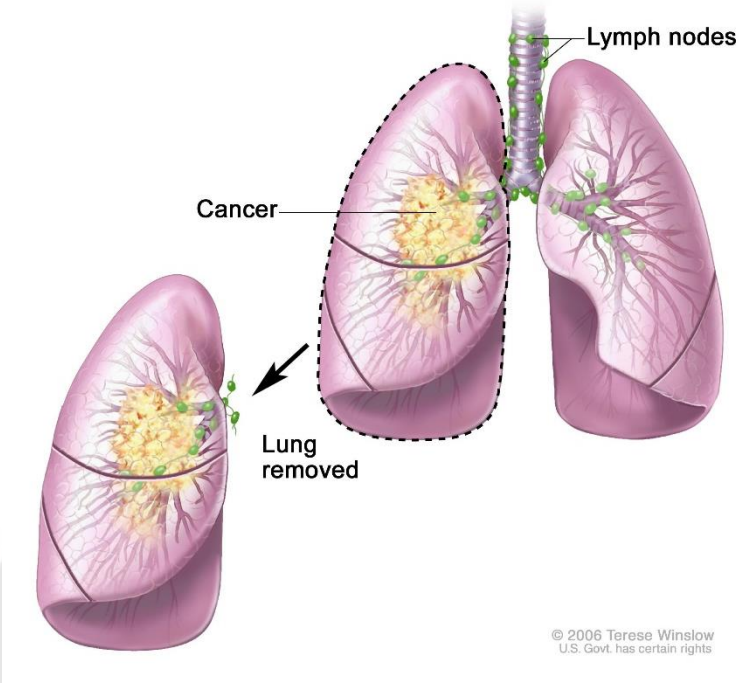


Figure 20 Pneumonectomy

Source: NATIONAL CANCER INSTITUTE. Non-Small Cell Lung Cancer Treatment (PDQ®)–Patient Version. Internet available from: http://wwwcancergov/types/lung/patient/non-small-cell-lung-treatment-pdq#section/all/.

- Sleeve resection: surgery to remove part of the bronchus [18].

Although the doctor removes all the cancer that can be found at the time of the surgery, some patients may be given chemotherapy or radiation therapy after surgery to kill the remaining cancer cells. The treatment given after the surgery to reduce the risk of cancer returning, which is called adjuvant therapy. Adjuvant therapy may include radiation therapy, chemotherapy, targeted therapy or immunotherapy [18]. Each therapy is described below.

2. Radiation therapy

Radiation therapy is a cancer treatment that uses high-energy x-rays or other types of radiation to destroy cancer cells. Radiation therapy can be divided into two types as **external radiation therapy** which is radiation given from a machine outside the body to send radiation toward the cancer and **internal radiation therapy** that uses a radioactive substance sealed in needles, seeds, wires, or catheters and placed directly into or near the cancer.

Radiation treatments are based on the type and stage of cancer being treated and position of cancer. For tumors in the airways, radiation is given directly to the tumor through an endoscope [18].

3. Chemotherapy

Chemotherapy is a cancer treatment that using anti-cancer drugs to stop the growth of cancer cells, both by killing or stopping the cells from dividing. Chemotherapy is taken by injected into a vein or by mouth. These drugs would enter the bloodstream and travel throughout the body to treat cancer cells. This treatment is beneficial for cancer anywhere in the body which called systemic chemotherapy. While chemotherapy that injects directly into specific area such as the cerebrospinal fluid, an organ, or a body cavity, these is called regional chemotherapy. Which one will be used is based on the type and stage of the cancer being treated [18].

4. Targeted therapy

Targeted therapy is a type of treatment that uses drugs or other substances that designed to attach specifically to the receptors and proteins unique to cancer cells. Targeted therapies usually cause less damage to normal cells than chemotherapy or radiation therapy. This type of therapy can be divided into two main types. Monoclonal antibodies therapy is antibody that specifically block the growth and spread of cancer by preventing cancer cells from dividing or directly destroying them. Another of targeted therapy is tyrosine kinase inhibitors, which are small-molecule drugs that go through the cell membrane and work inside cancer cells to block signals

that cancer cells need to grow and divide. These therapy are used to treat advanced, metastatic or recurrent non-small cell lung cancer [18].

2.3 Metastatic cancer

In metastasis, cancer cells can break away from the primary cancer, travel through the bloodstream or lymphatic system and form new tumors called secondary cancer (metastatic tumors) in other parts of the body. The metastatic tumor is the same type of cancer as the primary tumor as shown in Figure 21 [49].

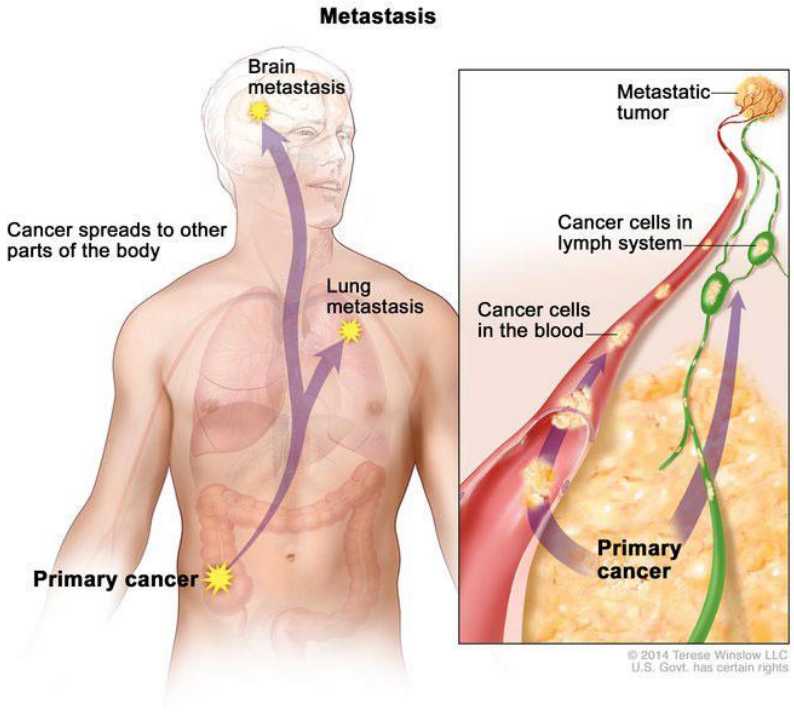


Figure 21 Metastasis

Source: NATIONAL CANCER INSTITUTE. Metastatic Cancer. Internet available from: https://wwwcancergov/types/metastatic-cancer. Updated 6 February 2017.

Metastasis is the leading cause of mortality for cancer patients, over 70% of lung cancer patients have advanced stages at time of diagnosis [5]. Cancer metastasis is a complex condition that consists of multi-step and continuous processes. These steps include [50, 51]:

1. Invasion and migration: malignant tumor cells dissociate from the primary tumor and invade nearby healthy tissues. During this process, several of proteolytic enzymes are secreted to degrade the basement membrane and extracellular matrix (ECM), so the cells can facilitate migration.

2. Intravasation: the cancer cells invade into the lymphatic and blood vessels. After the attachment on the endothelial cells via adhesion molecules, the malignant tumor cells secrete proteolytic enzymes and growth factors which enable them to penetrate the lymphatic and blood vessel.

3. Circulation: the cancer cells traveling through the bloodstream and has to withstand the conditions present in the blood, especially an attack from the immune system. Circulating cancer cells then bind to platelets and coagulation factors to protect themselves from immune detection, thus increase the chance of survival. A selection for particularly resistant and aggressive tumor cells takes place.

4. Extravasation: cancer cells can leave the blood stream by secret various factors to induce endothelial cell retraction. Vascular endothelial growth factor (VEGF) increases vascular permeability, causing cancer cell extravasation.

5. Colonization, proliferation and angiogenesis: the final steps of metastasis, the cancer cell settles at a distant tissue site and builds a secondary tumor. The cell proliferates and induces neo-angiogenesis in order to ensure sufficient vascularization to supply oxygen and nutrients.

2.3.1 Molecular of cancer metastasis

1. Cell migration

Cell migration plays a crucial role in various biological processes. It is important during organogenesis, embryonic development, immune response, tissue

repair response in both wound healing and angiogenesis. In the pathological situation, cell migration is crucial for cancer metastasis and cardiovascular disorders [52, 53].

The process of cellular migration is normally present as several steps and requires several mediators. In step one, the cell extends a protrusion of membrane at the leading edge to forward direction, which can called this protrusion structure as lamellipodia. Step two, the cell adheres the leading edge to the surface or substratum components, which at the same time de-adhere on the trailing edge. And the last step, the whole cell body is pulled forward by contractile forces generated at the cell body and rear of the cell (Figure 22) [54].

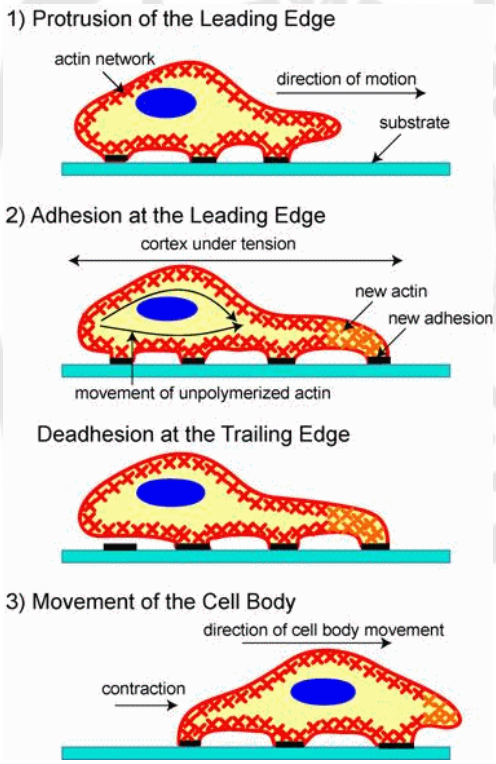


Figure 22 The process of cellular migration

Source: Ananthakrishnan R, Ehrlicher A. The forces behind cell movement. Int J Biol Sci. 2007; 3(5):303-17.

Regulation of cell migration

- **Lamellipodia**

Lamellipodia are flat, sheet-like membrane protrusions formed at the leading edge of migrating cells. Lamellipodia are cellular structure that have an important role in driving the migration of cells by attaching to the substrate and creating force to pull the cell body forward. Lamellipodia are enriched with a dendritic array of a branched network of actin filaments and molecular machinery that regulates polymerization/depolymerization and organization of actin filaments [55].

- **Mechanism of assembly actin filaments in lamellipodia protrusion**

The assembly of a branched network of actin filaments provides the mechanical propulsion that drives a range of dynamic cellular processes, including cell movement. The Arp2/3 (Actin related proteins 2 and 3) complex is an important component, which can nucleates new actin filaments from existing filaments leads to generating a branched network [56]. The initiation of new actin filaments start from activation of cell surface receptors generates signals including activated Rho family GTPases that activate SCAR or WASP (Wiskott–Aldrich syndrome protein) family. The WASP family composes of five members, including WASP, N-WASP (neural WASP), WAVE1 (WASP family verprolin homologous protein 1), WAVE2 and WAVE3 [57-59]. WASP-family proteins stimulate the Arp2/3 complex and consequently stimulate actin assembly, which Arp2/3 complex binds to G-actin results in a stable trimer and nucleus for the growth of a filament [60]. The filament grow at a fixed 70 degrees angle from the side of pre-existing actin filaments close to the plasma membrane and push the membrane forward by the addition of actin monomers to their barbed ends [59, 61]. The actin filament length is controlled by capping proteins, which binds to growing ends for block addition of new monomers and prevents further growth [62]. Another one of actin filament length control is actin depolymerizing factor (ADF) and the cofilin. The ADF/cofilin binds to ADP-F-actin subunits leads to sever of actin filaments and promotes dissociation of ADP-actin from the pointed end of the filament [63, 64]. The activity of

ADF/cofilin can be inhibited by LIM kinases through phosphorylation [65]. In addition, another important factor that obstruct activity of ADF/cofilin to severing and depolymerization is tropomyosin, which competes with ADF/cofilin for binding to ADP-F-actin [66]. After debranching, profilin protein catalyzing of ADP-bound actin monomers into ATP-bound monomers nucleotide exchange contribute to re-polymerization for rapid elongation of new barbed ends (Figure 23) [67, 68].

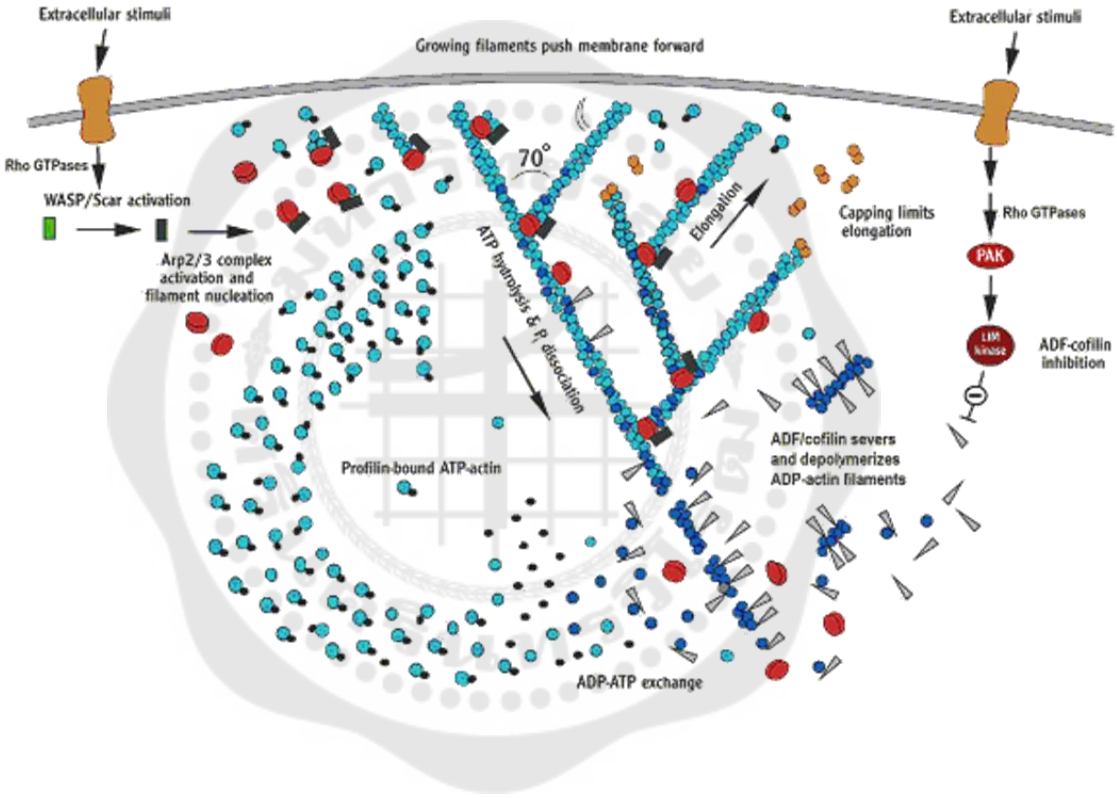


Figure 23 The dendritic nucleation model at the leading edge of motile cells

Source: Pollard TD, Borisy GG. Cellular motility driven by assembly and disassembly of actin filaments. Cell. 2003;112(4):453-65.

2. Cell invasion

Cell invasion, a one of important step in cancer metastasis, which is the primary cause of cancer death. Cell invasion is associated to cell migration and determines the capability of cancer cells to become motile and move through the

extracellular matrix within a tissue or to infiltrate neighboring tissues. Cancer cells that become invasive may spread to secondary sites and form metastases [69].

Regulation of cell invasion

- **Invadopodia/podosomes**

Cancer cells need to degrade ECM, which contained in the basement membrane, tumor stroma and blood vessel walls, in order to migrate from primary tumor sites and invade neighboring tissues for spreading to distant organs [70]. Invadopodia are ECM-degrading protrusions activity formed by highly invasive cancer cells on thick substrates [71]. Invadopodia contains a variety of proteins such as actin and actin regulatory proteins, adhesion molecules, membrane remodeling and signaling proteins and matrix degradation enzymes. Additionally, they have N-WASP and cortactin, which are main structure and found increasing in malignant cancer cells [72, 73]. Podosomes are structures similar to invadopodia, which are formed by cell types of monocytic origin such as macrophages, dendritic cells and osteoclasts that also have ECM degradation activity [74].

- **Extension mechanism of the Invadopodia**

Several of intracellular and extracellular components such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) and reactive oxygen species (ROS), can induce the formation and promote the function of podosomes and invadopodia [75]. Invadopodia formation can be divided into three stages - initiation, assembly and maturation [76, 77]. Initiation stage, signaling transduction is stimulated from growth factors and activate Src family kinases secreted from the focal adhesion [75]. These kinases play a pivotal role in the formation of invadopodia and when activated, Src binds and activates scaffolding protein tyrosine kinase substrate with five SH3 domains (TKS5) and other kinases such as protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K). TKS5 is also associated with the production of invadopodia in response to ROS. Activation of TKS5 is associated with cortactin to promote actin filament polymerization [78, 79]. Cortactin plays a crucial role in regulation of the actin cytoskeleton by activation the actin nucleation

activity of Arp2/3 complex. In addition, cortactin also stabilizes branched actin filaments produced by Arp2/3 complex via directly binds cofilin and inhibits its severing activity [55, 80]. In assembly stage, the actin cytoskeleton undergoes reorganization into an invasive structure via recruitment of WASP and N-WASP which regulated by upstream signaling such as Cdc42, SH3 domain-containing proteins (Nck and Grb2) and phosphoinositides [55]. Activated N-WASP works together with the Arp2/3 complex and fascin along with kinase activity to promote actin polymerization [81]. The last stage is maturation, which invadopodia could degrade the extracellular matrix by membrane-bound enzyme (MT1-MMP) and secreted matrix metalloproteinases (MMP2 and 9) as shown in Figure 24 [75].

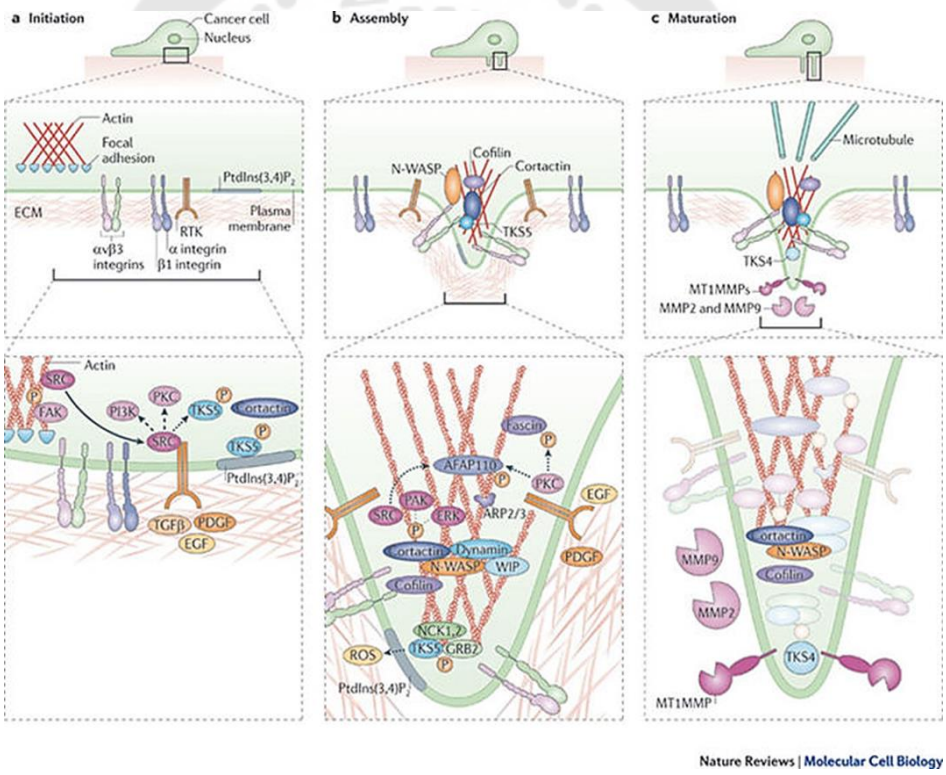


Figure 24 Invadopodia assembly

Source: Murphy DA, Courtneidge SA. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol. 2011;12:413-26.

3. Cell adhesion

Cell adhesion is the capacity of a single cell to attach to other cell (cell-to-cell adhesion) or to an ECM (cell-to-substrate adhesion). This process is implicated in stimulating signaling pathway that control of cellular morphology, migration, proliferation, survival and differentiation [82, 83]. For cancer metastasis, the loss of cell-cell adhesion ability in tumor cells allows tumor cells to dissociate from the primary tumor mass and changes in cell-matrix interaction facilitate the cells to invade the surrounding stroma and leading to the spreading of other organs.

The interaction between cells and ECM normally occurs by using several key adhesion molecules (CAM) including integrins, selectins, cadherins, immunoglobulin superfamily (IgSF), CD44 and focal adhesions.

Cell adhesion molecules (CAMs)

Cell adhesion process requires cell adhesion molecules or CAMs to bind with other cells or with ECM. These proteins are receptors that embedded in the cellular membrane or known as transmembrane receptors. They are consisted of three domains, an intracellular domain that interacts with the cytoskeleton, a transmembrane domain and an extracellular domain that interacts with other CAMs. Most of the CAMs belong to four protein families including cadherins, integrins, selectins and the immunoglobulin superfamily (IgSF) (Figure 25). They are responsible for facilitating cellular processes such as cell division, migration and differentiation, and are critical for the development and maintenance of tissue structure integrity in adults [84]. In addition, CAMs also play a crucial role in cancer progression and metastasis. Interaction between cancer cells and vascular endothelium determines the metastatic spread. Additionally, cancer cells are directly interact to platelets, leukocytes and soluble components leading to cancer cell adhesion, extravasation and the establishment of metastatic lesions [85].

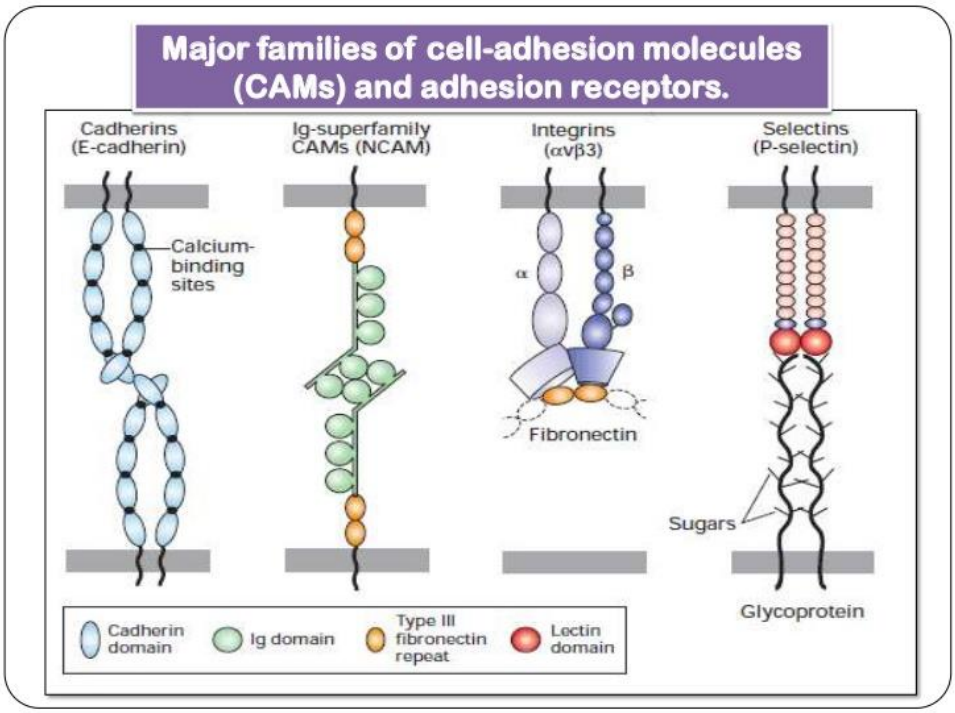


Figure 25 Families of CAMs

Source: https://www.slideshare.net/aljeirou/adhesion-molecules

1. Cadherins

The cadherins are calcium-dependent cell adhesion proteins. Their structure has a space at each repeat for binding with calcium. This structures help the chain rigid and easily to connect with a chain of another cell. Cadherins can be classified as E-cadherin, N-cadherin and P-cadherin. They are concentrated at the intermediate cell junctions between the actin filament network and specific linking proteins called catenins (β-catenin, a components of the Wnt signaling pathway). E-cadherin plays a pivotal role in the formation of epithelium. The loss of E-cadherin promotes tumor cell invasion, migration and spread from primary tumor to other part of the body [86].

2. Immunoglobulin superfamily (IgSF)

The immunoglobulin superfamily (IgSF) is a large protein superfamily of cell surface that involved the recognition, binding or adhesion processes of the cells. IgSF consists of cell surface antigen receptors, co-receptors of the immune system, cell adhesion molecules, some cytokine receptors and molecules involved in antigen presentation to lymphocytes. Generally, IgSF plays an important role associated with the immune system [87].

3. Selectins

Selectins are families of cell surface lectins that mediate leucocyte-endothelial cell interactions in inflammatory and immune responses. Member of selectins include P-selectin (expressed on platelets and leukocytes), E-selectin (expressed on endothelial cells) and L-selectin (expressed on leukocytes, monocytes, neutrophils and eosinophils) [88]. They are important for binding of circulating leukocytes to vascular endothelium during the inflammatory response. In addition, selectins regulate the adhesion of circulating cancer cells to the wall of blood vessels in metastasis process [89].

4. Integrins

Integrins are transmembrane glycoproteins that consist of 18 α and 8 β forming as 24 heterodimeric pairs. Integrins are mediators of cell adhesion that directly bind to ECM elements such as fibronectin, vitronectin, laminin or collagen and provide anchorage for cell motility and invasion [85]. Integrins mediate a bidirectional signaling by forming a bent conformation. When adaptor proteins such as talin, vinculin and α-actinin binds to the tail of β-subunit, this refers to “inside-out signaling”, while binding of ligand to the head of integrin is called “outside-in signaling”. Attachment of cells to ECM components induce integrins cluster and their downstream signaling molecules such as focal adhesion kinase (FAK) or Src family kinases, which affect the cellular shape and migratory properties of cells as shown in Figure 26 [85, 90].

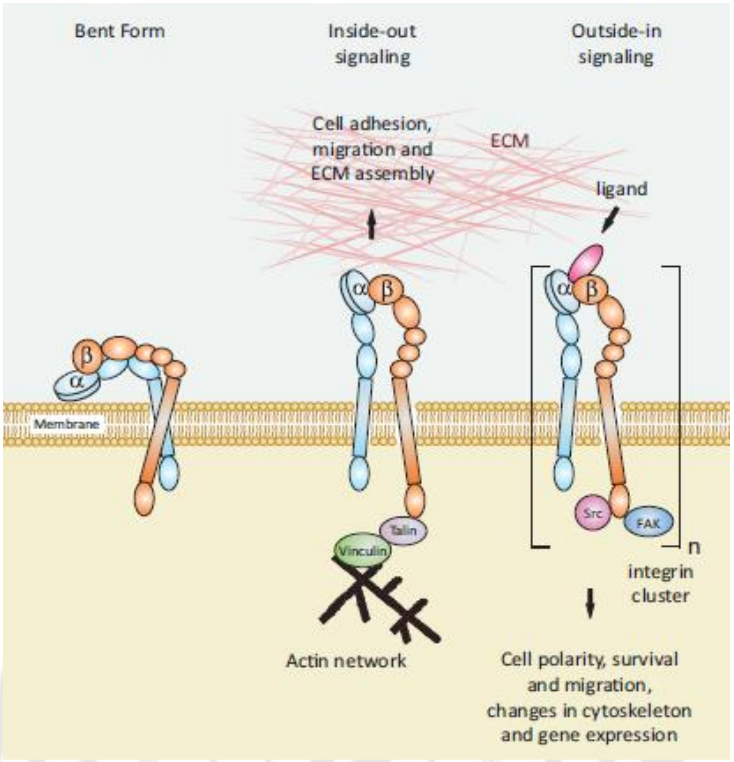


Figure 26 Integrin activation states

Source: Mas-Moruno C, Rechenmacher F, Kessler H. Cilengitide: the first anti-angiogenic small molecule drug candidate design, synthesis and clinical evaluation. Anticancer Agents Med Chem. 2010;10(10):753-68.

Focal adhesion

Upon binding of ECM ligands to the adhesion receptors, a number of signal transduction cascades are activated. Cellular proteins and signaling proteins are recruited to the inner surface of the plasma membrane, where they form structures called focal adhesions (FAs). The proteins in FAs such as vinculin, talin, paxillin, tensin, p130Cas and α-actinin provide strong linkage to the actin cytoskeleton as well as connect the cells tightly to ECM (Figure 27) [91, 92]. Formation of FAs also recruit signaling proteins such as FAK and Src. FAs play a pivotal role in integrin-mediated signaling cascades of cellular processes; proliferation survival and migration [92].

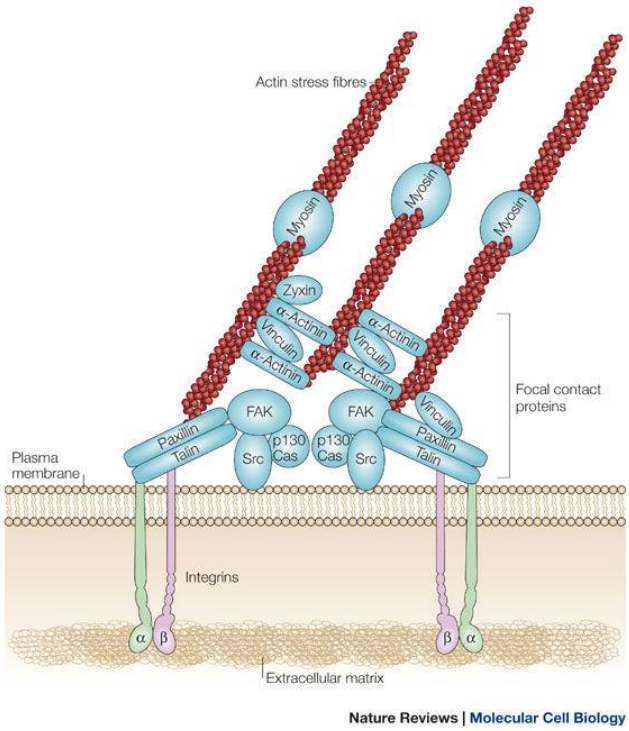


Figure 27 Formation of focal adhesions

Source: Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005;6(1):56-68.

1. Focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase that plays an important role in signal transduction pathway. Overexpression or activation of FAK can be found in metastatic stage of solid tumor. In addition, kinase-dependent and kinase-independent functions of FAK control cell movement, invasion, survival, gene expression and cancer stem cell self-renewal [(93)].

2. FAK activation and phosphorylation

The mechanism that promotes FAK activation involves integrin receptor clustering. The binding of cells to ECM proteins leads to FAK autophosphorylation which Tyr397 is the most prominent phosphorylation. This

activation leads to a formation of FAK-Src complex which initiates multiple downstream signaling pathways [53]. Overexpression of FAK has been shown to associate with invasive and metastatic potential of several human tumors [93, 94]. FAK/Src complex facilitates the binding of p130Cas and Rac activation resulting in lamellipodia formation and cell migration [91]. In addition, FAK interactions with p85 subunit of PI3K, which activated PI3K can be stimulated cell migration via downstream effector Rac [91]. FAK is also induces both the expression of MMP2 and MMP9 for ECM degradation through the Src-Cas-Crk-Dock180 signaling cascade and activation of Rac1 and JNK, which promotes cancer cells invasion [95, 96]. Moreover, FAK could regulate angiogenesis signaling by association with PI3K and increase stimulation of vascular endothelial growth factor receptor-2 (VEGFR2) [97] and pro-angiogenesis growth factor angiopoietin-1 (Ang1) [98].

2.4 Angiogenesis

2.4.1 Angiogenesis in tumor development

Angiogenesis is an essential process for promoting tumor growth and metastasis. It is a process of new blood vessels formation upon activation by growth factors. This process continues even as the tumor matures. Therefore, up-regulation of angiogenesis is an important step in sustained tumor growth and tumor metastasis [99].

2.4.2 Regulation of tumor angiogenesis

Cancer cells produce several angiogenic factors for regulation of angiogenesis. For example, vascular endothelial-derived growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-β), tumor necrosis factor (TNF) and angiopoietins (Angs) [100].

VEGFs are main regulators of vascular development and function of blood and lymphatic vessel. VEGF family members are the most important factors to induce angiogenesis. VEGF family includes VEGF A, B, C, D and placental growth factor (PIGF). The angiogenic effects of VEGF families activate through interaction with

different affinities to VEGF receptor tyrosine kinases (VEGFR), which VEGFR2 has been shown the main signal transducer. The interaction of VEGF with VEGFR2 resulting in biological responses such as cell proliferation, survival, migration and tumors proliferation [101, 102].

2.4.3 VEGF signaling in angiogenesis

VEGF ligands mediate their angiogenic effects by specifically bind to VEGFR, leading to subsequent signal transduction. Biological responses are mediated mainly by VEGFA-activated VEGFR2, through complex network of intracellular signal transduction pathways. Binding of VEGFA to extracellular Ig-like domains 2 and 3 of VEGFR2 promotes receptor dimerization which activates further through tyrosine phosphorylation in the intracellular domain of VEGFR2 and downstream mediators as shown in Figure 28. Major phosphorylation sites in VEGFR2 are Tyr951, Tyr1175 and Tyr1214, which have been proved as crucial sites for migration of endothelial cells in angiogenesis [103]. Phosphorylation of Tyr1175 (pTyr1175) has also been shown to involve with the pathway of cell proliferation by activation the signaling molecules of the RAS/RAF/ERK/MAPK pathway [103-105]. In addition, pTyr1175 could promote cell migration by phosphorylation of cAMP-response-element-binding protein (CREB) and heat shock protein 27 (HSP27) [106-108]. In the process of cell migration, it has also been shown that pTyr1175 induced the binding of adapter proteins SHB (SH2-domain-containing adaptor protein B), which resulting in FAK phosphorylation [109].

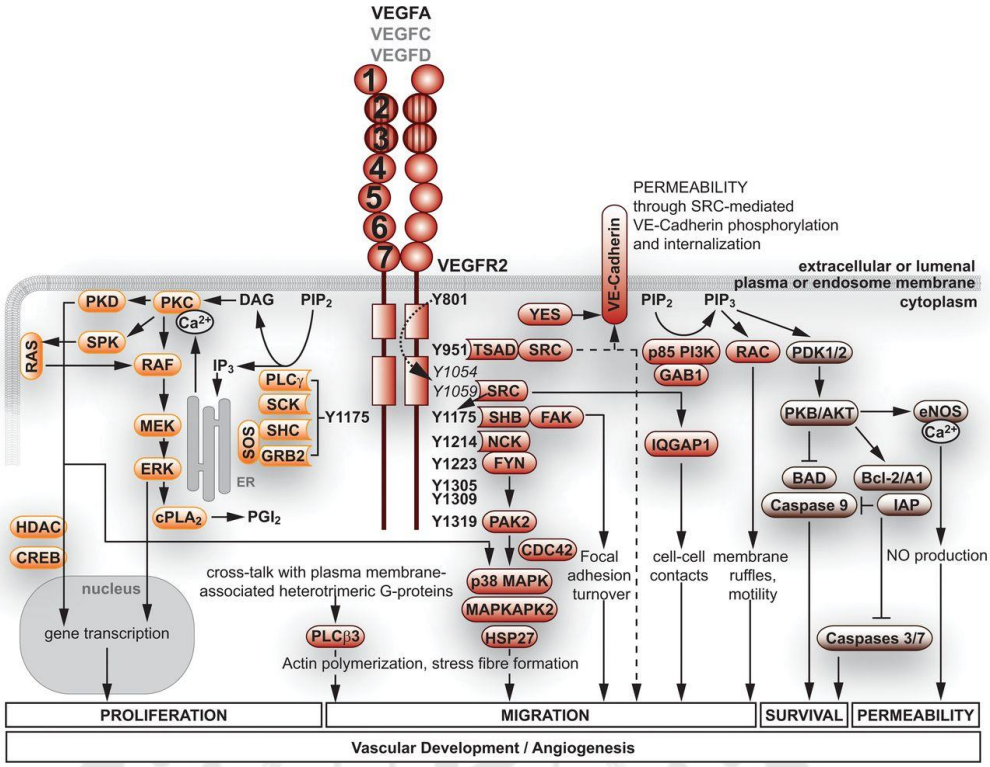


Figure 28 VEGFR2 tyrosine phosphorylation sites and signal transduction

Source: Koch S, Claesson-Welsh L. Signal Transduction by Vascular Endothelial Growth Factor Receptors. Cold Spring Harbor Perspectives in Medicine. 2012;2(7):162-83.

2.5 Hypoxia

2.5.1 Definition of hypoxia

Oxygen is essential for living organisms to produce adequate amounts of ATP that necessary for metabolic activities. Hypoxia or the condition of low oxygen concentration is a common phenomenon that develops in solid tumors due to uncontrolled growth of cancer cells and form large solid tumor masses, resulting in obstruction and compression of the blood vessels surrounding tumor masses. These abnormal blood vessels frequently dysfunction which resulting in poor oxygen supply to the center tumor regions [110-112]. Oxygen level in hypoxic tumor usually defined at ≤ 2% O_2 which normally oxygen concentration (normoxia) is about 21% [113]. Cancer cells

respond differently to oxygen reduction either death or survival depending on the time of oxygen depletion. Short-term hypoxia allows cells to survive by stimulating the cells to autophagy, an apoptotic and metabolic adaptation. Acute hypoxia (exposure to hypoxia in a few minutes or up to 72 hours) was associated with more aggressive tumor patterns by induction of spontaneous metastasis [114-116]. Chronic hypoxia, exposure to hypoxia between a few hours and as long as several weeks [114] is associated with high frequency of DNA fragmentation and accumulation of DNA replication errors. Lack of oxygen leads to the damage of DNA repairing system including homologous recombination and mismatch repair causing the genetic instability and mutagenesis [117-119].

2.5.2 Molecular and mechanisms of hypoxia

The effect of hypoxia mainly involves with hypoxia inducible factor (HIF), which is a heterodimeric protein composed of a constitutively expressed β-subunit and an oxygen regulated α-subunit. It is a transcription factor that plays an important role in mediating the capability to adapt to low oxygen concentrations [120]. There are several member proteins that belongs to HIF family such as HIF-1α, HIF-1β, HIF-2α, HIF-2β etc. However, HIF-1α is mainly response to the level of oxygen [121]. Under normal oxygen condition, HIF-1α is hydroxylated by prolyl hydroxylase domain (PHD). This hydroxylation is recognized by the von Hippel-Lindau (VHL), a part of ubiquitin E3-ligase complex, causing rapid proteosomal degradation of HIF-1α [9, 122]. While in hypoxic conditions, the PHD enzymes lose their activity and the hydroxylation of the HIF-1α subunit could not occur. This makes HIF-1α stabilization and translocates to the nucleus where it dimerizes with constitutively expressed the HIF-1β subunit and forms the active HIF-1. Activation of HIF-1 subsequently leads to the recruitment of transcriptional co-activators. This protein complex binds to consensus hypoxia-responsive elements (HREs) within the promoter regions of target genes and initiates the transcription of a variety of hypoxia-responsive genes (Figure 29) involving tumor

progression such as cell survival/proliferation, metabolism, angiogenesis and invasion/metastasis [123].

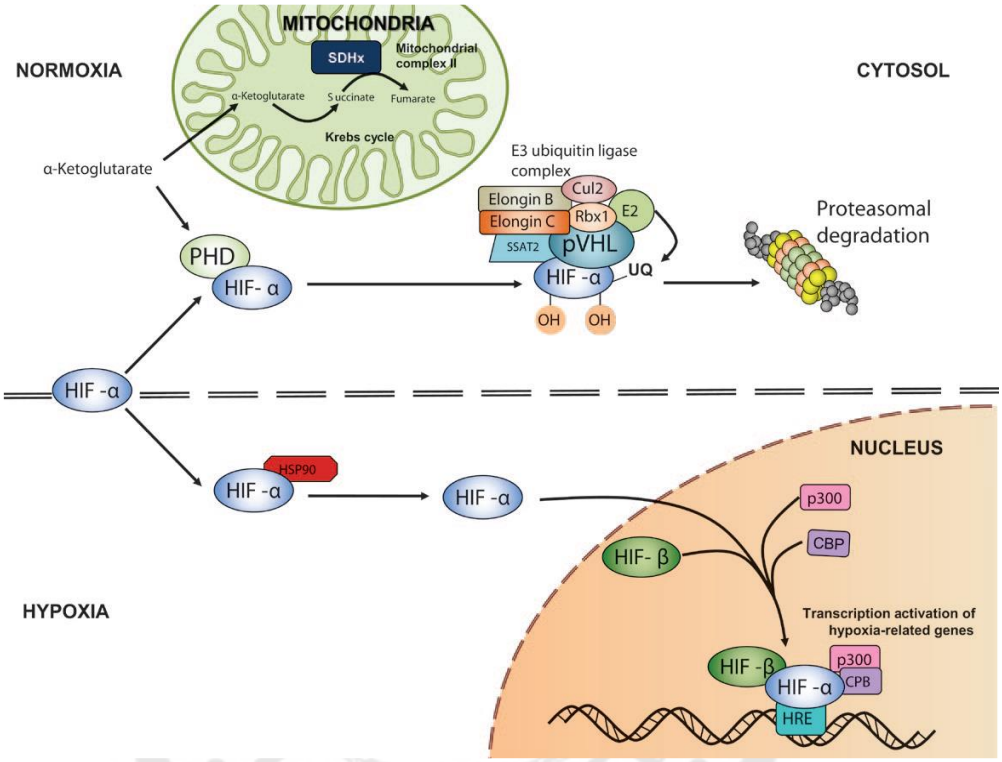


Figure 29 Regulation of signaling molecules under normoxic and hypoxic conditions

Source: Jochmanova I, Yang C, Zhuang Z, Pacak K. Hypoxia-Inducible Factor Signaling in Pheochromocytoma: Turning the Rudder in the Right Direction2013.

As described above, hypoxia condition that occurs because of lack of oxygen within the cell environment. In addition, this condition also could be induce by some chemicals.

Cobolt chloride ($CoCl_2$) is a mimic of hypoxia that can enhance the stability of HIF-1α. $CoCl_2$ could prevent the pVHL, which an ubiquitin-protein ligase is binding to HIF-1α that lead to preventing subsequent ubiquitination and degradation of HIF-1α [124]

(Figure 30) [125]. $CoCl_2$ was used to induce epithelial-mesenchymal transition (EMT) that promote the progression of tumors [126].

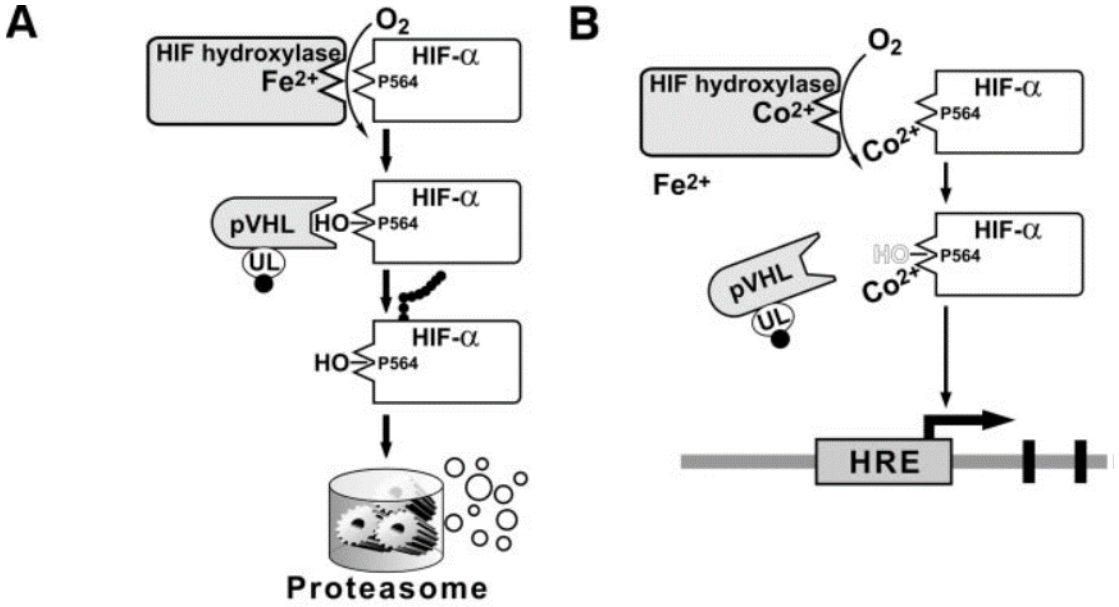


Figure 30 $CoCl_2$ inhibits HIF-1α-pVHL interaction

Source: Yuan Y, Hilliard G, Ferguson T, Millhorn DE. Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. J Biol Chem. 2003;278(18):15911-6.

2.5.3 Signaling pathways related to hypoxia

1. Effects of HIF-1α on cell proliferation and survival

HIF-1α plays a crucial role in cellular proliferation. HIF-1α induce growth factors such as insulin-like growth factor-2 (IGF2) and transforming growth factor-α (TGF-α), which binding of these factors to their receptors as insulin-like growth factor receptor-1 (IGFR1) and epidermal growth-factor receptor (EGFR), respectively, contribute to activates signal transduction pathways resulting in both increased cell proliferation and survival as well as stimulates the expression of HIF-1α itself. These autocrine signaling pathways are essential for cancer progression [127, 128].

2. Effects of HIF-1α on angiogenesis

HIF-1α up-regulates the expression of important molecules that induce angiogenesis include VEGF-R2, members of the FGF family and PDGF, which important in the vascular formation [129]. Additionally, HIF-1α also induce enzymes expression such as MMPs in order to sprout and split the existing blood vessels, at the same time, it induces the endothelial cells proliferation by regulation of VEGF-R1, Ang-1 and Ang-2 expression. Finally, HIF-α support vessel maturation via induction of Ang-1, PDGF and TGF-β to recruit supporting cells such as smooth muscle cells and pericytes creating mature and stable new blood vessels [113, 130].

3. Effects of HIF-1α on metastasis

Hypoxia affects the invasion and migration of cancer cells via epithelial-mesenchymal transition (EMT). During this process, epithelial cell characteristics are reduced by down-regulation of epithelial-associated gene expression such as E-cadherin, β-catenin that involved in adherence cell junctions and up-regulation of mesenchymal-like gene expression such as N-cadherin and vimentin, which facilitates cells to become more motile [5, 8].

4. Effects of HIF-1α on metabolism

The uptake of glucose by metastatic cancer cells is significant when compared to normal cells. Under hypoxic conditions, the cells switch from the tricarboxylic acid (TCA) cycle which is oxygen-dependent metabolic pathway to the oxygen-independent pathway as glycolysis [128]. HIF-1α stimulates glycolysis by induces the expression of pyruvate dehydrogenase kinase (PDK), which inhibits the enzyme pyruvate dehydrogenase via phosphorylation leads to repress mitochondrial function and oxygen consumption. This phenomenon causes a shut-down of the formation of mitochondrial acetyl-CoA and oxidative phosphorylation (OXPHOS) [131]. In these condition, mitochondrial reactive oxygen species (ROS) is reduced which contributes to the formation of lactate. This acidify can be used in the microenvironment leading to an

increasing of invasion and migration [132]. HIF-1α plays a very important role in this metabolic response to hypoxia by up-regulating the expression of glycolytic enzymes such as aldolase A, phosphoglycerate kinase 1 and lactate dehydrogenase to convert glucose into lactate [123]. In addition, HIF-1α also induces the over-expression of glucose transporters 1 and 3 (GLUT1 and GLUT3) that facilitate the uptake of glucose in cells [128].

2.6 Terrein

Terrein ($C_8H_{10}O_3$; MW= 154.06 kDa) is a secondary bioactive fungal metabolite, which was first isolated from the *Aspergillus terreus* by Raistrick and Smith in 1935 [10]. This type of fungus is commonly found in soil, marine and grasslands, as well as contaminants of stored and peanuts as shown in Figure 31. The correct structure and an absolute stereochemistry of terrein was revealed in 1954 by Grove [133] and also by Barton [134]. The chemical structure of terrein contains free hydroxyl groups at positions 4 and 5 of the cyclopentenone ring (Figure 32) [135].

Figure 31 Morphology of *Aspergillus terreus*

Source: https://en.wikipedia.org/wiki/Aspergillus_terreus.

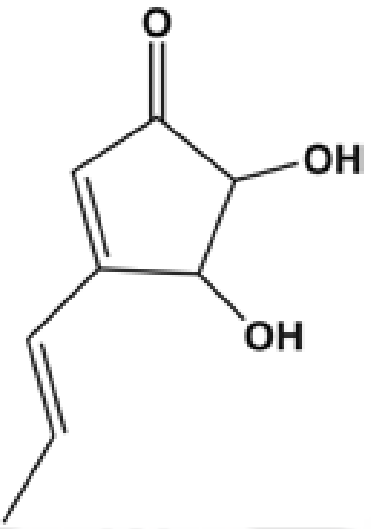


Figure 32 The chemical structure of terrein

Source: Demasi M, Felicio A, Pacheco A, Leite H, Lima C, Andrade L. Studies on terrein as a new class of proteasome inhibitors. J Braz Chem Soc. 2010;21:299-305.

Terrein has a variety in biological activities. The biological effect of terrein was first studied in 2004 by Park et al [(11)] in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab). The result was found that terrein reduced melanin production by stimulating via ERK pathway which causing down-regulation of micropthalmia-associated transcription factor (MITF) that normally controls the expression of tyrosinase. In 2007, Kim et al [(136)] demonstrated the antiproliferative activity of terrein in human skin equivalents (SEs), which can be developed as a medicine for hyperproliferative skin disease such as psoriasis vulgaris or psoriasis. In the year after, Kim et al, was revealed a strong antiproliferative effect of terrein on human epidermal keratinocytes. And, it is exhibited that terrein reduced human keratinocyte proliferation by inhibiting ERK pathway and decreased the expressions of cyclin B1 and Cdc2 complex which resulting in G2/M cell cycle arrest [(137)]. In 2009, Kim et al [(138)] further investigated the long-term hypopigmenting action of terrein in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab). The results suggested that terrein

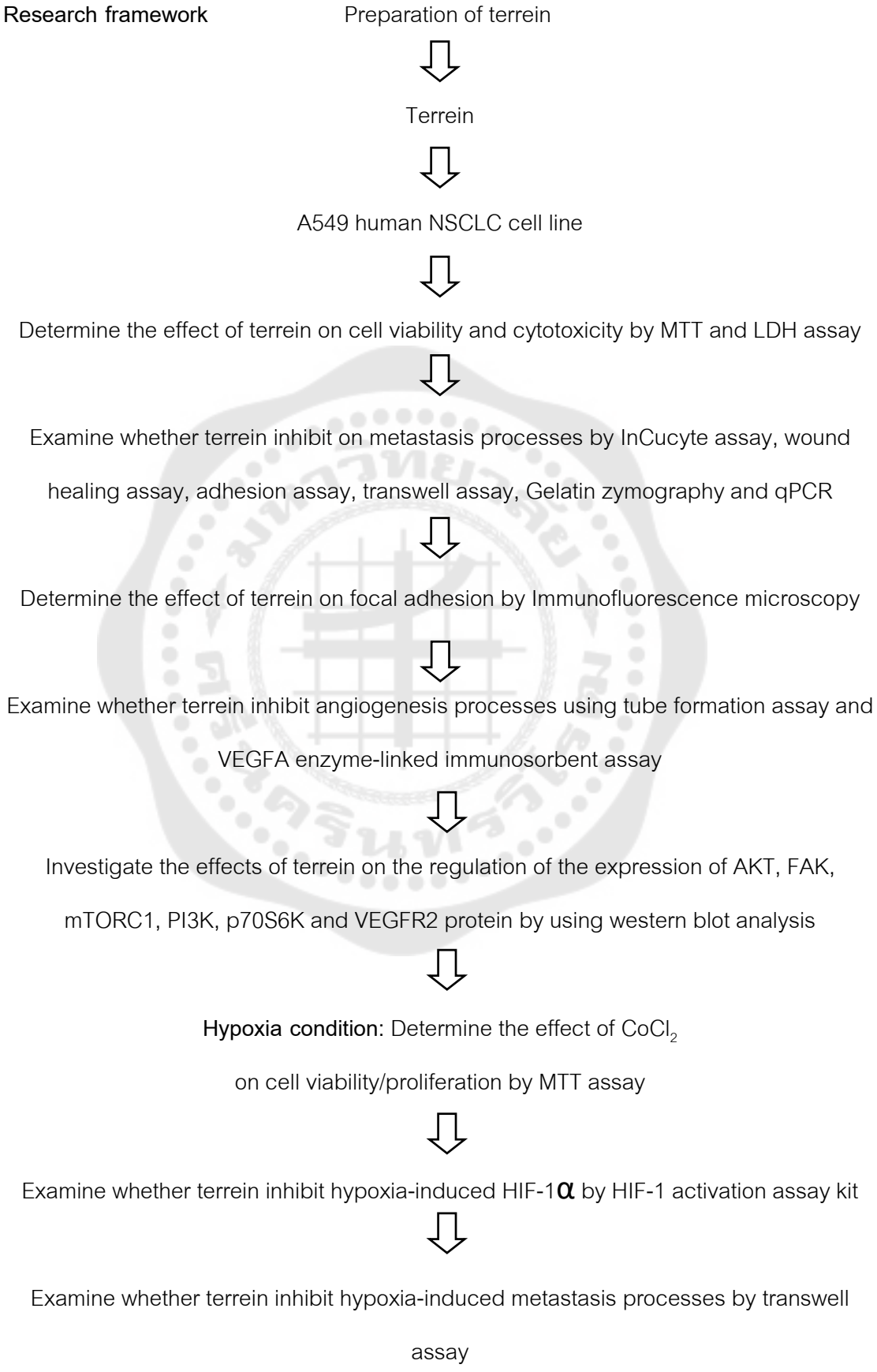
reduced melanogenesis through ubiquitin-dependent proteasomal degradation of tyrosinase as well as via decreased expression of its mRNA.

Anti-inflammatory properties of terrein was studied in 2008 by Lee et al [12], which showing that terrein could down-regulate intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressed in LPS-induce inflammatory of human dental pulp cells. In addition, terrein could suppress NF-kB activation by blocking the activation of Akt. In 2015, Lee et al [139] also showed that terrein act as anti-inflammatory and anti-oxidant through the Nrf2/ERK1/2/HO-1 pathway in aged human diploid fibroblast (HDF) cells.

Apart from anti-inflammatory effect of terrein, several studies have also shown that terrein has the ability of anticancer. Terrein affects cancer by inhibition of angiogenin secretion from androgen-dependent prostate cancer cells (LNCaP-CR) and the tube formation of human umbilical vein endothelial cell (HUVEC). This suggests that terrein is effective agent to act as angiogenesis inhibitors [140]. Additionally, terrein acted as proteasome inhibitors and promoted cell death on pulmonary cancer cell line [135]. The effects of terrein also found as anti-proliferative in ABCG2 breast cancer cell (MCF-7 cells), which the mechanism was via caspase-7 pathway and the inhibition of the Akt signaling pathway [141]. In 2013, there were more discovered by Porameesanaporn et al [13] demonstrated that terrein has anti-cancer effect in human cervical carcinoma cells (HeLa) and the mechanism was via apoptosis induction through p53 and ERK regulation. Chen et al demonstrated the effect of terrein in human ovarian cancer cells, and showing that terrein could inhibit the proliferation of the ovarian cancer cells by suppressing the expression of cyclin B1 and Cdc2 proteins and causing G2/M phase cell cycle arrest. More importantly, terrein has also been shown the ability to inhibit the *LIN28* gene, which is an important gene in ovarian cancer stem cells [142]. In 2015, Zhang et al [14] investigated activity of terrein on human hepatoma (Bel-7402), and the result showed that terrein inhibited Bel-7402 cells proliferation through cell cycle arrest. Terrein could inhibit cell proliferation and angiogenesis of head and neck cancer through suppressing angiogenin production [15]. In 2017, Wu et al [143] reported that

terrein could suppress the proliferation of Eca109 human esophageal cancer cells through the G2/M phase cell cycle arrest induction. At the same year, Chawsoun et al [144] showed that terrein induced breast cancer cell death via oxidative stress induction and antioxidant GSH level reduction. And recently, Kasorn et al demonstrated terrein could inhibit metastasis processes that include adhesion, migration and invasion of human breast cancer cells via Rho and Rac signaling pathways inhibition [145]. From these data, it is suggested that terrein may be a potential agent as having the activity of melanogenesis inhibitor, anti-inflammation and anti-cancer.

Research framework



CHAPTER III
METHODOLOGY

3.1 Materials

Materials used in this study are divided into; (1) chemicals and reagents, (2) antibodies, (3) Equipments and (4) other suppliers.

3.1.1 Chemicals and reagents

1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
2. 4′,6-diamidino-2-phenylindole (DAPI)
3. 4% paraformaldehyde (Thermo fisher)
4. Bovine serum albumin (BSA) (Sigma)
5. Cobalt chloride ($CoCl_2$) (Sigma)
6. Coomassie Brilliant Blue R-250 (Bio-Rad)
7. Crystal violet (Sigma)
8. Dimethyl sulfoxide (DMSO) (Biobasic)
9. Disodium hydrogen phosphate (Na_2HPO_4)
10. Dulbecco's Modified Eagle Medium (GIBCO)
11. ECL plus™ western blotting detection reagents (Bio-Rad)
12. Ethyl acetate (EtOAc)
13. Ethanol (Merck)
14. Fetal bovine serum (FBS) (GIBCO)
15. HIF-1 alpha transcription factor assay kit (Abcam-ab133104)
16. Hydrochloric acid (HCl) (Merck)
17. LDH assay (G-Biosciences, St. Louis, MO)
18. Matrigel matrix (Corning)
19. Methanol (MeOH)
20. Micro cover glasses, round (VWR®)
21. Novex™ Zymogram Developing Buffer (10X) (Invitrogen™)
22. Novex™ Zymogram Renaturing Buffer (10X) (Invitrogen™)

23. Nuclear extraction kit (Abcam-ab113474)
24. Penicillin-Streptomycin (GIBCO)
25. Potassium chloride (KCl) (Merck)
26. Potassium dihydrogen phosphate (KH_2PO_4) (Merck)
27. ProLong Gold Antifade Mountant (Life Technologies)
28. RevertAid Reverse Transcriptase kit (Thermo fisher)
29. Rhodamine phalloidin (Life Technologies)
30. RNeasy Mini Kit following manufacturer's instructions (QIAGEN)
31. Sephadex LH-20 (GE Healthcare)
32. Sodium carbonate (Na_2CO_3) (Merck)
33. Sodium chloride (NaCl) (Merck)
34. Sodium hydroxide (NaOH) (Merck)
35. Triton X-100
36. Trypsin (GIBCO)
37. VECTASHIELD® Mounting Medium with DAPI (Vector Lab)
38. VEGF human enzyme-linked immunosorbent assay kit (Abcam-ab100662)

3.1.2 Antibodies

1. Akt Antibody (Cell Signaling Technology)
2. Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Life Technologies)
3. Anti-mouse IgG, HRP-linked Antibody (Cell Signaling Technology)
4. Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology)
5. GAPDH Antibody (Cell Signaling Technology)
6. Integrin αM Antibody (Thermo fisher)
7. Phospho-Akt (Ser473) Antibody (Cell Signaling Technology)
8. Phospho-Akt (Thr308) Antibody (Cell Signaling Technology)
9. Phospho-FAK (Tyr397) Polyclonal Antibody (44-624G) (Invitrogen™)
10. Phospho-mTOR (Ser2448) Antibody (Cell Signaling Technology)

11. Phospho-p70 S6 Kinase (Thr389) Antibody (Cell Signaling Technology)

12. Phospho-PI3K p85

13. Phospho-VEGFR2 (Tyr1175) Antibody (Cell Signaling Technology)

14. PI3K Antibody (Cell Signaling Technology)

15. VEGFR2 Antibody (Cell Signaling Technology)

3.1.3 Equipments

1. Bench top centrifuge (Z32HK, Hermle)

2. Chromo4™ Detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA)

3. CO_2 incubator (Autoflow 4750, NuAire)

4. Column chromatography

5. Confocal microscope (Nikon)

6. Evaporator

7. Gel documentation (UVITEC, Cambridge)

8. Hemacytometer (Boeco)

9. IncuCyte S3 (Essen Bioscience)

10. Inverted microscope (OLYMPUS CKX41)

11. Microscope slides (VWR®)

12. Multi-Mode Microplate Reader (Synergy HT, BioTek)

13. NanoDrop spectrophotometers (Thermo Scientific)

14. pH meter (Thermo Scientific)

15. Spectronic GENESYS 10 UV Spectrophotometer (Thermo Scientific)

16. Vacuum

17. Vortex mixer (Genie 2, Scientific Industries)

3.1.3 Other supplies

1. 1000 mL collection flask

2. 1000 mL round bottom flask

3. 1000 mL separatory funnel
4. 100 mL round bottom flask
5. 1.5 mL microcentrifuge tube (Bioline)
6. 15 mL centrifuge tube (Corning)
7. 16x150 mm test tube
8. 2000 mL flask
9. 24-well culture plate (Nunc)
10. 25 cm cell culture flask (Nunc)
11. 3000 mL flask
12. 4000 mL flask
13. 50 mL round bottom flask
14. 6-well culture plate (Nunc)
15. 75 cm cell culture flask (Nunc)
16. 8-well culture slides (Falcon)
17. 96-well culture plate (Nunc)
18. Amicon Ultra-0.5 Centrifugal Filters 3K (Merck)
19. Amicon Ultra Centrifugal Filters 10K (Merck)
20. Bradford protein assay (Bio-Rad)
21. Cover slips
22. Pasteur pipettes
23. Transwell inserts 0.8 μm pore size (Falcon)

3.2 Methods

3.2.1 Preparation of terrein

3.2.1.1 Aspergillus terreus culture

The fungus *Aspergillus terreus* CRI301 was cultivated with 200 ml sabouraud dextrose agar (30 grams SDB, 1 liter sea water-filtered) in Erlenmeyer flask - Pyrex 2L incubated at room temperature for 34 days.

3.2.1.2 Aspergillus terreus extraction

The fermentation broth was added with ethyl acetate (EtOAc) as a solvent. After hard shaker, the fermentation broth was filtrated in cotton wool to remove the mycelia. The supernatant was extracted three times with an equal volume of EtOAc in order to separate more of the substance. The extract (EtOAc layer) was concentrated in evaporator and then in vacuum to produce solid material.

3.2.1.3 Purification

The EtOAc extract was fractionated and purified by using a Sephadex LH-20 (2 cm inner diameter and 125 cm long), and eluted with methanol (MeOH). Each fraction was further separated with thin-layer chromatography (TLC) and the same components of the dye mixture running at same rates were collected. This crude extract was concentrated in evaporator and then in vacuum in order to obtain the pure compound. Purity evaluation and characteristics of terrein compound were detected with NMR Spectrometer.

3.2.2 Cell culture

The A549 human NSCLC cell line was purchased from ATCC (ATCC® CCL-185™). The cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin and incubated in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidified atmosphere. Cell concentration was maintained culture at an initial

density between 6 X $10^3$and 6 X 10^4 cells/cm^2 in culture flask. Culture medium was changed 2 to 3 times per week.

African green monkey kidney (Vero) cell line was purchased from ATCC. The vero cell line is epithelial cells, which was extracted from the kidney of a normal adult African green monkey. The cell line was cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin and incubated in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidified atmosphere. Culture medium was changed 2 to 3 times per week.

L6 skeletal muscle cell line was obtained from Sweeney lab. The L6 cell line is myoblast cells, which was derived from rat skeletal muscle. The cell line was cultured in AMEM containing 10% FBS, 1% Antibiotic-Antimycotic and incubated in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidified atmosphere. Culture medium was changed 2 to 3 times per week.

H9C2 cardiomyoblast cell was obtained from Sweeney lab. The H9C2 cell line is cardiomyoblast cells, which was derived from rat heart tissue. The cell line was cultured in DMEM containing 10% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin and incubated in a CO_2 incubator at 37°C with 5% CO_2 and 95% humidified atmosphere. Culture medium was changed 2 to 3 times per week.

3.2.3 MTT assay for cell viability

Cytotoxicity of terrein against lung cancer cells, A549 cells and normal cell line include Vero cells, L6 cells and H9C2 cells were determined by using a colorimetric MTT assay as previously described [5]. A549 cells, Vero cells, L6 cells and H9C2 cells were harvested by 0.25% trypsin containing 1 mM EDTA and plated on 96-well plate at a density of 1 × 10^4, 1.8 × 10^4, 3 × 10^4, 2 × 10^4 cells/well, respectively and allowed overnight to attach. Then the cells were treated with various concentrations of terrein at 0.1% dimethyl sulphoxide (DMSO) highest concentration and then incubated for 24 hours at 37°C. After that, the medium was removed and MTT solution at 0.5 mg/ml was added each well. The reaction was incubated further for 4 hours and the supernatant

was discarded after incubation. Then, the formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple formazan was determined by using ELISA microplate reader the absorbance at 595 nm. All measurements were carried out in triplicate. Statistical analysis was performed by one way ANOVA test.

3.2.4 Cell proliferation assay

IncuCyte proliferation assay was used to determine the effect of terrein on human lung cancer cell proliferation as previously described [146]. Real-time live-cell imaging was conducted using IncuCyte S3 (Essen Bioscience). A549 cells were seeded on 96-well plate at a density of 4×10^3 cells/well and allowed overnight to attach. Then the cells were treated with various concentrations of terrein and the plates were placed in the IncuCyte for imaging every 3 hours for 3 days. Proliferation curves were generated using Incucyte proliferation analysis with confluences as the parameter.

3.2.5 Lactate dehydrogenase (LDH) enzyme assay

The cytotoxicity effect of terrein on A549 cells using LDH assay that was conducted using a kit following manufacturer's instructions (G-Biosciences, St. Louis, MO). Briefly, the cells were treated with various concentrations of terrein for 24 hours. Then 25 µl of supernatant each sample was transferred to new 96-well plate and added 25 µl reaction mixture to each well. After incubate at 37°C for 30 minutes, 25 µl stop reaction was added to each well and measured the absorbance at 490 nm. All measurements were carried out in triplicate. Statistical analysis was performed by one way ANOVA test.

3.2.6 Wound healing assay

The effect of terrein on A549 cell migration was determined by monolayer wound healing assay as previously described [147]. A549 cells were harvested by 0.25% trypsin containing 1 mM EDTA and plated on 6-well plate at a density of 5×10^5 cells/well in DMEM media containing 2% FBS and allowed to attach overnight. After forming a confluent monolayer, the cells were scratched with 200 µl sterile pipette tip

from side to side of 6-well culture plate. Subsequently, the cells were washed three times with DMEM media to remove cell debris. Then, the cells were immediately replaced with 0, 20, 40 and 80 μM (0, 6.25, 12.5 and 25 IC_{50} respectively) of terrein in DMEM media containing 10% FBS in each well. Cell migration was monitored and imaged under inverted microscope (OLYMPUS CKX41) for 0, 6, 12 and 24 hours. The distance of wound (compared with control at 0 hour) was measured in three-independent wound sites per group. Relative cell motility was calculated as the wound width at 6, 12 and 24 hours.

3.2.7 Transwell migration assay

The effect of terrein on A549 cell migration was performed as previously described method [5, 148]. A549 at 5×10^4 cells were plated in 200 μl serum-free medium with 0, 20, 40 and 80 μM of terrein in the upper chamber of a non-coated transwell insert. For the lower chamber, 750 μl of medium containing 10% fetal bovine serum was used as a chemoattractant to encourage cell migration. Then, the plate was incubated at 37°C for 24 hours. After that, transwell insert was separated from 24-well plate and then washed twice times with cold phosphate-buffered saline (PBS). The cells were fixed with 100% ice-cold methanol for 20 minutes following with twice times washed in cold PBS. The cells were stained with 0.5 % crystal violet for 15 minutes and then washed with distilled water (dH_2O) for several times until dye stop coming off. The cells that do not migrate at the inside of upper chamber were removed using a cotton swabs. The cells that migrate through the pore of insert chamber into the lower chamber was photographed under an inverted microscope and quantifying from at least five randomly selected fields by Image J software (version 1.47, National Institutes of Health (NIH), Bethesda, MD, USA). The percentage of cell migration was compared to the control group which represents 100%.

3.2.8 Transwell invasion assay

The effect of terrein on A549 cell invasion was performed by following the method as previously described [5, 148]. The bottom of each upper chambers were coated

with 50 µl of 2.0 mg/mL Matrigel and incubated at 37°C for 2 hours. After that, the matrigel was removed and 5×10^4 A549 cells were further plated in 200 µl of serum-free medium with 0, 20, 40 and 80 µM of terrein. In the lower chamber, 750 µl of medium containing 10% fetal bovine serum was used as a chemoattractant to encourage cell invasion. The plate was incubated at 37°C for 24 hours. Transwell insert was separated from 24-well plate and then washed twice times with cold PBS. The cells were fixed with 100% ice-cold methanol for 20 minutes after that washed twice times with cold PBS. The cells were stained with 0.5% crystal violet for 15 minutes and then washed with dH_2O for several times until dye stop coming off. The cells that do not invade at the inside of upper chamber were removed using a cotton swabs. The cells that invade through the pore of insert chamber into the lower chamber were photographed under an inverted microscope and quantifying from at least five randomly selected fields by Image J software. The percentage of cell invasion was compared to the control group which represents 100%.

3.2.9 Adhesion assay

The effect of terrein on A549 cell adhesion was performed by following the method as previously described [149]. The 96-well plate was coated with 50 µl of 2.0 mg/mL matrigel and incubated at 37°C for 2 hours. After that, the matrigel was removed and cells at density of 3×10^4 were plated each well in 100 µl of medium containing 10% FBS with 0, 20, 40 and 80 µM of terrein. After incubation for 30 minutes at 37°C, non-adherent cells were removed by washing four times with 50 µl of PBS. Then, the MTT dye was added at 0.5 mg/ml to each well and incubated for 4 hours. After removing the supernatant, the formazan crystals in each well were dissolved in 100 µl of DMSO. The amount of purple formazan was determined by measuring the optical density using ELISA microplate reader at 595 nm.

3.2.10 Gelatin zymography

Gelatin zymography assay was used to examine the effect of terrein on proteolytic enzymes activity that released from human lung cancer cells as previously described [150]. A549 cells were seeded on 6-well plate at a density of 8×10^5 cells/well in DMEM media containing 10% FBS overnight. The cells were treated with 0, 20, 40 and 80 μM of terrein and incubated at 37°C for 24 hours. After treatment, the supernatant was collected and concentrated by using Amicon Ultra-0.5 Centrifugal Filters 3K. The protein concentration was measured by using Bradford protein assay. Bovine serum albumin (BSA) was used as standard protein sample. After calculation of protein concentration, each sample was adjusted to having the same concentration.

The protein samples were separated by 10% SDS-polyacrylamide gels containing 0.73 mg/ml gelatin. After electrophoresis, the gels were washed with 1X renaturing buffer (Novex™ Zymogram Renaturing Buffer (10X) Invitrogen™) for 1 hour at room temperature with gentle agitation on a bench rocker. The gels were washed 4 times with ddH_2O for 20 minutes each and then incubated with 1X developing buffer (Novex™ Zymogram Developing Buffer (10X) Invitrogen™) for 30 minutes at room temperature with gentle agitation on a bench rocker. After that gels were incubated with new 1X developing buffer at 37°C for overnight. Subsequently, the gels were stained with staining solution (0.5% Coomassie Brilliant Blue R-250 in methanol:acetic acid:water, 4:1:5, v/v/v), and washed with destaining solution. Finally, the bands indicating MMP-2 (68 kDa) and MMP-9 (82 kDa) were clearly visible in contrast with the blue background and detected by a DNR Bio-Imaging Systems MiniBIS Pro (Bio Sciences). Intensity of bands were quantitated by Image J software. The MMP-2 and MMP-9 activity was expressed as a percentage relative to the control group.

3.2.11 Quantitative real-time PCR

A549 cells were seeded on 6-well plate at a density of 4×10^5 cells/well in DMEM media containing 10% FBS overnight. The cells were treated with 0, 20, 40 and 80 μM of terrein and incubated at 37°C for 24 hours. Total RNA was isolated from cells using RNeasy Mini Kit following manufacturer's instructions (QIAGEN) and measured

RNA concentration using NanoDrop spectrophotometers (Thermo Scientific). First-strand cDNA was synthesized from 400 ng/µl RNA using the Thermo Scientific RevertAid Reverse Transcriptase kit (Thermo fisher). Quantitative real-time PCR was conducted using a Chromo4™ Detection system (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, CA) according to cycling conditions outlined by the PCR array manufacturer. All genes were detected listed in the table 2 and analyzed through real-time PCR using the following cycling conditions: 95°C/2 minutes, followed by 40 cycles of [95°C/15 second, 61°C/15 second, 72°C/1 minute], then 72°C/2 minutes. Melting curve analysis was used to ensure primer specificity. Data were analyzed using Optical Monitor 3 software and normalized to GAPDH mRNA expression.

Table 2 Sequence-specific primers for RT-PCR

Gene name		Primer sequence	Product size
MMP2	Forward (5' > 3')	CTCATCGCAGATGCCTGGAA	104
	Reverse (5' > 3')	TTCAGGTAATAGGCACCCTTGAAGA	
MMP9	Forward (5' > 3')	ATCCGGCACCTCTATGGTC	121
	Reverse (5' > 3')	CTGAGGGGTGGACAGTGG	
MT1-MMP	Forward (5' > 3')	AAGAGGAGAAGAGCAAACAG	91
	Reverse (5' > 3')	CGGTAGGCACTGAACTTG	
GAPDH	Forward (5' > 3')	ATCTTCTTTTGCGTCGCCAG	51
	Reverse (5' > 3')	TTCCCCATGGTGTCTGAGC	

3.2.12 VEGF determination

To examine whether terrein could inhibit angiogenesis processes, the function of VEGF was evaluated by using the VEGF human enzyme-linked immunosorbent assay kit (Abcam-ab100662). Briefly, the cells were treated with 0, 20, 40 and 80 μM of terrein for 24 hours. The supernatant was collected and concentrated by using Amicon Ultra-4 Centrifugal Filters 10K. The protein concentration was measured by using Bradford protein assay. BSA was used as standard protein sample.

After calculation of protein concentration, each sample was adjusted to having the same concentration.

For the assay plate, an antibody specific for human VEGF was coated prior to adding with the standards and protein samples and then incubation at 4°C overnight with gentle shaking. The solution was discarded and washed four times with 1X wash solution, then biotinylated anti-human VEGF antibody was added to each well and incubated for 1 hour at room temperature. After that unbound biotinylated antibody was washed, HRP conjugated streptavidin was added to each well and incubated for 45 minutes at room temperature. Then, the TMB substrate solution was added and incubated for 30 minutes at room temperature in the dark room. The color was developed in proportion to the bound VEGF in samples. The stop solution was added to each well that color is changed from blue to yellow. The intensity of the color was measured at 450 nm immediately.

3.2.13 Tube formation assay

The tube formation assay is a rapid and quantitative method for examination of cell differentiation and modulations that involved in angiogenesis processes. The *In vitro* capillary-like tube formation assay was performed by following the method as previously described [151]. The 24-well plate was coated with 10 mg/ml of matrigel and incubated at 37°C for 1 hour. The remaining liquid was carefully removed from the culture plate without disturbing the layer of matrigel matrix. Then, A549 cells 5×10^4 cells in 1 ml of DMEM media containing 2% FBS were plated each well. After that, terrein at 0, 20, 40 and 80 μM were added and incubated at 37°C for 24 hours. Tube formation was photographed by inverted microscope (OLYMPUS CKX41) and quantifying the tubular structures in five randomly selected fields using Angiogenesis Analyzer plugin for ImageJ software [152]. The percentage of tube length was compared to the control group.

3.2.14 Western blot analysis

A549 cells were seeded on 60-mm culture dished at a density of 8 × 10^5 cells/dishes in DMEM media containing 10% FBS overnight. The cells were treated with 0, 20, 40 and 80 μM of terrein and incubated at 37°C for 24 hours. After treatment, cells were lysed with RIPA buffer (5 ml of 1M tris-HCl PH 7.4, 30 ml of 5M NaCl, 5 ml of 20% NP-40, 5 ml of 10% sodium deoxycholate, 0.5 ml of 20% SDS, 50 mL of dH_2O and protease inhibitor cocktail) on ice. Subsequently, cell lysates were centrifuged at 15,000 *g* at 4°C for 10 minutes. Protein concentration was measured by using Bradford protein assay. The protein extracts (40 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were blocked with 5% BSA in TBS-T buffer (5% w/v BSA, 1X Tris-buffered saline solution and 0.1%Tween-20) at room temperature for 1 hour and then probed with primary antibody against Integrin αM, Akt, p-Akt (Ser473), p-Akt (Thr308), FAK, p-FAK (Tyr397), p-mTORC1 (Ser2448), pP70 S6 Kinase (Thr389), PI3K p85, VEGFR2, p-VEGFR2 (Tyr1175), and GAPDH and incubated at 4°C overnight. After washing three times with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody for 1 hour. The membranes were visualized by enhanced chemiluminescence using ECL plus™ western blotting detection reagents and recorded on Gel documentation (UVITEC, Cambridge). Band density was quantified by Image J software.

3.2.15 Immunofluorescence microscopy

The effect of terrein on pFAK and actin cytoskeleton colocalization in A549 cells using immunofluorescence and confocal microscope by Nikon. A549 cells were seeded on micro cover glasses, round (VWR®) in 24-well plate at a density of 3 × 10^3 cells/well in DMEM media containing 10% FBS overnight. Cells were treated with terrein at 20, 40 and 80 μM for 24 hours. Cells were washed with PBS^{++} (calcium and magnesium) three times and fixed in 4% paraformaldehyde for 20 min at room temperature. After wash with PBS^{++} three times, cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature and then permeabilized cells were washed

three times with PBS^{++}. Cells were blocked with blocking buffer (3% BSA in PBS^{++}) for 1 hour at room temperature and then sequentially incubated with pFAK (Tyr397) polyclonal antibody (44-624G) (Invitrogen™) (1:200) for overnight. Then cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:800) (Life Technologies) and rhodamine phalloidin (1:320) (Life Technologies) for 1 hour at room temperature. Stained cells were washed three time with in PBS^{++} and then cells on glass coverslips were mounted onto microscope slides (VWR®) in ProLong Gold Antifade Mountant (Life Technologies) and VECTASHIELD® Mounting Medium with DAPI (Vector Lab) (1:1). Stained cells were observed with a Nikon A1R confocal laser scanning microscope system (Nikon Corp., Tokyo, Japan) assigned with a X60 objective.

3.2.16 Hypoxia condition

3.2.16.1 Cytotoxicity of cobalt chloride

A549 cells were harvested by 0.25% trypsin containing 1 mM EDTA and plated on 96-well plate at a density of 1×10^4 cells/well and allowed overnight to attach. Then the cells were treated with various concentrations of cobalt chloride ($CoCl_2$) and then incubated for 24 hours at 37°C. The medium was removed. Then the MTT dye was added at 0.5 mg/ml each well. After incubation for 4 hours, the supernatant was discarded. The formazan crystals in each well was dissolved in 100 µl of DMSO. The amount of purple formazan was determined by measuring the optical density using ELISA microplate reader at 595 nm.

3.2.16.2 HIF-1 alpha transcription factor assay

A549 cells were seeded overnight on 60-mm culture dished at a density of 8×10^5 cells/dishes in DMEM media containing 10% FBS. The cells were induced hypoxia with $CoCl_2$ for 24 hours and further treated with terrein at 0, 20, 40 and 80 µM at 37°C for 24 hours. Then, after removing the growth medium and washing, the cells were harvested and cellular nuclear protein was extracted by nuclear extraction kit (Abcam-ab113474). To further analyze the level of HIF-1 alpha, the HIF-1 alpha transcription

factor assay kit (Abcam-ab133104) was used. By following the instruction, nuclear extract was added into each well and incubated overnight at 4°C without agitation. Unbound reagent was washed five times with 1X wash buffer and after that HIF-1α primary antibody was added and incubated for 1 hour at room temperature without agitation. After washing unbound antibody, transcription factor goat anti-rabbit HRP conjugate was added and incubated for 1 hour at room temperature without agitation. The reaction was washed and then transcription factor developing solution was added to each well and incubated for 15-45 minutes at room temperature with gentle agitation and protection from light. The stop solution was added to each well that color is changed from blue to yellow. The intensity of the color was measured at 450 nm within 5 minutes of adding the stop solution.

3.2.16.3 Transwell migration assay

In hypoxia condition, A549 cells were seeded overnight on 60-mm culture dished at a density of 8×10^5 cells/dishes in DMEM media containing 10% FBS. The cells were induced hypoxia with adding $CoCl_2$ for 24 hours. Then, the cells were harvested and 5×10^4 cells in 200 µl serum-free medium with or without 0, 20, 40 and 80 µM of terrein was plated in the upper chamber of a non-coated transwell insert. In the lower chamber, 750 µl medium containing 10% fetal bovine serum was used as a chemoattractant to encourage cell migration. The plate was incubated at 37°C for 24 hours. Transwell insert was separated from 24-well plate and washed twice with cold PBS. Each samples were fixed with 100% ice-cold methanol for 20 minutes and washed twice with cold PBS. Then, the samples were stained with 0.5% crystal violet for 15 minutes and washed with dH_2O for several times until dye stop coming off. The cells that do not migrate of upper chamber were removed using a cotton swabs. The cells that migrate through the pore of insert chamber into the lower chamber were photographed under an inverted microscope and quantifying from at least five randomly selected fields by Image J software. The percentage of cell migration was compared to the control group which represents 100%.

3.2.16.4 Transwell invasion assay

In hypoxia condition, A549 cells were seeded overnight on 60-mm culture dishes at a density of 8 × 10^5 cells/dishes in DMEM media containing 10% FBS. The cells were induced hypoxia with $CoCl_2$ for 24 hours. Then, the cells were harvested by 0.25% trypsin containing 1 mM EDTA. For the bottom of each upper chamber, it was coated with 50 µl of 2.0 mg/mL of matrigel and incubated at 37°C. After 2 hours, the uncoated matrigel was removed. Then, A549 at 5×10^4 cells in 200 µl serum-free medium with 0, 20, 40 and 80 µM of terrein were plated on this coated upper chamber. In the lower chamber, 750 µl medium containing 10% fetal bovine serum was used as a chemoattractant to encourage cell invasion. The plate was incubated at 37°C for 24 hours. Transwell insert was separated from 24-well plate and then washed twice with cold PBS. The cells were fixed with 100% ice-cold methanol for 20 minutes after that washed twice again with cold PBS. The cells were stained with 0.5% crystal violet for 15 minutes and then washed with dH_2O for several times until dye stop coming off. The cells that do not invade were removed using a cotton swabs. The cells that invade through the pore of insert chamber into the lower chamber were photographed under an inverted microscope and quantifying from at least five randomly selected fields by Image J software. The percentage of cell invasion was compared to the control group which represents 100%.

3.2.17 Statistical analysis

All experiments were performed with at least triplicates in each group. The data were shown in means ± SEM and analyzed by GraphPad Prism version 5.01 software. Statistical significance was calculated using ANOVA with Dunnett's multiple comparision posthoc test. At $p < 0.05$ was considered statistically significant.

CHAPTER IV
FINDINGS

4.1 Characteristics of terrein

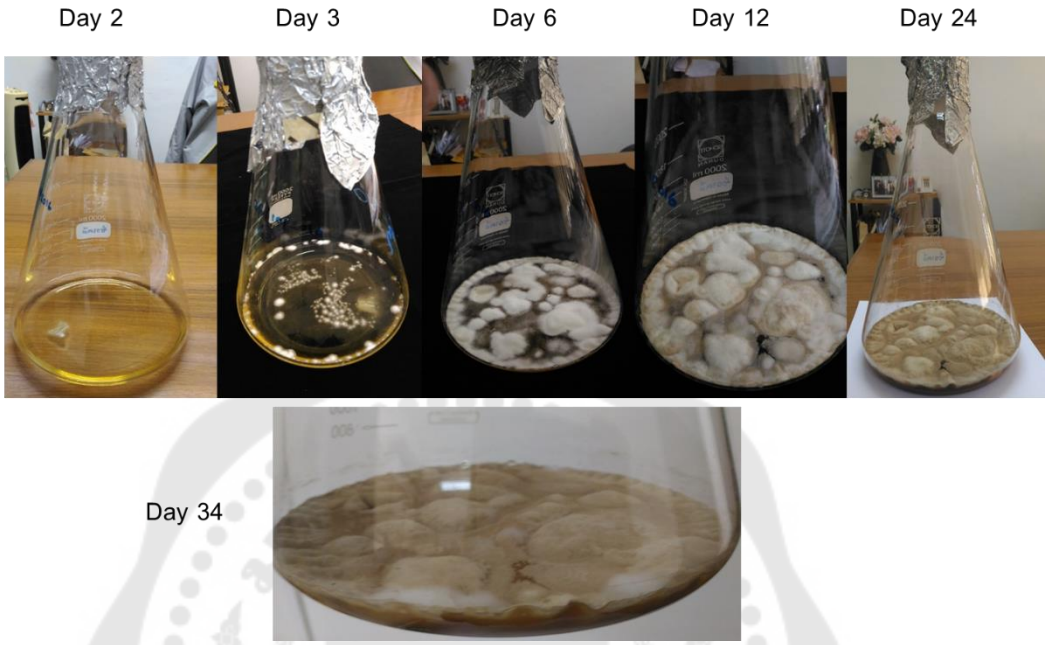
The fungus *Aspergillus terreus* CRI301 was cultivated in sabouraud dextrose agar under stationary condition at room temperature for 34 days. As shown in Figure 33A, the increased number of fungi was correlated to the days of cultivation. The culture (3.6 Litters) of *Aspergillus terreus* CRI301 was filtered to separate cells and broth. The culture broth was extracted three times with an equal volume of EtOAc. The EtOAc layers were combined and evaporated to dryness, yielding 2.535 grams of a crude extract. The crude EtOAc extract was purified by a Sephadex LH-20 column chromatography that eluted with MeOH to afford 30 fractions (Figure 33B). On the basis of their TLC characteristics, similar fractions were combined to afford similar fractions were combined to afford 10 fractions (Figure 33C-E).

Fraction 4 was obtained as pale yellow powder which was crystallized from dichloromethane (CH_2Cl_2) to obtain terrein (258.7 mg) as white needles.

The structure of terrein was characterized by ^{1}H NMR spectroscopic data, and finally confirmed by literature data comparison (Figure 33F).

Terrein was obtained as a white needles. The ^{1}H NMR spectrum of terrein (Figure 33G) exhibited three methylene protons at δ_H 6.83 (1H, *m*, H-2'), 6.44 (1H, *d*, *J* = 15.9 Hz, H-1') and 5.97 (1H, *s*, H-2), two oxygenated methine protons at δ_H 4.73 (1H, s, H-4) and 4.08 (1H, s, H-5) and a methyl group at δ_H 1.91 (3H, *d*, *J* = 15.9 Hz, H-3') as shown in Table 3.

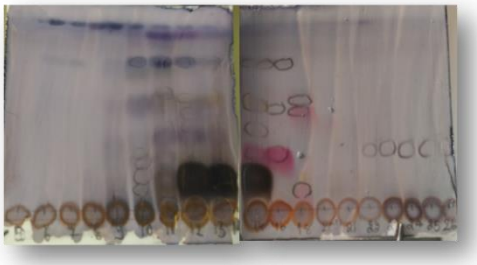
A



B

C

D



E

G

F

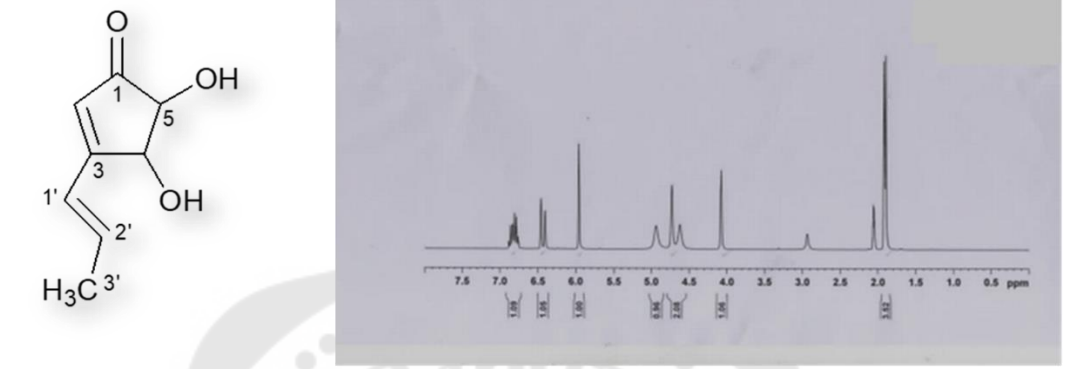


Figure 33 Characteristics of terrein. (A) *Aspergillus terreus* was cultured for 34 days. (B) Separation of pure compound from Sephadex LH-20 column chromatography. (C) All fraction was collected. (D, E) Thin layer chromatography (TLC). (F) The structure of terrein was characterized by ^{1}H NMR spectroscopic data. (G) The 1H NMR spectrum of terrein.

Table 3 1H NMR (300 MHz) data of terrein in Acetone-d_6

Position	Terrein	Terrein[153]
	δ_H (ppm)	δ_H (ppm)
1	-	-
2	5.97 (s)	5.97 (br s)
3	-	-
4	4.73 (1H, br *s*)	4.89 (1H, *d*)
5	4.08 (1H, br *s*)	4.28 (1H, *d*)
1’	6.44 (1H, *d*, *J* = 15.9 Hz)	6.40 (1H, *d*, *J* = 15.8 Hz)
2’	6.83 (1H, *m*)	6.84 (1H, *m*)
3’	1.91 (3H, *d*, *J* = 15.9 Hz)	1.96 (3H, *d*, *J* = 15.9 Hz)

4.2 The effects of terrein on cell viability of different cell lines

First, we examined the cytotoxic effect of terrein on different cell lines include A549 lung cancer cells, African green monkey kidney (Vero) cells, L6 skeletal muscle cells and H9C2 cardiomyoblast cells using a MTT assay. All cell lines were treated with various concentrations of terrein for 24 hours. The maximum final concentration of DMSO was 0.1%. The data demonstrated that terrein significantly inhibited cell viability of A549 cells, Vero cells, L6 cells and H9C2 cells with an IC_{50} at 229 µM, 870 µM, 1,240 µM and 579 µM, respectively as shown in Figure 34.

Selective index (SI) values of terrein on A549 cells were compared with normal cell lines as shown in Table 4. SI was determined according to the following equation: SI= IC_{50} of normal cells/ IC_{50} of A549 cells. SI values of terrein were 3.8, 5.4 and 2.5 for A549 cells in comparison to Vero cells, L6 cells and H9C2 cells, respectively, indicating high cytotoxic selectivity for terrein against cancer cells versus normal cells.

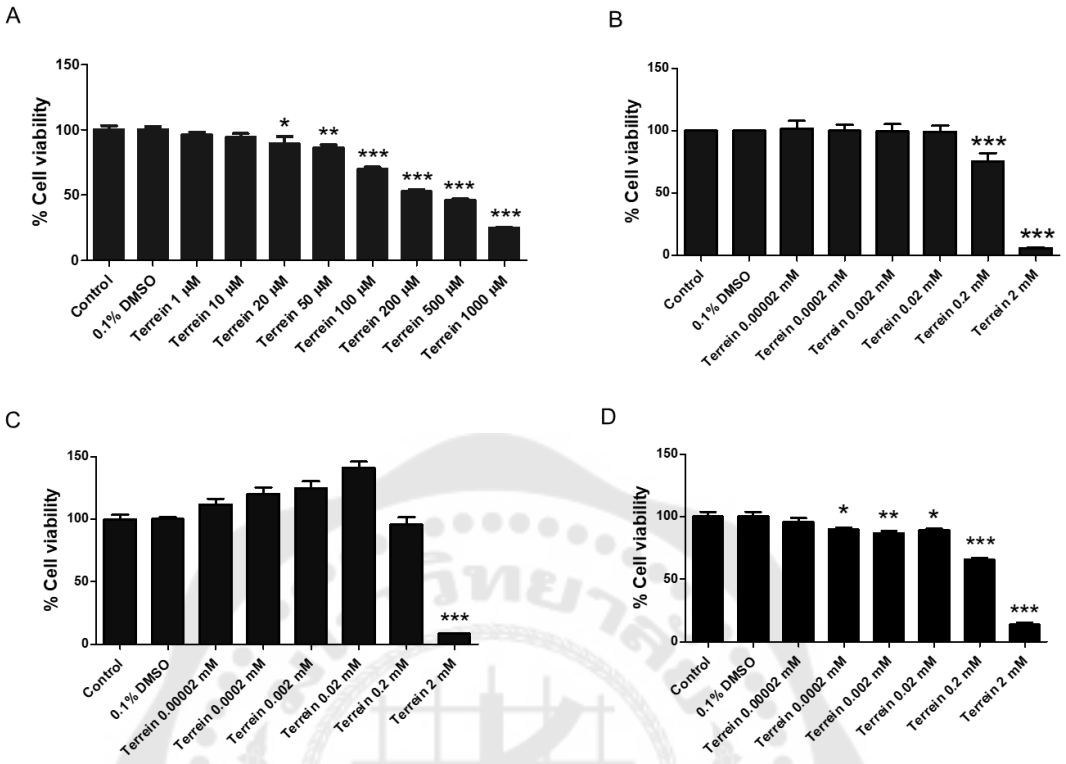


Figure 34 Terrein inhibits cell viability in different cell type. Cell viability was assessed by MTT assay after 24 hours treatment with various concentrations of terrein. (A) A549 cells (B) Vero cells (C) L6 cells (D) H9C2 cells Values are expressed as Mean±SEM of three experiments. *P<0.05, **P<0.01, ***P<0.001, as compared with vehicle control (0.1% DMSO).

Table 4 The half maximal inhibitory concentration (IC_{50}) and selective index (SI) of terrein

Types of cell	IC_{50} (µM)	SI A549 cells
A549 cells	229	-
Vero cells	870	3.8
L6 cells	1,240	5.4
H9C2 cells	579	2.5

4.3 Terrein inhibits proliferation of A549 lung cancer cells

To examine the effects of terrein on A549 cell proliferation. Cells were treated with different concentrations of terrein 1-1000 μM. Cell proliferation was monitored every 3 hours for 3 days. Proliferation curves were generated using IncuCyte proliferation analysis with cell confluences as the parameter. The result found that terrein at 20-1000 μM exhibited a dose-dependent inhibitory effect on A549 cell proliferation as shown in Figure 35.

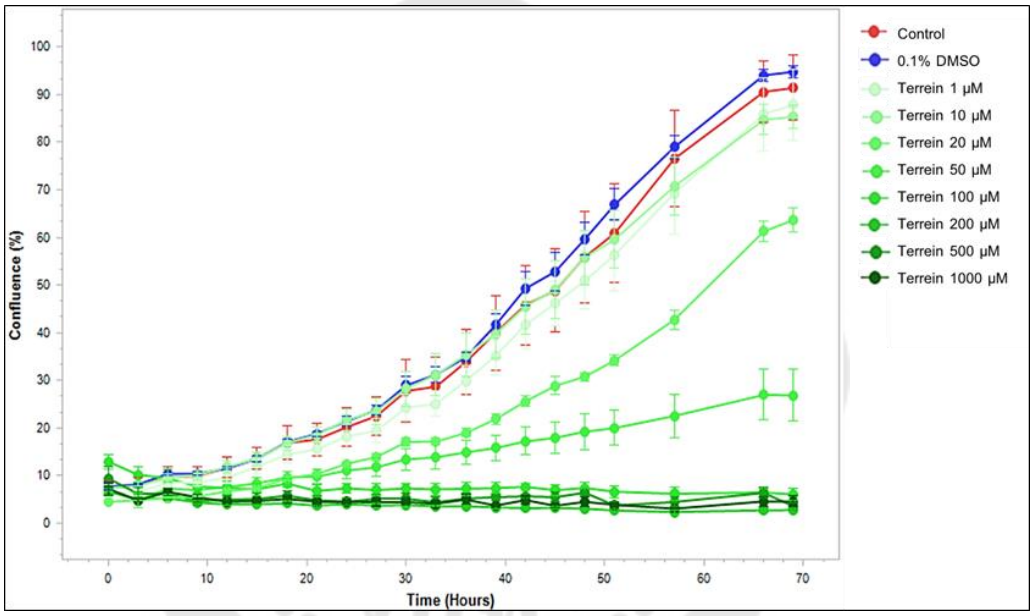


Figure 35 Effects of terrein on A549 cell proliferation. Cells were plated on 96-well plate and treated with various concentration of terrein ranging from 1-1000 μM and monitored for 3 days in the IncuCyte (n=4; Mean±SEM).

4.4 The effect of terrein on A549 cell cytotoxicity

The effect of terrein on A549 cytotoxicity was determined using LDH assay. Lactate dehydrogenase (LDH) enzyme is normally used as biomarker for cellular

cytotoxicity and cytolysis as it will be released from damaged cells. The result showed that terrein at 100, 200 and 1000 μM significantly cytotoxicity on A549 cells (Figure 36).

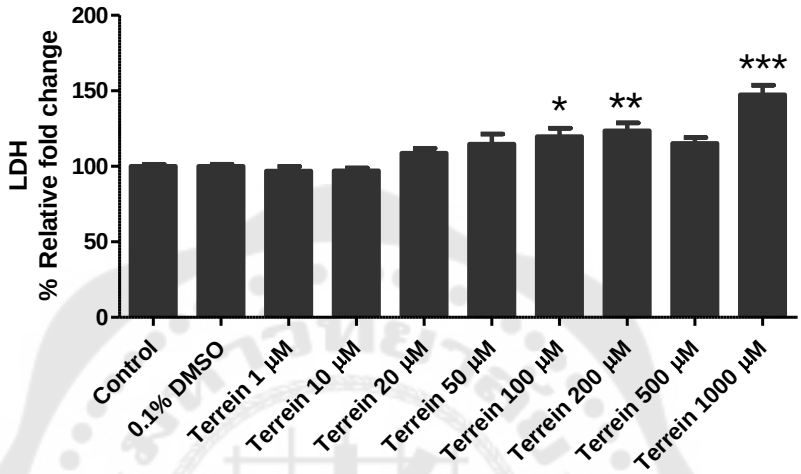
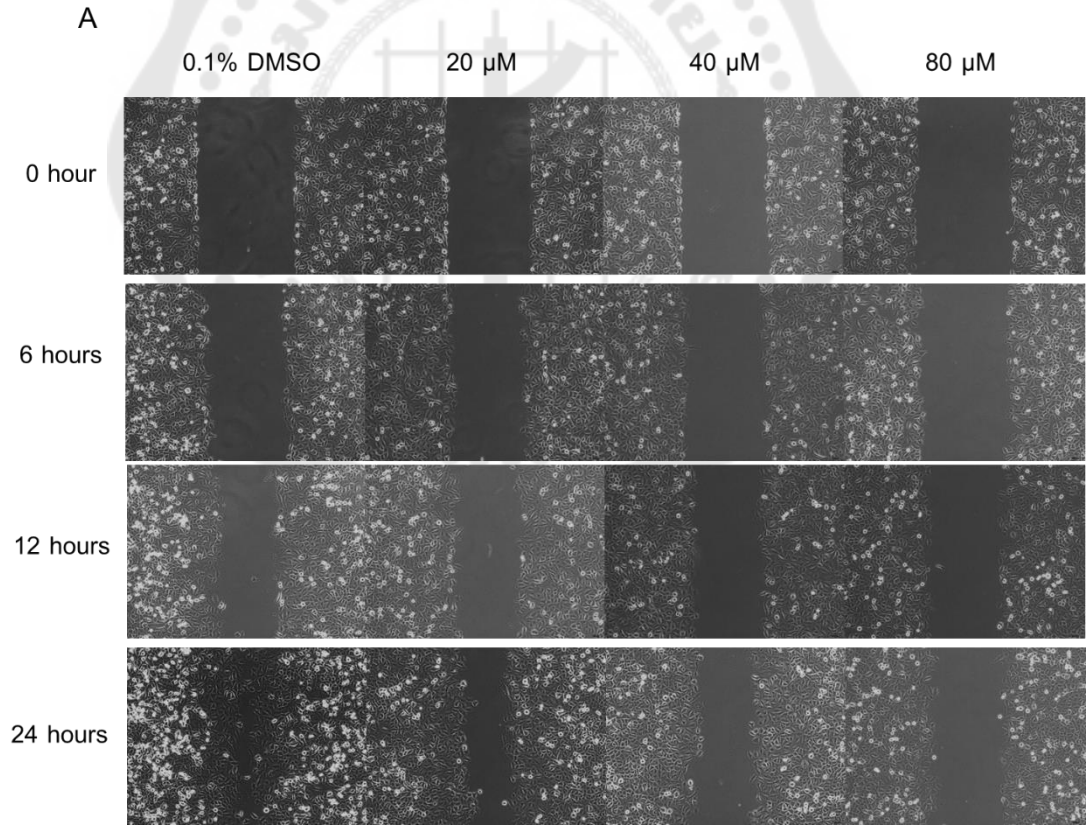


Figure 36 Cytotoxicity of terrein on A549 cells. A549 cells were treated with various concentrations of terrein (0-1000 μM) for 24 hours and determined using LDH assay. Values are expressed as Mean±SEM of three experiments. *P<0.05, **P<0.01, ***P<0.001, as compared with vehicle control (0.1% DMSO).

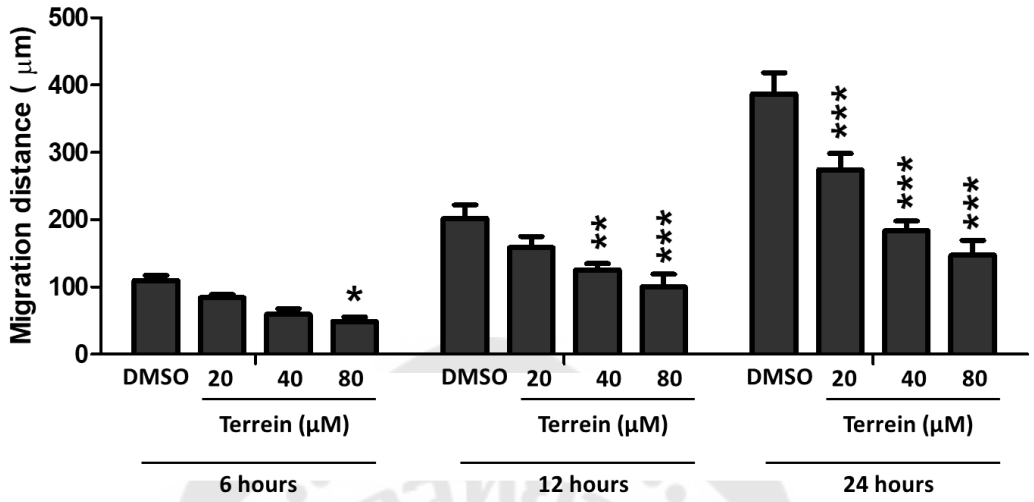
4.5 Terrein inhibits metastasis process of A549 lung cancer cells

To determine the effect of terrein metastasis processes of lung cancer cells, the inhibitory on lung cancer cell migration was firstly evaluated. The migration of cells were measured using a wound healing assay. In this method, the doses of terrein that were selected to investigate should be in the range that is not toxic to cell samples. By using MTT and LDH assay, the concentrations of 0, 20, 40 and 80 μM of terrein were selected and treated on A549 cells for 0, 6, 12 and 24 hours. The results showed that terrein significantly inhibited cells moving towards at 6, 12 and 24 hours (Figure 37A, B).

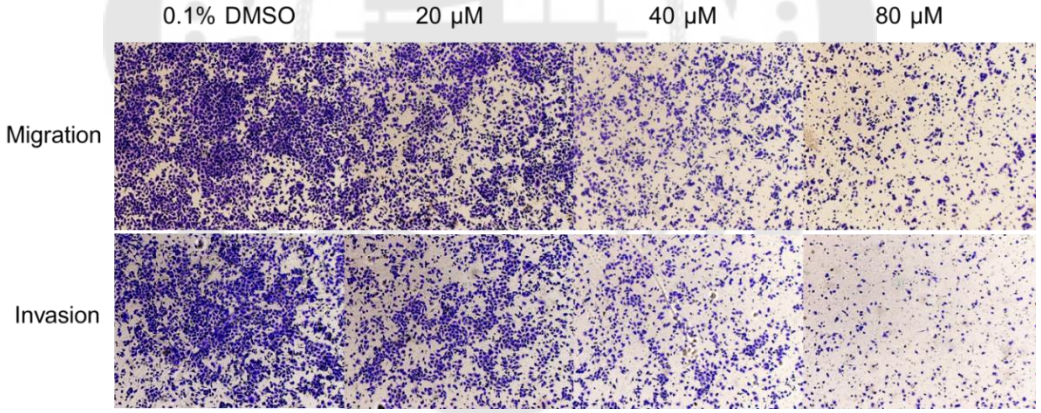
The suppression ability of terrein on the migration and invasion of A549 lung cancer cells were further examined. A549 cells were treated with 0, 20, 40 and 80 μM of terrein for 24 hours and transwell assay was used to observe the impact of terrein on A549 cells migration and invasion. The results found that terrein significantly decreased the number of cells migration and invasion which reduced by as much as 78% and 82%, respectively when compared with untreated control (Figure 37C, D). Adhesion of cancer cells also associate metastasis at an early attachment step. As shown in Figure 36E, terrein decreased A549 cells adhesion by approximately 19% at the highest concentration of terrein when compared with vehicle control.



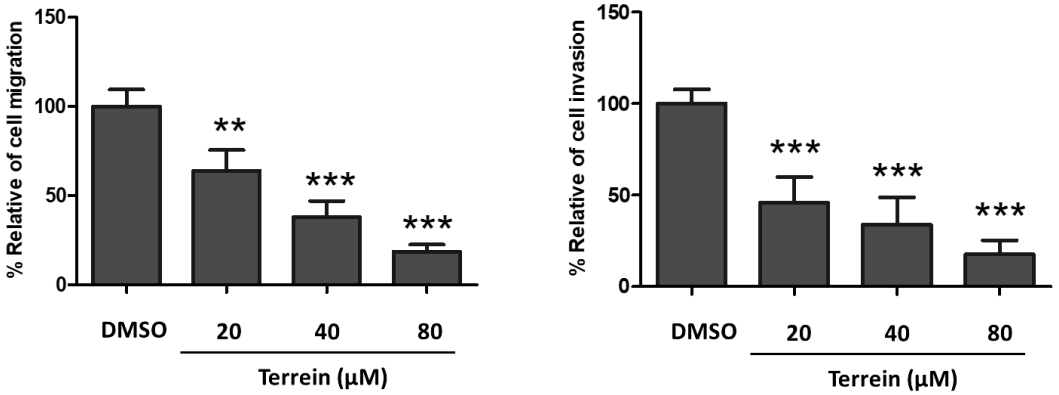
B


Migration distance (μm)
500
400
300
200
100
0
*
**
***
***
***
***
DMSO
20
40
80
Terrein (μM)
6 hours
12 hours
24 hours

C


0.1% DMSO
20 μM
40 μM
80 μM
Migration
Invasion

D


% Relative of cell migration
150
100
50
0
**
***
***
DMSO
20
40
80
Terrein (μM)
% Relative of cell invasion
150
100
50
0
***
***
***
DMSO
20
40
80
Terrein (μM)

E

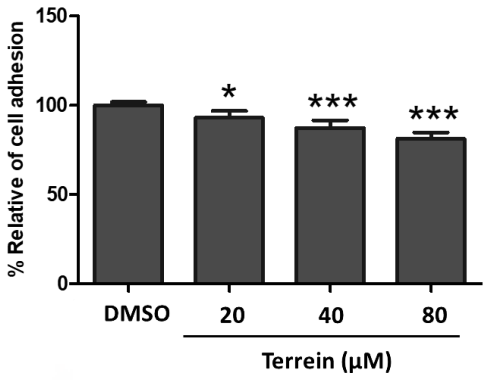


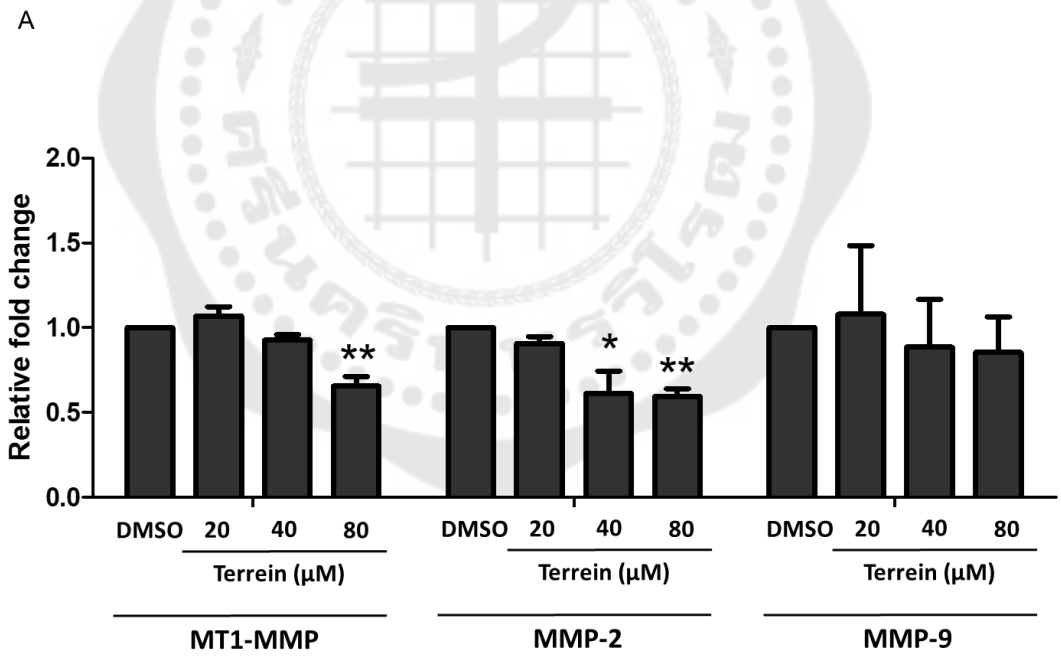
Figure 37 The effect of terrein on A549 cells metastasis process. Wound healing assay was performed to check the migration of A549 cells after 0, 6, 12 and 24 hours of terrein treatment. (A) A representative image of scratch- wound healing assay of A549 cells using a magnification of 10x. (B) Migration distance of treated and untreated samples were measured in at least three independent locations in each wound. (C) Effects of terrein on A549 lung cancer cell migration and invasion after 24 hours of terrein treatment and crystal violet staining. Representative images were captured at a magnification of 10x. (D) The migrate and invade cells were quantified by using imageJ. (E) Adhesion of A549 cells on Matrigel-coated plates. Effect of terrein on cell adherence were measure by MTT assay after 30 minutes of terrein incubation. Significance was measured as Mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001, as compared with vehicle control.

4.6 Terrein inhibits expression and activity of MMPs in A549 lung cancer cells

Matrix metalloproteinases (MMPs) are proteolytic enzymes that play an important role in ECM remodeling and crucially associated in the regulation of multiple stages of cancer progression including cell growth, differentiation, apoptosis, migration, invasion and immune surveillance [154, 155]. Quantitative real-time PCR was used to

examine the effect of terrein on A549 lung cancer cell invasive property. This property was related with altered expression levels of MT1-MMP, MMP-2 and MMP-9. As shown in Figure 38A, terrein significantly decreased the mRNA levels of MT1-MMP and MMP-2. Meanwhile, terrein tends to reduced mRNA levels of MMP-9 but not significant different.

We further examined the effect of terrein on A549 lung cancer cell invasive property. Gelatin zymography was used to determine the effect of terrein on MMP-2 and MMP-9 activity that released from A549 lung cancer cells after treated with 0, 20, 40 and 80 μM of terrein for 24 hours. The results showed that terrein significantly suppressed gelatinase activity of both MMP-2 and MMP-9. The bands indicating MMP-2 (68 kDa) and MMP-9 (82 kDa) were clearly visible, with significant inhibition at 40 and 80 μM of MMP-2 and 80 μM of MMP-9 as shown in Figure 38B and 38C, respectively.



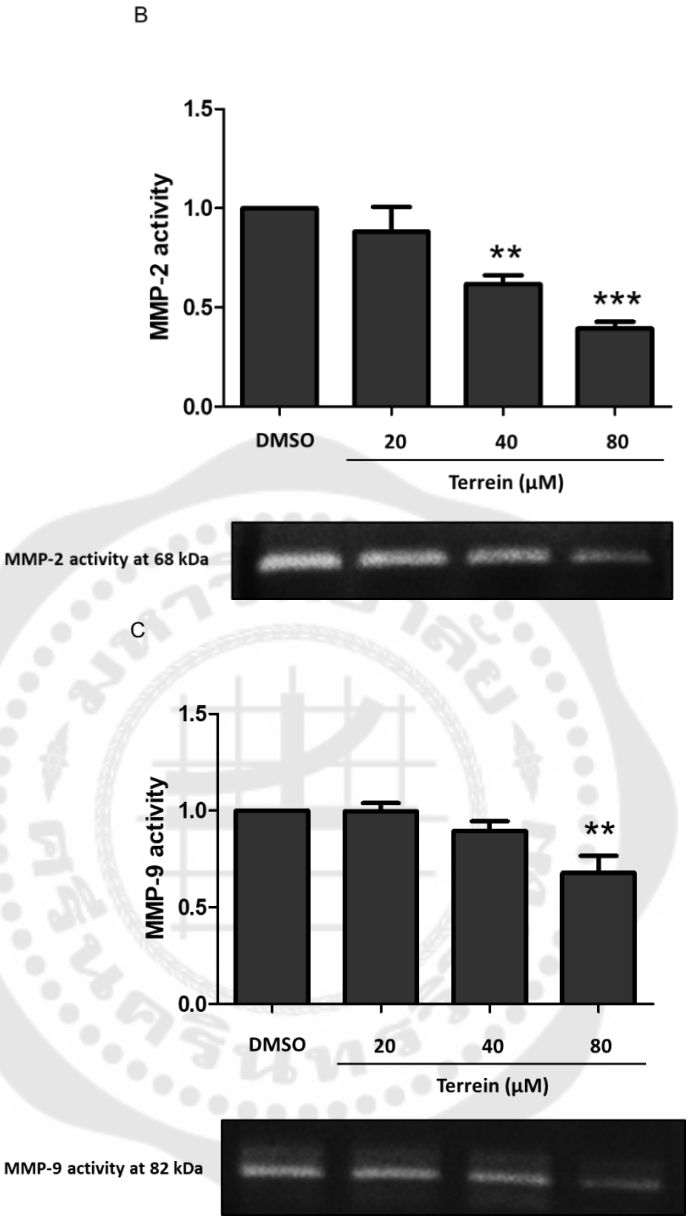


Figure 38 Effects of terrein on expression and activity of MMPs in A549 lung cancer cells. (A) The expression of MMPs were determined by qPCR after 24 hours of terrein treatment. (B) Representative of gelatin zymography of MMP-2 after 24 hours of terrein treatment. (C) Representative of gelatin zymography of MMP-9 after 24 hours of terrein treatment. The activity of MMP-2 and MMP-9 were quantified by Image J software. Significance was measured as Mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01, as compared with vehicle control.

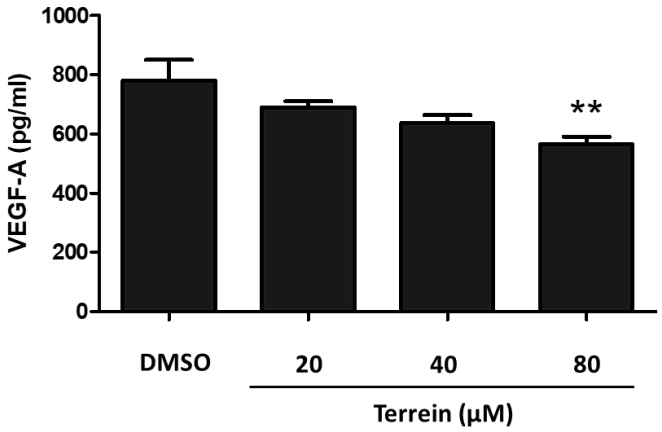
4.7 Terrein inhibits angiogenesis process of A549 lung cancer cells

Angiogenesis is an essential process for promoting tumor growth and metastasis. It is a process of new blood vessels formation upon activation by growth factors. VEGFs are the most important factors to induce angiogenesis [(99)]. To examine whether terrein could inhibit angiogenesis process, A549 cells were treated with 0, 20, 40 and 80 μM of terrein for 24 hours. The VEGF-A that secreted from A549 cells in medium was collected, concentrated and observed by VEGF-A Human ELISA assays. The result showed that terrein significantly reduced secretion of VEGF-A from A549 lung cancer cells when compared with vehicle control (Figure 39A).

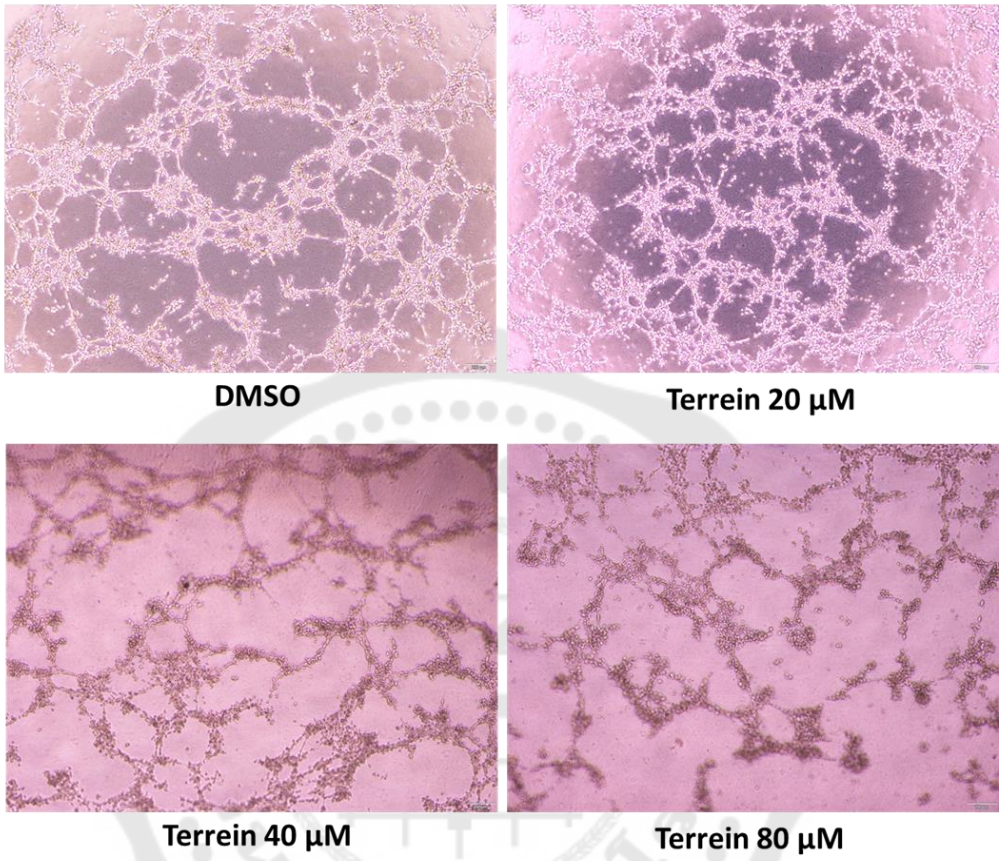
The tube formation assay is a rapid and quantitative method for examination of cell differentiation and modulations that involved in angiogenesis processes. The *In vitro* capillary-like tube formation assay was performed. A549 cells were treated with 0, 20, 40 and 80 μM of terrein which culture on Matrigel-coated plates for 24 hours. Tube formation was photographed by inverted microscope. The results demonstrate that terrein significantly suppressed formation of a tube-like structures when compared with vehicle control (Figure 39B).

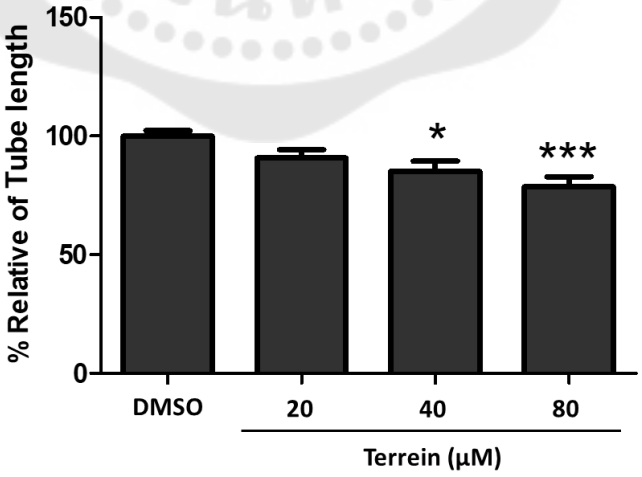
In addition, we further observed the VEGFR2 phosphorylation on Tyr1175, which is crucial sites for migration of cells in angiogenesis. The results showed that terrein could inhibit phosphorylation of VEGFR2 as shown in Figure 39C.

A



B


DMSO
Terrein 20 µM
Terrein 40 µM
Terrein 80 µM


% Relative of Tube length
150
100
50
0
*
***
DMSO
20
40
80
Terrein (µM)

C

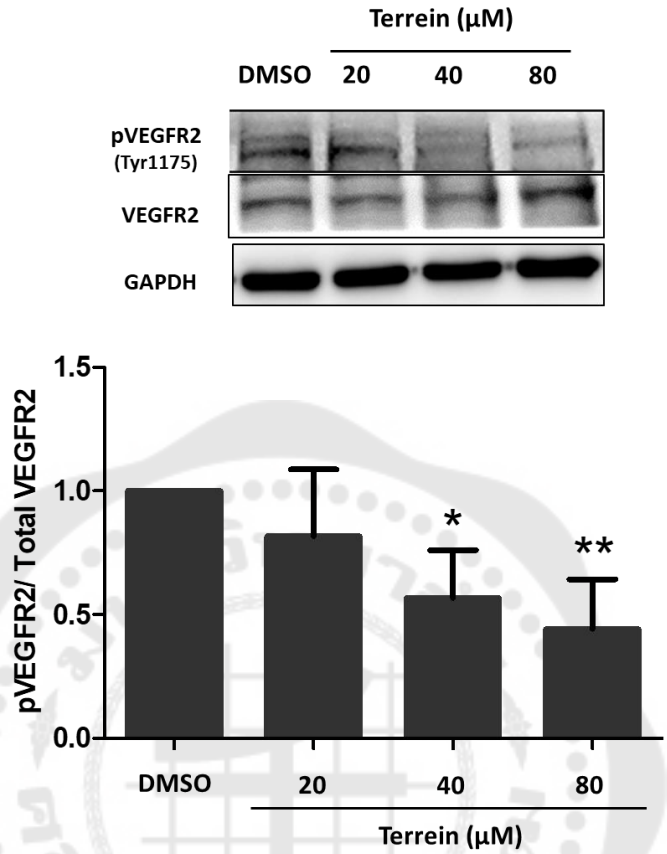


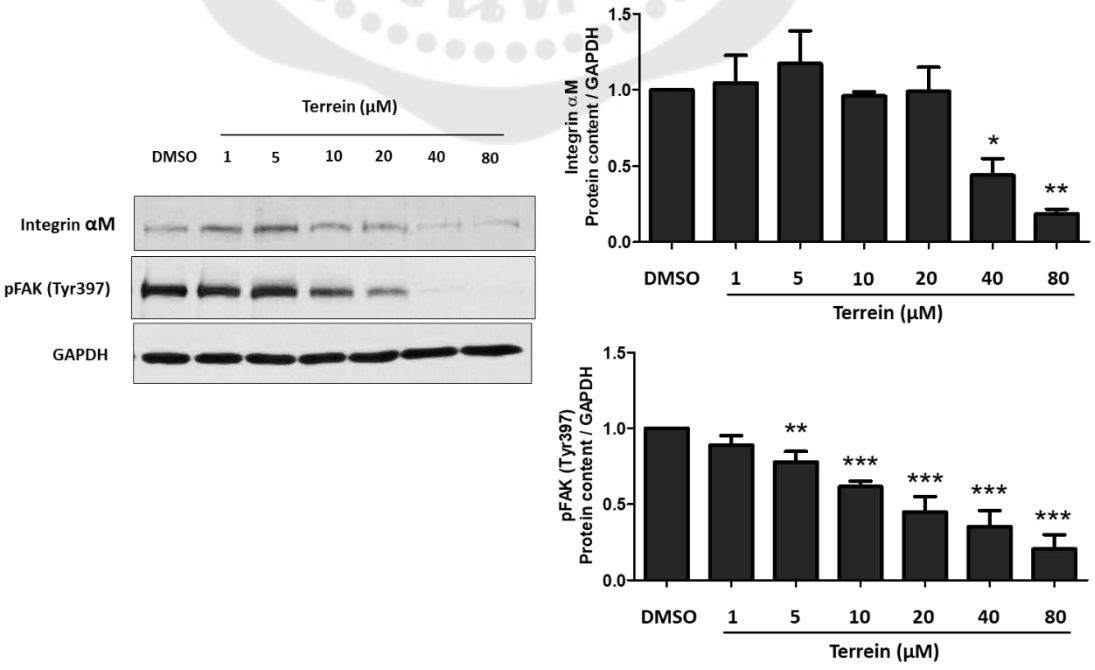
Figure 39 Terrein inhibits angiogenesis process of A549 lung cancer cells. (A) Effects of terrein on release of VEGF-A from A549 lung cancer cells that examined by VEGF-A Human ELISA assays after 24 hours of terrein treatment. (B) Terrein inhibited A549 lung cancer cells tube formation. After 24 hours of terrein treatment, tubular structures were photographed under an inverted microscope at a magnification of 10x. (C) Representation of protein level of VEGFR2 was measured by western blot after 24 hours of terrein treatment and quantified by Image J software. Significance was measured as Mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001as compared with vehicle control.

4.8 Terrein inhibits activity of metastasis mediators in A549 lung cancer cells

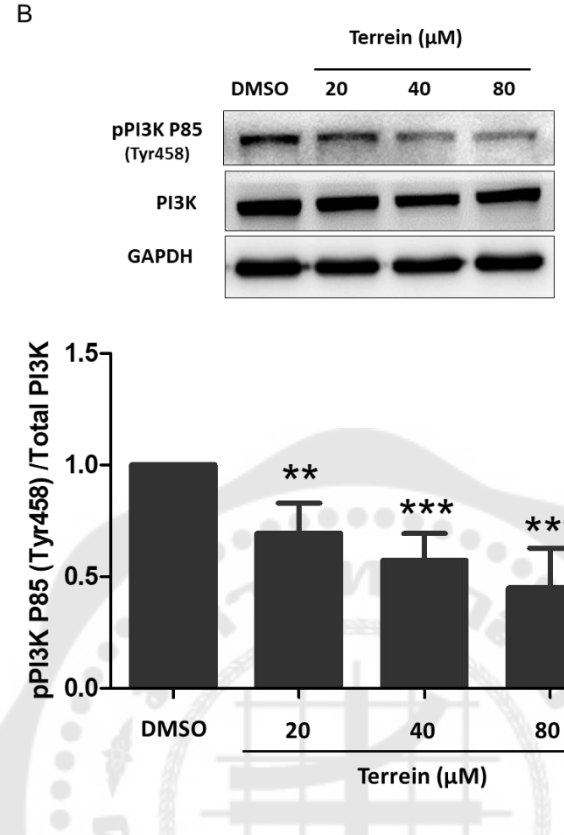
To investigate the effect of terrein on the expression of metastasis mediators such as integrin αM, FAK, mTORC1, PI3K, AKT and P70S6K, a western blotting was performed. A549 cells were treated with different concentrations of terrein for 24 hours. As shown in Figure 40A, the results indicated that terrein significantly inhibited integrin αM and phosphorylation of FAK at Tyr397 when compared with vehicle control. Interestingly, low concentration of terrein could clearly inhibit phosphorylation of FAK.

Active integrins recruit various proteins to the FA, including FAK to stimulate cell signaling. When FAK is activated, it further stimulates downstream proteins to express and active. FAK's downstream signaling proteins are PI3K, AKT, mTORC1 and P70S6K. These proteins affect cell survival, proliferation, angiogenesis, invasion, migration and metastasis. Thus, the inhibition of FAK leads to suppression of downstream signaling mediators. The phosphorylation of PI3K p85 at Tyr458 (Figure 40B), AKT at Ser473 (Figure 40C and 40D), AKT at Thr308 (Figure 40C and 40E), mTORC1 at Ser2448 (Figure 40C and 40F) and pP70S6K at Thr389 (Figure 40C and 40G) were decreased by terrein in a dose dependent manner when compared with vehicle control.

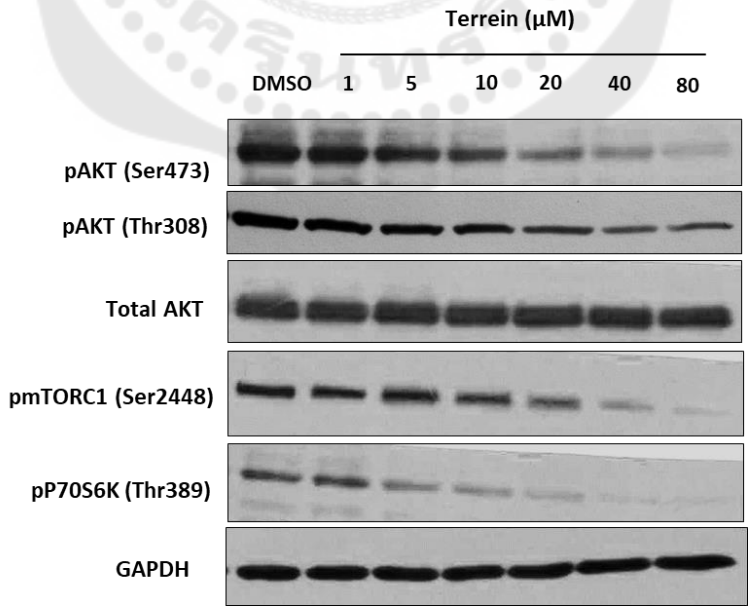
A



B


Terrein (μM)
DMSO
20
40
80
pPI3K P85
(Tyr458)
PI3K
GAPDH
pPI3K P85 (Tyr458) /Total PI3K
1.5
1.0
0.5
0.0
**
***
***
DMSO
20
40
80
Terrein (μM)

C


Terrein (μM)
DMSO
1
5
10
20
40
80
pAKT (Ser473)
pAKT (Thr308)
Total AKT
pmTORC1 (Ser2448)
pP70S6K (Thr389)
GAPDH

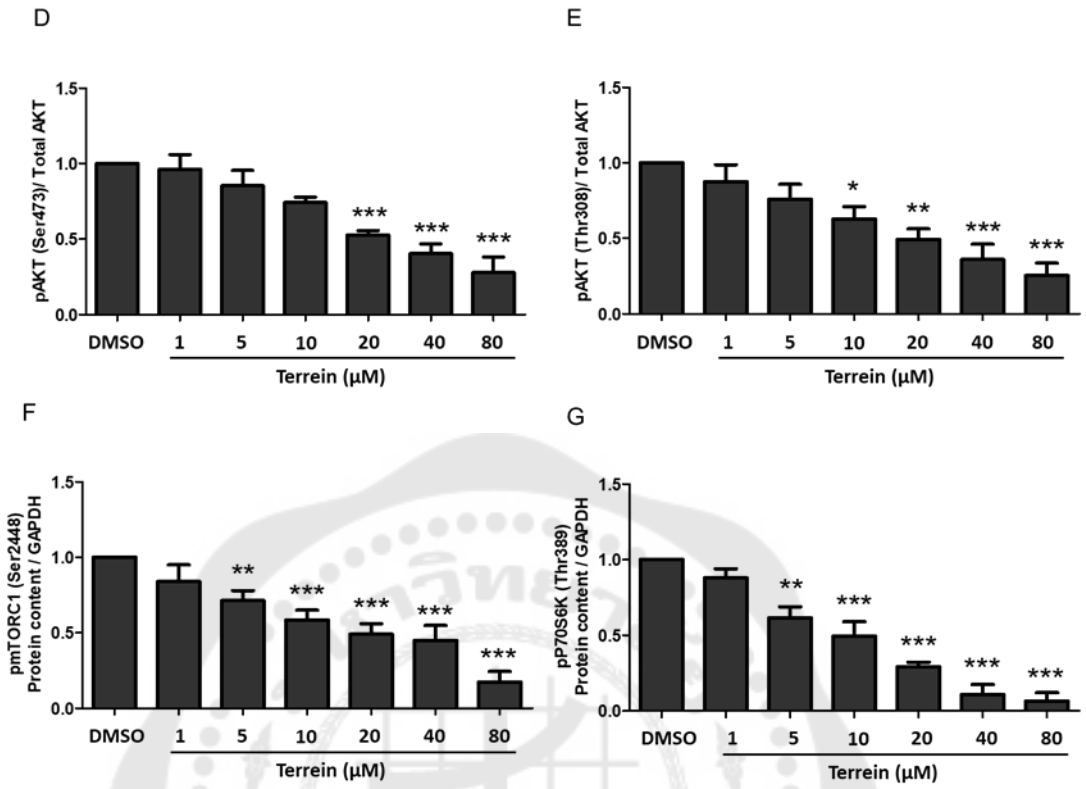
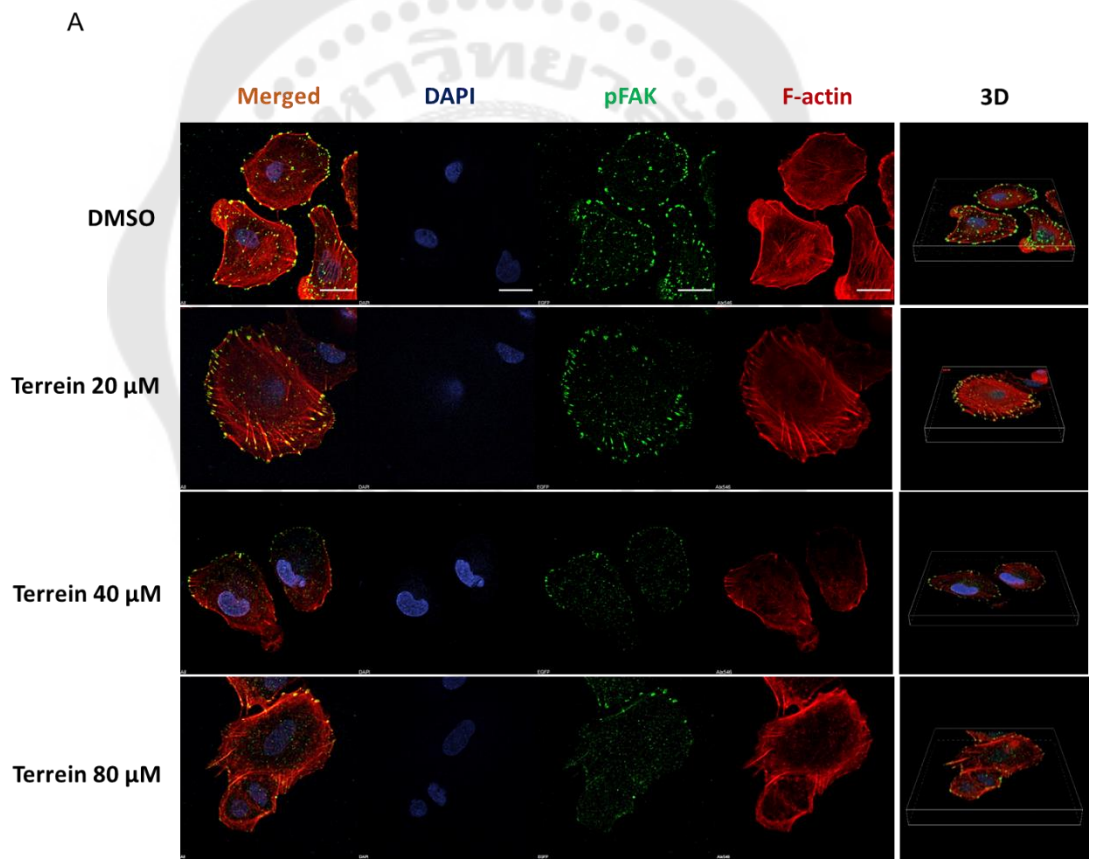


Figure 40 Effects of terrein on the expression of signaling mediators associated with metastatic process in A549 lung cancer cells. Representative of protein levels were examined by western blot after 24 hours of terrein treatment and the quantified bar graph were demonstrated. (A) Integrin αM and FAK phosphorylation. (B) PI3K p85 phosphorylation. (C) AKT, mTORC1 and P70S6K. (D) AKT phosphorylation. (E) mTORC1 phosphorylation. (F) P70S6K phosphorylation. Significance was measured as Mean ± SEM of at least three separate experiments. *P<0.05, **P<0.01, ***P<0.001, as compared with vehicle control.

4.9 Terrein inhibits phosphorylation of FAK in A549 lung cancer cells

To further confirm the effect of terrein on phosphorylation of FAK at Tyr397 in A549 cells using immunofluorescence. The representative and quantitative images (Figure 41A-B) showed that terrein at 40 and 80 µM significantly suppressed phosphorylation of FAK at Tyr397 when compared with vehicle control. When combining the results with the western blot data, it is clearly indicate that terrein has the ability to inhibit phosphorylation of FAK at Tyr397, which is an upstream regulator of many proteins that involved cancer metastasis.



B

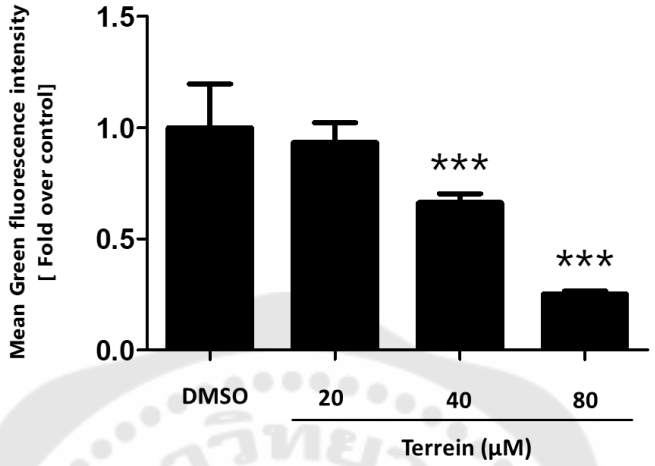


Figure 41 Effects of terrein on phosphorylation of FAK at Tyr397 in A549 cells. Cells were treated with terrein at 0, 20, 40 and 80 μM for 24 hours. (A) Representative confocal images of phosphorylation of FAK at Tyr397. (B) Its quantification. Significance was measured as Mean ± SEM of at least three separate experiments. ***P<0.001, as compared with vehicle control. Scale bar = 20 μm.

4.10 The effect of terrein on hypoxia condition

4.10.1 Cytotoxicity of $CoCl_2$ on A549 cells

The hypoxic condition is recognized as the factor to enhance the aggressive ability of cancer cell metastasis. Also, the chemical cobalt chloride ($CoCl_2$) has a property to induce hypoxic condition in several cell types. Then, in order to see the effect of terrein on the aggressive metastasis lung cancer cells, the hypoxic model induced by $CoCl_2$ was firstly performed. In addition, the cytotoxic effect of $CoCl_2$ itself was evaluated before further treatment with terrein. With MTT assay, the data revealed that $CoCl_2$ at 1-800 μM had no toxicity on A549 cells, except $CoCl_2$ at 1,000 μM that

decreased cell viability of A549 cells at 43.57% when compared with vehicle control (Figure 42).

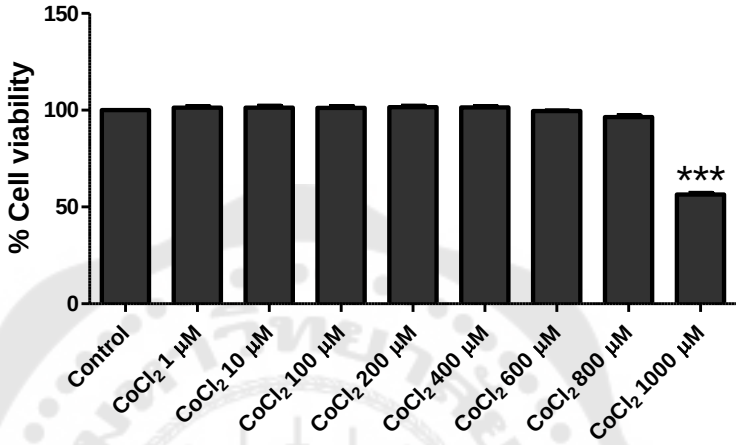


Figure 42 Cytoxicity of $CoCl_2$ on A549 cells. Cell viability was assessed after 24 hours treatment with various concentrations of $CoCl_2$. Significance was measured as Mean ± SEM of at least three separate experiments. ***P<0.001, as compared with control.

4.10.2 Terrein suppressed HIF-1 alpha expression in $CoCl_2$ induced-A549 cells

To further investigate the effect of terrein on the aggressive metastatic lung cancer of which $CoCl_2$-induced hypoxia was used as a model, the level of HIF-1α expression was selected to examine. HIF-1α is a transcription factor that normally increased in its expression in respond to hypoxia. Therefore, HIF-1α was used in this study as an indicator of successfully hypoxic model after induced by $CoCl_2$. In addition, it was used as a marker to indicate the effect of terrein in the hypoxic condition. Then, A549 cells were treated with $CoCl_2$ for 24 hours before added with terrein at 0, 20, 40 and 80 µM for another 24 hours. The results showed that $CoCl_2$ significantly induced expression of HIF-1α in A549 cells when compared with control. Additionally, terrein

demonstrated the inhibition effect on the expression of HIF-1α after induced by $CoCl_2$ for 24 hours. Interestingly, terrein could reduce the expression of HIF-1α into the level that almost the same as treated with terrein alone (Figure 43).

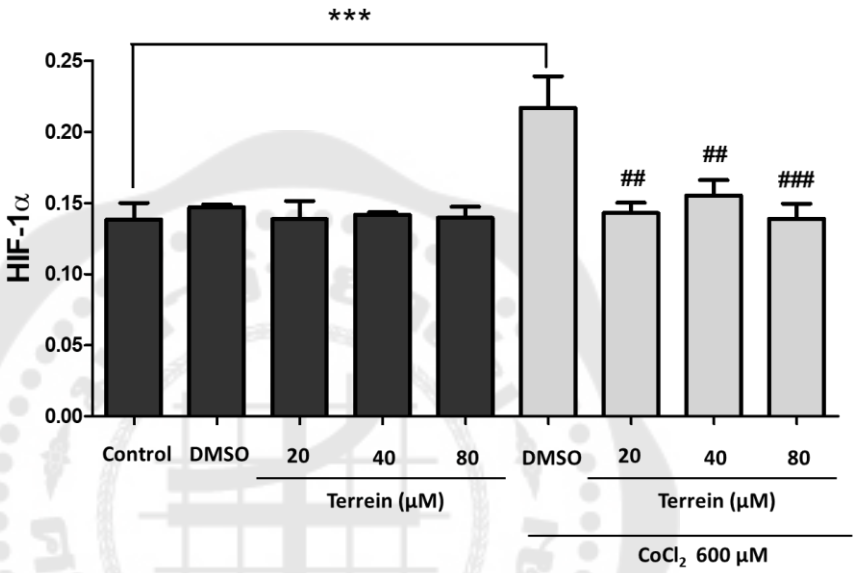


Figure 43 Terrein suppressed HIF-1α expression in A549 cells. Cells were induced HIF-1α with $CoCl_2$ for 24 hours and further treated with terrein at 0, 20, 40 and 80 μM for 24 hours. Significance was measured as Mean ± SEM of at least three separate experiments. ***P<0.001, as compared with control. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, as compared with $CoCl_2$.

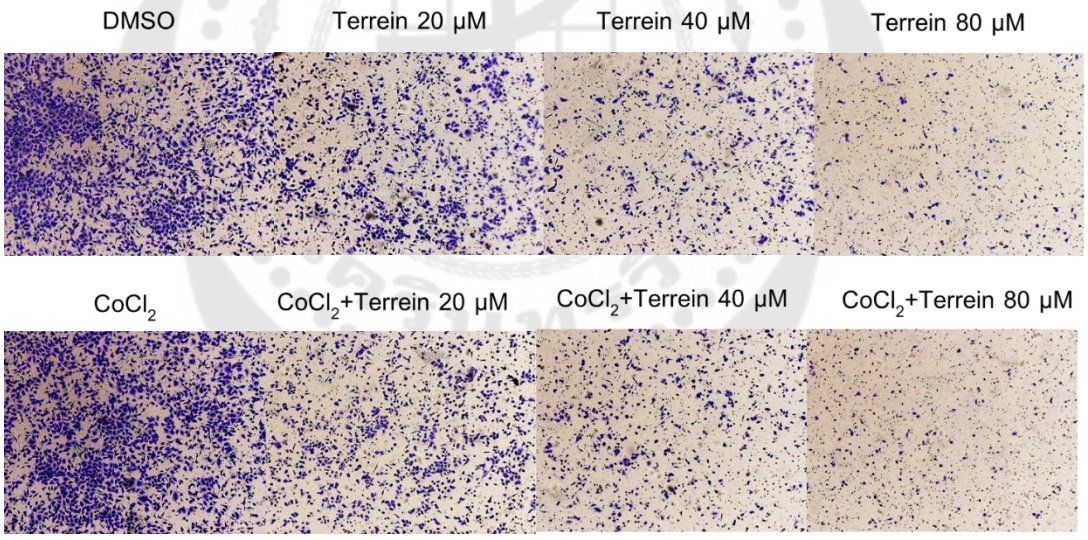
4.10.3 Terrein affected to the metastatic process of A549 lung cancer cells but not significant difference from normal condition

Subsequently, the effect of terrein on cell migration and invasion in A549 cells-induced hypoxic condition was detected by transwell assay. Cells were induced hypoxia with $CoCl_2$ for 24 hours and further treated with terrein at 0, 20, 40 and 80 μM for 24 hours. The ability of A549 cell migration was showed in Figure 44A and 44B.

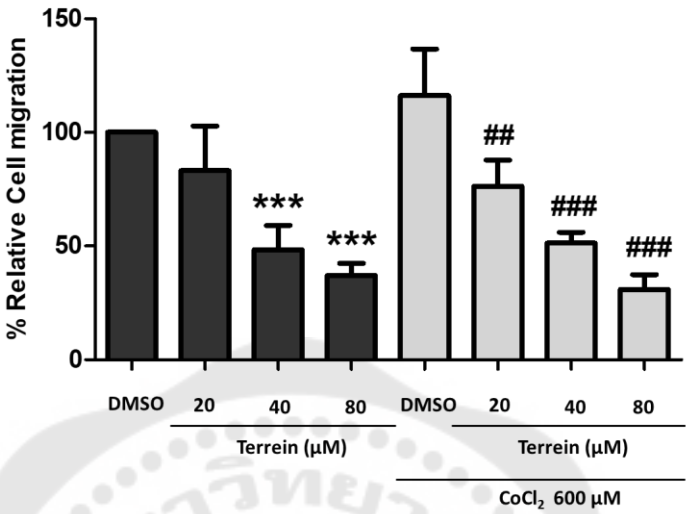
$CoCl_2$ could induce migration of A549 cells but not significant difference when compared with cells in normal condition. However, the number of cell migration in hypoxic condition was significantly decreased after treated with terrein when compared with cells treated with $CoCl_2$ alone.

The effect of terrein on A549 cell invasion was shown in Figure 44C and 44D. The results demonstrated that $CoCl_2$ could induce invasion of A549 cells which increased as much as 51.82% but not significant difference when compared with cells in normal condition. The number of invading A549 cells in hypoxic condition after treated with terrein were significantly reduced when compared with cells treated with $CoCl_2$ alone.

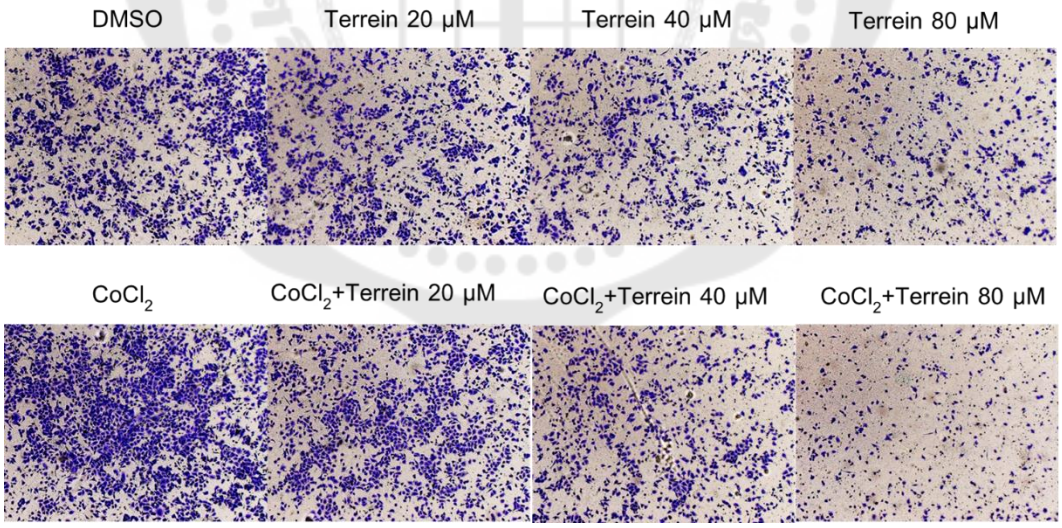
A



B


% Relative Cell migration
150
100
50
0
***
***
##
###
###
DMSO
20
40
80
DMSO
20
40
80
Terrein (μM)
Terrein (μM)
CoCl2 600 μM

C


DMSO
Terrein 20 μM
Terrein 40 μM
Terrein 80 μM
CoCl2
CoCl2+Terrein 20 μM
CoCl2+Terrein 40 μM
CoCl2+Terrein 80 μM

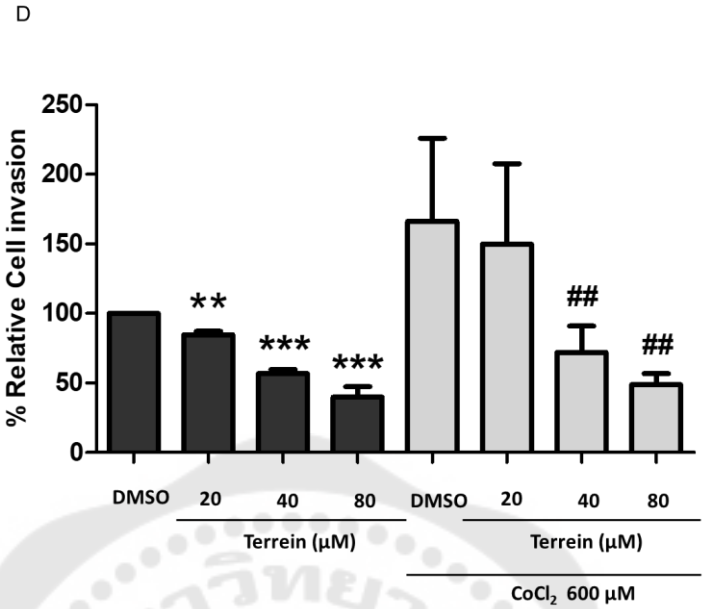


Figure 44 Terrein suppressed migration and invasion of A549 cells-induced hypoxia. Cells were induced HIF-1α with $CoCl_2$ for 24 hours and further treated with terrein at 0, 20, 40 and 80 μM for 24 hours. (A) Effects of terrein on cell migration of A549 cells-induced hypoxia after crystal violet staining. (B) The migrate cells were quantified by using imageJ. (C) Effects of terrein on cell invasion of A549 cells-induced hypoxia after crystal violet staining. (D) The invade cells were quantified by using imageJ. Significance was measured as Mean ± SEM of at least three separate experiments. **P<0.01, ***P<0.001, as compared with control. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, as compared with $CoCl_2$.

CHAPTER V
CONCLUSION AND DISCUSSION

Human lung cancer is the leading cause of cancer death both worldwide and Thailand [4]. The most common cause of lung cancer-related death patients is diagnosed at metastatic stages that cancer cells spread to nearby tissue or other parts of the body [5]. Therefore, suppression of metastatic cancer is the effective way to reduce the death of cancer patients. Lung cancer treatment is currently a combination of surgery, radiotherapy and chemotherapy, which is still very limited in many aspects such as side effects from the treatment, drug resistance, and the high expense of treatment. Nowadays, the world has turned attention to use natural substances for cancer treatment because they have less in side effects. Terrein is a bioactive natural substance that has been reported to induce various biological functions, including melanogenesis inhibition [11], an anti-inflammation [139] and anticancer [13]. However, the effect of terrein on the underlying molecular mechanisms in metastatic human lung cancer has not been observed.

In the present study, the biological effects of terrein is therefore investigated by targeting through the molecular mechanism on metastasis processes, including cell proliferation, adhesion, migration, invasion and angiogenesis of A549 human lung cancer cells. This study revealed that terrein exhibited cytotoxic effects on different cell types; A549 cells, Vero cells, L6 cells and H9C2 cells by inhibiting cell viability with an IC_{50} at 229 µM, 870 µM, 1,240 µM and 579 µM, respectively (Figure 34). From MTT result suggested that terrein had a specific effect on A549 lung cancer cells relative to normal cells with high selectivity index values. As shown in other reports, terrein was cytotoxic to human cervical cancer cells (HeLa) with IC_{50} at 0.29 mM (290 µM) [13], human breast cancer cell lines, MCF-7 and MDA-MB-231 cells, were at 2.34 mM (2,340 µM) and 700 µM, respectively [145]. These data indicated that terrein was more toxic to A549 lung cancer cells than to other cancer cells with the lowest IC_{50} value. This cytotoxic effect of terrein on A549 cells was further confirmed using LDH assay. LDH is a soluble cytoplasmic enzyme found in all cells. This enzyme normally located intracellular except

for the damaged cells that LDH will release out. Therefore, to investigate the damaged cells *in vitro*, LDH detection in the culture medium is well-known as one crucial marker. To determine LDH activity that is measured, using a coupled enzymatic reaction, which results in the conversion of a tetrazolium salt into a red formazan product. The reaction involves conversation of lactate to pyruvate by LDH and reduction of NAD+ to NADH was performed. This number of formazan is directly proportional to the amount of LDH in the culture supernatant, which is directly proportional to the number of cell death or damaged cells [156]. As indicated in Figure 36, the high concentration of terrein significantly increased cytotoxic effect on A549 cells, which is correlated well with the result of MTT assay. These results suggest that high concentration of terrein enlarged cell damage which resulting in reduced cell viability.

Cancer metastasis is consisted of several steps, including cell proliferation, adhesion, migration, invasion and angiogenesis [50]. All of them are important for cancer cell progression. To determine the effect of terrein on these processes, low concentrations of terrein (20, 40 and 80 μM) that are not toxic to lung cancer cells were selected. As shown in Figure 37, the results revealed that terrein significantly inhibited wound healing, cell adhesion, cell migration and cell invasion of A549 lung cancer cells. In addition, the proliferation analysis clearly exhibited the inhibition effect of terrein (Figure 35). These results indicate that terrein could suppress multi-steps in cancer metastatic processes.

To further examine on the anti-metastatic properties of terrein on lung cancer cells, the activity of matrix-metalloprotenases (MMP) was investigated. Numerous studies have demonstrated that MMPs play special roles in tumor progression, invasion, metastasis and angiogenesis [157, 158]. Increased expression of MMPs have been associated with poor prognosis in several types of tumors including breast cancer, gastric cancer and osteosarcoma [159-161]. All invasive malignant tumors, including lung cancer cells have been known to express high levels of MMPs, especially, MMP-2 and MMP-9. MMP-2 plays an essential role in the progression of cancer because it cleaves several ECM components and basement membranes[162]. Activating MMP-2 promotes

secondary growth and sustainability of cells in metastatic sites [163]. Moreover, MMP-2 plays a major role in tumor development and angiogenesis, and thus the development of potential MMP-2 inhibitor becomes an important goal in the lung cancer therapy [164]. MMP-9 is a gelatinase enzyme that digests gelatin and various ECM molecules. The expression and activity of MMP-9 are upregulated in NSCLC and are associated with the pathologic type and clinical stage of NSCLC [165]. MMP-9 also tends to increase the tumor size. The expression levels of MMP-9 were higher in advanced stages III and IV compared with primary stages I and II tumors in NSCLC cases and higher in NSCLC metastasis than without metastasis as well as a reduced 5-year survival rate [166]. Both MMP-2 and MMP-9 are classified as soluble enzymes that are secreted in the extracellular milieu. However, there are some MMPs that are classified as membrane-type MMP (MT-MMPs) in which their C-terminal regions are tethered to the plasma membrane and have ability to cleave pericellular ECM. In these MT-MMPs group, membrane-type 1 MMP (MT1-MMP) has been indicated to be involved in degrading extracellular matrix and accelerating angiogenesis [167]. It has also been shown to regulate the turnover of various ECM components such as fibrin, fibronectin, vitronectin, laminin, collagen type I, II, III and IV, and proteoglycan [168]. In addition, MT1-MMP is participated in the activation of secreted MMPs such as MMP-2. Overexpression of MT1-MMP was directly related to the increased cell migration [169] which has been demonstrated in several types of human cancer cells such as lung cancer [170], breast cancer [171], colon cancer [171], cervical cancer [172], prostate cancer [173] and glioblastomas [174].

Therefore, blocking the expression and activity of these enzymes has the potential to suppress lung cancer cell metastasis and increases the possibility of NSCLC patients' survival. As shown in this work, it is demonstrated that terrein could inhibit expression of MT1-MMP, MMP-2 and MMP-9 that evaluated by qPCR (Figure 38A). Downregulation of MT1-MMP leads to reduce expression of MMP-2. The effect of terrein was also confirmed by gelatin zymography that MMP-2 and MMP-9 activities

were significantly suppressed (Figure 38B-C). These findings indicate that terrein has the potential role of the anti-metastatic agent that has never been reported.

In cancer cells, angiogenesis or the creation of new blood vessels is another mechanism approved to be essential for their survival. New blood vessels will support the growth of tumors, by specifically feeding their hypoxic and necrotic areas to provide them with essential nutrients and oxygen [175]. Then, this study further investigated the effect of terrein on angiogenesis by determination of VEGF-A expression. VEGF is a major chemotactic factor during angiogenesis that initiates cell migration, cell adhesion and the interaction between endothelial cells and ECM followed by the formation of tubular network. In mammals, VEGF has several types including VEGF-A, VEGF-B, VEGF-C, VEGF-D; however, VEGF-A is widely studied and has a major role in angiogenesis by acting through VEGFR2 [102]. As demonstrated in Figure 39A, terrein at 80 μM significantly suppressed secretion of VEGF-A from A549 lung cancer cells when compared with vehicle control. To confirm this effect, the expression of VEGFR2, a member of tyrosine kinase receptor family, responsible for VEGF-A activation was investigated. Also, the phosphorylated site at Tyr1175 of carboxy terminal region of VEGFR2 was determined. As seen in Figure 39C, the ratio of phosphorylated VEGFR2/VEGFR2 was decreased comparing to vehicle control, thus indicating that the downstream signaling events were inhibited. These data support the tube formation results as indicated in Figure 39B. The characteristics of tube-like structure were reduced with a dose-dependent manner when treated with terrein.

From all data shown, it could be concluded that terrein has the effect of anti-metastasis in lung cancer cells as all processes involved including cell proliferation, migration, invasion, adhesion and angiogenesis are inhibited. Therefore, it is interesting to further explore on its metastatic inhibition mechanism. Based on the recent knowledge of cancer cell migration, adhesion and invasion, it is known that the formation of focal adhesion complexes are crucial sites for cancer metastasis. The focal adhesions are integrins-containing, multi-proteins assemblies spanning the plasma membrane that linked intracellular cytoskeleton to the extracellular matrix. The

interaction of multiple proteins at focal adhesions site served as the holding points to promote the protrusion of the leading edge as the structure called invadopodia and lamellipodia. At the focal adhesions, the signal transduction is initiated by the interaction of integrin to ECM which further promotes the assembly of cytoplasmic scaffold and kinase proteins [176]. FAK or focal adhesion kinase is one of the principal integrin signaling regulators that are recruited at the adhesion site and autophosphorylated at Tyr397 site. This contributes the activation of its intrinsic kinase function and create the docking sites for several downstream signaling molecules [177]. FAK is increased in many highly malignant human cancers [178]. Overexpression of FAK in cancer cells leads to a resistance in apoptotic process. In addition, an increasing of FAK was found to contribute through the activation of PI3K/AKT and MEK-ERK1/2 signaling pathways, resulting in increasing cancer cell survival and proliferation [179]. To inhibit integrin signaling, FAK expression and its phosphorylation are therefore another target of the effective anti-metastatic agent. As shown in this work, terrein could inhibit integrin αM expression and the phosphorylation of FAK at Tyr397 with significantly (Figure 40A, 41)

In addition, downstream mediators of FAK including PI3K, AKT, mTOR and P70S6K were further investigated. PI3K is divided into 2 subunits, which consists of the p110 catalytic subunit and the p85 regulatory subunit [180]. PI3K activation is associated with the receptor through one or two Src homology 2 domains in the regulatory subunit, which contributes to the activation of the catalytic subunit. Activated PI3K pathway leads to the phosphorylation of PIP2 (phosphatidylinositol 3,4-bisphosphate) and produces PIP3 (phosphatidylinositol 3,4,5-trisphosphate). PIP3 activates 3-phosphoinositide-dependent kinase 1 (PDK1) and its major downstream effector, which is AKT [181]. AKT is a serine–threonine kinase that upon activated by PI3K, AKT transfers to the plasma membrane, leads to its conformational change. AKT consists of a central kinase domain with a threonine residue (T308) that binds to the PDK1 and a C-terminal tail domain (S473) that binds to the second mTOR complex 2 (mTORC2). Phosphorylation of AKT promotes many of molecular functions in the cell, such as cell proliferation, survival, migration, and differentiation [181]. One major downstream effector of AKT is mTOR

(mammalian target of rapamycin). mTOR is a highly conserved protein kinase that contains two protein complexes, including mTORC1 (mTOR, mLST8, and raptor) and mTORC2 (mTOR, mLST8, mSIN1, and Rictor). mTORC1 induces protein synthesis and cell growth by phosphorylating ribosomal p70S6 kinase 1 (S6K1) and eukaryotic translation factor 4E-binding protein 1 (4EBP1). While mTORC2 regulates the organization of actin cytoskeleton through F-actin stress fiber, paxillin, RhoA, Rac1, Cdc42 and PKCα. So, mTOR plays a critical role in the regulation of cancer cell motility and metastasis [182]. The inhibition of the PI3K/AKT/mTOR pathway by an mTOR inhibitor has been shown to suppress cancer cell invasion and migration and promotes apoptosis in tumors [183, 184]. Recent work has demonstrated that inhibited the FAK/PI3K/AKT/mTOR pathway and reduced the MMP-2 and MMP-9 protein expressions, suppressed gastric cancer cell migration and invasion [185]. As shown in Figure 40, terrein significantly decreased the phosphorylation of PI3K p85 at Tyr458, AKT at Ser473, AKT at Thr308, mTORC1 at Ser2448 and pP70S6K at Thr389, which indicates the inhibition of PI3K/AKT/mTOR pathway. Therefore, all these data indicate that terrein has ability to suppress important proteins in signaling transduction pathway that regulates cancer cell proliferation, survival, migration, metastasis, differentiation and angiogenesis.

The effect of terrein was further investigated on hypoxic condition which known to promote the invasive potential of cancer cells [126].To do so, $CoCl_2$ was used to mimic hypoxia in A549 lung cancer cells and HIF-1α transcription factor that normally responsible for hypoxia as a marker was determined. Figure 43 demonstrated that $CoCl_2$ itself significantly induced expression of HIF-1α in A549 lung cancer cells when compared with control. These results indicated that the hypoxic condition was successfully induced. Moreover, different doses of terrein were not induced the hypoxic condition in lung cancer cells, the levels of HIF-1α were in the same range as untreated control cells. When A549 cells treated with $CoCl_2$ and terrein, it was clearly showed that the level of HIF-1α expression significantly reduced to the level as based-line of untreated samples. HIF-1α is a transcription factor that has one important role of

inducing cancer cells transition from the epithelial morphology into mesenchymal features (EMT), the characteristic of cells that increasing migratory and invasive abilities [186]. The linkage of HIF-1α expression and EMT changes has been clearly observed in breast cancer cells in which $CoCl_2$ treatment induced HIF-1α expression and EMT marker genes, vimentin and fibronectin [126]. In gastric cancer, HIF-1α was shown to express about 90% of human gastric cancer biopsies at the front edge of the invading tumor compared to HIF-1α normal tissues. Inhibition of HIF-1α significantly decreased the gastric cancer cell metastasis *in vivo* and HIF-1α deficient cells were reduced in cell migration, invasion and adhesion *in vitro* [187]. Therefore, a reduction of HIF-1α from the effect of terrein should result in the reduction of migration and invasion abilities. As demonstrated in Figure 44, the effects of terrein were clearly inhibited both the migration and invasion of A549 cells with a dose-dependent manner. However, these inhibitory effects in cell migration assay were not significantly different between $CoCl_2$ treated and untreated samples. For cell invasion assay, $CoCl_2$ could increase invasion of A549 cells by 51.82% but no significant differences between samples with and without $CoCl_2$ treatment. These data probably indicate that the augmentation of HIF-1α after $CoCl_2$ treatment may have the effect only on the invasive property of lung cancer cells.

In conclusion, the present study demonstrates a novel finding of the potency of terrein as an anticancer agent in lung cancer. Terrein could inhibit lung cancer cell proliferation and metastatic processes, including adhesion, migration and invasion through inhibiting integrin/FAK pathway along with its downstream signaling, PI3K/AKT/mTOR/S6K pathway. In addition, terrein could inhibit angiogenesis processes by decreasing VEGF secretion and tube formation via reducing VEGF/VEGFR2 interaction. Moreover, terrein could suppress the aggressive phenotype of human lung cancer cell invasion in the hypoxic condition. All these data suggested that terrein is a potential new compound that is worth to develop as an anticancer agent and to be further tested in clinical trial.

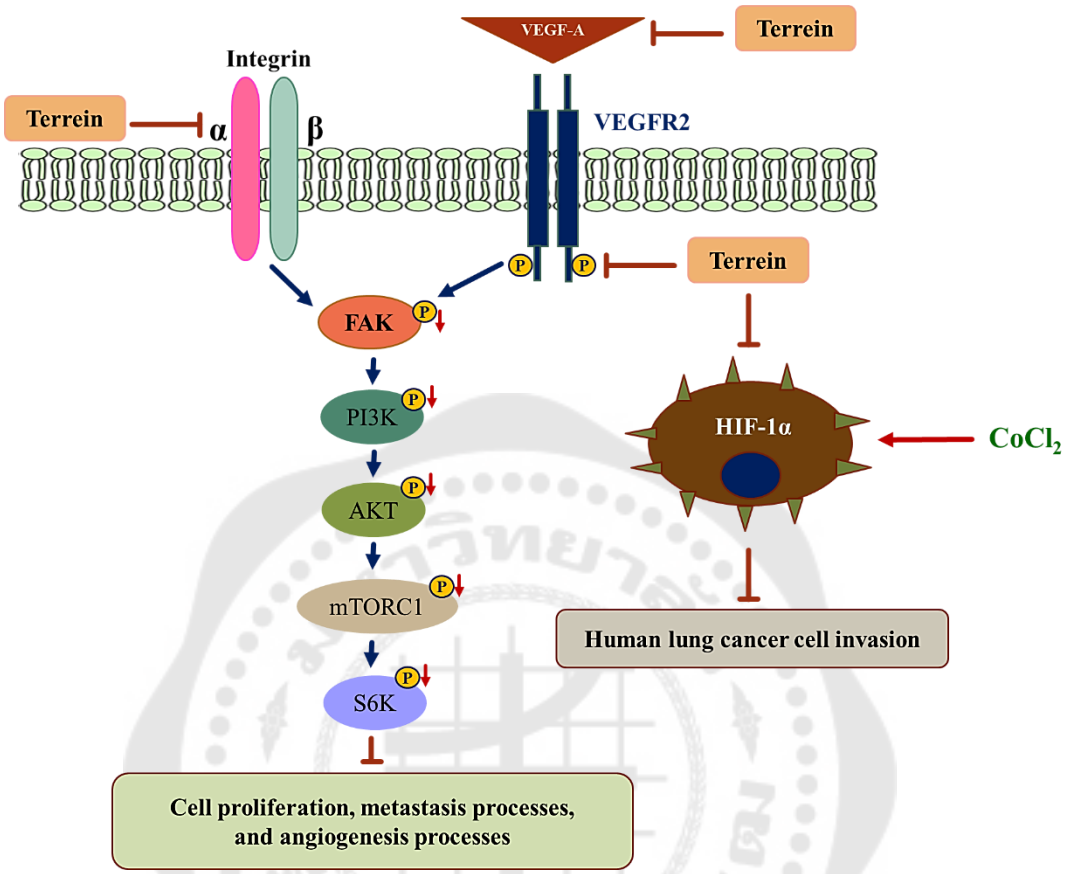


Figure 45 Summary of terrein effects on lung cancer cell metastasis and angiogenesis.

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