ผลของวานิลลินต่อฟีโนไทป์คล้ายเซลล์ต้นกำเนิดมะเร็งในเซลล์มะเร็งปอดชนิดไม่เล็กผ่านการยับยั้งวิถีเอเคที

นายทรงพล ศรีนวล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

EFFECTS OF VANILLIN ON CANCER STEM-LIKE PHENOTYPES IN NON-SMALL CELL LUNG CANCER THROUGH THE INHIBITION OF AKT PATHWAY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology and Toxicology
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Thesis Title	EFFECTS OF VANILLIN ON CANCER STEM-LIKE PHENOTYPES IN NON-SMALL CELL LUNG CANCER THROUGH THE INHIBITION OF AKT PATHWAY
By	Mr. Songpol Srinual
Field of Study	Pharmacology and Toxicology
Thesis Advisor	Assistant Professor Varisa Pongrakhananon, Ph.D.

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ทรงพล ศรีนวล : ผลของวานิลลินต่อฟีโนไทป์คล้ายเซลล์ต้นกำเนิดมะเร็งในเซลล์มะเร็งปอดชนิดไม่เล็กผ่านการยับยั้งวิถีเอเคที (EFFECTS OF VANILLIN ON CANCER STEM-LIKE PHENOTYPES IN NON-SMALL CELL LUNG CANCER THROUGH THE INHIBITION OF AKT PATHWAY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ. ดร. วริษา พงศ์เรขนานนท์, 76 หน้า.

การแพร่กระจายของมะเร็งและความไม่ประสบความสำเร็จของการรักษามะเร็งในปัจจุบันมีสาเหตุหลักมาจากเซลล์ต้นกำเนิดมะเร็งซึ่งเป็นประชากรส่วนน้อยที่พบในก้อนมะเร็งทั้งหมด โดยเซลล์ดังกล่าวทำให้มะเร็งกลับมาเป็นซ้ำและดื้อต่อยาเคมีบำบัดมากขึ้นโดยเฉพาะอย่างยิ่งในมะเร็งปอด จากการศึกษาพบว่าเอเคทีและโปรตีนที่เกี่ยวข้องในวิถีสัญญาณ มีบทบาทสำคัญต่อพฤติกรรมและการแสดงออกของโปรตีนต่างๆ ของเซลล์ต้นกำเนิดมะเร็ง ดังนั้นเอเคทีและวิถีระดับโมเลกุลที่เกี่ยวข้องจึงเป็นเป้าหมายที่น่าสนใจในการคิดค้นวิจัยสารที่จะพัฒนาไปเป็นยา อย่างไรก็ตามการหาสารจากธรรมชาติเพื่อยับยั้งการแสดงออกของลักษณะดังกล่าวยังมีงานวิจัยไม่มากนัก วานิลลินเป็นสารหนึ่งที่พบในวานิลา (*Vanilla planifolia)* มีการศึกษาถึงฤทธิ์ทางเภสัชวิทยาที่น่าสนใจ รวมทั้งมีฤทธิ์ต้านการแพร่กระจายของเซลล์มะเร็ง อย่างไรก็ตามยังไม่มีการศึกษาฤทธิ์ของวานิลลินต่อการแสดงออกของเซลล์ต้นกำเนิดมะเร็ง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของวานิลลินต่อฟีโนไทป์คล้ายเซลล์ต้นกำเนิดมะเร็งและวิถีสัญญาณที่เกี่ยวข้องในเซลล์มะเร็งปอดชนิดไม่เล็กรวมถึงกลไกที่เกี่ยวข้องกับวิถีเอเคที ผลการศึกษาพบว่า วานิลลินในขนาดที่ไม่เป็นพิษต่อเซลล์มะเร็งปอด สามารถลดปริมาณของโปรตีนบ่งชี้ของเซลล์ต้นกำเนิดมะเร็งและพฤติกรรมที่คล้ายเซลล์ต้นกำเนิดมะเร็ง รวมถึงทรานสคริปชั่นแฟคเตอร์ที่เกี่ยวข้องอย่างมีนัยสำคัญ นอกจากนี้วานิลลินยับยั้งการทำงานของเอเคทีจากการลดการแสดงออกของเอเคที โดยผ่านทางการเพิ่มการทำลายเอเคทีโดยยูบิควิตินโปรตีโอโซม จากการศึกษาแสดงให้เห็นถึงคุณสมบัติใหม่ของวานิลลินในการยับยั้งฟีโนไทป์คล้ายเซลล์ต้นกำเนิดมะเร็งและโปรตีนที่เกี่ยวข้องเพื่อเป็นข้อมูลพื้นฐานในการพัฒนาสารนี้เป็นยารักษามะเร็งในอนาคต

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยาและพิษวิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559

5876111733 : MAJOR PHARMACOLOGY AND TOXICOLOGY
KEYWORDS: CANCER STEM CELLS / OCTAMER-BINDING TRANSCRIPTION FACTOR 4 / PROTEIN KINASE B / UBIQUITIN / VANILLIN / PROTEASOMAL DEGRADATION

SONGPOL SRINUAL: EFFECTS OF VANILLIN ON CANCER STEM-LIKE PHENOTYPES IN NON-SMALL CELL LUNG CANCER THROUGH THE INHIBITION OF AKT PATHWAY. ADVISOR: ASST. PROF. VARISA PONGRAKHANANON, Ph.D., 76 pp.

Metastasis of cancer and failure of current treatment is mostly caused by cancer stem cells (CSCs) which is a minor population within whole tumor. CSCs lead to cancer relapse and chemotherapeutic resistance especially in lung cancer. Previous study reported that protein kinase B (Akt) and its signaling pathways govern both protein signalings and the CSCs phenotypes. Hence, Akt and its molecular pathway become promising target for the development of anticancer drug. However, the study regarding on the negative effect of natural compound on CSCs in cancer have been less discovered. Vanillin, the natural compound from *Vanilla planifolia*, posed attractive pharmacological activities which have been recently found to suppress cancer metastasis. Hence, the effects of vanillin on CSCs have not been previously studied. This study aimed to investigate the effects of vanillin on cancer stem-like phenotypes and related signaling pathway in non-small cell lung cancer H460 cells. The results indicated that non-toxic concentration of vanillin suppresses CSC markers, CSC behaviors and related transcription factors significantly. Focusing on the underlying mechanism, vanillin inhibits Akt activation through a down-regulation of Akt. Interestingly, the decrease of Akt level is caused by vanillin- enhancing Akt degradation via ubiquitin-proteasomal pathway. This finding provides a promising biological activity of vanillin against CSCs which might be benefit for anticancer drug development.

Department: Pharmacology and Physiology
Field of Study: Pharmacology and Toxicology
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Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	=	percentage
μM	=	micromolar
^{o}C	=	degree Celsius
ABCG2	=	ATP-binding cassette subfamily member 2
Akt	=	protein kinase B
ALDH1A1	=	aldehyde dehydrogenase
ANOVA	=	analysis of variance
CO_2	=	carbon dioxide
CSCs	=	cancer stem cells
DMSO	=	dimethylsulfoxide
EDTA	=	ethylenediamine
FBS	=	fetal bovine serum
h	=	hour, hours
min	=	minute (s)
ml	=	milliliter (s)
mM	=	millimolar
MTT	=	3-(4,5-dimethylthiazol-2-Yl)-2,5 diphenyltetrazolium bromide
NSCLC	=	non-small cell lung cancer
Oct4	=	octamer-binding transcription factor 4
P-Akt	=	active Akt (Phosphorylated at Ser473 and Thr308 residues)
PBS	=	phosphate-buffered saline
PI	=	propidium Iodide
PMSF	=	phenylmethylsulfonyl fluoride
RPMI	=	Roswell Park Memorial Institute
S.D.	=	standard deviation
TBST	=	tris-buffered saline solution with 0.1% Tween 20

CHAPTER I
INTRODUCTION

Lung cancer has been reported as a major cause of mortality in cancer which is expressed high metastatic rate than other types of cancers in worldwide (1). Approximately 80 - 85% of lung cancer patients have been defined as non-small cell lung cancer which is highly metastatic potential. Major types of pathological findings are adenocarcinoma, squamous cell carcinoma and large cell carcinoma (2, 3). In spite of surgery, radiofrequency ablation or radiotherapy, systemic chemotherapy and targeted therapy have been used for treatment of NSCLC; however, numerous number of treatments are failure, which is affected on mortality rate has been increased (4). The most important cause behind is an aggressive metastasize of the cancer.

Cancer metastasis is the spreading of cancer cells from the site of primary tumor to other distant organs. This event was related to over 90% of death in cancer patients (5). Recent research discovered the subpopulation of tumor initiating cells or cancer stem cells (CSCs) in the tumor mass that have greatly tumorigenic and self-renewal properties resulting in maintain tumor status, and cancer relapse (6). Clinical observation, *in vitro* and *in vivo* study revealed that lung cancer stem cells is remaining viable after treatment and resist to cisplatin which is the first line therapy for lung cancer (7-10). Lung cancer stem cells are able to survive under stress condition and exhibit the aggressiveness behaviors including motility, invasion and anchorage-independent growth (11). Therefore, the attenuation or removal of CSCs might improve patient outcome.

CSCs express specific surface markers similar with other types of stem cells including Octamer-binding transcription factor 4 (Oct4), Nanog, ATP-binding cassette sub-family G member 2 (ABCG2), and CD133 (12). Oct4 and Nanog are transcription

factors which are responsible in maintaining of pluripotency, self-renewal proliferation and tumorgenicity in both normal stem cells and cancer stem cells (13, 14). Numerous studies reported the relationship between elevation of Oct4 and Nanog and low patient's survival rate including high incidence of metastases cancer (15, 16). In lung cancer, both Oct4 and Nanog are required for maintain CSC-like phenotypes. Overexpression of Oct4 and Nanog enhances a number of spheroid formations *in vitro* and significantly increases of new tumor formation *in vivo*. Likewise, CD133, a transmembrane glycoprotein, was found in human stem cells and its expression is associated with self-renewal, tumorigenesis and differentiation (17). The CD133-positive lung cancer cells isolated from the patients exhibit high level of Oct4 and ABCG2, which obviously display the resistance to chemotherapy and radiotherapy (18). Conversely, an attenuation of Oct4 and Nanog using RNA interference in CSCs leads to the loss of an ability to form spheroid and enhancing the sensitivity of cells to chemotherapy and inhibits tumorigenesis in nude mice (13, 19).

Numerous researches identify the underlying mechanism promoting cancer stemness properties. Lately, Akt and its related pathway were identified as an upstream signaling of Oct4 and augmented CSC-like phenotypes (20). Akt-phosphorylation enhances the stemness through the upregulation of both mRNA and protein level of Oct4 and Nanog (21). The Akt-positive cancer cells, establish the colony formed in soft-agar *in vitro* and *in vivo* nude mice along with an enhancing of CSC markers indicating tumorigenic ability (22, 23). On the other hands, Akt-knockdown experiment through RNA silencing exhibited the massive suppression of Oct4 and stemness behaviors (24).

Natural substances, being one of the fruitful sources, have gain enormously interested as an alternative therapy due to their several pharmacological activities including vanillin (25). Vanillin, 4-Hydroxy-3-methoxybenzaldehyde, is the main active ingredients found in *Vanilla planifolia* seed. It is widely used as a flavoring agent in many products including food, cosmetic and others (26). Previous reports exhibited

the various pharmacological activities of vanillin including antimicrobial, hypotriglyceridemic, anti-inflammatory and antimutagenic in rodent and human (27-29). Vanillin has been shown the efficacy on the inhibition of cancer invasion and migration through the abolishment of matrix metalloprotease activity and downregulation of Nuclear Factor-**K**B in hepatocellular carcinoma cells (30, 31). Vanillin could also attenuate the formation of lamellipodia and angiogenesis in lung cancer through the suppression of PI3K activity and also induce apoptosis of various cancer types such as human cervical cancer and breast cancer (32, 33). Nevertheless, the pharmacological effect of vanillin and its mechanism on CSC-like phenotypes in lung cancer has not been enlightened. In this study, the negative regulator of vanillin on CSC phenotypes and its underlying mechanism were investigated in non-small cell lung cancer H460 cells. The result of this study could provide important information for further development of this compound as anticancer substance.

Research Questions

1. Does vanillin could suppress CSC-like phenotypes including cell surface markers and their behaviors?
2. Does vanillin inhibit CSC-like phenotypes through Akt and its downstream transcription factors related to CSCs?

Objectives

1. To investigate the effect of vanillin on CSC-like phenotype including cell surface markers and their behaviors
2. To determine the effect of vanillin on Akt pathway and its downstream transcription factors related to CSC-like phenotypes

Hypotheses

1. Vanillin is able to diminish CSC-like phenotypes both cell surface markers expression and their behaviors.
2. The mechanism of vanillin on inhibition of CSC-like phenotypes is through Akt and its downstream signaling.

Expected benefits

The results of this study may provide the scientific information of vanillin on regulation of lung cancer stem like phenotypes and its molecular mechanisms including related transcription factors, which benefit to the further development of this novel anticancer substance.

CHAPTER II

LITERATURE REVIEWS

1. Lung cancer

Lung cancer is one of the most common causes of death among many types of cancer. Lung cancer is categorized into two major groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) based on the differentiation under the microscopic examination. NSCLC, the most common type of lung cancer (approximately 85% of lung cancer patients), has three subgroups: adenocarcinoma, squamous cell carcinoma and large cell carcinoma (4, 34). In 2015, the cancer statistic reported that the incidence rate of lung cancer diminished in 2.2% per year in women and 3.0% in men. Also, the death of lung cancer patients was decreased at rates 2.9% per year; however, lung cancer is still the top of cancer ranking (Figure 1). The risk factors of lung cancer are cigarette, asbestos, radiation, air pollution and diesel exhaust (2, 35, 36).

Estimated Death	Male		Female	
Lung & bronchus	85,920	27%	72,160	26%
Prostate	26,120	8%	40,450	14%
Colon & Rectum	26,020	8%	23,170	8%
Pancreas	21,450	7%	20,300	7%
Liver & intrahepatic bile duct	18,280	6%	14,240	5%
Leukemia	14,130	4%	10,470	4%
Esophagus	12,720	4%	10,270	4%
Urinary Bladder	11,820	4%	8,890	3%
Non-Hodgkin lymphoma	11,520	4%	8,630	3%
Brain & other nervous system	9,440	3%	6,610	2%
All Sites	31,290	100%	281,400	100%

Figure 1 Ranking various by cancer types for the deaths by sex, United States, 2016 (1)

Treatments of lung cancer are included surgery, radiation chemotherapy and targeted therapy (37). Although the new strategies to cure the lung cancer were

develop, the clinical outcome still be failure. One of the causes behind is due to the presence of aggressive subpopulation within tumor, called cancer stem cells (38).

2. Cancer stem cells

Cancer stem cells (CSCs) or tumor initiating cells (TICs), are sub-population identified in hematopoietic malignancy and in many solid tumors including breast, colon and lung cancer (39). CSCs hypotheses were firstly proposed in acute myeloid leukemia (AML). The population of cells with $CD34^{+}CD38^{-}$ surface markers, transplanted into severe immunodeficiency (SCID) mice, showed capability of proliferation and tumor initiation extensively (40, 41). Later, CSCs in solid tumor was founded in brain and breast cancers (42, 43). Following these finding, many researchers were initiated to identify the characteristic and properties of cancer stem cells.

2.1 Characteristics of CSCs

One of these proposed that CSCs was originated from normal stem cells mutation (44). On the contrary, progenitor cells and differentiated cell mutation were also played role as original of cancer stem cells (45). They hypothesize that some population become CSCs but not all trait of cancer cells are expresses this capability (46). CSCs express the capability of self-renewal, immortality, resistance to chemotherapy, and high colonogenicity, and they are able to propagate and generate new malignancy both *in vitro* and *in vivo* (47-49). Moreover, CSCs are thought as potential cause of therapy resistance, minimal residual disease, relapse after initial successful therapy, and evade conventional treatment modalities (50). To define these population, the cancer stem cells markers which is expressed differently among different types of cells were able to use as a tool to isolate these population (51).

2.2 Cancer stem cell markers

Many strategies to isolate these population form whole tumor were developed as essential tools including cancer stem cells markers. The isolation of CSCs from other tumor cells or normal stem cells were used those markers specifically for each types of cancer (52). Many CSCs markers phenotypes were summarized in Table 1 (51).

Table 1 CSCs markers in distinctly in various tumors (51)

Breast	Colon	Pancreatic	Prostate	Lung	Ovarian
ALDH1	ABCB5	ABCG2	ALDH1	ABCG2	CD24
CD133	ALDH1	ALDH1	CD133	ALDH1	CD44
CD44	CD133	CD44	$\alpha_2\beta_1$integrin	CD133	CD117
CD90	CD44	CD133	Trop2	CD90	CD133

2.2.1 ATP-binding cassette subtype G2

ATP-binding cassette (ABC) transporters are membrane transporter, which is able to efflux of many substances including cytotoxic drugs. ABCG2 is one of subtype expressed in both normal tissue and cancer stem cells population which is transcribed from *ABCG2* gene located on chromosome 4q22 (53). Elevation of this transporter is highly prominent in cancer stem cells resulted in chemotherapeutic resistance (54). Relationship between ABCG2 and CSCs have been reported that isolated cells which are highly express of ABCG2 ($ABCG2^+$) exhibited self-renewal properties maintaining the cancer cell population and developed the progression of cancer (55). In lung cancer, elevated expression of ABCG2 was founded in stem cells isolated from six human lung cancer cell lines which exhibited drug resistance (56). In cisplatin-treated lung cancer cell lines (A549 and H460), which turned out to cancer stem-like phenotypes, exhibit the elevation of ABCG2 and contribute to drug resistance (57).

2.2.2 Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) is an enzyme comprised of 19 isoforms which located in various parts of cell such as cytoplasm, mitochondria and nucleus (58). ALDH was normally responsible for detoxification of aldehyde which are generated by many processes of cells including lipid peroxidation into carboxylic acid (59, 60). Overexpression of ALDH found in various cancers which enhance cancer aggressiveness and relapse (61). ALDH isoform 1A1 (ALDH1A1) was found to regulate the signaling pathway of stemness (62, 63). ALDH1A1 exhibited function on metabolism and detoxification of chemotherapeutic agents such as cyclophosphamide (64, 65). In CSCs population, ALDH expression and activity were extensively enhanced and resisted to chemotherapy (66). In lung cancer, the ALDH1A1 were regulated by notch signaling pathway, an important pathway of CSCs, which resulted in the reduction of survival rate (67).

2.2.3 CD133

CD133 or Prominin-1 is a five-membered transmembrane glycoprotein translated form gene located on chromosome 4 (4q15.33) in human (68). Expression of CD133 was activated by various stimuli including hypoxia, mitochondrial dysfunction, transforming growth factor beta (TGF-β)-induced environment and epigenetic factors such as demethylation in cancers (69, 70). Numerous literatures indicate that CD133 was involved in several behaviors of cancer such as metastasis, chemo- or radio-resistance and also properties of cancer stem cells (71). Overexpression of CD133 ($CD133^+$) was found in many types of cancers such as colorectal, ovarian cancer and others which related to f spheroid formation and tumorgenesis capacity both *in vitro* and *in vivo*, indicating its role on an enhancing of CSC-like phenotype (72, 73). Upregulation of signaling pathway related to survival of stemness included mitogen-activated protein kinase (MAPK) and Protein kinase B (PKB, Akt) also founded in $CD133^+$

colon cancer cells (74). Isolation of $CD133^+$ from lung cancer cell line (A549 and H157 cells) expressed cisplatin-resistant phenotypes, positive spheroid formation and upregulation of transcriptions and markers-related to CSCs (75, 76).

2.3 Transcription factors

Cancer stem cells (CSCs) and embryonic stem cells (ESCs) shared several signaling pathways including transcription factors, Oct4 and Nanog (77). Overexpression of these proteins were defined higher in tumor cells and CSCs than ESC or normal tissue which effect on proliferation and self-renewal properties of CSCs (78).

2.3.1 Octamer-binding transcription factor 4

Oct4 was stranded for octamer binding transcription factor 4 or POU5F1. This protein was transcribed by *POU5F1* gene located on chromosome 6 of human chromosome to 4 isoforms; Oct4A, Oct4B-190, Oct4B-265, and Oct4B-164 (79). Oct4A was found in oocytes, embryonic stem cells and embryonic carcinoma cells which involved in self-renewal property. Whereas Oct4B and Oct4B1 differed from Oct4A in N-transactivation domain play a role in cell stress and cell differentiation (79-81). Many literatures were reported the relationship between Oct4 and cancers both *in vitro* and *in vivo*. High level of oct4 expression have been indicated as high aggressive tumor, poor prognosis of patient, tumorgenicity and cancer relapse. Clinical data reported that Oct4 expressed at high level in patients who had cancer stage 3 and 4 or metastatic stage (82, 83). Meanwhile, *in vitro* and *in vivo* experiments also exhibited the relationship between high Oct4 expression and CSCs-like phenotypes. In $Oct4^+$ cells, the formation of tumorsphere with the expression of cancer stem markers such as CD133, ALDH1 and tumorigenic potential were greater compared with $Oct4^-$ cells (18). In the same way, elevated Oct4 expression was correlated with poor prognosis and chemotherapeutic resistance in head and neck carcinoma (84). Lu and colleagues

demonstrated that Oct4 knockdown CSCs lead to the decreasing of proliferation, migration and tumorgenesis (19). In lung cancer, Oct4 was played as crucial role in maintain stem-like phenotypes and linked to CD133 upregulated (18). While overexpression of Oct4 was extremely correlated with poor clinicopathological and prognostic phenotypes of non-small cell lung cancers (NSCLC) (85).

2.3.2 Nanog

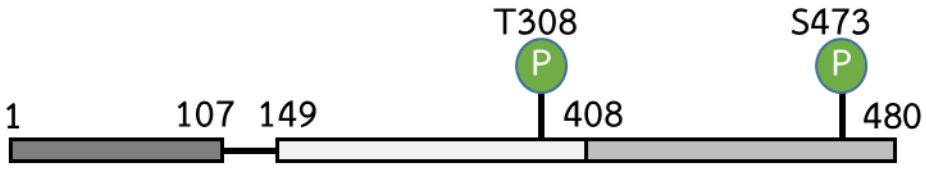
Nanog is a transcription factor responsible for self-renewal and reprogramming of pluripotent in both normal stem cells and cancer stem cells (78). Nanog was transcribed from *NANOG* gene located on chromosome 12 of human (86). Many literatures focused on role of Nanog expression and its signaling proteins in cancer including cancer stem cells. Captivating evidence demonstrates that Nanog enhances CSC phenotype by saturating subset or cancer cells with self-renewal capacity and enhancing of immune-resistance of cancer cells (87). $CD44^+$ and $CD133^+$ exhibits positive correlation with enriching of Nanog expression in various cancer types including breast, prostate and ovarian cancers (88-90). Furthermore, Nanog expression level was showed to be correlated with an induction of CSC phenotypes and immuno-resistance through an upregulation of T-cell leukemia/lymphoma 1A/Akt (Tcl1A/Akt) pathway in human cervical cancer (24). Down-regulated Nanog expression in human gastric cancer cells diminishes proliferation, invasion and migration capability of cancer cells (91). In lung cancer, the expression levels of Nanog were increased compared to the nearby normal lung tissues, and positively correlated with elevation of advance stage of cancer which becomes worse prognosis for lung cancer patients (92).

3. Protein kinase B / Akt in CSCs

Several signaling pathways have been demonstrated as essential regulators on the capacity of cancer stem cell. The phosphatidylinositol 3-kinase/Akt, Janus-activated kinase/signal transducer and activator of transcription, Jak/Stat, Wnt, Hedgehog, Notch and nuclear factor-κB signaling pathways have all been upregulated to mediate numerous cancer stem cell properties including self-renewal, survival, proliferation, and expression of CSCs markers (93). Massive studies have emphasized the linkage between PI3K/Akt/mTOR signaling and cancer stem cells (94). Phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways are crucial in many physiological and pathological conditions, including cell proliferation, angiogenesis, metabolism, differentiation and survival (95). In Lung cancer, the expression of Akt and its activity were pronounced and strongly related to the cancer aggressiveness (96).

3.1 Akt structure and family

Protein kinase B (PKB), known as Akt, was serine/threonine kinase protein which is related to many processes of both normal cells, normal stem cells, cancer cells and cancer stem cells included cell proliferation, metabolism, survival, cell cycle progression and self-renewal capacity. Akt consists of 480 amino acid divided to 3 constructive domains, an N-terminal pleckstrin homology (PH) domain, central catalytic kinase and carboxy-terminal extension that contains a hydrophobic motif for characterization of AGC kinase protein (97). Three isoforms of human Akt are Akt1, Akt2 and Akt3 which are transcribed by *AKT* gene located at human chromosome 14, 19 and 1, respectively. The major domain and its homology among these isoforms was described in Figure 2 (98).



Pair	PH	LINK	CAT	EXT
Akt1/Akt2	80	46	90	66
Akt1/Akt3	84	40	88	76
Akt2/Akt3	76	17	87	70

Figure 2 Akt structure and % identity between each isoforms. Domain definition using Akt1 residue numbers (upper). The homology compared between each isoform of Akt. PH - pleckstrin homology (PH) domains, LINK – Linker region, CAT – Catalytic kinase domain, EXT – C-terminal extension (lower) (98).

3.2 The biological functions of Akt

Akt isoforms contribute to various cellular responses, including cell growth, cell survival and metabolism (97). This multiplicity of Akt functions might be caused by the variation and specificity of its substrates. Recently, proteins which are regulated by Akt was found more than 50 proteins including cell cycle regulators, apoptotic proteins and cancer stem cells regulating pathway (98). For instance, p21, cyclin-dependent kinase inhibitors, have been reported as a direct substrate of Akt. The p21 functions to mediate cell cycle arrest, the phosphorylation of this protein at Thr145 and Ser146 by Akt can suppress p21 activity (99). Furthermore, Akt was also phosphorylated GSK3β which prevent β-catenin degradation, and was a mechanistic target of rapamycin complex (mTOR) to enhance cell proliferation and survival (100). BAD and caspase 9 which are the signaling proteins in apoptotic pathway was also regulated by Akt through

phosphorylation at Ser136 and Ser196 resulting in a reduction of apoptotic activity (101). The regulation and target of Akt were summarized in Figure 3.

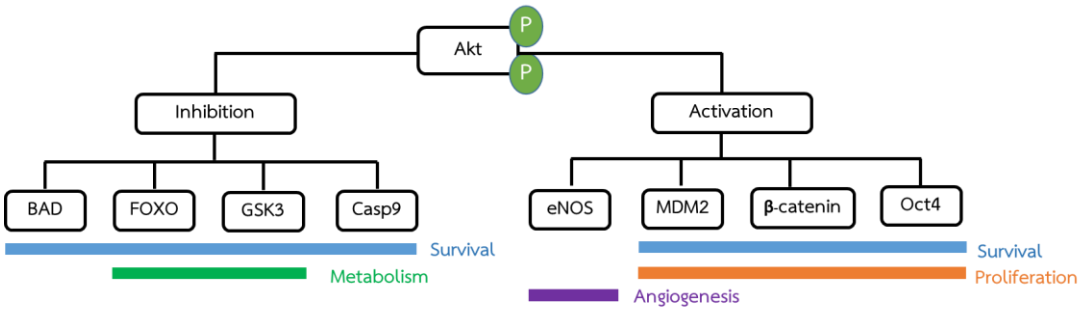


Figure 3 Cellular function and the target of Akt (102)

3.3 Akt signaling pathway and cancer stem cells

Massive studies exhibited the role of Akt in prolongation of CSCs. In prostate cancer, activation of PI3K/Akt/mTOR signaling pathway was connected with elevation of CSC phenotypes and chemotherapy resistance. It has reported that Akt regulate the CSCs markers and their behavior through the transcription factor-related to CSCS (103). Deleterious of Akt downstream signaling through suppression of Akt could decrease aldehyde dehydrogenase 1 (ALDH1) activity and others CSC markers in colorectal cancer and hepatocellular cancer stem cells (104). ABCG2 expression was regulated by PI3K/Akt signaling pathway in glioma tumor stem-like cells (23). Activation of PI3K could enhance expression of ABCG2 in both acute myeloid leukemia and acute lymphatic leukemia (105). In contrast, Inhibiting PI3K activity which is also reducing Akt activity could eliminate the generation and growth of $CD44^{High}/CD24^{Low}$ spheroid formation (106). Regulation of Nanog and Oct4 through phosphorylation has been reported and founded that downregulation of Akt could diminish Oct4 and Nanog dramatically. Inhibition of Akt could suppress activity of Oct4 which is resulted in stem-

like phenotype dwindle (21). The correlation between Akt, Nanog and Oct4 has been showed in Figure 4.

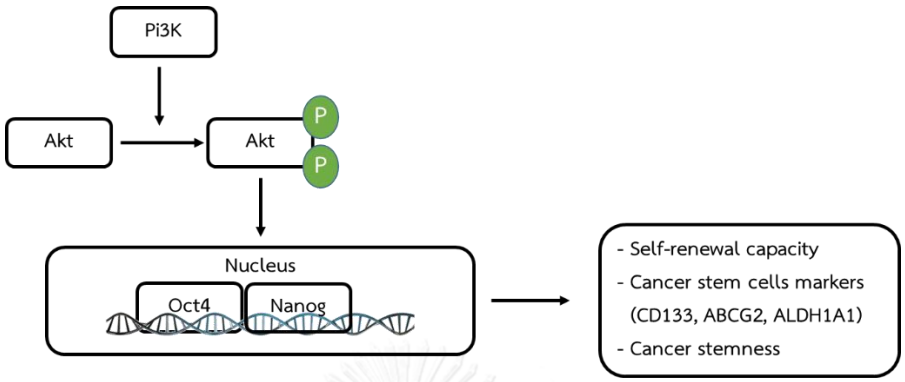


Figure 4 Regulation of Oct4 and Nanog through Akt-mediating pathway (107).

3.4 Physiological activation and regulation of Akt

The functions of Akt can be regulated by 2 major processes, its activation and degradation. Akt could be activated by phosphorylation at Thr308 through phosphatidylinositoal-3 kinase (PI3K), and phosphorylation at Ser473 in C-terminal domain is accounted for full activation of Akt (108). Meanwhile, Akt could be phosphorylated at other region by various proteins including β-arrestin-2, Src, mTORC2, PDK1, Integrin link kinase (ILK) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), CDK2 and cyclin A (109). In contrast, dephosphorylated inactivation of Akt can be caused by phosphatase and tensin homolog (PTEN), Protein phosphatase 2A (PP2A) and leucine rich repeat protein phosphatase (PHLPP). PTEN also prevents membrane recruitment of Akt and inhibits its activity. PP2A dephophorylates Akt directly at Thr308, while PHLPP affects on Ser473 dephosphorylation (110). Furthermore, SUMOylation and acetylation could regulate the activation of Akt. SUMOylation of Akt at K276 is aroused by SUMO E3 ligase leading to an activation of Akt, but actylation at K20 and K14 within PH domain leading to Akt inactivation (111). In case of Akt degradation, it is caused by various

processes including ubiquitin-proteasome degradation through BRCA1, TTC3 and MULAN ubiquitin ligase, which are responsible for K48-linked ubiquitin formation and terminate its activity (112, 113).

4. Vanillin

Vanillin, 4-Hydroxy-3-methoxybenzaldehyde, is a major active ingredient found in *Vanilla planifolia* seed (Figure 5). It is wildly used as a flavoring agent in many products including food, cosmetic and others. Natural vanillin can be collected from the seed while synthetic vanillin was synthesized by using ferulic acid as a substrate (114). Physicochemical properties of vanillin have been reported. Vanillin is freely soluble in water (1 g/ 100 mL) and others organic solvent such as acetone, hot benzene and chloroform (26). Vanillin is highly oxidized in alkaline condition which is saturated with oxygen and high temperature. The oxidation process was occurred through various process including enzymatic oxidation by dominantly aldehyde oxidase but minorly xanthine oxidase or peroxidase enzyme that is produce the vanillin acid (115). Vanillin also has been reported the ability to interact with many proteins such as bovine serum albumin and ovalbumin (116). The application of vanillin in pharmacological aspect has been aroused. Pharmacokinetic study of vanillin *in vivo* has been studied and the parameters after intravenous and oral administration are described in Table 2.

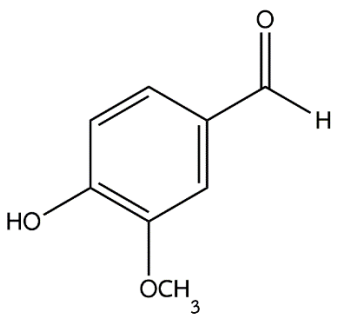


Figure 5 Vanillin (4-Hydroxy-3-methoxybenzaldehyde)

Table 2 Pharmacokinetic parameters of vanillin in plasma following intravenous (I.V.) and oral (P.O.) administration in male Sprague-Dawley rats (117)

PK parameters	**I.V.**	**P.O**
Dose	20 mg/kg	100 mg/kg
AUC_{o-t} (ng h mL^{-1})	3,915.27 ± 620.05	1,304.41 ± 74.64
$AUC_{o-\infty}$ (ng h mL^{-1})	4,298.21 ± 575.32	1,634.87 ± 231.88
C_{max} (ng/mL)	N/A	290.24 ± 44.30
$T_{1/2}$	5.10 ± 1.57	10.32 ± 2.62
CL	4.72 ± 0.65	62.17 ± 8.50
V_{ss} (L)	29.79 ± 13.86	N/A
Bioavailability (F)	N/A	7.6%

Later, numerous reports demonstrated the several biological activities of vanillin such as antimicrobial, hypotriglyceridemic, anti-inflammatory and antimutagenic in rodent and human (27-29, 118). Vanillin exhibits neuroprotective effect including attenuation of behavioral impairment, neurotransmitter defect, oxidative stress and apoptosis in both rotenone-induced Parkinson's disease in rat model and neuroblastoma cell line (119). Antioxidant properties of vanillin has been reported *in vitro* that it has higher potency against oxidative stress than standard substance such as Trolox or ascorbic acid after expose to the UV irradiation (118, 120). Massive reports on the effect of vanillin in cancers have been elevated. Invasion and migration which is occurred through enhancing of matrix metalloprotease activity and upregulation of Nuclear Factor-**κ**B could be inhibited by treatment of vanillin in hepatocellular carcinoma (30). Other mechanisms related to cancer metastasis such as lamellipodia and angiogenesis could also be suppressed by treatment with vanillin. The apoptosis induction of vanillin has been reported in various cancer types such as human cervical cancer and breast cancer (32, 33). The synergistic effect of co-treatment between vanillin and doxorubicin in MCF-7 breast cancer cell has been reported. The

mechanism occurred through induction of apoptosis and inhibition of cell growth (121). Nevertheless, the CSC-like property in lung cancer which is a major cause of cancer aggressiveness has remaining undiscovered.

CHAPTER III
MATERIALS AND METHODS

Materials

1. Cell culture

Human non-small cell lung cancer cells (NCI-H460) were purchased from American Type Culture Collection, ATCC (Manassas, VA, USA). NCI-H460 were cultured in monolayers with Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin and streptomycin and 10% fetal bovine serum (FBS). Cell cultures were incubated at 37°C in a humidified incubator fulfilled with 5% CO2. Cells were sub-cultured routinely with 0.25% Trypsin in 0.53 mM EDTA and were seeded as recommended by the supplier around 70% confluence.

2. Chemicals and Reagents

Vanillin in USP-secondary standard purity grade (> 99.0% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutamate, penicillin-streptomycin antibiotics, phosphate buffered saline (PBS) were purchased from GIBCO (Grand Island, NY, USA). Methanol, Dimethyl sulfoxide (DMSO) and mouse monoclonal antibodies to Oct-4, ubiquitin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit monoclonal antibodies to ABCG2, beta-catenin, Nanog, phosphorylated AKT (P-AKT) in Ser473 and Thr308 were from Cell Signaling Technology, Inc. (MA, USA). Goat monoclonal antibody against ALDH1A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and rabbit monoclonal antibody against CD133 was purchased from United State biological (USbiological, MA, USA).

3. Equipments

Automated cell counter (TC20 Bio-Rad, Singapore), autopipette 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl (pipetman, Gilson, Middleton, WI, USA), Fluorescence microplate reader (Anthros, Durham, USA), cell culture plate: 6-well, 24-well, 96-well, ultra-low attachment plate and pipette tips specified for each volume of autopipette (Corning, NY, USA), glass bottle: 100, 250, 500, and 1,000 ml (Duran, Mainz, Germany), fluorescence microscope (Olympus IX51 with DP70, Japan), Centrifuge (CF-10 Wise spin, Korea), laminar air flow cabinet (Bosstech, Bangkok Thailand), Humidified incubator (Thermo scientific, Waltham, MA, USA), pH meter (SevenCompactS220, Mettler-Toledo, Zürich, Switzerland) and vortex mixer (Scientific industries, NY, USA)

Methods

1. Sample preparation

Vanillin in USP-secondary standard (> 99.0% purity) grade obtained from Sigma-Aldrich was prepared to the 1mM stock solution. A 1.55 mg of vanillin was freshly diluted with RPMI supplemented with 10% FBS media. Treated solution was prepared by dilution of stock solution with culture media to working concentrations.

2. Cell viability and cell proliferation assays

Cell viability and Proliferation assays were performed by using the colorimetric MTT assay. Briefly, H460 cells were seeded onto 96-well plate in amount of 10,000 cells and 5,000 cells per well for cell viability and proliferation assay respectively. After cell attachment, various concentrations of vanillin (0-400 µM) were treated for 24 h for cell viability assay. Pretreated cells with the same concentration of vanillin for 1 day and 3 days were sub-cultured and leaved for 24, 48 and 72 h for cell proliferation assays. At the end of each incubation time, the medium was removed and replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (life

technologies, Carlsbad, CA, USA) and re-incubated for 3 h at 37°C, then the MTT solution was substituted with 100 µl dimethyl sulfoxide (DMSO) to dissolve the occurring formazan crystal and measure the absorbance at 570 nm by using microplate reader. The absorbances were calculated and represented as % viability and % relative proliferation compared to the 100% of non-treated cells.

$$\% \text{ cell viability} = \frac{\text{A570 of treatment group}}{\text{A570 of control group}} \times 100$$

$$\% \text{ relative proliferation} = \frac{\text{A570 of treatment group at each time point}}{\text{A570 of control group at each time point}} \times 100$$

3. Nuclear staining assay

Hoechst 33342 and propidium iodide (PI) (sigma-aldrich, St. Louis, MO, USA) were used to stain the nuclei to identify necrotic and apoptotic cell death respectively. Cells were treated with vanillin for 24 h and after washing with phosphate buffer saline (PBS), cells were incubated with either 5 µM PI or 10 µM Hoechst 33342 at 37°C for 30 min. Cells were visualized and captured under fluorescent microscope at 10x magnifier (Olympus IX5; 10X with DP70 digital camera system, Tokyo, Japan). PI-positive necrotic cells and nuclear condensation of apoptotic cells were scored and analyzed.

4. Anchorage-independent growth assay

Human non-small cell lung cancer cells were pretreated with vanillin (0-50 µM) for 1 and 3 days before subject to the assay. Soft agar colony-formation assay was used to determine the anchorage independent growth property of cancer stemness. Soft agar was prepared by the combination between complete media (RPMI 1640 supplemented with 10% FBS) and 1% agarose in the 1:1 ratio. This mixture was left for

solidify in 24-well plate as a bottom layer. Then, pretreated cells were suspended in RPMI complete media is mixed with 0.3% agarose, added onto the prepared bottom layer, and incubated at 37°C. Complete media would be applied every 2 days to prevent the soft agar dryness. After 2 weeks, the colony was examined under phase-contrast microscope with 4X magnification (Olympus IX5; 4X with DP70 digital camera system, Tokyo, Japan). Relative colony number and size were analyzed compared to the control group.

$$\text{Relative colony size or number} = \frac{\text{colony size or number of each treatment}}{\text{colony size or number of control}} \times 100$$

5. Spheroid formation assay

Human NCI-H460 lung cancer cells were pretreated with vanillin in various concentration (0 – 50 μM) for 1 and 3 days. Later, pretreated cells at the number of 2,500 cells/well were cultured onto 24-well ultralow attachment plate in RPMI serum-free medium for 7 days. After that, spheroids were captured under phase-contrast microscope with 4X magnification as primary spheroid (Olympus 1X51 with DP70). Primary spheroids were disaggregated by trypsinization, resuspensed in RPMI serum-free medium as a single cell, and cultured onto 24-well ultralow attachment plate. After further 14 days, secondary spheroids were investigated by phase-contrast microscope with 4X magnification (Olympus 1X51 with DP70). Relative spheroid number and size were analyzed compared to the control group.

$$\text{Relative spheroid size or number} = \frac{\text{spheroid size or number of each treatments}}{\text{spheroid size or number of control}} \times 100$$

6. Western blot analysis

Cells are lysed using lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10% glycerol, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich, MO, USA), and protease inhibitor cocktail (Merck Millipore, MA, USA), sonicated and incubated on ice bath for 45 min. Proteins content were measured by BCA protein assay kit (Pierce™ Thermo Fisher Scientific Inc, IL, USA). An equally amount of denatured protein was separated by 10% SDS-Poly-acrylamide and transferred to 0.45 um nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat-milk in Tris buffer solution with 0.1% Tween 20 (TBST) for at least 30 min and incubated with indicated primary antibody at 4 °C overnight. The antibody concentrations used were shown in the Table 3). After that, membranes were washed with TBST for 3 times and incubated with secondary antibody at room temperature for 2 h. The antigen-antibody complexes were detected using chemiluminescent solution (Pierce Biotechnology, Inc., IL, USA), and exposed to the film (Carestream Health, Inc., Rochester, NY, USA). The densitometry of target protein was measured by imageJ and quantified as a relative expression to control group.

Table 3 The concentrations of antibody used in Western blot analysis.

Primary antibody	**Dilution**
CSC markers: CD133, ABCG2, ALDH1A1	1:1000
Transcription factors: Oct4, Nanog	1:1000
Akt and its active form: P-Akt (Ser473, Thr308), Akt	1:1000
Anti-ubiquitin	1:1000
GAPDH	1:1000

7. Immunoprecipitation assay

Cells were pretreated cells with Lactacystin 10 μM for 1 h and treated with vanillin. Later, cells were collected and lysed by lysis buffer containing TMN buffer (50 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.5 mM $MgCl_2$) supplemented with 10 % glycerol, 10% protease inhibitor cocktail, 1 % nonylphenylpolyethylene glycol (NP-40) and 1 % PMSF for 30 mins and centrifugation at 12,000 rpm at 4°C for 15 min. The supernatants were blocked with protein G agarose bead (GE Healthcare, Little Chalfont, UK) to remove non-specific binding. After centrifugation at 3,000 rpm at 4°C for 5 min, protein contents were measured. The supernatants were collected and stored at 4°C as an input for immunoblotting. An amount of 300 μg of protein was incubated with anti-Akt antibody (how much?) at 4°C overnight, and incubated with protein G agarose beads for 2 h at 4°C. The immunoprecipitates were collected, washed with TMN buffer and resuspended in 30 μl of 2x SDS sample buffer. Samples were boiled at 95°C for 5 min, and subjected to Western blot analysis using antibodies against ubiquitin (1:1000).

8. Statistical analysis

Collected data from at least 4 independent-experiments were normalized by control groups and presented as mean ± standard deviation (SD). The statistical difference among the groups was analyzed by using one-way ANOVA followed by Scheffe's the post hoc test. SPSS software version 22 (IBM Inc., NY, USA) is used to perform statistical analysis. The statistical significant was considered at $p < 0.05$.

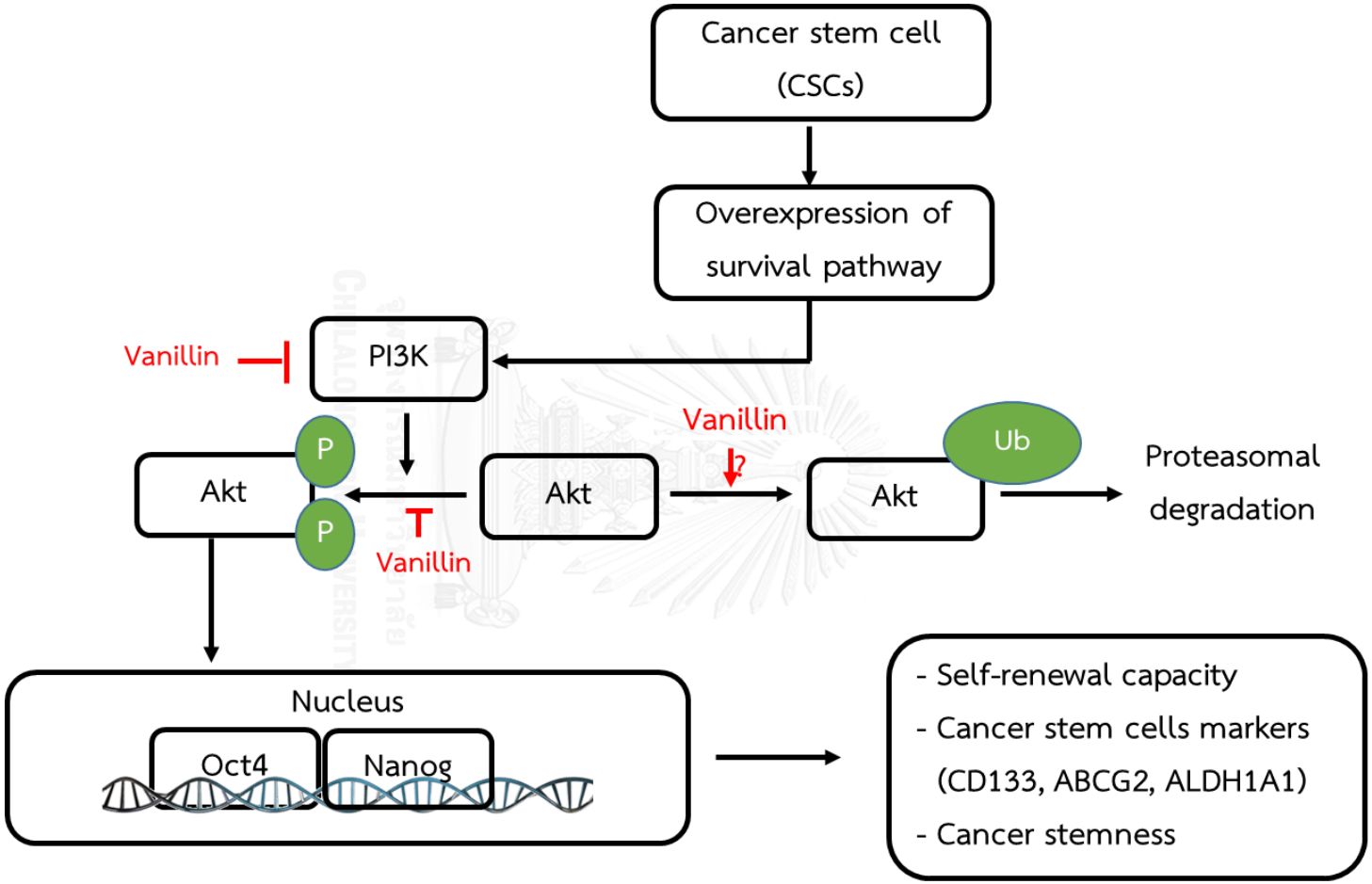


Figure 6 Conceptual framework of this study

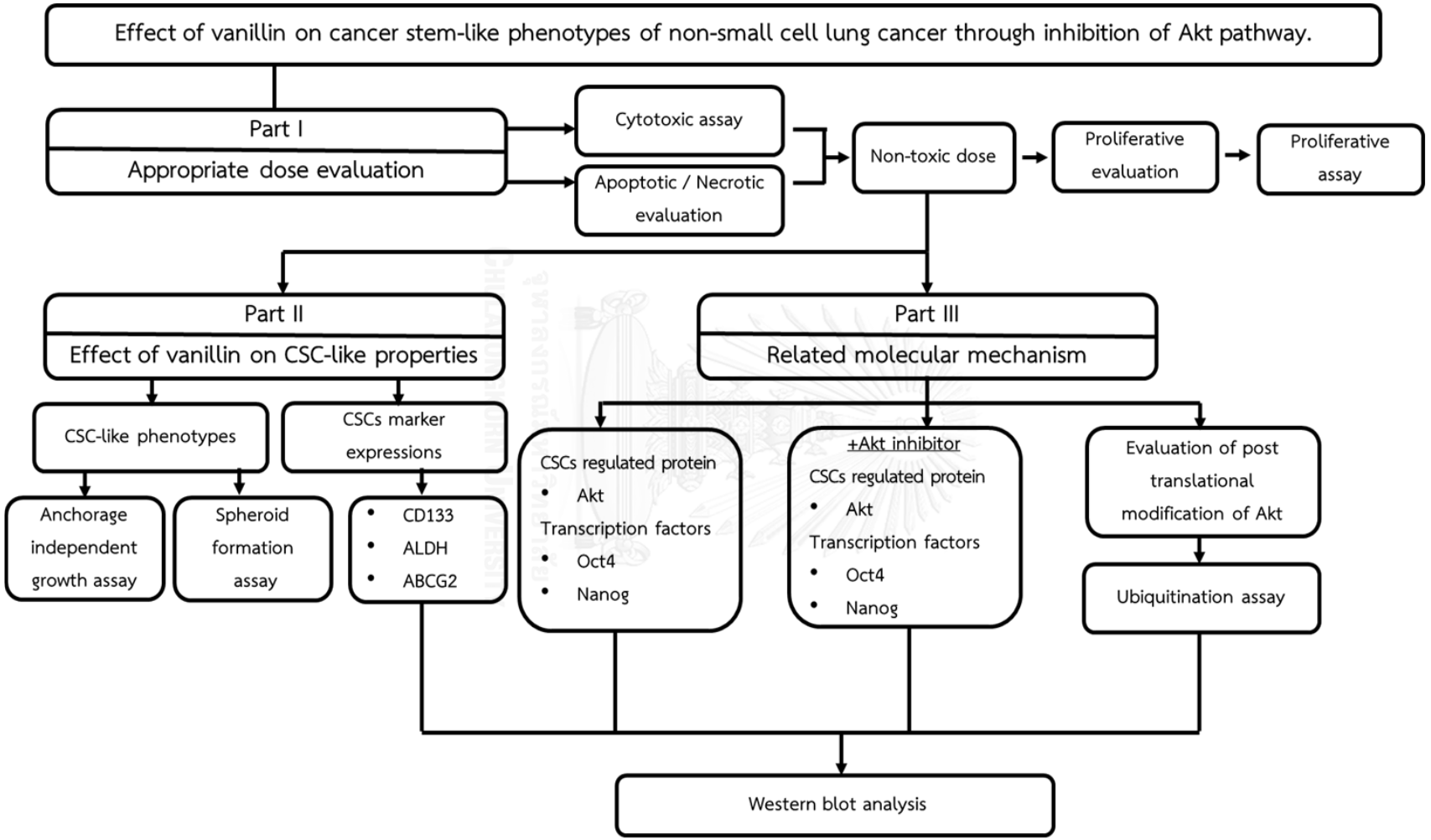


Figure 7 Experimental designs

Part I: Investigation of cytotoxic effects of vanillin in human non-small cell lung cancer H460 cells

1. **Evaluating the cytotoxic effects of vanillin in human lung cancer cell line**

Initially, cytotoxic assay was performed to determine appropriate concentrations of vanillin that cause no effect on human lung cancer cell lines. Cells were treated with vanillin (0-200 μM) for 24 h. Percent cell death was calculated from the optical density measured at 570 nm. Also, apoptotic nuclei and necrotic nuclei were determined by staining with Hoechst 33342 and Propidium Iodide compared with control, respectively. Concentration of vanillin which has percent viability more than 80% and does not cause apoptosis or necrotic nuclei will be used in the following experiments.

2. **Investigating the proliferative effect of vanillin in human lung cancer cell line**

Concentration of vanillin which is effect on proliferation of NCI-H460 cells were ruled out to eradicate confounding effect on stemness dwindling. H460 cells were treated with non-cytotoxic concentration of vanillin. Then, MTT colorimetric assay were performed to determine proliferation effect of vanillin at 24, 48 and 72 hour. Vanillin dose that showed no effect on cell proliferation was used to further study cancer stemness assay and other experiments.

Part II: Determination of effects of vanillin on cancer stem-like phenotypes in human non-small cell lung cancer H460 cells

1. **Determining the effect of vanillin on CSC-like phenotypes in human lung cancer cell line**

Because self-renewal and tumorgenicity were the distinctive behaviors of cancer stem-like phenotypes, anchorage-independent growth and spheroid formation had been used to characterize these features (122). To investigate the suppressive effect of vanillin on colony and spheroid formation, H460 cells were pretreated with non-toxic doses of vanillin for 1 and 3 days, followed by the examination of anchorage-independent growth and spheroid formation.

2. **Investigating of the effect of vanillin on CSC marker expressions in human lung cancer cell line**

Cancer stem cells markers were expressed differently in each types of cancer by which CD133, ABCG2, and ALDH1A1 were distinctly expressed in lung cancer stem cells (51). NCI-H460 cells were incubated with non-toxic concentration of vanillin for 1 and 3 days, and the expression of CSCs markers was determined by western blot analysis.

3. **Investigating the effect of vanillin on CSC-related transcription factors and signaling proteins in human lung cancer cell line**

CSCs phenotypes displayed not only the elevation of stemness markers, but also had high level of the related transcription factor including Nanog and Oct4 (123). To assess whether the negative regulation of vanillin on CSCs had an involvement on these transcription factors, H460 cells were treated with non-toxic concentrations of vanillin for 1 and 3 days, and the expressions of Oct4 and Nanog were evaluated.

Part III: Investigation of Akt-related mechanism of vanillin on cancer stem-like phenotypes in human non-small cell lung cancer H460 cells

1. Investigating the effect of vanillin on Akt in human lung cancer cell line

Because the expressions of Nanog and Oct4, contributing to the stemness behaviors, were regulated directly by Akt (20). Evidences suggest that Akt played a vital role on cell survival and proliferation. Akt had been shown to drives cancer cells to CSCs-like phenotypes in many aspects including proliferation, migration, and self-renewal properties through the transcription factors Oct4 and Nanog. Blockage of Akt inhibited *in vitro* proliferation of spheroid formation and enhanced Oct4 degradation. Hence, the role of vanillin in regulating CSC was further identified whether Akt expression and its activity were disturbed. H460 cells were either treated with vanillin or perifosine (1,1-Dimethylpiperidinium-4-yl octadecyl phosphate), an Akt inhibitor as a positive control, prior to protein expression analysis by western blot assay.

2. Investigating the effect of vanillin on Akt degradation

The rate of Akt degradation is a dominant mechanism that related to the function of Akt, by which an ubiquitin-proteasomal is a major pathway responsible for Akt degradation (113). Enhancing of this degradation pathway affected both stability and activity of this protein and consequently altered its downstream signaling (112). To assess the effect of vanillin on of Akt-ubiquitin degradation, H460 cells were treated with proteasome inhibitor, lactacystin, prior to treated with vanillin. Then, the ubiquitin-Akt complexes were examined by Immunoprecipitation assay.

CHAPTER IV
RESULTS

Part I Cytotoxic evaluation of vanillin in NCI-H460 human lung cancer cell line

To evaluate cytotoxic effect of vanillin in NCI-H460 human lung cancer cell line, cell viability assay was performed using MTT colorimetric assay. Cells were treated with vanillin in various concentrations (0 – 200 μM) for 24 h. Figure 8A demonstrated that the concentrations between 0 – 50 μM had non-cytotoxic effect, whereas the concentration more than 100 μM significantly decreased cell viability of on NCI-H460 lung cancer cells. To further confirm mode of cell death after treated with vanillin, apoptotic and necrotic cell deaths were examined by Hoechst 33342 and Propidium Iodide (PI) nuclear-staining assay. The result demonstrated that vanillin less than 50 μM did not cause either necrotic or apoptotic cells, consistency with MTT assay (Figure 8B and C). Apoptotic and necrotic nuclei were significantly found in response to vanillin higher than 100 μM compared with non-treated group. These non-toxic doses of vanillin were used in further experiments.

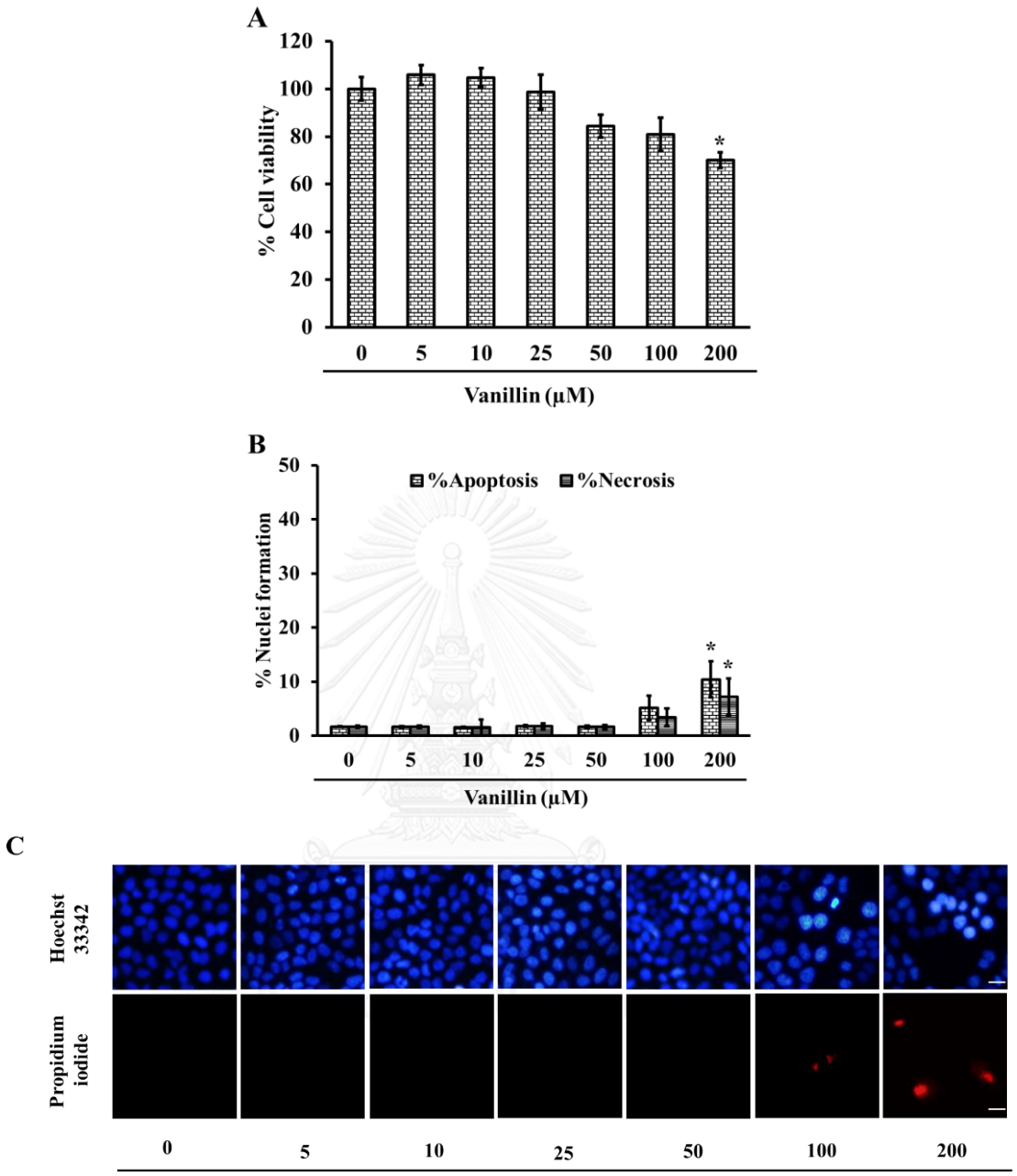


Figure 8 Cytotoxic evaluation of vanillin on NCI-H460 cells. Cells were treated with vanillin in various concentrations for 24 h. **A.** cytotoxicity of vanillin was evaluated by MTT-colorimetric assay **B.** Percentage of apoptotic and necrotic cells were quantified. **C.** Apoptotic nuclei and necrotic cells treated with vanillin were examined by Hoechst 33342/propidium iodide staining assay and scale bar represented 20 µm. Data was shown as mean ± SD (N=4) and *, $p < 0.05$ compared with non-treated group.

Effects of vanillin on NCI-H460 human lung cancer cell proliferation.

Since the proliferation of the cells in response to the treatment may affect the stemness evaluation, the vanillin concentrations that used to characterize the suppression capability on cancer stem-like phenotypes of lung cancer would not cause anti-proliferative effect. Anti-proliferative effect of vanillin was thus identified in NCI-H460 lung cancer cells, NCI-H460 cells were pretreated with various concentrations of vanillin (0 – 100 μM) for 1 and 3 days. Later, pretreated cells were subcultured and subjected to cell proliferation assay using MTT-colorimetric assay at indicated time (0, 24, 48 and 72 h).

Figure 9 shows that pretreatment with vanillin at low dose (0 - 50 μM) for 1 and 3 days did not effect the proliferation of lung cancer cells, whereas the high dose (100 and 200 μM) exhibited the suppressive effect significantly after pretreatment for 3 days. This suggested that the appropriated doses of vanillin, that further examined the stemness-like phenotypes, were 0 – 50 μM.

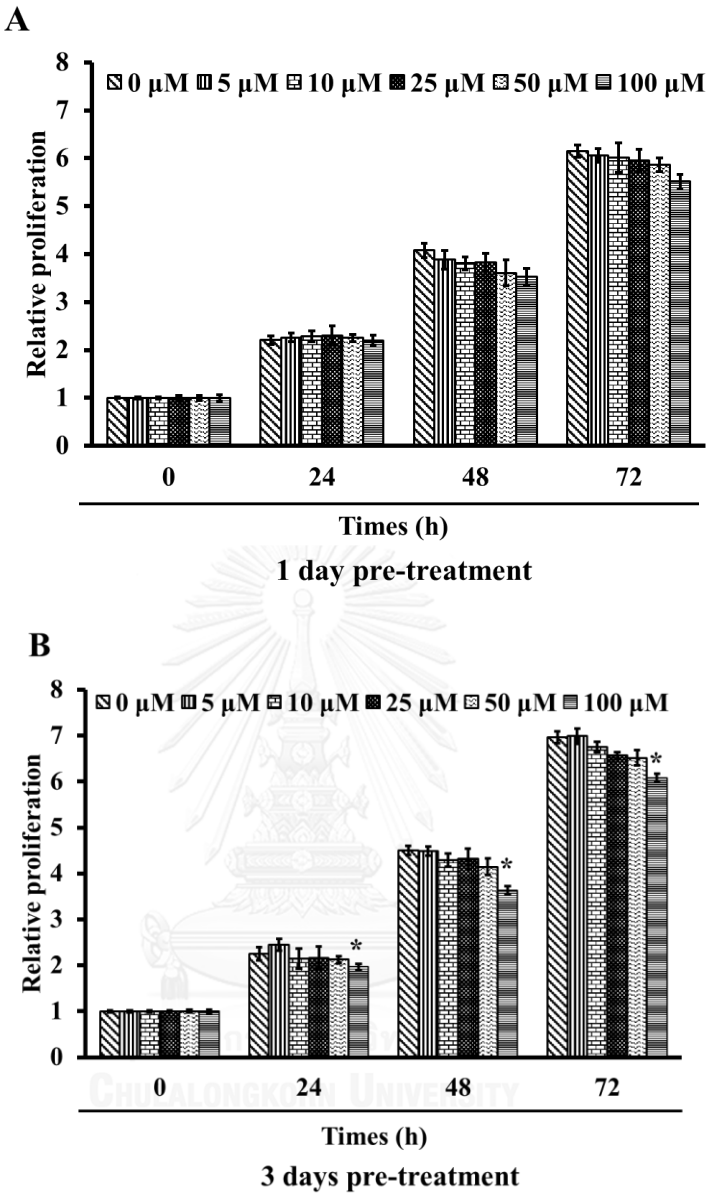


Figure 9 Anti-proliferative evaluation of vanillin on NCI-H460 cells. Cells were pretreated with various concentrations of vanillin for 1 day **(A)** and 3 days **(B)**. Proliferative effect was performed by MTT-colorimetric assay at each indicated time point. The relative proliferation of the cells was quantified. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

Part II Evaluation the suppressive capability of vanillin on cancer stem-like phenotypes of human non-small cell lung cancer H460 cells

Vanillin exhibits an inhibitory effect on CSC-like phenotypes in human lung H460 cancer cells

Anchorage-independent growth assay were normally used as a behavioral assay to determine the self-renewal and tumorgenic capability which are represented the cancer stem-like phenotypes (124). Pretreated cells with various concentrations of vanillin for 1 and 3 days were subjected to an anchorage-independent growth assay. Figure 10A illustrated the representative images of colony in response to vanillin pretreatment. Vanillin-attenuated anchorage-independent growth were significantly in the cells pretreated for 3 days, whereas the 1 day pretreatment did not affect obviously. The reduction of colony size and number were observed in a dose-dependent manner (Figure 10B and C).

To confirm above finding, the spheroid formation assay was performed. Consistency with an anchorage-independent growth assay, pretreatment with vanillin for 3 days was able to inhibit the formation both primary and secondary spheroids (Figure 11). The size and number of colony were extensively decreased in secondary spheroid (Figure 11E and F). These results indicated that vanillin have a suppressive capability on cancer stem-like phenotypes.

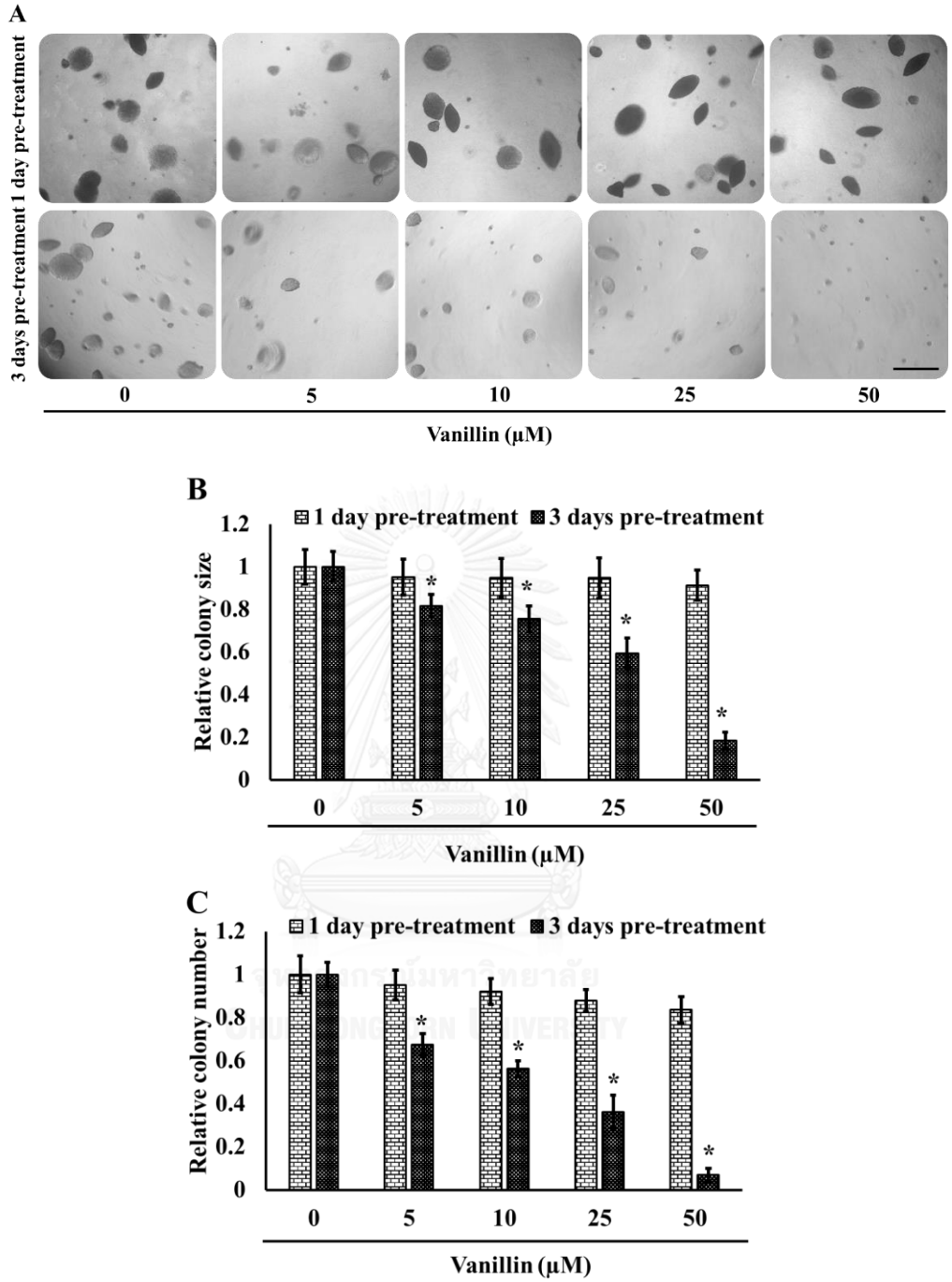
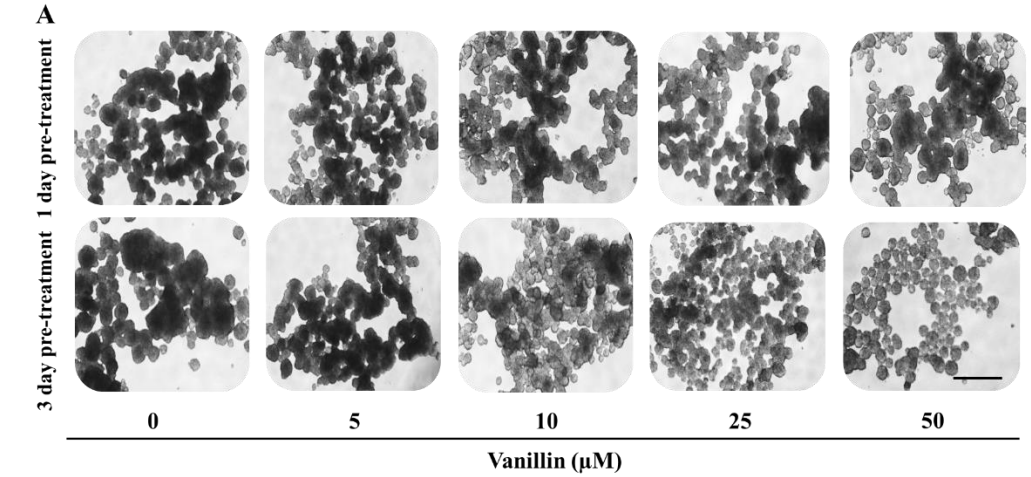
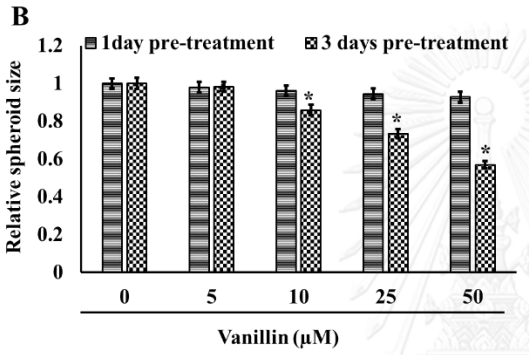


Figure 10 Vanillin suppressed an anchorage-independent growth property of NCI-H460 cells. Human NCI-H460 Cells were pretreated with vanillin in various concentrations for 1 day and 3 days and subjected to an anchorage-independent growth assay. **A.** Colonies were captured under microscopic with magnificent 4x. **B.** Relative colony size and **C.** number were quantified. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group. Scale bar represented 500 µm.


A
1 day pre-treatment
3 day pre-treatment
0
5
10
25
50
Vanillin (µM)


B
1day pre-treatment
3 days pre-treatment
Relative spheroid size
1.2
1
0.8
0.6
0.4
0.2
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5
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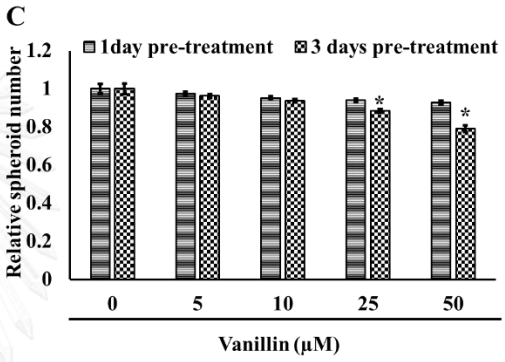
Vanillin (µM)


C
1day pre-treatment
3 days pre-treatment
Relative spheroid number
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1
0.8
0.6
0.4
0.2
0
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5
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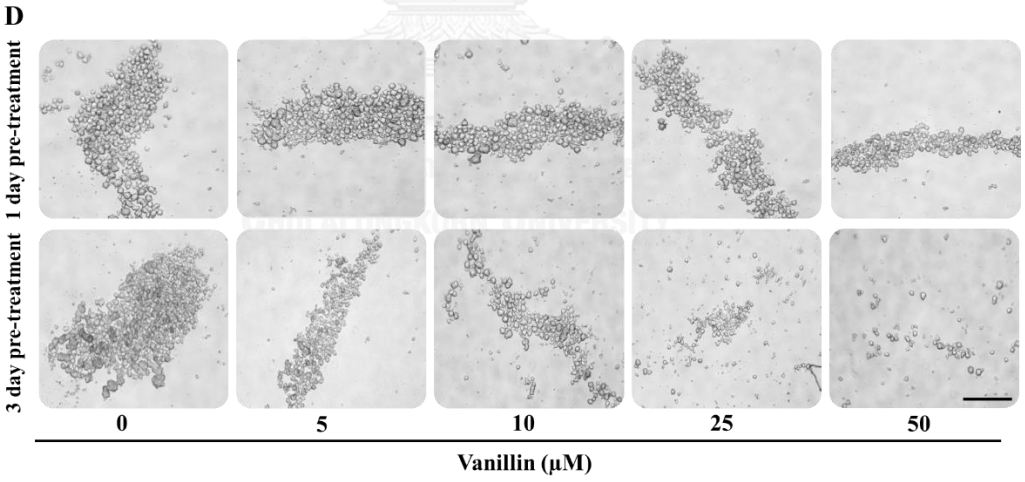
25
50
Vanillin (µM)


D
1 day pre-treatment
3 day pre-treatment
0
5
10
25
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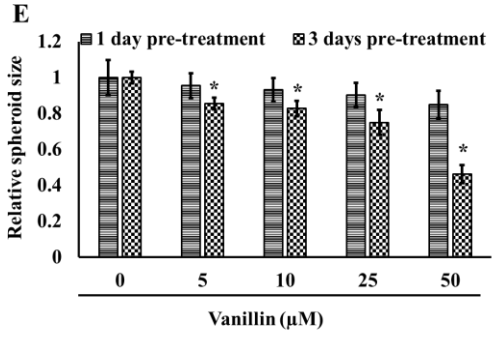
Vanillin (µM)


E
1 day pre-treatment
3 days pre-treatment
Relative spheroid size
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1
0.8
0.6
0.4
0.2
0
0
5
10
25
50
Vanillin (µM)

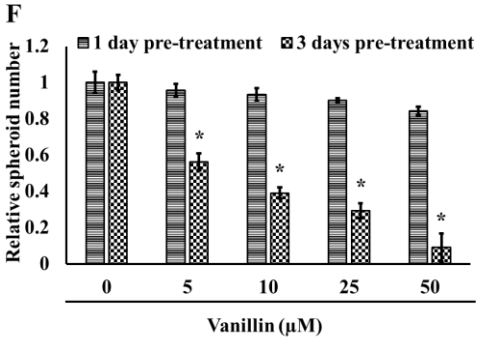
F
1 day pre-treatment
3 days pre-treatment
Relative spheroid number
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
25
50
Vanillin (µM)

Figure 11 Vanillin suppressed spheroid characteristic of NCI-H460 cells Human NCI-H460 Cells were pretreated with vanillin in various concentration for 1 and 3 days and subjected to spheroid formation assay. **A.** Primary spheroids were captured under microscopic with 4x magnification. Relative spheroid size **(B)** and number **(C)** were quantified. **D.** Secondary spheroids were also captured with 4x magnification. The relative size **(E)** and number **(F)** were quantified. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group. Scale bar represented 500 μm.

Vanillin downregulated cancer stemness markers in NCI-H460 cells

Cancer stem cell markers, CD133, ABCG2 and ALDH1A1, are expressed differently compared between each types of cancer including lung cancer (51). Not only the suppression of an anchorage-independent growth and spheroid formation was evaluated, inhibition of those markers by vanillin in NCI-H460 lung cancer cell was also identified. NCI-H460 cells were treated with vanillin (0 – 50 μM) for 1 and 3 days prior to determine the CSC markers expression by western blot analysis. Figure 12 showed that the expression of CSC markers was not significant changes in cells pretreated with vanillin 1 day. Interestingly, pretrement with vanillin for 3 days exhibited a dose-dependent attenuation of those cancer stem cells markers, CD133, ABCG2, ALDH1A1 significantly ($p=0.021$) (Figure 13). These results indicated that vanillin could suppress not only CSC-like phenotypes, but also CSC markers in NCI-H460 cells.

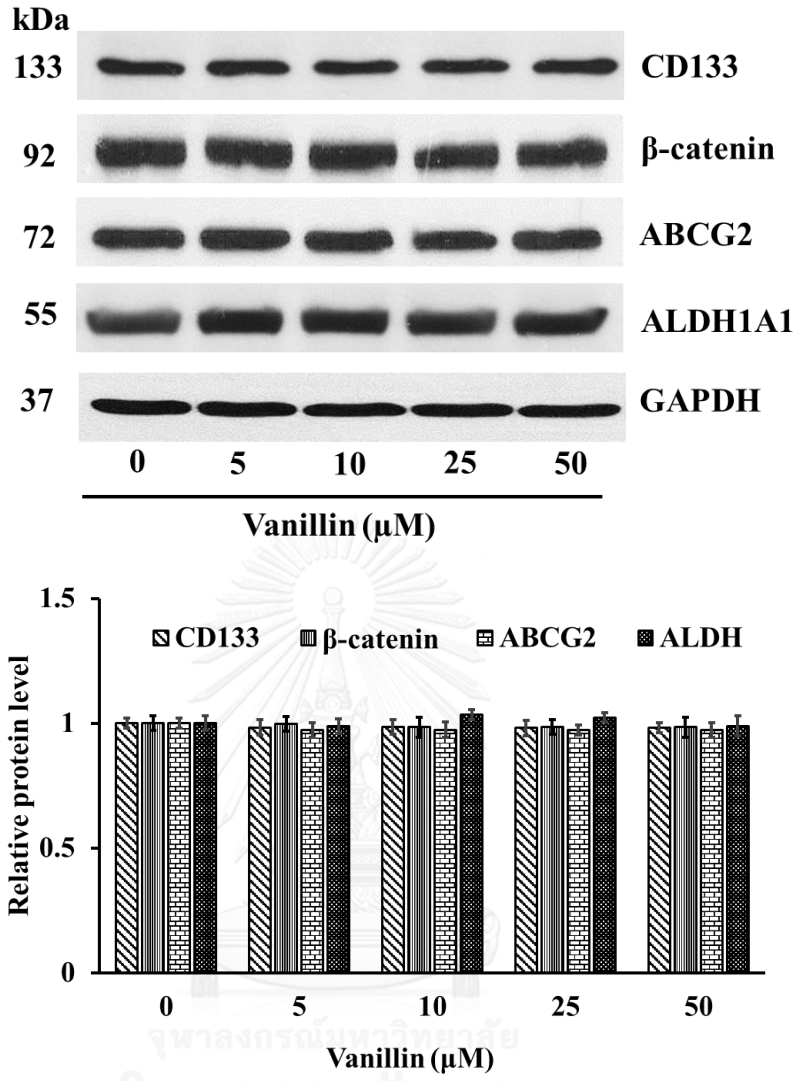

Figure 12 Vanillin attenuated expression of cancer stem markers in NCI-H460 cells. Human NCI-H460 Cells were pretreated with various concentrations of vanillin for 1 day and subjected to Western blot analysis. Relative protein expression of cancer stem cell markers and transcription factors was quantified. Data was shown as mean ± SD (N=4).

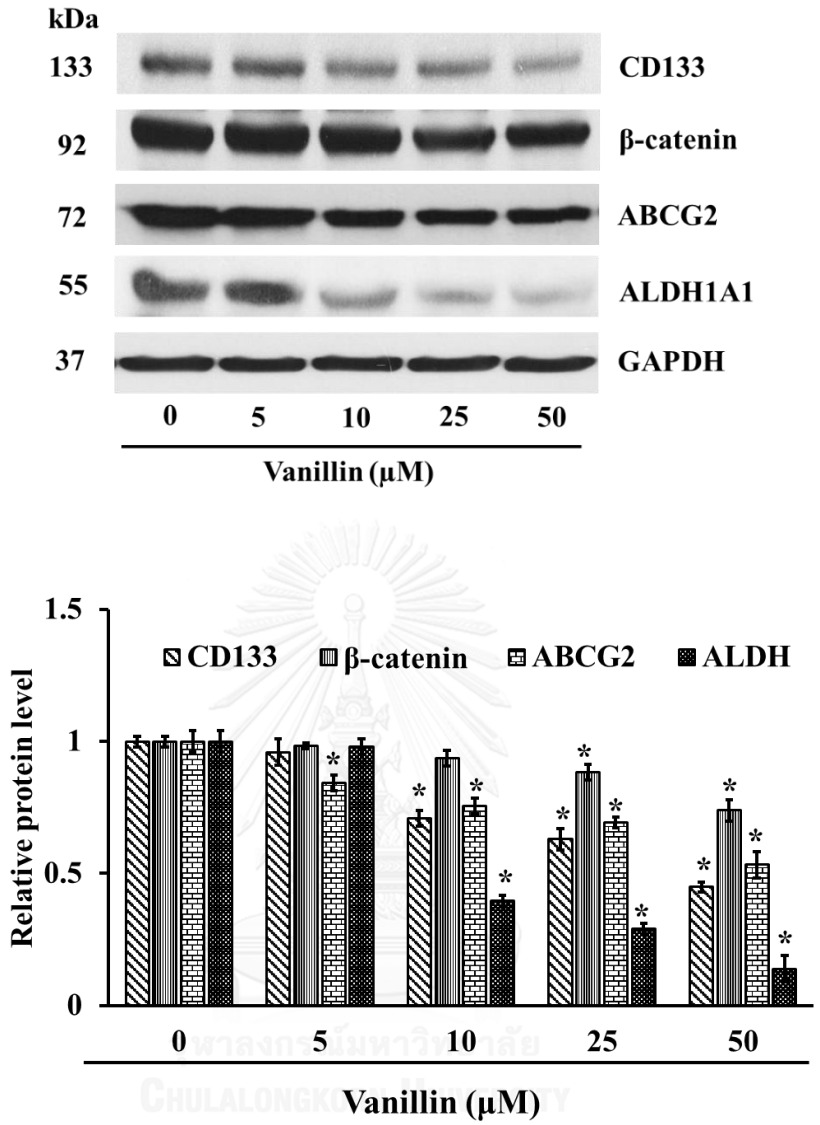

Figure 13 Vanillin attenuated expression of cancer stem markers in NCI-H460 cells. Human NCI-H460 cells were pretreated with various concentrations of vanillin for 3 days and subjected to Western blot analysis. Relative protein expression of cancer stem cell markers and transcription factors was quantified. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

Part III: Investigation of Akt-related mechanism of vanillin on cancer stem-like phenotypes of human non-small cell lung cancer H460 cells

Vanillin downregulated CSCs transcription factor, Nanog and Oct4 expressions.

Numerous reports have been suggested that high levels of transcription factors, Nanog and Oct4, are founded in cancer cells which exhibited major characteristics including elevation of cancer stem markers, cancer stem-like phenotypes and enhancing of self-renewal property (13, 123). Assessment of vanillin on suppression of those transcription factors in NCI-H460 cells were performed. NCI-H460 cells were treated with vanillin for 1 and 3 days and evaluated the protein expression level using Western blot analysis. Figure 14 exhibited the significantly decreasing of Oct4 and Nanog in the cells, which are treated with vanillin for 3 days whereas the expression level was not detectable changes in response to treatment with vanillin for 1 day. These results show the consistency with above data that the effect of vanillin on cancer stemness phynotypes was clearly exhibited after 3 days-treatment.

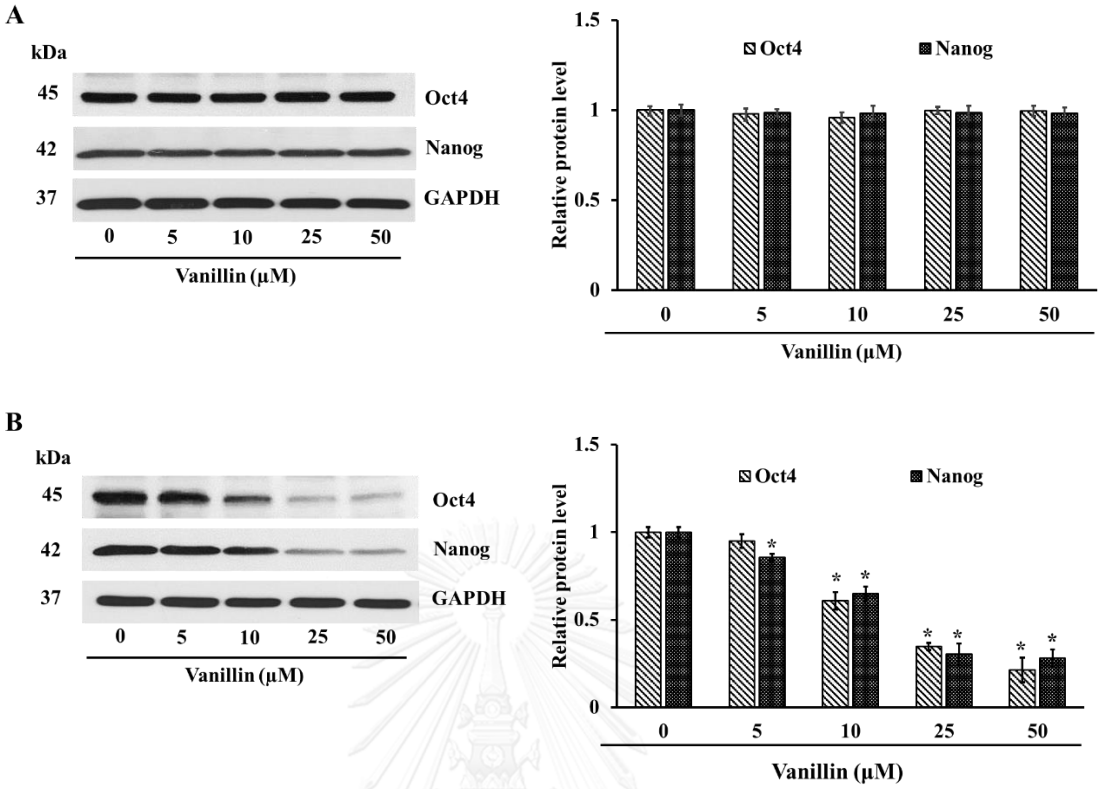

Figure 14 Vanillin attenuated transcription factors in NCI-H460 cells. Human NCI-H460 cells were pretreated with various concentrations of vanillin for 1 **(A)** and 3 **(B)** days and subjected to Western blot analysis. Relative protein expression of cancer stem cell markers and transcription factors was quantified. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

Vanillin decreased CSCs transcription factors through an Akt-dependent mechanism

Nowadays, numerous reports suggested that transcription factors, Nanog and Oct4, is modulated by Akt (16, 20, 21) Effects of vanillin on Akt activity and expression were further evaluated. Pretreated NCI-H460 cells with vanillin for 3 days were subjected to Western blot analysis. Figure 15 exhibited that active Akt, in term of phosphorelated Akt (P-Akt) at Ser473 and Thr308, was decreased significantly in dose dependent manner. The result also showed that total Akt expression level was obviously downregulated in the same pattern. Quantitative result obtained from densitometer indicated that ratio of P-Akt over total Akt is similarly changed, suggesting that vanillin attenuated cancer stem-like phenotypes through suppression of both activity and expression of Akt.

To confirm the hypothesis that downregulation of Oct4 and Nanog is a consequence of an inhibition of Akt, NCI-H460 were pretreated with non-toxic concentrations of perifosine (1,1-dimethyl piperidinium-4-yl octadecyl phosphate) (107, 125, 126), an Akt inhibitor, for 3 days and the expression of P-Akt, transcription factors and cancer stem cell markers were determined using Western blot analysis. Interestingly, inactivation of Akt caused by Akt inhibitor downregulated its signaling cascade, including Oct4, Nanog and cancer stem markers which showed similar effect as vanillin (Figure 16). These results suggested that vanillin, at least in part, inhibits Akt activation and subsequently suppressed of CSC-transcription factors, CSC- markers and its related phenotype.

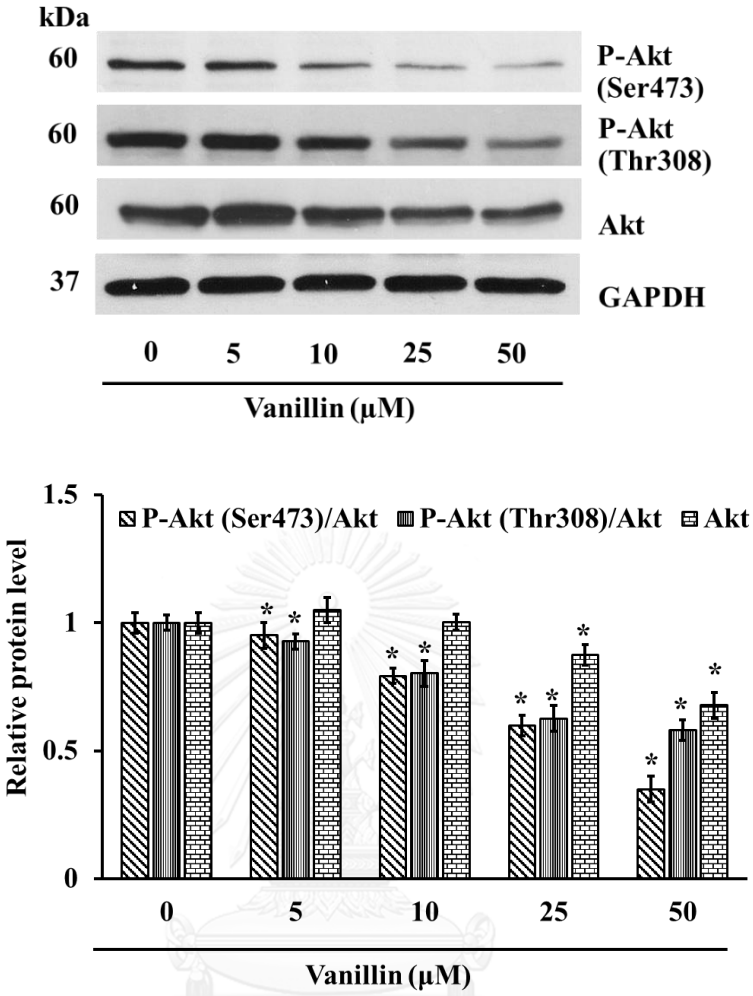

Figure 15 Vanillin attenuated Akt activity and the expression of total Akt in NCI-H460 cells. Human NCI-H460 Cells were pretreated with vanillin in various concentration (0 – 50 μM) for 3 days. The active Akt and its parent form were examined by Western blot analysis. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

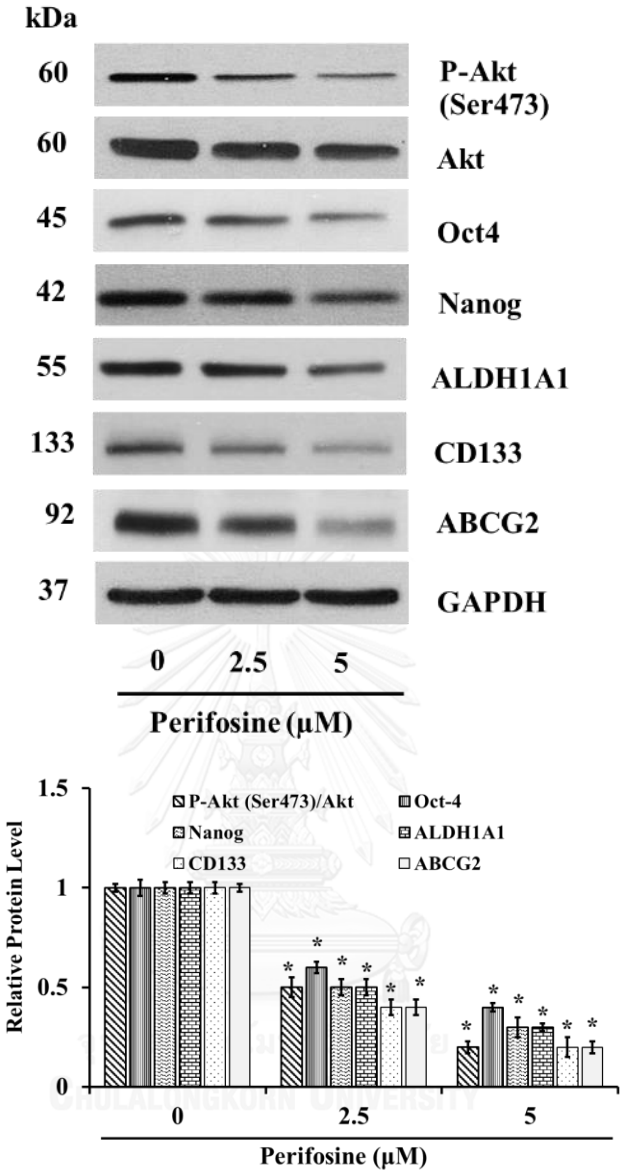

Figure 16 Perifosine downregulated Akt activity, the expression of total Akt, transcription factors and CSC markers in NCI-H460 cells. Human NCI-H460 Cells were pretreated with perifosine in various concentration (0 – 5 μM) for 3 days. The active Akt and its parent form, and CSC-related proteins were examined by Western blot analysis. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

Vanillin enhances Akt degradation through an ubiquitin-proteasomal mechanism

Numerous reports have suggested that the regulation of existing Akt is caused by ubiquitin-proteasomal pathway (112). To assess the possibility that vanillin might effect on Akt degradation through this pathway, NCI-H460 cells were pretreated with proteasomal inhibitor, Lactacystin (Lac, 10 μM), prior to treatment of vanillin (50 μM) for 3 h. Akt-ubiquitin complex was identified by immunoprecipitation method. Figure 17 demonstrated that vanillin promotes Akt-ubiquitin interaction significantly compared with control group, indicating the vanillin enhanced the Akt degradation via ubiqutination, which caused the suppression of cancer stemness.

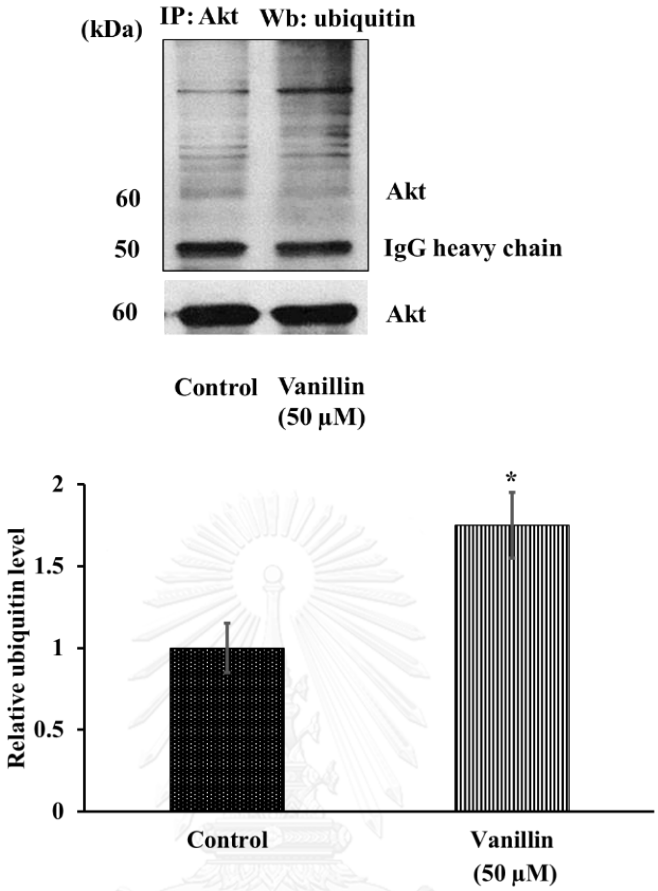

Figure 17 Vanillin enhanced the Akt-ubiquitin interaction in NCI-H460 cells Human NCI-H460 Cells were pretreated with 10 µM of Lactacystin, proteasome inhibitor, prior to 50 µM of vanillin for 3 h. Akt was immunoprecipitated antibody against Akt and pulled down by protein G agarose bead. The immunocomplex was observed by Western blot analysis using antibody against ubiquitin. Data was shown as mean ± SD (N=4) and * = $p < 0.05$ compared with non-treated group.

CHAPTER V
DISCUSSION

Recently, cancers have been reported as the most common cause of death within human population. Even if the newly technique and drug for treatment have been continuously developed, rate of successful treatment is remaining low because of the resistance of conventional chemotherapy, metastasis, self-renewal capacity of cancer cells and cancer stem cells (6, 51). Cancer stem cells (CSCs) are a small population within tumors and metastasis cancer which exhibited the overexpression of cancer stem cells markers, CD133, ALDH1A1 and ABCG2, and kinase proteins such as Akt (127). At present, drug research and development have focused on finding novel natural compounds i.e. curcumin, resveratrol and pomegranate targeted on these populations and downstream signaling pathways (128-130). Herein, this is the first research of vanillin, major component found in *Vanilla planifolia*, which exhibits the suppressive capability of cancer stem-like phenotypes and related molecular mechanism. Compared with the time-course of pretreatment, suppression of CSC phenotypes was clearly observed in 3 days treatment while the 1 day treatment did not exhibit the effects, suggesting that the unaffected period is caused by compensation mechanism for example mitogen activated protein kinase (MAPK) which play a major role in the maintenance of cell survival and proliferation of cancer cells in response to stress condition (131). Previous report explored that MEK inhibitor was able to activate Akt through enhancing of epidermal growth factor stimulation (132, 133). This result might be a reason why the 1 day-treatment of vanillin did not express the prominent result at that time. Moreover, half-life of some transcription factor including Oct4 is more than 36 h, suggesting that the effects of substance which alter this protein might take times more than 1 day (134). However, our finding

demonstrated the correlated data that vanillin could suppresses the pluripotency of cancer stem cells and CSC markers after treatment for 3 days.

Not only the *in vitro* behavior assays were used to evaluate the stem-like phenotypes, CD133, ALDH1A1 and ABCG2 have been defined as vulnerable markers for cancer stem-like phenotypes both *in vitro* and *in vivo* lung cancer (17, 107, 135). Lung cancer with overexpression of one to all of those markers, $CD133^{+}$, $ABCG2^{+}$, $ALDH^{hi}$ exhibited a high tumorgenicity, self-renewal property and chemoresistance (136, 137). We further evaluated the effect of vanillin on such markers and founded that vanillin-treated group exhibited the downregulation of these markers (Fig. 13). Also, transcription factors, Oct4 and Nanog, are normally upregulated in various types of cancers including lung cancer and used as protein markers to evaluate the stemness capability of cancer cells. These transcription factors have been reported as the major role in self-renewal and pluripotency properties and regulated by major kinase Akt within the cells (20, 21, 56). Suppression of these transcription factors was shown to diminish the proliferation and spheroid formation of cancer cells (19). Herein, Oct4 and Nanog were enormous decreased in vanillin-pretreated group, demonstrating the positive pharmacological property on suppression of cancer stem-like phenotypes correlated with behavioral changes.

Protein kinase B, known as an Akt, regulates in various cellular process in both cancer cells and CSCs including cell proliferation, migration, self-renewal capacity and pluripotent property (94). Previous report suggested that Akt regulates action of transcription factors, Oct4 and Nanog. For example, in breast cancer and prostate cancer, inhibition of PI3k or Akt activity led to pluripotent loss or interfere the CSC phenotypes (138, 139). We also demonstrated the important role of Akt in CSCs that is a target of vanillin in both activity and its expression contributing to downregulation of CSC markers and transcription factors. The phenomenon was also observed in perifosine treatment, an Akt inhibitor which has been proven widely as Akt inhibitor

both *in vitro* and *in vivo* (140, 141). In lung cancer, perifosine less than 10 μM shows non-cytotoxic effect (107, 125, 126) which use to confirm the important role of Akt on CSC properties in this study. Perifosine was able to suppress CSC markers in corresponding to the inhibition of Akt activation (Figure 16), similarly observed in vanillin treatment (Figure 13 – 15). We also found that Akt expression was downregulated in vanillin-treated group, it might be possible that vanillin suppresses Akt activation, at least in part of attenuation of Akt expression in this cell system.

It has been widely known that the entire protein level within the cells is also regulated by ubiquitin-proteasomal pathway, a catabolic pathway for several signaling molecules including Akt through multi-step ubiquitin-ligase enzyme (142, 143). The result of this study exhibit that poly-ubiquitinated Akt is increased obviously in vanillin-pretreated group (Figure 17). However, the exact mechanism which explained this phenomenon caused by vanillin is remain unknown. Numerous literatures suggested that enhancing of poly-ubiquitin formation is caused by reactive oxygen species (ROS) and the alteration of oxidative status within the cells by exogenous exposure could interfere this process (144, 145). Vanillin, which is reported to have both antioxidant and pro-oxidant effect depended on cell types and doses, might alter the redox status of the cells; therefore, vanillin may enhance Akt-ubiquitin complex through this mechanism (146).

Finally, it could be concluded that suppression on CSC-like phenotypes in NCI-H460 cells caused by vanillin is through the increasing of Akt-ubiquitin complex, prior to Akt degradation. This Akt degradation causes the downregulation of transcription factors, Oct 4 and Nanog as well as CSC phenotype in lung cancer (Figure 18). This finding provides new strategy and novel anticancer targeting to suppress CSCs in lung cancer.

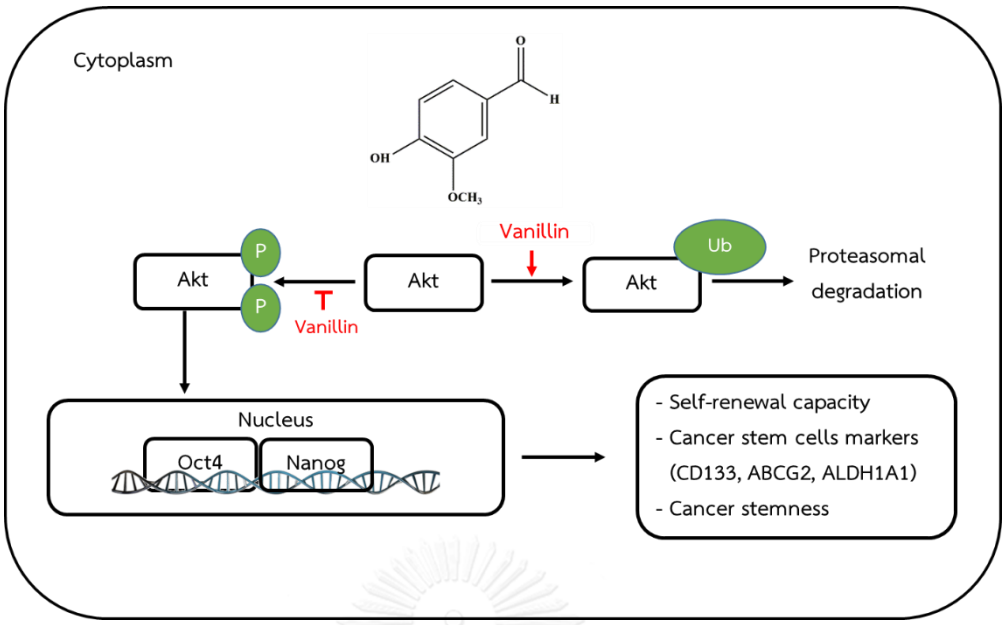

Figure 18 Schematic overview of vanillin on lung cancer stem-like phenotypes and its related pathway. Suppression of both activation and expression of akt by vanillin through enhancing ubiquitin-proteasomal pathway, dwindle the CSC transcription factors Oct4 and Nanog and suppresses self-renewal capacity and cancer stem cell markers.

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APPENDIX

APPENDIX

TABLES AND FIGURES OF EXPERIMENTAL RESULTS

Table 4 Cell viability of NCI-H460 cells determined by MTT-colorimetric assay after treated with various concentrations of vanillin (0 – 200 μM)

Vanillin (μM)	Cell viability (%)
Control	100.00 ± 4.89
5	105.82 ± 4.09
10	104.79 ± 3.83
25	98.60 ± 7.25
50	84.38 ± 4.71
100	80.87 ± 6.95
200	70.11 ± 3.25*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 5 Relative proliferation of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 day and determined by MTT-colorimetric assay

Vanillin (μM)	Relative proliferation			
	Time 0	Time 24	Time 48	Time 72
Control	1.00 ± 0.01	2.21 ± 0.09	4.08 ± 0.15	6.15 ± 0.12
5	1.00 ± 0.02	2.26 ± 0.09	3.89 ± 0.20	6.06 ± 0.14
10	1.00 ± 0.02	2.28 ± 0.11	3.80 ± 0.13	6.01 ± 0.30
25	1.00 ± 0.04	2.30 ± 0.19	3.82 ± 0.19	5.95 ± 0.24
50	1.00 ± 0.05	2.25 ± 0.07	3.61 ± 0.27	5.86 ± 0.14
100	1.00 ± 0.07	2.20 ± 0.10	3.53 ± 0.17	5.51 ± 0.15

All values represented as mean ± SD (N=4).

Table 6 Relative proliferation of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days and determined by MTT-colorimetric assay

Vanillin (μM)	Relative proliferation			
	Time 0	Time 24	Time 48	Time 72
Control	1.00 ± 0.03	2.24 ± 0.14	4.50 ± 0.10	6.96 ± 0.13
5	1.00 ± 0.02	2.44 ± 0.13	4.49 ± 0.10	6.98 ± 0.17
10	1.00 ± 0.02	2.14 ± 0.22	4.29 ± 0.14	6.75 ± 0.11
25	1.00 ± 0.03	2.16 ± 0.24	4.32 ± 0.22	6.56 ± 0.08
50	1.00 ± 0.03	2.12 ± 0.07	4.15 ± 0.18	6.51 ± 0.17
100	1.00 ± 0.04	1.96 ± 0.07*	3.64 ± 0.09*	6.09 ± 0.09*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 7 Relative colony number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 day determined by anchorage-independent growth assay

Vanillin (μM)	Relative colony size	Relative colony number
Control	1.00 ± 0.08	1.00 ± 0.09
5	0.95 ± 0.09	0.95 ± 0.07
10	0.94 ± 0.09	0.92 ± 0.09
25	0.94 ± 0.10	0.88 ± 0.10
50	0.91 ± 0.07	0.84 ± 0.09

All values represented as mean ± SD (N=4).

Table 8 Relative colony number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days determined by anchorage-independent growth assay

Vanillin (μM)	Relative colony size	Relative colony number
Control	1.00 ± 0.07	1.00 ± 0.05
5	0.82 ± 0.05*	0.67 ± 0.05*
10	0.76 ± 0.06*	0.56 ± 0.04*
25	0.59 ± 0.07*	0.36 ± 0.08*
50	0.18 ± 0.04*	0.07 ± 0.03*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 9 Relative primary spheroid number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 day determined by spheroid formation assay.

Vanillin (μM)	Relative spheroid size	Relative spheroid number
Control	1.00 ± 0.03	1.00 ± 0.03
5	0.98 ± 0.02	0.96 ± 0.01
10	0.96 ± 0.03	0.93 ± 0.02
25	0.93 ± 0.03	0.90 ± 0.01
50	0.92 ± 0.03	0.86 ± 0.02

All values represented as mean ± SD (N=4).

Table 10 Relative primary spheroid number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days determined by spheroid formation assay.

Vanillin (μM)	Relative spheroid size	Relative spheroid number
Control	1.00 ± 0.03	1.00 ± 0.03
5	0.95 ± 0.03	0.96 ± 0.02
10	0.85 ± 0.03*	0.94 ± 0.02
25	0.73 ± 0.02*	0.85 ± 0.01*
50	0.57 ± 0.02*	0.78 ± 0.02*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 11 Relative secondary spheroid number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 day determined by spheroid formation assay.

Vanillin (μM)	Relative spheroid size	Relative spheroid number
Control	1.00 ± 0.10	1.00 ± 0.06
5	0.95 ± 0.07	0.96 ± 0.03
10	0.93 ± 0.06	0.92 ± 0.01
25	0.90 ± 0.07	0.89 ± 0.01
50	0.86 ± 0.08	0.85 ± 0.02

All values represented as mean ± SD (N=4).

Table 12 Relative secondary spheroid number and size of NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days determined by spheroid formation assay.

Vanillin (μM)	Relative spheroid size	Relative spheroid number
Control	1.00 ± 0.03	1.00 ± 0.04
5	0.86 ± 0.03*	0.56 ± 0.05*
10	0.82 ± 0.04*	0.39 ± 0.03*
25	0.75 ± 0.07*	0.29 ± 0.04*
50	0.46 ± 0.05*	0.09 ± 0.08*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 13 Relative protein level values of CSC markers in NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 days.

Vanillin (μM)	Relative protein levels			
	CD133	β-Catenin	ABCG2	ALDH1A1
Control	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.04
5	0.98 ± 0.05	0.99 ± 0.03	0.94 ± 0.03	0.97 ± 0.03
10	0.99 ± 0.03	0.98 ± 0.03	0.95 ± 0.03	0.93 ± 0.02
25	0.98 ± 0.04	0.99 ± 0.02	0.93 ± 0.02	0.93 ± 0.02
50	0.97 ± 0.02	0.99 ± 0.05	0.93 ± 0.05	0.93 ± 0.05

All values represented as mean ± SD (N=4).

Table 14 Relative protein level values of CSC markers in NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days.

Vanillin (μM)	Relative protein levels			
	CD133	β-Catenin	ABCG2	ALDH1A1
Control	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.04	1.00 ± 0.04
5	0.95 ± 0.05	0.98 ± 0.03	0.84 ± 0.03*	0.97 ± 0.03
10	0.70 ± 0.03*	0.93 ± 0.03	0.75 ± 0.03*	0.40 ± 0.02*
25	0.62 ± 0.04*	0.88 ± 0.03*	0.69 ± 0.02*	0.29 ± 0.02*
50	0.49 ± 0.02*	0.73 ± 0.04*	0.53 ± 0.05*	0.13 ± 0.05*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 15 Relative protein level values of CSC transcription factors in NCH-H460 cells pretreated with vanillin in non-toxic concentration for 1 days.

Vanillin (μM)	Relative protein levels	
	Oct4	Nanog
Control	1.00 ± 0.03	1.00 ± 0.03
5	0.95 ± 0.04	0.98 ± 0.02
10	0.94 ± 0.05	0.97 ± 0.04
25	0.94 ± 0.02	0.93 ± 0.06
50	0.94 ± 0.07	0.93 ± 0.05

All values represented as mean ± SD (N=4)

Table 16 Relative protein level values of CSC transcription factors in NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days.

Vanillin (μM)	Relative protein levels	
	Oct4	Nanog
Control	1.00 ± 0.03	1.00 ± 0.03
5	0.94 ± 0.04	0.85 ± 0.02*
10	0.61 ± 0.05*	0.64 ± 0.04*
25	0.34 ± 0.02*	0.30 ± 0.06*
50	0.31 ± 0.07*	0.28 ± 0.05*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group.

Table 17 Relative protein level values of downstream signaling compared with its parents in NCH-H460 cells pretreated with vanillin in non-toxic concentration for 3 days.

Vanillin (μM)	Relative protein levels	
	P-Akt (Ser473) / Akt	P-Akt (Thr308) / Akt
Control	1.00 ± 0.04	1.00 ± 0.03
5	0.95 ± 0.05*	0.92 ± 0.03*
10	0.79 ± 0.03*	0.80 ± 0.05*
25	0.60 ± 0.04*	0.62 ± 0.05*
50	0.35 ± 0.05*	0.58 ± 0.04*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group

Table 18 Relative protein level values of CSC markers, transcription factors and its related signaling proteins in NCH-H460 cells pretreated with perifosine for 3 days.

Perifosine (μM)	Relative protein levels			
	Nanog	ALDH1A1	CD133	ABCG2
Control	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.02
2.5	0.53 ± 0.04*	0.58 ± 0.04*	0.42 ± 0.02*	0.48 ± 0.04*
5	0.32 ± 0.05*	0.39 ± 0.02*	0.27 ± 0.04*	0.22 ± 0.03*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group

Table 19 Relative protein level values of CSC markers, transcription factors and its related signaling proteins in NCH-H460 cells pretreated with perifosine for 3 days (cont.)

Perifosine (μM)	Relative protein levels	
	P-Akt (Ser473) / Akt	Oct4
Control	1.00 ± 0.02	1.00 ± 0.04
2.5	0.53 ± 0.05*	0.64 ± 0.03*
5	0.29 ± 0.03*	0.42 ± 0.02*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group

Table 20 Relative protein level values of ubiquitin-proteasome complex in NCI-H460 cells pretreated with vanillin (50 μM).

Vanillin (μM)	Relative protein levels
	ubiquitin
Control	1.00 ± 0.15
50	1.75 ± 0.18*

All values represented as mean ± SD (N=4); * $p < 0.05$ versus non-treated group

VITA

Songpol Srinual was born on December, 2nd 1988 in Bangkok. In 2012, he was graduated from Khon Kaen University, Khon Kaen, Thailand in Pharmaceutical Sciences majoring in pharmacognosy and toxicology. He has been employed as forensic toxicologist at Department of Forensic Medicine, Faculty of Medicine, Chulalonkorn University for 3 years prior to entered as Master's student of Program in Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.