



รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาลักษณะความรุนแรงและกลไกการควบคุมในเซลล์มะเร็งต้นกำเนิด เซลล์มีเซนไคม์
และเซลล์ชนิด quiescent ในเซลล์มะเร็งปอดมนุษย์

โดย ผศ. ภก. ดร.ฉัตรชัย เชาว์ธรรม

รศ. ภก. ดร.ปิติ จันทร์วรโชติ

ภาควิชาชีวเคมีและจุลชีววิทยา

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชาเภสัชวิทยาและสรีรวิทยา

คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สิ้นสุดโครงการ วันที่ 1 พฤษภาคม พ.ศ. 2561

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ผู้วิจัย

1. ผศ. ภก. ดร.ฉัตรชัย เขาว์ธรรม ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
2. รศ. ภก. ดร.ปิติ จันทรรพโชค ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และ จุฬาลงกรณ์มหาวิทยาลัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: MRG5980143

ชื่อโครงการ : การศึกษาลักษณะความรุนแรงและกลไกการควบคุมในเซลล์มะเร็งต้นกำเนิด เซลล์มีเซนไคม์ และเซลล์ชนิด quiescent ในเซลล์มะเร็งปอดมนุษย์

ชื่อนักวิจัย และสถาบัน : 1. ผศ. ภก. ดร.ฉัตรชัย เชาว์ธรรม ภาควิชาชีวเคมีและจุลชีววิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
2. รศ. ภก. ดร.ปิติ จันทรรพโชค ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

อีเมล : chatchai.c@chula.ac.th

ระยะเวลาโครงการ : 2 ปี (2 พฤษภาคม 2559 ถึง 1 พฤษภาคม 2561)

บทคัดย่อ : ความแตกต่างของเซลล์มะเร็งภายในก้อนเนื้อออกเป็นสาเหตุสำคัญของความหลากหลายในการแพร่กระจาย ความล้มเหลวของเคมีบำบัด การกลับมาของโรคและอัตราการรอดชีวิตที่ลดลงในผู้ป่วยโรคมะเร็งปอด ด้วยวิธีการเพาะเลี้ยงเซลล์เดี่ยวในภาชนะเลี้ยงเซลล์ชนิด 96 หลุม สามารถแยกกลุ่มประชากรเซลล์มะเร็งปอดที่มีรูปร่างต่างกันได้ 3 กลุ่มหลัก การประเมินพฤติกรรมความรุนแรง ได้แก่ ความดื้อต่อยาเคมีบำบัด การเจริญโตในสภาวะไร้การยึดเกาะ และความสามารถในการเกิดก้อนมะเร็งในกลุ่มประชากรเซลล์มะเร็งทั้งสามกลุ่ม พบว่ากลุ่มประชากรเซลล์รูปร่างใหญ่แสดงความดื้อต่อการตายที่เหนียวแน่นด้วยยาเคมีบำบัดสูงสุด เพื่อศึกษาลักษณะจำเพาะของเซลล์กลุ่มนี้ได้ทำการศึกษาการเปลี่ยนแปลงของโปรตีนที่เกี่ยวข้องกับกระบวนการเปลี่ยนจากเซลล์เยื่อบุผิวไปเป็นเซลล์มีเซนไคม์และเซลล์มะเร็งต้นกำเนิด นอกจากนี้ยังพบรูปแบบที่จำเพาะของข้อมูลเมแทบอลิซึมในกลุ่มประชากรที่ดื้อต่อยาเคมีบำบัดเปรียบเทียบกับกลุ่มเซลล์มะเร็งดั้งเดิม เพื่อค้นหาเคมีบำบัดใหม่ได้ทำการศึกษาความสามารถในการต้านมะเร็งของเปปไทด์สกัดจากเห็ดขอนขาวและสารอะวิซีควิโนน บี สกัดจากต้นโกงกาง ข้อมูลที่ได้จากการศึกษาจะนำไปสู่การค้นหากลไกควบคุมการดื้อยาในเซลล์มะเร็งปอดและนำมาซึ่งข้อมูลเชิงลึกเกี่ยวกับความแตกต่างของเซลล์มะเร็ง

คำหลัก : มะเร็งปอด, ความดื้อต่อยา, พฤติกรรมความรุนแรง, มะเร็งต้นกำเนิด, การเปลี่ยนแปลงจากเยื่อบุผิวเป็นมีเซนไคม์, เมแทบอลิซึม

Abstract

Project Code : MRG5980143

Project Title : Investigation on aggressive behaviors and regulatory mechanisms in cancer stem-like cells (CSCs), mesenchyme-like cells and quiescent sub-population of human lung cancer cells

Investigator : 1. Asst. Prof.Chatchai Chaotham, Ph.D. Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

2. Assoc. Prof.Pithi Chanvorachote, Ph.D. Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University

E-mail Address : chatchai.c@chula.ac.th

Project Period : 2 years (2nd May 2016 – 2nd May 2018)

Abstract: The heterogeneity of cancer cells in tumor is an important rationale for the verities in metastasis, chemotherapeutic failure, relapse of tumor pathology following with decrease of survival in lung cancer patients. Three main populations exhibiting distinct morphology were isolated from human lung cancer H292 cells through a single cell clone selection in 96-well plate. The aggressive behaviors including chemotherapeutic resistance, anchorage-independent growth and tumor initiation were evaluated in these three populations. Results showed that the sub-population exhibiting large cell morphology possessed the highest capability to resist to chemotherapeutic drug-induced apoptosis. In order to provide in-depth cellular signature of this specific cell population, the alteration of proteins involving in epithelial-to-mesenchymal transition (EMT) and cancer stem cell markers were characterized by western blot analysis. Furthermore, the unique pattern of metabolomics profile was also elucidated in chemotherapeutic resistant sub-population in comparison to their parental total population. In searching for novel chemotherapeutic compounds, potential anticancer activities of peptide extracted from edible mushroom; *Lentinus squarrosulus* and avicequinone B extracted mangrove tree; Avicenniaceae was investigated. The obtained information will be further analyzed and proposed as potential regulatory pathway mediating drug resistance in human lung cancer cells and provide more insight regarding cancer cell heterogeneity.

Keywords : Lung cancer, Drug resistance, Aggressive behaviors, Cancer stemness, Epithelial-mesenchymal transition (EMT), Metabolomics

1. Introduction to Research

Lung cancer is one of the leading causes of death in cancer patients (Siegel, et al., 2014). The spreading of tumor pathology to vital organs associating with chemotherapeutic failure are considered as main reason for low survival rate in lung cancer patients (Spano, et al., 2012; Chaffer and Weinberg, 2011). Although, the investigation on cancer biology has been preceded for several decades, key regulatory machinery in cancer aggressiveness is still ambiguous. The discovery of heterogeneous population in tumor tissue demonstrates side-population of cancer cells expressing stem cell-like phenotype with aggressive tumorigenic activity (Bonnet and Dick, 1997). This side-population is recognized as tumor-initiating cells, tumor-propagating cells or cancer stem-like cells (CSCs) which have been found in many types of cancer (Li, et al., 2009; Eramo, et al., 2008; Ma, et al., 2007; Collins, et al., 2005). CSCs have been suggested as a rationale behind chemo/radio-resistance and cancer relapse (Zhang, et al., 2012; Bertolini, et al., 2009; Levina, et al., 2008; Bao, et al., 2006). The overexpression of transcription factors regulating self-renewal and pluripotency, such as nanog, sox2 and oct4 is indicated in CSCs. (Liu et al., 2013). Moreover, CSCs can up-regulate CD133 and ALDH1A1, markers for CSCs, which involve in tumorigenicity and drug resistant activity (Bertolini et al., 2009; Grosse-Gehling et al., 2013; Li et al., 2011; Perona et al., 2011). Meanwhile, a novel therapeutic strategy concentrates on the eradication of CSCs; the regulatory molecules to mediate stemness phenotype are unrevealed.

Interestingly, the investigation on population of CSCs unveils quiescent sub-population that presents unique rate of cell cycle. Despite other aggressive characters still under examination, chemotherapeutic resistance in quiescent CSCs gains more attention (Zeuner, 2015; Moore and Lyle, 2011). Higher aggressiveness against chemotherapeutic treatment has been evaluated in quiescent sub-population (Zeuner, et al., 2014). However, molecular biology modulating chemotherapeutic sensitivity in quiescent CSCs of lung cancer cells is largely unknown (Zeuner, 2015).

Recent evidences demonstrate that highly metastatic cancer cells are mediated through epithelial to mesenchymal transition (EMT) (Mehlen and Puisieux, 2006; Scheel and

Weinberg, 2012). EMT is a typical process in embryogenesis; however, it can be occasionally stimulated during wound healing and tumor metastatic process (Iwatsuki et al., 2010). Aggressive migration and invasion are obviously elucidated in mesenchyme-like cancer cells (De Craene and Berx, 2013; Iwatsuki et al., 2010; Thiery et al., 2009; Yang and Weinberg, 2008). Furthermore, numerous inducers can trigger cancer stem-like cells (CSCs) phenotype through EMT process as evidence of EMT characters in CSCs (Scheel and Weinberg, 2012; Voon et al., 2013; Yongsanguanchai et al., 2015; Zhou et al., 2014). Nevertheless, whether the cancer cells with EMT morphology is characterized to be the quiescent CSCs and such EMT characteristics have an influence on chemotherapeutic resistant activity has been unclear.

According to aggressiveness and interrelation between these distinct populations in lung cancer cells, the effective therapeutic treatment should mediate the key regulatory mechanism in all type of cells including CSCs, mesenchyme-like cells and quiescent sub-population. Using microarray technique, this study aims to identify the epigenetic variation and targeted key molecules involving with aggressive behaviors in CSCs, mesenchyme-like cells and quiescent sub-population of human lung cancer cells. These would lead to a fundamental knowledge that essentially describes molecular biology of lung cancer cells and development of effective chemotherapeutic drugs.

As a unique natural source of diverse bioactive compounds, edible mushrooms are a potential resource for novel anticancer drug discovery. Apoptosis induction has been demonstrated in cancer cells cultured with peptides isolated from edible mushrooms (Patel and Goyal, 2012; Dan et al. 2016). Due to its safety as a traditional food and its potential therapeutic effects, *Lentinus squarrosulus* Mont. (Polyporaceae) has been highlighted. Beside carbohydrates, proteins, vitamins and minerals, *Lentinus squarrosulus* also contains various bioactive compounds, including phenolic compound, immunostimulatory glucans and lectins peptide (Mhd Omar et al. 2011; Sen et al. 2013; Das et al. 2017). This study aimed to evaluate the anticancer activity and the underlying mechanism of peptide extracted from the Thai edible mushroom *Lentinus squarrosulus* in human lung cancer cells.

Avicennone B, naphtho [2, 3-b] furan-4, 9-dione isolated from mangrove tree such as *Avicennia alba* and *Avicennia marina* has been shown to possess several pharmacological activities (Ito, et al., 2000). Anticancer activity of naphthoquinone derivatives have been

illustrated through the induction of apoptosis and the inhibition on migration and invasion [Lin, et al.,2010; Tsai, et al., 2014). So far, the potentials of these furanonaphthoquinone compounds for sensitizing anoikis and their regulatory approaches are largely unknown. We aimed to investigate the anoikis sensitizing effect and the underlying mechanisms of action of avicequinone B in human lung cancer cells. The information obtained from this study will emphasize the therapeutic benefits of avicequinone B for further development as an effective anticancer drug.

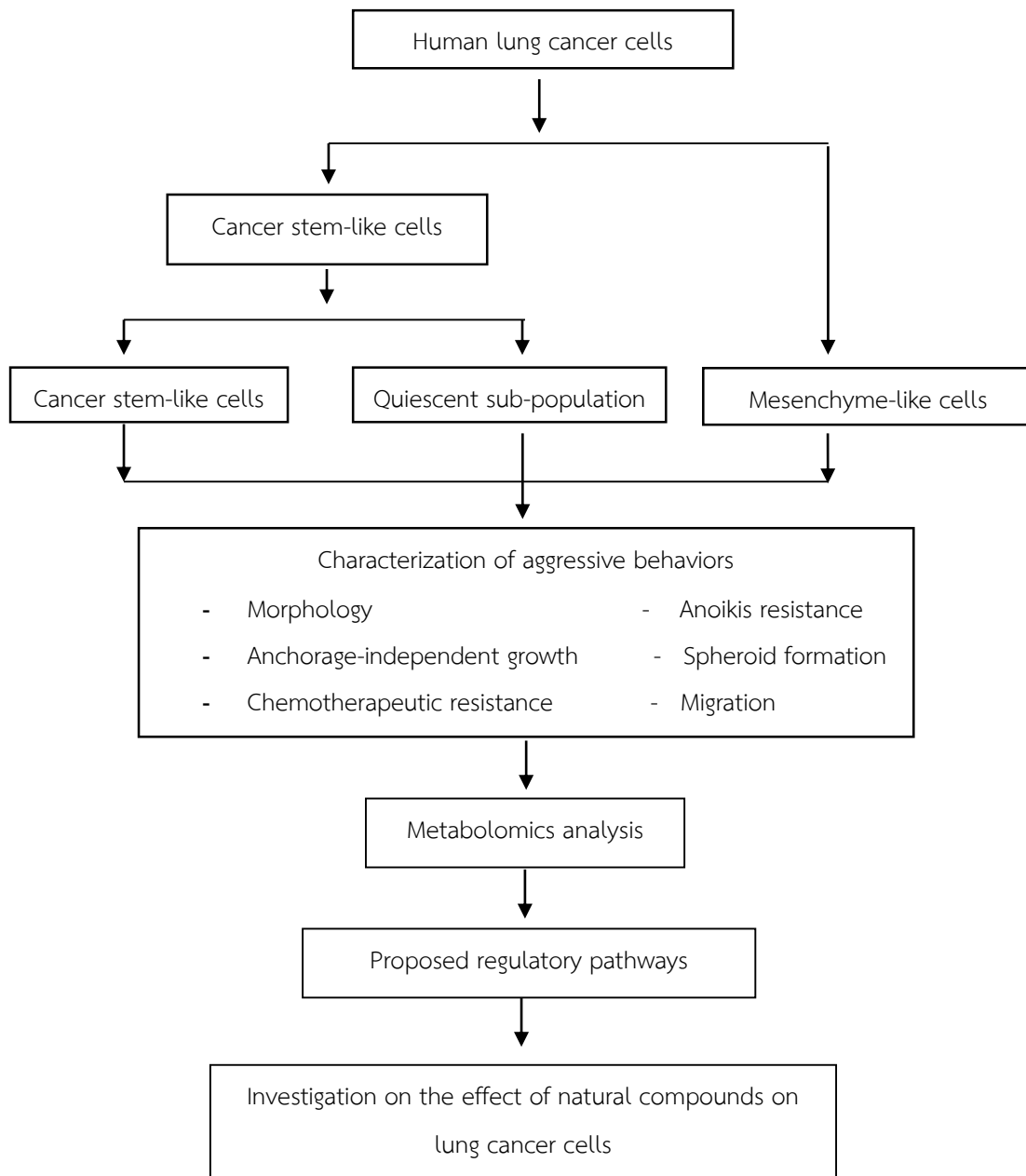
2. Literature review

3. Objective

- 3.1 To isolate and identify CSCs, mesenchyme-like cells and quiescent sub-population of human lung cancer cells
- 3.2 To investigate the aggressive behaviors of CSCs, mesenchyme-like cells and quiescent sub-population of human lung cancer cells
- 3.3 To identify key regulatory molecules mediating CSCs, mesenchyme-like cells and quiescent sub-population of human lung cancer cells

4. Research methodology

4.1 Experimental design



4.2 Method

1) Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, phosphate buffer saline (PBS), trypsin-EDTA, were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), Hoechst 33342, propidium iodide (PI), dimethyl sulfoxide (DMSO), cisplatin, agarose and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Doxorubicin hydrochloride and etoposide were purchased from Merck Millipore (Darmstadt, Germany). Antibody of ALDH1A1, β -actin, anti-goat IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CD133 primary antibody is obtained from Cell Applications (San Diego, CA, USA). Antibody of E-cadherin, N-cadherin, Vimentin, Snail, Slug, Nanog, Sox2, Oct4, ABCG2 and specific secondary-HRP antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Avicelquinone B was kindly provide by Asst. Prof. Supakarn Chamni, Ph.D., Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Peptide extracted from *Lentinus squarrosulus* was prepared by Assoc. Prof. Maneewan Suksomthip, Ph.D., Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2) Cell culture

Human non-small cell lung cancer NCI-H292, NCI-H460 and NCI-H23 cells were obtained from the American Type Culture Collection ATCC® (Manassas, VA). They and maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin at 37°C in a 5% CO₂ humidified incubator.

3) Isolation and identification of CSCs, EMT and quiescent sub-population in lung cancer cells

In order to isolate sub-population in human lung cancer cells, H292 cells were seeded into 96 well-plates at density of 1 cell/well in RPMI containing 10% FBS. Monitoring on different rate of cell cycle and morphology is observed under

microscope every 24 h for 7 days. The colony formed from single cell was separated and maintained in 6 well-plate in 10% FBS of RPMI until reach 70-80% confluence before using in experiments.

4) Cytotoxicity assay

After seeding the cells at the density of 1×10^4 cells/well into 96 well-plates for 12 h, the cells were incubated with indicated treatment or left untreated as control for 24 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for evaluation of cell viability. Briefly, after 24 h of treatment, the medium was replaced with 100 μ L of 0.5 mg/mL MTT solution and incubated at 37°C in dark place for 4 h. MTT solution was then replaced with 100 μ L of DMSO in order to dissolve the formed formazan crystals. Absorbance of formazan color was detected at a wavelength of 570 nm by Anthros microplate reader (Durham, NC, USA). The cell viability was calculated by dividing the optical density (OD) of the treated cells by that of the control cells.

5) Detection on mode of cell death

After 24 h of indicated treatments, the cells were then incubated with 10 μ g/mL of Hoechst 33342 and 5 μ g/mL of propidium iodide (PI) for 30 minutes at 37 °C. Hoechst stains the apoptotic cells with fragmented nuclei and/or condensed chromatin while PI positively stains dead necrotic cells. The fluorescence images were visualized and captured under a fluorescence microscope (Olympus IX51 with DP70).

6) Annexin V/PI flow cytometric analysis

Mode of cell death was also evaluated by flow cytometry using an Annexin V-FITC apoptosis kit. Human lung cancer cells were collected and prepared as single cell suspension in phosphate buffer saline (PBS) with a pH of 7.4. Annexin V-FITC/PI staining was performed according to the manufacturer's instructions. Briefly, cell suspensions were centrifuged and resuspended in 100 μ L of binding buffer then 10 μ L of Annexin V-FITC (1 μ g/mL) and 5 μ L of PI (2.5 μ g/mL) were added into the cell suspensions. After incubation at 37 °C for 15 minutes, the cell suspensions were evaluated via a FACScan flow cytometer using CellQuest software (Becton-Dickinson, Redlands, CA, USA) to determine percentages live, apoptosis and necrosis cells.

7) Cell proliferation assay

In order to evaluate proliferative activity, human lung cancer cells were prepared at density of 2×10^3 cells/well in 96 well-plate. Cell viability was examined at 24, 48 and 72 h of culture period by MTT assay.

8) Spheroid formation assay

Primary and secondary spheroids were grown as previously described (Kantara et al., 2014). Cells were washed with PBS and detached by 1 mM EDTA. Then, the cells were subjected into made into 12-wells ultralow attachment plate at density of 5×10^3 cells/well in serum free-RPMI. Primary spheroids were observed at day 7 of culture (37°C, 5% CO₂) using a phase contrast microscope (Olympus IX51 with DP70). Primary spheroids were disaggregated into single cells by using 1mM EDTA and resuspended with serum free-RPMI. The cells were placed again in 12-wells ultralow attachment plate at density of 5×10^3 cells/well. After incubation for next 30 days, secondary spheroids were observed under a phase contrast microscope (Olympus IX51 with DP70).

9) Anoikis assay

Detachment-induced cell death was evaluated in single-cell suspension culture onto separated Costar ultralow attachment plates (Corning Life Sciences) at a density of 15×10^5 cells/mL in RPMI supplemented with 10% FBS. The suspended cells were incubated at 37°C in a 5% CO₂ incubator. Cell viability is analyzed by XTT assay at 0, 3, 6, 9, 12 and 24 h of incubation time.

10) Anchorage-independent growth assay

Soft agar colony formation was used for examination of anchorage-independent growth. For bottom layer, mixture of RPMI containing 10% FBS and 1% agarose at 1:1 ratio was seeded into 24-well plate at 500 µL/well. After setting for 5 minutes at 4 °C, an upper layer consisting of 3×10^3 cells/well in 2:1 agarose gel mixture of RPMI 1640 containing 10% FBS : 1% agarose was added on top of the bottom layer. The upper layer was left to solidified at 37°C in a 5% CO₂ humidified incubator for 2 h before 500 µL of 10% FBS in RPMI medium. Colony formation was analyzed under microscope (Olympus IX51 with DP70) after 14 days of incubation at 37°C and 5% CO₂.

11) Western blot analysis

The related-protein level was evaluated via western blot analysis. Cells were collected and lysed at 4°C for 60 minutes. An equal amount of protein content analyzed by BCA protein assay kit (Pierce, Rockford, IL, USA) was loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for size separation. After transferring protein onto nitrocellulose membrane (Bio-Rad Laboratory, Hercules, CA, USA), non-specific protein binding was blocked by incubation of membrane with 5% skim milk at 37°C for 1 h. Then, the membrane was washed three times with TBST (25 mmol/l Tris-HCl, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 5 minutes following with the incubation with specific primary antibody at 4°C overnight. Before further incubation with HRP-conjugated secondary antibody at 37°C for 2 h, the membrane was washed three times with TBST for 5 minutes. Chemiluminescence (Supersignal West Pico; Pierce) was used to enhance the immunoreactive signal. The quantitative analysis is performed via analyst/PC densitometric software (Bio-Rad Laboratory, Hercules, CA, USA).

12) Metabolomics analysis

All metabolite extraction steps were performed at 4 °C in a cold room. After wash three time with cold PBS, cells were harvested in 80% cold ethanol. Then, the cell suspension was mixed thoroughly by sonicator for 10 min. After centrifugation at 1,600xg for 3 min, the supernatant was collected and further centrifuge evaporated overnight. Cellular metabolite extracts were derivatized by CH₃ONH₂ at 37°C for 1 h. Oximes and alklyoximes derivatives was then incubated with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 70°C for 30 min. The metabolite extracts were collected into a glass vial for GC-MS and kept at -80 °C until analysis via GC-MS/MS (Agilent 7000 Series Triple Quad GC/MS).

13) Statistical analysis

All data are expressed as mean $\pm$ S.D. The reproducibility of the results is confirmed in at least three independent experiments. Data are from a representative set of experiments after normalized to the results of controls. Statistical differences are determined using one-way ANOVA following with a post hoc test. $P < 0.05$ is considered as statistically significant.

Results

1. Sub-population containing in human lung cancer cells

Single-cell of human lung cancer H292 cells was successfully isolated through single-cell seeding in 96-well plate. Distinct two main populations composing with small (H292-S) and large (H292-L) morphology cells were revealed in H292 cells. It was worth nothing that isolated H292S and H292L could be maintained for 2-3 culture passage then mix population as H292 wild type (H292-WT) was found in both sub-populations.

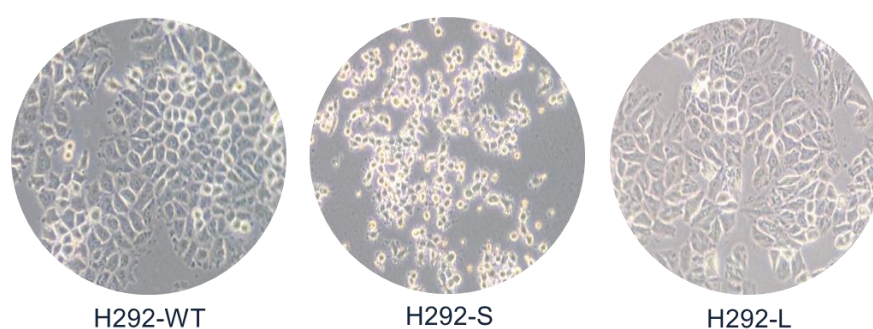


Figure 1 Human lung cancer cells composed with at least three sub-population with different morphology; wild type (WT), small (S) and large (L) cells.

2. Aggressive behaviors in sub-population of human lung cancer cells

Aggressive features including drug sensitivity, anchorage-independent growth and cancer stemness were further investigated in isolated sub-populations. Both H292-S and H292-L possessed chemotherapeutic resistance. Higher %cell viability was observed in H292-S and H292-L sub-population incubated with cisplatin (50 μ M), doxorubicin (10 μ M) and etoposide (100 μ M) for 24 h compared with H292-WT (fig. 2a). Capability to growth under detachment, a critical step in cancer metastasis was evaluated in lung cancer H292 sub-populations. Figure 2b indicates the increase colony size formed in H292-S compared with H292-WT. Surprisingly, colony formation was rare detected in H292-L sub-population.

To determine stemness phenotype in isolated sub-populations, spheroid forming assay was performed. Tightly packed colonies were notified in H292-L sub-population while comparable size and number was presented in H292-WT and H292-S (fig. 2c)

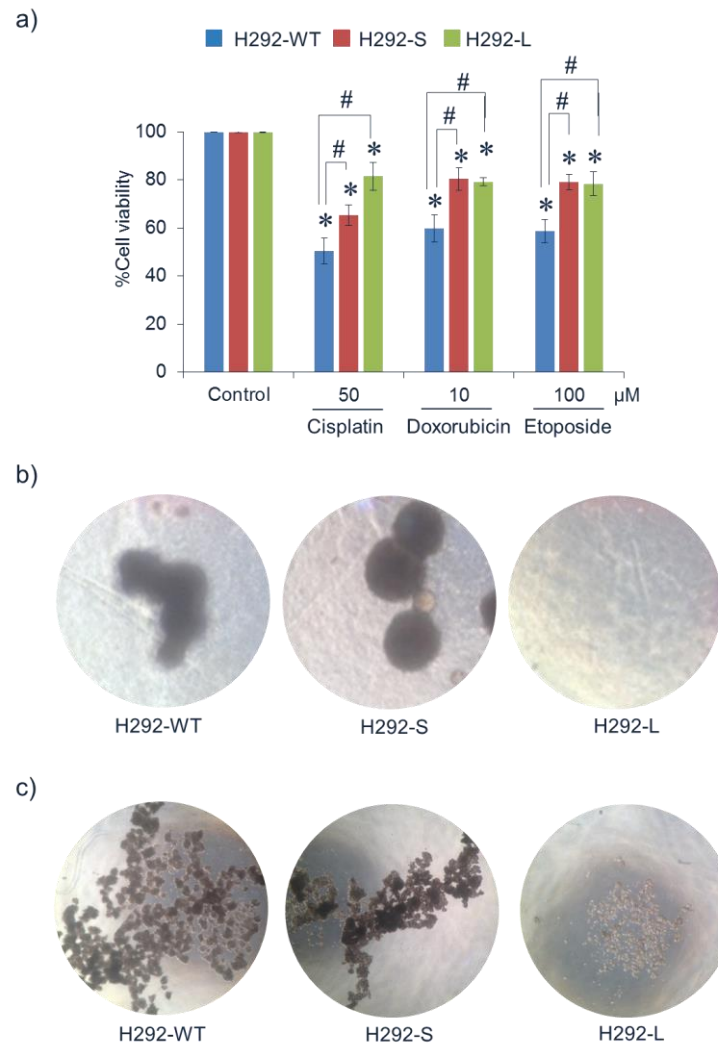


Figure 2 a) Chemotherapeutic resistant activity of lung cancer sub-population towards current chemotherapeutic drugs b) Capability to growth under detachment condition of sub-population in H292 cells. c) Capability to generate new tumor was evaluated through spheroid formation assay. Tightly packed colonies were observed in H292-L sub-population. * $p \leq 0.05$ versus non-treated control cells, # $p \leq 0.05$ versus H292-WT.

3. Alteration on stemness and EMT regulating proteins in sub-populations of human lung cancer cells

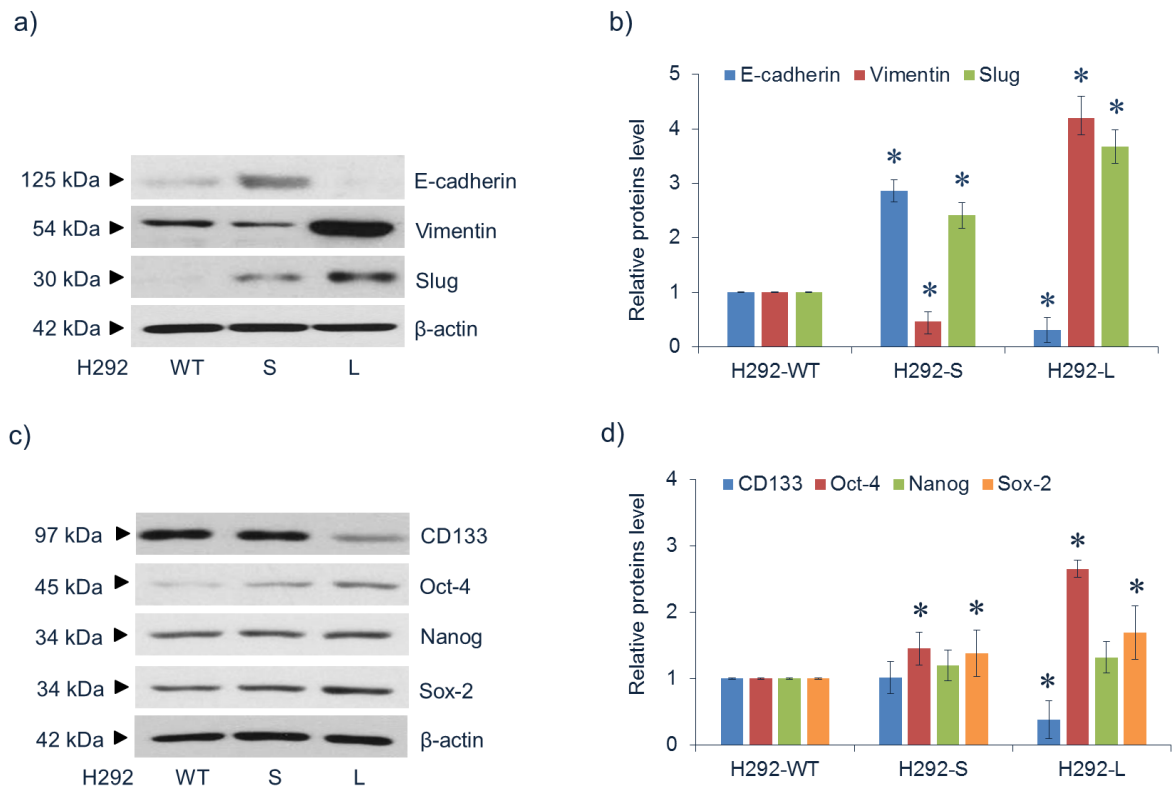


Figure 3 Western blot analysis revealed a and b) the over-expression of EMT regulating protein including vimentin and slug in H292-L sub-population. c and d) Despite lower expression of stemness marker protein; CD133, up-regulation of stemness transcription factor; oct-4 and sox-2 were presented in H292-L cells. * $p \leq 0.05$ versus H292-WT

Protein markers of epithelial to mesenchymal transition (EMT) which involves with metastasis activity highly expressed in H292-L sub-population (fig. 3a). It should be noted that the up-regulation on EMT transcription factors, vimentin and slug in H292-L did not correlated with capability to survival under detachment condition (fig. 2b). There was the significant augmentation of stemness regulating proteins; Oct-4 and Sox-2 in both H292-S and H292-L compared with H292-WT (fig. 3c). Notably, the level of CD133, stemness marker protein was down-regulated in H292-L sub-population (fig. 3b).

4. Metabolomic analysis of sub-population of lung cancer cells

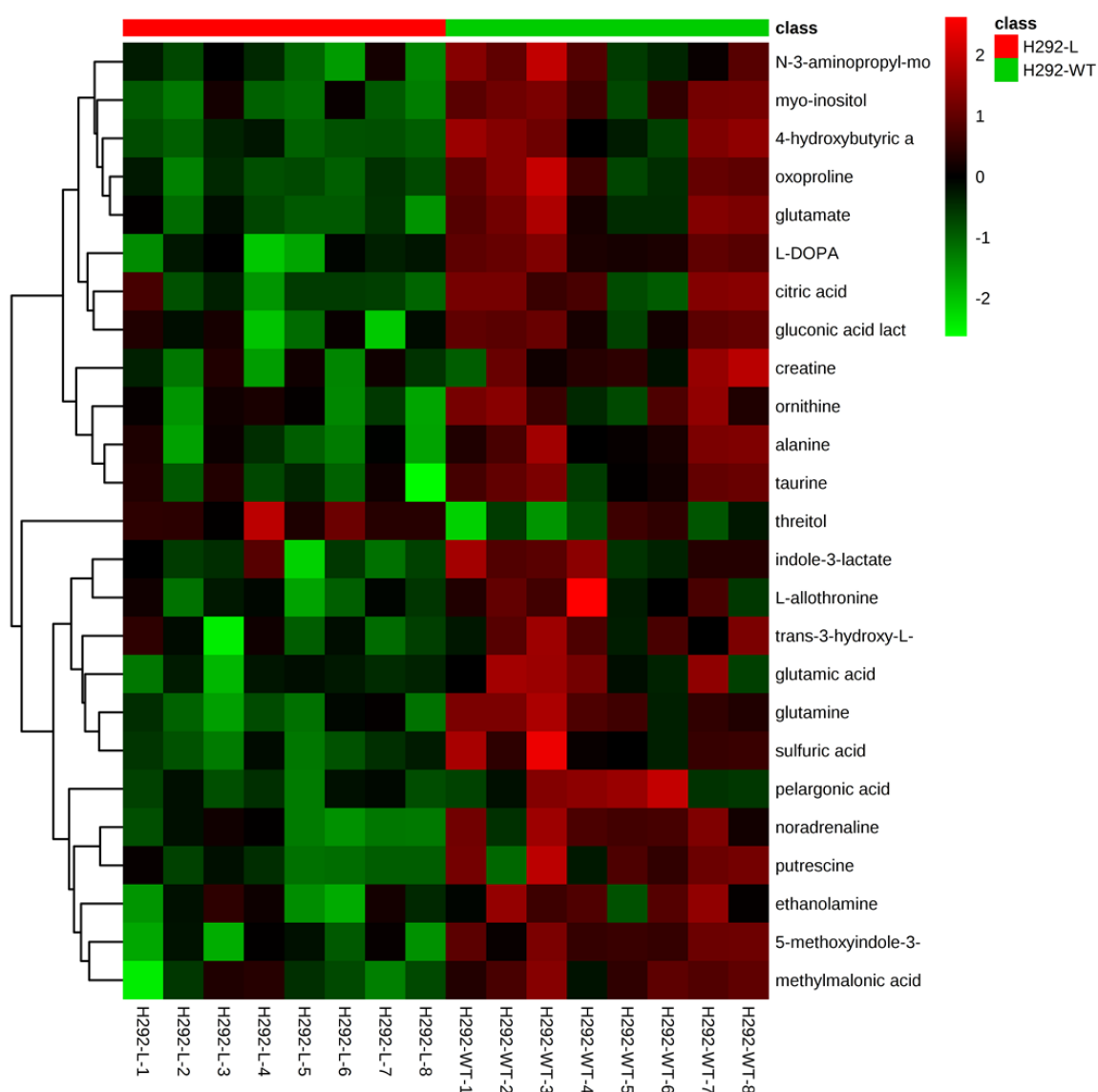


Figure 4 Heatmap of top 25 metabolic compounds ranked by p -value

In order to clarify the underlying mechanisms regulating distinct phenotype in sub-population of human lung cancer cells, metabolomics analysis was performed in H292-L sub-population. Figure 4 demonstrates the significant difference of top 25 metabolite level in H292-L compared with H292-WT. The reduction of overall metabolites was reveal in H292-L sub-population. The unique pattern of metabolomics profile was elucidated in H292-L sub-population (fig. 5).

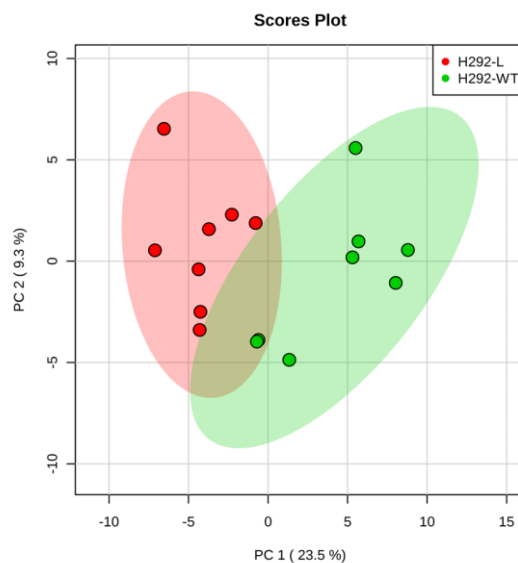


Figure 5 Principal component analysis of metabolite profiles of H292-WT and H292-L sub-population

5. Peptides extracted from *Lentinus squarrosulus* Mont. induces apoptosis in human lung cancer cells

5.1 Cytotoxicity of the peptide extracts in human lung cancer cells

The reduction of cell viability was early observed at 6 h after treatment of H460 lung cancer cells with 20 µg/ml peptide extracts (fig. 6a). After 24 h of incubation, 40% reduction in viability of the cells was found in the cell treated with 20 µg/ml peptide extracts. Figure 2d presents dose-dependent cytotoxicity of the peptide extracts in human lung cancer cells. Notably, 85% of viable cell was suppressed after treatment of the H460 cells with peptide extracts at the concentration of 40 µg/ml for 24 h.

Mode of cell death detected by co-staining of Hoechst33342/PI indicated apoptotic cell death after incubation H460 with the peptide extracts at 5-20 µg/ml for 24 h, meanwhile there was notification of necrosis (fig.6b and 6e). However, Figure 6f illustrated the highest concentration (40 µg/ml) of peptide extracts caused both apoptosis and necrosis in human lung cancer cells with DNA condense stained by Hoechst33342 and red fluorescence of PI, respective. Thus, peptide extracts at

5-20 µg/ml were selected for further evaluation of anticancer activity in human cancer cells.

5.2 Apoptosis induction in lung cancer cells treated with peptide extracts

In order to confirm peptide extracts-induced apoptosis, the evaluation on apoptosis marker proteins was performed via western blot analysis. The activation of caspase3 indicating with the increase of cleaved-caspase3 was observed in treatment of H460 cells with peptide extracts (5-20 µg/ml) for 24 h (fig. 7). There were also the significant reduction of PARP-1 (Poly(ADP-ribose) polymerase-1) and the augmentation of cleaved-PARP in lung cancer cells treated with 10-20 µg/ml (fig. 7b).

5.3 Peptides extracted form *L.squarrosulus* stimulate both intrinsic and extrinsic apoptosis pathway

There are two major apoptotic pathways, intrinsic and extrinsic machinery. The alteration of Bcl-2 family proteins involving in intrinsic or mitochondrial pathway was significantly observed in H460 cells treated with the peptide extracts. Figure 8a showed that there was a reduction of Bcl-2, an anti-apoptotic protein, while a pro-apoptotic protein, BAX obviously increased after incubation of lung cancer cells with 10-20 µg/ml of peptide extracts for 24 h. The induction of extrinsic or death receptor signaling in peptide extracts-treated H460 cells was presented by the reduction of c-FLIP, an inhibitor of death receptor-activated caspase cascade, associating the augmentation of cleaved-caspase8 (Fig. 8b).

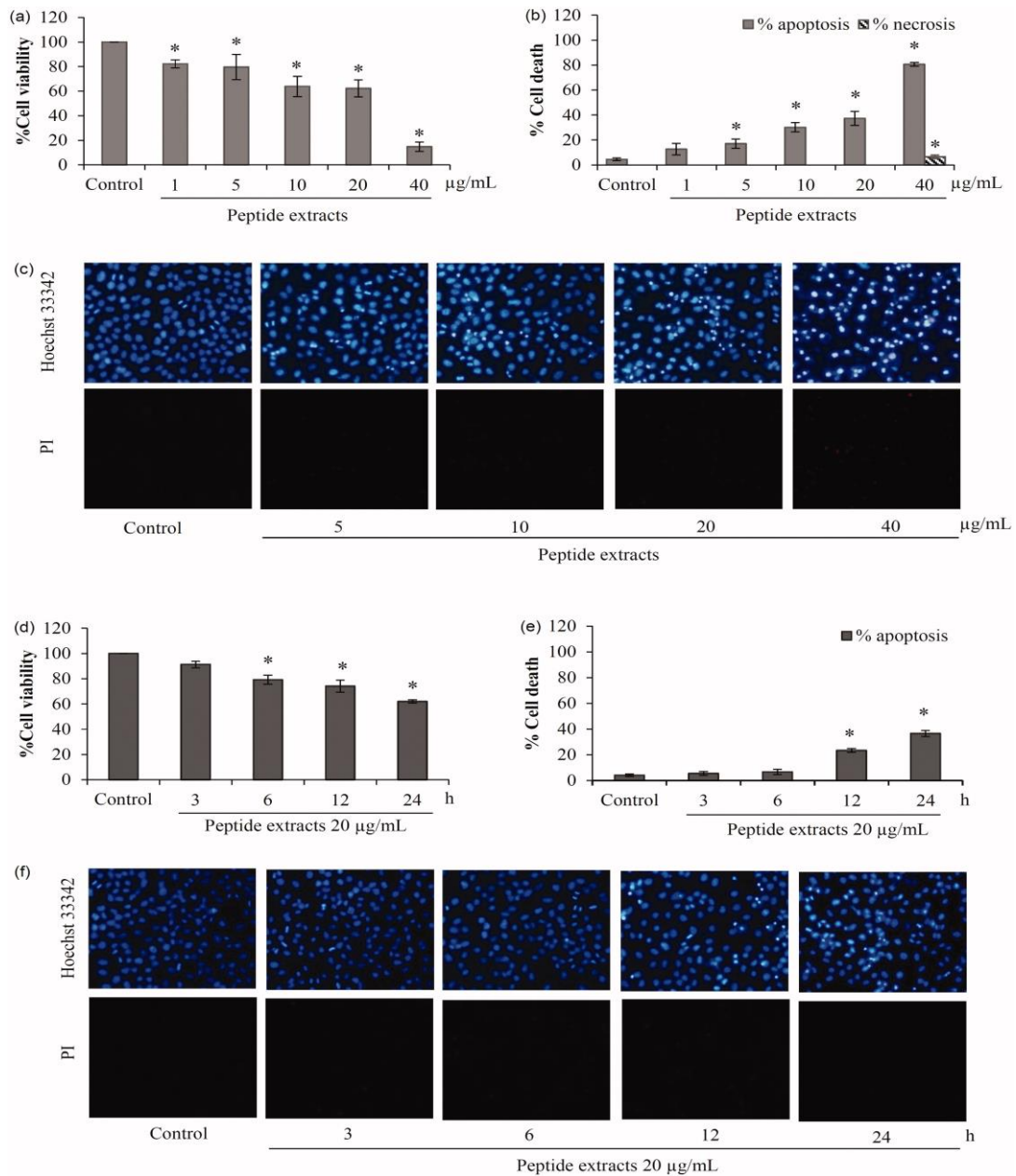


Figure 6 Apoptosis induction of peptide extracts from *Lentinus squarrosulus* Mont. Cell viability of H460 lung cancer cells was evaluated after treatment with the extracts in (a) time-dependent and (c) dose-dependent manner. (b) The effect of peptide extracts on apoptosis induction was early notified at 12 h of treatment lung cancer cells with 20 μg/mL. (e) Additionally, there was a significant augmentation of apoptotic cell death in the incubation of peptide extracts at 5-40 μg/mL for 24 h. (c and f) Apoptosis and necrosis cells were presented via co-staining of Hoechst and propidium iodide. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

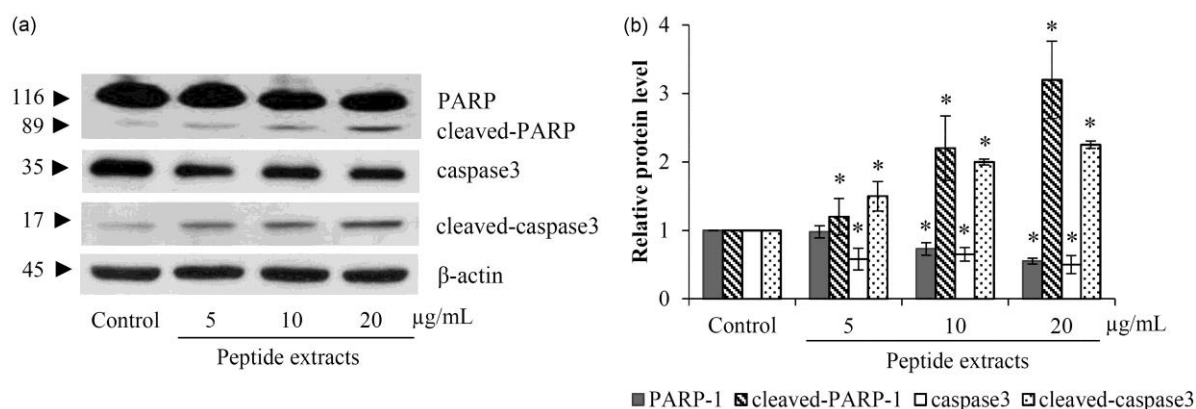


Figure 7 The apoptosis cells death was confirmed by the alteration of caspase3 and PERP proteins. (a) Western blot analysis indicated the increasing of active caspase3 (cleaved-caspase3) in peptide extracts-treated H460 cells. (b) As substrate of activated casapase3, the significant reduction of PARP was associated with the level of cleaved-caspase3. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

5.4 Selective anticancer activity of peptides extracted from *L. squarrosulus*

To evaluate the selective anticancer activity, cytotoxicity of the peptide extracts in human dermal papilla DPCs cells was investigated. Dermal papilla cells are one of the most affected normal cells induced by current chemotherapeutic drugs. Damage of dermal papilla cells leads to hair loss and a low quality of life in lung cancer patients. The selective anticancer effect and human safety profile of *L. squarrosulus* peptides are presented in figure 9. After 24 h of treatment, the peptide extracts at 1–40 μg/mL caused no significant alteration of % cell viability in DPCs cells compared with non-treated control cells (fig. 9(a)).

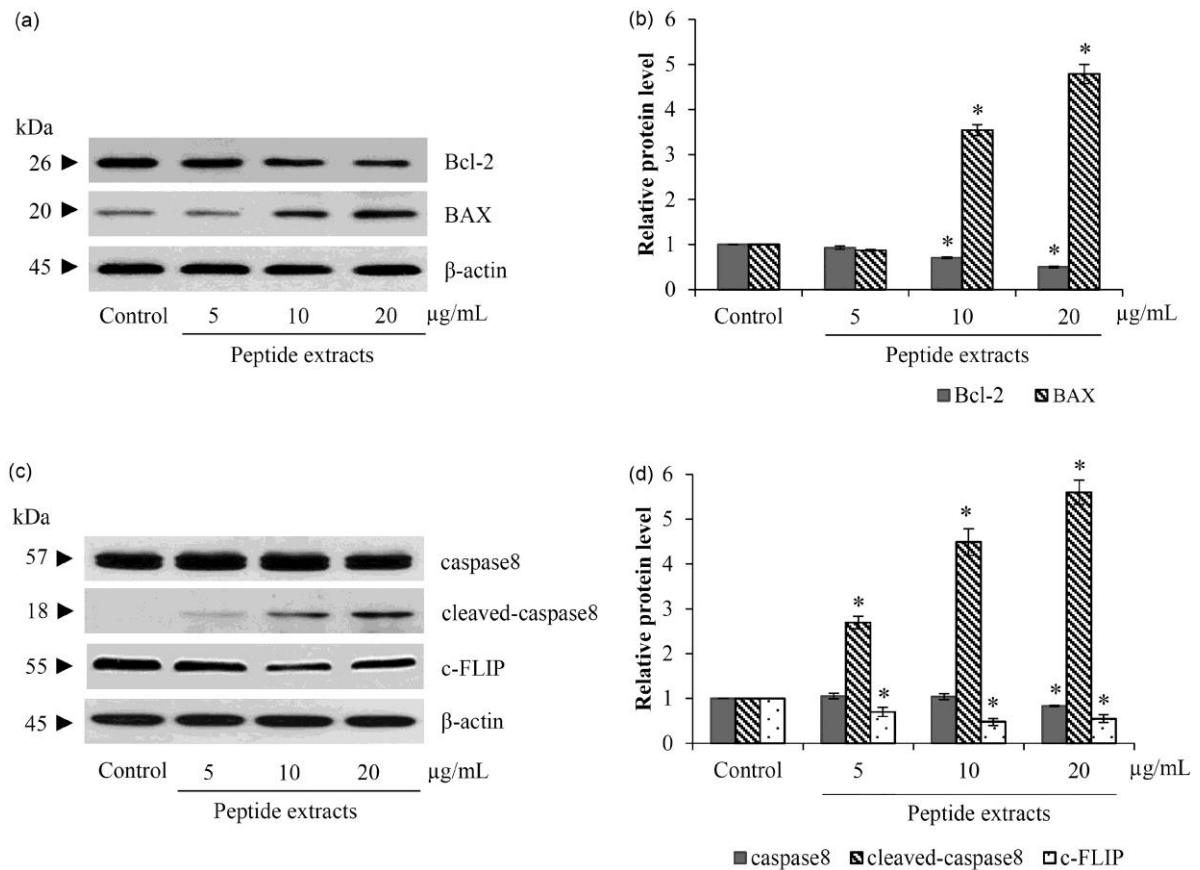


Figure 8 Peptide extracts stimulated both mitochondrial and death-receptor apoptotic pathway. (a and b) There were the reduction of Bcl-2 and the up-regulation of BAX after treatment of lung cancer cells with 10-20 μ g/ml of peptide extracts from *Lentinus squarrosulus*. (c and d) Protein-relating death-receptor pathway, c-FLIP and caspase8 were decreased in peptide extracts-incubated H460 cells. Values are means of the independent triplicate experiments $\pm$ SD. * $p < 0.05$ versus non-treated control.

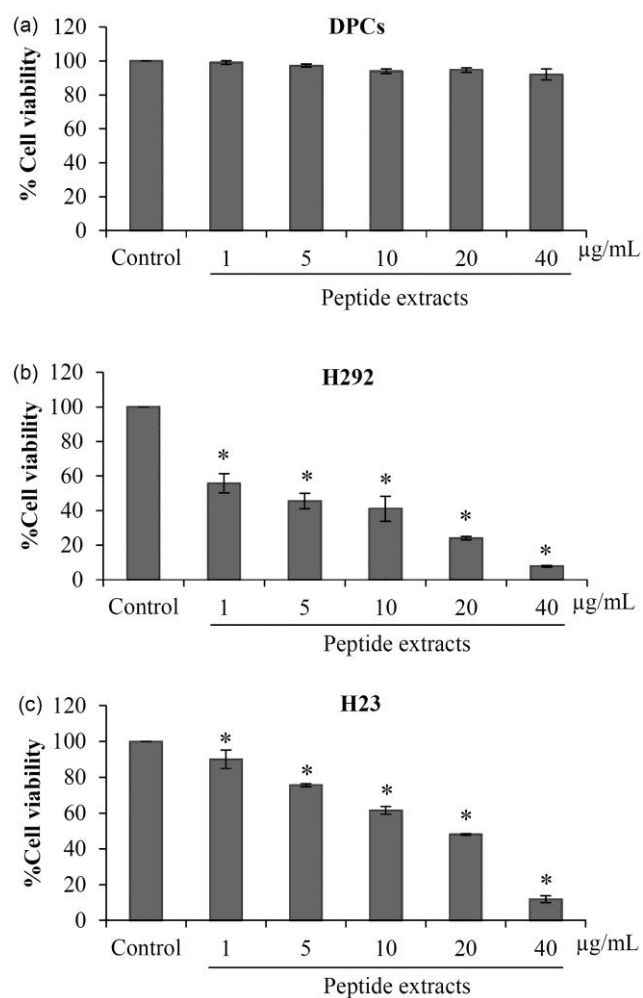


Figure 9. Selective anticancer activity of peptide extracts from *Lentinus squarrosulus*. (a) Low toxicity of the peptide extracts to non-cancer cells was indicated in human dermal papilla DPCs cells treated with 1–40 µg/mL peptides for 24 h. (b and c) Peptide extracts from *L. squarrosulus* induced cell death in various lung cancer cells. The anticancer activity of the extracts was also evaluated in H292 and H23 lung cancer cells. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

6. Avicequinone B sensitizes anoikis in human lung cancer cells

6.1 Cytotoxicity of avicequinone B in human lung cancer cells

To investigate the effect of avicequinone B on anoikis, the cytotoxicity of the compound in lung cancer H460 cells was firstly elucidated. Cell viability was examined by MTT assay after treatment of the cells with avicequinone B at 0-10 μM for 24 h. Cytotoxic profile of avicequinone B was shown in figure 10. In detail, the significant reduction of %cell viability was observed in the cells treated with 8-10 μM of avicequinone B (fig. 10a). Figure 2b indicates the increase of apoptosis cell death in H460 cells after treatment with 10 μM of avicequinone B. There was no observation of necrosis cells stained with red fluorescence of PI in all treatment of avicequinone B (fig. 10c). These results demonstrated that non-toxic concentrations of avicequinone B in human lung cancer H460 cells were between 0.5 to 4 μM .

The inhibitory effect of avicequinone B on proliferation in human lung cancer cells was further evaluated. Figure 10d indicates that treatment with 2-4 μM of avicequinone B for 72 h significantly suppressed %cell proliferation in lung cancer H460 cells compared non-treated control cells. Notably, the anti-proliferative activity of avicequinone B (4 μM) was early observed after 48 h of incubation time.

6.2 Anoikis sensitizing effect of avicequinone B in H460 lung cancer cells

Detachment-induced cell death was assessed in human lung cancer cells through the culture of H460 cells as single-cell suspension in non-adhesive poly-HEMA coated-plates. After 0-24 h of incubation with non-toxic concentrations (0-4 μM) of avicequinone B, the survival of the cells was determined by XTT assay. The reduction of cell survival was detected in the control cells as early as 6 h after detachment (fig. 11a). For treated cells, avicequinone B at 4 μM significantly diminished viability of H460 cells compared with non-treated control cells at the same time point. Co-staining with Hoechst33342 and PI confirmed the anoikis sensitizing effect of avicequinone B. Induction of apoptosis without presenting of necrosis was illustrated in avicequinone B-treated H460 cells (fig. 11c). Figure 11b

shows the significant augmentation of anoikis in H460 cells after incubation with 4 μM of avicequinone B for 24 h.

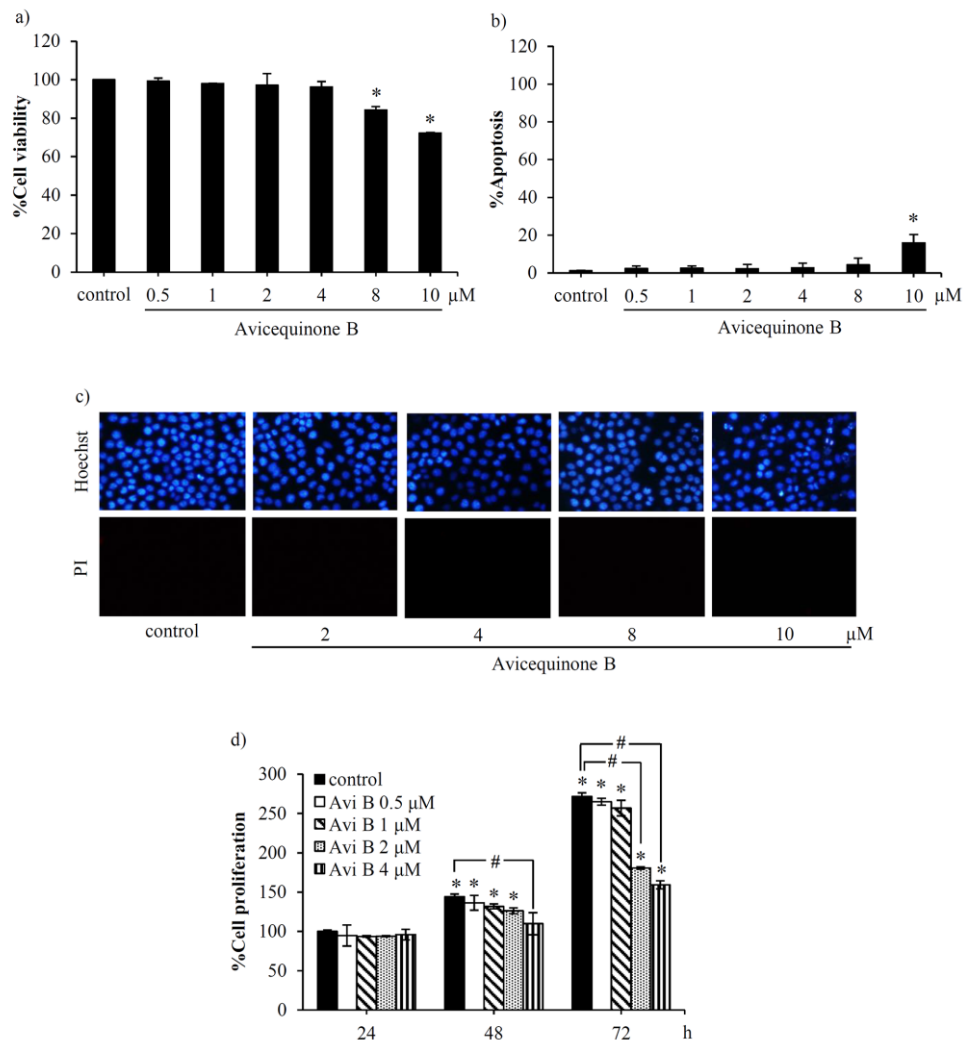


Figure 10 Cytotoxicity of avicequinone B in human lung cancer cells. (a) MTT assay revealed the significant reduction of cell viability in lung cancer H460 cells after treatment with 8-10 μM of avicequinone B for 24 h. (b) Avicequinone B at 10 μM induced apoptosis cell death in human lung cancer cells. (c) Co-staining with Hoechst33342/PI demonstrated that no necrosis cell death was detected in H460 cells at all treatment of avicequinone B. (d) The suppression on proliferation in adherent lung cancer cells was significantly notified in H460 cells incubated with 2-4 μM of avicequinone B for 72 h. Data represent the means $\pm$ SD ($n = 3$). *, # $p \leq 0.05$ versus untreated control cells.

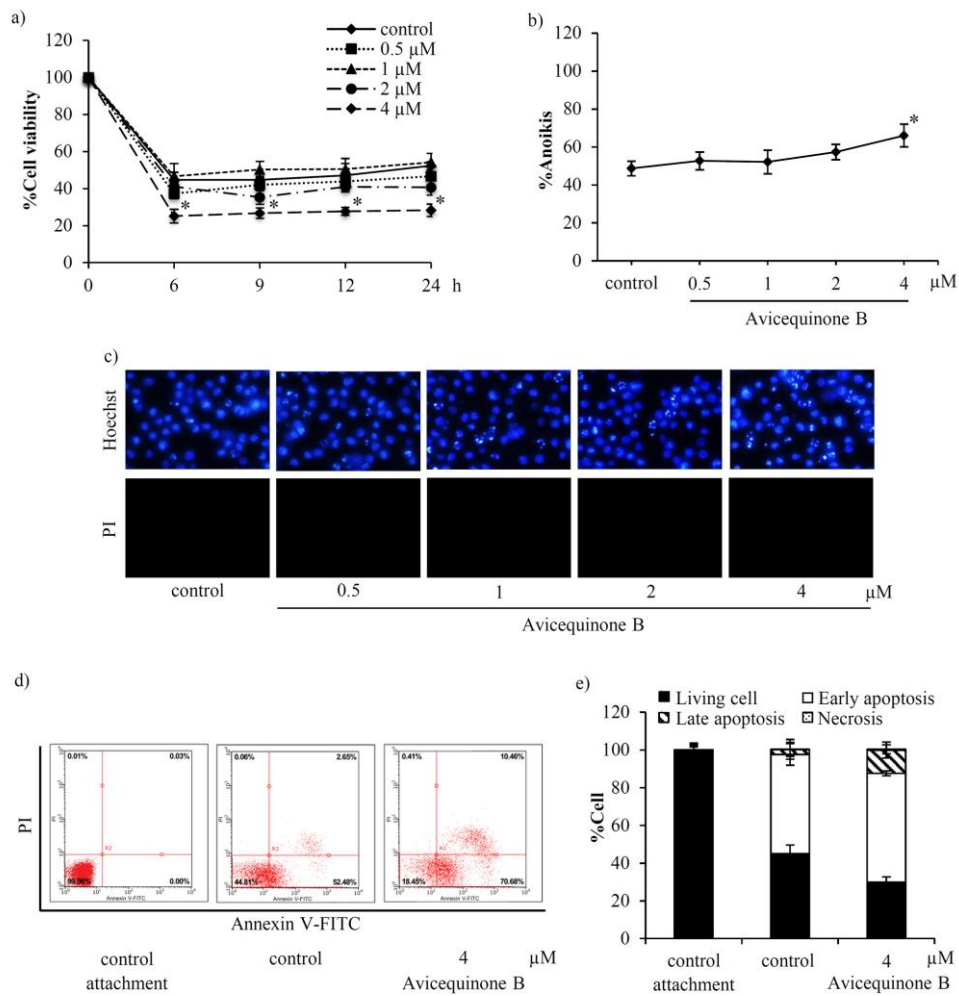


Figure 11 Avicequinone B sensitized anoikis in human lung cancer cells. (a) The reduction of viability was early observed in lung cancer H460 cells after culture under detachment condition for 6 h. (b) Avicequinone B at 4 μ M significantly induced anoikis in H460 cells that were culture under detachment condition for 24 h. (c) Bright blue fluorescence of Hoechst33342 indicated DNA condense and apoptosis body was indicated in H460 cells treated with 4 μ M of avicequinone B. (d) Detachment-induced apoptosis was evidenced with histograms obtained from flow cytometry analysis of H460 cells at anchorage culture (control attachment), detachment without treatment for 24 h (control) and detachment with 4 μ M of avicequinone B for 24 h. (e) The detection early and late apoptosis without necrosis cells was remarkably increased after incubation of non-adherent H460 cells with avicequinone B at 4 μ M. * $p \leq 0.05$ versus untreated control cells at the same time point.

Flow cytometry analysis also indicated the presence of anoikis in human lung cancer cells. Annexin V-FITC which interacts with phosphatidylserine on the cell membrane of apoptosis cells was dramatically detected in H460 cells cultured in ultra-low attachment plate for 24 h (fig. 11d). The higher number of early (Annexin V-FITC positive) and late (Annexin V-FITC positive and PI positive) apoptosis were obviously notified in detached H460 cells incubated with 4 μ M of avicequinone B compared with the non-adherent control cells (fig. 11e). These results evidenced the anoikis sensitizing activity of avicequinone B in human lung cancer cells.

6.3 Avicequinone B suppresses cancer cell growth in anchorage-independent condition

The effect of avicequinone B on capability to growth and survive under detachment condition was further evaluated in soft-agar assay. Human lung cancer H460 cells were anchorage-independently grown in 0.33% agarose gel supplemented with culture medium in presence or absence of avicequinone B (0.5-4 μ M). Figure 12a presents the colony formation initiating from a single cell of H460 after 14 days of culture under detachment condition. The reduction of both relative colony number and size was significantly observed in H460 cells treated with 2-4 μ M of avicequinone B (fig. 12b and 12c). Intriguingly, avicequinone B at 4 μ M obviously suppressed proliferation in lung cancer cells at both anchorage-dependent (Fig.3d) and - independent condition.

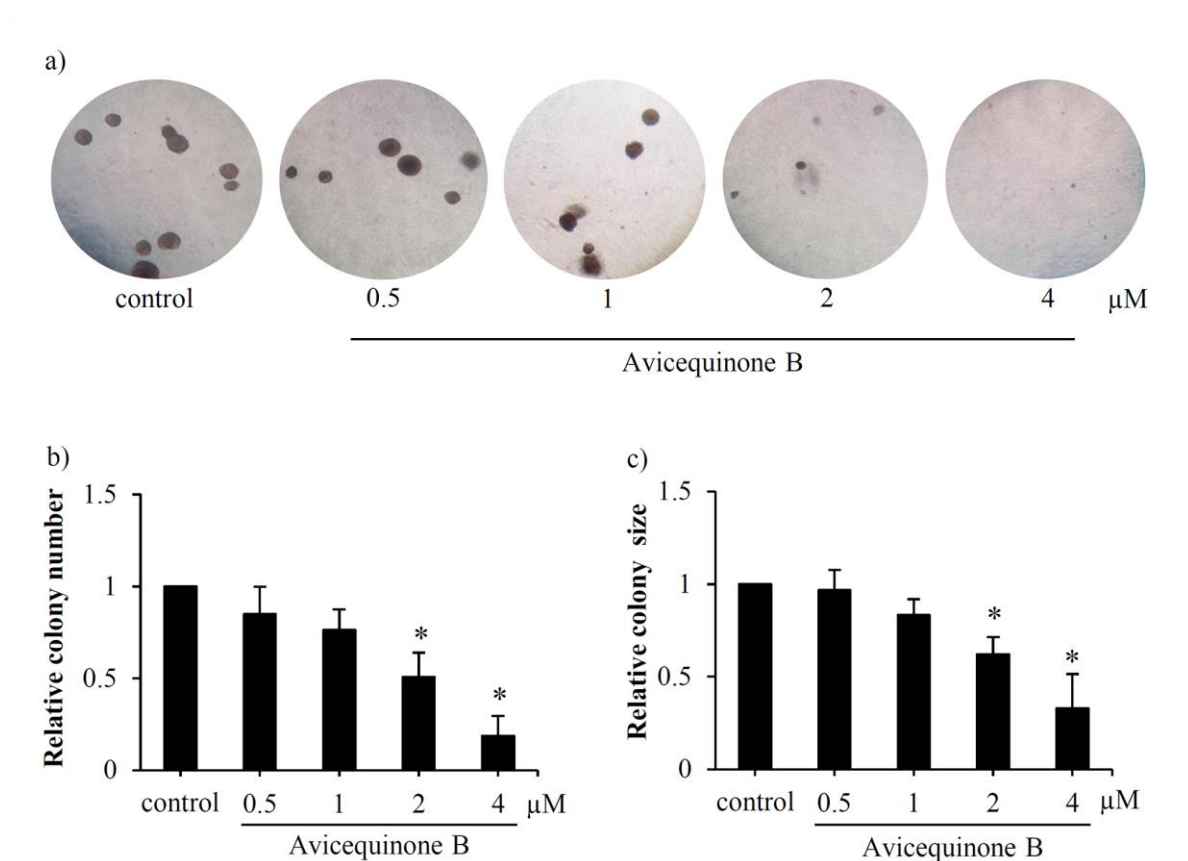


Figure 12 Effect of avicequinone B on anchorage-independent growth. (a) Colony formation of single-cell H460 cells was evaluated under a microscope after culture in soft agar for 14 days. (b) Relative colony number and (c) relative colony size were gradually decreased incubation of single-cell H460 with 1-4 μM of avicequinone B. Data represent the means ± SD (n = 3). *p ≤ 0.05 versus untreated control cells.

6.4 Avicequinone B decreases anti-apoptotic proteins in non-anchorage lung cancer cells

Anoikis is an apoptosis cell death induced by detachment condition, the alteration of apoptosis-regulating proteins including Bcl-2, Mcl-1, Bax and caveolin-1 was examined in H460 cells treated with avicequinone B. In order to escape from anoikis, human lung cancer H460 cells can sustain the level of anti-apoptosis proteins during non-adherent circumstance. Avicequinone B at 4 μM significantly reduced the level of caveolin-1 in lung cancer cells detached for 12 h (fig. 13a). Moreover,

treatment with 2-4 μM of avicequinone B significantly declined anti-apoptotic Bcl-2 family proteins, Bcl-2 and Mcl-1 (fig. 13b). It is worth noting that the expression of Bax, a pro-apoptosis protein was not altered in response to the treatment of avicequinone B (1-4 μM) for 12 h (fig. 13a and b).

6.5 Down-regulation of integrin mediated-survival signal by avicequinone B

In order to proliferate under non-adherent condition, anoikis resistant cancer cells substantially activate pro-survival pathways [9]. Thus, the alteration on integrin mediated-survival signal was investigated in H460 cells cultured with avicequinone B. Western blot analysis obviously revealed the down-regulation of integrin $\beta 1$ and $\beta 3$ in human lung cancer cells treated with 4 μM of avicequinone B under detachment condition (Fig. 5c). Interestingly, the reduction of integrin $\beta 3$ was also notified at low concentrations (1-2 μM) of avicequinone B. Figure 5d indicates the reduction of p-FAK and p-Src, down-stream signaling molecules of integrins in H460 cells treated with avicequinone B. The alteration on level of AKT and ERK protein, pro-survival molecules activated by p-Src was further investigated [30]. As presented in figure 5e and f, the diminution of activated ERK (p-ERK) was notified in detached H460 cells incubated with avicequinone B (1-4 μM). Meanwhile there were no significant alteration of AKT and p-AKT expression in avicequinone B treated-H460 cells compared with non-treated control. This inhibitory effect on integrin/FAK/Src survival pathway corresponded with sensitization to anoikis (Fig.3) and low colony formation (Fig. 4) in human lung cancer cells exposed with avicequinone B.

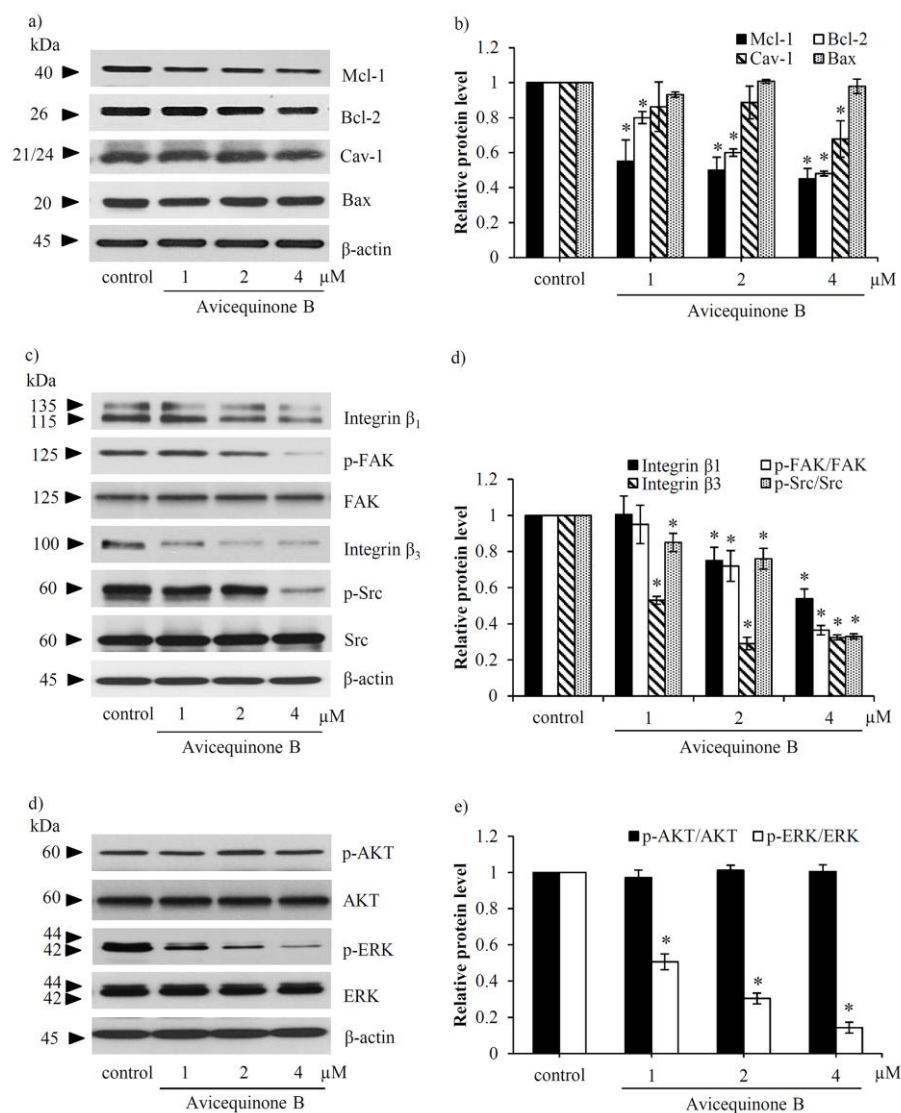


Figure 13 Avicequinone B down-regulated anti-apoptosis proteins and pro-survival signaling. (a) Western blot analysis revealed the reduction of anti-apoptosis proteins, Mcl-1 and Bcl-2 in H460 cells treated with avicequinone B (1-4 μM) under detachment condition for 12 h. (b) The decrease of caveolin-1 (Cav-1) was significantly notified in non-adherent H460 cells incubated with avicequinone B at 4 μM. (c) Low expression level of integrin β_1 and β_3 were demonstrated in avicequinone B-treated lung cancer cells. (d) Downstream pro-survival signaling of integrin including p-FAK (Y397) associating with p-Src (Try416) were also down-regulated after incubation of detached H460 cells with avicequinone B (1-4 μM). (e) The reduction of p-ERK (Thr 981) was significantly notified while (f) there was no alteration of p-AKT (Ser 473)/AKT in avicequinone-treated lung cancer cells. Data represent the means $\pm$ SD (n = 3). * $p \leq 0.05$ versus untreated control cells.

6.6 Avicequinone B sensitizes anoikis and suppresses anchorage-independent growth in various lung cancer cells

In order to confirm anoikis sensitizing effect of avicequinone B, the detachment-induced cell death was performed in human lung cancer H292 and H23 cells. Figure 14a and b indicate that avicequinone B at non-toxic concentrations (2-4 μ M; data not shown) significantly reduced viability in non-adherent H292 and H23 cells, respectively. Inspiringly, the sensitizing activity of avicequinon B on detachment-induced cell death was distinctly observed at lower dose (2 μ M) in both H292 and H23 lung cancer cells compared with H460 cells which only responded to 4 μ M of avicequinon B. Soft agar assay demonstrated the diminution of both colony number and size in H292 (fig. 14c) and H23 (fig. 14d) treated with 2-4 μ M of avicequinone B. These data strengthened the anti-metastasis activity of avicequinone B on the induction of anoikis and the inhibition on survival under detachment condition in human lung cancer cells.

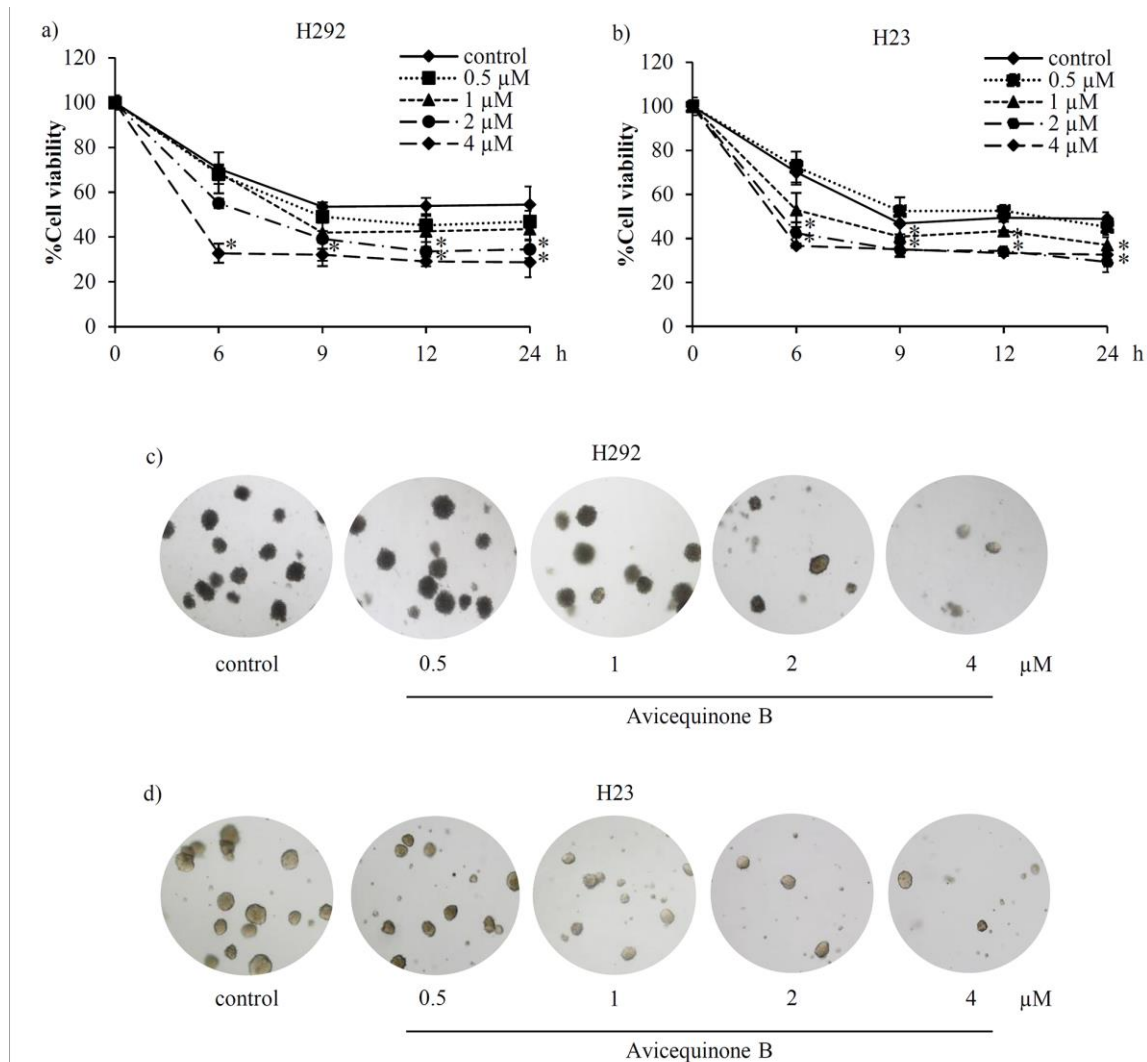


Figure 14 Avicequinone B restrained survival under detachment condition in various lung cancer cells. The lower viability of non-adherent (a) H292 and (b) H23 lung cancer cells was significantly notified in the cells incubated with avicequinone B at 2-4 μ M compared with non-treated control. Anchorage-independent growth assay revealed the reduction of both number and size of forming colonies in lung cancer (c) H292 and (d) H23 cells after culture with 2-4 μ M of avicequinone B for 14 days. Data represent the means $\pm$ SD (n = 3). *p $\leq$ 0.05 versus untreated control cells at the same time point.

Discussion

Human lung cancer cells composed with various sub-populations which possessed aggressive behaviors and distinct phenotypes. The sub-population of H292 lung cancer cells exhibiting large cell morphology showed highest chemotherapeutic resistance and *in vitro* tumor initiation activity. The characterization on regulatory proteins involving with aggressiveness revealed that this large cell sub-population contained CSC and EMT property. The unique metabolomics profile discovered in large cell sub-population inspired for further study on the metabolites regulating aggressive phenotypes in lung cancer cells. However, the alteration of metabolite compounds in sub-population was initially screened in this study. The metabolic pathway that might regulate specific feature in lung cancer sub-population was not successfully proposed. The focusing on specific metabolite groups should be proceeded in order to get the precise information for pathway analysis. Moreover, proteomic analysis should be performed to strengthen the metabolomics data. The obtained information about regulatory proteins and metabolomics will be further analyzed to propose the potential regulatory pathways mediating drug resistance in human lung cancer cells and provide more insight regarding cancer cell heterogeneity.

The stability of cell population critically impeded the investigation on aggressive behaviors and regulatory mechanisms in sub-populations. The reversion of mix population composing with various cell morphology was detected in isolated sub-population that culture for 1-2 month. The optimal condition including culture medium and sub-culture period should be further modified.

Cancer research has focused on a search for novel active compounds with high efficacy and low toxicity. Thus, this study has demonstrated the promising anticancer effect of peptides extracted from the *L. squarrosulus* mushroom against human lung cancer cells. Although drug resistance in lung cancer is complex and frequently caused by several factors, the increase of anti-apoptotic proteins such as Bcl-2 and c-FLIP has been shown to be dominant (Indran et al., 2011). The over expression of Bcl-2 and accumulation of cellular Bcl-

2 proteins have been shown to mediate lung cancer cell resistance to several chemotherapies as well as death stimuli (Wesarg et al., 2007; Yip and Reed 2008; Yang et al., 2009). Likewise, the increase in the level of c-FLIP has been shown to inhibit the death of cancer cells in response to immune cell-mediated apoptosis (Wang et al., 2008; Bagnoli et al., 2010; Safa & Pollok 2011). This investigation demonstrated that peptides extracted from *L. squarrosulus* at concentrations of 1–40 µg/mL significantly decreased viability and induced apoptosis of various human lung cancer cells including H460, H23 and H292 cells. The mechanism of apoptosis demonstrated in H460 cells showed that the peptide extracts mediated apoptosis by increasing BAX while decreasing Bcl-2 protein. Although many chemotherapeutic drugs are currently prescribed for the treatment of lung cancer, their usage is frequently limited by severe side effects to non-cancer cells (Lemjabbar-Alaoui et al., 2015). The high safety profile in humans of *L. squarrosulus* mushroom has been proven by the long time it has been consumed as a traditional food as well as a toxicity study of an animal model (Mhd Omar et al. 2011; Das et al. 2017). Selective anticancer activity presented in this study strengthens the potential development of extracts from *L. squarrosulus* with less side-effect than current chemotherapeutic drugs. In summary, the findings from this study highlight the potent effects of peptides extracted from fruiting bodies of *L. squarrosulus* mushroom, in mediating apoptosis in lung cancer cells through the decrease of anti-apoptotic Bcl-2 and c-FLIP proteins and increase of pro-apoptotic protein Bax. Nevertheless, the purification, examination and identification of amino acid sequencing of active peptide with a high efficacy and safety profile in extracts of *L. squarrosulus* should be further studied.

The capability to growth and escape from cell death under detachment condition has been found in non-adherent tumor cells circulating in blood and lymphatic system (Kim et al., 2012). In order to resist to detachment-induced cell death, cancer cells acquire the high expression of anti-apoptosis proteins and anoikis resistant mechanisms (Kim et al., 2012; Geiger and Peeper, 2005). The up-regulation of anti-apoptotic proteins and pro-survival signal has been revealed in lung cancer cells with anoikis resistant phenotype (Liu, et al., 2008). Down-regulation of caveolin-1, Mcl-1 and Bcl-2 as well as suppression on AKT and ERK activation successfully initiate detachment-induced cell death in anoikis-resistant lung cancer

cells (Sirimangkalakitti et al., 2016; Chanvorachote et al., 2015). The suppression on integrin/FAK/Src pathway consequence with the reduction of pERK/ERK and diminution of anti-apoptosis Bcl-2 family proteins evidence the sensitizing effect of avicequinone B in anoikis resistant lung cancer cells.

Phosphorylation on FAK and Src leading to formation FAK-Src complex which modulates metastasis features such as migration and anchorage-independent growth (M. A. et al., 2004). Therefore, the suppression on integrin/FAK/Src signaling has been recognized as targeted pathway for treatment metastasis cancer (Mitra and Schlaepfer, 2016). Inhibition on migration and invasion of naphthoquinone compound has been demonstrated in cancer cells (Lin et al., 2010). Taken together with the anoikis sensitizing activity of avicequinone B obtained from this study, these data strengthened the possibility to develop naphthoquinones and their derivatives as novel anti-metastasis drugs.

Conclusion

The results gained from this study lightened the attempts to clarify the correlation and regulating role on cellular metabolite and aggressive behavior in sub-population of human lung cancer cells. The potential anticancer activities of nature extracts would urge the finding a novel chemotherapy focusing modulating metabolism in cancer cells.

Output

Publication : Prateep A, Sumkhemthong S, Suksomtip M, Chanvorachote P, Chaotham C. Peptides extracted from edible mushroom: *Lentinus squarrosulus* induces apoptosis in human lung cancer cells. *Pharm Biol.* 2017;55(1):1792-1799. doi: 10.1080/13880209.2017.1325913.

Submitted manuscript : Prateep A, Sumkhemthong S, Karnsomwan W, De-Eknamkul W, Chamni S, Chanvorachote P, Chaotham C. Avicquinone B sensitizes anoikis in human lung cancer cells

References

- Bagnoli M, Canevari S, Mezzanzanica D. Cellular FLICE-inhibitory protein (c-FLIP) signalling: a key regulator of receptor-mediated apoptosis in physiologic context and in cancer. *Int J Biochem Cell Biol.* 2010; 42 :210–3.
- Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature.* 2006; 444(7120):756-60.
- Bertolini G, Roz L, Perego P, Tortoreto M, Fontanella E, Gatti L, Pratesi G, Fabbri A, Andriani F, Tinelli S, Roz E, Caserini R, Lo Vullo S, Camerini T, Mariani L, Delia D, Calabrò E, Pastorino U, Sozzi G. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci U S A.* 2009; 106(38):16281-6.
- Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med.* 1997; 3(7):730-7.
- Chaffer CL and Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011; 331:1559-64.
- Chanvorachote P, Pongrakhananon V, Halim H. Caveolin-1 regulates metastatic behaviors of anoikis resistant lung cancer cells. *Mol Cell Biochem.* 2015; 399: 291-302.
- Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* 2005; 65(23):10946-51.
- Dan X, Liu W, Wong JH, Ng TB. 2016. A ribonuclease isolated from wild *Ganoderma lucidum* suppressed autophagy and triggered apoptosis in colorectal cancer cells. *Front Pharmacol.* 2016; 7: 217.
- Das AR, Borthakur M, Saha AK, Joshi SR, Das P. 2017. Molecular characterization and antioxidant potential of three wild culinary-medicinal mushrooms from Tripura, northeast India. *Int J Med Mushrooms.* 2017; 19: 55–63.
- De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer.* 2013; 13(2):97-110.

Eramo A, Lotti F, Sette G, Piloizzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C, De Maria R. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ.* 2008; 15(3):504-14.

Geiger TR, Peeper DS. The neurotrophic receptor TrkB in anoikis resistance and metastasis: a perspective. *Cancer Res.* 2005; 65: 7033-6.

Grosse-Gehling P, Fargeas CA, Dittfeld C, Garbe Y, Alison MR, Corbeil D, Kunz-Schughart LA. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *J Pathol.* 2013; 229(3):355-78.

Indran IR, Tufo G, Pervaiz S, Brenner C. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta.* 2011; 1807:735–45.

Ito C, Katsuno S, Kondo Y, Tan HT, Furukawa H. Chemical constituents of *Avicennia alba*. Isolation and structural elucidation of new naphthoquinones and their analogues. *Chem Pharm Bull.* 2000; 48: 339-43.

Iwatsuki M, Mimori K, Yokobori T, Ishi H, Beppu T, Nakamori S, Baba H, Mori M. Epithelial-mesenchymal transition in cancer development and its clinical significance. *Cancer Sci.* 2010; 101(2), 293-299.

Kim YN, Koo KH, Sung JY, Yun UJ, Kim H. Anoikis resistance: an essential prerequisite for tumor metastasis. *Int J Cell Biol.* 2012; 2012: 306879.

Lemjabbar-Alaoui H, Hassan OU, Yang YW, Buchanan P. Lung cancer: biology and treatment options. *Biochim Biophys Acta.* 2015; 1856: 189–210.

Levina V, Marrangoni A, Wang T, Parikh S, Su Y, Herberman R, Lokshin A, Gorelik E. Elimination of human lung cancer stem cells through targeting of the stem cell factor-c-kit autocrine signaling loop. *Cancer Res.* 2010; 70(1):338-46.

Levina V, Marrangoni AM, DeMarco R, Gorelik E, Lokshin AE. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties. *PLoS One.* 2008; 3(8):e3077.

Li C, Lee CJ, Simeone DM. Identification of human pancreatic cancer stem cells. *Methods Mol Biol.* 2009; 568:161-73.

Li W, Liu C, Tang Y, Li H, Zhou F, Lv S. Overexpression of Snail accelerates adriamycin induction of multidrug resistance in breast cancer cells. *Asian Pac J Cancer Prev*. 2011; 12(10), 2575-2580.

Lin KL, Su JC, Chien CM, Tseng CH, Chen YL, Chang LS, Lin SR. Naphtho[1,2-b]furan-4,5-dione induces apoptosis and S-phase arrest of MDA-MB-231 cells through JNK and ERK signaling activation. *Toxicol In Vitro*. 2010; 24: 61-70.

Liu X, Fan D. The epithelial-mesenchymal transition and cancer stem cells: functional and mechanistic links. *Curr Pharm Des*. 2015; 21(10):1279-91.

Liu G, Meng X, Jin Y, Bai J, Zhao Y, Cui X, Chen F, Fu S. Inhibitory role of focal adhesion kinase on anoikis in the lung cancer cell A549. *Cell Biol Int*. 2008; 32: 663-70.

M. A. Westhoff, B. Serrels, V. J. Fincham, M. C. Frame, N. O. Carragher. Src-Mediated Phosphorylation of Focal Adhesion Kinase Couples Actin and Adhesion Dynamics to Survival Signaling. *Mol Cell Biol*. 2004; 24: 8113-33.

Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, Zheng BJ, Guan XY. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology*. 2007;132(7):2542-56.

Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer*. 2006 Jun;6(6):449-58.

Mhd Omar NA, Abdullah N, Kuppusamy UR, Abdulla MA, Sabaratnam V. Nutritional composition, antioxidant activities, and antiulcer potential of *Lentinus squarrosulus* (Mont.) mycelia extract. *Evid Based Complement Alternat Med*. 2011; 2011: 539356.

Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Bio*. 2006; 18: 516-23.

Moore N, Lyle S. "Quiescent, Slow-Cycling Stem Cell Populations in Cancer: A Review of the Evidence and Discussion of Significance," *J Oncol*. 2011;2011. pii: 396076.

Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: a review. *3 Biotech*. 2012; 2:1–15.

Perona R, López-Ayllón BD, de Castro Carpeño J, Belda-Iniesta C. A role for cancer stem cells in drug resistance and metastasis in non-small-cell lung cancer. *Clin Transl Oncol*. 2011; 13(5):289-93.

Safa AR, Pollok KE. Targeting the anti-apoptotic protein c-FLIP for cancer therapy. *Cancers (Basel)*. 2011; 3 :1639–71.

Sen IK, Maji PK, Behera B, Mallick P, Maiti TK, Sikdar SR, Islam SS. Structural characterization of an immunoenhancing heteroglycan of a hybrid mushroom (pfls1h) of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer. *Carbohydr Res*. 2013; 371: 45–51.

Scheel C, Weinberg RA. Cancer stem cells and epithelial-mesenchymal transition: concepts and molecular links. *Semin Cancer Biol*. 2012; 22: 396-403.

Siegel R, Ma J, Zou Z, Jemal A. Cancer Statistics, 2014. *CA Cancer J Clin* 2014;64:9-29.

Sirimangkalakitti N, Chamni S, Suwanborirux K, Chanvorachote P. Renieramycin M Sensitizes Anoikis-resistant H460 Lung Cancer Cells to Anoikis. *Anticancer Res*. 2016; 36: 1665-71.

Spano D, Heck C, Antonellis P, Christofori G, Zollo M (2012). Molecular networks that regulate cancer metastasis. *Sem Cancer Biol* 2012;22: 234–49.

Thiery, J. P., Acloque, H., Huang, R. Y., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell*, 139(5), 871-890. doi:10.1016/j.cell.2009.11.007

Tsai PC, Chu CL, Fu YS, Tseng CH, Chen YL, Chang LS, Lin SR. Naphtho[1,2-b]furan-4,5-dione inhibits MDA-MB-231 cell migration and invasion by suppressing Src-mediated signaling pathways. *Mol Cell Biochem*. 2014; 387: 101-11.

Voon, D.C., H. Wang, J.K. Koo, J.H. Chai, Y.T. Hor, T.Z. Tan, Y.S. Chu, S. Mori, and Y. Ito. 2013. EMT-induced stemness and tumorigenicity are fueled by the EGFR/Ras pathway. *PLoS One*. 8:e70427.

Wang X, Chen W, Zeng W, Bai L, Tesfaigzi Y, Belinsky SA, Lin Y. Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. *Mol Cancer Ther*. 2008; 7: 1156–63.

Wesarg E, Hoffarth S, Wiewrodt R, Kröll M, Biesterfeld S, Huber C, Schuler M. Targeting BCL-2 family proteins to overcome drug resistance in non-small cell lung cancer. *Int J Cancer*. 2007; 121: 2387–94.

Yang TM, Barbone D, Fennell DA, Broaddus VC. Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. *Am J Respir Cell Mol Biol*. 2009; 41: 14–23.

Yip KW, Reed JC. Bcl-2 family proteins and cancer. *Oncogene*. 2008; 27: 6398–406.

Yongsanguanchai, N., V. Pongrakhananon, A. Mutirangura, Y. Rojanasakul, and P. Chanvorachote. Nitric oxide induces cancer stem cell-like phenotypes in human lung cancer cells. *Am J Physiol Cell Physiol*. 2015; 308:C89-100.

Zeuner A, Francescangeli F, Contavalli P, Zapparelli G, Apuzzo T, Eramo A, Baiocchi M, De Angelis ML, Biffoni M, Sette G, Todaro M, Stassi G, De Maria R. Elimination of quiescent/slow-proliferating cancer stem cells by Bcl-XL inhibition in non-small cell lung cancer. *Cell Death Differ*. 2014;21(12):1877-88.

Zeuner A. The secret life of quiescent cancer stem cells. *Molecular & Cellular Oncology*. 2015; 2(1), e968067.

Zhang Y, Wang Z, Yu J, Shi Jz, Wang C, Fu Wh, Chen Zw, Yang J. Cancer stem-like cells contribute to cisplatin resistance and progression in bladder cancer. *Cancer Lett*. 2012; 322(1):70-7.

Appendix

ประวัติผู้วิจัย/อาจารย์ที่ปรึกษา

ชื่อหัวหน้าโครงการ

(ภาษาไทย) นายฉัตรชัย เชาว์ธรรม ตำแหน่งทางวิชาการ ผู้ช่วยศาสตราจารย์ ดร.

(ภาษาอังกฤษ) Mr.Chatchai Chaotham

ภาควิชา ชีวเคมีและจุลชีววิทยา คณะ เกษตรศาสตร์ โทรศัพท์ 02-218-8372

โทรสาร - E-mail chatchai.c@chula.ac.th

ที่อยู่ปัจจุบัน 308/561 ถนนประชาชื่น เขตบางซื่อ กรุงเทพฯ 10800 โทรศัพท์ 091-951-4623

มหาวิทยาลัย	ปริญญา	สาขาวิชา	ปีที่ได้รับ พ.ศ.
จุฬาลงกรณ์มหาวิทยาลัย	เกษตรศาสตรบัณฑิต	-	2548
จุฬาลงกรณ์มหาวิทยาลัย	วิทยาศาสตรดุษฎีบัณฑิต	เทคโนโลยีเกษตรกรรม (นานาชาติ)	2556

ผลงานวิจัยที่พิมพ์เผยแพร่ (ระบุแหล่งพิมพ์และปีที่พิมพ์)

1. **Chaotham C**, Chanvorachote P. A bibenzyl from *Dendrobium ellipsophyllum* inhibits migration in lung cancer cells. J Nat Med. 2015; 69(4):565-74
2. Ausawasamrit A, Itthiwarapornkul N, **Chaotham C**, Sukrong S, Chanvorachote P. Lupalbigenin from *Derris scandens* Sensitizes Detachment-induced Cell Death in Human Lung Cancer Cells. Anticancer Res. 2015;35(5):2827-34.
3. Tanagornmeatar K, **Chaotham C**, Sritularak B, Likhitwitayawuid K, Chanvorachote P. Cytotoxic and anti-metastatic activities of phenolic compounds from *Dendrobium ellipsophyllum*. Anticancer Res. 2014;34(11):6573-9.
4. **Chaotham C**, Pongrakhananon V, Sritularak B, Chanvorachote P. A Bibenzyl from *Dendrobium ellipsophyllum* inhibits epithelial-to-mesenchymal transition and sensitizes lung cancer cells to anoikis. Anticancer Res. 2014;34(4):1931-8.

5. **Chaotham C**, Chivapat S, Chaikitwattana A and De-Eknamkul W. Acute and chronic oral toxicity of a partially-purified plaunotol extract from *Croton stellatopilosus* Ohba. BioMed Research International 2013 (303162): 12 pages
6. **Chaotham C**, De-Eknamkul W, Chanvorachote P. Protective effect of plaunotol against doxorubicin-induced renal cell death. J Nat Med. 2013;67(2):311-9.
7. Pongjit K, Ninsontia C, **Chaotham C**, Chanvorachote P. Protective effect of Glycine max and Chrysanthemum indicum extracts against cisplatin-induced renal epithelial cell death. Hum Exp Toxicol. 2011;30(12):1931-44.
8. Ninsontia C, Pongjit K, **Chaotham C**, Chanvorachote P. Silymarin selectively protects human renal cells from cisplatin-induced cell death. Pharm Biol. 2011;49(10):1082-90.

ชื่ออาจารย์ที่ปรึกษา

(ภาษาไทย) นายปิติ จันทร์วรโชติ ตำแหน่งทางวิชาการ รองศาสตราจารย์ ดร.

(ภาษาอังกฤษ) Mr.Pithi Chanvorachote

ภาควิชา เภสัชวิทยาและสรีรวิทยา คณะ เภสัชศาสตร์ โทรศัพท์ 02-218-8345

โทรสาร 02-218-8340

E-mail: pithi_chan@yahoo.com

ที่อยู่ปัจจุบัน 40-41 หมู่บ้านปรีชา 1 ถนนบรมราชชนนี เขตตลิ่งชัน กรุงเทพฯ 10170

มหาวิทยาลัย	ปริญญา	สาขาวิชา	ปีที่ได้รับ พ.ศ.
จุฬาลงกรณ์มหาวิทยาลัย	เภสัชศาสตรบัณฑิต	-	2545
จุฬาลงกรณ์มหาวิทยาลัย	วิทยาศาสตร์ดุขฎิบัณฑิต	เทคโนโลยีเภสัชกรรม (นานาชาติ)	2549
Meiji Pharmaceutical University	Post-doctoral training	Biotechnology	2550

ผลงานวิจัยที่พิมพ์เผยแพร่ (ระบุแหล่งพิมพ์และปีที่พิมพ์)

งานวิจัย

1. Pongrakhananon V, Chunhacha P, **Chanvorachote P***, 2013. Ouabain suppresses the migratory behavior of lung cancer cells. *PloS one*, 8(7):e68623.
2. **Chanvorachote P**, Kowitdamrong A, Ruanghirun T, Sritularak B, Mungmee C, Likhitwitayawuid K., 2013. Anti-metastatic activities of bibenzyls from *Dendrobium pulchellum*. *Natural Product Communications*, 8(1):115-118.
3. **Chanvorachote P**, Chunhacha P., 2013. Caveolin-1 regulates endothelial adhesion of lung cancer cells via reactive oxygen species-dependent mechanism. *PloS one*, 8(2):e57466.
4. Wongvaranon P, Pongrakhananon V, Chunhacha P, **Chanvorachote P***, 2013. Acquired resistance to chemotherapy in lung cancer cells mediated by prolonged nitric oxide exposure. *Anticancer Research*, 33(12):5433-5444.

5. Sanuphan A, Chunhacha P, Pongrakhananon V, **Chanvorachote P***, 2013. Long-term nitric oxide exposure enhances lung cancer cell migration. *BioMed Research International*, 2013:186972.
6. Wanakhachornkrai O, Pongrakhananon V, Chunhacha P, Wanasuntronwong A, Vattanajun A, Tantisira B, **Chanvorachote P***, Tantisira MH., 2013. Neuritogenic effect of standardized extract of *Centella asiatica* ECa233 on human neuroblastoma cells. *BMC Complementary and Alternative Medicine*, 13:204.
7. Cheun-Arom T, **Chanvorachote P***, Sirimangkalakitti N, Chuanasa T, Saito N, Abe I, Suwanborirux K., 2013. Replacement of a quinone by a 5-O-acetylhydroquinone abolishes the accidental necrosis inducing effect while preserving the apoptosis-inducing effect of renieramycin M on lung cancer cells. *Journal of Natural Products*, 76(8):1468-1474.
8. Dhumrongvaraporn A, **Chanvorachote P***, 2013. Kinetics of ultraviolet B irradiation-mediated reactive oxygen species generation in human keratinocytes. *Journal of Cosmetic Science*, 64(3):207-217.
9. Kowitdamrong A, **Chanvorachote P**, Sritularak B, Pongrakhananon V., 2013. Moscatilin inhibits lung cancer cell motility and invasion via suppression of endogenous reactive oxygen species. *BioMed Research International*, 2013:765894.
10. Chunhacha P, Sriuranpong V, **Chanvorachote P***, 2013. Epithelial-mesenchymal transition mediates anoikis resistance and enhances invasion in pleural effusion-derived human lung cancer cells. *Oncology Letters*, 5(3):1043-1047.
11. Powan P, Saito N, Suwanborirux K, **Chanvorachote P***, 2013. Ecteinascidin 770, a tetrahydroisoquinoline alkaloid, sensitizes human lung cancer cells to anoikis. *Anticancer Research*, 33(2):505-512.
12. Luanpitpong S, **Chanvorachote P**, Stehlik C, Tse W, Callery PS, Wang L, Rojanasakul Y., 2013. Regulation of apoptosis by Bcl-2 cysteine oxidation in human lung epithelial cells. *Molecular Biology of the Cell*, 24(6):858-869.
13. **Chanvorachote P**, Pongrakhananon V., 2013. Ouabain downregulates Mcl-1 and sensitizes lung cancer cells to TRAIL-induced apoptosis. *American Journal of physiology, Cell Physiology*, 304(3):C263-272.
14. Choochuay K, Chunhacha P, Pongrakhananon V, Luechapudiporn R, **Chanvorachote P***, 2013. Imperatorin sensitizes anoikis and inhibits anchorage-independent growth of lung cancer cells. *Journal of Natural Medicines*, 67(3):599-606.

15. Chairuangkitti P, Lawanprasert S, Roytrakul S, Aueviriyavit S, Phummiratch D, Kulthong K, **Chanvorachote P***, Maniratanachote R., 2013. Silver nanoparticles induce toxicity in A549 cells via ROS-dependent and ROS-independent pathways. *Toxicology in vitro : An international journal published in association with BIBRA*, 27(1):330-338.
16. Chaotham C, De-Eknamkul W, **Chanvorachote P***, 2013. Protective effect of plaunotol against doxorubicin-induced renal cell death. *Journal of natural medicines* 2013, 67(2):311-319.
17. **Chanvorachote P***, Chunhacha P, Pongrakhananon V: Anoikis: a potential target to prevent lung cancer metastasis? *Lung Cancer Management*, 2(3):169-171.
18. Plaibua K, Pongrakhananon V, Chunhacha P, Sritularak B, **Chanvorachote P***, 2013. Effects of Artonin E on Migration and Invasion Capabilities of Human Lung Cancer Cells. *Anticancer Research*, 33(8):3079-3088.
19. Ninsontia C, **Chanvorachote P***, 2014. Ouabain mediates integrin switch in human lung cancer cells. *Anticancer Research*, 34(10): 5495-5502.
20. **Chanvorachote P**, Pongrakhananon V, Chunhacha P., 2014. Prolonged nitric oxide exposure enhances anoikis resistance and migration through epithelial-mesenchymal transition and caveolin-1 upregulation. *BioMed Research International*, 2014:941359.
21. **Chanvorachote P**, Chunhacha P, Pongrakhananon V., 2014. Caveolin-1 induces lamellipodia formation via an Akt-dependent pathway. *Cancer cell International*, 14:52.
22. Charoenrungruang S, **Chanvorachote P**, Sritularak B, Pongrakhananon V., 2014. Gigantol, a bibenzyl from *Dendrobium draconis*, inhibits the migratory behavior of non-small cell lung cancer cells. *Journal of Natural Products*, 77(6):1359-1366.
23. Chaotham C, Pongrakhananon V, Sritularak B, **Chanvorachote P***, 2014. A Bibenzyl from *Dendrobium ellipsophyllum* inhibits epithelial-to-mesenchymal transition and sensitizes lung cancer cells to anoikis. *Anticancer Research*, 34(4):1931-1938.
24. Meksawan K, Sermsri U, **Chanvorachote P***, 2014. Zinc supplementation improves anticancer activity of monocytes in type-2 diabetic patients with metabolic syndrome. *Anticancer Research*, 34(1):295-299.
25. Pongrakhananon V, Stueckle TA, Wang HY, O'Doherty GA, Dinu CZ, **Chanvorachote P**, Rojanasakul Y., 2014. Monosaccharide digitoxin derivative sensitize human non-small cell lung cancer cells to anoikis through Mcl-1 proteasomal degradation. *Biochemical Pharmacology*, 88(1):23-35.

26. Maiuthed A, **Chanvorachote P***, 2014. Cisplatin at sub-toxic levels mediates integrin switch in lung cancer cells. *Anticancer Research*, 34(12):7111-7117.
27. Tanagornmeatar K, Chaotham C, Sritularak B, Likhitwitayawuid K, **Chanvorachote P***, 2014. Cytotoxic and anti-metastatic activities of phenolic compounds from *Dendrobium ellipsophyllum*. *Anticancer Research*, 34(11):6573-6579.
28. Winitthana T, Lawanprasert S, **Chanvorachote P***, 2014. Triclosan potentiates epithelial-to-mesenchymal transition in anoikis-resistant human lung cancer cells. *PLoS one*, 9(10):e110851.
29. Ruanghirun T, Pongrakhananon V, **Chanvorachote P***, 2014. Ouabain enhances lung cancer cell detachment. *Anticancer Research*, 34(5):2231-2238.
30. Powan P, **Chanvorachote P.**, 2014. Nitric oxide mediates cell aggregation and mesenchymal to epithelial transition in anoikis-resistant lung cancer cells. *Molecular and Cellular Biochemistry*, 393(1-2):237-245.
31. Charoenrungruang S, **Chanvorachote P**, Sritularak B, Pongrakhananon V. 2014. Gigantol-induced apoptosis in lung cancer cell through mitochondrial-dependent pathway. *TJPS*, 38(2):67-73.
32. Wongvaranon P, Pongrakhananon V, **Chanvorachote P***, 2014. Molecular mechanisms of chemotherapeutic resistance in lung cancer. *TJPS*, 38(1):1-4.
33. Luanpitpong S, Li J, Manke A, Brundage K, Ellis E, McLaughlin SL, Angsutararux P, Chanthra N, Voronkova M, Chen YC, **Chanvorachote P**. 2015. SLUG is required for SOX9 stabilization and functions to promote cancer stem cells and metastasis in human lung carcinoma. *Oncogene*.
34. Busaranon K, Plaimee P, Sritularak B, **Chanvorachote P***, 2015. Moscatilin inhibits epithelial-to-mesenchymal transition and sensitizes anoikis in human lung cancer H460 cells. *Journal of Natural Medicines*.
35. Chaotham C, **Chanvorachote P***, 2015. A bibenzyl from *Dendrobium ellipsophyllum* inhibits migration in lung cancer cells. *Journal of Natural Medicines*, 69(4):565-574.
36. Petpiroon N, Suktap C, Pongsamart S, **Chanvorachote P***, Sukrong S., 2015. Kaempferol-3-O-rutinoside from *Afgekia mahidoliae* promotes keratinocyte migration through FAK and Rac1 activation. *Journal of Natural Medicines*, 69(3):340-348.
37. Pengpaeng P, Sritularak B, **Chanvorachote P***, 2015. Dendrofalconerol A sensitizes anoikis and inhibits migration in lung cancer cells. *Journal of Natural Medicines*, 69(2):178-190.

38. **Chanvorachote P**, Pongrakhananon V, Halim H. 2015. Caveolin-1 regulates metastatic behaviors of anoikis resistant lung cancer cells. *Molecular and Cellular Biochemistry*, 399(1-2):291-302.
39. Ausawasamrit A, Itthiwarapornkul N, Chaotham C, Sukrong S, **Chanvorachote P***, 2015. Lupalbigenin from *Derris scandens* Sensitizes Detachment- induced Cell Death in Human Lung Cancer Cells. *Anticancer Research*, 35(5):2827-2834.
40. Pengpaeng P, Sritularak B, **Chanvorachote P***, 2015. Dendrofalconerol A suppresses migrating cancer cells via EMT and integrin proteins. *Anticancer Research*, 35(1):201-205.
41. Bhummaphan N, **Chanvorachote P***, 2015. Gigantol Suppresses Cancer Stem Cell-Like Phenotypes in Lung Cancer Cells. *Evidence-based Complementary and Alternative Medicine : eCAM*, 2015:836564.
42. Tawinwung S, Ninsontia C, **Chanvorachote P***, 2015. Angiotensin II Increases Cancer Stem Cell-like Phenotype in Lung Cancer Cells. *Anticancer Research*, 35(9):4789-4797.
43. Luanpitpong S, **Chanvorachote P***, 2015. Nitric Oxide and Aggressive Behavior of Lung Cancer Cells. *Anticancer Research*, 35(9):4585-4592.
44. Klongkumnuankarn P, Busaranon K, **Chanvorachote P**, Sritularak B, Jongbunprasert V, Likhitwitayawuid K. , 2015. Cytotoxic and Antimigratory Activities of Phenolic Compounds from *Dendrobium brymerianum*. *Evidence-based complementary and alternative medicine : eCAM*, 2015:350410.
45. Awaiwanont V, Tantituvanont A, Suwakul W, Meksawan K, and **Chanvorachote P***, 2015. Scavenging Activity of Whey Protein Hydrolysates in HaCaT Cells. *Chiang Mai Journal of Science*, 42(4): 907-917.
46. Yongsanguanchai N, Pongrakhananon V, Mutirangura A, Rojanasakul Y, **Chanvorachote P***, 2015. Nitric oxide induces cancer stem cell-like phenotypes in human lung cancer cells. *American Journal of Physiology, Cell Physiology*, 308(2):C89-100.
47. Hasatsri S, Yamdech R, **Chanvorachote P**, Aramwit P., 2015. Physical and biological assessments of the innovative bilayered wound dressing made of silk and gelatin for clinical applications. *Journal of Biomaterials Applications*, 29(9):1304-1313.
48. Unahabhokha T, Sucontphunt A, Nimmannit U, **Chanvorachote P**, Yongsanguanchai N, Pongrakhananon V., 2015. Molecular signalings in keloid disease and current therapeutic approaches from natural based compounds. *Pharmaceutical Biology*, 53(3):457-463.

49. Tantituvanont A, Jiamchaisri P, Chunhacha P, Pongjit K, Ninsontia C, Meksawan K, Sritularak B, **Chanvorachote P***, 2015. Silymarin inhibits cisplatin-mediated apoptosis via inhibition of hydrogen peroxide and hydroxyl radical generation. *Songklanakarin Journal of Science & Technology*, 37(2).
50. Kiratipaiboon C, Tengamnuay P, **Chanvorachote P***, 2015. Glycyrrhizic acid attenuates stem cell-like phenotypes of human dermal papilla cells. *Phytomedicine*, 22(14):1269-1278.
51. Kiratipaiboon C, Tengamnuay P, **Chanvorachote P***, 2016. Ciprofloxacin Improves the Stemness of Human Dermal Papilla Cells. *Stem Cells International*.
52. Pramchu-em C, Meksawan K, **Chanvorachote P***, 2016. Zinc sensitizes lung cancer cells to anoikis through down-regulation of Akt and caveolin-1. *Nutrition and Cancer*, 68(2):312-319.
53. Unahabhokha T, **Chanvorachote P**, Pongrakhananon V., 2016. The attenuation of epithelial to mesenchymal transition and induction of anoikis by gigantol in human lung cancer H460 cells. *Tumor Biology*, 1-9.
54. Sirimangkalakitti N, Yokoya M, Chamni S, **Chanvorachote P**, Plubrukarn A, Saito N, Suwanborirux K., 2016. Synthesis and Absolute Configuration of Acanthodendrin, a New Cytotoxic Bromotyrosine Alkaloid from the Thai Marine Sponge *Acanthodendrilla* sp. *Chemical and Pharmaceutical Bulletin*, 64(3):258-262.
55. Busaranon K, Plaimee P, Sritularak B, **Chanvorachote P***, 2016. Moscatilin inhibits epithelial-to-mesenchymal transition and sensitizes anoikis in human lung cancer H460 cells. *Journal of Natural Medicines*, 70(1):18-27.
56. **Chanvorachote P**, Luanpitpong S., 2016. Iron induces cancer stem cells and aggressive phenotypes in human lung cancer cells. *American Journal of Physiology- Cell Physiology*, 310(9):C728-C739.
57. Phiboonchaiyanan PP, Kiratipaiboon C, **Chanvorachote P***, 2016. Ciprofloxacin mediates cancer stem cell phenotypes in lung cancer cells through caveolin-1-dependent mechanism. *Chemico-biological Interactions*, 250:1-11.
58. Sirimangkalakitti N, Chamni S, Suwanborirux K, **Chanvorachote P***, 2016. Renieramycin M Sensitizes Anoikis-resistant H460 Lung Cancer Cells to Anoikis. *Anticancer Research*, 36:1665-1671.
59. Sirimangkalakitti N, Olatunji OJ, Changwichit K, Saesong T, Chamni S, **Chanvorachote P**, Ingkaninan K, Plubrukarn A, Suwanborirux K., 2016. Bromotyrosine Alkaloids with

Acetylcholinesterase Inhibitory Activity from the Thai Sponge *Acanthodendrilla* sp. *Natural Product Communications*.

60. Ninsontia C, Phiboonchaiyanan PP, and **Chanvorachote P***, 2016. Zinc induces epithelial to mesenchymal transition in human lung cancer H460 cells via superoxide anion-dependent mechanism. *Cancer Cell International*, 16: 48. Published online 2016 Jun 17. doi: 10.1186/s12935-016-0323-4.
61. Saisongkorh V, Maiuthed A, and **Chanvorachote P***, 2016. Nitric oxide increases the migratory activity of non-small cell lung cancer cells via AKT-mediated integrin α_v and β_1 upregulation. *Cellular Oncology*, 39 (5): 449-462. doi:10.1007/s13402-016-0287-3
62. Sirimangkalakitti N, Chamni S, Charupant K, **Chanvorachote P**, Mori N, Saito N, Suwanborirux K, 2016. Chemistry of Renieramycins. Part 15: Synthesis of 22-O-Ester Derivatives of Jorunnamycin A and their Cytotoxicity against Non-Small Cell Lung Cancer Cells. *Journal of Natural Products*. 79 (8): 2089-2093.
63. Puchsaka PW, Chaotham C, **Chanvorachote P.**, 2016. α -Lipoic acid sensitizes lung cancer cells to chemotherapeutic agents and anoikis via integrin β_1/β_3 downregulation. *International Journal of Oncology*, 49 (4):1445-1456.
64. Phiboonchaiyanan PP, Busaranon K, Ninsontia C, **Chanvorachote P.**, 2016. Benzophenone-3 increases metastasis potential in lung cancer cells via epithelial to mesenchymal transition. *Cell Biology and Toxicology*.
65. Pinkhien T, Maiuthed A, Chamni S, Saito N, **Chanvorachote P.**, 2016. Bishydroquinone renieramycin M induces apoptosis of human lung cancer cells through a mitochondria-dependent pathway. *Anticancer Research*, 36 (12): 6327-6333.

หนังสือ

1. **ปิติ จันทรรวโรชิตี**. 2555. *ความรู้เบื้องต้นเกี่ยวกับผิวหนังและเส้นผม* พิมพ์ครั้งที่ 1. กรุงเทพมหานคร : สำนักพิมพ์นานะรงค์, พฤษภาคม 2555 จำนวน 130 หน้า ศูนย์หนังสือจุฬาลงกรณ์มหาวิทยาลัย จัดจำหน่าย
2. **Chanvorachote P.** and Chunhasha P., 2016. Lung cancer metastasis. In *Introduction to Cancer Metastasis*, p.61-71.Elsevier

บทความวิชาการ

1. **Chanvorachote P**, Chamni S, Ninsontia C, Phiboonchaiyanan PP. 2016. Potential anti-metastasis natural compounds for lung cancer. *Anticancer Research*, 36(11): 5707-5717.

Peptides extracted from edible mushroom: *Lentinus squarrosulus* induces apoptosis in human lung cancer cells

Arisara Prateep, Somruethai Sumkhemthong, Maneewan Suksomtip, Pithi Chanvorachote & Chatchai Chaotham

To cite this article: Arisara Prateep, Somruethai Sumkhemthong, Maneewan Suksomtip, Pithi Chanvorachote & Chatchai Chaotham (2017) Peptides extracted from edible mushroom: *Lentinus squarrosulus* induces apoptosis in human lung cancer cells, *Pharmaceutical Biology*, 55:1, 1792-1799, DOI: [10.1080/13880209.2017.1325913](https://doi.org/10.1080/13880209.2017.1325913)

To link to this article: <https://doi.org/10.1080/13880209.2017.1325913>



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Published online: 23 May 2017.



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RESEARCH ARTICLE



Peptides extracted from edible mushroom: *Lentinus squarrosulus* induces apoptosis in human lung cancer cells

Arisara Prateep^a, Somruethai Sumkhemthong^a, Maneewan Suksomtip^a, Pithi Chanvorachote^{b,c} and Chatchai Chaotham^{a,c}

^aDepartment of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; ^bDepartment of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; ^cCell-based Drug and Health Products Development Research Unit, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

ABSTRACT

Context: *Lentinus squarrosulus* Mont. (Polyporaceae) is an interesting source of diverse bioactive compounds.

Objective: This is the first study of the anticancer activity and underlying mechanism of peptides extracted from *Lentinus squarrosulus*.

Materials and methods: Peptides were isolated from the aqueous extract of *L. squarrosulus* by employing solid ammonium sulphate precipitation. They were further purified by ion-exchange chromatography on diethylaminoethanol (DEAE)-cellulose and gel filtration chromatography on Sephadex G25. Anticancer activity was investigated in human lung cancer H460, H292 and H23 cells cultured with 0–40 µg/mL of peptide extracts for 24 h. Cell viability and mode of cell death were evaluated by MTT and nuclear staining assay, respectively. Western blotting was used to investigate the alteration of apoptosis-regulating proteins in lung cancer cells treated with peptide extracts (0–20 µg/mL) for 24 h.

Results: The cytotoxicity of partially-purified peptide extracts from *L. squarrosulus* was indicated with IC₅₀ of 26.84 ± 2.84 , 2.80 ± 2.14 and 18.84 ± 0.30 µg/mL in lung cancer H460, H292 and H23 cells, respectively. The extracts at 20 µg/mL induced apoptosis through the reduction of anti-apoptotic Bcl-2 protein (~0.5-fold reduction) and up-regulation of BAX (~4.5-fold induction), a pro-apoptotic protein. Furthermore, *L. squarrosulus* peptide extracts (20 µg/mL) also decreased the cellular level of death receptor inhibitor c-FLIP (~0.6-fold reduction).

Conclusions and discussion: This study provides the novel anticancer activity and mechanism of *L. squarrosulus* peptide extracts, which encourage further investigation and development of the extracts for anti-cancer use.

ARTICLE HISTORY

Received 19 February 2017

Revised 14 April 2017

Accepted 26 April 2017

KEYWORDS

Mushroom extract; selective anticancer activity; human safety; Bcl-2; BAX; c-FLIP

Introduction

Lung cancer is currently a leading cause of cancer mortality, worldwide (Siegel et al. 2015). For several decades, attempts have been made to search for novel active compounds and strategies with improved efficacy and safety profiles (Bailly 2009). Although several available chemotherapies are currently prescribed for the treatment of lung cancer, their usage is frequently limited by severe side effects as well as drug resistance (Lemjabbar-Alaoui et al. 2015). Apoptosis induction is currently the most focused activity in anticancer-based natural product research. Apoptosis is a programmed cell death that eliminates unwanted, harmful and damaged cells to maintain homeostasis and cell population (Samali et al. 2010). The deregulation or disruption of apoptosis leads to an aberrant of cell population, tumour initiation and eventually, cancer pathology (Brown & Attardi 2005). Therefore, apoptosis is considered a key mechanism for inhibition as well as elimination of cancer. For a mechanistic approach, apoptosis is regulated through two main pathways, which are intrinsic (mitochondrial) and extrinsic (death receptor). The intrinsic pathway causes the alteration in the balance of Bcl-2 family proteins such as Bcl-2 (B-cell lymphoma 2), Mcl-1 (Myeloid Cell Leukaemia 1),

Bcl-xL (B-cell lymphoma-extra large) and BAX (BCL2 Associated X Protein) (Indran et al. 2011). The shift in cellular Bcl-2 family proteins toward an increase of pro-apoptotic proteins and/or a decrease of anti-apoptotic proteins results in the instability of mitochondrial membrane causing the release of cytochrome c to cytoplasm (Portt et al. 2011; Czabotar et al. 2014). The cytosol cytochrome c then stimulates caspase activation cascade (McDonnell et al. 2003). In terms of drug discovery, several natural product-derived compounds have been reported to exert anticancer activities by modulating the level of pro- and anti-apoptotic proteins of Bcl-2 family (Elmore 2007; Halim et al. 2011; Powan et al. 2013). An extrinsic apoptosis pathway induced by death ligand binding to its receptor, frequently fails to execute the cancer cells because of the high cellular level of c-FLIP (FLICE-like inhibitory protein), a potent inhibitor for caspase-8 (Wang et al. 2008; Safa & Pollok 2011). Taken together, targeting these anti-apoptosis members of the Bcl-2 family and c-FLIP are a promising way to sensitize the cancer cells to apoptosis.

As a unique natural source of diverse bioactive compounds, edible mushrooms are a potential resource for novel anticancer drug discovery. Apoptosis induction has been demonstrated in

CONTACT Chatchai Chaotham  cchoatham@gmail.com  Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, 10330 Bangkok, Thailand

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cancer cells cultured with peptides isolated from edible mushrooms (Patel & Goyal 2012; Dan et al. 2016). Due to its safety as a traditional food and its potential therapeutic effects, *Lentinus squarrosulus* Mont. (Polyporaceae) has been highlighted. Beside carbohydrates, proteins, vitamins and minerals, *Lentinus squarrosulus* also contains various bioactive compounds, including phenolic compound, immunostimulatory glucans and lectins peptide (Mhd Omar et al. 2011; Sen et al. 2013; Das et al. 2017). This study aimed to evaluate the anticancer activity and the underlying mechanism of peptide extracted from the Thai edible mushroom *Lentinus squarrosulus* in human lung cancer cells.

Materials and methods

Chemical reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, phosphate-buffered saline (PBS) pH 7.4, trypsin, L-glutamine, foetal bovine serum (FBS) and penicillin/streptomycin solution were obtained from Gibco (Gaithersburg, MA). Prigrow III medium for human dermal papilla DPCs cells was purchased from Applied Biological Materials Inc. (Richmond, Canada). Hoechst33342, propidium iodide (PI), dimethylsulphoxide (DMSO), ethyl alcohol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TRIS hydrochloride (Tris-HCl), sodium chloride (NaCl), Tween 20, skim milk, bovine serum albumin (BSA) and ammonium sulfate were purchased from Sigma Chemical Inc. (St. Louis, MO). A bicinchoninic acid (BCA) protein assay kit was purchased from Thermo scientific (Waltham, MA). Protease inhibitor cocktail was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Antibodies for Bcl-2, BAX, caspase-3, caspase-8, PARP, c-FLIP, β -actin and peroxidase-labelled secondary antibodies were obtained from Cell Signalling Technology Inc. (Denver, MA). Immobilon Western chemiluminescent HRP substrate was purchased from Millipore, Corp (Billerica, MA).

Preparation of peptide extracts

Isolation of crude peptides

Partially-purified peptide extracts from *Lentinus squarrosulus* were prepared and kept in -80°C . Briefly, fresh fruiting bodies of *L. squarrosulus* (Figure 1) were homogenized in deionized sterile water (3 mL/g). The clear supernatant was collected after centrifugation at 12,000g, 4°C for 30 min. Then, Tris-HCl buffer (pH 7.4) was added until the concentration reached 10 mM. Solid ammonium sulfate was slowly added to reach 40–80% saturation. After 1 h at 4°C , the protein pellet was collected by centrifuge at 12,000g, 4°C for 30 min. The pellets were re-solubilized in 50 mL of 10 mM Tris-HCl buffer (pH 7.4) and dialyzed overnight with 10 mM Tris-HCl buffer at 4°C to eliminate ammonium sulfate. Freeze-drying method was used to obtain the concentrated crude peptides.

Peptide purification

For further purification, ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose (Sigma Chemical, St. Louis, MO) column (5×30 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4) was used. The bound peptides were eluted with step-wise salt concentration gradient (0, 0.1, 0.5 and 1 M NaCl) in 10 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.2 mL/min. The eluted fractions with highest absorbance at 280 nm were



Figure 1. Thai edible mushroom, *Lentinus squarrosulus* Mont.

pooled and concentrated using freeze-drying. Further purification of these pooled fractions was carried out through size exclusion chromatography on Sephadex G25 (Amersham Bioscience, Piscataway, NJ) column (5×30 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.4). The peptides were eluted with 10 mM Tris-HCl buffer (pH 7.4) of the previously described flow rate. All steps of purification were performed at 4°C . Fractions with highest absorbance at 280 nm were pooled and concentrated using freeze-drying. The concentrated peptides were further determined for protein content, homogeneity, cytotoxicity, mode of cell death and western blot analysis.

Determination of protein content

The concentrated purified fractions of size exclusion chromatography were further determined for total protein content by a BCA assay kit to acquire an equal amount of peptides for further experiments. The concentrated purified fractions were freshly dissolved in deionized sterile water, then incubated with the mixer between BCA Reagent A and B at a ratio 50:1 in a dark place at 37°C for 30 min. The optical density of the purple colour product was evaluated via microplate reader (Anthros, Durham, NC) at 562 nm. The protein concentration was calculated from the calibration curve of bovine serum albumin (BSA) at 0–12 $\mu\text{g}/\mu\text{L}$.

Evaluation on homogeneity of purified fractions

The homogeneity of purified fractions of size-exclusion chromatography was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The method was carried out as described by Laemmli and Favre (1973), using a 15% (w/v) gel (Laemmli & Favre 1973). The gels were stained with 0.1% Coomassie brilliant blue R-250 solution and destained with methanol: acetic acid: water (30:10:60% v/v) solution.

Cell culture

All cell lines (passage number of 30–50) were cultured in an attachment cell culture plate at the optimum condition in the incubator supplied with 5% CO_2 at 37°C until they reached 70–80% confluence before using for further experiments. Human lung cancer H460, H292 and H23 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium while human dermal

papilla DPCs cells (Applied Biological Materials Inc., Richmond, Canada) were cultured in Prigrow III medium. The cultured mediums were replaced with fresh completed mediums supplemented with 2 mM L-glutamine, 10% FBS and 100 units/mL of penicillin/streptomycin every two days.

Cytotoxicity assay

Cells were seeded onto a 96-well plate at a density of 1×10^4 cells/well. After cultured for 12 h, the cells were further incubated with peptide extracts at 0–40 µg/mL for 0–24 h. At indicated time point, cell viability was determined by MTT assay. The cells were incubated with 0.4 mg/mL of MTT in a dark place at 37 °C for 4 h. Then, the supernatant was replaced with DMSO to dissolve the formazan product. The intensity of formazan colour was examined by microplate reader (Anthros, Durham, NC) at 570 nm. The optical density ratio of treated to non-treated control cells was calculated and presented in term of relative cell viability.

Nuclear staining assay

Apoptotic and necrotic cell death were evaluated by co-staining of Hoechst33342 and propidium iodide (PI). After incubation with concentrated peptides (0–40 µg/mL) for 0–24 h, the cells at a density of 1×10^4 cells/well in a 96-well plate were stained with 10 µM of Hoechst33342 and 5 µg/mL PI dyes for 30 min at 37 °C. The apoptotic and necrotic cells were visualized under a fluorescence microscope (Olympus IX51 with DP70) as condensed chromatin and/or fragmented nuclei and red fluorescence-positive cells, respectively.

Western blot analysis

Cells were seeded at a density of 5×10^5 cells/well onto a 6-well plate for 12 h and cultured in a completed medium containing peptide at 0–20 µg/mL for 24 h. After washing with cold PBS, the cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride,

100 mM phenylmethylsulphonyl fluoride and a protease inhibitor cocktail for 45 min at 4 °C. Then, the supernatant was collected, and the protein content was determined using the BCA protein assay kit. An equal amount of 40 µg protein from each sample was denatured by heating at 95 °C for 5 min with Laemmli loading buffer and, subsequently, loaded onto a 10% SDS-PAGE. After separation, proteins were transferred onto 0.45 µM nitrocellulose membranes (Bio-Rad, Hercules, CA). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl pH 7.5, 125 mM NaCl and 0.05% Tween 20) and incubated with the appropriate primary antibodies, either Bcl-2 (dilution of 1:1000 in TBST containing 5% BSA), BAX (dilution of 1:1000 in TBST containing 5% BSA), caspase-3 (dilution of 1:1000 in TBST containing 5% skim milk), caspase-8 (dilution of 1:1000 in TBST containing 5% BSA), PARP (dilution of 1:1000 in TBST containing 5% skim milk), c-FLIP (dilution of 1:1000 in TBST containing 5% BSA) or β-actin (dilution of 1:1000 in TBST containing 5% BSA) at 4 °C overnight. Then, the membranes were washed three times with TBST for 5 min and incubated with horseradish peroxidase-labelled isotype-specific secondary antibodies (dilution of 1:2000 in TBST containing 5% skim milk) for 2 h at room temperature. The immune complexes were detected by enhancement with chemiluminescence substrate and quantified by analyst/PC densitometric software (Bio-Rad Laboratory, Hercules, CA).

Statistical analysis

Mean data were averaged from three independent experiments. Statistical analysis was performed on SPSS Statistic 22 version (Armonk, NY) using one-way ANOVA followed by Bonferroni's *post hoc* test. A *p*-value ≤ 0.05 was considered as statistically significant.

Results

Partially-purified peptide extracts isolated from *Lentinus squarrosulus*

Crude proteins were precipitated from a crude extract of *L. squarrosulus* fruiting-bodies with 40–80% (w/v) saturation of

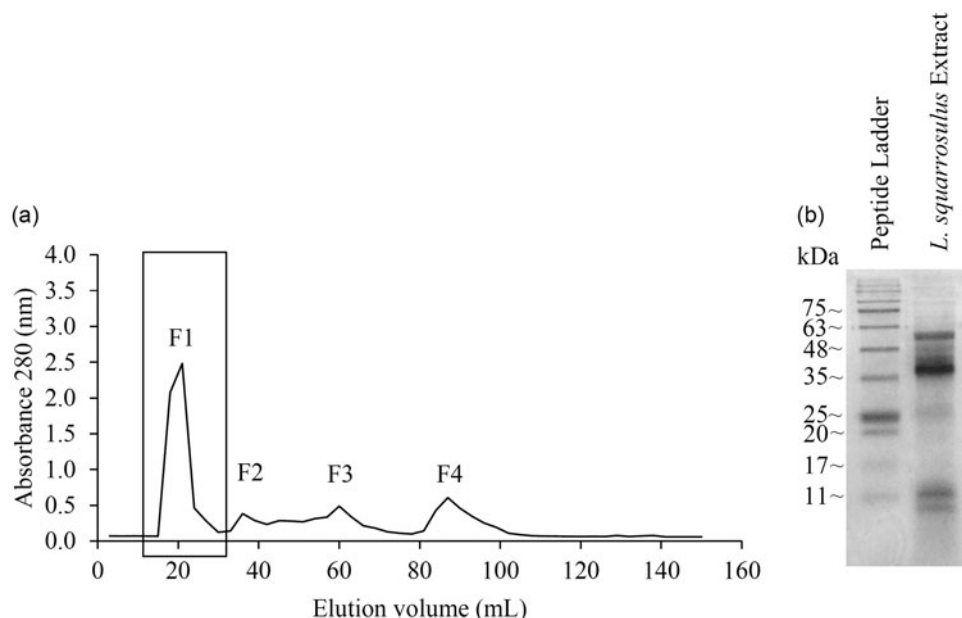


Figure 2. Isolated peptide extracts from *Lentinus squarrosulus*. (a) Peptide elution profile obtained from Sephadex G-25 gel filtration chromatography. The rectangular box indicates the highest peptide containing fraction (F1) which was used for further evaluation. (b) SDS-PAGE of F1 fraction of gel filtration chromatography.

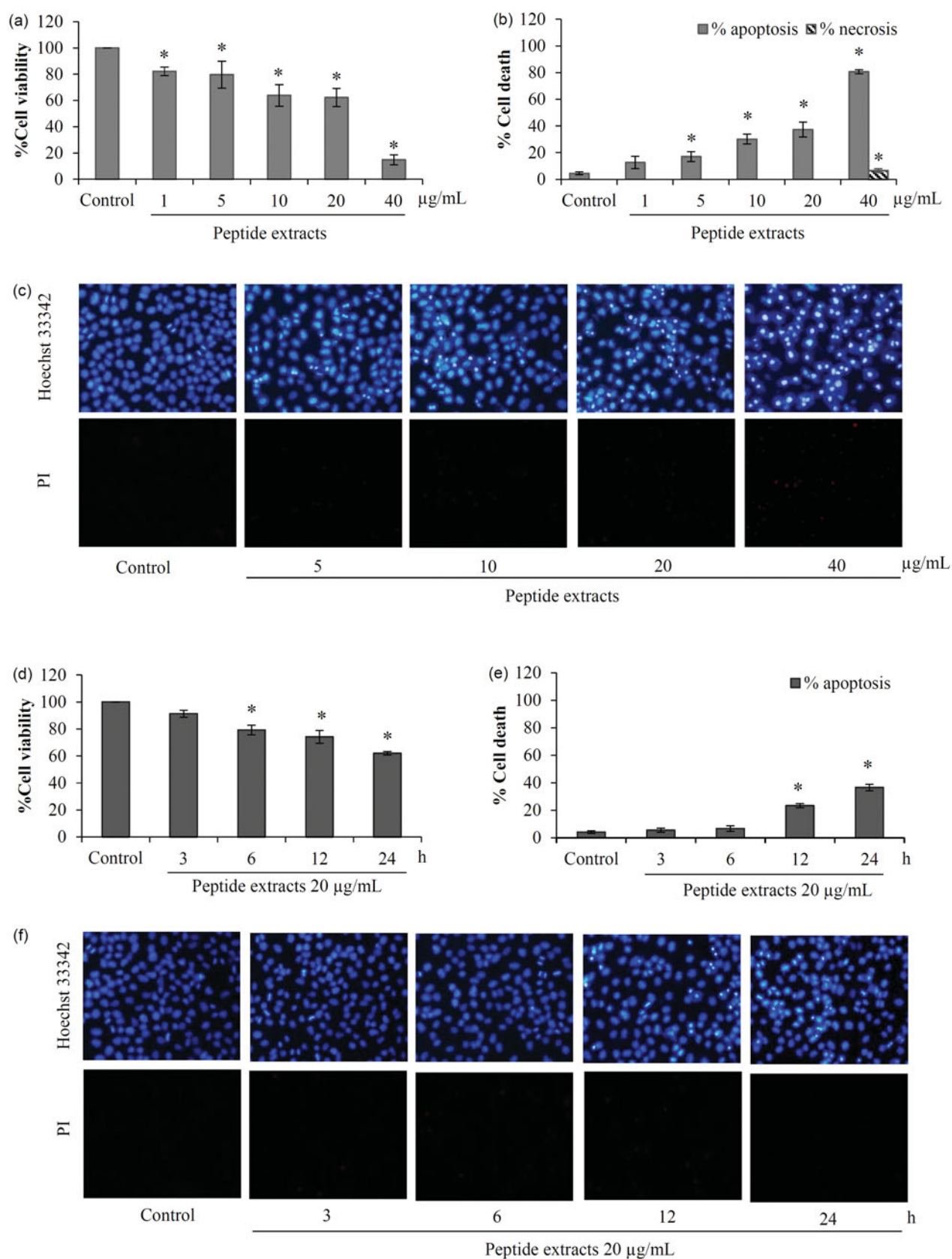


Figure 3. Apoptosis induction of peptide extracts from *Lentinus squarrosulus*. Cell viability of H460 lung cancer cells was evaluated after treatment with the extracts in (a) dose-dependent and (d) time-dependent manner. (b) There was a significant augmentation of apoptotic cell death in the incubation of peptide extracts at 5–40 μg/mL for 24 h. (e) Additionally, the effect of peptide extracts on apoptosis induction was early notified at 12 h of treatment of lung cancer cells with 20 μg/mL. (c and f) Apoptosis and necrosis cells were presented via co-staining of Hoechst33342 and propidium iodide. Values are means of the independent triplicate experiments ± SD. * $p \leq 0.05$ versus non-treated control.

ammonium sulfate. The solubilized crude proteins were further purified by ion-exchange chromatography on a DEAE-cellulose column. After, the adsorbed protein was eluted with a step-wise salt concentration gradient of NaCl at 0, 0.1, 0.5 and 1 M in 10 mM Tris-HCl buffer (pH 7.4); only fractions eluted with 0.1 M NaCl showed the highest absorbance at 280 nm. They were then further purified by gel filtration on a Sephadex G-25 column. The elution profile of protein with a total four peaks, designated

as F1 to F4, were obtained (Figure 2(a)). Sample from F1 with the highest absorbance at 280 nm was pooled and concentrated before further analysis. The SDS-PAGE analysis of the concentrated sample from F1 showed various bands in the size range of 10–50 kDa. Among these, there were four shape distinctions in size of around 9, 11, 38 and 56 kDa. The most intensive protein band was ≈ 38 kDa (Figure 2(b)). The recovery of protein in this step was about $0.159 \pm 0.004\%$ w/w.

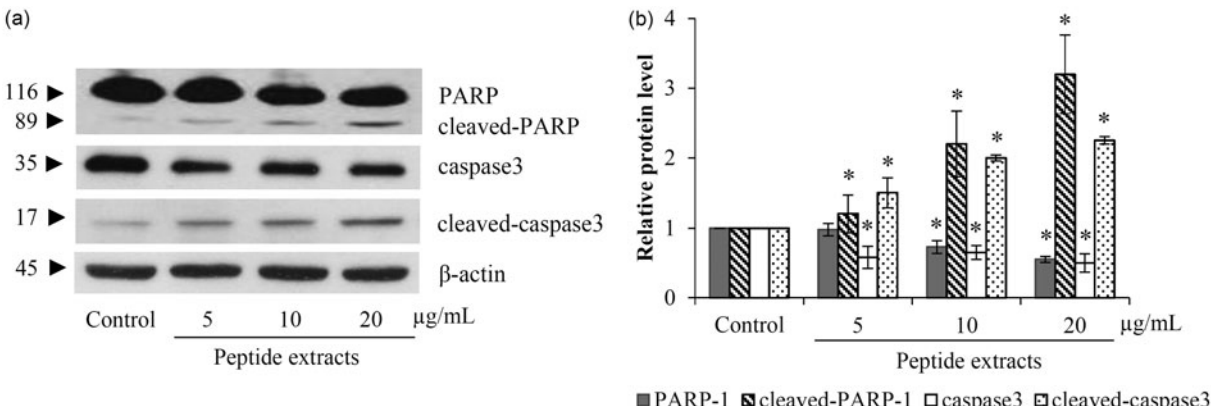


Figure 4. The apoptosis cells death was confirmed by the alteration of caspase-3 and PERP proteins. (a) Western blot analysis indicated an increase of active caspase-3 (cleaved- caspase-3) in peptide extracts-treated H460 cells. (b) As a substrate of activated caspase-3, the significant reduction of PARP was associated with the level of cleaved-caspase-3. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

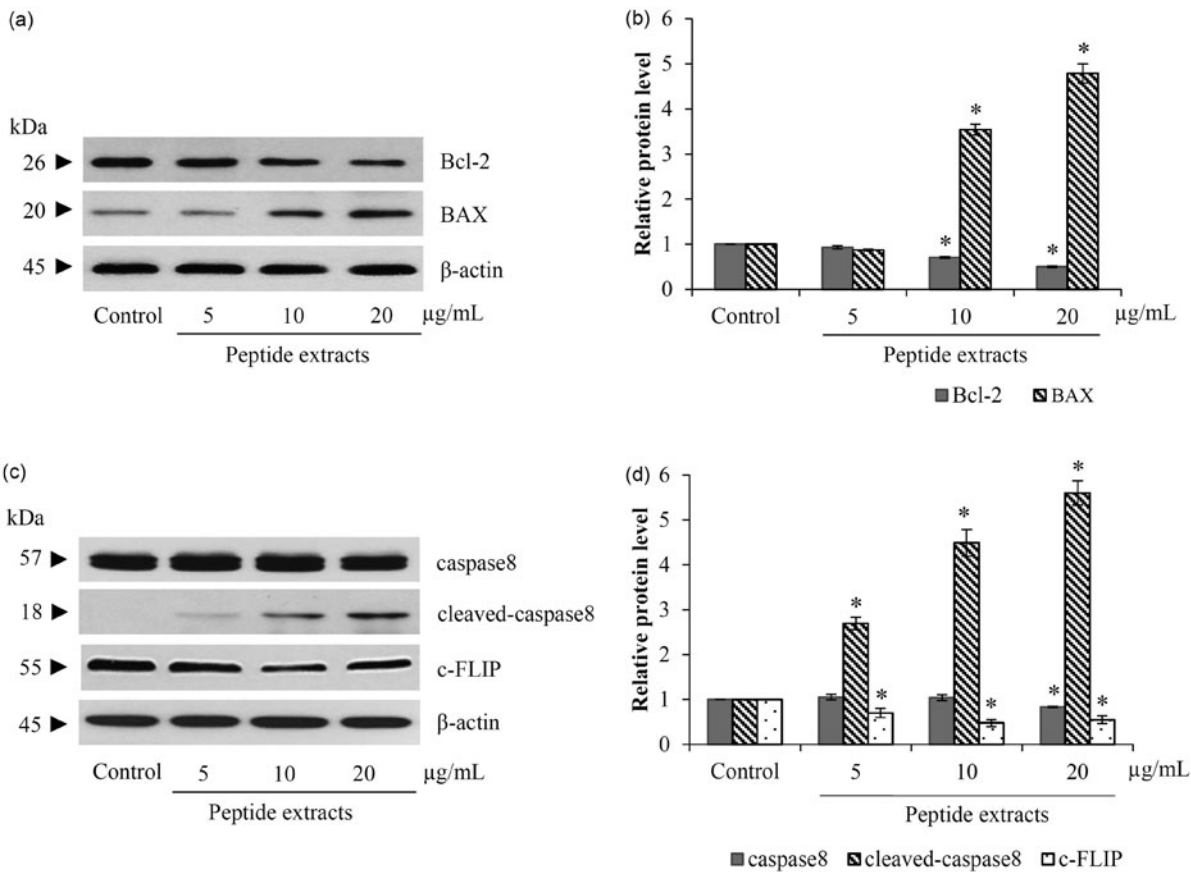


Figure 5. Peptide extracts stimulated both mitochondrial and death-receptor apoptotic pathway. (a and b) There were the reduction of Bcl-2 and up-regulation of BAX after treatment of lung cancer cells with 10–20 $\mu\text{g/mL}$ of peptide extracts form *Lentinus squarrosulus*. (c and d) Protein-relating death-receptor pathway, c-FLIP and caspase-8 were decreased in peptide extracts-incubated H460 cells. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

Cytotoxicity of the peptide extracts in human lung cancer cells

The reduction of cell viability was observed early at 6 h after treatment of H460 lung cancer cells with 20 µg/mL peptide extracts (Figure 3(d)). After 24 h of incubation, 40% reduction in viability of the cells was found in the cell treated with 20 µg/mL peptide extracts. Figure 3(a) presents dose-dependent cytotoxicity of the peptide extracts in human lung cancer cells. Notably, 85% of viable cell was suppressed after treatment of H460 cells with peptide extracts at the concentration of 40 µg/mL for 24 h.

Mode of cell death detected by co-staining of Hoechst33342/PI indicated apoptotic cell death after incubation H460 cells with the peptide extracts at 5–20 µg/mL for 24 h. Meanwhile, there was no indication of necrosis (Figure 3(b,e)). However, Figure 3(c) illustrated that the highest concentration (40 µg/mL) of peptide extracts caused both apoptosis and necrosis in human lung cancer cells with DNA condense stained by Hoechst33342 and red fluorescence of PI, respectively. Thus, peptide extracts at 5–20 µg/mL were selected for further evaluation of anticancer activity in human cancer cells.

Apoptosis induction in lung cancer cells treated with peptide extracts

To confirm peptide extracts-induced apoptosis, the evaluation on apoptotic marker proteins was performed via western blot analysis. The activation of caspase-3 indicating an increase of cleaved-caspase-3 was observed in treatment of H460 cells with peptide extracts (5–20 µg/mL) for 24 h (Figure 4). There was also significant reduction of PARP (Poly(ADP-ribose) polymerase-1) and the augmentation of cleaved-PARP in lung cancer cells treated with 10–20 µg/mL (Figure 4(b)).

Peptides extracted from *L. squarrosulus* stimulate both intrinsic and extrinsic apoptosis pathways

There are two major apoptotic pathways, intrinsic and extrinsic machinery (Indran et al. 2011). The alteration of Bcl-2 family proteins involved in the intrinsic or mitochondrial pathway was significantly observed in H460 cells treated with the peptide extracts. Figure 5(a) shows that there was a reduction of Bcl-2, an anti-apoptotic protein, while a pro-apoptotic protein, BAX obviously increased after incubation of lung cancer cells with 10–20 µg/mL of peptide extracts for 24 h. The induction of extrinsic or death receptor signalling in peptide extracts-treated H460 cells was presented by the reduction of c-FLIP, an inhibitor of death receptor-activated caspase cascade which is associated with the augmentation of cleaved-caspase-8 (Figure 5(c)).

Selective anticancer activity of peptides extracted from *L. squarrosulus*

To evaluate the selective anticancer activity, cytotoxicity of the peptide extracts in human dermal papilla DPCs cells was investigated. Dermal papilla cells are one of the most affected normal cells induced by current chemotherapeutic drugs. Damage of dermal papilla cells leads to hair loss and a low quality of life in lung cancer patients (Herbst et al. 2004; Chie et al. 2004; Koizumi et al. 2016). The selective anticancer effect and human safety profile of *L. squarrosulus* peptides are presented in Figure 6. After 24 h of treatment, the peptide extracts at 1–40 µg/mL caused

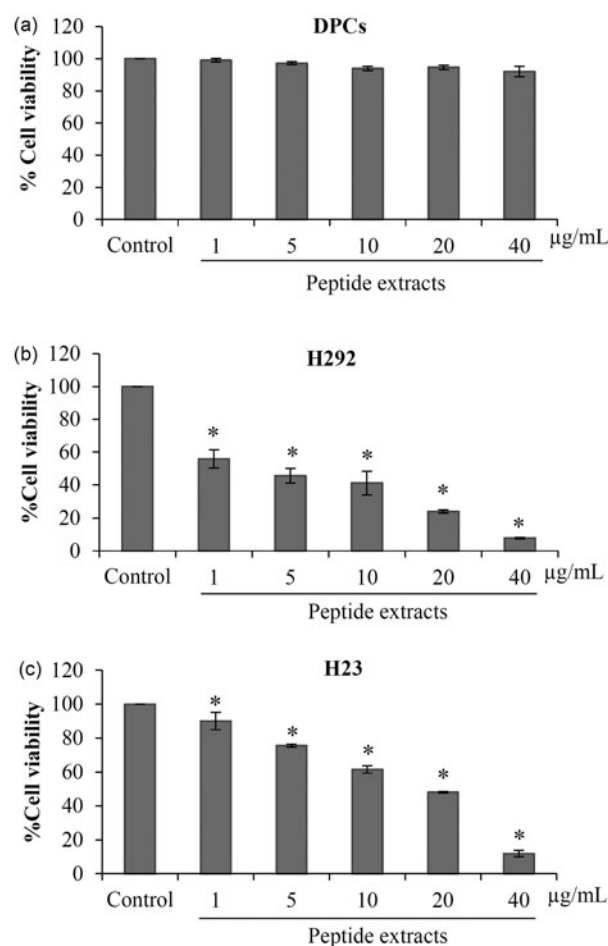


Figure 6. Selective anticancer activity of peptide extracts from *Lentinus squarrosulus*. (a) Low toxicity of the peptide extracts to non-cancer cells was indicated in human dermal papilla DPCs cells treated with 1–40 µg/mL peptides for 24 h. (b and c) Peptide extracts from *L. squarrosulus* induced cell death in various lung cancer cells. The anticancer activity of the extracts was also evaluated in H292 and H23 lung cancer cells. Values are means of the independent triplicate experiments $\pm$ SD. * $p \leq 0.05$ versus non-treated control.

no significant alteration of % cell viability in DPCs cells compared with non-treated control cells (Figure 6(a)).

Due to various phenotypes of lung cancer cells (Boiarskikh et al. 2011), the anticancer effect of the peptide extracts was further examined in different types of human lung cancer (H292 and H23 cells). After 24 h of incubation with 1–40 µg/mL of peptide extracts, the cell viability of H292 and H23 obviously decreased (Figure 6(b,c)). Notably, H292 cells seem to be more sensitive to the peptide extracts than H460 and H23 cells. The half inhibitory concentration (IC₅₀) was approximately of 26.84 ± 2.84 , 2.80 ± 2.14 , 18.84 ± 0.30 µg/mL for H460, H292 and H23 cells, respectively. These results confirm the effect of peptide extracts from *L. squarrosulus* against human lung cancer cells.

Discussion

Among leading cancers, lung cancer is recognized as an important cause of cancer-related deaths, with a significant cause of death being failure of drug treatment (Dziedzic et al. 2016). Cancer research has focused on a search for novel active compounds with high efficacy and low toxicity. Thus, this study has demonstrated the promising anticancer effect of peptides extracted from the *L. squarrosulus* mushroom against human lung cancer cells. Although drug resistance in lung cancer is

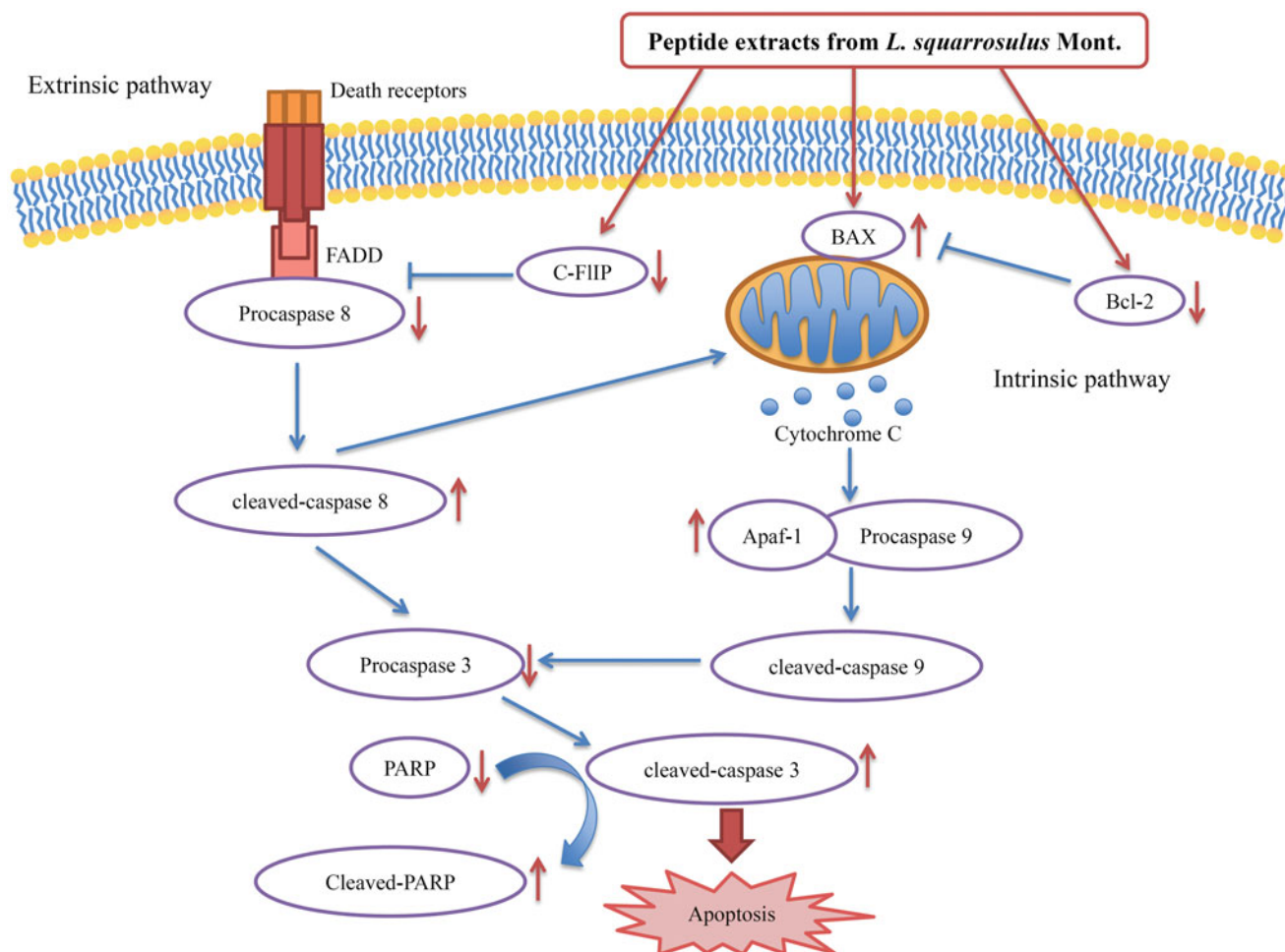


Figure 7. Proposed mechanistic scheme of anticancer activity of peptide extracts from *Lentinus squarrosulus* in human lung cancer cells. The extracts mediated a mitochondrial or intrinsic apoptotic pathway through the reduction of Bcl-2 and increase of BAX. Meanwhile, the extracts also decrease c-FLIP, an inhibitor for death-receptor regulating apoptosis.

complex and frequently caused by several factors, the increase of anti-apoptotic proteins such as Bcl-2 and c-FLIP has been shown to be dominant (Indran et al. 2011). The over expression of Bcl-2 and accumulation of cellular Bcl-2 proteins have been shown to mediate lung cancer cell resistance to several chemotherapies as well as death stimuli (Wesarg et al. 2007; Yip & Reed 2008; Yang et al. 2009). Likewise, the increase in the level of c-FLIP has been shown to inhibit the death of cancer cells in response to immune cell-mediated apoptosis (Wang et al. 2008; Bagnoli et al. 2010; Safa & Pollok 2011). This investigation demonstrated that peptides extracted from *L. squarrosulus* at concentrations of 1–40 µg/mL significantly decreased viability and induced apoptosis of various human lung cancer cells including H460, H23 and H292 cells. The mechanism of apoptosis demonstrated in H460 cells showed that the peptide extracts mediated apoptosis by increasing BAX while decreasing Bcl-2 protein. The result from this study is consistent with others in which several natural-derived compounds have anticancer effects by suppressing Bcl-2 protein (Halim et al. 2011; Woo et al. 2012; Vizetto-Duarte et al. 2016). Although c-FLIP has been perceived to have a principle role as a death receptor-mediating apoptosis, studies also suggest that this protein increases cell survival and proliferation (Bagnoli et al. 2010; Dickens et al. 2012). For example, the increase of cellular c-FLIP has been shown to activate NF-κB (Baratchian et al. 2016), and inhibition of such a pathway by dominant expression of its inhibitory subunit IκB decreased cell

survival (Chanvorachote et al. 2005). This study has shown that treatment of the cells with peptides extracted from *L. squarrosulus* results in a significant depletion of c-FLIP. Together with the above context, the extract may not only induce apoptosis, but also suppress cancer cell survival and proliferation. However, the efficacy of the peptides should be investigated in lung cancer cells with aggressive behaviours of chemotherapeutic resistance and metastasis characters.

Although many chemotherapeutic drugs are currently prescribed for the treatment of lung cancer, their usage is frequently limited by severe side effects to non-cancer cells (Lemjabbar-Alaoui et al. 2015). The high safety profile in humans of *L. squarrosulus* mushroom has been proven by the long time it has been consumed as a traditional food as well as a toxicity study of an animal model (Mhd Omar et al. 2011; Das et al. 2017). Selective anticancer activity presented in this study strengthens the potential development of extracts from *L. squarrosulus* with less side-effect than current chemotherapeutic drugs.

In summary, the findings from this study highlight the potent effects of peptides extracted from fruiting bodies of *L. squarrosulus* mushroom, in mediating apoptosis in lung cancer cells through the decrease of anti-apoptotic Bcl-2 and c-FLIP proteins and increase of pro-apoptotic protein Bax (Figure 7). Nevertheless, the purification, examination and identification of amino acid sequencing of active peptide with a high efficacy and safety profile in extracts of *L. squarrosulus* should be further studied.

Disclosure statement

The authors report no conflicts of interest.

Funding

The work was supported by the Thailand Research Fund under Grant [MRG5980143]; and Chula Research Scholar, Ratchadaphiseksomphot Endowment Fund under a Grant for International Research Integration.

References

- Bagnoli M, Canevari S, Mezzananza D. 2010. Cellular FLICE-inhibitory protein (c-FLIP) signalling: a key regulator of receptor-mediated apoptosis in physiologic context and in cancer. *Int J Biochem Cell Biol.* 42:210–213.
- Bailey C. 2009. Ready for a comeback of natural products in oncology. *Biochem Pharmacol.* 77:1447–1457.
- Baratchian M, Davis CA, Shimizu A, Escors D, Bagneris C, Barrett T, Collins MK. 2016. Distinct activation mechanisms of NF-kappaB regulator inhibitor of NF-kappaB Kinase (IKK) by isoforms of the cell death regulator cellular FLICE-like Inhibitory Protein (cFLIP). *J Biol Chem.* 291:7608–7620.
- Boiarskikh UA, Kondrakhin IV, Evshin IS, Sharipov RN, Komel'kov AV, Musatkina EA, Chevkina EM, Sukoian MA, Kolpakov FA, Kashkin KN, et al. 2011. Prediction of a non-small cell lung cancer sensitivity to cisplatin and paclitaxel based on the marker genes expression. *Mol Biol.* 45:600–607.
- Brown JM, Attardi LD. 2005. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer.* 5:231–237.
- Chanvorachote P, Nimmannit U, Wang L, Stehlik C, Lu B, Azad N, Rojanasakul Y. 2005. Nitric oxide negatively regulates Fas CD95-induced apoptosis through inhibition of ubiquitin-proteasome-mediated degradation of FLICE inhibitory protein. *J Biol Chem.* 280:42044–42050.
- Chie WC, Yang CH, Hsu C, Yang PC. 2004. Quality of life of lung cancer patients: validation of the Taiwan Chinese version of the EORTC QLQ-C30 and QLQ-LC13. *Qual Life Res.* 13:257–262.
- Czabotar PE, Lessene G, Strasser A, Adams JM. 2014. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol.* 15:49–63.
- Dan X, Liu W, Wong JH, Ng TB. 2016. A ribonuclease isolated from wild *Ganoderma lucidum* suppressed autophagy and triggered apoptosis in colorectal cancer cells. *Front Pharmacol.* 7:217.
- Das AR, Borthakur M, Saha AK, Joshi SR, Das P. 2017. Molecular characterization and antioxidant potential of three wild culinary-medicinal mushrooms from Tripura, northeast India. *Int J Med Mushrooms.* 19:55–63.
- Dickens LS, Powley IR, Hughes MA, MacFarlane M. 2012. The 'complexities' of life and death: death receptor signalling platforms. *Exp Cell Res.* 318:1269–1277.
- Dziedzic DA, Rudzinski P, Langfort R, Orlowski T. 2016. Risk factors for local and distant recurrence after surgical treatment in patients with non-small-cell lung cancer. *Clin Lung Cancer.* 17:157–167.
- Elmore S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* 35:495–516.
- Halim H, Chunhacha P, Suwanborirux K, Chanvorachote P. 2011. Anticancer and antimetastatic activities of Renieramycin M, a marine tetrahydroisoquinoline alkaloid, in human non-small cell lung cancer cells. *Anticancer Res.* 31:193–201.
- Herbst RS, Giaccone G, Schiller JH, Natale RB, Miller V, Manegold C, Scagliotti G, Rosell R, Oliff I, Reeves JA, et al. 2004. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial-INTACT 2. *J Clin Oncol.* 22:785–794.
- Indran IR, Tufo G, Pervaiz S, Brenner C. 2011. Recent advances in apoptosis, mitochondria and drug resistance in cancer cells. *Biochim Biophys Acta.* 1807:735–745.
- Koizumi T, Fukushima T, Gomi D, Kobayashi T, Sekiguchi N, Sakamoto A, Sasaki S, Mamiya K. 2016. Alectinib-induced alopecia in a patient with anaplastic lymphoma kinase-positive non-small cell lung cancer. *Case Rep Oncol.* 9:212–215.
- Laemmli UK, Favre M. 1973. Maturation of the head of bacteriophage T4. I. DNA packaging events. *J Mol Biol.* 80:575–599.
- Lemjabbar-Alaoui H, Hassan OU, Yang YW, Buchanan P. 2015. Lung cancer: biology and treatment options. *Biochim Biophys Acta.* 1856:189–210.
- McDonnell MA, Wang D, Khan SM, Vander Heiden MG, Kelekar A. 2003. Caspase-9 is activated in a cytochrome c-independent manner early during TNFalpha-induced apoptosis in murine cells. *Cell Death Differ.* 10:1005–1015.
- Mhd Omar NA, Abdullah N, Kuppusamy UR, Abdulla MA, Sabaratnam V. 2011. Nutritional composition, antioxidant activities, and antitumor potential of *Lentinus squarrosulus* (Mont.) mycelia extract. *Evid Based Complement Alternat Med.* 2011:539356.
- Patel S, Goyal A. 2012. Recent developments in mushrooms as anti-cancer therapeutics: a review. *3 Biotech.* 2:1–15.
- Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. 2011. Anti-apoptosis and cell survival: a review. *Biochim Biophys Acta.* 1813:238–259.
- Powan P, Saito N, Suwanborirux K, Chanvorachote P. 2013. Ecteinascidin 770, a tetrahydroisoquinoline alkaloid, sensitizes human lung cancer cells to anoikis. *Anticancer Res.* 33:505–512.
- Safa AR, Pollok KE. 2011. Targeting the anti-apoptotic protein c-FLIP for cancer therapy. *Cancers (Basel).* 3:1639–1671.
- Samali A, Fulda S, Gorman AM, Hori O, Srinivasula SM. 2010. Cell stress and cell death. *Int J Cell Biol.* 2010:Article ID 245803.
- Sen IK, Maji PK, Behera B, Mallick P, Maiti TK, Sikdar SR, Islam SS. 2013. Structural characterization of an immunoenhancing heteroglycan of a hybrid mushroom (pf1sh) of *Pleurotus florida* and *Lentinus squarrosulus* (Mont.) Singer. *Carbohydr Res.* 371:45–51.
- Siegel RL, Miller KD, Jemal A. 2015. Cancer statistics, 2015. *CA Cancer J Clin.* 65:5–29.
- Vizetto-Duarte C, Custodio L, Gangadhar KN, Lago JH, Dias C, Matos AM, Neng N, Nogueira JM, Barreira L, Albericio FRauter AP, et al. 2016. Isolololide, a carotenoid metabolite isolated from the brown alga *Cystoseira tamariscifolia*, is cytotoxic and able to induce apoptosis in hepatocarcinoma cells through caspase-3 activation, decreased Bcl-2 levels, increased p53 expression and PARP cleavage. *Phytomedicine.* 23:550–557.
- Wang X, Chen W, Zeng W, Bai L, Tesfagzi Y, Belinsky SA, Lin Y. 2008. Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. *Mol Cancer Ther.* 7:1156–1163.
- Wesarg E, Hoffarth S, Wiewrodt R, Kröll M, Biesterfeld S, Huber C, Schuler M. 2007. Targeting BCL-2 family proteins to overcome drug resistance in non-small cell lung cancer. *Int J Cancer.* 121:2387–2394.
- Woo CC, Kumar AP, Sethi G, Tan KH. 2012. Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol.* 83:443–451.
- Yang TM, Barbone D, Fennell DA, Broadus VC. 2009. Bcl-2 family proteins contribute to apoptotic resistance in lung cancer multicellular spheroids. *Am J Respir Cell Mol Biol.* 41:14–23.
- Yip KW, Reed JC. 2008. Bcl-2 family proteins and cancer. *Oncogene.* 27:6398–6406.

Avicquinone B sensitizes anoikis in human lung cancer cells

Arisara Prateep^{1†}, Somruethai Sumkhemthong^{1†}, Wiranpat Karnsomwan²,
Wanchai De-Eknamkul², Supakarn Chamni², Pithi Chanvorachote^{3,4*},
Chatchai Chaotham^{1,4*}

†Contributed equally, *Co-corresponding author

¹Department of Biochemistry and Microbiology, Faculty of Pharmaceutical
Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

²Departments of Pharmacognosy and Pharmaceutical Botany, Chulalongkorn
University, 10330 Bangkok, Thailand.

³Department of Pharmacology and Physiology, Faculty of Pharmaceutical
Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

⁴Cell-based Drug and Health Products Development Research Unit, Faculty of
Pharmaceutical Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

Correspondence: Chatchai Chaotham, Department of Biochemistry and Microbiology,
Faculty of Pharmaceutical Sciences, Chulalongkorn University, 10330 Bangkok,
Thailand. Email: cchoatham@gmail.com Tel: +66 2 218 8372

Authors' email

AP: arissmint@gmail.com; SS: somthai.pla@gmail.com;

WK: noonwiranpat@gmail.com; WD: dwanchai@gmail.com;

SC: supakarn.c@pharm.chula.ac.th; PC: pithi_chan@yahoo.com;

CC: cchoatham@gmail.com

Abstract

Background: During metastasis, cancer cells require anoikis resistant mechanism to survive until reach the distant secondary tissues. As anoikis sensitization may benefit for cancer therapy, this study demonstrated the potential of avicequinone B, a natural furanonaphthoquinone found in mangrove tree (Avicenniaceae) to sensitize anoikis in human lung cancer cells.

Methods: Anoikis inducing effect was investigated in human lung cancer H460, H292 and H23 cells that were cultured in ultra-low attachment plate with non-cytotoxic concentrations of avicequinone B. Viability of detached cells was evaluated by XTT assay at 0-24 h of incubation time. Soft agar assay was performed to investigate the inhibitory effect of avicequinone B on anchorage-independent growth. The alteration of anoikis regulating molecules including survival and apoptosis proteins were elucidated by western blot analysis.

Results: Avicequinone B at 4 μ M significantly induced anoikis and inhibited proliferation under detachment condition in various human lung cancer cells. The reduction of anti-apoptotic proteins including anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia 1 (Mcl-1) associating with the diminution of integrin/focal adhesion kinase (FAK)/Proto-oncogene tyrosine-protein kinase (Src) signals were detected in avicequinone B-treated cells.

Conclusions: Avicequinone B sensitized anoikis in human lung cancer cells through down-regulation of anti-apoptosis proteins and integrin-mediated survival signaling.

Keywords: Lung cancer, Anoikis, Survival pathway, Avicequinone B, Furanonaphthoquinone

Background

Lung cancer is a high prevalence and leading cause of death in cancer patients worldwide [1]. Metastasis or the spreading of cancer cells from primary site to secondary vital organs is a major cause of mortality in the lung cancer [2, 3]. According to highly metastatic features, most of lung cancer patients are frequently diagnosed at advanced stage presenting dissemination of tumor pathology [4, 5]. Such concepts lead to the urgent need for anti-metastasis therapy for lung cancer.

In the process of metastasis, most population of detached cancer cells should die by the mechanism of detachment-induced apoptosis termed “anoikis” [6]. This specific pattern of cell death is caused by the reduction of cellular survival signals providing by the adhesion of the cells to appropriate surface or membrane through integrins [7]. Loss of interaction between integrins, cellular adhesive molecules and extracellular matrix (ECM) leads to the deprivation of survival signals following with apoptosis cell death in non-adherent cells [8].

Anoikis is believed to be a critical mechanism in preventing non-adherent cell growth and the growth of cells in an inappropriate environment [9]. In order to survive in blood or lymphatic circulation, certain cancer cells acquire anoikis resistance mechanisms. In metastatic cells, anoikis is inhibited by the increase of survival signals through the modulation of integrins expression contributing activation of FAK (focal adhesion kinase)/Src (Proto-oncogene tyrosine-protein kinase) and PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (Phospho kinase B) pathway [10-14]. Caveolin-1 was previously shown to play a role in attenuating anoikis response in lung cancer cells by maintain the level of Mcl-1 [15-17]. Moreover, the up-regulation of anti-apoptosis Bcl-2 family proteins including Bcl-2 (B-cell lymphoma 2) and Mcl-1

(myeloid cell leukemia 1) has been associated with anoikis resistance and highly metastasis cancer cells [18-20].

Avicequinone B, naphtho [2, 3-b] furan-4, 9-dione isolated from mangrove tree such as *Avicennia alba* and *Avicennia marina* has been shown to possess several pharmacological activities [21]. Anticancer activity of naphthoquinone derivatives have been illustrated through the induction of apoptosis and the inhibition on migration and invasion [22, 23]. So far, the potentials of these furanonaphthoquinone compounds for sensitizing anoikis and their regulatory approaches are largely unknown. We aimed to investigate the anoikis sensitizing effect and the underlying mechanisms of action of avicequinone B in human lung cancer cells. The information obtained from this study will emphasize the therapeutic benefits of avicequinone B for further development as an effective anticancer drug.

Material and Method

Chemical reagents

All chemical reagents used for synthesis of avicequinone B and cell culture including XTT (2,3-b-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide salt), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Hoechst33342, propidium iodide (PI), DMSO (dimethylsulfoxide) and agarose were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Annexin V-FITC for apoptosis detection was provided by Thermo Fisher Scientific (Waltham, MA, USA.). Primary antibody of Bcl-2, Mcl-1, Bax (Bcl-2-associated X protein), caveolin-1, integrin β 1, integrin β 3, FAK, p-FAK (Try 397), Src, p-Src (Try 418), AKT, p-AKT (Ser 473), ERK (extracellular signal-regulated kinase), p-ERK (Thr 981), β -actin and specific

horseradish peroxidase (HRP)-link secondary antibody were obtained from Cell Signaling Technology, Inc. (Danver, MA, USA). Supersignal West Pico, a chemiluminescence substrate for western blot analysis was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Protease inhibitor cocktail and Bicinchoninic acid (BCA) protein assay kit were obtained from Roche Applied Science (Indianapolis, IN, USA) and Pierce Biotechnology (Rockford, IL, USA), respectively.

Preparation of avicequinone B

Avicequinone B was prepared from chemical synthesis using a facile synthesis as previous report [24]. Briefly, anhydrous solvents were dried over 4Å molecular sieves. Methyl vinyl sulfone (4.71 mmol, 500 mg) was dissolved in dry dichloromethane (CH₂Cl₂, 10 ml) in a 50-mL oven-dried round-bottomed flask. The reaction mixture was stirred at room temperature under an argon atmosphere. Next, neat bromine (Br₂, 7.07 mmol, 0.2 ml) was slowly added into the reaction. Then, the reaction mixture was refluxed for 6 h, concentrated under reduce pressure and reconstituted in dry tetrahydrofuran (THF, 20 ml). The reaction solution was then cooled at 0°C and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 7.07 mmol, 1.1 ml) was slowly added dropwise over 20 min. The reaction mixture was stirred at 0°C for 30 min. Next, lawsone (4.71 mmol, 820.2 mg) was added and another portion of DBU (7.07 mmol, 1.1 ml) was slowly added dropwise over 20 min. The reaction mixture was stirred at 0°C for 30 min. The reaction was warmed up to room temperature and heated to reflux for 6 h. The reaction was then concentrated under reduced pressure and the residue was dissolved in dichloromethane (100 ml), washed with water (100 ml) and saturated aqueous ammonium chloride (100 ml). The organic layer was separated and the aqueous layer was extracted

with dichloromethane (50 ml x 3 times). The combined organic layer was dried over anhydrous sodium sulfate and concentrated to obtain the crude product. The crude product was purified over silica gel column chromatography using dichloromethane: hexanes (3:1 v/v) as eluent to provide avicequinone B as a pale yellow solid at a yield of 69 mg (12%). ¹H-NMR (CDCl₃, 300 MHz) δ 8.22 (1H, m, 5-H), 8.22 (1H, m, 8-H), 7.77 (1H, m, 6-H), 7.77 (1H, m, 7-H), 7.77 (1H, d, J = 1.5 Hz, 2-H), 7.01 (1H, d, J = 1.5 Hz 3-H); ¹³C-NMR (CDCl₃, 300 MHz) δ 180.6 (C-4), 173.6 (C-9), 152.7 (C-9a), 148.6 (C-2), 132.5 (C-4a), 134.0 (C-7), 133.9 (C-6), 133.2 (C-8a), 130.5 (C-3a), 127.1 (C-8), 127.0 (C-5), 108.7 (C-3); IR (KBr) 3142, 2853, 1683, 1585, 1566, 1478, 1365, 1206, 1182, 952, 714 cm⁻¹; HRMS-ESI m/z 221.0212 ([M+Na]⁺, calcd for C₁₂H₆O₃Na⁺ 221.0215). Spectroscopic data of avicequinone B were matched with the previous report. The synthetic scheme of avicequinone B was demonstrated in figure 1.

Cell culture

Human lung cancer H460, H292 and H23 cells were obtained from ATCC, American Type Culture Collection (Manassas, VA, USA). They were maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and 100 units/ml penicillin/streptomycin (Gibco, Gaithersburg, MA, USA) under 5% CO₂ at 37°C. The cells that reached 70-80% confluence were used for next experiments.

Cell viability assay

Cell viability of adherent cell was evaluated by MTT assay. Briefly, lung cancer cells were cultured in complete RPMI medium containing various concentrations (0-10

μM) of avicequinone B for 24 h. The cells were then incubated with 0.4 mg/ml of MTT solution at 37°C in a dark place for 4 h. The MTT solution was replaced with DMSO (100 μl/well) to dissolve the purple formazan crystal. The intensity of formazan color was measured at 570 nm using a microplate reader (Anthros, Durham, NC, USA). Percentage of cell viability in relation to the non-treated controls was calculated from the optical density (OD) ratio of treated to non-treated control cells.

In order to evaluate anti-proliferative activity of avicequinone B, human lung cancer H460 cells were prepared at density of 2×10^3 cells/well in 96 well-plate. The cells were incubated with non-toxic concentrations of avicequinone B or left untreated for 24, 48 and 72 h then cell viability was examined via MTT assay. The %cell proliferation was obtained from the calculation between OD of the cells at each time point divided by OD of non-treated control at 24 h.

Detachment-induced cell death

A single-cell suspension of human lung cancer cells at a density of 1.5×10^5 cells/ml was prepared in RPMI culture medium. In order to prevent anchorage, lung cancer single-cell suspension was culture in an ultra-low attachment plate (Corning, Acton, MA, USA). The cells were treated with non-toxic concentrations of avicequinone B for 0, 6, 9, 12 and 24 h or left untreated. After indicated time point, the cells were harvested for evaluation of cell viability through the incubation with 20 μM of XTT at 37°C for 4 h. The intensity of the formazan product from XTT was measured at 450 nm using a microplate reader.

Detection of mode of cell death

Nuclear staining with Hoechst33342 (10 μ M) and PI (5 μ g/ml) was performed to detect apoptosis and necrosis cell death in human lung cancer cells treated with avicequinone B. Bright blue fluorescence of Hoechst33342 and red fluorescence of PI were observed and evaluated under fluorescent microscope (Olympus IX51 with DP70; Olympus, Melville, NY, USA) as apoptosis and necrosis cells, respectively. For evaluation on detachment-induced apoptosis or anoikis, single-cell suspension of human lung cancer cells treated non-toxic concentrations of avicequinone B for 24 h in ultra-low attachment plate were collected and co-stained with Hoechst33342/PI [25, 26]. Mode of cell death were examined and counted under fluorescent microscope.

The relative ratio of amount of apoptosis cells to total cell number was presented as %apoptosis and %anoikis for attachment and detachment culture condition, respectively.

Annexin V/PI flow cytometry analysis

Anoikis in non-adherent cells was further evaluated through flow cytometry using an Annexin V-FITC apoptosis assay kit. Single-cell suspension of H460 cells cultured in an ultra-low attachment 6 well-plate at a density of 1.5×10^5 cells/ml were collected after incubated with 0-4 μ M of avicequinone B for 24 h. The cells were wash and resuspended in phosphate buffer saline (PBS), pH 7.4. The single-cell suspensions were then centrifuged and dispersed in binding buffer (100 μ l). Annexin V-FITC (1 μ g/ml) and PI (2.5 μ g/ml) were added into the cell suspensions as recommended in the manufacturer's instructions. Living, apoptosis and necrosis cells were analyzed via a FACScan flow cytometer using CellQuest software (Becton-Dickinson, Redlands, CA, USA).

Anchorage-independent growth assay

The ability to proliferate under detachment condition of human lung cancer cells was investigated in soft agar assay. Each well of a 24 well-plate was covered with 500 μ l of 0.5% agarose in complete RPMI medium. In order to prevent spontaneous aggregation of detached cells, the single-cell suspension was dispersed in 0.33% agarose in culture medium which is the optimum condition to prevent cell aggregation and permit cell growth [27]. The single-cell suspensions of lung cancer cells in 0.33% agarose (1,500 cells/250 μ l) with different concentration of avicequinone B were prepared and placed on solidified 0.5% agarose gel. After the upper layer was left to set in the incubator at 37°C for 4 h, 250 μ l of culture medium was added on top and every 3 days. The formation of cancer colonies was investigated under a microscope (Olympus IX51 with DP70) after 14 days.

Western blot analysis

The alteration of anoikis-regulating proteins in lung cancer cells was analyzed by western blot analysis. Single-cell suspensions of H460 cells at a density of 1.5×10^5 cells/ml were incubated with avicequinone B (0-4 μ M) for 12 h. Then, the cells were harvested and lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Applied Science) at 4°C for 60 min. Cell lysates were collected and determined for total protein content by using the BCA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein from each sample was resolved under denaturing conditions by 10% SDS-PAGE and transferred onto a nitrocellulose

membrane. The membranes are blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM sodium chloride, 0.05% Tween 20) before the incubation with specific primary antibody at 4°C for 12 h. After washing three times (5 min) with TBST, the membranes were probed with HRP-conjugated secondary antibody for 2 h at room temperature. The signal of immunoreactive proteins was detected by enhanced chemiluminescence (Supersignal West Pico; Thermo Fisher Scientific, Waltham, MA, USA). The quantitative analysis was performed with the analyst/PC densitometry software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Mean data from three independent experiments were normalized to result of non-treated control. Statistical analysis was performed using one-way ANOVA following with post-hoc test. $p < 0.05$ was considered as statistically significant.

Results

Cytotoxicity of avicequinone B in human lung cancer cells

To investigate the effect of avicequinone B on anoikis, the cytotoxicity of the compound in lung cancer H460 cells was firstly elucidated. Cell viability was examined by MTT assay after treatment of the cells with avicequinone B at 0-10 μ M for 24 h. Cytotoxic profile of avicequinone B was shown in figure 2. In detail, the significant reduction of %cell viability was observed in the cells treated with 8-10 μ M of avicequinone B (Fig. 2a). Figure 2b indicates the increase of apoptosis cell death in H460 cells after treatment with 10 μ M of avicequinone B. There was no observation of necrosis cells stained with red fluorescence of PI in all treatment of avicequinone B (Fig. 2c).

These results demonstrated that non-toxic concentrations of avicequinone B in human lung cancer H460 cells were between 0.5 to 4 μ M.

The inhibitory effect of avicequinone B on proliferation in human lung cancer cells was further evaluated. Figure 2d indicates that treatment with 2-4 μ M of avicequinone B for 72 h significantly suppressed %cell proliferation in lung cancer H460 cells compared non-treated control cells. Notably, the anti-proliferative activity of avicequinone B (4 μ M) was early observed after 48 h of incubation time.

Anoikis sensitizing effect of avicequinone B in H460 lung cancer cells

Detachment-induced cell death was assessed in human lung cancer cells through the culture of H460 cells as single-cell suspension in non-adhesive poly-HEMA coated-plates. After 0-24 h of incubation with non-toxic concentrations (0-4 μ M) of avicequinone B, the survival of the cells was determined by XTT assay. The reduction of cell survival was detected in the control cells as early as 6 h after detachment (Fig. 3a). For treated cells, avicequinone B at 4 μ M significantly diminished viability of H460 cells compared with non-treated control cells at the same time point. Co-staining with Hoechst33342 and PI confirmed the anoikis sensitizing effect of avicequinone B. Induction of apoptosis without presenting of necrosis was illustrated in avicequinone B-treated H460 cells (Fig. 3c). Figure 3b shows the significant augmentation of anoikis in H460 cells after incubation with 4 μ M of avicequinone B for 24 h.

Flow cytometry analysis also indicated the presence of anoikis in human lung cancer cells. Annexin V-FITC which interacts with phosphatidylserine on the cell membrane of apoptosis cells [28] was dramatically detected in H460 cells cultured in ultra-low attachment plate for 24 h (Fig. 3d). The higher number of early (Annexin V-

FITC positive) and late (Annexin V-FITC positive and PI positive) apoptosis were obviously notified in detached H460 cells incubated with 4 μ M of avicequinone B compared with the non-adherent control cells (Fig. 3e). These results evidenced the anoikis sensitizing activity of avicequinone B in human lung cancer cells.

Avicequinone B suppresses cancer cell growth in anchorage-independent condition

The effect of avicequinone B on capability to growth and survive under detachment condition was further evaluated in soft-agar assay. Human lung cancer H460 cells were anchorage-independently grown in 0.33% agarose gel supplemented with culture medium in presence or absence of avicequinone B (0.5-4 μ M). Figure 4a presents the colony formation initiating from a single cell of H460 after 14 days of culture under detachment condition. The reduction of both relative colony number and size was significantly observed in H460 cells treated with 2-4 μ M of avicequinone B (Fig. 4b and 4c). Intriguingly, avicequinone B at 4 μ M obviously suppressed proliferation in lung cancer cells at both anchorage-dependent (Fig.3d) and -independent condition.

Avicequinone B decreases anti-apoptotic proteins in non-anchorage lung cancer cells

Anoikis is an apoptosis cell death induced by detachment condition, the alteration of apoptosis-regulating proteins including Bcl-2, Mcl-1, Bax and caveloin-1 was examined in H460 cells treated with avicequinone B. In order to escape from anoikis, human lung cancer H460 cells can sustain the level of anti-apoptosis proteins during non-adherent circumstance [29]. Avicequinone B at 4 μ M significantly reduced the level of caveolin-1 in lung cancer cells detached for 12 h (Fig. 5a). Moreover, treatment with 2-4 μ M of avicequinone B significantly declined anti-apoptotic Bcl-2 family proteins, Bcl-2

and Mcl-1 (Fig. 5b). It is worth noting that the expression of Bax, a pro-apoptosis protein was not altered in response to the treatment of avicequinone B (1-4 μ M) for 12 h (Fig. 5a and b).

Down-regulation of integrin mediated-survival signal by avicequinone B

In order to proliferate under non-adherent condition, anoikis resistant cancer cells substantially activate pro-survival pathways [9]. Thus, the alteration on integrin mediated-survival signal was investigated in H460 cells cultured with avicequinone B. Western blot analysis obviously revealed the down-regulation of integrin β 1 and β 3 in human lung cancer cells treated with 4 μ M of avicequinone B **under detachment condition** (Fig. 5c). Interestingly, the reduction of integrin β 3 was also notified at low concentrations (1-2 μ M) of avicequinone B. Figure 5d indicates the reduction of **p-FAK** and **p-Src**, down-stream signaling molecules of integrins in H460 cells treated with avicequinone B. **The alteration on level of AKT and ERK protein, pro-survival molecules activated by p-Src was further investigated [30]. As presented in figure 5e and f, the diminution of activated ERK (p-ERK) was notified in detached H460 cells incubated with avicequinone B (1-4 μ M). Meanwhile there were no significant alteration of AKT and p-AKT expression in avicequinone B treated-H460 cells compared with non-treated control.** This inhibitory effect on integrin/FAK/Src survival pathway corresponded with sensitization to anoikis (Fig.3) and low colony formation (Fig. 4) in human lung cancer cells exposed with avicequinone B.

Avicequinone B sensitizes anoikis and suppresses anchorage-independent growth in various lung cancer cells

In order to confirm anoikis sensitizing effect of avicequinone B, the detachment-induced cell death was performed in human lung cancer H292 and H23 cells. Figure 6a and b indicate that avicequinone B at non-toxic concentrations (2-4 μ M; data not shown) significantly reduced viability in non-adherent H292 and H23 cells, respectively. Inspiringly, the sensitizing activity of avicequinon B on detachment-induced cell death was distinctly observed at lower dose (2 μ M) in both H292 and H23 lung cancer cells compared with H460 cells which only responded to 4 μ M of avicequinon B (Fig. 3a). Soft agar assay demonstrated the diminution of both colony number and size in H292 (Fig. 6c) and H23 (Fig. 6d) treated with 2-4 μ M of avicequinone B. These data strengthened the anti-metastasis activity of avicequinone B on the induction of anoikis and the inhibition on survival under detachment condition in human lung cancer cells.

Discussion

The capability to growth and escape from cell death under detachment condition has been found in non-adherent tumor cells circulating in blood and lymphatic system [31]. In order to resist to detachment-induced cell death, cancer cells acquire the high expression of anti-apoptosis proteins and anoikis resistant mechanisms [6, 31, 32]. Previously, caveolin-1 was shown to inhibit anoikis through the preservation of anti-apoptosis Mcl-1 protein in detached lung cancer cells [19]. Caveolin-1 was shown to be declined in response to the detachment of cells from ECM and the sustained level of the protein confers anoikis resistance [33]. Furthermore, the augmentation of Bcl-2 and diminution of Bax also manipulate anoikis in cancer cells [34]. In this study, anoikis sensitizing effect of avicequinone B in human lung cancer cells involved with the down-regulation of caveolin-1 together with the reduction of Mcl-1 and Bcl-2 (Fig. 5). Although

the decrease of Mcl-1 and Bcl-2 was notified after treatment with avicequinone B at 1-4 μ M, the significantly reduction of caveolin-1 and induction of anoikis were only observed in human lung cancer cells incubated with avicequinone B at 4 μ M. These results support the critical role of caveolin-1 on modulation of anoikis resistance in human lung cancer cells [16, 17].

Integrins are transmembrane molecules that regulate not only cell adhesion but also various cellular signaling pathways [8]. The interaction between integrins and protein components in ECM or specific ligands activate downstream FAK at focal adhesion complex consequence with phosphorylation of pro-survival proteins. In spite of loss of anchorage with ECM, highly metastatic cancer cells are able to generate integrin survival signals resulting in resistance to anoikis [10, 12, 35]. The up-regulation of integrin β 1 and β 3 influences with various aggressive behaviors in cancer cells [36, 37]. Recent study revealed that the diminution of β 1 and β 3 integrins suppresses survival and growth under detachment condition in human lung cancer cells [38]. Herein, suppression on integrin survival pathway in lung cancer cells induced by avicequinone B was elucidated. There was the reduction of integrin β 1 and β 3 level consequence with the restrain of downstream signaling molecules, p-FAK and p-Src in non-adherent lung cancer cells treated with avicequinone B.

The up-regulation of anti-apoptotic proteins and pro-survival signal has been revealed in lung cancer cells with anoikis resistant phenotype [39]. Down-regulation of caveolin-1, Mcl-1 and Bcl-2 as well as suppression on AKT and ERK activation successfully initiate detachment-induced cell death in anoikis-resistant lung cancer cells [40, 41]. The suppression on integrin/FAK/Src pathway consequence with the reduction

of pERK/ERK and diminution of anti-apoptosis Bcl-2 family proteins evidence the sensitizing effect of avicequinone B in anoikis resistant lung cancer cells.

Phosphorylation on FAK and Src leading to formation FAK-Src complex which modulates metastasis features such as migration and anchorage-independent growth [42]. Therefore, the suppression on integrin/FAK/Src signaling has been recognized as targeted pathway for treatment metastasis cancer [43]. Inhibition on migration and invasion of naphthoquinone compound has been demonstrated in cancer cells [22]. Taken together with the anoikis sensitizing activity of avicequinone B obtained from this study, these data strengthened the possibility to develop naphthoquinones and their derivatives as novel anti-metastasis drugs.

Conclusions

In conclusion, our study provided evidence indicating that avicequinone B suppressed survival and induced anoikis in human lung cancer cells under detachment condition through inhibition on integrin/FAK/Src signaling and down-regulation of anti-apoptosis protein including cavelolin-1, Mcl-1 and Bcl-2 (Fig.7). These data support the further development of avicequinone B as an effective treatment to overcoming cancer metastasis.

Abbreviations

Bcl-2: B-cell lymphoma 2; Mcl-1: myeloid cell leukemia 1; FAK: Focal adhesion kinase; p-FAK: Phosphorylated focal adhesion kinase; Src: Proto-oncogene tyrosine-protein kinase; p-Src: Phosphorylated proto-oncogene tyrosine-protein kinase ECM: Extracellular matrix; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT: Phospho kinase B; p-AKT: Phosphorylated phospho kinase B; ERK: Extracellular signal–

regulated kinase; : p-ERK: Phosphorylated Extracellular signal-regulated kinase; PBS: phosphate buffer saline; Cav-1: Caveolin-1; XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI: Propidium iodide; DMSO: Dimethylsulfoxide; Bax: Bcl-2-associated X protein; HRP: Horseradish peroxidase; BCA: Bicinchoninic acid; THF: Tetrahydrofuran; DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene; RPMI: Roswell Park Memorial Institute; FBS: Fetal bovine serum; OD: Optical density; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Declarations

Acknowledgments

Not applicable.

Funding

The work was supported by the Thailand Research Fund under Grant [MRG5980143] and a research grant from the 90th Anniversary of Chulalongkorn University Ratchadaphiseksomphot Endowment Fund.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Authors' contributions

Conceived and designed the experiments: PC and CC. Synthesized the compound: WK, WD and SC. Performed the experiments: AP, SS PC and CC. Analysis and interpretation of data: PC and CC. Wrote the paper: SC, PC and CC. All authors read and approved the final manuscript.

Ethics approval and consent to participate

409 Not applicable.

410 ***Consent for publication***

411 All the authors have read and approved the paper for publication.

412 ***Competing interests***

413 The authors declare that they have no competing interests.

414 ***Authors' information***

415 ¹Department of Biochemistry and Microbiology, Faculty of Pharmaceutical
416 Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

417 ²Departments of Pharmacognosy and Pharmaceutical Botany, Chulalongkorn
418 University, 10330 Bangkok, Thailand. Email: noonwiranpat@gmail.com

419 ³Department of Pharmacology and Physiology, Faculty of Pharmaceutical
420 Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

421 ⁴Cell-based Drug and Health Products Development Research Unit, Faculty of
422 Pharmaceutical Sciences, Chulalongkorn University, 10330 Bangkok, Thailand.

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424 **References**

425 1. Vansteenkiste J, De Ruyscher D, Eberhardt WE, Lim E, Senan S, Felip E, et al.
426 Early and locally advanced non-small-cell lung cancer (NSCLC): ESMO Clinical
427 Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2013;24
428 suppl:vi89-98.

429 2. Riihimäki M, Hemminki A, Sundquist K, Hemminki K. Causes of death in patients
430 with extranodal cancer of unknown primary: searching for the primary site. BMC
431 Cancer. 2014;14:439.

432 3. Nichols L, Saunders R, Knollmann FD. Causes of death of patients with lung cancer.

- 433 Arch Pathol Lab Med. 2012;136:1552-7.
- 434 4. Popper HH. Progression and metastasis of lung cancer. *Cancer Metastasis Rev.* 2016;
435 35:75-91.
- 436 5. D'Antonio C, Passaro A, Gori B, Del Signore E, Migliorino MR, Ricciardi S, Fulvi
437 A, de Marinis F. Bone and brain metastasis in lung cancer: recent advances in
438 therapeutic strategies. *Ther Adv Med Oncol.* 2014;6:101-14.
- 439 6. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis.
440 *Cancer Lett.* 2008;272:177-85.
- 441 7. Frisch SM, Ruoslahti E. Integrins and anoikis. *Curr Opin Cell Biol.* 1997;9:701-6.
- 442 8. Vachon PH. Integrin signaling, cell survival, and anoikis: distinctions, differences,
443 and differentiation. *J Signal Transduct.* 2011;2011:738137.
- 444 9. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer
445 progression. *Biochim Biophys Acta.* 2013;1833:3481-98.
- 446 10. Felding-Habermann B, Fransvea E, O'Toole TE, Manzuk L, Faha B, Hensler M.
447 Involvement of tumor cell integrin alpha v beta 3 in hematogenous metastasis of
448 human melanoma cells. *Clin Exp Metastasis.* 2002;19:427-36.
- 449 11. Díaz-Montero CM, Wygant JN, McIntyre BW. PI3-K/Akt-mediated anoikis
450 resistance of human osteosarcoma cells requires Src activation. *Eur J Cancer.*
451 2006;42:1491-500.
- 452 12. Haenssen KK, Caldwell SA, Shahriari KS, Jackson SR, Whelan KA, Klein-Szanto
453 AJ, Reginato MJ. ErbB2 requires integrin alpha5 for anoikis resistance via Src
454 regulation of receptor activity in human mammary epithelial cells. *J Cell Sci.*
455 2010;123:1373-82.
- 456 13. Lee YC, Jin JK, Cheng CJ, Huang CF, Song JH, Huang M, Brown WS, Zhang S,

Yu-Lee LY, Yeh ET, McIntyre BW, Logothetis CJ, Gallick GE, Lin SH. Targeting constitutively activated $\beta 1$ integrins inhibits prostate cancer metastasis. *Mol Cancer Res.* 2013;11:405-17.

14. Stucci S, Tucci M, Passarelli A, Silvestris F. $\text{Av}\beta 3$ integrin: Pathogenetic role in osteotropic tumors. *Crit Rev Oncol Hematol.* 2015;96:183-93.

15. Fiucci G, Ravid D, Reich R, Liscovitch M. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. *Oncogene.* 2002;21:2365-75.

16. Rungtabnapa P, Nimmannit U, Halim H, Rojanasakul Y, Chanvorachote P. Hydrogen peroxide inhibits non-small cell lung cancer cell anoikis through the inhibition of caveolin-1 degradation. *Am J Physiol Cell Physiol.* 2011;300:235-45.

17. Halim H, Luanpitpong S, Chanvorachote P. Acquisition of anoikis resistance up-regulates caveolin-1 expression in human non-small cell lung cancer cells. *Anticancer Res.* 2012;32:1649-58.

18. Ravid D, Maor S, Werner H, Liscovitch M. Caveolin-1 inhibits cell detachment-induced p53 activation and anoikis by upregulation of insulin-like growth factor-I receptors and signaling. *Oncogene.* 2005;24:1338-47.

19. Chunhacha P, Pongrakhananon V, Rojanasakul Y, Chanvorachote P. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. *Am J Physiol Cell Physiol.* 2012;302:1284-92.

20. Tang W, Feng X, Zhang S, Ren Z, Liu Y, Yang B, lv B, Cai Y, Xia J, Ge N.. Caveolin-1 Confers Resistance of Hepatoma Cells to Anoikis by Activating IGF-1 Pathway. *Cell Physiol Biochem.* 2015;36:1223-36.

21. Ito C, Katsuno S, Kondo Y, Tan HT, Furukawa H. Chemical constituents of

Avicennia alba. Isolation and structural elucidation of new naphthoquinones and their analogues. Chem Pharm Bull. 2000;48:339-43.

22. Lin KL, Su JC, Chien CM, Tseng CH, Chen YL, Chang LS, Lin SR. Naphtho[1,2-b]furan-4,5-dione induces apoptosis and S-phase arrest of MDA-MB-231 cells through JNK and ERK signaling activation. Toxicol In Vitro. 2010;24:61-70.

23. Tsai PC, Chu CL, Fu YS, Tseng CH, Chen YL, Chang LS, Lin SR. Naphtho[1,2-b]furan-4,5-dione inhibits MDA-MB-231 cell migration and invasion by suppressing Src-mediated signaling pathways. Mol Cell Biochem. 2014;387:101-11.

24. Tseng CH, Chen YL, Yang SH, Peng SI, Cheng CM, Han CH, Lin SR, Tzeng CC. Synthesis and antiproliferative evaluation of certain iminonaphtho[2,3-b]furan derivatives. Bioorg Med Chem. 2010;18:5172-82.

25. Chunchacha P, Pongrakhananon V, Rojanasakul Y, Chanvorachote P. Caveolin-1 regulates Mcl-1 stability and anoikis in lung carcinoma cells. Am J Physiol Cell Physiol. 2012;302:C1284-92.

26. Ke X, Li L, Dong HL, Chen ZN. Acquisition of anoikis resistance through CD147 upregulation: A new mechanism underlying metastasis of hepatocellular carcinoma cells. Oncol Lett. 2012;3:1249-54.

27. Borowicz S, Van Scoyk M, Avasarala S, et al. The Soft Agar Colony Formation Assay. J Vis Expe. 2014:51998.

28. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry. 1998; 31:1-9.

- 503 29. Pongrakhananon V, Nimmannit U, Luanpitpong S, Rojanasakul Y, Chanvorachote
 504 P. Curcumin sensitizes non-small cell lung cancer cell anoikis through reactive
 505 oxygen species-mediated Bcl-2 downregulation. *Apoptosis*. 2010;15:574-85.
- 506 30. Kim LC, Song L, Haura EB. Src kinases as therapeutic targets for cancer. *Nat Rev*
 507 *Clin Oncol*. 2009 Oct;6(10):587-95.
- 508 31. Kim YN, Koo KH, Sung JY, Yun UJ, Kim H. Anoikis resistance: an essential
 509 prerequisite for tumor metastasis. *Int J Cell Biol*. 2012;2012:306879.
- 510 32. Geiger TR, Peeper DS. The neurotrophic receptor TrkB in anoikis resistance and
 511 metastasis: a perspective. *Cancer Res*. 2005;65:7033-6.
- 512 33. Chanvorachote P, Chunchacha P. Caveolin-1 regulates endothelial adhesion of lung
 513 cancer cells via reactive oxygen species-dependent mechanism. *PLoS One*. 2013;
 514 8:e57466.
- 515 34. Martin SS, Vuori K. Regulation of Bcl-2 proteins during anoikis and amorphosis.
 516 *Biochim Biophys Acta*. 2004;1692:145-57.
- 517 35. Maiuthed A, Chanvorachote P. Cisplatin at sub-toxic levels mediates integrin switch
 518 in lung cancer cells. *Anticancer Res*. 2014;34:7111-7.
- 519 36. Velling T, Nilsson S, Stefansson A, Johansson S. beta1-Integrins induce
 520 phosphorylation of Akt on serine 473 independently of focal adhesion kinase and Src
 521 family kinases. *EMBO Rep*. 2004;5:901-5.
- 522 37. Bhattacharya S, Ray RM, Johnson LR. Integrin beta3-mediated Src activation
 523 regulates apoptosis in IEC-6 cells via Akt and STAT3. *Biochem J*. 2006;397:437-47.
- 524 38. Puchsaka P, Chaotham C, Chanvorachote P. α -Lipoic acid sensitizes lung cancer
 525 cells to chemotherapeutic agents and anoikis via integrin $\beta 1/\beta 3$ downregulation. *Int*
 526 *J Oncol*. 2016;49:1445-56.

39. Liu G, Meng X, Jin Y, Bai J, Zhao Y, Cui X, Chen F, Fu S. Inhibitory role of focal adhesion kinase on anoikis in the lung cancer cell A549. *Cell Biol Int*. 2008;32:663-70.
40. Sirimangkalakitti N, Chamni S, Suwanborirux K, Chanvorachote P. Renieramycin M Sensitizes Anoikis-resistant H460 Lung Cancer Cells to Anoikis. *Anticancer Res*. 2016;36(4):1665-71.
41. Chanvorachote P, Pongrakhananon V, Halim H. Caveolin-1 regulates metastatic behaviors of anoikis resistant lung cancer cells. *Mol Cell Biochem*. 2015;399:291-302.
42. M. A. Westhoff, B. Serrels, V. J. Fincham, M. C. Frame, N. O. Carragher. Src-Mediated Phosphorylation of Focal Adhesion Kinase Couples Actin and Adhesion Dynamics to Survival Signaling. *Mol Cell Biol*. 2004;24:8113-33.
43. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Bio*. 2006;18:516-23.

Figure legends

Figure 1 Synthetic scheme of avicequinone B

Figure 2 Cytotoxicity of avicequinone B in human lung cancer cells. (a) MTT assay revealed the significant reduction of cell viability in lung cancer H460 cells after treatment with 8-10 μ M of avicequinone B for 24 h. (b) Avicequinone B at 10 μ M induced apoptosis cell death in human lung cancer cells. (c) Co-staining with Hoechst33342/PI demonstrated that no necrosis cell death was detected in H460 cells at all treatment of avicequinone B. (d) The suppression on proliferation in adherent lung cancer cells was significantly notified in H460 cells incubated with 2-4 μ M of avicequinone B for 72 h. Data represent the means $\pm$ SD (n = 3). *, # $p \leq 0.05$ versus untreated control cells.

Figure 3 Avicequinone B sensitized anoikis in human lung cancer cells. (a) The reduction of viability was early observed in lung cancer H460 cells after culture under detachment condition for 6 h. (b) Avicequinone B at 4 μ M significantly induced anoikis in H460 cells that were culture under detachment condition for 24 h. (c) Bright blue fluorescence of Hoechst33342 indicated DNA condense and apoptosis body was indicated in H460 cells treated with 4 μ M of avicequinone B. (d) Detachment-induced apoptosis was evidenced with histograms obtained from flow cytometry analysis of H460 cells at anchorage culture (control attachment), detachment without treatment for 24 h (control) and detachment with 4 μ M of avicequinone B for 24 h. (e) The detection early and late apoptosis without necrosis cells was remarkably increased after incubation of non-adherent H460 cells with avicequinone B at 4 μ M. * $p \leq 0.05$ versus untreated control cells at the same time point.

Figure 4 Effect of avicequinone B on anchorage-independent growth. (a) Colony formation of single-cell H460 cells was evaluated under a microscope after culture in soft agar for 14 days. (b) Relative colony number and (c) relative colony size were gradually decreased incubation of single-cell H460 with 1-4 μ M of avicequinone B. Data represent the means $\pm$ SD (n = 3). $*p \leq 0.05$ versus untreated control cells.

Figure 5 Avicequinone B down-regulated anti-apoptosis proteins and pro-survival signaling. (a) Western blot analysis revealed the reduction of anti-apoptosis proteins, Mcl-1 and Bcl-2 in H460 cells treated with avicequinone B (1-4 μ M) under detachment condition for 12 h. (b) The decrease of caveolin-1 (Cav-1) was significantly notified in non-adherent H460 cells incubated with avicequinone B at 4 μ M. (c) Low expression level of integrin β 1 and β 3 were demonstrated in avicequinone B-treated lung cancer cells. (d) Downstream pro-survival signaling of integrin including p-FAK (Y397) associating with p-Src (Try416) were also down-regulated after incubation of detached H460 cells with avicequinone B (1-4 μ M). (e) The reduction of p-ERK (Thr 981) was significantly notified while (f) there was no alteration of p-AKT (Ser 473)/AKT in avicequinone-treated lung cancer cells. Data represent the means $\pm$ SD (n = 3). $*p \leq 0.05$ versus untreated control cells.

Figure 6 Avicequinone B restrained survival under detachment condition in various lung cancer cells. The lower viability of non-adherent (a) H292 and (b) H23 lung cancer cells was significantly notified in the cells incubated with avicequinone B at 2-4 μ M compared with non-treated control. Anchorage-independent growth assay revealed the reduction of both number and size of forming colonies in lung cancer (c) H292 and (d) H23 cells after

culture with 2-4 μ M of avicequinone B for 14 days. Data represent the means $\pm$ SD (n = 3). $*p \leq 0.05$ versus untreated control cells at the same time point.

Figure 7 Proposed mechanism of avicequinone B-induced anoikis in lung cancer cells. Avicequinone B mediated anoikis through the reduction of anti-apoptosis proteins caveloin-1, Mcl-1 and Bcl-2 as well as down-regulation of integrin/FAK/Src pro-survival pathway.