รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาลักษณะความรุนแรงและกลไกการควบคุมในเซลล์มะเร็งต้นกำเนิด เซลล์มีเซ็นไคม์ และเซลล์ชนิด quiescent ในเซลล์มะเร็งปอดมนุษย์

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สัญญาเลขที่ MRG5980143

โครงการ การศึกษาลักษณะความรุนแรงและกลไกการควบคุมในเซลล์มะเร็งต้นกำเนิด เซลล์มีเซ็นไคม์ และเซลล์ชนิด quiescent ในเซลล์มะเร็งปอดมนุษย์

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และ จุฬาลงกรณ์มหาวิทยาลัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: MRG5980143

ชื่อโครงการ : การศึกษาลักษณะความรุนแรงและกลไกการควบคุมในเซลล์มะเร็งต้นกำเนิด เซลล์มีเซ็นไคม์ และเซลล์ชนิด quiescent ในเซลล์มะเร็งปอดมนุษย์

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บทคัดย่อ : ความแตกต่างของเซลล์มะเร็งภายในก้อนเนื้องอกเป็นสาเหตุสำคัญของความหลากหลายในการแพร่กระจาย ความล้มเหลวของเคมีบำบัด การกลับมาของโรคและอัตราการรอดชีวิตที่ลดลงในผู้ป่วยโรคมะเร็งปอด ด้วยวิธีการเพาะเลี้ยงเซลล์เดี่ยวในภาชนะเลี้ยงเซลล์ชนิด 96 หลุม สามารถแยกกลุ่มประชากรเซลล์มะเร็งปอดที่มีรูปร่างต่างกันได้ 3 กลุ่มหลัก การประเมินพฤติกรรมความรุนแรง ได้แก่ ความดื้อต่อยาเคมีบำบัด การเจริญโตในสภาวะไร้การยึดเกาะ และความสามารถในการเกิดก้อนมะเร็งในกลุ่มประชากรเซลล์มะเร็งทั้งสามกลุ่ม พบว่ากลุ่มประชากรเซลล์รูปร่างใหญ่แสดงความดื้อต่อการตายที่เหนี่ยวนำด้วยยาเคมีบำบัดสูงสุด เพื่อศึกษาลักษณะจำเพาะของเซลล์กลุ่มนี้ได้ทำการศึกษาการเปลี่ยนแปลงของโปรตีนที่เกี่ยวข้องกับกระบวนการเปลี่ยนจากเซลล์เยื่อบุผิวไปเป็นเซลล์มีเซ็นไคม์และเซลล์มะเร็งต้นกำเนิด นอกจากนี้ยังพบรูปแบบที่จำเพาะของข้อมูลเมแทบอโลมิกส์ในกลุ่มประชากรที่ดื้อต่อยาเคมีบำบัดเปรียบเทียบกับกลุ่มเซลล์มะเร็งดั้งเดิม เพื่อค้นหาเคมีบำบัดใหม่ได้ทำการศึกษาความสามารถในการต้านมะเร็งของเปปไทด์สกัดจากเห็ดขอนขาวและสารอะวิซีควิโนน บี สกัดจากต้นโกงกาง ข้อมูลที่ได้จากการศึกษาจะนำไปสู่การค้นหากลไกควบคุมการดื้อยาในเซลล์มะเร็งปอดและนำมาซึ่งข้อมูลเชิงลึกเกี่ยวกับความแตกต่างของเซลล์มะเร็ง

คำหลัก : มะเร็งปอด, ความดื้อต่อยาม พฤติกรรมความรุนแรง, มะเร็งต้นกำเนิด,การเปลี่ยนแปลงจากเยื่อบุผิวเป็นไปมีเซ็นไคม์, เมแทบอโลมิกส์

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

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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

Executive Summary

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 pluripotentcy, 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 stemmness 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, weather 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 mediates 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 squrrosulus* 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.

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 (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

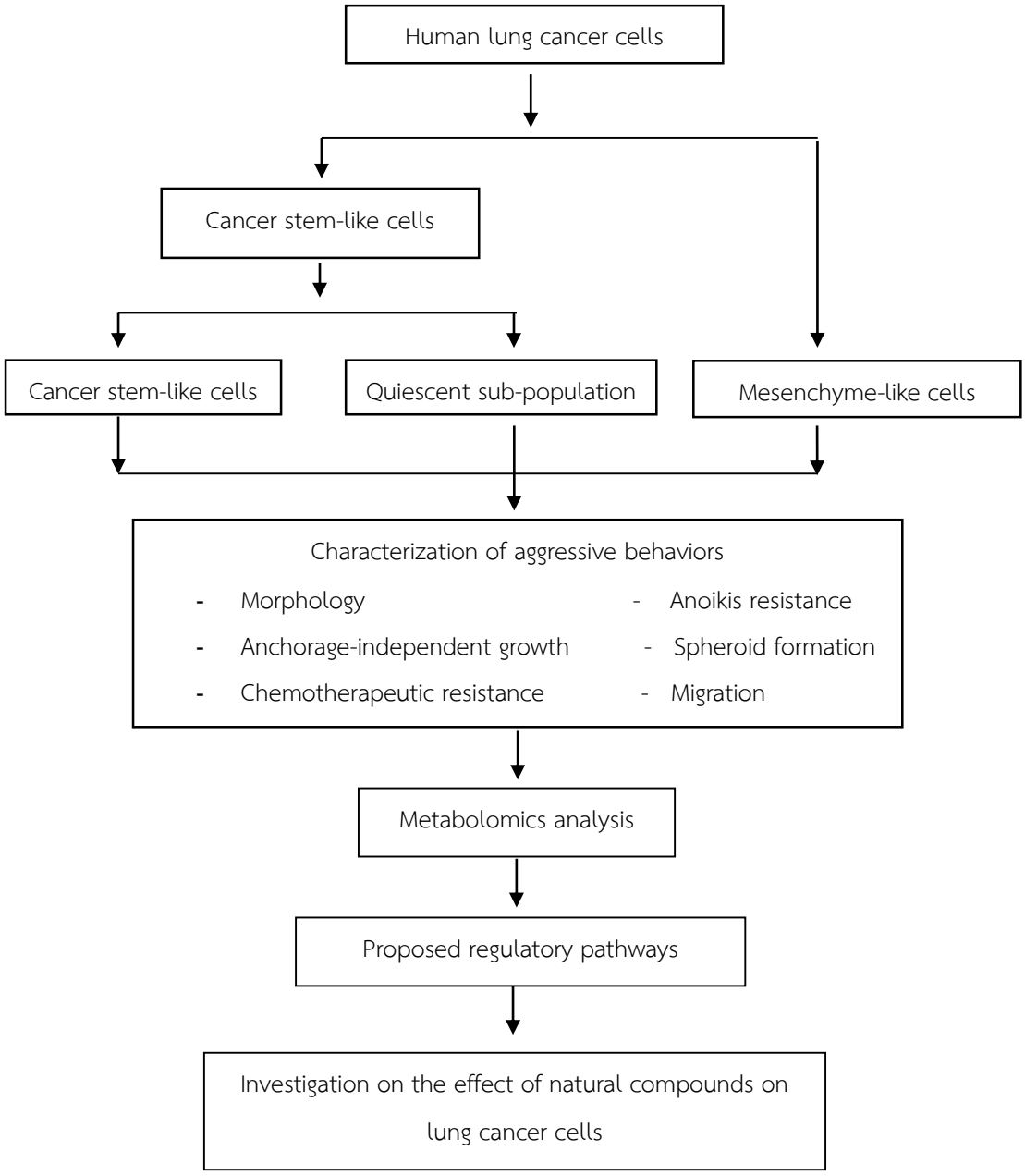
3.1 To isolate and identify CSCs, mesenchyme-like cells and quiescent sub-population of human lung cancer cells

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-sulfophenyl)-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). Avicequinone 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% CO_2 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 $1x10^4$ 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 µL 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 µL 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 µg/mL of Hoechst 33342 and 5 µg/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 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 µl of binding buffer then 10 µl of Annexin V-FITC (1 µg/ml) and 5 µl of PI (2.5 µg/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% CO_2 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% CO_2 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% CO_2.

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. Chemiluminutesescence (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 CH_3ONH_2 at 37°C for 1 h. Oximes and alklyoximes derivatives was then incubated with N-methyl-N-(trimethylsilyl) triflouroacetamide (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 ± 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.

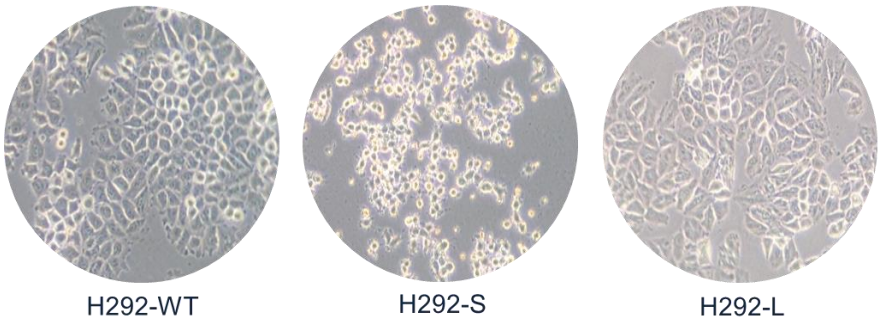


Figure 1 Human lung cancer cells composed with at least three sub-population with different morphology; wild type (WT), small (S) and large (L) cells.

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 μM), doxorubicin (10 μM) and etoposide (100 μM) 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)

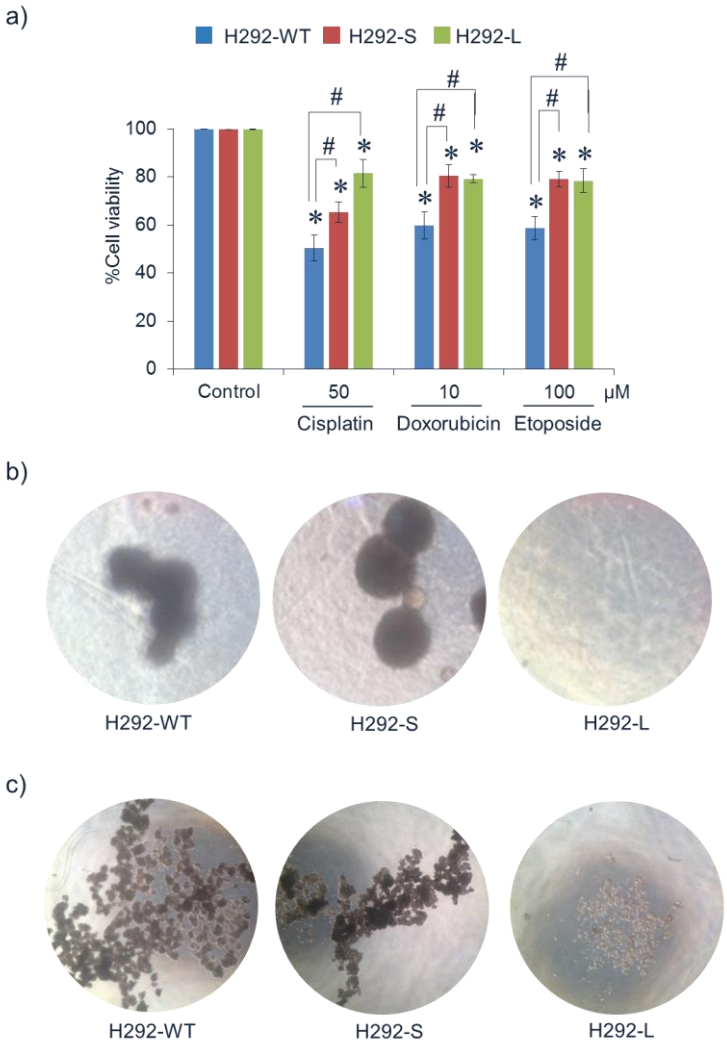


Figure 2 a) Chemotherapeutic resistant activity of lung cancer sub-population towards current chemotherapeutic drugs b) Capability to growth under detachment condition of sub-population in H292 cells. c) Capability to generate new tumor was evaluated through spheroid formation assay. Tightly packed colonies 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

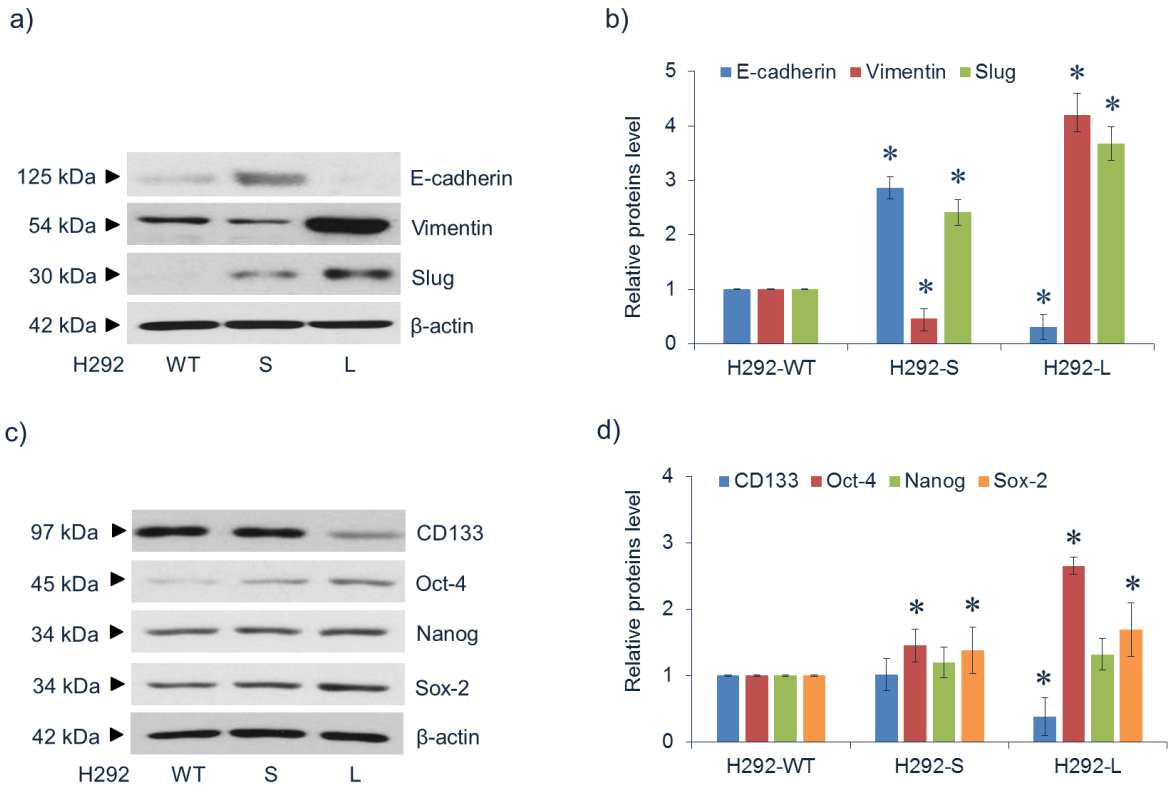


Figure 3 Western blot analysis revealed a and b) the over-expression of EMT regulating protein including vimentin and slug in H292-L sub-population. c and d) Despite lower expression of stemness marker protein; CD133, up-regulation of stemness transcription factor; oct-4 and sox-2 were presented in H292-L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

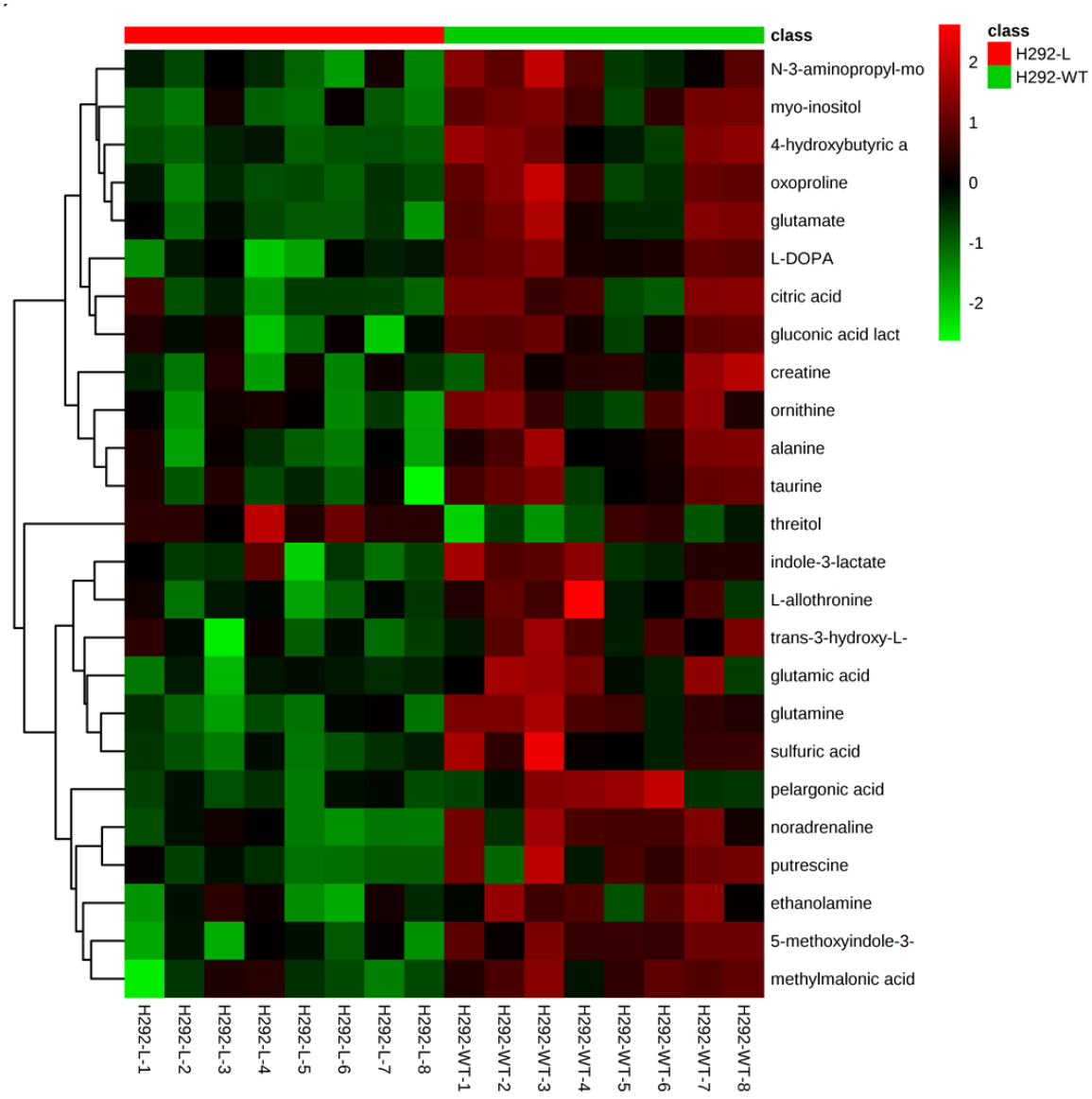


Figure 4 Heatmap of top 25 metabolic compounds ranked by *p*-value

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).

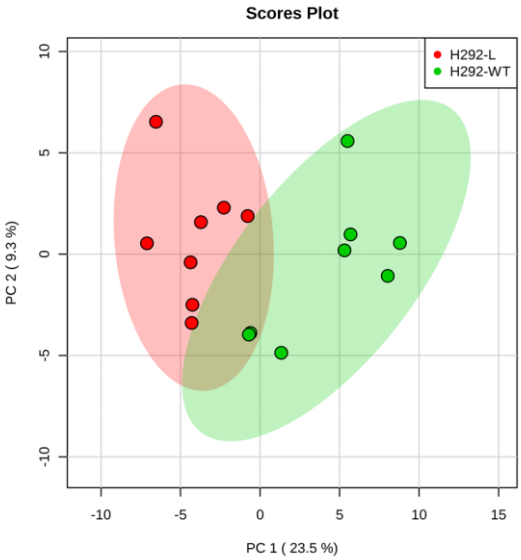


Figure 5 Principal component analysis of metabolite profiles of H292-WT and H292-L sub-population

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 Hoechse33342 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).

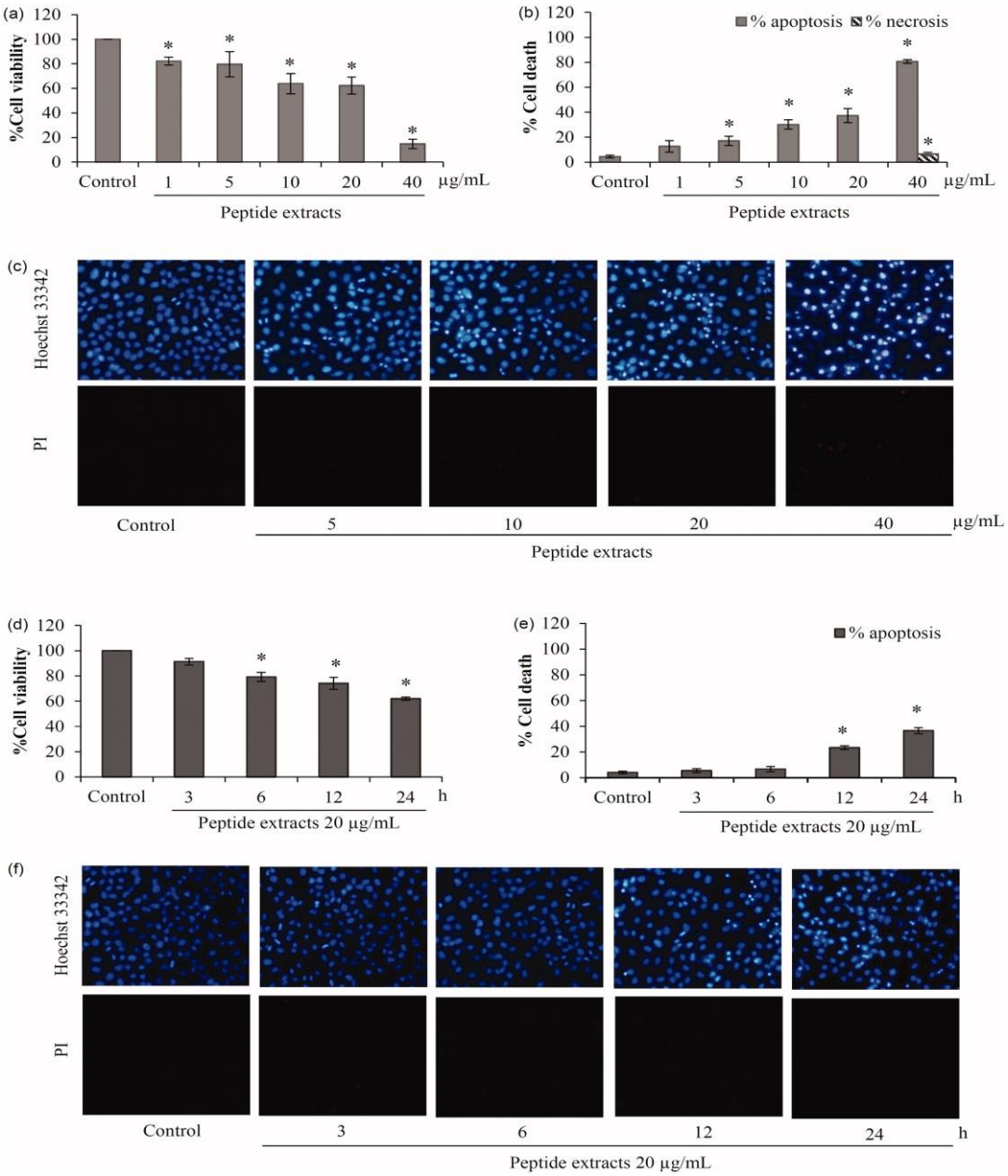


Figure 6 Apoptosis induction of peptide extracts from *Lentinus squarrosulus* Mont. Cell viability of H460 lung cancer cells was evaluated after treatment with the extracts in (a) time-dependent and (c) dose-dependent manner. (b) The effect of peptide extracts on apoptosis induction was early notified 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 ± SD. * $p \leq 0.05$ versus non-treated control.

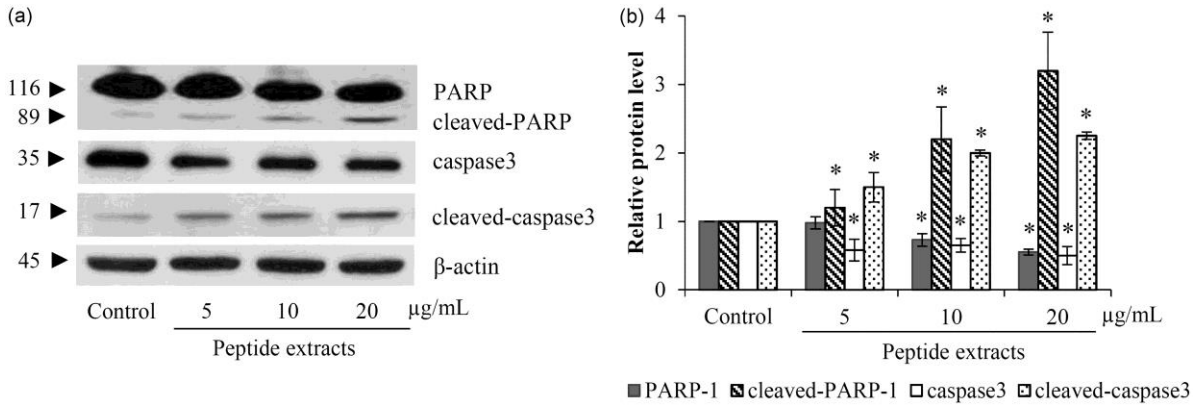


Figure 7 The apoptosis cells death was confirmed by the alteration of caspase3 and PERP proteins. (a) Western blot analysis indicated the increasing of active caspase3 (cleaved-caspase3) in peptide extracts-treated H460 cells. (b) As substrate of activated casapase3, the significant reduction of PARP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

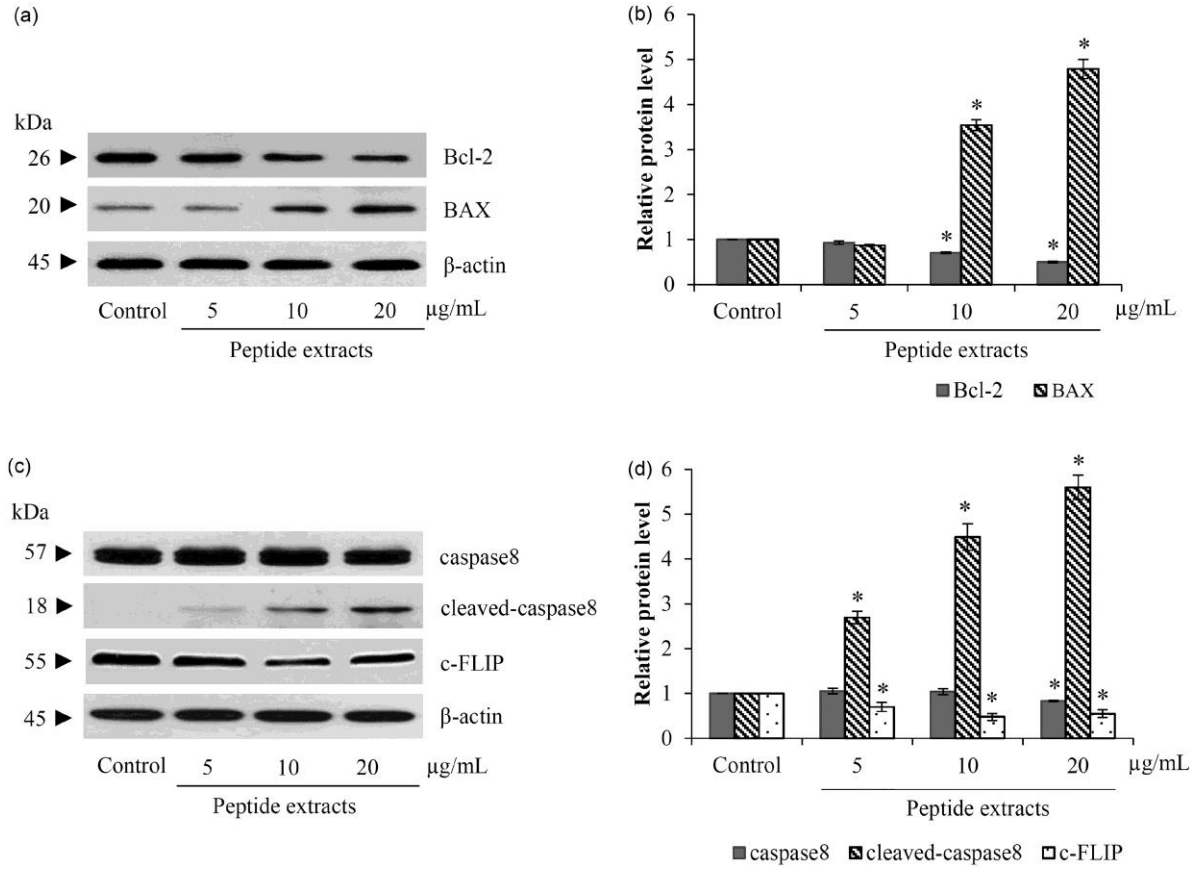


Figure 8 Peptide extracts stimulated both mitochondrial and death-receptor apoptotic pathway. (a and b) There were the reduction of Bcl-2 and the up-regulation of BAX after treatment of lung cancer cells with 10-20 μg/ml of peptide extracts form *Lentinus squarrosulus*. (c and d) Protein-relating death-receptor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

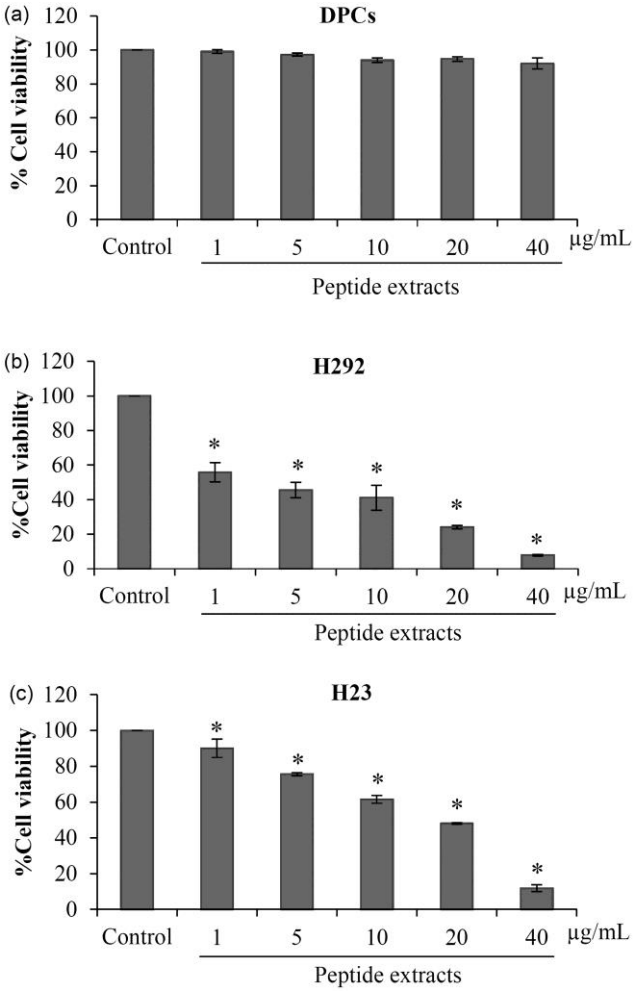


Figure 9. Selective anticancer activity of peptide extracts from *Lentinus squarrosulus*. (a) Low toxicity of the peptide extracts to non-cancer cells was indicated in human dermal papilla DPCs cells treated with 1–40 µg/mL peptides for 24 h. (b and c) Peptide extracts from L. squarrosulus induced cell 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 ± 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.

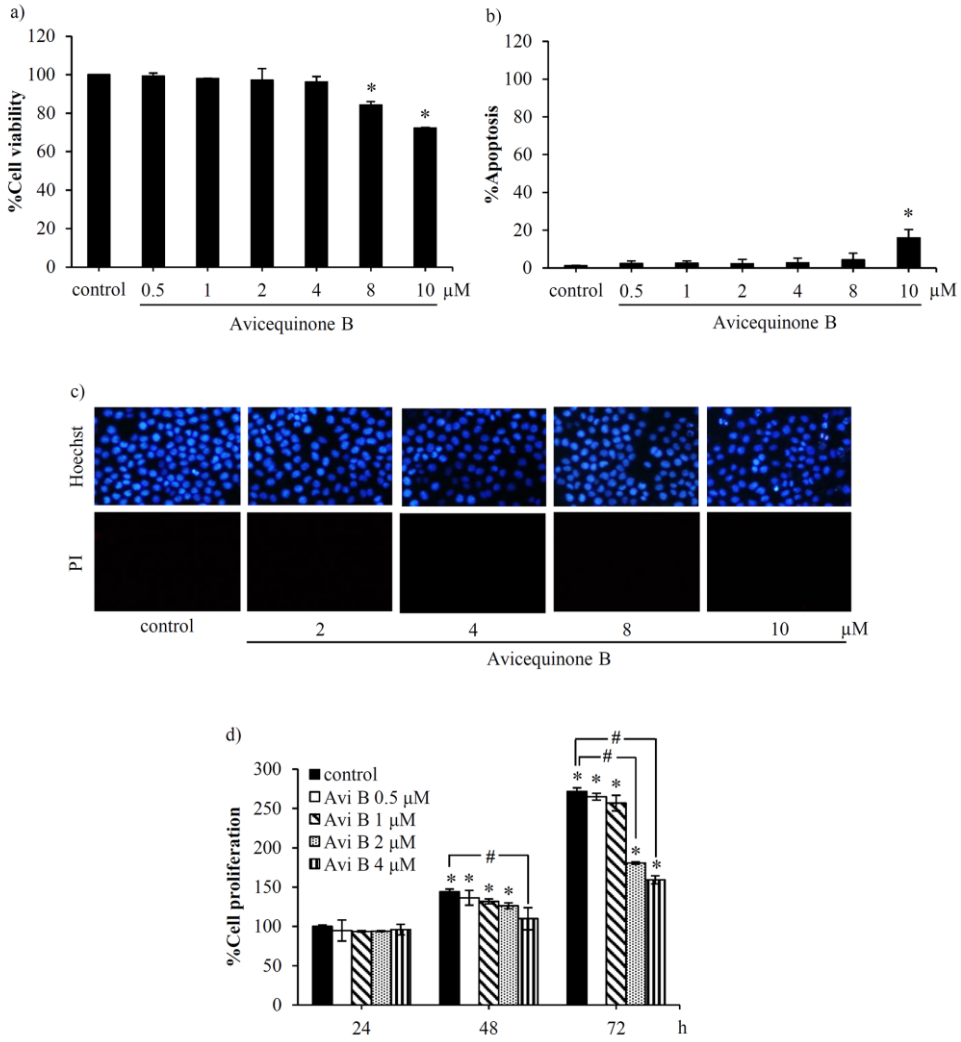


Figure 10 Cytotoxicity of avicequinone B in human lung cancer cells. (a) MTT assay revealed the significant reduction of cell viability in lung cancer H460 cells after treatment with 8-10 μM of avicequinone B for 24 h. (b) Avicequinone B at 10 μM induced apoptosis cell death in human lung cancer cells. (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 ± SD (n = 3). *, # $p \leq 0.05$ versus untreated control cells.

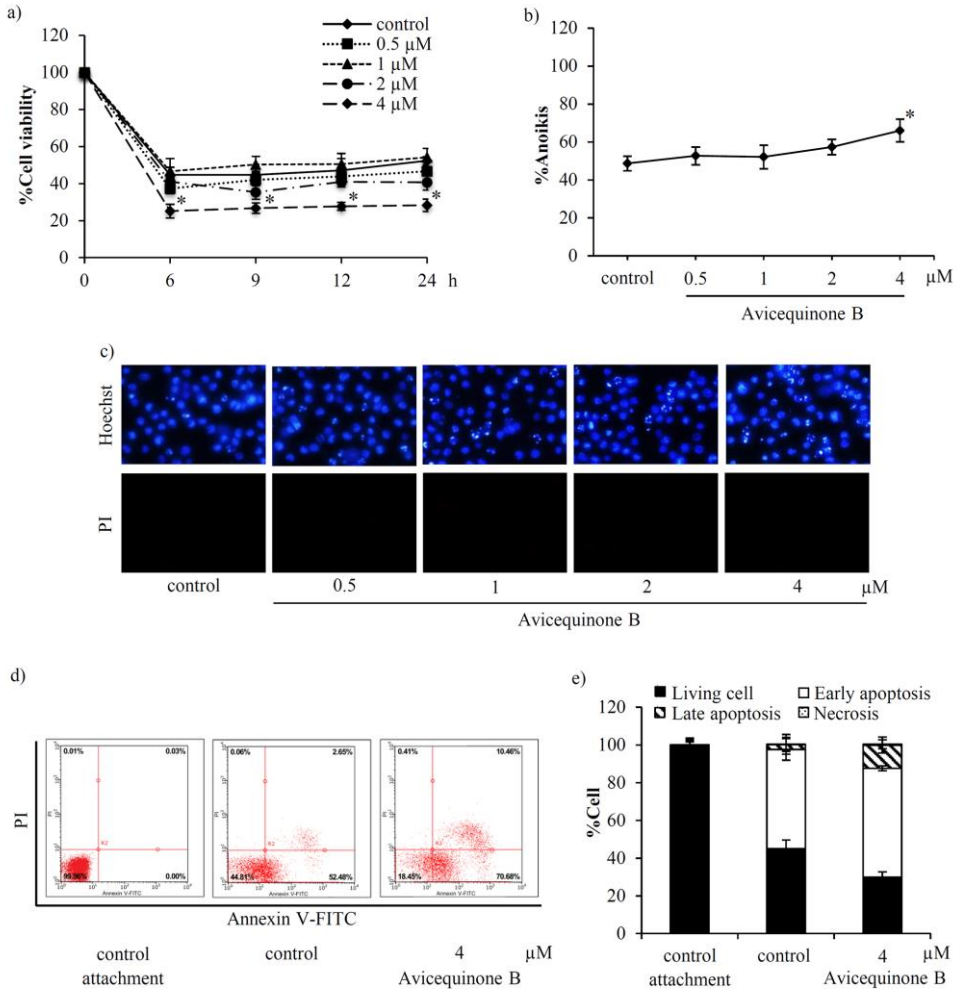


Figure 11 Avicequinone B sensitized anoikis in human lung cancer cells. (a) The reduction of viability was early observed in lung cancer H460 cells after culture under detachment condition for 6 h. (b) Avicequinone B at 4 µM significantly induced anoikis in H460 cells that were culture under detachment condition for 24 h. (c) Bright blue fluorescence of Hoechst33342 indicated DNA condense and apoptosis body was indicated in H460 cells treated with 4 µM 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 µM 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 µM. *$p \leq 0.05$ 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 μM 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 μM). 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 μM of avicequinone B (fig. 12b and 12c). Intriguingly, avicequinone B at 4 μM obviously suppressed proliferation in lung cancer cells at both anchorage-dependent (Fig.3d) and -independent condition.

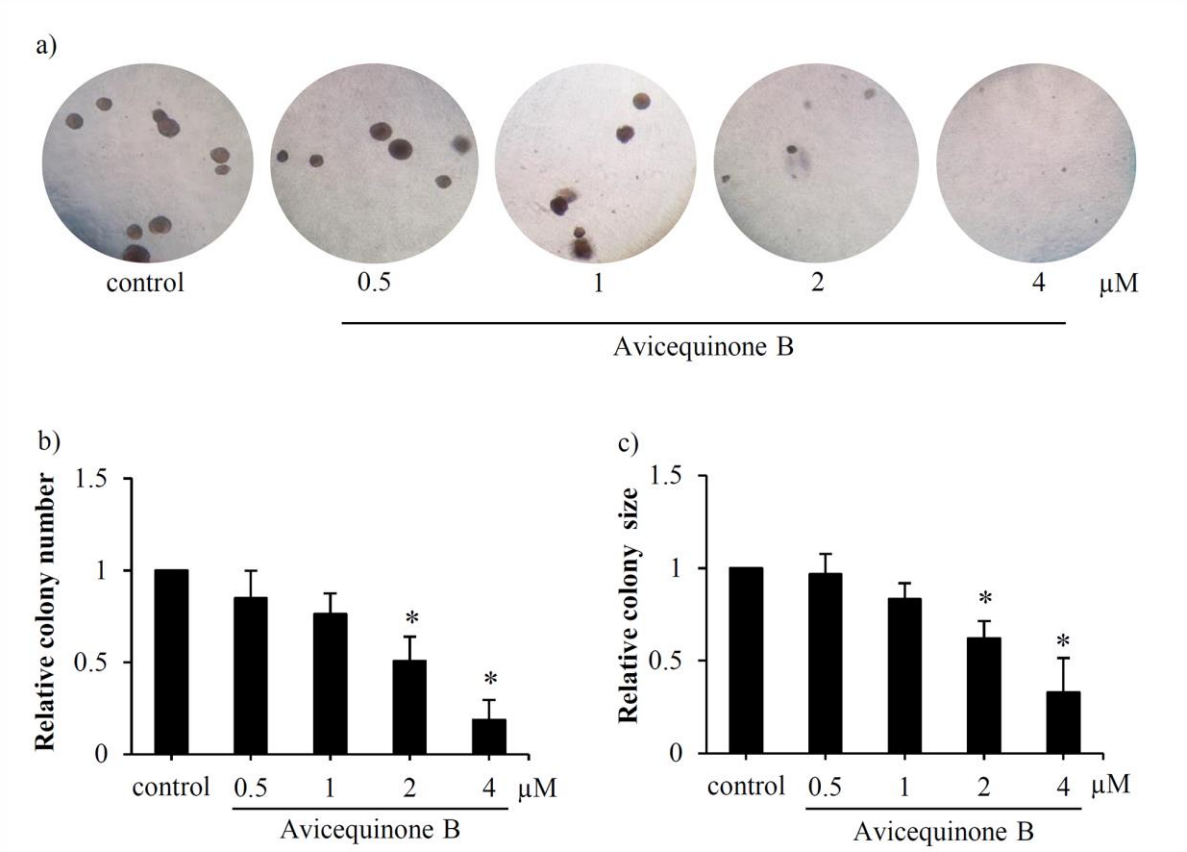


Figure 12 Effect of avicequinone B on anchorage-independent growth. (a) Colony formation of single-cell H460 cells was evaluated under a microscope after culture in soft agar for 14 days. (b) Relative colony number and (c) relative colony size were gradually decreased incubation of single-cell H460 with 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 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. 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 β1 and β3 in human lung cancer cells treated with 4 μM of avicequinone B under detachment condition (Fig. 5c). Interestingly, the reduction of integrin β3 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.

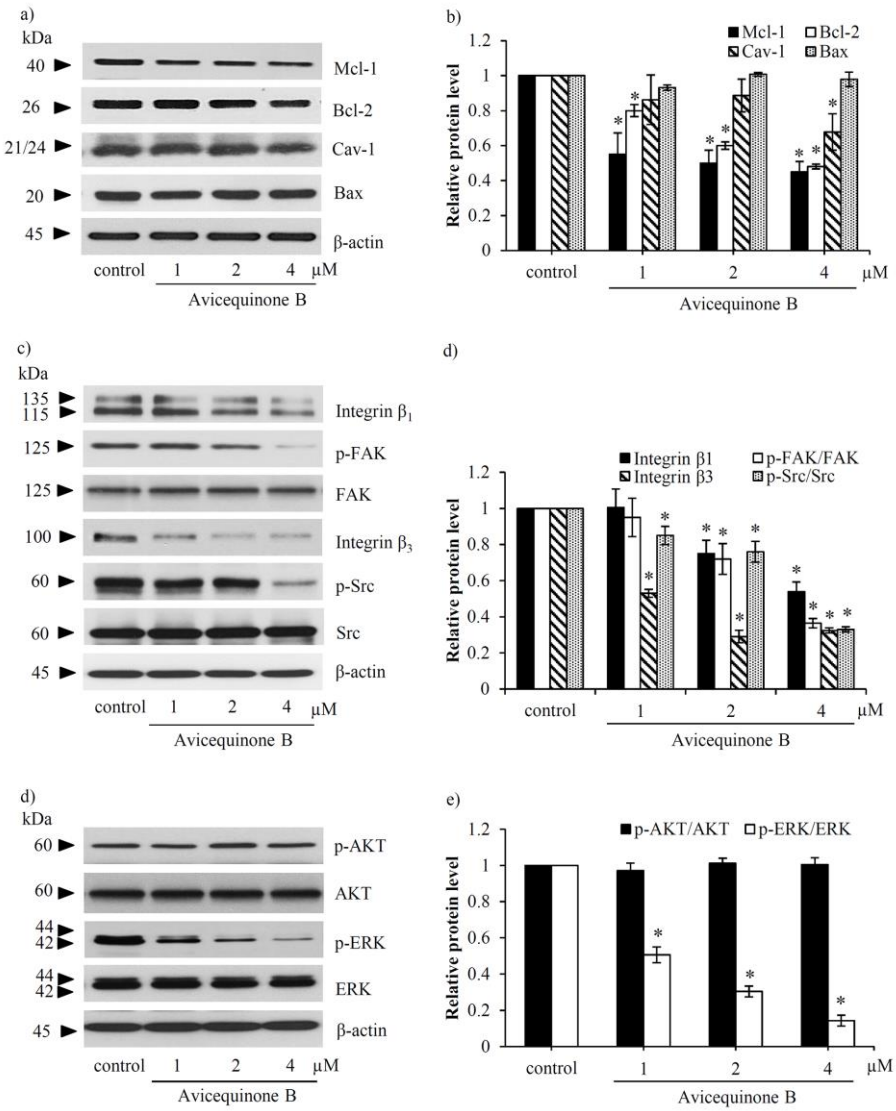


Figure 13 Avicequinone B down-regulated anti-apoptosis proteins and pro-survival signaling. (a) Western blot analysis revealed the reduction of anti-apoptosis proteins, Mcl-1 and Bcl-2 in H460 cells treated with avicequinone B (1-4 μM) under detachment condition for 12 h. (b) The decrease of caveolin-1 (Cav-1) was significantly notified in non-adherent H460 cells incubated with avicequinone B at 4 μM. (c) Low expression level of integrin β1 and β3 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 ± 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 μM; 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 μM) in both H292 and H23 lung cancer cells compared with H460 cells which only responded to 4 μM 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 μM 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.

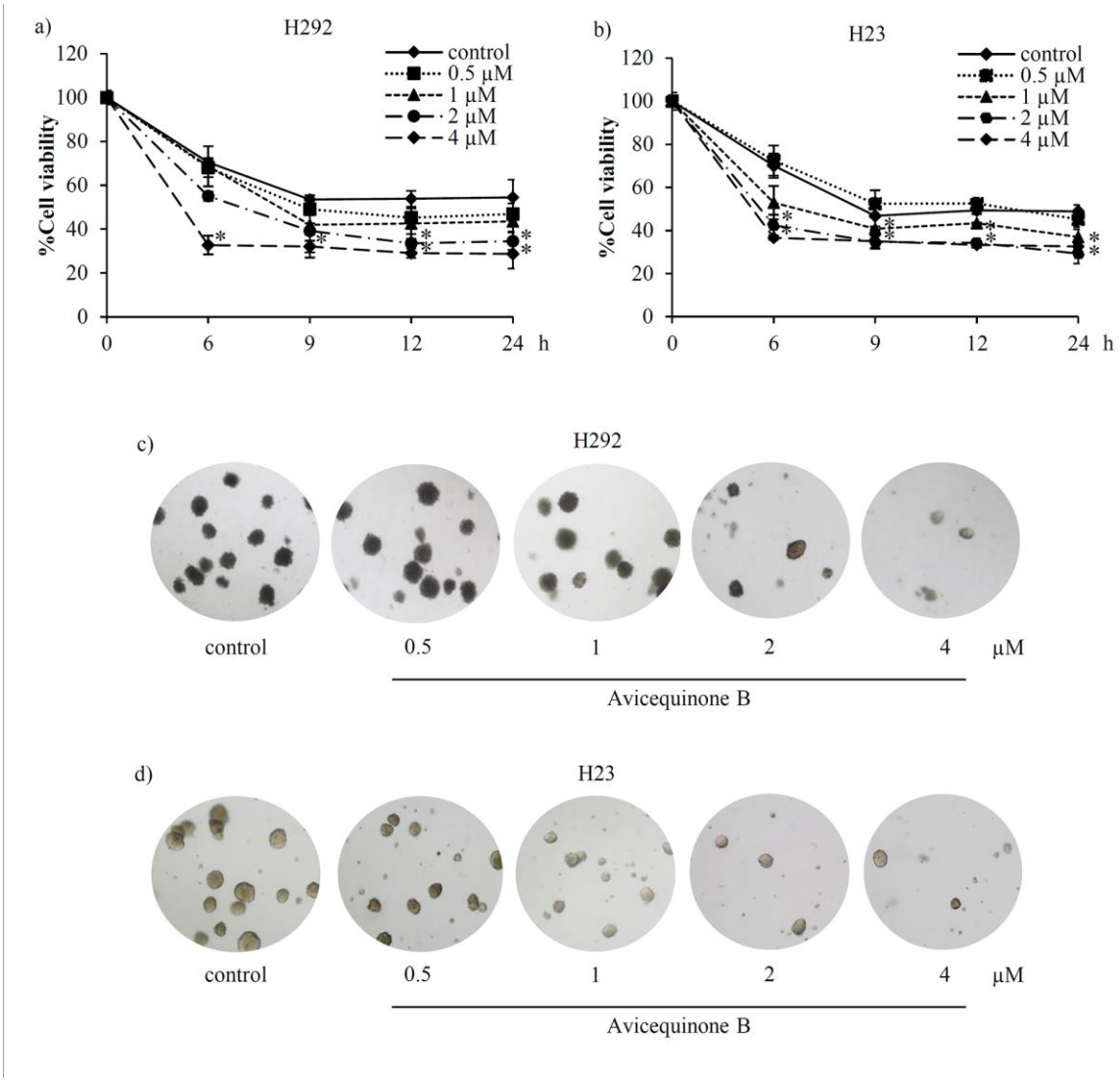


Figure 14 Avicequinone B restrained survival under detachment condition in various lung cancer cells. The lower viability of non-adherent (a) H292 and (b) H23 lung cancer cells was significantly notified in the cells incubated with avicequinone B at 2-4 µM compared with non-treated control. Anchorage-independent 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 µM of avicequinone B for 14 days. Data represent the means ± SD (n = 3). *p ≤ 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 contianed 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 form this study lightened the attempts to clarified 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.

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Appendix

ประวัติผู้วิจัย/อาจารย์ที่ปรึกษา

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จุฬาลงกรณ์มหาวิทยาลัย	วิทยาศาสตร์ดุษฎีบัณฑิต	เทคโนโลยีเภสัชกรรม (นานาชาติ)	2556

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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.

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จุฬาลงกรณ์มหาวิทยาลัย	วิทยาศาสตร์ดุษฎีบัณฑิต	เทคโนโลยีเภสัชกรรม (นานาชาติ)	2549
Meiji Pharmaceutical University	Post-doctoral training	Biotechnology	2550

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

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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 squarrosuls*.

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 IC_{50} 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 anticancer use.

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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 programed 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

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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 squrrosulus* 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), dimethysulphoxide (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 12,000 *g*, 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 12,000 *g*, 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 stepwise 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 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.

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

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 μg/μL.

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

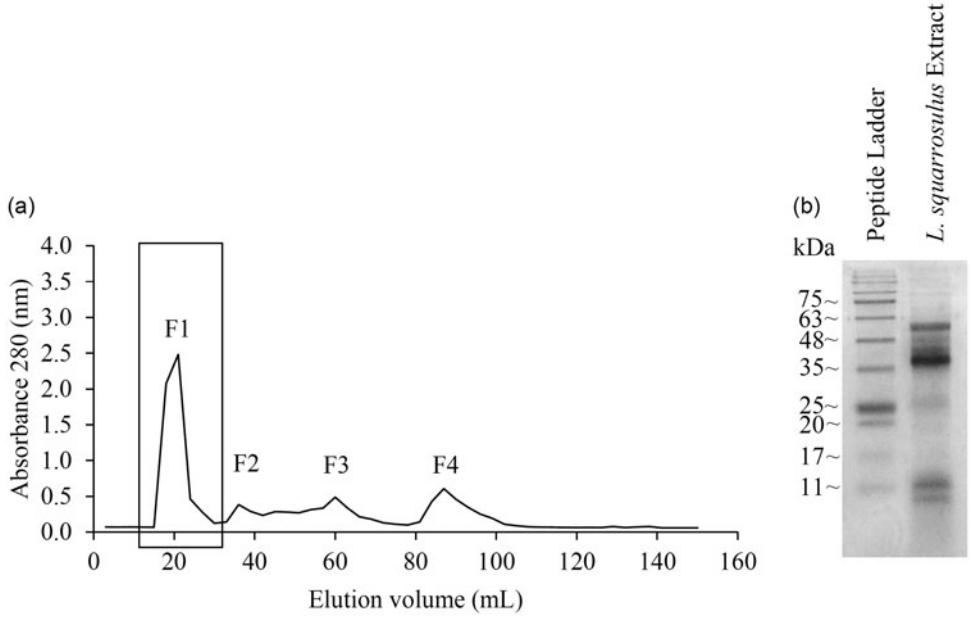

Figure 2. Isolated peptide extracts from *Lentinus squarrosulus*. (a) Peptide elution profile obtained from Sephadex G-25 gel filtration chromatography. The rectangular box indicates the highest peptide containing fraction (F1) which was used for further evaluation. (b) SDS-PAGE of F1 fraction of gel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

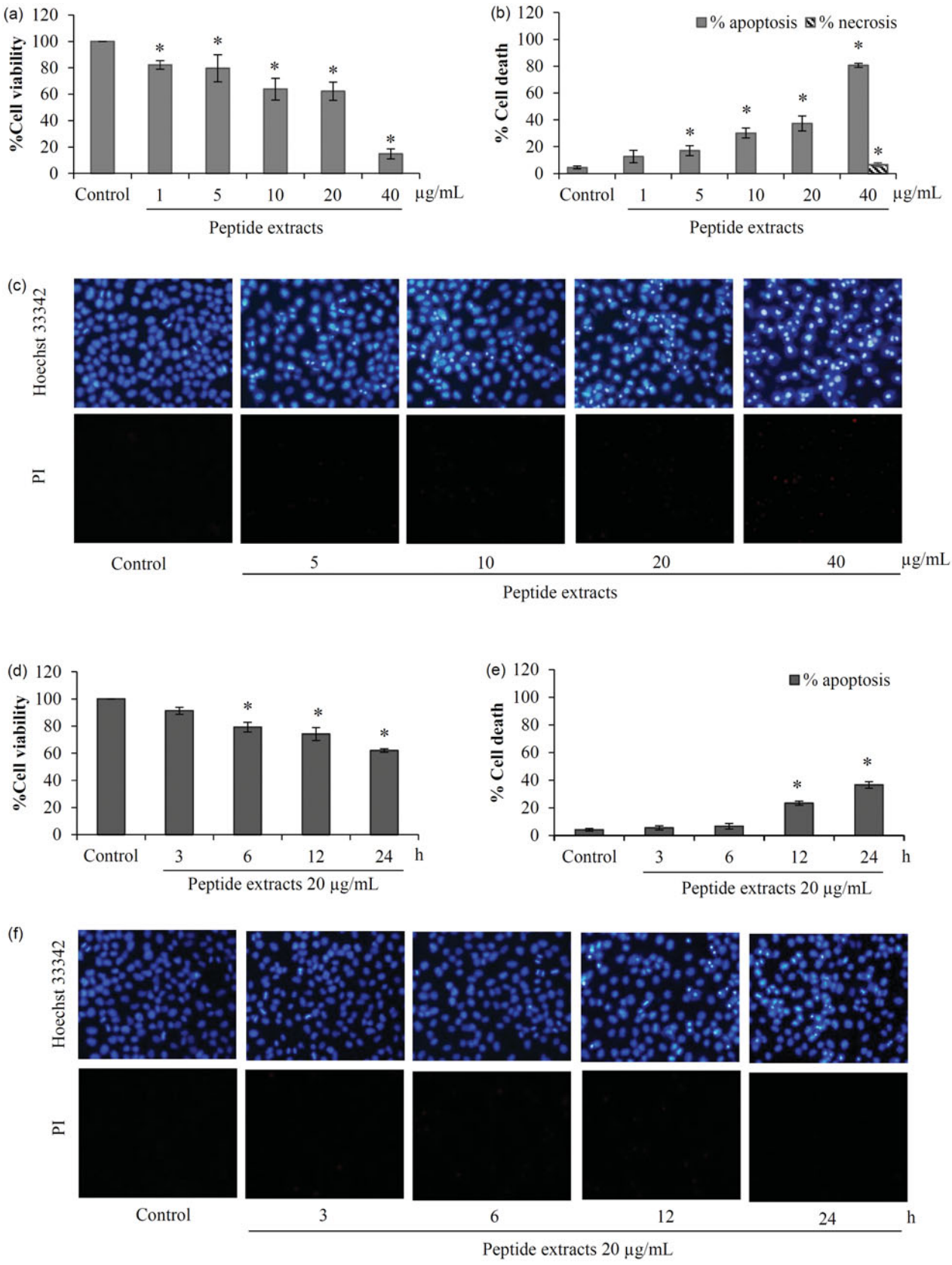

Figure 3. Apoptosis induction of peptide extracts from *Lentinus squarrosulus*. Cell viability of H460 lung cancer cells was evaluated after treatment with the extracts in (a) dose-dependent and (d) time-dependent manner. (b) There was a significant augmentation of apoptotic cell death in the incubation 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 ± 0.004% w/w.

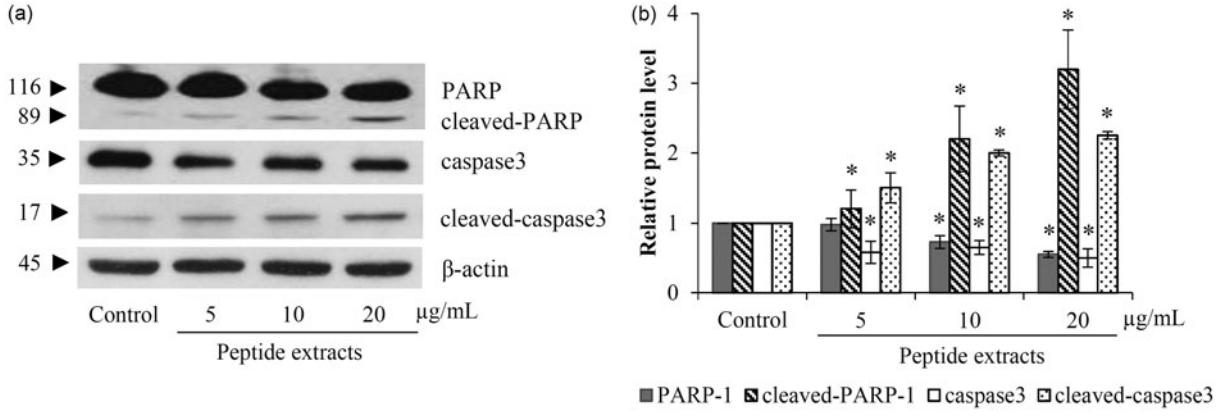

Figure 4. The apoptosis cells death was confirmed by the alteration of caspase-3 and PERP proteins. (a) Western blot analysis indicated an increase of active caspase-3 (cleaved- caspase-3) in peptide extracts-treated H460 cells. (b) As a substrate of activated caspase-3, the significant reduction of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

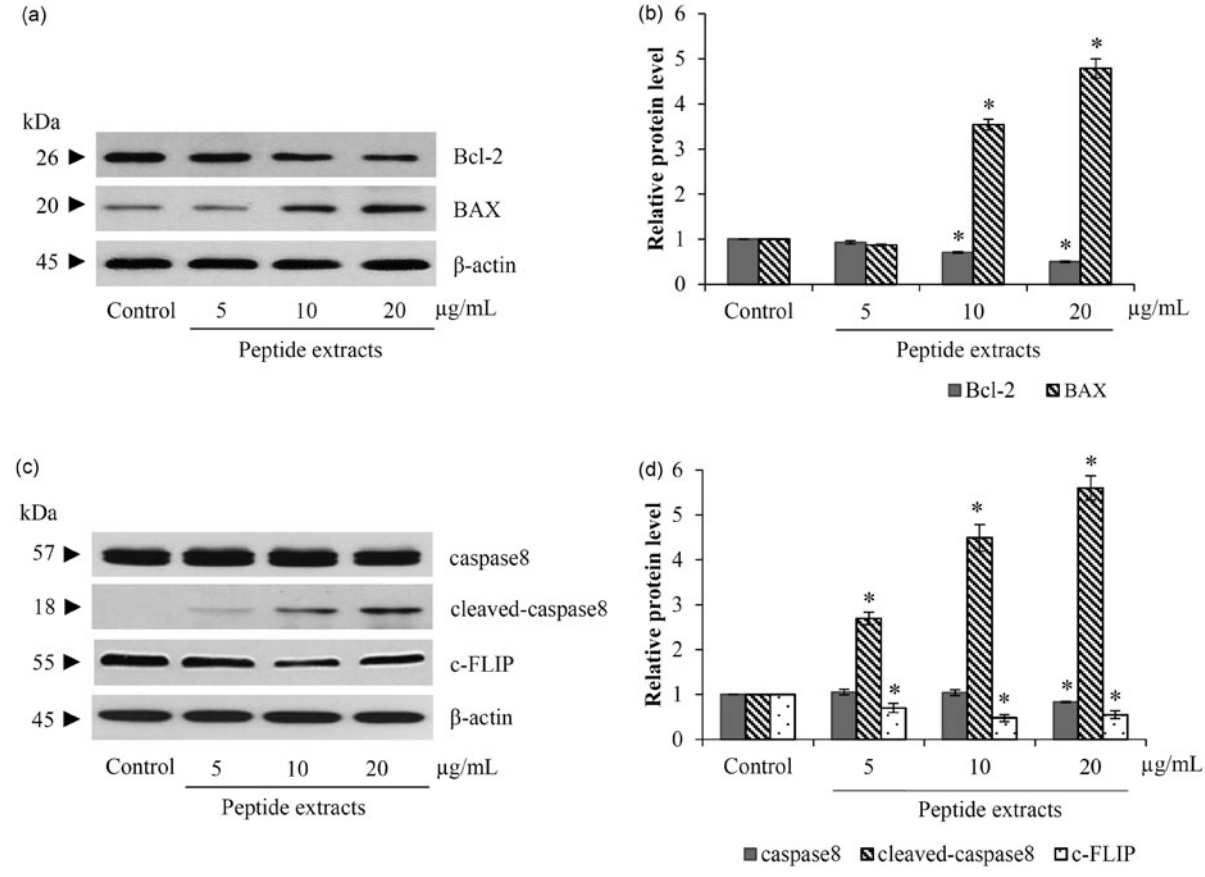

Figure 5. Peptide extracts stimulated both mitochondrial and death-receptor apoptotic pathway. (a and b) There were the reduction of Bcl-2 and up-regulation of BAX after treatment of lung cancer cells with 10–20 μg/mL of peptide extracts form *Lentinus squarrosulus*. (c and d) Protein-relating death-receptor pathway, c-FLIP and caspase-8 were decreased in peptide extracts-incubated H460 cells. Values are means of the independent triplicate experiments ± 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 Hoechse33342 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

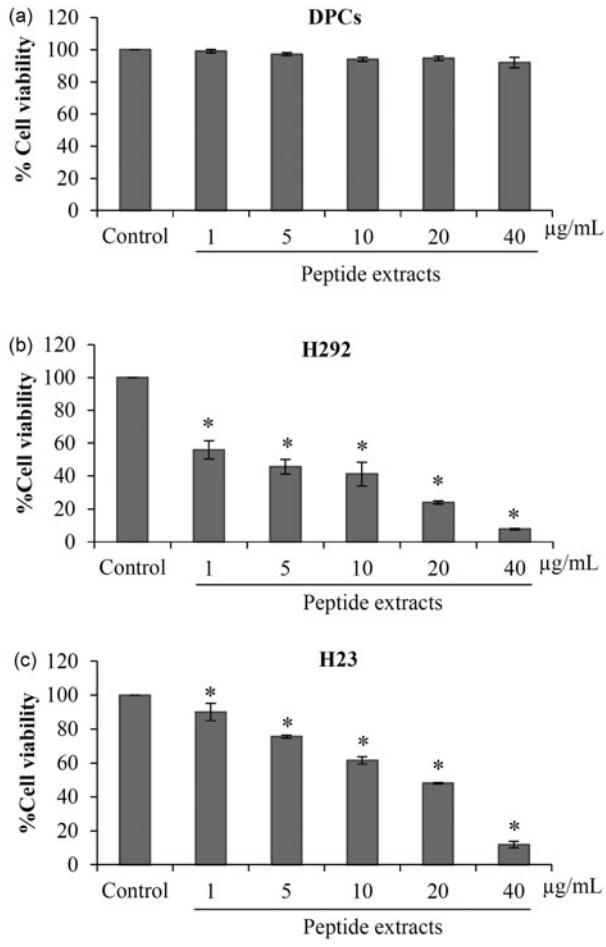

Figure 6. Selective anticancer activity of peptide extracts from *Lentinus squarrosulus*. (a) Low toxicity of the peptide extracts to non-cancer cells was indicated in human dermal papilla DPCs cells treated with 1–40 µg/mL peptides for 24 h. (b and c) Peptide extracts from *L. squarrosulus* induced 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 ± 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

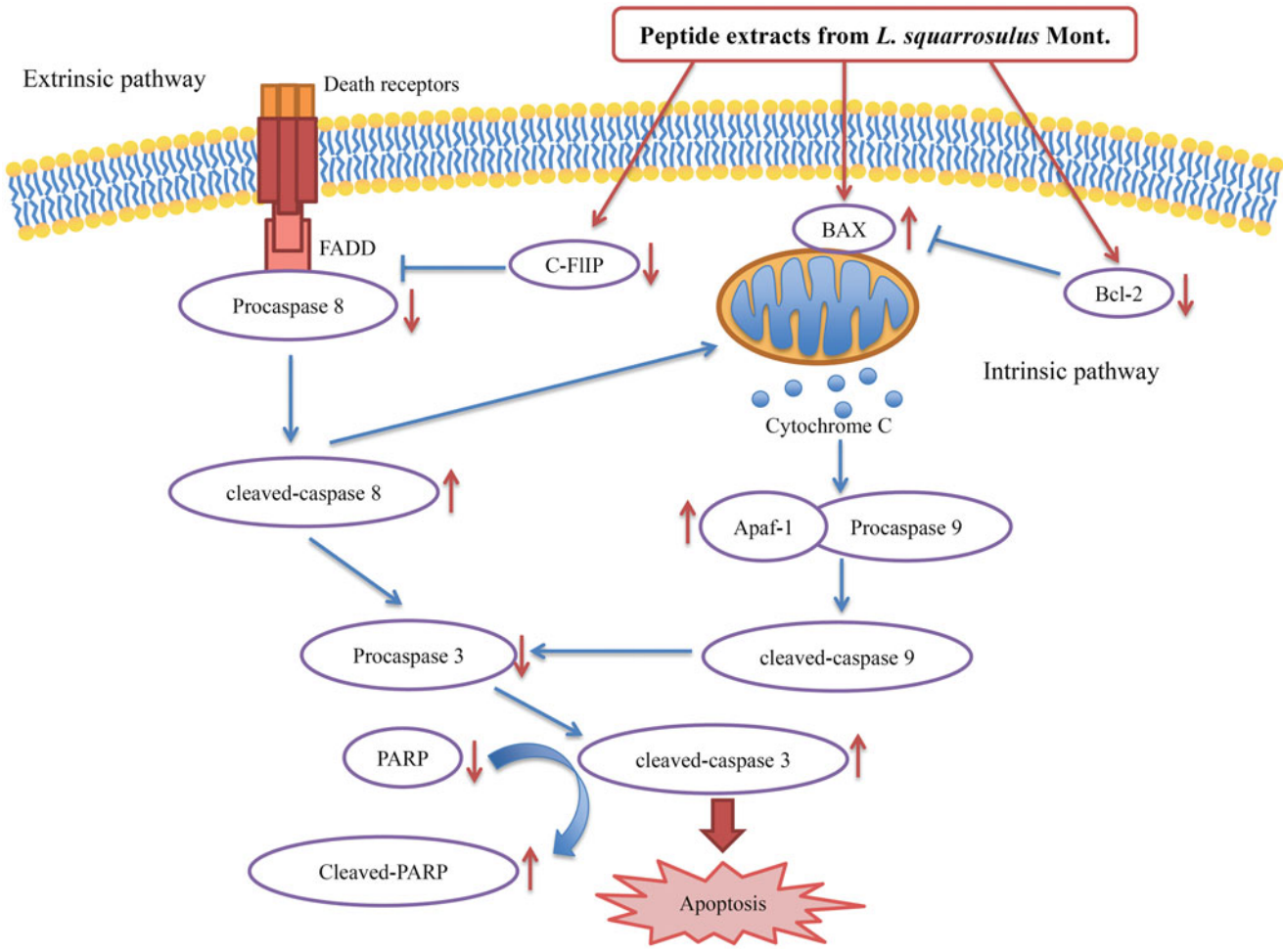

Figure 7. Proposed mechanistic scheme of anticancer activity of peptide extracts from *Lentinus squarrosulus* in human lung cancer cells. The extracts mediated a mitochondrial or intrinsic apoptotic pathway through the reduction of Bcl-2 and increase of BAX. Meanwhile, the extracts also decrease c-FLIP,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

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Avicequinone B sensitizes anoikis in human lung cancer cells

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Abstract

Background: During metastasis, cancer cells require anokis 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 μM 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-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Hoechst33342, propidium iodide (PI), DMSO (dimethysulfoxide) 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 β1, integrin β3, 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 (CH_2Cl_2, 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 (Br_2, 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%). ^{1}H-NMR ($CDCl_3$, 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); ^{13}C-NMR ($CDCl_3$, 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 $C_{12}H_6O_3Na^+$ 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% CO_2 at 37ºC. 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 37ºC 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 μM) and PI (5 μg/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 μM 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 μl). Annexin V-FITC (1 μg/ml) and PI (2.5 μg/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 µl 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 µl) 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 37ºC for 4 h, 250 µl 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 µM) 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 4ºC 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 µM 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 µM of avicequinone B (Fig. 2a). 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. 2c).

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 2d 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.

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. 3a). 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. 3c). Figure 3b shows the significant augmentation of anoikis in H460 cells after incubation with 4 µM 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 μM 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 μM). 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 μM of avicequinone B (Fig. 4b and 4c). Intriguingly, avicequinone B at 4 μM 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 μM significantly reduced the level of caveolin-1 in lung cancer cells detached for 12 h (Fig. 5a). Moreover, treatment with 2-4 μM of avicequinone B significantly declined anti-apoptotic Bcl-2 family proteins, Bcl-2

289 and Mcl-1 (Fig. 5b). It is worth noting that the expression of Bax, a pro-apoptosis protein
290 was not altered in response to the treatment of avicequinone B (1-4 μM) for 12 h (Fig. 5a
291 and b).
292
293 *Down-regulation of integrin mediated-survival signal by avicequinone B*
294 In order to proliferate under non-adherent condition, anoikis resistant cancer cells
295 substantially activate pro-survival pathways [9]. Thus, the alteration on integrin
296 mediated-survival signal was investigated in H460 cells cultured with avicequinone B.
297 Western blot analysis obviously revealed the down-regulation of integrin β1 and β3 in
298 human lung cancer cells treated with 4 μM of avicequinone B under detachment condition
299 (Fig. 5c). Interestingly, the reduction of integrin β3 was also notified at low
300 concentrations (1-2 μM) of avicequinone B. Figure 5d indicates the reduction of p-FAK
301 and p-Src, down-stream signaling molecules of integrins in H460 cells treated with
302 avicequinone B. The alteration on level of AKT and ERK protein, pro-survival molecules
303 activated by p-Src was further investigated [30]. As presented in figure 5e and f, the
304 diminution of activated ERK (p-ERK) was notified in detached H460 cells incubated with
305 avicequinone B (1-4 μM). Meanwhile there were no significant alteration of AKT and p-
306 AKT expression in avicequinone B treated-H460 cells compared with non-treated
307 control. This inhibitory effect on integrin/FAK/Src survival pathway corresponded with
308 sensitization to anoikis (Fig.3) and low colony formation (Fig. 4) in human lung cancer
309 cells exposed with avicequinone B.
310
311 *Avicequinone B sensitizes anoikis and suppresses anchorage-independent growth in*
312 *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 µM; 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 µM) in both H292 and H23 lung cancer cells compared with H460 cells which only responded to 4 µM 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 µM 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

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

342 Integrins are transmembrane molecules that regulate not only cell adhesion but
343 also various cellular signaling pathways [8]. The interaction between integrins and protein
344 components in ECM or specific ligands activate downstream FAK at focal adhesion
345 complex consequence with phosphorylation of pro-survival proteins. In spite of loss of
346 anchorage with ECM, highly metastatic cancer cells are able to generate integrin survival
347 signals resulting in resistance to anoikis [10, 12, 35]. The up-regulation of integrin β1 and
348 β3 influences with various aggressive behaviors in cancer cells [36, 37]. Recent study
349 revealed that the diminution of β1 and β3 integrins suppresses survival and growth under
350 detachment condition in human lung cancer cells [38]. Herein, suppression on integrin
351 survival pathway in lung cancer cells induced by avicequinone B was elucidated. There
352 was the reduction of integrin β1 and β3 level consequence with the restrain of downstream
353 signaling molecules, p-FAK and p-Src in non-adherent lung cancer cells treated with
354 avicequinone B.

355 The up-regulation of anti-apoptotic proteins and pro-survival signal has been
356 revealed in lung cancer cells with anoikis resistant phenotype [39]. Down-regulation of
357 caveolin-1, Mcl-1 and Bcl-2 as well as suppression on AKT and ERK activation
358 successfully initiate detachment-induced cell death in anoikis-resistant lung cancer cells
359 [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-b-(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: Dimethysulfoxide; 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

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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

Not applicable.

Consent for publication

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

Competing interests

The authors declare that they have no competing interests.

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551 **Figure legends**

552 **Figure 1** Synthetic scheme of avicequinone B

553

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

562

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

574

575 **Figure 4** Effect of avicequinone B on anchorage-independent growth. (a) Colony
576 formation of single-cell H460 cells was evaluated under a microscope after culture in soft
577 agar for 14 days. (b) Relative colony number and (c) relative colony size were gradually
578 decreased incubation of single-cell H460 with 1-4 μM of avicequinone B. Data represent
579 the means ± SD (n = 3). *$p \leq 0.05$ versus untreated control cells.
580
581 **Figure 5** Avicequinone B down-regulated anti-apoptosis proteins and pro-survival
582 signaling. (a) Western blot analysis revealed the reduction of anti-apoptosis proteins,
583 Mcl-1 and Bcl-2 in H460 cells treated with avicequinone B (1-4 μM) under detachment
584 condition for 12 h. (b) The decrease of caveolin-1 (Cav-1) was significantly notified in
585 non-adherent H460 cells incubated with avicequinone B at 4 μM. (c) Low expression
586 level of integrin β1 and β3 were demonstrated in avicequinone B-treated lung cancer cells.
587 (d) Downstream pro-survival signaling of integrin including p-FAK (Y397) associating
588 with p-Src (Try416) were also down-regulated after incubation of detached H460 cells
589 with avicequinone B (1-4 μM). (e) The reduction of p-ERK (Thr 981) was significantly
590 notified while (f) there was no alteration of p-AKT (Ser 473)/AKT in avicequinone-
591 treated lung cancer cells. Data represent the means ± SD (n = 3). *$p \leq 0.05$ versus
592 untreated control cells.
593
594 **Figure 6** Avicequinone B restrained survival under detachment condition in various lung
595 cancer cells. The lower viability of non-adherent (a) H292 and (b) H23 lung cancer cells
596 was significantly notified in the cells incubated with avicequinone B at 2-4 μM compared
597 with non-treated control. Anchorage-independent growth assay revealed the reduction of
598 both number and size of forming colonies in lung cancer (c) H292 and (d) H23 cells after

599 culture with 2-4 μM of avicequinone B for 14 days. Data represent the means ± SD (n =
600 3). *$p \leq 0.05$ versus untreated control cells at the same time point.
601
602 **Figure 7** Proposed mechanism of avicequinone B-induced anoikis in lung cancer cells.
603 Avicequinone B mediated anoikis through the reduction of anti-apoptosis proteins
604 caveloin-1, Mcl-1 and Bcl-2 as well as down-regulation of integrin/FAK/Src pro-survival
605 pathway.
606