CHAPTER 2

Materials and Methods

2.1 Reagents and chemicals

Name of reagents and chemicals used in this study experiments are shown in Appendix A. The detail preparation of the reagents or buffer used in the experiments is shown in Appendix C.

2.2 Preparation of alkaloids from *S. venosa*

The tuber part of *S. venosa* was obtained from Prachuapkhirikhan province of Thailand and identified by Forest Herbarium. A voucher specimen (BKF No. 140583) has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. The isolation and purification of alkaloids CN, OMBC, THP and NMTHP from the tuber of *S. venosa* were performed by Dr. Wilart Pompimon using silica gel column chromatography method at the Laboratory of Natural Products, Center for Innovation in Chemistry, Department of Chemistry, Faculty of Science, Lampang Rajabhat University.

2.3 Ovarian cancer cell lines and maintenance

SKOV3 ovarian carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). SKOV3 cells were derived from the ascetic fluid from patient with ovarian tumor and resistant to cis-platinum, adriamycin, diphtheria toxin and tumor necrosis factor.

A2780 ovarian carcinoma cell lines were purchased from Health Protection Agency Culture Collections (Salisbury, UK). A2780 cells were established from ovarian tumor tissue from an untreated patient. Cisplatin resistance (A2780/cis) ovarian carcinoma cell lines were purchased from European Collection of Authenticated Cell Cultures (ECACC) operated by Public Health England. This cisplatin-resistant cell line had been developed

by addition of increasing concentrations of cisplatin to the parent cisplatin-sensitive A2780 cell line. In order to maintain resistance, cisplatin has to be exposed to the media every 2-3 passages.

These cell lines were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 5 mM L-glutamine, 50 μg/mL penicillin and 50 μg/mL streptomycin. The cell lines were kept in a humidified incubator with 5% CO_2 at 37°C. When the cells reached 70–80% confluence, they were collected and used for subsequent investigation.

2.4 Patients and samples

Solid tumor tissues were collected from consented patients undergoing surgery for ovarian cancer at the Department of Gynecologic Oncology, Division of Surgery, Maharaj Nakorn Chiang Mai Hospital, Thailand. This study was approved by Chiang Mai University Ethics Committee for Human Research (Ethic number 194/2556). Clinical details were documented and specimens were handled according to the Human Tissue Act. All patients were found to be chemo naïve and the effect of chemotherapy was evaluated after six cycles of first-line chemotherapy. Platinum resistance was defined as patients who showed progression or relapsed of cancer during the first-line platinum chemotherapy treatment or within 6 months after treatment. Complete or partial response were regarded as responsive to chemotherapy. Stable or progressive disease were considered as non-responsive**.**

2.5 Primary cell culture from solid tumor tissues of ovarian cancer patients

Solid tumor tissue collected at surgery was confirmed by a pathologist from the Department of Pathology. Solid tumor tissue sample was transported by placing into a sterile conical tubes containing iced-cold DMEM in a bucket of ice. In the laboratory, the solid tumors were cut into 2 mm3 pieces with a sterile scalpel and then placed in a Petridish containing 5 ml of DMEM with 20% streptomycin/penicillin. After that, the tissue samples were put into an enzyme mixture (collagenase A 0.15 U/mL and dispase II 2.4 U/mL diluted in DMEM) and incubated for 60 minutes at 37°C, in order to gain enzymatic digestion. For mechanical digestion, the tissue and enzyme mixture were vortexed at 10-minute intervals. Then, the cell filtrate was centrifuged at 100 × g for 7

minutes. The culture flasks were coated with type-1 collagen coating matrix (Invitrogen, CA) in order to augment the cell attachment to the flasks. After resuspension of the cells in DMEM containing 20% FBS, samples were assessed for cell viability. The medium was changed 24 hours after first plating and every two to three days for the following two weeks. When 80% confluency was attained, subculturing was performed by using 0.25% trypsin. The experiments were performed between passages 4 and 9.

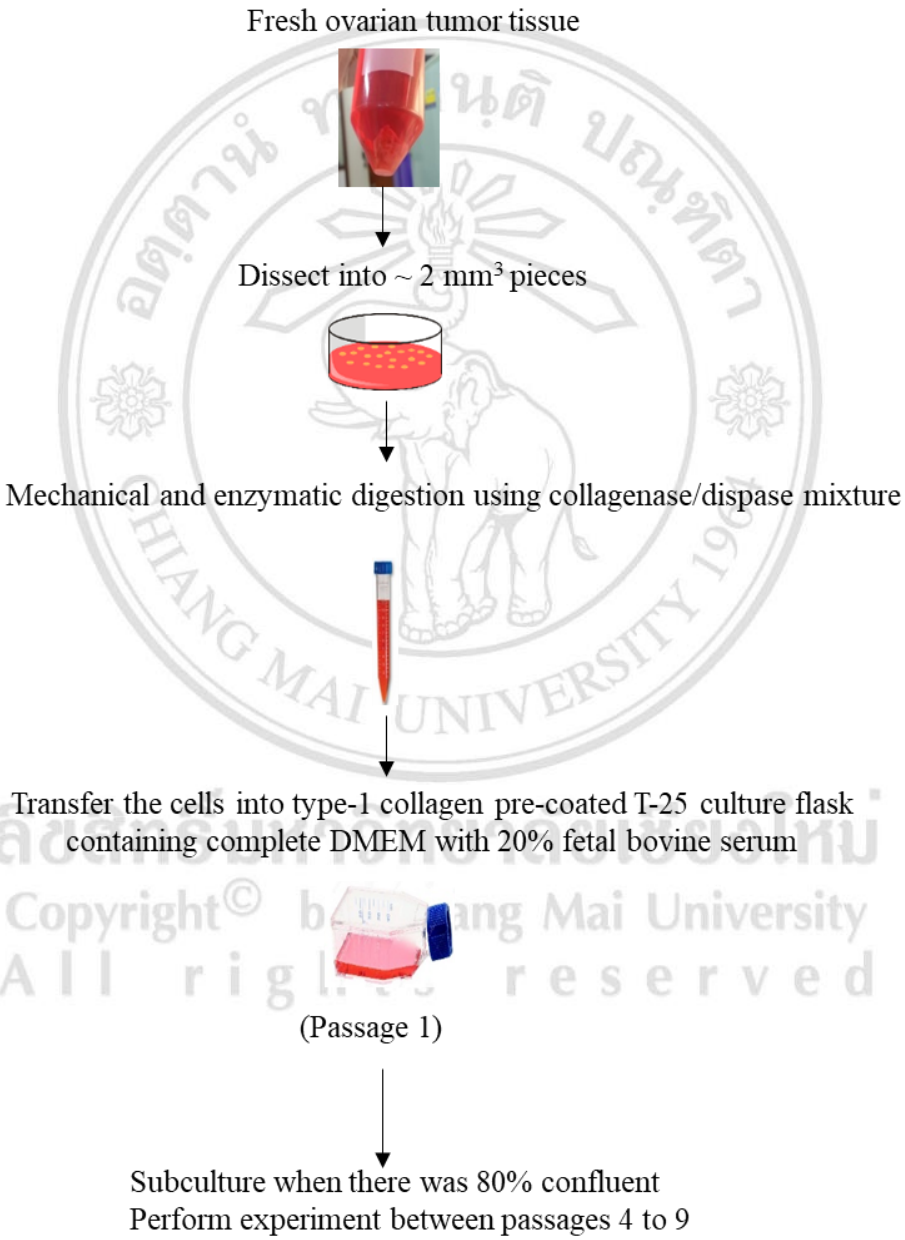

Figure 2.1 Steps involved in establishing primary cell culture from solid tumor tissues of ovarian carcinoma patients

2.6 Treatment with alkaloids and drugs

For experiment, crystal forms of CN, OMBC, THP and NMTHP were dissolved in DMSO at concentration 40 mM as a stock solution which was used within one month. Cisplatin was dissolved using normal saline at concentration 3 mM as a stock solution. For cell viability assay, the cells were treated with alkaloids 0-200 μM concentrations for 48 hours or various concentrations of cisplatin for 72 hours.

For apoptotic assay, non-toxic concentrations of alkaloids were used and the cells were pretreated with non-toxic concentration of CN or OMBC (20, 40, 60 μM) for 2 hours, followed by treatment with cisplatin (7.5 μM) for 36 hours.

For molecular signaling study, the cells were treated with cisplatin (7.5 μM) for various time of incubation (0, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours) or pretreated with CN or OMBC for 2 hours followed by cisplatin treatment (7.5 μM) for 30 minutes.

2.7 Measurement parameter

2.7.1 MTT cell viability assay

Ovarian cancer cells (3 x10^3cells/well) were seeded in a 96-well plates and kept at 37 °C, 5% CO_2 overnight in RPMI containing 10% FBS. After indicated time point of different treatment, the supernatant was discarded and MTT dye was added and incubated for an additional 4 h. The precipitated formazan crystals were dissolved in DMSO and optical density was measured at 540 nm with a reference wavelength of 630 nm using a microplate reader. Percent cell survival was calculated by using the following formula:

$$\% \text{ cell survival} = \frac{\text{OD of control cells} - \text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

2.7.2 Combination index analysis

Combination index (CI) was analyzed for evaluating the character of drug interactions in combination chemotherapy. In an attempt to assess potential drug interactions, the Chou-Talalay median effect principle was applied [72]. CI is a numerical value obtained from calculation by using the following formula:

$$CI = \frac{D1}{D1x} + \frac{D2}{D2x}$$

D1 and D2 in the numerator denote the concentrations of compounds 1 and 2 in combination that are used to reach x% inhibition. D1x and D2x in the denominator represent the concentrations of compounds 1 and 2 which display x% inhibition when present separately. CI value less than 1 exhibits a synergistic effect, CI value equal to 1 represents an additive effect, and CI value more than 1indicates an antagonistic effect.

2.7.3 Guava apoptotic assay

Cell apoptosis were evaluated with flow cytometry assay by using guava nexin reagent (Merck Millipore Corporation, USA). The assay depends on an event related with the onset of apoptosis in which the translocation of phosphatidylserine (PS) to the external membrane of apoptotic cells was detected by using two dyes in this assay: 1) annexin V FITC to identify PS on the external membrane of apoptotic cells and 2) a cell impermeant dye 7-aminoactinomycin D (7-ADD), which indicates structural integrity of the membrane. Annexin V binds with high affinity to phosphatidylserine which is a membrane phospholipid normally localized to the inner face of the cell membrane. During early apoptotic process, phosphatidylserine are translocated to the external surface of the cell membrane favoring to bind easily with Annexin V. Although 7-AAD is able to exclude from healthy cells and early apoptotic cells, it cannot exclude from the late-stage apoptotic and dead cells. Briefly, ovarian cancer cells (2 $x10^5$ cells/well) were seeded in a 6-well plates and incubated at 37 °C, 5% CO_2 overnight in RPMI containing 10% FBS. The cells were treated with platinum drugs with or without alkaloids for appropriate time to induce apoptosis. After that, cells were washed, suspended in binding buffer, and incubated for 20 minutes with annexin V-FITC (Merck Millipore Corporation,

USA) at room temperature in the dark. Measurement of fluorescence was done by using Guava easyCyte HT flow cytometer (Merck Millipore Corporation, USA) with a minimum of 5,000 events per sample. The early apoptotic cells with Annexin V positive only and late apoptotic cells which showed both Annexin V and 7-AAD positive were computed.

2.7.4 Determination of IL-6 by ELISA assay

The level of IL-6 upon exposure to cisplatin with or without alkaloids in ovarian cancer cells was identified by sandwich ELISA assay (Biolegend, San Diego, USA). Ovarian cancer cells were seeded, incubated overnight and treated with CN or OMBC and cisplatin for 48 hours. Then, the supernatant were collected to determine the production of IL-6 by the sandwich ELISA assay which determines the amount of antigen between capture antibody and detection antibody. This assay is based on the capture of IL-6 by specific IL-6 monoclonal antibody (first antibody) immobilized on a 96-well microtiter plate. After unbound materials had been washed out, biotinylated anti-human IL-6 detection antibody (second antibody) were placed creating an antibody-antigen-antibody 'sandwich'. Avidin-horseradish peroxidase was subsequently added, followed by tetramethylbenzidine (TMB) substrate solution, showing a blue color which was proportionate to the amount of IL-6 present in the sample. Finally, the color development was stopped with the addition of stop solution and the optical density of the calibrators and specimens were measured at 450 nm with a microplate reader.

2.7.5 Tumor cell invasion assay

A Transwell system (24 wells, 8 mm pore size with polycarbonate membrane; Corning Costar, Lowell, MA, USA) coated with 200 μg/mL Matrigel (BD Biosciences, MA, USA) was used to determine tumor cell invasion. First, 2.0×10^5 cells were seeded in an upper Transwell chamber in 900 μL medium containing 0.5% FBS, +/- 50 ng/mL IL-6 and +/- CN. The lower chamber contained 600 μL medium complemented with 10% FBS. The cells were cultivated for 24 hours and at the end of experiment, cells residual on the upper chamber were scrubbed with a cotton swab, whereas cells that had passed into the lower surface were fixed with ethanol for 5 minutes and stained with 0.05% crystal violet for

30 minutes. The number of cells that invaded into the lower surface was analyzed statistically by Image J software.

2.7.6 Colony formation assay

SKOV-3 cells were harvested at 300 cells/well on 12-well plates for overnight. The attached cells were treated with IL-6 (50 ng/mL) and/or CN for 48 hours. Following incubation, the treated media were removed and fresh media were added every 3 days. Cells were seeded for 10 days until visible colonies were formed. The cells were fixed with ethanol and stained with 0.05% crystal violet for 30 min. The plates were washed 3 times with tap water and air-dried. The number of colonies was analyzed statistically by TotalLab Quant software.

2.7.7 Gelatin zymography

The expression status of MMPs in cell culture medium was determined by gelatin zymography. SKOV3 cells were sowed in 12-well plates overnight and then cultured in medium containing anti-IL-6 antibodies (15 μg/mL) with or without CN. After 24 hours, the cell culture supernatants were collected for assay procedure. For loading sample preparation, equal amount of sample volume was mixed with 4X SDS sample buffer without heating nor adding reducing agents, and then loaded onto an SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. Electrophoresis was run to separate the proteins. Following electrophoresis, the gel was washed with 2.5% Triton X-100 to eliminate SDS, and incubated with gelatinase buffer (50 mM Tris-HCl buffer, pH 7.4, and 10 mM $CaCl_2$) for overnight at 37˚C. Then, staining of the gels were performed by treating with 0.1% Coomassie Brilliant Blue R-250 for 4 hours. After that, the gels were washed with destaining solution until the appearance of clear bands against an intensely stained background.

2.7.8 Extraction of whole cell proteins and nuclear proteins

For extraction of whole cell proteins, the cultured cells were washed with iced-cold PBS and the cell pellet was retained by centrifugation at 13,000 × g, 4ºC for 10 minutes. Then the cell pellet was lysed with RIPA buffer containing protease inhibitors for 15 minutes on ice. The insoluble matter was removed by centrifugation for 13,000 × g, 10 minutes at 4 °C and the supernatant was then collected.

The nuclear lysate was prepared for determination of the translocation of NF-κB from cytosol to the nucleus. Ovarian cancer cells were cultured and treated with the alkaloids at indicated concentration and time period. For preparation of the nuclear extract fraction, the treated cells were collected and washed twice with iced-cold PBS. The cell pellet was suspended with 400 µl of lysis buffer (10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KC1, 0.5 mM PMSF, 1 mM DTT, 1 µg/mL leupeptin, 1 µg/mL aprotinin). After allowing the cells to swell on ice for about 20 min, 5 µl of 10 % of Nonidet P-40 was added. The tubes were vortexed for 15 s and centrifuged at 13,000 × g for 1 min. The supernatant represents the cytoplasmic extract. The nuclear pellets were suspended in iced-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin) with intermittent vortex for 30 minutes. The nuclear extract was centrifuged at 17,800 × g for 12 minutes, and the supernatant was collected for determination of the nuclear proteins. Determination of protein concentrations were assessed by the Bradford method.

2.7.9 Western blotting

For loading sample preparation, equal amount of sample volume was mixed with 4X loafing buffer and heat at 95°C for 5 minutes. Then, the samples were separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane by electroblotting. Membranes were incubated with specific primary antibody in 5% nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C overnight and then exposed to peroxidase-conjugated appropriate secondary antibody. Finally, antibody binding was visualized by treating with an enhanced chemiluminescence detecting substrate (Thermoscientific, USA). To confirm equal

volume of protein loading, the membrane was stripped and re-probed with anti-β-actin. The band density was analyzed by using ImageJ software.

2.7.10 Statistical analysis

All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, USA). Data were presented as mean ± standard deviation (SD) of three independent experiments. Statistical analyses were calculated by one-way ANOVA and Student's *t*-test. Association between *ex-vivo* IL-6 levels and clinicopathological parameters was assessed by Chi Square test. A difference between the test groups was considered statistically significant when the *p* value was <0.05.