CHAPTER III
MATERIALS AND METHODS

Materials

1. Chemicals and reagents

Trypsin, Hoechst 33342, 2,3-b-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethysulfoxide (DMSO) and Polyhydroxyl- ethylmethacrylate (poly-HEMA) and agarose are obtained from Sigma Chemical, Inc. (St. Louis, MO, USA). Bcl-2, Mcl-1, Cav-1, pAkt (Ser 473), Akt and β-actin rabbit antibody are obtained from Cell Signaling Technology, Inc. (Danver, USA). Bax-HRP rabbit antibody is obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, USA).

2. Equipments

Fluorescence microplate reader (SpectraMax® M5, Molecular Devices, CA, United States), Fluorescence microscope (Olympus IX51 with DP70), Automated cell counter (Bio-Rad), Flow cytometer (FACSort, Becton Dickinson, Rutherford, NJ, USA), Autopipette: 0.2-2 µl, 2-20 µl, 20-200 µl and 100-1,000 µl, Pipet tips: 2-10 µl,10-100 µl, 20-200 µl and 200-1000 µl, Cell culture plate: 6-well ultralow-attached plate, 24-well plate and 96-well plate (Costar), Conical tube: tube 15 ml and 50 ml (Corning), Centrifuge, Laminar flow cabinet, humidified incubator, pH meter, Vortex mixer, Balance, Blotting cell and membranes

Methods

1. DF-A preparation

Dendrofalconerol A (>98%) was isolated from the aerial parts of *Dendrobium falconeri*. Briefly, the dried stems of this plant (800 g) were powdered and extracted with MeOH at room temperature. The MeOH extract was filtered and evaporated under reduced pressure to give a viscous mass (73 g). This material was subjected to vacuum-liquid chromatography on silica gel (EtOAc-hexane gradient) to give fractions A-K. Fraction I (1.51 g) was separated by CC (silica gel; EtOAc-haxane, gradient and EtOAc-hexane, 1:4) to give 35 fractions. Fraction 25 (216 mg) was further purified by Sephadex LH20 (MeOH) to give dendrofalconerol A (29 mg). The structure of DF-A was determined through analysis of its spectroscopic data (32).

2. Cells culture

Non-small cell lung cancer H460 and keratinocyte HaCat cells used in this study were obtained from American Type Culture Collection (Manassas, VA). H460 cells were cultured in RPMI medium, while HaCat cells were cultured in DMEM medium. All media were supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin. Cells were maintained in a humidified incubator containing 5%CO_2 at 37^{0}C.

3. DF-A treatment

DF-A was dissolved in DMSO, and diluted with sterile PBS to achieve the working concentrations. The percentage of DMSO in working solution was less than 0.1 which showed no toxic in H460 cell.

4. Cell viability assay

To determine DF-A-mediated cytotoxicity, cell viability is determined by MTT assay which measures cellular capacity to reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to purple formazan crystal by mitochondria dehydrogenase enzyme. After treatment, the medium is replaced by 100 µl/well of MTT solution (0.4 mg/ml) and the cells are incubates for 4 h at 37 °C. After that, MTT solution is removed and a 100 µl/well of DMSO is added to dissolve the formazan crystal. At the end of the assay period, the intensity is measured at 570 nm using microplate reader (SpectraMax® M5, Molecular Devices, California, United States). All analyses are established in at least three independent replicate cultures. The cell viability is calculated from optical density (OD) ratio of treated to non-treated control cells and is presented as a percentage that of the non-treated controls.

$$\text{Cell viability (\%)} = \frac{\text{OD570 of treated cells}}{\text{OD570 of control cells}} \times 100$$

5. Anoikis assay

To determine the ability of DF-A on detached cells. H460 cells in the culture plate are detached into a single-cell suspension in RPMI-serum free medium and then seeded into a poly-HEMA coated plate at a density of 1.5×10^5 cells/ml. Cells are then harvested at 0, 6, 9, 12, and 24 h. Cells are then incubated with 20 µM of MTT reagent for a further 4 h at 37°C. The intensity of the formazan product is measured at 450 nm using a microplate reader. All analyzes are performed in

triplicate. The percentage of viable cells that is estimated by comparing with the untreated control cells in time- and concentration dependent manner.

$$\text{Cell viability (\%)} = \frac{\text{OD570 of treated cells}}{\text{OD570 of control cells}} \times 100$$

6. Nuclear staining assay

Apoptotic and necrotic cell death is determined by Hoechst 33342/propidium iodide (PI) co-staining. Then, cells are incubated with 10 μM of the Hoechst 33342 dye and 5 μg/mL of the PI dye for 30 min at 37°C in the dark. Finally, cells are visualized under a fluorescence microscope (Olympus IX51 with DP70). The blue fluorescent Hoechst dye detects apoptotic cells while the red fluorescent propidium iodide detects necrotic cells. At the end of the assay period, cells are visualized under a fluorescence microscope. All analyzes are performed in triplicate. The percentage of Apoptotic cells is estimated by comparing with the untreated cells and calculated as follow

$$\text{Apoptotic cell (\%)} = \frac{\text{Apoptotic cells}}{\text{Total cell number}} \times 100$$

$$\text{Necrotic cell (\%)} = \frac{\text{Necrotic cells}}{\text{Total cell number}} \times 100$$

7. Analysis of cell cycle by flow cytometry

Cell cycle analysis is performed by flow cytometry to determine sub-G_0 fraction. Briefly, suspended cells are treated with various concentrations of DF-A in RPMI serum free medium for 24 h at 37°C. Then, cells are collected after

centrifugation, re-suspended and fixed with cool absolute EtOH, and then kept it for 24 h at -20°C. Lastly, pellets are incubated with propidium iodide (PI) buffer (10X PBS, 1 mg/ml RNase A, 100X Triton, 0.5 mM EDTA, 10 mg/ml PI) for 30 min at 37°C under dark condition. After that, the pellets are immediately analyzed using FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA). All analyzes are performed in triplicate. The relative sub-G_0 fraction of treated cells is estimated by comparing with the untreated control cells

8. Anchorage-independent growth assay

Anchorage-independent growth assay is performed in two-layer soft agar. Briefly, aliquot of melted 1% agarose (1 g of agarose in 100 ml of sterile water) and cool in 55°C water bath before use. To make the lower layer, an equal volume of melted 1% agarose (55°C) and complete RPMI medium (1:1) are mix to give 0.5% agarose and 0.5% complete RPMI medium. Then, the mixture is put in 24-wells plate at 500 µl/well and allowed solidify at 4°C for 5 min. To prepare the upper layer (0.33% agarose), melted 1% agarose (55°C) is mixed with complete RPMI medium containing trypsinized adherent cells at a density of 1500 cells/well under various concentrations. Following that, it is added to the lower layer at 250 µl/well and allowed to gel at room temperature before placing in incubator at 37°C for 2 weeks. Fresh complete RPMI medium (250 µl/well) is added every 3 days over the upper layer. After 2 weeks of growth colonies are photographed in order to count the colonies and measured the colony size. All analyzes are performed in triplicate. The percentage of colony number is estimated by comparing with the untreated control cells and calculated as follow

$$\text{Colony number (\%)} = \frac{\text{Colony number of treated cells}}{\text{Colony number of control cells}} \times 100$$

For the percentage of colony size is estimated by comparing with the untreated control cells and calculated as follow

$$\text{Colony size (\%)} = \frac{\text{Diameter of treated cells}}{\text{Diameter of control cells}} \times 100$$

9. Westren blot analysis

Cells are harvested and lysed on ice for 60 min. After the sample had been prepared, the protein content of cell lysate is determined using BSA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein of each sample is separated by size using SDS-PAGE and then transferred into nitrocellulose membranes. The membrane is blocked in 5% skim milk in TBST (25 mmol/l Tris-HCL, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature, and then probe with appropriate primary antibodies at 4°C overnight and washed three times with TBST for 8 min. After that, the membrane is incubated with horseradish peroxidase-conjugated secondary antibodies according to the primary antibodies for 2 h at room temperature. Lastly, the signal of immunoreactive proteins is detected by enhance chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA). Protein expression (Bcl-2, Mcl-1, Bax, Cav-1, Akt, pAkt,) is investigated and ß-actin is used as a loading of control in each treatments. The immune complexes are detected by enhanced with chemiluminescence substrate (Supersignal West Pico; Pierce) and quantified using analyst/PC densitometry software (Bio-Rad) normalized to the level of ß-actin protein. All analyzes are performed in triplicate.

10. Cell proliferation assay

Cells were seeded at a density of $5x10^3$ cells/well in a 96-well plate overnight. Cells were incubated with various non-toxic concentrations of DF-A for 24, 48, or 72 h. Cell proliferation was determined by MTT assay and calculated from optical density (OD) ratio of treated group to non-treated control group at the indicated time using the following formula;

$$\text{Cell viability (\%)} = \frac{\text{OD570 of treated cells}}{\text{OD570 of control cells}} \times 100$$

11. Wound healing assay

Cells were seeded at a density of $3.5x10^4$ cells/well. After the cell reach 90% confluence, a micropipette tip was used to scratch the cells to generate wound space. After scratching, the cells were gently washed with PBS and replenish with fresh RPMI supplement with 10%FBS containing indicated non-toxic concentration of DF-A and allowed to migrate for 12, 24 or 48 h. The progress of cell migration into the wound was photographed by inverted microscope (Olympus IX51 with DP70, Melville, NY) at indicated times. The average wound space was calculated from the random field of view and represent as the relative cell migration. Relative cell migration was calculated by dividing the change of wound space of treated cells by that of the untreated cells in each experiment using the following formula;

$$\text{Migration level} = \frac{\text{(average space at time 0 h) - (average space at specific time)}}{\text{(average space at time 0 h)}}$$

$$\text{Relative migration} = \frac{\text{migration level of treatment}}{\text{migration level of control}}$$

12. Drug sensitivity assay

To determine the effect of DF-A on susceptibility of the cells to cisplatin and etoposide. Cells were seeded at a density of 7×10^3 cells/well onto 96-well plate overnight. After that, cells were pre-treated or left untreated with non-toxic concentrations of DF-A for 24 h and treated with 100 μM cisplatin (IC_{50} =100 μM) or 100 μM etoposide (IC_{70} =100 μM) . After 24 h, cell viability was investigated by MTT assay. The percentage of viable cells that is estimated by comparing with the either cisplatin- or etoposide-treated cells using the following formula;

$$\text{Cell viability (\%)} = \frac{\text{OD570 of treated cells}}{\text{OD570 of control cells}} \times 100$$

13. Statistical Analysis

Mean data from at least three independent experiments are normalized to result in the non-treated control. Statistical differences between means are determined using an analysis of variance (ANOVA) and post hoc test at a significance level of $p < 0.05$, and presented as the mean ± SE.

14. Experimental designs

14.1 Conceptual Framework

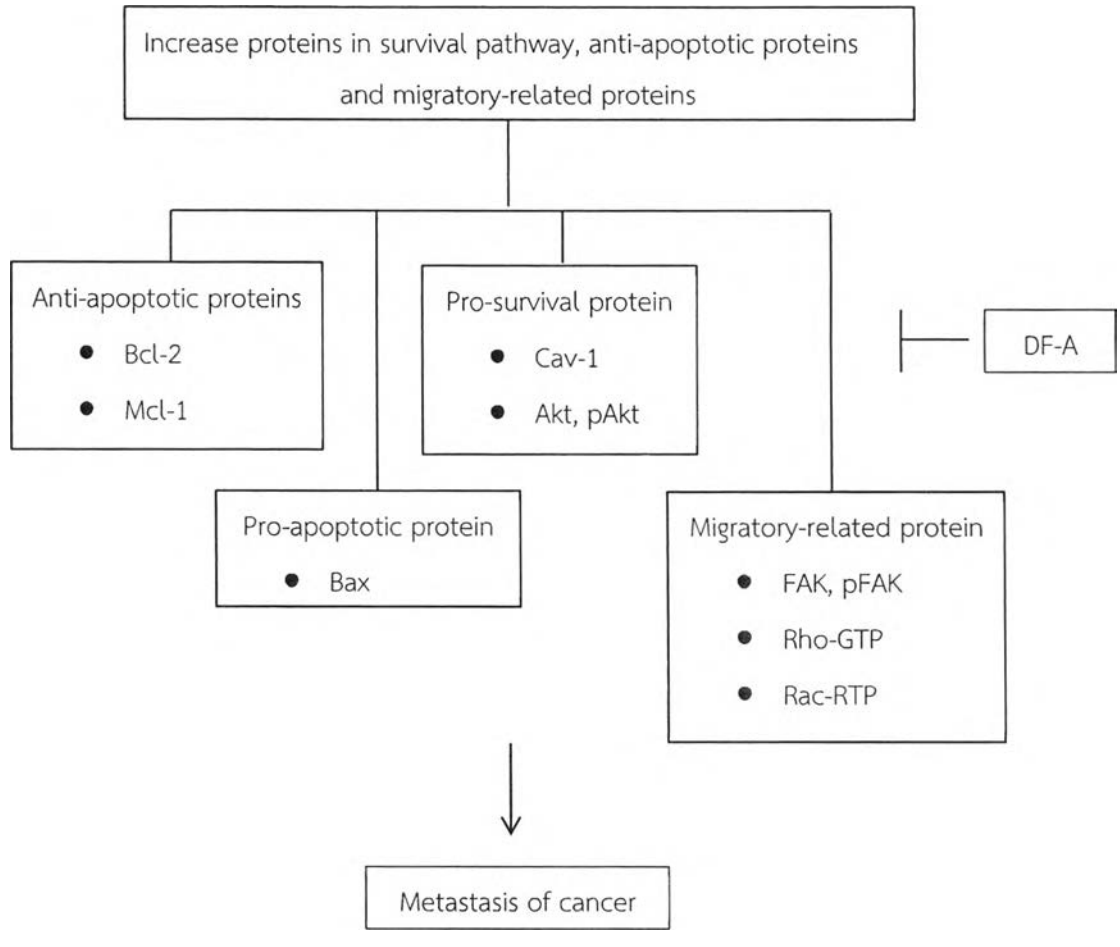
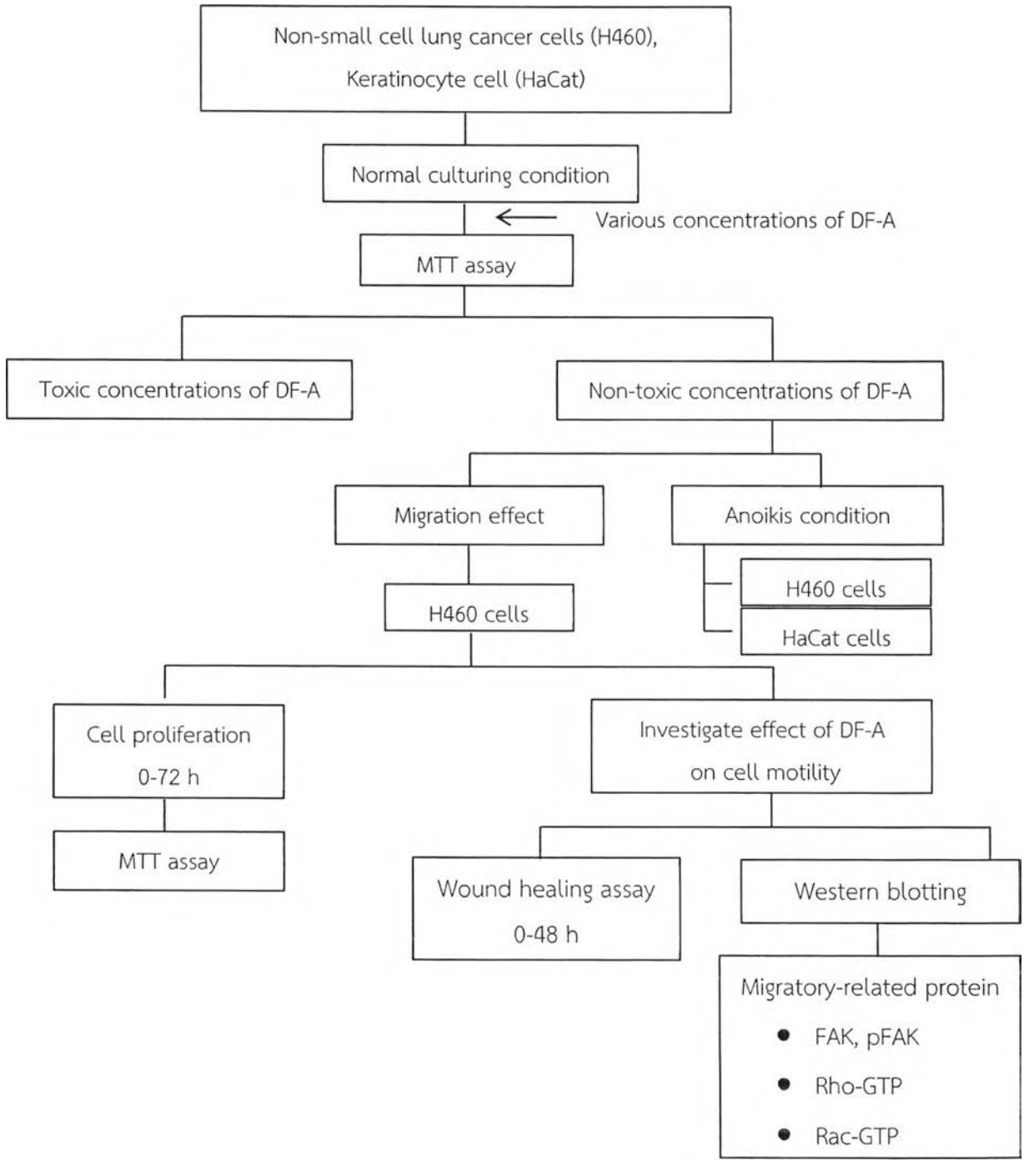


Figure 17 Conceptual framework

14.2 Experimental designs

Part 1



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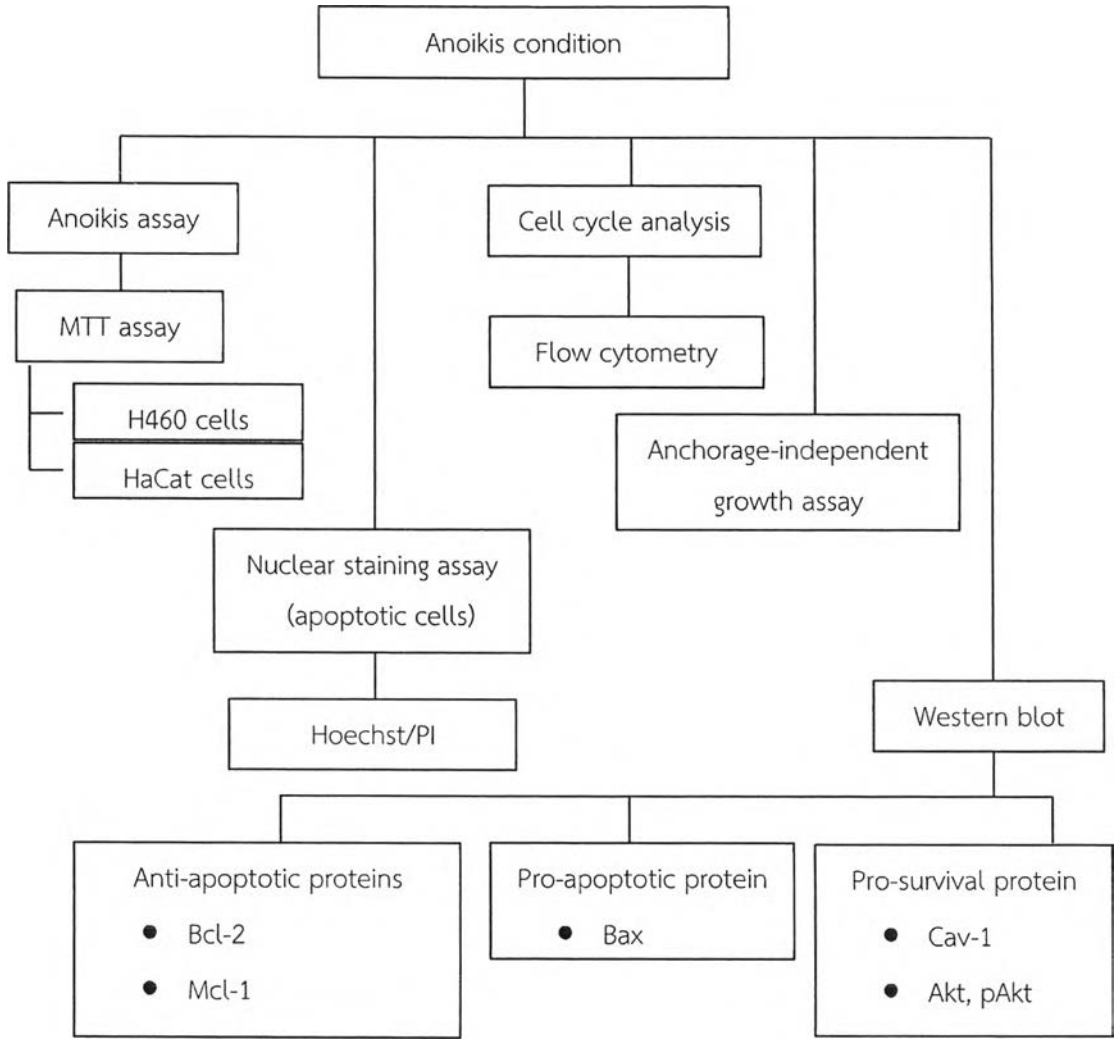

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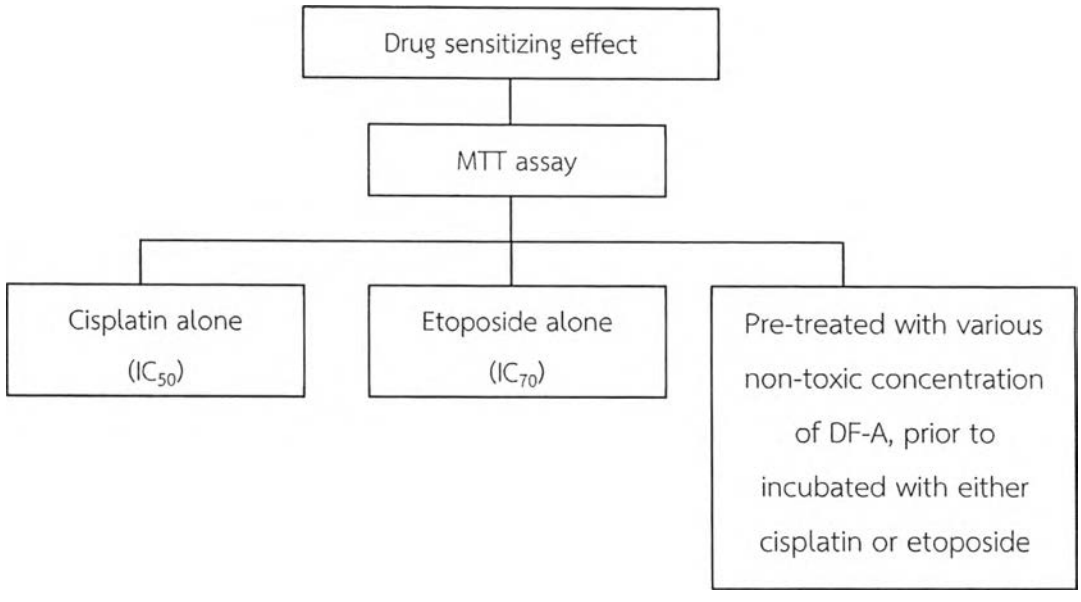

Figure 18 Experimental designs

14.3 Investigation on the toxicity by dendrofalconerol A.

H460 and HaCat cells were seeded at the density of 1 x 10^4 cells per well in a 96-well plate overnight. Cells were treated with various concentrations of DF-A (0-100 μM) and incubated for 24 h and cell viability was investigated by MTT assay.

14.4 Investigation the effect of dendrofalconerol A on anoikis.

To determine the ability on anoikis response of detached H460 and HaCat cells. Adherent cells in the culture plate were detached and made into a single-cell suspension in RPMI-serum free medium and then seeded into a poly-HEMA coated plate at a density of 1.5×10^5 cells/ml. Cells were then harvested at 0, 6, 9, 12, and

24 h. Then, cells were incubated with 20 μM of MTT reagent for a further 4 h at 37°C. The intensity of the formazan product was measured at 450 nm using a microplate reader. In addition, to confirmed characteristic of detachment-induce apoptosis, detached cells were treated with or without DF-A in RPMI serum free medium for 24 hours at 37°C. Apoptotic and necrotic cell death were determined by Hoechst 33342/propidium iodide (PI) co-staining. Then, cells are incubated with 10 μM of the Hoechst 33342 dye and 5 μg/mL of the PI dye for 30 min at 37°C in the dark. Finally, cells were visualized under a fluorescence microscope (Olympus IX51 with DP70).

14.5 Investigation the effect of dendrofalconerol A on Sub-G_0 fraction.

Cell cycle analysis was performed by flow cytometry to determine sub-G_0 fraction. Briefly, suspended cells were treated with various concentrations of DF-A in RPMI serum free medium for 24 h at 37°C. Then, cells were collected after centrifugation at 4,500 rpm for 5 min at 4°C, re-suspended in PBS and fixed with cool absolute EtOH while 1 ml of cell suspension on vertex, and kept it for 24 h at -20°C. The pellets were washed in PBS and centrifuged as the same velocity and time. Lastly, pellets were incubated with propidium iodide (PI) buffer (10X PBS, 1 mg/ml RNase A, 100X Triton, 0.5 mM EDTA, 10 mg/ml PI) for 30 min at 37°C under dark condition. After that, the pellets were immediately analyzed using FACScan flow cytometer (Becton Dickinson, Rutherford, NJ, USA) using a 488 nm excitation beam and a 630 nm band-pass filter with CellQuest software (Becton Dicknison).

14.6 Investigation the effect of dendrofalconerol A on H460 cells anchorage-independent growth by soft agar assay.

Anchorage-independent growth assay was performed in two-layer soft agar. Briefly, aliquot of melted 1% agarose (1 g of agarose in 100 ml of sterile water) and cool in 55°C water bath before use. To make the lower layer, an equal volume of melted 1% agarose (55°C) and complete RPMI medium (1:1) were mix to give 0.5% agarose and 0.5% complete RPMI medium. Then, the mixture was put in 24-wells plate at 500 µl/well and allowed solidify at 4°C for 5 min. To prepare the upper layer (0.33% agarose), melted 1% agarose (55°C) was mixed with complete RPMI medium containing trypsinized adherent cells at a density of 1500 cells/well under various concentrations. Following that, it was added to the lower layer at 250 µl/well and allowed to gel at room temperature before placing in incubator at 37°C for 2 weeks. Fresh complete RPMI medium (250 µl/well) was added every 3 days over the upper layer. After 2 weeks of growth colonies were photographed in order to count the colonies and measured the colony size.

14.7 Investigation effect of DF-A on proliferation of human lung cancer H460 cells.

Cells were seeded at a density of $5x10^3$ cells/well in a 96-well plate overnight. Cells were incubated with various non-toxic concentrations of DF-A for 24, 48, or 72 h. Cell proliferation was determined by MTT assay and calculated from optical density (OD) ratio of treated group to non-treated control group.

14.8 Investigation the effect of DF-A on human lung cancer H460 cell migration.

Cells were seeded at a density of 3.5×104 cells/well. After the cell reach 90% confluence, a micropipette tip was used to scratch the cells to generate wound space. After scratching, the cells were gently washed with PBS and replenish with fresh RPMI supplement with 10%FBS containing indicated non-toxic concentration of DF-A and allowed to migrate for 12, 24 or 48 h. The progress of cell migration into the wound was photographed by inverted microscope (Olympus IX51 with DP70, Melville, NY) at indicated times. The average wound space was calculated from the random field of view and represent as the relative cell migration. Relative cell migration was calculated by dividing the change of wound space of treated cells by that of the untreated cells in each experiment.

14.9 Investigation on molecular mechanisms of dendrofalconerol A sensitized anoikis and inhibited cell migration

Cells were harvested and lysed on ice for 60 min. After the sample had been prepared, the protein content of cell lysate is determined using BSA protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein of each sample was separated by size using SDS-PAGE and then transferred into nitrocellulose membranes. The membrane was blocked in 5% skim milk in TBST (25 mmol/l Tris-HCL, pH 7.4, 125 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature, and then probe with appropriate primary antibodies at 4^{o}C overnight and washed three times with TBST for 8 min. After that, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies according to the primary antibodies for 2 h at room temperature. Lastly, the signal of immunoreactive proteins was detected

by enhance chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL, USA). Protein expression (Bcl-2, Mcl-1, Bax, Cav-1, Akt, pAkt, FAK, pFAK, Rho-GTP, Rac-GTP) was investigated and β-actin is used as a loading of control in each treatments. The immune complexes are detected by enhanced with chemiluminescence substrate (Supersignal West Pico; Pierce) and quantified using analyst/PC densitometry software (Bio-Rad) normalized to the level of β-actin protein. All analyzes were performed in triplicate.

14.10 Investigation the effect of DF-A on susceptibility of the cells to cisplatin and etoposide.

Cells were seeded at a density of 7×10^3 cells/well onto 96-well plate overnight. After that, cells were pre-treated or left untreated with non-toxic concentrations of DF-A for 24 h and treated with 100 μM cisplatin (IC_{50} =100 μM) (33, 34) or 100 μM etoposide (IC_{70} =100 μM) (34). After 24 h, cell viability was investigated by MTT assay.