CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and Reagents

Troclosan (Irgasan®) (TCS) from Sigma Chemical, Inc. (St. Louis, MO, USA) was kindly supplied by Dr. Phisit Khemawoot, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

These following chemicals were used in the study.

Bovine serum albumin (BSA), dimethylsulfoxide (DMSO), Hoechst33342, phalloidin tetramethylrhodamine B isothiocyanate, propidium iodide (PI), skim milk, Trizma® base and Tween 20 were purchased from Sigma-Aldrich Co. LLC (MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), Glutamax, Penicillin-Streptomycin, PrestoBlue® reagent, phosphate buffered saline (PBS), RPMI-1640 medium, Trypsin-EDTA were purchased from Life technologies (MD, USA). Hydrochloric acid and sodium chloride obtained from Labscan Asia (Thailand). Acrylamide, agarose, ammonium persulfate, Bradford assay kit, nitrocellulose membranes, sodium dodecyl sulfate and tetramethylethylenediamine were purchased from Bio-Rad Laboratories, Inc. (CA, USA). Matrigel was obtained from BD Biosciences, Inc. (MA, USA). BCA assay kit and Supersignal west pico chemiluminescent was obtained from Thermo Fisher Scientific, Inc. (IL, USA). Protease inhibitor mixture was purchased from Roche Diagnostics (IN, USA). Antibodies for E-cadherin, N-cadherin, vimentin, slug, snail, phosphorylated Akt (S473), Akt, phosphorylated FAK (Y397), FAK, β-actin, Cell Lysis Buffer and peroxidase conjugated secondary antibodies were obtained from Cell Signaling

Technology Inc. (MA, USA). Active Rac1-GTP and Active Rho-GTP were obtained from NewEast Biosciences (PA, USA). Immobilon Western chemiluminescent HRP substrate was obtained from Millipore Corp (MA, USA) and Thermo Fisher Scientific, Inc. (IL, USA).

2. Equipments

These following equipments were used: Automated cell counter, Analyst/PC densitometry software, Blotting cell and membranes (Bio-Rad, CA, USA), Autopipette: 0.2-2 µl, 2-20 µl, 20-200 µl and 200-1,000 µl, Autopipette tips: 0.2-2 µl, 2-20 µl, 20-200 µl and 200-1000 µl (Gilson, France), Cell culture plate: 6-well, 24 well and 96-well, ultra-low attachment plate: 6 well, 24 well and 96-well (Coming, NY, USA), Centrifuge Hermle Labnet Z383K (HERMLE Labortechnik, Germany), Fluorescence microscope (Olympus IX51 with DP70, Olypus America Inc., Center valley, PA, USA), Fluorescence Reader VICTOR3V (Perkin Elmer, MA, USA), Microplate reader (Anthros, Durham, NC, USA), Phase contrast microscope with camera (Meiji TC5100, Japan), pH meter (Beckman Instrument, CA, USA), Vortex mixer (Clay Adams, NJ, USA), Laminar flow cabinet (BossTech, Thailand), Humidified incubator (Thermo Fisher Scientific, Inc., IL, USA).

Methods

1. Preparation of TCS solution

TCS was dissolved in DMSO as a stock solution at 10 mM by dissolving 0.00289 g of TCS in 1,000 µl of DMSO. This stock solution (10 mM) was then diluted with RPMI-1640 medium to obtain various final concentrations of TCS solutions (2.5, 5, 7.5 and 10 µM) containing 0.1% DMSO.

2. Cell culture

NCI-H460 human lung carcinoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin in 37°C with 5% CO_2 humidified incubator. Cells were trypsinized when cells reached 80–90% confluence.

3. Cell viability assay

To determine the non-cytotoxic concentrations of TCS for using the subsequent experiments, cells viability following TCS treatment was tested by MTT assay. The assay was based on the conversion of the yellow tetrazolium salt, MTT to form a soluble blue formazan product by mitochondrial dehydrogenase enzymes in living cells. After the indicated treatment, the medium was replaced with MTT solution (5.0 mg/ml in PBS) and incubated at 37°C for 4 h. Then the medium was replaced with 100 µl DMSO to solubilize formazan product and the intensity of the formazan product was measured at 570 nm using a microplate reader. All analyzes were performed in three independent triplicate cultures. Cell viability was expressed

as the percentage calculated from the optical density (OD) of treated cells relative to the controlled cells as the following equation;

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

4. Nuclear staining assay

Apoptotic and necrotic cells were determined by Hoechst33342 and PI co-staining. Hoechst 33342 is blue-fluorescence dye that can permeate through cell membrane and stain the chromatin in cells. For apoptotic cells, the condensed chromatin and/or fragment of nuclei in apoptotic cells express more brightly blue fluorescent color of Hoechst 33342 than the chromatin in normal cells. PI has been widely used for fluorescent DNA staining but it cannot enter into normal viable cell and apoptotic cells. This dye can pass into nucleus only in necrotic cells which have membrane disruption (Figure 21).

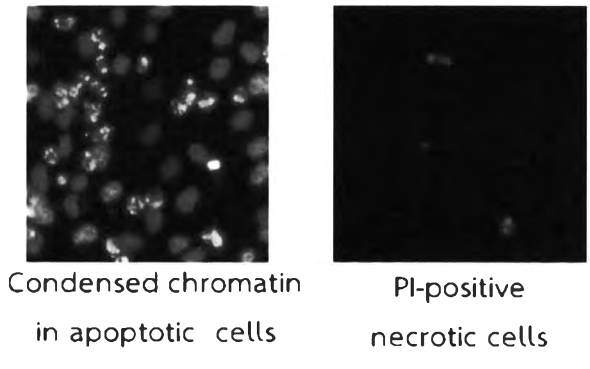


Figure 21 Apoptotic cells and necrotic cells in Hoechst33342 and PI co-staining

After the specific treatments, cells were incubated with 10 µg/ml of Hoechst33342 and 5 µg/ml of PI for 30 min at 37°C. The apoptotic cells having condensed chromatin were detected by light blue fluorescent color of Hoechst33342. The necrotic cells were detected by light red fluorescent color of PI.

The apoptotic cells having condensed chromatin and PI-positive necrotic cells were visualized and scored under a fluorescence microscope. All analyzes were performed in three independent triplicate cultures. The percentage of apoptotic cells was calculated as following and the result was compared to that of the non-treated cells;

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

5. Anoikis assay

To determine the effect of TCS on anoikis of H460 cells in detached condition, H460 cells were detached into a single-cell suspension in RPMI-serum free medium and seeded into ultra-low attachment 6-well plate at a density of 1.5×10^5 cells/ml. Suspended cells were treated with TCS at non-toxic concentrations and incubated at 37 °C. Cells were then harvested at 0, 3, 6, 9, 12, and 24 h and allowed to attach on 96-well plate for 7 h at 37°C. Cells were incubated with MTT solution (5.0 mg/ml in PBS) for a further 4 h at 37°C. The intensity of the formazan product is measured at 570 nm using a microplate reader. All analyzes were performed in three independent triplicate cultures. The percentage of viable cells that was estimated by comparing with the untreated control cells in time- and concentration-dependent manner.

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

6. Anoikis resistant cells (AR cells)

Anoikis resistant cell (AR cell) culture was carried out according to the method of Sakuma et al. (102) and Khongmanee et al. (103) with minor modifications. In brief, attached H460 cells were trypsinized when cells reached 80–90% confluence with

0.05% trypsin/0.02% EDTA. Then cells were cultured in ultra-low attachment 6-well plate in RPMI-1640 medium and maintained condition as described in attachment culture. Cells were cultured at a density of 2×10^5 cells/ml for 48 h. Suspended cells were collected and prepared into a single cell suspension by 1 mM EDTA treatment. Then cells were washed with complete RPMI-1640 medium. Cell viability was measured using automated cell counter. Viable cells were used for further experiments.

7. PrestoBlue assay

Cells viability of AR cells is determined by PrestoBlue assay. PrestoBlue™ Cell Viability Reagent is supplied as a 10X solution. This reagent is a nontoxic resazurin-based solution which can permeate into cells. This compound is blue and nonfluorescent and then is modified by the reducing power of living cells to become red and highly fluorescent compound. The fluorescence intensity was calculated to obtain percentage of cell viability (Figure 22).

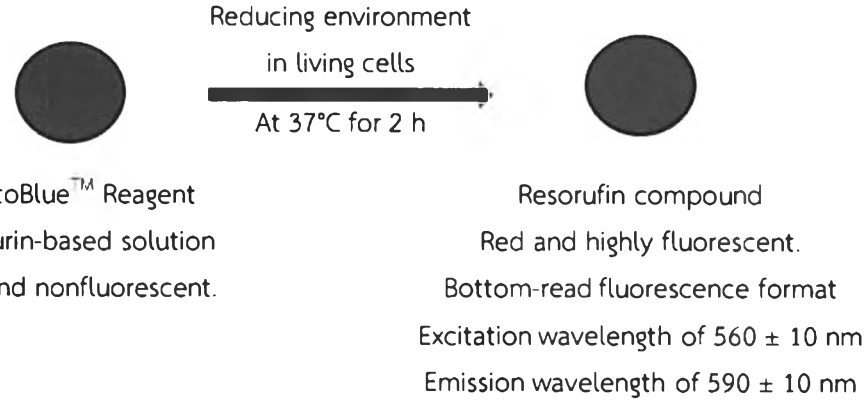


Figure 22 The principle of PrestoBlue assay

After the indicated treatment, the suspended cells were incubated with the recommended concentration of PrestoBlue reagent at 37°C for 2 h. The fluorescence intensity was read in bottom-read fluorescence format using the excitation

wavelength of 560 ± 10 nm and emission wavelength of 590 ± 10 nm. Cell viability was expressed as the percentage calculated from fluorescence intensity of the treated cells relative to the controlled cells.

$$\text{Cell viability (\%)} = \frac{\text{Fluorescence intensity of the treated cell}}{\text{Fluorescence intensity of the controlled cell}} \times 100$$

8. Colony formation assay

Upon treatment with TCS, tumorigenic ability of cells was examined via colony formation assay in accordance with the method of Koleske et al. (104) with minor modifications. Colony formation assay determined the tumorigenic ability of single cancer cells in detached condition. This assay was based on the ability of a single cell to overcome anoikis and grow into the colonies. Cancer cells were seeded as singe cells in second agarose layer to prevent cell attachment and cell-cell contact and cultured with appropriate conditions in soft agar for several days.

In this study, cells were treated with TCS at the non-toxic concentrations for 24 h and then treated with 1 mM EDTA to prepare single cell suspension. Cells were suspended in RPMI-1640 containing 10% FBS and 0.33% agarose, then 250 µl containing 1×10^3 cells were embedded as a second layer in a 24-well plate over a 500 µl of base layer containing 10% FBS and 0.5% agarose. The cells were fed every 3 days by adding 250 µl of complete medium. After 7 and 10 days, the resulting colonies were photographed at ×4 magnification. Colony number and colony size were determined on 10^{th} day of culture. All analyzes are performed in three independent triplicate cultures (Figure 23).

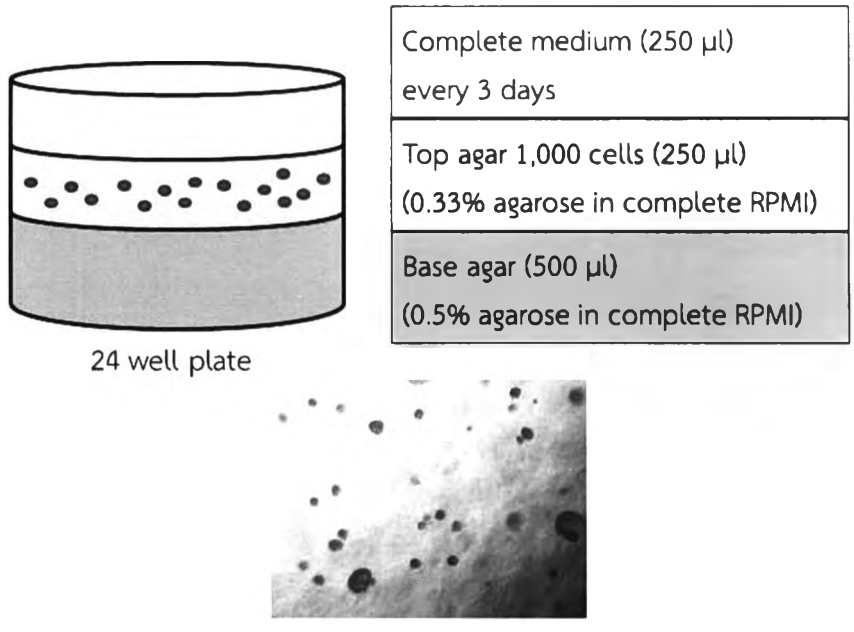


Figure 23 Colony formation assay

Colony number and colony size were estimated by comparing with the untreated controlled cells and expressed as the percentage calculated as the following equations.

$$\% \text{ Colony number} = \frac{\text{Colony number of treated cells}}{\text{Colony number of controlled cells}} \times 100$$

$$\% \text{ Colony size} = \frac{\text{Mean of colony size (diameter) of treated cells}}{\text{Mean of colony size (diameter) of controlled cells}} \times 100$$

9. Filopodia characterization

Filopodia was characterized by phalloidin-rhodamine staining assay as described by Kowitdamrong et al. (49). Phalloidin has a high-affinity to bind to F-actin and also preventing its depolymerization. Rhodamine is red-orange fluorescent dye conjugated with phalloidin molecule. After cells were fixed, phalloidin-rhodamine

permeated into cells and bound to F-actin bundles which are the primary composition of filopodia of cells as shown in Figure 24.

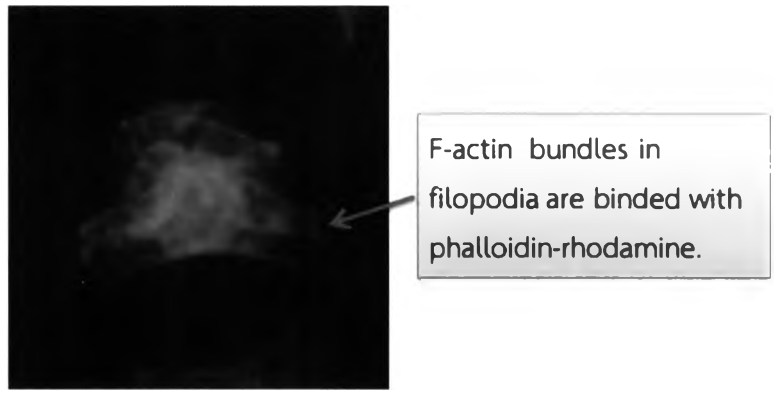


Figure 24 The characterization of filopodia by phalloidin-rhodamine staining assay

In this study, cells were treated with TCS at the non-toxic concentrations for 24 h in detached condition and seeded at a density of 2×10^3 cells/well onto 96-well plate for 4 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at 37°C, permeabilized with 0.1% Triton-X100 in PBS for 4 min, and blocked with 0.2% BSA for 30 min. Then cells were incubated with 1 : 100 phalloidin-rhodamine in PBS for 15 min and washed with PBS 3 times. Filopodia was then imaged by a fluorescence microscope.

10. Migration assay

Migration was determined by Boyden chamber assay as previously described by Kowitdamrong et al. (49). This assay was based on the ability of cell to move through a porous membrane by chemotactic responses of cells. In this assay, the upper chamber and the lower chamber contained medium with serum at different concentrations. Cells were seeded in medium in the upper chamber and migratory cells vertically moved through the pores of the membrane into the lower chamber containing

medium with higher serum content. Cells that migrated to the underside of the membrane were fixed on the membrane, stained and quantified (Figure 25).

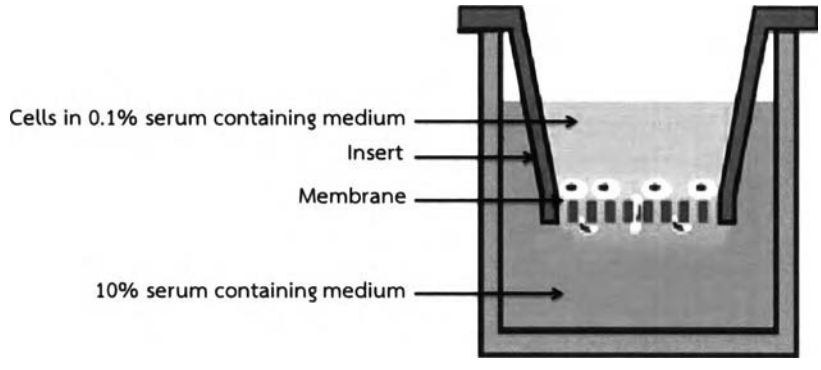


Figure 25 Boyden chamber assay (Transwell migration assay)

In this study, cells were pretreated with TCS at the non-toxic concentrations for 24 h in detached condition. Cells were seeded at a density of 5 $\times 10^4$ cells/well onto an upper chamber of 24-transwell plate of the transwell filter (8-μM pore) in the medium containing 0.1% serum and 500 μl of complete medium was added at lower chamber. After 24 h, the non-migrated cells in the upperside membrane were removed by cotton-swab wiping. Cells that migrated to the underside of the membrane were stained with 10 μg/ml Hoechst33342 for 30 min. Cells were then visualized and scored under a fluorescence microscope. All analyzes were performed in triplicate. Cell migration was represented as relative migration calculated by dividing an average number of migratory cells in each field at the basolateral side of membrane of treated group by that of non-treated group in each experiment using the following formula;

$$\text{Relative migration} = \frac{\text{An average number of migratory cells of treated group}}{\text{An average number of migratory cells of non-treated group}}$$

11. Invasion Assay

The invasion assay was carried out using 24-transwell chambers as previously described in Kowitdamrong et al. (49). The principle of this assay was as same as transwell migration assay. Porous membrane was coated with a thin layer of matrigel, a basement membrane extract, which was used as a model of ECM. Only invasive cells could degrade this matrix and moved through the matrigel and the porous membrane into the bottom of membrane. Invasive cells were fixed and quantified by staining assay (Figure 26).

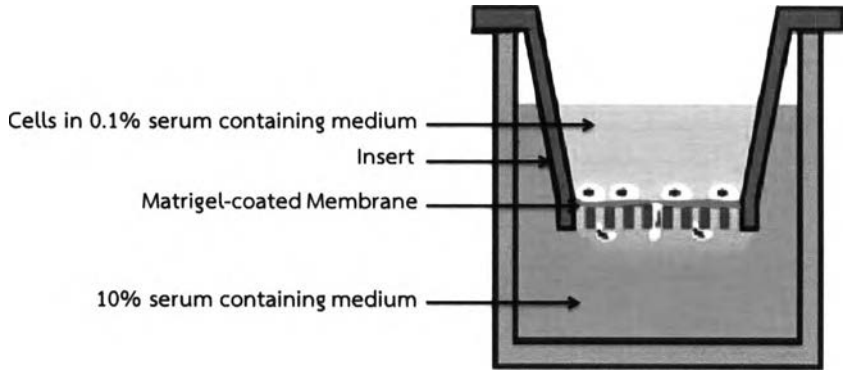


Figure 26 Boyden chamber assay (Transwell invasion assay)

In this study, transwells were coated with 50 µl of 0.5% matrigel on the upper surface of chamber and incubated overnight at 37°C in a humidified incubator. After treatment with TCS at the non-toxic concentrations for 24 h in detached condition, cells were seeded at a density of 5×10^4 cells/well onto the upper chamber in medium containing 0.1% serum and 500 µl of complete medium was added at the lower chamber. After 24 h, the non-invaded cells in the upperside of membrane were removed by cotton-swab wiping. Invaded cells at the basolateral side of membrane were fixed with cold absolute methanol for 10 min and stained with 10 µg/mL Hoechst33342 for 30 min. Cells were then visualized and scored under a

fluorescence microscope. All analyzes were performed in triplicate. Cell invasion was represented as relative invasion calculated by dividing an average number of invaded cells in each field at the basolateral side of membrane of treated group by that of non-treated group in each experiment using the following formula;

$$\text{Relative invasion} = \frac{\text{An average number of invaded cells of treated group}}{\text{An average number of invaded cells of non-treated group}}$$

12. Western blot analysis

After the specific treatments, cells were incubated in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1.5% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture for 2 h on ice. Cell lysates were collected and protein content was determined using the Bradford assay kit. Equal amounts of protein from each sample were denatured by heating at 95°C for 5 min with loading buffer and subsequently loaded onto a 7.5% SDS-polyacrylamide gel electrophoresis for the detection of EMT markers and E-cadherin expression or 10% SDS-polyacrylamide gel electrophoresis for the detection of migratory-related protein expression. After separation, proteins were transferred onto 0.45 μM nitrocellulose membranes. The transferred membranes were blocked in 5% non-fat dry milk in TBST (25 mM Tris-HCL (pH 7.5), 125 mM NaCl, 0.1% Tween 20) for 1 h, and then incubated with a specific primary antibody overnight at 4°C. Membranes were washed three times with TBST for 5 min and incubated with Horseradish peroxidase-conjugated anti-rabbit IgG for 2 h at room temperature. Membranes were washed three times with TBST for 5 min and the immune complexes were detected by enhancement with a chemiluminescent substrate and quantified using analyst/PC

densitometry software normalized to the level of β-actin protein. All analyzes are performed in three independent triplicate cultures.

13. Statistical Analysis

Data were obtained from three independent experiments and presented as means ± standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA and post hoc test (Tukey's test) at a significance level of *P*-values < 0.05. SPSS 17.0 was used for all statistical analyses.

14. Experimental designs

14.1 Conceptual Framework

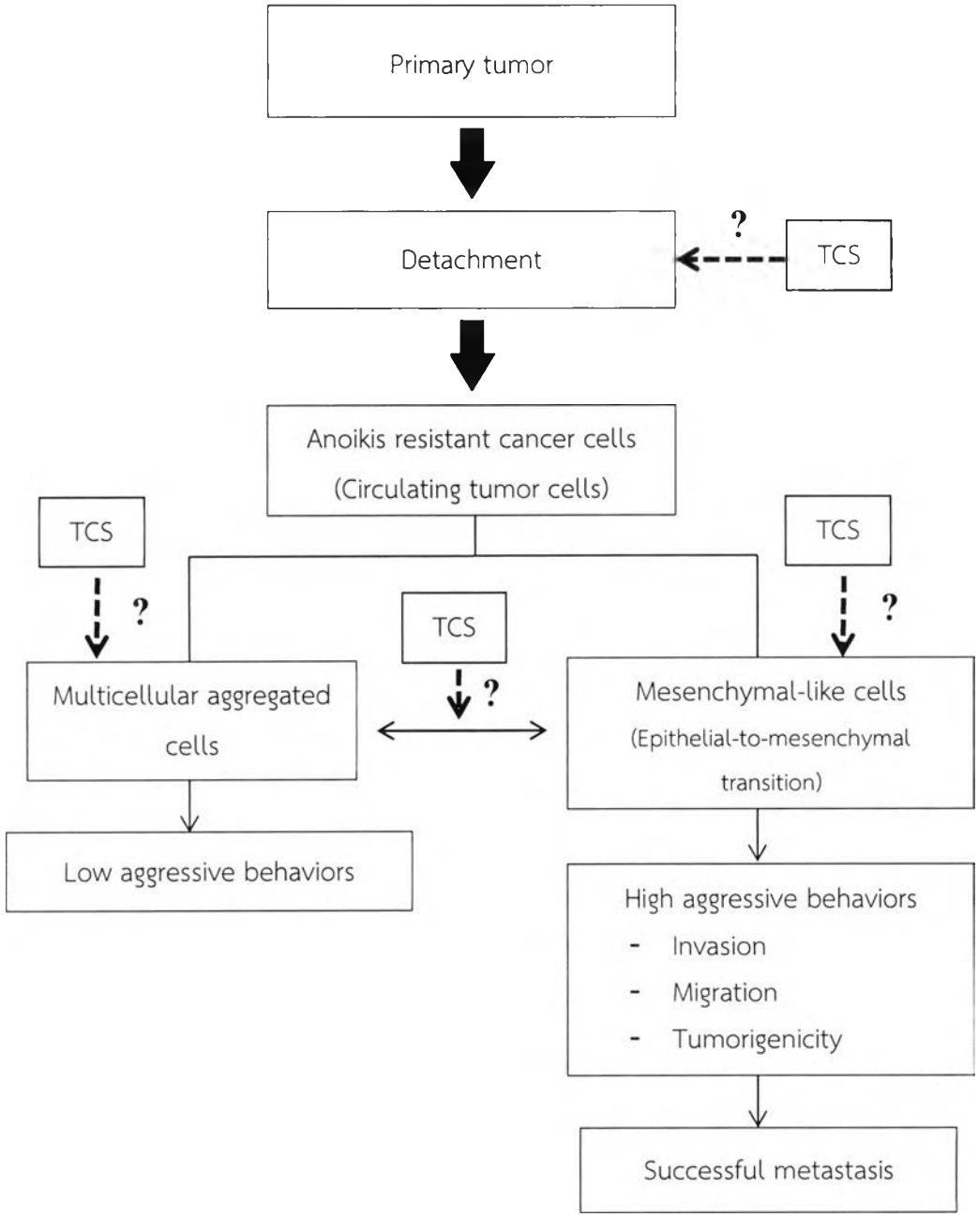
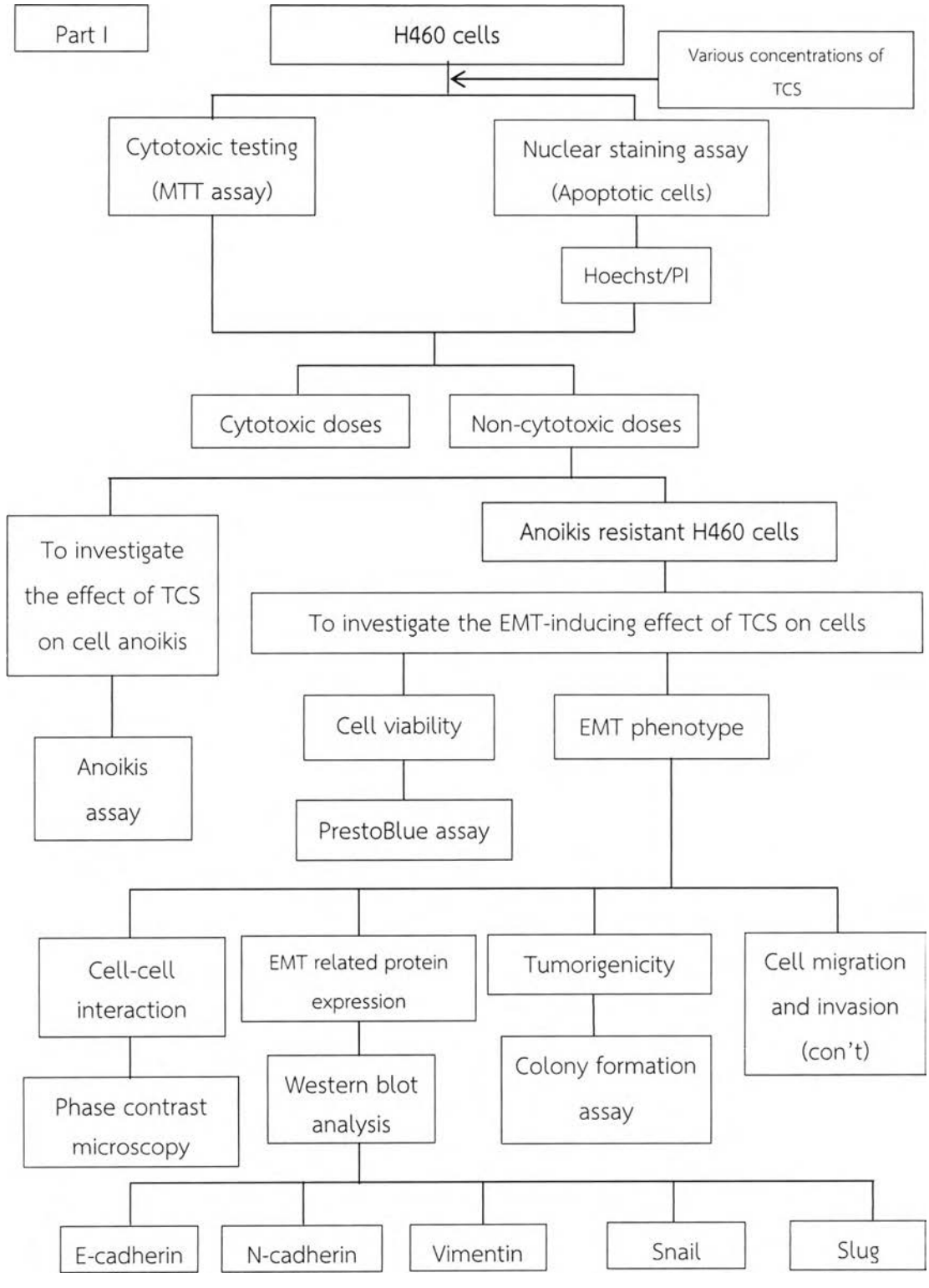


Figure 27 Conceptual Framework

14.2 Experimental designs



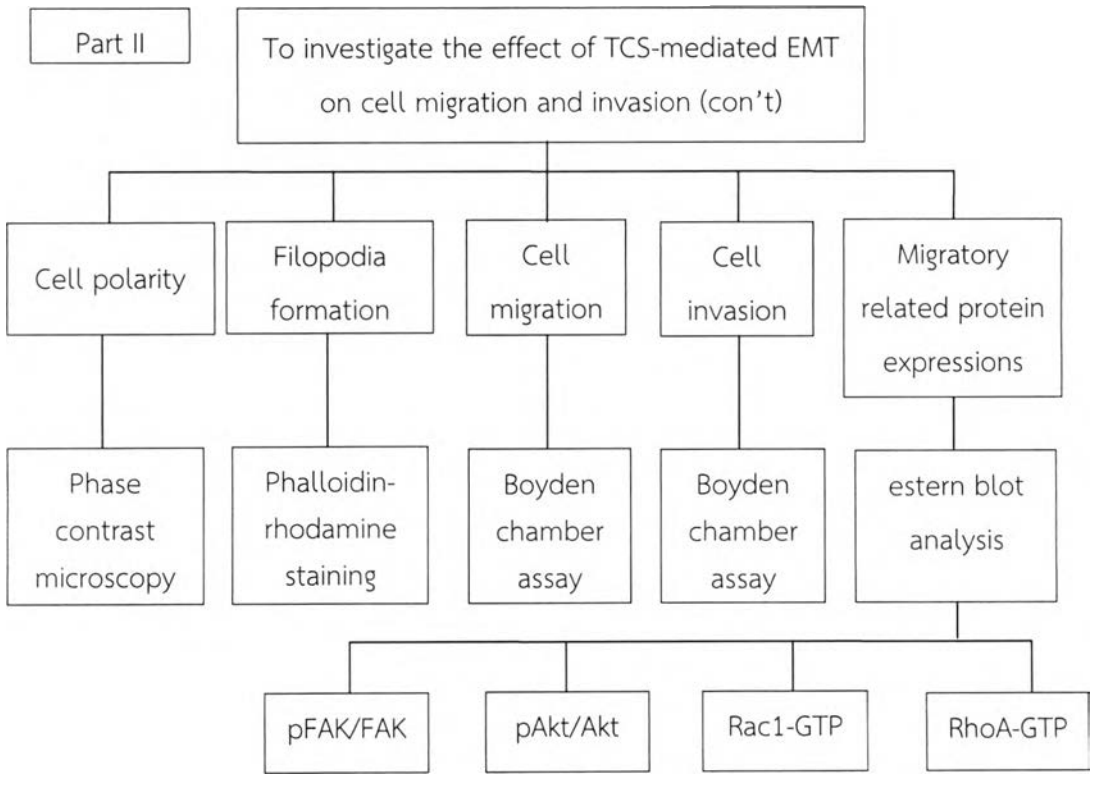


Figure 28 Experimental designs

14.3 Investigation of cytotoxic effect of TCS on H460 cells

To determine cell viability in response to TCS in H460 cells, cells were seeded at a density of 1×10^5 cells/ml onto 96-well plate overnight. After that cells were treated with various concentrations of TCS for 24 h. Then cell viability was investigated by MTT assay. Non-toxic concentrations were used for following experiments.

14.4 Investigation on mode of cell death induced by TCS in H460 cells

To investigate the mode of cell death after TCS treatment, cells were seeded at a density of 1×10^5 cells/ml onto 96-well plate and incubated overnight. Cells were then treated with TCS for 24 h. Nuclear morphology of the cells were detected by Hoechst33342/PI co-staining assay. Apoptotic and necrotic cell death were visualized under fluorescence microscope. Non-toxic concentrations were used for following experiments.

14.5 Investigation the effect of TCS on anoikis of H460 cells

To study the effect of TCS on cell anoikis, cells from monolayer were trypsinized into single cells suspension in serum free medium and seeded 5 ml of cells in ultra-low attachment 6-well plate at density of 1.5×10^5 cells/ml. Cells were treated with non-toxic concentrations of TCS incubated at 37 °C for 3, 6, 9, 12 and 24 h. At each time points, cells were harvested and seeded into 96-well plate (100 µl/well). Then cells were allowed to attach plate for 7 h at 37 °C. Cells viability was determined by using MTT assay as described in Methods.

14.6 Investigation of cell viability in response to TCS on AR cells

To confirm the non-cytotoxic effect of TCS on AR cells, cells which survive in suspension culture for 48 h were prepared into single-cell suspension by 1 mM EDTA. Then cells were seeded in ultra-low attachment 96-well plate at density of 1.5×10^5 cells/ml and treated with non-toxic concentrations of TCS at 37 °C for various times up to 48 h. Cell viability was detected by PrestoBlue assay.

14.7 Investigation the effect of TCS on cell-cell interaction of AR cells

To examine the effect of TCS on cell-cell interaction of AR cells, cells were seeded onto ultra-low attachment 24-well plate at the density of 1.5×10^5 cells/ml and treated with non-toxic concentrations of TCS for 0-48 h. The formation of cell aggregation was observed and photographed using a phase contrast microscope. Aggregate size and number was determined by image analyzer and calculated relative to the non-treated control as the percentage of aggregate size and aggregate number, respectively.

14.8 Investigation the molecular mechanisms of EMT inducing effect of TCS on AR cells

To identify the underlying mechanism of EMT induction in response to TCS treatment in AR cells, western blot analysis were performed to evaluate the expression of E-cadherin and several EMT markers including N-cadherin, vimentin, snail and slug. AR cells were treated with non-toxic concentrations of TCS for 24 h in suspension cultures at density 1.5×10^5 cells/ml in ultra-low attachment 6-well plate. After indicated treatment, cells were centrifuged at 4,500 rpm for 15 min and then supernatant was removed. Then cells were washed with cold PBS and centrifuged at 4,500 rpm for 15 min and cold PBS was removed. After that cells were

collected and incubated with lysis buffer on ice for 2 h. Protein expressions were identified by western blot analysis as described in Methods. The β-actin was used as a loading control of each treatment.

14.9 Investigation the effect of TCS-mediated EMT on tumorigenic ability of AR cells

To investigate tumorigenic ability of TCS-treated AR cells, this effect was investigated by using colony formation assay. Cells were seeded at a density of 1.5 × 10^5 cells/ml onto ultra-low attachment 6-well plate and treated with TCS at non-toxic concentrations for 24 h. Then cells were collected and prepare to be single cell suspension by 1 mM EDTA before subjected to colony formation assay.

14.10 Investigation the effect of TCS-mediated EMT on cell polarity of AR cells in attachment condition

To study the effect of TCS-mediated EMT on cell polarity of AR cells in attachment condition, cells were pretreated with non-toxic concentrations of TCS for 24 h in suspension cultures at density 1.5 × 10^5 cells/ml in ultra-low attachment 6-well plate. Then cells were allowed to attach on conventional culture dishes for 4 h. Cell morphology was detected by phase contrast microscopy.

14.11 Investigation the effect of TCS-mediated EMT on filopodia formation of AR cells in attachment condition

To study the effect of TCS-mediated EMT on filopodia formation of AR cells in attachment condition, cells were pretreated with non-toxic concentrations of TCS for 24 h in suspension cultures at density 1.5 × 10^5 cells/ml in ultra-low attachment 6-well plate. Then cells were seeded at a density of 2 × 10^3 cells/well onto 96-well

plate for 4 h. After that filopodia and viable cells were detected by phalloidin-rhodamine and Hoechst33342 staining, respectively. Cells were visualized under fluorescence microscope.

14.12 Investigation the effect of TCS-mediated EMT on AR cell migration

To study the effect of TCS-mediated EMT on AR cell migration, the migratory ability of cells was evaluated by using Boyden chamber assay. Cells were pretreated with non-toxic concentrations of TCS for 24 h in suspended condition at a density of 1.5×10^5 cells/ml in ultra-low attachment 6-well plate. Then cells were collected and prepare to be single cell suspension by 1 mM EDTA before subjected in transwell migration assay as described in Methods.

14.13 Investigation the effect of TCS-mediated EMT on AR cell invasion

To study the effect of TCS-mediated EMT on AR cell invasion, the invasion behavior of cells was evaluated by using Boyden chamber assay with matrigel-coated transwells on the upper surface of chamber. Cells were pretreated with TCS at non-toxic concentrations in suspended condition at a density of 1.5×10^5 cells/ml in ultra-low attachment 6-well plate for 24 h. After that cells were collected and prepare to be single cell suspension by 1 mM EDTA before subjected in transwell invasion assay as described in Methods.

14.14 Investigation the molecular mechanisms of TCS-mediated EMT on AR cell motility

To identify the underlying mechanism of cell motility in response to TCS-mediated EMT in AR cells, western blot analysis were performed to evaluate the expression of migratory-related proteins including activated FAK (phosphorylated FAK,

Tyr 397), FAK, activated Akt (phosphorylated Akt, Ser 473), Akt, activated Rac1 (Rac1-GTP), and activated RhoA (RhoA-GTP). AR cells were pretreated with non-toxic concentrations of TCS for 24 h in suspension cultures at density 1.5×10^5 cells/ml in ultra-low attachment 6-well plate. After treatment, cells were allowed to attach on conventional culture dishes for 4 h. Then the medium was removed and cells were washed with cold PBS. Cells were harvested and lysed with lysis buffer on ice for 2 h. Protein expressions were identified by western blot analysis as described in Methods. The β-actin was used as a loading control of each treatment.