Chapter III Materials and Methods

This chapter explains materials and methods that be used during experiment. Two strategy approaches explained in this part; cellular studies using U2OS cell line and computational studies. The strategies were prepared to understand three big issues in this study; the role of AIF to menadione induced death of U2OS cells, the lethal function of AIF, and the role of AIF in menadione metabolism. The work was divided by three parts; characterization the effect of menadione-induced death on the response of U2OS cells, characterization the effect of AIF depletion on the response of the cell to cytotoxicity of drugs, and *in silico* approach to study functional group interaction between AIF and menadione. The strategy of this study describes on the scheme of research (Figure III.1).

III.1 Materials

This section explains materials used in cellular studies. They are cell line, chemicals, kit reagents, antibodies, and *si*RNA.

*Cell line***.** U2OS cell lines were derived from human osteosarcoma (ATCC n°HTB-96). Medium for cell culture is DMEM modified containing Dubecco's Modified Eagle Medium (DMEM; Life Technologies), Fetal Bovine Serum (FBS; Life Technologies), and Penicillin-Streptomycin (PS; Life Technologies). Medium for transfection is Reduced Serum Media (Opti-MEM; Life Technologies). Cell lysates were prepared using Dubelcco's Phosphate-Buffered Saline (DPBS; Life Technologies), TrypLE (Life Technologies), and SDS (BioRad). Tryphan blue (sigma) was used for cell counting. Fluorescent dye 4',6-diamidino-2-phenylindole (DAPI, D1306; Invitrogen) was used for cell death assay. Mitochondrial membrane permeabilization (MMP) was assayed using 1,1',3,3,3',3'-hexamethylindodicarbocyanine iodide ($DilC_1$ (5), Molecular Probes M34151) and Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP, tochris) as positive control.

Chemicals. Drugs used for cytotoxicity assay were 2-methyl-1,4-naphthoquinone (menadione, M 5625; Sigma), 2,3-dymethoxy-1,4-naphthoquinone (DMNQ, sigma-aldrich D5439), 1,4-benzoquinone (benzoquinone, BZQ, sigma-aldrich

51386), and 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-antracenedione dihydrochloride (mitoxantrone, MTX, sigma-aldrich M6545). Interruption of antioxidant system in the cell was assayed by γ-L-Glutamyl-L-cysteinyl-glycine (L-gluthatione reduced, GSH, G 6013; Sigma) and N-acetylcysteine (NAC, 616-91; Sigma). Caspase-inhibitor VI carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (ZVAD.fmk, Cat#219007; Calbiochem) was used for caspase dependency assay. Rotenone (R8875; Sigma-aldrich) was used as inhibitor of Complex I of respiratory chain activity. 1H,7H-Phyrazolo(1,2-α)pyrazole-1,7-dione,3-(bromomethyl)-2,5,6-trimethyl-71418-44-5 (Monobromobimane, MBB; Life Technologies) was used as fluorescent dye to quantify GSH level in the cell.

Kits. The proteins in the cell lysate were quantified using the colorimetric assay kit "Dc Protein Assay" which contains three reagents A, B, and S (Bio-Rad) based on the protocol of Lowry. Western blot materials for detecting protein consist of NuPAGE Bis-Tris Gels 4-12% (Life Technologies), Tris Buffered Saline (TBS; Euromedex), Tris-Glycine Buffer (migration buffer, Euromedex), tween 20 (Sigma-Aldrich), 4-morpholine ethane sulfonic acid (MES) SDS Running Buffer (Bioland scientific), methanol RPE (Carlo Erba), and ethanol RPE (Carlo Erba). Western blot analysis using ECL Prime Western Blotting Detection Reagent (Life Sciences) and strep-Tactin-HRP (Bio-Rad).

Antibodies and small interference RNA (siRNA). Antibodies that used include β-actin (mouse mAb; Ab49900 Abcam), AIF (mouse mAB; Santa Cruz), Hif1α (mouse mAb; 610958 BD transduction), secondary antibodies anti-mouse and anti-rabbit (Southern Biotech). *si*RNA transfections were performed using the Lipofectamine 2000 reagent (Life Technologies). The following *si*RNA sequences were used, for negative control: Co1: CCG UGC UCC UGG GGC UGG G [dT][dT] (hemerin1); Co2: AUG CAG AAC UCC AAG CAC G [dT][dT] (mAIF19); for human AIF, AIF1: GGG CAA AAU CGA UAA UUC U[dT][dT] (AIFvar1); AIF2: GCA GAC UUU CUC UGU GUA U[dT][dT] (AIFV) (Delettre et al., 2006).

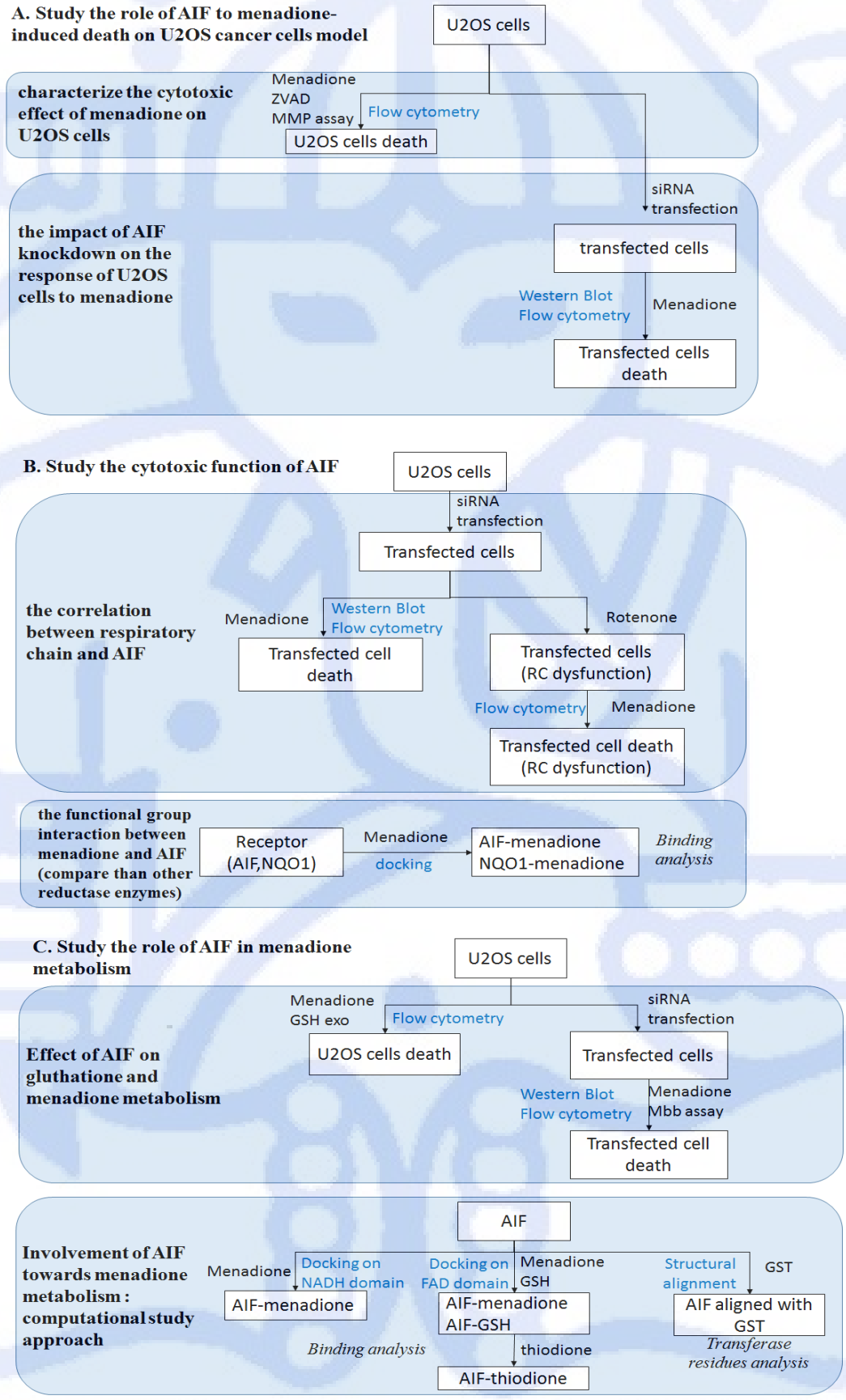

Figure III. 1 Scheme of research

III.2 Place and Time of Research

Mammalian cell culture, cellular manipulation experiments, drug/antioxidant treatments, flow cytometry and western blot analysis were performed at the Laboratory directed by Prof. Kroemer (INSERM U848) at the Institut Gustave Roussy-France, for two years (since January 2013-January 2015). In silico experiments were performed at Biochemistry laboratory of ITB-Indonesia, for one year and six months (January 2015-July 2016).

III.3 Cell culture and Treatment

III.3.1 Cell Culture

Human Osteosarcoma U2OS cells (ATCC n°HTB-96) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) PS. Culturing should always be done with aseptic technique in sterile conditions. Cells were cultured in flask 8 cm, were placed into 37^{o}C 5% CO_2 incubator, and medium was changed at 3-4 days intervals. Cells should be checked microscopically daily to ensure they are healthy and growing as expected. Attached cells should be mainly attached to the bottom of the flask, round and elongated in shape and refracting light around their membrane. Suspension cells should look round, plump, and refracting light around their membrane. Some suspension cells may clump. Media should be pinky orange in color.

III.3.2 Cell Counting

Culturing cells for drug treatment involved plating 120,000 cells per well (6 well plate). The bottles contain culture medium, DPBS, and trypLE were warm in 37^{o}C water bath for at least 30 minutes. When ready, media from one flask of the required cells was aspirated into a waste pot (containing laboratory disinfectant). Cells were washed with DPBS 10 mL, three times. Then, trypLE 2 mL was added to cells in order to detach cells from the bottom of flask. Cells were incubated into incubator for around 5 minutes. Trypsinized-cells were moved from flask to falcon 50 mL. Flask was washed with 10 mL medium, then the cells were added to same falcon. Cells ready for quantifying and further experiments. For cell counting, cell suspension need to be mixed well by gentle agitation of the falcon. Before the cells

have a chance to settle at all, about 1 mL of cell suspension was taken out using pipette, was placed into new falcon, and was added with tryphan blue. Cells were quantified using hemocytometer. One drop of cell suspension was transferred to hemocytometer under the coverslip. The suspension will be drawn under the coverslip by capillary action and cells are ready to be counted. Cells were counted in each of four squares of hemocytometer. The average cell was multiplied by 50,000. The final value is the number of viable cells/mL in the original cell suspension. Then, the value was divided by the number of cells in each well (in this case 120,000 cells) in order to calculate dilution value of cell suspension.

III.3.3 Seed Cell

Suspension cells were diluted using complete medium in order to get 120,000 cells per well (6 well plate). Fresh complete medium 1 mL was added to each well. Suspension cells 1 mL were taken by pipet 10 mL and were added drop by drop to each wells. Cell culture plates were placed into incubator for 20 hours before using for an assay.

III.3.4 Drug Treatment

The drugs are menadione, DMNQ, BZQ, and MTX. All drugs were prepared to get appropriate concentration for cell treatment. The menadione powder stock (molecular weight 172.2 g/mol) was freshly prepared in ethanol to get final concentration 50 µM. First, 50 mM menadione stock solution was prepared by dissolving 25.8 mg of menadione in 3 mL of ethanol, then it was vortexed to mix the solution. The falcon of menadione solution was wrapped with aluminium foil to prevent light exposure. Menadione intermediate solution was prepared by diluting 50 µL of menadione stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells without treatment in ethanol containing medium used as negative control. Cells in plate with menadione treatment were incubated in cell incubator for 3 hours.

The 20 µM DMNQ final concentration was prepared in DMSO. The DMNQ powder stock (molecular weight 218.21 g/mol) was freshly prepared in DMSO to get final concentration 20 µM. First, 46 mM DMNQ stock solution was prepared by dissolving 30 mg of DMNQ in 3 mL of DMSO, then it was vortexed to mix the solution. The falcon of DMNQ solution was wrapped with aluminium foil to prevent light exposure. DMNQ intermediate solution was prepared by diluting 17.9 µL of DMNQ stock solution in 50 mL of complete medium in order to get 20 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells without treatment in DMSO containing medium used as negative control. Cells in plate with DMNQ treatment were incubated in cell incubator for 48 hours.

BZQ was prepared in H_2O. The BZQ powder stock (molecular weight 108.09 g/mol) was freshly prepared in H_2O to get final concentration 120 µM. First, 90 mM BZQ stock solution was prepared by dissolving 30 mg of DMNQ in 3 mL of H_2O, then it was vortexed to mix the solution. The falcon of BZQ solution was wrapped with aluminium foil to prevent light exposure. BZQ intermediate solution was prepared by diluting 66.7 µL of BZQ stock solution in 50 mL of complete medium in order to get 120 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells without treatment in complete medium used as negative control. Cells in plate with BZQ treatment were incubated in cell incubator for 3 hours.

MTX was prepared in H_2O. The MTX powder stock (molecular weight 517.4 g/mol) was freshly prepared in H_2O to get final concentration 50 µM. First, 20 mM MTX stock solution was prepared by dissolving 30 mg of MTX in 3 mL of H_2O, then it was vortexed to mix the solution. The falcon of MTX solution was wrapped with aluminium foil to prevent light exposure. MTX intermediate solution was prepared by diluting 125 µL of MTX stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells without treatment in

complete medium used as negative control. Cells in plate with MTX treatment were incubated in cell incubator for 3 hours.

III.3.5 Antioxidant Treatment

Antioxidants stock solution prepared in order to get 50 µM GSH (NAC). The GSH stock solution (molecular weight 307.32 g/mol) was prepared in H_2O to get final concentration 50 µM. First, 163 mM GSH stock solution was prepared by dissolving 150 mg of GSH in 3 mL of H_2O, then it was vortexed to mix the solution. GSH intermediate solution was prepared by diluting 15.3 µL of GSH stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Complete medium used as negative control. Cells in plate with GSH treatment were incubated in cell incubator for 3 hours. The NAC stock solution (molecular weight 163.19 g/mol) was prepared in H_2O to get final concentration 50 µM. First, 613 mM NAC stock solution was prepared by dissolving 300 mg of NAC in 3 mL of H_2O, then it was vortexed to mix the solution. NAC intermediate solution was prepared by diluting 4.1 µL of NAC stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells without treatment in complete medium used as negative control. Cells in plate with NAC treatment were incubated in cell incubator for 3 hours.

For combination experiment GSH_menadione treatment, intermediate solution was prepared by diluting 50 µL of menadione stock solution and 15.3 µL of GSH stock solution in 50 mL of complete medium in order to get 50 µM of menadione and 50 µM of GSH in intermediate medium. For combination experiment NAC_menadione treatment, intermediate solution was prepared by diluting 50 µL of menadione stock solution and 4.1 µL of NAC stock solution in 50 mL of complete medium in order to get 50 µM of menadione and 50 µM of NAC in intermediate medium. Cells in complete medium, ethanol in complete medium, 50 µM of GSH in complete medium, and 50 µM NAC in complete medium. used as

negative control. Cells in plate with combination treatment were incubated in cell incubator for 3 hours.

III.3.6 Caspase Inhibitor Treatment

Caspase inhibitor ZVAD.fmk stock solution was prepared in H_2O. The ZVAD.fmk powder stock (molecular weight 453.5 g/mol) was prepared in H_2O to get final concentration 50 µM. First, 111 mM ZVAD.fmk stock solution was prepared by dissolving 5 mg of ZVAD.fmk in 1 mL of H_2O, then it was vortexed to mix the solution. ZVAD.fmk intermediate solution was prepared by diluting 227 µL of ZVAD.fmk stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells pre-treated with 50 µM ZVAD.fmk in medium for 1 hour. Then, medium containing cells was replaced with medium solution that containing of drugs (50 µM menadione or 20 µM DMNQ or 120 µM BZQ) in presence or absence 50 µM ZVAD.fmk. Cells were treated at the indicated times of incubations. Cells in complete medium used as negative control. Cells in menadione treatment used as positive control.

III.4 Analysis of Morphology and Autofluoresence of the Cell

The morphology of cells were observed and analyzed by FACS machine, BD^{TM}LSR II Flow Cytometer. After treatment, culture medium in each well were transferred to falcon tube. Then, cells were washed with PBS and transferred to the same tube. Cells were trypsinized with 300 µL of trypLE each well, and transferred to the same tube. Pelleted cells by centrifugation at 1,600 rpm for 8 minutes. The cell pellet was re-suspended into 100 µL complete medium and was transferred to FACS tube. Enough DAPI were prepared for sample in each tube. DAPI powder stock was prepared in deionized water. First, 14.3 mM DAPI stock solution was prepared by dissolving 10 mg of DAPI in 1 mL of solvent. Then it was vortexed to mix the solution. DAPI intermediate solution was prepared in order to get 2 µM DAPI in each tube. 1.5 µL DAPI (14.3 mM) was added to 5 mL of complete medium. 100 µL of DAPI intermediate medium was added to suspension cells in each tube just before each passage in the FACS machine. FACS machine was set to count 10,000

cells. For cell morphology, Flow cytometry analysis were achieved by measuring cell size (FSC) related to granularity of the cell (SSC) and by studying the membrane permeabilization (DAPI staining). Autofluoresent product was achieved by measuring cell size (FSCFSC) related to granularity of the cell (SSC) and auto-fluoresencence (FITC).

III.5 Measurement of GSH in the Cell

After treatment, medium of the cells in each wells were transferred to falcon tube. Then, cells were washed with PBS and transferred to the same tube. Cells were trypsinized with 300 µL trypLE per well, and transferred to the same tube. Cells were pelleted by centrifugation at 1,600 rpm for 8 minutes. Pellet washed with 2 mL of DMEM in order to discard cysteine was containing in the culture medium, then cells suspension were centrifuged at 1,600 rpm for 8 minutes. 10 mM MBB stock solution was prepared in DMSO. 2.7 g MBB powder was diluted in 1 mL DMSO, it was vortexed to mix the solution. MBB intermediate solution was prepared by adding 5 µL MBB to 1 mL of DMEM, in order to get 50 µM MBB in each tube. The pellet was re-suspended into 50 µM MBB in 200 µL DMEM. Cell suspensions incubate for 10 minutes (37°C, 5% CO_2), and then transferred to the FACS tube. Flow cytometry analysis were achieved by measuring the autofluorescence of the cells (FITC and Pacific Orange) and cell size (FSC) related to granularity of the cell (SSC). Cells without treatment in ethanol containing medium used as control.

III.6 Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was determined using Mitoprobe DilC1(5) Assay kit. After menadione treatment, cells medium in each well were transferred to falcon tube. Then, cells were washed with PBS and transferred to the same tube. Cells were trypsinized with 300 µL trypLE each well, and transferred to the same tube. The cell pellet was prepared by centrifugation at 1,600 rpm for 8 minutes. The cell pellet was re-suspended into 200 µL DMEM or 200 µL of 50 nM DilC1, and transferred to FACS tube. Then, cells were incubated for 30 minutes. 100 µL DAPI intermediate solution was added to each tube just before each passage in the

machine. Flow cytometry analysis were achieved by measuring cell size (FSC) related to granularity of the cell (SSC) and cells autofluorescent (FITC/APC). Treatment cells with CCCP was used as positive control. The CCCP stock solution was prepared in DMSO. 10.2 mg of CCCP powder was diluted in 1 mL DMSO in order to get 50 mM CCCP stock solution. Then it was vortexed to mix the solution. CCCP intermediate solution was prepared by diluting 50 µL of CCCP stock solution in 50 mL of complete medium in order to get 50 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells in DMSO containing medium used as negative control. Cells in plate with menadione treatment were incubated in cell incubator for 3 hours. Then, cells were prepared for FACS machine.

III.7 Rotenone Assay

Cells pre-incubated with 1 µM rotenone for 1.5 hours, then followed by menadione treatment for 3 hours. Rotenone stock solution was prepared in DMSO. 0.5g rotenone powder (molecular weight 394.41 g/mol) was diluted in 1 mL DMSO in order to get 1 mM stock solution. Then it was vortexed to mix the solution. Rotenone intermediate solution was prepared by diluting 5 µL of rotenone stock solution in 50 mL of complete medium in order to get 1 µM intermediate medium. Old medium of cells in plate was discarded and was replaced with intermediate medium. Cells in DMSO containing complete medium, ethanol containing complete medium, rotenone in complete medium used as negative control. Cells in menadione treatment in absence of rotenone used as positive control.

Cells were trypsinized with 300 µL per well, and transferred to the same tube. The cell pellet was prepared by centrifugation at 1,600 rpm for 8 minutes. The pellet was re-suspended into 100 µL complete medium and transferred to facs tube. 3 µM DAPI solution was added to each tube just before each passage in the machine. For cell morphology, Flow cytometry analysis were achieved by measuring cell size (FSC) related to granularity of the cell (SSC) and by studying the membrane permeabilization (DAPI staining).

III.8 Cell Transfection

The day before transfection, cells were seeded at a density of 160 000 per well (6 wells plates) in order to obtain an approximate confluency of 60 to 70% at the time of transfection. The ratio of *si*RNA to Lipofectamine that we used was 1:3 (meaning that for 1 µg of siRNA, 3 µL of lipofectamine were added). For each transfection, lipofectamine/*si*RNA complexes were prepared as follows: the solution A, prepared by diluting 1.3 µg of siRNA in 500 µL of Optimem medium, was added to the solution B (3.9 µL of lipofectamine diluted in 500 µL of Optimem medium) and incubated for 15 minutes at room temperature. Then, the culture medium was aspirated from the wells and replaced with the siRNA/lipofectamin mixture. After 2 hours of incubation, 1 mL of DMEM medium supplemented by 20% (v/v) FBS was added to each well and cells were re-incubated for 16 hours. The day after transfection, the transfection medium was aspirated and cell was incubated for 2-3 days in presence of the regular culture medium (complete medium).

III.9 Cell Lysate Preparation

Total cell lysates were prepared as follows: culture medium was transferred to the tube. Then, cells were washed with PBS and transferred to the same tube. Cells were trypsinized with 300 µL trypLE each well, and transferred to the same tube Cell suspension was centrifuged 1,600 rpm, 8 minutes to get the pellet. After washing with PBS 2 times, then cells were lysed with 1% SDS in PBS. The lysates were collected in Eppendorf tubes and then heated to 100°C for 4 minutes. After, a sonication step (3x 10 seconds) that is necessary to break the viscosity due to high molecular weight DNA, lysates were stored frozen at -20°C. The proteins in the lysate were quantified using the colorimetric assay kit "Dc Protein Assay" based on the protocol of Lowry.

III.10 Protein Quantification

30 µL cell lysate was prepared for protein quantification. After thawing at room temperature, aliquot was diluted (five times for transfection cell lysate; 10 times for non-transfection cell lysate) with SDS 1% (5µL aliquot + 15µL SDS 1%), then was vortexed. In protein quantification BSA used as a standard. BSA standard solution

was prepared with 0;0.2;0.4;0.6;0.8;1.0;1.5 (μg/μL) in SDS 1%. Solution A+S was prepared by adding 20 μL solution S into 1 mL solution A. Then all solutions were prepared in the plate with 96 well. 5 μL of BSA standard solution or sample was added in the well. Then, 25 μL solution A+S was added to the same well, followed by 200 μL solution B. The surface of well was covered by aluminium foil. The solution was incubated for 20 minutes before passage to the machine (absorbance microplate reader, BMG Labtech FluoStar Optima Plate Reader).

III.11 Western blot

Western blot was used to visualize proteins that have been separated by gel electrophoresis. The gel was placed next to a nitrocellulose membrane and an electrical current caused the proteins to migrate from the gel to the membrane. The membrane then be probe by antibodies and detection reagents. Solutions and reagents that were prepared are TBST, milk, migration buffer, and transfer buffer. Migration buffer consist of 950 mL miliQ and 50 mL MES SDS running buffer. For each experiment used 200 mL of migration buffer. Transfer buffer consist 10% (v/v) Tris-HCl buffer, and 20% (v/v) methanol. TBST consist of 900 mL miliQ, 100 mL TBS, and 500 mL tween 20% (v/v). 5 % (v/v) milk solution was prepared by adding 2 g milk powder into 40 mL TBST. For sample preparation, 50 μL cell lysate was thaw at room temperature. To each sample to be analyzed, the loading buffer (4% (v/v) SDS, 20% (v/v) glycerol, 125 mM Tris pH 6.8, 0.2 M dithiothreitol (DTT) and FAQs H_2O) was added 1:1 (v/v). After heating for 4 minutes at 100°C, samples were loaded on a SDS-PAGE pre-cast (NUPAGE gel - Invitrogen) gel and the proteins were separated by migration in 1x MES buffer (Invitrogen) for about 2 hours at 100 V before being electro-transferred to a nitrocellulose membrane (Biorad - 0.2 microns). The membrane was activated with methanol for 1 minute and rinsed with transfer buffer before preparing the stack. The stack was prepared as follows; the back side of sandwich box was placed at the bottom. The gels then were placed in the 'transfer sandwich' (whatman paper-gel-membrane-whatman paper). The transfer was placed in the transfer box. Transfer process for 1 hour at 100 V in the cold room. Then, the membrane was washed with DPBS 1x5 minutes followed by coloring with *Rounge Pounceau* for 5 minutes. The membrane was

washed with PBS 3x5 minutes and continued with TBST 1x5 minutes. After blocking non-specific binding sites in 5% (v/v) milk/TBST-0.05% (v/v) Tween (for 1 hour at room temperature), and then for further 16 hours at 4°C with the specified primary antibody diluted in the same incubation mixture supplemented with 0.02% v/v Na-azide. The day after, the membrane was washed 3x5 minutes with TBST and incubated for 1 hour with 1:5000 (v/v) the secondary antibody coupled to Horseradish peroxidase (source mouse). The membrane was incubated for 1 hour at room temperature (on shaker). Strep T actin HRP 1:5000 was added to the membrane 10 minutes before incubation completely. After discarding medium, the membrane was washed with TBST 3x5 minutes. Antibody binding was revealed by chemiluminescence using an ECL detection kit as follows: the membrane was placed on the paper wathman, 2 mL ECL reagent A was added to 2 mL ECL reagent B and was mixed well, 2 mL mixed ECL reagent was added to the surface of nitrocellulose membrane. After incubating for 4 minutes, the membrane ready to check to the ECL chemiluminescence system Premium/Select and the Image Quant LAS 4000 camera (GE healthcare).

III.12 Computational Study

Computational study were applied in order to confirm the involvement of AIF in menadione cytotoxicity, especially to predict the interaction of menadione to AIF compare to other mitochondrial reductase enzyme, NQO1. This study also applied to investigate the specific role of AIF in the modulation of menadione arylation, that could not be revealed by in vitro cellular experiments. Computational approaches were applied by structural alignment which was employed using *FATCAT* software and molecular docking studies which were performed by *AutoDock Vina* software. Docking studies restricted using rigid structure of each receptor, which were retrieved from the Protein Data Bank (http://www.rscb.org/pdb).

III.12.1 Protein and Ligand Preparation for *In Silico* study

The crystal structure of Apoptosis Inducing Factor (AIF), Cytochrome P450 reductase (CPR), NQO1, and gluthatione-S-transferase (GST) with PDB code

subsequently 3GD4, 1AMO, 2F10, and 1B48 in complex with its ligands were retrieved from the Protein Data Bank (http://www.rscb.org/pdb) (Sevrioukova, 2009; Xiao et al., 1999). The complexes, heteroatoms and water molecules bound to the receptor molecules were removed from each protein structure. Finally hydrogen atoms were merged to the target receptor molecules. Protein preparation have done using *AutoDock Vina* software (Trott and Olson, 2010). Ligands used in this study; oxidized Flavin Adenine Dinucleotida (FAD), Nicotinamide Adenine Dinucleotide (NADH), Nicotinamide Adenine Dinucleotide Phosphate ($C_{21}H_{28}N_7O_{17}P_3$, NADPH), 2-methyl-1,4-naphthoquinone (menadione), 1,4-benzoquinone (BZQ), 2,3-dimetoxy-1,4-naphthoquinone (DMNQ), reduced gluthatione ($C_{10}H_{17}N_3O_6S$, GSH), menadione-S-SG (thiodione), BZQ-S-SG, and bishydroxy [2H-1-benzopyran-2-one,1,2-benzopyrone] ($C_{19}H_{12}O_6$, Dicumarol, DTC). All ligands were sketched using *Marvin Sketch* software. Dreiding energy lowest was built to optimize ligands using Marvin sketch and further used for computational studies.

III.12.2 Validation Methods for Docking Study

Validation process of docking program was applied to confirm the feasibility of parameters are used in this study. Receptor-ligand interaction (AIF-NADH or NQO1-DTC or CPR-NADPH) of computational study compared to receptor-ligand interaction of crystal structure. Docking study was applied using *AutoDock Vina* software. Validation parameters used such as the amount and type of contact residues, and Root Mean Standard Deviation (RMSD) of ligands between reference and experiment. Experiment method will be validated when the value of RMSD≤2.

III.12.3 Docking Studies using *AutoDock Vina* Software

The docking analysis of receptors with ligands was carried out by *AutoDock Vina* docking software which is most commonly available software. Grid resolution was set to 1 Å, located on NADH (or FAD) binding domain of AIF, CPR, and NQO1. Affinity energy indicated the easy binding of ligand and receptor.

III.12.4 Interaction Receptor-Ligand Analysis

Interaction between docked ligand and its receptor analyzed by *AutoDock Vina* software Energy analysis of AIF with ligands was carried out by *AutoDock Tools*. Parameters that measured are a number of close residues, hydrogen bond, and the distance between functional groups of ligand and close residues of receptor. Further analysis was applied to investigate the suitability of functional group and residues in order to measure the type of other interaction.

III.12.5 Superposition and Active Site Analysis

3D structure alignment of AIF and GST was employed in order to know the folding similarity of both protein, especially on transferase binding domain. This analysis was performed by *FATCAT*-3D structural alignment (Ye and Godzik, 2003). Alignment result become a standard to create grid boxes for investigating transferase domain on AIF. This domain used as target domain of menadione binding. Then, interaction analysis was applied to find contact residues of menadione on AIF. Then, transferase residues of GST used as standard for finding the transferase catalytic site of menadione binding on AIF.