CHAPTER III

MATERIALS AND METHODS

Animals

Male Wistar rats obtained from the National Laboratory Animal Center of Salaya Campus, Mahidol University. Each weighed 100-120 g.

Cells

Leukocytic cell lines used were as follows:

- MonoMac 6 (MM6), a human mature monocytic cell line (provided by Prof. Dr. H.-W. Löms Zeigler-Heitbrock, University of München, Germany)

- THP-1, a human monoblastic cell line; Jurkat, a human leukemic T cell line, and Raji, a human lymphoma B cell line (All were provided by the MRC Toxicology Unit, University of Leicester, U.K.)

Chemicals

Methomyl (obtained with courtesy of Du Pont (Thailand) Co. Ltd.; RPMI 1640 medium with GLUTAMAX II, phosphate buffer saline (PBS), penicillin, and streptomycin (Life Technologies), RNase A (Promega); fetal calf serum, acetonitrile, ethanol, disodium ethylenediaminetetraacetic acid (EDTA), 2,3-diphosphoglycerate (2,3-DPG) test kit, and propidium iodide (Sigma); annexin V-FITC and annexin buffer (Alexis Biochemicals); tetramethylrhodamine ethyl ester (TMRE) (provided by the MRC Toxicology Unit, University of Leicester); benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk) (Enzyme Systems Products); interleukin-6 (IL-6) (R&D Systems Europe, Ltd.); milk; polyclonal caspase-3 antibody, anti-rabbit polyclonal antibody conjugated with horse radish peroxidase, and ECL kit (MERCK); 30% acrylamide mix (Protogel®), ammonium persulfate, β-mercaptoethanol, bromophenol blue, commassie brilliant blue R250, glacial acetic acid, glycerol, glycine, hydrochloric acid (HCl), methanol, sodium dodecylsulfate (SDS), TEMED, Tris, and tween 20 (provided by Lab. 231, Department of Microbiology and Immunology,

University of Leicester, U.K.); buffered ammonium chloride-potassium (ACK) solution (provided by Allergy and Clinical Immunology Department, Faculty of Medicine, Chulalongkorn University), dichlorophenol-indolphenol (DCIP) reagent (see "*In Vivo* Assays, Secion 2.2."); and gluteraldehyde, osmium tetroxide, uranyl acetate, and lead citrate (provided by the Scientific and Technological Research Equipment Center, Chulalongkorn University).

Equipment

Clean incubator set up at 37 °C supplied with 5% CO_2; laminar air flow hood; Becton-Dickinson flow cytometry; spectrophotometry; refrigerated centrifuge; JEOL (JEM-200CX) transmission electron microscope.

In Vitro Assays

1. Roles of Methomyl and Acetonitrile on Apoptosis

1.1. Condition for Cell Cultures

These cell lines were maintained in RPMI 1640 medium with GLUTAMAX II supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 ° C with 5% carbon dioxide (CO_2).

1.2. Treatments

Cell lines were treated with the following conditions:

1.2.1. Methomyl at 0, 0.2, 0.4, 0.8, 1.5, 3, 6, 12, 18, and 30 mM for 6 hours.

1.2.2. Methomyl at 0, 0.2, 0.4, 0.8, 1.5, 3, 6, 12, 18, and 30 mM for 24 hours.

1.2.3. Acetonitrile at 0, 0.1, 0.5, 1, 5, 10, and 30% for 6 hours.

The cells were collected at 5 x 10^5 to 1 x 10^6 cells per milliliter for flow cytometric analysis.

1.3. Flow Cytometric Analysis (See Appendix C)

1.3.1. Detection of Cell Cycle Profiles and Sub-G_1 Population in Propidium Iodide Stained Cells (Hotz et al., 1994)

1 x 10^6 cells were resuspended in 200 µl phosphate buffer saline (PBS), pH 7.2, and fixed by 2 ml of 70% ethanol in PBS, at -20 °C overnight, washed with PBS, resuspended in 800 µl PBS, and incubated with 100 µl RNase A (1 mg/ml) and 100 µl propidium iodide (PI) (50 µg/ml PBS) for 30 minutes at 37 °C prior to flow cytometric analysis. Cell cycle profiles were demonstrated as DNA content frequency histograms, the population of apoptotic cells with fractional DNA content would present itself as a sub-G_1 area.

1.3.2. Detection of Exposed Phosphatidyl Serine on the Plasma Membrane Using Annexin V-FITC and Propidium Iodide (Piqué et al., 2000)

Cell viability was determined by analysing phosphatidylserine exposure and membrane integrity by double staining 1 x 10^6 cells (in 1 ml annexin buffer) with 1.5 µl of annexin V-FITC for 10 min. and 10 µl of PI (50 µg/ml PBS) for 5 min. at room temperature prior to flow cytometric analysis. Apoptotic cell death was measured as the percentage of annexin V- or annexin V- and PI-positive cell population.

1.3.3. Detection of Changes in Mitochondrial Transmembrane Potential ($\Delta\Psi m$) Using Tetramethylrhodamine Ethyl Ester (Piqué et al., 2000)

Changes in $\Delta\Psi$m were determined by staining 1 x 10^6 cells (in medium) with 1 µl of 100 µM tetramethylrhodamine ethyl ester (TMRE) for 10 min. at 37 °C prior to flow cytometric analysis. The loss in $\Delta\Psi$m was seen as a shift to lower red fluorescence.

2. Determination of Role of Caspases in Apoptosis

2.1. Benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone

Role of caspases in methomyl induced apoptosis was determined by preincubation of THP-1 cells with a nonspecific caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(Ome)-fluoromethylketone (zVAD-fmk), at 0, 1, 10, and 100 μM for 30 minutes prior to adding 0, 12, 24, 30 mM of methomyl. After 6 hour incubation, the inhibition of apoptosis by zVAD-fmk was detected by flow cytometric analysis with TMRE and annexin V-FITC (see section 1.3.).

2.2. Western Blot Analysis of Caspase-3 Cleavage

2.2.1. Jurkat cells were treated with the following conditions:

- Methomyl at 0, 6, 18 mM for 6 hours.
- Acetonitrile at 0, 1, 5, 10 % for 6 hours.

2.2.2. Cells were collected and kept as the following procedure:

Collected cells
↓
Centrifuge 400 g, 4 min
↓
Pellets
↓
Washed with PBS (centrifuge 400 g, 4 min)
↓
Pellets
↓
Kept at -80 °C

2.2.3. Samples were prepared as the following procedure:

Frozen samples
↓
Adding 50 μl of sample buffer
↓ Vortex
Sonication (2x5 sec.) — Keep on ice
↓
Place in boiling water for 4 min.
↓
SDS gel electrophoresis

2.2.4. SDS gel electrophoresis and detection

SDS gel electrophoresis
(100 volts for the stacking gel, 130 volts for the lower gel)
↓
Transferring to hybond membrane
(80 volts for 2h)
↓
Blocking overnight by PBS-Tween + 5% milk at 4 °C
↓ Washing in PBS-Tween
Agitating with polyclonal caspase-3 Ab (diluted 1/1000 in PBS-Tween) for 1h
↓ Washing step 1
Agitating with anti-rabbit polyclonal antibody conjugated with horse radish peroxidase
(diluted 1/2000 in PBS-Tween + 5% milk) for 1h
↓ Washing step 2
ECL kit (1 minute exposure)
↓
Showing of the detection by autoradiography film

*** Note:** See recipes for SDS gel electrophoresis and washing protocol in "Appendix D".

3. Determination of Role of Interleukin-6 in Apoptosis

Role of interleukin-6 (IL-6) in methomyl induced apoptosis was determined by incubation of MM6, THP-1, and Jurkat cells with IL-6 (50 ng/ml) and methomyl (0, 6, 12, 18, 24, and 30 mM) for 6 hours prior to flow cytometric analysis with TMRE (see Section 1.3.).

In Vivo Assays

1. Flow Cytometric Analysis of Lymphocyte Apoptosis

1.1. Treatments

Rats were divided into 3 groups (n=6) and treated with the following conditions:

Group 1: Control

Group 2: Methomyl 4 mg/kg body weight (single dose, p.o.)

Group 3: Methomyl 8 mg/kg body weight (single dose, p.o.)

After 6 hours, blood was collected and leukocytes were obtained by following the procedure of sample preparation:

1.2. Sample Preparation (a method provided by Allergy and Clinical Immunology Department, Faculty of Medicine, Chulalongkorn University)

Blood was collected using 1.5 mg/ml EDTA as an anti-clotting agent
↓
Red blood cells were lysed using buffered ACK solution
↓ 5 min.
PBS added
↓
Centrifugation 1500 rpm for 5 minutes
↓
Pellet
↓
Washed 3 times in PBS (1500 rpm, 5 minutes)
↓
Resuspending in annexin buffer
↓
Flow cytometric analysis

1.3. Flow Cytometric Analysis of Lymphocyte Apoptosis by Detection of Exposed Phosphatidyl Serine on the Plasma Membrane Using Annexin V-FITC and Propidium Iodide (Piqué et al., 2000)

Similar to the *in vitro* assay, lymphocyte cell viability was determined by analysis of phosphatidylserine exposure and membrane integrity by double staining cells with annexin V-FITC and PI prior to flow cytometric analysis. Apoptotic cell death was measured as the percentage of annexin V- or annexin V- and PI-positive cell population.

2. Analysis of Erythrocyte NADH-DCIP Reductase Activity

2.1. Treatments

Rats were divided into 3 groups (n=6) and treated with the following conditions:

Group 1: Control

Group 2: Methomyl 4 mg/kg body weight (single dose, p.o.)

Group 3: Methomyl 8 mg/kg body weight (single dose, p.o.)

After 6 hours, blood was collected (with 1.5 mg/ml EDTA as an anti-clotting agent) and followed the procedure of sample preparation:

2.2. Preparation of 0.19 mM DCIP (Dichlorophenol-indophenol) Reagent

Trizma Base	4.36	g
EDTA $Na_2.2H_2O$	2.68	g
DCIP (M.W. 290.1 g)	0.0276	g
Saponin	0.05	g
Distilled water q.s. to	500	ml
Adjust pH to 7.5 with HCl		

2.3. Determination of NADH-DCIP Reductase Activity (Adapted from Zurbriggen and Dreyer, 1996)

In a cuvet, 20 µl of erythrocytes was added into 100 µl of 1.3 mM NADH, followed after 30 seconds by the addition of 2.5 ml of 0.19 mM DCIP. After incubation for 90 seconds at room temperature, the absorbance change at 600 nm was measured over 5 minutes.

The decrease in absorbance overtime indicated an increase in NADH-DCIP reductase activity. The difference between the absorbance at 5 minutes and 0 minutes in the treated group was compared to that of control. Result was shown as -fold increase.

3. Analysis of 2,3-Diphosphoglycerate (2,3-DPG) Levels in Blood

3.1. Treatments

Rats were divided into 3 groups (n=6) and treated with the following conditions:

Group 1: Control

Group 2: Methomyl 4 mg/kg body weight

Group 3: Methomyl 8 mg/kg body weight

After 24 hours, blood was collected and followed the procedure of sample preparation:

3.2. Determination of 2,3-DPG levels (Sigma Diagnostics, Inc., 2000)

Sigma Diagnostics 2,3-DPG reagents were used. The reagents include: trichloroacetic acid solution 8% w/v; nicotinamide adenine dinucleotide (reduced form; NADH); triethanolamine buffer solution; adenosine-5'-triphosphate (ATP) solution; glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate phosphokinase (GAPD/PGK) enzyme mixture; phosphoglycerate mutase; and phosphoglycolic acid solution.

After 24 hours of treatments, blood was collected from rats and followed the following process:

3.2.1. Preparation of Protein-Free Supernatant

1) Pipet 1.0 mL of freshly drawn blood (with 1.5 mg/ml EDTA as an anti-clotting agent) into 3.0 mL of cold trichloroacetic acid 8%. Shake vigorously for several seconds. Keep mixture cold for an additional 5 minutes to assure complete protein precipitation.

2) Centrifuge 10 minutes at approximately 3000 rpm to obtain clear supernatant.

3.2.2. Analysis

3) Into a 1-mg NADH vial, pipet 8.0 mL triethanolamine buffer solution. Cap and invert several times to dissolve the NADH.

4) Into a cuvet, pipet 2.5 mL solution from Step 3, 0.1 mL ATP solution and 0.25 mL protein-free supernatant (from Step 2). Mix by inversion.

5) Then, add 0.02 mL GAPD/PGK enzyme mixture and 0.02 mL phosphoglycerate mutase. Mix by inversion. Wait 5 minutes.

6) Read and record absorbance at 340 nm vs water as reference. This is INITIAL A.

7) Add 0.1 mL phosphoglycolic acid solution and mix by inversion.

8) Let stand for 30 minutes at room temperature or 15 minutes at 37 °C to allow reaction to go to completion.

9) Read and record absorbance at 340 nm vs water as reference. This is FINAL A.

10) To determine 2,3-DPG concentration, refer to the "Calculations" section.

3.2.3. Calculations

ΔA = INITIAL A - FINAL A

Corrected ΔA = ΔA - 0.030

Blood 2,3-DPG (μmol/mL) = Corrected ΔA x 7.7

The factor is derived as follows: $\frac{2.99}{6.22 \times 0.0625} = 7.7$

Where:

2.99 = Volume (mL) of reaction mixture in cuvet

6.22 = Absorbance at 340 nm of a solution containing 1 NADH/mL

0.0625 = Volume (mL) of original sample in reaction mixture

The three enzymatic reactions involved in the described procedure are as follows:

1) 2,3-DPG is hydrolysed to 3-phosphoglycerate (3-PGA) and inorganic phosphorus. The enzyme which catalyzes this reaction is present in purified preparations of phosphoglycerate mutase (PGM) and is termed 2,3-DPG phosphatase. 2-Phosphoglycolic acid is needed as a stimulator for this reaction.

$$\text{2,3-DPG} \xrightarrow[\text{2-Phosphoglycolic acid}]{\text{2,3-DPG phosphatase}} \text{3-PGA} + \text{Pi}$$

2) 3-PGA reacts with ATP in the presence of PGK to form 1,3-diphosphoglycerate (1,3-DPG) and ADP.

$$\text{3-PGA} + \text{ATP} \xrightarrow{\text{PGK}} \text{1,3-DPG} + \text{ATP}$$

3) 1,3-DPG oxidizes NADH to NAD in the presence of GAPD and is reduced to G-3-P.

$$\text{1,3-DPG} + \text{NADH} \xrightarrow{\text{GAPD}} \text{G-3-P} + \text{NAD}$$

Measuring the decrease in absorbance at 340 nm caused by the oxidation of NADH to NAD reflects the amount of 2,3-DPG originally present.

4. Electron Microscopy

4.1. Treatments

Rats were divided into 3 groups (n=6) and treated with the following conditions:

Group 1: Control

Group 2: Methomyl 4 mg/kg body weight (single dose, p.o.)

Group 3: Methomyl 8 mg/kg body weight (single dose, p.o.)

4.2. Sample Preparation (a method of the Scientific and Technological Research Equipment Center, Chulalongkorn University)**:**

After 6 hours, spleens were removed, cut into small pieces and fixed in a solution containing 2.5% of gluteraldehyde in 0.1 M PBS pH 7.4 at 4 °C. The samples were processed as follows:

Rinsing of samples with PBS 3 times (15 min. each time)
↓
Fixation in 1% osmium tetroxide in 0.1 PBS for 1 hour at room temperature
↓
Washing in distilled water for 15 minutes
↓
Dehydration in 35%, 50%, 70%, and 95% ethanol for 15 minutes each time
and dehydration in absolute ethanol 3 times (15 min. each time)
↓
Infiltration in absolute ethanol: spurr resin (3:1 for 2 h; 1:1 for 2 h; and 1:3 for 2 h)
and infiltration in spurr resin 3 times (2 h each time)
↓
Embedding of samples in beam capsule
↓
Polymerization at 70 °C for 8 hours
↓
Sectioning by LKB ultramicrotome
(Sections obtained were in the range of 60-90 nm)
↓
Collecting of sections with 300 meshed-copper grids
↓
Section staining in uranyl acetate and lead citate
before viewing in the transmission electron microscope
with accelerating voltage 100 KV

Data Analysis

- Flow cytometric results were expressed as the mean percentage of apoptotic cell death of treated cells $\pm$ S.E. compared to that of the control by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant. The same statistical analysis was used in the comparison of percentage of cells with the reduced $\Delta\Psi m$ and of percentage of cells in the sub-G_1 area between the control and treated groups.
- Percentage of cell death at the same dose of methomyl exposure between cells treated and untreated with zVAD-fmk was compared by using the unpaired student t-test, p-value of less than 0.05 was regarded as significant.
- Percentage of cell death at the same dose of methomyl exposure between cells treated and untreated with IL-6 was compared by using the unpaired student t-test, p-value of less than 0.05 was regarded as significant.
- The increase in NADH-DCIP reductase activities of the treated groups (expressed as numbers of fold-increased) was compared with controls by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant.
- The 2,3-DPG levels of treated and control groups were compared by using the ANOVA coupled with Dunnett test, p-value of less than 0.05 was regarded as significant.