CHAPTER III
RESEARCH METHODOLOGY

3.3 Chemicals and materials

Andrographolide, L-buthionine-(S,R)-sulfoximine (BSO), and glutathione (GSH, reduced form) were supplied by Wako Pure Chemical (Tokyo, Japan). Beta-naphthoflavone (β-NF) and N-acetyl-L-cysteine (NAC) were obtained from Sigma Chemical Co (St. Louis, MO). 3-methylchrolanthene (3-MC) was a product of Eastman Kodak Co., (Rochester, NY). Testosterone propionate was purchased from Kasei TCI (Tokyo, Japan). ReverTraAce® is a product of Toyobo Co., Ltd. (Osaka, Japan). SYBR® Green I and G-Taq Labo Pass™ were products of Cambrex Bio Science Rockland, Inc. (East Rutherford, NJ) and Hokkaido System Science Co., Ltd. (Hokkaido, Japan), respectively. The TaqMan® Gene Expression Assays were products of Applied Biosystems (Branchburg, NJ).

Materials for culturing hepatocytes were purchased from Gibco® Invitrogen Cell Culture (Carlsbad, CA), BioWhittaker™ Cambrex Bio-Sciences (Walkerville, MD), and Wako Pure Chemical (Osaka, Japan). Percoll and collagenase (Type I) were products of GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and Sigma Chemical Co., respectively.

Materials for assessment of enzyme activities, namely ethoxyresorufin, methoxyresorufin, β-nicotinamide adenine dinucleotide phosphate (NADPH), and resorufin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibody against rat CYP1A1/CYP1A2 for Western blotting was a gift from Dr. Y. Funae (Osaka City University, Osaka, Japan). The Amersham Pharmacia Biotech Co. (Uppsala, Sweden) supplied Hybond-C membranes for blotting. All other laboratory chemicals were of the highest available purity from commercial suppliers.

3.4 Preparation of primary cultures of mouse hepatocytes and treatments

The livers of C57BL/6 male mice (Sankyo Laboratories, Shizuoka, Japan) at 7 weeks of age were perfused with collagenase, and viable hepatocytes were isolated by

Percoll isodensity centrifugation (Nemoto & Sakurai, 1995). Standard culture conditions were as follows: the cells were dispersed in Waymouth MB752/1 medium containing bovine serum albumin (2 g/L), insulin (0.5 mg/L), transferrin (0.5 mg/L), and selenium (0.5 μg/L), and seeded at a density of 5×10^5 cells/1.5mL/35-mm collagen-coated dish. The Waymouth medium did not contained phenol red, a pH indicator, to exclude the possibility of estrogen-like action. Depending on the presence of cell attachment factors, the hepatocytes were anchored to the dishes within 3 h and subsequently formed a monolayer. The culture dishes were maintained at 37°C in a CO_2-humidified incubator. The medium was renewed 3 h after plating and then, 24 h later, treatments were started as follows to study effect of andrographolide on cytochrome P450 expression. Total RNA was harvested at 24 h after starting of the treatment.

1) 0.2% dimethyl sulfoxide (DMSO, control)
2) 10 μM beta-naphthoflavone (β-NF)
3) 25 μM andrographolide (Andro)
4) 10 μM β-NF + 25 μM Andro

To study the effect of glutathione (GSH) on synergistic CYP1A1 induction, hepatocytes were treated with 0.2% DMSO, 25 μM andrographolide, 10 μM β-NF, 10 μM β-NF+ 25 μM andrographolide in the presence of either GSH-content modulators, 10-20 mM GSH, 1-10 mM NAC (a prototype of GSH), or a GSH inhibitor (75-300 μM BSO) as follows. Total RNA was harvested at 24 h after starting of the treatment.

1) DMSO
2) Andrographolide (Andro)
3) β-NF
4) β-NF+Andro
5) β-NF+Andro+GSH
6) β-NF+Andro+NAC
7) β-NF+Andro+BSO

Measurement of lactate dehydrogenase (LDH) activity was carried out to indicate non-toxic compound at those concentrations of the treatments. Total RNA was prepared by the method of guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987). The concentration of total RNA was measured before kept at -20°C for further studies.

3.5 Animals designs and treatments

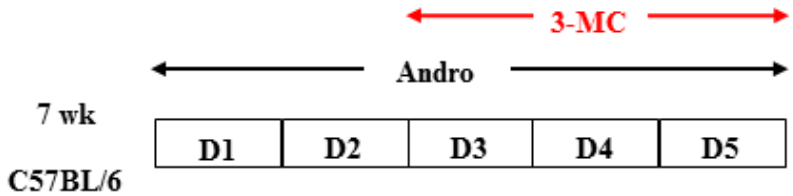
Male and female C57BL/6 mice at 5-7 weeks of age were supplied by National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. All mice were housed in animal unit of faculty of Pharmaceutical Sciences, Khon Kaen University under the supervision of the Khon Kaen University Animal Ethics Committee for Use and Care, Khon Kaen, Thailand (Approval No. AEKKU 0101/2550 and AEKKU 09/2553). At all times, the mice were housed on wood shaving bedding in stain-less steel cages, with water and a commercial mouse diet supplied *ad libitum*. The mice quarters were air conditioned (22 ± 2ºC) and had a 12 h light/dark cycle.

3.5.1 Experiment in the normal mice

Seven weeks-old mice of both sexes (n=5) were subcutaneously injected daily with 5 mg/kg/day of andrographolide for 5 days, and/or intraperitoneally given 20 mg/kg/day of 3-methylcholanthrene (3-MC) for 3 days. Treatments in the first experiment (normal male and female mice) were as follows.

1) Corn oil (control)
2) Andrographolide (Andro) 5 mg/kg/day, s.c., for 5 days
3) 3-MC 20 mg/kg/day, i.p., for 3 days
4) 3-MC+Andro (Co-tx)

The mice were sacrificed at 24 h after the last treatments, and the livers were immediately excised for preparation of total RNA and microsomes as described elsewhere (Nemoto & Sakurai, 1992).

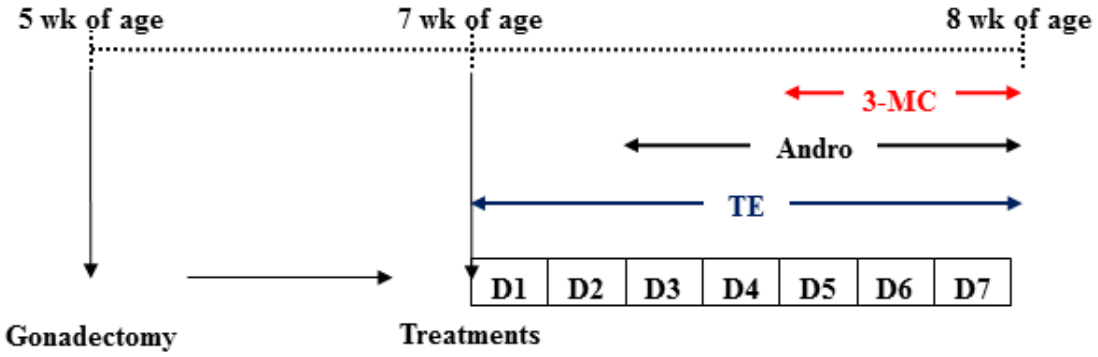


3.3.2 Experiment in the gonadectomized mice

In the second part, mice at 5 weeks of age were orchiectomized or ovariectomized. Two weeks after the gonadectomy, treatments were started. The treatments with andrographolide (Andro) and/or 3-MC were as same as the first part described above. In some cases, testosterone propionate (TE) at 5 mg/kg/day was subcutaneously injected daily for 7 days, starting two days before administration of andrographolide. These compounds were dissolved in corn oil and the final administration volume is 0.1 ml.

Treatments in the second experiment (gonadectomized mice) were as follows.

1) 3-MC
2) 3-MC+TE
3) 3-MC+Andro
4) 3-MC+Andro+TE



The control group was intraperitoneally given 20 mg/kg/day of 3-MC for 3 day to compare synergism of CYP1A1 induction in the castrated mice. The mice were sacrificed at 24 h after the last treatments, and the livers were immediately excised for preparation of total RNA and microsomes as described elsewhere (Nemoto & Sakurai, 1992).

3.6 Microarray analysis

Total RNA from mouse hepatocytes was re-purified using a FastPure™ RNA kit (TaKaRa®, Shiga, Japan), and its concentration and quality were examined using a NanoDrop ND-1000 (NanoDrop Technologies, DE, USA) and Agilent® 2100 Bioanalyzer (Agilent Technologies, CA, USA). Total RNA samples were submitted to Bio Matrix Research, Inc. (Chiba, Japan) for analysis using a GeneChip® Mouse Exon 1.0 ST array (Affymatreix Technologies, Tokyo, Japan) according to the standard protocol. The microarray data was analyzed using GeneSpring GX10 software (Agilent Technologies).

3.7 Quantitative real-time RT-PCR

After the microarray analysis, the expression of mouse CYPs and GAPDH mRNAs was determined by quantified real-time reverse transcription polymerase chain reaction (RT-PCR). Complementary DNA (cDNA) was synthesized by ReverTraAce® and G-Taq Labo Pass™ under the conditions recommended by the supplier (Applied Biosystems, Branchburg, NJ) using specific TaqMan® Gene Expression Assays (Inventoried) for *Cyp1a2*, *Cyp1b1*, *Cyp2b10*, *Cyp3a11*, *Ugt1a6*, and specific TaqMan® MGB Gene Expression Detection kits for *Cyp1a1*, *Cyp2b9*, *and Cyp3a41*, or SYBR® Green I for *Cyp2a4* and GAPDH (Table 2). Real-time PCR was performed using the ABI Prism® 7000/7500 Sequence Detection System (Applied Biosystems) with ABI Prism® 7000/7500 SDS software. The PCR conditions were as followed: denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The amplified products of CYP1A1, CYP1A2, CYP1B1, CYP2B9, CYP2B10, CYP3A11, CYP3A41, and UGT1A6 were directly detected by monitoring the fluorescence of the reporter dye (FAM), for which an increase in fluorescence signal was detected only if the target sequence is complementary to the probe and amplified by the PCR. The amplified PCR products of CYP2A4 and GAPDH were monitored directly by measuring the increase in SYBR® Green that is bound to double-stranded DNA amplified by PCR.

3.8 Western blotting

Livers were immediately excised and cut into small pieces before homogenization with ice-cold 1.15% KCl. Microsomal fractions were prepared after ultracentrifugation of the 10,000×*g* supernatant at 104,000×*g* for 60 min at 4°C. The microsomal protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (Nemoto & Sakurai, 1992). Five or twenty micrograms of microsomal proteins were resolved by 10% SDS–PAGE and then transferred to a Hybond-C membrane. The CYP species localized were detected using a rabbit polyclonal antibody against rat CYP1A1 protein, which cross-reacted with CYP1A2, followed by a horseradish peroxidase conjugated goat anti-rabbit IgG antibody, and then were visualized with 3,3′-diaminobenzidine and hydrogen peroxide (Sakuma et al., 1999).

3.9 Assessment of ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) activities

Ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) activities were determined by the method of Sakuma et al. (1999) with some modifications. A reaction mixture containing 10 mM Tris-HCl (pH 7.8), 200 µM NADPH, 0.5 mg of hepatic microsomes, and 50 µM ethoxyresorufin or methoxyresorufin in a final volume of 3.5 ml were incubated at 37°C for 3 min. Subsequently, the formation of resorufin was measured by spectrofluorometry with an excitation wavelength of 530 nm and emission wavelength of 585 nm every 30 seconds for 10 times. An increase of intensity (Y-axis) was plotted versus time (X-axis) and the slope of regression line was employed to calculated EROD or MROD activities in pmol/min/mg protein, compared to the intensity of resorufin standard.

3.10 Determination of glutathione content

Hepatocytes were cultured at a density of 1.5×10^6 cells/4 mL/60 mm collagen-coated dishes, and scraped after adding 5% metaphosphoric acid. Cell pellets were collected in 1.5 mL-microtube, followed by sonication, and centrifugation at 10,000 x*g* at 4°C for 10 min. After centrifugation, the supernatant was serially diluted and

transferred to a 96-wells microplate for determination of the GSH contents. Total GSH, GSH (reduced form), and GSSG (oxidized form) were measured using the HT glutathione assay kit (Trevigen®, Gaithersburg, MD). After adding the reaction mixture to each well, absorbance was measured at wavelenght 405 nm, compared to the standard GSSG. Glutathione reductase reduces GSSG to GSH. The sulfhydryl group of GSH reacts with DTNB (5,5′-dithiobis-2-nitrobenzoic acid) to produce a yellow color of 5-thio-2-nitrobenzoic acid (TNB) with possessed absorbance at wavelength 405 nm, and the mixed disulfide (GSTNB). GSTNB is reduced by glutathione reductase to recycle the glutathione and produces more TNB. The rate of TNB production was directly proportional to the recycling reaction which in turn proportional to the concentration of glutathione in the sample. The measurement of TNB absorbance of at 405 nm provided an accurate estimation of glutathione in the sample (Trevigen, Inc., 2007).

3.11 Determination of reactive oxygen species (ROS)

Hepatocytes, at 3 or 24 h after starting of the treatment with β-NF and/or andrographolide, were stained by 2′,7′-dichlorodihydrofluorescein diacetate (DFC-DA, 5μM) (Royall & Ischiropoulos, 1993), BES-H_2O_2 (10 μM: H_2O_2 specific), or BES-So (10 μM: O_2- specific) for 30 min, and then they were detached by Accutase (Innovative Cell Technologies). The cells were stained by propiodium iodide (PI) for determination of dead cells. After staining, the fluorescence was measured at filter list 1 (530/30 nm, FL1) and filter list 3 (long pass 650 nm, FL3) wavelength by FACSCanto II flow cytometer (BD Biosciences, NJ, USA). PI-negative and ROS-positive cells were gated and the percentages of the fluorescent high cells in the gated cells were calculated.