CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Forty male Wistar albino rats and forty female Wistar albino rats (250-300 g) were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakon Pathom, Thailand. Animals were housed three per cage at Medical Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Bangkok and acclimatized for at least a week prior to the experiment. All animals were in a controlled humidify room at a constant temperature of 22-25°C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to standard diet and water throughout the study.

2. Equipments

The following instruments were used in the experimentation.

1. Autopipettes 20, 100, 200 and 1000 µl (Rainin Instrument, USA)
2. Autopipettes 5000 µl (Gibson, France)
3. Fluorescence, Absorbance and Luminescence reader VICTOR^3 V model (Perkin Elmer, USA)
4. Metabolic shaker bath (Heto, Denmark)
5. 96-well black plates (Perkin Elmer Life Sciences, USA)
6. pH meter (Beckman Instruments, USA)
7. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Heidolph, Germany)
8. Reagent reservoir (Rainin Instrument, USA)
9. Refrigerated centrifuge (Allegra X-12R, Beckman Coulter, USA)
10. Refrigerated superspeed centrifuge (Beckman Instruments, USA)
11. Refrigerated ultracentrifuge (Beckman Instruments, USA)
12. Sonicator (Elma, Germany)
13. Spectrophotometer (Shimadzu, Japan)
14. Timer (Citizen®)

15. Multi-chanel pipette (Rainin Instrument, USA)
16. Multi-chanel Tips (Rainin Instrument, USA)
17. Autopipettes Tips (Gibson, France)
18. Ultra-low temperature freezer (Forma Scientific, USA)
19. Vortex mixer (Clay Adams, USA)

3. Enzymes and Chemicals

The Vivid® CYP450 Blue Screening Kits was obtained from Invitrogen Corporation, USA. It consists of five components as following:

- CYP450 BACULOSOMES® Reagents which consist of recombinant human cytochrome P450 (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) and rabbit NADPH-cytochrome P450 reductase (CYP2E1 also contains human cytochrome *b*5)

- Vivid® Substrate which may be 7-benzyloxymethyloxy-3-cyanocoumarian (BOMCC) or ethyloxymethyloxy-3-cyanocoumarian (EOMCC) ether of fluorescent dyes depending on CYP isoforms

- Regeneration System which consists of Glucose-6-phosphate (G6P) and Glucose-6-phosphate dehydroginase (G6PD) in potassium phosphate pH 8.0

- $NADP^+$ in potassium phosphate pH 8.0

- Vivid® CYP450 Reaction Buffer which may be Buffer I, II, III depending on CYP isoforms. All beffer are potassium phosphate pH 8.0 but they are different in concentration.

The standardized extract of *C. asiatica* (ECa 233) was supplied by Associate Professor Dr. Ekarin Saifah, Faculty of Pharmaceutical Sciences, Chulalongkorn University. ECa 233 is a white to off white extracted powder of *C. asiatica* containing triterpenoid glycosides not less than 80% and the ratio of medecassoside and asiaticoside content should be within 1.5 ± 0.50.

The following chemicals were purchased from Sigma Chemical Co. Ltd., USA: Adenosine 3′-phosphate-5′-phosphosulfate (PAPS), bovine serum albumin (BSA), cupric sulfate, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenol-indophenol (DCPIP), dicumarol, dimethylsulfoxide (DMSO), ethylene diaminetetraacetic acid (EDTA), Folin & Ciocalteu's phenol reagent, glutathione reduced form (GSH), magnesium chloride ($MgCl_2$), nicotinamide adenine dinucleotide reduced form (NADH), α-naphthoflavone, *p*-nitrophenol sulfate,

potassium phosphate monobasic anhydrous (KH_2PO_4), sulfaphenazole, sodium carbonate (Na_2CO_3), sodium citrate, sodium phosphate dibasic anhydrous (Na_2HPO_4), triton X-100, Trizma® base, UDP-glucuronic acid.

Acetonitrile anhydrous was purchased from Labscan Asia, Thailand.

Ethanol, hydrochloric acid (HCl), *p*-nitrophenol, potassium chloride (KCl) and trichloroacetic acid (TCA) were purchased from Merck, Germany.

Ketoconazole was obtained from Siam Pharmaceutical, Thailand.

Imipramine was obtained from Siam Chemi-Pharm (1997), Thailand.

Miconazole nitrate was obtained from the Government Pharmaceutical Organization, Thailand.

2-Naphthol was purchased from Aldrich, USA.

Potassium phosphate dibasic anhydrous (K_2HPO_4) and sodium hydroxide (NaOH) were purchased from Univar, USA.

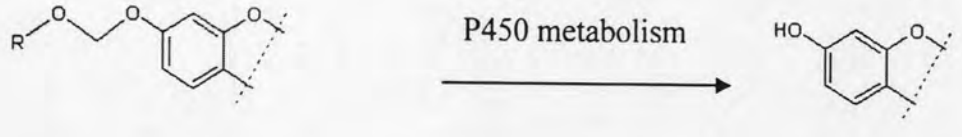
Sodium chloride was purchased from Ajax Finechem, Australia.

Ultrapure water was prepared by ELGASTAT MAXIMA UF® (ELGA, England).

Methods

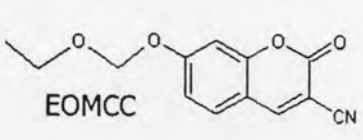
1. *In vitro* assessment of inhibitory effects of ECa 233 on human cytochrome P450

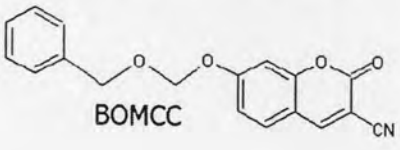
Inhibition of CYP450 activities were determined by using the Vivid® CYP450 Screening Kits (www.invitrogen.com). The Vivid® substrates were BOMCC and EOMCC are metabolized by specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions.



BOMCC, EOMCC

3-cyano-7-hydroxycumarin





Reagent

1. ECa 233 solution

ECa 233 solution for CYP1A2, CYP2C19 or CYP2B6 was freshly prepared by dissolving 0.05 g of the powder in 150 µl of DMSO and dilute to 10 ml with ultrapure water. This stock solution was then diluted to various final concentrations with DMSO/water as following.

Isozyme Type	Final Concentrations of ECa 233 solution (µg/ml)	DMSO in Final Concentrations (%v/v)
1A2	15.63, 31.25, 62.5, 125, 250, 500, 750 and 1,000	0.3
2C19	62.5, 125, 250, 375, 500, 600, 750 and 1,000	0.3
2B6	15.63, 62.5, 125, 250, 500, 750, 1,000 and 2,000	0.6

ECa 233 solution for CYP2D6, CYP2C9, CYP3A4 or CYP2B6 was freshly prepared by dissolving 0.025 g of the powder with 50 µl of DMSO and dilute to 10 ml with ultrapure water. This stock solution was then diluted to various final concentrations with DMSO/water as following.

Isozyme Type	Final Concentrations (µg/ml)	DMSO in Final Concentrations (%v/v)
2D6	62.5, 125, 250, 375, 500, 600, 750 and 1,000	0.2
2C9	62.5, 125, 250, 300, 375, 500, 600 and 750	0.15
3A4	62.5, 125, 250, 300, 375, 500, 600 and 750	0.15
2E1	25, 37.5, 50, 75, 100, 150, 200 and 250	0.05

The final concentrations of ECa 233 solution was limited by ECa 233 solubility and solvent tolerances for different CYP isoforms.

2. Master pre-mix for 100 wells

Dilute CYP450 BACULOSOMES® Reagents and Regeneration System in Vivid® CYP450 Reaction Buffers as following. The dilution was done on ice and mix well by inversion. The solutions were kept on ice until ready to use.

Isozyme Type	Vivid® CYP450 Reaction Buffer (µl)	Regeneration System (µl)	CYP 450 BACULOSOME® Reagent (µl)
1A2	4850 (buffer I)	100	50
2B6	4800 (buffer I)	100	100
2C9	4800 (buffer II)	100	100
2C19	4850 (buffer II)	100	50
2D6	4800 (buffer I)	100	100
2E1	4850 (buffer III)	100	50
3A4	4850 (buffer I)	100	50

3. Reconstituted substrate

Reconstitute the Vivid® substrate using anhydrous acetronitrile as following. The solutions were kept at room temperature for immediate use or stored at -20°C.

Isozyme Type	Vivid® Substrate (0.1 mg/tube)	Acetronitrile added per tube (µl)
1A2	EOMCC	205
2B6	BOMCC	160
2C9	BOMCC	160
2C19	EOMCC	205
2D6	EOMCC	205
2E1	EOMCC	205
3A4	BOMCC	160

4. Pre-mixture of reconstituted substrate and $NADP^+$ for 100 wells

Mix reconstituted substrate, $NADP^+$ and Vivid® CYP450 Reaction Buffers as following. The solutions were prepared on ice and mixed by vortex

Isozyme Type	Vivid® Substrate (mg/tube)	Reconstituted Substrate (µl)	$NADP^+$ (µl)	Vivid® CYP450 Reaction Buffers (µl)
1A2	EOMCC	15	100	885
2B6	BOMCC	25	100	875
2C9	BOMCC	50	100	850
2C19	EOMCC	50	100	850
2D6	EOMCC	50	100	850
2E1	EOMCC	50	100	850
3A4	BOMCC	50	100	850

5. 0.5 M Tris-HCl buffer pH 10.5 (MW of Trizma base = 121.1)

Trizma base 6.06 g. was dissolved and made up to 100 ml with ultrapure water. The solution was adjusted to pH 10.5 with NaOH or HCl.

Procedures

1. Measure fluorescence intensity of the 96-well microplate before use for background correction to corresponding wells.
2. Microplate set-up.

   Each plate includes triplicates for test compound (ECa 233 solution) at each test compound concentration, background of test compound (corrected for fluorescence intensity due to the test compound), solvent control (corrected for inhibition due to the solvent) and background of solvent control (corrected for fluorescence intensity due to the solvent). A typical plate set-up is described and represented schematically below.

<table>
<tr><td colspan="12">Well Number</td></tr>
<tr><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td colspan="8" rowspan="2"></td><td colspan="3">← Triplicates for solvent control →</td><td></td></tr>
<tr><td colspan="8">← Triplicates for test compound at 8 different concentrations →</td><td colspan="3">←Triplicates for background→ of solvent control</td><td></td></tr>
<tr><td colspan="8"></td><td colspan="3"></td><td></td></tr>
<tr><td colspan="8"></td><td colspan="3"></td><td></td></tr>
<tr><td colspan="8">← Triplicates for background of test compound →</td><td colspan="3"></td><td></td></tr>
<tr><td colspan="8"></td><td colspan="3"></td><td></td></tr>
</table>

Test compound: 40 µl of ECa 233 solution at different concentration and 50 µl of Master pre-mix

Background of test compound: 40 µl of ECa 233 solution at different concentration and 50 µl of Reaction Buffers

Solvent control: 40 µl of solvent used in ECa 233 solution and 50 µl of Master pre-mix

Background of solvent control: 40 µl of solvent used in ECa 233 solution and 50 µl of Reaction Buffers

The solution mixture in the plate was orbitally shaken for 15 seconds and linearly shaken for 15 seconds. The plate was preincubated for 20 minutes at room temperature.

3. The reaction was initiated by an addition of 10 µl of pre-mixture of reconstituted substrate and $NADP^+$ into each well. The solution mixture in the plate was shaken for 30 seconds as previously.
4. The plate was protected from light and incubated at room temperature for 30 minutes (CYP1A2, CYP2B6, CYP3A4, CYP2C9, CYP2C19 and CYP2E1) and 60 minutes (CYP2D6).
5. At the end of incubation, the reaction was stopped by addition of 10 µl of 0.5 M Tris-HCl buffer pH 10.5 to each well and shaken for 30 seconds as previously.
6. The plate was transferred into the fluorescent plate reader and the fluorescence intensities were read immediately using the excitation wavelength 390 ± 20 nm and emission wavelength 450 ± 10 nm.

Calculations

Percent inhibition was calculated for each concentration of test compound by using the following equation.

$$\% \text{ inhibition} = \left(1 - \frac{(RFU_{\text{test compound}} - RFU_{\text{background of test compound}})}{(RFU_{\text{solvent control}} - RFU_{\text{background of solvent control}})} \right) \times 100$$

RFU - relative fluorescence unit or fluorescent intensity

The median inhibition concentration (IC_{50}) was calculated for the test compound using Probit analysis.

Verification of the Vivid® CYP450 Screening Kits Protocol

The procedure in the protocol was verified by determination of IC_{50} of known inhibitors. The reaction was performed as mentioned above using α-naphthoflavone, miconazole, sulfaphenazole, miconazole, miconazole, imipramine and ketoconazole as an inhibitor for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 isoforms, respectively (Mark and Larson, online; Mark et al., 2002). Each plate includes duplicates for an inhibitor at each inhibitor concentration, background of an inhibitor (corrected for fluorescence intensity due to the inhibitor), solvent control (corrected for inhibition due to the solvent) and background of solvent control (corrected for fluorescence intensity due to the solvent).

2. Determination of phase II drug metabolizing enzymes activities

2.1 Animal treatment

Male or female rats were randomly divided into 4 treatment groups. Each treatment group comprised 10 rats as following:

1. Control group: Rats were orally administered with 1 ml/kg/day ultrapure water once daily for 90 days.
2. ECa 233 treatment group 1: Rats were orally administered with ECa 233 at a daily dose of 10 mg/kg/day for 90 days.
3. ECa 233 treatment group 2: Rats were orally administered with ECa 233 at a daily dose of 100 mg/kg/day for 90 days.
4. ECa 233 treatment group 3: Rats were orally administered with ECa 233 at a daily dose of 1,000 mg/kg/day for 90 days.

ECa 233 was freshly prepared by dissolving 1 g of the powder extract with 1 ml of distilled water to make a concentration of 10, 100 and 1,000 mg/ml of ECa 233 suspension.

The oral dosages of ECa 233 (10, 100 and 1,000 mg/kg/day) used in this study were based on the finding dosage that ECa 233 demonstrated a positive effect on learning and memory impairment at the dose of 10 and 30 mg/kg (unpublished data).

2.2 Liver cytosolic and microsomal preparation

At the end of the treatment, the overnight-fasted rats were anesthesized with diethyl ether by inhalation before collecting livers. Rat liver cytosolic and microsomal

fractions were prepared according to the method described by Lake (1987) with some modifications.

Reagents

1. 0.1 M Phosphate buffer pH 7.4

   1 L of 0.1 M phosphate buffer pH 7.4 containing 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4 and 11.50 g of KCl. The solution was adjusted to pH 7.4 with NaOH or HCl.

2. 0.1 M Phosphate buffer pH 7.4 containing 20% v/v glycerol

   1 L of 0.1 M phosphate buffer pH 7.4 containing 200 ml of glycerol, adjusted to pH 7.4 with NaOH or HCl.

3. 0.9% w/v of NaCl

Procedures

1. After anesthesized with diethyl ether, rat liver was quickly perfused through the portal vein with ice-cold 0.9% w/v NaCl until the entire organ become pale, then the liver was immediately removed form the body.
2. Liver was rinsed with ice-cold 0.9% w/v NaCl and blotted dry with gauze.
3. The whole liver was weighed, cut into prices and homogenized in 3 fold volume of ice-cold 0.1 M phosphate buffer pH 7.4.
4. The liver homogenate was centrifuged at 10,000 *g* for 30 minutes at 4°C, using refrigerated superspeed centrifuge to pellet intact cells, cell debris, nuclei and mitochondria.
5. The supernatant was transferred into ultracentrifuge tube and further centrifuged at 100,000 *g* for 60 minutes at 4°C, using refrigerated ultracentrifuge.
6. The supernatant (cytosolic fraction) was aliquoted collected in microtubes and stored at -80°C until the time of enzyme activity assay.
7. The pellets (microsomal fraction) were resuspended with 5 ml of 0.1 M phosphate buffer pH 7.4 containing 20% v/v glycerol. The microsomal suspensions were aliquoted, kept in microtubes and stored at -80°C until the time of enzyme activity assay.

2.3 Determination of protein concentrations

The protein concentrations of liver cytosolic and microsomal fractions were determined according to the method modified from the method of Lowry et al. (1951).

Reagents

1. 2% w/v Na_2CO_3

   30 g of anhydrous Na_2CO_3 was dissolved with 1.5 L of ultrapure water.

2. 0.5 M NaOH

   5 g of NaOH was made up to 250 ml in ultrapure water.

3. 2% w/v Sodium citrate

   0.4 g of sodium citrate was dissolved in ultrapure water 20 ml.

4. 1% w/v Cupric sulfate

   0.2 g of cupric sulfate was dissolved with 20 ml of ultrapure water.

5. 1 mg/ml BSA in 0.5 M NaOH

   0.02 g of BSA was dissolved with 20 ml of 0.5 M NaOH.

6. Folin & Ciocalteu's phenol reagent

7. Working protein reagent

   The solution was freshly prepared in a sufficient amount for all tubes in the assay (6.5 ml of the solution was required for each tube). This reagent comprised 2% w/v Na_2CO_3, 0.5 M NaOH, 2% w/v sodium citrate and 1% w/v cupric sulfate solutions in a 100:10:1:1 ratio by volume, respectively. This reagent must be clear during the assay.

Procedures

1. 16 x 125 mm tubes were labeled in duplicate for 8 standards (0, 50, 100, 150, 200, 250, 300 and 400 µg) and for each unknown sample.

2. 0.5 M NaOH and 1 mg/ml BSA were added into each standard tubes as following:

Standard tube (µg)	0	50	100	150	200	250	300	400
1 mg/ml BSA (µl)	0	50	100	150	200	250	300	400
0.5 M NaOH (µl)	500	450	400	350	300	250	200	100

   After addition of the reagents, each tube was vortex-mixed thoroughly.

3. To each unknown sample tube, 490 µl of 0.5 M NaOH and 10 µl of cytosol or microsome were added and then mixed thoroughly.

4. After adding 6.5 ml of working protein reagent to each tube, the tubes were allowed to stand at room temperature for 10 minutes.

5. Then, 200 µl of Folin & Ciocalteu's phenol reagent was added to each tube and immediately vortexed for at least 30 seconds.
6. After the tubes were placed at room temperature for at least 30 minutes, the absorbance of the solution in each tube was measured spectrophotometrically at 500 nm using the 0 µg standard tube as a blank.

Calculations

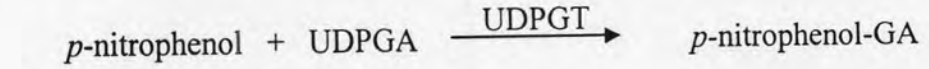
1. The average absorbance of each standard was plot against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.
2. The protein concentration (mg/ml or µg/µl) in each unknown sample was obtained by dividing its amount of protein (from step 1) with the volume of cytosolic or microsomal sample used in the reaction.

2.4 Determination of enzyme activities

All enzyme assays were performed in duplicate. Every method was verified for linearity and precision before using for determination of the enzyme activity in the cytosolic or microsomal samples.

2.4.1 Determination of UDP-glucuronosyltransferase (UDPGT) activity

Liver microsomal UDPGT activity was assayed using *p*-nitrophenol as a substrate and UDPGA as a co-enzyme according to the method of Bock et al (1973) with some modifications. The reaction was shown as following:



The absorbance of a remained *p*-nitrophenol was measured spectrophotometrically at 405 nm.

Reagents

1. 1 M Tris-HCl buffer pH 7.4 (MW of Trizma base = 121.1)
   121.1 g of trizma base was dissolved and made up to 1 L with ultrapure water. The solution was adjusted to pH 7.4 with NaOH or HCl.
2. 50 mM $MgCl_2 \cdot 6H_2O$ (MW = 203.31)

1.017 g of $MgCl_2 \cdot 6H_2O$ was dissolved and made up to 100 ml with ultrapure water.

3. 5% w/v Trichloroacetic acid (MW = 163.39)

   50 g of trichloroacetic acid was dissolved with 1 L of ultrapure water.

4. 2 M NaOH (MW = 40)

   20 g of NaOH was dissolved and made up to 250 ml with ultrapure water.

5. 0.25% w/v Triton X-100

   0.05 g of triton X-100 was dissolved with 20 ml of ultrapure water.

6. 5 mM *p*-nitrophenol (MW = 139.11)

   0.0174 g of *p*-nitrophenol was dissolved and made up to 25 ml with ultrapure water. This reagent was freshly prepared and kept in a light protected container.

7. 30 mM UDPGA (MW = 646.2)

   0.01939 g of UDPGA was dissolved with 1 ml of ultrapure water.

Procedures

1. 0.5 ml of total reaction volume contained 600 µg of microsomal protein, 180 µl of ultrapure water, 1 M Tris-HCl buffer pH 7.4, 20 µl of 0.25% w/v Triton X-100, 50 µl of 50 mM $MgCl_2$ and 50 µl of 5 mM *p*-nitrophenol.
2. The solution in each tube was mixed and preincubated for 2 minutes at 37°C in a metabolic shaker bath.
3. The reaction was initiated with the addition of 50 µl of 30 mM UDPGA whereas 50 µl of 1 M Tris-HCl buffer pH 7.4 was added in the corresponding blank tube. The tubes were mixed and incubated for 10 minutes at 37°C in a metabolic shaker bath.
4. At the end of incubation, the reaction was stopped by addition of 1 ml of ice-cold 5% trichloroacetic acid, each tube was mixed and kept on ice.
5. The protein pellets were removed from the reaction mixture by centrifugation at 3,400 rpm for 15 minutes at 0°C.
6. 1 ml of the supernatant was transferred into another new tube.
7. 250 µl of 2 M NaOH and 2 ml of ultrapure water were added into the tube and mixed throughly. The tubes were protected from light.
8. The absorbance of *p*-nitrophenol in the solution was measured spectrophotometrically at 405 nm.

9. A standard curve of *p*-nitrophenol was generated by 5 concentrations of 5 mM *p*-nitrophenol solutions, 120 µl of 1 M Tris-HCl buffer pH 7.4 and 666 µl of 5% trichloroacetic acid were added into each standard tube containing various concentrations of *p*-nitrophenol which were prepared as following:

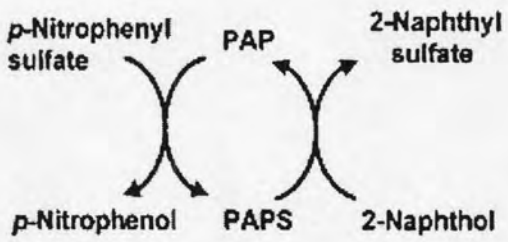
p-nitrophenol concentration (nmol/ ml)	90	110	130	150	170
5 mM *p*-nitrophenol (µl)	18	22	26	30	34
ultrapure water (µl)	196	192	188	184	180

Calculation of UDPGT activity

Amount of *p*-nitrophenol remaining in the reaction tube was determined by comparing its absorbance against a *p*-nitrophenol standard curve. The disappearance of *p*-nitrophenol was determined by subtracting the measured remaining *p*-nitrophenol in the sample tube from that in the blank tube. Microsomal UDPGT activity was calculated from amount of the disappered *p*-nitrophenol divided by the time of incubation (10 minutes) and amount of microsomal protein used in the reaction (0.6 mg).

2.4.2 Determination of sulfotransferase (SULT) activity

SULT activity was measured using 2-naphthol as a substrate as well as adenosine 3′-phosphate-5′-phosphosulfate (PAPS) and *p*-nitrophenylsulfate as co-enzymes according to the method described by Frame et al. (2000) with some modifications. The reaction was shown as following:



The absorbance of product (*p*-nitrophenol) was measured spectrophotometrically at 405 nm.

Reagents

1. 2% w/v $MgCl_2$

   1 g of $MgCl_2 \cdot 6H_2O$ was dissolved with 50 ml of ultrapure water.

2. 0.05% w/v 2-Naphthol (MW = 144.17)

   0.005 g of 2-naphthol was dissolved with 0.3 ml of ethanol and adjusted to 10 ml with ultrapure water. This solution was freshly prepared.

3. 5% w/v *p*-Nitrophenylsulfate

   0.05 g of *p*-nitrophenylsulfate was dissolved with 1 ml of ultrapure water. This solution was freshly prepared.

4. 0.025% w/v PAPS

   0.005 g of PAPS was dissolved with 20 ml of ultrapure water.

5. 0.0668 M Potassium phosphate buffer pH 6.5 (KH_2PO_4 MW = 136.09, K_2HPO_4 MW = 174.18)

   22.88 ml of 0.1336 M KH_2PO_4 and 10.52 ml of 0.1336 M K_2HPO_4 were mixed and made up to 1 L with ultrapure water. The solution was adjusted to pH 6.5 with NaOH or HCl (Deangelis, 2007).

6. 0.25 M Tris-HCl buffer pH 8.7 (MW of Trizma base = 121.1)

   30.275 g of trizma base was made up to 1 L with ultrapure water. The solution was adjusted to pH 8.7 with NaOH or HCl.

Procedures

1. The final volume of 1.5 ml reaction mixture contained 750 µg of cytosomal protein, 0.668 M potassium phosphate buffer pH 6.5, 76 µl of 2% w/v $MgCl_2$, 62 µl of 0.025% w/v PAPS and 76 µl of 2.5% w/v *p*-nitrophenylsulfate.
2. The tubes were protected from light and preincubated for 2 minutes at 37 °C in a metabolic shaker bath.
3. The reaction was initiated by an addition of either 44 µl of 0.05% w/v 2-naphthol or 44 µl of solvent as a blank. The tubes were mixed.
4. After 15 minutes incubation at the same conditions, the reaction was stopped by adding 1.5 ml of ice-cold 0.25 M Tris-HCl pH 8.7. The tube was kept on ice.
5. The reaction mixture was centrifuged at 3,500 rpm for 15 minutes at 2°C.
6. The supernatant was transferred into another new tube and protected from light.
7. The *p*-nitrophenol formation was determined at 405 nm by spectrophotometer within 30 minutes.

Calculation of SULT activity

The concentration of *p*-nitrophenol formed was calculated based on the extinction coefficient of 18.4 $mM^{-1}cm^{-1}$, which directly correlated to the concentration of 2-naphthylsulfate formed (Mulder et al., 1977). Results were expressed as nmol/min/mg protein by subtracting the blank absorbance from that of the sample, dividing the amount of *p*-nitrophenol by the time of 15 minutes incubation and amount of cytosolic protein (0.75 mg) used in reaction.

2.4.3 Determination of glutathione-*S*-transferase (GST)

Cytosolic GST activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and glutathione reduced form (GSH) as a co-enzyme according to the method described by Habig et al. (1980) with some modifications. The reaction was shown as following:

$$GSH + CDNB \xrightarrow{GST} \text{conjugation of CDNB with glutathione} + H_2O$$

The absorbance of product (conjugation of CDNB with glutathione) was measured spectrophotometrically at 340 nm.

Reagents

1. 20 mM CDNB in 95% ethanol (MW = 202.6)

   0.0406 g of CDNB was dissolved with 10 ml of 95% ethanol. This solution was freshly prepared and kept in light-protected container.
2. 20 mM GSH (MW = 307.33)

   0.0614 g of GSH was dissolved with 10 ml of ultrapure water. This solution was freshly prepared and protected from light.
3. 0.1 M Sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium (Na_2HPO_4 MW = 142, EDTA sodium MW = 372.2)

   7.1 g of sodium phosphate and 0.1861 g of EDTA sodium were dissolved and made up to 500 ml with ultrapure water. The solution was adjusted to pH 6.5 with NaOH or HCl.

Procedures

1. For each 1 ml of reaction mixture comprised 890 µl of 0.1 M sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium, 50 µl of 20 mM CDNB and 50 µl

of 20 mM GSH. The reagents were added to both the reference and sample cuvettes.

2. Both cuvettes were covered with paraffin and mixed well.
3. Both cuvettes were put in a spectrophotometer and the absorbance was adjusted to zero at 340 nm.
4. 10 µl of cytosol was pipetted into the sample cuvette to initiate the reaction, whereas 10 µl of 0.1 M sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium was pior added into the reference cuvette.
5. Both cuvettes were put back immediately into the spectrophotometer and then started measuring the absorbance.
6. The absorbance was recorded for 10 seconds.

Calculation of GST activity

Cytosolic GST activity was calculated by dividing the concentration of 2,4-dinitrophenylglutathione by the time and amount of cytosolic protein used in the reaction. The concentration of 2,4-dinitrophenyl-glutathione was calculated from the extinction coefficient of 9.6 mM^{-1} cm^{-1} (Jakoby, 1985). GST activity was expressed as nmol/min/mg protein.

2.4.3 Determination of NAD(P)H quinoneoxidoreductase (NQOR) activity

NQOR activity was mesured using 2, 6-dichlorophenol-indophenol (DCPIP) as a substrate and NADH as a co-enzyme according to the method of Ernster (1990) with slightly modifications. The reaction was shown as following:

$$\text{DCPIP} + \text{NADH} \longrightarrow \text{Reduced DCPIP}$$

The absorbance of product (reduced DCPIP) at 600 nm was measured using spectrophotometer.

Reagents

1. 0.1% w/v DCPIP (MW = 290.08)

   0.05 g of DCPIP was dissolved with 50 ml of ultrapure water and kept the solution in light protected container.

2. 0.0125% w/v Dicumarol (MW = 336.3)

   0.0125 g of dicumarol was dissolved with 1,000 µl of 5 M NaOH and made up to 100 ml with ultrapure water.

3. 2% w/v NADH (MW = 709.4)

   0.03 g of NADH was dissolved with 1.5 ml of ultrapure water. This solution was freshly prepared.

4. 5 M NaOH (MW = 40)

   20 g of NaOH was dissolved and made up to 100 ml with ultrapure water.

5. 0.68% w/v Tris-HCl buffer pH 7.5 (MW of Trizma base = 121.1)

   6.8 g of trizma base was dissolved with 1 L of ultrapure water and adjusted to pH 7.5 with NaOH or HCl.

6. 5% w/v Triton X-100

   2.5 g of triton X-100 was dissolved with 50 ml of ultrapure water.

Procedures

1. The reaction mixture was prepared in a cuvette.
2. For each 3 ml of reaction mixture comprised 0.68% w/v Tris-HCl buffer pH 7.5, 48 µl of 5% w/v Triton X-100, 54 µl of 2% w/v NADH and 45 µl of 0.1% w/v DCPIP. The reagents were added into the sample cuvette. The cuvette was covered with paraffin and mixed well. 3 ml of ultrapure water was added into the reference cuvette.
3. The reaction was initiated by an addition of cytosol (containing 500 µg of the cytosolic protein) into the sample cuvette.
4. The sample cuvette was immediately placed back to the spectrophotometer and the absorbance was recorded for 10 seconds.
5. In this study, cytosolic samples were nonpurified preparations and may contain other diaphorase enzymes therefore, dicumarol which was a selective inhibitor of the NQOR activity was added into the reaction. The parallel reaction was performed in the presence of 81 µl of 0.0125% w/v dicumarol instead of ultrapure water.

Calculation of NQOR activity

NQOR activity was determined from the extinction coefficient of 21 mM^{-1} cm^{-1} at 600 nm (Ernster, 1967, 1990). Absorbance difference between uninhibited sample and dicumarol-inhibited sample was divided by the time and amount of cytosolic protein (500 µg) used in the reaction. Unit of NQOR activity was expressed as nmol/min/mg protein.

Verification of the methods used for enzyme activity assay

Before using the methods for assay activities of UDPGT, SULT, GST and NQOR, the methods were verified for the linearity with respect to amount of protein used in the reaction. The reactions were performed as mentioned above. Linearity assays for UDPGT, GST and NQOR were performed by analyzing activity of the enzymes using liver microsome or cytosols from β-naphthoflavone-, phenobarbital- and estradiol-induced rat, respectively. Also, linearity assays for SULT was performed by analyzing activity of the enzymes using liver cytosols from normal rat. Coefficient of determination (R^2) between amounts of microsomal/cytosolic protein and the corresponding absorbance of the final reaction product was then calculated.

3. Statistical analysis

The data were presented as means ($\overline{X}$) ± standard error of the means (SEM). Mean differences between ECa 233 treated groups and control group were compared using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test and $p < 0.05$ was considered to be significant.