APPENDIX

Appendix 1. List of the chemicals and materials used in the study.

The chemicals used were analytical grade unless specified.

Chemicals/Materials	Source
Absolute ethanol	E. Merck, Germany
Absolute methanol (Analytical grade and HPLC grade)	J.T. Baker Inc., U.S.A
Ampicillin (U.S.P)	I.B.I.
Bacto agar	Difco laboratories, U.S.A
d-Biotin	Sigma chemical Co., U.S.A.
Corn oil (Commercial grade)	Sigma chemical Co., U.S.A.
Citric acid monohydrate	Sigma chemical Co., U.S.A.
Crystal violet	E. Merck, Germany
Dimethysulfoxide (spectroscopic grade)	E. Merck, Germany
Dipotassium hydrogenphosphate	Fluka A.G., Buchs, Switzerland
Disodium hydrogenphosphate ($Na_2HPO_4.12\ H_2O$)	E. Merck, Germany
EMEM (Eagle minimum essential medium)	Gibco laboratories, USA.
Filter paper No.1	Whatman International Ltd., England
5-Fluorouracil (U.S.P.)	ABIC, Isarael
Glucose anhydrous	Fluka A.G., Buchs, Switzerland

Chemicals / Materials	Source
D-Glucose 6-phosphate (monosodium salt)	Sigma Chemical Co., U.S.A.
HEPES Buffer	Amresco, U.S.A.
L(+) Histidine monohydrochloride	Matheson Coleman and Bell, U.S.A
Magnesium chloride	May and Baker Ltd., England.
Magnesium sulfate ($MgSO_4.7H_2O$)	Fluka A.G., Buchs, Switzerland
Millipore membrane	Nihon Millipore, Kogyo, Yonezawa, Japan
β-Naphthoflavone	Aldrich Chemical Company Inc., U.S.A.
Newborn calf serum	Gibco laboratories, USA.
β-Nicotinamide adenine dinucleotide reduced form (β-NADH)	Orental Yeast Company, Japan
β-Nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH)	Oriental Yeast Company, Japan
Oxoid nutrient broth No.2	Oxoid Ltd., England
Phenobarbital sodium (U.S.P.)	Wako Pure Chemical Industries, Ltd. Japan
Potassium chloride	May and Baker Ltd., England
RPMI 1640 powder	Gibco laboratories, U.S.A.
Sodium bicarbonate	Fluka A.G., Buchs, Switzerland
Sodium hydroxide	Reidel-de-Haen, Germany

Chemicals / Materials	Source
Sephadex LH-20	Pharmacia, Sweden
Sodium ammonium hydrogenphosphate ($NaNH_4HPO_4.4H_2O$)	Fluka A.G.,Buchs, Switzerland
Sodium dihydrogen phosphate ($Na\ H_2PO_4.2H_2O$)	E. Merck, Germany
Sodium chloride	E. Merck, Germany
Trypan Blue	BDH Chemical Ltd., England

Appendix 2. List of instruments used in the study

Instrument	Model	Source
Autoclave	SS-240	Tomy Sciko Co.Ltd., Tokyo, Japan
Analytical balance	AC 100	Mettler Instrument A.G., Switzerland
Circulating aspirator	WJ-15	Sibata, Japan
Dispenser	Dispet TM	Nichiryo Co., Ltd., Japan
Disposable tissue culture flask	-	Costar, U.K.
Incubator	B 5050	Heraeus, West Germany
5 % CO_2 Incubator		
Freeze dryer	ALPHAI-6	Martin Christ GMBM & Co., K.G., Germany
Hemocytomer		Fisher, USA.

Instrument	Model	Source
High Performance Liquid Chromatography		LCD Analytical
UV detector (Chrom-A-Scape)		Bio-Rad
Laminar flow	H-25	ISSCO
Millipore, Holder	SX001300	Millipore Corporation, U.S.A.
Phase contrast microscope	-	NIKON, Japan
pH meter	701A	Orioh Inc., U.S.A.
Polytron homogenizer	GH-6010	Kinematica, Switzerland
Refrigerator	GR-2000 TG	Toshiba, Thailand
Refrigerator (-80^{o} C)		Sanyo, Thailand
Stereomicroscope	VMZ-4SA-2W	Olympus, Japan
Superspeed Centrifuge	RC2-B	Ivan Sorval Inc., U.S.A.
Vacuum ratatory evakporator	Eyela	Tokyo Rikakikai Co., Ltd, Japan.
Water bath	Type 1 No. D 7095	Yazama, Japan

Appendix 3 Induction of rat liver microsomal enzymes

The induction procedure was prepared as described by Matsushima, et al, (1976). Five male Sprague-Dawley rats weighing approximately 200 g from the Animal House, Faculty of Medicine, Chiang mai University were used. The rats were given drinking water ad libitum and regular complete diet. The rats were given sodium phenobarbital and 5, 6-benzoflavone (β-napthoflavone) by intraperitoneal injection each day as follows:

First day, morning : a single injection of 10 mg/ml of sodium phenobarbital in saline solution, at a dosage of 30 mg per kg body weight

Second day, morning : a single injection of 20 mg/ml of sodium phenobarbital in saline solution, at a dosage of 60 mg per kg body weight

Third day, morning : the same dose of sodium phenobabrital as the second day.

afternoon : a single injection of 10 mg/ml of 5,6-benzoflavone in com oil, at a dosage of 80 mg per kg body weight.

Fourth day, morning : the same dose of sodium phenobarbital as the second day. That evening rats were starved about 12 hours before sacrificed

Preparation of liver homogenate fraction (S9)

On the fifth day after the induction of the liver enzymes, the rats were sacrificed by cervical dislocation and the livers were removed with sterile technique.

The freshly excised livers were placed in preweighted beaker containing approximately 1 ml of chilled 0.15 M KCl. After weighing, the livers were

washed in chilled 0.15 M KCl for several time. The washed livers were transferred to a beaker containing 3 volumes of chilled 0.15 M KCl (3 ml/g wet liver) and were minced with sterile scissors, and homogenized with a Polytron Homogenizer. The homogenate was centrifuged for 10 minutes at 9,000 g, the supernatant (S9 fraction) was saved. The freshly prepared S9 fraction was distributed in 1-2 ml portions in cryogenic vials (Wheaton), quickly freeze and stored immediately at -80° C. The protein concentration in S9 fraction was determined by BCA assay and approximately 30 mg/ml.

As required for mutation assay, a sufficient S9 fraction was thawed at room temperature and S9 mix was prepared as soon as the S9 had thawed and the S9 mix was kept in ice. The remaining S9 fraction was discarded.

The S 9 mix (rat liver microsomal enzymes + cofactors)

S9 mix was freshly prepared each day and usually kept in ice. The components of 1 ml the standard S9 mix were

0.2 M Sodium phosphate buffer, pH 7.4	0.5	ml.
0.4 M MgCl - 1.65 M KCl	0.02	ml.
1 M Glucose-6-phosphate	0.005	ml.
0.1 M NADPH	0.04	ml.
0.1 M NADH	0.295	ml.
Sterile distilled H_2O	2.295	ml.
Rat liver S9 fraction	0.1	ml.

The ingredients should be added in the order and should be chilled. Any left S9 fraction or S9 mix was discarded.

Appendix 4 Preparation of some reagents

4.1 Preparation of minimal glucose agar plate

The components of 1000 ml minimal glucose agar medium were

Bacto-Difco agar	15	g
Distilled water	850	ml
10 x Vogel-Bonner medium E	100	ml
40 % Glucose	50	ml

The ingredients should be autoclaved separately, when the solution has cooled slightly, added together, mixed well and poured 30 ml into each plate.

The components of 1,000 ml. the Vogel-Bonner medium E (ten-fold solution) are

$MgSO_4.7H_2O$	2	g
Citric acid. H_2O	20	g
K_2HPO_4	100	g
$NaNH_4HPO_4.4H_2O$	35	g

4.2 Preparation of top agar containing histidine and biotin

A : The components of 10 ml top agar

Bacto-Difco agar	0.6	g
NaCl	0.5	g
Distilled water	100	ml

The solution was steriled by autoclave at 1 lb, 120° C. 20 min

B : The components of 100 ml. 0.5 mM histidine/biotin

Ingredient	per liter	
D-Biotin	124	mg
L-Histidine HCl H_2O	105	mg
Distilled H_2O	100	ml

Dissolve histidine and biotin by stirring in water. Sterilize by filteration through membrane filter (0.22 um pore size)

Depending on numbers of plate for each test, calculate a total volume of top-agar required (according to the amount of top agar used = 2 ml per plate). The Histidine/biotin solution will be added to the Top agar before starting the mutagenic experiment by the ratio of 10 ml of solution B : 100 ml solution A.

4.3 Preparation of tissue culture media and straining

A : Preparation of RPMI medium

RPMI 1640 medium powder (with L-glutamine)	1	bag.
Hepes buffer	5.9	g.
$NaHCO_3$	1	g.
Distill water	1	l.

- Combine and adjust pH to 7-7.2
- Sterilize by pass through Millipore filter membrane (0.22 mm).

B : Preparation of EMEM (Eagle minimum essential medium)

EMEM (with Earle's Salts and L-glutamine)	1	bag.
Hepes buffer	1.5	g.
$NaHCO_3$	0.9	g.
Distill water	1	l.

- Dissolve in water and adjust pH to 7-7.2
- Sterilize by pass through Millipore filter membrane (0.22 mm.)

C : Preparation of 0.4 % Trypan blue

Trypan Blue	0.4	g.
NaCl	0.81	g.
KH_2PO_4	0.06	g.
Distill water	100	ml.

- Dissolve by water, and then heat its to boiling
- Adjust pH to 7.2 - 7.3
- Adjust volume to 100 ml.

Appendix 5 Dye exclusion technique (Freshney, 1987)

Dye exclusion technique is one of technique for cell counting by using principle, as follow : viable cells are impermeable to nigrosin,trypan blue, and a number of other dyes (Kaltenbach, et al, 1958)

Method

1. Prepared cell suspension by resuspension
2. Take a clean hemocytometer slide (Improved Neubauer) and the coverslip in pace (figure A)
3. Add 100 ul of cell suspension to 100 ul of 0l4 % trypan blue on the open surface of the slide, mix, transfer to the edge of the coverslip, and allow to run into the counting chamber.
4. Leave 10 minutes (do not leave too long or viable cells with deteriorate and take up stain)
5. Place on microscope under a x10 objective
6. Count the number of unstained cells
7. Wash hemocytometer and return to box
8. Calculate the percentage of unstained cells by calculate the average of the two counts , and derive the concentration of sample as follow :

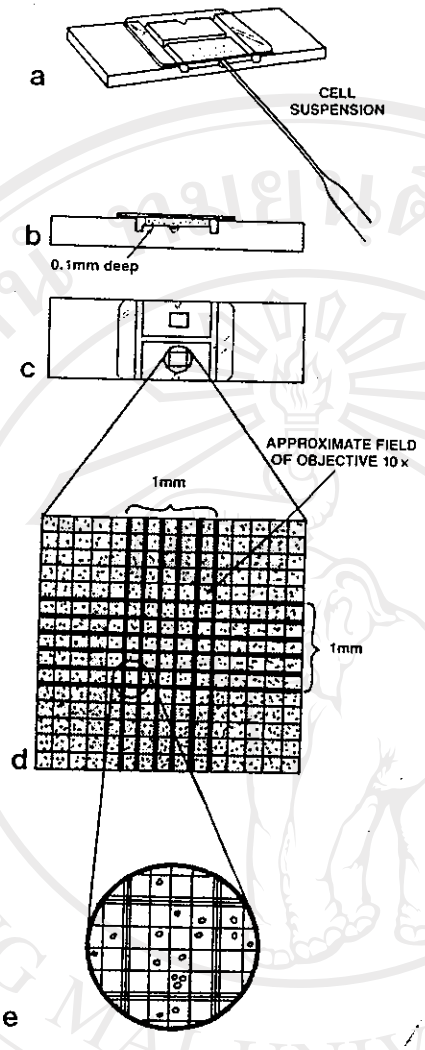

Figure A. *Hemocytometer slide (Improved Neubauer). a. Adding cell suspension to the assembled slide. b. Longitudinal section of slide showing position of cell sample in 0.1-mm-deep chamber. c. Top view of slide. d. Magnified view of total area of grid. Light central area is that area which would be covered by the average × 10 objective (depending on field of view of eye piece). This covers approximately the central 1 mm^2 of the grid. e. Magnified view of one of the 25 smaller squares, bounded by triple parallel lines, making up the 1-mm^2 central area. This is subdivided by single grid lines into 16 small squares to aid counting.*

$$C = 2n/V$$

c = cell concentration (cells/ml.)

n = number of cell counted

v = volume counted (ml.)

2 = 2 fold concentration (cell suspension was dituted by trypan blue)

For the Improved Neubauer slide, the depth of the chamber is 0.1 mm. and assuming only the central 1 mm^2 is used, V is 0.1 mm^3 or 10^{-4} ml. The formula becomes :

$$C = 2n \times 10^4$$

Appendix 6 Preparation tissue sampling for histopathological study.

1. Whole bodies of mice were fixed in 10 % neutral-buffer formalin.
2. Livers, lungs, spleen, kidneys and tumor were resected and fixed in 10 % neutral-buffer formalin.
3. Tumor was measured it size(diameter) and the sample from midportion in whole block was evalvated tissue necrosis.
4. Other organs were dissected for organ metastasis study, as follow;

Liver	6 peices	
Lung	6 peices	(3 peices from each lobe)
Spleen	2 peices	
Kidney	2 peices	(1 peices from each kidney)

VITA

Name : Miss Rawiwan Puatanachokchai

Date of birth : December 8, 1968

Education

March, 1983 Certificate of Mathayom III ,
The Demonstration School of Chiang Mai University

March,1986 Certificate of Mathayom VI
The Demonstration School of Chiang Mai University

February, 1990 Bachelor of Nursing
Faculty of Nursing, Chiang Mai University

Publication :

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