CHAPTER III

RESEACH METHODOLOGY

Materials

CUR and HHC

CUR and HHC were synthesized and provided by Prof. Dr. Apichart Suksamrarn, Department of Chemistry, Faculty of Sciences, Ramkhamhaeng University.

Cell culture

The HT-29 human colon adenocarcinoma cell lines purchased from the American Type Culture Collection (ATCC). HT-29 cells were used up to 20 passages.

Animal model

Male 4-week-old Wistar rats (NationalAnimalCenter, Salaya, NakornPathom, Thailand) weighing between 100-120 g were used. Animals were housed at 25±2°C under a 12-hrs light/dark cycle. All procedures were carried out in accordance with the Guidelines of Animal Care described by AnimalCenter, Faculty of Medical Science, NaresuanUniversity, Phitsanulok, Thailand.

Methods

Invitro studies

1. HT-29 cell culture

HT-29 human colon cancer cells were cultured in RPMI 1640 media (GIBCO^{TM}, New Zealand) supplemented with 10% fetal bovine serum (FBS) (GIBCO^{TM}, New Zealand), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technology, Inc) and cultured in 60 mm culture plate (Nunc, Denmark) at 37°C in an atmosphere of 95% humandified air and 5% CO_2. Cells were subcultured every two days by 0.025% trypsin/EDTA.

2. Preparation of curcuminiods and HHC

Curcuminoids and HHC were synthesized and gracefully provided by Prof. Dr. ApichartSuksamrarn and colleagues (Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Thailand). The curcuminoid mixture obtained

from the rhizomes of *Curcuma longa* was subjected to silica gel column chromatography, using hexane-dichloromethane, dichloromethane and dichloromethane-methanol as eluents to afford CUR as the major constituent. Recrystalization was accomplished by dissolving the evaporated eluate with a small quantity of dichloromethane and ethanol was then added. CUR crystallized out as yellow needles, melting point (m.p.) 181-183°C. HHC was synthesized from CUR by catalytic hydrogenation reaction in ethanol for 5 h, with palladium on charcoal as a catalyst. The product was isolated from THC and octahydrocurcumin by silica gel column chromatography which followed by recrystallization with dichloromethane-*n*-hexane to give a 45% yield of HHC as white amorphous solid, m.p. 81–82°C. The spectroscopic (IR, ^{1}H-NMR and mass spectra) data of the synthesized HHC were consistent with those in the previous report (Changtam, et al., 2010).

When preparing the stock concentration of the CUR and HHC, all compounds were initially dissolved in absolute dimethyl sulfoxide (DMSO) and prepared as 20 mM stock solutions. Next, the solution was aliquot, stored in the dark and kept frozen as a stock solution at -50°C. The stock solution was diluted in RPMI 1640 free serum before being used to achieve the final concentration of each treatment. Throughout the experiments, the concentration of DMSO was not more than 0.1%.

3. Assessment of cell viability

A MTT reduction assay was used for assessing cell viability by measuring cellular mitochondrial dehydrogenase activity. MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) yellow tetrazolium salt is absorbed into cells and reduced to a dark blue formazan by mitochondrial succinate dehydrogenase. Formazanaccumulation directly reflects mitochondrial activity, which is an indirect measure of cell viability. The reduction of MTT is proportional to the number of active mitrochondria in the live cells.

HT-29 cells were plated in 96-well plates at a density of $1x10^4$ cells/well in a final volume of 100 µl RPMI 1640 medium and plate was incubated overnight at 37°C in humidified incubator, 5% CO_2. This study was divided the HT-29 cells into 6 groups as followed; control, 5-FU (5 µM), CUR, HHC (5, 10, and 25 µM) and 5-FU in combination with CUR and HHC.Then, cells were exposed to test compounds and

vehicle control (RPMI free serum medium) was used as a negative control according to the experimental design. After 24, 48 and 72h of experiment, the medium was removed and the new medium containing 10 mg/ml of MTT was added to each well. Cells were furthered incubated with MTT for 2h at 37°C in an atmosphere of 95% humidified air and 5% CO_2. The formazan crystal was solubilized by DMSO and the absorbance was measured at 540 nm on a microtiter plate reader (Bio-Tek, Instruments, Winooski, VT, USA) and the values of the different absorbance were expressed as a percentage of control. Each measurement was done in triplicate.

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance of sample x 100}}{\text{Mean absorbance of control}}$$

For analysis the effectiveness of 5-FU combined with CUR and HHC in inhibiting growth of HT-29 cells. This study was evaluated by measure the combination index (CI) (Chen et al., 1995)which adapted from the method described by Chou and Talalay(Chou and Talalay, 1984). The CI was calculated by dividing the concentration of the drug in the combination at IC_{50} by the IC_{50} of the individual drugs.

$$CI = \frac{\text{Dose of 5-FU}}{IC_{50}\text{ (5-FU)}} + \frac{\text{Dose of CUR /HHC}}{IC_{50}\text{ (CUR /HHC)}}$$

In this equation, the sum of the dose of 5-FU and the dose of CUR or HHC give 50% inhibition of cell growth. CI<1 indicates a synergistic effect; CI=1, additive effect; and CI>1, antagonistic effect.

4. Assessment of mRNA expression

4.1 RNA extraction

Cells were plate in a 6 well plate at a density of 1.5 $X10^5$ cells/well. This study was divided the HT-29 cells into 6 groups as followed; control, 5-FU (5 μM), CUR, HHC (25 μM) and 5-FU in combination with CUR and HHC. After 7h,

cells were exposed to compound according to the experimental designs and then, the cells were harvested and the total RNA was isolated using the TRIzol® reagent (Invitrogen, USA). The purity and yield of the isolated RNA were determined by using a spectrophotometer.

4.2 cDNA synthesis

A volume of 1 μg RNA from each sample was treated with DNase to digest any contaminate genomic DNA and converted to cDNA by M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to manufacturer's instructions. The final concentration of the components in the reaction compose 1x ImProm-II™ reaction buffer, 6 mM $MgCl_2$, 1 mM each dNTP, 1 u Recombinant RNasin® ribonuclease inhibitor, ImProm-IIM reverse transcriptase, 1 μg RNA containing 25 μg/ml oligo (dT) primer and the volume was adjusted by nuclease free water. The process of cDNA synthesis was performed by annealing at 25°C for 5 min, then extension at 42°C for 60 min and inactivation of the reverse transcriptase at 70°C for 15 min. The cDNA was stored at -70°C until used.

4.3 Polymerase chain reaction

Specific DNA sequences were amplified with a PCR mixture (Promega, Madison, WI, USA) containing 1x Green GoTaq® Flexi buffer, 1.5 mM $MgCl_2$, 0.2 mMdNTP, 10 μM oligonucleotide primers, 0.625 μl of GoTaq® DNA polymerase, 2 μl of cDNA and the volume was adjusted by nuclease free water (Promega, USA). Each PCR primer used in this study which is shown in Table 1. The temperature cycling conditions for amplification were performed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for COX-1, COX-2 and GAPDH. Amplificated products were separated by electrophoresis with 1% agarose gel in a 1x TAE buffer, visualized by ethidium bromide staining. The gel images were photographed by an image analysis system (GelDoc 1000; Bio-Rad, Hercules, CA, USA). The expression of cytotoxic factor genes were indirectly examined by measuring the intensity of specific PCR bands which were quantitated in relation to GAPDH bands in the same cDNA using Gene Tool analysis software (Syngene, Cambridge, UK). Percent mRNA expression was determined by normalizing the band intensity of COX-1 or COX-2 with GADPH for each sample.

Table 1 Primers for genes amplification and expected size of PCR products

Primers		Sequences	Product size (bp)
GAPDH	sense	5'-CGG ATT TGG TCG TAT TGG GC-3'	200
	antisense	5'-AAA TGA GCC CCA GCC TTC TCC-3'	
COX-1	sense	5′-TGC CCA GCT CCT GGC CCG CCG CTT-3′	304
	antisense	5′-GTG CAT CAA CAC AGG CGC CTC TTC-3′	
COX-2	sense	5′-TTC AAA TGA GAT TGT GGG AAA AT-3′	305
	antisense	5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′	

5. Assessment of protein extraction

The cells ($1x10^6$ cells/ml in 60 mm. dish) were collected and washed with PBS. After centrifugation, cell lysis was carried out at 4°C by vigorous shaking for 15 min in a RIPA lysis buffer (50 mMTrisHCl pH 8, 150 mMNaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS). After centrifugation at 13,000 rpm for 20 min, supernatant was separated and stored at -20°C. The protein concentration was determined by using BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). After the addition of a sample loading buffer, protein samples were electrophoresed in a 8-15% SDS-polyacrylamide gel. Proteins were transferred to PVDF membranes at 400 mA for 30 min. The blots were blocked for 1 h at room temperature in fresh blocking buffer (0.1% Tween-20 in Tris-buffered saline, pH 7.4, containing 5% skim milk). The primary antibody was used diluted to 1:1000 of anti-COX-1, 1:500 of anti-COX-2 (Cayman Chemical Co., Ann Arbor, MI, USA). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies in a TBST buffer for 1 h at room temperature. The blots were washed again three times in TBST buffer and the transferred proteins were incubated in a ECL substrate solution (Pierce Biotechnology, Inc., Rockford, IL, USA) for 5 min, according to the manufacturer's instructions and visualized with radiographic film.

6. **Assessment of nuclear change**

For assessing the nuclear morphological changes of apoptotic cells, HT-29 cells cultured on poly-D-lysine coated cover slips were treated with CUR, HHC, 5-FU and their combination for 48 h and then washed with PBS. Cells were stained with 50 µg/ml of Hoechst 33342 at 37 °C for 15 min in the dark. The nuclear morphology was observed under a fluorescence microscope (x40) (Nikon, Melville, NY, USA). Two cover slips were used per experimental group, with at least 200 cells in four random fields being counted on each slide and then determining the percentage of cells with characteristic morphologic changes by fluorescence microscopy (x40) (Nikon, Melville, NY, USA). Each experiment was repeated two independent studies (Allen, et al., 2001).

***Invivo*studies**

Methods

1. DMH-induced colorectal cancer in rat

DMH was dissolved in 0.9% NaCl containing 1.5 mg EDTA/100 ml and final pH was adjusted to pH 6.5 (Dai, et al., 2009). The animals were randomly divided into seven groups (Figure 18). Group 1, negative control (normal group, n= 10) was fed oral daily with propylene glycol (PG) plus DMH vehicle. Group 2-7 served as carcinogenic groups. In these groups, animals were treated with the DMH 40 mg/kg twice a week by subcutaneous (s.c.) injection for two weeks (Katsuki, et al., 2006; Kinjo, et al., 2006) in order to induce ACF. Group2, positive control (untreated group, n= 12) was fed oral daily with PG plus DMH; group 3 (5-FU group, n= 12), was treated with weekly intraperitoneal injection (i.p.) of 5-FU at a dose of 50 mg/kg BW; group 4 (CUR group, n= 12) and 5 (HHC group, n= 12) was treated with daily intragastric administration of CUR or HHC at a dose of 50mg/kg, respectively; group 6 (5-FU+CUR, n= 12) was treated with 5-FU combination with CUR and group 7 (5-FU+HHC, n= 12) was treated with 5-FU combination with HHC. Body weights were weekly recorded. All animals were sacrificed by Nembutal injection overdose. Colon, liver, spleen, and kidney weight were measured. Organ weight ratios were calculated by dividing the total organ weight by total body weight in each rat.

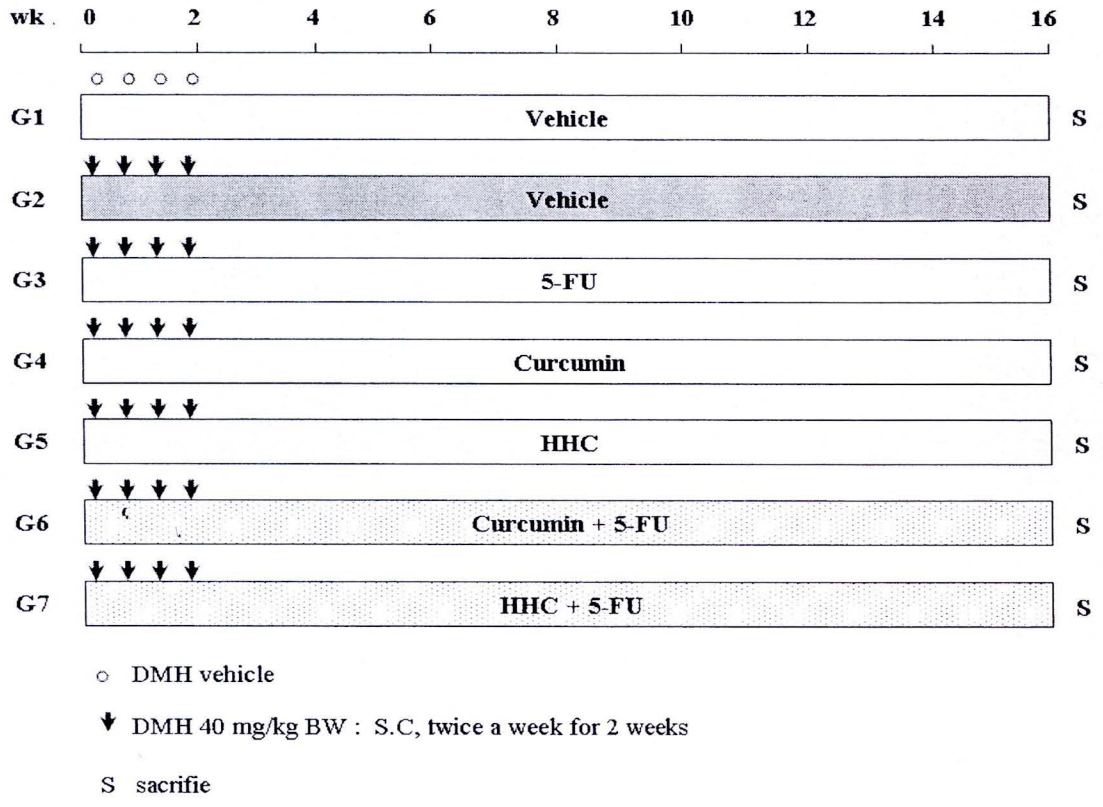

Figure 18 Experimental protocol

2. Assessment of aberrant crypt foci (ACF)

The colons were harvested and washed with cold normal saline. After that the colon tissues were fixed in 10% buffer neutral formalin for 24 h.The colon tissues were stained with 2% methylene bluefor 2 min and placed on a microscope slide with the mucosal surface up. ACF were counted in 7 rats from each group under a light microscope at 40X magnification for each rat. ACFs were distinguished from surrounding normal crypts by their (1) have increased size (2) large luminal openings (3) thickened epithelial and (4) larger than adjacent normal crypts (McLellan and Bird, 1988; Papanikolaou, et al., 1998; Bouzourene, et al., 1999). Large ACF were defined as containing 4 crypts or more (>3 crypts/ACF) (Tache, et al., 2007). In this study, the number of ACF and large ACF were measured.

3. COX-2 immunohistochemical analysis

Paraffin sections from distal 5 cm. of colon were dewaxed and rehydrated through xylene and a graded alcohol series. Antigen retrieval was incubated with Tris-EDTA pH 9.0 at 95-100 °C for 40 min and then removed the slides to room temperature and allowed the slides to cool for 20 min. Endogenous peroxidase activity was blocked with3% hydrogen peroxide in methanol for 15 min at roomtemperature. After washing in water, nonspecific binding siteswere blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 min at room temperature. The primarypolyclonal antibody COX-2 (1:500 dilution) was diluted in PBST and incubated at 4°C overnight. Then, rinsed the slide gently with PBS and developed by the Envision system/HRP as followed: the slides incubated in peroxidase labeled polymer for 30 min and substrate-chromogen for 10 min at room temperature. The nucleiwere counterstained with modified hematoxylin solution. For determination of COX-2 expression, the positive stained cells were counted by image analysis (Image Pro®-plus). COX-2 expression was defined expressed by the percentage of number of positive-stained cells to the total numberof cells counted.

4. COX-1 immunohistochemical analysis

The paraffin sections from distal colon were dewaxed and rehydrated through a graded alcohol series. Endogenous peroxidase activities were blocked with3% hydrogen peroxide in methanol for 15 min at roomtemperature. After washing in water, nonspecific binding siteswere blocked with 5% bovine serum in phosphate-buffered saline (PBS) for 30 min at room temperature.The primarymonoclonal antibody COX-1 (1: 1000 dilution) was diluted in PBST and incubated at 4°C overnight. Then, rinsed the slides with PBS and incubated with the secondary antibody conjugated with biotin for 1 h at room temperature. Incubated the slides with the ABC reagent for 30 min at room temperature and developed by DAB peroxidase substrate. The nucleiwere counterstained with modified hematoxylin solution. For determination of COX-1 expression, the positive stained cells were counted by image analysis software (Image Pro®-plus). COX-1 expression was expressed by the percentage of number of positive-stained cells to the total numberof cells counted.

5. Cell death detection (apoptosis)

Apoptotic cells in distal colon were visualized by using the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) method with FragEL^{TM}DNA fragmentation Detection kit. The deparaffinized tissue sections were inactivated by endogenous peroxidases with 3% H_2O_2 in methanol at room temperature for 5 min. The tissue sections were incubated with TUNEL reaction mixture containing the TdT at 37 °C for 60 min. Slides were rinsed twice in PBS for 10 min and dried around the sample. The labeled DNA was detected by DAB solution for 10-15 min at room temp. The nuclei were counterstained immediately with methygreen solution. Finally, the slides were washed and analyzed under light microscope. Apoptotic index (AI) was determined as the percentage of the labeled nuclei with respect to the total number of nuclei counted.

Statistical analysis

All values are represented as mean ± SEM. The difference in mean values among different groups was analyzed by one-way analysis of variance (ANOVA) followed by Post Hoc Duncan's test to compare the significance between individual groups. All the statistical calculation were carried out using SPSS and *P*-value <0.05 was considered significant.