Coptis chinensis inhibits hepatocellular carcinoma cell growth through nonsteroidal anti-inflammatory drug-activated gene activation

KATHY KA-WAI AUYEUNG and JOSHUA KA-SHUN KO

Pharmacology and Toxicology Laboratory, School of Chinese Medicine, Hong Kong Baptist University, Hong Kong SAR, P.R. China

Received May 25, 2009; Accepted July 14, 2009

DOI: 10.3892/ijmm_00000267

Abstract. Conventional chemotherapy of liver cancer fails to provide satisfactory remission and may cause serious side effects, thus it is crucial to derive alternative treatments that effectively inhibit cancer cell growth with known mechanisms of action. In the present study, we investigated the anti-carcinogenic effects of Coptis chinensis and its major constituent, berberine, in HepG2 hepatocellular carcinoma (HCC) cells and attempted to elucidate the underlying mechanism, including involvement of the nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1). Inhibition of cell proliferation, induction of apoptosis and cell cycle arrest at the G2/M phase were observed in HepG2 cells treated with Coptis chinensis or berberine. The pro-apoptotic effects were associated with corresponding down-regulation of Bcl-2, activation of procaspase-3 and -9 as well as cleavage of poly (ADP-ribose) polymerase. We further demonstrated the involvement of NAG-1 in the pro-apoptotic events following prior activation of its upstream transcriptional factor early growth response gene (Egr-1). This was confirmed by increased NAG-1 promoter activity preceded by the elevation of Egr-1/DNA binding activity. Our results suggest that both Coptis chinensis and berberine are potential anti-carcinogenic agents in treating HCC by inducing cell cycle arrest and promotion of apoptosis, while NAG-1 is a molecular target during the drug-induced pro-apoptotic action in HepG2 cells.

Introduction

More than 90% of primary liver cancers are hepatocellular carcinoma (HCC). Although the incidence of HCC is relatively low in Western countries such as the United States, it represents 10-50% of malignancies in Africa and parts of Asia. According to reports from the Department of Health, Cancer Registry and Hospital Authority of Hong Kong in 2005, liver cancer was the second most common cancer and third leading cause of cancer-related deaths in the region. Around 50-75% of primary liver cancer patients have an abnormally high blood level of α-fetoprotein. High blood level of α-fetoprotein occurs when there is HCC or other metastatic cancers in the liver. Conventional chemotherapy of liver cancers, such as HCC, involves a combination of interferon-α and 5-fluorouracil (1). Unfortunately, the response of HCC to current chemotherapy is not consistent and has many side effects (e.g. neutropenia and immunosuppression). The discovery of novel biomarkers that help identify potential chemotherapeutic agents is crucial.

Coptis chinensis (‘Huanglian’) is found mainly in the province of Szechwan in mainland China. It is frequently used in treating diabetes mellitus (2), and is particularly helpful in the treatment of diarrhea, acute enteritis and dysentery, delirium due to high fever, leukemia and otitis media (3). The initial discovery of the anti-tumorigenic potential of Coptis chinensis came from the finding that it inhibits the formation of azoxy-methane-induced aberrant crypt foci, a putative pre-neoplastic lesion of colon cancer in rats (4). However, the anti-carcinogenic effect of the medicinal herb in liver cancer (HCC in particular) is not well defined. Coptis chinensis contains many alkaloids, mainly berberine, coptisine and palmatine, as well as phenolic compounds such as ferulic acid, chlorogenic acid and 3,4-dihydroxyphenethyl glucoside. By using high performance liquid chromatography (HPLC), berberine (Fig. 1A) was shown to be the key component of Coptis chinensis (Fig. 1B). Hence, the anti-cancer potential of Coptis chinensis could be associated with the effects of this major alkaloid. Nevertheless, the precise mechanism of action during their anti-tumorigenic activities in HCC and the presence of any known molecular targets or biomarkers have not been elucidated.

One of the objectives of current cancer therapy is to reduce the number of tumor cells and prevent further accumulation. The regulatory circuits of the cell cycle enforce a series of checkpoints, allowing passage only after completion of critical events, which require the activation of intracellular cyclin-dependent kinases (cdk or cdc) (5). Overexpression of pro-apoptotic proteins and/or down-regulation of anti-apoptotic proteins could augment the effectiveness of chemotherapy. The mitochondria-dependent apoptotic pathway involves...
cleavage of procaspase-9 and activation of caspase-3. This process involves cleavage of an inhibitor for caspase-activated DNAses, which subsequently produces poly (ADP-ribos) polymerase (PARP), an enzyme identified as a substrate for the caspasess (6). DNA repair is prevented following cleavage of PARP by caspase-3, leading to DNA fragmentation, an essential event for programmed cell death. Nonetheless, members of the bcl-2 family are important in regulating the mitochondrial pathway (7).

Nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) is a divergent member of the transforming growth factor-ß superfamily that is identified as a novel therapeutic target during the anti-tumorigenic and pro-apoptotic activities in various cancer cell lines (8). It is highly expressed in the mature normal human colonic epithelial cell surface but is significantly reduced in most human tumor biopsies. Anti-carcinogenic treatment with NSAIDs, such as sulindac, could increase NAG-1 expression in the colon and liver tissues obtained from Min mice (9). It was suggested that the transcription factor early growth response gene (Egr-1) is required for full activation of NAG-1 upon stimulation by chemotherapeutic drugs (10). Nevertheless, according to our recent findings, this correlation is not always true (11). In the present study, we aimed to investigate the precise mechanism that governs the growth-inhibitory and pro-apoptotic effects of Coptis chinensis and berberine. In particular, we identified NAG-1 as an important molecular target for the drugs.

Materials and methods

Materials. Extracted Coptis chinensis powder was obtained from Sheng Foong Pharmaceutical (Taiwan). Crude herbal extract concentrate (500 mg) was dissolved in 20 ml of ultrapure water at 50°C to prepare a 100 mg/ml stock, which was further diluted into the working concentrations. Berberine hemisulphate with a purity of ≥95% was purchased from Sigma (St. Louis, MO). Working concentrations of berberine were prepared from a 12.5 mM stock in ultrapure water. The cell proliferation enzyme-linked immunosorbent assay (ELISA) kit was purchased from Roche Mannheim, Germany), the enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Biosciences (Piscataway, NJ), the mouse monoclonal anti-bcl-2 from Zymed Laboratories Inc. (San Francisco, CA), the rabbit polyclonal anti-NAG-1, mouse monoclonal anti-caspase-3 and -9 from Upstate (Charlottesville, VA), the mouse monoclonal anti-PARP from Beckton Dickinson (San Jose, CA), the rabbit polyclonal anti-Egr-1 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the mouse monoclonal anti-ß-actin from Sigma. 3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide (MTT) and other chemicals were also obtained from Sigma unless specified.

Assessment of Coptis chinensis extract by HPLC. The Coptis chinensis extract was analyzed in our laboratory by reverse HPLC (Agilent 1100, Agilent Technologies, Palo Alto, CA), using a Zorbax Eclipse XDB-C18 analytical 4.6x150 mm column with a mobile phase of 25% acetonitrile/acetate acid (1% v/v) at a flow of 1 ml/min. The HPLC profile is shown in Fig. 1B. Among the dominant peaks, the largest one (detected at 21 min) is berberine.

Cell culture. HepG2 cells (HB-8065) were obtained from American Type Culture Collection (Rockville, MD), which originated from human liver tissues with HCC. Cells were cultured in Dulbecco's modified Eagle's medium (D-MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, NY). Cells were sub-cultured every 2-4 days according to the previous subculture ratio.

Cell viability assay. Cell viability was measured by the MTT assay. HepG2 cells were seeded at a density of 2x10^3 cells/well in 96-well plates. After the treatment with various concentrations of Coptis chinensis (0.125-4 mg/ml) and berberine (3.125-100 μM) for 24, 48 or 72 h, cells in each well were incubated with 30 μl of MTT at 37°C for 3 h. The culture medium was then refreshed and the intracellular formazan product was dissolved in 100 μl of dimethyl sulfoxide (DMSO). Absorbance was determined spectrophotometrically at 540 nm. Six independent experiments were conducted for each test.

Cell proliferation assay. The rate of cellular proliferation was determined using an ELISA kit via 5-bromo-2'-deoxyuridine (BrdU) incorporation into newly synthesized DNA. Cells were seeded at a density of 1x10^3 cells in each well of 96-well plates. After treatment with Coptis chinensis or berberine for 72 h, BrdU was added at a final concentration of 10 μM and cells were incubated for an additional 2 h at 37°C. Cells were then fixed for 30 min at room temperature, followed by incubation with 100 μl of anti-BrdU-POD-labeled antibody for 90 min. After 15 min following addition of substrate solution, the reaction was stopped by 1 M H2SO4. Colorimetric
absorbance was measured spectrophotometrically at 450 nm. Three independent experiments were conducted for each test.

Flow cytometry. Cells were seeded in 100-mm petri dishes at a density of 3.0x10⁵ cells/dish. After treatment with Coptis chinensis or berberine for 72 h, harvested cells were fixed overnight with 75% ethanol. Cells were then stained with 50 μg/ml propidium iodide (PI) in a buffer containing 0.1% Triton X-100 and 100 μg/ml RNase A (Invitrogen, Carlsbad, CA) at 4°C for 1 h. Cell cycle distribution and subdiploid population were detected by flow cytometry (FACS Canto™, Beckton Dickinson Biosciences). Phase distributions were calculated from the resultant DNA histogram using the ModFit LT version 3.0 software, and expressed as a percentage of cells in the respective phases. In addition, the percentage of apoptotic cells was defined as those found in the sub-G1 region.

Analysis of DNA fragmentation by DNA gel electrophoresis. Cells were seeded in 100-mm petri dishes at a density of 3.0x10⁵ cells/dish. After treatment with Coptis chinensis or berberine for 72 h, cells were harvested and lysed in 300 μl of a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 150 mM NaCl (Fisher, Leicestershire, UK) and 0.5% Triton X-100 at 4°C for 30 min. The mixture was then incubated with 100 μg/ml RNase A (Invitrogen) at 37°C for 2 h, followed by another 2 h of incubation with 200 μg/ml proteinase K at 50°C. DNA was extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated overnight at -20°C by adding 3 M sodium acetate and 100% ethanol (Merck, Whitehouse Station, NJ). The pellet was then dissolved in a buffer containing 500 mM Tris (pH 8.0) and 50 mM EDTA, with the amount of DNA measured by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Equal amount of DNA (10 μg) was prepared for each sample. Samples were then subjected to electrophoresis on a 2% agarose gel containing 0.5 μg/ml ethidium bromide with the DNA marker (GeneRuler™ 100 DNA Ladder Plus; Fermentas, Hanover, MD), and visualized under UV light using a gel documentation system (AlphaImager 2200, Alpha Innotech Corp., San Leandro, CA).

Western blotting. Western blotting was performed as described previously (11). Cells were seeded in 100-mm petri dishes at a density of 3.0x10⁵ cells/dish and received drug treatments for 72 h. The nitrocellulose membrane was incubated with the respective primary antibodies (1:500-1:1,000) and the appropriate secondary antibodies (1:5,000) conjugated with horseradish peroxidase. The immunoreactive proteins were detected by using the ECL kit. The membrane was then stripped by stripping buffer containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl at 50°C for 30 min for quantification and normalization with β-actin (1:10,000).

Transfection and luciferase assay. Cells were cultured at a density of 1x10⁵ cells/well for 16 h. Mixture containing 0.4 μg of NAG-1 construct (pNAG133) and 0.02 μg of pRL-null (internal control) were co-transfected by Lipofectamine Plus™ reagent (Gibco) according to the manufacturer's protocol (10). The transfection mixture was refreshed after 24 h and AST was added and incubated for another 6 h. Cells were then harvested in 1x luciferase lysis buffer. Luciferase activities were detected by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Electrophoretic mobility shift assay. Nuclear proteins were isolated to perform electrophoretic mobility shift assay in determining Egr-1/DNA binding activity. Nuclear proteins (10 μg) were incubated with reaction buffer for 15 min, followed by incubation with 32P end-labeled oligonucleotide containing a sequence for the Egr-1/DNA binding site (5'-GGA CTT AGC GGG GGC GAG CGG GGG CGA-3'). The reaction mixture was separated in a non-denaturing 6% polyacrylamide gel that was later exposed to radiographic film at -80°C.

Statistical analysis. Data were expressed as mean ±SEM. Statistical significance of at least P<0.05 was determined by one-way analysis of variance (ANOVA) followed by a post-hoc Turkey's test using the SPSS version 10.0 software.

Results

Coptis chinensis and berberine reduce HepG2 cell viability. Treatment of HepG2 cells with Coptis chinensis extract and berberine for 24-72 h induced concentration-dependent growth-inhibitory effect at different treatment times (Fig. 2). Cell viability was decreased by >80% by treating cells with the highest concentration of either Coptis chinensis (4 mg/ml) or berberine (100 μM). In addition, results after 72 h of drug treatment indicate that the median inhibitory concentration (IC₅₀) of Coptis chinensis is ~1 mg/ml, of which the 25 and 75% inhibitory concentrations (IC₂₅ and IC₇₅) are 0.3 and 3 mg/ml, respectively. From our chromatographic analysis of Coptis chinensis, it was found that 0.3, 1 and 3 mg/ml of its extract contain ~10, 30 and 100 μM of berberine, respectively. In fact, this is in accordance with the MTT results of berberine. Hence, these concentrations were used to demonstrate the anti-carcinogenic effects of Coptis chinensis and berberine throughout the study.

Coptis chinensis and berberine inhibit HepG2 cell proliferation and cause G2/M phase arrest. Compared with untreated control HepG2 cells, significant inhibition of cell proliferation in cells treated with Coptis chinensis and berberine was observed (Fig. 3A and B). In addition, results from flow cytometry also revealed that the HepG2 cell population at G2/M phase was significantly increased after treated with 1 and 3 mg/ml of Coptis chinensis or 30 and 100 μM of berberine for 72 h, with concomitant reduction of cell numbers in the G1 phase (Fig. 3C). All these effects were shown to be concentration-dependent.

Coptis chinensis and berberine induce DNA fragmentation and apoptotic cell death in HepG2 cells. Coptis chinensis and berberine treatments resulted in concentration-dependent DNA fragmentation (in the form of DNA laddering in agarose gel), indicating the presence of double-strand breaks in the DNA (Fig. 4A). Moreover, flow cytometric analysis using PI-staining also shows that both Coptis chinensis and berberine induced accumulation of cells in the sub-G1 phase in a
concentration-dependent manner after 72 h of treatment, which is a characteristic of active apoptosis (Fig. 4B). These data could be used to substantiate the findings in the DNA gel electrophoresis test, since the extent of apoptosis represents the amount of nucleosomes formed, which results in DNA degradation.

Coptis chinensis and berberine modulate expression of apoptosis-related proteins in HepG2 cells. Compared with untreated control HepG2 cells, treatment of Coptis chinensis and berberine for 72 h significantly decreased the protein expression of Bcl-2 (Fig. 5A). This concentration-dependent Bcl-2 down-regulation was found to be in concert with caspase-3 activation as indicated by the decrease in procaspase-3 expression (Fig. 5B). Reduced protein expression of procaspase-9 was also observed in Coptis chinensis- (3 mg/ml) and berberine- (100 μM) treated cells (Fig. 5B), designating the involvement of the intrinsic apoptotic pathway. As a result, these events induced cleavage of PARP (Fig. 5B), the substrate of caspase-3, subsequently leading to defective DNA repair and resulted in apoptotic cell death. Furthermore, pretreatment with the caspase 3 inhibitor Z-DEVD-FMK (40 μM) for 45 min attenuated the drug-induced PARP cleavage (Fig. 5C).

Coptis chinensis and berberine induce overexpression of NAG-1 protein in HepG2 cells through Egr-1 activation. Coptis chinensis and berberine inhibited cell proliferation and induce cell cycle arrest at the G2/M phase in HepG2 cells. Cell proliferation following treatment of Coptis chinensis (CC) (A) or berberine (B) for 72 h was determined by using BrdU ELISA. A concentration-dependent inhibition of cell proliferation was caused by both drugs. Data were obtained from 2-3 independent experiments. *P<0.05, **P<0.01 vs. corresponding control. (C) Distribution of HepG2 cells at different phases of the cell cycle with or without drug treatment for 72 h was analyzed by flow cytometry using ModFit 3.0 LT software to analyze. Results are expressed as the percentage of cells in each phase ±SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. control cells at the corresponding phase of the cell cycle.
activity was also investigated after 6 h incubation with *Coptis chinensis* and berberine. Results have demonstrated that *Coptis chinensis* and berberine increase NAG-1 promoter activity by 2- and 1.9-fold, respectively (Fig. 6B). We then examined whether NAG-1 induction requires Egr-1 activation. Time-course study of Egr-1/DNA binding activity and protein expression were investigated. It was indicated that incubation of *Coptis chinensis* and berberine in the HepG2 cell culture increase the protein expression of Egr-1 (Fig. 6A) at an earlier time than that of NAG-1. The Egr-1/DNA binding activity was also found to be elevated markedly (Fig. 6C). All this information suggests that overexpression of NAG-1 protein by *Coptis chinensis* and berberine involve prior activation of Egr-1 at the transcriptional level.

**Discussion**

Chinese medicinal herbs have been used for the treatment of human diseases for thousands of years. However, it is not until recently that herbal formulations and extracted active constituents have been systematically studied in order to elucidate their effectiveness in the chemotherapy of cancers. Despite this effort, their precise modes of action during the anti-tumorigenic processes remains unclear. In this investigation, we focused on the pro-apoptotic and anti-proliferative mechanisms of the medicinal herb *Coptis chinensis* and its major constituent berberine in human HepG2 cells. Growth-inhibitory effects were introduced in HepG2 cells following treatment with either *Coptis chinensis* or berberine, as indicated by the concentration- and time-dependent reduction of the number of viable cells. The remarkable down-regulation of α-fetoprotein (data not shown), a specific biomarker for liver cancer, also signifies the successful prevention of tumor progression by both agents in HepG2 cells.

The observed anti-proliferative effects of *Coptis chinensis* and berberine in HepG2 cells could be due to the induction of cell cycle arrest at the G2/M phase. This finding is coherent with earlier studies in other cancer cells (12,13). However, it
Figure 6. *Coptis chinensis* (CC) and berberine (Ber) induce overexpression of NAG-1 protein in HepG2 cells through Egr-1 activation. (A) Time-course study of NAG-1 and Egr-1 expression following drug treatment was determined by Western blotting. Expression of NAG-1 and Egr-1 proteins was significantly elevated following drug treatments. Representative immunoblots from four independent experiments after 72 h (NAG-1) or 12 h (Egr-1) of drug treatment are shown. Intensity of the target bands was normalized with ß-actin re-probed on the same immunoblot. (B) NAG-1 promoter activity (after 6 h of drug treatments) was evaluated using the Luciferase reporter assay. Results indicate fold induction in drug-treated promoter activity was normalized with ß-actin re-probed on the same immunoblot. (B) NAG-1 promoter activity (after 6 h of drug treatments) was evaluated using the Luciferase reporter assay. Results indicate fold induction in drug-treated promoter activity was evaluated using the normalized with ß-actin re-probed on the same immunoblot. (C) Nuclear proteins were isolated and EMSA was performed to determine the Egr-1 DNA binding activity, which has been shown to be time-dependently increased by the test drugs (peaked at 6-12 h).

is the first time that such action has been reported in HepG2 cells. The anti-proliferative gene responses during growth arrest of cancer cells, including inhibition of the cyclin-dependent kinases and/or down-regulation of growth-related factors such as the protooncogene c-myc. Regulation of the cyclin-dependent kinase activity is strictly controlled by diverse mechanisms, including changes in the level of cyclins, phosphorylation of positive and negative regulatory sites of kinases and interaction with stoichiometric inhibitors (14). We demonstrated overexpression of the inactive phosphorylated form of cdc 2 (data not shown), the cyclin-dependent kinase gaging the G2 phase and the mitotic switch, by treating HepG2 cells with *Coptis chinensis* and berberine. Accumulation of p-cdc 2 has also been suggested to be an important contributor in causing drug-mediated G2/M arrest in other cancer cells (15).

Remarkable cleavage of chromosomal DNA into nucleosomal units is observed with increasing concentrations of *Coptis chinensis* and berberine, implicating the effects on DNA fragmentation in HepG2 cells. Degradation of DNA in the nuclei of apoptotic cells occurs after activation of caspases. This could be owing to the initial release of cytochrome c from the mitochondria, which in turn facilitates changes in the Apaf-1 structure to allow procaspase-9 recruitment and processing (16-18). As a result, procaspase-9 is activated to become caspase-9, leading to subsequent activation of caspase-3. Actually, release of cytochrome c could be inhibited by anti-apoptotic proteins such as bcl-2 and bcl-xL (7). Nevertheless, bcl-2 expression was found to be down-regulated by *Coptis chinensis* and berberine in HepG2 cells, although no alteration of bcl-xL expression was observed (data not shown). The inhibitors of apoptotic proteins (IAP) are also anti-apoptotic factors that act by binding to and inhibiting executioner caspases in both extrinsic and intrinsic pathways (19). We tested the effects of *Coptis chinensis* and berberine on the protein expression of IAP survivin in HepG2 cells, but again no modulation was found.

NAG-1 is a novel gene that was first identified in the human colorectal adenocarcinoma cell line HCT 116 upon NSAID induction, and is devoid of cyclooxygenase-2 activity (20). It has been found that NAG-1 can be induced by different compounds other than NSAID, such as the peroxisome proliferator-activated receptor ß (PPARß) ligands troglitazone and 1,1-bis(3’-indolyl)-1-(p-substitutedphenyl) methanes (C-DIM), which subsequently promotes apoptosis and inhibits the growth of HCT 116 colon cancer cells through a receptor-independent pathway (10,21). Moreover, compounds obtained from natural sources such as resveratrol (from grape skin) and genistein (from soybean) have also been recognized as potent inducers of NAG-1 (8,22). Based on our findings on *Coptis chinensis* and berberine, NAG-1 indeed plays an imperative role in the pro-apoptotic and anti-tumorigenic actions of herbal medicinal compounds in treating liver cancer. Furthermore, we previously demonstrated that NAG-1 is also a target for chemotherapeutic treatments by other novel herbal adjuvant agents (11,23). Hence, we suggest that NAG-1 is a good biomarker for the pro-apoptotic and anti-carcinogenic activity of herbal based anti-cancer drugs. Initially, NAG-1 was proposed to be induced in a p53-dependent manner, but it was later discovered that basal transcription of NAG-1 is also regulated by several regulatory elements in its promoter region (24). A study on NAG-1 induction by the PPARß ligand troglitazone have suggested that expression of NAG-1 requires the transcription factor Egr-1, but not the Sp-1 (10). This proposition was confirmed by our data, of which Egr-1 activation by *Coptis chinensis* and berberine appeared to be essential in causing NAG-1 overexpression.

Taken together, our results show that both *Coptis chinensis* and its major constituent berberine inhibit proliferation and
promote apoptosis in HepG2 cells through a similar mode of action, including facilitation of cell cycle arrest in the G2/M phase as well as bcl-2 inhibition and caspase activation. Since the concentrations of *Coptis chinensis* containing similar contents of berberine induce comparable degrees of pro-apoptotic actions, we propose that the anti-carcinogenic activity of the herb is mainly due to its berberine content. On the other hand, we also identified a significant contribution of the novel pro-apoptotic protein NAG-1 in the anti-carcinogenic processes, which requires activation of an upstream transcriptional factor Egr-1. This highlights NAG-1 as a potential new biomarker in the treatment of HCC and related cancers, particularly by herbal-based chemotherapeutic agents.

**Acknowledgements**

The plasmid containing NAG-1 vector was kindly offered by Dr Seung J. Baek of the University of Tennessee. This study was supported by the Hong Kong Baptist University Faculty Research Grant FRG/06-07/I-02.

**References**