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Synergistic Effect of Combined Etodolac Plus 5-Fluorouracil in Human Hepatoma Therapy--an *In vitro* Evaluation by Cell Lines HepG2, HA22T and KELFIB

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Abstract: Hepatocellular Cancer Cell (HCC) lines HepG2, HA22T and KEL FIB were used to test the therapeutic effect of different combined protocols for etodolac plus 5-FU: 1). the selective COX 2 inhibitor etodolac alone at 0.0~0.16 μg μL^{-1}; 2). 5-FU alone at 0.0~1.25 μg mL^{-1}; 3). etodolac (0.31 μg μL^{-1}) plus 5-FU (1.25 μg mL^{-1}) in a simultaneous-, or an alternative sequential protocol. Cell viability was measured using MTT assay and flow cytometric analysis. An inverse dose dependent survival rate was observed for both drugs, while the apoptotic data of the three HCC cell lines have favored the simultaneous administration of etodolac plus 5-FU, which revealed to be more effective than administering etodolac or 5-FU alone. We conclude that in view of the therapeutic efficacy with a minimized cytotoxicity, a simultaneous administration of etodolac plus 5-FU, each at much more reduced dosages than usually prescribed alone, could be strongly recommended for treatment of HCC.

Key words: Hepatoma, COX-2 inhibitor etodolac, 5-FU, chemotherapy

INTRODUCTION

Hepatocellular Carcinoma (HCC) have become common worldwide malignant tumors in the world, the majority of which occurs in Asia and Africa (Bosch, 1997). HCC represents more than 4% of total worldwide cancer incidences with increasing deaths at least 315,000 per year (Harris and Sun, 1984). Recently, the incidence and mortality are spreading in North America and Europe (Taylor *et al*., 1997; EI-Serag *et al*., 2003) in the United States (El-Serag and Mason, 1999) and in Taiwan. HCC actually has contributed a 42.5% of liver cancer incidence in the world, while amounting to 90% of Primary Hepatoma (HP) in China (Ye, 2004). Such an increase has been attributed primarily to chronic infections of viral hepatitis C (HCV) (El-Serag and Mason, 2000; Falcon *et al*., 2005) and B (HBV), particularly in children (Kao, 2003). HCC often develops in patients with liver cirrhosis (Akriviadis *et al*., 1998). Surgical resection usually is the standard modality with a 5-year survival rate of only 25~29%, yet only limited to a small proportion of patients (Kondo *et al*., 1999). Alternatively, non-surgical therapy has been frequently administered to patients with inoperable HCC (Huo and Lee, 2004). However, the long-term prognosis of HCC generally is poor.

5-Fluorouracil (5-FU) was the first chemotherapeutic agent reported to be effective for treatment of HCC (Friedman, 1983; Lin *et al*., 1997) whose cytotoxic effect on cancer cells has been ascribed to its capability of inducing cancer cell apoptosis. Such an apoptosis occurs in hepatoma cell lines via both Fas-dependent and Fas-independent pathways (Jiang *et al*., 1999). Cyclooxygenase (COX) is a rate limiting enzyme to covert arachidonic acid to Prostaglandins (PGs), thromboxanes and other eicosanoids (Kujubu *et al*., 1991; Xie *et al*., 1991; Hla and Neilson, 1992) during inflammation. COX has two isoforms, COX1 and COX2 (Eberhart and Dubois, 1995). While COX-1 constitutively expressed in a variety of tissues, being important for the maintenance of homeostatic functions including synthesis of cytoprotective gastrointestinal prostaglandins and the modulation of

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platelet aggregation (Eberhart and Dubois, 1995; O'Neill and Ford, 1993; Fosslien, 2000) COX-2 is induced by several growth factors and cytokines including Tumor Necrosis Factor α (TNF-α), facapentanene-12, interleukin 1β and platelet activating factor, PAF) (Plummer *et al*., 1999) primarily responsible for PGs production in inflammatory sites (Hla, 1992; Eberhart and Dubois, 1995; Dewitt and Smith, 1988; Funk, 1991; Kujubu *et al*., 1991; O'Banion *et al*., 1992; Sano *et al*., 1992; Crofford *et al*., 1994) is involved in the regulation of cell growth and blastocyst implantation (Vane *et al*., 1998) and angiogenesis (Tsujii *et al*., 1998). The overexpression of COX2 has been reported in various types of tumors, such as colorectal (Kargman *et al*., 1995) gastric (Ristimmaki *et al*., 1997) lung (Hida *et al*., 1998) prostate (Liu *et al*., 1998) breast (Liu and Rose, 1996) and malignant epithelia cancer, pancreatic and Hepatocellular Carcinoma (HCC) (Sano *et al*., 1995; Molina *et al*., 1999; Koga *et al*., 1999; Kern *et al*., 2002). The COX2 gene encodes an inducible prostaglandin synthase enzyme that is overexpressed in adencarcinomas and tumors (Liu *et al*., 2001). COX-2-induced tumor tissue expressed reduced levels of the proapoptotic proteins Bax and Bcl-x (L) and an increase in the anti-apototic proteins Bcl-2, enhanced COX2 expression is sufficient to induce mammary gland tumorigenesis (Tsujii and Dubois,1995; Liu *et al*., 2001) which can inhibit apoptosis, therefore, inhibition of COX2 may represent a mechanism-based chemopreventive approach for cancer therapy (Liu *et al*., 2001).

Tumor cell apoptosis is associated with rapid activation of the caspases-3, -6, -9, but independent of Bcl-2 and BAX and the phosphorylation status of AKT/PKB and BAD (Kern *et al*., 2002). Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) can reduce the risk of colon cancer (Kune *et al*., 1998; Thun *et al*., 1991; Giovannucci *et al*., 1995; Suh *et al*., 1993; Peleg *et al*., 1994) and decrease, in number and size, the polyps in patients with Familial Adenomatous Polyposis (FAP) (Waddel and Loughry, 1983; Giardiello *et al*., 1993; Steinbach *et al*., 2000).

The well-documented antiproliferative effect of NSAID was first identified on hepatoma cells (Hial *et al*., 1997). Present study demonstrated that sulindac can induce apoptosis in human HepG2 cells, associated with the inhibition of cell proliferation and DNA synthesis. Apoptosis caused by sulindac is associated with the activation of caspase-3 (Liu *et al*., 2002). Etodolac, a member of the pyranocarboxylic acid group of NSAIDs, exhibits anti-inflammatory, analgesic and antipyretic activities in animal models. Yamazaki *et al*. (2002) indicated celecoxib, another selective COX-2 inhibitor, induced apoptosis and inhibits proliferation of cancer cells to express COX-2. For decades, cytotoxic chemotherapeutics have been dominating in the systemic cancer therapy, especially by the so called The Maximum Tolerable Dose (MTD). However, the toxic side effects are frequently observed to be associated with MTD-based chemotherapy, which normally include acute myelosuppression and many other well known side effects including the long lasting cardiac, renal, neurological and reproductive complications. Whether to use a combination of medicine with a designed administration protocol, each in a much smaller dosages than those that are usually prescribed alone, can be more effective yet less toxic? Such a concept is called The Metronomic Chemotherapy (Pietras and Hanahan, 2005).

In present study, the COX-2 inhibitor etodolac was selected in this trial, because of its commercial availability, ease of administration, excellent side effect profile and putative anti-angiogenic effects (Masferrer *et al*., 2000; Gately and Kerbel, 2003). On the other hand, 5-FU was selected to be used in such a combination, because a number of synergistic interactions had already been demonstrated previously between 5-FU and other antineoplastic drugs in clinical investigations (Chen *et al*., 2004).

This study investigates the strategy for combined administration of 5-etodlac plus 5-FU, with an aim to discover a novel and safer synergistic therapy in treatment of HCC.

MATERIALS AND METHODS

Cell lines and culture methods: Human HCC cell lines HepG2, HA22T and KELFIB (human skin keloid fibroblast cell line) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in a complete medium containing DMEM (Gibco BRL Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Isreal), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Celox Corporation, Hopkins, MN). KELFIB was used as the control.

Medicine: Etodolac, a racemic mixture of [+] S and [-] R enantiomers of (±) 1,8-diethyl-1,3,4,9-tetrahydropyrano-[3,4-b] indole-1-acetic acid [C17H21NO3, MW = 287.37], was gifted by TTY Biopharm. 5-fluorouracil (5-FU) was provided free by ICN. Etodolac (5 mg mL^{-1}) and 5-FU (50 mg mL^{-1}) were suspended in PBS solution, respectively for in vitro experiments.

Procedure

Cell viability: IC50 and IC20: In simple dose-dependent studies, cells (104 well) of HepG2, HA22T and KEL FIB were seeded to 96-well plates and exposed to 5-FU or etodolac. The experiments were performed to search the values of IC50 (the 50% viability inhibitory concentration) and IC20 (the 20% viability inhibitory concentration) for 5-FU and etodolac, respectively. Thus, cells were added with 5-FU ranging from 1.25×10-3 to 1250 μg mL^{-1} and etodolac ranging from 0.02~1.25 μg μL^{-1}, respectively and incubated for 48 h, MTT assay was used to evaluate the % cell viability, from which values of IC50 and IC20 were chosen to proceed the next experimentations, with an aim to minimize the therapeutic dosages and related toxicity in clinical prescription.

Administration protocols: Simple simultaneous combined therapy (SSCT): Two drugs interacting at IC50 Cells (1×104/well) of HepG2, HA22T and KEL FIB were seeded to 96-well plates and added with 5-FU at IC50 (9.70 μg mL^{-1}) plus etodolac at IC50 (0.22 μg μL^{-1}) and incubated for 0, 12, 24 and 48 h, respectively. MTT assay was performed to compare the cell growth status.

Optimization of a combined therapy: To investigate the drug interaction in more detail, a combined therapy was performed for each cell lines, which actually involved a Serial Paired Simultaneous Administration (SPSA) and a Serial Paired Intermitted Sequential Administration (SPISA) protocols.

Serial Paired Simultaneous Administration (SPSA): In the protocol SPSA, different levels of 5-FU and etodolac at concentrations around the marginal IC20 (5-FU at 1.25×10-3, 1.25×10-2, 0.125, or 1.25 μg mL^{-1} and etodolac at 0.02, 0.04, 0.08, to 0.16 μg μL^{-1}, respectively) were fed in pair simultaneously.

Serial Paired Intermitted Sequential Administration (SPISA): In the protocol SPISA, different levels of 5-FU and etodolac at dosages around IC20 (5-FU at 1.25×10-3, 1.25×10-2, 0.125, or 1.25 μg mL^{-1} and etodolac at 0.02, 0.04, 0.08, to 0.16 μg μL^{-1}, respectively) were administered by pairs in a sequential manner, with 5-FU added first then followed by etodolac, or the vice versa. The intermitted periods were all fixed at 24 h. Thus cells (1×104 well) of HepG2, HA22T and KEL FIB were seeded to 96-well plates and incubated, respectively for 24 h at 37oC in a 5% CO2 atmosphere, the supernatant was completely aspirated, then treated with 25 μL of drug(s), respectively as indicated in different protocols. The MTT assay was used to determine the cell viability.

MTT assay: Cell (105 cells mL^{-1}) of HepG2, HA22T and KEL FIB were seeded into 96-well plates and after incubated for 24 h at 37°C in a 5% CO2 atmosphere, the supernatant was completely aspirated. In the simultaneous protocol, the tested cells in 96 well plates were then treated with 25 μL of either drugs at various concentrations Medium DMEM was added to make a final volume of 200 μL and incubated for 48 h, the medium containing drugs was washed off and replaced with fresh medium. The number of cells was quantified by a colorimetric MTT assay according to Mosmann (1985) using MTT (Sigma Chemical, St. Louis, MO) (5 mg mL^{-1} in phosphate-buffered saline) for a further 4 h incubation. In the sequential protocols, the tested cells in 96-well plates were treated first with either kind of drug (either etodolac or 5-FU) at serial concentrations study for 24 h, the first drug in the culture medium was then washed off, followed by treatment with the second medicine (either 5-FU or etodolac, or the vice versa) at selected concentrations for another 24 h. The cells were then subjected to MTT assay after 4 h incubation. Acid-isopropanol (120uL of 0.04 N HCl in isopropanol) was added to all wells, thoroughly mixed for few minutes at ambient temperature to dissolve the dark blue crystals. The plates were read on EIA reader at 590 nm and 630 nm, respectively. Cell survival was determined by MTT assay and plotted in percentage of the control (untreated cells). Independent triplicates were performed.

TUNEL Assay (DNA fragmentation and trypan blue exclusion assay for cell death): Cells (106 mL^{-1}) were treated with etodolac or 5-FU in a dose-dependent or a time-dependent manner at 37°C in a humidified 5% CO2 incubator, then determined by Trypan Blue exclusion and washed three times with phosphate-buffered saline supplemented with 2% FCS, fixed with 1% formaldehyde at 4°C for 20 min, washed twice with ice-cold phosphate-buffered saline and finally suspended in 70% ice-cold ethanol. The whole TUNEL assay was performed using an ApoAlertTm DNA fragmentation assay kit (Clotech, CA, USA) as directed by the manufacturer.

Briefly, cells (2×106 cells mL^{-1}) were collected, washed twice with phosphate buffer saline and resuspended in a reaction mixture containing 45μL equilibration buffer, 5μL nucleotide mix and 1μL terminal deoxynucleotidyl transferase enzyme. The reaction was performed in a dark, humidified 37°C incubator for 60 min and terminated by adding 1mL of 20 mM EDTA. Terminal deoxynucleotidyl transferase catalyzes the incorporation of Fluorescein Isothiocyanate (FITC)-labeled dUTP at the free 3'-hydroxyl ends of fragmented DNA. The apoptotic cells were monitored based on green fluorescence at

520±20 nm by FACScan (Becton Dickinson, CA, USA). Data were collected and treated with the CELLQuest software. Specific apoptosis was calculated by subtracting the background apoptosis observed in the control cells from the total apoptosis observed in the experimental. Triplicate experiments were repeated and data obtained were treated statistically.

Annexin V assay for relocated phosphatidylserine: The interactive effects of drugs on HepG2 and HA22T cells were also evaluated for apoptosis induction with the Annexin V assay. HepG2 and HA22T cells were treated with etodolac in combination with 5-FU at doses of IC20 (5-FU, 0.016 μg mL^{-1}; etodolac, 0.05 μg L^{-1}), respectively, according to the simultaneous or the sequential protocols as mentioned above. The Annexin V assay was performed by following the manufacturer's protocol using an ApoAlertTm Annexin V apoptosis kit (Clotech, CA., USA), which is based on the principle to observe redistribution of phosphatidylserine from the interior face of the plasma membrane to the cell surface within the early-stage post induction of apoptosis. Annexin V, a strong anticoagulant capable of conjugating with FITC, is able to bind onto the phosphatidylserine molecules having been relocated onto the cell surface. The number of apoptotic cells is then measurable from the intensity of the emitted fluorescence.

Briefly, the drug-treated cells (200 μL) were added with 10 μL of Annexin V-FITC (1 μg mL^{-1}). After incubated at room temperature for 15 min in the dark, the cells were analyzed by FACScan (Becton Dickinson, CA, USA) using a single laser-emitting excitation light at 488 nm and finally analyzed with a CELLQuest software. Specific apoptosis was calculated by subtracting the background apoptosis observed in the control cells from the total apoptosis observed in the drug-treated cells. The population Annexin-V+/PI- (early apoptotic) represents the apoptotic cells; the Annexin-V+/PI+ population, the secondary apoptotic cells; and the Annexin-V-/PI+ indicates the necrotic cells.

Flow-cytometric analysis: Cells (2×104) were cultured in 6-cm culture plates and treated with etodolac or etodolac plus 5-FU at concentrations as mentioned in this study, The floating and adherent cells post trypsinization were collected and washed with ice-cold PBS, fixed and permeabilized with 70% ethanol at -20oC overnight, then incubated with 30 μg mL^{-1} Propidium Iodide (PI) and 100 μg mL^{-1} RNase for 30 min at ambient temperature in the dark after washing with ice-cold PBS on the next day. Cell cycle analysis was performed with a flow cytometer (FACS Calibur; BD Biosciences, CA, USA). Data acquisition and analysis were performed in the flow cytometer with the accompanying software (CellQuest; BD Biosciences, USA). Appropriate gating was used to select the easily distinguished single population. Ten thousand events per sample were counted and at least triplicate determinations were performed to assure each cell cycle distribution. The final percentages of cells in each phase were calculated from the triplicates with the data treated statistically.

Statistical analysis: Data were taken from at least triplicate independent determinations and expressed in mean±SD.

RESULTS

Dose-and time-dependent inhibitory effects on cell growth exerted by 5-FU and/or etodolac: A dose-dependent growth-inhibitory effect on cell lines HepG2, HA22T and KELFIB were observed after 48 h of incubation with both 5-FU (Fig. 1) and etodolac (Fig. 2). The level of 20 and 50% inhibitions (IC20 and IC50) by 5-FU occurred at 0.031 and 9.70 μg mL^{-1}, respectively (Fig. 1), compared to 0.05 and 0.22 μg μL^{-1}, respectively for etodolac (Fig. 2). In term of cell viability inhibitory effect per unit concentration, 5-FU was more potent than etodolac (Fig. 1 and 2), Alternatively, the time-course studies showed that growth inhibition on all cell lines was apparently conceivable at 24 h post incubation despite the protocols used (Fig. 3).

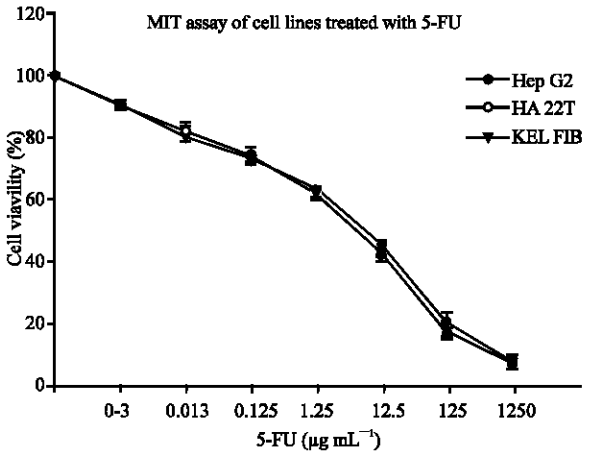

Fig. 1: Dose-dependent growth inhibitory effect of 5-FU on cell lines HepG2, HA22T and KELFIB. The cell growth rate (%) was calculated by dividing the number of 5-FU-treated cells by that of untreated cells. Data from triplicate experiments are shown in the mean±SD

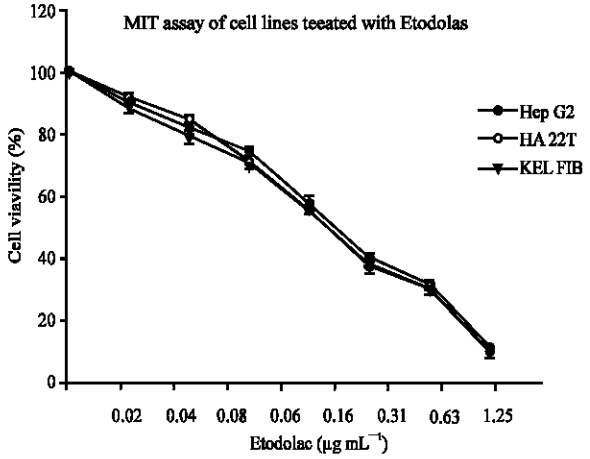

Fig. 2: Dose-dependent growth inhibitory effect of etodolac on cell lines HepG2, HA22T and KELFIB. Cell growth rate (%) was calculated by dividing the number of etodolac-treated cells by that of untreated cells. Data from triplicate experiments are shown in mean±SD

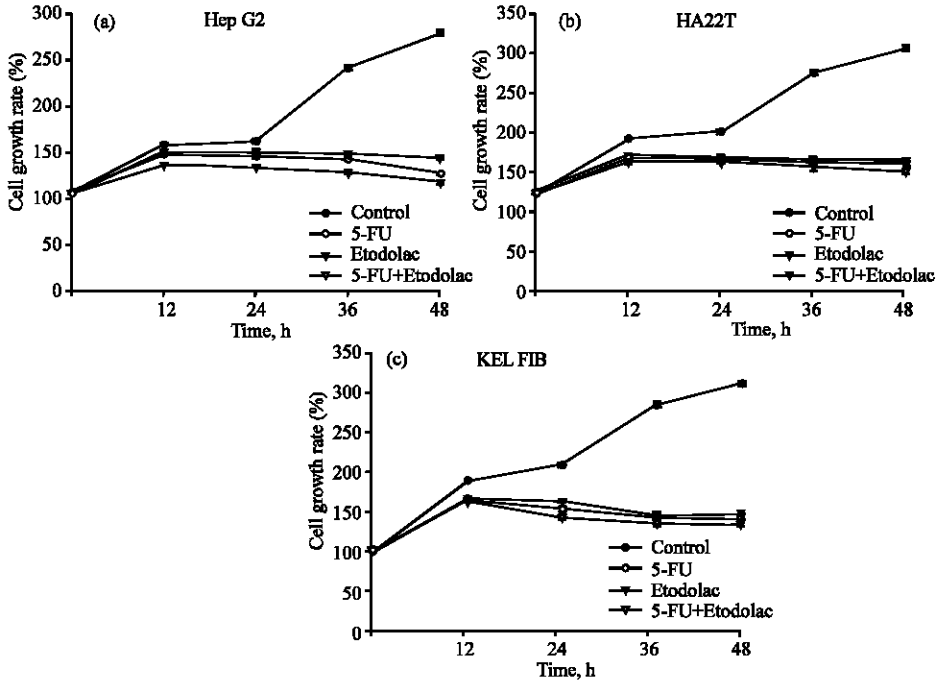

Fig. 3: The time-dependent growth-inhibitory effects on HepG2, HA22T, KEL FIB and FIB treated with either 5-FU (9.70 μg mL^{-1}) or etodolac (0.22 μg μL^{-1}) alone; and 5-FU (9.70 μg mL^{-1}) plus etodolac (0.22 μg μL^{-1}), respectively. Data from triplicate experiments are shown in mean±SD

Dose responsive interaction between etodolac and 5-FU: Results obtained from all administration protocols in cell cultures HepG2, HA22T and KELFIB are shown in Fig. 4, respectively. Taking 20% viability inhibition (IC20) as the criteria, a simultaneous administration protocol treating with 5-FU (1.25 μg mL^{-1}) plus etodolac (0.02~0.16μg μL^{-1}) showed a percent inhibition more than 40% in any cell line (Fig. 4 a-c), most prominently on the HepG2. In the sequential protocol, with 5-FU added first and followed by etodolac, results seemed to have been slightly improved with cell lines HepG2 (Fig. 4d) and HA22T (Fig. 4e). The worst outcome from the same combination was obtained with the protocol with etodolac added first and followed by 5-FU (Fig.4 g-i).

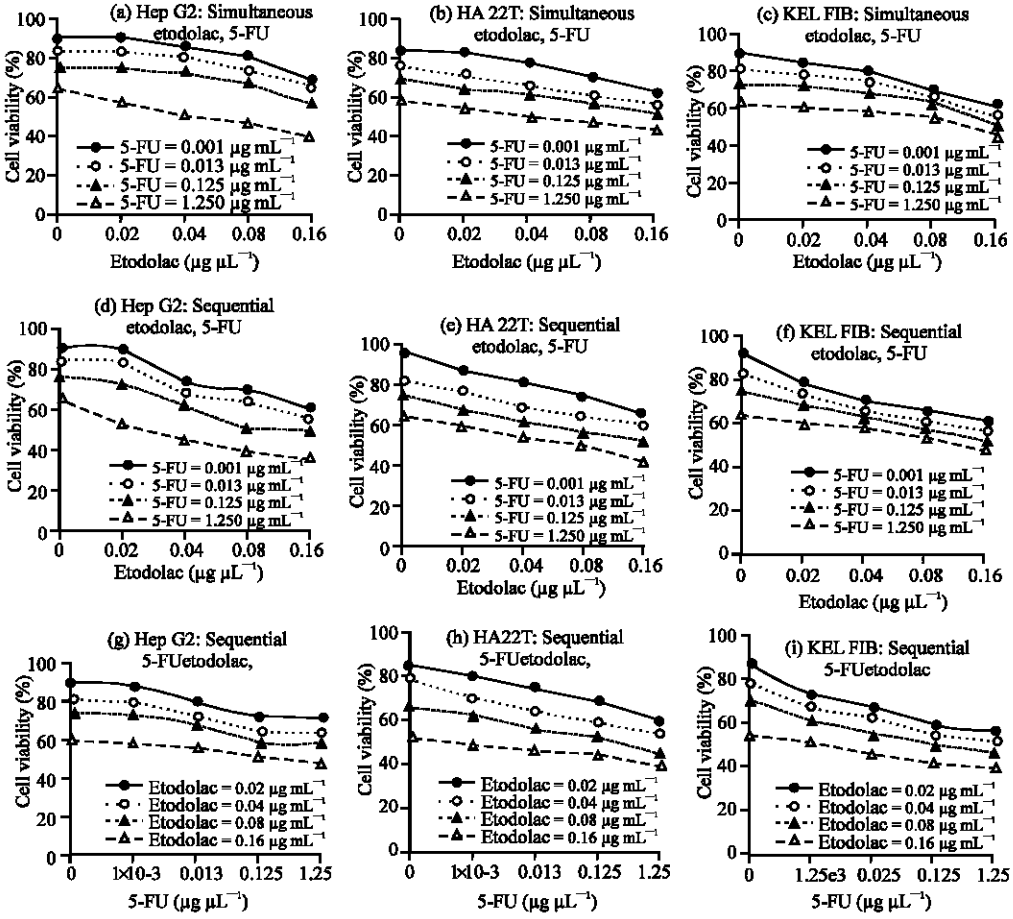

Fig. 4: Drug interaction curves demonstrated by Serial Paired Intermitted Sequential Administration (SPISA) on HepG2, HA22T and KEL FIB cell lines by etodolac plus 5-FU. Simultaneous protocol (A, B and C, respectively), 5-FU added first followed by etodolac (d, e and f, respectively) and 5-FU sequentially after etodolac (g., h and i, respectively). % cell viability was measured using the MTT assay and plotted in percentage of the control (cell without exposure to drugs). Data are expressed in mean±S.D. of triplicates

Dose-dependent induction of DNA fragmentation: DNA was cleavaged into oligonucleosomal fragmentations (180-200 bp), from which a typical feature of apoptosis was observed. For HepG2, the % apoptotic cells were 17.8, 36.5 and 47.8, comparing to those of cell HA22T 19.0, 37.0 and 48%, at etodolac 100, 200 and 300 μg mL^{-1}, respectively (Fig. 5).

Induction of apoptosis by etodolac in combination with 5-FU administered simultaneously or sequentially: All 5-FU, etodolac and 5-FU plus etodolac induced apoptosis in cell lines HepG2 and HA22T (Fig. 6). In HepG2, the extent of apoptosis was more effectively affected by 5-FU than etodolac (5-FU, 6.5%; etodolac: 0.5%, Fig. 6). As can be seen, although the sequential protocol 5-FU administered first, then followed by etodolac induced more apoptosis (11.6%) than the vice versa (1.2%) in HepG2, yet the simultaneous administration showed the most prominent effect (29.5%) (Fig. 6b), being significantly larger comparing with any of the single or sequential exposures (Fig. 6b).

Similarly in HA22T, 5-FU showed stronger effect (9.0%) on apoptosis than etodolac (7.45%) if used alone (Fig. 6b). In the sequential protocol with 5-FU added first, then followed by etodolac, more apoptosis (5.0%) was found than the vice versa (3.5%). Among the protocols, the simultaneous administration still revealed the most strongest effect (17.8%); again, evidencing the fact that the simultaneous exposure to 5-FU plus etodolac can be the most potent and feasible protocol (Fig. 6b).

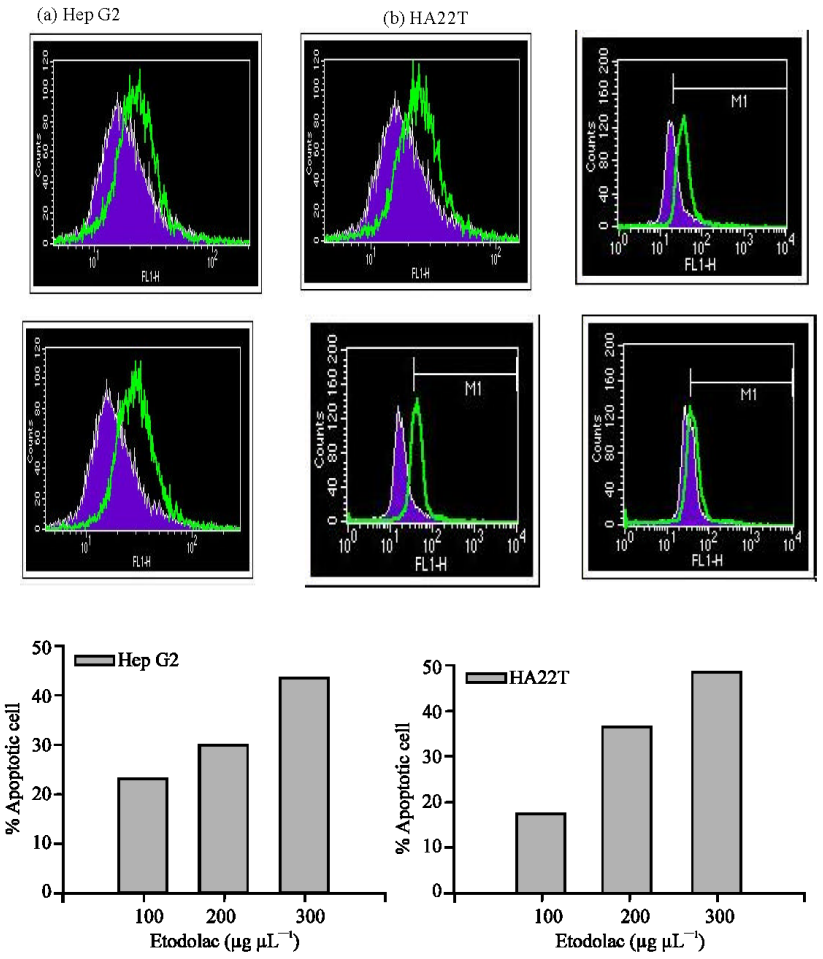

Fig. 5: Dose-dependent induction of DNA fragmentation in HepG2 (a) and HA22T (b) cell lines. Cells (1×105 cells mL^{-1}) were treated with etodolac at 100 to 300 μg mL^{-1} for the time period and cultured under the same condition as indicated. By performing TUNEL assay, the amount of Fluorescein Isothiocyanate (FITC) labeled DNA was analyzed using FACScan

DISCUSSION

A dose-dependent growth-inhibitory effect on cell lines HepG2, HA22T and KELFIB were observed after 48 h of incubation with both the 5-FU and the etodolac treated groups (Fig. 1 and 2). 5-FU (Fig. 1) appeared to be more potent than etodolac (Fig. 2) in view of the cell growth inhibitory capability by per unit concentration of medicine, i.e., a 50% inhibition was caused by etodolac at 0.22 μg μL^{-1} (Fig. 2), comparing to that of 5-FU at 9.70 μg mL^{-1} (Fig. 1), these data together with the IC20 values (for 5-FU, 0.031μg mL^{-1}; and for etodolac, 0.05 μg μL^{-1}) were thus selected as the critical concentrations for performing further experimentations as described in study, Alternatively, the time-course studies showed that growth inhibition on all cell lines was apparently observed at 24 h post incubation despite the protocol and the cell line used (Fig. 3). After 24 h of incubation, the % cell growth rates of all cell lines were seen distinctly affected and the % suppression on HepG2 at 48 h were 145, 158 and 167% by etodolac, 5-FU and 5-FU plus etodolac, respectively, comparing with the control (Fig. 3). Similar results were found for cell lines HA22T and KELFIB; for HA22T, the % suppression by etodolac, 5-FU and 5-FU plus etodolac were 147, 160 and 167%, comparing to those for KELFIB, 174, 180 and 182%, respectively. Results revealed that the most potent effect was by protocol with simultaneous administration of etodolac (0.22 μg μL^{-1}) plus 5-FU (9.70 μg mL^{-})[1] an implication of a more beneficial effect can be obtained by a combined therapy than any medicine if administered alone (Fig. 3).

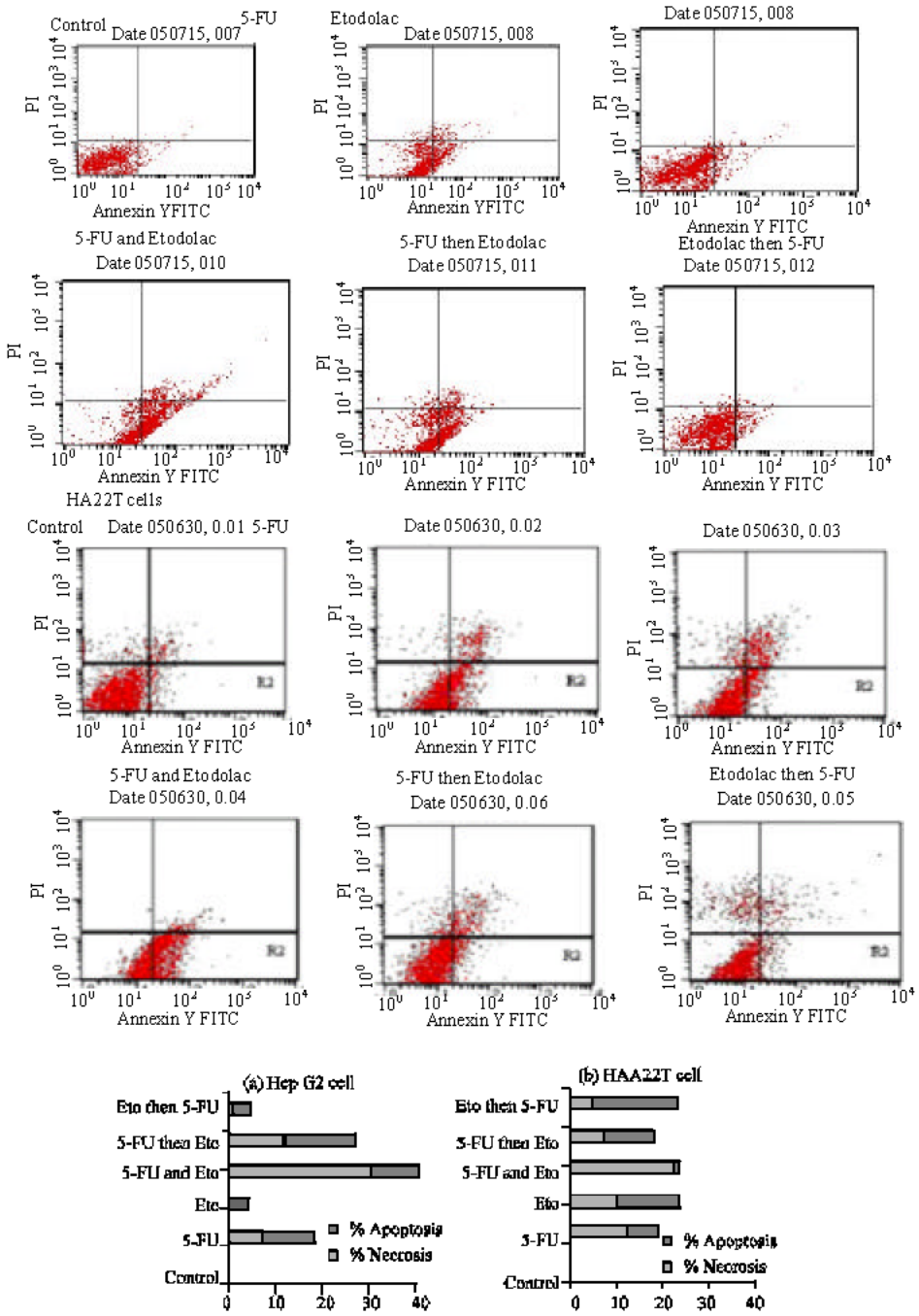

Fig. 6: Quantification of cell deaths of HepG2 and HA22T cells growing at exponential phase by Annexin-V assay. (a) Right lower quadrant in each small figure represents the population of apoptotic cells with low intensity of propidium iodide, but high Annexin-V expression and Right upper quadrant in each small figure represents the population of necrotic cells with high intensity of propidium iodide, but low Annexin-V expression. The control representing cells without exposure to drugs is show in the upper row of HepG2, HA22T. Cells were treated at IC20 (etodolac, 0.05 μg μL^{-1} in combination with 5-FU, 0.016 μg mL^{-1}) according to the simultaneous or the sequential schedules as mentioned above. Annexin-V assay was used to determine apoptotic cells (a). Cell death percentage of HepG2, HA22T cells treated with the indicated protocols are presented. HepG2, HA22T cells showed significant increments in apoptotic percentage when treated with simultaneous schedules compared to those treated with sequential schedules (b)

The interaction among various drug concentrations in cell HepG2, HA22T and KELFIB are shown in Fig. 4 (Fig. 4 a~I, respectively). Taking individual IC20 value as a criterium, around which proper drug concentrations (5-FU, 0.01~1.250 μg mL^{-1}; etodolac, 0.02~0.16 μg μL^{-1}) were selected. It can be seen that any concentration of 5-FU lower than 1.250 μg mL^{-1}, failed to suppress viability to a level lower than 50%, despite the cell line used. Either a simultaneous paired addition of etodolac with 5-FU at 1.250 μg mL^{-1} plus etodolac (0.02~0.16 μg μL^{-1}) (Fig. 4a-c), or a sequential treatment of cells with 5-FU (1.250 μg mL^{-1}) first and then followed by etodolac (0.02~0.16 μg μL^{-1}) (Fig. 4 d-f) has shown rather satisfactory effect. Whereas, the vice versa, etodolac at concentrations below 0.16μg μL^{-1} was found totally ineffective for cell viability suppression (Fig. 4 g-i). As a contrast, less effect was revealed by combinations of etodolac (0.16 μg μL^{-1}) added first then followed by 5-FU (1.25×10-3~1.25 μg mL^{-1}).

As well known, 5-Fluorouracil (5-FU) was the first chemotherapeutic agent reported to be effective for treatment of HCC (Friedman, 1983; Lin *et al*., 1997), whose cytotoxic effect on cancer cells has been ascribed to its capability of inducing cancer cell apoptosis. Such an apoptosis occurs in hepatoma cell lines via both Fas-dependent and Fas-independent pathways (Jiang *et al*., 1999). Three possible mechanisms underlying 5-FU have been proposed: By inhibition of thymidylate formation and blocking of DNA synthesis; synthesis of malfunctioning RNA due to the replacement of uracil by 5-FU and 3) inhibition of pyrimidine nucleotide synthesis, with resultant interference with the production of RNA or DNA, or with both (Reeves and Cailleau, 1969). Uridine Phosphorylase (UPase) in human tumor tissues is usually elevated in various tumor tissues and is closely correlated with the differentiation stage of tumors (Katsumata *et al*., 2003). In conferring the advantage of 5-FU in cancer therapy, i.e., Upase plays an important role in the activation of 5-FU via anabolism of 5-FU through pyrimidine salvage pathway (Cao and Pizzorno, 2004).

In contrast, the action mechanism of etodolac, like other NSAIDs, is still not completely understood, yet is speculated to be related with the prostaglandin synthase inhibition. Recently, it has been reported to inhibit the HCC cell growth with cell cycle arrests at phases G1S and G2M by the expressions of p21WAF1/Cip1 and p27Kip1 and simultaneously by inhibiting the expressions of Cdc2, CDK2/4 and Cyclin D1 (Cheng *et al*., 2002). Etodolac also inhibits cell growth by blocking the MAP kinase signaling pathway and modulates the cell cycle via the ERK pathway to induce apoptosis in HCC cells (Cheng *et al*., 2004). Its action mechanism against HCC is independent upon the expression levels of COX-2 mRNA in HCC (Cheng *et al*., 2004). Yamazaki *et al*. (2002), in comparing the different COX-2 inhibitors on cancer cell proliferation, has indicated that celecoxib, another selective COX-2 inhibitor, induced apoptosis and inhibits proliferation of cancer cells to express COX-2, whereas etodolac behaved unlikely, a result being contradictory to Matsunaga *et al*. (2004). Chen *et al*. (2001) ascribed such varying responses to the cell-line-dependent.

The application of a combined therapy for HCC is based on the rationale that by effectively introducing various different antitumor mechanisms, a combined therapy may become synergistically more effective. Multiple drugs to be used in treatment and prevention of diseases can be traced back for thousands of years beginning from the use of the compound medicinal herbs in ancient China. The popularity of using drug combinations for the treatment of cancer necessitates the quest at the laboratory level for the best choice of drugs to be used, for the quantitative evaluation of experimental data to provide the bases for the design of clinical trial protocols and finally for the implementation of the rationale into clinical practice.

The exact molecular mechanisms of hepatocarcinogensis and means for effective prevention and treatment are still unclear. Although long-term survival in patients with HCC is possible in selected patients, the overall prognosis remains unsatisfactory especially in those with aggressive tumor behavior. Clinically, some patients with impaired liver function and HCC are not therapeutically eligible for aggressive chemotherapy. Such cases include those with advanced cirrhosis and low platelet counts and being unable to tolerate myelo-suppressive therapy, those with uncontrolled ascites who are intolerable to i.v. hydration required for treatment with platinum analogs and those whose diseases failed to respond to previously administered regimens.

CONCLUSION

In conclusion, selective inhibition of COX-2 by eodolac, in combination of 5-FU, has lead to a remarkable growth inhibition of HCC cells. In treatment of HCC, etodolac plus 5-FU at proper doses can be a promising safer and more effectively chemopreventive than either etodolac or 5-FU alone as conventionally prescribed clinically, thus a simultaneous combined administration protocol can be a very promising alternative suggestive for the HCC therapy.

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