Research Paper

Antitumor Activity of Combination Treatment of *Lentinus edodes* Mycelium Extracts with 5-Fluorouracil against Human Colon Cancer Cells Xenografted in Nude Mice

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AIM: **5-Fluorouracil (5-FU) is one of the widely used chemotherapeutic drugs targeting various cancers, but its chemoresistance remains as a major obstacle in clinical settings. In this study, we evaluated the *in vivo* efficacy of *Lentinus edodes* mycelium extracts (designated as LEM), an edible mushroom extracts, as a 5-FU adjuvant agent. Furthermore, we intended to study the underlying mechanisms to account for the role of LEM.**

METHODS: **Human colon cancer COLO 205 cells were treated with 5-FU, LEM, or combination of 5-FU with LEM. Induction of apoptosis and cell cycle arrest was demonstrated by DNA ladder electrophoresis and flow cytometry, respectively. Additionally, COLO 205 cells were transplanted into athymic nude mice as a tumor model for evaluation of the antitumor effect of combination treatment with LEM plus 5-FU. The mechanisms for altered cell cycle progression were investigated by immunoblotting analyses of the G_0/G_1-phase regulatory proteins.**

RESULTS: **COLO 205 cells were markedly sensitized to apoptosis and G_0/G_1-phase arrest by combination treatment of 5-FU with LEM when compared with 5-FU alone. Our results furthermore indicated that LEM markedly enhanced the 5-FU-mediated up-regulation of the p53, p21/Cip1 and p27/Kip1 proteins in COLO 205 cells-xenografted tumor tissues. In contrast, although the expression levels of cyclins B and D3 proteins were down regulated in the 5-FU-treated tumor tissues, no significant potentiation effect was observed in the tumors with 5-FU and LEM combination treatment.**

CONCLUSION: **Our results suggest that combination of 5-FU with LEM may represent a novel chemotherapeutic strategy in colon cancers and that p53, p21/Cip1 and p27/Kip1 may play some important roles for the involvement in antitumor activity.**

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***Lentinus edodes* mycelium extracts**

G_0/G_1 arrest

apoptosis

p53

Introduction

Colorectal cancer is one of the most commonly diagnosed cancers in the world and the second most common cause of cancer death in the Western countries [1]. The management of primary colorectal cancer is determined according to the site of the tumor and surgery remains the mainstay of treatment. Postoperatively, patients should be considered for adjuvant chemotherapy based on the final staging diagnosis. Combination chemotherapy is now a mainstay in the treatment of advanced and disseminated colon neoplasia [2]. The rationale for combination therapy is based on the principle that resistance occurred in a heterogeneous tumor cell population to any single agent could be overcome by using multiple agents with different mechanisms of action [3]. In addition, rational delivery of combination chemotherapy may induce an additive or synergistic response, for example, curcumin, a major yellow pigment in turmeric used widely all over the world, synergistically inhibits the growth of human colon cancer cell line HT-29 if in combination with 5-fluorouracil (5-FU[2]), and significantly and specifically inhibits the expression of cyclooxygenase-2 protein [4]. In other studies, high-dose therapy with the antioxidants such as pyrrolidinedithiocarbamate and L-N-acetylcysteine (NAC) is reported to increase the efficacy of 5-FU, producing complete resolution of colorectal cancer cell line xenografts in nude mice [5,6]. It must be noted, however, that overlapping toxicities between combinations may preclude their clinical utility.

An edible mushroom *Lentinus edodes* is one of the popular foods in several Far Eastern countries. The water soluble extract of the mycelial culture of *Lentinus edodes* (designated as LEM) was isolated from solid medium of sugar-cane bagasse and defatted rice bran [7]. Several studies have

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[2]**Abbreviations:** 5-FU, 5-fluorouracil; NAC, L-N-acetylcysteine; LEM, *Lentinus edodes* mycelium extracts; LAP, alcohol precipitate of LEM.

shown that LEM and its purified fractions have many physiological activities including antitumor [8,9], antiviral [10,11], and immunomodulating activities [12]. The antitumor activity of LEM has been demonstrated by activation of macrophages *in vitro* [13,14]. Many investigators have demonstrated that polysaccharides from various fungi, especially mushrooms, act as anticarcinogenic immunomodulators [9,15-21]. The suggested mechanisms for antitumor effects of these mushroom-derived polysaccharides include activation of caspase-mediated cancer cells apoptosis [19,22], inhibition of cancer cell growth cycle [17], and induction of antiangiogenesis [16,18]. More detailed mechanisms for LEM-induced antitumor response still need more investigations.

In this study, we observed that LEM could induce cancer cell apoptosis and G_0/G_1-phase arrest, thus suggesting a potential opportunity for combination therapy with 5-FU. Our data demonstrated that at least additive cytotoxicity could be achieved by combination treatment of LEM with 5-FU, in different human cancer cell lines with various *p53* status. In addition, combination treatment of LEM with 5-FU exhibited an inhibitory efficacy toward the colorectal cancer cell xenografts in nude mice, suggesting that promising antitumour activity may be achieved against colorectal cancer by such combination therapy. Furthermore, we disclosed the underlying mechanisms by investigating the effects of this combination treatment on the expression of G_0/G_1-phase regulatory proteins in the tumor tissues.

Materials and Methods

Preparation of LEM

Lentinus edodes mycelium was purchased from the SUN-LEM Co., Ltd. (Taipei, Taiwan). The spores of *Lentinus edodes* were germinated and cultured in liquid medium containing 20 mg/ml malt extract, 2.5 mg/ml yeast extract and 2 mg/ml ammonium tartrate. The resulting mycelia pellet was cultured in the solid medium which was composed of bagasse and defatted rice bran (5:1, w/w). The culture medium was disrupted before the formation of fruit bodies. The disrupt material was incubated in water at 40-50°C for 60 h to promote autolysis of the mycelia and partial digestion of the culture medium with mycelia enzymes. The digest was then extracted with water at 60°C. The extract was aseptically filtered and lyophilized. The resultant pale brownish powder was designated as LEM [7]. To the aqueous solution of LEM, 4 volumes of ethyl alcohol were added and the resulting precipitate (designated as LAP) was pooled and lyophilized. The LAP was easily dissolved in cancer cell culture medium to evaluate its effect on cancer cell proliferation.

Cell culture

The HT 29 (p53 mutant) [23] and COLO 205 (p53 wild-type) [24] cell lines were isolated from human colon adenocarcinoma (American Type Culture Collection HTB-38 and CCL-222). Hep 3B (p53 partially deleted)[25] and Hep G2 (p53 wild-type) [25] cell lines were derived from human hepatocellular carcinoma (ATCC HB-8064 and HB-8065)[26]. The HL 60 cell line (p53 null) was derived from human myeloid leukemia cells (59170; American Type Culture Collection). The cell line FCH, a homozygous familial hypercholesterolemia cell (CRL 1831; American Type Culture Collection), was derived from primary cultures of normal colon epithelial cells [27]. The cell lines were grown in Eagle's minimal essential medium (for Hep 3B and Hep G2 cells) or RPMI 1640 (for COLO 205, HT 29 and HL 60 cells) supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycin, and 0.3 mg/ml glutamine in a humidified incubator (37°C, 5% CO_2). The CRL 1831 cells were grown in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12, 1:1 with 2.5 mM L-glutamine, 1.2 mg/ml sodium bicarbonate, 15 mM HEPES, and 0.5 mM sodium pyruvate supplemented with 10% FCS, 10 ng/ml cholera toxin, 5 μg/ml insulin, 5 μg/ml transferrin, 100 ng/ml hydrocortisone and 10 mM HEPES in a humidified incubator (37°C, 5% CO_2).

Determination of cell growth curve

Human cancer cells (1×10^4) and CRL 1831 cells (1×10^5) were plated in 35-mm Petri dishes. On the next day, the medium was replaced with or without LAP (0.5-1.5 mg/ml) and the incubation medium was renewed every day during the experiment. At the end of incubation, cells were harvested for cell count with a hemaetocytometer.

Cell synchronization, drug treatment, and flow cytometric analysis.

At 24 h after plating of cells, medium was removed. Cells were washed 3 times with medium (without FCS), and then incubated with medium containing 0.04% FCS for 24 h. Under such a condition, cells were arrested in G_0/G_1 as determined by flow cytometric analysis. The low serum medium was removed and the cells were then stimulated by the addition of medium containing 10% FCS. LAP solutions were added at the indicated concentrations (0.1-20 mg/ml) for 15 h. The stages of cell cycle in LAP- and mock-treated groups were then measured by flow cytometric analysis.

Analysis of apoptosis

Apoptosis of the COLO 205 cells subjected to various treatments were detected by analyzing the internucleosomal DNA cleavage [24]. Briefly, LAP-treated and control cells were collected and lyzed, and cellular DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamyl alcohol (24:1, v:v), precipitated with 0.1 volume of sodium acetate, pH 4.8, and 2.5 volumes of ethanol at -20°C for overnight, and finally centrifuged at 13,000 ×g for 1 h. Equal amount of DNA samples were resolved by 2% agarose gel electrophoresis. The DNA ladder pattern was visualized by ethidium bromide staining.

Treatment of COLO 205 cell xenografts in vivo

Male BALB/c-nu mice were purchased from the National Laboratory Animal Center of Taiwan. They were maintained under Specific Pathogen Free conditions and supplied with sterilized food and water. After trypsinization, the COLO 205 cells (10^6 cells/0.2 ml) were injected subcutaneously into the flanks of BALB/c-nu mice (4-6 weeks old, 18-20 g). Once the tumor reached a volume of 200 mm^3, the mice were divided randomly into five groups of eight mice each. Mice were treated daily with 0.1 ml saline orally as the control or treated with a 5-FU derivative, which was converted to 5-FU *in vivo*, by using gavage tube at a dose of 18 mg/kg/day for 42 consecutive days. In addition, combination treatment of 5-FU with dried LEM powder (1-5% of daily diets) was administered orally at a dose previously described [28]. The tumor size was measured using calipers and the tumor volume was estimated by the formula: tumor volume (mm^3) $=1/2 \times L \times W^2$ (where L is the length and W is the width of the tumor)[29]. To monitor the drug toxicity, the body weights of mice were measured every week. After 42 days, the mice were sacrificed, and the tumor were removed and weighted. In addition, the pathological examination was performed on the major organs of each mouse including liver, lung and kidney.

Protein extraction and Western blot analysis

As described previously [30], frozen tumor tissues were pulverized in liquid nitrogen, and then mixed with lysis buffer (Tris-HCl 0.5 M, pH 6.8, SDS 0.4%). Western blot analyses were performed by probing with proper dilutions of specific antibodies at room temperature for 2 h. Anti-p21/Cip1, anti-p27/Kip1, anti-p53, anti-GAPDH monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-cyclin B1, D1, and D3, anti-cdk2 and cdk4 monclonal antibodies (Transduction Laboratories, Lexington, KY, USA) were used at a concentration of 1:1,000 dilution. Anti-

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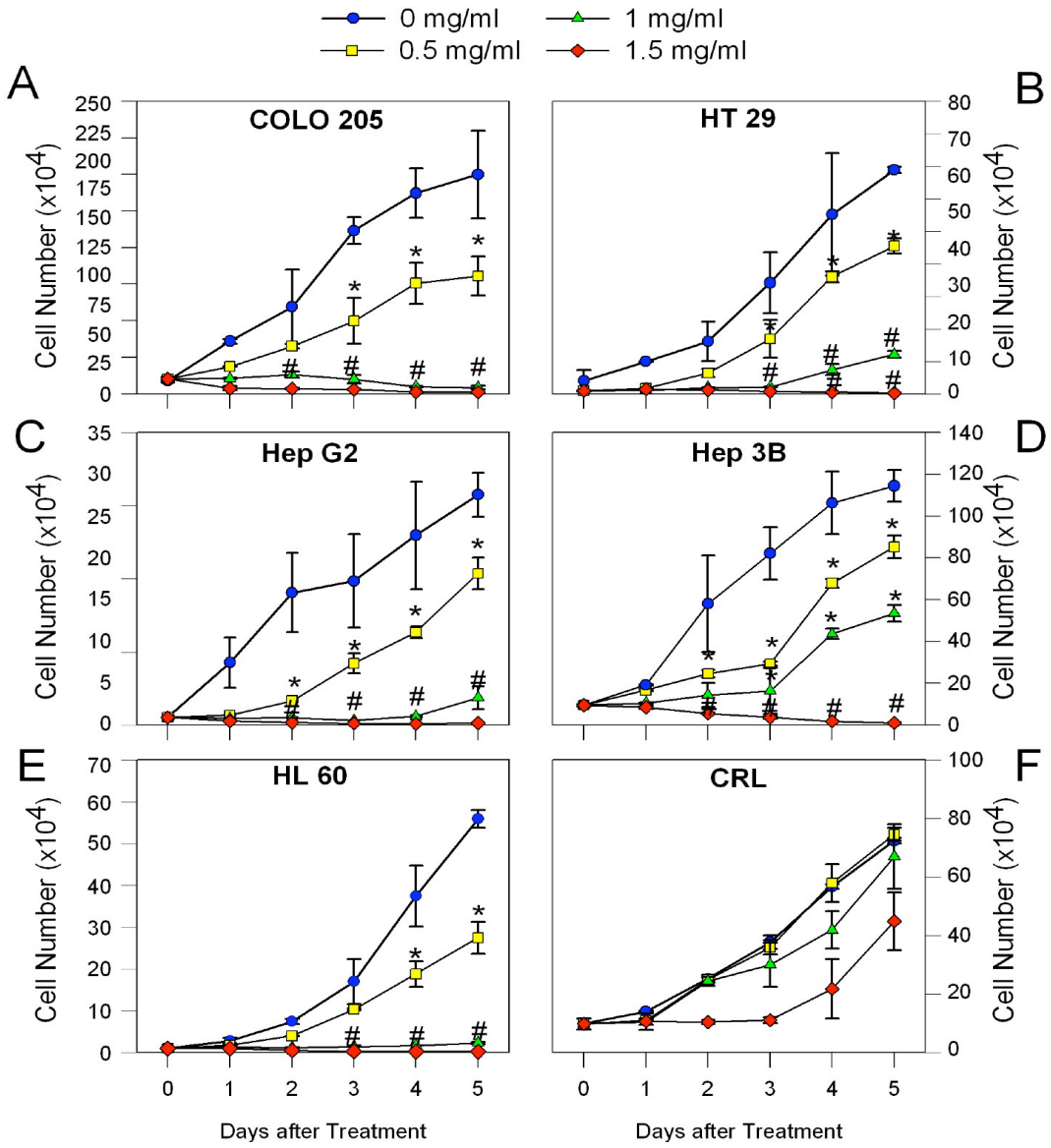

Figure 1: Growth inhibition of human cancer cells treated with LAP. (A) COLO 205, (B) HT 29, (C) Hep G2, (D) Hep 3B, (E) HL 60, and (F) CRL cells were treated with various concentrations of LAP (0.5 to 1.5 mg/ml). The media containing various concentrations of LAP were refreshed everyday and the total cell numbers were counted at the indicated time points. Three samples were analyzed in each group and the results were presented as means ± SE. Comparisons were subjected to Student's *t* test. Significance was accepted if $P < 0.05$. $^{*}P < 0.05$. $^{\#}P < 0.01$.

cyclin A polyclonal antibody (Transduction, San Diego, CA, USA) was used at a concentration of 1:250 dilution. The secondary antibodies, alkaline phosphatase-coupled anti-mouse and anti-rabbit antibodies (Jackson, Westgrove, PA, USA), were incubated at room temperature for 1 h at 1:5,000 and 1:1,000 dilutions, respectively. Specific protein bands were developed by incubating with the colorigenic substrates (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate; Sigma Chemical Co., St. Louis, MO, USA). In each experiment, membranes were also probed with anti-GAPDH antibody to correct the differences in protein loading.

Statistics

All data were expressed as mean ± SE. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

Results

LAP-induced G_0/G_1-phase arrest in human cancer cells

Our data indicated that both the alcohol precipitate and water-soluble fractions of LEM (designated as LAP) induced significant growth inhibition of human cancer cells including hepatoma (Hep G2 and Hep 3B), colon cancer (HT 29 and COLO 205), and leukemia (HL 60) cells (Figure 1A-1E). Distinctly, significant cell growth inhibition was only observed in human non-cancerous colon epithelial (CRL) cells when treated with higher concentration (1.5 mg/ml) of LAP (Figure 1F), indicating that LAP-induced cell growth inhibition and cytotoxicity was preferentially observed in human cancer cells.

In order to determine whether LAP could induce cell growth cycle arrest in human cancer cells, COLO 205 cells were synchronized at the G0/G1 phase by 0.04% serum starvation for 24 h [30], and then were replaced with complete medium (with 10% FCS) containing LAP (0.1-20 mg/ml). Flow cytometric analysis was performed at 15 h after cells were released from synchronization. Our previous reports have

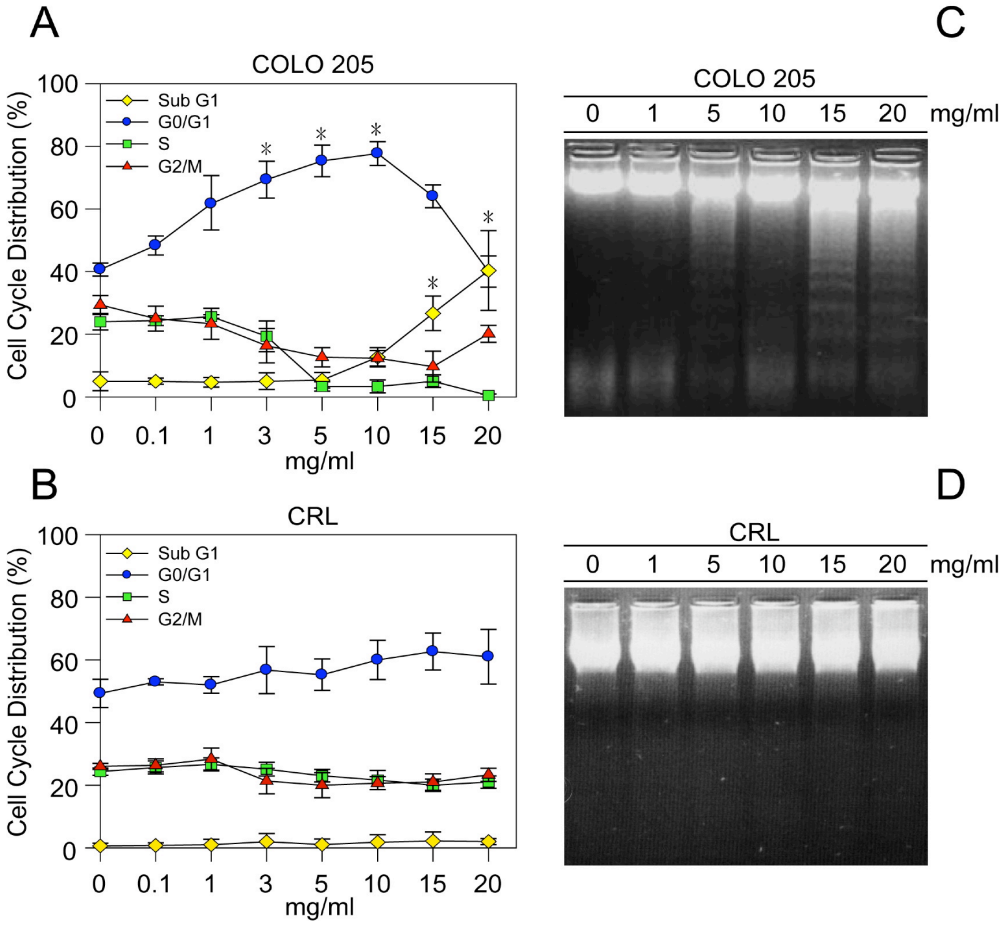

Figure 2: LAP-induced G_0/G_1-phase arrest and apoptosis in human colon cancer cells. (A) Human colon cancer cells (COLO 205) and (B) human non-cancerous colon epithelial cells (CRL) were synchronized with 0.04% FCS for 24 h as described in Materials and Methods. After synchronization, cells were then released into complete medium (10% FCS) containing various concentrations of LAP (0.1-20 mg/ml). Cell cycle arrest in both cells was measured by flow cytometry at 15 h after exposure to LAP. Three samples were analyzed in each group and the results were presented as mean ± SE. Comparisons were subjected to Student's *t* test. Significance was accepted when $P < 0.05$. $^*P < 0.05$. (C) COLO 205 and (D) CRL cells were treated with various concentrations of LAP (1-20 mg/ml) for 24 h. After treatments, cells were harvested and DNA fragmentation effects were then detected by agarose gel electrophoresis.

demonstrated that the population of COLO 205 cells entered the S phase at 15 h after re-stimulated with complete medium. The cells in S phase were the most suitable for analysis of concentration-dependent effects of G_0/G_1 arrest [30,31]. Accordingly, this time point (15 h) was chosen for studying the efficacies of LAP-induced G_0/G_1 arrest in human colon cancer COLO 205 cells and human non-cancerous colon epithelial (CRL) cells. Our results showed that 1 mg/ml LAP caused an apparent G_0/G_1-phase arrest in COLO 205 cells (61.3%); in contrast, no significant cell cycle change was observed in the LAP-treated CRL cells (Figure 2A and 2B).

LAP-induced apoptosis in human colon cancer cells

As shown in Figure 2A, apoptotic COLO 205 cells appeared as sub-G_1 population were demonstrated by flow cytometric analyisis when cells were treated with higher concentrations of LAP (> 15 mg/ml) for 15 h (Figure 2A). The COLO 205 cells treated with LAP (> 15 mg/ml) exhibited morphological changes (data not shown) as well as progressive internucleosomal DNA degradation yielding a ladder of DNA fragments (Figure 2C). Importantly, neither apoptosis nor cell growth cycle arrest were observed in CRL cells even at higher concentrations (> 15 mg/ml) of LAP treatment (Figure 2B and 2D). From these data, we found that the G_0/G_1-phase arrest and apoptosis were induced preferentially in COLO 205 cells than the non-cancerous colon epithelial (CRL) cells when exposed to LAP.

LEM potentiates the antitumor effect of 5-FU in nude mice

Combination treatment of cancer cells with the drugs affecting cell cycle checkpoints has been suggested to be one of the approaches to enhance the drug-induced apoptotic effect in human malignant cells [29,32]. Accordingly, COLO 205 cells were treated with LAP (5 mg/ml) in combination with 5-FU (500 nM) which caused G_0/G_1 arrest [33], and examined the degree of the occurrence of apoptosis. Our results revealed that combination treatment of LAP and 5-FU significantly potentiated the occurrence of DNA laddering in COLO 205 cells (Figure 3A, lane 4). As a control group shown in Figure 3A, lower concentration of LAP (5 mg/ml) or 5-FU (500 nM) alone did not induce significant DNA laddering in COLO 205 cells (Figure 3A, lanes 2 and 3).

Given the enhancement of LAP in the 5-FU-induced apoptosis of COLO 205 cells *in vitro*, we next determined whether administration of LEM could affect the 5-FU-induced tumor regression in an *in vivo* setting. 5-FU (18 mg/kg/day) [34] was administered orally by gavage tube with or without the dried LEM powder (1 and 5%) supplemented in daily diet according to the previous papers described [35]. The tumor volumes in the mice given LEM, 5-FU or combination of LEM

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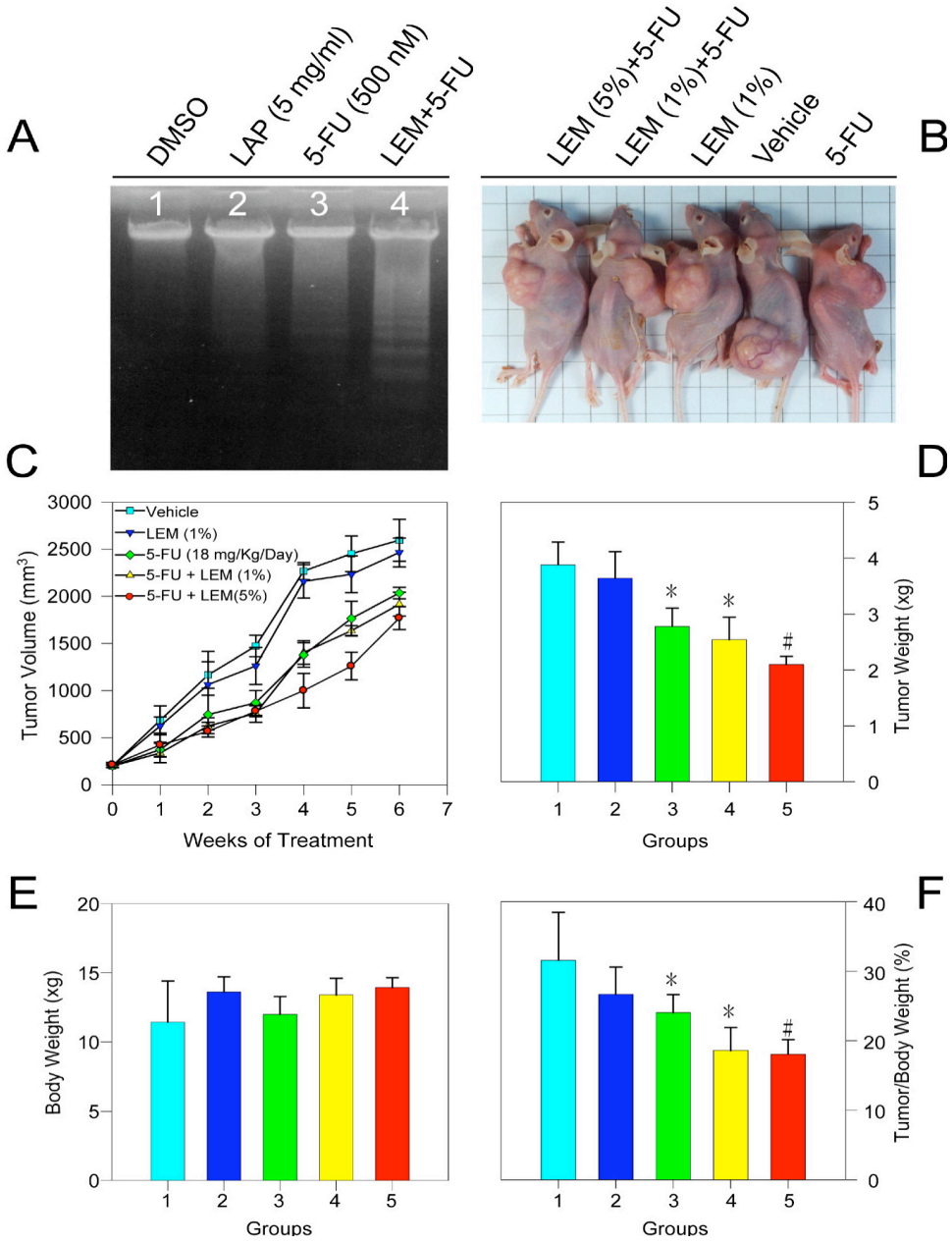

Figure 3: LEM potentiates the anti-tumor activity of 5-FU in nude mice. (A) Human COLO 205 cells were treated for 24 h with LAP (5 mg/ml), 5-FU (500 nM) or LAP plus 5-FU. DNA fragmentation was examined by using agarose gel electrophoresis. Lane 1, cells were mock treated with DMSO as a control. (B-F) COLO 205 cells were inoculated into nude mice and treated with LEM (1 or 5%), 5-FU (18 mg/kg/day) or combination treatment as described in Materials and Methods. (B) The outward status of COLO 205-tumors in the nude mice with different treatments. The COLO 205-tumors were then dissected from nude mice for protein extraction at 6 weeks after vehicle or drug treatment. (C) Average tumor volumes of vehicle *vs.* drug-treated nude mice (n = 8). The tumor weight (D), animal body weight (E), and tumor/body weight ratio (F) were measured at the end of experiment. Five samples were analyzed in each group, and values represent the mean ± SE. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted when $P < 0.05$. $^{*}P < 0.05$ if compared with vehicle-treated mice. $^{\#}P < 0.05$ if compared with the mice treated with LEM or 5-FU alone. The mice were grouping as: vehicle-treated (group 1), 1% LEM-treated (group 2), 5-Fu (18 mg/Kg/day)-treated (group 3), 5-Fu plus 1% LEM-treated (group 4), and 5-Fu plus 5% LEM-treated (group 5).

with 5-FU and those given vehicle (ethanol plus peanut oil) were determined. As illustrated in Figure 3B-3F, the tumor volume and tumor weight of the mice treated with LEM (5%) plus 5-FU were significantly reduced as compared with those treated with LEM (1%) plus 5-FU, suggesting that higher dose of LEM was able to enhance 5-FU-induced tumor regression (Figure 3C and 3D). In mice receiving these treatment regimens, no toxicity was observed in the gross signs such as body weight, visible inspection of general appearance and microscopic examination of individual organs (Figure 3E and data not shown). The tumor/body weight ratio was significantly reduced in the combination treatment group (Figure 3F). Our results provide further evidence indicating that such therapeutic regimens may have significance in application for cancer chemotherapeutic purposes.

Combination treatment of LEM with 5-FU induces p53, p21/Cip1, and p27/Kip1 expression in tumor

In order to further investigate the molecular mechanisms for such therapeutic regimens that inhibited tumor growth *in*

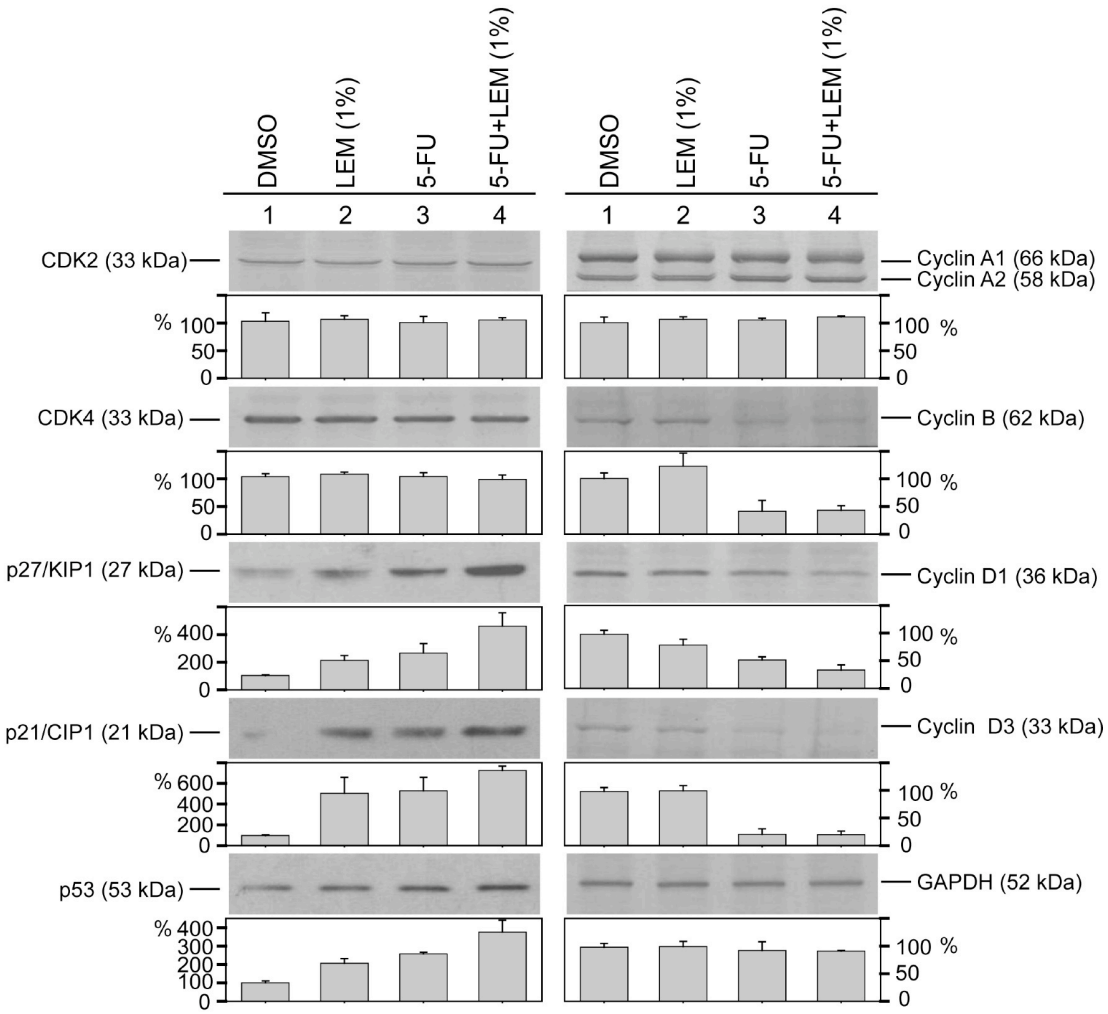

Figure 4: Combination treatment of 5-FU with LEM increases the expression levels of p53, p21/Cip1 and p27/Kip1 proteins in the COLO 205-xenografted tumor tissues. Western blot analyses were performed to determine the levels of p53, p27/Kip1 and p21/Cip1 proteins in the tumors. The data shown were the representative from 8 different tumor samples in the same group. Membranes were also probed with anti-GAPDH antibody to correct for differences in protein loading.

vivo, the protein lysates were extracted from tumor tissues in each group and the cell cycle regulatory proteins were then determined by immunoblotting analyses. We found that the expression of p53, p21/Cip1 and p27/Kip1 proteins in tumor tissues was elevated in the groups treated with LEM or 5-FU alone (Figure 4, lanes 2 and 3). Surprisingly, in the combination treated group, the p53, p21/Cip1 and p27/Kip1 protein levels were drastically induced in the tumor tissues (Figure 4, lane 4). Our results also showed that the expression of cyclins B and D3 was significantly inhibited in both 5-FU and 5-FU plus LEM treated tumor tissues. The levels of cyclins A1, A2, E and D1 expression in tumor tissues were not significantly changed in all of these treatments. Our results implied that the cell cycle inhibition effect induced by LEM was through elevation of the p53, p21/Cip1 and p27/Kip1 levels.

Discussion

5-FU is the most commonly used chemotherapeutic agent in advanced colorectal cancer. However, due to its clinical toxicity, additional experiments were performed to define an optimal schedule for drug administration enabling 5-FU to be administered at a lower dosage. On the other hand, recent studies in cyclooxygenase-2 inhibitors from clinical trials of colon cancer therapy highlights the need to develop other agents for cancer chemoprevention trials [36]. Thereby, high-dose therapy with the antioxidants such as NAC and pyrrolidinedithiocarbamate were reported to increase the efficacy of 5-FU, producing complete inhibition of colorectal cancer cells-xenografted tumors in nude mice [6,37].

The antioxidative properties of the mushroom extracts LEM have been demonstrated by intracellular oxidative stress inhibition according to different assays based on lipid peroxidation, deoxyribose, and peroxidase activities [38]. The aim of this study was to investigate whether LEM could increase the efficacy of 5-FU against colorectal cancer cells *in vitro* and *in vivo*. In combination with 5-FU, LEM potently enhanced the antitumor effects of 5-FU against COLO 205-xenografted tumors in nude mice through induction of apoptosis and cell growth cycle inhibition (Figure 3). In this study, LEM (1-5%) was added to the daily diet of the nude mice (around 25 g body weight per mice) with free access to food (approximately of 2.5 g intake per day) and water. According to this regimen, the daily consumption of LEM (1%) in nude mice was around 1 g/Kg body weight. The doses of LEM employed in the nude mouse experiments were 6-10 fold higher than those used in human prostate and breast cancer

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therapy [39,40] to ensure that we would not miss important biological effects. Preliminary experiments showed that our strain of nude mice could tolerate up to 10 g/Kg/day of LEM with no toxic responses including gross morphology, pathological examination, and serum biochemical analysis (data not shown).

Evidence emerging from many different types of experimental designs continues to support the concept that dietary habits and nutritional status play important roles in determining the risk of developing colorectal cancer [41]. For example, intervention strategies with selenium compounds represent a viable option to reduce colon cancer [36,42,43]. In addition, broccoli selenium has been identified and demonstrated to exhibit their anti-colon cancer activity in rats [44]. Currently, selenium (in the form of high selenium containing yeast or selenomethionine) is being evaluated for anticancer effects against human colon polyp recurrence [43]. Induction of apoptosis and inhibition of cell proliferation are considered important cellular events that can account for the cancer preventive effects of selenium [45]. In LEM, high concentration of selenium was identified [46-48] and such results implied that LEM might have a potential role to act as a chemopreventive agent against colon cancer. Although we have no direct evidence indicating that the selenium present in LEM was involved in potentiation of the 5-FU-mediated anticancer effects, the selenium-induced apoptosis in different human cancer cell lines were observed in the previous papers [49-53]. The principal action of LEM, as determined by this study, was to enhance the apoptosis induced by 5-FU therapy. When administered as a single agent, LEM did not induce sustained apoptosis; however, in combination with 5-FU a significant increase in apoptosis beyond control values occurred both *in vitro* (at 24 h) and *in vivo* (at 6 weeks). All these results implied that LEM might have potential for clinical application in combination with 5-FU for colon cancer patients.

In this study, significant induction of p53 protein expression was observed in both tumor tissues treated with LEM and LEM plus 5-FU. The p53 tumor suppressor is a predominantly nuclear transcriptional factor, activated by various stresses including chemotherapeutic and chemopreventive agents [54]. According to the recent study, p53 has been demonstrated to play an antioxidant role in prevention of cellular oxidative stress [55]. Down regulation of p53 results in excessive oxidation of DNA, increased mutation rate and karyotype instability, which are prevented by incubation with the antioxidant (such as NAC) [55]. Previous study in clinical trials observed in colon cancer patients has suggested a link between *p53* status and anticancer drug sensitivity [56,57]. In this study, human cancer cell lines with different *p53* status were selected for studying the mechanisms of LEM-induced anticancer activity. As shown in Figure 1, we demonstrated that the LAP-induced cell cycle arrest effect was similar in all of cancer cells when compared to non-cancerous epithelial cells. We still do not know what mechanism was responsible for the ability of LEM and 5-FU to induce *in vivo* p53 expression, however, p53-mediated signaling pathway may be of importance. Our *in vivo* study demonstrated that the p53-regulated p21/Cip1 protein was also induced in the COLO 205-xenografted tumors (Figure 4). p21/Cip1 is a negative regulator of cell cycle progression and is a transcriptional target of p53. Increase of p21/Cip1 protein expression by LEM in the tumor tissues might be via a p53-dependent signaling pathway. The previous report has indicated that ectopic overexpression of p21/Cip1 in human colon cancer HT 29 cells was associated with inhibition of CDK2-associated kinase activity and caused an appreciable growth inhibition in monolayer and soft agar cultures [58]. More experiments are needed to clarify the role of p21/Cip1 protein expression in the LEM-induced antitumor effects in COLO 205-xenografted tumors.

The cell proliferation-inhibitory effects of anti-cancer drugs used clinically are usually reversible [59,60]. Therefore, once the drug treatment has been terminated, then the growth of tumor cell rebounds. On the other hand, due to the clinical toxicity observed in long-term treatment of anticancer drugs, scientists and clinicians are searching for new agents exerting irreversible cell proliferation-inhibitory effects. Moreover, agents with additive anticancer effects can be applied in lower doses for a longer time period to achieve the same effect but with less side effects. Accordingly, additional analysis needs to be performed in our further study to demonstrate that the 5-FU-induced clinical toxicity could be significantly attenuated by LEM. Our results provide the molecular basis of LEM-induced cancer cell growth inhibition *in vitro* and further animal experiments will be important to demonstrate the potential anti-cancer effect of LEM *in vivo*.

Acknowledgments

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