Mechanism of Growth Inhibitory Effect of Blumea balsamifera Extract in Hepatocellular Carcinoma

Toshio Norikura,1 Akiko Kojima-Yuasa,1 Miki Shimizu,1 Xuedan Huang,1 Shenghui Xu,1 Saeda Kametani,1 Sook-Nyung Rho,2 David Opare Kennedy,1 and Isao Matsui-Yuasa1;1

1Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan
2Department of Food and Nutrition, College of Human Ecology, Chung Ang University, 72-1, Naeri, Daeduk-myun, Ansung-si, Kyunggi-do 456-756, Korea

Received September 13, 2007; Accepted January 24, 2008; Online Publication, May 7, 2008
[doi:10.1271/bbb.70586]

Blumea balsamifera is known to improve physiological disorders such as rheumatism and hypertension, but its anticancer activity has not been well elucidated. In this study, we found that Blumea balsamifera MeOH extract (BME) induced growth-inhibitory activity in rat and human hepatocellular carcinoma cells without cytotoxicity in rat hepatocytes which were used as a normal cell model. BME induced cell cycle arrest at the G1 phase via decreases in the expression of cyclin-E and phosphorylation of retinoblastoma protein. Furthermore, BME reduced the level of a proliferation-inducing ligand, that stimulates tumor cell growth. These findings suggest that BME has possible therapeutic potential in hepatoma cancer patients and that depletion of cellular APRIL is an important mechanism in the growth-inhibitory effect of BME.

Key words: Blumea balsamifera; cell cycle; retinoblastoma protein (Rb); cyclin-E; proliferation-inducing ligand (APRIL)

Hepatocellular carcinoma is the third most common cancer worldwide. It causes more than 600,000 deaths annually1) as it is known to be a type of tumor highly resistant to available chemotherapeutic agents.2) The high prevalence and high death rate have spurred a search for novel strategies in the prevention and treatment of liver cancer.

Natural products are excellent sources of lead compounds in developing new medicaments for the treatment of diseases. This is particularly evident in the treatment of cancers, in which more than 60% of drugs are of natural origin.3) Hence a new natural source with anticancer activities would be a valuable tool in cancer therapy. Blumea balsamifera is a medicinal plant that grows in Southeast Asia, including South China and the Philippines. This leaves are also used as a tea, and as a cure for certain disorders such as rheumatism and hypertension. Recently, its leaves have attracted attention as a part of the plant with various physiological activities, including plasmin-inhibitory,4) antifungal,5) and liver-protective effects.6) Furthermore, recent study indicates that dihydroflavonol, extracted from Blumea balsamifera, abrogates tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance in leukemia cells,7) but its anticancer function, especially its anti-tumor-proliferative activity, has not been defined thus far.

Chemoprevention is gaining more prominence. These approaches aim to decrease overall cancer morbidity and mortality by using substances that are capable of preventing cancer progression. We have found that extracts of green tea,8) evening primrose,9) and Zizyphus jujuba10) have anti-cancer activities in various tumor cell lines.

A proliferation-inducing ligand, APRIL, has been identified as a new member of the tumor necrosis factor (TNF) family. The expression of APRIL mRNA and protein was high in various tumor cell lines and tissues, but was almost undetectable in various normal tissues.11) APRIL has been reported to stimulate tumor cell growth via its signal in an autocrine and/or paracrine mode.12) Furthermore, a previous study found that expression of APRIL protein was detected in HepG2 cells, where it can promote neovascularization, as estimated by human umbilical vein endothelial cell (HUV EC) tube formation.13) Hence APRIL has received increasing attention as a therapeutic target in cancer therapy, but the...
functions of APRIL in tumor cells, especially human hepatocarcinoma cells, have not been well elucidated.

The aim of the present study was to examine the anticancer activities of *Blumea balsamifera* and related mechanisms in human liver cancer. To achieve this, leaves were extracted with MeOH and 50% EtOH, yielding *Blumea balsamifera* MeOH extract (BME) and 50% EtOH extract (BEE) respectively. In order to compare the effects as between derived from normal and tumor cells of the same origin, the effects of these extracts were examined in rat hepatocytes and rat hepatocellular carcinoma cells (McA-RH7777). Furthermore, we examined the effect of BME in human hepatocellular carcinoma cells (HepG2) and the mechanisms underlying the growth-inhibitory effect of BME. To address this, we examined the effect of BME on tumor-cell growth-related factors such as the expression levels of cyclin-E and APRIL, and the phosphorylation of retinoblastoma (Rb) protein in HepG2.

**Materials and Methods**

**Materials.** Leaves of *Blumea balsamifera* were collected in the Pinatubo area of the Philippines in July, 2005. Rb and cyclin-E antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). APRIL antibody was purchased from ProSci. (Poway, CA). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, Texas). The other chemicals used in this study were special-grade commercial products.

**Extraction.** The leaves of *Blumea balsamifera* were dried under sunlight for 2 d. and processed for powder. The dried powder was mixed with 50 parts of MeOH or 50% EtOH (v/v) for 24 h at room temperature respectively to extract the active components. Each of the fluid extracts was evaporated, and the remaining residue was freeze-dried to yield *Blumea balsamifera* MeOH extract (BME) and 50% EtOH extract (BEE) respectively.

**Total phenolic content.** The amount of total soluble phenolics was determined by the Folin-Ciocalteu method. The reaction mixture consisted of 0.25 ml of sample solution and 1.25 ml of 10% Folin-Ciocalteu’s Reagent. After a period of 3 min, 1 ml of 7.5%-sodium carbonate solution was added. The mixtures were shaken and allowed to stand for 30 min. Absorbance was measured at 760 nm with a spectrophotometer. Total phenolic contents were calculated by catechin equivalent value.

**Cell culture (HepG2).** HepG2 cells were cultured in DMEM medium supplemented with 10% FBS in a humidified incubator containing 5% CO₂ in air at 37 °C before use. The cells were washed and cultured again at a concentration of 2.0 × 10⁵ cells/ml in fresh medium. BME was dissolved in DMSO and diluted in cultured medium immediately before use (final DMSO concentration, 0.25%). In all the experiments, control cultures were made up of medium, DMSO, and the cells only.

**Cell culture (McA-RH7777).** McA-RH7777 cells were cultured in αMEM medium supplemented with 10% FBS in a humidified incubator containing 5% CO₂ in air at 37 °C before use. The cells were washed and cultured again at a concentration of 2.0 × 10⁵ cells/ml in fresh medium. BEE and BME were dissolved in DMSO and diluted in cultured medium immediately before use (final DMSO concentration, 0.25%).

**Hepatocyte preparation and culture.** Hepatocytes were isolated by the collagenase perfusion method from 10 week-old male Wister rats anesthetized with diethyl ether. The viability of the isolated hepatocytes was over 90% as determined by 0.2% trypan blue exclusion. The cells were plated in 35-mm plastic dishes at a density of 2.5 × 10⁵ cells/ml in 2 ml of Williams’ Medium E supplemented with 10% FBS, and were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C overnight. After overnight incubation, the culture medium was changed to fresh medium, and cultures were incubated with varying concentrations of BME.

**Number of viable cells.** The number of viable cells was measured by Neutral Red assay, as described previously. Neutral Red stock solution (0.4% Neutral Red in water) was diluted 1:80 in phosphate-buffer saline. Cells were incubated with Neutral Red solution for 2 h at 37 °C to allow the lysosomes of the viable cells to take up the dye. The Neutral Red solution was then removed, and the cultures were washed rapidly (in less than 2.5 min) with a mixture of 1% formaldehyde and 1% calcium chloride. A mixture of 1% acetic acid and 50% ethanol was added to the cells to extract the Neutral Red from the hepatocytes at room temperature for 30 min. Each sample was then measured at 540 nm with a spectrophotometer.

**Measurement of cell cycle.** At the end of 24 h of incubation, the cell cycle distribution was analyzed with a Laser Scanning Cytometer (Olympus LSC101, Tokyo) by propidium iodide (PI) staining. Briefly, after the designated treatments, the cells were collected by trypsinization and washed twice in PBS. They were centrifuged at 300 × G for 5 min, and then fixed with ice-cold 70% ethanol at 4 °C overnight. The fixed cells were incubated with freshly prepared RNase solution (200 µg/ml in PBS) for 30 min at 37 °C, and then stained with PI solution (200 µg/ml in PBS) on ice for 30 min.

**Evaluation of DNA synthesis.** The evaluation of DNA synthesis was performed according to the method of Ko et al. with minor modifications. The capacity for DNA synthesis was evaluated by bromodeoxyuridine
(BrdU) immunohistochemical staining. At the end of incubation with 0.05 M of BrdU with or without 50 μg/ml BME for 24 h, HepG2 cells were fixed with 5% acetic acid in EtOH overnight at 4 °C. We used anti-BrdU antibody (1:50 dilution) as the primary antibody. The fixed cells were re-incubated with formamide at 70 °C for 45 min and then with biotinylated anti-mouse IgG for 1 h, followed by incubation with HRP-labeled streptavidin for 1 h. For peroxidase-reaction, 0.2 mg/ml of DAB with nickel chloride color modification was incubated for 5 min until the desired color intensity developed.

**Western blotting analysis of Rb, cyclin-E, and APRIL.**

After the designated treatments, HepG2 cells were washed twice with PBS. Then they were dissolved for 30 min with lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 1 mM Na3VO4, 50 mM NaF, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin, 2 μg/ml of pepstatin A, and 1 mM phenylamethane sulfonyl fluoride). Finally, the solution was centrifuged at 13,000 × G for 10 min at 4 °C. The supernatant was collected, and protein concentrations were determined by the Bradford method.19) Equal amounts of protein were loaded onto each lane of 7.5% (Rb), 10% (cyclin-E), and 13% (APRIL) SDS–PAGE gels respectively, and the separated proteins were blotted to 0.45 μm PVDF membranes (Amersham, Hybond-P, Piscataway). After overnight blocking with 5% non-fat milk, and 0.1% Tween-20 in TBS, each membrane was stained with primary antibody for 1 h at room temperature. After washing, the membrane was re-incubated with 1:500 diluted biotinylated anti-mouse IgG (Rb) or anti-rabbit IgG (cyclin-E and APRIL) for 1 h at room temperature. After several washing steps, the color reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB). Densitometry analysis of the protein bands was performed with software Scion Image (Scion Corporation, Frederick).

**Results**

**Effect of BME and BEE on numbers of viable cells**

In order to compare the effects between derived normal and tumor cells of the same origin, the numbers of viable cells were measured in rat hepatocytes and rat hepatocellular carcinoma cells (McA-RH7777). As shown in Fig. 1A, BME decreased the number of viable cells in a dose-dependent manner, but BEE did not show any apparent effect on McA-RH7777. Furthermore, BME did not decrease the numbers of viable cells in rat hepatocytes, used as a normal cell model (Fig. 1B). Hence we regarded BME as a suitable extract to examine the anticancer activities of *Blumea balsamifera*. To confirm the anticancer activities of BME in humans, we measured the numbers of viable cells treated with varying concentrations of BME in human hepatocellular carcinoma cells (HepG2). BME decreased the numbers of viable cells in HepG2 in a dose-dependent manner (Fig. 1C). Hence the growth inhibitory effect of BME on HepG2 and its mechanisms were examined in subsequent experiments.

**Statistical analysis.** Data were expressed as mean ± SD and statistical differences in assay values were evaluated by ANOVA, followed by the Dunnett’s multiple comparison post hoc test. A p value of less than 0.05 was considered significant.

**Effect of BME on cell-cycle distribution in HepG2**

The changes in cell-cycle profile induced by BME are shown in Fig. 2. Cells incubated with BME showed an accumulation of G1 phase cell-cycle restriction points in a dose-dependent manner. In contrast, the
number of cells in the S phase and a subsequent progression of the cell cycle in the G2/M phase decreased significantly.

**Effect of BME on DNA synthesis in HepG2**

The changes in DNA synthesis were evaluated by bromodeoxyuridine (BrdU) immunohistochemical staining. BrdU, an analog of thymidine, can be incorporated specifically into DNA in place of thymidine. Anti-BrdU can then be used to identify cells that undergo DNA synthesis during exposure to BrdU. After treatment with BrdU for 24 h, control cells were almost all positive for BrdU, but the rate of BrdU-positive cells in HepG2 was decreased by treatment with 50 \( \mu \)g/ml BME (Fig. 3).

**Effect of BME on intracellular protein expression and posttranslational modification in HepG2**

In order to identify the mechanisms in anti-proliferative activities of BME, changes in intracellular protein status were examined by western blotting analysis. As shown in Fig. 4A, BME (50 \( \mu \)g/ml) time-dependently decreased the ratio of hyperphosphorylated Rb (ppRb) versus hypophosphorylated Rb (pRb), an effect that was most prominent at 24 h. BME also decreased this ratio at 24h in a dose-dependent manner (Fig. 4B). In accordance with these results, BME (50 \( \mu \)g/ml) decreased cyclin-E expression in both a time- (Fig. 5A) and a dose-dependent manner (Fig. 5B).

**Effect of BME on the expression of APRIL in HepG2**

In order to identify the mechanisms underlying the growth-inhibitory effect of BME, changes in intracellular APRIL status were examined by western blotting analysis. APRIL, is synthesized as transmembrane protein, and is proteolytically cleaved at a multibasic cleavage motif prior to secretion of the biologically active soluble form (S-APRIL).\(^{20}\) As shown in Fig. 6A, BME (50 \( \mu \)g/ml) decreased the expression of L-APRIL (unprocessed form) and S-APRIL (soluble form) in a time-dependent manner. Furthermore, BME also decreased these proteins at 24h in a dose-dependent manner (Fig. 6B).
Discussion

The results obtained in the present study demonstrate for the first time, to the best of our knowledge, that an extract of *Blumea balsamifera*, BME, causes a dose-dependent inhibition of tumor cell growth (Fig. 1A, C) and an accumulation of cells in the G1 phase in human hepatocellular carcinoma cells (HepG2) (Fig. 2) without cytotoxicity in rat hepatocytes which were used as a normal cell model (Fig. 1B). Furthermore, BME reduced cellular APRIL levels in HepG2 cells (Fig. 6A, B). This might be one of the mechanisms responsible for its growth-inhibitory activities. To the best of our knowledge, no reduction of APRIL in tumor cell lines by the treatment of extracts derived from a natural plant has recognized thus far. Furthermore, we investigated this mechanism in BME, and found that the anti-proliferative activities were derived from a decrease in the ratio of hyperphosphorylated Rb (ppRb) versus hypophosphorylated Rb (pRb) (Fig. 4A, B) and the expression level of cyclin-E (Fig. 5A, B).

Control of cell proliferation is crucial in maintaining cellular homeostasis, and loss of this mechanism is a principle hallmark of cancer cells. Hence the inhibition of tumor cell growth without side effects is recognized as an important target in cancer therapy. Indeed, to develop inhibitors of cell-cycle progression, Flavopiridol and UNC-01 are being explored in cancer patients in phase-one and phase-two clinical trials. Furthermore, other more specific inhibitors are current in preclinical development. The present study indicates that BME inhibit cell growth in human hepatocellular carcinoma cells (HepG2) in a dose-dependent manner (Fig. 1C). BME showed growth-inhibitory activity in rat hepatocellular carcinoma cells (McARH7777), similarly to HepG2, in a dose-dependent manner (Fig. 1A). On the other hand, BME did not induce cytotoxicity in rat hepatocytes (Fig. 1B). These results suggest that BME have cancer-specific selectivity.

The search for molecular differences between a particular tumor and its normal tissue origin started about two decades ago. It has been found that normal cell proliferation is under strict regulation, governed by checkpoints located at distinct positions in the cell-cycle. Deregulation of these checkpoint events and the molecules associated with them can transform a normal cell into a cancer cell. It has been reported that retinoblastoma (Rb) pathway, is frequently nonfunctional in human cancer. Cell-cycle progression is regulated by cyclin-dependent kinases (Cdks) which during G1 phase progressively phosphorylate the Rb protein. Upon phosphorylation, Rb is inactivated, and
releases transcription factors of the E2F family, which subsequently induce transcription of genes needed for S-phase entry. In this study, BME inhibited cell proliferation and DNA synthesis due to an accumulation of the G1 phase in a dose-dependent manner (Figs. 2, 3). In accordance with these results, BME decreased the ratio of hyperphosphorylated Rb (ppRb) versus hypophosphorylated Rb (pRb) in both a time- (Fig. 4A) and a dose-dependent manner (Fig. 4B). Rb is known to be one of the targets of cyclin-E/Cdk2 phosphorylation. On the other hands, it has been reported that expression of cyclin-E is a requisite for the initiation of centrosome duplication, which occurs as cells progress through the G1 phase. In this study, BME decreased the expression level of cyclin-E in both a time- (Fig. 5A) and a dose-dependent manner (Fig. 5B). These results suggest that BME induced changes of cell-cycle regulated proteins, such as inhibition of Rb phosphorylation and expression of cyclin-E. These changes inhibited the initiation of centrosome duplication and resulted in an accumulation of cells in the G1 phase.

APRIL mRNA levels have been reported to be low in normal tissues. In contrast, high mRNA levels have been detected in several tumor cell lines and tissues. Growth-promoting effects of APRIL on tumors have been detected in various tumor cell lines. A previous study indicated that expression of APRIL protein occurred in HepG2 cells, where it can promote neovascularization, as estimated by the human umbilical vein endothelial cell (HUVEC) tube formation. Therefore, the neutralization of APRIL function is receiving increasing attention as a potential therapeutic target in cancer. APRIL is synthesized as a transmembrane protein (L-APRIL), and is proteolytically cleaved at a multibasic cleavage motif prior to secretion of the biologically active soluble form (S-APRIL). As shown in Fig. 6A, the expression of L-APRIL and S-APRIL was detected in HepG2 cells, and these levels were decreased by the treatment with BME in a dose- and time-dependent manner. S-APRIL has been reported to stimulate tumor cell growth via its signal in an autocrine and/or paracrine mode. These results suggest that the growth-inhibitory effects of BME involve the decrease of S-APRIL level.

Different effects as between cancer and normal cells, were observed under treatment with BME. The cancer-specific selectivity shown in this study suggests that BME may become a tool to inhibit cancer growth without side effects. Although we identified the involvement of APRIL, Rb, and cyclin-E proteins in this study, the precise roles of BME in HepG2 and the components of BME responsible for the effects observed here is currently under study. The greater part of BME is the phenolic component (29%, w/w), and the anticancer activity of BME is superior to that of BEE (Fig. 1A). Hence it is possible that the effective component of BME consists of lipophilic and phenolic materials.

**Acknowledgment**

This study was conducted with funds from the Japan Society for the Promotion of Science, and this is gratefully acknowledged.

**References**


