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Asian Ginseng Extract Inhibits In Vitro and In Vivo Growth of Mouse Lewis Lung Carcinoma via Modulation of ERK–p53 and NF-κB Signaling

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ABSTRACT

Asian ginseng (AG) is the most commonly used medicinal herb in Asian countries. It is often prescribed for cancer patients as a complementary remedy. However, whether AG in fact benefits cancer patients remains unknown because some studies reported that AG facilitates tumor growth, which contradicts its usage as a dietary remedy to cancer patients. In addition, most of research works on ginseng for anti-cancer were using single ginsenoside rather than whole root extracts used in clinics. Thus, intensive studies using the type of ginseng as its clinical form are necessary to validate its benefits to cancer patients. In this study, anti-tumor potency and underlying molecular mechanisms of the ethanol extract of AG (EAG) were examined in mice with Lewis lung carcinoma (LLC-1). We showed that EAG significantly suppressed tumor growth in LLC-1-bearing mice with concomitant down-regulation of PCNA proliferative marker, and it exhibited specific cytotoxicity to cancer cells. EAG also induced MAPK and p53 signaling in LLC-1 cells, which suppressed cyclin B–cdc2 complex and in turn induced G2–M arrest and apoptosis. Although EAG could activate NF-κB signaling, the proteasome inhibitor of MG-132 could effectively prevent NF-κB targeted gene expression induced by EAG and then sensitize LLC-1 cells to induce EAG-mediated apoptosis. Collectively, EAG in a relatively high dose significantly suppressed tumor growth in LLC-1-bearing mice, indicating that AG may benefit lung cancer patients as a dietary supplement. This is the first report demonstrating possible combination of EAG with proteasome inhibitors could be a novel strategy in anti-cancer treatment. J. Cell. Biochem. 111: 899–910, 2010.

KEY WORDS: ASIAN GINSENG; ANTI-CANCER; LLC-1 CARCINOMA; CELL-CYCLE ARREST; APOPTOSIS

Asian ginseng (AG) has been used for centuries and is arguably the most valuable tonic herb in Asian countries. According to Shenong's report in the Liang Dynasty of China (sixth century DC), long-term usage of AG can prolong the life span of humans. More recent pharmacological studies have demonstrated that AG possesses anti-tumor and other activities, such as immunostimulation, improving heart functions, blood vessels vasodilation, and anti-oxidation [Block and Mead, 2003]. Phytochemical analysis of the roots, stems, and leaves of AG have showed that it contains 36 ginsenosides and other constituents such as phytosterols, amino acids, peptides, and vitamins [Wilkie and Cordess, 1994]. White AG contains more ginsenosides Rb1, Rb2, Rc, and Rd; while red AG has new ginsenosides Rh1, Rh2, and Rg3 created in the steaming process of dried whole roots of white AG [Xiaoguang et al., 1998; Shibata, 2001]. In the current study, white AG without artificial treatment process was chosen for preparing the ethanol extract of AG (EAG).

Cancer patients, particularly in the late stages and particularly those who receive Western medical treatments, represent a unique population for the study of AG. Cancer occurrences are inherently from a state of immunosuppression in the body while the standard cancer therapies (notably chemotherapy) are likewise immunosuppressive [Xiaoguang et al., 1998]. Thus, herbal immune stimulants like AG are often used by patients to stimulate their immune system or counteract the side effects caused by conventional chemo/radiotherapy. In clinical studies, patients with nasopharyngeal carcinoma receiving radiotherapy plus injections of AG polysaccharides were

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found to have increased activities of NK and LAK cells [Xie et al., 2001]. Patients with stage 3 gastric cancer taking red AG were observed to have a higher 5-year survival rate than the placebo-taking patients [Block and Mead, 2003]. Recently, a 5-year epidemiological study conducted in South Korea showed that long-term ingestion of AG had preventive effect on cancer occurrences [Yun, 2003]. Experimentally, numerous studies have examined the anti-cancer and cytotoxic activities of the fractions and compounds of AG against a wide variety of cancer cell lines or in vivo neoplasms [Chang et al., 2003]. However, contradictory reports of AG have been demonstrated with very weak or without cytotoxic effect on Ehrlich ascites cells, JTC-26 cells, HRT-18 rectal carcinoma cells, WiDr colon cancer cells, HepG2 liver carcinoma cells, and HS-578-T cells [Chang et al., 2003]. And, even, a stimulatory effect on tumor growth has been noted in MCF-7 cells in mouse models [Lu Pingcheng et al., 1997; Amato et al., 2002]. Studies on herb–drug interactions showed that either the extract of AG or ginsenosides was found to decrease the catalytic activity of human P450 enzymes (CYP1A2) [Chang et al., 2002] and inhibit P-glycoprotein activity [Sparreboom et al., 2004]. Suppression of either P450 enzyme or P-glycoprotein activity may trigger a rise of plasma concentrations of chemotherapy agents, leading to exaggerated toxicity similar to the reaction of a drug overdose. Furthermore, AG was reported to exhibit phytoestrogenic effect that discourages to be administrated in women who suffer from breast and endometrial cancers [Weiger et al., 2002].

Taking all of these evidences together, whether ginseng could facilitate carcinoma growth and metastasis along with its tonic action to the body remains an open question. We aimed to address these issues by using whole root EAG, rather than single or multiple ginsenosides, in treating Lewis lung carcinoma cells (LLC-1)-bearing mice. Results showed that EAG possessed significant specific cytotoxicity to cancer cells and suppressive potency on tumor growth in LLC-1-bearing mice through down-regulation of the PCNA proliferative marker. Further mechanistic studies showed that EAG activated MAPK signaling and tumor suppressor proteins p53 and p21 in LLC-1 cells, by which G2–M cell cycle (cyclin B–cdc2 complex) was suppressed, and in turn induced G2–M arrest and apoptosis. Our work also showed that the proteasome inhibitor MG-132 could effectively down-regulate EAG-mediated NF-κB activation and then potentiate EAG-induced cell death in LLC-1 cells. Collectively, these findings verify, on the animal, cellular, and molecular levels, how AG benefits lung cancer patients.

MATERIALS AND METHODS

CHEMICALS, ANTIBODIES, AND *siRNAs*

All compounds were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. U0126 and SP600125 were purchased from Cell Signaling Technologies, Inc. (Beverly, MA). Etoposide (VP-16), MG-132, and COX2 inhibitor II were obtained from Calbiochem (San Diego, CA). Primary antibodies against P-Stat3, Chk-1, Chk-2, P-Chk1^{Ser345}, P-Chk2^{Thr68}, P-cdc2^{Tyr15}, P-p53^{Ser15}, P-p38, total p38, P-ERK, total ERK, P-JNK, total JNK, P-Akt^{Ser473}, P-IKKα^{Ser180}/β^{Ser181}, P-p65^{Ser536}, Bcl_{xL}, and PARP were purchased from Cell Signaling Technologies, Inc. PCNA, cyclin B1, cdc2, Egr-1, p21, p53, Caspase 3, Akt, IκBα, ICAM-1, PECAM-1, VEGF, COX2, and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK, COX-2, and non-targeting control *siRNA* were obtained from Santa Cruz Biotechnology.

PREPARATION OF THE STANDARDIZED ETHANOL EXTRACTS OF AG (EAG)

A standardized EAG will be used in all experiments of the project. In brief, the root of *Panax ginseng* C.A. Meyer was purchased from the wholesale market in Tonghua country of Jilin province, PR China, the major cultivation and production area of white ginseng. The quality of EAG conforms to the requirement of Hong Kong Standard of Chinese Materia Medica. The EAG has been prepared by refluxing the ginseng powder 2 h with 5 volumes (vs. ginseng's weight) of 70% ethanol for three times, and then the ethanol extracts were pooled and concentrated and finally dried to powder (EAG) under low temperature. The extraction rate reached at around 28%, meaning that 1 kg AG produces 280 g of EAG. The above ethanol extraction method was well established and optimized for recovery of major ginsenosides.

HPLC/DAD ANALYSIS OF EAG

The chemical fingerprints of EAG were analyzed by high-performance liquid chromatography (HPLC; 1100, Agilent Technologies, CA) with the Phenomenex ODS column (250 mm × 4.6 mm i.d.; particle size 5 μm; Phenomenex, Inc., USA) protected by a Security Guard Cartridge (C18, 4 mm × 3.0 mm i.d.; Phenomenex, Inc.) and connected with G1312A Binary Pumps, G1379A degasser, G1315B Diode-Array Detector, and G1313A Autosampler. The mobile phase was a gradient elution of KH_2PO_4 buffer (2 mM, pH 6.8) and acetonitrile, starting isocratically with 21% of acetonitrile for 15 min and increasing to 38% over 55 min. The flow rate of the mobile phase was 1.0 ml/min, and the detector wavelength was 203 nm. The fingerprint similarity of five different batch samples (S1–S5) of EAG was 0.9496, indicating consistent quality of EAG in batch to batch (Supplementary Fig. 1). In the fingerprints of EAG six ginsenosides, Rg1, Re, Rb1, Rc, Rb2, and Rd, were identified by comparing the retention time with the authentic compounds. The contents of Rg1, Re, Rb1, Rc, Rb2, and Rd were 7.63, 6.90, 12.21, 10.65, 7.24, and 4.59 mg/g, respectively.

ANIMAL EXPERIMENTS

Male C57BL/6J mice at the age of 6–8 weeks were obtained from the Chinese University of Hong Kong, Hong Kong, China. Animal care and treatment procedures are conformed to the Institutional Guidelines and Animal Ordinance (Department of Health, HKSAR). Mice were randomly divided into vehicle control and EAG treatment (0.25, 0.5, and 1 g/kg/day) groups ($n = 20$–22). All animals in different groups received a pretreatment for 10 days followed by subcutaneously tumor cells injection and then the mice were subjected to vehicle or EAG treatment for another 20 days. In order to make sure all groups were appropriate blinded, all the experimental procedures such as subcutaneous tumor cells injection, treatment, and tumor size measurement were performed by three individual persons who were blinded for animal group identity.

For EAG pretreatment, 0.25, 0.5, and 1 g/kg/day of EAG was orally administered to the animals by gavage feeding for 10 consecutive days, while drinking water was applied as control. After pretreatment, the mice from all groups were subcutaneously injected with 0.5×10^5 of mouse LLC-1 to the right dorsal region. After pretreatment and tumor cells injection, EAG (0.25, 0.5, and 1 g/kg/day) were gavage fed to the animals of treatment groups for another 20 days. Body weight and tumor volume (length $\times$ width$^2 \times$ 0.52) were measured in alternative days. After 20-day treatment course, all mice were scarified and the dissected tumors were weighted and subjected to immunohistochemical analysis.

IMMUNOHISTOCHEMISTRY

The dissected tumors were fixed and then processed into paraffin blocks for sectioning at 7 μm. Sections were deparaffinized in xylene, and subsequently rehydrated in graded ethanol and ddH_2O. Tumor sections were then treated with 20 μg/ml of Proteinase K (Roche Diagnostics, Mannheim, Germany), followed by 3% of hydrogen peroxide to block the endogenous peroxidase activity. After serum blocking, sections were incubated with anti-PCNA antibody for 1 h, followed by 10 min incubation with *SuperPicture*™ HRP Polymer conjugate (Zymed Lab, Invitrogen, Carlsbad, CA). After washing, slides were incubated in DAB substrate solution until the desired stain intensity was developed. The slides were then counterstained with hematoxylin, dehydrated, and mounted. Quantification of PCNA signal (a proliferative index) was counted by PCNA immunostaining of each cell. Images taken with Nikon ECLIPSE 80*i* microscope were captured by CCD digital camera Spot RT3™ (Diagnostic Instruments, Inc., Melville, NY).

CELL CULTURE AND *siRNA* TRANSFECTION

All cells were obtained from the American Type Culture Collection (Rockville, MD) unless otherwise specified. Cells were characterized by ATCC according to their guidelines on cell line verification test recommendations. Human peripheral T lymphocytes were isolated from the buffy coat blood (Blood Bank-Red Cross, Hong Kong) using methods of the previous report [Li et al., 2009]. *siRNA* knockdown was performed using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics).

CYTOTOXICITY ASSAY, WESTERN BLOTTING, CELL-CYCLE ANALYSIS, APOPTOSIS DETECTION, AND INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

Cell viability, Western blot detection, cell-cycle progression, and immunofluorescence staining were measured and monitored as described previously [Wong et al., 2005]. Apoptosis was detected by Annexin V staining kit (BD Biosciences).

STATISTICAL ANALYSIS

The data are expressed as means ± SD as indicated. The difference was considered statistically significant when the *P*-value was <0.05. Student's *t*-test or one-way ANOVA analysis was used for comparison among different groups.

RESULTS

EAG SUPPRESSES TUMOR GROWTH IN LLC-1-BEARING MICE

AG is usually consumed as a water or alcoholic extract of whole roots, or as powdered root in clinics. Thus, the EAG was used in order to replicate as closely as possible the clinical dosage and to achieve maximum clinical relevance. As lung cancer ranks at the first occurrence among cancer patients while ginseng is often used by lung cancer patients, our study was therefore undertaken with lung cancer cells. Besides, AG is commonly used by cancer patients to boost their immune system, suggesting that AG may manifest its therapeutic effects through normal immune system. Therefore, the normal mice with an intact immune system is preferred for the development of mouse carcinoma bearing mice model instead of using human carcinoma bearing nude mice model to evaluate the therapeutic efficacy of EAG. To examine whether AG exacerbates or suppresses tumor growth, we pretreated mice with EAG (0.25, 0.5, and 1 g/kg/day) or drinking water for 10 days, which mimicked the situation of people who consume ginseng as a complementary remedy or for tonic purposes. After pretreatment, we injected the aggressive mouse lung carcinoma cells, LLC-1, subcutaneously into the dorsal region of the mice. The vehicle or EAG treatment was continued for another 20 days. The results indicated that treatment with two lower dosages of EAG (0.25 g and 0.5 g/kg/day body weight) showed no significant effect (data not shown), while EAG treatment with 1 g/kg/day demonstrated a marked suppression on tumor volume (vehicle: 1341.5 ± 596.5; EAG treatment: 981.3 ± 457.3) and tumor mass (vehicle: 1.22 ± 0.6; EAG treatment: 0.88 ± 0.41; Fig. 1A,B) without drop of body weight (Supplementary Fig. 2). These results indicate that EAG treatment with an optimum dosage has an anti-tumor effect without toxicity in mice.

EAG INHIBITS CELL PROLIFERATION IN VIVO AND IN VITRO

To further investigate the underlying mechanisms of EAG in suppression of tumor progression in vivo, we isolated the solid tumors from EAG (1 g/kg/day)-treated LLC-1-bearing mice and determined the proliferative index of tumors by immunohistochemical analysis using PCNA antibodies. Results showed that EAG-treated LLC-1-bearing mice had significantly lower cell proliferative index compared to the vehicle-treated animals (Fig. 1C, left panel). These results coincided with the in vitro anti-proliferative effect found in LLC-1 cells with EAG treatment. In addition, Western blot results demonstrated that both signal transducers and activators of transcription (P-Stat3) and proliferating cell nuclear antigen (PCNA) expression were time-dependently down-regulated upon EAG treatment (Fig. 1C, right lower panel). Collectively, these data suggest that EAG suppressed tumor growth through inhibition of cell proliferation.

EAG EXHIBITS SPECIFIC CYTOTOXICITY TO DIFFERENT CANCER CELLS IN VITRO

An ideal anti-cancer remedy should eliminate cancer cells, specifically without notable adverse effects to the adjacent normal tissues. To determine if EAG exhibits its cytotoxicity specifically on cancer cells, the normal human T lymphocytes and lung fibroblasts (CCD-19Lu), together with the cancer cell lines of human cervical

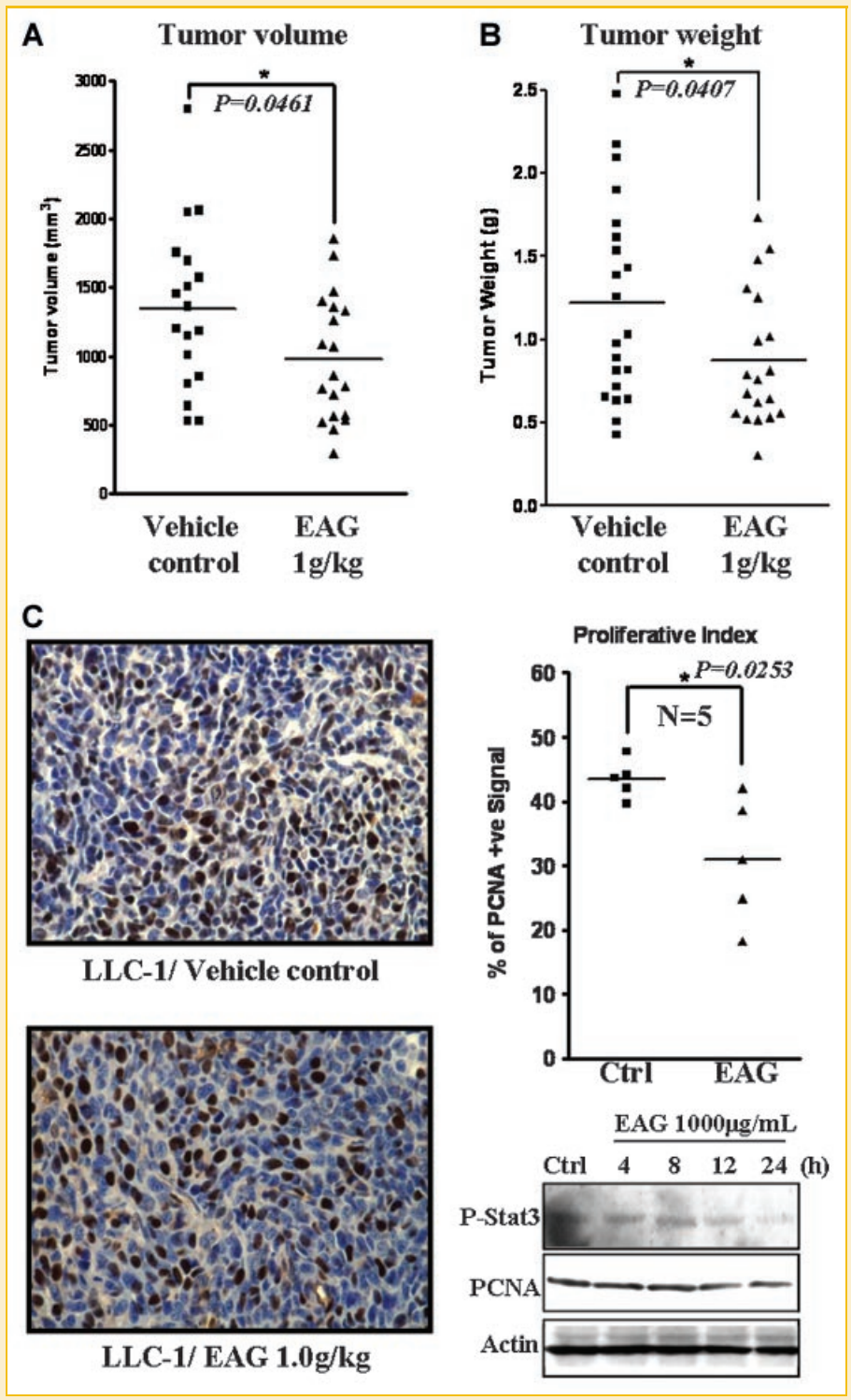


Fig. 1. Efficacy of EAG ginseng extract in tumor mice model system. A,B: The tumor volume and weight of vehicle control and EAG-treated groups of animals on the day of sacrifice. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with vehicle treatment. C: Effect of EAG on tumor proliferation. Left panel: PCNA staining images are representative of five tumor sections from five animals of each group. More than 3,000 cells were counted from five views of each section taken with 20× magnifications. Right upper panel: Statistical analysis of PCNA staining (proliferative index) from vehicle and EAG-treated animals. Chart represents mean of PCNA signal, $^{*}P < 0.05$ by Student's *t*-test. Right lower panel: Western blotting analysis of cell proliferative markers (P-Stat3 and PCNA) in EAG-treated LLC-1 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cancer (HeLa), liver cancer (HepG2), breast cancer (MCF-7), and mouse lung cancer (LLC-1) were incubated with different concentrations of EAG. Results showed that EAG exhibited significant cytotoxicity to all these cancer cells, but not to the normal T lymphocytes and lung fibroblasts (Fig. 2), suggesting that EAG induces cell cytotoxicity specifically to cancer cells. Interestingly, EAG seems to have significant dose-dependent cytotoxic effect on LLC-1 lung cancer cells, but not on other cancer cells from different origins, suggesting that lung cancer cells might be more susceptible to EAG treatment. This result provides evidence that ginseng is probably more suitable for lung cancer patients as a complementary remedy. However, the underlying mechanisms remain to be elucidated.

EAG SPECIFICALLY INDUCES G2–M CELL-CYCLE ARREST IN CANCER CELLS BUT NOT IN NORMAL CELLS

In addition to cell proliferation, cell-cycle progression is an important parameter of anti-tumor drug action. Thus, we determined whether EAG affects cell-cycle progression in normal and cancer cells. Flow cytometry data indicated that EAG treatment arrested the LLC-1 cells in the G2–M phase in a time- and dose-dependent manner. Concomitantly, the cell death-presenting sub-G1 population was found increasing gradually after EAG treatment (Fig. 3A). However, EAG treatment did not arrest normal lung fibroblasts CCD-19Lu in the G2–M phase. Instead, EAG delayed only the entry of G1 phase to S phase of cell cycle after 24 h treatment; normal cell-cycle distribution resumed at 36 h. Meanwhile, the sub-G1 population could not be detected in both dosages of EAG treatment, and there was no change in CCD-19Lu cell density and morphology after EAG treatment (Fig. 3B). These data coincided with the results of EAG having no cytotoxicity on normal cells shown in Figure 2. Collectively, our data strongly suggest that the EAG-mediated anti-cancer effect is tumor-specific. Next, to elucidate how EAG induces G2–M arrest, changes in the cell-cycle-related markers in response to EAG treatment were investigated. Unlike results with UV or topoisomerase II inhibitor (VP-16) treated

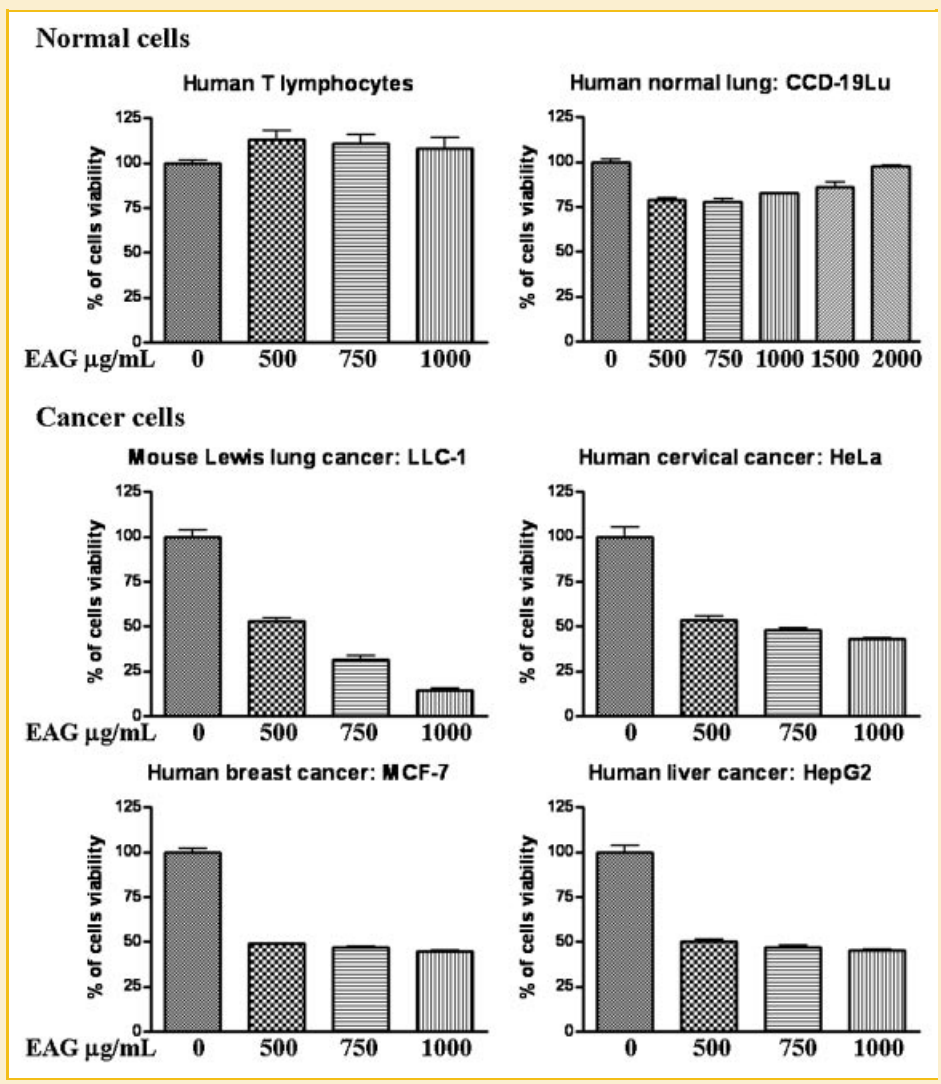
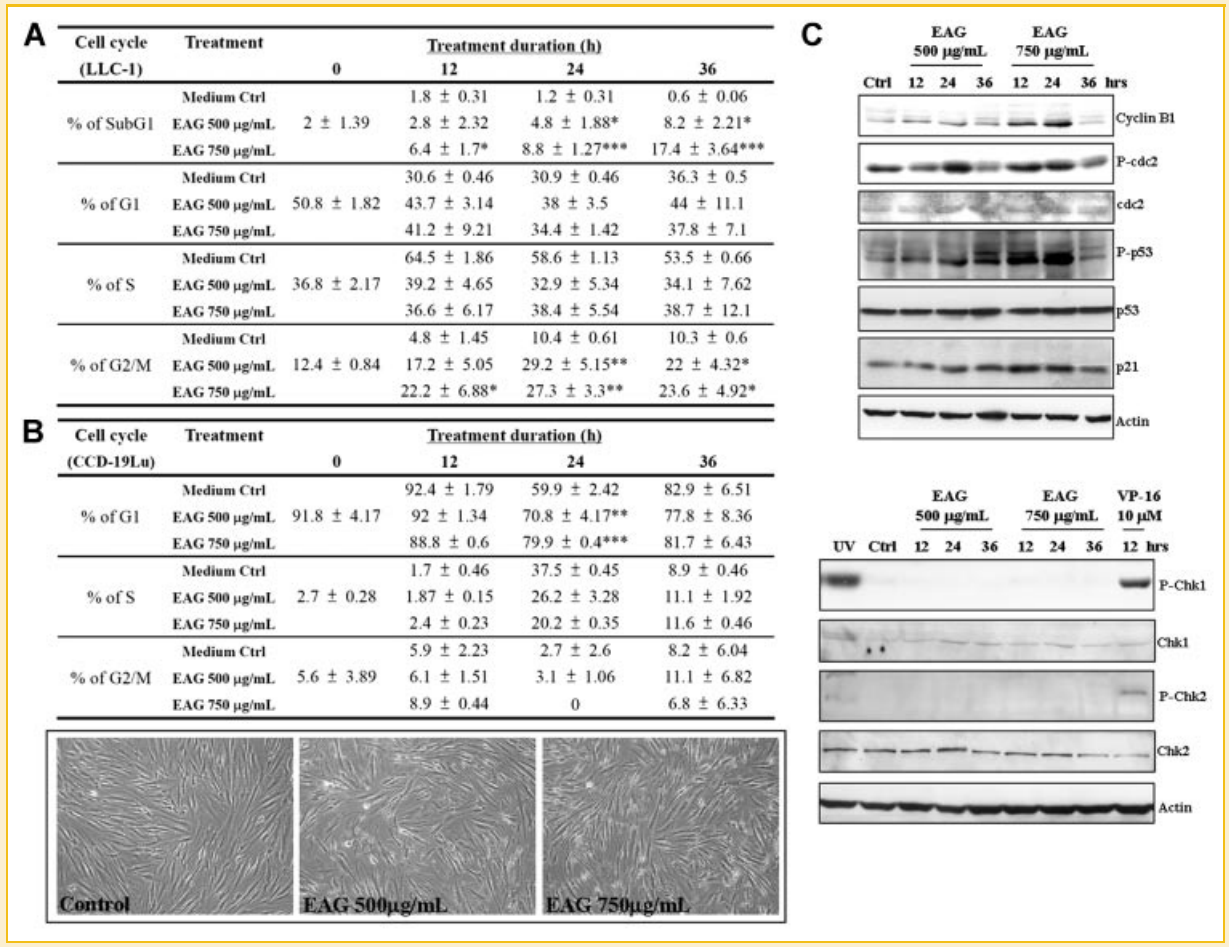


Fig. 2. Cytotoxicity assay of EAG on normal and cancer cells. The cell viability was measured by MTT assay after 72 h incubation.



A

Cell cycle (LLC-1)	Treatment	Treatment duration (h) 0	12	24	36
% of SubG1	Medium Ctrl	2 ± 1.39	1.8 ± 0.31	1.2 ± 0.31	0.6 ± 0.06
	EAG 500 μg/mL		2.8 ± 2.32	4.8 ± 1.88*	8.2 ± 2.21*
	EAG 750 μg/mL		6.4 ± 1.7*	8.8 ± 1.27***	17.4 ± 3.64***
% of G1	Medium Ctrl	50.8 ± 1.82	30.6 ± 0.46	30.9 ± 0.46	36.3 ± 0.5
	EAG 500 μg/mL		43.7 ± 3.14	38 ± 3.5	44 ± 11.1
	EAG 750 μg/mL		41.2 ± 9.21	34.4 ± 1.42	37.8 ± 7.1
% of S	Medium Ctrl	36.8 ± 2.17	64.5 ± 1.86	58.6 ± 1.13	53.5 ± 0.66
	EAG 500 μg/mL		39.2 ± 4.65	32.9 ± 5.34	34.1 ± 7.62
	EAG 750 μg/mL		36.6 ± 6.17	38.4 ± 5.54	38.7 ± 12.1
% of G2/M	Medium Ctrl	12.4 ± 0.84	4.8 ± 1.45	10.4 ± 0.61	10.3 ± 0.6
	EAG 500 μg/mL		17.2 ± 5.05	29.2 ± 5.15**	22 ± 4.32*
	EAG 750 μg/mL		22.2 ± 6.88*	27.3 ± 3.3**	23.6 ± 4.92*

B

Cell cycle (CCD-19Lu)	Treatment	Treatment duration (h) 0	12	24	36
% of G1	Medium Ctrl	91.8 ± 4.17	92.4 ± 1.79	59.9 ± 2.42	82.9 ± 6.51
	EAG 500 μg/mL		92 ± 1.34	70.8 ± 4.17**	77.8 ± 8.36
	EAG 750 μg/mL		88.8 ± 0.6	79.9 ± 0.4***	81.7 ± 6.43
% of S	Medium Ctrl	2.7 ± 0.28	1.7 ± 0.46	37.5 ± 0.45	8.9 ± 0.46
	EAG 500 μg/mL		1.87 ± 0.15	26.2 ± 3.28	11.1 ± 1.92
	EAG 750 μg/mL		2.4 ± 0.23	20.2 ± 0.35	11.6 ± 0.46
% of G2/M	Medium Ctrl	5.6 ± 3.89	5.9 ± 2.23	2.7 ± 2.6	8.2 ± 6.04
	EAG 500 μg/mL		6.1 ± 1.51	3.1 ± 1.06	11.1 ± 6.82
	EAG 750 μg/mL		8.9 ± 0.44	0	6.8 ± 6.33

Fig. 3. A,B: Effect of EAG on cell-cycle progression of mouse Lewis lung carcinoma, LLC-1 and human normal lung fibroblasts, CCD19-Lu. Exponentially growing LLC-1 and CCD19-Lu cells were synchronized in the serum-free medium for 24 h. Then the cells were incubated in the medium control or EAG containing medium for indicated times. The cell cycle was evaluated using propidium iodide and flow cytometry analysis. The charts indicated the quantitative analysis of cell-cycle distribution (% of cell population). Means ± SD are from three independent experiments (one-way ANOVA: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Significantly increased compared to medium control treatment. B, lower panel: Morphology of CCD19-Lu treated with or without EAG for 36 h. Magnification 20×. C: Effect of EAG on G2–M cell-cycle markers expression.

LLC-1 cells, it showed no genotoxic stress-induced activation of checkpoint kinase 1, P-Chk1 and checkpoint kinase 2, P-Chk2 after EAG treatment. However, the tumor suppressor proteins p21 and p53 were activated by EAG treatment together with concomitant up-regulation of cyclin B1 and the inactive phosphorylated form of cdc2 kinase (Fig. 3C). Modulation of these markers leads to disturbance of cell-cycle progression [Innocente et al., 1999], which in turn induces apoptosis [Liu et al., 2003]. Taken together, these data suggest that EAG-induced cell-cycle arrest and apoptotic cell death result from cell-cycle disturbance and activation of p53 signaling.

EAG INDUCES APOPTOTIC CELL DEATH

Apoptosis induction by EAG was further characterized by Annexin V and DAPI staining because they can reveal the loss of membrane integrity, chromatin condensation, and nuclear fragmentation that are common in the phenotypes of cells undergoing apoptosis [Kondo et al., 2005]. Results of Annexin V-FITC staining showed that EAG induced early apoptosis in a dose-dependent manner (Fig. 4A). Besides, chromatin condensation and nuclear fragmentation were observed in the DAPI-stained nuclear region of the EAG-treated LLC-1 cells (Fig. 4B). Furthermore, apoptotic markers such as PARP and Caspase-3 were used to validate this apoptotic event. Results demonstrated that EAG treatment activated the cleavage of PARP and pro-caspase 3 in a time-dependent manner (Fig. 4C). Thus, these results conclusively demonstrate that EAG activated apoptotic markers PARP and caspase 3, which in turn induced apoptosis with loss of membrane asymmetry, chromatin condensation, and nuclear fragmentation.

EAG INDUCES APOPTOTIC CELL DEATH THROUGH MODIFICATION OF MAPKs AND p53 SIGNALING

It has been demonstrated that activation of mitogen-activated protein kinases (MAPKs) and p53 signaling pathways are correlated with cell-cycle arrest and apoptosis induction [Wu, 2004]. We explored the contribution of MAPK family proteins ERK, JNK, p38, and p53 signaling to EAG-induced apoptotic cell death. Western blot data demonstrated that the levels of p-ERK, p-JNK, p-p38, and

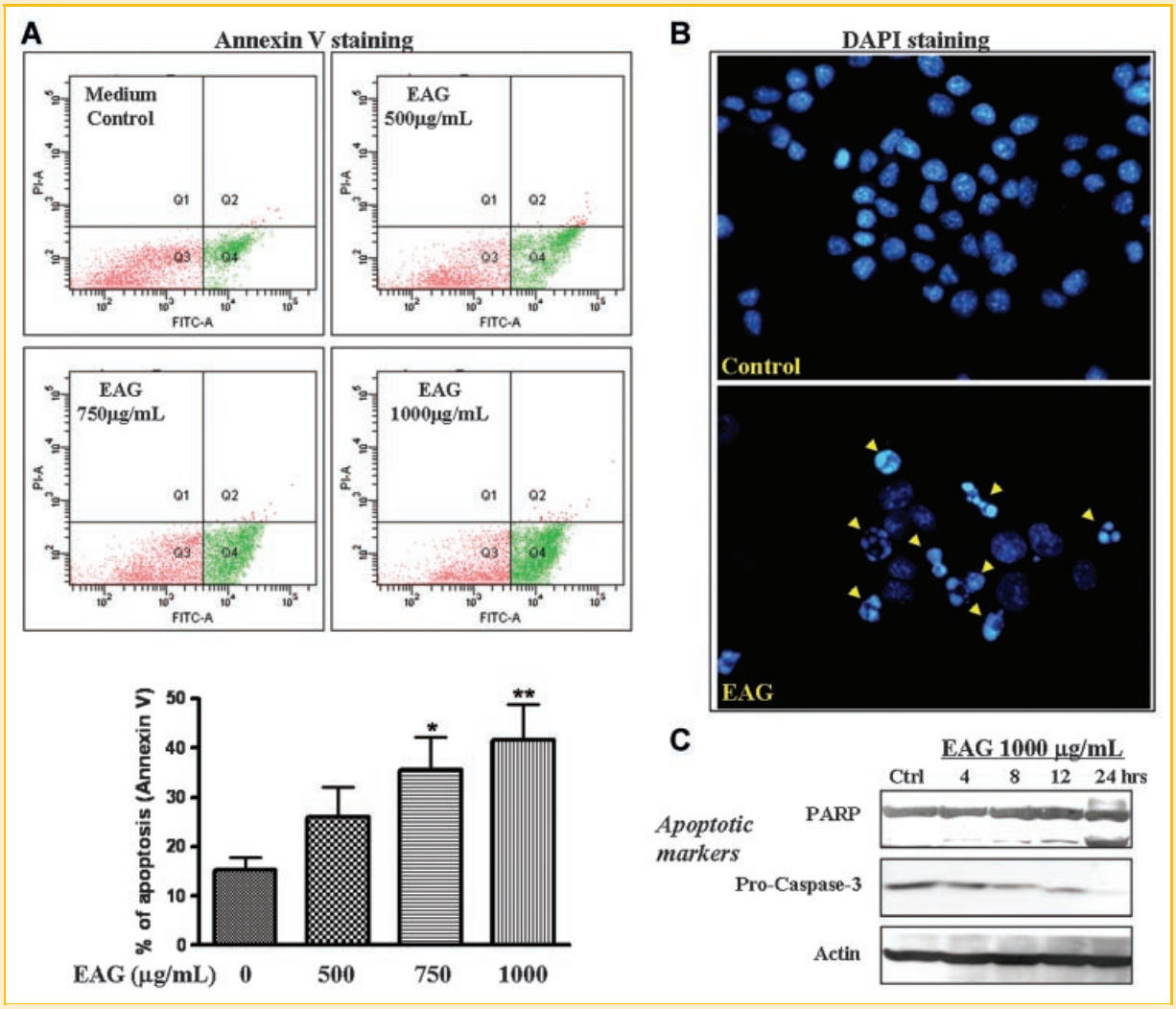


Fig. 4. EAG induces apoptosis in LLC-1 cells. A: Early apoptosis detected by Annexin V staining. Cells were incubated with medium control or 500–1,000 μg/ml of EAG for 24 h. The cells were then stained with Annexin V-FITC and PI, followed by flow cytometry analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with medium control treatment. B: DNA/nuclear fragmentation detected by DAPI staining. Cells were incubated with or without 1,000 μg/ml of EAG for 24 h. The cells were then fixed and stained with DAPI. Arrow heads: Cells undergo apoptosis with chromatin condensation and nuclear fragmentation morphologies. Images shown are representative of two independent experiments. C: Western blot detection of apoptotic markers in EAG-treated LLC-1 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

p-p53 were markedly elevated in a time-dependent manner after EAG treatment, indicating that EAG activated MAPKs as well as p53 signaling (Fig. 5A). To further confirm activation of these signaling pathways is necessary for EAG-mediated cell cytotoxicity, LLC-1 cells were incubated with EAG in the presence of MAPKs and p53 specific inhibitors, for example, ERK inhibitor, U0126 [Favata et al., 1998]; JNK inhibitor, SP600125 [Gao et al., 2009]; p38 inhibitor, SB203580 [Ho et al., 2008]; and p53 inhibitor, Pifithrin-α [Katiyar et al., 2009]. Cytotoxicity assay revealed that, except for SP600125 (data not shown), both U0126 and Pifithrin-α exhibited a protective effect on EAG-treated cells; this coincides with the results of EAG-treated ERK*si* knockdown cells, and the viability of cells was increased to a certain extent. In contrast, blocking of p38 activation by SB203580 enhanced EAG-mediated cell cytotoxicity (Fig. 5B), suggesting that MAPKs activation plays a controversial role in cell cytotoxicity in response to EAG treatment. Since MAPKs are involved in the regulation of p53 activation, we investigated whether p53 is activated by ERK in EAG-treated LLC-1 cells. As shown in Figure 5C, pretreatment of the cells with U0126, but not Pifithrin-α, resulted in a decrease in both ERK and p53 activation as determined by the decreased ERK and p53 phosphorylation. Moreover, the inhibition of p53 activity by Pifithrin-α was associated with a decrease in the cleavage of PARP and pro-caspase 3 (Fig. 5C, middle panel). The same effect was observed in the suppression of ERK activity (data not shown). Furthermore, human non-small cell lung cancer cells, H1299 with p53 deletion, were less sensitive to EAG-mediated cytotoxicity (Fig. 5C, lower panel), suggesting a crucial role of p53 in EAG-induced apoptosis. It has been reported that transcription of the p21 gene is activated by early growth response-1 (Egr-1) transcription factor through ERK/Elk-1 pathway [Choi et al., 2008]. Here, we demonstrated that blockage of EAG-induced ERK activation by U0126 resulted in a

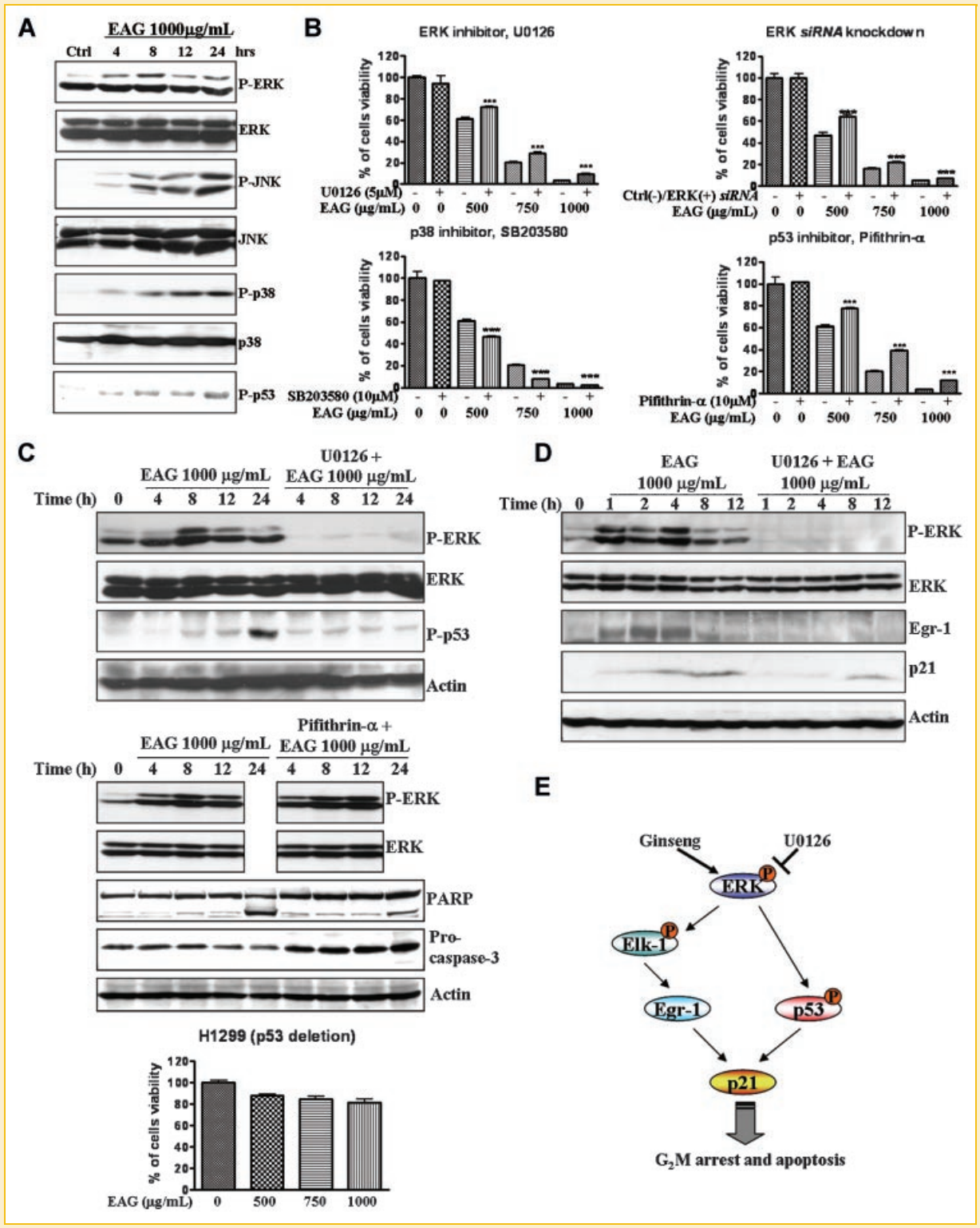


Fig. 5. A: Effect of EAG on p53 and MAPKs signaling pathway. B: EAG-mediated cell death through modification of MAPKs and p53 signaling. LLC-1 cells were preincubated with or without specific inhibitors for 1 h; or transfected with control or ERK *siRNA* for 48 h, the cells were then treated with EAG together with or without specific inhibitors. After 72 h, the cell viability was measured by MTT assay. Means ± SD are from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Significantly altered compared to non-inhibitors or control *siRNA* treated cells. C: Involvement of ERK and p53 signaling in EAG-mediated apoptosis in LLC-1 cells. Cells were pretreated with or without 5 μM U0126 (upper panel) or 10 μM of Pifithrin-α (middle panel) for 1 h, followed by EAG treatment and then the cells lysates were subjected to Western blotting analysis. Lower panel: Cytotoxicity assay of EAG on H1299 (p53-null cells). D: Involvement of ERK–Egr-1 signaling in EAG-induced p21 expression. Serum-starved LLC-1 cells (0.5% FBS for 24 h) were pretreated with or without 5 μM U0126 for 1 h, followed by EAG treatment and then the cells lysates were subjected to Western blotting analysis. E: Schematic diagram of EAG-mediated apoptosis through ERK–Egr-1/p21 and -p53/p21 signaling. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

decrease of both Egr-1 and p21 expression (Fig. 5D). Collectively, these data suggest that ERK is involved in EAG-induced apoptosis through Egr-1/p21- and p53/p21-dependent pathways (Fig. 5E).

EAG ACTIVATES NF-κB SIGNALING PATHWAY AND ITS TARGETED GENES EXPRESSION

EAG contains ginsenosides Rg1 and Re (Supplementary Fig. 1), which have been shown to exhibit angiogenic effect [Yu et al., 2007; Lin et al., 2008]. NF-κB signaling is constitutively involved in the expression of genes that participate in tumor promotion, angiogenesis, and metastasis [Karin et al., 2002]. To determine whether EAG treatment induces angiogenic genes expression through NF-κB signaling, the EAG-treated LLC-1 cells were screened for the NF-κB signaling markers and its targeted gene products. Western blot results showed that the phosphorylation of Akt, IKKα/β, and the NF-κB subunit, p65, were detected with concomitant degradation of IκBα (Fig. 6A). In addition, NF-κB targeted gene products ICAM-1, PECAM-1, VEGF, COX-2, and Bcl_{XL} were elevated in a time-dependent manner after EAG treatment (Fig. 6A). Taken together, these results suggest that EAG can activate NF-κB signaling, thereby inducing NF-κB targeted genes expression.

PROTEASOME INHIBITOR, MG-132, SUPPRESSES EAG-INDUCED NF-κB TARGETED GENE EXPRESSION AND SENSITIZES LLC-1 CELLS TO EAG-MEDIATED APOPTOSIS

Activation of NF-κB signaling contributes to anti-apoptotic gene expression such as Bcl_{XL}, which may retard the apoptotic effect of EAG in LLC-1 cells. It seems plausible that treatment of cells with agents capable of inhibiting NF-κB signaling would increase the anti-cancer potency of EAG. Proteasome inhibitor, MG-132, has been shown to inhibit NF-κB activation by preventing degradation of IκBα [Traenckner et al., 1994]. We therefore investigated whether MG-132 suppresses the EAG-induced NF-κB activation and sensitizes cells to EAG-mediated apoptosis. Surprisingly, cytotoxicity assay demonstrated that co-administration of EAG with minimal toxic dose of MG-132 resulted in an extreme decrease in cell viability as compared with the treatment of each agent alone (Fig. 6B, upper panel). This synergistic effect was also observed in

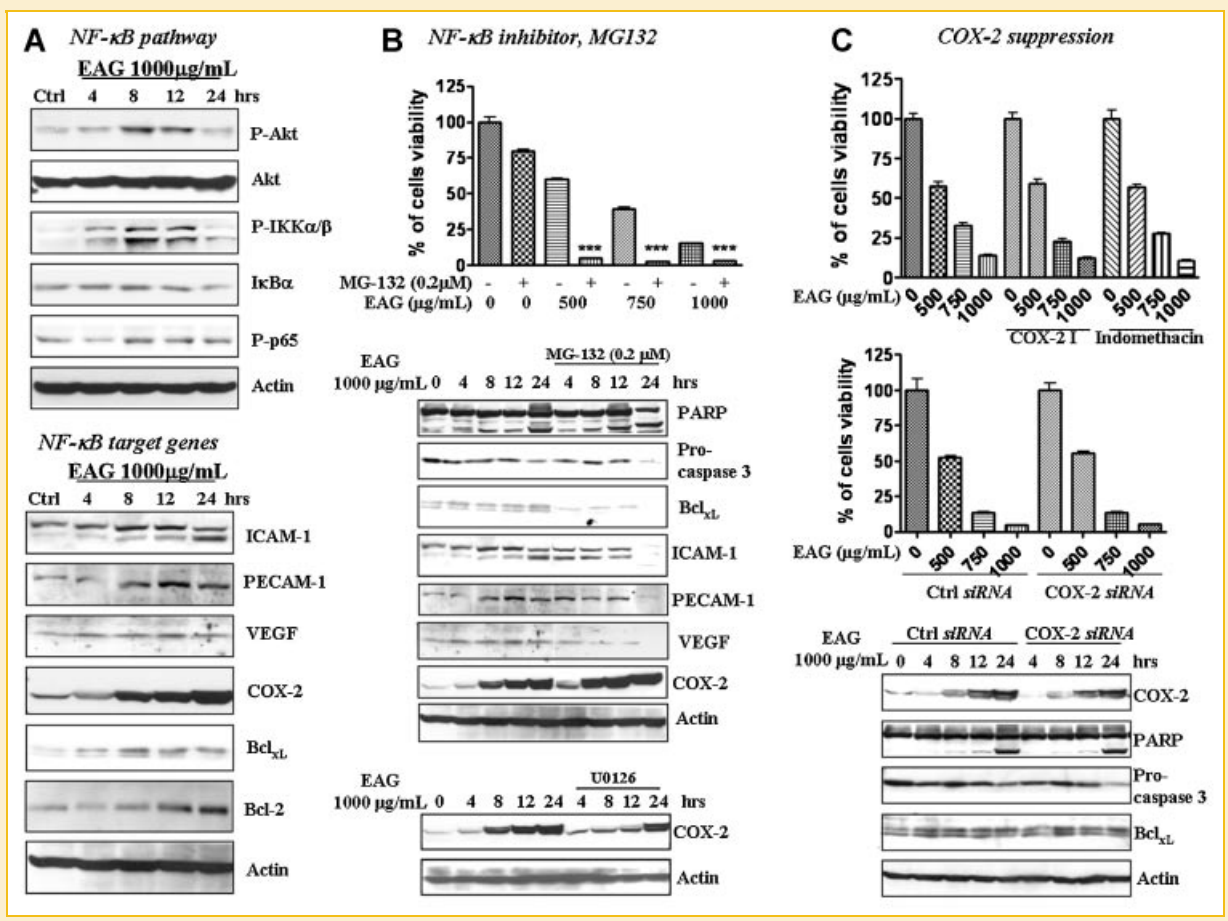


Fig. 6. A: Effect of EAG on NF-κB signaling and NF-κB targeted gene expression. B: Effect of proteasome inhibitor, MG-132 on EAG-treated LLC-1 cells. Upper panel: MG-132 enhanced the EAG-mediated cytotoxicity in LLC-1 cells. Means ± SD are from three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Significantly suppressed compared to non-inhibitors treated cells. Lower panel: Effect of MG-132 on apoptotic markers and NF-κB targeted genes expression in EAG-treated LLC-1 cells. C: Role of COX-2 expression in EAG-mediated apoptosis. Upper panel: LLC-1 cells were preincubated with or without COX-2 inhibitors (10 μM COX-2 inhibitor II or 10 μM indomethacin) for 24 h, followed by EAG treatment. Middle panel: LLC-1 cells were either transfected with control *siRNA* or COX-2 *siRNA* for 48 h, the cells were then treated with EAG. The cell viability was then measured by MTT after 72 h. Lower panel: Effect of COX-2 knockdown on EAG-induced apoptotic markers expression.

human HepG2, HeLa, and MCF-7 cancer cells (data not shown). Then, we further verified the effects of MG-132 and EAG on NF-κB signaling. Interestingly, except COX-2 all other EAG-induced NF-κB targeted genes expression, for example, ICAM-1, PECAM-1, VEGF, and Bcl_{XL} were suppressed upon MG-132 treatment. Furthermore, this simultaneous treatment induced the proteolytic cleavage of caspase-3 and PARP apoptotic markers (Fig. 6B, middle panel). Collectively, these results suggest that MG-132 inhibits the EAG-induced NF-κB signaling and potentiates the EAG-mediated apoptotic cell death.

EAG-MEDIATED APOPTOTIC CELL DEATH IS INDEPENDENT OF COX-2 EXPRESSION

COX-2 has been reported to suppress apoptosis and thus to provide a survival advantage to neoplastic cells [Fujimura et al., 2006]. It therefore becomes the major molecular target for the prevention of cancers. In the current study, COX-2 expression was induced by EAG treatment, and its expression level was elevated with increasing apoptotic cell death during addition of MG-132 (Fig. 6B), and down-regulated under the protective treatment of U0126 (Fig. 6B, bottom panel). These apparent contradictory results prompted us to further explore the potential role of COX-2 in EAG-mediated cell death. As shown in Figure 6C, treatment with COX-2 inhibitors and EAG together had no appreciable effect on cell viability as compared to EAG treatment alone. Alternatively, COX-2 specific knockdown by COX-2 *siRNA* did not sensitize the cells in response to EAG treatment (Fig. 6C, middle panel). Western blot data further confirmed that the cleavage of apoptotic markers (PARP and Caspase-3) or the anti-apoptotic marker Bcl_{XL} had no significant difference in their protein expression between COX-2 and control *siRNA* knocked down cells (Fig. 6C, lower panel). These findings indicate that EAG-induced apoptotic cell death is independent of COX-2 expression.

DISCUSSION

Does AG have anti-cancer properties? To address this question, we used the ethanol extract of Asian ginseng (EAG) containing total ginsenosides, instead of individual ginsenosides in our current study, because EAG is more equivalent to what is used in clinics. Although study of single active components from AG can reveal aspects of its efficacy, it cannot tell the whole story, and thus is a necessary but not sufficient approach for studying traditional Chinese medicine. In practice, people do not take pills or capsules containing pure ginsenosides. So, the goal of our current study was not to determine which, if any, particular compound is responsible for ginseng's action. We hypothesized that the action is, rather, the result of complex interaction of many compounds and many targeted molecules interacting with a living system. As ginseng may exhibit its anti-cancer effect through boosting the immune system, thus, instead of using a human carcinoma-bearing nude mice model, we used a mouse carcinoma-bearing mice model as it offered an intact immune system which enables the evaluation of the beneficial effects of EAG during the preclinical stage. The results demonstrated that EAG had a significant inhibitory effect on LLC in mice at a dosage of 1 g/kg body weight without observable side effects but had no discernible effect with lower dosages. Clearly, in our studies, consumption of EAG did not facilitate tumor growth. Instead, our studies suggest that pretreatment of EAG may help suppress cancer to a certain extent or delay the onset of lung tumor growth.

Mechanistic studies of EAG showed that expression of PCNA and the activated form of Stat-3 were down-regulated in vivo and in vitro, respectively. PCNA is the common biological marker for revealing the proliferative activity, mitotic activity, percentage of DNA-synthesizing cells, and the frequency of PCNA-positive cells in lung tumors [Noguchi and Shimosato, 1995]. Additionally, Stat3 is constitutively activated in a number of human tumors and processes oncogenic potential and anti-apoptotic activities [Catlett-Falcone et al., 1999]. Therefore, monitoring these biological markers allowed us to validate the drug potency and therapeutic potential of EAG in both in vivo and in vitro model systems. We further showed that EAG could arrest the growth of cancer cells in the G2–M phase with concomitant cleavage of PARP and caspase-3 apoptotic markers. Given the cell cycle checkpoint can be activated by various stresses (e.g., UV) that lead to DNA breaks, which in turn activate both genotoxic stress induced activation of Chk1 and Chk2 [Abraham, 2001]. In fact, both Chk1 and Chk2 were not activated, suggesting that EAG-induced G2–M cell-cycle arrest is not the consequence of DNA damage. Moreover, the progression of cell-cycle from G2 to M phase is mainly regulated by cyclin B–cdc2 complex [Koepp et al., 1999]. Our data clearly demonstrated that EAG activated p53, which in turn induced the expression of p21, resulting in the sequestration of cdc2 to the cytoplasm and direct inhibition of cyclin B–cdc2 complexes. All these events contribute to G2–M cell-cycle arrest [Innocente et al., 1999] as well as induction of apoptosis [Liu et al., 2003]. Consistently, previous studies showed that ginsenoside Rb1, one of the components found in EAG, was demonstrated to induce the cleavage of PARP and caspase 3; and the up-regulation of p53 and p21 [Helms, 2004], which coincided with our current findings on EAG. In addition, the MAPKs signaling pathway also participates in cell-cycle arrest and apoptosis induction [Wu, 2004]. Constitutive MAPK activity contributes to p53-dependent apoptosis and phosphorylation of p53 on serine residue 15 [Brown and Benchimol, 2006]. Besides, the p53 protein can functionally interact with the MAPK family proteins. Upon exposure to stressful stimuli, MAPKs phosphorylate and activate p53, leading to p53-mediated cellular responses. In fact, ERK was found not only as an upstream activator to p53, it also activated p21 expression through the Egr-1 pathway, thereby promoting EAG-induced apoptosis involving Egr-1/p21 and p53/p21-dependent mechanisms. In contrast, polysaccharides from ginseng were found to inhibit cancer cell growth through suppression of the MAPKs pathway [Ahn et al., 2006]. However, our findings showed that EAG induced the activation of MAPKs. Suppression of MAPKs signaling with their specific inhibitors altered the EAG-mediated cell cytotoxicity. These contradictory results imply that the drug actions of EAG indeed result from the complex interaction of many compounds and many targeted molecules.

Previous studies demonstrated that Akt can effectively activate IKK [Ozes et al., 1999] and induce p65 phosphorylation [Sizemore et al., 1999]. Phosphorylation of p65 is required for its transcriptional activity and subsequent translocation to the nucleus [Zhong

et al., 1998]. Obviously, EAG was demonstrated to activate NF-κB signaling through Akt, IKK, and NF-κB p65 phosphorylation. Furthermore, EAG-mediated p38 activation may also contribute to NF-κB activation. A specific inhibitor of p38 kinase, SB203580, has been demonstrated to suppress NF-κB activation and increase cytotoxicity in cells exposed to gliotoxin [Hur et al., 2008]. Therefore, suppression of NF-κB activity may be the reason for the decreased cell viability in co-administration of EAG and SB203580. Collectively, these results suggest that EAG could activate NF-κB signaling through activation of p38 and Akt pathway, which promote the expression of genes involved in cell proliferation, survival (Bcl_{XL}), metastasis (ICAM-1), inflammation (COX-2), and angiogenesis (PECAM-1 and VEGF), and eventually contribute to oncogenesis. Suppression of NF-κB signaling and expression of its targeted genes may be a way to potentiate anti-cancer drug efficacy. Besides, the anti-tumor activity of proteasome inhibitor, bortezomib, could correlate with suppression of NF-κB activity [Richardson et al., 2006]. Consistent with these findings, we found that the treatment of LLC-1 cells with another proteasome inhibitor MG-132 could effectively prevent NF-κB targeted gene expression induced by EAG. Moreover, MG-132 strongly potentiated the decrease in cell viability accompanied by the activation of caspase-3 and PARP cleavage upon EAG administration. These results are in agreement with those reported in studies of other types of cancer cells [Domingo-Domenech et al., 2008]. Alternatively, we showed that the role of COX-2 expression was independent of EAG drug actions. Inhibition of ERK with U0126 blocked EAG-mediated COX-2 induction, whereas suppression of NF-κB by MG-132 failed to block EAG-induced COX-2 expression, suggesting the involvement of an ERK–COX-2 pathway independent to NF-κB signaling. Besides, chemopreventive agents have been demonstrated to induce oxidative stress in cancer cells leading to COX-2 over-expression and COX-2-independent cell death [Sun et al., 2009], suggesting that EAG may also induce oxidative stress, thereby increasing COX-2 expression.

In fact, EAG was found to contain mainly Rg1, Re, Rb1, Rb2, Rc, and Rd ginsenosides. The anti-cancer effect of Rb1 has been shown to reduce lung metastasis in mice [Hasegawa and Uchiyama, 1998], while Rd is a potential agent for cancer prevention due to its specific 26S proteasome inhibitory effect [Chang et al., 2008]. However, Rb1 ginsenoside can act as a phytoestrogen to activate estrogen receptors in human breast carcinoma MCF-7 cells [Lee et al., 2003b], while Rc is found to induce c-fos oncogene expression in MCF-7 [Lee et al., 2003a]. In addition, Rg1 and Re have been found to exhibit angiogenic properties [Huang et al., 2005; Liang et al., 2005]. Therefore, these ginsenosides contained in EAG may counteract with each other during angiogenic process and rendered the EAG to either exacerbate or suppress tumor growth. In fact, EAG could suppress tumor growth in vivo, but it also enhanced angiogenic gene expression in vitro. These controversial findings could be explained by the balance hypothesis of the angiogenic switch during angiogenesis [Hanahan and Folkman, 1996]. The stability of the angiogenic switch determines the time of initiation of the subsequent angiogenic process. When there are more anti-angiogenic factors such as IFN-γ [Gohji et al., 1994] than angiogenic stimulators such as TNF-α [Hanahan and Folkman, 1996], the angiogenic switch will not promote tumor growth. For instance, our unpublished data showed that TNF-α was slightly down-regulated, while IFN-γ was slightly up-regulated in both lymphocytes and splenocytes isolated from the EAG-treated LLC-1-bearing mice. This suggests that EAG treatment contributes more to anti-cancer activity, leading to tumor suppression in mice, cell-cycle arrest, and apoptosis in cells.

In respect of the translational values to clinical usage, the results reported in the current animal studies involved the use of an effective dose of EAG that is equivalent to approximately six times of the dosage of ginseng roots prescribed in clinic. For a person with 60 kg body weight it is often to prescribe 10 g ginseng roots per day, calculating as about 0.17 g per kg of body weight. Our current study showed that EAG at 1 g/kg of body weight could effectively suppress tumor growth in LLC-1-bearing mice. So, the study done in such a manner has provided proof-of-principle evidence that EAG could suppress lung cancer growth in vivo. Nevertheless, one cannot simply extend our findings to the clinical setting until more adequate scientific and rigorous human trials are done. On the other hand, the EAG-activated NF-κB signaling could be abrogated by the proteasome inhibitor (MG-132) and then potentiated EAG-mediated apoptosis in LLC-1 cells. In fact, another proteasome inhibitor, bortezomib, is now under clinical trials for the treatment of solid tumors [Richardson et al., 2006]. In this connection, study on a combinative remedy using EAG and proteasome inhibitors may be a new strategy for the study of anti-cancer.

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