

***Agaricus blazei* Murill enhances doxorubicin-induced apoptosis in human hepatocellular carcinoma cells by NFκB-mediated increase of intracellular doxorubicin accumulation**

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Abstract. It has been demonstrated that the *Agaricus blazei* Murill (ABM) mushroom, which primarily consists of polysaccharides, possesses anti-tumor activities. However, the mechanisms by which ABM inhibits human hepatocellular carcinoma growth remain unknown. Our study demonstrates that ABM acts as an enhancer to sensitize doxorubicin (Dox)-mediated apoptotic signaling, and this sensitization can be achieved by enhancing intracellular Dox accumulation via the inhibition of NFκB activity. These findings suggest that ABM, when combined with low doses of Dox, has the potential to provide more efficient therapeutic effects against drug-resistant human hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the fourth leading cause of cancer-related mortality worldwide, with the highest incidence in Asia (1). Despite surgical management and application of non-surgical therapeutic modalities, the incidence of HCC continues to rise (2). Doxorubicin (Dox) is an effective chemotherapeutic drug for the treatment of patients with HCCs (3). However, Dox has been shown to cause development of drug resistance to apoptosis, thereby limiting successful outcomes of cancer chemotherapy (4). One feature that cytotoxic treatments of cancer have in common is their activation of the nuclear transcription factor κB (NFκB), which regulates cell survival (5,6). NFκB activation suppresses the apoptotic potential of chemotherapeutic drugs and contributes to resistance. In this approach, the ability to sensitize apoptotic signaling pathways implicated in enhancing chemosensitivity of chemotherapeutic drugs has important therapeutic implications (7).

NFκB is a heterodimeric complex composed of two subunits p50 and p65. It is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of κB (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50-p65 heterodimer is exposed, leading to nuclear translocation of NFκB. The p50-p65 heterodimer binds to specific DNA sequences on the promoter of target genes (8,9). BAY 11-7082 is shown to inhibit IκBα phosphorylation thereby blocks proteasomal degradation of IκBα, and hence sequestering NFκB in the cytoplasm in an inactivated state (10). Treatment of cell-permeable inhibitor peptide (NFκB SN50) has shown to inhibit nuclear translocation of NFκB and to inhibit NFκB-dependent reporter gene transcription (11).

In recent years many natural polysaccharides and polysaccharide-protein complexes, isolated from mushrooms, have been used as therapeutic drugs (12). Many studies have demonstrated that polysaccharides from basidiomycetes mushrooms had highly beneficial therapeutic effects including: i) preventing oncogenesis after the administration of peroral medications developed these mushrooms or their extracts, ii) direct anti-tumor activity against various tumors, iii) synergistic anti-tumor activity in combination with chemotherapy, and iv) preventive effects on tumor metastasis (13-16). Data on polysaccharides isolated from *Agaricus blazei* Murill (ABM) are highly impressive, and these polysaccharides show promise for use as cancer therapeutics. Studies have shown that ABM extracts strongly suppress the growth of various tumors *in vitro* and *in vivo* (17,18). However, the molecular signaling involved in ABM-mediated anti-tumor activity has never been investigated in HepG2 human hepatocellular carcinoma cells.

Apoptosis (programmed cell death), is an important physiological process of cell death and occurs during tissue remodeling, immune regulation and tumor regression. Most of the chemotherapeutic drugs kill cancer cells by inducing apoptotic death pathways (19). Cells undergoing apoptosis show a sequence of cardinal morphological features, including membrane blebbing, cellular shrinkage and condensation of chromatin (20). The intrinsic mitochondria-mediated pathway, when stimulated, leads to the release of cytochrome c from the mitochondria and to the activation of the death signal (21). Apoptotic signaling and execution through this pathway depends on caspases, or aspartate-specific cysteine

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proteases, which are the key effector molecules in the apoptotic process (22).

Although there are many therapeutic strategies to treat cancer, including chemotherapy, drug resistance have limited the successful outcomes in most cases. Recently, TNF-related apoptosis-inducing ligand (TRAIL) and non-steroidal anti-inflammatory drugs (NSAIDs) have been found to sensitize Dox-induced apoptosis in various types of tumors (23,24). In addition, the combination treatment of Dox and natural products dramatically augments the therapeutic effects against breast cancer, prostate cancer and hepatocellular carcinoma (25,26). In this study, we investigated the ability of ABM to synergize the inhibitory action of Dox in human hepatocellular carcinoma HepG2 cell line. We also evaluated the molecular mechanisms underlying this synergistic effect and sought evidence of synergy between Dox and ABM.

Materials and methods

Materials. The human hepatocellular carcinoma cell line, HepG2 (ATCC HB-8065), was obtained from Korean Cell Line Bank (Seoul, Korea). Fetal bovine serum, penicillin G, streptomycin and RPMI-1640 were obtained from Gibco (Grand Island, NY, USA). Dox, acridine orange and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell-permeable inhibitor peptide (NF κ B SN50) and BAY 11-7082 were obtained from Calbiochem (La Jolla, CA, USA). General caspase inhibitor z-VAD-fmk was purchased from BD Bioscience (San Diego, CA, USA). All other chemicals were of Sigma grade. Antibodies to caspase 3, p53, phospho-p53, NF κ B p105/p50, NF κ B p65 and horseradish peroxidase (HRP)-linked anti-rabbit IgG were obtained from Cell Signaling (Beverly, MA, USA). Antibodies to cytochrome c, Bax, Bcl-2, β -actin and HRP-linked goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Mushroom extracts and the chemotherapeutic drug. The crude water-soluble polysaccharide, which was obtained from the fruiting body of ABM by hot water extraction and ethanol precipitation, was fractionated by DEAE-cellulose and Sepharose CL-6B column chromatography, as previously reported (27). The purified components of ABM primarily consisted of polysaccharide, which was β -glucan. ABM contained a level of endotoxin below the detection limits (0.0015 EU/ml) as assessed by an E-TOXATE kit (Sigma) (unpublished data). Dox was dissolved in 1X phosphate-buffered saline (PBS) for injection at a concentration of 5 mg/ml and stored in aliquots at 4°C. Immediately before each experiment, serial dilutions of this drug were performed in culture media to obtain the required final concentrations.

Cell culture. HepG2 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a 5% CO₂ incubator, and the media were changed twice weekly.

In vitro cytotoxic assay; colorimetric MTT assay. The cytotoxicity of ABM and Dox on the viability of HepG2 cells was measured using the MTT assay, which relies on the ability of viable cells to metabolically reduce the tetrazolium salt MTT to a purple formazan product, which can be quantified colorimetrically (28). Briefly, cell suspensions (2x10⁵ cells/ml) were seeded in 96-well plates and incubated at 37°C to allow for cell attachment. After 48 h, the cells were treated with serum-free medium containing the indicated additives. The plates were incubated for 1 h (day 0) and 72 h (day 3) at 37°C. After the end of each exposure time, 50 μ l of the MTT stock solution (5 mg/ml) in serum-free medium were added to each well to reach a total reaction volume of 200 μ l. After incubation for 2 h at 37°C, the supernatants were aspirated. The formazan crystal in each well was dissolved in isopropyl alcohol and the absorbance was determined at 570 nm on a scanning multiwell spectrophotometer. The relative percentage of survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment. Percent cytotoxicity was calculated using the following formula: percent cytotoxicity = [1 - (absorbance of experimental wells/absorbance of control wells)] x 100%.

Cell morphology assessment. Cells (2.5x10⁵) were subcultured for 48 h on coverslips in 6-well plates. After combination treatment of ABM (100 μ g/ml) with Dox (1 μ g/ml) for 72 h, cell cytological morphology changes were observed using an Olympus optical microscope. Cells were fixed with 3.7% paraformaldehyde for 20 min, washed with PBS, and stained with acridine orange (5 μ g/ml) for 5 min. After washing with PBS, cells were mounted under glass coverslips with Vectashield (Brunschwig, Amsterdam, The Netherlands) and observed. Photographs were taken under a fluorescence microscope (24).

Annexin V/propidium iodide staining. Apoptosis-mediated cell death of tumor cells was examined using a FITC-labeled annexin V/propidium iodide (PI) apoptosis detection kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Briefly, cells were harvested by trypsinization, washed with PBS, and centrifuged to collect the cell pellet. The number of cells was adjusted to 1x10⁶ cells/ml. Cells were then resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4), and stained with FITC-labeled annexin V and PI at room temperature for 15 min in the dark. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) within 1 h after supravital staining. FITC-labeled annexin V was analyzed using excitation and emission setting of 488 and 535 nm. PI was analyzed using excitation and emission setting of 488 and 575 nm. For each flow cytometer run, 10,000 cells were required. The percentages of cells were calculated by Cell Quest software (Becton-Dickinson). The cells in the early stages of apoptosis were annexin V-positive and PI-negative; however, the cells in the late stages of apoptosis were both annexin V- and PI-positive. The apoptotic index (%) was calculated as the sum of early and late apoptotic cells divided for the total number of events (29).

Preparation of subcellular fractionation. The mitochondrial fraction was prepared using a mitochondria isolation kit (Sigma) according to the manufacturer's instructions. Briefly, after various treatments, HepG2 cells were harvested and resuspended in 0.65-2 ml of lysis buffer per 2.5×10^7 cells. The homogenate was incubated on ice for 5 min, two volumes of 1X extraction buffer were added, and the solution was centrifuged at $600 \times g$ for 10 min at 4°C . After centrifugation, the supernatant was transferred to fresh 1.5 ml tubes and centrifuged at $11,000 \times g$ for 10 min at 4°C . The supernatant was removed, and the pellet was suspended in a CellLytic M cell lysis reagent with protease inhibitor cocktail (1:100 [v/v]).

Nuclear extracts were prepared by lysing nuclei in a high salt buffer supplemented with protease and phosphatase inhibitors using a nuclear extraction kit (Panomics Inc., Fremont, CA, USA) according to the manufacturer's protocol. Protein concentrations were quantified by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. After treatment, cells were washed in 1X PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi, 1 mM phenylmethylsulphonyl fluoride, 2 $\mu\text{g/ml}$ pepstatin A] for 30 min on ice. Lysates were centrifuged at $12,000 \times g$ for 20 min at 4°C . The supernatant was collected, and the protein content of the supernatant was measured using a Bio-Rad protein assay kit before analysis. The cytosolic, mitochondrial, or nuclear protein samples were loaded at 10 $\times g$ of protein/lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10-15% gel, and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF membrane, 0.2 μm ; Bio-Rad). Membranes were blocked with 5% non-fat powdered milk in 1X Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h, and they were incubated with primary antibodies at 4°C overnight. Finally, the membranes were treated with HRP-linked secondary antibodies for 1 h at 4°C . The membranes were washed with TBS-T after each antibody binding reaction. Detection of each protein was performed using an enhanced chemiluminescence kit (Millipore Co., Billerica, MA, USA) (29).

Assay for caspase 3 activities. After various treatments, HepG2 cells were lysed in 500 μl lysis buffer consisting of 10 mM Tris-HCl (pH 7.5), 10 mM $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$ (pH 7.5), 130 mM NaCl, 1% Triton X-100, and 10 mM NaPPi. Caspase 3 activity in the lysates was evaluated using a caspase assay kit (BD Bioscience) according to the manufacturer's instructions, with some modifications (30). For each sample, 50 μl of the cell lysate, 5 μl of Ac-DEVD-AMC (1 mg/ml; a synthetic fluorogenic substrate), and 50 μl of reaction buffer [20 mM HEPES (pH 7.5), 10% glycerol, and 2 mM DTT] were transferred to a 96-well plate, mixed and incubated at 37°C for 1.5 h. Caspase 3 cleaves Ac-DEVD-AMC to release the fluorescent AMC. The release of AMC in the cell lysates was measured using the Fluoroskan Ascent FL with Ascent software version 2.6 (Thermo scientific, Langenselbold, Germany) using an excitation filter with a wavelength of 360 nm and an emission filter with a wavelength of 460 nm.

Assay for caspase inhibitor activity. The cells were seeded at a density of 5×10^5 cells/well, and cultured for 48 h in RPMI-1640 medium. The cells were pre-incubated with the indicated concentrations of z-VAD-fmk for 2 h, and they were treated with the combination treatment of ABM (100 $\mu\text{g/ml}$) and Dox (1 $\mu\text{g/ml}$) for 72 h. The viability of HepG2 cells was measured using the MTT assay (30).

Dox accumulation assay. Cells (2.5×10^5) were subcultured on coverslip in 6-well plates and incubated overnight, after which additives were added for 24 h. Subsequently, the culture medium was removed, and the cells washed three times with PBS. Cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature, washed three times with PBS, and mounted under glass coverslip with Vectashield. The cells were observed, and photographs for Dox fluorescence were taken under fluorescence microscopy (31).

Statistical analysis. Data were expressed as means \pm standard error (SEM), and the results were taken from at least three independent experiments performed in triplicate. The data were analyzed by Student's t-test to evaluate significant differences. A level of $p < 0.05$ was regarded as statistically significant.

Results

ABM and Dox, at a low dose, synergise to induce apoptosis in human hepatocellular carcinoma cells. To investigate the anti-tumor effect of ABM on hepatocellular carcinoma, we tested the effect of ABM or/and Dox treatment on human hepatocellular carcinoma cells. HepG2 cells were either left untreated, or treated with different doses of ABM or/and Dox for 72 h. After treatment with ABM alone, a moderately dose-dependent growth-inhibitory effect on HepG2 cells was observed. A similar phenomenon was observed following Dox treatment. Interestingly, the combination of ABM and 1 $\mu\text{g/ml}$ of Dox had a significantly ($p < 0.05$) greater dose-dependent inhibitory effect than that of ABM or Dox alone at comparable concentrations (Fig. 1A). To evaluate whether or not the growth-inhibitory effect of the combination treatment of ABM and Dox was associated with apoptosis, a double-staining method using FITC-labeled annexin V and PI was performed. During the early stages of apoptosis, cells display phosphatidylserine on their outer cell membranes, which is readily detectable by annexin V. During the later stages of apoptosis, as the plasma membrane becomes increasingly permeable, PI can move across the cell membrane to bind to cellular DNA. Double staining the cells with annexin V and PI allowed us to detect apoptotic cells by flow cytometry. The combination of ABM (100 $\mu\text{g/ml}$) and Dox (1 $\mu\text{g/ml}$) had an apoptotic effect on the cells. Moreover, the growth-inhibitory effect of the combination treatment on HepG2 cells was found to be time-dependent, because the inhibitory effect became gradually stronger with the passage of time after treatment; the most significant effect was observed at 72 h (Fig. 1B). To further confirm the cytotoxic synergy between ABM and Dox, we performed a morphology study. Cells in dishes supplemented with 100 $\mu\text{g/ml}$ of ABM and 1 $\mu\text{g/ml}$ of Dox became sparse, rounded and detached from

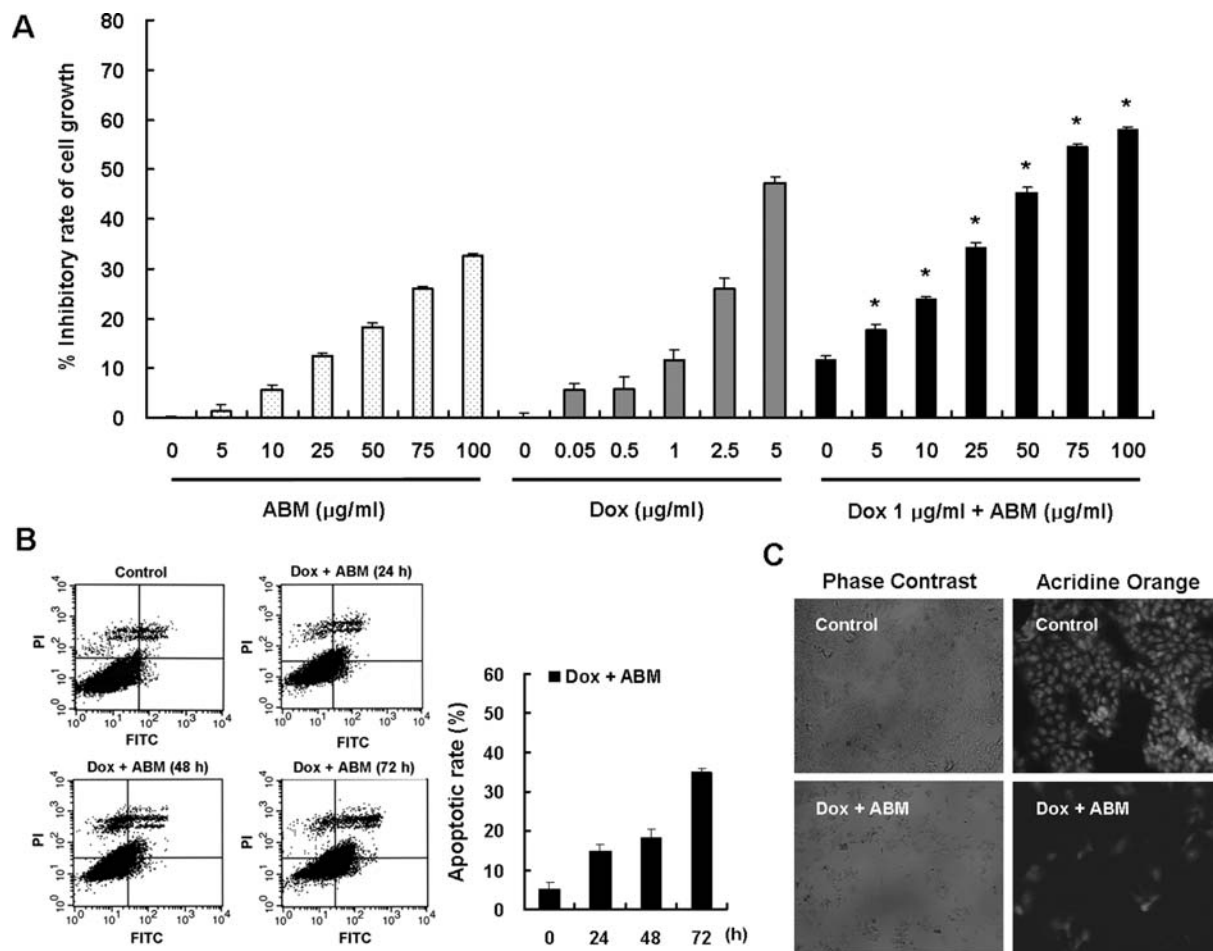


Figure 1. Synergistic cell growth inhibition and apoptosis in HepG2 cells treated with ABM in combination with Dox. (A) The concentration-dependent synergistic cell growth-inhibitory effect of various concentrations of ABM in combination with Dox. Cells were incubated with various concentrations of ABM or/and Dox for 72 h. Cell viability was measured by the MTT reduction assay. Each value represents the mean \pm SE of three independent experiments. Significant differences were compared with the control at $p < 0.05$ by Student's t-test. (B) Percentages of apoptotic cells after the combination treatment of ABM (100 $\mu\text{g/ml}$) and Dox (1 $\mu\text{g/ml}$) in HepG2 cells at 0, 24, 48 and 72 h were measured by the annexin V/PI flow cytometry analysis. (C) Morphological characteristics of HepG2 cells before and after the combination treatment. The HepG2 cells were treated with or without ABM (100 $\mu\text{g/ml}$) and Dox (1 $\mu\text{g/ml}$) for 72 h and stained with acridine orange. The cell morphology was observed under optical microscope (magnification, $\times 100$) and fluorescence microscope (magnification, $\times 200$).

the dishes. We observed the normal morphology of drug-untreated HepG2 cells by staining cells with acridine orange. In this case, cells exhibited a shape that contained tri- or multi-angles with abundant cytoplasm and large oval nuclei with dispersed chromatin. Combination-treated HepG2 cells had the typical morphology of apoptotic cells, including cell shrinkage, chromatin condensation and formation of apoptotic bodies (Fig. 1C). Overall, the results indicated that low doses of ABM and Dox synergistically enhanced Dox-induced apoptosis in HepG2 cells. We conducted further experiments to understand the molecular mechanism by which ABM sensitizes Dox-induced apoptosis in HepG2 cells.

Involvement of caspases in the synergistic effect of ABM and Dox. The caspase family plays an important role in many types of apoptosis, including Dox-induced apoptosis (32). At high doses, Dox can induce apoptosis, possibly through caspase-mediated apoptotic signaling (33,34). To study the mechanisms of the combination treatment of ABM and Dox, we examined whether or not caspase was involved in the synergistic induction of apoptosis. Upon activation, caspases

are cleaved into small, active forms (22). Therefore, we analyzed changes in the levels of pro-caspase 3 by Western blot analysis. Treatment with ABM or Dox alone did not induce caspase 3 cleavage. In comparison, pro-caspase 3 cleavage was induced in response to combination treatment with ABM and Dox (Fig. 2A). To further determine the activation of caspase 3 by the combination treatment of ABM and Dox, the activity of caspase 3 was analyzed using a commercial caspase 3 assay kit. The combination treatment significantly ($p < 0.05$) increased the activity of caspase 3 (Fig. 2B). To confirm whether or not the apoptosis induced by the combination treatment occurs via the caspase-dependent pathway, a general caspase inhibitor z-VAD-fmk was added to the combination treatment. Pretreatment of caspase inhibitor significantly ($p < 0.05$) reduced cytotoxicity by the combination treatment in a dose-dependent manner (Fig. 2C). Thus, these results indicated that low doses of ABM and Dox could synergistically enhance caspase activity in HepG2 cells.

Combination treatment-mediated apoptosis via the mitochondria-mediated apoptotic pathway. The mitochondria-

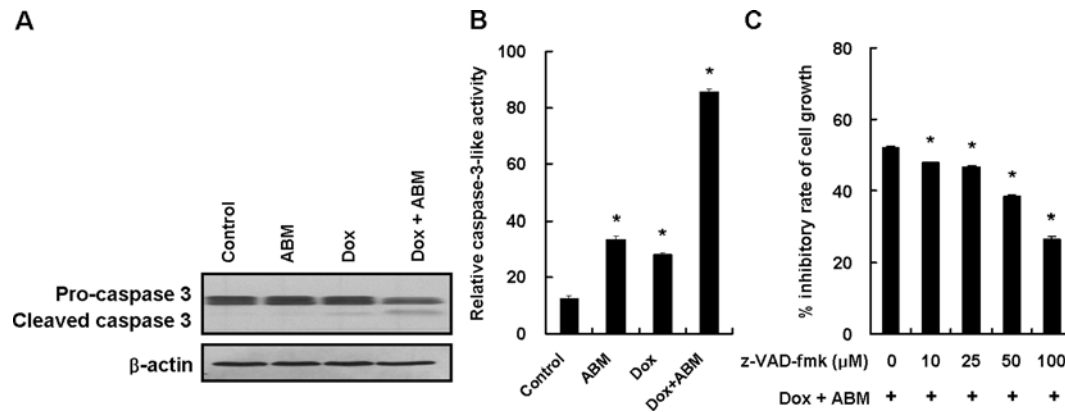


Figure 2. Activation of caspase 3 following the combination treatment of ABM and Dox. (A) After treating with ABM (100 μg/ml), Dox (1 μg/ml), or ABM plus Dox for 72 h, HepG2 cells were harvested, and lysates were prepared. The cleavage of pro-caspase 3 and existence of the activated form of caspase 3 were determined by Western blot analysis. Equal loading of total proteins in each sample was verified by β-actin expression. (B) Effect of the treatment of ABM (100 μg/ml), Dox (1 μg/ml), or ABM plus Dox on caspase 3-like activity in HepG2 cells. After treatment for 72 h, preparation of cell lysates and measurement of caspase 3-like activity was performed. Each value represents the mean ± SE of three independent experiments. Significant differences were compared with the control at * $p < 0.05$ by Student's t-test. (C) Effects of the caspase inhibitor z-VAD-fmk on the synergistic cell growth inhibition in HepG2 cells treated with ABM plus Dox. HepG2 cells were pre-incubated with the indicated concentrations of z-VAD-fmk for 2 h then treated with ABM (100 μg/ml) plus Dox (1 μg/ml) for 72 h. Cell viability was measured by the MTT reduction assay. Each value represents the mean ± SE of three independent experiments. Significant differences were compared with the control at * $p < 0.05$ by Student's t-test.

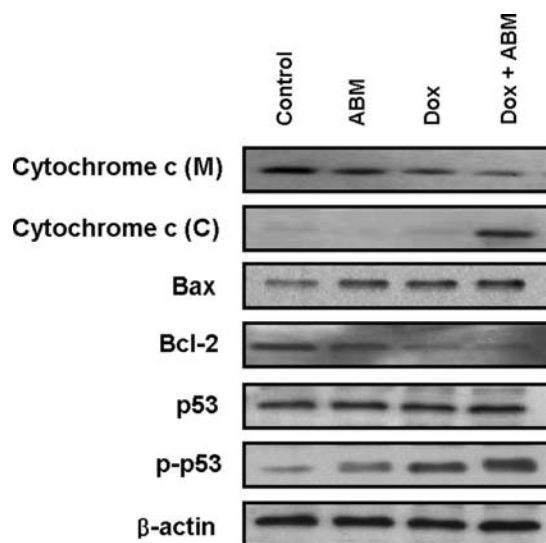


Figure 3. Altered expression of apoptotic-related proteins in HepG2 cells treated with ABM, Dox, or ABM plus Dox. After treating with ABM (100 μg/ml), Dox (1 μg/ml), or ABM plus Dox for 72 h, HepG2 cells were harvested, and lysates were prepared. The mitochondrial (M) and cytosolic (C) fraction from the indicated additive-treated cells were isolated and analyzed for the expression of cytochrome c using Western blot analysis. The levels of Bax, Bcl-2, p53 and phospho-p53 were assessed using Western blot analysis. Equal loading of total proteins in each sample was verified by β-actin expression.

mediated apoptotic pathway plays a critical role in apoptosis. The intrinsic apoptotic pathway is dependent on the balance between Bcl-2 and Bax affecting the release of cytochrome c and second mitochondria-derived activator of caspase (19). To examine whether the combination treatment of ABM and Dox contributed to mitochondrial damage and to the activation of the downstream apoptosis cascade, the release of cytochrome c into the cytosolic fraction (an indicator of compromised mitochondrial integrity) was assessed using

Western blot analysis. Cytochrome c was present in the cytosolic fraction and absent in the mitochondrial fraction isolated from the cells treated with the combination of ABM and Dox (Fig. 3). Bcl-2 family proteins serve as critical regulators of the mitochondria-mediated apoptosis pathway, functioning as either inhibitors (e.g., Bcl-2, Bcl-XL, Bcl-W and Mcl-2) or promoters (e.g., Bax, Bid, Bad and Bim) of cell death (35). To further confirm that mitochondrial apoptotic events were involved in the combination treatment-induced apoptosis, we analyzed changes in the levels of Bax and Bcl-2 by Western blot analysis. The combination treatment of ABM and Dox increased expression of the pro-apoptotic protein Bax, but decreased expression of the anti-apoptotic protein Bcl-2 in HepG2 cells (Fig. 3). The efficacy of Dox on cancer cells is in large part p53-dependent (33,34). As a result of the effect of p53 on the combination treatment-induced apoptosis using Western blot analysis, the phosphorylation of p53, a modulator of Bax, was affected in co-treated HepG2 cells (Fig. 3). Overall, these results demonstrated that the intrinsic apoptotic pathway was involved in the combination treatment-induced apoptosis in HepG2 cells.

ABM increased the intracellular accumulation and retention of Dox. Dox is DNA-damaging agent. DNA damage is caused by ATM and Chk2 kinases, which phosphorylate and stabilize p53, allowing it to function as a transcription factor inducing apoptosis (36). To understand the enhancement of the phosphorylation of p53 in the combination treatment of ABM and Dox, we investigated the effect of ABM on intracellular accumulation of Dox. Dox is an auto-fluorescent compound that allows for the visualization of its intracellular presence by fluorescence microscopy. To investigate the effect of ABM on intracellular accumulation of Dox, we examined the fluorescence of intracellular-accumulated Dox in HepG2 cells after incubation for 24 h in the absence or presence of ABM (100 μg/ml). The intracellular fluorescence, which was primarily visible in the nuclei, was remarkably higher in cells

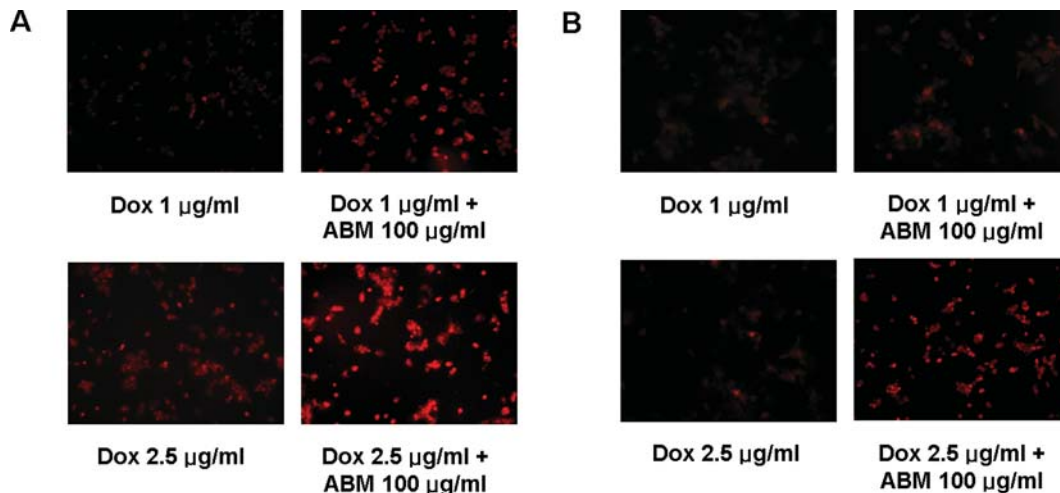


Figure 4. Effect of ABM on the intracellular accumulation and retention of Dox. (A) Intracellular Dox accumulation: fluorescent microscopic pictures of HepG2 cells that were incubated for 24 h in a medium containing Dox (1 and 2.5 µg/ml) in combination with ABM (100 µg/ml). Magnification, x200. (B) Intracellular Dox retention: fluorescent microscopic pictures of HepG2 cells treated with Dox (1 and 2.5 µg/ml) for 24 h, followed by 24 h incubation in control medium or medium containing ABM (100 µg/ml). Magnification, x200.

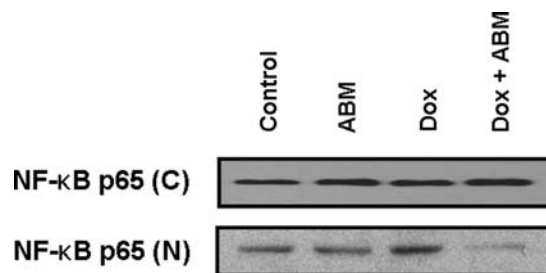


Figure 5. Suppression of NFκB subunit p65 translocation following combination treatment with ABM and Dox. After treating with ABM (100 µg/ml), Dox (1 µg/ml), or ABM plus Dox for 24 h, the nuclear (N) and cytosolic (C) fractions from the indicated additive-treated cells were isolated and analyzed for the expression of p65 using Western blot analysis.

that were co-treated with ABM (Fig. 4A). To determine the effect of ABM on the intracellular retention of Dox, HepG2 cells were incubated for 24 h with Dox (1 and 2.5 µg/ml) followed by incubation for an additional 24 h in a control medium or in a medium containing ABM (100 µg/ml). Cells treated with ABM contained more Dox than those that were incubated in the control medium during the second 24 h culture period (Fig. 4B). Therefore, these results indicated that ABM enhanced Dox-induced cytotoxicity in HepG2 cells was due to an increase in intracellular Dox accumulation.

The combination treatment of ABM and Dox suppress NFκB translocation. NFκB has been shown to be involved in chemoresistance in different cell types, suggesting a possible role of this transcription factor in the chemosensitising effect of agents (5,7). To unravel the molecular mechanisms involved in Dox accumulation, we examined the effect of the combination treatment of ABM and Dox on the translocation of NFκB by Western blot analysis using an NFκB subunit p65 antibody. Dox strongly induced the translocation of nuclear p65 into the nuclei; however, the combination treatment remarkably inhibited Dox-enhanced nuclear p65 translocation (Fig. 5).

The inhibition of NFκB activation was involved in modulating intracellular Dox accumulation. To further investigate the role of NFκB in the enhanced anti-tumor efficacy of Dox, we examined the effects of Dox in the absence or presence of NFκB inhibitors, BAY 11-7082 (10 µM) or SN50 (25 µM), on HepG2 cell growth and intracellular Dox accumulation. The NFκB inhibitors, BAY 11-7082 or SN50, had a greater dose-dependent anti-proliferative effect than that of Dox alone, and they also increased intracellular Dox accumulation (Fig. 6A and B). Furthermore, Western blot analysis of another NFκB subunit p50 in nuclear (N) and cytosolic (C) extracts from HepG2 cultured for 24 h in the treatment with Dox (alone or in combination with BAY 11-7082 or SN50) showed that BAY 11-7082 and SN50 inhibited Dox-enhanced nuclear p50 translocation (Fig. 6C). Thus, these results showed that the inhibition of NFκB activation might be involved in modulating intracellular Dox accumulation.

Discussion

In this study, we showed that low doses of ABM and Dox synergize to induce apoptosis in human hepatocellular carcinoma HepG2 cells. Caspase 3 was activated in the apoptotic process induced by the combination treatment of ABM and Dox. The suppression of caspase activity by a general caspase inhibitor z-VAD-fmk confirmed that the promotion of apoptosis by combination treatment involved a caspase-dependent pathway. Moreover, reducing the ratio of Bcl-2/Bax protein expression through the phosphorylation of p53 induced the release of cytochrome c from the mitochondria to cytosol. Therefore, we have been able to show that the mitochondria-mediated apoptotic pathway was involved in the combination treatment-induced apoptosis in HepG2 cells.

Cancer progression is partially affected by the development of cancer cell resistance to apoptosis induced by chemotherapeutic drugs. NFκB has been shown to be involved in chemoresistance in different cell types, suggesting a possible role of this transcription factor in the chemosen-

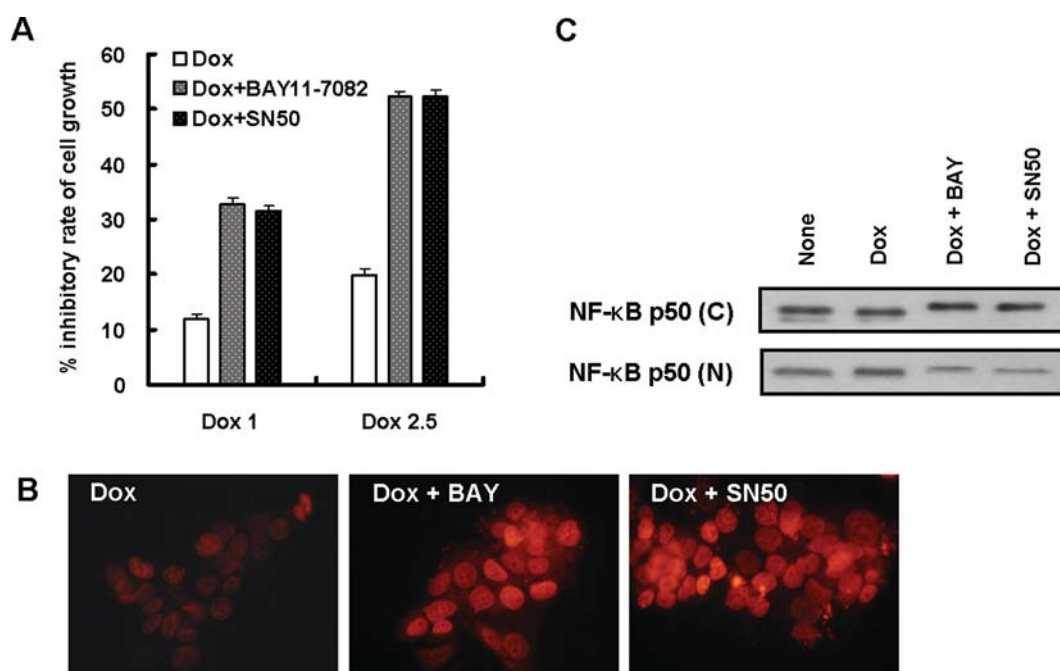


Figure 6. Effect of Dox, alone or in combination with NFκB inhibitor on HepG2 cell growth and effect of NFκB inhibitor on intracellular Dox accumulation. (A) A dose-dependent synergistic cell growth-inhibitory effect of BAY 11-7082 or SN50 in combination with Dox. HepG2 cells were pre-incubated with BAY 11-7082 (10 μM) or SN50 (25 μM) for 2 h then treated with Dox (1 and 2.5 μg/ml) for 72 h. Cell viability was measured by the MTT reduction assay. Each value represents the mean ± SE of three independent experiments. (B) Fluorescent microscopic pictures of HepG2 cells that were incubated for 24 h in a medium containing Dox (1 μg/ml) or in combination with BAY 11-7082 (10 μM) or SN50 (25 μM). Magnification, ×400. (C) Suppression of the NFκB subunit p50 translocation following combination treatment with NFκB inhibitor and Dox. HepG2 cells were pre-incubated with BAY 11-7082 (10 μM) or SN50 (25 μM) for 2 h then treated with Dox (1 μg/ml) for 24 h. The nuclear (N) and cytosolic (C) fractions from the indicated additive-treated cells were isolated and analyzed for the expression of p50 using Western blot analysis.

sitising effect of drugs (5,7). In this study, ABM increased the intracellular accumulation and retention of Dox. We also showed that, like ABM, NFκB inhibitors (BAY 11-7082 or SN50) increased the intracellular accumulation of Dox and augmented the Dox-induced cytotoxicity mediated via suppression of NFκB activity. Therefore, the combination treatment of low doses of ABM and Dox, which enhanced chemosensitivity by suppression of NFκB translocation, would have similar implications for the treatment of patients with HCCs.

In response to diverse stimuli, pro-apoptotic Bcl-2 family proteins such as Bax initiate the intrinsic apoptosis pathway by forming channels on assimilating into the mitochondria, thus increasing outer mitochondrial membrane permeability, and thereby facilitating the release of cytochrome c and other pro-apoptotic factors from the mitochondrial intermembrane space. Released cytochrome c forms an apoptosome complex with Apaf-1, which activates caspase 9, and in turn, its downstream caspase 3, resulting in the morphological features of apoptosis (22). Dox is DNA-damaging agent. DNA damage is caused by ATM and Chk2 kinases, which phosphorylate and stabilize p53, allowing it to function as a transcription factor inducing apoptosis (36). The efficacy of a low dose of Dox on cancer cells is in large part p53-dependent (33,34). It was found that p53 mediated apoptosis by transcriptionally regulating many other genes, including Bax, Apaf-1, Fas, or killer/DR5 (36). In addition, recent studies support a transcriptionally independent pathway involving localization of p53 to the mitochondria, followed by binding and inhibi-

tion of Bcl-xL and/or Bcl-2 or by activating Bax directly (37). In agreement with this, the combination treatment of ABM and Dox upregulated the phosphorylation of p53, increased the expression of the pro-apoptotic protein Bax, and decreased the expression of anti-apoptotic Bcl-2, increased the release of cytochrome c from the mitochondria to cytosol, and subsequently activated caspase 3. A universal caspase inhibitor blocked this synergistic cytotoxic effect on HepG2 cells, further suggesting the involvement of caspase activity in the cytotoxicity rendered by the combination treatment with ABM and Dox. Therefore, these results support the involvement of the intrinsic mitochondria-mediated apoptotic pathway in combination treatment-induced apoptosis in HepG2 cells.

One feature that cytotoxic treatments of cancer have in common is their activation of the transcription factor NFκB, which suppresses the apoptotic potential of chemotherapeutic drugs and contributes to resistance (5,6). Inhibition of NFκB translocation has been implicated in enhanced chemosensitivity of chemotherapeutic drugs (7). Using Western blot analysis we found that combination treatment with ABM and Dox could inhibit NFκB nuclear translocation, where Dox increased it. To further confirm the role of NFκB in the enhanced cytotoxic efficacy of Dox, we combined NFκB inhibitors BAY 11-7082 or SN50 with Dox in the MTT and fluorescent Dox accumulation assays. NFκB inhibitors inhibited Dox-enhanced p50 nuclear translocation in Western blot analysis, increased the cytotoxic effect of Dox in the MTT assay in a dose-dependent manner, and increased the intracellular accumulation of Dox. The molecular mechanism

linking inhibition of NF κ B activity with enhanced Dox accumulation may be associated with the membrane-related actions of Dox and the decrease high basal expression of multi-drug resistance gene and transglutaminase (38-40).

In conclusion, chemotherapeutic resistance to drugs is a major obstacle to the successful treatment of human hepatocellular carcinoma. We have demonstrated that ABM acts as an enhancer to sensitize Dox-mediated apoptotic signaling by enhancing intracellular Dox accumulation via the inhibition of NF κ B activity. Therefore, ABM, when combined with low doses of Dox, has the potential to provide more efficient therapeutic effects against drug-resistant human hepatocellular carcinoma.

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