RESEARCH ARTICLE

# Blocking Bcl-2 Leads to Autophagy Activation and Cell Death of the HEPG2 Liver Cancer Cell Line

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## Abstract

**Background: Apoptosis may be induced after Bcl-2 expression is inhibited in proliferative cancer cells. This study focused on the effect of autophagy activation by ABT737 on anti-tumor effects of epirubicin. Methods: Cytotoxic effects of ABT737 on the HepG2 liver cancer cell line were assessed by MTT assay and cell apoptosis through flow cytometry. Mitochondrial membrane potential was measured by fluorescence microscopy. Monodansylcadaverin (MDC) staining was used to detect activation of autophagy. Expression of p53, p62, LC3, and Beclin1, apoptotic or autophagy related proteins, was detected by Western blotting. Results: ABT737 and epirubicin induced growth inhibition in HepG2 cells in a dose- and time-dependent manner. Both ABT737 and epirubicin alone could induce cell apoptosis with a reduction in mitochondrial membrane potential as well as increased apoptotic protein expression. Further increase of apoptosis was detected when HepG2 cells were co-treated with ABT373 and epirubicin. Furthermore, our results demonstrated that ABT373 or epirubicin ccould activate cell autophagy with elevated autophagosome formation, increased expression of autophagy related proteins and LC3 fluorescent puncta. Conclusions: ABT737 influences cancer cells through both apoptotic and autophagic mechanisms, and ABT737 may enhance the effects of epirubicin on HepG2 cells by activating autophagy and inducing apoptosis.**

**Keywords:** Bcl-2 - ABT737 - autophagy - chemotherapy

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## Introduction

Hepatoma is the sixth most common cancer worldwide. Its incidence has increased rapidly, and it has emerged as the leading cause of cancer-related death worldwide (Poon et al., 2009). To date, chemotherapy is the most frequently used treatment for liver cancer and other cancers. However, the toxic effects of the chemotherapy medicines to normal tissues and normal cells have been one of the major obstacles to successful cancer chemotherapy, along with mechanisms of drug resistance (Vero et al., 2009). Progression towards drug resistance can be achieved through the inactivation of apoptosis and is generally considered a hallmark of cancer (Hanahan and Weinberg, 2000). Understanding these drug resistance mechanisms facilitates the identification of methods to therapeutically reactivate the death response and develop clinical trials to treat the disease (Abou-Alfa and Venook, 2008; Karantza-Wadsworth and White, 2008).

BCL-2 is involved in autophagy inhibition by binding to the BH3 (BCL-2 homology, BH) domain of Beclin 1 (BECN1, autophagy related) and by negatively regulating the autophagy-promoting BECN1-VPS34 complex (Pattingre et al., 2005). BECN1 and BCL-2 complex formation is primarily dependent on the BH3 domain of BECN1 and the BH3-receptor domain of BCL-2 (Wei et al., 2008). Pharmacologic interference with this interaction by BCL-2 knockdown, BH3-mimetics activation results in autophagy induction and provides a novel therapeutic strategy targeting cancer cells with high treatment resistance because of BCL-2 overexpression (Erlich et al., 2007).

Autophagy is intricately implicated in both health and disease. Autophagy defects play a role in the pathogenesis of diverse diseases, including myopathy, neuronal degeneration, microbial infection, inflammatory bowel disease, aging, and cancer (Cadwell et al., 2008; Malicdan et al., 2008; Saitoh et al., 2008; Winslow and Rubinsztein, 2008; Yen and Klionsky, 2008; Orvedahl and Levine, 2009). Recent studies have elucidated the functional role of autophagy in different cellular processes and the potential of autophagy modulation as a novel therapeutic strategy for different pathologic conditions, including cancer (Carew et al., 2007; Mathew et al., 2007; Chen and Karantza-Wadsworth, 2009). Anticancer therapies, such as hormonal agents, chemotherapy, and irradiation, frequently induce autophagy, in most cases as a prosurvival response potentially contributing to treatment

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resistance. However, autophagy activation in particular genetic backgrounds and/or completion of the autophagic process beyond reversibility of cell viability can also lead to cell death, thus enhancing treatment efficacy (Buytaert et al., 2006; Gewirtz, 2007; John et al., 2008; Al-Ejeh et al., 2010).

We predict that activation of autophagy by blocking Bcl-2 may contribute to anti-tumor actions. In this study, we examined the effects of the Bcl-2 inhibitor ABT737 on apoptosis and autophagy activation as well as the contribution of autophagy to chemosensitivity effects of epirubicin on HEPG2 liver cancer cell line. The results showed that blocking Bcl-2 leads to activation of apoptotic and autophagic pathways, and autophagy activation contributes to the enhancement of epirubicin-induced death of cancer cells.

## Materials and Methods

*Drug preparation*

ABT737 (Santa Cruz, USA) was diluted in distilled sterilized water to create a stock solution that was stored according to the manufacturers' suggestions. Epirubicin (Merk, Darmstadt, Germany) was diluted in saline to create a stock solution that was stored according to the manufacturers' suggestions. The final solution of ABT737 used in the experiments was 1 $\mu$mol/L, and epirubicin was 20 $\mu$g/mL. This ABT737 concentration was selected based on our pilot experiments on HEPG2 cells, and the concentration of epirubicin was selected based on the manufacturers' suggestions.

*Cell culture and viability assay*

HepG2 liver cancer cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HEPG2 cells were maintained in an RPMI1640 medium containing 10% heat-inactivated fetal bovine serum and 0.03% L-glutamine, followed by incubation in a 5% $CO_2$ atmosphere at 37 °C. Cells in the mid-log phase were used in the experiments. Cell viability was assessed by MTT assay. To determine the time-course of HEPG2 cell response to ABT737 and epirubicin, HEPG2 cells were planted into 96-well microplates ($7\times10^4$ cells/well). ABT737 (1 μmol/L) and epirubicin (20 $\mu$g/mL) were added to culture medium. Cell viability was assessed with MTT assay at 24, 48, and 72 h post-drug treatment. MTT (Sigma, St Louis, MO, USA) solution was added to the culture medium (500 μg/mL final concentration) for 4 h before the end of treatment, and the reaction was stopped by adding 100 μL 10% acidic SDS. The absorbance value (A) at 570 nm was read using an automatic multiwell spectrophotometer (Bio-Rad, Richmond, CA, USA). The percentage of cell death was calculated as follows: cell death (%) = [(1 - A of experimental well)/ A of positive control well] × 100.

*Apoptosis detection*

To analyze the effects of ABT737 and epirubicin on cell apoptosis progression, we incubated HEPG2 cells with ABT737 (1 μmol/L) and epirubicin (20 $\mu$g/mL) for 12 or 24 h. The cells were harvested using 0.25% trypsin, washed with phosphate-buffered saline (PBS), counted, and adjusted to $1\times10^6$ cells/mL. The cells were fixed in 70% ethanol, treated with 100 mg/L RNase at 37 °C for 30 min, and stained with 50 mg/L propidium iodide (Sigma) for 30 min. The cells were analyzed using flow cytometry (Epics XL; Beckman Coulter, Fullerton CA, USA). Percentage of apoptotic cells was considered as the percentage of cells with DNA content lower than that of cells in G0-G1 in the propidium iodide intensity-area histogram plot (Alvarez-Tejado et al., 2001). These hypodiploid cells were considered to represent apoptotic cells, and the rate of apoptosis was analyzed using Multicycle software (Beckman Coulter).

*Mitochondrial potential ($\Delta\Psi$) detection*

Mitochondrial $\Delta\Psi$ was determined using the KeyGEN Mitochondrial Membrane Sensor Kit (KeyGEN, Nanjing, China). The mitosensor dye aggregates in the mitochondria of healthy cells and emits red fluorescence against a green monomeric cytoplasmic background staining. However, in cells with a collapsed mitochondrial $\Delta\Psi$, the dye cannot accumulate in the mitochondria and remains as monomers throughout the cells with green fluorescence (Rashid et al., 2001). HEPG2 cells were incubated with ABT737 and epirubicin in 24-well plates for the indicated times and then pelleted, washed with PBS, and re-suspended in 0.5 mL of diluted mitosensor reagent (1 μmol/mL in incubation buffer). After incubating cells with mitosensor reagent for 20 min, 0.2 mL of incubation buffer was added, and cells were centrifuged then resuspended in 40 μL of incubation buffer. Finally, cells were washed and resuspended in 1 mL PBS for flow cytometry analysis.

*Visualization of MDC-labeled vacuoles*

Exponentially growing cells were plated on 24-chamber culture slides, cultured for 24 h, and then incubated with the drug in 10% FCS/RPMI1640 for 6, 12, and 24 h. Autophagic vacuoles were labeled with MDC (Biederbick et al., 1995) (Sigma, St Louis, MO, USA) by incubating cells with 0.001 mmol/L MDC in RPMI1640 at 37 °C for 10 min. After incubation, cells were washed twice with PBS and immediately analyzed with fluorescence microscopy (Nikon Eclipse TE 300, Japan) equipped with a filter system (V-2A excitation filter: 380 nm to 420 nm, barrier filter: 450 nm). Images were captured with a CCD camera and imported into Photoshop.

*Total cell and nuclear protein extraction and Western blot analysis*

For extraction of total cell proteins, cells were washed with pre-cooled PBS and subsequently lysed in pre-cooled RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.25% sodium deoxycholate, and 0.1% NP-40) containing 1 mM phenylmethysulfonyl fluoride, 50 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 1 mM NaF, 5 mM EDTA, 5 mM EGTA, and a combination of protease inhibitors. Cell lysis was performed on ice for 30 min. Clear protein extracts were obtained by centrifugation for 30 min at 4 °C. For nuclear protein extraction, the Nuclear Protein Extraction Kit (KeyGEN, Nanjing, China) was used

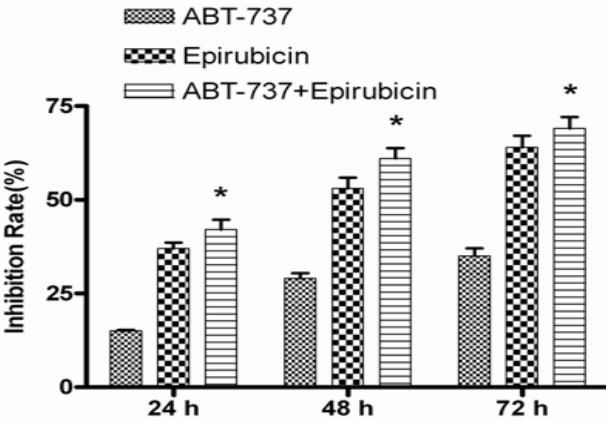


**Figure 1. Viability of HEPG2 Cells Exposed to ABT737 or Epirubicin Treatment.** (A) Reduced viability of HEPG2 cells after ABT737 or Epirubicin treatment. HEPG2 cells ($7\times10^4$ cells/mL) were cultured with ABT737 or Epirubicin for the indicated times and cell viability was analyzed using an MTT assay. ABT737 has a effect when used in combination with Epirubicin than when used alone. ABT737 inhibited the proliferation of HEPG2 Liver cancer cells and enhanced the Chemosensitivity of Epirubicin. values are mean±SD of 3 independent experiments. $^bP < 0.05$, $^cP < 0.01$ compared to the control group; $^*P < 0.05$ compared to the ABT737 alone treatment group

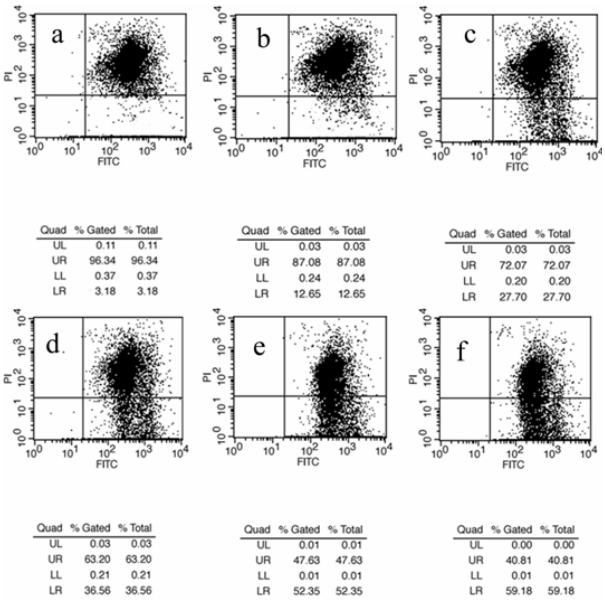


**Figure 2. Mitochondrial Dysfunction was Induced after ABT737 or Epirubicin Treatment.** HEPG2 cells were incubated with ABT737 (1 μmol/L) or Epirubicin (20 μg/mL) for indicated times. (a) control, (b) 6 h, (c) 12 h, (d) 24 h after ABT737 treatment (×400) (N=3). (e) 12 h with Epirubicin treatment. (f)12 h with ABT737 and Epirubicin treatment

according to the manufacturer's recommendations. Protein was extracted from HEPG2 liver cancer cells as previously described. Protein concentration was determined with a Bradford protein assay kit. Proteins were resolved on 8.5% polyacrylamide gels and subsequently transferred onto nitrocellulose membranes. For immunoblot analysis, nitrocellulose membranes were incubated with specific antibodies recognizing target proteins overnight at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibody (1:3000) for 1 h at room temperature, subsequently analyzed by ECL detection system (Amersham Pharmacia Biotech) and visualized by autoradiograpy. Protein β-actin (1:5000; Sigma) was used as loading controls.

*Statistical analysis*

All data were presented as means ± SD. Statistical analysis was conducted using ANOVA followed by a Dennett's-test, with $P < 0.05$ considered statistically significant.

## Results

*ABT737 inhibited cell viability and enhanced epirubicin tumor cell growth inhibition*

ABT737 reduced HEPG2 viability in a time-dependent manner. MTT assays revealed that 24 h post-treatment, the rate of inhibition reached 15%±0.31% at the dose (1 μmol/L) used. The rate of inhibition increased when the incubation time was prolonged, reaching 29%±1.46% and 35%±2.17% at 48 and 72 h post-treatment, respectively (Figure 1). We used the chemotherapy drug epirubicin (20 μg/mL) to assess the clinical value of the Bcl-2 inhibitor in tumor treatment and test the synergistic inhibitory effect of the Bcl-2 inhibitor on growth in combination with a chemotherapy drug. We found that ABT737 has an effect when used in combination with epirubicin than when used alone (Figure 1). Thus, ABT737 inhibited the proliferation of HEPG2 liver cancer cells and enhanced the chemosensitivity of epirubicin.

*ABT737 induced mitochondrial dysfunction and enhanced the effects of inducing mitochondrial dysfunction of epirubicin*

Mitochondrial ΔΨ was examined using the fluorescent dye JC-1. We detected a collapse in mitochondrial ΔΨ as early as 6 h post-ABT737 or -epirubicin treatment, as indicated by increased emission of green fluorescence. This change reached a maximum after 24 h ABT737 treatment or 12 h epirubicin treatment (Figure 2). A collapse in mitochondrial ΔΨ always indicates cell apoptosis or necrosis. When used in combination with epirubicin, contrary to that when used alone, ABT737 induced mitochondrial dysfunction and activated cell apoptosis in HEPG2 cells. The results prove that ABT737 enhanced the effects of inducing mitochondrial dysfunction in HEPG2 cells by epirubicin.

*ABT737 induced apoptosis and enhanced the effects of inducing apoptosis of HEPG2 cells by epirubicin*

The effect of ABT737 on the cell apoptosis progression of HEPG2 cells was studied after 6, 12, and 24 h of ABT737 (1 μmol/L) exposure. Flow cytometry indicated that ABT737 cell apoptosis occurred after 6, 12, and 24 h treatment. A significant difference was found between 1 μmol/L ABT737 and epirubicin group and the control group at 12 h. When the cells were treated with ABT737 and epirubicin for 12 h, the apoptosis of HEPG2 cells was more significantly induced than when ABT737 was used alone (Figure 3). The results indicate that ABT737

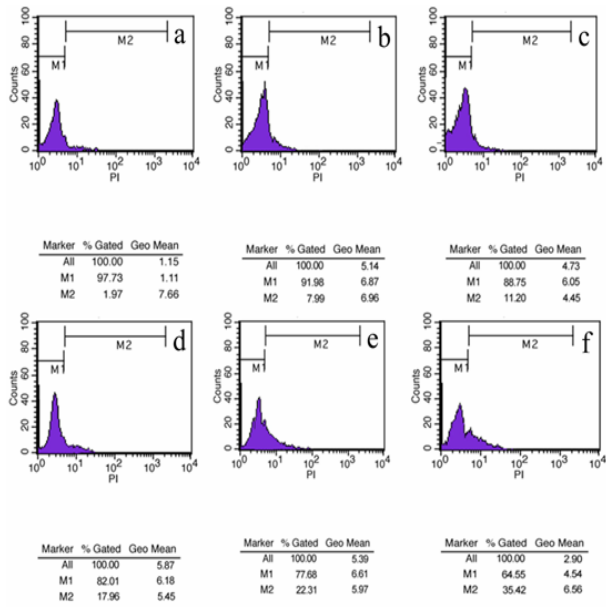


**Figure 3. Apoptosis was Induced after ABT737 or Epirubicin Treatment.** HEPG2 cells were incubated with ABT737 (1 μmol/L) or Epirubicin (20 μg/mL) for indicated times. (a) control, (b) 6 h, (c) 12 h, (d) 24 h after ABT737 treatment (×400) (N=3). (e) 12 h with Epirubicin treatment. (f) 12 h with ABT737 and Epirubicin treatment

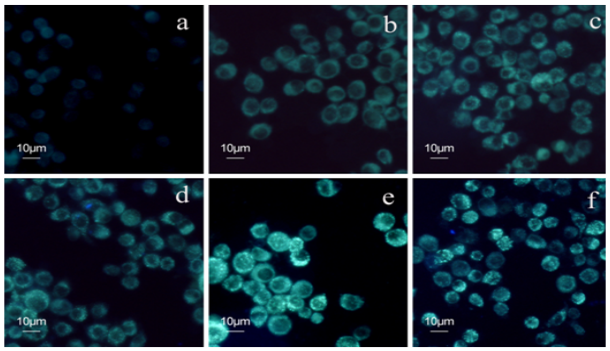


**Figure 4. Autophagy was Activated after ABT737 or Epirubicin Treatment.** HEPG2 cells were incubated with ABT737 (1 μmol/L) or Epirubicin (20 μg/mL) for indicated times and stained with MDC (100 μmol/L). Fluorescence particles indicate L-AVs. (a) Control, (b) 6 h, (c) 12 h, (d) 24 h after ABT737 treatment (×400) (N=3). (e) 12 h with Epirubicin treatment. (f) 12 h with ABT737 and Epirubicin treatment

enhanced the cell apoptosis-inducing effects on HEPG2 cells by epirubicin.

*ABT-737 increased autophagic vacuoles and enhanced the effects of inducing autophagy of HEPG2 cells by epirubicin*

The autofluorescent substance MDC has recently been shown to be a marker for late autophagic vacuoles (L-AVs) but not endosomes (Biederbick et al., 1995). The dye is trapped in acidic, membrane-rich organelles and also exhibits increased fluorescence quantum yield in response to the compacted lipid bilayers present in L-AVS (Niemann et al., 2000). When cells are viewed with a fluorescence microscope, AVs stained by MDC appear as distinct dot-like structures distributed within the cytoplasm or localized in the perinuclear regions. We found an increased number of MDC-labeled vesicles after

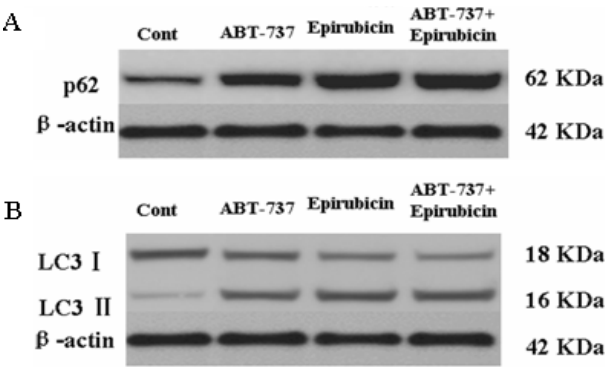


**Figure 5. Western Blotting Analysis of p62 and LC3 of the Control and ABT737 or Epirubicin Treated HEPG2 Cells.** Cells were treated with ABT737 (1 μmol/L) or Epirubicin (20 μg/mL) for 12h and were harvested for total proteins. It indicates that ABT737 may up-regulating the expression of p62 and LC3II. ABT737 enhanced the effects of Epirubicin in up-regulating the expression of p62 and LC3II

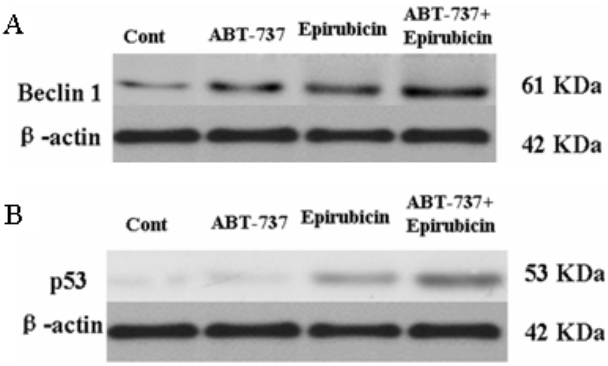


**Figure 6. Western Blotting Analysis of Beclin 1 and p53 of the Control and ABT737 or Epirubicin Treated HEPG2 Cells.** Cells were treated with ABT737 (1 μmol/L) or Epirubicin (20 μg/mL) for 12h and were harvested for total proteins. It indicates that ABT737 may up-regulating the expression of Beclin 1 and p53. ABT737 enhanced the effects of Epirubicin in up-regulating the expression of Beclin 1 and p53

ABT-737 and epirubicin treatment compared with sole ABT-737 treatment (Figure 4). The results indicate that ABT737 enhanced the cell autophagy-inducing effects on HEPG2 cells by epirubicin.

*ABT737 increased the expression of p62 and LC3 and enhanced the effects of epirubicin in activating autophagy*

To distinguish the specific Bcl-2-mediated cell proliferation blockage from autophagy, we measured the expression of autophagic proteins LC3 and p62 after treatment with ABT737 or epirubicin. As shown in Figure 5A, p62 expression in the HEPG2 cell line was activated by ABT737 or epirubicin treatment. ABT737 may have enhanced the effects of epirubicin in activating autophagy. Western blot analysis was used to detect the protein levels of LC3-I and LC3-II (Figure 5B). The results showed that the LC3 levels, particularly LC3-II, increased, leading to an increased ratio of LC3-II/LC3-I after ABT737 or epirubicin treatment. ABT737 may have also enhanced the effects of epirubicin in activating autophagy. These results suggest that ABT737 may induce tumor cell apoptosis by regulating p62 and LC3-II.

*ABT737 upregulated p53 and BECN1 and enhanced the effects of epirubicin in upregulating p53 and BECN1*

To distinguish the specific Bcl-2-mediated cell proliferation blockage from autophagy and apoptosis, we measured the expression of apoptotic proteins, p53 and BECN1, after treatment with ABT737 or epirubicin. As shown in Figure 6A, the BECN1 expression in the HEPG2 cell line was activated by ABT737 or epirubicin treatment. ABT737 may have enhanced the effects of epirubicin in activating autophagy and inducing apoptosis. As shown in Figure 6B, p53 expression in the HEPG2 cell line was also upregulated, and ABT737 may have enhanced the effects of epirubicin in activating apoptosis. These results suggest that ABT737 may induce tumor cell apoptosis by regulating BECN1 and p53.

## Discussion

The Bcl-2 family of proteins plays critical roles in human cancers, including hepatic cancer. Bcl-2 activation has been shown to enhance tumor growth, invasion, motility, tumor spreading and metastasis, and inhibition of apoptosis. The overexpression of Bcl-2 family proteins in hepatic cancer may also play important roles in resistance to a wide spectrum of chemotherapeutic agents. Therefore, identification of an inhibitor targeting the Bcl-2 family of proteins is likely to provide a therapeutic benefit for hepatic cancer.

The tumor suppressor p53 plays a central role in sensing various genotoxic stresses and important roles in apoptosis by regulating expression of pro-apoptotic proteins. In this study, we demonstrated that ABT737, the inhibitor of Bcl-2, significantly upregulated the p53 levels, indicating that apoptosis may be triggered by ABT737. We found that mitochondria $\Delta\Psi$ collapsed after ABT737 or epirubicin treatment. This study proved that ABT737 enhanced the effects of epirubicin-induced mitochondrial dysfunction in HEPG2 cells. Mitochondria play a central role in regulating cell death and survival. Diverse proapoptotic stimuli act on the mitochondria, triggering mitochondrial $\Delta\Psi$ collapse, cytochrome c release, and caspase activation. The mitochondrial permeability transition represents an important event initiating apoptotic cell death.

Increasing evidence has suggested that autophagy plays important roles in tumor cell growth, differentiation, and response to anti-tumor drugs (Gozuacik and Kimchi, 2004). Many classic anti-tumor drugs have now been found to exert their cytotoxic actions through autophagic mechanisms (Bursch et al., 1996; Kanzawa et al., 2004; Wong et al., 2009). In the present study, Bcl-2 inhibition using ABT737 resulted in significantly increased levels of p62, LC3, and BECN1, particularly the increased production of LC3-II. LC3 is an autophagosomal ortholog of yeast Atg8. LC3 has been best characterized as an autophagosomal marker in mammalian autophagy, and the LC3 levels may also reflect the levels of autophagy (Pankiv et al., 2007). BECN1 is the mammalian ortholog of the yeast ATG6-Vps30 gene. It can complement the defect in autophagy present in ATG6-/- yeast strains and stimulate autophagy when overexpressed in mammalian cells (Lum et al., 2005). p62 is also found in protein aggregates that are positive for both ubiquitin and microtubule-associated protein 1 light chain3 (LC3), a well-characterized marker of autophagy. Subsequent experiments demonstrated the direct interaction of p62 and LC3 through a 22-amino acid sequence known as the LC3-interacting region (Kirkin et al., 2009). Therefore, p62 is inferred to be the link between polyubiquitinated proteins and autophagy (Kirkin et al., 2009). The present results suggest that autophagy is induced by ABT737, and its activation may contribute to the anti-tumor effects of epirubicin.

In summary, the present study revealed a new mechanism associated with Bcl-2 inhibition-triggered impairment of cell proliferation and induction of cell death of cancer cells. Blocking Bcl-2 increases p53 expression as well as induces pro-apoptotic and autophagic proteins p62, LC3, and BECN1. ABT737 induces death of cancer cells through both apoptotic and autophagic mechanisms, and ABT737 may enhance the effects of epirubicin of activating autophagy and inducing apoptosis. Further investigation on the relationship of autophagy activation and anti-tumor effects of Bcl-2 inhibitors will unveil new strategies for tumor therapy.

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