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ORIGINAL ARTICLE

ABT-263 sensitizes TRAIL-resistant hepatocarcinoma cells by downregulating the Bcl-2 family of anti-apoptotic protein

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Abstract
Purpose Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent due to its selective cytotoxicity to transformed cells. However, most human hepatocellular carcinomas (HCC) develop resistance to TRAIL. Thus, there is an urgent need to investigate the molecular targets and the underlying mechanisms that may be involved in overriding the resistance of tumor cells to TRAIL.
Methods Cell viability analysis was performed in HCC cells after treatment with TRAIL and/or ABT-263. Flow cytometry was used to assess apoptosis. The expression of caspases and members of the Bcl-2 family was examined through immunoblot analysis. Finally, the viability of cancer cells transfected with a plasmid containing HBx (hepatitis B virus X protein) following treatment with TRAIL was also measured.
Results In this study, we demonstrate that ABT-263, a potent and orally bioavailable inhibitor of the Bcl-2 family, was able to reverse the resistance of hepatocarcinoma cell lines to TRAIL-induced apoptosis, while sparing normal liver cells. The molecular mechanism of the reversal in resistance may be attributed to the inhibition by ABT-263 of anti-apoptosis proteins of the Bcl-2 family. In addition, we determined that HBx was able to sensitize TRAIL-resistant hepatocarcinoma Huh7 cells.
Conclusions These findings provide a novel insight into the clinical application of TRAIL-induced apoptosis of HCC cells.

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Introduction

Human hepatocellular carcinoma (HCC) is the third most common deleterious disease and has a considerably high cancer mortality rate [9, 24]. Curative therapies for HCC are limited to surgery, such as resection and liver transplantation, and chemotherapy. However, the surgical outcomes are not satisfactory, as surgery is not suitable for patients with advanced disease stages [4, 19]. Therefore, investigations into novel therapeutic approaches to cure HCC are necessary.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a member of the TNF family of proteins, has widely been regarded as a promising chemotherapeutic drug to treat a variety of cancers due to its selective cytotoxicity in cancer cells [5, 31]. TRAIL binds to its receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and recruits the intracellular adaptor Fas-associated protein with death domain (FADD) and pro-caspase-8 to form the death-inducing signaling complex (DISC), wherein caspase-8 is self-cleaved and initiates an extrinsic apoptosis cascade. Activated caspase-8 can further cleave Bid and thus trigger the mitochondria apoptosis pathway [15, 23]. Unfortunately, despite promising, TRAIL is ineffective in approximately half of the tumor cell lines tested. In particular, most of HCC liver cancer cell lines show resistance to TRAIL treatment.

Many chemotherapeutic drugs, such as HDACi MS275 and SAHA, the proteasomal inhibitors MG132 and bortezomib, and the Raf/MEK/Erk signaling kinase inhibitor sorafenib, can sensitize resistant cancer cells to TRAIL-induced cell death by targeting different molecules [6, 10,

14, 16, 18, 20, 21, 32]. Therefore, TRAIL has potential to treat human carcinoma in combination with other drugs [13, 33]. ABT-263 is a potent, orally bioavailable Bcl-2 inhibitor and has been demonstrated to have clinical value in treating leukemia, lymphoma, small cell lung cancer and other types of cancers. Thus, TRAIL has a potential to treat cancer either as a single agent or as part of a combinational regimen [1, 2, 30].

In the present study, we demonstrate that the human liver cancer cell lines HepG2, Huh7, BEL-7402 and FHCC98 show strong resistance to TRAIL-induced apoptosis. However, the TRAIL-resistant liver cancer cells were highly sensitive to the ABT-263 and TRAIL combination treatment and were killed by these agents via an apoptotic pathway. Moreover, ABT-263 combined with TRAIL did not kill normal liver cells. In addition, we demonstrated that the HBx gene increased the sensitivity of hepatocarcinoma cells to TRAIL. Thus, these findings provide a new mechanism for combinational TRAIL chemotherapy of human hepatocellular carcinoma.

Materials and methods

Cell lines and cell culture

The human hepatocarcinoma cell lines HepG2, Huh7, FHCC98 and BEL-7402 and the human normal liver cell line L02 were purchased from the China Center for Type Culture Collection (CCTCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 Units ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin in a 5% CO2 incubator (Sanyo). The human acute T-cell leukemia cell line Jurkat was purchased from the CCTCC and cultured in suspension in RPMI-1640 medium (GIBCO) supplemented with 10% FBS and 100 Units ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin in a 5% CO2 incubator.

Antibodies and reagents

The mouse monoclonal anti-GAPDH antibody was purchased from Beyotimes. The rat monoclonal anti-PARP, pro-caspase-8, caspase-9, pro-caspase-3, DR-5, c-FLIP(L), Bcl-2, Bcl-xl, Mcl-1 and Bax antibodies were purchased from Cell Signal Technology (CST). The polyclonal goat anti-mouse and goat anti-rabbit HRP-anchored secondary antibodies were purchased from Beyotimes. The ECL reagent was purchased from Millipore. The recombinant human TNF-related apoptosis-inducing ligand (rhTRAIL) was purchased from R&D Systems and reconstituted at 20 μg ml^{-1} in sterile PBS containing 0.1% bovine serum albumin. After reconstitution, TRAIL was divided into 20 μl aliquots into different microcentrifuge tubes and stored at −80°C. ABT-263 was purchased from Active Biochemicals Co, Hong Kong, diluted with DMSO to a concentration of 1 μM and then stored at −80°C.

Cell lysis and Western blot analysis for detection of apoptosis

For the Western blot assay, cells were collected and lysed with 1% SDS (Amresco) to break down the membranes, and then, whole proteins were heated at 95°C for 20 min to inactivate the proteases. The samples were centrifuged for 15 min, and the protein concentration of each sample was assessed using the BCA reagent (Thermo Scientific). Equivalent amounts of protein were loaded into each lane and separated using SDS-PAGE on 10 or 15% gels (Amresco) and then transferred to a PVDF membrane (Millipore) using a semi-dry transfer (Bio-Rad) and blocking with 5% non-fat milk for 1 h. Finally, membranes were incubated with the specific primary antibody overnight at 4°C. After washing the membrane with Tris-buffered saline containing Tween-20 (TBST) 3 times for 10 min each, membranes were incubated with HRP-conjugated secondary antibodies for an hour and then washed 3 times with TBST. The target protein bands were visualized using an ECL reagent kit (Millipore) and exposure to an X-ray film (Fuji).

Plasmids and transfection of HBx

For cell transfection, Huh 7 cells were cultured overnight, and the FuGENE® HD transfection reagent (Roche) was used for transfection following the manufacturer's instructions. Briefly, Huh7 cells were seeded at a concentration of 1×10^6 cells per well into 6-well plates and cultured at 37°C in 5% CO_2 incubator for 12 h. Prior to transfection, the FuGENE® HD transfection reagent was warmed to room temperature, at which point the plasmid DNA was diluted with serum-free medium to 2 μg per 100 μl and added with the appropriate concentration of transfection reagent to the mixture. The mixture was incubated at room temperature for 15 min, followed by addition to the transfection wells, gentle mixing and incubation for 48 h. Expression levels of HBx following TRAIL treatment of HBx overexpressing and control cells were determined with Western blot analysis. The pcDNA3.1-HBx and pcDNA3.1 plasmid were kindly provided by Professor Yin Zhu (College of Life Sciences, Wuhan University, China).

Cell viability assay

For the cell viability assay, the cells were treated with TRAIL at the specified concentration (50, 100 or 200 ng ml^{-1}) and

were incubated overnight in the presence or absence of 0.9 μM ABT-263 for 24 h. Cells were then centrifuged and washed using PBS, and 0.4% trypan blue was added. Cells were counted using a hemocytometer. The dead cells stain blue, while the live ones remain unstained and transparent [28]. A minimum of four independent experiments were performed.

Flow cytometry for apoptosis

Huh7, HepG2 and Jurkat cells were cultured at a concentration of 1×10^6 cells/well overnight and treated with 50 or 100 ng ml^{-1} TRAIL for 24 h. Subsequently, cells were collected, washed in PBS 3 times and assayed with the Annexin V-FITC apoptosis detection kit (Bipec Biopharma, Cambridge) according to the manufacturer's instructions. Briefly, cells were pretreated with binding buffer and then incubated with Annexin V-FITC and propidium iodide (PI) in the dark. Apoptotic cells were examined via flow cytometry (Beckman), and a minimum of 10,000 events were counted.

Statistical analysis

All experiments were performed a minimum of 3 times, unless otherwise stated. Experimental differences were analyzed for statistical significance using the Student's *t* test. A P value of <0.05 was considered significant.

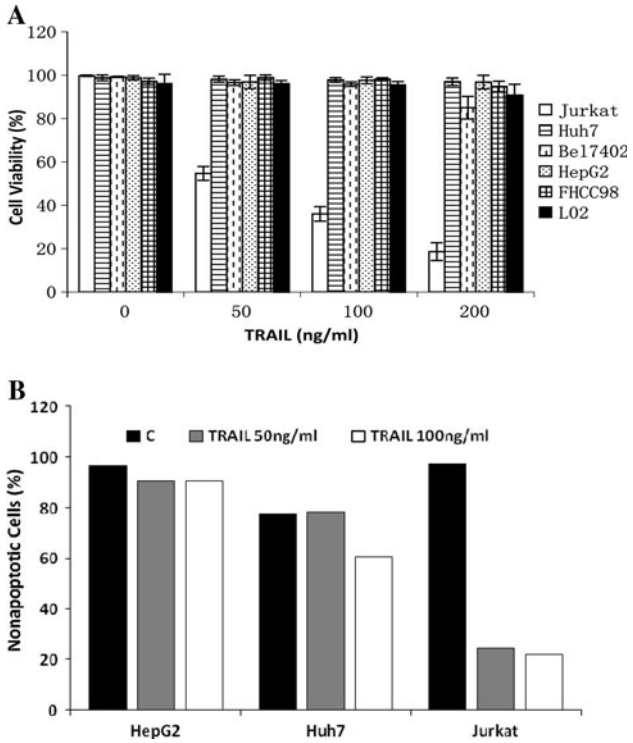

Fig. 1 Failure of TRAIL to induce apoptosis in hepatoma cell lines. **a** Cell viability assays after treatment with TRAIL for 24 h. Four HCC cell lines (Huh7, BEL7402, HepG2 and FHCC98) and one normal liver cell line (L02) were treated with 50, 100 and 200 ng ml^{-1} TRAIL for 24 h, with Jurkat cells used as positive controls. Cell viability was evaluated using trypan blue exclusion analysis [11, 28]. Data are presented as mean ± SD ($n \geq 4$). **b** Apoptosis was assessed using Annexin V-FITC and PI staining via flow cytometric analysis of apoptosis after treatment with TRAIL (50 and 100 ng ml^{-1}) for 24 h

Results

Hepatocellular cancer cells exhibit high resistance to TRAIL-induced apoptosis

The sensitivity of HCC cell lines to TRAIL was investigated using the four liver cancer cell lines, Huh7, BEL7402, HepG2 and FHCC98, with Jurkat cells used as a positive control [22]. Cells were treated with an increasing dose of recombinant human TRAIL (rhTRAIL). The results demonstrate that a relatively high concentration of TRAIL (200 ng ml^{-1}) could not induce significant cell death in the four liver cancer cell lines after 24 h treatments but was able to reduce the viability of Jurkat cells in a dose-dependent manner (Fig. 1a). The percentage of Jurkat cell death was greater than 80% when exposed to 200 ng ml^{-1} of TRAIL. However, the four liver cancer cell lines maintained cell survival upon TRAIL treatment, with cell viabilities consistently above 90%, except for the BEL7402 cell line, which decreased to 85% in the presence of 200 ng ml TRAIL. The result showed that TRAIL failed to induce hepatic cancer cell apoptosis as a potential chemotherapeutic agent. Next, we use a normal liver cell line L02 to detect the cytotoxicity of TRAIL on normal cell. Actually, L02 cell line was obtained by transforming SV40 T antigen into normal liver cell to establish the immortal ability. So, even though there are some different features to real normal liver cell, we can generally consider L02 cell line normal liver cells. There are some authors have regarded L02 as normal control cell line [17]. It can be drawn from Fig. 1a that TRAIL had no effect on L02, indicating that TRAIL has the ability of selective cytotoxicity to some types of tumors. Flow cytometry analysis results agreed with the trypan blue results (Fig. 1b). Thus, TRAIL failed to induce apoptosis both in hepatoma cell lines and in normal liver cells.

The Bcl-2 family inhibitor ABT-263 potentiates the cytotoxic effect of TRAIL to cancer cells

As an inhibitor of the anti-apoptotic Bcl-2 family members, ABT-263 induced apoptosis in tumor cells, both as a single agent and as part of a synergistic regimen [1–3, 30]. In this study, ABT-263 was utilized in combination with TRAIL to treat liver cancer cells. After 24 h of treatment, ABT-263

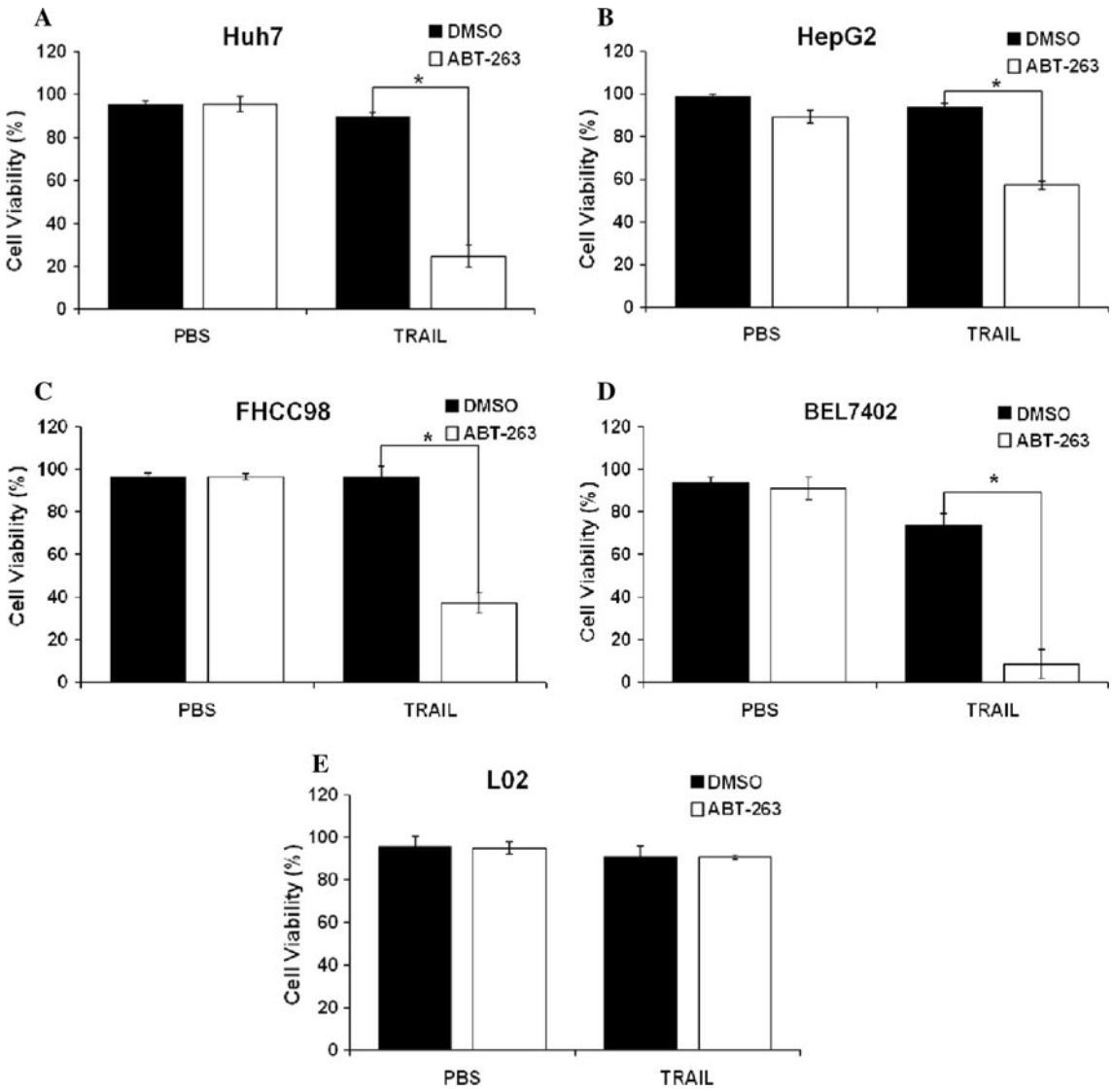

Fig. 2 ABT-263 potentiates TRAIL-induced apoptosis in HCC cells. TRAIL-resistant HCC cells **a** Huh7, **b** HepG2, **c** FHCC98, **d** BEL7402 and **e** normal liver cells L02 were treated with ABT-263 (0.9 μM), TRAIL (200 ng ml^{-1}) or TRAIL (200 ng ml^{-1}) + ABT-263 (0.9 μM) for 24 h. Cell viability was evaluated using a trypan blue exclusion experiment. Data are presented as mean ± SD, ($n \geq 4$). *bars*, SD. *$P < 0.05$

promoted TRAIL-induced cell death in hepatic cancer cells. However, neither of the two agents alone was able to induce cell death significantly (Fig. 2a–d). HepG2 and FHCC98 were more resistant to the combination treatment, with 57.2 and 37.3% cell survival rate, respectively. ABT-263 strongly promoted TRAIL-induced cytotoxicity in BEL7402 and Huh7, as the cell viability decreased to nearly 20% in both cell lines. Importantly, treatment with the two agents alone or in combination did not have a toxic effect on the L02 normal liver cell line, suggesting that the combination regimen would be a safe therapy for hepatocellular carcinoma (Fig. 2e). Therefore, a synergistic chemotherapeutic regimen for TRAIL in the treatment for human hepatocellular carcinoma, while sparing normal liver, was demonstrated.

Treatment with ABT-263 in combination with TRAIL-activated caspases

To determine the molecular mechanism of the synergistic effect of TRAIL and ABT-263 to the HCC cells, Western blot analysis was used to detect proteins of specific apoptotic pathways in Huh7 and BEL7402 cell lines after treatment with ABT-263 and TRAIL for 24 h. The results indicated that PARP, an apoptotic protein marker, was cleaved upon treatment (Fig. 3), which further confirmed that cells underwent apoptosis. Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis and inflammation [25]. We found that (Fig. 3) pro-caspase-3, pro-caspase-8 and caspase-9 were activated after treatment with ABT-263 and TRAIL for 24 h. These data suggest that

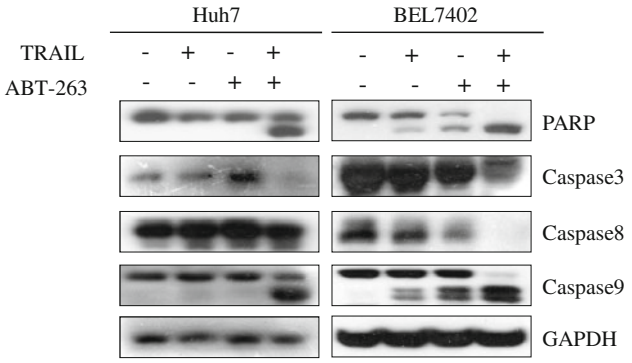

Fig. 3 ABT-263 treatment in combination with TRAIL activates caspases. Western blot analysis of PARP and pro-caspase-3, pro-caspase-8, and caspase-9 after treatment with ABT-263 (0.9 μM), TRAIL (200 ng ml^{-1}) or TRAIL (200 ng ml^{-1}) + ABT-263 (0.9 μM) for 24 h. GAPDH was used as loading control

ABT-263 is capable of sensitizing HCC cells to undergo TRAIL-induced apoptosis.

ABT-263 sensitizes HCC cells through inhibition of the pro-survival Bcl-2 family members

The level of the TRAIL receptor DR5 and the intracellular anti-apoptotic molecule c-FLIP(L) are closely associated with the sensitivity of TRAIL-induced apoptosis [6, 7, 16, 18, 32]. However, treatment of Huh7 and BEL7402 cells with TRAIL and/or ABT-263 did not alter the protein levels of the DR5 and c-FLIP(L) (Fig. 4a), indicating that DR5 and c-FLIP(L) did not influence the sensitization of hepatoma cells to TRAIL via ABT-263.

Members of the Bcl-2 family of proteins are critical regulators of the intrinsic apoptosis pathway. Thus, we determined whether ABT-263, a Bcl-2 family inhibitor, altered the sensitivity of HCC cells to TRAIL through activation of Bcl-2 family members. As shown in Fig. 4b, we found that select anti-apoptotic Bcl-2 family members, especially Mcl-1 and Bcl-xL, were downregulated when the Huh7 and BEL7402 cells were treated with TRAIL in combination with ABT-263. Therefore, decreased expression of select members of the Bcl-2 family may be essential in the regeneration of TRAIL-sensitivity of HCC cell lines via ABT-263.

HBx sensitizes TRAIL-treated HCC cells

Studies have demonstrated a relationship between chronic infection of hepatitis B virus (HBV) and the occurrence of hepatocellular carcinoma. Moreover, the hepatitis B Virus X protein (HBx) is essential for the activation of many vital signaling pathways in hepatoma cells, as well as in regulating the sensitivity of cells to chemotherapy. However, the

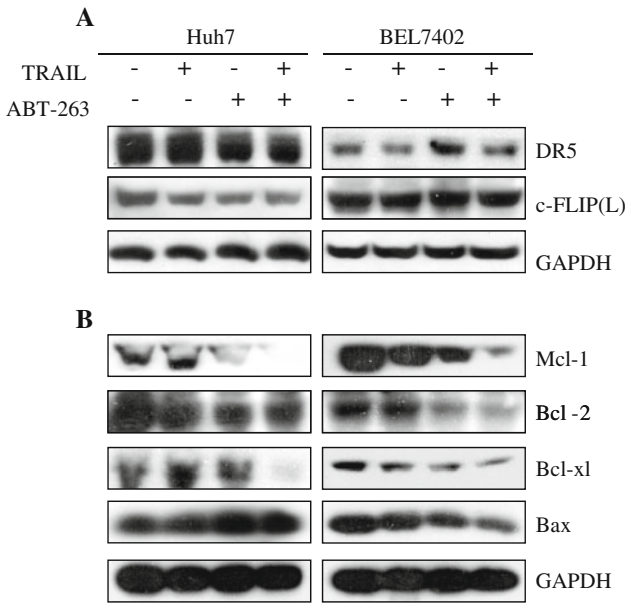

Fig. 4 ABT-263 sensitizes HCC cells through inhibition of the pro-survival Bcl-2 family members. The expression of **a** DR5 and c-FLIP(L), and **b** the pro-survival Bcl-2 family proteins members Bcl-2, Bcl-xL, Mcl-1 and pro-apoptosis member Bax were examined using Western blot analysis after treatment with ABT-263 (0.9 μM), TRAIL (200 ng ml^{-1}) or TRAIL (200 ng ml^{-1}) + ABT-263 (0.9 μM) for 24 h. GAPDH was used as loading control

role of HBx in sensitizing cancer cells to TRAIL-induced apoptosis is controversial [8, 26, 34]. After 48 h of transfection, Huh7 cells transfected with pcDNA3.1-HBx had a greater expression of HBx compared with Huh7 cells transfected with vector pcDNA3.1 (Fig. 5a). Thus, we treated HBX- and vehicle-transfected Huh7 cells with 200 ng ml^{-1} TRAIL. The results indicated that, compared with the vehicle-transfected cells, Huh7 cells that overexpress HBx are more sensitive to the TRAIL treatment, with cell viability sharply decreasing to 48.8% in the HBx-transfected cells (Fig. 5b). Therefore, these results indicated that HBx has the potential to sensitize TRAIL-resistant hepatocellular carcinoma cells to undergo apoptosis upon TRAIL treatment.

Discussion

TRAIL is a promising anticancer drug, first discovered in the 1990s [5, 31], as it can induce apoptosis in transformed cancer cells while sparing most normal human cells. Unfortunately, many cancer cells develop mechanisms that overcome TRAIL-induced cell death. Many factors contribute to the development of cancer cell resistance to TRAIL. For example, death receptors and c-FLIP(L) influence the extrinsic apoptosis cascade triggered by TRAIL [7, 32].

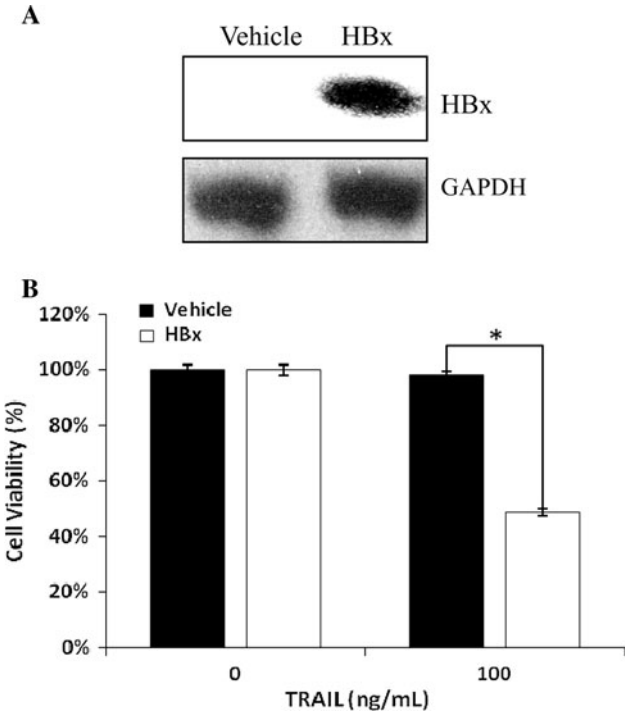

Fig. 5 HBx promotes HCC cell apoptosis with treated TRAIL. **a** HBx protein level in Huh7 cells as assayed using Western blot after cells were transfected with vehicle and pcDNA3.1-HBx for 48 h. **b** Cell viability of Huh7 cells transfected with vehicle and pcDNA3.1-HBx for 48 h followed by treatment with ABT-263 (0.9 μM), TRAIL (200 ng ml^{-1}) or TRAIL (200 ng ml^{-1}) + ABT-263 (0.9 μM) for 24 h. Cell viability was evaluated using trypan blue exclusion. Data are presented as mean ± SD ($n \geq 4$). *Bars*, SD. *$P < 0.05$

Previously, we have demonstrated that c-FLIP(L) is the major component that sustains resistance to TRAIL for prostate cancer and that the c-Fos/c-Jun heterodimer binds to the promoter region of c-FLIP(L) and represses its expression [18].

Many researchers have found that combining TRAIL with other anticancer agents contributes to the sensitization of cancer cells to it, thus overriding cancer cell resistance to this agent. Baicalein, a naturally occurring flavonoid, promotes DR5 expression in an ROS-dependent or independent pathway and thus sensitizes cancer cells to TRAIL [29]. Additionally, previous studies reported by we and others demonstrated that the proteasomal inhibitors MG132 and bortezomib promote TRAIL-induced apoptosis [18, 20]. The Bcl-2 anti-apoptotic family members, such as Bcl-2, Bcl-xL and Mcl-1, are critical in the regulation of the release of Cytochrome c from mitochondria and thus influence the apoptosis process [35]. This led to the analysis of combining combination of Bcl-2 family inhibitors and TRAIL. In our study, we demonstrate that the combination of ABT-263 and TRAIL induced potent extrinsic and intrinsic apoptosis in HCC cell lines.

The clinical prospect of ABT-737 is impaired by its poor physiochemical and pharmaceutical characteristics [12, 27]. However, a second-generation BH3 mimic, termed ABT-263, has been discovered, and like its precursor, it can bind to Bcl-2, Bcl-xL and Bcl-w with high affinity, promoting apoptosis [30]. Moreover, due to its orally bioavailability, ABT-263 has a better clinical prospect either as a single anticancer drug or in combination use [1, 2]. Actually, BEL7402 cell is more sensitive than Huh7 when treated with TRAIL alone or combination with ABT263. In Fig. 3, TRAIL alone can induce weak PARP cleavage and the Western blot results are coincidence with the data of cell viability (Fig. 2d). This reflects the different HCC cell lines is not the same sensitivity to TRAIL-induced cell death. Furthermore, our findings suggest that the combination of ABT-263 and TRAIL induces HCC cells apoptosis through regulation of the extrinsic and intrinsic apoptotic pathways.

In summary, our study demonstrated that ABT-263 decreased the expression of the anti-apoptotic Bcl-2 family proteins and contributed to the enhancement of TRAIL-induced apoptosis in HCC cells. Moreover, the HBx gene renders the liver cancer cell Huh7 responsive to TRAIL treatment. Our findings provide a novel insight into the clinical application of TRAIL-induced apoptosis in human cancer cells.

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