

# ABT-737, a Bcl-2 family inhibitor, has a synergistic effect with apoptosis by inducing urothelial carcinoma cell necroptosis

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**Abstract.** ABT-737 is a recently reported inhibitor of members of the Bcl-2 family of apoptosis regulators. However, to the best of our knowledge, its necroptosis-inducing function in bladder cancer has not yet been researched. Thus, the present study aimed to investigate whether this Bcl-2 family inhibitor can induce both apoptosis and necroptosis of urothelial carcinoma cells. The proliferation and survival of urothelial carcinoma cell lines treated with a combination of both Z-VAD-FMK as a pan-caspase inhibitor and ABT-737 were assessed in vitro. Z-DNA binding protein 1 (ZBP1), receptor-interacting protein (RIP)1 and RIP3 were knocked down using small interfering RNA in urothelial carcinoma cell lines. The protein expression levels of ZBP1, RIP1 and RIP3 following cell transfection were measured via western blot analysis. Cell viability was determined using an MTT assay. Cell invasion was examined using cell invasion assays. The expression levels of necroptosis-related proteins, high mobility group box 1, ZBP1, mixed-lineage kinase domain-like protein (MLKL) and RIP3, were measured via western blotting. It was found that ABT-737 inhibited the proliferation and invasion of bladder cancer cells by inducing cell necrosis. The data demonstrated that ZBP1 and RIP3 have main roles in the cell necrosis induced by ABT-737. In addition, RIP3 and ZBP1, without interacting with RIP1, directly induced MLKL-mediated programmed cell necrosis. Thus, understanding how urothelial carcinoma cells react to Bcl-2 family inhibitors may accelerate the discovery of drugs to treat bladder cancer.

## Introduction

In the United States and Europe, bladder cancer is the second most common urological malignancy and is the eighth leading cause of all cancer-related mortalities in humans (1). Moreover, men are four times more likely than women to

develop bladder cancer (2). Urothelial cell carcinoma (UCC) is the most common form of this neoplasm, accounting for 90% of bladder cancer (2,3). Due to the high recurrence rate, the treatment of bladder cancer is difficult and requires long-term follow-up (4). Thus, bladder cancer is one of the most expensive cancer types to treat, costing \$4 billion in healthcare costs in the US alone (1).

Apoptosis and necrosis are two pathologically relevant types of cell death. Apoptosis is a type of programmed cell death that is tightly controlled during development and in physiological cellular turnover, while necrosis is considered to occur predominantly in an uncontrolled manner (5,6). The initiator caspase that controls the intrinsic pathway of apoptosis is caspase 9, which is able to bind to adapter protein apoptotic protease activating factor 1 (APAF1) following exposure of its caspase recruitment domain (6). The extrinsic pathway, also known as the death receptor (DR) pathway of apoptosis (7), is initiated by patrolling natural killer cells or macrophages when they produce death ligands, which upon binding with DRs in the target cell membrane, induces the extrinsic pathway via the activation of procaspase 8 to caspase 8 (8). DRs are members of the TNF superfamily and include several members such as tumor necrosis factor receptor 1 and nerve growth factor receptor. DRs activate caspase 8, resulting in recruitment of monomeric procaspase 8 via its death-inducing domain to a death-inducing signal complex (DISC) located on the cytoplasmic domain of the ligand-bound DR (9). The DISC also includes either an adaptor protein, known as the FAS-associated death domain (FADD), or a TNF receptor-associated death domain (8).

Necroptosis involves the loss of membrane integrity and the release of damage-associated molecular pattern molecules, and is therefore closely associated with the inflammatory response (10). Necroptosis involves the activation of specific death mediators, such as receptor-interacting protein (RIP) kinases and mixed-lineage kinase domain-like protein (MLKL) (11,12). CYLD lysine 63 deubiquitinase is essential for necrosis and serves as a target for proteolysis by caspase-8 (13). When caspase-8 is inactivated or absent, RIP1 and RIP3 are not cleaved and become phosphorylated. RIP1 then recruits RIP3 via RIP homotypic interaction motif (RHIM) domain-mediated interactions (14). RIPK3 is reported to activate a number of different downstream signals, such as phosphoglycerate mutase and dynamin-related protein (Drp1), to induce reactive oxygen species production in the mitochondria (11,12). These

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DRs are considered to be TNF-related apoptosis-inducing ligand receptors that can induce apoptosis in cancer cells (12). A death-inducing signaling complex is formed at the DR (15). Moreover, FADD, DRs and procaspase-8 are the constituents of the multicomponent machinery formed and involved in the activation of caspase-8 in bladder cancer cells (15).

Bcl-2 is an anti-apoptotic protein member of the Bcl-2 family. ABT-737 is a Bcl-2 homology (BH)3-mimetic drug, and an inhibitor of Bcl-2, Bcl-xL and Bcl-w (16). ABT-737 competes with Bim to bind Bcl-2, leading to the release of Bim and triggering Bax/Bak-mediated apoptosis (16). There are four distinct BH domains in mammalian cells, including BH1, BH2, BH3 and BH4 (17). Programmed cell death, or apoptosis, is controlled by a number of proteins, among which are members of the widely expressed Bcl-2 family (17). The BH3 domain promotes Bcl-2 family member dimerization. Homodimerization of Bcl-2 involves a head-to-tail interaction in which the N-terminal region interacts with the more distal region of Bcl-2 (18). Previous studies have reported ABT-737 antitumor effects in HCC cell lines and its use as a treatment for patients with leukemia in clinical trials (19). Bcl-2 upregulation has been detected in a variety of human cancer types, including bladder cancer (20). Bcl-2 also serves a role in regulating normal cellular proliferation and occupies a critical position in the biochemical pathways important for the transduction of mitogenic signals from a variety of growth factor receptors in bladder cancer (21).

Therefore, the present study was performed to investigate the antitumor effects of ABT-737 (an inhibitor of Bcl-2) on urothelial carcinoma cells and its potential mechanisms in necroptosis. The principal objective of this study was to determine whether ABT-737 inhibits the proliferation and invasion of bladder cancer cells. In total, two types of human urothelial carcinoma cell lines, UMUC3 and 5637 cells, were used in this study. These are the most widely used human urothelial carcinoma cell in the research field. The stem line modal chromosome number was 67 occurring at 36% in the UMUC3, and the modal chromosome number was 80, occurring in 42% in 5637 cells (21). Z-VAD-FMK is an irreversible caspase inhibitor (22) with no cytotoxic effects and is used for studying the potential mechanisms of ABT-737 that reduce caspase activity and promote necroptosis (23). Based on these results, the second objective of the current study was to determine whether RIP1/ZBP1/RIP3 activation regulates urothelial carcinoma cell necrosis; therefore, ZBP1 small interfering (si)RNA, RIP1 siRNA and RIP3 siRNA were used to knock down the target proteins and determine their potential roles in necroptosis. The summary of the experimental design is presented in Table I.

## Materials and methods

**Cell culture.** In total, two types of human urothelial carcinoma cell lines from American Type Culture Collection, UMUC3 and 5637, were cultured in RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Ameresco, Inc.) at 37°C in 5% CO<sub>2</sub>. For special treatment, ABT-737 or Z-VAD-FMK (R&D Systems, Inc.) were used for cell culture. UMUC3 and 5637

human bladder cancer cell lines treated with ABT-737 at 2.5, 5, 10, 20 and 40  $\mu$ mol/l for 12 h before cell viability assay.

**Transfection assay.** siRNA sequences targeting human Z-DNA binding protein 1 (ZBP1; Gene ID:81030), RIP1 (cat. no. 856689) and RIP3 (cat. no. 824503) were constructed by Shanghai GeneChem Co., Ltd. Transfections with the siRNAs were as follows: 5637 and UMUC3 cells were plated into 6-well plates upon reaching 60-70% confluency and transfected the next day with 4  $\mu$ g each siRNA, using 10  $\mu$ l Lipofectamine<sup>®</sup> 2000 (1  $\mu$ g/ $\mu$ l; Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C, for 4 h. Cells were harvested 48 h after transfection and whole cell lysates were isolated for western blotting.

**Reverse transcription-quantitative (RT-qPCR).** Total RNA was isolated from  $\sim 1 \times 10^6$  cultured cells using the RNagents in a total RNA isolation system (Promega Corporation) and was measured using a NanoDrop-1000 system (Thermo Fisher Scientific, Inc.). Then, 10  $\mu$ g total RNA was reverse-transcribed to single-stranded cDNA by using SuperScript<sup>™</sup> IV first-strand cDNA synthesis reaction kit (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 30 min. RT-qPCR was performed in 20  $\mu$ l reaction volumes using 50 ng cDNA. The primer pair sequences were as follows: High mobility group box 1 (HMGB1), forward: 5'-GAGCCACCACTCACCTACT-3' and reverse: 5'-CCAGGCATTCGGCAATGTG-3'; ZBP1, forward: 5'-GCTTTGCTGCGTACTTCCA-3' and reverse: 5'-GTCCACACGGGTTCCAGA-3'; RIP3, forward: 5'-GGCTTCGACACCCGTGTAA-3' and 5'-CGTCAAACCTCTTGTCATCCA-3'; MLKL, forward: 5'-GTAGAGGACACGGCAAGAT-3' and reverse: 5'-TTCACGAACTGTCAACTGCAC-3'; and cytochrome (Cyt)-C, forward: 5'-TTGACCTACGTGGCTTGGAAG-3' and reverse: 5'-GGTAACGGAATCGGGCTGAAT-3'. The thermocycling conditions were as follows: Initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; followed by a final extension at 72°C for 2 min using a MJ Mini Personal thermocycler (Bio-Rad Laboratories, Inc.) with the same primers.  $\beta$ -actin 5'-CTCCATCCTGGCCTCGCTGT-3' and 5'-GCTGTCACCTTCACCGTTCC-3' was as the internal control. The expression levels of genes were determined with SYBR-Green fluorophore using the RT2 SYBR-Green Master Mix (SABiosciences; Qiagen GmbH). Relative quantification was performed using the 2<sup>- $\Delta\Delta C_q$</sup>  method (24,25).  $\beta$ -actin was used as the endogenous reference gene for all experiments.

**Western blot analysis.** Cells were lysed with lysis buffer (BD Biosciences) and incubated on ice for 30 min. The cell lysates were centrifuged at 12,000  $\times$  g (10 min; 4°C). The concentration of the protein was quantified with a Pierce BCA kit (Thermo Fisher Scientific, Inc.) using a NanoDrop-1000 system (Thermo Fisher Scientific, Inc.). The protein samples (50  $\mu$ g) were separated on 8% polyacrylamide gels and transferred onto PVDF membranes, followed by blocking with 5% non-fat milk in buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl and 0.1% Tween-20] room temperature for 1 h. The membranes were probed with for 1 h at room temperature and incubated overnight at 4°C with each of the primary antibodies: Anti-HMGB1

Table I. A comprehensive summary of experimental design (including Figs. 1-6 experiments).

Group	Cell type	Treatment
Fig. 1		
Control group	WT	N/A
Experimental group	WT	ABT-737
Fig. 2		
Control group	WT	N/A
Experimental group	WT	ABT-737 + Z-VAD-FMK
Fig. 3A		
Control group	WT	siRNA-NC
Experimental group	WT	RIP1 siRNA
Fig. 3B		
Control group	RIP1 knockdown	N/A
Experimental group	RIP1 knockdown	ABT-737 + Z-VAD-FMK
Fig. 4A		
Control group	WT	siRNA-NC
Experimental group	WT	ZBP1 siRNA
Fig. 4B		
Control group	ZBP1 siRNA	N/A
Experimental group	ZBP1 siRNA	ABT-737 + Z-VAD-FMK
Fig. 5A		
Control group	WT	siRNA-NC
Experimental group	WT	RIP3 siRNA
Fig. 5B		
Control group	RIP3 knockdown	N/A
Experimental group	RIP3 knockdown	ABT-737 + Z-VAD-FMK
Fig. 6		
Experimental group	WT	ABT-737 + Z-VAD-FMK

WT, wild-type; siRNA, small interfering RNA; NC, negative control; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein.

(cat. no. MA5-17278; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.), anti-ZBP1 (cat. no. AF6309; 1:1,000; R&D Systems, Inc.), anti-RIP3 (cat. no. MAB7604; 1:1,000; R&D System, Inc.), anti-RIP1 (cat. no. MAB3585; 1:1,000; R&D Systems, Inc.), anti-MLKL (cat. no. MAB9187; 1:1,000; R&D Systems, Inc.) and anti-GAPDH (cat. no. 2275-PC-100; 1:5,000; R&D Systems, Inc.). On the second day, the membranes were washed with TBS-Tween-20 (1%) and then incubated with anti-rabbit-HRP (cat. no. CTS005; 1:5,000; R&D Systems, Inc.) or anti-mouse-HRP antibody (cat. no. CTS002; 1:5,000; R&D Systems, Inc.) or 1 h at room temperature. Immunodetection was performed using ECL reagents (Thermo Fisher Scientific, Inc.). Images were captured using a Syngene Bio Imaging system (Synoptics Ltd.). Densitometry was analyzed with Empiria Studio® Software (9141-500E) <https://www.licor.com/bio/empiria-studio/resources>. The results were presented as the mean  $\pm$  SD of three independent experiments.

**Co-immunoprecipitation.** For whole-cell extracts, two 10-cm dishes of 5637 and UMUC3 cells (at 80% confluency) were washed with cold PBS and then solubilized for 30 min on

ice in 1% NP-40 lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1% NP40 and 0.02%  $\text{NaN}_3$ ) with proteinase cocktail inhibitor (Cell Signaling Technology, Inc.) and 1 mM PMSF (Sigma-Aldrich; Merck KGaA). Cells were then centrifuged for 10 min at room temperature as  $16,000 \times g$  to remove the precipitate, and 20  $\mu\text{l}$  supernatant was reserved for subsequent analysis. Then, 1  $\mu\text{g}$  primary antibodies (anti-ZBP1; cat. no. AF6309; 1:1,000; R&D Systems, Inc., anti-RIP3; cat. no. MAB7604; 1:1,000; R&D System, Inc.) or normal control IgG were incubated with 50  $\mu\text{l}$  Dynabeads (R&D Systems, Inc.) at room temperature for 10 min. The remaining supernatant was then incubated with antibody-coated Dynabeads at 4°C overnight. The medium was collected and washed four times with cold washing solution. The medium was resuspended in 1X SDS loading buffer and boiled, and the precipitate was removed via centrifugation at  $112 \times g$  at 4°C for 20 min to obtain the protein sample. The protein samples were separated by SDS-PAGE (8% gel) and transferred to PVDF membranes. Anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, Inc.).

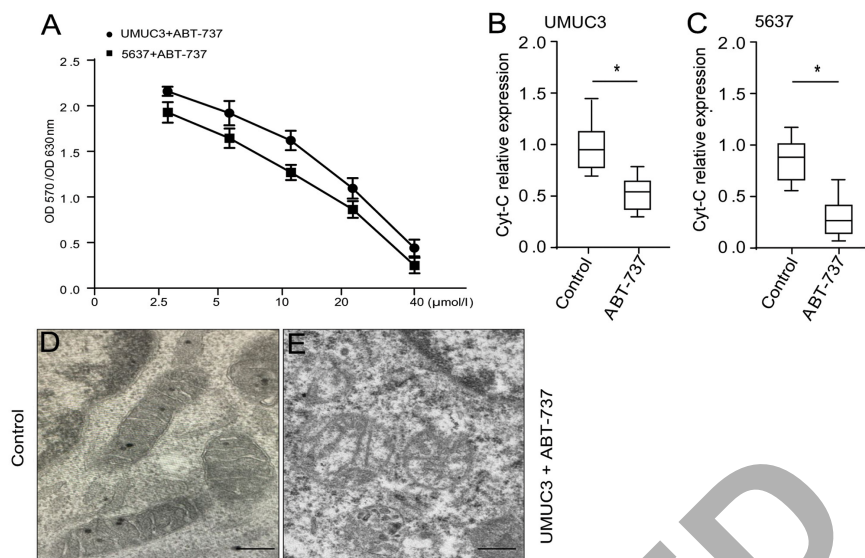


Figure 1. ABT-737 inhibits the proliferation of bladder cancer cells, which promotes mitochondrial injury. (A) MTT assays were performed to examine the viability of UMUC3 and 5637 cells treated for 12 h with various concentrations of ABT-737. Expression levels of cytosolic Cyt-C genes in mitochondria of (B) UMUC3 and (C) 5637 cells. (D and E) Representative images of transmission electron microscopy photomicrographs of UMUC3 treated with ABT-737 for 12 h (scale bar=0.5 μm). \*P<0.05. Cyt-C, cytochrome C; OD, optical density.

*Cell viability was detected using a MTT assay.* 5637 and UMUC3 cells were seeded in 96-well plates at 10,000 cells per well and incubated for 24 h at 37°C in 5% CO<sub>2</sub>. The cells were treated with caspase inhibitor Z-VAD-FMK (1 μmol/l, Bio-Techne) combined with ABT-737 (5 μmol/l) at 37°C for 12 h. To measure cell viability, after treatment, MTT (20 μl) was added to each well and incubated for 4 h at 37°C at 37°C in 5% CO<sub>2</sub>. Then, 150 μl DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance was measured at 570 nm using an automated microplate reader (Bio-Rad Laboratories, Inc.).

*Matrigel invasion assay.* Invasion assays were performed in triplicate in 6-well Transwell units with 8 μm filters coated with Matrigel (BD Biosciences) at 1:6 dilution at 37°C in 5% CO<sub>2</sub>. Each well was loaded with 2x10<sup>6</sup> cells with culture medium (containing 2% FBS) into the upper chambers and total culture medium (with 10% FBS) into the lower chambers. After incubation for 36 h at 37°C in 5% CO<sub>2</sub>, cells passing through the filters into bottom wells were fixed in formalin (10%) at room temperature for 2 min and stained with crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 10 min. Cell numbers in 10 randomly selected fields (magnification, x200) from each well were counted by a phase contrast microscope (Olympus Corporation).

*Electron microscopy.* UMUC3 cells treated with ABT-737 and control specimens were rinsed in PBS and then post-fixed in cacodylate-buffered 1% osmium tetroxide solution at 4°C for 2 h, dehydrated in an ethanol series (30,50,70,80, 90 and 100%) and embedded in Poly/Bed 812. Cells were immersed immediately in fixative (3.0% glutaraldehyde buffered in 0.1 M sodium cacodylate, pH 7.2) overnight at 4°C. Cells were randomly selected for analyses without prior knowledge of genotype. Sections were stained at room temperature for four hours and observed under a transmission electron microscope

(magnification, x200,000; HT7700; Hitachi, Ltd.) and statistical analyses were determined using GraphPad Prism 7 (GraphPad Software, Inc.).

*Statistical analysis.* The sample size for each experiment was determined based on a previous study (8). Data are presented as the mean ± SD. Statistical analyses were conducted with GraphPad Prism 7 (GraphPad Software, Inc.) and an unpaired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

## Results

*ABT-737 inhibits the proliferation of bladder cancer cells, which promotes mitochondrial injury.* An MTT assay was used to detect the growth inhibition of UMUC3 and 5637 human bladder cancer cell lines treated with ABT-737 at 2.5, 5, 10, 20 and 40 μmol/l for 12 h. The growth inhibition rates of the treated UMUC3 cells were 5.6, 23.5, 31.2, 55.8 and 72.7%, respectively. The growth inhibition rates of the treated 5637 cells were 4.7, 20.6, 35.7, 51.2 and 79.6%, respectively. ABT-737 significantly inhibited the proliferation of the UMUC3 and 5637 cells in a concentration-dependent manner (Fig. 1A). Mitochondrial gene RT-qPCR analysis demonstrated that the mitochondrial Cyt-C content in the treated group was significantly decreased (Fig. 1B and C) compared with that of the control group. Transmission electron microscopy observations of the UMUC3 cells treated with ABT-737 in culture revealed that mitochondrial morphology was markedly damaged compared with that of the control (Fig. 1D and E). These data suggested that ABT-737 promoted mitochondrial injury and caused the outflow of Cyt-C from bladder cancer cells.

*ABT-737 inhibits the proliferation and invasion of bladder cancer cells by inducing cell necrosis.* Z-VAD-FMK is an



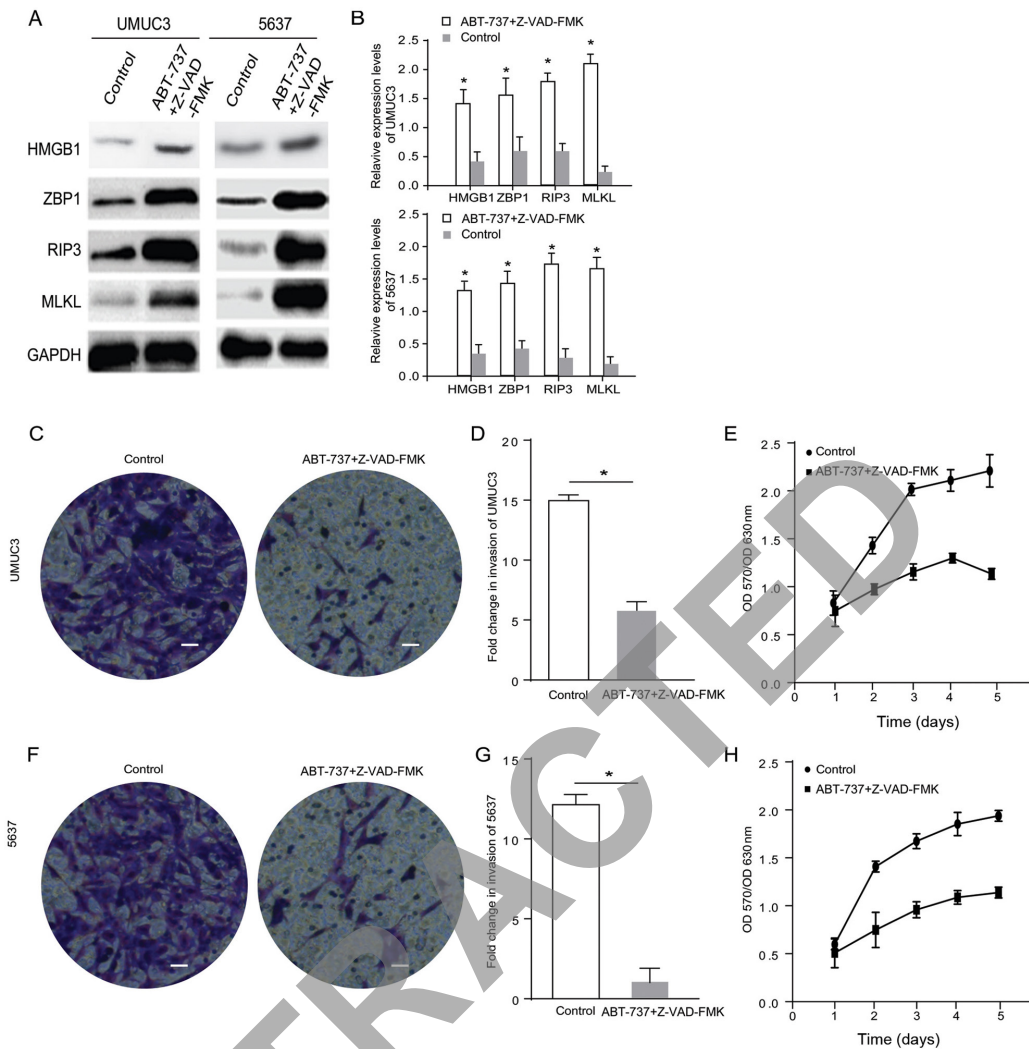


Figure 2. ABT-737 inhibits the viability and invasion of bladder cancer cells by inducing cell necrosis. (A) Expression levels of HMGB1, ZBP1, RIP3 and MLKL were analyzed via western blotting and the results were (B) semi-quantified. (C) Transwell invasion assays were performed with UMUC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (D) Quantification of Transwell assay results (E) MTT assays were performed to examine the viability of UMUC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (F) Transwell invasion assays were performed with 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (G) Quantification of Transwell assay results (H) MTT assays were performed to examine the viability of 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (scale bar=0.5  $\mu$ m). \*P<0.05 vs. control. OD, optical density; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein; HMGB1, high mobility group box 1; MLKL, mixed-lineage kinase domain-like protein.

irreversible caspase inhibitor (11) with no cytotoxic effects and is used for studying the potential mechanisms of ABT-737 that reduce caspase activity and promote necroptosis (12). Based on the results of proliferation experiments and relevant literature, the caspase inhibitor Z-VAD-FMK (1  $\mu$ mol/l) combined with ABT-737 (5  $\mu$ mol/l) was used to treat UMUC3 and 5637 cells for 12 h and then the expression levels of necrosis-related proteins HMGB1, MLKL and RIP3, and the nucleic acid sensing protein ZBP1 were detected (Fig. 2A). The semi-quantifications obtained via western blot analysis indicated that the relative expression levels of HMGB1, ZBP1, MLKL and RIP3 were three-fold higher in the ABT-737/Z-VAD-FMK treatment group compared with the blank control group 12 h after treatment (Fig. 2B).

MTT assays and cell invasion assays were used to measure the effects of ABT-737/Z-VAD-FMK on the invasive ability and proliferation of the UMUC3 and 5637 cell lines. Compared with the control group, the proliferation (Fig. 2D and G) was notably decreased. The invasive ability

of the UMUC3 and 5637 cells after treatment (Fig. 2C and F, quantified in E and H) was significantly decreased compared with that of the control group. These results indicated that ABT-737 inhibited cell proliferation and invasion by inducing cell necrosis.

*ABT-737 induces cancer cell necrosis and inhibits cell proliferation and invasion when the RIP1 gene is knocked down in bladder cancer cells.* RIP1 is an important protein associated with cancer cell necrosis (16). The current study knocked down RIP1 in the UMUC3 and 5637 cell lines using siRNA. The western blot analysis results demonstrated that the RIP1 expression level was significantly reduced after cell transfection (Fig. 3A and B). The expression levels of the necrosis-related proteins HMGB1, RIP3, MLKL and ZBP1 were detected in the RIP1-knockdown cells treated with ABT-737/Z-VAD (Fig. 3C). The results indicated that the expression levels of HMGB1, ZBP1, MLKL and RIP3 were significantly (2-fold) highly expressed in the RIP1-knockdown

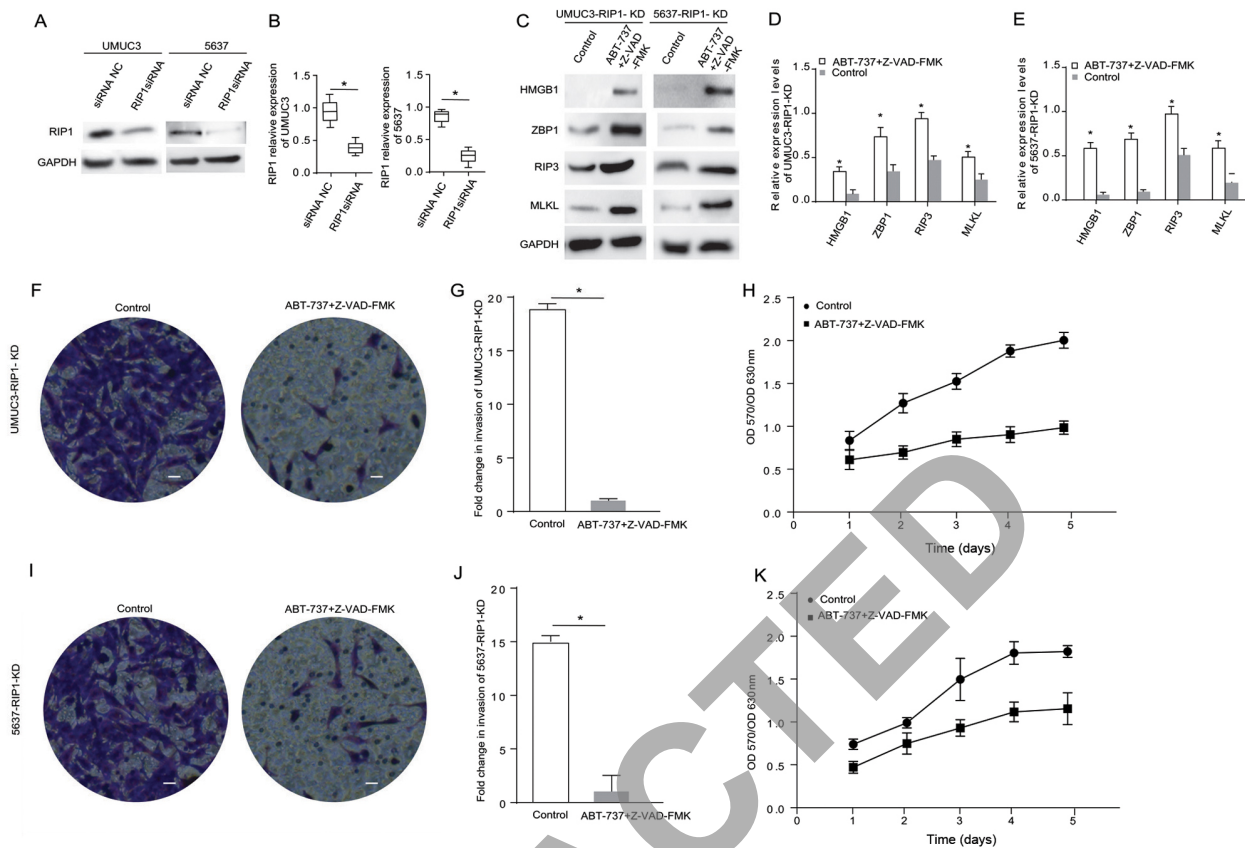


Figure 3. ABT-737 induces cancer cell necrosis and inhibits cell viability and invasion when the RIP1 gene is knocked down in bladder cancer cells. (A) Expression levels of RIP1 in UMC3 and 5637 cells were analyzed via western blotting after treatment with RIP1 siRNA for 12 h, and the results were (B) semi-quantified. (C) Protein expression levels of HMGB1, ZBP1, RIP3 and MLKL were analyzed in RIP1-KD UMC3 and RIP1-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. The results were semi-quantified in (D) UMC3 and (E) 5637 cells. (F) Transwell invasion assays were performed with RIP1-KD UMC3 cells. (G) Quantification of Transwell assay results (H) MTT assays were performed to examine the viability of RIP1-KD UMC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (I) Transwell invasion assays were performed with RIP1-KD 5637 cells. (J) Quantification of Transwell assay results. (K) MTT assays were performed to examine the viability of RIP1-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. \* $P < 0.05$  vs. control or siRNA NC. OD, optical density; scale bar = 50  $\mu$ m; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein 1; HMGB1, high mobility group box 1; MLKL, mixed-lineage kinase domain-like protein; siRNA, small interfering RNA; NC, negative control; KD, knockdown.

5637 cells and RIP1-knockdown UMC3 cells treated with ABT-737/Z-VAD (Fig. 3D and E). Transwell and MTT assays identified that, compared with the blank control group cells, the proliferative and invasive abilities of the RIP1-knockdown UMC3 cells (Fig. 3F-H) and the RIP1-knockdown 5637 cells (Fig. 3I-K) were significantly decreased after ABT-737/Z-VAD treatment. These data suggested that RIP1 did not affect the induction of cell necrosis induced by ABT-737 treatment.

**ABT-737 induces cancer cell necrosis and inhibits cell proliferation and invasion when the ZBP1 gene is knocked down in bladder cancer cells.** ZBP1 is another important protein in the Bcl-2 pathway associated with cancer cell necrosis (17). Therefore, ZBP1 was also knocked down in UMC3 and 5637 cells using siRNA. The western blot analysis results demonstrated that the ZBP1 expression level was significantly reduced after cell transfection (Fig. 4A and B). The expression levels of the apoptosis-related proteins HMGB1, RIP3, RIP1 and MLKL were measured in the ZBP1-knockdown cells treated with ABT-737/Z-VAD-FMK (Fig. 4C). The results indicated that the expression levels of HMGB1, RIP3 and MLKL were significantly higher in the ZBP1-knockdown

5637 cells and the ZBP1-knockdown UMC3 cells treated with ABT-737/Z-VAD-FMK (Fig. 4D and E). The Transwell and MTT assays demonstrated that, compared with the control group, the proliferative and invasive abilities of the ZBP1-knockdown UMC3 cells (Fig. 4F-H) and the ZBP1-knockdown 5637 cells (Fig. 4I-K) were significantly reduced after ABT-737/Z-VAD-FMK treatment. These data suggested that ZBP1 did not affect the necrosis induction of bladder cancer cells treated with ABT-737.

**RIP3 serves a major role in the cell necrosis induced by ABT-737 treatment.** In another experiment, the role of RIP3 in necrosis was examined by knocking down the RIP3 gene in UMC3 and 5637 cells. The western blot analysis results demonstrated that the RIP3 expression level was significantly decreased after cell transfection (Fig. 5A and B). The expression levels of necrosis-related proteins HMGB1, ZBP1 and MLKL were measured in the RIP3-knockdown cells treated with ABT-737/Z-VAD-FMK (Fig. 5C). The results suggested that the expression levels of ZBP1 were significantly changed in the RIP3-knockdown 5637 cells following ABT-737/Z-VAD-FMK treatment (Fig. 5D). The expression levels of ZBP1 and MLKL were significantly higher in the RIP3-knockdown UMC3

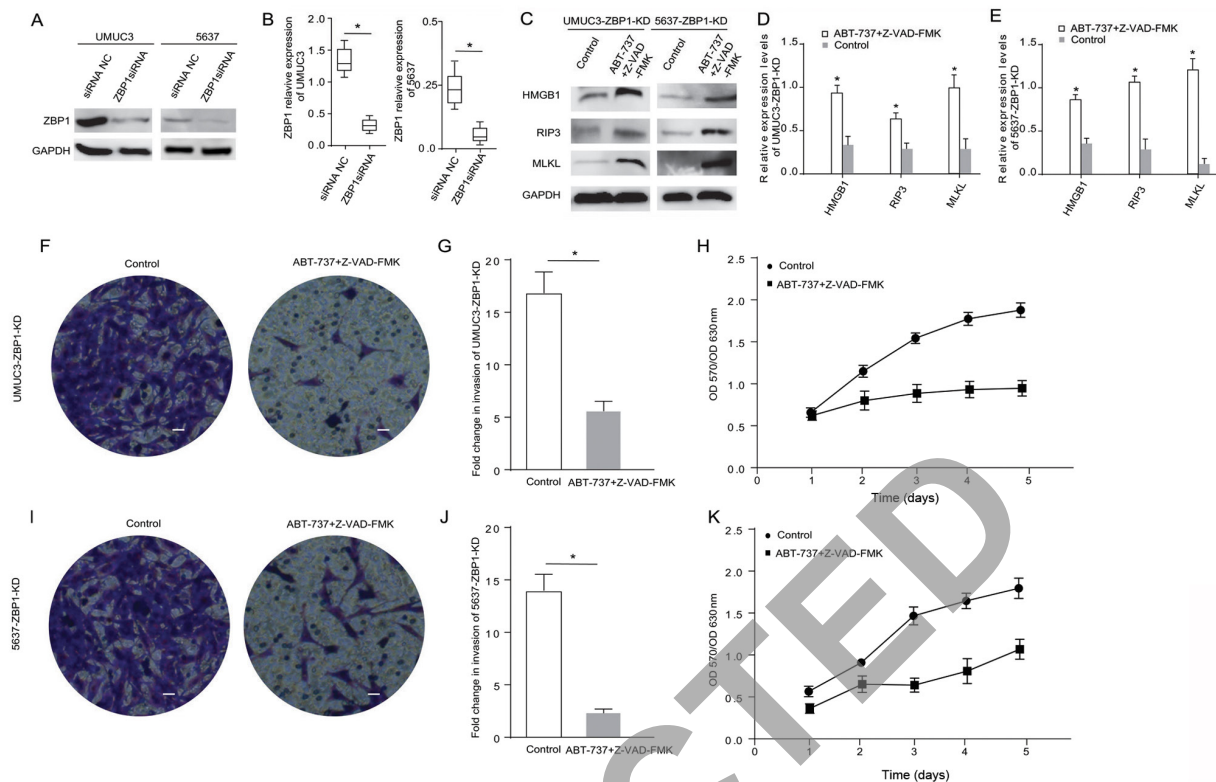


Figure 4. ABT-737 induces cancer cell necrosis and inhibits cell viability and invasion when the ZBP1 gene is knocked down in bladder cancer cells. (A) Expression levels of ZBP1 were analyzed in UMUC3 and 5637 cells via western blotting after treatment with ZBP1 siRNA for 12 h, and the results were (B) semi-quantified. (C) Protein expression levels of HMGB1, RIP3 and MLKL were analyzed in ZBP1-KD UMUC3 and ZBP1-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. The results were semi-quantified in (D) UMUC3 and (E) 5637 cells. (F) Transwell invasion assays were performed with UMUC3-KD cells. (G) Quantification of Transwell assay results. (H) MTT assays were performed to examine the viability of ZBP1-KD UMUC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (I) Transwell invasion assays were performed with ZBP1-KD 5637 cells. (J) Quantification of Transwell assay results. (K) MTT assays were performed to examine the viability of ZBP1-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. \* $P < 0.05$  vs. siRNA NC or control. OD, optical density; scale bar=50  $\mu$ m; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein; HMGB1, high mobility group box 1; MLKL, mixed-lineage kinase domain-like protein; siRNA, small interfering RNA; NC, negative control; KD, knockdown.

cells after ABT-737/Z-VAD-FMK treatment (Fig. 5E). The Transwell and MTT assays identified that the proliferative and invasive abilities of the RIP3-knockdown UMUC3 cells (Fig. 5F-H) and the RIP3-knockdown 5637 cells (Fig. 5I-K) showed no significant changes after ABT-737/Z-VAD-FMK treatment. These results verified that RIP3 knockdown can counteract the ABT-737-induced necrosis of bladder cancer cells.

**ABT-737 upregulation of ZBP1 interaction with RIP3 induces bladder cancer cell necrosis.** To investigate the mechanism of ABT-737-induced cell necrosis, the communication between the key proteins, RIP3 and ZBP1, was determined in bladder cancer cells treated with ABT-737/Z-VAD. RIP3 and ZBP1 were examined via immunoprecipitation. The immunoprecipitation results revealed that RIP3 directly combined with ZBP1 to induce the necrosis of both cell lines (Fig. 6A and B).

## Discussion

Malignant tumour cell apoptosis rapidly leads to cell loss and slows tumour growth (26). Numerous anticancer medications (e.g., Myc, E1a and cyclin-D1) have been recently developed by inducing the apoptosis of cancer cells (25). When the

apoptosis process is dysregulated, cell sensitivity to treatment decreases (27). Some researchers have shown that the main characteristic of malignant tumour is resistance to the classical caspase-dependent pathway (28), in which cell apoptosis is inhibited when intracellular caspase is prevented from executing apoptosis (29).

ABT-737, a small-molecule inhibitor used in the current study, is a BH3 simulator developed by the IDUT laboratory and Abbott Laboratory, and is the first class of small molecule inhibitors (SMIs) that causes tumour-specific killing (30). ABT-737 can simulate the BH3 domain of proapoptotic proteins, which is the critical event in the mitochondrial apoptosis pathway. The mitochondrial outer membrane permeability is increased, and Cyt-C is released from the mitochondrial intermembrane space into the cytoplasm. Then, ABT-737 binds to the cytoplasmic factor APAF1, promoting its oligomerization to form an apoptotic body and activate caspase 9, further activating other downstream caspase family proteins, and ultimately promoting apoptosis (31). The current study found that ABT-737 induced the apoptosis of tumour cells, and this action was accompanied by necrosis.

Procedural necrosis has been recently described as a previously unknown type of cell death that is regulated by a death signal that does not rely on caspase (7). RIP1, RIP3



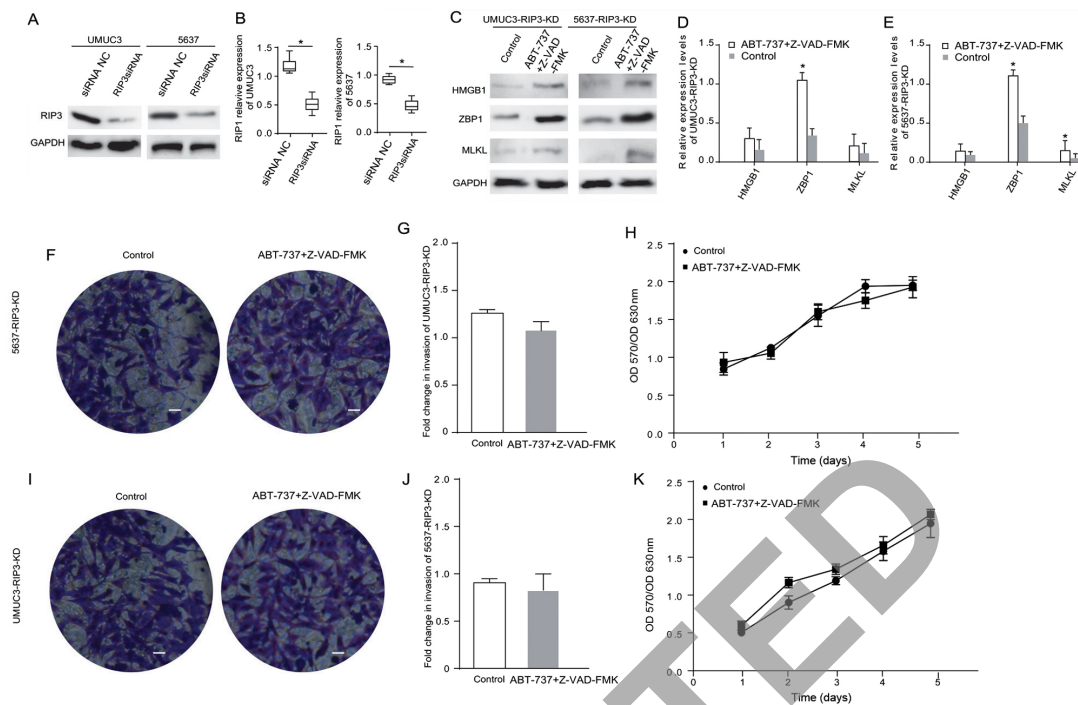


Figure 5. RIP3 serves a major role in the cell necrosis induced by ABT-737 treatment. (A) Expression levels of RIP3 in UMUC3 and 5637 cells after treatment with RIP3 siRNA for 12 h were analyzed via western blotting, and the results were (B) semi-quantified. (C) Protein expression levels of HMGB1, ZBP1 and MLKL were analyzed in RIP3-KD UMUC3 and RIP3-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. The results were semi-quantified in (D) UMUC3 and (E) 5637 cells. (F) Transwell invasion assays were performed with RIP3-KD UMUC3 cells. (G) Quantification of Transwell assay results. (H) MTT assays were performed to examine the viability of RIP3-KD UMUC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (I) Transwell invasion assays of RIP3-KD 5637 cells. (J) Quantification of Transwell assay results. (K) MTT assays were performed to examine the viability of RIP3-KD 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (scale bar=50  $\mu$ m) \* $P$ <0.05 vs. siRNA NC or control. OD, optical density; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein; HMGB1, high mobility group box 1; MLKL, mixed-lineage kinase domain-like protein; siRNA, small interfering RNA; NC, negative control; KD, knockdown.

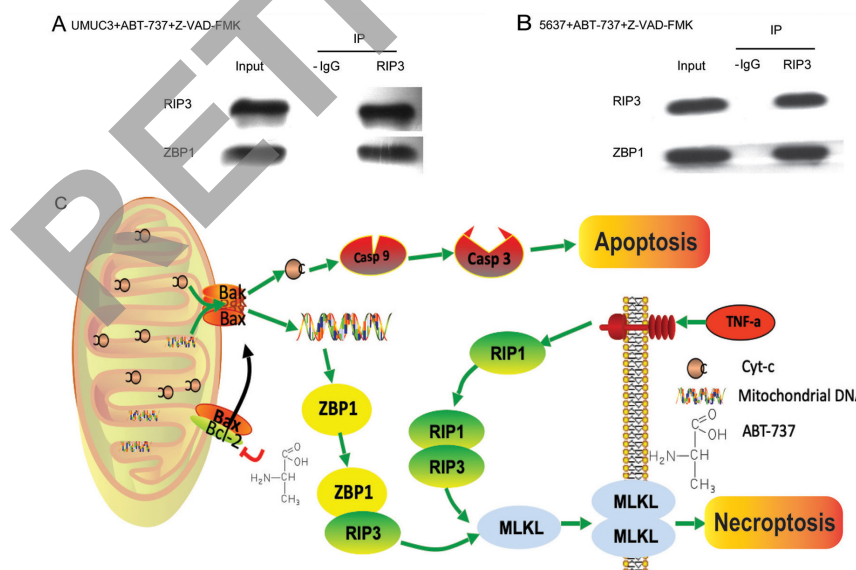


Figure 6. Co-IP results and a schematic diagram of the mechanism underlying ABT-737 upregulation of the interaction of ZBP1 with RIP3 in inducing bladder cancer cell necrosis. (A) Co-IP was performed using anti-RIP3 antibody with UMUC3 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (B) IP was performed using anti-RIP3 antibody with 5637 cells treated with Z-VAD-FMK combined with ABT-737 for 12 h. (C) A schematic diagram showing the mechanisms via which ABT-737 induced necrosis. IP, immunoprecipitation; ZBP1, Z-DNA binding protein 1; RIP, receptor-interacting protein; MLKL, mixed-lineage kinase domain-like protein; Cyt-C, cytochrome C.

and MLKL are the key control factors in programmed necrosis (32). The present study focused on the role of necrosis in cells treated with ABT-737. The use of the

apoptosis inhibitor Z-VAD-FMK reduced the apoptotic rate and inhibited cell necrosis, but not apoptosis, which help to identify the mechanism via which ABT-737 induced the



necrosis of UMUC3 and 5637 cells. This study confirmed that in UMUC3 and 5637 cells, the mitochondrial DNA released by ABT-737-mediated mitochondrial swelling and rupture, entered the cytoplasm and activated ZBP1. The data also demonstrated that ZBP1 served an important role in the process of necrosis. ZBP1, as a solenocyte DNA sensor, binds to RIP1 or RIP3 via its RHIM domain after binding to double stranded DNA (33).

RIP3-mediated cell necrosis is a programmed and controlled form of cell necrosis (34). RIP3-regulated cell necrosis is initially manifested by changes in the cell membrane, leading to swelling and rupture of organelles and membranes (14). Previous studies established that phosphorylated RIP3 binds and phosphorylates MLKL (p-MLKL). Then, p-MLKL proteins can combine with each other via their N-terminal regions to form homo-oligomers and are transferred to the cell membrane, promoting  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx and inducing necrosis (35). Moreover, MLKL was found to contain the phosphorylated mitochondrial protein PGAM family member 5, mitochondrial serine/threonine protein phosphatase and to activate the mitochondrial lysis protein Drp1, ultimately inducing necrosis (12). A limitation of the present study was that phosphorylation of RIP1 and RIP3 expression was not measured. ABT-737 has been demonstrated to bind to Bcl-2 with a high affinity and disrupts the interaction with these proteins.

In conclusion, the present results indicated that RIP3 and ZBP1, without interacting with RIP1, directly caused MLKL-mediated programmed cell necrosis (Fig. 6C). These results suggested that ABT-737-induced necrosis had a synergistic effect with apoptosis in urothelial carcinoma cells, which provided novel evidence to evaluate the relationship between apoptosis and necrosis. Furthermore, the current results provided new research ideas and directions for targeted chemotherapy for clinical drug-resistant tumours.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

RC and XL contributed to the conception of the study and analyzed the data. ZW performed the experiments and wrote the manuscript. RC, ZW and KT analyzed the data and wrote the manuscript. XL and KT analyzed the data and provided constructive criticism. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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