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ABT-737 overcomes resistance to immunotoxin-mediated apoptosis and enhances the delivery of Pseudomonas Exotoxin-based proteins to the cell cytosol

Roberta Traini^{*}, Gal Ben-Josef^{*}, Diana V. Pastrana, Elizabeth Moskatel, Ashima K. Sharma, Antonella Antignani, and David J. FitzGerald[^]

Laboratory of Molecular Biology, CCR, NCI, NIH, HHS. 37 Convent Dr Room 5124. Bethesda, MD 20892

Abstract

Pseudomonas exotoxin (PE)-based immunotoxins (antibody-toxin fusion proteins) have achieved frequent complete remissions in patients with hairy cell leukemia but far fewer objective responses in other cancers. To address possible mechanisms of resistance, we investigated immunotoxin activity in a model system using the colon cancer cell line, DLD1. Despite causing complete inhibition of protein synthesis, there was no evidence that an immunotoxin targeted to the transferrin receptor caused apoptosis in these cells. To address a possible protective role of prosurvival Bcl-2 proteins, the BH3-only mimetic, ABT-737, was tested alone or in combination with immunotoxins. Neither the immunotoxin nor ABT-737 alone activated caspase 3, while the combination exhibited substantial activation. In other epithelial cell lines, ABT-737 enhanced the cytotoxicity of PE-related immunotoxins by as much as 20-fold, but did not enhance diphtheria toxin or cycloheximide. Because PE translocates to the cytosol via the endoplasmic reticulum (ER) and the other toxins do not, ABT-737-mediated effects on the ER were investigated. ABT-737 treatment stimulated increased levels of ER stress response factor, ATF4. Because of its activity in the ER, ABT-737 may be particularly well suited for enhancing the activity of immunotoxins that translocate from the ER to the cell cytosol.

Introduction

Immunotoxins are antibody-toxin chimeric proteins targeted to kill cancer cells (1–3). Design features include an antibody or antibody fragment and a toxin moiety that is directed to kill specific cells displaying a surface receptor or antigen. Recombinant immunotoxins composed of antibody Fvs linked to domains II and III of PE have shown great promise for the treatment of hematologic malignancies but have performed less well against cancers derived from epithelial cells (4–9). A feature of protein immunotoxins is the potency associated with the enzymatic component of the toxin. PE and diphtheria toxin (DT) are both cytotoxic because they gain access to the cytosol and inhibit protein synthesis via the ADP-ribosylation of EF2. However, there is a significant difference in the intracellular route taken by each toxin. DT uses the low pH of endosomes to escape from this organelle to the cytosol (10,11). PE does not translocate from endosomes but rather uses a KDEL retrieval sequence at its C-terminus to traffic to the ER. Once there, an active fragment translocates to the cytosol, possibly using the ERAD system (12–15). In the cytosol, both toxins act catalytically to ADP-ribosylate EF2 and shut down protein synthesis. It has been shown that one or only a few of these toxin

[^]Corresponding author: djpf@helix.nih.gov.

^{*}Co-first authors

molecules delivered to the cytosol are sufficient to kill a target cell (16). Much of that argument is based on early experiments where killing was equated solely with inhibition of protein synthesis. Recently, assays that focus more precisely on the mechanisms of cell death have been developed. These have led to the appreciation that some growth inhibitory treatments, while apparently causing biochemical damage, do not result in efficient death (17). Resistance to apoptosis is an important reason why cells fail to die. In part this is because prosurvival proteins, such as those within the BCL-2 family of proteins, can keep cells alive even in the face of harsh treatments. In fact the basal level expression of the Bcl-xl was the only one of 10 candidate genes matched with 122 standard chemotherapy agents that negatively correlated with sensitivity in the NCI 60 cell line screen (18).

ABT-737 and ABT-263 were developed as agents to neutralize the prosurvival effects of Bcl-2 proteins (19,20). Because ABT-737 is a BH3-only mimetic, it can bind to the hydrophobic groove in Bcl-2 proteins and liberate Bax or Bak allowing the process of apoptosis to proceed efficiently. Binding experiments have revealed that ABT-737 has high affinity for Bcl-2, Bcl-xl and Bcl-w but little or no affinity for Mcl-1 (21). Thus in cancers where Mcl-1 is a pivotal prosurvival protein, ABT-737 is less effective (21,22). Several reports therefore suggest that combination treatments of ABT-737 with agents that degrade or neutralize Mcl-1 have a better chance of being effective than ABT-737 alone (23,24) (25). Mcl-1 has a short half-life of approximately 30 min. Agents that inhibit protein synthesis will lead to loss of Mcl-1 over time (26,27). Thus by combining ABT-737, an agent that neutralizes three major BCL2 proteins, and an immunotoxin, an agent that causes a loss in Mcl-1, apoptosis may be achieved selectively in cells displaying target antigens.

Toxin and immunotoxin activities have been associated with apoptosis in some cell systems but mechanisms of cell death have not been extensively studied (28–30) (31) (26,32). To study immunotoxin-cell outcomes, we have employed two PE-based immunotoxins, HB21-PE40 (33) (directed to the human transferrin receptor) and SS1P (4) (directed to surface expressed mesothelin) and a variety of epithelial cell lines.

As a combination treatment with agents that inhibit protein synthesis, we report here that ABT-737 has two activities: one is common to agents that inhibit protein synthesis and one is specific to PE-based proteins. In the first instance, there is an enhancement of apoptosis and in the second PE is rendered more potent because ABT-737 disrupts the ER and liberates more toxin into the cell cytosol.

Materials and Methods

Compounds

ABT-737 was purchased from Selleck Chemicals LLC and dissolved in DMSO at 10 mM stock concentration, stored frozen at –20°C. ABT-263 was obtained from Toronto Research Chemicals Inc, North York, Canada and dissolved in DMSO at 3 mM and stored frozen at –20°C. Velcade (bortezomib) was obtained from the NIH pharmacy. HB21-PE40 and SS1P were produced recombinantly in *E. coli* as described previously (33,34). HB21-CET40 was described recently(35). DT was purchased from List Biological Laboratories, Campbell CA, PE was from a lab stock and cycloheximide (CHX) from Sigma.

Antibodies

PARP, BD (556494), Caspase3, Santa Cruz (7148), Mcl-1, Cell Signaling (4572), Tubulin, Sigma (T6074) and ATF4 Santa Cruz (SC-200).

Cells

DLD1, and SKOV3 were each obtained from ATCC and grown in RPMI-1640 plus 10% FBS, Pen-Strep and Pyruvate. The KB3-1 cells were obtained from Michael Gottesman, NCI and grown in DMEM plus 10% FBS.

Assays

Treated cells were assayed for inhibition of protein synthesis by the addition of ^3H -leucine (2 $\mu\text{Ci}/\text{ml}$) for 4 hr in 96-well plates. Cells were collected on filter mats and samples counted using a Wallac beta plate reader. To measure cytotoxicity, WST-1 (Roche) was added to 96-well plates at a final concentration of 10% and absorbance measured at 450 nm. ATP assays were conducted using the CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega, Madison WI). Caspase 3/7 was detected using the Caspase-Glo kit from Promega (G8091). Caspase-3 activity was measured using one of two fluorescent substrates Caspase-3 Fluorometric Assay Kit (R and D systems Cat # BF1100) or Caspase-3 Fluorometric-KIT (Invitrogen Cat# KHZ0012). Data are reported in fluorescent units per μg of cell protein.

A short exposure cell-killing assay was carried out as follows. KB3-1 cells were seeded in 6-well plates at a density of $5 \times 10^4/\text{ml}$. The next day, cells were treated for 4 hr with either immunotoxin alone, ABT-737 alone or combinations of both as indicated. At the end of the treatment, cells were washed with PBS, trypsinized and replated. Replated cells were incubated for a further 6 days. Finally, cells were washed with PBS and then stained with methylene blue (0.5%) in methanol-water (50:50 by volume)

Western blots

Immunotoxin-treated cells in the presence or absence of ABT-737, were washed with PBS and then solubilized with RIPA buffer containing both protease and phosphatase inhibitors. Precast Tris-glycine 8–16% gels were used to separate cell lysates. Primary antibodies were routinely detected with Donkey anti-mouse-HRP or Donkey anti-rabbit-HRP (Jackson ImmunoResearch).

Results

ABT-737 overcomes resistance to immunotoxin-mediated apoptosis in DLD1 cells

Resistance to apoptosis is a major obstacle to effective cytotoxic cancer therapy (18). Colorectal cancer cells are among the most difficult to kill (36). DLD1 cells (a colon cancer cell line) are susceptible to growth inhibition (Fig 1A–C) by an immunotoxin directed to the human transferrin receptor (HB21-PE40). HB21-PE40 inhibited protein synthesis of DLD1 cells in a dose-response manner with complete inhibition being achieved at concentrations at, or exceeding, 1 ng/ml (Fig 1A). In cytotoxicity assays, HB21-PE40 was active with an IC_{50} of ~ 0.1 ng/ml (Fig 1B & C). However, using either a WST-1 or an ATP depletion assay, there was incomplete killing at higher concentrations (Fig 1B & C). Further, when cells were treated with HB21-PE40 (10 ng/ml) for 72 hr and then monitored for viability, approximately 20% of cells excluded Trypan Blue, further indicating incomplete killing (data not shown). When caspase 3/7 activity was measured, immunotoxin-treated DLD1 cells showed no evidence of apoptosis (Fig 1D). However, apoptosis, as evidenced by increased caspase 3/7 activity, was noted when velcade (a proteasome inhibitor) was added as a positive control (Fig 1D). We conclude that DLD1 cells are resistant to immunotoxin-mediated apoptotic death.

To address the role of prosurvival Bcl-2 proteins in mediating resistance, we investigated the outcome of combining the BH-3 only mimetic, ABT-737, with immunotoxins. ABT-737 neutralizes Bcl-2, Bcl-xl and Bcl-w but does not bind Mcl-1. However, agents that inhibit protein synthesis, including immunotoxins, result in the loss of Mcl-1, due to its short half life

(27). Thus, combination treatments of an immunotoxin and ABT-737 could neutralize or eliminate all the relevant prosurvival Bcl-2 family proteins. In Fig 2A the addition of ABT-737 increased the cytotoxicity of all concentrations of the HB21PE40 immunotoxin for DLD1 cells. To investigate the basis for this, we probed elements of the apoptosis pathway. Neither immunotoxin alone (at 1 or 10 ng/ml) nor ABT-737 (at 10 μ M) caused a large increase in caspase 3 activity. However the combination resulted in a substantial increase in activity compared with either treatment alone (Fig 2B). That activation of caspase 3 was primarily due to immunotoxin-mediated inhibition of protein synthesis, in combination with ABT-737, was confirmed by showing that CHX in combination with ABT-737 produced a similar result (Fig 2C). Further analysis of key members of the intrinsic apoptosis pathway was conducted. RIPA extracts were probed for Mcl-1 and cleavage of PARP. Samples from immunotoxin treated cells exhibited a loss of Mcl-1 while only those that were also treated with ABT-737 resulted in the cleavage of PARP (Fig 2D). Of note was the apparent increase in Mcl-1 levels in samples treated with ABT-737 alone (Fig 2D lane 5). A similar observation was reported very recently in lymphoma cells (37). From our initial experiments we conclude that immunotoxin treatment of DLD1 cells does not by itself result in apoptosis despite a complete reduction in protein synthesis and loss of Mcl-1. However, when ABT-737 was added in combination, there was a significant increase in caspase 3 activity, PARP cleavage, loss of Mcl-1 and cell death (Fig 2B, D -lanes 3 and 4).

Effect of ABT-737 on other cell lines

To determine the broad utility of immunotoxin-ABT-737 combinations, we expanded our testing to include other cell lines and other immunotoxins. Combinations of ABT-737 and HB21-PE40 were added to SKOV3, an ovarian cancer cell line reported to be resistant to certain toxin-based agents (38). As noted with DLD1 cells, when immunotoxin and ABT-737 combinations were used, there was an enhancement of killing (by ~ 20-fold) over the addition of immunotoxin alone (Fig 3A). Next we tested the cervical cancer cell line KB3-1 (an immunotoxin sensitive cell line) with immunotoxins targeted either to the transferrin receptor (Fig 3B) or to mesothelin (Fig 3C). Again, there was a profound increase of immunotoxin activity with an approximate 20-fold greater toxicity when ABT-737 was present compared with immunotoxin alone. For KB3-1 cells, we extended the analysis to look at western blots of apoptosis-related proteins after treatments (for 24 hr) with single agents or combinations of immunotoxin and ABT-737. As shown in Fig 3D, the presence of ABT-737 resulted in PARP cleavage, Mcl-1 degradation and loss of pro-caspase 3. When the same treatments were extended to 48 hr, immunotoxin alone resulted in PARP and pro-caspase 3 cleavage (data not shown), indicating that KB3-1 cells are not resistant to apoptosis but in the presence of ABT-737 cell death is evident earlier.

ABT-737 specifically enhances toxin translocation from the ER

Because KB3-1 exhibited no apparent resistance to immunotoxin treatment, the 20-fold enhancement of HB21-PE40 and SS1P cytotoxicity by ABT-737 was unexpected. In order to explore the mechanism of the ABT-737 effect, we performed additional experiments using the same WST-1 where ABT-737 was added in combination with three other agents that inhibit protein synthesis. These included native DT, CHX and HB21-CET40, a newly described immunotoxin made from a truncated exotoxin derived from *Vibrio cholerae* also, like PE, ending in a KDEL-like sequence (35). Only ABT-737 in combination with PE- or CET-immunotoxins produced a greater than 10-fold enhancement of toxicity (Fig 3A–C and Fig 4C). Both these agents end in KDEL-like sequences and are reported to translocate to the cytosol from the ER (12,14). With DT and CHX, which reach the cytosol by ER-independent routes, there was no evidence of ABT-737-mediated enhancement (Fig 4A, B). This result does not contradict the observation reported in Fig 2C where there was an increase in caspase 3 activity with ABT-737 and CHX, but reflects what each assay measures (see discussion). The

enhancement of PE cytotoxic activity could be due to increased delivery of toxin or increased susceptibility of ABT-treated cells for ADP-ribosylated EF2. The lack of enhancement of DT cytotoxicity appeared to rule out the latter. Therefore we sought to investigate the delivery of the enzymatic domain of PE to the cytosol. We reasoned that if PE were delivered in greater amounts, there would be a greater reduction in protein synthesis. Alternatively, if ABT-737 was acting downstream of ADP-ribosylation, then protein synthesis levels would be the same regardless of the presence of ABT-737. Results indicated that for two PE-based immunotoxins (SS1 is shown), there was a 25-fold greater reduction in the level of protein synthesis in the presence of ABT (Fig 4D and data with HB21-PE40, not shown) than in its absence. This result suggests that ABT-737 can promote the delivery of greater number of PE molecules from the ER to the cytosol.

ABT-737 treatment produces stress within the ER

Because the cellular uptake of immunotoxins, added at sub-nanomolar concentrations, is well below the detection limit for immunoblot analysis, we could not document directly that additional molecules of toxin were delivered to the cytosol. Instead, we sought evidence that ABT-737 was interacting with the ER. In various cell types, Bcl-2 family members are found associated with the ER (39,40) and thus the addition of ABT-737 could cause ER stress in these cells. To test if ABT-737 interacted with the ER and provoked an ER stress response, we incubated KB3-1 or DLD1 cells for 4 hrs with either ABT-737 or with DTT; the latter being a well-known mediator of ER stress. Treated cell extracts were analyzed for the transcription factor ATF-4 (41). Results indicate that DTT strongly upregulated the ER stress response in both cell lines, while ABT-737 produced a strong stress response in KB3-1 cells and a moderate one in DLD1 cells (Fig 5A). Overall our results are consistent with ABT-737 acting on the ER and causing either directly or indirectly an increased translocation of PE- or CET-based immunotoxins to the cytosol.

Clinical considerations for Immunotoxin-ABT combination therapy

Assays for cell viability routinely involve exposing cells to toxins continuously for 48 hrs. However, PE-based recombinant immunotoxins have plasma half-lives of only 2–7 hr (7) (8) (9) (4) and thus may not remain in contact with target cells for more than a few hours. To simulate short-term exposures, immunotoxin-ABT-737 combinations were applied for 4 hrs. Surviving cells were then visualized after 6 days (Fig 6A,B). When the immunotoxin HB21-PE40 was used, the combination with ABT-737 resulted in more than a 10-fold enhancement of killing (Fig 6A). Similarly, ABT-737 enhanced SS1P activity (Fig 6B). To compare immunotoxin treatment with another agent that inhibits protein synthesis, a parallel experiment was conducted with CHX. By contrast with the immunotoxins, a 4 hr exposure with CHX and ABT-737 did not result in any significant cell killing (data not shown). Because immunotoxins act catalytically and have no known intracellular inhibitors, they will continue to be active once delivered to cytosol. In contrast, CHX is a reversible inhibitor of protein synthesis and therefore its effect will only be noted if it is present continually. From the above data we conclude that ABT-737 may be particularly useful in promoting immunotoxin killing even with short term exposures.

While ABT-737 itself is not destined for clinical use its 'orally available' variant, ABT-263, is currently under clinical evaluation. To confirm that ABT-263 has a similar toxin-enhancing activity as ABT-737, we performed additional cytotoxicity and caspase 3 activity assays with this compound (Fig 6C and D). DLD1 cells were incubated with HB21-PE40 in the presence or absence of ABT-263. Our results indicate that ABT-263 has similar toxicity-enhancing activities as those noted with ABT-737 (Fig 6C and D). ABT-263 also enhanced KB3-1 and SKOV3 killing (data not shown). We conclude that immunotoxin resistance can be overcome

using ABT-263 and that future *in vivo* investigations can proceed with this orally-available compound.

Discussion

Here we show that the BH3-only mimetic, ABT-737, has two properties: one, it can overcome resistance to immunotoxin-mediated apoptosis and, two, it can increase immunotoxin delivery from the ER to the cytosol resulting in enhanced killing by as much as 20-fold. Both activities were achieved using concentrations (3 or 10 μ M) of ABT-737 that were non-toxic when added alone. Combinations of immunotoxin and ABT-737 were effective at overcoming resistance because each agent targeted a distinct Bcl-2 family member. By inhibiting protein synthesis, immunotoxin treatment promotes a decline in Mcl-1 levels. This was reported previously for both CHX (27) and PE-immunotoxins (26) and confirmed here. ABT-737 is a peptide mimetic modeled on a BH3-only domain and exhibits high binding affinity binding for Bcl-2, Bcl-xl and Bcl-w (19). Binding of ABT-737 to either Bcl-2 or Bcl-xl neutralizes their prosurvival activity, allowing Bax or Bak to initiate the intrinsic arm of the apoptosis pathway. ABT-737 binds to Mcl-1 poorly and because of this, is not effective as a single agent against cancers expressing high levels of Mcl-1 (42). For these reasons, the effectiveness of immunotoxin-ABT combinations in overcoming resistance to apoptosis was not unexpected.

However, ABT-mediated enhancement of PE delivery to the cytosol was completely unexpected. In our study, this was noted in several ways: there was a 20-fold enhancement of the cytotoxic activity of two PE-immunotoxins and one CET-immunotoxin made from a KDEL-ending toxin from *Vibrio cholerae*. In addition, ABT-737 enhanced the delivery of toxin as monitored with an inhibition of protein synthesis assay. This suggested that ABT-737 was acting at a step in the toxin pathway prior to the ADP-ribosylation of EF2. Further, in the same assays, there was little enhancement of DT toxicity suggesting that endosomal delivery was not involved. Because PE escapes from the ER to the cytosol, we speculated that ABT-737 was acting by altering ER structure or function, perhaps by interacting with Bcl-2 proteins in the ER and causing a stress response. We attempted to confirm this by probing for increased ATF4 expression, a classic indicator of ER stress, and elevations of this transcription factor were noted within four hours of adding ABT-737 to either DLD1 or KB3-1 cells. ABT-737-mediated ER stress was not observed by Hermanson et al (43) when analyzing several leukemic cell lines. In our unpublished results we did not see any enhancement of PE-immunotoxin cytotoxicity when we investigated responses in the Burkitt lymphoma cell lines CA46 or Raji. Thus, ABT-737 may interact with epithelial and lymphoid-derived cell lines in different ways. ER stress is usually initiated by the release of the chaperone Bip/Grp78 from one of three monitor transmembrane proteins, PERK, ATF6 and IRE1. Release of Bip/Grp78 usually initiates ERAD and the retrieval of poorly folded proteins from the ER to the cytosol. We note that one theory of toxin egress from the ER is to mimic an unfolded protein and thereby act as bait for ERAD (13). By stressing the ER, ABT-737 appears to increase the efficiency of toxin transport to the cytosol. The mechanistic details of how ABT-737 interacts with the ER are currently under investigation.

In immunotoxin-resistant DLD1 cells, enhancement of caspase 3 activity was seen with both CHX and HB21-PE40 in combination with ABT-737 because ABT-737 removed the block in the apoptotic pathway caused by high levels of Bcl-2 family prosurvival proteins. In this capacity, ABT-737 demonstrated its first property of overcoming resistance to apoptosis in the presence of agents that inhibit protein synthesis. However, in immunotoxin-sensitive KB3-1 cells, when inhibition of cell growth was measured using a WST-1 assay, there was no ABT-737-mediated enhancement of CHX or DT activity. Because these cells are sensitive to protein synthesis inhibitors, the cells were killed similarly in the presence or absence of ABT-737 and no enhancement was noted because ABT-737 did not enhance delivery of these

toxins to the cell cytosol. In contrast, cells treated with combinations of HB21-CET40 and SS1P with ABT-737 were killed more efficiently and with lower doses of immunotoxin because ABT-737 not only removes the prosurvival block, but also increases the delivery of these toxins from the ER to the cytosol.

In vivo, recombinant immunotoxins are short-lived proteins with half-lives of 2–8 hrs and might not be in contact with tumor cells very long. When we conducted a short-term assay, where cells and agents were in mutual contact for only four hours, we found that the combination of immunotoxin and ABT-737 was at least 10-fold more cytotoxic than the immunotoxin alone. Because ABT-737 was removed after only four hours, it must act quickly to allow increased toxin egress to the cytosol. Immunotoxins are reported to be stable in the cytosol, although that is challenging to prove experimentally, our data from this experiment support this contention.

ABT-737 is not readily available as an orally administered compound, so ABT-263 was developed (20). Here we extend our initial findings to ensure that the clinically relevant compound also enhances PE-immunotoxin activity.

In summary, our major findings include the discovery that complete inhibition of protein synthesis and loss of Mcl-1 does not always lead to cell death. To overcome this resistance we employed the BH3-only mimetic, ABT-737, which implicates Bcl-2, Bcl-xl or Bcl-w as the proximate cause of resistance. Further, ABT-737 enhanced the delivery of active PE to the cytosol suggesting that prosurvival Bcl-2 proteins are located in the ER and that ABT-737 disrupts ER function via the neutralization of one or more of these proteins. Our data support the preclinical development of ABT-263-immunotoxin combinations for cancer therapy.

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Abbreviations

DT	diphtheria toxin
PE	pseudomonas exotoxin
CET	cholera exotoxin
ERAD	endoplasmic reticulum associated degradation
CHX	cycloheximide
WST	water soluble tetrazolium

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Fig 1.

Response of DLD1 cells to immunotoxin treatment. The immunotoxin, HB21-PE40, was added at various concentrations as indicated, cells harvested and assayed as indicated below. A. Inhibition of protein synthesis was determined by measuring the incorporation of 3 H-leucine into cells 24 hrs post treatment. Results are reported as CPM/well with error bars indicating one standard deviation. B. Cell viability using the WST-1 reagent was assayed after 48 hr. Shown are absorbance readings at 450 nm with error bars indicating one standard deviation. C. Cell energy levels, by measuring ATP, were determined after 48 hr. D. Apoptosis was assessed by measuring caspase 3/7 activity at 48 hr.

Fig 2.

ABT-737 overcomes resistance of DLD1 cells. Immunotoxin or immunotoxin-ABT-737 combinations were added for 48 hr to DLD1 cells as indicated and assayed for viability, caspase activity or PARP cleavage. Cycloheximide (CHX) was added to inhibit protein synthesis. A. Cell viability using the WST-1 reagent shows enhanced killing with ABT-737. B. Caspase 3 activity in DLD1 cells treated with immunotoxin, immunotoxin plus ABT-737 or ABT-737 alone. C. Caspase 3 activity following CHX, CHX plus ABT-737 or ABT-737. D. Western blot of DLD1 lysates following treatments with immunotoxin alone, immunotoxin plus ABT or ABT alone. PVDF membranes were probed for PARP cleavage or the presence of Mcl-1. Equal protein amounts (~30 µgs) were loaded in each lane.

Fig 3. ABT-737 enhances immunotoxin activity in SKOV3 and KB3-1 cells. In panels A, B and C, cells were treated as indicated for 48 hr and viability was assessed using a WST-1 assay. D. KB3-1 cells were treated as indicated for 24 hr, cell lysates prepared and probed with antibodies to PARP, procaspase 3, Mcl-1 and tubulin.

Fig 4.

ABT-737-mediated enhancement of KDEL-ending toxins. KB3-1 cells were incubated with various concentrations of (A) DT, (B) CHX or (C) an immunotoxin made with a truncated exotoxin from *Vibrio cholerae* and assayed for viability 48 hr post treatment. Toxin treatments were made alone or in combination with ABT-737 at the concentrations indicated. C. The SS1P immunotoxin was added to cells either alone or in combination with ABT-737 for 18 hr and then assayed for inhibition of protein synthesis.

Fig 5. ABT-737 causes ER stress. A. Lysates of ABT-treated DLD1 and KB3-1 cells were probed for the ER stress marker, ATF4. Lysates were prepared after 4 hr of treatment with either ABT-737 10 μ M or 10 mM DTT.

Fig 6. ABT-737 and ABT-263 both exhibit immunotoxin-enhancing activity. A. ABT-737 enhances immunotoxin activity with even a short (4 hr) exposure to the combination. ABT-737 was added in combination with immunotoxins HB21-PE40 or SS1P for 4 hr, cells trypsinized and replated for 6 days. Cells that survived were visualized using methylene blue as the stain. B. ABT-263 enhances immunotoxin activity against DLD1 cells.