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ABT-737 promotes the dislocation of ER luminal proteins to the cytosol, including *Pseudomonas* exotoxin

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Abstract

Impaired apoptosis is often a key element in tumor development. Therefore, drugs mimicking prosurvival antagonists offer promise as cancer therapeutics. When ABT-737, a BH3-only mimetic, was added to KB3-1 human cervical adenocarcinoma cells we noted an induction of an Endoplasmic Reticulum (ER) stress response and the dislocation of ER luminal proteins including chaperones to the cell cytosol. Further, when immunotoxin (antibody-toxin chimeric molecule) and ABT-737 combinations were added to cells, there was enhanced toxin-mediated inhibition of protein synthesis, consistent with enhanced translocation of toxin to the cytosol. A similar enhancement was not seen with thapsigargin, suggesting that ER stress alone was not responsible for enhanced translocation. Cytosol preparations from ABT-737-treated but not from thapsigargin-treated cells revealed the presence of greater amounts of processed 37 kDa toxin fragment compared with the addition of immunotoxin alone. As early as 4 hr after the addition of ABT-737 and immunotoxin, there was release of mitochondrial cytochrome c and activation of caspase 3/7 indicating that the combination caused apoptotic cell death. These results were reflected in decreased cellular ATP levels that were noted with combinations of ABT-737 and immunotoxin but not with either agent alone or with combinations of thapsigargin and immunotoxin. We conclude that ABT-737 increases ER permeability, promoting the dislocation of toxin from the ER to the cytosol resulting in early apoptotic cell death. These mechanistic insights suggest why this class of BH3-only mimetic synergizes in a particular way with PE-based immunotoxins.

Keywords

immunotoxin; BH3-only mimetic; membrane permeability; ER stress; apoptosis

Introduction

Antibody-toxin fusion proteins (immunotoxins) target antigens of the surface of cancer cells causing cytotoxic damage when the toxin is internalized (1,2). Immunotoxins derived from *Pseudomonas* exotoxin (PE) are cytotoxic for mammalian cells via their ADP-ribosylation of elongation factor 2 (EF2) leading to cessation of protein synthesis (1,2,3). Native PE or

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PE-derived immunotoxins are taken into cells via endocytosis, are proteolytically processed by the cellular protease furin and are then routed to the cell cytosol via the endoplasmic reticulum (ER). The involvement of the ER was established many years ago with the discovery that PE had a KDEL-like sequence (REDLK) at its C-terminus that was required for cellular toxicity (4). Likewise, this KDEL-like sequence is needed for immunotoxin-mediated killing of cancer cells (5). While the need for a KDEL sequence implicates the ER in the toxin pathway, it does not provide information regarding the molecular pathway of toxin translocation from the ER to the cytosol. One candidate transporter, Sec61, was suggested several years ago (6). In this model, toxin unfolding in the ER led to retrotranslocation via the pathway usually taken by newly synthesized secreted proteins. And then, presumably, upon arrival in the cytosol the toxin's ADP-ribosylating domain would refold and begin the process of enzymatically modifying EF2. Further, it has been established that the toxicity of PE is directly related to its ADP-ribosylating action. A toxin mutant lacking a key amino acid (glutamic acid 553 - using the numbering system corresponding to the amino acids of mature PE) in the NAD binding pocket also reaches the cytosol but is non-toxic for cells (7,8).

PE-based immunotoxins are currently being evaluated in clinical trials targeting B and T cell malignancies and a variety of epithelial tumors (9,10). Only in patients with Hairy Cell Leukemia has there been a high complete response rate following immunotoxin treatment (11). In patients with other cancers, responses have been less encouraging (12,13). Evidence of immunotoxin resistance therefore has spurred the testing of combination treatments to increase the killing efficacy of target cells (14,15). Impaired apoptosis is often a critical step in tumor development (16). The key regulators of apoptosis are the proteins of the Bcl-2 family (17). In many tumors, one or more of its prosurvival members, Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 are overexpressed promoting tumor formation, survival and resistance to chemotherapy (18). Therefore, drugs mimicking prosurvival antagonists, the Bcl-2 homology 3 (BH3) only proteins, offer promise as anticancer agents. ABT-737, a BH3-only peptide mimetic, was developed as a therapeutic cancer agent because it neutralizes the prosurvival proteins: Bcl-2, Bcl-xl and Bcl-w (19). However, ABT-737 does not interact with Mcl-1 and cells with sufficient levels of Mcl-1 can resist ABT-737-mediated apoptosis. Because Mcl-1 has a short half-life (~30 min), agents that inhibit protein synthesis can promote the loss of Mcl-1; yet, in the case of the PE-based immunotoxins, in several instances the loss of Mcl-1 was not enough to kill cells (15,20). Hence, we and others have reported on combination treatments with ABT-737 and PE-based immunotoxins leading to increased apoptotic death with strong evidence of synergistic cell killing (20,21). Similar results were also shown for ABT-263 (14), the orally-available clinical version of ABT-737. Enhanced killing was attributed to an increase in toxin-mediated inhibition of protein synthesis leading to apoptosis. However, no mechanistic insights were uncovered.

Here we focus on the translocation of the immunotoxin from the ER membrane system, attempting to explain how ABT-737-immunotoxins combinations produce strong synergistic cell killing. Because different cell types and surface targets can make comparisons difficult, we have chosen to characterize the effects of ABT-737 on the activity of a model immunotoxin directed to the transferrin receptor. The transferrin receptor is expressed on essentially all growing cells and this will allow results obtained in the current KB3-1 cell

system to be tested in other human cell lines including cancer cells of different tissue types. Here we report on a new function for the BH3 mimetic, ABT-737, namely it can increase the permeability of ER membranes, which we argue aids in the translocation of the toxic portion of PE-based immunotoxins from the ER to the cytosol.

Materials and Methods

Reagents

Two immunotoxins, HB21scFv-PE40 (HB21PE40, enzymatically active) and HB21scFv-PE40 553 (HB21PE40 553, enzymatically inactive), were produced recombinantly in *E. coli* as described previously (22). ABT-737 was purchased from Selleck Chemicals LLC (Houston, TX), dissolved in dimethyl sulphoxide (DMSO) at 10 mmol/L stock concentration, and stored frozen at -20°C . Thapsigargin was purchased from Life Technology (Grand Island, NY). MG132 was from Sigma-Aldrich (St. Louis, MO).

Cell Lines and Transfection

The cervical adenocarcinoma cell line KB3-1 (here called KB cells) was obtained from Michael Gottesman (National Cancer Institute, Bethesda, MD). No authentication was done by the authors. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technology Grand Island, NY) plus 10% fetal bovine serum. For transfection studies, KB cells were incubated with Effectene (Qiagen, Valencia CA) according to the manufacturer's instructions and a plasmid encoding full length but enzymatically inactive PE (23). Transfections were for 16 hours followed by the addition of various chemical agents for an additional 4 hours.

Cytotoxicity Assays

Viability was determined with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison WI). This assay quantifies the amount of ATP present, which signals the presence of metabolically active cells. ATP was measured as luminescence produced by the mono-oxygenation of luciferin catalyzed by the Ultra-Glo-luciferase. Caspase 3/7 protease activity was measured using the Caspase-Glo 3/7 Assay kit (Promega, Madison WI) that quantifies the cleavage of the substrate, Ac-DEVD-pNA. Following the addition of immunotoxin for 1, 2 or 3 hours, protein synthesis inhibition was quantified by incubating cells in a 96-well format with $2\text{ }\mu\text{Ci/ml}$ ^3H -leucine (Perkin Elmer, Boston, MA) and then counting samples on a filter mat using a Wallac Beta plate reader (Perkin Elmer, Boston MA).

Subcellular fractionation and immunoblot analysis

Treated or control cells were collected, washed with phosphate buffered saline solution (PBS), and solubilized in RIPA buffer (Thermo Scientific Pierce, Rockford, IL) with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the Nanodrop2000c Spectrophotometer (Thermo Scientific, Waltham, MA). Equal amounts of protein were loaded onto NuPAGE 4-12% Bis-Tris gels (Life Technology Invitrogen, Grand Island, NY) and transferred to nitrocellulose membranes (Life Technology Invitrogen, Grand Island, NY). The following primary

antibodies were used: eIF2 α (Cell Signaling Technology, Danvers, MA), pEIF2 α (Epitomics, Burlingame, CA), CREB-2/ATF-4 (Santa Cruz Biotechnology, Santa Cruz, CA), Mcl-1, Bcl-xL and Bcl-2 (Cell Signaling Technology, Danvers, MA), tubulin (Sigma-Aldrich, St. Louis, MO). Primary antibodies were routinely detected with donkey anti-mouse horseradish peroxidase or donkey anti-rabbit horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific Pierce, Rockford, IL).

For the subcellular fractionation, treated or control cells were harvested and washed once with a glycine 'stripping' buffer (glycine 50mM, NaCl 150mM, pH 3). Cells were resuspended in cellular fractionation buffer (20 mM Hepes/KOH, pH 7.5, 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 1.5 mM EGTA, 250 mM sucrose, 1mM DTT), passed through a 25-gauge needle 10 times, and centrifuged for 10 min at $600 \times g$ to remove unbroken cells and nuclei and to gain post-nuclear supernatant. The post-nuclear supernatant was centrifuged at $7000 \times g$ for 10 min to pellet mitochondrion-enriched heavy membranes (HM) and heavy membrane supernatants. Heavy membrane supernatants were centrifuged in a fixed angle S100AT4-542 rotor at $100,000 \times g$ for 1.30 hours to separate the ER-containing light membrane (LM) fraction from the cytosol. The heavy and the light membranes were then solubilized with RIPA buffer containing 1mM DTT and both protease and phosphatase inhibitors. Equal amounts of protein from the cytosol and membranes were analyzed by Western blot using the primary antibodies for calnexin (Stressgen, Ann Arbor, MI), actin, cytochrome c (BD Biosciences, San Jose, CA) and TOMM20 (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-PE monoclonal antibody, M40-1, was described previously from this lab (24). Measurement of protein band intensities was performed using Image J software (NIH).

Results

Kinetics of ABT-737 and immunotoxin-mediated ER stress response

Previously, it was reported that the BH3-only mimetic, ABT-737, overcame resistance to PE immunotoxin-mediated apoptosis causing ER stress and inducing an unfolded protein response (UPR), as evidenced by the expression of ATF4 (20,21). Therefore, we sought to understand if the UPR was involved in the synergy between the immunotoxin and ABT-737. To address this, we carried out a time course of treatments with this compound either alone or in combination with an immunotoxin directed to the human transferrin receptor. For comparisons with a classic ER stress-producing agent, thapsigargin was included in many parallel experiments. When assayed alone on KB cells, ABT-737 provoked an increase in phosphorylated eIF2 α and expression of ATF4 at 2 and 4 hours, that peaked at 8 hours post addition and then declined (Fig 1). However, these early increases were less pronounced than the response seen with thapsigargin. (Fig 1). The active immunotoxin induces eIF2 α phosphorylation at 8 hours and later times. In contrast to ABT-737 alone, ABT-737 in combination with an active immunotoxin produced very different results. The phosphorylation of eIF2 α by the combination was greater than with either ABT-737 or immunotoxin alone, confirming a high level of ER stress. Despite the high level of phosphorylated eIF2 α in the combination, there was no evidence of ATF4 expression, mostly likely due to

toxin-mediated inhibition of protein synthesis. Thus, in combination treatments, the ER stress program is thwarted because ATF4 is not expressed. ABT-737 plus the inactive immunotoxin produced responses that were essentially identical to ABT-737 alone, confirming that the absence of ATF4 in combination treatments depended on the ADP-ribosylation of EF2 leading to the inhibition of protein synthesis.

When we performed a time course of the immunotoxin and ABT-737 action, we found that the immunotoxin alone produced a modest increase in caspase 3/7 activity after 16 hours (Fig 2a), which increased slightly after 24 hours with 40% cell survival (Fig 2b). ABT-737 alone did not stimulate apoptosis and, after 24 hours, reduced cell viability by approximately 50%. By contrast, a combination of the two agents increased caspase 3/7 activity starting at 2 hours with a major peak at 4 hours (Fig 2a). The peak in apoptosis was followed by substantial cell death at 8 hours (Fig 2b). In contrast, when cells were treated with thapsigargin in combination with the immunotoxin, we noticed only a modest increase in caspase 3/7 activity at 4 hours which was much less than the ABT-737-immunotoxin combination. Further, the thapsigargin-immunotoxin combination resulted in relatively poor cell killing with near complete cell survival at 8 hours. Thus even though thapsigargin produced a more robust ER stress response compared to ABT-737, this was not associated with either rapid caspase activation or increased cell death. In fact, only prolonged (24 hours) treatments of immunotoxin and thapsigargin resulted in reduced cell proliferation and any cell death (Fig2 a,b). From these results we conclude that, by itself, ER stress is not sufficient to enhance immunotoxin killing. It also suggests that ABT-737 harbors additional properties beyond the ability to provoke an ER stress response.

Immunotoxin-mediated inhibition of protein synthesis was enhanced in the presence of ABT-737

Previously, we showed that ABT-737 in combination with PE-based immunotoxins reduced cellular protein synthesis to a greater extent than either agent alone (20). One interpretation of that result postulated that ABT-737 enhanced toxin translocation from the ER to the cytosol. However, other interpretations are possible including that ABT-737 provoked greater toxicity (ie an ER stress response leading to reduction in protein translation rates) because of its activity at the level of ER. If the latter were the case, then thapsigargin treatment would also result in increased reductions in protein synthesis. To address this issue, we measured immunotoxin-mediated inhibition of protein synthesis kinetically in the presence and absence of ABT-737 and compared results using similar treatments with thapsigargin. The results clearly show that immunotoxin-mediated inhibition of protein synthesis was enhanced in the presence of ABT-737, suggesting that ABT-737 treatment resulted in the delivery of more toxin molecules to the cytosol (Fig 3). In this assay, combination effects could be seen as early as 2 hours post addition of both agents. However, similar results were not achieved when combinations of thapsigargin and immunotoxin were added to cells (Fig 3). From this we conclude that ER stress alone is not responsible for enhanced immunotoxin delivery to the cytosol.

ABT-737 increases ER membrane permeability to luminal proteins

Next we addressed the mechanism of enhanced immunotoxin translocation in the presence of ABT-737. Previous reports indicated that ER stress, induced by thapsigargin or tunicamycin, could increase ER membrane permeability allowing ER luminal proteins to be released into the cytosol with no loss of ER membrane integrity (25). To determine whether the permeability of the ER changed in the presence of ABT-737 alone, immunotoxin alone or both agents together, we probed microsomal and cytosolic fractions for evidence of dislocation of two ER luminal proteins, protein disulfide isomerase (PDI) and the 78-kDa glucose-regulated protein (GRP78/BiP). We chose a 4 hours time point because this coincided with the maximum activation of caspase produced by combinations of ABT-737 and immunotoxin. When cytosolic preparations were probed for ER luminal proteins, the amounts of PDI and GRP78 were notably higher in the cytosol of ABT-treated cells than in those of untreated or thapsigargin treated cells (Fig 4a). This suggested that ABT-737 increased ER membrane permeability to luminal proteins, possibly including any toxin that was present. However, the mechanisms of permeabilization induced by ABT-737 and thapsigargin are clearly different because thapsigargin does not enhance immunotoxin action. Moreover, ER permeability could be considered a feature of ER stress-induced apoptosis and GRP78 and PDI could be considered markers of this effect.

In the same cytosolic preparations there was little or no evidence of ER membrane proteins, as determined by blots detecting calnexin, confirming selective release of soluble components and not ER destruction. Of interest were cytosolic samples from combination treatments. When these were analyzed, we noticed reduced amounts of PDI and Grp78 in the combination with ABT737 compared to ABT-737 alone. While experiments aimed at understanding the nature of the dislocation process are undergoing, since we were monitoring a cytotoxic combination of immunotoxin and a BH3-mimetic at the peak of apoptotic induction and protein synthesis inhibition (Figs 2 and 3), we speculate that the channels or pores opened by ABT-737 change their conformation or are deregulated resulting in reduced translocation of chaperones. Similar accumulations of PDI and Grp78 were seen with combinations of immunotoxin and thapsigargin compared to thapsigargin alone. Minor reductions in protein synthesis levels and/or a small amount of apoptosis apparently did not alter the changes in permeability caused by thapsigargin. Another possible explanation could involve channels or pores with the immunotoxin competing for the same channels as those specifically regulated by ABT-737.

Our results show that there is a unique connection between ABT-737 treatment and the selective dislocation of luminal proteins. When membrane fractions were analyzed at 4 hr, there was evidence of chaperone up-regulation in cells treated with ABT-737 or thapsigargin. However, as noted already a similar level of chaperone upregulation (with ABT-737 and thapsigargin) did not produce the same amount of protein dislocation to the cytosol. In that regard, ABT-737 was far superior: suggesting that the increased levels of ER luminal proteins in the cytosol were due to increased dislocation rather than increased expression.

To confirm the contents of the cytosol were derived from apoptotic cells, we assessed samples from combination treatments for the release of cytochrome c (Fig 4b). As shown,

cytochrome c is recovered in the cytosol only in samples from the combination treatment with ABT-737 and not with immunotoxin alone or immunotoxin plus thapsigargin. Activation of caspase and release of cytochrome c provide two independent markers that apoptosis begins within 4 hours of adding ABT-737 and immunotoxin. The potency of PE-based immunotoxins depends on the presence of a functional ADP-ribosylating domain, leading to cessation of protein synthesis. But only small amounts of immunotoxin (below the detection level of western blot analysis) are needed to completely inhibit cellular protein synthesis. Enhanced ABT-mediated dislocation of the immunotoxin might be confirmed by immunoblotting if higher amounts of toxin protein could be detected in the cytosol. Therefore we generated an enzymatically inactive mutant of the immunotoxin. The mutant HB21PE40 differs from the wild type version by one amino acid: it lacks glutamic acid (critical for NAD binding) at position 553 (and is here called 'ntIT' for non-toxic immunotoxin). To quantify toxin translocation, relatively high concentrations (250ng/ml) of ntIT were added to cells in the presence and absence of ABT-737; and, after 4 hours, cytosol was prepared from treated and control cells. We measured the intensity of the ntPE band in the cytosol and in the membrane fraction for each treatment and then compared this ratio with the ratio of ntPE in the cytosol and membranes of the 'ntPE-only' treated cells. We determined that ABT-737 treatment resulted in four-fold more immunotoxin in the cytosol (Fig4b, supplemental Fig1). The presence of the 37kD in the cytosol indicated that the immunotoxin was correctly processed and only this form of the toxin was selectively translocated from the ER.

In order to show that the enhanced dislocation of the toxin predominantly occurred from the ER compartment, we transfected KB cells with a plasmid encoding full length *Pseudomonas* exotoxin (PE). Similar to the experiments with the immunotoxin, we used an active site mutant of PE lacking glutamic acid 553 (ntPE). Because we employ a mammalian secretion sequence, ntPE is delivered directly into the ER where a small fraction of the protein retrotranslocates to the cytosol while the remainder is mostly secreted. Transfected cells were allowed to express ntPE for 16 hours at which time ABT-737 or no treatment was applied for a further 4 hours. Cytosol preparations from these cells were then probed with antibodies to PE. From these we confirmed that ABT-737 treatment resulted in the same fold increase of ntPE in the cytosol that we had seen with the ntIT treated cells (Fig 5a).

Net accumulation of toxin could be due to enhanced delivery or interference with the normal degradation process. To investigate proteasome-mediated toxin degradation, we incubated cells with the inhibitor, MG132. There was no change in toxin accumulation in the cytosol, nor did MG132 influence the action of ABT-737, confirming that ABT-737 action was via enhanced delivery of toxin to the cytosol (Fig 5a). Further, treatments with thapsigargin in either system, at concentrations that cause ER stress, did not result in toxin accumulation in the cytosol (Fig 4b and Fig 5b), again confirming that the action of ABT-737 is specific for enhanced toxin translocation. From these results we conclude that ABT-737 alters the permeability of the ER, allowing more toxin molecules to reach the cytosol.

Regulation of the Bcl2 prosurvival proteins

Several studies have reported that the PE-based immunotoxin treatment leads to a rapid and marked decrease in the levels of the anti-apoptotic protein, Mcl-1 (14,20,26), suggesting that immunotoxin-induced apoptosis may be triggered by degradation of Mcl-1 that cannot be replaced due to inhibition of protein synthesis. However, when we monitored Mcl-1 levels following combination treatments of immunotoxin and ABT-737, there was no loss at 4 hr indicating that apoptosis did not depend on loss of this prosurvival protein (Fig 6). In combination treatments, complete loss of Mcl-1 was not noted until 8 hours post treatment while cells treated with the immunotoxin alone did not lose Mcl-1 until 16 hours post treatment. The earlier loss of Mcl-1, seen with the combination treatment compared with the immunotoxin alone, was consistent with ABT-737-mediated increase of toxin delivery to the cytosol inducing a faster shutdown of protein synthesis.

Discussion

A significant obstacle to the efficacy of PE-based immunotoxins is the inability of these agents to induce apoptosis despite causing inhibition of protein synthesis and the loss of the prosurvival protein, Mcl-1 (15,20). However, here we show that immunotoxin combinations with the BH3 mimetic ABT-737 caused a rapidly lethal synergism, with strong caspase activation being evident as early as 4hr post treatment and complete loss of viability as early as 8 hours. We reported previously an enhanced inhibition of protein synthesis in the presence of ABT-737 that was not seen with diphtheria toxin, suggesting a possible effect of ABT-737 on the ER that could help the PE-based toxins translocate into the cytosol (14,20). Here we report on a new feature of ABT-737 activity, namely the capacity to increase the permeability of soluble ER proteins leading to dislocation to the cell cytosol, including also PE and PE-derived immunotoxins. Dislocation was noted with both PDI and GRP78 and the increased retrotranslocation of those soluble proteins was not associated with the destruction of the ER membranes. Previously, it was reported that ER stress inducers, such as thapsigargin and tunicamycin, could provoke the release of endogenous ER luminal proteins to the cytosol during apoptosis in a process that was dependent on the proapoptotic Bcl-2 members Bax and Bak (25). Further, luminal proteins released into the cytosol were accompanied by the translocation and anchorage of Bax to the ER membrane (27). ABT-737 seems to have a similar effect on ER membranes confirming its action as ER stress inducer, but it seems also to trigger a specific permeabilization for immunotoxins since thapsigargin did not produce the same rapid or extensive translocation of the toxin into the cytosol. ABT-737 binds with high affinity to Bcl-xL and Bcl2, which are also localized on the ER membranes, and as a BH3 mimetic it causes apoptosis, priming the intrinsic pathway characterized by Bax translocation to mitochondrial and ER membranes (27). The evidence that ER permeabilization seems to be very specific for PE-based immunotoxins opens the hypothesis that there could be an interaction between the Bcl2 proteins on the ER and the toxin. This interaction could interfere with the suggested exit pathway from the ER to the cytosol through the Sec61p ER translocation pore (28) since the immunotoxin alone is able to kill the cells but the process is much slower than in the presence of ABT-737. Moreover, considering that there are similarities between the structure of the translocation domain of bacterial toxins and the transmembrane domain of the prosurvival members of the Bcl-2

family (29), the analysis of the possible protein interactions of the immunotoxin at the level of the ER compartment will be the next rational step in our study on the mechanisms of the PE-based immunotoxins action. The addition of ABT-737 causes ER stress activating the unfolded protein response. We extended the study on the ER stress induced by ABT-737 including also the immunotoxin alone and the combination with the ABT. We focused on the activation of the PERK kinase because this branch of the unfolded protein response is strictly correlated to the regulation of the protein synthesis in the cell (30). As mentioned above under ER stress conditions, PERK is activated and phosphorylates eIF2 α causing a translational arrest. This rapid response normally operates as an important pro-survival mechanism. Indeed, the deficiency of PERK or the expression of a non-phosphorylatable form of eIF2 α results in cells that are hypersensitive to ER stress (31) or hypoxic conditions during tumor growth (32,33), supporting the idea of a physiological role of translational attenuation on supporting cell survival via decreasing the un-folded protein load. Under prolonged ER stress, PERK signaling can trigger cell death. In fact, the artificial sustained activation of PERK, but not IRE1 α , induces proliferation arrest and cell death. One major event responsible for this switch between pro-survival and pro-apoptotic responses is the induction of C/EBP homologous protein (CHOP: also named growth arrest and DNA-damage-inducible 153 GADD153), by ATF4 (34). The most studied mechanism of cell death induced by CHOP is the regulation of several Bcl-2 members; the best-known effect is the down regulation of Bcl-2, sensitizing cells to apoptosis (35). When we treated the cells with the immunotoxin alone and in combination, we noticed a clear phosphorylation of eIF2 α but no expression of ATF4. In the presence of the immunotoxin, the persistent phosphorylation of eIF2 α and the consequent absence of ATF4 can delay the cytotoxic effect of the protein synthesis inhibition; however, eventually the cells will overcome this protection and die. In the presence of ABT737, there is at least four fold more toxin in the cytosol and protein synthesis inhibition is robust and the protective effect of eIF2 α phosphorylation is null. Moreover ABT-737, as BH3 mimetic, can sensitize the cells to apoptosis. Thus no adaptive UPR is possible and cells succumb rapidly to apoptotic cell death. Lastly, considering the outcome of combination treatments, it is noteworthy to point out that the degradation of Mcl-1 followed the strong activation of caspase and release of cytochrome c at 4 hours and probably amplified the induction apoptosis leading to complete cell death at 8 hours. From our study we conclude that the key to the synergy seen with the co-treatments of the immunotoxin and ABT-737 is a new feature attributed to ABT-737 action, namely the ability to permeabilize ER membranes. This causes a rapid and substantial delivery of toxin in the cytosol. As a consequence, the strong inhibition of protein synthesis in the first few hours of treatment annuls the temporary survival response due to the phosphorylation of eIF2 α and lack of ATF4. Moreover, loss of Mcl-1 complements ABT-737 action. Thus, in cells treated with both immunotoxin and ABT-737, prominent prosurvival proteins are either absent or neutralized, leading to enhanced apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ER	endoplasmic reticulum
PE	Pseudomonas exotoxin A
IT	immunotoxin
ntIT	non toxic immunotoxin
EF2	elongation factor 2, HM, heavy membranes
LM	light membranes

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Figure 1. Time course of ER stress induced by ABT-737 and immunotoxin treatment

KB cells were exposed for various times to the immunotoxin 10ng/ml, ABT-737 10μM, a combination of the immunotoxin and ABT-737, thapsigargin 5μM, and a combination with the non toxic immunotoxin and ABT-737. The level and the posttranslational modifications of the ER stress proteins were visualized by western blot with the indicated antibodies.

Figure 2. Apoptosis and cell death following ABT-737 and the immunotoxin treatment
 KB cells were treated for 2, 4, 8, 16 and 24 hours with the immunotoxin 10ng/ml, ABT-737 10μM, a combination of the immunotoxin and ABT-737, thapsigargin 5μM, and a combination of the immunotoxin and thapsigargin. A) Caspase 3/7 activity was determined by adding Ac-DEVD-pNA to each well and incubating the plate for 1 hour at room temperature. B) Cell viability was determined as quantitation of the ATP present in metabolically active cells using a thermostable mutant of *Photuris pennsylvanica* luciferase (LucPpe2^m), which improves performance in ATP assays. The luminescence of each well was measured and the values are presented as a percentage relative to PBS-treated cells. The mean values were determined from three different experiments where each point in each experiment was derived from triplicate plates. The *error bars* represent the standard deviation of the mean.

Figure 3. ABT-737 increases the rate of toxin-mediated inhibition of protein synthesis
KB cells were treated for 1, 2 and 3 hours with the immunotoxin 10ng/ml, ABT-737 10 μ M, a combination of the immunotoxin and ABT-737, thapsigargin 5 μ M, and a combination of the immunotoxin and the thapsigargin. Inhibition of protein synthesis was determined by measuring the incorporation of 3 H-leucine into cells.

Figure 4. Cytochrome c release and dislocation of ER chaperones and toxin following treatment with ABT-737

A) Cells were treated for 4 hours with immunotoxin 10ng/ml, ABT-737 10 μ M or immunotoxin plus ABT-737. Additionally, control cells were treated with either thapsigargin alone or in combination with immunotoxin at 10 ng/ml. After subcellular fractionation of lysed cells, cytosol and membranes were probed for the following ER chaperones, Grp78, PDI and calnexin. B) Cytosol and mitochondrial pellet were probed for cytochrome c, TOMM20 or actin. C) KB cells were treated for 4 hours with HB21PE40 553 (ntIT) 250ng/ml, ABT-737 10 μ M, a combination of ntIT and ABT-737, and a combination of ntIT and thapsigargin 5 μ M. After subcellular fractionation of the lysate, the cytosol and membranes were probed for the presence of the toxin using anti-PE antibodies. To quantify the reactive bands of the 37kDa PE fragment, scanned blots were analyzed using Image J. The amount of the 37kDa PE fragment, in the cytosol and the membranes of each treatment, was quantified relative to intensity of the corresponding band in the membrane fraction. The graph represents the ratio between the amount of the PE fragment in the cytosol and in the membranes expressed as fold increase over the cells treated with the ntIT alone. The mean values are determined from three different experiments and the error bars represent the standard deviation of the mean.

Figure 5. Enhanced translocation of non-toxic PE

A) Cells were transfected with ntPE or a vector control for 16 hours followed by the addition of ABT-737 10 μ M, MG132 0.2 μ M or combinations of both for 4 hours. B) Cells were transfected with ntPE or a vector control for 16 hours followed by the addition of thapsigargin 5 μ M for 4 hours. For all the conditions, cell cytosol and membranes were isolated and probed with antibodies to PE, GRP78, PDI, calnexin or actin. To quantify ntPE scanned blots were analyzed using Image J. The graph represents the fold ratio of band intensity in the cytosol over the corresponding band in the membrane fraction. The mean values are determined from two different experiments and the error bars represent the standard deviation of the mean.

Figure 6. Intracellular level of the Bcl-2 prosurvival family members with the immunotoxin and ABT-737

KB cells were incubated with the immunotoxin 10ng/ml, ABT-737 10mM, a combination of the immunotoxin and ABT-737, thapsigargin 5mM, and a combination of the non toxic immunotoxin and ABT-737, at different time points. The amounts of the Bcl-2 family proteins were visualized by western blot with the indicated antibodies.