

Paclitaxel and TRAIL Synergize to Kill Paclitaxel-resistant Small Cell Lung Cancer Cells through a Caspase-independent Mechanism Mediated through AIF

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Abstract. *Background: Small cell lung cancer (SCLC) is the most aggressive form of lung cancer with poor disease outcome. The chemotherapeutic agent paclitaxel (PA) is commonly used as a second-line treatment in SCLC, but response rates are low. Materials and Methods: 86M1 SCLC cells were treated in the presence or absence of paclitaxel and TRAIL or the combination for 24 hours. Western blot analysis was utilized to examine protein expression, cell surface protein expression and membrane integrity were elucidated by flow cytometry, and immunofluorescence microscopy was used to demonstrate translocation of proteins to the cell nucleus. Results: Human 86M1 SCLC cells were found to be resistant to PA killing in vitro. This resistance is mediated by up-regulation of pro-survival*

protein BCL-xl. However, PA also increases surface expression of death receptors 4 and 5 (DR4 and DR5, respectively). The death receptors' ligand increased SCLC killing by PA through an apparent caspase-independent route involving activation/translocation of AIF. Conclusion: The addition of TRAIL to PA can potentiate apoptosis in a relatively PA-resistant SCLC line (specifically 86M1 cells). More importantly, we are the first to report an active method of resistance to paclitaxel in SCLC via BCL-xl up-regulation.

Abbreviations: 7AAD: 7-Aminoactinomycin D; AIF: apoptosis inducing factor; BAK: Bcl-2 homologous antagonist/killer; BAX: Bcl-2-associated X; BCL-2: B-cell leukemia/lymphoma 2; BCL-xl: B-cell lymphoma-extra large; BIM: Bcl-2 interacting mediator of cell death; CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CTL: cytotoxic T lymphocyte; DR4: death receptor 4; DR5: death receptor 5; DTT: dithiothreitol; ES: extensive stage; FADD: Fas-associated protein with death domain; FAS L: FAS ligand; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; FLICE: FADD-like interleukin-1 beta-converting enzyme (caspase 8); GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LDH: lactate dehydrogenase; MCL-1: induced myeloid leukemia cell differentiation protein; MHC: major histocompatibility complex; OD: optical density; PARP: poly (ADP-ribose polymerase); PMSF: phenylmethanesulphonylfluoride; PUMA: p53 upregulated modulator or apoptosis; PVDF: polyvinylidene fluoride; SCLC: small cell lung cancer; SDS: sodium dodecyl sulfate; TNF: tumor necrosis factor; TRAIL: tumor necrosis factor related apoptosis inducing ligand; XIAP: X-linked inhibitor of apoptosis protein.

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Small cell lung cancer (SCLC) is an aggressive form of lung cancer. Although SCLC is a highly chemosensitive disease, outcome is generally poor and the 5-year survival rate is <10% (1). Diagnosis of extensive stage (ES) comprises approximately two-thirds of new SCLC cases, and the median survival of these patients is only 2-4 months if untreated, with survival increasing to 6-8 months with chemotherapy. This disease is very responsive to first-line chemotherapy with response rates of greater than 50% routinely observed. However, these responses are often short-lived and disease recurrence in the ES patient population is frequent. Patients with relapsed disease, or patients who fail to respond to chemotherapy generally succumb to their disease within a few months (1). Treatment of patients with relapsed SCLC is especially challenging if the disease is platinum-resistant, when disease progression occurs within 3 months of completion of a platinum containing regimen. In these patients, median survival ranges from 3.7 to 4.7 months (2-7). In SCLC, paclitaxel is primarily considered a second-line therapy after the failure of platinum-based treatment regimens (8). Several modes of action of paclitaxel have been described. The drug is most well known as a microtubule stabilizer. Specifically, paclitaxel binds to tubulin and interferes with spindle formation in mitosis, ultimately arresting cells in G₁ and G₂/M phases of the cell cycle, leading to cell death (9-13). In addition to stabilizing microtubules paclitaxel may act to sequester free tubulin, effectively depleting the cell's supply of tubulin (14). Beyond these effects on microtubules, more recent research has indicated that paclitaxel also induces

programmed cell death in cancer cells by binding to the pro-survival protein Bcl-2, blocking its function (15, 16). A variety of pharmacoinmunologic effects have also been attributed to paclitaxel (17-19).

There are two major pathways of apoptotic cell death. One pathway involves changes in mitochondrial membrane potential and the translocation of proteins from the mitochondria into the cytoplasm, including translocation of cytochrome *c*, ultimately triggering the activation of caspases and other proteins, subsequently leading to apoptosis (20, 21). BCL family proteins play key roles in the mitochondria and are central to regulating mitochondrial membrane integrity. The family consists of both pro-survival proteins, such as the well characterized BCL-2 and BCL-xl (22), as well as pro-apoptotic proteins including BAX and BAK (23). The other major apoptotic pathway is primarily dependent on caspase-8 activation driven by the binding of death receptors on the cell surface by death ligands. Two well characterized death receptors (DR) are DR4 and DR5 which are both activated by tumor necrosis factor related apoptosis inducing ligand (TRAIL). TRAIL is present on activated cytotoxic T lymphocytes (CTL), but not on unstimulated peripheral blood T-cells (24). Binding of DR4/5 by TRAIL results in the recruitment of Fas-Associated protein with Death Domain (FADD) and FADD-like interleukin-1 beta-converting enzyme (FLICE) to the intracellular portion of the DR. The resulting complex can then cleave and activate caspase-8, leading to apoptosis through mitochondrial dependent (BID cleavage) or mitochondrial independent (caspase-3 cleavage) pathways (25-28). It has been reported that paclitaxel exerts effects on both of the aforementioned pathways (29-34). Additionally, paclitaxel has been shown to regulate the expression of DR4 and DR5 (35-37). However, much of this remains controversial and there is disagreement in the literature relating to the exact mechanism of paclitaxel induced cancer cell apoptosis. This discord is primarily due to the variation in the concentration of paclitaxel used in the studies – some studies use biologically relevant doses of paclitaxel (25-150 nM), whereas other studies describe results obtained from cells treated with 10 to 20-fold the aforementioned doses (38).

In the study described herein we sought to elucidate mechanisms by which SCLC are resistant to PA as well as assessing a potential treatment strategy to overcome these mechanisms.

Materials and Methods

Cell propagation. 86M1 SCLC cells (39, 40) were maintained in complete RPMI (RPMI-1640 media, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and penicillin-streptomycin; Invitrogen, Carlsbad, CA, USA). Cells were enumerated, and live cells counted, using a hemacytometer and trypan blue exclusion.

Reagents. Paclitaxel was obtained as a 7 mM stock from Cardinal Health (Dublin, OH, USA) supplied through the Moffitt Pharmacy. Recombinant human TRAIL was obtained from R&D Systems (Minneapolis, MN, USA).

Cell death assay. Sensitivity of 86M1 SCLC to paclitaxel was determined by lactate dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). Briefly, SCLC cells were culture for 48 hours with or without different concentrations of paclitaxel ranging from 0.1 to 1000 nM. Following drug exposure, the plates were gently centrifuged and 50 µl of the culture supernatant was added to a new 96 well flat bottom plate. Kit substrate, 50 µl, was added and the plate was incubated for 30 minutes. Stop solution was then added, and the samples were gently mixed. Absorbance at 490 nm was recorded. Media background controls were also performed and subtracted from all of the recorded OD values. Specific cell lysis was quantitated using the following formula: (sample release minus spontaneous release) divided by (maximum release minus spontaneous release) × 100.

Death receptor expression. Surface expression of DR4 and DR5 was analyzed by flow cytometric analysis. Cells were treated with or without 100 nM paclitaxel for 20 hours. Cell culture suspensions were then harvested and cell washed in phosphate-buffered saline (PBS) and stained with phycoerythrin (PE)-conjugated anti-DR4 or -DR5 antibodies (EBiosciences, San Diego, CA, USA) diluted 1:50 in PBS. The cells were stained at room temperature for 20 minutes. The cells were then washed twice in PBS. A viability dye (7AAD) was then added. Live cells were gated as 7AAD-negative and this population was used to evaluate DR expression.

Acridine orange/ethidium bromide staining. Apoptosis was visualized by chromatin condensation. Cells were treated with or without 100 nM paclitaxel for 20 hours. Where indicated, cells were then treated with 50 ng/ml TRAIL (R&D Systems) for the final 1-4 hours of culture, following which the cells were harvested. The cells were then incubated with 100 µg/ml acridine orange (Roche, San Francisco, CA, USA), a DNA fluorochrome which is able to cross the intact plasma membrane, for 5 minutes as previously described (41). Since these were suspended cells, the cells were then placed on a slide and coverslipped. Chromatin morphology was analyzed using an Olympus inverted fluorescence microscope and Q Capture Pro Software (Olympus, Center Valley, PA, USA).

Annexin V staining. After treatment with paclitaxel and/or TRAIL, cells were harvested and washed in cold PBS and then resuspended at a density of 1-10×10⁶ cells per ml in 1X Annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). 100 µl of the cells were labelled with 10 µl Annexin V-Pacific Blue conjugate. The cells were incubated for 15-20 minutes in the dark, after which 400 µl 1X Annexin binding buffer was added to each tube. Cells were immediately analyzed using an LSRII cytometer (BD Biosciences). Annexin staining was analyzed using Flow Jo software (Tree Star Inc., Ashland, OR, USA). Apoptosis was quantified by the percentage of positive cells falling under the defined positive curve.

Cell fractionation. Mitochondrial and cytosolic cell fractions were obtained using the Mitochondria/Cytosol Fractionation kit from BioVision (Mountain View, CA, USA). Fractionation was carried

out according to the manufacturer's instructions. An antibody raised against a mitochondrial-specific protein, Cox IV (Cell Signal Technologies, Danvers, MA, USA), was detected by Western blot to validate fractionation and as a loading control for mitochondrial fraction protein loading. Proteins were subjected to Western blot analysis as described below.

Western blot analysis. Following treatment with or without 100 nM paclitaxel for 20 hours, and, where indicated, 50 ng/ml of TRAIL for the final 1-4 hours of culture, cells were harvested, washed, and resuspended in 1X CHAPS buffer (Cell Signal Technologies) supplemented with 5 mM dithiothreitol (DTT) (Cell Signal Technologies) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MI, USA). Protein concentration was determined using the BioRad (Hercules, CA, USA) protein assay and the lysates were diluted to equal concentrations. 3X or 10X sodium dodecyl sulfate (SDS)-loading buffer (BioRad) was added to a final concentration of 1X and samples were boiled for 5 minutes. The proteins were resolved on 4-20 or 8-16% gradient pre-cast SDS-Hepes polyacrilamide gels (Pierce/Fisher, Pittsburgh, Pennsylvania, USA) and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with Tris-buffered saline containing 5% non-fat milk (Carnation, Glendale, CA, USA) and 0.5% Tween (Fisher, Pittsburgh, PA, USA). For phospho-protein blotting, the 5% non-fat milk was replaced with 5% bovine serum albumin (BSA) (Sigma). The membranes were incubated in primary antibodies over night at 4°C (antibodies indicated in figures and figure legends). The membranes were then washed and incubated for 1 hour in horseradish peroxidase-conjugated secondary goat anti-rabbit or rabbit anti-mouse antibodies (Millipore). Proteins were detected using ECL plus (Amersham) and hyperfilm (Amersham). All antibodies were purchased from Cell Signal Technologies, except anti-AKT and anti-pAKT which were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Flow cytometric analysis of mitochondrial membrane potential and caspase activation. Mitochondrial membrane potential and caspase activation were determined by flow cytometry using the Dual Sensor MitoCasp kit (Cell Technology Inc., Santa Monica, CA, USA). The cells were acquired using a FACSCalibur cytometer (BD Biosciences) and staining intensity was analyzed using Flow Jo software (Tree Star Inc.).

AIF fluorescence microscopy. SCLC cells were treated as indicated and then harvested. The cells were permeabilized and fixed using Fix and Perm (Calbiochem, San Diego, CA, USA). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-AIF antibody (Abcam, Cambridge, MA, USA) and washed in PBS. The cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) and allowed to settle on a slide under a coverslip. Fluorescence microscopy was performed using a Zeiss LSM 510 Laser Scanning Confocal Microscope (Zeiss, Thornwood, NY, USA). Images and co-localization were evaluated using Image Pro Plus 6.2 (Media Cybernetics, Silver Spring, MD, USA).

Results

86M1 SCLC cells are resistant to killing by paclitaxel. We determined the effect of paclitaxel monotherapy on SCLC cell line 86M1. The cells were not sensitive to biologically

relevant doses (100-200 nM) of paclitaxel *in vitro* (Figure 1A). In fact, the lethal dose, 50% (LD₅₀) was not achieved with 5-10 fold times this dose. For all remaining experiments, a biologically relevant concentration of 100 nM (which resulted in the killing of approximately one quarter of the cells) was used.

DR expression on SCLC is increased following culture with paclitaxel. Paclitaxel has been shown to induce apoptosis of a cancer cells, however, the exact mechanism of this activity has not been fully elucidated, although paclitaxel was first described as disrupting microtubules and inhibiting mitosis. With a recent report suggesting that chemotherapy can promote the CTL-dependent induction of tumor cell apoptosis (42), it was a logical next step to determine if paclitaxel enhances the expression of DR that CTL bind to initiate apoptosis, namely DR4 and DR5. Flow cytometry was employed to show that DR4 and DR5 expression is upregulated on the surface of SCLC cells following 20 hours of treatment with 100 nM paclitaxel (Figure 1B).

PA and TRAIL synergize to kill SCLC. CD8⁺ T-cells are thought to be the principle mechanism by which the immune system recognizes and kills tumor cells, and the goal of a large proportion of proposed immunotherapies for cancer are based on promoting anti-tumor T-cell responses (43). The two major modes by which T-cells kill tumor cells are through the release of granzymes that enter the cells and set off a cascade of caspase activation and by the ligation of DR on the target cells. The most characterized death receptor ligands used by cytotoxic T-cells to induce apoptosis of target cells are FAS ligand (FAS L), tumor necrosis factor (TNF), and TRAIL (44). Since the receptors for TRAIL (DR4 and DR5) were upregulated upon treatment with paclitaxel (Figure 1B), we next determined if paclitaxel would sensitize SCLC to killing *via* TRAIL. Cells were cultured alone or with paclitaxel for 20 hours. Following this incubation, the cells were then treated with TRAIL for 1-4 hours. We then evaluated apoptosis in two different experiments. Annexin staining was used to identify membrane instability (exposure of phosphatidyl serine). Paclitaxel alone induced only modest apoptosis, however when SCLC cells were treated with paclitaxel and TRAIL in combination, the number of apoptotic cells dramatically increased (Figure 2A). Concordant with the Annexin staining results, the visualization of chromatin condensation was also seen only in doubly treated cells. Either treatment alone only resulted in a minimal number of cells displaying condensed chromatin upon microscopic examination (Figure 2B).

Paclitaxel/TRAIL combination induces apoptosis of SCLC through an apparent caspase-3 independent mechanism. The roles of mitochondria and caspases in the paclitaxel and TRAIL

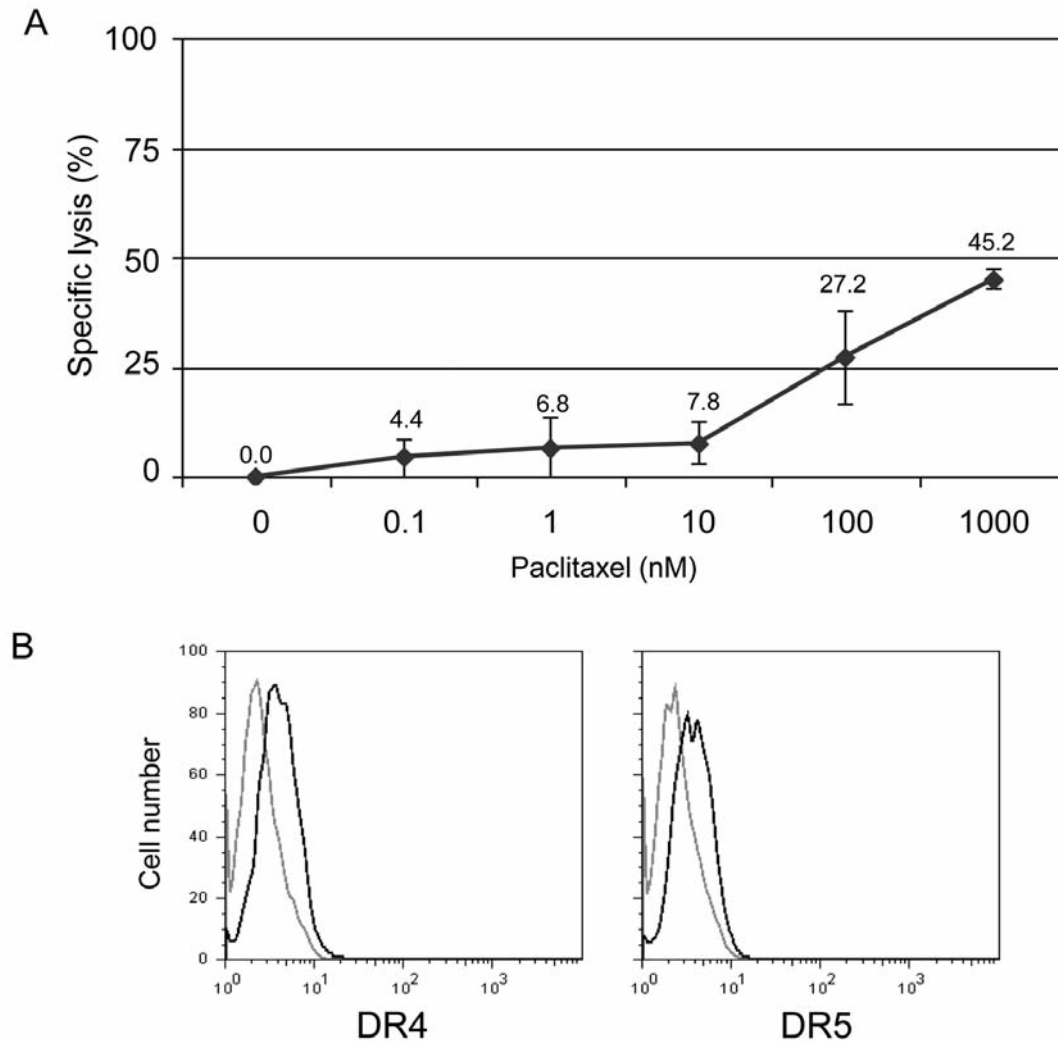


Figure 1. Effect of paclitaxel on SCLC 86M1 cells. A: SCLC cells were cultured with the indicated concentration of paclitaxel for 24 hours. Following the incubation, specific lysis was assessed by LDH release. Percentage specific lysis was calculated as: (sample release minus spontaneous release) divided by (maximum release minus spontaneous release) $\times 100$. LD50 was not achieved even at 1000 nM paclitaxel. B: Death receptor 4 and 5 expression is up-regulated on SCLC 86M1 cells following treatment with paclitaxel. Cells were treated with paclitaxel (100 nM) for 20 hours. Cells were harvested and labelled with PE-conjugated anti-DR4 or -DR5 antibodies. Live cells were gated using exclusion of 7AAD labelling. The light grey histogram shows untreated cells and the black histogram corresponds to SCLC treated with paclitaxel.

initiated apoptosis was examined using a MitoCasp assay kit, as well as examining caspase activation. Mitochondrial membrane potential and caspase activation was determined using flow cytometry. Cells were treated with a fluorescently labeled cationic dye to detect membrane potential, or loss thereof. In healthy cells, the cationic dye is accumulated by the mitochondria in proportion to the membrane potential. In apoptotic cells, where the mitochondrial membrane potential is compromised, the cationic dye does not accumulate in the mitochondria and these cells exhibit a lower fluorescence signal. Cells were also treated with carboxyfluorescein (FAM) labeled fluoromethyl ketone (FMK)-peptide inhibitors of

caspases that specifically bind active caspases. Using this assay we found that there was a loss of membrane potential in cells treated with paclitaxel, TRAIL and paclitaxel with TRAIL. There is no binding of the FAM-FMK molecules, indicating no caspase activation (Figure 3A). The loss of membrane potential in the paclitaxel and TRAIL singly treated cells was curious, as use of the two agents combined resulted in increased apoptosis as compared to either agent alone.

To further confirm these results and understand the mechanism involved in the increased apoptosis induced by the paclitaxel/TRAIL combination, Western blot analysis was performed. We confirmed that paclitaxel and TRAIL (both

as single agents and in combination) do not induce activation of caspases 3, 8, or 9 in 86M1 SCLC cells. We also demonstrated that the induced apoptosis, as described above, does not appear to be mediated through Poly (ADP-ribose) polymerase (PARP) cleavage (Figure 3B).

Paclitaxel actively induces resistance in SCLC tumor cells by up-regulating pro-survival BCL-xl expression. Huisman, *et al.* demonstrated that paclitaxel treatment of non-small cell lung cancer (NSCLC) cells triggers cell death through a caspase-independent, mitochondrial-dependent route (30). Therefore, we examined proteins that have been demonstrated to play significant roles in mitochondrial-dependent apoptosis. Most notably we observed that paclitaxel up-regulates the expression of the pro-survival protein BCL-xl (Figure 4). This increased expression was abrogated when TRAIL was combined with paclitaxel. In cells treated with both agents, another pro-survival protein, pBAD, was down-regulated as compared to paclitaxel treatment alone (Figure 4). Interestingly, there was also a decrease in BID expression following treatment with the paclitaxel/TRAIL combination (Figure 4). This was in contrast to a recently published report in which Huisman *et al.* demonstrated that paclitaxel killed non-small cell lung cancer (NSCLC) in a caspase-independent manner in which the cleavage of BID appears to play an important role in paclitaxel-specific killing (45). However, we did not observe BID cleavage with paclitaxel alone, but rather saw reduced expression of full length BID with only the doubly treated cells (Figure 4). However, as outlined below, upon mitochondrial and cytosolic fractionation, a role for BID becomes evident (Figure 5A). No changes in expression were observed for XIAP, MCL-1, Survivin, PUMA, and BIM. pAKT expression did increase upon treatment with either paclitaxel or TRAIL, as well as the combined treatment as compared to untreated cells (Figure 4). The significance of this is unknown, however Zhou *et al.* have demonstrated that active AKT (pAKT) plays an important role in suppressing apoptosis at the post-mitochondrial stage, *i.e.* downstream of cytochrome c release, and before activating caspase-9 (46). This may explain the lack of caspase activation in SCLC following treatment with paclitaxel and/or TRAIL. There was, however, low level endogenous caspase-8 activity in the cell lysates, but there was not a detectable difference in the levels of cleaved caspase-8 in untreated or singly/doubly-treated SCLC cells.

Paclitaxel/TRAIL combination induces AIF-dependent apoptosis in paclitaxel-resistant SCLC cells. Since PARP cleavage did not appear to be crucial in the apoptotic effect mediated by the paclitaxel/TRAIL combination, we determined which molecule was likely mediating the ultimate DNA fragmentation necessary to complete the cell

death process. After separating mitochondrial and cytosolic proteins and performing immunoblotting, it was observed that although the mechanism of killing of SCLC by the paclitaxel/TRAIL combination is mitochondrial dependent, cytochrome c release is not part of the death pathway induced by this combination treatment (Figure 5A). Interestingly, the influx of BID, and to a much lesser extent of BAX, into the mitochondria was observed with doubly treated cells as compared to either paclitaxel or TRAIL treatment alone. We also found that apoptosis inducing factor (AIF) was cleaved into a mature form following co-treatment of SCLC cells with paclitaxel and TRAIL (Figure 4). Further investigation demonstrated that in PA and TRAIL doubly-treated SCLC cells, AIF does indeed leave the mitochondria, translocate through the cytosol, and localize to the nucleus of the cell where it is able to initiate DNA degradation (Figure 5A-B). No changes in Endo G expression or cellular localization were observed (Figure 5A).

Discussion

SCLC is frequently responsive to first-line chemotherapy. However, the response is often short-lived and response rates to second-line therapies are poor. We sought to determine the mechanism of resistance to paclitaxel and how this resistance can be overcome. Western blot analysis demonstrated that BCL-xl expression increases in response to paclitaxel, suggesting that this is a mechanism by which SCLC cells become resistant to this common second-line chemotherapy agent. Additionally, it was determined that biologically relevant doses of paclitaxel induced the surface expression of DR4 and DR5. Although there was not a robust increase in surface expression of the DR, in the case of DR5, the expression went from none to dim as detected by fluorescently labelled antibodies. Addition of the DR4/DR5 ligand, TRAIL, lead to the abrogation of paclitaxel-induced BCL-xl expression, and also resulted in a decrease in the expression of the pro-survival protein pBAD (47) and activation of pro-apoptotic BID. Cleaved BID translocates to mitochondria and induces cytochrome c release and mitochondrial damage (48). The combination also resulted in increased apoptosis of SCLC cells as determined by both Annexin V staining and chromatin condensation. With this treatment combination, the induced apoptosis was mediated, at least in part, through AIF and was independent of significant caspase activation and increases in PARP (Poly (ADP-ribose) polymerase) cleavage. The lack of caspase involvement in killing mediated by paclitaxel has been reported elsewhere for other cancer types, such as NSCLC (30). However, we now report that the addition of TRAIL to paclitaxel can potentiate apoptosis in a paclitaxel-resistant SCLC cell line, as well as the novel observation that the mechanism

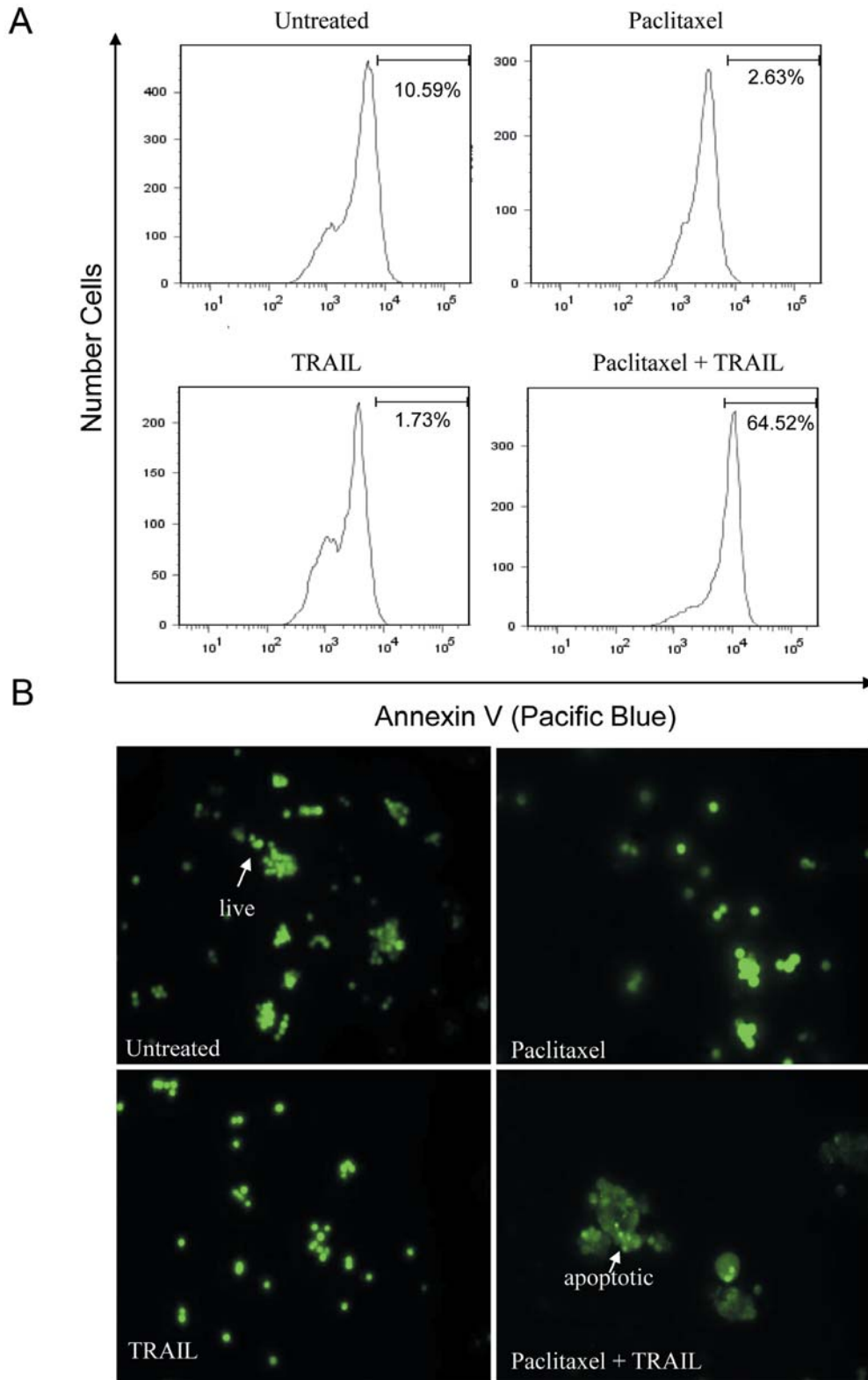


Figure 2. Paclitaxel and TRAIL synergize to kill SCLC cells. A: Cells were treated in the presence or absence of paclitaxel (100 nM) for 20 hours. Where indicated, 50 ng/ml TRAIL was added for the final 1-4 hours of culture. Apoptosis was also analyzed using Annexin V staining. A significant increase in Annexin staining was observed, as indicated by the histogram shift to the right. B: Cells were cultured as above, harvested, and then stained with acridine orange to visualize chromatin. Live cells have intact nuclei, whereas apoptotic cells have fragmented and condensed nuclear content.

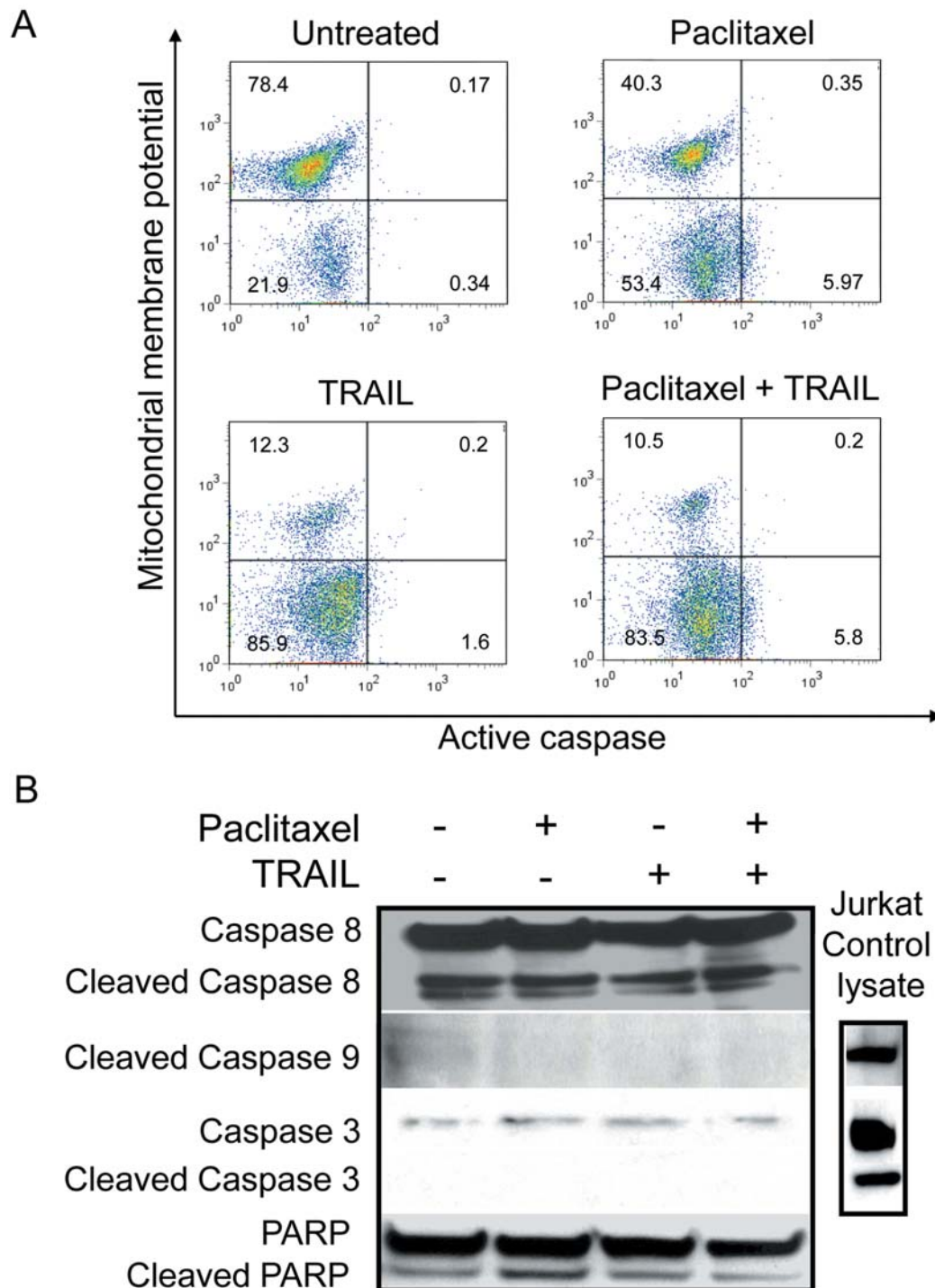


Figure 3. SCLC apoptosis induced by paclitaxel/TRAIL. SCLC apoptosis induced by paclitaxel/TRAIL combination is concomitant with loss of mitochondrial membrane potential and independent of caspase activation. A: Cells were treated with or without paclitaxel (100 nM) for 20 hours. Where indicated, 50 ng/ml TRAIL was added for the final 1-4 hours of culture. Cells were harvested and stained using MitoCasp reagents. Following paclitaxel, TRAIL, or combined treatment there was a loss of mitochondrial membrane potential. However, active caspases were not identified. Jurkat cells treated with cycloheximide and TRAIL were used as a positive control (data not shown). B: Western blot analysis was performed to verify the flow cytometric analysis of caspase activation: 100 μ g of protein was loaded per well of the SCLC lysates, 50 μ g of control Jurkat lysate (cycloheximide and TRAIL treated) was loaded. SCLC cells have lower levels of caspase-3 and -9 expression than do Jurkat-cells and no changes in cleaved caspases were observed in extracts from SCLC cells left untreated or treated with either paclitaxel, TRAIL, or the combined treatment. A representative GAPDH blot is shown to demonstrate equal protein loading.

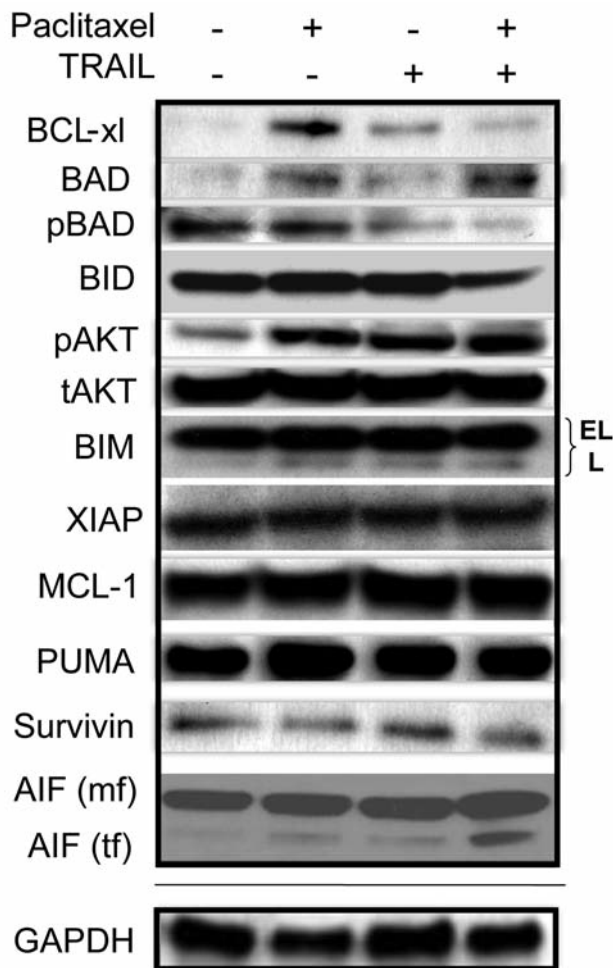


Figure 4. Paclitaxel induces the upregulation of the expression of pro-survival BCL-xl in SCLC. SCLC cells were treated with single agent or paclitaxel/TRAIL combination (100 nM and 50 ng/ml, respectively) for 24 hours. Proteins from cell lysates were resolved on a gel and transferred to PVDF. Immunoblotting was performed using the indicated primary antibodies. Paclitaxel treatment resulted in the up-regulation of BCL-xl. BCL-xl expression decreased in SCLC treated with both paclitaxel and TRAIL. The expression of full length BID was modestly decreased in doubly treated cells. Truncated, active AIF (tf) can be seen only in doubly treated cells (mf – mitochondrial form). Apoptosis mediated by the paclitaxel/TRAIL combination occurs, at least in part, through AIF activation. A representative GAPDH blot is shown to demonstrate equal loading of protein.

of this potentiation is through a reversal of paclitaxel-induced BCL-xl expression. Also involved in this synergistic killing, as determined by Western blot, is an up-regulation of the expression of pro-apoptotic pAKT and pBAD, concomitant with a decrease in pro-survival BAD (Figure 4), in the lysates from cells treated with the combination of paclitaxel and TRAIL. It is likely that the

regulation of these apoptosis-associated proteins also contribute to the synergistic killing observed with paclitaxel and TRAIL.

Our research group recently conducted a clinical trial with a therapeutic cancer vaccine, Ad.p53-DC, for SCLC (49). Although there were some clinical responses, the majority of patients, 23 out of 27, had disease progression and were treated with additional chemotherapy. Interestingly, the patients who received additional chemotherapy subsequent to the vaccine responded at an unusually high rate and clinical response to salvage chemotherapy was closely associated with the induction of an immunological response to vaccination. This synergy has been observed by others as well (50-52). The findings presented here offer an explanation not only for the resistance of SCLC to second-line treatment with paclitaxel, but also provide a possible explanation for the increased sensitivity to second-line treatment in patients vaccinated in our Ad.p53 trial. We hypothesize that TRAIL-mediated CTL killing of SCLC cells is inadequate, however with the addition of paclitaxel, AIF-mediated killing can occur. The converse also occurs. Paclitaxel is inadequate in the absence of TRAIL, due to the induced resistance mediated through BCL-xl up-regulation. It is only when TRAIL and paclitaxel are combined that BCL-xl expression is reduced and AIF is activated and translocated. When co-delivered *via* polymeric micelles, the two agents are further shown to have synergistic focused anticancer activity (53). This bivalent activity reflects the widespread potential of TRAIL in countering chemoresistance, such as in combination with PPAR γ ligands against ovarian cancer (54).

There is an emerging paradigm in cancer immunotherapy in which immunotherapeutic treatments may be more effective when used in direct combination with chemotherapy (49-51, 55-57). Specifically, immunotherapy alone for the treatment of SCLC is likely inefficacious for a variety of reasons. For decades, studies have suggested that tumor cells develop mechanisms to avoid immune-mediated rejection. Over twenty years ago, Doyle *et al.* showed that SCLC can have decreased expression of MHC I at both the mRNA and protein level, which could dramatically limit the expression of tumor-associated antigens (TAA) on the surface of the tumor cells (58). More recent studies show that there is a decrease in FAS expression on SCLC cells (59, 60) and that some lung cancer cells produce a soluble decoy receptor, DcR3, that mimics FAS and binds FAS L (61). DcR3 could act to bind FAS L on CTL and obstruct the interaction between the CTL ligand and the tumor cell receptor, allowing the tumor to escape T-cell-mediated killing. Another mechanism of immuno-evasion involving a relevant receptor pathway utilized by CTL to kill tumor cells has been described and involves the silencing or loss of DR5 and/or caspase-8 expression in SCLC (60, 62).

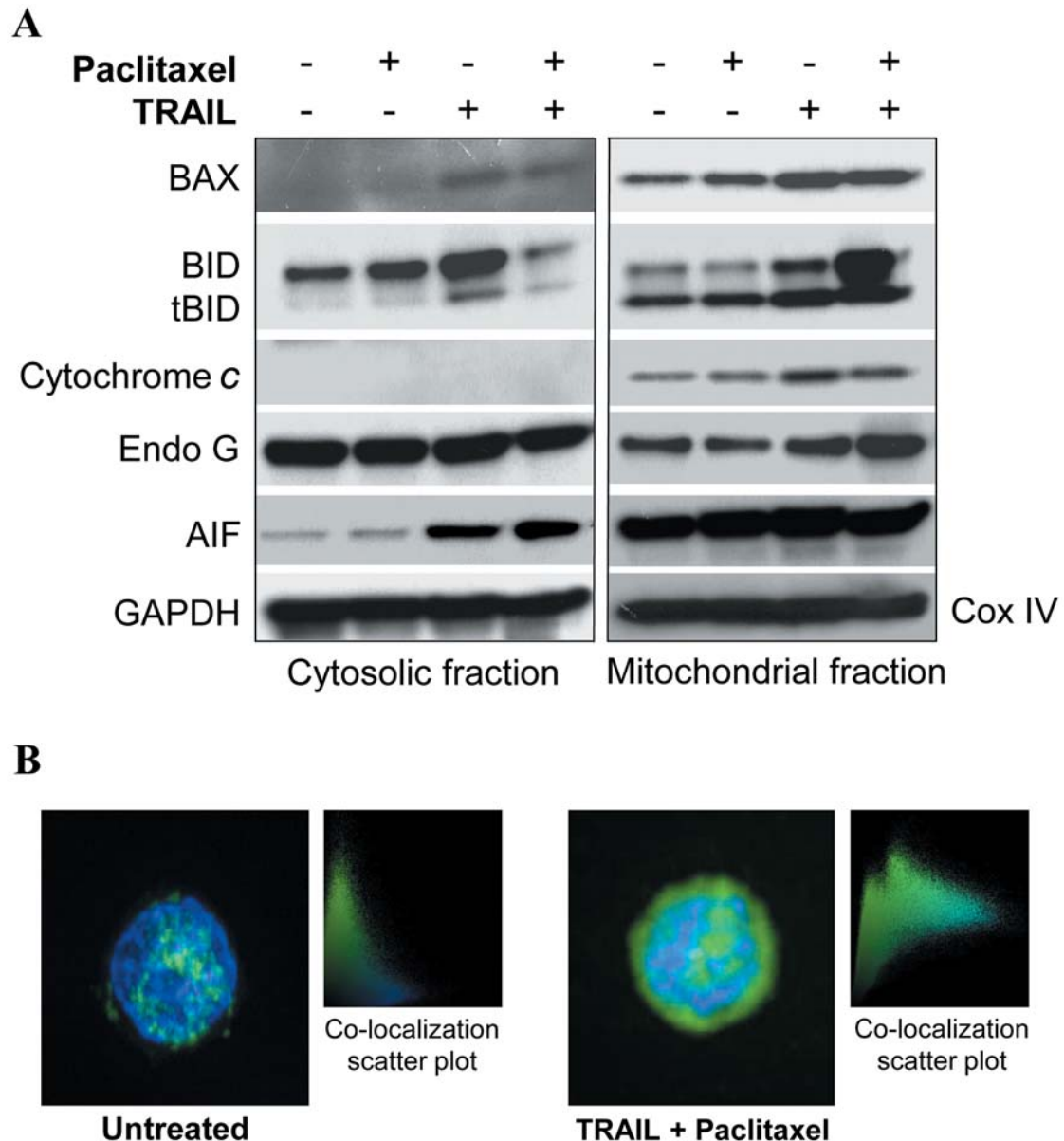


Figure 5. Paclitaxel and TRAIL synergize to kill SCLC cells. Killing of SCLC cells by paclitaxel with TRAIL is independent of cytochrome *c* release and appears to be mediated through the translocation and ultimate nuclear localization of AIF. A: Mitochondrial and cytosolic fractions of SCLC treated as indicated were resolved on polyacrylamide gels, transferred and then subjected to immunoblotting with the indicated antibodies. Most notably, pro-apoptotic BID was demonstrated to leave the cytosol and enter the mitochondria, cytochrome *c* release from the mitochondria into cytosol was not observed, and AIF translocated into the cytosol following treatment with paclitaxel and TRAIL. GAPDH and CoxIV were used as loading controls for the cytosolic and mitochondrial fractions, respectively. B. AIF was also determined to localize in the nucleus of doubly-treated cells. Briefly, treated cells were stained with FITC- labeled AIF and DAPI. In untreated as well as singly treated cells (data not shown) AIF did not co-localize in the nucleus, however in doubly treated cells, AIF was found in the nucleus.

Additionally, it has recently been shown that there is an up-regulation of BCL-2 in SCLC and that this increased expression potentiates chemotherapy resistance in SCLC cell lines (63), possibly by blocking TRAIL-induced apoptosis (64). Interestingly, each of these described mechanisms of

tumor immuno-evasion relate directly to pathways utilized by CTL to recognize and induce apoptosis of tumor cells. Emerging literature, in concordance with our data represented herein, points to a need to consider combining chemotherapy with immunotherapy. Understanding the mode of actions and

the pharmaco-immunologic effects of chemotherapeutic drugs will allow for thoughtful development of combination treatment strategies, specifically with immunotherapeutic regimens, to treat cancer.

Competing Interests

The Authors declare that they have no competing interests.

Authors' Contribution

TBH participated in conception of the experiments and project design, performed cell death assays, cell fractionation, and microscopy, and drafted the manuscript. NM, KL and TC performed Western blot analyses. SJA assisted in conception of the project and design of the experiments and assisted in drafting of the manuscript. All authors have read and approved the final manuscript.

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