

Taxanes Sensitize Prostate Cancer Cells to TRAIL-Induced Apoptotic Synergy via Endoplasmic Reticulum Stress **A** **C**



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ABSTRACT

Docetaxel and cabazitaxel are guideline-chemotherapy treatments for metastatic castration-resistant prostate cancer (mCRPC), which comprises the majority of prostate cancer deaths. TNF-related apoptosis inducing ligand (TRAIL) is an anticancer agent that is selectively cytotoxic to cancer cells; however, many human cancers are resistant to TRAIL. In this study, we sensitized androgen-independent and TRAIL-resistant prostate cancer cells to TRAIL-mediated apoptosis via taxane therapy and examined the mechanism of sensitization. DU145 and PC3 cells displayed no significant reduction in cell viability when treated with soluble TRAIL, docetaxel, or cabazitaxel alone indicating that both cell lines are resistant to TRAIL and taxanes individually. Taxane and TRAIL combination synergistically amplified apoptosis strongly suggesting that taxanes sensitize prostate cancer cells to TRAIL. A Jun N-terminal kinases (JNK) inhibitor

inhibited apoptosis in treated cells and significantly reduced death receptor expression indicating JNK activation by ER stress sensitizes PCa cells to TRAIL-induced apoptosis by upregulating DR4/DR5 expression. In addition, suppression of C/EBP homologous protein (CHOP) reduced TRAIL sensitization in both cell lines indicating that ER stress-related apoptosis is mediated, in part, by CHOP. Cytochrome *c* knockdown showed a significant decrease in sensitivity in PC3 cells, but not in Bax-deficient DU145 cells. A computational model was used to simulate apoptosis for cells treated with taxane and TRAIL therapy as demonstrated in *in vitro* experiments. Pretreatment with taxanes sensitized cells to apoptosis induced by TRAIL-mediated apoptosis, demonstrating that combining TRAIL with ER stress inducers is a promising therapy to reverse TRAIL resistance to treat mCRPC.

Introduction

Prostate cancer is the second leading cause of death in U.S. men (1, 2). Once prostate cancer has reached an advanced stage that no longer responds to hormone treatment or chemotherapy, a situation called metastatic castration-resistant prostate cancer (mCRPC), treatment options are limited (3). FDA-approved chemotherapies for mCRPC are taxanes, docetaxel (DTX) and cabazitaxel. Docetaxel is the first line of treatment given to patients with mCRPC who stop responding to conventional therapies (4–6). Unfortunately, most patients develop resistance to docetaxel. Cabazitaxel (CBZ), the dimethoxy derivative of docetaxel, becomes the second line of treatment demonstrating a survival benefit for patients with docetaxel-refractory mCRPC (6, 7). Effective treatment options for patients with mCRPC still remain a significant clinical problem because resistance to chemotherapy treatment and metastatic progression still occur. In the last decade, there has been more attention and focus on cancer stem cells (CSC) and their role in tumor recurrence and poorer prognosis in patients with mCRPC. Like stem cells, CSCs constitute a small subpopulation in tumors that have clonogenic and self-renewal abil-

ities that can help explain their tumorigenicity, metastatic potential, and chemotherapy/radiotherapy resistance (8–11).

A promising anticancer therapeutic agent is TNF-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein that selectively kills cancer cells while sparing normal cells. Apoptosis is induced when TRAIL binds to death receptors DR4 and DR5 on the surface of a cell (12, 13). Previous studies in our lab have used TRAIL liposomes to effectively target and kill circulating tumor cells (CTC) while reducing metastatic tumor burden in various animal models (14–16). TRAIL does have a limitation due to the variety of mechanisms cancer cells undergo to develop TRAIL resistance (17–19).

The endoplasmic reticulum (ER) is a highly dynamic organelle that is responsible for protein and lipid synthesis in eukaryotic cells (20, 21). Protein production, folding, transport, and maintaining protein homeostasis is one of the main functions of the ER and is important to the viability of cells. When the normal functions of the ER are disturbed, a cellular stress response is initiated called the unfolded protein response (UPR), that attempts to reestablish normal ER function and restore homeostasis (22–24). If ER stress is chronic or prolonged and irreversible cell damage occurs, there is a switch from pro-survival to proapoptotic signaling pathways involving distinct ER stress sensors (25, 26).

Previous studies have shown the sensitization of prostate cancer cells to TRAIL-induced apoptosis when treated with different small molecules (27–36). Although there have been numerous studies that have examined TRAIL sensitization in prostate cancer cells with clinically-relevant chemotherapies, these studies do not fully explore the role of ER stress in the mechanism behind TRAIL sensitization using these taxanes (37–41). In this study, we investigated the role of ER stress in the mechanism of sensitization of prostate cancer cells to TRAIL-induced apoptosis via taxane pretreatment, and we adopted a computational model to predict this synergistic effect of apoptosis in each cell line. We confirmed that pretreatment with taxanes

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significantly enhanced the susceptibility of DU145 and PC3 cells to TRAIL-induced apoptosis compared with taxane or TRAIL alone. This synergistic effect was mediated by CHOP and JNK, proapoptotic factors associated with chronic ER stress. Taxane treatment resulted in a marked increase in DR5 expression. This study provides new insight into the mechanism of TRAIL sensitization by ER stress and mitochondrial pathways *in vitro* and presents a useful computational model of these processes.

Materials and Methods

Cell culture

Prostate cancer cell lines DU145 (ATCC #HTB-81) and PC3 (ATCC #CRL-1435) were obtained from ATCC. DU145 cells were maintained in Eagle minimum essential medium (EMEM) cell culture media (Corning). PC3 cells were cultured in F12 cell culture media (Gibco, Thermo Fisher Scientific). Media was supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (both purchased from Gibco) under humidified conditions at 37°C and 5% CO₂. All cell lines were obtained in Spring 2018 and placed in liquid nitrogen until required for use. Cells were tested twice using a Mycoplasma Detection Kit (ATCC 30–1012K) following manufacturer's protocol. Testing occurred once after thawing at passage 3 and then near the end of experiments at passage 15. Test results were negative. Cells were last tested on April 1, 1978.

Chemicals/reagents

Cabazitaxel (ADV465749196) and docetaxel (01885) were purchased from Sigma Aldrich. Both taxanes were dissolved in a 1:10 solution of DMSO:PBS (pH 7.4) to a final concentration of 100 µmol/L. Dimethylsulfoxide (DMSO) was purchased from ATCC. Soluble histidine-tagged TRAIL (BML-SE721–0100) was purchased from Enzo Life Sciences. Azoramidate (5796), SB 202190 p38 MAPK inhibitor (1264), and SP 600125 JNK inhibitor (1496) were all purchased from Tocris.

Small interfering RNA transfection

Two small interfering RNA (siRNA) oligonucleotide sequences were used to target Smac (siDiablo/Smac; Assay ID: 290077 and 182519), Cytochrome *c* (siCYCS; Assay ID: 132742 and 132743), and CHOP (siDDIT3; Assay ID: s3995 and s3996). A negative control siRNA (Scr; AM4611) was used to control for siRNA delivery effects. All were purchased from Thermo Fisher Scientific. Cells were plated in 12-well plates and transfected using the siRNAs, Lipofectamine RNAiMAX reagent (Invitrogen), and Opti-MEM (Gibco) according to the manufacturer's instructions. Cell media were replaced after 48 hours and then cells were treated with taxanes alone (24 hours), sTRAIL (24 hours) alone or in combination (24 + 24 = 48 hours). The final concentration for each siRNA was 30 nmol/L.

Annexin V/PI apoptosis assay

Human prostate cancer cell lines DU145 and PC3 (5×10^4 cells) were seeded in 24-well plates overnight and then exposed to TRAIL (100 ng/mL), taxane drug (0.25 µmol/L docetaxel or cabazitaxel), inhibitor (20 µmol/L azoramidate, SB202190, or SP600125), or a combination for 24 hours. For pretreatment studies, cells were treated with inhibitor for 24 hours, then taxane for 24 hours, then TRAIL for 24 hours. For posttreatment studies, cells were treated with a taxane for 24 hours, then inhibitor for 24 hours, then TRAIL for 24 hours. The sequential treatment plan was chosen to determine the ability and capacity of sensitization that occurs with taxane alone without TRAIL

treatment and to allow adequate time for protein translation. Cells were harvested using Accutase (StemCell Technologies) and analyzed by an Annexin V/propidium iodide (AV/PI) flow cytometry assay to assess cell viability. FITC Annexin V (556419) and propidium iodide (556463) staining solutions were purchased from BD Biosciences. Staining of untreated and treated cells was carried out according to the manufacturer's instructions. Cells were incubated for 15 minutes with reagents at room temperature in the dark and immediately analyzed using a Guava EasyCyte 12HT benchtop flow cytometer (Millipore Sigma). Viable cells were classified as AV⁻/PI⁻, early apoptotic cells as AV⁺/PI⁻, late-stage apoptotic cells as AV⁺/PI⁺, and necrotic cells as AV⁻/PI⁺. Flow cytometry plots were analyzed using FlowJo software (FlowJo). The following control samples were used to calibrate the instrument: unlabeled cell samples to evaluate the level of autofluorescence and adjust the instrument accordingly, and cell samples labeled individually with AV and PI to define the boundaries of each cell population.

Synergistic evaluation by Jin formula and Chou-Talalay method

The synergistic effect of combined taxane and TRAIL was analyzed by Jin formula (36, 42) and Chou-Talalay method (43, 44). For Jin formula, $Q = E_{a+b}/(E_a + E_b - E_a \times E_b)$, where E_{a+b} , E_a and E_b are the average inhibitory effects of the combination treatment, taxane only and TRAIL only, respectively. In this method, $Q < 0.85$ indicates antagonism, $0.85 < Q < 1.15$ indicates additive effects and $Q > 1.15$ indicates synergism. The E_{a+b} , E_a and E_b values were obtained from the apoptosis assay. These values were analyzed further using CompuSyn software (45) to obtain combination index (CI) values for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations.

CSC identification in surviving fraction

CSCs were characterized as CD44⁺/CD24⁻ subpopulations. Following the apoptosis assay protocol described previously, cells from each treatment group were also labeled using anti-CD44-APC (338805) and anti-CD24-Brilliant Violet 421 (311121) mAbs (BioLegend). Mouse IgG1 APC (400119) and mouse IgG2a Brilliant Violet 421 (400259) constituted isotype controls (BioLegend). The following control samples were used to calibrate the instrument: unlabeled, AV only, PI only, AV/PI, mouse IgG1 APC only, mouse IgG2a Brilliant Violet 421 only, anti-CD44-APC only, and anti-CD24-Brilliant Violet 421. Flow cytometry plots were analyzed using FlowJo software (FlowJo).

Endoplasmic reticulum stress assay

PC3 and DU145 cells were seeded on 6-well plates at a density of 400,000 cells per well. The cells were incubated at 37°C and allowed to adhere overnight. The next day, the cancer cells were treated with 0.25 µmol/L of docetaxel or cabazitaxel, or left untreated for 24 hours. After treatment, the cells were washed with Hank balanced salt solution (HBSS) without calcium or magnesium (Thermo Fisher Scientific; 14170112) and lifted with trypsin (Thermo Fisher Scientific; 25200072). The cells were collected and fixed with 4% paraformaldehyde (Electron Microscopy Sciences; 15714-S). After fixation, the cells were permeabilized using Triton X-100 (Sigma-Aldrich; T8787). The cells were then blocked using 5% BSA (Sigma-Aldrich; A4161), and subsequently stained with a phycoerythrin (PE)-conjugated antibody against XBP1 (BioLegend; 647503) and an Alexa-Fluor 488-conjugated antibody against GRP78 (Thermo Fisher Scientific; 53-9768-80). Fluorescence was measured using a flow cytometer and

median fluorescence intensity was used to determine expression of the ER stress markers XBP1 and GRP78.

MTT assay

PC3 and DU145 cells were seeded on a 96-well plate at a density of 10,000 cells per well. The cells were incubated at 37°C and allowed to adhere overnight. The next day, the cancer cells were either left untreated or treated with 0.25 $\mu\text{mol/L}$ of docetaxel or cabazitaxel for 24 hours. The cells were then washed with HBSS without calcium or magnesium. After washing, the cells were treated with or without 25 $\mu\text{mol/L}$ Z-VAD-FMK (Tocris; 2163) for 30 minutes before treating cells with or without 100 ng/mL TRAIL. The cells were incubated with combinations of Z-VAD-FMK and TRAIL for 24 hours. After the treatment, cell viability and proliferation were measured using the MTT assay (Abcam; ab211091) according to the manufacturer's instructions. Cells were incubated with 50 μL of serum-free media and 50 μL of MTT reagent for 3 hours. The media was then aspirated and the cells were incubated with the MTT solvent for 15 minutes. Finally, the absorbance was measured at OD 570 nm using a microplate reader.

Death receptor expression

TRAIL receptor surface expression was analyzed using Human TruStain blocking solution (422302), PE Mouse IgG1 isotype control (400112), PE anti-human DR4 (307206), and PE anti-human DR5 (307406). Reagents were purchased from BioLegend. Cells were plated in 24-well plates and treated with cabazitaxel and docetaxel for 24 hours. Cell were then lifted, fixed with 4% paraformaldehyde, blocked with TruStain in BSA, and incubated with respective antibodies before performing flow cytometry. The median fluorescence intensity was measured and analyzed to determine relative expression.

Antibodies and Western blot analysis

DU145 and PC3 cells were transfected with siRNA oligonucleotides or incubated overnight and subsequently treated with different treatments for the indicated times. Afterwards, cells were rinsed with sterile PBS and lysed with 4 \times Laemmli sample buffer (Bio-Rad; 1610747) and then subjected to SDS-PAGE [7% (w/v) for DR4 and DR5, 15% (w/v) for Diablo/SMAC, cytochrome *c*, and CHOP] and transferred to polyvinylidene difluoride membranes. After transfer, membranes were blocked with 5% milk (Boston BioProducts) in Tris-buffered saline supplied with 0.1% Tween (Thermo Fisher Scientific). Primary antibodies were prepared at 1:500 dilution in 5% milk in the case of DR5 (Abcam; ab199357) and DDIT3 (Abcam; ab1419). Smac (Cell Signaling Technology, 15108) primary antibody was prepared at 1:1,000 dilution in 5% milk. In the case of DR4 (Abcam; ab8414), cytochrome *c* (Abcam; ab133504), GAPDH (Millipore; MAB374), primary antibodies were prepared at 1:5,000 dilution in 5% milk. Anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Rockland) were prepared at 1:2,000 dilution in 5% milk. Membranes were imaged with West Pico (Thermo Fisher Scientific) as per their respective protocols, using an ImageQuant LAS-4000 system (GE Healthcare).

Computational model

The computational simulation was performed in MATLAB using the systems-ODE solving function "ode15s". Figures utilizing the simulation were created in MATLAB. The systems of ODEs used in the simulation were derived from the Hope-King and Albeck-Sorger models (46, 47). The biochemical reactions were modeled using the mass-action kinetic equations shown by Hope and colleagues. A cell

was considered apoptotic when the concentration of cPARP reached half the initial condition of PARP. Mitochondrial permeability was determined by measuring the concentration of cytochrome *c* present in the cytosol. To create an ER-stressed or taxane-treated condition, the initial conditions of Bcl-2, cIAP1/2, and DR4/5 were altered as shown in Supplementary Table S1. To account for the different apoptotic responses of DU145 and PC3 cells, different initial conditions were used for Bax and x-linked inhibitor of apoptosis protein (XIAP) as shown in Supplementary Table S2.

Statistical analysis

GraphPad Prism 8 software was used to plot and analyze datasets. Student *t* test was used for comparisons between two groups, with $P < 0.05$ considered significant. ANOVA was used for comparing multiple groups with $P < 0.05$ considered significant. Data are presented as mean \pm SD with at least three independent replicates used for each experiment.

Results

Pretreatment with CBZ and DTX sensitize DU145 and PC3 cells to TRAIL-induced apoptosis synergistically

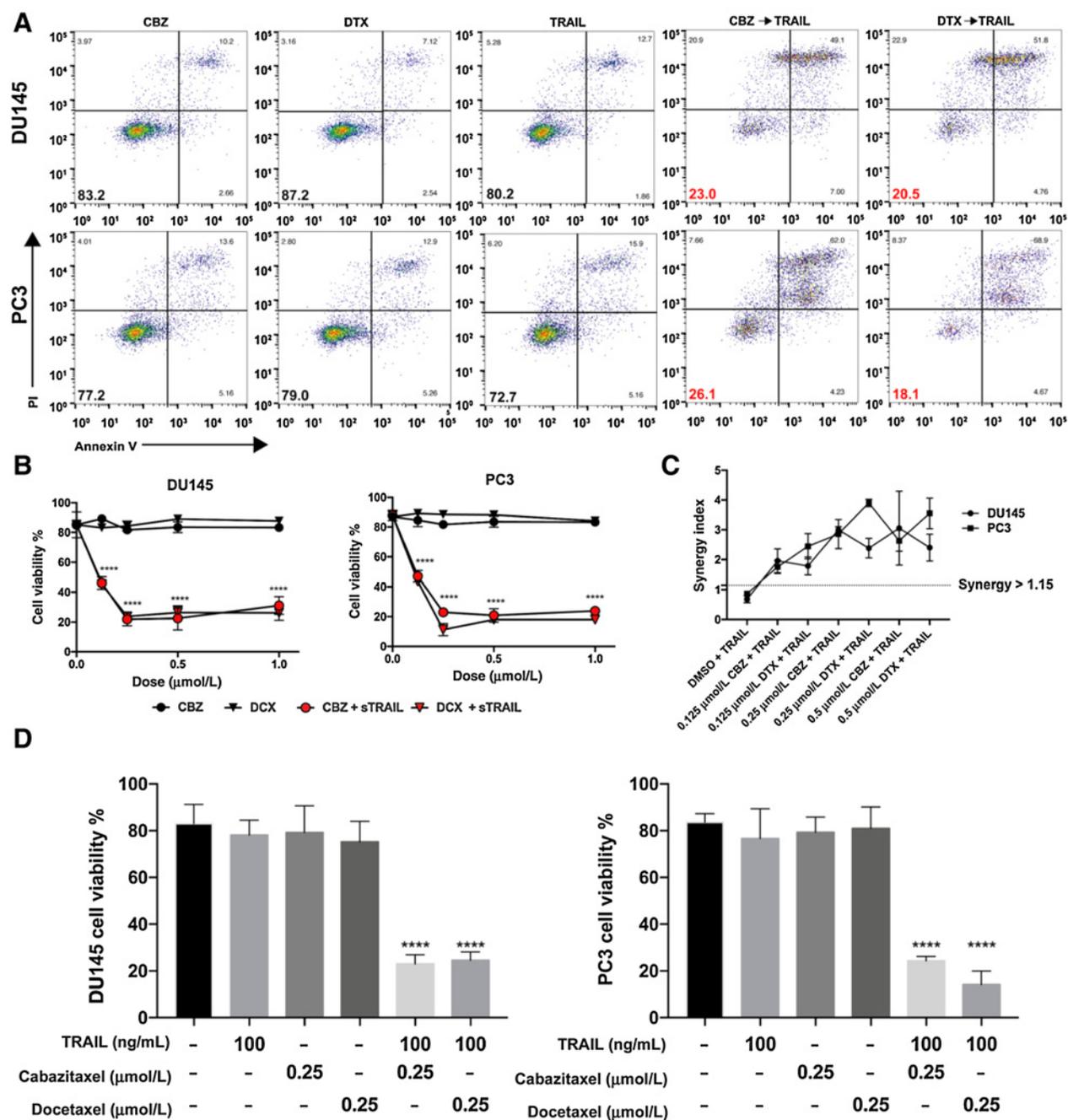
To determine whether metastatic, hormone-independent DU145 and PC3 cells were resistant to TRAIL, cells were treated with 100 ng/mL of soluble TRAIL for 24 hours and analyzed by flow cytometry (Supplementary Fig. S1A). PC3 cells displayed no significant reduction in cell viability compared with the untreated control cells, confirming their resistance to TRAIL-induced apoptosis. DU145 cells displayed a more significant reduction in cell viability compared with untreated control cells, indicating an increased susceptibility to apoptosis although cell viability remained high at approximately 80% (Supplementary Fig. S1B).

Because taxanes can inhibit cell growth through G₂-M arrest, cell proliferation was explored and measured using an MTT assay to capture growth inhibition as well as cell death. In addition, a caspase inhibitor, Z-VAD-FMK, was used to illustrate growth inhibition through the inhibition of apoptosis (Supplementary Fig. S4). For both cell lines, MTT absorbance decreased when treated with cabazitaxel only, docetaxel only, or cabazitaxel/docetaxel in combination with TRAIL demonstrating the growth-inhibitory effects of taxanes alone and of the combination. For DU145 cells, Z-VAD-FMK had no effect on any treatment including the combination. For PC3 cells, increased MTT absorbance was seen in Z-VAD-FMK cells illustrating that growth inhibition is impeded by the apoptosis inhibition (Supplementary Fig. S4A and S4B).

To determine cell viability when exposed to taxanes, DU145 and PC3 cells were treated for 24 hours with cabazitaxel and docetaxel at concentrations ranging from 0.01 $\mu\text{mol/L}$ to 1 $\mu\text{mol/L}$. At these dosages, cell viability for both cell lines was >80% when treated with taxanes alone (Supplementary Fig. S1C). Cells were also treated with each taxane over a 24- to 96-hour period to assess apoptosis over prolonged exposure times (Supplementary Fig. S1D). The cell lines exhibited a time-dependent response to each taxane, demonstrating decreasing cell viability over time, with DU145 cells showing increased cell death compared to PC3 cells. When treated with either docetaxel or cabazitaxel, DU145, and PC3 cells showed rounder morphologies and were moderately detached from the plates but still viable after 24 hours.

In both cells, pretreatment with various concentrations (0–1 $\mu\text{mol/L}$) of each taxane for 24 hours and then treatment with 100 ng/mL of TRAIL was efficient in inducing apoptosis in DU145 and PC3 cells

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**Figure 1.**

Synergistic effect of taxane plus TRAIL treatment in TRAIL-mediated apoptosis of DU145 and PC3 cells. **A**, Representative flow cytometry plots of DU145 and PC3 cells after treatment with taxane alone, TRAIL alone, or taxane then TRAIL. **B**, Cell viability of DU145 and PC3 cells when treated with varying concentrations of taxane alone or in combination with TRAIL. **C**, Synergistic antitumor effect of combined taxane and TRAIL in DU145 and PC3 cells by Jin formula. **D**, Cell viability of DU145 and PC3 cells when treated with 0.25 μmol/L taxane alone or in combination with TRAIL. The values represent the mean ± SD ($n = 6$). ****, $P \leq 0.0001$ compared with control.

(Fig. 1A). Cell viability decreased two-fold when exposed to the lowest concentration of taxane at 0.125 μmol/L in combination with TRAIL. At 0.25 μmol/L, cell viability decreased almost 4-fold in DU145 and remained at this level with increasing concentration of taxane (Fig. 1B). We determined the most effective concentration to be 0.25 μmol/L to produce a significant response of TRAIL-induced apoptosis. Further increasing the concentration did not have an

additional effect on cell viability for the sequential therapy. Pretreating cells with taxanes for 24 hours followed by TRAIL significantly decreased cell viability from approximately 80% down to approximately 20% (Fig. 1D). Furthermore, taxane and TRAIL exerted a synergistic inhibitory effect ($Q > 1.15$, $CI < 1$) in both cell lines. Such synergy was observed when TRAIL was combined with either taxane (Fig. 1C; Supplementary Table S3). These data confirm that

TRAIL-induced apoptosis is promoted in DU145 and PC3 cells pretreated with either cabazitaxel or docetaxel, and demonstrates a significant synergistic effect.

CSCs comprise the surviving fraction in DU145 and PC3 cells

We used flow cytometry to help characterize the surviving fraction of stem-like tumor cells in the taxane plus TRAIL-treated groups. We identified the cancer cells with stem-like characteristics as the CD44⁺/CD24⁻ subpopulation (Fig. 2A and D). In the total cancer cell population of DU145 cells, the CSCs subpopulation is relatively low at approximately 12% in the control group and linearly increases to approximately 40% in both combination treatment groups. The CSC subpopulation in the viable cell population follows the same pattern of approximately 11% in the control group and approximately 40% in both combination treatment groups (Fig. 2B). There was a significant difference when compared to the control of each population and their respective treatment groups; however, no significant difference was observed between total population versus viable subpopulation in each treatment group suggesting that CSCs in this cell line may slowly differentiate and proliferate into more CSCs in response to each progressive therapy (Fig. 2C).

In the total cancer cell population of PC3 cells, the CSCs subpopulation is moderately higher at approximately 35% in the control group and remains at this percentage even in both combination treatment groups. Surprisingly, the CSC subpopulation in the viable cell subpopulation was relatively high at approximately 65% in all groups except the combination groups in which there was a 10% decrease (Fig. 2E). A significant difference was observed between the total population and the viable subpopulation in each group, but none was observed when comparing each population with their respective controls except for in the viable combination group. Despite the difference in CSCs percentage compared with DU145 cells, PC3 cells displayed the same pattern in distribution among populations and treatment groups (Fig. 2F).

Mitochondrial and ER stress pathways of apoptosis affect sensitization

DU145 and PC3 cells were transfected with the siRNA targeting SMAC and CYCS and then treated with TRAIL alone or TRAIL with taxane to calculate the degree of TRAIL sensitization. The efficiency of each siRNA knockdown in each cell line was confirmed via Western blot analysis (Fig. 3A and D). The relative expression of SMAC and CYCS showed a significant decrease compared to control indicating the knockdown was effective (Fig. 3B and E). Inhibition of SMAC showed no significant difference in cell death when compared with control samples of both cell lines (Fig. 3C and F). In DU145 cells, cytochrome *c* inhibition resulted in no significant sensitization effect; while PC3 cells showed a significant decrease in taxane-mediated TRAIL sensitization (Fig. 3C and F). Sensitization of these cells was reduced to 29.3% and 23.04% for cabazitaxel- or docetaxel-treated cells with knocked down cytochrome *c* compared with 72% and 71.3% in scrambled-siRNA cabazitaxel- or docetaxel-treated cells, respectively. DU145 cells are deficient in Bax, indicating that mechanisms other than mitochondrial apoptosis induction are activated (48).

To demonstrate the activation of ER stress, the modulation of the expression of GRP78 (a biomarker for ER stress) and XBP1 (downstream transcription factor of JNK) were analyzed (Supplementary Fig. S4C and S4D). In DU145 cells, GRP78 expression significantly increased upon treatment with cabazitaxel and docetaxel and XBP1 expression was more pronounced. In PC3 cells, only docetaxel-treated cells showed an increase in expression compared with untreated

control and XBP1 expression did not significantly change. These results indicate that ER stress is occurring for both cell lines when treated with docetaxel. PC3 cells treated with cabazitaxel showed no significant increase in the ER stress surface markers (Supplementary Fig. S4C and S4D).

Chronic ER stress can initiate apoptotic mechanisms, like CHOP, that lead to cell death (49). DU145 and PC3 cells were transfected with CHOP siRNA, and then treated with TRAIL and/or taxane to calculate sensitization. The efficiency of each siRNA knockdown in each cell line was confirmed via Western blot analysis (Fig. 3A and D). The relative expression of CHOP displayed a significant decrease compared with control indicating that the knockdown was effective (Fig. 3B and E). In both cell lines, CHOP inhibition showed a significant effect on TRAIL sensitization and effectively attenuated taxane/TRAIL-induced death. Knockdown of CHOP in DU145 cells reduced sensitization from 74% and 72.8% for scrambled-siRNA cabazitaxel- or docetaxel-treated cells, compared with 26.6% and 21.7% in siCHOP cabazitaxel- or docetaxel-treated cells, respectively (Fig. 3C). Knockdown of CHOP in PC3 cells reduced sensitization from 72% and 71.7% for scrambled-siRNA cabazitaxel- or docetaxel-treated cells compared to 35% and 38.1% in siCHOP cabazitaxel- or docetaxel-treated cells, respectively (Fig. 3F).

Increased DR5 expression in prostate cancer cells after treatment with cabazitaxel and docetaxel

Treatment with taxanes alone caused a marked increase in cell surface expression of DR5 compared with no treatment for both DU145 and PC3 cells, but not DR4 (Fig. 4A). There is a higher baseline expression of DR5 in DU145 when compared with PC3 innately; however, both cell lines express higher levels of DR5 when treated with taxanes (Fig. 4B). The relative fluorescence intensity for DR5 in DU145 cells exhibited a 24.5% increase when treated with cabazitaxel and a 42.3% increase when treated with docetaxel compared to control. The relative fluorescence intensity for DR5 in PC3 cells exhibited a 58.7% increase when treated with cabazitaxel and a 61.8% increase when treated with docetaxel compared to control. When measuring the protein expression using Western blot analysis, we observed a significant increase in DR5 expression upon exposure to 0.25 μ mol/L of each taxane in both cell lines (Fig. 4C). DR4 expression did not significantly change after exposure to each taxane (Fig. 4D).

There have been conflicting reports about DR5 regulation by CHOP in relation to ER stress and TRAIL sensitization (50, 51). An increase in DR5 expression was observed in both cell types in response to taxane exposure with negligible effects on DR4 upregulation (Fig. 4E and F). The inhibition of CHOP expression did not attenuate DR5 upregulation, suggesting that taxane-induced DR5 expression is independent of CHOP. This further implies that CHOP is not the only or even a major regulator of DR5 induction in DU145 and PC3 cells; however, CHOP does have a role in the initiation of apoptosis in this taxane-induced ER stress-sensitization to TRAIL.

Death receptor regulation and synergistic apoptosis is mediated by JNK activation

The posttranscriptional regulation of CHOP is mediated by the p38 MAPK family coinciding with JNK activation. In this study, we inhibited p38 MAPK and JNK with SB202190 and SP600125 inhibitors. We also used a small-molecule modulator of UPR, azoramidate (52). Azoramidate pre- and posttreatment displayed a significant decrease in cell viability in both cell lines similar to taxane plus TRAIL alone (Supplementary Fig. S2A and S2B). These data illustrate that azoramidate does not improve ER-stressed cells that are committed to

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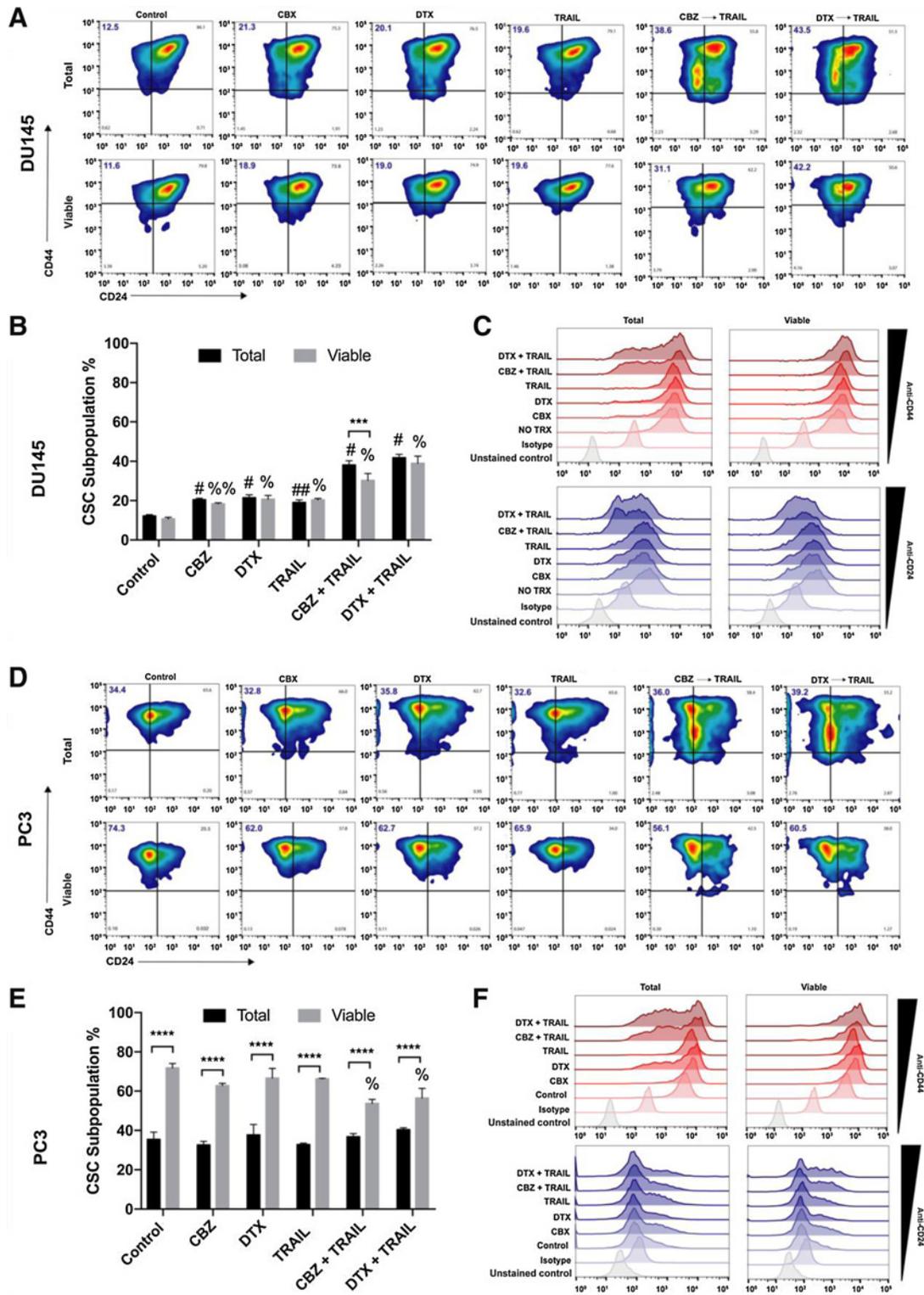
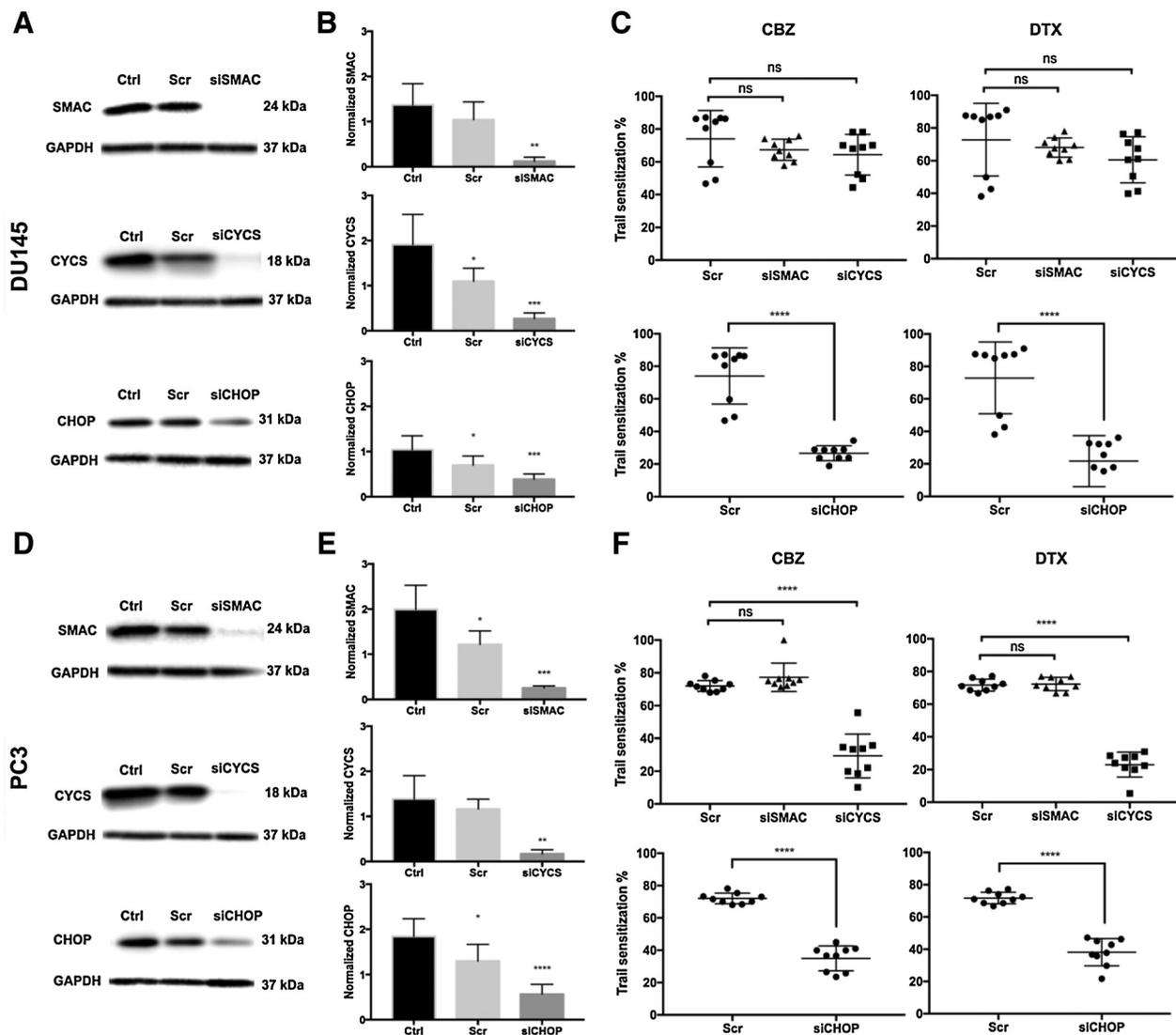


Figure 2.

Analysis of CD44⁺/CD22⁻ stem cell population in viable percentage of DU145 and PC3 cells. **A** and **D**, Representative CD44/CD24 flow cytometry plots of DU145 and PC3 cells after each treatment displaying total percentage of CD44⁺/CD22⁻ stem cells in first quadrant. **B** and **E**, Mean fluorescence intensity of CD44⁺ and CD24⁺ cell populations in DU145 and PC3 comparing total and viable populations. **C** and **F**, Stacked histograms displaying cell CD44⁺ and CD24⁺ cell population shifts in DU145 and PC3 cells across treatment groups. The values represent the mean \pm SD ($n = 6$). ***, $P < 0.001$; ****, $P \leq 0.0001$, significantly different from total versus viable. ##, $P \leq 0.001$; #, $P \leq 0.0001$, significantly different from total population control. %, $P \leq 0.001$; %%, $P \leq 0.0001$, significantly different from viable subpopulation control.

Taxanes Sensitize Prostate Cancer Cells to TRAIL Synergy

**Figure 3.**

Cytochrome c knockdown reduces TRAIL-induced apoptosis in PC3 cells, but not in Bax-deficient DU145 cells. CHOP knockdown reduces TRAIL-induced apoptosis in DU145 and PC3 cells. **A** and **D**, Western blot analysis of SMAC, cytochrome c, and CHOP expression in DU145 and PC3 cells after siRNA knockdown. **B** and **E**, Relative expression of SMAC, cytochrome c, and CHOP after siRNA knockdown compared with control. **C** and **F**, TRAIL sensitization of DU145 and PC3 cells when treated with respective taxane and TRAIL after scrambled siRNA, SMAC/Diablo, and cytochrome c knockdown. The values represent the mean \pm SD ($n = 6$ or 9). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; ****, $P \leq 0.0001$.

apoptosis. Pre- and posttreatment with SB202190 displayed a significant decrease in cell viability indicating that the p38 MAPK pathway does not contribute to the sensitization of DU145 and PC3 cells (Supplementary Fig. S2A and S2B). Posttreatment and not pretreatment with JNK inhibitor SP600125 was sufficient to abrogate the effects of TRAIL-mediated apoptosis after taxane sensitization (Fig. 5A and B). These data demonstrate that the JNK signaling pathway is involved and necessary for the apoptotic response to ER stress.

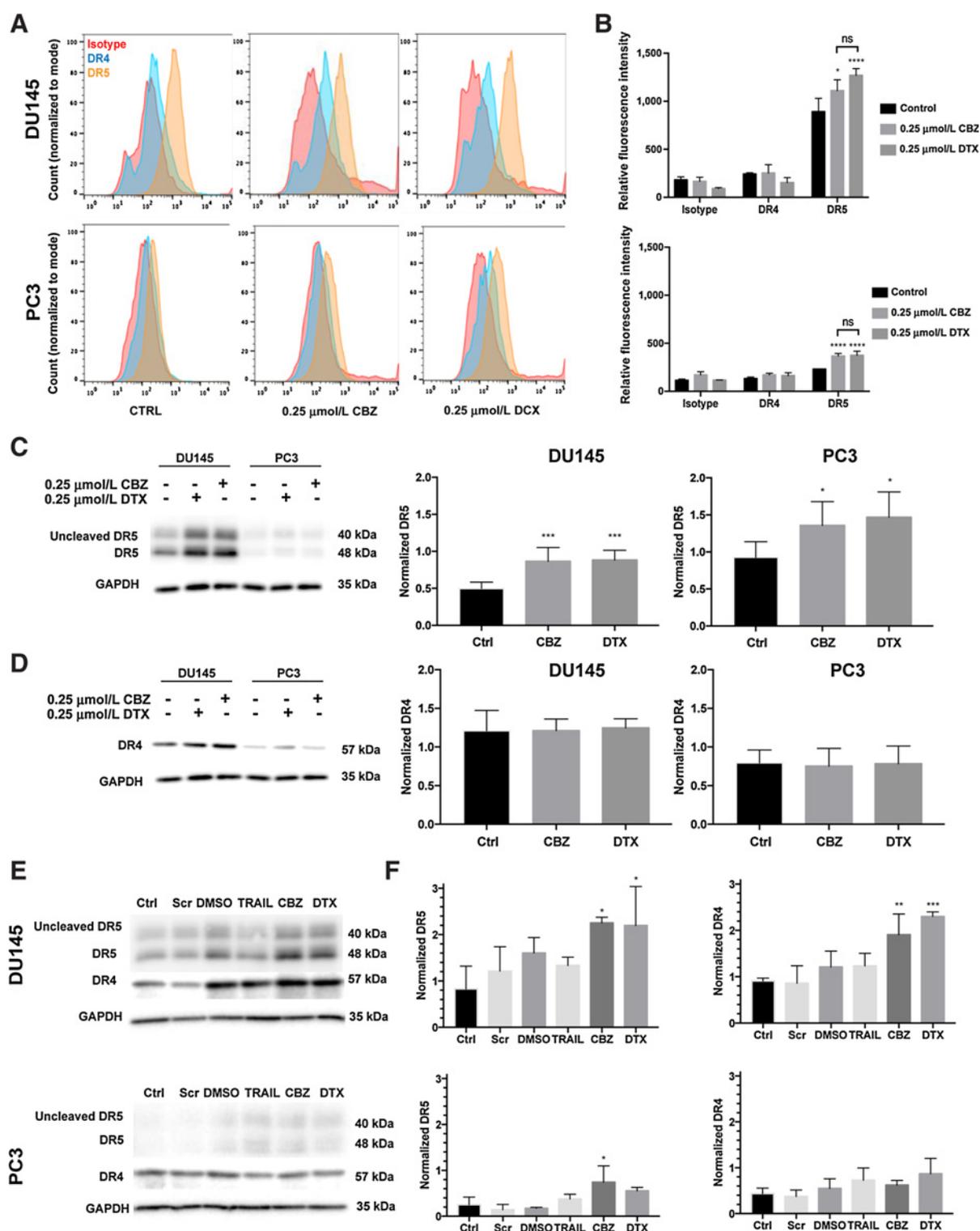
To determine whether JNK is responsible for the upregulation of DR5, DU145 and PC3 cells were treated with SP600125 and their lysates were analyzed for DR4 and DR5 expression via Western blot analysis (Fig. 5C). We observed a decrease in DR4 and DR5 expression

in DU145 and PC3 cells treated with the JNK inhibitor after taxane exposure (Fig. 5D). These data illustrate that cabazitaxel and docetaxel induce death receptor expression via JNK activation with JNK acting as a regulator of DR4 and DR5 expression. It is proposed that posttreatment with the JNK inhibitor attenuated JNK activation and inhibited DR4 and DR5 upregulation.

Computational model of taxane and TRAIL synergy

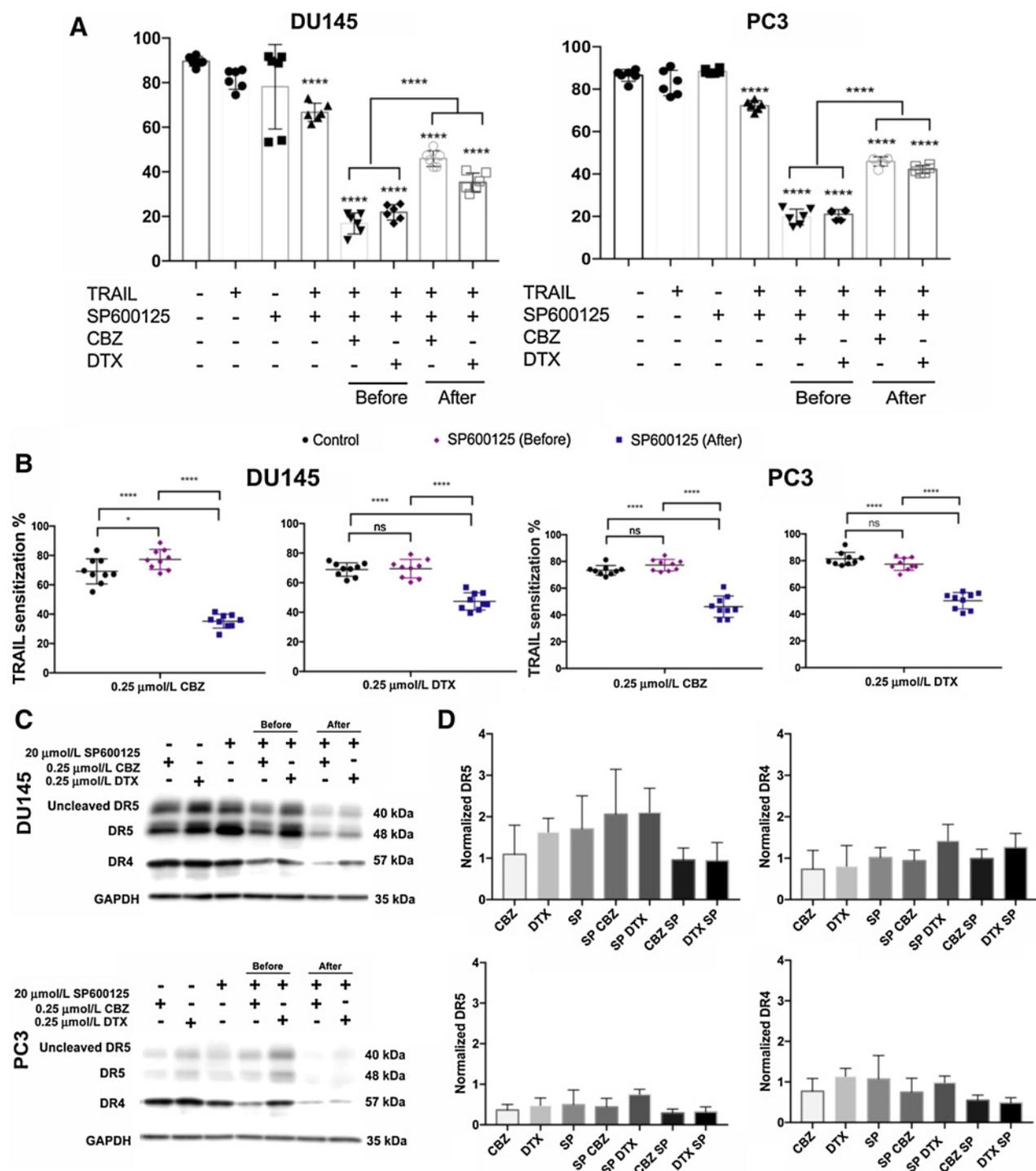
We further interrogated the observed mechanism *in silico* using an adapted computational model (Fig. 6A; refs. 46, 47). An ER-stressed condition was modeled for taxane-treated cells by altering the initial conditions of Bcl-2, DR4/5, and cIAP1/2 expression (Supplementary Table S1; refs. 53, 54). DU145 and PC3 cells showed different modes of

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**Figure 4.**

Taxane pretreatment increases death receptor expression. **A**, Representative histograms of death receptor expression using flow cytometry. **B**, Mean fluorescent intensity of DR4 and DR5 compared with isotype control. **C**, Western blot analysis and relative expression of DR5 in DU145 and PC3 cells treated with 0.25 $\mu\text{mol/L}$ cabazitaxel (CBZ) or docetaxel (DTX). **D**, Representative Western blot and relative expression of DR4 in DU145 and PC3 cells treated with 0.25 $\mu\text{mol/L}$ cabazitaxel or docetaxel. **E**, Western blot analysis of death receptor expression in siCHOP-knockdown DU145 and PC3 cells treated with DMSO, TRAIL, cabazitaxel or docetaxel. **F**, Relative death receptor expression in siCHOP-knockdown DU145 and PC3 cells treated with DMSO, TRAIL, cabazitaxel, or docetaxel. The values represent the mean \pm SD ($n = 6$). *, $P < 0.05$; **, $P < 0.01$; ***, $P \leq 0.005$; ****, $P < 0.0001$.

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**Figure 5.**

JNK activation is significant in ER stress-related apoptosis and death receptor regulation. **A**, Cell viability of DU145 and PC3 cells after pre- and posttreatment with 20 μmol/L SP600125 (JNK inhibitor). **B**, TRAIL sensitization of DU145 and PC3 cells treated with JNK inhibitor before or after taxane (0.25 μmol/L) therapy followed by TRAIL treatment. **C**, Western blot analysis of death receptor expression in DU145 and PC3 cells treated with either cabazitaxel (CBZ), docetaxel (DTX), and JNK inhibitor alone or in combination. Cells were also treated with the JNK inhibitor before or after taxane exposure. **D**, Relative death receptor expression in DU145 and PC3 cells treated with either cabazitaxel, docetaxel, and JNK inhibitor alone or in combination. Cells were also treated with the JNK inhibitor before or after taxane exposure. The values represent the mean ± SD ($n = 6$ or 9). *, $P < 0.05$; ****, $P < 0.0001$.

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apoptosis in response to taxane plus TRAIL treatment. To account for this, DU145 and PC3 cells were given different initial conditions for Bax and XIAP (Supplementary Table S2). DU145 cells were modeled as having no Bax expression (55). XIAP expression was also reduced for DU145 cells, as DU145 apoptosis was not dependent on cytochrome *c* or SMAC, indicating that DU145 cells undergo purely extrinsic apoptosis.

Cleaved PARP (cPARP) was used as a measure of apoptosis, as its cleavage is indicative of late-stage apoptosis. When the cPARP concentration equaled half the initial condition of noncleaved PARP, cancer cells were considered apoptotic. Cytochrome *c* was used to monitor mitochondrial permeability (Fig. 6B and C). When DU145 cells were treated with combination therapy, the time until apoptosis was reduced. However, the overall amount of cPARP generated was not increased. This suggests that taxane treatment enhances the

potency of TRAIL. For Bax-deficient DU145 cells treated with TRAIL with or without taxane therapy, no mitochondrial permeability took place (Fig. 6B).

PC3 cells treated with TRAIL and without taxane therapy did not pass the apoptotic threshold, whereas PC3 cells treated with combination therapy did. This suggests that taxane therapy had a substantial effect in increasing the potency of TRAIL applied to PC3 cells. PC3 cells are positive for Bax expression, allowing for mitochondrial permeability and the subsequent release of the proapoptotic protein cytochrome *c*. When taxanes were used in combination with TRAIL, mitochondrial permeability was observed, but not for cancer cells treated with TRAIL only. This simulation further suggests that taxanes enhance TRAIL-mediated apoptosis by enhancing the occurrence of mitochondrial permeability (Fig. 6C).

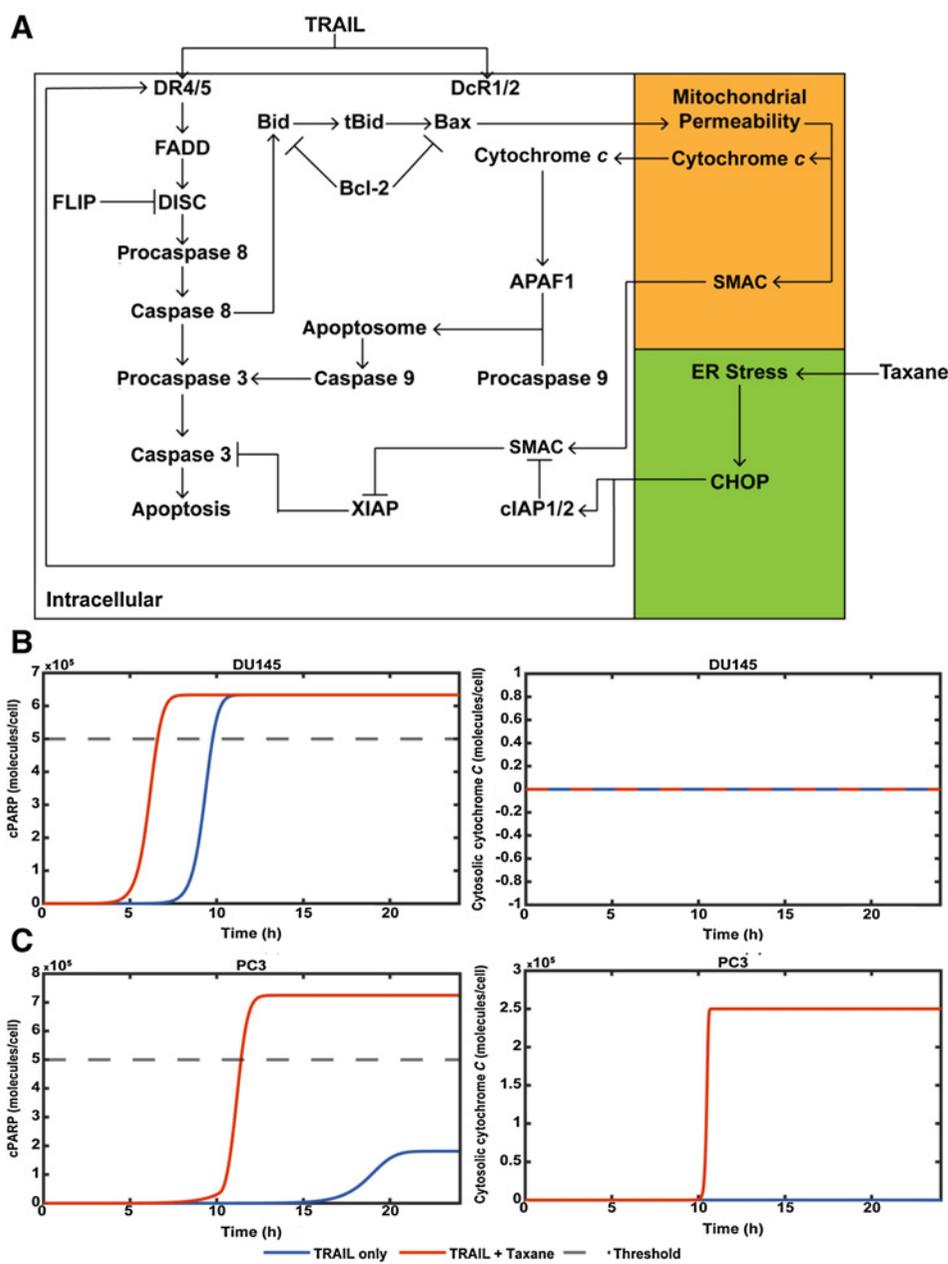


Figure 6.

Computational model of TRAIL-mediated apoptosis of DU145 and PC3 cells. **A**, Schematic of apoptosis signaling pathway of DU145 and PC3 cells in response to taxane and TRAIL treatment. **B**, cPARP concentration of DU145 cells treated with or without taxane therapy and TRAIL. The concentration of cytosolic cytochrome *c* to represent mitochondrial permeability. **C**, cPARP and cytochrome *c* concentrations for PC3 cells treated with or without taxane therapy and TRAIL.

The computational model was also used to probe how the differential expression of certain proteins will affect chemotherapy-induced sensitization of cancer cells to TRAIL. When mitochondrial outer membrane permeabilization (MOMP) or cell death did not occur, the time was plotted as 0 for visibility. For both cancer cell lines, increasing DR4/5 expression reduced the time until apoptosis occurred. For PC3 cells it also reduced the time until MOMP. Interestingly, MOMP preceded the time of cell death. This indicates that cell death continued to be dependent upon MOMP for PC3 cells. There was no MOMP for DU145 cells. To plot this, MOMP was set equal to 0 (Supplementary Fig. S3A).

When XIAP expression was modified for DU145 cells, the time until cell death increased with increasing XIAP. At a certain point, XIAP prevented DU145 cells from undergoing apoptosis. This was represented by the time of death being set equal to 0 on the plot (Supplementary Fig. S3B). This suggests that when XIAP reaches a certain level of expression, MOMP is necessary to induce apoptosis. However, due to DU145 cells being resistant to mitochondrial dysfunction, this is not possible. When XIAP expression was reduced for PC3 cells, MOMP would occur after the time of cell death. This shows that when XIAP is reduced, PC3 cells are no longer reliant on MOMP for apoptosis to occur. As XIAP increased, MOMP no longer contributes to PC3 cells undergoing apoptosis (Supplementary Fig. S3B).

Cytochrome *c* expression was varied as well for DU145 cells and PC3 cells. As expected, the cell death time of DU145 cells was not altered by cytochrome *c* expression (Supplementary Fig. S3C). For PC3 cells, cytochrome *c* expression dramatically affected the time of cell death. When cytochrome *c* expression was low, PC3 cells would not undergo apoptosis in response to combination therapy. When cytochrome *c* expression was increased, the time of cell death was not significantly altered (Supplementary Fig. S3C). Finally, when SMAC expression was increased or reduced for PC3 and DU145 cells, there was no change in the time of cell death (Supplementary Fig. S3D).

Discussion

In this study, we demonstrated that pretreatment with clinically used taxanes sensitizes prostate cancer cells to TRAIL-induced apoptosis through JNK pathway activation and induction of DR4 and DR5 expression (Supplementary Fig. S1; Fig. 1). These results are consistent with previous studies of human cancers where TRAIL-induced apoptosis was enhanced via DR4/DR5 regulation after exposure to DNA damage and chemotherapeutic agents (56–61). For the first time, we established the role of the ER stress pathway in apoptosis induced by taxane and TRAIL combination in DU145 and PC3 cells accompanied by a new computational model that can predict responses to taxane plus TRAIL treatment. We used docetaxel and cabazitaxel as first-line therapies for the sensitization of prostate cancer cells to TRAIL. We found no significant difference in the TRAIL-mediated apoptotic response from either taxane pretreatment (Fig. 1). This finding could potentially help bring clarity to the treatment sequencing in mCRPC based on patient-specific responses to each chemotherapeutic.

Current treatments target the bulk of the tumor and do not address the heterogeneity of cancer cells, including CSCs (62, 63). Previous studies using *in vivo* and *in vitro* models have demonstrated that cells positive for CD44, but lacking in CD24, are useful in identifying the CSC population (10, 11, 40). Although there was a higher number of CSCs in PC3 cells, PC3 CSCs moderately responded to the combina-

tion therapy. DU145 CSCs appeared more resistant as the chemotherapy and combination treatments induced more phenotypic changes of non-CSCs to CSCs in the total population and viable subpopulation (Fig. 2). There is a need for the development of therapies that specifically target stem cell subpopulations to reduce their tumor-initiating potential, once more prostate stem cell markers are identified and standardized.

The mechanism by which taxanes increase the TRAIL sensitivity of prostate cancer cells is still not completely understood and deserves further exploration. In this study, DU145 cells showed growth inhibition when exposed to taxanes or the combination treatment. However, DU145 cells showed no pronounced effect when treated with apoptosis inhibitor, Z-VAD-FMK, suggesting that DU145 cells undergo a predominantly caspase-independent form of apoptosis (64). On the other hand, PC3 cells displayed increased cell survival when treated with Z-VAD-FMK, indicating that the combination induces caspase-dependent apoptosis (Supplementary Fig. S4). The activation of ER stress was seen in both cell lines when looking at GRP78 regulation specifically in docetaxel treatment groups (Supplementary Fig. S4C and S4D).

Both DU145 and PC3 cells showed increased expression of DR5 compared with negative control cells when exposed to taxanes alone (Fig. 4). In PC3 cells, there was a significant decrease in TRAIL sensitization in cells with knockdown cytochrome *c*, indicating that the intrinsic mitochondrial pathway is one of the moderators of apoptosis in these cells (Fig. 3). The difference in TRAIL sensitization via the mitochondrial apoptotic pathway observed in this study further supports the idea that patient-specific therapy tailored to treatment response is needed and deserves further exploration in clinical trials.

Recent studies have shown that ER stress-mediated apoptosis is associated with autophagy through CHOP in breast cancer and human glioblastoma cells (65, 66). Inhibiting CHOP in prostate cancer cells reduced the apoptotic effect of the combination treatment in both cell lines (Fig. 3). These findings suggest that each taxane may function at least partly through ER stress-induced apoptosis via CHOP. CHOP has been shown to activate other apoptotic pathways as a multifunctional transcription factor in the ER stress response (67). CHOP can activate caspase-3 and BH3-only apoptotic proteins as well as inactivate BCL2 antiapoptotic proteins through inhibition (49). CHOP induces the downstream target gene GADD34 and dephosphorylates eIF2 α , which in turn leads to protein translation recovery increasing ER stress and apoptosis (68). Production of reactive oxygen species and activation of cellular calcium signaling also leads to CHOP-mediated apoptosis (67).

Because CHOP expression can be mediated by JNK, we explored the inhibitory response of SP600125 (JNK inhibitor; refs. 39, 69). We observed an increase in cell viability that significantly differed from controls as well as pretreatment with the inhibitor (Fig. 5). Furthermore, TRAIL sensitization was attenuated in the group treated with the JNK inhibitor after taxane exposure, suggesting that JNK must be activated in this ER stress-induced apoptotic pathway by each taxane. DR4 and DR5 were markedly reduced following a taxane then inhibitor treatment scheme (Fig. 5). This finding suggests that JNK activation is one of the regulators of death receptor induction by taxanes. DU145 cells follow the ER stress-induced apoptotic pathway primarily via death receptor induction. In PC3 cells, death receptor induction is achieved by the cooperation of cell-intrinsic and cell-extrinsic pathways. In CHOP knockdown cells, PC3 but especially DU145 cells exhibited an increase in DR4/5 expression suggesting that

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CHOP is not an essential regulator of death receptor expression but has a major role in the ER stress apoptotic pathway. CHOP and JNK represent good targets to overcome TRAIL resistance in new drug therapies.

The experimental results from this study were used to inform the basis of the computational model. Both the model and the *in vitro* experiments showed that cytochrome *c* expression significantly affects chemotherapy-induced sensitization for PC3 cells (Fig. 6). Using the simulation, DR4/5 expression was found to have a significant effect on cell death times for both DU145 and PC3 cells (Supplementary Fig. S3). This model could be further used to examine the apoptotic response in different cell types based on cell-specific protein expression that affects apoptosis. In summary, we have demonstrated the synergistic effect of taxanes enhancing TRAIL-mediated apoptosis in two different prostate cancer cells via ER stress and DR5 induction. Initially TRAIL resistant, the degree of apoptosis in these cells increased significantly when exposed to taxanes first, followed by TRAIL. Combination therapies and computer models such as these could help overcome tumor resistance mechanisms leading to better clinical outcomes for patients with mCRPC.

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Authors' Contributions

K.A. Grayson: Conceptualization, formal analysis, investigation, methodology, writing—original draft, writing—review and editing. **J.M. Hope:** Investigation, methodology. **W. Wang:** Investigation, methodology. **C.A. Reinhart-King:** Resources, methodology. **M.R. King:** Conceptualization, resources, supervision, funding acquisition, project administration, writing—review and editing.

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