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ORIGINAL ARTICLE

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The topoisomerase I inhibitor topotecan increases the sensitivity of prostate tumor cells to TRAIL/Apo-2L-induced apoptosis

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Abstract *Purpose*: TRAIL/Apo-2L is cytotoxic against numerous prostate tumor cell lines; however, some lines are more resistant than others. Identification of an agent that increases prostate tumor cell sensitivity to TRAIL/Apo-2L would prove valuable for TRAIL/Apo-2L-mediated tumor therapy. Thus, we examined the effect of combining five clinically approved chemotherapeutic agents with TRAIL/Apo-2L for treating prostate tumor cells. *Methods*: Four human prostate tumor cell lines were initially tested for TRAIL/Apo-2L sensitivity. Subsequent studies examined whether the TRAIL/Apo-2L-induced killing of DU-145 cells was augmented in the presence of the chemotherapeutic molecules, as measured by annexin V-FITC/propidium iodide staining. Furthermore, caspase 8 activation and BID cleavage were examined by immunoblotting. RT-PCR and flow cytometry were performed to monitor TRAIL-R1 and TRAIL-R2 levels after chemotherapeutic treatment. *Results*: DU-145 cells were the least responsive of the prostate tumor cell lines tested to TRAIL/Apo-2L-induced death. Surprisingly, only topotecan, a topoisomerase I inhibitor, when used in combination with rTRAIL/Apo-2L led to significant apoptosis of DU-145 cells, as measured by caspase 8 activation, BID cleavage, and annexin V-FITC/PI staining. Topotecan alone had little to no toxicity on the DU-145 cells. Furthermore, the increase in TRAIL/Apo-2L sensitivity following topotecan treatment correlated with increased expression of TRAIL-R1 and TRAIL-R2 and decreased intracellular levels of the antiapoptotic protein survivin. *Conclusions*: Our results define a promising direction for alternative therapies against androgen-independent prostate cancers. The sensitivity of DU-145 cells to TRAIL/Apo-2L was dramatically increased when combined with topotecan, suggesting that low-dose topotecan treatment to upregulate TRAIL-R1 and TRAIL-R2 and downregulate survivin, followed by TRAIL/Apo-2L administration, may be a viable therapy for treating cancer of the prostate.

Keywords TRAIL/Apo-2L · Apoptosis · Prostate · Topotecan · Survivin

Abbreviations *BID* Bcl-2-interacting domain death agonist · *FADD* Fas-associated death domain · *FLIP* FADD-like IL-1β-converting enzyme-inhibitory protein · *IAP* Inhibitor of apoptosis protein · *MDR* Multidrug resistance · *PrEC* Prostate epithelial cells · *RT-PCR* Reverse transcription-polymerase chain reaction · *TRAIL* TNF-related apoptosis-inducing ligand

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Introduction

TNF-related apoptosis inducing ligand (TRAIL, or Apo-2L) is cytotoxic against many different types of tumor cell lines; however, there have been a number of tumor cell lines identified that are partially or completely resistant to TRAIL/Apo-2L-induced apoptosis [3, 10, 16, 34, 42]. This resistance has been attributed to increased levels of intracellular inhibitors of the apoptotic machinery, such as Bcl-2 family members, FLIP, and survivin, defects in the apoptotic signaling machinery, and the absence or

mutation of the death-inducing TRAIL/Apo-2L receptors (TRAIL-R1 and TRAIL-R2) [12, 13, 17, 35], suggesting that similar alterations in primary tumors may prevent TRAIL/Apo-2L from having any cytotoxic effect when used clinically. Consequently, a variety of studies have demonstrated that tumor cell responsiveness to TRAIL/Apo-2L can be enhanced by chemotherapeutic agents and radiation therapy [3, 7]. These agents alter the levels of the intracellular apoptosis inhibitors and/or TRAIL/Apo-2L receptor expression, such that the apoptotic signal can be generated when TRAIL-R1 and/or TRAIL-R2 ligation occurs. With prostate cancer being the most common visceral cancer and the second-leading cause of death from cancer in men in the United States [25], the identification of a chemotherapeutic agent that augments prostate tumor cell sensitivity to TRAIL/Apo-2L is of great importance.

Topotecan, a derivative of camptothecin, was developed as a new chemotherapeutic that acts to inhibit topoisomerase I, the enzyme required to alleviate the tension on the DNA strand generated during the replication process, resulting in the breaking of the DNA strands [23]. Topotecan has been tested in several clinical trials, where it has proved effective in small-cell lung cancer and cervical cancer at appropriate doses [27]; however, it can cause several undesirable side effects such as anemia, alopecia, and neutropenia [27]. In this study, we examined the ability of several chemotherapeutic agents to enhance the responsiveness of prostate tumor cells to TRAIL/Apo-2L. Our investigation revealed that topotecan greatly increased the sensitivity of the human prostate tumor cell line DU-145 to TRAIL/Apo-2L, and identified a potential mechanism of action by which topotecan alters the response of prostate tumor cells to TRAIL/Apo-2L.

Materials and methods

Reagents and antibodies

Reagents and sources were as follows. MOPC-21, non-specific IgG1 isotype control, and HOPC-2, non-specific IgG2a isotype control, were from Sigma (St. Louis, Mo.). The mAb against the four TRAIL/Apo-2L receptors (M271, IgG2a anti-TRAIL-R1; M413, IgG1 anti-TRAIL-R2; M430, IgG1 anti-TRAIL-R3; M444, IgG1 anti-TRAIL-R4) were produced at Immunex Corporation (now Amgen, Seattle, Wash.). The anti-caspase-8 mAb (provided by Dr. Marcus Peter, University of Chicago), anti-BID antiserum (R&D Systems, Minneapolis, Minn.), anti-actin mAb (ICN Biochemicals, Aurora, Ohio), anti-cytochrome *c* mAb (Pharmingen, San Diego, Calif.), and anti-survivin antiserum [19] were used for Western blotting according to the manufacturers' instructions. Recombinant soluble TRAIL/Apo-2L was provided by Genentech (San Francisco, Calif.), and used at the indicated concentrations. Actinomycin D, cisplatin, methotrexate, and etoposide were purchased from Sigma. Mitoxantrone and topotecan were obtained from the University of Iowa Hospitals and Clinics pharmacy.

Cell lines

The human prostate tumor cell lines ALVA-31, DU-145, LNCaP, and PC-3 were obtained from Dr. Timothy Ratliff (University of Iowa) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, sodium pyruvate, non-essential amino acids, and HEPES (hereafter referred to as complete RPMI). Normal human prostate epithelial cells (PrEC) were obtained from Clonetics Corporation (San Diego, Calif.) and cultured as directed.

In vitro killing with TRAIL

Sensitivity to TRAIL/Apo-2L was assayed by incubating the cells in 96-well plates (2×10^4 cells/well) with recombinant TRAIL/Apo-2L (at the indicated concentrations) for 24 h. In some experiments, actinomycin D (797, 79.7, and 7.97 n*M*), cisplatin (33.3, 16.7, and 3.33 μg/ml), methotrexate (110, 11.0, and 1.1 μ*M*), mitoxantrone (193.3, 96.6, and 19.3 μ*M*), topotecan (2183, 218.3, and 21.83 n*M*), or etoposide (17.0, 8.5, and 1.7 μ*M*) were added to the culture medium immediately before the addition of TRAIL/Apo-2L. Cell death was determined by crystal violet staining, with results presented as percent cell death: [1−(OD cells treated with hTRAIL/Apo-2L per OD untreated cells)×100]. The cell death induced by any of the chemotherapeutic agents alone was insignificant compared to background levels.

Isobologram analysis

Calculations of synergistic cytotoxicity following treatment with TRAIL/Apo-2L and topotecan were determined by isobologram analysis, as described by Berenbaum [4]. Briefly, the IC_{30} for each agent alone at 24 h was calculated after assuming a linear dose-response relationship and was used to derive the line of additivity. Interaction indices were calculated as previously described [8], using the CalcuSyn software package (Biosoft). Points falling below the line of additivity (<1.0) represent synergism.

In vitro apoptosis assay

For analysis of apoptosis, tumor cells were incubated as described above and apoptotic cell death was measured by flow cytometry using FITC-conjugated annexin V (R&D Systems) and propidium iodide (PI; Sigma). Briefly, tumor cells were incubated with TRAIL/Apo-2L (100 ng/ml), topotecan (218 n*M*), or both for 16 h, after which the cells were collected and resuspended in 100 μl incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 m*M* NaCl, 5 m*M* $CaCl_2$) containing 10 μl annexin V-FITC and 10 μl PI (50 μg/ml) solution for 10 min. After adding an additional 400 μl incubation buffer, cells were immediately analyzed on a FACScan (Becton Dickinson, San Jose, Calif).

Western blotting

Cells were lysed in PBS containing 1% Nonidet P-40 and Complete Mini protease inhibitors (Roche, Mannheim, Germany; one tablet per 10 ml solution). The lysed cells were centrifuged at 14,000 *g* to remove cellular debris, and protein concentrations of the lysates were determined by the colorimetric bicinchoninic acid analysis (Pierce Chemical Company, Rockford, Ill.). Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane (Novex, San Diego, Calif.), and blocked with 5% nonfat dried milk in PBS/Tween-20 (0.05% v/v) overnight. The membrane was incubated with the anti-caspase-8 mAb, anti-BID antiserum, or anti-survivin antiserum (diluted according to the manufacturers' instructions) for 1 h. After washing, the membrane was incubated with an anti-mouse (Amersham, Arlington Heights, Ill.; diluted 1:1000), anti-goat (Jackson ImmunoResearch, West Grove, Pa.; diluted 1:5000), or anti-rabbit (Jackson ImmunoResearch; diluted 1:5000) HRP-conjugated antibodies for 1 h. Following several washes, the blots were developed by chemiluminescence according to the manufacturer's protocol (SuperSignal West Pico Chemiluminescence Substrate, Pierce).

Cytochrome *c* release as measured by digitonin-based subcellular fractionation

The release of cytochrome *c* from mitochondria was measured by Western blotting as described previously [41]. Following treatment with TRAIL/Apo-2L and/or topotecan for various periods of time, cytosolic and pellet (mitochondrial) fractions were generated using a digitonin-based subcellular fractionation technique. Briefly, 10^7 cells were harvested by centrifugation at 800 *g*, washed in PBS, pH 7.2, and re-pelleted. Cells were digitonin-permeabilized for 5 min on ice at a density of 3×10^7 cells/ml in cytosolic extraction buffer (250 m*M* sucrose, 70 m*M* KCl, 137 m*M* NaCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, pH 7.2, 100 μ*M* PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin) containing 200 μg/ml digitonin. Plasma membrane permeabilization of cells was confirmed by staining in a 0.2% trypan blue solution. Cells were then centrifuged at 1000 *g* for 5 min at 4°C. The supernatants (designated the cytosols) were saved and the pellets solubilized in the same volume of mitochondrial lysis buffer (50 m*M* Tris, pH 7.4, 150 m*M* NaCl, 2 m*M* EDTA, 2 m*M* EGTA, 0.2% Triton X-100, 0.3% Nonidet P-40, 100 μ*M* PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin), followed by pelletting at 10,000 *g* for 10 min at 4°C. The resultant supernatant was designated the mitochondrial pellet fraction. Supernatants were mixed with 6× reducing SDS-PAGE sample buffer, and extracts from equal cell numbers were separated by SDS-PAGE.

Flow cytometry

Untreated or topotecan-stimulated DU-145 cells were incubated with the following unlabeled primary mAb for 1 h at 4°C: MOPC-21, HOPC-2, M271, M413, M430, M444. After three washes, primary antibody binding was detected with a PE-conjugated, Fc-specific, mouse anti-human $F(ab')_2$ (Jackson ImmunoResearch). Cells were analyzed immediately following staining on a FACScan.

RT-PCR

Total RNA was isolated from untreated or topotecan-stimulated DU-145 cells with TRIzol reagent (Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions. RNA samples (1 μg each) were tested for DNA contamination by 30 cycles of PCR with human *β*-actin primers. After it was shown that there was no DNA contamination, cDNA synthesis was performed using an RNA PCR kit (Perkin Elmer, Norwalk, Ct.) with the supplied oligo $d(T)_{16}$ primer. Reverse transcription was performed using a thermal program of 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min. PCR reactions were performed using the following primers: human *β*-actin (*forward* 5′-GAAACTACCTTCAACTC CATC-3′; *reverse* 5′-CGAGGCCAGGATGGAGCCGCC-3′), human TRAIL-R1 (*forward* 5′-CTGAGCAACGCAGACTCGC TGTCCAC-3′; *reverse* 5′-TCCAAGGACACGGCAGAGCCTG TGCCAT-3′), and human TRAIL-R2 (*forward* 5′-GCCTCAT GGACAATGAGATAAAGGTGGCT-3′; *reverse* 5′-CCAAATC TCAAAGTACGCACAAAC GG-3′), giving products of 219, 506, and 502 bp, respectively. *β*-Actin PCR cycle conditions were 95°C for 45 s, 55°C for 1 min, and 72°C for 45 s for 25 cycles. TRAIL-R1 and TRAIL-R2 cycle conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 25 cycles. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide.

Results

Sensitivity of human prostate tumor cell lines to rTRAIL/Apo-2L

Initial studies examining the responsiveness of four human prostate tumor cell lines, ALVA-31, DU-145, LNCaP, and PC-3, revealed that ALVA-31 and PC-3 were the most sensitive to TRAIL/Apo-2L-induced death, with LNCaP demonstrating intermediate responsiveness, and DU-145 being the least susceptible to rTRAIL/Apo-2L (Fig. 1). While these results are consistent with previous findings [30, 46], some studies have shown the susceptibility of LNCaP and DU-145 to TRAIL/Apo-2L to be quite different. This disparity may be due to differences in the recombinant TRAIL molecules, or culture conditions used to maintain the different cell lines. Normal PrEC were completely resistant to TRAIL/Apo-2L-induced death, just as previously demonstrated [15].

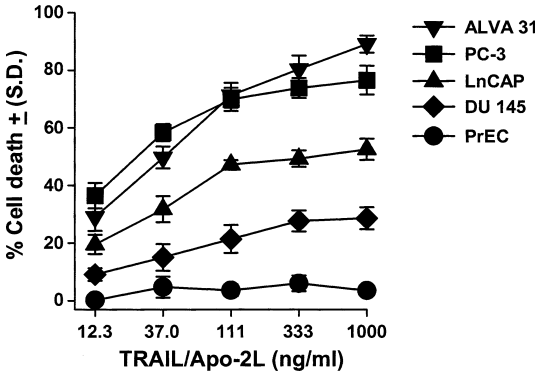

Fig. 1 Sensitivity of human prostate tumor cell lines and normal prostate epithelial cells (PrEC) to TRAIL/Apo-2L-induced death. Cells were seeded at 2×10^4/well in 96-well flat-bottomed microtiter plates and allowed to adhere for at least 6 h before the addition of TRAIL/Apo-2L at the indicated concentrations. Cell viability was determined after 24 h by crystal violet staining. Each value represents the mean of three wells. Experiments were performed at least three separate times with each cell line

Topotecan enhances the rTRAIL/Apo-2L-induced death of DU-145 cells

Since DU-145 was the least TRAIL/Apo-2L-sensitive prostate tumor line in our hands, we sought to identify a chemotherapeutic agent that would increase the responsiveness of these cells to TRAIL/Apo-2L. Like many other cancer types, the contribution of the P-glycoprotein-mediated drug efflux (multidrug-resistance; MDR) can be related to the stage of prostate cancer [40]. Thus, actinomycin D (transcription inhibitor), cisplatin (DNA-alkylating agent), methotrexate (dihydrofolate reductase inhibitor), mitoxantrone (topoisomerase II inhibitor), etoposide (topoisomerase II inhibitor), and the camptothecin analogue topotecan (topoisomerase I inhibitor) were tested for their ability to increase the sensitivity of DU-145 cells to TRAIL/Apo-2L. At the doses used, none of the agents was toxic to the cells alone. Both topotecan and actinomycin D dramatically increased the TRAIL/Apo-2L-induced death of DU-145 cells, with topotecan providing the strongest enhancement (Fig. 2A, E). In contrast, cisplatin, methotrexate, mitoxantrone, and etoposide had no effect on TRAIL/

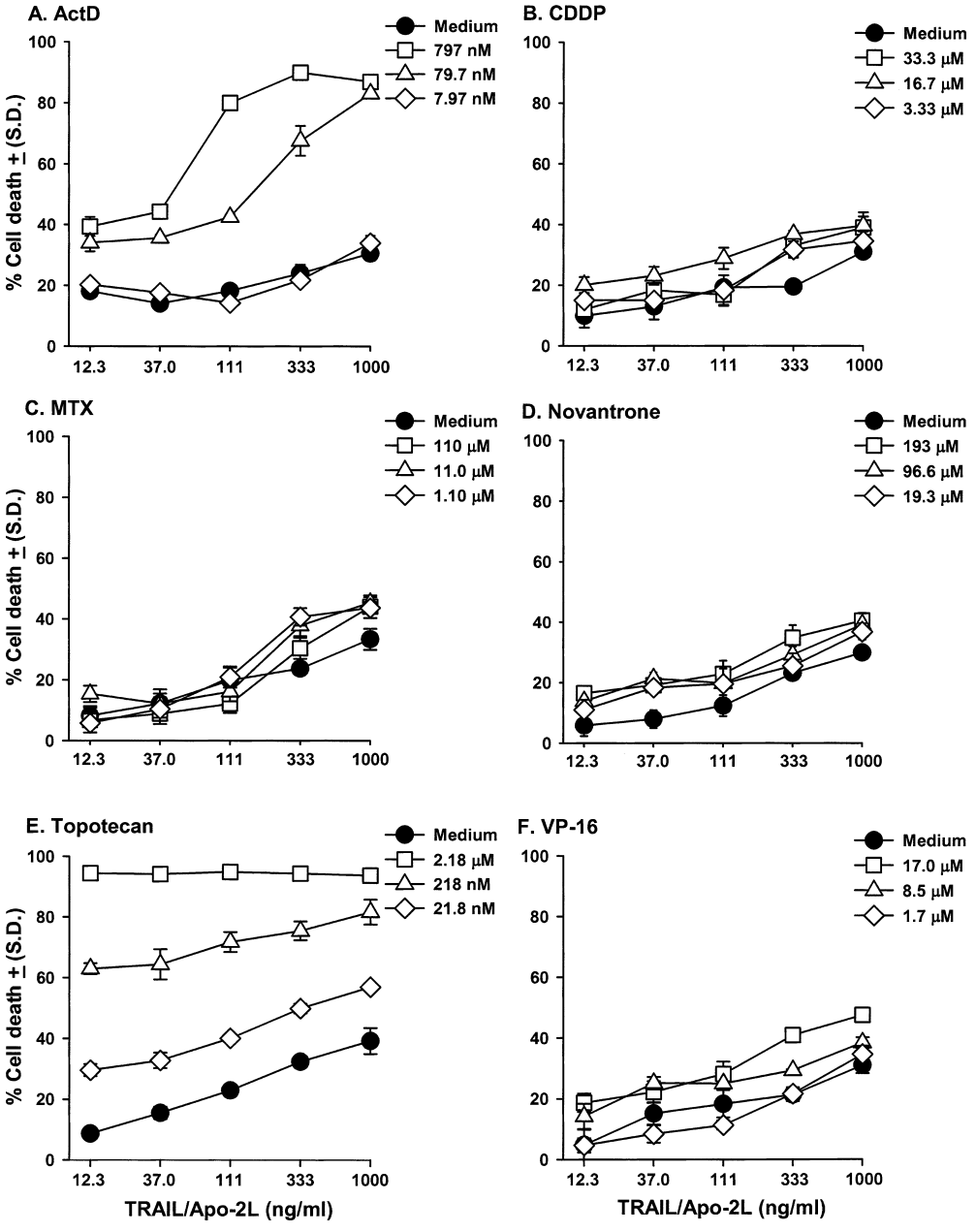

Fig. 2A–F Augmentation of TRAIL/Apo-2L-induced death of DU-145 cells by actinomycin D and topotecan. DU-145 cells were seeded at 2×10^4/well in 96-well flat-bottomed microtiter plates and allowed to adhere for at least 6 h. Actinomycin D (*ActD*, **A**), cisplatin (*CDDP*, **B**), methotrexate (*MTX*, **C**), mitoxantrone (*Novantrone*, **D**), topotecan (**E**), or etoposide (*VP-16*, **F**) were added to the cells at the indicated concentrations immediately before adding TRAIL/Apo-2L (*medium* DU-145 cells treated with TRAIL/Apo-2L alone). Cell viability was then determined after 24 h by crystal violet staining. Each value represents the mean of three wells. Experiments were performed at least three separate times with similar results

Apo-2L-induced killing (Fig. 2B, C, D, F). Since topotecan provided the strongest augmentation of TRAIL/Apo-2L-induced cell death, further studies focused on this compound. In order to determine whether the combination of topotecan and TRAIL/Apo-2L in DU-145 cells resulted in synergistic cytotoxic effects, isobologram analysis was performed. In this analysis, synergism occurs when the interaction index is less than 1.0. As shown in Fig. 3, the interaction indices indicated a high degree of synergism between TRAIL/Apo-2L and topotecan against DU-145 cells. Because of high tumoricidal activity of TRAIL/Apo-2L and topotecan, subsequent studies were designed to investigate the mechanism by which topotecan increases the TRAIL/Apo-2L sensitivity of DU-145 cells.

While the crystal violet staining used to evaluate DU-145 cell death following exposure to TRAIL/Apo-2L and topotecan, as presented in Figs. 1 and 2, is a suitable method for quantifying the amount of cell death, it does not distinguish between apoptotic and necrotic death. TRAIL/Apo-2L-induced cell death occurs through an apoptotic mechanism characterized by caspase activation and cleavage of numerous intracellular proteins, alterations in the cell membrane, and DNA fragmentation [17, 34, 42]. To confirm that the TRAIL/Apo-2L/topotecan-induced death of DU-145 cells was mediated through an apoptotic mechanism, DU-145 cells were exposed to TRAIL/Apo-2L (100 ng/ml), topotecan (218 n*M*), or both for various periods of time, after which the cells were lysed and the cellular

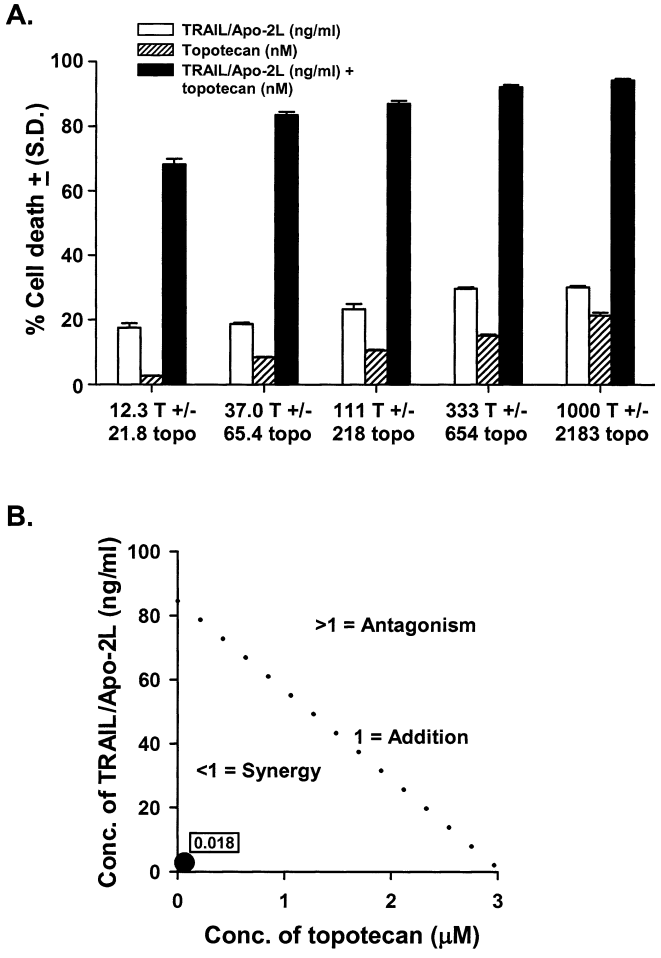

Fig. 3A, B The synergistic cytotoxic effects of TRAIL/Apo-2L and topotecan in DU-145 cells. **A** DU-145 cells were seeded at 2×10^4/well in 96-well flat-bottomed microtiter plates and allowed to adhere for at least 6 h. The effects of TRAIL/Apo-2L and topotecan on cell viability, as measured by crystal violet staining, were measured after 24 h. The concentration of TRAIL/Apo-2L alone (*T*, ng/ml), topotecan alone (*topo*, n*M*), and the two agents in combination are indicated beneath each set of combinations. **B** Isobologram analysis of the combination of TRAIL/Apo-2L and topotecan demonstrates a synergistic interaction in DU-145 cells (● concentrations of both drugs that result in cell death that is 70% of control, IC_{30}). The line of additivity is also shown. The *boxed number* is the CI, which was calculated using the CalcuSyn software package

proteins separated by SDS-PAGE for Western blot analysis of caspase-8 activation and BID cleavage. Caspase-8 is the most proximal caspase in the apoptosis signaling pathway, as it interacts with the death domains of TRAIL-R1/-R2 through the adapter protein FADD [26, 36]. Caspase-8 activation yields an 18-kDa active subunit and p42/41 intermediate cleavage products from the 55-kDa inactive procaspase-8 protein [29].

BID is one of the various proteins cleaved by active caspase-8, and serves as the molecular link between TRAIL-R1/-R2 and the mitochondria [18]. In the present case of DU-145 cells, caspase-8 activation and BID cleavage were detected only when DU-145 cells were treated with both TRAIL/Apo-2L and topotecan (Fig. 4A). As mentioned, full-length (p22) BID is cytosolic, where it serves as a substrate for active caspase-8. The carboxy terminal p15 fragment of BID, produced by caspase-8 cleavage, translocates from the cytosol to the mitochondria, where it induces mitochondrial membrane permeabilization with the resultant release of apoptosis-inducing proteins (i.e. cytochrome *c*) from the mitochondrial intermembrane space into the cytosol. Analysis of DU-145 cells treated with TRAIL/Apo-2L, topotecan, or both revealed the release of cytochrome *c* into the cytosol fraction of DU-145 cells only when they were treated with both TRAIL/Apo-2L and topotecan (Fig. 4A). Collectively, these results show the activation of the molecules important in the apoptotic process.

To further demonstrate the induction of apoptosis, the ability of annexin V to bind to DU-145 cells treated with TRAIL/Apo-2L or topotecan alone or in combination was assessed (Fig. 4B). Annexin V preferentially binds to phosphatidylserine, a phospholipid component of the inner leaflet of the plasma membrane that is rapidly externalized during apoptosis [11, 28]. At the concentrations used, apoptotic DU-145 cells were only detected when both TRAIL/Apo-2L and topotecan were added. These results clearly demonstrate that the TRAIL/Apo-2L-induced death of DU-145 cells is via an apoptotic mechanism, and significantly enhanced by topotecan.

Topotecan increases TRAIL/Apo-2L receptor expression on DU-145 cells

TRAIL/Apo-2L interacts with four distinct receptors: DR4 [32], DR5/TRAIL-R2 [33], TRID/DcR1/TRAIL-R3 [33], and TRAIL-R4/DcR2 [10] (hereafter referred to as TRAIL-R1, -R2, -R3, and -R4, respectively). Both TRAIL-R1 and TRAIL-R2 contain a cytoplasmic death domain, and crosslinking by TRAIL/Apo-2L or receptor-specific mAb activates the apoptosis signaling pathway in sensitive cells [32, 33]. In contrast, neither TRAIL-R3 (which is GPI linked) nor TRAIL-R4 (which is a type I membrane protein) contains a complete cytoplasmic death domain, and neither can mediate apoptosis upon ligation [10, 33]. Because they bind to TRAIL/Apo-2L without directly signaling for cell death, it was proposed that TRAIL-R3 and TRAIL-R4 may serve as protective receptors, acting either as antagonistic receptors [33] or as receptors transducing an antiapoptotic signal [10]. The differential expression of the TRAIL/Apo-2L receptors may, therefore, determine whether a cell is resistant or sensitive to TRAIL/Apo-2L-induced apoptosis [33]. Thus, TRAIL/Apo-2L receptor expression on DU-145 cells was analyzed by flow cytometry. The data demonstrate TRAIL-R1 and TRAIL-R2 are expressed on DU-145 cells (Fig. 5A), but not TRAIL-R3 or TRAIL-R4 (data not shown).

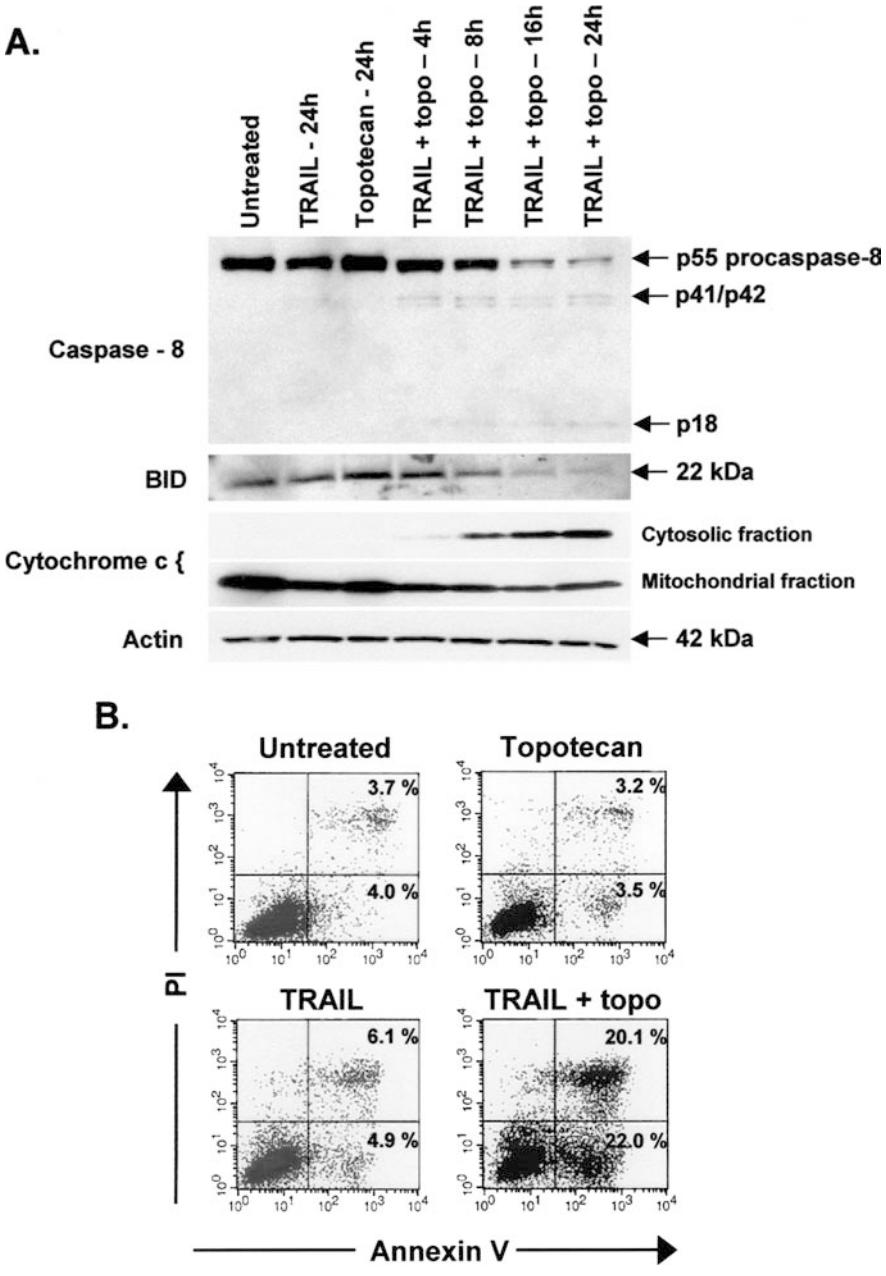

Fig. 4A, B TRAIL/Apo-2L induces apoptosis in DU-145 cells. **A** Kinetics of caspase-8 activation, BID cleavage, and cytochrome *c* release following TRAIL/Apo-2L treatment. DU-145 cells at 5×10^5/well in 24-well plates were treated with TRAIL/Apo-2L (100 ng/ml) alone or in combination with topotecan (218 n*M*), cell lysates were prepared at the indicated times after infection, and caspase-8 activation, BID cleavage, and cytochrome *c* release were determined by Western blot analysis. For comparison, lysates from untreated DU-145 cells were also examined. The blot for actin is included to demonstrate equal protein loading. **B** Annexin V staining of DU-145 cells. Phosphatidylserine externalization on DU-145 cells following TRAIL/Apo-2L treatment. DU-145 tumor cells were cultured for 16 h in medium alone or in the presence of TRAIL/Apo-2L (100 ng/ml) alone or in combination with topotecan (218 n*M*). Cells were then stained with FITC-annexin V and propidium iodide (*PI*) and analyzed by flow cytometry. The percent of FITC-annexin V^+/PI^- tumor cells is indicated for each condition. Histograms represent 10^4 gated tumor cells. Experiments were repeated at least three times with similar results

TRAIL/Apo-2L receptor levels were also measured after incubating DU-145 cells for 24 h with topotecan. Interestingly, the addition of topotecan to DU-145 cells increased the expression of both TRAIL-R1 and TRAIL-R2 at the cell surface (Fig. 5A). This increase also corresponded with an increase in TRAIL-R1 and TRAIL-R2 mRNA levels (Fig. 5B). The observed modulation of TRAIL-R1 and TRAIL-R2 levels by topotecan provides a possible explanation for the increase in responsiveness of DU-145 cells to rTRAIL/Apo-2L. Yet, the normal low-level expression of TRAIL-R1 and TRAIL-R2 on DU-145 cells suggests that there are additional regulatory mechanism(s) that suppress TRAIL/Apo-2L-induced apoptosis.

Modulation of intracellular levels of the IAP survivin by topotecan correlates with increased TRAIL/Apo-2L sensitivity

There are a number of intracellular proteins capable of inhibiting death receptor-mediated apoptosis when present at sufficient levels [1, 17, 22, 39]. One such molecule is survivin, a member of the inhibitor of apoptosis protein (IAP) family, which is expressed in tissues during development but not in terminally differentiated mature tissue [1]. High intracellular levels of survivin have also been shown to correlate with tumors of poor prognosis [37]. Moreover, survivin inhibits the apoptosis signaling pathway through the direct

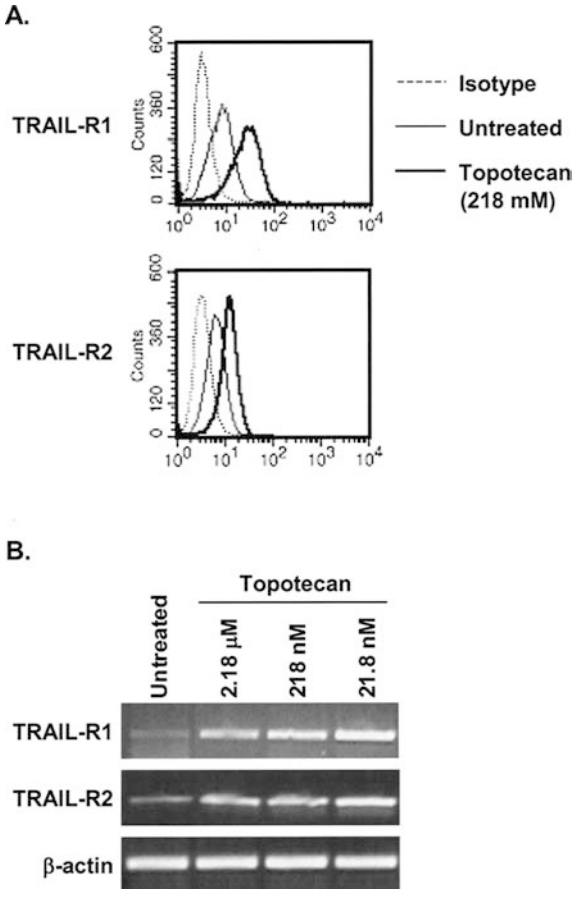

Fig. 5A, B Increase in TRAIL-R1 and TRAIL-R2 expression on DU-145 cells occurs with topotecan treatment. **A** Surface levels of TRAIL-R1 and TRAIL-R2 were examined in DU-145 cells after 24 h culture in medium alone or medium containing topotecan (218 n*M*) (*thick solid lines* staining by anti-TRAIL-R1 mAb M271 or anti-TRAIL-R2 mAb M413 in topotecan-treated cells, *thin solid lines* staining by the same mAb without actinomycin D, *dotted line* staining with isotype control mAb). Histograms represent 10^4 gated tumor cells. **B** DU-145 cells were treated as in **A**, and then TRAIL receptor mRNA levels were determined by RT-PCR as described in Materials and methods

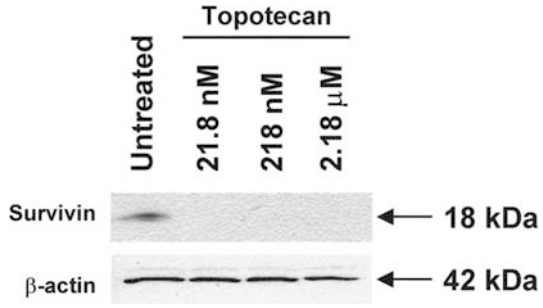

Fig. 6 Susceptibility of DU-145 cells to TRAIL/Apo-2L-induced apoptosis is related to levels of survivin. Six-well plates containing 5×10^5 cells were incubated in medium alone or with topotecan (2183, 218, or 21.8 n*M*) for 24 h. Cell lysates were then separated by SDS-PAGE, transferred to nitrocellulose, and intracellular levels of survivin protein were measured in equivalent cell lysate amounts by Western blot analysis. The blot for β-actin is included to demonstrate equal protein loading

inhibition of downstream effector caspases, and when overexpressed in cancer cells leads to aberrant proliferation through mitosis [1]. Thus, survivin expression was assessed in DU-145 cells by immunoblotting. As demonstrated in Fig. 6, survivin was normally expressed at high levels in DU-145 cells. We hypothesized that if survivin was involved in protecting DU-145 cells from TRAIL/Apo-2L-mediated death, then topotecan would decrease survivin levels over time. DU-145 cells were incubated with increasing concentrations of topotecan (2183, 218.3, and 21.83 n*M*) for 24 h, after which survivin levels were again determined by immunoblotting. As predicted, survivin levels were found to decrease with topotecan exposure. Interestingly, survivin levels were equally altered by both the lowest and highest doses of topotecan. These results suggest that the expression of apoptosis-inhibiting molecules such as survivin, coupled with low expression levels of the death-inducing TRAIL/Apo-2L receptors, influence the susceptibility of DU-145 cells to TRAIL/Apo-2L-mediated apoptosis.

Discussion

The investigation and development of immunologically based treatment strategies for prostate cancer has increased in recent years due to the increasing incidence rate of the disease. Members of the TNF superfamily exert a variety of physiological effects on numerous cell types, including the induction of cellular activation, proliferation, and death [2]. Since its identification in 1995 [34, 42], TRAIL/Apo-2L has attracted much attention because it induces apoptosis in a wide variety of transformed cells but does not display cytotoxicity to normal cells. Although TRAIL/Apo-2L is a potent inducer of apoptosis, many tumor cell types remain resistant. Consistent with the findings of previous studies, we found that in different tumor cell lines within a specific tumor type sensitivity to TRAIL/Apo-2L can be variable [3, 17]. ALVA-31 and PC-3 displayed the greatest sensitivity to the cytotoxic effects of TRAIL/Apo-2L, while LNCaP was moderately sensitive, and DU-145 was the least sensitive.

We then extended previously reported observations [30, 46] to examine potential mechanisms that regulate TRAIL/Apo-2L sensitivity in DU-145 cells, and to identify agents that could increase TRAIL/Apo-2L sensitivity in these cells. In many tumor systems, including prostate cancer, there have been reports describing chemotherapeutic methods of increasing the response of the TRAIL/Apo-2L-resistant tumor cells to the cytotoxicity of TRAIL/Apo-2L [31, 44, 47]. Our investigation revealed that DU-145 cells could be converted to TRAIL/Apo-2L-sensitive by blocking transcription with actinomycin D, demonstrating that this line was not fundamentally defective in the apoptotic signaling machinery. Additionally, there was a dramatic increase in TRAIL/Apo-2L sensitivity when DU-145

cells were treated with topotecan, but this augmentation did not occur when cisplatin, methotrexate, mitoxantrone, or etoposide were used. Investigation into the mechanism of the effect of topotecan in DU-145 cells revealed that it increased expression of both death-inducing TRAIL/Apo-2L receptors (TRAIL-R1 and TRAIL-R2) and decreased intracellular levels of the apoptosis inhibitor survivin.

There are potentially multiple regulatory components in the TRAIL/Apo-2L/TRAIL receptor system that influences a cell's sensitivity to TRAIL-induced apoptosis. It is apparent from our findings that both TRAIL/Apo-2L receptor expression and the presence of the intracellular inhibitor of apoptosis, survivin, are critical determinants of the sensitivity of DU-145 cells to TRAIL/Apo-2L. The four TRAIL/Apo-2L receptors bind TRAIL/Apo-2L with comparable affinities [10], and when combined with the initial observation that the non-death signaling TRAIL-R3 and TRAIL-R4 are primarily expressed in normal tissues and absent in tumor cells [33], it was convenient to conclude that expression of either or both of these receptors confers resistance to TRAIL/Apo-2L-induced apoptosis. While TRAIL-R3 and TRAIL-R4 may indeed still function as the "decoy" receptor as originally hypothesized, subsequent studies have clearly revealed that sensitivity or resistance to TRAIL/Apo-2L-induced apoptosis is not controlled simply by the lack or presence of these receptors [17]. The intracellular proteins that function to inhibit various steps in the apoptotic signaling pathway, such as cFLIP, Bcl-2 family members, and IAP family members [1, 12, 17, 22, 39], are also key molecules that help to regulate the signals generated at the death-inducing TRAIL/Apo-2L receptors after ligation.

In the present study, the intracellular levels of survivin in DU-145 cells correlated with the degree of TRAIL/Apo-2L responsiveness. High levels of survivin were observed in normal, untreated DU-145 cells, but survivin was undetectable after incubation with topotecan. While survivin is normally expressed during fetal development, the expression pattern of survivin is quite restricted in normal adult cells and tissues [1, 37]. Survivin is expressed only in the G_2/M phase of the cell cycle, where it associates with the centromeres and mitotic spindle. Expression of survivin, however, has been noted in a high proportion and variety of human tumors, suggesting that increased levels of survivin contribute to accelerated cell proliferation and tumorigenesis [1, 37]. Thus, it is possible that survivin may counteract the apoptotic pathway by preventing the tumor cell from staying in a particular phase of the cell cycle. In addition to regulating cell division, survivin also inhibits apoptosis by interfering with caspase activation, namely caspases 3, 7 and 9 [38].

It is also important to remember that the change in TRAIL/Apo-2L responsiveness of DU-145 cells following treatment with topotecan was marked by an increase in surface expression of TRAIL-R1 and TRAIL-R2, suggesting that the apoptotic signaling pathway can only be initiated when a certain surface level of TRAIL-R1 and/or TRAIL-R2 has been reached. The promoter regions of both TRAIL-R1 and TRAIL-R2 have recently begun to be examined to identify potential transcriptional regulatory mechanisms. Analysis of the 5′-flanking region of the human TRAIL-R1 and TRAIL-R2 genes has identified several putative binding sites for a number of transcription factors, including NF-AT, Sp1, and AP-1 [21, 45]. Moreover, TRAIL-R1 and TRAIL-R2 expression is enhanced by DNA damage and regulated by p53 [20, 43]. Of the chemotherapeutics tested in this study, methotrexate was the only agent that did not directly target the cellular DNA, as it exerts its chemotherapeutic effect by its ability to counteract and compete with folic acid in cancer cells resulting in folic acid deficiency within the cells causing their demise [24]. In contrast, actinomycin D intercalates into the DNA, where it serves to inhibit transcription by preventing RNA polymerase from traveling along the surface of the DNA molecule [6]. Cisplatin is believed to kill cancer cells by covalently binding to DNA and interfering with its repair mechanism, eventually leading to cell death [9]. Topotecan is a topoisomerase I inhibitor [23], while etoposide and mitoxantrone are topoisomerase II inhibitors [5, 14].

The DNA topoisomerases are the enzymes involved in regulating the extent of DNA supercoiling. Type I topoisomerases alter the degree of DNA supercoiling by causing single-strand breaks and religation. In contrast, type II topoisomerases cause double-strand breaks. The different roles of DNA topoisomerase I and II may indicate an opposing pair of roles in the regulation of DNA supercoiling. Both activities are especially crucial during DNA transcription and replication, when the DNA helix must be unwound to allow proper function of large enzymatic machinery, and topoisomerases have indeed been shown to maintain both transcription and replication. It is unclear how blocking the action of topoisomerase I with topotecan had such a profound effect on the level of TRAIL/Apo-2L sensitivity in DU-145 cells compared to inhibiting topoisomerase II function with etoposide or mitoxantrone. Perhaps it is key that DU-145 cells contain mutant p53. These unique in vitro observations may have important clinical implications for the application of TRAIL/Apo-2L in the treatment of prostate cancer.

There are potentially multiple regulatory components in the TRAIL/Apo-2L receptor system that influences a cell's sensitivity to TRAIL/Apo-2L-induced apoptosis. It is apparent from our findings that both TRAIL/Apo-2L receptor expression and the presence of inhibitors of apoptosis, such as survivin, are critical to prostate tumor cell sensitivity. Exploiting these pathways may prove critical to finding more effective therapies for this disease. Our findings would suggest that using the adjuvant effects of topotecan and TRAIL/Apo-2L may be a start to finding such cures.

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