

# Mifepristone Pretreatment Overcomes Resistance of Prostate Cancer Cells to Tumor Necrosis Factor $\alpha$ -related Apoptosis-inducing Ligand (TRAIL)

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## Abstract

**Examination of the effects of TRAIL (tumor necrosis factor  $\alpha$ -related apoptosis-inducing ligand) showed higher apoptotic response in LNCaP C4–2, whereas LNCaP were resistant. However, treatment of LNCaP with Mifepristone, an antiprogestin, before TRAIL induced significant apoptosis, similar to the levels observed in LNCaP C4–2. Experiments to determine the reasons for altered response of the cell lines showed no significant differences in death/decoy receptors and caspase-8 activity. However, treatment induced increased truncation of Bid and activation of caspases -9, -7, and -3 in LNCaP C4–2. Time course experiments showed that caspase-8 was activated before the involvement of mitochondrial pathway, and caspase-9 was responsible for activation of caspases -7 and -3. Use of specific caspase inhibitors demonstrated the presence of a short-loop feedback activation of Bid. Published reports suggested that increased phosphorylation of Akt was responsible for resistance of LNCaP to TRAIL. However, no significant differences were noticed in the levels of phosphorylated Akt in TRAIL-resistant LNCaP and TRAIL-sensitive LNCaP C4–2. On the basis of our results, it is suggested that the differences in response of the two cell lines to TRAIL is at the mitochondrial level.**

## Introduction

The goal of antihormone therapy, chemotherapy, or other cancer therapies is to induce apoptosis in tumor targets. Prostate cancer is a multifocal disease with clones of androgen-sensitive and androgen-refractory cells (1–3). Commonly used androgen deprivation therapies induce apoptotic cell death in androgen-responsive cells (4–7), whereas effective therapy for refractory cancer is not available (8–10). However, the androgen-refractory prostate cancer cells retain the capacity to undergo apoptosis (11–13). In prostate cancer cells, apoptosis has been induced by variety of agents such as staurosporine (14–16), levostatin (17), thapsigargin (11), okadaic acid (18), Mifepristone (19, 20), camp-

tothecin (12), and TRAIL<sup>2</sup> (21–23). These agents induce apoptosis through the involvement of transforming growth factor  $\beta$  (24, 25), Bcl2 (19, 25), sphingolipid (26), and death receptor (20–22) pathways.

TRAIL induced apoptosis in a variety of cancers when treated individually and demonstrated synergistic effects in combination with other apoptotic agents, such as etoposide and *cis*-diamminedichloroplatinum II. TRAIL induced apoptosis through its interaction with death receptors DR4 (TRAIL-R1; Ref. 27) and DR5 (TRAIL-R2, TRICK2, or KILLER; Refs. 28–31). The function of death receptors may be blocked by the expression of decoy receptors DcR1 (TRID/TRAIL-R3; Ref. 32) and DcR2 (TRUNDD/TRAIL-R4; Ref. 32), which compete for the binding of TRAIL. Death receptors, Fas-associated death domain and procaspase-8 (FLICE, MACH, and Mch5) form the death-inducing signaling complex. On recruitment of procaspase-8 by Fas-associated death domain, several molecules of procaspase-8 aggregate into close proximity, which enables the low intrinsic protease activity of caspase-8 to cleave downstream proteins necessary for the induction of apoptosis (32). In type I cells, caspase-8 propagates the death signal directly through the activation of procaspase-3 and procaspase-7. In type II cells, because death-inducing signaling complex does not result in efficient activation of caspases directly, the apoptotic signal is amplified via the mitochondria. Caspase-8 cleaves BID (a Bcl<sub>2</sub> family protein), which translocates into the mitochondria and promotes release of cytochrome *c* from the mitochondria, which binds to cytoplasmic Apaf-1 and activates caspase-9, forming the apoptosome (33–38).

Cancer cells have been shown to be sensitive to apoptotic stimulus of TRAIL, whereas normal cells showed very little response (31, 39). Differential response of cancer cells to apoptotic stimulus enormously increased the usefulness of TRAIL as a therapeutic agent for cancer. However, the reasons for differential response of normal and cancer cells to TRAIL are not known, although differences in decoy receptors were suggested. But, recent observations indicate that decoy receptors may not be responsible for differences in the sensitivity to TRAIL (40, 41). Alternately, inhibition of activation of caspase-8 by intracellular regulators such as cellular FLICE inhibiting protein (40) may be responsible.

Prostate cancer cells differ in their response to various apoptotic stimuli. On induction of apoptosis by staurosporine or FTY120, PC3 cells were more resistant to apoptosis compared with LNCaP cells (12, 13). Treatment with okadaic

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<sup>2</sup> The abbreviations used are: TRAIL, tumor necrosis factor  $\alpha$ -related apoptosis-inducing ligand; PI3k, phosphatidylinositol 3'-kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBS, Tris-buffered saline; NF $\kappa$ B, nuclear factor  $\kappa$ B.

acid showed that DU145 cells responded differently compared with TSU-Prl, LNCaP, and PC3 cells (18). Differences in apoptotic response have been attributed to differential activation of the Bcl2 pathway, sphingolipid signaling, activation of caspases, and/or other key proteins. An early report suggested that LNCaP, PC3, and DU145 were resistant to TRAIL (21), which were not supported by recent reports (22, 42). It was shown recently that resistance of LNCaP prostate cancer cells to TRAIL might be because of elevated PI3k/Akt activity in these cells (23, 43). Inhibition of PI3k using wortmannin, LY 294002, or by transfecting dominant-negative Akt suppressed constitutive Akt activity and sensitized LNCaP cells to TRAIL treatment (23, 43) indicating that Akt may be responsible for resistance to TRAIL. However, when LNCaP cells were treated with androgens or epidermal growth factor, the cells were protected from apoptosis without activation of PI3k/Akt activity, indicating that androgens or epidermal growth factor activated a PI3k/Akt-independent survival pathway (44).

Mifepristone, an antiprogestin, was developed for the inhibition of progesterone-dependent reproductive processes (45) but demonstrated antitumor activity in *in vitro* and animal models (46–52). Currently, Mifepristone is being tested in a Phase II trial to treat recurrent epithelial ovarian carcinoma and a Phase III study for treating meningioma. Mifepristone induced 20–40% cell death depending on the cancer and the model system used, which suggest the need for a more effective treatment regimen. In cervical carcinoma, treatment of radioresistant cells with Mifepristone induced radiation-induced apoptosis (53). Similarly, sensitivity to Adriamycin in Adriamycin-resistant breast cancer cells was increased by Mifepristone (54). We have demonstrated recently that treatment of prostate cancer cells with Mifepristone and the antiestrogen tamoxifen induced apoptosis. However, combined treatment did not yield a significant increase in apoptosis in prostate cancer cells (20). We have demonstrated that, among other pathways, Mifepristone activated the TRAIL apoptotic pathway.

In this study, we demonstrate that unlike LNCaP, LNCaP C4–2, derived from LNCaP, are sensitive to TRAIL. Our results demonstrate no significant differences in phosphorylation of Akt in LNCaP and LNCaP C4–2, although the latter were sensitive to TRAIL. Results show that the differences in response to TRAIL may be because of differential activation of mitochondrial apoptotic response. Finally, we demonstrate that pretreatment of LNCaP with Mifepristone sensitized these cells to TRAIL by increased activation of apoptotic machinery.

## Materials and Methods

### Cell Culture

LNCaP cells were obtained from American Type Culture Collection (Rockville, MD), and LNCaP C4–2 cells were purchased from Urocor Inc. (Oklahoma City, OK). LNCaP C4–2 were derived from a coinjection of parental LNCaP with normal human bone fibroblasts in athymic male nude mice and passaged in the mice twice. Cells were grown in RPMI 1640 supplemented with 10% (LNCaP) or 5% (LNCaP C4–2) fetal bovine serum (Hyclone, Logan, UT) and grown in the

presence of 5% CO<sub>2</sub> at 37°C. Cells were treated with 10 μM Mifepristone (Sigma, St. Louis, MO) for 3 days or with increasing concentrations (from 200 ng/ml to 600 ng/ml) of TRAIL (Biomol Research Laboratories, Inc, Plymouth Meetings, PA) for various time intervals 2, 4, 6, 8, 16, 20, and 24 h. Some cells were treated with Mifepristone for 3 days and then with TRAIL for indicated periods. On completion of the experiment, cells were harvested, and total proteins, cytosol, or mitochondrial fractions were isolated as described below. At least three experiments were conducted with a minimum of four plates per treatment per experiment.

For experiments using inhibitors, the cells were pretreated with 100 μM caspase-8-specific (Z-IETD-FMK) or 50 μM caspase-9-specific (Z-LEHD-FMK) inhibitors (Enzyme Systems Products, Livermore, CA). The inhibitors were dissolved in DMSO; to limit cellular toxicity, care was taken not to exceed 0.2% DMSO in the culture medium. Appropriate controls included vehicle-treated cells with or without the inhibitors.

### Apoptosis Assays

We have used two methods to measure induction of apoptosis by drug treatment: MTT and Apoptosense assays. In the MTT assay tetrazolium is bio-reduced by live cells into colored diformazon. This reduction is catalyzed by NADH and NADPH produced by dehydrogenases in metabolically intact cells and, therefore, is indicative of cell survival. Apoptosense assay is an ELISA for quantitation of apoptosis-associated caspase-mediated cleavage of cellular protein cytokeratin-18. Horseradish peroxidase-conjugated monoclonal antibody M30 recognizes a neoepitope (amino acids 387–396) in the COOH-terminal domain that is exposed after cleavage by caspase. This test measures the amount of M30 antibody bound to the neoepitope, which is directly proportional to the amount of activated caspase that is indicative of apoptosis. Results are expressed as M30 antibody bound to the cleaved cytokeratin-18 expressed per μg of protein.

**MTT Assay.** Cells were seeded in 96-well plates (6000 cells/well), and treatment was started 24 h after seeding the cells. Cells were incubated at 37°C with the MTT reagent for 3 h and processed according to the manufacturer's instructions (Promega Corporation, Madison, WI). Color development was measured at 490 nm on a Spectra MAX 340 microplate reader (Molecular Devices, Menlo Park, CA).

**Apoptosense Assay.** Cells were plated in Petri dishes and treated as described above. On completion of the experiments, cells were harvested and total protein extracted as described below. Protein extract was added to the wells coated with mouse monoclonal M30 antibody (Peviva, DiaPharma, West Chester, OH); horseradish peroxide tracer solution was added to the wells and incubated for 4 h. Color was developed by adding tetramethyl benzidine solution, and absorbance was determined at 450 nm on a Spectra MAX 340 microplate reader (Molecular Devices). Standard solution supplied by the supplier was used for generating standard curves.

Table 1 Antibodies used, their manufacturers, and dilution for immunoblots

| Antibody         | Manufacturer                           | Catalog no. | Dilution     |
|------------------|--|-------------|--------------|
| Actin            | Sigma Chemical Co., St. Louis, MO      | A5441       | 1:5000       |
| Akt, Phospho     | Cell Signaling Technology, Beverly, MA | 9271        | 1:800        |
| Bid              | BioSource International, Camarillo, CA | 44-433      | 1 $\mu$ g/ml |
| Caspase-3        | BD Pharmingen, San Diego, CA           | 556425      | 1:1000       |
| Caspase-8        | Cell Signaling Technology, Beverly, MA | 9746        | 1:700        |
| Caspase-9        | Cell Signaling Technology, Beverly, MA | 9502        | 1:700        |
| Cytochrome C     | Imgenex, San Diego, CA                 | IMG-101     | 1:2000       |
| Death Receptor 4 | Imgenex, San Diego, CA                 | IMG-109     | 1:500        |
| Death Receptor 5 | Imgenex, San Diego, CA                 | IMG-120     | 1:400        |

### Preparation of Cell Lysates for Western Blotting

Cells were harvested by trypsinization, washed in 1 $\times$  PBS, and cell pellets were resuspended in lysis buffer [100 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, and protease inhibitor mixture from Roche Diagnostic Corporation, Indianapolis, IN]. Cells were incubated over ice for 30 min and centrifuged at 10,000  $\times$  *g* at 4°C for 10 min. The supernatant was collected and the protein concentration estimated using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA).

### Separation of Mitochondrial and Cytosolic Fractions

Cells were trypsinized, centrifuged at 600  $\times$  *g* for 10 min at 4°C, washed twice in ice-cold PBS, and resuspended in buffer A [20 mM HEPES-KOH (pH 7.2), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium-EDTA, 1 mM sodium-EGTA, 250 mM sucrose, and protease inhibitor mixture]. Cells were homogenized on ice with a glass Dounce homogenizer (30 strokes) and were centrifuged at 750  $\times$  *g* for 5 min at 4°C. The supernatant was centrifuged for 15 min at 10,000  $\times$  *g* at 4°C to pellet mitochondria, which was washed and resuspended in lysis buffer. The supernatant was centrifuged at 100,000  $\times$  *g* for 60 min at 4°C, and S-100 cytosol was collected.

### Western Blotting

Proteins (50  $\mu$ g, unless stated otherwise) were separated on NuPAGE 10% Bis-Tris gels (Novex precast mini gels; Invitrogen, Carlsbad, CA) at 100 V for 1 h in the presence of 1 $\times$  4-morpholinepropanesulfonic acid-SDS running buffer (Invitrogen, Carlsbad, CA). Separated proteins were transferred to (polyvinylidene difluoride) membranes (Bio-Rad Laboratories) at 42 V for 2.5 h using a Novex XCell II blotting apparatus in 4-morpholinepropanesulfonic acid transfer buffer in the presence of NuPAGE antioxidant. Transfer of the proteins to the polyvinylidene difluoride membrane was confirmed by staining with Ponceau S (Sigma). The blots were blocked in 5% nonfat dry milk in TBS, washed twice for 10 min each with TBS containing 0.1% Tween 20 and incubated for 2 h at RT with primary antibody diluted in TBS containing 0.5% milk. The antibodies used for the immunoblots, their suppliers, and dilution are shown in Table 1. Immunoreactive bands were visualized using the ECL detection system (Amersham, Pharmacia Biotech, Arlington Heights, IL), and signals were developed after exposure to X-ray film (X-Omat films; Eastman Kodak Company, Rochester, NY).

### Assay for Caspase-9 Activity

Caspase-9 activity was assayed using a colorimetric substrate, Ac-LEHD-pNA, using a kit from Chemicon International, Inc. (Temecula, CA). Cleavage of the COOH-terminal peptide bond by the enzyme released *p*-nitroaniline, which was measured at 405 nm. Pure recombinant human caspase-9 was used as a positive control.

### Results

**TRAIL-induced Differential Apoptosis in Prostate Cancer Cells: Pretreatment with Mifepristone Sensitized Cells to TRAIL.** Treatment of LNCaP cells with 400 ng/ml TRAIL did not alter cell survival significantly compared with controls (Fig. 1A), which agree with published data. Similar treatment of LNCaP C4-2 reduced cell survival as early as 8 h and continued the trend through out the experiment (Fig. 1B). LNCaP C4-2 survival was reduced by 20% by 8 h, which additionally decreased to 40% by 24 h, suggesting that LNCaP C4-2 were sensitive to TRAIL. Treatment of LNCaP with Mifepristone followed by TRAIL decreased cell survival significantly. By 16 h of combined treatment ~45% of the cells were alive (Fig. 1A). Treatment of LNCaP C4-2 with Mifepristone and TRAIL for 8 h decreased cell survival by 30%, and by 24 h only 40% of the cells survived the combined treatment (Fig. 1B). Treatment of cells with Mifepristone affected the survival of LNCaP more than LNCaP C4-2, which agreed with our earlier observations (20).

Results described above were generated by using MTT assay, which measures mitochondrial enzyme activity. Although MTT assay is an accepted indicator of cell survival, the results may differ with the number of cells in the wells. In these experiments, extreme care was taken to seed a similar number of LNCaP and LNCaP C4-2. However, it is well known that LNCaP do not attach to the wells as stringently as LNCaP C4-2, and also the rate of multiplication of the cells differ. Therefore, to obtain more accurate measurement of induction of apoptosis by drug treatment, an ELISA-based Apoptosense assay kit was used. This assay measures the cleavage of cytokeratin 18 by caspases that are activated in response to apoptotic stimuli. Values obtained using this assay were expressed as activity of M30 antibody against the cleaved cytokeratin 18 per unit of protein instead of cell number, which diminished the adverse effects of cell number/adhesion to plates and growth rate. Therefore, representative assays were carried out by treating cells in 100-mm

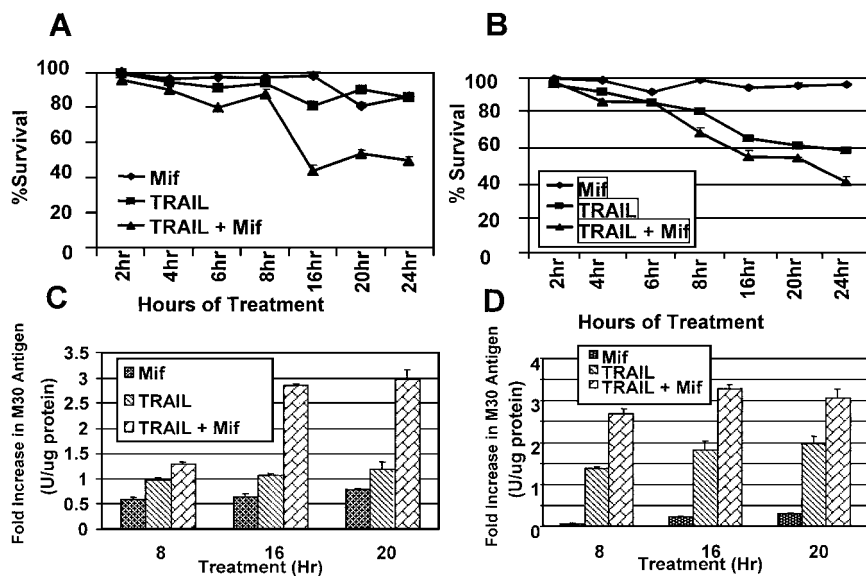


Fig. 1. Pretreatment with Mifepristone facilitates TRAIL-induced apoptosis. LNCaP (A and C) or LNCaP C4-2 (B and D) were treated with 10  $\mu$ M Mifepristone for 3 days and treated with TRAIL for the indicated time periods. The effects on cells were assayed using MTT (A and B) or Apoptosense (B and D) assays. *Mif*, cells treated with Mifepristone. The values are compared with respective controls; bars,  $\pm$ SD.

Petri dishes with drugs for 8, 16, and 20 h. Treatment of LNCaP with TRAIL for 8 h resulted in a 0.9-fold increase in M30 antigen, whereas treatment up to 20 h yielded 1.2-fold increase (Fig. 1C). Treatment of LNCaP C4-2 with TRAIL showed higher M30 antigen at 8 h (1.4-fold), 16 h (1.8-fold), and 20 h (2.0-fold) compared with LNCaP (Fig. 1D). LNCaP treated with both Mifepristone and TRAIL showed significantly increased activity compared with TRAIL alone at each time point. Similarly, TRAIL treatment of LNCaP C4-2 resulted in higher M30 activity compared with LNCaP. Thus, the data obtained by MTT and Apoptosense assays showed similar results.

Experiments with lower concentration of TRAIL (200 ng/ml) individually or with Mifepristone yielded lower cell death, whereas similar treatment with higher dose of TRAIL (600 ng/ml) did not increase apoptosis significantly compared with cells treated with 400 ng/ml TRAIL (data not shown). Therefore, all of the additional experiments were conducted by treating the cells with 400 ng/ml of TRAIL.

**Induction of Apoptosis Is Associated with Up-Regulation of Death Receptors.** Apoptotic response of cells to TRAIL is mediated through increase in the expression of death receptors DR5 and/or DR4, with possible decrease in expression of decoy receptors, DcR1 and DcR2. Therefore, to determine whether the differences in the response of LNCaP and LNCaP C4-2 to TRAIL is mediated through death or decoy receptors, cells were treated with TRAIL (400 ng/ml) with or without Mifepristone for 2, 4, 6, 8, 16, 20, and 24 h. Constitutive expression of death receptors DR4 and DR5 was higher in LNCaP C4-2 compared with LNCaP. Treatment with Mifepristone significantly increased the expression of DR5 in both cell lines (Fig. 2), whereas DR5 expression in LNCaP increased more compared with LNCaP C4-2 confirming our earlier results (20). Treatment of cells with TRAIL alone did not change the expression of DR5 in either cell line, whereas combining Mifepristone with TRAIL up-regulated the expression of DR5 significantly within 2 h in

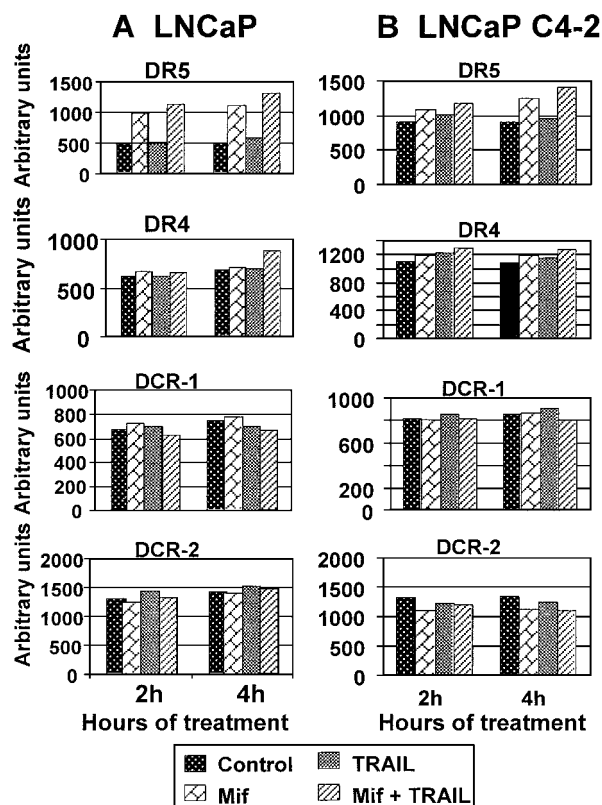
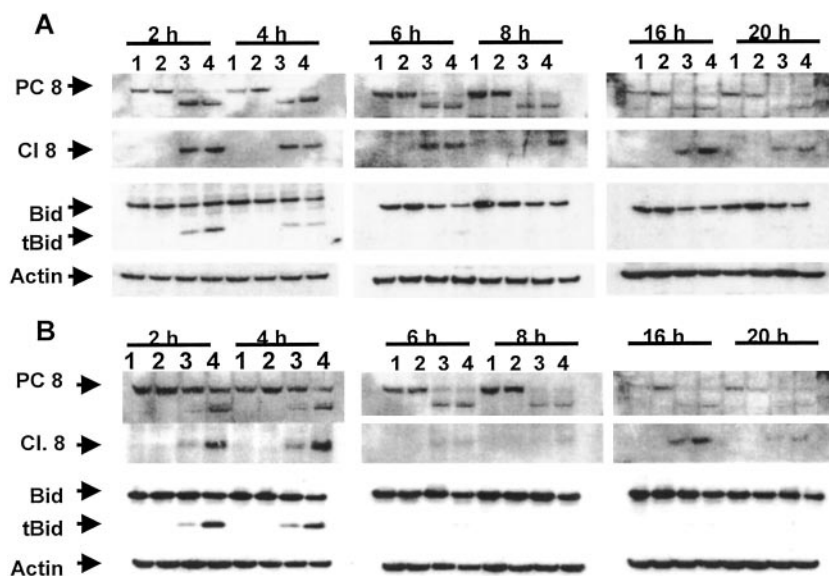


Fig. 2. Expression of death and decoy receptors in response to TRAIL and Mifepristone. LNCaP (A) and LNCaP C4-2 (B) cells were treated with Mifepristone and TRAIL for 2, 4, 6, 8, 16, or 20 h, as described. The blots were scanned, stripped, probed with actin antibody, and scanned. The values were normalized to respective values of actin.

LNCaP (Fig. 2A). DR4 did not show significant changes in treated LNCaP and LNCaP C4-2 (Fig. 2, A and B). Decoy receptor DcR1 decreased slightly by 6–8 h only in LNCaP





**Fig. 3.** Activation of caspase-8 and truncation of Bid. Activation of procaspase-8 and truncation of Bid in response to TRAIL and Mifepristone. LNCaP cells (A) were treated with Mifepristone and TRAIL for 2, 4, 6, 8, 16, or 20 h, as described. Note the  $M_r$  57,000 procaspase-8, intermediate  $M_r$  43,000 product, and activated caspase-8 at  $M_r$  18,000 (Cl. 8, Caspase-8). tBid denotes truncated Bid. LNCaP C4-2 cells (B) were treated with Mifepristone and TRAIL for 2, 4, 6, 8, 16, or 20 h, as described. 1, controls; 2, Mifepristone treated; 3, TRAIL treated; and 4, TRAIL and Mifepristone treated.

C4-2 treated with TRAIL and Mifepristone, whereas DcR2 did not change in any cell line. Continued treatment up to 24 h did not demonstrate significant differences compared with results described above (data not shown).

**Induction of Apoptosis Involves Activation of Caspase-8 and Truncation of Bid.** Recent publications demonstrate that caspase-8 activation is a prerequisite for the effects of TRAIL (40, 55–59), suggesting the possibility that its differential activation may account for altered response of cells to TRAIL. Western blot analysis was conducted to determine the expression of procaspase-8 ( $M_r$  57,000 protein), activated caspase ( $M_r$  18,000), or intermediate form ( $M_r$  46,000). Levels of procaspase-8 in LNCaP cells treated with TRAIL decreased as early as 2 h, with concomitant appearance of intermediate and activated products of  $M_r$  46,000 and  $M_r$  18,000 (Fig. 3A). Treatment of LNCaP with both Mifepristone and/or TRAIL, increased  $M_r$  18,000 activated product suggesting enhanced caspase-8 activity when treated with both drugs. Similar analysis of caspase-8 in LNCaP C4-2 did not show significant differences compared with LNCaP (Fig. 3B) indicating that altered activation of procaspase-8 is not responsible for differences in apoptotic responses of these cell lines.

BID is a  $M_r$  22,000 BH3 domain only proapoptotic member of the Bcl<sub>2</sub> family, which is truncated into a  $M_r$  15,000 tBid by activated caspase-8 (34, 59). When LNCaP and LNCaP C4-2 were treated with TRAIL individually or with Mifepristone, tBid was noted by 2 h (Fig. 3, A and B) although no cell-specific differences were noted. Pretreatment with Mifepristone before TRAIL increased truncation of Bid in both cell lines compared with TRAIL alone, although it was significantly higher in LNCaP C4-2 compared with LNCaP. Furthermore, tBid was detected for a longer period in LNCaP C4-2 compared with LNCaP, which correlated with increased apoptosis in LNCaP C4-2.

**Activation of Caspases -3, -9, and -7 by TRAIL.** Activation of caspase-8 leads to either direct activation of pro-

caspase-3 or its indirect activation through mitochondrial pathway involving tBid, cytochrome c, and caspase-9. Activation of caspase-3 yields two cleaved products: an initial  $M_r$  17,000 and a mature  $M_r$  12,000 proteins. Treatment of LNCaP and LNCaP C4-2 with TRAIL alone or with Mifepristone activated caspase-3 within 2 h of treatment (Figs. 4, A and B). TRAIL treatment of LNCaP yielded cleaved  $M_r$  17,000 caspase-3 product by 2 h that increased by 6 h. However, caspase-3 was activated maximally (as observed by the appearance of a p12 product) when LNCaP were treated with both TRAIL and Mifepristone for 6 h (Fig. 4A). Similar treatment of LNCaP C4-2 showed a significantly increased p17 band by 2 h (Fig. 4B), which increased significantly with time. In LNCaP C4-2, p12 form appeared as early as 2 h and continued throughout the experiment. These results indicate that caspase-3 activity was sustained and robust in LNCaP C4-2 compared with LNCaP.

Treatment of LNCaP with TRAIL activated caspase-7  $\times$  20 h, whereas pretreatment with Mifepristone activated caspase-7  $\times$  4 h (Fig. 4A). Similar to observations with caspase-3, activated caspase-7 was noted in LNCaP C4-2 within 2 h of Mifepristone and TRAIL treatment (Fig. 4B). Time course studies indicate that activation of caspase-3 precedes that of caspase-7.

As caspase-9 is a key protein in the formation of apoptosome, in addition to immunoblots, *in vitro* assays were performed to determine the activity of caspase-9. Treatment of LNCaP with TRAIL increased caspase-9 activity within 2 h, which additionally increased by 4 h (Fig. 5). However, caspase-9 activity was higher in LNCaP C4-2 compared with LNCaP with the same treatment regimen. Mifepristone alone increased caspase-9 activity in both cell lines, although the activity was lower than TRAIL alone. When the two drugs were combined, caspase-9 activity increased very significantly, and no differences were noticed in LNCaP and LNCaP C4-2. Thus, the activity of caspase-9 in response to

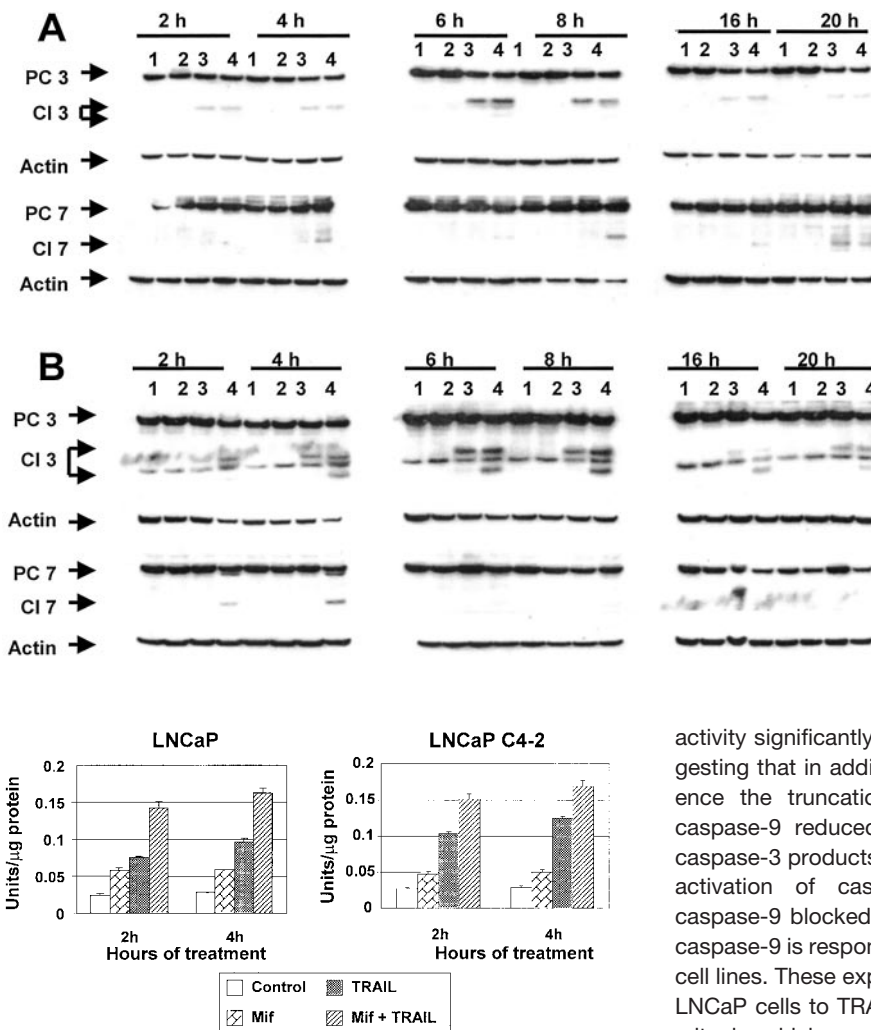


Fig. 5. Caspase-9 was activated by treatment. LNCaP (A) and LNCaP C4-2 (B) cells were treated as described, and caspase-9 activity was determined using calorimetric assays. The values are expressed as caspase activity units/ $\mu$ g protein. Results are mean with  $n = 4$ ; bars,  $\pm$ SE.

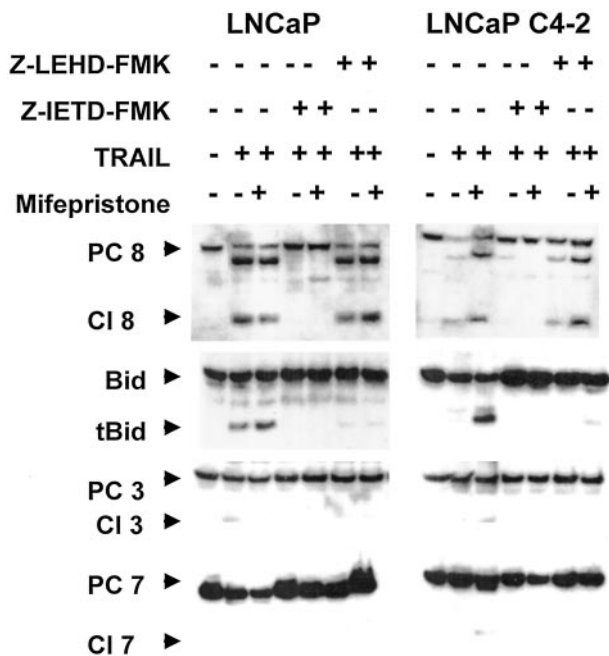
treatment correlated very well with apoptotic response of the cell lines (as seen in Fig. 1).

**Use of Inhibitors to Examine the Role of Specific Caspases in Apoptotic Response.** The above results suggest that mitochondrial apoptotic pathway may be responsible for differential response of LNCaP and LNCaP C4-2 to TRAIL treatment. To additionally confirm the role of specific caspases, inhibitors were used to specifically block caspases. The caspase-8 inhibitor Z-IETD-FMK blocked activation of caspase-8 completely, as noted by the absence of both intermediate and p18-cleaved products of caspase-8 in LNCaP and LNCaP C4-2 (Fig. 6). Not surprisingly, tBid was not present in the cells where caspase-8 function was blocked. Furthermore, caspase-3 and caspase-7 were not activated in cells with caspase-8 inhibition. Inhibition of caspase-9 with Z-LEHD-FMK, and treatment with TRAIL and/or Mifepristone, did not affect caspase-8 activity in either cell line. However, interestingly, inhibition of caspase-9

Fig. 4. Activation of procaspase-3 and -7. Activation of procaspase-3 and -7 in LNCaP (A) and LNCaP C4-2 (B). The treatment and lanes are as described for Fig. 3. PC3, procaspase-3; CI 3, cleaved caspase-3; PC7, procaspase-7; CI 7, cleaved caspase-7.

activity significantly reduced the levels of tBid (Fig. 6), suggesting that in addition to caspase-8, caspase-9 may influence the truncation of Bid. As expected, inhibition of caspase-9 reduced caspase-3 activity, although cleaved caspase-3 products were noted, probably because of direct activation of caspase-3 by caspase-8. Inhibition of caspase-9 blocked activation of caspase-7, indicating that caspase-9 is responsible for activation of caspase-7 in these cell lines. These experiments suggested that sensitization of LNCaP cells to TRAIL treatment by Mifepristone involved a mitochondrial apoptotic pathway.

**Differential Response of LNCaP and LNCaP C4-2 to TRAIL Is Not Mediated through Akt.** Recent publications have examined the response of LNCaP to TRAIL, although this is the first report on the response of LNCaP C4-2 to TRAIL. Published reports suggested that lack of response of LNCaP to TRAIL may be because of increased levels of Akt, a protein implicated in cell survival pathways (23, 43, 60). Therefore, to determine whether phosphorylation of Akt accounted for differential response to TRAIL, its levels in LNCaP and LNCaP C4-2 were compared. Our results demonstrated no significant differences in the levels of phosphorylated Akt in control or TRAIL-treated LNCaP and LNCaP C4-2. Treatment of cells with TRAIL for 15 min slightly decreased phosphorylated Akt in both LNCaP and LNCaP C4-2 (Fig. 7), which returned to control levels by 60 min and exceeded control levels by the end of experimental period (4 h). These results demonstrate that decreased response of LNCaP to TRAIL (compared with LNCaP C4-2) is not because of differences in the levels of phosphorylated Akt. When the cells were treated with both TRAIL and Mifepristone, levels of phosphorylated Akt decreased significantly compared with similar response in TRAIL-treated cells. The decrease in Akt levels was greater in Mifepristone and



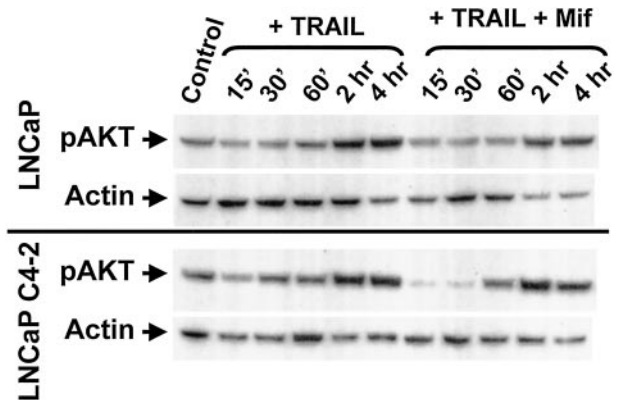
**Fig. 6.** Use of inhibitors to examine the role of specific caspases in apoptotic response. Cells were treated with drugs in the presence or absence of inhibitors for caspases -8 or -9. Z-IETD-FMK is a specific inhibitor for caspase-8, whereas Z-LEHD-FMK is specific to caspase-9. All of the other abbreviations are as described in earlier figures.

TRAIL-treated LNCaP C4-2 (by 15 min) compared with LNCaP. We are presently investigating the possibility that initial decrease in the levels of protective Akt may be sufficient to allow mitochondria to commit to apoptosis in treated cells.

**Cytochrome C Was Released in Response to Treatment.** As LNCaP C4-2 are sensitive to TRAIL although the levels of phosphorylated Akt were same in LNCaP and LNCaP C4-2, we investigated whether differential release of cytochrome *c* from mitochondria was responsible for altered apoptotic response. Cells were treated, and cytosolic fractions were carefully isolated and analyzed for the presence of cytochrome *c*. The absence of mitochondrial contamination in cytosol was determined by immunoblots for mitochondrial protein Cox II (data not shown). Treatment of LNCaP with TRAIL increased cytochrome *c* levels in cytosol within 15 min, which increased additionally by 30 min (Fig. 8). Cytochrome *c* levels in TRAIL-treated LNCaP C4-2 cytosol were significantly higher compared with LNCaP, suggesting that mitochondrial pathway was more sensitive in these cells. More cytochrome *c* was released into cytosol when cells were treated with both Mifepristone and TRAIL. Similar to the results with TRAIL alone, cytosolic cytochrome *c* were higher in LNCaP C4-2 compared with LNCaP. These results indicate that increased response of LNCaP C4-2 to TRAIL is because of higher sensitivity of mitochondria.

## Discussion

TRAIL is a potent new drug that is being tested for the treatment of brain, colon, and mammary cancer. Studies



**Fig. 7.** Reduced response of LNCaP to TRAIL is not mediated through Akt. Cells were treated with TRAIL alone or with Mifepristone for different durations, and the cytosol was examined for the levels of phosphorylated Akt (pAKT).

have indicated that TRAIL preferentially kills tumor cells and demonstrate no measurable adverse effects in animal studies. An earlier report suggested that three popular prostate cancer cell lines, LNCaP, PC3, and DU145 were resistant to the effects of TRAIL (21). Subsequent experiments in our laboratory and others (22, 42, 43) demonstrate that TRAIL is capable of inducing death of several prostate cancer cell lines. However, LNCaP cells have been shown to be consistently resistant to TRAIL (23, 43). Similar resistance to TRAIL has been demonstrated in other cancer cell lines. Some of the reasons identified for the resistance to TRAIL are: absence or lowered expression of death receptors, loss of caspase-8, and inhibition of caspase-3 function (61). In LNCaP, elevated levels of Akt have been credited with responsibility for the resistance to TRAIL (23, 43). To sensitize TRAIL-resistant cells, several new drugs are being used, which induce up-regulation of TRAIL receptors, inhibit NF $\kappa$ B activation, down-regulate inhibitor of apoptosis proteins, or potentiate mitochondrial apoptotic pathways (61). As it is known that normal cells are resistant to TRAIL, it is important to determine the reasons for resistance of LNCaP and compare the response of it subline TRAIL-sensitive LNCaP C4-2. In the past, we have demonstrated that Mifepristone induced apoptosis in LNCaP by activating the TRAIL pathway (20). Therefore, experiments were conducted to determine whether treatment of cells with Mifepristone and TRAIL altered the apoptotic response.

The effect of drugs on cells was measured using two different methods. As explained earlier, MTT assays measure cell survival more than cell death. Therefore, MTT data were supplemented with data from Apoptosense, which measured the cleavage of cytokeratin 18 in response to activated caspases. It was satisfying that in these experiments, both MTT and Apoptosense assays yielded comparable results, providing confidence in these assays. Initial experiments with TRAIL showed that in LNCaP C4-2, apoptosis increased progressively, whereas LNCaP showed very little apoptosis. LNCaP cells are androgen-dependent, noninvasive prostate cancer cells, whereas LNCaP C4-2 cells were derived from LNCaP, and are invasive and more metastatic.



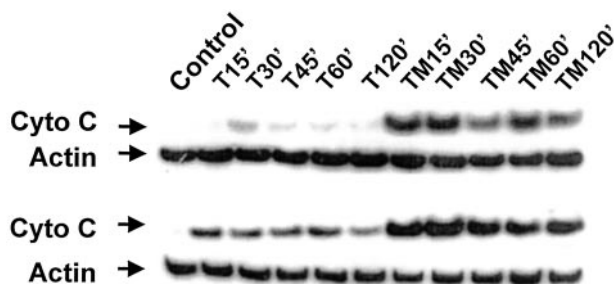


Fig. 8. Cytochrome c was released in response to treatment. Control and treated cells were harvested, and cytosol was isolated as described. Release of cytochrome c into the cytoplasm was measured by immunoblot. Cyto C, cytochrome c.

As LNCaP C4-2 cells were originally described as androgen-independent cell lines, we considered whether the differences to androgen response was responsible for differential apoptotic effects of TRAIL. However, a recent publication demonstrated functional androgen receptors in both these cells (62), indicating that differences in response of the cells to TRAIL were not androgen mediated.

To determine the reasons for differential response of the cells to treatment, we examined the expression of death/decoy receptors, activation of caspases and truncation of Bid. It is well established that treatment of cells with TRAIL activates DR4 and DR5 (occasionally, with concomitant decrease in decoy receptors). Examination of levels of death and decoy receptor in TRAIL-treated prostate cells showed marginal changes in death and decoy receptors, which were not significant enough to account for differences in apoptotic response, suggesting that altered response to TRAIL may be because of down-stream proteins.

Recent publications showed that caspase-8 activation is a prerequisite for the effects of TRAIL (40, 55–59). Treatment of colon cancer cells with doxorubicin and cisplatin sensitized the cells to TRAIL-induced cleavage of procaspase-8 and downstream proteins (57), suggesting that anticancer drugs increased the ability of TRAIL to activate caspase-dependent cell death. Treatment of LNCaP and LNCaP C4-2 with TRAIL alone or in combination with Mifepristone activated caspase-8; however, no significant differences in caspase-8 activation were noted between the cell lines, suggesting that caspase-8 may not be responsible for altered apoptotic response of the cells. The key intermediary between death receptors and caspase-8 are the cFLIP proteins, which block activation of caspase-8. cFLIP did not show significant changes between cell lines in control and treated cells (data not shown), which agrees with published data that in LNCaP, cFLIP did not influence response to TRAIL (23).

As Bid is truncated by activated caspase-8, we next examined the levels of tBid in response to treatment. Levels of tBid were higher in treated LNCaP C4-2 compared with LNCaP and lasted longer. However, it is difficult to explain the differences in levels of tBid, when caspase-8 activation was similar in both cell lines. One possibility is that in addition to caspase-8 some downstream factor(s) influenced the truncation of Bid. Bid is known to be truncated by down-

stream caspases such as caspase-3 and caspase-9 and, therefore, activation of mitochondrial apoptotic pathway may induce a short-loop feedback on Bid truncation. To accomplish this we should be able to observe increased activation of caspases-9, -3, and -7. As expected, our results demonstrate increased activation of caspases-9 and -3 in TRAIL- and/or Mifepristone-treated LNCaP C4-2 compared with LNCaP. To determine whether caspase-9-mediated activation of caspase-3 resulted in truncation of Bid under our experimental conditions, the function of caspase-9 was blocked. The specific inhibitor for caspase-9 effectively blocked the function of caspase-9, resulting in decreased activation of caspase-3 and lower levels of tBid. Thus, enhanced levels of tBid in LNCaP C4-2 may be because of increased activation of caspase-3, which, in turn, truncated Bid. Thus, our results support our hypothesis that poor response of LNCaP to TRAIL was because of decreased response of mitochondrial machinery. Pretreatment with Mifepristone probably sensitized LNCaP by activating the TRAIL pathway (20) and by activating other mitochondria-based apoptotic pathways, such as the Bcl2 family.

In LNCaP, similar to other cells, members of cell survival pathway provide protection from apoptosis. In LNCaP the tumor suppressor gene *PTEN* is inactivated because of a frameshift mutation, thus subjecting the cells to continuous stimulus by the PI3k/Akt pathway (63). Treatment of LNCaP with inhibitors of PI3k increased drug-induced apoptosis, indicating that cells lacking *PTEN* are less sensitive to anti-cancer drugs (63), although this response was blocked by other survival stimuli such as treatment with epidermal growth factor or androgens (44, 64, 65). Recently, publications suggested that resistance of LNCaP to TRAIL is because of enhanced constitutive expression of phosphorylated Akt (23, 43). These authors used inhibitors to block PI3k function and showed that resistance of LNCaP to TRAIL is PI3k-dependent. If the levels of phosphorylated Akt are the main reason for TRAIL resistance of LNCaP, we expected to observe significant differences between phosphorylated Akt in LNCaP and TRAIL-sensitive LNCaP C4-2. However, Western blots showed no significant changes in the levels of phosphorylated Akt in LNCaP and LNCaP C4-2, indicating that although Akt may promote cell survival, Akt may not be responsible for the differences in response of LNCaP and LNCaP C4-2 to TRAIL. Differences in apoptotic response of these cells may be mediated by other agents, such as Bcl<sub>2</sub> and NF $\kappa$ B. Recently, NF $\kappa$ B is known to influence the function of X-linked inhibitor of apoptosis protein, which inhibits TRAIL-induced apoptosis (66). In these experiments, the levels of cytochrome c in cytosol was significantly higher in TRAIL-treated LNCaP C4-2 suggesting that differences in the response of LNCaP mitochondria to TRAIL may be responsible for altered apoptotic response. Furthermore, highly significant increase in cytosolic cytochrome c in cells treated with both Mifepristone and TRAIL suggests increased involvement of mitochondria in Mifepristone-mediated apoptosis.

In summary, our data demonstrate that closely related LNCaP and LNCaP C4-2 respond differently to TRAIL. Differences in response to TRAIL are not because of altered



levels of phosphorylated Akt but may be because of differential response of mitochondrial apoptotic pathways. Furthermore, we demonstrate that Mifepristone sensitized TRAIL-resistant cells at the mitochondrial level.

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