# Curcumin (Diferuloyl-Methane) Enhances Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis in LNCaP Prostate Cancer Cells[1]

**Dorrah Deeb, Yong X. Xu, Hao Jiang, Xiaohua Gao, Nalini Janakiraman, Robert A. Chapman, and Subhash C. Gautam[2]**

Division of Hematology/Medical Oncology [D. D., Y. X. X., X. G., N. J., R. A. C., S. C. G.], and William T. Gossett Neurology Laboratories [H. J.], Department of Neurology, Henry Ford Health System, Detroit, Michigan 48202

## Abstract

**The role of natural food products in prevention of prostate cancer has been confirmed in recent epidemiological studies; however, the mechanism of chemoprevention by the dietary constituents largely remains unknown. Curcumin, the yellow pigment and active component of turmeric (Curcuma longa), exhibits chemopreventive and growth inhibitory activity against several tumor cell lines. The androgen-sensitive human prostate cancer cell line LNCaP is only slightly susceptible to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor family of cell death-inducing ligands. In this study, we investigated whether curcumin and TRAIL cooperatively interact to promote death of LNCaP cells. At low concentrations (10 μM curcumin and 20 ng/ml TRAIL), neither of the two agents alone produced significant cytotoxicity (curcumin, <10%; TRAIL, ~15%) in LNCaP cells, as measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium dye reduction assay. On the other hand, cell death was markedly enhanced (2–3-fold) if tumor cells were treated with curcumin and TRAIL together. The combined curcumin and TRAIL treatment increased the number of hypodiploid cells and induced DNA fragmentation in LNCaP cells. The combined treatment induced cleavage of procaspase-3, procaspase-8, and procaspase-9, truncation of Bid, and release of cytochrome *c* from the mitochondria, indicating that both the extrinsic (receptor-mediated) and intrinsic (chemical-induced) pathways of apoptosis are triggered in prostate cancer cells treated with a combination of curcumin and TRAIL. These results define a potential use of curcumin to sensitize prostate cancer cells for TRAIL-mediated immunotherapy.**

## Introduction

Prostate cancer is the most commonly diagnosed cancer in men and is the second leading cause of cancer-related deaths in North America (1, 2). Current therapies, such as surgery, chemotherapy, and radiation therapy, are of limited efficacy, especially in advanced disease, and metastatic disease remains incurable (3, 4). Androgen reduction therapy is commonly used to control hormone-sensitive tumor cells; however, hormone refractory clones often emerge after hormonal therapy (5). Therefore, novel therapies, including biotherapy, are needed to treat adenocarcinoma of prostate while it is still confined to the gland and potentially curable.

Failure to undergo programmed cell death (apoptosis) has been implicated in tumor development and resistance to cancer therapy (6, 7). Promotion of apoptosis in prostate cancer cells may lead to the regression and improved prognosis of refractory disease. Indeed, prostate cancer cells have been shown to remain somewhat sensitive to apoptosis induced by chemotherapeutic agents (8, 9) and members of the TNF[3]-α superfamily of death inducing ligands, including TNF-α and FasL (Apo-1L or CD95L; Refs. 10–12). However, the use of these apoptosis-inducing drugs and cytokines is limited by unacceptable systemic toxicity. TRAIL (Apo-2L), the most recently identified member of the TNF family of apoptosis-inducing ligands, has been shown to induce apoptosis in various cancer cell types *in vitro* with little or no cytotoxicity to the normal cells (13–15). Furthermore, unlike TNF-α and FasL (16–18), TRAIL exhibits antitumor activity *in vivo* without systemic toxicity (19). Thus, induction of cell death by TRAIL might be an attractive new treatment option for advanced prostate cancer.

In contrast to the high incidence of prostate cancer in North America, the incidence of this disease in Japan and China is very low (20, 21). The low incidence of prostate cancer in Asian men has been attributed to the dietary consumption of large amounts of plant-based foods rich in phytochemicals (22–24). Because of these observations, nutritional supplements such as soybean, garlic, green tea, and so on, which are rich in polyphenolic compounds, have been used to augment the anticancer therapies (25, 26). Curcumin, the active component of turmeric, and resveratrol (3,5,4′-

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[2] To whom requests for reprints should be addressed, at Oncology Research Laboratory, 4D, Henry Ford Health System, One Ford Place, Detroit, MI 48202. Phone: (313) 874-6998; Fax: (313) 874-3770; E-mail: sgotam@msn.com.

[3] The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium; FACS, fluorescent-activated cell sorting; CHX, cycloheximide; NF-κB, nuclear factor κB.

trihydroxystilbene), found in grapes, are two such dietary constituents that have received a great deal of attention recently as chemoprotective agents (27–29). We have previously shown that both of these phytochemicals inhibit proliferation and induce apoptosis in human and mouse leukemia cell lines (30, 31). Whether these agents also chemosensitize tumor cells to the apoptosis-inducing effects of cytokines has not been explored. In this report, we demonstrate that curcumin enhances the TRAIL-induced apoptosis in human prostate cancer cell line LNCaP. No such cooperation was observed between resveratrol and TRAIL or curcumin and TNF-$\alpha$. Cell death after combined treatment with curcumin and TRAIL correlated with DNA fragmentation and activation of initiator caspase-8 and caspase-9 and effector caspase-3. The combined curcumin/TRAIL treatment also induced the truncation of proapoptotic Bid and release of cytochrome *c* from the mitochondria.

## Materials and Methods

**Reagents.** *Trans*-resveratrol (*trans*-3,4′,5-trihydroxy stilbene) and curcumin were purchased from Sigma Chemical Co. (St. Louis, MO). Anticaspase-3, caspase-8, and caspase-9 antibodies were purchased from BD PharMingen (San Diego, CA). Anti-Bid antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). TRAIL was purchased from R&D Systems (Minneapolis, MN), and TNF-$\alpha$ was purchased from Pepro Tech Inc. (Rocky Hill, NJ). A 100-mM solution of resveratrol or curcumin was prepared in DMSO, and all test concentrations were prepared by diluting the stock solution in tissue culture medium.

**Cell Lines.** LNCaP cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), 1% penicillin/streptomycin, 1 $\mu$g/ml hydrocortisone, and 100 nM testosterone as described previously (32). DU145 and NIH 3T3 cells (American Type Culture Collection) were grown in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Hyclone, Logan, UT) and 1% penicillin/streptomycin. The mouse bladder tumor cell line MBT-2 obtained from Dr. James A. Hampton (Medical College of Ohio, Toledo, OH) was grown in RPMI 1640 as described for LNCaP cells but without hydrocortisone and testosterone. All cell lines were cultured at 37°C in a humidified atmosphere consisting of 5% $CO_2$ and 95% air.

**Measurement of Cell Viability.** Cells were seeded into 96-well plates at a density of $2 \times 10^4$ cells/well in 100-$\mu$l tissue culture medium in triplicate. After 24-h incubation to allow cells to adhere, cells were treated for 48 h either with curcumin, resveratrol, TRAIL, or TNF-$\alpha$ separately or in different combinations, as described in individual experiments. Cell viability was determined by the colorimetric MTS using CellTiter 96 $AQ_{ueous}$ Assay System from Promega (Madison, WI). In this assay, the quantity of formazan product formed is directly proportional to the number of viable cells in the cultures.

**Flow Cytometry.** To study the expression of TRAIL-R, LNCaP cells ($10^6$ cells) were pretreated with Fc block (anti-CD16/CD32) and reacted with primary goat anti-TRAIL-R1, TRAIL-R2, TRAIL-R3, or TRAIL-R4 antibodies (50 $\mu$g/ml) for 45 min on ice. Cells were washed once with PBS and were then incubated with phycoerythrin-conjugated donkey anti-goat secondary antibody (1:50 dilution) for 45 min at 4°C. Cells were washed and analyzed by FACS on a FACScan flow cytometer (Becton Dickinson).

**DNA Fragmentation.** To measure DNA fragmentation, $6 \times 10^6$ untreated or treated LNCaP cells were washed twice with 1× PBS and then lysed in cold lysis solution [5 mM of Tris (pH 7.4), 20 mM of EDTA, 0.5% Triton X-100) for 20 min. Cell lysates were centrifuged at 27,000 × *g* for 15 min, and DNA was extracted from the aqueous phase with phenol:chloroform:isoamyl alcohol (25:24:1, by volume) containing 0.1% hydroxyquinoline. DNA was precipitated with 0.3 M sodium acetate and 2 volumes of cold 100% ethanol. DNA samples isolated from equal number of untreated and treated cells were fractionated on 1.2% agarose gel in Tris-acetate (40m M Tris-acetate, 1 mM EDTA) electrophoresis buffer. After fractionation of DNA, gels were treated with RNase (20 $\mu$g/ml) for 3 h and stained with ethidium bromide.

**Western Blot Analysis.** Total cellular proteins were isolated by detergent lysis (1% Triton X-100, 10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 2 mM sodium vanadate, 5 $\mu$g/ml leupeptin, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml pepstatinin, and 10 $\mu$g/ml 4–2-aminoethyl-benzenesulfinyl fluoride) as described by Keane *et al.* (33). Lysates were clarified by centrifugation at 14,000 × *g* for 10 min at 4°C, and protein concentration was determined by Bio-Rad colorimetric assay (Bio-Rad, Hercules, CA). Samples (50 $\mu$g) were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% Bromphenol Blue, 125 mM Tris-HCl, and 640 mM $\beta$-mercaptoethanol) and separated on 10% SDS-polyacrylamide gels. Proteins resolved on the gels were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl with 0.05% Tween 20 (TPBS) and probed with antibodies to caspase-3, caspase-8, caspase-9, Bid, cytochrome *c*, or actin. Immune complexes were visualized with enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

**Measurement of Caspase-3 Activity.** LNCaP cells were grown to confluence in 100-mm diameter culture dishes and treated with test agents for 20 h. Cell lysates were prepared using the lysis buffer included in the ApoAlert Caspase Assay kit (Clonetech Laboratories, Inc., Palo Alto, CA) and clarified by centrifugation at 14,000 × *g* for 5 min. Caspase-3 activity in cell lysates (100 $\mu$g each lysate) was measured in 96-well microtiter plates using reagents and instructions provided in the assay kit. Plates were read at 405 nm in a microplate reader.

**Measurement of Cytochrome *c*.** To determine whether combination treatment induces release of cytochrome *c* from mitochondria, LNCaP cells were treated with TRAIL (20 ng/ml), curcumin (10 $\mu$M), or a combination of TRAIL and curcumin for 20 h. Using ApoAlert Cell Fractionation Kit (Clonetech Laboratories, Inc.), mitochondria and cytosol fractions were prepared from treated cells after instructions and reagents included in the kit. Ten $\mu$g of cytosolic or mitochondrial protein were separated on a 12% SDS-PAGE gel and after protein transfer membrane was probed with cytochrome *c* antibody.

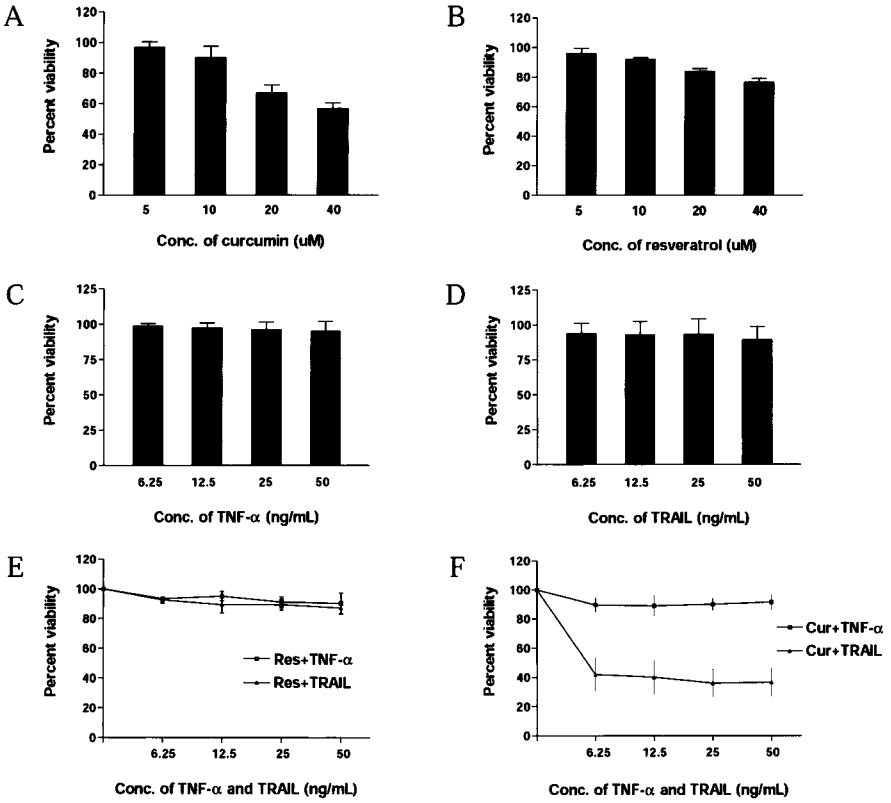


*Fig. 1*. Effect of curcumin, resveratrol, TNF-$\alpha$, or TRAIL separately and in combination on viability of LNCaP cells. A total of $2 \times 10^4$ LNCaP cells was seeded in each well of a microtiter plate in 0.1 ml of culture medium. Cells were allowed to adhere for 20 h and then washed to remove nonadherent cells. Cells were treated with curcumin (*A*), resveratrol (*B*), TNF-$\alpha$ (*C*), or TRAIL (*D*) for 48 h at concentrations as shown. For the combined treatment, cells were treated with 10 $\mu$M resveratrol and TNF-$\alpha$ or TRAIL (*E*) or 10 $\mu$M curcumin and TNF-$\alpha$ or TRAIL (*F*) for 48 h. Cell viability was measured by the MTS assay using CellTiter $AQ_{ueous}$ assay system from Promega. Similar results were observed in five independent experiments.

## Results

**Curcumin, but not Resveratrol, Chemosensitizes LNCaP Cells for TRAIL-mediated Cytotoxicity.** Androgen-sensitive LNCaP prostate cancer cells are largely resistant to TRAIL-induced apoptosis. To test whether curcumin and resveratrol sensitize prostate tumor cells to TNF-$\alpha$ or TRAIL-mediated cytotoxicity, we first examined the effect of these phytochemicals individually on the viability of LNCaP cells in MTS assay. As shown in Fig. 1*A*, curcumin at 20–40 $\mu$M concentration reduced cell viability by 40–45%. The effect of curcumin on cell viability was minimal at 5–10 $\mu$M concentration (10–15% reduction). In contrast, LNCaP cells were less sensitive to the cytotoxic effect of resveratrol. A small reduction in cell viability (15–20%) was observed at 20–40 $\mu$M resveratrol (Fig. 1*B*). Next, we tested the effect of TNF-$\alpha$ and TRAIL separately and in combination with curcumin or resveratrol on the viability of LNCaP cells. Treatment with TNF-$\alpha$ or TRAIL at concentrations from 6.25 to 50 ng/ml for 48 h produced very little loss in cell viability ($<$10%; Fig. 1, *C* and *D*, respectively). In the combined treatment, resveratrol (10 $\mu$M) in combination with TNF-$\alpha$ or TRAIL over a concentration range of 6.25- 50 ng/ml produced cytotoxicity, which was no more than that observed by treating cells with each of these agents individually (Fig. 1*E*). Cytotoxic effect of treatment with curcumin (10 $\mu$M) and TNF-$\alpha$ together was also not different from that of each agent alone (Fig. 1*F*). In contrast, treatment with a combination of curcumin and TRAIL produced greater cytotoxicity compared with the sum of toxicity produced by each as a single agent (Fig. 1*F*). Overall, the reduction in cell viability with combined treatment was 2.5–3-fold higher than the sum of the loss of cell viability by either agent alone.

**Combined Curcumin and TRAIL Treatment Inhibits Clonal Growth of LNCaP Cells.** Whether a combination of curcumin and TRAIL affects the clonal expansion of LNCaP cells was measured by incorporating curcumin (10 $\mu$M) or TRAIL (20 ng/ml), or curcumin and TRAIL together (10 $\mu$M and 20 ng/ml, respectively) in culture medium containing 0.3% agar and $10^3$ LNCaP cells/ml in 35-mm Petri dishes. Fig. 2 shows the colony formation by LNCaP cells under different treatment conditions. Separately, curcumin or TRAIL suppressed clonal growth by 25–35%; however, growth inhibition with combined curcumin and TRAIL treatment was $>$95%. A few colonies that developed in the presence of curcumin and TRAIL together were smaller in size and reduced in cellularity.

**Curcumin Selectively Augments TRAIL-induced Cytotoxicity in LNCaP Cells.** To determine whether enhanced cytotoxicity by combined curcumin and TRAIL treatment is a phenomenon common to all cell types, we tested the sensitivity of DU145 prostate cancer cells, MBT-2 murine bladder cancer cells, and NIH3T3 fibroblasts to combined curcumin (10 $\mu$M) and TRAIL (20 ng/ml) in MTS assay. Fig. 3 demonstrates that only LNCaP cells, but not DU145, MBT-2 or NIH3T3 cells exhibit enhanced sensitivity to the combined treatment.

**LNCaP Cells Express TRAIL Receptors.** The cell surface expression of TRAIL-R1, TRAIL-R2, TRAIL-R3, and

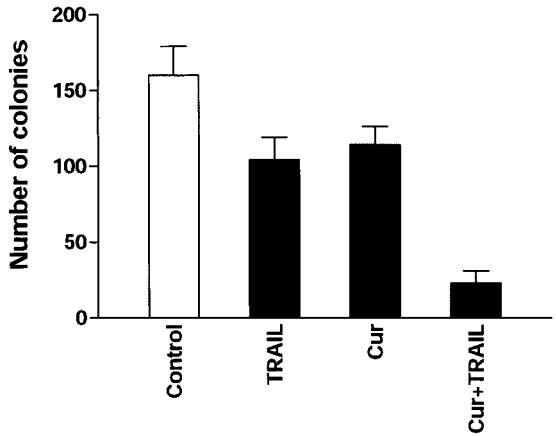


*Fig. 2*. Effect of combined curcumin and TRAIL treatment on clonal growth of LNCaP cells. A total of $10^3$ LNCaP cells was resuspended in 1 ml of culture medium containing 0.3% agar, 10 $\mu$M curcumin or 20 ng/ml TRAIL or curcumin + TRAIL. Cell suspension was added to 35-mm culture dishes in duplicate. After incubation for 7 days, the number of colonies formed by tumor cells was determined using an inverted microscope. Similar results were observed in three independent experiments.

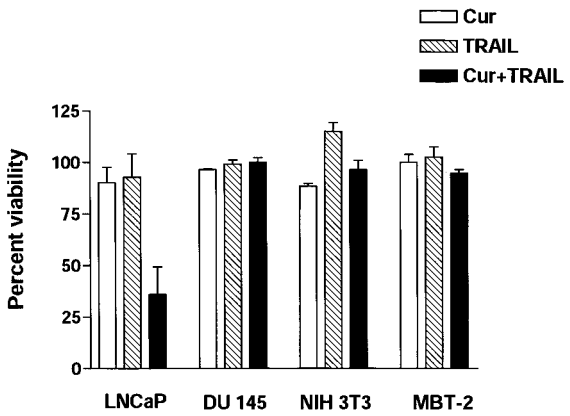


*Fig. 3*. Selectivity of combined curcumin and TRAIL treatment. LNCaP, DU145, NIH 3T3, or MBT-2 cells were treated with curcumin and TRAIL for 48 h as described in Fig. 1*F*. Cell viability was determined by MTS assay. Similar results were observed in three independent experiments.

TRAIL-R4 receptors on LNCaP cells was analyzed by flow cytometry. Fig. 4 shows high expression of both death signal transducing (TRAIL-R1 and TRAIL-R2) and decoy (TRAIL-3 and TRAIL-4) receptors at the surface of LNCaP cells. Compared with TRAIL-R1 (68% cells), the high affinity TRAIL-R2 was more abundantly expressed on LNCaP cells (99% cells). The expression of both of the decoy receptors was also very high (95–98%) on these cells.

**Combined Curcumin and TRAIL Treatment Induces Apoptosis in LNCaP Cells.** To determine the mechanism of the cytotoxic effect of combined treatment, LNCaP cells treated with curcumin or TRAIL or curcumin + TRAIL for 48 h were stained with propidium iodide. FACS analysis of cells stained with propidium iodide revealed very little increase in hypodiploid cells in sub-$G_1$ phase after treatment with curcumin or TRAIL alone (3.5 and 1.6%, respectively; Fig. 5, *top panel*). On the other hand, treatment with a combination of curcumin and TRAIL resulted in marked increase in cells in sub-$G_1$ phase (61%). It is interesting to note that cells treated with curcumin alone showed enrichment in $G_2$-M phase of cell cycle. The results of FACS analysis of DNA were corroborated by significant loss of cells in cultures, as visualized by light microscopy (Fig. 5, *bottom panel*).

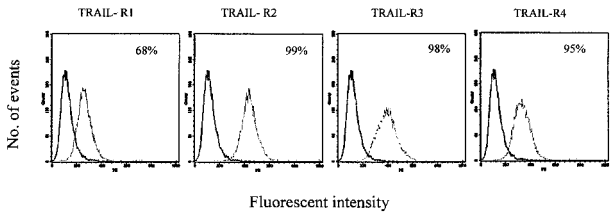


*Fig. 4*. Flow cytometric analysis of TRAIL- R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 expression on LNCaP cells.

To determine whether combination treatment induces DNA fragmentation, DNA was isolated from treated cells and fractionated by agarose gel electrophoresis. A typical ladder pattern of internucleosomal fragmentation was observed in cells treated with curcumin and TRAIL together (Fig. 6, *Lane 5*). Treatment with curcumin alone did not induce DNA fragmentation (*Lane 4*), whereas some DNA fragmentation was observed in cells treated with TRAIL alone (*Lane 3*). DNA fragmentation induced by combined curcumin and TRAIL treatment was completely blocked by caspase-3 inhibitor z-DEVD-fmk (*Lane 6*). Furthermore, z-DEVD-fmk was found to rescue cells from curcumin + TRAIL-induced cell death (data not shown). Taken together, these data indicate that combination treatment induces apoptosis in LNCaP cells.

**Augmentation of Apoptosis by Curcumin and TRAIL Combination Involves Activation of Caspases.** Because caspase-8 is a key upstream caspase, the activation of which mediates the cleavage of procaspase-3, we analyzed the processing of procaspase-8 and procaspase-3 in treated cells by Western blotting. Fig. 7 demonstrates little effect of TRAIL or curcumin on the processing of procaspase-3 or procaspase-8. In contrast, cells treated with curcumin and TRAIL together showed significant cleavage of both procaspases. The bar graphs compare the relative intensities of procaspase-3 and procaspase-8 bands after various treatments. The activation of caspase-3 was completely blocked by caspase-3 inhibitor z-DEVD-fmk (Fig. 8*A*). In addition, we also measured the relative proteolytic activity of caspase-3 in cell lysates by the proteolytic cleavage of DEVD-pNA. No cleavage of DEVD-pNA was observed with cell lysate prepared from curcumin-treated cells (Fig. 8*B*). Treatment with TRAIL alone increased the cleaving activity; however, cleavage of DEVD-pNA was most pronounced by cell lysate prepared from LNCaP cells treated with curcumin and TRAIL together. These data are consistent with the results of Western blotting analysis of caspase-3 activation in treated cells.

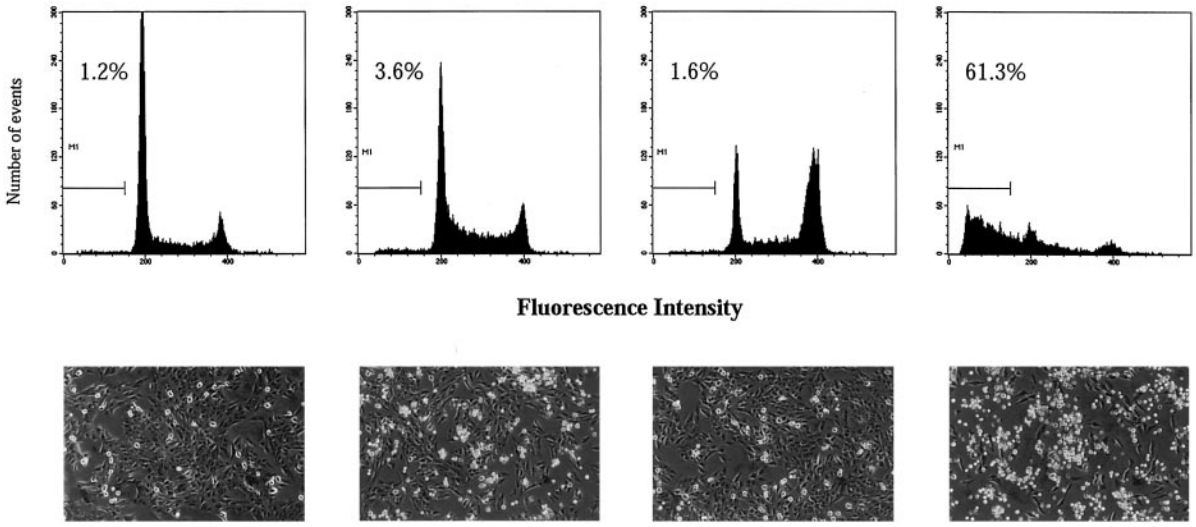


*Fig. 5*. *Top panel:* histograms of flow cytometric analysis showing effect of curcumin or TRAIL or curcumin + TRAIL treatment on apoptosis in LNCaP cells. *Bottom panel:* cellular changes in cultures as visualized by light microscopy. Similar results were observed in two independent experiments.

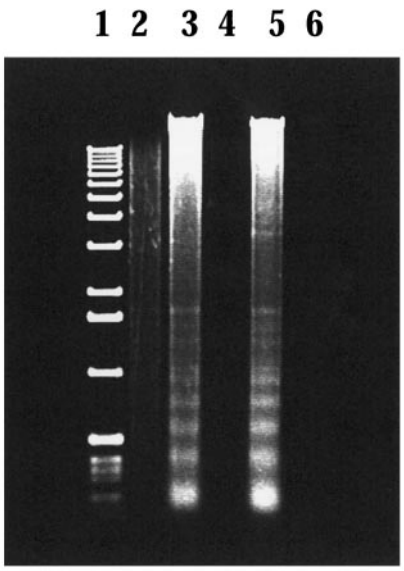


*Fig. 6*. Combined curcumin/TRAIL treatment induces DNA fragmentation. DNA isolated from LNCaP cells that were untreated (*Lane 2*), or treated with TRAIL (*Lane 3*), or curcumin (*Lane 4*), or curcumin + TRAIL (*Lane 5*), or curcumin + TRAIL in the presence of z-DEVD-fmk (*Lane 6*) was fractionated by electrophoresis. Oligonucleosomal length DNA fragments were visualized by staining gels with ethidium bromide. *Lane 1*, DNA ladder. Similar results were obtained in two different experiments.

**CHX Enhances Curcumin/TRAIL-induced Activation of Caspase-3 and Cytotoxicity.** To determine whether augmentation of apoptosis by combined curcumin and TRAIL treatment requires *de novo* protein synthesis, LNCaP cells were pretreated with CHX (1 $\mu$g/ml) for 1 h before applying curcumin and TRAIL. The cleavage of procaspase-3 by curcumin/TRAIL treatment was enhanced by pretreating cells with CHX. In fact, procaspase-3 was completely cleaved by combined curcumin/TRAIL in cells pretreated with CHX (Fig. 9*A*). Furthermore, enhancement of procaspase-3 cleavage in cells pretreated with CHX also resulted in reduced cell survival (Fig. 9*B*). These results indicate that inhibition of either the constitutively expressed inhibitory proteins or those induced by the treatment itself (curcumin/TRAIL) results in augmentation of caspase-3 activation and cell death.

**Combination of Curcumin and TRAIL Induces Cleavage of Bid and Release of Cytochrome *c* from Mitochondria.** Because Bid, a proapoptotic member of the Bcl-2 family, can be activated through cleavage by activated caspase-8 and induce release of cytochrome *c* from mitochondria, we measured Bid and cytochrome *c* in cells treated with TRAIL or curcumin or curcumin/TRAIL together. The levels of native Bid were slightly reduced after treatment with TRAIL or curcumin alone; however, the most reduction in Bid was seen in cells treated with a combination of curcumin and TRAIL (Fig. 10*A*). Whether truncated Bid induced the release of cytochrome *c* from mitochondria was examined next. As shown in Fig. 10*B*, treatment of LNCaP cells with TRAIL or curcumin alone partially reduced mitochondrial cytochrome *c*; however, mitochondrial cytochrome *c* was most reduced in cells treated with curcumin/TRAIL together. Consistent with the treatment-dependent reduction in cytochrome *c* levels in mitochondria, there was a corresponding increase in cytochrome *c* in cytosol, the highest being in cytosol fraction of cells treated with the combination of curcumin and TRAIL (Fig. 10*C*).

Cytochrome *c* in association with Apaf-1, also released from mitochondria, activates procaspase-9. To determine whether combination of curcumin and TRAIL induces processing of procaspase-9, cells were treated with curcumin or TRAIL or curcumin/TRAIL together for 20 h. Cell extracts were prepared and analyzed by Western immunoblotting as described before. Treatment with TRAIL alone reduced the procaspase-9 somewhat, whereas no change in the levels of procaspase-9 was detected in cells treated with curcumin

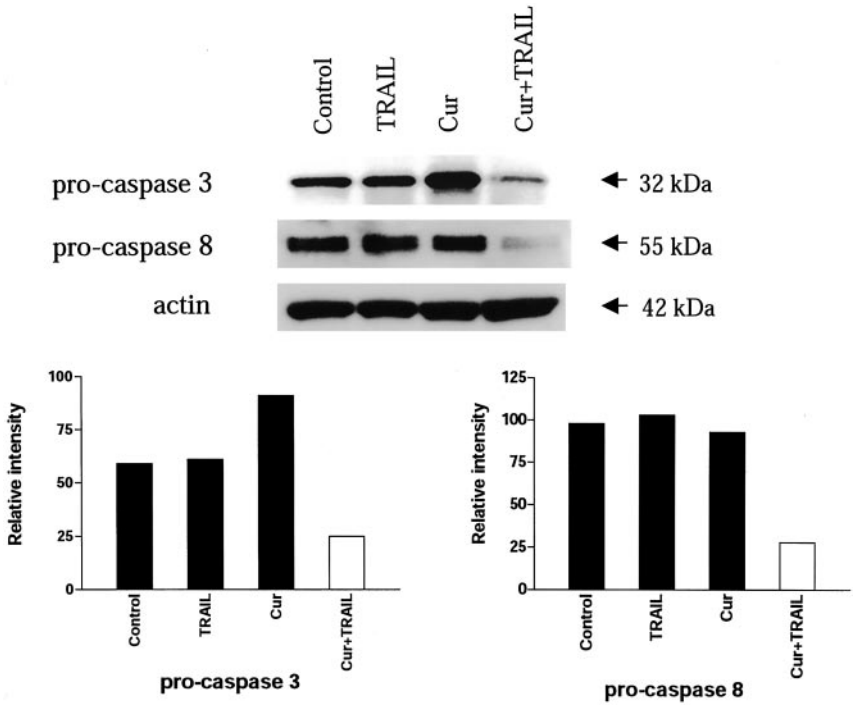


*Fig. 7.* Combined treatment with curcumin and TRAIL cleaves procaspase-3 and procaspase-8. Total cellular protein (50 $\mu$g/lane) isolated from untreated LNCaP cells or those treated with TRAIL, or curcumin, or curcumin + TRAIL for 20 h was fractionated on 10% SDS-PAGE gel. Proteins were transferred from the gel to nitrocellulose membrane and probed with antibody to caspase-3, caspase-8, or actin and horseradish peroxidase-conjugated second antibody and visualized with enhanced chemiluminescence. Histograms compare the band densities of procaspase-3 and procaspase-8 after different treatments. Similar results were obtained in three independent experiments.

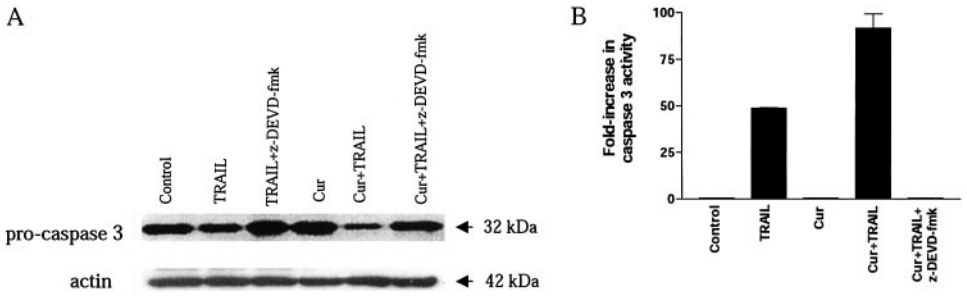


*Fig. 8.* Cleavage of procaspase-3 by combined curcumin and TRAIL treatment is inhibited by caspase-3 inhibitor z-DEVD-fmk. LNCaP cells were treated in the absence or the presence of z-DEVD-fmk (100 $\mu$M), as described in Fig. 6. *A*, analysis of procaspase-3 by Western blotting. *B*, colorimetric measurement of caspase-3 proteolytic activity of cell lysates using ApoAlert Caspase Assay kit from Clonetech Laboratories. Similar results were observed in two independent experiments.

alone (Fig. 10*C*). On the other hand, procaspase-9 was most reduced in cells treated with curcumin and TRAIL together (Fig. 10*C*).

## Discussion

Dietary constituents of food have been shown to provide protection against many diseases and reduce the severity of others (34, 35). We have previously reported that two such ingredients of food, resveratrol and curcumin, inhibit the clonogenic growth and induce apoptosis in several human and murine leukemia cell lines (30, 31). In this study, we demonstrated that moderate concentrations of resveratrol or curcumin (5–10 $\mu$M) do not induce appreciable cell death in LNCaP cells. Similarly, LNCaP cells were also resistant to both TNF-$\alpha$ and TRAIL at concentrations ranging from 5 to 50 ng/ml. In contrast, curcumin, but not resveratrol, in combination with TRAIL, but not TNF-$\alpha$, caused 2–3-fold enhanced cell death in LNCaP cells at concentrations that each agent alone is ineffective or only poorly effective. Cooperation between curcumin and TRAIL was also observed in inhibition of the clonal growth of these cells.

Several recent reports have shown the ability of nontoxic concentrations of chemotherapeutic agents to sensitize tumor cells for apoptosis by death-inducing ligands such as FasL, TRAIL, or TNF-$\alpha$ (8–11). The enhanced cytotoxicity of combined curcumin and TRAIL treatment may result from augmentation of TRAIL-induced apoptosis by curcumin. Indeed, treatment of LNCaP cells with curcumin and TRAIL together caused a decrease in the diploid cells with a corresponding increase in the hypodiploid (sub-$G_1$) population, suggesting that cytotoxicity induced by curcumin and TRAIL is mediated by induction of apoptosis. This observation was also supported by the induction of internucleosomal DNA fragmentation and its inhibition in the presence of caspase-3 inhibitor z-DEVD-fmk. These results are consistent with the previously reported data by other investigators that anticancer agents such as doxorubicin, 5-fluorouracil, cisplatin, paclitaxel, synthetic retinoids, etoposide, and so on enhance

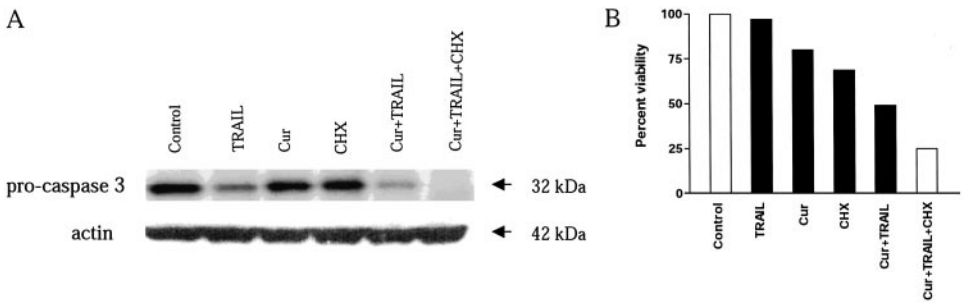


*Fig. 9.* Pretreatment of LNCaP cells with CHX augments cleavage of procaspase-3 and cell death by combined curcumin and TRAIL treatment. *A*, cells were treated with CHX (1 $\mu$g/ml) starting 1 h before treatment with curcumin + TRAIL. After incubation for 20 h, cellular proteins were analyzed for procaspase-3 levels by Western blotting. *B*, cell viability (MTS assay) after pretreatment of cells with CHX for 1 h and then treatment with curcumin + TRAIL for 48 h. Similar results were obtained in two different experiments.

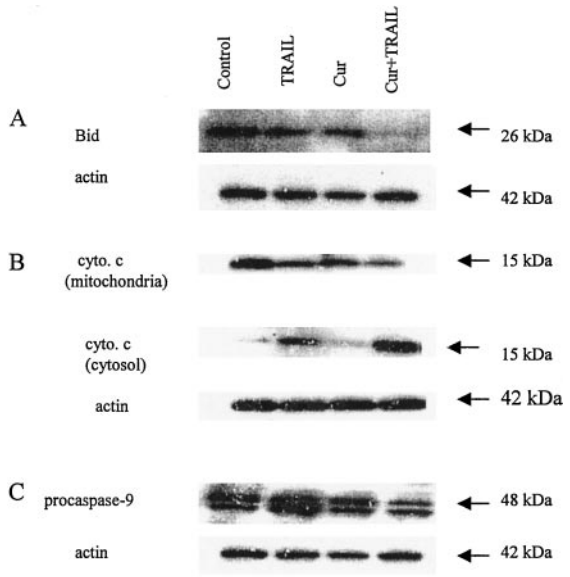


*Fig. 10.* Combined treatment with curcumin and TRAIL truncates Bid, induces release of cytochrome *c* from mitochondria, and cleaves procaspase-9. LNCaP cells were treated with curcumin, TRAIL, or curcumin and TRAIL combined as described previously. For analysis of Bid (*A*) or procaspase-9 (*C*) by Western blotting, cell extracts were prepared and processed as described in Fig. 7. Mitochondrial and cytosolic cytochrome *c* (*B*) was analyzed by using ApoAlert Cell Fractionation Kit (Clonetech Laboratories, Inc.).

TRAIL-mediated apoptosis in tumor cells at subtoxic concentrations (8, 33, 36, 37). In the present studies, curcumin lowered the signaling threshold required for TRAIL-induced cell death in LNCaP cells. As low as 6.25 ng/ml TRAIL induced significant cell death in combination with subtoxic concentration of curcumin, whereas treatment with TRAIL alone, up to a concentration of 50 ng/ml, was inconsequential.

FACS analysis revealed that LNCaP cells express death signal transducing receptors TRAIL-R1 and TRAIL-R2, suggesting that these cells are capable of responding to TRAIL. However, LNCaP cells are not killed when treated with TRAIL alone. This is most likely because these cells also express decoy (inhibitory) receptors TRAIL-R3 and TRAIL-R4. Unlike signaling by FasL, little is known about the events associated with binding of TRAIL to its receptors. Because combination of curcumin and TRAIL induced the cleavage of the most apical initiator caspase-8, this suggests that apoptosis in LNCaP cells is, at least in part, mediated via the death receptor-signaling pathway. Whether increased cell death in cultures treated with curcumin and TRAIL together results from the enhancement of the expression or binding affinity of death receptors TRAIL-R1 and TRAIL-R2 by curcumin has not been established. Similarly, the argument can also be made that curcumin may increase the sensitivity of LNCaP cells to TRAIL by reducing the expression of inhibitory TRAIL-R3 and TRAIL-R4.

Two major modes of apoptotic cell death program have been identified, namely receptor-mediated and chemical-induced apoptosis. In both cases, caspases, a family of cysteine proteases, play an important role (38). Binding of the death-inducing ligands (*e.g.,* TNF-$\alpha$, FasL, and TRAIL) with their cognate receptors activates caspase-8 and caspase-3 leading to apoptosis (39). In chemical-induced (chemotherapeutic agents) apoptosis, undefined signals induce release of cytochrome *c* from mitochondria, which in conjunction with Apaf-1 causes activation of caspase-9. Activated caspase-9, in turn, can activate effector caspase-3, caspase-6, and caspase-7 (38). Recent evidence also suggests existence of cross-talk between caspase-8- and caspase-9-mediated apoptotic pathways through Bid, a pro-apoptotic Bcl-2 family member (40, 41). Our studies have demonstrated enhanced cleavage of procaspase-3, procaspase-8, and procaspase-9 in LNCaP cells treated with curcumin and TRAIL together. Furthermore, the results also demonstrated that combined curcumin/TRAIL treatment induces cleavage of Bid and release of cytochrome *c* from the mitochondria. These finding are consistent with the previously reported effect of curcumin in which it was shown to induce cleavage of Bid and release of cytochrome *c* from mitochondria in HL-60 leukemia cells (42). The release of cytochrome *c* from mitochondria and activation of caspase-9 indicate that the intrinsic pathway is also activated in cells treated with combination of curcumin and TRAIL. The activation of initiator/effector caspases (caspase-8, caspase-9, caspase-3) and proapoptotic Bid seen in LNCaP cells treated with combined curcumin and TRAIL treatment may result from the enhancement by curcumin of TRAIL-initiated cleavage processes. Alternatively, curcumin and TRAIL may

promote apoptosis by inducing two independent but complementary activating signals.

Chemotherapy agents can augment TRAIL-induced apoptosis in cancer cells by inducing p53 or lowering NF-κB activity. Expression of NF-κB transcription factor in tumor cells has been shown to inhibit apoptosis by TNF-α (43). We have previously shown that curcumin inhibits the activation and translocation of NF-κB to the nucleus (44). This suggests that up-regulation of TRAIL-induced apoptosis by curcumin may result from the suppression of NF-κB activity in LNCaP cells. Further, suppression of the endogenous inhibitors of apoptosis such as cFLIP or XIAP is another mechanism by which curcumin could augment TRAIL-mediated apoptosis (45). It remains undetermined at present whether curcumin abrogates the activity of these intracellular negative regulators of apoptosis.

In summary, this study demonstrates that curcumin augments TRAIL-mediated apoptosis in androgen-sensitive prostate cancer cells. The induction of apoptosis by combined curcumin and TRAIL treatment involves the activation of initiator/effector caspases (caspase-8, caspase-9, caspase-3), cleavage of proapoptotic Bid, and the release of cytochrome *c* from the mitochondria. Thus, combination of TRAIL with curcumin, a pharmacologically safe compound, may provide a more effective adjuvant treatment for prostate cancer.

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