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ORIGINAL ARTICLE

Green tea polyphenol EGCG sensitizes human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and synergistically inhibits biomarkers associated with angiogenesis and metastasis

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) is a promising candidate for cancer therapy, however, emergence of drug resistance limits its potential use. Here, we report for the first time that epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea, sensitizes TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis through modulation of intrinsic and extrinsic apoptotic pathways. When combined with EGCG, Apo2L/TRAIL exhibited enhanced apoptotic activity in LNCaP cells characterized by three major molecular events. First, apoptosis induction was accompanied by the upregulation of poly(ADP-ribose) polymerase cleavage and modulation of pro- and antiapoptotic Bcl2 family of proteins. A synergistic inhibition of inhibitors of apoptosis with concomitant increase in caspase cleavage was observed. Second, pretreatment of cells with EGCG resulted in modulation of death-inducing signaling cascade complex involving DR4/TRAIL R1, Fas-associated death domain and FLICE-inhibitory protein proteins. Last, we observed a synergistic inhibition in the invasion and migration of LNCaP cells. This effect was observed to be mediated through inhibition in the protein expression of vascular endothelial growth factor, uPA and angiopoietin 1 and 2. Further, the activity and protein expression of MMP-2, -3 and -9 and upregulation of TIMP1 in cells treated with a combination of EGCG and TRAIL was observed. These data might have implications for developing new strategies aimed at eliminating prostate cancer cells resistant to TRAIL.
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Keywords: EGCG; TRAIL; intrinsic pathway; extrinsic pathway; angiogenesis; metastasis

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Introduction

Prostate cancer (CaP) has become the most frequently diagnosed, noncutaneous neoplasm and second leading cause of cancer-related deaths among men in the United States. It is estimated that in the year 2007, 218 890 new cases will be diagnosed and 27 050 men will die from CaP in the United States alone (Jemal *et al*., 2007). Thus, developing novel treatment options for CaP has become an important medical need. The use of phytonutrient as anticancer agents has gained considerable importance in recent years. Several studies from our laboratory and by others have suggested that green tea, especially its constituent polyphenols possess chemopreventive and therapeutic potential against CaP (Jian *et al*., 2004; Bettuzzi *et al*., 2006; Siddiqui *et al*., 2006, 2007). Much of the anticancer and/or cancer chemopreventive effects of green tea are attributed to be mediated by its major polyphenol(−)epigallocatechin-3-gallate (EGCG) (Adhami *et al*., 2007; and the references therein).

Tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL), is a cytokine of TNF family and is capable of inducing apoptotic cell death in a variety of cancer type, while producing negligible effects on normal cells (Zhang *et al*., 2000; Ozawa *et al*., 2001; He *et al*., 2004). Studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (Deeb *et al*., 2004; Yamaguchi *et al*., 2005; Hu *et al*., 2006), however, these cells can be sensitized by combination of TRAIL with different chemotherapeutic drugs, indicating that combinatorial approach can be utilized to overcome the resistance of cancer cells to TRAIL. Thus, development of novel strategies to sensitize cancer cells to undergo TRAIL-mediated apoptosis has become an important strategy for effective cancer therapy.

Angiogenesis is a physiological process involving the growth of new blood vessels from preexisting vessels. It is a fundamental step in the transition of tumors from a dormant state to a malignant state followed by its potential metastasizing to different organs (Cantarella *et al*., 2006; and the references therein). Inhibiting the angiogenesis and metastasis of human CaP cells could serve as an effective strategy against the progression of CaP.

LNCaP is an androgen-responsive human CaP cell line, which secrete prostate-specific antigen and express

prostate-specific membrane antigen and prostatic acid phosphatase due to a functional active androgen receptor. These cells are tumorigenic and metastatic in castrated host and have been originated from the lymph node metastasis of a CaP patient (Koshida *et al*., 2004). LNCaP cells are also known to be resistant to apoptosis induction by TRAIL (Deeb *et al*., 2004; Yamaguchi *et al*., 2005; Hu *et al*., 2006).

This study was designed to investigate if EGCG could sensitize LNCaP cells to TRAIL-mediated apoptosis and to establish the molecular mechanisms underlying these effects. Here, for the first time we present evidence that combination of nonapoptosis-inducing doses of EGCG and TRAIL synergistically led to the apoptosis of LNCaP cells. Synergism shown here is the combination of individual effects of EGCG and TRAIL so the total effect seen is greater than the sum of the two. We also demonstrate that both extrinsic and intrinsic pathways are involved in the EGCG-mediated apoptosis of TRAIL-resistant CaP cells and also made clear that angiogenesis and metastasis is inhibited by the combination of TRAIL and EGCG.

Results

EGCG sensitizes LNCaP cells to TRAIL-mediated cell growth inhibition and induction of apoptosis

LNCaP cells are known to be refractory to apoptosis induction by TRAIL (Deeb *et al*., 2004; Yamaguchi *et al*., 2005; Hu *et al*., 2006; Lakshmikanthan *et al*., 2006). We confirmed this in a dose-escalation experiment (Figure 1a) and observed that LNCaP cells were refractory to TRAIL-induced cell death to a dose as high as 150 ng ml^{-1} while in PC3 cells 10 ng ml^{-1} TRAIL treatment resulted in about 70% inhibition of cell growth. LNCaP cells pretreated with EGCG (20 or 40 μM) for 24 h followed by treatment with TRAIL (100 ng ml^{-1}) for additional 24 h resulted in 22 and 28% more inhibition compared to each agent alone suggesting a possible synergism between the two agents (Figure 1b). We next determined if the growth-inhibitory effects of EGCG and TRAIL correlated with enhanced induction of apoptosis. Using an Annexin-labeled fluorescent kit, we observed enhanced apoptosis of LNCaP cells treated with TRAIL and EGCG in combination compared with individual effects of each agent alone (Figure 1c).

Effect of TRAIL treatment to EGCG sensitized LNCaP cells on modulation of intrinsic apoptotic pathway

To understand the molecular mechanism underlying the effects of TRAIL treatment on EGCG sensitized cells, we studied several molecules involved in the initiation and execution of apoptosis. We observed cleavage of

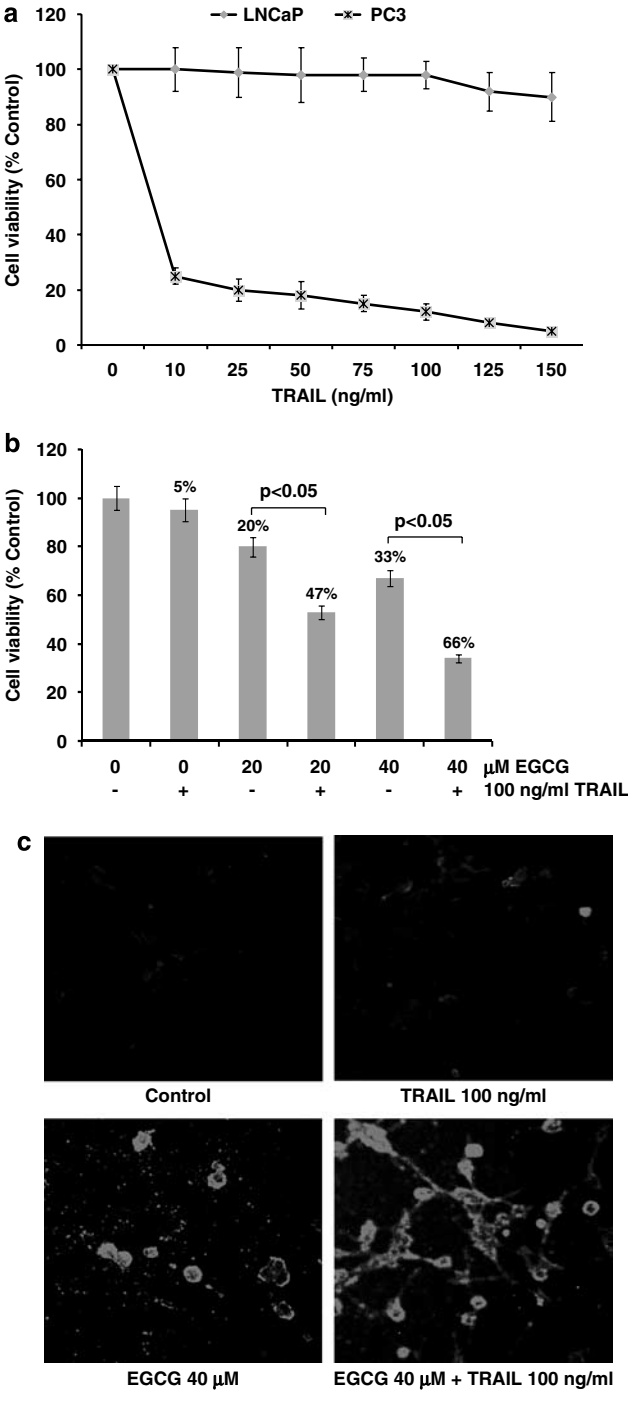


Figure 1 Effect of EGCG and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on cell growth and apoptosis of human prostate cancer cells. Cell growth analysis: (**a**) LNCaP and PC3 cells were treated with escalating doses of TRAIL and cell growth was determined by MTT assay in a 96-well plate as detailed in 'Materials and methods'. (**b**) LNCaP cells were treated as given under 'Materials and methods', and cell growth was determined by MTT assay. Columns represent mean of three separate experiments in which each treatment was repeated in 10 wells; bars + s.e. Values above each column reflect percentage change compared with control (no treatment). At each concentration of EGCG (20 or 40 μM), the effects seen with TRAIL (100 ng ml^{-1}) were statistically significant ($P<0.05$). (**c**) Apoptosis detection: LNCaP cells were grown on cell culture slides and treated as described in 'Materials and methods' and apoptosis was determined by a commercially available kit. Representative photomicrographs from each treatment group showing induction of apoptosis (green fluorescence). Data shown are from one experiment repeated three times with similar results.

116 kD death substrate poly(ADP-ribose) polymerase (PARP) into a stable 85 kD fragment. This effect was more pronounced in cells that were treated with combination as compared to the individual treatments alone (Figure 2a). Levels of proapoptotic Bak and Bax were found to be increased by EGCG and TRAIL and this increase was augmented when the two agents were combined together (Figure 2b). Concomitant to this, the

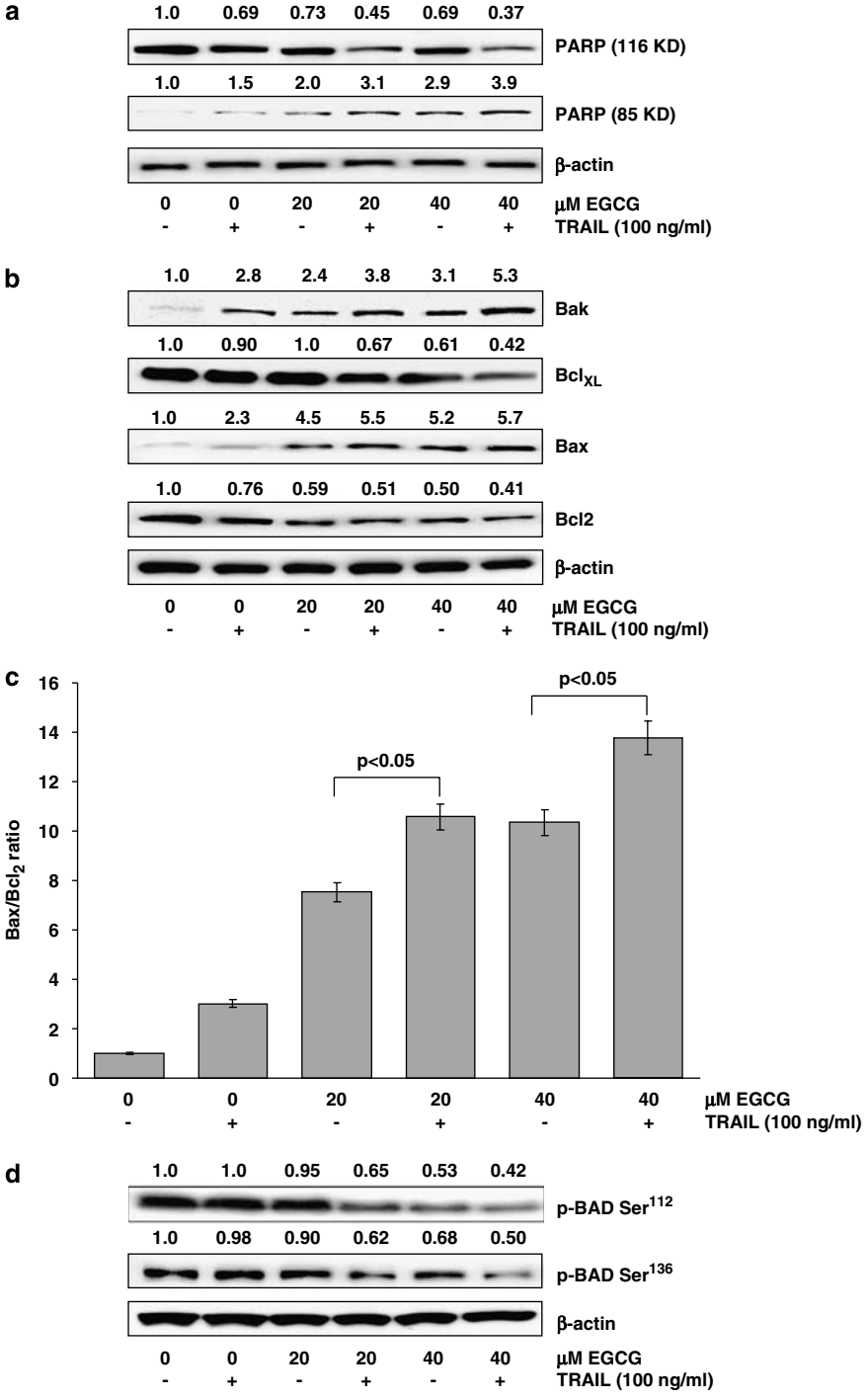


Figure 2 Effect of epigallocatechin-3-gallate (EGCG) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on the protein expression of 85 and 116 kD poly(ADP-ribose) polymerase (PARP) (**a**), Bak, Bcl_{XL}, Bax and Bcl2 (**b**), on the Bax to Bcl2 ratio (**c**) and Bad (**d**) in LNCaP cells. The cells were treated and harvested as described under 'Materials and methods'. A total of 40 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel followed by western blot analysis using specific antibodies and secondary horseradish peroxidase conjugates. Equal loading was confirmed by stripping the membrane and reprobing it with β-actin. Values on top of bands represent relative densities normalized to β-actin. Data from a typical experiment repeated three times with similar results. At each concentration of EGCG (20 or 40 μM), the effects seen with TRAIL (100 ng ml^{-1}) were statistically significant ($P<0.05$).

protein expressions of prosurvival Bcl_2 and Bcl_{XL} were observed to be synergistically inhibited by EGCG and TRAIL in combination. Evaluation of relative Bax and Bcl_2 levels suggested a shift in the ratio that favored apoptosis (Figure 2c). We further observed a synergistic inhibition in the phosphorylation of proapoptotic Bad protein at Ser^{112} and Ser^{136} (Figure 2d).

The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic proteins that block cell death, in part, by inhibiting the downstream caspase activation pathways and thus play a critical role in determining cell fate (Liston *et al*., 2003; Schimmer, 2004). As shown in Figure 3a, we observed a synergistic inhibition of IAP proteins XIAP, cIAP1, Survivin and Smac/Diablo. This inhibition was more pronounced in the groups treated with combination as compared to each agent alone. A synergistic activation of effector caspases-3 and -6 and initiator caspases-8 and -9 was observed in the combination treated cells as compared to cells treated with each agent alone (Figure 3b).

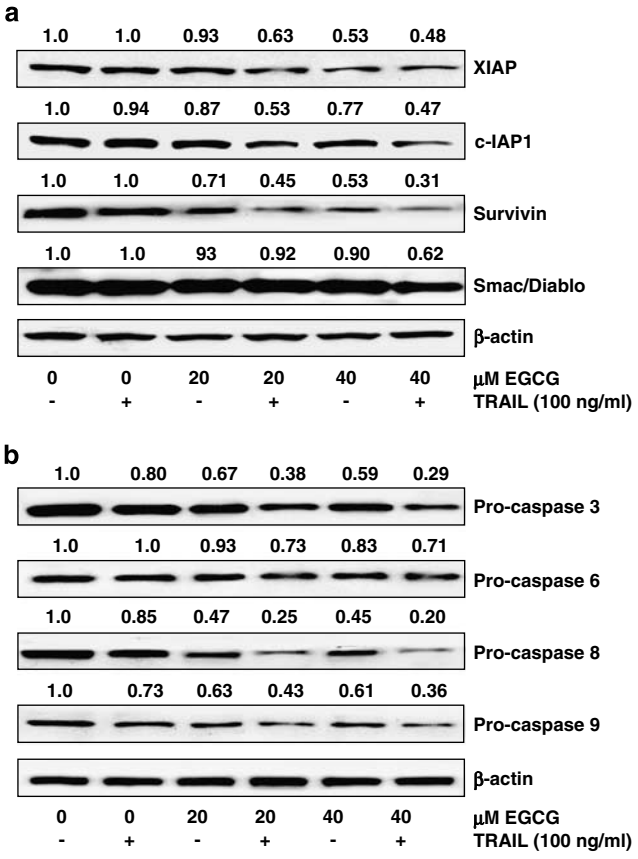


Figure 3 Effect of epigallocatechin-3-gallate (EGCG) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on the protein expression of XIAP, c-IAP1, Survivin and Smac/Diablo (**a**), and Procaspase 3, 6, 8 and 9 (**b**) in LNCaP cells. The cells were treated and harvested as described under 'Materials and methods'. Western blot analysis was performed as given under Figure 2. Values on top of bands represent relative densities normalized to β-actin. Data from a typical experiment repeated three times with similar results.

Effect of TRAIL treatment to EGCG sensitized LNCaP cells on modulation of extrinsic apoptotic pathway

Cell surface death receptors trigger apoptosis upon ligand binding. As shown in Figure 4, treatment with EGCG and TRAIL synergistically upregulated the protein expression of DR4, a death receptor that transduces TRAIL-mediated signals to the intracellular apoptotic machinery. DR4 upon activation interacts with Fas-associated death domain (FADD), an intracellular adapter molecule and with FLICE-inhibitory protein (FLIP), a key regulator of death receptor signaling and an endogenous inhibitor that interacts with FADD to antagonize apoptosis. We observed a significant inhibition in protein expression of FADD and FLIP and this effect was more profound in the cells treated with combination of EGCG and TRAIL (Figure 4).

EGCG pretreatment of LNCaP cells increases TRAIL-mediated inhibition of markers of angiogenesis and metastasis

An array of regulatory molecules including TNF-α and TRAIL contribute substantially to angiogenesis (9). TRAIL is known to act as a mitogen for primary endothelial cells at a degree comparable to vascular endothelial growth factor (VEGF), a major regulator of both physiological and pathological angiogenesis. We, therefore, determined the effect of EGCG and TRAIL, alone and in combination on VEGF expression. We observed a significant inhibition in the protein expression of VEGF in the group treated with EGCG and TRAIL (Figure 5). We further analysed the effect of EGCG and TRAIL on urokinase plasminogen activator (uPA), which is involved in the metastatic phenotype of many types of cancers (de Bock and Wang, 2004). As shown in Figure 5, EGCG and TRAIL synergistically inhibited the protein expression of uPA. The angiopoietins are protein growth factors that promote angiogenesis (Thurston, 2003) and angiopoietin 1 and 2 are known to be required for the formation of mature blood vessels. We observed a significant and synergistic

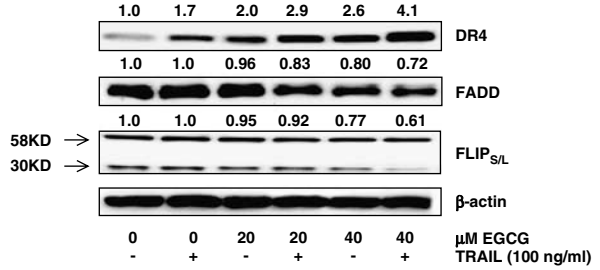


Figure 4 Effect of epigallocatechin-3-gallate (EGCG) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on the protein expression of death-inducing signaling cascade involving DR4, Fas-associated death domain (FADD) and FLIP in LNCaP cells. The cells were treated and harvested as described under 'Materials and methods'. Western blot analysis was performed as given under Figure 2. Values on top of bands represent relative densities normalized to β-actin. Data from a typical experiment repeated three times with similar results.

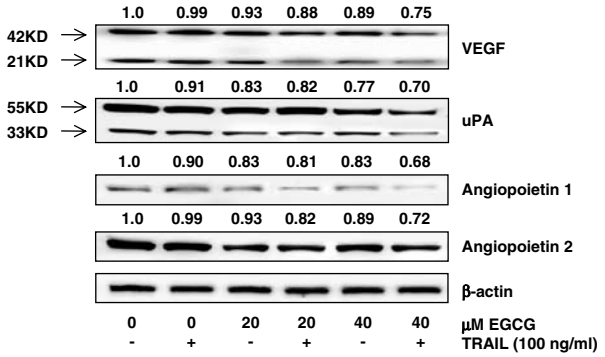


Figure 5 Effect of epigallocatechin-3-gallate (EGCG) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on the protein expression of vascular endothelial growth factor (VEGF), uPA and Angiopoietin 1 and 2 in LNCaP cells. The cells were treated and harvested as described under 'Materials and methods'. Western blot analysis was performed as given under Figure 2. Values on top of bands represent relative densities of the bands normalized to β-actin. Data from a typical experiment repeated three times with similar results.

downregulation in the protein expression of these proteins in the cells treated with combination of EGCG and TRAIL (Figure 5).

The matrix metalloproteinases (MMPs) play a key role in matrix degradation thus allowing tumor propagation (Adhami *et al*., 2004; and the references therein). Thus, we investigated the protein expression and activity of MMPs and observed a synergistic inhibition in the protein expression of MMP-2, -3 and -9 in the cells treated with EGCG and TRAIL (Figures 6a and b). Similar results were obtained by gelatin zymography for activity analysis of MMPs. Significant inhibition in the activity of MMP-2 and -9 was observed (Figure 6d). The expression of MMPs and TIMPs has been proposed to be coregulated and their imbalance has been shown to be an essential feature of invasive cancers (Adhami *et al*., 2004). We observed a significant upregulation in the protein expression of TIMP1, the tissue inhibitor of MMP-9. Further, as can be seen by data in Figure 6c, analysis of MMP-9 to TIMP1 ratio indicated that the balance was shifted in such a way that favored TIMP1 expression suggesting inhibition of MMP-9 expression.

EGCG co-treatment increases TRAIL-mediated inhibition of invasion and migration

In the next set of experiments, we determined the effect of EGCG and TRAIL in combination on the invasion and migration potential of LNCaP cells. As shown in Figure 7a, TRAIL (100 ng ml^{-1}) and EGCG (40 μM) in combination synergistically inhibited cell invasion. The quantification of the invaded cells suggested that TRAIL and EGCG alone were able to impede cell invasion by about 9 and 48%, respectively. However, these agents in combination significantly inhibited invasion by 72% as compared to untreated control (Figure 7b). This inhibition in cell invasion might be in part due to the cytotoxicity induced by the agents as shown in Figure 1b (33 and 66% inhibition in cell viability with EGCG 40 μM alone and combination, respectively); however, invasion assay showed a inhibition of 48 and 72% at the same doses which is 15 and 8% more than inhibition in cell viability demonstrating that the effect seen is only partially due to the cytotoxicity of the agents. In addition the scratch wound assay was performed to evaluate the migratory potential of the LNCaP cells when treated with TRAIL (100 ng ml^{-1}) and EGCG (40 μM) alone and in combination. We observed synergistic inhibition of cell migration in groups treated with EGCG and TRAIL as compared to untreated controls and each agent alone (Figure 7c).

Discussion

In this study we report for the first time, sensitization of LNCaP cells to TRAIL-mediated apoptosis by green tea polyphenol EGCG. Androgen-responsive LNCaP cells are more resistance to TRAIL-mediated apoptosis compared to their androgen nonresponsive counterparts suggesting a restrictive role of the androgen receptor in TRAIL-mediated induction of apoptosis. A critical step in apoptosis that follows the activation of death receptor by TRAIL is the recruitment of FADD downstream that results in the activation of caspases leading to cell death. Androgens have been shown to induce expression of the c-FLIP gene, which is a potent inhibitor of Fas/FasL-mediated apoptosis (Gao *et al*., 2005). This largely explains the fact that LNCaP cells are resistant to TRAIL-mediated apoptosis compared to their AR negative counterpart PC3 (Figure 1a). Due to the development of TRAIL resistance, in recent years there has been considerable interest in combinatorial therapies that results in pronunciation of TRAIL effect on various cancer cell types including CaP. Munshi *et al*. (2002) demonstrated that treatment of CaP cells with doxorubicin, a well-known chemotherapeutic agent, enhances TRAIL-mediated apoptosis (Munshi *et al*., 2002). However, chemotherapeutic drugs such as doxorubicin are often associated with general cytotoxic effects, which are highly undesirable and warrant the exploration of other nontoxic agents to achieve TRAIL sensitivity.

In addition to the involvement of death inhibitors, such as c-FLIP, several studies indicate constitutively active AKT as an important regulator of differential sensitivity to TRAIL (Chen *et al*., 2001; Falschlehner *et al*., 2007). LNCaP cells express highest level of constitutive active AKT, which has been thought to be directly related to their TRAIL resistance. EGCG, a natural phytochemical has been shown to have multimodal mechanisms of action. We and others have shown that at cellular level it inhibits AKT expression and activation while interfering with the bcl2 expression presumably by decreasing its stability (Siddiqui *et al*., 2004; Bigelow and Cardelli, 2006; Qin *et al*., 2007). These observations strongly advocate employment of

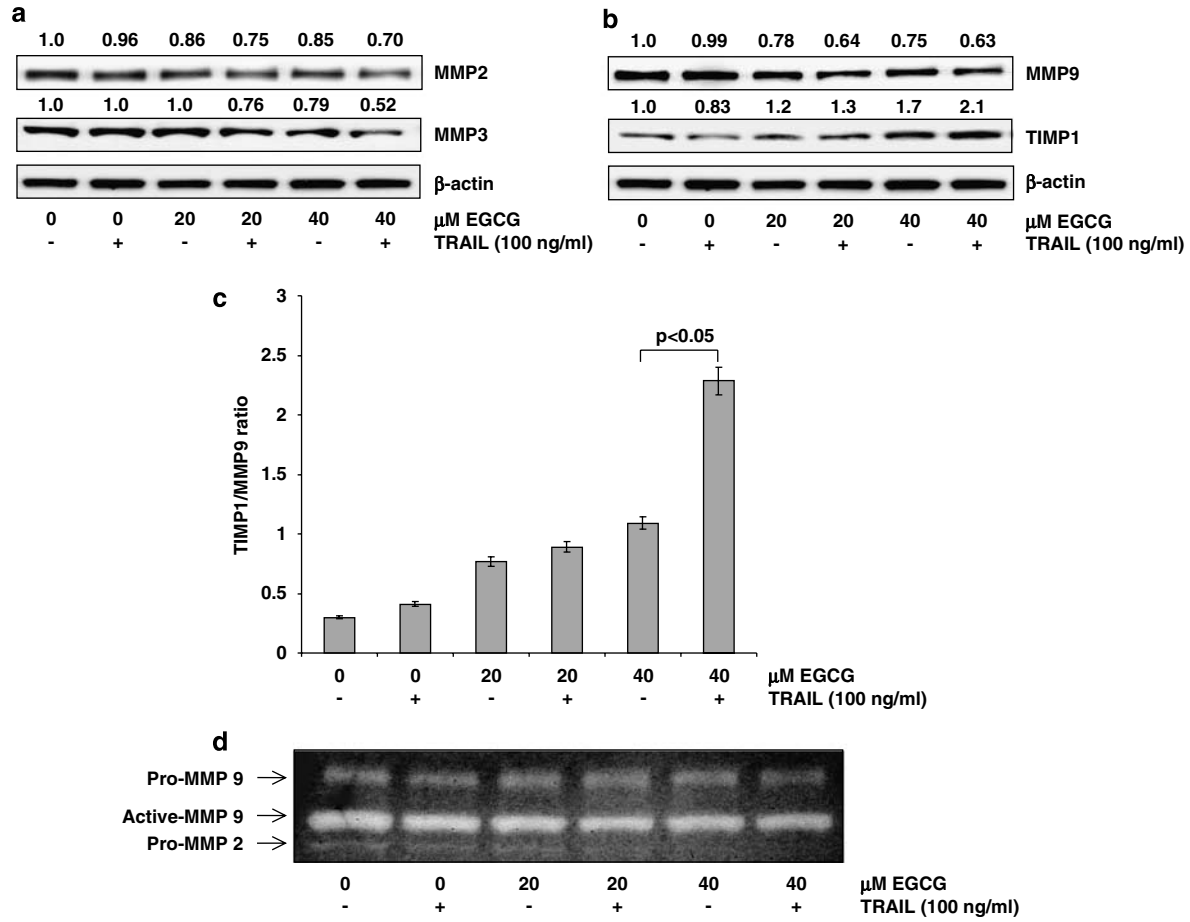
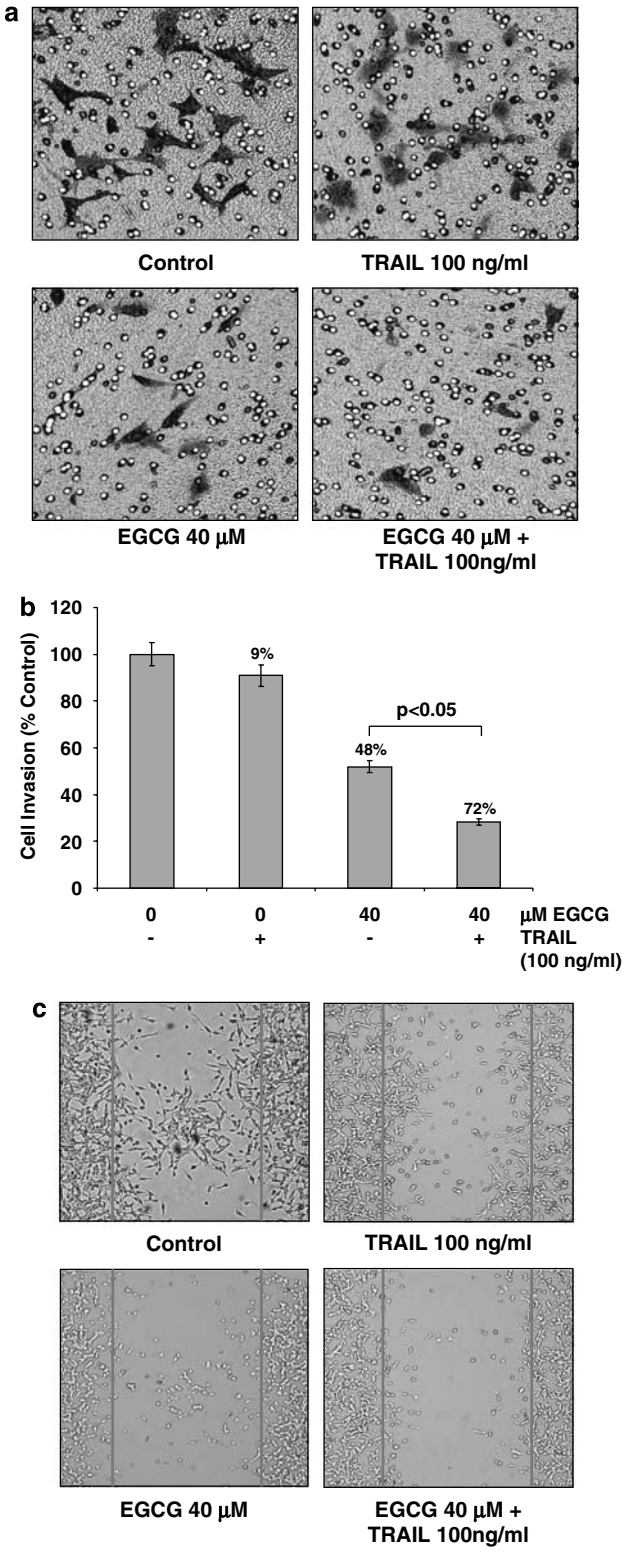


Figure 6 Effect of epigallocatechin-3-gallate (EGCG) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) treatment, alone and in combination, on the protein expression of matrix metalloproteinases (MMP)-2 and MMP-3 (**a**), MMP-9 and TIMP1 (**b**), on the TIMP1 to MMP-9 ratio (**c**) and MMP activity (**d**) in LNCaP cells. Protein expression: (**a** and **b**) The cells were treated and harvested as described under 'Materials and methods'. Western blot analysis was performed as given under Figure 2. Values on top of bands represent relative densities of the bands normalized to β-actin. Data from a typical experiment repeated three times with similar results. (**c**) Effect of EGCG and TRAIL on TIMP1 to MMP-9 ratio. The ratios were determined from the data obtained by immunoblot analyses of MMP and TIMP in Figure 6b. Data are mean ± s.e. of three different experiments. At each concentration of EGCG (20 or 40 μM), the effects seen with TRAIL (100 ng ml^{-1}) were statistically significant ($P<0.05$). Activity analysis: (**d**) Media collected from treatment groups were run on gelatin gels and developed as described under 'Materials and methods'. Data are from a typical experiment repeated three times with similar results.

EGCG to overcome TRAIL resistance in various cancers including CaP especially those with constitutively active AR-AKT-bcl2 expression. We found that pretreatment of LNCaP cells with EGCG at 20 and 40 μM dramatically converts a previously sublethal dose of TRAIL (100 ng ml^{-1}) into a potentially effective apoptosis inducer (Figures 1a and b). There exists a synergism in the apoptotic process presumably by downregulation of prosurvival signaling molecule AKT in addition to modulation of other important anti/proapoptotic molecules. It has been shown that proteasome inhibitors cooperate with TRAIL in inducing apoptosis in cancers with over expression of bcl2 (Nencioni *et al*., 2005). Our results demonstrate that EGCG that has also been shown to act as a proteasome inhibitor (Lam *et al*., 2004; Landis-Piwowar *et al*., 2005) might actually synergize with TRAIL in inducing apoptosis in LNCaP cells. Interestingly our results suggest that pretreatment of cells with either EGCG or TRAIL does not result in modulation of expression of antiapoptotic molecules such as Bcl_{XL}, and Bcl_2, however, a combinatorial treatment led to twofold decrease in their expression (Figures 2b and 4) suggesting that both compounds act synergistically in repressing their expression. Also, a decrease in expression of pro- and anti-apoptotic molecules such as Bcl_{xL}, XIAP, c-IAP1 and bcl2 might result from stabilization of corresponding inhibitory proteins by EGCG proteasome inhibitory activity. Although TRAIL by itself failed to induce significant apoptosis in the cells, it significantly modulated the levels of PARP, Bak, Bax and Bcl2 (Figure 2), procaspases 3, 8 and 9 (Figure 3b), and DR4 (Figure 4), but not XIAP, c-IAP1, survivin, Smac/Diablo (Figure 3), FADD and FLIP (Figure 4). This observation suggests that TRAIL does induce changes in apoptosis-related elements, however, the resistance to TRAIL-induced apoptosis in LNCaP cells may be very well associated with the prosurvival

elements that TRAIL fails to modulate, especially XIAP, c-IAP1 and survivin. When these prosurvival factors are inhibited by pretreatment with EGCG, the threshold for TRAIL resistance is overcome, which may explain why pretreatment of EGCG synergistically increases TRAIL-induced apoptosis.



As a strategy of survival many cancers epigenetically repress the expression of DR hence bypassing apoptosis (He *et al*., 2002). A twofold increase in expression of DR4 which was observed in EGCG treated cells may be attributed to DNA demethylation activity associated with EGCG (Stresemann *et al*., 2006) allowing the regain of enhanced DR expression in LNCaP thereby making them prone to TRAIL-mediated apoptosis**.** Further assessment of the combinatorial treatment on migration capacity of LNCaP was also performed. Interestingly, EGCG and TRAIL co-treatment to LNCaP cells resulted in the decreased capacity of LNCaP cells to migrate through the extracellular matrix with concomitant decrease in the protein markers of cell migration and invasion such as MMPs, uPA VEGF and so on (Figures 5, 6, and 7), indicating that the co-treatment not only results in restoration of TRAIL sensitivity but also leads to a dramatic decrease in invasion and migratory capacity of LNCaP cells, a critical strategy to contain the spread of CaP metastasis that might result in enhanced efficacy of presently employed therapeutic regimen.

Our results suggest that EGCG sensitizes LNCaP cells to TRAIL-mediated apoptosis via modulation of extrinsic and intrinsic apoptotic pathways and inhibition of markers of angiogenesis and metastasis. These results have implications for developing new strategies aimed at elimination of TRAIL resistance in CaP cells.

Materials and methods

Materials

Antibodies to procaspases-3, -6, -8 and -9, MMP-3 and -9, FADD, VEGF, uPA and DR4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PARP (85 kDa), XIAP, FLIP, p-Bad, c-IAP1, Smac/Diablo and Bcl2 antibodies were procured from Cell Signaling Technology (Beverly, MA, USA). PARP (116 kD), Bax, Bcl_{XL} and Bak antibodies were purchased from Upstate (Lake Placid, NY, USA). Antibody to Survivin was obtained from Novus Biologicals (Littleton, CO, USA), MMP-2 antibody was obtained from Neo Marker (Fremont, CA, USA) and Angiopoietin 1 and 2 antibodies were from Abcam (Cambridge, MA, USA). Antimouse or antirabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Pharmacia Life Sciences (Arlington Heights, IL, USA). The Bicinchoninic acid (BCA) Protein Assay Kit was purchased from Pierce (Rockford, IL, USA). Novex precast Tris-Glycine gels were obtained from Invitrogen (Carlsbad,

Figure 7 Epigallocatechin-3-gallate (EGCG) co-treatment increases tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated inhibition of invasion and migration. (**a**) The invasion assay was performed by commercially available kit as described under 'Materials and methods'. Photomicrographs of the invaded cells were taken after specified time. (**b**) Quantification of the invaded cells was done by dissolving the invaded cells in 10% acetic acid and reading the OD at 540 nm. With EGCG 40 μM, the effects seen with TRAIL (100 ng ml^{-1}) were statistically significant ($P<0.05$). (**c**) Wound healing assay was performed as described in 'Materials and methods'. Photomicrographs were taken after the specified time after washing the plates with cold PBS. Data are from a typical experiment repeated three times with similar results.

CA, USA). EGCG was a kind gift from Dr Yukihiko Hara (Mitsui Norin, Shizuoka, Japan) and TRAIL was from Sigma (St. Louis, MO, USA).

Treatment of cells
Human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI 1640 (ATCC, Rockville, MD, USA) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and 5% CO_2. Cells were pretreated with EGCG (20 or 40 μM) for 24 h; EGCG was dissolved in PBS (pH 7.4) as 5 mg ml^{-1} stock and immediately added to the complete media for the treatment followed by the treatment with TRAIL (100 ng ml^{-1}) for another 24 h. Cells that were used as controls were incubated with the maximum used amount of PBS only.

Cell growth and viability
The effect of EGCG (20 and 40 μM) and TRAIL (100 ng ml^{-1}) on the viability of cells was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in Adhami *et al*. (2007). The effect of each compound alone and in combination on growth inhibition was assessed as percentage of cell viability in which vehicle-treated cells were taken as 100% viable.

Apoptosis detection by fluorescence microscopy
The annexin V-FLUOS staining kit (Roche Applied Biosciences, Indianapolis, IN, USA) was used for the detection of apoptotic cells. Assay was performed as described previously (Adhami *et al*., 2007).

Protein extraction and western blotting
Protein extraction and western blotting was performed as described previously (Siddiqui *et al*., 2004).

Gelatin zymography
Gelatin zymography was performed as described earlier by Saleem *et al*. (2006). Areas of protease activity appeared as clear bands against a dark blue background.

Invasion assay
The cell Invasion assay kit (Chemicon International, Temecula, CA, USA) was used for detection of cell invasion as per vendor's protocol. Cells were resuspended in culture medium, and incubated in a chemoinvasion chamber. Treatment with TRAIL (100 ng ml^{-1}) and EGCG (40 μM) was done in the inner chamber either alone or in combination as a co-treatment and the cells were incubated for 20 h. The invasive capability of cells was measured as per vendor's protocol. Photomicrographs of the invaded cells were taken in five predetermined fields at a magnification of $\times 200$ and quantification of the stained cells was done by dissolving the cells in 10% acetic acid and reading the OD at 540 nm.

Wound healing assay for cell migration
The cells were allowed to grow to ~70% confluency in tissue culture plates and three parallel wounds were made using a pipette tip with a diameter of ~1.5 mm. Treatment was given as described under invasion assay and wound healing was observed under microscope. After 48 hours cells were washed with ice cold 1 × PBS and imaged under microscope.

Statistical analysis
All assays were repeated in three independent experiments, and only representative blots are presented. Immunoblots were scanned by HP Precisionscan Pro 3.13 (Hewlett-Packard, Palo Alto, CA, USA). Densitometry measurements of the scanned bands were done using digitalized scientific software program UN-SCAN-IT. Data were normalized to β-actin and expressed as mean ± s.e. followed by appropriate statistical analysis. Intergroup comparison were performed by one-way analysis of variance (ANOVA) followed by appropriate *post hoc* analysis and P-values <0.05 were considered to be statistically significant.

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