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# Delphinidin, a component of pigmented fruits and vegetables may act as an inhibitor of prostate cell carcinoma

KEYWORDS: Prostate cancer, Delphinidin, TRAIL, Apoptosis, chemoprevention


| Arshi Malik | Adel Al Hazzani | Basiouny El-Gamal |
|---|---|---|
| Department of Clinical Biochemistry, King Khalid University, Kingdom of Saudi Arabia | Department of Internal Medicine, King Khalid University, Kingdom of Saudi Arabia | Department of Clinical Biochemistry, King Khalid University, Kingdom of Saudi Arabia |

| Mohamed Abd Ellatif | Sarah Afaq | Waleed N Hassan |
|---|---|---|
| Department of Clinical Biochemistry, King Khalid University, Kingdom of Saudi Arabia | Department of Clinical Biochemistry, King Khalid University, Kingdom of Saudi Arabia | Department of Clinical Biochemistry, King Khalid University, Kingdom of Saudi Arabia |


**ABSTRACT** *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 that Delphinidin the major constituent of pigmented fruits and vegetables, sensitizes TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis through modulation of apoptotic pathway. When combined with Delphinidin, Apo2L/TRAIL exhibited enhanced apoptotic activity in LNCaP cells characterized by major molecular events.*

*These data might have implications for developing new strategies aimed at eliminating prostate cancer cells resistant to TRAIL.*

## INTRODUCTION

Prostate cancer (CaP) has become the most frequently diagnosed, noncutaneous neoplasm and second leading cause of cancer-related deaths among men . It is estimated that in the year 2015, 1,658,370 new cases will be diagnosed and 589,430 men will die from CaP (1). 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 various laboratories have suggested that pigmented fruits and vegetables, especially its constituent Delphinidin possess chemopreventive and therapeutic potential against CaP (2).

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 (3,4,5). Studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (6,7,8), 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.

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 (9). LNCaP cells are also known to be resistant to apoptosis induction by TRAIL (6,7,8).

This study was designed to investigate if Delphinidin could sensitize LNCaP cells to TRAIL-mediated apoptosis. Here, we present the evidence that combination of nonapoptosis-inducing doses of delphinidin and TRAIL synergistically led to the apoptosis of LNCaP cells. Synergism shown here is the combination of individual effects of Delphinidin and TRAIL so the total effect seen is greater than the sum of the two.

## MATERIALS AND METHODS

**Reagents and cell lines:** Poly (ADP-ribose) polymerase (PARP) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate was obtained from Amersham Pharmacia Life Sciences. The Bio-Rad DC Protein Assay Kit was purchased from Bio-Rad. Novex precast Tris-Glycine gels were obtained from Invitrogen. The Annexin-V-FLUOS Staining Kit was purchased from Roche. Delphinidin (>99% pure) was obtained from Extrasynthese.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Sigma.

PC3, LNCaP cells were obtained from American Type Culture Collection and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotics.

### 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 Delphinidin (20,40 or 80 mM) for 24 h; 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 Delphinidin (20,40 and 80 mM) and TRAIL (100 ng $ml^{-1}$) on the viability of cells was determined by

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3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in (10). 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 (10).

**Protein Extraction and Western Blotting**
After treatment, the cells were incubated in ice-cold lysis buffer (50 mmol/liter Tris-HCl/150 mmol/liter NaCl/1 mmol/liter EGTA/1 mmol/liter EDTA/20 mmol/liter NaF/100 mmol/liter $Na_3VO_4$/0.5% Nonidet P-40/1% Triton X-100/1 mmol/liter PMSF, pH 7.4) with freshly added protease inhibitors (Calbiochem) over ice for 20 min. For Western blotting, 40 µg of protein was resolved over 12% polyacrylamide gels, transferred onto a nitrocellulose membrane, probed with appropriate monoclonal primary antibody, incubated with appropriate secondary antibody horseradish peroxidase conjugate, and detected by chemiluminescence and autoradiography.

**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.

## RESULTS

**Delphinidin 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. We confirmed this in a dose-escalation experiment (Figure 1) and observed that LNCaP cells were refractory to TRAIL-induced cell death to a dose as high as 125 ng ml$^{-1}$ while in PC3 cells 10 ng ml$^{-1}$ TRAIL treatment resulted in about 80% inhibition of cell growth.

**Figure 1**. LNCaP cells are resistant to growth inhibition by TRAIL. 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'.

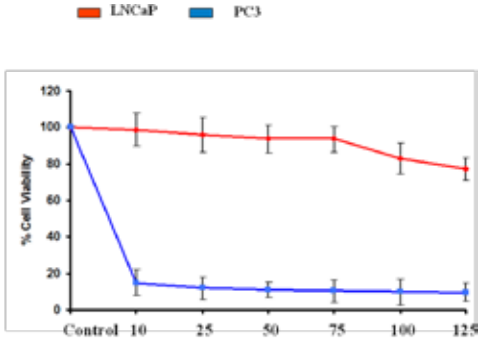


Figure 1

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LNCaP cells pretreated with Delphinidin (20,40 or 80 mM) for 24 h followed by treatment with TRAIL (100 ng ml$^{-1}$) for additional 24 h resulted in 22,28 and 41% more inhibition compared to each agent alone suggesting a possible synergism between the two agents (Figure 2).

**Figure 2**. Delphinidin sensitizes LNCaP cells to TRAIL mediated cell death as assessed by the MTT assay. 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. At each concentration of Delphinidin (20,40 or 80 mM), the effects seen with TRAIL (100 ng ml$^{-1}$) were statistically significant ($P$<0.05).

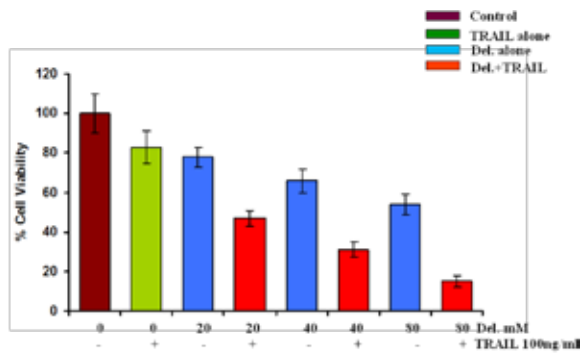


Figure 2

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We next determined if the growth-inhibitory effects of Delphinidin 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 DELPHINIDIN in combination compared with individual effects of each agent alone (Figure 3). We used this method because it identifies the apoptotic (green fluorescence) as well as necrotic (red fluorescence) cells.

**Figure 3.** Delphinidin + TRAIL treatment to LNCaP cells resulted in the induction of apoptosis as evident from the morphology of cells, assessed by fluorescence microscopy. 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.

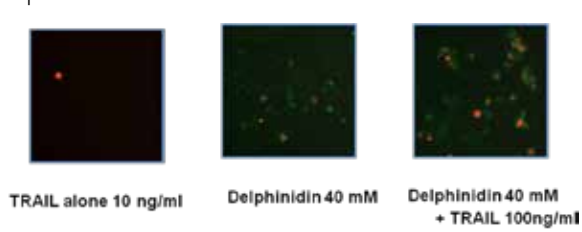


Figure 3

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To understand the molecular mechanism underlying the effects of TRAIL treatment on Delphinidin sensitized cells, we studied the most important molecule involved in the initiation and execution of apoptosis. We observed cleavage

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of 116kD death substrate poly(ADP-ribose) polymerase (PARP) into a stable 85kD fragment. This effect was more pronounced in cells that were treated with combination as compared to the individual treatments alone (Figure 4).

**Figure 4.** Delphinidin pretreatment of LNCaP cells increases TRAIL-mediated apoptosis as evidenced by the cleavage of PARP. Effect of Delphinidin 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). The cells were treated and harvested as described under 'Materials and methods'. A total of 40 ■g 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. Data from a typical experiment repeated three times with similar results. At each concentration of Delphinidin (20,40 or 80 mM), the effects seen with TRAIL (100 ng $ml^{-1}$) were statistically significant ($P<0.05$).

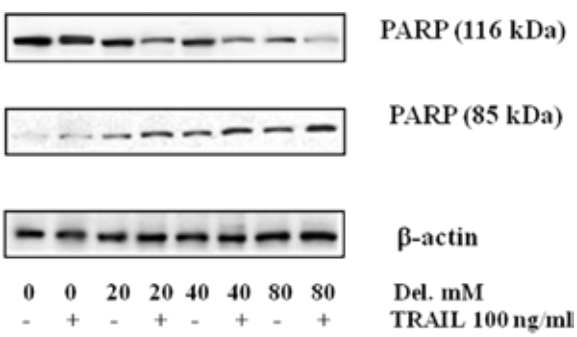


**Figure 4**

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## DISCUSSION

In this study we report the sensitization of LNCaP cells to TRAIL-mediated apoptosis by Delphinidin. 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 (11). This largely explains the fact that LNCaP cells are resistant to TRAIL-mediated apoptosis compared to their AR negative counterpart PC3 (Figure 1). 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.demonstrated that treatment of CaP cells with doxorubicin, a well-known chemotherapeutic agent, enhances TRAIL-mediated apoptosis (12). 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 (13, 14). LNCaP cells express highest level of constitutive active AKT, which has been thought to be directly related to their TRAIL resistance. Many natural phytochemicals have been shown to have multimodal mechanisms of action. It has been shown that at cellular levels, they inhibits AKT expression and activation while interfering with the bcl2 expression presumably by decreasing its stability (15,16). These observations strongly advocate employment of Delphinidin 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 Delphinidin at 20,40 and 80mM dramatically converts a previously sublethal dose of TRAIL (100ng$ml^{-1}$) into a potentially effective apoptosis inducer (Figure 2). 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.

Our results suggest that Delphinidin sensitizes LNCaP cells to TRAIL-mediated apoptosis via modulation of apoptotic pathway. These results have implications for developing new strategies aimed at elimination of TRAIL resistance in CaP cells.

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