Tetrandrine (TET) Induces Death Receptors: Apo Trail R1 (DR4) and Apo Trail R2 (DR5) and Sensitizes Prostate Cancer Cells to TRAIL Induced Apoptosis

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Abbreviations: PCa- Prostate Cancer, Tetrandrine- TET derivative, TRAIL- Tumor necrosis factor-Related Apoptosis-Inducing-Ligand, DR- Death Receptors, shRNA- small hairpin Ribonucleic Acid, Scrambled- Scrl, Double knockdown- DK

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There is no conflict of interest.

A patent application describing the use of TET for sensitizing cancer cells to TRAIL is in process.
Abstract

TNF-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells, but not in normal cells, as such is a promising therapeutic agent. However, therapeutic resistance limits its clinical use in many malignancies including prostate cancer (PCa). Strategies to sensitize cancer cells to TRAIL are urgently needed. We demonstrate here that small-molecule Tetrandrine (TET) potentially sensitizes previously resistant (LNCaP and C4-2B cells) and mildly sensitive (PC3 cells) PCa cells to TRAIL-induced apoptosis, and they do so by up-regulating mRNA expression and protein levels of death receptors Apo Trail R1 (DR4) and Apo Trail R2 (DR5). Using shRNA knockdown, we show critical requirement of DR4 and DR5 in sensitization of PCa cells to TRAIL. We show that double knock down of DR4 and DR5 abrogated the apoptotic effects of TET and TRAIL. We also demonstrate that TET induced DR4 and DR5 expression is independent of p53 status. Given that loss of p53 is associated with progression of PCa to CRPC and NEPC, our results show that TET by acting as a TRAIL sensitizing agent in PCa could serve as a potential therapeutic agent in CRPC and NEPC for which there is no cure to date.
Introduction

Prostate cancer (PCa) is the most common cancer in males and is responsible for about 26,000 deaths each year in the USA [1]. Current treatment approaches for PCa include surgery, chemotherapy, radiation therapy, hormonal therapy, cryosurgery and high-intensity focused ultrasound produce a high rate of cure for localized organ confined disease[2]. Androgen deprivation therapies yield a high 5-year survival rate, however, most patients relapse with metastatic castration resistant prostate cancer (mCRPC) and to date there is no effective therapeutic strategy available for mCRPC [3]. There is an urgent and yet unmet need for developing effective therapeutic strategy for PCa.

TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the TNF superfamily of cytokines [4]. The soluble extracellular domain of TRAIL triggers apoptosis by binding to its cognate agonistic death receptors (DR)- TRAIL receptor 1 (TRAIL-R1)/DR4 (Apo2, TNFRSF10A) [5] and TRAIL-R2/DR5 (KILLER, TNFRSF10B) [6]. Unlike TNF, TRAIL is preferentially cytotoxic to cancer cells over normal cells [7], as such TRAIL has been used to selectively kill cancer cells [8, 9]. Interestingly, TRAIL signaling pathway is blocked in normal cells due to a high expression of decoy receptors and FLICE-like inhibitory protein, which inhibits caspase 8, caspase 10, and apoptosis [10]. Though TRAIL is a promising anticancer agent, cancer cells develop resistance to TRAIL, which has limited its therapeutic efficacy [11]. Overcoming TRAIL resistance has recently received considerable attention to treat PCa.

The inability of prostatic epithelial cells to undergo apoptosis leads to the transformation and progression towards malignancy in PCa cells [12, 13]. Resistance to apoptosis is one of the mechanisms used by cancer cells to overcome toxicity of therapeutic agents. Cancer cells have evolved numerous strategies to resist cell death, such as the expression of anti-apoptotic proteins or the downregulation or mutation of pro-apoptotic cell death components [14]. As such novel approaches to overcome therapeutic resistance by way of sensitizing cancer cells to apoptosis inducing agents is one of the important strategies for increasing the effectiveness of chemotherapeutic agents [15]. Apoptosis may be triggered by two pathways: extrinsic (involves the binding of death ligands to death receptors) and intrinsic (initiates the mitochondrial pathway) to induce apoptosis [16]. TRAIL induces apoptosis mainly through death receptor-
mediated pathways. TRAIL binding to the death receptors- DR4 and DR5 leads to the assembly of a death-inducing signaling complex [17] and recruitment of adaptor protein FAS-associated death domain, which results in caspase activation [18] and activation of apoptotic pathway selectively in cancer cells. Specific targeting/stimulation of the extrinsic pathway to trigger apoptosis in tumor cells seems to be a better option for cancer therapy since death receptors have a direct link to the cell’s death machinery [17].

The molecular basis for the TRAIL resistance in PCa is not completely understood [11]. Commonly used PCa cells, like many other cancer cells, are also resistant to TRAIL-induced apoptosis [19, 20]. Studies with several agents that have been investigated for their effectiveness to sensitize cancer cells to TRAIL (including natural products such as curcumin [21], sulforaphane [22], and cardamonin [23]) suggest that resistance to TRAIL could be reversed at least under experimental conditions. Also, the antitumor activity of natural compounds has been extensively studied in several cancers [24]. Tetrandine (TET), a bis-benzylisoquinoline alkaloid and a natural compound, isolated from the roots of *Stephania tetrandra*, has been reported to treat hypertension and inflammation for hundreds of years. TET has been shown to have multiple pharmacological activities including immunosuppression, anti-hypertensive and anti-tumor activity [25]. There is an increasing body of literature describing the anti-cancer effects of TET alone or in various combinations with other agents that demonstrates the effectiveness of TET in modulating apoptosis including in PCa [26-30], however, none of the studies to-date have been conducted to evaluate the cytotoxic effects of TET in combination with TRAIL.

In the present study, we tested the effectiveness of TET in sensitization of PCa cells to TRAIL. We show for the first time that treatment of PCa cells with TET induces expression of DR4, DR5 and TNFR1, and sensitizes these cells to TRAIL-mediated cell death. To the best of our knowledge, this is the first report describing sensitization of any type of cancer cells to TRAIL by TET. An abstract describing these studies has been recently published [31].

**Materials and Methods**

**Cell lines, media components and other chemicals**

Cell lines used in this study LNCaP, PC3, RWPE-1 and HEK293T were procured from ATCC (Rockville, MD) between 2004 and 2017. C4-2B cells were generated by Dr. Chung [32].
These cells were routinely tested for mycoplasma. The latest mycoplasma test, performed on 1/31/2018 using abm- Mycoplasma PCR Detection Kit (Applied Biological Materials Inc. Richmond, BC) as per manufacturer recommendations, was negative. The cell lines were not independently authenticated. L-glutamine, penicillin- streptomycin and sodium pyruvate were purchased from Hyclone/Caisson. Media components and Fetal Bovine Serum (FBS) were procured from Caisson and Atlanta Biologicals respectively and all other chemicals were procured from Corning. Keratinocyte-Serum Free Medium (K-SFM), bovine pituitary extract (BPE) and epidermal growth factor (EGF) were obtained from GIBCO (Germantown, MD). Crystal violet was obtained from Sigma-Aldrich (St. Louis, MO), TET (3B2-0454) and TRAIL (CYT-443) were obtained from 3B Scientific Corporation (Libertyville, IL) and ProSpec-Tany Technogene Ltd. (East Brunswick, NJ) respectively. TRAIL was dissolved in distilled water and kept at -20°C until further use. All other chemicals were purchased from Sigma.

Antibodies

Antibodies are described in (Table-S1).

**shRNA and special reagents**

Specific lentiviral expression constructs for short hairpin RNA (shRNA) targeting human DR4 and DR5 (MISSION shRNA Plasmid DNA) were purchased from Sigma- Aldrich (St. Louis, MO). Constructs used were NM_003844.2-943s21c1 and NM_003844.2-421s1c1 (labeled as human DR4 shRNA1 and shRNA2 respectively), and NM_003842.3-822s1c1 and NM_003842.3-599s1c1 (labeled as human DR5 shRNA1 and shRNA2 respectively). pMD2.G envelope plasmid and psPAX2 packaging plasmid were purchased from Addgene. Scrambled shRNA [labeled as MISSION® pLKO.1-puro Non-Target shRNA Control Plasmid DNA (SHC016-1EA)] was used as a control. Plasmid DNA were purified using QIAGEN plasmid midi kit as per manufacturer’s instructions (Hilden, Germany).

**Cell culture**

LNCaP and C-4-2B cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin-Streptomycin and 1% Sodium Pyruvate. PC3 cells were maintained in DMEM-F12 medium, supplemented with 10% FBS and 1% Penicillin-Streptomycin. RWPE-1 cells were maintained in K-SFM supplemented with 1% Penicillin-Streptomycin, 0.05 mg/ml.
BPE and 5ng/ml EGF. 293T cells were maintained in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin. All cells were maintained in humidified atmosphere of 95% air, 5% CO₂ and 37°C temperature in cell incubator.

**Dot Blot Array**

Proteome Profiler™ Human Apoptosis Array Kit, catalog no. ARY009, (R&D systems, Minneapolis, MN) was used to detect apoptosis-related proteins. Sample preparation of PCa cells and dot blot array procedure was done as per manufacturer’s instructions. Briefly, untreated and 20μM TET treated (24h) LNCaP cells were washed with PBS and solubilized in lysis buffer (provided in kit). 400μg of each whole cell lysate was incubated with antibody cocktail (supplied in kit), and proceeded according to manufacturer’s protocol. Membranes were exposed to X-ray film and developed as per manufacturer instructions. Pixel density was calculated using Image Studio Lite Ver 5.2.

**Cell Viability Assay**

Cell viability was determined by crystal violet assay. Cells were seeded in 24-well plates. At least 18h after plating, where indicated, the cells were treated with different concentrations of TET (5-80µM), TRAIL (10-100ng/ml) and combination of TET (5-20µM) with TRAIL (20-80ng/ml TRAIL) for different time-points (48-72h). At indicated time-points, 0.4% crystal violet in 0.2 M citrate buffer was added per well for 30 minutes and washed with water 2-3 times for removal of excessive dye. Plates were air dried for overnight and scanned with HP Scanjet Pro 7400 scanner (HP, Palo Alto, CA). Incorporated crystal violet was solubilized with 2-ethoxy ethanol for overnight and the absorbance was measured at a wavelength of 570nm using Biotek Synergy plate reader (Winooski, VT).

**Creation of shRNA containing lentiviral particles and lentiviral delivery of shRNA**

We found NM_003844.2-421s1c1 (DR4 shRNA2) and NM_003842.3-599s1c1 (DR5 shRNA2) to be most effective shRNA constructs for knock down of DR4 and DR5 respectively in LNCaP cells. As such we used these two constructs to study the double knockdown (DK) effects of DR4/DR5. DR4 and DR5 purified shRNA plasmids or non-target shRNA control (scrl) plasmids along with a packaging and envelope plasmid were transfected into 293T cells using Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. After 24h,
the medium was changed (20mM HEPES, 30% FBS/DMEM with antibiotics, 2mM sodium butyrate) and kept at 37°C, 5% CO₂ for 24h. Next day, the medium was collected, filtered through 0.45µm filters, and kept at -80°C. Cells were infected with lentiviral particles as described previously [33].

**RNA isolation, cDNA synthesis and quantitative Real-Time PCR**

Total RNA was isolated from LNCaP, scr1 and DR4/DR5 DK cells using E.Z.N.A Total RNA Kit I (Omega Bio-tek, Norcross, GA) as per manufacturer’s instructions. 1µg total RNA was used to synthesize cDNA using iScript cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA) as per manufacturer’s instructions. Real Time PCR was performed using a Light Cycler 480 (Roche Diagnostics, Indianapolis, IN) as described previously [34]. Briefly, a multiwell 96-well plate containing 10µM of each primer, 10µl of 2× master mix, and 2µl of cDNA template in a final reaction volume of 20µl was used to amplify the specific mRNA sequences. Following cycle parameters were used: 95°C for 10 min; 45 cycles of 95°C for 10s, 63°C for 10s and 72°C for 10s. GAPDH primers were included to normalize variation from sample to sample. Data were expressed as fold change (2^(-ΔΔCt)) with respect to untreated PCa cells. Primers were procured from Integrated DNA Technologies (Coralville, IA) and the sequence of primers is given in (Table –S2). All experiments were repeated three times.

**Western blot analysis**

For western blotting assays, PCa cells were seeded in 60mm dishes. At least 18h after plating, where indicated, the cells were treated with different concentrations of TET (5-20µM), TRAIL (80-100ng/ml) and combination of TET and TRAIL (10-20µM TET, and 80-100ng/ml TRAIL) for different time-points (24-48h). At the end of experimental period, media was aspirated, the cells were washed with ice cold PBS and solubilized with RIPA buffer (Sigma-Aldrich, St. Louis, MO). For preparation of total cell lysate, the samples were homogenized by sonicator and centrifuged at maximum speed for 5 minutes for removal of cell debris. Samples containing equal amounts of protein were heated to 95°C for 5 minutes, separated on either 4-12% SDS-PAGE. Samples were transferred to Immobilon-FL PVDF (Millipore, Billerica, MA) membrane using standard electroblotting procedures, and were blocked with 5% milk in PBS for 1h at room temperature with gentle agitation. Membranes were washed with 0.1% T-20 in TBS and probed with respective primary antibodies, as indicated, for overnight at 4°C. Next day after
washing the membranes were kept in secondary antibodies for 2h at room temperature in dark. The membranes were visualized for protein signals by Odyssey CLx imaging system (LI-COR, Lincoln, NE) using Image Studio Lite Ver 5.2.

**Apoptosis assay**

For these experiments, PCa cells were seeded in 100mm dishes. At least 18h after plating, where indicated, the cells were either untreated, or treated with TET (20µM) or TRAIL (100ng/ml), and incubated for 48h. At the end of experimental periods, cells were collected after trypsinization and were processed using FITC Annexin-V/PI Apoptosis Detection kit I (BD Pharmingen) as per manufacturer’s instructions. Cells were analyzed by BD Biosciences LSRII flow cytometer (San Jose, CA) as per standard protocols at LSUHSC core facility.

**Determination of combination effect of TET and TRAIL**

Combination effects of TET and TRAIL on cell death were calculated by Jin’s formula [35]. The formula is $Q = \frac{E_{a+b}}{E_a \times E_b}$, where $E_{a+b}$, $E_a$ and $E_b$ are the average inhibitory effects of the combination treatment, TET only and TRAIL only, respectively. $Q > 1.15$ indicates synergism, $Q < 0.85$ indicates antagonism and $0.85 < Q < 1.15$ indicates additive effects according to this formula. The $E_{a+b}$, $E_a$ and $E_b$ values were obtained from readings of the crystal violet assay.

**Statistical analysis**

The GraphPad Prism software package (version 6.0; La Jolla, CA, USA) was used for preparation of graphs and data analysis, with data presented as the mean ± standard deviation (SD). After determining equal variance, comparisons among the means of multiple groups were performed using two-way analysis of variance test. $p <0.05$ was considered to indicate a statistically significant difference.

**Results**

**TET upregulates TRAIL receptors in PCa cells:** To assess the effects of TET (Figure 1A) on apoptosis-related proteins, PCa cells LNCaP were treated with TET (20µM, 24h) and dot blot
array was performed as per manufacturer’s instructions. In response to TET treatment, LNCaP cells expressed high levels of death receptors- DR4 and DR5 and TNFR1 in comparison to untreated control cells (Figure 1B and 1C). Next, we analyzed DR4 and DR5 protein levels following TET treatment in LNCaP cells. Our western blot analysis revealed that DR4 and DR5 protein levels increased in response to TET in concentration and time-dependent manner with an increase starting at 5µM concentration for 24h and increase in expression started at 1h when TET concentration was 20µM (Figure 1D & 1E). Our real time PCR results showed similar increase in DR4 and DR5 mRNA levels after TET treatment in the time-dependent and concentration-dependent manner (Figure 1F & 1G).

**TET and TRAIL show synergistic effects in decreasing/reducing cell viability:** Since TET increased expression of DR4 and DR5, the receptors for TRAIL, we investigated the effects of TET on viability of PCa cells in response to TET, TRAIL and combination of TET and TRAIL by crystal violet assay. TRAIL treatment did not affect the cell viability even at higher dose (100ng/ml) while TET treatment resulted in cell death at higher concentrations (Figure 1H). Our results show that pretreatment of PCa cells with TET (12h) followed by TRAIL treatment resulted in significant increase in cell death (II). The mode of interaction of TET and TRAIL was observed synergistic in nature as calculated by Jin’s formula (Figure 1J). Our crystal violet data showed that RWPE1 cells are resistant to TET and TRAIL treatment (Supplementary Figure S1A & S1B) at 48h & 72h. We also found that these normal prostate cells have negligible levels of DR4 and DR5 in presence or absence of TET as revealed by our western blot analysis (Supplementary Figure S1C). Our results suggest that TET reduces cell viability and also sensitizes PCa cells to TRAIL causing irreversible cell death.

**DR4 and DR5 knock down cells are less sensitive to TET and TRAIL:** To elucidate the functional role of death receptors DR4 and DR5 in mediating the effects of TET on sensitizing LNCaP cells to TRAIL, we used lentiviral shRNA constructs to knock down DR4 or DR5 in combination. Western blot analysis revealed that DK cells (LNCaP cells transfected with lentiviral shRNA for DR4 and DR5 together) showed markedly reduced DR4/DR5 protein levels, and TET treatment (20µM for 24h) did not affect their protein levels (Figure 2A). Next, we analyzed the concentration-dependent effects of TET on DR4/DR5 protein levels in LNCaP
scrl-control and DK cells. We observed that effects of TET on DR4 and DR5 protein levels in LNCaP scrl cells were concentration dependent (Figure 2B) whereas DK cells failed to show any change in their protein levels in response to such treatment (Figure 2C). We also found that the increase of DR4/DR5 is in time-dependent fashion in LNCaP scrl-control cells (Figure 2D) whereas there was no such effect in DK cells (Figure 2E). In addition results presented in (Figure 2F & 2G) show that TET treatment resulted in time dependent increase in DR4 and DR5 mRNA levels in LNCaP scrl-control cells but not in DK cells.

We then determined the effects of combination of TET and TRAIL in LNCaP scrl-control and DK cells on cell viability. For this experiment, we treated LNCaP scrl-control and DK cells with TET (5-10µM), TRAIL (10-100ng/ml) and combinations of TET and TRAIL for 24-72h and cell viability was assessed by crystal violet assay.

We observed that TRAIL alone had no effect on viability in either LNCaP scrl-control or DK cells (Figure 3A). Treatment of LNCaP scrl-control cells with 10µM TET decreased cell viability whereas DK cells were not sensitive to TET (Figure 3B). Moreover, TET in combination with TRAIL resulted in increased cell death of scrl-control cells to TRAIL-induced cell death, whereas DK cells were relatively resistant (Figure 3C and 3D). We also observed that TET treatment resulted in increase of DR4 and DR5 protein levels. On the other hand, TRAIL did not show any effect on their levels. Moreover, the combination of TET and TRAIL resulted in considerable increase in protein levels of DR4 and DR5 in LNCaP scrl control cells (Figure 3E) whereas DK cells did not show any response in protein levels in regard to any of the above treatment (Figure 3F). These results indicate that DR4 and DR5 are required for TET-induced sensitization of PCa cells to TRAIL.

**DR4 and DR5 are required for PCa cell apoptosis in response to TET and TRAIL:** To understand the mechanism of TRAIL-induced cell death in TET treated PCa cell death, first we checked caspase activation following treatment of PCa cells with various concentrations of TET, TRAIL and combination of TET and TRAIL for different time-points (24-48h) in LNCaP scrl-control and DR4/DR5 DK cells by western blot. Our results show that pretreatment with TET followed by TRAIL resulted in activation of caspases-8 and 3 in LNCaP scrl-control cells (Figure 4A) but these treatments had no effect on caspase activation in DR4/DR5 DK cells.
Next we performed apoptosis assays to determine the cell death in response to TET, TRAIL and combination of TET and TRAIL in LNCaP scrl-control and DK cells. Our apoptosis data showed that pretreatment of PCa cells with TET followed by TRAIL treatment enhanced the cell death in LNCaP scrl-control cells at 48h (Figure 4C) whereas DK cells were less sensitive (Figure 4D). As shown in Fig. 4E, upon combined treatment of TET and TRAIL, apoptotic cell death was significantly higher in scrl cells as compared to DK cells (96.4% vs 32.15% respectively, p<0.0001) (Figure 4E). These results demonstrate that DR4 and DR5 activation is required for caspases activation which contributes to the TET-induced cell death of PCa cells.

TET-induced sensitization of PCa cells to TRAIL is independent of p53 status: To determine whether the effects of TET on expression of DR4 and DR5 are dependent on expression status of p53, we treated LNCaP-derived C4-2B cells with TET (0-20µM) for 24h and 20µM TET for 0-48h. Western blotting analysis of these cells revealed an increased levels of p53. We also found that TET treatment results in upregulation of DR4 and DR5 in both concentration and time-dependent manner (Figure 5A & 5B). Interestingly, p53 null PC3 cells also depicted upregulation of DR4 and DR5 protein levels whereas p53 expression was not observed upon TET treatment (Figure 5C & 5D). We also evaluated the expression of DR4 and DR5 in response to TET, TRAIL and combination of TET and TRAIL in PC3 cells. These cells were treated with different concentrations of TET, TRAIL and combinations of TET and TRAIL for 24h. Following 24h, western blot was done to check DR protein levels. We found that treatment of PC3 cells with TET resulted in upregulation of DR4 and DR5 protein levels. Surprisingly, treatment of PC3 cells with TRAIL alone resulted in decline of DR4 and DR5 protein levels, whereas, the combination of TET and TRAIL showed increase in DR4 and DR5 levels, similar to C4-2B cells. These results suggested that TET could potentially sensitize PC3 cells to TRAIL (Supplementary Figure S2). Next, we evaluated the effects of TET, TRAIL and combination of these two on cell viability in C4-2B and PC3 cells. C4-2B cells were resistant to TRAIL treatment even at 100ng/ml TRAIL concentration. 5µM TET showed no effect on cell viability whereas 10µM TET showed decline of cell viability (Figure 5E) and combination of TET sensitized these cells to TRAIL at 72h. This decline in cell viability was observed in time and dose-dependent manner whereas 10µM TET with TRAIL had irreversible effect on cell viability.
viability. The combination of TET and TRAIL had synergistic action as revealed by Jin’s formula (Figure 5F). PC3 cells were sensitive to TRAIL as TRAIL showed some decline in cell viability in concentration-dependent manner, however, these cells were largely resistant to TET since TET treatment alone did not show much effect on cell viability (Figure 5G). Interestingly, sub-lethal dose of TET (5µM) in combination with TRAIL treatment sensitized PC3 cells and resulted in increased PC3 cell death. PC3 cells also expressed synergistic nature of action of combination of two drugs for cell viability (Figure 5H). Taken together, these data reveal that TET induces expression of DR4 and DR5 and sensitizes PCa cells to TRAIL irrespective of p53 status.

Discussion

In the present study, the potential anticancer effect of TET, a bioactive product derived from *Stephania tetrandra*, a Chinese herb, and its ability to overcome resistance to TRAIL was explored in TRAIL resistant PCa (LNCaP, C4-2B) and minimal sensitive (PC3) cells as the model system. We observed that pretreatment of PCa cells with TET followed by TRAIL treatment results in massive cell death. Treatment of PCa cells with TET resulted in increased mRNA and protein levels of DR4 and DR5. We also observed that knockdown of DR4 and DR5 abrogated the effects of TET in sensitizing PCa cells to TRAIL, suggesting a critical requirement of DR4 and DR5 for TRAIL-mediated activation of caspase and apoptosis of PCa cells. We also found that TET-induced upregulation of DR4 and DR5 is independent of p53 status.

TRAIL has attracted considerable attention as a novel anticancer agent. Although many types of cancer cells are sensitive to TRAIL-induced apoptosis, other cells including PCa are resistant [36], but success with TRAIL as a single agent has been limited owing to evolution of resistance mechanisms. TRAIL in combination with ionizing radiation has been shown to improve tumor eradication in breast cancer [37]. It is important to point out here that both TRAIL and agonistic antibodies to TRAIL receptors are currently in clinical trials for treatment of cancer patients and resistance of tumor cells to apoptosis is one of the major hurdles in the application of TRAIL. Our results show that PCa cells (LNCaP and C4-2B) were completely insensitive to the effects of TRAIL and PC3 cells were partially responsive to TRAIL. Taken together, these studies highlight the need for new strategies to sensitize cancer cells, in general, and PCa cells in particular to TRAIL.
TET has been used for a long time in Chinese medicines. It is a cytotoxic compound capable of exerting remarkable antitumor activity against various cancer cells \textit{in vitro} and \textit{in vivo}. However, little is known about its effect on human PCa cells. Our crystal violet data showed that TET induces cell death in PCa cells in time-dependent and concentration-dependent manner which is consistent with previous studies [30]. Our data showed that LNCaP and C4-2B cells are resistant to TRAIL treatment but pretreatment of TET followed by TRAIL treatment results in increased cell death of PCa cells \textit{in vitro}. We also found that as low as 10µM concentration of TET is able to sensitize TRAIL at 72h. However, the normal prostate epithelial (RWPE1) cells were resistant to similar dose of TET alone and combination of TET and TRAIL as well, which suggests that TET is non-toxic to normal cells. This observation is also in accordance with other studies [38, 39]. There are several reports on sensitizing cancer cells to TRAIL using other compounds e.g. etoposide [40], but until now no report has been published on sensitizing cancer cells using combination of TET and TRAIL. Results presented herein provide evidence for the first time in any system that treatment with TET sensitizes resistant cancer cells to TRAIL, suggesting that combination of TET and TRAIL could serve as an effective therapeutic option for CRPC and perhaps other malignancies that are resistant to current therapeutic regiments.

Resistance to TRAIL-induced apoptosis in cancer cells occurs via different mechanisms like downregulation of death receptors- DR4 and DR5; upregulation of antagonistic decoy receptors that bind TRAIL but do not contain the functional domains necessary to transduce apoptotic signals; upregulation in cell survival proteins such as Bcl-xL, Bcl-2, XIAP, survivin, cellular FLICE-like inhibitory protein (c-FLIP, a caspase-8 inhibitor also known as I-FLICE) and Mcl-1, and downregulation in pro-apoptotic proteins [41]. Our results show levels of DR4 and DR5 are marginally expressed in PCa cells (LNCaP and C4-2B) and treatment of these cells with TET increased the expression of both DR4 and DR5 at mRNA and protein levels. Decreased DR4 and/or DR5 protein levels can lead to TRAIL resistance [10]. We observed that TET induces DR4 and DR5 mRNA and protein level with increase in dose of TET and in time-dependent manner. As of present, there is no study which reflects the effect of TET on death receptors in any cell type. Combined treatment of TET and TRAIL resulted in enhanced upregulation of DR4 and DR5 in concentration-dependent and time-dependent manner in our study. Our knockdown experiments using shRNA against DR4 and DR5 in LNCaP cells resulted
in the muted expression of DR4 and DR5 and TET did not enhance DR4 and DR5 expression in these cells. DR4/DR5 double knockdown cells were also less sensitive to apoptosis as revealed by apoptosis experiment. Recently, a study by Cheng et al., showed that Mitomycin-C (MMC), an antibiotic that has demonstrated antitumor activity in preclinical and clinical studies and is widely used to treat various cancers, induces DR4 and DR5 expression in a dose-dependent manner, and gene silencing of DR5 or DR4 and DR5 abolishes TRAIL-induced apoptosis [42]. These data indicate that DR4 and DR5 expression is critical for sensitization of cancer cells to TRAIL.

It has been well understood that activation of caspases is critical in apoptosis by various apoptotic stimuli [43]. Our results demonstrate that TET induces cleavage of caspase-8 and 3 and pretreatment of TET followed by TRAIL treatment enhances the cleavage at 48h in scrl cells whereas DK cells failed to reveal the cleavage of caspases. Consistent to our results, other study have also shown that TET is required for activation of caspases in cancer [44]. Our apoptosis data showed a significant increase (41.25%) in apoptotic cell number in response to TET and combination of TET and TRAIL (96%) treatment in scrl cells whereas DK cells’ apoptotic population was low (32%). TRAIL in combination with TET, therefore, induced apoptosis to a much greater extent than either did alone. This finding again suggesting the synergistic action of TET and TRAIL in inducing apoptosis in PCa cells. These data also suggest that TET induces binding of TRAIL to death receptors which causes caspases activation and ultimately apoptosis of PCa cells. Also, these data provide clues that death receptors are required for TET-induced apoptosis of PCa cells.

Several previous attempts using agonistic agents or TRAIL itself or agonistic antibodies to DR4/DR5 in tumor therapy did not progress to clinic, perhaps underlining the fact that in advanced tumors there is downregulation /loss of expression of DR4/DR5 [45]. Interestingly, several studies suggest that inactivation of DR4/DR5 receptor leads to tumor promotion [46]. Recent studies also suggest that down regulation of DR4/DR5 helps tumor cells evade immune surveillance [47], highlighting the role of DR4/DR5 in tumor immunotherapy. Thus our observations that TET induces robust expression of DR4 and DR5 in PCa cells, suggest that TET may play an important role not only in sensitizing PCa cells to TRAIL, but also play a role in tumor suppression and perhaps help sensitize such tumors to immunotherapy.
TET has been reported to activate various signaling molecules and apoptosis-related genes e.g. TET increases p53 protein levels in different cancer cells [28, 48]. As shown in fig. 5, TET also upregulated p53 in C4-2B PCa cells in our study. It has been reported that p53 serves as a regulator of the apoptotic process that can modulate the extrinsic pathway [49]. Interestingly, three DNA-binding sites of p53 have been identified in the genomic locus of DR5 [50]. However, p53 was not required for TET-induced upregulation of death receptors in all cell types, as revealed by our results of upregulation of DR4 and DR5 in PC3 which are p53 null as well as AR negative PCa cells. This observation is consistent to a previous study in colon cancer cells [41] where the authors showed that the upregulation of death receptors by azadirone, a limonoid tetranortriterpene, is independent of p53 status. These observations suggest that p53 is not required for TET-induced apoptosis in cancer cells. We also found that TRAIL reduces DR4/DR5 protein levels in PC3 cells. Interestingly, TET blocks TRAIL-induced DR4/DR5 loss which suggests the opposing effects of TET and TRAIL in inducing DR4/DR5 expression levels in p53 null cells.

In conclusion, to the best of our knowledge, the results of our present study show for the first time, in any system, that TET potentiates the TRAIL-induced apoptosis, in-part, by DR4 and DR5 upregulation in human PCa cells by activating caspases (Figure 6). These data provide the proof-of-principle for use of TET as TRAIL sensitizer and novel therapeutic strategy to sensitize cancer cells in general and PCa cells in particular to apoptosis. Our data that TET induced DR4 and DR5 expression and TRAIL mediated apoptosis is independent of p53 status are quite exciting, given that loss of p53 is associated with PCa progression to CRPC and NEPC. Taken together, our results suggest that TET by acting as a TRAIL sensitizing agent in PCa could serve as a potential therapeutic agent in CRPC, for which there is no cure to date.

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References:


41. Gupta, S.C., et al., Azadirone, a limonoid tetranortriterpene, induces death receptors and sensitizes human cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)


Figure legends

Figure 1- TET synergistically sensitizes LNCaP cells to TRAIL and results in upregulation of death receptors- DR4 & DR5 in concentration and time-dependent manner. A. The chemical structure of Tetrandrine. B & C. Dot blot array (B) and quantitation (C) showing the effect of TET treatment on DR4 and DR5 protein levels in LNCaP cells. D&E. Representative immunoblots showing the DR4 and DR5 expression in response to indicated concentrations of TET and at different time-points. LNCaP cells were treated with 5-20µM TET for 24h (Fig. 1D) and 20µM TET for 0- 24h (Fig. 1E). After the treatment cells were lysed and DR4 and DR5 levels were detected by Western blotting. GAPDH was used as loading control. F&G. Real time PCR showing the effects of TET on DR4 and DR5 mRNA levels. LNCaP cells were treated with 5-20µM TET for 24h (Fig. 1F) and 20µM TET for 0- 24h (Fig. 1G). After the treatment cells were lysed, total RNA was extracted and DR4 and DR5 mRNA levels were detected by Real Time PCR. GAPDH was used as loading control. C is control. H. Cell viability as revealed by crystal violet staining of LNCaP cells showing the effects of various concentrations of TRAIL and TET for different time-points (48h and 72h). Wells are representative of at least three repeats. Respective graphs showing the cell viability in response to dose treatments. I. Cell viability as revealed by crystal violet staining of LNCaP cells showing the effects of various concentrations of combination of TET and TRAIL at 48-72h. Wells are representative of at least three repeats. Respective graphs showing the cell viability in response to dose treatments. J. Representative graph showing the synergistic effect of TET and TRAIL in LNCaP cells. Synergy was calculated by Jin’s formula. Data are normalized to the untreated control at the respective concentrations of TET and TRAIL, and are representative of at least three repeats. Error bars represent standard deviation. *p value <0.05; **p value <0.01; ***p value <0.001, ****p value <0.0001.

Figure 2- Dual knock down of DR4 and DR5 results in muted expression of DR4 and DR5 in PCa cells in response to TET. A. Western blot analysis showing the effects of knockdown of DR4 and DR5 in PCa cells. LNCaP cells were transfected using shRNA against both DR4 and DR5 (Fig. 2A) death receptors. Non-target shRNA (scrl) was used as control. Control and transfected cells were treated with 20µM TET for 24h and DR expressions were analyzed by western blotting. B&C. Representative immunoblots showing the concentration-dependent (5-
20µM) effects of TET (for 24h) on DR4 and DR5 expression levels in scrl (Fig. 2B) and DK cells (Fig. 2C). D&E. Representative immunoblots showing the time-dependent (0-48h) effects of TET on DR4 and DR5 expression levels in scrl (Fig. 2D) and DK cells (Fig. 2E). F&G. Quantitative Real Time PCR for DR4 (Fig. 2F) and DR5 (Fig. G) after 20µM TET treatment for different time-points (0-8h) in scrl and DK cells. Fold change was calculated with the ct value of untreated LNCaP cells as a reference. GAPDH was used as loading control. Averages are representative of 3 independent experiments. Error bars represent standard deviation. *p value <0.05; **p value <0.01; ***p value <0.001, ****p value <0.0001.

Figure 3- TET does not sensitize DR4 and DR5 knock down cells to TRAIL. A, B, C&D. Crystal violet assay showing the effect of TRAIL (Fig. 3A), TET (Fig. 3B), and combinatorial treatment of TET (5µM) and TRAIL (Fig. 3C) and combinatorial treatment of TET (10µM) and TRAIL (Fig. 3D) in LNCaP scrl and DK cells. PCa cells were treated with different concentrations of TET (0-10µM), TRAIL (0-100ng/ml) and combination of TET with TRAIL. Respective graphs showing the cell viability upon different treatments. E&F. Representative immunoblots showing the effect of TET, TRAIL and combination of TET and TRAIL in scrl (Fig. 3E) and DK cells (Fig. 3F) at different time points (0-48h). After the indicated treatments cells were lysed and DR4 and DR5 levels were detected by Western blotting. GAPDH was used as loading control. Averages are representative of 3 independent experiments. Error bars represent standard deviation. *p value <0.05; **p value <0.01; ***p value <0.001, ****p value <0.0001.

Figure 4- DR4 and DR5 are required for combined effects of TET and TRAIL in apoptosis and activation of caspases in TET sensitized PCa cells. A&B. Western blot analysis showing the effect of TET, TRAIL and combination effect of TET with TRAIL on cleaved caspases (caspase-8 and 3) in scrl (Fig. 4A) and DK cells (Fig. 4B). Different concentrations of TET (10-20µM) and TRAIL (80ng/ml) and combinations of TET and TRAIL were used for 24h and 48h. C&D. Apoptosis assay showing the effect of TET, TRAIL and combination treatment of TET and TRAIL on cell death in LNCaP scrl (Fig. 4C) and DK cells (Fig. 4D) at 48h. E. Representative graph showing the percent live and apoptotic scrl and DK cells after TET, TRAIL and combination treatment of TET and TRAIL at 48h. Averages are representative of 3
independent experiments. Error bars represent standard deviation. *p value <0.05; **p value <0.01; ***p value <0.001, ****p value <0.0001.

Figure 5- TET-induced expression of death receptors DR4 and DR5 is independent of p53 status: TET sensitizes PC3 cells to TRAIL-induced apoptosis. A&B. Western blot analysis showing the effect of TET on DR4, DR5 and p53 in concentration-dependent (Fig. 5A) and time-dependent fashion (Fig. 5B) in C4-2B cells. C&D. Western blot analysis showing the effect of TET on DR4, DR5 and p53 in concentration-dependent (Fig. 5C) and time-dependent fashion (Fig. 5D) in PC3 cells. Different concentrations of TET (0-20µM) were used for 24h and 20µM TET was used for different time points (0-48h). E. Crystal violet staining showing the effects of TRAIL and TET on cell viability for different time-points at 48h and 72h in C4-2B cells. F. Crystal violet staining showing the effects of combination of TET and TRAIL on cell viability for different time-points at 48h and 72h in C4-2B cells. Wells are representative of at least three repeats. Respective graphs showing the cell viability in response to dose treatments. Averages are representative of 3 independent experiments. Error bars represent standard deviation. Representative graph showing the synergistic effects of TET and TRAIL as revealed by Jin’s formula in C4-2B cells. G. Crystal violet staining showing the effects of TRAIL and TET on cell viability for different time-points at 48h and 72h in PC3 cells. H. Crystal violet staining showing the effects of combination of TET and TRAIL on cell viability for different time-points at 48h and 72h in PC3 cells. Wells are representative of at least three repeats. Respective graphs showing the cell viability in response to dose treatments. Averages are representative of 3 independent experiments. Error bars represent standard deviation. Representative graph showing the synergistic effects of TET and TRAIL as revealed by Jin’s formula in PC3 cells. *p value <0.05; **p value <0.01; ***p value <0.001, ****p value <0.0001.

Figure 6- Schematic diagram for TET-sensitized TRAIL induced upregulation of death receptors and induction of apoptosis in PCa cells. PCa cells survive because of absence of death receptors hence no apoptosis takes place. These cells develop resistance to TRAIL therefore TRAIL is unable to induce cell death. Upon TET treatment, there is an increase in DR4 and DR5 mRNA and protein levels which cause apoptosis and further treatment of PCa cells with TRAIL sensitizes these cells to TRAIL which enhances activation of caspases and massive cell death.
Figure 2

A. TET (µM) concentration and protein expression levels for DR4, GAPDH, and DR5 under Scrl and DK conditions.

B. TET (µM) concentration and protein expression levels for DR4 and GAPDH under Scrl and DK conditions.

C. TET (µM) concentration and protein expression levels for DR4, GAPDH, and DR5 under Scrl and DK conditions.

D. TET (20 µM) concentration and protein expression levels for DR4 and GAPDH under Scrl and DK conditions.

E. TET (20 µM) concentration and protein expression levels for DR5 and GAPDH under Scrl and DK conditions.

F. DR4 mRNA fold change over time (h) for Scrl and DK conditions.

G. DR5 mRNA fold change over time (h) for Scrl and DK conditions.
Figure 3

A. TRAIL (ng/ml)

- 0
- 80
- 100

48h

- 0
- 80
- 100

72h

Cell Viability

B. TET (μM)

- 0
- 5
- 10

48h

- 0
- 5
- 10

72h

Cell Viability

C. TET+TRAIL

- 0
- 5+20
- 5+40
- 5+80

48h

- 0
- 5+20
- 5+40
- 5+80

72h

Cell Viability

D. TET+TRAIL

- 0
- 10+40
- 10+80

48h

- 0
- 10+40
- 10+80

72h

Cell Viability

E. TRAIL + TET

- 24h

- 48h

DR4

GAPDH

DR5

GAPDH

F. TRAIL + TET

- 24h

- 48h

DR4

GAPDH

DR5

GAPDH
Figure 6

- **With TRAIL**
  - TRAIL → Inactive Caspase-8 → Inactive Caspase-3 → Cell Survival

- **Untreated Ca cells**
  - TRAIL → Inactive Caspase-8 → Inactive Caspase-3 → Cell Survival

- **With TET**
  - TET → DR4/DR5 protein → mRNA DR4/DR5 → Cell Death

- **With TET & TRAIL**
  - TET & TRAIL → Cleaved Caspase-8 → Cleaved Caspase-3 → Massive Cell Death
Molecular Cancer Therapeutics

Tetrandrine (TET) Induces Death Receptors: Apo Trail R1 (DR4) and Apo Trail R2 (DR5) and sensitizes Prostate Cancer Cells to TRAIL induced apoptosis

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