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DELTA-TOCOTRIENOL IS THE MOST BIOACTIVE ANTICANCER AGENT AGAINST GASTRIC CANCER

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ABSTRACT

This study evaluated the anticancer activity of δ-tocotrienol (DT3), a bioactive form of vitamin E, in the inhibition of gastric cancer growth and survival compared with other three forms α-, β- and ϒ-tocotrienol (AT3, BT3 and GT3). DT3 is the most bioactive form of vitamin E in inhibiting the cell proliferation of non-metastatic (AGS) and metastatic (746T) gastric cancer cells followed by GT3 and BT3. However, AT3 is devoid of any inhibitory effect on gastric cancer cell viability. The estimated IC50 values are 80±5, 55±5 and 40±5 µM for BT3, GT3 and DT3, respectively in both cell lines. DT3 (40 µM) significantly inhibited malignant transformation (p <0.02, p <0.01), cell migration (p <0.01) and invasion (p < 0.05, p < 0.02) compared to vehicle in AGS and 746T cells. DT3 inhibited markers for epithelial (E-cadherin) to mesenchymal (vimentin) transition (EMT), metastasis (matrix metalloproteinase 9 [MMP9]), angiogenesis (Vascular endothelial growth factor [VEGF]), inflammation (Nuclear factor-kappa B [NF-kB]), and Wnt signaling (β-catenin) and its downstream transcriptional targets C-MYC, Cyclin D1 and survivin compared to vehicle. In addition DT3 also induced apoptosis in gastric cancer cells (AGS and 746T). Taken together, these data demonstrate that DT3 is a potential therapeutic agent in advanced gastric cancer and warrants further investigation for its clinical use in the prevention and treatment of gastric cancer.

KEYWORDS: DT3, Gastric cancer, Apoptosis, Migration, EMT, Invasion.

INTRODUCTION

Gastric cancer is one of the leading causes of the mortality worldwide and is responsible for over millions of death every year.[1] The estimated new cases for gastric cancer in United States are over twenty six thousands.[2] The majority of these cancers are diagnosed at an advanced stage and outcomes remain poor for metastatic disease.[3] It is estimated that the metastatic spread of cancer is responsible for 90% of human cancer-related deaths. The current therapeutic strategies for most gastric cancer patients include surgical resection of the tumor and chemotherapy. Approximately one-third of patients undergo radical resections relapse with a recurrence rate as high as 70% in advanced gastric cancer.[4] Chemotherapy provides only modest benefit, with median overall survival of 10 months.[5] Despite therapeutic advances,[6] overall options remain limited due to toxic side effects of the chemotherapy and development of chemo resistance,[7,8] Thus, novel and safe therapeutic agents are urgently needed for patients with advanced gastric cancer.

Several studies have suggested that increased intake of dietary fruits, vegetables, and cereal grains may prevent gastrointestinal cancers, including gastric cancer,[9,12] Tocotrienols are a unique family of four natural vitamin E compounds (α-, β-, δ-, γ-tocotrienols), which are found in fruits, vegetables, cereal grains, and essential oils,[13,14] and have distinct biologic activity from tocopherols (α-, β-, δ-, γ),[15,17] Preclinical studies have shown that, in contrast to tocopherols, tocotrienols have unique bioactive properties against cancer cells.[13,20] Furthermore our preclinical and clinical studies in pancreatic cancer showed no obvious toxicity to the host.[21,22] Although GT3 have been reported to inhibit the gastric cancer cells growth.[23,26] However the current study aimed to investigate the comparative anticancer activity of α-, β-, δ-, γ-tocotrienols (AT3, BT3, DT3, and GT3) in both metastatic and non-metastatic gastric cancer cells.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Alpha, beta, gamma, and delta tocopherols and tocotrienols (97%) were obtained from Davos Life Ltd (Helios, Singapore). L-glutamine, penicillin, streptomycin, and HEPES buffer were purchased from Life Technologies, Carlsbad, CA. Fetal bovine serum (FBS) was purchased from Atlanta Biological, Atlanta, GA. Dulbecco's modified minimal essential medium (DMEM), and DMEM/F-12K, phosphate buffered saline (PBS) and 0.05% Trypsin/EDTA were purchased from Life Technologies, Carlsbad, CA. Ethanol (100%) was purchased from Aaper Alcohol and Chemical, Shelbyville, KY. Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma-Aldrich Company, St. Luis, MO. Human gastric cancer cell line AGS and human metastatic gastric cancer cell line 746T were purchased from ATCC, Manassas, VA.

Cell Culture and Growth

Human gastric cancer cells (AGS) and metastatic gastric cancer cells 746T were grown in DMEM/F-12K and DMEM, respectively, which were supplemented with 10% FBS, penicillin (50 IU/ml) and streptomycin (50 mg/ml). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Cell Proliferation MTT Assay

Cells were seeded in 96-well plates at a density of 3000 cells per well and allowed to attach overnight. Cells were then incubated for 72 hours with various concentrations of AT3, BT3, GT3, and DT3 (10^{-5}-10^{-4} M) or ethanol (< 5%) vehicle as control. media were aspirated and replaced with 20 µL of 1 mg/mL MTT and incubated for 2 to 4 hours at 37°C in a humidified atmosphere of 5% CO_2. Media was aspirated and 200 µl of DMSO added to each well and incubated for 5 min with shaking and absorbance was read at 540 nm.

Colonogenic Survival (Anchorage Independent) Growth Assay

Standard soft agar colony formation assays were performed in AGS and metastatic 746T cells. The cells were seeded at a density of 5000 per well in a 12-well plate in 0.3% agar over a 0.6% bottom agar layer. Colonies were fed with growth media with DT3 (5 x 10^{-5} M) and growth of colony formation was observed for 10 to14 days. Colonies were photographed after overnight incubation with 1 mg/mL MTT in the wells. The colonies were counted under stereo microscope and compared with control ethanol vehicle. Each experiment was done in triplicate, at least twice.

Cell Migration and Invasion Assay

Cell migration was performed by scratch test or wound healing assay. AGS and 746T cells were seeded in 6-well plates and cultured to 100% confluence. Wounds were generated in the cell monolayer using small plastic pipette tip. The cells were then rinsed with PBS, treated with DT3 (4x 10^{-5} M) and cultured for another 24 hours. The spread of wound closure was observed and photographed under light microscope. For invasion assays, 1×10^5 cells (AGS and 746T) treated with DT3 (4 x 10^{-5} M) in serum-free media were added into the upper chamber of an insert precoated with Matrigel (BD Bioscience). The lower chamber was filled with DMEM with 10% FBS. After 48 hours of incubation, the cells remaining on the upper surface of the membrane were removed, whereas the cells that had invaded through the membrane were stained with 20% methanol and 0.2% crystal violet, imaged, and counted under light microscope.

Apoptosis assay

Human non-metastatic gastric cancer cells (AGS) and metastatic gastric cancer cells (746T) were plated and treated concurrently with vehicle (5% ethanol) or DT3 (40 µM) for 24 hours. Cells were harvested and 10^5 cells were transferred to 5 mL tubes in PBS (100 µL) then 2 µL of propidium iodide and 5 µL of Annexin V–FITC (BD Bioscience) were added and mixed. The tubes were incubated for 15 minutes at room temperature in the dark then 400 µL of binding buffer was added and tubes were analyzed for apoptosis by flow cytometry. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), with analysis using FLOW-JO software (Tree Star, Inc., Ashland, OR) to assess the Annexin-positive cell population.

Cell protein extraction and quantification

Cells were washed 3 times in cold PBS (pH 7) then lysed in protein extraction reagent RIPA buffer (Thermo Scientific, Rockford, IL) containing an EDTA and protease inhibitor cocktail. Protein concentration was determined using BCA reagents (Pierce, Rockford, IL), according to the manufacturer's instructions.

Western blot analyses

Extracted proteins from DT3- or vehicle-treated cells (40 µg) were resolved on 12.5% SDS polyacrylamide gel (SDS PAGE) running gel and a 5% stacking gel. Proteins were then electrotransferred onto nitrocellulose membranes. After blocking in 5% nonfat powdered milk for 1 hour, the membranes were washed and then treated with antibodies to β-catenin, cyclin D1,C-MYC, surviving, E-cadherin, vimentin, c-PARP, NF-κB/p65, VEGF, MMP9, and β-actin (1:1000 and 1: 5000) overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA; Cell Signaling, Danvers, MA). After washing, the blot was incubated with horseradish peroxidase-conjugated secondary antibody IgG (1:5000 and 1:10000) for 1 hour at room temperature. The washed blot was then treated with Super Signal West Pico chemiluminescent substrate (Pierce) for positive antibody reaction. Membranes were exposed to X-ray film (KODAK) for visualization and densitometric

quantization of protein bands using AlphaEaseFC software (Alpha Innotech).

Statistical analyses

The data were expressed as mean ± standard error of the mean. The data were analyzed statistically using unpaired *t* tests or 1-way analyses of variance (ANOVA) where appropriate. ANOVA was followed by Duncan's multiple range tests using SAS statistical software for comparisons between different treatment groups. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of different forms of tocotrienols on gastric cancer cell growth

We first analyzed the concentration-dependent response of tocotrienols (AT3, BT3, GT3 and DT3) on cell proliferation by MTT assay in gastric cancer cells (AGS and 746T). Data show that BT3, GT3, and DT3 significantly inhibited the cell proliferation of AGS and 746T cells (Figure 1A and B). The inhibition of cell proliferation with DT3 was.

Figure 1 A, Effects of α-tocotrienol (AT3), β-tocotrienol (BT3), δ-tocotrienol (DT3), and γ-tocotrienol (GT3) on non-metastatic (AGS) gastric cancer cell growth (MTT assay). BT3, DT3, and GT3 significantly inhibited the proliferation of gastric cancer cells in a concentration-dependent manner (10 to 100 µM). In contrast, no significant effect was observed with AT3-treated cells at concentrations of 10 to 100 µM. Points, means; bars, standard errors (SEs; n = 3-5, **p* <0.01, ***p* <0.05). **B,** Effects of α-tocotrienol (AT3), β-tocotrienol (BT3), δ-tocotrienol (DT3), and γ-tocotrienol (GT3) on metastatic (746T) gastric cancer cell growth (MTT assay). BT3, DT3, and GT3 significantly inhibited the proliferation of gastric cancer cells in a concentration-dependent manner (10 to 100 µM). In contrast, no significant effect was observed with AT3-treated cells at concentrations of 10 to 100 µM. Points, means; bars, standard errors (SEs; n = 3-5, **p* <0.01, ***p* <0.05). greater than BT3 and GT3. We have earlier reported that DT3 was the most bioactive form of vitamin E against pancreatic cancer cells.[19] Interestingly DT3 significantly inhibited the growth of all gastric cancer cells in a concentration-dependent manner. The IC_{50} value of DT3 in gastric cancer cells was 40 µM.

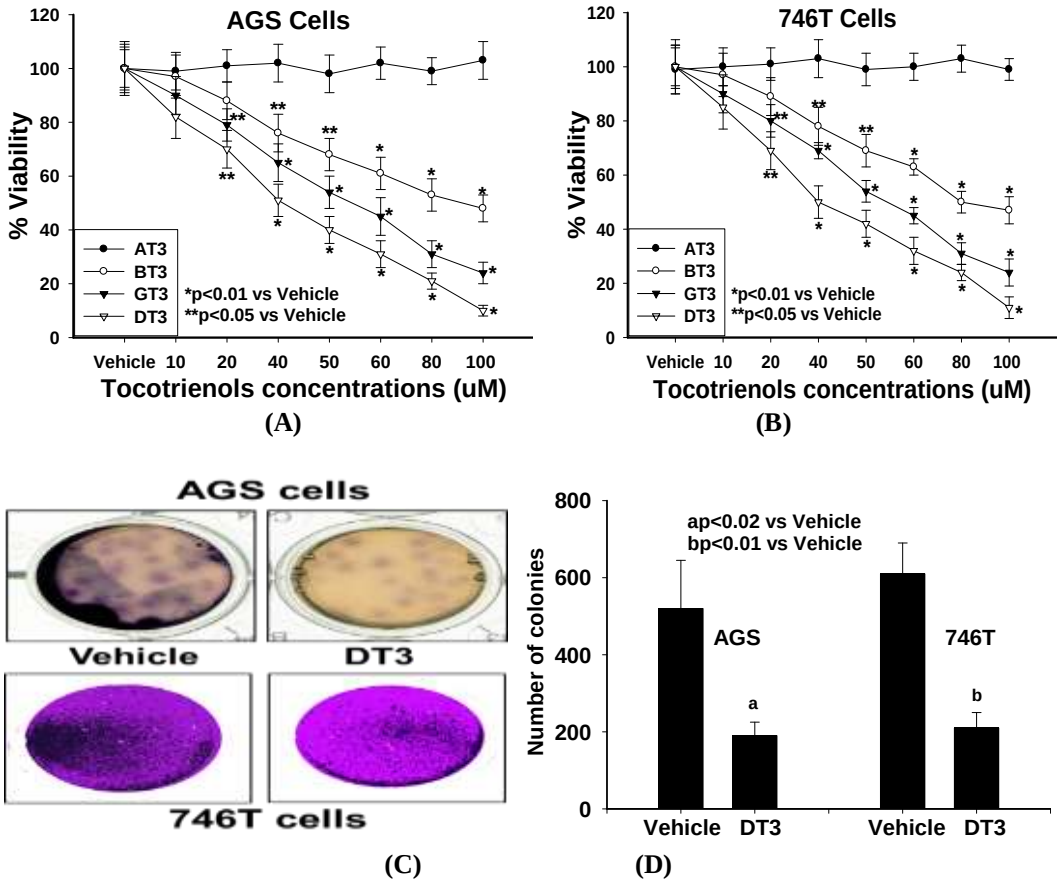

Figure 1: C and D, Effects of DT3 (40 µM) on anchorage-independent growth (soft agar colony formation assay) for 14 days in non-metastatic (AGS) and metastatic (746T) gastric cancer cells. D, DT3 at 40 µM significantly inhibited the malignant transformation of (AGS) and (746T) gastric cancer cells (a*p* <0.02 and b*P*<0.01). Bars, SE (n = 3).

We further analyzed the effects of DT3 (40 µM) on anchorage-independent growth of AGS and 746T cells using soft agar colony formation assay. DT3 significantly inhibited the colony formation in both cells (Figures 1C and D), but the inhibition was greater (73%) in 746 cells than (64%) in AGS cells, indicating the inhibition of gastric cancer malignant transformation.

DT3 inhibits migration and invasion of colon cancer cells

We further investigated whether DT3 could also inhibit cell migration and invasion in AGS and 746T cells. Using the wound-healing assay, we found that DT3 significantly (ap < .02; bp < .05) suppressed tumor cell mobility in both cells compared with their corresponding vehicle controls (Figures 2A and B).

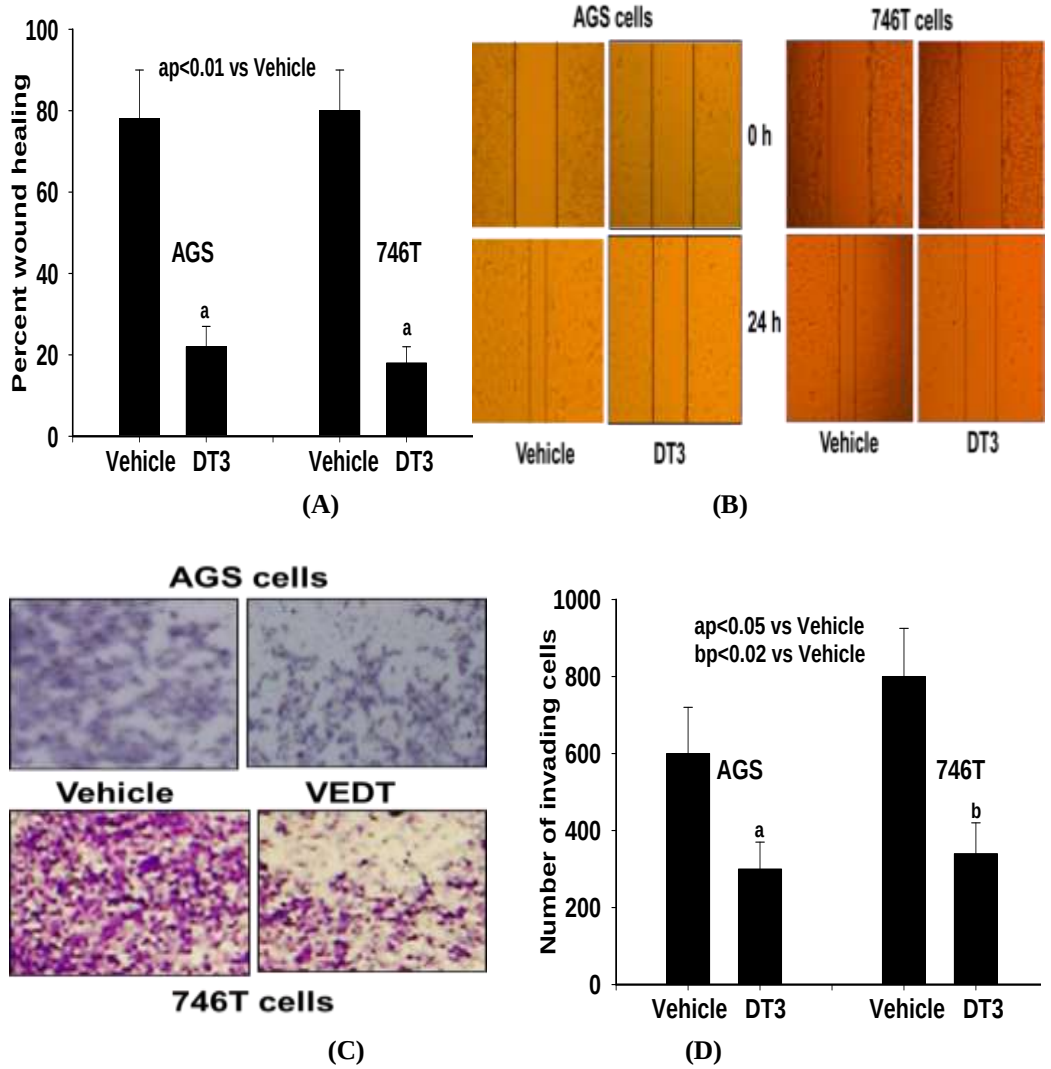

Figure 2: A & B, Effects of δ-tocotrienol (DT3) on cell migration of non-metastatic (AGS) and metastatic (746T) gastric cancer cells using wound-healing assay. DT3 at 40 µM concentration significantly suppressed tumor cell mobility in both cells compared with their corresponding vehicle controls (ap <0.01). Bars, SE (n = 3). C & D, Effects of DT3 on invasion assay with Matrigel in non-metastatic (AGS) and metastatic (746T) gastric cancer cells. DT3 at 40 µM concentration significantly decreased the invasive capacity of AGS cells (ap <0.05) and 746T cells (bp <0.02). Bars, SE (n = 3).

Similarly, invasion assay with Matrigel demonstrated that DT3 significantly decreased the invasive capacity of both cells compared to vehicle (Figures 2C and D). Taken together, these results suggest that DT3 can suppress the metastatic processes of gastric cancer.

DT3 induces apoptosis in gastric cancer cells

To investigate the effects of DT3 on cell survival, gastric cancer cells AGS and 746T were treated with DT3 (40 µM) for 24 hours and apoptosis was analyzed using Annexin V/PI staining by flow cytometry. The percentage of apoptotic cell death was greater in 746T cells (44%) than in AGS cells (15%) compared to vehicle (Figure 3). Western blot analysis further confirmed the apoptotic activity of DT3 with increased PARP1 cleavage in both AGS and 746 gastric cancer cells (Figure 4).

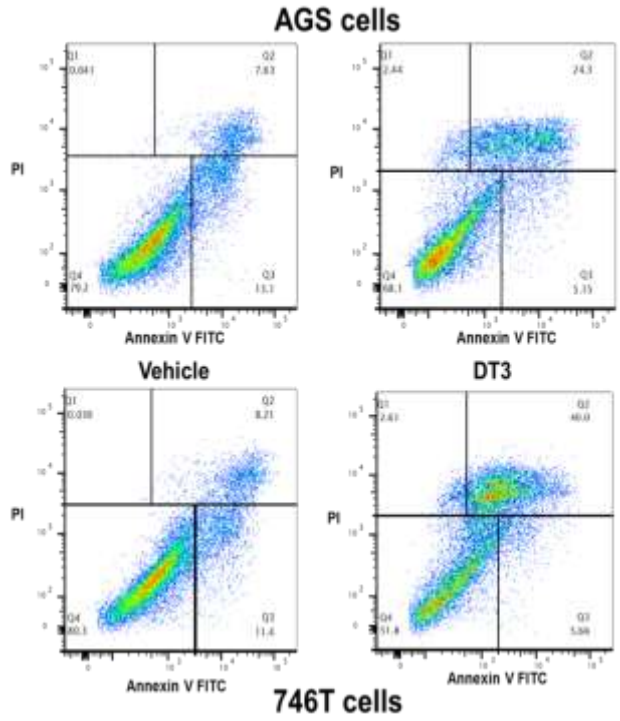

Figure 3: Effects of δ-tocotrienol (DT3) on apoptosis (Annexin V/PI staining) in non-metastatic (AGS) and metastatic (746T) gastric cancer cells. DT3 at 40 µM concentration for 24 hours increased apoptosis in AGS cells (24%), and 746T cells (40%) compared to vehicle. Data are from 3 independent experiments.

DT3 inhibits epithelial to mesenchymal transition (EMT) in colon cancer cells

To further explore the effect of DT3 on EMT *in vitro* using non-metastatic (AGS) and metastatic (746T) gastric cancer cells, we used Western blot analyses to examine the expression of epithelial marker (E-cadherin) and mesenchymal marker (vimentin). DT3 increased E-cadherin expression in AGS and 746T cells compared to vehicle control (Figure 4). In contrast, there were profound decreases in the expression of vimentin in both AGS and 746T cells compared to vehicle control (Figure 4).

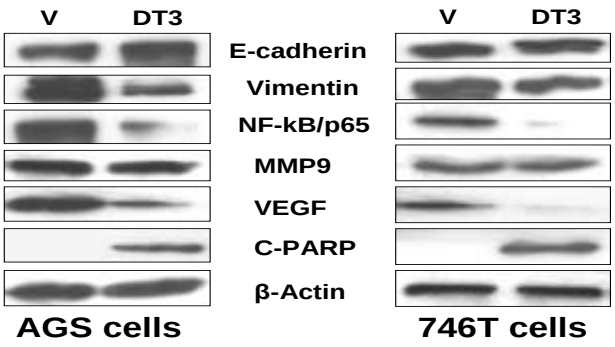

Figure 4: Effects of DT3 on protein expression of the markers of EMT (E-cadherin and Vimentin), inflammation (NF-kB), metastasis (MMP9), angiogenesis (VEGF) and apoptosis (cleaved PARP) in non-metastatic (AGS) and metastatic (746T) gastric cancer cells using Western blot analysis. DT3 at 40 µM concentration depleted vimentin, NF-kB, MMP9 and VEGF protein expression, and increased E-cadherin and cleaved PARP protein expression compared with vehicle (V) in both cell lines. Data are from 3 independent experiments.

DT3 inhibits markers of cancer angiogenesis, inflammation, and metastasis in gastric cancer cells

To investigate the effect of DT3 on angiogenesis, inflammation, and metastasis, we used Western blot analyses to examine the expression of angiogenesis marker vascular endothelial growth factor (VEGF), inflammatory transcription factor (NF-kB/p65) and metastasis marker-matrix metalloproteinase 9 (MMP9). DT3 decreased VEGF, NF-kB/p65 and MMP9 protein expression in gastric cancer cells compared to vehicle control (Figure 4), indicating the potential of DT3 as an antiangiogenic, anti-inflammatory, and antimetastatic agent (Figure 4).

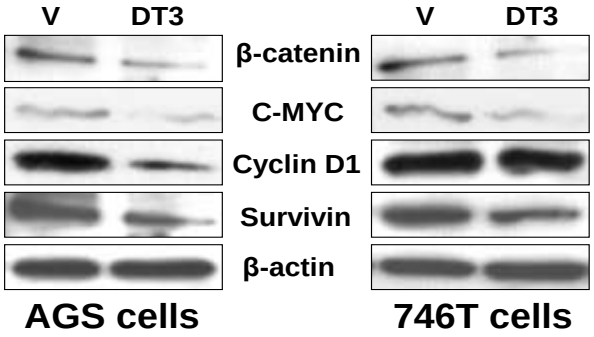

Figure 5: Effects of DT3 on protein expression of β-catenin and its downstream transcriptional targets C-MYC, cyclin D1 and survivin in non-metastatic (AGS) and metastatic (746T) gastric cancer cells using Western blot analysis. DT3 at 40 µM concentration depleted β-catenin, C-MYC, cyclin D1 and survivin protein expression compared with vehicle (V) in both cell lines. Data are from 3 independent experiments.

DT3 inhibits Wnt/β-catenin signaling in gastric cancer cells

Beta-catenin mutational activation is known to be implicated in the gastric carcinogenesis.[27,29] Therefore we investigated the effect of DT3 on Wnt/β-catenin pathway in gastric cancer cells. Interestingly DT3 decreased Wnt pathway protein β-catenin as well as its downstream transcriptional targets C-MYC, cyclin D1 and survivin expression compared to vehicle control (Figure 5).

DISCUSSION

The therapeutic strategies for most gastric cancer patients include surgical resection of the tumor and chemotherapy. Approximately one-third of gastric cancer patients undergo surgical resections relapse with a

recurrence rate as high as 70% in advanced gastric cancer.[4] Chemotherapy provides only modest benefit, with median overall survival of 10 months.[5] Despite therapeutic advances, overall options remain limited due to toxic side effects of the chemotherapy and development of chemo resistance.[7,8] In the present study we used micronutrient DT3 to impede the gastric cancer cell growth and survival. Our data provide the first report of a direct comparison of the effects of 4 isoforms of vitamin E tocotrienol on human gastric cancer cells. Results of this study show that DT3 is most active agent to inhibit the gastric cancer cell (both metastatic and non-metastatic) growth than BT3 and GT3. DT3 also inhibited the malignant transformation (anchorage-independent growth) of gastric cancer cells. Our earlier studies have shown superior antitumor activity of DT3 than BT3 and GT3 in pancreatic cancer both in vitro as well as in vivo.[19] In another study we used AKT inhibitor MK2206 combined with standard chemotherapy agent carboplatin and paclitaxel showed growth inhibition of gastric cancer cells AGS, SNU-1 and SNU-16 in a synergistic manner.[30] Moreover we have shown that DT3 has negligible toxicity both in mice and humans,[21,22] and may be a better agent for clinical use over chemotherapeutic drugs that are known to have side effects.[31] Several studies indicate that inflammatory events contribute in the gastric carcinogenesis,[32-34] Proinflammatory cytokines, cyclooxygenase 2 (COX2) as well as transcription factor NF-κB signaling involved in gastric tumor formation.[35-38] Our results demonstrate that the activity of DT3 in gastric cancer cells related to the inhibition of NF-κB activity. We and others have also reported that tocotrienol inhibited NF-κB activity and the expression of NF-κB–regulated gene products in pancreatic cancer and gastric cancer *in vitro* as well as *in vivo*.[17,19,24] These results strongly suggest that the bioactivity of DT3 against cancer cells is due in part to inhibition of the activity of the inflammatory transcription factor NF-κB.

In gastric cancer patients, relapse and cancer reoccurrence was reported to be as high as 70% in advanced gastric cancer,[4] generally in the form of metastasis.[36,39] Metastasis is a highly organ-specific pathophysiological activity involving multiple steps such as proliferation, angiogenesis, invasion, and extravasation into liver and lung.[40,41] Our data clearly show that DT3 treatment significantly inhibited the migration as well as invasion of gastric cancer cells, likely limiting the process of metastasis *in vivo*. Furthermore, EMT is a program in which epithelial cells are transformed to motile mesenchymal cells.[42] Therefore, induction of EMT can lead to invasion, intravasation, dissemination, and colonization of tumor cells in the liver and lung.[43,44] Our data demonstrated that DT3 treatment inhibited the EMT induction by enhancing the expression of epithelial marker E-cadherin and decreasing the expression of mesenchymal marker vimentin in AGS and 746T cells. EMT also plays an important role during metastatic tumor progression through enhanced angiogenesis.[44,45] Furthermore the down-regulation of E-cadherin and increased activity of MMP9 has been reported in metastatic tumors.[46] Our data show that DT3 prevented the EMT and decreased MMP9 and VEGF expression in the gastric cancer cells further reflect the antiangiogenic activity of DT3. Furthermore Wnt/ β-catenin pathway activation contributes to carcinogenesis in gastric adenocarcinomas.[27-29]

The accumulated cytoplasmic *β*-catenin translocate into the nucleus and interacts with T-cell factor/lymphoid-enhancing factor (TCF/LEF) to activate the downstream target genes (C-MYC, Cyclin D1 and survivin) which are related with cell proliferation, survival, and progression.[39,47,48] Our data demonstrate that DT3 inhibited the growth and survival of gastric cancer cells through depletion of β-catenin and its downstream transcriptional targets C-MYC, cyclin D1 and survivin expression in gastric cancer cells.

Besides uncontrolled growth and proliferation of cancer cells, evasion of programmed cell death (apoptosis) is also one of the hallmarks of cancer.[49] Our data demonstrate that DT3 increased in the percentage of gastric cancer cells showing externalization of phosphatidylserine (PS), a marker for apoptosis detected by FITC-annexin V binding. Apoptotic cell death induced by DT3 is also confirmed by increased cleaved PARP1 protein expression in gastric cancer cells. Earlier reports demonstrated the induction of apoptosis in gastric cancer cells by GT3.[23,26] We report in this study for the first time the induction of apoptosis by DT3 in both metastatic and non-metastatic gastric cancer cells.

In summary, we found the micronutrient DT3 is the most bioactive against gastric cancer cells through inhibition of growth, EMT, migration, invasion, angiogenesis, inflammation, Wnt/ *β*-catenin signaling as well as induction of apoptosis. These data provide a novel anticancer activity of DT3, which may be used for treatment of advanced gastric cancer in the clinic.

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