Chemoprevention of Azoxymethane-Induced Colon Carcinogenesis by Delta-Tocotrienol

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Abstract

This study evaluated the preclinical activity of δ-tocotrienol (DT3), a bioactive form of vitamin E, in the inhibition of colorectal cancer growth and development in vitro and in vivo. DT3 is the most bioactive isomer of vitamin E in inhibiting growth of colorectal cancer cells. However, it had little effect on the proliferation of normal colon mucosal cells NCM460. In HCT-116 and SW-620 colorectal cancer cells, DT3 (50 µM) significantly inhibited malignant transformation ($P < .02, P < .001$), cell migration ($P < .02, P < .05$) and invasion ($P < .05, P < .01$) compared to vehicle. DT3 inhibited markers for epithelial (E-cadherin) to mesenchymal (vimentin) transition, metastasis (matrix metalloproteinase 9), angiogenesis vascular endothelial growth factor (VEGF), inflammation (NF-kB), and Wnt signaling (β-catenin) compared to vehicle in colorectal cancer cells. DT3 induced apoptosis selectively in colorectal cancer cells (SW-620 cells, HCT-116 cells, and HT-29) without affecting the normal colon cells. In the Aoxymethane-induced colorectal carcinogenesis model in rats, DT3 (200 mg/kg orally twice a day) for 20 weeks significantly inhibited colorectal polyps by 70% and colorectal cancer by almost 99% compared to the vehicle treatment group ($P < .02, P < .001$), and the cancer inhibition effect was more potent than sulindac (50%). Taken together, these data demonstrate that DT3 is a potential chemopreventive agent in colorectal cancer, warranting further investigation into its clinical use in the prevention and treatment of colorectal cancer.
Introduction

Colorectal cancer is the third most commonly diagnosed cancer worldwide with over 1 million new cases annually, often found in people aged 50 or older, with a median age at diagnosis of 70 years (1, 2). Furthermore, it is one of the most common causes of cancer-related deaths at over half a million annually (1, 3). The histopathologic and molecular processes underlying the transition from a normal epithelium to an invasive adenocarcinoma “adenoma-carcinoma sequence” is the stepwise accumulation of genetic and epigenetic changes in oncogenes and tumor suppressor genes (4). The loss of function of Wnt components is critically involved in the pathogenesis of colorectal cancer ((5)). Inactivation of the adenomatous polyposis coli (APC) gene or activating mutations of β-catenin is reported in all colorectal cancer patients and is the critical initiating step in malignant transformation (6, 7). The current therapeutic strategies for most colorectal cancer patients include surgical resection of the tumor and chemotherapy. After curative treatment, cancer reoccurs in 60% of all cases, generally in the form of metastasis (8). Moreover, about 30% of patients with colorectal cancers have new polyps at follow-up colonoscopy (9). Chemoprevention has, therefore, emerged as a complementary strategy for patients who are predisposed to colon cancer because of hereditary disease, ulcerative colitis, or family history (10, 11). The most commonly investigated chemopreventive agents are nonsteroidal anti-inflammatory drugs. They have been shown to reduce the incidence of colorectal cancer when used long-term. However, due to cardiotoxicity and gastrointestinal side effects in the average-risk population of predisposed patients, their clinical utility is limited (11, 12).
Thus, novel and safe chemopreventive agents are urgently needed for colorectal cancer patients.

Several studies have suggested that increased intake of dietary fruits, vegetables, and cereal grains may prevent gastrointestinal cancers, including colon cancer (13-18). It has been estimated that dietary changes have the potential to decrease colorectal cancer incidence by 60% to 70% (19), and reduce the risk of colorectal cancer polyp formation by 40% (20). The possible effect of vitamin E in the prevention of colorectal cancer has been studied in animal models and humans (21,22). Tocotrienols, which are found in cereal grains, comprise one of the most compelling groups of antitumor bioactive compounds (23,24). The vitamin E family consists of 2 groups of compounds: 4 forms of tocopherols, α- (AT), β- (BT), δ- (DT), and γ- (GT); and 4 forms of tocotrienols: α- (AT3), β-(BT3), δ- (DT3), and γ- (GT3) (25). We previously demonstrated that DT3 exhibited the most potent antitumor activity among the 4 tocotrienol isoforms in pancreatic cancer, both in vitro and in vivo (26,27). Furthermore, our preclinical and clinical studies in pancreatic cancer showed no obvious toxicity to the host (28,29). However, the study of chemoprevention using DT3 against colorectal cancer is sparse. The current study aimed to investigate the tumor prevention effects of orally administered DT3 in an azoxymethane (AOM)-induced colorectal cancer rat model.

**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 Mediatech, Herndon, VA. Fetal bovine serum (FBS) was purchased from Atlanta Biological, Atlanta, GA. Dulbecco’s modified minimal essential medium (DMEM), RPMI, McCoy 5A, 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. Human colon cancer cell lines HCT-116, HT-29 and SW480, human metastatic colon cancer SW620, and immortalized normal colonic mucosal cells NCM460 were obtained from ATCC, Manassas, VA.

Cell Culture and Growth

Human colon cancer cells lines HCT-116, HT-29 and SW480 and SW620, and immortalized normal colonic mucosal cells NCM460 were obtained from ATCC in 2017. These cell lines are well characterized and tested at regular intervals using RT-PCR and Mycoplasma kit. SW620 and NCM460 cells were grown in RPMI and McCoy 5A media, respectively, whereas HCT-116, SW480, and HT-29 were grown in DMEM and McCoy 5A media, 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% CO₂.

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 AT, BT, GT, and DT (10^{-5}-10^{-4} M) and 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% CO₂. Media were aspirated and 200 µl of dimethyl sulfoxide was 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 HCT-116 and metastatic SW620 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 to 14 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. HCT-116 and SW620 cells were seeded in 6-well plates and cultured to 100% confluence. Wounds were generated in the cell monolayer using small plastic pipette tips. The cells were then rinsed with PBS, treated with DT3 (5 x 10^{-5} M) and cultured for another 24 hours.
The spread of wound closure was observed and photographed under light microscope. For invasion assays, $1 \times 10^6$ cells (HCT-116 and SW620) treated with DT3 ($5 \times 10^{-5}$ 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 or RPMI 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 normal colon cells (NCM460), human colon cancer cells (HCT-116 and HT-29) and metastatic SW620 cells were plated and treated concurrently with vehicle (5% ethanol) or VEDT (50 μM) for 24 hours. Cells were harvested and $10^6$ 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) and 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 (40µg) 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 treated with antibodies to APC, β-catenin, 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 SuperSignal 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).

**Animals and Treatments**

**Rat AOM-induced colon carcinogenesis model**
Female Fisher 344 rats (6 weeks old, 120-140 g) were obtained from Charles River (Wilmington, MA, USA) and kept in the institute’s animal facility for 1 week for quarantine. They were housed in plastic cages and maintained on 12-hour light/dark cycles and provided food and water ad libitum. Rats (n = 40) were injected with a dose of the chemical carcinogen azoxymethane (15 mg/kg, SC) once a week for two weeks to develop colon polyps and cancer. The animals were randomized into 4 groups and treated as follows: 1) untreated, 2) vehicle (oral olive oil), 3) sulindac (20 mg/kg orally) and 4) DT3 (200 mg/kg orally twice a day) for 20 weeks. After 20 weeks, they were sacrificed; colons isolated and examined for evidence of aberrant crypt foci (ACF) and number of polyps and tumors/cancers. Our power analysis for this protocol yielded 10 animals per group for this experiment. Earlier studies used fewer than 10 rats (n = 6, 7, 8 or 10) per group for chemoprevention studies (30-37).

Colons were stained with methylene blue (0.05%) for the gross evidence of ACF and were fixed in buffered formalin for histopathological analysis. The care and use of the animals reported in this study were approved by Institutional Laboratory Animal Care and Use Committee and as per guidelines of the National Institute of Health.

**Histological analyses**

Formalin-fixed, paraffin-embedded colon tissues were sectioned (4 µm) and stained with hematoxylin-eosin. Immunohistochemistry was performed using the Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) per manufacturer’s protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution. Sections were heated for antigen retrieval.
Tissue sections were stained with hematoxylin and eosin (H & E). Detection was performed using the Ventana OmniMap kit. H & E staining was quantified by the Moffitt Anatomic Pathology Core. Colons were stained with methylene blue (0.05%) for detection of aberrant crypt foci (ACF).

**Immunohistochemical staining**

Slides were deparaffinized on the automated system with EZ Prep solution (Ventana Medical Systems, Tucson, AZ). Enzymatic retrieval method was used with Protease 1 (Ventana). The rabbit primary antibody that reacts to APC (#ab40778, Abcam, Cambridge, MA) was used at a 1:400 concentration in Dako antibody diluent (Carpenteria, CA) and incubated for 32 minutes. The Ventana OmniMap Anti-Rabbit Secondary Antibody was used for 16 minutes. The detection system used was the Ventana ChromoMap kit and slides were counterstained with Hematoxylin. Slides were then dehydrated and cover slipped.

**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 $P < .05$. 
Results

Effects of different forms of tocopherols and tocotrienols on colon cancer cell growth

We first analyzed the concentration-dependent response of tocopherols (AT, BT, GT and DT) and tocotrienols (AT3, BT3, GT3 and DT3) on cell proliferation by MTT assay in colon cancer cells (HCT-116). Data show that BT3, GT3, and DT3 significantly inhibited the cell proliferation of HCT-116 cells, but AT3 and tocopherols (AT, BT, GT and DT) had little effect on colon cancer cell growth (Figure 1A). The inhibition of cell proliferation with DT3 was greater than BT3 and GT3. We have earlier reported that DT3 was the most bioactive isomer of vitamin E in pancreatic cancer cells (26). We further analyzed the antiproliferative effect of DT3 on immortalized human normal colon mucosa cells (NCM460), colon cancer cells SW620, HCT-116, HT-29, and SW 480. Interestingly, DT3 significantly inhibited the growth of all colon cancer cells in a concentration-dependent manner, with little effect on the growth of normal colon mucosal cells (Figure 1B). The IC$_{50}$ value of DT3 in all colon cancer cells was 50 to 60 μM. We further analyzed the effects of DT3 (50 μM) on anchorage-independent growth of HCT-116 and SW620 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 in SW620 cells (ap < .001) than in HCT-116 cells (bp < .02), indicating the inhibition of colon cancer malignant transformation.

DT3 induces apoptosis in colon cancer cells but not in normal cells
To investigate the effects of DT3 on cell survival, colon cancer cells HCT-116, HT-29, and SW-620 and normal colon cells NCM460 were treated with DT3 (50 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 SW-620 cells (44%) than in HCT-116 (13%) and HT-29 (15%) compared to vehicle (Figure 2). Interestingly, DT3 did not induce apoptosis in normal colon cells NCM460. Western blot analysis further confirmed the apoptotic activity of DT3 with increased PARP1 cleavage in both SW-620 and HCT-116 colon cancer cells (Figure 4).

**DT3 inhibits migration and invasion of colon cancer cells**

We further investigated whether DT3 could also inhibit cell migration and invasion in SW620 and HCT-116 cells. Using the wound-healing assay, we found that DT3 significantly ($a p < .02; b p < .05$) suppressed tumor cell mobility in both cells compared with their corresponding vehicle controls (Figures 3A and B). Similarly, invasion assay with Matrigel demonstrated that DT3 significantly decreased the invasive capacity of both cells compared to vehicle (Figures 3C and D), but the inhibition of invasion was greater in SW620 cells ($a p < .01$) than in HCT-116 cells ($b p < .05$). Taken together, these results suggest that DT3 can suppress the metastatic processes of colon cancer.

**DT3 inhibits epithelial to mesenchymal transition in colon cancer cells**

To further explore the effect of DT3 on epithelial to mesenchymal transition (EMT) in vitro using SW620 and HCT-116 colon 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 SW620 and HCT-116 cells.
compared to vehicle control (Figure 4). In contrast, there were profound decreases in the expression of vimentin in both SW-620 and HCT-116 cells compared to vehicle control (Figure 4).

**DT3 inhibits markers of cancer angiogenesis, inflammation, metastasis, and β-catenin expression in colon cancer cells**

To investigate the effect of DT3 on angiogenesis, inflammation, and metastasis in vitro using both SW-620 and HCT-116 cells, we used Western blot analyses to examine the expression of angiogenesis marker (VEGF), inflammatory transcription factor (NF-kB/p65), metastasis marker-matrix MMP9, and Wnt pathway proteins (APC and β-catenin). DT3 decreased VEGF, NF-kB/p65, and MMP9 protein expression in SW-620 and HCT-116 colon cancer cells compared to vehicle control (Figure 4), indicating the potential of DT3 as an antiangiogenic, anti-inflammatory, and antimetastatic agent. Of note, DT3 also decreased the β-catenin expression without affecting the levels of APC compared to vehicle control (Figure 4). Beta-catenin mutational activation is known to be implicated in the colon carcinogenesis ((38)).

**DT3 prevents colon polyps/premalignant lesions/tumors formation in AOM-carcinogenesis model in rats**

To further determine the effect of DT3 on chemoprevention of colon polyps and cancer formation in vivo, AOM carcinogenesis rat models were treated with DT3, vehicle, or anti-inflammatory drug sulindac for 20 weeks. The results showed that sulindac treatment significantly inhibited polyp formation (78%, *P < .001) better than tumor
formation (50%, **P < .02), and DT3 almost completely inhibited tumor formation (99%, *P < .001) but had less effect on polyp formation (70%, **P < .02) in the colon of AOM rats (Figures 5 and 6A). Our data show slightly better polyp prevention by sulindac and significantly better cancer prevention by DT3. We hypothesize that sulindac and DT3 protect against AOM-induced cancer formation by different mechanisms. Although sulindac and DT3 have been shown to have multiple mechanisms of action, sulindac primarily targets inflammatory pathways, whereas DT3 primarily induces neoplastic cell apoptosis. It is possible that DT3 is more effective at targeting the cancer-forming pathways that might develop, without going through the classic adenoma-carcinoma sequence. The histopathological data showed adenocarcinoma plus signet ring (9+1) in the untreated (NT) group, adenocarcinoma plus signet ring (8+2) in the vehicle-treated group, adenocarcinoma plus signet ring (4+2) in the sulindac-treated group, and signet ring hyperplasia and normal mucosa in the DT3-treated group (Figure 5B, Supplementary Table S1, and Supplementary Figures S1, S2, S3, and S4). The histopathological data further revealed that AOM rats in the NT group showed adenoma plus signet ring (9+1); vehicle treatment showed evidence of adenoma (10) in the vehicle-treated group (Figure 6B, Supplementary Table S1, Supplementary Figure S5). The aberrant crypt foci (ACF), the earliest stage of development of pre-malignant lesions were seen in vehicle-treated, NT, and sulindac-treated groups (Figure 6B, Supplementary Figures S5), whereas AOM rats treated with DT3 had normal colonic mucosa (Figure 6B). Furthermore, Methylene blue and APC staining for ACF were observed in vehicle, NT, and sulindac-treated rats, but no ACF was observed in DT3-treated rat colons (Supplementary Figures S5). We did not quantify the ACF in each
group because our study endpoint was to quantify the number of adenomas/polyps and cancers/tumors in each group of rats. These data provide the potential of DT3 in the chemoprevention of colon cancer formation.

Discussion

The chemoprevention strategies for most of the colorectal cancer patients include using nonsteroidal anti-inflammatory drugs. Although long-term use of these agents has been shown to reduce the incidence of colorectal cancer, their use is limited due to cardiotoxicity and gastrointestinal side effects (11,12). Therefore, we evaluated the inhibitory activity of different forms of tocopherols and tocotrienols in colorectal cancer cells. Our results clearly demonstrate that these vitamin E forms have different abilities to inhibit colon cancer growth and survival. Tocotrienols (BT3, GT3, and DT3) show significant inhibition of colorectal cancer cell growth, whereas AT3 and tocopherols (AT, BT, GT and DT) show little effect on cancer cell growth (22). Interestingly DT3 was the most active isomer to inhibit colon cancer cell growth without affecting the growth of human normal colon mucosal cells. Our data provide the first report of a direct comparison of the effects of 8 forms of vitamin E on human colon cancer cells. The present data are consistent with our earlier studies demonstrating the inhibition of pancreatic cancer growth and survival in vitro and in vivo with tocotrienols ((26,27,39,40)). Our results agree with other investigators’ who reported DT3 inhibited the proliferation of colorectal cancer cells in vitro (41-45). This is also the first report to show that DT3 inhibits the formation of colorectal polyps of adenoma and carcinoma in a living animal model. Interestingly, our data further show that DT3 is superior to sulindac in inhibiting colorectal cancer carcinogenesis. sulindac, an anti-inflammatory
drug, is known to inhibit colorectal polyp and colorectal cancer formation (46). The results suggest that DT3, with negligible toxicity (28,29), may be a better agent for clinical use over anti-inflammatory drugs that are known to have side effects. Several studies indicate that inflammatory events contribute in the colorectal carcinogenesis and inflammatory transcription factor NF-κB signaling involved in colorectal cancer formation (47-50). Other reports also show that colon cancer cell lines have abnormally high NF-κB activity and low IκB levels (47,51,52), suggesting that dysregulation of NF-κB contributes to colon carcinogenesis. Our results demonstrate that the activity of DT3 in colorectal 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 in vitro as well as in vivo (27,53). These results strongly suggest that the bioactivity of tocotrienols against cancer cells is due in part to inhibition of the activity of the inflammatory transcription factor NF-κB.

In colorectal cancer patients, cancer reoccurrence was reported to be over 60% after curative treatment, generally in the form of metastasis (8). Metastasis is a highly organ-specific pathophysiological activity involving multiple steps such as proliferation, angiogenesis, invasion, and extravasation into liver and lung (54,55). Our data clearly show that DT3 treatment significantly inhibited the migration as well as invasion of colorectal cancer cells (HCT-116 and SW-620), likely limiting the process of metastasis in vivo. Furthermore, EMT is a program in which epithelial cells are transformed to motile mesenchymal cells (56). Therefore, induction of EMT can lead to invasion, intravasation, dissemination, and colonization of tumor cells in the liver and lung (57,58). 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 HCT-116 and SW-620 cells. EMT also plays an important role during metastatic tumor progression through enhanced angiogenesis (58,59). Furthermore the down-regulation of E-cadherin and increased activity of MMP9 has been reported in metastatic tumors (60). Our data show that DT3 prevented the EMT and decreased MMP9 and VEGF expression in the colorectal cancer cells further reflect the antiangiogenic activity of DT3.

Besides uncontrolled growth and proliferation of cancer cells, evasion of programmed cell death (apoptosis) is also one of the hallmarks of cancer (61). Our in vitro results demonstrate that DT3 increased in the percentage of colon cancer cells showing externalization of phosphatidylserine (PS), a marker for apoptosis detected by FITC-annexin V binding without affecting the normal colon mucosal cells NCM460. Apoptotic cell death induced by DT3 is also confirmed by and increased cleaved PARP1 protein expression in colon cancer cells. Our results agree with other investigators who reported DT3 induced cell death including apoptosis in colon cancer cells (41,45). Zhang et al (45) reported that cell death caused by DT3 in colorectal cancer cells was associated with inhibition of Wnt/β-catenin expression. Our data also demonstrated that DT3-induced apoptosis is associated with depletion of β-catenin and VEGF in colon cancer cells confirming the down regulation of Wnt pathway by DT3 in colorectal cancer.

In summary, we found the natural vitamin E isomer DT3 is the most bioactive against colorectal cancer through inhibition of EMT, migration, invasion, angiogenesis, inflammation as well as induction of apoptosis in vitro and inhibition of precancerous
and cancerous lesions in vivo. These data provide a novel chemopreventive activity of DT3, which may be used for treatment of colorectal cancer in the clinic.
Acknowledgments

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References


Figure Legends

Figure 1.

Inhibition of colorectal cancer cell proliferation with DT3 (A) Effects of α-tocopherol (AT), β-tocopherol (BT), δ-tocopherol (DT), and γ-tocopherol (GT), α-tocotrienol (AT3), β-tocotrienol (BT3), δ-tocotrienol (DT3), and γ-tocotrienol (GT3) on colon cancer cell growth (MTT assay). BT3, DT3, and GT3 significantly inhibited the proliferation of colorectal cancer HCT-116 cells at concentrations of 10 to 100 μM. In contrast, no significant effect was observed with AT3-, AT-, BT-, GT-, and DT-treated cells at concentrations of 10 to 100 μM. Points, means; bars, standard errors (SEs; n = 3-5, *p < .001, **p < .05). (B) Effects of DT3 on immortalized human normal colon mucosa, metastatic, and other colon cancer cell proliferation (MTT assay). DT3 significantly decreased the proliferation of colorectal cancer (SW620, HCT-116, HT-29, and SW480) cells in a concentration-dependent manner without a significant effect on the growth of normal colon mucosa cells (NCM460). Points, means; bars, SE (n = 3-5, ap < .001, bp < .05). All statistical analyses were performed using ANOVA with Duncan multiple range test. (C) Effects of DT3 on malignant transformation assay in SW-620 and HCT-116 cells’ anchorage-independent cell growth (soft agar colony formation assay) were investigated for 14 days. (D) Effects of DT3 on number of colon cancer malignant colonies in SW-620 and HCT-116 cells. DT3 at 50 μM significantly inhibited the malignant transformation of colorectal cancer SW-620 and HCT-116 cells (ap <.001 and bP < .02). Bars, SE (n = 3). All statistical analyses were performed using ANOVA with Duncan multiple range test.

Figure 2.

Effects of δ-tocotrienol (DT3) on apoptosis (Annexin V/PI staining) in colorectal cancer cells and immortalized human normal colon mucosa cells. DT3 at 50 μM concentration for 24 hours increased apoptosis in SW-620 cells (44%), HCT-116 cells (13%), and HT-29 cells (15%).
compared to vehicle. DT3 did not induce apoptosis in normal colon cells NCM460 compared with vehicle. Data are from 3 independent experiments.

Figure 3.
Effects of DT3 on colorectal cancer cell migration
(A) Effects of δ-tocotrienol (DT3) on cell migration of colorectal cancer SW620 and HCT-116 cells using wound-healing assay. DT3 at 50 μM concentration significantly suppressed tumor cell mobility in both cells compared with their corresponding vehicle controls (a < .02 and b < .05). Bars, SE (n = 3). (B) Effects of DT3 on invasion assay with Matrigel of colorectal cancer SW620 and HCT-116 cells. DT3 at 50 μM concentration significantly decreased the invasive capacity of SW620 cells (a < .01) and HCT-116 cells (b < .05). Bars, SE (n = 3).

Figure 4.
Effects of DT3 on protein expression of APC, β-catenin, and markers of EMT (E-cadherin and Vimentin), inflammation (NF-kB), metastasis (MMP9), angiogenesis (VEGF) and apoptosis (cleaved PARP) in colorectal cancer SW620 and HCT-116 cells using Western blot analysis. DT3 at 50 μM concentration depleted β-catenin, vimentin, NF-kB, MMP9 and VEGF protein expression, and increased E-cadherin and cleaved PARP protein expression, without affecting acyl carrier protein expression compared with vehicle in both cell lines. Data are from 3 independent experiments.

Figure 5.
DT3 prevents colon polyps/premalignant lesions/tumors formation in AOM-carcinogenesis model in rats (A) Effects of δ-tocotrienol (DT3) (200 mg/kg orally twice a day) and an anti-inflammatory drug Sulindac (20 mg/kg orally) for 20 weeks on chemoprevention of colon polyps in AOM carcinogenesis model of Fisher 344 rats. DT3 significantly inhibited polyp formation (**p
< .02) in the colon of AOM rat compared to no treatment or vehicle group. Whereas, Sulindac significantly inhibited polyp formation (*p < .001) in the colon of AOM rat compared to no treatment or vehicle group. Data: means; bars, standard errors (SEs; n = 10). (B) Effects of DT3 (200 mg/kg orally twice a day) and an anti-inflammatory drug Sulindac (20 mg/kg orally) for 20 weeks on chemoprevention of colorectal cancer in colon AOM carcinogenesis model of Fisher 344 rats. The histopathological data show invasive cancer in untreated group (NT), invasive cancer singlet ring type in vehicle-treated group (V), normal to adenoma in Sulindac-treated group (Sulindac), and hyperplasia and normal mucosa in DT3 treated group (DT3). H & E staining of colon (10 X). H & E - hematoxylin and eosin.

Figure 6.
Effects of DT3 and Sulindac (A) Effects of DT3 (200 mg/kg orally twice a day) and an anti-inflammatory drug Sulindac (20 mg/kg orally) for 20 weeks on AOM-induced carcinogenesis in Fisher 344 rats. DT3 significantly inhibited tumor formation (*p < .001) in the colon of AOM rats compared to no treatment or vehicle group, and Sulindac had a significant but weaker activity (**p < .02). Data: means; bars, standard errors (SE; n = 10). (B) Effects of DT3 (200 mg/kg orally twice a day) and an anti-inflammatory drug Sulindac (20 mg/kg, orally) for 20 weeks on chemoprevention of colon premalignant ACF and carcinoma in colon AOM carcinogenesis model of Fisher 344 rats. The histopathological data show that AOM rats untreated (NT) or treated with vehicle only (V) show evidence of a very rare signet ring carcinoma invading the colonic submucosa (arrows). The ACF, the earliest stage of development of premalignant lesions, were seen in the vehicle only (V) and Sulindac treated animals (arrows). Whereas, DT3-treated rats had completely normal colonic mucosa. H & E staining of colon (10 X). H & E - hematoxylin and eosin.
Figure 1.

A. Graph showing the percentage of viable cells vs DT3 concentration (μM) for different cell lines (AT, BT, GT, DT, AT3, BT3, GT3, DT3).

B. Graph showing the percentage of viable cells vs DT3 concentration (μM) for different cell lines (NCM-460, HCT-116, SW-620, SW-480, HT-29).

C. Images of cell colonies for HCT-116 and SW-620 under vehicle and DT3 conditions.

D. Bar chart showing the number of colonies for HCT-116 and SW-620 under vehicle and DT3 conditions.
Figure 2.
Figure 3.

A

HCT-116

SW-620

0 h

24 h

Vehicle

DT3

Vehicle

DT3

B

Percent wound closure (%)

ap<0.02 vs Vehicle
bp<0.05 vs Vehicle

HCT-116

SW-620

Vehicle

DT3

Vehicle

DT3

C

HCT-116

Vehicle

DT3

SW-620

D

Number of invading cells

ap<0.01 vs Vehicle
bp<0.05 vs Vehicle

HCT-116

SW.620

Vehicle

DT3

Vehicle

DT3
Figure 4.

<table>
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<th>Vehicle</th>
<th>DT3</th>
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HCT-116   SW-620
Figure 5.
Figure 6.
Chemoprevention of Azoxymethane-Induced Colon Carcinogenesis by Delta-Tocotrienol

Kazim Husain, Anying Zhang, Steven C Shivers, et al.

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