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δ-Tocotrienol treatment is more effective against hypoxic tumor cells than normoxic cells: potential implications for cancer therapy

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RUNNING TITLE: Tocotrienols potently prevent cancer growth
**Abbreviations:** Akt, protein kinase B; CDK4, cyclin dependent kinase 4; DLD-1, human colorectal adenocarcinoma cell line; H&E, hematoxylin and eosin; HIF-1α, hypoxia inducible factor-1α; NIH, National Institutes of Health; PBS, phosphate-buffered saline; PECAM-1, platelet endothelial cell adhesion molecule-1; RBT3, rice bran tocotrienols; RT-PCR, reverse transcription-PCR; T3, tocotrienol; Toc, tocopherol; VEGF, vascular endothelial growth factor; WST-1, water-soluble tetrazolium salt.
Abstract

Tocotrienols, unsaturated forms of vitamin E, inhibit the proliferation of a variety of cancer cells and suppress angiogenesis. However, the mechanisms underlying those effects on cancer cell growth remain unclear especially under hypoxic conditions. In this study, we demonstrated that δ-tocotrienol (δ-T3) could be used as a novel anti-cancer agent against human colorectal adenocarcinoma (DLD-1) cells under both normoxic and hypoxic conditions. δ-T3 inhibited the growth of DLD-1 cells in a dose-dependent fashion by inducing cell cycle arrest and apoptosis. This effect was more potent under hypoxic than normoxic conditions. The anti-cancer effect of δ-T3 was achieved by its upregulation of cyclin dependent kinase inhibitors (p21 and p27), the activation of caspases and the suppression of phosphorylation of protein kinase B (Akt) at Thr\textsuperscript{308} and Ser\textsuperscript{473}. In \textit{in vivo} studies, oral administration of rice bran tocotrienols (RBT3, mainly γ-T3) (ten mg/mouse/day) significantly inhibited tumor growth in nude mice. In tumor analyses, RBT3 activated p21, p27, caspase-3, and caspase-9 and decreased Akt phosphorylation. Furthermore, immunostaining revealed that RBT3 decreased the number of cells positive for CD31/platelet endothelial cell adhesion molecule-1 in microvessels in the tumor. Taken together, these data suggest that tocotrienols are potent anti-tumor agents capable of inducing apoptosis and inhibiting angiogenesis under both hypoxic and normoxic conditions. Tocotrienols could have significant therapeutic potential in the clinical treatment of tumors.

\textit{Key words}: angiogenesis; tocotrienol; tocopherol; normoxia; hypoxia

Conflicts of interest: None.
1. Introduction

Vitamin E occurs naturally in eight different forms: α-, β-, γ- and δ-congeners of both tocopherol (Toc) and tocotrienol (T3). The two differ structurally in that tocopherols have a saturated phytlyl side chain attached to its chroman ring, whereas tocotrienols possess an unsaturated isoprenoid side chain. Tocotrienols have recently gained increasing scientific interest due to their health-related biological properties, including their anti-cholesterolemic [1], anti-hypertensive [2], neuroprotective [3], immunomodulatory [4] and anti-cancer properties [5] that differ somewhat from those of tocopherols. Tocotrienols, which are naturally present in rice bran and palm oil, have anti-cancer properties with low toxicity both in vitro and in vivo [6 - 9]. For example, T3, especially the δ- and γ-congener, effectively suppressed the proliferation of colorectal adenocarcinoma cells, prostate cancer, and hepatoma cells in vitro [10 - 12]. Furthermore, γ-T3 showed potent apoptotic activity in preneoplastic, neoplastic, and highly malignant mouse mammary epithelial cells. Importantly, at the same dose, γ-T3 had almost no adverse effects on the growth or function of normal cells [13]. The molecular mechanisms underlying the anti-cancer activity of δ- and γ-T3 include activation of caspases [11], inhibition of nuclear factor-κB [14,15] and telomerase [10], downregulation of c-Myc [10] and modulation of the Bax/Bcl-2 ratio [6]. Furthermore, δ- and γ-T3 are potent inhibitors of several protein tyrosine kinases, including epidermal growth factor receptor [13], vascular endothelial growth factor receptor-2 [16] and extracellular signal-regulated kinase 1/2 that are involved in the development and progression of cancer [17]. In a rat tumor model, tocotrienols reduced the severity and extent of neoplastic transformation in 2-acetylaminofluorene-induced hepatocarcinogenesis [9]. Dietary supplementation of tocotrienols-rich oil inhibited the
growth of human breast cancer MCF-7 tumors implanted in athymic nude mice [18]. Data from a clinical trial suggested that δ-T3 in combination with tamoxifen may have potential against breast cancer-related survival. Consequently, several clinical trials of tocotrienols are being conducted with the expectation that they will have therapeutic potential against cancer cell growth [5].

Most solid human tumors contain hypoxic regions due to poor microcirculation within the tumor mass [19]. Although hypoxia is toxic to both tumor cells and normal cells, tumor cells undergo adaptive changes that allow them to survive and proliferate in hypoxic environments, enabling aggressive tumor behavior [20]. These changes include transcriptional induction of genes involved in glycolysis, hematopoiesis, angiogenesis, apoptosis, and tissue invasion [21]. In several types of tumors (e.g., mammary carcinoma and glioma), tumor hypoxia is highly correlated with the expression of an angiogenic transcriptional factor, hypoxia inducible factor-1α (HIF-1α) [22], which in turn stimulates tumor progression. Therefore, inhibition of hypoxic adaptation (e.g., HIF-1α expression) in tumor cells might be an effective strategy for controlling malignant tumor growth [23].

We have previously shown that δ-T3 inhibited the expression of HIF-1α in cancer cells and suppressed cancer cell proliferation under hypoxic conditions [24]. Similarly, a T3 analogue reportedly suppressed survival and invasion of tumor cells under hypoxia [25]. Thus, these studies suggest a novel mechanism by which T3 achieves its anti-cancer effects, i.e., a reduction of tumor cell adaptation to hypoxia. However, to the best of our knowledge, few studies have analyzed the effect of tocotrienols under hypoxia [24, 26]. Also, the anti-tumor effects of tocotrienols in an animal model are not well understood. Therefore, in this study, we aimed to evaluate the anti-cancer effects and mechanisms of T3 (mainly δ- and γ-T3) in human colorectal adenocarcinoma cells
(DLD-1) especially under hypoxic conditions and in a nude mouse model.

2. Materials and Methods

2.1. Reagents

δ-T3 was purchased from Chromadex (Santa Ana, CA). Water-soluble tetrazolium salt (WST-1) reagent was obtained from Dojindo Laboratories (Kumamoto, Japan). Rice bran tocotrienols (RBT3) was kindly provided by Sanwa Yushi Co., Ltd (Yamagata, Japan). RBT3 was composed of 97.5% tocotrienols (3.5% α-T3, 89.9% γ-T3, and 4.1% δ-T3) and 2.1% tocopherols (1.4% γ-Toc and 0.7% δ-Toc) (wt/wt). All other reagents used in this study were of analytical grade.

2.2. Cell cultures

DLD-1 cells were obtained from the Cell Resource Center for Biomedical Research at Tohoku University School of Medicine (Sendai, Japan). The cells were cultured in RPMI-1640 (containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Dainippon Sumitomo Pharmaceutical, Osaka, Japan), 100 kU/L penicillin (GIBCO, BRL, Rockville, MD), and 100 mg/L streptomycin (GIBCO). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Hypoxia (< 1% O₂) was achieved using an anaerobic jar (AnaeroPack Series, Mitsubishi Gas Chemical Co., Tokyo, Japan) equipped with an AnaeroPack, disposable O₂-absorbing and CO₂-generating agent.
2.3. Preparation of test medium

δ-T3 was dissolved in ethanol at a concentration of 20 mM. The stock solution was diluted with serum-free UltraCulture medium (Cambrex, Walkersville, MD) to achieve the desired final concentration (0 - 20 μM). The final concentration of ethanol in the test medium was less than 0.1% (v/v), which did not affect cell viability. Medium with ethanol alone was similarly prepared and used as the control medium.

2.4. Proliferation assays

Cell proliferation was evaluated by use of the WST-1 assay [27]. DLD-1 cells were plated at 5 × 10^3 cells/well in 96-well culture plates and pre-incubated in growth medium (RPMI-1640 medium containing 10% FBS). After 24 h, the medium was replaced with the test medium (UltraCulture medium with 0 - 20 μM δ-T3). After incubation for 24 - 48 h under hypoxic or normoxic conditions, cell viability was determined using WST-1 reagent according to the manufacturer’s instructions.

2.5. Hoechst staining

Nuclear morphological changes were evaluated using Hoecht 33258 (Sigma). DLD-1 cells (5 × 10^5 cells) were plated in 60-mm dishes and pre-incubated in growth medium for 24 h. After the medium was replaced with the test medium, the cells were further incubated for 24 - 48 h under hypoxia or normoxia. The cells were fixed with 4% paraformaldehyde (Wako, Osaka, Japan), rinsed with phosphate-buffered saline
(PBS) and stained with Hoechst 33258 (0.1 g/L PBS). Apoptotic nuclear morphology was observed under UV illumination with an IX-FLA fluorescence microscope (Olympus, Tokyo, Japan).

2.6. Flow cytometric analysis for cell cycle distribution

DLD-1 cells (5 × 10^5 cells) were plated in 60-mm dishes and pre-incubated in growth medium for 24 h. After replacement with test medium, the cells were further incubated for 24 - 48 h under hypoxic or normoxic conditions and then collected by trypsinization. Cells were washed with PBS, fixed with 70% ice-cold ethanol overnight at 4°C and stained with one mL of propidium iodide/RNase solution (Becton Dickinson, Bedford, MA) for 30 min at 37°C in the dark. The cell cycle distribution was analyzed by flow cytometry (Becton Dickinson). Percentages of cells in different phases of the cell cycle were determined by ModFit LT cell cycle analysis software (Becton Dickinson).

2.7. Total RNA isolation and mRNA analysis

DLD-1 cells (5 × 10^5 cells) were plated in 60-mm dishes and pre-incubated in growth medium for 24 h. The cells were further incubated for 12 - 48 h under hypoxia or normoxia. Total RNA was isolated with RNeasy plus Mini kit (Qiagen, Valencia, CA) for quantitative reverse transcription-PCR (RT-PCR). Then, cDNA was synthesized using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare, Piscataway, NJ). Quantitative RT-PCR amplification was performed with a DNA Engine Opticon 2 system (MJ Research, Waltham, MA) using SYBR Premix Ex Taq II (Takara Bio, Otsu,
Japan) and gene-specific primers for p21 (Cdkn1a), p27 (Cdkn1b), caspase-3, caspase-9, vascular endothelial growth factor A (Vegfa) and β-actin (Actb) (Table 1). PCR conditions for these primers were 95°C for one min, 95°C for five s and 60°C for 20 s over 40 cycles.

2.8. Western blot analysis

DLD-1 cells (1 × 10^6 cells) were plated in 100-mm dishes and pre-incubated in growth medium for 24 h. After replacement of the medium with test medium, the cells were further incubated for 8 - 48 h under hypoxic or normoxic conditions. Cellular proteins (80 μg/well) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 - 20% e-PAGE; Atto, Tokyo, Japan) and protein bands were then transferred to polyvinylidene fluoride membranes (GE Healthcare). Following blocking, the membranes were incubated with primary antibodies targeted against HIF-1α (Becton Dickinson), p21 (Upstate Biotechnology, Lake Placid, NY), p27 (Medical Biological Laboratories, Nagoya, Japan), cyclin dependent kinase 4 (CDK4), cleaved caspase-3, cleaved caspase-9, phospho-protein kinase B (Akt) (Thr^{308}), phospho-Akt (Ser^{473}), Akt and β-actin (Cell signaling Technology, Beverly, MA), followed by horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). ECL Plus reagents (GE Healthcare) were used for detection. Western blot band intensities were calculated by densitometric analyses with National Institutes of Health (NIH) image software. Representative pictures are shown with average densitometric data.

2.9. Ethics statement
The animal study was performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, which was granted by the Tohoku University Ethics Review Board (2007-Noudou-32) and the Guide for Care and Use of Laboratory Animals published by the U.S. NIH (NIH publication 85-23, revised 1996). All animals were anesthetized to minimize their suffering.

2.10. Animal treatment

Male athymic nude mice (BALB/cA Jcl-nu nu/nu, four weeks of age) were obtained from CLEA (Tokyo, Japan) and maintained in a clean, pathogen-free environment. The mice were caged individually, housed in a temperature- (23°C) and humidity-controlled room with a 12 h light/dark cycle and acclimatized for one week. A DLD-1 cell suspension (5 × 10^6 cells in 100 µL of serum- and phenol red-free RPMI-1640 medium (Gibco)) was injected into the dorsal region of each mouse using a 21 gauge needle. Two days after tumor cell inoculation, mice were randomly divided into two groups. For the T3 group (n = 10), each mouse received 10.3 mg/day RBT3 (0.36 mg/day α-T3, 9.22 mg/day γ-T3, 0.42 mg/day δ-T3, 0.14 mg/day γ-Toc, and 0.07 mg/day δ-Toc) dissolved in 50 mg vitamin E-stripped corn oil (MP Biomedicals Inc., Santa Ana, CA) once a day for a total of 21 successive days. For the control group (n = 10), each mouse received only vehicle (50 mg vitamin E-stripped corn oil). RBT3 dissolved in vitamin E-stripped corn oil and vehicle (vitamin E-stripped corn oil) were administered by gastric intubation. During the treatment period, the mice were allowed free access to basal diet containing a minimal required amount of α-Toc (15 mg/kg diet) and distilled water. After the treatment period, the mice were anesthetized, and blood
was drawn from the abdominal vena cava using a heparinized syringe. The tumors were excised, weighed, and divided into three pieces. One piece was snap-frozen in liquid nitrogen and stored at -80°C for vitamin E and Western blot analysis. The second piece was treated with RNA-later (Ambion, Austin, TX) and stored at -80°C for mRNA analysis. The third piece was fixed with a solution of 4% paraformaldehyde in PBS for immunohistochemical staining.

2.11. Vitamin E analysis

For vitamin E analysis, plasma (0.05 mL) was mixed with 1 mL of 6% ethanolic pyrogallol, 1 mL of 1 μM ethanolic 2,2,5,7,8-pentamethyl-6-hydroxychroman (internal standard), and 0.95 mL of 0.9% NaCl aqueous solution. The mixture was extracted with 5 mL of hexane/ethyl acetate (9:1). After centrifugation, the upper layer was collected. The extraction with hexane/ethyl acetate (9:1) was then repeated. The upper layers were combined and dried. The residue was dissolved in hexane, and a portion of the extract was subjected to fluorescence-high performance liquid chromatography as described previously [28]. Separation was performed at 35°C using a silica column (ZORBAX Rx-SIL, 4.6 × 250 mm; Agilent, Palo Alto, CA). A mixture of hexane/1,4-dioxane/2-propanol (988:10:2) was used as a mobile phase at a flow rate of 1.0 mL/min. Vitamin E congeners were detected by an RF-10AXL fluorescence detector (excitation 294 nm, emission 326 nm; Shimadzu, Kyoto, Japan). The concentrations of vitamin E congeners were calculated against calibration curves for standard vitamin E congeners, and then corrected by using the peak area ratios of the vitamin E congeners to 2,2,5,7,8-pentamethyl-6-hydroxychroman (internal standard). For tumor analysis, tumor tissues (50 mg) were homogenized with 0.5 mL of 0.9% NaCl aqueous solution.
To the homogenate (0.5 mL), 1 mL of 6% ethanolic pyrogallol, 1 mL of 1 μM ethanolic 2,2,5,7,8-pentamethyl-6-hydroxychroman, and 0.2 mL of 60% KOH aqueous solution were added and mixed. The resulting mixture was incubated at 70°C for 30 min. The saponified solution was cooled, and 2.5 mL of 0.9% NaCl aqueous solution was added. The mixture was extracted with hexane/ethyl acetate (9:1), and vitamin E congeners in the extract were determined by fluorescence-high performance liquid chromatography as described above.

2.12. mRNA and protein expression analysis

For mRNA analysis, total RNA was extracted from tumor tissues (30 mg), and mRNA expression was evaluated by quantitative RT-PCR as described above. For protein expression, tumor tissues (100 mg) were disrupted in lysis buffer by using a Teflon homogenizer. Extracted proteins were subjected to Western blot analysis as described above.

2.13. Immunohistochemical staining

For immunohistochemical analysis, five μm-thick sections from tumor embedded in paraffin were stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed as previously described [29]. Primary antibodies used were anti-phospho Akt (Ser473) (Cell Signaling Technology) and anti-CD31/platelet endothelial cell adhesion molecule-1 (PECAM-1) (Spring Bioscience, Pleasanton, CA).

2.14. Statistical analysis
3. Results

3.1. δ-T3 inhibited proliferation and induced apoptosis in DLD-1 cells, especially under hypoxic conditions

To clarify the differences between the effects of δ-T3 under hypoxic and normoxic conditions, proliferation assays were first conducted using DLD-1 cells. δ-T3 inhibited cell proliferation in a dose-dependent manner (Fig. 1). At low to medium concentrations, the inhibitory effect was greater under hypoxic conditions. With Hoechst staining, condensed chromatin and formation of apoptotic small bodies were observed in δ-T3-treated cells (Supplemental Fig. 1). Treatment of DLD-1 cells with δ-T3 for 48 h induced apoptosis in a dose-dependent fashion. Similar to the anti-proliferative effect, apoptotic activity was also greater under hypoxic conditions.

To investigate the mechanism by which cell proliferation was inhibited, we examined the effect of δ-T3 on the cell cycle. After a 24 h treatment, δ-T3 caused DLD-1 cells to accumulate in the G1 phase, with decreased proportions of S and G2 phase cells (Fig. 2). After a 48 h incubation, δ-T3 increased the frequency of apoptotic cells (sub-G1 phase) in a dose-dependent manner. When the highest dose of δ-T3 (20 μM) was used, sub-G1 cells increased more under hypoxia (44.4 ± 2.9%) than normoxia
3.2. δ-T3-mediated changes in cell cycle arrest and apoptotic pathways were greater when DLD-1 cells were treated under hypoxic condition

The results of quantitative RT-PCR showed that δ-T3, especially at 20 μM, upregulated mRNA expression of cell cycle arrest genes (p21/Cdkn1a) and p27/Cdkn1b and proapoptotic genes (caspase-3 and caspase-9), except for caspase-9 after 24 h normoxic incubation conditions (Fig. 3). Similarly, δ-T3 increased p21, p27 proteins, and cleaved caspase proteins (Fig. 4). The increase of CDK4 and phosphorylation of Akt (Thr\textsuperscript{308} and Ser\textsuperscript{473}) was suppressed by δ-T3. In concordance with the suppression of cell proliferation and the induction of apoptosis (Figs. 1 and 2), greater changes in the expression of genes and in the proteins involved in cell cycle arrest and apoptosis were observed under hypoxia than normoxia. This could be related to strong induction of HIF-1α protein under hypoxic conditions, and its increment could be suppressed by δ-T3 treatment (Fig. 4).

3.3. RBT3 supplementation inhibited tumor growth after implantation of DLD-1 cells into nude mice

To investigate the effects of T3 \textit{in vivo}, we orally administered RBT3 to nude mice transplanted with DLD-1 cells. No sign of toxicity was observed, as judged by monitoring body weight (Fig. 5) and clinical signs. Tumors that did not successfully implant in two mice of the T3 group were excluded from evaluation. The final tumor volumes and weights in RBT3-fed mice (10.3 mg/mouse/day) averaged 515 ± 96 mm\textsuperscript{3}
and 450 ± 54 mg, respectively. These values were 64% and 44% lower, respectively, compared with those of vehicle-fed mice (1,424 ± 226 mm³ and 810 ± 48 mg). After supplementation with RBT3, tocotrienols accumulated in the plasma (4.6 ± 1.3 μmol/L) and in the tumor (122.1 ± 18.2 nmol/g) (Table 2). In contrast, tocotrienols were not detectable in vehicle-fed mice. RBT3 supplementation scarcely affected plasma and tumor levels of the tocopherols (Table 2).

We next assessed the expression of genes and proteins in the mouse tumors. In tumors from RBT3-treated mice, mRNA expression of p21/Cdkn1a (279%), p27/Cdkn1b (385%), caspase-3 (415%), and caspase-9 (167%) was elevated compared with expression in tumors from the control group (Fig. 6A). In Western blot analysis, cleaved caspase-3 and cleaved caspase-9 levels were elevated in RBT3-fed mice compared with vehicle-fed mice (Fig. 6B). Also, RBT3-fed mice showed a decreased phosphorylation of Akt (Thr³⁰⁸ and Ser⁴⁷³). In histological analyses, tumors from RBT3-fed mice showed a lower number of phospho-Akt-positive cells, CD31/PECAM-1-positive endothelial cells and red blood cells compared with tumors from the control group (Fig. 6C). These results indicated that T3-treatment suppressed tumor growth in vivo via cell cycle arrest and activation of apoptosis under normoxia and hypoxia and also inhibited hypoxia-induced angiogenesis of tumor tissue.

4. Discussion

In this study, we clearly showed that δ-T3 treatment caused growth inhibition and induced apoptosis in DLD-1 cells. Hypoxia is one of the key regulators in tumor growth and survival, and it is also associated with poor prognosis and resistance to chemotherapy [20]. Importantly, in the present study, we have demonstrated that δ-T3
could inhibit colon cancer cell growth even under hypoxic conditions. Concordant with the phenotypic results (Figs. 1 and 2), δ-T3 induced cell cycle arrest and proapoptotic signals at the level of both mRNA and protein under hypoxia as well as normoxia (Figs. 3 and 4). The cell cycle is regulated by a series of checkpoints employing cyclins, CDKs and CDK inhibitors [30]. Cyclins associate with CDKs to form active kinase complexes. These complexes are inhibited by binding with CDK inhibitors such as p21 and p27 [30]. p21 inhibits the CDK4/cyclin D complex and the CDK2/cyclin E complex and thereby blocks proliferation via G1-phase arrest [31]. In addition to the cell cycle regulatory activity, p27 is also involved in the regulation of cancer cell differentiation and apoptosis [32]. Our data showed that δ-T3 induced cell cycle arrest via increasing p21 expression after 24 h, then induced apoptosis via upregulation of p27 after 48 h. p21 and p27 protein degradation and caspase-9 activation are regulated by Akt activity [33]. Therefore, our findings (δ-T3-induced DLD-1 cell cycle arrest in G1 phase, Fig. 2) could be explained by upregulation of p21 and/or p27 and also by suppression of CDK4 and Akt under both normoxia and hypoxia.

Since δ-T3 exerted anti-cancer effects in hypoxia, we analyzed whether δ-T3 could inhibit the expression of HIF-1α. Hypoxic tumor cells are believed to become resistant to most anti-cancer agents as their gene expression profile changes as they adapt to hypoxic conditions [34]. The majority of the genes required for hypoxia adaptation are regulated by HIF-1α [35]. HIF-1α is therefore a key regulator for adaptation of tumor cells to hypoxia. In this study, Western blots showed that δ-T3 could suppress hypoxia-induced HIF-1α expression. Hänze et al. reported that RNA interference of HIF-1α inhibited its downstream signaling and affected cellular proliferation [36]. We believe that T3’s potent anti-cancer effect under hypoxia is attributed to its suppression of HIF-1α.
We found that orally administered RBT3 inhibited the growth of human colon tumors in vivo (Fig. 5). When we assessed the possible mechanisms by which RBT3 manifested its anti-tumor effects in vivo, we found that Akt phosphorylation (a survival marker) as well as CD31/PECAM-1 (a microvessel density indicator) were downregulated by RBT3 (Fig. 6C). Further investigation revealed the upregulation of caspase-3 and caspase-9 as well as the downregulation of Akt phosphorylation. Expression of p21 and p27 was also increased in the tumors (Fig. 6A-B). Inhibition of tumor volume and upregulation of apoptotic genes/proteins showed a correlation with high concentrations of tocotrienols in the tumors (Table 2).

The concentrations of δ-T3 used in the in vitro experiments (10 and 20 µmol/L) were above physiological concentrations. In the present data, the concentration of tocotrienols in the tumor was 122 nmol/g, and our results are in accord with a previous report that found that γ- and δ-T3 inhibited tumor growth due to in vivo accumulation in tumors (54 and 70 nmol/g) [37]. Therefore, we speculate that the accumulation of tocotrienols within the tumor inhibited the Akt signal pathway and induced apoptosis signaling in both cancer and endothelial cells. These results suggested that the in vivo anti-cancer mechanism was similar to the in vitro mechanism. At present, the reason for the accumulation of tocotrienols in tumors is not entirely clear, and the mechanism of tocotrienol accumulation needs further investigation. In our in vivo study, we showed an anti-tumor effect of RBT3 at ten mg/mouse. This dose means 400 mg/kg body weight for a 25 g mouse. For a 60 kg human, this would be the equivalent of 2,000 mg/day even if it were adjusted for the body-surface area coefficient (human, 370 mg/m²; mouse, 30 mg/m²). Other studies demonstrated that γ- and δ-T3 suppressed growth of various tumors in mouse models at lower doses (e.g., 3 - 100 mg/kg body weight) [37 - 39]. We will evaluate the efficacy of the tocotrienols at lower doses in a future study.
Most diseases, especially cancer, are a result of the deregulation of as many as 500 gene products, and inhibition of a single target or single pathway is unlikely to treat or control the disease [40]. Therefore, drugs that modulate multiple targets (multi-targeted therapy) are needed to inhibit the growth of cancer cells. In the present study, δ-T3 and RBT3 inhibited cancer cell growth under both hypoxic and normoxic conditions in vitro as well as in transplanted tumors. In addition to the above mentioned mechanisms of action of tocotrienols, previous studies demonstrated multiple anti-cancer properties of δ-T3, including induction of cell cycle arrest [39], activation of pre-miRNA-34a [41] and inhibition of Wnt signaling [42]. γ-T3, similar to δ-T3, has multiple anti-cancer activities such as activation of cell death receptors [43], inhibition of signal transducer and activator of transcription 3 [44] and inhibition of angiogenic pathways [29, 38, 45]. In particular, Siveen et al. reported that γ-T3 significantly reduced tumor growth and tumor-induced angiogenesis in a hepatocellular carcinoma mouse model. This was achieved through the induction of tumor cell apoptosis and suppression of various angiogenic markers [38]. This report supports our in vivo data, the latter indicating that γ- and δ-T3 have great potential for the treatment of cancers by induction of apoptosis and by inhibition of angiogenesis. Furthermore, Ananthula et al. reported that a δ-T3 derivative showed potent anti-cancer effects compared to that of δ-T3 under cobalt-induced hypoxic conditions and its anti-cancer effects were achieved by both the reduction of HIF-1α levels and the inhibition of Akt signaling [46]. This mechanism was concordant with our in vitro data. Other studies reported the design and synthesis of T3 analogues that had improved inhibitory effects on the proliferation and invasion of cancer cells when compared with natural forms of the tocotrienols [47]. Thus, the use of analogues could lead to T3-based anti-cancer therapy in the future. We propose that the tocotrienols, especially γ- and δ-T3, are promising anti-cancer agents, and oral
administration of these tocotrienols might be beneficial in preventative/therapeutic treatment of cancer cells.

5. Conclusion

Previous studies [24 - 26] suggest a mechanism by which T3 achieves its anti-cancer effects, i.e., an inhibition of tumor cell adaptation to hypoxia. Therefore, in this study, we evaluated the anti-cancer effects and mechanisms of T3 in human colorectal adenocarcinoma cells (DLD-1) especially under hypoxic conditions and in a nude mouse model. As results, δ-T3 caused growth inhibition, apoptosis, and gene/protein expression changes in DLD-1 cells. This effect was found to be more potent under hypoxic than normoxic conditions. We believe that the potent anti-cancer effect of δ-T3 under hypoxia is attributed to its suppression of HIF-1α. Rice bran tocotrienols supplementation inhibited tumor growth in xenografted mice. The in vivo anti-cancer mechanism is considered to be similar to the in vitro mechanism. Overall, our data suggest that tocotrienols are potent anti-tumor agents capable of inducing apoptosis and inhibiting angiogenesis under both hypoxic and normoxic conditions. Further studies are needed to validate whether tocotrienols have significant therapeutic effects (e.g., inhibition of tumor cell adaptation to hypoxia) for clinical treatment of malignant tumors such as colon cancer.

References


[2] Newaz MA, Nawal NN. Effect of γ-tocotrienol on blood pressure, lipid


Table 1. Primer sequences for quantitative RT-PCR amplification of selected human genes

<table>
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<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sequence (5’-3’)</th>
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<td>CCTTGCTGCTCTACCTCCAC</td>
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<td>β-actin (Actb)-R</td>
<td>NM_001101</td>
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Table 2. Concentrations of tocotrienols and tocopherols in plasma and tumors of xenografted mice fed on rice bran tocotrienols (RBT3) [10.3 mg/day (0.36 mg/day α-T3, 9.22 mg/day γ-T3, 0.42 mg/day δ-T3, 0.14 mg/day γ-Toc, and 0.07 mg/day δ-Toc)] or vehicle (vitamin E-stripped corn oil) for 21 days

<table>
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<tr>
<th></th>
<th>Plasma (µmol/L)</th>
<th>Tumor (nmol/g)</th>
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<tbody>
<tr>
<td></td>
<td>(Control)</td>
<td>(Tocotrienol, T3)</td>
</tr>
<tr>
<td>Tocotrienols</td>
<td>N.D.(^1)</td>
<td>4.6 ± 3.8</td>
</tr>
<tr>
<td>α-Tocotrienol</td>
<td>N.D.</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>β-Tocotrienol</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>γ-Tocotrienol</td>
<td>N.D.</td>
<td>4.2 ± 3.5</td>
</tr>
<tr>
<td>δ-Tocotrienol</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Tocopherols</td>
<td>3.8 ± 0.3(^2)</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>3.8 ± 0.3</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

\(^1\)N.D., not detectable

\(^2\)Values represent mean ± S.D.

\(^3\)There were no significant differences between control and T3 groups.
Figures Legends

**Fig. 1.** Effect of δ-tocotrienol (δ-T3) on the proliferation of DLD-1 cells under conditions of normoxia (95% air and 5% CO₂) or hypoxia (< 1% O₂). DLD-1 cells were incubated with test medium under normoxia or hypoxia, and the viable cell numbers were assessed with the water-soluble tetrazolium salt (WST-1) assay. Cell viability data are expressed as the percentage of control under normoxic conditions (24 h). Values are means ± SD (n = 6). *# P < 0.05 compared with control.

**Fig. 2.** Effects of δ-tocotrienol (δ-T3) on cell cycle distribution of DLD-1 cells. DLD-1 cells were cultured in test medium containing δ-T3 (0 - 20 µM) under normoxia (95% air and 5% CO₂) or hypoxia (< 1% O₂). After incubation for 24 - 48 h, the cells were harvested and stained with propidium iodide and subjected to flow cytometric assays. Percentages of cells in sub-G1 and G1 phases were calculated using Cell Quest & ModFit cell cycle analysis software, represented in the right side of the histogram. Values are means ± SD (n = 3).

**Fig. 3.** Effects of δ-tocotrienol (δ-T3) on p21 (Cdkn1a), p27 (Cdkn1b), caspase-3 and caspase-9 mRNA expression. DLD-1 cells were treated with test medium containing δ-T3 (0 - 20 µM) under normoxia (95% air and 5% CO₂) or hypoxia (< 1% O₂). After incubation for 12 - 48 h, mRNA levels were measured by quantitative RT-PCR. Gene expression levels (normalized to β-actin/Actb) are expressed as the percentage of control in normoxia. Values are means ± SD (n = 6). Means without a common letter differ, P < 0.05.
Fig. 4. Effect of δ-tocotrienol (δ-T3) on changes in cell cycle and apoptotic protein levels. DLD-1 cells were incubated with test medium containing δ-T3 (0 - 20 µM) under conditions of normoxia (95% air and 5% CO₂) or hypoxia (< 1% O₂) for 8 - 48 h, after which Western blot analysis was conducted. Each Western blot is a representative example of data from three replicate experiments. Band intensities represent average data of densitometric analyses. Values are means (n = 3).

Fig. 5. Effect of oral administration of rice bran tocotrienols (RBT3) on DLD-1 xenografts in nude mice. DLD-1 cells (5 × 10⁶ cells) were injected into the subcutaneous tissue on the backs of mice. Twenty mice were randomly divided into two groups of ten animals each: a control group that was fed vehicle (vitamin E-stripped corn oil) and an experimental group that was fed T3 [RBT3 10.3 mg/day (0.36 mg/day α-T3, 9.22 mg/day γ-T3, 0.42 mg/day δ-T3, 0.14 mg/day γ-Toc, and 0.07 mg/day δ-Toc)]. RBT3 dissolved in vitamin E-stripped corn oil and vehicle (vitamin E-stripped corn oil) were orally administered once a day by gastric intubation for 21 days. Twenty one days after injection, tumor weight, tumor volume and body weight were measured in control and T3 groups. Means ± SD (n = 8 - 10), P < 0.05 versus control.

Fig. 6. Quantitative RT-PCR, Western blots and histochemical analyses of tumors in nude mice administered rice bran tocotrienols (RBT3). For (A) and (B), data are from tumors of mice given the following by gastric intubation for 21 days: vitamin E-stripped corn oil (control group) or 10.3 mg/day RBT3. The latter contained 0.36 mg/day α-T3, 9.22 mg/day γ-T3, 0.42 mg/day δ-T3, 0.14 mg/day γ-Toc and 0.07 mg/day δ-Toc dissolved in vitamin E-stripped corn oil. Values are means ± SD (control, n = 10; RBT3, n = 8), P < 0.05 versus control. (A) mRNA expression levels (normalized to
β-actin/ACTB) of p21 (Cdkn1a), p27 (Cdkn1b), caspase-3, caspase-9 and vascular endothelial growth factor A (Vegfa). (B) Protein levels of phospho-protein kinase B (Akt) (Thr308 and Ser473), Akt, caspase-3 and caspase-9. In Western blot analysis, β-actin was used for normalization to ensure equal sample loading in each lane. Band intensities were evaluated by densitometric analysis and results are shown as bar graphs. Means ± SE. (C) Tissue sections from tumors were fixed in paraformaldehyde and embedded in paraffin for immunohistochemical staining of phospho-Akt (Ser473) and CD31/platelet endothelial cell adhesion molecule-1 (PECAM-1), and hematoxylin and eosin (H&E). For detailed analytical protocols, refer to Materials and Methods. Representative sections are shown.
Fig. 2.

24 h

<table>
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<th>δ-T3 (μM)</th>
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<th>20</th>
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<tbody>
<tr>
<td>normoxia</td>
<td></td>
<td></td>
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<tr>
<td>G1: 49.32 ± 1.43</td>
<td>Sub G1: 0.08 ± 0.09</td>
<td>G1: 49.97 ± 1.29</td>
<td>Sub G1: 0.02 ± 0.01</td>
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<tr>
<td>hypoxia</td>
<td></td>
<td></td>
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<tr>
<td>G1: 50.84 ± 1.32</td>
<td>Sub G1: 0.09 ± 0.05</td>
<td>G1: 51.84 ± 0.50</td>
<td>Sub G1: 0.07 ± 0.04</td>
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48 h

<table>
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<tr>
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<tr>
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<tr>
<td>G1: 77.22 ± 1.24</td>
<td>Sub G1: 0.08 ± 0.04</td>
<td>G1: 75.95 ± 0.64</td>
<td>Sub G1: 0.18 ± 0.06</td>
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<tr>
<td>G1: 77.41 ± 0.92</td>
<td>Sub G1: 0.15 ± 0.08</td>
<td>G1: 48.09 ± 4.45</td>
<td>Sub G1: 27.08 ± 4.25</td>
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Fig. 4.

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<td>0.9</td>
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<tr>
<td>p27</td>
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<tr>
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<td>1.0</td>
<td>0.9</td>
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</tbody>
</table>
Fig. 5.
Fig. 6.

A

 mRNA 
(%) of control)

 p21/Cdkn1a  p27/Cdkn1b  Caspase-3  Caspase-9  Vegfa

 B

 Protein 
(%) of control)

 Phospho Akt (Thr^308)  Phospho Akt (Ser^473)  Akt (total)  Cleaved caspase-3  Cleaved caspase-9  β-actin

 C

 Control #1 Control #2 T3 #1 T3 #2

 Phospho Akt (Ser^473)
 Normal mouse IgG
 (negative control)
 H&E

 CD31/PECAM-1
 Normal rabbit IgG
 (negative control)
 H&E