Delta-tocotrienol Augments Cisplatin-induced Suppression of Non-small Cell Lung Cancer Cells *via* Inhibition of the Notch-1 Pathway

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Abstract. *Non-small cell lung cancer (NSCLC), accounting for 80% of lung cancers, is the leading cause of all cancer deaths. Previously, we demonstrated that delta-tocotrienol inhibits NSCLC cell proliferation, invasion and induces apoptosis by down-regulation of the Notch-1 signaling pathway. The objective of this study was to investigate whether delta-tocotrienol, could enhance the anticancer effects of cisplatin. Treatment with a combination of delta-tocotrienol and cisplatin resulted in a dose-dependent, significant inhibition of cell growth, migration, invasiveness, and induction of apoptosis in NSCLC cells, as compared to the single agents. This was associated with a decrease in NF-κB DNA binding activity, decrease in Notch-1, Hes-1, Bcl-2 and increase in cleaved Caspase-3 and PARP expressions. These results suggest that down-regulation of Notch-1, via inhibition of NF-κB signaling pathways by delta-tocotrienol and cisplatin, in combination, could provide a potential novel approach for tumor arrest in NSCLC, while lowering the effective dose of cisplatin.*

Lung cancer is the leading cause of death among all malignant diseases. It is estimated that 226,160 people (116,470 men and 109,690 women) will be diagnosed with this disease and out of these, 160,340 will die of cancer of the lung and bronchus in 2012 (1). Non-small cell lung cancer (NSCLC) accounts for 80% of all reported lung cancers and has a poor five-year survival rate of only 16% (2). This is partly attributed to the fact that NSCLC progresses undetected (asymptomatically) untill it has metastasized. Some of the common symptoms for lung cancer include persistent cough, chest pain, coughing up

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blood, hoarseness, weight loss, fatigue and recurrent respiratory infections, all of which can be related to other respiratory diseases in addition to lung cancer and hence are not specific (3). The invasive NSCLC rapidly establishes distant metastases in organs including the bones, contralateral lung, liver and brain, ahead of diagnosis, which are lethal for the patients rather than the primary tumors in lung themselves (4).

Another factor contributing to the poor prognosis of NSCLC patients is the lack of effective therapy to battle the aggressive disease. According to the American Society of Clinical Oncology-Clinical Practice, platinum (cisplatin and carboplatin) and non-platinum combination therapies are the standard first line treatments for NSCLC patients (12), with cisplatin being the most frequently used chemotherapeutic agent for the treatment of NSCLC. However, the utility of cisplatin for the clinical management of NSCLC patients is limited by its dose-related drug resistance. This disappointing outcome strongly suggests that innovative research is required to manage this fatal disease. The different mechanisms proposed to be involved in cisplatin resistance, are changes in cellular uptake and efflux of the drug, increased detoxification of the drug, inhibition of apoptosis and increased DNA repair (13). Also, it has been reported that the Notch pathway may play a role in cisplatin induced drug resistance. For example, it has been shown that the Notch-1 expression is negatively correlated to cisplatin sensitivity of head and neck squamous cell carcinoma, and could be used to predict cisplatin sensitivity (14). Moreover, Notch-1 was highly expressed in cisplatin-resistant head and neck squamous cell carcinoma patients suggesting that the overexpression of Notch-1 induces the reprogrammed survival pathways in head and neck squamous cell carcinoma responding to chemotherapy (15). Similarly, up-regulation of Notch-1 is associated with the cisplatin resistance in ovarian cancer cell lines (16). In addition, concurrent inhibition of Notch-1 pathway and use of cisplatin elicits a striking induction of colorectal cancer cell death (17). These results support the notion that inactivation of the Notch pathway could sensitize the patients who are likely to respond to cisplatin.

Notch signaling plays an important role in cell proliferation and apoptosis (5). Since Notch signaling regulates critical cell fate decisions, alterations in Notch signaling are associated with tumorigenesis. It has been found that Notch signaling is frequently dysregulated with up-regulated expression in different types of cancers such as lung, colon, head and neck, pancreatic (6-9). Overexpression of Notch-1 has been shown to inhibit apoptosis in different types of cancers (10, 11). Clinical data has demonstrated that 30% of NSCLCs have increased Notch activity and 10% of NSCLCs have gain-of-function mutation on *Notch-1* gene (9). These data suggest that Notch could be considered as a therapeutic target.

Previously, we demonstrated that treatment of NSCLC cells with delta-tocotrienol resulted in a dose- and time-dependent inhibition of cell growth, cell migration, tumor cell invasiveness, and induction of apoptosis (18). Real-time RT-PCR and western blot analysis showed that antitumor activity by delta-tocotrienol was associated with a decrease in expression of Notch-1, Hes-1, Survivin, MMP-9, VEGF, and Bcl-2. In addition, there was a decrease in nuclear factor-kappaB (NF-κB) DNA binding activity (18). It is of interest to see whether a combination of delta tocotrienol and cisplatin may be an effective therapy against NSCLC, while sensitizing the cells against acquired drug resistance. The objective of this study was to investigate the growth inhibitory effects of a low cisplatin dose in combination with delta-tocotrienol and to further demonstrate the effects of their combination on intracellular signaling mechanisms.

Materials and Methods

Cell culture, reagents and antibodies. Human NSCLC cell lines, including A549, H1650 obtained from ATCC, were grown in DMEM medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, in 5% CO_2 atmosphere. Pure delta-tocotrienol was a kind gift from American River Nutrition, Inc (American River Nutrition, Hadley, MA). Protease inhibitor cocktail was obtained from Sigma (St. Louis, Mo). Primary antibodies for cleaved caspase-3, PARP, β-actin and cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl , 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) were purchased from Cell Signaling Technology (Danvers, MA). Primary antibodies against Notch-1, Hes-1, Bcl-2 were bought from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were bought from Bio-Rad Laboratories (Hercules, CA).

Cell viability studies by MTS assay. The A549 and H1650 cells (5×10^3) were seeded in a 96-well culture plate. After overnight incubation, the medium was removed and replaced with a fresh medium containing DMSO (vehicle control), delta-tocotrienol alone, cisplatin alone, or the combination of delta-tocotrienol and cisplatin. After 72 h of incubation, 20 μl of CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) was added to each well. After 2 h of incubation at 37°C in a humidified, 5% CO_2 atmosphere, the absorbance at 490 nm was recorded on an EL×800 plate reader (Bio-Tek, Winooski, VT). Each variant of the experiment was performed in triplicate.

Clonogenic assay. One million cells per well were seeded in a 100 mm dish and incubated overnight. Subsequently, the cells were cultured in the presence of control medium, delta-tocotrienol (15μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM), grown for 72 h. Later, the viable cells were counted and plated in 100 mm dishes in a range of 1,000 cells per plate. The cells were then incubated for 21 days at 37°C in a 5% CO_2 incubator. All the colonies were fixed in 4% paraformaldehyde and stained with 2% crystal violet.

Histone/DNA ELISA for detection of apoptosis. The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in NSCLC cells. Briefly, 10^5 cells were seeded in six-well plates. After 24 h of incubation, cells were treated in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h. The cells were then lysed, and cytoplasmic histone/DNA fragments were extracted and incubated in microtiter plate modules coated with anti-histone antibody. In order to detect the immobilized histone/DNA fragment, peroxidase-conjugated anti-DNA antibody was used before color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using an EL×800 plate reader (Bio-Tek, Winooski, VT) at 405 nm.

Annexin V-FITC method for apoptosis analysis. Annexin V-FITC apoptosis detection kit (BD, San Jose, USA) was used to measure the apoptotic cells. Briefly, A549 and H1650 cells were incubated in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h. Cells were trypsinized, washed twice with ice-cold PBS and re-suspended in 1× binding buffer at a concentration of 10^5 cells/ml in a total volume of 100 μl. After that, 5 μl of Annexin V-FITC and 5 μl of PI (Propidium Iodide) were added. All the samples were kept in the dark for 20 min at room temperature. Finally, 400 μl of 1× binding buffer was added to each tube and the number of apoptotic cells was analyzed by flow cytometry (BD, San Jose, CA).

Wound healing assay. A549 and H1650 were seeded in a six well plate at the concentration of 4×10^5 cells per well. After overnight incubation, the culture media were removed and a scratch wound across each well was made using fine tips. All the wound areas were washed by PBS for three times to make sure no loosely held cells attached. Subsequently, the cells were cultured in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM). The wound images were taken as 0 h. After 20 h, wound healing pictures were taken under a microscope.

Cell invasive assay. BD Biocoat invasion kit (BD, San Jose, CA) was used to evaluate the tumor invasive ability. Briefly, approximately 2.5×10^5 A549 or H1650 cells with basal media were transferred in each 6-well upper chamber in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol and cisplatin. In the meantime, 3 ml of culture medium with 10% FBS was added into each lower chamber of each 6-well plate. After 20 h incubation, the cells on the upper chamber were removed using a cotton stick. Each experiment was performed in duplicate. The cells were fixed in 4% paraformaldehyde and stained with 2% crystal violet. The spectrophotometric absorbance of the samples was determined by using an EL×800 plate reader (Bio-Tek, Winooski, VT), at 570 nm.

Protein extraction and western blotting. A549 and H1650 cell lines were treated in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h to evaluate the effects of treatment on Notch-1, Hes-1, PARP, Survivin, Bcl-2, and β-actin expressions. Cells were lysed in the cold lysis buffer for 30 min on ice. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, CA). Each sample contained 50 μg of total cell lysates. The samples were loaded on 10% SDS-polyacrylamide gel electrophoresis. After that, the gel was transferred to a nitrocellulose membrane (Whatman, Clifton, NJ) using transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) in a Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 1 h at room temperature with 5% nonfat dried milk in 1×TBS buffer containing 0.1% Tween. After that, membranes were incubated over night at 4°C with primary antibodies (1:1000). The membranes were washed 3 times with TBS-T, and subsequently incubated with the secondary antibodies (1:5000) containing 2% BSA for 2 h at room temperature. The signal intensity was then measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA)

Real-time quantitative PCR for gene expression analysis. Total RNA was isolated using the RNeasy Mini Kit from QIAGEN (Valencia, CA, USA) according to the manufacturer's protocols. Two micrograms of total RNA from each sample were subjected to first strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Reverse transcription reactions were performed at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. Real-time PCR analyses were performed using the Eppendorf Realplex 4 system (Hauppauge, NY). The sequences of the primers sets used for this analysis are as follows: MMP-9, forward primer (5'-CGG AGT GAG TTG AAC CAG-3') and reverse primer (5'-GTC CCA GTG GGG ATT TAC-3'); VEGF, forward primer (5'-GCC TTG CCT TGC TGC TCT AC-3') and reverse primer (5'-TTC TGC CCT CCT CCT TCT GC-3'); GAPDH, forward primer (5'-CAG TGA GCT TCC CGT TCAG-3') and reverse primer (5'-ACC AGA AGA CT GTG GAT GG-3'); All these primers are checked by running them on virtual PCR, and primer concentration was optimized to avoid the primer dimer formation. Real-time PCR amplifications were performed using 2× SYBR Green PCR Master Mix (Applied Biosystems). Two microliters of RT reaction were used for a total volume of 25 microliters of quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95°C 10 min, followed by 50 cycles of 95°C 15 s and 60°C 1 min. Data were analyzed according to the comparative fold increases or decreases in gene expression determined quantitation of normalized GAPDH expression in each sample.

Microwell colorimetric NF-κB assay for measuring NF-κB activity. TransAM™ Transcription Factor ELISAs kit for P65 (Avtive Motif, Carlsbad, CA) was used to evaluate the binding activity of NF-κB according to the protocol. Briefly, one million of A549 and H1650 cells were seeded in an 100 mm dish. After 24 h of incubation, cells were treated in the presence of control medium, delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, or the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h. After that, nuclear protein was extracted from each sample using the nuclear protein extraction kit according to the protocol (Pierce, Rockford, IL). Two micrograms of each sample were incubated in the microplate coated

with anti-p65 DNA sequence. In order to detect the p65-DNA binding complex, peroxidase-conjugated anti-DNA antibody was used before color development with ABTS substrate for peroxidase. The chemilluminance of the samples was determined by using chemiDoc XRS (Bio-Rad Laboratories, CA). The volume of each sample was determined by Quantity One software (Bio-Rad Laboratories, CA).

Data analysis. Results were expressed as means±SEM and analyzed using GraphPad Prism 4.0 (Graph pad Software, La Jolla, CA). Statistical comparisons between groups were done using one-way ANOVA. Values of $p < 0.05$ were considered to be statistically significant and individual *p*-values are reported in the figures, separately. Calcusyn (Biosoft, United Kingdom) was used to analyze the combination effect of delta-tocotrienol and cisplatin.

Results

In order to test the effects of delta-tocotrienol, cisplatin and their combination on cell growth, A549 and H1650 cells were treated with control medium, delta-tocotrienol alone, cisplatin alone, or the combination of delta-tocotrienol and cisplatin for 72 h separately, followed by the MTS assay. As shown in Figure 1 A (A549) and B (H1650), a significant potentiation in the inhibition of cell growth was observed by combination of delta-tocotrienol and cisplatin compared to single agents in both A549 and H1650 cells respectively. In the A549 cell line, combination treatment with 7.5 μM of delta-tocotrienol and 2 μM of ciplatin, 15 μM of delta-tocotrienol and 4 μM of ciplatin, and 30 μM of delta-tocotrienol and 8 μM of ciplatin for 72 h resulted in 25%, 55%, and 92% inhibition of cell growth relative to control, respectively. Similarly, treatment of H1650 cell line with these combinations for 72 h resulted in 32%, 56%, and 91% inhibition of cell growth, respectively, relative to control. Based on the MTS results, we selected 15 M of delta-tocotrienol and 4 μM of ciplatin to perform further experiments.

In order to confirm the effects of delta-tocotrienol and cisplatin on cell growth, A549 and H1650 cells were treated with each of the single agents or their combination and assessed for cell viability by clonogenic assay. As shown in the Figure 1 C (A549) and D (H1650), the combination treatment of delta-tocotrienol and cisplatin resulted in a significant inhibition of colony formation compared to either agent alone or the control in both NSCLC cell lines. Overall, the results from the clonogenic assay were consistent with the MTS shown in Figure 1A and 1B. The molecular mechanisms involved in NSCLC cell growth inhibition were further investigated, and the results are presented in the following sections.

Since inhibition of cell growth could also result from apoptosis, induced by delta-tocotrienol and cisplatin, we further investigated whether delta-tocotrienol, cisplatin and in combination could induce apoptosis in both cell lines by two different approaches, histone/DNA ELISA and the Annexin V/PI staining. The effects of delta-tocotrienol (15 μM), cisplatin (4 μM) individually and in combination, were tested
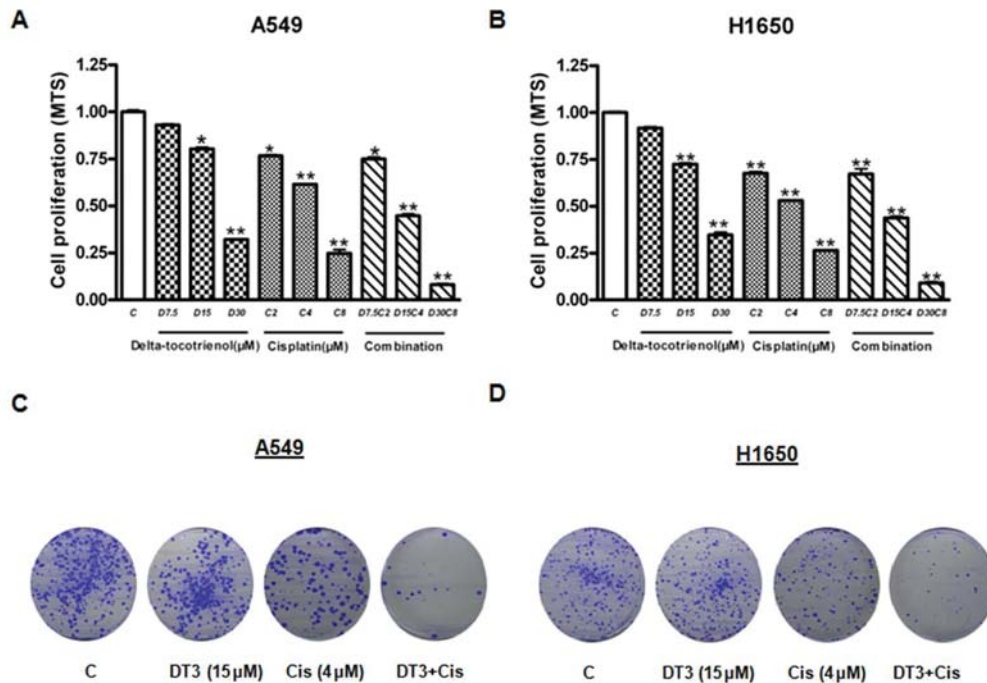
Figure 1. *Antiproliferative effects of delta-tocotrienol and/or cisplatin on NSCLC cells. Cell viability of human NSCLC cell lines, A549 (A) and H1650 (B). Both A549 (A) and H1650 (B) cells were initially plated at a density of 5×10^3 cells/well (3wells/group) in 96-well plates and grown in experimental medium with delta-tocotrienol (D), cisplatin (C) and the combination of DC for 72 h. Viable cell number was determined using the MTS colorimetric assay. Vertical bars indicate the mean cell count±SEM (n=3). *p<0.05 is considered as significant as compared with vehicle-treated controls. Cell survival of human NSCLC cell lines A549 (C) and H1650 (D). A549 (C) and H1650 (D) cells treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and the combination of delta-tocotrienol (15 μM), cisplatin (4 μM) were evaluated by the clonogenic assay. Colony formation in control and treated A549 and H1650 cells is shown. A significant reduction in colony formation between treated and untreated cells of both A549 and H1650 cells was observed by photomicrographic analysis.*
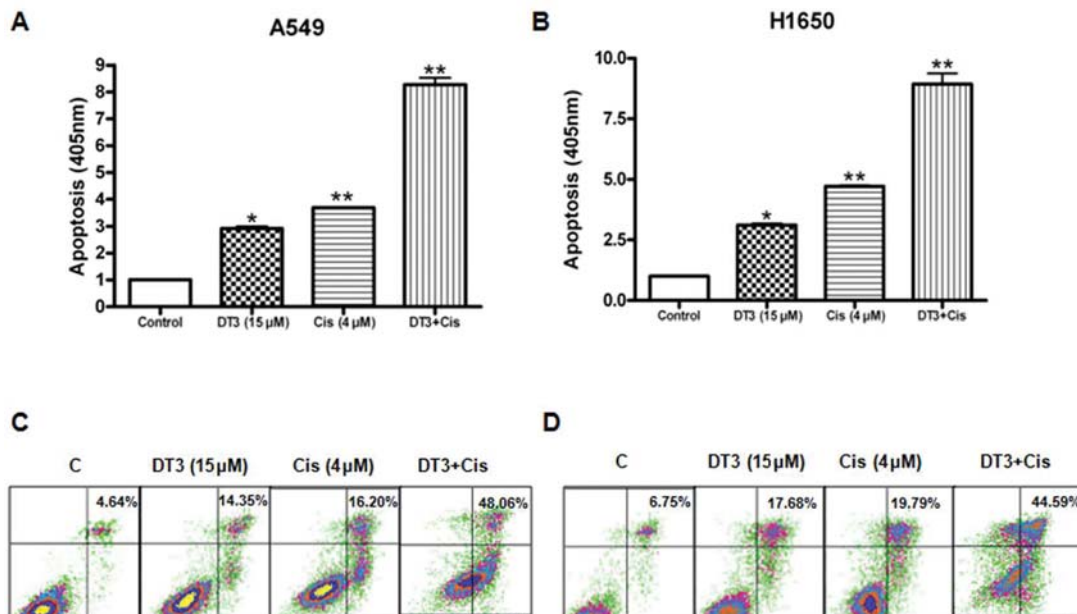

Figure 2. *Induction of apoptotic effects of delta-tocotrienol and cisplatin NSCLC cells. A549 (A) and H1650 (B) cells were treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and their combination for 72 h. Then apoptosis for both cell lines was determined by histone/DNA ELISA. *p<0.05, **p<0.01. A549 (C) and H1650 (D) cells were treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and their combination for 72 h. Then apoptosis for both cell lines was determined by Annexin V-FITC staining.*
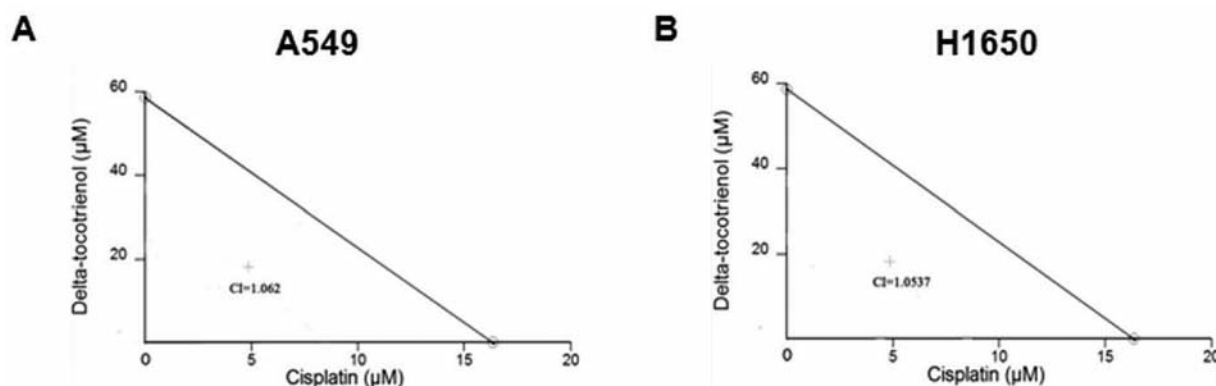
Figure 3. *Isobologram plots for the combination analysis of delta-tocotrienol and cisplatin. Isobologram plots for combination treatments with delta-tocotrienol (7.5, 15, 30 μM) and cisplatin (2, 4, 8 μM) in A549 (A) and H1650 (B) were evaluated by the MTT assay. The combination index (CI) shows an additive effect of the two agents.*

using ELISA in both cell lines. As shown in Figure 2A (A549) and Figure 2B (H1650), exposure of A549 and H1650 to delta-tocotrienol (15 μM) and cisplatin (4 μM) for 72 h significantly enhanced apoptosis. In addition, the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) further increased apoptosis in both cell lines.

The Annexin V/PI staining data confirmed apoptosis-inducing effect of delta-tocotrienol and/or cisplatin treatment in both tested cell lines (Figure 2C and D), respectively. In A549 cell line (Figure 2C), the combination treatment of delta-tocotrienol and cisplatin induced 48.06% apoptosis as compared with 14.35% with delta-tocotrienol (15 μM) and 16.20% in cisplatin (4 μM) treatments alone. Similarly, in H1650 cell line (Figure 2D), the combination treatment of delta-tocotrienol and cisplatin induced 44.59% apoptosis as compared with 17.68% with delta-tocotrienol (15 μM) and 19.79% in cisplatin (4 μM) treatments alone. These results are consistent with those from the MTS assay, suggesting that the potentiation in overall cell growth inhibition by the combination of cisplatin and delta-tocotrienol, could in part be due the induction of apoptosis in both NSCLC cell lines.

As both delta-tocotrienol and cisplatin alone induce apoptosis in various cancer cells, we wanted to verify the effect of the combination of delta-tocotrienol and cisplatin treatment in A549 and H1650 cell lines. As shown in Figure 3A and Figure 3B, the combination index of ED$_{75}$, as calculated by the calsusyn software, was found to be 1.06 (A549) and 1.05 (H1650) respectively. According to the combination definitions, these results confirm the additive effects between delta-tocotrienol and cisplatin in NSCLC cells.

Thus far, our results have shown that delta-tocotrienol, cisplatin and their combination inhibited cell growth and induced apoptotic cell death in NSCLC cells. Our previous data demonstrated that delta-tocotrienol induced apoptosis through the Notch-1 pathway in NSCLC cells (18). In order

to further understand the molecular mechanism involved in delta-tocotrienol- and cisplatin-induced apoptosis of NSCLC cells, modifications in the cell death pathway were investigated. Using western blotting analysis, we found that combination treatment of delta-tocotrienol and cisplatin significantly suppressed the protein expression of Notch-1 and its downstream signaling molecule Hes-1 in NSCLC A549 (Figure 4A) and H1650 (Figure 4B), compared to treatment by either delta-tocotrienol or cisplatin alone. Given that Notch signaling and its gene products are known to regulate cell proliferation, cell cycle distribution and apoptosis, we further explored the apoptosis-related genes in both NSCLC cell lines. Our data showed that the combination treatment of delta-tocotrienol and cisplatin suppressed the Notch-1 pathway as compared with either delta-tocotrienol or cisplain alone in the A549 and H1650 cell line respectively. This suppression of Notch-1 was associated with the higher expression of cleaved caspase 3, cleaved PARP and inhibition of Bcl-2 expression.

To further confirm the result on changes in protein expression, we also conducted real-time PCR to assess Notch-1 and its target genes such as *Hes-1* in NSCLC cells upon the treatment of delta-tocotrienol, cisplatin and their combination. Our data clearly demonstrated that the combination treatment of delta-tocotrienol and cisplatin caused a more potent suppression of Notch-1 signaling pathway in A549 (Figure 4 C) and H1650 (Figure 4D) cells as compared to either compound or control. Taken together, our findings strongly suggest that the combination treatment of delta-tocotrienol and cisplatin suppressed transcription and translation of Notch-1 and its target genes such as *Hes-1, Bcl-2* leading to reduced proliferation, and enhanced apoptosis in NSCLC cells.

The NF-κB pathway plays important roles in cancer cell transformation, cell invasion, and apoptosis. Further, NF-κB has been shown to cross-talk with Notch signaling (19).
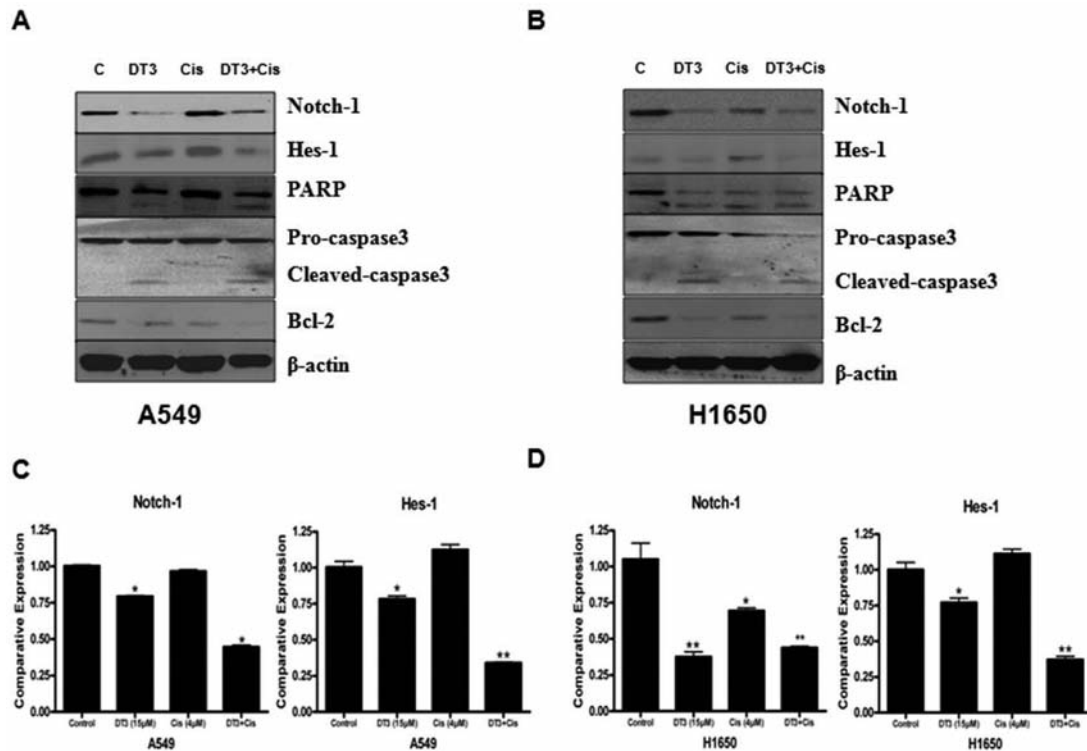
Figure 4. *Down-regulation of Notch-1 and its target genes by delta-tocotrienol and cisplatin. A549 and H1650 cells were treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and their combination for 72 h. The expressions of Notch-1, Hes-1, PARP, caspase-3 (pro- and cleaved-), Bcl-2 protein were detected by western blotting analysis in A549 (A) and H1650 (B) NSCLC cells. The relative expression of mRNA by real time PCR of Notch-1 (left panel) and Hes-1 (right panel) for A549 (C) and H1650 (D) cells is shown. *p<0.05, **p<0.01.*

Cisplatin, a DNA-damaging drug, is known to increase the NF-κB activity (20, 21). This increasing activity of NF-κB is associated with drug resistance. Interestingly, our previous data demonstrated that delta-tocotrienol could successfully reduce NF-κB DNA binding activity in NSCLC cells (18). The effect of combination treatment of delta-tocotrienol and cisplatin on NF-κB DNA-binding activity in NSCLC cells was determined by subjecting nuclear extracts from treated A549 and H1650 cells to p65 ELISA. As shown in Figure 5 A and B, compared to the control, the combination treatment significantly inhibited the DNA-binding activity of NF-κB for both cell lines. These results suggested that the treatment with delta-tocotrienol decreased the cisplatin-induced NF-κB activation, which further confirmed the potential benefit of delta-tocotrienol as a sensitizing agent to cisplatin.

Since *VEGF* and *MMP9* are known to be the downstream target genes of NF-κB signaling pathway, the relative expressions of *VEGF* and *MMP9* were evaluated by RT-PCR. As shown in Figure 5 C (A549) and 5 D (H1650), the combination treatment of delta-tocotrienol and cisplatin significantly inhibited the expression of *VEGF* and *MMP9* in both cell lines, respectively. The results clearly demonstrated

that delta-tocotrienol sensitized the NSCLC cells to cisplatin by inhibiting NF-κB activity and the expressions of its target genes.

Although the effect of delta-tocotrienol and cisplatin on anti-proliferation and induction of apoptosis has been shown in certain cancers, their effects on tumor cell migration and invasion has not been evaluated thus far. Since the combination treatment of delta-tocotrienol and cisplatin inhibited MMP-9 and VEGF expression, which are important factors for cell migration and invasion, we conducted a Matrigel invasion assay in order to assess its effect on the invasive capacity of A549 and H1650 NSCLC cells. As shown in Figure 6 A and B, the combination treatment of delta-tocotrienol and cisplatin in A549 and H1650 cells significantly decreased their invasive capability as compared with either the untreated control, or delta-tocotrienol, and ciplatin alone.

In order to determine the combination effect of delta-tocotrienol and cisplatin on the tumor migration ability in NSCLC cells, we performed the wound healing assay. As demonstrated in Figure 6 C (A549) and D (H1650), the combination of delta-tocotrienol and cisplatin inhibited cell migration compared with the untreated control, delta-tocotrienol, and cisplatin alone.
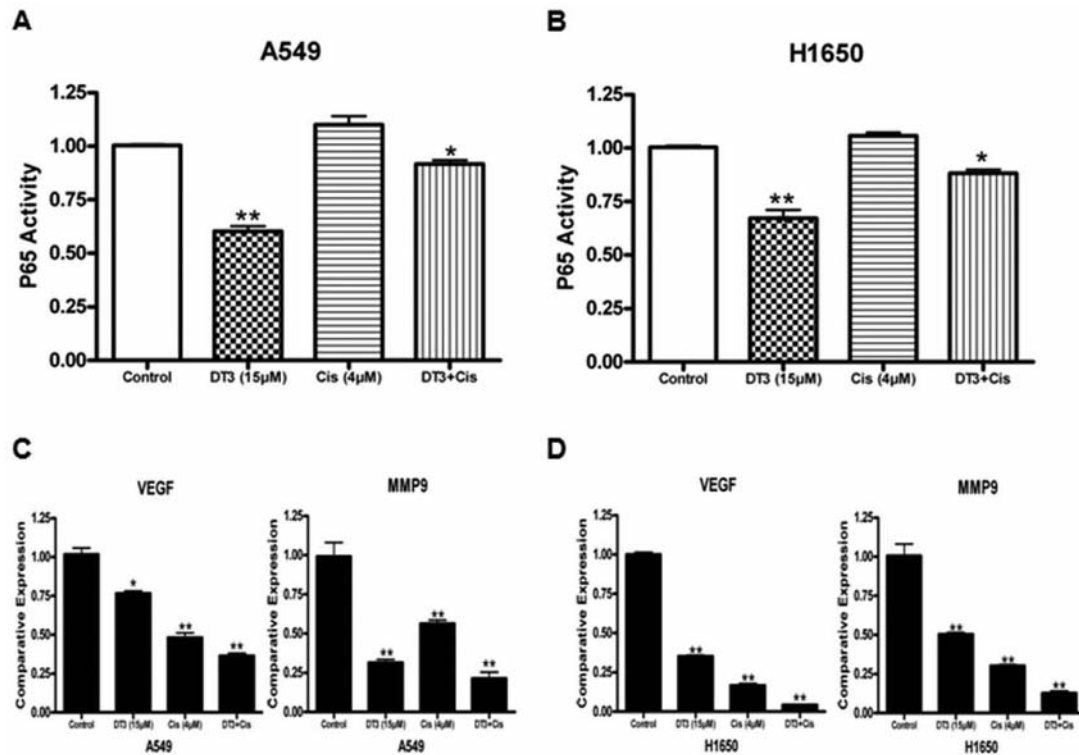
Figure 5. *The effects of delta-tocotrienol and cisplatin on NF-κB activity and its down-stream genes. A549 (A) and H1650 (B) cells were incubated with delta-tocotrienol (15 μM), cisplatin (4 μM), and their combination or DMSO-control for 72 h, and nuclear proteins were subjected to ELISA assay for the evaluation of NF-κB DNA binding activity. A549(C) and H1650 (D) cells were treated with delta-tocotrienol (15 μM), cisplatin (4 μM), and their combination for 72 h. The expressions of VEGF (left) and MMP9 (right) were analyzed by real-time-RT-PCR. Relative gene expressions are presented as means±S.E.M of three independent experiments.*p<0.05, **p<0.01.*

Discussion

Cisplatin, is one of the most widely used chemotherapy drugs, and has been approved for the treatment of different types of human solid carcinomas including lung, ovarian, bladder, and testicular cancers. Unfortunately, drug resistance and toxicity during chemotherapy remains a major hurdle and challenge for the usage of cisplatin in cancer therapy. Our previous data showed treatment of NSCLC cells with delta-tocotrienol results in a dose-dependent inhibition of cell growth, cell migration, tumor cell invasiveness, and induction of apoptosis (18). Real-time RT-PCR and western blot analysis showed that antitumor activity of delta-tocotrienol was associated with a decrease in Notch-1, Hes-1, survivin, MMP-9, VEGF, and Bcl-2 expression (18). The aim of this study was to determine whether delta-tocotrienol has potential in combinational therapy with cisplatin for the treatment of NSCLC and further, to elucidate its molecular mechanism. We demonstrated that treatment of NSCLC cells with delta-tocotrienol and cisplatin in combination resulted in a dose-dependent inhibition of cell proliferation. Also, this is the first study to report the effectiveness of delta-tocotrienol in inducing apoptosis in

NSCLC cell lines, when used in combination with cisplatin. Abnormal overexpression of Notch-1 pathway has been found in a subset of NSCLC patients (9). Interestingly, activated forms of Notch-1 and its down-stream molecule Hes-1 can be stimulated by cisplatin. Delta-tocotrienol mitigated the activation of Notch-1, leading to greater expression of apoptotic proteins such as cleaved caspase-3, cleaved PARP and the inhibition of Bcl-2 expression as compared with either delta-tocotrienol or cisplatin alone in the A549 and H1650 cell lines, respectively. Therefore, the inhibition of Notch-1 signaling by delta-tocotrienol augmentation is a rational strategy against NSCLC cells to reduce the escape from the cell death phenomenon by cisplatin.

Our previous data also demonstrated that delta-tocotrienol could decrease NF-κB-DNA binding activity. NF-κB plays important roles in many cellular processes including cell proliferation, invasion, and angiogenesis, all of which are crucial for cancer development and progression (22). Growing data indicates that there is cross-talk between the Notch-1/Hes-1 pathways and the NF-κB pathway. Notch ligands induce NF-κB activation in leukemia cells, and decreased Notch-1 expression in these cells has been shown to be accompanied
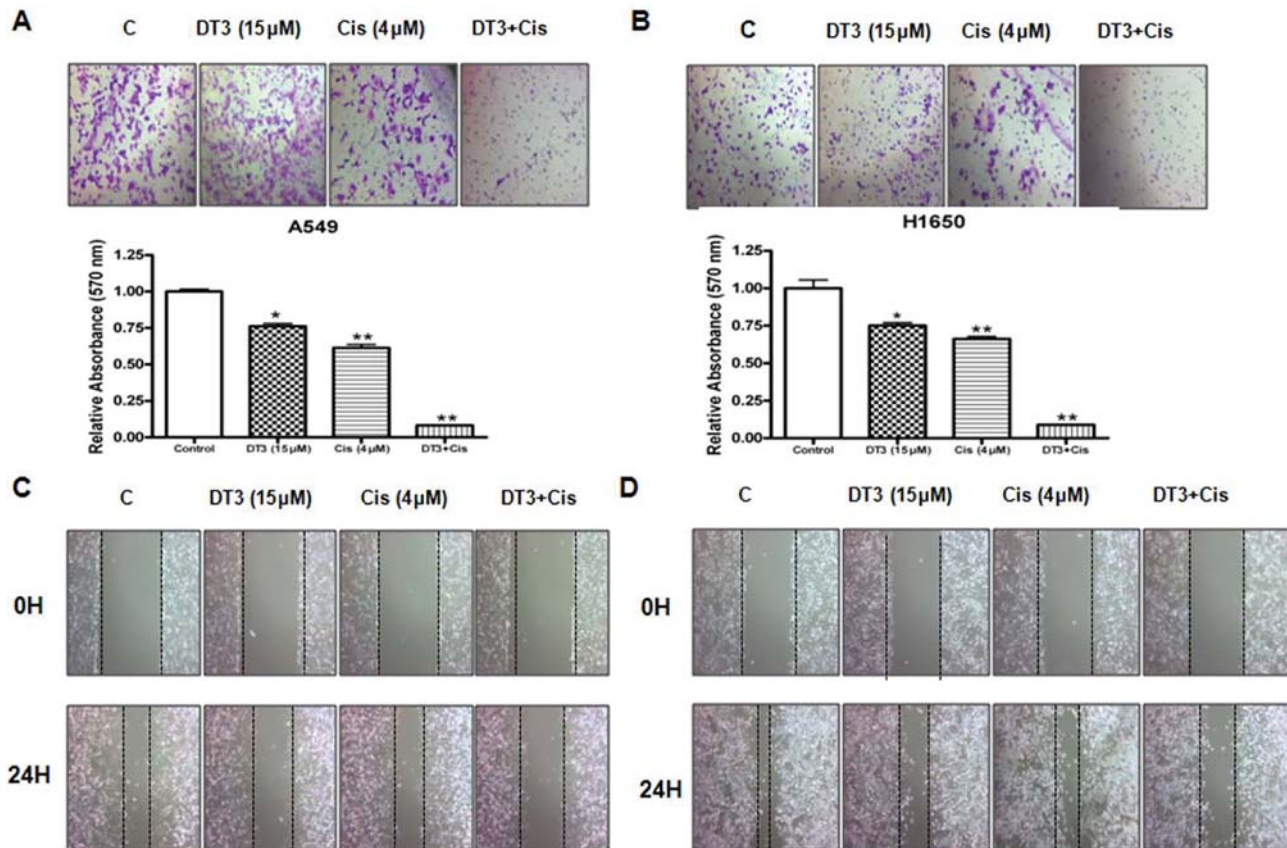
Figure 6. *Delta-tocotrienol inhibits cell migration and invasion. Inhibition of NSCLC cell invasion by delta-tocotrienol, cisplatin, and their combination for 72 h. Upper panel: A549 (A) and H1650 (B) cells were seeded into Matrigel-coated inserts with delta-tocotrienol (15 μM), cisplatin (4 μM), the combination or DMSO (C). Cells that invaded to the lower surface of the insert over a period of 20 h were stained with crystal violet dye. Lower panel: The photometric intensity of the invaded cells was determined for the number of A549 and H1650 cells. Cell invasion is presented as means±S.E.M of three independent experiments. *p<0.05, **p<0.01. Inhibition of NSCLC cell migration by delta-tocotrienol (15 μM), cisplatin (4 μM), their combination or DMSO (C) using the wound healing assay. Uniform wounds were made by scratching the confluent cultures which were then treated with delta-tocotrienol (15 μM) alone, cisplatin (4 μM) alone, the combination of delta-tocotrienol (15 μM) and cisplatin (4 μM) or DMSO (C) for over 20 h. Then the wound healing images were captured using a microscope at 10× objective.*

with concomitant decrease in NF-κB binding activity (23). Moreover Notch-1 has been found to induce sustained NF-κB activity by facilitating its nuclear retention (24). Recently, Notch-1/Hes-1 pathways were found to be upstream mechanisms for maintainence of NF-κB activation in leukemia *in vivo* and *in vitro* (25). Interestingly, cisplatin, a DNA damaging agent, has been shown to induce the NF-κB activity *in vitro* and *in vivo* (26, 27). The activated NF-κB plays important roles in drug resistance and tumor relapse. Consistent with previous data, we found that treatment with delta-tocotrienol could reduce the NF-κB activity (Figure 5A and B) in NSCLC cells. Moreover, delta-tocotrienol also suppressed the NF-κB activity induced by cisplatin in both NSCLC cell lines (Figure 5A and B). These molecular findings lend support in favor of simultaneous targeting of Notch-1 and NF-κB for effectively sensitizing NSCLC cells to cisplatin.

Furthermore, we wanted to explore the anti-metastatic effect of delta-tocotrienol and of cisplatin in NSCLC cells. Indeed, we showed that in both A549 and H1650 cells, migration and invasiveness were significantly reduced under treatment of delta-tocotrienol and cisplatin (Figure 6). Previously, it has been reported that delta-tocotrienol suppressed hypoxia-induced VEGF and IL-8 expression at both mRNA and protein levels which in turn suppressed tumor angiogenesis (28). Consistent with previous studies, our study confirmed that the anti-metastatic effects induced by delta-tocotrienol and cisplatin were associated with a decrease in VEGF and MMP-9 expressions (Figure 5C and D).

In conclusion, in this study, we provided evidence to support that delta-tocotrienol could enhance sensitivity of cisplatin in NSCLC cells. The combined treatment of delta-tocotrienl and cisplatin significantly suppressed both NSCLC cells growth,

colony formation, cell migration and invasion. Delta-tocotrienol suppressed cisplatin-caused activation of NF-κB pathway. Although the data needs to be substantiated further in a valid *in vivo* animal model for lung cancer, our findings indicate the potential of this combination of delta-tocotrienol and cisplatin as a novel therapeutic strategy for NSCLC.

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