Sage components enhance cell death through nuclear factor kappa-B signaling

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1. ABSTRACT

The sage components linalyl acetate (Ly) and alpha-terpineol (Te) exhibit synergistic anti-proliferative effects. We investigated the effects of Ly and Te on NF-κB signaling in HCT-116 colon cancer cells. Ly and Te combinations dose-dependently reduced HCT-116 viability at non-cytotoxic concentrations. Combination treatment induced 30%-60% increase in PreG1 through induction of apoptosis and necrosis. DNA binding assays revealed that combination treatment suppressed both basal and TNF-α-induced NF-κB activation. This suppression correlated with the inhibition of p65 nuclear translocation and IkB-alpha degradation. The lack of change in IκK expression levels or inhibition in IκB-alpha phosphorylation suggest the involvement of an IκK-independent mechanism. Ly and Te combination was found to downregulate the expression of NF-κB-regulated antiapoptotic and proliferative gene products. Separate treatments and drug combinations significantly decreased DNA binding activity of NF-κB which led to the potentiation of cell death induced by the colon cancer drugs oxaliplatin and 5-FU. These results indicate that Ly and Te anticaner activities are partly mediated through the suppression of NF-κB activation, suggesting their use in combination with chemotherapeutic agents to induce apoptosis.

2. INTRODUCTION

Medicinal plants have been used for thousands of years for the treatment of many diseases as mild as colds and as severe as cancer. Today, and worldwide, at least 120 distinct chemical substances derived from plants are currently in use and are considered as important drugs (1). Increasing evidence from animal and cell culture studies suggests that combining plant-derived compounds with conventional therapeutics is one of the most promising applications for these compounds. The Lebanese sage Salvia libanotica (also known as S. triloba) is a medicinal shrub endemic to the Mediterranean region and is one of the most widely used plants in folk medicine (2). The three compounds in sage essential oil (alpha-terpineol, linalyl acetate, camphor) are responsible for the oil’s antitumor effects (3). Linalyl acetate has been ascribed anti-inflammatory (4), antibacterial (5), and antifungal activities (6) and is used in aromatherapy and in the flavoring and fragrance industries. Alpha terpineol has been shown to exhibit anti-proliferative effects on human erythroleukemic cells (7) in addition to anti-inflammatory (8), antibacterial (9) and anti-fungal activities (10). Alpha terpineol is also a potent inhibitor of superoxide production and selective regulator of cell function during inflammation (11).

Our studies show that Ly and Te synergize to induce cell cycle arrest and apoptosis mainly via
mitochondrial damage (cytochrome c release), caspase activation, and PARP cleavage, in human colorectal cancer cells (3). Recently, we have shown that protein kinases and/or the NF-κB pathway are possible targets for linalyl acetate cytotoxicity (12), suggesting that the bioactive sage components may be mediating their antitumor properties through the regulation of the NF-κB pathway.

The transcription factor NF-κB is involved in cancer development and progression, as well as in resistance to chemotherapy and radiotherapy (13, 14). NF-κB is activated in response to various carcinogens, growth factors, inflammatory stimuli, and pro-oxidants (13). It consists of p50 and p65 subunits and is sequestered in the cytoplasm through interacting with the inhibitory protein IkB-alpha (13). TNF-alpha, an important upstream activator of NF-κB signaling plays a major role in carcinogenesis (14). In response to TNF-alpha, IkB kinase (IKK) phosphorylates IkB at serine residues 32 and 36, triggering its ubiquitination and proteasomal degradation, thereby unmasking the nuclear localization signal of NF-κB (15). In the nucleus, NF-κB binds DNA thus enhancing the transcription of various gene products that regulate proliferation, apoptosis, angiogenesis, inflammation, and metastasis (16).

The induction of apoptosis in tumor cells is a key mechanism for the effectiveness of anticancer drugs. Apoptotic cell death can be initiated through the mitochondrial pathway by modulation of several Bcl-2 family proteins (e.g. Bax and BH3 only proteins) that are major regulators of mitochondria integrity and permeabilization (17, 18). Bcl-2 family proteins are classified into two groups according to their function: anti-integrity and permeabilization (17, 18). Bcl-2 family proteins including the anti-apoptotic Bcl-2 proteins that sequester proapoptotic proteins and prevent their activation. Once activated, these Bcl-2 family members cause the loss of mitochondrial membrane potential and subsequent release of cytochrome-c from the mitochondria (20).

In this study we aimed to investigate the effect of Ly and Te on NF-κB activation pathway and NF-κB-regulated cellular responses. We show that the drug combination reduced colon cancer cell viability, caused PreG 1 increase, induced apoptosis and necrosis and inhibited NF-κB translocation, and NF-κB/DNA binding. Furthermore, Ly and Te combination potentiated apoptosis induced by 5-FU and oxaliplatin, thus providing the possibility for combining the sage-derived compounds with conventional chemotherapeutic drugs used for the treatment of colon cancer.

3. METHODS

3.1. Cell culture and Treatments

HCT116 human colon cancer cells were obtained from ATCC and maintained in RPMI 1640 with 25mm Hepes and L-Glutamine in a humidified atmosphere of 5% CO₂ and 95% air. Cell media were supplemented with 1% Penicillin-Streptomycin (100U/ml) and 10% heat-inactivated FBS. Alpha terpineol (0.9375 g/ml) and Linalyl acetate (0.901g/ml) from Acros Organics (Geel, Belgium) were obtained in liquid form and were further diluted in media. Recombinant human-TNF-alpha (R&D Systems, Minneapolis, US) was added at 20ng/ml for 4hrs. In studies with TNF-alpha, drugs were added 4hrs prior to TNF-alpha.

3.2. Cell viability and LDH cytotoxicity assay

HCT-116 (1.2 x 10⁵ cells/ml) were cultured in 96-well plates and treated with drugs 24hrs after plating. Effects on cell viability were studied 24hrs after treatment by the non-radioactive cell proliferation kit (Promega Corporation, Madison, USA) and by the trypan blue dye exclusion method, while toxicity effects were assayed 6hrs post-treatment using the CytoTox 96 non-radioactive cytotoxicity kit (Promega) as described previously (21). Experiments were repeated three times and were performed in triplicates.

3.3. Flow cytometric analysis of DNA content

Cells were plated in 60-mm dishes (1.2 x 10⁵ cells/ml), treated with drug combinations for 24hrs, then harvested and fixed in 70% ethanol. Supernatants containing the dead cells were collected and attached live cells were harvested by 2x trypsin and added to the supernatant. Flow cytometry analysis of Propidium Iodide-stained DNA was done as described previously (21). Cell Quest program was used to determine the percentages of cells in various cell cycle phases. Pre-G1 cells with DNA content <2n represent apoptotic or necrotic cells.

3.4. Annexin V Assay

Cells were collected along with the supernatant and centrifuged at 1500 rpm for 10min, 4 degrees Celsius. The pellet was washed with PBS and centrifuged at 1500 rpm for 10min, 4 degrees Celsius. The pellet was resuspended in 100microlic Annexin-V-Fluo labeling solution (20microlic annexin reagent and 20microlic PI (50microlic/ml) in 1000µl incubation buffer pH 7.4 (10mM Heps/NaOH, 140mM NaCl, 5mM CaCl2). The samples were incubated for 15min at room temperature and 0.4ml incubation buffer was added. The cellular fluorescence was then measured by flow cytometry using a Fluorescence Activated Cell Sorter (FACS) flow cytometer (Becton Dickinson, Research Triangle, NC).

3.5. Preparation of nuclear extracts.

Cells were washed twice with 1x PBS, collected by scraping, and centrifuged at 4 degrees Celsius at 300g for 7min. The pellet was resuspended in 250microlic of Buffer A (10mM Tris-HCl pH 7.9, 10mM KC1, 1.5mM MgCl₂, 1mM Dithiothreitol (DTT), and water) and incubated on ice for 10min. The samples were centrifuged at 4, 600g for 10min, 4 degrees Celsius. The collected pellet was resuspended in 35microlic hypertonic Buffer C (20mM Hepes pH 7.9, 0.4M NaCl, 1.5mM MgCl₂, 25% (v/v) glycerol, 0.2mM EDTA, 1mM DTT, 0.5mM PMSF, and water). The samples were then gently agitated for 30min, 4 degrees Celsius, and centrifuged at 14,000 rpm.
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for 20min, 4 degrees Celsius. The supernatant was collected and diluted with 30microl Buffer D (20mM Hepes, 50mM KCl, 20% (v/v) Glycerol, 0.2mM EDTA, 1mM DTT, 0.5mM PMSF, and water). Samples were then stored at -80 degrees Celsius until immunoblotting or EMSA.

3.6. Electrophoretic mobility shift assay (EMSA) and supershift analysis.

10microg of nuclear proteins were incubated with 32P-labeled double strand NF-κB consensus oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGGC-3') for 30min at room temperature. Mutant NF-κB oligonucleotide with a single mutation in the NF-κB DNA binding site (5'-AGT TGA GGC GAC TTT CCC AGGC-3') was used as a control for specificity. In control conditions, 1microl of unlabeled normal or unlabeled mutant oligonucleotide were added to separate tubes. The reaction was stopped by adding 6microl of DNA loading buffer. The DNA: protein complexes were separated by electrophoresis on 10% nondenaturing polyacrylamide gel with a running buffer of 1x TBE. Gel was fixed for 10-15min in 5% acetic acid and 10% ethanol. The fixed gel was dried for 2hrs at 80 degrees Celsius, and the DNA: protein complexes were visualized by autoradiography. In case of supershift analysis, nuclear protein extracts were incubated with goat anti-p65 (C-20), p-50 (SC114X) or p-65 (Santa-Cruz X) for 30min, followed by incubation with labeled wild type NF-κB consensus oligonucleotide for another 30min to identify NF-κB protein complexes. The NF-κB mutant oligonucleotide and NF-κB consensus oligonucleotide were from Santa-Cruz (California, US). The T4 Polynucleotide kinase was from Promega (Madison, US).

3.7. Protein Extraction and Western Blot Analysis.

Cellular protein extracts were prepared and proteins were quantified as described previously (21). 50microg of whole cell lysate was separated by SDS-PAGE (12% gels) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) in cold transfer buffer (14.4g of Glycine, 3g Tris, and 1g SDS (Amersham Pharmacia Biotech, Buckinghamshire, UK)) at 30 Volts overnight. The membranes were blocked with 1% BSA (C-21), goat anti-p65 (C-20X), p-50 (SC114X) or p-65 (Santa-Cruz X), and incubated with appropriate secondary FITC-conjugated antibody. The membranes were probed with the primary antibodies IκB-α (C-21)-G, IκB-α/beta (H-470) or Lamin A/C (H-110), Bcl-2alpha, Bcl-Xl, cyclin D1, c-myc followed by horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG-HRP (all primary and secondary antibodies from Santa-Cruz, California, US). GAPDH (Biogenesis, Poole, UK) or Lamin A/C was used to ensure equal protein loading. The immunoreactive bands were visualized on X-ray film with chemiluminescent substrate (Santa-Cruz). To quantify protein bands, densitometry was done using LabWorks 4.0 software.

3.8. Immunocytochemistry.

Cells were plated at 1.2 x 10^6 cells/ml on glass coverslips in 6-well plates and treated at 50% confluency. On collection day, cells were washed with 1x PBS, fixed using 70% ethanol and stored at -20 degrees Celsius for at least 12hrs. Fixed cells were washed three times with PBS for 5min each and blocked for 1hr in PBS and 3% (vol/vol) normal goat serum (NGS) (Santa Cruz Biotechnologies, Santa Cruz, CA). Blocking was followed by three 5min washes with PBS and incubation for 2hrs with the primary antibody NF-κB p65 (C-20X) (Santa-Cruz, California, US) at a dilution of 1:300 in PBS containing 0.1% NGS. Cells were washed three times with PBS and incubated for 1hr with appropriate secondary FITC-conjugated antibody diluted 1:2000, followed by another three washes. The slides were then mounted on microscope slides using one drop of FluorSave™ reagent (Calbiochem, EMD Biosciences, Inc. La Jolla, CA) and stored at 4 degrees Celsius overnight. Slides were analyzed under LSCM fluorescent confocal microscope (LSM 410, Zeiss, Germany).

4. RESULTS

4.1. Ly and Te synergize to cause cell death at non-cytotoxic concentrations.

We have previously investigated potential anti-tumor effects of Ly and Te when used alone or in combination on human colon cancer HCT-116 cells (3). We showed that separate treatments with Ly and Te (0.8-1mM) reduced cell viability by only 10-20%, yet combining them caused significant anti-neoplastic effects with minimal toxicity to FHs74Int normal intestinal epithelial cells (3), suggesting that <1mM concentrations are non-cytotoxic to normal tissue. Here, we assessed the viability of HCT-116 in response to a range of drug concentrations (0.1mM-1mM). As shown in Figure 1A, drug combinations caused dose-dependent inhibition of cell viability with IC50 of 0.8mM. To investigate whether drug combination exert synergistic effects at the non-cytotoxic dose of 0.6mM, HCT-116 cells were treated with Ly, Te, or combinations and cell viability was determined by the trypan blue dye exclusion assay. Separate treatments by Ly and Te reduced cell viability by 20% and 2%, respectively, whereas the combination reduced it by 42% (Figure 1B), suggesting synergistic effects. Cell cycle changes by PI staining of cellular DNA content revealed an accumulation of cells in the pre-G1 phase upon treatment with Ly+Te at 0.6 or 1mM for 24hrs. The pre-G1 population increased from 1% in the control to 32% and 59% at 0.6 and 1mM, respectively (Figure 1C). Annexin V staining showed that the mode of cell death was by apoptosis and necrosis. At 24hrs post-treatment, Ly+Te (0.6mM) resulted in 12% necrotic cells, 4% apoptotic cells, and 19% mixed cells.

4.2. Ly and Te inhibit nuclear levels of NF-κB and reduce NF-κB gene products.

Our recent findings indicate that NF-κB is a possible target for linalyl acetate cytotoxicity (12). Therefore, we investigated the effect of Ly and Te combination on basal nuclear levels of p65. HCT-116 cells were treated with Ly+Te over a time-course of 3hrs-24hrs. Nuclear extraction and immunoblotting against the NF-κB subunit p65 showed a decrease in the nuclear levels of p65 after 24hrs of combined or separate treatments (Figure 2A). To determine the effect of Ly+Te on the upstream inhibitor of NF-κB, IκB, HCT-116 cells were treated with Ly+Te for 3hrs-24hrs followed by total protein extraction and immunoblotting against IκB-alpha. We observed a time-
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Figure 1. Ly and Te synergized to cause cell death at non-cytotoxic concentrations. Ly and Te combinations dose-dependently inhibited HCT-116 viability (A) and acted synergistically to reduce cell viability (B). Cell Viability was determined by the Cell Titer 96 non-radioactive assay (MTT-based), and by trypan blue. Flow cytometric analysis of PI-stained DNA revealed an accumulation of cells in the pre-G1 phase when Ly and Te were combined (C). Annexin V (means ± SD) showed that both apoptosis and necrosis are the mechanisms of cell death (D). In all experiments, cells were treated with drugs at 50% confluency for 24 hrs and the experiment was repeated three times each in triplicates. Significance was examined using one-way ANOVA test. Bars with (*) are statistically different from the control (p<0.01).

dependent increase in the protein I\textsubscript{\text{KB}}-alpha (Figure 2B), which may represent an increase in expression levels or an inhibition of protein degradation. To investigate which of these two possibilities is at play, we studied the modulation of the phosphorylated form of I\textsubscript{\text{KB}}-alpha. Cells were pretreated with the proteasomal inhibitor PS341 at a dose of 10μM to block the rapid degradation of pI\textsubscript{\text{KB}}-alpha and then subjected to treatment with Ly+Te. Contrary to our expectations, the combination increased the expression levels of pI\textsubscript{\text{KB}}-alpha protein, suggesting the involvement of an IKK-independent mechanism that blocks I\textsubscript{\text{KB}}-alpha degradation. This was confirmed by the lack of change in the expression levels of the IKK-alpha/beta protein (Figure 2B). We then aimed at determining whether Ly+Te affect the protein expression level of NF-κB-regulated genes. NF-κB is known to inhibit apoptosis by inducing the expression of multiple anti-apoptotic proteins and interfering with the expression or activity of proapoptotic proteins. The anti-apoptotic proteins regulated by NF-κB include IAPs 1 and 2, XIAP, Bcl-2, and Bcl-x\textsubscript{L} (22). In addition, numerous gene products that mediate cellular proliferation, such as cyclin D\textsubscript{1}, and c-Myc, have NF-κB binding sites in their promoters (23). To study the effect of Ly+Te on the basal level of expression of NF-κB target genes, HCT-116 cells were treated with Ly+Te (0.6mM) for 3hrs-24hrs. This was followed by total protein extraction and immunoblotting against Bcl-2, Bcl-x\textsubscript{L}, cIAP1, cIAP2, cyclin D1, and c-Myc. Interestingly, Ly+Te reduced the protein expression levels of all NF-κB regulated genes that were investigated and the decrease was most pronounced at 24hrs after treatment; although a clear decrease in the protein expression levels of c-IAP1, cyclin D1 and c-Myc occurred at earlier times.

4.3. Ly and Te inhibit TNF-alpha-induced NF-κB activity and suppress NF-κB-regulated genes

TNF-alpha is one of the major mediators of NF-κB activation and increasing evidence point to a protumorigenic role of the TNF-alpha-NF-κB activation axis (24). Therefore, we aimed to investigate the effect of drug
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Figure 2. Ly and Te inhibited nuclear levels of NF-κB and reduced NF-κB gene products. Combining Ly and Te at 0.6mM decreased basal p65 nuclear levels (A), increased IκB-alpha and pIκB-alpha protein expression and induced no changes in IKK-alpha/beta (B). HCT-116 cells were treated at 50% confluency for 24hrs followed by extraction of nuclear protein lysates or total protein lysates and immunoblotting with p65 or pIκB-alpha/IκB-alpha, respectively. In pIκB-alpha experiments, cells were incubated overnight with the proteasomal inhibitor PS341 before drug treatment. Ly and Te decreased the protein expression levels of NF-κB regulated genes involved in apoptosis and cellular proliferation (C). 24hrs after treatment with Ly and Te (0.6mM), total cell protein lysates were prepared and immunoblotted with Bcl-2, Bcl-xL, cIAP1, cIAP2, cyclin D1, c-Myc and GAPDH antibodies. Lamins and GAPDH antibodies were used to ensure equal loading.

combinations on TNF-alpha-induced NF-κB activation and specifically on the nuclear translocation of p65. Cells were treated either with 20ng/ml of recombinant TNF-alpha for 1hr or with Ly+Te (0.6 mM) for 4hrs prior to TNF-alpha treatment. Nuclear extracts were immunoblotted for the p65 subunit of NF-κB and compared to nuclear extracts derived from control cells or cells treated with drugs alone. TNF-alpha enhanced the nuclear translocation of p65 as expected, and Ly+Te caused a significant decrease in TNF-alpha-induced nuclear translocation of p65 (Figure 3A). To
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Figure 3. Ly and Te inhibited TNF-alpha-induced NF-κB activity and suppressed NF-κB-regulated genes. Ly and Te combination inhibited TNF-alpha-induced nuclear translocation of p65 (A). The block in p65 nuclear translocation was confirmed by immunocytochemistry using anti-p65 antibody (B). Cells were treated with 0.6mM drugs for 4hrs, 20ng/ml TNF-alpha for 15min or with drugs for 4hrs prior to treatment with TNF-alpha. Nuclear cell protein lysates were immunoblotted with p65 and lamins antibodies 24hrs later. White arrows indicate cells with p65 nuclear translocation. Ly and Te also blocked TNF-alpha-induced IκB-alpha degradation (C) and reduced Bcl-2 and Bcl-xL protein expression levels (D). Cells were either treated with TNF-alpha for 5min-60min or with 0.6mM drugs for 4hrs prior to TNF-alpha treatment. Whole cell protein lysates were immunoblotted with IκB-alpha, Bcl-2, Bcl-xL and GAPDH antibodies.

further validate this decrease, immunocytochemistry with p65 antibody was carried out in treated cells. Indeed, the drug combination significantly blocked the nuclear translocation of p65 induced by 15min incubation with TNF-alpha (Figure 3B), thus confirming the protein expression results. Subsequently, the drugs effects on TNF-alpha-induced phosphorylation of IκB-alpha were studied, as TNF-alpha is known to activate NF-κB signaling through phosphorylation-dependent degradation of IκB proteins (25). IκB-alpha degradation is known to result in NF-κB translocation to the nucleus where it upregulates the expression of Bcl-2 and Bcl-xL. Cells were either treated with TNF-alpha (10ng/ml) or with Ly+Te (0.6mM) for 4hrs prior to TNF-alpha. The combination treatment was capable of blocking TNF-alpha-induced degradation of IκB-alpha resulting in an increase in IκB-alpha protein expression (Figure 3C). The increase in IκB-alpha was obvious at all time points after TNF-alpha treatment. Interestingly, Ly+Te combinations did also modulate the downstream targets of NF-κB induced by TNF-alpha. While the Bcl-2 and Bcl-xL proteins increased in response to TNF-alpha alone, the expression levels of both proteins was reduced significantly upon incubation with drugs for 4hrs prior to TNF-alpha (Figure 3D).
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Figure 4. Ly and Te inhibited basal and TNF-alpha-induced DNA-binding activity of NF-κB. The DNA binding activity of NF-κB was determined by EMSA and in response to treating HCT-116 cells at 50% confluency with Ly and Te combinations (0.6mM) for 5hrs. To investigate drug effects on TNF-alpha-induced DNA-binding activity of NF-κB, cells were treated with Ly and Te (0.6mM) for 5hrs, 20ng/ml TNF-alpha for 1hr, or with Ly and Te for 4hrs followed by 20ng/ml TNF-alpha for 1hr. To test for the specificity of binding, either non-labeled normal NF-κB consensus or non-labeled mutant consensus was added separately in excess. For the supershift conditions, 1microl of p65, p50 or p52 antibodies were added to separate reaction tubes.

4.4. Ly and Te inhibit DNA-binding activity of NF-κB

In order to investigate the effect of Ly and Te on the promoter binding activity of NF-κB, Electrophoretic Mobility Shift Assay (EMSA) was performed. Ly+Te combination treatments significantly decreased the basal and TNF-alpha-induced DNA binding activity of NF-κB (Figure 4). The binding reaction was highly specific as shown by the competition when the non-labeled normal oligonucleotide was added. This competition was absent when the non-labeled mutant oligonucleotide was added to the reaction. Although the p50 antibody induced a moderate supershift, the p65 antibody induced a major one indicating that the NF-κB heterodimer is mostly comprised of p65 subunits.

4.5. Ly and Te chemosensitize cells exposed to oxaliplatin and 5-FU

Oxaliplatin and 5-FU, two chemotherapeutic drugs used against colorectal cancer, act through the selective induction of apoptosis in colon cancer cells (26). The clinically achievable doses of oxaliplatin range from 5-50 microM (27), while those of 5-FU from 0.5-500microM (28). We investigated if the drug combination chemo-sensitizes cells exposed to 5-FU and oxaliplatin. Cells were treated with different combinations of drugs and cell viability (live/dead cells) was measured by the trypan blue exclusion assay. Oxaliplatin (10microM) treatment alone for 24hrs reduced cell viability by 42%, while combining it with Ly and Te decreased the viability by 93% (Figure 5A). A dose of 1microM oxaliplatin, which is much lower than the pharmacologically achievable dose, caused 30% reduction in cell viability when used alone and 81% when combined with Ly and Te (Figure 5A). To study the type of interaction between Ly, Te and oxaliplatin, cells were treated with Ly+oxaliplatin, Te+oxaliplatin or Ly+Te+oxaliplatin followed by measuring the effect on cell viability. Our results confirmed that 1microM oxaliplatin chemo-sensitized cells when used in combination with both Ly and Te (Figure 5B). The three compounds synergized to result in 81%
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Figure 5. Ly and Te chemosensitize cells exposed to oxaliplatin and 5-FU. Combining Ly and Te potentiated the cytotoxic effects induced by high or low doses of oxaliplatin (A) or 5-FU (C). Cells were treated with oxaliplatin (1 microM or 10 microM), 5-FU (5 microM or 50 microM) separately or in combination with Ly+Te (0.6 mM). Separate or combined treatments with Ly and Te also acted synergistically to chemosensitize cells exposed to low doses of oxaliplatin (B) or low doses of 5-FU (D). Cells were treated at 50% confluency with 1 microM oxaliplatin alone, 5 microM 5-FU alone or in combination with Ly, Te or Ly+Te (0.6 mM). In all experiments, treatment was at 50% confluency and viability was determined by trypan blue exclusion method 24 hrs later. Each value is the mean ± SD of three separate experiments each done in triplicates. Bars with (*, ANOVA) or (†, two-tailed Tukey) are statistically different from their respective controls (p<0.01). (E) Annexin V assay showed that the mechanism of chemosensitization was by apoptosis and necrosis. Percentages of cells (± SD) of two independent experiments each in duplicates were determined using the Cell Quest program.

reduction in viability which is significantly greater than what is expected from an additive effect.

The concentration of 5-FU that results in 50% growth inhibition (GI₅₀) in HCT-116 has been reported to be 90 microM (28). Treatment of HCT-116 with 5-FU at 50 microM for 24 hrs decreased cell viability by 13% (Figure 5C), while combining it with Ly and Te reduced viability by 77%. Lowering the dose of 5-FU to 5 microM, which is significantly lower than GI₅₀, reduced cell viability by only 8% when applied alone. If combined with Ly and Te, a significant decrease in cell viability from 8% to 64% was observed (Figure 5C). To study the type of interaction between Ly, Te and 5-FU, cells were treated with Ly+5-FU, Te+5-FU or Ly+Te+5-FU and results showed that Ly and Te chemo-sensitized cells when combined with 5 microM of 5-FU (Figure 5D). These three compounds caused a synergistic decrease in viability of 65% which is significantly more than the expected additive effect.

To study the cellular mechanism of chemosensitization, cells were treated with Ly+Te in combination with 5-FU or oxaliplatin and then subjected to Annexin V staining after 24 hrs.
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A significant increase in the apoptotic and mixed (apoptotic and necrotic) populations was observed when Ly and Te were used in combination with oxaliplatin or 5-FU (Figure 5E) as compared to separate treatments, suggesting that both apoptosis and necrosis are the potential mechanisms of chemo-sensitization.

5. DISCUSSION

Previous work from our laboratory has shown that combining Ly and Te significantly inhibits the growth of a panel of colon cancer cell lines without major cytotoxicity to normal intestinal cells (3). In this study, we showed that Ly and Te, if combined, exert a synergistic anti-proliferative effect, induce apoptosis and necrosis, and the mechanism appears to be through the suppression of NF-κB activity. NF-κB inhibition by the drugs correlated with the upregulation of IκB-alpha conferring stabilization to the cytosolic IκB/NF-κB complex. In addition, drug combination blocked the DNA-binding activity of NF-κB. This inhibition was also observed in response to TNF-alpha induction. Ly and Te downregulated TNF-alpha-induced nuclear translocation of p65 and both basal and TNF-alpha-induced NF-κB-dependent gene products involved in anti-apoptosis and proliferation. The combination treatment also potentiated cell death induced by the colon cancer chemotherapeutics oxaliplatin and 5-FU.

In a recent study an integrated drug and gene expression database was used in data analysis to correlate the activity pattern of Ly to the activity pattern of 400 standard drugs and investigational substances in a panel of 10 cell lines (12). The results of the drug-gene database showed a high correlation between the activity pattern of Ly, and the activity pattern of the tyrosine kinase inhibitor PKC412, as well as to the activity pattern of the NF-κB inhibiting agents pyrithione zinc and disulfiram (12). A Connectivity Map for Ly using the breast cancer cell line MCF7 identified strong connection of Ly signature with several protein kinase inhibitors including rottlerin and tyrphostin AG-1478, in addition to several NF-κB inhibiting agents such as MG-132 and resveratrol. Ly and Te were also shown to downregulate the expression of the NF-κB regulated genes, Bcl-xL and Bcl-2, in human colon cancer cells (3). Because of these findings, and the importance of NF-κB in cancer progression, its constitutive activation in colorectal cancer (29) and its involvement in resistance to chemotherapy and radiotherapy (30), we investigated the effects of Ly and Te on the NF-κB pathway.

The canonical NF-κB pathway, which mostly targets p50/p65 is known to be responsible for blocking apoptosis through the expression of anti-apoptotic target genes under most conditions. Here we show that Ly and Te if used as separate agents or when combined inhibit basal NF-κB p65 nuclear translocation after 24hrs of treatment. This led us to investigate the effects on upstream regulators of NF-κB activity. The level of expression of the NF-κB inhibitor, IκB-alpha, was upregulated in the presence of drugs suggesting that the observed inhibition in p65 nuclear translocation is through stabilizing the IκB-alpha/NF-κB complex.

The ability of drug combination to upregulate the expression of IκB-alpha can occur through several mechanisms. The classical mechanism is through the inhibition of IKK complex which would result in blocking the phosphorylation and subsequent degradation of IκB-alpha. To investigate whether Ly and Te combination inhibited NF-κB nuclear translocation through inhibiting IKK-mediated phosphorylation of IκB-alpha, we studied their effect on the pIκB-alpha levels. Unexpectedly, we found that the level of pIκB-alpha increased in response to combining Ly and Te, suggesting that these drugs do not target IKK activity. Rather, other IKK-independent mechanisms which involve the inhibition of proteasomal activity may be at play (29). The effect on proteasomal activity requires further investigation and is the subject of a future study. Interestingly, Ly and Te, if combined, resulted in concomitant decrease in the expression of NF-κB target genes involved in apoptosis (Bcl-xl, Bcl-2, cIAP1, cIAP2) and cell proliferation (c-Myc and cyclinD1), suggesting that the observed enhancement of cell death in response to combination treatment is related to the modulation of key markers of apoptosis and proliferation.

Importantly, the TNF-alpha- NF-κB activation axis which is known to have a pro-tumorigenic role is suppressed by Ly and Te. Pretreatment with drugs for 4hrs significantly inhibited the nuclear levels of NF-κB in response to 1hr induction with TNF-alpha. This was further confirmed by immunofluorescence showing a decrease in the nuclear localization of p65. The drug combination was also capable of reversing TNF-alpha effects and increasing the expression level of IκB-alpha, suggesting that the observed inhibition in p65 nuclear translocation is through stabilization of the IκB-alpha/NF-κB complex. More so, combination treatment blocked the upregulation in the protein expression levels of Bcl-2 and Bcl-xL both of which are TNF-alpha-induced NF-κB-regulated gene products.

The inhibition of NF-κB nuclear translocation determined by western blotting on nuclear extracts of drug-treated cells is not sufficient to conclude their effect on NF-κB activity. In this respect, and in order to assess the effect of combinations of Ly and Te on DNA-binding activity of NF-κB, we performed the gel shift assay. The results of EMSA clearly demonstrate a significant decrease in both basal and TNF-alpha induced NF-κB DNA-binding activities in HCT-116 cells. Supershift analysis showed that complexes involved in the interaction consist mostly of p65 subunits.

As Ly and Te suppressed the expression of NF-κB-regulated genes that block apoptosis, it was expected that they would enhance apoptosis induced by chemotherapeutic agents. Chemosensitivity to oxaliplatin and 5-FU, two of the main chemotherapeutics used in the treatment of colorectal cancer, is highly influenced by the level of expression of anti-apoptotic Bcl-2 proteins (31, 32). Using Ly and Te in combination with either oxaliplatin or 5-FU resulted in a significant synergistic cytotoxic effect in HCT-116. This synergistic effect allows the use of doses of
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oxaliplatin or 5-FU that are lower than the range of clinical doses reducing the risk of adverse side effects that accompany administering high doses of these chemotherapeutic agents.

NF-κB activation has been shown to mediate the suppression of apoptosis through the expression of several antiapoptotic gene products. Downregulating Bcl-xL has previously shown to enhance apoptosis induced by oxaliplatin (31) and 5-FU (33) in colorectal cancer cell lines. Hence, by downregulating the NF-κB-dependent apoptosis gene products such as cIAP1, cIAP2, Bcl-2 and Bcl-xL, Ly and Te combination significantly enhanced the apoptotic effects induced by oxaliplatin and 5-FU.

The role of NF-κB and the signaling pathways that control its activity in carcinogenesis as well as in resistance to chemotherapy has become clear (29, 30). Although there are possible complications that may result from targeting NF-κB, the therapeutic potential and the benefits of such an approach in cancer therapy make it worth performing further studies in potential NF-κB inhibitors. Overall, our results show that Ly and Te combination may act as a potential suppressor of tumor cell survival through the inhibition of NF-κB activity. This also provides a lead toward the use of Ly and Te in combination with chemotherapeutic drugs that act through the induction of apoptosis. Our future attempt is to further elucidate the effect of Ly and Te on the IκB/NF-κB network of action and determine if they act as proteasomal inhibitors that block IκB degradation. Future studies would also aim to study the efficacy of combining Ly and Te with oxaliplatin or 5-FU in colon cancer animal models.

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7. REFERENCES


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Footnote: The term synergistic used in this article only refers to our gross observation that treatment with two drug combinations exerted an effect that was greater than what was expected from the sum of effects of the individual compounds used. Isobologram analysis for synergy was not done.

Key Words: Colon Cancer, Herbal Medicine, Linalyl Acetate, NF kappab, Alpha-Terpeniol, Sage

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