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Mechanisms of Cytotoxicity-Inducing Effect of 1,8-Cineole, a Plant Terpenoid, on Lepidopteran (*Spodoptera frugiperda*) Cell Line

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Abstract

Introduction: To the best of our knowledge, there is little information in regards to the cytotoxicity of 1,8-cineole. Insect cells exhibit wide resistance to the lethal effects of chemical compounds. Activation of p53 by acetylation can cause cell toxicity. This study has been carried out in order to investigate whether the use of 1,8-cineole in the *Spodoptera frugiperda* SF-9 cell line can induce cytotoxicity by increasing p53 acetylation expression or not.

Materials and Methods: SF-9 cell line was cultured in TC100 insect culture medium and treated with 1,8-cineole at concentration of 850.45 µmol/L, based on the half-maximal inhibitory concentration (IC_{50}) index at different times (24, 48 and 72h). The IC_{50} value was estimated for 1,8-cineole in SF-9. The percentage of alive cells was measured by MTT assay. The ELISA and Bradford protein techniques were used to detect endogenous levels of total and acetylated p53 protein generated in SF-9 cells.

Results: The findings of the present study indicated that treatment with 850.45 µmol per liter of 1,8-cineole shows a time-dependent increase in the number of dead cells of SF-9 cell line. The effect of severe toxicity on SF-9 was observed after 72 h of incubation with 1,8-cineole, with approximately 4% of SF-9 cells alive. We observed a significant increase in the level of p53 acetylation up to 48 h in SF-9, indicating that 1,8-cineole resulted in up-regulation of acetylated P53 and consequently p53 activation in SF-9 cells. Results showed that there is relationship between acetylated p53 protein levels and 1,8-cineole toxicity in SF-9 cell line.

Conclusions: 1,8-cineole, may function through common pathways and mediate their cytotoxic effects through targeting p53 and its acetylation in SF-9 cells.

Keywords: 1,8-cineole, p53, SF-9 Cell Line, Cytotoxicity

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Introduction

The monoterpene Oxide, 1,8-cineole (Eucalypol) is the main constituent of eucalyptus oil, an essential oil isolated from *Eucalyptus globulus* Labill used for food flavoring, as a fragrance in cosmetics and used as an insecticide.[1] Also, 1,8-cineole has an important role in the treatment of upper and lower air diseases due to its anti-inflammatory properties.[2] In addition, in vitro experiments in Swiss and Wistar rats have shown an analgesic effect of 1,8-cineole.[3] Further studies have shown the protective effect of 1,8-cineole in rodents againstethanol-induced gastric mucosal injury and liver failure.[4,5] In addition,adirectantibacterialactivityof1,8-cineole has beenreported.[6]

Only limited data are available on the toxicity and cytotoxicity of 1,8-cineole.[7] Lepidopteran insect cells exhibit wide resistance to the lethal effects of ionizing radiation, and to a number of chemicals including radiometric drugs, alkylating agents[8, 9] and peroxides such as H_2O_2 and coumain hydroperoxide.[10] In contrast, these cells have been shown to be more sensitive to DNA cross-linking agents such as mitomycin-C[8], as well as to actinomycin-D.[11] Investigating the molecular stress responses responsible for such inappropriate responses of lepidopteran cells is important for revealing the different alternating mechanisms present in this higher eukaryotic model system.

Studies have shown a distinct role of unusual protection against DNA damage as well as cell death in the radio resistance of lepidopteran samples.[12] Interestingly, alternative mechanisms for regulating radiation-induced apoptosis have recently been reported[13], strongly suggesting a need for a more detailed understanding of the mechanisms regulating cell death. Lepidopteran insect cells have been identified as

a peculiar chromatin organization that can be affected by numerous small-sized chromosomes. It is well known that compression of chromatin can play an important role in radiation resistance or some chemicals because DNA-bound histones along with some nuclear proteins protect against DNA damage.[14] However, chromatin compression may also affect other processes, including transcription as well as repairing and processing DNA damage.[15]

Chromatin remodeling can be of two types, the first involving ATP-dependent chromatin remodeling and the second involving histone modifications mainly acetylations / deacetylations.[16] A pair of enzymes that work to maintain dynamic equilibrium inside the cell, doing the acetylation / diacetylation activities. Histone acetyltransferases (HATs) are enzymes responsible for the acetylation of lysine residues of histones and other proteins, while deacetylase activities are performed by histone deacetylases (HDACs).[17]

Tumor suppressor protein p53 plays an important role in cellular response to DNA damage and other genomic abnormalities. Activation of p53 can cause cell cycle arrest, DNA repair and apoptosis. Succeeding DNA damage, p53 is acetylated at Lys382. HDACs mediate deacetylation of p53 and regulate the activity of this protein negatively.[18]

Following exposure to DNA damaging agents, the level of intracellular p53 increases primarily via inhibited degradation, and is associated with nuclear translocation and increased transcriptional activity. Accumulation of p53 in the nucleus activates a variety of downstream signaling pathways including cell cycle checkpoints that facilitate DNA repair, or alternatively the intrinsic pathway of cell death when damage is irreparable.[19]

A new study has shown that in lepidopteran insect cells, a separate hypoacetylated state is maintained in the core histones, which protects them from DNA damage and consequently cell death.[17] Therefore, we hypothesize that the use of 1,8-cineole in the SF-9 cell line can induce cytotoxicity by increasing p53 acetylation expression.

Materials and Methods

Cell line, treatment and culture conditions

The cell line (Sigma) used in the present study was cultured in the TC100 insect culture medium with 10% fetal bovine serum (FBS, sigma) and 1% penicillin streptomycin and incubated at 37°C and in humidified atmosphere containing 5% CO_2. The 1,8-cineole was dissolved in stock solutions and diluted to DMSO for appropriate concentrations. Cells> 80% confluent were growing exponentially in 25 cm^2 culture flasks and were regularly sub-cultured twice a week in exponential phase by seeding 40,000–50,000 cells/cm^2 area in 25 cm^2 culture flasks. Exponentially growing cells were used for all treatments with 1,8-cineole. Cells were kept in insect culture medium for 24 h and were then incubated with specific concentrations of 1.8-cineole based on IC_{50} at different times (24, 48 and 72 h).

MTT Assay (Cell sensitivity assay)

The MTT assay was commonly used to assess cell proliferation and viability by measuring the reduction of yellow MTT by mitochondrial dehydrogenases in viable cells.[20] This yield purple formazan crystals that detected colorimetrically at 570 nm. The IC_{50} values (concentration of 1,8-cineole required to inhibit cell viability by 50%) were obtained after 24 h of treatment. In summary, 10^4 cells were counted and placed into each well of a 24-well micro plate and treated with different concentrations of 1,8-cineole (0, 75, 150, 300, 600,1200, 2400 µM doses) for 24 h, and MTT survival assays werethen performed to evaluate cell viability with different drug concentrations.[20]

Bradford protein assay and ELISA

Total (intracellular) protein concentration was indicated by the Bradford method. Bradford Protein Quantification is an accurate method for determining protein concentration in solution based on the binding of Coomassie Blue dye to proteins. This procedure was performed before the Enzyme-Linked Immune Sorbent Assay (ELISA).Total protein extracted from SF-9 cells before and after 1,8-cineole treatment is described later in the ELISA method.[21]

Briefly, the Bovine Serum Albumin (BSA) was used at 9 different concentrations (0.25, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 mg/mL) for standard protein preparation. After diluting the protein, stock staining reagent was prepared (500 mg Coomassie Blue was dissolved in 500 ml methanol and added to 100 ml phosphoric acid and 50 ml ddH_2O) and diluted in 8 ml ddH_2O. Two ml of dye reagent was added to each standard protein tube and was incubated at room temperature for at least 5 min. Absorption of protein standards and experimental samples was performed by spectrophotometry (Bauss & Lamb, Germany) at 595 nm and finally a standard curve was plotted.

Sandwich ELISA assay was used to specifically identify endogenous levels of total and acetylated p53 protein production in SF-9 cells in the presence or absence of 1,8-cineole at different times (24, 48 and 72 h). In summary, cells were removed under 1,8-cineole-treated conditions at different times, and cells were washed with cold PBS. The PBS was removed and 0.5 mL ice-cold cell lysis buffer with 1 mM Phenylmethyl Sulfonyl Fluoride (PMSF) was added to each plate and incubated on ice for 5 min. Cells were separated from the plate and transferred to the appropriate tube and freezing and thawing experiments were performed three times. The tubes were centrifuged at 4°C for 10 min and the supernatant was transferred to a new tube. This supernatant was the cell lysates. For the ELISA assay, at first the total protein extract concentration in both cell lysates was determined by the Bradford method. The Sandwich ELISA was performed according to the manufacturer's protocol. Finally, the absorbance of samples were read in ELISA reader (Hyperion, Germany) at 450 nm wavelength and ELISA analysis was calculated based on control index**.** All experiments were processed independently three times for each group.

Statistical analysis

Quantitative data were presented as mean ± standard deviation and student t-test were used for statistical comparison. The SPSS 16.0 software was used for data analysis. Significance

was accepted at $P<0.05$.

Results

MTT assay and IC_{50}

After treatment of SF-9 cells with MTT solution in this method, dark blue formazan crystals were observed in the cells indicating their metabolic activity. The decrease in the number of cells was shown by the half-maximum inhibitory concentration (IC_{50}). The IC_{50} values for 1,8-cineole were estimated. The results showed that the basic concentration of 1,8-cineole for achieving IC_{50} in SF-9 cells at 24 h was 850.45 µmol/L (Figure 1).

The MTT results showed that based on the IC_{50} index 1,8-cineole at concentration of 850.45 µmol/L could significantly induce cytotoxicity in SF-9 cells which increased with time (Figure 2). The SF-9 dead cells showed a sharp increase at all times (≥50% at 24h, $P<0.05$ and ≥75% at 48h, $P<0.01$), so 1,8-cineole treatment arrested SF-9 cell proliferation (≥95% of inhibition) at 72 h. In the control samples that DMSO was used, a slight but non-significant cell death was observed in the cell line ($P>0.05$).

Bradford protein

The Bradford experiment was performed to determine total protein concentration in SF-9 cells lysates within 48 hours treatment with 1,8-cineole. The standard curve was linear in the range of 0.25 to 6 mg/mL of BSA. The total proteinconcentration for SF-9 cells was 5.17 µg/mL.

Acetylated and total p53 sandwich ELISA

To investigate the effects of 1,8-cineole on cell death, ELISA analysis was performed on SF-9 cells. Cells were treated with 850.45 µmol/1,8-cineole at different times (0, 24, 48 and 72 h) to investigate their effects on p53 acetylation status. Results of ELISA analysis were calculated based on control index. The results showed that 1,8-cineole can induce p53 acetylation in SF-9 cells and significantly increase in the total protein levels with ascending time up to 48 h treatment in SF-9 cells ($P<0.05$). Interestingly, between 48 and 72 h, a decrease in acetylated protein levels was observed in SF-9 cells (Figure 3a). Consistently, we also performed the above described procedure to evaluate the total p53 protein in the cell line. The results showed an increase in the total protein levels up to 48 h ($P<0.001$) (Figure 3b). In control samples (using DMSO without 1,8-cineole), there were negligible effects on total and acetylated p53 induction in the cell line at different time points of the study ($P>0.05$) (Figure 3a and Figure 3b). Also, a direct relationship was discovered between acetylated p53 protein levels and 1,8-cineole toxicity in SF-9 cell line.

Discussion

The SF-9 cells are highly resistant to a variety of stressors including DNA damage and ionizing radiation. It has been thought that relatively higher resistance to stressors may be due to its high-grade chromatin density in SF-9 cells compared to mammalian cells.[14, 15]

The potency and functional mechanisms of 1,8-cineole at the concentration evaluated were confirmed by IC_{50} in the SF-9 cell line. In this study, treatment with 850.45 µmol per liter of 1,8-cineole at different times showed a time-dependent increase in the number of dead cells of SF-9 cell line. The effect of severe toxicity on SF-9 was observed after 72 h of incubation with 1,8-cineole, with approximately 4% of SF-9 cells alive. Therefore, it was hypothesized that in SF-9 cells, 1,8-cineole alone could induce cytotoxicity in these cells in a time-dependent manner.

When it was found that 1,8-cineole could induce cytotoxicity in these cells, we decided to study the molecular mechanisms involved in this process. To further investigate and determine the total and acetylated status of p53 in response to 1,8-cineole in cells, ELISA analysis was performed after ensuring total protein concentration using the Bradford method. We observed a significant increase in the level of p53 acetylation up to 48 h in SF-9, indicating

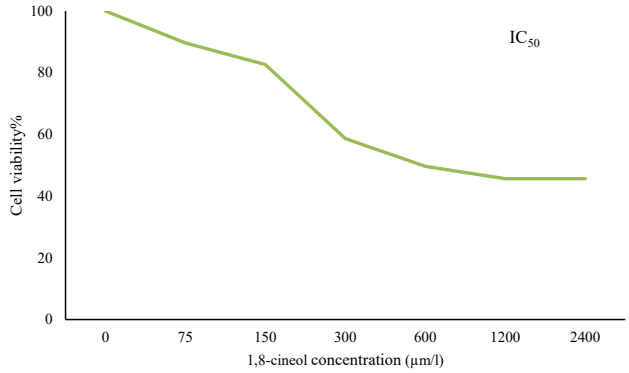

Figure 1. IC_{50} assay for the analysis of half-maximal inhibitory concentration of 1,8-cineole in SF-9 cell line after 24-h of treatment. Cells were incubated with or without 1,8-cineole using 0,75, 150, 300, 600, 1200, 2400 µM doses and the relative amount of viable cells were estimated by measuring the absorbance of the cell suspension after incubation with MTT. The graph of viability versus drug concentration was used to calculate IC_{50} values base on trend line equation for SF-9 cell line (850.45 µM).

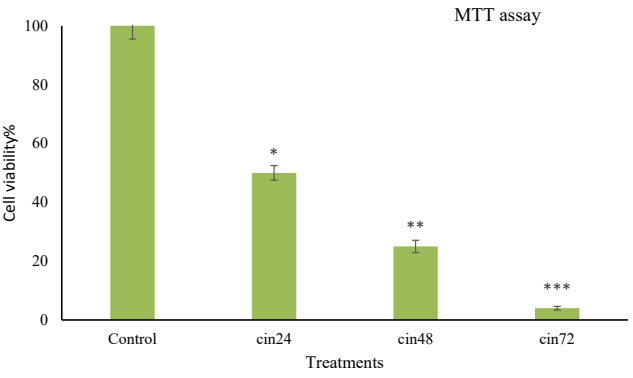

Figure 2. Relative levels of alive cells in SF-9 cells treated with 850.45 µM/L 1,8-cineole for different times. Cells incubated with the vehicle (DMSO) were used as a control. Values are the means+SD for triplicate experiments. Results are representatives of 3 independent experiments (n=3)
*$P<0.05$, **$P<0.01$, ***$P<0.001$
Cin24: Effect of 1,8-cineole after 24 hours
Cin48: Effect of 1,8-cineole after 48 hours
Cin72: Effect of 1,8-cineole after 72 hours

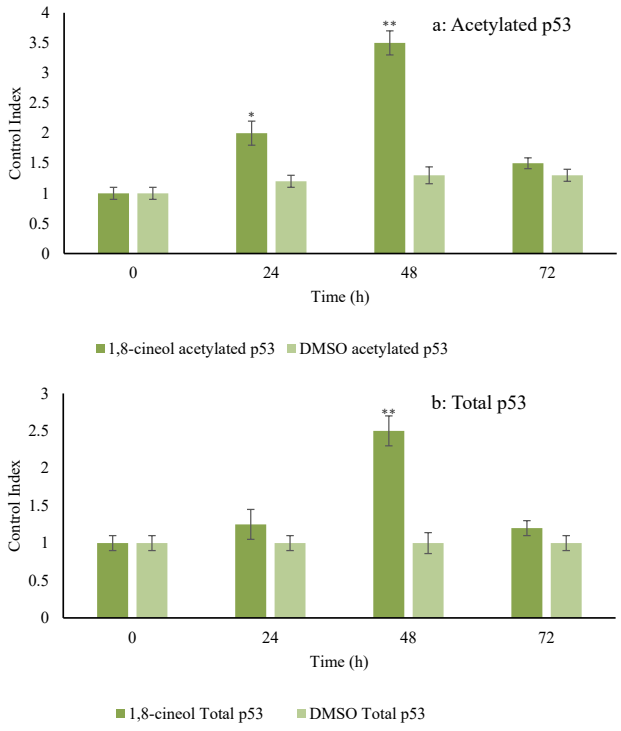

Figure 3. The results of ELISA assay of acetylated (a) and total (b) p53 protein generated in Sf-9 cells based on control index. Cells were treated with 850.45 µM of 1, 8-Cineol for 0, 24, 48 and 72 h. Between 48 and 72 h, a decrease in total and acetylated protein levels was observed in Sf-9 cells. No significant difference was shown in total and acetylated p53 content of in other groups. Results are representatives of 3 independent experiments (n=3). Values are the means + SD for triplicate experiments.
*$P<0.01$, **$P<0.001$

that 1,8-cineole resulted in up-regulation of acetylated p53 and consequently p53 activation in SF-9 cells. Also, a direct relationship was discovered between acetylated p53 protein levels and 1,8-cineole toxicity in SF-9 cell line. These results suggest that incubation of SF-9 with 1,8-cineole may increase p53 protein acetylation and cytotoxicity in SF-9 cells. On the other hand, however, 1,8-cineole can effectively activate p53 and subsequent cell death in SF-9 cell line. This finding is consistent with previous findings.[22]

Tumor suppressor p53 is one of the most extensively studied DNA damage responsive protein, which involves hundreds of genes and proteins which plays an important role in maintaining genomic stability, tumor suppression as well as in cellular responses to various types of genotoxic agents.[23]

In response to DNA damaging factors, p53 is known to be regulated spatially and temporally by many post-translational modifications. Acetylation is an important covalent alteration following p53 that has been observed in response to DNA damage. Acetylated p53 levels may be an important regulator of p53 function.[24]

In normal cells, p53 is a short-lived protein due to the activity of Mdm2 (its negative regulator), as ubiquitin ligase, which inhibits and destabilizes p53, thus rendering p53 levels undetectable and inactive to induce cytotoxicity.[19] Response to different types and levels of stress that cause DNA damage, the HAT family mediates p53 acetylation at the C terminus and blocks some important sites of p53 ubiquitination by Mdm2.[25] This function results in the stabilization of p53 protein and a significant increase in the amount and activity of p53 protein .The balance between p53 acetylation and deacetylation is mediated by HATs and HDACs.[26] Studies show that drug inhibition of HDACs may induce apoptosis by direct acetylation of p53 in some cells.[26]

Eukaryotic chromatin compression is regulated by the status of histone acetylation, which may induce DNA damage and processing in response to stress. Loss of chromatin compaction after treatment with HDAC inhibitors has been reported previously.[27] The ROS generation has been identified as a mediator of HDAC inhibitor-induced cell death.[28] It is demonstrated that HDAC inhibition induces apoptosis in SF-9 cells, along with increased intracellular ROS, increased mitochondrial membrane lipid oxidation (cardiolipin), and mitochondrial membrane potential loss. Intracellular ROS production after exposure to stress results from mitochondrial electron transport chain dysfunction and can thus induce apoptosis.[29,30]

In addition, increased accumulation of DNA damage through disruption in response to DNA repair leading to cell cycle arrest in combination with mitochondrial dysfunction has been observed in many cell types after exposure to HDAC inhibitors. Also it has been reported that release of cytochrome c from mitochondria in SF-9 cells following cardiolipin oxidation is primarily mediated by Bax-dependent mechanism.[31,32] Therefore, 1,8-cineole may act as a HDAC inhibitor in SF-9 cells . Further studies dissecting signaling events are required to understand 1,8-cineole-induced stress signaling in these cells.

The results of the present study may suggest that the higher degree of p53 acetylation in SF-9 cells in response to 1,8-cineole is largely mediated by the balance between histone acetyltransferase and histone dactylase which needs further studies.

Although an increase has been observed in the acetylated levels of p53 in response to 1,8-cineole, the p53 protein levels was decreased because of proteasome release and destruction within cells after 48 h treatment of the cells.

Conclusions

Results showed that there is a significant increase in the level of p53 acetylation up to 48h in SF-9, indicating that 1,8-cineol resulted in up-regulation of acetylated P53 and consequently p53 activation in SF-9 cells. Also, a relationship existed between acetylated p53 protein levels and 1,8-cineol toxicity in SF-9 cell line. It is suggested that 1,8-cineole, may function through common pathways and mediate its cytotoxic effects through targeting p53 and its acetylation in SF-9 cells.

Authors' Contributions

All authors equally contributed to the present study

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

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