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Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line, A549

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Abstract

Carvacrol, the predominant monoterpene in many essential oils of Labitae including Origanum, Satureja, Thymbra, Thymus, and Corydothymus has substantial antibacterial, antifungal, antihelmintic, insecticial, analgesic and antioxidant activities. Approximately 75% of lung cancer is non-small cell carcinoma (NSCLC) which comprises several histologic types squamous cell, adenocarcinoma and large cell carcinoma. It was reported that the portion of lung tumors diagnosed as denocarcinoma has increased. Thus a human non-small cell lung cancer (NSCLC) cell line, A549 was chosen for this study.

To investigate the effects of carvacrol on cell morphology, apoptosis and total protein amount, the cells incubated with various concentration of carvacrol in DMSO for 24 h. In carvacrol applied A549 cell line, increase in dose of carvacrol caused a decrease in cell number, degeneration of cell morphology and a decrease in total protein amount. To characterize carvacrol induced changes in cell morphology, cells were examined by light microscopy. Cells were treated with carvacrol were seen to have detached from the disk, with cell rounding, cytoplasmic blebbing and irregularity in shape. The data demonstrate that carvacrol is very potent inhibitor of cell growth in A549 cell line.

Abbreviations: DMSO – dimethyl sulfoxide; EDTA – ethylenediaminetetraacetic acid; IFO – Institute for Fermentation, Osaka; PBS – phosphate buffered saline

Introduction

Carvacrol is a phenolic compound present in the essential oil fraction of oreganum and thyme. It has been shown to exhibit antibacterial and antifungal activity and its primary site of toxicity is the membrane, causing damage and collapse of the integrity of this membrane and leakage of vital intracellular constituents (Periago and Moezelaar 2001).

A549, a malignant cell line, which was derived from human lung carcinoma (Raghu et al. 1986). The lungs include the respiratory bronchioles, alveolar ducts, alveolar sacs, and the alveoli. The alveoli are lined with two types of cells, type I and type II pneumocyte. The type I pneumocyte is a very large thin cell strectched over a very large area. This cell can not replicate and is susceptible to a large number of toxic insults. The type II pneumocyte is smaller, roughly cuboidal cell that is usually found at the alveolar septal junctions. The type II pneumocyte will replicate in the alveoli and will replicate to replace damaged type I pneumocytes. A549 cells resemble

type II cells in a number of important features, because they are readily cultured and derived from a human source, and they are widely used as a model of type II cells (Umimo et al. 2000). For this reason A549 cell line was chosen for this study.

Apoptosis is a physiological process leading to cell death (Chen et al. 2002). It plays an important role as a protective mechanism in the organism by removing damaged cells or over-proliferating cells due to improper mitotic stimulus (Lee et al. 2002). The earliest morphological changes in apoptotic cells include cytoplasmic shrinking, loss of cell–cell contacts and active membrane blebbing (Sutherland et al. 2001). The induction of apoptosis has become a target strategy for antitumor drug discovery in recent years, and an apoptosis-inducing agent specific for tumor cells may be an ideal antitumor drug (Chen et al. 2002).

In this study, we report that the carvacrol was cytotoxic towards a human non-small cell lung cancer (NSCLC) cell line (A549) and caused the cells to undergo apoptotic cell death.

Materials and methods

Plant material and the carvacrol

The plant material, *Origanum onites L.*, was collected from West Anatolia. Carvacrol (2-methyl-5-(1-methylethyl)phenol) was obtained by fractional distillation of *Origanum onites L.* essential oil. Carvacrol was dissolved in DMSO (Merck) and stored at +4 °C.

Cell culture and exposure to carvacrol

A549 cell line was obtained from Institute for Fermentation, Osaka (IFO, Japan). The cells were maintained as a monolayer in nutrient mixture F-12 HAM (Sigma) Medium containing 10% (v/v) foetal bovine serum (Sigma) and were incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. The medium was replaced with fresh medium and carvacrol was added. Carvacrol was diluted in the culture medium so that DMSO did not exceed 0.1% of total incubation volume. Negative control group was untreated A549 cells and positive control group was treated with DMSO alone. To investigate the effects of carvacrol on cell morphology, cell number, protein concentration and apoptosis, the cells were incubated with various concentration of carvacrol (100, 250, 500 and 1000 μM) for 24 h. These were added to the flasks at the time of plating cells. All incubation sets were repeated at least three times with duplicate samples.

Cell number and viability

To assess the effects of carvacrol on cell number, A549 cells were plated onto 25 cm^2 tissue culture flask at a seeding density 1×10^6 cells and allowed to attach for 24 h. After 24 h, the medium was removed and the monolayers were washed twice with sterile PBS solution (137 mM NaCl, 2.7 mM KCl, 15 mM KH_2PO_4, 8 mM Na_2HPO_4; pH 7.3) and then incubated with a trypsin-EDTA solution (Sigma) at 37 °C for 2 min. The cells were resuspended in PBS solution. The cell number and viability were determined by counting the cell using a hemocytometer after the cell suspension was mixed equally with 0.4% of trypan blue solution.

Cell staining

The morphology of the cells treated with carvacrol was examined after staining MayGrunwald (Fluka)-Giemsa (Sigma) method which was modified from Clark (1981). Cell monolayers grown on coverslips were washed three times sterile PBS solution for 1 min, and fixed in cold metanol for 1 min, then washed again steril PBS solution. Then, coverslips were stained in MayGrunwald solution for 2 min, after that half of the MayGrunwald solution was removed and replaced with same amount of distilled water. After 1 min, solution was discarded and coverslips were washed with distilled water and coverslips were stained with 50% Giemsa solution for 5 min. After three times distilled water washes, one drop of Entellan (Sigma, USA) was put on a microscope slide and coverslip was mounted. Subsequently, cells were analyzed using Olympus BX 50 microscope and photographed using Kodak 100 ASA film.

Protein determination

Protein was measured by the method of Bradford (1976), using bovine serum albumin as a standard.

Results and discussion

A549 cells were treated with carvacrol at the concentrations of 100, 250, 500 and 1000 μM for 24 h. A549 cell normally has a polygonal shape and sheetlike pattern in normal monolayer culture, which is compatible with its epithelial origin. The cells treated with DMSO and 100 μM carvacrol did not show any morphological changes. However, after 250 μM carvacrol treatment, the shape of cells was changed dramatically, when compared with that of (+)–control and (−)–control cells. The treated cells acquired a spindle shape and resembled mesenchymal cells. The cells treated with 500 and 1000 μM carvacrol showed some apoptotic characteristics as well as morphological changes (Figure 1). Carvacrol also affected cell number and total protein content. The cell number decreased in 250, 500 and 1000 μM carvacrol treated cell plates whereas the cell number increased in 100 μM carvacrol treated plates and (+) and (−)–controls (Figure 2). Similarly, the protein content of the treated cells decreased when they were treated with 250, 500 and 1000 μM carvacrol (Table 1).

Natural products have been shown to be excellent and reliable sources for the development of new drugs. Carvacrol is a *p*-menthane type aromatic monoterpene which can be found in the essential oils of many aromatic plants (Martins et al. 1999). Monoterpenes are compounds found in the essential oils extracted from many plants, including fruits, vegetables, spices and herbs. These compounds contribute to the flavor and aroma of plant from which they are extracted. Monoterpens are acyclic, monocyclic, or bicyclic C 10 compounds synthesized by monoterpene synthases using geranyl pyrophosphate (GPP) as substrate. GPP is also the precursor in the synthesis of farnesyl pyrophosphate (FPP) and geranyl–geranyl pyrophosphate (GGPP), two important compounds in cell metabolism of animals, plants and yeast. Monoterpene cyclases produce cyclic monoterpenes through a multistep mechanism involving a universal intermediate, a terpinyl cation which can be transformed to several compounds. Experimental studies, using animal cancer models, have demonstrated that some monoterpens possess anticarcinogenic properties, acting at different cellular and molecular levels. Loza-Tavera (2001) claimed that monoterpens could be considered as effective, non-toxic dietary antitumorigenic agents that hold promise as a novel of anticancer drugs.

Inhibitory effect of carvacrol on DMBA-induced tumorigenesis in rats was shown (Zeytinoglu et al. 1998). Furthermore to support this, curing effect of carvacrol on DMBA-induced tumorigenesis in rat lung was conducted from the histological point. After DMBA treatment, deformation on the structure of alveolar occured, the number of type I cells decreased. and number of type II cells increased. This deformation was curred with carvacrol. After carvacrol treatment the number of of type I cells increased and the number of type II cells decreased. According to our results, carvacrol inhibited viability and proliferation of A549 cells and induced early apoptotic features in a dose-dependent manner. This result is in good agreement with the histological events described above Stammatia et al. (1999) and He et al. (1997) also reported similar effects of carvacrol on Hep-2 cells derived from a human larynx carcinoma and B16 melanomas, respectively. To understand the effectiveness of carvacrol on normal lung cells, HFL1 cell line was used. The same procedure was applied to HFL1 cells. It was shown that carvacrol has no significant effects on HFL1 cells. In this study, A549 cells treated with 500 and 1000 μM carvacrol exhibited cytoplasmic shrinkage and loss of cell–cell contacts which are the earliest changes seen in apoptotic cells. The cytoplasm and nuclear chromatin of these cells became condensed and fragmented as reported in (Sutherland et al. 2001). Carvacrol treated A549 cell line showed differences in cell number, cell morphology and total protein amount depending on carvacrol doses when compared with untreated (control) A549 cell line. In carvacrol applied A549 cell line, increased dose of carvacrol caused a decrease in cell number, total protein content, and degeneration of cell morphology.

In conclusion, it can be suggested that carvacrol may have an anticancerogenic effect and it could be used as a drug substance to cure cancer. However, more research concerning the possible

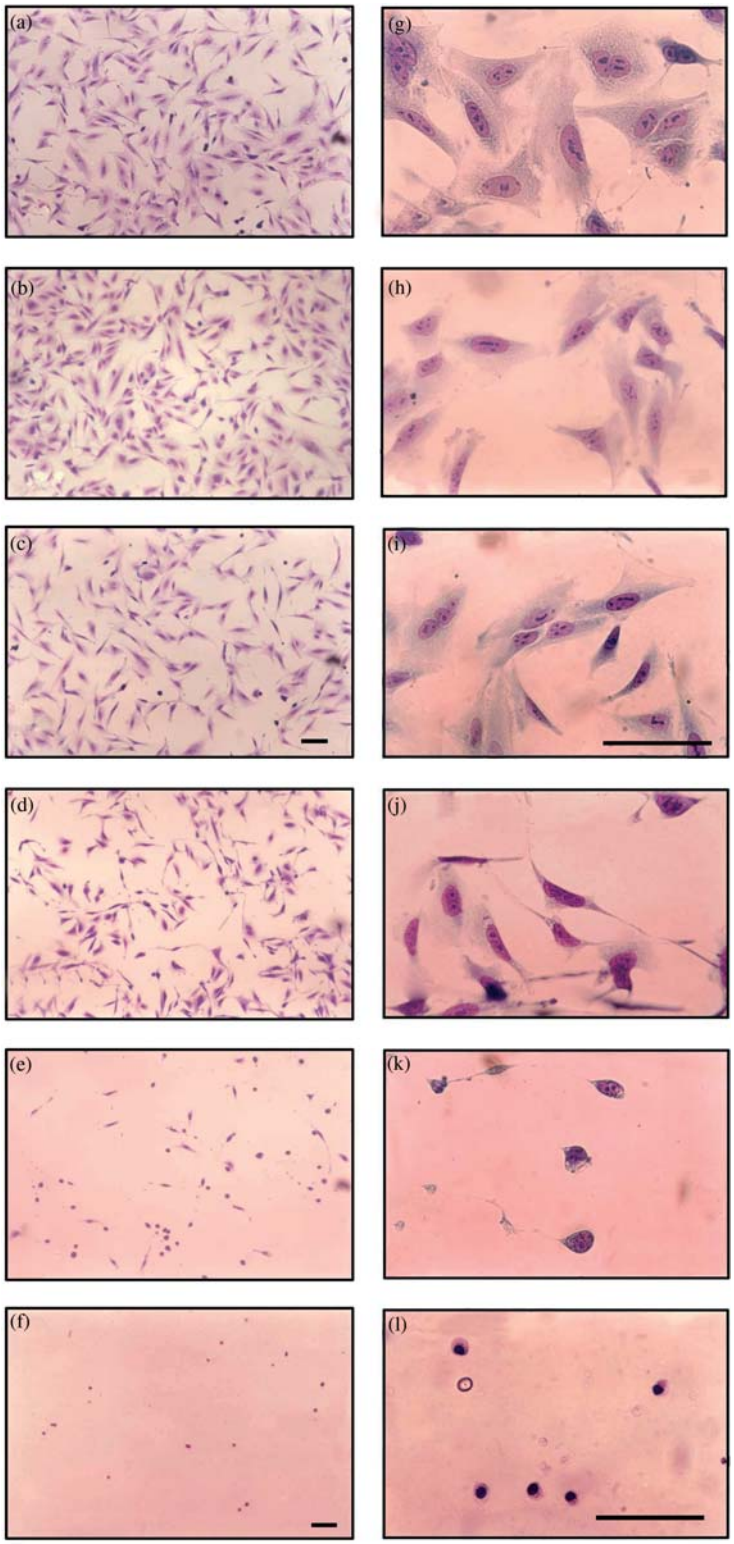

Figure 1. Effects of carvacrol on untreated A549 cells (a,g), treated with only DMSO (b,h), treated with 100 μM carvacrol (c,i), 250 μM carvacrol (d,j), 500 μM carvacrol (e,k) and 1000 μM carvacrol (f,l). Scale bar: 125 μm.

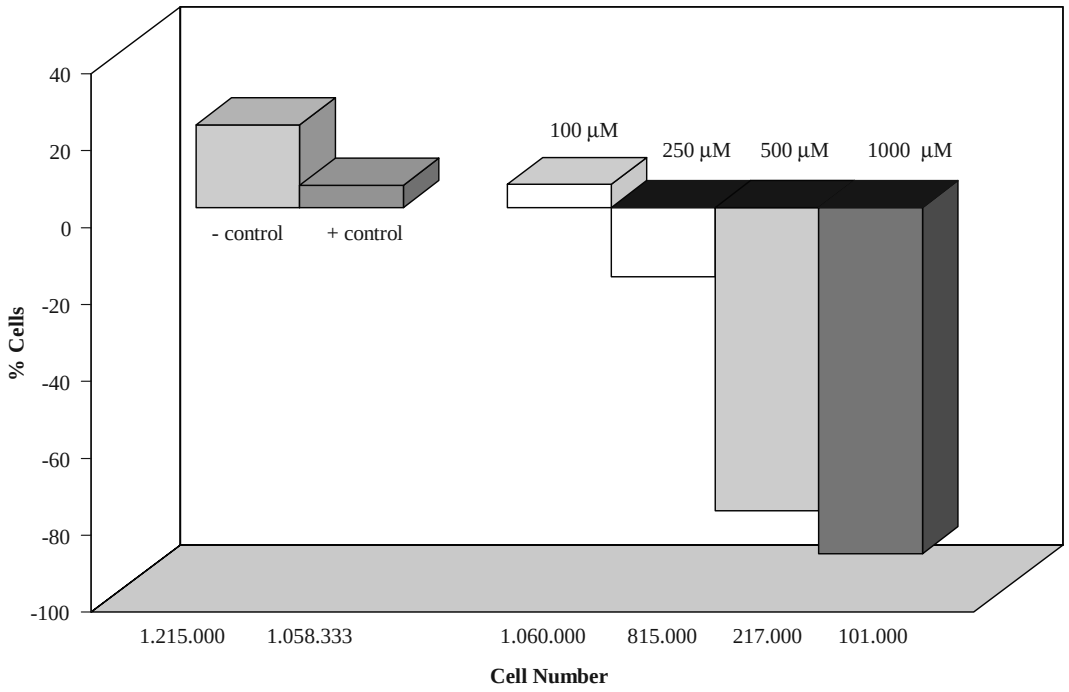

Figure 2. A representitive evaluation of dose-dependent effect of carvacrol on the proliferation of A549 cells.

Table 1. Spectrophotometric measurement of A549 cells

	Total protein content
Negative control	26.2 μg/ml
Positive control	29.5 μg/ml
100 μM carvacrol	20.0 μg/ml
250 μM carvacrol	15.2 μg/ml
500 μM carvacrol	4.7 μg/ml
1000 μM carvacrol	–

utility of carvacrol in cancer treatment is warranted.

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