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Synergistic antiproliferative and anticholesterogenic effects of linalool, 1,8-cineole, and simvastatin on human cell lines

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ABSTRACT

Monoterpenes are naturally occurring plant hydrocarbons with multiple effects on the mevalonate pathway (MP), while statins competitively inhibit hydroxymethylglutarylcoenzyme-A reductase (HMGCR), the rate-limiting enzyme in the MP. Monoterpenes and statins proved capable of inhibiting both proliferation and cholesterogenesis. In the present study we assess the *in vitro* antiproliferative and anticholesterogenic effects of two monoterpenes: linalool and 1,8-cineole—either alone, in combination with each other, or combined individually with simvastatin—on liver-derived (HepG2) and extrahepatic (A549) cell lines. The three compounds alone inhibited cell proliferation in a dose-dependent fashion, while their pairwise combination produced synergistic antiproliferative effects in both cell lines. Incorporation experiments with [^{14}C]acetate revealed that linalool and 1,8-cineole inhibited the MP, probably at different points, resulting in a reduction in cholesterogenesis and an accumulation of other MP intermediates and products. Linalool or 1,8-cineole, either together or individually with simvastatin, synergistically inhibited cholesterol synthesis. At low concentrations both monoterpenes inhibited steps specifically involved in cholesterol synthesis, whereas at higher concentrations HMGCR levels became down-regulated. Added exogenous mevalonate failed to reverse the inhibition of proliferation exerted by linalool and 1,8-cineole, suggesting that HMGCR inhibition alone is not responsible for the antiproliferative activity of those agents. This work demonstrates that monoterpenes in combination with each other, or individually in combination with simvastatin synergistically inhibits proliferation and cholesterogenesis in the human cell lines investigated, thus contributing to a clearer understanding of the action of essential-oil components, and their combination with the statins, in the targeting of specific points within a complex metabolic pathway.

1. Introduction

The mevalonate pathway (MP) is a highly branched metabolic sequence that provides cells with bioactive molecules crucial in multiple cellular processes. The end products of the MP include sterol isoprenoids, such as cholesterol, and nonsterol isoprenoids, such as heme-A, dolichol, and ubiquinone [1,2].

The major branch point of this pathway resides at farnesyl diphosphate (FPP), precursor of the different final products. Furthermore, the addition of an isoprene unit to FPP yields geranylgeranyl diphosphate (GGPP). FPP and GGPP can be post-translationally adducted onto regulatory proteins by protein prenyltransferases to enable protein anchoraging to internal cell membranes and a consequent functional activation. Most of the known prenylated proteins are small GTP-binding species, including the Ras family—with those controlling cell growth and proliferation—and the Rho family—those being crucial mediators of cell migration—[3].

The rate-limiting point of the MP is the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonic acid, a reaction catalyzed by HMG-CoA reductase (HMGCR), one of the most extensively regulated enzymes in nature [2]. This regulation of the MP, however, can occur at multiple levels throughout the pathway [4].

Various drugs capable of interfering with the MP have been developed, among which pharmacons the statins (*e.g.*, lovastatin, simvastatin, and atorvastatin) competitively inhibit HMGCR [5,6] and deplete cells of downstream isoprenoids, including FPP and GGPP [7]; thus resulting in a reduction in FPP and a decreased *de novo* cholesterol synthesis. Although the statins are used abundantly and effectively in the treatment of hypercholesterolemia, side effects are associated with their use, such as myopathy and hepatotoxicity [8].

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Statins also exhibit antitumor activities in culture against various tumor cells of different origin, which action results primarily from a suppression of proliferation and an induction of apoptosis [9,10]. Nevertheless, conflicting results have been reported on the application of statins as anticancer agents in clinical practice [11–13]. The high concentrations needed to inhibit cell proliferation have been associated with an elevated toxicity, thus restricting their use as a monotherapeutic agent for cancer treatment.

In contrast, monoterpenes—components of the essential oils of many plants including herbs, vegetables, and fruits—are naturally occurring hydrocarbons produced by the condensation of two isoprene units that are used as raw materials in many fields; including spices, phytotherapy, perfumes, and cosmetics [14].

Since monoterpenes are relatively nontoxic, inexpensive, and available in an ingestive form; an increasing interest has arisen in the potential use of essential oils for treating different pathologies of relevant social impact such as diabetes [15], cancer [16], and hypercholesterolemia [17,18]. Certain monoterpenes and essential oils exhibit anticholesterogenic, antiproliferative, and proapoptotic activities in culture as well as moderate hypocholesterolemic, chemopreventive, and chemotherapeutic actions *in vivo* [16–21] These compounds have been suggested as exerting their action through multiple effects on the MP, including an inhibition of protein prenylation and a noncompetitive suppression of HMGCR activity [20,22] along with the targeting of certain other loci specifically involved in cholesterol synthesis [23,24].

Given that the mechanisms of action by which monoterpenes and statins inhibit the MP are different, we hypothesized that a combined treatment between one or more monoterpene and a statin might exert synergistic antiproliferative and anticholesterogenic effects.

Simvastatin, a compound derived synthetically from a fermentation product of *Aspergillus terreus*, is one of the most widely used statins in the treatment of hypercholesterolemia because of the ability to decrease cholesterol synthesis by acting primarily at the hepatic level.

Linalool is a naturally occurring monoterpene alcohol with a pleasant scent present in more than 200 species of plants such as mint, laurel, cinnamon, and citrus fruits and is also either a major or usual compound in most herbal essential oils and in both green and black teas [25].

The cyclic monoterpene oxide 1,8-cineole (cineole, eucalyptol) is present in many essential oils of plants including the eucalyptus and is traditionally used as a food flavoring agent, for treating symptoms of airway diseases, and in aromatherapy [26].

The present study was designed to determine the action of linalool and 1,8-cineole—both in combination or one of the two along with simvastatin—on cholesterogenesis and cellular proliferation in the liver-derived (HepG2) and extrahepatic (A549) tumor cells in culture.

2. Materials and methods

2.1. Reagents

Solvents were obtained from Carlo Erba (Milan, Italy); linalool >95%, 1,8-cineole 99%, mevalonolactone 97%, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma–Aldrich (St. Louis, MO, USA); neutral red from Anedra (Argentina); [^{14}C]acetate (56.8 Ci/mol) from Perkin Elmer Life Science, Inc. (Boston, MA); streptomycin Richet (Argentina); while Merck, Sharp and Dohme (Argentina) kindly provided simvastatin. The sodium salt of simvastatin was prepared by dissolving the drug in ethanol at 60 °C, adding equimolar amounts of NaOH, and then incubating at 60 °C for 1 h. The ethanol was finally evaporated under a stream of nitrogen and the salt dissolved in distilled water at a final concentration of 10 mg/ml [19].

2.2. Cell culture and treatment

The HepG2 human-hepatoma cells were purchased from the American Type Culture Collection. The A549 human-alveolar-adenocarcinoma cells were kindly provided by Dr. Amada Segal-Eiras (CINIBA, UNLP. Argentina). The cells were maintained in 75-cm^2 flasks in filter-sterilized Eagle's Minimal Essential Medium (MEM; Gibco, Invitrogen Corporation) supplemented with (Natocor, Córdoba, Argentina) 10% (v/v) fetal-bovine serum plus 0.1 mg.l^{-1} streptomycin in a humidified incubator at 5% (v/v) CO_2/air and 37 °C.

Cells were grown under standard conditions for 48 h. The medium was changed to fresh MEM plus 10% fetal-bovine serum containing increasing concentrations of linalool, 1,8-cineole, or simvastatin (to determine the half maximal inhibitory concentrations—the IC_{50}—for cell growth and cholesterol biosynthesis) or a combination of those compounds in pairs (to evaluate possible synergistic effects). For the structures of the two monoterpenes and the statin cf. Fig. 1. After 24 h, the cells were washed twice with phosphate-buffered saline (PBS: NaCl 137 mM; KCl 2.7 mM, 10.0 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4) and incubated in serum-free MEM Zinc option (IMEM-Zo) with the same additions for another 24 h.

In experiments with mevalonate, cells were treated with linalool, 1,8-cineole, or simvastatin plus mevalonate for 48 h under the same conditions as described for the monoterpenes and the statin.

The linalool, 1,8-cineole, and simvastatin added to the media had been previously dissolved in dimethyl sulfoxide. The final concentration of that vehicle in the control and supplemented media was 0.2% (v/v).

2.3. Cell viability and cell proliferation

2.3.1. MTT assay

Cell viability was measured by the MTT assay [27]. Since the cell lines employed in the present study had different proliferation rates, the number of cultured cells was adjusted to a density such that the cells grew exponentially before initiating the experimental incubations and that insured at the same time a linear relationship between cell number and the optical density as measured in the MTT assay at end of all treatments—that is, the HepG2 and A549 cells were seeded in 24-well plates at densities of 1.5×10^4 and 0.75×10^4 cells per well, respectively.

Cells were treated as described in Section 2.2 and incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 0.5 mg/ml in PBS for 3 h. The resulting formazan was dissolved in 0.04 M HCl in isopropanol and the absorbance at 560 nm measured with an Elisa reader (Beckman Coulter DTX 880 Multimode Detector).

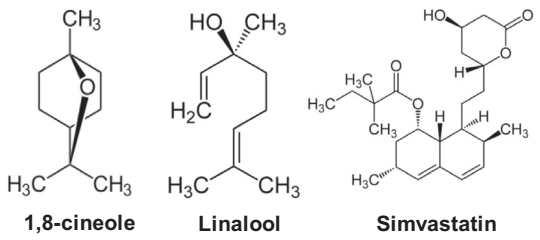

Fig. 1. Structural formulas of 1,8-cineole, linalool, and simvastatin.