

Geraniol and simvastatin show a synergistic effect on a human hepatocarcinoma cell line

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Simvastatin is a competitive inhibitor of 3-hydroxymethylglutaryl coenzyme A reductase activity, whereas geraniol is a monoterpene with multiple pharmacologic effects on mevalonate metabolism. Both of them inhibit growth and proliferation of many cell lines. The present study was designed to determine the action of geraniol, in combination with simvastatin, by assessing their effects *in vitro* on human hepatocarcinoma cell line (Hep G2). The treatment of Hep G2 cells with concentrations of simvastatin or geraniol that did not inhibit cell proliferation (5 $\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin and 50 $\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol) resulted in a significant inhibition of cell proliferation. We also examined the effect of simvastatin, geraniol and the combination of both on the biosynthesis of lipids from [¹⁴C]-acetate. Our results demonstrate that the combination of simvastatin and geraniol synergistically inhibited cholesterol biosynthesis and proliferation of Hep G2 cell line, contributing to a better understanding of the action of a component of essential oils targeting a complex metabolic pathway, which would improve the use of drugs or their combination in the fight against cancer and/or cardiovascular diseases. Copyright © 2011 John Wiley & Sons, Ltd.

KEY WORDS—geraniol; simvastatin; cholesterologenesis; cell proliferation; phospholipids

INTRODUCTION

The mevalonate pathway plays a critical role in cell growth and proliferation. Mevalonic acid is synthesized from 3-hydroxymethylglutaryl coenzyme A (HMG-CoA) in a reaction catalysed by HMG-CoA reductase, which is the rate-limiting enzyme in this pathway.¹ Its metabolism yields a series of sterol and nonsterol isoprenoids including cholesterol and nonsterol products that are vital for diverse cellular functions.^{1–4}

Statins are drugs that inhibit HMG-CoA reductase competitively. They are the first-choice agents for the treatment of hypercholesterolemia with demonstrated efficacy in reducing mortality and morbidity in the prevention of coronary disease and long-term reduction in cerebrovascular events.^{5–8} In addition to cholesterol-lowering properties, statins exhibit lipid-independent immunomodulatory and anti-inflammatory actions,^{9,10} stimulation of bone formation and inhibition of growth of tumor cells.¹¹ These effects primarily involve the inhibition of isoprenoid lipid production and subsequent protein prenylation and activity of signalling proteins such as the small GTPases, which affect the function of most cell types.¹² However, high concentrations are typically required to induce some of these effects *in vitro*, raising questions concerning therapeutic relevance.¹³

The effect of statins as anticancer agents has been evaluated extensively in cell culture; animal models and preclinical evaluation of their chemotherapeutic potential required high doses of the drug. Although an extremely uncommon side effect, myopathy has been long recognized as a potential risk of these drugs (muscle pain and weakness may affect 1–5% of statin-treated patients)¹⁴ and high-dose statin-associated toxicity also includes gastrointestinal dysfunction, elevated creatine phosphokinase, anorexia and ulcerative lesions.

Another class of compounds that affect cholesterol levels and cell proliferation is the monoterpenes. These plant isoprenoids from the mevalonate pathway are widely distributed in fruits and vegetables. It has been suggested that these compounds inhibit cancer growth and development through multiple effects on mevalonate pathway including inhibition of protein isoprenylation at the level of prenyl-protein transferase enzymes and HMG CoA reductase activity in a noncompetitive way.¹⁵

Monoterpenes, including geraniol, have been evaluated as potential anticancer agents. The anticancer effects of monoterpenes in cell culture and rodent models are well documented; however, their effects in clinical trials have been more difficult to demonstrate, and they presented multiple side effects and drug intolerance.¹⁶

Because the mechanisms of action by which statins and monoterpenes interfere on mevalonate pathway are different, it was hypothesized that combined low-dose treatment may have synergistic antiproliferative and hypocholesterogenesis effects.

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The present study was designed to determine the action of a monoterpene, geraniol, in combination with a statin, simvastatin, by assessing their effects *in vitro* on human hepatocarcinoma cell line (Hep G2).

MATERIALS AND METHODS

Reagents

[¹⁴C] Acetate (54.7mCi·mmol⁻¹) was purchased from Perkin Elmer Life Science, Inc. (Boston, MA). Inorganic reagents and solvents were of analytical grade. Dimethylsulphoxide (DMSO) was supplied by Analyticals Carlo Erba (Milan, Italy). Geraniol 98% was supplied by Sigma (St Louis, MO). Merck, Sharp and Dohme (Argentina) kindly provided simvastatin. Sodium salt of simvastatin was prepared by dissolving the drug in ethanol at 60°C; equimolar amounts of NaOH were added and incubated at 60°C for 1 h. Ethanol was then evaporated under nitrogen, and the salt was dissolved to a final concentration of 10 mg·ml⁻¹ in distilled water.¹⁷

Cell culture

Hep G2 cells were obtained from American Type Culture Collection and maintained at 37°C in confluent layers attached to 95-cm² flasks on Eagle's minimal essential medium (MEM) with 10% heat-inactivated fetal bovine serum (both from Gibco, Grand Island, NY).

To initiate experiments, cells (3×10^6 per flask) were seeded in 95cm² tissue culture flasks. When the cells were at logarithmic growth phase, the medium was changed to fresh MEM with simvastatin, geraniol, or a combination of both. Geraniol was dissolved in DMSO (final concentration of DMSO in control and treated bottles was 0.5%). After 24 h, the medium was removed; cells were washed three times with Hank's solution and incubated in serum-free MEM Zinc option (IMEMZo) with the same treatment for 24h. At the end of the experiment, the attached cells were washed three times with 5 ml ice-cold saline solution, detached from the growing surface mechanically with a rubber-tipped spatula and resuspended in 5 ml of the same solution. An aliquot of the resulting suspension was used to determine cellular protein content.¹⁸ The remaining cell material was centrifuged at 500 g for 10 min, and the pellet was processed for further analysis.

Viability and cell proliferation were determined via trypan blue dye exclusion cell counts in a hemocytometer,¹⁹ and also evaluated by measuring the mitochondrial-dependent reduction of colourless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a coloured blue formazan.²⁰ Cells were seeded in 24-well sterile plates at a density of 25000 cells per well for 48 h. After exposure of the cells to the test drugs, the medium was removed, and the cells were incubated with MTT (Sigma Chemical Co., St Louis, MO) (0.5 mg·ml⁻¹ in PBS) for 2 h. Formazan was dissolved in 0.04 mol·l⁻¹ of HCl in isopropanol, and the absorbance at a $\lambda=560$ nm was measured with an Elisa reader (Beckman Coulter DTX 880 Multimode Detector).

The number of viable cells is directly proportional to the production of formazan. All tests were performed three times in quadruplicate.

Lipid extraction and analysis

Total lipids from cell pellets were extracted with methanol/chloroform 2:1.²¹ An aliquot was used to separate and quantify free and esterified cholesterol by thin layer chromatography (TLC) using acidic ferric chloride solution as spray reagent,²² and a curve was constructed with pure standards run on the same plate. Spot images were analysed with a Kodak 1D Image Analysis Software Version 3.5 (Scientific Imaging Systems Eastman Kodak Company Rochester, NY). Another aliquot was saponified with 10% KOH in methanol at 85°C for 45 min. Nonsaponifiable lipids were extracted with petroleum ether (bp 30–40°C). Saponified fatty acids were extracted from the methanolic phase with light petroleum (bp 30–40°C) after acidification

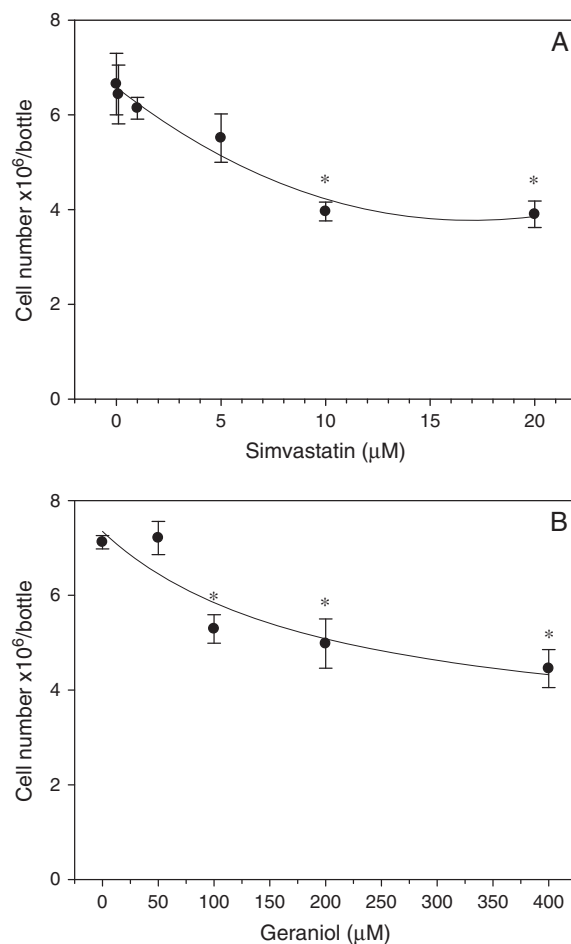


Figure 1. Effect of simvastatin (A) and geraniol (B) on growth rate of Hep G2 cells. Exponential growth culture cells in complete medium were treated with simvastatin or geraniol for 48 h. Viability and cell proliferation were determined using a trypan blue dye exclusion cell counts in a hemocytometer. Data are means \pm SD of four individual samples * $p < 0.01$ versus control (0 mmol·l⁻¹)

with HCl. The remaining lipid extract was used to analyse neutral and polar lipid species using TLC.

Incorporation of ^{14}C -acetate

Cells were incubated as previously described (see Section on *Cell Culture*), with the addition of [^{14}C] acetate ($3\mu\text{Ci}\cdot\text{ml}^{-1}$ culture medium) over the last 3 h. Lipid extraction was performed as previously described. Cholesterol and its precursors present in the nonsaponifiable fraction were separated using TLC on silica gel G developed in chloroform 100%²³ and identified by comparison with a standard mixture containing (Running factor (Rf) values in parenthesis) cholesterol (0.34) lanosterol (0.52) and

squalene (0.81) applied simultaneously onto the plate. Neutral lipids and polar lipids were separated by TLC on silica gel G using hexane–diethylether–acetic acid (80:20:1 v/v/v)²⁴ and chloroform–methanol–acetic acid–water (50:37.5:7.5:2 v/v/v/v),²⁵ respectively, as mobile phases. All lipid classes were identified by comparison with a standard mixture, and the appropriate zones of the plate were scraped off and recovered for radioactivity measurement.

Statistical analyses

Statistical analyses were performed by means of ANOVA GB-STAT Professional Statistics and Graphics Version 4.0. Dynamic Microsystems Inc. The Tukey *t*-test was used for mean comparisons. Statistical significance was assigned to $p < 0.05$.

RESULTS

Effect of simvastatin, geraniol and the combination of both on cellular proliferation and the content of cholesterol

When different concentrations of simvastatin or geraniol were added to the FBS containing conditioned medium of Hep G2, the compounds significantly inhibited the proliferation of cells in a dose-dependent manner starting from 10 and $100\mu\text{mol}\cdot\text{l}^{-1}$, respectively (Figure 1). The content of neither free nor esterified cholesterol was affected by the treatment (data not shown).

The treatment of Hep G2 cells with combined low doses of simvastatin and geraniol that significantly did not inhibit

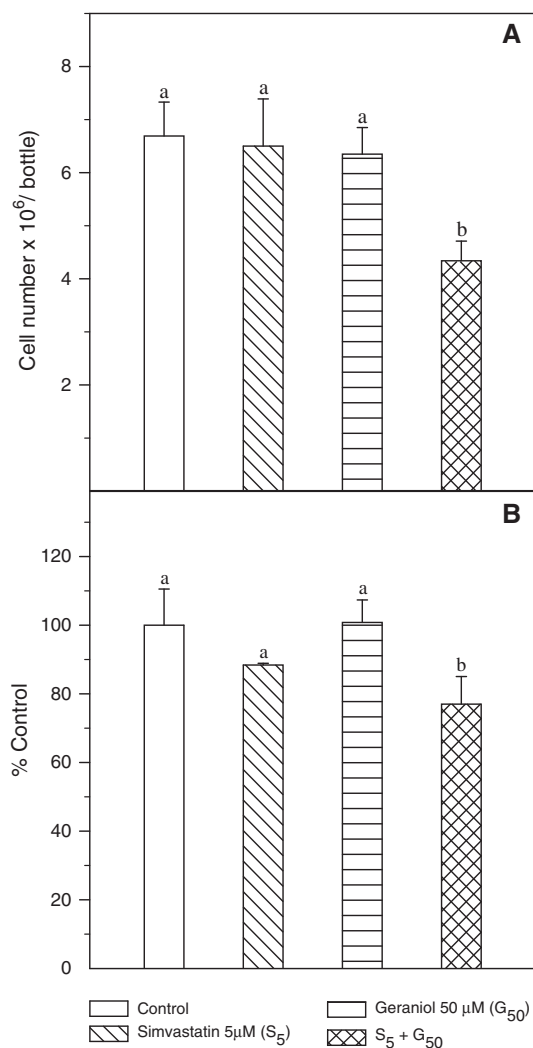


Figure 2. Effect of simvastatin+geraniol on cell proliferation of Hep G2 cells. Exponential growth culture cells in complete medium were treated with $5\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin, $50\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol and a combination of $5\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin and $50\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol for 48 h. Viability and cell proliferation were determined using trypan blue dye exclusion cell counts in a hemocytometer (A) and MTT assay (B). Data are means \pm SD of four individual samples. Means without common letters are different ($p < 0.05$)

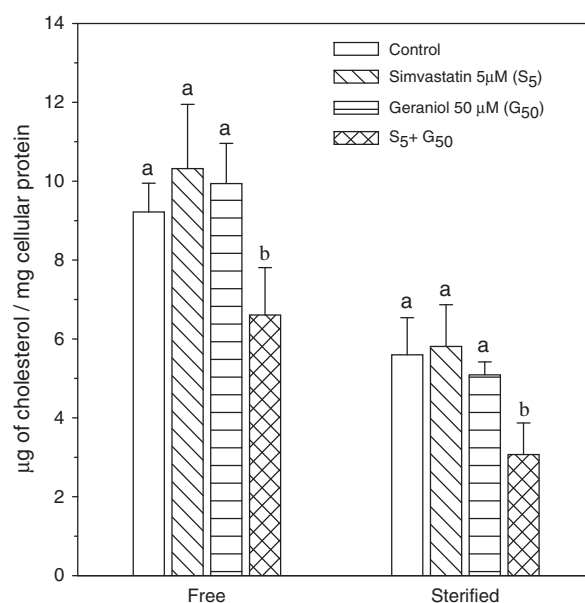


Figure 3. Effect of simvastatin+geraniol on free and esterified cholesterol of Hep G2 cells. Exponential growth culture cells in complete medium were treated with $5\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin, $50\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol and a combination of $5\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin and $50\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol for 48 h. Free and esterified cholesterol were separated and quantified using TLC. Data are means \pm SD of four individual samples. Means without common letters are different ($p < 0.05$)

cell proliferation individually ($5\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin and $50\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol) resulted in statistically significant inhibition of cell proliferation (35.12% by cell counting and 23% by MTT test) (Figure 2) and free and esterified cholesterol content reduction (26.51% and 45.7%, respectively) (Figure 3).

The viability of control cells as well as that of those treated with simvastatin or geraniol remained high during the experiment ($\geq 90\%$).

[^{14}C]-Acetate incorporation into lipids

We examined the effect of simvastatin, geraniol and the combination of both on the biosynthesis of lipids from [^{14}C]-acetate in Hep G2 cell line.

Figure 4 shows the incorporation of [^{14}C]-acetate into nonsaponifiable lipids. Simvastatin and geraniol inhibited acetate incorporation into nonsaponifiable lipids starting from 0.025 and $1\mu\text{mol}\cdot\text{l}^{-1}$, respectively. The combination of $0.01\mu\text{mol}\cdot\text{l}^{-1}$ simvastatin and $0.1\mu\text{mol}\cdot\text{l}^{-1}$ geraniol inhibited

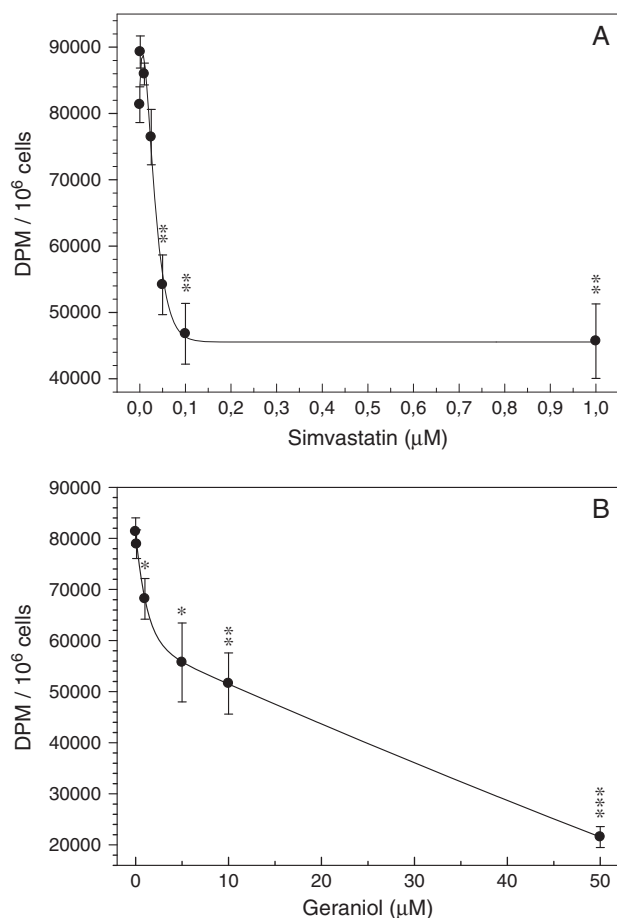


Figure 4. Acetate incorporation into nonsaponifiable lipids on Hep G2 cells. Exponential growth culture cells were treated with simvastatin (A) or geraniol (B) for 48h. After 24h of treatment, cells were incubated in MEM Zinc option (IMEMZo). [^{14}C]-acetate was added for the last 3h. Data are means \pm SD of four individual samples. * $p < 0.01$; ** $p < 0.001$.

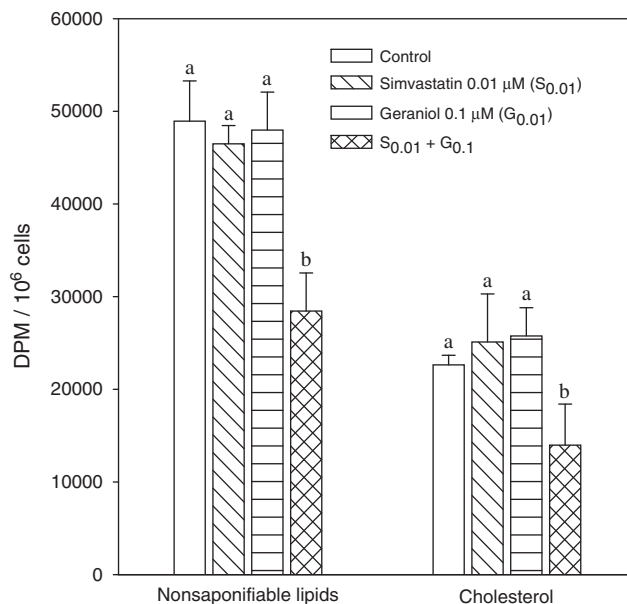


Figure 5. Acetate incorporation into nonsaponifiable lipids and cholesterol on Hep G2 cells. Exponential growth culture cells were treated with $0.01\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin, $0.1\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol or the combination of $0.01\mu\text{mol}\cdot\text{l}^{-1}$ of simvastatin and $0.1\mu\text{mol}\cdot\text{l}^{-1}$ of geraniol for 48h. After 24h of treatment, the cells were incubated in MEM Zinc option (IMEMZo). [^{14}C]-acetate was added over the last 3h. Data are means \pm SD of four individual samples. Means without common letters are different ($p < 0.05$).

acetate incorporation into nonsaponifiables by 41.9% and cholesterol by 38.2% (Figure 5).

To further examine the effect on the biosynthesis of nonsaponifiable and saponifiable lipids from [^{14}C]-acetate, the cells were incubated with doses of simvastatin and geraniol, which inhibited cholesterol synthesis without inhibiting cell proliferation. Simvastatin ($1\mu\text{mol}\cdot\text{l}^{-1}$), geraniol ($10\mu\text{mol}\cdot\text{l}^{-1}$) or the combination of both increased the incorporation of [^{14}C]-acetate into total fatty acids. Incorporation into cholesterol and its metabolic intermediates was significantly reduced after simvastatin treatment; however, although incorporation into cholesterol was reduced after geraniol treatment, incorporation into squalene was significantly increased (Table 1). The increment of

Table 1. [^{14}C]-acetate incorporation into insaponifiable lipids and total fatty acid

	Percentage of control			
	Control	Simvastatin $1\text{mol}\cdot\text{l}^{-1}$	Geraniol $10\text{mol}\cdot\text{l}^{-1}$	Simvastatin 1 $\text{mol}\cdot\text{l}^{-1}$ + geraniol $10\mu\text{mol}\cdot\text{l}^{-1}$
Total fatty acids	100 \pm 20 ^a	169 \pm 17 ^b	161 \pm 11 ^b	232 \pm 31 ^c
Total insaponifiable lipids	100 \pm 18 ^a	70 \pm 1 ^{b,d}	135 \pm 15 ^c	89 \pm 3 ^{a,d}
Cholesterol	100 \pm 14 ^a	59 \pm 11 ^b	70 \pm 5 ^b	67 \pm 15 ^b
Lanosterol	100 \pm 18 ^a	45 \pm 11 ^b	78 \pm 21 ^a	64 \pm 18 ^a
Squalene	100 \pm 9 ^a	80 \pm 8 ^a	216 \pm 73 ^b	104 \pm 31 ^a

Note: Values are means \pm SD ($n=4$). Means without common letters within a common lipid species are different ($p < 0.05$).

Table 2. [14 C]-acetate incorporation into saponifiable lipids

	Percentage of control			
	Control	Simvastatin 1 mol·l ⁻¹	Geraniol 10 mol·l ⁻¹	Simvastatin 1 mol·l ⁻¹ + geraniol 10 μmol·l ⁻¹
Cholesterol ester	100 ± 10 ^a	134 ± 26 ^b	153 ± 4 ^b	158 ± 8 ^b
Triacylglycerides	100 ± 6 ^a	178 ± 46 ^{b,c}	156 ± 12 ^b	220 ± 45 ^c
Sphingomyelin	100 ± 24 ^a	172 ± 25 ^b	181 ± 10 ^b	292 ± 60 ^c
Phosphatidylcholine	100 ± 8 ^a	165 ± 31 ^{b,c}	121 ± 12 ^{a,c}	213 ± 41 ^b
Phosphatidylserine	100 ± 44 ^a	187 ± 72 ^a	128 ± 38 ^a	215 ± 67 ^b
Phosphatidylinositol	100 ± 23 ^a	143 ± 65 ^a	187 ± 56 ^a	478 ± 231 ^b
Phosphatidylethanolamine	100 ± 19 ^a	190 ± 36 ^b	158 ± 22 ^{a,c}	194 ± 69 ^{b,c}

Note: Values are means ± SD ($n=4$). Means without common letters within a common lipid species are different ($p<0.05$).

[14 C]-acetate incorporation into squalene disappeared with the combination of simvastatin and geraniol, and this treatment only showed a diminution in cholesterol incorporation.

The [14 C]-acetate incorporation into phospholipid and neutral lipids is shown in Table 2. Treatment with simvastatin increased acetate incorporation into most lipid classes. Treatment with geraniol also increased acetate incorporation into neutral lipids and sphingomyelin, but the incorporation into phospholipids did not show any significant difference. Combined treatment with both drugs increased acetate incorporation into all the lipid species.

DISCUSSION

HMG CoA reductase inhibitors have been shown to interact additively or synergistically with other chemotherapeutic agents, such as 5-fluorouracil,^{26,27} N,N'-bis-2-chloroethyl-N-nitrosourea,²⁸ cisplatin, doxorubicin, paclitaxel,^{29–32} 1-β-D-arabinofuranosylcytosine^{33,34} and gemcitabine.³⁵ A synergistic impact of lovastatin and γ-tocotrienol also was shown in prostate carcinoma cells,³⁶ murine melanoma cells,³⁷ human DU145 prostate carcinoma and A549 lung carcinoma cells³⁸ and mammary tumour cells.^{39,40} Llaverías *et al.* have reported an additive effect between an acyl-CoA: cholesterol acyltransferase inhibitor and a statin on reduction in cell cholesterol ester content of human macrophages.⁴¹ This effect was attributed to the inhibition of hydroxymethyl glutaryl-CoA reductase and the depletion of some mevalonate derivatives.

In this study, we demonstrated that the combination of simvastatin and geraniol synergistically inhibits cholesterol biosynthesis and proliferation of Hep G2 cell line. Subeffective concentrations of simvastatin or geraniol that did not inhibit cholesterol synthesis if used alone were effective when used in combination. As we have already reported, a low concentration of geraniol does not inhibit HMG CoA reductase in Hep G2 cells, but it is likely to evoke an inhibition at a certain metabolic step between lanosterol and cholesterol.⁴² Ren and Gould⁴³ demonstrated that another monoterpene, perillyl alcohol, blocks the conversion of lanosterol to cholesterol in NIH3T3 cells. The synergistic effect produced by the subeffective

concentration of the compounds could be the result of two mechanisms, partial inhibitions of HMG CoA reductase by simvastatin and of cholesterol synthesis at a late stage of the metabolic pathway by geraniol.

Also, a subeffective concentration of simvastatin or geraniol that did not inhibit cell proliferation if used alone was effective when used in combination. As shown for different cell systems,^{44,45} the concentration of simvastatin required to inhibit Hep G2 cell proliferation is several times higher than the concentration required to inhibit cholesterol synthesis. Sinensky *et al.* have already described that the inhibition of HMG CoA reductase required to completely inhibit isoprenylation of proteins involved in cellular proliferation is within the order of 500-fold greater than that required for 50% inhibition of cholesterol synthesis. It seems that, as the mevalonate pool decreases, this intermediate is preferentially directed to the synthesis of nonsterol isoprenoids required for cell proliferation rather than cholesterol. In our system, the concentration of geraniol required to achieve an inhibition of cell proliferation is 100-fold greater than the concentration required to inhibit cholesterol synthesis significantly. We have previously reported that at concentrations that inhibit cell proliferation, geraniol inhibits HMG CoA reductase and ³H-mevalonolactone incorporation into protein fraction in Hep G2 cells.⁴² The inhibition of reductase by geraniol could counteract the well-known fact that statins upregulate HMG CoA reductase expression,⁴⁶ and this fact would explain in part the synergistic effect of the subeffective concentration of both compounds used in combination. The depleted mevalonate pool would, in consequence, diminish pools of farnesyl and other phosphorylated products, which isoprenylate various cellular proteins such as small G-proteins, nuclear lamins and heteromeric G-protein γ-subunit that are vital for cellular growth and proliferation. Nevertheless, we cannot rule out other described effects of geraniol to be involved in this synergistic effect. Changes in cell permeability, membrane and ion channel perturbation have been described as geraniol effects on human colon cancer cells *in vitro*⁴⁷ and *in vivo*.²⁷

Clinical application of statins has been limited because of the high-dose toxicity that can lead to rhabdomyolysis and even death.⁴⁸ Our results may potentially represent an

interesting approach for future clinical trials using lower doses of statins in combination with monoterpenes.

To provide data on the effects of the combination of simvastatin and geraniol on lipid metabolism in Hep G2 cells, we studied the acetate incorporation into the lipid classes. We used doses of simvastatin and geraniol that inhibited cholesterol biosynthesis but not cell proliferation. The inhibition of acetate incorporation into all the insaponifiable lipids reflected the inhibition of the incorporation into mevalonate because of the inhibition of HMG CoA reductase caused by statin. In contrast, as previously mentioned, the concentration of geraniol lower than $50 \mu\text{mol}\cdot\text{l}^{-1}$ did not inhibit HMG CoA reductase activity in Hep G2 cells. The increased incorporation into squalene and the decreased incorporation into cholesterol without any effect in lanosterol would reflect an inhibition of the metabolic pathway between this intermediate and lanosterol. When both compounds were incubated together, there was no longer an accumulation of acetate incorporation into squalene. This would reflect again the decreased incorporation into mevalonate because of the effect of simvastatin coupled to an inhibition of the metabolic pathway between squalene and cholesterol.

The incorporation of acetate into triglycerides and sphingomyelin reflected an increased incorporation into fatty acids produced by the incubation of cells with simvastatin, geraniol or their combination. The same applies to the phospholipids of cells incubated with simvastatin. In contrast, the majority of the phospholipids of cells incubated with geraniol showed a nonsignificant increase in the incorporation of acetate despite the increase in total cellular fatty acids. We attributed this to a possible inhibition of phosphotransferases as reported by Michel *et al.* for farnesol and geranylgeraniol in A549 cells.⁴⁹ Incubation of geraniol-treated Hep G2 cells with ^3H -choline demonstrated an accumulation of P-choline in aqueous medium and a decrease in ^3H -choline incorporation into phosphatidylcholine (data not shown). This effect disappeared when cells were incubated with the combination of both compounds, suggesting that the mevalonate pathway leading to cholesterol or prenylated proteins is potentially linked to the regulation of the biosynthesis of phospholipids.

Further studies are required to characterize the exact mechanism, especially the role of protein prenylation in mediating the effects of geraniol and simvastatin on cell proliferation and lipid metabolism. Nevertheless, our data contribute to a better understanding of the action of a common component of essential oils targeting a complex metabolic pathway, which would improve the use of drugs or a combination of drugs in the fight against cancer and/or cardiovascular diseases.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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