

Perturbation by Geraniol of Cell Membrane Permeability and Signal Transduction Pathways in Human Colon Cancer Cells

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ABSTRACT

Geraniol, a natural component of plant essential oils, has antiproliferative effects on human colon cancer cells. To obtain more insight into its mechanism of action, we studied its effect on the resting membrane potential and on the expression of proteins involved in cell signaling pathways. Since geraniol is a well known inhibitor of mevalonate metabolism, the effect of mevalonate supplementation on geraniol-triggered growth inhibition was also determined. Geraniol (400 μM) induced membrane depolarization with a decrease of membrane resistance due to local perforation of the cell membrane. Incubation of Caco-2 cells with geraniol

(400 μM) for 6 h caused a 60% reduction of protein kinase C (PKC) activity. After 16 h of incubation, geraniol decreased by 50% the amount of active forms of p44/p42 extracellular signal-regulated protein kinases (ERK). Mevalonate supplementation did not reverse inhibition of cell growth by geraniol. These results indicate that the antiproliferative effect of geraniol on Caco-2 cells was not related to a limitation of the mevalonate pool but was directly linked to the perturbation of cell membrane function leading to the reduction of PKC activity and to the decreased expression of p44/p42 ERK active forms.

Geraniol, an acyclic dietary monoterpene, has *in vitro* and *in vivo* antitumor activity against various cancer cell lines (Shoff et al., 1991; Yu et al., 1995; Carnesecchi et al., 2001). Geraniol is a well known inhibitor of mevalonate (MVA) metabolism. It inhibits 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity in human liver (Elson and Yu, 1994). It was presumed that this effect may account for its antitumor activity (Elson, 1995; Yu et al., 1995). Inhibition of HMG-CoA reductase activity leads to a limitation of MVA pathway intermediates, which are necessary for post-translational processing of growth-associated proteins (Elson and Yu, 1994; Elson, 1995).

We have previously reported that geraniol sensitizes Caco-2 cells to an anticancer drug (Carnesecchi et al., 2002). It was also reported that geraniol interferes with the membrane functions of *Candida albicans* and *Saccharomyces cerevisiae* (Tsuchiya, 2001) and increases fluidity of liposome membranes (Bard et al., 1998). Thus, geraniol-triggered changes of cell membrane lipid fluidity may provoke conformational changes of ion channels leading to increased or decreased *trans*-membrane ionic flow (Warber, 1998). In ad-

dition, alterations of membrane lipid fluidity may change the conformation of integral membrane proteins (e.g., protein kinases) and perturbate intracellular signaling pathways leading to changes in gene expression (Butler et al., 2002).

In this study, we have evaluated effects of geraniol on the resting potential of the cell membrane, on the expression of proteins involved in cell signaling pathways, particularly of membrane-bound protein kinase C (PKC), and p44/p42 extracellular signal-regulated protein kinases (ERK). The effects of MVA supplementation on cell growth inhibition triggered by geraniol was also determined.

Materials and Methods

Cell Culture. Caco-2 cells were obtained from the European Collection of Animal Cell Culture (CERDIC, Sophia Antipolis, France). They were cultured in 75-cm² Falcon flasks in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, supplemented with 10% heat-inactivated horse serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and subcultured after trypsinization (0.5% trypsin/2.6 mM EDTA). Cells were used up to 40 passages.

In experiments, cells were seeded at 6×10^5 cells on culture dishes (100 mm in diameter) and at 4500 cells per well in 96-well plates and were grown in DMEM supplemented with 3% horse serum, transferrin (5 $\mu\text{g}/\text{ml}$), selenium (5 ng/ml), and insulin (10 $\mu\text{g}/\text{ml}$) (TSI-defined medium; Invitrogen, Cergy-Pontoise, France). Geraniol (Sig-

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ABBREVIATIONS: MVA, mevalonate; HMG, 3-hydroxy-3-methylglutaryl; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated kinases.

ma-Aldrich, St. Louis, MO) and DL-mevalonic acid lactone (Sigma-Aldrich) were dissolved in absolute ethanol and added to the culture medium 24 h after seeding (the final concentration of ethanol was 0.1%).

In all experiments, culture medium and geraniol were replaced every 24 h. Cells were harvested after various times, washed three times with phosphate-buffered saline (pH 7.2), and kept frozen at -70°C until assays were performed.

Determination of PKC Activity. Caco-2 cells were treated for 6 h with or without geraniol ($400\ \mu\text{M}$). Then they were washed with cold phosphate-buffered saline buffer, harvested by scraping, and collected by centrifugation. The cell pellet was suspended in the sample preparation buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM benzamide) and sonicated. The cytosol fraction was obtained by centrifugation at $100\ 000g$ for 1 h at 4°C . PKC activity was measured in the cytosol fraction using a nonradioisotopic commercial kit (Mesacup PK assay kit; Medical and Biological Laboratories Co., Naka-ku Nagoya, Japan).

Western Blot Analysis. Cells were lysed in Tris-HCl buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 1% Triton X-100) by sonication. Lysates were centrifuged ($100\ 000g$ for 30 min at 4°C), and the protein content was determined (Lowry et al., 1951). Equal amounts of proteins were submitted to 15% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Pall Gelman Sciences, Ann Arbor, MI), blocked with bovine serum albumin (3%), and then incubated with nonphosphorylated p44/42 mitogen-activated protein kinase (MAPK) antibody (Thr202/Tyr204; Calbiochem, San Diego, CA) and phosphorylated p44/42 MAPK antibody (Thr202/Tyr204, Thr185/Tyr187; BioSource International, Camarillo, CA). Then the membranes were incubated with a peroxidase-conjugated goat anti-rabbit IgG (Calbiochem). The immune complexes were visualized using Supersignal West Pico chemiluminescent substrate (Perbio Science, Bezons, France), and intensity of the bands was measured with a Geldoc image analyser using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Electrophysiological Recordings. In these experiments, cells were seeded at 2×10^5 cells on culture dishes (25 mm in diameter) and used after 2 to 4 days after seeding. Electrodes were pulled from soft glass by a vertical two-stage puller (L/M-3P-A; Darmstadt, Germany). Pipettes had a resistance between 2.5 and 4 M Ω .

In a first experimental approach, patch-clamp recordings were performed on the whole cell. Patch pipettes were filled with standard KCl internal solution (130 mM KCl, 10 mM NaCl, 10 mM HEPES, and 2.5 mM MgCl_2 , pH 7.2). Geraniol was added to the external solution. Membrane potentials were measured with and without geraniol.

In the second experimental procedure, the cells were maintained continuously under perfusion at a rate of 3 to 4 ml/min at room temperature, and patch-clamp recordings were performed in a perforated patch configuration. The electrode tip was filled by dipping it into a small beaker containing the internal solution without geraniol. Pipettes were filled with KCl internal solution with $250\ \mu\text{M}$ geraniol. The extracellular solution had the following composition: 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 11.1 mM glucose, and 10 mM HEPES. Voltage clamp was achieved using an amplifier (patch-clamp L/M-EPC7). Whole-cell current, perforated patch current, and capacity transients were recorded in response to voltage steps by using pClamp 6.0 software (Axon Instruments, Foster City, CA).

Statistical Analysis. Data are reported as means \pm S.E. Significant differences between control and geraniol-treated cells were evaluated using the Student's *t* test. Differences were considered significant at $p < 0.05$.

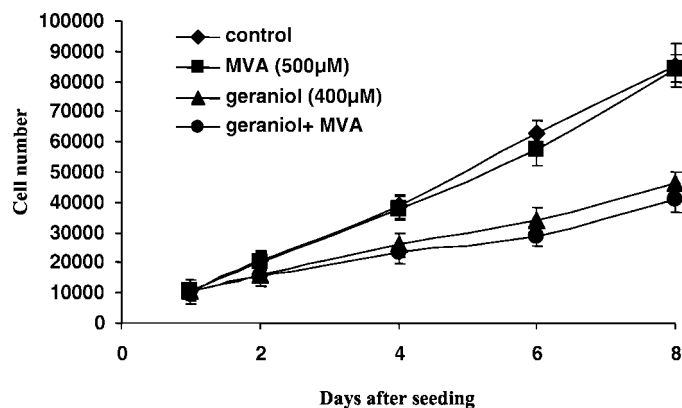


Fig. 1. Effect of mevalonate supplementation on the growth of Caco-2 cells treated with geraniol. Cells were seeded at 4500 cells/well in 96-well plates in DMEM medium supplemented with 3% horse serum, transferrin ($5\ \mu\text{g}/\text{ml}$), selenium ($5\ \text{ng}/\text{ml}$), and insulin ($10\ \mu\text{g}/\text{ml}$). Geraniol or/and mevalonate were added 24 h after seeding for 8 days. The culture medium (containing 0.1% ethanol, with and without geraniol and/or mevalonate) was replaced every 24 h. Values represent mean \pm S.E. ($n = 8$).

Results

Mevalonate and Caco-2 Cell Growth. Since geraniol is a well known inhibitor of MVA metabolism, the effect of MVA supplementation on geraniol-triggered growth inhibition was determined (Flach et al., 2000). Twenty-four hours after seeding, cells were exposed to $500\ \mu\text{M}$ of MVA, $400\ \mu\text{M}$ geraniol, or a mixture of geraniol and MVA for 8 days. MVA did not reverse inhibition of cell growth by geraniol (Fig. 1). Similar results were obtained even with higher doses of MVA (1 and 2 mM; results not shown).

Inhibition of PKC Activity by Geraniol. We have evaluated effects of geraniol on the expression of proteins involved in cell signaling pathways, particularly of membrane-bound PKC. As indicated in Fig. 2, PKC activity was reduced by 60% in the membrane of cells exposed to geraniol ($400\ \mu\text{M}$) for 6 h. This effect was unrelated to a direct interaction between geraniol and the PKC protein because geraniol exerted no inhibitory effects when added directly to cell homogenates.

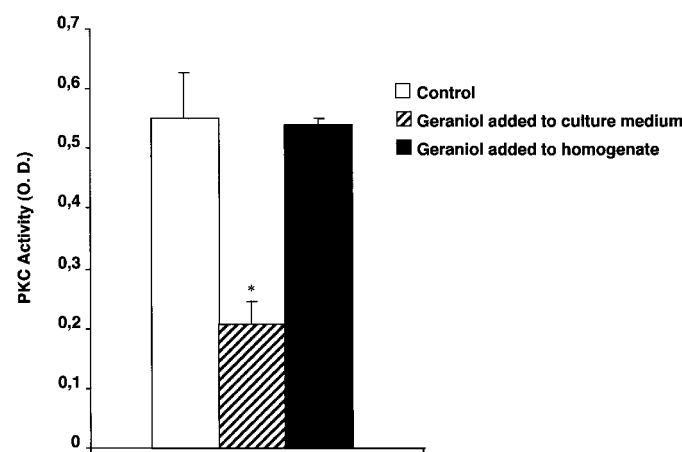


Fig. 2. Effect of geraniol on PKC activity in Caco-2 cells. Cells were treated without (open column) or with $400\ \mu\text{M}$ of geraniol for 6 h. Geraniol was added either to the cell culture medium (hatched column) or to the cell homogenate (dark column). Cells were harvested and PKC activity was measured in three separate experiments. Values represent mean \pm S.E. ($n = 3$); *, $p < 0.05$ (Student's *t* test).

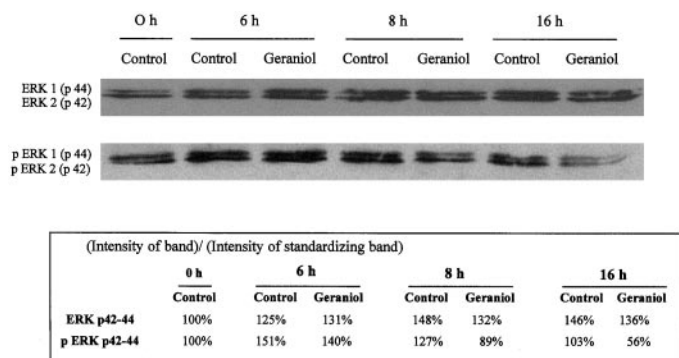


Fig. 3. Effect of geraniol on the amount of p44/p42 ERK in Caco-2 cells. Cells were treated for 6, 8, 16, and 24 h in the absence or presence of 400 μ M geraniol. At the times indicated, cell extracts (20 μ g) were analyzed after Western blotting by using anti-phospho-specific p44/p42 ERK antibody and control anti-p44/p42 ERK antibody. Intensity values were calculated using Quantity One software (Bio-Rad Laboratories). Three independent experiments gave similar results.

p44/42 ERK Reduction by Geraniol. To assess the effect of the monoterpene on the involvement of MAPKs in signal transduction, the amount of inactive and active phosphorylated forms of ERK were determined after 6, 8, and 16 h of exposure to geraniol (400 μ M). The nonphosphorylated form of ERK was detected in comparable amounts in both control and geraniol treated cells. The amount of the nonphosphorylated form of ERK was higher from 6 to 16 h when compared with the starting amount (0 h) because the Caco-2 cells enter after plating an exponential phase of growth and these proteins are key regulators of cell growth. The amount of the nonphosphorylated form of ERK, however, did not change significantly from 6 to 16 h (6 h: 125.4 ± 5 ; 8 h: 133 ± 7 ; 16 h: 137 ± 6 ; $n = 3$). In contrast, the amount of phosphorylated p44/42 ERK decreased by 30 and 50%, respectively, after 8 and 16 h of incubation with geraniol (Fig. 3).

Geraniol Depolarizes Caco-2 Membrane and Decreases Membrane Resistance. To assess whether the perturbation of the signal transduction pathway by geraniol might be related to an effect on cell membrane permeability, we have studied the effects of geraniol (in the external solution) on the resting potential of intact Caco-2 cells (Fig. 4). At a holding potential of -60 mV, the mean resting membrane potential was -57 ± 2 ($n = 12$). Geraniol (400 μ M) depolarized the membrane potential to a mean value of -10 mV ± 6 ($n = 7$).

Figures 5 and 6 illustrate the effect of geraniol (added to the internal pipette) on the access resistance when patch-clamp recording was performed in a perforated patch configuration. In Fig. 5, a series of whole-cell capacity transients, obtained every 30 s, are shown. Such transients were recorded every 30 s after a G Ω seal. In Fig. 6, 1/access resistance versus time after the seal was plotted (mean \pm S.E. of eight experiments). The access resistance (R_a) from each trace was calculated from the time 0 value of the exponential after curve fitting the decreasing phase of capacity transients (I_c) (Fig. 5). The current value following the voltage step V_p was $I_c = V_p/R_a$, where V_p was 20 mV in our experiments. This relationship was used to calculate R_a . We found that geraniol partitioning into the membrane patch begins within a minute after making a G Ω seal, and access resistance below 10 M Ω was observed within 3 min.

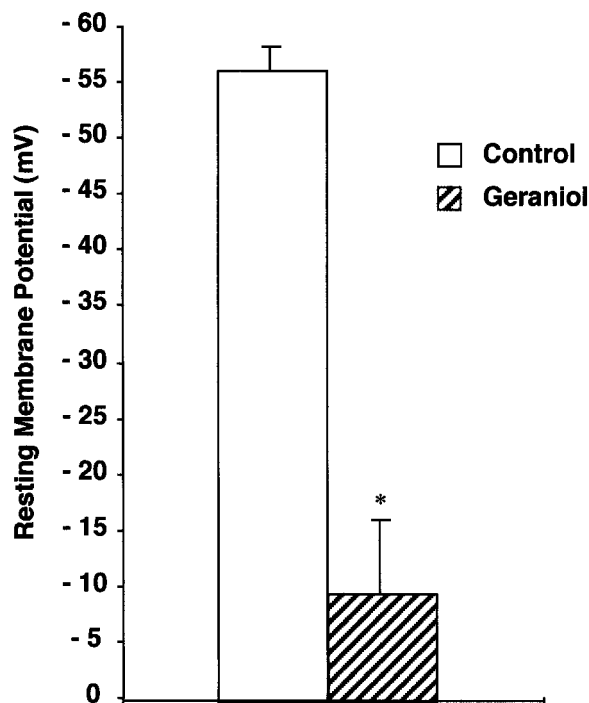


Fig. 4. Effect of geraniol on the resting membrane potential of Caco-2 cells, recorded in the whole-cell patch clamp mode. Patch electrodes were filled with standard internal saline. Mean resting membrane potentials were obtained from Caco-2 cell incubated with standard extracellular saline ($n = 12$, open column) or with standard extracellular saline + 400 μ M geraniol (hatched column, $n = 7$). Data are expressed as mean \pm S.E.; *, $p < 0.05$ (Student's t test).

Discussion

Monoterpenes belong to a family of isoprenoid constituents of fruits and plants. Their anticarcinogenic action has mainly been attributed to the inhibition of the MVA pathways (Elson and Yu, 1994; Elson, 1995). Proteins are isoprenylated by the covalent attachment of a lipophilic farnesyl or geranylgeranyl group to a cysteine residue at or near the terminal carboxyl group (Clarke, 1992). Known mammalian prenylated proteins include p21 Ras, prelamin A, and lamin B, which play an essential role in cell proliferation (Goldstein and Brown, 1990; Maltese, 1990; Cuthbert and Lipsky, 1995). When these proteins are not prenylated, cells do not proliferate.

Mevinolin, a competitive inhibitor of HMG-CoA reductase activity, depletes cells of intermediate products of the MVA pathway that are required for the post-translation modification of cell growth proteins and inhibits cell growth (Fairbanks et al., 1986; Elson and Yu, 1994; Elson, 1995). Similarly, geraniol suppresses hepatic HMG-CoA reductase activity and causes the deprivation of MVA essential for the isoprenylation of cell growth proteins (Clegg et al., 1980, 1982; Elson et al., 1989). Our data show that MVA (500 μ M) supplementation did not reverse the antiproliferative effect observed with geraniol, and even if we treated Caco-2 cells with higher concentrations of MVA (1 mM and 2 mM, data not shown), there was no reversion of growth inhibition. Thus, the results obtained with the colon cancer cell line Caco-2 do not support the report by Elson and Yu (1994), who showed that MVA reversed the effect of geraniol. Our results suggest that the antiproliferative activity of geraniol is not

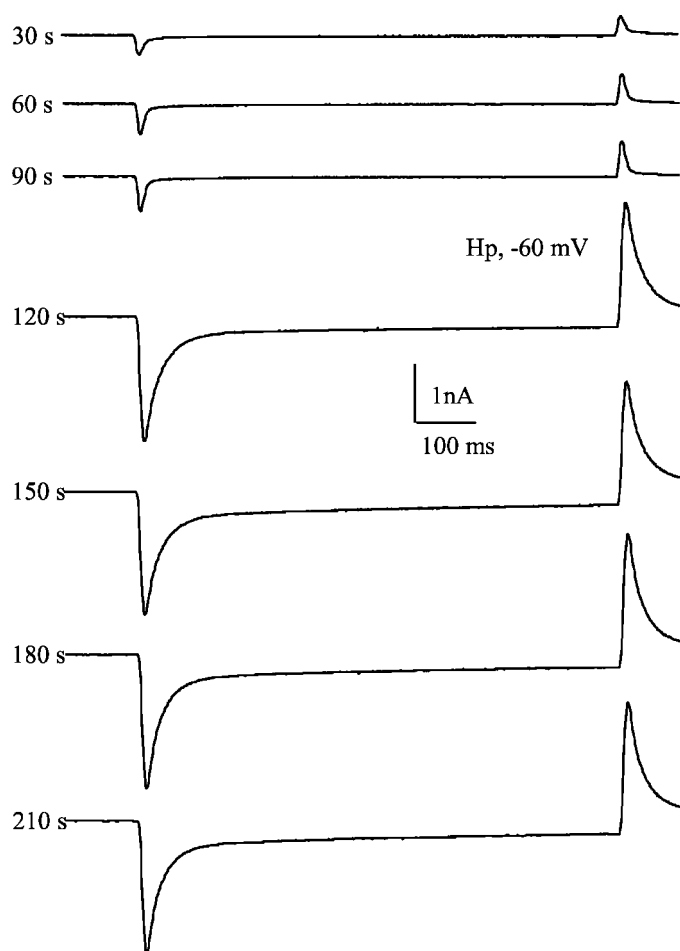


Fig. 5. Capacity transients obtained following a 20 mV command to a geraniol (250 μM) containing pipette $\text{G}\Omega$ sealed to Caco-2 cells. Traces from bottom to top were obtained at 30, 60, 90, 120, 150, 180, and 210 s, respectively, following seal formation. One division of current = 1 nA. Bandwidth = 100 ms.

due to a limiting pool of MVA and inhibition of protein prenylation is not the only target of the anti-proliferative properties of geraniol.

As was shown in the present study, the dietary monoterpene geraniol caused a significant change in the resting membrane potential. Perfusion with 400 μM geraniol caused the depolarization of the cell membrane. Moreover, geraniol decreased the access resistance (i.e., it increased membrane permeability) in function of time. These observations suggest that geraniol interacts reversibly with the cell membrane, probably by acting as pore-forming molecule and/or by affecting ion channel function.

Geraniol may act as a permeabilizing agent, as is the case with amphotericin B, an antibiotic used as a reference in electrophysiological experiments (Rae et al., 1991). Previous studies have shown that amphotericin B permeabilizes the cell membrane by partitioning into cholesterol containing lipids and forms narrow channels that allow monovalent cations and anions to permeate, while excluding multivalent ions and nonelectrolytes (Cass et al., 1970; Holz and Finkelstein, 1970). In comparison with the access resistance of amphotericin B (Rae et al., 1991), geraniol led to a rapid decrease of the access resistances 3 min after $\text{G}\Omega$ seal and reached a stable value more rapidly than amphotericin B.

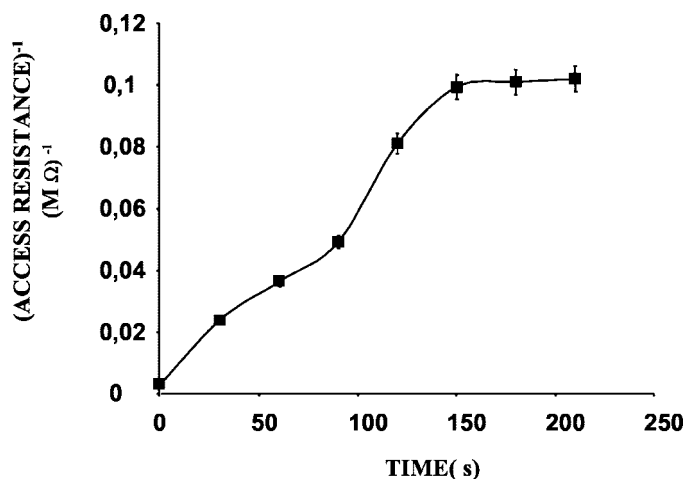


Fig. 6. Normalized access resistances (R_a) obtained in eight experiments with a pipette tip filled with 250 μM of geraniol. We plotted $1/\text{access resistance}$ versus time after $\text{G}\Omega$ seal. R_a from each trace was obtained from the time 0 value of the exponential obtained by curve fitting the falling phase of capacity transients (I_c) (see Fig. 5). The current value following the voltage step V_p was $I_c = V_p/R_a$ where V_p was 20 mV in our experiments. This relationship was used for the calculation of R_a .

Therefore, geraniol may become a new reference as a perforating agent in electrophysiological studies.

The present study confirmed previous results (Bard et al., 1998) with *C. albicans* and *S. cerevisiae*, indicating that geraniol affects bilayer membrane fluidity and increases the membrane bilayer permeability to erythritol. It was also previously shown that monoterpenes affect the structure of biological membranes and modify their lipid packing density, which in turn causes an increase in ion permeability and perturbs membrane-bound enzyme functions (Warber, 1998).

To understand the membrane depolarizing effect provoked by geraniol, it may be suggested that geraniol interacts with receptor channels, thus inducing a modification of ion conductance. Recently, a specific membrane receptor for the monoterpene menthol has been identified (McKemy et al., 2002). It is an excitatory ion channel expressed by neurons and is a member of the long transient receptor potential channel subfamily. A homologous receptor to this transient receptor potential is expressed by a variety of human tumors, including prostate, melanoma, colorectal, and breast carcinoma (Tsavaler et al., 2001). Thus, the mechanism of action of geraniol appears to be similar to that of menthol (i.e., by acting on the same type of receptor or on a different receptor) expressed in tumor cells.

Changes in the bioelectric potential of cell membrane modify or initiate several signal transduction pathways (Sanders and Bethke, 2000; Butler et al., 2002). Membrane perturbation affects PKC activity (Huang et al., 1999) and mitogen-activated protein kinases transduction (Butler et al., 2002). In the present study, geraniol induced the inhibition of PKC activity after 6 h. This was not caused by a direct effect on PKC molecules since geraniol did not alter PKC activity when added to a cell homogenate in vitro. Furthermore, a 50% reduction of ERK active forms was observed in Caco-2 cells exposed to geraniol during 16 h.

In conclusion, our results suggest that the antiproliferative effects of geraniol are essentially due to membrane and ion channels perturbations causing modifications of membrane-

bound protein activity and alterations of the intracellular signaling pathways. Considering the present results, it will be of interest to determine the precise nature of the molecular interactions between geraniol and the cell membrane.

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