Geraniol, a Component of Plant Essential Oils, Sensitizes Human Colonic Cancer Cells to 5-Fluorouracil Treatment

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ABSTRACT
Differentiation of human colonic cancer cells at confluency has been correlated to their increased resistance to chemotherapeutic agents. The aim of this study was to determine whether blocking Caco-2 cell differentiation could sensitize the cells to 5-fluorouracil (5-FU) treatment. We show that in cells at confluency, geraniol (400 μM) prevented the formation of brush-border membranes and inhibited the expression of intestinal hydrolases (sucrase, lactase, alkaline phosphatase). The anti-proliferative effect of geraniol (400 μM) together with 5-FU (5 μM) was twice that of 5-FU alone. The cytotoxicity induced by 5-FU was enhanced in the presence of geraniol, as shown by a 50% increase of lactate dehydrogenase release in the culture medium. These effects are related to enhanced intracellular accumulation of 5-FU in the presence of geraniol as shown by a 2-fold increase in intracellular 5-[^3H]FU (1.5 μCi/ml). It is concluded that geraniol sensitizes colonic cancer cells to 5-FU treatment, by increasing the cytotoxicity of the drug, and that this results from the facilitated transport of 5-FU and the blockade of the morphological and functional differentiation of the cancer cells.

Colonic cancer is a major cause of death by cancer in humans (Boring et al., 1993), which is largely due to the fact that this cancer is highly resistant to chemotherapy. Tumor cell heterogeneity has been proposed to be a factor responsible for the resistance of colon cancer to antineoplastic agents (Brattain et al., 1984). It has been shown that clusters of cells which express differentiation characteristics of enterocytes are present to variable extents in all colonic cancers “in situ” and that resistance to high concentrations of chemotherapeutic agents seems to be restricted to cells with an enterocytic phenotype (Lesuffleur et al., 1998). This is supported by the observation that exposure of HT29 cells to increasing concentrations of methotrexate or 5-fluorouracil completely eliminates undifferentiated cell types, generating a differentiated population with enterocytic phenotype (Lesuffleur et al., 1991).

The human colon cancer cell line Caco-2 spontaneously undergoes structural and functional enterocytic differentiation in culture at late confluency (Pinto et al., 1983). Phenotypic changes that occur after confluence include the formation of brush-border membranes and expression of intestinal hydrolases, which are markers of functional differentiation also found in enterocytes and human fetal colonocytes (Rousset, 1986). It has also been shown that the differentiated Caco-2 cells retain all their malignant potentialities. Indeed, late postconfluent differentiated cells remain tumorigenic in nude mice, and differentiated cells are able to dedifferentiate in vitro (Pandrea et al., 2000). Similarly, recent results obtained with a human hepatoma cell line have shown that, as for Caco-2 cells, the differentiation process was reversible and did not prevent the cells from reentering the cell cycle (Glaise et al., 1998).

Since prevention of colonic cancer cell differentiation might be an important factor in the treatment of colonic cancer, the present study attempted to determine whether inhibiting Caco-2 cell differentiation by treatment with geraniol, a natural compound with chemopreventive properties (Carnescichi et al., 2001), could sensitize Caco-2 cells to treatment with 5-fluorouracil (5-FU), an anticancer drug used in colorectal therapy. Thus, we investigated the effects of geraniol on cell morphology and on several differentiation markers that are normally expressed in Caco-2 cells after confluence. We also evaluated the effects of combining geraniol and 5-FU on cancer cell growth and cytotoxicity.

Materials and Methods

Cell Culture. Caco-2 and SW620 cells were obtained from the European Collection of Animal Cell Culture (CERDIC, Sophia Antipolis, France) and were cultured in 75-cm² Falcon flasks containing DMEM and 25 mM glucose supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells

ABBREVIATIONS: 5-FU, 5-fluorouracil; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; FITC, fluorescein isothiocyanate; CI, combination index; ERK, extracellular signal-regulated kinase.
were incubated at 37°C in a humidified atmosphere of 5% CO₂ and subcultured after trypsinization (0.5% trypsin/2.6 mM EDTA). They were used for up to 30 to 40 passages.

In all experiments, cells were seeded at 6 × 10⁵ cells on culture dishes (100 mm in diameter), or at 4500 cells/well in 96-well plates. Caco-2 and SW620 cells were grown in DMEM supplemented with 3% horse serum, 5 µg/ml transferrin, 5 ng/ml selenium, and 10 µg/ml insulin (TSI defined medium; Invitrogen, SARL, Cergy-Pontoise, France). Geraniol (Sigma-Aldrich, Saint Louis, MO) was dissolved in absolute ethanol, and 5-FU (Teva Pharmachemie B.V., Mijdrecht, The Netherlands) was diluted in PBS at a final concentration of 50 mg/ml. The compounds were added to the culture medium 24 h or 7 days after cell seeding (final concentration of ethanol, 0.1%).

In all experimental conditions, culture medium, geraniol, and 5-FU were replaced every 24 h. Cells were harvested after various times, washed three times with PBS (pH 7.2), and kept at −70°C until assays were performed.

Electron Microscopy. Caco-2 cells were seeded on plastic coverslips in Petri dishes, and culture medium was changed every 24 h. At day 7, confluent Caco-2 cells were fixed for 2 h in sodium cacodylate-buffered 2% glutaraldehyde (0.125 M, pH 7.4) at room temperature. Cells were rinsed in sodium cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, and then washed overnight. They were subsequently dehydrated in graded ethanolos and embedded in Spurr resin by classical methods (Spurr, 1969). Ultrathin sections post-stained with 2% uranyl-ace- tate were observed at 60 kV with a Hitachi H-7500 transmission electron microscope, and pictures were obtained using the Advantage CCD camera system of AMT (Advanced Microscopy Techniques Corp., Danvers, MA).

Isolation of Brush-Border Membranes and Hydrolase Assays. Caco-2 cells were homogenized in 4 ml of Tris-mannitol buffer (50 mM mannitol, 2 mM Tris, pH 7.1) by sonication. Brush-border membranes were isolated as described by Schmitz et al. (1973).

Sucrase activity was determined according to Messer and Dahlqvist (1966) and lactase activity according to Koldovsky et al. (1969). Alkaline phosphatase activity was assayed by the method of Garen and Levinthal (1960), and N-aminopeptidase activity was determined according to Maroux et al. (1973).

Enzyme activities were expressed as specific activities (milliunits/milligram of protein), 1 U of activity corresponding to 1 µmol of product formed/min at 37°C.

Cell Growth. Cells were seeded in 96-well plates and incubated for different times. Cell growth was stopped by addition of 50% trichloroacetic acid (50%, v/v), and the protein content of each well was determined by staining with sulforhodamine B (Skehan et al., 1990). Absorbance was determined at 490 nm. The relationship between cell number (protein content per well) and absorbance was evaluated using the Student t test. Differences were considered to be significant for values of p < 0.05.

Determination of Apoptosis and Cytotoxicity. Caco-2 cells (7 × 10⁵ cells/10-ml Petri dish) were seeded and treated with 5-FU (5 µM) alone or together with geraniol (IC₅₀: 400 µM) and 5-FU for 24 h, at day 7. After trypsinization, cells were collected by centrifugation and stored at −80°C. Apoptotic DNA was separated from genomic DNA, using the Suicide Track DNA ladder kit (Oncogene Research Products, Cambridge, MA). DNA fragments were separated by electrophoresis and stained with ethidium bromide.

Apoptosis was further tested by evaluating phosphatidylserine membrane externalization by measuring annexin V-conjugated fluorescein isothiocyanate (FITC) binding using an Annexin V-FITC kit (Medsystems Diagnostics GmbH, Vienna, Austria). Briefly, cells were washed in cold PBS without calcium and, for each sample, 5 × 10⁶ cells were resuspended in 100 µl of reaction buffer (10 µl of binding buffer 10-fold concentrated, 10 µl of propidium iodide, 1 µl of annexin V-FITC, and 79 µl of deionized water. After incubation for 15 min in the dark at room temperature, each sample was diluted with binding buffer to obtain an appropriate final volume for flow cytometry.

To determine cytotoxicity, cells (4 × 10⁵/well) were seeded on culture dishes (100 nm diameter) and incubated in DMEM culture medium supplemented with 3% FCS. On the 7th day after seeding, cells were incubated with 5-FU (5 µM) alone or together with geraniol (IC₅₀: 400 µM) and 5-FU for 1, 2, 3, and 4 days. Then, cytotoxicity was assessed by determining the release of lactate dehydrogenase (LDH) (Skehan et al., 1990) into the culture medium using the cyto Tox R nonradioactive cytotoxicity assay kit (Promega, Madison, WI).

Measure of 5-FU Uptake. Intracellular accumulation of 5-FU (5 µM) in cells treated with or without geraniol (400 µM) was determined by measuring the amount of 5-[³H]-5-FU (1 µCi/µmol; specific activity: 312.9 GBq/nmol; PerkinElmer Life Sciences, Boston, MA), taken up by Caco-2 cells. Approximately 5 × 10⁵ cells were seeded in culture dishes (100 mm diameter) and incubated at 37°C for 24 h. The culture medium was replaced every 24 h. At day 7 after seeding, when cells reached confluency, they were incubated for 9 h in a culture medium containing 5-FU (5 µM) and 1.5 µCi/ml [³H]-5-FU with or without geraniol. Cells were then collected, washed three times with cold PBS, and sonicated. The radioactivity present in the trichloroacetic acid-precipitable fraction was determined by liquid scintillation spectrometry.

Analysis of Combined Effects of 5-FU and Geraniol. The effectiveness of the combination effects of geraniol and 5-FU at inhibiting the growth of Caco-2 and SW620 cells was evaluated through the measure of the combination index (CI) (Chou and Talalay, 1984). The fractional inhibitory concentration was calculated by dividing the concentration of the drug in the combination inhibiting cell growth by 50% (IC₅₀) by the amount of the drug that is required to reach the same degree of inhibition (IC₅₀) by itself.

\[ CI = \frac{\text{dose of 5-FU} + \text{dose of geraniol}}{\text{IC₅₀ of 5-FU} + \text{IC₅₀ of geraniol}} \]

In this equation, the sum of the dose of 5-FU and the dose of geraniol give 50% inhibition of cell survival. CI > 1 indicates synergism, CI = 1, additivism, and CI < 1, antagonism.

Statistical Analysis. Data are reported as means ± S.E.M Statistical differences between control and geraniol-treated cells were evaluated using the Student’s t test. Differences were considered to be significant for values of p < 0.05.

Results

Effect of Geraniol on Caco-2 Cell Morphological Differentiation. Caco-2 cells undergo phenotypic changes after confluence, which are characterized by an enterocyte morphology and by the expression of various hydrolases in the brush-border membrane typical of the differentiated state. At day 4 after confluence (7 days after seeding), cells form a monolayer showing apical microvilli and tight junctions characteristic of the differentiated state. Brush-border microvilli were numerous, and they were long in nontreated Caco-2 cells (Fig. 1, A and A’). After treatment with geraniol (400 µM), the brush border was modified; microvilli were scarce, and they were shorter (Fig. 1, B and B’). Treatment with 5-FU (5 µM) alone did not modify brush-border membranes (Fig. 1, C and C’). Microvilli at the apical surface were short, swollen, and scarce in cells treated with 5-FU (5 µM) together with geraniol (400 µM), and cells had irregular nuclei with condensed chromatin (Fig. 1, D and D’).

Geraniol and the Functional Differentiation of Caco-2 Cells. The treatment of Caco-2 cells at confluence (7 days after seeding) with 400 µM geraniol inhibited the increase of sucrase and lactase activities normally observed at this stage (Fig. 2). The inhibition was approximately 90% for
Effect of Geraniol and 5-Fluorouracil on Colon Cancer Cells

Fig. 1. Ultrastructure of confluent Caco-2 cell monolayers. Cells (7 days after seeding) were treated with drugs or vehicle for 4 days. Transmission electron micrographs show the brush-border microvilli on the apical surface of control Caco-2 cells (0.1% ethanol) (A and A'), cells treated with geraniol (400 μM) (B and B'), cells treated with 5-FU (5 μM) (C and C'), or cells treated with geraniol and 5-FU (D and D'). Note that geraniol appears to reduce the length and density of microvilli. Nuclei have a very invaginated aspect after the combined treatment. Bar represents 2 μM.

sucrase and 70% for lactase. In addition, geraniol also significantly inhibited the increase in alkaline phosphatase and aminopeptidase activities by about 50%. In fact, the level of all the differentiation markers (brush-border hydrolases) remained at their initial level measured at day 7 after plating, just before the treatment with geraniol.

Effect of Geraniol and 5-FU on Caco-2 Cell Growth. The effects of increasing doses of 5-FU alone or in combination with geraniol were determined after treatment for 8 days. The concentration of 5-FU ranged between 1 and 25 μM; geraniol was used at its IC₅₀ value (400 μM). As shown in Fig. 3, the antiproliferative effects of 5-FU were significantly enhanced in the presence of geraniol. Geraniol alone provoked a 30% cell loss, and treatment with 1 μM 5-FU alone caused a cell loss of 25%. When combined, 5-FU (1 μM) and geraniol caused a 55% cell loss. Similarly, at higher concentrations of 5-FU, the number of surviving cells was reduced by half in the presence of geraniol. As shown in Table 1, when geraniol was added to the culture medium, the amount of 5-FU required to reach the IC₅₀ value was significantly reduced. Thus, with 5-FU alone, the amount of 5-FU necessary was 25 μM, and this amount was reduced to 1 μM in the presence of geraniol.

Effect of Geraniol on Caco-2 Cell Death. To determine whether geraniol caused apoptosis, DNA fragmentation assays and annexin V labeling were performed. Exponentially growing cells and confluent cells (7 days after seeding) were treated with 5-FU (5 μM) with or without geraniol for 24 h did not exhibit apoptotic DNA fragmentation ladders (results not shown). These results were confirmed by the absence of annexin V labeling using flow cytometry analysis (results not shown). These results show that 5-FU treatment induced nonapoptotic cell death characterized by the condensation of nuclear chromatin, cytoplasmic vacuolation, and absence of annexin staining or DNA fragmentation (Sperandio et al., 2000).

The effect of geraniol on 5-FU cytotoxicity was assessed after confluency (7 days after seeding) by measuring LDH release in the culture medium after 4 days of treatment with the drugs. As shown in Fig. 4, the presence of geraniol, which alone showed no cytotoxic effect, enhanced the cytotoxic effects of 5-FU (5 μM) by a factor of 2.

Cellular Uptake of 5-FU. Intracellular accumulation of 5-[6-³H]FU, determined in the presence or absence of geraniol, after 9 h of treatment, showed (Fig. 5) that the uptake of 5-FU by Caco-2 cells was enhanced 2-fold in the presence of geraniol.

Evaluation of Cell Resistance to 5-FU Treatment. To evaluate cell resistance to 5-FU treatment, we tested increasing doses of 5-FU on both differentiated and exponentially growing Caco-2 cells, and also on growing SW620 cells (another human colon cell line). Table 1 shows that confluent cells were more resistant than exponentially growing cells. For confluent Caco-2 cells, the IC₅₀ for 5-FU was 25 μM, whereas for growing Caco-2 and SW620 cells the IC₅₀ values for 5-FU were, respectively 0.4 and 2 μM.

Effect of Geraniol on Growing Caco-2 and SW620 Cells Treated with 5-FU. The comparative effects of geraniol and 5-FU were studied on exponentially growing Caco-2 and SW620 cells (Fig. 6). For growing Caco-2 cells, the concentration of geraniol (200 μM) necessary to reach the IC₅₀ value was reduced 2-fold compared with cells at confluency (400 μM). The effects of graded doses of 5-FU alone or in combination with geraniol were determined. Cells were treated for 8 days with geraniol at its IC₅₀ value (200 μM) 24 h after seeding. For both cell lines, geraniol potentiated the inhibition of cell growth observed with 5-FU alone (Fig. 6). As illustrated in Table 1, growing SW620 cells were more resistant to 5-FU treatment than proliferating Caco-2 cells (IC₅₀ for 5-FU: 2 and 0.4 μM, respectively). In the presence of geraniol, the sensitivity of growing cells to 5-FU treatment was also significantly increased. Furthermore, measurement of CI (mean ± S.E.) at the IC₅₀ iso-effect level, which determines whether the interactions of the two drugs are synergistic, additive, or antagonistic, indicated that the association of 5-FU and geraniol presented a synergistic effect on Caco-2 cells (CI = 0.9 ± 0.014), whereas the effect appeared to be additive for SW620 (CI = 0.97 ± 0.10).
Discussion

This study shows that geraniol, a component of essential plant oils, sensitizes human colonic cancer cells to 5-FU treatment. The effect of geraniol was greater on cells which were more resistant to 5-FU (i.e., differentiated Caco-2 cells). We demonstrate here that geraniol acts on two major targets involved in the resistance of colon cancer cells to chemotherapeutic agents: the process of cell differentiation and membrane permeability to the drug. Geraniol induced cell membrane modifications that prevented differentiation of colonic cancer cells and enhanced their uptake of 5-FU.

The two cell lines, Caco-2 and SW620, used in the present study, differ in their sensitivity to 5-FU treatment, and they also responded differently to geraniol and to the combination of 5-FU with geraniol. Indeed, the combination index at the IC₅₀ iso-effect level, which determines whether the interactions of the two drugs are synergistic, additive, or antagonistic, indicated that the association of 5-FU and geraniol presented a synergistic effect on Caco-2 cells, whereas the effect appeared to be additive for SW620. As shown by studies on thymidylate synthase kinetics, differences in both substrate affinity (Kₘ) and conversion rate (Vₘ) may be largely responsible for different responses in cell lines to 5-FU (Mans et al., 1999). However, several other factors such as trans-membrane transport or changes in metabolic pathways are probably involved in 5-FU sensitivity (Peters et al., 2000). On the other hand, intracellular catabolism of 5-FU does not seem to be involved in variations in cell sensitivity, since activity of dihydropyrimidine dehydrogenase, the first enzyme in the pathway of 5-FU catabolism, is low in Caco-2 and SW620 cell lines (Katona et al., 1999).

Our data favor a direct relationship between the differentiation state of colonic cancer cells and their increased resistance to chemotherapy. We show that differentiated Caco-2 cell were more resistant to 5-FU treatment than both undifferentiated Caco-2 and SW620 cells. Previous studies have shown that resistance to high concentrations of chemotherapeutic agents appears to be restricted to cells with differentiation characteristics of enterocytes, which are also present

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<th>Cell Lines</th>
<th>Caco-2</th>
<th>Growing Caco-2</th>
<th>SW620</th>
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<tr>
<td>IC₅₀ 5-FU</td>
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<tr>
<td>5-FU alone</td>
<td>25 ± 0.04</td>
<td>0.4 ± 0.009</td>
<td>2 ± 0.1</td>
</tr>
<tr>
<td>5-FU + geraniol (IC₅₀)</td>
<td>1 ± 0.05</td>
<td>0.2 ± 0.007</td>
<td>0.5 ± 0.22</td>
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in varying proportions in all colonic cancers “in situ” (Lesuf-fleur et al., 1998). More recently, it has also been reported that the response of Caco-2 cells to butyrate depends on their phenotype (Mariadason et al., 2001) and that differentiated cells are essentially resistant to butyrate treatment (Ho et al., 1994).

We show that interaction of geraniol with the cell membrane prevents the differentiation process and facilitates the uptake of the chemotherapeutic agent by cancer cells. Recent studies have shown that geraniol interferes with the membrane function of Candida and Saccharomyces (Tsuchiya, 2001) and increases fluidity of liposomal membranes (Bard et al., 1998). Changes in membrane fluidity induced by heptacaine, a component of the Capsicum fruit, have been attributed to the insertion of its lipophilic fragment into phospholipid acyl chains to create a free volume in hydrophobic membrane regions (Gallova et al., 1995). Phospholipid acyl chains bind cooperatively and fill the free volume to fluidize membranes. A similar mechanism of action may be proposed for geraniol. The control of differentiation is mediated by interactions of signaling molecules at the cell surface, which ultimately lead to long-term changes in gene expression. The mitogen-activated protein kinase kinase/ERK pathway is known to participate in a number of cellular processes, including differentiation (Ding et al., 2001; Aliaga et al., 1999), which appears to require an appropriate balance between activation and inhibition of mitogen-activated protein kinase signaling molecules. In addition, opposing effects may occur in the same cell, depending on the strength and duration of the signal transmitted through the ERK cascade (Gredinger et al., 1998; Schaeffer and Weber, 1999). Recently, Butler et al. (2002), have reported that ERK is intimately associated with membrane perturbation. We suggest that interactions of geraniol with the membrane may alter signal transduction via the mitogen-activated protein kinase pathway and prevent cell differentiation.

The present data question the “differentiation” therapeutic approach (Lotan, 1990), which is based on “the demonstration that cancer is reversible and that the transformed phenotype can be suppressed by cytostatic agents and by differentiation-inducing physiological and pharmacological agents” (Lotan, 1990, p. 3460). This approach claims that inducing the differentiation of colonic cancer cells would result in a less malignant phenotype. However, it has been reported that differentiated Caco-2 cells keep all their malignant potentiality, since they can reenter the cell cycle (Glaise et al., 1998; Pandrea et al., 2000). We suggest that the che-

![Fig. 4. Effects of geraniol on 5-FU cytotoxicity. Cells were exposed to 5-FU (5 μM) (A) or to geraniol (400 μM) together with 5-FU (B) between days 7 and 10 after seeding. The medium was changed every day. Cytotoxicity was assessed by determining the release of LDH into the culture medium. Data are means ± S.E. (n = 4), p < 0.05.](image)

![Fig. 5. Intracellular accumulation of 5-FU in the presence of geraniol. At day 7, cells were incubated for 9 h with medium containing 5-FU (5 μM) and 1.5 μCi/ml 5-[6-3H]FU (squalo) or 5-FU (5 μM) and 1.5 μCi/ml 5-[6-3H]FU in the presence of geraniol (400 μM) (C). The radioactivity present in the trichloroacetic acid-precipitable fraction was measured by liquid scintillation spectrometry. Values represent means ± S.E. (n = 3). * p < 0.05.](image)

![Fig. 6. Inhibition of Caco-2 (A) and SW620 (B) cell proliferation by 5-FU alone or together with geraniol. Cells were exposed for 8 days to 5-FU alone (●) and various combinations of 5-FU and geraniol (■). Geraniol and 5-FU were replaced every 24 h. Values represent means ± S.E.M. (n = 8), p < 0.05.](image)
moterapeutic approach for colorectal cancer should be modified, favoring the use of differentiation blockers.

By fluidizing the membrane, geraniol may favor cellular uptake of anticancer drugs. This could permit the use of lower concentrations of chemotherapeutic drugs and, at the same time, lower their secondary effects. Investigations are in progress with different colonic cancer models in rodents to determine whether the combination of geraniol and 5-FU may offer a promising approach for optimizing the treatment of colorectal cancer.

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