Farnesol and geraniol chemopreventive activities during the initial phases of hepatocarcinogenesis involve similar actions on cell proliferation and DNA damage, but distinct actions on apoptosis, plasma cholesterol and HMGCoA reductase

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Chemopreventive activities of farnesol (FOH) and geraniol (GOH) were evaluated during the initial phases of hepatocarcinogenesis. Rats received during eight consecutive weeks 25 mg/100 g body weight FOH (FOH group) or GOH (GOH group), or only corn oil (CO group, controls). Incidence (%) and mean number of visible hepatocyte nodules/animal were inhibited in FOH group (13% and 4 ± 1; P < 0.05), but not in GOH group (42% and 18 ± 17, P > 0.05), compared to CO group (100% and 42 ± 17). Mean area (mm²) and % liver section area occupied by total hepatic placental glutathione S-transferase positive preneoplastic lesions (PNLs) were reduced in FOH group (0.09 ± 0.06; 2.8 ± 1.3; P < 0.05) compared to CO group (0.18 ± 0.12; 10.0 ± 2.8), while in GOH group only the mean area of these PNL was reduced (0.11 ± 0.09; P < 0.05), but not the % liver section area occupied by them (5.1 ± 1.1; P > 0.05). Compared to CO group, FOH and GOH groups showed reduced (P < 0.05) PNL cell proliferation and DNA damage, but only GOH group showed increased PNL apoptosis (P < 0.05), FOH group, but not GOH group, presented reduced (P < 0.05) total plasma cholesterol levels and increased (P < 0.05) hepatic levels of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity. Inhibition of cell proliferation and DNA damage relate to both isoprenoids’ anticarcinogenic actions while induction of apoptosis specifically relates to GOH protective actions. Inhibition of HMGCoA reductase activity could be associated with FOH, but not GOH anticarcinogenic actions. FXR does not seem to be involved in the isoprenoids’ chemopreventive activities.

Introduction

Several epidemiologic studies consistently showed that high intake of fruits and vegetables presents a protective action against different cancer types (1). Diet-derived isoprenoids represent promising cancer chemopreventive agents (2). They comprise a class of substances with over 22 000 constituents widely distributed in fruits and vegetables and originate from the mevalonate pathway. Although cell culture studies utilizing diverse cancer cell lines demonstrated the pronounced effects of farnesol (FOH) (Figure 1A) and geraniol (GOH) (Figure 1B) on the inhibition of cell proliferation (3), there is scarce information on the *in vivo* cancer chemopreventive potential of these acyclic isoprenoids (2). In rats FOH inhibited chemically induced colon (4,5) and pancreatic (6) carcinogenesis and GOH inhibited mammary carcinogenesis (7).

FOH and GOH cell proliferation inhibition is based on their ability to posttranscriptionally inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, thus reducing synthesis of cholesterol and intermediaries of the mevalonate pathway, such as farnesyl and geranylgeranyl pyrophosphates (8). These mevalonate derivatives are important for protein farnesylation and geranylgeranylation of certain proto-oncogenes (2). Differently from normal cells, preneoplastic and neoplastic liver lesions present a loss in the transcriptional downregulation mechanism of HMG-CoA reductase by sterols and of cholesterologenesis (9). However, in these lesions this enzyme retains sensitivity to isoprenoid-mediated inhibition (2). Thus isoprenoids could represent potential chemopreventive agents against hepatocarcinogenesis (10).

It was proposed that FOH could exert anticarcinogenic actions through farnesoid X activated receptor (FXR)-mediated gene expression (11). This nuclear receptor was initially shown to be activated by FOH but not by GOH (12). Subsequently bile acids were identified as the endogenous

**Abbreviations:** 2-AAF, 2-acetylaminofluorene; AB, apoptotic bodies; AI, apoptotic indices; BrdU, 5-bromo-2′-deoxyuridine; CO, corn oil; DEN, diethylnitrosamine; FOH, farnesol; FXR, farnesoid X activated receptor; GOH, geraniol; GST-P, Polyclonal anti-placental glutathione S-transferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LI, labeling index; PNLs, preneoplastic lesions; RH, resistant hepatocyte.

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ligands for FXR, which plays a central role in bile acids and cholesterol metabolism (13). Activators of FXR such as FOH are potent cell proliferation inhibitors and apoptosis inducers (14).

The objective of this study was to evaluate the chemopreventive effects of FOH and GOH during initiation and promotion phases of chemical hepatocarcinogenesis in rats submitted to the ‘resistant hepatocyte’ (RH) model (15). Hepatocytes were initiated with diethylnitrosamine (DEN) and promoted with 2-acetylaminofluorene (2-AAF) coupled with a partial hepatectomy (PH). Evaluated parameters included hepatic preneoplastic lesion (PNL) development, cell proliferation, apoptosis, DNA damage, plasma cholesterol concentration, HMG-CoA reductase mRNA levels and FXR protein levels.

Materials and methods

Chemicals
FOH (trans, trans-3, 7, 11-trimethyl-2, 6, 10-dodecatrien-1-ol; 96%) was purchased from Aldrich (Buchs, Switzerland). 2-AAF, 5-bromo-2'-deoxyuridine (BrdU), DEN, dimethylsulfoxide, 3, 3'-diaminobenzidine, GOH (trans-3, 7-dimethyl-2, 6-octadien-1-ol, 98%), MOPS and agarose were purchased from Sigma (St. Louis, USA). The commercial diet was purchased from Purina (Campinas, Brazil). Corn oil (CO) was Mazola® (São Paulo, Brazil). Polyclonal anti-placental glutathione S-transferase (GST-P) rabbit antibody was purchased from Medical and Biological Laboratories Co. (Tokyo, Japan). Polyclonal anti-BrdU rat antibody, secondary biotinylated antibody and streptavidin-biotin-peroxidase complex (StrepABComplex/HRP Duet, Mouse/Rabbit) were purchased from Dako (Glostrup, Denmark). The total plasma cholesterol kit was purchased from BioSystems (Barcelona, Spain). Hamster HMGCoA reductase cDNA probe was obtained from ATCC pRed 227 plasmid. Trizol™ was purchased from Gibco (Gaithersburg, USA). Duralose UV nitrocellulose membranes were purchased from Stratagene (La Jolla, USA). Multiprime DNA Labelling System Kit, [α-32P]dCTP (6000 Ci/mmol), Nick™ columns, Hybond™-C extra nitrocellulose membranes and ECL chemoluminescence kit were purchased from Amersham Biosciences (Piscataway, USA). N-PEI™ and BCA protein assay kit were purchased from Pierce (Rockford, USA). Polyclonal anti-FXR antibody and secondary antibody conjugated to horse radish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Other chemicals were of the highest available quality.

Animals and experimental protocol
Male Wistar rats from the colony of the Faculty of Pharmaceutical Sciences, initially weighing 45–50 g, maintained in cages of 4 animals, at a constant temperature (22°C), with 12 h light-dark cycle and receiving water and commercial diet ad libitum, were used.

Figure 2 illustrates the experimental protocol design. At the end of a 7 day acclimatization period, with the exception of 11 Wistar rats not submitted to any experimental procedure [normal (N) group], 36 animals were randomly divided into three experimental groups. Those of FOH and GOH groups were treated with 25 mg/100 g body wt FOH or GOH dissolved in CO (0.25 mL/100 g body wt). Animals receiving only CO (0.25 mL/100 g body wt) were used as controls (CO group). All treatments were conducted by gavage, daily for eight consecutive weeks. Rats were submitted to the RH model of hepatocarcinogenesis as follows (16): two weeks after the beginning of the different treatments the animals received an intraperitoneal dose of DEN (20 mg/100 g body wt) for initiation. Two weeks later the animals received six intragastric doses of 2-AAF, the first 4 (3 mg/100 g body wt) on four consecutive days before partial (2/3) hepatectomy and the remaining two (2.5 mg/100 g body wt), on day 2 and 4 thereafter. All animals were euthanized by light ether anesthesia and exsanguination 6 weeks after DEN administration. One hour before sacrifice the rats received a single intraperitoneal injection of BrdU (10 mg/100 g body wt) dissolved in dimethylsulfoxide and saline (1:3 v/v). The study was conducted in accordance with NIH guidelines for the care and use of laboratory animals.

Visible hepatocyte nodules counting
After the sacrifice the liver was removed from each animal, weighed and examined grossly on the surface and in 3 mm cross-sections for the presence

![Fig 2. Experimental design.](https://academic.oup.com/carcin/article-abstract/27/6/1194/2390981)
of visible hepatocyte nodules of varied sizes and a generally whitish or yellowish color, different from the hepatic parenchyma.

Histopathologic examination

Representative fragments of each liver lobe were fixed in Metacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for 24 h and included in paraffin. Five micrometre sections were hematoxylin-eosin (H&E) stained for histopathologic examination that was conducted by an experienced pathologist. Hepatic PNL were classified as mixed (acidophilic and vacuolated or acidophilic and amorphophilic) foci or nodules according to the criteria established in the literature (17). Hepatocyte foci were considered PNL smaller than one hepatocyte lobule, whereas hepatocyte nodules comprised spherical PNL larger than one or more hepatocyte lobules (18).

Immunohistochemistry for GST-P and BrdU

Histological sections of the liver samples were also processed to be submitted to immunohistochemical reactions in order to detect PNL (foci/nodules) positive for GST-P or hepatocytes positive for BrdU, according to the method described by Hsu et al. (19). After removal of paraffin the endogenous peroxidase was blocked by 3% hydrogen peroxide in PBS for 5 min. Thereafter the sections were incubated overnight at 4 °C with primary anti-GST-P rabbit antibody to a 1:1000 dilution or primary anti-BrdU rat antibody at a 1:60 dilution, in 1% BSA. Finally, the sections were incubated for 1 h with secondary biotinylated antibody, thereafter applying the streptavidin-biotin-peroxidase complex. Peroxidase binding sites were detected by incubation with 3,3′-diaminobenzidine (0.5%) and hydrogen peroxide (0.1%) dissolved in PBS, for ~2 min at room temperature. Sections were counterstained with hematoxylin.

Hepatocyte foci and nodules staining uniformly and non-uniformly for GST-P were classified as the persistent and remodeling type, respectively (10,20,21). They were measured by the KS-300 program (Kontron Elektronik, Munich, Germany) using a Nikon Microphot-FXA, Tokyo, Japan) photomicroscope connected with a microcomputer. Data were expressed as GST-P positive PNL (n cells/mm²) and area (mm²), and % liver section occupied by these PNL.

In order to evaluate the BrdU labeling index (LI) 1000 hepatocytes were analyzed per animal, of which 500 in PNL (foci/nodules) areas and 500 in the surrounding normal tissue (10,22), using a light microscope (Carl Zeiss, Munich, Germany) equipped with a Nikon Microphot-FXA, Tokyo, Japan) photomicroscope connected with a microcomputer. Data were expressed as GST-P positive hepatocytes/n (n cells/mm²) and area (mm²), and % liver section occupied by these PNL.

In order to evaluate the BrdU labeling index (LI) 1000 hepatocytes were analyzed per animal, of which 500 in PNL (foci/nodules) areas and 500 in the surrounding normal tissue (10,22), using a light microscope (Carl Zeiss, Göttingen, Germany). BrdU LI was expressed as the number of BrdU-positive hepatocyte nuclei × 100/total number of analyzed hepatocytes.

Apoptosis evaluation

Hepatic apoptotic bodies (AB) were quantitated by fluorescence microscopy as described by Stinchcombe et al. (23) using a Nikon microscope (Tokyo, Japan) equipped with an epifluorescence unit. This method is based on the strong eosin fluorescence of AB in H&E-stained liver tissues submitted to blue light (450–490 nm). Identification of AB was confirmed by switching the microscope system from blue to transmitted light and using morphological criteria established by Goldsworthy et al. (24). AB were represented by acidophilic bodies with fragmentation or lack of chromat in an acidophilic background. They were measured by the KS-300 program (Kontron Elektronik, Munich, Germany) using a Nikon Microphot-FXA, Tokyo, Japan) photomicroscope connected with a microcomputer. Data were expressed as the number of acidophilic bodies/mm² of PNL or surrounding normal tissue areas (23). Total plasma cholesterol concentration

Blood was collected by puncture of the abdominal aorta at the time of sacrifice. Immediately after collection it was placed in centrifuge tubes containing 5 mg EDTA and centrifuged at 3500 g, 4 °C for 10 min. Total plasma cholesterol concentration, an indirect measure of HMGCoA reductase activity (10), was determined using an enzymatic-spectrophotometric technique. Analysis was performed at 500 nm with a Model U 110 (Hitachi, Tokyo, Japan) spectrophotometer.

HMGCoA reductase mRNA dot blot analysis

HMGCoA mRNA dot blot analysis was performed basically according to Di Groce et al. (25). Total RNA was isolated from liver samples of the experimental rats, previously stored at −80 °C, using Trizol™ reagent. Samples of total RNA (75 µg) were then transferred to Duralose UV nitrocellulose membranes by a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories, California, USA) and fixed by baking at 80 °C for 2 h. Prehybridization and hybridization with HMGCoA reductase cDNA probes were carried out as described by Sambrook and Russell (26). Radioiodination of probes was performed with the Multiprime DNA Labelling System Kit and [γ-32P]dCTP (6000 Ci/mmol) and the labelled probes purified using Nick™ columns. Routinely probes with specific activities of (1–3) × 10⁶ c.p.m./µg were obtained. Autoradiographies were carried out at −80°C during periods between 2–4 days using X-ray films in X-ray cassettes fitted with intensifying screens. Quantitative densitometry of the autoradiograms was performed using Bio-Rad densitometer (Imaging Densitometer, Model GS-700) with a specific software (Molecular Analyst). Control of the relative amount of the mRNA of interest was made by previous staining of the nitrocellulose membrane with methylene blue (27).

Hepatic DNA strand breakage (Single cell gel electrophoresis—comet assay)

Hepatic DNA strand breakage was evaluated in liver samples previously stored at −80 °C, using the comet assay essentially as described by Toledo et al. (28) and Fonseca et al. (20). The tissues were smoothly homogenized in PBS (2.7 mM KCl, 81 mM NaPO₄, 1.5 mM KH₂PO₄, 0.14 M NaCl; pH 8.0), under refrigeration, and filtered. The isolated cells were then immobilized in a low-melting agarose (Sigma, USA) matrix on a glass slide. Thereafter, these cells were lysed in the following solution: TBE buffer [90 mM Tris; 90 mM H₂BO₃; 2 mM Na₂-EDTA; pH 8.4] and 2.5% SDS, and submitted to electrophoresis in TBE buffer at 62 V during 2 min. The resulting comets were stained with silver nitrate (10,20,28). Normal rat liver tissues, challenged or not with hydrogen peroxide (10% final concentration, 5 min at room temperature and sonication) were used as negative and positive controls, respectively (10,20,28).

Length of the comets was evaluated using the previously described image analysis system. One hundred nucleoids per animal were randomly analyzed (50 images per slide). Coded slides were scored blindly. The viability of the liver cells was indirectly determined by analyzing the comet images after electrophoresis (29). The comet image was considered to be from a non-viable cell when it presented a ‘cloudy’ appearance or a very small head and a tail like a balloon (necrotic or apoptotic cells). The viability of the cell suspension was considered acceptable when the frequency of such images was <2% (29).

FXR western blot analysis

Nuclear protein extracts were prepared from the liver samples of the experimental rats, using the N-PER™ reagent (10). Protein concentration was determined with the BCA protein assay kit. Samples of nuclear protein extracts (50 µg) were separated by electrophoresis in 10% denaturing polyacrylamide gel (SDS-PAGE), in 1 × Tris–glycine buffer, according to the method by Laemmli (30). The proteins were then transferred from the gel to a nitrocellulose membrane. Nitrocellulose membrane blockade was performed with PBS containing 5% powdered milk during 1 night at 4 °C. After washing with PBS buffer containing 0.1% Tween-20, it was incubated for 2 h at room temperature with the primary anti-FXR antibody (1:1000) in PBS buffer containing 0.5% powdered milk, followed by incubation with the secondary antibody conjugated to horseradish peroxidase. The membrane was developed using the ECL chemiluminescence kit. An X-ray film was then exposed to the membrane, resulting in approximately 54 kDa bands, corresponding to the expected molecular weight of FXR (12). In order to quantify band intensities, the Bio-Rad densitometer (Imaging Densitometer, Model GS-700) with a specific software (Molecular Analyst) was used. Control of the relative amount of the protein of interest was made by previous staining of the nitrocellulose membrane with Coomassie blue (10,31).

Statistical analysis

Sigma Stat 2.0 (Jandel, San Rafael, USA) program was used for the statistical analysis. Fisher’s exact test was used when indicated. One-way ANOVA and Student’s t-tests were used when the results presented a normal distribution and, in the case this did not occur, Kruskall-Wallis and Mann-Whitney’s tests were used. To all cases a level of significance of P < 0.05 was applied.

Results

Body and liver weights, and incidence and mean number of visible hepatocyte nodules

Table I presents the data on body and liver weights and incidence and mean number per rat of visible hepatocyte nodules after daily treatment during eight consecutive weeks with 25 mg/100 g body wt FOH (FOH group) or GOH (GOH group), or only CO (0.25 mL/100 g body wt; controls; CO group) of animals submitted to the RH model. No statistically significant differences (P > 0.05) were observed between the different experimental groups regarding the final body weights and absolute and relative liver weights. These results indicate that FOH and GOH did not present toxicity at the used doses.
FOH and GOH groups presented total (persistent + remodeling), persistent or remodeling GST-P positive PNL. Also compared to CO group, FOH and GOH groups presented slight proliferation of these cells. FOH and GOH groups presented moderate apoptosis of these cells.

\[ \text{GST-P} \] is a marker of foci and nodules that can demonstrate more numbers and larger sizes of these putative PNL than other markers in various rat liver carcinogenesis studies (32). A typical and critical property of hepatocyte foci and nodules is their capability of expressing one of two options: spontaneously remodeling to a normal appearing liver by the majority (95–98%) or persistence with cell proliferation and evolution of hepatocyte foci and nodules (17). In addition, CO group presented moderate apoptotic activity of these cells.

Histopathological examination of H&E stained liver sections revealed the presence of mixed (acidophilic and vacuolated or acidophilic and amphophilic) hepatocyte foci and nodules (17) in the CO group. FOH and GOH groups presented mostly mixed (acidophilic and vacuolated or acidophilic and amphophilic) hepatocyte foci (17). In addition, CO group presented slight proliferation of these cells.

Table II shows the values obtained by morphometric quantification of the number and mean area of total (persistent + remodeling), persistent or remodeling GST-P positive PNL, as well as percentage of the histological section area occupied by these PNL. Compared to CO group, FOH and GOH groups did not present differences (P > 0.05) regarding the number of total (persistent + remodeling), persistent and remodeling GST-P positive PNL. Also compared to CO group, FOH and GOH groups presented total (persistent + remodeling), persistent and remodeling GST-P positive PNL with smaller (P < 0.05) sizes. FOH group presented total (persistent + remodeling), persistent and remodeling GST-P positive PNL that occupied a smaller (P < 0.05) area of the liver section when compared to CO group. GOH group remodeling but not total (persistent + remodeling) or persistent GST-P positive PNL occupied a smaller (P < 0.05) area of the liver section when compared to CO group.

Liver histopathological examination

Histopathological examination of H&E stained liver sections revealed the presence of mixed (acidophilic and vacuolated or acidophilic and amphophilic) hepatocyte foci and nodules (17) in the CO group. FOH and GOH groups presented mostly mixed (acidophilic and vacuolated or acidophilic and amphophilic) hepatocyte foci (17). In addition, CO group presented slight proliferation of these cells.

Cell proliferation and apoptosis

Figure 3 shows BrdU LI of normal liver tissue and areas surrounding PNL, as well as of the PNL areas themselves at the end of the 8 weeks of experiment. BrdU LI of PNL
surrounding normal tissue areas in CO group was higher ($P < 0.05$) than the BrdU LI of N group. This increase in CO group normal tissue area LI is likely to be related to the fact that these animals were subjected to a 2/3 PH while they were on 2-AAF, a mitoinhibitor. As the mitoinhibitory effects of 2-AAF decrease, the LI in the suppressed liver begins to increase (10). BrdU LI of PNL areas tended to be higher than BrdU LI of the respective surrounding normal tissue areas in CO group. These results agree with the information that during hepatocarcinogenesis cell proliferation increases (10,20).

Compared to CO group, FOH and GOH groups presented smaller BrdU LI in normal tissue areas surrounding PNL ($P < 0.05$, except for GOH group) and in PNL areas ($P < 0.05$). There were no differences ($P > 0.05$) in FOH and GOH groups between BrdU LI in PNL areas and normal tissue areas surrounding PNL. In the present study hepatic AB were quantitated by the fluorescence microscopy method described by Stinchcombe et al. (23) and also used in rats submitted to RH model (21). Advantages of this method are the fast identification of AB due to their strong fluorescence in H&E-stained liver sections and increased sensitivity, since small fluorescent AB, usually not recognized by transmitted light microscopy, can also be identified (23). Figure 4 shows examples of the fluorescent hepatic AB observed in the present study.

Figure 5 shows AI of normal liver tissue and areas surrounding PNL, as well as of the PNL areas themselves at the end of the 8 weeks of experiment. No differences ($P > 0.05$) were observed between AI of PNL surrounding normal tissue areas in CO group and AI of N group. AI of PNL areas was higher ($P < 0.05$) than AI of the respective surrounding normal tissue areas in CO, FOH and GOH groups. These results agree with the information that during hepatocarcinogenesis apoptosis increases (10,20). Compared to CO group, FOH group did not present differences regarding AI in normal tissue areas surrounding PNL ($P > 0.05$) and in PNL areas ($P > 0.05$). Also compared to CO group, GOH group presented higher AI both in normal tissue areas surrounding PNL ($P < 0.05$) and in PNL areas ($P < 0.05$).

**Total plasma cholesterol**

Figure 6 shows total plasma cholesterol concentration values of all experimental groups at the end of the 8 weeks of experiment. Compared to the N group, CO group tended to present higher total plasma cholesterol concentrations (10). Compared to CO group, FOH group presented smaller ($P < 0.05$) total plasma cholesterol concentrations. There were no differences ($P > 0.05$) between CO group and GOH group regarding total plasma cholesterol concentrations.
**HMGCoA reductase mRNA dot blot analysis**

Measuring hepatic HMGCoA reductase mRNA may involve technical difficulties because of the low amount of this transcript (34). Thus in order to detect and quantify HMGCoA reductase mRNA in the obtained liver samples, we decided to use dot blot-based methodology, as recommended by Di Groce et al. (25).

Figure 7 shows HMGCoA reductase mRNA dot blot analysis performed with total RNA of normal rat livers and entire livers (nodules + non-nodular surrounding tissues) of animals from CO, FOH and GOH groups. Compared to N group, CO group did not present differences ($P > 0.05$) regarding hepatic levels of HMGCoA reductase mRNA. Compared to CO group, FOH group presented increased ($P < 0.05$) hepatic levels of HMGCoA reductase mRNA while GOH group did not present differences ($P > 0.05$). Since whole livers were used to evaluate HMGCoA reductase mRNA by dot blot analysis, it should be mentioned that the obtained results could be a reflection of the different proportions between the PNL area and the PNL-free liver area observed in the different groups.

**Hepatic DNA strand breakage**

In this study we opted for staining the formed comets with silver nitrate because of the advantages of the method, for example, to allow the permanent record of the experiment and independent verification of the results, as well as to avoid problems associated with fluorescence such as decay. In addition, staining with silver allows the comets to be analyzed using a simple light microscope instead of expensive and complex equipment such as fluorescence microscopes (10,20,28).

Table III shows comet length values of normal rat livers challenged or not with hydrogen peroxide (HP), as well as of livers of rats treated with CO, FOH or GOH and submitted to the RH model of hepatocarcinogenesis. Results are expressed in relation to normal animals’ hepatic HMGCoA reductase mRNA levels considered to be 100%. Values are means ± SEM, $n = 6$ (all groups). Statistics by Student’s $t$-test: statistically significant differences ($P < 0.05$) are represented by ‘a’ when compared to CO group (controls).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Final number of animals</th>
<th>Comet length ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>86.4 ± 6.0</td>
</tr>
<tr>
<td>N(HP)*</td>
<td>6</td>
<td>205.6 ± 4.2</td>
</tr>
<tr>
<td>CO</td>
<td>7</td>
<td>123.8 ± 8.9</td>
</tr>
<tr>
<td>FOH</td>
<td>8</td>
<td>98.7 ± 2.6</td>
</tr>
<tr>
<td>GOH</td>
<td>7</td>
<td>98.1 ± 5.5</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

*Positive comet assay controls.

*Negative comet assay controls.

Statistically significant difference compared to N group (Student’s $t$-test, $P < 0.05$).

Statistically significant difference compared to CO group (Student’s $t$-test, $P < 0.05$).
In the present study, FOH inhibited the incidence and mean number per rat of visible hepatocyte nodules, as well as the size of total, persistent and remodeling GST-P positive PNL and the percentage liver section area occupied by remodeling ones. To our knowledge, these are the first reports on chemopreventive activities of FOH and GOH on hepatocarcinogenesis.

Similarly, in a previous study of our group the isoprenoids geranylgeraniol and β-ionone also presented chemopreventive activities when administered at the dose of 16 mg/100 g body wt to rats during the initial phases of the RH model (10).

Treatment of rats with FOH prior to the initiation with azoxymethane inhibited the incidence of aberrant crypt foci (4). In a study also conducted with rats submitted to a colon carcinogenesis model, treatment with FOH before and after initiation with azoxymethane inhibited both incidence and multiplicity of aberrant crypt foci (5). Protective FOH actions were also reported in a study in hamsters where treatment for 48 weeks with the isoprenoid starting 5 weeks after initiation with 7 N-nitrosobis(2-oxopropyl)amine resulted in reduction of pancreatic carcinoma incidence (6). Female Sprague-Dawley rats treated with the GOH for 2 weeks prior to initiation with 7,12-dimethylbenz[a]anthracene and 22 weeks afterwards presented inhibition of mammary tumor multiplicity (7).

Besides being well-characterized and particularly adapted to compare effects of compounds potentially able to modulate an ongoing carcinogenic process, one of the most positive features of the RH model is its ability to distinguish the few persistent nodules from the large number of remodeling ones and thus to allow studies of the nodule to cancer sequence (35). In our study FOH and GOH reduced the sizes of both remodeling GST-P positive PNL and the persistent ones, considered to be the precursors of hepatocellular carcinomas (15).

In the present study FOH and GOH were administered continuously for 8 consecutive weeks starting 2 weeks before initiation with DEN and in a period comprising promotion with 2-AAF and partial hepatectomy. FOH inhibited in vitro rabbit liver CYP2E1 (36) and GOH induced in vivo GST (37). The isoprenoids’ protective actions in our study could be eventually related to inhibition of DEN and 2-AAF metabolism, as also suggested for geranylgeraniol and β-ionone (10). Thus it would be important to confirm FOH and GOH chemopreventive actions in other hepatocarcinogenesis models using different initiators and promoters, and by administering the isoprenoids specifically during the promotion phase of the RH model.

Tumor growth suppression by FOH and other isoprenoids is attributed to both the suppression of cell division and the induction of apoptosis (2). FOH has been shown to inhibit the proliferation of pancreatic cancer cells in vitro and in vivo (11) and to induce apoptosis in leukemia (38), lung (39) and pancreatic (6) cancer cells. GOH has been shown to mainly inhibit the proliferation of leukemia (40), hepatoma (41), melanoma (41) and pancreatic cancer (6) cells in vitro and/or in vivo.

Inhibition of cell proliferation was observed in the present study both in PNL and in the surrounding normal tissues after treatment with FOH or GOH at the dose of 25 mg/100 g body wt. Thus both isoprenoids’ chemopreventive activities could be attributed to inhibitory effects on cell proliferation. Similarly, rats treated with geranylgeraniol or β-ionone at a dose of 16 mg/100 g body wt during the initiation and promotion phases of the RH model presented inhibition of hepatic PNL cell proliferation (10).
Treatment of birds, rats or mice with isoprenoids such as GOH, β-ionone or limonene resulted in HMGCoA reductase activity inhibition and in total plasma cholesterol concentration decrease (42–44). Geranylgeraniol and β-ionone inhibited total plasma cholesterol levels in rats submitted to the RH model (10). In the present experiment treatment of rats with FOH, but not with GOH, resulted in significant decrease of total plasma cholesterol concentration. Considering that reduction in total cholesterol concentrations in rats treated with isoprenoids would reflect inhibition of HMGCoA reductase activity (10,45), we suggest that in our experiment cell proliferation inhibition by FOH, but not by GOH, could be related to inhibition of this enzyme as also proposed for geranylgeraniol and β-ionone (10). Isoprenoids’ actions on HMGCoA reductase occur at the posttranscriptional level (46,47). FOH markedly inhibited HMGCoA reductase activity in vitro through stimulation of its degradation (48,49). Interestingly, HMGCoA reductase mRNA hepatic levels were increased in FOH group. This increase could be due to a compensatory up-regulation of HMGCoA reductase gene expression following an eventual degradation of the enzyme by this isoprenoid. Such up-regulation has been described in livers of rats treated with lovastatin, a competitive inhibitor of HMGCoA reductase (34). On the other hand, cell proliferation inhibition by GOH could be due to mechanisms not related to the mevalonate pathway such as inhibition of ornithine decarboxylase activity (50).

FOH apoptosis induction has been shown mainly in tumor cell lines (6,38,39). Treatment with the sesquiterpene clearly induced apoptosis in human BxPC3 pancreatic cancer cells, but resulted in somewhat higher apoptotic rates in chemically induced hyperplastic pancreatic ductal neoplasms (6). In the present study FOH did not induce apoptosis either in the PNL area or in the normal surrounding area. Thus it would be important to evaluate FOH apoptosis induction potential in vivo, especially during preneoplastic phases. On the other hand, the monoterpene GOH induced apoptosis both in PNL area and in the normal surrounding area. This suggests that apoptosis induction seems to be relevant only for GOH’s chemopreventive activities observed in the present study. We are unaware of data in the literature showing in vivo apoptosis induction by this monoterpene. In vitro GOH induced apoptosis in pancreatic cancer cells (6).

In the present study both FOH and GOH inhibited cell proliferation and in addition, GOH but not FOH induced apoptosis. Based on these results it would be expected that GOH should be a better chemopreventive agent than FOH. However, the results of macroscopic analysis of the livers and morphometric analysis of GST-P positive PNL indicate that FOH was more effective than GOH. FOH inhibition of DEN metabolism (36) would decrease initiation and result in an attenuated carcinogenic process since the beginning of the experiment. GOH, by influencing 2-AAF metabolism (37), would present an impact only during the promotion phase. This would explain why FOH in addition to inhibiting the growth (size) of the foci, also inhibited the incidence and number of nodules per rat, whereas GOH only inhibited the growth (size) of the foci. FOH and GOH were administered by weight basis (25 mg/100 g body wt). If this dose is converted to a molar basis, GOH group rats received daily more micromoles (162 μmol/100 g body wt) of the respective isoprenoid compared to FOH group rats (112 μmol/100 g body wt). This reinforces the observations that FOH was more effective than GOH.

As previously observed (10,20,28), compared to normal rats DNA damage was increased in rats submitted to the RH model (CO group), as measured by the comet assay which enables the evaluation of breaks in the DNA strands and oxidized bases due to reactive oxygen species action (51). DNA damage was inhibited by treatments with FOH and GOH, isoprenoids without pronounced antioxidant activity. Similar effects were described for geranylgeraniol and β-ionone, also non-antioxidant isoprenoids (10). As suggested for these latter isoprenoids inhibition of DNA damage by FOH and GOH could involve induction of the DNA repair system (10).

During rat hepatocarcinogenesis hepatic FXR mRNA levels were not altered (52). Similarly, in our study hepatic FXR protein levels were also not altered in the RH model. Thus, contrary to what was suggested for nongenotoxic hepatocarcinogenesis (53), this nuclear receptor does not seem to be involved in DEN-induced hepatocarcinogenesis. Moreover, treatments with FOH and GOH did not alter hepatic FXR protein levels. This suggests that neither FOH nor GOH chemopreventive activities involve this nuclear receptor. In vitro FOH-induced keratinocyte differentiation involved alteration of peroxisome proliferator activated receptor α expression but not of FXRs (54).

In the presented study FOH and GOH showed pronounced inhibitory effects during the initial phases of the RH model, as also described for geranylgeraniol and β-ionone (10). This indicates that isoprenoids could represent a promising class of chemopreventive agents against hepatocarcinogenesis. Furthermore, inhibition of cell proliferation and DNA damage seems to be important for both isoprenoids’ anticarcinogenic actions, while induction of apoptosis seems to be specifically related to GOH protective actions. Inhibition of HMGCoA reductase activity could be associated with FOH, but not GOH protective actions. FXR does not seem to be involved in the isoprenoids’ chemopreventive activities.

Acknowledgements

The authors thank Miss Silvia M.P. Neves for providing the care and maintenance of the animals. T.P.O. was supported by a fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 00/00918-8). This study was also supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of Interest Statement: None declared.

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