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Prevention and Therapy of Mammary Cancer by Monoterpenes

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Abstract Monoterpenes, found in a wide variety of plants, are a major component of plant essential oils. The unsubstituted monocyclic monoterpene limonene has been shown to prevent carcinogen-induced mammary cancer at both the initiation and the promotion/progression stages. This terpene also causes the complete regression of the majority of advanced rat mammary cancer when added to the diet. Modification of limonene by hydroxylation at various positions increases both its chemopreventive and therapeutic efficacy. For example, the naturally occurring hydroxylated limonene analog perillyl alcohol is 5–10 times more potent than limonene and has a similar therapeutic index.

Several cellular, metabolic and molecular activities are associated with terpene exposure. These include induction of phase I and II hepatic detoxification enzymes, selective inhibition of protein isoprenylation, inhibition of CoQ synthesis, and induction of the mannose 6-phosphate/IGFII receptor and TGFβ.

Due to the therapeutic efficacy of monoterpenes in experimental model systems, clinical evaluation of this class of compounds has begun in advanced cancer patients. A Phase I trial of limonene is in progress in the UK. Efforts in the US will target perillyl alcohol for Phase I testing. Pre-IND toxicology is currently being completed. Phase I trials are anticipated to begin in the Spring of 1995. We feel that the results of these therapeutic trials, if positive, will facilitate the development of current terpenes and more potent analogs for future chemoprevention trials.

Key words: Breast cancer, cancer prevention, cancer therapy, monoterpene

Progress in treating most common solid malignancies has slowed in recent years, due at least in part to the lack of new, efficacious agents. This has led to a new emphasis on cancer prevention and on developing therapeutic agents with novel mechanisms. Recent data from our laboratory and others suggest that monoterpenes, a class of non-toxic compounds that act through novel mechanisms, may be useful for both chemoprevention and treatment of cancer.

Monoterpenoids, formed by the condensation of two isoprene molecules, are commonly and primarily produced by plants. They occur in monocyclic, bicyclic and acyclic forms in many commonly consumed fruits and vegetables, including citrus fruits and food flavoring such as mints, and are either simple or oxygenated hydrocarbons. Limonene, the simplest monocyclic monoterpene, has been shown to inhibit a variety of organ-specific cancers in rodent models including mammary [1], stomach, lung, skin and liver cancers [reviewed in 2].

The potential of monoterpenes to prevent cancer was first reported in 1971 by Homburger *et al.* [3], who showed that when limonene was co-administered with the carcinogen benzo[*rst*]-pentaphene, developing tumors were reduced [3]. The ability of these compounds to prevent formation of carcinogen-induced rat mammary cancer was independently reported by both our group [1] and that of Wattenberg [4]. Wattenberg's group, while screening diverse natural

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products, found that orange peel oil, composed mainly of limonene, could prevent 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary cancer [4]. Our group was evaluating limonene among various compounds with the ability to inhibit HMG-CoA reductase activity to prevent mammary carcinogenesis. This early experiment [5] showed that of inhibitors tested at 20 ppm in the diet, only limonene reduced mammary tumor yield following DMBA. Interestingly, this dose of limonene did not inhibit hepatic HMG-CoA reductase. Subsequent experiments using higher non-toxic doses of limonene confirmed its preventative activities [1].

The possibility of extending the monoterpenes' utility beyond prevention to therapy was first suggested by a rigorous statistical analysis of a mammary chemoprevention experiment which found that mammary carcinomas in the limonene-fed groups had a greater tendency to subsequently regress than those in the control group [1]. This suggestion was first confirmed in experiments in which small mammary tumors in the rat (~3 mm in diameter) were treated with limonene [6,7] and later extended to the therapy of large, stable, advanced mammary carcinomas [7,8]. Phase I therapeutic trials have just begun as a starting point to evaluate limonene's ability to treat human malignancies [9].

MONOTERPENE CHEMOPREVENTION

In addition to preventing DMBA-induced mammary carcinogenesis, as discussed above, we showed that limonene could also prevent carcinomas induced by nitrosomethylurea (NMU), a directly acting carcinogen. Furthermore, cancer prevention with dietary limonene intervention occurred only during promotion/progression of NMU-induced carcinomas, not during initiation [10]. The finding that the terpenes could prevent cancer associated with directly acting carcinogens suggested that the terpenes were not only blocking agents but were also suppressing agents in their prevention role.

In order to extend our mammary prevention studies beyond those associated with chemical exposure, we evaluated and established a model in our laboratory [11] which directly introduces activated oncogenes into *in situ* ductal mammary cells using amphotropically packaged retroviral vectors. In the first study using this model to evaluate a chemopreventive agent, we inserted the activated v-Ha-*ras* oncogene into *in situ* mammary cells. *ras* was chosen for this first study because monoterpenes were shown to inhibit the post-translational farnesylation of RAS p21 (see below). Carcinomas that developed following retroviral transfer of *ras* were more locally invasive than chemically induced cancer; unlike chemically induced cancer, they were capable of metastatic spread [11]. We showed that dietary limonene could prevent mammary carcinomas induced by the direct transfer of activated *ras*. At necropsy, control rats had developed 7.6 carcinomas/rat; limonene-fed rats had developed only 1.2 carcinomas/rat [12,13].

MONOTERPENE TUMOR THERAPY

In the first follow-up of our initial observation of monoterpene therapeutic efficacy as discussed above, rats were given either DMBA or NMU. When mammary tumors of 3 mm diameter were palpated, rats were assigned to a 10% limonene or control diet. Limonene caused a significant increase in regression of tumors initiated by both DMBA and NMU when compared to the occurrence of spontaneously regressing tumors. These regressions likely were due to a cytostatic rather than cytotoxic effect of limonene; a significant percentage of regressed tumors reappeared if limonene treatment was stopped, but did not recur during continuous limonene treatment [7]. Limonene also prevented the development of secondary tumors, *i.e.*, carcinomas developing after dietary assignment.

This study of small early tumors bridged the areas of prevention and therapy. Since the tumors were quite small and still subject to spontaneous regression, it was important to test the effects of limonene on large, advanced, stable mammary carcinomas. In this study, tumors were initiated with DMBA or NMU. Rats with carcinomas at least 10 mm in diameter were randomly assigned to a 10% limonene or control diet group. In the DMBA study, no spontaneous complete regressions were observed and only 7% of these tumors had partial regressions. In contrast, 87% of the limonene-treated tumors showed regressions (complete ~60% and partial ~27%). Similar results were found in the NMU-induced mammary carcinomas [7,8].

MECHANISMS UNDERLYING TERPENE PREVENTION OF DMBA-MAMMARY CARCINOMAS DURING THE INITIATION PERIOD

Limonene and related monoterpenes fed only at the time surrounding carcinogen administration (–2 weeks to +1 week) were shown to prevent DMBA- but not NMU-initiated mammary carcinomas. This suggests that limonene can act as a "blocking" agent, and led to the hypothesis that limonene could alter DMBA metabolism or its adduction to DNA. [^{3}H]-DMBA was orally gavaged into rats eating control or limonene diets. Twenty-four hours post-DMBA, rats were necropsied and DNA extracted from a variety of tissues. DNA from all tissues in the limonene-fed rats showed a similar (~50%) reduction in bound ^{3}H from DMBA. This suggested a systemic effect of limonene. These observations led to the hypothesis that limonene alters the systemic (hepatic) metabolism of DMBA, reducing the available active DNA-binding metabolites of DMBA [14].

In order to test this hypothesis, we quantified the effects of two monocyclic monoterpenes on hepatic phase I and phase II DMBA-metabolizing enzymes. Rats were fed either limonene (1% or 5% in AIN76A) or sobrerol (1% in AIN76A). Five percent limonene and 1% sobrerol have approximately equal chemopreventive activities when fed during DMBA administration. Control rats received either AIN76A diets or AIN76A diets with phenobarbital (PB). The 5% limonene diet raised total CYP (P-450) to the same extent as PB, whereas 1% sobrerol did not raise CYP levels. Both 5% limonene and 1% sobrerol had similar effects on raising microsome epoxide hydratase (EH) protein and associated hydrating activity toward benzo(*a*)pyrene 4,5-oxide. These monoterpenes also modified the rate and regioselectivity of *in vitro* microsomal DMBA metabolism when compared to PB treatment or control diets. We concluded that while terpene diets did modify phase I metabolism of DMBA, these changes did not explain the chemopreventive activity under study [14]. We thus extended these studies to hepatic phase II enzymes. We investigated the potential roles of hepatic glutathione-S-transferase (GST) and uridine diphosphoglucuronosyl transferase (UDPGT) in monoterpene-mediated chemoprevention. Both diets containing the isoeffective anticarcinogenic terpenes limonene (5%) or sobrerol (1%) elevated the hepatic GST activity. Both terpene diets also significantly increased the activity of the methylcholanthrene-inducible and the phenobarbital-inducible UDPGT isozymes. One percent sobrerol was as effective as 5% limonene in these enzyme inductions. We thus conclude that much of the anticarcinogenic activity of these monocyclic monoterpenes during the initiation phase of DMBA carcinogenesis is mediated through the induction of hepatic detoxification enzymes GST and UDPGT [15].

ACTIVITIES (POTENTIAL MECHANISMS) ASSOCIATED WITH PREVENTION AT THE PROMOTION/PROGRESSION STAGE AND THERAPY OF MAMMARY CARCINOMAS

We have shown in a wide variety of rodent and human cell lines that the monoterpenes reversibly inhibit cell growth [16]. Recent experiments with NIH 3T3 cells suggest that the terpenes cause a G_1 cell cycle block. This inhibition of growth can also be seen in *in vivo* mammary carcinomas. We also compared the histopathology of tumors that were collected when they regressed to half their maximal diameter with control tumors. Control tumors were mostly anaplastic and very cellular with little stromal tissue. Regressing tumors had areas that resembled control histopathology but also had areas characterized as having a simple epithelium surrounding duct-like structures embedded in a dense stroma. This latter histopathology dominated as regression proceeded. Finally, this simple epithelium was for the most part replaced by stromal tissue as the tumor completely regressed [7].

No endocrine mechanism (estrogen/prolactin) appeared to be involved in limonene's anticancer activity, so we turned to the mevalonolactone-lipid pathway, since monocyclic monoterpene limonene derives from it. Though limonene structure is tertiary, like members of this pathway, it is constrained by its ring structure. Hypothesizing that limonene could act as a prenyltransferase inhibitor in mammalian cells, we investigated the potential of monoterpenes to inhibit the synthesis of various products of this pathway.

A small but important subset of cellular proteins undergoes post-translational modification when lipids are added to the carboxyl terminus

by adding either farnesyl (C-15, 3 isoprenes) or geranylgeranyl C-20, 4 isoprenes) to cysteine. These proteins include small G proteins such as RAS p21, the γ subunit of the heterotrimeric G proteins, and the nuclear structural lamins A and B [reviewed in 2].

We used cultured cells to study the effects of monoterpenes on protein isoprenylation. Limonene inhibited isoprenylation of a class of cellular proteins of 21–26 kD, including RAS p21 and possibly other small GTP-binding proteins, in a dose-dependent manner in both cell lines, but did not affect the isoprenylation of several other proteins, including the nuclear lamins. Limonene is metabolized extensively *in vivo* (see below) but not in cultured cells. When tested, rat serum metabolites of limonene were found to be more potent than limonene in the inhibition of isoprenylation in NIH 3T3 cells. These results demonstrate that limonene selectively inhibits isoprenylation of 21–26 kD proteins at a point in the mevalonic acid pathway distal to 3-hydroxy-3-methylglutaryl coenzyme A reductase [17].

In addition to altering the isoprenylation of protein, we have detected other changes in the mevalonolactone-lipid pathway. After [^{14}C]-mevalonolactone labeling in HMG-CoA reductase-blocked (lovastatin) cells, we found a dramatic reduction in ubiquinone (CoQ) synthesis upon terpene addition. While analyzing CoQ levels by thin layer chromatography (TLC), we noticed that the spot we previously thought was cholesterol shifted slightly on our high resolving TLC system following terpene exposure. Further analysis showed that while the control spot was indeed cholesterol, the shifted spot in perillyl alcohol-treated cells was identified as lathosterol, a sterol precursor to cholesterol. In NIH 3T3 cells treated with perillyl alcohol, conversion of lathosterol to cholesterol is inhibited 75%. Interestingly, there was no inhibition in the synthesis of the combined pool of cholesterol and lathosterol, suggesting a specific inhibition by the terpenes near the end of the cholesterol synthetic pathway between lathosterol and cholesterol [18].

Based on the finding that terpenes could cause the regression of frank malignancies via a cytostatic/differentiating mechanism, we hypothesized that this effect could be mediated via the up- or down-regulation of genes that are important in this process. Such gene regulation could be mediated through a signaling pathway perturbation after even modest changes in protein isoprenylation.

Sice many phase I and phase II hepatic detoxification enzymes which were up-regulated in response to terpene exposure were specific isoforms whose up-regulation was also associated with PB treatment [15,16], we started our search with known PB-inducible genes that might have relevance to mammary cancer. Jirtle *et al.* [19] had demonstrated that PB could up-regulate protein levels of the mannose-6-phosphate/insulin-like growth factor II (M6P/IGFII) receptor and TGF-β_1. Collaborating with Professor Jirtle, we compared control growing and limonene-treated regressing mammary carcinomas for M6P/IGFII receptor and TGF-β_1 using immunohistochemical methods. Both these proteins increased dramatically in limonene-treated regressing tumors when compared to control tumors. Interestingly, the increases co-localized, confined mostly to tumor parenchymal (epithelial) cells and not found in stromal cells. We next measured cellular mRNA for these two proteins using a RNase protection assay, and found a consistent increase (~2–3X) in mRNA for the M6P/IGFII receptor. No change in TGF-β_1 message was found, not unexpected since TGF-β_1 is often post-translationally controlled. Interestingly, the mRNA from two mammary carcinomas that were resistant to limonene therapy did not have elevated levels of mRNA for either the M6P/IGFII receptor or TGF-β_1 [8]. The M6P/IGFII receptor has at least two functions related to mammary cancer regression. It internalizes IGFII for lysosomal degradation. In addition, the latent form of TGF-β_1 has two mannose residues which bind to the M6P/IGFII receptors. This binding facilitates activation of the negative mammary growth factor TGF-β. Together, these two receptor-mediated activities could lead to mammary carcinoma regression via a cytostatic/differentiation mechanism.

PRECLINICAL PHARMACOLOGIC STUDIES OF MONOTERPENES

We characterized circulating metabolites of limonene in female rats and determined their effects on cell growth. The two major circulating metabolites of limonene were perillic acid and dihydroperillic acid. Two minor metabolites were

detected and identified as the methyl esters of the two major acid metabolites [20].

We subsequently showed that all four metabolites of limonene were more effective than limonene in inhibiting protein isoprenylation in cultured cells. The rapid metabolism of limonene in rats, together with the biological activity of its metabolites, suggests that limonene is acting as a prodrug, *i.e.*, its antitumor activity may be derived from its metabolites. If this is the case, it is important to identify and quantify the metabolites of limonene in humans to use it therapeutically.

We performed an IRB-approved study on healthy human volunteers to identify plasma metabolites of limonene. On-line capillary gas chromatography/mass spectrometry (GC/MS) analysis indicated that at least five additional compounds were present after 4 hours. Two major peaks were identified as the rat limonene metabolites dihydroperillic acid and perillic acid. Two minor peaks were respective methyl esters of these acids as found in the rat. A third major peak was identified as limonene-1,2-diol. Limonene was thus metabolized by humans and rats in a similar manner. These facts and the high therapeutic ratio of limonene in the therapy of rodent cancers has led to clinical trials of limonene in patients with various malignancies. These Phase I trials are in progress in England [9,21].

While limonene has an excellent therapeutic index in our rat model and shows little acute or chronic toxicity in many species including humans, clinical tumor therapy would require large multiple daily doses (~1 g/kg/day divided into 3 or 4 doses). While this dosing is likely achievable, it would complicate compliance and might cause gastrointestinal disturbance. On-going Phase I studies will help clarify these concerns. To develop more potent analogues, we chose to investigate perillyl alcohol in our first *in vivo* rat study. It is a potent inhibitor of protein prenylation in cultured cells, is on the FDA GRAS (generally regarded as safe) list, and is commercially available at high purity >96% in bulk quantities.

When tested for therapy in advanced, stable, rat mammary carcinomas, 2% dietary perillyl alcohol caused the regression of 75% of these tumors (50% complete, 25% partial). Significant levels of regression were obtained at dietary levels as low as 0.5%. Thus perillyl alcohol was greater than five times more potent than limonene in this model system.

Rats with DMBA-induced mammary carcinomas fed diets of 1% perillyl alcohol had a tumor regression rate of 55%. We estimated that they consumed 0.88 g/kg-bw/day of perillyl alcohol. This converts to 5.2 g/m^2. This g/m^2 dose in the rat converts to 10 g/day for a 70 kg human or three daily doses of 3.3 g/dose. We feel that this may be a tolerable dose in a cancer clinical trial [21]. We are currently working with the National Cancer Institute to complete formal preclinical testing and formulation for perillyl alcohol. We anticipate starting clinical trials at our Cancer Center as well as other institutions within 6–12 months.

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