PREVENTION AND TREATMENT OF MAMMARY CANCER WITH MONOTERPENES¹

Michael N. Gould

Department of Human Oncology, University of Wisconsin-Madison

Abstract: The monoterpenes are a new class of anticancer drugs having efficacy in both cancer prevention and treatment. We have shown that the monocyclic monoterpenes limonene and perillyl alcohol can prevent chemically-induced rat mammary carcinomas if fed either during the initiation stage or the promotion/progression stage of carcinogenesis. In addition, these terpenes can induce the complete regression of the majority of advanced mammary carcinomas without toxicity. The activities associated with tumor regression include the inhibition of the prenylation of small G proteins and the synthesis of ubiquinone. In addition, regressing tumors have elevated levels of TGF β and its activating receptor. Both rodents and humans metabolize limonene to similar compounds. Based on these findings limonene is currently undergoing clinical testing in cancer patients. (*J Toxicol Pathol* 7: 237~241, 1994)

Key words: Breast cancer, Terpenes, Cancer therapy, Cancer prevention, Review

Introduction

Advancements in the therapy of cancer has slowed over the last several decades. This has caused a redirection in oncology toward a new focus on both cancer prevention and therapy with agents which act through novel mechanisms. The monoterpenes represent a new class of cancer drugs which may be efficacious in both preventing and treating malignant disease.

Monoterpenes are made by plants by the condensation of two isoprenes in the mevalonate-lipid pathway. Monoterpenes are simple or substituted 10 carbon hydrocarbons. They can be in an acyclic, monocyclic or bicyclic form. Many monoterpenes are the major component of plant essential oils which are often used as flavoring agents.

Our laboratory has shown that many monoterpenes can both prevent and treat experimental cancers. We have focused most of our work on

two monocyclic monoterpenes limonene, a simple hydrocarbon and its oxygenated analogue perillyl alcohol. The first report indicated that the monoterpenes could function to prevent cancer was in the early 1970's. Hamburger et al. showed that limonene inhibited dibenzpyrene-induced subcutaneous tumors¹. In the mid-1970's Duuren and Goldschmidt showed that limonene inhibited benzo(a)pyrene induced skin tumors². In the early 1980's, our group and Wattenburg's group independently demonstrated that limonene³ and orange oil⁴, from which it is derived, could prevent DMBA initiated rat mammary cancer.

Prevention of Mammary Cancer

Our prevention studies have focused on a series of related rat mammary carcinoma models. In most studies mammary cancer is initiated by either the directly acting carcinogen NMU or the indirectly acting carcinogen DMBA. The latter, a polycyclic aromatic hydrocarbon, requires metabolic activation. For prevention studies, this model can be divided into two stages: an initiation stage which surrounds a single carcinogen administration (–2 weeks to +1 week); and a promotion/progression stage which follows initia-

Accepted for publication: March 8, 1994

Mailing address: Michael N. Gould University of Wisconsin-Madison Department of Human Oncology, K4/332 600 Highland Avenue Madison, WI 53792, USA

¹ Supported by PHS, NIH Grant CA38128

tion and ends at the appearance of a recognizable carcinoma (+2 weeks to approximately 18-20 weeks). As will be discussed below, these models can also be used to study therapy. Carcinomas of below 5 mm in diameter often (~25%) spontaneously regress whereas tumor of 10 mm diameter or larger are almost always stable.

We have shown that limonene can prevent DMBA⁵ but not NMU⁶ induced mammary carcinomas if fed only surrounding initiation. This observation suggested that limonene might alter DMBA metabolism. Rats fed limonene surrounding the administration of [³H]-labeled DMBA had approximately a 50% reduction in carcinogen-DNA adducts in all tissues examined⁷. This data was interpreted to suggest that limonene increases the systemic (hepatic) detoxification of DMBA. Limonene was found capable of increasing the levels of both phase I and phase II detoxification enzymes. Phase I enzymes that increase included both cytochrome P450 and epoxide hydratase⁷. Both the phase II enzymes glutathione transferase and glucuronosyl transferase were also found to increase⁸. A structure activity study suggested that the ability of the monoterpenes to prevent DMBA-initiated carcinomas when fed during initiation was mainly due to their ability to induce phase II detoxification enzymes⁸.

The monoterpenes have also been shown to be able to prevent mammary carcinomas at the promotion/progression stage. Specifically limonene can prevent both DMBA⁵ and NMU⁶ initiated carcinomas if fed only during the promotion/progression stage. The mechanism underlying prevention at this stage will be discussed below.

Therapy of Mammary Cancer

In our first study of limonene's chemoprevention of mammary cancer, we fed this terpene from one week before DMBA treatment to 25 weeks post treatment. Not only did we observe that limonene could prevent mammary cancer but a statistical analysis of the data confirmed that limonene treatment increased the rate of regression of carcinomas after their detection by palpation³. This observation was first confirmed in an experiment in which rats with small (~3 mm diameter) tumors were placed in limonene diets⁹. Rats treated with 10%

dietary limonene¹⁰ in a third study showed a 68% complete regression of carcinomas. A steep dose response was seen with 7.5% being an effective treatment while 5% limonene diets did not induce a significant level of regression. Even at 10% dietary levels limonene did not cause significant toxicity.

Even though limonene had an excellent therapeutic index a large amount of this compound was needed for successful treatment. This large volume might inhibit human compliance to a prescribed therapy. We thus attempted to identify more potent monoterpenes.

Perillyl alcohol was chosen for evaluation because it was found to be more effective than limonene in various *in vitro* screens (see below), and was readily available at high purity. When tested, perillyl alcohol was found to be at least five times as potent as limonene. One to two percent dietary perillyl alcohol was as effective as 10% dietary limonene in causing tumor regression. Significant levels of regression was observed even following 0.5% dietary perillyl alcohol. Doses as high as 2% could be chronically fed to rats without toxicity¹¹.

Metabolism of Monoterpenes

The large amounts of limonene required for both therapy and prevention during the latter stages of carcinogenesis suggested the hypothesis that limonene was a prodrug with active metabolites. The urinary metabolites of limonene were extensively characterized in several species¹². However, it is the serum metabolites that are more likely to have a therapeutic function. We thus characterized the serum metabolites of limonene in the rat. Following extraction, the terpene content of the serum was analyzed by gas chromatography with both mass and infrared spectroscopy. Structures deduced from the spectroscopy were then synthesized and their GC and MS characteristics were compared to target metabolites for verification.

Soon after oral administration of limonene, it is rapidly metabolized to two major and two minor metabolites. The major metabolites were shown to be perillic acid and dihydroperillic acid while the minor metabolites were the methyl esters of

these two acids. Using similar methods, we also characterized the metabolites of perillyl alcohol in the rat. Interestingly, the oxygenated monoterpene was metabolized to the same four metabolites as was limonene. This raised the question of why then was this alcohol more potent than limonene in its *in vivo* therapeutic action? The answer was found in the relative pharmacokinetics of these terpenes. Feeding perillyl alcohol at a 2% dietary level resulted in a higher level of these metabolites than feeding rats with a 10% limonene diet.

This rat data suggests that limonene and perillyl alcohol are prodrugs. Thus if they are to be used for human therapy, it is important to ask how they are metabolized in humans. Subjects were fed 0.1 g/kg of limonene. Blood was collected four hours after feeding and analyzed for limonene and terpene metabolites. Human serum contained all four rat metabolites. In addition, humans metabolized limonene to a unique serum metabolite the limonene 1,2-diol¹³. The similarities in metabolism between rat and humans suggest that the prodrug limonene and perillyl alcohol are candidates for human therapeutic trials.

Cellular, Biochemical and Molecular Activities of Terpenes During Therapy

The monoterpenes and their metabolites have been shown to have several *in vitro* and *in vivo* activities. These have the potential of being mechanistically involved in late-stage prevention and therapy of mammary cancer. The mechanism underlying the terpenes anticancer activity is not organ-specific in that terpenes can modulate carcinogenesis in organs other than mammary such as liver¹⁴. In addition, we have thus far failed to link the effects of monoterpenes to any mammary-endocrine effect. We thus sought alternative activities within the mevalonate-lipid pathway in which limonene is synthesized.

The initial effect that we reported for monoterpenes in this pathway was for the isoprenylation of proteins. An important but small subset of cellular proteins are post-translationally modified by the addition of farnesyl or geranylgeranyl to the carboxyl end of the protein through a covalent linkage with cysteine. We have shown that when cultured cells are treated with monoterpenes pro-

tein isoprenylation is inhibited¹⁵. This inhibition includes proteins which are prenylated with either farnesyl or geranylgeranyl, and is confined mainly to proteins between 21 and 26 kd. Many of these proteins are members of the small G protein family which includes the *ras* proteins.

The above experiments were done by labeling cells with C14-mevalonate. Thus the exact mechanism underlying the inhibition of protein isoprenylation could lie anywhere between mevalonic acid and the isoprenylated protein. In order to begin to define specific targets we first studied the effects of the monoterpenes and their metabolites on isolated protein farnesyl transferase (PFT) and protein geranylgeranyl transferase (PGGT). Both mammalian and yeast enzyme were studied *in vitro*. Limonene, perillyl alcohol and their major metabolite perillic acid were weak inhibitors of both enzymes from both species. In contrast, the minor metabolite of these terpenes perillic acid methyl ester was a potent inhibitor of both enzymes with an IC₅₀ of approximately 10 μ M. This methyl ester was shown to be a competitive inhibitor of yeast PFT in regards to farnesyl pyrophosphate¹⁶.

We next asked which protein whose prenylation was affected by the terpenes was a functional target for prevention and therapy. *Ras* was the first small G protein to be studied. We showed that when 3T3 cells were treated with 1 mM perillyl alcohol a 50% inhibition of protein prenylation was obtained. By immunoprecipitation experiment, we found that the farnesylation of *ras* was inhibited by 40%.

In order to determine if this effect of *ras* was functionally involved *in vivo*, we asked if dietary limonene could prevent mammary carcinomas induced specifically by activated *ras*. Mammary carcinomas were induced by the direct transfer of v-Ha-*ras* into *in situ* ductal mammary cells by the use of retroviral vector¹⁷. Limonene feeding did not affect the rate of integration or stability of expression of retrovirally delivered genes¹⁸. Limonene, however, was very efficient at preventing tumor development subsequent to *ras* delivery¹⁸. While this finding demonstrates that limonene is effective in preventing *ras* associated tumors, it does not implicate *ras* as a primary

target.

In order to ask if *ras* was a primary target, we initiated mammary carcinomas with NMU under a protocol which would activate *ras* in half the tumors which developed¹⁹. We hypothesized that if *ras* was a primary target for terpene treatment then tumors with activated *ras* should be preferentially prevented. This was, however, not observed. While limonene did prevent tumors in a NMU treated rat, it did not alter the percentage of tumors with activated *ras*¹⁸.

In addition to affecting protein isoprenylation, two other biochemical effects of terpenes in the mevalonate-lipid pathway were found in terpene treated cultured cells. We found that terpene treatment was highly effective in inhibiting the synthesis of ubiquinone²⁰. This compound is required for mitochondrial oxidative phosphorylation. This may contribute to the positive terpene therapeutic ratio in that tumors unlike animal tissue are often deficient in this process.

While investigating ubiquinone synthesis using thin layer chromatography of cellular lipids, we noticed that the cholesterol spot had a slight shift in migration when isolated from terpene treated cells. This shift in location was due to the inhibition in the final synthesis of cholesterol from the sterol lathosterol. The shifted spot was thus lathosterol. The sum of lathosterol plus cholesterol was, however, constant in control and terpene treated cells, only the ratio of these two sterols was altered²⁰.

As described above terpene treatment could increase liver enzymes *in vivo*. Based on this, we hypothesized that terpenes could alter key protein levels in treated tumors. These changes could possibly occur at least in part via alteration in the processing of small G proteins which are involved in signal transduction. In order to test this hypothesis, we began studying mammary relevant proteins in terpene treated regressing mammary carcinomas.

We found that regressing mammary tumors had dramatic increases in TGF β_1 and the mannose 6-phosphate/IGF II receptor. The increase in both proteins was co-localized and found exclusively in the tumor parenchymal cells²¹. TGF β is a negative mammary growth factor for mammary

gland and carcinoma derived cells. It is secreted in a latent form which requires activation for biological activity. This activation is facilitated by its binding to the M6P/IGFII receptor via two mannose residues in the latent complex. In addition to this activity this receptor also acts to degrade IGFII, a mammary co-mitogen²¹.

Conclusions

The monoterpenes represent a new class of anticancer compounds that are effective in preventing and treating mammary carcinomas in the rat. These compounds act via novel mechanisms in causing the regression of experimental cancers. Pharmacokinetic studies suggest that limonene and perilly alcohol may be prodrugs which act via their metabolites. Fortunately, humans have similar metabolites. Based on these data, phase I cancer therapeutic trials with limonene have begun in England²². In addition, perilly alcohol is in pre-clinical development and formulation in anticipation of U.S. clinical trials in advanced cancer patients.

References

1. Homburger, F, Treger, A, and Boger, E: Inhibition of murine subcutaneous and intravenous benzo (rst) pentaphene carcinogenesis by sweet orange oils and d-limonene. *Oncology* **25**: 1-20, 1971.
2. Van Duuren, BL and Goldschmidt, BM: Cocarcinogenic and tumor-promoting agents in tobacco carcinogenesis. *J Natl Cancer Inst* **56**: 1237-1242, 1976.
3. Elegbede, IA, Elson, CE, Qureshi, A, Tanner, MA, and Gould, MN: Inhibition of DMBA-induced mammary cancer by the monoterpene d-limonene. *Carcinogenesis* **5**: 661-664, 1984.
4. Wattenberg, LW: Inhibition of neoplasia by minor dietary constituents. *Cancer Res (Suppl)* **43**: 2448-2453, 1983.
5. Elson, CE, Maltzman, TH, Boston, JL, Tanner, MA, and Gould, MN: Anti-carcinogenic activity of d-limonene during the initiation and promotion/promgression stages of DMBA-induced rat mammary carcinogenesis. *Carcinogenesis* **9**: 331-332, 1988.
6. Maltzman, TH, Hurt, LM, Elson, CE, Tanner, MA, and Gould, MN: The prevention of nitrosomethylurea-induced mammary tumors by d-limonene and orange oil. *Carcinogenesis* **10**: 781-783, 1989.
7. Maltzman, TH, Christou, M, Gould, MN, and Jefcoate, CR: Effects of monoterpenoids on *in vivo*

- DMBA-DNA adduct formation and on phase I hepatic metabolizing enzymes. *Carcinogenesis* **12**: 2081-2087, 1991.
8. Elegbede, JA, Maltzman, TH, Elson, CE, and Gould, MN: Effects of anticarcinogenic monoterpenes on phase II hepatic metabolizing enzymes. *Carcinogenesis* **14**: 1221-1223, 1993.
 9. Elegbede, JA, Elson, CE, Tanner, MA, Qureshi, A, and Gould, MN: Regression of rat primary mammary tumors following dietary d-limonene. *J Natl Cancer Inst* **76**: 323-325, 1986.
 10. Haag, JD, Lindstrom, MJ, and Gould, MN: Limonene-induced regression of mammary carcinomas. *Cancer Res* **52**: 4021-4026, 1992.
 11. Haag, JD and Gould, MN: Mammary carcinoma regression induced by perillyl alcohol, a hydroxylated analog of limonene. *Cancer Chemother Pharmacol*, submitted, 1993.
 12. Kodama, R, Yano, T, Furukawa, K, Noda, K, and Ide, H: Studies on the metabolism of d-limonene (p-mentha-1,8-diene): IV. Isolation and characterization of new metabolites and species differences in metabolism. *Xenobiotica* **6**: 377, 1976.
 13. Crowell, PL, Elson, CE, Bailey, HH, Elegbede, A, Haag, JD, and Gould, MN: Human metabolism of the experimental cancer therapeutic agent d-limonene. *Cancer Chemother Pharmacol*, submitted, 1993.
 14. Dietrich, DR and Swenberg, JA: The presence of $\alpha 2\mu$ -globulin is necessary for d-limonene promotion of male rat kidney tumors. *Cancer Res* **51**: 3512, 1991.
 15. Crowell PL, Chang, RR, Ren, Z, Elson, CE, and Gould, MN: Selective inhibition of isoprenylation of 21-26 kDa proteins by the anticarcinogen d-limonene and its metabolites. *J Biol Chem* **226**: 17679-17685, 1991.
 16. Gelb, MF, Tamanoi, F, and Gould, MN: The inhibition of protein prenyltransferases by oxygenated metabolites of limonene. in preparation, 1994.
 17. Wang, B, Kennan, WS, Yasukawa-Barnes, J, Lindstrom, MJ, and Gould, MN: Carcinoma induction following direct *in situ* transfer of v-Ha-ras into rat mammary epithelial cells using replication-defective retrovirus vectors. *Cancer Res* **51**: 2642-2648, 1991.
 18. Gould, MN, Moore, CJ, Zhang, R, Wang, B, Kennan, WS, and Haag, JD: Limonene chemoprevention of mammary carcinoma induction following direct *in situ* transfer of v-H-ras into rat mammary epithelial cells using replication-defective retroviral vectors. *Cancer Res* submitted, 1993.
 19. Zhang, R, Haag, JD, and Gould, MN: Reduction in the frequency of activated *ras* oncogenes in rat mammary carcinomas with increasing N-methyl-N-nitrosourea doses or increasing prolactin levels. *Cancer Res* **50**: 4286-4290, 1990.
 20. Ren, Z and Gould, MN: Inhibition of ubiquinone and cholesterol synthesis by the monoterpene perillyl alcohol. *Cancer Lett*, in press, 1994.
 21. Jirtle, RL, Haag, JD, Ariazi, EA, and Gould, MN: Increased levels of M6P-IGF-II receptor and TGF β 1 levels during monoterpene-induced regression of mammary tumors. *Cancer Res* **53**: 3849-3852, 1993.
 22. McNamee, D: Limonene trial in cancer. *Lancet* **342**: 801, 1993.