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Chemopreventive Effect of Perillyl Alcohol on 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Induced Tumorigenesis in (C3H/HeJ X A/J)F_1 Mouse Lung

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Abstract This study was designed to test the chemopreventive potential of perillyl alcohol, an inhibitor of farnesyltransferase, in a mouse lung tumor bioassay. Perillyl alcohol is a naturally occurring monoterpene found in lavender, cherries, and mint. We have shown previously that the majority of lung tumors in this bioassay have an activating mutation in the K-*ras* gene, which occurs early in the development of mouse lung carcinogenesis. The Ras protein undergoes a series of post-translational modifications, the first of which is farnesylation at the cysteine of the C-terminal CAAX motif. These modifications lead to the anchoring of Ras p21 to the plasma membrane in its biologically active state. Activated Ras p21 couples growth regulatory signals from receptor tyrosine kinases to cytoplasmic second messengers. In a preliminary study, we determined the maximum tolerated dose of perillyl alcohol to be 75 mg/kg body weight. For the bioassay, 5-week-old male (C3H/HeJ X A/J) F1 hybrid mice were randomized into trial groups, and treated with perillyl alcohol three times per week i.p., starting 1 week prior to initiation with the carcinogen NNK, and continuing for 22 weeks after initiation. Our results show a 22% reduction in tumor incidence, and a 58% reduction in tumor multiplicity. Our study demonstrates that perillyl alcohol is an effective chemopreventive compound in the mouse lung tumor bioassay. J. Cell. Biochem. Suppl. 27:20–25.

Key words: CAAX motif; farnesyltransferase inhibitor; K-*ras;* lung cancer; monoterpene

Lung cancer continues to be one of the most prevalent cancers in the western world, and is predicted to increase in developing countries with the rise in tobacco smoking [1]. Tobacco smoke has been shown to contain many potent carcinogens. One such carcinogen is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which shows a preference for inducing lung tumors in several species [2], and is a suspected etiologic agent in tobacco-related human cancer [3]. NNK is metabolically activated by cytochrome P450 enzymes to metabolites which can alkylate DNA [4,5]. Alkylation of DNA results in a G→A transition at the second base of codon 12 of the K-*ras* gene in mouse lung tissues [6,7]. Previous studies have demonstrated that oncogenic K-*ras* is frequently associated with human lung adenocarcinomas [8–10].

The *ras* p21 is a small G-protein (21 kDa) involved in signal transduction at the plasma membrane, coupling signals from cell surface tyrosine kinase receptors to cytosolic second messengers (MEK, MAPK), ultimately resulting in an increase in cell growth [8,11]. In order for *ras* to be biologically active, the nascent protein undergoes a series of post-translational modifications. The first modification begins with

Abbreviations: CAAX, protein sequence in which C = cysteine, A = (aliphatic), and X = (any amino acid); DCC, deleted in colon cancer; FTase, farnesyltransferase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase kinase; MTD, maximum tolerated dose; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PBMC, peripheral blood mononuclear cells; POH, perillyl alcohol. C_3AF_1, (C_3H/HeJXA/J)F_1.

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recognition of a consensus carboxyl-terminal CAAX motif by farnesyltransferase (FTase), where the terminal amino acid is generally methionine (K-*ras*4A/4B, N-*ras*) or serine (H-*ras*) [12,19]. In all forms of *ras*, FTase adds a C15 farnesyl group to the cysteine of the CAAX motif, which is followed by proteolytic removal of the terminal AAX residues, and finally carboxymethylation [13–16]. In H-*ras*, N-*ras*, and K-*ras*4A, these modifications are followed by palmitoylation on cysteine residues immediately upstream of the terminal cysteine, increasing the strength of membrane binding. The alternatively spliced K-*ras*4B lacks this cysteine sequence, and instead contains a string of six lysine residues thought to increase the avidity of binding to the membrane [14].

Chemoprevention strategies are designed to inhibit critical pathways of carcinogenesis, as assessed by biomarkers such as *ras*, p53, and APC. One such strategy is to target the post-translational modification of oncogenic *ras* [17]. By inhibiting isoprenylation of *ras* with FTase inhibitors, all subsequent modifications will also be inhibited, leaving the mutant *ras* protein in an inactive cytosolic form [14,15]. In addition, work with CAAX mutants has shown that oncogenic *ras* proteins trapped in the cytosol act as dominant inhibitory proteins, possibly by competing for limited regulatory and/or effector molecules [18,19].

Perillyl alcohol (POH) is a naturally occurring monoterpene found in lavender, cherries, and mint. In vitro, POH has been shown to be a potent inhibitor of small G protein (21–26 kDa) isoprenylation [20,21]. In the rat, dietary POH was shown to be chemotherapeutic, causing regression of mammary tumors [22,23] and pancreatic tumors [24]. Other investigators demonstrated that POH exerted its chemopreventive effect via apoptosis for colon [25] and hepatic carcinogenesis [26]. POH was also shown to effectively inhibit isoprenylation of proteins required for cell cycle progression in human lymphocytes [27].

The present study was designed to test the chemopreventive potential of systemic administration of POH, given throughout the test period, including initiation and promotion phases of lung tumorigenesis, in a well–characterized mouse model [28,29]. The (C3H x A/J) F_1 (C3A F_1) hybrid was chosen because induction by NNK leads to pulmonary tumors with an oncogenic K-*ras* species, which is also found in most human cancers [8].

MATERIALS AND METHODS

Chemicals

NNK was purchased from Chemsyn Science Laboratories (Lenexa, KS). POH (96%), equal to 1.04 µl/mg, was purchased from Aldrich Chemical (Milwaukee, WI). The treatment vehicle was Tricaprylin, purchased from Sigma (St. Louis, MO).

Animals

All mice were purchased from Jackson Laboratories (Bar Harbor, ME). The maximum tolerated dose (MTD) study was carried out on 4–5-week-old male A/J mice. Male C3A F1 hybrids were used in the lung tumor bioassay starting at 5 weeks of age. Animals were housed 4 per cage, in plastic cages with hardwood bedding and dust covers, in a HEPA-filtered, environmentally controlled room (24 ± 1°C, 12/12 h light/dark cycle). Animals received Rodent Lab Chow, #5001 (Purina, St. Louis, MO) and water ad libitum. Following a 7-day quarantine, the animals were randomized into treatment groups, with weights monitored weekly for the duration of the study.

Maximum Tolerated Dose

To determine the MTD for the bioassay, 4–5-week-old male A/J mice were randomized into 4 treatment groups (4 mice per group). Initial testing included the following doses of POH: 250, 500, 1,000, 2,000 mg/kg body weight, to be administered in 0.1 ml total volume, i.p., three times per week. As a consequence of its limited solubility in aqueous medium, POH was suspended in Tricaprylin at the appropriate dose immediately prior to injection. Due to inherent toxicity, age-matched groups for 50 and 100 mg/kg body weight were added to the MTD study. The surviving animals were kept on the treatment schedule for a total of 6 weeks, followed by a 4-week observation period. The animals were terminated by CO_2 asphyxiation and inspected for signs of toxicity, including edema and enlargement of internal organs (liver, spleen, heart).

Lung Tumor Bioassay

Five-week-old male C3A F_1 hybrid mice were randomized into groups as follows: Group 1,

POH/NNK (n = 16); Group 2, POH only (n = 16); Group 3, NNK only (n = 10); Group 4, vehicle only (n = 10). As seen schematically in Figure 1, POH treatment began 1 week prior to administration of carcinogen (week -1), and continued for 22 weeks after delivery of the first dose of carcinogen. POH was delivered i.p. at the MTD dose of 75 mg/kg body weight, 0.1 ml total volume, three times per week. A stock solution of NNK was prepared by solubilizing 30 mg/ml in warmed PBS, and kept protected from light at 4°C between use. The NNK was administered i.p. at 100 mg/kg body weight in 0.1 ml sterile PBS. A total of two injections were given 1 week apart, 24 hours after the fourth and seventh doses of POH, respectively. All animals were monitored for signs of toxicity, including weight loss, fur loss or roughened appearance, abdominal edema, and other abnormalities on a weekly basis. Animals were euthanized by CO_2 asphyxiation at week 23 after the start of POH. The lungs were removed, and up to 3 tumors per lung were flash-frozen in liquid nitrogen for future analysis. The remaining tissues were fixed in 10% buffered formalin overnight, followed by 70% EtOH treatment. Before embedding in paraffin, each lung was examined with the aid of a dissecting microscope to obtain the surface tumor count. The embedded tissues were serially sectioned at 5 m for current and future study. Selected sections were stained by H&E, and examined by light microscopy. Unpaired Student's *t*-tests were run to test for significance of surface tumor counts.

RESULTS

Maximum Tolerated Dose

Although previous studies have established the MTD for dietary POH, our bioassay was designed to test the efficacy of POH by i.p. administration. The initially presumed LD_{50} of 2,000 mg/kg i.p. (manufacturer's MSDS), and the 1,000 mg/kg dose both were immediately lethal; the 500 mg/kg dose proved lethal immediately following the second i.p. dose. We then added two age-matched dose groups, at 50 and 100 mg. The non-lethal doses (250, 100, and 50 mg/kg) produced an immediate response, including disoriented movement, dysplasia of hind limbs, and finally a "drunken" appearance with rapid, shallow breathing followed by 4–6 hours of sleep. Animals that received the 250 mg/kg dose continued to exhibit signs of intoxication for up to 2 hours post-injection, and showed less overall weight gain when compared to the 50 mg/kg dose group (17 g vs. 22 g). Animals in the two lowest dose groups remained awake and alert by the fourth i.p. injection of POH. At the end of this MTD study, the 100 mg/kg dose group showed slight signs of clinical toxicity, including a roughened coat. Gross inspection of tissues at the time of sacrifice did not show any

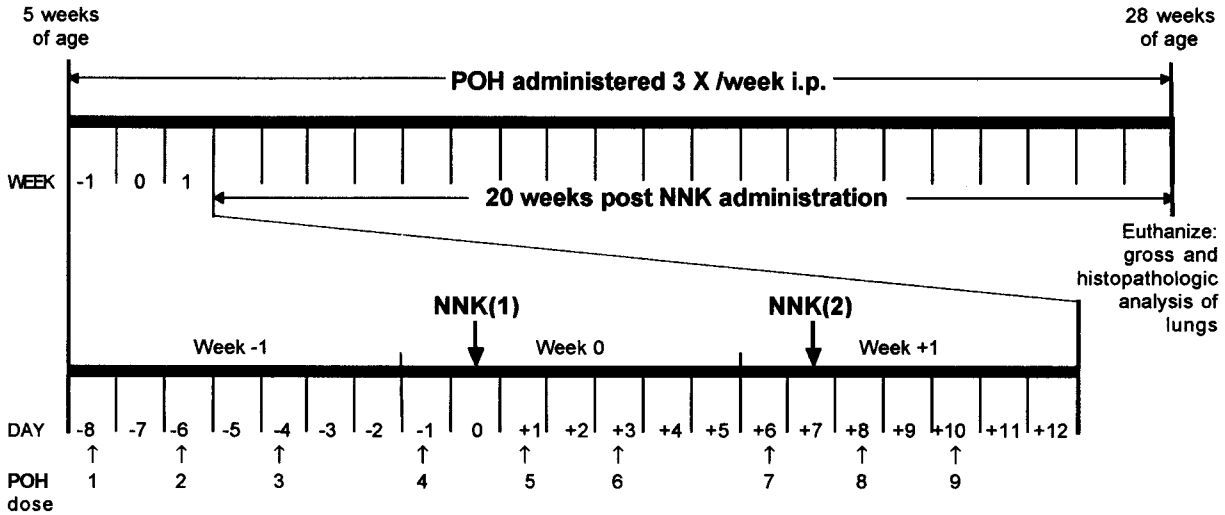

Fig. 1. Experimental design and protocol to test the chemopreventive efficacy of POH on NNK-induced lung tumorigenesis. Male C3A F_1 hybrid mice were started on a schedule of 75 mg/kg body weight perillyl alcohol administered in three i.p. doses per week, starting 1 week prior to NNK administration (study week -1), and continuing until study termination, 22 weeks post the start of NNK treatment. NNK was given in two i.p. doses, at 100 mg/kg body weight, for a total dose of 200 mg/kg body weight.

overt signs of toxicity in the 50 or 100 mg/kg dose groups. We, therefore, chose an intermediate dose between the 50 and 100 mg/kg, designating 75 mg/kg the MTD dose for the bioassay.

Bioassay

The average weight of mice in Groups 1 and 3 was greater than the average weight of mice in the vehicle control, Group 4 (8 and 4%, respectively) while the average weight of animals in Groups 2 and 4 differed by less than 1%. In addition, POH alone (Group 2) was well tolerated, with all animals surviving to the end of the study. Two animals from Group 3 were terminated 1 month prior to the end of the study to conduct preliminary tumor evaluation, and were not included in the final statistics. Two animals from Group 1 (POH/NNK), and one animal from Group 4 (vehicle only) were lost within the first half of the study. Necropsies were performed on these animals, and the cause of death was determined to be due to investigator's error at the time of injection. The incidence of tumors in Group 3 animals (NNK only) was 100%, with a multiplicity of 4.38 ± 1.60 tumors/mouse. POH treatment of Group 1, significantly reduced tumor incidence to 78.6% ($P < 0.005$), and tumor multiplicity to 1.86 ± 1.66 tumors/mouse ($P < 0.001$). The tumors have been confirmed as adenomas by light microscopic examination of H&E stained sections. The adenomas had non-pleomorphic nuclei, with no morphologic evidence of apoptosis within the tumors by routine H&E. These results indicate that POH is an effective chemopreventive compound in the mouse lung tumor bioassay, with a 22% inhibition of tumor incidence, and 58% reduction in tumor multiplicity in the NNK–treated animals receiving POH ($P < 0.001$). The results from this bioassay are summarized in Table I.

DISCUSSION

This study was designed to assess the efficacy of POH as a chemopreventive compound, and was part of a larger study of several potential FTase inhibitors. A unique feature of the experimental design included chemopreventive treatment prior to NNK initiation, as well as during initiation, promotion, and progression phases. The mouse hybrid that we employed has been shown to produce lung tumors with an oncogenic K-*ras* mutation (G→A transition at the second base of codon 12) when induced by NNK [6,7], which is contributed by the A/J allele [30]. Furthermore, oncogenic K-*ras* requires farnesylation, and thus is subject to inhibition by FTase inhibitors like POH [16,31,32]. Finally, this system was chosen for its relevance to human lung cancer, which frequently has activated K-*ras* mutations [8].

POH produced a significant decrease in overall incidence and tumor multiplicity in this mouse lung tumor bioassay. Our study was designed based on previous work demonstrating that POH is a potent inhibitor of small G-protein prenyltransferases [21,33]. However, there are other possible mechanisms that could

TABLE I. Effect of Perillyl Alcohol on Lung Tumorigenesis Induced by NNK in Male C3A F_1 Hybrid Mice[†]

Group no.	Treatment with chemopreventive	No. of surviving mice/no. of initial mice[a]	Total dose of NNK (mg/mouse)[b]	Body weight (g/mouse)[c]	Incidence of mice with tumors (%)[d]	Lung tumor multiplicity[e]
1	POH	14/16	3.9	37.6 ± 1.9	78.6*	1.86 ± 1.66**
2	POH	16/16	None	34.4 ± 2.2	6.3	0.06 ± 0.25
3	None	8/10	3.9	36.0 ± 3.0	100.0	4.38 ± 1.60
4	None	9/10	None	34.7 ± 3.0	0.0	0.00 ± 0.00

[†]Six-week-old C3A F_1 hybrid mice were given NNK administered in two i.p. doses. NNK dose 1 was given at week 0, 24 hours after the fourth dose of Perillyl alcohol (POH); NNK dose 2 was given at week 1, 24 hours after the seventh dose of POH. POH treatments were administered at 75 mg/kg i.p. three times per week starting 1 week prior to NNK (week −1) and continuing until week +21.

[a]Two mice in group 3 were terminated at week +18 to gauge tumor progression, and are not included in the final statistics.

[b]Total NNK dose (100 mg/kg) per mouse: week 1 = 1.9 mg, week 2 = 2.0 mg; total = 3.9 mg/mouse.

[c]Mean ± S.D. (at week +21). No statistical difference by Student's *t*-test.

[d]Percent incidence of mice with lung tumors larger than 0.5 mm. Statistically different between group 1 and group 3 (*$P < 0.005$, Student's *t*-test).

[e]Mean ± S.D. Lung tumors larger than 0.5 mm were counted. Statistically different between group 1 and group 3 (**$P < 0.001$, Student's *t*-test).

explain the decrease in NNK-induced tumors in Group 1 (POH/NNK). First, there could be direct inhibition of the carcinogen, with POH inhibiting the metabolic activation of NNK via inhibition of cytochrome P450 enzymes. In their recent paper, Morse and Toburen have shown inhibition of metabolic NNK activation at 1 and 4 hours post–treatment with various monoterpenes, including POH [34]. However, their data indicate that by 24 hours the inhibitory effects were undetectable [34]. Wattenberg and Coccia reported inhibition of NNK by d-limonene, which like POH is metabolized to perillic acid; however, their study design administered the NNK 1 hour after monoterpene treatment, and should now be interpreted as a direct inhibition of metabolic activation of NNK [35]. In the present study, NNK was administered 1 day after the fourth and seventh dose of POH. Based on this treatment modality, it is possible that POH inhibits the metabolic activation of NNK, thereby inhibiting lung tumorigenesis. Second, POH could be indirectly inducing free radical scavenging mechanisms like glutathione-*S*-transferase, as demonstrated in 7,12-dimethylbenz(A)anthracene-induced mammary carcinogenesis [36]. Third, as seen in liver tumors, POH could be promoting apoptosis [26]; however, our morphologic examination of H&E sections of adenomas does not support this mechanism in lung tumors. Fourth, Hohl and Lewis, unable to detect an increase in cytosolic nonfarnesylated *ras* p21, hypothesized that POH and other monoterpenes may actually decrease transcription/translation, or increase degradation of *ras* [37].

Finally, POH and other monoterpenes have been demonstrated to interact with the mevalonate pathway, which produces precursors of cholesterol, as well as a number of isoprenoids [27,38]. For example, POH could inhibit tumor cell proliferation by inhibiting cellular synthesis of ubiquinone (CoQ9), an energy source for oxidative phosphorylation-deficient tumor cells relying on glycolysis for ATP production [38]. In a recent study to test the effect of POH, mevalonate was added exogenously to POH–treated peripheral blood mononuclear cells (PBMC), but failed to rescue the cells from a G1 block, whereas addition of mevalonate to PBMC treated with compactin, a genuine inhibitor of hydroxymethylglutaryl coenzyme A reductase, did overcome the POH-induced inhibition. This would indicate that POH may inhibit isoprenylation of other proteins required for cell cycle progression but distal to the synthesis of mevalonate [27].

The objective of ongoing studies is to elucidate the mechanism or mechanisms responsible for the chemopreventive effect of POH in the mouse lung tumor model. To determine whether POH is acting as an inhibitor of FTase in this system, we will be analyzing the location of *ras* p21 protein by immunohistochemistry, as well as the extent of post-translational processing of *ras* p21 in normal tissues and tumors by 2D protein gel electrophoresis. Accumulation of cytosolic *ras* p21 would indicate an inhibition of *ras* isoprenylation, and one could argue that the inhibition, in conjunction with a dominant-negative response to this increase in cytosolic *ras*, could account for the preventive effect of POH [18,19]. Our present study demonstrates that POH significantly inhibits NNK-induced oncogenesis in the C3A F_1 mouse lung model. Based on these findings, and others, POH should be strongly considered for future chemopreventive trials.

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