Research Article

Cancer Prevention Research

Chemoprevention of Lung Carcinogenesis by Dietary Nicotinamide and Inhaled Budesonide

Arthur R. Galbraith[1], Donna E. Seabloom[2,3], Beverly R. Wuertz[2,3], Jennifer D. Antonides[1], Vernon E. Steele[4], Lee W. Wattenberg[1], and Frank G. Ondrey[1,2,3]

Abstract

Nicotinamide, the amide form of vitamin B3, and budesonide, a synthetic glucocorticoid used in the treatment of asthma, were evaluated to determine their individual and combinational chemopreventive efficacy on benzo(*a*)pyrene-induced lung tumors in female A/J mice. Nicotinamide fed at a dietary concentration of 0.75% significantly inhibited tumor multiplicity. Nicotinamide by aerosol inhalation at doses up to 15 mg/kg/day did not result in a statistically significant reduction in tumor multiplicity. Finally, dietary nicotinamide was administered with aerosol budesonide and tumor multiplicity reduced by 90% at 1 week and 49% at 8 weeks post last carcinogen dose. We conclude nicotinamide is an effective and safe agent for lung cancer dietary prevention at both early- and late-stage carcinogenesis and that efficacy is increased with aerosol budesonide. Combination chemoprevention with these agents is a well-tolerated and effective strategy which could be clinically advanced to human studies.

Introduction

The leading cause of human cancer mortality in the United States is lung cancer, with an estimated 155,870 deaths in 2017 (1). In spite of continued improvements in surgical, radiation, and chemotherapeutic strategies, more than 70% of all patients with lung cancer still die from this disease (1). One of the major problems in reducing lung cancer deaths is that carcinogen-induced genotoxic damage and initiation have already taken place long before symptoms of cancer appear. With the recognition that defined steps in the carcinogenesis process exist (2–6), it was realized a number of chemopreventive strategies could be employed, especially suppressive or post carcinogen interventions (7–10).

Nicotinamide exerts a wide range of effects in DNA damage, mutagenesis, and DNA repair (11), and is capable of exerting a strong chemopreventive effect on tumor development and multiplicity in animals and humans (12, 13). Nicotinamide-containing preparations for skin cancer prevention have gained renewed recent interest. Originally, significant preclinical work was performed on the ability of nicotinamide to aid UV-induced photoprotection (14–19). In addition, a large recently reported clinical trial, the phase III ONTRAC trial, preselected high-risk patients with two nonmelanoma skin cancers in the past 5 years and at 1 year there was a 23% rate reduction in new skin cancers in nicotinamide-treated patients versus placebo patients ($P = 0.02$; ref. 20). In preclinical skin carcinogenesis models, an intriguing effect of immune stimulation with nicotinamide is counteraction of photo-immune suppression (14, 16, 21, 22). Additional preclinical data demonstrate nicotinamide efficacy in malignancies including: bladder, posttransplant hepatocarcinogenesis, and urethane-induced lung tumors (13, 23–26). Ostensibly, nicotinamide is a pleotropic vitamin precursor, which acts at many metabolic levels, so there is not a simple receptor or precise mechanism of action. However, as a vitamin B precursor, nicotinamide supplementation has the capacity to interact and alter cellular NADH/NAD+ ratios, a potential cellular biosensor, which may downregulate cancer-associated transcription mechanisms involving SIRT1 (27, 28). Strategies utilizing nicotinamide SIRT1 suppression activities could have an inhibitory effect on NF-κB actions, including those influenced by cigarette smoke products (29, 30). These interactions may favorably reduce NF-κB activity and chronic inflammation (31).

Research demonstrated glucocorticoids can also exert a strong delaying or inhibitory effect on induced tumors in

[1]Carcinogenesis and Chemoprevention Research Program, Masonic Cancer Center, University of Minnesota, Minneapolis, Minnesota. [2]AeroCore Testing Services, Department of Otolaryngology, University of Minnesota, Minneapolis, Minnesota. [3]Department of Otolaryngology, Medical School, University of Minnesota, Minneapolis, Minnesota. [4]Division of Cancer Prevention, National Cancer Institute, Rockville, Maryland.

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Corresponding Author: Frank G. Ondrey, University of Minnesota, 420 Delaware St, SE, Box 396, Minneapolis, MN 55455. Phone: 612-625-3200; Fax: 612-626-9871; E-mail ondre002@umn.edu

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animals (8, 32–39), and more recent studies have shown some of these, especially budesonide, were capable of reducing pulmonary tumor multiplicity in A strain mice when administered by diet (40) and by aerosol inhalation (41–43). When administration of budesonide by aerosol inhalation was combined with dietary feeding of myo-inositol, a synergistic additive chemopreventive effect on tumor multiplicity was observed (42).

We report here the results of several chemoprevention studies in the benzo(*a*)pyrene (B[a]P)-induced pulmonary carcinogenesis model in female A/J mice. Dietary nicotinamide shows promising inhibitory effects in both the early- and late-stage lung carcinogenesis and when combined with aerosolized budesonide, chemoprevention was enhanced. An important feature of this study is the utilization of regional budesonide delivery via aerosol. We have recently shown aerosol pioglitazone was a safe and effective agent for A/J mouse lung chemoprevention (44). Previously our group has published on budesonide, five fluorouracil, and difluoromethylornithine (DFMO) as effective agents for lung cancer prevention (41, 45). Others have shown phosphor-sulindac, Polyphenon E, and retinoids are also efficacious in lung cancer prevention (46–48). Translationally, a human phase IIb study was conducted with inhaled budesonide at 1,600 mcg/day for 6 months and the trial result did not reverse histology in endobronchial lesions, often squamous carcinoma precursor lesions accessible by standard bronchoscopy (49). However, in COPD populations there is indeed a protective effect of inhaled steroids in the prevention of lung carcinoma (50, 51). With the large patient populations now undergoing screening and follow up for peripheral nodules, and the advent of navigational bronchoscopy, additional studies could be carried out for the examination of smaller airway lesions that could transition to adenocarcinomas (52). With an updated paradigm provided by recent screening advances, anti-inflammatory drugs (such as budesonide and sulindac derivatives) could be used in clinical trials via aerosol and effects in the lung periphery more effectively interrogated.

Materials and Methods

Materials

The chemicals used were budesonide (>99% purity), nicotinamide (≥98% purity; Sigma Aldrich Chemical Co.) and B[a]P (>95% purity; TCI America).

Animals and diets

Seven-week-old female A/J mice (The Jackson Laboratories) were fed pellet diet NIH-07 7022 (Harlan Teklad Diets) and acclimated to the facility for 2 weeks. Mice were weighed 1 day after arrival and then weekly. Mice were then switched to D00062001 semipurified diet (Research Diets Inc.) consisting of 27% vitamin-free casein, 59% corn starch, 10% corn oil, 4% salt mix (USP XIV), and a complete mixture of vitamins. We use the D62 diet for chemoprevention studies as other diet preparations, providing a more complete complement of nutrients and vitamins, have been found to be chemopreventive in their own right (e.g., soy inositols; refs. 53, 54). Animal diet was replenished thrice weekly. All animals were housed in a constant temperature facility with controlled lighting (12 hours light/12 hours dark). Animals were observed in their cages daily, including weekends and holidays, for lethargy, body hunching, attenuation, and rough hair/coat. Animals appearing distressed or moribund, or lost 2 g or more of body weight in a week, were sacrificed early and necropsied.

Pulmonary tumor formation

B[a]P-induced pulmonary tumor formation in female A/J mice was used as the carcinogenesis model for all the chemoprevention studies. For tumor induction, at 11 weeks of age, all mice were given three administrations of 3 mg B[a]P/kg of body weight in 0.2 mL cottonseed oil by oral gavage on days 1, 4, and 8. Mice were randomized into treatment groups by weight the day prior to the first test agent administration and reweighed once per week.

Test agent administration

Administration of the test compounds was started 1 or 8 weeks following the last dose of B[a]P, depending on experimental group, and was continued for the duration of the study. For aerosol administration, mice were exposed individually to aerosolized budesonide or the solvent (ethanol) by placing the animal's nose into the cone of the apparatus. Aerosol treatments were administered 5 days/week, Monday to Friday. Aerosol generation apparatus (MiniHeart Nebulizer; Westmed, Inc.), and associated evaporator and solvent removal systems were used as described previously (41, 42), as were aerosol doses delivered (43). Budesonide aerosols were generated from an ethanol solution by jet nebulization with solution concentration and exposure times set to give an inhaled dose of 25 μg/kg/day. Aerosol generation apparatus completely removes the solvent, allowing exposure to treatment agent as dry particles. Control animals were exposed to air only following removal of nebulized ethanol (budesonide solvent) by the aerosol apparatus. Administration of nicotinamide aerosols was performed in the same manner except the solvent used was water.

Experimental diets were started 1 or 8 weeks after last dose of B[a]P. Nicotinamide was thoroughly mixed into D62 meal at 0.25% or 0.75% by weight. The animals were fed 4 g of diet per mouse per day as past studies have indicated approximately 2 g/day is actually consumed. Diet was replenished thrice weekly. Mice were weighed weekly, care was taken to handle the animals gently throughout to minimize stress. Mice were closely observed in their cages daily, including weekends and holidays, for lethargy, body hunching, attenuation, and rough hair coat.

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Mice appearing distressed or moribund during the experiment based on the signs just listed, or that lost 2 g or more of body weight in a week, were sacrificed early and necropsied. Experiments were terminated 16 weeks after the last carcinogen dose. All groups were sacrificed and underwent necropsy. The lungs were collected for analysis. The final body weights, number of animals with lung tumors, and the number of lung tumors per tumor-bearing animal were recorded.

Statistical analysis

Data were analyzed in a group wise fashion for differences in tumor counts by ANOVA analysis. Dunnett's posttesting was used to determine which groups were statistically different from the control groups or if the combination treatments were statistically different from the single agent treatments. In addition, a two-tailed Student *T* test was performed for each treatment group versus the control group (absolute control and/or the pertinent solvent control group). Data in the charts were presented as a mean ± SEM for each group. Graphpad PRISM software version 5 was used for the statistical analysis. *P* values on two sided *t* tests were considered significant at $P < 0.05$.

Compliance

All experimental procedures are carried out according to University of Minnesota Department of Environmental Health and Safety requirements which abide by regulatory requirements set at the local, state, and federal level. All studies were conducted with the approval of the Institutional Animal Care and Use Committee at The University of Minnesota, under NIH Animal Welfare Assurance number A3456.

Results

A number of studies with various forms of delivery of nicotinamide and/or budesonide were conducted. The timeline for the studies is demonstrated in the infographic in Fig. 1. We first conducted a series of dietary nicotinamide alone studies. For the nicotinamide dietary studies, two dose levels of nicotinamide were administered [0.25% and 0.75% (w/w) of agent in diet], beginning at 1 week (early-stage treatment) or 8 weeks (late-stage treatment) after the last B[a]P administration. In this first series of experiments, we observed 0.75% dietary nicotinamide reduced lung surface adenoma counts by 41% to 48% from control values in the early-stage treatment experiment after 16 total treatment weeks. For 0.25% dietary nicotinamide, adenoma inhibition ranged from 13% to 46% in the two experiments we performed. A representative set of experiments are depicted in Fig. 2. In addition, we analyzed the dose responses in these groups and found significant dose-dependent decreases for nicotinamide, as judged by ANOVA analysis (Fig. 2A). The animals experienced a slight decrease in rate of weight gain throughout the experiment,

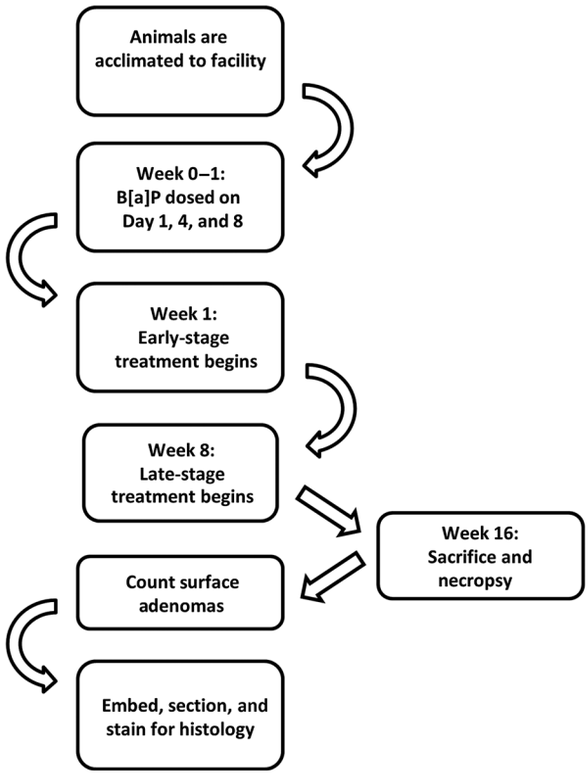

Figure 1.
Infographic depiction of experimental timeline.

which was only significant at 0.75% nicotinamide (Fig. 2B).

We continue to be interested in longer postinitiation chemoprevention, so we initiated agent treatment 8 weeks after carcinogen administration. These experiments allow tumor initiation to progress for 8 weeks before treatment begins. Treatment continues from this point through the end of the 16-week experiment. We performed two experiments and found 55% reduction in lung surface adenoma formation in the initial experiment using 0.75% dietary nicotinamide ($P < 0.05$; Fig. 2C). In the second experiment, lung surface adenomas were reduced by 21%, which was not a statistically significant reduction ($P = 0.07$). These additional data from the second set of early- and late-stage treatment studies are presented in Supplementary Fig. S1 (SF1). For the late-stage experiments, at 0.25% dietary nicotinamide, adenoma counts were not significantly reduced (Fig. 2C). The late-stage animals also experienced a slight decrease in rate of weight gain, which was only significant in the high-dose group (Fig. 2D). These data demonstrate 0.75% nicotinamide was an effective agent at early-stage carcinogenesis. In addition, 0.75% nicotinamide reduced pulmonary adenomas at late-stage carcinogenesis; however, the likely biologically relevant effects only met statistical significance in one of two experiments

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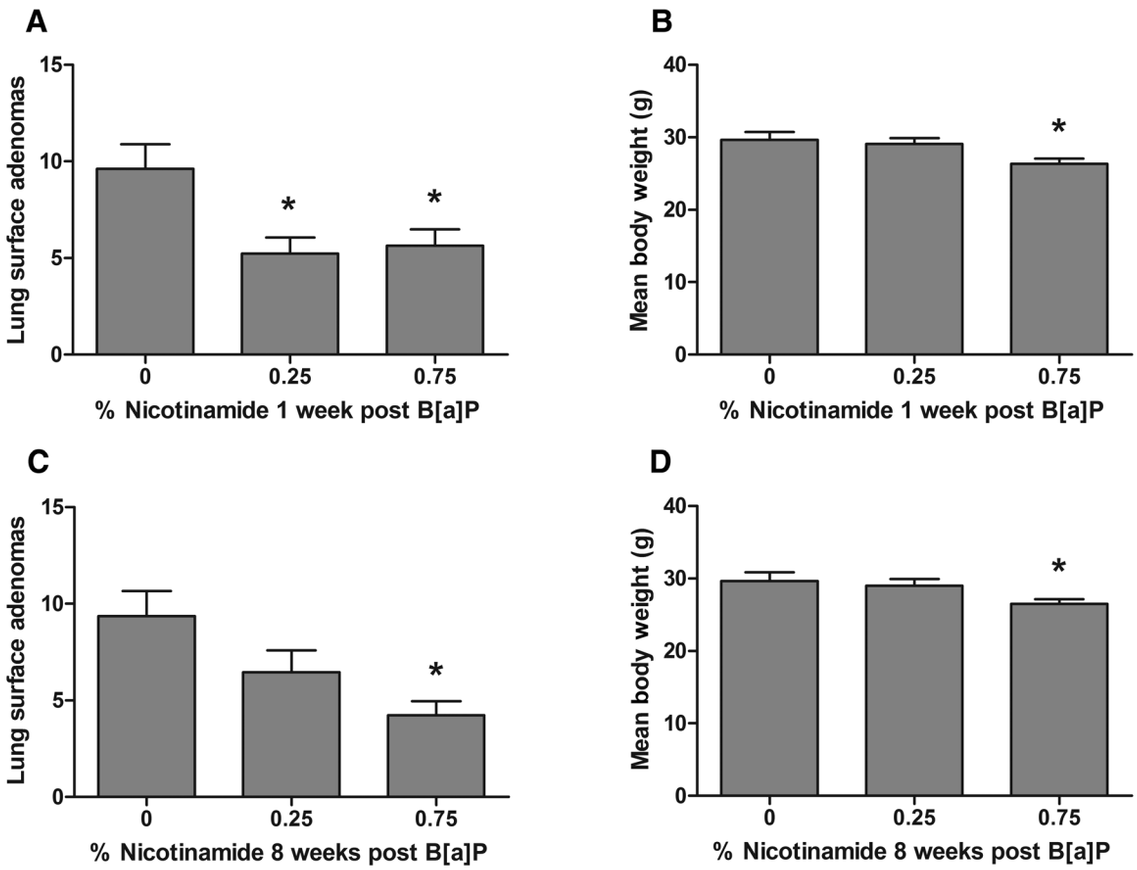

Figure 2.

Effect of dietary nicotinamide in an early- and late-stage intervention model. Treatment with nicotinamide at 1 week post-carcinogen resulted in statistically significant decreases in lung adenoma formation at both 0.25% and 0.75% versus control (**A**). Endpoint mean body weights show decrease weight gain in early-stage intervention at high dose (**B**). Nicotinamide treatment at 8 weeks post carcinogen resulted in a significant decrease in adenoma formation only at the 0.75% dose (**C**). Endpoint mean body weight decrease in weight gain seen at high dose (**D**). *, $P < 0.05$. Graphs are from representative experiment using 14 animals per group. Dose-dependent decrease in treatments by ANOVA analyses show significance in the early-stage intervention, $F(2,37) = 5.730$, $P = 0.0068$ and at the late-stage intervention, $F(2,37) = 4.116$, $P = 0.0243$. Dunnett's posttest demonstrated significance between groups at $P < 0.05$.

performed. For 0.25% dietary nicotinamide, there was a biologically relevant decrease in pulmonary adenomas at the early stage; less significant reductions were observed for late-stage carcinogenesis, and statistically there were trends for decreased adenoma formation although only one of the studies met statistical significance.

Based on prior successful experience administering other agents via aerosol chemoprevention, we conducted two experiments with aerosol nicotinamide. An aerosol solution of nicotinamide was administered in 60-second exposure times for a dose of either 1, 2, 5, or 15 mg/kg starting either 1 or 8 weeks after last B[a]P administration. The agent was well tolerated by the mice at all concentrations. The animals appeared healthy and with no respiratory ill effects. At all concentrations of nicotinamide for both experiments, there were no significant changes in surface lung adenoma counts in any of the groups at either one or eight weeks post initiation (Fig. 3A). The animal weights were stable throughout the study (Fig. 3B).

In the past, we have had success with inhaled steroids, including budesonide, as a clinically translatable strategy for lung cancer prevention (41–43, 55). Consequently, we postulated that combining an anti-inflammatory steroid along with a dietary supplement affecting NADH metabolism would allow for two noncompeting strategies to potentially augment the chemoprevention effect of nicotinamide alone. We administered 0.75% (w/w) dietary nicotinamide and aerosol budesonide (25 μg/kg body weight) individually and in combination, at 1 and 8 weeks post-carcinogen in the A/J mouse model. Compared with control groups, nicotinamide alone gave a 54% adenoma reduction at early-stage administration ($P < 0.0001$) and a 39% reduction at late-stage administration ($P = 0.0231$). Budesonide alone inhibited adenoma formation by 77% at early stage ($P < 0.0001$) and 41% at late stage ($P = 0.0149$) compared with controls. When nicotinamide was combined with aerosolized budesonide, the two agents resulted in 90% ($P < 0.0001$) adenoma inhibition at

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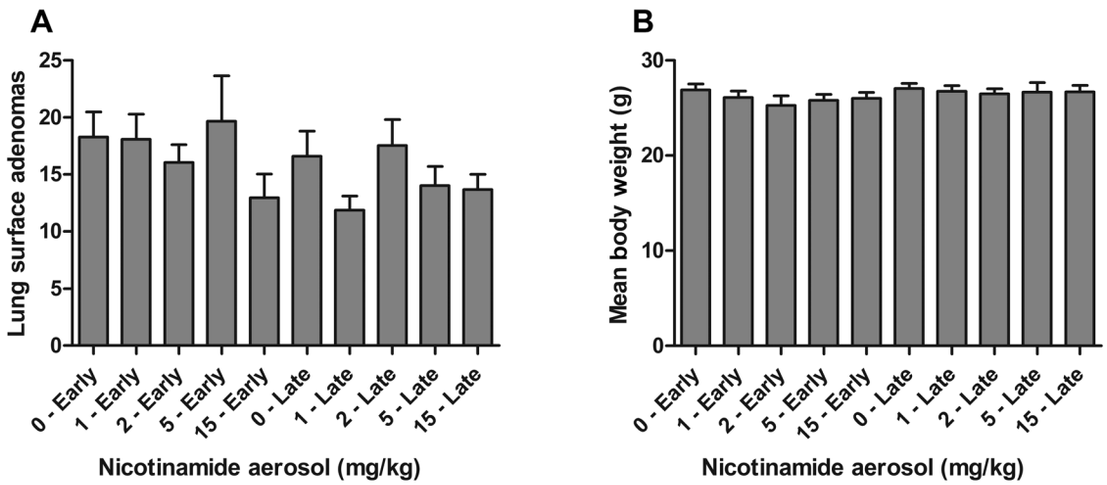

Figure 3.

Effect of aerosol nicotinamide in an early- and late-stage intervention model. Doses of aerosol nicotinamide ranged from 1 to 15 mg/kg. There were no statistically significant decreases in tumor formation however at the early-stage intervention there was a 29% decrease at the highest dose (**A**). Eighteen animals per group were used for the early-stage intervention and 15 animals per group for the late-stage intervention. No toxicity was noted and endpoint weights of the animals were not changed (**B**).

1-week post-carcinogen and 49% ($P = 0.0039$) at 8 weeks post-carcinogen, versus the corresponding solvent control groups (Fig. 4A). When compared with single-agent treatment, the combination treatment reduced adenoma multiplicity. Nicotinamide/budesonide significantly reduced adenoma multiplicity at the early stage compared with nicotinamide alone ($P < 0.0001$) or budesonide alone ($P = 0.0015$). At the late-stage treatment, nicotinamide/budesonide combination treatment significantly reduced adenoma multiplicity compared with nicotinamide alone ($P = 0.0170$); however, the decrease from budesonide alone was not statistically significant at the late-stage treatment ($P = 0.4668$). It is important to note, in Fig. 4B, the nicotinamide/budesonide combination

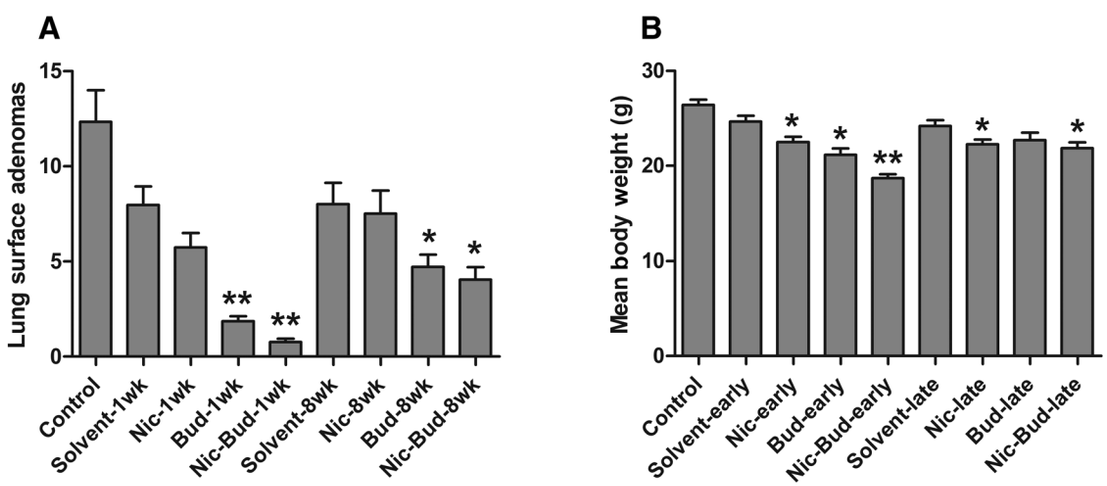

Figure 4.

Effect of dietary nicotinamide and aerosol budesonide in an early- and late-stage intervention model. We used 0.25% and 0.75% nicotinamide in the diet with 25 μg/kg by daily aerosol delivery. There were significant decreases in adenoma formation with either dietary nicotinamide or aerosol budesonide and this was enhanced by the simultaneous delivery of both agents (**A**). Statistically significant differences across treatment groups were seen by ANOVA analyses in the early stage intervention, $F(3,84) = 26.70$, $P < 0.0001$ and at the late-stage intervention, $F(3,82) = 4.324$, $P = 0.007$. Dunnett's posttest demonstrated significance between groups at $P < 0.05$. Combination therapy significantly decreased adenoma multiplicity versus nicotinamide ($P < 0.0001$) and budesonide ($P = 0.0015$) at the early stage (1 week) and versus only nicotinamide ($P = 0.0170$) at the late stage (8 weeks). There were 25 animals per group and no toxic effects noticed in the animals for the duration of the experiment; however, there was a decrease in the end body weights (**B**). *T* test versus solvent controls: *, $P < 0.05$; **, $P < 0.0001$.

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treatment early group experienced an approximate 10% weight loss from the starting weights for the group. The animals did not appear unhealthy in any fashion; however, the weight loss may be partially responsible for the approximate 90% inhibition of adenoma formation in this group.

Discussion

Nicotinamide, as a dietary element, has the potential to be associated with cancer prevention. The effects are pleotropic due to the nature of nicotinamide on various vitamin B and related pathways. We performed a series of dietary experiments with nicotinamide at 0.25% and 0.75% (w/w) in the diet of B[a]P exposed mice. We found a dose-dependent decrease in pulmonary adenoma formation with both concentrations, and even discovered nicotinamide was effective at late-stage carcinogenesis prevention (treatment beginning 8 weeks post carcinogen dose). The results with 0.25% (w/w) nicotinamide approached statistical significance and biological relevance with 20% to 30% reductions in tumor formation in general. We had originally performed dose finding experiments with 0.125%, 0.25%, and 0.375% nicotinamide (Supplementary Fig. S2), which although a statistically significant change in adenoma formation was not seen, these doses were nontoxic. This gave us the 0.25% dose as a safe starting point and we added the 0.75% dose above the levels originally tested. As we found no ill effects on the mice, in future studies additional higher doses of nicotinamide will be attempted, and because nicotinamide is a congener of vitamin B3, which can undergo modification *in vivo*, it will likely be well tolerated at higher doses. Because this was the initial set of studies of this agent in the A/J mouse model, we did want to investigate the effects at early- and late-stage carcinogenesis, and as an aerosol. We found nicotinamide delivered as an aerosol was well tolerated; however, at doses up to 15 mg/kg, we found no statistically significant effects in adenoma reduction at either early- or late-stage treatment. However, we did observe tumor reductions of up to 29% ($P = 0.09$) at 15 mg/kg dosing. This would suggest future studies with this agent at higher concentrations or by alternative delivery techniques (e.g., engineered nanoparticles) might be promising. The water soluble nature of nicotinamide decreases residency time in the lung.

In the nicotinamide-budesonide combination study, all treatment groups showed statistically significant tumor inhibition when compared with the corresponding controls. Importantly, both nicotinamide and budesonide were shown to be effective inhibitors at a late stage in the carcinogenesis process (all groups with treatment beginning 8 weeks post-carcinogen, with 8-week treatment duration). As would be expected, the treatment groups showed increased inhibition when administered at an early-stage treatment beginning 1-week post-carcinogen (16-week treatment duration). At each of the two time points, the decrease in adenoma multiplicity was strongest in the combination nicotinamide-budesonide group, followed by the budesonide alone group, nicotinamide alone group, then by the solvent control group, respectively. Therefore, the highest tumor inhibition was found with the nicotinamide/budesonide combination group at 1 week post-carcinogen (90% vs. SC). This trend was also predicted based on results of our previous studies with nicotinamide in this project as well as published results with budesonide (40–42). One other feature of the project was a lack of weight gain in the animals administered aerosol budesonide versus corresponding controls. The animals appeared healthy and were simply a bit smaller than their cohorts. The decrease in rate of weight gain in the treatment groups may play some role in the extent of tumor inhibition observed. However, weight differences alone cannot account for high degree of adenoma inhibition observed (up to 90%). Several pairs of treatment groups do exhibit weight differences, however; have highly significant differences in tumor counts. At times solvent effects in aerosol studies are attributable to an increase the stress of the animals caused by handling during administration of the aerosol, preventing weight increases in those cohorts. We recently published an aerosol toxicology study where the aerosol itself was not toxic and neither the treatment or the handling (solvent control groups) caused decrease in rate of body weight gain (44).

The nicotinamide–budesonide combination study involved administration of previously successful chemopreventive agents tested for the first time together and for their late-term chemopreventive effects in the murine lung. Dietary nicotinamide and aerosolized budesonide both showed statistically significant chemopreventive efficacy at early- and late-stages, and when administered in combination yielded very significant tumor inhibition, indicating the two agents work by different mechanisms which appear to be additive. However, this result must be interpreted carefully due to a weight loss of approximately 10% in the nicotinamide–budesonide combination treatment early group from the starting weights, which could have contributed to the 90% decrease in adenoma counts. This result can inform future studies regarding doses and schedules of aerosol budesonide and dietary nicotinamide.

In our studies, administration of the putative agent occurs subsequent to all administrations of carcinogen. Inhibition of carcinogenesis under these conditions can be very significant and useful; animal studies have lead directly into lung cancer prevention clinical trials, as in the cases of dietary myo-inositol and aerosolized budesonide, for example (49, 56). In general, efficacy achieved post-carcinogen administration is not a common property of chemopreventive agents. This is especially true in so-called late-term chemoprevention, which attempts to achieve inhibition with agents which begin administration

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relatively shortly before experiment termination. Improved efficacy at an early intervention stage is seen in other chemoprevention models as well. F344 rats in a NMBA esophageal carcinogenesis model show vitamin E, selenium, and α-tocopherol are more effective at reducing tumor volume at early-stage intervention versus later-stage intervention (57–59). Reddy and colleagues found celecoxib decreases tumor volume of colon adenomas and early adenocarcinomas at both early and late interventions, however, is more effective at the early stage (60). Effect of rapamycin treatment was more pronounced at the early-stage intervention in an additional A/J mouse lung carcinogenesis model utilizing NNK (61). Recently, late-term chemoprevention has been a primary focus of our laboratory; identifying compounds which are effective at a late stage in the carcinogenesis process could be particularly important and valuable as the time period required for administering the agents is decreased. In addition, treatment efficacy for high-risk patients and patients with high-grade dysplasia or early cancers may experience benefits from these treatments. It is notable now that budesonide and nicotinamide, especially in combination, have been shown to have late-term efficacy. Advances in computing and technology would now allow analysis of additional and molecular markers beyond tumor volume and histology. Field carcinogenesis could be studied using high throughput multi-omic approaches much more efficiently and economically.

Because of their valuable potential for the treatment and prevention of human cancers, agents with post-carcinogen-administration chemoprevention properties, especially those effective in the late stage of carcinogenesis such as nicotinamide and budesonide, warrant further study.

For example, the randomized phase IIb clinical lung cancer prevention study, based on our work with budesonide, did not find a significant effect on regression or progression rates of bronchial dysplasia between the treatment or placebo group because the study was limited by the amount of budesonide able to be delivered within the FDA-approved dose range (49). However, there was a statistically significant decrease in p53 and BclII expression in bronchial biopsies of treated patients compared with placebo. And a small but statistically significant decrease in the proportion of computed tomography–detected lung nodules after budesonide compared with placebo ($P = 0.024$). A principal constraint on such prevention studies is that maximum dosing should not exceed FDA-approved levels. However, it would potentially be possible to continue inhaled steroid pulmonary prevention studies as combination studies with safe compounds, increasing their efficacy. Nicotinamide, as a dietary supplement, could be attempted in such a study due to what should be a high safety profile. With renewed interest in this agent as a skin cancer prevention drug, there may be additional interest in moving the compound forward in clinical prevention strategies modeled after what was accomplished in this preclinical study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B.R. Wuertz, J.D. Antonides, V.E. Steele, L.W. Wattenberg

Development of methodology: A.R. Galbraith, J.D. Antonides, V.E. Steele, L.W. Wattenberg

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.R. Galbraith, B.R. Wuertz, J.D. Antonides

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.R. Galbraith, B.R. Wuertz, J.D. Antonides, L.W. Wattenberg, F.G. Ondrey

Writing, review, and/or revision of the manuscript: A.R. Galbraith, D.E. Seabloom, B.R. Wuertz, V.E. Steele, L.W. Wattenberg, F.G. Ondrey

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.R. Galbraith, D.E. Seabloom, B.R. Wuertz, J.D. Antonides, V.E. Steele, F.G. Ondrey

Study supervision: V.E. Steele, L.W. Wattenberg

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