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Original Article

Effects of talactoferrin alpha on lung adenoma prevention in A/J mice June 2, 2016

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Abstract: Talactoferrin alpha is a promising non-toxic solid tumor cancer agent that met with success in the treatment of early-stage lung cancer clinically in humans. It is well-tolerated, anddendritic cell-stimulation is a target. We tested the efficacy of this agent in a chemoprevention setting in A/J mice. All groups received benzo[a]pyrene (B[a]P) by oral gavage in three doses of 3 mg/kg body weight over the course of one week. Animals were then randomized into 5 groups of 24 mice per group based on weight. Experimental diets oftalactoferrin alpha (Agennix Inc., Indianapolis, IN), at 1.40% and 0.42% of the diet, were started one week or eight weeks after the last dose of B[a]P. Animals were continued on the feeding schedule, weighed weekly, and monitored for toxicity. The study was concluded 16 weeks after administration of B[a]P. The agent was well-tolerated for the duration of the experiment and there was no observable toxicity or weight change. The average number of adenomas per animal was 14.04 ± 0.93 (N=24) in the control group, 18.14 ± 1.45 (N=22) in the early low-dose group, 16.70 ± 1.30 (N=23) in the late low-dose group, 15.09 ± 1.41 (N=23) in the early high-dose group and 14.46 ± 1.21 (N=24) in the late high-dose group. We conclude talactoferrinalpha is well-tolerated. However, it did not inhibit carcinogenesis at a dose of 1.4% or 0.42% of the diet, which equates to human doses of 1.12 g/kg/day or 0.336 g/kg/day.

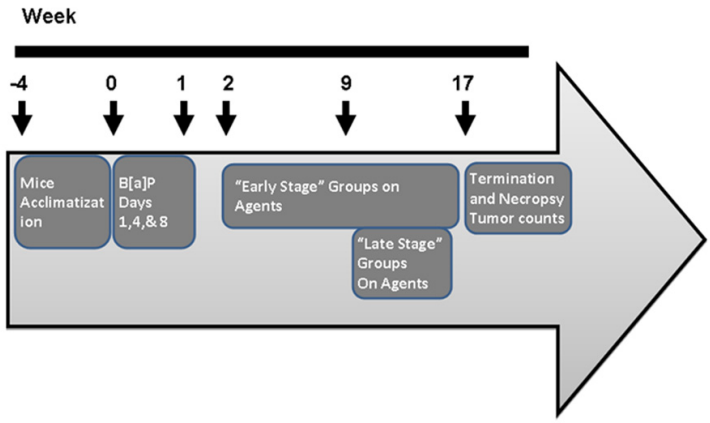
Keywords: Talactoferrin alpha, lung carcinogenesis, A/J mice, chemoprevention

Introduction

Lung cancer is a leading cause of cancer deaths worldwide. In the United States in 2014, there were an estimated 224,000 newly diagnosed cases with over 159,000 deaths from this malignancy [1]. The overall survival for the disease has not shown great strides in improvement in many years, and no effective cancer chemoprevention strategies presently exist. Therefore, there is a significant unmet need for improvements in disease outcome, which could be found in more effective therapies and prevention.

The concept of advancing the field of immune modulation for the treatment of solid tumors is not new. The first efficacious treatment with "Coley's toxins" was reported in 1893 [2-4]. However, though immune therapies and vaccines have not become first-line treatment for solid tumors, they have been utilized in relapsed cases or in an adjuvant setting [5-7]. One agent, talactoferrin alpha, is an orally available recombinant drug derived from natural lactoferrin [8, 9]. The use of oral talactoferrin has met significant success for the treatment of aerodigestive malignancies in animal models, and this has prompted its use in clinical trials. Earlier results in both relapsed solid tumors and renal cell carcinoma showed small response rates and disease-free survival, extending its use in further trials [10-12]. Both Phase I and Phase II human clinical trials of oral talactoferrin for relapsed lung cancers showed safety, efficacy, and improved survival rates in the intent to treat populations of 6.1 versus 3.8 months [13, 14]. A larger follow-up study for lung cancer treatment has been completed (FORTIS-M) and has been recently published. Unfortunately, in relapsed patients, talactoferrin alpha was not

A **Timeline for A/J Mouse Lung Chemoprevention using Talactoferrin alpha**



B Experimental group description.

Treatment Group Description	Animals (per group)	Diet Initiation (Weeks Post-B[a]P Administration)	Treatment Duration (Weeks)
Control	24	1	15
Talactoferrin 0.42%-1 wk	24	1	15
Talactoferrin 1.4%-1 wk	24	1	15
Talactoferrin 0.42%-8 wk	24	8	8
Talactoferrin 1.4%-8 wk	24	8	8

Figure 1. Treatment schema for talactoferrin alpha experiment. A. Mice are acclimated to the environment in the lab for three weeks before the initiation of Benzo[A]pyrene. During week 0, B[a]P was given on days 1, 4, and 8. One week post-initiation, the "early stage" groups were given talactoferrin alpha in the diet. Eight weeks after initiation, at the beginning of week nine, the "late-stage" groups were given talactoferrin alpha. At the end of either eight or fifteen weeks of treatment, the animals were sacrificed and tumor counts performed. B. The amount of agent and description of the experimental group is listed.

shown to have efficacy in the Phase III setting in patients who have failed prior therapy twice [15].

A principal mechanism of action of the agent, since it is not well-absorbed, is to act as a GI immune stimulatory agent that promotes dendritic cell maturation. Dendritic cells have been shown to then recirculate and target tumor cells through improving innate adaptive immune responses in the local tumor microenvironment and regional lymphatic drainage [16-20]. Since the agent is well-tolerated orally with minimal side effects [12], it is potentially an agent for chemoprevention of cancer, assuming the same immune responses would be stimulated at the preinvasive site of initial tumor formation. Additionally, since the agent was effective in several early-phase lung cancer trials, it was reasonable to expect the agent might work in the prevention of aerodigestive cancers as well.

In the current study, we examined talactoferrin alpha in an A/J mouse model of pulmonary carcinogenesis. We found the agent to be well-tolerated; however, it was ineffective at doses employed for both early and late post-initiation stages of carcinogenesis.

Materials and methods

Regulatory compliance

All experimental procedures are carried out according to approved standard operating procedures detailing personnel protective equipment, exposure guidelines, proper reagent handling procedures, waste disposal, and step-by-step experimental procedure details. Staff receives training annually in laboratory safety, chemical handling, and hazardous waste disposal. Our program is audited annually and is compliant with the 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 performed were conducted with the approval of the Institutional Animal Care and Use Committee at The University of Minnesota, NIH Animal Welfare Assurance number A3456.

Pulmonary tumor model

Seven-week-old female A/J mice were fed pellet diet NIH-07 7022 (Harlan Teklad Diets, Madison WI) and acclimated to the facility for three weeks. Mice were then switched to a semi-purified diet (Research Diets Inc, New Brunswick, NJ) consisting of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (USP XIV), and a complete mixture of vitamins. At 11 weeks of age, the mice were given three doses of 3 mg of benzo[a]pyrene (B[a]P; TCI America)/kg of body weight in 0.2 mL of cottonseed oil by oral gavage [21]. The time interval

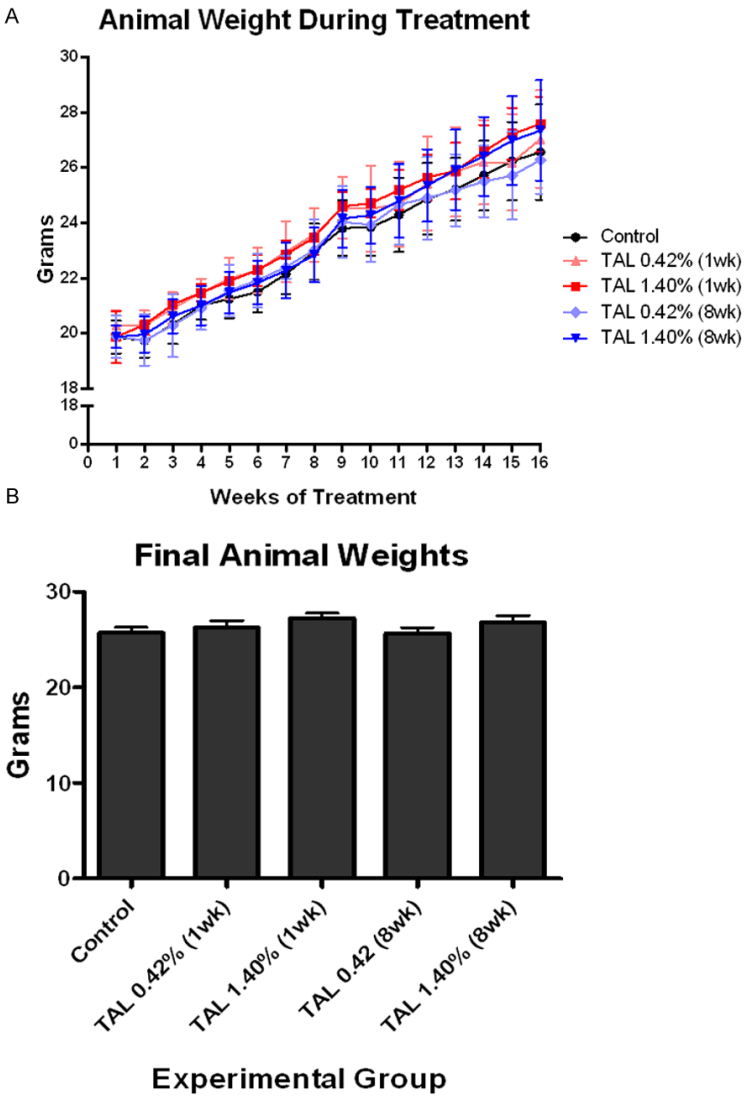


Figure 2. Weight changes of mice given talactoferrin alpha treatment. A. The animals consistently gained weight throughout treatment. Animals were weighed weekly, and there were no statistical differences in weight changes for any of the groups in early-stage (Tal 0.42% 1 wk or Tal 1.4% 1 wk) or late-stage (Tal 0.42% 8 wk or Tal 1.4% 8 wk). The agent was well-tolerated for the duration of the experiment, and there was no observable toxicity. Animals were monitored for weight loss, lethargy attenuation, rough hair coat, or other signs of ill health for the duration of the study. B. Final animal weights. There were no statistical differences in weights between groups (one-way ANOVA, all groups P=0.8903).

between the first and second dose was three days, while between the second and third dose, it was four days (Days 1, 4 and 8). Mice were randomized into five groups of 24 animals by weight the day prior to the first administration of test agents and reweighed once a week.

Talactoferrin alpha administration

Experimental diets oftalactoferrin alpha (generously provided by Agennix Inc., Indianapolis, IN) at 1.40% and 0.42% of the diet were started one week (early stage) or eight weeks (late stage) after the last dose of B[a]P (**Figure 1**) [21]. Diets were prepared by adding 14 mL of a 100 mg/mL solution of talactoferrin alpha (for 1.4 g talactoferrin alpha) per 100 g of diet for 1.4% diet and 4.2 mL of the solution for the 0.42% diet. Dosages were based on an average 25 g mouse consuming 2 g of diet per day. Animals were continued on the feeding schedule, weighed weekly, and monitored for weight loss, lethargy, rough hair coat, or other signs of ill health. The study was concluded 16 weeks after administration of B[a]P. All groups were sacrificed and underwent necropsy.

Statistics

Data were analyzed in a group-wise fashion for differences in tumor counts between control and individual experimental groups by ANOVA testing (one-way) for the early and late-stage experiments as well as any changes in weights between groups. Dunnett's post-testing was employed as well. The Stat Mate module of Graph Pad Prism software Version 5 (Carlsbad, CA) was used for analysis. ANOVA analyses and Graph Pad itself was used for the calculations of the Mean and SEM for each group. $P<0.05$ was used as a cutoff for significance on 2-sided testing of standard error of the mean for all the ANOVA and post tests.

Results

Talactoferrin alpha was well-tolerated in the diet at all doses for the duration of the experiment. There were no significant differences in animal weight (one-way ANOVA P=0.9353 early groups versus control, P=0.9595 late groups versus control, Dunnett's multiple comparison

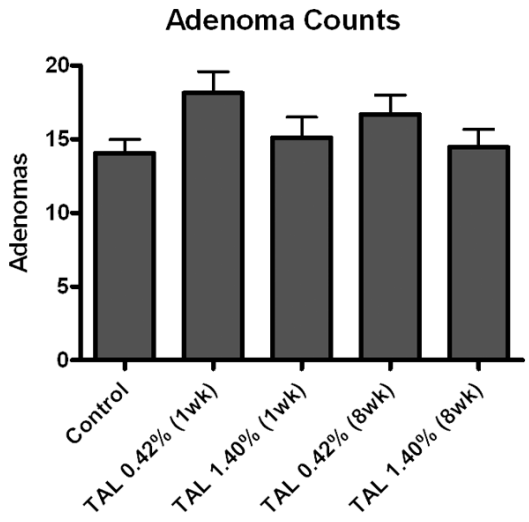


Figure 3. Adenoma counts in animals undergoing talactoferrin alpha treatment. Talactoferrin alpha treatment resulted in no significant decrease in adenoma formation in any of the early or late-stage experimental groups. The Y-axis demonstrates average adenomas per animal, and the x-axis demonstrates the experimental group.

post-testing not significant), behavior, or physical parameters up through and including the 16-week time point at the time of sacrifice (**Figure 2**).

The number of adenomas formed per animal was typical for our A/J mouse experimental carcinogenesis model [21]. The control group (Group 1, **Figure 1B**) formed on average 14.04 ± 0.93 (N=24) lung adenomas/animal. The low-dose early-stage animals (Group 2, **Figure 1B**) formed 18.14 ± 1.45 adenomas (N=22). This represented a 19% increase in the number of adenomas/animal compared to controls, which, according to ANOVA analysis, was not a significant difference (P=0.1368; Dunnett's multiple comparison post-testing not significant). The high-dose early-stage animals (Group 3, **Figure 1B**) formed 15.09 ± 1.41 adenomas/animal on average, which was similar to the control animals (one-way ANOVA P=0.8864). In the late-stage groups, the low-dose (Group 4, **Figure 1B**) and high-dose (Group 5, **Figure 1B**) animals formed 16.70 ± 1.30 and 14.46 ± 1.21 adenomas/animal respectively, which was not statistically different from controls (one-way ANOVA P=0.9387) (**Figure 3**). On Dunnett's multiple testing, there were no statistically significant differences in any of the comparisons between groups.

Discussion

Lactoferrins are members of a larger group of compounds referred to as "cationic antimicrobial peptides". Bovine Lactoferrin is the most studied of the group, and active research on structure function relationships of lactoferrin family members is ongoing to identify more efficacious members and their capacity to act as anticancer peptides [22, 23]. Talactoferrin alpha was one of the more promising compounds of the group which had strong preclinical data and early-phase clinical trial results to the extent that the agent was fast-tracked through the cancer drug pipeline for lung cancer treatment [9, 24-27]. However, after insignificant results from a recent phase III clinical trial for lung cancer [15], the drug is no longer in the cancer drug pipeline for treatment [28].

In the present study, we examined dietary talactoferrin alpha for the prevention of pulmonary carcinogenesis in A/J mice. We conducted experiments which tested low and high doses of the agent in both early and late-stage (post-initiation) carcinogenesis in this mouse model. Although we found the agent was very well-tolerated in the diet, we did not observe a clinically significant decrease in pulmonary adenomas in the model. In the low-dose early-stage group of animals, there was a slightly enhanced adenoma multiplicity (19% increase), which was not statistically significant on ANOVA testing. This has led us to conclude that this lactoferrin derivative has an insignificant effect in the A/J mouse lung tumorigenesis model, a standard preclinical model of lung chemoprevention.

Thus, in the present study, a formerly promising clinical lung cancer treatment agent, talactoferrin alpha, was tested and shown to be unsuccessful in the preclinical setting of pulmonary chemoprevention. Our data is consistent with recent human clinical trial results for talactoferrin alpha in lung cancer patients. It is possible talactoferrin alpha may provide utility in either a different disease setting, or as an immune stimulating adjuvant in a combined treatment setting for chemoprevention. In a similar sense to the utility of combination chemotherapy, there has been interest in utilizing agents combinatorially in the prevention setting [29, 30].

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Disclosure of conflict of interest

None.

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