Chemoprevention with Acetylsalicylic Acid, Vitamin D and Calcium Reduces Risk of Carcinogen-induced Lung Tumors

HANS-CHRISTIAN POMMERGAARD[1], JAKOB BURCHARTH[1], J. ROSENBERG[1] and HANS RASKOV[2]

[1]*Herlev Hospital, University of Copenhagen, Department of Surgery, Herlev, Denmark;*
[2]*The Specialist Medical Center at Diakonissestiftelsen, Frederiksberg, Denmark*

Abstract. *Background/Aim: Research has shown that chemoprevention may be effective against the development of lung cancer. The purpose of the present study was to evaluate the effect of oral chemoprevention in a mouse model of tobacco carcinogen-induced lung tumor. Materials and Methods: A total of 60 A/J mice were randomized to a normal diet, a diet with low calcium, or a chemoprevention diet with acetylsalicylic acid, 1-α 25(0H)$_2$-vitamin D$_3$ and calcium. In addition to the diet, mice received carcinogens by oral gavage for ten weeks. Results: The chemoprevention diet significantly reduced the number of animals with tumors [1 vs. 13, (p<0.001)] and the median number (range) of tumors [0 (0-1) vs. 1 (0-4), (p<0.001)] compared to controls. No signs of toxicity in relation to the diets were observed. Conclusion: The chemoprevention diet had a protective effect against tumor development in the mouse lungs.*

With approximately 3,000 deaths per day, lung cancer is the leading cause of cancer death. In addition, the prognosis is poor with a 5-year survival of 15% (1). Smoking is considered the main reason for 90% of lung carcinomas. Even though 70% of all smokers attempt smoking cessation each year, only 5% succeed (2) and despite cessation of smoking, the risk of lung cancer continues to be elevated (1). In light of this knowledge, chemoprevention of lung cancer seems reasonable. Different drugs have shown beneficial effects as lung cancer chemoprevention, *e.g.* isothiocyanates, myoinositol, green tea, silibinin and dexamethasone (1). Treatment with acetylsalicylic acid (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) have successfully prevented lung tumors in mice (3-5). Vitamin D may possesses anticancer properties (6) and an inverse relationship between calcium with vitamin D and both colonic (7, 8) and lung cancer (9) may exist. However, a combination of these three drugs has never been investigated as chemoprevention for lung cancer. A combination treatment may allow for the dosage of the individual drug, *e.g.* ASA, to be reduced due to a possible potentiating effect, thereby minimizing the risk of adverse effects (10).

The purpose of this study was to investigate the effect of orally-administered ASA, vitamin D and calcium as combination chemoprevention in a mouse model of tobacco carcinogen-induced lung cancer.

Correspondence to: Hans-Christian Pommergaard, Herlev Hospital – University of Copenhagen, Department of Surgery, Herlev Ringvej 75 – DK-2730 Herlev, Denmark. Tel: +45 23241821, email: hcpommergaard@gmail.com

Key Words: Chemoprevention, lung cancer, experimental model, tobacco carcinogens, combination therapy, acetylsalicylic acid, vitamin D, calcium.

Materials and Methods

Animals. In 60 A/J mice (Charles River laboratories, Kungsbacka, Sweden) with specific pathogen-free (SPF) status, lung tumors were induced by tobacco carcinogens as previously described (11). The animals were caged in polypropylene cages in a negative pressure isolator equipped with HEPA filters (class EU10). A light-cycle of 12 hours' light/dark and a temperature of between 18˚ and 22˚C were maintained. Drinking water was UV-irradiated for sterilization. The animals were handled by educated personnel with veterinary supervision and welfare for each animal was evaluated on a daily basis. These animals were also used to evaluate the chemoprevention regimen to prevent bladder tumors, and the weight, water and diet consumption of the animals have been previously reported (12). Prior to initiation, the study was approved by the Danish agency for animal experiments (license number 1997-101-27).

Diet and chemoprevention. Mice were randomized to one of the three diets. Group A (control group) received a normal diet containing 5,000 μg/g calcium; group B (low-calcium group) received a diet containing 2,500 μg/g calcium; and group C (intervention group) received a diet containing 300 μg/g ASA, 0.02 μg/kg 1-α 25(0H)$_2$-vitamin D$_3$ (vitamin D) and 7,500 μg/g calcium.

After two weeks feeding on the respective diets *ad libitum*, tumor induction was initiated with carcinogens administered orally. The precise dosages of the chemopreventive drugs were decided on the basis of previous pilot studies where both toxicology and efficacy were tested (13). The pilot studies discovered that a dosage lower than 300 μg/g ASA had no effect and a dosage higher than 0.02 μg/kg vitamin D$_3$ resulted in kidney damage to the animals.

Table I. *Summarized results for the three groups.*

	Group A (control)	Group B (low calcium)	Group C (intervention)
No.of animals	20	20	20
No. of deaths	2	2	2
No. of animals with tumors	13	7	1 (**$p<0.001$**)[a], (**$p=0.041$**)[b]
No. of tumors in the animals, median (range)	1 (0-4)	0 (0-2)	0 (0-1) (**$p<0.001$**)[a], ($p=0.085$)[b]

[a]Compared with group A, [b]compared with group B, significant results are shown in bold.

Induction of tumors. Benzo[a]pyrene (Sigma-Aldrich, Brøndby, Denmark) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Chemos GmbH, Regenstauf, Germany) were administered with oral gavage at a dosage of 3 μmol per mouse for each substance with cotton oil as a vehicle as described elsewhere (11). All mice received both carcinogens, which were administered once a week for a total of ten weeks.

Evaluation of effect. The experiment was terminated two weeks after the last carcinogen administration. The lung tissue of each animal was dissected and the number of tumors was counted. Body weight, diet consumption and drinking water consumption were recorded for each animal once a week for the entire experiment. Tissues from random tumor samples was evaluated for histology to confirm the presence of adenoma. This experiment was designed as a hypothesis-testing study. Hence, no evaluation of the biochemical pathways responsible for any effect was performed.

Statistics. To evaluate the distribution of deaths between the groups, Chi-square test was used. The Fischer's exact test was used to compare the presence (yes/no) of tumors between the diet groups. The Mann-Whitney test was used to compare the number of tumors between the groups and Friedman test was used to determine differences over time between the groups for weight, diet and water consumption data, since these data were not normally distributed. Data in figures are presented as median values. A *p*-value below 0.05 was considered statistically significant.

Results

The number of deaths was comparable in the three groups (Table I).

Development of tumors. There were significantly fewer animals with tumors in the intervention group (group C) compared with the control group ($p<0.001$). Moreover, the number of tumors was significantly lower in the intervention group compared with controls ($p<0.001$) (Table I).

The number of animals with tumors was significantly lower in the intervention group compared with the low-calcium group ($p=0.041$). The number of tumors in these animals was also lower, however, without reaching statistical significance ($p=0.085$) (Table I).

Weight gain and consumption of diet and water. The weight gain during the treatment period was different between group A and group C ($p=0.019$), which may be due to a higher initial weight for animals in group C. The median weights were comparable throughout the remaining part of the experiment (Figure 1). The weight gain was comparable between group B and group C ($p=0.414$).

The water consumption was different between group A and group C ($p=0.049$), which may result from a higher initial consumption in group C. The consumption was comparable between group B and group C ($p=0.082$). (Figure 2).

The diet consumption was comparable between the three groups (group A *vs*. group C ($p=0.306$), group B *vs*. group C ($p=0.447$)) (Figure 3).

Histological evaluation. Tissue samples confirmed that all analyzed tumors were adenomas.

Discussion

In this study, we found that chemoprevention with a novel combination of ASA, vitamin D and calcium had a preventive effect on the number of tumors and the number of animals with tumors. Based on the results for weight, diet and water consumption, the chemoprevention treatment showed complete absence of toxic effects.

NSAIDs may be effective against lung cancer development due to inhibition of the cyclooxygenase (COX)-2 enzyme. Studies have shown that this enzyme is expressed in human non-small cell lung cancer, and both the COX-1 and COX-2 enzymes are expressed in lung tumors in mice (14, 15). Previous research has shown an effect of NSAIDs, including ASA, on experimentally-induced lung tumors in mice (3-5). Vitamin D has anti-proliferative and pro-apoptotic properties and studies have suggested that this vitamin may protect against different types of cancers (6, 16), including lung cancer (17). Vitamin D in combination with calcium has been shown to be protective against lung cancer in an observational study (9). Calcium alone was effective as chemoprevention for colorectal cancer, and in

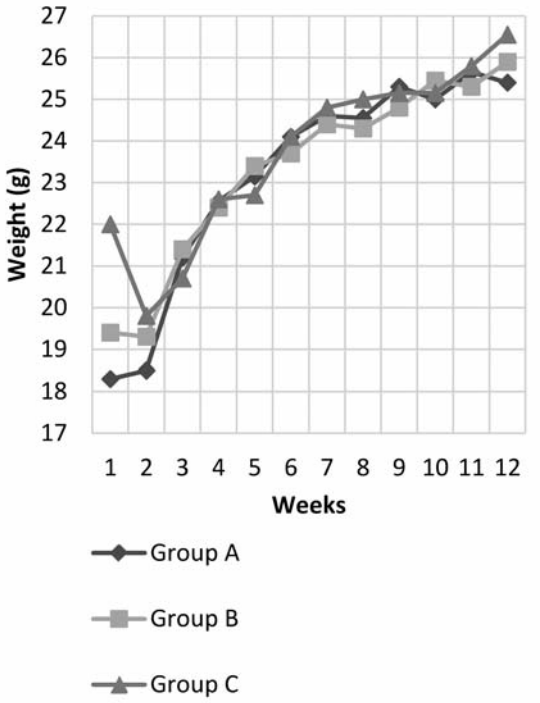

Figure 1. *Median weight in grams of mice for the three groups: A: Control; B: low calcium; C: intervention. Permission to reuse the figure is covered by the copyright transfer agreement of Cancer Investigation (12). Except for layout, no modifications have been made.*

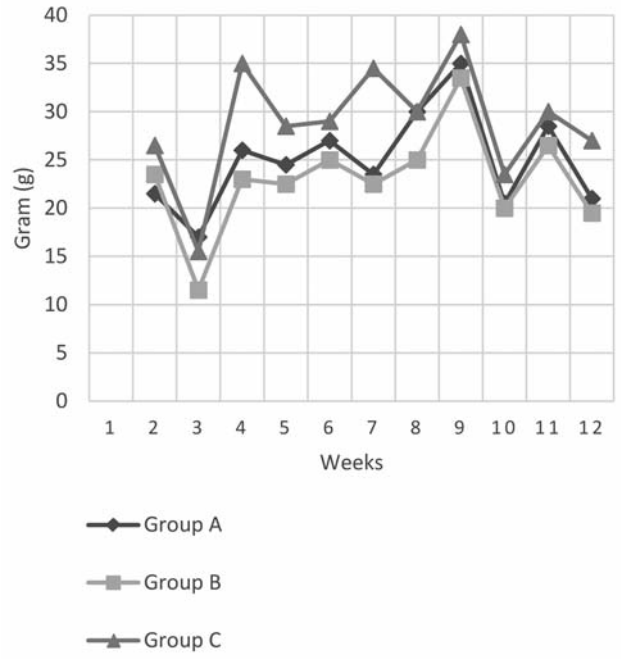

Figure 2. *Median water consumption in grams for the three groups (A (control), B (low calcium), C (intervention)). Permission to reuse the figure is covered by the copyright transfer agreement of Cancer Investigation (12). Except for layout, no modifications have been made.*

particular colorectal adenomas, which is thought to be due to its involvement in the regulation of various genes and cellular pathways (18, 19). However, the evidence that calcium alone may be protective against lung cancer is still scarce (20).

A limitation of this study was that the affected biochemical pathways were not investigated. Moreover, this study did not evaluate the tested agents both as monotherapy and in combination. Thus, the agent(s) responsible for the effect are unknown. The effect in the intervention group was less pronounced in comparison with the low-calcium group when compared with the control group. This may imply that a low calcium level in the diet is favorable over a high level. However, whether a lower level of calcium in the diet of the intervention group would have created a greater effect remains unknown.

Results from the present study indicate that a combination therapy might be suited as chemoprevention in high-risk patients, such as smokers and ex-smokers. However, prior to clinical evaluation, a study investigating which drugs are responsible for the effect should be initiated. Furthermore, it should be considered if this combination treatment has long-term adverse effects in humans, *e.g.* gastrointestinal bleeding (10).

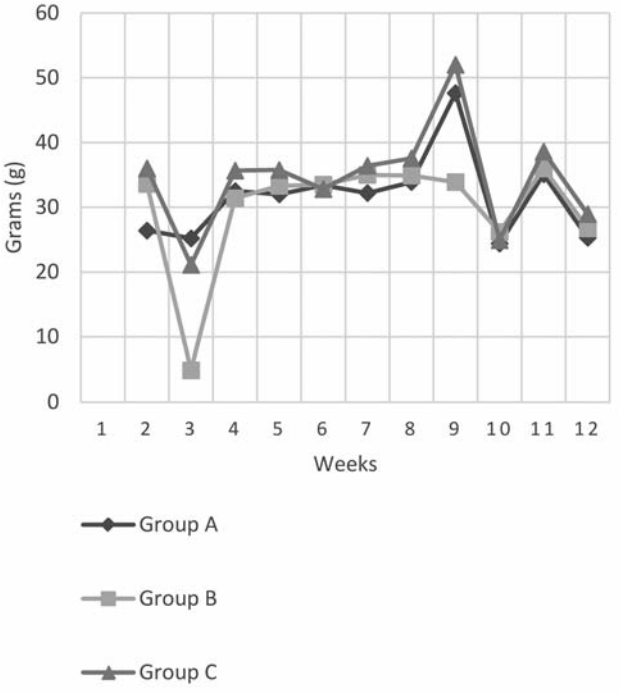

Figure 3. *Median diet consumption in grams for the three groups (A (control), B (low calcium), C (intervention)). Permission to reuse the figure is covered by the copyright transfer agreement of Cancer Investigation (12). Except for layout, no modifications have been made.*

In conclusion, we found a chemopreventive effect of the combination of ASA, vitamin D and calcium against tobacco carcinogen-induced lung tumors in mice. The effect of these drugs alone and in combination should be evaluated together with biochemical analyses, in order to determine the compounds and pathways responsible for the effect.

Declaration of Interest

The Author declare that they have no conflicts of interest or financial interest associated with the study and no financial interest in the patents cited in reference 13.

Acknowledgements

The experiment was conducted by Pipeline Biotech A/S, Roevedvej 1,8380 Trige, Denmark. No funding was used for this study

References

1 Hecht SS, Kassie F and Hatsukami DK: Chemoprevention of lung carcinogenesis in addicted smokers and ex-smokers. Nat Rev Cancer *9*: 476-88, 2009.
2 Gilpin EA and Pierce JP: Demographic differences in patterns in the incidence of smoking cessation: United States 1950-1990. Ann Epidemiol *12*: 141-50, 2002.
3 Duperron C and Castonguay A: Chemopreventive efficacies of aspirin and sulindac against lung tumorigenesis in A/J mice. Carcinogenesis *18*: 1001-6, 1997.
4 Jalbert G and Castonguay A: Effects of NSAIDs on NNK-induced pulmonary and gastric tumorigenesis in A/J mice. Cancer Lett *66*: 21-8, 1992.
5 Rioux N and Castonguay A: Prevention of NNK-induced lung tumorigenesis in A/J mice by acetylsalicylic acid and NS-398. Cancer Res *58*: 5354-60, 1998.
6 Deeb KK, Trump DL and Johnson CS: Vitamin D signalling pathways in cancer: Potential for anticancer therapeutics. Nat Rev Cancer *7*: 684-700, 2007.
7 Cho E, Smith-Warner SA, Spiegelman D, Beeson WL, van den Brandt PA, Colditz GA, Folsom AR, Fraser GE, Freudenheim JL, Giovannucci E, Goldbohm RA, Graham S, Miller AB, Pietinen P, Potter JD, Rohan TE, Terry P, Toniolo P, Virtanen MJ, Willett WC, Wolk A, Wu K, Yaun SS, Zeleniuch-Jacquotte A and Hunter DJ: Dairy foods, calcium, and colorectal cancer: A pooled analysis of 10 cohort studies. J Natl Cancer Inst *96*: 1015-22, 2004.
8 Touvier M, Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, Riboli E, Hercberg S and Norat T: Meta-analyses of vitamin D intake, 25-hydroxyvitamin D status, vitamin D receptor polymorphisms, and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev *20*: 1003-16, 2011.
9 Koo LC: Dietary habits and lung cancer risk among Chinese females in Hong Kong who never smoked. Nutr Cancer *11*: 155-72, 1988.
10 Lanas A and Sopeña F: Nonsteroidal anti-inflammatory drugs and lower gastrointestinal complications. Gastroenterol Clin North Am *38*: 333-52, 2009.
11 Hecht SS, Isaacs S and Trushin N: Lung tumor induction in A/J mice by the tobacco smoke carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[a]pyrene: A potentially useful model for evaluation of chemopreventive agents. Carcinogenesis *15*: 2721-5, 1994.
12 Pommergaard HC, Burcharth J, Rosenberg J and Raskov H: Oral chemoprevention with acetyl salicylic Acid, vitamin D and calcium reduces the risk of tobacco carcinogen-induced bladder tumors in mice. Cancer Invest *31*: 490-3, 2013.
13 patft.uspto.gov (Internet). Patent no. 7,851,461 and 6,703,380. Last accessed on 20 February 2013.
14 Bauer AK, Dwyer-Nield LD and Malkinson AM: High cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) contents in mouse lung tumors. Carcinogenesis *21*: 543-50, 2000.
15 Lee JM, Yanagawa J, Peebles KA, Sharma S, Mao JT and Dubinett SM: Inflammation in lung carcinogenesis: New targets for lung cancer chemoprevention and treatment. Crit Rev Oncol Hematol *66*: 208-17, 2008.
16 Garland CF, Garland FC, Gorham ED, Lipkin M, Newmark H, Mohr SB and Holick MF: The role of vitamin D in cancer prevention. Am J Public Health *96*: 252-61, 2006.
17 Norton R and O'Connell MA: Vitamin D: Potential in the prevention and treatment of lung cancer. Anticancer Res *32*: 211-21, 2012.
18 Chu DZ, Hussey MA, Alberts DS, Meyskens FL Jr., Fenoglio-Preiser CM, Rivkin SE, Mills GM, Giguere JK, Blanke CD and Goodman GE: Colorectal chemoprevention pilot study (SWOG-9041), randomized and placebo controlled: The importance of multiple luminal lesions. Clin Colorectal Cancer *10*: 310-6, 2011.
19 Wang JL, Lin YW, Chen HM, Kong X, Xiong H, Shen N, Hong J and Fang JY: Calcium prevents tumorigenesis in a mouse model of colorectal cancer. PLoS One *6*: e22566, 2011.
20 Peterlik M, Grant WB and Cross HS: Calcium, vitamin D and cancer. Anticancer Res *29*: 3687-98, 2009.

Received September 17, 2013
Revised October 19, 2013
Accepted October 21, 2013