Abstract. Background: Concurrent and sequential administration of combinations of budesonide, bexarotene, suberoylanilide hydroxamic acid (SAHA) and atorvastatin were evaluated in A/J mice for prevention of lung tumors initiated by 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol, NNK). Materials and Methods: Individual drugs and their combinations were administered for 26 weeks after NNK initiation. For sequential administration, budesonide was given for 21 weeks followed by a second drug. Results: Alone, budesonide, bexarotene, and SAHA caused a significant decrease in total and large tumors at 21 and 26 weeks. Concurrent treatment with budesonide and bexarotene or SAHA caused a significantly greater decrease in total tumors and large tumors than either drug administered alone. Sequential administration of all combinations (except budesonide/atorvastatin) gave a significant reduction in total and large tumors. Budesonide followed by SAHA and SAHA with atorvastatin yielded a greater reduction in large tumors. Conclusion: Combinations of drugs demonstrated a greater efficacy in preventing mouse lung tumors than did the individual agents.

In the USA, lung cancer is the most common cause of cancer deaths in both men and women (1); therefore, the development of preventive strategies for this cancer continues to be a vital public health priority. Using combinations of chemopreventive agents that have different modes of action is a promising strategy to increase the efficacy of chemopreventive agents. Such combinations could act on more than one molecular pathway or steps within the carcinogenic process, resulting in an additive or synergistic effect in preventing cancer. Furthermore, the use of combinations may allow the drugs to be administered at lower doses resulting in a decreased potential toxicity (2). This latter justification is highly important, since chemopreventive agents must be administered for long periods of time to relatively healthy individuals who are at high risk of developing disease.

Some combinations of drugs, with different modes of action, have been reported to have greater efficacy in preventing mouse lung tumors than the same drugs given individually. Atorvastatin and polyphenon E have been shown to act synergistically to reduce the occurrence of mouse lung tumors (3). A combination containing 1,4-phenylenebis(methylene) selenocyanate, phenethyl isothiocyanate, d-limonene, and indole-3-carbinol were significantly more inhibitory than indole-3-carbinol given independently (4). In other studies, combinations containing myo-inositol with either dexamethasone, budesonide, or beclomethasone significantly inhibited lung tumors in A/J mice (5-7). Budesonide, administered in the diet, in combination with oral gavage of zarnesta MT (a farnesyl transferase inhibitor) prevented mouse lung tumors more effectively than did the individual agents (8). Synergistic or enhanced relationships for inhibition of mouse lung tumors were also found for combinations of N-acetylcysteine (NAC) and ascorbic acid (9) or N-acetyl-S-(N-2-phenethylthiocarbamoyl)-l-cysteine and myo-inositol (10, 11).

In summary, the above data show that the use of two or more drugs/agents with different pharmacologic properties exhibit additive or even synergistic effects in preventing lung cancer.

In the current study, we have investigated the efficacy of combinations of four different chemopreventive agents, having diverse pharmacologic activity, by using two different treatment regimens, namely, concurrent and sequential. This approach was based on the expectation that mouse lung tumors will exhibit sensitivity to a particular agent only if the involved molecular pathway in the tumor is modulated by that agent. Thus, the approach of using a combination containing two chemopreventive agents that target different molecular pathways is very likely to increase prevention of
tumors, since it should inhibit tumors that are dependent on pathways modulated by one of the agents, as well as those that are modulated by the other agent. In addition, to obtain the same level of efficacy in preventing lung tumors, the use of two agents in combination may allow them to be employed at lower doses than the individual agents used alone, i.e., the use of lower dose levels should result in less potential toxicity than the use of higher dose levels of a single agent. Furthermore, the sequential administration of two agents that target different molecular pathways is also expected to further reduce the potential for toxicity because this regimen does not have the potential for enhanced toxicity that could possibly result from the interaction of the two agents. In summary, the rationale for the concurrent and sequential use of combinations of chemopreventive agents is to target a greater number of tumors by using two or more agents that act on different molecular pathways while reducing the potential for toxicity.

**Materials and Methods**

*Carcinogens, chemopreventive agents, and diets.* The carcinogen, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNK) was purchased from Toronto Research Chemicals (North York, ON, Canada). Budesonide was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and bexarotene and suberoylanilide hydroxamic acid (SAHA) from Chemie Tek (Indianapolis, IN, USA). Atorvastatin was obtained from the National Cancer Institute, DCP Repository (Rockville, MD, USA). The AIN-76A diet was purchased from Dyets Inc. (Bethlehem, PA, USA).

*Animal experiments.* Female strain A/J mice at 7-8 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained in The Ohio State University Laboratory Animal Facility under (IACUC) approved protocols and were provided AIN-76A pelleted diet and water, ad libitum. Mice at 8-9 weeks of age were administered 100 mg/kg body weight of NNK in sterile saline by intraperitoneal injections, once a week for four consecutive weeks. Two weeks after the fourth dose of NNK, the mice were weighed and randomly assigned to one of the treatments groups listed in Table I. They then began to receive the chemopreventive drugs (budesonide, bexarotene, SAHA and atorvastatin) and combinations containing two of the drugs in AIN-76A diets. The number of mice in each treatment group at the start of the experiment is presented in Table I. After 21 weeks, some mice stopped receiving budesonide in their diet and started to receive a second drug (Table I). Mice were euthanized by CO2 asphyxiation followed by cervical dislocation after receiving the chemopreventive agents for 21 or 26 weeks. At necropsy, the lungs were harvested, fixed in phosphate buffered formalin, transferred within two days to 70% alcohol and evaluated under a dissecting microscope for the number and size of tumors. The lungs were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). H&E stained sections of the lungs were evaluated for the number of tumors and for histopathologic diagnosis of adenoma and adenocarcinoma. Carcinomas were distinguished from adenomas by the presence of large undifferentiated cells, cellular atypia, loss of normal alveolar architecture, increased nuclear/cytoplasmic ratio, and nuclear pleomorphism.

**Statistical analysis.** Body weights were analyzed by ANOVA followed by Dunnett’s test; tumor multiplicity by ANOVA followed by the Bonferroni t-test; and the incidence of animals with tumors by ANOVA on ranks followed by Dunnett’s test. For all tests a p-value <0.05 was considered significant.

**Results**

There were no significant treatment-related deaths or toxicity; in two groups, two mice died during the study, i.e., in the control diet and in diet with budesonide followed by bexarotene. The body weights of the mice were monitored over the duration of the study. There were no significant treatment-related alterations in the body weights of mice sacrificed at week 21 or at week 26. The body weights of the

---

**Table I. Effect of combinations of chemopreventive agents on the yield of mouse lung tumors.**

<table>
<thead>
<tr>
<th>Treatment period 1:</th>
<th>Treatment period 2:</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>weeks 0-21</td>
<td>weeks 21-26</td>
<td>week 21</td>
</tr>
<tr>
<td>1) 1.6 mg/kg budesonide</td>
<td>160 mg/kg bexarotene</td>
<td>20</td>
</tr>
<tr>
<td>2) 160 mg/kg bexarotene</td>
<td>600 mg/kg SAHA</td>
<td>20</td>
</tr>
<tr>
<td>3) 600 mg/kg SAHA</td>
<td>500 mg/kg SAHA+ 180 mg/kg atorvastatin</td>
<td>20</td>
</tr>
<tr>
<td>4) 180 mg/kg atorvastatin</td>
<td>1.6 mg/kg budesonide</td>
<td>0</td>
</tr>
<tr>
<td>5) 1.6 mg/kg Budesonide + 160 mg/kg bexarotene</td>
<td>600 mg/kg SAHA</td>
<td>0</td>
</tr>
<tr>
<td>6) 1.6 mg/kg Budesonide + 600 mg/kg SAHA</td>
<td>500 mg/kg SAHA+ 180 mg/kg atorvastatin</td>
<td>0</td>
</tr>
<tr>
<td>7) 500 mg/kg SAHA+ 180 mg/kg atorvastatin</td>
<td>Control diet</td>
<td>0</td>
</tr>
<tr>
<td>8) 1.6 mg/kg budesonide</td>
<td>Control diet</td>
<td>25</td>
</tr>
<tr>
<td>9) 1.6 mg/kg budesonide</td>
<td>Control diet</td>
<td>20</td>
</tr>
<tr>
<td>10) 1.6 mg/kg budesonide</td>
<td>Control diet</td>
<td>20</td>
</tr>
</tbody>
</table>

SAHA: suberoylanilide hydroxamic acid.
mice treated with the chemopreventive agents for the longer duration of treatment (26 weeks) are presented in Figure 1.

Lung tumors were found in all the mice except for one mouse at week 21 and two mice at week 26 that received budesonide and budesonide plus SAHA, respectively. The multiplicity of lung tumors was evaluated both as the total number of tumors (total lung tumors) and the number of large tumors (>1 mm in diameter) per mouse. The multiplicity of total lung tumors per mouse increased from week 21 to week 26, but was not statistically significant for large tumors, i.e., 4.04±0.41 and 5.07±0.66 at weeks 21 and 26, respectively (Figure 2A and B). At both time points, budesonide and bexarotene and their combination reduced the multiplicity of both total and large lung tumors at weeks 21 and 26. However, only the combination prevented a statistically significant increase in total and large tumors between weeks 21 and 26. At week 26, but not week 21, the combination had significantly greater efficacy in preventing both total and large tumors. Thus, the combination containing budesonide and bexarotene was more efficacious in preventing lung tumors and their increase in size than either drug administered alone.

SAHA reduced the yield of total and large tumors at weeks 21 and 26, as well as their further increase between week 21 and 26 in both classifications of lung tumors (Figure 3A and B). The preventive effect of the combination of budesonide and SAHA (Figure 3A and B) was similar to that of the combination of budesonide and bexarotene. The combination of budesonide and SAHA demonstrated a significantly greater efficacy in preventing both total and large tumors at both time periods. In fact, large tumors were completely prevented at week 21 and were reduced in frequency by 91% in the week 26 harvest. Thus, the combination of budesonide and SAHA, similar to the previous combination, was more efficacious at week 26 in preventing lung tumors and their increase in size, but unlike the previous combination was also more efficacious at week 21, than either drug administered alone.

The ability of sequential treatment with two drugs to prevent lung tumor development was evaluated by administering budesonide for the first 21 weeks of treatment followed by a second drug for six weeks. Treatment with budesonide followed by treatment with six more weeks of budesonide, or of a different agent, i.e. bexarotene, SAHA, or a combination of SAHA plus atorvastatin reduced the multiplicity of total lung tumors and large tumors at week 26, while treatment with budesonide followed by atorvastatin did not (Figure 4A and B). Interestingly, budesonide treatment followed by SAHA alone or SAHA plus atorvastatin reduced the yield of large tumors to a greater extent than continuous treatment with budesonide alone (Figure 4B). Thus, sequential treatment with drugs that target different molecular pathways can have a greater efficacy in preventing lung tumors than continuous treatment with a single drug.

Discussion

Combinations of two or more chemopreventive agents have demonstrated additive and in some cases synergistic activity in preventing lung tumors in mice (3-11). Examples of the
combinations that exhibited greater efficacy include: i) a mixture of 1,4-phenylenebis(methylene)selenocyanate, phenethyl isothiocyanate, indole-3-carbinol, and d-limonene that was significantly more efficacious than indole-3-carbinol given alone (4); ii) combinations of myo-inositol and dexamethasone (5, 6); iii) combinations of budesonide or beclomethasone with myo-inositol (7); iv) combinations of budesonide with a farnesyl transferase inhibitor (zarnestra MT) (8); v) combination of N-acetylcysteine (NAC) and ascorbic acid (9), and vi) combinations of N-acetyl-S-(N-2-phenethylthiocarbamoyl)-l-cysteine and myo-inositol and of N-acetyl-S-(N-2-phenethylthiocarbamoyl)-l-cysteine, indole-3-carbinol, and myo-inositol (10, 11). In the study reported herein, we determined the ability of combinations containing budesonide with one of three other chemopreventive agents with different modes of action to prevent mouse lung tumors. Budesonide, a synthetic anti-inflammatory glucocorticoid used to control mild-to-moderate persistent asthma has been shown to prevent the formation of lung adenomas and adenocarcinomas in mice when administered either by inhalation or in the diet (7, 12-14). In the experiments reported herein, budesonide (acting alone) effectively inhibited both total and large tumors at 21 weeks after NNK administration. One of the other agents evaluated in combination with budesonide was bexarotene (Targretin®, an RXR agonist) (15). As a chemopreventive agent, bexarotene has proven to be highly effective in preventing lung and mammary tumors in animal models (16-18). In the current studies, bexarotene...
administered alone and in combination with budesonide inhibited both total and large tumors in mouse lung at 21 and 26 weeks. Interestingly, the combination of bexarotene with budesonide demonstrated greater efficacy in preventing large lung tumors at 26 weeks, indicating at least additive activity of the two drugs.

The other two drugs evaluated in combination with budesonide, SAHA (a histone deacetylase inhibitor) and atorvastatin (a statin), have been investigated for the prevention of various types of cancers including lung cancer in mice (19-21). Combinations containing a statin with another chemopreventive agent have suggested synergistic activity in lowering the risk of cancer in humans. Hoffmeister et al. (22) found that use of either statins or low dose aspirin (100 mg) resulted in a lowering of the risk for colorectal cancer, but also found that the use of low-dose aspirin in concert with statins caused a greater reduction in risk than either agent acting alone. Correspondingly, among those individuals involved in the California Men’s Health Study, Flick et al. (23) found that use of statins for more than 5 years resulted in a 28% reduction in risk of prostate cancer. The authors implied that this reduction was likely associated with the concurrent use of nonsteroidal anti-inflammatory drugs.

In the current studies, SAHA and the combinations of budesonide and SAHA when given concurrently reduced the yield of total lung tumors and of large tumors. The combination of SAHA and budesonide was more efficacious in preventing both total and large lung tumors than either drug acting alone. Thus, similar to the combination of budesonide and bexarotene, the combination of budesonide and SAHA demonstrated greater efficacy, indicating at least additive activity of the two drugs in preventing total and large lung tumors.

Administration of budesonide for 21 weeks followed by the sequential administration for six weeks of bexarotene, SAHA, or SAHA plus Atorvastatin was also effective in preventing both total and large lung tumors. Administration of budesonide followed by bexarotene demonstrated the same degree of prevention of lung tumors as continuous 26 weeks of treatment with budesonide, while budesonide followed by SAHA or SAHA plus atorvastatin was of similar efficacy in preventing total lung tumors, but of significantly greater efficacy in preventing large tumors. This would indicate that SAHA targeting of tumors is dependent on histone deacetylase activity and targets tumors that are resistant to budesonide. Although, the major pharmacologic activity of SAHA is inhibition of histone deacetylase, it is possible that another activity of SAHA is responsible for the prevention of lung tumors, especially their increase in size. In contrast, sequential treatment with budesonide followed by atorvastatin did not significantly lower the multiplicity of total or large lung tumors relative to mice that did not receive a chemopreventive agent. This would suggest that atorvastatin does not prevent lung tumors, similar to the findings of Lu et al. (24), who have also reported the inability of atorvastatin to prevent lung tumors in mice.

In summary, concurrent treatment with combinations containing budesonide and bexarotene or budesonide and SAHA demonstrated a greater efficacy in preventing mouse lung tumors than the individual agents, supporting the concept of using combinations of chemopreventive agents for enhanced prevention of lung cancer. Additionally, primary treatment with budesonide followed by sequential treatment with SAHA or SAHA plus atorvastatin caused a significantly greater reduction in large tumors than continuous treatment with budesonide indicating that sequential administration of chemopreventive drugs with different targets is an effective regimen that has the potential to increase efficacy while
reducing toxicity that can result from long-term treatment with high doses of a single chemopreventive agent.

Acknowledgements

Supported in part by grant, 5R21CA135335 from the NCI.

References


Received June 10, 2011
Revised August 5, 2011
Accepted August 8, 2011