Prevention of mouse lung tumors and modulation of DNA methylation by combined treatment with budesonide and R115777 (Zarnestra\textsuperscript{MT})

Fadel S. Alyaqoub, Lianhui Tao, Paula M. Kramer, Vernon E. Steele, Ronald A. Lubet, William T. Gunning and Michael A. Pereira

1 Medical University of Ohio, Toledo, OH 43614, USA, 2 Ohio State University, College of Medicine and Public Health, Columbus, OH 43210, USA and 3 National Cancer Institute, Bethesda, MD 20892, USA

*To whom correspondence should be addressed at: Ohio State University, College of Medicine and Public Health, Department of Internal Medicine, 300 West Tenth Avenue, 1148 CHRI, Columbus, OH 43210, USA. Tel: +1 614 293 6864; Fax: +1 614 293 4072; Email: Pereira.25@osu.edu

Budesonide (an anti-inflammatory glucocorticoid), R115777 (a farnesyl transferase inhibitor, Zarnestra, Tipifarnib) or combinations of them were evaluated for prevention of lung tumors and for modulation of DNA methylation in tumors. Lung tumors were induced by vinyl carbamate in female Strain A mice. One week later, mice received 60 or 100 mg/kg R115777 by oral gavage and 5 days/week, 0.8 or 1.6 mg/kg of budesonide in their diet, or their combined treatment until killed at 20, 28 and 36 weeks after administering the vinyl carbamate. Other mice were administered the drugs for 2 weeks before killing at 20 weeks. At Week 20, the rank order for prevention of lung tumors was the combined treatment > budesonide > R115777. At later killings, R115777 was no longer effective, whereas budesonide and the combinations continued to prevent tumors, albeit at a reduced efficacy. DNA hypomethylation in lung tumors was prevented by treatment with R115777, budesonide and the combinations. When administered starting at Week 18 to tumor-bearing mice, the drugs reversed DNA hypomethylation in the tumors. In summary, combined treatment with budesonide and R115777 produced the following results: (i) it was more efficacious in preventing lung tumors than the individual drugs; and (ii) it prevented and reversed DNA hypomethylation in lung tumors. These results support the combined use of budesonide and R115777 in prevention of lung tumors and suggest that reversal of DNA hypomethylation in lung tumors would be useful as a surrogate endpoint biomarker for prevention.

Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women. The utilization of chemopreventive agents to suppress or reverse the process of lung carcinogenesis appears to be a useful strategy to reduce lung cancer deaths. Budesonide, a synthetic anti-inflammatory glucocorticoid used to control mild-to-moderate persistent asthma (1), has been shown to prevent the formation of lung adenomas and adenocarcinomas in mice (2–7). As a chemopreventive agent, budesonide appears to decrease both the growth rate of tumors and the progression of adenomas to adenocarcinomas; lung tumors from budesonide-treated mice were smaller and contained fewer carcinomas than tumors from mice not administered the drug (4). A Phase IIb clinical study of inhaled budesonide administered to current cigarette smokers with bronchial dysplastic lesions has been conducted (8). Although budesonide did not cause regression of the lesions, it did decrease the protein expression of p53 and Bcl-2, suggesting that it might have some activity in precancerous lesions.

Farnesyl transferase inhibitors are another class of drugs under evaluation for prevention of lung cancer. These inhibitors prevent post-translational farnesylation of numerous proteins, including the Ras oncoproteins (9). Mouse lung tumors induced by vinyl carbamate, the carcinogen used in the present study, have mutations in the Ki Ras gene (5, 10). Inhibition of farnesylation of the Ras proteins prevents their incorporation into the cellular membranes and their activity in signal transduction (11–13). R115777 (Zarnestra, Tipifarnib; (+)-6-[amino(4-chlorophenyl)(1-methyl-1H-imidazol-5-y)]methyl]-4-(3-chlorophenyl)-1-methyl-2-[1H]-quinolinone) is an example of a farnesyl transferase inhibitor that has prevented benzo(a)pyrene-induced lung tumors in mice (14). Adverse effects of R115777 in clinical trials have included neutropenia, anemia, myelosuppression, mild peripheral neuropathy and fatigue (15, 16).

Combinations of drugs are being evaluated in attempts to increase the efficacy of cancer prevention, while not increasing toxic side effects. Combined treatment with drugs having different mechanisms is most attractive, since they could attack the carcinogenic process at more than one site or pathway. This could result in the combined treatment having an additive, if not a synergistic activity in preventing cancer. Budesonide and R115777 are two such chemopreventive drugs having different modes of action so that combined treatment with them could modulate different biological and molecular alterations in lung carcinogenesis resulting in greater efficacy.

Surrogate endpoint biomarkers are being developed for demonstrating efficacy of chemopreventive agents. These biomarkers are likely to be epigenetic alterations that can be modulated by chemopreventive agents. One epigenetic alteration found in most tumors is global DNA hypomethylation (17–20). Modulation of DNA hypomethylation in tumors by chemopreventive agents has been proposed as a biomarker for chemoprevention (4, 5, 21–24). We have previously reported that agents effective in preventing cancer are also effective in preventing and reversing DNA hypomethylation in tumors, while agents that do not prevent cancer were ineffective in modulating DNA hypomethylation (4, 21–24).
One example of a chemopreventive agent that prevents both mouse lung tumors and DNA hypomethylation in the tumors is budesonide (4,21). We report here an evaluation of the ability of combined treatment with budesonide and R115777 to prevent and to modulate DNA hypomethylation in mouse lung tumors.

Materials and methods

Chemicals
Vinyl carbamate (purity >99%) was obtained from Toronto Research Chemicals (North York, Ontario, Canada); AIN-76A diet (casein 20%, DL-methionine 0.3%, cornstarch 15%, sucrose 50%, corn oil 5%, cellulose 5%, AIN mineral mixture 3.5%, AIN vitamin mixture 1.0% and choline bitartrate 0.2%) from Dyets (Bethlehem, PA); and budesonide and carboxymethylcellulose from Sigma Chemical (St Louis, MO). The NCI DCP Repository provided the R115777.

Prevention of lung tumors: experimental design
Female strain A/J mice from Jackson Laboratory (Bar Harbor, ME) were housed in the AAALAC accredited laboratory animal facility at the Medical University of Ohio. At 8 weeks of age, the mice were administered 16 mg/kg vinyl carbamate by intraperitoneal injection once a week for two consecutive weeks. One week later, the mice were administered: 0 (2% carboxymethylcellulose vehicle control), 60 or 100 mg/kg R115777 by oral gavage, 5 days/week; 0.8 or 1.6 mg/kg budesonide in their diet; 0.8 mg/kg budesonide + 60 mg/kg R115777; or 1.6 mg/kg budesonide + 100 mg/kg R115777. The dose levels of R115777 and budesonide were chosen since we have previously demonstrated that the high dose levels prevented lung tumors without toxicity in female strain A mice (4,14). The volume of the vehicle administered to the mice was 0.2 ml/mouse. Mice were killed at 20, 28 and 36 weeks after the last dose of vinyl carbamate. Each treatment group contained 16 mice except for the diet control group that contained 48 mice at the time of killing (Week 36). To determine the ability of the drugs to modulate biomarkers in tumors, groups of eight mice each were administered the various treatments for only 2 weeks before killing at Week 20.

DNA hypomethylation: dot blot analysis
DNA was isolated from lung tissue and tumors by digestion with RNase A and proteinase K followed by organic extraction with phenol and chloroform. Purified DNA (1 µg) was denatured in 100 µl of 0.4 M NaOH/10 mM EDTA at 100 °C for 10 min, neutralized with 2 M ammonium acetate and dotted onto a Hybond™ nitrocellulose membrane. The membranes were dried, incubated with 7% milk in Tris-buffered saline + Tween-20 (pH 7.6, TBST) for 1 h and then incubated with a 1:10 000 dilution of rabbit polyclonal primary antibody specific against 5-methylcytosine in DNA (Megabase Research Products, Lincoln, NE) for 2–3 h. They were then washed with 7% milk-TBST and TBST followed by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h. After washing again with 7% milk-TBST and TBST, the membrane was treated with enhanced-chemiluminescence western blotting detection reagents (Amersharm Pharma- ciabio Tech, Arlington Heights, IL) and exposed to Kodak autoradiograph films. Optical density of the dots was quantified with a Scion Image Analysis System (Scion, Frederick, MD). Equal DNA loading of the membrane was demonstrated by methylene blue staining.

Statistical analysis
The results were analyzed for statistical significance by ANOVA followed by the Bonferroni t-test or when the data were not normally distributed with equal variance by ANOVA on Ranks followed by Dunn’s test. Statistical significance was indicated by a P-value < 0.05.

Results

Prevention of vinyl carbamate-induced lung tumors
The different treatments did not cause mortality; none of the groups had more than one death during the 36 weeks of study. However, the high dose of R115777 (100 mg/kg), as well as its combination with 1.6 mg/kg budesonide, decreased the body weight of the mice (Figure 1A and B). At the time of killing (Week 36), the body weight of the mice that received the vehicle, 100 mg/kg R115777, and the combination of 100 mg/kg R115777 + 1.6 mg/kg budesonide were 23.1 ± 0.39, 20.3 ± 0.38 and 19.7 ± 0.65, or decreased by 12.2 and 14.7%, respectively. At the two earlier killings of 20 and 28 weeks, the body weight of the mice in these two treatment groups was decreased by only 8–9%. This and the fact that the low dose level of R115777 and of the combination did not decrease the body weight of the mice while decreasing tumor yield would indicate that the treatment-related affects upon tumor yield were independent of the decrease in body weight. The 0.8 mg/kg budesonide increased the body weight of the mice, i.e. from 23.1 ± 0.39 to 25.8 ± 0.84 at 36 weeks.

The effect of budesonide, R115777, and the two combinations on lung tumor multiplicity is presented Figure 2A and B and Table I. At all three killings, >95% of the tumors were solid adenomas with the remainder being either papillary adenomas or adenocarcinomas. At the Week 20 killing, both dose levels of budesonide and R115777 significantly reduced lung tumor multiplicity with budesonide being more effective. At Weeks 28 and 36, R115777 was no longer effective in preventing lung. The high dose level of
R115777 caused a non-significant 0 and 16% increase in tumor multiplicity at Weeks 28 and 36, respectively. At Week 36, budesonide was also less effective in preventing lung tumors than at Week 20. The low dose level of budesonide at Week 36 was not effective in decreasing tumor multiplicity, i.e. the 19% (from 100 to 81%) decrease in multiplicity was not statistically significant. The high dose level of budesonide also appeared to be less effective at Week 36, causing a 35% reduction in tumor multiplicity compared with a 48% reduction at Week 20.

Combined treatment with budesonide and R115777 was more effective in preventing lung tumors than either drug administered alone. At Week 20, the high dose combination caused a 70% decrease in tumor multiplicity, while the high dose of budesonide and R115777 caused only a 52 and 30% reduction in tumor multiplicity. Tumor multiplicity in the high dose combination was 2.00 ± 0.25 that was statistically less than in both the high dose of budesonide and R115777, i.e. 3.50 ± 0.41 and 4.69 ± 0.42 (P-value <0.05). At Week 36, tumor multiplicity in the high dose combination (3.33 ± 0.53) was again significantly less than either the high dose of budesonide (5.56 ± 0.59) or R115777 (10.0 ± 1.69). With respect to the low dose combination at Weeks 20 and 36, it was significantly less than the low dose of R115777, but not the low dose of budesonide. However at Week 36, this combination was the only low dose group to be significantly different from the control group.

The size of the tumors at all three killings did not differ among any of the treatment groups. For example at Week 36, the average diameter of the tumors in the control group was 1.44 ± 0.04 mm and range of the average diameter for the other treatment groups was between 1.27 ± 0.09 (high dose budesonide group) and 1.57 ± 0.07 (low dose combination).

Modulation of DNA hypomethylation
The effect of budesonide, R115777 and the combinations on DNA hypomethylation in lung tumors at the three killing times is presented in Figure 3A–C. At all three killings, DNA methylation in lung tumors from the control group was significantly lower relative to normal lung tissue. Also, at all three killings, both dose levels of budesonide, R115777 and their combinations significantly increased the level of DNA methylation in lung tumors, with the exception of the low dose level of R115777 (60 mg/kg gavage) at 36 weeks. The level of DNA methylation in lung tumors was increased by treatment with the two drugs and the combinations to the extent that it was no longer significantly less than the level of methylation in normal lung DNA (P-value <0.01, except for the low dose combination at 36 weeks, P-value <0.05).

The ability of budesonide, R115777 and the combinations to reverse the DNA hypomethylation in lung tumors was determined by administering the chemopreventive agents to mice with adenomas (Figure 4). The chemopreventive agents were administered for the 2 weeks before the mice being killed at Week 20. Both dose levels of R115777 and the combinations and the high dose level of budesonide reversed

### Table 1. Effect of budesonide, R115777 and their combinations on lung tumor multiplicity

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Week 20</th>
<th>Week 28</th>
<th>Week 36</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1.6 mg/kg Budesonide</td>
<td>3.50 ± 0.41 (52)*</td>
<td>2.43 ± 0.43 (37)*</td>
<td>5.56 ± 0.59 (65)*</td>
</tr>
<tr>
<td>2 0.8 mg/kg Budesonide</td>
<td>2.81 ± 0.36 (42)*</td>
<td>—</td>
<td>7.00 ± 0.72 (81)</td>
</tr>
<tr>
<td>3 100 mg/kg R115777</td>
<td>4.69 ± 0.42 (70)*</td>
<td>6.50 ± 0.58 (100)</td>
<td>10.0 ± 1.69 (116)</td>
</tr>
<tr>
<td>4 60 mg/kg R115777</td>
<td>3.75 ± 0.82 (56)*</td>
<td>—</td>
<td>8.40 ± 0.60 (98)</td>
</tr>
<tr>
<td>5 100 mg/kg R115777 + 1.6 mg/kg Budesonide</td>
<td>2.00 ± 0.25 (30)*†</td>
<td>1.40 ± 0.33 (22)*</td>
<td>5.33 ± 0.53 (39)*†</td>
</tr>
<tr>
<td>6 60 mg/kg R115777 + 0.8 mg/kg Budesonide</td>
<td>2.93 ± 0.46 (44)*</td>
<td>—</td>
<td>5.50 ± 0.65 (64)*</td>
</tr>
<tr>
<td>7 Control</td>
<td>6.72 ± 0.45</td>
<td>6.48 ± 0.58</td>
<td>8.60 ± 0.39</td>
</tr>
</tbody>
</table>

Results are mean ± SE with the number in parentheses indicating the percentage of the control group.

*Indicates a significant difference from the control group, by ANOVA followed by a Bonferroni t-test (P-value <0.05).

†Indicates that the combination was significantly different from both of its constituents, by ANOVA followed by Bonferroni test (P-value < 0.05).
DNA hypomethylation in tumors. DNA methylation in the tumors was increased by both dose levels of R115777 and the low dose combination to the extent that it was no longer hypomethylated relative to normal lung. Although both dose levels of budesonide and the high dose combination did significantly increase DNA methylation in the tumors, it was not sufficient to reach the level of methylation in normal lung.

**Discussion**

The interest in evaluating chemopreventive agents in combination comes from the conjecture that targeting multiple pathways associated with carcinogenesis may increase the effectiveness of cancer prevention without increasing toxicity. Budesonide, a glucocorticoid, and R115777, a farnesyl transferase inhibitor, have been demonstrated to prevent lung tumors in mice (2–7,14). We report here that the combined use of budesonide and R115777 resulted in an apparent synergistic increase in efficacy in preventing mouse lung tumors. Combinations of the two drugs were more efficacious than either drug alone in decreasing the yield of lung tumors. This included the two killings at Weeks 28 and 36 where R115777 no longer demonstrated an ability to decrease the yield of lung tumors. Hence, the combinations were more active than the sum of the activity of the two drugs.

The apparent synergy of the combination would suggest that there is an effect on different pathways or on different moieties of the same pathway by budesonide and R115777. Moderate to advanced lung cancer apparently becomes resistant to inhibition of cell proliferation by glucocorticoids. The resistance has been attributed to an increase in the level of c-Jun in response to activation of the Ki-ras gene (25,26). c-Jun apparently inhibits the binding of glucocorticoids to their receptor (26). Hence, the high incidence of K-ras mutations in vinyl carbamate-induced tumors (5,10) could decrease their sensitivity to glucocorticoids, including budesonide. K-ras is farnesylated in order to bind to the inner face of the plasma membrane and become biologically active. The enzyme, farnesyl transferase catalyzes the addition of a farnesyl group to the cysteine of CAAX tetrapeptide motif at the C-terminus of proteins (11–13). R115777, by inhibiting the farnesylation of K-ras, could increase the sensitivity of the lung tumors to inhibition by budesonide. This could result in the apparent synergistic activity of
combined budesonide and R115777 treatment in preventing lung tumors.

In a previous study, budesonide was demonstrated to slow the growth and progression of lung tumors to cancer (4). 5-Leukotriene pathway inhibitors (Accolate, MK-886 and Zileuton) also appear to slow the progression of mouse lung tumors (27). In the present study, R115777, budesonide and the combined treatment were less effective in preventing lung tumors at 36 weeks than at the two earlier killings, i.e. at 36 weeks there was a smaller percentage reduction in tumor multiplicity. In fact R115777 only reduced tumor multiplicity at 20 weeks and not at 28 or 36 weeks. Hence, R115777 similar to budesonide and 5-leukotriene inhibitors was less effective in decreasing tumor multiplicity with time.

We also evaluated the ability of budesonide, R115777 and the combinations to modulate a potential surrogate endpoint biomarker in lung tumors, i.e. DNA hypomethylation that is found in most cancers including mouse and human lung tumors (4,17–24). We have previously shown that budesonide, administered for a short-term before killing, reversed DNA hypomethylation in mouse lung tumors (4,5). A similar 14-day treatment with R115777 and the combinations restored the level of DNA methylation in lung tumors to that observed in normal lung tissue (Figure 4). Budesonide, R115777 and the combinations also prevented DNA hypomethylation at all three killings (Figure 3A–C), when treatment began 1 week after vinyl carbamate, long before the occurrence of overt tumors. This suggests that lung tumors can develop without the DNA being hypomethylated indicating that hypomethylation is not an absolute requirement for mouse lung tumor formation. However, DNA hypomethylation is believed to accelerate the development of lung tumors, since all tumor promoters and non-genotoxic carcinogens that accelerate the occurrence of tumors and progression to cancer have consistently induced DNA hypomethylation (22,28). In confirmation, we have shown that methionine prevents DNA hypomethylation and delays the occurrence of mouse liver tumors (22). Therefore, budesonide and R115777 could decrease the rate at which lung tumors occur by preventing DNA hypomethylation in the tumors.

In summary, combined treatment with budesonide and R115777 was shown to (i) be more efficacious in preventing lung tumors than when the drugs were used independently; and (ii) prevent and reverse DNA hypomethylation in lung tumors. These results would support the combined use of budesonide and R115777 in prevention of lung tumors. The results also suggest that reversal of DNA hypomethylation in lung tumors by chemopreventive agents could be used as a surrogate endpoint biomarker for prevention of tumors.

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References


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