Chemoprevention of Benzo(a)pyrene-induced Lung Tumors in Mice by the Farnesyltransferase Inhibitor R115777


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ABSTRACT

Purpose: Inhibitors of farnesyltransferase (e.g., R115777) are being developed for therapy and prevention of various cancers. The efficacy of R115777 [Zarnestra; (B)-6-[amino(4-chlorophenyl)(1-methyl-1H-imidazol-5-yl)-methyl]-4-(3-chlorophenyl)-1-methyl-2(1H)-quinolinolone] to prevent the development of lung tumors in mice was determined.

Experimental Design: Female strain A mice (7–8 weeks of age) were given 100 mg/kg benzo(a)pyrene [B(a)P] by i.p. injection, and 4 or 14 weeks later, they were given 50 or 100 mg/kg R115777 by oral gavage 5 days/week. The mice were sacrificed 22 weeks after they received the B(a)P.

Results: Tumor multiplicity was 5.0 ± 0.85, 4.5 ± 0.52, 2.1 ± 0.31, and 1.5 ± 0.31 tumors/mouse in mice that received 0, 50, 100 (weeks 4–22), or 100 (weeks 14–22) mg/kg R115777. Thus, 100 mg/kg R115777 was similarly effective in preventing lung tumors when administered during the promotional phase of carcinogenesis [that is, either 4 or 14 weeks after B(a)P], whereas the lower dose of 50 mg/kg R115777 was ineffective. The proliferating cell nuclear antigen labeling index was also significantly reduced in lung tumors from mice treated with 100 mg/kg R115777 starting at 4 or 14 weeks.

Conclusions: These results demonstrated that R115777 can prevent the development of lung tumors in the A/J mouse model, where tumors routinely have mutations in the Ki-Ras oncogene.

INTRODUCTION

It is well established in the literature that a significant number of all human cancers, including lung tumors, have a mutation of a Ras oncogene. Ras proteins are membrane-associated GTPases that are involved in the regulation of signal transduction pathways that control cell differentiation, proliferation, and apoptosis (1). In fact, because of their association with a number of oncogenic murine viruses, the Ras oncogenes were the first group of oncogenes identified. Although all three Ras oncogenes (Ha, Ki, and N) have been found to be mutated in different cancers, mutations in Ki-Ras are the most common in human lung, colon, and pancreatic cancers. Oncogenic mutations of Ras at codons 12, 13, and 61 have been reported to decrease GTPase activity, resulting in persistent constitutively GTP-bound signaling (2).

Because of the known involvement of Ras proteins in cancer and their early isolation, blocking of Ras activity became an early molecular target for developing therapeutic or preventive drugs (3). There are indeed a number of potential targets for blocking Ras activity. Thus, one approach is to directly block the interaction of Ras with one of its downstream elements. For Ras protein to translocate to the cell membrane and become active, it must be prenylated, typically farnesylated. Thus, a variety of drug companies and investigators have taken the approach of developing inhibitors directed against the enzyme farnesyltransferase, which farnesylates the Ras proteins, as a method of blocking Ras activity (4–7). Although the Ras proteins are farnesylated, a number of additional proteins can be farnesylated as well. One group of FTIs,3 which includes R115777[Zarnestra;(B)-6-[amino(4-chlorophenyl)(1methyl-1H-imidazol-5-yl)-methyl]-4-(3-chlorophenyl)-1-methyl-2(1H)-quinolinolone (Fig. 1)], competes for the CAAX peptide binding site to prevent farnesylation of the Ras protein and its subsequent translocation to the membrane (8). R115777 has been shown to have antitumor effects in four different human tumor xenograft models in nude mice (9). R115777 has also entered Phase II clinical trials for chemotherapy of solid malignancies (10). The purpose of the study reported here was to evaluate the efficacy of R115777 in preventing the development of lung tumors in strain A mice, which routinely have mutations in the Ki-Ras oncogene.

MATERIALS AND METHODS

Chemicals. B(a)P (purity >99%) was purchased from Sigma (St. Louis, MO). The FTI R115777 was provided by Janssen Research Foundation (Spring House, PA).

Animals. Female A/J mice (5–6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The

1 The abbreviations used are: FTI, farnesyltransferase inhibitor; B(a)P, benzo(a)pyrene; PCNA, proliferating cell nuclear antigen.
mice were housed in our American Association of Laboratory Animal Care-accredited laboratory animal facility. Mice were housed in polycarbonate solid-bottom, shoebox-type cages (13 x 18 x 28 cm) with Andersons Bed-o-Cob 1/8 bedding (Andersons, Maumee, OH). The mice were quarantined for 2 weeks before the bioassay. The environment in the animal rooms was maintained at a temperature of 72 ± 2°F, relative humidity of 40–60%, at least 10–15 air changes per hour, approximately 30 foot-candles of light (cage level), and a light cycle of 12 h on/12 h off. The diet was a semipurified AIN-76A containing 20% casein, 0.3% d,L-methionine, 52% cornstarch, 13% dextrose, 5% corn oil, 5% alphacel fiber, 3.5% AIN mineral mixture, 1.0% AIN vitamin mixture, and 0.2% choline bitartrate (Dyets, Inc., Bethlehem, PA). The diet and drinking water were provided ad libitum.

**Experimental Design.** The experimental design is illustrated in Table 1. When the mice were 7–8 weeks old, they were given a single i.p. injection of 100 mg/kg B(a)P in 0.2 ml of corn oil to induce lung tumors. Starting at 4 or 14 weeks after administration of B(a)P, the mice were given 50 or 100 mg/kg R115777 by gavage in 0.2 ml of a 20% β-cyclodextrin solution. The R115777 was administered 5 days/week for the duration of the experiment. These dose levels of R115777 were chosen because they were previously reported to be effective in human tumor xenograft studies in nude mice (9).

Mice were weighed weekly for the first 8 weeks of the experiment, and then they were weighed every 2–4 weeks until sacrifice. Mice were sacrificed by carbon dioxide asphyxiation at 22 weeks after B(a)P administration. Thus, they were given R115777 for 8 or 18 weeks. The lungs were harvested, fixed overnight in formalin, transferred to 70% alcohol, and evaluated for tumors before embedding in paraffin for histology.

**Histopathological Evaluation.** Lung tumors were classified as being solid/alveolar adenomas, papillary adenomas, and undifferentiated carcinomas. The criteria used for solid/alveolar adenoma classification required well-differentiated cuboidal-shaped cells obliterating at least three contiguous alveolar spaces (11). Papillary tumors were identified as well differentiated columnar cells with pleomorphic nuclei expanding within the lung parenchyma as finger-like projections.

**PCNA Labeling Index.** To determine the PCNA labeling index, sections of adenomas were deparaffinized with xylene and placed in 2N HCl at 50°C for 30 min. Endogenous peroxidase was quenched with 3% hydrogen peroxide (Sigma) for 30 min. The sections were blocked with diluted horse serum (Vector Laboratories, Burlington, CA) for 30 min and incubated with 100 μl of monoclonal mouse anti-PCNA (dilution, 1:300; Sigma) at room temperature for 1 h. The sections were washed and incubated with biotinylated antimouse IgG (Vector Laboratories) for 30 min at room temperature, followed by incubation with Vectastain ABC KIT reagent (Vector Laboratories) for 30 min. Stain was developed with 3,3’-diaminobenzidine tetrahydrochloride for 15 min. The slides were counterstained with hematoxylin. Nuclei of PCNA-labeled cells stained brown, whereas unlabeled nuclei were blue.

The PCNA-stained sections were analyzed using NIH Image 1.57 software with threshold density filters to negate user bias. A x40 objective lens was used. The labeling index was determined by enumerating PCNA-positive cells and then evaluating the total number of tumor cells present within the high-power field, dividing the PCNA-labeled cells by the total cell count and multiplying by 100. The total number of cells evaluated per tumor ranged from 600 to 4800 cells, depending on the tumor size.

**Statistical Analysis.** Results are presented as means ± SE and were analyzed by an ANOVA followed by a Tukey test, with significance indicated by P < 0.05.

### RESULTS AND DISCUSSION

The FTI R115777 was evaluated for its efficacy in preventing B(a)P-induced lung tumors in mice. The mice showed no signs of morbidity or toxicity during the experiment; there was no loss of body weight, no sign of animal malaise, and no change in behavior or appearance. The 100 mg/kg dose of R115777 decreased the yield of lung tumors (Fig. 2). Tumor multiplicity was 5.0 ± 0.85, 4.5 ± 0.52, and 2.1 ± 0.31 tumors/mouse in mice that received 0, 50, and 100 mg/kg R115777, respectively. Thus, the high dose of R115777 reduced the yield of lung tumors by 58%, whereas the lower dose of 50 mg/kg did not significantly reduce the yield of tumors, causing only a 10% reduction. Histological classification of tumor morphology showed that the B(a)P-induced lung tumors were predominantly solid/alveolar neoplasms; only one tumor was found to have papillary morphology (data not shown). We have reported previously (12) that B(a)P induces more solid than papillary lung adenomas in strain A mice.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>N</th>
<th>Duration of treatment</th>
<th>Chemopreventive agent dose (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R115777</td>
<td>16</td>
<td>4–22</td>
<td>100</td>
</tr>
<tr>
<td>R115777</td>
<td>16</td>
<td>14–22</td>
<td>100</td>
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<tr>
<td>R115777</td>
<td>16</td>
<td>4–22</td>
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</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>16</td>
<td>4–22</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mice were given R115777 beginning 4 or 14 weeks after administration of B(a)P and sacrificed 22 weeks after B(a)P administration.

All mice received B(a)P in a β-cyclodextrin vehicle (0.2 ml) by i.p. injection at 7–8 weeks of age.

R115777 was administered by oral gavage times/week.
The PCNA labeling index showed that lung tumors from animals given 100 mg/kg FTI had significantly reduced numbers of proliferating cells (roughly 60%) when compared with tumors from the untreated control group (Fig. 3). Although 50 mg/kg R115777 did reduce the PCNA labeling index compared with the control group, it was not statistically significant. The development of lung tumors appears to be sensitive to chemoprevention long after the administration of the initiating carcinogen. R115777 decreased tumor multiplicity almost 65% when administered starting 10 weeks after B(a)P and for only 8 weeks (Fig. 4). This finding implies that most of the effect of R115777 is on later stages of tumor development in this adenoma model. If a significant portion of the effects of R115777 related to the earlier portions of the tumor process, then one would expect greater preventive activity when treatment with the agent was initiated at 4 weeks versus 14 weeks post-B(a)P. This finding that prevention can be initiated later during the prevention process is in agreement with a previous report using a peptidomimetic FTI, FTI-276 (13), and supports the rationale for proposed clinical trials with this agent in smokers with previously existing lesions. Budesonide has similarly been shown to be highly effective in preventing lung tumors when administered starting 10 weeks after vinyl carbamate (14). Thus, the promotional phase of lung carcinogenesis appears to be sensitive to chemoprevention. The decreased tumor multiplicity and decreased proliferation rates that we observed in R115777-treated tumors are consistent with the primary effects of the FTI being on the growth rate and progression of lung lesions.

The three types of RAS proteins are primarily farnesylated at the CAAX motif to be transported to the cell membrane. However, the amino acids adjacent to this motif can alter the affinity of the farnesyltransferase protein for the Ras protein. Because of the differing amino acid binding sequences, the farnesyltransferase enzyme has different affinities for different Ras proteins. The affinity is relatively low for Ha-Ras and N-Ras, meaning that these interactions can be readily competed off, whereas the affinity is much higher for Ki-Ras, meaning that it cannot easily be competed off. This correlates with known activities of the FTIs as a class and R115777 in particular. R115777 is profoundly effective against tumors with mutated Ha-Ras or N-Ras but variably effective against tumors with Ki-Ras (9), raising the question of whether Ras is the primary target of the FTIs in tumors with Ki-Ras mutations. In the case of tumors with Ha-Ras or N-Ras mutation, inhibition of the farnesyltransferase enzyme is proposed to prevent the binding of the Ras protein to the plasma membrane. This results in the down-regulation of downstream pathways including the mitogen-activated protein kinase pathway and the phosphatidylinositol 3'-kinase/AKT pathways, which in turn affect cell proliferation and apoptosis.
Virtually all lung tumors induced by B(a)P in strain A mice have previously been shown to have mutations in codon 12 of the Ki-Ras oncogene (15). In a previous study with another FTI, FTI-276, the percentage of tumors with Ki-Ras mutations was similarly high in tumors from control and FTI-276-treated mice (13). The question therefore arises whether FTIs, including FTI-276 and R115777, are working directly against mutated Ki-Ras or working against a number of other farnesylated proteins including Rho, nonmutated forms of Ha-Ras or N-Ras, and so forth. To test this hypothesis, we are presently testing R115777 in an A/J tumor model using azoxymethane, an agent that induces few, if any, lung tumors with Ki-Ras mutations.

The present study demonstrates the efficacy of the FTI R115777, an imidazole analogue that can be administered p.o. and is presently in multiple Phase II trials for cancer therapy. Furthermore, the present studies also examined both a true prevention model (R115777 treatment from weeks 4–22) and a mixed prevention/therapy model (R115777 treatment from weeks 14–22). The similar effects of both models imply that most or all of the effects of R115777 occur during the progression/promotion phase of tumor development. Furthermore, the most likely scenario for Phase II prevention trials would more closely parallel the prevention/therapy model using former or present smokers. These results support the potential use of R115777 in such prevention trials.

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REFERENCES

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