N-(4-Hydroxyphenyl)Retinamide in the Chemoprevention of Squamous Metaplasia and Dysplasia of the Bronchial Epithelium

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ABSTRACT

Lung cancer remains the number one cause of cancer-related deaths in the United States. To reduce the mortality associated with this disease, individuals at risk must be identified prior to the development of lung cancer, and effective prevention strategies must be developed. One such strategy is to use retinoids like N-(4-hydroxyphenyl)retinamide (4-HPR), which has been found to possess chemopreventive activities in preclinical studies. In this study, 139 smokers were registered and 82 were randomized onto a double-blinded, placebo-controlled chemoprevention trial of 4-HPR administered p.o. (200 mg once daily). Of these, 70 participants were eligible for response evaluation. Biopsies were obtained at six predetermined sites in the bronchial tree from participants before and at the completion of 6 months of treatment. 4-HPR treatment had no measurable effect on histopathology (squamous metaplasia and dysplasia) in the bronchial epithelium of current smokers. 4-HPR was detected (104.5 ± 64.0 ng/ml, mean ± SD) in the serum of participants, supporting its potential bioavailability. Serum retinol levels decreased markedly (44% of placebo-treated patients) as a consequence of 4-HPR treatment. Notably, the mRNA level of retinoic acid receptor β, which is typically increased by retinoid treatment, did not change in the bronchial epithelium of 4-HPR-treated participants. Clonal populations of bronchial epithelial cells were detected by analysis of loss of heterozygosity at putative tumor suppressor loci on chromosomes 3p, 9p, and 17p, and these changes were not altered by 4-HPR treatment. In conclusion, at this dose and schedule, 4-HPR was not effective in reversing squamous metaplasia, dysplasia, or genetic and phenotypic abnormalities in the bronchial epithelium of smokers.

INTRODUCTION

Large areas of bronchial epithelium seem to undergo histological changes as a result of carcinogen exposure, suggesting a “field cancerization” phenomenon with multifocal primary lesions (1). Thus, efforts to control the primary cancer must also include treatment of surrounding bronchial epithelia. Several investigators have shown that retinoids are potent inhibitors of field carcinogenesis and can reverse aerodigestive tract carcinogenesis (2–4). Although these studies demonstrate the potential of retinoids in cancer “chemoprevention,” the beneficial effects of retinoids have been limited by treatment-related toxicities that define the duration of treatment and reduce the compliance of patients. Clearly, retinoids with a more favorable therapeutic index are needed to enhance the activity of retinoids in cancer chemoprevention.

Efforts to identify retinoids that are more effective in lung cancer chemoprevention have been hampered by the lack of an appropriate “intermediate end point” to demonstrate a reduction in lung cancer risk. Lung carcinogenesis is a multistep process that involves the progression of a normal bronchial epithelial cell through a sequence of histopathological entities, culminating in a fully transformed, invasive cancer. Whereas the sequence of events has not been completely defined, the classical progression includes normal, hyperplasia, squamous metaplasia, dysplasia, carcinoma in situ, and invasive cancer. We previously used this model in the design of a randomized, placebo-controlled chemoprevention trial, testing the efficacy of 13cRA3 in the reversal of squamous metaplasia and dysplasia (5). Current smokers underwent bronchoscopic examination with biopsies, and individuals with detectable squamous metaplasia and/or dysplasia were randomized to 6 months of treatment with 4-HPR.

3 The abbreviations used are: 13cRA, 13-cis-retinoic acid; RARE, RA response element; RAR, RA receptor; 4-HPR, N-(4-hydroxyphenyl)retinamide; MI, metaplasia index; 4-MPR, 4-methoxyphenylretinamide; LOH, loss of heterozygosity; GEE, generalized estimating equation.
13cRA or placebo and evaluated for response by repeat bronchoscopy with biopsies. We found that 13cRA did not reduce the incidence or severity of squamous metaplasia or dysplasia in active smokers (5). Whereas there was no effect on bronchial histopathology, we found evidence that 13cRA activated retinoid signaling pathways in the bronchial epithelium (6). Retinoid signaling is activated by binding of retinoids to retinoid nuclear receptors, which are a family of transcription factors that bind to specific DNA sequences termed RAREs (7). The RAR-β gene contains a RARE in its gene promoter region that functions as a positive regulatory element. Presumably, 13cRA increased the expression of the RAR-β gene in the bronchial epithelium through this mechanism (6).

The inactivity of 13cRA in the reversal of squamous metaplasia and dysplasia in active smokers raises the possibility that premalignant bronchial foci are resistant to retinoid treatment. A growing body of laboratory studies supports the theory that, during the process of malignant transformation, human bronchial epithelial cells become resistant to the growth inhibitory effects of retinoids (8, 9). Contributing to the development of retinoid refractoriness, retinoid nuclear receptors are dysfunctional in a proportion of non-small cell lung carcinomas, exhibiting reduced transcriptional activation in response to retinoid binding (10). Because this retinoid signaling abnormality could limit the lung cancer chemopreventive effects of conventional retinoids, alternative approaches should be considered. Toward this aim, a variety of synthetic retinoids have been developed that function through novel mechanisms (11). One of these retinoids is 4-HPR, which has demonstrated chemopreventive activity in animal models of mammary, prostate, lung, and bladder cancer (11). In clinical trials, 4-HPR demonstrated activity in the reversal of premalignant oral leukoplasia and preventive effects against ovarian cancer and, among premenopausal breast cancer patients, contralateral breast cancer (12). In tissue culture, 4-HPR induces apoptosis in a variety of lung cancer cell lines through both retinoid receptor-dependent and -independent mechanisms (13–15).

In this study, we tested the hypothesis that squamous metaplasia and dysplasia revert to normal bronchial epithelium in 4-HPR-treated participants through stimulation of retinoid nuclear receptor-dependent signaling pathways, as shown by an increase in RAR-β gene expression. We examined the activity of 4-HPR in the chemoprevention of bronchial dysplasia and squamous metaplasia in current and former smokers. Following screening by bronchoscopic examination with biopsies, individuals with detectable squamous metaplasia and/or dysplasia were randomized to 6 months of treatment with 4-HPR or placebo. Response to treatment was determined by repeat bronchoscopy with biopsies. We found that 4-HPR had no detectable effect on squamous metaplasia or dysplasia under these conditions. Potential explanations for its inactivity are discussed.

MATERIALS AND METHODS

Clinical Trial Design. This study was conducted among chronic and former smokers who had a minimum smoking history of 20 pack-years. Participants must have had: (a) adequate renal, hematological, and hepatic function; (b) not taken >25,000 IU of vitamin A or other retinoids per day within 3 months of study entry; and (c) detectable bronchial squamous metaplasia and/or dysplasia at the time of screening bronchoscopy. Participants may have had a prior tobacco-related cancer but must have been tumor free for 6 months before participation in the study. 4-HPR was supplied by R.W. Johnson Pharmaceutical Research Institute.

All eligible participants underwent a screening bronchoscopy with biopsies at six predetermined sites in the bronchial tree, including the main carina, the bifurcation of the right upper lobe and mainstem bronchus, the bifurcation of the right middle lobe and right lower lobe, the bifurcation of the left upper lobe and the lingula, the medial bronchus of the right lower lobe, and the anterior bronchus of the left lower lobe. The biopsies were routinely processed and stained with H&E, and 10 sections were evaluated per site for the presence of dysplasia and squamous metaplasia by referee pathologists (J. Y. R. and B. L. K.) blinded to the timing of the specimens and the treatment group. MI was calculated as described previously (5) by dividing the number of biopsy sections exhibiting squamous metaplasia by the total number of sections examined, and multiplying the result by 100. Participants found to have evidence of dysplasia or a MI value of >15% were randomly assigned to receive 4-HPR (200 mg p.o. per day) or placebo for 6 months, with a monthly 3-day drug holiday to prevent 4-HPR-related ocular toxicity. Participants were seen at monthly intervals and evaluated for compliance, drug-related toxicity, and serum drug levels. Toxicity was graded, as described previously, using National Cancer Institute Common Toxicity Criteria (5). At the end of 6 months, bronchoscopic examination was repeated with biopsies at the same sites to evaluate the bronchial tree for evidence of change.

Analysis of Serum Retinol and Drug Levels. Levels of 4-HPR and its major metabolite 4-MPR were measured in serum samples obtained at baseline, 2, and 6 months of treatment that had been frozen at −70°C and protected from exposure to light by performing high-performance liquid chromatography using 4-ethoxyphenylretinamide as an internal standard (16). The chromatographic separation was performed on a Vydac 201TP column (0.46 × 25 cm). The isocratic mobile phase was 55% acetonitrile, 10% n-butyl alcohol, 35% water, and 0.01 mol/liter ammonium acetate. The detector was programmed at 364 nm for the first 12 min, 325 nm for the next 3 min, and 364 nm for the last 8 min to correspond to the elution times of 4-HPR, 4-MPR, and 4-ethoxyphenylretinamide. Serum retinol levels were measured in serum samples taken from patients at the same time points by high-performance liquid chromatography analysis performed at Smith Kline Beecham Laboratories, as described previously (17).

In Situ mRNA Hybridization Studies. Nonradioactive in situ hybridization of RAR-β mRNA was performed on formalin-fixed, paraffin-embedded sections as described previously (18). The binding specificity of the antisense riboprobes was verified using sense probes as controls. Staining was scored as either detectable or undetectable. RNA quality was verified by in situ hybridization of retinoid X receptor-α mRNA, which is expressed constitutively in bronchial epithelium (6).

Microsatellite Analysis. For microdissection, four 4-μm thick tissue sections were mounted on glass slides and stained with H&E. The epithelial part of each biopsy was microdis-
containing 3% DMSO, 200 ng DNA polynucleotide kinase (New England Biolabs, Beverly, MA) and T4 polynucleotide kinase (ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). PCR reactions were carried out in a 12.5-μl volume containing 3% DMSO, 200 μM dNTP, 1.5 mm MgCl2, 0.4 μM PCR primers including 0.1 μM [γ-32P]ATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) and T4 DNA polynucleotide kinase (New England Biolabs, Beverly, MA). PCR products were separated on a 6% polyacrylamide-urea-formamide gel and then exposed to film. LOH was defined as a reduction of the intensity by visual inspection in one of the two products.

RESULTS

One hundred thirty-nine participants were registered into the clinical trial. Of these, 82 participants were eligible and randomized. Inadequate MI was the most frequent justification for ineligibility. Treatment groups (4-HPR and placebo) were well matched for age, gender, squamous metaplasia, dysplasia, and smoking status (Table 1). Seventy participants were evaluable for response, having completed at least 3 months of treatment, followed by a second bronchoscopic examination with biopsies. The most frequent causes of early treatment withdrawal were non-drug related (i.e., conflicts with work schedule, moving away). Of the 70 evaluable participants, 62 had detectable squamous metaplasia but no dysplasia, 1 had dysplasia but no squamous metaplasia, and 7 had both squamous metaplasia and dysplasia (Table 3). No evidence of ocular toxicities (i.e., night blindness) was observed in participants who took 4-HPR in this trial.

Treatment was well-tolerated; treatment-related toxicities were grade 1 only, and there was no attrition of participants from the study due to toxicity. The most frequently observed toxicities included skin reaction, cheilitis, conjunctivitis, and headaches (Table 2). No evidence of ocular toxicities (i.e., night blindness) was observed in participants who took 4-HPR in this trial.

Overall, 4-HPR had no detectable effect on squamous metaplasia or dysplasia. This was true when the unit of analysis was participant (Table 3) or biopsy site (Table 4). Table 3 shows that the mean MI was reduced over time by 7.1% and 4.9% in the 4-HPR and placebo groups, respectively. The difference in MI reduction between the 4-HPR and placebo groups was not statistically significant \( P = 0.79 \) by two-sided Wilcoxon's rank-sum test.
metaplasia was only marginally significant \( (P = 0.08\) by McNemar test). On the other hand, there was no indication of reduction in metaplasia in the placebo group. However, the change of metaplasia status was not significantly different between the 4-HPR and placebo groups \( (P = 0.57\) by GEE method).

Only 3.0% and 2.4% of the sites showed dysplasia in the baseline biopsy for the 4-HPR and placebo group, respectively (Table 4). No change of dysplasia status from baseline to 6 months was found in either the 4-HPR or placebo group. The difference between the two treatment groups was not statistically different \( (P = 0.83\) by GEE method).

*Fig. 1* Scatter plot of MI at 6 months versus baseline by smoking status. A, smoking at baseline, placebo group. B, smoking at baseline, 4-HPR group. C, nonsmoking at baseline, placebo group. D, nonsmoking at baseline, 4-HPR group. ● and ●, smoking at 6 months; ○ and ○, nonsmoking at 6 months.

<table>
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<th>End point</th>
<th>Treatment</th>
<th>No. of biopsies (baseline/6 months)</th>
<th>Time</th>
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<td>(-/-)</td>
<td>(-/+)</td>
<td>(+/-)</td>
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<td>35</td>
<td>51</td>
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<tr>
<td></td>
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<tr>
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<td>6</td>
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<tr>
<td></td>
<td>Placebo(^b)</td>
<td>199</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) The change of metaplasia status was not significantly different between treatment groups \( (P = 0.57\), GEE method).

\(^b\) The change of dysplasia status was not significantly different between treatment groups \( (P = 0.83\), GEE method).
measurable activity in the chemoprevention of bronchial dysplasia and squamous metaplasia in current or former smokers. The incidence of dysplasia in this population was low, relative to that reported in other chemoprevention trials (23, 24), but it was similar to what we reported in our previous trial (5). Because of the low incidence of dysplasia in this study, there was not sufficient statistical power to evaluate the efficacy of 4-HPR in the reversal of bronchial dysplasia. Bronchial squamous metaplasia is a common histological abnormality in current smokers, which, in some cases, reverts to normal histology on smoking cessation (5). Lesions that revert most likely represent an acute reaction to the presence of cigarette smoke and are not stable premalignant foci. However, squamous metaplasia has been detected in former smokers who have quit for more than 1 year (19, 25), raising the possibility that certain squamous metaplastic lesions are genuine changes in the bronchial epithelium. Supporting this theory, genetic abnormalities detected in bronchial dysplasia and carcinoma in situ, such as LOH at chromosome 3p14, are more frequent in bronchial fields with high MI than in fields with low MI (19). Overall, the incidence of LOH at chromosomes 3p, 9p, and 17p in this study was similar to that previously reported (19) Furthermore, areas of squamous metaplasia are hyperproliferative and express increased levels of epidermal growth factor receptor (26), demonstrating biological abnormalities that distinguish them from normal bronchial mucosa.

The mechanism by which 4-HPR mediates its chemopreventive effect has been investigated extensively. In tissue culture, 4-HPR treatment of cancer cells activates transcription of RAREs through retinoid nuclear receptor-dependent pathways. Supporting this hypothesis, the modulation of RAR-β expression by biopsy site and participant is shown in Table 5. In this randomized, placebo-controlled trial, 4-HPR had no measurable activity in the chemoprevention of bronchial dysplasia and squamous metaplasia in current or former smokers. The incidence of dysplasia in this population was low, relative to that reported in other chemoprevention trials (23, 24), but it was similar to what we reported in our previous trial (5). Because of the low incidence of dysplasia in this study, there was not sufficient statistical power to evaluate the efficacy of 4-HPR in the reversal of bronchial dysplasia. Bronchial squamous metaplasia is a common histological abnormality in current smokers, which, in some cases, reverts to normal histology on smoking cessation (5). Lesions that revert most likely represent an acute reaction to the presence of cigarette smoke and are not stable premalignant foci. However, squamous metaplasia has been detected in former smokers who have quit for more than 1 year (19, 25), raising the possibility that certain squamous metaplastic lesions are genuine changes in the bronchial epithelium. Supporting this theory, genetic abnormalities detected in bronchial dysplasia and carcinoma in situ, such as LOH at chromosome 3p14, are more frequent in bronchial fields with high MI than in fields with low MI (19). Overall, the incidence of LOH at chromosomes 3p, 9p, and 17p in this study was similar to that previously reported (19). Furthermore, areas of squamous metaplasia are hyperproliferative and express increased levels of epidermal growth factor receptor (26), demonstrating biological abnormalities that distinguish them from normal bronchial mucosa.

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One possible explanation for the apparent inactivity of 4-HPR on the bronchial epithelium is that 4-HPR drug levels were either undetectable or insufficient to be biologically active. Serum levels of 4-HPR and its major metabolite 4-MPR were 104.5 ± 64.0 ng/ml and 201.6 ± 136.6 ng/ml, respectively. As an additional marker of 4-HPR biological activity, we examined serum retinol levels, which are reduced by 4-HPR treatment (22). Serum retinol levels were drawn at baseline, 2 months, and 6 months. Nadir retinol levels were lower after the 4-HPR treatment (P = 0.0001) but remained at a similar level in the placebo group (Table 8). The mean nadir retinol level in the 4-HPR group was only 44% of the mean level in the placebo group. These findings suggest that, in this study, 4-HPR was biologically active from the standpoint of its effect on retinoid metabolism, inducing a reduction in serum retinol, but had no effects on squamous metaplasia or dysplasia.

**DISCUSSION**

In this randomized, placebo-controlled trial, 4-HPR had no measurable activity in the chemoprevention of bronchial dysplasia and squamous metaplasia in current or former smokers. The incidence of dysplasia in this population was low, relative to that reported in other chemoprevention trials (23, 24), but it was similar to what we reported in our previous trial (5). Because of the low incidence of dysplasia in this study, there was not sufficient statistical power to evaluate the efficacy of 4-HPR in the reversal of bronchial dysplasia. Bronchial squamous metaplasia is a common histological abnormality in current smokers, which, in some cases, reverts to normal histology on smoking cessation (5). Lesions that revert most likely represent an acute reaction to the presence of cigarette smoke and are not stable premalignant foci. However, squamous metaplasia has been detected in former smokers who have quit for more than 1 year (19, 25), raising the possibility that certain squamous metaplastic lesions are genuine changes in the bronchial epithelium. Supporting this theory, genetic abnormalities detected in bronchial dysplasia and carcinoma in situ, such as LOH at chromosome 3p14, are more frequent in bronchial fields with high MI than in fields with low MI (19). Overall, the incidence of LOH at chromosomes 3p, 9p, and 17p in this study was similar to that previously reported (19). Furthermore, areas of squamous metaplasia are hyperproliferative and express increased levels of epidermal growth factor receptor (26), demonstrating biological abnormalities that distinguish them from normal bronchial mucosa.

The mechanism by which 4-HPR mediates its chemopreventive effect has been investigated extensively. In tissue culture, 4-HPR treatment of cancer cells activates transcription of RAREs through retinoid nuclear receptor-dependent pathways and increases the expression of RAR-β (27, 28). However, we
found no effect of 4-HPR on RAR-β mRNA levels in the bronchial epithelium. This stands in contrast to the effect of 13cRA on RAR-β mRNA in the bronchial epithelium observed previously (6). Potential explanations for this finding include: (a) activation of retinoid nuclear receptor-dependent pathways was not a prominent effect of 4-HPR in these patients; or (b) the 4-HPR levels were not sufficient enough to activate this pathway. Retinoid nuclear receptor-dependent pathways are not necessary for some of the biological effects of 4-HPR. For example, retinoid receptor transcriptional activation is not necessary for the apoptotic effects of 4-HPR on cancer cells (29, 30). Although the pathways that mediate the apoptotic effects of 4-HPR have not been defined, it has been shown that induction of apoptosis by 4-HPR occurs coincidently with the production of reactive oxygen species and apoptosis is blocked by pyrrolidine dithiocarbamate, an oxygen radical scavenger (31).

The inactivity of 4-HPR on pathological changes in the bronchial mucosa raises the possibility that serum 4-HPR levels in participants in this study were not high enough to achieve a biological effect. The apoptotic effects of 4-HPR in tissue culture typically require 4-HPR concentrations of >1 μM (14, 32). The mean serum level of 4-HPR observed in this clinical trial was 104.5 ng/mL (0.26 μM). Higher serum levels might be achieved by increasing the dose of 4-HPR. The tolerability of higher dose is not known and awaits the results of ongoing Phase I studies. Previously, the dose-limiting toxicities of 4-HPR were visual and ophthalmological toxicities, which occur in 20% and 8%, respectively, of patients at 5 years (33, 34). These toxicities are hypothesized to occur because of a reduction in serum retinol levels (22). Similar to previous studies, we found that serum retinol levels are reduced in 4-HPR-treated individuals. 4-HPR binds to retinol-binding protein in the liver and thereby competes with retinol, decreasing the affinity of retinol-binding protein for transthyretin, reducing serum retinol and thereby competes with retinol, decreasing the affinity of retinol-binding protein for transthyretin, reducing serum retinol levels (35, 36). Retinol is necessary to maintain the normal growth and differentiation of the bronchial epithelium (37); therefore, loss of serum retinol may counterbalance the chemopreventive effects of 4-HPR. However, we have not observed any clinical signs of vitamin A deficiency in 4-HPR-treated participants.

Several clinical trials examining the effectiveness of conventional retinoids in the chemoprevention of lung cancer have been completed. Similar to the outcome of this trial, 13cRA demonstrated no activity in the reversal of bronchial squamous metaplasia and dysplasia in current smokers (5). Two large, randomized trials that were recently completed (Alpha-Tocopherol Beta Carotene and Carotene and Retinol Efficacy Trial) demonstrated no efficacy of β-carotene plus retinol (38) or β-carotene alone (39) in the chemoprevention of lung cancer in current smokers. Another trial that tests the efficacy of 13cRA in the chemoprevention of second primary tumors in patients with resected lung cancers has reached its targeted accrual and is nearing completion. If this trial has a similar outcome, there will be clear evidence that conventional retinoids are not effective in the chemoprevention of lung cancer in smokers.

Whereas conventional retinoids have demonstrated minimal activity as lung cancer chemopreventive agents, the activity of 4-HPR and other novel retinoids should be further explored. Combinations of 4-HPR with retinol should be investigated to examine whether the chemopreventive effects of 4-HPR can be enhanced by restoring normal retinol levels. Phase I studies with high-dose 4-HPR are ongoing, which will determine whether 4-HPR can be tolerated at doses that reach serum concentrations of >1 μM. Long-term administration may be required to attain a chemopreventive effect; five years of administration at the same dose used in this study revealed chemopreventive activity against breast and ovarian cancer (12, 40). Once the pathways that mediate the apoptotic effects of 4-HPR are understood, biomarkers of 4-HPR activity might be developed for clinical trials to study its activity in tissues-at-risk (41), and strategies might be developed to target specific tumor types that are susceptible to 4-HPR actions.

**REFERENCES**


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