Chemoprevention of Lung Carcinogenesis: Aerosol Administration and Deposition in the Mouse Lung

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Chemoprevention of Lung Carciogensis: Aerosol Administration and Deposition in the Mouse Lung

By

Jingjie Zhang

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2013
Saint Louis, Missouri
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Jingjie Zhang

Washington University in St. Louis

March 2013
Dedicated to

My Parents, Jinsheng Zhang and Gaiqin Liu
INTRODUCTION Chemoprevention is the use of natural or synthetic agents to inhibit either the initial development or the further progression of early lesions. Chemoprevention of lung cancer aims to decrease lung cancer morbidity and mortality, especially for former smokers. Many synthetic and natural compounds have been investigated for their potential chemopreventive efficacy. Conventional administration of these compounds (especially oral administration) is convenient, but may potentially result in adverse side effects. Aerosol delivery, on the other hand, offers many advantages over conventional routes of administration for diseases of the respiratory...
tract and the lungs. These advantages include the extensive pulmonary surface area available for drug deposition, the avoidance of first-pass metabolic degradation by the liver and/or intestines, the noninvasive nature of administration, and the localized effect of low doses of drugs, all of which lead to a rapid response with fewer systemic side effects. The efficacy of a given drug via aerosol administration depends on many factors such as particle size distribution, total particle mass concentration, and the physiochemical properties of drug particles. Genetically-engineered mice play an important role in drug screening and preclinical studies. However, because mice have much smaller lungs than those of human beings, the lung deposition data attained for humans cannot be applied to the mouse. Hence few studies have focused on particle deposition in the mouse lung. It is thus necessary to measure the mass deposition of particles in the mouse lung.

**METHOD** A spray-drying process was used to study the inhibitory effects of potential chemopreventive agents on carcinogen-induced tumors in the A/J mouse. The carcinogen in the studies was benzo[a]pyrene, unless otherwise specified. Synthetic and natural compounds were investigated individually or in combination. The compounds were aerosolized with a custom-built Collison atomizer. The resultant drug aerosols were delivered to the mice that were retained in a nose-only exposure chamber. Four small molecular inhibitors (gefitinib, erlotinib, lapatinib and wortmannin), and four natural agents (resveratrol, caffeine, anthocyanins, and protocatechuic acid), were considered as examples of single agents. Gefitinib and erlotinib were delivered in an aerosol form to reduce the cutaneous side effects. Lapatinib and wortmannin were each administered both via aerosol and oral gavage to compare the efficacy and toxicity as they were administered via different routes. Resveratrol was evaluated in two models
with either vinyl carbamate or benzo[a]pyrene as the carcinogen. It was delivered via aerosol to avoid fast clearance in the blood before it reached the lung. Caffeine was delivered in aerosol to assess only its inhibitory effects and to avoid its negative effects on body weight. Anthocyanins were delivered via aerosol due to their poor bioavailability. Protocatechuic acid is a metabolite of anthocyanins and was also delivered via aerosol for comparison with anthocyanins. The combinations of aerosolized budesonide (a synthetic glucocorticoid) and dietary polyphenon E (a well-defined mixture of green tea extract) was discussed as one example of the combinational treatment.

In addition to the bioassays, drug deposition in the mouse lung was evaluated for both polydispersed and monodispersed drug particles for a better understanding of the delivery process and for future applications. Gefitinib was selected as the model agent. Polydispersed gefitinib particles were generated with the Collison atomizer used in the animal studies. Monodispersed particles were generated using the single-capillary electrospray technique. Lung and blood samples were harvested immediately after the aerosol treatment. The lung and plasma levels of gefitinib were measured with varied solution concentrations, exposure durations, and particle sizes. The aerial mass concentration in the chamber was also measured to estimate the doses.

**RESULTS** Aerosolized erlotinib (5 mg/ml) did not inhibit tumor multiplicity but reduced tumor load by 63.8% \((P < 0.05)\). Aerosolized gefitinib in three separate doses (5, 10, and 15 mg/ml) inhibited tumor multiplicity by ~30% for all three doses when the tumors were induced by one dose of benzo[a]pyrene (100 mg/kg body weight), but the results were not statistically significant. Aerosolized gefitinib showed consistent inhibitory effects on tumor load, and the inhibition rate increased as the dose increased.
The tumor load was reduced by 39.0%, 46.2%, and 56.4% ($P < 0.05$) for 5, 10, and 15 mg/ml gefitinib solutions, respectively. The highest dose (15 mg/ml) of gefitinib was repeated in mice whose tumors were induced by two doses of benzo[a]pyrene (100 mg/kg body weight, one week apart) and it inhibited both tumor multiplicity (by 49.8%, $P < 0.001$) and tumor load (by 57.0%, $P < 0.001$). No visible skin alteration was observed in mice treated with aerosolized gefitinib or erlotinib. Both aerosolized lapatinib (50 mg/ml) and orally-administered lapatinib (100 mg/kg body weight) showed inhibitory effects. Aerosolized lapatinib reduced tumor multiplicity by 39.6% ($P < 0.05$) and tumor load by 41.7% ($P < 0.05$). Orally-dosed lapatinib reduced tumor multiplicity by 37.6% (not significant) and tumor load by 42.4% ($P < 0.05$). At the current doses of lapatinib, no adverse side effect was observed in either the aerosol group or the orally-dosed group. Wortmannin showed striking inhibitory effects via aerosol inhalation and per os. Oral wortmannin (1.0 mg/kg body weight) inhibited tumor multiplicity by 85.5% ($P < 0.001$) and tumor load by 77.9% ($P < 0.05$). In the same model, aerosolized wortmannin (2.0 mg/ml) inhibited tumor multiplicity by 50.8% ($P < 0.05$) and tumor load by 79.7% ($P < 0.05$). Despite the efficacy of oral wortmannin, the accompanying systemic adverse effects were not negligible. Reduced body weight and death were observed in the orally-dosed mice, but not in the aerosol treated mice. Thus, aerosolized wortmannin was evaluated a second time in the bioassay with two doses of benzo[a]pyrene, and it was found to reduce tumor multiplicity and tumor load by 66.7% ($P < 0.001$) and 80.4% ($P < 0.0001$), respectively, with a slight decrease in body weight. Resveratrol inhibited the proliferation of cells in the human lung cancer cell lines A549 and H1129, which indicates that resveratrol could possibly be an effective inhibitor of human lung cancer. Aerosolized resveratrol was shown to inhibit
the tumor load in both vinyl carbamate- and benzo[a]pyrene-induced models. The decrease in tumor load was 26.3% \((P < 0.05)\) and 36.0% \((P < 0.01)\) for 7.5 and 15 mg/ml solutions, respectively, in the vinyl carbamate-induced model. In the benzo[a]pyrene-induced model, aerosolized resveratrol (15 mg/ml) significantly reduced tumor multiplicity by 37.1% \((P < 0.05)\) and tumor load by 72.0% \((P < 0.01)\). Pharmacokinetic studies showed that more resveratrol was delivered to the lung by aerosol inhalation than by oral gavage. Aerosolized caffeine (10 mg/ml) inhibited tumor multiplicity by 31.9% \((P < 0.05)\) and tumor load by 44.3% \((P < 0.05)\) without causing a reduction in the body weight gain, in contrast to the orally-administered caffeine, which did cause body weight loss. Aerosolized protocatechuic acid (12 mg/ml) reduced tumor multiplicity by 47.8% \((P < 0.05)\) and tumor load by 44.9% \((P < 0.05)\). However, the inhibitory effects of anthocyanins (5 mg/ml, extracted from black raspberries) were marginal (14.5% on the tumor multiplicity and 30.4% on the tumor load, not significant).

The particle deposition in the mouse lung was estimated using gefitinib as the model compound. For the Collison atomizer, the aerosol mass concentration in the exposure chamber increased linearly from 12.3 to 179.8 \(\mu\)g/L as the solution concentration increased from 1 to 50 mg/ml. The lung and plasma levels of gefitinib increased monotonically with increased solution concentration and exposure time, and the concentration in the lung was much higher than that in the plasma. The deposition efficiency is defined as the ratio of the mass deposited in the lung to the dose, and it is a function of particle size. In general, monodispersed particles have a higher delivery efficiency than polydisperse particles. For polydisperse particles, the 2.5 mg/ml solution (with a mass mean aerodynamic diameter, MMAD, at 120 nm) had the highest...
efficiency. For monodispersed particles, 100 nm particles showed the highest deposition efficiency.

CONCLUSIONS Aerosol delivery is a promising approach for the chemoprevention of lung cancer. Many natural and synthetic compounds showed inhibitory effects on benzo[a]pyrene-induced lung tumorigenesis in A/J when they are delivered via aerosol inhalation. In contrast to oral administration, aerosol delivery of the agents mitigated systemic toxicities with comparable inhibitory effects and improved the efficacy of some agents by increasing their bioavailability in the lung. The current aerosol delivery system was characterized and the mass deposition in the mouse lung was positively correlated with both the solution concentration and the exposure time. Aerosols with an MMAD around 100 nm may have the highest delivery efficiency, for both polydispersed and monodispersed distributions.
Chapter 1

Introduction and Overview
Chapter 1 Introduction and Overview

1.1 Introduction

In 2012, lung cancer remained the leading cause of cancer death in the United States, accounting for 29% of cancer deaths in men and 26% in women (American Cancer Society, 2012). Based on its histopathological features, lung cancer is grouped into small cell lung cancer (SCLC; ~20%) and non-SCLC (NSCLC; ~80%). NSCLC is further divided into three subtypes: squamous cell carcinoma (~30%), adenocarcinoma (~50%), and large cell lung carcinoma (Wang et al., 2012). The 5-year survival rate for all stages combined is only 16%, despite improvements in surgical techniques and combined therapies (American Cancer Society, 2012). So far, an early detection method has not been perfected. Consequently, lung cancer is difficult to detect at an early stage and hard to cure at a developed stage. Unfortunately, there is a vast population at high risk of contracting this malignant disease, especially smokers and ex-smokers (Doll and Hill 1954; Herzog et al., 1997; Witschi et al., 1997a; Wingo et al., 1999). Therefore, it is imperative to prevent lung carcinogenesis. Although smoking cessation remains essential for lung cancer prevention, additional preventive and control strategies are still necessary to implement the intervention more completely, particularly for individuals already at risk due to past exposure.

Chemoprevention is the use of natural or synthetic agents to inhibit the initial development or further progression of early lung lesions (Hong and Sporn, 1997). This concept has been applied to many types of cancers including, but not limited to, breast, colorectal, esophageal, skin, and lung cancer. In the case of lung cancer, it is more
effective to reduce the risk of developing lung cancer than to treat established or advanced disease (Wattenberg, 1985; Yang et al., 2002; William et al., 2009). Aside from the enormous benefit of preventing the malignant disease, there are three other reasons that lung cancer is an attractive target for chemopreventive strategies (Cohen and Khuri, 2002). First, lung cancer has a long latency period, about 20-30 years, between regular smoking exposure and the onset of the disease, which provides a long period of opportunity to initiate effective chemoprevention strategies to reverse premalignant lesions or even delay progression sufficiently to improve survival. Second, there are identifiable precursor lesions that provide a readily identifiable clinical endpoint to judge the effectiveness of chemoprevention. Last, but equally as important, there are numerous biomarkers available as potential intermediate endpoints for chemoprevention studies (Cohen and Khuri, 2002). Therefore, lung cancer is a promising target, and preventive intervention on carcinogenesis should be more effective than cancer treatment, considering the natural progression of lung cancer (Hecht et al., 2009). People at high risk of contracting lung cancer, mainly addicted smokers and ex-smokers, are the subjects for chemoprevention, as 90% of lung carcinogenesis is due to tobacco smoke exposure (Hecht et al., 2009). In the past 30 years, many advances have been made in studies on the chemoprevention of lung carcinogenesis, which increases the feasibility that chemoprevention will be an effective approach for the control of human lung cancer (Hong and Sporn, 1997; Cohen and Khuri, 2002; Winterhalder et al., 2004; Vignot et al., 2005; Gray et al., 2007; Blesa et al., 2008; Hecht et al., 2009). This progress includes understanding the mechanisms of carcinogenesis and the inhibitory effects of the drugs at the molecular level, developing suitable animal models such as transgenic mice, searching and screening for potentially
Chapter 1 Introduction and Overview

effective agents, developing biomarkers, and designing new drug delivery systems.

In current studies of lung cancer prevention in animal models, drugs are usually administered through an oral route (for long term treatment) or an intraperitoneal (i.p.) injection, which is mostly for short term observation on xenografts in animals but is not suitable for lung cancer chemoprevention in humans. Targeted delivery of preventive agents at the site of action is desired to maximize their inhibitory effects on carcinogenesis and to avoid the exposure of other healthy organs and surrounding cells to the agents, especially those agents with poor metabolic profiles and systemic toxicities. The conventional oral route might not be effective for the chemoprevention of lung cancer. Instead, the administration of drugs directly to the lungs via inhalation offers several advantages over oral administration. These include the extensive pulmonary surface area available for drug contact, the avoidance of first-pass metabolic degradation by the liver and/or intestines, and the noninvasive nature of administration. Inhalation also enables locoregional drug delivery of low doses of an aerosolized drug to its site of action for a localized effect, which leads to a rapid response with fewer systemic side effects (Hershey et al., 1999; Dolovich and Dhand, 2011; Zarogoulidis et al., 2012). Aerosol chemotherapy for lung cancer has been investigated in vitro, in animal models, and in human trials (Zarogoulidis et al., 2012). However, limited studies exist documenting the feasibility of delivering agents by inhalation for lung cancer chemoprevention. Our group evaluated the inhaled administration of polyphenon E (Fu et al., 2009), budesonide (Fu et al., 2011), and bexarotene (targretin) (Zhang et al., 2011) in a mouse lung cancer model and achieved significant inhibitory effects on lung tumorigenesis. We also reported higher concentrations of drugs in the lung and mitigated side effects regarding bexarotene when compared with oral administration.
(Zhang et al., 2011). In this dissertation, the studies of the chemoprevention of lung carcinogenesis are extended to aerosol administration of small molecular inhibitors and more natural compounds to prevent primary lung tumors induced by the tobacco-derived carcinogen, benzo[a]pyrene, in the mouse model.

Furthermore, studies on single agents offer limited insight into the chemoprevention of lung carcinogenesis. Tobacco smoke contains multiple lung carcinogens, toxicants, co-carcinogens, tumor promoters, and inflammatory compounds (Hecht et al., 2009), which play different roles in the initiation, promotion, and progression of lung cancer. Moreover, lung carcinogenesis is a complex mix of miscellaneous mutations and lesions at different stages of development (Yamaguchi and Perkins, 2012). Therefore, targeting one molecular pathway or step to inhibit carcinogenesis is difficult and may not always be possible. Accordingly, we reason that a logical combination of multiple agents that target different pathways can enhance the inhibition of lung tumorigenesis and reduce adverse side effects. To demonstrate this concept, I tested several combinational groups of agents and will discuss one of them in detail in Chapter 6. The combination of aerosolized budesonide, a synthetic glucocorticoid for anti-inflammation, and dietary polyphenon E, a well-defined natural mixture extracted from green tea, was investigated for their chemopreventive efficacy on lung carcinogenesis.

Besides lung cancer prevention, pulmonary delivery of drug particles is also preferred for other diseases in the respiratory tract and the lung, especially for cystic fibrosis, asthma, and COPD (chronic obstructive pulmonary disease) (reviewed in refs: Sham et al., 2004; Kleinstreuer et al., 2008). Recently, drug-aerosol inhalation has also been used to deliver medicine, such as growth hormone, calcitonin, etc. (Siekmeier and Scheuch, 2009) as a novel approach to systemic therapy. To maximize medical effectiveness and
to minimize the potential side effects, aerosol delivery of drugs must be targeted, which means delivering drug aerosols to the specified sites in the respiratory system. The desired sites are related to specific diseases. For example, regarding lung cancer treatment, adenocarcinomas are usually seen peripherally in the lungs, as opposed to small cell lung cancer and squamous cell lung cancer, which both tend to be centrally located. Therefore, for adenocarcinomas, most of the drug aerosol needs to be delivered to the peripheral airways, even to the alveoli. For small cell lung cancer and squamous cell lung cancer, most of the drug aerosol needs to be delivered to the central airways. For the chemoprevention of lung carcinogenesis, the drug aerosols need to cover the whole respiratory tract, since the types and the sites of tumors cannot be anticipated.

Table 1-1 Comparison of Murine and Human Lung Anatomy (Fox et al., 2007)

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alveoli</td>
<td>38-80 μm</td>
<td>200-400 μm</td>
</tr>
<tr>
<td>Diameter main bronchus</td>
<td>1 mm</td>
<td>10-15 mm</td>
</tr>
<tr>
<td>Airway branching pattern</td>
<td>Monopodial</td>
<td>Dichotomous</td>
</tr>
<tr>
<td>Diameter bronchioli</td>
<td>0.01-0.05 mm</td>
<td>&lt; 1 mm</td>
</tr>
<tr>
<td>Diameter terminal bronchioli</td>
<td>0.01 mm</td>
<td>0.6 mm</td>
</tr>
</tbody>
</table>

The efficacy of a given drug delivered via aerosol administration depends on many factors, such as the physiochemical properties of the drug, the anatomy of the respiratory tract, and the airflow pattern in the lung airways. For a given drug, the particle size distribution is the most important property controlling the particle deposition. Genetically-engineered mice play an important role in drug screening and preclinical studies, while only limited studies have focused on particle deposition in the mouse lungs. The success or failure of pulmonary administration of drug aerosols in mouse models cannot be elucidated explicitly until we have a better understanding of the particle transport and deposition behavior in vivo. Because the mouse respiratory
system is much smaller than that of a human being (Table 1-1), the deposition data obtained for human respiratory systems should not be applied to the case of mice. It is, thus, necessary to measure the mass deposition of particles in the mouse lung. In this dissertation, the mass deposition of drug particles was measured for both polydispered and monodispered particles.

Since no chemopreventive agent has been validated for lung cancer, more effort is needed to screen agents. Aerosol delivery may provide new opportunities to revive otherwise effective agents with adverse side effects and to increase the locoregional quantity of drugs in the lung. Additionally, characterization of the aerosol delivery system is also needed for data interpretation and system improvement. There are two major objectives of this dissertation:

1. To study the chemopreventive effects of synthetic and natural compounds via aerosol administration, singly or in combination with another agent, in the mouse model.
2. To evaluate drug particle deposition in mouse lungs.

1.2 Dissertation Structure

The whole dissertation contains eight chapters.

Chapter 1 is a general introduction to the background, the motivations, and the objectives of this study.

Chapter 2 is dedicated to reviews of three aspects. First, some evolved concepts of carcinogenesis and chemoprevention are reviewed, and the key factors in chemoprevention studies in animal models are discussed. Promising targets and agents in preclinical studies and clinical trials are only briefly discussed since there are many
incisive and comprehensive reviews on these topics. Second, agents that have been tested in mice for their chemopreventive effects via aerosol inhalation are reviewed as is progress achieved and lessons learned. Third, the advances in the aerosol delivery of drug particles to the mouse respiratory tract are reviewed.

Chapter 3 introduces the experimental setup and the protocols of animal experiments used in this study. A complete list of agents and agent combinations that have been tested is presented.

In Chapter 4 through Chapter 6, potential chemopreventive agents are evaluated via inhalation, either singly or in combination, in mice. Chapter 5 evaluates four small molecular inhibitors. First, aerosolized gefitinib and erlotinib, which target the tyrosine kinase domain of the epidermal growth factor receptor (EGFR), are examined for their inhibition on tumorigenesis in mouse lungs, and their toxicities are evaluated. Then, lapatinib, a novel dual inhibitor of both EGFR and Her2 (human EGFR type II, also known as Neu, ErbB2) is studied. Lapatinib was administered via both inhalation and oral gavage, and the comparative efficacy is noted. Last, a covalent inhibitor of phosphoinositide 3-kinase (PI3K), wortmannin, is used as a model drug to demonstrate the advantages of aerosol drug delivery. Wortmannin is a potent PI3K inhibitor, but it failed clinical translation due to drug-delivery challenges and toxicities. Wortmannin was delivered via both inhalation and oral gavage, and the efficacy and systemic toxicity are compared. Chapter 5 gives the results of the evaluation of four interesting natural compounds. Resveratrol, a phytoalexin from plants, and anthocyanins, dyes extracted from black raspberries, were delivered in the form of aerosols, because resveratrol has a fast clearance in the blood and anthocyanins have low bioavailability. Protocatechuic acid, as a major metabolite of anthocyanins and tea polyphenols, was
also studied to compare with anthocyanins. Caffeine, one component of tea extract and an interesting agent prevalent in our daily life, was also studied. In Chapter 6, the combination of aerosolized budesonide and dietary polyphenon E was studied.

In Chapter 7, mass deposition of drug aerosols in mouse lungs is measured and discussed. Gefitinib was used as a model drug. The lung and plasma levels of the drug delivered via inhalation are correlated to the solution concentration, exposure duration, and particle sizes.

In Chapter 8, the accomplishments of this dissertation are summarized. The issues and challenges in current studies are also discussed for future research efforts.
Chapter 2

Review
Chapter 2 Review

This work focuses on the aerosol administration of synthetic and natural agents that may potentially be chemopreventive of lung carcinogenesis and drug particle deposition in the mouse lung. Thus, we will review several principles and current progress regarding these two topics.

2.1 Chemoprevention of lung carcinogenesis

2.1.1 Carcinogenesis

Carcinogenesis is the process by which the growth, development, identity, and proliferation of normal cells becomes dysregulated and life-threatening, and is experimentally categorized in three broad stages – initiation, promotion, and progression (Wu et al., 2011).

There are two important concepts regarding carcinogenesis: multistep carcinogenesis and the “field of cancerization”. The multistep carcinogenesis theory, which is supported by recent molecular findings (Gomperts et al., 2011), describes a stepwise accumulation of genotypic and phenotypic changes progressing through pre-invasive histological changes to invasive disease (Cohen and Khuri, 2002; Soria et al., 2003). The field of cancerization refers to areas of histologically normal-appearing tissue adjacent to neoplastic lesions that display molecular abnormalities, some of which are the same as those in tumors (Gomperts et al., 2011). Malignant lesions can develop from multiple genetically distinct clones in diverse areas, and lesions in one part of the
exposed area imply increased risks of developing cancer in other sites within the area (Soria et al., 2003; Gomperts et al., 2011).

2.1.2 Chemoprevention

Chemoprevention is defined as the use of pharmacologic or natural agents that inhibit the development of invasive cancer, either by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of premalignant cells in which such damage has already occurred (Hong and Sporn, 1997). The essence of chemoprevention is intervention within the multistep carcinogenic process and throughout a wide field (Cohen and Khuri, 2002). The stepwise accumulation of precancerous alterations at the molecular level reflected in the macroscopic view is the latency period of cancer. The field of cancerization implies the uncertainty of the type and the location of tumors. Thus, chemoprevention aims to extend the latency period to a meaningful length to achieve a lifetime suppression of malignancy by targeting the whole epithelial surface of the respiratory tract with single or multiple chemopreventive agents.

Before clinical trials are begun, the efficacy and safety of new agents should be validated in experimental models. Appropriate animal models are essential for cancer chemoprevention studies. To build a sound scientific premise for an animal bioassay that shows the efficacy of chemopreventive agents, we must consider three critical factors. First, we need an appropriate animal model for specific forms of cancer. Then we need an appropriate test agent (plausible mechanism, defined dose, route, and duration). Finally, we need biomarker(s)/intermediate lesions that can be evaluated to
provide insights into the mechanisms of the agent’s efficacy or toxicities. Since the analysis of biomarkers was not involved in this dissertation, the following review is not intended to cover this topic.

2.1.2.1 Animal models

Animal tumor/cancer models fall into three categories: (1) well-established chemically-induced; (2) spontaneous; or (3) transgenic (Steele and Lubet, 2010). The endpoint of an animal bioassay is not as controversial as that of clinical trials (Wu et al., 2011) and is typically the tumor, which is the primary endpoint in most phase III clinical prevention trials. Experimental data of cell lines and animal models are the cornerstone of clinical trials and further development of chemopreventive agents. The animal bioassays not only provide evidence of agent efficacy, but also help to generate dose-response, toxicity, and pharmacokinetic data prior to clinical trials. An ideal animal model for chemoprevention testing should have the ability to assemble or approximate intermediate and cancerous lesions of human lung cancer both molecularly and histologically, and should bear similar genetic and epigenetic alterations to those found in humans. Besides, the animal model should “be capable of producing consistent tumor burden in greater than 60% of animals developing the endpoint (typically cancerous lesions) within a reasonable period of time (< 6 months)” (Steele and Lubet, 2010), and should have high predictive validity in humans. No current animal model is ideal, and better animal models are sought.
Table 2-1 Carcinogen-induced and genetically-engineered animal models in current use for the screening and development of lung cancer chemopreventive agents (Steele and Lubet, 2010; Wang et al., 2012; Dragnev et al., 2013)

<table>
<thead>
<tr>
<th>Species</th>
<th>Carcinogen/Genetic Modification</th>
<th>Endpoint Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster</td>
<td>MNU</td>
<td>Squamous cell carcinomas</td>
</tr>
<tr>
<td>Mouse</td>
<td>B[a]P, NNK, vinyl carbamate, uracil, urethane, cigarette smoke</td>
<td>Adenomas and adenocarcinomas</td>
</tr>
<tr>
<td>Mouse</td>
<td>NTCU</td>
<td>Squamous cell carcinomas</td>
</tr>
<tr>
<td>Mouse</td>
<td>TP53 mutants plus B[a]P or NNK</td>
<td>Adenocarcinomas</td>
</tr>
<tr>
<td>Mouse</td>
<td>Altered TP53 and GPCR5a knockout (NNK)</td>
<td>Adenocarcinomas (no K-ras mutation)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Floxed Rb and p53 allele</td>
<td>Small cell lung cancer</td>
</tr>
</tbody>
</table>

Abbreviations: MNU, methylnitrosourea; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NTCU, N-nitroso-tris-chloroethylurea; B[a]P, benzo[a]pyrene; TP53, tumor protein 53 gene; GPCR5a, G-protein-coupled receptor family C, group 5, member a gene; Rb, retinoblastoma protein.

For testing potential lung cancer prevention agents, carcinogen-induced and genetically engineered animal models that are commonly used are summarized in Table 2-1. The animal models in Table 2-1 represent all histological types of human NSCLC. A potential chemopreventive agent needs to be evaluated comprehensively for each lung cancer type in the relevant animal model. Transgenic animal models are not included in Table 2-1, but they are useful for studying the mechanism of carcinogenesis and chemopreventive agents, especially agents that act on specific molecular and cellular targets, because transgenic animal models bear certain genetic abnormalities which are similar to those found in humans, such as insertions, deletions, and mutations at targeted gene sites.
Figure 2-1 Lung adenoma induced by one dose of B[a]P, 100 mg/kg body weight, intraperitoneal injection (i.p.), 20 weeks, A/J female mouse with normal lung as control: A, gross view of lung with adenoma; B, gross view of lung with adenocarcinoma induced by vinyl carbamate, 0.32 mg per mouse, i.p., 20 weeks; C, histopathology of lung adenomas. (Figures adopted from Wang et al., 2012)
In this dissertation, the mouse lung adenoma model in A/J mice (Figure 2-1) was used because it is efficient, consistent, and reliable. It provides helpful comparison since it has been frequently used in previous studies. In this model, lung adenomas (most harboring K-ras mutations) can be induced by various carcinogens such as vinyl carbamate, benzo[a]pyrene (B[a]P), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), diethylnitrosamine, uracil mustard, and urethane. Among these carcinogens, B[a]P, NNK, vinyl carbamate, and urethane are related to cigarette smoke and are frequently used in animal models to simulate cancerous lesions normally caused by cigarette smoke in humans. A more relevant carcinogen is the cigarette smoke itself, which mimics the cancer induction process in humans by a complex mixture of chemicals. The doses and schedule of the carcinogens in each model listed in Table 2-1 are described in detail (Steele and Lubet, 2010; Wang et al., 2012).

### 2.1.2.2 Protocols

![Commonly Used Chemoprevention Protocols](image)

*Figure 2-2 Commonly used Chemoprevention Protocols (Adopted from Wang et al., 2006)*

There are three frequently used protocols (Figure 2-2) for testing the efficacies of chemopreventive agents using A/J mice based on the agent’s mechanism of action and
the stages of tumor progression. In the complete protocol, intervention begins 1-2 weeks prior to carcinogenic initiation and continues thereafter until 20-24 weeks. This model is used to examine agents that either block carcinogen initiation and/or suppress tumor growth. However, only limited lung tumor progression from adenomas to adenocarcinomas occurs at 20-24 weeks, when animals are usually terminated. In the post-initiation protocol, intervention begins 2-3 weeks post-carcinogen initiation and continues until termination of the animals at 20-24 weeks. This model is used to determine tumor suppressing effects independent of tumor initiation, mimicking the status of ex-smokers who quit smoking and receive preventive treatment. In the tumor progression protocol, intervention begins 12-20 weeks post-carcinogen initiation and continues to approximately 30 weeks. This model is used to determine the effects of chemopreventive agents on progression from adenomas to carcinomas. The tumor progression protocol is more relevant to clinical chemoprevention trials in humans with tumor histology as the endpoint.

2.1.2.3 Agents

The identification of promising chemopreventive agents usually relies on three approaches: epidemiological studies that show a positive correlation between dietary patterns and the risk of developing cancer, such as β-carotene (Hennekens et al., 1996; Omenn et al., 1996); traditional and historical pharmacopeia that show antitumor effects clinically, such as Chinese herbal medicine (reviewed by Li et al., 2013); and a more targeted approach towards promising molecular and cellular targets, as reviewed by Thiery-Vuillemin A et al (2005).
Empirical approaches based on epidemiological data are unpredictable (Sporn and Suh 2012). The efficacy and toxicities of agents that correlate with a reduced cancer risk should be validated in experimental models before they move on to clinical trials. Traditional prescriptions and therapies encounter similar or even problematic situations due to lack of statistical significance and convincing mechanisms. Many studies are testing the chemopreventive efficacy and toxicities of these agents in cell lines and animal models. These studies seek to determine the effective ingredients or active components, revealing the active mechanisms within the affected sites. Such agents are mostly naturally occurring, including nutrients or nutritional supplements from food and beverages, and compounds or complexes derived from animals, plants, herbs, etc. It is worth noting that a new trend in studying these agents focuses not only on the single “active” ingredient/compound, but also on the existence of other ingredients as a complex, such as (-)-epigallocatechin-3-gallate (EGCG) in polyphenon E (Fu et al., 2009; Zhang et al., 2010), or herbs in Chinese medicine, used singly or in compounds.

The most rational approach is to design and test potential chemopreventive agents that act on specific molecular and cellular targets (Sporn and Suh 2012). Examples of these targets or pathways include retinoid receptor signaling, mutations in and overexpression of transmembrane receptors (epidermal growth factor receptor [EGFR] and insulin-like growth factor receptor [IGFR]), inflammation and inflammation related pathways (cyclooxygenase-2 [COX-2] and nuclear factor kappa-light-chain-enhancer of activated B cells [NF-kB]), angiogenesis (vascular endothelial growth factor [VEGF]), histone deacetylase, cyclin D1, etc. The emerging studies of targeted agents are accompanied by the accumulation of knowledge concerning carcinogenesis. Promising
targets and potential agents for lung cancer prevention have been reviewed extensively (Keith 2012; Sporn and Suh, 2012; Dragnev et al., 2013), thus, they will not be examined here.

Only agents with the most potent efficacy and lowest toxicities in the cell line studies and animal models are considered to be tested in clinical trials. However, to date the clinical lung cancer prevention trials have shown minimal efficacy (Dragnev et al., 2013). Thus, there is currently no validated agent for lung cancer chemoprevention. Previous and current lung cancer prevention trials are summarized in Table 2-2. The list of potential agents for clinical lung cancer prevention trials is growing. Several agents that hold promise are suggested for future clinical trials, including myo-inositol, rapamycin, bexarotene, gefitinib and erlotinib (based on unpublished data in this dissertation), triterpenoid, etc (Keith 2012; Dragnev et al., 2013). More effort is needed to screen effective agents in preclinical animal studies and clinical trials.
### Table 2-2 Previous and current clinical trials testing potential agents for lung cancer chemoprevention (Keith 2012; Dragnev et al., 2013)

<table>
<thead>
<tr>
<th>Agents</th>
<th>Mechanism</th>
<th>Cohort</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-carotene*</td>
<td>antioxidant</td>
<td>Current and former smokers</td>
<td>Lung cancer incidence</td>
</tr>
<tr>
<td>Vitamine E*</td>
<td>antioxidant</td>
<td>Current and former smokers</td>
<td>Lung cancer incidence</td>
</tr>
<tr>
<td>Vitamine A and N-acetyl cysteine*</td>
<td>Retinoic acid agonist (RAR)/antioxidant</td>
<td>Patients who had received treatment for primary lung cancer</td>
<td>Recurrence, death or second lung cancer</td>
</tr>
<tr>
<td>Anethole dithioethione*</td>
<td>Increases glutathione-S-transferase and additional phase II enzymes</td>
<td>Current and former smokers with bronchial dysplasia</td>
<td>Bronchial dysplasia</td>
</tr>
<tr>
<td>Inhaled budesonide*</td>
<td>Anti-inflammation</td>
<td>Smokers with dysplasia</td>
<td>Dysplasia histology</td>
</tr>
<tr>
<td>Inhaled budesonide*</td>
<td>Anti-inflammation</td>
<td>Current and former smokers with lung nodules</td>
<td>Target nodule size</td>
</tr>
<tr>
<td>Myo-inositol†</td>
<td>PI3K inhibitor</td>
<td>Smokers with bronchial dysplasia</td>
<td>Dysplasia histology</td>
</tr>
<tr>
<td>Celexocib†</td>
<td>COX-2</td>
<td>Current and former smokers</td>
<td>Proliferative marker Ki-67</td>
</tr>
<tr>
<td>Iloprost†</td>
<td>Prostacyclin analog; anti-inflammation, anti-proliferation</td>
<td>Current and former smokers</td>
<td>Endobronchial histology</td>
</tr>
<tr>
<td>Pioglitazone‡</td>
<td>PPARγ</td>
<td>Current and former smokers</td>
<td>Endobronchial histology</td>
</tr>
</tbody>
</table>

Abreviations: COX-2, cycloxygenase-2; PPAR, peroxisome proliferator-activated receptor. *: no effect; †: positive effect; ‡: ongoing trials.

### 2.2 Aerosolized agent to avoid side effects

The typical routes of administration include oral (in the diet, in the drinking water or by gavage), intravenous, intraperitoneal, and aerosol routes. Oral administration is preferred by humans for its convenience; however, aerosol administration hold major
advantages over other routes for agents with known toxicities to gastrointestinal organs and for those with poor metabolic profiles (i.e., they are rapidly metabolized and excreted) (Steele and Lubet, 2010).

Aerosol administration may be as effective as other methods, or potentially increase the efficacy of multiple agents in animal models. For example, budesonide, a synthetic glucocorticoid, delivered by aerosol for very short periods of time, showed striking inhibitory effects in the lung adenoma model in A/J mice, both alone and combined with other orally-administered agents (Wattenberg et al., 1997; Wattenberg et al., 2000; Fu et al., 2010). Another example is Polyphenon E (PPE) and its active component (-)-epigallocatechin-3-gallate (EGCG), which are from green tea extract. Witschi et al (2004) did a pilot study on EGCG in A/J mice and showed that aerosolized (EGCG) had minor inhibitory effects. Yan et al (2007) and Fu et al (2009) investigated the inhibitory effects of PPE as a mixture (i.e., EGCG with other components) on lung tumorigenesis in A/J mice and found a consistent reduction in tumor load and multiplicity. Effective agents assessed in animal studies are candidates for clinical trials. However, inhaled budesonide failed in two Phase II clinical prevention trials (Lam et al., 2004; Veronesi et al., 2011). PPE in the diet showed similar inhibitory effects in a mouse model (Zhang et al., 2010). As an oral route is more convenient for humans, aerosol delivery is applicable but not necessary in the case of PPE.

Aerosol delivery may be able to reduce the toxicity without losing efficacy. Two recent studies provided some evidence. Aerosolized bexarotene significantly reduced tumor multiplicity and tumor load without increasing total plasma cholesterol and triglyceride level compared with oral administration (Zhang et al., 2010). Inhaled
3-bromopyruvate (at a dose of 10 mg/ml) significantly decreased tumor multiplicity and tumor load by 49% and 80%, respectively, without causing any liver toxicity (Zhang et al., 2012). Inhaled 3-bromopyruvate and bexarotene are promising agents for potential future clinical prevention trials. Thus, in the case of agents with associated toxicities when delivered by other methods, the aerosol approach is both applicable and necessary.
Table 2-3 Testing conditions and efficacy of agents evaluated in previous work (Fu 2009, PhD dissertation)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Route</th>
<th>Dose</th>
<th>Carcinogen</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Multiplicity</td>
</tr>
<tr>
<td>PPE</td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>53 %</td>
</tr>
<tr>
<td>PPE without EGCG</td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>20 %</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Aerosol</td>
<td>1 mg/ml</td>
<td>VC</td>
<td>6 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td></td>
<td>6 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>38 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mg/ml</td>
<td></td>
<td>27 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 mg/ml</td>
<td></td>
<td>33 %</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Aerosol</td>
<td>1 mg/ml</td>
<td>VC</td>
<td>0 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td></td>
<td>-12 %</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>VC</td>
<td>-5 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.5 mg/ml</td>
<td></td>
<td>7 %</td>
</tr>
<tr>
<td>Budesonide</td>
<td>Aerosol</td>
<td>1 mg/ml</td>
<td></td>
<td>35 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 mg/ml</td>
<td>B[a]P</td>
<td>42 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 mg/ml</td>
<td></td>
<td>57 %</td>
</tr>
<tr>
<td>PPE</td>
<td>Diet</td>
<td>1.5 w.t.% in diet</td>
<td>B[a]P</td>
<td>27%</td>
</tr>
<tr>
<td>Budesonide</td>
<td>Aerosol</td>
<td>3 mg/ml</td>
<td>B[a]P</td>
<td>58%</td>
</tr>
<tr>
<td>+ PPE</td>
<td>+ Diet</td>
<td>1.5 w.t.% in diet</td>
<td>B[a]P *</td>
<td>36%</td>
</tr>
<tr>
<td>I3C</td>
<td>Diet</td>
<td>10 μmol/g diet</td>
<td>B[a]P</td>
<td>36%</td>
</tr>
<tr>
<td>Budesonide</td>
<td>Aerosol</td>
<td>3 mg/ml</td>
<td>B[a]P</td>
<td>42%</td>
</tr>
<tr>
<td>+ I3C</td>
<td>+ Diet</td>
<td>10 μmol/g diet</td>
<td>B[a]P</td>
<td>6%</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Gavage</td>
<td>10 mg/kg B.W.</td>
<td>B[a]P</td>
<td>55.5%</td>
</tr>
<tr>
<td>Budesonide</td>
<td>Aerosol</td>
<td>3 mg/ml</td>
<td>B[a]P</td>
<td>55.5%</td>
</tr>
</tbody>
</table>

Abbreviations: PPE, polyphenol E; EGCG, (-)-epigallocatechin-3-gallate; B[a]P, benzo[a]pyrene; VC, vinyl carbamate; I3C, indole-3-carbinol; B.W., body weight. The exposure duration of all the aerosol treatment was 15 min except that budesonide was given for 2 min.
To provide more candidates for clinical trials, more agents were evaluated with the rationale that the aerosol approach may revive agents in which the oral approach causes side effects or low bioavailability. Former work is presented in Table 2-3. The results and testing conditions are summarized. A part of the work for this dissertation is the extension and development of previous studies: gefitinib was evaluated again in order to verify the efficacy and toxicity; a pharmacokinetic study of resveratrol was conducted to explain the efficacy shown previously; and the three combination groups were repeated with lower doses of each single agent to weaken the overwhelming efficacy of budesonide.

2.3 Particle deposition in the mouse lung

Particle deposition in the mouse lung is studied for two purposes: toxicology and drug delivery. In contrast to the extensive studies on particle deposition in the human lung, there are only limited studies on particle deposition in the mouse lung. However, the data for the human lung cannot be applied to studies of the mouse lung due to the differences between human lungs and mouse lungs (Table 1-1). Despite the differences, the mechanisms of particle deposition in the respiratory system are the same for the human and the mouse. In general, five mechanisms are involved in the process of particle deposition in the lungs. The most important of these mechanisms are inertial impaction, sedimentation, and diffusion. Interception and electrostatic precipitation are generally less important mechanisms (Hinds, 1999). These mechanisms are directly related to the particle size (Hinds, 1999; Friedlander, 2000). Other factors that exert an impact on the delivery efficiency and particle deposition pattern in the respiratory
system include the physiochemical properties of aerosols, the anatomy of the respiratory tract, and the airflow pattern in the lung airways. When the animal model and the drug are fixed, the particle size distribution is the most important factor.

It is challenging to measure the mass deposited in the lung and to map the deposition pattern in the central and peripheral lung because the mouse lung is much smaller than a human lung. Nevertheless, recent developments and advances in imaging, mass spectrometry, and data analysis tools have made it possible to evaluate the intra organ regional deposition patterns of pharmaceutical aerosols in preclinical species. Progress was achieved in two aspects. One was the development and validation of new methods with improvement in instrumentation, and the other was that the particle size was extended down to 150 nm for the particle size-deposition efficiency relationship. The achievements are summarized in Table 2-4.
Many efforts, including both computational modeling and experimental measurements, have been made to figure out the optimal size range for particle deposition in the mouse lung. One report tested particles ranging from 150 nm to 2.5 \( \mu m \) (mass median aerodynamic diameter, MMAD) generated from an ultrasonic nebulizer, and by measuring the mass, it was found that smaller particles have a much higher deposition fraction in rodent animal models (Zhang et al., 2008). A later study provided supportive results that the deposition patterns of aerosols between 0.5 and 5.0 \( \mu m \) showed an increase in both overall and peripheral deposition as the particle size decreased.
µm (MMAD) showed an increase in both overall and peripheral deposition as the particle size decreases using polydispersed particles followed by SPECT/CT imaging for analysis (Kuehl et al., 2012). Another report using positron emission tomography measured the distribution and transport of ~100 nm polystyrene nanoparticles labeled with $^{64}$Cu and showed that the inhaled nanoparticles primarily deposited in the lung and stayed in the lung steadily for 48 hr (Palko et al., 2010). Combining this data with the data from Raabe’s study (Raabe, 1988), the trend of changes of deposition efficiency with particle size can be depicted: for particles larger than 5 µm, over 90% of particles will deposit in nasal-pharyngeal zone, and for particles from 150 nm to 5 µm deposition increases as the particle size decreases.

Factors other than particle size were also studied. An ex vivo fluorescent imaging approach using aluminum phthalocyanine tetrasulfonic acid (AIPCS) particles of varied sizes was developed to measure the particle distribution inside the lung, and showed that the aerosol deposition is clearly linked to lung function (Yi et al., 2012). The relationship between drug particle deposition and cancer phenotypes were studied ex vivo using MALDI instrumentation in imaging mode. This provided a method to measure the mass deposition in the mouse lung, although the original study did not deliver the drug via aerosol (Marko-Varga G et al., 2011). Instead of mapping the particle distribution patterns, a novel technique of synchrotron phase-contrast X-ray imaging (PCXI) was developed to track a single particle down to 5 µm in a live mouse trachea (Donnelley et al., 2010). This technique might facilitate the modeling of a single particle’s trajectory within the mouse trachea if the resolution could be refined.

Most of the reports mentioned above focused on developing novel measurement and
imaging techniques. Only one focused on the particle generation process (Zhang et al., 2008). In this dissertation, I made additional efforts and attempted to characterize both polydisperse and monodispersed particles for future improvement of particle generation methods.
Chapter 3

Experimental Methods
Chapter 3 Experimental Methods

3.1 Animal Experiment

In this study, a benzo[a]pyrene (B[a]P)-induced female A/J mouse model was used to evaluate the inhibitory effects of potential chemopreventive agents. A/J mice are an inbred strain\(^\text{①}\) of mice that are extremely sensitive to the development of both spontaneous and chemically induced lung tumors. The formation of adenocarcinoma in the A/J mouse lung involves K-ras (Kirsten rat sarcoma viral oncogene homologue) mutations which are detected in 30% of lung adenocarcinomas in humans and are, by far, the most common mutations other than \(p53\) (Belinsky et al., 1993; Wang et al., 2012). B[a]P is a tobacco smoke-related carcinogen that has been proven to induce lung tumors in mice. B[a]P-induced tumors bear a point mutation predominately in Codon 12 with a GC to TA transversion\(^\text{②}\) compared with the random mutation pattern detected in spontaneous lung tumors. This point mutation is detected in 40% of human lung tumors containing an activated K-ras gene (Belinsky et al., 1993). Thus, B[a]P-induced lung tumors share both morphological phenotypes and K-ras genotypes in common with corresponding human lung tumors.

\(^{①}\) An inbred strain is one that is produced using at least 20 consecutive generations of sister × brother or parent × offspring matings, or is traceable to a single ancestral pair in the 20\(^{th}\) or subsequent generation (The Jackson Laboratory, Bar Harbor, ME, US). Website: http://research.jax.org/grs/type/inbred/index.html

\(^{②}\) A transversion is the replacement of a base of one chemical category by a base of the other. Transversion occurs when either a pyrimidine is replaced by a purine or a purine is replaced by a pyrimidine (Anthony et al., 2008).
The use of animals was approved by Washington University’s Institutional Animal Care and Use Committee. Female A/J mice at 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed with wood chip bedding in an environmentally-controlled, clean-air room with a 12-hour light-dark cycle at a constant temperature and a relative humidity of 50%. Drinking water and diet were supplied \textit{ad libitum}.

To mimic the exposure-intervention timing in former smokers who are supposed to take drugs after smoke cessation, a post-initiation protocol (Figure 3-1) was used in the study. To initiate the tumors, in general, the mice were given either a single intraperitoneal (i.p.) dose of B[a]P (100 mg/kg body weight) in 0.2 ml tricaprylin or two doses of B[a]P by i.p. injection (each at 100 mg/kg body weight, one week apart). Two weeks after the last dose of B[a]P, the mice were randomly divided into control and treatment groups with 12 mice per group. Theoretically, the larger the sample size, the better it can represent the population. However, the number of mice in each group was limited by time and cost. Based on empirical experience, 12 mice were used for each group, to take into account possible variance among mice and possible mortality during treatment. Each treatment group had a counterpart control group. The control groups...
were treated in exactly the same way as the treatment groups except that the control groups receive no drugs during the experiment. Oral B[a]P (by gavage) is also used to initiate lung tumorigenesis (Steele and Lubet, 2010). However, oral B[a]P can also induce esophageal (Wen and Walle, 2007), forestomach (Gangar et al., 2011), and gastric tumorigenesis (Goyal et al., 2010). Primary tumors in organs other than the lung can highly complicate data interpretation; thus, i.p. injection of B[a]P is preferred as it only induces lung tumorigenesis.

Three administration methods were used in the study, including aerosol inhalation, oral gavage, and diet. For aerosol inhalation, the mice were exposed to aerosols in a custom-built nose-only exposure chamber which could hold a maximum of 12 mice at the same time (Fu et al., 2009). For dietary administration, the drug was well-mixed with AIN-76A purified powder diet (Dyets, Inc., Bethlehem, PA) and 3% sugar in a KitchenAid (St. Joseph, MI) mixer for one hour. For gavage administration, the drug was evenly dissolved or dispersed in a vehicle. The drug solutions or suspensions were fed to the mouse in a dose of 0.01ml per gram body weight. The diet was prepared twice per week, and fresh diet in the cages was supplied daily.

The body weight of the mice was monitored and recorded on the same day every week during treatment. At the end of the treatment, mice were euthanized by CO₂ asphyxiation and dissected. Larger lung tumors and specimens of non-tumorous lung tissue were flash frozen in liquid nitrogen and stored at -80°C for further analysis. The rest of the lungs from each mouse were fixed in Tellyesniczky’s solution (90% ethanol, 5% glacial acetic acid, and 5% formalin) overnight (Zhang et al., 2000) and then stored in 70% ethanol. The fixed lungs were evaluated under a dissecting microscope to obtain
a fixed surface tumor count and individual tumor diameters. Individual tumor volumes (V) were calculated based on the following formula: $V = \frac{\pi d^3}{6}$ (Wang et al., 2003), where d is the diameter assuming a spherical tumor. The total tumor volume of each mouse was defined as the sum of the volumes of all frozen and fixed individual tumors. Tumor load was determined by calculating the mean value of the total tumor volume per mouse in each group.

### 3.2 Aerosol Procedure

Drugs were dissolved in a 50:50 solution of dimethylsulfoxide (DMSO) and ethanol. The solution was nebulized into droplets by custom-built Collison nebulizers. As shown schematically in Figure 3-2, generated aerosols were directed to two tandem diffusion scrubbers containing activated granulate charcoals (Sigma-Aldrich, St. Louis, MO) to remove the ethanol and DMSO.

![Figure 3-2 Schematic diagram of the aerosol delivery system used in the animal study](image-url)
Then, the resultant dry aerosols were introduced into the nose-only exposure chamber from the top inlet. Effluent aerosols were discharged from the outlet at the bottom of the chamber and then collected with a high efficiency particulate air filter (HEPA filter; Pall Co., Port Washington, NY). A vacuum pump and an orifice were connected to the HEPA filter to control the total air flow through the chamber at 2 L/m.

The major differences between this delivery system and the system used in previous work (Fu 2009) are the orifice and vacuum pump at the exit of the exposure chamber. The orifice guaranteed a constant flowrate and pressure in the system, thereby minimizing the bias caused by flow variations inside the chamber.

The size distribution of the generated aerosols were measured by a scanning mobility particle sizer (SMPS) spectrometer which included an electrostatic classifier (TSI model 3080), a differential mobility analyzer (DMA, TSI model 3081), and a condensation particle counter (CPC, TSI model 3025). The geometric median diameter, mass median aerodynamic diameter (MMAD), geometric standard deviation (GSD), and the order of magnitude of the particle number were obtained. The mass concentration of the drug aerosols inside the chamber was measured with off-line and on-line methods. In the off-line method, a gravity method, the drug aerosols were loaded on a piece of environmentally balanced filter medium at the exit of the exposure chamber during the treatment. The filter medium was then re-balanced and weighed to determine the mass of the drug aerosols. The total volume of air was determined by the exposure time and the air flow rate. The mass concentration inside the chamber was calculated as follows:

\[
[C]_{\text{aerosol, mg/L}} = \frac{M_{\text{filter loading, mg}}}{(\text{exposure time}_{\text{min}} \times \text{air flow rate}_{\text{L/min}})}
\]  

(1)
where \([C]_{\text{aerosol}}\) is the aerosol mass concentration inside the exposure chamber, mg/L, and \(M_{\text{filter loading}}\) is the mass of particles loaded on the filter media, mg.

The on-line method used the Kanomax piezobalance aerosol mass monitor (Kanomax, Japan, Inc.). The two methods provided similar results in a certain concentration range, although the off-line gravity method was more accurate for large mass concentrations.

The mass of inhaled drugs was calculated as follows:

\[
M_{\text{inhaled}} = [C]_{\text{aerosol}} \times \text{RMV} \times \text{exposure time},
\]

where RMV is the respiratory minute volume of the mouse. The RMV was determined by plethysmography either just prior to or during the exposure. In the present study the RMV was empirically determined to be 0.025 mg/min, using Guyton’s formula as follows (Guyton, 1947):

\[
\text{RMV}_{\text{ml}} = 2.1(\text{body weight}_{\text{g}})^{0.75}.
\]

The dose could be estimated as follows:

\[
\text{Dose} = \frac{M_{\text{inhaled}}}{\text{body weight}_{\text{g}}} = \frac{[C]_{\text{aerosol}, \text{mg/L}} \times \text{RMV}_{\text{L/min}} \times \text{exposure time}_{\text{min}}}{\text{body weight}_{\text{g}}}. \tag{4}
\]

### 3.3 Statistical Analyses

The statistical analyses were done with PSAW® Statistics 18 (IBM, Somers, NY) and Microsoft® Excel™ 2007 (Microsoft, Redmond, WA). Tumor multiplicity and tumor load were analyzed by a two-sided Student’s t-test. For the combination treatment, tumor multiplicity and tumor load were analyzed by a one-way analysis of variance (ANOVA) followed by the Tukey’s HSD (honestly significant difference) test for multiple comparisons to protect against an inflated alpha level, thereby limiting the risk
Chapter 3 Experiment Methods

of a Type I error.

3.4 Summary of Agents Evaluated in the Dissertation

Twenty four agents were evaluated alone, including synthetic and natural compounds. Thirteen pairs of agents were evaluated in combinations. A simple strategy was developed to select agents suitable for aerosol delivery (Figure 3-3). The hypothesis was proposed and tested that aerosol inhalation could reduce adverse side effects and might potentially increase the efficacy of the agent.

![Figure 3-3](image)

A strategy for selecting agents for aerosol inhalation.

Eight single agents and one combination of agents (Table 3-1) are discussed in great detail in the following chapters.
### Table 3-1 Potential chemopreventive agents evaluated using a post-initiation protocol in the B(a)P-induced A/J mouse model

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Concentration</th>
<th>Completion Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>Aerosol</td>
<td>5 mg/ml</td>
<td>Nov. 2009</td>
</tr>
<tr>
<td></td>
<td>Aerosol</td>
<td>10 mg/ml</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td></td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td></td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>Aerosol</td>
<td>5 mg/ml</td>
<td>Nov. 2009</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>Aerosol</td>
<td>50 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>100 mg/kg body weight</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Aerosol</td>
<td>2 mg/ml</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td></td>
<td>Gavage</td>
<td>1 mg/ml</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td></td>
<td>Aerosol</td>
<td>2 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Aerosol</td>
<td>15 mg/ml</td>
<td>Nov. 2009</td>
</tr>
<tr>
<td>Protocatechuic Acid</td>
<td>Aerosol</td>
<td>12 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Anthocyanins</td>
<td>Aerosol</td>
<td>5 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Aerosol</td>
<td>10 mg/ml</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Budesonide + Polyphenon E</td>
<td>Aerosol + Diet</td>
<td>1.125 mg/ml + 0.75 %</td>
<td>Sep. 2010</td>
</tr>
</tbody>
</table>

Their molecular structures are shown in Figure 3-4. Other agents and combinations are discussed in the Appendix.
A. Gefitinib

B. Erlotinib

C. Lapatinib

D. Wortmannin
Chapter 3 Experiment Methods

E. Resveratrol

F. Protocatechuic Acid

G. Caffeine

H. Budesonide

I. Anthocyanins (Black raspberry Extract, molar%)

cyanidin-3-O-glucoside (12%)

cyanidin-3-O-(2''-xylosylrutinoside) (34%)

cyanidin-3-O-rutinoside (53%)
Figure 3-4 Molecular structures of the evaluated agents (I and J were adopted from Pubchem Compound: http://www.ncbi.nlm.nih.gov/pccompound).
Chapter 4

Chemopreventive Effects of

Small Molecular Inhibitors via Inhalation
Chapter 4 Chemopreventive Effects of Small Molecular Inhibitors via Inhalation

Four small molecular inhibitors are discussed in this chapter. Three of them (gefitinib, erlotinib, and lapatinib) are synthetic compounds, and the last one (wortmannin) is a natural compound. Orally-administered gefitinib, erlotinib, and wortmannin are able to inhibit lung tumorigenesis in mice, but their efficacy is accompanied by toxicities which are not acceptable for long term chemoprevention. Lapatinib has similar toxicity to gefitinib and erlotinib but, currently, has not shown inhibitory effects on lung tumorigenesis in animal models or clinical trials. Thus, this chapter tests the hypothesis that aerosol delivery of small molecular inhibitors reduces the toxicities of the agents without compromising their efficacy.

4.1 Gefitinib and Erlotinib – EGFR Inhibitors

EGFR is a transmembrane tyrosine kinase (TK) type I receptor of the ErbB family that plays an important role in processes such as cell growth, proliferation, survival, and differentiation. Selective blockade of EGFR via newly developed EGFR tyrosine kinase inhibitors (TKIs) has been an effective therapeutic approach specifically in non-small-cell lung cancer (NSCLC) (Hida et al., 2009). Gefitinib (Iressa™, ZD1839, AstraZeneca Pharmaceuticals, DE) (Figure 3-4 A) is the first orally active inhibitor of

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① The manuscript has been prepared and will be submitted to Molecular Carcinogenesis.
Chapter 4 Chemopreventive Effects of Small Molecular Inhibitors via Inhalation

EGFR (HER1 or ErbB1). EGFR-specific gefitinib blocks the nucleotide-binding pocket of ErbB proteins, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction (Muhsin et al., 2003; Citri and Yarden, 2006). Gefitinib has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with the most common form of lung cancer, NSCLC, and it is now approved in 36 countries worldwide as a first-, second-, or third-line treatment option for NSCLC (Hida et al., 2009). The possibility of using gefitinib as a chemopreventive agent has also recently been studied using murine animal models (Fujimoto et al., 2005; Yan et al., 2006; Kishino D et al., 2008; Ohashi et al., 2009, 2010; Keith RL et al., 2010). Gefitinib administered by oral gavage shows inhibitory effects in murine cancer models.

Erlotinib (Tarceva®, OSI774, OSI Pharmaceuticals, LLC, Farmingdale, NY) (Figure 3-4 B) is also an orally available reversible ATP-competitive inhibitor of wild-type EGFR TK. The mechanism of erlotinib is similar to that of gefitinib. Erlotinib was approved by the U.S. FDA in 2004 for the treatment of patients with locally advanced or metastatic NSCLC after failure of at least one previous chemotherapy regimen (Pao and Miller, 2005). The results of the clinical trials using erlotinib for the treatment of early stage, locally advanced, and metastatic NSCLC were reviewed and summarized (Gridelli et al., 2010; Lyer and Bharthuar, 2010). Erlotinib has also been examined for lung carcinogenesis chemoprevention (Liby et al., 2008; Zerbe et al., 2008; Dragnev et al., 2011).

Although these two small molecular inhibitors have already shown anti-tumor activity in a variety of tumor types, alone and in combination with chemotherapy, side effects, especially visible mucocutaneous alterations such as rashes, are frequently seen.
in gefitinib- and erlotinib-treated patients. Owing to the uniform expression of EGFR, its high dependence on pathway activity, and ease of access, the skin has been used as a surrogate marker of EGFR signaling as similar effects are expected to occur in tumor tissue (Lacouture 2006; Gridelli et al., 2010). For chemoprevention, the side effects definitely exert negative influences on quality of life and are not acceptable for long-term intervention. Therefore, for both chemoprevention and clinical applications, the delivery target of these drugs should be controlled with a promise of an effective local level in the lung and a minimum systemic level. Aerosol delivery is, clearly, the best administration route in this case. Previous studies showed that tumor growth was inhibited with no observable side effects (Yan et al., 2006). Further studies performed in this dissertation using the same animal model have demonstrated the feasibility of delivering small molecular inhibitors by the inhalation route without visible side effects. This study represents an effort to determine the efficacy and safety of pulmonary–delivered gefitinib and erlotinib with B[a]P-induced lung tumors in mice.

### 4.1.1 Animal Bioassays

#### 4.1.1.1 Efficacy Assays

Three sets of bioassays were conducted with an incremental dose of gefitinib, and one group of erlotinib was added in the first set to ensure the generality of our results. The protocols are illustrated in Figure 4-1, and the groups are presented as described in Table 4-1. In the first set, female A/J mice were given one single intraperitoneal (i.p.) injection of B[a]P (100 mg/kg body weight) in 0.2 ml of tricaprylin. Two weeks after
B[a]P injection, the mice were randomly divided into control and treatment groups and subjected to aerosol treatment. The control group received DMSO/EtOH (the solvent of gefitinib and erlotinib) via inhalation while the treatment groups were given either aerosolized gefitinib (0.8 mg/kg body weight) or erlotinib (0.8 mg/kg body weight). The mice were treated once a day, 5 days per week for 20 consecutive weeks. In the second set, we tested only gefitinib. All the conditions were the same as the first set, except that we increased the dose of gefitinib from 0.8 to 1.6 and 2.5 mg/kg body weight. In the third set, we repeated the gefitinib dose of 2.5 mg/kg body weight but increased the number of induced tumors by using two doses of B[a]P (each at 100 mg/kg body weight, one week apart). The treatment lasted for 19 weeks. In all three sets of the bioassay, all the solutions were freshly prepared on the same day they were used. The aerosolized drug exposure was conducted using the custom-built nose-only exposure chamber. The mice inhaled aerosols for 15 minutes by placing their noses into the cone of each sub-compartment.
4.1.1.2 Skin toxicity evaluation

In the toxicity evaluation assay, female A/J mice at 8 weeks of age were randomly divided into 2 groups with 5 mice in each group: 1) Aerosol 15 (15 mg/ml solution as the feeding fluid for the nebulizer); 2) Gavage 100 (100 mg/kg body weight); 3) Gavage 200 (200 mg/kg body weight). The mice were either gavaged with a dose of 200 mg/kg body weight in 1% Tween 80 solution or exposed to gefitinib aerosols for 15 min. Both treatments were given 5 days per week for 10 consecutive weeks (Figure 4-1). Photos were taken of each individual mouse for comparison.
Table 4-1 Experimental design for the gefitinib and erlotinib bioassay

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice</th>
<th>Carcinogen</th>
<th>Treatment</th>
<th>Dose (mg/kg body weight)†</th>
<th>Comments on solution preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>12</td>
<td>B[a]P*</td>
<td>DMSO/EtOH‡</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1-2</td>
<td>12</td>
<td>B[a]P *</td>
<td>Gefitinib</td>
<td>0.8</td>
<td>Powdered gefitinib was dissolved in the vehicle at 5 mg/ml.</td>
</tr>
<tr>
<td>1-3</td>
<td>12</td>
<td>B[a]P *</td>
<td>Erlotinib</td>
<td>0.8</td>
<td>Powdered erlotinib was dissolved in the vehicle at 5 mg/ml.</td>
</tr>
<tr>
<td>2-1</td>
<td>12</td>
<td>B[a]P *</td>
<td>DMSO/EtOH</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2-2</td>
<td>12</td>
<td>B[a]P *</td>
<td>Gefitinib</td>
<td>1.6</td>
<td>Powdered gefitinib was dissolved in the vehicle at 10 mg/ml.</td>
</tr>
<tr>
<td>2-3</td>
<td>12</td>
<td>B[a]P *</td>
<td>Gefitinib</td>
<td>2.5</td>
<td>Powdered gefitinib was dissolved in the vehicle at 15 mg/ml.</td>
</tr>
<tr>
<td>3-1</td>
<td>12</td>
<td>B[a]P †</td>
<td>DMSO/EtOH</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3-2</td>
<td>12</td>
<td>B[a]P †</td>
<td>Gefitinib</td>
<td>2.5</td>
<td>Powdered gefitinib was dissolved in the vehicle at 15 mg/ml.</td>
</tr>
</tbody>
</table>

B(a)P*: one single injection at 100 mg/kg body weight in 0.2 ml tricaprylin.
B(a)P+: two injections and each at 100 mg/kg body weight in 0.2 ml tricaprylin. The second one was given one week after the first one.
Dose (mg/kg body weight)†: the dose is estimated using Equation (4).
DMSO/EtOH‡: DMSO and EtOH were mixed at 50:50 ratio volumetrically as the vehicle for gefitinib and erlotinib.

4.1.2 Results

4.1.2.1 Particle size characterization of the drug aerosols

We first determined the size distribution of the gefitinib (Figure 4-2 A) and erlotinib (Figure 4-2 B) aerosols. Particles generated with a Collison type atomizer are
characterized by a poly-disperse distribution as a result of the mechanism. The drug particles have a size distribution ranging from 0.02 μm to 0.6 μm with $\sigma_g$ (geometric standard deviation) of 1.8. The mass median aerodynamic diameter (MMAD) of the aerosols ranges from 0.12 μm to 0.2 μm with an ascending mass solution concentration from 5 to 15 mg/ml. Over 90% of the particles were smaller than 0.3 μm in diameter which is a favorable range for drug delivery in a mouse inhalation study (Raabe et al., 1977, 1988). Additionally, smaller size particles with a diameter down to 0.1 μm have a higher chance of being delivered into peripheral alveoli where adenomas and adenocarcinomas commonly occur.

We also measured the mass concentration of the drugs in the chamber by collecting the particles at the exit of the exposure chamber on a piece of filter media. The aerosol mass concentrations for 5, 10, and 15 mg/ml solutions were approximately 50, 110, and 170 μg/L, respectively. The respiratory minute volume (RMV) was estimated to be 0.025 L/min by Guyton’s formula (Guyton, 1947). Based on the information, we
calculated the equivalent dose to be 0.8, 1.6, and 2.5 mg/kg body weight.

4.1.2.2 Inhibitory effect of aerosolized gefitinib and erlotinib individually on lung tumor multiplicity and tumor load

Reduced body weight and mortality are clear signs of systemic toxicity. Based on general observations, treatment with either gefitinib or erlotinib aerosols did not have any observed adverse effect. No visible skin alterations were observed throughout the experiment. Body weight gain was normal. No mouse died in the treatment groups during the experiment. This indicates that the inhalation method was well-tolerated at the given doses of gefitinib.

In the first set of the bioassay, B[a]P induced an average of 5.0 ± 0.63 (n = 12) tumors per mouse in the solvent control group. The average tumor load of the control group was 0.30 ± 0.07 mm³. Aerosolized gefitinib showed some, although not significant, inhibitory effects on tumor multiplicity, while aerosolized erlotinib showed no effects on tumor multiplicity. However, both drugs significantly decreased the tumor load. Gefitinib decreased the tumor load by 39%, and erlotinib showed an even stronger effect with a decrease of 63.8% (P < 0.05).
Figure 4-3 Inhibitory effects of aerosolized gefitinib and erlotinib. A, Aerosolized gefitinib at 0.8 mg/kg body weight (5 mg/ml solution) after a single B[a]P injection; B, Aerosolized erlotinib at 0.8 mg/kg body weight (5 mg/ml solution) after a single B[a]P injection; C, Gefitinib at 1.6 and 2.5 mg/kg body weight (10 and 15 mg/ml solution, respectively) after one B[a]P injection; and D, Aerosolized gefitinib at 2.5 mg/kg body weight (15 mg/ml solution) after two B[a]P injections, one week apart. *$P < .05$, ***$P < .001$, compared with the vehicle control group. Inhibition rate percentage is also labelled in the figures presented as #%.

In the second bioassay, only gefitinib was tested with higher doses. The tumor incidence was 91.7% and 83.3% for the groups treated with gefitinib at 1.6 and 2.5 mg/kg body weight, respectively, compared with 100% in the vehicle control groups. The incidence was dose-dependent in the mice. B[a]P induced an average of 4.1 ± 0.62 (n = 12) tumors per mouse in the control group with an average tumor load of 0.78 ± 0.17 mm$^3$. Again, gefitinib (1.6 and 2.5 mg/kg body weight) showed an insignificant
trend to reduce the tumor multiplicity but decreased the tumor load by 46.2% and 56.4% 
($P < 0.05$), respectively.

Table 4-2 Effects of aerosolized gefitinib at varied doses (5, 10, and 15 mg/ml solution) on 
tumor incidence, tumor multiplicity, and tumor load.

<table>
<thead>
<tr>
<th>Dose mg/kg body weight</th>
<th>Tumor incidence (%)</th>
<th>Tumor multiplicity (mean±SE)</th>
<th>Inhibition (%)</th>
<th>Tumor load (mm³,mean±SE)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay1 (one dose of B[a]P)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
<td>5.0 ± 0.6</td>
<td>-</td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td>Erlotinib 0.8</td>
<td>100</td>
<td>4.73 ± 0.3</td>
<td>5</td>
<td>0.11 ± 0.02</td>
<td>63.8*</td>
</tr>
<tr>
<td>Gefitinib 0.8</td>
<td>100</td>
<td>3.1 ± 0.4</td>
<td>38.0</td>
<td>0.18 ± 0.02</td>
<td>39.0</td>
</tr>
<tr>
<td><strong>Assay 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>100.0</td>
<td>4.09 ± 0.62</td>
<td>-</td>
<td>0.78 ± 0.17</td>
</tr>
<tr>
<td>Gefitinib 1.6</td>
<td>91.7</td>
<td>3.00 ± 0.48</td>
<td>26.7</td>
<td>0.42 ± 0.08</td>
<td>46.2</td>
</tr>
<tr>
<td>Gefitinib 2.5</td>
<td>83.3</td>
<td>2.75 ± 0.57</td>
<td>32.8</td>
<td>0.34 ± 0.09</td>
<td>56.4*</td>
</tr>
<tr>
<td><strong>Assay 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
<td>16.6 ± 1.10</td>
<td>-</td>
<td>3.65 ± 0.36</td>
</tr>
<tr>
<td>Gefitinib 2.5</td>
<td>100</td>
<td>8.17 ± 1.49</td>
<td>49.8***</td>
<td>1.57 ± 0.31</td>
<td>56.9***</td>
</tr>
</tbody>
</table>

*: $P < 0.05$, **: $P < 0.001$. The solvent control for the three assays is the same 50% DMSO in 
EtOH.

Since there were fewer tumors in the first and second sets, two B[a]P injections were 
given in the third assay. The incidence rate in this case was 100% for both the control 
group and the gefitinib group. Two doses of B[a]P induced an average of 16.6 ± 1.1 
(n = 12) tumors per mouse with a tumor load of 3.65 ± 0.36 mm³. Gefitinib (2.5 mg/kg body 
weight) inhibited tumor multiplicity by 49.8% ($P < 0.001$) and the tumor load by 56.9% 
($P < 0.001$).

The results are summarized in Table 4-2 and presented in Figure 4-3. The combined 
results of gefitinib in the first and second assays showed a positive dose-response, and
in the three different sets of bioassays, gefitinib showed consistent inhibitory effects on the tumor load.

![Figure 4-4](image)

**Figure 4-4** Morphological alterations in female A/J mice treated with gefitinib. **A,** Alopecia at the region near the eyelid with average severity in gavage groups (200 mg/kg body weight). **B,** No alopecia occurred in the aerosol group (2.5 mg/kg body weight). **C,** Thinning hair in mice with gefitinib by oral gavage.

### 4.1.2.3 Observation of skin toxicity in the mice treated with gefitinib by gavage and via inhalation

We further compared the impact of administration methods on the toxicity of gefitinib. We observed changes in each group during the 10-week treatment (**Figure 4-4**). Alopecia at the region near the eyelid occurred in all the mice treated by oral gavage at doses of 100 and 200 mg/kg body weight (**Figure 4-4 A**). No alopecia was observed in the aerosol inhalation group (**Figure 4-4B**). One mouse out of five in the gavage group at a dose of 100 mg/kg body weight had thinning hair, while all of the mice in the gavage group at a dose of 200 mg/kg body weight had thinning hair at later stages of the experiment (**Figure 4-4 C**). Alternatively, mice in the aerosol groups had a normal
appearance. Interestingly, the severity of the alopecia and thinning hair seemed to be dose-dependent in those mice in the gavage groups. We also observed abnormal white spots on the liver upon gross examination in orally-administered gefitinib groups.

4.1.2.4 Pharmacokinetics of gefitinib and erlotinib

Gefitinib and erlotinib were both prepared in a 5 mg/ml 50% DMSO/EtOH solution. Mice were exposed to aerosols for 15 min and were sacrificed at designated time points after exposure. The time at the end of exposure was marked as time zero. Blood and lung samples were obtained at time 0, 10, 20, 30, 45, and 60 min, homogenized, extracted, and analyzed using HPLC. The following parameters were estimated as described earlier (Liao et al., 2004): maximum lung concentration ($C_{\text{max}}$); the mean area under the lung concentration-time curve (AUC); the area under the first moment curve (AUMC$_\infty$); the mean resident time (MRT) and the lung half time ($t_{1/2}$). The results are shown in Figure 4-5 and Table 4-3.

Table 4-3 Pharmacokinetic parameters of gefitinib and erlotinib for aerosol administration (Data shown in Mean ± SD).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gefitinib</th>
<th>Erlotinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/g)</td>
<td>1.81 ± 0.38</td>
<td>4.59 ± 0.70</td>
</tr>
<tr>
<td>AUC (µg/g·h)</td>
<td>2.37 ± 0.83</td>
<td>2.92 ± 0.52</td>
</tr>
<tr>
<td>AUMC$_\infty$ (µg/g·h$^2$)</td>
<td>2.14 ± 1.11</td>
<td>2.06 ± 0.23</td>
</tr>
<tr>
<td>MRT$_\infty$ (h)</td>
<td>0.90 ± 0.52</td>
<td>0.71 ± 0.21</td>
</tr>
<tr>
<td>$t_{1/2}$ (min)</td>
<td>93.3 ± 0.60</td>
<td>28.88 ± 1.31</td>
</tr>
</tbody>
</table>

The maximum concentration was achieved at time zero. The concentration in the lung decreased with time (Figure 4-5). The half-life of gefitinib and erlotinib in the lung was
about 90 min and 30 min, respectively. For erlotinib, low plasma concentration (0.11 μg/ml) was detected one hour after exposure, which was one tenth of the lung concentration (1.1 μg/g lung tissue). For gefitinib, a similar low plasma concentration was detected. The results implied that after aerosol delivery gefitinib and erlotinib mainly stayed in the lung for the first hour.

Figure 4-5 Concentration-time curves of A, gefitinib and B, erlotinib in the mouse lung following aerosol inhalation (Mean ± Standard Deviation).

4.1.3 Discussion

Tremendous progress in our knowledge of cancer at the cellular and molecular levels has been made in the past few decades. This knowledge initiates the rise of a novel class of drugs that are useful in targeted anti-cancer therapies. These drugs act on a well-defined target or biological pathway that, when activated or inactivated, causes regression or destruction of the malignant process (Hamilton et al., 2010). Although, at present, only a small set of anti-cancer therapies are administered based on the genetic alterations present in individual tumors in clinical practice, more targeted anti-cancer agents are being widely investigated in preclinical models and clinical trials. Studies of some agents even extend to cancer prevention studies.
EGFR (epidermal growth factor receptor) mutations currently represent one of the best-studied models as a target for anti-cancer therapies. Gefitinib and erlotinib are specific inhibitors of the EGFR kinase which effectively binds to the ATP-binding site of EGFR tyrosine kinase and blocks the downstream Ras signal transduction cascade.

Patients with EGFR-mutant non-small cell lung cancer who are treated with EGFR TKIs have the longest survival times. Gefitinib and erlotinib have been studied in vivo in limited cases for their chemopreventive effects on lung carcinogenesis. Five reports for gefitinib in murine models of lung cancer chemoprevention exist (Fujimoto et al., 2005; Yan et al., 2006; Ohashi et al., 2008, 2009; Kishino et al., 2009; Keith et al., 2010) as well as three other reports for erlotinib on its preventive effects (Liby et al., 2008; Zerbe et al., 2008; Dragnev et al., 2011). The studies are summarized in Table 4-4. Most of the models achieved positive results. Gefitinib dosages ranged from 5 mg/kg to 250 mg/kg/day, and erlotinib dosages ranged from 10 mg/kg to 200 mg/kg; however, dosage is not the only factor that affects the efficacy. Three reports provided direct evidence that gefitinib and erlotinib inhibited the phosphorylation of mouse EGFR in vivo (Zerbe et al., 2008; Ohashi et al., 2009; Keith et al., 2010). Although one of them used an activating EGFR transgenic mice model (Ohashi et al., 2009) to develop tumors driven by EGFR mutations, another one injected EGF into the mice to stimulate EGFR autophosphorylation (Keith et al., 2010). However, two others reported a feeble signal from phosphorylated EGFR in the collected tumors and uninvolved lung tissue (Yan et al., 2006; Kishino et al., 2009). These results from different models indicate that gefitinib and erlotinib can partly inhibit carcinogenesis independently of the expression level or phosphorylation status of EGFR in the mice, but their efficacy is strengthened
dramatically in models with activated EGFR mutations. Moreover, the cancer genome is complex and may harbor multiple mutations besides EGFR mutations. In the smoking-related lung cancer models, EGFR may not be closely associated with tumorigenic signaling. Even in the cancer genome in which EGFR mutations exist, the interference between the EGFR TKIs and the cancer genome is mutual. Mutations, besides activating EGFR signaling and determining the existence of specific proteins and enzymes, may either enhance or cancel the efficacy of EGFR TKIs. Thus, EGFR TKIs can only partially inhibit carcinogenesis due to the heterogeneity of mutant cells. This is the biological limit for the efficacy of EGFR TKIs.

In this study, we used a typical smoking-related lung cancer model in which B[a]P was the carcinogen. We observed a dose-response efficacy of aerosolized gefitinib ranging from 0.8 to 2.5 mg/kg body weight. With a much lower dose compared with other reports, we achieved 56.4% and 63.8% inhibition on tumor load by gefitinib and erlotinib, respectively, which is comparable with other reported results with higher oral doses (See refs in Table 4-4). It is the first time for gefitinib and erlotinib to show significant inhibitory effects at a dosage lower than 5 mg/kg body weight in smoking-related models. This may be attributed to the locoregional delivery via inhalation. Improvement of efficacy can be expected within the biological limit with increased dosages. However, 200 mg/kg body weight/day for the mouse is equivalent to twice that of 500 mg/day for humans (assuming the body weight of a human is 60 kg) (Yan et al., 2006), which is exactly the daily dose of gefitinib used for anti-cancer chemotherapy. Thus, although a relatively high dose of 200 mg/kg body weight (p.o.) showed striking inhibition on tumors (~90% on tumor load; Yan et al., 2006), lower
doses are preferred in the chemoprevention of lung cancer. For higher chemoprevention efficacy, except for increasing the dosage, a combination of EGFR TKIs and other agents is a promising strategy. There have been several attempts made with a variety of drugs such as bexarotene combined with erlotinib (Dragnev et al., 2011) and gefitinib combined with prostacyclin synthase overexpression (Keith et al., 2010). Further studies are needed in the future.

Despite the anti-cancer benefits of targeted therapies, their specificity also brings about concerns regarding frequently occurring adverse effects, such as skin, vascular, cardiac, and gastrointestinal toxic effects, because many of the signaling pathways exist not only in premalignant cells but also in normal healthy cells (Keefe and Bateman, 2012). In cancer treatment therapies, the balance between tumor control and adverse effects is usually tipped in favor of anti-cancer effects; however, in chemopreventive intervention, adverse effects should be managed, minimized, and avoided if possible. In general, chemopreventive agents must be easily tolerated in addition to being effective. They must cause no decline in the quality of life of the high-risk, but otherwise normal, individuals because people may take them for a prolonged time period. This is the toxicity limit for small molecular inhibitors, including gefitinib and erlotinib. Reduced body weight (Zerbe et al., 2008), light alopecia (Yan et al., 2006), and hepatotoxicity (Zerbe et al., 2008) were observed in mouse models. These symptoms appeared in the mice treated with relatively high doses of the agents (≥ 50 mg/kg body weight for gefitinib, and ≥ 30 mg/kg body weight for erlotinib, p.o. or i.p. injection). In our study, visible alopecia and thinning hair was observed in the A/J mouse treated with gefitinib (100 mg/kg body weight p.o.) for 10 weeks. We also found white spots in the mouse
liver which was reported in another study of erlotinib (Zerbe et al., 2008). The results showed that the adverse side effects of gefitinib were not coincidental.

To reduce the adverse side effects, one of the strategies is to use lower doses with conventional oral and intravenous routes. If the dose of the two inhibitors is lowered to 5 mg/kg body weight, no toxicities are shown when they are administered by gavage or i.p. injection (Zerbe et al., 2008; Kishino et al., 2009). However, with such a low dose, the efficacy of the inhibitors cannot be guaranteed unless tumorigenesis is totally driven by EGFR mutations (See Table 4-4).

Our strategy was to use a locoregional delivery method instead of systemic absorption of the agents. In this study, we showed the efficacy of aerosolized gefitinib and erlotinib, and, next, we tried to demonstrate the advantage of aerosol delivery from a pharmacokinetic (PK) perspective. The plasma, lung, and liver concentration of erlotinib following one single injection of 10 mg/kg erlotinib in male and female A/J mice was measured in a chemoprevention study (Zerbe et al., 2008). In that particular study, the maximum plasma erlotinib concentration appeared at 0.5 hr in male and female mice at 4.5 and 5.5 μg/ml, respectively. The maximum liver erlotinib concentration was also observed at 0.5 hr in males and females at 3.6 and 6.0 μg/g, respectively. The lung erlotinib concentration was only about half of that in the liver at each time point with 1.3 and 1.5 μg/g in males and females, respectively, with 0.5 hr as the peak level. In our study, erlotinib was given to female A/J mice via inhalation at a dose of 0.8 mg/kg. The maximum lung erlotinib concentration was observed at 0 min at 4.6 μg/g. At 0.5 hr, the lung concentration of erlotinib dropped to 2.1 μg/g, but was still higher than the peak lung level after oral administration. During the first hour, there was
no erlotinib detected in the plasma. By directly delivering erlotinib into the lung, we had a nearly two-fold higher lung erlotinib concentration with on tenth of the dose compared with the reported data. This demonstrates the advantage of aerosol delivery in the case of erlotinib. However, in the case of gefitinib, the advantage of aerosol inhalation is not as evident as it is in the case of erlotinib. Gefitinib was also aerosolized and given via inhalation at a dose of 0.8 mg/kg body weight. The maximum lung gefitinib concentration was also observed at 0 min at 1.81 μg/g and decreased with time to 1.0 μg/g at 1 hr. The blood, lung, and liver gefitinib concentration at 2 hrs after a single oral dose of 5 mg/kg body weight [¹⁴C]-gefitinib was reported to be 0.71, 8.33, and 13.42 equivalent μg/g (by converting radioactivity to concentration) in the rat (McKillop et al., 2004). Although the oral dose was about 5 times higher than the aerosol dose, the lung concentration of gefitinib in the orally-dosed rat was also 4.6 times higher than that in the aerosol-dosed mouse. The only advantage is that aerosol delivery leads to a much lower plasma concentration and avoided gefitinib accumulation in the liver before being transported to the lung. The differences in the PK properties between gefitinib and erlotinib might be due to the chemical properties of the agents and the measurement methods. Therefore, we showed that the inhalation route could deliver more or comparable amounts of EGFR TKIs into the lung, which is the target organ in lung cancer prevention, at a relatively low dose.

The last control factor in aerosol delivery is the device and technology limit. There is no doubt that particle generation methods and design of delivery systems are very important technical points in aerosol delivery studies. Once the efficacy and toxicity limit is understood for a certain drug, the next step is to improve the delivery efficiency
with controllable particle properties. One of the most important parameters is the particle size of the drug. The particle size is the key factor for particle transportation in the human body, especially in the pulmonary system and lung. Particles with a larger size have a much higher chance of becoming lodged in the oropharynx, nasopharynx, and trachea. Particles with smaller sizes have a much higher chance of being effectively transported and reaching the lung tissue. Furthermore, in our model, even smaller particle sizes are required so that the drug particles can reach the peripheral alveolar where adenoma mostly occurs. It is worth noting the differences between the pulmonary system in humans and in mice (Table 1-1). Human alveoli are 200-400 μm while mouse alveoli are only 39-80 μm. The diameter of the terminal bronchioli that directly connects to the alveoli is 0.6 mm for humans but only 0.01 mm for mice (Fox et al., 2007). In other words, mice have finer structures and, consequently, particles with much smaller sizes, down to nanometer size, are required while submicron can go far in the human pulmonary system. In this study, particle sizes generated using the Collison atomizer range from 20 nm to 0.3 micron which is better for the delivery of aerosolized drugs deep into mouse lungs compared with those used in previous studies (Dahl et al., 2000; Liao et al., 2004). However, for nanoparticles, supplying a large enough amount of mass for effectiveness might be a problem. More effort needs to be devoted to the design of particle generation and delivery systems so the size and the mass throughput requirement can be both controlled and satisfied for the applications.

In summary, the present study successfully delivered the EGFR TKIs via inhalation and demonstrated that aerosolized EGFR TKIs, gefitinib and erlotinib, inhibited B[a]P-induced tumorigenesis in an A/J mouse model individually without any
observable systemic and cutaneous side effects. Therefore, this preclinical study indicated that aerosol delivery can potentially offer significant advantages over oral administration against human lung cancer and provides a basis for future clinical evaluation.
## Table 4-4 Reports on gefitinib and erlotinib for the chemoprevention of lung carcinogenesis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dosage (mg/kg body weight)</th>
<th>Route</th>
<th>Mouse strain</th>
<th>Carcinogen</th>
<th>Inhibition on Lung Tumors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Multiplicity</td>
<td>Load</td>
</tr>
<tr>
<td>gefitinib</td>
<td>200</td>
<td>gavage</td>
<td>Wild-type</td>
<td>B(a)P, 100 mg/kg body weight, i.p. single injection</td>
<td>62.5% ***</td>
<td>89.3% ***</td>
</tr>
<tr>
<td>gefitinib</td>
<td>200</td>
<td>gavage</td>
<td>Female A/J</td>
<td>B(a)P, 100 mg/kg body weight, i.p. single injection</td>
<td>74.6% ***</td>
<td>94.9% ***</td>
</tr>
<tr>
<td>gefitinib</td>
<td>5</td>
<td>gavage</td>
<td>Female A/J</td>
<td>NNK, 10 mmol, i.p. single injection</td>
<td>59.7% ***</td>
<td>81.4% ***</td>
</tr>
<tr>
<td>gefitinib</td>
<td>50</td>
<td>gavage</td>
<td>Activating EGFR transgenic mouse</td>
<td>N/A</td>
<td>27%</td>
<td>N/A</td>
</tr>
<tr>
<td>gefitinib</td>
<td>50 i.p. injection</td>
<td>Wild-type FVB/N</td>
<td>urethane, 1 mg/kg body weight, i.p. single injection</td>
<td>100%</td>
<td>100%</td>
<td>Ohashi et al., 2009</td>
</tr>
<tr>
<td>gefitinib</td>
<td>100 i.p. injection</td>
<td>PGIS verexpressor</td>
<td>urethane, 1 mg/kg body weight, i.p. single injection</td>
<td>No significant change</td>
<td>No significant change</td>
<td>Keith et al., 2010</td>
</tr>
<tr>
<td>erlotinib</td>
<td>10 i.p. injection</td>
<td>Female A/J</td>
<td>urethane, 1 mg/g body weight i.p. single injection</td>
<td>-21%</td>
<td></td>
<td>Zerbe et al., 2008</td>
</tr>
<tr>
<td>erlotinib</td>
<td>100 mg/kg diet</td>
<td>Female A/J</td>
<td>Vinyl carbamate, two doses (0.32 mg/mouse), 1 week apart</td>
<td>52% (14 week); 36% (20 week)</td>
<td>57% (20 week)</td>
<td>Liby et al., 2008</td>
</tr>
</tbody>
</table>

*: P < 0.05, **: P < 0.001; ***: P < 0.0001. N/A: Not available in the references. PGIS: prostaglandin I2 (prostacyclin) synthase. PGIS overexpressors: transgenic mice with PGIS overexpression.
4.2 Lapatinib - A Dual Inhibitor of EGFR and Her2/Neu

Similar to gefitinib and erlotinib, lapatinib (GW-572016; GlaxoSmithKline, Middlesex, UK) is also a member of the 4-anilinoquinazoline group of tyrosine kinase inhibitors (TKIs). Lapatinib may have a therapeutic advantage over gefitinib and erlotinib because it is a dual inhibitor of EGFR (ErbB1) and HER2 (ErbB2). Lapatinib dytosylate (Tykerb®, GlaxoSmithKline) was approved for the treatment of Her2-positive advanced or metastatic breast cancer in 2007 and was further approved for the treatment of hormone-positive and her2-positive advanced breast cancer in 2010. The use of lapatinib for the treatment of lung cancer is still under clinical investigation. Lapatinib induced apoptotic cell death in lung cancer cell line A549 singly (Kurtze et al., 2011; Diaz et al., 2010) and in combination with other agents (Olaussen et al., 2009), and lapatinib significantly reduced tumor size and angiogenesis in the A549 tumor-bearing mice (Diaz et al., 2010). Another report showed that a single treatment with lapatinib could induce deaths in K Ras-mutated A549 NSCLC adenocarcinoma cells that are likely to be resistant to gefitinib and erlotinib (Kurtze et al., 2011). It is also reported that lapatinib alone was effective in two other cell lines, human lung cancer cell line H1650 (harboring E746-A750 deletion in exon19 of EGFR) and human lung cancer cell line H1781 (harboring G776V,C in-frame insertions in exon 20 of ErbB2 with wild-type EGFR), but was ineffective in human lung adenocarcinoma cell line H1975 (carrying two missense mutations of EGFR, L858R and T790M in exons 20 and 21 of EGFR) and H1975 xenograft in vivo (Suzuki et al., 2009). Instead, the combination of lapatinib and cetuximab was able to induce down-regulation of EGFR and apoptotic cell death in
H1975 cells and completely inhibited tumor growth in the H1975-bearing xenografts (Kim et al., 2008). However, although it has gained great success in the Her2 overexpression of breast cancer, lapatinib monotherapy had a low response in patients with advanced or metastatic NSCLC in a randomized Phase II trial (Ross et al., 2010).

Despite the disappointing results of the clinical trial, lapatinib is still worth investigating for the inhibition of lung cancer based on the success of in vitro studies in NSCLC cell lines. Lapatinib has not been studied to test its chemopreventive effects on lung carcinogenesis. In this work, lapatinib (obtained from National Cancer Institute) was administered both by oral gavage and aerosol administration for its inhibitory effects on lung tumorigenesis in a mouse model. This is the first time that lapatinib was evaluated in a chemoprevention model.

Figure 4-6 Lapatinib treatment protocol.

Female A/J mice at 6 weeks of age were given two doses of B[a]P (100 mg/kg body weight, one week apart) by i.p. injection in 0.2 ml of tricaprylin. The mice were randomly divided into 4 groups with 12 mice per group: 1) Aerosol control group (50% DMSO in ethanol); 2) Lapatinib aerosol group (50 mg /ml); 3) Gavage control group [the vehicle: 0.5 % hydroxypropylmethylcellulose (w/w) with 0.1% Tween 80 in water]; 4) Lapatinib gavage group (100 mg/kg body weight). The treatment started two weeks after the second B[a]P injection. The aerosol procedure was the same as in the case of
gefitinib and erlotinib. The mice were treated once a day, five days a week for 19 consecutive weeks (Figure 4-6).

![Figure 4-7](image)

*Figure 4-7* Body weight changes of lapatinib-treated groups compared to their corresponding control groups during treatment. Error bar: SE.

The body weight was monitored during the 19-week treatment. There was no significant difference between the treatment group and the control group for both the aerosol and the gavage administration (Figure 4-7). Two mice died in the gavage control group and one died in the gavage lapatinib group. The cause of death is unknown. None of the mice died in the aerosol treated groups. There was no visible skin alteration or other side effects in the mice during the treatment, although, lapatinib has been associated with common toxicities of EGFR inhibitors, such as skin rashes and diarrhea (Moy and Goss, 2007).
The tumor incidence was 100% for all the groups with two B[a]P injections. Lapatinib inhibited tumor multiplicity and tumor load by both means of administration, p.o. and inhalation (Figure 4-8). The results are summarized in Table 4-5. B[a]P induced an average of 13.70 ± 2.06 (n = 10) tumors per mouse in the gavage control group, with a tumor load of 4.34 ± 0.75 mm$^3$ per mouse. In the lapatinib-treated group (p.o.), tumor multiplicity and tumor load were 8.55 ± 1.49 tumors and 2.50 ± 0.46 mm$^3$ per mouse (n = 11), respectively. Lapatinib by oral gavage reduced tumor multiplicity and tumor load by 37.6% ($P = 0.0537$) and 42.4% ($P < 0.05$), respectively.

For the aerosol groups, B[a]P induced an average of 16.27 ± 1.15 tumors per mouse in the aerosol control group, with a tumor load of 3.65 ± 0.63 mm$^3$ per mouse. In the lapatinib-treated group (p.o.), tumor multiplicity and tumor load were 9.83 ± 2.48 tumors and 2.12 ± 0.55 mm$^3$ per mouse (n = 11), respectively. Lapatinib via inhalation inhibited the tumor multiplicity and tumor load by 39.6% ($P < 0.05$) and 41.7% ($P < 0.05$).
Table 4-5 Effects of lapatinib by oral gavage and aerosol inhalation on tumor multiplicity and tumor load. Tumor multiplicity and tumor load were presented as mean ± SE.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Tumor multiplicity</th>
<th>Inhibition (%)</th>
<th>Tumor load (mm³)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gavage Ctrl</td>
<td>-</td>
<td>13.70 ± 2.06</td>
<td>4.34 ± 0.75</td>
<td></td>
</tr>
<tr>
<td>Gavage L 100 mg/kg body weight</td>
<td>8.55 ± 1.49</td>
<td>37.6</td>
<td>2.50 ± 0.46</td>
<td>42.4*</td>
</tr>
<tr>
<td>Aerosol Ctrl</td>
<td>-</td>
<td>16.27 ± 1.15</td>
<td>3.65 ± 0.63</td>
<td></td>
</tr>
<tr>
<td>Aerosol L 50 mg/ml</td>
<td>9.83 ± 2.48</td>
<td>39.6*</td>
<td>2.12 ± 0.55</td>
<td>41.7*</td>
</tr>
</tbody>
</table>

*: P < 0.05. The aerosol solvent control group was given 50% DMSO in EtOH. The gavage control group was given 0.5 w.t.% hydroxypropylmethylcellulose (w/w) with 0.1 % Tween 80 in water.

In this study, aerosol lapatinib (50 mg/ml) is equivalent to approximately 9 mg/kg body weight (estimated using Equation 4), which is much lower than the gavage dose (100 mg/kg body weight). The fact that aerosolized lapatinib showed comparable inhibitory effects to that of orally-dosed lapatinib at a much higher dose (100 mg/kg body weight) indicated that aerosol delivery may have advantages over oral administration. Pharmacokinetic data are needed as the next step to interpret the efficacy results. It is also important to investigate the mechanism of lapatinib on B[a]P-induced tumors.

4.3 Wortmannin - A PI3K Inhibitor

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is crucial to many aspects of cell growth and survival. It is more frequently targeted by genomic aberrations including mutation, amplification, and rearrangement (Hennessy et al., 2005; Yap et al., 2008). Several abnormalities in the lung cancer pathways are located on or related to the PI3K/AKT (AKT: the human homologue of the viral oncogene v-akt)
signaling pathway including deletion mutations in P55γ (isoform of p85, found in lung cancer cell line HCC15), mutation and amplification of PI3KCA (up to 50%), translocations in the forkhead family (> 50% in alveolar rhabdomyosarcoma), mutations in PTEN, alternate splicing deletion AA6-273 in EGFRvIII (40%), activating mutation (10%) and amplification (variable) of EGFR, mutations in HER2/neu (10% in lung adenocarcinoma), and mutations in Ras (~30%) (Hennessy et al., 2005; Yap et al., 2008). Some of the abnormalities are acquired and frequently lead to therapy resistance. Thus, the PI3K/AKT pathway is an attractive target for the treatment of lung cancer and chemoprevention of lung carcinogenesis. Furthermore, as an activated pathway in cancer, it is easier to target than the lost tumor suppressors.

Wortmannin is a steroidal furanoid toxin originally isolated from the fungus Penicillium wortamanni in the 1950s (Brian et al., 1957). Later in 1993, it was discovered that wortmannin could potently, durably, and irreversibly inhibit phosphoinositide 3-kinase (PI3Ks) by covalent inactivation of the enzyme (reviewed by Knight 2010; Liu et al., 2012). Wortmannin has been shown to effectively inhibit the growth of human NSCLC in vitro and in vivo (Boehle et al., 2002; Hemstrom et al., 2006). It also enhanced the sensitivity of NSCLC cells to ionizing radiation by inhibiting PI3K (Zhang et al., 2010). However, it is restricted to preclinical studies as a tool because of its liver toxicity, low stability, and poor pharmacologic properties. Wortmannin is one example of the many abandoned drugs that encounter toxicity or delivery problems during the translation from preclinical to clinical studies. To overcome the disadvantages of wortmannin, two strategies have been proposed and conducted. One is modifying the molecular structure of wortmannin to produce PX-866
Chapter 4 Chemopreventive Effects of Small Molecular Inhibitors via Inhalation

(Ihle et al., 2004), and the other is binding wortmannin to water-soluble polymers to form conjugates (Varticovski et al., 2001; Gu et al., 2006). A recent report improved the polymer-conjugate method by altering the chain-structure of the conjugate to the core-shell structure and finalizing the conjugates into the nanoparticle form (Karve et al., 2012) for better solubility and lower toxicity. The same approaches can be applied to other drugs with similar disadvantages.

In this study, a different strategy was evaluated for the delivery of wortmannin into the lung. Wortmannin was nebulized into nanoparticles and directly delivered into the lung via inhalation, avoiding the potential systemic delivery barrier and toxicity. The inhibitory effects of wortmannin on lung tumorigenesis were compared between oral administration and inhalation administration in a mouse model.
Figure 4-9 Wortmannin treatment protocols. A, Tumors were induced by one B[a]P injection. For the oral gavage groups, the mice were treated once a day, five days per week. A-I, a dose of 1.4 mg/kg body weight was given to the mice for two weeks until a severe decrease in the body weight was observed; A-II, all treatment was suspended for four days including weekends; A-III, the oral dose was reduced to 1.0 mg/kg body weight until the end of the treatment. For the aerosol group, the solution was 2 mg/kg body weight. The mice were treated with aerosols for 10 min a day, five days per week for 20 consecutive weeks. B, Tumors were induced by two doses of B[a]P, one week apart. Only aerosolized wortmannin was evaluated using 2 mg/ml solutions. The mice received aerosol treatment five days per week for 19 consecutive weeks. B-I, the mice were subjected to wortmannin aerosols for 10 min per day for 5 weeks. B-II, the exposure duration was reduced to 5 min per day for the rest of the treatment.
Two bioassays were designed. In the first assay, female A/J mice at 6 weeks of age were given a single intraperitoneal (i.p.) injection of B[a]P (100 mg/kg body weight) in 0.2 ml of tricaprylin. Two weeks after the B[a]P injection, the mice were randomly divided into 4 groups with 12 mice per group: 1) Aerosol control group (50% DMSO in ethanol); 2) Aerosol wortmannin group (2 mg wortmannin/ml vehicle); 3) Gavage vehicle control group (0.4 % DMSO in saline); 4) Gavage wortmannin group (1.4 mg wortmannin / kg body weight for the first 2 weeks, and after that, adjusted to 1.0 mg / kg body weight). Two weeks after the i.p. injection, the mice were treated once a day, five days a week for 20 consecutive weeks (Figure 4-10). The solutions for aerosol groups were prepared weekly and stored at -20°C in aliquot. The solutions for gavage were freshly prepared on the same day of use. The inhalation exposures were conducted using a custom-built nose-only exposure chamber. The mice were exposed to aerosol for 10 minutes by placing their noses into the cone of each sub-compartment. The body

Figure 4-10 Body weight changes of wortmannin-treated groups compared to the control groups during treatment. The body weight was monitored as an indicator of the systemic toxicity of the drug. A and B refer to protocols in Figure 4-9. Error bar: SE.
weight of the mice was recorded on the same weekday every week during treatment. For the gavage groups, the amount of administered solutions was adjusted based on body weight.

In the first bioassay, aerosol treatment did not cause mortality during the 22 weeks of study. Aerosolized wortmannin (2 mg/ml solution, Wort-A I) caused little adverse effects on the body weight of mice (Figure 4-10 C). At the end of 22-weeks, the mean body weight of the mice in the Wort-A I group was 23.2 ± 0.58 g, compared with 21.8 ± 0.53 g in its corresponding solvent control group. There was no significant difference in the body weight between these two groups. For the gavage wortmannin group (Wort-G), a dose of 1.4 mg/kg body weight was given in the first two weeks until a dramatic decrease in the mean body weight as well as the deaths of two mice were observed. Medication administration was then modified so that the mice could recover their body weight for up to four days (including weekends). Additionally, the dose was reduced to 1.0 mg/kg body weight for the remainder of the experiment. Even with the lower dose, the body weight gain in the gavage wortmannin group was much lower than the gavage control group (Figure 4-10 C). At the end of the experiment, the mean body weight in Wort-G group was 19.74 ± 0.44 g, which was significantly lower ($P < 0.001$) than the average body weight of 23.30± 0.61 g for the control group, which was about 3.50 g less on average.
In the first assay, both aerosol and oral administered wortmannin were evaluated. In spite of the side effect causing body weight loss, orally dosed wortmannin showed a significant inhibitory effect on B[a]P induced tumors. The incidence rate of tumors in the Wort-G group was 50% (n = 10) while all the mice in the gavage control group (n = 12) had tumors by the end of the experiment (Table 4-6). As shown in Figure 4-11, wortmannin p.o. decreased tumor multiplicity by 85.5% (0.70 ± 0.26 tumors, n = 10, \( P < 0.001 \)) and tumor load by 78.0% (0.15 ± 0.14 mm\(^3\), n = 10, \( P < 0.05 \)) compared with 4.83 ± 0.82 tumors and 0.70 ± 0.19 mm\(^3\) in the gavage control group (n = 12).
Interestingly, wortmannin seemed to strikingly reduce the tumor multiplicity, with only a few larger tumors.

**Table 4-6** Effects of wortmannin by oral gavage and aerosol inhalation on tumor multiplicity and tumor load. Tumor multiplicity and tumor load are presented as mean ± SE.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Incidence</th>
<th>Tumor multiplicity</th>
<th>Inhibition</th>
<th>Tumor load (mm³)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>-</td>
<td>100%</td>
<td>4.83 ± 0.81</td>
<td>0.70 ± 0.18</td>
<td>-</td>
</tr>
<tr>
<td>Wort-G</td>
<td>1.0 mg/kg B.W. a</td>
<td>50%</td>
<td>0.70 ± 0.26</td>
<td>85.5%**</td>
<td>0.15 ± 0.14</td>
</tr>
<tr>
<td>AC 1</td>
<td>-</td>
<td>100%</td>
<td>3.73 ± 0.85</td>
<td>0.77 ± 0.22</td>
<td>-</td>
</tr>
<tr>
<td>Wort-A 1</td>
<td>2 mg/ml b</td>
<td>91.7%</td>
<td>1.83 ± 0.32</td>
<td>50.8%*</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>AC 2</td>
<td>-</td>
<td>100%</td>
<td>17.27 ± 1.15</td>
<td>3.65 ± 0.36</td>
<td>-</td>
</tr>
<tr>
<td>Wort-A 2</td>
<td>2 mg/ml c</td>
<td>91.7%</td>
<td>5.42 ± 2.12</td>
<td>66.7%**</td>
<td>0.72 ± 0.19</td>
</tr>
</tbody>
</table>

*: P < 0.05; **: P < 0.001; ***: P < 0.0001. The aerosol solvent control group was given 50% DMSO in EtOH. The gavage control group was given 0.4 % DMSO in saline. a: 1.4 mg/kg body weight for the first 2 weeks, 1.0 mg/kg body weight from the 5th week to the end of the protocol. b: Mice were subjected to aerosol treatment for 10 min. c: The exposure duration was 10 min for the first 5-week treatment, and then was reduced to 5 min until the end of the protocol.

The tumor incidence rate in the aerosol control group was 100%, while the incidence rate in the Wort-A group was 91.6%. No visible surface tumors were observed in one out of twelve mice (Table 4-6). Aerosolized wortmannin showed inhibitory effects on lung tumor multiplicity and tumor load significantly (Figure 4-11). Aerosolized wortmannin (2 mg/ml solution) decreased tumor multiplicity by 50.8% (1.83 ± 0.32, n = 12, P < 0.05) and tumor load by 79.7% (0.16 ± 0.05 mm³, n = 12, P < 0.05) compared with the solvent control group in which, on average, tumor multiplicity and tumor load were 3.73 ± 0.85 and 0.77 ± 0.22 mm³ (n = 12), respectively. All of the observed tumors in the aerosol wortmannin group were smaller than 1.0 mm in diameter.

In the second bioassay, only aerosolized wortmannin was examined. Two B[a]P
injections induced many more tumors than one single B[a]P injection (Figure 4-11). The incidence of tumors in the control group and the wortmannin treatment group were 100% and 91.7%, respectively. The aerosol control group had 16.27 ± 1.15 tumors (n = 12) per mouse on average, and the tumor load was 5.42 ± 2.12 mm$^3$ per mouse. Aerosolized wortmannin significantly reduced the tumor multiplicity and tumor load by 66.7% ($P < 0.001$) and 80.4% ($P < 0.0001$), respectively. The results of the second bioassay confirmed the conclusion from the first bioassay. However, the body weight in the wortmannin treatment group was 7.3% less than that of the control group ($P < 0.05$). Although the body weight difference was within 10% compared to the control group, it is unknown whether the decrease in the body weight gain was contributive to the inhibition of the tumors or not.

Pharmacokinetic data are necessary to interpret the inhibitory effects of wortmannin and the differences in toxicities when it is administered via oral gavage and aerosol inhalation. Since there was still a toxicity concern when using aerosol delivery, as shown in the second bioassay, a safer approach might be the combination of polymer coating and aerosol delivery.
Chapter 5

Chemopreventive Effects of

Natural Compounds via Inhalation
Chapter 5 Chemopreventive Effects of Natural Compounds via Inhalation

In this chapter, four plant-based natural compounds, resveratrol, caffeine, anthocyanins, and protocatechuic acid, are evaluated. These compounds exist ubiquitously in nature and are found in our daily diet. Intake of an appropriate amount of these compounds is generally non-toxic and beneficial to human health. All of the four compounds have shown cancer prevention effects \textit{in vitro} and \textit{in vivo}. However, their lung cancer prevention effect is controversial. It is suspected that their anti-cancer effect is related to their metabolism and pharmacokinetics. Thus, the hypothesis to be tested in this chapter is that aerosol delivery reduces exposure of other organs to the test agent and may increase the locoregional quantity of the compound in the lung.

5.1 Resveratrol

Resveratrol, trans-3,5,4'-trihydroxy-trans-stilbene (Figure 3-4 E), is a natural phytoalexin produced by a variety of plants (Whyte \textit{et al.}, 2007). It is particularly abundant in the skin of grapes and is, consequently, an ample component of red wine and grape juice. It is synthesized \textit{de novo} by plants in response to stress, injury, fungal infection, ozone exposure, or UV irradiation (Amri \textit{et al.}, 2012; Neves \textit{et al.}, 2012). The compound was first isolated in 1940 from the roots of hellebore and then from the roots

\footnote{The manuscript is under preparation. The evaluation of resveratrol in VC-induced mice was done by Dr. Huijing Fu as part of her PhD work.}
of Polygonum Cuspidatum, a plant used traditionally in Chinese and Japanese medicines. Since the 1990’s, there have been many studies that show the multiple beneficial health effects of resveratrol, including cancer prevention (Athar et al., 2007; Bishayee 2009), cardioprotection (Penumathsa et al., 2009; Wu et al., 2011), and prolongation of lifespan (Das et al., 2010; Lekli et al., 2010) in several species. There are many in vitro and in vivo studies providing a rationale to support the use of resveratrol in human cancer chemoprevention. The chemopreventive properties of resveratrol are attributed to its anti-oxidant, anti-inflammatory effects, and its ability to modulate multiple genes and enzymes (Whyte et al., 2007; Bishayee 2009; Bae et al., 2011).

Oral administration is the most convenient way to take resveratrol since the compound exists in wine and other foods. Nevertheless, bioavailability of resveratrol is always a concern. Because resveratrol has a short half-life in vivo due to its rapid metabolism and elimination, its application as a chemopreventive or a chemotherapeutic agent is limited. The most convincing in vivo evidence of the anti-tumor activity of resveratrol exists in the tumors it can contact directly. Topical application of resveratrol against non-melanoma skin cancer in the rodent model always shows consistent inhibitory effects (Athar 2007; Bishayee 2009). Moreover, oral administration of resveratrol significantly inhibits gastric and colorectal carcinogenesis in various rodent models (Bishayee 2009; Sengottuvelan et al., 2009). However, oral administration of resveratrol shows conflicting results or minor efficacy in other organs, such as the lung, despite the positive results from in vitro studies. This controversy remains unsolved, and the variation in efficacy of results is often attributed to the low bioavailability of
resveratrol. Strategies to improve the bioavailability of resveratrol include examining alternative delivery routes, developing innovative formulations, and modulating the metabolism of resveratrol (Bishayee 2009; Amri et al., 2012).

In this study, we addressed the inhibitory effects of resveratrol (Fisher Scientific Service, Thermo Fisher Scientific Inc., US) on lung carcinogenesis in A/J mice. We used inhalation delivery to increase the concentration of free resveratrol in the lung. The results were encouraging. Furthermore, we compared the pharmacokinetic profiles of the oral and inhalation routes. The results suggest that aerosol administration of resveratrol has significant advantages in comparison to oral administration, as shown in lung and plasma concentration-time curves.

5.1.1 Experimental Methods

5.1.1.1 Animal Bioassay

We first examined the effect of aerosolized resveratrol on lung tumors. Chemoprevention studies were performed using vinyl carbamate (VC)-induced and benzo[a]pyrene-induced (B[a]P) female A/J mouse models. Then, we conducted a pharmacokinetic study to determine the plasma and lung levels of resveratrol following oral and inhalation delivery to a Swiss mouse model.

In the chemoprevention study, we used the post-initiation protocol schematized in Figure 5-1 in carcinogen-induced A/J mouse models.
Chapter 5 Chemopreventive Effects of Natural Compounds via inhalation

Figure 5-1 Resveratrol treatment protocol. The aerosol treatment started two weeks after the 1st injection of vinyl carbamate or the single injection of benzo[a]pyrene.

At the age of eight weeks, to initiate tumor growth, carcinogen was administered to mice. The B[a]P group received one single intraperitoneal (i.p.) injection of B[a]P (100 mg/kg body weight) in 0.2 ml of tricaprylin. The VC group received two i.p. injections of VC (0.32 mg per injection in 0.2 ml sterile saline without pH adjustment) once a week for two consecutive weeks. The mice then were randomly divided into control and treatment groups with 12 mice per group. To dissolve resveratrol, 50% DMSO in ethanol was used as the vehicle. The treatment group was given resveratrol, while the corresponding control group was given vehicle only. To eliminate bias, all the other procedures were the same for the control and treatment groups. Specifically, we had five groups: 1) VC-Control group; 2) VC-Res7.5 (7.5 mg/ml Resveratrol in vehicle); 3) VC-Res15 (15 mg/ml resveratrol in vehicle); 4) BP-control group; 5) BP-Res15 (15 mg/ml resveratrol in vehicle). All the solutions were prepared fresh daily. The body weight of the mice was recorded on the same weekday every week during the treatment.

Two weeks after the first i.p. injection of either carcinogen, the treatment regimen began. The mice were treated with aerosolized resveratrol for 15 min per day, five days
a week, for 18 or 20 consecutive weeks. The mice in the VC groups were sacrificed by CO₂ asphyxiation after 18 weeks of treatment, and the mice in the B[a]P groups were euthanized by CO₂ asphyxiation after 20 weeks of treatment.

In the pharmacokinetics study, we used female NIH Swiss mice. For the aerosol administration, mice were exposed to the aerosol cloud for 15 min using a solution of 15 mg/ml resveratrol in the vehicle. Following exposure, the animals were sacrificed by cervical dislocation at 1 of the 7 designated time points (0, 5, 10, 15, 30, 60, and 120 min) in groups of three, where the end of the exposure marked zero time. Blood was obtained from the retro-orbital sinus and was collected into EDTA-pretreated eppendorf tubes. The tubes were centrifuged and the plasma removed and stored at -80 °C until assayed. The lung was severed at the carina, the esophagus and trachea were removed, and the lung was collected in a cryogenic vial, snap-frozen in liquid nitrogen, and later stored at -80 °C until assayed.

For the oral administration, groups of three animals received resveratrol by gavage at a dose of 75 mg/kg body weight in 0.2 ml solution (equivalent to 500 ppm in diet if a single mouse consumed 3 grams of food per day). The animals were sacrificed, and the plasma and lung samples were collected and processed as described above.

5.1.1.2 Tissue Assay Method

The lung tissue was weighed, frozen in liquid nitrogen, and crushed with a pre-cooled tissue pulverizer. The crushed tissue was collected in an eppendorf tube with 200 μl of distilled water and vortexed to form a uniform homogenate. After the addition of 500 μl of acetonitrile, the samples were vortexed for two minutes and centrifuged at 13,200
rpm for 15 min at 4 °C to precipitate the proteins. The supernatant was removed by a syringe, through a 0.2 μm Nylon syringe filter (Whatman GD/X, 13mm, and disposable), dried using nitrogen, and then reconstituted with 200 μl of methanol. The blood plasma was processed in the same way as the lung tissue homogenate. The mobile phase consisted of 50 mM ammonium acetate (pH = 2.2) and acetonitrile, with a ratio of 70:30. The flow rate of the mobile phase was 1 ml/min. The detection wavelength was 308 nm.

Resveratrol was quantified by HPLC. The HP 1100 series HPLC system that was used for separation consisted of an autosampler, a quaternary pump, a thermostatted column compartment, and a UV detector (Agilent Tech, Santa Clara, CA). An Agilent Zorbax SB-C18 (4.6 × 125 mm, 5.0 μm) column was used.

5.1.1.3 MTT Proliferation Method

Inhibitory effects on the growth and viability of cells were determined using the tetrazolium dye (MTT; 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay described in Horiuchi et al., 1988. An MTT kit was purchased from Promega Corporation (Madison, WI, U.S.). Human lung cancer cell line A549 was cultured in 100 μl medium, plated in 96-microwell plates with 5,000 cells / well, and incubated for 24 hr. Resveratrol at 15 μM and 30 μM was tested. The cells were incubated further for 48 hr with the drug. 100 μl of thawed PMS (phenazine methosulphate) solution was added to 2.0 ml thawed MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2-tetrazolium) solution, and was immediately well mixed. The MTS/PMS solution (20 μl) was pipetted to each well of a 96-well assay containing 100 μl of culture medium. After incubation for 4 hr at 37 °C in humidified 5% CO₂, the
optical density (OD) of the wells was determined using an ELISA microplate reader at an absorbance wavelength of 490 nm. Each plate contained “blank” background control wells holding an appropriate volume of media but no cells. All experiments were performed three times. The control cells were grown under the same conditions without the addition of resveratrol. Cell survival (% of control) was calculated relative to untreated control cells. We also validated the results using a second human lung cancer cell line, H1129, employing the same method as described above.

5.1.2 Results

5.1.2.1 Inhibitory effects of aerosol resveratrol on VC- and B[a]P-induced lung carcinogenesis in female A/J mice

The body weight of the mice was recorded weekly. In general, for both VC- and B[a]P-induced mice, there was no significant difference between the control groups and the resveratrol groups. Aerosolized resveratrol was well-tolerated at the given dose and showed no adverse systemic effect.

VC induced more tumors with a larger tumor load than B[a]P did. In VC-induced groups, aerosolized resveratrol showed no inhibitory effect on tumor multiplicity but did show a significant effect on tumor load (Figure 5-2 A). The decrease in tumor load was 26.3 % ($P < 0.05$) for VC-Res7.5 and 36.0 % ($P < 0.01$) for VC-Resv15. Increased inhibition was achieved with a higher dose. In the B[a]P-induced group, resveratrol (15 mg/ml resveratrol solution) showed significant inhibitory effects on both tumor multiplicity and tumor load (Figure 5-2 B). The average tumor multiplicity and tumor load were decreased by 37.1% ($P < 0.05$) and 72.0% ($P < 0.01$), respectively.
Figure 5-2 Inhibitory effects of resveratrol on VC- or B[a]P-induced tumors in A/J mice. A, vinyl carbamate-induced lung tumorigenesis; B, benzo[a]pyrene-induced lung tumorigenesis.

5.1.2.2 Effect of resveratrol on human non-small cell cancer cell line A549 in the MTT assay

The inhibitory effect on tumor cells as a function of resveratrol concentration was determined using human lung cancer cell line A549, and this was repeated in three experiments. For A549 cells incubated for 48 hrs, 15 μM resveratrol achieved around 10% reduction of the formazan product, while 30 μM resveratrol resulted in about a 30% reduction (Figure 5-3). Resveratrol showed an inhibitory effect on the proliferation of lung cancer cell line A549, and the effect was dose-responsive. We also validated the results using a second human lung cancer cell line, H1299. The maximum inhibition rate occurred at 24 hr.
Figure 5-3  Inhibitory effects of resveratrol in the cell proliferation of human non-small cell lung cancer cell line A549. Inhibition rate percentage is shown on top of each bar.

### 5.1.2.3 Lung distribution of resveratrol following oral and inhalation delivery

The exposure chamber provided an aerosol cloud of dry particles of resveratrol at a concentration of $0.169 \pm 0.014 \text{ mg/L}$ for 15 mg/ml resveratrol solution. This is equivalent to 2.5 mg/kg body weight. The size of the aerosols ranged from 0.02 μm to 0.5 μm. The MMAD was 0.2 μm and the GSD was 1.8.

In Figure 5-4 B, the concentrations in the lung and plasma are given as a function of time following aerosol treatment. For the lung, the concentration immediately following exposure, which was taken as time 0, was 8.9 nmol / g lung. This concentration fell slowly with time, so that even at 2 hours, the concentration was 0.45 nmol / g. The plasma level following inhalation delivery, at time 0, had a concentration of 0.89 nmol / mL, which was about one tenth of the lung concentration. This was the highest concentration observed. With time, the plasma concentration decreased. At 2 hours, the concentration was 0.1 nmol/L, which was low but detectable.
Figure 5-4 Blood and lung concentrations of resveratrol in mice dosed by gavage (A) and via inhalation (B), respectively.

Figure 5-4 A shows the lung and plasma levels following oral administration. For the lung, the peak concentration of 1.43 nmol/g appeared at 5 min after treatment. Within 10 min, the concentration fell rapidly to about 0.21 nmol/g. At one hour, the level of resveratrol was not measurable. For the blood, the peak concentration of 3.78 nmol / mL also appeared at 5 min, which was about 2.5 times higher than the peak lung concentration. It also fell rapidly within 10 min, to a level of 0.16 nmol / mL.

Table 5-1 The pharmacokinetic parameters calculated for gavage dosing (75 mg/kg body weight) and aerosol inhalation (equivalent to 2.5 mg/kg body weight).

<table>
<thead>
<tr>
<th></th>
<th>Gavage</th>
<th>Aerosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Lung</td>
</tr>
<tr>
<td>AUC, nM·min</td>
<td>40</td>
<td>18.6</td>
</tr>
<tr>
<td>AUC_{lung}/AUC_{plasma}</td>
<td>0.465</td>
<td>28.7</td>
</tr>
<tr>
<td>AUMC_{∞}, nM·min²</td>
<td>228.2</td>
<td>69</td>
</tr>
<tr>
<td>MRT, min</td>
<td>12.3</td>
<td>17.3</td>
</tr>
<tr>
<td>t_{1/2}, min</td>
<td>2.2</td>
<td>33.8</td>
</tr>
<tr>
<td>C_{max}, nM/g or nM/ml</td>
<td>3.78</td>
<td>1.43</td>
</tr>
<tr>
<td>t_{max}, min</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
Chapter 5 Chemopreventive Effects of Natural Compounds via inhalation

Other parameters estimated from the concentration-time curve are given in Table 5-1. The AUC\textsubscript{lung} following aerosol delivery (at a dose of 2.5 mg/kg body weight) was 284 nM·min, whereas the corresponding parameter following oral dosing (at a dose of 75 mg/kg body weight) was only 19 nM·min. Thus, the AUC\textsubscript{lung} was 14 times greater when the drug was administered by aerosol, even with a lower dose. Conversely, the AUC\textsubscript{plasma} following aerosol delivery was 10 nM·min, which was lower than that of 40 nM·min following gavage.

For aerosol delivery, the \((\text{AUC}_{\text{lung}}/\text{AUC}_{\text{plasma}})_{\text{inhalation}}\) value was 28.7. However, after gavage, the \((\text{AUC}_{\text{lung}}/\text{AUC}_{\text{plasma}})_{\text{gavage}}\) was only 0.47. Thus, the therapeutic advantage of these two delivery modalities can be estimated as follows:

\[
\left(\frac{\text{AUC}_{\text{lung}}}{\text{AUC}_{\text{plasma}}}\right)_{\text{inhalation}} / \left(\frac{\text{AUC}_{\text{lung}}}{\text{AUC}_{\text{plasma}}}\right)_{\text{gavage}} = 28.7 / 0.47 = 61 \quad (5)
\]

\((\text{AUC}_{\text{lung}}/\text{AUC}_{\text{plasma}})\) is like a partition coefficient which describes the distribution of a substance in the lung and the blood \textit{in vivo}. When \((\text{AUC}_{\text{lung}}/\text{AUC}_{\text{plasma}})\) is greater than 1, it means that more of the test agent is delivered to the lung than to the blood. The ratio obtained using Equation (5) is a measure of the difference in the partition coefficients of two delivery approaches, inhalation and oral gavage. When the ratio is greater than 1, it means that aerosol inhalation can deliver more resveratrol to the lung than oral gavage. Thus, the greater the ratio, the larger is the advantage of aerosol inhalation. In the case of resveratrol, it is clear that aerosol delivery has a significant advantage over the oral route.
5.1.3 Discussion

In this study, resveratrol delivered by inhalation was evaluated as a chemopreventive agent in a VC- and a B[a]P-induced A/J mouse model. The results of our study show that resveratrol delivered by inhalation is effective in reducing the load of VC-induced tumors and in reducing both the number and the load of B[a]P-induced lung tumors in A/J mice. As a supplement, we also evaluated the effect of resveratrol on the proliferation of cancer cells in vitro using an MTT assay. Consistent inhibition rates were obtained in repeated experiments.

Positive results are not rare in most studies of resveratrol as a cancer preventive agent. However, where lung cancer is concerned, negative results have predominated in the in vivo studies. Hecht et al (1999) and Berge et al (2004) both failed to show an effect of dietary resveratrol on lung carcinogenesis in vivo. Hecht et al (1999) did not explain the reason. The results described by Berge et al (2004) resembled the findings of Hecht et al (1999); however, they suspected that the bioavailability of resveratrol might be the key. In their studies, no resveratrol or its conjugates were found in the tissues of animals receiving the compound in the diet in their studies. Interestingly, in some other studies, intact resveratrol has been detected in mouse lung tissue after a single intragastric delivery (Vitrac et al., 2003; Sale et al., 2004). Other studies argued that it is the rapid clearance in the mouse tissue rather than the poor bioavailability that adversely affects the prevalence of resveratrol (Asensi et al., 2002; Yu et al., 2002). In this study, we also observed measurable levels of resveratrol in mouse lung tissue after a single dose by gavage. Thus, the negative results in previous in vivo studies can possibly be attributed to the hypothesis that resveratrol given in the diet may not have reached the lung “in
sufficient concentrations or biological active form” (Berge et al., 2004) due to its low bioavailability and poor metabolism (if the measurement method that was used was sensitive enough).

To solve the delivery problem, three strategies are often considered: looking for a substitute for resveratrol, such as DMU (trans 3,4,5,4’-tereamethoxystilbene) (Sale et al., 2004) which has a better metabolism profile; exploring proper formulation solutions to overcome the delivery limitation; and trying other administration routes, as we did in our study. Inhalation is not a novel route for lung and respiratory diseases; however, it is novel for resveratrol. In this study, we demonstrated that resveratrol has inhibitory effects on lung carcinogenesis *in vivo* and further interpreted the results from the perspective of pharmacokinetics. Plasma and lung levels of resveratrol were determined in mice following oral and inhalation delivery. The results showed that inhalation delivery of resveratrol resulted in obviously, significantly higher levels in the lung when compared with oral dosing. Analysis of the concentration as a function of time suggests that a pharmacokinetic advantage of 61 is obtained in the mouse between inhalation and oral routes. Further studies on aerosol delivery of resveratrol are merited for verification and improvement. Ultimately, resveratrol can certainly be considered as a potential chemopreventive agent for lung carcinogenesis.

### 5.2 Caffeine

Studies on caffeine’s antitumor effects date back to the 1970s (Kakunaga, 1975; Nomura, 1976; Nomura, 1977; Theiss and Shimkin, 1978). In the last 20 years, caffeine has always been studied together with tea or tea polyphenols, such as EGCG, to
elucidate the mechanisms of the cancer chemopreventive effects of the tea (reviewed by Yang et al., 2011). Caffeine itself has been studied extensively by Conney et al. for its chemopreventive effects on UVB-induced skin carcinogenesis in mouse models (reviewed by Conney et al., 2007, 2008, 2013). The study was further extended to caffeine analogues (Rogozin et al., 2008) and caffeine sodium benzoate (Lu et al., 2007), which might potentially inhibit UVB-induced skin tumorigenesis. Caffeine was also shown to prevent carcinogen-induced lung tumorigenesis in mouse models. Urethane and NNK, two chemicals existing in tobacco smoke, are two commonly used carcinogens in the evaluation of caffeine. In earlier studies, varied schedules and doses of urethane and caffeine were tested, which lead to dissimilar conclusions on its inhibitory effects on lung adenoma (Theiss and Shimkin, 1978; Armuth V and Berenblum I, 1981). Later, NNK became the focal carcinogen in animal assays. Caffeine consistently showed inhibitory effects on NNK-induced lung tumorigenesis in murine models, independently of other components of black tea or green tea (Xu et al., 1992; Chung et al., 1998; Lu et al., 2006). It also inhibited dibenzo[a,l]pyrene (DBP) transplacental carcinogenesis in the lungs of mouse offspring born to mothers administered with DBP (Castro et al., 2008). However, its inhibitory effect was confounded by its suppressive effects on metabolism and negative effect on body weight. The exact mechanism of the inhibition produced by caffeine is still not entirely known. One postulated mechanism stated that the increased metabolism of NNK or DBP in the liver due to caffeine-induced hepatic cytochrome P450 enzymes will result in the decreased bioavailability of the carcinogens to the lung (Chung et al., 1998; Castro et al., 2008). Other mechanisms are possibly involved in the activities of
In all the reports above, caffeine was given systemically (via i.p. injection or p.o.) or topically. In this study, caffeine (purchased from Sigma-Aldrich, St. Louis, MO) was delivered by aerosol inhalation in the hope of reducing systemic exposure to caffeine and removing the confounding effects mentioned above.

Figure 5-5 Caffeine treatment protocol.

Figure 5-6 Body weight changes of caffeine-treated mice compared with mice in the control group during treatment. Error bar: SE.

Aerosolized caffeine was evaluated using a post-initiation protocol in a
B[a]P-induced A/J mouse model. Female A/J mice at 6 weeks of age were given two doses of B[a]P (100 mg/kg body weight, one week apart) by i.p. injection in 0.2 ml of tricaprylin. The mice were randomly divided into two groups, with 12 mice per group: 1) Aerosol control group (50% DMSO in ethanol); 2) Caffeine group (10 mg/ml). The treatment started two weeks after the second B[a]P injection. The aerosol procedure was the same as in the case of gefitinib and erlotinib. The mice were treated once a day, five days a week, for 19 consecutive weeks (Figure 5-5). Aerosolized caffeine did not negatively influence the body weight at this dose (Figure 5-6).

Table 5-2 Effects of aerosolized caffeine on tumor incidence, tumor multiplicity, and tumor load. Tumor multiplicity and tumor load are presented as mean ± SE.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Incidence (%)</th>
<th>Tumor multiplicity</th>
<th>Inhibition (%)</th>
<th>Tumor load (mm³)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DMSO/EtOH</td>
<td>100</td>
<td>16.27 ± 1.15</td>
<td>-</td>
<td>3.65 ± 0.36</td>
<td>-</td>
</tr>
<tr>
<td>Caffeine 10 mg/ml</td>
<td>100</td>
<td>11.08 ± 2.02</td>
<td>31.9*</td>
<td>2.03 ± 0.43</td>
<td>44.3*</td>
</tr>
</tbody>
</table>

*: P < 0.05. The aerosol solvent control group was given 50% DMSO in EtOH.

The incidence of lung tumors was 100% in the aerosol control group and the caffeine treatment group (Table 5-2). Aerosolized caffeine significantly reduced the tumor multiplicity and tumor load by 31.9% (P < 0.05) and 44.3% (P < 0.01). Aerosolized caffeine reduced the number of relatively large tumors in each category (Figure 5-7). It seems that caffeine could suppress tumor growth but was not able to inhibit tumor initiation.
The results are presented in Figure 5-7. This study showed that aerosolized caffeine partly, but significantly, inhibited the tumor multiplicity and the tumor load in the mouse lungs without decreasing body weight; it is indicated that inhibitory effect of caffeine on lung tumorigenesis is related to other mechanisms rather than by decreasing the body weight. Caffeine tended to accumulate in the blood. The serum level was about 4 times higher than that in the liver and in the lung (Che et al., 2011). Consequently, aerosol delivery may be the solution to effectively increase the bioavailability of caffeine in the mouse lungs.
5.3 Anthocyanins and Protocatechuic Acid (PCA)

Anthocyanins are a class of water-soluble highly pigmented flavonoids which could impart peculiar red-orange to blue-violet colors to many fruits and vegetables, such as berries, red grapes, purple sweet potatoes, and red cabbages. The produce appears red, purple, or blue according to the pH and the plant’s structural features (Hou et al., 2004; Li et al., 2012; Massela et al., 2012). The anthocyanins are glycosides of anthocyanidins. Complex glycosylation patterns result in hundreds of anthocyanins, but fewer than a dozen aglycones (anthocyanidins) are identified. Seven anthocyanidins - delphinidin, luteolinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin - are found in commonly consumed plant food, containing the same flavylium ion backbone (Figure 5-8). The bioactivity of the resulting anthocyanins may vary with the molecular structures. Diet rich in anthocyanins has been suggested to prevent from a variety of disorders, such as cancer, cardiovascular disease, aging, inflammation, diabetes, arthritis, and obesity, as well as bacterial infections (Hou et al., 2004; Aqil et al., 2012; Li et al., 2012). PCA has demonstrated similar health benefits as anthocyanins.

Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA; Figure 3-4) is a simple
phenolic compound ubiquitously distributed in nature. PCA exists in many daily consumed foods, such as bran and grain, brown rice, fruits, nuts, and spices, as well as plant-derived beverages, and is also the biologically active component of some medicinal plants (reviewed by Tanaka et al., 2011; Masella et al., 2012). Although the concentration of PCA in fruits and vegetables is very low (PCA food content is summarized by Masella et al., 2012), complex polyphenols, such as anthocyanins, are good sources of PCA in vivo because PCA is the main metabolite of these polyphenols.

The cancer chemopreventive activities of anthocyanins have been studied in vitro using multiple cancer cell lines and in vivo in animal models for esophageal, colon, skin and lung cancer. Many mechanisms have been proposed, including antioxidant effects, Phase II enzyme activation, anti-cell proliferation, induction of apoptosis, anti-inflammatory effects, anti-angiogenesis, anti-invasiveness, and induction of differentiation (reviewed by Wang and Stoner, 2008). Based on search results from Pubmed (pubmed.com), in vitro studies in cell lines are far more than prevalent in vivo studies in animal models. Low bioavailability might hinder the in vivo evaluation of anthocyanins because the amount of anthocyanins that reach the target tissue might be insufficient to have biological activity despite the strong effects in vitro. The chemopreventive effects of PCA have also been evaluated extensively in vitro (Yin et al., 2009) and in vivo in several models of chemically induced carcinogenesis in laboratory animals (Reviewed by Tanaka et al., 2011). Most of the studies in animal models focus on carcinogenesis in the digestive system, and inhibition of tumorigenesis was observed when PCA was administered in the initiation phase and in the promotion/progression of carcinogenesis at doses of 200-2000 ppm in the diet (Tanaka et al., 2011). However,
there is only one report about lung carcinogenesis, and its disappointing result is that PCA did not inhibit NNK-induced lung tumorigenesis in mice (Mori et al., 1999). The authors did not provide an explanation for this result.

Current studies on the chemopreventive effects of anthocyanins and PCA on lung carcinogenesis are not enough. There are still several questions: Does an anthocyanin or PCA inhibit lung carcinogenesis in vivo? Does the carcinogen or protocol matter? If there is an inhibitory effect, is it attributed to the intact agents or their metabolites? What is the main mechanism? If it does not have any inhibitory effect, is the lack due to the bioavailability or to the agents themselves? In this study, we started with the first question. Anthocyanins extracted from black raspberries and PCA were evaluated in a B[a]P-induced mouse model. Intact agents were delivered via aerosol inhalation to avoid complications from metabolites. The core hypothesis is that aerosol inhalation can increase the bioavailability of anthocyanins in the lung.
Anthocyanins were prepared by Dr. Gary Stoner’s group from black raspberries (Wang et al., 2009) and are mainly glycosylated cyanidin. The molar percents of the three major anthocyanins were 12% cyanidin-3-O-glucoside, 34% cyanidin-3-O-(2β-xylosylrutinoside, and 53% cyanidin-3-O-rutinoside (Figure 3-4). PCA was purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Female A/J mice at six weeks of age were given two doses of B[a]P (100 mg/kg body weight, one week apart) by i.p. injection in 0.2 ml of tricaprylin. The mice were randomly divided into three
groups with 12 mice per group: 1) Aerosol control group (50% DMSO in ethanol); 2) Anthocyanins group (5 mg/ml); 3) PCA group (12 mg/ml). The treatment started two weeks after the second B[a]P injection. The aerosol procedure was the same as described before. The mice were treated once a day, five days a week, for 19 consecutive weeks (Figure 5-9).

![Figure 5-10](image)

**Figure 5-10** Inhibitory effects of aerosolized PCA and aerosolized anthocyanins individually. **A**, Inhibitory effects of aerosolized anthocyanins and aerosolized PCA on tumor multiplicity and tumor load. **B**, Tumor size distribution in the control group, anthocyanins, and PCA groups. The tumor number clumps the tumors in all the mice. *: $P < 0.05$. Inhibition rate percentage is labeled on top of the bars.

The body weight was monitored during the 19-week study. There was no significant difference between the treatment groups and the control group for the mice treated with anthocyanins or PCA (Figure 5-9). There was no mortality in all the groups. The tumor incidence was 100% for the aerosol control group. In the anthocyanins-treated mice, one out of twelve had no visible surface tumors in the lung, with a tumor incidence of 91.7%. The PCA-treated group had the same tumor incidence of 91.7%.
The results are summarized in Table 5-3. In the anthocyanins-treated group, the tumor multiplicity and tumor load were 13.92 ± 3.24 tumors and 2.54 ± 0.30 mm\(^3\) per mouse (n = 12), respectively. As presented in Figure 5-10 A, aerosolized anthocyanins showed marginal, but insignificant, inhibitory effects on both tumor multiplicity (reduced by 14.5\%) and tumor load (reduced by 30.4\%). From the tumor size distributions (Figure 5-10 B), we can find an indication that anthocyanins reduced the number of tumors with a diameter greater than 0.7 mm. Thus, the inhibitory effect of aerosolized anthocyanins is not conclusive. Higher doses of anthocyanins may be required to achieve significant inhibition.

In the PCA-treated group, the tumor multiplicity and tumor load were 8.50 ± 0.48 tumors and 2.01 ± 0.45 mm\(^3\) per mouse (n = 12). PCA significantly reduced the tumor multiplicity and tumor load, by 47.8\% (P < 0.01) and 44.9\% (P < 0.01), respectively. As shown in Figure 5-10 B, tumors with a diameter between 0.5 to 0.8 mm were reduced by PCA. It is suggested that the inhibitory effect of PCA on B[a]P-induced lung tumorigenesis at least partly depends on the intact PCA itself. There are two reasons that might explain the negative results in Mori’s study (Mori et al., 1999). One is the
difference between carcinogens, and the other is that the amount of PCA in the bioactive form in the lung is insufficient. Pharmacokinetic and immunohistological studies are necessary to interpret the results.
Chapter 6

Chemopreventive Effects of the Combination of Aerosolized Budeosnide and Dietary Polyphenon E
Chapter 6 Chemopreventive Effects of the Combination of Aerosolized Budesonide and Dietary Polyphenon E

Lung carcinogenesis is a complex mixture of miscellaneous mutations and lesions at different stages of development. It is reasoned that a combination of multiple agents that target different pathways can enhance the inhibition of lung tumorigenesis and reduce adverse side effects. Thirteen combinations of agents were evaluated in this dissertation. Since aerosolized budesonide and aerosolized gefitinib were shown to inhibit B[a]P-induced tumorigenesis in the A/J mice consistently, these two agents were selected to be combined with other agents that were also effective. Additionally, other combinations, such as a combination of oral pioglitazone and aerosolized 2-deoxy-D-glucose, which target the glucose metabolism, were also assessed in the mouse model. In this chapter, the combination of aerosolized budesonide and dietary polyphenon E is discussed in detail as an example of the combination group. Other combinations were briefly discussed in the Appendix.

Budesonide is a synthetic glucocorticoid that has been used for the treatment of bronchial asthma and chronic obstructive pulmonary disease in inhalation therapy for over two decades. Budesonide as a single agent has been shown to prevent the formation of lung tumors in mice treated with smoke-associated compounds, such as Benzo[a]pyrene (Wattenberg et al., 1997; Wattenberg et al., 2000; Wang et al., 2003; Estensen et al., 2004; Balansky et al., 2006), vinyl carbamate (Pereira et al., 2002), and

① The manuscript has been prepared and will be submitted to Molecular Carcinogenesis.
4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNK) (Casto et al., 2011), per os (Pereira et al., 2002; Pereira et al., 2006; Wang Y et al., 2003; Balansky et al., 2006) and via inhalation (Wattenberg et al., 1997; Wattenberg et al. 2000; Estensen et al., 2004; Fu et al., 2011).

Polyphenon E (PPE) is a well-defined, pharmaceutical-grade mixture of green tea polyphenols that contains at least five catechins. Epigallocatechin-3-gallate (EGCG) is the most abundant catechin comprising 65% of PPE. PPE administered by diet (Clark and You, 2006; Zhang et al., 2010; Yan et al., 2006) and by aerosol, also significantly inhibited B[a]P induced lung tumorigenesis in A/J mice (Yan et al., 2007; Fu et al., 2009). PPE in drinking water can also inhibit NNK-induced lung tumorigenesis in A/J mice (Lu et al., 2006). PPE and budesonide are capable of inhibiting tumorigenesis induced by multiple carcinogens associated with cigarette-smoking.

The use of budesonide or PPE in combination with other agents is a new and emerging approach to lung cancer chemoprevention. There are four reports for budesonide and two for PPE. Inhaled budesonide combined with other agents, such as dietary myo-inositol (Wattenberg and Estensen, 1997) and orally-administered pioglitazone (Fu et al., 2011), achieved greater inhibitory effects in B[a]P-induced A/J mouse models. Budesonide in the diet combined with orally-administered R115777 (ZarnestraMT) was shown to inhibit tumors in a vinyl carbamate-induced A/J mouse model (Alyaqoub et al., 2007). A recent study investigated the efficacy of concurrent and sequential administration of combinations of budesonide, suberoylanilide hydroxamic acid (SAHA), bexarotene (targretin), and atorvastatin on NNK-induced lung tumors in A/J mice and showed that budesonide combined with other agents
achieved a greater efficacy than did the individual agents (Casto and Pereira, 2011). Dietary PPE and aerosolized difluoromethylornithine (DFMO) combined were not more effective than PPE alone (Anderson et al., 2008). However, PPE in drinking water combined with atorvastatin in the diet synergistically inhibited NNK-induced lung tumorigenesis.

In this study, we investigated the chemopreventive efficacy of the combination of aerosolized budesonide and dietary PPE. Budesonide (purchased from Sigma-Aldrich, St. Louis, MO) inhibited both tumor multiplicity and tumor load in our experiment as a single agent. With the addition of PPE (obtained from National Cancer Institute, Division of Cancer Prevention, Bethesda, MD), the combination led to greater efficacy.

### 6.1 Experimental Methods

#### 6.1.1 Animal Bioassay

Animals were housed with wood chip bedding in the environmentally-controlled, clean-air room with a 12-hour light-dark cycle at constant temperature and a relative humidity of 50%. Drinking water and diet were supplied ad libitum. Female A/J mice at seven weeks of age were given a single intraperitoneal (i.p.) injection of B[a]P (100 mg/kg body weight) in 0.2 ml of tricaprylin. Two weeks after the B[a]P injection, the mice were randomly divided into 6 groups with 12 mice per group: 1) Aerosol control group (50% DMSO in ethanol); 2) Budesonide group (1.125 mg/ml); 3) Diet control group (AIN-76A diet, Dyets, Inc., Bethlehem, PA, with 0.3% sugar); 4) PPE group (7.5g/kg diet); 5) Aerosol-and-diet control group; 6) Budesonide and PPE combination
Chapter 6 Chemopreventive Effects of the Combination

group (bud + PPE). The treatment started two weeks after the B[a]P injection. The mice were treated once a day, five days a week for 20 consecutive weeks (Figure 6-1).

Figure 6-1 Treatment protocol. All treatment began two weeks after the i.p. injection of B[a]P. Aerosol groups were subjected to aerosol delivery treatment for two minutes per day, five days per week. All diet control groups received an AIN-76 diet with 3% sugar (wt/wt), while all polyphenon E groups received an AIN-76 diet with 3% sugar and 0.75% PPE. The total treatment duration continued for 20 weeks. All mice were sacrificed 22 weeks after the B[a]P injection.

All the solutions were fresh prepared on the same day they were used. The inhalation exposures were conducted using a custom-built nose-only exposure chamber. The mice were exposed to aerosols for two minutes by placing their noses into the cone of each sub-compartment. The body weight of the mice was recorded on the same weekday every week during the treatment. For the dietary PPE groups, mice were fed AIN-76A purified powder diet at a dose of 7.5 g/kg diet. A formulated diet was prepared using a KitchenAid mixer (St. Joseph, MI), mixing for at least 40 min twice a week, and the diet available in the cages was changed daily.

6.1.2 Cell Proliferation Assay

Human NSCLC cell lines A549 and H1299 were purchased from American Type
Culture Collection (Manassas, VA) and cultured in RPMI 1640 media with 10% fetal bovine serum (Gibco®, Invitrogen, Carlsbad, CA) and a penicillin and streptomycin cocktail (Gibco). All cells were cultured in a humidified incubator at 37 °C at 5% CO₂. A549 and H1299 cells were seeded in 96-well plates (BD Falcon®, Franklin Lakes, NJ) at a density of 2×10³ cells per well. Twenty-four hours after seeding, cells were exposed to a different concentration of budesonide, PPE, or the combination of the two as indicated for 24, 48, or 72 hr. The proliferation rate was measured by Alamar Blue, a cell variability indicator with resazurin as its active ingredient, which could be converted to the fluorescent molecule, resorufin, by active cells. Alamar Blue (Invitrogen, Carlsbad, CA) was added to the culture media at 10% of the media volume during the last 10 hr of the exposure period. Fluorescence was detected on a Synergy HT microplate reader (Biotek, Winooski, VT) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

### 6.1.3 Combination Effects Analysis

Data for the combination effect analysis were obtained from experiments in two lung cancer cell lines, A549 and H1129. A linear model was used to test for combination effects of budesonide and PPE (i.e., antagonism, additivity, and synergy). The model is

\[ y_{ijkl} = B_i + P_j + BP_{ij} + C_k + e_{ijkl}, \]  

where \( y \) is live cell numbers for the \( k \)th cell line and the \( l \)th replication, \( i = 1, 2, \) or \( 3; B \) is the budesonide effect, \( i = 1 \) or \( 0; P \) is the PPE effect, \( j = 1 \) or \( 0; C \) represents the difference between two cell lines, \( k = 1 \) for A549 and 0 for H1299; \( e \) is an error term. The statistical analyses were performed in R (www.r-project.org).
6.2 Results

6.2.1 Inhibitory effects of aerosolized resveratrol on B(a)P-induced lung tumors in A/J mice

We first determined the characteristics of the budesonide aerosols generated by our custom-made Collison type atomizer. The size distribution of budesonide aerosols from 1.125 mg/ml solution was measured. The GMD was 0.065 μm, and the GSD was 1.8. The size falls in the window of breathable particles for mice to ensure as high a delivery efficiency as possible.

The mass concentration in the exposure chamber was measured at 13.8 μg/L. The dose of budesonide to the animal was estimated to be 25 μg/kg body weight per day (calculated from a published method assuming that the average mouse body weight is 25 g; ref: Wattenberg et al., 1997). The dose of PPE to the animal was estimated to be 900 mg/kg body weight per day, assuming that the average mouse body weight is 25 g and one mouse consumes 3 gram of diet each day.

At the end of the 22-week study, the body weight of the mice in the treatment groups were 21.9 ± 0.5, 26.6 ± 0.69, and 24.2 ± 1.08 g, for budesonide, PPE, and the combination group, respectively. The corresponding control groups (the aerosol control group, the diet control group, and the aerosol-and-diet control group) were 22.6 ± 0.47, 25.6 ± 1.28 and 24.6 ± 0.72 g, respectively. There is no significant difference in the body weight between the treatment groups and the control groups, which means that the treatment of budesonide and PPE did not cause systemic toxicity.
Figure 6-2 Inhibitory effects of aerosolized budesonide, dietary polyphenon E and their combination on lung tumor multiplicity and tumor load. A, Dietary PPE (0.75 w.t.% in diet); B, Aerosolized budesonide (1.125 mg/ml, 2 min/day); C, The combination of aerosolized budesonide and dietary PPE; D, Decreased percentage of tumor multiplicity and tumor load by the three treatments. BUD: budesonide; PPE: Polyphenon E; Diet Ctrl: Diet control; Sol_Ctrl: Solvent control; Sol_Diet_Ctrl: Solvent and diet control. Inhibition rate percentage is labeled on the top of each bar. Error: SE.

The tumor incidence rate in all the control groups and PPE group is 100%, while the incidence rate in the groups treated with budesonide, PPE, and their combination is 75%, 100%, and 83.3% (Table 6-1), respectively. Tumor multiplicity and tumor load were both reduced in the treatment groups (Figure 6-2). As a single agent, aerosolized budesonide showed inhibitory effects on lung tumor multiplicity and tumor load compared with their own control groups. Budesonide decreased tumor multiplicity by 55.2% (1.83 ± 0.28, n = 12) and tumor load by 66.7% (0.26 ± 0.09 mm$^3$) compared with
the solvent control group in which, on average, tumor multiplicity and tumor load are $4.1 \pm 0.62$ and $0.78 \pm 0.17 \text{mm}^3$ ($n = 11$), respectively (Figure 6-2 B). However, PPE showed no significant difference from that of the diet control group with a 7.5 g/kg diet. B[a]P induced an average of $5.67 \pm 0.94$ and $6.45 \pm 1.17$ (n=11) tumors per mouse in the diet control group and PPE group, respectively. The average tumor load was $1.42 \pm 0.25 \text{mm}^3$ per mouse in the diet control group, while the average tumor load in the PPE group was $1.35 \pm 0.28 \text{mm}^3$ (n = 12) (Figure 6-2 A).

Table 6-1 Effects of aerosolized budesonide, dietary polyphenon E and their combination on tumor incidence, tumor multiplicity, and tumor load.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Tumors</th>
<th>Inhibition</th>
<th>Tumor load</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>incidence (%)</td>
<td>multiplicity (mean±SE)</td>
<td>(%)</td>
<td>(mean±SE) (mm$^3$)</td>
<td>(%)</td>
</tr>
<tr>
<td>SolCtrl</td>
<td>100</td>
<td>4.09 ± 0.62</td>
<td>-</td>
<td>0.78 ± 0.17</td>
</tr>
<tr>
<td>BUD</td>
<td>75</td>
<td>1.83 ± 0.53</td>
<td>55.2*</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td>Diet Ctrl</td>
<td>100</td>
<td>5.67 ± 0.94</td>
<td>-</td>
<td>1.42 ± 0.25</td>
</tr>
<tr>
<td>PPE</td>
<td>100</td>
<td>6.45 ± 1.17</td>
<td>-13.8</td>
<td>1.35 ± 0.28</td>
</tr>
<tr>
<td>Sol_Diet_Ctrl</td>
<td>90.9</td>
<td>6.00 ± 1.00</td>
<td>-</td>
<td>1.58 ± 0.46</td>
</tr>
<tr>
<td>BUD+PPE</td>
<td>83.3</td>
<td>2.08 ± 0.42</td>
<td>65.3*</td>
<td>0.2 ± 0.06</td>
</tr>
</tbody>
</table>

* $P < 0.05$. Abbreviation: SolCtrl: solvent control; BUD: budesonide; Diet Ctrl: diet control; PPE: polyphenon E; Sol_Diet_Ctrl: solvent+diet control.

In the aerosol and diet control group, the B[a]P-induced lung tumor multiplicity and tumor load were $6.00 \pm 1.00$ (n = 11) and $1.58 \pm 0.46 \text{mm}^3$, respectively. With the treatment of budesonide combined with PPE, tumor multiplicity and tumor load were reduced to $2.08 \pm 0.42$ (n= 12) and $0.20 \pm 0.06 \text{mm}^3$, respectively. The combination of budesonide and PPE was more effective in preventing lung tumors than either drug
alone, inhibiting tumor multiplicity by 65.3% and tumor load by 87.3% (Figure 6-2 C).

Figure 6-3 Tumor size distribution in aerosolized budesonide, dietary Polyphenon E and their combination groups compared with the corresponding control groups.

The tumor size distributions are shown in Figure 6-3. Aerosolized budesonide, individually, completely inhibited tumors greater than 1.0 mm and reduced the number of medium sized tumors (0.7-0.8 mm). The combination of the two agents also eliminated tumors greater than 1.0 mm and reduced the number of tumors with sizes from 0.7 to 1.0 mm (Figure 6-3).

6.2.2 An additive inhibitory effect of budesonide and PPE on the proliferation of human lung cancer cells

Human non-small lung cancer cells A549 and H1299 were exposed to budesonide or PPE. Our results showed that the proliferation of A549 and H1299 cells decreased significantly in a time-dependent manner following either 50 μM budesonide treatment, 20 μg/ml PPE, or their combination. Additionally, at any time point, the combination
inhibited cell proliferation more effectively.

![Figure 6-4](image)

**Figure 6-4** An additive combined effect of budesonide and polyphenon E in the inhibition of cell proliferation of human non-small lung cancer cells: A, A549 cell lines; B, H1299 cell lines.

When cells were treated with the combination of budesonide and PPE, a strong growth-inhibition was observed (**Figure 6-4**). 50 μM budesonide inhibited proliferation to 48.4% in A549 cells and 61.6% in H1299 cells, while 20 μg/ml PPE inhibited the proliferation to 81.5% in A549 cells and 67.6% in H1299 cells. When the agents were combined (at the same dose as in individual dosing), proliferation rates decreased to 32.9% and 40.0% in A549 and H1299 cells, respectively.

| Estimate | SE   | t-value | Pr (>|t|)  |
|----------|------|---------|--------|
| Budesonide | 1.053 | 0.056   | 18.823 | < 2e-16 *** |
| Polyphenon E | -0.237 | 0.079   | -2.992 | 0.00383 ** |
| Cell      | -0.132 | 0.079   | -1.675 | 0.098       |
| Budesonide:Polyphenon E | -0.120 | 0.079   | -1.518 | 0.133       |

*** < 0.001, ** < 0.01, * < 0.05.

To further demonstrate the combinational effects of these two agents, the linear model was used. As shown in **Table 6-2**, both budesonide and PPE had significant effects in inhibiting cell growth, but there was no agent interaction effect. Thus, the two agents
exhibited independent, additive inhibitory effects on cell growth.

6.3 Discussion

Lung carcinogenesis is most likely associated with multiple pathways and steps. Thus, using combinations of multiple agents that work on different pathways or steps is a promising strategy and has advantages over the single-agent strategy. First, combinations of two or more agents may lead to additive or synergistic effects on tumorigenesis inhibition. Second, combinations of agents allow lower doses of one or more agents in the combination to minimize potential adverse side effects.

Many mechanisms have been proposed for the chemopreventive efficacy of budesonide, but none of them have been identified. It is suggested that budesonide exerts its effects through growth arrest via Mad2/3 and through apoptosis via Bim/Blk and by inference caspase-8/9 (Yao et al., 2004). Moreover, budesonide prevented DNA hypomethylation (Tao et al., 2002; Wang et al., 2003; Pereira et al., 2006) and protected the lung from environmental-cigarette-smoke-induced alterations of microRNA expression (Izaotti et al., 2010). The mechanisms of PPE’s chemopreventive efficacy of lung carcinogenesis have been proposed based on studies with EGCG, which is believed to be the most active compound in PPE in cell lines (Yang et al., 2009), but recent studies have also shown that the preventive efficacy of EGCG on mice lung tumorigenesis requires the presence of other tea catechins in both dietary and aerosol administration (Fu et al., 2009; Zhang et al., 2010). PPE can interact and bind with multiple proteins (Bode and Dong, et al., 2009) to arrest or trigger signaling or metabolic pathways that lead to the inhibition of carcinogenesis (reviewed in Yang et al.,
That budesonide and PPE act by different mechanisms provides the rationale for the combination of the two agents.

Moreover, budesonide is shown to be hepatotoxic in murine models. Orally-dosed budesonide at 2.4 mg/kg in diet (equivalent to 288 μg/kg body weight, assuming that one mouse with a body weight of 25 g consumes 3 g of diet a day) for 6 weeks was shown to induce intense microRNA variations in the liver of mice (Izzotti et al., 2010). Budesonide in the drinking water at 50 μg/kg body weight was tumorigenic in male rat livers (Ryrfeldt et al., 1992). PPE at a high dose of 2000 mg/kg body weight p.o. is toxic in the mouse (Chang et al., 2003). However, PPE has poor bioavailability, and high doses are required for its efficacy in the rodent (Chen et al., 1997; Lamber et al., 2003). Adverse side effects should be avoided for chemopreventive purposes. Therefore, the combination of these two agents at lower doses could potentially reduce the adverse side effects with similar or even stronger efficacy compared with the individual agent.

In the present study, we studied the combination of budesonide and PPE both in vitro and in vivo. The in vitro test using human cell lines A549 and H1299 provides evidence that these two agents exhibit independent and additive inhibitory effects on tumor cell growth. In vivo, budesonide was administered by aerosol to the mice, which has been demonstrated to inhibit lung carcinogenesis individually, and PPE was given in the diet. Aerosolized budesonide (1.125 mg/ml) inhibited tumor multiplicity and tumor load by 55.2% and 66.7%, while dietary PPE (7.5 mg/kg diet) did not show its inhibitory effects. When the two agents were combined, the combination inhibited tumor multiplicity by 65.4% and tumor load by 87.3% without exerting an additional impact on mouse body weight compared with the control group. It is noticeable that budesonide aerosolized
with the same nebulizer at a higher dose (2.25 mg/ml, 2 min) inhibited tumor load in the same mouse model by 78% (Fu et al., 2011). The efficacy of the combination is slightly higher than budesonide alone at a higher dose. It is reported that dietary PPE inhibited tumor load dose-dependently by 65% and 78% at 0.5 and 1.0 mg/kg diet, but showed no effect on tumor multiplicity (Yan et al., 2006). However, in our study, PPE showed minor inhibitory effects on tumor load. The reason is unknown and might be due to the variations in animals and batches of agents. Compared with the reported results, the combination still has the advantage. Thus, we have demonstrated that the combination is more effective than either agent alone in decreasing tumor multiplicity and tumor load.

In humans, inhaled budesonide does not show inhibitory effects on tumors as strongly as in the mice. In a randomized Phase II clinical trial, inhaled budesonide (Pulmicort Turbuhaler®) administered to current cigarette smokers with bronchial dysplastic epithelium had no effect on the regression of bronchial dysplastic lesions or on the prevention of new lesions in current smokers compared with the placebo group, but it did result in a modest decrease in p53 and Bc/II protein expression in bronchial biopsies (Lam et al., 2004). Another randomized Phase II trial (Lazzeroni et al., 2010; Veronesi et al., 2011) of inhaled budesonide in high-risk individuals with CT screen-detected lung cancer revealed a significant effect of budesonide on the regression of existing target nodules by per-lesion analysis but no effect on new lesions. The results from these randomized clinical trials suggested that budesonide had some activity in precancerous lesions. Consequently, combination of these agents to produce a stronger, collaborative effect is a promising strategy for the future.

In summary, combined aerosolized budesonide and dietary PPE was demonstrated to
be more efficacious in inhibiting lung tumors than the individual agent. Their additive effects were observed both \textit{in vivo} and \textit{in vitro}. The low dose combination of these two agents is preferable for chemoprevention use since the low dose combination exhibits stronger inhibitory effects on tumor multiplicity and tumor load with no observable side effects.
Chapter 7

Evaluation of Drug Particle Deposition

in the Mouse Lung
Chapter 7 Evaluation of Drug Particle Deposition in the Mouse Lung

The systemic dose is the quantity of the drugs administered into the body. In the aerosol delivery system, the systemic dose is the total amount of drugs (mass or amount of substances) inhaled by the mouse, measured by mass or molars of the drug per kg of body weight. For the gavage and the parenteral administration, the systemic dose is explicit, and could be calculated accurately from the drug concentration in the formulation and the fed/injected/infused volume. For the drug mixed in the diet or dissolved in the beverage, the systemic dose could be estimated when the daily consumption of diet or beverage is assumed based on observation. The consumption might be gender and species specific but should not vary considerably for the commonly-used animal models. However, for the aerosol delivery system, the relationship between the systemic dose and the operative parameters, mainly the solution concentration and the exposure time, is implicit and device-dependent. Thus it is necessary to characterize the specific experimental setup and aerosol generation device used in the aerosol delivery system and, to establish the relationship between the systemic dose and the operative parameters. The dose for aerosol delivery could be estimated from the aerosol concentration, the exposure time, and the respiratory minute volume of the lab animals (Section 3.2).

The efficacy of the drugs in the lung depends on the level of the bioactive form of the drug in the lung. The lung level is determined by the amount of drugs that finally reach and stay in the lung, measured by the mass or moles of the drug per unit mass of the lung. The measurement method for the mass in the lung is the same for all the
administration routes, but the factors that influence the lung level in the cases of oral or aerosol administrations are different. For oral administration, the lung level depends on metabolism and clearance, thus the formulation, i.e. the molecular structure or the drug carriers (such as nanoparticles and polymers), is critical to oral administration. Conversely, for aerosol administration, the lung level is not quite related to the metabolism. The properties of the drug particles (clusters of drug moleculars) and transport behavior of the particles inside the respiratory tract determine the lung deposition of the drug aerosols and, subsequently, the lung level of the drug.

So far, many agents have been evaluated using the current experimental set-up (Figure 3-2), and encouraging results were achieved. In the current delivery system, the only two operable parameters are solution concentration and exposure time. The relationship between the operable parameters and the lung deposition has not been established for the current system. Therefore, it is necessary to characterize the system to better understand it and for to eventually improve on its design.

Concerning the particle mass deposition in the mouse lung, there are three levels of information to obtain with ascending resolution: the total mass deposited in the lung, the mass deposited in each lung lobe, and the mass distribution pattern in the lung. In this chapter, the total mass deposited in the lung was measured as the first step to give an insight into the problem.

7.1 Materials and Methods

7.1.1 Particle generation and characterization

The anticancer drug gefitinib was selected as the model medicine. The aerosol mass
concentration in the exposure chamber and the mass deposited in the lung were measured with varied solution concentrations and exposure time. The testing matrix is laid out in Table 7-1.

Table 7-1 Testing matrix for the current aerosol delivery system.

<table>
<thead>
<tr>
<th>Solution Concentration, mg/ml</th>
<th>1</th>
<th>2.5</th>
<th>7.5</th>
<th>15</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure Time, min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td></td>
<td></td>
<td></td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td></td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>20 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>√</td>
</tr>
</tbody>
</table>

The custom-built Collison atomizer was used to generate polydispersed particles. The applied air pressure was 20 psi. This same atomizer and conditions were used in previous animal bioassays as the aerosol generator. Gefitinib powder was dissolved in 5 ml 50% DMSO in ethanol with concentrations of 1.0, 2.5, 7.5, 25, and 50 mg/ml, which covers the frequently-used concentration range in the animal bioassays, to evaluate the effect of solution concentration. In addition, two vials of 5 ml solutions at 15 mg/ml were also prepared to evaluate the effect of the exposure time. The aerosol mass concentration was estimated using the gravity method. The aerosol residue was collected in the filter media at the exit of the animal exposure chamber. The filter media was conditioned under a vacuum overnight before and after the particle loading, and then weighed. The air flow rate through the filter media was controlled at 2.0 liters per minute with an orifice.

The single-capillary electro spray technique was used to generate monodispersed particles. Three sizes were selected: 50, 100, and 200 nm. Since the mass throughput of the single-capillary electro spray is very low, the gravity method is not sensitive enough for the measurement. A piezobalance dust monitor (KD12, Kanomax, Japan, Inc.) was
used to measure the aerosol mass concentration in the chamber.

### 7.1.2 Animal experiment and tissue assay methods

Female NIH-Swiss mice were used in this study. The mice were divided into seven groups (Iressa1, Iressa2.5, Iressa7.5, Iressa25, Iressa50, Iressa10min, and Iressa20min), corresponding to seven testing conditions, with four mice per group. The mice were exposed to drugs with different aerosol concentrations in the chamber and for a designated duration. They were sacrificed immediately following the aerosol treatment. Blood was collected from their retro-orbital sinuses. The whole lung was harvested from each mouse and snap-frozen in liquid nitrogen after removing the trachea and blood. The liver, spleen, and kidney were also harvested for future analysis. To separate the plasma from the hemoglobin, the blood samples were centrifuged at 13,200 rpm for 15 min. The plasma was transferred to another labelled eppendorf tube and stored in -80°C. The lung and other organs were also stored at -80°C for future analysis.

The lung tissue was weighed, frozen in liquid nitrogen, and crushed with a pre-cooled tissue pulverizer. The crushed tissue was collected in an eppendorf tube containing 200 μl of DMSO and vortexed to form a uniform homogenate. After the addition of 500 μl of acetonitrile, the samples were vortexed again for two minutes and centrifuged at 13,200 rpm for 15 min at 4°C to precipitate the proteins. The supernatant was removed and dried using nitrogen and then reconstituted with 200 μl of methanol. The plasma was processed in the same way as the lung tissue homogenate. The mobile phase consisted of 50 mM ammonium acetate (pH = 2.2) and acetonitrile, with a ratio of 80:20. The flow rate of the mobile phase was 1 ml/min. The detection wavelength was 348 nm.

Gefitinib was quantified by HPLC. The HP 1100 series HPLC system consisted of an
autosampler, a quaternary pump, a thermostatted column compartment, and a UV detector (Agilent Tech, Santa Clara, CA). An Agilent Zorbax SB-C18 (4.0 × 150 mm, 3.5 μm) column was used for separation.

### 7.2 Results and Discussion

For each testing condition listed in Table 7-1, the mass concentration of gefitinib aerosols was estimated. The results are shown in Table 7-2 and Figure 7-1.

![Figure 7-1 Mass concentration of gefitinib aerosols in the animal exposure chamber.](image)

The aerosol mass concentration positively correlated with the solution concentration, and the correlation was almost linear. The aerosol mass concentration ranged from 12.3 to 179.8 μg/L. The maximum aerosol concentration was achieved with a solution of 50 mg/ml, which was the solubility limit in the current solvent system (50% DMSO in ethanol). For the 15 mg/ml solution, the mass concentration was measured for 10 min and 20 min as a simple verification for the measurement method. The aerosol mass concentrations were 0.160 and 0.180 μg/L for 10 min and 20 min, respectively. The two
values were close to each other, and the deviation was acceptable.

Table 7-2: Mass concentrations of gefitinib aerosols in the animal exposure chamber with varied solution concentrations and exposure time using the custom-made Collison atomizer.

<table>
<thead>
<tr>
<th>Group</th>
<th>Filter Loading μg</th>
<th>Aerosol Concentration μg/L</th>
<th>Solution Concentration mg/ml</th>
<th>Consumed Solution Volume, ml</th>
<th>Consumed Mass, mg</th>
<th>Solution Utilization Efficiency ‡, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iressa1*</td>
<td>370</td>
<td>12.3</td>
<td>1.0</td>
<td>4.00</td>
<td>4.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Iressa2.5*</td>
<td>380</td>
<td>18.0</td>
<td>2.5</td>
<td>3.50</td>
<td>8.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Iressa7.5*</td>
<td>2340</td>
<td>78.0</td>
<td>7.5</td>
<td>3.75</td>
<td>28.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Iressa 25*</td>
<td>7680</td>
<td>256.0</td>
<td>25.0</td>
<td>3.00</td>
<td>75.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Iressa50*</td>
<td>18120</td>
<td>604.0</td>
<td>50.0</td>
<td>3.25</td>
<td>162.5</td>
<td>11.2</td>
</tr>
<tr>
<td>Iressa10min†</td>
<td>3190</td>
<td>159.5</td>
<td>15.0</td>
<td>2.50</td>
<td>37.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Iressa20min‡</td>
<td>7190</td>
<td>179.8</td>
<td>15.0</td>
<td>4.25</td>
<td>63.7</td>
<td>11.3</td>
</tr>
</tbody>
</table>

*: The number stands for the solution concentrations. For example, Iressa1 means the solution concentration is 1 mg/ml, and so on. The dosing time, or exposure time, was 15 min. †: The solution concentration is 15 mg/ml, and the exposure time is 10 and 20 min, respectively. ‡: The utilization rate was calculated by (aerosol mass concentration × exposure time × flowrate)/Consumed mass × 100%.

The liquid consumption was also measured by gross observation. A 5 ml solution was added to the atomizer in each case. 3.5 ml on average was consumed during a 15 min of atomization process, and the liquid consumption did not correlate with the solution concentration. A 2.5 ml and a 4.25 ml solution was sprayed out for a 10 min and a 20 min of atomization, respectively. Compared to the aerosol loaded in the filter media, only 9.0% of the drugs on average consumed by the atomizer were utilized. The results suggested that most of the drug particles were lost in the tubings and the diffusion dryers before they entered the chamber.
Table 7-3 The concentration and total deposition of gefitinib in the mouse lung for polydispersed particles.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung Concentration, nmol/g tissue</th>
<th>Lung Weight, g</th>
<th>Total lung deposition, μg</th>
<th>Plasma Concentration, nmol/ml</th>
<th>Body weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iressa1</td>
<td>1.20±0.24</td>
<td>0.21±0.01</td>
<td>0.11±0.02</td>
<td>0.03±0.03</td>
<td>25.8±0.8</td>
</tr>
<tr>
<td>Iressa2.5</td>
<td>2.63±0.64</td>
<td>0.17±0.02</td>
<td>0.19±0.05</td>
<td>0.18±0.07</td>
<td>26.0±1.6</td>
</tr>
<tr>
<td>Iressa7.5</td>
<td>6.10±1.01</td>
<td>0.17±0.02</td>
<td>0.46±0.07</td>
<td>0.40±0.11</td>
<td>26.0±1.2</td>
</tr>
<tr>
<td>Iressa 25</td>
<td>14.16±5.19</td>
<td>0.18±0.02</td>
<td>1.06±0.32</td>
<td>0.77±0.09</td>
<td>26.6±1.0</td>
</tr>
<tr>
<td>Iressa50</td>
<td>24.22±8.63</td>
<td>0.16±0.00</td>
<td>1.74±0.62</td>
<td>0.96±0.18</td>
<td>26.5±0.9</td>
</tr>
<tr>
<td>Iressa10min</td>
<td>11.04±1.36</td>
<td>0.18±0.03</td>
<td>0.87±0.08</td>
<td>0.71±0.06</td>
<td>26.7±1.5</td>
</tr>
<tr>
<td>Iressa20min</td>
<td>16.52±2.52</td>
<td>0.17±0.01</td>
<td>1.23±0.18</td>
<td>1.00±0.13</td>
<td>25.8±0.5</td>
</tr>
</tbody>
</table>

Figure 7-2 Concentrations of gefitinib in the lung or plasma as a function of A, B, solution concentrations, and C, D, exposure time.

The lung concentration and plasma concentration were measured, and the total
amount of gefitinib in the lung and blood was estimated. The results were shown in Figure 7-2 and Table 7-3. The concentration of gefitinib in the lung rose with increased solution concentration non-linearly, but monotonically. So did the concentration in the plasma. The concentration in the lung was 15-25 times higher than in the plasma. This proved that the majority of the drug delivery via aerosol inhalation reached the lung, with only a miniscule portion entering the circulation following treatment. The concentration in the lung and plasma also increased with longer exposure time. However, the concentrations did not double with doubled exposure time. The total mass deposition for each testing condition was also shown in Table 7-3.

Table 7-4 The mass concentration of gefitinib aerosols in the animal exposure chamber with varied solution concentrations and exposure time using the single-capillary electrospray nebulizer.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aerosol Concentration μg/L</th>
<th>Solution Concentration mg/ml</th>
<th>Consumed Solution Volume, ml</th>
<th>Consumed Mass, mg</th>
<th>Solution Utilization Efficiency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 nm</td>
<td>0.15</td>
<td>0.42</td>
<td>0.39</td>
<td>0.16</td>
<td>142.86</td>
</tr>
<tr>
<td>100 nm</td>
<td>0.18</td>
<td>1.66</td>
<td>0.41</td>
<td>0.67</td>
<td>48.36</td>
</tr>
<tr>
<td>200 nm</td>
<td>0.6</td>
<td>12.6</td>
<td>0.60</td>
<td>7.56</td>
<td>11.90</td>
</tr>
</tbody>
</table>

For the monodispersed particles, the aerosol mass concentrations were 0.15, 0.18, and 0.6 μg/l for 50, 100, and 200 nm aerosols, respectively. The solution utilization efficiency for 50 nm measured higher than 100%, probably due to an overestimation of aerosol mass concentration in the chamber. The average lung deposition, for the 50, 100, and 200 nm case were 0.047, 0.087, and 0.103 μg, respectively.
Figure 7-3 Size distribution of the generated gefitinib aerosols. A, B, and C, monodispersed aerosols generated by electrospray (ES) with an MMAD of 50, 100, and 200 nm. A, ES 50; B, ES 100; C, ES 200; D, polydispersed aerosols generated with a custom-built Collison atomizer. The solution was 15 mg/ml, and was atomized at 20 psi. The shape of the size distribution for the other solutions was similar to the one shown in D, with a shifted MMAD.

The delivery efficiency for the custom-built Collison atomizer fluctuated from 10 to 20% for different testing conditions (Figure 7-4 A). The 2.5 mg/ml solution had the highest delivery efficiency. The variation of the delivery efficiency might be a result of the shift in the mass mean diameter (MMD) of the aerosols, although the aerosols generated by the Collison atomizer were polydisperse. The MMD for a 2.5 mg/ml solution was around 120 nm (geometric standard deviation was 1.9). The delivery efficiency for the electrospray nebulizer is higher than that of the Collison atomizer.
The putative peak appeared at 100 nm. The combined results of the two generators suggested that 100 nm or so might be the optimal size for the maximum delivery efficiency.

![Figure 7-4 B](image)

**Figure 7-4** Delivery efficiency for the custom-built Collison atomizer and the single-capillary electrospray system. The delivery efficiency is defined as the mass deposited in the lung/dose (Zhang et al. 2011).

The aerosol mass concentration and the lung deposition measured in this work made it possible to compare the aerosol inhalation method to other administration routes, providing basic information for future improvements. The Collison atomizer has two advantages: high mass throughput and relatively simple setup. The disadvantages are the low delivery efficiency and the lack of delivery accuracy due to polydispersity of the aerosols. Conversely, single-capillary electrospray has a higher delivery efficiency and probably a higher delivery accuracy as well, but the low mass throughput is the bottleneck. Obviously, monodispersed particles with a controlled size are more desirable for drug delivery. Therefore, developing a novel electrospray system with a high mass throughput is both necessary and urgent.

Besides the improvements in the nebulizer, system optimization is equally important. At present, the custom-built Collison atomizer is still the primary aerosol generator in the animal bioassays. The tubing and desiccant/scrubber system needs to be optimized.
to overcome the low efficiency of solution utilization.
Chapter 8

Dissertation Accomplishments and

Recommendations for Future Work
Chapter 8 Dissertation Accomplishments and Recommendations for Future Work

8.1 Summary of accomplishments

In this dissertation, a broad range of agents were evaluated, either alone or combined, for their chemopreventive effects on lung tumorigenesis. These agents include synthetic inhibitors, plant-derived natural compounds, anti-inflammatory drugs, cytokines, etc. Their chemopreventive effects were examined. Throughout this process, the advantages of aerosol administration over conventional delivery methods were emphasized. The accomplishments of this study are summarized accordingly.

An aerosol delivery system, consisting of a custom-built Collison atomizer, diffusion dryers and/or scrubbers, a nose-only mouse exposure chamber, an orifice, and a vacuum pump were used to deliver potentially chemopreventive agents in the form of aerosols to the mouse via inhalation. Eight single agents and one combination of agents were evaluated using the post-initiation protocol in female A/J mice.

The eight single agents include four small molecular inhibitors and four natural compounds from plant sources. The four inhibitors are gefitinib, erlotinib, lapatinib, and wortmannin. Small molecular inhibitors have clear and specific mechanisms. The studies showed that gefitinib and erlotinib, two EGFR tyrosine kinase inhibitors (TKIs), consistently inhibited B[a]P-induced lung tumorigenesis in A/J mice. Aerosol delivery avoided the cutaneous side effects commonly found in this type of inhibitors. No visible skin alteration was observed. The pharmacokinetic study showed that aerosol delivery held an advantage over oral administration to deliver more or equivalent amount of agents into the mouse lung at a relatively low dose. Lapatinib, a dual inhibitor of EGFR
and Her2/neu, was studied \textit{in vivo} as a chemopreventive agent for lung carcinogenesis for the first time to the best of our knowledge. Lapatinib delivered either via inhalation or via oral gavage showed significant inhibitory effects on tumor load, and aerosolized lapatinib also inhibited tumor multiplicity. Wortmannin is a highly potent PI3K inhibitor but was abandoned in clinical evaluation due to its toxicity and poor pharmacokinetic properties. This study compared the efficacy and toxicity when wortmannin was delivered via oral gavage and via inhalation, and demonstrated that aerosolized wortmannin is as potent as orally-dosed wortmannin but with reduced adverse side effects. Aerosol delivery is a worthy strategy to potentially revive wortmannin-like agents, which are highly toxic or poorly metabolized when administered orally.

The four natural agents are resveratrol, caffeine, anthocyanins, and protocatechuic acid (PCA). The natural agents usually have nonspecific mechanisms and tend to react with or bind to multiple sites of action. The chemopreventive effects of resveratrol and caffeine on lung tumorigenesis are debatable based on reported data. In this study, aerosolized resveratrol has been shown to inhibit B[a]P- and VC- induced tumorigenesis in the mouse lung. The pharmacokinetic profile of resveratrol demonstrated that the aerosol route is a better way to deliver resveratrol, which may have a fast blood clearance rate \textit{in vivo}. Aerosolized caffeine also showed significant inhibitory effects on tumor multiplicity and tumor load. The results were not confounded by the negative effects of caffeine on the body weight, since the mice treated with aerosolized caffeine maintained a similar body weight to the control group. Anthocyanins are a class of compounds with ubiquitous existence in nature, and are one of the main sources of PCA in the human body. Anthocyanins marginally inhibited lung tumorigenesis in the mouse model, whereas PCA showed significant inhibitory effects on tumor load and tumor
multiplicity.

From the evaluation of the single agents, the advantages of aerosol delivery were demonstrated. Specifically for the agents with systemic toxicity and/or poor metabolic profiles, aerosol delivery helped to reduce the dose, minimize the toxicity, and maximize the amount of drugs in the lung.

The combinational treatment of aerosolized budesonide and dietary polyphenon E were studies in the mouse model. An in vitro study showed that budesonide and polyphenon E have additive effects in two human cancer cell lines, A549 and H1129. An in vivo study showed that the combination of aerosolized budesonide and dietary polyphenon E had stronger inhibitory effects on tumor load and tumor multiplicity than the single agent. The study provided evidence that treatment with a combination of multiple agents that act on different mechanisms is a promising approach to lower the single agent dosage and the toxicity as well, and enhance the inhibitory effects on tumorigenesis.

More single agents and other combinational treatments are summarized and discussed in the Appendix.

For a better understanding of the current aerosol delivery system, the dose and mass deposition of the drug in the mouse lung were measured. Establishing relationship between the dose and operation parameters, specifically the solution concentration and the exposure time, were established and could be used to compare with other administration routes. Polydispersed particles were generated with the custom-built Collison atomizer. The aerosol mass concentration in the chamber increased linearly with the increasing solution concentration. The mass deposited in the mouse lung also increased with the increasing solution concentration monotonically, but in a non-linear
manner. Longer exposure time lead to a higher dose and more mass deposited in the lung. However, the drug utilization efficiency, as well as the delivery efficiency, was quite low. The 2.5 mg/ml solution, which leads to an MMAD of 120 nm, had the highest delivery efficiency among other solutions. To further investigate the influence of particle size, monodispersed particles were generated and used. It was found that particles with an MMAD of 100 nm had the highest delivery efficiency, which seemed to be in agreement with the testing results for the polydisperse particles.

8.2 Recommendations for future work

In this dissertation, most of the work focused on the evaluation of agents in the animal bioassay. However, the bioassay is only the first step, and is accompanied by multiple unknowns, and followed by more questions. Chapter 2 reviews the three key factors for designing a sound chemoprevention animal bioassay: the agent, the animal, and the biomarker (introduced but not involved in depth). Furthermore, to study aerosol administration for chemoprevention in animal bioassay, other factors should be added – technologies of aerosol generation and transportation. In this chapter, the discussion or recommendation for future work will start from the accomplishments and lessons learned from the finished bioassays, focusing on the four key factors mentioned above.

8.2.1 Pharmacokinetics

One of the hypotheses tested in this dissertation is that aerosol delivery is capable of delivering the drugs directly into the lung, reducing exposure of other healthy organs.
For most of the agents discussed in Chapter 4 and Chapter 5, this statement is implied, but not explicitly addressed. Only the pharmacokinetic data can provide direct evidence. A comparison between the oral route and pulmonary route is preferred. Upon a literature search, there are only limited pharmacokinetic studies on pulmonary administration in mice. More published studies reflect the pharmacokinetic data of orally administered drugs than inhaled ones, yet not all of the drugs were studied. Therefore, it is necessary to measure the pharmacokinetics of the inhaled drugs. In this dissertation, only three of the testing agents, gefitinib, erlotinib, and resveratrol, had time-concentration curves and the steady-state lung and plasma concentration measured for aerosol delivery. Currently I’m working on the measurement of the steady-state concentration of four other drugs, lapatinib, wortmannin, anthocyanins, and PCA, in lung and plasma, via oral gavage and aerosol inhalation. The time-concentration curves of the lung and plasma are necessary for these agents, as well as for caffeine.

The current workflow is to test the \textit{in vivo} efficacy of a drug in an animal model first and whether the pharmacokinetic study will be carried out or not depends on the efficacy of the agent. The drug with a higher efficacy has a higher priority. The safest workflow measures the pharmacokinetics and toxicity of the agent before the agent is tested for its efficacy, if time and cost allows.

A more in depth analysis requires assistance from chemists and pharmacologists. Not only the intact drugs, but also the metabolites and related proteins, are part of the pharmacokinetic study.

\textbf{8. 2. 2 Biomarkers Analysis}

The biomarker analysis could be both explanatory for the animal bioassays alone and
predictive for the clinical trials. Positive results obtained from animal bioassays are not enough for an agent to be a candidate for clinical trials. For an agent to be a candidate, an appropriate biomarker – instead of a lung tumor – is needed as an endpoint to begin Phase II chemoprevention trials. The ideal condition might be to find a fingerprint biomarker for each type of lung cancer or to find a fingerprint biomarker for a certain agent. However, either one is difficulty.

The carcinogenesis is generally related to alterations in two distinct pathways: the suppression/deactivation of apoptosis and the promotion of proliferation. The efficacy of an agent is related to reversing this directions: the induction of apoptosis and the suppression of proliferation. Two approaches to biomarker analysis were discussed in a review of lung cancer chemoprevention (Dragnev et al., 2012). One is called a ‘window-of-opportunity model’, which is more like a ‘fixed-spectrum’ method and focuses on the effect of the agent. There are several pre-selected candidates for this approach, which are expected to be related to the efficacy and the mechanism of the agent. In my opinion, this approach is suitable for the agents with known mechanisms. The other approach focuses on the effect of carcinogenesis and looks for alterations in several pre-selected relevant pathways. This approach is more like a ‘scanning-spectrum’ method and might be suitable for agents with unclear mechanisms or effects. Either approach has risks. For the first approach, even the agents with clear molecular targets may encounter confounding conditions in data interpretation, such as gefitinib which was discussed in Section 4.1. Gefitinib is an EGFR inhibitor which is supposed to inhibit the phosphorylation of EGFR, and consequently the level of EGFR expression and level of EGFR phosphorylation are considered to be suitable biomarkers. Unfortunately, it seems that neither EGFR overexpression or EGFR mutation occurred
in the B[a]P-induced lung tumors in A/J mice (data not shown in this dissertation). Then
the expected biomarkers of gefitinib did not work, although gefitinib did inhibit lung
tumorigenesis in the same model. Thus, in this case, the second approach may help to
explore other mechanisms of gefitinib besides the commonly known one as an
EGFR-TK inhibitor. The related limitations of the first approach in clinical trials include
recruitment difficulties. However, the second approach may provide too many
biomarkers, making it difficult to gauge. I also questioned that this method may risk
falsely relating one biomarker with the efficacy and/or the mechanism of the agent.

In brief, a biomarker analysis is necessary to give insight into the mechanisms of the
agents and to provide endpoints for future clinical trials.

8.2.3 Design and Analysis of Drug Combinations

For the combination treatment, the concept and hypothesis are attractive, but the
details should be carefully designed. The best example of suitable combination
treatment probably may come from nature, such as polyphenon E and traditional
Chinese herb medicines. Understanding the interactions of single agents when
combined is a complex process, rather than a simple mixture. A screening strategy is
necessary for the experimental design. What agents could be combined together? What
dose level could maximize the efficacy? What type of administration route is proper for
each agent? Should they be administered concurrently or sequentially? All of these
questions need answers, or at least considerations, before a combination group could be
tested in the animal model. Statistical methods may bring some new insights to the
experimental design of animal assays.
8.2.4 Improvement in Aerosol Instrumentations

Through animal studies, the current aerosol delivery system has been proven to be effective in delivering the agents directly into the mouse lung. However, as indicated in Chapter 7, the drug utilization and delivery efficiency were both very low. The whole system requires improvement in both the particle generation and the mass transfer. The desired particle generation technique should have the capability to generate enough particles with controllable sizes and physiochemical properties. At present, neither the Collison atomizer nor the single-capillary electrospray system could satisfy this requirement. Therefore, it is necessary to discover and develop novel particle generation methods. In terms of mass transfer in the aerosol delivery system, the tubings and the diffusion dryers/scrubbers should be optimized to minimize particle loss in the system for future applications. Since the aerosol delivery process is more device-dependent, it is necessary to characterize the specific system before it is used in drug screening or for any evaluation. The characterization could help in data interpretation and in determining the true reasons for the success or failure of the agent or the method.

Additionally, the current aerosol generation methods are not effective enough to atomize/nebulize protein aerosols. I learned this lesson from the limited cases of protein delivery in my experiment, the chemokine CXCL12 [chemokine (C-X-C motif) ligand 12, EGF (epidermal growth factor), and insulin. Since the proteins used in this experiment were hard to purify, sold in small amounts, and cost a lot, the loss in the aerosol delivery system could not be afforded. Another concern with protein atomization/nebulization is how to maintain the protein activity. This also presents an interesting area to explore for future studies.
Appendix: Other Agents Evaluated in This Dissertation

Work

9.1 Single Agent

9.1.1 Non-Steroidal Anti-Inflammatory Drugs

Six NSAIDs (non-steroidal anti-inflammatory drugs, Table 9-1), including aspirin (ASA), naproxen, licofelone, celecoxib, piroxicam, and sulindac, were evaluated. All of them except celecoxib act as nonselective inhibitors of the enzyme cyclooxygenase (COX)-1 and COX-2, while celecoxib is a selective COX-2 inhibitor. Each drug was nebulized by a custom-built Collison atomizer with a controlled liquid feeding (100 μl/min controlled by a syringe pump). The exposure time is was 10 min, and flowrate was 1.0 L/min. The solution concentration is the same in molar/ml using 30 mg/ml sulindac as a standard because sulindac showed an inhibitory effects in a prior experiment. All other drugs except sulindac showed no inhibitory effect. There might be two reasons: 1) the molar concentration of the solution should vary based on their IC50; 2) the solvent control group had much fewer tumors than in normal conditions. An inflammation related pathway is an interesting approach to cancer prevention, and these agents should be re-assessed with a more thoughtful design if possible.
Table 9-1  Efficacy and testing conditions of aerosolized NSAIDs (non-steroidal anti-inflammatory drugs) using a post-initiation protocol in female A/J mice

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Solution Concentration</th>
<th>Carcinogen</th>
<th>Efficacy</th>
<th>Complete</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA</td>
<td>Aerosol</td>
<td>15 mg/ml B[a]P</td>
<td>-38.6%</td>
<td>-25.1%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Aerosol</td>
<td>19 mg/ml B[a]P</td>
<td>-58.5%</td>
<td>-20.6%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Licofelone</td>
<td>Aerosol</td>
<td>32 mg/ml B[a]P</td>
<td>-11.8%</td>
<td>20.8%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>Aerosol</td>
<td>32 mg/ml B[a]P</td>
<td>-34.0%</td>
<td>-33.9%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>Aerosol</td>
<td>28 mg/ml B[a]P</td>
<td>-38.6%</td>
<td>-12.1%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Sulindac</td>
<td>Aerosol</td>
<td>15 mg/ml B[a]P</td>
<td>8.3%</td>
<td>44.9%</td>
<td></td>
<td>Sep. 2010 †</td>
</tr>
<tr>
<td></td>
<td>Aerosol</td>
<td>30 mg/ml B[a]P</td>
<td>6.1%</td>
<td>46.6%</td>
<td></td>
<td>Aug. 2011</td>
</tr>
</tbody>
</table>

‡: Mice at 6 weeks of age received one dose of B[a]P (100 mg/kg body weight, i.p. injection). All the aerosol groups, in Aug. 2011 (the same below), shared the same aerosol control group. B[a]P induced an average of 3.73 ± 0.85 tumors/mouse, with a tumor load of 0.77 ± 0.22 mm³/mouse.

†: The aerosol control group for sulindac in Sep. 2010 (the same below) has the tumor multiplicity of 4.09 ± 0.62 tumors/mouse, and the tumor load of 0.78 ± 0.17 mm³/mouse. The exposure time is 8 min.
9.1.2 Other Inhibitors

Table 9-2 Efficacy and testing conditions of other inhibitors using a post-initiation protocol in female A/J mice

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Solution Concentration</th>
<th>Carcinogen</th>
<th>Efficacy</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B[a]P</td>
<td><em>Efficacy</em></td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>Aerosol 8 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>18.5%</td>
<td>41.3%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 mg/ml</td>
<td>B[a]P</td>
<td>0.0%</td>
<td>53.3%*</td>
</tr>
<tr>
<td>Akt-1/2i</td>
<td>Aerosol 10 min</td>
<td>20 mg/ml</td>
<td>B[a]P</td>
<td>-39.0%</td>
<td>-4.7%</td>
</tr>
<tr>
<td>CDDO</td>
<td>Aerosol 10 min</td>
<td>2.5 mg/ml</td>
<td>B[a]P</td>
<td>-22.0%</td>
<td>-31.6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>9.8%</td>
<td>62.4%</td>
</tr>
<tr>
<td>AZD6244</td>
<td>Aerosol 10 min</td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>17.3%</td>
<td>34.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mg/ml</td>
<td>B[a]P</td>
<td>21.9%</td>
<td>37.1%</td>
</tr>
<tr>
<td>SAHA</td>
<td>Aerosol 15 min</td>
<td>5 mg/ml</td>
<td>B[a]P*</td>
<td>33.5%*</td>
<td>42.9%*</td>
</tr>
</tbody>
</table>

Abbreviation: SAHA, suberoylanilide hydroxamic acid; B[a]P, benzo[a]pyrene.

*: P < 0.05

†: Mice received two doses of B[a]P (100 mg/kg body weight, i.p. injection), one week apart.

Tumor multiplicity in the control group (Oct. 2012) was 16.60 ± 1.10 tumors/mouse, and the tumor load was 3.65 ± 0.36 mm³/mouse.
9.2 Combination Treatment

9.2.1 Aerosolized Budesonide in Combination with Other Agents

Aerosolized budesonide combined with six other agents, including polyphenon E (Chapter 6), indole-3-carbinol, pioglitazone, myo-inositol, targretin, and deguelin, were evaluated. Three combinations (Polyphenon E, indole-3-carbinol, and pioglitazone) were a continuation of the former bioassay. In the former assay, the efficacy of budesonide overpowered the effects of the other agents with which it was combined to the extent that no combination effects were observed. When the combination groups were repeated, the dose of every agent was reduced by half in hope that its efficacy could also be cut by half. However, the results of combinations with dietary I3C and pioglitazone (by gavage) were disappointing. The efficacy of budesonide was only slightly weakened compared with that in the former assay (78.4% of tumor load by 2 mg/ml budesonide, Fu et al., 2010).

For the other three combinations, each single agent, myo-inositol, targretin, and deguelin, showed significant inhibitory effects. However, there was no combination effect when they were combined with budesonide. Either budesonide counteracts the efficacy of the other agent, or the dose of each agent was not optimal. These guesses need supportive data from mechanistic studies.
### Table 9-3: Efficacy and testing conditions of aerosolized budesonide in combinations with other agents

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Concentration</th>
<th>Carcinogen</th>
<th>Efficacy Multiplicity</th>
<th>Efficacy Load</th>
<th>Complete Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Budesonide</strong></td>
<td>Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>51.1%</td>
<td>64.1%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>I3C</strong></td>
<td>Diet</td>
<td>5 µmol/g</td>
<td>B[a]P</td>
<td>8.8%</td>
<td>15.5%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Budesonide</strong></td>
<td>+ Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>40.8%</td>
<td>46.2%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>I3C</strong></td>
<td>+ Diet</td>
<td>5 µmol/g</td>
<td>B[a]P</td>
<td>8.8%</td>
<td>15.5%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Pioglitazone</strong></td>
<td>Gavage</td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>8.8%</td>
<td>15.5%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Budesonide</strong></td>
<td>+ Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>39.3%</td>
<td>57.4%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Pioglitazone</strong></td>
<td>+ Gavage</td>
<td>5 mg/kg</td>
<td>B[a]P</td>
<td>39.3%</td>
<td>57.4%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Myo-inositol</strong></td>
<td>Aerosol 8 min</td>
<td>30 mg/ml</td>
<td>B[a]P</td>
<td>0.0%</td>
<td>53.3%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Budesonide</strong></td>
<td>+ Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>45.0%</td>
<td>77.3%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>myo-inositol</strong></td>
<td>+ Aerosol 8 min</td>
<td>30 mg/ml</td>
<td>B[a]P</td>
<td>45.0%</td>
<td>77.3%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Targretin</strong></td>
<td>Aerosol 8 min</td>
<td>10 mg/ml</td>
<td>B[a]P</td>
<td>20.5%</td>
<td>58.5%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td><strong>Budesonide</strong></td>
<td>+ Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>42.2%</td>
<td>63.2%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Targretin</strong></td>
<td>Aerosol 8 min</td>
<td>10 mg/ml</td>
<td>B[a]P</td>
<td>42.2%</td>
<td>63.2%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Deguelin</strong></td>
<td>Aerosol 8 min</td>
<td>1 mg/ml</td>
<td>B[a]P</td>
<td>4.2%</td>
<td>56.3%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td><strong>Budesonide</strong></td>
<td>+ Aerosol 2 min</td>
<td>1.125 mg/ml</td>
<td>B[a]P</td>
<td>45.0%</td>
<td>62.2%</td>
<td>Sep. 2010</td>
</tr>
<tr>
<td><strong>Deguelin</strong></td>
<td>Aerosol 8 min</td>
<td>1 mg/ml</td>
<td>B[a]P</td>
<td>45.0%</td>
<td>62.2%</td>
<td>Sep. 2010</td>
</tr>
</tbody>
</table>

Abbreviation: I3C, indole-3-carbinol.

*: P < 0.05.
### 9.2.2 Aerosolized Gefitinib in Combination with Other Agents

Table 9-4 Efficacy and testing conditions of aerosolized gefitinib in combination with other agents

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Concentration</th>
<th>Carcinogen</th>
<th>Efficacy</th>
<th>Complete Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>Aerosol 15 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>26.8%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>CDDO</td>
<td>Aerosol 10 min</td>
<td>2.5 mg/ml</td>
<td>B[a]P</td>
<td>-22.0%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Gefitinib + CDDO</td>
<td>Aerosol 10 min</td>
<td>15 mg/ml + 2.5 mg/ml</td>
<td>B[a]P</td>
<td>12.2%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>AZD6244</td>
<td>Aerosol 10 min</td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>17.3%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Gefitinib + AZD6244</td>
<td>Aerosol 10 min</td>
<td>15 mg/ml + 5 mg/ml</td>
<td>B[a]P</td>
<td>43.9%</td>
<td>Aug. 2011</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>Aerosol 15 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>49.8%***</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Aerosol 10 min</td>
<td>2 mg/ml</td>
<td>B[a]P</td>
<td>66.7%**</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Gefitinib + Wortmannin</td>
<td>Aerosol 15 min</td>
<td>15 mg/ml + 2 mg/ml</td>
<td>B[a]P</td>
<td>74.4%*****</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Aerosol 15 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>50.6%***</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>Gefitinib + Rapamycin</td>
<td>Aerosol 15 min</td>
<td>15 mg/ml + 2 mg/ml</td>
<td>B[a]P</td>
<td>41.1%**</td>
<td>Oct. 2012</td>
</tr>
</tbody>
</table>

*: P < 0.05; **: P < 0.001; ***: P < 0.0001; ****: P < 0.00001.
### 9.2.3 Other Combinations

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Concentration</th>
<th>Carcinogen</th>
<th>Efficacy</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Multiplicity</td>
<td>Load</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Aerosol 8 min</td>
<td>1 mg/ml</td>
<td>B[a]P</td>
<td>20.0%</td>
<td>28.2%</td>
</tr>
<tr>
<td>3BrPA</td>
<td>Aerosol 8 min</td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>55.5%</td>
<td>70.5%*</td>
</tr>
<tr>
<td>Rapamycin +</td>
<td>Aerosol 8 min+</td>
<td>1 mg/ml +</td>
<td>B[a]P</td>
<td>41.3%</td>
<td>60.3%</td>
</tr>
<tr>
<td>3BrPA</td>
<td>Aerosol 8 min</td>
<td>5 mg/ml</td>
<td>B[a]P</td>
<td>64.5%*</td>
<td>26.9%</td>
</tr>
<tr>
<td>DFMO</td>
<td>Aerosol 8 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>8.3%</td>
<td>44.9%</td>
</tr>
<tr>
<td>Sulindac</td>
<td>Aerosol 8 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>12.5%</td>
<td>16.7%</td>
</tr>
<tr>
<td>DFMO +</td>
<td>Aerosol 8 min+</td>
<td>15 mg/ml +</td>
<td>B[a]P</td>
<td>36.0%*</td>
<td>40.8%*</td>
</tr>
<tr>
<td>Sulindac</td>
<td>Aerosol 8 min</td>
<td>15 mg/ml</td>
<td>B[a]P</td>
<td>-28.7%</td>
<td>-24.9%</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>Gavage</td>
<td>10 mg/kg B.W.</td>
<td>B[a]P</td>
<td>19.3%</td>
<td>43.1%</td>
</tr>
</tbody>
</table>

Abbreviations: 3BrPA: 3-Bromopyruvate acid; DFMO, difluoromethylornithine; 2-DG, 2-deoxy-D-glucose; B.W., body weight. *: \( P < 0.05 \).
9.3 Intranasal delivery of interleukin 17A to prevent primary lung tumors

Figure 9-1 Treatment protocol of interleukin 17A

Mice in Group 1 received an early treatment, two weeks after the second B[a]P injection. Mice in Group 2 received a late treatment, two weeks before the termination of the experiment. The efficacy of intranasally-delivered interleukin 17A was first compared with the IgG control group, and compared between the two treatment groups. The control groups received four times of treatment, while each of the treatment group receives twice treatment. This design is conservative because the control group had more stress from the dripping treatment than the treatment groups, which may negatively influence the tumor growth. The results were presented in Table 9-6.
### Table 9-6 Efficacy and testing conditions of intranasally-delivered interleukin 17A (IL 17A)

<table>
<thead>
<tr>
<th>Agents</th>
<th>Administration</th>
<th>Concentration</th>
<th>Carcinogen</th>
<th>Efficacy</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Control</td>
<td>Intranasal dripping (30 μl)</td>
<td>0.1 mg/ml</td>
<td>B[a]P</td>
<td>-</td>
<td>Oct. 2012</td>
</tr>
<tr>
<td>IL17A-Early</td>
<td>Intranasal dripping (30 μl)</td>
<td>0.1 mg/ml</td>
<td>B[a]P</td>
<td>16.7%</td>
<td>49.3%</td>
</tr>
<tr>
<td>IL17A-Late</td>
<td>Intranasal dripping (30 μl)</td>
<td>0.1 mg/ml</td>
<td>B[a]P</td>
<td>-0.5%</td>
<td>40.5%</td>
</tr>
</tbody>
</table>
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Journal Article in Preparation


Posters and Presentations in Conferences:


5. **Jingjie Zhang**, Huijing Fu, and Da-Ren Chen. Inhibitory effects on lung carcinogenesis in A/J mice by aerosolized budesonide combined with dietary pholyphenon E. The 7th Asian Aerosol Conference, Xi’an, China, August 2011


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