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PREVENTION AND TREATMENT OF LUNG CANCER
BY GREEN TEA POLYPHENOLS

By

Pengxiao Cao
M.B., Shandong Medical University
M.S., University of Louisville

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky

August 2011

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Pengxiao Cao
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A Dissertation Approved on

June 6, 2011

By the following Dissertation Committee:

Ramesh C. Gupta, Ph.D.

Teresa W. Fan, Ph.D.

W. Glenn McGregor, M.D.

David Schultz, Ph.D.

Steven R. Myers, Ph.D.

DEDICATION

This dissertation is dedicated to my wife, Junling Li, for her unparalleled love, support and encouragement. Moreover, this dissertation is dedicated to our lovely daughter, Addie Y. Cao, and son, Charles R. Cao, who open a totally new world for me, a world of joy and surprise.

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ABSTRACT

PREVENTION AND TREATMENT OF LUNG CANCER BY GREEN TEA POLYPHENOLS

Pengxiao Cao

June 6, 2011

Green tea polyphenols (GTPs) are gaining increasing attention because of their potential anti-tumor effects. However, poor oral bioavailability limits their efficacy *in vivo*. In this dissertation, two hypotheses were tested: 1) GTPs administered systemically by a sustained release system will circumvent the problem of limited bioavailability and lower the effective dose compared to the traditional oral route, and 2) adjuvant treatment of GTPs with a standard chemotherapeutic agent (*e.g.* cisplatin) will enhance efficacy of the therapeutic agent.

In our lab, a polymeric implant delivery system was developed, in which GTPs were uniformly embedded within a polycaprolactone matrix to provide sustained release of GTPs. The release profile of GTP implants was further investigated both *in vitro* and *in vivo*.

To test the first hypothesis, polyphenon E (poly E), a standardized green tea extract, was administered by PCL implants grafted subcutaneously or via the drinking water to

S/D rats. The animals were challenged by benzo[*a*]pyrene (BP) via subcutaneous implant. Results showed a significant reduction of BP-induced DNA adducts in the implant group; however, only a modest but insignificant reduction occurred in the drinking water group. Notably, the total dose of poly E administered was >100-fold lower in the implant group. Analysis of selected phase I, phase II, and DNA repair enzymes at the mRNA, protein levels and enzymatic activity showed no significant modulation by poly E. The effect of poly E on DNA adduct formation was presumably due to scavenging of the reactive intermediates of BP by GTPs, which was illustrated by other experiments in this dissertation.

To test the second hypothesis, the combined effects of the GTPs, anthocyanidins and cisplatin on the growth of lung cancer H1299 cells in cell culture and also a xenograft nude mouse model were investigated. In both studies, combination of GTPs-anthocyanidins with cisplatin exerted a more dramatic anti-cancer effect. However, systemic toxicity was found in the animals receiving the combination treatment. Possible mechanism of action was investigated.

Together, these data demonstrated that sustained systemic delivery of poly E lower the effective dose by overcoming oral bioavailability and combination of GTPs-anthocyanidins and suboptimal doses of cisplatin may be effective in neo-adjuvant chemotherapy.

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CHAPTER I: GENERAL INTRODUCTION

Epidemiology of lung cancer

Data from the National Cancer Institute, the Center for Disease Control and Prevention, the North American Association of Central Cancer Registries and the National Center for Health Statistics indicate that, in the United States, lung cancer is the second most common cancer diagnosed in 2010, only less than prostate cancer in men and breast cancer in women. Numerically, this translates to 116,750 males and 105,770 females diagnosed with lung cancer in 2010, which accounts for 15% and 14% of all the cancer types respectively (1). Moreover, lung cancer also accounts for the highest cancer-related death rate, 29% death in men and 26% in women, which corresponds to the death of 86,220 male patients and 71,080 female patients (1).

Worldwide estimates of the incidence and mortality of lung cancer provided by the International Agency for Research on Cancer (IARC) indicated that lung cancer contributed to 13% of the total cancer cases and 18% of cancer-related deaths in 2008, which represents 1.6 and 1.4 million cases respectively. Worldwide, lung cancer is the leading cancer type diagnosed in males, accounting for 17% of the total new cancer cases and 23% of the cancer-related deaths. In females, it's the fourth most commonly diagnosed cancer only less than breast, colon & rectum and cervical cancers, and the second leading cause of cancer-related deaths (2).

Treatment of lung cancer

The treatment regimens for lung cancer are based on the type and stage of lung cancer and the patients' overall health. To date, standard treatment options include surgical resection, chemotherapy and radiation therapy with chemotherapy comprising the backbone of the treatment strategies. Despite the development of various chemotherapeutic agents in 1980s and 1990s, platinum containing therapy was the most commonly selected regimen largely a result of its efficacy, yielding a responsive rate of 30~40% and a median survival time estimated to be one year for patients in advanced stages (3).

The standard chemotherapeutic treatments discussed above have a somewhat non selective mechanism, i.e. both cancer and non cancer cells can be affected by these treatments. However, the recent appearance of molecular target therapy has provided an opportunity to more selectively target cancer cells. Cancer cells acquire some common phenotypes including unlimited proliferation, resistance to apoptotic signals and metastasis through the accumulation of a variety of genetic and epigenetic changes. Advancements in molecular biology and biochemistry make it possible to identify and target these molecular mechanisms. One such promising class of therapeutics is inhibitors of epidermal growth factor receptor (EGFR) such as gefitinib and erlotinib which have been found to exert dramatic clinical effects, resulting in a prolonged (approximately 4 months) progression-free survival time of patients with lung cancer compared with patients treated with platinum doublet therapy. Several other molecular-targeting drugs have also demonstrated significant promise towards lung cancer treatment or are currently under investigation in clinical trials including the anti-angiogenic drug

bevacizumab, the anti-epidermal growth factor receptor antibody cetuximab and the anti-vascular endothelial growth factor antibody bevacizumab (4, 5).

Lung cancer prognosis

Despite the progress in cancer research and cancer treatment strategies, the prognosis of lung cancer remains relatively poor as reflected in the mortality rate. In developed countries, the overall 5-year relative survival rate for lung cancer patients was 12-18%, in comparison with other most commonly diagnosed malignancies including 73-89% for breast cancer, 50-99% for prostate cancer and 43-63% for colorectal cancer (6). Furthermore, this prognosis has improved only marginally over the last few decades. Clearly, more effective therapeutic strategies are urgently needed.

Risk factors for lung cancer

The majority of lung cancers have been attributed to environmental factors. The incidence of lung cancer strongly correlates with cigarette smoking, with 90% of lung cancers associated with tobacco use (7). Kentucky has the highest adult smoking rates in the U.S., with 30% or more of the population identified as smokers; a number that greatly surpasses other states (8). Not surprisingly, lung cancer incidence and mortality rates in Kentucky are among the highest in United States (9).

Although tobacco smoke contains more than 5,000 chemicals, several dozens of them are carcinogenic, mutagenic or tumor promoters. Two classes of compounds have been identified and most investigated as animal carcinogens, namely nitrosamines and polycyclic aromatic hydrocarbons (PAHs) (10, 11).

Another significant source of these environmental carcinogens, predominantly found in developing countries, is the emission from coal combustion associated with cooking and heating, particularly in rural areas of China. These combustion processes result in higher PAH levels in air indoor and outdoor and have been associated with an elevated lung cancer risk to populations in these regions (12, 13).

Benzo[a]pyrene and carcinogenesis

Benzo[a]pyrene (BP) is a model PAH, and also one of the most potent environmental carcinogens found ubiquitously in tobacco smoke, automobile exhaust emissions and grilled foods (14, 15). Numerous studies have demonstrated the association of BP exposure and induction of carcinogenesis in many organs including lung, skin, mammary gland and others (16-18).

Enzymatic activation is needed before BP can be metabolized to its ultimate carcinogenic metabolite, *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide (*anti*-BPDE) and other potentially carcinogenic and/or mutagenic agents. Certain types of cytochrome P450s (CYPs) found in the subcellular microsomal fraction, especially CYP1A1, CYP1B1 are involved in this process (19). *Anti*-BPDE exerts its carcinogenic activity by alkylating nucleosides in DNA molecules at the structurally named bay region of the *anti*-BPDE moiety. The reaction predominates with the purine bases, particularly deoxyguanosine in DNA (20). As a result, both bulky stable and depurinating DNA adducts are formed (21, 22). Insufficient removal of stable DNA adducts prior to replication can create mutational hot spots in the gene which may result in deactivation of tumor suppressor genes or activation of oncogenes leading to tumor initiation (23-26).

Need for prevention

In the United States, annual age-adjusted incidence rates for lung and bronchus cancer has reportedly decreased from 1984 through 2006 in males. However, this observation has not been found in females. One of the most significant reasons for this disparity is believed to be the historical differences in cigarette smoking between men and women. Cigarette smoking by women appeared to peak approximately 20 years later than men (*1*). This peak pattern coupled with widespread and significant lung cancer incidence, substantial mortality and poor prognosis strongly suggest the importance of developing prevention strategies, including tobacco control, in the control of lung cancer development. As previously discussed, current treatment strategies for lung cancer only have limited effects in improving the prognosis of lung cancer patients. Therefore, cancer prevention should be put in a more prominent position which could dramatically influence the incidence and death rate of lung cancer. Further, for high-risk individuals including heavy smokers (e.g., people with 50-100 pack-year history) and long term workers expose to smoke or fumes occupationally, specific chemopreventive intervention may be necessary.

Natural products with chemopreventive/chemotherapeutic activity

One promising pathway towards preventing cancers including those of the lung is the use of natural products. Natural products are part of human lives historically and currently. Many of them have been found to bear medicinal functions. Studies have demonstrated the chemopreventive/chemotherapeutic effects of many natural compounds, e.g. green tea and tea catechins, curcumin in the yellow spice turmeric, resveratrol in the

skin of red grapes, isoflavones and soy preparations from beans, tanshinone in the Chinese folklore tanshen, the red carotenoid pigment lycopene in tomatoes, water melon, etc., quercetin from apples and other fruits, etc. These compounds are able to inhibit tumor cell proliferation, induce cell apoptosis in cell culture and inhibit the formation and development of tumors at various stages in animal studies, suggesting their potential use in cancer patients or cancer survivors (27-32). For example, pomegranate fruit extracts inhibited the growth of colon, prostate and lung cancer cells in culture and also in animal models (28). Apple juice and apple extracts can modulate signaling transduction pathways and prevent skin, mammary and colon carcinogenesis (30).

Some natural compounds have been tested or are under investigation in clinical trials with promising results. For example, administration of green tea catechins was found to be very effective for treating premalignant lesions of prostate in a double-blinded, placebo-controlled study. The incidence of prostate cancer in treatment group is 3% verse 30% in the placebo group (33). In another clinical trial, the combination of curcumin and quercetin reduced the size and number of intestinal adenomas in patients with familial adenomatous polyposis (34).

Other possible applications of these natural compounds include, but are not limited to, use as adjuvants to standard chemotherapeutic agents for the purpose of decreasing toxicity of standard chemotherapeutic agents or increasing efficacy, which will be discussed in the section of “dilemma of chemotherapy” in this chapter (35-39).

Green tea polyphenols’ antitumor effects and safety profile

Green tea is one of the most popular drinks in the world. Studies are now suggesting that green tea has many health benefits, which are attributed to its bioactive components, including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (40, 41). GTPs are highly potent antioxidants and may help prevent atherosclerosis, particularly coronary artery disease (42, 43). Also, using a murine model, GTPs have been found to have beneficial effects in the treatment of neurodegenerative diseases (44).

More recently, green tea has been attracting attention because of its possible application in cancer prevention. The anticarcinogenic effects of GTPs have been tested using a variety of cancer cell lines *in vitro* (45-49). More importantly, in the majority, but not all, of published animal studies, green tea preparations were found to decrease lung tumor incidence and tumor multiplicity in chemically-induced lung tumor models, including both PAHs (e.g., BP) and nitrosamines (e.g., N-nitrosodiethylamine (NDEA) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (41, 50-54). Green tea preparations were found to be effective when administered to mice either during or after carcinogen exposure to reduce tumor incidence and multiplicity (50, 51). Studies also showed that green tea extracts are effective in decreasing tumor multiplicity and tumor incidence by approximately 60% and 25% in animal models of esophageal and gastric carcinogenesis (41). These results suggest the anti-tumor potential of GTPs.

GTPs are usually not perceived as “medicine” by people who drink tea or even those who don’t drink tea. One reason is because of the level of safety of GTPs. The adverse events in humans are mild after daily administration of 800 mg EGCG for four weeks, which are no different with those reported in the placebo group. Additionally,

repeated administration of GTPs has no significant impact on blood counts and blood chemistry profiles (55). The systemic review performed by US Pharmacopeia (USP) Dietary Supplement Information Expert Committee (DSI EC) showed that no significant safety issues were found when dietary supplement products containing green tea extracts are used and formulated (56).

Mechanisms of action of green tea polyphenols

The possible mechanisms of action of green tea catechins have been extensively studied *in vitro*. GTPs were found to induce apoptosis in cancer cells and inhibit the proliferation of a variety of cancer cells types *in vitro*, including lung cancer cells (45-49). GTPs have also been shown to inhibit epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factor 2 (FGF2) dependent signaling pathways, and interfere with enzyme activities of JUK, JUN, MEK1, MEK2, EK1, EK2, CDK2 (57). They are also highly potent antioxidants (58). In the presence of transition metal ions or alkalis, GTPs can generate reactive oxygen species, including hydrogen peroxides which are believed to help to kill cancer cells (59, 60). However, the broad *in vitro* and *in vivo* anti-cancer effects of GTPs are not attributed to a single mechanism but most likely are due to a combination of mechanisms that results in the efficacy of GTPs. The translation of mechanisms of action found from *in vitro* studies to *in vivo* studies, however, remains inconclusive.

Inconsistent data

Although GTPs have exhibited chemopreventive effects in most published animal studies, these data have been somewhat inconsistent. In some studies, GTPs failed to show beneficial effects in the prevention of lung tumorigenesis in tobacco smoke-induced lung tumor models in A/J mice (61, 62). It is also reasonable to speculate that even more studies have not been published due to negative results. Furthermore, reviews of epidemiological and clinical studies indicate only a marginal beneficial association between green tea consumption and lung cancer risk (63, 64). Similarly, green tea did not show definitive effects on other cancer types, including gastric, colorectal and breast cancer (65-69).

Bioavailability of green tea polyphenols

One likely reason for the inconsistency of these studies with GTPs is their lack of bioavailability following oral exposure, which ultimately results in low blood levels of the bioactive components (70-74). EGCG and ECG are the most abundant and active compounds in GTPs. However, only about 0.1% of EGCG was bioavailable in rats following intragastric (*i.g.*) administration, resulting in a maximum plasma concentration (C_{max}) of EGCG only at ng/ml level (13.2 - 16.3 ng/ml) (72). Bioavailability for ECG ranged from 1 to 3.3% in rats following oral administration. Again, the C_{max} of ECG was also only determined to be at the ng/ml level (49.6 - 464.0 ng/ml) (73). In clinical studies, the blood levels of EGCG were found to range from pg/ml to ng/ml, even after large doses (75, 76). The lack of bioavailability indicated from these studies coupled with the demonstrable efficacy, low toxicity and known chemoprotective mechanisms of GTPs in several animal studies strongly suggests that these agents may exhibit greater

efficacy under conditions in which bioavailability can be increased by avoiding oral dosing routes.

The dilemma of chemotherapy

The basic principle of chemotherapy is to kill as many tumor cells as possible by treatment with chemicals in order to minimize the tumor burden. However, the majority of these drugs also affect normal cells such that each drug has its maximum tolerable dosage considering the toxicity and side effects. In practice, usually two or more chemotherapeutic agents with a lower dose in combination are needed in order to maximize efficacies and minimize toxicities and side effects (77, 78). The principle for combination of these therapeutics is to choose drugs with differing molecular targets, mechanisms of action and side effects (78). The most commonly used chemotherapeutic drugs in lung cancer treatment and their mechanisms of action and molecular targets are as follows: cisplatin, carboplatin (crosslinking with DNA) (79, 80); paclitaxel, docetaxel (anti-microtubule) (81, 82); doxorubicin (interact with DNA by intercalation) (83); gemcitabin (nucleoside analog, anti-metabolite) (84); vinorelbin (anti-mitotic, anti-microtubule) (85); etoposide (topoisomerase II inhibitor) (86); irinotecan and topotecan (topoisomerase I inhibitor) (87, 88). As noted previously, GTPs bear many mechanisms of action which are different from traditional chemotherapeutic agents, such as induction of ROS production and interference with signal transduction (57). Clearly, the molecular targets of GTPs are different from the chemotherapeutic drugs mentioned above (57). Additionally, as noted previously, GTPs are relatively safe as phytochemicals (56).

Therefore, it's reasonable to hypothesize that GTPs can be used as a neo-adjuvant treatment in the prevention and treatment of lung cancer.

In limited studies, it has been shown that green tea catechins enhanced the effect of gemcitabin, dacarbazine, doxorubicin or taxol in different cancer cell lines both *in vitro* and in xenograft animal models (37, 89-91). The combination of EGCG or ECG with doxorubicin increased intracellular doxorubicin accumulation in the chemoresistant hepatocellular carcinoma cell line BEL-7404/DOX, significantly inhibited cell proliferation *in vitro*, and hepatoma growth in a xenograft mouse model (37). EGCG was found to be able to sensitize human cholangiocarcinoma cell line Mz-ChA-1 to gemcitabine-induced apoptosis *in vitro* and *in vivo* (89). Similarly, the enhanced effects of EGCG and dacarbazine on B16-F3m melanoma cells and those of EGCG and paclitaxel on 4T1 breast carcinoma were observed (90, 91). No chemosensitizing effect of GTPs seem to have been reported in a lung cancer cell line and/or lung cancer animal model.

Hypotheses

My first hypothesis is that GTPs administered systemically, by a sustained-release system, will circumvent the problem of limited bioavailability and lower the effective dose compared to the traditional oral route. We have developed a novel delivery system in which GTPs are uniformly embedded with a polymer matrix to provide sustained release of GTPs. In the following studies, I will test this delivery system both *in vitro* and *in vivo*, and eventually test the hypothesis.

My second hypothesis is that adjuvant treatment of GTPs with a standard cancer chemotherapeutic agent (e.g. cisplatin) will enhance efficacy of the drug and lower its toxicity.

Specific Aims

The following specific aims will be pursued to achieve my goals:

Aim 1: To characterize and optimize the delivery of green tea polyphenols by polymeric implants. Polymer formulations will be developed using biodegradable polymers and GTPs and tested to determine the rate of release of GTPs *in vitro* and *in vivo*.

Aim 2: To determine the efficacy of green tea polyphenols against carcinogen-induced DNA adducts *in vitro*. Microsomal and microsome-free systems will be employed to determine the relative efficacy of green tea catechins (EC, EGC, ECG and EGCG) to inhibit DNA adducts induced by BP as measured by ³²P-postlabeling. Spectroscopic methods will be used to determine scavenging of electrophilic metabolites of BP by GTPs.

Aim 3: To determine short-term chemoprotective effects of green tea polyphenols administered by subcutaneous polymeric implants or orally *in vivo*. Rats will be treated with BP by subcutaneous implants alone or with BP co-administered with GTPs by polymeric implants or by oral. Animals will be euthanized at different intervals to determine the effects of GTPs on tissue BP-DNA adducts as measured by ³²P-postlabeling. Effects on selected phase I, phase II enzymes and other related enzymes

will be measured by quantitative RT-PCR and protein levels by Western blotting. The plasma and tissue levels of the GTPs will be determined by LC/MS.

Aim 4: To determine efficacy and potential mechanisms of adjuvant treatment of green tea polyphenols and a standard chemotherapeutic drug on lung cancer development using rodent models. Human lung cancer cell lines (H1299 and A549) will be used in cell culture studies first to test whether EGCG can enhance the effect of a standard chemotherapeutic drug. Nude mice will then be injected with human lung cancer cells and then subcutaneously treated with GTPs and the chemotherapeutic agent individually and as an adjuvant. Efficacy of the various treatments will be determined by measuring tumor indices and modulation of selected biomarkers.

Significance of the studies

Data resulting from these studies will identify the potential role of GTPs in lung cancer intervention and the possible mechanisms by which these agents mediate the carcinogenic process. Importantly, these studies provide an alternative approach to oral exposure that can circumvent bioavailability problems following oral administration of prospective chemopreventive or chemotherapeutic agents as well as lower the effective dose compared with the traditional oral route. Furthermore, adjuvant treatment of GTPs and a chemotherapeutic drug may increase the drug's efficacy by lowering its effective dose, reducing its toxicity and/or circumventing drug resistance. Future prospects of positive results of this proposed work include clinical trials using polymeric devices trapped with GTPs in the treatment of human lung cancer with standard chemotherapeutic drugs.

CHAPTER II: CHARACTERIZATION AND OPTIMIZATION OF THE DELIVERY OF GREEN TEA POLYPHENOLS BY POLYMERIC IMPLANTS

Introduction:

The concept of drug delivery by implants has been applied in a clinical setting for many years. One of the most successful applications of drug delivery by this method to date is sub-dermal contraceptive implants. These implant types have been accepted by millions of women over the past 30 years due to their long lasting efficacy and moderate side effects. However, insertion and removal of the implants has proven to be the greatest barrier to their clinical applications (92). One method to eliminate the necessity of removal of these implants is to use biodegradable materials. This concept has been tested in animal models in recent studies to deliver sex-related hormones and has been proven to be very promising (93, 94). However, biodegradable materials for sub-dermal delivery have not been tested to deliver chemopreventive agents.

The exceptionally low bioavailability of epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which are also the most active components of green tea polyphenols (GTPs), following oral administration, substantially limits their efficacy *in vivo* as discussed (Chapter I) (70, 72, 74). The use of subcutaneous biodegradable polymeric implants for delivery of GTPs to circumvent low bioavailability following oral exposure is explored here.

Poly (epsilon-caprolactone) (PCL) is one of most common biodegradable materials used in sub-dermal implant and importantly, its medical application has been approved by the FDA (95, 96). *In vivo*, the hydrolytic degradation of PCL polymer molecules into a smaller molecular weight polymer will lead to conversion of the implant to small particles which will then be excreted. This process usually takes few months to years based on the molecular weight of polymer. Polycaprolactone implants with 65,000 molecular weight are expected to take two or more years before they will degrade completely and be excreted (97). The drug is released in concert with the physical erosion of the implant, and more importantly, up taken into the blood and tissues as interstitial fluid penetrates into the polymer (98). PCL is ideally suitable for long-term delivery because of its slow degradation rate (96).

In our laboratory, we successfully prepared PCL implants containing GTPs, and these implants can be administered subcutaneously (99). This dosing method is hypothesized to eliminate the poor bioavailability of GTPs following oral administration found in previously published studies (72-74). I expect that in these studies the pharmacological effects of GTPs will be greatly enhanced due to their expected increase in plasma and tissue levels. The studies performed in this chapter were conducted to characterize and optimize the delivery of GTPs from PCL implants.

Materials and Methods:

Chemicals

PCL, GTPs, F68, cyclodextrin, polyethylene glycol, molecular weight 8000 (PEG 8000), ECG, EC, EGC and GTP60 (a green tea extract preparation containing 60%

catechins) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for all *in vitro* studies in this chapter unless mentioned otherwise. EGCG was purchased from LKT laboratories, Inc. (St. Paul, MN, USA). Polyphenon E (a standard green tea extract preparation containing approximately 90% catechins) was obtained from National Cancer Institute and used for the *in vivo* study.

Preparation of GTP-PCL implants

Polymeric implants were prepared using the methodology described elsewhere (99). Briefly, PCL and GTPs were dissolved in dichloromethane and ethanol respectively before mixing, followed by removal of the solvents under reduced pressure (Savant SpeedVac) overnight. The polymeric material was then filled in a disposable syringe attached to silastic tubing (I.D. 3.2 mm), heated at 70°C and extruded. The implants were removed from the silastic tubing mould and excised to desired length (0.5-3 cm length).

Polymer composition (PCLs with different molecular weights), supplements in the implant (F68, cyclodextrin, PEG8000) and GTP load were variable based on the purpose of studies.

Calibration curves of GTP60 *in vitro*

A calibration curve of GTP60 in phosphate-buffered-saline (PBS) with 10% bovine serum was established by spiking the medium with a series of known concentrations of GTP60 and measuring the O.D. values spectrophotometrically at 540 nm after reaction with a dye solution containing 0.1% ferrous sulfate and 0.5% potassium sodium tartrate

tetrahydrate (100). The O.D. values were plotted against the concentration of GTPs. At least three individual experiments were performed to generate a calibration curve.

The effect of polymer composition on GTP release *in vitro*

Implants of PCL of mol. wt. 65,000 (P65) and 15,000 (P15) with different compositions (0%, 10%, 30% or 50% P65) and 10% GTPs were prepared. Release of GTPs from 1 cm implants was investigated by stirring the implants in 5 ml phosphate-buffered-saline (PBS) with 10% bovine serum, pH 7.4 at 37°C to mimic the *in vivo* environment. The amount of catechins released was measured spectrometrically as mentioned above.

Overall degradation rate of GTP60 and poly E *in vitro*

The overall degradation rate of GTP60 in an *in vitro* environment was measured as follows. GTP60, with a known concentration was dissolved in PBS containing 10% serum and kept in amber colored vials at 37°C. An aliquot of this GTP60 solution was sampled and measured spectrophotometrically at different time points.

The overall degradation rate of poly E was obtained in the same method as above.

Degradation of GTPs determined by HPLC

Poly E solution in pure water, PBS or PBS with KOH (pH 12.4) was prepared and analyzed by HPLC (Shimadzu Corp., Columbia, MD) coupled with a C18 Sonoma column, 25 cm×4.6 mm, particle size of 5 µm (ES industries, West Berlin,, NJ) and detected using a UV detector. Mobile Phase A was water with 0.05% trifluoroacetic acid

(TFA), Phase B was acetonitrile with 0.05% TFA. The column was eluted with a linear gradient from 12% to 21% mobile Phase B in 25 min, and increased to 29% for another 10 min at a flow rate of 1 ml/min.

The effect of water soluble polymeric supplements (F68, Cyclodextrin, PEG8000) in the PCL implants on GTP release *in vitro*

In order to determine whether incorporation of water-soluble polymeric supplements into PCL implants can modify GTP release into the medium, a total of four groups were assigned. Implants containing 90% P65 and 10% GTP served as controls. The other three groups employed implants containing 81% P65, 10% GTP and 9% of either F68, cyclodextrin or PEG8000. Release of GTPs from 1 cm implants *in vitro* was measured as mentioned above.

The effects of drug load and implant surface area on GTP release *in vitro*

In order to determine the effects of the level of GTPs embedded in the implants as well as variable levels of implant surface areas on GTP release into the medium, I tested GTP release from 0.5-2 cm implants (corresponding to a surface areas of 2.30-1.075 mm²/mg) with 5-20% of drug loads. A total of 9 groups were assigned containing 20% GTPs of 0.5, 1.0 or 2.0 cm in length; 10 or 5 % GTPs of 1.0, 1.5 or 2.0 cm in length. All test implants contained P65 and F68 with a ratio of 9:1. Release of GTPs from implants *in vitro* was measured spectrophotometrically as described previously.

GTP release *in vivo*

In this study, I aimed to determine the rate of GTP release from subcutaneous implants in ACI rats. The implants were composed of the active component, 20% poly E (total of 40 mg), and the inert ingredients P65 and F68 with a ratio of 9:1. A 2-cm implant was surgically placed onto the back of the rat subcutaneously. At 1, 2, 3, 5, 8 and 19 wks, rats were euthanized and the implants were removed for further analysis. The residual amounts of poly E in the implants were measured by dissolving the implant in a mixture of dichloromethane and ethanol, extraction of the poly E in PBS, followed by reaction with a dying solution and spectrophotometric detection described above and back calculated based on the calibration curve generated by poly E in PBS without serum. The amount of poly E released at selected time points was calculated by subtracting the residual amounts of poly E from the initial amount.

GTP release from implant with PCL coating *in vitro*

In order to determine whether coating implants with a thin layer of blank PCL can improve the sustained release profile of GTP from implants, the following experiment was performed. GTP-PCL implants were prepared as described above. Then these implants were dipped in 8% PCL solution in dichloromethane for 1 sec followed by air dry and repeated sequentially six times. The release profile of these implants was tested *in vitro* comparing with the release profile from implants without PCL coating.

Results:

Calibration curve of GTPs in PBS with 10% serum

The calibration curve of O.D. versus GTP concentration (0 to 250 µg/ml) was found to be linear at the given range with a correlation factor greater than 0.999 (Figure 2-1).

Effect of polymer composition on GTP release *in vitro*

Release of GTPs from the implants showed a continuous release of the GTPs as a function of time (Fig. 2-2A). For example, the release of the GTPs from the implants containing 50% P65 fits very well to a mathematic equation $y = 547.02x^{-0.8637}$, with a R^2 equal to 0.9781, in which y is the daily GTP release and x is the time expressed in days (Fig. 2-2B). Initially, the observed release of the GTPs is comparatively high during the early time points, while the drug release is much slower at the later time points. A theoretical cumulative release of GTPs was obtained by summing up the daily release calculated by the equation above (Fig. 2-2C), however, only approximately 50% of the initial amount of the drug infused in the implant was released during the 9 month study. The plot of cumulative GTP release versus the square root of time was expressed as Figure 2-2D. In this study, the PCL implants remained intact during the entire nine months duration. A higher percentage of P65 was also found to increase the plasticity of the implants which makes them less fragile. For these reasons, implants with a higher percentage of P65 were used in all subsequent experiments.

Overall degradation rate of GTP60 and poly E *in vitro*

Degradation of GTPs varied as a function of time, faster initially and slower at later time point (Fig. 2-3). The rate of degradation of GTP60 in PBS with 10% serum was

found to be approximately 50% in 24 h. Under the same conditions, the degradation of poly E was similar with degradation levels of 58% in a 24 h duration (Fig. 2-3).

Degradation of GTPs determined by HPLC

The degradation rate of GTPs was confirmed by HPLC (Fig. 2-4). The four catechins clearly separated in the elution profile (Fig. 2-4A). The degradation of catechins in PBS occurred within 1 h (Fig. 2-4B) and faster rates were observed in PBS with potassium hydroperoxide (Fig. 2-4C). Results also showed EGCG and EGC were more labile to degradation than EC and ECG (Fig. 2-4).

Effect of implant supplements (F68, Cyclodextrin, PEG8000) on GTP release *in vitro*

In this study, I tested GTP release from implants with different supplement compositions. The purpose of adding these water-soluble polymers was to facilitate the preparation of the implants as well as obtain more sustained release of GTP. Our results indicate that these supplements do not significantly alter the GTP release pattern from the implants (Fig. 2-5) although the presence of these supplemented did facilitate extrusion of the polymeric material into silastic tubing mould.

Effects of drug load and implant surface area on GTP release *in vitro*

The results showed that drug load is the determining factor of GTP release (Figure 2-6). One cm implants with 20% GTP load released more GTP than implants with 10% and 5% drug load (Figure 2-6A). Similarly, two cm implants with 20% GTP load

released more GTP than implants with 10% and 5% drug load (Figure 2-6B). The drug release is proportional to the drug load, and the correlation is high (data not shown).

Comparison of implant sizes, 0.5, 1 and 2 cm containing 20% GTP load resulted in higher release of GTPs initially from the smaller implants, but then the release declined for the next several days (Fig. 2-7). Similar results were observed in 1, 1.5 and 2 cm implants with lower drug loads of GTPs (Fig. 2-7). It should be noted that these data have been normalized by implant weight, therefore, the shorter the implants are, the larger the surface area of the implants. I found that the implants with a larger surface area initially resulted in higher release. However, subsequently, the rate of GTP release was dictated by the drug load.

GTP release from subcutaneous implants *in vivo*

Our results showed that *in vivo* release of GTPs from the implants follows a similar pattern as found *in vitro*, initially a burst release followed by a decreased but continuous release (Fig. 2-8). The total amount of poly E released following 1, 2, 3, 5, 8, 19 week(s) was approximately 18%, 30%, 38%, 42%, 47% and 60%, respectively.

GTP release from implants with blank PCL coating

The result showed that the release of GTPs from implants with a PCL coating was much less initially as compared with implants without the coating, dropping from approximately 4.8% to 1.9% on the 1st day and 2.5% to 1.2% on the 2nd day. In fact, the GTP release from these two groups was almost identical after 9 days, with levels of 0.6% and 0.5% respectively, which indicates that the implants coated with a blank layer of

PCL released GTP at more constant rate than those implants without the PCL coating (Figure 2-9).

Discussion:

The studies in this chapter were conducted to characterize and optimize the release of GTPs from polymeric implants both *in vitro* and *in vivo*. *In vitro* studies were conducted using a mock environment to mimic the *in vivo* situation in order to establish a baseline for further *in vivo* evaluation. *In vitro* release of the GTPs from the GTP60 implant, as measured spectrophotometrically, showed a continuous decline with time. Interestingly the release of GTPs from implants can be expressed as $y = Ax^B$, in which y is the daily GTP release, x is the time expressed in days and A and B are constants (Fig. 2-2B). This mathematical expression of GTP release describes firstly that the release of GTPs from the implant is comparatively high during the early time points, while the drug release is much slower at the later time points. Secondly, it suggests that release is a simple diffusion mediated, in which the readiness of GTP molecules released from implants is inversely proportional to the square of the distance between the molecules and the implant surface. Thus, a plot between the cumulative GTP release versus square root of time should result in a straight line, which is indeed the case as illustrated in Fig. 2-2D. The deviation of the initial time points from the straight line are presumably due to the elapsed time needed for water to penetrate into the implants thoroughly to flush out the GTPs.

Another interesting finding is that the cumulative release of the GTPs measured spectrophotometrically was approximately 50% of drug load (Fig. 2-2C) suggesting degradation of the GTPs may be occurring once they are released into an aqueous environment. The loss of GTPs can be attributed to many environmental factors,

including light and air exposure, which make the accurate determination of the degradation rate of these compounds problematic. A preliminary study was therefore conducted to determine the rate of degradation of GTPs. The result indicates that approximately 50% of GTPs are degraded within 24 hrs (Fig. 2-3), which explains our previous result that approximately 50% of the total GTPs were released from the implant following a 9 month exposure to an aqueous environment at biological pH. It also indicates that the daily release of GTPs from the implant is actually double the value initially found in the previous experiment. The degradation of GTPs is confirmed by HPLC, in which EGCG is more labile to degrade (Fig. 2-4).

Other factors that may affect GTP release, including polymer composition, supplements (F68, cyclodextrin, PEG8000), drug load and surface area of the implants have been studied here, and indicate only drug load plays a key role. Polymer composition and use of supplements (F68, cyclodextrin, PEG8000) do not significantly affect the drug release. The surface area of the implants exhibited some effect on GTP release only during the first several days. At later time points, the drug load appeared to dictate the release. Although F68 does not have significant effect on GTP release, it facilitates the preparation of the implants because it is a surfactant and aids the molding process by decreasing the viscosity of the polymers. In the animal study, 10% F68 was included in the formulations for these reasons.

The release of GTP *in vivo* follows the same pattern as *in vitro*. Although some differences of GTP60 used for *in vitro* studies and poly E used for the *in vivo* study were observed, it is still reasonable to compare the *in vitro* and *in vivo* release. Essentially it

can be concluded that the *in vitro* studies correlated well with the *in vivo* situation and are thus reasonable predictors of the rate of drug release *in vivo*.

In these studies, one drawback of the GTP polymeric implants is that the release of the GTPs is comparatively high during the early time points, while the drug release diminishes significantly at the later time points. The variation in drug concentration could possibly result in either toxicity or lack of efficacy in future animal studies. In order to improve the release profile of the implants, a PCL coating was used to limit the initial burst release. Therefore, it was hypothesized that the initial burst rate of GTP release be lower resulting in an overall more uniform release rate. In the last experiment in this chapter, I demonstrated that release profile of GTPs can be modified by coating GTP-PCL implants with blank PCL and that indeed resulted in a more uniform rate of GTP release (Fig. 2-9).

In conclusion, my studies showed that GTPs are released from these PCL implants in a continuous fashion and the *in vitro* and *in vivo* release rates follow a similar fashion and the release profile of GTPs can be optimized by coating GTP-PCL implants with blank PCL.

Figure 2-1. Calibration curve of GTPs in PBS with 10% serum. GTPs: green tea polyphenols.

Figure 2-2A. Daily release of GTPs in PBS with 10% serum. Data are expressed as mean $\pm$ SD (n=3). P65: Poly (epsilon-caprolactone) of mol. wt. 65,000; P15: Poly (epsilon-caprolactone) of mol. wt. 15,000; GTPs: green tea polyphenols.

Fig. 2-2B. Daily release of GTPs from implants containing 50% P65. Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Fig. 2-2C. Theoretical cumulative GTPs release profile from implants containing 50% P65. Values were calculated from the equation given in Fig. 2B. GTPs: green tea polyphenols.

Fig. 2-2D. Theoretical cumulative GTP release from implants containing 50% P65 versus square root of time. GTPs: green tea polyphenols.

Fig. 2-3. Degradation of GTPs (polyphenon E) in PBS with 10% serum. Data are expressed as mean $\pm$ SD. (n=3). GTPs: green tea polyphenols.

Fig. 2-4. Degradation of GTPs (polyphenon E) in PBS or PBS with KOH (pH 12.4). A: poly E in water; B: poly E in PBS; C: poly E in PBS with potassium peroxide. EC: (-)-epicatechin; EGC: (-)-epigallocatechin; ECG: (-)-epicatechin gallate; EGCG: (-)-epigallocatechin gallate.

Fig. 2-5. Effect of supplements (F68, CD, PEG) on daily GTP release. Data are expressed as mean $\pm$ SD (n=3). CD: cyclodextrin; PEG: polyethelene glycol (PEG used was of mol. wt. 8000).

Fig. 2-6. Effect of GTP load on daily release based on 1 cm (A) and 2 cm (B) implants.

Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Fig. 2-7. Effect of surface area on daily release (normalized by implant weight). Data are expressed as mean $\pm$ SD ($n=3$). GTPs: green tea polyphenols.

Fig. 2-8. GTP release from subcutaneous implants in ACI rats. Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols.

Fig. 2-9. GTP released from GTP-PCL implants with and without coating of blank PCL. Data are expressed as mean $\pm$ SD (n=3). GTPs: green tea polyphenols; PCL: Poly (epsilon-caprolactone).

CHAPTER III: EFFECT OF GREEN TEA CATECHINS AND HYDROLYSABLE TANNINS ON BENZO[a]PYRENE-INDUCED DNA ADDUCTS AND STRUCTURE ACTIVITY RELATIONSHIP

Introduction:

Benzo[a]pyrene (BP) is a polycyclic aromatic hydrocarbon (101), which is present ubiquitously in tobacco smoke, automobile exhaust emissions and grilled foods (14, 15). It is one of the most potent environmental carcinogens. Numerous studies have demonstrated the association of BP exposure and induction of carcinogenesis in many organs including lung, skin, mammary gland and others (16-18).

Enzymatic activation of BP by certain types of cytochrome P450s (CYPs) found in the subcellular microsomal fraction, especially CYP1A1, are needed to produce the ultimate carcinogen *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide (*anti*-BPDE) (19). *anti*-BPDE exerts its carcinogenic activity by alkylating nucleosides on DNA molecules at the bay region of *anti*-BPDE. The reaction primarily happens with the purine bases, deoxyguanosine and deoxyadenosine in DNA (20). As a result, bulky stable and depurinating DNA adducts are formed (21, 22). Insufficient removal of these DNA adducts prior to replication creates hot spots in the gene and can result in deactivation of tumor suppressor genes or activation of oncogenes leading to tumor initiation (23).

Green tea is one of the most popular drinks in the world with some beneficial effects on cardiovascular (42, 43) and neurodegenerative diseases (44). Green tea is now

drawing increasing attention because of its possible application in cancer prevention (102, 103). Green tea preparations were found to decrease tumor incidence and tumor multiplicity in chemically-induced tumor models, including BP and other PAHs (41, 50-54, 104). Interestingly, green tea preparations were effective when administered to mice either during or after carcinogen exposure (50, 51), suggesting their chemopreventive effects in different phases of carcinogenesis.

The catechins in green tea are thought to be the bioactive components, including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG), which bear close structural similarities (Fig. 3-1A). EGCG is the predominant catechin (105). Many mechanisms of action of green tea catechins have been proposed based on studies *in vitro*. Green tea catechins were found to induce apoptosis and inhibit the proliferation of a variety of cancer cell types (45-49). Green tea catechins can generate reactive oxygen species (ROS), including hydrogen peroxides which are responsible for death of cancer cells (59, 60). However, these mechanisms of action are more relevant to the chemotherapeutic effects of green tea catechins, rather than their chemopreventive effects (57).

As noted above, DNA adduct formation is the initial key step in the BP-induced carcinogenesis process. Since green tea manifests its chemopreventive effects in almost all the animal studies conducted, I hypothesize that green tea should be able to inhibit the DNA adduct formation induced by BP. This notion has been supported by a limited number of studies, in which green tea components decreased BP-induced DNA damage in the Chang liver cell line evaluated by the comet assay (106) and EGCG inhibited the

formation of [³H]-BP-derived DNA adducts in a cell-free system (107). However, the mechanism behind these effects is not known.

Back in the early 80s, Conney and co-workers showed that the plant phenolic ellagic acid was highly potent in inhibiting the mutagenesis by *anti*-BPDE (108). Subsequently this group demonstrated that this inhibition occurred due to covalent interaction of ellagic acid with *anti*-BPDE (109). This finding was later supported by inhibition of *anti*-BPDE-induced DNA adducts (110). Green tea extract which contains several catechins with *cis*-diol groups, like in ellagic acid, was reported to decrease *anti*-BPDE-induced DNA strand breaks (111), presumably by the same mechanistic action of ellagic acid reported by Sayer *et al.* (109). Additionally, Bors and Michel (112) and Rice-Evans *et al.* (113) demonstrated that the *cis*-diol groups in green tea catechins could scavenge free radicals such as hydroxyl radicals, azide radicals and superoxide anions, thus correlate with their antioxidant activities.

I hypothesized that green tea catechins will inhibit BP-induced DNA adduct formation by direct quenching of *anti*-BPDE produced in the metabolism of BP, and that the potency of different catechins will vary with the number of their *cis*-diol groups. This structure activity relationship (SAR) study will help to further identify the mechanism of action of green tea catechins. It might also be beneficial for drug modification and drug development based on catechins or compounds bearing similar groups.

This hypothesis cannot be readily tested in a whole cell system because many factors such as the lipid solubility of the catechins, etc. could bias the interpretation. I therefore used a microsomal system to assess the capacity of various catechins in green tea to inhibit DNA adduct formation and determine SAR. Two hydrolysable tannins,

pentagalloylglucose (5GG) and tannic acid (penta-m-digalloyl-glucose), which have more number of *cis*-diol in their structures (Fig. 3-1B) than the catechins were also included to further test the SAR.

Materials and Methods:

Caution: *Both BP and anti-BPDE are mutagenic and carcinogenic. Protective clothing should be worn, and appropriate safety procedures should be followed when working with these compounds.*

Chemicals. EC, EGC, ECG, tannic acid, glucose-6-phosphate, glucose-6-phosphate dehydrogenase from baker's yeast (G6PDH), NADP⁺, BP and salmon testis (st)-DNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG was from LKT laboratories, Inc. (St. Paul, MN, USA). 5GG was obtained from Sinova, Inc. (Bethesda, MD, USA). *Anti*-BPDE was kindly provided by Dr. Subodh Kumar, State University of New York College at Buffalo. Chemicals used in ³²P-postlabeling DNA adduct analysis were the same as described previously (114).

Microsomal BP-induced DNA Adducts. Green tea catechins and hydrolysable tannins were dissolved in Me₂SO and prepared freshly. St-DNA (300 µg/ml) was pre-incubated with 50 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 2.5 mM glucose-6-phosphate, 1 U/ml G6PDH, 0.5 mM NADP⁺, β-naphthflavone-induced microsomal proteins (1 mg/ml) in 1ml for 10 min, in the presence of vehicle alone or green tea catechins or hydrolysable tannins (1-200 µM). BP dissolved in Me₂SO was added at a final concentration of 1 µM,

and incubation was continued for another 30 min at 37°C. The reaction was terminated by addition of EDTA and centrifugation (7500 rpm; 10 min). DNA was isolated from the supernatant by removal of RNA and proteins by digestions with RNases A and T1 and proteinase K, and a series of extractions with phenol, phenol:Sevag (chloroform:isoamyl alcohol, 24:1) and Sevag, followed by precipitation of the DNA with ethanol (114). The DNA concentration was estimated spectrophotometrically.

Reaction of st-DNA with *anti*-BPDE. St-DNA (200 µg/ml) was pre-incubated with 50 mM Tris-HCl (pH 7.5) in a total of 0.2 ml solution for 10 min, in the presence of vehicle or green tea catechins or hydrolysable tannins. Then, *anti*-BPDE was added at a final concentration of 0.5 µM and incubated at 37°C for another 30 min. The reaction was terminated by precipitating DNA with ethanol, and the DNA concentration was measured spectrophotometrically.

Analysis of DNA Adducts. DNA adducts were analyzed by ³²P-postlabeling as described (114). Briefly, 10 µg DNA was digested with micrococcal nuclease and spleen phosphodiesterase (MN/SPD). Before further treatment with nuclease P1 to enrich DNA adducts, an aliquot was used for evaluation of normal nucleotide levels. DNA adducts and normal nucleotides were labeled with [γ -³²P]ATP and T4 polynucleotide kinase. Labeled DNA adducts were separated by multi-directional polyethyleneimine (PEI)-cellulose TLC in the following solvents: D1 = 1.0 M sodium phosphate, pH, 6.0; D3 = 4 M lithium formate/7 M urea, pH 3.5; D4 = 4 M ammonium hydroxide/isopropanol (1.1:1), D5 = 1.7 M sodium phosphate, pH 6.0. Normal nucleotides were resolved in 180

mM sodium phosphate, pH 6.0 by one-directional PEI-cellulose TLC. DNA adducts and normal nucleotides were detected and quantified by Packard InstantImager.

ESI/MS and ESI/MS/MS Study. *Anti*-BPDE and test compounds (green tea catechins and hydrolyzable tannins) were incubated at 37°C at equimolar concentration (500 μ M) in H₂O and acetonitrile (9:1) for 40 min. Reaction products were diluted with 50% acetonitrile/0.1% formic acid and analyzed by ESI/MS in positive ion mode and mass resolution of 10,000 with a Q-TOF API-US mass spectrometer from Waters (Milford, MA). Samples were infused with a syringe pump at 1 μ L/min. Data acquisition lasted for at least 1 min after the signal was stabilized and the spectra were summed, smoothed, and stored. For MS/MS analysis, the collision energy was adjusted to a level such that the intensities of the precursor ions were decreased by 80 to 90%.

LC/MS and LC/MS/MS Study. *Anti*-BPDE and EGCG were incubated at 37°C at equimolar concentration (100 μ M) in H₂O and acetonitrile (9:1) for 40 min. DNA adduct separation was performed by Accela LC from Thermo Scientific (San Jose, CA) with a Hypersil GOLD 50 $\times$ 2.1 mm C18 column. A 15 min gradient with 5% acetonitrile/0.1% formic acid (Solvent A) and 95% acetonitrile/0.1% formic acid (Solvent B) at 0.1 mL/min was used. The gradient started from 5% Solvent B that increased linearly to 50% in 12 min and then increased linearly to 75% in 3 min. Elute from LC was coupled to a LTQ Orbitrap XL mass spectrometer from Thermo Scientific (San Jose, CA) via an ESI source. MS and MS/MS spectra were acquired in positive ion mode at 30,000 mass resolution.

Statistical Analysis. Results were reported as means $\pm$ SEM. The Students t-test was used for the determination of statistical significance between two individual groups. A p-value less than 0.05 with a 95% confidence interval was considered to give the level of significance.

Results:

Before testing the efficacy of these various phenolic compounds, I first determined the lowest concentration of BP in a microsomal reaction that would produce measurable levels of DNA adducts detected by the highly sensitive ^{32}P -postlabeling assay. Incubation of st-DNA with β -naphthaflavone-induced rat liver microsomes, which exhibit increased expression of CYP1A1 and CYP1B1, in the presence of varying concentrations of BP (0.5 to 10 μM) and co-factors resulted in the formation of two major DNA adducts (Fig. 3-2). These DNA adducts have previously been characterized as the products of the interaction of *anti*-BPDE (DNA adduct 1) and 9-OH-BP-4,5-epoxide (9-OH-BPE) (DNA adduct 2) with dG (20, 115).

Total DNA adduct levels increased with increasing concentration of BP (15 ± 6 to 467 ± 49 DNA adducts/ 10^7 nucleotides). The relative levels of DNA adducts 1 and 2 varied with BP concentrations. At the highest concentration of BP (10 μM), 9-OH-BPE-dG levels were slightly greater than *anti*-BPDE-dG levels (9-OH-BPE-dG/*anti*-BPDE-dG = 1.6). However, the ratio of the two DNA adducts increased with decreasing BP concentrations (9-OH-BPE-dG/*anti*-BPDE-dG = 4.5 at 0.5 μM BP), indicating that metabolism of BP to DNA-reactive metabolites is dose-dependent and reflective of the

relative amount of substrate. All subsequent reactions in the presence of the phenolic compounds were performed using relatively low concentration of BP (1 μ M).

Effect of Green Tea Catechins on Microsomal BP-DNA Adducts

Incubation of st-DNA with BP (1 μ M) in the absence or presence of varying concentrations (1 – 200 μ M) of EC, EGC, ECG and EGCG produced qualitatively the same DNA adduct profile (data not shown). Quantitatively, however, BP-DNA adduct levels varied with the type of catechin (Fig. 3-3A). Compared with BP alone (25.2 ± 1.8 DNA adducts/ 10^7 nucleotides; $n = 4$), each catechin tested (100 μ M) resulted in significant inhibition of BP-induced DNA adducts, with EGCG (75%) > ECG (66%) > EGC (39%) > EC (27%). Further, the DNA adduct inhibition observed with each compound was dose dependent (Fig. 3-3A).

When the percent DNA adduct inhibition was plotted against the various catechin concentrations, a clear dose-response was observed in the form of a sigmoid curve (Fig. 3-3B). EGCG and ECG were the most potent components of green tea catechins, with half maximal inhibitory concentration (IC_{50}) values of 16 and 24 μ M, respectively. The other two compounds, EGC and EC were least effective showing IC_{50} values of 146 and 462 μ M, respectively.

To determine the structure-activity relationship, $1/IC_{50}$ was plotted against the number of adjacent OH groups in their molecular structure. A clear relationship was evident (Fig. 3-3C), suggesting that the activity may reside in the *cis*-diol groups.

Effect of Hydrolysable Tannins on Microsomal BP-DNA Adducts

In order to further prove that the activity lies in the *cis*-diol groups, I investigated the effect of 5GG and tannic acid, which are hydrolysable tannins. The rationale for the use of these compounds is that they contain a higher number of adjacent OH groups in their molecular structure, as compared to the green tea catechins, 15 for 5GG and 25 for tannic acid (see Fig. 3-1B). Therefore, it is reasonable to expect that these compounds may be more efficacious than green tea catechins against BP-DNA adduction. As shown in Figures 3-4A and 3-4B, both of these compounds showed effective inhibition of microsomal BP-DNA adducts, and the inhibition was dose-dependent. Both 5GG and tannic acid elicited almost complete DNA adduct inhibition, with tannic acid being much more potent than 5GG ($IC_{50} < 4$ and $26 \mu M$, respectively). It is also interesting to note that the dose-response sigmoid curves were parallel to each other presumably due to their extreme structural similarities.

Effect of Green Tea Catechins and Hydrolysable Tannins on *anti*-BPDE-DNA Adducts

To determine the mechanism by which the test *cis*-diol-containing green tea catechins and the hydrolyzable tannins inhibit microsomal BP-DNA adduction, these compounds were studied in a non-enzymatic reaction, i.e., *anti*-BPDE ($0.5 \mu M$), the ultimate carcinogenic metabolite of BP was incubated with st-DNA ($200 \mu g/ml$) in the presence of vehicle alone or EGCG, ECG, 5GG and tannic acid ($200 \mu M$ each), followed by analysis of the DNA adduct levels by ^{32}P -postlabeling. As shown in Figure 3-5, all compounds showed effective inhibition of *anti*-BPDE-dG. However, the degree of inhibition with the test compounds varied: tannic acid (98% inhibition) > 5GG (68%) >

EGCG (64%) > ECG (39%). These data further support our earlier conclusion that the higher the number of adjacent OH groups, the greater the DNA adduct inhibition. These data also suggest that inhibition of microsomal BP-DNA adducts by the catechins and test hydrolyzable tannins is at least, in part, due to their direct interaction with the electrophilic metabolites of BP.

Detection of *anti*-BPDE-catechin Complex by ESI/MS/MS

This analysis was performed to detect the reaction products in the reaction mixtures of *anti*-BPDE and test compounds (green tea catechins and hydrolysable tannins). All showed clear peaks with the expected mass for the complexes formed. For example, a peak with a m/z ratio of 761 corresponding to *anti*-BPDE-EGCG complex was found in the *anti*-BPDE-EGCG reaction mixture; MS/MS spectrum of the complex further suggested a direct covalent interaction of *anti*-BPDE and EGCG (data not shown).

Investigation of Fragmentation Pattern of *anti*-BPDE-EGCG Complex by LC/MS/MS

LC/MS/MS analysis was performed to further rule out the possibility of non-covalent interaction between EGCG and *anti*-BPDE, and also to investigate the fragmentation pattern of *anti*-BPDE-EGCG complex. Several peaks with m/z ratio of 761 in the spectrum, which corresponding to *anti*-BPDE-EGCG complex, were found. Their retention times were 5.70, 8.51, 9.56, 10.17, 10.51, 10.59, 11.30, 11.47, 11.66 and 11.96 min (Fig. 3-6A). The MS/MS studies of each peak were performed and two major fragmentation patterns were found in the spectrum. The peaks with retention times of

8.51, 9.56, 10.17, 11.30, 11.47, 11.66 and 11.96 all generated a fragmentation pattern in which a fragment with m/z ratio of 591 and another fragment with m/z ratio of 303 exist suggesting *anti*-BPDE attacks the hydroxyl groups on the B ring of EGCG molecules (Fig. 3-6B). The peaks with retention times of 10.51 or 10.59 min generated a different fragmentation pattern in which a fragment with m/z ratio of 455 and another fragment with m/z ratio of 307 exist suggesting *anti*-BPDE attacks the hydroxyl groups on the D ring of EGCG molecules (Fig. 3-6C). Based on the data gathered, two possible *anti*-BPDE-EGCG complexes are proposed (Figs. 3-6B, 3-6C).

Discussion:

In this study, I used a range of EGCG (116) and other catechins and hydrolysable tannins to show dose-dependent inhibition of microsome-mediated BP-induced DNA adducts. Some of the catechins (e.g., EGCG) and hydrolysable tannins (tannic acid) showed nearly 50% inhibition of the adduct formation at as low as 16 and 4 μ M concentrations, respectively. The higher concentrations of test agents were necessary to combat the somewhat high concentration of BP used in order to be able to reliably quantify the resultant DNA adducts. The plasma concentration of EGCG in rodents and in human volunteers is reported to vary with the dosing of green tea extracts. For example, when high pharmacological doses of EGCG was given to mice (2000 mg/kg) (117) or polyphenon E (containing 1200 mg EGCG) given to human volunteers (118) orally, peak plasma concentrations found were approximately 9 μ M and 7.5 μ M in mice and humans, respectively. Typical achievable plasma EGCG concentration of submicro molar has also been reported after two or three cups of tea consumption in humans (74).

A study by Bors and Michel found the reaction rates of green tea catechins and gallate esters against hydroxyl radicals, azide radicals or superoxide anions correlate with catechol and pyrogallol groups in their molecular structures (112), which may explain the antioxidant properties of these compounds. In this study, I demonstrate a clear correlation of adjacent aromatic hydroxyl groups in the molecular structure of green tea catechins and hydrolysable tannins with the inhibitory effects of these compounds on DNA adduct formation induced by BP. Interestingly, it is nearly an exponential relationship between the number of adjacent aromatic hydroxyl groups and IC_{50} of these catechins. There are at least two possible mechanisms through which these compounds can decrease BP-DNA adduct formation, either through interacting with reactive intermediates or interfering with microsomal enzyme activities (eg. CYP1A1). Green tea catechins have inhibitory effects on CYP1A1 activity with the following descending order: ECG $\approx$ EGCG $>$ EC $\approx$ EGC (119). In our study, all the catechins interacted with *anti*-BPDE directly, indicating an exponential relationship with EGCG and ECG being much more potent than the other two catechins studied.

The higher efficacy of the two hydrolysable tannins is due to a greater number of functional hydroxyl groups in their molecular structures. With regards to the potency, the more functional hydroxyl groups in green tea catechins correspond to lower IC_{50} values. This conclusion also holds true in hydrolysable tannins with tannic acid being more potent than 5GG. However, the problem arises when we compare the IC_{50} of EGCG and 5GG, which are about 16 μ M and 26 μ M, respectively, while apparently 5GG has more functional hydroxyl groups than EGCG. This is probably because the potency of the compounds could also be affected by the basic structures. The molecular structure of

green tea catechins and hydrolysable tannins are quite different although some similarities exist, so the comparison of these two compounds may not be appropriate.

In this study, the covalent reaction of *anti*-BPDE and EGCG was demonstrated through ESI/MS/MS and LC/MS/MS. The hydroxyl groups on either B ring or D ring of EGCG molecules (Figs. 3-6B, 3-6C), but not both, were found to react with *anti*-BPDE thus sequestering *anti*-BPDE. This finding suggests that EGCG share the same mechanism of action with ellagic acid which also interacts directly with *anti*-BPDE and lead to sequestration of *anti*-BPDE (109).

Our MS studies on *anti*-BPDE-EGCG complex did not provide information on the exact position of the hydroxyl groups which react with *anti*-BPDE. The hydroxyl group on EGCG could be the 3',4' or 5' on the B ring or the 3'',4'' or 5'' on the D ring (see Fig 3-1A). NMR studies may be necessary to address this question. It is interesting to note that there are several peaks corresponding to m/z ratio of 761 in LC-MS spectrum in *anti*-BPDE-EGCG reaction (Fig. 3-6A). This is probably because *anti*-BPDE has two optical enantiomers and also *anti*-BPDE can attack different positions on B ring or D ring of EGCG molecules, which produce different complexes with different retention times as shown in our results.

The significance of the present study is to demonstrate a new mechanism of action of test catechins. Structure activity relationship of green tea catechins and hydrolysable tannins illustrated in this study may help us discover other chemopreventive reagents. It will also be useful in drug modification and development based on these compounds or compounds with similar molecular structures.

In conclusion, our data demonstrate that green tea catechins and the hydrolysable tannins are highly effective in inhibiting BP-DNA adduct formation at least, in part, due to direct interaction of adjacent hydroxyl groups in their structures, and that the activity is higher with an increasing number of functional hydroxyl groups.

Fig. 3-1. Chemical structures of catechins and two hydrolysable tannins. **A:** (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG). **B:** pentagalloylglucose (R=H) and tannic acid (R=galloyl group as shown on the right).

Fig. 3-2. Representative autoradiographs of ^{32}P -postlabeling analysis of microsome-mediated BP induced DNA adducts. (a) vehicle (2% DMSO) and (b) 1 μM BP. OR, origin. DNA adducts were resolved by multi-directional polyethyleneimine (PEI)-cellulose TLC using the following solvents: D1 = 1.0 M sodium phosphate, pH, 6.0; D3 = 4 M lithium formate/7 M urea, pH 3.5; D4 = 4 M ammonium hydroxide/isopropanol (1.1:1), D5 = 1.7 M sodium phosphate, pH 6.0. DNA adducts were detected by Packard InstantImager.

Fig. 3-3. Effect of indicated green tea catechins on microsomal BP-DNA adducts. **A:** Dose response of test catechins. Data are expressed as mean $\pm$ SEM (n=4). EGCG, (-)-epigallocatechin gallate; ECG, (-)-epicatechin gallate; EGC, (-)-epigallocatechin; EC, (-)-epicatechin. **B:** Estimation of IC_{50} of green tea catechins. **C:** The correlation between $1/\text{IC}_{50}$ with the number of adjacent aromatic hydroxyl groups.

Fig. 3-4. Effect of tannic acid (TA) and pentagalloylglucose (5GG) on microsomal BP-DNA DNA adducts. **A:** Data are expressed as mean $\pm$ SEM (n=4). **B:** Denotes estimated IC₅₀ of TA and 5GG.

Fig. 3-5. Effect of tannic acid (TA), pentagalloylglucose (5GG), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) (200 μ M) on *anti*-BPDE (0.5 μ M)-induced DNA adduct formation. Data are expressed as mean $\pm$ SEM (n=4). (**p<0.01, *p<0.05)

Fig. 3-6. The LC/MS and LC/MS/MS spectrum. **A:** LC/MS spectrum of *anti*-BPDE-EGCG complex. **B** and **C:** LC/MS/MS spectrum of *anti*-BPDE-EGCG complex.

**CHAPTER IV: SUSTAINED SYSTEMIC DELIVERY OF GREEN TEA
POLYPHENOLS BY POLYMERIC IMPLANTS SIGNIFICANTLY DIMINISHES
BENZO[*a*]PYRENE-INDUCED DNA ADDUCTS**

Introduction:

Lung cancer is the second most common cancer type in men and women, only less than prostate cancer in men and breast cancer in women. However, lung cancer accounts for the highest cancer-related deaths, 31% in men and 26% in women (120). Effective prevention and treatment strategies are therefore urgently needed.

Green tea is one of the most popular drinks in the world. It is now drawing more attention because of its possible chemoprotective effects. Green tea polyphenols (GTPs), including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) are believed to be the active components. These bioactive components of green tea along with green tea preparations have been shown to exert their chemopreventive effects *in vitro* (58, 103).

In vivo, green tea consumption decreased lung tumor incidence and tumor multiplicity in chemically-induced lung tumor models, including polycyclic aromatic hydrocarbon (101)- and nitrosamine-induced tumor models (41, 50-54). However, reviews on epidemiological and clinical studies indicated only a marginal beneficial association between green tea consumption and lung cancer risk (63, 64). Similarly,

green tea did not show definite effects on other cancer types, including gastric, colorectal and breast cancer (65-69). One major reason for the lack of consistent protection with GTPs is their lack of bioavailability following oral intake, which ultimately affects their blood levels (70, 74). Studies have shown that only about 0.1%~3% of EGCG and ECG were bioavailable following oral administration (72, 73, 75). In an effort to circumvent the lack of bioavailability associated with oral delivery of GTPs as well as many other chemopreventive agents, this laboratory has developed a novel delivery approach. In this approach, test agents are embedded in cylindrical polymeric implants, which upon subcutaneously grafting provide continuous systemic delivery of the test agents for an extended duration (99).

Benzo[a]pyrene (BP) represents a model PAH to study lung cancer. It is also one of the most potent and environmental carcinogens present ubiquitously in tobacco smoke, automobile exhaust emissions, grilled foods and other sources (14, 15). In this study, polyphenon E (poly E), a standardized green tea extract, was administered by polymeric implants or via drinking water to female Sprague-Dawley (S/D) rats. Animals were challenged with continuous low-dose BP via subcutaneous polymeric implants (121). Effects of poly E administered by the two routes were compared to determine its efficacy against DNA adducts induced by continuous exposure to BP. Potential mechanisms of action of poly E were also investigated by analysis of mRNA and enzyme activity of several phase I, phase II and nucleotide excision repair enzymes.

Materials and Methods:

Chemicals. Poly E was a generous gift from Pharma Foods International Co., LTD (Kyoto, Japan). EC, EGC and ECG were purchased from Sigma-Aldrich (St. Louis, MO). EGCG was from LKT laboratories, Inc. (St. Paul, MN). Chemicals used in ³²P-postlabeling DNA adduct analysis were the same as described previously (114). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Anti-CYP1A1 rabbit polyclonal IgG, GSTM1 rabbit polyclonal IgG, UGT1A mouse monoclonal IgG, goat anti-rabbit secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). *Anti-β-actin* mouse monoclonal antibody was from Sigma-Aldrich (St. Louis, MO). *Anti-CYP1B1* rabbit polyclonal IgG was obtained from BD Biosciences (San Jose, CA). Horse anti-mouse secondary antibody was from Cell Signaling Technology (Danvers, MA). Other reagents used were from the following sources: Trizol (Invitrogen Corporation, Carlsbad, CA), high capacity reverse transcription kit and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL.), and ECL plus detection kit (Amersham Biosciences, Piscataway, NJ).

Polymeric implants. Polymeric implants of poly E and BP were prepared by embedding them in a polymeric matrix comprised of a water-insoluble polymer, polycaprolactone (P65) (mol. wt. 65,000) using the methodology described elsewhere (99). Briefly, the polymer P65 was dissolved in dichloromethane and added to a solution of poly E in ethanol. For BP implants, the polymer and BP were both dissolved in dichloromethane. Following evaporation of the solvent(s) at 70°C with agitation with a glass rod, the polymer matrix was completely freed of the solvents under reduced

pressure overnight. The polymeric material was then filled in a disposable syringe attached to a silastic tubing (I.D. 3.2 mm), heated at 70°C and extruded. The implants were removed from the silastic tubing mould and excised to desired length.

***In vitro* release of poly E from the implants.** Release of poly E was investigated by stirring the implants (2 cm, 200 mg implant containing 40 mg poly E) in phosphate-buffered-saline (PBS) supplemented with 10% bovine serum, pH 7.4 at 37°C to simulate the *in vivo* environment. The amount of catechins released was measured spectrophotometrically at 540 nm after reaction with a dyeing solution containing 0.1% ferrous sulfate and 0.5% potassium sodium tartrate tetrahydrate (100).

Animal handling. Six week-old female S/D rats were purchased from Harland-Sprague-Dawley (Indianapolis, IN). After acclimation for 3 d, animals were randomized into six groups and provided 4% Teklad diet. One week later, animals were treated with BP, poly E or sham treatments as follows:

Group 1, No treatment

Group 2, Sham implants

Group 3, BP implant (2 cm, 200 mg implant containing 20 mg BP)

Group 4, BP implant + poly E implants (two, 2-cm 200 mg implant containing a 40 mg poly E/implant)

Group 5, BP implant + poly E in drinking water (0.8% w/v)

Group 6, Poly E implants

Sham, BP and poly E implants were grafted subcutaneously under anesthesia as described previously (122). Poly E was given in drinking water two days prior to BP implantation. Poly E solution was prepared in deionized water every other day by heating the solution at 90°C with stirring for 3 min. This solution was cooled and stored at 4°C until use. Animals from Groups 1-6 (n = 5) were euthanized one week following BP implantation (Group 6 had only 2 animals). Additional animals in Groups 1-4 (n = 5) were euthanized 4 weeks following BP implantation. Lung and liver tissues were collected and stored at -80°C until use. Blood was collected by cardiac puncture and plasma was collected by mixing with heparin and centrifugation. One ml plasma sample was mixed with 20 µl of 0.4 M NaH₂PO₄ containing 20% ascorbic acid and 0.1% EDTA (pH 3.6) and stored at -80°C until analysis as described (76). All animal experiments were performed after seeking approval from the Institutional Animal Care and Use Committee (IACUC).

Stability of GTPs in polymeric implants. GTPs were released from the implants by dissolving them in 10 ml of dichloromethane and ethanol (1:1), followed by extraction with water and centrifugation. The supernatant containing poly E catechins was filtered by passing through a 0.22 µm centrifugal filter (Millipore Corp., Billerica, MA). Finally, the eluate was analyzed by HPLC (Shimadzu Corp., Columbia, MD) coupled with a C18 Sonoma column, 25 cm×4.6 mm, particle size of 5 µm (ES industries, West Berlin, NJ) and detected by a diode array detector. Mobile Phase A was water containing 0.05% trifluoroacetic acid (TFA), and Phase B was acetonitrile Containing 0.05% TFA. The column was eluted with a linear gradient from 12% to 21% mobile Phase B in 25 min,

and increased to 29% for another 10 min at a flow rate of 1 ml/min. Unused implants and implants recovered from the animals were stored under argon until analysis of poly E catechin levels.

Measurement of poly E doses. The total doses of poly E administered by the implants or via drinking water were calculated as follows:

The dose of poly E in the implant group = initial amount of poly E per two implants
– residual amounts in two implants

The initial and residual amounts of poly E in the implants were measured by dissolving implants in a dichloromethane:ethanol mixture and extraction of the mixture with water as described above, followed by derivatization of the poly E catechins and spectrophotometric measurements (100).

The total dose of poly E in the drinking water group = the concentration of poly E (0.8%) x the amount of water consumed daily per animal x duration of the study in days.

Isolation of DNA, RNA, and microsomes and cytosolic fractions

DNA isolation. DNA from lung tissue was isolated by a solvent extraction procedure involving isolation of crude nuclei, removal of RNA and protein by sequential treatments with RNases and proteinase K, respectively, extractions with phenol, phenol:Sevag and Sevag and finally precipitation of DNA with ethanol (114). DNA concentration was estimated spectrophotometrically.

RNA isolation. RNA from lung tissue was isolated using Trizol reagent following the Vendor's protocol. The quantity and purity of RNA was tested by Nanodrop. The integrity of RNA was tested by agarose gel.

Microsome and cytosolic fractions. Lung tissue (300 mg) was homogenized in 0.25 M sucrose buffer with 0.1 mM EDTA (pH 7.4), centrifuged at 11,000×g for 20 min, followed by centrifugation of the supernatant at 100,000×g for 60 min. The supernatant was collected as cytosolic fraction. The pellet containing microsomes was resuspended in sucrose buffer. Protein concentrations of microsomal and cytosolic fractions were determined by using a BCA protein assay kit.

Analysis of DNA adducts. DNA adducts were analyzed by ^{32}P -postlabeling as described (114). Briefly, 10 μg DNA was digested with a mixture of micrococcal nuclease and spleen phosphodiesterase. Before further treatment with nuclease P1 to enrich adducts, an aliquot was used for evaluation of normal nucleotide levels. Adducts and normal nucleotides were labeled in parallel with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase. Labeled adducts were separated by multi-directional polyethyleneimine (PEI)-cellulose TLC in the following solvents: D1 = 1.0 M sodium phosphate, pH, 6.0; D3 = 4 M lithium formate/7 M urea, pH 3.5; D4 = 4 M ammonium hydroxide/isopropanol (1.1:1), D5 = 1.7 M sodium phosphate, pH 6.0; D2 development was omitted. Normal nucleotides were resolved in 0.18 M sodium phosphate, pH 6.0 by one-directional PEI-cellulose TLC. Adducts and normal nucleotides were detected and quantified by Packard InstantImager. The adduct levels were calculated as relative adduct labeling (RAL), i.e.,

RAL = cpm of adduct/cpm of normal nucleotides x 1/dilution factor. The levels were expressed as adducts/10⁹ nucleotides.

mRNA expression. mRNA levels in the lung tissues were determined by qPCR. Briefly, cDNA was synthesized by using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA). Polymerase Chain Reaction primers for CYP1A1, CYP1B1, Ephx1, UGT1A1, UGT1A6, SULT1A1, GSTM1, ERCC5, ERCC6, XPC and β -Actin were designed using Primer Express (Version 3.0, Applied Biosystems, Foster City, CA). Primer sequences for 18S RNA was obtained from literature (123). The primer sequences were as follows:

	Forward primer	Reverse primer	Amp (bp)
CYP1A1	5'-TGGAGACCTTCCGACATTCAT-3'	5'-GGGATATAGAAGCCATTCAGACTTG-3'	88
CYP1B1	5'-AACCCAGAGGACTTTGATCCG-3'	5'-CGTCGTTTGCCCACTGAAAA-3'	101
Ephx1	5'-ACTTACACATCCAAGCCACCAA-3'	5'-GGCCACGGGAGAGTCA-3'	66
UGT1A1	5'-ACACAGATCGCATGAACTTCCTG-3'	5'-AGGACTCAGAAGGTCCTTGACAGTC-3'	151
UGT1A6	5'-AGACCACATGACATTTCCCAA-3'	5'-AGAGTTCTGGTGTAAGGCAGGTAGG-3'	151
SULT1A1	5'-AGCTGAGACACACTCACCCTGTT-3'	5'-ATCCACAGTCTCCTCGGGTAGA-3'	122
GSTM1	5'-TCTTGACCAGTACCACATTTTGTAG-3'	5'-TCGAAAATATAGGTGTTGAGAGGTAGTG-3'	143
ERCC5	5'-GCCGTGGATATTAGCATTTGG-3'	5'-GGAGCATCACCATCAAATACAAAA-3'	161
ERCC6	5'-CTCCAATGCTTCCCCAGTACA-3'	5'-CGGGTTTATCGTGTCTCTCAAGA-3'	73
XPC	5'-GGAGGAGGTGGAAGAATTACTGA-3'	5'-CCGCGAGGTAGCAGAATTTT-3'	64
18S-RNA	5'-GGGAGGTAGTGACGAAAAATAACAAT-3'	5'-TTGCCCTCCAATGGATCCT-3'	101
β -Actin	5'-GCCAACCGTGAAAAGATGAC-3'	5'-ACCTCATAGATGGGCACAG-3'	165

All primer pairs have been tested for their amplification efficiency. β -Actin and 18S RNA were determined to be good reference genes in this study. Polymerase chain reaction was performed with a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). The comparative CT method was used to determine the difference in mRNA expression between samples by normalizing to housekeeping genes (β -actin and 18S-RNA). The fold differences were calculated as ($2^{-\Delta\Delta C_t}$).

Western blotting. 10% SDS polyacrylamide gel was used for separation of microsomal and cytosolic proteins (12 µg each)/well. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes. After co-incubation with primary and secondary antibodies, detection of proteins was performed using an ECL plus detection kit (Amersham Biosciences, Piscataway, NJ). Primary antibodies were anti-CYP1A1 rabbit polyclonal IgG (1:2000), anti-CYP1B1 rabbit polyclonal IgG (1:2000), GSTM1 rabbit polyclonal IgG (1:400), UGT1A mouse monoclonal IgG (1:2000) and anti-beta-actin mouse monoclonal antibody (1:4,000). All secondary antibodies were used at 1:3,000.

Enzymatic activities

CYP1A1 and CYP1B1. The assay was performed using the conditions described (124, 125). Briefly, the reaction mixture contained 100 mM potassium phosphate, pH 7.6, 5 µM ethoxyresorufin, 25 µg of microsomal protein and 250 µM NADPH. Excitation wavelength of spectrometer was set at 530 nm and emission at 585 nm. Readings were taken immediately after the addition of NADPH and continued for over 30 min. The activity was calculated from the linear portion of the plot.

GST activity. The activity was measured spectrophotometrically as described (125, 126). Briefly, the reaction mixture contained 100 mM potassium phosphate, pH 6.5, 1 mM 1-chloro-2,4-dinitrobenzene, 5 µg of cytosolic protein and 5 mM reduced glutathione. Absorbance was measured at 340 nm. Readings were taken immediately after the addition of reduced glutathione and continued for over 40 min. The activity was calculated from the linear portion of the plot.

Plasma levels of Poly E catechins by LC/MS. Quantification of poly E catechins was performed using LC/MS. Plasma sample preparation followed the literature (76). Briefly, 200 μ l plasma was mixed with 10.5 μ l of glacial acetic acid and 20 μ l of a mixture of β -glucuronidase (2,000 units) and sulfatase (43 units) (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 45 min. The reaction mixture was extracted with dichloromethane followed by ethyl acetate extraction twice. Ten μ l of 2.5% ascorbic acid was added to the combined ethyl acetate extracts. The mixture was lyophilized and the residue was dissolved in 40 μ l 8% acetonitrile. LC/MS was performed by Accela LC from Thermo Scientific (San Jose, CA) with a Hypersil GOLD 50 $\times$ 2.1 mm C18 column. A 15 min gradient with 5% acetonitrile/0.1% formic acid (Solvent A) and 95% acetonitrile/0.1% formic acid (Solvent B) at 0.1 ml/min was used. The gradient started from 5% Solvent B that increased linearly to 50% in 12 min and then increased linearly to 75% in 3 min. Elute from LC was coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) via an ESI source. MS and MS/MS spectra were acquired in positive ion mode at 30,000 mass resolution. Plasma from untreated animals served as blank. Blank plasma spiked with 1, 2, 5, 10, 25, 50, 100, 200, 400 pmol of each catechin standards (EGC, EC, EGCG and ECG), along with 50 pmol quercetin as an internal standard, were used to generate a calibration curve.

Tissue levels of GTPs by LC/MS. Lung tissue (200 mg) was mixed with 0.84 ml of PBS and 40 μ l of glacial acetic acid and homogenized. After centrifugation (16,000g, 6 min), 0.62 ml of the supernatant was processed essentially as described above for plasma GTP levels.

Statistical analysis. Results are reported as mean $\pm$ SD. Generalized linear model (GLM) was used to investigate the effect of treatment and time. SARS v.9.2 was used for statistical analyses. A p-value less than 0.05 was considered statistically significant.

Results:

***In vitro* release of poly E from the implants.** Agitation of poly E polymeric implants in PBS in the presence of serum showed a continuous release of the poly E catechins from the implants. There was a burst release initially and then it declined slowly but continuously. For example, more than 3.6 mg catechins were released on day 1 before declining to nearly 0.27 mg daily release on day 7, thus an exponential decrease occurred as a function of time (Figure 4-1). The cumulative release after 7, 28 and 56 days were 19%, 26% and 29% of the initial amount, respectively. In a separate study I determined that poly E was unstable in the aqueous environment degrading by 58% over 24-h period. Therefore, the above data of catechin release have been corrected by multiplying with the factor 2.38.

Effect of poly E on BP-induced DNA adduct levels and its rate of release and stability *in vivo*. Treatment of S/D rats with a subcutaneous BP implants produced two major DNA adducts in the lung (Figure 4-2A-b); no adducts spots were detectable in sham-treated animals (Figure 4-2A-a). These adducts have been characterized previously as derived by interaction of deoxyguanosine (127) with *anti*-BP-7,8-diol-9,10-epoxide (*anti*-BPDE) (adduct 1) (20) and 9-OH-BP-3,4-epoxide (9-OH-BP) (adduct 2) (115). Both adducts were found to have similar adduct levels in lung tissue following 1 week of

BP treatment, 11.8 ± 5.2 adducts/ 10^9 nucleotides for BPDE-derived and 13.8 ± 4.5 adducts/ 10^9 nucleotides for 9-OH-BP-derived adducts. The total adduct levels increased modestly by 20% from 1 week (25.6 ± 3.9 adducts/ 10^9 nucleotides) through 4 weeks (31.1 ± 5.2 adducts/ 10^9 nucleotides) of BP treatment (Figure 4-2). Adduct levels of both BPDE- and 9-OH-BP-derived adducts increased proportionately with time indicating that both adducts accumulated at similar rates.

Intervention with Poly E administered by the implant route (12.9 ± 2.3 adducts/ 10^9 nucleotides) resulted in a significant reduction (50% decrease; $p=0.023$) of total adduct levels after 1 week of BP treatment (Figure 4-2B1); the levels of both adducts were diminished similarly. The implant route of poly E administration was also effective in reducing the adduct burden after 4 weeks. *Anti*-BPDE-dG adduct levels were significantly reduced (35%) while 9-OH-BP-dG adduct levels were decreased (20%) but the reduction was statistically insignificant (Figure 4-2B2). Poly E administered via the drinking water modestly (34%) diminished the total adduct burden after 1 week, with similar effects on both adduct levels, however, this decline was not significant ($p=0.20$) (Figure 4-2B1); the effect of poly E via the drinking water route after 4 weeks was not investigated.

There was no significant difference in either the body weight or the lung and liver weights between any of the groups (data not shown) suggesting that BP or poly E administration, irrespective of the route of administration, had no detectable adverse effects.

To determine if poly E catechins embedded in the polymeric matrix were stable during the course of the treatment, extracts of the implants recovered from the animals

were analyzed by HPLC. Analysis of the extracts from implants before and after the animal treatment and their comparison with reference catechins at a wide range from 250 to 650 nm UV absorption showed no qualitative difference in the major catechins detected in the implant extracts (Figure 4-3), indicating that the catechins remained stable in the implant during the preparation of implants as well as during the course of the study *in vivo*.

To determine the total dose of poly E administered via the implants, I measured the residual amount of poly E in the implants recovered from the animals. The total dose administered via the drinking water was estimated based on the approximate daily water intake. Comparison of the total dose administered during the course of the 1 week study via the implants (15.7 mg) and the drinking water (1,632 mg) indicated that the oral dose was over 100-fold higher than the implant route. Additionally, the doses of poly E released from implants *in vivo* (15.7 mg) and *in vitro* (15.2 mg) at 1 week time point are in excellent agreement, indicating that the *in vitro* release system can predict the *in vivo* rate of release of poly E catechins.

Plasma levels of GTPs. The calibration curve generated by spiking plasma from untreated animals with 1, 2, 5, 10, 25, 50, 100, 200, 400 pmol of each reference catechin (EGCG, ECG, EGC, EC) and 50 pmol of quercetin as an internal standard was found to be linear in this range ($R^2 > 0.997$ for all compounds tested).

There were no detectable GTPs in animals (groups 1, 2 and 3) without any poly E treatment based on the LC/MS analysis of the plasma samples. However, animals treated with poly E implants for 1 week clearly showed the presence of all the four GTPs in the

plasma samples (Figure 4-4), with EGCG predominating (Table 4-1). In the drinking water group, however, EGC and EC were found at significantly higher levels (Table 4-1). The plasma levels of EGCG in the implant (60.6 ± 25.4 ng/ml) and drinking water (96.9 ± 43.9 ng/ml) groups were not significantly different. EGCG was also the most prominent GTP detected after 4 weeks of poly E implantation; however, the levels were lower compared with one week treatment (Table 4-1).

Tissue levels of GTPs. The detection limits for EGCG, EGC, EC and ECG were established first in the lung tissue environment and found to be approximately 1, 3, 3 and 4 ng/g, respectively. Lung levels of EGCG at 1 week time point were found to be 17.2 ± 8.50 and 19.8 ± 6.87 ng/g tissue in animals treated with poly E via implants (Group 4) and drinking water (Group 5), respectively. However, none of the other catechins EGC, EC and ECG were detected in the lung tissue presumably they were below the detection limits.

Effect of poly E on xenobiotic-metabolizing and DNA repair enzymes in lung tissue. Compared with sham treatment, BP treatment by low-dose continuous exposure resulted in substantial overexpression of CYP1A1 (192 ± 8.5 fold) and CYP1B1 (15 ± 1.2 fold) after 1 week as determined by qPCR. The effect on CYP1A1 expression after 4 weeks was even more pronounced (852 ± 25.9 fold) though the effect on CYP1B1 expression (8 ± 0.68 fold) was less pronounced (Figure 4-5). Poly E administration alone or together with BP treatment showed no significant effect on the expression of the selected phase I enzymes (CYP1A1, CYP1B1 and Epxh1), phase II enzymes (UGT1A1,

UGT1A6, SULT1A1 and GSTM1) or DNA repair enzymes (XPC, ERCC5 and ERCC6) (Figure 4-5).

Consistent with the findings at the mRNA level, sham implant treatment did not affect the expression of CYP1A1, CYP1B1, UGT1A and GSTM1 at the protein levels. However, treatment with BP implants showed higher levels of CYP1A1 and CYP1B1 after 1 week of BP treatment consistent with their overexpression at the mRNA levels. These proteins still remained overexpressed following 4 weeks of BP treatment compared with sham treatment, though the levels were less pronounced; poly E administration alone (group 6) showed no significant effects on any of the phase I and Phase II enzymes studied (Figure 4-6).

At the activity level also, BP treatment greatly induced the activity of CYP1A1 and CYP1B1 compared with sham treatment in the lung microsomes consistent with their overexpression at the mRNA and protein levels. However, no significant inhibition of the activity was observed by poly E administered by implants together with BP. The enzymatic activity was, in fact, significantly increased ($p=0.035$) by poly E when administered via the drinking water together with BP implant (Table 4-2). No significant change in the GST activity was observed following any of the treatment given individually or in combination (Table 4-2).

Discussion:

GTPs have been shown to have significant chemoprotective activity using *in vitro* models, however, their efficacy *in vivo* is inconsistent. A primary limiting factor of this discrepancy is believed to be lack of oral bioavailability of GTPs. In this study we have

investigated the application of a novel sustained-release system via subcutaneous polymeric implants in rats by poly E containing a mixture of GTPs as an alternative to the oral administration of these chemoprotective compounds to increase their bioavailability and efficacy *in vivo*. The rate of release of GTPs from these polymeric implants was first studied *in vitro* using a mock environment to mimic the *in vivo* situation in order to establish a baseline for further *in vivo* evaluation. *In vitro* release of the GTPs from the poly E implant, as measured spectrophotometrically, showed a continuous decline with time. This observation in the initial release appears to be a simple diffusion process. The surface-bound GTP molecules were readily released from the implant as the rate of release was inversely proportional to the square of the distance between the molecules and the implant surface. In this sense, surface-bound drug of the implants is released more readily compared with the drug molecules embedded in the inner layers of the implant. Further, GTPs are not stable under alkaline or near neutral pH, mainly because of oxidation at these pHs. In the *in vitro* release experiment, degradation of the GTPs occurs as they are released from the implants and enter into the surrounding medium (PBS with 10% bovine serum). In this study the rate of degradation of the GTPs was determined separately and the rate of release was adjusted using a correction factor. Although degradation of the GTPs occurs following their release from the implant, no degradation was observed during preparation of the implant due to the stability of GTPs in the organic solvents used in the preparation process. Once the GTPs are embedded in the polymeric matrix, they are sheltered from any aerobic oxidation.

BP treatment was also provided by subcutaneous polymeric implants in order to

provide a continuous low-dose carcinogen exposure instead of a bolus dose as this would represent a more realistic *in vivo* exposure situation.

Previous studies have indicated that green tea and its bioactive components have protective effects against PAH-mediated DNA damage and carcinogenesis, including BP. Preparations of green tea, administered during or after PAH exposure, have been shown to decrease tumor incidence and multiplicity in animal models including mouse forestomach and lung (50), mouse transplacental lung tumors (128), mouse skin (129) and hamster buccal pouch (130). Inhibition of PAH-mediated DNA damage (106, 129), including my own studies (131) and mutagenesis (132) have been shown to mediate green tea's antitumorigenic and anticarcinogenic effects. In this study, the GTP-containing poly E implants were found to be more effective in reducing BP-induced DNA adducts in rat lung tissue following a 1 week exposure, compared to poly E administered via the drinking water (50% versus 34% reduction). More importantly, I determined that the total dose of poly E administered via the implants was >100 times lower than that administered by the oral route (15.7 mg versus 1,632 mg). This observation strongly supports my hypothesis that delivery of GTPs by subcutaneous polymeric implants decreases the effective dose dramatically while eliciting an equal to or greater biological effect. Bioavailability of EGCG was also found to be improved by transdermal delivery compared to oral dosing (133). Poly E implants also continued to be an effective inhibitor of BP-induced DNA adduct formation following 4 weeks of exposure of the rats to continuous low doses of BP. However, the DNA adduct inhibition activity was reduced compared to the 1 week treatment, presumably due to decreased

release of the GTPs from the implants; the poly E effect via the drinking water route after 4 weeks was not investigated.

In order to further investigate the mechanism(s) of poly E's observed BP-induced DNA adduct inhibition, analysis of several enzymes involved in PAH metabolic activation (CYP1A1, CYP1B1 and Ephx1) and detoxification (UGT1A1, UGT1A6, SULT1A1, GSTM1) as well as others involved in nucleotide excision repair (ERCC5, ERCC6, XPC) was conducted. Reports from cell culture studies have shown that green tea extracts can act as an agonist of the AhR and induce the expression of CYP1A at the mRNA and protein levels (134, 135), while simultaneously as an antagonist to inhibit CYP1A expression induced by TCDD (134). GTPs are also known to inhibit the activity of CYP1A in liver microsomes isolated from phenobarbital and 3-methylcholanthrene treated rats (136). However, other studies suggested that the effect of GTPs on P450 monooxygenase varies with cell type (137) and tissue type (138). Many confounding factors, including but not limited to, the differences in composition of GTP preparations in various studies, *in vitro* versus *in vivo* administration routes and dosage may contribute to the observed inconsistency. Presumably due to similar reasons, there were conflicting reports of the effect of GTPs effects on phase II enzymes such as GST (130, 139, 140). In this study, continuous exposure to low dose BP via polymeric implants dramatically increased the expression of CYP1A1 and 1B1 at both mRNA and protein levels, while having no effect on GSTM1 expression. In agreement with this, the enzyme activity of CYP1, but not GST was significantly higher following BP implant treatment. Poly E, administered via the drinking water, but not by the implants, was found to further increase CYP1 enzymatic activity induced by BP, which may, in part, account for the

decreased efficacy of the oral exposure route as compared to the implant route regarding the inhibition of DNA adduct formation between these two groups. However, with the exception of CYP1 enzymatic activity, poly E, administered either by implant or orally, had no effects on the expression or activity of any of the metabolizing or DNA repair enzymes studied, suggesting that poly E inhibits BP-induced DNA adduct formation through non-enzymatic pathways. One likely pathway for the inhibition of BP-induced DNA damage is direct binding of the electrophilic metabolite of BP with the adjacent hydroxyl groups of the green tea polyphenols. In a recent study published from this laboratory (131), a correlation between the number of adjacent aromatic hydroxyl groups in the structure of various GTPs and hydrolyzable tannins and their potencies for inhibiting BP-induced DNA adduction was found. Further, electrospray ionization mass spectrometry and liquid chromatography-mass spectrometry analysis confirmed the direct covalent interaction of the hydroxyl groups of a model GTP, EGCG, with *anti*-BPDE, the ultimate carcinogenic metabolite of BP. EGCG is the most active and also the most abundant component in poly E. The biological effects in this experiment are hypothesized to be predominantly mediated by EGCG and possibly ECG albeit to a lesser degree. The dramatic difference in plasma levels of EC and EGC in the poly E implant group and drinking water group at the 1 week time point reflects the large difference (>100 times fold) in the total dose administered, as noted previously, while the absorption of EC and EGC from the digestive tract is relatively high (31.2% and 13.7%, respectively as reported) (72). The plasma level of EGCG is comparable between these two groups despite the large dose difference, presumably due to the relatively poor digestive absorption of EGCG (0.1%) (72). Considering the short half lives (<3-4 h) of these

catechins *in vivo* (72), the plasma level of the catechins primarily reflects the poly E released into blood stream by the implant or absorbed from the digestive tract on the last day or even a shorter time duration. The release profile from the implants *in vitro* might mimic the *in vivo* release pattern, so it is reasonable to speculate that the plasma concentration of GTPs in the implant group may be higher initially. In the drinking water group, the plasma concentration of GTPs somewhat fluctuated based on the water intake. The significant reduction in the DNA adduct levels observed after 7 d by poly E implants seem to result from its cumulative release during the 7 day period, not from just the 7th day. This notion may also explain the higher degree of adduct inhibition observed in the implant versus the drinking water groups.

In conclusion, my study demonstrates that sustained systemic delivery of GTPs by subcutaneous polymeric implants decreases the effective dose dramatically while eliciting a greater biological effect as compared to the traditional oral route. Further, mechanistic studies of poly E, at submicromolar plasma levels achieved in this study, suggest that its efficacy at inhibiting BP-induced DNA damage was not a result of modulation of metabolic or DNA repair pathways but from direct scavenging of the electrophilic metabolites. Thus, subcutaneous polymeric implants may provide a novel viable sustained release system for chemopreventive/chemotherapeutic agents with poor oral bioavailability such as GTPs.

Table 4-1. Plasma concentrations of individuals GTPs in S/D rats treated with polyphenon E (poly E) via polymeric implants or the drinking water together with benzo[*a*]pyrene implant for indicated periods. Poly E catechin levels were measured by LC-MS.

Poly E catechins	Plasma poly E catechin levels (ng/ml)		
	BP implant + poly E	BP implant + poly E in	BP implant + poly E
	implants (1 wk)	drinking water (1 wk)	implants (4 wk)
EGC	13.3 ± 3.8	277.8 ± 225.3*	7.2 ± 1.5
EC	8.8 ± 2.6	336.5 ± 300.9*	3.4 ± 0.3
EGCG	60.6 ± 25.4	96.9 ± 43.9	15.2 ± 6.3
ECG	8.2 ± 4.3	15.8 ± 8.3	0.9 ± 0.9

P<0.05

Table 4-2. Enzymatic activity of CYP1 and GST following treatment of S/D rats with benzo[a]pyrene (BP) implant or polyphenon E (poly E) implants or combination.

Treatment		CYP1 activity (fold change)	GST activity (fold change)
1 week	BP implant	38.74 ± 7.69	1.17 ± 0.14
	BP implant + Poly E implant	41.21 ± 6.37	1.18 ± 0.17
	BP implant + Poly E in drinking water	51.15 ± 8.89 ^a	1.21 ± 0.11
	Poly E implant	0.61 ± 0.11	1.23 ± 0.03
4 week	BP implant	33.72 ± 12.39	1.34 ± 0.20
	BP implant + Poly E implants	23.28 ± 7.88 ^b	1.23 ± 0.22

Enzymatic activity was expressed as fold change of sham treatment.

^a p<0.05 (BP implant + Poly E in drinking water group versus BP implant group at 1 week)

^b p<0.05 (BP implant + Poly E implant 1 week versus 4 week)

Fig. 4-1. Release of polyphenon E from polymeric implants *in vitro*. Implants (2 cm, 200 mg containing 40 mg polyphenon E) were suspended in 5 ml phosphate-buffered saline containing 10% bovine serum in a shaking water bath at 37°C. The release medium was changed daily and the amount of polyphenon E catechins released was measured as described in text.

Fig. 4-2. DNA adducts in lung tissue. **A**, Representative autoradiographs of ^{32}P -postlabeling analysis of benzo[*a*]pyrene (BP)-induced DNA adducts: a, one-week sham implant; b, one-week BP implant; c, one-week BP implant + polyphenon E implants; d, one-week BP implant + polyphenon E in drinking water; e, four-week BP implant; f,

four-week BP implant + polyphenon E implants. 1, *anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide-dG; and 2, 9-OH-benzo[*a*]pyrene-4,5-epoxide-dG. B1: DNA adduct levels after 1 week; and B2, DNA adduct levels after 4 weeks. * $p < 0.05$.

Fig. 4-3. HPLC profile of polyphenon E catechins. A, reference polyphenon E ; B, extract from freshly prepared polyphenon E implants; and C, extract from polyphenon E implants recovered from the animals after 4 weeks of treatment.

Fig. 4-4. Representative LC/MS spectrum of plasma of S/D rat treated with benzo[a]pyrene implant and polyphenon E implants for one week.

Fig. 4-5. mRNA expression of CYP1A1, 1B1, Ephx1, UGT1A1, UGT1A6, SULT1A1, GSTM1, ERCC5, ERCC6 and XPC in lung tissue of S/D rats treated with benzo[a]pyrene or polyphenon E or combination and indicated controls. Relative expression after 1 (A) or 4 (B) weeks of benzo[a]pyrene treatment. Group 1, No

treatment; Group 2, Sham implants; Group 3, BP implant; Group 4, BP implant + poly E implants; Group 5, BP implant + poly E in drinking water; and Group 6, Poly E implants.

Fig. 4-6. Protein expression of CYP1A1, CYP1B1, UGT1A and GSTM1 in the lung of S/D rat treated with benzo[*a*]pyrene and polyphenon E as analyzed by western blotting. Group 1, No treatment; Group 2, Sham implants; Group 3, BP implant; Group 4, BP implant + poly E implants; Group 5, BP implant + poly E in drinking water; and Group 6, Poly E implants.

CHAPTER V: EFFICACY AND POTENTIAL MECHANISMS OF GREEN TEA POLYPHENOLS AS AN ADJUVANT TREATMENT TO CISPLATIN-BASED LUNG CANCER THERAPY

Introduction:

In the United States, lung cancer is the second most common cancer type in men and women, only less than prostate cancer in men and breast cancer in women. However, lung cancer accounts for the highest cancer-related deaths (120). Effective prevention and treatment strategies are therefore urgently needed.

Currently, chemotherapy is one of the most important strategies in the treatment of lung cancer. To maximize the efficacy and minimize the side effects and toxicity of chemotherapeutic drugs, a combination of two or more chemotherapeutic drugs is often necessary. Based on multiple randomized clinical trials and meta-analyses, cisplatin-based chemotherapy is more preferred over other combinations if it is effective (141). The basic principle of combination chemotherapy is to select drugs with differing mechanisms of action, toxicity and side effect profiles in order to augment their efficacy and decrease their toxicity (78).

Green tea is one of the most popular drinks in the world. It is now drawing more attention because of its possible chemoprotective effects (41, 50-54). The possible mechanisms of action of green tea catechins have been extensively studied *in vitro*. GTPs

were found to induce apoptosis in cancer cells and inhibit the proliferation of a variety of cancer cells types *in vitro*, including lung cancer cells (45-49). GTPs can inhibit epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factor 2 (FGF2) dependent signaling pathway, and interfere with enzyme activities of JUK, JUN, MEK1, MEK2, EK1, EK2, CDK2 (57). They have also been shown to be highly potent antioxidants (58). Under certain conditions, GTPs can generate some types of reactive oxygen species, including hydrogen peroxides which are believed to help to kill cancer cells (59, 60).

Edible berries have demonstrated many biological effects in cardiovascular disorder, degenerative diseases, inflammatory responses and cancer intervention (142-144). Anthocyanidins presented in berries are responsible for the red, purple and blue color of these fruits. Studies have shown that diet supplemented with black raspberry powder inhibit azoxymethane-induced colon cancer and N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis (145, 146). Cherry anthocyanidins were able to inhibit tumor development in APC (min) mice (147, 148). These anthocyanidins are potent antioxidants as GTPs. They were found to inhibit proliferation and promote apoptosis of various cancer cell lines (149), decrease the expression of matrix metalloproteinase (MMP-2) and urokinase-plasminogen activator (u-PA), inhibit activation of c-Jun and NF-kappaB (150).

Based on these studies the mechanisms of action of GTPs and anthocyanidins appear to be different from known chemotherapeutics including but not limited to cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, gemcitabine and vinorelbine (79-85). For example, cisplatin and carboplatin exert their effects through crosslinking

with DNA and form DNA adducts (79, 80); paclitaxel and docetaxel are anti-microtubule agents (81, 82); gemcitabin is a nucleoside analog and an anti-metabolite (84); etoposide is a topoisomerase II inhibitor (86); irinotecan and topotecan are topoisomerase I inhibitors (87, 88). This mechanistic difference between GTPs, anthocyanidins and the standard chemotherapeutics currently used to treat lung cancer follows the principle of combination treatment. Importantly, the application of GTPs and anthocyanidins is relatively safe since it exhibits little to no toxicity or deleterious side effects *in vivo* (55).

In this study, I investigated the effects of these natural compounds as a neo-adjuvant treatment in combination with known chemotherapeutics including cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, gemcitabin, vinorelbin, etoposide and topotecan in cell culture. The effects of these combinations are various. One of the most promising combinations, which is GTPs, anthocyanidins and cisplatin, was further tested *in vivo* using a xenograft nude mouse model. The possible mechanisms of action of the combination therapy were also studied.

Materials and Methods:

Chemicals

EGCG was purchased from LKT laboratories, Inc. (St. Paul, MN, USA). Polyphenone E (poly E) was a gift from Pharma Foods International Co., Ltd (Kyoto, Japan). Anthocyanidins used in cell culture including delphinidin, cyanidin, malvidin, peonidin, petunidin were obtained from ChromaDex Inc. (Irvine, CA, USA). Anthocyanidins used in animal study were isolated from highly enriched bilberry extract by the laboratory of Dr. Inder Pal Singh, NIPER, Mohali, Punjab, India under a

collaborative arrangement. The anthocyanidins isolated contained 32.5% delphinidin, 28.6% cyanidin, 15.4% malvidin, 15% petunidin and 5% peonidin as analyzed by HPLC. Cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, gemcitabine, vinorelbine, etoposide, topotecan, quercetin, withaferin A, plumbagin, curcumin, resveratrol, cucurbitacin B, celastrol, ethidium bromide and acridine orange were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apoptosis kit with Annexin V Alexa Fluor 488 and PI for flow cytometry was purchased from Invitrogen Corporation (Carlsbad, California, USA). Rabbit anti-Bcl-2, anti-Bax, anti-cyclin B1, anti-cyclin D1, anti-cdc2, anti-p21 primary antibody and anti-rabbit HRP-linked secondary antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Rabbit anti-PARP antibody was from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti- β -actin antibody was from Sigma-Aldrich (St. Louis, MO). Mouse anti-PCNA primary antibody and anti-mouse secondary antibody was from Cell Signaling Technology (Danvers, MA). Other reagents used were from the following sources: Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL.), and ECL plus detection kit (Amersham Biosciences, Piscataway, NJ).

Cell culture

The human non-small cell lung cancer cell line, H1299, was obtained from ATCC. Cells between 5-20 passages were used and cultured in Glutamax medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

MTT assay

H1299 cells (5×10^3) were seeded in 96-well plate and received EGCG, anthocyanidins, EGCG plus anthocyanidins, cisplatin or EGCG/anthocyanidins/cisplatin the second day following seeding. After incubation for 48 hrs, the culture medium was replaced and 10 μ l of MTT (5 mg/ml in PBS) was added in each well for additional 3 hrs at 37 °C, the medium was discarded and replaced by 100 μ l of DMSO. Absorbance was determined by a SpectrMax M2 (Molecular Devices, Sunnyvale, CA) at 570 nm (151).

Cell cycle and apoptosis analysis by flow cytometry

2×10^5 cells were seeded in 60 mm culture dishes and received either vehicle, cisplatin, EGCG plus anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin) alone, or cisplatin in combination with EGCG and anthocyanidins the second day. Cells were lifted after incubation for 48 hrs and washed with PBS. For cell cycle analysis, cells were fixed in 70% ethanol and stained with PI only and measured by BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For apoptosis analysis, cells were re-suspended in binding buffer and stained with 5 μ l of Annexin Alexa Fluor 488 (Invitrogen Corporation, Carlsbad, California) and 1 μ l of PI for 15 min at room temperature and diluted with binding buffer before being measured by flow cytometry. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Ethidium bromide/acridine orange staining

1×10^5 cells/well were seeded in a 6-well plate culture dish and received either vehicle, 7.5 μ M of cisplatin, EGCG plus anthocyanidins (delphinidin, cyanidin,

malvidin, peonidin, petunidin) alone (7.5 μ M each) or cisplatin in combination with EGCG and anthocyanidins (7.5 μ M each) the second day. After 48 h, cells were detached and washed with PBS and re-suspended in 25 μ l of PBS followed by staining with 2 μ l of ethidium bromide (100 μ g/ml) and acridine orange (100 μ g/ml). Cells were viewed using a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) and pictures were taken with a Nikon Digital Sight camera (152).

Western blotting

5×10^5 cells were seeded in a 100 mm culture dishes and received either vehicle, 7.5 μ M of cisplatin, EGCG plus anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin) alone (7.5 μ M each) or cisplatin in combination with EGCG and anthocyanidins (7.5 μ M each) the second day. After 48 h, cells were harvested using RIPA buffer. Protein was quantified with Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL.). 10% SDS polyacrylamide gel was used for protein separation (30 μ g/well). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. After co-incubation with primary and secondary antibodies, detection of proteins was performed using an ECL plus detection kit (Amersham Biosciences, Piscataway, NJ). The concentration of anti-cyclin B1 and anti-p21 primary antibodies was 1:500, anti- β actin was 1: 3000, and all others were 1: 1000. The concentration of secondary antibodies was 1:1000 for detection of cyclin B1, p21, 1:5000 for β actin and 1:2000 for the rest.

Animal handling

Six week-old female nude mice were purchased from Charles (Indianapolis, IN). After acclimation for 7 d, animals were inoculated with 1×10^6 H1299 cells with matrigel on the left flank side. After tumor size reached approximately 30 mm^3 , animals were randomized and treated with poly E, anthocyanidins, cisplatin alone or in combination as in Table 5-1. Poly E was administered by intraperitoneal injection (0.1ml of 10 mg/ml poly E solution in H_2O containing 1.0% DMSO, every other day) or by polymeric implants (one, 1.5 cm implant containing 20% polyE). Anthocyanidins were given i.p. (0.1ml of 5 mg/ml bilberry extract solution in H_2O containing 1.0% DMSO, every other day). Cisplatin was administered i.p. (0.1 ml of 1mg/ml solution in PBS) once a week for 5 times. Body weights and tumors were measured weekly. Animals were euthanized after 5 weeks. Blood was taken by cardiac puncture. Skin tissue was fixed in 10% formaldehyde and removed to 70% ethanol before further processing. Tumor tissues were harvested for western blotting following the same protocol mentioned previously in the western blotting section except the protein loading is $50 \mu\text{g}/\text{well}$. The concentrations of primary antibody and secondary antibody remained the same.

All animal experiments were performed after seeking approval from the Institutional Animal Care and Use Committee (IACUC).

Results:

MTT assay for EGCG in combination with other phytochemicals

The MTT assay was conducted in order to assess the effects of various treatments on cell viability and proliferative activity in human lung cancer cells H1299. Firstly, a serial concentration of EGCG was used to determine the IC_{50} of EGCG, which was found

to be approximately 20 μM . At 10 μM , EGCG showed only a moderate effect (10% antiproliferative activity). Secondly, a serial concentration of anthocyanidins (comprised of an equal molar concentration of the following: delphinidin, cyanidin, malvidin, peonidin, petunidin), quercetin, withaferin A, plumbagin, curcumin, resveratrol, cucurbitacin B, celastrol alone or in combination with 10 μM of EGCG were tested for their effect on cell proliferation and viability. The results showed that EGCG enhanced the antiproliferative activity of the anthocyanidin mixture and plumbagin, but not the others (Figure 5-1). The antiproliferative activity of EGCG-anthocyanidins was determined to have an IC_{50} concentration of 6 μM . This value was found to be 2- and 3.3-fold lower compared with anthocyanidins (12 μM) and EGCG (20 μM) alone, respectively.

MTT assay for EGCG in combination with chemotherapeutics

A serial concentration of cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, gemcitabine, vinorelbine, etoposide and topotecan alone or in combination with 10 μM of EGCG were tested using MTT assay to assess cell viability of the human lung cancer cells H1299 following combination treatment with EGCG and these chemotherapeutics. The results showed that EGCG does not enhance the antiproliferative activity of any of these chemotherapeutics (data not shown).

However, when the mixture of EGCG and anthocyanidins (delphinidin, cyanidin, malvidin, peonidin and petunidin) was combined with cisplatin, the antiproliferative activity of cisplatin was found to be enhanced significantly. The IC_{50} of cisplatin alone was found to be ≈ 50 μM . However, when combined with the EGCG-anthocyanidin

mixture (10 μ M each), the IC₅₀ of cisplatin was significantly reduced (<3 μ M) (Figure 5-2).

Cell cycle analysis of cisplatin in combination with EGCG plus anthocyanidins

Cell cycle analysis was conducted in order to assess the effects of various treatments on cell cycle in human lung cancer cells H1299. Cells were treated with either vehicle (0.5% DMSO), 10 μ M of cisplatin, 10 μ M of EGCG- anthocyanidins or 10 μ M of cisplatin plus EGCG- anthocyanidins. The result showed that 46.7% of the cells treated with the vehicle alone were in G1 phase and 31.1% and 21.5% of the cells were in S or G₂ phase, respectively. After treatment with cisplatin, the number of cells in the G1 phase dropped to 25.8% while the number of cells in the G2 phase increased to 53.7%, suggesting that the cisplatin initiated a G2/M arrest. In contrast, cells treated with the EGCG-anthocyanidin mixture, exhibited no significant change in cell cycle distribution compared with vehicle treated cells, 50%, 26.9% and 21.2% in G1, S and G2 phase respectively. The effect of cisplatin in combination with EGCG plus anthocyanidins was similar to the effect of cisplatin alone, 24.5% in G1 and 56.5% in G2 phase (Figure 5-3).

Apoptosis analysis of cisplatin in combination with EGCG plus anthocyanidins

Apoptosis analysis was conducted in order to assess the effects of various treatments on apoptosis in human lung cancer cells H1299. In initial analysis, 10 μ M cisplatin did not cause significant apoptosis, therefore, cells were treated with either vehicle (0.5% DMSO), 25 μ M of cisplatin, 10 μ M of EGCG- anthocyanidins or 25 μ M of cisplatin plus EGCG- anthocyanidins. The results are depicted in Figure 5-4. In the

vehicle treated group the apoptotic cells and dead cells were 6.67% and 3.04% respectively, total of 9.71%. These levels increased following treatment with cisplatin to 12.9% apoptotic cells and 17% dead cells yielding a total of 29.9%. In the EGCG plus anthocyanidins group, the apoptotic cells accounted for 17.4% and dead cells, 9.48%, for a total of 26.88%. Treatment with the combination of cisplatin and EGCG-anthocyanidins resulted in a significant increase in apoptotic cells to 36.9% and dead cells to 22.8%, for a total of 59.7%. These data indicate that the combination treatment of cisplatin/EGCG/anthocyanidins was resulted in higher levels of apoptosis and cell death than treatment with cisplatin or EGCG-anthocyanidins alone.

Ethidium bromide / acridine orange staining

EB/AO staining was conducted to confirm the finding in flow cytometric study. No dead or apoptotic cells were found in vehicle (0.5% DMSO) treated samples (Figure 5-5). Further, cisplatin treatment (7.5 μ M) did not induce cell death or apoptosis. However, the size of cells appeared to be larger than those observed following vehicle treatment, suggesting that cisplatin may interfere with the cell cycle and cause cell cycle arrest. EGCG plus anthocyanidins (7.5 μ M) resulted in significant apoptosis which exhibited fragmented DNA staining with bright green or yellow. This effect was even more pronounced in the cisplatin and EGCG plus anthocyanidins combination treatment group, which confirms the finding in apoptosis analysis using flow cytometer.

Western blotting

Western blotting was conducted in order to assess the effects of various treatments on protein expression in human lung cancer cells H1299. Several molecular markers related to cell cycle (PCNA, p21, cdc2, cyclin B1, cyclin D1) and apoptosis (Bax, Bcl-2, PARP) have been investigated. The results showed that EGCG plus anthocyanidins down-regulated the expression of PCNA, cyclin B1, cyclin D1 and BCL-2, which are associated with the cell survival and proliferation; while up-regulated the expression of p21, which functions otherwise (Figure 5-6). Cisplatin treatment also decreased PCNA, cyclin D1 and BCL-2 expression, while increasing p21 expression. However, cisplatin treatment resulted in a significant increase of cyclin B1 expression. The combination treatment of cisplatin and EGCG plus anthocyanidins further decreased the expression of cyclin D1 and Bcl-2 compared with cisplatin alone or EGCG-anthocyanidins alone (Figure 5-6).

Xenograft nude mouse study An animal study was conducted in order to investigate whether the findings in cell culture can be translated to *in vivo*. A combination of poly E, anthocyanidins and cisplatin administered by i.p. (Group 6) significantly decreased the tumor volume compared to that of vehicle treatment group starting from the 1st week through the end of the study. At the 5th week, the tumor volume was decreased by 73% ($p < 0.001$) (Figure 5-7). Similarly, the combination of cisplatin-poly E-anthocyanidins in which poly E was given by polymeric implants (Group 7) decreased tumor volume by 73% ($p < 0.001$). Interestingly, other groups including poly E alone (15% reduction, $p > 0.05$), anthocyanidins (10% reduction, $p > 0.05$), poly E-anthocyanidins (6% reduction, $p > 0.05$) and cisplatin alone group (31%, $p > 0.05$) did not

show statistically significant reductions in tumor volume until the end of the study, suggesting the efficacy of cisplatin/poly E/anthocyanidins observed from the combination treatment may be synergistic not purely additive (Figure 5-7). Photographs of selected animals representing each of the treatment group are shown in Figure 5-8.

All the animals in Groups 1 - 5 were active and gaining body weight throughout the duration of the experiment. However the animals in Groups 6 and 7 appeared to exhibit decreased activity following i.p. injections and were losing body weight (Figure 5-9). The diet intake for these animals was measured. Groups 1-5 consumed, on average, 3.9, 3.4 3.9, 3.4 and 3.6 g/animal/day, respectively. Animals in Groups 6 and 7 consumed less diets compared with other groups, which were 2.9 and 2.6 g/animal/day, respectively, suggesting the possible toxicity of the combination treatment.

The blood chemistry was measured as shown in Table 5-2. The blood cells were counted as in Table 5-3. The blood analyses were conducted by Antech Diagnostics (New Hyde Park, NY). The combination treatment (Group 6) resulted in increased levels of ALT and WBC counts in the animals tested. Also, lower glucose levels and dramatic increased blood level of amylase and lipase were observed in these animals as well.

The skin samples of the animals in Groups 1 and 6 were fixed and HE staining was performed thereafter. The results showed that dramatic damage of the whole layers of the skin, including epidermis, dermis and also the subcutaneous tissue for Group 6 animals (Figure 5-10). Fewer hair follicles and shattered fat tissues cells were also observed in this group. Further, subcutaneous muscle layer was thinner in Group 6 comparing with that in Group 1.

The western blotting results of tumor tissue were consistent with the cell culture results. Specifically, cyclin D1 decreased in the combination treatment group compared with the vehicle treated group and other groups. Bcl-2 increased after combination treatment (Figure 5-11).

Discussion:

The studies in this chapter were conducted to test the feasibility of using GTPs as a neo-adjuvant treatment in the treatment of lung cancer. The MTT assay was used to study the effects of EGCG on cell viability and proliferation in human lung cancer cells. Quite surprisingly, we found that EGCG enhanced the antiproliferative effects of anthocyanidins and plumbagin, but not the other phytochemicals tested, including quercetin, withaferin A, curcumin, resveratrol, cucurbitacin B or celastrol. The reasons can be very complicated because these phytochemicals have been shown to interfere with many signal transduction pathways and molecular targets (153-162). Anthocyanidins, plumbagin and EGCG have all been shown to induce ROS production at specific dose ranges which results, subsequently, in cell death (60, 163, 164). This supports my observation that the combination of EGCG with either anthocyanidins or plumbagin results in greater loss of cell viability in lung cancer cells than either phytochemical alone.

Unexpectedly, EGCG was not found to enhance the antiproliferative effects of all the tested chemotherapeutics commonly used in lung cancer treatment, including cisplatin, carboplatin, paclitaxel, docetaxel, doxorubicin, gemcitabine, vinorelbine, etoposide and topotecan. The reasons remain unknown. Importantly, the antiproliferative

effects of cisplatin were significantly enhanced when this chemotherapeutic was combined with EGCG-anthocyanidins. The IC_{50} of cisplatin decreased approximately 20 fold (from 50 μ M to < 3 μ M) in the presence of EGCG-anthocyanidins. Cisplatin is a model drug in the treatment of lung cancer based on its efficacy as mentioned in the Introduction. Therefore, I aimed to further investigate the mechanisms of action of EGCG-anthocyanidins-cisplatin combination treatment and to determine if these effects were demonstrable using a xenograft nude mouse model.

Cell cycle analysis in the human lung cancer cells H1299 revealed that cisplatin induces cell cycle arrest at the G2/M phase. This observation corresponded to its significant increase of cyclin B1 at the protein level. Additionally, cisplatin was found to modulate other cell cycle or cell death related proteins including cyclin D1 and Bcl-2. Treatment of the cells with EGCG-anthocyanidins did not result in cell cycle arrest at the tested concentration. Further, the distribution of cells in cell cycle did not differ from that of the vehicle treatment. It's presumably because EGCG-anthocyanidins non-specifically inhibit the expression of cell cycle related protein (Figure 5-6). Both cisplatin and EGCG-anthocyanidins were found to induce cell death at the concentrations tested (Figure 5-4), and these effects on cell death appear to be additive in these *in vitro* studies. The morphological changes and apoptosis induced by cisplatin, EGCG-anthocyanidins alone or in combination further confirm these findings in flow cytometric analysis.

Using a xenograft nude mouse model, a combination of poly E, anthocyanidins and cisplatin was found to substantially decrease the tumor volume (73%), while poly E, anthocyanidins, poly E-anthocyanidins and cisplatin alone group had no observable effect. Regrettably, the toxicity caused by the combination treatment was also

noteworthy. The body weight of the animals in the combination treatment group was significantly lower than other treatment groups ($p < 0.01$). This observation was concurrent with a moderate glucose decrease, which correlated to lower diet consumption in the combination group. Additionally, amylase and lipase levels were also found to be increased in the combination group, indicating the animals are mobilizing their lipid and polysaccharides in the body or the possibility of pancreatitis caused by the treatment. Further, the animals in the combination groups were also observed to be less active compared with the animals in other groups. Elevated ALT, a liver specific enzyme, and WBC counts were also observed. AST, alkaline phosphatase and GGT which are not liver specific, however, were unaffected. These results suggest that the toxicity of the combination treatment is most likely a result of liver cell damage. The combination treatment also resulted in damage to the GI tract and disruption of the barrier function of GI tract causing the pathogens in the digestive tract to enter the blood stream. Pathogens in the blood can result in an elevated WBC count or possibly increased stress as a result of the increased handling during the combination treatment lead to the observed increase in WBC count, or both. In summary, the blood chemistry and blood count correlate with the toxicity found in the animal study.

Both, efficacy and toxicity were observed only in the cisplatin-poly E-anthocyanidins combination treatment group, suggesting the effects are a result of the interaction between cisplatin, poly E and anthocyanidins. Although the mechanism(s) of action through which the combination of these compounds exert their effect are unknown, indeed effects of cisplatin-poly E-anthocyanidins combination are arguably not possible to be explained by only one or two mechanisms considering the combination

mixture could interfere with a multitude of signaling transduction pathways and molecular targets. A Pubmed literature search on the interaction of cisplatin, green tea polyphenols and anthocyanidins found no similar studies have been conducted. Two publications were found in which green tea extracts showed protective effects of cisplatin-induced nephrotoxicity (165, 166). In this study, I investigated the effects of these drugs on the proteins involved in cell cycle regulation and cell apoptosis. Western blotting results in cell culture and in the xenograft animal study showed consistent findings. The combination treatment significantly inhibited the expression of cyclin D1, which is almost undetectable in cell culture and dramatically decreased in tumor tissues. Cyclin D1 is essential for cell proliferation and growth. The extreme low expression of cyclin D1 will most likely interfere with tumor cell proliferation and growth which was observable as a therapeutic effect as well as toxicity to normal cell growth which was reflected in decreased body weight of the treated animals. The expression of Bcl-2, which is an anti-apoptotic protein, increased after combination treatment both in cell culture and also in tumor tissue of the animals. Other cell cycle and cell apoptotic related proteins are also modulated by the combination treatment, which may partially explain the results I observed. All these results suggest that cisplatin-poly E-anthocyanidins combination has greater effects in terms of inhibiting cell proliferation and inducing cell apoptosis compared with individual treatments, thus decreasing the tumor volume. However, it is apparent that there is no selectivity of this combination treatment in that although it is efficacious against lung tumor growth, it is concurrently toxic to the host as well. This conundrum, however, is not uncommon of chemotherapeutic agents and requires a necessary optimization of treatment protocol for weighted beneficial versus toxic effects.

In conclusion, my data suggest that combination of EGCG-anthocyanidins may be effective as adjuvant chemotherapy in combination with cisplatin. However we must pay special attention to the toxicity caused by the combination treatment. The optimal dosage of each compound targeting to a moderate efficacy and minimal toxicity needs to be further studied. Further, the mechanisms of action of this combination therapy need to be further clarified.

Table 5-1: Grouping and treatment of the animals.

Group	Cisplatin	Poly E	anthocyanidins	No. of animals
1	-	-	-	12
2	-	+, i.p.	-	10
3	-	-	+, i.p.	10
4	-	+, i.p.	+, i.p.	10
5	+, i.p.	-	-	10
6	+, i.p.	+, i.p.	+, i.p.	10
7	+, i.p.	+, implant	+, i.p.	10

+, treatment; -, no treatment; i.p., intraperitoneal injection;

Table 5-2. Blood chemistry test.

Group	1 (veh.)	2 (poly E)	3 (anthos)	4 (poly E+ anthos)	5 (cisplatin)	6 (combination)
Total Protein	6.0 ± 0.3	5.4 ± 0.2	5.7 ± 0.5	5.3 ± 0.2	5.8 ± 0.4	5.6 ± 0.2
Albumin	3.7 ± 0.3	3.2 ± 0.1	3.5 ± 0.3	3.3 ± 0.3	3.5 ± 0.5	3.3 ± 0.1
Globulin	2.3 ± 0.3	2.2 ± 0.3	2.2 ± 0.2	2.1 ± 0.1	2.3 ± 0.2	2.3 ± 0.1
A/G Ratio	1.7 ± 0.3	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.3	1.4 ± 0.1
	216.3 ±	185.0 ±	227.5 ±	178.5 ±	242.0 ±	
AST (SGOT)	53.3	68.1 39.3 ±	88.6	42.1	158.2	234.5 ± 43.9
ALT (SGPT)	36.5 ± 6.4	13.4	33.5 ± 7.9	35.3 ± 7.3	35.0 ± 8.4	64.3 ± 24.1
Alkaline phosphatase	57.8 ± 18.6	17.0 79.8 ±	59.8 ± 19.3	70.8 ± 21.0	68.5 ± 13.5	69.3 ± 22.2
GGT	15.8 ± 8.8	8.3 ± 2.9	18.0 ± 17.5	9.3 ± 4.3	11.0 ± 12.7	9.0 ± 2.6
Total Bilirubin	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
BUN	28.0 ± 2.8	25.3 ± 2.2	23.8 ± 2.6	24.0 ± 3.6	22.0 ± 2.9	31.5 ± 3.1
Creatinine	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
BUN/Creatinine Ratio	87.5 ± 35.7	95.0 ± 38.9	82.5 ± 37.5	86.3 ± 45.9	82.5 ± 34.3	105.0 ± 62.3
Phosphorus	15.8 ± 1.2	13.6 ± 0.9 75.0 ±	12.8 ± 1.8	13.9 ± 1.5	11.2 ± 1.4	10.9 ± 1.9
Glucose	94.5 ± 50.9	25.9	27.4	82.5 ± 35.2	61.4	77.3 ± 18.4
Calcium	10.1 ± 0.3	9.2 ± 0.5	9.8 ± 0.2	9.6 ± 0.4	10.3 ± 0.3	7.2 ± 4.4
Magnesium	3.9 ± 0.2	3.4 ± 0.2	3.5 ± 0.2	3.6 ± 0.1	3.1 ± 0.3	2.9 ± 0.3
		149.0 ±	147.3 ±	151.0 ±		
Sodium	154.8 ± 9.4	12.1	11.0	11.6	152.0 ± 6.7	157.5 ± 7.7
Potassium	10.9 ± 1.0	7.7 ± 2.8 22.0 ±	6.9 ± 4.8	7.1 ± 4.9	9.7 ± 0.9	10.2 ± 1.4
Na/K Ratio	14.3 ± 1.3	10.0 105.8 ±	31.5 ± 20.2	30.5 ± 18.1 104.8 ±	16.0 ± 2.2	15.8 ± 1.7
Chloride	107.5 ± 6.6	7.5	108.0 ± 8.0	10.0	107.0 ± 3.5	109.5 ± 4.4
	146.5 ±	127.0 ±	128.8 ±		140.0 ±	
Cholesterol	10.2	3.2	20.6	129.5 ± 7.8	10.8	152.0 ± 17.7
	134.5 ±	112.3 ±		104.8 ±		
Triglyceride	41.4	14.5	97.3 ± 11.2	36.1	116.5 ± 5.7	92.0 ± 31.3
	691.0 ±	623.0 ±	644.3 ±	700.8 ±	758.8 ±	1459.8 ±
Amylase	91.3	108.1 85.5 ±	102.5	112.5	83.8	918.2 660.3 ±
Lipase	93.8 ± 18.2	23.4	90.0 ± 9.1	98.0 ± 11.2	98.8 ± 8.4	1125.2
	816.0 ±	630.5 ±	1088.5 ±	607.5 ±	1358.3 ±	
CPK	492.0	422.5	655.8	203.3	1316.6	522.8 ± 131.4

Table 5-3. Blood cell counts.

Group	1 (veh.)	2 (poly E)	3 (anthos)	4 (poly E+ anthos)	5 (cisplatin)	6 (combination)
WBC	2.3 ± 1.5	1.7 ± 0.5	3.6 ± 2.8	4.7 ± 1.3	3.5 ± 3.1	6.0 ± 1.3
RBC	7.3 ± 0.5		7.3 ± 0.8	7.8 ± 0.3	7.7 ± 0.1	7.0 ± 0.2
HGB	11.9 ± 0.5		12.4 ± 0.8	13.0 ± 0.4	13.1 ± 0.4	11.9 ± 0.3
HCT	39.8 ± 3.0	33.7 ± 2.5	38.3 ± 6.9	43.5 ± 2.9	36.3 ± 7.3	38.3 ± 1.7
MCV	56.0 ± 2.6		56.7 ± 1.5	55.8 ± 1.3	55.5 ± 0.7	54.8 ± 2.4
MCH	16.5 ± 0.4		17.2 ± 0.9	16.6 ± 0.5	17.0 ± 0.1	17.0 ± 0.4
MCHC	29.7 ± 1.2		30.7 ± 2.1	29.8 ± 1.5	30.5 ± 0.7	31.3 ± 1.0
<u>Differential</u>						
	452.8 ±	640.5 ±	686.3 ±	1448.8 ±	534.8 ±	2018.0 ±
Neutrophils	263.2	119.5	442.4	784.0	421.7	1101.9
	1601.3 ±	864.0 ±	2559.0 ±	2968.8 ±	2594.3 ±	3591.5 ±
Lymphocytes	1070.4	326.6	2068.4	798.0	2430.5	707.9
	234.8 ±	102.0 ±	322.0 ±	240.0 ±	343.5 ±	
Monocytes	169.1	112.0	366.5	232.4	385.0	390.5 ± 193.6
		43.5 ±				
Eosinophils	28.8 ± 9.1	37.1	43.0 ± 26.3	67.5 ± 52.4	38.3 ± 28.1	0.0 ± 0.0
Basophils	7.5 ± 15.0	0.0 ± 0.0	14.8 ± 29.5	0.0 ± 0.0	14.3 ± 28.5	0.0 ± 0.0
Platelet Estimate						
	1101.3 ±		1001.0 ±	1105.0 ±	925.0 ±	1199.5 ±
Platelet Count	185.8		325.7	189.4	240.4	301.3
<u>Differential (%)</u>						
Neutrophils	20.5 ± 7.2	40.0 ± 6.6	20.3 ± 8.5	29.8 ± 11.3	24.5 ± 17.6	32.8 ± 12.1
Lymphocytes	68.0 ± 4.2	52.0 ± 6.2	71.0 ± 7.7	63.5 ± 7.9	67.0 ± 12.7	60.8 ± 10.2
Monocytes	9.8 ± 3.6	5.3 ± 4.3	7.0 ± 4.8	5.5 ± 4.8	7.0 ± 5.3	6.5 ± 3.0
Eosinophils	1.5 ± 0.6	2.8 ± 2.5	1.5 ± 0.6	1.3 ± 1.0	1.3 ± 0.5	0.0 ± 0.0
Basophils	0.3 ± 0.5	0.0 ± 0.0	0.3 ± 0.5	0.0 ± 0.0	0.3 ± 0.5	0.0 ± 0.0

Fig. 5-1. MTT assay of anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration) at the presence/absence of EGCG.

Fig. 5-2. MTT assay of cisplatin at the presence/absence of EGCG and anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration).

Fig. 5-3. The effects of vehicle, cisplatin, EGCG+anthocyanidins alone or in combination on cell cycle. A. vehicle; B. 10 μ M of cisplatin; C. 10 μ M of EGCG- anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration); D. 10 μ M of cisplatin plus 10 μ M of EGCG-anthocyanidins.

Fig. 5-4. The effects of vehicle, cisplatin, EGCG-anthocyanidins alone or in combination on cell apoptosis. A. vehicle; B. 25 μ M of cisplatin; C. 10 μ M of EGCG-anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration); D. 25 μ M of cisplatin plus 10 μ M of EGCG-anthocyanidins.

Fig. 5-5. Ethidium bromide/acridine orange staining of the cells treated with vehicle, cisplatin, EGCG-anthocyanidins alone or in combination. A. vehicle; B. 7.5 μ M of cisplatin; C. 7.5 μ M of EGCG-anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration); D. 7.5 μ M of cisplatin plus 7.5 μ M of EGCG-anthocyanidins.

Fig. 5-6. Protein expression of H1299 lung cancer cells treated with vehicle, cisplatin, EGCG-anthocyanidins alone or in combination. A. vehicle; B. 7.5 μ M of cisplatin; C. 7.5 μ M of EGCG-anthocyanidins (delphinidin, cyanidin, malvidin, peonidin, petunidin with equal molar concentration); D. 7.5 μ M of cisplatin plus 7.5 μ M of EGCG-anthocyanidins.

Fig. 5-7. Effects of treatments (vehicle, poly E alone, anthocyanidins alone, poly E + anthocyanidins alone, cisplatin alone, cisplatin + poly E + anthocyanidins i.p. and cisplatin+anthocyanidins i.p. combined with poly E implant) on tumor volume in xenograft nude mouse model. Standard deviation coefficients range from 0.13 to 0.77, with an average of 0.43 and a median of 0.45 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 5-8. Pictures of nude mice treated with vehicle, poly E alone, anthocyanidins alone, poly E + anthocyanidins alone, cisplatin alone, cisplatin+poly E+anthocyanidins by i.p. and cisplatin+anthocyanidins i.p. combined with poly E implant.

Fig. 5-9. Effects of different treatments on body weights. Standard deviation coefficients range from 0.04 to 0.15, with an average of 0.092 and a median of 0.094. (* $p < 0.05$, ** $p < 0.01$).

Fig. 5-10. HE staining of animal skins. Upper panel: vehicle treated group; lower panel: cisplatin+poly E+anthocyanidins i.p. group.

Fig. 5-11. Protein expression in the tumor tissues. 1-6 represents the group number.

CHAPTER VI: SUMMARY

Green tea is one of the most popular drinks in the world. It's drawing increasing attention because of its potential chemopreventive effects. Green tea polyphenols (GTPs), including EGCG, ECG, EGC and EC are believed to be the active components, in which EGCG is the most active catechin.

Although numerous *in vitro* studies have demonstrated the anti-cancer effects of GTPs, epidemiological and clinic data showed mixed results. One major reason is due to lack of oral bioavailability of GTPs limits their efficacy *in vivo*.

In our laboratory, we developed a novel delivery system in which GTPs are uniformly embedded with a polymer matrix to provide sustained release of GTPs. I hypothesized that administration of these GTPs via subcutaneous polymeric implants will circumvent the problem of limited bioavailability of GTPs and lower the effective dose compared to the traditional oral route.

The release profile of these implants was investigated both *in vitro* and *in vivo* (Chapter II). The results showed that GTPs were released from these PCL implants in a continuous fashion and the *in vitro* and *in vivo* release follows a similar fashion. More importantly, GTPs remain stable during preparation of the implants and *in vivo* within the time duration tested (Chapter IV), although they are not stable at alkaline or near neutral condition once released from implants (Chapter II).

In animal study, polyphenon E (poly E), a standardized green tea extract, was administered by PCL implants grafted subcutaneously or via the drinking water. When challenged by continuous low dose of benzo[*a*]pyrene (BP) via subcutaneous polymeric implants, the GTP-polymeric implants showed a significant reduction of BP-induced DNA adducts; however, only a modest but insignificant reduction occurred when GTPs were administered via the drinking water. The implant delivery system also showed significant reduction of the known BP diolepoxide-derived DNA adduct after 4 weeks. Notably, the total dose of poly E administered was >100-fold lower in the implant group than the drinking water group (Chapter IV). This supports my hypothesis that sustained systemic delivery of poly E significantly reduced BP-induced DNA adducts by overcoming oral bioavailability issue and that the effective dose of poly E was substantially lower than oral delivery.

Mechanistic studies of poly E, at submicromolar plasma levels achieved in this study, suggest that its efficacy at inhibiting BP-induced DNA damage was not a result of modulation of metabolic or DNA repair pathways (Chapter IV) but from direct scavenging of the electrophilic metabolites, which has been detailed in my microsomal studies and LC/MS study in Chapter III. In the microsomal experiments, BP was incubated with rat liver microsomes and DNA in the presence of the green tea catechins or vehicle. DNA adduct formation was analyzed. The inhibitory activity of the catechins was in the following descending order: epigallocatechin gallate > epicatechin gallate > epigallocatechin > epicatechin, suggesting a correlation between the number of adjacent aromatic hydroxyl groups in the molecular structure and their potencies. To determine if the activity of these compounds was due to direct interaction of phenolic groups with

electrophilic metabolite(s) of BP, DNA was incubated with *anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (*anti*-BPDE). Significant inhibition of DNA adduct formation was found in the same order as shown above. Data from ESI/MS and LC/MS study confirmed and characterized the anti-BPDE-EGCG adduct. The hydroxyl groups on the B ring or D ring of EGCG molecule can interact with *anti*-BPDE. My data demonstrate that green tea catechins are highly effective in inhibiting BP-DNA adduct formation at least, in part, due to direct interaction of adjacent hydroxyl groups in their structures, and that the activity is higher with an increasing number of functional hydroxyl groups.

My second hypothesis is that adjuvant treatment of GTPs with a standard cancer chemotherapeutic agent will enhance efficacy of the drug because GTPs bear some distinct mechanisms of action which are different from other drugs.

To test the second hypothesis, I investigated the combined effect of EGCG and the berry anthocyanidins (EGCG-ANTHOS) alone and in combination with a common chemotherapeutic drug cisplatin on lung cancer H1299 cell using the MTT assay and also the combined effect of poly E, bilberry extract and cisplatin in xenograft nude mouse model. *In vitro*, the antiproliferative activity of EGCG-ANTHOS was found to be better than ANTHOS and EGCG alone. The combination of EGCG-ANTHOS and cisplatin increase the efficacy of cisplatin. Flow cytometric analysis for cell cycle distribution and apoptosis revealed cell cycle arrest and enhanced apoptosis induced by the combination mixture. Western blot analysis for the involved molecular targets showed that selected proteins (cyclin D1, B1, p21, PCNA, Bcl-2, PARP, etc.) associated with either cell cycle or apoptosis were more significantly modulated by the combination. *In vivo*, the combined effect of poly E-anthocyanidins and cisplatin caused significant inhibition on

tumor volume (73% reduction), which was significantly better than poly E, anthocyanidins and cisplatin alone groups, all of which do not show significant effect. However, concurrent with the efficacy from the combination treatment, toxicity is also obvious, manifested as the less activity of animals, body weight, elevated ALT and WBC count as well as the histological finding in skin. Clearly, no one generalized mechanism of action can explain the effects including both the efficacy and the toxicity effects. I investigated the effects of these compounds on the expression of cell cycle and cell apoptosis related proteins and the results showed the enhanced expression on some proteins including cyclin D1, Bcl-2 etc. which may explain the dramatic effects of the combination treatment in one respect. Taken together, my data suggest that EGCG-ANTHOS may be effective as adjuvant chemotherapy in combination with suboptimal doses of cisplatin. However, the potential toxicity effect of the combination treatment should be further addressed.

CHAPTER VII: IMPACT OF THE RESEARCH FINDINGS AND FUTURE PERSPECTIVES

We developed subcutaneous biodegradable polymeric implants which may provide a viable sustained release system for chemopreventive/chemotherapeutic agents with poor oral bioavailability. Chemopreventives are generally administered orally. On the other hand chemotherapeutic agents due to their poor bioavailability are usually given intravenously. For cancer patients, chemotherapy needs to be performed during a relatively long period of time. Therefore, oral administration appears to be burdensome and not cost-effective. Through this sustained systemic delivery system, not only can enhance the bioavailability but also be cost-effective. Further, patients with cancer might have a better compliance. One important thing for the success of cancer treatment is to prevent the growth of residual cancer cells after the surgery locally as well as systemically. Polymeric implants containing chemotherapeutic drugs will be a good choice to kill the possible residual cancer cells after surgery and also the floating cancer cells in the body. One approach is to leave the polymeric implants containing chemotherapeutic drugs at the surgical site, which might have residual cancer cells left before closing the wound. In that respect, no separate surgery needs to be done and the cost will be minimal. Before this sustained delivery system can be used in cancer patients, more work need to be performed. First, the release profile of these polymeric

implants is not optimized. Theoretically, a zero-order or near zero-order release profile will be more attractive. Second, an acceptable toxicity profile including both systemic and local toxicity should be the minimal requirement before any clinical application. Third, the dose of drugs can be precisely controlled for the purpose of efficacy and toxicity.

I also illustrated a new mechanism of action of green tea polyphenols, i.e. GTPs inhibit BP-induced DNA adduct formation through direct quenching of the electrophilic metabolites of BP, which partially closed the gap between the knowledge of chemopreventive effects of GTPs and the way they might work. It may help the mechanism studies of many other phytochemicals. Some phytochemicals bearing similar molecular structure may work in the same way, which will be a direction for future studies.

In addition, I found the synergistic effects of GTPs, anthocyanidins and cisplatin, suggesting the possibility of using GTPs and anthocyanidins as adjuvant treatment in combination with cisplatin. However, more studies need to be performed in order to figure out the components responding to the efficacy as well as the toxicity. The purpose of these studies is to find a more rational combination of these reagents which improves the response rate and/or reduces toxicity. The ultimate goal will be a clinical application.

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APPENDIX I: ABBREVIATIONS

5GG	pentagalloylglucose
9-OH-BPE	9-OH-benzo[<i>a</i>]pyrene-4,5-epoxide
ALT	alanine transaminase
<i>anti</i>-BPDE	<i>anti</i> -benzo[<i>a</i>]pyrene-7,8-diol-9,10-epoxide
AST	aspartate transaminase
BP	benzo[<i>a</i>]pyrene
BUN	blood urea nitrogen
CD	cyclodextrin
C_{max}	maximum plasma concentration
CPK	creatine phosphokinase
Cr	creatinine
CYPs	cytochrome P450s
DMSO	dimethyl sulphoxide
EC	(-)-epicatechin
ECG	(-)-epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGC	(-)-epigallocatechin
EGCG	(-)-epigallocatechin gallate
EGF	epidermal growth factor

FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF2	fibroblast growth factor 2
G6PDH	glucose-6-phosphate dehydrogenase
GGT	gamma-glutamyltransferase
GTP60	a green tea extract preparation containing 60% catechins
GTPs	green tea polyphenols
HCT	hematocrit
HGB	hemoglobin
HGF	hepatocyte growth factor
HPLC	high performance liquid chromatography
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MN/SPD	micrococcal nuclease and spleen phosphodiesterase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDEA	N-nitrosodiethylamine
NER	nucleotide excision repair
NNK	4-(methylnitrosamino)- 1 -(3-pyridyl)- 1- butanone
P15	poly (epsilon-caprolactone) with molecular weight of 15,000
P65	poly (epsilon-caprolactone) with molecular weight of 65,000
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline

PCL	poly (epsilon-caprolactone)
PEG	polyethylene glycol
poly E	polyphenon E
qPCR	real-time quantitative polymerase chain reaction
LC/MS	liquid chromatography-mass spectrometry
RBC	red blood cell
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
TA	tannic acid
WBC	white blood cell

CURRICULUM VITAE

Pengxiao Cao
Department of Pharmacology & Toxicology
University of Louisville School of Medicine
Louisville, KY 40202
p0cao001@louisville.edu
(502) 852-3684

EDUCATION AND TRAINING

2009-11	UNIVERSITY OF LOUISVILLE Ph.D. candidate in Pharmacology & Toxicology	Louisville, KY
2007-09	UNIVERSITY OF LOUISVILLE M.S. in Pharmacology & Toxicology	Louisville, KY
1994-99	QINGDAO CENTRAL HOSPITAL Residency	Qingdao, China
1989-94	SHANDONG MEDICAL UNIVERSITY M.B.	Jinan, China

EXPERIENCE

2001-05	CINKATE PHARMACEUTICAL CORPORATION Manager, Medical Department An Illinois chartered corporation with focus on drug discovery & development in China <ul style="list-style-type: none">Responsible for clinical trial design, planning, start-up, maintenance and closeout<ol style="list-style-type: none">A Phase II/III clinical trial of leflunomide in the treatment of lupus nephritisA Phase II/III clinical trial of leflunomide in prevention of transplant rejectionA Phase IV clinical trial of leflunomide for postmarketing surveillanceAnti-SARS coronavirus studyResponsible for product knowledge training and marketing support	Shanghai, China
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1999-00	QINGDAO CENTRAL HOSPITAL Attending doctor in Urology	Qingdao, China
	<ul style="list-style-type: none"> • Responsible for the diagnosis and treatment for the patients • Handle daily paperwork, including patient treatment forms • Conduct clinical trials • Database management • Supervise residents and medical students 	

TECHNIQUE SKILLS

CELL AND MOLECULAR BIOLOGY

Cell culture, cell staining, MTT assay, P450 assays, microplate reader, cell transfection, qRT-PCR, western blot, flow cytometry, ³²P-postlabeling

PK/PD RELATED TECHNIQUES

HPLC, LC/MS

IN VIVO

Animal models

AWARDS AND HONORS

2010-11	National Institute of Environmental Health Sciences Training Grant [T32-ES011564-07]
2007-09	Integrated Programs in the Biomedical Sciences (IPBS) Fellowship, University of Louisville, KY
1999	Best employee of the year, Qingdao Central Hospital, Qingdao, China
1990	Outstanding student award, Shandong Medical University, Jinan, China

MEMBERSHIP / AFFILIATIONS

2009-present	European Association for Cancer Research
2007-present	American Association for Cancer Research
2006-07	American Association of Pharmaceutical Scientists

2001-04 Chinese Rheumatology Association

1994-00 Chinese Urological Association

PUBLICATIONS

- **Cao, P.**, Vadhanam, M. V., Spencer, W. A., Cai, J., and Gupta, R. C. (2011) Sustained systemic delivery of green tea polyphenols by polymeric implants significantly diminishes benzo[a]pyrene-induced DNA adducts. *Chemical research in toxicology*. [Epub ahead of print]
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- **Cao, P.**, Ma, W., and Liu, C. (1998) Primary osteosarcoma of bladder. Chinese Journal of Urology, 19, 453.

NATIONAL MEETING PRESENTATIONS

- **Cao, P.**, Kausar, H., and Gupta, R.C. (2011) Potent chemotherapeutic activity of green tea polyphenols, blueberry anthocyanidin mixture and cisplatin: synergistic effects. Proc. Am. Assoc. Cancer Res., (Accepted).
- **Cao, P.**, Vadhanam, M.V., and Gupta, R.C. (2010) Green tea polyphenols implants are more effective than oral dosing in inhibiting benzo[a]pyrene-induced DNA adducts. Proc. Am. Assoc. Cancer Res., 51: 3914.
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- Vadhanam, M. V., Aqil, F., Bansal, S. S., **Cao, P.**, Jeyabalan, J., Ravoori, S., and Gupta, R. C. (2010) Controlled-release systemic delivery of chemopreventive agents – an update. Proc. Am. Assoc. Cancer Res., 51: 5673.
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