Chemical Research Method Grant Proposal
DNA Damage in Colon Cells Due to Exposure to ROS Molecules
Induced by Tobacco Smoke
University of San Diego

Part I

Part II. Introduction

A. General Introduction

Tobacco smoke inhalation has been identified as one of the most avoidable causes of mortality in the United States [13]. Smoking is accountable for a number of diseases, ranging from peripheral vascular disease, cerebrovascular disease, chronic pulmonary disease, lung cancer and other malignancies, to smoking's number one cause of death, myocardial infarction. According to the CDC, smoking is the cause of death for more than 480,000 individuals annually [15].

Tobacco inhalation can have the same effects as continuous exposure to mining environments, as well as exposure to smoke, pollutants and radiation, leading to imbalances of Reactive Oxygen Species (ROS) in biological systems. ROS molecules are produced by distinct biochemical oxidative pathways in organisms, and due to their reactivity are associated with pathological processes. The concentration of ROS in the body can either be beneficial through the regulation of homeostasis, or detrimental by causing damage in biological macromolecules. Imbalances in ROS levels can lead to oxidative stress and initiate carcinogenesis [11]. ROS are oxygen- containing small molecules that include oxygen radicals, such as superoxide (O2•−), hydroxyl (•OH), and peroxyl (RO2•). Due to the high energy contained in ROS molecules, their accumulation may lead to to DNA damage and mutagenesis.

B. Background

Tobacco in its gas phase contains molecules that can lead to increased oxidative stress in cells. These oxidative stresses cause damage in DNA, proteins and lipids. ROS targets guanine rich sequences due to its reductive properties, primarily 8-hydroxy-2'-deoxyguanosine [13(Valavanidis)]. This molecule functions in aqueous cigarette tar (ACT) solutions by forming adducts with DNA bases. In the gas phase of tobacco the ROS molecules promotes the degradation of endogenous antioxidants which significantly taxes cellular antioxidant defense.

Tobacco contains many toxic and carcinogenic chemical components, which have been known to function as stable and unstable free radicals as well as reactive oxygen species (ROS). Cigarette smoke (CS) is an aerosol, meaning a suspension of particles dispersed in air or gas, which contains 10E10 particles/mL [16]. The FDA has established a list of 93 harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke [12]. Although, this list only contains 93 components out of 5,000 chemical components found in tobacco and tobacco smoke. The mechanisms of action for all 5,000 components individually are therefore unclear or unknown, as well as their mechanism of action in comparison to that of CS, tar and nicotine. Contrastingly, Formaldehyde (FA), Benzene (Bz), and Isoprene (IP) are included in the typical components of CS[1]. FA is closely related to the activation of the oxidative stress to cells [3]. In general, half of the exposure of general population to benzene comes from CS, and it has been known to disturb the function of hormones [17,18]. IP has been reported to affect the reproductive and developmental processes in animals[7]. These factors and previous research on the metabolism of these molecules in other cells [12], suggest that they work through inducing the

creation of ROS and the imbalance in the oxidative levels in organisms, in addition to being confirmed carcinogens [12].

The primarily documented cancers related to CS exposure include laryngeal, esophagus, and gastric cancers. Colorectal cancer and how it relates to CS components is a relatively new research area. Previous studies have found relationships between CS and precursor growths in the large intestine [10], and nicotine has been linked to the proliferation or preventing effects of colon cancer cells [9]. Additionally, some research has found that components of CS including FA, Bz and IP have similar preventing properties to those of nicotine, by associating the growth of a cancer cell line inversely with components of CS [6], but the mechanism through which they work in cancerous colon cells is not yet known. Due to this, the proposed work intends to identify the exact biotransformation of FA, Bz and IP in colon cancer cells (SW620), using their known capabilities as ROS inducers. The previous work has shown that specific metabolites of these molelcuels may be resposnible for the DNA damage. Therefore, the role of metabolism will be examimed by assessing DNA damge done by both the known cacinogens as well as thei metabolites. Cells will also be treated with a metabolic activator to see if increaesed metabolic activity has an effect on DNA damge in the presence of these chemicals.

C. Goals Statement

If the proposed research into the chemical mechanisms of benzene, isoprene, and formaldehyde in tobacco inhalation is successful, it could provide more information on how DNA is damaged or mutated. This mechanism could potentially give rise to information regarding the risk of excess of mutation which could cause adverse effects of slowing down or stopping cell growth, specifically in cancerous cells.

This is an important factor in determining the carcinogenic nature of chemicals in tobacco smoke. Since tobacco smoke is ubiquitous in our environment it is essential to have a general understanding of how the chemicals can affect those who are exposed first hand as well as those who are exposed through second hand smoke. This research will also be beneficial in exploring at what concentrations these molecules have the greatest impact and what that impact is.

Part III. Proposed Research

A. Procedure

1. Materials and procedure outleine
    a. MDST8 Human colon carcinoma cells and healthy Human colon cells will be ordered from a scientific supplier and cultured in RPMI 1640 medium. RPMI 1640 is a common media for culturing mammalian cells, especially cancerous ones. Potential carcinogens along with a known metabolite will used in concentrations of 100mM and exposed to the colon cells to assess their ability to damage DNA and produce reactive oxygen species in the cell. Both MDST8 cells and healthy human colon cells will be exposed to both the carcinogen and a metabolite of each carcinogen. THe carcinogens are benzene, isoprene, and formaldehyde. Their metabolites are benzene-oxide, EPOX I, and formic acid, respectively. Healthy colon cells and MDST8 cells will both be treated with all of the carcinogens and metabolites both in the presence of a metabolism activator (S9 fraction) and not in the presence of an S9 activator and in the absence. In each condition, the DNA damage to each cell type will be assessed via comet assay, the cell viability will be assessed via the trypan blue exclusion technique, and oxidative sensitive dye will be used to measure the generation of ROS molecules in each condition.
2. Treatment of cell lines
    a. The cell suspensions (MDST8 and healthy human colon cells) will be treated with isoprene, EPOX I, benzene, benzene-oxide, formaldehyde, and formic acid (suspended at a concentration of 100 mM in DMSO) in RPMI 1640 medium either enriched or not with 5% FCS[5]. After incubation for 2 and 24 h (24 h of incubation is possible only in the presence of FCS) at 37 °C and 5% CO2, the cell viability and the DNA damage be evaluated by the trypan-blue exclusion technique and the comet assay, respectively. Experiments will be run run either with or without metabolic activation.In the experiments that will be carried out in the presence of metabolic activation the incubation system will be enriched with the S9 fraction properly diluted with 0.1 M phosphate buffer (pH 7.4) containing the following final concentrations of NADPH, 4 mM; glucose-6-phosphate, 5 mM; MgCl2, 8 mM; and KCl, 33 mM.
3. Comet assay-assess DNA damage
    a. The single-cell gel electrophoresis assay was performed essentially as described by Sing et al[8]. After treatment, aliquots of the cell suspension (50–100 μL, 0.5–1.0 × 105 cells) will be transferred to 1.5-mL Eppendorf tubes and centrifuged at 200 × *g* for 5 min. The supernatant will discarded and the pellet was mixed with 75 μL low-melting agarose (0.7% in PBS) and distributed onto conventional

microscope slides. The slides will be pre-coated with normal melting agarose (0.5% in PBS) and dried at 50 °C. After the agarose has solidified (4 °C for 10 min), a second layer of normal melting agarose will be applied similarly to the first. The slides were then immersed in the lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–HCl pH 10, containing freshly added 1% Triton ×100 and 10% DMSO) for 1 h at 4 °C and then placed into a horizontal electrophoresis apparatus filled with freshly made buffer (1 mM Na2EDTA. 300 mM NaOH, pH 13.0). After 20 min of pre-incubation (unwinding of DNA), the electrophoresis will be run for 20 min at a fixed voltage of 25 V and 300 mA which was adjusted by raising or lowering the level of the electrophoresis buffer in the tank. At the end of the electrophoresis, the slides will be washed three times with the neutralisation buffer (0.4 M Tris–HCl, pH 7.5), stained with 50 μL ethidium bromide (20 μg/mL), and kept in a moist chamber in the dark at 4 °C until analysis. All the above-reported steps will be carried out under red light to prevent any additional DNA damage.

4. ROS detection-dye to detect ROS production
    a. Oxidation-sensitive dye DCFDA (Molecular Probes/Invitrogen), will be used to measure ROS production by MDST8 and healthy colon cells. Cells were incubated at 37°C in RPMI 1640 in the presence of 2.5 μM DCFDA for 30 min[4]. Cells will be treated in the same twelve conditions (cells treated with 6 different chemicals and wither with or without metabolic activation) as in the comet assays. Cells were then labeled with APC-conjugated anti-Gr-1 and PE-conjugated anti-CD11b Abs on ice. Analysis will then be then conducted by flow cytometry. Procedure is described by Fabiani et. al 2007.

B. Anticipated Problems

It is possible that early stages of research will not yield useful information, such as in the case of all samples dying or no DNA damage being measured. In the scenario where no DNA damage can be measured the exposure times of the samples will be expanded from 2 hours to 24, and up to 48 if necessary. In the event that the concentrations are too high and are killing all of the cells in a given sample, the concentration can be lowered by a factor of 10. It is also possible that the mechanisms of DNA damage are different than the generation of ROS molecules, so we are performing Oxidation Sensitive Dye DCFDA to measure ROS generation in our cell samples.

C. Future Work

Potential future research opportunities include investigation into therapeutic uses of the three chemicals studied in our research. While they are known carcinogens, there is some evidence that in laboratory conditions they can slow down the growth of tumors [4]. The mechanism of this process is not well understood, but research into the process proposed may

yield more information about the mechanisms for cancer cell proliferation and the effects of carcinogens on cell viability, especially through ROS induction mechanisms.

Part IV. References

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