MODULATION OF PROSTATE AND PANCREATIC PHIP-DNA ADDUCTS BY CONSUMPTION OF CRUCIFEROUS AND APIACEOUS VEGETABLES IN MALE WISTAR RATS

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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MAY 2013

University of Minnesota

Acknowledgments

This thesis would not be complete without the help of numerous individuals who helped pave my path towards graduation. My sincere respect and appreciation for Sabrina Trudo is unexplainable. The opportunity she granted me with to seek further education at the University of Minnesota is only the beginning. Sabrina's vast knowledge, guidance, and patience made my graduation possible. I've grown to admire Sabrina's dedication, persistence, and fortitude, and am certain we will have a lasting friendship. Furthermore, I am grateful for my lab partner, Jae Kyeom Kim. Jae Kyeom is a wealth of knowledge, and spent many hours answering questions and assisting my research. Additionally, my sincere gratitude for my co-advisors, Dr. Dan Gallaher and Dr. Kristin Anderson cannot be undermined. Their support, feedback, and time provided important assistance for my thesis completion. I also need to thank my colleagues in lab 65: Scott Simpkins, Diane Nowak, Cynthia Gallaher, Ana Dos Santos, Drew Brockman, and Sanyub Kim; thank you for your willingness to help out and make lab life enjoyable! Lastly, I need to thank my family and amazing fiancé for supporting me and standing by my side through this entire journey.

Dedication

I dedicate this thesis to my loving parents. Without your support, motivation and belief in me, I would not be the woman I am today. You've been there through all the ups and downs, yet continue to believe in my ability to succeed and aspire.

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Chapter 1: Introduction

Introduction to the Thesis Project

Cancer will likely be the single largest cause of death worldwide by 2030, but unfortunately the cause for the majority of cancers is multifactorial and poorly understood (1). Several environmental carcinogens, such as tobacco smoke and food constituents, are known to contribute to carcinogenesis (2). Most of these carcinogens are handled in the body by a large number of biotransformation enzymes such as: cytochrome P450s (CYPs), UDP-glucoronyltransferases (UGTs), glutathione-S-transferases (GSTs), and sulfatases (SULTs) (3). Amongst environmental carcinogens, dietary factors such as the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), which is formed during the cooking of meat and fish, demonstrate carcinogenicity in the prostate and pancreas of rodents (4, 5). Epidemiological studies also indicate that consumption of cooked meat and meat products predispose individuals to neoplastic disease of the prostate and pancreas (6, 7).

Prostate cancer is the second leading cause of cancer death in adult men, and approximately 1 in 6 men will be diagnosed with prostate cancer during his lifetime (8, 9). Although prostate cancer has a good prognosis, its high prevalence makes prevention a key area of interest. The prostate is a gland found only in males and is located just below the bladder and in front of the rectum. The prostate contains cells responsible for making fluid that protects and nourishes the sperm (9). Prostate cancer is caused by changes in DNA of a normal prostate cell, but only about 5% to 10% of prostate cancer cases are linked to inherited DNA changes, with the remaining 90% due to somatic DNA changes that occurred during a person's lifetime (9). While it is unknown exactly what

causes prostate cancer, certain risk factors such as age, race, genetics, diet, obesity, smoking, and infections are known to be linked to the disease (9). Maintaining a healthy body weight, remaining physically active, eating a diet high in vegetables, and supplementing with vitamin E may lower the risk of prostate cancer (9).

Pancreatic cancer is estimated to be the fourth leading cause of death in men and women in the United States (8). Several risk factors such as smoking, male gender, older age, diabetes, family history, and dietary carcinogen exposure are known to increase one's susceptibility to pancreatic cancer. Recent research has shown the above risk factors affect the DNA of pancreatic cells, resulting in abnormal cell growth and tumor formation (10). Early diagnosis of pancreatic cancer is difficult because the pancreas lies deep inside the body, making it difficult to feel the tumors during a routine physical exam. By the time a person expresses symptoms (jaundice, pain, weight loss, digestive trouble, blood clots, etc.), the cancer is usually large and has metastasized to other organs (10). Even when diagnosed early, pancreatic cancer has a poor prognosis and is rapidly fatal, especially since the etiology is not fully understood and few therapeutic options are available (10). There are no established guidelines for preventing pancreatic cancer, but the current best approach is to avoid risk factors when possible. American Cancer Society recommends maintaining a healthy weight, eating well, and exercising (10).

Promising studies have provided incentive for developing effective chemoprevention strategies involving food and food compounds. Fruits and vegetables, due to their phytochemical makeup, have been touted as dietary sources that reduce one's risk of cancer. Two recent reviews support the idea that specific dietary compounds,

such as phytochemicals, confer a protective effect against certain cancers, through a variety of mechanisms such as acting as blocking or suppressing agents (11, 12). Amongst plant foods, cruciferous and apiaceous vegetables have been shown to be chemoprotective due to their unique phytochemical profiles (13). Specific to cruciferous vegetables, glucosinolate breakdown products induce biotransformation enzymes responsible for metabolizing carcinogens in the body, and therefore may reduce the formation of toxic metabolites (14, 15). Furanocoumarin compounds present in apiaceous vegetables inhibit carcinogen activating enzymes, and therefore may decrease procarcinogen activation (16).

The overall objective of this research project is to investigate the effects of intact cruciferous and apiaceous vegetable feeding, and their corresponding purified putative chemopreventive compounds, on PhIP-DNA adduct formation in male Wistar rat prostate and pancreas. Based on results from previous studies, we hypothesized that combined consumption of intact cruciferous and apiaceous vegetables would result in the greatest modulation of heterocyclic amine metabolism towards decreased carcinogen activation, and ultimately lead to a decrease in prostate and pancreatic cancer risk. The following thesis reports the effects of whole vegetable, and corresponding active compounds, from cruciferous and apiaceous vegetable supplemented diets on PhIP-DNA adduct formation in male rat prostate and pancreas. Chapter 2 will review the literature on heterocyclic aromatic amines, biotransformation enzymes, and prostate and pancreatic cancer. Chapter 3 reviews the literature on vegetable modulation of the biotransformation enzymes responsible for activating the procarcinogen PhIP. Chapter 4 describes the

methods used for this project, and chapter 5 presents the results of the analysis. Lastly, chapter 6 will discuss conclusions, implications of results, and future directions necessary in this field

Chapter 2 Literature Review: Heterocyclic Aromatic Amines and Biotransformation Enzymes

Overview

Human cancer is believed to be caused by the combined effects of genetic and environmental factors. With regards to environmental influences, dietary factors are estimated to account for approximately one-third of all human cancers (2, 17). Heterocyclic aromatic amines (HAA) are ubiquitous genotoxic mutagens present in our environment, and are widely distributed in components of the environment such as airborne particles, cigarette smoke, diesel exhaust particles, and cooked foods (18, 19). In our food environment, HAA are commonly and abundantly found in over-cooked foods, primarily meat and fish (20), but are also found in beer and wine (21). HAA are metabolized by biotransformation enzymes through different pathways in the body leading either to detoxification or activation of the procarcinogenic HAA. The purpose of this chapter is to review current information on HAA with an emphasis on PhIP, introduce the biotransformation enzymes that metabolize PhIP and other HAA, and then establish the relationship between HAA and prostate and pancreatic cancer.

Heterocyclic Aromatic Amines

Tremendous progress has been made in the field of HAA since their first discovery in 1977, when Japanese scientists showed that smoke condensates obtained from broiling fish showed mutagenic activity for *Salmonella typhimurium* TA100 and TA98 (22). There are now more than 20 HAA compounds identified as mutagenic in bacterial assays (23), and many of these HAA also exert carcinogenic effects in rodents (5, 24). Less compelling, but still noteworthy data, suggests that HAA intake is related to

human cancers (7, 25, 26). HAA are divided into five groups based on their chemical structures. The pyrolytic group forms mutagens when cooking temperature exceeds 300ºC. The other four groups are referred to as thermic mutagens, which form at <300ºC and can be further subdivided depending on which structure is attached to the imidazole ring (i.e., quinoline, quinoxaline, pyridine or furopyridine) (27).

HAA are formed via the Maillard reaction when creatine, free amino acids, and hexose sugar (all compounds found in raw meat) combine and form mutagenic products during the cooking process (28, 29). This HAA formation increases as temperature, fat percentage, and cooking time increase (30, 31). Knize et al. used a steel griddle to fry 100-g ground beef patties at 150, 190 or 230˚C for 2, 4, 6 or 10 minutes per side and determine effects on HAA formation. They found that mutagenic activity increased with both frying temperature and time. At 150˚C for 2 or 4 minutes, PhIP was not detectable, but at 190˚C and 230˚C for 10 minutes, PhIP reached 9.8 and 21 ng/g, respectively (32).

Furthermore, HAA content varies by meat product and cooking technique. When comparing bacon, chicken, hamburger, and steak, Sinha et al. found chicken was the meat with the highest HAA levels (33). Additionally, Sinha et al. measured HAA content in various pork products cooked by different techniques and varying degrees of doneness (31). They found HAA content was higher in bacon cooked very well done compared to bacon cooked just until done. They also found that oven-broiled bacon contained high HAA, but oven-broiled hot dogs had non-detectable HAA levels. Pan-fried sausage patties also showed higher levels of the HAA MeIQ than the pan-fried sausage links (5.4 ng/g vs. 1.3 ng/g). The authors mentioned this could possibly be due to the shape of the

meat product, i.e., patties are flat and contain a larger surface area for contact with heat (31). A similar study done by Sinha et al. showed pan-frying and barbecuing steak formed the highest amount of HAA compared to oven-broiled steak. Overall, the above studies indicate that an increase in cooking temperature and time increase the formation of HAA, and different meat products vary in their HAA content, especially with regards to their cooking method. This information needs to be considered when evaluating human intake for research purposes.

Felton et al. discovered that the specific HAA PhIP is found with the highest abundance in cooked foods (34), and that PhIP formation increases linearly with cooking time and temperature (32). Animal studies have shown that as little as 25 ppm of PhIP will induce cancerous lesions (35). It has been estimated that human intake of PhIP is approximately 6 ng/kg/day, but ranges dramatically across geographical regions, ethnicities, and cooking preferences (36).

Biotransformation Enzymes and HAA metabolism

Biotransformation enzymes are part of a complex enzymatic system that plays a central role in the metabolism, elimination, and detoxification of endogenous and exogenous compounds. Biotransformation is an enzymatic reaction that transforms a lipophilic compound to a more polar/water soluble metabolite, which is usually less active than the parent compound. However, in some instances this transformation leads to a more reactive metabolite, thus biotransformation enzymes are related to both detoxification and toxification processes in the body (3). There are many families and

subfamilies of biotransformation enzymes which are broadly categorized as either phase I or phase II biotransformation enzymes. With regards to PhIP metabolism, the CYP1As, SULTS, NATS, GSTs, and UGTs, are responsible for both the activation and detoxification of PhIP in the body (37-39).

Phase I enzymes and HAA

Phase I enzymes are commonly located in the endoplasmic reticulum of cells, and their purpose is to add or expose a functional group (i.e -OH,-NH_2,–COOH) by catalyzing oxidation, reduction, and hydroxylation reactions (40). These enzymes are found in both prokaryotes and eukaryotes, and are most abundant in the human liver and gastrointestinal tract (41). At least ten families of phase I enzymes have been identified (40), but the cytochrome P450 (CYP) monooxygenase family plays the most significant role (up to 80%) in phase I enzyme reactions (3, 12). These major CYP enzymes are involved in the metabolism of pharmaceuticals, endogenous toxins, and even steroids (40). The CYP enzymes are specifically notable for their role in metabolizing carcinogens, such as HAA, polycyclic aromatic hydrocarbons (PAH), cigarette smoke, and *N*-nitrosamines (3). Specifically, CYP1A1, CYP1A2 and CYP1B1 have all been shown to metabolize dietary HAA and PAH (3). The HAA PhIP is only carcinogenic after metabolic activation, which primarily involves *N*-hydroxylation by CYP enzymes to form N^2-hydroxy-PhIP (42). In a comparative study performed by Cross et al. where recombinant human P450 was expressed in insect cells, CYP1A2 showed the highest catalytic efficiency (V_{max}/k_m) for formation of the genotoxic N^2-OH-PHIP metabolite, followed by CYP1A1 and then CYP1B1 (3.2, 1.1, 0.1 nmol/min/nmol, respectively) (43).

Importantly, a difference in enzyme regioselectivity of PhIP also exists between humans and rodents. Turesky et al. demonstrated that recombinant human CYP1A2 catalytic activity towards N-oxidation of PhIP was 19-fold greater than in purified rat CYP1A2 (44). This is an important issue to consider when extrapolating animal evidence to humans.

Phase II enzymes and HAA

Phase II enzymes catalyze conjugation of phase I enzyme metabolites with additional moieties, including glutathione and glucuronic acid (3). This conjugation process usually increases polarity and inactivates the reactive metabolites formed by phase I enzymes by forming water-soluble compounds that can be excreted in urine or bile (40). Phase II enzymes are located in the cell's cytosol and predominantly reside in liver tissue in order to protect the cell from environmental and oxidative stress (3). Phase II enzymes consist of superfamilies of enzymes like the glutathione S-transferases (GST), acetyltransferases (NAT), sulfotransferases (SULT), and uridine 5'-diphospho (UDP) glucuronosyltransferases (UGT) (3). Numerous studies have shown that HAA are first activated by CYP1A2 to form bioactive *N*-hydroxy intermediates that are then either detoxified or further activated by Phase II enzymes. Glucuronidation is a major biotransformation pathway for xenobiotics, and the UGT1A1 isozyme has the highest capacity for converting mutagenic *N*-hydroxy-PhIP to a safer, excretable form, *N*-hydroxy-PhIP-N^2-glucouronide (38).

Alternatively, the *N*-hydroxy-PhIP can undergo sulfonylation by sulfotransferases

(SULT1A1) or esterification by N-acetylases (NAT2) to form highly reactive *N*-*O*-sulfonyl or -acetyl esters, which can then form covalent adducts with guanine of DNA (dG-C8-PhIP) and induce mutagenesis (45, 46). Eleven human SULT isoforms are known, and they strongly differ in tissue distribution and substrate specificity (47). SULT1A1 resides primarily in the liver and is thought to be primarily responsible for *0*-sulfonation of *N*-hydroxy-PhIP, followed by SULT1A2 (47).

Of the human NAT enzymes, the NAT2 isozyme is the principal enzyme responsible for the *O*-acetylation of *N*-hydroxy-PhIP, leading to electrophilic metabolites which can also proceed to form DNA adducts (48). Luckily, conjugation of the activated *N*-hydroxy-PhIP or *N*-acetoxy ester of PhIP with glucuronic acid or glutathione by phase II enzymes (primarily UGT1A and GSTA1) usually leads to safe metabolites and successful excretion of PhIP (37, 38). At least 5 glucuronidated forms of PhIP have been identified, but *N*-hydroxy-PhIP-N^2-glucoronide is thought to be the major urinary metabolite, accounting for roughly 50% of all the PhIP metabolites (49). Figure 2.1 outlines a detailed schematic of PhIP metabolism.

PhIP can be directly excreted from the body, but it typically gets extensively metabolized within 24 hours of ingestion (50). In addition to the safe excretion of active PhIP through metabolism, DNA repair enzymes are also known to play a role in cancer prevention (51). For instance, if PhIP-DNA adducts do form, DNA repair enzymes may offer another line of defense by removing DNA adducts. For example, one animal study found that mice missing the xeroderma pigmentosum group A (XPA) gene are defective in nucleotide excision repair, and showed higher levels of PhIP-DNA adducts in the liver

and distal small intestine, approximately 170% and 40% respectively, when given a single PhIP injection of 25 mg/kg body weight compared to wild-type mice (52).

Overall, since phase I enzymes are clearly involved in the activation of PhIP to a carcinogen, and certain phase II enzymes are known to allow safe excrete of PhIP, it is important to identify ways to lessen the activation stage and up-regulate the detoxification pathway, which will be further discussed in chapter 3.

PhIP-induced mutations and carcinogenesis

As discussed above, activation of PhIP can lead to covalent binding of its amino group to the C8 position of the DNA base guanine and form dG-C8-PhIP. Although studies outlining the exact mechanism of PhIP carcinogenicity are limited, mutations of the ß-catenin and adenomatous polyposis coli (APC) genes appear to be most common genetic alterations induced by PhIP, especially in a rodent model of colon cancer (53).

Briefly, APC is a tumor suppressor gene that is associated with various cellular processes and apoptosis (54, 55). ß-catenin is a protein involved in structural mediation of actin filaments, cadherins, and cell-junctions (56). With regards to cancer, ß-catenin's role seems to be related to its function as a transcriptional activator in the Wnt signaling system (54). The Wnt signaling transduction pathway is important for a number of developmental processes, including nervous system development, limb development, and apoptosis (55, 57). Upon activation of the Wnt signal, glycogen synthase is inhibited and causes the stabilization and accumulation of ß-catenin, which can then associate with the TCF/LEF family transcription factors and alter expression of target genes (54). APC and

ß-catenin work together to facilitate the Wnt signaling pathway process, as APC induces the degradation of ß-catenin. It has been shown that mutated APC are defective in their ability to degrade ß-catenin, leading to ß-catenin accumulation and constant activation of Wnt signaling (54). There is evidence that PhIP may interfere with Wnt signaling, potentially leading to tumorigenesis.

Ubagai et al. showed that intermittent administration of PhIP, in tandem with a high fat diet for differing time spans, induced intestinal tumors in rats (58). In their first protocol, F344 male rats were fed 400 ppm of PhIP for 2 weeks, followed with a high fat diet until 108 weeks. In protocol 2, the 2-week PhIP treatment was repeated three times with 4-week intervals of only the high fat diet for a total of 42 weeks. A total of 16 intestinal tumors were induced between all 39 experimental rats, and 3 of 9 tumors were found to harbor mutations in the ß-catenin gene, whereas two tumors harbored mutations in the APC gene (58). Another study also looking at colon carcinogenesis found that ß-catenin proteins accumulated in the cytoplasm of aberrant cypt foci, a preneoplastic lesion of the colon (59). Furthermore, Andreassen et al. exposed heterozygous nonsense (APC^{min}) neonatal mice to 50 mg/kg of PhIP and found that 55% of induced tumors showed a loss of the wild-type APC allele in the males, and 74% in females (60). It is important to note that the majority of research investigating genetic mutations after PhIP administration is limited to the intestinal tissue. To my knowledge, no studies exist that have investigated this phenomenon specifically in prostate and pancreatic tissue.

Evidence of HAA and prostate cancer risk

Animal Studies. Numerous animal studies have supported the link between PhIP intake and prostate cancer. Shirai et al. fed 70 male F344 rats a diet containing 400 ppm of PhIP for 52 weeks before euthanizing rats and removing the prostates. PhIP-DNA adduct levels were measured using ^{32}P-postlabeling analysis, and the prostate was found to have the highest adduct levels compared to colon and liver (5). Another study using F344 rats showed that with 4 weeks of PhIP feeding at 70 mg/kg, all lobes of the prostate had significantly elevated mutation frequencies compared to controls (61). G:C $\rightarrow$ T:A transversions were the predominant type of mutation, and histology confirmed an increased proliferation in response to PhIP. Li et al. showed that a single dose administration of 200 mg/kg body weight of PhIP by gavage to hCYP1A mice induced prostate lesions and other tissue molecular abnormalities similar to that observed in human prostate carcinogenesis (62). Similar results were found in other animal studies (63, 64).

Human Studies. PhIP intake has been correlated with human prostate cancer, but evidence is less compelling than in animal studies. Cross et al. conducted a large prospective study in 2005 and found that the highest quintile of PhIP intake (>269 ng/d) was associated with a 1.2-fold increased risk for prostate cancer (95% CI, 1.01-1.48) (7). Tang et al. conducted another case-control study and found that self-reported consumption of grilled meat was significantly associated with higher PhIP–DNA adducts in the prostate epithelial cells, but this association was limited to only grilled red meat consumption (P =.001) (65).

In 2007, the World Cancer Research Fund and American Institute for Cancer Research (WCRF/AICR) stated "there is limited evidence and inconsistent studies suggesting processed meat intake is a cause of prostate cancer"(66). Over the last decade, several case-control and cohort studies of meat intake and prostate cancer have been evaluated; a 2001 systematic review suggested a high intake of meat is positively associated with an increased risk of prostate cancer (67), but a more recent meta-analysis analyzing 15 prospective cohort studies found no association between red meat and prostate cancer risk (68). These inconsistencies may be due to the difficulties in estimating total HAA exposure in an individual, since HAA content varies according to cooking methods, type of meat, portion size, frequency of intake, and self-reported food frequency questionnaires used for data collection (69). Additional challenges include the correlation with other constituents in meats like heme iron, N-nitroso compounds, and animal fat, making it difficult to separate the effects of HAA from cooked meats on prostate cancer risk (70).

Evidence of HAA and pancreatic cancer risk

Animal Studies. Data from animal studies have shown that the pancreas is also highly susceptible to HAA exposure. After a single dose of orally administered PhIP to male rats, the pancreas yielded the highest PhIP-DNA adduct levels compared to colon, lung, heart and liver (71). Pfau et al. employed ^{32}P-postlabelling techniques to show that DNA adducts in the pancreas were 36-times higher than in the liver of F344 rats fed 400 ppm PhIP for 2 weeks (72). Yoshimoto et al. showed that eight different HAA, including PhIP fed at 0.04% of the diet, induced pancreatic duct lesions and carcinomas in female

hamsters (4). These results are also supported by other experimental animal studies (73-75).

Human Studies. HAA intake has also been associated with human pancreatic cancer risk (6, 76, 77), and PhIP-DNA adducts have specifically been detected in human pancreatic tissue samples (78). Zhu et al. found that individuals with the highest levels of PhIP-DNA adducts in their pancreatic tissue samples were 3.4 times more likely to be diagnosed with pancreatic cancer than those with lower PhIP-DNA adduct levels (CI, 1.4-7.5) (78). In a 2002 case-control study, Anderson et al. showed grilled and BBQ red meat intake was a risk factor for pancreatic cancer, yielding an OR of 2.2 (95% CI, 1.4-3.4) in the highest intake group compared to the reference group (6). In another analysis from the same case-control study, Anderson et al. found that after adjusting for numerous variables, the highest quintile of PhIP intake led to the highest odds ratio of pancreatic cancer (OR 1.8, CI 1.1-3.1) (25). In a similar case-control study performed by Li et al., intake of the HAA DiMeIQx was linearly associated with pancreatic cancer risk (P_{trend} = 0.02), but this association was not observed for PhIP (P_{trend} = 0.22) (76). Discrepancies between these case-control studies could be due to differences in the studies target population and/or the researchers' method for dietary data collection. For instance, Anderson et al. recruited study subjects from the general population, while Li et al. recruited patients from a hospital setting, leading to a potential for selection bias. Additionally, Anderson et al. performed in-person interviews to obtain information, while Li et al. used questionnaires, again leading to variation in data collection.

Similar results to what was found in the case-control studies above were found in a large prospective cohort study. Self-administered food frequency questionnaires were used to collect dietary information on over 600,000 American Association of Retired Persons (AARP) members at baseline and 6 months after baseline. Participants were then followed for 5 years, and during this time 836 incident pancreatic cancer cases were identified. High-temperature cooked meats showed a 52% increased pancreatic cancer risk in men for the highest intake quintile versus the lowest, but this trend was not observed in women (77). One other prospective cohort study showed well and very well done meat intake were generally associated with increased risk for pancreatic cancer, but the proportional hazard estimate for PhIP intake was not statistically significant (HR 1.15, 95% CI 0.76 -1.74) (79). Overall, the case-control and cohort studies above show a link between the intake of well-done meat and pancreatic cancer, suggesting diet and cooking methods are a potentially modifiable factor in reducing pancreatic cancer risk.

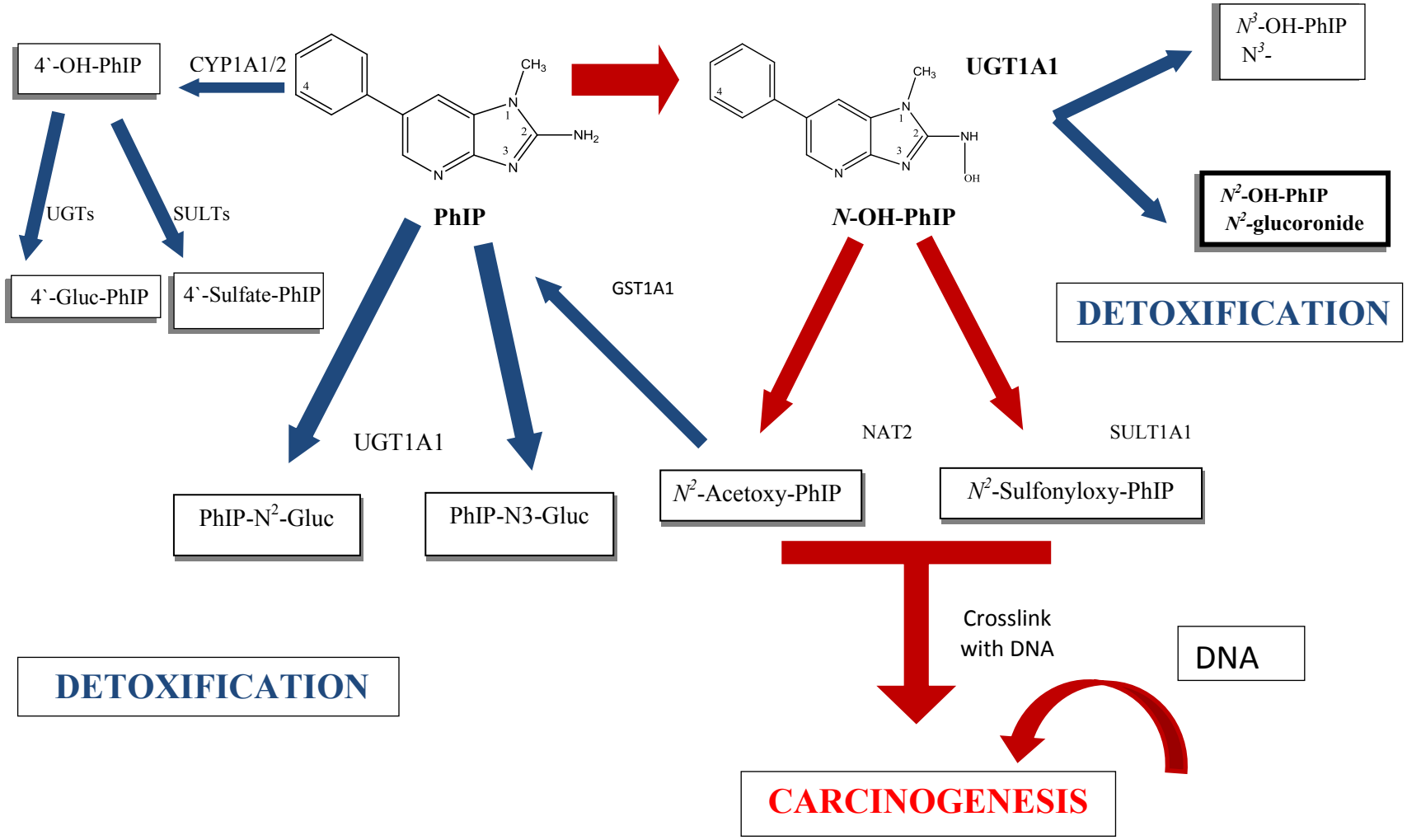


Figure 2.1. Major Biotransformation Pathways of PhIP. Adapted from Turesky et al. (80), Gooderham et al. (42), Zhou et al. (81), Patterson et al. (82).

Chapter 3 Literature Review: Vegetable Modulation of Biotransformation Enzymes

Overview

As reviewed in chapter 2, phase I enzymes are involved in the activation of dietary PhIP, and specific phase II enzymes are known to either further activate or safely excrete PhIP. Therefore, it is important to identify ways to lessen the activation stage and up-regulate the detoxification pathways of biotransformation in order to minimize carcinogenic burden. Two written reviews summarizing the existing research support the idea that plant foods confer a protective effect against certain cancers through a variety of mechanisms, including modulation of biotransformation processes (11, 12). The purpose of this chapter is to review the evidence of vegetables that influence the activating and detoxifying pathways of PhIP in the body, with emphasis on CYP1A1, CYP1A2, SULT1A1, NAT2, GST, and UGT enzymes.

Biotransformation Enzyme Modulation by intact Cruciferous Vegetables

The cruciferous (*Cruciferae)* vegetable family is comprised of broccoli, cauliflower, cabbage, Brussels sprouts, and other spices like mustard seed, and is one of the most widely consumed vegetable groups worldwide (83). Epidemiological studies provide evidence that cruciferous vegetables protect against human cancer risk, and animal studies show they reduce tumor formation (84). This protective property of cruciferous vegetables has been postulated to be due in part to their ability to influence phase I and II enzymes.

Phase I enzymes (CYP1A1 & CYP1A2)

Animal Studies. Influence of cruciferous vegetables on CYP1A enzymes has been well studied, especially in rodents. To investigate the influence of cruciferous vegetables on mRNA expression of CYP1A1 and CYP1A2, Robbins et al. fed mice 1 of 2 intervention diets, either freeze-dried blanched or unblanched purified Brussels sprouts at 20% (wt:wt) of the diet for 2 weeks (85). The expression of CYP1A1 and CYP1A2 was measured using real-time PCR, and it was found that both blanched and unblanched Brussels sprout diets increased liver mRNA expression of both CYP1A1 and CYP1A2, but the results were only statistically significant in the unblanched Brussels sprout group compared to the control (vegetable-free) diet. With lung tissue, mice fed the unblanched Brussels sprout diet also had statistically significantly higher expression of CYP1A1 and CYP1A2 than mice fed the control diet, while the blanched Brussels sprout diet only increased CYP1A1. These results suggest that cooking procedures may affect the bioavailability of cruciferous vegetable phytochemicals, and also that vegetable influence may be enzyme and tissue specific.

In one particular study where rats were supplemented with 2.5, 5, or 20% of their diet from cooked Brussels sprouts for up to 28 days, the authors reported that Brussels sprouts increased activity of CYP1A1 in a dose-dependent manner (86). The authors also noted that enzyme activity increased after 2 days with the 20% Brussels sprout diet, but took 14 days to see a similar increase with the 5% dosage, suggesting a dose-time response (87). Vang et al. analyzed the impact of a 7-day feeding with 10% (wt:wt) broccoli powder in male Wistar rats on CYP1A1/2 activity. Fifty rats were allocated to 1

of 4 different broccoli diet groups, where the broccoli varied only by cultivar and growing conditions. Overall, all broccoli diets increased EROD (7-ethoxyresorfurin-O-deethylase) and MROD (7-ethoxyresorfurin-O-demethylase) activity, which are indicators of CYP1A1 and CYP1A2 activity, respectively. Hepatic EROD activity increased by 3-fold across all broccoli diets, where MROD increased by 2.3 fold. Interestingly, MROD activity increased differently depending on the growing conditions of the broccoli, with a 2.5-fold MROD activity increase in rats fed broccoli grown in low-sulfur soil, compared to rats fed broccoli grown in sulfur soil. Furthermore, in rats fed broccoli grown without pesticides, a higher EROD activity was observed. These results suggest that modulation of CYP1A enzymes by broccoli may vary between commercially available produce. Additionally, these same authors investigated *in vitro* metabolism of PhIP metabolites in hepatic microsomes from these rats. It was found that the broccoli diets increased the N'-OH-PhIP metabolite from 1.6 to 1.9 fold, again indicating increased CYP1A1/2 activity (86).

Human Studies. In a randomized, controlled human intervention conducted by Lampe et al., healthy men and women aged 20-40 years, who consumed 428 grams of cruciferous vegetables for 6 consecutive days, showed a significant increase in the urine caffeine metabolites used to define CYP1A2 activity, when compared to the basal (vegetable-free) diet ($P < 0.04$) (88). Similar results were found with 500 g of broccoli consumption; after 12 days, CYP1A2 activity increased by 19% when compared to individuals in the vegetable-free diet group (89). Another human study that also utilized caffeine metabolism kinetics to monitor changes in CYP1A2 found similar results. After

a 12-day feeding period of 250 g of Brussels sprouts or broccoli at both breakfast and dinner, and consumption of 275 g well-cooked lean steak on day 13 followed by a 10 h urine collection, caffeine clearance had increased by 7% ($P < 0.001$), when compared to caffeine clearance during the washout period (12 day vegetable-free diet) (90). These same authors also measured unmetabolized PhIP in urine, and found that PhIP excretion was reduced by 21%; they attributed this to the induction of the PhIP-activating CYP1A2 enzyme. A related human study performed by Walters et al. showed that consumption of 250 g of broccoli and Brussels sprouts for 12 days induced CYP1A2, but simultaneously increased the N^2 and N^3 glucuronidated forms of PhIP, indicating that cruciferous vegetable consumption may induce both phase I and phase II enzymes (14).

Phase II enzymes

Fewer studies analyzing the influence of cruciferous vegetables on PhIP-metabolizing phase II enzymes have been conducted. In 2009, Navarro et al. performed a human study where bilirubin conjugation was used to determine UGT1A1 activity in humans. A 2-week dietary intervention indicated that higher consumption of cruciferous vegetables increased UGT1A1 activity, and this response was seen in a dose-response manner (i.e. single dose cruciferous vegetable intake was 7 g/kg body weight, double dose was 14 g/kg) (91). Also, the authors noted time-effects of crucifer feeding. It was found that on day 11 and day 14, serum bilirubin concentrations were lower than on day 7, indicating an increase in UGT1A1 activity, but by day 14 bilirubin concentration had increased since day 11 ($P = .004$), suggesting an adaptation to the vegetable diet.

With further regards to PhIP detoxifying enzymes, Bogaards et al. showed that in 10 healthy male subjects consuming 300 g of Brussels sprouts for 3-weeks plasma GST-α levels were elevated by a factor of 1.4 ($P = 0.002$) (92). Interestingly, another study that fed human subjects 436 g of fresh cruciferous vegetables for 7 days showed a similar effect as the above study with increased serum GST-α concentrations and GST activity, but the effect was more pronounced in women and varied by *GSTM1* genotype (93). In contrast to the increase in women's GST-α concentrations, another study found that 300 g of Brussels sprouts daily for 7 days increased GST-α in men ($P = .031$), but not in women ($P = .317$) (94), suggesting a gender and/or genetic role in vegetable response.

To my knowledge, only a few studies have evaluated NAT and SULT activity in response to intact cruciferous vegetable consumption. In a randomized cross-over study using non-smoking men and women, Lampe et al. found no effect on NAT2 activity in response to 6 days of 428 g of cruciferous vegetable intake when compared to a vegetable-free diet (88). Hoelzl et al. performed an intervention trial using 8 healthy male and female subjects and found that SULT1A1 activity decreased by approximately 30%, and protein expression in human lymphocytes was also reduced following consumption of 300 g/person/day of Brussels sprouts for 6 consecutive days (95). Unfortunately, the small sample size and short duration of this later experiment limits the confidence in the study.

In sum, the above studies show that cruciferous vegetables increase activity of the PhIP-activating CYP1A1/2 enzymes, but because they also induce specific phase II

enzymes, which may increase detoxification, cruciferous vegetables are still believed to be chemoprotective.

Biotransformation enzyme modulation by glucosinolates

As recently described, cruciferous vegetables modulate CYP1A1/2 and specific phase II enzymes, and this is thought to be due to the crucifer's composition of glucosinolates, a unique class of sulfur-containing compounds responsible for the aroma and bitter taste of the vegetables (96). These glucosinolates typically undergo cleavage reactions by the action of the enzyme myrosinase to form isothiocyanates (ITCs), indoles, and nitriles (97). Myrosinase (β-thioglucosidase) is an enzyme found compartmentalized near glucosinolate substrates in cell walls. Upon mechanical rupture such as cutting or chewing, myrosinase is released and free to interact with glucosinolates, whose reaction results in hydrolysis of parent glucosinolates into their bioactive metabolites. It is also postulated that humans can convert glucosinolates to their bioactive metabolites through microflora action in the gastrointestinal tract (83).

Over 120 glucosinolates have been identified in plants (98), but the breakdown products and concentration of the glucosinolates will vary according to vegetable and growing conditions (99). For example, watercress is known for its high content of phenethyl isothiocyanate (PEITC), which is a bioactive breakdown product of the parent glucosinolate, gluconasturtiin. Cabbage is known for its high content of glucobrassicin, whose breakdown product is indole-3-carbinol (I3C), and broccoli is dominant in glucoraphanin, which gives rise to sulforaphane (SFN) (100). Importantly, only a few

glucosinolates have been studied for their biotransformation enzyme modulating effect, and more research is warranted.

Phase I enzymes (CYP1A1 & CYP1A2)

A review published in 2004 found that purified glucosinolates may be associated with the inhibition of phase I enzymes but strong induction of phase II enzymes, and therefore can be classified as monofunctional inducers (101). Indole-3-carbinol (I3C) is one the most studied components of cruciferous vegetables. Wortelboer et al. fed male rats diets containing 0, 200, or 500 mg of I3C/kg of diet for 2, 7, 14 or 28 days and found that the I3C diet increased liver CYP1A1 activity in a dose-dependent manner (102). Hepatic EROD was enhanced 2-fold and 6.7-fold after 2 days, for 200 mg I3C/kg and 500mg I3C/kg dose, respectively. Interestingly, CYP1A protein levels increased in a dose-dependent manner, but not a time-dependent manner since similar protein content levels were seen across all time points for the different dosage groups (about 1.4-fold in the 500mg I3C/kg diet at all time points). He et al. performed an extensive study analyzing the effects of I3C on phase I enzyme modulation and PhIP-DNA adduct formation (103). Female F344 rats were fed one of 3 diets, the control AIN-76A, or a diet supplemented with either 0.02% or 0.1% (w/w) I3C for 23 days. During this period, animals were also intubated with 1 mg PhIP/kg of body weight per day. The authors found that in the organ tissues analyzed (e.g., stomach, pancreas, liver), both I3C diets resulted in inhibition of PhIP-DNA adduct formation ($P \leq 0$ .05), with up to 95.3% adduct reduction seen in the pancreas tissue with the 0.1% I3C diet. These same authors conducted a second experiment. In experiment 2, liver microsomes were prepared from

animals treated by gavage with I3C at 100 or 200 mg/kg body weight for 2 consecutive days. The authors found that both I3C diets resulted in an induction of CYP1A1 and CYP1A2 mRNA, along with increased EROD and MROD activity in liver microsomes in a dose-dependent manner. Specifically, the 100 mg I3C/kg diet let to a 2.8-fold and 9.6-fold increase in EROD and MROD activity, respectively, while the 200 mg I3C/kg diet led do a 3.9 fold and 11.5-fold increase, respectively.

However, there have been debates over their ability of metabolites of glucosinolates to induce CYP1A2, and it is now thought that their effect on CYP1A2 is dependent on the derivative of the parent glucosinolate (12, 101). For example, Marca et al. found that rat hepatocytes treated for 24 hours with 40 μM solutions of the aliphatic ITCs (e.g., SFN) inhibited both CYP1A1/2 mRNA expression and the associated EROD activity, while the aromatic ITCs, such as PEITC, increased both mRNA expression and enzyme activity (104). The same researchers also found that GST enzymes were induced only by specific ITCs, including PEITC and SFN. A review paper has further summarized the idea that the dietary effect of certain glucosinolates may either increase or decrease microsomal CYP1A content/activity depending on the specific glucosinolate and the experimental conditions, such as *in vitro* vs. *in vivo* environments, animal vs. human models, glucosinolate dose, and duration of glucosinolate exposure (105).

Phase II enzymes

There seems to be sufficient evidence to assume that induction of phase II enzymes by glucosinolates is also plausible (105). The effect of I3C on

biotransformation enzymes has been studied in animals. Wortelboer et al. fed male rats diets containing 0, 200, or 500 mg of I3C/kg of diet for 2, 7, 14, or 28 days and found that GST activity increased about 1.3 fold after 2 days on the 500 mg I3C/kg diet, and continued to increase in a dose-dependent manner, up to 1.5 fold increase at 28 days (102). Another rat study showed that 100 mg/kg body of I3C by oral administration for 4, 10, and 30 days increased GST expression and activity in rat liver similarly across all time points. Interestingly, no effect on enzyme activity was seen in kidney tissue, suggesting a tissue specific response to I3C (106). Sulforaphane (SFN) is a specific ITC that has also been shown to be chemoprotective. In one *in vitro* study, inducer potency of synthetic (R, S)-SFN, measured by quinone reductase (QR) and GST activities, in female CD-1 mice after administering daily doses of 15μmol for 5 days was performed. The results showed that SFN raised both QR and GST enzyme activities 1.6- to 3.1-fold in the liver, stomach, proximal small intestine, and lung (107). Influence by PEITC has also been reported in a few studies, and short-term administration of PEITC by gavage at doses higher than 0.25 mmol/kg/body weight has consistently been shown to increase GST activity and mRNA expression (106, 108-110). With regards to PhIP-adducts, Dingley et al. found that male rats dosed with 816 mg of PEITC/kg diet for 15 days significantly decreased PhIP-DNA adducts levels in the colon, liver, and prostate (111). The authors noted that GST enzymes were also up-regulated, but this induction was not seen in the UGT enzymes. Lastly, to my knowledge, no studies have been performed with regard to the effects of specific glucosinolates on modulation of SULT1A enzymes.

Proposed mechanism of action

CYP1A, GST, and UGT enzymes are thought to be regulated transcriptionally via the aryl hydrocarbon locus, involving the interaction of the Aryl hydrocarbon receptor (AhR) and aryl hydrocarbon nuclear translocator (ARNT) (112-114).

Cruciferous vegetables, and more specifically indole derivatives, are thought to act as a ligand for the AhR and influence gene expression of the respective enzymes (97). For instance, binding of I3C to AhR causes AhR translocation into the nucleus for interaction with the ARNT/XRE response element, ultimately leading to gene expression (115).

Alternatively, ITC compounds regulate gene expression of antioxidant and detoxification genes through the antioxidant response element (ARE) (116). The transcription factor NF-E2-related factor 2 (Nrf2) is known to activate phase 2 enzymes (e.g. GSTA1 & UGT1A1) expression through the ARE, following the dissociation of Nrf2 from kelch-like ECH-associated protein 1 (Keap 1) (117). ITCs such as PEITC and SFN are known to dissociate the Keap 1 protein from Nrf2 and allow it to translocate into the nucleus where it forms an Nrf2/Maf heterodimer and activates ARE (116, 118). Interestingly, studies have also shown that some ITCs induce phase I enzymes and others induce both phase I and phase II enzymes (through both XRE and ARE driven pathways) (12, 90, 119).

In addition to the two induction mechanisms of cruciferous vegetable constituents above, a variety of other mechanisms have also been postulated to account for the

vegetable chemoprotective properties, such as: inhibition of pro-inflammatory reactions by repression of NF-κB (nuclear factor-κB), inhibition of cytochrome P450 (CYP) enzyme activity, inhibition of histone deacetylase, and stimulation of cell cycle arrest and apoptosis (97).

Overall, the studies above favor the idea that glucosinolates induce both phase I and phase II biotransformation enzymes, and it seems that the majority of studies exploring the relationship between glucosinolates and biotransformation enzymes have been conducted *in vitro* or in animal studies. *In vitro* studies may not fully reflect the true state of metabolism in a human *in vivo* situation, and therefore more human studies are warranted. Furthermore, seeing that most glucosinolate constituents have been studied independently and for a short time duration (up to 28 days), further investigation is needed to determine long-term effects on biotransformation enzymes, as well as the synergistic effects of administering multiple glucosinolate constituents together.

Modulation of Biotransformation Enzymes by Apiaceous Vegetables

The apiaceous vegetable family (*Apiaceae*) is comprised of carrots, celery, parsnips, parsley, dill, and cilantro. A cancer protective property of apiaceous vegetables has been postulated due to their ability to influence phase I enzymes.

In general, fewer studies regarding apiaceous vegetables have been conducted compared to the cruciferous vegetable family, and to my knowledge, animal studies have only evaluated the effects of the vegetables' furanocoumarin compounds.

Phase I enzymes (CYP1A1 & CYP1A2)

In humans, consumption of apiaceous vegetables has been shown to inhibit phase I enzymes. In 2000, Lampe et al. performed a controlled intervention where human subjects were fed a 265 g apiaceous supplemented diet for 6 consecutive days, and found a 13-25% reduction in CYP1A2 activity, depending on which caffeine metabolite ratio was used (88). Another crossover feeding trial conducted by Peterson et al. found promising evidence that apiaceous vegetables inhibit CYP1A2. Healthy male and female subjects, fed 7 g/kg body weight of a cruciferous vegetable supplement diet (1C), had a 14% increase in CYP1A2 activity, but when 4 g/kg of apiaceous vegetables were added to the 1C diet, a notable 13% decrease in CY1A2 activity was seen ($P < 0.0001$) (120), suggesting the powerful inhibiting potential of apiaceous vegetables.

Phase II enzymes

Few human studies have been conducted to evaluate phase II enzyme modulation by apiaceous vegetables alone. Navarro et al. measured UGT1A1 activity through bilirubin levels after cruciferous and apiaceous vegetable feeding. The researchers found that a 2-week feeding period of ~7 g/kg crucifers plus ~4 g/kg apiaceous vegetables resulted in a reduction in bilirubin concentrations greater than that seen with the cruciferous vegetable diet alone ($P < 0.02$) (91). This suggests that constituents in apiaceous vegetables may increase UGT1A1 activity. However, the effects of these apiaceous vegetables alone on UGT1A1 induction have not been evaluated. Another study performed by Lampe et al. monitored the effects of apiaceous vegetable intake on

GST-α concentration (93). Fifty-seven men and women were randomized to a 6-day dietary controlled intervention (265g apiaceous vegetables/day) in order to monitor serum GST concentrations and activity. Among *GSTM1*+ men, GST-α blood serum concentrations decreased by 22% ($P = 0.03$), but no effects were seen in *GSTM1*-null individuals. Furthermore, the apiaceous diet increased GST-mu activity by 26% among GSTM1+ women ($P = .001$), but this affect was not observed in men.

With regards to the PhIP-activating phase II enzymes, Lampe et al. found no effect on NAT2 activity in response to 6 days of 265 g of apiaceous vegetable intake (88). Lastly, to my knowledge no studies have determined the effects of apiaceous vegetables on SULT1A enzymes.

Modulation of Biotransformation Enzymes by Furanocoumarins

Apiaceous vegetables are a rich source of furanocoumarins (121). Furanocoumarins are produced by plants as a defense mechanism against predators, but are stable during cooking (122). The core of the furanocoumarin structure consists of the psoralen or angelicin isomer. Derivatives of these two isomers are referred to as linear and angular furanocoumarins, respectively. Linear furanocoumarins include: psoralen, bergapten or 5-methoxypsoralen (5-MOP), xanthotoxin or 8-methoxypsoralen (8-MOP), imperatorin, and isopimpinellin. Angular furanocoumarins include: angelicin, pimpinellin, and isobergapten (123). Animal and *in vitro* studies have been performed to evaluate the effect of various furanocoumarins on phase I and II enzymes, but fewer human studies evaluating furanocoumarin compounds have been conducted.

Phase I (CYP1A1 & CYP1A2)

In an *in vitro* study by Peterson et al., the effects of three furanocoumarins (psoralen, 5-MOP, and 8-MOP) were investigated using yeast microsomes expressing *h*CYP1A2. It was found that all three furanocoumarin compounds were potent inhibitors of *h*CYP1A2-mediated MROD activity at physiologically relevant concentrations (124). In a similar study performed by Kang et al., the activity of CYP1A2 was investigated with four furanocoumarins: imperatorin, trioxsalen, isopimpinellin, and angelicin (16). A yeast strain expressing *h*CYP1A2 was used to determine MROD activity. Imperatorin and isopimpinellin significantly inhibited *h*CYP1A2 at concentrations less than 0.005 μM ($P = 0.05$) and less than 0.05μM ($P = 0.05$), respectively. Interestingly, isopimpinellin inhibited *h*CYP1A2 in a time and concentration dependent manner, and was ultimately categorized as a mechanism-based inhibitor (MBI) due to the observation of 60% inhibition of *h*CYP1A2 remained after 20 hours of dialysis. Overall, these results are consistent with previous studies that show linear furanocoumarins are more potent CYP inhibitors than angular furanocoumarins.

In contrast to these results, one animal study showed that a single dose (25 mg/kg body weight) of 8-MOP had biphasic effects on CYP1A1- and CYP1A2-mediated EROD and MROD activities, depending on length of time after treatment (125). Two hours after 8-MOP injection, EROD and MROD activities were depressed, 18 and 17%, respectively. However, at 24 hours, EROD and MROD activities were elevated by 727% and 932%, respectively. Interestingly, activity levels returned to baseline just 5 days after the single dose feeding. These results suggest that 8-MOP may function as both an

inhibitor and inducer of CYP1A1/A2 depending on time after treatment. Most studies investigating this effect have not considered time as an influencing factor, and this may account for such discrepancies between studies.

One possible explanation for the induction of EROD and MROD activity in the study above is the potential of furanocoumarins to induce CYP1A mRNA. Diawara et al. showed that synthetic psoralens induced the same mRNA of hepatic enzymes that are typically induced through the aryl hydrocarbon receptor (Ahr) and XRE pathway, including CYP1A1 & UGT1A6, in a dose-dependent manner (126). Another *in vitro* study that evaluated the effect of four furanocoumarins (angelicin, bergamottin, isopimpinellin, and 8-MOP) on expression and activity of CYP1A1 found interesting results. In the first series of experiments, the researchers found that 1nM to 1mM of the four furancoumarins inhibited EROD activity in both rat hepatocytes and microsomes under light and dark conditions after 48 hours (127). In the next series of experiments, the researchers investigated the effects of the furanocoumarins on CYP1A1 gene expression. It was found at most concentrations of the furanocoumarins, a decrease in CYP1A1 mRNA was seen, but at a concentration of 10 uM, angelicin induced CYP1A1 mRNA, and 1mM concentration of 8-MOP induced mRNA significantly greater than the control, as well as a known AhR agonist, TCDD. These results suggest that the mechanistic effects of furanocoumarins on biotransformation enzymes are multi-faceted, and more research is needed to clarify how the inhibitory and inducing effects prevail within natural circumstances (i.e., human studies).

A few human studies also showed inhibition of CYP1A2 activity when pharmacological doses of individual furanocoumarins, specifically 5-MOP (128) and 8-MOP (129), were administered. Bendriss et al. showed that in 8 human subjects, administration of 1.2 mg kg^{-1} of 5-MOP with 200 mg of caffeine resulted in a decreased clearance in oral caffeine ($P < 0.01$), and significantly decreased the CYP1A2 enzyme ratios (128). Mays et al. showed that in 5 human subjects, administration of 1.2 mg kg^{-1} of 8-MOP along with 200 mg of caffeine resulted in a decrease in caffeine metabolism, indicating a decrease in CYP1A2 (129).

Phase II enzymes

Few studies have been done regarding phase II enzyme modulation by apiaceous vegetables. In one *in vitro* study utilizing *Escherichia coli* expressing plasmids for SULT1A1, it was found that white grapefruit juice inhibited SULT1A1 activity by 95%. Subsequently, the specific furanocoumarins, bergamottin and dihydroxybergamottin, within the grapefruit juice were analyzed for their inhibiting potential, and it was found that only dihydroxybergamottin reduced SULT1A1 activity, in a dose-dependent manner, up to 40% reduction ($P = 0.05$ at 1μM, and $P = .001$ at 10μM) (130). As mentioned earlier, Diawara et al. showed that synthetic psoralens induced the same mRNAs of hepatic enzymes that are typically induced through the AhR and XRE pathway, including UGT1A6, in a dose-dependent manner (126). Bendriss et al. showed that in 8 human subjects, administration of 1.2 mg kg^{-1} of 5-MOP with 200 mg caffeine caused no difference in NAT2 activity when assessed by caffeine metabolism (128). However, in a more recent study, a 0.5 mmol/kg body weight single dose of 5-MOP was shown to

increase NAT activity in rat stomach, but when human stomach tumor cell lines were treated with 0.05-50 mM of 5-MOP, inhibition of NAT2 activity was observed after 72 hours (131). This observation demonstrates the idea that effects of 5-MOP may be cell/site specific, and more studies need to be conducted to conclude the various outcomes on NAT2, especially for *in vivo* situations.

Overall, the studies that investigated the effects of apiaceous vegetables and their respective furanocoumarins on the biotransformation enzymes involved in PhIP metabolism have mainly focused on CYP enzymes and more research needs to be conducted on their phase II enzyme modulation.

Proposed mechanism of action

Unlike the cruciferous vegetables mechanism of action, the biological role of apiaceous vegetables is not fully understood. Most studies suggest furanocoumarins act as AhR agonists to induce gene expression of phase I enzymes (126, 127). Although several studies show an inhibitory effect from apiaceous vegetables on CYP1A enzyme activity (124) one *in vitro* study suggests a biphasic effect (127). Baumgart et al. showed *in vitro* that angelicin, bergamottin, isopimpinellin, and 8-MOP induce CYP1A1 mRNA expression through the AhR receptor, but then inhibit the catalytic activity of CYP1A leading to an overall inhibitory effect on enzyme activity (127). In order to clearly elucidate the mechanism-based inhibition (or biphasic induction) by apiaceous vegetable constituents, further studies need to be conducted that investigate the time- and concentration-dependent outcomes of multiple regulating end-points.

Evidence of Vegetable Consumption and Prostate & Pancreatic Cancer Prevention

Vegetables have been touted as a dietary chemopreventative agent for years, and multiple study approaches have been used to study this effect. Animal studies allow for a more controlled, mechanistic look into vegetable components, while epidemiological studies allow researchers to form hypotheses and make associations. Multiple approaches are important for drawing conclusions and, as will be seen below, more studies of cruciferous vegetables on site-specific cancer prevention are still warranted.

Cruciferous vegetables and glucosinolates

Animal & *In vitro* Studies: In animal studies, most results are consistent in supporting the chemoprotective effect of cruciferous vegetables' respective constituents. One study that used a transgenic adenocarcinoma prostate mouse model, found that mice fed a diet containing 3 μmol PEITC/g for 19 weeks inhibited the progression of poorly differentiated carcinoma in prostate cells (132). Another study in mice found that 20 mg/kg body weight of I3C injected intraperitonially for 14 days resulted in a 78% decrease in prostate tumor volume, compared to control animals ($P = .001$). The same researchers also found that I3C decreased cell proliferation rate and promoted apoptosis in prostate cancer cells *in vitro* (133). Comparable effects on increased apoptosis were seen *in vitro* when LNCaP human prostate cancer cells were treated with 2.5 or 5 μM concentrations of PEITC (134).

Similar effects are suspected in the pancreas. One *in vitro* study found that administration of 10-15 μM of SFN inhibited pancreatic cancer cell growth and induced

apoptosis (135). Another *in vitro* study showed that 48 h treatment of SFN in 4 different pancreatic carcinoma cell lines induced apoptosis and blocked tumor growth, possibly though disruption of the NF-κB pathway (136). To my knowledge, no additional studies have been done investigating cruciferous vegetables and pancreatic cancer. In summary, the individual phytochemicals found in cruciferous vegetables have shown promising protective effects against prostate and pancreatic cancer development *in vitro* and in rodents, but further research is needed to confirm these results.

Human Studies: With human studies there is growing evidence that cruciferous vegetables reduce prostate and pancreatic cancer risk. Most case-control studies have found significant inverse associations between vegetables and prostate cancer, but prospective studies have been less promising. Consistent with this pattern, a recent meta-analysis evaluating the relationship between cruciferous vegetable intake and prostate cancer found a significantly decreased risk of prostate cancer with a high crucifer intake across all six of the case-control studies included in the meta-analysis (pooled RR = 0.79) (137). However, no significant effect was seen across the seven cohort studies included in the analysis. Furthermore, a 2012 case-control study found a slightly protective odds ratio (OR) of 0.87 (CI 0.70-1.09) when cruciferous vegetable consumption was greater than 1 portion a week (138), but a prospective study that evaluated self-reported dietary intake in over 130,000 men found no significant associations between cruciferous vegetable consumption and prostate cancer risk (P_{trend} = 0.953) (139).

With regards to pancreatic cancer, numerous case-control studies have suggested an inverse association with fruit & vegetable intake and pancreatic cancer (140-142), but

evidence of cruciferous vegetables intake is more limited. A European case-control study found that ≥1 portion of cruciferous/week resulted in a weak inverse association with crucifer intake (OR 0.9, CI 0.63-1.30) (138), but a similar case-control study conducted in the San Francisco Bay area found that cruciferous vegetable intake was associated with a stronger reduced risk of pancreatic cancer in the highest quartile of servings per day (OR 0.76, $P_{trend} = 0.06$) (143). The different outcomes of these two studies could be due to differences in data collection, in-person interview versus telephone interview, as well as study differences in how cruciferous vegetables were categorized and statistically analyzed. Furthermore, a multiethnic cohort study performed in the U.S. found that increased vegetable intake, as well as cruciferous vegetable intake, was not associated with a decreased pancreatic cancer risk (total vegetable intake $P_{trend} = 0.135$; cruciferous vegetable intake $P_{trend} = 0.156$) (144).

Variation in data collection, dietary recall, sample sizes, and vegetable classification may partially explain the disparity across these human studies. However, taken together, these studies suggest that cruciferous vegetables impart at least some protection against prostate and pancreatic cancer, with a stronger association for prostate cancer. The chemoprotective effects of cruciferous vegetables likely involves complex interactions and multiple mechanisms, and more human intervention trials need to be conducted in order to elucidate the specific associations.

Apiaceous vegetables and furanocoumarins

Animal & *In vitro* Studies: To my knowledge, no studies have evaluated the direct effect of fresh apiaceous vegetables on prostate and pancreatic cancer protection in animal models or *in vitro* studies.

Human Studies: One case-control study conducted in the San Francisco Bay area found that raw and cooked carrot intake was associated with a significantly reduced risk of pancreatic cancer in the highest quartile of servings per day (OR 0.56, $P_{trend} = 0.001$) (143). Furthermore, the authors noted that for ≥ 2 servings per week of raw carrots the OR was 0.62 (95% CI, 0.49-0.79), and 0.86 (95% CI, 0.65-1.1) for cooked carrots when compared to consumption of the vegetable ≤ 3 servings/month (143). In contrast, in a recent pooled analysis of 14 prospective studies, carrots were not statistically inversely associated with pancreatic cancer, even after multivariate adjustments (RR 0.99, 0.92-1.07) (145).

Together, the information on vegetable intake and prostate and pancreatic cancer risk is somewhat convincing, more so with regards to cruciferous vegetables. Animal and human data is limited with regards to apiaceous vegetables, creating a severe limitation in diet and cancer research. Furthermore, there is compelling evidence that bioactive food components influence phase I and phase II biotransformation of carcinogens, but there is little data comparing the effects of purified compounds versus their intact food sources on metabolic pathways and PhIP-DNA adduct formation.

Further insight into these effects will broaden our knowledge of the chemoprotective potential of fresh vegetables and their respective phytochemicals.

Summary

Prostate and pancreatic cancer are leading causes of cancer death in the U.S. today, making prevention a key area of research interest. It is thought that HAA are associated with increased risk of prostate and pancreatic cancer risk and cruciferous and apiaceous vegetables may impart a chemoprotective effect. This chemoprotective effect may be mediated by the vegetables' effect on biotransformation enzymes, leading to a beneficial balance between activation and inactivation of the procarcinogens. For example, PhIP-induced carcinogenicity is strongly dependent on specific phase I and phase II enzyme action. Cruciferous vegetables have been shown to induce phase I and II enzymes, while apiaceous vegetables have been shown to inhibit phase I enzymes. However, it is unknown if the combined intake of these two vegetable families result in a synergistic chemoprotective effect due to their complementary influence on PhIP metabolism. Therefore, we investigated the effect of these vegetable families, and their respective phytochemicals on PhIP-DNA adduct levels in male Wistar rats. To do this, we conducted two feeding studies and pursued the following objectives:

1.) Project I: Determine the effect of fresh cruciferous & apiaceous intake alone or combined on PhIP-DNA adduct formation in rat prostate and pancreatic tissue.

a. Hypothesis: Combined consumption of both vegetables will reduce adduct formation the greatest.

2.) Project II: Determine the effect of respective phytochemical groups from cruciferous or apiaceous vegetables alone or combined on PhIP-DNA adduct formation in rat prostate and pancreas.

a. Hypothesis: Combined consumption of both phytochemical families will reduce adduct formation the greatest.

Chapter 4: Materials and Methods

Overview

Most studies investigating vegetable modulation of biotransformation enzymes have focused on either cruciferous or apiaceous vegetable intake alone. In this study, we investigated the effect of intact cruciferous and apiaceous vegetables or respective purified compounds from the vegetables, either alone or combined, on PhIP-DNA adduct formation in prostate and pancreas of male Wistar rats. The information below describes the study design utilized in Project I, followed by Project II, and lastly the materials, methods, and other aspects common to both projects.

It is important to mention that other lab members carried out portions of this study. Jae Kyeom Kim performed the animal feeding study and furanocoumarin analysis, Cynthia M. Gallaher performed the glucosinolate analysis, and Daniel Gallaher and Sabrina Trudo assisted statistical analysis.

Project I

Experimental design

A total of 52 rats were divided into five feeding groups. Each vegetable-supplemented group included 10 rats, and positive and negative control groups contained 11 rats each. The two control groups were fed a basal diet, and remaining groups were fed the basal diet supplemented with one of three different vegetable combinations: cruciferous only, apiaceous only, or combination of both cruciferous and apiaceous. After 6 days of feeding, all animals (except those from the negative control group) were injected intraperitoneally with PhIP at 10 mg of PhIP per kg body weight, and urine was collected the next 24 hours. The following day (day 7) animals were sacrificed, and tissues were collected and stored at -80ºC.

Diets

The AIN-93G basal diet was used for both positive and negative PhIP control groups. The other three diets included: 21% cruciferous diet (AIN-93G + 21% w:w cruciferous vegetables), 21% apiaceous diet (AIN-93G + 21% w:w apiaceous vegetables), and 21% combination diet (10.5% w:w cruciferous vegetables + 10.5% w:w apiaceous vegetables). Certified organic vegetables used for the diets were purchased from a local market. Cruciferous vegetables included: watercress, broccoli, and green cabbage; and apiaceous vegetables included celery and parsnips. Vegetables were cut and ground with a food processor (Cuisinart Delux 11TM), and then added to the powdered AIN-93G diet accordingly. Diets were balanced for macronutrients to minimize confounding, and diet compositions are shown in Table 4.1. After diets were prepared, they were divided into separate plastic bags and stored at -80ºC until use. Each bag was thawed daily and provided to rats. Food intake was determined three times during the week from spillage and amount remaining in the food cup over a 24-hour period.

Project II

Experimental design

A total of 52 rats were divided into five feeding groups. Each phytochemical-supplemented group included 10 rats, and the positive and negative control groups contained 11 rats. Both control groups were fed a basal diet and the remaining three groups were fed the basal diet supplemented with a different phytochemical combination: glucosinolate metabolites only (GLSs), furanocoumarins only (FCs), or a combination of glucosinolate metabolites and furanocoumarins. After 6 days of feeding, all animals

(except those from the negative control group) were injected intraperitoneally with PhIP at 10 mg of PhIP per kg body weight; urine was then collected for the next 24 hours. The following day (day 7) animals were sacrificed, and tissues were collected and stored at -80ºC.

Diets

AIN-93G diets was used for both positive and negative control groups. The glucosinolate metabolite-supplemented diet included phenethyl isothiocyanate (PEITC) and indole-3-carbinol (I3C); the furanocoumarin-supplemented diet consisted of 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), and isopimpinellin. Concentrations of PEITC and I3C were matched with the total glucosinolate amount quantified within the cruciferous vegetables used in project I (21% w:w). 5-MOP, 8-MOP, and isopimpinellin were matched for their respective concentration quantified within the apiaceous vegetables used in project I (21% w:w). Diets included: basal (AIN-93G) for both positive (PhIP injection) and negative (vehicle injection) control groups, glucosinolate metabolite-supplemented diet (AIN-93G + 365 mg PEITC & I3C), furanocoumarin-supplemented diet (AIN-93G + 4.3 mg 5-MOP, 8-MOP, & isopimpinellin), and combination diet (AIN-93G + 185 mg GLSs & FCs). Diets were balanced for macronutrients to minimize confounding, and diet compositions are shown in Table 4.2. To prepare these diets, each compound was dissolved in corn oil and mixed with the AIG-93G powder diet. After diet preparation, food was divided into plastic bags and stored at -80ºC to minimize phytochemical degradation. Each bag was thawed daily and provided to rats. Food intake was determined three times during the week from spillage and diet remaining in the food cup during a 24-hour period.

Total GLS analysis

Total glucosinolates were analyzed by Cynthia M. Gallaher using a recently published method (146). Briefly, cruciferous vegetables (i.e., cabbage, broccoli, and watercress) were frozen in liquid nitrogen, lyophilized, ground, and stored at -80°C until extraction. When ready for extraction, 100% hot methanol (MeOH) at 80°C for 20 minutes was used to inactivate myrosinase. Next, 80% MeOH was added and samples were extracted for 30 minutes at 80°C. The samples were then cooled, homogenized, and rinsed with 90% MeOH. The remaining pellet was extracted again and pooled with the first extracts and dried under nitrogen gas. An anion exchange column was activated with MeOH followed by water, 0.5 M sodium acetate (pH 4.6), and water, and glucosinolates were reconstituted in water and applied to the column. Glucosinolates were eluted with 0.5 M NaCl and degraded with 2 M NaOH to release thioglucose. Absorbance was measured in a Varian Cary 50 Scan UV-spectrophotometer at 420 nm at 2 minutes after addition of the ferricyanide solution; final values were adjusted for interfering compounds. Sinigrin was used to construct the standard curve (detailed method found in Appendix A-1).

Furanocoumarin analysis

Furanocoumarins were analyzed by Jae Kyeom Kim using a modified method from Ostertag et al. (122). Briefly, celery and parsnips were homogenized in a food processor (Cuisinart Deluxe 11TM) and extracted with five volumes of 40% diethyl ether in water (v/v). Samples were sonicated and then centrifuged for 15 minutes. The extraction and centrifugation process were repeated four times, and supernatants were pooled. Next, collected organic phase was evaporated under nitrogen, with all sample vials covered with aluminum foil to avoid light exposure. Extracts were then reconstituted in 60%

acetonitrile in water (v/v) and applied to C18 columns, and finally to silica columns. The eluates were dried under nitrogen, re-suspended in 50% acetonitrile in water (v/v), and analyzed using a Gilson 612 HPLC system with UV diode array detection set at 310 nm. The column was a reverse phase C18 column (Agilent Poroshell 120 EC-C18, 150 × 4.6 mm, 2.7 μm). Initial mobile phase composition was 45% of 10 mM phosphoric acid and 55% MeOH (v/v). A flow rate of 0.5 mL/min was used for the separation over 45 min. The furanocoumarins were identified using authentic standards, retention time, and UV spectrum. Six samples per each vegetable were analyzed and three of these samples were spiked with various concentrations of one of the furanocoumarin standards (i.e., 8-MOP) to calculate recovery rate (full method found in Appendix A-2).

Project I & II

Overview

After the feeding period and removal of prostate and pancreas, DNA isolation, extraction and analysis of DNA adduct formation took place. DNA was isolated from rat tissues by enzymatic extraction, and further digested to individual bases under conditions shown to be highly efficient in recovery of the dG-C8 adducts of PhIP. These bases where then analyzed for adduct formation via LC-MS/MS technology. Methods are briefly described below, and detailed descriptions can be found in appendices.

Chemicals and reagents

Gentra Puregene DNA purification Kit was purchased from Qiagen Sciences (Maryland, USA). Enzymes used for enzymatic hydrolysis of DNA to deoxynucleosides included: DNase I, nuclease P1, phosphodiesterase I, and alkaline phosphatase; these

enzymes were purchased from Sigma-Aldrich® (St. Louis, MO). All other reagents and products were purchased from Honeywell (Muskegon, MI), Sigma-Aldrich® (St. Louis, MO), Fisher Scientific (St. Louis, MO) or Worthington Biochemical Corporation (Lakewood, NJ). Chemicals used for DNA adduct analysis were of analytical grade.

Animals

Male Wistar rats, 100-125g body weights, were obtained from Harland Laboratories (Indianapolis, IN), and were housed in wire-bottom stainless steel cages. Rats were fed the standard American Institute of Nutrition diet (AIN-93G) for five days in order to facilitate adaptation to a powder diet. Animals were allowed free access to water and diet, and the University of Minnesota Committee on Animal Care approved all experimental procedures.

DNA extraction

DNA was extracted using a Qiagen Gentra Puregene DNA Purification kit (protocol was provided by supplier; see detailed method in Appendix B-1). Briefly, prostate and pancreas were weighed and tissue cell membranes were disrupted through incubation with 600 µL lysis solution for 60 minutes. DNA was further extracted by incubation with 3 µL proteinase K for 2 hours, followed by 3 µL RNase A for 25 minutes. Next, proteins were sequestered by vortexing and centrifuging samples with 200 µL of protein precipitation solution. The DNA solution was removed from the protein precipitate, and 600 µL isopropanol was gently mixed in until the DNA formed an insoluble, visible mass. Isopropanol was decanted out of the microcentrifuge tube and then 600 µL of 70% ethanol was added to the tube in order to wash the DNA. The tubes were centrifuged with the ethanol for 1 minute and then ethanol was carefully removed;

remaining pellets were allowed to air dry for 10 minutes. Finally, 85 µL of DNA rehydration solution was added to the samples and incubated overnight at 55 ºC.

DNA quantification

After DNA extraction was complete, DNA concentration was determined using a UV spectrophotometer, assuming a concentration of DNA (50 µg/mL) is equal to 1.0 absorbance unit at 260 nm (147). In brief, one original DNA sample was diluted 1:50 and analyzed with a SmartSpec^{tm} Plus Bio-Rad UV spectrophotometer. With results from this sample, serial dilutions were made to include 200 µg, 100 µg, 50 µg, 25 µg, 12.5 µg, and 6.25 µg /ml concentrations that were then used to construct a standard curve. All original samples were then diluted 1:20 and transferred into a 96-well plate and analyzed using a Biotek Synergy HT microplate reader. Absorbance was read at 260/280 nm (see details in Appendix B-2).

DNA digestion

The internal standard [$^{13}C_{10}$]-dG-C8-PhIP was added to the isolated DNA at a level of 10 adducts per 10^7 bases prior to enzymatic digestion. The enzymatic digestion method was described previously (148, 149). Briefly, 50 µg of DNA was dissolved into 79 µL of Tris-HCL and 10 mM of $MgCl_2$. Next, 4 µL of DNase I was added to the samples and incubated for 1.5 hours. Nuclease P1 was added and samples incubated for 3 hours. Ten µL of alkaline phosphatase and 4 µL of phosphodiesterase were added and allowed to incubate for 18 hours. After incubation, 40 µL was transferred to an microcentrifuge tube and 60 µL of 20 mM ammonium acetate was added and centrifuged for 5 minutes. Ninety µL of supernatant from this test tube was transferred to an HPLC glass vial and analyzed for DNA digestion efficacy (method discussed below). Next, 120

μL of cold ethanol was added to the remaining solution in the original microcentrifuge tube and centrifuged for 5 minutes in order to precipitate the enzymes. The supernatant from these tubes was transferred to a glass vial and 5 μL of DMSO was added. Liquid from these samples was evaporated using a Savant SpeedVac concentrator (St. Louis, MO) for 2 hours. Lastly, samples were reconstituted with 15 μL of water (see full details in Appendix B-3).

HPLC assessment of the efficacy of enzymatic digestion of DNA

Complete DNA digestion was confirmed by analyzing DNA bases using HPLC (see Appendix B-4 for full description). In brief, DNA hydrolysate (2 μg) was assayed with a Gilson high performance liquid chromatography system (Middleton, WI), comprised of a 307 and 306 pump, a 231 XL auto sampler, and a Kratos Analytical Spectroflow 757 UV detector monitoring ultraviolet absorbance at 260 nm. A Phenomenex Synergi 4μ Fusion-RP 80Å column (150 x 4.6 mm; Torrance, CA) was employed for chromatography of the deoxynucleosides with a linear gradient starting at 95% 20 mM ammonium acetate (pH 4.5) and 5% acetonitrile and reaching 40% acetonitrile at 20 min. The flow rate was 1 mL/min.

Measurement of PhIP-DNA adducts

The method for determination of PhIP-DNA adducts was adapted and modified from Gu et al. (149). In brief, analyses were performed with an Exigent nanoLC Ultra system (Dublin, CA) interfaced with a triple quadropole mass spectrometer (TSQ Vantage MS; Thermo Fisher, San Jose, CA). A Waters Symmetry (Sutton, MA) trap column (180 μm x 20 mm, 5 μm particle size) was employed for online solid phase

enrichment of the PhIP-DNA adducts. The analytical column was a C18 AQ column (0.3 x 150 mm, 3 μm particle size) from Michrom Bioresources (Auburn,CA). The DNA digests were injected onto the trap column and washed with 0.2% formic acid in water containing 10% acetonitrile at a flow rate of 8 μL/min for 5 min. Next, the DNA adducts were back-flushed onto the C18 AQ column, and a linear gradient was employed to resolve the DNA adducts, starting at 10:90 solvent B:A (where solvent B is 0.01% formic acid in acetonitrile, and solvent A is 0.01% formic acid in water) and arriving at 95:5 solvent B:A at 28 min. The flow rate was set at 8 μL/min. The injection needle was washed with 0.01% formic acid in water containing 10% acetonitrile, then 0.01% formic acid in water containing 25% acetonitrile, and finally 100% DMSO. Adducts were measured by LC-ESI-MS/MS in the positive ionization mode. The mass spectral parameters were optimized as previously reported (148). Mass of the adduct was measured at *m*/z = 479, and the internal standard was detected with *m*/z value of 500 (see Appendix B-5 for solvent and buffer details).

Calibration curves

A standard curve was constructed using [$^{13}C_{10}$]-dG-C8-PhIP set at 10 adducts per 10^7 bases with unlabeled DNA adducts added at a range of 0-30 adducts per 10^7 bases. Calculations and protocol for constructing the standard curve are found in Appendix B-6. The data was fit to a straight line (area of response of adduct/internal standard versus the level of adduct per 10^7 bases) using ordinary least squares with equal weightings. The coefficient of determination (r^2) values of the slopes exceeded 0.990.

Verification of the PhIP-DNA adduct method

Accuracy and precision were confirmed using samples from positive and negative control animals. In brief, DNA from eight prostates and pancreas in each control group was isolated, and the respective microtubes were combined to form one tube of DNA for each control group; DNA was then quantified using a spectrophotometer. Next, 50 µg of DNA from each control sample was aliquoted into triplicates of each value for DNA digestion. Prior to DNA digestion, unlabeled DNA adducts were added at levels of 0, 5, 10, and 15 adducts per 10^7 bases to the negative control group samples only, and 10 adducts per 10^7 bases of the internal standard, [$^{13}C_{10}$]-dG-C8-PhIP, were added to both positive and negative control samples (see Appendix B-7 for details). Adducts were analyzed using LC-ESI-MS/MS, and performance is summarized in the results section of this paper.

Statistical analysis

All results were expressed as mean ± standard error mean (SEM). The data were initially analyzed using analysis of variance (ANOVA), and since statistically significant differences between diet groups were observed, further analysis using the least square means test was performed. All analyses were conducted using Statistical Analysis System software package (Cary, NC). A *P*-value less than 0.05 was considered statistically significant.

Table 4.1. Project I: Diet Composition

Diet Ingredients (g/kg)	AIN-93G	21% Cruciferous[†]	21% Apiaceous[‡]	21% Combination[‖]
Cornstarch	397.5	391.8 (5.7)[*]	382.3 (15.2)[*]	387.1 (10.4)[*]
Dextrinized cornstarch	132.0	132.0	132.0	132.0
Casein	200.0	195.5 (4.5)	198.0 (2.0)	196.8 (3.2)
Sucrose	100.0	100.0	100.0	100.0
Cellulose	50.0	46.1 (3.9)	43.2 (6.8)	44.6 (5.4)
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Soybean oil[⊥]	70.0	69.6 (0.4)	69.5 (0.5)	69.5 (0.5)
Apiaceous	N/A	N/A	210.0	105.0
Cruciferous	N/A	210.0	N/A	105.0
Total (g)	1000.0	1195.5	1185.5	1190.5

[†]Cabbage, watercress and broccoli. [‡]Celery and parsnip. [‖]Cabbage, watercress, broccoli, celery, & parsnips.
[*]The values in the parentheses represent corresponding macronutrients from the vegetables. All diets were balanced for macronutrients using the USDA National Nutrient Database. [⊥]The antioxidant, t-Butylhydroquinone (tBHQ; 0.02%) was included in soybean oil.

Table 4.2. Project II: Diet Composition

Diet Ingredients (g/kg)[*]	AIN-93G	PEITC & I3C[†]	FC[‡]	Combination[‖]
Cornstarch	397.5	397.5	397.5	397.5
Dextrinized cornstarch	132.0	132.0	132.0	132.0
Casein	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0	50.0
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Soybean oil[⊥]	70.0	70.0	70.0	70.0
PEITC (mg)	N/A	182.6	N/A	91.3 mg
I3C (mg)		182.6		91.3 mg
5-MOP (mg)	N/A	N/A	1.2 mg	0.6 mg
8-MOP (mg)			0.6 mg	0.3 mg
Isopimpinellin (mg)			2.5 mg	1.2 mg
Total (g)	1000.0	1000.4	1000.0	1000.2

[*]Concentrations of PEITC and I3C, and furanocoumarins were matched with the vegetable contents (21% cruciferous, 21% apiaceous, and 21% combination feeding) used in project I. Phytochemicals were dissolved in the vehicle (i.e., corn oil) and mixed into powder diet (AIN-93G). [†]PEITC and I3C were used for the PEITC & I3C supplemented diet. [‡]5-MOP, 8-MOP, and isopimpinellin were used for the furanocoumarin (FC) supplemented diet. [‖]PEITC, I3C, 5-MOP, 8-MOP, and isopimpinellin were used for the combination diet. [⊥]The antioxidant, tBHQ (0.02%) was included in soybean oil.

Chapter 5: Results

Overview

This chapter will present the findings of our study, including verification and validation of our method, animals' food intake and body weights over the course of the study, as well as tissue weights and PhIP-DNA adduct levels observed in prostate and pancreas.

HPLC Verification of DNA Digestion

DNA digestion was verified by HPLC analysis of DNA bases (adenosine (A), cytosine (C), guanine (G), and thymine (T)). DNA digestion efficacy was determined based on the ability to identify four clearly isolated peaks, each representing the individual DNA bases. Chromatograms of DNA digestion using purchased standards are presented in Figure 5.1, followed by examples from prostate and pancreatic tissue in Figure 5.2 and 5.3. Overall, DNA digestion was complete, with slightly more variability occurring within the pancreas tissue.

LC-MS/MS Method Validation for Detecting PhIP-DNA Adducts

Prostate tissue from negative control animals was used to determine accuracy and precision of the method. Tissue samples were run in triplicates for each adduct level (5, 10 or 15 adducts per 10^7 bases) to assess accuracy and precision of the LC-MS/MS instrumentation. Accuracy was determined by calculating percent error (observed value - true value)/true value *100). For precision, the coefficient of variation (% CV) was determined from the mean adduct level and standard deviation. Results are shown in Table 5.1. Overall, accuracy values were less than 15% and precision values were less than 20%.

Project I: Food Intake and Weight Gain

Table 5.2 displays mean food intake over the 7-day feeding trial. Food intakes of animals in the vegetable groups were statistically significantly different from the basal + PhIP group. Mean food intake in the cruciferous +PhIP group, followed by the combination + PhIP group, and the apiaceous + PhIP group was 14% ($P \leq 0.001$), 12.5% ($P = .01$), and 9% ($P = .05$) greater than the basal + PhIP group, respectively. This observed increase in food is likely due to the high water content of the vegetables. Overall, animals had similar weight gain and growth patterns. At day 7, the average weight gain ranged from 95 – 102 g. Table 5.2 also shows mean weight gain for each diet group.

Project I: Tissue Weights

Table 5.3 shows mean weights of prostate and pancreatic tissues for each diet group. No statistically significant differences amongst diet groups were observed.

Project I: PhIP-DNA Adducts

PhIP-DNA adduct levels in the prostate, shown as adducts per 10^7 bases, for the fresh vegetable + PhIP groups compared to the basal + PhIP group are shown in Figure 5.4. The apiaceous + PhIP group had decreased PhIP-DNA adduct formation by 33% ($P < .05$). PhIP-DNA adduct levels in the pancreatic tissue, shown as adducts per 10^7 bases, for the vegetable + PhIP groups compared to the basal + PhIP group are shown in Figure 5.5. No statistically significant differences were observed in the pancreas.

Project II: Food Intake and Weight Gain

Table 5.4 displays mean food intake over the 7-day feeding trial in project II. Observed food intake of animals in one phytochemical + PhIP group was statistically significantly different from the basal + PhIP group. Food intake in the furanocoumarin diet was 6.5% less than the basal + PhIP group ($P \leq 0.05$). However, animals had similar weight gain and growth patterns. Table 5.4 also shows mean weight gain for each diet group. At day 7, the average weight gain ranged from 98 – 102 g.

Project II: Tissue Weights

Table 5.5 shows mean weights of prostate and pancreatic tissues for each phytochemical + PhIP group. The mean prostate weight in the combination phytochemical + PhIP group was 16% less than the basal + PhIP group (P-value < 0.05).

Project II: PhIP-DNA adducts

PhIP-DNA adduct levels in the prostate, shown as adducts per 10^7 bases, in the phytochemical + PhIP groups compared to the basal + PhIP group are shown in Figure 5.6. The furanocoumarin + PhIP group and the combination + PhIP group decreased PhIP-DNA adduct formation by 45% ($P \leq 0.001$) and 30% ($P \leq 0.01$), respectively. PhIP-DNA adduct levels in the pancreatic tissue, shown as adducts per 10^7 bases, for the phytochemical + PhIP groups compared to the basal + PhIP group are shown in Figure 5.7. No statistically significant differences were observed in the pancreas.

Figure 5.1. HPLC Chromatogram of nucleotide standards (200 µg/ml concentration; 20 µL injection)

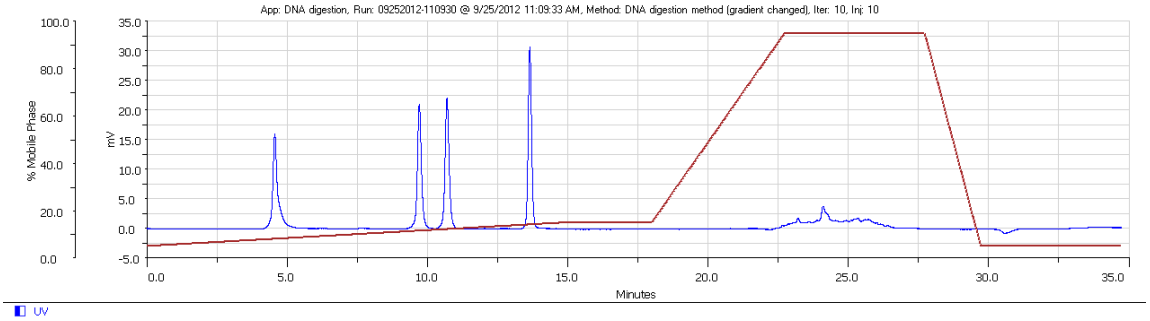


Figure 5.2. Example HPLC Chromatogram to verify DNA digestion for prostate sample #8 (Project I)

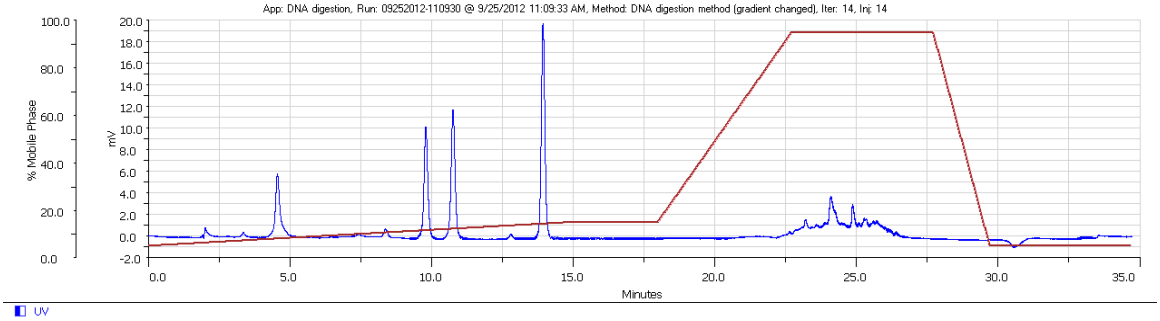


Figure 5.3. Example HPLC Chromatogram to verify DNA digestion for pancreas sample #43 (Project I)

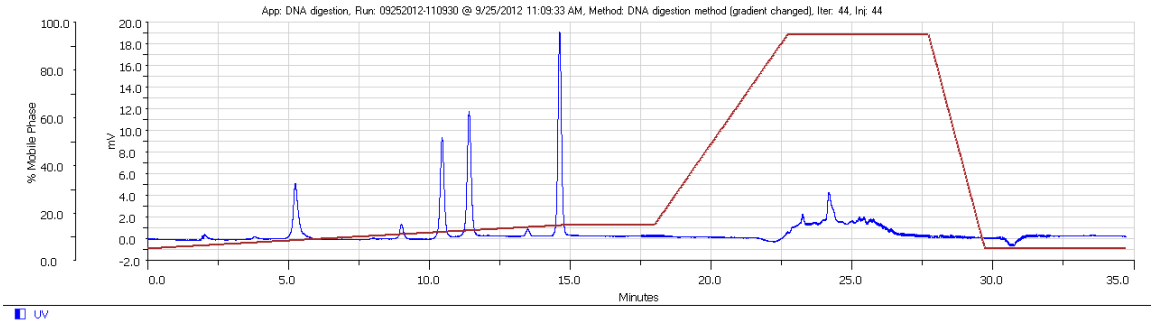


Table 5.1. Accuracy & Precision of PhIP-DNA Adducts by LC-MS/MS in Prostate

Negative Control spiked with:	Replicate 1	Replicate 2	Replicate 3	Mean	SD	Accuracy (% error)	Precision (% CV)
5 adducts per 10^7 bases	5.21	6.03	4.11	5.12	0.96	2.4	18.8
10 adducts per 10^7 bases	10.91	9.84	10.64	10.46	0.55	4.6	5.3
15 adducts per 10^7 bases	16.83	16.61	18.21	17.22	0.87	14.8	5.0

Table 5.2. Project I: Food Intake and Body Weight by Study Group

Group	Food Intake (g)	Weight Gain (g)
Basal diet + No PhIP	19.18 ± 0.58	102.19 ± 2.45
Basal diet + PhIP	19.96 ± 0.58	97.27 ± 2.44
Cruciferous diet + PhIP	23.21 ± 0.59***	99.18 ± 2.50
Apiaceous diet + PhIP	21.74 ± 0.58*	99.75 ± 2.44
Combination diet + PhIP	22.46 ± 0.58**	94.56 ± 2.44

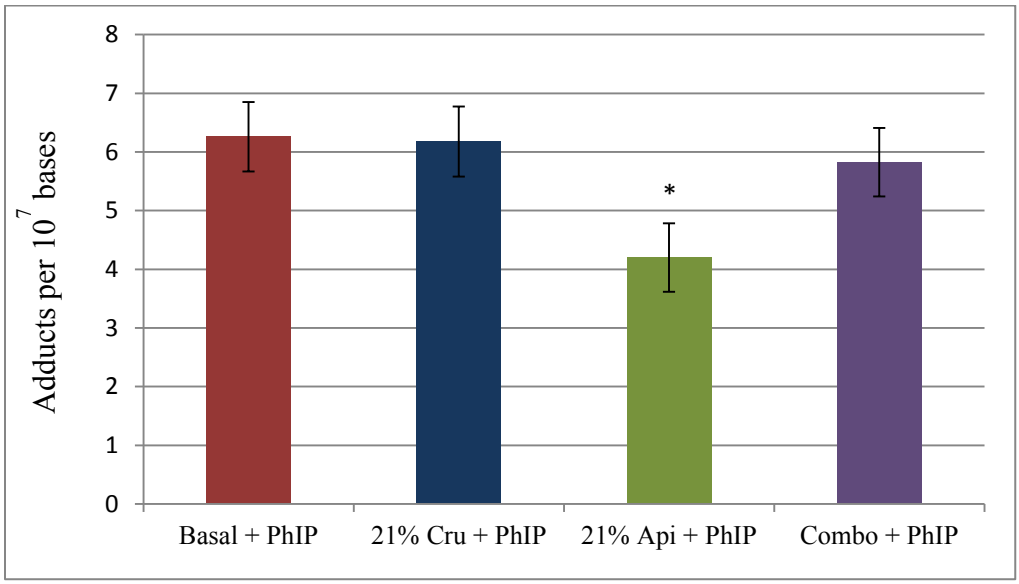
All results were expressed as Least Squares Means ± SE. Data were analyzed by ANOVA (Statistical Analysis System software package, Cary, NC). * *P*-value < 0.05. ** *P*-value < 0.01. *** *P*-value < 0.001.

Table 5-3. Project I: Tissue Weights (g)

Group	Prostate wt (g)	Pancreas wt (g)
Basal	0.21 ± 0.02	0.35 ± 0.03
Basal + PhIP	0.20 ± 0.02	0.36 ± 0.03
Cruciferous + PhIP	0.20 ± 0.02	0.36 ± 0.03
Apiaceous + PhIP	0.19 ± 0.02	0.36 ± 0.03
Combination + PhIP	0.24 ± 0.02	0.34 ± 0.03

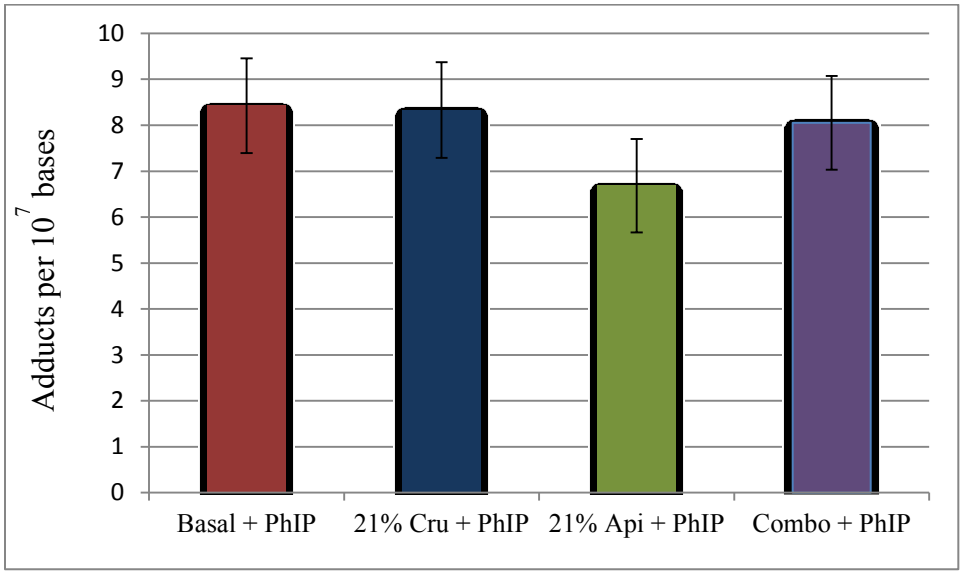
Values shown as Least Squares Means ± SE, n = 10-11. No difference amongst diet groups was observed.

Figure 5-4. Project I: PhIP-DNA Adducts in Prostate



Values are Least Square Means ± SEM, n = 10-11 per group. * $P < 0.05$.

Figure 5-5. Project I: PhIP-DNA Adducts in Pancreas



Values are Least Square Means ± SEM, n = 10-11 per group.

Table 5.4: Project II: Food Intake and Body Weight

Diet	Food Intake (g)	Weight Gain (g)
Basal diet + No PhIP	18.80 ± 0.37	100.17 ± 2.12
Basal diet + PhIP	18.98 ± 0.40	98.86 ± 2.27
GLS diet + PhIP	18.13 ± 0.42	99.40 ± 2.42
FC diet + PhIP	17.74 ± 0.41*	101.91 ± 2.37
Combo diet + PhIP	18.86 ± 0.41	98.36 ± 2.37

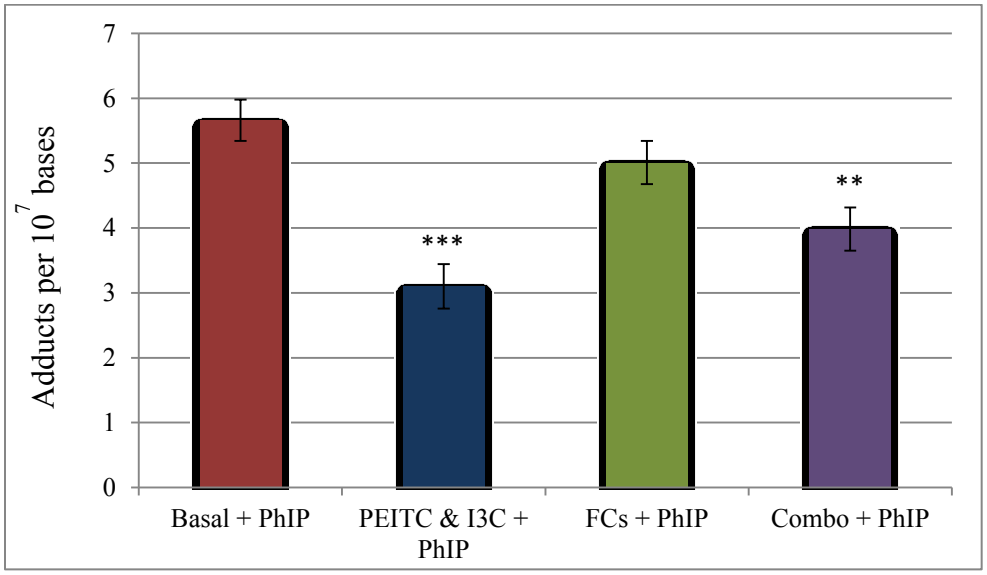
All results were expressed as Least Squares Means ± SE. Data were analyzed by ANOVA (Statistical Analysis System software package, Cary, NC). * Denotes a significant difference compared to the Basal + PhIP group when *P*-value < 0.05.

Table 5-5. Project II: Tissue Weights (g)

	Prostate wt (g)	Pancreas wt (g)
Basal	0.17 ± 0.01	0.28 ± 0.02
Basal + PhIP	0.19 ± 0.01	0.27 ± 0.02
PEITC & I3C + PhIP	0.18 ± 0.01	0.27 ± 0.02
Furanocoumarins+ PhIP	0.18 ± 0.01	0.27 ± 0.02
Combination + PhIP	0.16 ± 0.01*	0.28 ± 0.02

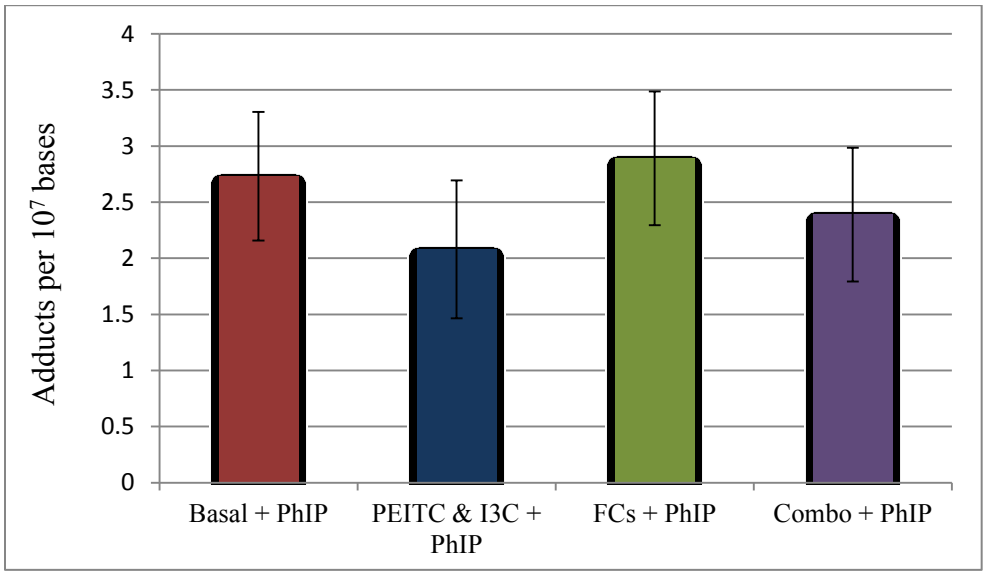
Values shown as Least Squares Means ± SE, n = 10-11. * *P*-value < 0.05.

Figure 5-6. Project II: PhIP-DNA Adducts in Prostate



Values are Least Square Means ± SEM, n = 10-11 per group. ** $P < 0.01$, *** $P \leq 0.001$

Figure 5-7. Project II: PhIP-DNA Adducts in Pancreas



Values are Least Square Means ± SEM, n = 10-11 per group.

Chapter 6: Discussion and Future Directions

Discussion

Previous vegetable feeding studies in animals and humans have predominantly focused on studying individual vegetable groups separately, and although these results can be informative, it hinders the discovery of the net effect of food combinations, which is more representative of a human diet. The purpose of our project was to determine if the combined effect of fresh apiaceous and cruciferous vegetables, or their respective phytochemical compounds, favorably influenced metabolism of the dietary carcinogen PhIP, as evidenced by PhIP-DNA adduct levels in prostate and pancreatic tissue of male Wistar rats.

We quantified PhIP-DNA adducts in both prostate and pancreatic tissue from a total of 6 different vegetable or phytochemical diet groups. In prostate tissue for project I, we saw a decrease in PhIP-DNA adduct levels with the apiaceous + PhIP group compared to the basal + PhIP group ($P < 0.05$). However, the cruciferous + PhIP and combination vegetable + PhIP group did not have decreased PhIP-DNA adduct levels when compared to the basal + PhIP group, as we had hypothesized. This lack of adduct reduction could be due to the ability of cruciferous vegetables to induce CYP1A2, which is the enzyme required for initially activating PhIP (88). It is also possible that hydrolysis of parent glucosinolates to their bioactive metabolites was incomplete and therefore the metabolites (isothiocyanates/indoles) were not fully absorbed. The degree of hydrolysis could be determined by analyzing metabolite levels in the blood or urine of the animals.

Results from project II also yielded interesting results. PhIP-DNA adduct levels in the prostate were reduced in the PEITC/I3C + PhIP group ($P < 0.001$), as well as the combination phytochemical + PhIP group when compared to the basal + PhIP group ($P < 0.01$). Contrary to our hypothesis, no effect was seen in the furanocoumarin + PhIP group. Since we did see a reduction in the fresh apiaceous + PhIP group, but not the furanocoumarin + PhIP group, this suggests that 8-MOP, 5-MOP, or isopimpinellin may not be the furanocoumarins responsible for the chemoprotective effect seen in the fresh apiaceous vegetable diet. It is possible that other furanocoumarins or different constituents within the apiaceous vegetable family are responsible for reducing PhIP-DNA adduct levels in the prostate. Furthermore, it is plausible that the concentrations of 5-MOP, 8-MOP, and isopimpinellin were not sufficient in project II to see an effect on PhIP-DNA adducts. For example, it is possible that our HPLC with UV detection method to quantify furanocoumarins was not as sensitive at deciphering/separating 5-MOP, 8-MOP and isopimpinellin as we had planned, potentially leading to a misinformed addition of furanocoumarins to the diet. Advanced technologies using 2-Dimensional HPLC show promise in better compound separation (150), and should be considered for use in future studies. In humans, pharmacological doses of 8-MOP and 5-MOP inhibit caffeine metabolism, indicating a decrease in CYP1A2 activity (128, 129), but to my knowledge, no studies have been done regarding the effect of furanocoumarins on PhIP-DNA adducts.

Interestingly, the PEITC/I3C + PhIP group resulted in a statistically significant reduction in PhIP-DNA adducts in the prostate tissue ($P < 0.001$) but, as mentioned, this

effect was not seen in the fresh cruciferous + PhIP group from project I. Again, these results posit a few possible explanations. First, the PEITC/I3C levels fed in the phytochemical diet were higher than in our fresh cruciferous feeding since we matched to the total glucosinolates concentrations from project I. It is possible that other glucosinolates are less potent than PEITC/I3C (104), meaning the dosage of PEITC/I3C in the fresh vegetables may not have been high enough to impart a beneficial impact on PhIP-DNA adduct levels. Another explanation could be that other glucosinolates or constituents within cruciferous vegetables negate the beneficial effect of PEITC and I3C bioactives. Although no negating effects have been studied in cruciferous vegetables themselves, it has been studied with the vitamin folate. Folate is a required vitamin for nucleotide synthesis of rapidly dividing cells, including cancer cells. The green tea polyphenol, epigallocatechin-3-gallate (EGCG) can interact with the folate pathway and inhibit folate synthesis, which is why the inhibitory effect of EGCG may have chemoprotective effects (151). Inoue et al. found that daily green tea consumption among women with low folate intake (<133.4 μg/day) was inversely associated with breast cancer compared to those with less green tea intake (OR 0.45, 95% CI 0.26-0.79). However, a high folate intake (>133.4 μg/day), negated the beneficial effects on green tea and no association with breast cancer was reported among daily tea drinkers with a high folate intake (152). This outcome highlights the possibility of interplay between various dietary constituents that may have occurred within our cruciferous vegetable diet.

No reduction in PhIP-DNA adduct levels were observed in the pancreas for both project I and II. We postulated this to be due to the nature of the pancreas tissue itself.

Pancreatic tissue is comprised of multiple layers, including both lobular and connective tissue (153). This variability in tissue histology may have introduced inconsistency to our DNA-extraction process, which led to the high intragroup variability in PhIP-DNA adduct levels that was observed. These findings are similar to that of other researchers. Pfau et al. found that PhIP-DNA adduct levels were 36-fold higher in the pancreas tissue of rats compared to liver tissue, but that there is very high variability within the pancreas (e.g., 508 ± 319 adducts per 10^9 in pancreas) (72). Furthermore, Turesky et al. showed that in nonhuman primates dosed with 20 mg/kg of 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone by gavage, the pancreas showed mean IQ-DNA adduct levels with widely varying standard deviations (15 ± 4; 13 ± 1; and 21 ± 7), again indicating high variability in pancreatic adduct levels (51). Further exploration into the makeup of pancreatic tissue, as well as reconsidering the methodology for DNA extraction from pancreas tissue, is needed.

Strengths

Our study has several strengths. First, our study includes those strengths inherent to an animal study, which allows for an ethically controlled intervention utilizing a complex biological system reflective of human complexity. Furthermore, we fed two classes of vegetables, both alone and combined, which is unlike most vegetable feeding studies that typically evaluate only one particular vegetable group or phytochemical. The combination diet more closely mimics the varied diet of a human. We also fed fresh cruciferous and apiaceous vegetables, as well as their respective phytochemical compounds, at physiologically relevant doses. In project I, a 21% vegetable diet equates

to ~101-106 g/day of fresh vegetables for a human (less than 4 ounces), which is certainly obtainable considering more than 25% of Americans reported consuming vegetables 3 or more times a day in 2009 (154). Additionally, the vegetables used for our diets were organic and purchased on a single day from a local market to avoid product variability. Macronutrients were also balanced in order to minimize confounding. Additionally, the sample size of our study was also a strength. We had 52 rats in each project, representing 10-11 rats per diet group, which allows for adequate statistical power. Lastly, our choice of LC-MS/MS instrumentation for PhIP-DNA adducts analysis allowed for high throughput sample analysis while still retaining accuracy, precision, and sensitivity.

Limitations

Despite the strengths of using an animal model to study endpoints, a rat-based model also has disadvantages. Most importantly are the interspecies differences between rodents and humans, especially with regards to HAA metabolism. There is a strong conservation among human and rat CYP1A1 and CYP1A2 enzymes, 83% and 80% respectively (155), but humans tend to have more hepatic CYP1A2 than rats -- estimated up to 240 pmol/mg protein more (156). Studies have also shown significant differences between rat and human CYP1A catalytic activity and specificity towards PhIP metabolism. Turesky et al. demonstrated that recombinant human CYP1A2 catalytic activity towards *N*-oxidation of PhIP was 19-fold greater than purified rat CYP1A2 (44). These researchers also showed that rats seem to favor detoxification routes of PhIP, as evidenced by 4'-OH-PhIP as the predominant urinary product (157). This necessitates

caution in the extrapolation of the data to humans, and it is important to question the generalizability of animal experiments to human populations.

Furthermore, our choice of PhIP-DNA adducts as the endpoint of our study has both advantages and disadvantages. DNA adduct formation is considered to be a necessary requirement for tumor induction since DNA adducts lie directly in the pathway of mutation by interacting with nucleic acids (158). Some researchers agree that DNA adducts may be an indicator of the carcinogenic dose which has reached the cellular level, potentially accounting for inter-individual variation in absorption, metabolism, and excretion of the carcinogen (159). For the purpose of our study, measuring PhIP-DNA adducts in the tissue allowed us to determine if vegetable consumption influenced the ability of PhIP to reach the respective tissues.

However, the current views on the use of DNA adducts in cancer prevention research are important to address, and data on PhIP-DNA adducts as a biomarker for prostate or pancreatic cancer are severely limited. A well summarized 2005 review article has provided some support suggesting DNA adducts (including PAH-DNA, PhIP-DNA, or bulky adducts) may be associated with a higher risk in humans for specific cancers, such as lung, liver, breast, and bladder (158). However, because of the huge variability in the studies' methodology, quantification of different adduct types, tissues assayed, and various cancer sites, the authors ultimately portrayed skepticism towards DNA-adducts as a biomarker of cancer risk.

Case-control studies analyzing associations between PhIP-DNA adducts and

cancer risks are also severely limited. Results from a recent article that reviewed 12 case-controls and prospective nested case-control studies suggest an association between cancer risk and DNA adducts. Poirier et al. found that various types of DNA adducts led to a 2- to 9-fold increase in odds ratios for lung, liver, colon, breast, bladder, or stomach cancer in individuals with the highest level of DNA adducts compared to matched counterparts with lowest adduct levels (160). However, only one of these 12 case-control studies analyzed PhIP-DNA adduct accumulation, and an OR of 4.03 (1.41-11.53) was reported only for breast cancer cases. Tang et al. conducted a case-control study that specifically analyzed PhIP-DNA adducts in tissues of 534 prostate cancer cases and matched benign prostate specimen and found that mean PhIP-DNA adduct levels were slightly elevated in cases, but the results were not statistically significant ($P = 0.32$). However, when the analysis was restricted to white males, the highest quartile of PhIP-DNA adducts presented an odds ratio of 1.73, but this outcome still did not reach statistical significance ($P = 0.10$) (161). Another case-control study analyzing PhIP-DNA adducts in pancreas tissue found that PhIP-DNA adduct levels in tissue of cancer subjects were significantly higher than PhIP-DNA adduct levels of pancreatic cancer free controls (OR = 3.4, 95% CI 1.5-7.5). Unfortunately, the results from the latter study should be taken as preliminary due to the study's small sample size (n = 122) (78). Together, results from the studies described above suggest that PhIP-DNA adducts *may* predict cancer risk, but results are not compelling.

Additionally, an individual's lifetime DNA adduct accumulation is variable and further dependent on DNA repair mechanisms and chemical stability, which must be

taken into consideration over time. For instance, repair mechanisms may impact the overall carcinogenic burden of HAA. Turesky et al. showed that continuous exposure to HAA does not lead to an accumulation of DNA adducts. When the HAA IQ was administered at 20 mg/kg five days per week for 9 years to nonhuman primates, only a marginal increase in liver DNA adduct was seen when compared to the adduct levels 24 h after a single dose of IQ (51). Turesky's results suggest that DNA repair processes were highly efficient at removing IQ-DNA adducts over time.

In our study, tissues were removed 24 hours after PhIP injection to reflect the early process of vegetable influence on biotransformation enzymes and PhIP-DNA adducts, and therefore we were unable to analyze any long-term beneficial impact. Perhaps, adaptation of biotransformation enzymes to the vegetable diets, or increased DNA repair mechanisms may have reversed the PhIP-DNA adducts from the tissue. Overall, current data suggests that the increased risk of cancer inferred by elevated DNA adducts appears to be modest at best. Even so, the utility of PhIP-DNA adducts to indicate how much active carcinogen reached the tissue levels is still relevant to our study.

Future Directions

Numerous animal and human studies have shown modulating effects from cruciferous and apiaceous vegetables and their respective constituents on biotransformation enzymes. Human case-control studies have also shown a protective effect from crucifers on prostate and pancreatic cancer risk. Why no influence from fresh

cruciferous vegetables on PhIP-DNA adducts was seen is uncertain, and to fall short of further investigation into this phenomenon is unwise. It would be advantageous to conduct a similar study to ours, using a higher dosage of vegetable concentrations, as well as investigating a variety of different constituents found within the vegetable families. For comparison, Walters et al. showed that consumption of 250 g of broccoli and Brussels sprouts and a well-cooked steak dinner for 12 days increased the N^2 and N^3 glucuronidated forms of PhIP, indicating that cruciferous vegetables induced phase II enzymes and enhanced PhIP detoxification (14). 250 g of broccoli and Brussels sprouts translates to over 100 g more from what we fed our animals, which may explain why we saw no reduction in PhIP-DNA adducts with fresh cruciferous vegetable feeding. Additionally, Dingley et al. found that male rats dosed with 816 mg (more than double our dosage) of PEITC/kg diet for 15 days significantly decreased PhIP-DNA adducts levels in the colon, liver, and prostate (111). To date, other vegetable constituents have not been studied for their effect on PhIP-DNA adducts. Future studies on vegetable intake on prostate and pancreatic cancer risk should consider monitoring a variety of cancer biomarkers over time, such as microRNA or circulating primary prostate cells for prostate cancer risk (162, 163), and serum CA-19-9 for pancreatic cancer risk (164).

To highlight even further just how complex the mechanism of action for vegetable chemoprevention is, one cannot rule out other factors that may mediate vegetable effects on biotransformation enzymes, such as duration of vegetable feeding, nutrient-nutrient interactions and genetic cross talk. The human diet includes a variety of constituents, which may act synergistically or negatively when handling carcinogenic burdens. This is

why future approaches should also include long-term controlled vegetable feeding interventions investigating diet-disease-genotype mechanisms. For example, employing a metabolomics or proteomics approach will allow the ability to study the food matrix and multiple points of controls and endpoints. Lastly, the use of PhIP-DNA adducts as a biomarker for prostate and pancreatic cancer risk needs to be further investigated. The lack of long term studies, as well as the concerns expressed from researchers, presents ambiguity for the use of PhIP-DNA adducts for cancer risk prediction.

Conclusion

We found that fresh cruciferous vegetables did not decrease PhIP-DNA adducts levels, but their glucosinolate constituents PETIC and I3C, did reduce adducts. We also showed that fresh apiaceous vegetables had the biggest impact on reducing PhIP-DNA adducts in the prostate; however their respective furanocoumarins (5-MOP, 8-MOP, and isopimpinellin) did not. Even though fresh apiaceous vegetables reduced PhIP-DNA adducts, we found that the combination of fresh apiaceous and cruciferous vegetables had no significant reducing effect. Overall, we concluded that cruciferous and apiaceous vegetables certainly play a role in modulation of biotransformation enzymes and may influence cancer risk, and the results of our study lend further insight into the complexity of diet and cancer risk.

Cancer is a growing health concern, particularly in the U.S (8). Prostate and pancreatic cancer prevalence reached over 2,000,000 and 35,000 cases, respectively, in 2009 (9, 10). The dietary carcinogen PhIP, which is formed during the cooking of meat

and fish, demonstrates carcinogenicity in the pancreas and prostate of rodents (4, 5), as well as an association with neoplastic disease of the pancreas and prostate in humans (6, 7). As previously described, multiple pathways are involved in the metabolism of procarcinogens, leading either to activation or detoxification. Activation of the dietary procarcinogen PhIP is initially dependent on the CYP1A1/1A2 enzymes, but the conjugating activity of specific phase II enzymes can form nonreactive PhIP metabolites for safe excretion before DNA damage occurs (37, 38, 42). Therefore, the biological potency for PhIP-induced carcinogenesis is strongly dependent upon the interaction of predominant biotransformation enzymes. Most of the biotransformation enzymes involved in PhIP metabolism can be modulated by plant constituents (12, 165, 166), which is why the American Cancer Society recommends eating a diet high in fruit and vegetables to reduce cancer risk (10). Understanding dietary modulation, nutrient interactions, and genetic difference in biotransformation enzymes is essential to achieving optimal chemopreventive effects from cruciferous and apiaceous vegetables. Since there is minimal long-term human data lending support to experimental observations on the relationship between diet and cancer, further studies investigating the carcinogenesis of PhIP, along with the modulating influence of cruciferous and apiaceous vegetables in humans, are warranted.

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Appendices

Appendix A: Diet Preparation Methods

Appendix A-1: Working Glucosinolate Extraction and Analysis

Solutions for Glucosinolate Extraction and Analysis

0.5M Sodium Acetate, pH 4.6

FW = 82.03 g/mole
0.5M = 0.5 * 82.03g/L = 41.015 g/L
Make 250 mL = **4.1015 g/250mL**

0.5N NaCl

3 mL are needed for elution through each SAX column.
FW = 58.45g/mole
0.5N = 0.5 * 58.45g/L = **29.23 g/L**

0.4 M Phosphate Buffer , pH 7

Use 0.2 M of sodium phosphate mono and dibasic
Monobasic: FW = 119.06 g/mole
0.2 * 119.06 g/L = 23.812 g/L = **5.953 g/250mL**
Dibasic: FW = 141.96 g/mole
0.2 * 141.96 g/L = 28.392 g/L = **7.098 g/250mL**
Dissolve in ddH_2O, adjust pH to 7 and bring to volume

2mM Ferricyanide (prepare fresh)

Used Potassium Ferricyanide, FW = 329.264 g/mole
Need 1.5 mL per assay
0.002 * 329.264 g/L = 0.65853 g/L = **0.06585 g/100mL**

1N NaOH (prepare fresh)

2N NaOH (prepare fresh)

Preparation of Sinigrin Standards:

Stock Solution Preparation Notes and Calculation

Prepare a **1.0mM stock** solution that is then used for serial dilutions to prepare the other standard levels. There is 2 X 1.5mL needed for each assay (blank and ferricyanide assays). To assure that there is enough plus some extra, prepare **4mL final for each standard level**. Also, the sinigrin concentration within the 4 ml of 1N NaOH will be diluted when the 0.308mL of concentrated HCl is added to neutralize the solution. Therefore, increase the amount of sinigrin to compensate for this dilution.
Compensation Calculation:

1mmole/L = Xmmole/3ml X=3umoles

3umoles * (415.49ug/umole) = 1246.47ug = 1.2465mg

This implies that the stock solution should contain 1.2465mg/1.5mL std volume used in assay

To compensate for the dilution effect:

1.2465mg Sinigrin/1.5ml assay vol = Xmg Sinigrin/(4mL + 0.308mL)

X = 3.581mg

*Make **8 mL of the 1mM** stock so that there is 4mL for the assay and 4mL available for the serial dilutions*

Weigh out 7.162mg Sinigrin and dissolve in 8ml 1N NaOH

Standard Levels to prepare:

0.03125mM, 0 .0625mM, 0 .125mM, 0.25mM, 0.5mM, 1.0mM

MW Sinigrin: 415.49g/mole

Dilution Calculations:

0.5mM: 0.5um/mL

Need 3.75mL (for dilution) + 4mL (assay) = 7.5mL

The dilution from the 1um/ml is 1:1

Use 3.875mL of the 1mM Stock + 3.875 mL 1N NaOH

0.25mM: 0.25um/ml

Need 3.5mL (for dilution) + 4mL (assay) = 7.5mL

The dilution from 0.5 to 0.25 is 1:1

Use 3.75mL of 0.5mM Std + 3.75mL 1N NaOH

0.125mM: 0.125um/ml

Need 3mL (for dilution) + 4mL (assay) = 7mL

The dilution from 0.25 to 0.125 is 1:1

Use 3.5mL of 0.25 + 3.5ml of 1N NaOH

0.0625mM: 0.0625um/mL

Need 2mL (for dilution) + 4mL (assay) = 7mL

Use 3mL of 0.125 + 3mL of 1N NaOH

0.03125mM: 0.03125um/mL

Need 4mL (1.5mL for each assay + 1mL extra)

The dilution from 0.0625 to 0.03125 is 1:1

Use 2mL of 0.0625 + 2mL of 1N NaOH

Vegetable processing:

1. Purchase **fresh** broccoli, cabbage, and watercress from produce department. Check with the produce manager for the freshest batch or when the store receives the vegetables and time your purchase and processing around this.

2. In the lab weigh a fresh sample of each vegetable, taking care to minimally damage. Place in a pilot plant hairnet, secure with a twist-tie and weigh. Flash freeze in liquid nitrogen then transfer the frozen vegetable on dry ice until it goes onto lyophilizer. From experience it was determined that the broccoli and cabbage require about 6 days to completely dry when using the center drying drum. This was confirmed by weight change over time. Weigh the vegetable when dry.
3. Grind the dried vegetable in a food processor to a powder and transfer to ziplock bags and store at -80c.
4. Weigh out approximately 200mg of the ground dried broccoli, or 100mg watercress, or 200mg cabbage and place in a 15ml glass screw-cap tube.

Deactivation of Myrosinase and Extraction of Glucosinolates

1. Pipette 4.5ml cold Methanol into a 15ml glass screw-cap tube. There should be 1 tube of methanol prepared for each tube containing plant material to extract. Place both sets of tubes in a heating block set at about 65-70c. Allow time (10min) for the tubes and content to equilibrate with the heating block.
2. Place a flask or capped tube with either ddH_2O or ddH_2O containing 0.5 mg sinigrin/1ml boiling water to preheat the solutions prior to addition to the extraction tubes
3. Transfer the 4.5ml of the 100% Methanol solution into the extraction tube by pouring it thru a small glass funnel Cap the tubes, lightly vortex, and then place it back in the heating block immediately.
4. Reflux the tubes with the methanol added for 20min at 70c. Do not tighten the caps completely. Pressure will build in the tube and if you open a tightened cap the solution will pop resulting in the loss of sample and methanol.
5. After 20min. carefully open the caps and add either 1 ml of 70°c water or 0.5ml sinigrin spike using the Microman Positive displacement pipette. Cap
6. Increase the heating block temperature to 80°c and extract for 30minutes.
7. Remove the tubes from the heating block. Cool the tube.
8. Homogenize the water –vegetable mix at speed 2.5 using the *PRO 200* homogenizer. 30 strokes
9. Transfer the homogenate to a high-speed centrifuge tube.
10. Add 2.5ml 90% methanol to the extraction tube and rinse the probe with a brief homogenization in this tube.
11. Centrifuge the primary homogenate at 18,000g for 15min. at room temperature. Transfer the supernatant to a 12ml round bottom Falcon tube.
12. Add the methanol rinse to the tube containing the pellet. Vortex and centrifuge again at 18,000g for 15min. Add this supernatant to the Falcon tube.

13. Add 2.5ml 90% methanol to the pellet, vortex, centrifuge and transfer the supernatant.
14. Store the tubes at -20c until further processing.

Important! Hydrolyzed samples should be assayed on the same day

Column and sample preparation:

1. Dry the tubes containing the pooled supernatants at 45c under nitrogen.
2. Prepare the Strong Anion Exchange (SAX) solid phase extraction cartridge (500mg from Supelco, Cat. #57017) on a vacuum manifold. It is important to never let the columns dry out.
   a. Activate the columns with 3 mL of Methanol
   b. Wash with 3 mL water.
   c. Wash SAX with 2 mL of 0.5M Sodium Acetate pH 4.6
   d. Wash SAX with 2 mL of H_2O
3. Reconstitute the dried extract with 3ml ddH_2O. Vortex
   a. ***Place a plug of glass wool into a 3ml syringe then load the supernatant into the syringe and through glass wool onto the SAX column.*** Note: If the solution looks relatively free of plant materials, begin loading the reconstituted extract onto the activated SAX column. The sulfate group on the glucosinolates should bind the compounds to the column matrix.
   b. Rinse the tube that had contained the extract with 2.5ml of ddH_2O water and load this rinse onto the column.
   c. Place collection tubes in the manifold rack.
   d. Elude the glucosinolates with a total volume of 3 mL of 0.5N NaCl. Add the NaCl in 1.5 mL increments and let it move slowly through the column.

Alkaline Treatment and Reaction with Ferricyanide

Alkaline Treatment:

1. Prepare the 1mM Sinigrin standard in 1N NaOH. This standard will be serially diluted and used for the standard curve in a 12ml Falcon tube.
2. Label 12 ml Falcon tubes to use for hydrolysis of the extraction. Add 2.0 mL of the eluted glucosinolate with an equal volume of 2 N NaOH to the appropriately labeled tube.
3. Incubate for 30 minutes at room temperature.
4. After about 20-25minutes, dilute the sinigrin standard preparation as described in the solution preparation portion of the method.
5. After 30 minutes, add 310ul of concentrated HCL to all tubes, vortex after each addition.

6. Transfer 1.5mL aliquots of the supernatant to 2 culture tubes for assaying the total glucosinolate content using the ferricyanide method. Confirm that there are no particulates.

Spectrophotometric Analysis of Glucosinolates using Ferricyanide:

1. Blank the spectrophotometer on water at 420nm.
2. Prepare duplicate tubes with 1.5ml of either standards or samples.
3. Add the phosphate or ferricyanide solution to the tube containing the sample. Vortex, transfer mixture to a glass cuvette, and read absorption at 2minutes (timed from the start of the addition of the phosphate or ferricyanide

Appendix A-2: Furanocoumarin Extraction and Analysis by HPLC

1. Cut at least 4-5 celery stalks in small pieces then homogenize in food processor. Peel parsnip, grate using food processor, and then homogenize parsnip using food processor. Weigh 5 g homogenate directly into 50 ml screw cap glass tubes. Add internal STD in this step; it could be one of furanocoumarins. Analyze the sample with and without internal STD.
2. Mix 5 g of homogenate with 15 ml water and 10 ml diethyl ether; vortex.
3. Homogenize mixture in Ultra-Turrax homogenizer, at setting 4, completing full up – down motion with tube 10 times.
4. Sonicate samples for 2 min.
5. Centrifuge in buckets at ~ 3500 x *g* for 15 min (repeat spin if poor separation with parsnip) and collect organic phase in 20 ml screw cap tubes.
6. Repeat extraction/centrifugation four more times, (vortexing for 1 minute; combine organic phases by adding only 10 ml of diethyl ether).
7. Evaporate solvent using N-evap during centrifugation. **Use foil under the hood.**
8. Resuspend residue in 1.8 ml acetonitrile then, sonicate 3 min.
9. Add 1.2 ml of water (to make 60% of acetonitrile).
10. Activate reversed-phase (C_{18}) columns with 3 ml methanol followed by 3 ml water.
11. Apply the sample into C_{18} column.
12. Elute with 3 ml 60% acetonitrile in water, and collect fraction in 15 ml screw top tubes.
13. Wash the tube inside with 3 ml 60% acetonitrile in water and apply this into column as well.

14. Remove solvents using N-evap with water bath at 40°C.

15. Reconstitute sample in 1 ml chloroform.

16. Activate SiOH column with 3 ml chloroform.

17. Apply the sample into SiOH column.

18. Elute with 3 ml 7.5% ethyl acetate in chloroform, collecting fraction in 15 ml screw cap tubes.

19. Wash the tube inside with 7.5% ethyl acetate in chloroform and apply this into column as well.

20. Remove solvent using N-evap.

21. Resuspend in 0.5 ml acetonitrile and sonicate samples for 3 min.

22. Add 0.5 ml water (or aqueous mobile phase of your separation; e.g., phosphoric acid); transfer with Pasteur pipet to HPLC vials.

23. Analyze using HPLC; UV diode array detector set at λ = 310 nm; Agilent Poroshell 120 EC-C18 column (150 x 4.6 mm; 2.7 μm); mobile phase: 10 mM phosphoric acid/methanol (45:55, v/v) at 40° C, flow rate 0.5 ml/min. (Isocratic method)

Note: Method adapted from Ostertag et al., JAFC 2002

Appendix B: Methods and Assays

Appendix B-1: Method for DNA Extraction

Qiagen Gentra Puregene DNA Purification Kit (written by Kim, 3/2/12)

1. Add 600 ul of Lysis Solution to a 1.5 ml microcentrifuge tube, and chill on ice.
2. Add 30 – 40 mg of tissue to the chilled Lysis Solution.
3. Incubate the lysate at 65°C for 60 min (or more)
4. Add 3 ul of Proteinase K, vortex samples, and incubate at 55°C for 2 hr or more and invert samples periodically.
5. Add 3 ul of RNase A, vortex samples, and incubate at 35°C for 25 min.
6. Cool down samples on ice for 5 min.
7. Add 200 ul of Protein Precipitation Solution and vortex for 20 sec.
8. Centrifuge for 10 min at 18,000 × g (the precipitated protein will form a tight white pellet. If the protein pellet is not tight, incubate on ice for 5 min more and repeat centrifuge).
9. Carefully transfer the supernatant, containing the DNA to a 1.5 ml microcentrifuge tube. Some supernatant may remain in the original tube containing protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with protein.
10. Add 600 ul of room temperature isopropanol.
11. Gently mix the solution by inversion until white thread-like strands of DNA form a visible mass. Longer incubation with isopropanol will yield higher amount of DNA.
12. Centrifuge for 1 min at 18,000 × g (DNA will be visible as a small white pellet) and carefully decant supernatant. You need to decant the supernatant toward opposite direction from where the DNA is precipitated to avoid losing DNA.
13. Add 600 ul of room temperature 70% ethanol and gently invert the tube several times to wash DNA.
14. Centrifuge for 1 min at 18,000 × g.
15. Carefully remove the ethanol using pipette.

16. Air dry the pellet for 10` min.
17. Add 100 ul of DNA Rehydration Solution and rehydrate the DNA by incubating at 65°C overnight
18. Check the purity and concentration. Take 2 ul and dilute with 98 ul of saline (i.e., 1:50dilution).

Appendix B-2: Method for DNA Quantification

DNA quantification using 96 well-plate (written by Kim, Apr 27 2012)

1. Dilute one of your sample (1:50, 2 ul of original solution and add 98 ul of saline)
2. Measure this sample using a spectrophotometer in the Mashek's lab
3. Dilute this original sample to 100 ug/ml concentration (for total volume 500 ul)
4. Make serial dilutions using the sample from step 3
   (i.e., 100 ug, 50 ug, 25 ug, 12.5 ug, and 6.25 ug/ml, respectively; make sure you will need 200 ul per each concentration for 96 well-plate, and a plain saline blank)
5. Dilute your other samples (1:20, take 15 ul of original sample and add 285 ul of saline)
6. Transfer 200 ul of each sample from step 5 into the 96 well-plate.
7. Measure at 260/280 nm using a microplate reader in the Chen lab.

Appendix B-3: Method for enzymatic hydrolysis of DNA to deoxynucleosides

Chemicals and Reagents (All enzymes are from Sigma.)

- **5 mM Tris-HCl/10 mM $MgCl_2$ (pH 7.4)**
  1. Weigh 605.7 mg of Tris (for 1 L solution)
  2. Weigh 2.03 g of $MgCl_2$
  3. Dissolve above in 950 ml of water and adjust pH with HCl
  4. Bring up to 1 L
  5. Store at 2-8°C
- **DNase I** (type IV from bovine pancreas; stored at -20°C). The unit ratio is 2000 U/mg protein (i.e., 2U/ug)
  1. Make normal saline (i.e., 0.9% NaCl). To make 0.9% NaCl (1 L), add 9 g of NaCl in 1 L water
  2. Make stock solution (2 mg of DNase I/ml saline solution).
  3. This stock solution will be stable up to a week (less than 10% activity loss at -20°C).
- **Nuclease P1** (from Peniccilium citrinum; stored at 2-8°C). The unit ratio is 200 U/mg protein (i.e., 0.2U/ug).

Make stock solution (1 mg of nuclease P1/ml of 1mM $ZnCl_2$). **To make 1 mM $ZnCl_2$ solution (10 ml):** make 2ml of 1mg/ml $ZnCl_2$ solution and take 1393 ul of this and add 8637 ul of water (to make 10 ml solution). Store at 2-8 °C

  1. You will use only 1 ul per each reaction (i.e., 50 ug of DNA); thus weigh as little as possible.
  2. Make this stock solution fresh and be careful to weigh.
- **Phosphodiesterase I** (from Crotalus adamanteus venom; stored at -20°C). Once you get a new vial, weigh the enzyme first and calculate Unit/weight ratio.
  1. Make stock solution[1] (40 mg (i.e., 0.4 U)/ml of solution; equivalent to 1 mg/25 ul).
  2. You will use only 10 ul per each reaction (i.e., 50 ug of DNA).

Make this stock solution fresh and be careful to weigh. **To make the solution for phosphodiesterase I (100 ml):**

  1. Weigh 642.4 mg of NaCl
  2. Weigh 305 mg of $MgCl_2$
  3. Weigh 1.33 g of Tris
  4. Adjust pH using HCl (pH 8.9)
  5. Add water up to 50 ml
  3. Add 50 ml of glycerol

- **Alkaline phosphatase** (from E. coli; stored at -20°C). The unit ratio is 30 U/mg protein (0.03 U/ug).
  1. Make stock solution (1 mg (i.e., 30 U)/ml of 1mM $MgCl_2$).

[1] 110 mM Tris-HCl (pH 8.9) containing 110 mM NaCl, 15 mM $MgCl_2$, and 50% glycerol

2. You will use only 4 ul per each reaction (i.e., 50 ug of DNA); thus weigh as little as possible.

Make this stock solution fresh and be careful to weigh. **To make 1 mM $MgCl_2$ solution (100 ml):**

1. Make 100 mg/ml stock solution
2. Take 203 ul of stock solution
3. Bring up to 100 ml

Procedures:

*Remove samples from -80°C and sonicate for 10 minutes.

1. Dissolve 50 ug of DNA in 79 ul of 5 mM Tris-HCl and 10 mM $MgCl_2$.
2. Add 2 ul of internal standard (thus 50 ug of DNA/81 ul concentration).
3. Add 4 ul of **DNase I** (8 ug (16 U) of DNase I/reaction (50 ug of DNA)).
4. Incubate for 1.5 hr at 37 °C.
5. Add 1 ul of **nuclease P1** (1 ug (0.2 U) of nuclease P1/reaction (50 ug of DNA)).
6. Incubate for 3 h at 37 °C.
7. Add 10 ul of **phosphodiesterase I** (0.004 U of phosphodiesterase I/reaction (50 ug of DNA)).
8. Add 4 ul of **alkaline phosphatase** (0.12 U of alkaline phosphatase/reaction (50 ug of DNA)).
9. Incubate for 18 h at 37 °C. *Centrifuge briefly when finished.*
10. Take 40 ul of mixture (contains 20 ug of DNA) and add 60 ul of 20 mM ammonium acetate.
11. Centrifuge the sample from step #10 for 5 min at 18,000 × g and transfer 90 ul of supernatant into HPLC glass vial.
12. Take remainder of original sample (30 ug of DNA/60 ul) and add 120 ul of cold EtOH (to precipitate enzymes) and centrifuge for 5 min at 18,000 × g.
13. Transfer supernatant to vile (150 ul; this fraction contains approximately 25 ug of DNA) and add 5 ul of DMSO.
14. Dry out sample using speedvac (appx. 2 hours, keeping pressure between 10-50).
15. Add 15 ul of water and analyze using LC/MS.

References:

The protocol was from Chem Res Toxicol, (Vol 8, No 8, 1995 and Vol 20, No 2, 2007)

Miscellaneous:

- In the references, DNase, nuclease P1, phosphodiesterase and alkaline phosphatase were applied at levels of 25.4U, 0.4 U, 0.00714 U, and 0.2 U/100 ug of DNA, respectively.
- In this protocol, we applied 23U, 0.4U, 0.0008U, and 0.24U of enzymes/100 ug of DNA, respectively.

Appendix B-4: Method for HPLC assessment of enzymatic DNA digestion efficacy

Reagents

- 20 mM ammonium acetate (pH 4.5)
- Acetonitrile

Column

- Phenomenex Synergy 4u Fusion-RP 80A (150 × 4.6 mm)

Methods

- Starts at 95% 20 mM ammonium acetate (pH 4.5) and 5% acetonitrile
- Reach 40% acetonitrile over 20 min

Flow rate
1 mg/min

Sample preparation

- Take 20 ul of DNA hydrolyzed solution and add 60 ul of 20 mM ammonium acetate
  (to make 20 ug of DNA/100 ul)
- Inject 20 ul of this mixture (i.e., 4 ug of DNA)

Analytical STD preparation

- Make 50 ug of each nucleotide/ml.
- Make 200 ug of all nucleotides/ml
- Inject 20 ul of STDs

Appendix B-5: Solution & buffer for LC-MS/MS quantification of DNA adducts

Mobile phase A: 0.01% formic acid in water (make 200 ml)

- Add 20 ul of formic acid
- Bring up to 200 ml with water

Mobile phase B: 0.01% formic acid in ACN (make 200 ml)

- Add 20 ul of formic acid
- Bring up to 200 ml with ACN

Trapping buffer: 0.2% formic acid in 10% ACN in water (make 2 L)

- Add 4 ml of formic acid in 200 ml of ACN
- Bring up to 2 L with water

Washing buffer A: 10% ACN in water (make 10 ml)

- Add 1 ml of ACN
- Add 9 ml of water

Washing buffer B: 25% ACN in water (make 10 ml)

- Add 2.5 ml of ACN
- Add 7.5 ml of water

Washing buffer C: 100% DMSO (make 10 ml)

- Add 10 ml of DMSO

Appendix B-6: Standard Preparation for LC-MS/MS calibration curves

Analytical standard (AS) calculation

1. You will have 25 ug of DNA/ 20 ul at the end (i.e., after the whole DNA digestion processes)

This is equivalent to 125 ug of DNA/ 100 ul

To convert the DNA amount into nucleosides

(125 ug of DNA / 1) × (1 mole / 330 g) = 0.379 umole nucleosides (in 125 ug of DNA)

2. We would like to make the adduct level as 5 adducts in 10^7 nucleosides

5 adducts: 10^7 nucleosides = x adducts: 0.379 umole nucleosides (equivalent to 125 ug of DNA)

x adducts = 5 × 0.379 umole ~~nucleosides~~/ 10^7 ~~nucleosides~~

x adducts = 1.895 umole/10^7

x adducts = 1.895×10^{-13} mole

If you convert this to fmole unit (i.e., 10^{-15}),

189.5×10^{-15} mole = 189.5 fmole (this is the amount of adduct in 125 ug of DNA in 100 ul)

In conclusion, this is equivalent to 5 adducts per 10^7 nucleosides concentration.

3. You need 189.5 fmole AS per 100 ul in order to make 5 adducts per 10^7 nucleosides concentration.

To convert fmole concentration to pg unit,

189.5 fmole of AS/100 ul
= 1895 fmole of AS/1000 ul
= 1895 ~~fmole~~ of AS/ml × (1×10^{-15} ~~mole~~)/~~fmole~~ × 489.5 g MW of AS /~~mole~~

= 928 pg of AS/ml

This is equivalent to 5 adducts per 10^7 nucleosides concentration (i.e., final concentration of your STD)

4. You want to make the intermediate solution (i.e., stock solution)

$(M_1) \times (2\ ul) = (928\ pg\ of\ AS/ml) \times (20\ ul)$

where
M_1 is the concentration of stock solution

2 ul is the amount of stock solution you need to take 5 adducts

928 pg of AS/ml is the final concentration of your STD (i.e., 5 adducts per 10^7 nucleosides)

20 ul is the final volume of your STD

$M_1 = 9275$ pg/ml

5. Thus, we need to make our stock solution (i.e., 9275 pg/ml concentration) from Turesky's STD solution (This is provided from Turesky's lab).

2. The concentration is 1 ug/ml.

$(1\ ug/ml) \times (V_1) = (9275\ pg/ml) \times (1\ ml)$

where

1 ug/ml is the concentration of Turesky's STD solution

V_1 is the volume you need to take AS from the Turesky's STD solution

9275 pg/ml is the concentration of stock solution you want to make

1 ml is the final volume of your stock solution you want to make.

Therefore, take 9.275 ul of original Turesky's AS solution and bring up to 1 ml by adding 990.725 ul of 50% DMSO in Tris buffer. (Note that you need to make 50% DMSO in the Tris buffer. This tris buffer is the one you use for the DNA digestion experiment).

Internal standard (IS) calculation

1. You want to make 10 adducts of IS per 10^7 nucleosides concentration

This is equivalent to 379 fmole/ 100 ul (see the calculation above for the 5 adducts per 100 ul concentration; 189.5 fmole per 100 ul)

To convert fmole concentration to pg unit,

379 fmole of IS/100 ul
= 3790 fmole of IS/1000 ul
= 3790 ~~fmole~~ of IS/ml $\times$ $(1 \times 10^{-15}$ ~~mole~~$)/$~~fmole~~ $\times$ 499.4 g MW of IS /~~mole~~

= 1892.73 pg of IS/ml

This is equivalent to 10 adducts per 10^7 nucleosides concentration (i.e., final concentration of your STD)

2. You want to make the intermediate solution (i.e., stock solution)

$(M_1) \times (2 \text{ ul}) = (1892.73 \text{ pg of IS/ml}) \times (20 \text{ ul})$

where
M_1 is the concentration of stock solution

2 ul is the amount of stock solution you need to take 10 adducts

1892.73 pg of IS/ml is the final concentration of your STD (i.e., 10 adducts per 10^7 nucleosides)

20 ul is the final volume of your STD

$M_1 = 18927.3$ pg/ml

3. Thus, we need to make our stock solution (i.e., 18927.3 pg/ml concentration) from Turesky's STD solution. (This is provided from Turesky's lab, the concentration is 5.16 ug/ml).

$(5.16 \text{ ug/ml}) \times (V_1) = (18927.3 \text{ pg/ml}) \times (1 \text{ ml})$

where

5.16 ug/ml is the concentration of Turesky's STD solution

V_1 is the volume you need to take IS from the Turesky's STD solution

18927.3 pg/ml is the concentration of stock solution you want to make

1 ml is the final volume of your stock solution you want to make.

Therefore, take 3.67 ul of original Turesky's IS solution and bring up to 1 ml by adding 996.33 ul of 50% DMSO in Tris buffer. (Note that you need to make 50% DMSO in the Tris buffer. This tris buffer is the one you use for the DNA digestion experiment).

STDs table (for total volume 20 ul)

	# of AS	Volume of AS stock solution	# of IS	Volume of IS stock solution	Volume of 50% DMSO in Tris buffer	Total volume
S0	0	0 ul	10	2 ul	18 ul	20 ul
S1	5	2 ul	10	2 ul	16 ul	20 ul
S2	10	4 ul	10	2 ul	14 ul	20 ul
S3	15	6 ul	10	2 ul	12 ul	20 ul
S4	20	8 ul	10	2 ul	10 ul	20 ul
S5	25	10 ul	10	2 ul	8 ul	20 ul
S6	30	12 ul	10	2 ul	6 ul	20 ul

STDs table (for total volume 100 ul)

	# of AS	Volume of AS stock solution	# of IS	Volume of IS stock solution	Volume of 50% DMSO in Tris buffer	Total volume
S0	0	0 ul	10	10 ul	90 ul	100 ul
S1	5	10 ul	10	10 ul	80 ul	100 ul
S2	10	20 ul	10	10 ul	70 ul	100 ul
S3	15	30 ul	10	10 ul	60 ul	100 ul
S4	20	40 ul	10	10 ul	50 ul	100 ul
S5	25	50 ul	10	10 ul	40 ul	100 ul
S6	30	60 ul	10	10 ul	30 ul	100 ul

Note.

1. Use silanized vials for the STDs and samples with pre-slit septa.

2. Make the stock solution in 50% DMSO in Tris buffer; do not decrease the DMSO concentration.

3. Once retrieve the original STD (either AS or IS) from Turesky's lab, thaw them at the room temperature for 5 min and sonicate 5 min. This is very important step!

4. When you dilute the original STD solutions to make stock solution, you also need to sonicate this again for 5 min.

5. Store the prepared serial concentrations of STD at -80°C until analyze

6. Before you bring the samples to the cancer center, sonicate them again

Appendix B-7: Sample preparation for accuracy and precision verification.

Verification of PhIP-DNA adduct LC-MS/MS method

Objective: To digest whole genome DNA into single nucleotides for further analysis using LC-MS/MS.

Method:

- Whole genome DNA was extracted from the colon.

-50 ug DNA was placed into epitube for both project I & II.

-Three negative and 3 positive controls were included in order to validate the instrument and method.

NEGATIVE Controls:

A1: Spike with 15 adducts/10^7 nucleotides (3 each) = 6 µL of AS stock

A2: Spike with 10 adducts/10^7 nucleotides (3 each) = 4 µL of AS stock

A3: Spike with 5 adducts/10^7 nucleotides (3 each) = 2 µL of AS stock

A4: Spike with 0 adducts/10^7 nucleotides (3 each) NO spike

*Spike all samples with 10 adducts/10^7 nucleotides of internal standard.

POSTIVE Controls:

B1: No spike with analytical standard, spike with 10 adducts/10^7 nucleotides of IS (3 each) *10 adducts/10^7 nucleotides = 2 µL of IS stock

Appendix C: Data Set and SAS Codes

```
data HFHL_PhIP_Study (label='all data')

veg_only (label='vegetable diets only')

pure_cmps (label='purified compounds only')

;

Title2 'HFHL PhIP Stdy by Kim (vegetable diet vs pure compound diet)';

Input Animal      Diet Block  Experiment  Weight_gain Food_intake Colon
      Pancreas    Prostate    Liver Lung  CYP1A1      CYP1A2
      Plate_number

UGT1A1      SULT1A1     CYP1A1_E    CYP1A2_E    UGT1A1_E    SULT1A1_E
      I     II    III   IV    V     VI    VII   VIII  IX X  XI    XII
      XIII  XIV   XV    XVI   XVII Glucuronidated_p Sulfated_p
      Methylated_p Five_hydroxylated_p

N2_hydroxylated_p I_p   II_p III_p  IV_p  V_p   VI_p  VII_p VIII_p
      IX_p  X_p   XI_p  XII_p XIII_p      XIV_p XV_p  XVI_p XVII_p
col_DNA_adduct col_DNA_adduct_P Prost_DNA_adduct Panc_DNA_adduct

Prost_DNA_adduct_p Panc_DNA_adduct_p

;


Label

Animal= 'Animal number'

Diet= 'Diet type'

Block= 'Block animal was sacrificed'

Experiment= '1. vegetable diet feeding; 2. pure compound diet feeding'

Weight_gain= 'final weight - initial weight'

Food_intake= 'Food intake per 24 hr'

Colon= 'Colon weight (g)'

Pancreas= 'Pancreas weight (g)'

Prostate= 'Prostate weight (g)'
```

```
Liver= 'Liver weight (g)'

Lung= 'Lung weight (g)'

CYP1A1= 'CYP1A1 EROD - pmol_resorfurin/min_mg_protein'

CYP1A2= 'CYP1A2 MROD - pmol_resorfurin/min_mg_protein'

Plate_number= 'Plate number of kinetic assay'

UGT1A1= 'Enzyme UGT1A1 assay'

SULT1A1= 'Cytosolic SULT1A1 Activity (nmol/min/ug prot)'

CYP1A1_E= 'CYP1A1 ELISA'

CYP1A2_E= 'CYP1A2 ELISA'

UGT1A1_E= 'UGT1A1 ELISA'

SULT1A1_E= 'SULT1A1 ELISA'

I= 'PhIP'

II= 'methylated PhIP - has not idenfied yet'

III= 'N2-methyl-PhIP'

IV= '4 prime-OH-PhIP'

V= 'N2-OH-PhIP'

VI= '5-OH-PhIP'

VII= 'N2-methyl-4 prime-OH-PhIP'

VIII= '4 prime-OH-PhIP sulfate'

IX= '5-OH-PhIP sulfate'

X= '5, 4 prime-DiOH-PhIP sulfate'

XI= 'PhIP-N3-glucuronide'

XII= 'PhIP-N2-glucuronide'

XIII= '4 prime-OH-PhIP glucuronide'

XIV= '5-OH-PhIP glucuronide'

XV= '4 prime-OH-PhIP-N2-glucuronide'

XVI= 'N2-OH-PhIP-N2-glucuronide'
```

```
XVII= 'N2-OH-PhIP-N3-glucuronide'

col_DNA_adduct= 'DNA adduct analysis'

col_DNA_adduct_P= 'DNA adduct analysis proportion to basal'

Glucuronidated_p = 'All glucuronidated metabolites proportion to basal'

Sulfated_p = 'All sulfated metabolite sproportion to basal'

Methylated_p = 'All methylated metabolites proportion to basal'

Five_hydroxylated_p = 'All 5-OH metabolites proportion to basal'

N2_hydroxylated_p = 'All N2_OH metabolites proportion to basal'

I_p = 'I proportion to basal'

II_p = 'II proportion to basal'

III_p = 'III proportion to basal'

IV_p = 'IV proportion to basal'

V_p = 'V proportion to basal'

VI_p = 'VI proportion to basal'

VII_p = 'VII proportion to basal'

VIII_p = 'VIII proportion to basal'

IX_p = 'IX proportion to basal'

X_p = 'X proportion to basal'

XI_p = 'XI proportion to basal'

XII_p = 'XII proportion to basal'

XIII_p = 'XIII proportion to basal'

XIV_p = 'XIV proportion to basal'

XV_p = 'XV proportion to basal'

XVI_p = 'XVI proportion to basal'

XVII_p = 'XVII proportion to basal'

Prost_DNA_adduct = 'Prostate DNA adduct (per 10^7 nucleosides)'

Panc_DNA_adduct = 'Pancreas DNA adduct (per 10^7 nucleosides)'
```

```
Prost_DNA_adduct_p = 'Pancreas DNA adduct (per 10^ nucleosides)'
Panc_DNA_adduct_p = 'Pancreas DNA adduct (per 10^ nucleosides)'
;

DNA_adduct_t=sqrt(col_DNA_adduct);


if experiment = 1 and diet = 2 then basalPhIP = 1;
if experiment = 2 and diet = 2 then basalPhIP = 2;

if experiment = 1 and diet = 1 then basalnoPhIP = 1;
if experiment = 2 and diet = 1 then basalnoPhIP = 2;

if experiment = 1 then output veg_only;
if experiment = 2 then output pure_cmps;
output HFHL_PhIP_Study;
datalines;

1     2     1     1     83.90 20.80 0.82  0.34  0.11  6.55  0.95  10.55
      6.48  1.00  13.7323216   11.63 1.40  0.50  1.09  0.99  27.72 8.43
      9.36  18.15 0.91  3.32  15.73 7.02  0.00  0.00  3.64  1.00  0.21
      0.00  0.00  0.77  3.74  0.69  0.89  0.79  0.59  0.81  1.58  0.89
      0.76  1.11  1.18  1.37  0.71  1.66  0.00  1.00  1.09  1.93  0.56
      0.00  0.00  0.87  0.39  7.06  0.95  7.87  8.13  1.19  0.88

2     2     1     1     85.10 19.40 0.99  0.34  0.22  7.50  1.23  6.66
      5.56  1.00  18.28637531 10.88 0.99  0.64  0.88  1.04  14.18 9.09
      11.06 15.01 2.74  2.31  22.70 4.25  0.20  0.00  4.20  1.13  0.28
      0.00  0.22  1.40  11.23 1.29  1.39  0.96  1.03  2.10  0.81  0.96
      0.90  0.91  3.54  0.95  1.03  1.00  2.18  1.00  1.26  2.18  0.75
      0.00  2.09  1.57  1.19  8.46  1.14  6.99  11.90 1.06  1.29

3     2     1     1     90.20 20.70 1.14  0.36  0.15  7.09  1.28  6.57
      6.65  1.00  19.84298795 10.39 1.13  1.05  1.41  1.56  16.52 7.91
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	8.63	23.95	1.02	2.62	19.19	3.79	0.16	0.00	2.90	1.03	0.38
	0.14	0.29	1.15	10.31	1.95	1.21	0.80	2.12	1.23	0.94	0.84
	0.70	1.46	1.32	1.08	0.87	0.90	1.74	1.00	0.87	1.99	1.02
	4.67	2.75	1.29	1.09	11.11	1.50	7.62	14.65	1.15	1.59	
4	2	1	1	102.70		24.25	1.06	0.54	0.31	7.36	1.38
	7.91	6.97	1.00	20.41603208		7.91	0.78	0.99	1.62	0.87	21.90
	8.27	7.65	18.27	0.00	2.12	23.89	4.77	0.00	0.00	3.72	0.00
	0.00	0.00	0.00	0.54	8.87	0.38	0.71	0.86	0.47	0.51	1.25
	0.88	0.62	1.11	0.00	0.87	1.08	1.13	0.00	1.00	1.12	0.00
	0.00	0.00	0.00	0.61	0.94	10.26	1.39	7.09	10.39	1.07	1.13
5	3	1	1	86.20	24.10	1.00	0.59	0.24	6.37	1.14	11.34
	12.67	1.00	18.01404274		10.98	1.01	1.05	1.17	1.28	19.49	7.10
	9.09	15.15	0.00	2.55	17.40	7.94	0.40	0.32	3.27	0.89	0.39
	0.00	0.34	0.91	14.75	1.36	2.18	0.76	1.43	0.86	1.11	0.75
	0.74	0.92	0.00	1.05	0.79	1.88	4.36	0.32	0.98	1.72	1.05
	0.00	3.22	1.02	1.56	8.75	1.18	9.56	11.88	1.45	1.29	
6	3	1	1	99.90	25.10	0.87	0.39	0.20	6.77	1.25	14.22
	10.56	1.00	29.73717412		10.83	1.48	1.47	0.78	1.28	15.82	6.35
	6.66	16.76	0.00	2.48	12.72	6.84	0.33	0.16	3.74	1.11	0.50
	0.24	0.43	2.18	23.68	3.09	1.79	0.60	3.19	1.65	0.90	0.67
	0.54	1.02	0.00	1.02	0.58	1.62	3.59	0.16	1.12	2.14	1.34
	8.00	4.08	2.45	2.50	7.94	1.07	7.97	8.22	1.21	0.89	
7	4	1	1	92.60	23.25	1.05	0.35	0.21	6.44	1.23	8.08
	8.26	1.00	30.57892557		6.85	0.80	1.28	0.76	1.09	17.95	10.44
	9.60	15.28	0.34	2.55	25.61	3.41	0.00	0.00	3.54	1.07	0.20
	0.00	0.00	1.12	8.88	0.84	0.27	1.02	0.26	0.88	1.02	1.11
	0.78	0.93	0.44	1.05	1.16	0.81	0.00	0.00	1.06	2.06	0.54
	0.00	0.00	1.26	0.94	7.58	1.02	2.74	7.56	0.41	0.82	
8	5	1	1	83.80	23.20	0.97	0.56	0.23	6.74	1.09	10.84
	8.65	1.00	20.95646556		10.55	1.46	0.84	2.21	1.36	16.61	6.87
	6.92	15.13	0.00	2.37	17.36	9.56	0.35	0.18	3.98	1.35	0.60
	0.27	0.49	0.64	17.32	3.09	2.08	0.69	3.49	0.85	0.95	0.73
	0.56	0.92	0.00	0.98	0.79	2.26	3.81	0.18	1.20	2.61	1.61
	9.00	4.65	0.72	1.83	8.99	1.21	7.96	7.34	1.20	0.80	
9	1	2	1	92.50	21.00	1.02	0.46	0.23	7.13	1.21	14.60
	7.07	2.00	25.06246331		12.30	0.88	0.89	1.06	1.13	.	.
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10	1	2	1	78.90	18.25	1.07	0.49	0.19	6.02	0.99	9.38
	8.88	2.00	24.60134288		12.31	0.86	1.25	1.39	1.08	.	.

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11	1	2	1	91.50	19.05	0.94	0.35	0.20	6.65	1.18	7.88
	6.37	2.00	21.34384669		11.96	0.72	0.69	1.30	0.48	.	.
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12	1	2	1	86.70	19.95	0.99	0.31	0.21	7.12	1.06	6.86
	5.06	2.00	22.80551532		9.08	0.86	0.59	1.47	0.03	.	.
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13	3	2	1	76.80	19.95	0.94	0.27	0.18	5.76	0.99	17.19
	16.77	2.00	17.13533962		10.70	0.78	1.33	0.82	1.22	18.86	5.93
	8.73	19.80	0.37	2.15	13.80	5.39	0.21	0.00	3.03	1.12	0.56
	0.21	0.33	1.65	17.88	2.63	1.19	0.66	2.54	1.41	1.07	0.63
	0.71	1.21	0.48	0.89	0.62	1.27	2.29	0.00	0.91	2.16	1.50
	7.00	3.13	1.85	1.89	5.21	0.70	8.80	9.09	1.33	0.99	
14	3	2	1	99.80	26.85	0.92	0.28	0.16	7.47	1.11	11.57
	15.08	2.00	16.62474981		11.11	1.50	1.55	1.03	1.39	12.92	8.24
	11.60	17.67	0.59	2.93	14.86	5.09	0.34	0.33	2.61	0.78	0.43
	0.25	0.42	2.14	18.80	2.88	1.74	0.83	3.39	1.72	0.74	0.87
	0.95	1.08	0.76	1.21	0.67	1.20	3.70	0.33	0.78	1.51	1.15
	8.33	3.98	2.40	1.98	4.72	0.64	4.24	5.67	0.64	0.62	
15	4	2	1	92.60	21.90	1.01	0.30	0.14	6.77	1.22	8.18
	9.13	2.00	25.24394104		11.26	1.35	1.44	0.90	0.85	14.19	10.16
	9.45	12.58	1.31	2.40	27.73	4.03	0.15	0.00	3.55	0.97	0.07
	0.01	0.21	1.18	12.02	1.15	0.86	1.03	0.74	1.43	0.81	1.08
	0.77	0.77	1.69	0.99	1.25	0.95	1.63	0.00	1.07	1.87	0.19
	0.33	1.99	1.33	1.27	4.23	0.57	3.88	0.55	0.59	0.06	
16	5	2	1	75.20	25.85	0.89	0.27	0.22	5.68	1.00	14.75
	13.31	2.00	13.90565743		11.87	0.96	2.18	0.62	1.12	14.64	8.23
	13.27	15.81	0.13	2.69	16.53	5.87	0.23	0.00	2.35	0.83	0.31
	0.16	0.30	2.23	16.42	2.22	1.30	0.90	2.24	1.47	0.83	0.87
	1.08	0.96	0.17	1.11	0.75	1.39	2.50	0.00	0.71	1.60	0.83
	5.33	2.84	2.51	1.73	4.18	0.56	3.92	5.42	0.59	0.59	
17	1	3	1	121.20		20.65	1.09	0.34	0.26	7.05	1.13
	8.12	3.15	3.00	11.38907186		11.77	0.72	0.46	0.75	2.55	.

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18	1	3	1	98.00	19.05	0.99	0.34	0.23	6.98	1.13	6.54
	4.45	3.00	14.05985302		11.20	0.98	1.77	1.04	1.63	.	.
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19	2	3	1	110.30		20.65	1.10	0.37	0.27	7.17	1.01
	12.27	4.61	3.00	14.71334846		11.76	0.96	1.93	1.42	0.61	15.62
	9.29	11.92	13.89	0.12	1.81	27.06	3.91	0.26	0.00	3.29	1.06
	0.50	0.12	0.22	0.74	10.18	1.77	1.59	1.06	2.14	0.69	0.89
	0.99	0.97	0.85	0.16	0.75	1.22	0.92	2.83	1.00	0.99	2.05
	1.34	4.00	2.09	0.83	1.07	7.61	1.03	5.53	0.34	0.84	0.04
20	2	3	1	96.30	22.35	1.05	0.29	0.17	6.18	1.08	11.02
	4.96	3.00	10.31559623		12.27	1.35	2.30	0.99	1.49	14.51	7.94
	12.67	17.58	2.52	2.22	22.51	4.25	0.23	0.00	2.83	0.75	0.26
	0.00	0.22	1.15	10.35	1.07	1.50	0.96	1.11	1.88	0.83	0.84
	1.03	1.07	3.26	0.92	1.02	1.00	2.50	1.00	0.85	1.45	0.70
	0.00	2.09	1.29	1.09	9.97	1.35	7.90	14.24	1.20	1.55	
21	3	3	1	94.70	23.25	1.00	0.32	0.21	7.12	1.17	14.14
	8.40	3.00	15.70114864		11.49	1.11	1.99	1.08	1.46	15.09	8.16
	10.28	18.69	1.11	3.52	17.95	7.19	0.26	0.00	2.84	0.94	0.25
	0.00	0.35	1.01	12.35	1.30	1.51	0.84	1.07	1.29	0.86	0.87
	0.84	1.14	1.43	1.45	0.81	1.70	2.83	0.00	0.85	1.81	0.67
	0.00	3.32	1.13	1.30	6.64	0.90	5.63	10.67	0.85	1.16	
22	3	3	1	116.90		25.30	0.93	0.30	0.25	9.02	1.19
	10.12	5.65	3.00	12.41499346		10.35	1.46	0.82	0.64	1.52	11.06
	9.82	15.24	15.59	0.31	2.17	20.96	4.53	0.18	0.00	2.95	0.84
	0.36	0.15	0.29	1.24	14.31	2.02	1.01	1.08	1.96	1.10	0.63
	1.04	1.24	0.95	0.40	0.90	0.95	1.07	1.96	0.00	0.89	1.62
	0.97	5.00	2.75	1.39	1.51	4.80	0.65	1.91	5.07	0.29	0.55
23	4	3	1	101.30		21.40	0.87	0.32	0.20	7.16	1.09
	9.99	6.04	3.00	22.24948234		12.57	1.05	1.45	1.12	1.67	16.53
	12.52	13.56	9.66	0.00	2.01	25.63	5.08	0.00	0.00	4.28	1.26
	0.26	0.00	0.00	0.00	9.21	0.77	0.40	1.20	0.21	0.32	0.94
	1.33	1.11	0.59	0.00	0.83	1.16	1.20	0.00	0.00	1.29	2.43
	0.70	0.00	0.00	0.00	0.97	4.94	0.67	4.55	6.90	0.69	0.75
24	5	3	1	103.40		24.55	1.06	0.37	0.31	7.18	1.07
	13.56	10.44	3.00	18.3898655		11.32	1.31	1.77	0.55	1.98	19.22

	6.98	10.18	16.16	0.04	2.04	17.20	7.28	0.31	0.00	3.39	1.04
	0.43	0.12	0.29	0.99	14.33	1.94	1.70	0.78	2.05	0.89	1.09
	0.74	0.83	0.98	0.05	0.84	0.78	1.72	3.38	0.00	1.02	2.01
	1.15	4.00	2.75	1.11	1.51	7.30	0.99	5.22	10.27	0.79	1.12
25	1	4	1	107.10		21.80	1.08	0.43	0.26	7.06	1.40
	7.81	4.07	4.00	18.87360893		10.71	0.62	1.26	1.22	1.04	.
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26	4	4	1	112.40		22.40	1.00	0.43	0.21	7.30	1.29
	9.66	5.48	4.00	12.44771346		12.53	1.11	1.18	0.86	1.09	11.23
	12.11	10.91	12.55	0.00	1.89	29.01	2.74	0.00	0.00	4.43	1.24
	0.23	0.00	0.00	1.13	12.53	0.99	0.22	1.16	0.19	0.86	0.64
	1.28	0.89	0.76	0.00	0.78	1.31	0.65	0.00	0.00	1.33	2.39
	0.62	0.00	0.00	1.27	1.32	3.83	0.52	4.45	7.84	0.67	0.85
27	4	4	1	123.90		20.05	1.17	0.46	0.20	9.16	1.29
	8.37	5.92	4.00	17.14747293		12.19	0.80	1.25	0.94	1.36	19.67
	10.69	10.56	9.77	1.41	1.93	31.54	2.11	0.00	0.00	3.04	1.07
	0.00	0.00	0.00	0.78	7.42	0.66	0.17	1.14	0.20	1.16	1.12
	1.13	0.86	0.60	1.82	0.80	1.43	0.50	0.00	0.00	0.91	2.06
	0.00	0.00	0.00	0.88	0.78	6.85	0.93	5.34	8.95	0.81	0.97
28	4	4	1	120.00		20.00	1.07	0.35	0.30	7.80	1.09
	10.82	6.48	4.00	18.05825484		11.10	1.17	1.42	0.86	1.31	13.72
	9.95	15.27	12.69	0.59	1.93	27.35	2.95	0.14	0.00	2.29	0.69
	0.26	0.08	0.00	0.99	11.10	1.10	0.74	1.18	1.25	1.02	0.78
	1.06	1.25	0.77	0.76	0.80	1.24	0.70	1.52	0.00	0.69	1.33
	0.70	2.67	0.00	1.11	1.17	4.94	0.67	4.88	5.88	0.74	0.64
29	5	4	1	107.90		23.55	1.15	0.43	0.20	7.86	1.11
	11.84	6.72	4.00	14.29804896		12.48	1.15	1.03	0.46	1.04	18.31
	9.50	18.00	13.75	0.67	2.73	16.92	3.33	0.19	0.00	3.00	0.65
	0.18	0.00	0.00	1.24	11.54	0.75	0.95	1.08	0.80	1.16	1.04
	1.01	1.47	0.84	0.87	1.13	0.77	0.79	2.07	0.00	0.90	1.25
	0.48	0.00	0.00	1.39	1.22	2.89	0.39	3.14	4.37	0.47	0.48
30	5	4	1	111.20		20.90	0.98	0.41	0.25	7.76	1.34
	12.04	6.63	4.00	13.14025652		11.67	0.64	0.81	0.66	1.29	13.87
	10.98	12.28	16.36	0.15	2.35	20.03	3.70	0.15	0.00	3.44	0.97
	0.42	0.17	0.17	1.02	13.95	1.99	0.84	1.02	2.07	0.94	0.79
	1.17	1.00	1.00	0.19	0.97	0.91	0.87	1.63	0.00	1.03	1.87
	1.13	5.67	1.61	1.15	1.47	5.59	0.76	4.89	13.11	0.74	1.43
31	5	4	1	117.80		21.70	1.08	0.30	0.24	7.89	1.09
	11.08	6.61	4.00	14.53915934		8.48	0.84	1.09	0.82	1.42	13.43

	10.29	12.01	17.02	0.54	3.05	19.55	4.33	0.00	0.00	3.12	1.44
	0.65	0.00	0.00	1.23	13.33	1.18	0.34	0.99	0.31	1.16	0.76
	1.09	0.98	1.04	0.70	1.26	0.88	1.02	0.00	0.00	0.94	2.78
	1.74	0.00	0.00	1.38	1.41	5.56	0.75	4.70	6.28	0.71	0.68
32	1	5	1	90.10	18.15	0.96	0.29	0.16	6.75	1.08	8.11
	5.45	4.00	15.53454967		11.39	0.86	0.69	0.33	0.17	.	.
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33	1	5	1	103.80		19.50	1.09	0.31	0.21	7.70	1.29
	6.03	4.01	5.00	18.19247844		10.82	0.92	0.23	0.76	1.45	.
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34	2	5	1	86.40	19.35	0.94	0.25	0.14	7.20	1.14	11.58
	6.97	5.00	17.61130071		8.62	0.96	0.75	0.87	1.19	15.22	10.18
	11.89	16.41	1.20	2.39	22.71	4.17	0.16	0.00	2.75	0.73	0.28
	0.07	0.21	0.95	10.68	1.36	1.24	1.03	1.52	1.25	0.87	1.08
	0.97	1.00	1.55	0.99	1.03	0.98	1.74	1.00	0.83	1.41	0.75
	2.33	1.99	1.07	1.13	5.73	0.77	6.94	11.63	1.05	1.27	
35	2	5	1	77.80	17.45	0.95	0.34	0.21	6.33	0.88	9.55
	6.22	5.00	16.76645752		10.08	1.29	0.86	0.72	0.82	15.23	9.99
	14.26	15.18	0.00	2.82	19.17	4.24	0.00	0.00	3.27	0.00	1.21
	0.00	0.00	1.09	13.55	0.98	0.67	1.03	0.54	0.88	0.87	1.06
	1.16	0.92	0.00	1.16	0.87	1.00	0.00	1.00	0.98	0.00	3.25
	0.00	0.00	1.22	1.43	5.13	0.69	5.28	7.49	0.80	0.82	
36	3	5	1	85.10	24.50	0.98	0.28	0.17	6.73	1.15	18.38
	8.03	5.00	23.29977329		12.42	0.80	0.42	0.80	1.37	11.39	9.89
	12.87	18.16	0.71	2.68	19.73	4.48	0.00	0.00	3.36	1.18	0.40
	0.00	0.22	1.51	13.41	1.37	0.35	1.00	0.28	1.34	0.65	1.05
	1.05	1.11	0.92	1.11	0.89	1.06	0.00	0.00	1.01	2.28	1.07
	0.00	2.09	1.70	1.42	3.37	0.45	5.47	6.10	0.83	0.66	
37	3	5	1	89.60	21.15	0.99	0.36	0.15	7.20	1.22	13.15
	9.76	5.00	20.61237862		10.45	0.94	1.11	0.58	1.56	17.71	8.51
	12.32	12.53	0.00	3.05	15.40	3.38	0.00	0.00	3.87	0.94	0.62
	0.00	0.00	1.79	19.88	1.25	0.27	0.87	0.31	1.37	1.01	0.90
	1.01	0.76	0.00	1.26	0.70	0.80	0.00	0.00	1.16	1.81	1.66
	0.00	0.00	2.01	2.10	5.47	0.74	7.58	6.81	1.15	0.74	
38	4	5	1	87.00	20.75	1.13	0.29	0.14	6.72	1.09	14.98
	9.08	5.00	19.83116282		7.62	1.29	1.26	1.30	1.26	14.21	10.54

	15.99	11.55	0.00	1.99	24.97	3.25	0.00	0.00	3.80	1.19	0.30
	0.00	0.00	1.29	10.89	0.98	0.26	1.18	0.21	0.87	0.81	1.12
	1.30	0.70	0.00	0.82	1.13	0.77	0.00	0.00	1.14	2.30	0.80
	0.00	0.00	1.45	1.15	5.42	0.73	1.87	8.00	0.28	0.87	
39	5	5	1	77.70	23.85	1.05	0.20	0.21	6.19	1.06	11.99
	11.50	5.00	17.82440987		12.81	1.11	1.24	1.74	0.91	23.49	8.42
	8.03	17.98	0.00	3.35	16.45	3.82	0.00	0.00	5.62	1.34	0.54
	0.00	0.00	0.00	10.97	0.98	0.30	0.76	0.35	0.39	1.34	0.89
	0.66	1.10	0.00	1.38	0.74	0.90	0.00	0.00	1.69	2.59	1.45
	0.00	0.00	0.00	1.16	6.28	0.85	8.04	6.91	1.22	0.75	
40	1	6	1	95.90	18.35	1.06	0.03	0.17	6.85	1.06	8.52
	10.56	5.00	24.68423311		13.31	0.60	1.55	0.16	0.56	.	.
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41	1	6	1	100.30		18.05	0.09	0.31	0.13	6.70	1.04
	6.36	4.35	6.00	22.51421979		13.04	0.64	0.30	0.24	0.96	.
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42	2	6	1	91.70	17.65	0.96	0.50	0.30	7.00	1.12	9.61
	6.65	6.00	19.9983649		12.30	0.90	0.52	0.65	0.62	20.07	10.71
	15.44	12.42	0.00	1.67	24.31	3.55	0.00	0.00	3.23	0.00	0.21
	0.00	0.00	1.03	7.34	0.50	0.61	1.17	0.42	0.64	1.14	1.14
	1.26	0.76	0.00	0.69	1.10	0.84	0.00	1.00	0.97	0.00	0.56
	0.00	0.00	1.16	0.77	6.72	0.91	6.64	8.38	1.00	0.91	
43	2	6	1	99.20	20.05	1.01	0.34	0.20	7.10	1.02	6.65
	5.61	6.00	19.97859624		12.43	0.64	0.54	0.74	0.93	15.64	11.50
	16.57	15.21	0.00	2.76	19.00	3.66	0.00	0.00	4.02	0.00	0.45
	0.00	0.00	0.00	11.18	0.51	0.62	1.14	0.53	0.39	0.89	1.22
	1.35	0.93	0.00	1.14	0.86	0.86	0.00	1.00	1.21	0.00	1.21
	0.00	0.00	0.00	1.18	4.85	0.66	5.37	6.43	0.81	0.70	
44	3	6	1	86.60	23.15	1.02	0.39	0.24	6.79	1.12	10.93
	8.76	6.00	25.4883268		13.28	0.78	0.67	0.37	1.67	14.45	8.64
	12.08	15.53	0.46	2.51	16.94	3.07	0.06	0.00	2.93	0.76	0.51
	0.14	0.00	1.86	20.07	1.80	0.46	0.89	1.59	1.60	0.82	0.92
	0.99	0.95	0.59	1.04	0.77	0.72	0.65	0.00	0.88	1.47	1.37
	4.67	0.00	2.09	2.12	4.69	0.63	5.00	8.92	0.76	0.97	
45	3	6	1	94.10	23.00	0.98	0.38	0.16	7.24	1.03	10.70
	8.40	6.00	30.95127498		12.71	0.72	0.93	0.40	2.11	13.69	8.30

	11.83	13.51	1.24	2.25	16.34	3.17	0.14	0.00	3.90	1.22	0.35
	0.14	0.18	2.36	21.39	2.25	0.76	0.86	1.78	2.17	0.78	0.88
	0.97	0.82	1.60	0.93	0.74	0.75	1.52	0.00	1.17	2.35	0.94
	4.67	1.71	2.65	2.26	6.15	0.83	5.73	10.30	0.87	1.12	
46	4	6	1	95.70	24.65	1.15	0.50	0.21	7.44	1.07	13.40
	12.88	6.00	25.96	102066	12.75	0.74	1.59	0.80	1.07	14.23	9.42
	18.42	10.20	0.38	1.75	25.84	2.60	0.00	0.00	2.90	1.03	0.15
	0.05	0.00	1.22	11.81	1.08	0.20	1.22	0.60	1.04	0.81	1.00
	1.50	0.62	0.49	0.72	1.17	0.61	0.00	0.00	0.87	1.99	0.40
	1.67	0.00	1.37	1.25	4.90	0.66	5.14	10.71	0.78	1.17	
47	5	6	1	88.20	21.00	1.17	0.32	0.16	6.98	1.09	.
	.		6.00	20.86204989		13.05	1.21	1.25	0.29	1.43	18.38
	7.12	9.87	9.94	1.10	2.42	15.39	5.51	0.35	0.27	3.75	1.06
	0.35	0.20	0.25	2.18	21.85	2.56	1.79	0.75	2.94	2.06	1.05
	0.76	0.81	0.61	1.42	1.00	0.70	1.30	3.81	0.27	1.13	2.05
	0.94	6.67	2.37	2.45	2.31	6.02	0.81	5.80	8.61	0.88	0.94
48	2	7	1	111.40		20.40	1.13	0.35	0.12	7.95	1.34
	7.49	7.42	6.00	26.20358584		12.40	0.60	0.92	0.60	0.89	16.54
	10.36	15.38	14.45	0.00	2.63	26.83	2.97	0.00	0.00	2.75	0.00
	0.32	0.00	0.00	0.97	6.80	0.50	0.57	1.19	0.52	0.60	0.94
	1.10	1.25	0.88	0.00	1.08	1.21	0.70	0.00	1.00	0.83	0.00
	0.86	0.00	0.00	1.09	0.72	4.50	0.61	5.50	7.51	0.83	0.82
49	4	7	1	104.90		20.50	1.02	0.33	0.17	7.15	1.23
	8.71	9.42	7.00	18.6172415		12.79	0.57	0.94	0.47	1.19	17.69
	11.41	13.97	14.92	0.00	1.53	25.69	2.56	0.14	0.00	3.25	0.00
	0.31	0.00	0.00	0.59	7.93	0.47	0.71	1.17	0.54	0.50	1.01
	1.21	1.14	0.91	0.00	0.63	1.16	0.60	1.52	0.00	0.98	0.00
	0.83	0.00	0.00	0.66	0.84	2.42	0.33	3.87	5.58	0.59	0.61
50	4	7	1	114.60		20.20	1.10	0.31	0.19	7.57	1.12
	11.12	9.48	7.00	18.31735023		11.19	0.60	1.19	0.76	1.28	19.56
	10.68	15.36	12.12	0.94	2.11	25.29	2.30	0.00	0.00	3.04	1.00
	0.17	0.00	0.00	0.97	6.48	0.72	0.18	1.18	0.22	1.00	1.11
	1.13	1.25	0.74	1.22	0.87	1.14	0.54	0.00	0.00	0.91	1.93
	0.46	0.00	0.00	1.09	0.68	3.64	0.49	4.78	5.55	0.72	0.60
51	5	7	1	116.80		21.05	1.00	0.31	0.22	7.67	1.10
	10.27	10.44	7.00	23.65973145		12.92	0.72	0.98	0.44	0.51	18.89
	7.46	11.21	18.83	0.46	2.44	16.52	3.90	0.12	0.00	3.37	1.04
	0.45	0.10	0.23	1.11	13.88	1.78	0.74	0.82	1.41	1.10	1.08
	0.79	0.91	1.15	0.59	1.01	0.75	0.92	1.31	0.00	1.01	2.01
	1.21	3.33	2.18	1.25	1.46	7.61	1.03	10.23	12.02	1.55	1.31
52	5	7	1	111.10		16.65	0.92	0.33	0.36	7.55	1.16
	11.22	11.13	7.00	17.76665445		12.69	0.84	1.23	0.56	0.28	14.14

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      11.27 13.76 12.84 1.04  2.54  23.16 3.47  0.14  0.00  2.71  0.75
      0.25  0.06  0.12  1.49  12.27 1.29  0.78  1.12  1.14  1.44  0.81
      1.20  1.12  0.78  1.34  1.05  1.05  0.82  1.52  0.00  0.81  1.45
      0.67  2.00  1.14  1.67  1.29  3.38  0.46  3.86  6.85  0.58  0.75

53    1     8     2     95.90 18.65 1.00  0.38  0.22  7.52  1.05  4.93
      3.24  1.00  25.55829698 9.18  0.31  0.46  0.52  0.34  .     .
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54    1     8     2     78.70 16.00 0.93  0.27  0.19  6.82  1.06  8.14
      4.31  1.00  23.90471727 12.32 0.96  0.74  0.69  0.63  .     .
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55    2     8     2     85.30 15.55 0.99  0.32  0.20  7.12  1.16  6.85
      4.01  1.00  26.63873791 10.53 0.63  0.94  0.55  0.39  19.73 8.91
      10.72 15.72 0.36  2.39  15.71 6.64  0.20  0.00  3.52  1.25  0.88
      0.00  0.29  1.00  12.67 1.71  0.95  0.80  0.99  1.28  1.06  0.83
      0.80  1.21  1.05  1.09  0.75  1.01  1.85  0.00  1.04  1.27  2.62
      1.00  3.26  1.34  1.46  5.74  1.43  9.39  6.18  1.59  2.34

56    2     8     2     90.50 18.50 1.03  0.25  0.19  6.62  1.22  7.22
      3.21  1.00  20.43312442 13.05 1.10  1.15  0.29  0.66  18.80 8.67
      9.49  18.50 0.48  2.78  14.84 9.58  0.23  0.09  3.21  1.05  0.37
      0.00  0.27  0.99  10.65 1.39  4.86  0.74  3.85  1.32  1.01  0.81
      0.71  1.42  1.40  1.27  0.71  1.45  2.13  11.00 0.94  1.07  1.10
      1.00  3.03  1.33  1.22  5.21  1.30  8.49  6.66  1.44  2.52

57    6     8     2     91.10 17.45 0.96  0.22  0.18  8.12  1.17  17.00
      6.02  1.00  27.1032187 12.94 0.63  0.96  1.20  1.31  28.11 4.51
      8.87  15.23 0.00  3.63  6.40  13.72 0.00  0.54  3.50  0.00  0.60
      0.00  0.00  0.00  14.89 0.65  22.69 0.46  16.92 0.57  1.51  0.42
      0.66  1.17  0.00  1.66  0.31  2.08  0.00  66.00 1.03  0.00  1.79
      0.00  0.00  0.00  1.71  2.96  0.74  4.47  3.21  0.76  1.21

58    6     8     2     94.50 19.30 0.97  0.35  0.15  7.92  1.06  53.10
      17.59 7.00  22.85534092 13.45 0.78  0.76  1.33  1.38  20.10 5.67
      7.34  20.40 2.14  4.05  11.11 11.30 0.00  1.15  0.00  0.00  0.54
      0.00  0.00  2.29  13.91 0.90  47.42 0.54  35.60 3.64  1.08  0.53
      0.55  1.57  6.26  1.85  0.53  1.71  0.00  140.56      0.00  0.00
      1.61  0.00  0.00  3.07  1.60  1.56  0.39  3.42  0.60  0.58  0.23

59    7     8     2     86.60 15.25 0.91  0.28  0.17  6.33  1.04  8.68
      4.21  1.00  27.22666431 12.85 0.90  1.22  1.02  1.21  30.41 9.28
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	11.92	11.07	0.00	2.20	12.87	10.27	0.21	0.11	4.45	1.27	1.02
	0.00	0.23	0.00	4.68	1.25	5.65	0.79	4.10	0.18	1.64	0.86
	0.89	0.85	0.00	1.01	0.62	1.56	1.94	13.44	1.31	1.29	3.04
	0.00	2.58	0.00	0.54	5.00	1.25	8.17	0.00	1.38	0.00	
60	8	8	2	80.60	17.85	1.07	0.25	0.19	6.69	1.00	49.22
	5.86	1.00	26.62944966		10.20	0.82	0.94	0.79	0.80	32.00	6.73
	8.57	17.55	0.00	5.04	11.20	11.81	0.00	0.50	3.27	0.00	0.00
	0.00	0.00	0.00	3.32	0.19	20.97	0.60	15.85	0.13	1.72	0.63
	0.64	1.35	0.00	2.31	0.54	1.79	0.00	61.11	0.96	0.00	0.00
	0.00	0.00	0.00	0.38	4.38	1.09	8.13	4.73	1.38	1.79	
61	1	9	2	90.70	18.30	0.86	0.34	0.15	6.93	0.99	8.60
	4.04	2.00	20.90643119		13.21	0.76	1.31	0.75	0.74	.	.
	.	.	.	.	.	.	.	.	.	.	.
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62	1	9	2	95.20	16.90	0.99	0.35	0.16	7.70	1.07	8.53
	2.92	2.00	21.00985842		10.51	0.47	1.02	1.21	1.55	.	.
	.	.	.	.	.	.	.	.	.	.	.
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63	2	9	2	96.10	18.75	1.09	0.24	0.18	8.20	1.19	6.82
	3.45	2.00	27.21612492		11.32	0.61	0.97	1.06	1.28	18.96	9.89
	13.62	11.80	0.33	2.33	19.89	7.89	0.21	0.00	2.66	0.77	0.34
	0.00	0.00	1.28	10.02	0.92	1.05	0.97	1.00	1.28	1.02	0.92
	1.02	0.91	0.97	1.07	0.96	1.20	1.94	0.00	0.78	0.78	1.01
	1.00	0.00	1.72	1.15	3.69	0.92	6.54	3.08	1.11	1.16	
64	2	9	2	69.10	15.10	0.87	0.30	0.17	5.87	0.92	6.34
	3.58	2.00	28.80395478		13.05	0.88	0.96	1.39	1.13	31.26	10.42
	10.78	11.74	0.00	2.89	20.80	7.29	0.00	0.00	4.82	0.00	0.00
	0.00	0.00	0.00	0.00	0.35	0.37	0.93	0.58	0.00	1.68	0.97
	0.81	0.90	0.00	1.32	1.00	1.10	0.00	0.00	1.42	0.00	0.00
	1.00	0.00	0.00	0.00	3.60	0.90	6.21	0.00	1.05	0.00	
65	6	9	2	82.30	15.45	0.91	0.28	0.12	6.60	0.97	56.44
	14.87	7.00	26.04440312		12.72	0.76	0.77	1.13	1.40	20.11	6.63
	8.68	20.48	1.81	3.52	8.09	10.73	0.30	0.66	2.27	0.00	0.52
	0.00	0.36	2.21	13.64	1.54	28.36	0.55	21.26	3.28	1.08	0.62
	0.65	1.58	5.30	1.61	0.39	1.63	2.77	80.67	0.67	0.00	1.55
	0.00	4.04	2.96	1.57	1.93	0.48	4.07	2.16	0.69	0.82	
66	6	9	2	96.40	16.20	0.93	0.27	0.19	8.40	1.07	59.45
	6.03	2.00	28.74267353		12.52	0.76	0.94	1.07	1.31	21.82	6.66

	10.34	15.09	0.00	3.18	8.50	13.27	0.00	0.18	2.99	0.74	1.04
	0.00	0.33	1.78	14.09	1.78	8.00	0.60	5.86	1.34	1.17	0.62
	0.77	1.16	0.00	1.46	0.41	2.01	0.00	22.00	0.88	0.75	3.10
	0.00	3.70	2.39	1.62	2.59	0.64	4.02	0.55	0.68	0.21	
67	7	9	2	96.60	16.65	1.20	0.21	0.18	8.30	1.02	7.70
	3.99	2.00	20.960114		13.03	0.53	0.75	1.27	0.91	18.99	11.43
	13.21	11.50	0.72	2.43	21.29	7.08	0.21	0.00	3.46	0.88	0.26
	0.00	0.11	0.78	7.64	0.84	1.00	1.03	0.76	1.34	1.02	1.06
	0.99	0.89	2.11	1.11	1.02	1.07	1.94	0.00	1.02	0.90	0.78
	0.00	1.23	1.05	0.88	5.45	1.36	5.50	4.01	0.93	1.52	
68	8	9	2	92.90	18.15	0.83	0.24	0.11	7.02	1.05	38.12
	21.37	8.00	27.45521556		12.42	0.61	0.80	0.88	0.97	33.01	5.43
	5.94	23.10	0.00	3.65	14.15	6.32	0.57	0.40	3.10	0.70	0.00
	0.00	0.00	0.00	3.62	0.29	18.37	0.54	13.96	0.14	1.78	0.51
	0.44	1.78	0.00	1.67	0.68	0.96	5.27	48.89	0.91	0.71	0.00
	0.00	0.00	0.00	0.42	2.52	0.63	2.66	0.00	0.45	0.00	
69	1	10	2	100.70		18.60	0.92	0.30	0.23	7.06	1.17
	6.57	4.42	2.00	21.06945297		12.54	0.98	0.69	1.64	0.84	.
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70	1	10	2	113.00		19.00	1.10	0.24	0.19	7.36	1.00
	7.50	3.63	2.00	24.87786237		13.09	0.92	0.95	1.23	0.85	.
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71	2	10	2	108.00		18.20	1.01	0.27	0.20	6.87	1.13
	5.87	3.39	2.00	29.5036804		8.35	0.53	0.70	0.83	0.88	15.99
	10.73	19.04	9.38	0.00	1.66	17.68	8.37	0.23	0.00	2.91	0.87
	0.27	0.00	0.17	0.83	11.86	1.13	1.13	1.09	0.97	0.83	0.86
	1.00	1.43	0.72	0.00	0.76	0.85	1.27	2.13	0.00	0.86	0.89
	0.80	1.00	1.91	1.11	1.36	4.68	1.17	7.81	1.19	1.32	0.45
72	2	10	2	101.00		18.35	0.87	0.21	0.19	7.31	0.97
	10.57	6.05	7.00	22.82054803		12.07	1.08	1.06	1.69	1.66	20.02
	9.98	10.76	15.51	0.00	2.80	25.17	4.94	0.00	0.00	2.83	0.92
	0.00	0.00	0.00	0.75	6.31	0.64	0.25	0.98	0.57	0.58	1.08
	0.93	0.81	1.19	0.00	1.28	1.21	0.75	0.00	0.00	0.83	0.94
	0.00	1.00	0.00	1.01	0.72	4.34	1.08	5.32	1.41	0.90	0.53
73	6	10	2	107.30		18.40	0.86	0.13	0.25	8.10	1.19
	65.87	16.68	7.00	27.66150005		12.64	1.10	0.77	1.15	1.26	19.43

	6.21	11.43	18.57	0.00	3.19	8.21	12.89	0.00	0.44	2.80	0.00
	0.68	0.00	0.00	0.00	16.15	0.67	18.58	0.61	13.81	0.62	1.05
	0.58	0.86	1.43	0.00	1.46	0.39	1.95	0.00	53.78	0.82	0.00
	2.03	0.00	0.00	0.00	1.86	2.21	0.55	3.52	1.34	0.60	0.51
74	6	10	2	93.90	16.05	0.95	0.29	0.23	7.90	1.18	85.89
	18.84	8.00	18.27303248		12.55	1.08	1.06	0.87	0.94	22.43	5.58
	8.73	19.16	0.28	3.85	11.73	7.79	0.30	3.69	1.66	0.00	0.51
	0.00	1.49	0.00	12.80	2.89	151.65		0.58	113.88		0.76
	1.21	0.52	0.65	1.48	0.82	1.76	0.56	1.18	2.77	451.00	
	0.49	0.00	1.52	0.00	16.72	0.00	1.47	2.68	0.67	2.79	2.82
	0.47	1.06									
75	7	10	2	116.80		17.35	1.04	0.25	0.13	7.60	1.05
	11.12	7.25	3.00	23.42825912		10.46	1.25	0.75	2.86	0.78	15.82
	11.60	13.99	13.95	0.54	1.33	28.09	3.62	0.13	0.00	1.93	0.00
	0.33	0.00	0.00	0.89	7.78	0.52	0.58	1.16	0.45	1.22	0.85
	1.08	1.05	1.07	1.58	0.61	1.35	0.55	1.20	0.00	0.57	0.00
	0.98	0.00	0.00	1.19	0.89	3.06	0.76	5.05	1.13	0.85	0.43
76	8	10	2	99.60	16.85	0.87	0.27	0.21	6.78	1.09	29.75
	10.43	8.00	19.89395349		12.52	1.23	0.92	1.07	1.30	24.45	7.26
	10.19	22.43	0.00	4.27	13.56	4.88	0.00	0.00	3.85	0.00	0.00
	0.00	0.00	0.00	9.12	0.31	0.25	0.70	0.49	0.35	1.32	0.68
	0.76	1.73	0.00	1.95	0.65	0.74	0.00	0.00	1.13	0.00	0.00
	0.00	0.00	0.00	1.05	3.02	0.75	4.25	0.00	0.72	0.00	
77	7	11	2	111.30		15.75	0.83	0.42	0.20	7.45	1.10
	8.21	5.81	3.00	16.86987149		12.29	1.98	0.84	0.75	0.74	18.38
	10.11	9.85	16.52	0.91	1.28	28.76	3.36	0.12	0.00	2.27	0.61
	0.22	0.00	0.14	0.77	6.71	0.76	0.54	1.02	0.42	1.49	0.99
	0.94	0.74	1.27	2.66	0.59	1.38	0.51	1.11	0.00	0.67	0.62
	0.66	0.00	1.57	1.03	0.77	3.70	0.92	4.64	5.34	0.79	2.02
78	7	11	2	105.70		17.55	0.96	0.32	0.27	7.49	1.12
	5.89	5.34	3.00	20.33548798		12.79	0.67	0.88	0.69	0.64	19.32
	10.58	16.68	11.15	0.70	1.96	20.38	5.51	0.21	0.00	2.13	0.60
	0.28	0.00	0.17	0.89	9.44	0.89	0.93	1.07	0.71	1.44	1.04
	0.98	1.25	0.86	2.05	0.90	0.98	0.83	1.94	0.00	0.63	0.61
	0.83	0.00	1.91	1.19	1.08	4.18	1.04	5.56	0.16	0.94	0.06
79	7	11	2	111.10		17.50	0.99	0.15	0.23	8.24	1.02
	6.33	4.21	3.00	13.55896754		13.33	0.82	0.79	0.76	0.76	15.66
	12.18	15.30	10.78	1.62	1.64	26.65	3.55	0.05	0.00	3.07	0.74
	0.25	0.00	0.00	1.07	7.43	0.67	0.33	1.19	0.30	2.34	0.84
	1.13	1.15	0.83	4.74	0.75	1.28	0.54	0.46	0.00	0.90	0.75
	0.75	0.00	0.00	1.44	0.85	3.74	0.93	3.41	1.69	0.58	0.64

80	8	11	2	90.20	16.95	1.07	0.33	0.20	6.68	0.97	32.14
	12.11	3.00	20.8360918		12.88	1.12	1.37	1.05	1.27	11.59	6.90
	9.51	16.19	2.37	3.71	10.99	10.60	0.35	0.21	1.91	0.59	0.62
	0.00	0.00	2.29	22.15	1.23	10.17	0.63	7.65	4.18	0.62	0.64
	0.71	1.25	6.93	1.70	0.53	1.61	3.24	25.67	0.56	0.60	1.85
	0.00	0.00	3.07	2.54	2.56	0.64	3.33	2.52	0.56	0.95	
81	8	11	2	115.40		16.85	1.22	0.34	0.20	7.41	1.20
	46.95	14.69	4.00	25.76455164		12.72	0.88	1.26	1.60	0.77	30.19
	4.94	5.80	23.10	0.00	4.40	10.74	9.51	0.19	0.00	4.17	0.72
	0.51	0.00	0.00	0.34	5.39	0.65	1.07	0.47	0.94	0.36	1.62
	0.46	0.43	1.78	0.00	2.01	0.52	1.44	1.76	0.00	1.23	0.73
	1.52	0.00	0.00	0.46	0.62	2.51	0.63	3.03	1.98	0.51	0.75
82	8	11	2	106.70		17.40	1.08	0.31	0.16	7.81	1.12
	14.79	13.43	4.00	17.33590623		12.57	0.86	1.52	1.19	1.29	43.31
	7.28	11.77	21.49	0.00	4.34	0.00	11.80	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.60	0.52	0.50	0.00	2.33
	0.68	0.88	1.65	0.00	1.99	0.00	1.79	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	3.09	0.77	3.30	0.00	0.56	0.00
83	1	11	2	107.40		16.70	1.15	0.25	0.15	6.86	1.16
	5.30	4.26	4.00	12.69516968		12.36	0.59	0.93	0.50	0.30	.
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84	2	11	2	100.70		18.40	1.17	0.25	0.20	7.87	1.21
	10.76	4.13	4.00	20.43807849		12.98	1.29	1.13	1.69	0.92	12.90
	12.33	17.98	10.82	1.25	1.48	27.78	3.47	0.13	0.00	2.78	0.80
	0.14	0.00	0.00	0.73	7.41	0.70	0.58	1.28	0.72	1.83	0.69
	1.15	1.35	0.83	3.66	0.68	1.33	0.53	1.20	0.00	0.82	0.81
	0.42	1.00	0.00	0.98	0.85	3.74	0.93	4.23	0.00	0.71	0.00
85	1	12	2	89.80	18.45	1.05	0.18	0.11	6.36	0.97	5.80
	3.59	4.00	19.30915879		11.96	0.98	1.45	1.11	1.37	.	.
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86	1	12	2	100.10		22.10	1.03	0.13	0.16	7.08	1.05
	5.64	4.88	4.00	21.7052629		12.06	1.10	0.56	1.59	2.21	.
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87	2	12	2	89.50	20.20	1.09	0.23	0.14	7.15	1.00	4.34
	2.88	4.00	18.12835565		11.31	1.08	1.11	0.44	1.16	21.98	10.26
	11.96	13.22	0.00	1.59	19.93	7.09	0.00	0.00	4.37	1.43	0.43
	0.00	0.00	0.31	7.42	0.90	0.36	0.94	0.43	0.42	1.18	0.95
	0.90	1.02	0.00	0.73	0.96	1.07	0.00	0.00	1.29	1.46	1.28
	1.00	0.00	0.42	0.85	4.14	1.03	4.09	4.27	0.69	1.61	
88	2	12	2	95.20	20.30	1.07	0.23	0.18	7.90	1.15	7.29
	5.33	4.00	16.06890241		9.28	1.72	1.28	1.07	0.56	17.02	11.49
	13.83	13.95	0.00	2.08	20.72	5.77	0.00	0.00	3.98	1.68	0.56
	0.00	0.00	0.00	8.93	0.94	0.29	1.03	0.49	0.34	0.92	1.07
	1.04	1.07	0.00	0.95	1.00	0.87	0.00	0.00	1.17	1.71	1.67
	1.00	0.00	0.00	1.03	3.31	0.82	5.15	4.01	0.87	1.52	
89	6	12	2	96.80	20.75	1.01	0.28	0.18	7.12	1.28	77.29
	17.35	8.00	21.83412816		12.36	1.37	1.03	2.07	0.90	27.89	6.90
	10.64	17.75	1.18	4.11	8.21	10.80	0.00	0.32	2.75	0.68	0.39
	0.00	0.40	1.54	6.44	1.42	13.58	0.61	10.25	2.09	1.50	0.64
	0.80	1.37	3.45	1.88	0.39	1.64	0.00	39.11	0.81	0.69	1.16
	0.00	4.49	2.07	0.74	2.11	0.53	3.35	0.30	0.57	0.11	
90	6	12	2	95.40	19.60	1.00	0.30	0.14	8.19	1.20	64.05
	23.15	8.00	16.61477044		9.47	1.41	0.71	1.77	1.79	25.31	6.81
	11.98	14.45	0.00	2.37	10.30	9.98	0.00	0.30	2.98	0.00	0.44
	0.00	0.43	0.93	13.70	1.41	12.73	0.68	9.44	0.94	1.36	0.63
	0.90	1.11	0.00	1.08	0.49	1.51	0.00	36.67	0.88	0.00	1.31
	0.00	4.83	1.25	1.57	2.24	0.56	2.54	4.49	0.43	1.70	
91	7	12	2	91.10	18.85	0.94	0.22	0.16	6.59	1.01	9.33
	6.34	4.00	18.77412673		10.14	1.29	0.83	1.10	1.36	25.65	10.78
	15.33	10.99	0.00	1.51	17.69	6.76	0.00	0.00	4.46	1.18	0.41
	0.00	0.00	0.00	5.25	0.62	0.34	1.00	0.17	0.20	1.38	1.00
	1.15	0.85	0.00	0.69	0.85	1.02	0.00	0.00	1.31	1.20	1.22
	0.00	0.00	0.00	0.60	2.63	0.66	3.63	4.03	0.61	1.52	
92	8	12	2	96.10	22.50	1.06	0.21	0.16	7.21	1.05	33.89
	7.80	4.00	25.89364389		12.75	0.92	0.68	0.34	0.54	22.87	5.90
	7.94	22.70	0.00	4.37	13.02	5.22	0.00	0.12	2.39	0.49	0.00
	0.00	0.00	1.80	13.20	0.73	5.15	0.59	4.17	1.31	1.23	0.55
	0.59	1.75	0.00	2.00	0.63	0.79	0.00	14.67	0.70	0.50	0.00
	0.00	0.00	2.41	1.52	2.06	0.51	2.80	3.13	0.47	1.18	
93	1	13	2	111.50		21.10	0.96	0.24	0.17	7.11	1.19
	8.82	6.65	5.00	20.54937919		12.58	0.98	0.82	0.57	0.43	.
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94	1	13	2	95.00	19.40	1.18	0.29	0.15	7.55	0.96	7.07
	6.23	5.00	17.43178804		12.08	0.78	0.94	1.46	1.26	.	.
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95	2	13	2	115.30		23.15	0.99	0.27	0.19	7.81	1.10
	7.76	5.20	5.00	25.60582946		10.48	1.04	0.66	0.64	0.55	13.13
	11.56	14.43	12.44	0.61	2.35	21.70	6.50	0.19	0.00	2.59	0.80
	0.32	0.00	0.25	1.39	11.75	1.36	0.91	1.07	0.96	1.67	0.71
	1.08	1.08	0.96	1.78	1.08	1.04	0.98	1.76	0.00	0.76	0.81
	0.95	1.00	2.81	1.86	1.35	2.72	0.68	2.81	2.88	0.48	1.09
96	2	13	2	108.70		20.10	0.97	0.29	0.21	7.94	1.16
	7.46	4.62	5.00	26.41064747		10.72	1.02	1.04	1.36	1.81	14.73
	13.94	14.32	9.76	0.73	1.69	24.76	5.05	0.00	0.00	3.73	1.24
	0.38	0.00	0.00	0.92	8.73	0.96	0.26	1.19	0.44	1.46	0.79
	1.30	1.07	0.75	2.14	0.77	1.19	0.77	0.00	0.00	1.10	1.26
	1.13	1.00	0.00	1.23	1.00	2.96	0.74	4.99	0.00	0.84	0.00
97	6	13	2	103.80		18.45	0.94	0.20	0.14	9.92	1.10
	57.49	29.35	8.00	21.05752014		12.80	0.82	0.47	1.13	1.41	10.76
	5.78	8.69	19.73	0.00	3.87	7.84	10.61	0.00	0.44	0.00	1.24
	0.58	0.00	0.00	3.22	27.25	1.49	18.46	0.52	13.89	2.48	0.58
	0.54	0.65	1.52	0.00	1.77	0.38	1.61	0.00	53.78	0.00	1.26
	1.73	0.00	0.00	4.32	3.13	2.67	0.66	3.56	3.81	0.60	1.44
98	6	13	2	98.40	18.70	1.11	0.30	0.17	8.09	1.22	85.38
	12.77	5.00	20.63903943		12.86	0.67	1.16	0.93	1.06	13.69	6.20
	9.58	15.03	4.34	3.56	8.88	9.72	0.00	0.18	2.17	0.63	0.76
	0.00	0.36	2.98	21.89	2.01	7.82	0.57	5.91	6.40	0.74	0.58
	0.72	1.16	12.70	1.63	0.43	1.47	0.00	22.00	0.64	0.64	2.27
	0.00	4.04	4.00	2.51	2.13	0.53	2.78	2.14	0.47	0.81	
99	7	13	2	100.30		17.80	0.90	0.29	0.13	8.46	1.06
	8.95	7.42	5.00	15.89648946		12.67	0.70	1.30	1.07	0.73	14.54
	11.39	17.25	9.76	3.70	2.59	20.18	8.39	0.29	0.14	1.85	0.00
	0.15	0.00	0.14	1.47	8.16	0.78	7.02	1.11	5.24	4.58	0.78
	1.06	1.29	0.75	10.82	1.19	0.97	1.27	2.68	17.11	0.54	0.00
	0.45	0.00	1.57	1.97	0.94	2.78	0.69	4.43	4.57	0.75	1.73
100	8	13	2	99.40	19.10	0.89	0.31	0.14	8.31	1.18	61.85
	9.61	5.00	22.95365171		12.72	0.55	1.01	0.73	0.71	27.40	6.45
	13.56	22.69	0.00	4.46	9.15	4.87	0.00	0.00	4.19	0.00	0.00
	0.00	0.00	0.00	7.23	0.29	0.25	0.69	0.51	0.28	1.47	0.60
	1.02	1.75	0.00	2.04	0.44	0.74	0.00	0.00	1.23	0.00	0.00
	0.00	0.00	0.00	0.83	3.09	0.77	3.16	3.67	0.53	1.39	

```
101    7      14     2      115.00       19.75 0.86  0.37  0.22  7.16  1.04
       13.82  9.65   6.00   17.79044721  13.15 0.53  0.96  0.88  0.97  17.24
       11.07  13.09  10.94  0.69  2.31   24.28 6.00  0.20  0.00  2.56  0.77
       0.19   0.00   0.00   0.96  9.71   0.64  0.92  1.06  0.73  1.47  0.93
       1.03   0.98   0.84   2.02  1.06   1.17  0.91  1.85  0.00  0.75  0.78
       0.57   0.00   0.00   1.29  1.12   3.38  0.84  4.01  3.08  0.68  1.16

102    7      14     2      107.80       20.20 1.03  0.29  0.20  7.66  1.41
       7.48   4.44   6.00   16.41844379  12.41 0.53  0.81  0.71  0.66  10.67
       12.67  12.48  12.52  1.41  2.01   24.76 4.11  0.09  0.00  2.96  0.71
       0.25   0.00   0.12   1.61  13.63  1.06  0.48  1.10  0.44  2.62  0.57
       1.18   0.93   0.96   4.12  0.92   1.19  0.62  0.83  0.00  0.87  0.72
       0.75   0.00   1.35   2.16  1.57   2.34  0.58  3.20  3.61  0.54  1.36

103    8      14     2      117.60       21.35 1.04  0.26  0.15  7.53  1.17
       23.38  9.58   6.00   17.66936596  13.07 0.59  0.91  0.74  0.73  22.43
       6.91   9.03   25.31  0.00  4.50   15.94 5.33  0.00  0.64  0.00  0.00
       0.60   0.00   0.00   1.28  8.02   0.63  26.34 0.69  20.07 0.88  1.21
       0.64   0.68   1.95   0.00  2.06   0.77  0.81  0.00  78.22 0.00  0.00
       1.79   0.00   0.00   1.72  0.92   2.77  0.69  2.80  3.51  0.47  1.33

104    8      14     2      108.30       19.05 1.03  0.34  0.15  7.73  1.17
       23.13  21.39  6.00   16.33296551  13.28 0.74  2.18  1.45  1.23  15.73
       6.30   11.01  16.47  1.94  4.15   9.24  10.42 0.00  0.41  3.05  1.25
       0.44   0.00   0.48   2.35  16.76  1.99  17.23 0.62  13.00 3.58  0.85
       0.59   0.82   1.27   5.68  1.90   0.44  1.58  0.00  50.11 0.90  1.27
       1.31   0.00   5.39   3.15  1.93   3.47  0.87  3.91  3.05  0.66  1.15

105    1      14     2      107.00       19.90 1.06  0.33  0.17  7.27  0.95
       10.53  4.38   6.00   16.01275748  12.15 0.63  0.93  0.71  0.67  .
       .      .      .      .     .      .     .     .     .     .     .
       .      .      .      .     .      .     .     .     .     .     .
       .      .      .      .     .      .     .     .     .     .     .
       .      .      .      .     .      .     .     .     .     .     .
```

```
proc sort data = HFHL_PhIP_Study; by diet;
```

```
proc format;
value Dietfmt
1='Basal diet without PhIP'
2='Basal diet with PhIP'
```

```
3='21% Cruciferous diet'
4='21% Apiaceous diet'
5='Combination diet'
6='ITC diet'
7='Furanocoumarin diet'
8='Combination of phytochem diet'
;

Value blockfmt
1='first day of microsome isolation from rats'
2='second day of microsome isolation from rats'
3='third day of microsome isolation from rats'
4='fourth day of microsome isolation from rats'
5='fifth day of microsome isolation from rats'
6='sixth day of microsome isolation from rats'
7='seventh day of microsome isolation from rats'
;
Value Experimentfmt
1='vegetable diet experiment'
2='phytochemical diet experiment'
;

Value BasalPhIPfmt
1='Experiment I Basal with PhIP'
2='Experiment II Basal with PhIP'
;
```

```
Value BasalnoPhIPfmt

1='Experiment I Basal no PhIP'

2='Experiment II Basal no PhIP'

;

proc print data=HFHL_PhIP_Study; by Diet experiment ; format Diet
Dietfmt. Experiment Experimentfmt.

;


proc means data=HFHL_PhIP_Study n mean stderr std min max; by diet
experiment;

TITLE 'ANOVA descriptive statistics';

format Diet dietfmt. Experiment experimentfmt.;

var Weight_gain--Panc_DNA_adduct_p DNA_adduct_t;

run;


*Proc glm will provide Least square means (under matrix for p-values),
Means (under multiple comparison for letter grouping);


proc glm data=HFHL_PhIP_Study;

title 'One Way Analysis of Variance in all rats';

format Diet Dietfmt.;

class Diet block;

model Weight_gain--Panc_DNA_adduct_p DNA_adduct_t=diet block;

Means Diet / duncan scheffe;

LSMeans Diet /stderr  pdiff;

run;

proc glm data=veg_only;

title 'One Way Analysis of Variance in veg diet rats';
```

```
format Diet Dietfmt.;

class Diet block;

model Weight_gain--Panc_DNA_adduct_p DNA_adduct_t=diet block;

Means Diet / duncan scheffe;

LSMeans Diet /stderr  pdiff;

run;

proc glm data=pure_cmps;

title 'One Way Analysis of Variance in pure compounds rats';

format Diet Dietfmt.;

class Diet block;

model Weight_gain--Panc_DNA_adduct_p DNA_adduct_t=diet block;

Means Diet / duncan scheffe;

LSMeans Diet /stderr  pdiff;

run;


proc glm data=HFHL_PhIP_Study;

title 'Basal groups with PhIP (comparison of basal groups with PhIP of
project I and II)';

format BasalPhIP BasalPhIPfmt.;

class BasalPhIP;

model  Weight_gain--Panc_DNA_adduct_p DNA_adduct_t=BasalPhIP;

Means BasalPhIP / duncan scheffe;

LSMeans BasalPhIP /stderr  pdiff;

run;


proc glm data=HFHL_PhIP_Study;

title 'Basal groups with no PhIP (comparison of basal groups no PhIP of
project I and II)';
```

```
format BasalnoPhIP BasalnoPhIPfmt.;
class BasalnoPhIP;
model  Weight_gain--Panc_DNA_adduct_p DNA_adduct_t=BasalnoPhIP;
Means BasalnoPhIP / duncan scheffe;
LSMeans BasalnoPhIP /stderr  pdiff;
run;
ods graphics on;

proc factor data=HFHL_PhIP_Study
PLOT
method=prin
simple
scree
priors=one
mineigen=2
round
rotate=varimax
;
title 'PCA analysis of PhIP metabolites';
var I_p--XVII_p;
ods graphics off;
```