TIME COURSE EFFECT OF DIETARY EXPOSURE FROM CRUCIFEROUS AND APIACEOUS VEGETABLES ON RAT HEPATIC CYP1A1, CYP1A2, AND SULT1A1 ENZYMES

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 MASTERS OF SCIENCE

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JULY 2011

-University of Minnesota

Acknowledgments

This thesis would not have been accomplished without the support of many people that I encountered along the way. I have the utmost gratitude for my adviser, Dr. Sabrina Peterson, not only for giving me the opportunity of my life to work and study in this university, but also for the mentorship throughout graduate school. Without her thoughtful guidance this thesis would not have been possible. I am also grateful for Dr. Dan Gallaher, who was the co-principal investigator of this project. His constant support and advice during the study was fundamental for its the completion. In addition his trust in my work and the ability to help me be calm was deeply appreciated.

Thank you to my committee member Dr. Vince Fritz, for his time reading my thesis and the feedback. Also thanks to Dr. Will Thomas for his help on the statistical analysis. I am thankful for the volunteers: Heidi Burns and Ranelle Kirchner who helped during the feeding study. I also would like to thank all my colleagues at lab 65, Cindy Gallaher, Jae Kyeom Kim, Drew Brockman, Lindsay Young, Scott Simpkins, Richard Shin, and MoonYeon Youn. Their presence during this journey made all of it so much more interesting and fun. Cindy provided delicious food and an environment that allowed me to grow into the person I was meant to be. Kim helped during the study and was willing to teach me lab assays. Drew provided steady support from the beginning until the end of my graduate school experience, his kindness has forever touched my heart.

Especially, I would like to express my immense gratitude to my family. To my mother, who even from far away kept me motivated to pursue my dreams. And my dear husband, for being a great partner in my life embracing all the challenges and adventures of living in another country. Your love healed and changed my life.

Dedication

I dedicate this thesis to the women who came before me. My mother, Elzira Vogel for teaching me the essence of being a strong woman. And to the loving memory of my grandmother, Noemia Vogel who could never dream for herself what life has presented to me. I feel her love and know that I carry her dreams inside of me.

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Chapter 1 Introduction

Introduction to the Thesis Project

The idea that fruits and vegetables may help reduce the risk of cancer has been studied for over 30 years.[1] Despite the link between diet and health being extremely complex,[2] research has emerged regarding the protection by some phytochemicals present in vegetables. Amongst them, cruciferous and apiaceous vegetables have been shown to be protective due to their phytochemical profiles.[3] More specifically, these vegetables have been shown to modulate biotransformation enzymes responsible for metabolizing carcinogens in the body. Humans are often exposed to carcinogenic factors, some of which are through the diet. Heterocyclic aromatic amines are commonly and abundantly found in the environment, particularly through the consumption of over-cooked meats. One of the most abundant heterocyclic aromatic amines is 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). Biotransformation enzymes are responsible for the activation and detoxification of PhIP in the body. Most studies that investigated the modulation by vegetables of these enzymes were short-term feeding studies.

Therefore, the overall objective of this research project was to investigate the effects of cruciferous and apiaceous vegetables, individually and with both groups combined, on biotransformation enzymes over time. More specifically, we aimed to determine these effects on enzymes that activate PhIP: CYP1A1, CYP1A2, and SULT1A1. On the basis of previous studies, we hypothesized that the apiaceous vegetables would decrease and cruciferous vegetables would increase CYP1A1 and CYP1A2 activity and protein levels. Regarding the SULT1A1 enzyme, no studies have investigated these vegetable effects therefore we set out to characterize their effects on this enzyme. In addition, we

hypothesized that the induction by cruciferous vegetables and the inhibition by apiaceous vegetables were not sustained over time.

The following thesis reports the effects of cruciferous- and apiaceous-supplemented diets on rat hepatic biotransformation enzymes over 7, 30, and 60 days of feeding. Chapter 2 reviews the literature on heterocyclic aromatic amines and biotransformation enzymes, and chapter 3 reviews the literature on vegetable modulation in regards to biotransformation enzymes with emphasis on those involved in activation of PhIP. Chapter 4 describes the methods employed in this project, and chapter 5 presents the results. Lastly, chapter 6 discusses our conclusions, the implications of the results, and the directions for future work in this area.

Chapter 2 Literature Review: Heterocyclic Aromatic Amines and Biotransformation Enzymes

Literature Review: Heterocyclic Aromatic Amines and Biotransformation Enzymes

Heterocyclic aromatic amines (HAAs), carcinogens found in cooked meats, are metabolized by biotransformation enzymes through different pathways in the body leading either to detoxification or activation. The purpose of this introductory chapter is to review overall information on HAAs with an emphasis on 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). Next, I will review information on biotransformation enzymes and PhIP metabolism, specifically the enzymes responsible for the pathways which lead to PhIP activation.

Heterocyclic Aromatic Amines

HAAs were discovered in the late 1930s when a Swedish chemist demonstrated that extracts of fried horse meat induced cancer when applied to mouse skin.[4] Later, scientists in Japan demonstrated mutagenic activity in bacteria, *Salmonella typhimurium* T98, from broiled fish smoke condensate.[5] Now more than 20 individual HAAs have been isolated as mutagens.[6] Besides the mutagenic activity in bacterial assays, HAAs also exert carcinogenic effects in lab animals such as rodents and nonhuman primates.[7] Based on sufficient data from animal studies but still insufficient data from human studies, the U.S. Department of Health and Human Services Report states that exposure to HAAs is *reasonably anticipated* to be carcinogenic to humans.[8]

HAAs are ubiquitous in the environment from tobacco smoke condensate and diesel exhaust[9] to foods, mainly cooked meats,[10] and drinks such as beer and wine.[9, 11] Particularly, exposure to them is often associated with meat consumption due to their abundant formation during heating processes such as frying, broiling, deep-fat frying or

roasting.[12] The heating process activates a Maillard reaction between creatine/creatinine, free amino acids, and sugars[12, 13] leading to the formation of HAAs in meat. The rate of formation varies over more than a 100-fold change[14] depending upon the type of meat, method of cooking, cooking duration, and temperature.[15] This is illustrated in a study done by Sinha et al.[16] where they studied different methods of cooking and temperatures in regards to HAA formation in pork meat products. Bacon had the highest content of HAAs in comparison to hot dog, which showed no detectable amount. In addition, bacon had a different formation rate depending on the cooking method; the highest was oven-broiled when compared to pan-frying or microwaving. Conversely, in a similar study also done by Sinha et al.,[17] steak pan-frying and barbecuing formed the highest amounts of HAAs. When comparing between bacon, chicken, hamburger, and steak, chicken was the meat that most produced HAAs. Amongst them, PhIP was the main compound formed at over 300 ng/g, compared to other HAAs that were less than 50 ng/g.[14] In general, an increase in temperature and time significantly increases the formation of HAAs.[15] HAAs are divided into five groups based on their chemical structures. One group is the pyrolytic mutagens, formed when cooking exceeds a temperature of 300ºC. The other four groups are referred to as thermic mutagens, which formed at <300ºC and are subdivided according to the structure attached to the imidazole ring such as quinoline, quinoxaline, pyridine or a furopyridine.[12] PhIP, one of the most abundant HAAs found in cooked meats, belongs to the pyridine class.[14]

A recent report in May 2011 regarding colorectal cancer from the World Cancer Research Fund and American Institute for Cancer Research (WCRF/AICR) stated that the link between red meat consumption and colorectal cancer is now under the category

of *"convincing,"* a change from the past report that showed the link was "*probable"*.[18] However, when it comes to analyzing the link for individual HAAs and colorectal cancer, the data is very limited and inconsistent. According to the National Toxicology Program Report only four out of eleven case-control studies that evaluated the effects of PhIP and risk of various cancers showed a statistically significant difference with positive associations found with breast, colorectal, and gastric cancers.[8] Fewer prospective studies have been published regarding colorectal cancer and HAAs; only one out of the three showed an association with PhIP intake.[19] This inconsistency could partially be due to the difficulties in estimating total HAA exposure in an individual, since they vary according to cooking methods, type of food, portion size, and frequency of intake.[20] Additional challenges include the correlation with other constituents in meats like protein, nitrosamines, and animal fat, making it difficult to separate the effects of HAAs from cooked meats. Consequently, there is a stronger link for overall meat intake as opposed to individual HAAs.[8] In addition to the broad individual exposure range of HAAs, their metabolism can take different pathways in the body and presumably play a major role in dictating their carcinogenic effects. This metabolism is carried out by biotransformation enzymes that are responsible for their detoxification and activation in the body. For example, the metabolism of PhIP can lead to a safe or to a reactive metabolite. Most HAAs are considered procarcinogens, where they may not exert mutagenicity in their native forms, however during their path to detoxification they could become activated and transformed into a potent carcinogenic/mutagenic metabolite in the body.

Biotransformation Enzymes

Biotransformation enzymes are part of a complex enzymatic system that plays a central role in the metabolism, elimination, and/or detoxification of endogenous and exogenous compounds.[21] 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.[22] 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.[22] Despite its complexity, this system can be simplified by dividing it into two general phases:

Functionalization phase or phase I enzymes. Usually located in the endoplasmic reticulum, the purpose of these enzymes is to add or expose a functional group (-OH,-NH_2,–SH) to the parent compound through oxidation, reduction, or hydrolysis reactions resulting in a more polar metabolite.[23] After this reaction, the metabolite generated could be highly reactive or inactivate (depending on what the parent compound was) and directly excreted, but in most cases it needs conjugation before it can be excreted.[24]

Conjugation phase or phase II enzymes. Primarily located in the cytosol, these enzymes covalently link a functional group on the parent compound or phase I metabolite with endogenously derived glucuronic acid, sulfate, glutathione, amino acids, or acetate. This conjugation usually increases polarity and inactivates the reactive metabolites from phase I enzymes, albeit in some instances conjugation might actually increase reactivity. Hence, depending on the route, this phase II metabolite can now either react with other proteins or, more commonly, be excreted through urine and feces.[24]

Phase I Enzymes

The phase I enzymes are mainly composed of a superfamily of monooxygenase enzymes that are heme-thiolate proteins called cytochrome P450 (CYP). Their name comes after their location in the membrane (*cyto*) and the heme pigment (*chrome P*) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide.[25] They are found in all organisms from bacteria to humans and are also incorporated into a wide range of tissues, but mainly in the liver, small intestine, kidney, and lungs.[26, 27] The CYP enzymes that metabolize endogenous substrates such as the body's prostaglandins, steroids, and cell wall proteins are found mainly in the mitochondria. The CYPs that metabolize exogenous substrates such as toxins, mutagens, carcinogens, and drugs, are mainly found in the smooth endoplasmic reticulum (ER).[28] These CYPs are anchored on the outer face of the ER by a hydrophobic amino acid, and adjacent to it is NAHPH-P450 reductase and cytochrome b_5 which provide the electron and the cofactor. Cytochrome P450 isoenzyme nomenclature was proposed by Nebert and colleagues.[29] They are identified by their amino acid homology sequence and divided into families of 40% homology using upper case letters and Arabic numerals, and subfamilies of 55% homology using a letter after the family designation (e.g., CYP1A, CYP1B).[27] The human genome encodes for more than 57 CYP isoforms in 21 families and 43 subfamilies with the individual genes denoted by Arabic numeral.[30] CYPs catalyze a variety of reactions including dealkylations, oxidations, epoxidations, and hydroxylations.

Phase II Enzymes

The phase II enzymes consist of many superfamilies of enzymes such as: uridine 5'-diphospho(UDP)-glucuronosyltransferases (UGT), glutathione S-transferases (GST), acetyltransferases (NAT), and sulfotransferases (SULT).[7] These enzymes are found in a variety of tissues but mainly in the liver. Their conjugation activity usually leads to detoxification of compounds, however more evidence is surfacing that it can also lead to toxic metabolites in some instances.

UGTs catalyze addition of a glycosyl group from a UDP-sugar to a small hydrophobic molecule through a process called glucuronidation. Glucuronidation is a major detoxification pathway for endogenous and exogenous compounds. Because UGTs conjugate glucuronides to a variety of functional groups, *e.g.*, -OH,-COOH, -NH_2, -SH and C-C,[31] the metabolites are allowed to be excreted into the urine or bile.[32] They are divided into two families (UGT1 and UGT2) and subsequently into 17 subfamilies based on their sequence similarities.[31] They are found in a variety of extrahepatic tissues including the gastrointestinal tract, kidney, lung, and nasal passages, however the liver is the main site.[31, 33] Their substrate specificity varies by isoform, and in general they are known to metabolize bilirubin, steroid hormones, bile acids, eicosanoids, nonsteroidal anti-inflammatory drugs, morphine, nitrosamines, and carcinogens.[31]

GSTs catalyze the nucleophilic addition of glutathione to a substrate, which usually has a carbon, nitrogen, oxygen, or sulfur center. The conjugated metabolite can be excreted into the bile or metabolized via the mercapturic acid pathway and excreted into the urine.[34] Currently, there are three distinct superfamilies of GSTs: cytosolic, mitochondrial, and microsomal, however the latter has no structural resemblance with the

other two.[35] The cytosolic and mitochondrial GSTs are involved in the metabolism of foreign chemicals or xenobiotics.[36] The cytosolic GSTs represent the largest family hence they are divided into 7 classes designated by Greek letters (e.g., alpha(A), mu(M), omega(O)), in addition they are highly polymorphic.[34] GSTs have a variety of roles apart from glutathione conjugations and metabolize a variety of substrates including herbicides, insecticides, carcinogens, by-products of oxidative stress, eicosanoids, etc.[35]

NATs are found on chromosome 8p22, catalyze the transfer of an acetyl group from acetyl coenzyme A to an amino group of a variety of endogenous and exogenous substrates.[37] Humans only have two isoenzymes, NAT1 and NAT2, and one pseudogene, NATP1.[37] Both isoenzymes are extremely polymorphic, with NAT2 having over 50 different alleles according to the most updated database from March 2011.[38] NATs undertake three main reactions: *O*- or *N*-acetylation and *N,O*-trans-acetylation. More specifically with HAAs, the *O*-acetylation usually is after *N*-hydroxylation from CYP enzymes; the *N*-acetylation is directly to the parent compound[39] and the *N,O*-trans-acetylation after *N*-acetylarylamines have been formed.[40] In general *N*-acetylation forms a less active metabolite because this bond is energetically more difficult to break. However *N,O*-trans-acetylation or *O*-acetylation reactions facilitate the displacement of the leaving group on the HAAs giving rise to a reactive metabolite.[41] Due to the many functional polymorphisms, usually this class of enzymes are referred to in the literature according to their activity (slow versus rapid acetylator).[39] Numerous studies have attempted to establish whether NAT polymorphic acetylation rate and cancer risks are associated, however the data are still inconclusive, less for bladder but more so for

colorectal, pancreatic, and breast cancer.[37] For example, in a study looking at PhIP-DNA adduct formation, levels did not differ between slow or rapid acetylators.[42]

The SULT enzymes catalyze *N*- or *O*-sulfation[43] from its donor, 3-phosphoadenosine 5-phosphosulfate (PAPS), to small planar phenol or hydroxyl/amino groups. [44] In humans, more than 11 SULT forms have been detected. They are classified similar to CYP enzymes by their amino acid sequences into families, subfamilies, and individual genes by using number, capital letter, and number, respectively (e.g., SULT1A1).[45] The SULTs are divided into two broad classes depending on their localization:

Golgi apparatus. These SULTs are membrane-bound and sulfonate peptides, proteins, lipids, and glycosaminoglycans.

Cytosolic. These SULTs are responsible for xenobiotics and small endogenous substrates.

Amongst the SULT enzymes, SULT1A1 is the major one in adult liver though it is also expressed in extrahepatic tissues.[46] SULT1A1 has the highest affinity for N-hydroxylation sulfonation.[47] Generally sulfonation is considered a detoxification pathway leading to a more hydrophilic compound and therefore aiding in excretion through the urine or bile. However, there are some instances where SULT activity results in more toxic metabolites.

As noted above, most of the biotransformation enzymes are involved in the detoxification and activation pathways of a variety of compounds including HAAs. More than 20 HAAs are known and PhIP is one of the most abundant found in cooked meats and consequently humans are exposed to it more frequently. PhIP can be metabolized through different pathways in the body, and the role of biotransformation enzymes varies

according to the metabolic pathway. The major enzymes involved in the activation of PhIP seem to be CYP1A1, CYP1A2, and SULT1A1.

Metabolism of PhIP

PhIP can be directly excreted by the body but is usually extensively metabolized; less than 1% is usually eliminated as unaltered compound (see Figure 2.1).[48, 49] PhIP is also metabolized very quickly by the body; its metabolites are usually excreted within less than 24 hours, 35% within the first 12 hours and 60-85% by 24 hours.[48] The metabolism of PhIP has been studied extensively in a variety of animal-based models (in vivo and in vitro): rabbit and mouse,[50] other rodents,[42, 51, 52] nonhuman primates,[53] as well as in human-based models through in vitro studies[54-57] using recombinant human microsomes,[56, 58] or in vivo studies.[49, 59] There are also a few studies comparing human metabolism with other primates such as monkeys, cynomolgus,[54, 60] and most importantly rodents since the majority of studies utilize this model.[48, 61-65] PhIP can be metabolized into a variety of compounds. In a recent review by Teunissen et al. looking at PhIP metabolites in biological matrices, a total of 23 metabolites were found. [66] PhIP can be directly conjugated by phase II enzymes through UGTs at the N^2- or N^3- position in the triazine ring nitrogen[67, 68] and can be excreted that way. Another class of enzymes known to metabolize PhIP are GSTs, which have been shown to have a protective effect against PhIP-induced carcinogenesis.[69] However, humans have a regioselectivity for *N*-oxidation of PhIP. Therefore, the main excretory form for PhIP is formed after an activation step via CYP1A enzymes to 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyrimidine (N^2-OH-PhIP).[59] N^2-OH-PhIP has the potential to be further esterified by NATs and

SULTs to N^2-acetoxy-PhIP and N^2-sulfonyloxy-PhIP, respectively; these metabolites are known to create DNA adducts.[62] Therefore, CYP1A enzymes play a pivotal role in PhIP metabolism.[60]

CYP1A Enzymes

The CYP1A family, CYP1A1 and CYP1A2, are a superfamily of enzymes known to interact with more than 240 and 430 xenobiotics, respectively.[70] In addition to their xenobiotic substrates they also metabolize endogenous compounds such as bilirubin, melatonin, estrogens and other lipophilic compounds.[71] Nevertheless they are usually referred to for their important role in metabolism of carcinogens, not only HAAs but also polycyclic aromatic hydrocarbons (PAHs), mycotoxins, and nitrosamines.[72] Even though they share 70% of their amino acid sequence[71] they are mainly expressed in different tissues; CYP1A1 is not limited to hepatic tissues whereas CYP1A2 is mainly expressed in the liver.[56] Besides CYP1A1 and CYP1A2 contributing to the bioactivation of HAAs in different tissues,[44] their individual roles towards HAA metabolism have also shown some significant differences, although this conclusion is limited to mainly in vitro studies.[59] In an in vitro study[73] with COS-1 cells expressing *hCYP1A1*or hCYP1A2 cDNA the metabolism of five different HAAs was compared between CYP1A1 and CYP1A2 by assessing the number of revertants/mg of protein. For most of these HAAs, CYP1A2 had at least one order of magnitude more activity than CYP1A1, though for PhIP the activity was similar for both enzymes.[56] But in another in vitro study utilizing *Salmonella typhimurium* with human microsomes, CYP1A2 showed a higher activity for metabolizing PhIP.[74]

Notably, CYP1A1 and CYP1A2 seem to favor PhIP metabolism over other HAAs such as 2-amino-3-methyl-imidazo[4,5-f]-quinolines (IQ), 2-amino-3,4-dimethyl-imidazo[4,5-f]-quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (8-MeIQx), and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx).[73] As mentioned above, individual roles of CYP1A1 and CYP1A2 in the metabolism of HAAs vary and this also applies to PhIP metabolism. In an in vitro study utilizing human recombinant CYP expressed in Sf9 insect cells,[58] it was found that CYP1A1 favors the formation of 4`-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (4`OH-PhIP) compared to CYP1A2 which favors the N^2-OH-PhIP formation. Although when looking at catalytic efficiency for PhIP, CYP1A1 was more efficient at PhIP *N*-hydroxylation than CYP1A2, Vmax/Km = 3.2 and 1.1, respectively.[58] Thus, CYP1A1 and CYP1A2 can activate PhIP through *N*-hydroxylation reactions leading to N^2-OH-PhIP which is mutagenic.[58] They can also hydroxylate the ring structure of PhIP leading to the 4`-OH-PhIP product which some believe is not a reactive metabolite due to the lack of mutagenic activity towards *Salmonella.*[50] This metabolite (4`-OH-PhIP) is usually generated from CYP1A1 and does not seem to be relevant for humans because it was mainly seen in rodents' metabolites.[75] N^2-OH-PhIP could be further conjugated by phase II enzymes in the liver, or transported to other organs for further conjugation as well, or excretion.[51] As mentioned above, *N*-hydroxylation seems to be the primary oxidative route in humans and since the liver is the most active organ that metabolizes HAAs, it is mainly done by CYP1A2.[76, 77] A few phase II enzymes are able to conjugate N^2-OH-PhIP; the major detoxification route is via UGTs and the further activation route is via esterification from NATs or SULTs.

SULT1A1 and NAT2 Enzymes

Three sulfotransferases (SULT1A1, 1A3, 1E1) have been shown to sulfate *N*-OH HAAs. The data is limited regarding individual roles but it seems that SULT1A1 is the most active isoform.[77-79] Another class of enzymes known to esterify PhIP is NATs, mainly NAT2. In an in vitro study utilizing human liver cytosol with N^2-OH-PhIP, when the cofactor for NATs was added, DNA adducts increased significantly compared to control where the cofactor was not added.[65] Even though NAT2 esterifies N^2-OH-PhIP, SULTs are more efficient at this reaction[80] and as a result acetylation plays a minor role in PhIP activation.[81] This was demonstrated by a study utilizing *Salmonella typhimurium* with heterologous expression of *h-NAT1*, *h-NAT2,* and *h-SULT1A1*. After incubation of N-hydroxy-PhIP, the bacterial cells expressing *h-SULT1A1* showed a significant strong mutagenic activity compared to others.[78] Interestingly, the SULT enzymes might also play a role in detoxification of HAAs. Even though 4`-OH-PhIP is a minor metabolite in humans, SULT enzymes can esterify it consequently leading to excretion as a 4`-PhIP-sulfate ester metabolite.[59] SULT enzymes can also perform *N*-sulfonations leading to the formation of sulfamates. This reaction may sequester procarcinogens leading to a non-reactive metabolite that competes with *N*-hydroxylation reactions from CYP enzymes. Therefore SULTs could also be considered a detoxification enzyme.[45] However, sulfamates were only shown with MeIQx and only to a minor degree.[82] The sulfoxy metabolites are unstable so as a result it is difficult to assess their role in PhIP metabolism.[59] In summary N^2-OH-PhIP can be further *O*-acetylated via acetyltransferase (NAT1/2) or *O*-sulfonated via sulfotransferase (SULT1A1), which forms an unstable ester.

These unstable esters can undergo heterolytic cleavage and produce a nitrenium ion-acetate anion pair which is able to form DNA adducts or react with other cellular constituents.[83] The carcinogenic effect is derived from the cleavage of the leaving group of the esterified PhIP, consequently forming the nitrenium ion which attaches to the guanine base of DNA, mainly at the C-8 position (dG-C8).[51] This is considered the initial step in carcinogenesis[48] and is usually used as a biomarker of potential mutagenesis and carcinogenesis. But the DNA can still undergo repair by nucleotide excision repair mechanisms[84] to remove the adduct thereby prevent mutagenesis.

However, N^2-OH-PhIP and even the nitrenium ion-acetate can be conjugated by UGT or reduced by glutathione via GST producing safer non-mutagenic metabolites and rendering them safe for excretion.[85]

UGT1A1 and GST1A1 Enzymes

As mentioned above conjugation by phase II enzymes usually leads to a more hydrophilic metabolite and supports excretion. UGT1A1 and GST1A1 have been shown to produce a non-reactive metabolite in excretion studies utilizing PhIP.

At least five glucuronides for PhIP have been reported in urinary metabolites, the major one being 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-N^2-glucuronide (N^2-OH-PhIP-N^2-glucuronide). Alternatively, a glucuronic acid can be attached at the N^3-position although this is a minor metabolite in humans and seen mainly in rodents.[85] In one study the ratio between glucuronidation at N^2 position versus N^3 position was 6:1, respectively.[59] Also, glucuronidation tends to favor *N*-hydroxylated PhIP versus the parent compound, PhIP. A 53-fold difference has been noted for glucuronidation of the metabolite as opposed to the parent compound.[68] UGT1A1 is

thought to be the major enzyme to glucuronidate PhIP [59] and more recently it was found that UGT1A10 may be the more efficient enzyme amongst this subfamily.[57] Interestingly, even during the path to excretion in the intestine or colon bacterial β-glucuronidase could cleave this metabolite. This would allow the metabolite to regain direct mutagenicity and consequently damage the intestinal or colon walls. Noteworthy, N^2-glucuronides are resistant to β-glucuronidase therefore mainly N^3-glucuronides, the major metabolite in rodents, are cleaved by this enzyme.[51, 85]

The GSTs also have a protective role against N^2-OH-PhIP, more so after its acetylation via NAT2. Six out of eight human hepatic cytosols incubated with *N*-acetoxy-PhIP showed a significant reduction in PhIP-DNA binding capacity after adding glutathione.[65] In another experiment done with purified enzymes instead this reduction was more predominantly with the GST1A1 isoform when compared to other GSTs in humans and rats. No studies have investigated the role of GSTs after sulfation of PhIP.

In summary, multiple pathways are involved in the metabolism of PhIP in humans leading either to activation or detoxification. Activation is initially dependent on CYP1A but due primarily to the conjugating activity of UGT an unreactive metabolite can be formed and excreted. Therefore, the biological potency for PhIP-induced carcinogenesis is strongly dependent upon the interaction of biotransformation enzymes and which pathways predominate.[61] Most studies have focused on urinary excretion or used other species to understand PhIP metabolism, however extrapolations need to be viewed with caution. In one of the in vivo studies, after a subject received a known dosage of PhIP only 50% of the PhIP was excreted in the urine, where the other two subjects showed ~90% excretion in the urine. This illustrates that there is great variation in PhIP

metabolism that cannot be understood only by observing urinary metabolites. However, this was a small study and the subject, with only 50 % excretion, was an elder undergoing colorectal cancer surgery. [59] Also, interspecies differences have been shown regarding PhIP metabolism pathways [64] suggesting that the effects of PhIP are much more potent in humans compared to rodents.[48]

Finally, many of the biotransformation enzymes that play a major role in PhIP metabolism are known to be modulated by short-term exposure to plant constituents. Therefore, understanding this modulation is important for future studies regarding the carcinogenic effects of PhIP.

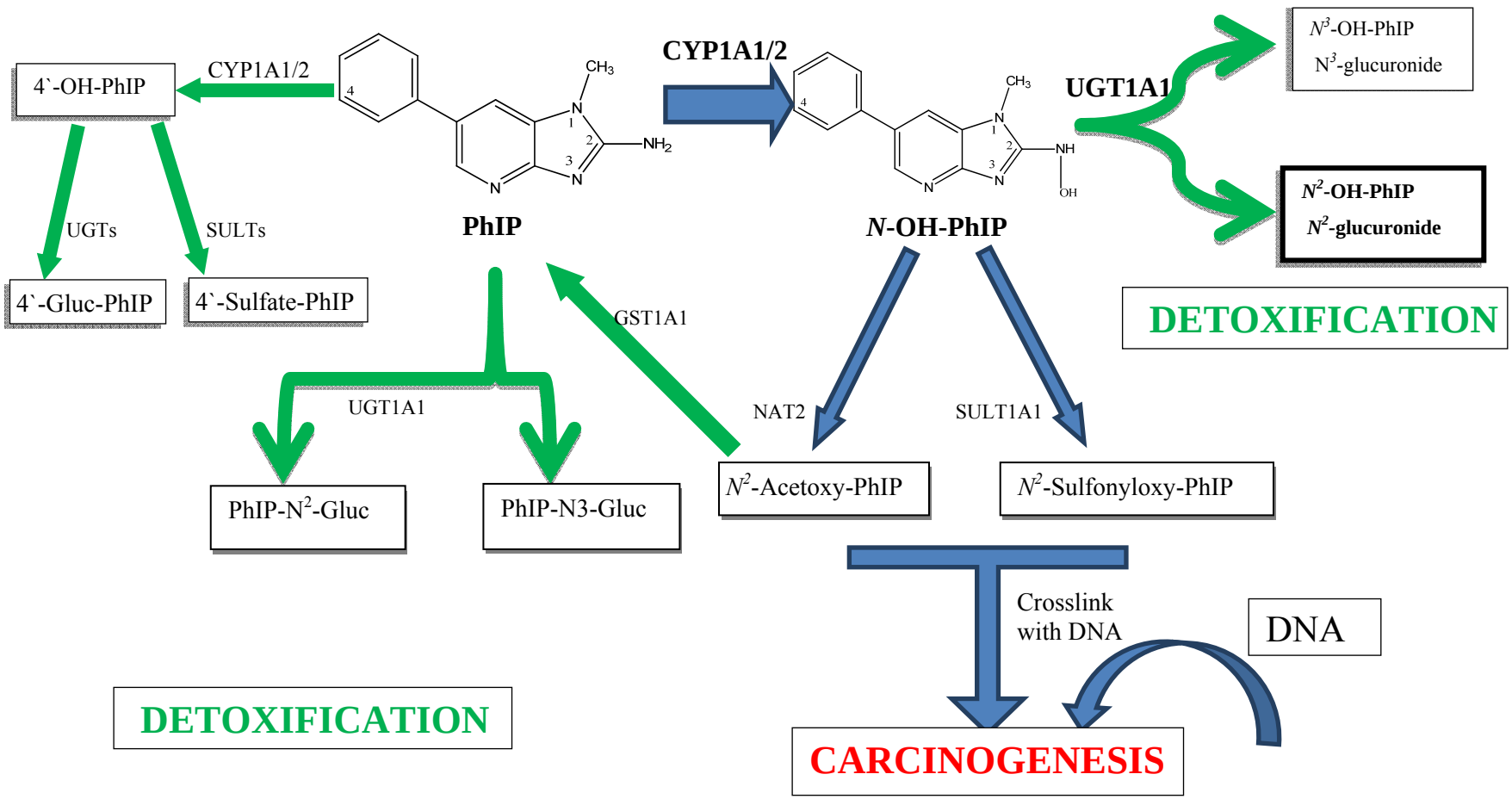

Figure 2-1. Major Biotransformation Pathways of PhIP. Adapted from Turesky et al.,[77] Gooderham et al.,[76] Zhou et al.,[86] Patterson et al.[62]

Chapter 3 Literature Review: Modulation by Vegetable of PhIP-metabolizing Biotransformation Enzymes

Vegetable Modulation of PhIP-metabolizing Biotransformation Enzymes

Several biotransformation enzymes are involved in PhIP metabolism, therefore their regulation is extremely important. In particular, the equilibrium between the rate of formation of the reactive metabolite and the rate of inactivation is critical to its carcinogenic potential. It has been shown that several biotransformation enzymes respond to an individual's environment or lifestyle choices such as diet.[87] Notably, studies have shown that vegetables are able to modulate biotransformation enzymes. The purpose of this chapter is to review information on vegetables that modulate the activating pathways of PhIP in the body hence CYP1A1, CYP1A2, SULT1A1, and NAT2 enzymes.

Vegetable Effects on HAAs

Even though the evidence is less compelling than in the early 1990s, it is still well established that diets high in plant foods, specifically fruits and vegetables, have a protective role against certain cancers.[88] Recently, a few studies have also investigated the effects of vegetables on HAA genotoxicity. In an in vitro study utilizing Chinese hamster lung fibroblasts genetically engineered to express human biotransformation enzymes (V79-hCYP1A2-hSULT1A1*1), the genotoxicity of PhIP and N-OH-PhIP was minimized after the incubation with certain teas and juices from fruits and vegetables.[80] This result shows the protective role of phytonutrients on carcinogenesis against PhIP even after it has been metabolically activated. Similar results were found in a crossover feeding study.[89] Subjects consumed for two weeks meats heated at 250ºC and for another two weeks a combination of foods known to inhibit HAAs was added to the diets such as:

cruciferous vegetables, chlorophyllin tablets, and yogurt. This combination yielded a significant reduction in urine and fecal mutagenicity in addition to less DNA damage in colon cells measured by the comet assay.[89]

HAA genotoxicity can be prevented by different approaches such as:

-Prevention of HAA formation in cooking through the use of antioxidants, phenolic-compounds, or ascorbic acid.

-Direct inactivation of HAAs through binding with other compounds such as chlorophyllin, fibers and α-cellulose.

-Improving DNA repair mechanisms.

-Modulation of particular biotransformation enzymes. In the case of PhIP, this could be inhibition of CYP1A1, CYP1A2 , SULT1A1, and NAT2 activity or induction of GST and UGT.[90]

CYP1A1 and CYP1A2 Enzymes

The CYP1A enzymes are known to be modulated by xenobiotic compounds such as those found in vegetables. Amongst all vegetables, the cruciferous family has received the most attention regarding this modulation.[91] Nevertheless, apiaceous vegetables have also been shown to have this ability.

Modulation by Cruciferous Vegetables

Modulation by cruciferous vegetables of CYP1A enzymes has been studied extensively, mainly in rodents. Mice fed 20% freeze dried radish for 2 weeks showed an increase in hepatic mRNA levels of CYP1A1/1A2.[92] Utilizing another type of cruciferous vegetable, Brussels sprouts, male Wistar rats were supplemented with 2.5%,

5%, or 20% cooked vegetable for 2, 7, 14, and 28 days. The activity of CYP1A1, measured as 7-ethoxyresorfurin-O-deethylase (EROD) activity, was increased after 2 days of 20% Brussels sprouts. For the 5% dosage it took 14 days to see a similar increase,[91] suggesting a dose-time response. Vang et al. investigated the effects of a 10% (wt:wt) broccoli diet for 7 days in female Wistar rats. Regarding CYP1A1 and CYP1A2 enzymes, the rats showed an increase in protein levels in the liver, but not in the colon, while both organs had significant increases in mRNA levels.[93] In a subsequent study the same investigator analyzed glucosinolate levels, a class of compounds present in the cruciferous vegetables[94] in different samples of broccoli that were grown in different conditions and different cultivars and then examined their effects on CYP1A1/1A2 activity in male Wistar rats. The diet consisted of 10% (wt:wt) broccoli powder fed for 7 days. Overall, broccoli samples did increase EROD and 7-methoxyresorfurin-O-demethylase (MROD) activity (measures for CYP1A1 and CYP1A2, respectively). The different growing conditions did change the profile of GLS in broccoli and also changed the activity of hepatic CY1A2 enzyme. Hepatic MROD was increased 2.5-fold in rats fed the broccoli grown in low sulfur soil compared to rats fed broccoli grown with sulfur. Amongst the 8 different samples of broccoli, the *Shogun* cultivar grown from June to August showed the most activity when compared to other broccoli samples and the control group. In addition, they analyzed in vitro metabolism of PhIP in hepatic microsomes. The broccoli diets increased the N'-OH-PhIP metabolites from 1.6- to 1.9-fold.

Bonnesen et al.[95] investigated the effect of the following glucosinolates found in broccoli: glucobrassicin, neoglucobrassicin, 4-methoxyglucpbrassicin, and 4-

hydroxyglucobrassicin. They administered to male Wistar rats the glucosinolates degraded or intact at a dosage of 88 mmol/kg body weight for 3 days. Although not all of the glucosinolates showed an effect, overall they induced both protein expression and enzyme activity of CYP1A1/1A2. Perocco et al.[96] gave Sprague-Dawley rats glucoraphanin, an intact glucosinolate, at two different dosages (120 and 240 mg/kg body weight) for 4 days. Both females and males showed an increase in EROD and MROD activity.

Besides the liver, other organs also show a similar response to a cruciferous diet. In the lungs of male Sprague-Dawley rats fed 120 mg or 240 mg/kg body weight of glucoraphanin in a single or repeated dose, EROD and MROD were increased when compared to control groups.[97] In the colon of male Wistar rats fed 10% broccoli, overall EROD and MROD activity were increased.[94] EROD was increased as well in the small intestines of male Wistar rats due to the intake of Brussels sprouts for 2 and 28 days.[91] In the kidney, MROD and EROD were also increased in male Wistar rats from 7 days of feeding 10% broccoli.[94]

CYP1A2 activity can be measured in humans by caffeine metabolism using urinary caffeine metabolite ratios. Using this approach, Kall et al.[98] investigated the effect of 12 days of consumption of 500g of broccoli in humans. At the end of the feeding trial CYP1A2 activity was increased by 19% compared to when subjects were not eating cruciferous vegetables. Similar results were found in a crossover study design with consumption of ~430g/day of a cruciferous vegetable mixture. When compared to basal diet (no vegetables included), cruciferous increased CYP1A2 activity by 18-37%, as assessed by caffeine metabolite ratio.[99] Although cruciferous vegetables increase activity

of CYP1A1 and CYP1A2, which tend to activate carcinogens, they are thought to be chemoprotective because they also induce phase II enzymes which would change the balance towards detoxification.

Even though most studies show CYP1A induction after the consumption of cruciferous vegetables or glucosinolates, a few studies have shown a reduction. Canistro et al.[100] provided another glucosinolate, gluconasturtiin, found in higher amounts in watercress, in a single or repeated dosage of 24 and 48mg/kg body weight for 4 days in male Swiss Albino CD1 mice. The hepatic MROD and EROD activity were significantly reduced, and more so with the single dose when compared to the repeated dose. However, in the kidney EROD was increased and MROD was decreased compared to the control group. In an in vitro study utilizing secondary metabolites of glucosinolates such as isothiocyanates, sulforaphane and two analogs were assessed with a concentration range of 0.5 - 2.5 μM in two cell lines.[101] It was noted that EROD and MROD was inhibited in benzo[*a*]pyrene induced mcf7 cells. On the other hand, when looking at induced HepgG2 cells EROD was not inhibited, and at a concentration of 2.5 μM a trend towards induction was observed. Therefore, the effect of a particular glucosinolate seems to be cell and dose dependent. In addition, the chemical structure of glucosinolates also seems to influence the results. It has been speculated the inhibitory effects of certain glucosinolates depends on the length of the alkyl chain, for example the increase to C_6 on the arylalkyl isothiocyanate resulted in a greater inhibitory effect in certain CYP1A enzyme activity.[102]

Overall, the evidence indicates that cruciferous vegetables have the ability to increase CYP1A activity. This could be due to the content of a unique class of compounds called

glucosinolates, which is responsible for the pungent aromas and bitter taste of the vegetables.[103]

Glucosinolates

Most cruciferous vegetables originate from the genus *Brassica* (e.g., broccoli, cabbage, cauliflower, kale, Brussels sprouts, radish), and have been widely recognized as a rich source of carotenoids, vitamin C, folate, soluble fiber, and glucosinolates, all of which may play a role in chemoprevention.[104] The amount of glucosinolates (β-thioglucoside N-hydroxysulfates) can approach 1% of their dry weight. Glucosinolates are synthesized and stored in an inactive form in plant cells, and only released when the cells are damaged from chewing, food preparation, or microbial attack.[105] The glucosinolates are then hydrolyzed by myrosinase (β-thioglucoside glucohydrolase; E.C 3.2.3.1) giving rise to glucose and a number of bioactive products such as isothiocyanates or indoles.[106] More than 120 glucosinolates with unique hydrolysis products have been indentified in plants[107] and the profile and concentration of them vary according to the specific vegetable and growing conditions.[108] Usually the cruciferous vegetables are distinguished by the glucosinolate present in the most quantity, although many are present. As such, watercress is known for its high content of gluconasturtiin which is the parent compound of the bioactive product phenethyl isothiocyanate (PEITC). Cabbage is known for a higher content of glucobrassicin and its major product is indole-3-carbinol (I3C), and broccoli for glucoraphanin which gives rise to sulforaphane.[106] Importantly, only a few glucosinolate products have been extensively studied for their chemoprevention effect[109] and the data show mixed results. Glucosinolates have several

biological effects, both from the intact glucosinolates and their products, which are produced either through an enzymatic reaction or non-enzymatic reaction.[110] Although, it is well accepted that the chemoprotective effect of cruciferous vegetables are likely due to these bioactive products, a recent in vitro study of the effects of the parent compound glucoraphanin versus the bioactive product sulforaphane has challenged this view. Rat liver slices were incubated with either glucoraphanin or sulforaphane. Glucoraphanin treatment led to a significant increase in EROD and MROD while sulforaphane did not.[111] The lack of effect persisted even after the addition of myrosinase to the glucoraphanin medium. But the relevance of this outcome in humans is uncertain since mammalians, although not as efficient as plant, also have myrosinase in the gastrointestinal tract which could process the degradation of glucosinolates.[112]

In summary, studies have shown that intact cruciferous vegetables increase CYP1A1 and CYP1A2 activity while decreased activity has been observed with a few of the purified compounds. These vegetables are thought to modulate these enzymes by acting on CYP1A regulation mechanisms.

CYP1A Regulation

CYP1A enzymes are thought to be mainly regulated transcriptionally via the Ah (aryl hydrocarbon) locus, involving the interaction of the Ah receptor (AhR) and aryl hydrocarbon nuclear translocator (ARNT).[113] Cruciferous vegetables, more specifically glucosinolates, are thought to act as a ligand for this AhR and consequently influence gene expression.[114] *CYP1A1* and *CYP1A2* are located on chromosome 15q24.1. They are separated only by 23 kb with no open reading frame between them and share the 5'

flanking region[115] oriented head-to-head.[116] The spacer contains regulatory regions for individual genes and alternatively these regions might overlap with one another.[116] The most well-studied ligands for CYP1A1 are planar polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD).[117] Once these AhR ligands enter the cell, they bind to the AhR in the cytosol and then act as a transcription factor in the cytosol.[118] The AhR is bound to two 90-kDa heat-shock protein (HSPs), the cochaperone p23, and a 43-kDa immunophillin-like protein hepatitis B virus X-associated protein 2 (XAP2). All of these proteins allow the stabilization of the receptor. Upon binding with the ligand, it is activated and disassociates from the complex allowing it to translocate to the nucleus where it will bind to ARNT (AhR-heterodimer).[86] This complex now has a classical recognition motif (5'-GCGTG-3' DNA) which can bind to the consensus sequence of the xenobiotic response element (XRE) or dioxin response element (DRE) of *CYP1A1* located 1kb upstream in the promoter region.[119, 120] This process is affected by numerous coactivators, other transcription factors, and an Ahr repressor. In addition, several negative regulatory elements are located in the *CYP1A1* promoter region.[71]

CYP1A2 is also regulated by the Ah receptor[21] but the dioxin-response enhancer is located in a different position than *CYP1A1*; it is downstream and further away, ~15kb, from the gene.[120] Furthermore, the details of the specific mechanism are still unclear and it has been suggested that pre- and post-transcriptional regulatory pathways play a role.[21, 121] *CYP1A2* contains several putative XRE or DRE sites and recently in HepG2 cells another *CYP1A2* enhancer was shown that is not dependent upon AhR binding.[120] Therefore, the expression of *CYP1A2* probably depends upon the interactions between enhancers and promoters[115, 120] as well as the other factors that also affect *CYP1A1*.

CYP1A1 has the potential to be highly induced in extrahepatic tissues where it is mainly expressed, such as the lung, larynx, kidney, placenta, lymphocytes, and fetal liver.[122] It is also somewhat inducible in the liver where it is usually found at low concentration levels.[123] Human CYP1A2 has a much greater variety of substrates, despite the homology with CYP1A1, metabolizing such substrates as drugs, aromatic amines, heterocyclic amines, nitrosamines, mycotoxins, nitroaromatics, and estrogens. [122] Important to note is that endogenous factors also play a role in its regulation (e.g., hormones), and may influence *CYP1A* directly or indirectly via regulation of crosstalk of other transcription factors.[123] Cruciferous vegetables, specifically glucosinolates, are thought to activate CYP1A enzymes transcriptionally by acting as AhR ligands. In addition, mRNA and protein stabilization by glucosinolates have been speculated to play a role as well.[111]

Furthermore, humans are usually exposed to a combination of vegetables and the interactions amongst them also need to be taken into consideration to make conclusions from chemoprotective studies. Peterson et al. investigated the interaction between cruciferous and apiaceous (carrot family) vegetables on CYP1A2 activity in a randomized, cross-over feeding trial in humans.[124] Despite the concurrent intake of cruciferous vegetables, the net inhibitory effect seemed to favor apiaceous vegetables, showing a reduction of CYP1A2 activity. Only two other studies were found analyzing the effects of combined intake of cruciferous and apiaceous vegetables on biotransformation enzymes, both done on phase II enzymes, more specifically GSTs.[99, 125] Overall, this combination diet had no effect on GST serum concentration and a decrease was only noticed with certain genotypes such as *GSTM1*+ and *GSTM1*-

null/GSTT1null. These results suggest that the combined vegetables might have a greater impact in detoxification of carcinogens due to reduced CYP activity, which are known to activate certain carcinogens, in addition to not altering GSTs, which are known to detoxify certain carcinogens. However, these results warrant further investigation and caution since these enzymes are also known to metabolize other substrates.

Apiaceous Vegetables

Apiaceous vegetables, *Apiaceae,* include carrots, celery, parsnips, parsley, and cilantro. They are a rich source of furanocoumarins, which are further divided according to their chemical structure: linear and angular. Linear furanocoumarins are: psoralen, bergapten or 5-methoxypsoralen (5-MOP), xanthotoxin or 8-methoxypsoralen (8-MOP), imperatorin, and isopimpinellin. Angular furanocoumarins are: angelicin, pimpinellin, and isobergapten.[126] Furanocoumarins do not degrade with cooking and are increased by environmental stress like seasonal trends and fungal and bacterial infections.

As mentioned above, fewer studies regarding apiaceous vegetables have been done with biotransformation enzymes. The studies that have been conducted mostly used individual furanocoumarins due to their medicinal benefits in treating psoriasis[127, 128] and vitiligo.[128] Nevertheless, a few human studies showed inhibition of CYP1A2 activity by consumption of the whole plant[99] in addition to the individual furanocoumarins, more specifically 5-MOP[129] and 8-MOP.[130] The above two studies utilizing furanocoumarins were in concordance with the in vitro study done by Peterson et al.[131] They investigated the effects of three furanocoumarins, psoralen, 5-MOP, and 8-MOP, on MROD utilizing yeast expressing *hCYP1A2.* All three compounds were potent inhibitors at physiologically relevant concentrations. Similar results were found with other

furanocoumarins, imperatorin and isopimpinellin utilizing cDNA-expressed P450 microsomes.[132] The inhibitory effect was greater for CYP1A1 than CYP1A2 when compared to control group but this could partially be due to the substrate of choice for CYP1A2 which was 7-ethoxyresorufin. CYP1A2 can also utilize 7-ethoxyresorfurin as a substrate but has a higher affinity for 7-methoxyresorfurin. In another in vitro study using a hepatic preparation from mice, 5-MOP, imperatorin, and isopimpinellin were potent inhibitors of EROD activity.[133] Similar to these inhibitors, 1 nM to 1 mM concentrations of 8-MOP, isopimpinellin, and angelicin were also reported to decrease EROD activity in rat intact hepatocytes and isolated microsomes, ultimately resulting in complete inhibition.[134] This effect was accompanied by a decrease in mRNA level of the CYP1A1 enzyme at most concentrations of the furanocoumarins. However, 8-MOP and angelicin showed an induction of CYP1A1 mRNA levels at higher doses.

In another study with male Sprague-Dawley rats, a time-course and dose-response experiment were conducted with 8-MOP.[135] For the time course experiment, rats were administered a single dose (25 mg/kg) and sacrificed 2, 4, 8, 16, 24, 48, or 72 hours after treatment. EROD and MROD activity were depressed 18% and 17% after 2 hours and remarkably elevated 727% and 932% after 24 hours, respectively. Levels returned to normal values after 5 days from this single dose. This biphasic response suggests that 8-MOP could be considered both an inducer and inhibitor. For the dose-response experiment rats were treated with 0.1, 1, 5, 25, or 50 mg/kg and sacrificed after 24 hours. Both EROD and MROD were increased in a dose-dependent manner reaching a maximum level at 25 mg/kg. These results suggest that the effect of 8-MOP is dose and time dependent.

It is important to note that furanocoumarins are also present in other foods such as oranges, lemons, and grapefruit. Grapefruit have received considerable attention due to their capability for inhibiting several P450s, more specifically CYP3A4, hence the caution not to drink the juice when taking certain drugs due to increased bioavailability and decreased clearance.[136] Also, inhibitors of CYP1A1 and CYP1A2 have been shown from the flavanoid class of phytochemicals, more specifically the methoxy and hydroxyl flavones, although with some discrepancies between the CYP1A isoforms.[137]

A number of furanocoumarins in apiaceous vegetables have been identified as mechanism-based inactivators (MBI) of various CYPs while others are still being investigated for such a mechanism.[138] MBI play a major role in drug metabolism and cancer detoxification systems because they irreversibly inhibit the enzyme, resulting in prolonged inhibition even after the MBI has been cleared from the system. De novo synthesis of enzyme is required to regain enzymatic activity.

Thus, cruciferous and apiaceous vegetables modulate the CYP1A1 and CYP1A2 enzymes via different mechanisms. Regarding the other enzymes related to PhIP activation (SULT1A1 and NAT2) less is known about dietary modulation of their activity but there is some indication of diet effects.

SULT1A1 and NAT2 Enzymes

Less research has been done regarding the mechanism controlling SULT1A1. SULTs are usually not considered to be "xenobiotic inducible," however similar inducers for CYP enzymes also seem to have a significant effect on SULT1 regulation. Runge et al,[139, 140] investigated the effect of 2,3,7,8-tetrachloro-p-dioxin (TCDD), which is an inducer of CYP1A1 enzymes via the Ah receptor. After incubation for 72 hours of

primary cultured rat hepatocytes in TCDD, SULT1A1 mRNA levels decreased by 72-80% suggesting that AhR agonists may decrease SULT1A1 gene transcription via an AhR-mediated mechanism.[139]

In an in vitro study utilizing *Escherichia coli* expressing plasmids for SULT1A1, it was found that grapefruit juice inhibited SULT1A1 activity by 80%.[141] In a subsequent assay, the same group looked at specific compounds found in grapefruit juice such as flavonoids (naringin, naringenin, and quercetin) and furanocoumarins (bergamottin and 6`7`-dihydrobergamottin). It was found that among the furanocoumarins only 6`7`-dihydrobergamottin was able to reduce SULT1A1 activity. The most potent inhibitor was quercetin, a flavonoid, which reduced activity almost completely. Parsnip and celery, apiaceous vegetables, contain some quercetin but the amounts are relatively low compared to other vegetables, according to USDA food composition tables.[142] Therefore, it seems that SULT might share similar transcription factors with other biotransformation enzymes, however the specific modulation by certain vegetables like apiaceous remains unclear. Based on the studies described above, it appears that the combination of all constituents present in the plant might provide a greater effect than the individual compounds.

Few studies have been done regarding NAT modulation. In one study, subjects were given caffeine with and without 1.2 mg/kg body weight of 5-MOP (a furanocoumarins). Among all subjects, no differences were noted in NAT2 activity as assessed by metabolism of caffeine.[129] In a recent study, the effects of 5-MOP on N-acetylation of 2-aminofluorene by NAT was investigated in rats and in human cell lines.[143] In the rat experiment, a 0.5 mmol/kg body weight single dose resulted in an overall decreased N-

acetylation. The experiment from human colon and stomach tumor cell lines demonstrated that the treatment with 5-MOP (0.05-50 mM) decreased NAT activity in a dose-dependent manner. However, with the colon cell line the 50 mM concentration an induction was noticed. This demonstrates that the effects of 5-MOP are cell and dose dependent. Due to the lack of studies regarding modulation of NAT by vegetables, it is difficult to conclude what effects, if any exist.

In summary, the studies that investigated the effects of vegetables on biotransformation enzymes, more specifically the ones involved in activating PhIP, have mainly focused on cruciferous and to some extent on apiaceous vegetables.

Long versus Short Term Studies

Most of the studies mentioned above provided cruciferous and apiaceous vegetables over a short-term exposure time. For cruciferous vegetables the studies varied from a single dose to a few days of intake. Only one animal study provided the vegetables for a somewhat longer timeframe (28 days of feeding). For apiaceous vegetables, most studies used only the purified compounds found in the vegetables. Few studies provided the whole apiaceous vegetable, and even then the maximum exposure length was 6 days.

The only long-term cruciferous feeding study was done by Arikawa et al.[144] For one of the experiments rats were fed fresh broccoli, green cabbage, and watercress at 14.4%, 22.6%, and 10% of diet, respectively, for 14 weeks. For their additional experiment rats were fed lyophilized watercress, red cabbage, and green cabbage at 4%, 10%, and 10% of diet respectively, for 9 weeks. In both experiments CYP1A1 activity was not increased

when compared to the control diet group. This result suggests the increase in CYP1A1 activity observed in short-term feeding studies may not be sustained over periods of chronic consumption.

In addition, some of these vegetables are known to modulate the activity of biotransformation enzymes very quickly. This was illustrated in 20 nonsmoking subjects who consumed cruciferous vegetables and underwent a pre- and post-washout with no vegetables for 12 days.[145] At the end of 12 days of consuming vegetables, CYP1A2 activity was increased compared to the pre-washout period. Moreover, after 12 days of not eating cruciferous vegetables the CYP1A2 activity returned to pre-washout levels. This illustrates the body's ability to adapt very rapidly to diet changes and suggests the need for chronic consumption to maintain any effects on biotransformation. However, long-term consumption of vegetables has not been investigated with regard to modulation of biotransformation enzymes; only assumptions have been made from short-term studies. Therefore, it is important to understand the effects of these vegetables on biotransformation enzymes over longer feeding periods.

Summary

A diet rich in fruits and vegetables has been shown to be protective against certain cancers. One of the mechanisms for this protective role could be the ability of plant compounds, phytonutrients, to reduce the mutagenicity and carcinogenicity of HAAs.[90] This may be mediated by influencing biotransformation enzymes and the balance between activation and inactivation of procarcinogens. Humans are exposed to HAAs in a wide variety of daily staples, therefore research regarding protection from HAAs is

imperative for prevention of certain cancers. The biological potency for PhIP-induced carcinogenicity is strongly dependent upon biotransformation enzymes and the pathways by which the metabolites are processed. Therefore, a better understanding of how diet affects carcinogen metabolism, and more specifically the activating pathways of PhIP in humans, is essential for human risk assessment of this carcinogen. Cruciferous and apiaceous vegetables have been shown to modulate some of the enzymes involved in the activation of PhIP, however most studies were short-term feeding studies. As a result, we investigated the time effect of these vegetable families individually and combined on biotransformation enzymes involved in the PhIP activation pathway. In particular, our specific aims were:

1. Determine the effects of dietary exposure from a mixture of cruciferous and apiaceous vegetables on:
    a. Rat hepatic CYP1A1 and CYP1A2 protein and activity levels
    b. Rat hepatic SULT1A1 activity levels.
2. Evaluate whether the effects on these enzymes change over time.

Chapter 4 Materials and Methods

Introduction

Most of the studies regarding vegetable modulation of biotransformation enzymes have been of short duration. In this study we investigated the modulation of cruciferous and apiaceous vegetables over time on two microsomal enzymes (CYP1A1/CYP1A2) and one cytosolic enzyme (SULT1A1). We utilized an animal model of 120 rats divided into three feeding times: 7-, 30-, and 60-days. This chapter describes the study design, materials, and methods utilized in analyzing the activity and protein expression of the microsomal enzymes and activity of the cytosolic enzyme. In addition, a method to analyze total glucosinolates in cruciferous vegetables is described.

Chemicals and Reagents

The following chemicals were purchase from Sigma-Aldrich®: ethoxyresorufin, methoxyresorufin or resorufin methyl ether, β-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium salt (NADPH), resorufin sodium salt, albumin from bovine serum (BSA), Bradford reagent, potassium hexacyanorerrate III (ferricyanide) 244023, sinigrin hydrate from horseradish, 2-naphthol, adenosine 3'-phosphate 5'-phosphosulfate A1651(PAPS), and 4-nitrophenyl sulfate from Acros organics New Jersey.

The following were purchased from Santa Cruz Biotechnology Inc, Santa Cruz CA: β-actin primary antibody (R-22 rabbit polyclonal IgG 100ug/ml sc-130657), goat anti-rabbit secondary antibody (IgG-HRP 200ug/0.5 ml), CYP1A1 primary antibody (G-18 goat polyclonal IgG 200ug/ml sc-9828), CYP1A2 primary antibody (K-15 goat

polyclonal IgG 200 ug/mlsc-9836), donkey anti-goat secondary antibody (IgG-HRP 200ug/0.5ml), and western blotting luminol reagent.

Prestained SDS-PAGE standards (low range) and Tween 20 (polyoxyethylene sorbitan monolaurate 170-6531) were purchased from BioRad (Hercules, CA). Restore™ Western Blot stripping buffer was purchased from Thermo Scientific (Rockford, IL). The TEMED (GR151-25) was purchased from Hoefer, Inc (San Francisco, CA). The isoflurane was purchased from Phoenix Pharmaceutical, Inc (St Joseph, MO).

Animals

Male Wistar rats, 225-249 g body weight for 7-day feeding group and 150-174 g for the 30-day and 60-day day feeding groups, were purchased from Harlan Laboratories (Indianapolis, IN) and were housed separately in wire-bottom stainless steel cages. Upon arrival, animals were fed AIN-93G diet for approximately 7 days to allow adaptation to a semi-purified diet. Animals were allowed free access to water and food. Animal housing and use complied with the University of Minnesota Policy on Animal Care.

Experimental Design

A total of 120 rats were ordered and subsequently divided into 3 feeding time points (7-, 30-, and 60-days). Each time point had a total of 40 rats that were subsequently divided into four types of diets (n=10 each). The diets were: basal (AIN-93G), 21% cruciferous diet (AIN-93G+ cruciferous vegetables), 9% apiaceous diet (AIN-93G + apiaceous vegetables), and 15% combination diet (10.5% cruciferous vegetables + 4.5% apiaceous vegetables). The 7-day feeding group arrived and was sacrificed before the arrival of the 30- and 60-day feeding groups.

Diets

Upon arrival animals were fed AIN-93G diet[146] (Table 4.1). Vegetables were purchased from the local market Lund Food Holdings, Inc. The cruciferous diet consisted of: hydroponically grown watercress (Live Gourmet®), USDA organic biologique broccoli (Earthbound Farm®), and USDA organic green cabbage™. The apiaceous diet consisted of USDA organic celery (Earthbound Farm®) and the parsnips came from Mississippi Market store, also organically grown (no brand).

Vegetables were cut and ground with a food processor (Cuisinart Delux 11™) and added to the powdered diet accordingly: 21% wt:wt cruciferous diet (70 g/kg diet of each vegetable), 9% wt:wt apiaceous diet (45 g/kg of each vegetable), 15% wt:wt combination diet (35 g/kg of each cruciferous vegetable plus 22.5 g/kg of each apiaceous vegetable). Diets were made every 10-13 days and aliquots on each diet stored in bags at -80 ^{o}C. One bag was thawed daily and given to animals.

Food intake was determined from spillage and diet remaining in the food cup during 24 hours. Data are shown as dry food intake from the vegetable containing diets and were calculated using the following formula where: DW= dry weight, FW=fresh weight: DW = (FW) - (FW x vegetable amount) + ((FW x vegetable amount) x (The sum of all the dry weight vegetables)*

* Dry weight was determined according to the USDA nutrient database of each individual vegetable and summed according to diet.

Isolation of Liver Microsomes and Cytosol

At the end of each feeding time point animals were fasted for 12 hours and were anesthetized with isoflurane and microsomes isolated using the method described by Prasad et al.[147] Briefly, livers were perfused *in situ* with ice-cold 50 mM Tris base – 150 mM KCl buffer (pH7.5) and homogenized in Tris-KCl buffer containing phenylmethylsulphonyl fluoride (1 mM final concentration). The microsomal and cytosolic fractions were obtained by utltracentrifugation of the 10 000 *g* supernatant at 105 000 *g* for 70 minutes. The microsomal pellet was resuspended in Tris-glycerol buffer (pH 7.5) and the supernatant stored for cytosolic assay. Both samples were kept at -80^{o}C. Prior to measuring CYP1A1 and CYP1A2 activity, microsomes were washed by ultracentrifugation at 105 000*g* with 100mM sodium pyrophosphate/10 mM EDTA buffer and resuspended in 500 µl of 58 mM Tris buffer, and the remaining was used for blotting. Appendix A-1 and A-2.

Protein Content

Protein concentration was determined according to the method proposed by Bradford using bovine serum albumin as a standard.[148] Microsomal protein or standard (5 µl) was added to 200 µl of Bradford reagent Sigma® in an assay plate. For the cytosolic samples 10 µl protein or standard was added to 990 µl reagent in a cuvette. Both concentrations were determined spectrophotometrically at 595 nm. Appendix A-3.

CYP1A1 and CYP1A2 Activity

To assess the activity of CYP1A1 and CYP1A2 in the microsomal fraction a kinetic assay for 7-ethoxyresorfurin O-deethylation and 7-methoxyresorfurin O-demethylation

was performed after modification of previous methods.[149, 150] Briefly, the final reaction mixture consisted of 5 μM corresponding substrates, 0.05 M Tris buffer pH 7.5, 0.025 M $MgCl_2$, and 500 μM NADPH. Later, microsomal protein was added to a final concentration of 300 μg/ml. The experiment was conducted in 96-well plates kept cold until inserted in a Biotek Synergy HT microplate reader (excitation: 530/25 nm; emission: 590/35 nm). The protocol consisted of a 5 minute lag time at 37°C, followed by measurement of resorufin formation spectrofluorimetrically every 1.15 minutes for 15 minutes. Results were quantitated by comparison to fluorescence of the known amounts of authentic resorufin. The plate consisted of duplicate samples aliquotted into 4 wells/sample. Appendix A-4.

CYP1A1 and CYP1A2 Protein Expression

Protein expression was assessed immunoblotting.[151] Liver microsomes (1 mg/ml) were dissolved in 1x sample buffer including 10% sodium dodecyl sulphate, heated for 15 minutes, resolved by polyacrilamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked for 2 hours in blocking buffer containing 5% bovine serum albumin in a Tris-buffered saline solution (0.5 M Tris base, 9% NaCl, 1.0% Tween 20 pH 7.8). After blocking, the membranes were incubated with B-Actin primary antibody, CYP1A1 and CYP1A2 goat polyclonal primary antibodies for about 13-15 hours and 17-19 hours respectively, at 4°C. Incubation with corresponding peroxidase-conjugated secondary antibody was carried out for 1 hour at room temperature. The bands were visualized by the enhanced chemiluminescence method according to the manufacturer's instructions (Santa Cruz

Biotechnology, Inc Santa Cruz, CA). After each visualization, membranes were stripped of antibodies using stripping buffer according to the Thermo Scientific protocol. The intensity of protein bands was quantified using the ImageJ software program. Each membrane had duplicates of each sample and a normalized sample (sample used in all blots) therefore the final number was calculated by the ratio of enzyme/β-Actin then divided by the ratio of the normalized sample. Appendix A-5.

SULT1A1 Activity

Cytosolic liver SULT1A1 was assessed by Maria Nelson (undergraduate student) via a modification of previous methods.[152] Final reaction mixture consisted of the following reagents: 800 ul from 50 mM potassium phosphate buffer pH 6.5, 5 mM magnesium chloride, 20 uM PAPS (5'-phosphoadenosine 3'-phosphosulfate), 5 mM p-nitrophenyl sulfate, then the addition of 100 µl of 0.1mM Naphthol, and 400 µg/100 µl protein sample for a total final volume of 1 ml. SULT1A1 catalyzes the synthesis of 2-naphthylsulfate from PAPS and 2-nathphtol. P-nitrophenyl sulfate is needed to regenerate PAS and produce p-nitrophenol during the assay. The mixture was incubated for 15 minutes at 37ºC and terminated with Tris-HCl buffer, pH 8.7. P-nitrophenol was quantified colorimetrically at 405 nm. Appendix A-6.

Glucosinolate Analysis

Glucosinolates were analyzed by Cynthia Gallaher (research scientist) according to the new method by Gallaher et al (submitted for publication) modified from Jezek et al.[153] Briefly, cruciferous vegetables were frozen by liquid nitrogen, lyophilized and ground then treated with 100% methanol (MeOH) for 20 minute at 80ºC to prevent myrosinase

(thioglucoside glucohydrolase) activity. Water was added (80% MeOH) and the sample extracted for an additional 30 min. This homogenate was centrifuged for 20 minute at 18,000 g and the supernatant dried under nitrogen. Glucosinolates were reconstituted with water and isolated using an anion exchange column. They were hydrolyzed with 2 N NaOH to release 1-thioglucose and ferricyanide was added (reacts with 1-thioglucose). The decrease in absorbance was measured at 2 min, at 420 nm and final values were adjusted for interfering compounds. Sinigrin was used to make the standard curve. Appendix A-6.

Statistical Analysis

The data were analyzed by one-way analysis of variance using Proc mixed (SAS Institute, Cary, NC) for enzyme activity and protein expression and p-values were not adjusted. Proc GLM was used for food intake and body and liver weight. P-values were adjusted for multiple comparisons using Duncan's method. Utilizing linear regression analysis, the correlation between CYP1A protein and activity was analyzed.

Table 4-1. Diet Composition

AIN-93G Diet	**Basal (g/Kg)**	**21% Cruciferous (g/Kg)**	**15% Combination (g/Kg)**	**9% Apiaceous (g/Kg)**
Cornstarch	397.5	387. 9 (9.6g)*	388 (9.5)*	388 (9.5)*
Casein	200.00	195.5 (4. 5g)*	196 (4.0)*	196.5 (3.5)*
Dextrinized Cornstarch	132.00	132.00	132.00	132.00
Sucrose	100.00	100.00	100.00	100.00
Fiber	50.00	46.1 (3.9g)*	46.5 (3.5)*	47.1 (2.9)*
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
L-Cystine	3.00	3.00	3.00	3.00
Choline Bitartrate	2.50	2.50	2.50	2.50
Soybean oil	70.00	69.6 (0.4g)*	69.7 (0.31)*	69. 8 (0.22)*
Cruciferous	N/A	210g	105g	N/A
Apiaceous	N/A	N/A	45 g	90 g
TOTAL	**1000g**	**1191. 6**	**1132. 7**	**1073. 9**
% Carbohydrate	62.95	62. 95	62.95	62.95
% Protein	20.00	20.00	20.00	20.00
% Fat	7.00	7.00	7.00	7.00
% Dietary fiber	5.00	5.00	5.00	5. 00

* Nutrient contribution from the vegetable according to USDA National Nutrient Database for Standard Reference, Release 22 (2009)[154].

- The antioxidant BHT was not needed due to soybean oil with 0.02% tBHQ

Chapter 5 Results

This chapter presents the results of our study, including food intake, body and liver weight, as well as CYP1A activity and protein expression and SULT1A activity. Further, the total glucosinolate content of cruciferous vegetables is also presented.

Food Intake

There were some statistically significant differences in food intake between diet groups, but the actual differences were small. Furthermore, no particular diet group was consistently different from the others within each time point as detailed below.

Figure 5.1 shows food intake for the 7-day time point. The cruciferous diet group consumed less than basal and apiaceous diet groups (p=0. 02 and 0.03, respectively), but the difference was only 2 grams (g) dry weight/day (d). Figure 5.2 shows food intake for the 30-day time point. No statistically significant difference was found across the groups within any week period. Figure 5.3 shows food intake for the 60-day time point; four out of eight measurements indicate statistically significant differences, however this was not consistent for any particular diet. During the 1st week the apiaceous diet group consumed about 2 g/d more than basal and cruciferous diet groups (p= 0.02 and 0.01 respectively). During the 4th week the basal group consumed about 3 g/d more than cruciferous and combination groups (p= 0.006 and 0.03) and the apiaceous group consumed more than the cruciferous group (p=0.02). At the 5th week the basal diet group consumed more than the cruciferous group (p=0.03) and at week 8 the basal group consumed about 3 g more than the apiaceous diet group (p=0.03).

Body and Liver Weight

The 7-day group (n=40) had an initial weight of 238 – 246 g (figure 5.4). The 30- and 60-day groups arrived shortly after completion of the first group and had initial weights of 164 – 171 g and 160 – 168 g, respectively (Figures 5.5 and 5.6). Overall, across the diets within each time point animals had a similar growth pattern.

Table 5.1 shows liver weight for all animals across each time point. No statistically significant difference among the diet groups within each time point was observed.

CYP1A1 Activity

The cruciferous diet significantly increased CYP1A1 activity ($p<0.0001$), nevertheless this increase was diminished over time with a trend-like p-value of 0.08 for interaction between cruciferous vegetables and time. That is, the difference between the basal and cruciferous groups decreased over time. There were no statistically significant differences in enzyme activity among the other diets at any time point. Time significantly influenced CYP1A1 activity ($p=0.04$), overtime, CYP1A1 activity increased. The individual differences Awithin a time point are shown in the figure 5.7 A.

CYP1A1 Protein Expression

The cruciferous diet group significantly increased CYP1A1 expression at the 7-day period ($p=0.007$), but this induction was not sustained over time. Even though at 7 days the combination diet significantly induced protein expression, overall it had no statistically significant difference. The apiaceous diet had no effect on protein expression at any time point. Protein expression significantly decreased over time ($p<0.0001$). The individual differences within a time point are shown in Figure 5.7 B.

CYP1A2 Activity

The cruciferous diet significantly increased CYP1A2 activity compared to the basal diet ($p=0.0005$) and this increase was sustained over time. The apiaceous diet did not have an effect on CYP1A2 activity and the combination diet had a trend towards increased activity ($p=0.085$), relative to the basal diet. There was no interaction between time and any of the vegetable diets. CYP1A2 activity decreased significantly overtime ($p= 0.01$). The individual differences within a time point are shown in Figure 5.8 A.

CYP1A2 Protein Expression

The cruciferous diet and combination diet groups significantly increased expression compared to the basal diet at 7-days ($p=0.0059$ and $p=0.004$ respectively). But the induction was not sustained after 7 days. No interaction between time and vegetable effect was noted. All the other diets had no effect on expression and therefore no interactions were noted. There was a significant decrease in protein expression over time ($p=<0.0001$). The individual differences within a time point are shown in Figure 5.8 B.

Correlations

To explore the mechanism of regulation of CYP1A enzymes we analyzed correlation. There was no correlation between enzyme activity and protein expression for either CYP1A1 (Figure 5.9) or CYP1A2 (Figure 5.10). The correlation between enzyme activity and protein expression for only the basal groups (graph not shown) were CYP1A1 $R^2= 0.015$ and CYP1A2 $R^2= 0.011$. Overall, no correlation was noted between protein expression and enzyme activity.

SULT1A1 Activity

In 7-day animals, the apiaceous diet significantly inhibited SULT1A1 activity ($p=0.02$). However, there was no inhibition at 30-day or 60-day such that across all time points, there was no significant difference between the apiaceous diet and the basal diet ($p=0.15$). No other diets had a significant effect and consequently no interactions were noted. See figure 5.11.

Glucosinolate Content

Throughout our feeding study we had a total of 5 batches of diet prepared therefore 5 samples of vegetables. We analyzed the total glucosinolate content in four batches; the fifth batch was not analyzed due to lyophilization problems with the watercress sample. The results were: 1st batch = 628.7, 2nd batch = 897. 2, 3rd batch = 1009.4 and 4th batch = 850.6 µmol total glucosinolates /100 g fresh weight.

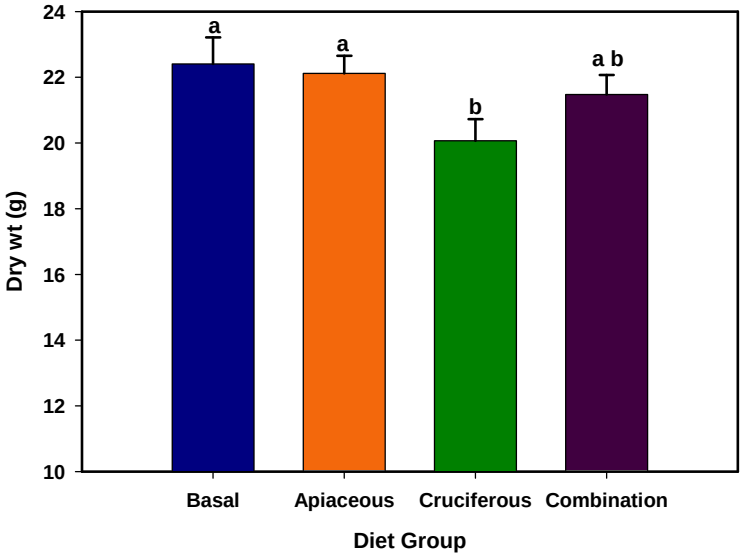

Figure 5-1. 7-day Food Intake Based on Dry Weight. Values shown as means ± SEM. Bars sharing the same letter are not different from each other ($p>0.05$), n = 10 per group.

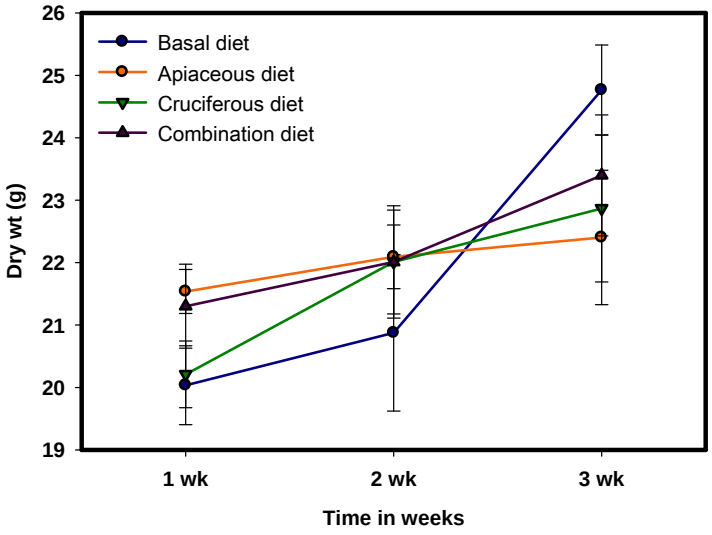

Figure 5-2. 30-day Food Intake Based on Dry Weight. Values shown as means ± SEM of n = 10 per group. There was no difference within each week across the diet groups.

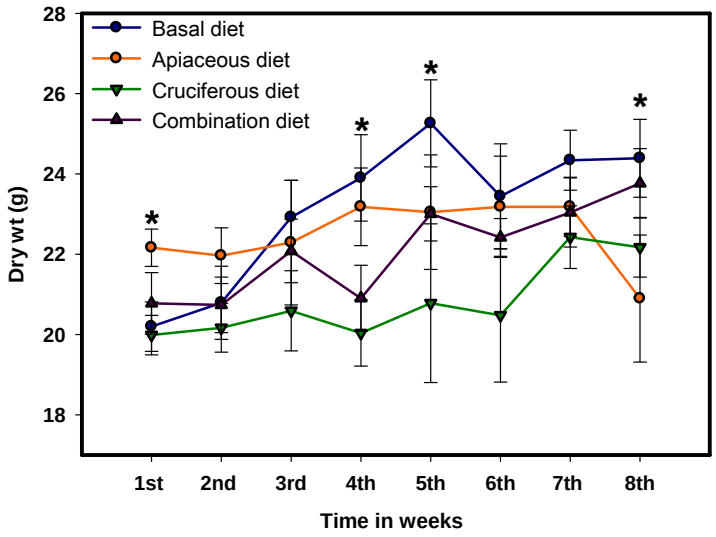

Figure 5-3. 60-day Food Intake Based on Dry Weight. Values are shown as means ± SEM, n = 10 - 11 per group. Asterisks represent differences across diet groups within the week ($p<0.05$). P-values were adjusted for multiple comparisons using Duncan method.

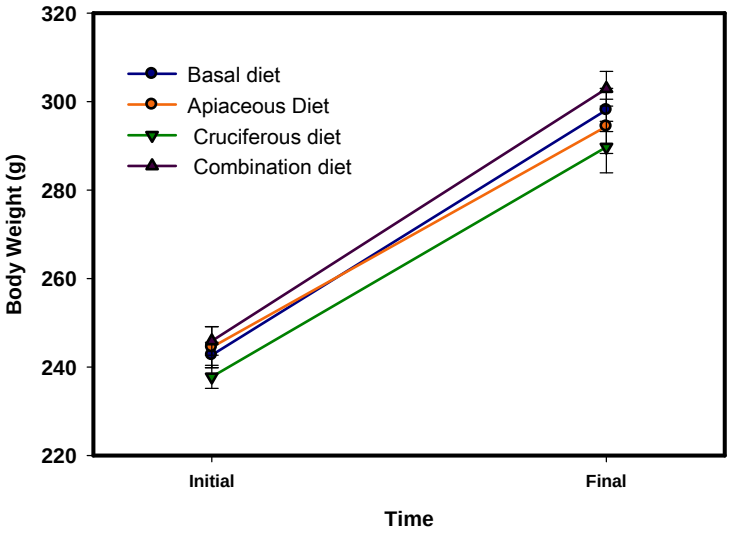

Figure 5-4. 7-day Body Weight. Values shown as means ± SEM, n = 10.

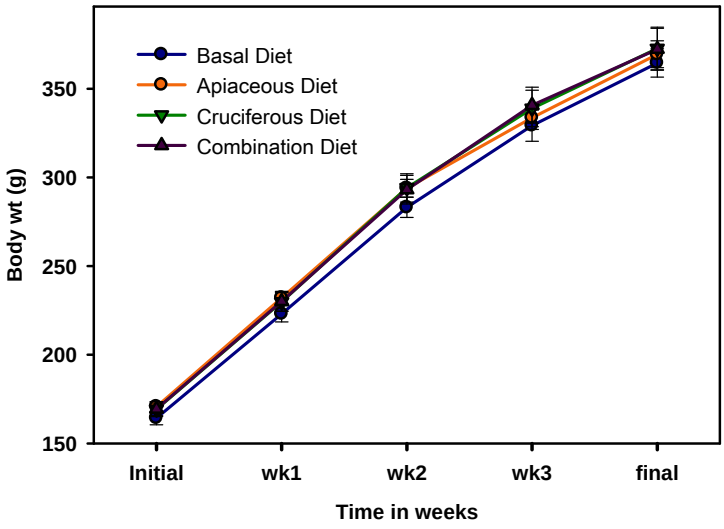

Figure 5-5. 30-day Body Weight. Values shown as means ± SEM, n = 10.

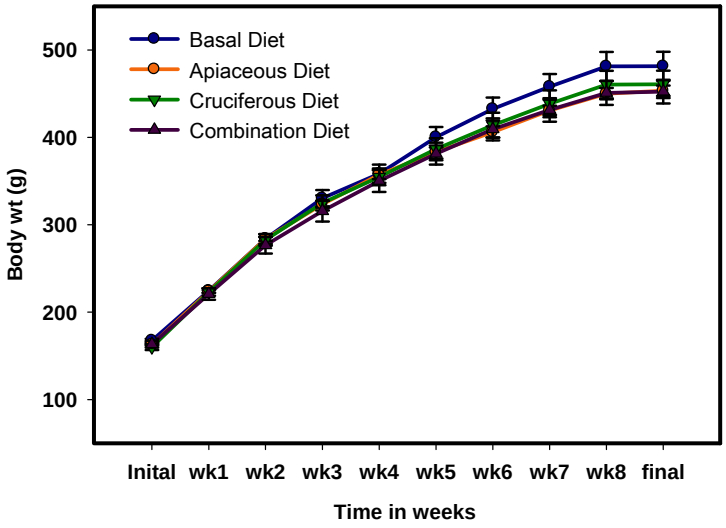

Figure 5-6. 60-day Body Weight. Values shown as means ± SEM, n = 10.

Table 5-1. Liver Weight (g). Values shown as means ± SEM, n = 10. No difference among the diet groups within each time point.

Liver wt (g)	Basal	Apiaceous	Cruciferous	Combination
7 day	10.66 ± 0.37	9.86 ± 0.28	10.07 ± 0.38	10.12 ± 0.33
30 day	12.07 ± 0.44	11.82 ± 0.36	12.06 ± 0.40	11.98 ± 0.70
60 day	14.20 ± 0.65	13.25 ± 0.56	12.86 ± 0.56	14.34 ± 0.76

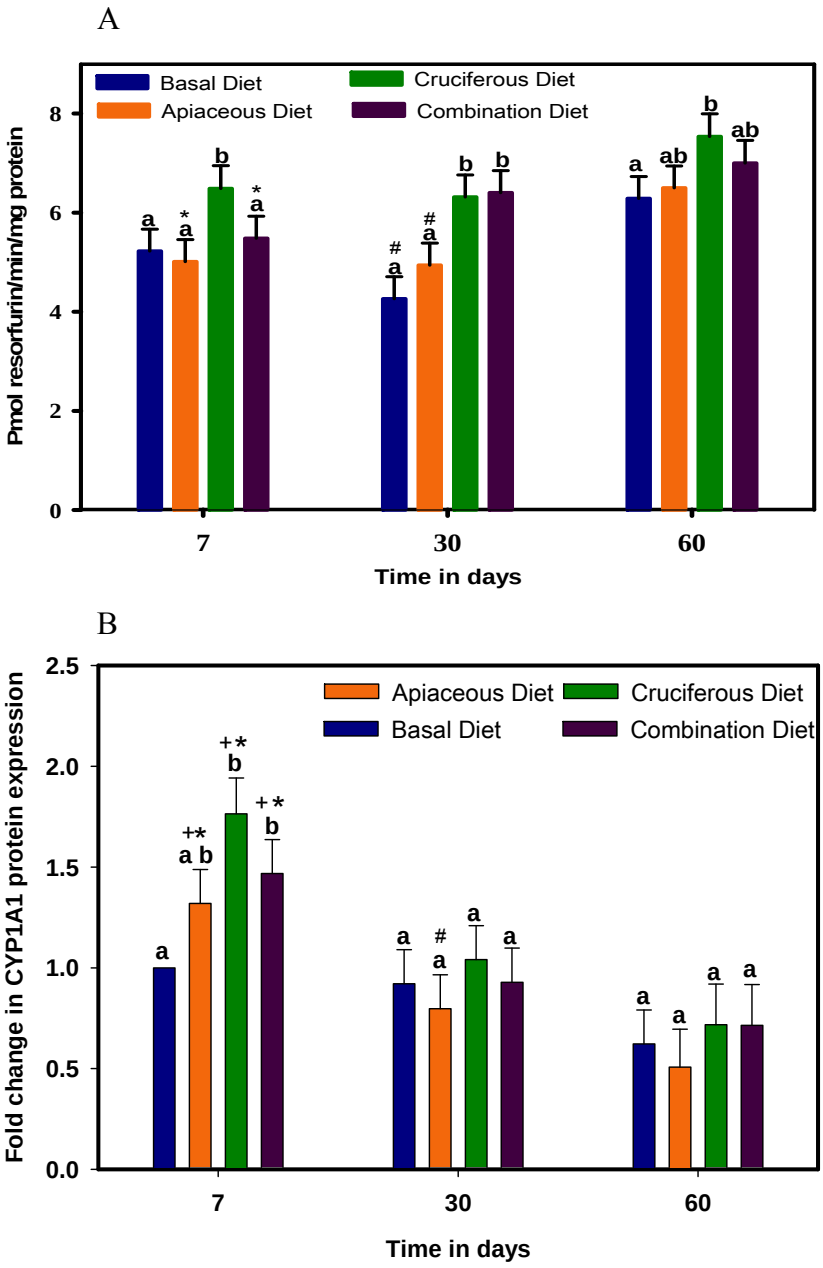

Figure 5-7. CYP1A1 Enzyme Activity and Protein Expression. Results are shown as least squares means ± SEM. Bars sharing the same letters within a time group are not different from each other. The signs represent differences amongst time points: 7- vs 30-day plus (+), 7- vs 60-day asterisk (*), 30- vs 60-day pound (#). A) **CYP1A1 activity,** n = 9 - 11. B) **CYP1A1 expression** n= 7 - 8. Results are shown as change in fold from 7 day basal diet.

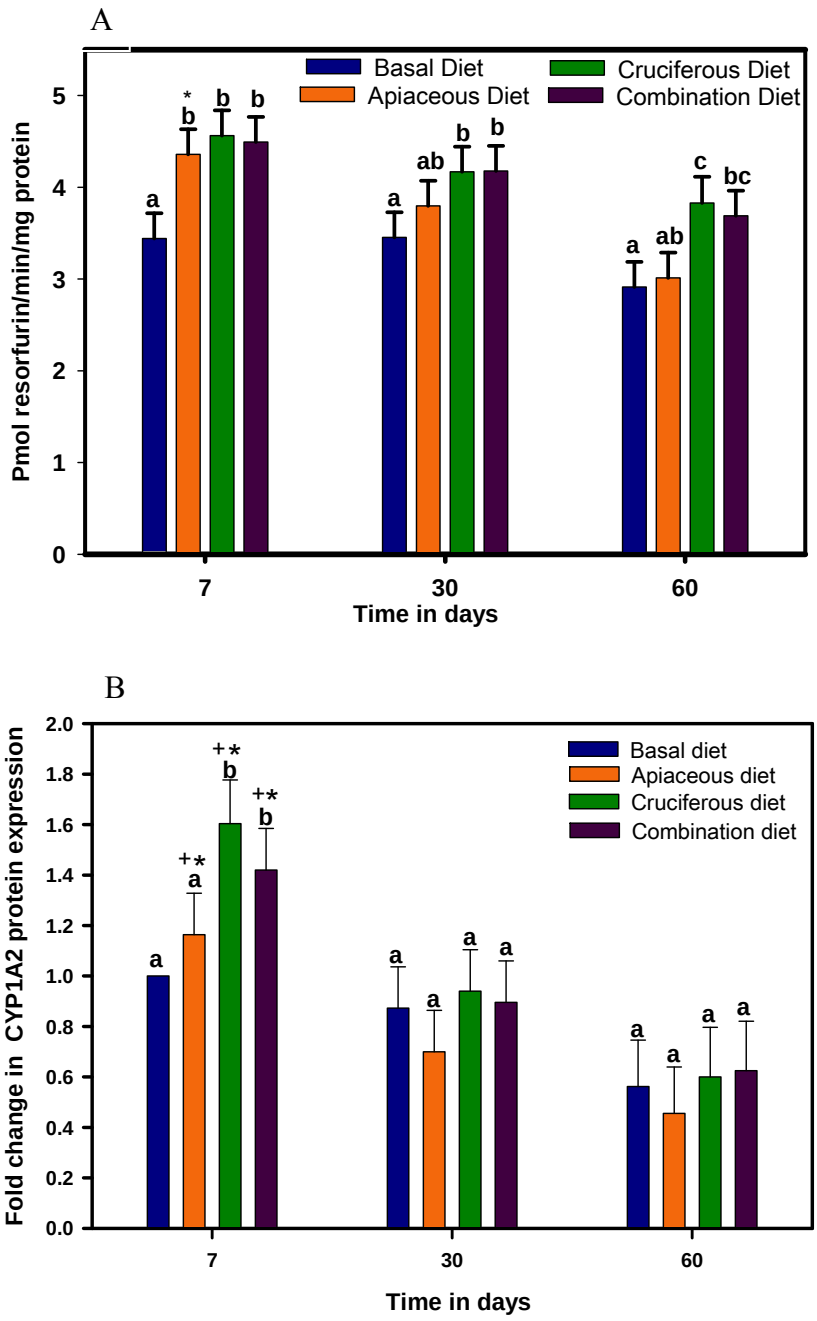

Figure 5-8. CYP1A2 Enzyme Activity and Protein Expression. Results are shown as least squares means ± SEM. Bars sharing the same letters within a time group are not different from each other. The signs represent differences amongst time points: 7- vs 30-day plus (+), 7- vs 60-day asterisks (*). A) **CYP1A2 activity** n = 9 - 11. B) **CYP1A2 expression** n = 7 - 8. Results are shown as change in fold from 7 day basal diet.

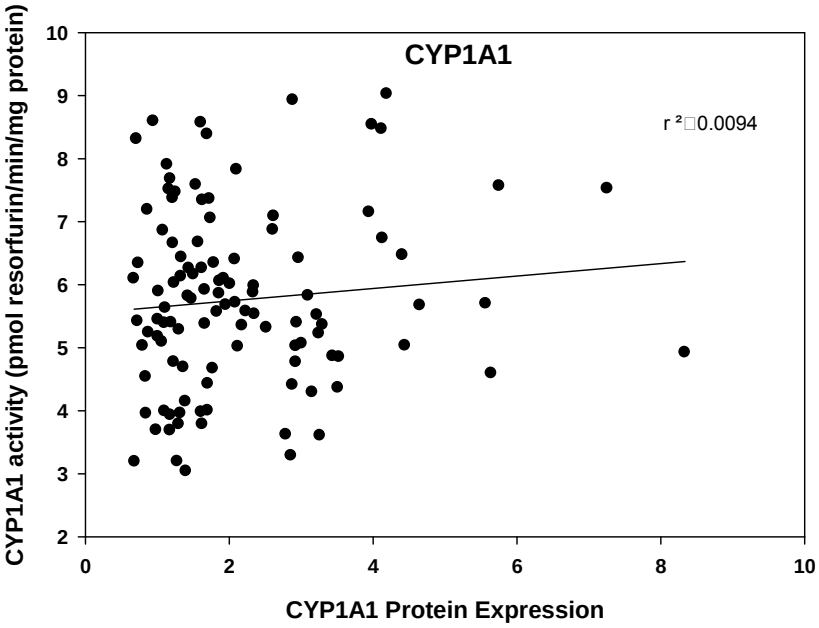

Figure 5-9. CYP1A1 Enzyme Activity and Protein Expression Correlation.

Correlation for protein expression, presented in densitometry units, and protein activity presented as amount of product, n = 109.

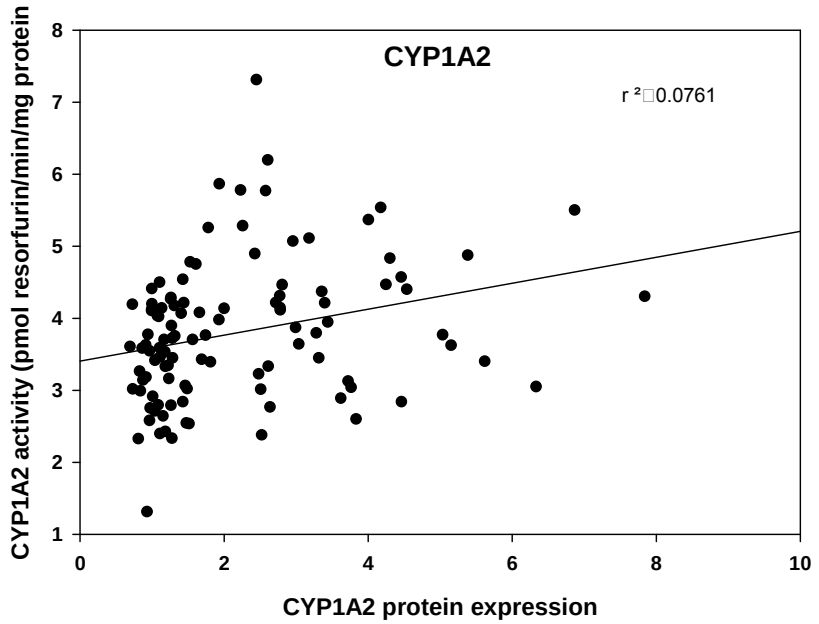

Figure 5-10. CYP1A2 Enzyme Activity and Protein Expression Correlation.

Correlation for protein expression, presented in densitometry units, and protein activity presented as amount of product, n = 109.

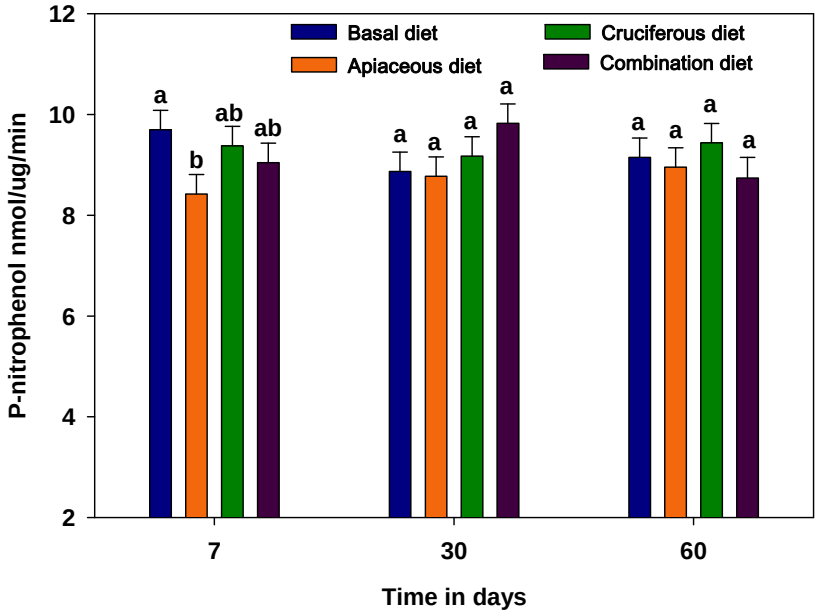

Figure 5-11. SULT1A1 Activity. Results are shown as least squares means ± SEM, n= 10 - 11. Bars sharing the same letters within a time group are not different from each other. No difference over time.

Chapter 6 Discussion and Future Directions

Discussion

In previous chemoprevention studies utilizing vegetables as a modulator of biotransformation enzymes, the exposures were usually short-term. The major goal of this project was to determine if the effects of vegetables on biotransformation enzymes changed over longer exposure times.

We looked at three enzymes (CYP1A1, CYP1A2, and SULT1A1) and found no statistically significant interactions between vegetable effects and time. As expected and in agreement with several studies, cruciferous vegetables did increase CYP1A1 and CYP1A2 activity and protein expression. Contrary to our expectations, apiaceous vegetables did not inhibit CYP1A1 or CYP1A2. This lack of an overall effect from apiaceous vegetables could be due to dosage. As mentioned above, few studies have been done regarding this class of vegetables and CYP1A enzymes. Most studies utilized purified furanocoumarins and a few human studies utilized actual apiaceous vegetables. Consequently, extrapolation of vegetable doses was challenging when designing this vegetable feeding study in rats. In addition, the diet needed to be palatable, avoiding a dietary concentration of apiaceous vegetables that would negatively affect food intake (which was accomplished in this study). Therefore we chose 9% (wt:wt) apiaceous vegetables supplemented to the rodent diet, which is the equivalent of 46 g/day fresh weight for human consumption based on human energy equivalence calculations. For comparison, one of the studies that showed an inhibitory effect on CYP1A2 in humans provided 265.5 g/day of apiaceous vegetables.[99] We recommend a higher but tolerable dose for future rodent studies.

Hence, regarding the question of whether these vegetable effects on biotransformation enzymes change over time we were only able to answer for cruciferous vegetables since the other diets did not show any effect. For CYP1A1 activity there was a trend for interaction between time and cruciferous vegetables. This result warrants further investigation and caution in the interpretation of short-term feeding studies. Because if time is interacting with the effect, short-term studies will not capture the true results that humans have from a long exposure to these vegetables. For the SULT1A1 enzyme, only a decrease in activity at 7-day from the apiaceous vegetables was observed, but overall none of the diets significantly changed its activity. To our knowledge, this is the first report of apiaceous vegetables inhibiting SULT1A1 and warrants further investigation, given the role of SULT1A1 in drug and other xenobiotic metabolism.

In addition, time was an influential variable on CYP1A1 and CYP1A2 for both activity and protein expression. At the end of the study rats were approximately 10, 11, and 13 weeks old for the 7-, 30-, and 60-day feedings, respectively. Time, in this model, accounts for age and other non-specific variables. Hepatic CYP expression is known to change with age in an isoform-specific manner.[155] Sprague-Dawley rats showed an increase in mRNA levels of CYP1A2 and EROD activity up to 30 postnatal days but no differences were noted when comparing between 8 and 12 weeks of age.[156] In another study, also with Sprague-Dawley rats, looking at CYP1A protein expression for 3, 12, 26, and 104 weeks, CYP1A1 and CYP1A2 protein expression and enzyme activity were decreased at the 12th week compared to the 3rd week.[155] Protein expression for CYP1A2 did not change from the 12th to 26th week, however EROD and MROD were further decreased. In another study with male and female Wistar rats looking at 1, 3, 7, 14, 26,

52, and 104 weeks, CYP1A1 and CYP1A2 did not change in protein expression overall.[157] These results show some changes in CYP enzymes by age but mainly when comparing to developmental time points, such as postnatal versus adult; no studies have looked at the differences in the 8-13 week time frame. More studies are needed to determine if this little difference in age in our rats had an influence on CYP1A enzymes.

Few studies have compared correlations between CYP1A1 and CYP1A2 protein and activity levels after exposure to these vegetables, but the lack of correlation in this study is not unprecedented. For example, some studies have showed that CYP1A1 protein expression was not detected despite EROD activity in the same sample.[95,91, 155] Discrepancies also arise between other enzyme regulation points such as mRNA level versus protein level. After a 10% broccoli diet fed to female Wistar rats, CYP1A1 and CYP1A2 had similar mRNA induction however very different protein expression levels.[93] And in another study with glucosinolates, discrepancies were shown between protein and activity levels for both CYP1A1 and CYP1A2 where it showed higher activity on samples with lower protein levels.[111] Most studies typically only analyze activity levels of these enzymes.[94, 96, 97, 100] This lack of correlation between protein and activity levels could be due to a few reasons. First, is the issue of assay limitations which will be described later. Another reason could be due to other mechanisms involved in CYP regulation from cruciferous vegetables. Some investigators already alluded to the fact that post-translational protein stabilization might be another important means of CYP regulation.[111, 158] This effect was already shown with CYP2E1 by xenobiotic inducers.[159] The results of this study showed cruciferous vegetables are enhancing the activity of CYP1A1 and CYP1A2. It also insinuates that the body is responding in a "negative

feedback mechanism" and therefore less protein is needed as shown by lower protein levels at 30- and 60-day feeding groups.

Strengths

There are several strengths to our study. We attempted to simulate similar vegetable preparation from humans for rats by feeding them fresh vegetables as opposed to lyophilized vegetables or purified compounds. In addition, the diets were balanced for macronutrients to minimize confounding by these variables. The dosage was physiologically possible to achieve in a day, for the cruciferous and combination diets were equivalent to 106g/day and 76g/day for humans. It is important to study the effects of vegetables in relative amounts that people are likely to consume given that the latest data of US consumption of vegetables from 2009 indicates that only about 26. 3% consumed vegetables three or more times per day.[160]

As opposed to pharmacological agents, nutrition interventions usually have less drastic results therefore it is important to have a large enough sample size for statistical power. We had a total of 120 rats which provided 10 rats per group to provide this statistical power. Another strength of this study was the in vivo approach which allows the vegetables to interact with the complex biological system and the competing reactions by CYP enzymes. This approach reflects the similar interaction also seen in humans. In addition, we incorporated two classes of vegetables in contrast to most studies that usually study single agents or foods. Human diets consist of a mixture of compounds from a variety of foods present at each meal; therefore it is important to start to investigate interactions amongst them.

Limitations

Utilizing a rat-based model has its advantages and disadvantages. The advantage is the ability to harvest the liver and analyze protein and activity levels directly. Human studies typically require reliance on excretion of products, probes or biopsies from usually sick people that may not be representative of the general population. Nonetheless, the assessment of these enzymes from harvested rat livers also has limitations. For protein expression most studies use Western blotting. This technique allows specificity for CYP isoforms however it is questionable if it is sufficiently sensitive to detect the small differences usually noted in nutrition intervention studies. More so, the assessment of activity also has its limitations. Older studies referred to EROD as an assay for the CYP1A family in general; however, CYP1A2 metabolizes 7-ethoxyresorfurin at a much lower rate than CYP1A1.[150] Therefore, MROD was recently introduced as a more specific assay for CYP1A2, although other CYPs can also act on it.[161] A review of the literature indicates that most studies investigate activity levels more than protein expression. When interpreting CYP activity results, it is important to keep in mind the caveat of cross-reactivity amongst CYP isoforms for the same substrate, nevertheless assessing activity can be a good and widely accepted measure.

More importantly, an animal model may raise some concerns about interspecies differences. Despite the strong conservation among human and rat CYP1A1 and CYP1A2 enzymes, 83% and 80% for CYP1A1 and CYP1A2, respectively,[162] studies have shown significant differences between rat and human CYP1A catalytic activity and regioselectivity. For example, the drug omeprazole was shown to induce CYP1A2 in humans but not in rodents.[163] Also differences in inhibitory potency were shown with

furafylline, an inhibitor of CYP1A2; rodents need a dose 1000-fold higher for similar inhibition to humans.[164] More importantly, interspecies differences have been shown in regards to PhIP metabolism. It is difficult to compare total amount of CYP protein between humans and rats due to their inducibility. Nevertheless, humans tend to have more hepatic CYP1A2 than rats, with estimations of 5-245 pmol/mg protein and 5-35 pmol/mg protein, respectively.[165] But controlling for total protein and given similar MROD results, it was shown that humans *N*-hydroxylate PhIP much more efficiently than rats.[63] Likewise, Langouët et al.[64] found that hepatocytes from humans were significantly more active in the conversion of PhIP to the genotoxic metabolites than rats. In addition, it was observed that N-oxidation (activation pathway) predominates in humans. Rats seem to favor detoxification routes of PhIP; the 4`-OH-PhIP metabolite alone accounts for 20% of total metabolites.[64] Regarding SULT enzymes, one study showed DNA binding of N-OH-PhIP was significantly higher in human liver cytosols and recombinant SULTs than in the rat counterparts.[166] These differences suggest an underestimation of human risk regarding PhIP metabolism and therefore interspecies differences need to be taken into consideration for risk assessments.

Another limitation was the fact that we purchased vegetables from local vendors at different points in time and not from a controlled environment. This was an attempt to mimic similar human exposure to the phytochemicals in the vegetables. However, phytochemicals in the vegetables are known to be affected by environmental stress and growing conditions. For that reason we analyzed glucosinolate content of the cruciferous vegetables and are planning to analyze the furanocoumarin content of the apiaceous vegetables. Although the glucosinolate content varied from batch to batch, the levels in

each batch were sufficient to elicit the expected effects on CYP1A1 and CYP1A2 based on previous publications.[94-97,111]

Future directions

CYP1A enzymes are thought to be mainly regulated transcriptionally however recent evidence suggests other important control mechanisms might significantly impact overall activity. One mechanism that has not been given much attention is post-translational stabilization of these enzymes. In addition, an integration of other enzyme systems, pathways, or compounds are needed to interpret results. CYP1A enzymes not only have detrimental effect, but also protective effects against other carcinogens as shown by an increased BaP-DNA adduct levels in Cyp1a1(-/-) knockout mice when compared to Cyp1a1(+/-) mice.[167] Besides, they might act in concert with other biotransformation enzymes, such as phase II enzymes.[72] Therefore, a future approach should include a collaboration of molecular-cellular biologists and horticulturists in combination with robust technologies that study whole biological systems such as proteomics or metabolomics.

Given the interspecies differences and the complexity of detoxification systems it is important to conduct human intervention studies. Investigating the long-term effect of whole plants individually or in a combination diet at normally consumable levels in humans is a project worth pursuing to confirm results from animal studies and putative mechanisms identified through in vitro studies.

Conclusion

Cancer is a growing health problem around the world. This year alone 569,490 Americans are expected to die of cancer, 18% from colon and rectal cancer alone.[168] A large body of literature indicates that 30% of cancer cases are linked to poor dietary habits and the estimate reaches 70% when referring to gastrointestinal tract cancers.[169] Red meat consumption has been reported as a "convincing" risk factor for colorectal cancer.[18] The carcinogens, HAAs, formed during cooking or processing of meats has been postulated as one of the culprits for this association. PhIP is one of the most common HAAs formed during cooking.[14, 170] PhIP formed the most DNA-adducts in the colon when compared to other tissues, such as liver, mammary, lung, and bladder tissues.[42] The biological potency for PhIP-induced carcinogenicity is strongly dependent upon it metabolism from biotransformation enzymes and the pathways by which the metabolites are processed.

The American Institute for Cancer Research recommends eating a diet high in plant foods such as fruits and vegetables to prevent against cancers, particularly of the mouth, pharynx, larynx, esophagus, stomach, lung, pancreas, and prostate.[171] And more recently the WCFR/AICR update report continues to present data showing an inverse relationship between consumption of non-starchy vegetables and fruits, and colorectal cancer.[18] One of the mechanisms for this protective role could be the ability of many plant compounds, phytonutrients, to modulate biotransformation enzymes.

Accumulating data suggest the ability of an individual to deal with the chemical stress from the environment plays a role in the etiology or exacerbation of a range of chronic conditions and diseases.[23] Diets high in plant foods have a protective role in the body.

However an individual's diet consists of a large variety of compounds, including carcinogens and mutagens, which may act as additive, synergistic, or inhibitory agents towards chemical insults. It is very significant to understand the modulation effects from our lifestyle, such as diet, on cancer and disease risks. For example, whether there is greater benefit from sporadic but frequent short-term exposures or sustained long-term exposures to putative chemoprotectants.

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APPENDICES

APPENDIX A: Methods and Assays

APPENDIX A-1 Method for Microsomal Isolation

Equipment and materials

Homogenizer (large and small probe)

Centrifuge + rotor for 20 ml tubes (10,000 X g) 6 tubes per centrifugation

Ultracentrifuge + 70Ti rotor (20 ml ultracentrifuge tubes) 8 tubes per centrifugation

Syringe with 21G needles + glass wool

Razor blades and Kim wipes

Glass plate mounted on a bucket with ice

Pipettes (1 ml + 50 ul)

Container with ice to keep the homogenizer probe cold

Beakers with ice cold water

Set aspirator and Culture tubes

Name 10 aliquots (250ul) per animal for liver + 6 aliquots of 400 ul cytosol

Liquid nitrogen for remaining liver.

Buffers

Isolation buffer: 50 mM Tris base/150 mM KCl (**pH = 7.5 at 0-4 C**)

Storage buffer: 50 mM Tris base/50% glycerol (**pH =7. 5 at 0-4 C**). Make 100 mM Tris solution and dilute with an equal volume of glycerol. Adjust the pH the next day in ice water bath and bring to final volume.

Phenylmethylsulfonyl fluoride (PMSF) dissolved in ethanol or methanol for a final concentration of 1 mM. Protease inhibitor.

Butylated hydroxytoluene (BHT) for a final concentration of 0.01%. It is kept as a 10% stock in ethanol (w/v). Antioxidant.

Procedures:

NOTE: For this procedure it is VERY IMPORTANT to keep everything cold during all times. Chill homogenizer probe using a container with ice and keep it cold. For one ultracentrifuge tube the max is ~10.5g of liver.

• Kill the animal w/ isoflurane.

- Cut the animal open, insert the 21G needle into the renal artery, and perfuse the liver with 35 mls of isolation buffer. Liver and kidney will clear if the appropriate vessel has been entered.
- Cut the blood vessels below heart. This will release the backpressure and further clear the tissues.
- Remove liver, blot dry with Kim wipes, weigh, and mince with razor blades on a glass plate mounted on a bucket with ice.
- Add 50 uL of PMSF while mincing.
- Rinse the minced liver twice with isolation buffer (we use about 1 mL) to further remove residual 10/16/03blood. Use Kim wipes to remove excess buffer.
- Add the minced liver to a centrifuge tube (Oakridge tubes) containing 3 mls of isolation buffer to 1 gram wet weight of liver plus 50 uL of PMSF. (5g per tube=15ml adjust as needed – we used two tubes per rat)
- Total liver wt 10. 5g the remains drop into liquid nitrogen for later usage.
- Homogenize the minced liver with the pre-cooled probe while keeping the centrifuge tube inside a beaker with ice-cold water. The time for homogenization will depend on how well the liver is minced. Usually 10-20 seconds is enough. Use the thicker probe homogenizer.
- Centrifuge at 10,000 X g for 15 minutes at 4C. (8300 rpm)
- Aspirate off very carefully the lipid layer that forms on the surface of the supernatant.
- Decant supernatant through a syringe (12 ml w/ Luer lock tip) containing glass wool, approximately 1/3 full to remove excess lipids collecting into the ultracentrifuge tubes. Add second tube
- Balance the tubes carefully. By adding solution buffer. Ultracentrifuge tubes at 100,000 X g (37 K rpm) for 70 minutes. Make sure to remove the tubes from the ultracentrifuge as soon as it stops spinning.
- Aspirate off and discard floating lipid.
- Keep the supernantant (cytosol), aspirating off the last drops.
- Add 1.75 mls for (10.5g) of storage buffer (Tris/glycerol) to the tube containing the pellet. Use a cold spatula to remove pellet from the surface of the glycogen pellet,

which looks like a yellowish gel. Waiting a few minutes after addition of the storage buffer might cause the microsomes to slide down the glycogen pellet, making it easier to leave the glycogen pellet behind. Glycogen will be the layer sticking to the tube. Use spatula to remove the reddish layers (where the microsomes are). Try to leave the glycogen in the tube

- Transfer the microsomes plus the storage buffer to a different tube (we used culture tubes), and quickly homogenize – small probe (5 seconds), trying to keep the tube in ice-cold water.
- Add BHT (final concentration should be 0.01%) and homogenize for 5 more seconds. For 1.75 ml you should add 17-18 ul.
- Transfers the homogenate to 250ul to vials using a positive displacement pipette, flush with nitrogen, and store at –80 C until it is time to run your assays.

APPENDIX A-2 Method for Microsomal Wash

Done immediately before running your assay. Microsomes need to be store with glycerol buffer. This step will wash glycerol, proteases and albumin.

- Wash buffer: 100 mM $Na_4P_2O_7$ (Tetrasodium pyrophosphate or *Sodium Pyrophosphate MW:446. 06*)/10 mM EDTA or *Disodium Salt MW372. 24* (**pH = 7. 8 at 0-4 C**).
- Chill equipment as necessary: deaggregator, tubes, solutions and spatulas.
- Check pH of washing buffer (tetrasodium pyrophosphate/EDTA). It should be around 7. 8
- Take ~200 ul of the microsomes in storage buffer (wash the epi tube to max protein amt) and diluting to 6 mls of washing buffer. This sample provided a protein concentration of 5-10 mg/ml. The washing buffer removes soluble protein by mass action so total protein concentration needs to be low.
- Balance the ultracentrifuge tubes by weighing to ± 0.1 g.
- Ultracentrifuge at 37 K rpm for 70 min.
- Decant and discard supernatant.
- Use a spatula to remove pellet from ultracentrifuge tube. Place microsomes in 1.7ml epi tube.
- Re-suspend washed pellet in 500 ul of 58 mM Tris buffer
- Use the deaggregator (Pellet Pestle ® Motor Kontes) gently until suspension is homogeneous
- Do Bradford assay for protein quantification and immediately the assay

APPENDIX A-3 Method for CYP1A Enzymes Protein Quantification by Bradford Assay

Microassay- Clear plate. 3 replicates

(1)Gently mix the Bradford reagent (Sigma) in the bottle and bring to room temperature.

(2)Prepare a series of tubes containing (00 Tris Buffer) 0. 250, 0. 500, 750, 1000, 1500 mg/ml protein. (With Tris buffer and BSA=Bovine Serum Albumin)

(3)Add 5 ul buffer of for 1 row. Add 5 ul protein standards to separate wells in the plate

(4)Prepare microsomes dilution: 1/10, 1/15, 1/20, dilution.

(5)Add 200ul of Bradford reagent. No need to place in ice. Cover clear plate.

(6) Wait 10 min and read absorbance at 595 nm, Intensity 3, shaking time 10 sec.

BSA Std Curve: 3 replicates = 15ul total Made on 07-27-2010

1. 00 mg/ml: Make 800 ul (533.3 ul 1.5mg + 266.7 ul buffer)

0.750 mg/ml: Make 400 ul (200 ul of 1.5 mg + 200 ul buffer)

0. 500 mg/ml: Make 600 ul (300 ul 1mg + 300 ul buffer)

0. 250 mg/ml: Make 400 ul (200 ul 0.5mg + 200 ul buffer)

Microsomes dilution: 3 replicates = 15 ul total

1/5: Make 200 ul (40 ul Microsomes + 160 ul buffer) - Will not use

1/10: Make 200 ul (100 ul of 1/5 + 100 ul buffer)

1/15: Make 100 ul (33. 3 ul of 1/5 + 66.7 ul buffer)

1/20: Make 100 ul (50 ul of 1/10 + 50 ul buffer)

APPENDIX A-4 Method for CYP1A1 and CYP1A2 Activity

- 7-Ethoxyresorfurin O-deethylase EROD and 7-Methoxyresorfurin O-demethylase MROD for CYP1A1 and CYP1A2 activity respectively.
- Final concentration in 1 ml aliquot: 0.05M Tris buffer, 0.025M MgCl2, 5uM Ethoxyresorufin or Methoxyresorufin, 500uM NADPH
- Stock solutions concentration: 0.058MTris buffer (pH7.5 at37C), 0.833M MgCl2, 1000uM Ethoxyresorufin or Methoxyresorufin, 50 mM NADPH (added 4.38ml instead of 4.15ml of DMSO so final concentration 1055uM for EROD)
- Volume per sample: 865ul Tris buffer, 30 ul MgCl2, 5ul Ethoxyresorufin or Methoxyresorufin, 100 ul protein concentration

1) Open protocol on plate reader. Everything on ice and light sensitive.
2) For Standard Curve Resorufin: Stock solution: 100,000 uM. Use 10,000 uM
   Dilut. Make 1000uM = Take 10ul 10,000 + 90ul DSMO = 100 ul 1000 uM
   Dilut. Make 100uM = Take 10ul 1,000 + 90ul DMSO = 100 ul 100uM
      (50 nM) Make 10uM= Take 10 ul 100 + 90 ul DMSO = 100 ul 10uM
      (40 nM) Make 8uM =Take 8 ul of 100 +92 ul DMSO = 100 ul 8 uM
      (25 nM) Make 5uM= Take 20 ul 10 + 20 ul DMSO = 40 ul 5uM
      (20 nM) Make 4uM= Take 20 ul 8 + 20 ul DMSO = 40 ul 4uM
      (10 nM) Make 2uM= Take 10 ul 4 + 10 ul DMOS= 20 ul 2uM
Add 5 ul dilution + 995ul of supplement (6055 ul buffer+210ul MgCl2, 70 ul NADPH)
3) Add 865ul Tris buffer, 30 ul MgCl2, 5 ul Ethoxyresorufin/Methoxyresorufin substrate (Cover in aluminum) per sample, 10 ul NADPH (0.004165g/100ul fresh stock solution 50mM) (15570 ul buffer, 540ul MgCl, 90 ul MROD, 180 ul NADPH
4) Aliquot into epi tube 900ul
5) Add 100 ul protein concentrations – Vortex gently (Level 1) until for 5-10sec.
Final volume 1 ml Final concentration: 300 ug/ml.
   For control: Add 100 ul denatured microsomes (water bath for 15 minutes)
6) Place 200 ul into the black.

7) Black plate, on ice. Read plate: Kinetic assay: Lag time 5 minute reading (every 1:15 minutes). Protocol excitation 530/25, emission 590/35. Sensitivity 75. Shaking 5 secs intensity 3. Calculations from 13 points. RFU/min

APPENDIX A-5 Western Blots

1- Sample= 1.25mg of protein/ml concentration. Add 200ul to epi tube + 50ul X5sample load buffer for final concentration of 1.0 mg/ml). Do not dilute sample buffer

X5 Sample buffer	Amount
0.6M Tris-HCl (pH 6.8)	1 ml
Glycerol	5 ml
10% SDS	2 ml
2-mercaptoethanol	0.5 ml
1% bromophenol (make 0.01g/ml)	1 ml
Deionized distilled H_2O	0.9 ml

Heat up epi tube ~15 min.

2- Gel preparation: Resolving gel – use 50ml tube. Using 1.5mm glass plate (wash w/ DDW or methanol)

Final gel concentration	15%
Deionized distilled H_2O	4.6 ml
30% Acrylamide (in bottle)	10.0 ml
Resolving gel buffer pH 8.8	5.0 ml
10% SDS	0.2 ml
10% (w/v) ammonium persulfate	0.2 ml
TEMED	12 ul
Total volume	20 ml

Add APS and TEMED last (Otherwise gel will become hard before pouring the solution into the kit-both are catalyst of gel polymerization). Handle the acrylamide with latex gloves (neurotoxic). After adding SDS only shake do not vortex due to bubbles.

3- Pour the resolving gel solution (~8 ml or until half one into green line) into glass plate and seal with 1 ml of MeOH (takes 15 min)

4- When gel has set, rinse the surface of gel with deionized water (2-3 times). Tip plate so you get the rest of the water from it (use a dry paper) OK to unclamped.

5- Pour the stacking gel solution (≈ 2-3 ml) into glass plate and <u>place the comb</u> in (takes 15 min) use Kim wipes to clean outside glass.

5% Stacking gel (10 ml)

Deionized distilled H_2O	3.4 ml
30 % Acrylamide	0.83 ml (830 ul)
Stacking gel buffer pH6.8	0.63 ml (630 ul)
10% SDS	0.05 ml (50 ul)
10% (w/v) ammonium persulfate	0.05 ml (50 ul)
TEMED	0.006 ml (6 ul)

Observe the 15ml tube, when that is hardened gel is done.

6- Unclamped plate then take comb off slowly, add used buffers until bubbles are on top. Higher side out. Place plate into the clear bucket, make sure inside full of buffer. Add 20 ul of your sample – standard letter only 5ul.

7- Fill the electrophoresis kit with tank/running buffer (inside: new buffer; outside: used buffer), new buffer dilute (50 ml buffer + 450ml water). Supply the power/voltage (for the stacking gel: up to 75 V (15 min); for the running gel: up to 150 V (90 min at 150V)

8- Prepare the membrane

Before using, activate the membrane with methanol (90 ml for 1 min), then add 810 ml DDW + 100ml X10 Transfer buffer (up to 1 L) inside the tapeware (plastic container). Use the forceps to move membrane (gently).

9- Take plates and immerse into the transfers buffer for few seconds, lift plate and get rid of stacking gel using green plastic and immerse the running gel only into the transfer buffer.

10- Take "sandwich" apparatus, clear side up, black bottom, white thing up. You will immerse all the below into the transfer buffer in this order: Placing into clear side

- Sponge, white paper, membrane, gel (cut up right + std letter on left both), paper, clear roll, sponge again.

11- Place membrane into the tank (Clear – red). Current (45 mA for 3 hours+ 90mA for 1 hour) Place stir bar into the tank, add the left over transfer buffer into tank. Place ice pack inside and the whole apparatus in the cold room.

12- After transfer, immerse the membrane in blocking buffer for 2 hr (to minimize background)

13- Rinse 3 times with 10 % TBS (50ml X10 TBS + 450ml water).

14- Make antibody dilutions. Incubate the membrane with primary antibody (AB) diluted in Blocking buffer.
This order: B-Actin, wash membrane, CYP1A1, wash membrane, CYP1A2, wash membrane (all fresh buffers)
15- Rinse the membrane with TBST (50 ml X10 + 450ml DDW) for 10 min using shaker (2 times) + 10 min using TBS.

16- Incubate the membrane with secondary AB diluted in Blocking buffer for 1 hour.

17- Wash the membrane with TBST for 10 min (2 times). Wash the membrane with TBS for 10 min (1 times)

18- Mix the ECL solution A and B (1:1) and react with the membrane (about 1.4 ml) Take transparency film, cut in half create a sandwich add tape on top. Place few papers and Kim wipes under film on cast. Place membranes between films. Std ladder on left, check on membrane the glossy side is where the protein is and that should be facing up.

19- Developing film: At the dark room. 2 minutes incubation time. Exposures for B-Actin ~3-5 min, for CYP1A enzymes 10-30 sec.

20-Stripping the membrane: Wash 3 times with TBS.

Add stripping buffer for 30 minutes at 37C, place box on shake incubator.

21- Wash 3 times with TBS then add blocking buffer. Place membrane in cold temperature. Go back to Step 12.

Dilutions: Steps 14 and 16

CYP's dilutions:	1^{st}: 1:2000 15-30sec	2^{nd} 1:4000
	4 ul anti + 8ml BSA	3ul anti + 12ml BSA
B-Actin	1^{st}: 1:2000 3 or 5 min	2^{nd} 1:4000
	4 ul + 8ml BSA	3ul +12ml BSA

Solutions:

X10 Running buffer (=tank buffer)

Tris base (FW 121.1)	30.3 g
Glycine (1.92 M)	144 g
SDS (powder)	10 g
Deionized distilled H_2O	1 L

Store at room temp.; dilute with water for using (1:10)

X10 Transfer buffer

Tris base (FW 121.1)	30.3 g
Glycine (1.92 M)	144 g
Deionized distilled H_2O	1 L

Store at room temp.; dilute with 10% methanol in water for using (1:10)

X10 TBS (0.5 M Tris base, 9% NaCl, pH 8.4)

Tris base (FW 121.1)	61 g
NaCl	90 g
Deionized distilled H_2O	1 L

Store at room temp.; dilute with water for using (1:10); Adjust pH with HCl if necessary

X10 TBST (0.5 M Tris base, 9% NaCl, 1.0% Tween 20, pH 8.4)

Tris base (FW 121.1)	61 g
NaCl	90 g
Tween 20 (use wt boat)	10 ml
Deionized distilled H_2O	1 L

Store at room temp.; dilute with water for using (1:10); Adjust pH with HCl if necessary

Blocking Buffer (5% skim milk or BSA)

Skim milk or BSA	5 g
TBST	100 ml

Use 10ml of X10 TBST + 90ml DDW

APPENDIX A-6 Method for Cytosolic SULT1A1 Activity

PROTEIN QUANTIFICATION

Protein reagent:

1- Dissolve 100mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol

2- Add 100 ml of 85% (w/v) phosphoric acid

3- Dilute to 1 liter with water and filter

4-Store in amber bottle as full as possible

Standard: Bovine serum albumin (BSA): 1mg/mL in water

Cuvette Assay:

- Prepare a series of standards containing 2.5-35 µg/ml protein

-Add 10ul of cytosolic sample

-Add 1990µl of reagent to each cuvette and vortex. Total volume 2 ml

- Wait 10 minutes and read absorbance at 595 nm.

SULT1A1 ACTIVITY

Principal of Assay:

SULT1A1 is a thermostable phenol sulfotransferase that catalyses the synthesis of 2-naphthylsulfate from 2-naphthol and 3'-phosphoadenosine 5'-phosphosulfate (PAPS). The addition of p-nitrophenyl sulfate to the assay mix contributes to an effective PAPS regenerating system (Frame et al, 2000). Consequently 3'-phosphohoadenosine 5'phosphate (PAPS) does not accumulate during the incubation to interfere with enzyme activity, but instead serves as a cofactor to cause the removal of sulfate from p-nitrophenyl sulfate to regenerate PAPS. The reaction produces p-nitrophenol that can be quantified colorimetrically at 405nm.

Method Using Liver Cytosol:

Volume of reaction mixture = 1ml

Containing

- 50 mM potassium phosphate buffer (pH 6.5)
- 5 mM magnesium chloride
- 20 uM PAPS
- 5 mM p-nitrophenyl sulfate

- 0.1 mM Naphthol
- 0.4 mg cytosolic protein

Reagents

1. 50mM Potassium phosphate, 5mM Magnesium chloride buffer (pH 6.5)
   FW Potassium phosphate = 136.09 FW Magnesium chloride = 203.3

   Reagent 1 is used as the foundation to prepare the following other components of the reaction:

   5mM ρ-Nitrophenyl sulfate

   20uM PAPS

   Naphthol – the enzyme substrate

2. 0.1mM Naphthol (umoles/ml rx vol, delivered in 100ul)
   FW = 144.17g/mole

   For example: 0.1um/ml (144.17ug/um)= 14.42ug (in each rx mixture)

   Therefore, prepare a solution containing 14.42ug in 100ul of reagent 1.

   Note: *Naphthol is not very soluble in aqueous solutions, therefore dissolve the Napththol in 100% ethanol then add and equal volume of water to give 50% ETOH. The final ethanol concentration should be less than 5 % in the reaction tube. 100ul of 50% ethanol added in a total volume of 1 ml is 5%.*

3. 5mM ρ-Nitrophenyl sulfate (umoles/ml rx vol, delivered in 800ul)
   MW = 275.5

   For example:

   Prepare 0.8 ml of this solution per sample. Therefore for 30 samples, 30 x 0.8ml = 24ml are needed. Prepare 30ml so that you have 6ml (20%) extra.

   For example: 5um/0.8ml=X/30ml X=187.5umoles(275.27ug/umole) = 51.61mg

   Dissolve 51.61mg in 30ml reagent 1

4. 20uM PAPS (0.02 umoles/ml rx vol, delivered in 20ul)
   MW = 507.26

Note: This is a very small amount to weigh out, even if making enough for multiple assays and is a relatively expensive reagent. At some point test to determine if a stock can be prepared and frozen in assay aliquots to be diluted.

5. Liver cytosol (400ug protein) and a volume of reagent 1 such that the summed volumes = 100ul

6. 0.25M Tris-HCL, pH 8.7 (100ul needed to terminate the reaction)
   FW = 157.6

Vortex and incubate covered for 15 minutes at 37C. Reaction is terminated with 100ul of 0.25M Tris-HCl buffer, pH 8.7

Read absorbance at 405nm

Method References:

1. Mulder G, Hinson J, and Gillette JR. 1977 Biochemical Pharmacology, Vol 26. Pp189-196Generation of Reactive Metabolites of N-Hydroxy-Phenacetin by Glucuronidation and Sulfation
2. Frame, L, et al. 2000, Drug Metabolism and Disposition, 28: 1063-1068. A Simple Colorimetric Assay for Phenotyping the Major Human Thermostable Phenol Sulfotransferase (Sult1A1) Using Platelet Cytosols
3. Maiti S, et al. 2004 Biochemical and Biophysical Research Communications 323, 235-241. Stress regulation of sulfotransferases in male rat liver.

APPENDIX A-7 Method for 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/mole0.5N = 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

<u>Column and sample preparation:</u>

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 B: Data Set and SAS Codes

```
data HFHL_TimeStudy_Analysis (label='all data')
seven_day (label='7 day data only')
thirty_day (label='30 day data only')
sixty_day (label='60 day data only')
basal_only (label='basal diet only');
;
Title2 'February 2011 Analysis: Kinetics (EROD-MROD), Body weight, Food
Intake, Liver weight';
Input  Animal  Diet Time Block  Initial_wt_g  week_1wt  week_2wt
  week_3wt  week_4wt  week_5wt  week_6wt  week_7wt  week_8wt
Final_wt  Wt_Change_Int_FinalF_FI_1_week D_FI_1_week F_FI_2_week
  D_FI_2_week F_FI_3_week D_FI_3_week F_FI_4_week D_FI_4_week
F_FI_5_week D_FI_5_week F_FI_6_week D_FI_6_week F_FI_7_week D_FI_7_week
  F_FI_8_week D_FI_8_week Liver_wt_g  EROD MROD Plate  Api  Cru CYP1A1
CYP1A2 SULT1A1
;

Label Animal= 'Animal number'
Diet= 'Diet type'
Time= 'Feeding time'
Block= 'Block animal was sacrificed'
week_1wt= '1st week animal wt (g)'
week_2wt= '2nd week animal wt (g)'
week_3wt= '3rd week animal wt (g)'
week_4wt= '4th week animal wt (g)'
week_5wt= '5th week animal wt (g)'
week_6wt= '6th week animal wt (g)'
week_7wt= '7th week animal wt (g)'
week_8wt= '8th week animal wt (g)'
Final_wt= 'Animal wt (g) one day before liver harvest'
Wt_Change_Int_Final=  'Initial wt - Final wt (g)'
F_FI_1_week=   'Food intake based on fresh wt content(g) week1'
D_FI_1_week=   'Food intake based on dry wt content (g) week1'
F_FI_2_week= 'Food intake based on fresh wt content(g) week2'
D_FI_2_week= 'Food intake based on dry wt content (g) week2'
F_FI_3_week=   'Food intake based on fresh wt content(g) week3'
D_FI_3_week= 'Food intake based on dry wt content (g) week3'
F_FI_4_week=   'Food intake based on fresh wt content(g) week4'
D_FI_4_week=   'Food intake based on dry wt content (g) week4'
F_FI_5_week=   'Food intake based on fresh wt content(g) week5'
D_FI_5_week= 'Food intake based on dry wt content (g) week5'
F_FI_6_week=   'Food intake based on fresh wt content(g) week6'
D_FI_6_week= 'Food intake based on dry wt content (g) week6'
F_FI_7_week=   'Food intake based on fresh wt content(g) week7'
D_FI_7_week= 'Food intake based on dry wt content (g) week7'
F_FI_8_week=   'Food intake based on fresh wt content(g) week8'
D_FI_8_week= 'Food intake based on dry wt content (g) week8'
EROD= 'Enzyme CYP1A1 EROD kinetics assay -
pmol_resorfurin/min_mg_protein'
MROD= 'Enzyme CYP1A2 MROD kinetics assay -
pmol_resorfurin/min_mg_protein'
Plate= 'Plate group-day of kinetic assay'
```

```
Api= 'Diet with Apiaceous vegetables'
Cru= 'Diet with Cruciferous vegetables'
CYP1A1= 'Western data for CYP1A1'
CYP1A2= 'Western data for CYP1A2'
SULT1A1= 'Cytosolic SULT1A1 Activity nmol/min/ug'
;

*WESTERN BLOT Value was divided by the mean basal group of 7 day to
provide change in fold of expression;
if time=1 then CYP1A1f=cyp1a1/2.1657;
if time=1 then cyp1a2f=cyp1a2/2.3674;
if time=2 then CYP1A1f=cyp1a1/2.1657;
if time=2 then cyp1a2f=cyp1a2/2.3674;
if time=3 then CYP1A1f=cyp1a1/2.1657;
if time=3 then cyp1a2f=cyp1a2/2.3674;

output HFHL_TimeStudy_Analysis;
if Time = 1
then output seven_day;
if Time = 2
then output thirty_day;
if Time = 3
then output sixty_day;
if diet=1 then output basal_only;

CARDS;
1  1  1  1  226  226  .  .  .  .  .  .  .  .  281  56 23.2 23.2 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 11.74  8.31 4.18 1  0  0  0.712  0.739  9.35
2  1  1  1  244  244  .  .  .  .  .  .  .  .  285  41 21.7 21.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.46  5.02 3.97 1  0  0  2.122  1.940  8.38
3  1  1  2  239  250  .  .  .  .  .  .  .  .  284  46 27.8 27.8 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 11.73  5.03 3.25 2  0  0  0.799  0.840  8.88
4  1  1  2  240  235  .  .  .  .  .  .  .  .  278  38 20.5 20.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.37  5.67 3.04 2  0  0  4.654  6.345  8.96
5  1  1  3  250  268  .  .  .  .  .  .  .  .  300  51 20.2 20.2 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  8.77 5.39 4.30 3  0  0 2.942  2.784  10.01
6  1  1  3  256  271  .  .  .  .  .  .  .  .  301  45 23.4 23.4 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  8.97 4.77 4.11 3  0  0  1.231  1.008  8.61
7  1  1  4  239  272  .  .  .  .  .  .  .  .  316  78 24.3 24.3 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 11.49  5.38 3.33 4  0  0  1.666  1.234  10.39
8  1  1  4  254  278  .  .  .  .  .  .  .  .  321  67 23.3 23.3 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 12.28  3.04 2.63 4  0  0  1.401  1.164  10.46
9  1  1  5  246  278  .  .  .  .  .  .  .  .  313  67 18.9 18.9 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.53  5.22 2.59 5  0  0  3.249  3.844  10.90
10 1  1  5  235  263  .  .  .  .  .  .  .  .  302  67 20.8 20.8 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.29  4.41 3.03 5  0  0  2.881  3.776  11.01
11 2  1  1  259  259  .  .  .  .  .  .  .  .  290  31 22.7 20.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.05  6.87 7.30 1  1  0  2.610  2.462  8.38
12 2  1  1  260  260  .  .  .  .  .  .  .  .  312  52 26.0 23.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.73 4.14 3.69 1  1  0 1.394  1.574  5.20
13 2  1  2  242  250  .  .  .  .  .  .  .  .  285  43 26.0 23.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.28 6.10 4.77 2  1  0 1.926  1.541  7.05
14 2  1  2  254  261  .  .  .  .  .  .  .  .  302  48 23.8 21.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  . 10.11  5.86 6.18 2  1  0  1.863  2.620  7.04
```

```
15 2  1  3  237  249  .  .  .  .  .  .  .  .  281  44 23.8 21.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  8.29 5.06 4.13 3  1  0  3.008  2.792  6.25
16 2  1  3  260  278  .  .  .  .  .  .  .  .  317  58 25.5 23.2 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.20 4.43 3.74 3  1  0  1.704  1.329  8.15
17 2  1  4  222  237  .  .  .  .  .  .  .  .  260  38 20.3 18.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  11.22  3.62 2.76 4  1  0  2.790  2.651  10.20
18 2  1  4  254  276  .  .  .  .  .  .  .  .  322  68 26.2 23.9 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  10.88  3.78 2.78 4  1  0  1.627  1.275  10.58
19 2  1  5  226  250  .  .  .  .  .  .  .  .  297  71 24.9 22.7 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  10.57  4.92 4.29 5  1  0  8.338  7.854  11.51
20 2  1  5  231  246  .  .  .  .  .  .  .  .  278  47 23.6 21.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.24 5.36 3.94 5  1  0  3.298  3.452  9.85
21 3  1  1  231  231  .  .  .  .  .  .  .  .  298  67 25.3 20.1 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.73 4.59 3.39 1  0  1  5.645  5.631  7.43
22 3  1  1  245  245  .  .  .  .  .  .  .  .  267  22 27.9 22.1 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  11.10  5.35 4.06 1  0  1  2.181  1.416  10.16
23 3  1  2  234  242  .  .  .  .  .  .  .  .  279  45 23.3 18.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  8.26 9.02 5.52 2  0  1  4.192  4.186  7.30
24 3  1  2  229  235  .  .  .  .  .  .  .  .  264  35 25.2 20.0 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  11.91  6.09 3.76 2  0  1  .    .    8.19
25 3  1  3  225  240  .  .  .  .  .  .  .  .  271  46 22.1 17.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.02 7.57 5.49 3  0  1  5.754  6.879  8.10
26 3  1  3  243  257  .  .  .  .  .  .  .  .  290  47 26.7 21.2 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  8.95 6.42 5.85 3  0  1  2.968  1.946  8.62
27 3  1  4  249  270  .  .  .  .  .  .  .  .  313  64 30.9 24.5 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  10.98  7.82 5.06 4  0  1  2.104  2.967  10.40
28 3  1  4  244  262  .  .  .  .  .  .  .  .  301  57 23.4 18.6 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.79 5.57 4.07 4  0  1  1.831  1.671  11.44
29 3  1  5  244  274  .  .  .  .  .  .  .  .  313  69 24.7 19.6 .  .  .  .  .  .  .  .
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30 3  1  5  233  259  .  .  .  .  .  .  .  .  301  68 23.4 18.6 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.67 6.73 4.82 5  0  1  4.134  4.315  10.20
31 4  1  1  246  246  .  .  .  .  .  .  .  .  295  49 24.9 21.2 .  .  .  .  .  .  .  .
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33 4  1  2  255  264  .  .  .  .  .  .  .  .  313  58 25.8 22.0 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.97 5.71 4.10 2  1  1  2.087  2.793  7.25
34 4  1  2  246  252  .  .  .  .  .  .  .  .  291  45 21.2 18.1 .  .  .  .  .  .  .  .
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37 4  1  4  245  272  .  .  .  .  .  .  .  .  320  75 28.4 24.2 .  .  .  .  .  .  .  .
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38 4  1  4  239  258  .  .  .  .  .  .  .  .  292  54 24.6 21.0 .  .  .  .  .  .  .  .
   .  .  .  .  .  .  .  9.08 5.02 5.10 4  1  1  2.928  3.193  10.81
39 4  1  5  245  271  .  .  .  .  .  .  .  .  310  65 23.0 19.6 .  .  .  .  .  .  .  .
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41 1  2  1  183  242  294  342  .  .  .  .  .  364  181  20.9 20.9 20.3 20.3
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43 1  2  2  165  225  292  343  .  .  .  .  .  370  205  18.7 18.7 22.5 22.5
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44 1  2  2  170  235  310  375  .  .  .  .  .  406  236  20.5 20.5 24.7 24.7
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45 1  2  3  157  217  283  337  .  .  .  .  .  376  220  22.2 22.2 26.5 26.5
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46 1  2  3  168  230  283  315  .  .  .  .  .  339  171  23.5 23.5 19.8 19.8
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47 1  2  4  153  208  266  306  .  .  .  .  .  338  185  18.5 18.5 20.1 20.1
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49 1  2  5  146  201  253  282  .  .  .  .  .  322  176  18.0 18.0 11.6 11.6
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   10.62
50 1  2  5  155  208  267  313  .  .  .  .  .  357  201  17.8 17.8 19.9 19.9
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72 4  2  1  185  250  319  362  .  .  .  .  .  395  210  28.4 24.2 29.4 25.1
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73 4  2  2  168  229  282  332  .  .  .  .  .  360  192  25.0 21.3 24.4 20.8
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74 4  2  2  163  233  309  363  .  .  .  .  .  388  225  26.2 22.3 29.3 25.0
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90 1  3  3  168  223  265  298  325  353  380  406  423  423  255  17.9 17.9
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91 2  3  1  160  222  295  338  384  416  427  440  452  452  292  26.9 24.5
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93 2  3  1  162  225  291  337  369  405  430  452  476  476  314  25.2 23.0
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101  3  3  1  145  205  260  293  321  322  334  352  382  382  237  23.9
   19.0 23.9 19.0 23.8 18.9 24.9 19.8 5.6    4.4    26.0 20.6 23.9 19.0
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70 3  3  1  159  236  286  342  347  404  447  471  500  500  341  29.6 23.5
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105  3  3  2  156  216  266  304  336  367  395  417  440  439  283  24.0
   19.0 23.9 19.0 23.4 18.6 23.9 19.0 25.3 20.1 26.2 20.8 27.0 21.4 30.3
   24.0 12.09  6.01 3.44 13 0  1  2.014  1.297  9.60
106  3  3  2  181  252  325  373  404  448  474  508  535  536  355  28.5
   22.6 29.2 23.2 29.7 23.6 27.6 21.9 29.8 23.6 25.1 19.9 28.9 22.9 31.6
   25.1 .    .    .    13 0  1  .    .    .
107  3  3  2  167  226  296  331  364  388  406  436  465  462  296  24.9
   19.8 27.3 21.7 29.1 23.1 24.3 19.3 27.8 22.1 31.8 25.2 31.4 24.9 29.4
   23.3 12.00  7.37 3.75 14 0  1  1.217  1.756  7.78
108  3  3  2  158  215  271  308  338  367  390  411  429  432  274  23.6
   18.7 23.0 18.2 25.3 20.1 22.2 17.6 26.4 20.9 21.6 17.1 24.6 19.5 26.6
   21.1 12.05  6.13 3.13 14 0  1  1.330  0.887  9.88
109  3  3  3  145  207  265  298  326  349  380  404  412  414  269  25.5
   20.2 21.1 16.7 16.0 12.7 21.7 17.2 17.6 14.0 22.8 18.1 27.0 21.4 27.7
   22.0 11.32  8.10 4.15 15 0  1  .    .    11.00
110  3  3  3  159  220  279  323  360  386  420  444  463  465  306  24.3
   19.3 27.7 22.0 25.6 20.3 25.4 20.2 30.0 23.8 11.1 8.8    30.8 24.4 28.1
   22.3 14.22  8.53 4.24 15 0  1  .    .    10.42
122  3  3  3  163  220  275  312  345  371  397  413  430  430  268  25.0
   19.8 24.9 19.8 25.1 19.9 22.9 18.2 28.4 22.5 23.4 18.6 23.9 19.0 23.7
   18.8 12.24  .    .    .  0  1  .    .    10.05
112  4  3  1  166  231  293  339  379  409  437  464  486  486  320  24.1
   20.5 26.4 22.5 29.0 24.7 25.5 21.7 28.1 23.9 25.9 22.1 33.1 28.2 32.0
   27.3 14.63  7.90 3.54 11 1  1  1.140  0.977  8.75
```

```
113  4  3  1  161  223  283  328  361  390  419  440  455  455  294  20.8
  17.7 22.7 19.3 25.3 21.6 23.5 20.0 26.5 22.6 27.7 23.6 27.3 23.3 28.9
  24.6 19.91  5.92 2.52 12 1  1  1.663  1.527  7.61
114  4  3  1  158  221  285  330  361  397  428  449  468  468  310  24.5
  20.9 23.2 19.8 25.5 21.7 23.8 20.3 32.1 27.4 27.1 23.1 25.8 22.0 28.0
  23.9 15.01  8.57 3.42 12 1  1  1.608  1.700  7.64
115  4  3  2  160  221  283  321  348  379  409  427  448  449  289  24.4
  20.8 26.1 22.2 23.6 20.1 22.8 19.4 27.2 23.2 26.2 22.3 22.8 19.4 24.6
  21.0 14.29  3.96 3.01 13 1  1  1.324  1.499  7.93
116  4  3  2  166  223  280  323  360  389  412  440  468  470  304  25.1
  21.4 25.2 21.5 25.0 21.3 25.2 21.5 26.3 22.4 23.5 20.0 28.9 24.6 32.2
  27.4 13.55  4.54 2.39 13 1  1  0.840  1.121  8.40
117  4  3  2  186  254  321  373  415  448  480  505  525  528  342  28.8
  24.5 28.3 24.1 29.7 25.3 31.0 26.4 28.4 24.2 27.5 23.4 27.6 23.5 26.7
  22.8 15.96  7.08 3.00 14 1  1  2.620  2.521  10.04
118  4  3  2  165  211  265  299  331  366  389  417  434  436  271  28.4
  24.2 25.6 21.8 28.5 24.3 26.4 22.5 27.8 23.7 29.3 25.0 28.5 24.3 26.3
  22.4 13.58  7.34 4.40 14 1  1  1.630  1.009  10.05
119  4  3  3  171  235  290  330  366  396  419  441  458  459  289  23.5
  20.0 24.8 21.1 25.6 21.8 25.3 21.6 26.6 22.7 24.6 21.0 28.1 23.9 24.6
  21.0 13.29  7.84 4.19 15 1  1  .    .    9.96
120  4  3  3  164  215  258  279  314  340  365  380  385  387  223  24.6
  21.0 21.3 18.1 19.7 16.8 22.6 19.3 24.2 20.6 26.8 22.8 26.6 22.7 31.7
  27.0 11.73  9.62 6.72 15 1  1  .    .    8.27
77 4  3  3  137  172  207  234  266  300  334  351  381  385  248  19.6 16.7
  19.8 16.9 27.2 23.2 19.2 16.4 22.8 19.4 24.5 20.9 21.7 18.5 23.9 20.4
  11.48  .    .    . 1  1  .    .    .
;


proc sort data = HFHL_TimeStudy_Analysis; by Diet;

proc format;
  value Dietfmt
    1='Basal diet'
    2='9% Apiaceous'
    3='21% Cruciferous'
    4='15% A+C';
  Value Timefmt
    1='7 day'
    2='30 day'
    3='60 day';
  Value Apifmt
    1= 'W/ Api'
    0= 'No Api';
  Value Crufmt
    1='W/ Cruci'
    0='No Cruci';


proc print data=HFHL_TimeStudy_Analysis; by Diet; format Diet Dietfmt.;
*Proc means= Will provide the mean and std error to make graphs;
proc means data=HFHL_TimeStudy_Analysis n mean stderr std min max;
TITLE 'ANOVA descriptive statistics';
format Diet dietfmt.;
```

```
format Time timefmt.;
by diet time;
var Initial_wt_g--Cru;
run;
*Proc glm will provide Least square means (under matrix for p-values),
Means (under multiple comparison for letter grouping);

proc glm data=seven_day;
title 'Seven Day -One Way Analysis of Variance in all rats';
format Diet Dietfmt.;
format Time Timefmt.;
class Diet  ;
model Initial_wt_g--Cru=Diet;
Means Diet / Duncan;
LSMeans Diet /stderr  pdiff;
run;

proc glm data=thirty_day;
title 'Thirty Day- One Way Analysis of Variance in all rats';
format Diet Dietfmt.;
format Time Timefmt.;
class Diet  ;
model Initial_wt_g--Cru=Diet;
Means Diet / Duncan;
LSMeans Diet /stderr pdiff;
run;

proc glm data=sixty_day;
title 'Sixty Day - One Way Analysis of Variance in all rats';
format Diet dietfmt.;
format Time timefmt.;
class Diet  ;
model Initial_wt_g--Cru=Diet;
Means Diet / Duncan;
LSMeans Diet /stderr pdiff;
run;
*KINETIC ASSAY DATA ANALYSIS;
* Proc mixed was used for analyzis of kinetic assay because we are able
to add plate into the class. Also, no adjustment for multiple
comparison.;

 proc mixed method=ml data=HFHL_TimeStudy_Analysis;
   title 'EROD-CYP1A1 Full data results';
  class Plate Api Cru Time;
   model erod = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru  /
ddfm=bw;
   random plate / subject=plate;* v vcorr;
   lsmeans  Time*Api*Cru / pdiff diff slice=Time;

 proc mixed method=ml data=HFHL_TimeStudy_Analysis;
   title 'MROD-CYP1A2 Full data results';
  class Plate Api Cru Time;
   model mrod = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru  /
ddfm=bw;
   random plate / subject=plate ;*v vcorr;
```

```
   lsmeans  Time*Api*Cru / diff slice=Time;

   run;
* WESTERN DATA ANALYSIS: We Rank data due to failed normality;
*Create a new data with ranks;

proc rank data=HFHL_TimeStudy_Analysis out=RankWesterns;
title 'Rank western blot values';
var CYP1A1 CYP1A2;
ranks rankCYP1A1 rankCYP1A2;
proc print;
proc sort data=RankWesterns; by Diet Time;
proc print;

*Using similar analysis from the kinetics to Western Blot data by
ranking, MIXED is very similar to GLM when we do not have the randon
effects (class);
proc mixed method=ml data=RankWesterns;
title 'CYP1A1 Western Blot data results (ranked)';
class Api Cru Time;
model rankCYP1A1 = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru
/ ddfm=bw;
lsmeans Time*Api*Cru / diff slice=Time;


proc mixed method=ml data=RankWesterns;
title 'CYP1A2 Western Blot data results (ranked)';
class Api Cru Time;
model rankCYP1A2 = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru
/ ddfm=bw;
lsmeans Time*Api*Cru / diff slice=Time;
run;


proc glm data=RankWesterns;
class Api Cru Time;
model rankCYP1A1 CYP1A1  = Api Cru Api*Cru Time Time*Api Time*Cru
Time*Api*Cru / ddfm=bw;

* 3-21-11 Using CYPfolds with Mixed function: Purpose was to make
graphs;
proc mixed method=ml data=RankWesterns;
title 'CYP1A1 Western Blot Fold Data-graph only';
class Api Cru Time;
model CYP1A1f = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru /
ddfm=bw;
lsmeans Time*Api*Cru / diff slice=Time;
run;


proc mixed method=ml data=RankWesterns;
title 'CYP1A2 Western Blot Fold Data-graph only';
class Api Cru Time;
model CYP1A2f = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru /
ddfm=bw;
```

```
lsmeans Time*Api*Cru / diff slice=Time;
run;

*4-15-11 Analize SULT1A1 activity. Utilized the same model as activity
even though no plate in the model ;
proc mixed method=ml data=HFHL_TimeStudy_Analysis;
title 'SULT1A1';
class Api Cru Time;
model SULT1A1 = Api Cru Api*Cru Time Time*Api Time*Cru Time*Api*Cru /
ddfm=bw;
lsmeans Time*Api*Cru / diff slice=Time;
*lsmeans api cru time api*cru api*time api*cru / pdiff;
run;

*5-13-11 Analize the correlation CYP activity vs CYP protein
expression;

proc corr data=HFHL_TimeStudy_Analysis;
title 'CYP1A1 correlation';
var CYP1A1f;
with EROD;
run;

proc plot data=HFHL_TimeStudy_Analysis;
plot EROD * CYP1A1f ;
run;

proc corr data=HFHL_TimeStudy_Analysis;
title 'CYP1A2 correlation';
var CYP1A2f;
with MROD;
run;

proc plot data=HFHL_TimeStudy_Analysis;
plot MROD * CYP1A2f ;
run;


proc corr data=basal_only;
title 'Cyp1A1 basal diet only';
var CYP1A1f;
with EROD;
run;
proc plot data=basal_only;
plot EROD * CYP1A1f;
run;

proc corr data=basal_only;
title 'Cyp1A2 basal diet only';
var CYP1A2f;
with MROD;
run;
proc plot data=basal_only;
plot MROD * CYP1A2f;
run;
```

```
quit;

proc plot data=basal_only;
plot MROD * CYP1A2;
run;

proc corr data=basal_only;
title 'Cyp1A2 not folded basal diet only';
var CYP1A2;
with MROD;
run;
```