ETHNIC DIFFERENCES IN THE METABOLISM OF 1,3-BUTADIENE AND LUNG CANCER RISK

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Dedication

This dissertation is dedicated to my mother, Jennifer Paula Zittnan, fixer of all things broken or bent.

ABSTRACT

Cigarette smoking remains one of the most preventable causes of death in the world, and is the leading cause of lung cancer. Epidemiological studies show inherent differences in lung cancer risk among smokers of different ethnic groups, with Native Hawaiian and African Americans have the highest risk, European Americans having an intermediate risk, and Latinos and Japanese Americans having the lowest risk. It has been proposed that these disparities in risk are due to ethnic differences in the metabolism, and ultimately bioactivation, of carcinogens in present in cigarette smoke.

1,3-butadiene (BD) is one of the most abundant and potent carcinogens present in cigarette smoke. BD is metabolically activated to the reactive species 3,4-epoxy-1-butene (EB), hydroxymethylvinylketone (HMVK), 3,4-epoxy-1,2-butanediol (EBD), and 1,2,3,4-diepoxybutane (DEB), which have the ability to form pro-mutagenic DNA adducts. These species can be detoxified through glutathione conjugation and excreted in urine; EB and HMVK are excreted as 2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene/ 1-(*N*-acetyl-L-cystein-S-yl)-2-hydroxybut-3-ene (MHBMA) and 4-(*N*-acetyl-L-cystein-S-yl)-1,2-dihydroxybutane (DHBMA). The research presented in this thesis focuses on ethnic differences in BD metabolism and the initial development of a DEB specific biomarker. A high throughput HPLC-ESI$^-$-MS/MS method for the simultaneous quantitation of MHBMA and DHBMA in humans previously developed in our laboratory was applied to quantify these mercapturic acids in smokers of different ethnic groups.

In a large multi-ethnic study composed of African American, European American, and Japanese American smokers (N = 1,072). Urinary MHBMA and

MHBMA/MHBMA+DHBMA were highest in African Americans, followed by European Americans, and Japanese Americans, and strongly influenced by *GSTT1* genotype. A genome wide association study (GWAS) revealed strong associations between MHBMA and GSTT1: associations with 136 SNPs were detected, and all of them were located between 24.2—24.4 Mb near the *GSTT1* gene on chromosome 22q11. Additional experiments with recombinant human GSTT1 and GSTT2 confirmed EB as a substrate for the first time.

The same method was also applied to a separate smaller study of African American and European American smokers (N = 151). In contrast to the previous work, urinary MHBMA was higher in European Americans than African Americans in this study; this is likely due to decreased sample size. Statistical analyses revealed no correlation between urinary MHBMA or DHBMA and urinary N7-(1-hydroxy-3-buten-2-yl)guanine (EBGII), as well no significant associations between urinary EBGII, MHBMA, or DHBMA and various specific SNPs from BD-metabolizing genes (*EPHX1* and *CYP2E1*) and DNA repair genes (*FANCE*). *GSTT1* copy number was also included in this analysis, and showed a significant association with urinary MHBMA.

Urinary MHBMA and DHBMA were also quantified in smokers (N=79) receiving treatment with the chemopreventative agent 2-phenethyl isothiocyanate (PEITC). Overall, PEITC treatment resulted in only slight increases in MHBMA and DHBMA as compared to treatment with a placebo, but was found to significantly increase MHBMA in individuals null for *GSTT1* or both *GSTT1* and *GSTM1*, indicating a potential protective effect of PEITC in these individuals.

Lastly, an HPLC-ESI$^+$-MS/MS method for the of detection of a novel DEB-specific biomarker, N^{ε}, N^{ε}-(2,3-dihydroxybutan-1,4-diyl)-L-lysine (DHB-Lys) was explored. Initial development focused on the use of an ion-pairing agent, perfluoroheptanoic acid (PFHA), which was chosen to increase HPLC retention of DHB-Lys. Though addition of PFHA to the aqueous mobile phase during HPLC-ESI$^+$-MS/MS analysis did result in increased retention of the analyte, its use also presented additional challenges with analyte carryover, sample contamination, and ion suppression. Methodology utilizing derivatization of DHB-Lys through the addition of a 6-aminoquinolyl group (6-AQ) at the alpha nitrogen was tested on DEB-treated O^6-alkylguanine DNA alkyltransferase (AGT), and showed a dose dependent increase in DHB-Lys formed.

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LIST OF ABBREVIATIONS

6-AQ	6-aminoquinolyl
A	Adenine
ADH	alcohol dehydrogenase
AQC	6-aminoquinolyl-N-hydroxysuccinimdyl carbamate
BD	1,3-butadiene
bis-BDMA	1,4-*bis*-(*N*-acetylcysteinyl)butane-2,3-diol
C	Cytosine
CYP 450	cytochrome P450 monoxygenase
DEB	1,2,3,4-diexpoxybutane
DHBMA	dihydroxybutyl mercapturic acid [4-(*N*-acetyl-L-cystein-S-yl)-1,2-dihydroxybutane]
DHB-Lys	dihydroxybutyl-lysine [N^{ε}, N^{ε}-(2,3-dihydroxybutan-1,4-diyl)-L-lysine]
DNA	deoxyribonucleic acid
EB	3,4-epoxy-1-butene
EBD	3,4-epoxy-1,2-diol
EBGII	N7-(1-hydroxy-3-buten-2-yl)guanine
EH	epoxide hydrolase
G	Guanine
GST	glutathione S-transferase
GWAS	genome wide association study

Hb	hemoglobin
HB-Val	*N*-(2-hydroxy-3-butenyl)-valine
HILIC	hydrophilic interaction liquid chromatography
HMVK	hydroxymethylvinylketone
HPLC	high performance liquid chromatography
HPLC-ESI-MS/MS	high performace liquid chromatography-electrospray ionization tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MHBMA	monohydroxybutenyl mercapturic acid [2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene / 1-(*N*-acetyl-L-cystein-S-yl)-2-hydroxybut-3-ene]
MS/MS	tandem mass spectrometry
NMR	nuclear magnetic resonance
PEITC	2-phenethyl isothiocyanate
ppm	parts per million
pyr-Val	*N*,*N*-(2,3-dihydroxy-1,4-butadiyl)-valine
SNP	single nucleotide polymorphism
T	Thymine
THBMA	Trihydroxybutyl mercapturic acid [4-(*N*-acetyl-L-cystein-S-yl)-1,2,3-trihydroxybutane]
THB-Val	1,2,3-trihydroxybutyl-valine

I. LITERATURE REVIEW

1.1 Chemical Carcinogenesis

1.1.1 Role of DNA Adducts in Carcinogenesis

Over their lifetime, humans are exposed to a range of inherently reactive species from exogenous and endogenous sources including, air,[1] water,[2] food,[3] reactive oxygen species,[4] and products of lipid peroxidation.[5] Unless detoxified by the body, these compounds have the ability to react with cellular macromolecules such as DNA, proteins, RNA, and lipids, which can lead to genetic mutations or cellular toxicity (Figure 1.1).[6] Agents that directly react with DNA include reactive oxygen species, deaminating agents, alkylating agents, and compounds containing electrophilic groups such as α,β-unsaturated carbonyls; these types of molecules are inherently carcinogenic and require no metabolic activation to a reactive species.[6, 7] Alternatively, compounds that do require metabolic activation to an electrophilic species are termed "procarcinogens".[8]

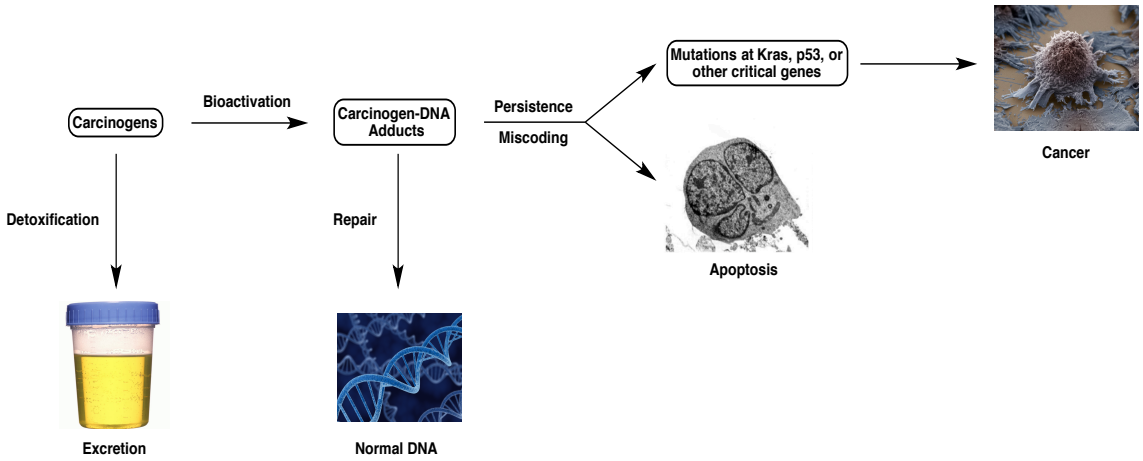


Figure 1.1 Role of DNA adducts in chemical carcinogenesis

DNA is an electron rich biomolecule that can be modified at all four nucleobases—guanine (G), cytosine (C), adenine (A), and thymine (T)—to form covalent DNA adducts.[9] If not repaired, these adducts can lead to heritable mutations and ultimately cancer. The chemical stability of DNA adducts is determined by their chemical structure.[10] Alkylation at the N^2, N^4, and N^6 positions of G, C, and A, respectively, the N1 positions of G and T, and the O^6 and O^4 positions of G and T, respectively, produces chemically stable adducts, whereas alkylation at the N7 and N3 positions of G, the N7, N3, and N1 positions of A, and the N3 position of C can destabilize the *N*-glycosidic bond, potentially leading to spontaneous depurination and the formation of abasic sites.[11] Alternatively, N7-dG adducts and N7-dA adducts can undergo ring opening reactions to form formamidopyrimidine lesions.[12] DNA adducts can be repaired through a variety of enzymatic repair mechanisms including base excision repair, nucleotide excision repair, homologous recombination, and non-homologous end joining, restoring DNA structure.[13] However, if an adduct persists, it can lead to miscoding events during DNA replication.[14] Bypass of bulky DNA adducts during DNA replication is achieved through translesion synthesis (TLS) polymerases, which take over when normal replicative polymerases blocked by a DNA adduct.[15] As compared to normal replicative polymerases, TLS polymerases have larger active sites, which allow them to accommodate bulky lesions, and lack proofreading activity, which decreases their fidelity during replication.[15, 16] If a TLS polymerase incorporates an incorrect base opposite a DNA adduct, a mutation is introduced. Non-viable mutations can lead to programmed cell death, or apoptosis.[17] Viable mutations, however, can lead to

cancer, particularly if they occur within a tumor suppressor gene, such as *p53*,[18] or a proto-oncogene, such as *Kras* or *Bcl-2*.[9, 19]

Because of DNA adducts are directly involved in cancer initiation, they can be used as mechanism based biomarkers of carcinogen exposure and bioactivation.[20] Alternatively, carcinogen detoxification products present in blood or urine can also be used as biomarkers of carcinogen exposure,[8] representing the fraction of toxic metabolites that did not result in DNA damage. By quantifying both DNA damage and detoxification products, the fate of a carcinogenic species can be more accurately assessed, potentially providing an increased understanding of the role they play in cancer development.

1.1.2 Using Carcinogen-DNA Adducts and Urinary Metabolites as Biomarkers of Carcinogen Exposure

Human exposure to carcinogens can be measured by quantifying specific biomarkers in tissues, hair, and biological liquids such as blood, urine, and saliva. Carcinogen-DNA adducts reflect the extent of carcinogen bioactivation to reactive metabolites, while urinary biomarkers like mercapturic acids represent pathways of detoxification of those reactive metabolites. The ability to accurately quantify such biomarkers can give insight into a particular carcinogen's ability to cause cancer as well as its overall toxicity.

Many carcinogens require enzymatic bioactivation to reactive species, and the enzymes involved in their metabolism can be broadly categorized as Phase I or Phase II enzymes.[21, 22] Arguably the most important Phase I enzymes are the cytochrome P450 monoxygenases (CYP450s). These enzymes have the ability to catalyze oxidation,

reduction, and hydrolytic reactions of a variety of substrates, which typically increases their solubility in water and facilitates their excretion. These modifications can also act as a chemical handle for further metabolism by Phase II enzymes. Phase II enzymes catalyze the conjugation of a compound to a highly polar or charged molecule; this process aids in the excretion of these compounds. Some examples of Phase II metabolism include glucuronidation, sulfonation, amino acid conjugation, and glutathione (GSH) conjugation. In particular, GSH conjugates are often further processed by γ-glutamyltranspeptidase, a dipeptidase, such as cysteinyl glycinase, and *N*-acetyltransferase (known as the mercapturic acid pathway) to form mercapturic acids, which are excreted in urine.[23] With respect to their role in carcinogenesis, Phase I metabolism can lead to carcinogen bioactivation or detoxification, whereas Phase II metabolism is generally regarded as a pathway of detoxification.

One of the earliest compounds linked to cancer was benzo[*a*]pyrene (BaP), a polycyclic aromatic hydrocarbon formed from an incomplete combustion of organic compounds and found in coal tar, automobile exhaust, and cigarette smoke.[24] In 1775, Dr. Percivall Pott reported an association between occupational exposure to soot and incidences of scrotal cancer in chimney sweeps, and hypothesized that the cancer was caused by prolonged exposure to soot.[25] His observations were proved correct in the 1930s, when Kennaway and coworkers isolated BaP from coal tar and identified it as a highly carcinogenic compound.[26]

Today, the metabolism of BaP is well established (Figure 1.2). BaP is oxidized by CYP450s 1A1 and 1B1 to form BaP-7,8-epoxide and BaP-9,10-epoxide.[27] Both these metabolites can be hydrolyzed to their respective diols by epoxide hydrolase (EH);[27, 28]

these diols can then undergo glucuronidation and be excreted in the urine.[29] If BaP-7,8-diol is not detoxified, it can undergo a second round of oxidation by CYP450s 1A1 and 1B1 to form its ultimate carcinogenic species, BaP-7,8-dihydrodiol,-9,10-epoxide (BPDE).[27] This metabolite can react at the N^2 position of G to form (+/-)-*cis*/*trans*-N^2-BaP dihydrodiol epoxide-2'-deoxyguanosine (N^2-BPDE-dG) adducts.[30, 31] BPDE can be detoxified through hydrolysis by EH to form BaP-tetrols[32] or by conjugation with GSH and the mercapturic acid pathway to form tetrahydro-trihydroxy-BaP-*S*-*N*-acetylcysteine (BPDE-NAC).[33, 34]

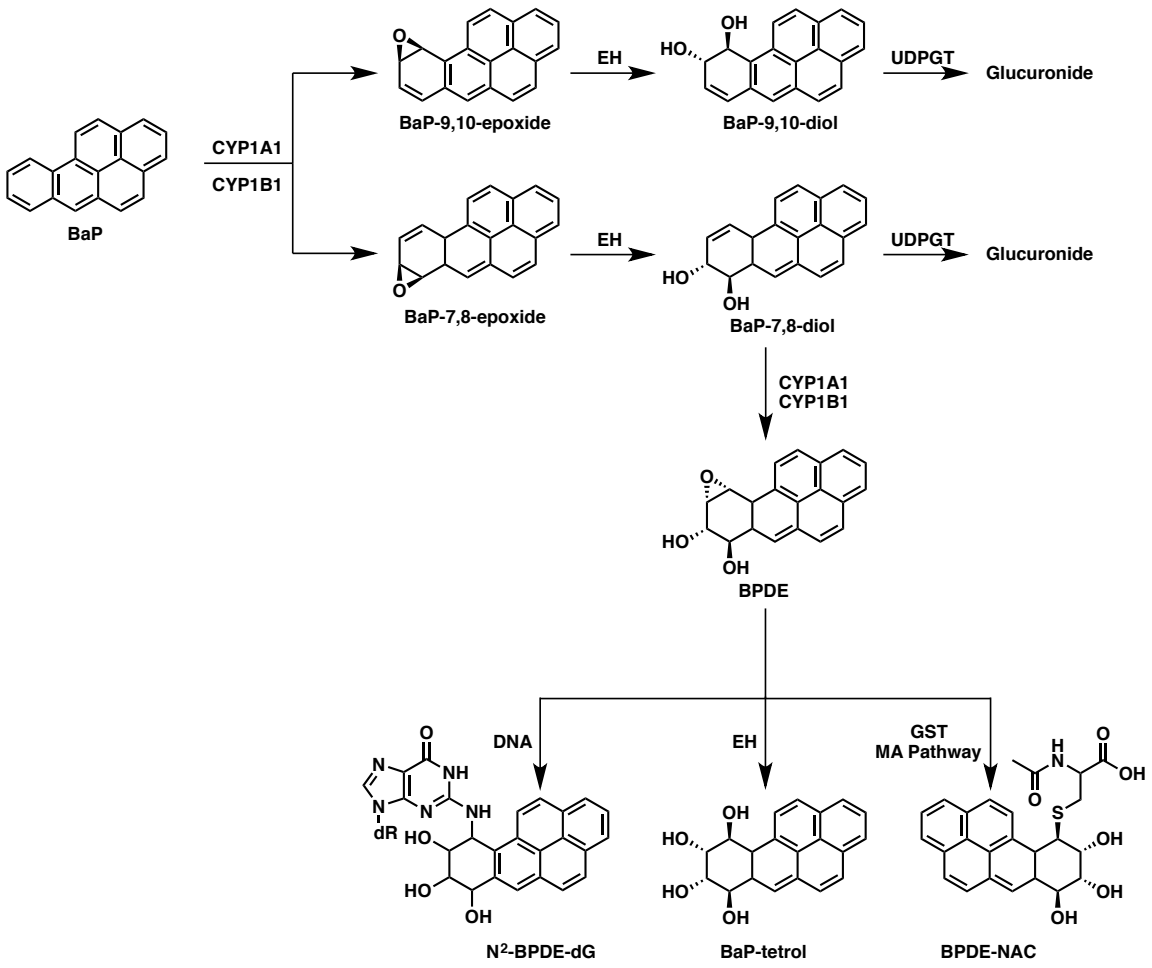


Figure 1.2 Metabolic pathways of BaP. UDPGT: uridine diphosphate glucuronyltransferase; GST: glutathione S-transferase; MA: mercapturic acid

Quantitation of urinary metabolites of BaP, such BPDE-NACs, can provide insight into the extent of carcinogen exposure within an individual as well as the level of detoxification of BaP versus its bioactivation. While BaP-tetrols represent biomarkers of BaP exposure, BPDE-DNA adducts such as N^2-BPDE-dG reflect the extent of BaP bioactivation to DNA reactive species and ultimately, its ability to cause mutations and cancer. Several studies have shown significantly higher levels of BPDE DNA adducts in DNA extracted from white blood cells in occupationally exposed workers such as roofers or foundry workers and smokers as compared to unexposed individuals.[35-37] Furthermore, in male smokers, BPDE-DNA adducts were significantly associated with age, years of smoking, and pack years.[37]

The toxicants acrolein and crotonaldehyde, found in tobacco smoke, automobile exhaust, and smoke from wood burning, also produce both DNA adducts and urinary mercapturic acid metabolites, which can be used to biomarkers of exposure.[38] As Michael acceptors, acrolein and crotonaldehyde do not require metabolic activation to form reactive intermediates. Both compounds can directly react with deoxyguanosine (dG) to form exocyclic 1,N^2-propanodeoxyguanosine (PdG) adducts (Figure 1.3),[39] which have been shown to cause G → T and G → C transversions, as well as G → A transitions.[40-43] PdG adducts have been used as biomarkers of exposure in oral tissue of smokers; in particular, concentrations of acrolein PdG adduct 1 (Figure 1.3) were found to be 3-fold higher in smokers as compared to nonsmokers, whereas crotonaldehyde PdG adducts 1 and 2 (Figure 1.3) were found to be 8.8 and 5.5 times higher, respectively, in smokers as compared to nonsmokers.[44] Overall, the total amounts of PdG adducts was 4.4 times higher in smokers as compared to nonsmokers.[44]

Additionally, acrolein and crotonaldehyde can form mercapturic acid metabolites that are excreted in urine (Figure 1.4). Acrolein can undergo glutathione conjugation followed by mercapturic acid pathway processing to form 3-oxopropylmercapturic acid (OPMA).[45] Reduction of OPMA yields 3-hydroxypropylmercapturic acid (HPMA), which is the main metabolite of acrolein found in urine.[45, 46] Similarly, crotonaldehyde can be conjugated with glutathione, processed by the mercapturic acid pathway, and subsequently reduced to form 3-hydroxy-1-methylpropylmercapturic acid (HMPMA).[47] In smoking cessation studies, the concentrations of urinary HPMA and HMPMA were shown to decrease by approximately 87% just three days after smoking cessation, indicating that the formation of these mercapturic acids is dependent on exposure to their respective parent compounds in cigarette smoke.[48]

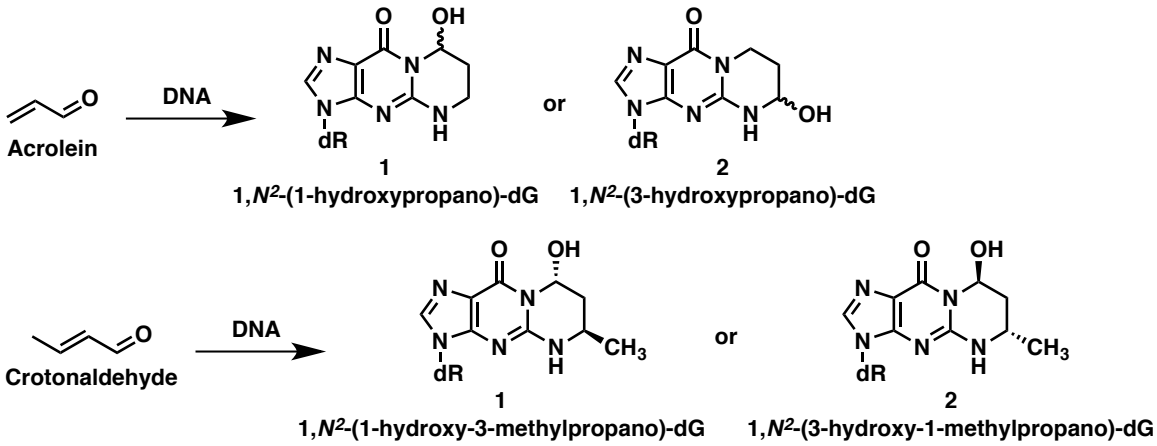


Figure 1.3 DNA adducts formed from acrolein and crotonaldehyde

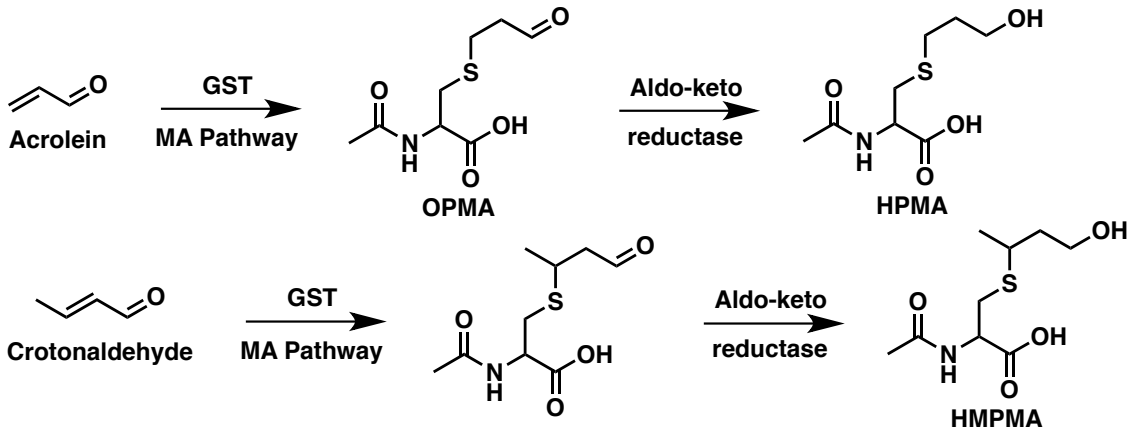


Figure 1.4 Mercapturic acids formed from acrolein and crotonaldehyde

Additional examples of carcinogen-DNA adducts used as biomarkers of exposure include 8,9-dihydro-8-(N7-guanyl)-9-hydroxy aflatoxin B1 (AFB1-N7-Gua), formed from liver carcinogen aflatoxin B1,[49] O^6-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O^6-POB-dG) and 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (7-POB-dG), formed from metabolites of tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone,[50] and N-(2'-deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (dG-C8-PhIP), formed from 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP)[51] (Figure 1.5). Additional examples of mercapturic acids used as biomarkers of exposure include *S*-phenyl mercapturic acid (SPMA)[52] and 2-hydroxyethyl mercapturic acid (HEMA),[53] metabolites of benzene and ethylene oxide, respectively (Figure 1.5). Overall, both DNA adducts and mercapturic acids formed from carcinogen exposure are of significant biological interest as they can be used as biomarkers of exposure, metabolic activation, and detoxification, and their quantitation in human samples can be used to determine the levels of exposure and potential cancer risk.

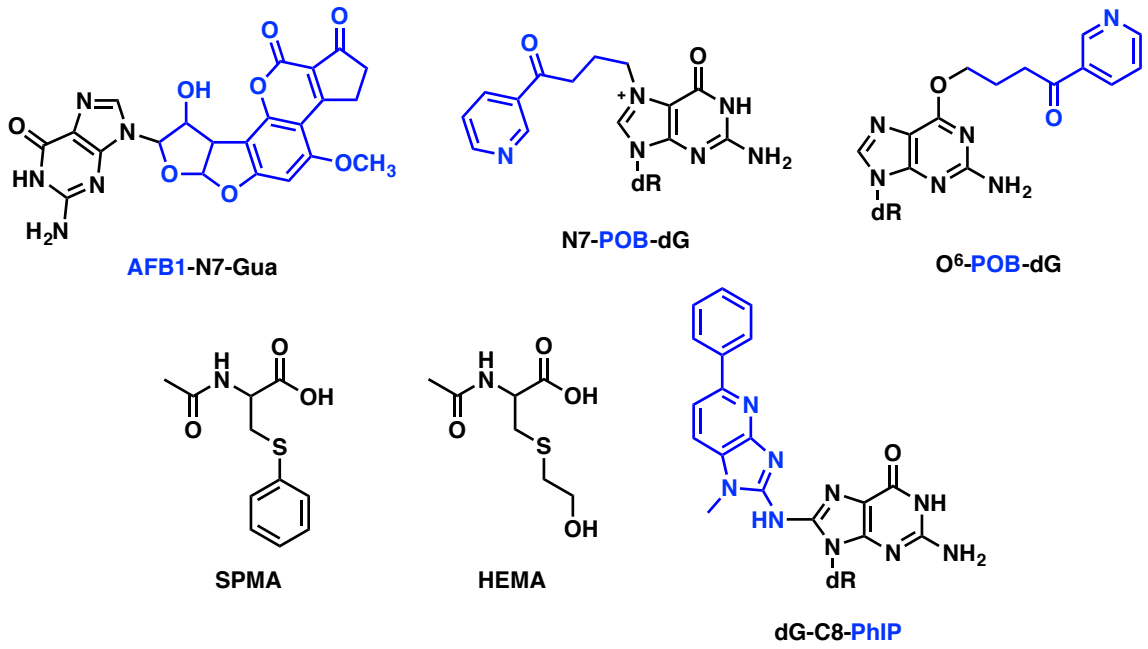


Figure 1.5 Examples of DNA adducts and mercapturic acids used as biomarkers of carcinogen exposure

1.2 Lung Cancer

Cancer refers to a group of diseases that are characterized by abnormal cell growth. The uncontrolled growth of these cells forms masses called tumors; malignant tumors have the ability to spread to other parts of the body. Mutations in DNA that lead to this uncontrolled growth can be caused by exogenous factors, such cigarette smoking, or endogenous ones, such as genetic mutations. In the United States (US) in 2016, an estimated 1,685,210 individuals were diagnosed with some form of cancer, while 595,690 individuals were estimated to die from the disease.[54] Cancer is the second leading cause of death in the United States, and the relative five-year survival rate for all forms of cancer diagnosed between 2005-2011 was 69%.[54] However, survival rates vary depending on cancer type and stage at diagnosis, with survival rates increasing when the cancer is diagnosed at an earlier stage.

Lung and bronchus cancer accounted for approximately 13.5% of the estimated new cancer cases for 2018 and 25.3% of the estimated deaths, and approximately 6.2% of men and women will be diagnosed with lung and bronchus cancer in their lifetime.[55] For individuals diagnosed with lung cancer between 2008-2014, the relative five-year survival rate was only 18.6%.[55] This low survival rate can be attributed to the fact that the majority of these cases (57%) were diagnosed at an advanced stage, meaning that the cancer had metastasized, or spread, to other parts of the body.[55] Lung cancer can be caused by a variety of factors, including exposure to radon gas and asbestos, however the leading cause of lung cancer is cigarette smoking.[54]

1.2.1 Smoking and Lung Cancer

Smoking is the most preventable cause of death in the world.[54] The link between smoking and human disease was first established in the US Surgeon General's report in 1964.[56] Since then, there have been an estimated 20 million deaths that can be attributed to smoking in the US, and as of 2014, 40 million adults in the US identified as current smokers.[54] Excluding secondhand smoke, smoking accounts for 32% of all cancer deaths; with regard to lung cancer specifically, smoking causes 83% of lung cancer deaths in men and 76% of lung cancer deaths in women.[54] Secondhand smoke, however, can also be dangerous, and exposure to secondhand smoke does increase the risk of lung cancer.[14, 54] Exposure to secondhand smoke in nonsmoking adults leads to an estimated 7,330 lung cancer deaths each year.[54]

Cigarette smoke is a complex mixture that contains over 60 compounds classified as known human carcinogens, as well as over 180 compounds that are reasonably anticipated to be human carcinogens.[14] Known carcinogens in tobacco smoke include aromatic amines, heterocyclic aromatic amines, heterocyclic compounds, inorganic compounds, metals, *N*-nitrosamines, nitrohydrocarbons, phenolic compounds, polycyclic aromatic hydrocarbons (PAHs), various small molecules, and volatile hydrocarbons;[57] some examples can be found in Figure 1.6. It is the prolonged exposure to these carcinogens, even in the relatively low amounts found in cigarette smoke, which can lead to the formation of DNA adducts and cancer initiation.

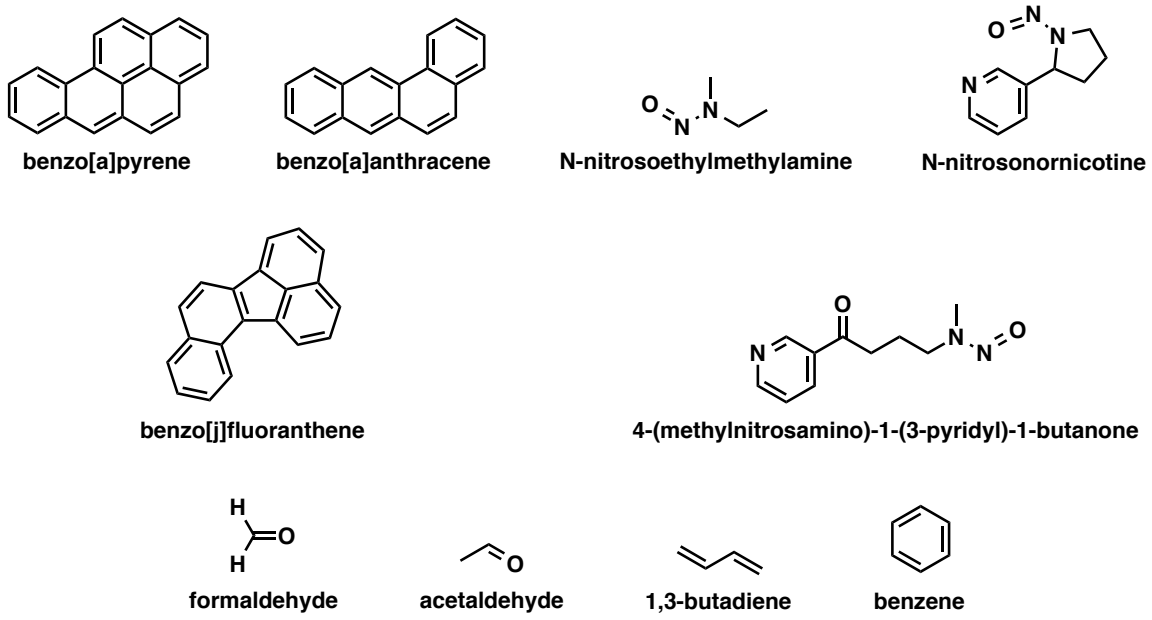


Figure 1.6. Examples of known carcinogens present in cigarette smoke

1.2.2 Ethnic Differences in Smoking Associated Lung Cancer

While smoking is a well-established cause of lung cancer, the risk of developing lung cancer in smokers varies widely between different ethnic groups. One of the earliest studies that examined ethnic differences in smoking induced lung cancer was published by Hinds *et. al.* in 1981.[58] Their study examined lung cancer risk among 176 Japanese, 67 Chinese, and 132 Hawaiian female lung cancer patients from Hawaii, as well as 2,404 controls. When adjusted for age and socioeconomic status, they found that the smoking-associated lung cancer risk was highest in Hawaiian women, followed by Japanese and Chinese women.[58] A similar study was published in 1992 by Le Marchand *et. al.*,[59] examining lung cancer risk among 740 lung cancer patients from Hawaii diagnosed between 1979 and 1985, as well as 1,616 controls in a greater number of ethnicities. After adjusting for pack years of smoking, occupation, education, and age, their findings showed that Hawaiian, Filipino, Caucasian, and Chinese male smokers had a 121%, 53%, 46%, and 20% greater risk, respectively, for developing lung cancer as compared to Japanese male smokers.[59] This trend was different for women, and after adjusting for pack years of smoking, age, and residual effect of ethnicity, their findings showed that Filipino, Caucasian, and Hawaiian female smokers had a 273%, 176%, and 67% greater risk, respectively, for developing lung cancer as compared to Japanese female smokers.[59] However, Chinese female smokers were 59% less likely to develop lung cancer as compared to Japanese female smokers.[59] It was hypothesized that these ethnic disparities could be due to differences in dietary antioxidants and/or genetic risk factors among these ethnic groups.[59]

Further work on this subject was conducted by Schwartz *et. al.*[60] in 1997 and included 5,588 African American and white American individuals in Detroit, Michigan diagnosed with lung cancer between 1984 and 1987 and 3,692 controls. In individuals aged 55-84 years old, the difference in lung cancer risk between African Americans and whites was explained by smoking habits; however, in 40-54 year old males, African Americans were found to be 2-4 times more likely to develop any type of lung carcinoma as compared to whites, even after adjusting for smoking habits.[60] In 40-54 year old females, a similar increase in risk was seen for African Americans, but only for squamous cell and small cell carcinomas.[60] Interestingly, another study conducted in 2003 by Stellman *et. al.*[61] comparing black and white Americans found no differences in lung cancer risk in either males or females with similar smoking habits. However, given that the median age for the participants in this study was approximately 60 years old, it is possible that had the authors compared risk based on age group, they might have seen similar results to those reported by Schwartz *et. al*. Stellman *et. al.* also compared lung cancer risk in males from the United States (New York City and Washington, DC) and Japan (Nagoya).[62] This study included 781 lung cancer patients diagnosed between 1992 and 1998 and 625 hospital controls and found that the odds ratio for lung cancer in current US smokers relative to controls was 10 times the odds ratio for lung cancer in current Japanese smokers relative to controls.[62]

A more recent and much larger study published by Haiman *et. al.* in 2006[63] involved 183,813 smokers belonging to five different ethnic groups: African American, Japanese American, Latino, Native Hawaiian, and European American. In individuals smoking 10 or fewer cigarettes per day, African Americans had the highest risk of lung

cancer among current and former smokers, followed by Native Hawaiians, European Americans, Japanese Americans, and Latinos (relative risk being 1.00, 0.88, 0.45, 0.25 and 0.21, respectively, and adjusted for duration of smoking, sex, and time since quitting).[63] The differences in these risks are statistically significant, and have a p-value less than 0.001.[63] However, as the amount of smoking increases, the ethnic differences in lung cancer risk begin to decrease. In individuals smoking 31 or more cigarettes per day, the relative risks adjusted for duration of smoking, sex, and time since quitting are 1.00, 0.95, 0.82, 0.79, and 0.75 for African Americans, Native Hawaiians, European Americans, Latinos, and Japanese Americans, respectively.[63] The ethnic differences in risk in heavy smokers are not statistically significant, having a p-value of 0.31.[63] Adjustment for daily fruit and vegetable intake as well as socioeconomic status did not explain the large difference in lung cancer risk among populations, leading the authors to hypothesize that variations in the metabolism of nicotine between the groups could account for differences in smoking behavior, and therefore differences in exposure to carcinogens in cigarette smoke.[63] Furthermore, among individuals smoking 30 or fewer cigarettes per day, differences in metabolic activation and detoxification of these carcinogens between the groups may play a role in lung cancer risk.[63] However, as the amount of cigarettes smoked per day increases, these pathways become saturated and thus less significant.

1.3 1,3-Butadiene Overview

1,3-Butadiene (BD) is a volatile, colorless gas that is primarily used as a monomer in the production of synthetic rubbers.[64] Worldwide, over 95% of BD is produced as a byproduct of steam cracking, a petrochemical process used to generate

ethylene and other alkenes, but BD can also be produced from the catalytic dehydrogenation of *n*-butane and *n*-butene and the oxidative dehydrogenation of *n*-butene.[64] In 2004, the global demand for BD was approximately 9 million metric tons; 54% of that amount was for its use in styrene-butadiene rubber and polybutadiene, which are used in tires, adhesives, and resins.[64] Individuals involved in the production of BD can be exposed via inhalation, with typical levels estimated to be 2 ppm.[65] BD is also present, albeit in smaller amounts, in smoke from burning wood (approximately 15 ppb),[66] urban air (approximately 1—10 ppb),[67] and cigarette smoke (approximately 20-75 μg in main-stream smoke and 205-260 μg in side-stream smoke).[14, 68] Among the 50+ known human carcinogens present in cigarette smoke, BD was shown to have the highest cancer risk index.[69]

Inhalation exposure of laboratory mice and rats to BD leads to the development of tumors at multiple sites, including the heart, lymphomas, mammary glands, and lungs.[70-73] In humans, epidemiological studies have shown an increased risk of lymphatic and hematopoietic cancers in occupationally exposed individuals, as well as elevated numbers of lung cancer cases in occupationally exposed women.[74-79] Based on results from both animal and human studies, BD has been classified as a known human carcinogen by the International Agency for Research on Cancer (IARC)[80] and the US National Toxicology Program (NTP).[81]

1.3.1 Metabolism of 1,3-Butadiene

BD undergoes metabolic activation to reactive intermediates (Figure 1.7). BD is first oxidized by CYP450 monooxygenases 2E1 and 2A6 to form 3,4-epoxy-1-butene (EB).[82, 83] EB can undergo further oxidation, again by CYP450s 2E1 and 2A6, to form

1,2,3,4-diepoxybutane (DEB),[82-84] or it can be detoxified by epoxide hydrolase (EH) to form 1-butene-3,4-diol (EB-diol).[85] Oxidation of EB-diol by CYP2E1 forms 3,4-epoxy-1,2-butanediol (EBD); EBD can also be formed through the hydrolysis of DEB by EH.[86-88] EB-diol can also be oxidized by alcohol dehydrogenase (ADH) to form hydroxymethyl vinyl ketone (HMVK).[89] These metabolites can be detoxified through GSH conjugation and excreted in urine as mercapturic acids, or they can form adducts with DNA and proteins. These pathways will be discussed in greater detail in Section 1.4.

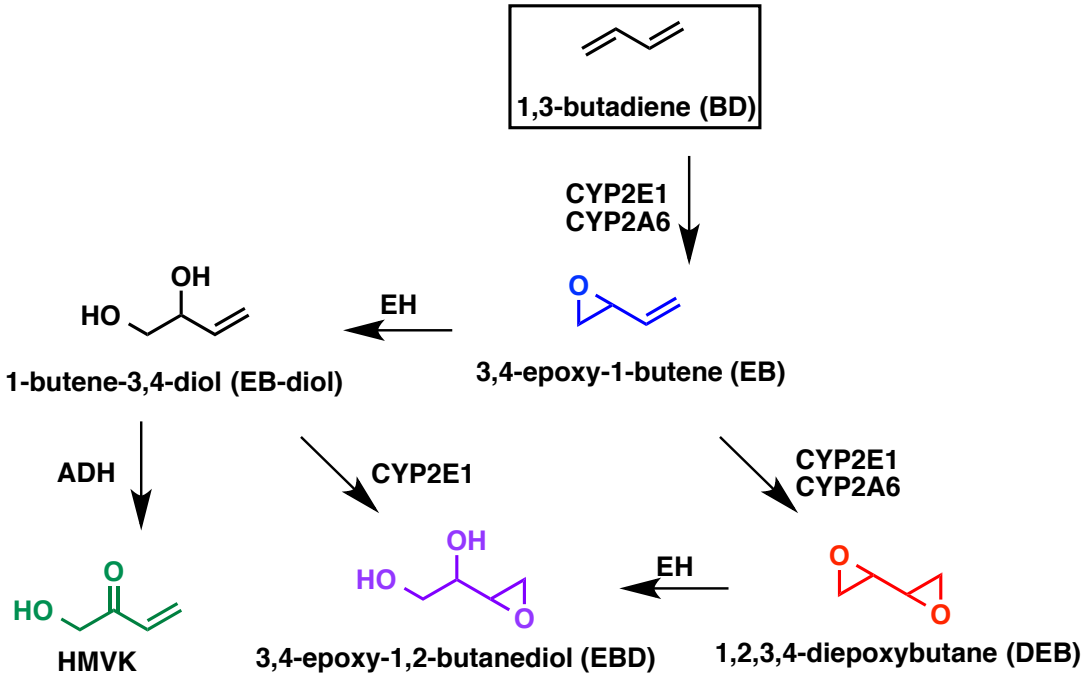


Figure 1.7 Metabolism of BD

1.3.2 Toxicity and Mutagenicity of 1,3-Butadiene

Toxicity of BD was first established through animal studies, where exposure of laboratory rodents to BD via inhalation led to tumors at multiple sites. Sprague-Dawley rats exposed to 0, 1,000, or 8,000 ppm of BD for 6 hours a day, 5 days a week for 105 weeks (females, n = 110) or 111 weeks (males, n = 110) developed tumors of pancreas, testicle, thyroid gland, uterus, Zymbal gland, and mammary gland.[73] Similarly, B6C3F1 mice exposed to 0, 6.25, 20, 62.5, 200 (n = 70, each), or 625 (n = 90) ppm of BD for 6 hours a day, 5 days a week for 104 weeks developed tumors of the lung, heart, forestomach, liver, mammary gland, Harderian gland, ovary, preputial gland and lymphomas.[71]

The genotoxic effects of BD are due to the formation of reactive metabolites—specifically EB, EBD, and DEB—that have the ability to form covalent DNA and protein adducts. In humans, EBD is the most abundant epoxide formed, followed by EB and DEB. Though it is the least abundant epoxide metabolite of BD, DEB is the most mutagenic and genotoxic; this is likely due to its ability to form DNA-DNA crosslinks as a *bis*-electrophile.[90] *In vitro* experiments in which TK6 human lymphoblastoid cells were treated with DEB, EB, and EBD showed that DEB was mutagenic at concentrations of 1—5 μM, whereas EB and EBD were mutagenic at concentrations of 100-800 μM; these results indicate that DEB is 100 to 200 fold more mutagenic than EB or EBD.[88] Comparison of mutational spectra of the *HPRT* gene in these cells revealed that treatment with DEB (4 μM for 24 hours) lead to AT→TA transversions and partial deletions, whereas treatment with EB (400 μM for 24 hours) lead to AT→TA transversions and GC→AT transitions.[91]

In vivo studies in which B6C3F1 *laci* transgenic mice were exposed to 62.5, 625, or 1250 ppm BD for 6 hours a day, 5 days a week, for 4 weeks, revealed *laci* gene AT→GC transitions and AT→TA transversions in the spleen and bone marrow, as well as GC→AT transitions at non-CpG sites in the spleen.[90] Additional studies looking at mutations of the *HPRT* gene in BD exposed humans have also been performed, but the results are conflicting. In occupationally exposed workers, both Ma *et. al.*[92] and Liu *et. al.*[93] reported significantly higher levels of exon deletions in the *HPRT* gene as compared to controls. Additionally, Ma *et. al.* also found increased frameshift mutations and AT→TA transversions in BD exposed workers as compared to controls.[92] However, studies by Hayes *et. al.*[94] and Tates *et. al.*[95] did not show any significant differences in *HPRT* mutation frequencies between BD exposed and control workers.

The ability of BD to induce mutations in other cancer related genes has also been explored. Zhuang *et. al.*[96] analyzed mutations of *p53* and *ras* genes in 40 and 31 lymphomas from B6C3F1 mice, respectively, that had been exposed to BD for two years (details of their exposure can be found in the first paragraph of this section). Of the 31 mice analyzed for *ras* mutations, 32% were found to contain point mutations on K-*ras* and 13% had point mutations on N-*ras*.[96] All mutations were located in codons 12, 13, and 61; point mutations on these codons are associated with rodent and human cancers. The majority of the mice that received lower doses of BD (62.5 ppm) exhibited *ras* mutations, leading the authors to hypothesize that these mutations might play a role in the early development of BD-induced lymphoma.[96] Of the 40 mice analyzed for *p53* mutations, 7 were shown to have both point mutations and frameshift mutations resulting from deletions and insertions.[96] These mutations were found in *p53* exons 5-8, which is

where 90% of reported *p53* mutations occur. All of *p53* mutations were found in mice that received higher doses of BD (625 ppm), indicating that these mutations might play a role in the progression of BD-induced lymphoma.[96]

1.4 Biomarkers of Exposure to 1,3-Butadiene

Biomarkers of BD exposure can be developed by following the bioactivation and detoxification pathways of BD. As described above, reactive epoxides formed upon BD metabolism can directly react with DNA and proteins, or they can undergo detoxification through GSH conjugation, conversion to mercapturic acids, and excretion in urine. BD adducts and detoxification products can be used as biomarkers of BD exposure and potential indicators of cancer risk.

1.4.1 Detoxification/Urinary Biomarkers

The first step in the detoxification of BD is the conjugation of electrophilic BD metabolites with endogenous nucleophilic molecules like GSH; this reaction is facilitated by glutathione *S*-transferases (GSTs). Further processing of these BD metabolite-GSH conjugates by γ-glutamyltranspeptidase, a dipeptidase, such as cysteinyl glycinase, and *N*-acetyltransferase (known as the mercapturic acid (MA) pathway, Figure 1.8) forms mercapturic acids that are ultimately excreted in urine. The use of urine samples over blood samples for analysis of BD biomarkers in humans can be advantageous, as urine is readily available, and its collection is non-invasive. Four mercapturic acids of BD have been reported in the literature; BD-metabolites EB, HMVK, EBD, and DEB are ultimately excreted in urine as 2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene/1-(*N*-acetyl-L-cystein-S-yl)-2-hydroxy- but-3-ene (together referred to as MHBMA),[97] *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine (DHBMA),[48] 4-(*N*-acetyl-L-cystein-S-yl)-1,2,3-

trihydroxybutane (THBMA)[98] and 1,4-*bis*-(*N*-acetyl-L-cystein-S-yl)butane-2,3-diol (*bis*-BDMA),[99] respectively (Figure 1.9).

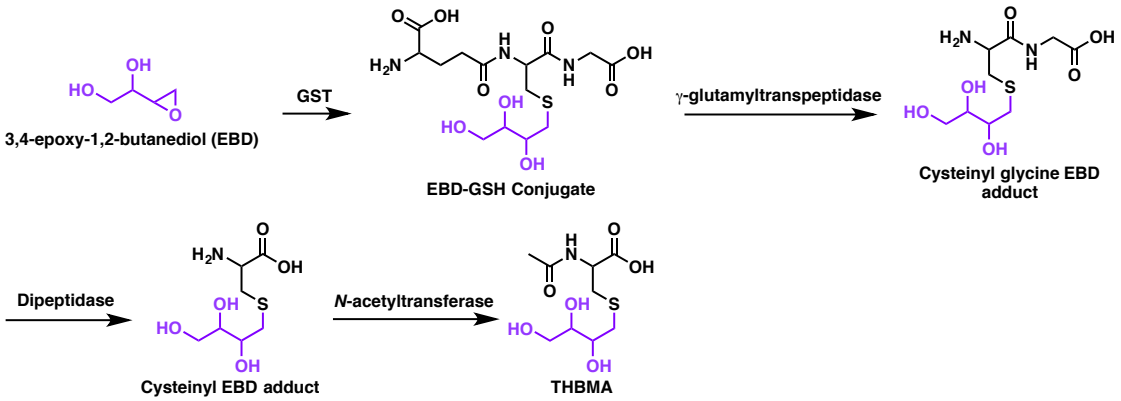


Figure 1.8 MA pathway of BD metabolite EBD

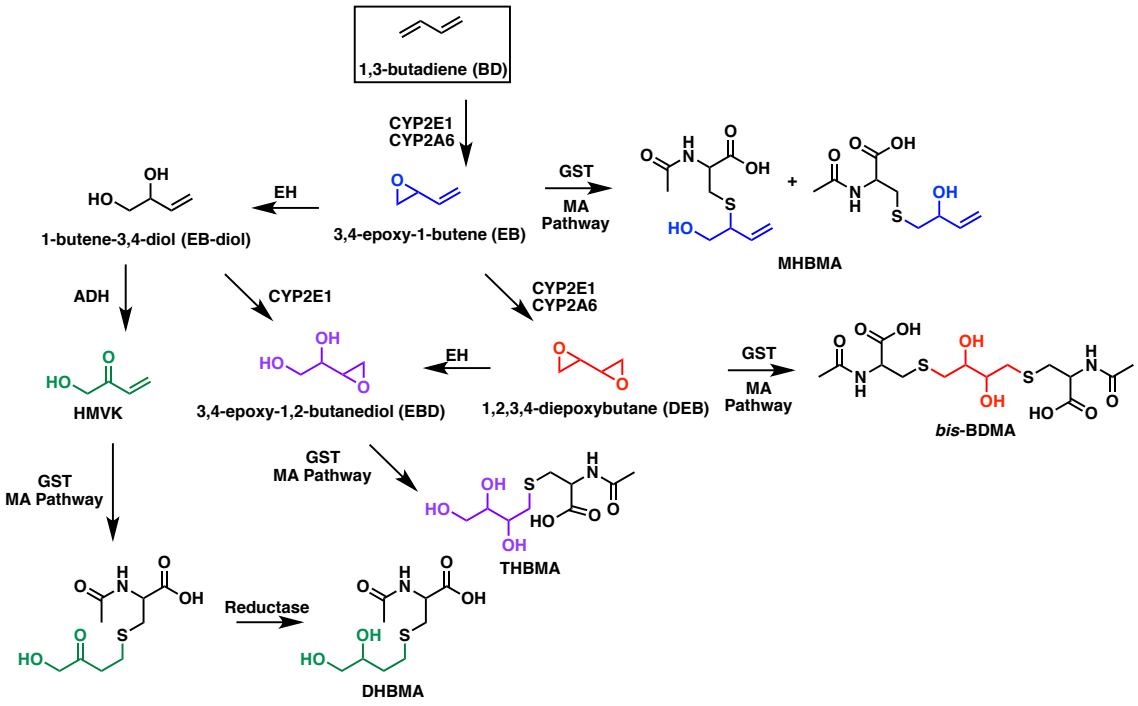


Figure 1.9 Detoxification products formed from BD metabolites

Sabourin *et. al.*[100] identified MHBMA and DHBMA as the major urinary metabolites in F344/N rats, Spraque-Dawley rats, B6C3F1 mice, and Syrian hamsters exposed to 8000 ppm of [^{14}C]-BD and cynomolgus monkeys exposed to 10, 300, and 8000 ppm of [^{14}C]-BD. At the highest BD exposure concentrations, mice, rats, and hamsters excreted 1.5-4 times more MHBMA than DHBMA, whereas monkeys excreted primarily DHBMA.[100] In addition to being the first study to identify DHBMA as a BD metabolite, the data also showed an inverse relationship between the amount of MHBMA excreted and the EH activity in each species, providing an early foundation for understanding BD detoxification in humans. As a follow up to this study, Bechtold *et. al.*[97] subjected B6C3F1 mice and F344/N rats to a more relevant concentration of 11.7 ppm BD and quantified urinary MHBMA and DHBMA. Mice excreted similar amounts of urinary MHBMA and DHBMA, whereas the rats produced greater amounts of urinary DHBMA, indicating that the hydrolysis of EB becomes saturated at higher BD exposures in rats.[97] Looking to compare these data to those from humans, the authors also quantified DHBMA in occupationally exposed workers (3-4 ppm BD), control workers (< 0.1 ppm BD), and outside control individuals, all of whom were nonsmokers.[97] Occupationally exposed workers excreted the highest levels of DHBMA (3200 ng/mL urine), followed by control workers (620 ng/mL urine), and the outside controls (320 ng/mL urine; $p = 0.05$ when comparing exposed workers to outside controls).[97] However, no MHBMA was detected in any human samples in these early studies (LOD: 100 ng MHBMA/mL urine), signifying that in humans, MHBMA is a minor detoxification product, and that EB is predominantly cleared through hydrolysis versus GSH conjugation.

More sensitive methodology for MHBMA detection in humans was developed by Van Sittert *et. al.*[101] (LOD: 0.1 ng/mL urine), who were able to quantify MHBMA (2 mg/mL) and DHBMA (524 ng/mL) in the urine of occupationally exposed workers from the rubber industry in the Netherlands and Czech Republic. Overall, the median values of both MHBMA and DHBMA were highest in polymer workers, followed by monomer workers, and then controls, who surprisingly did show detectable background levels of both mercapturic acids. Upon expanding the study to include rats, mice, and monkeys, the authors found that humans produced the greatest amounts of DHBMA, and therefore had the highest rates of EB hydrolysis of all species examined.[101] Ultimately, the study concluded that the increased hydrolysis of BD epoxides in humans puts them at a lower cancer risk associated with BD exposure as compared to rats, mice, and monkeys. Albertini *et. al.*[102] continued this work to examine correlations between urinary MHBMA and DHBMA levels and genetic polymorphisms, gene mutations, levels of other BD-induced adducts, and BD exposure in occupationally exposed males. Though the authors found no association between MHBMA and DHBMA levels and polymorphisms in BD-metabolizing enzymes *CYP2E1*, *EH*, and *ADH* or *hrpt* gene mutations, they did see positive correlations with BD-hemoglobin adducts and BD exposure.[102]

Further examination between the association of BD exposure and urinary MHBMA/DHBMA levels was carried out by Urban *et. al.*,[103] who quantified the BD-mercapturic acids in smokers and nonsmokers (n = 10 each). MHBMA levels were much higher in smokers (86.4 ± 14.0 μg/24 hours) than in nonsmokers (12.5 ± 1.0 μg/24 hours; $p < 0.001$), but DHBMA levels were similar between the two groups (smokers: 644 ± 90 μg/24 hours, nonsmokers: 459 ± 72 μg/24 hours), signaling that MHBMA

might be a better biomarker in humans of low levels of BD exposure, such as those found in cigarettes.[103] Carmella *et. al.*'s[48] work regarding smoking cessation further supports this claim: urinary MHBMA levels decreased > 80% in smokers three days after quitting and > 95% fifty-six days after quitting, whereas DHBMA levels remained constant even after quitting. Roethig *et. al.*[104] quantified both MHBMA and DHBMA in a study with a much larger sample size (n = 3,585 smokers and 1,077 nonsmokers) and found that MHBMA levels were 12 times higher in smokers than nonsmokers, while DHBMA levels were only 1.4 times higher in smokers. Interestingly, when the authors further categorized their subjects by ethnic group, they found that European American smokers excreted significantly higher amounts of both MHBMA and DHBMA as compared to African American smokers, despite African American smokers having the larger lung cancer risk.[104]

Our laboratory further investigated BD's role in lung cancer risk among smokers of different ethnic groups by developing an HPLC-ESI$^-$-MS/MS methodology for the quantitation of MHBMA and DHBMA in urine, and applying it to urine samples from native Hawaiian, European American, and Japanese American smokers (n = 193, 195, and 196).[105] In this study, Park *et. al.* found that urinary MHBMA levels differed significantly between ethnic groups (p = 0.0001), with European Americans excreting the highest amounts and Japanese Americans excreting the lowest.[105] Urinary MHBMA levels among the ethnic groups did not correlate with lung cancer risk, however, the differences were significantly associated with the *GSTT1*-null genotype.[105]

Though MHBMA and DHBMA are some of the most extensively studied BD-mercapturic acids, they are not the only detoxification products that can be used as

biomarkers of BD exposure. Richardson *et. al.* first identified THBMA in Spraque-Dawley rats and B6C3F1 mice exposed to 200 ppm [^{14}C]-BD, where it comprised 10% of the total urinary radioactivity in the exposed rodents.[106] The formation of THBMA at lower levels of BD exposure (1-20 ppm) was later confirmed by Booth *et. al.* using [^{14}C]-BD in rodents.[107] THBMA was first detected in human urine by Kotapati *et. al.*[98] in our laboratory, where median urinary THBMA levels were significantly higher in smokers (21.6 ng/mg creatinine) than nonsmokers (13.7 ng/mg creatinine; $p < 0.01$). Following smoking cessation, urinary THBMA levels decreased 25-50%, suggesting that exposure to BD from cigarettes is an important source for the formation of this metabolite.[98] Boogaard *et. al.* were the first to find support for the formation of a fourth BD-mercapturic acid, *bis*-BDMA, when they observed evidence of a *bis*-glutathione conjugate of DEB.[108] Kotapati *et. al.* quantified *bis*-BDMA in rats exposed to BD (0, 62.5, and 200 ppm) in a dose dependent manner, but it has yet to be detected in human urine samples.[99]

1.4.2 Butadiene-DNA Adducts

Because DNA adducts represent biologically relevant cellular damage that can lead to mutations and cancer, their quantification and use as biomarkers of exposure and risk is of high importance. BD epoxides have the ability to form covalent bonds at the nucleophilic sites on the DNA bases adenine (A), guanine (G), thymine (T), and cytosine (C), forming DNA adducts. The most common sites of adduct formation by BD are the N7 position of G, the N1, N3, and N^6 positions of A, and the N3 position of T. EB can react with G at the N7 position to form N7-(2-hydroxy-3-buten-1-yl)guanine (EBGI) and N7-(1-hydroxy-3-buten-2-yl)guanine (EBGII) or at the N^6 position of A to form N^6-(2-

hydroxy-3-buten-1-yl)-2'-deoxyadenosine (N^6-HB-dA I) and N^6-(1-hydroxy-3-buten-2-yl)-2'-deoxyadenosine (N^6-HB-dA II) (Figure 1.9).[109-111] Similarly, EBD can also react at the N7 position of G to give N7-(2',3',4'-trihydroxybut-1'-yl)guanine (N7-THBG) (Figure 1.9).[112] As a *bis*-electrophile, DEB can react with two different DNA bases to form interstrand and intrastrand crosslinks, or twice with one DNA base to form exocyclic DNA adducts. Examples of DNA-DNA crosslinks formed by DEB include 1,4-*bis*-(guan-7-yl)-2,3-butandiol (*bis*-N7G-BD) and 1-(guany-7-yl)-4-(aden-1-yl)-2,3-butandiol (N7G-N1A-BD);[113, 114] examples of exocyclic adducts formed from DEB include N^6-(2-hydroxy-3-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine (1,N^6-γ-HMHP-dA), N^6-(1-hydroxy-2-hydroxymethylpropan-1,3-diyl)-2'-deoxyadenosine (1,N^6-α-HMHP-dA), and N^6,N^6-(2,3-dihydroxybutan-1,4-diyl)-2'-deoxyadenosine (N^6,N^6-DHB-dA) (Figure 1.10).[115]

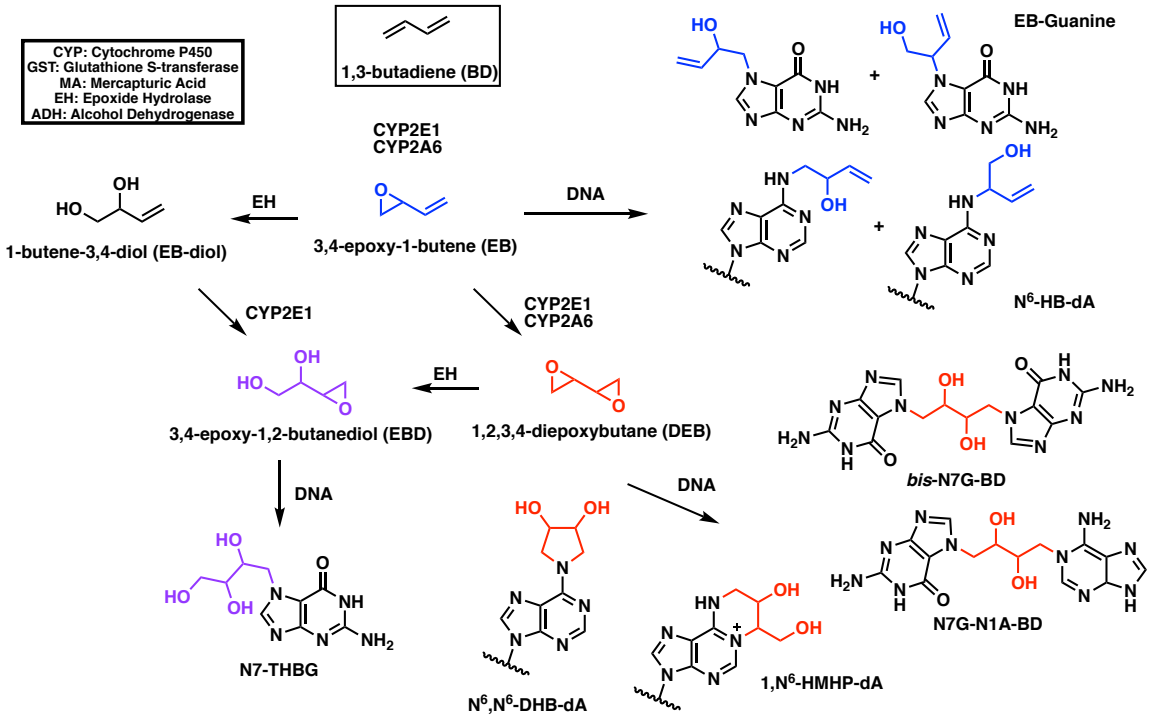


Figure 1.10 DNA adducts formed from BD metabolites

Methodology employing ^{32}P-postlabeling and high performance liquid chromatography–mass spectrometry has been used to quantify BD-DNA adducts. Koivisto *et. al.* reported the formation of EBGI, EBGII, and N7-THBG in the liver, lung, and testis tissues of mice and rats exposed to BD.[116-119] Using isotope dilution mass spectrometry, Tretyakova *et. al.* quantified EBGI, EBGII, and N7-THBG in the liver tissue of rodents exposed to 1250 ppm BD for ten days.[120] Expansion of this study by Koc *et. al.* showed a clear dose dependent relationship of these adducts in the liver, lung, and kidney tissues of mice and rats exposed to increasing concentrations of BD (20, 62.5, or 625 ppm).[112]

DNA crosslinks formed from DEB have been extensively studied in our laboratory. In C57BL/6 mice exposed to 625 ppm BD for seven hours a day, five days a week, Goggin *et. al.* found significantly higher levels of *bis*-N7G-BD adducts in both liver (3.2 ± 0.4 adducts/10^6 dG) and lung (1.8 ± 0.5 adducts/10^6 dG) tissues as compared to controls.[121] N7G-N1A-BD adducts were also detected in DNA from liver tissue in B6C3F1 mice exposed to 625 ppm BD for two weeks.[122] Levels of both *bis*-N7G-BD and N7G-N1A-BD adducts in liver tissues increased linearly with BD exposure (0-625 ppm) in mice, whereas adduct levels plateaued in rats at an exposure of 62.5 ppm BD, indicating metabolic saturation.[123] Goggin *et. al.*[10] also quantified the DEB specific exocyclic adducts 1,N^6-HMHP-dA in liver tissues of B6C3F1 mice exposed to 625 ppm BD (0.44 0.08 adducts/10^8 nucleotides). Persistence of these various DEB specific DNA adducts varied, with N7G-N1A-BD and 1,N^6-HMHP-dA adducts having a half-life > 30 days in lung, liver, and kidney tissues.

1.4.3 BD-Hemoglobin Adducts

Electrophilic BD epoxides can react with the N-terminal valine in hemoglobin to form BD-hemoglobin adducts.[124] Reaction of this N-terminus with EB generates N-(2-hydroxy-3-butenyl)-valine (HB-Val);[125] reaction with EBD forms N-(2,3,4-trihydroxybutyl)-valine (THB-Val),[126] and reaction with DEB forms *N*,*N*-(2,3,-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val)[127] (Figure 1.11). Unlike BD-DNA adducts, these protein adducts do not undergo repair and therefore accumulate over the lifetime of red blood cells; while they do not represent the risk of mutation or cancer due to BD exposure, their quantitation can act as a sensitive biomarker for BD dosimetry studies.[128]

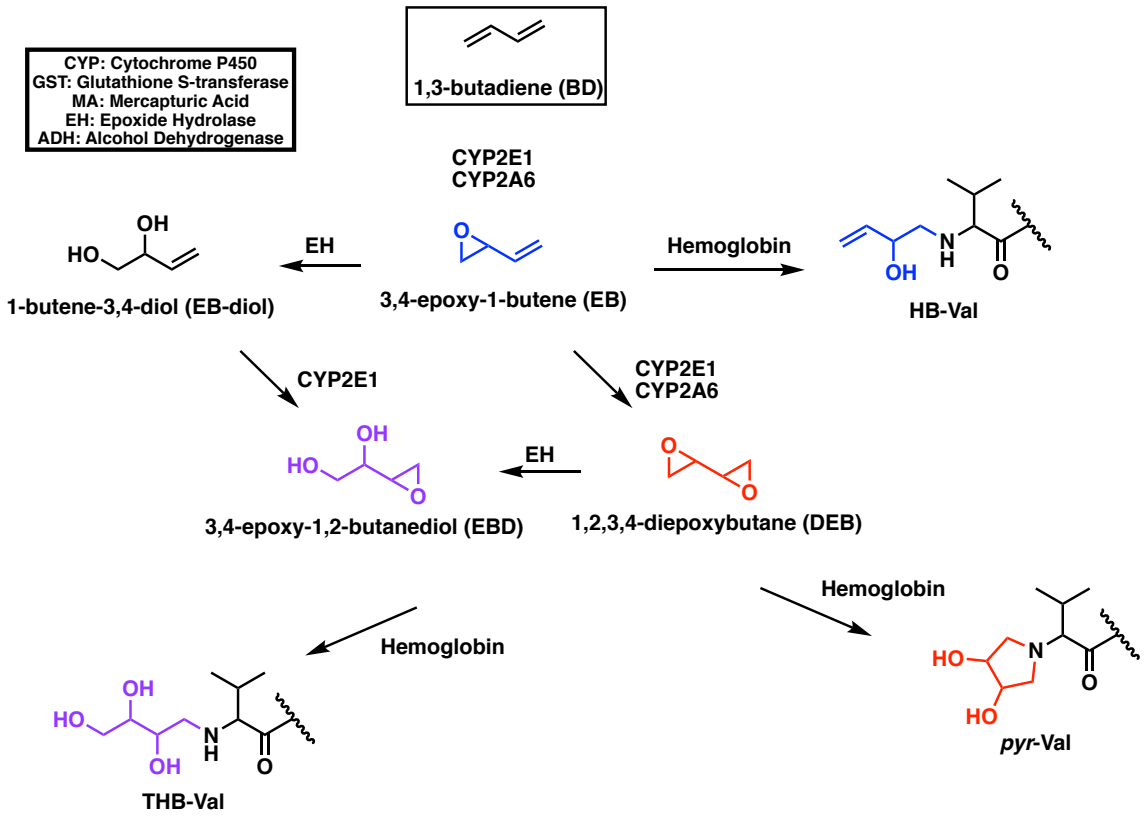


Figure 1.11 Hemoglobin adducts formed from BD metabolites

All three BD-hemoglobin adducts (HB-Val, THB-Val, and *pyr*-Val) have been detected in blood samples from laboratory animals exposed to BD by inhalation and humans exposed to BD in occupational settings. The first reported detection of HB-Val and THB-Val *in vivo* was by Osterman-Golkar *et. al.* who saw a linear dose response relationship between HB-Val levels and BD exposure (0, 250, 500, or 1000 ppm BD for six hours/day for two weeks) in Wistar rats. Rats exposed to 1000 ppm had an average of 3 nmol HB-Val/g hemoglobin.[125] Similar results were seen for THB-Val in a follow up study in which the adduct was detected in both mice and rats exposed to BD (0, 2, 10, or 200 ppm).[126] Additionally, both HB-Val and THB-Val were quantified in occupationally exposed workers (1 ppm BD), with levels ranging from 1-3 pmol/g hemoglobin and 10-14 pmol/g hemoglobin, respectively.[129] *pyr*-Val was first reported by Kautianine *et. al.* in 2000,[127] who observed its formation in mice treated with large doses of DEB; similar results in rats were confirmed later in an expanded study by Fred *et. al.*[130]

Because BD is metabolized differently by different species, there are significant interspecies differences with regard to BD-hemoglobin adduct levels. In B6C3F1 mice and Spraque-Dawley rats exposed 1000 ppm BD for six hours/day five days/week, HB-Val and THB-Val levels were 2-8 times higher in mice.[131] The same study also quantified THB-Val in occupationally exposed and unexposed workers in China, reporting statistically significantly higher levels in exposed workers (88 ± 59 pmol/g hemoglobin) as compared to controls (39 ± 13 pmol/g hemoglobin; $p < 0.001$).[131] Further studies in humans compared HB-Val and THB-Val levels in men working in administration (0.010 ppm BD), monomer production (0.290 ppm BD), and polymer production (0.811 ppm BD) at a BD and styrene-BD rubber production facility in the Czech Republic.[132] Both

HB-Val and THB-Val levels increased with increasing BD exposure (polymer production > monomer production > administration), and the differences in adduct levels between the groups were statistically significant ($p < 0.001$).

Boysen *et. al.* quantified HB-Val, THB-Val, and *pyr*-Val in B6C3F1 mice and Sprague-Dawley rats exposed to 3, 62.5, and 1250 ppm BD six hours/day for two weeks.[133] THB-Val was found to be the most abundant adduct in both species, however, mice formed larger amounts of *pyr*-Val than rats, further highlighting the differences in BD metabolism between the two species. Similar studies in which mice and rats were exposed to 0, 0.5, 1, or 1.5 ppm BD confirmed that pyr-Val does form during exposure to sub ppm concentrations of BD.[134] Boysen *et. al.* were also the first to report the quantitation of *pyr*-Val in occupationally exposed humans.[135] Concentrations were lowest in control workers (0.11 ± 0.07 pmol/g hemoglobin), higher in monomer production workers (0.16 ± 0.12 pmol/g hemoglobin), and highest in polymer production workers (0.29 pmol/g hemoglobin), showing a positive correlation between adduct formation and BD exposure.

1.5 Summary and Thesis Goals

BD is a well-known industrial and environmental chemical. Humans can be exposed to BD occupationally during the production of synthetic rubber or environmentally through exposure to automobile exhaust, wood burning fires, or cigarette smoke. BD is metabolically activated to reactive epoxides, which can react with DNA and hemoglobin to form adducts, or be detoxified through conjugation with GSH. These compounds can act as biomarkers of BD exposure and can be used in risk assessment of BD and can provide insight into interethnic and inter-individual differences in BD

metabolism. The first goal of this thesis is to elucidate the role that BD specifically plays in cancer risk differences among smokers of different ethnic groups. By quantifying MHBMA and DHBMA in the urine of these smokers using HPLC-ESI-MS/MS methodology developed in our laboratory, we can look for associations with single nucleotide polymorphisms in their genomes, correlations with other biomarkers of BD exposure, such as BD DNA-adducts, or the effect of chemopreventative agents such as phenethyl isothiocyanate on BD metabolism.

MHBMA and DHBMA represent BD biomarkers that have been comprehensively studied, and while their continued use is vital in uncovering new information regarding BD's role in smoking induced lung cancer, there still remains a need to develop new biomarkers of BD exposure. By focusing on the development of biomarkers formed specifically from DEB, we can create a way to measure formation of the most mutagenic of the BD metabolites. Though DEB specific biomarkers exist, only one—*pyr*-Val—has ever been quantitated in humans, and it requires blood samples to be collected. Therefore, the second goal of this thesis is to develop a DEB-specific biomarker of BD exposure in humans that can be measured in urine, a more easily obtainable biological liquid than blood.

II. GENETIC DETERMINANTS OF 1,3-BUTADIENE METABOLISM AND DETOXIFICATION IN THREE POPULATIONS OF SMOKERS WITH DIFFERENT RISKS OF LUNG CANCER

Adapted from:

Boldry, E. J., Patel, Y.M., Kotapati, S., Esades, A., Park, S.L., Tiirikainen, M., Stram, D. O., Le Marchand, L., and Tretyakova, N.; Genetic Determinants of 1,3-Butadiene Metabolism and Detoxification in Three Populations of Smokers with Different Risks of Lung Cancer. *Cancer Epidemiol. Biomarkers Prev.* **2017**, 26, 1034-1042.

This work was performed in collaboration with Yesha Patel, Dr. Srikanth Kotapati, Amanda Esades, Dr. Lani Park, Dr. Maarit Tiirikainen, Dr. Daniel Stram, and Dr. Loic Le Marchand, under the direction of Dr. Natalia Tretyakova. Urine samples were provided by Loic Le Marchand. HPLC-ESI$^-$-MS/MS methodology was developed by Srikanth Kotapati and used by Emily Boldry, Srikanth Kotapati, and Amanda Esades for urine sample analysis. Enzyme experiments were carried out by Emily Boldry. Statistical analysis was carried out by Yesha Patel with assistance from Lani Park under the supervision of Daniel Stram. Genotyping was performed by Maarit Tiirikainen. Emily Boldry, Yesha Patel, Lani Park, Maarit Tiirikainen, Loic Le Marchand, and Natalia Tretyakova wrote the manuscript.

2.1 Introduction

Cigarette smoking is a leading cause of lung cancer, with 14-28% of male smokers and 13-28% of female smokers above the age of 35 at risk for developing the disease in the United States.[136] Smoking is responsible for 87% and 70% of lung cancer deaths in men and in women, respectively.[137] However, the risk for the development of lung cancer in smokers varies greatly between ethnic groups, with African American and Native Hawaiian smokers having the highest risk, followed by White, Japanese American, and Latino smokers.[63] These ethnic differences remain after adjustment for reported smoking history.[63] While the exact origins of the pronounced ethnic differences in smoking-induced lung cancer risk remain to be established, the frequencies of genetic polymorphisms in xenobiotic metabolism genes differ significantly between racial groups, potentially affecting the extent of carcinogen bioactivation to DNA-reactive species.[105, 138-144]

The mechanism of smoking-induced lung cancer involves irreversible binding of metabolically activated tobacco carcinogens to DNA, forming covalent DNA adducts which cause mutations in critical genes.[57] Cigarette smoke contains 69 known carcinogens, including 1,3-butadiene (BD) (20-75 μg and 205-360 μg per cigarette in mainstream and side stream smoke, respectively).[14, 68] BD is a multi-site carcinogen in laboratory rats and mice.[70-73] Epidemiological studies have uncovered an association between occupational exposure to BD and the development of leukemia and lymphoma in humans,[74-79] leading to its classification as a Group 1 agent by IARC and as a known human carcinogen by the National Toxicology Program.[80, 81]

BD is metabolically activated to several DNA reactive species, including 1,2-

epoxybutene (EB), 1,2,3,4-diepoxybutane (DEB), hydroxymethylvinyl ketone (HMVK), and epoxy-1,2-butanediol (EBD) (Figure 1.7).[82-84] Epoxidation of BD to EB is catalyzed by cytochrome P450 monooxygenases 2E1 and 2A6 (CYP2E1 and 2A6).[83] Epoxide hydrolase (EH)-mediated hydrolysis of EB gives rise to 1-butene-3,4-diol (EB-diol), which is subsequently converted to HMVK by alcohol dehydrogenase (ADH).[85, 89] Alternatively, EB can be further epoxidized by CYP2E1 to 1,2,3,4-diepoxybutane (DEB),[84] which in turn can be hydrolyzed to epoxy-1,2-butanediol (EBD).[86, 87]

Analysis of urinary BD-mercapturic acids can be employed to monitor human exposure to BD and the extent of its bioactivation to electrophilic species. EB, HMVK, EBD, and DEB can be conjugated with glutathione (GSH) and further processed via the mercapturic acids pathway to form 2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene) (together referred to as MHBMA), *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), 4-(*N*-acetyl-L-cystein-S-yl)-1,2,3-trihydroxybutane (THBMA) and 1,4-*bis*-(*N*-acetyl-L-cystein-S-yl)butane-2,3-diol (*bis*-BDMA), respectively (Figure 1.7).[48, 97-101] It has been proposed that glutathione *S*-transferases theta 1 and mu 1 (GSTT1 and GSTTM1) can catalyze this reaction,[145, 146] however, direct biochemical evidence for their involvement has not been provided. Urinary concentrations of MHBMA and DHBMA are elevated in smokers as compared to nonsmokers and decrease upon smoking cessation; of the two, MHBMA is more strongly associated with smoking.[48, 103, 104]

Significant interspecies differences in response to butadiene have been observed, with laboratory mice being significantly more sensitive than rats towards BD-induced cancer.[71, 73] Mice developed lung tumors following BD exposure to as low as 6.25 ppm,

while rats developed only minor tumors at BD exposures as high as 1000 ppm.[71, 73] These differences are thought to be a result of more efficient bioactivation of BD to EB and DEB and less efficient detoxification of BD-epoxides in mice.[82, 147-149] Similarly, the balance of BD bioactivation and detoxification in a given individual is likely to be dependent upon competing enzymatic reactions mediated by CYP2E1, CYP2A6, EH, ADH, and GST proteins (Figure 1.7). Specifically, single nucleotide polymorphisms (SNPs) in genes encoding for butadiene metabolism genes may affect the metabolic pathways of BD in smokers and workers occupationally exposed to BD, potentially modifying lung cancer risk.[150-153]

The main goals of the present study were to compare urinary excretion of butadiene metabolites MHBMA and DHBMA in a large cohort of African American, White, and Japanese American smokers and to examine the associations between urinary MHBMA and DHBMA excretion and specific genetic variants via a large scale genome wide association study (GWAS). By identifying variants associated with MHBMA and DHBMA excretion, we can begin to establish the mechanisms by which differences in BD metabolism may modify the risk of smoking-induced lung cancer.

2.2 Experimental

2.2.1 Materials

MHBMA, DHBMA, $^{2}H_{6}$-MHBMA, and $^{2}H_{7}$-DHBMA were purchased from Toronto Research Chemicals (Toronto, Canada). Recombinant human GSTT1 and GSTT2 were purchased from MyBioSource (San Diego, CA). LC/MS grade formic acid was obtained from Sigma Aldrich (St. Louis, MO), and LC/MS grade water and acetonitrile were acquired from Fisher Scientific (Pittsburgh, PA). All other reagents

were purchased from Sigma Aldrich (St. Louis, MO). Oasis HLB 96 well plates were procured from Waters Corporation (Milford, MA).

2.2.2 Study Population

Subjects for this study were participants in the Multiethnic Cohort Study (MEC), which consists of 215,251 men and women from five ethnic groups: Whites, African Americans, Hawaiians, Japanese Americans, and Latinos.[63] Participants, aged 45-75 years old, from Hawaii and California enrolled in the MEC between 1993 and 1996 by completing a detailed questionnaire that outlined dietary habits, demographic factors, education level, occupation, personal behavior, prior medical conditions, and family history of cancer.

This specific study employed urine samples from Japanese American, African American and white individuals who were current smokers at time of urine collection and had no personal history of cancer. Blood and first morning urine samples were collected from participants in California; blood and an overnight urine sample were collected from participants in Hawaii. All urine was kept on ice until processing; aliquots were stored at -20 ºC until analysis.

2.2.3 Data Collection

A total of 1,072 samples were analyzed for MHBMA and DHBMA (n= 327 African Americans, 396 Whites, and 349 Japanese Americans). MHBMA and DHBMA concentrations were adjusted for age, sex, total nicotine equivalents (TNE), and in some cases, for urinary creatinine. The methods of measuring creatinine and TNE (the sum of nicotine, cotinine, 3′-hydroxycotinine and their glucuronides, and nicotine *N*-oxide) have been previously described.[141, 154, 155]

2.2.4 HPLC-ESI$^-$-MS/MS Analysis of MHBMA and DHBMA in Human Urine

Urinary concentrations of MHBMA and DHBMA were determined using previously published HPLC-ESI$^-$-MS/MS methods.[105, 156] Samples that showed no MHBMA or DHBMA signal were assigned a value corresponding to the limit of detection divided by 2 (0.1 ng/mL urine for MHBMA [9 samples] and 2.5 ng/mL urine for DHBMA [2 samples]).

Sixteen sets of quality control samples (48 samples total) were included in the analyses. These positive controls were used to account for inter-batch variation. When necessary, data were adjusted for batch using the values for urinary MHBMA and DHBMA concentration in these samples. Overall, the mean coefficient of variation for these replicates was 8.87% and 8.49% for MHBMA and DHBMA, respectively.

In addition to calculating urinary MHBMA and DHBMA concentrations, a metabolic ratio was also determined as part of the analyses. Metabolic ratio is calculated as the total amount of urinary MHBMA divided by the total amount of MHBMA plus the total amount of DHBMA [MHBMA / (MHBMA + DHBMA)]. For this investigation, the metabolic ratio can be representative of a fraction of non-hydrolyzed EB and provide an understanding into the metabolic processing of BD in an individual.

2.2.5 Genotyping and Quality Control

DNA was extracted from blood leukocytes using a QiaAmp DNA blood extraction kit (Qiagen, Valencia, CA). Samples were genotyped using the Illumina Human1M-Duo BeadChip (1,199,187 SNPs) as described previously.[157] The genotyping quality control consisted of removing individual samples with ≥2% of genotypes not called, removing SNPs ≤98% call rate and known duplicate samples, excluding samples

with close relatives (as determined by estimated IBD status), and samples with conflicting or indeterminate sex. Imputation was performed using SHAPEIT and IMPUTE2 to a reference panel from the 1000 Genomes Project (1KGP; March, 2012).[158, 159] We included SNPs with an IMPUTE2 info score of ≥0.30 and minor allele frequency (MAF) >1% in any MEC ethnic group. A total of 11,892,802 SNPs/indels with a frequency >1% in any single ethnic population (1,131,426 genotyped and 10,761,376 imputed) were included in the analysis.

GSTT1 and *GSTM1* gene copy number assays were run using TaqMan copy number assays Hs00010004_cn Hs02575461_cn, respectively. All assays were run on the 7900HT FAST Real-Time System (Life Technologies, Carlsbad, CA). SNPs were called using the TaqMan Genotyper software, and copy number calls were determined using the CopyCaller v2.0 software (Life Technologies, Carlsbad, CA). Approximately 5% blind duplicate samples were included for quality control. Genotyping of the *GSTT1* and *GSTM1* deletion polymorphisms was successful in 1,009 and 1,068 individuals, respectively. For the purposes of this study, the deletion or null genotype is represented as (0/0), one copy of the gene is represented as (1/0), and two copies of the gene is represented as (1/1).

2.2.6 Statistical Methods

Association of each variant with geometric mean MHBMA, DHBMA or metabolic ratio levels was evaluated using linear regression models, with adjustment for age, sex, race, TNE, BMI, and the first 10 principal components. Principal components were estimated using 19,059 randomly selected autosomal SNPs with frequency ≥ 2% in the combined multiethnic sample.[69] A p-value cut-off of 5×10^{-8} was used for genome-

wide significance. In regions with multiple associated variants, conditional models were used to evaluate individual signals at $p < 5 \times 10^{-8}$. In a like manner, ethnic-specific analyses were performed in each of the three individual populations. Percentage variation of MHBMA, DHBMA or the metabolic ratio was assessed using R^2 values. To further assess associations with variants located in the deleted region, analyses among subjects homozygous for the *GSTT1* non-null alleles were performed.

2.2.7 GSTT1 and GSTT2 catalyzed conjugation of EB with glutathione

1,2-epoxybutene (EB; final concentration: 2 mM) was incubated with glutathione (GSH; final concentration: 5 mM) in the presence or in the absence of GSTT1 or 2 (0, 2.5, or 5 μg) in a 0.1 M phosphate buffer, pH 7.4 (50 μL total volume). The mixtures were allowed to incubate for 2 hours at 37 °C and quenched with 15% (w/w) trichloroacetic acid (50 μL). Reaction mixtures were filtered using Amicon Ultra Centrifugal Filters (0.5 mL, 10K; EMD Millipore, Billerica, Massachusetts), and the filters were subsequently washed with 100 μL water.

HPLC-ESI^+-MS/MS analyses were performed using an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 1100 Series LC/MSD Trap SL (Agilent Technologies, Santa Clara, CA). An Agilent Zorbax SB300 C18 column (150 x 0.5 mm, 5 μm) column was maintained at 25 °C and eluted with 15 mM ammonium acetate (A) and methanol (B) with a linear gradient of (time, %B): 0-20 min, 2 to 20% B. Under these conditions, the epoxybutene-glutathione conjugate (EB-GSH) eluted at approximately 3.8 minutes. Extracted ion chromatograms of the conjugate, *m/z* 317.2, were used to determine the fold increase in EB-GSH formation as compared to the corresponding non-enzymatic reaction.

2.3 Results

A total of 1,072 smokers (327 African Americans, 349 Japanese Americans, and 396 Whites) were included in the analysis (Table 2.1). Overall, there were significant differences in smoking habits between these groups, with Whites smoking the greatest numbers of cigarettes per day (CPD), followed by Japanese Americans and African Americans (Table 2.1). Racial/ethnic differences were observed also for TNE, with, however, a different ordering: African American smokers had the highest levels, followed by Whites and Japanese Americans (Table 2.1). These trends with respect to CPD and TNE were the same when the smokers were categorized by sex. Significant differences were also seen in the creatinine levels between the groups, with African Americans having much higher levels than Japanese Americans and Whites (Table 2.1). Because of this large variability, MHBMA and DHBMA levels can appear artificially low for African Americans when adjusted for creatinine. Therefore, unadjusted values (ng/mL urine) were employed in our final analyses.

Table 2.1 Summary of study population stratified by race/ethnicity and sex.			
	African Americans	**Japanese Americans**	**Whites**
	Median [Interquartile Range]		
All	n = 327	n = 349	n = 396
Age (years)	64 [59- 69]	63 [59-69]	62 [59-68]
BMI (kg/m^2)	26.9 [23.2 - 30.7]	24.3 [21.9 - 26.6]	24.8 [22.0 - 28.1]
Creatinine (mg/dL)	88 [54-138]	54 [33-81]	53 [33-83.2]
Cigarettes per day	10 [5-18]	13 [10-20]	17 [10-20]
Total nicotine equivalents (nmol/mL)	44.2 [26.8-73.8]	26.5 [15.4-41.2]	35.8 [21.9-60.8]
Males	n = 94	n = 181	n = 169
Age (years)	63 [58-66]	63 [59-68]	62 [59-67]
BMI (kg/m^2)	25.7 [23.0-28.2]	25.0 [23.0-26.9]	25.8 [23.3-27.9]
Creatinine (mg/dL)	124.5 [81.5-165.1]	66 [40-95]	71.0 [46.3-104.0]
Cigarettes per day	10 [6-20]	15 [10-20]	20 [12.5-20]
Total nicotine equivalents (nmol/mL)	54.4 [29.2-95.5]	29.3 [17.9-45.6]	39.9 [24.6-73.0]
Females	n = 233	n = 168	n = 227
Age (years)	65 [60-71]	64 [59-70.5]	62 [58-69]
BMI (kg/m^2)	27.5 [23.6-31.6]	23.4 [20.6-26.5]	24.0 [21.0-28.3]
Creatinine (mg/dL)	79 [50-126]	44.5 [28.0-63.5]	46 [29-65]
Cigarettes per day	10 [5-15]	10 [7.5-20]	15 [7-20]
Total nicotine equivalents (nmol/mL)	41.3 [26.1-65.3]	22.5 [13.0-35.0]	31.2 [20.2-50.5]

Geometric means for urinary concentrations of MHBMA and DHBMA in African American, Japanese American, and White smokers (ng/ml urine) are given in Table 2.2. Urinary levels of MHBMA were significantly different between the three ethnic groups overall ($p = 4.0 \times 10^{-25}$) and gender-specific (males: $p = 7.5 \times 10^{-11}$, females: $p = 1.7 \times 10^{-15}$). African American smokers excreted the highest amounts of MHBMA, followed by White and Japanese American smokers (6.4, 5.7, and 3.3 ng/mL urine, respectively). Urinary levels of DHBMA also differed across ethnic groups ($p = 3.3 \times 10^{-4}$), with African Americans excreting the highest amounts of the metabolite, followed by White and Japanese American smokers (362.0, 294.6, and 292.7 ng/mL urine, respectively). These overall differences extended to females only (males: $p = 0.07$, females: $p = 4.1 \times 10^{-13}$); the DHBMA levels of African Americans in males were significantly different from Whites ($p < 0.05$). In female smokers, DHBMA levels also only differed significantly between African Americans and Whites ($p < 0.05$). The ethnic differences for the metabolic ratios (calculated as MHBMA / (MHBMA + DHBMA)) were similar to those for MHBMA ($p = 1.7 \times 10^{-14}$) overall, and similar ethnic differences were observed for each sex (males: $p = 2.2 \times 10^{-6}$, females: $p = 2.8 \times 10^{-9}$). For both sexes, the metabolic ratios in Japanese Americans were significantly lower than those of Whites ($p < 0.05$), while no differences were observed for other ethnic groups.

Table 2.2 Geometric means (95% confidence limits) for urinary MHBMA and DHBMA by race/ethnicity and sex. Values are given in ng/mL urine.

	All		African Americans		Japanese Americans		Whites		
	Geometric means [a]	(95% CL)[a]	Geometric means [a]	(95% CL)[a]	Geometric means [a]	(95% CL)[a]	Geometric means [a]	(95% CL)[a]	p-value [b]
All									
N	1072		327		349		396		
MHBMA	4.8	(4.5 - 5.2)	6.4	(5.9 - 7.0)	3.3	(3.0 - 3.6) [c]	5.7	(5.2 - 6.1)	4.0×10^{-25}
DHBMA	308.8	(292.8 - 325.8)	362.0	(332.5 - 394.1) [c]	292.7	(270.1 - 317.3)	294.6	(274.1 - 316.7)	3.3×10^{-4}
MHBMA / (MHBMA + DHBMA)	0.017	(0.016 - 0.018)	0.018	(0.016 - 0.019)	0.012	(0.011 - 0.012) [c]	0.020	(0.018 - 0.021)	1.7×10^{-14}
Males									
N	444		94		181		169		
MHBMA	5.3	(4.9 - 5.7)	7.6	(6.5 - 8.9)	3.9	(3.4 - 4.3) [c]	6.4	(5.7 - 7.2)	7.5×10^{-11}
DHBMA	345.3	(322.3 - 370)	411.5	(355.8 - 475.8) [c]	335.0	(300.5 - 373.4)	341.5	(306.7 - 380.3)	0.07
MHBMA / (MHBMA + DHBMA)	0.016	(0.015 - 0.018)	0.019	(0.016 - 0.023)	0.012	(0.011 - 0.014) [c]	0.020	(0.017 - 0.022)	2.2×10^{-6}
Females									
N	628		233		168		227		
MHBMA	4.6	(4.3 - 4.8)	5.6	(5.0 - 6.2)	2.9	(2.5 - 3.2) [c]	5.1	(4.6 - 5.7)	1.7×10^{-15}
DHBMA	287.2	(271.2 - 304.1)	322.6	(291 - 357) [c]	258.2	(299.1 - 291.1)	257.7	(233.7 - 284.1)	4.1×10^{-3}
MHBMA / (MHBMA + DHBMA)	0.017	(0.016 - 0.018)	0.018	(0.016 - 0.021)	0.012	(0.010 - 0.014) [c]	0.021	(0.019 - 0.023)	2.8×10^{-9}

[a] P-values and geometric least square means have been adjusted for BMI, age, batch, TNE (and sex where appropriate).
[b] P-values are comparing overall differences across ethnic groups
[c] P-values across ethnic groups (with Whites as the reference) were indicated where significant with $p < 0.05$

MHBMA and DHBMA concentrations across ethnic groups were stratified by *GSTT1* copy number as GST catalyzed glutathione conjugation is the major metabolic pathway leading to MHBMA and DHBMA (Figure 1.7, Table 2.3). Of the three ethnic groups, Japanese Americans had the highest frequency of the null genotype (48%), followed by African Americans (23%) and Whites (19%) (Table 2.3). For all ethnic groups, both MHBMA levels and metabolic ratios were strongly associated with *GSTT1* deletion copy number genotype ($p < 0.0001$). Individuals with the null deletion genotype (0/0) excreted the lowest amount of MHBMA, followed by those with one copy of *GSTT1* (1/0) and those with two copies of the gene (1/1; 3.1, 5.4, and 6.9 ng/mL urine, respectively) (Table 2.3). Among individuals null for *GSTT1*, Japanese American smokers had significantly lower MHBMA levels than Whites ($p < 0.05$). No significant association between *GSTT1* deletion and excretion of DHBMA was seen in any of the ethnic groups (Table 2.3).

To confirm that GSTT proteins can catalyze conjugation of EB glutathione, EB was incubated with glutathione in the presence of increasing amounts of recombinant human GSTT1 and GSTT2 enzymes, and the conjugation products were analyzed by HPLC-ESI-MS/MS (Figure 2.1). We found that both enzymes can catalyze this reaction, with GSTT1 exhibiting a faster rate, confirming the mechanistic involvement of these genes in MHBMA/DHBMA formation. In addition, MHBMA values were associated with *GSTM1* genotype, suggesting that another isoform of glutathione *S*-transferase may be involved in metabolism of butadiene (Table 2.3).

Table 2.3 Geometric means (95% CI) of BD metabolites stratified by GSTT1 and GSTM1 CNV and race/ethnicity

GSTT1* copy number genotype	**All**		**Whites**		**African Americans**		**Japanese Americans**		
	N	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**p-value**
MHBMA (ng/mL)									
1/1	232	6.9 (6.3 -7.6)	126	7.6 (6.8 - 8.6)	78	9.7 (8.4 - 11.2) [b]	28	4.1 (3.0 - 5.6) [b]	1.6×10^{-3}
1/0	482	5.4 (5.1 - 5.8)	187	5.7 (5.1 - 6.2)	155	8.2 (7.4 - 9.1) [b]	140	3.6 (3.1 - 4.1) [b]	3.2×10^{-6}
0/0	295	3.1 (2.9 - 3.4)	72	3.0 (2.6 - 3.5)	71	5.1 (4.4 - 5.9)	152	2.0 (1.8 - 2.3) [b]	2.8×10^{-6}
p-value	<.0001		<.0001		<.0001		<.0001		
DHBMA (ng/mL)									
1/1	232	293.9 (266.7 - 323.8)	126	284.8 (250.2 - 324.2)	78	406.2 (341.6 - 483.0)	28	222.3 (172.6 - 286.2)	0.28
1/0	482	322.4 (302.1 - 344.1)	187	302.3 (272.0 - 335.9)	155	455.1 (402.3 - 514.7) [b]	140	241.1 (215.3 - 270.0)	1.7×10^{-3}
0/0	295	325.5 (290.0 - 354.4)	72	337.7 (284.6 - 400.7)	71	425.4 (355.5 - 509.1)	152	244.6 (219.5 - 272.6)	0.19
p-value	0.226		0.293		0.536		0.790		
MHBMA / (MHBMA + DHBMA) ratio									
1/1	232	0.025 (0.022 - 0.027)	126	0.028 (0.024 - 0.032)	78	0.025 (0.021 - 0.030)	28	0.020 (0.014 - 0.027) [b]	9.0×10^{-3}
1/0	482	0.018 (0.017 - 0.019)	187	0.020 (0.018 - 0.022)	155	0.019 (0.017 - 0.022)	140	0.016 (0.014 - 0.018) [b]	0.04
0/0	295	0.010 (0.009 - 0.011)	72	0.010 (0.008 - 0.011)	71	0.013 (0.010 - 0.015)	152	0.009 (0.008 - 0.010)	4.7×10^{-5}
p-value	<.0001		<.0001		<.0001		<.0001		

***GSTM1* copy number genotype**	**All**		**Whites**		**African Americans**		**Japanese Americans**		
	N	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**N**	**Geometric means (95% CL)** [a]	**p-value**
MHBMA (ng/mL)									
1/1	137	5.5 (4.8 - 6.2)	207	7.4 (5.8 - 9.3)	75	7.7 (6.6 - 9.0)	27	3.0 (2.1 - 4.1) [b]	4.2×10^{-3}
1/0	452	5.3 (5.0 - 5.7)	153	6.4 (5.7 - 7.2)	157	7.6 (6.8 - 8.5)	142	3.2 (2.7 - 3.6) [b]	1.3×10^{-11}
0/0	479	4.4 (4.1 - 4.8)	35	4.9 (4.4 - 5.4)	94	7.6 (6.6 - 8.7) [b]	178	2.6 (2.3 - 3.0) [b]	1.6×10^{-12}
p-value	<.0001		<.0001		0.986		0.134		
DHBMA (ng/mL)									
1/1	137	310.7 (274.8 - 351.3)	207	294.0 (230.1 - 375.5)	75	412.5 (347.4 - 489.9)	27	245.9 (190.1 - 318.1)	0.39
1/0	452	318.3 (297.7 - 340.4)	153	302.1 (268.6 - 339.7)	157	434.1 (383.8 - 490.1) [b]	142	245.7 (219.7 - 274.7)	0.01
0/0	479	315.4 (295 - 337.3)	35	300.1 (271.2 - 332.1)	94	436.1 (370.9 - 512.6) [b]	178	244.5 (221.3 - 270.1)	0.05
p-value	0.94		0.098		0.868		0.997		
MHBMA / (MHBMA + DHBMA) ratio									
1/1	137	0.019 (0.014 - 0.016)	207	0.026 (0.020 - 0.035)	75	0.020 (0.016 - 0.024)	27	0.013 (0.009 - 0.018) [b]	8.8×10^{-4}
1/0	452	0.018 (0.016 - 0.019)	153	0.022 (0.019 - 0.025)	157	0.018 (0.016 - 0.021) [b]	142	0.014 (0.012 - 0.016) [b]	5.8×10^{-8}
0/0	479	0.015 (0.014 - 0.016)	35	0.017 (0.015 - 0.019)	94	0.018 (0.015 - 0.022)	178	0.011 (0.010 - 0.013) [b]	1.1×10^{-6}
p-value	<.0001		<.0001		0.808		0.157		

*(0/0) is equivalent to the gene deletion

[a] Adjusted for age, sex, BMI, batch and TNE

[b] P-value <0.05 when compared to Whites

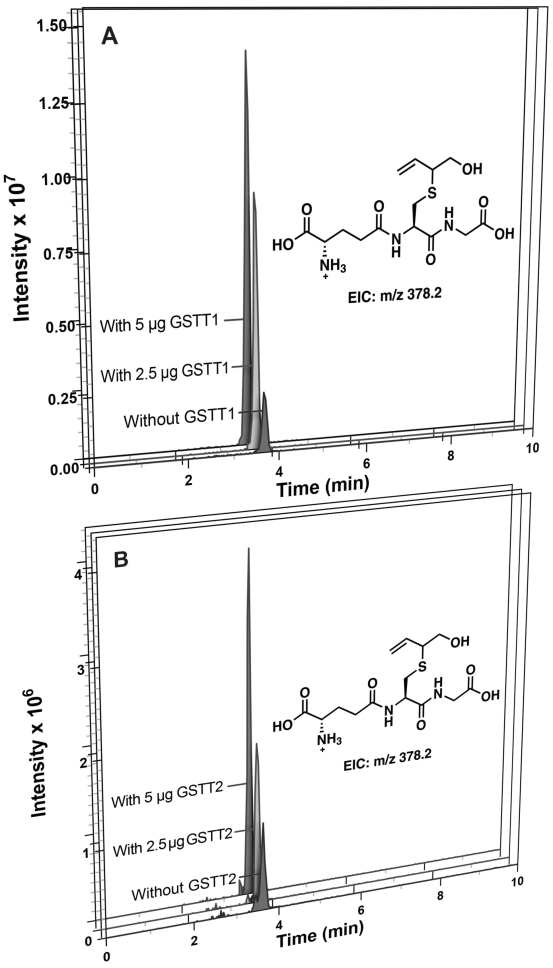


Figure 2.1 Extracted ion chromatograms showing formation of EB-GSH (m/z 378.2) in the presence of GSTT1 (A) and GSTT2 (B)

Additional analyses were conducted to identify factors responsible for the variability noted in BD metabolite excretion. We examined the associations of urinary MHBMA and DHBMA concentrations in relation to smokers' sex, age, BMI, batch, TNE and CPD (Table 2.4). We found that together, these factors explain 44.23% of the variability in MHBMA and 32.12% of the variability in DHBMA concentrations. For both MHMBM and DHBMA models, significant differences in adjusted means were observed for Japanese Americans ($p < 0.0001$) when compared to Whites. The same factors explained 11.05% of the variance in the metabolic ratio and were also significant for Japanese Americans ($p < 0.0001$), compared to the Whites.

Table 2.4. Percent Variation Explained

MHBMA (ng/mL) N=1009	Overall Percent Variation Explained	Whites		African Americans		Japanese Americans	
		n	Mean[a]	n	Mean[a]	n	Mean[a]
Sex + Age + BMI + Batch+ TNE+CPD	44.23%	385	5.73	304	6.15	320	3.29
P-values		-		0.27		<0.0001	
+ GSTT1_deletion	7.30%	385	5.30	304	5.95	320	3.76
P-values		-		0.05		<0.0001	
+ GSTM1_deletion	0.88%	385	5.56	304	5.92	320	3.91
P-values		-		0.29		<0.0001	
DHBMA (ng/mL) N=1009							
Sex + Age + BMI + Batch+ TNE+CPD	32.12%	385	306.1	304	352.2	320	294.7
P-values		-		0.02		0.506	
+ GSTT1_deletion	0.16%	385	306.7	304	350.3	320	288.3
P-values		-		0.03		0.31	
+ GSTM1_deletion	0.00%	385	305.3	304	349.2	320	286.8
P-values		-		0.03		0.29	
MHBMA / (MHBMA + DHBMA) N=1009							
Sex + Age + BMI + Batch+ TNE+CPD	11.05%	385	0.020	304	0.018	320	0.012
P-values		-		0.32		<0.0001	
+ GSTT1_deletion	11.40%	385	0.018	304	0.018	320	0.014
P-values		-		0.77		<0.0001	
+ GSTM1_deletion	1.12%	385	0.019	304	0.018	320	0.015
P-values		-		0.28		<0.0001	

[a] All Means are expressed as geometric least squares means adjusted for age, gender, BMI, batch, TNE (CPD, GSTT1, and GSTM1 where appropriate)

After adjusting for age, gender, batch, BMI, and race, MHBMA and DHBMA showed strong correlations with TNE (r = 0.55 and 0.47, respectively; Table 5), while the metabolic ratio showed a moderate association (r = 0.14, Table 2.5). Upon adjustment for the same variables and TNE, CPD accounted for only 0.48%, 1.27%, and 0.09% of the variability in MHBMA concentration, DHBMA concentration, and the metabolic ratio, respectively (Table 2.5), suggesting that measured TNE is a better predictor than self-reported CPD of the formation of these biomarkers. These correlations did not differ greatly when categorized by ethnic group or by *GSTT1* copy number (Table 2.5).

Table 2.5. Partial Correlations

	N	MHBMA & TNE[a]	N	DHBMA & TNE[a]	N	MHBMA / (MHBMA + DHBMA) & TNE[a]
Overall	1072	0.55	1072	0.47	1072	0.14
African Americans	327	0.60	327	0.54	327	0.03
Whites	396	0.59	396	0.41	396	0.23
Japanese Americans	349	0.48	349	0.46	349	0.12
GSTT1 = 1/1	232	0.70	232	0.51	232	0.25
GSTT1 = 1/0	482	0.56	482	0.44	482	0.15
GSTT1 = 0/0	295	0.54	295	0.54	295	0.07

[a] All partial correlations have been adjusted for age, gender, batch, BMI (and race where appropriate)

Aside from the *GSTT1* deletion (which explains 7.3% of the variability in MHBMA levels) we also investigated the association between the metabolites and the *GSTM1* deletion, which explains 0.88% of the variability in MHBMA and 1.1% of the variability in the metabolic ratio ($p < 0.0001$, Table 2.4). The *GSTT1* deletion explained 0.16% of variability in DHBMA and the *GSTM1* explained close to zero percent of the variability in DHBMA (0.01%). The *GSTT1* deletion explains 11.4% of the variability in metabolic ratio values, with an additional 1.12% of the variability explained by the *GSTM1* deletion.

Table 2.4 also provides tests for ethnic differences in metabolite levels before and after adjustment for the two deletion genotypes. For MHBMA, tests for heterogeneity give a t-statistic value of 1.15 for African Americans before adjustment for the two deletion genotypes, and 1.05 after adjustment, a 9% variability attributed to racial groups provided by the deletion genotypes. Importantly, strong differences of MHBMA levels between Japanese and White smokers remained even after adjustment for *GSTT1* deletion ($p < 0.0001$), suggesting that additional factors contribute to ethnic differences in BD metabolism.

Besides *GSTT1* and *GSTM1* deletions, GWAS analyses were conducted to identify any other genomic determinants of BD metabolism. For the GWAS of MHBMA, we detected associations at $p < 5 \times 10^{-8}$ with 136 variants. However, all of them were located between 24.2—24.4 Mb near the *GSTT1* gene on chromosome 22q11. There was one other rare association on 2p22.3 that was globally significant; but this was a potentially unreliable rare variant that was not further considered. The significant associations at 22q11 were explained by the *GSTT1* deletion, as no secondary signal was

detected after conditioning on the *GSTT1* deletion genotype. The deletion allele was significantly associated with lower MHBMA levels, found to be lowest among Japanese Americans (Table 3).

In ethnic-specific analyses, 108 globally significant associations were observed in Whites near *GSTT1*. As in the overall analyses, the significance of the SNP associations was greatly diminished when the analyses were conditioned on the *GSTT1* deletion genotype. The strongest remaining ethnic-specific association after conditioning on the *GSTT1* deletion was in Whites for rs62241865 ($p=6.6x10^{-5}$). The neighboring gene for this SNP is *SYN3*. The minor allele frequency of this SNP was 6 percent in Whites and 1 percent or less in the other ethnic groups. A single association in Japanese Americans in rs6004031 (our top-most significant SNP in overall analysis) near *GSTT1* was noted to be globally significant at $p = 5.13 \times 10^{-9}$, and four globally significant associations were observed for African Americans. None of these associations remained strongly significant after conditioning on *GSTT1*.

For DHBMA levels, no genome-wide significant associations were observed in either overall or ethnic specific analyses for any of the genotyped GWAS variants, or for SNPs and other variants that were imputed based on the GWAS.

For the metabolic ratio between MHBMA and DHBMA, there were 144 associations at $p < 5 \times 10^{-8}$ located between 24.2-24.4 Mb near *GSTT1* gene on chromosome 22q11. Like MHBMA, these associations were explained by the *GSTT1* deletion, and no secondary signal was noted after conditioning on the deletion in any ethnic group. In ethnic-specific analyses, 54 globally significant associations in Whites were observed near *GSTT1*. A single association in Japanese Americans in rs6004031

(our top-most significant SNP in overall analysis) near *GSTT1* was noted to be globally significant at $p = 1.37 \times 10^{-9}$. No globally significant associations were observed for African Americans. Again, the associations observed near *GSTT1* were explained by the *GSTT1* deletion genotype.

2.4 Discussion

Of over 60 known carcinogens present in tobacco smoke, 1,3-butadiene (BD) has the highest cancer risk index.[14, 69] BD inhalation shows potent carcinogenicity in laboratory mice and rats,[70-73] and there is a strong association between occupational exposure to BD and the development of lymphoma and leukemia in humans.[74-79] However, epidemiological studies have reported a weak association between lung cancer cases in women and occupational exposure to BD.[160, 161] Overall, the role of BD in smoking induced lung cancer has yet to be fully understood.

We quantified urinary BD-mercapturic acids MHBMA and DHBMA (Figure 1.7) as biomarkers of BD exposure and metabolic activation in African American, White, and Japanese American smokers.[103, 104] Our results show significant ethnic differences in the excretion of MHBMA, with African American smokers excreting the highest levels, followed by Whites and Japanese Americans (Table 2.2). These results correlate with the high lung cancer risk of African Americans and low lung cancer risk of Japanese Americans as compared to Whites,[63] suggesting that BD could play a role in the differences in lung cancer etiology seen between these groups. Interestingly, a similar trend was recently reported for mercapturic acids derived from acrolein, crotonaldehyde, and benzene.[154, 162] Furthermore, levels of the mercapturic acid formed from benzene are strongly influenced by *GSTT1* deletion, highlighting the important role of this GST gene

in the detoxification of structurally distinct carcinogens.[162]

In the present study, individuals with the *GSTT1* deletion excreted the lowest levels of MHBMA, followed by individuals with one copy of the gene and those with two copies (Table 2.3). This is consistent with our recent smaller study, which also reported the lowest MHBMA levels from individuals with the *GSTT1* deletion, followed by those with one and two copies of the gene.[105] Adjusting for *GSTT1* deletion explained the difference in urinary MHBMA between Japanese Americans and Whites; however, the difference between Whites and African Americans remained (Table 2.4).

The effect of *GSTM1* deletion on urinary MHBMA concentrations was also investigated, and among null individuals, the same trend was seen, with African Americans excreting the most MHBMA, followed by Whites and Japanese Americans (Table 2.3). Other glutathione *S*-transferases such as GSTT2 could contribute to MHBMA formation and account for the differences in MHBMA excretion between African Americans and Whites. However, we did not see evidence supporting a role of other GST genes in our GWAS, which found no additional signal after conditioning on *GSTT1* deletion genotype.

To the best of our knowledge, this study is the first to employ a GWAS to identify single nucleotide polymorphisms (SNPs) or other genetic variants associated with MHBMA and DHBMA excretion. With regard to MHBMA, the GWAS showed significant associations of 136 SNPs located near the *GSTT1* gene. However, when the GWAS was conditioned on *GSTT1* deletion, these SNPs were no longer detected in any ethnic group. Similar results were seen in the GWAS for the metabolic ratio, which was significantly associated with 144 SNPs near the *GSTT1* gene; these SNPs were also

explained by the *GSTT1* deletion. Experiments with recombinant GSTT1 and GSTT2 have confirmed the ability of these enzymes to catalyze glutathione conjugation with EB (Figure 2.1).

The strong relationship between the *GSTT1* deletion and MHBMA levels may potentially complicate the use of MHBMA as a biomarker of BD exposure since this protein is required for MHBMA formation. However, in our study, the ethnic differences in MHBMA excretion remained regardless of *GSTT1* genotype (Table 2.3). For studies where genotyping is not available, biomarkers directly reflecting BD damage, such as a BD-DNA adducts, might be a better choice to evaluate cancer risk specifically caused by BD.

Urinary concentrations of DHBMA also differed by ethnic group, with African Americans excreting the highest amounts, followed by Whites and Japanese Americans (Table 2.2). With respect to *GSTT1*, individuals with the deletion excreted the highest amounts of DHBMA, followed by individuals with one and two copies of *GSTT1* (Table 2.3), but these differences were not significant ($p = 0.226$). These findings are analogous to those reported by Fustinoni et al., who did not see a difference in urinary DHBMA levels between occupationally exposed workers with the *GSTT1* or *GSTM1* deletion genotype and workers containing one or more copies of either gene.[146]

Overall, this study is the first of its kind to use a GWAS to identify potential SNPs associated with urinary MHBMA and DHBMA levels, clearly showing an association between *GSTT1* genotype and MHBMA levels in smokers from three different ethnic groups. Furthermore, our results reveal that MHBMA levels, expressed in ng/mL urine, are highest in African Americans and lowest in Japanese Americans as

compared to Whites, which is consistent with their respective lung cancer risks.

III. HPLC-ESI$^-$-MS/MS ANALYSIS OF URINARY MHBMA AND DHBMA AND ITS CORRELATION TO URINARY N-7-(1-HYDROXY-3-BUTEN-2-YL) GUANINE IN TWO POPULATIONS OF SMOKERS

Reprinted (adapted) with permission from Sangaraju, D., Boldry, E. J., Patel, Y.M., Walker, V., Stepanov, I., Stram, D., Hatsukami, D., and Tretyakova, N.; Isotope Dilution nanoLC/ESI$^+$-HRMS^3 Quantitation of Urinary N7-(1-Hydroxy-3-buten-2-yl) Guanine Adducts in Humans and Their Use as Biomarkers of Exposure to 1,3-Butadiene. *Chem. Res. Toxicol.* **2017**, 20, 678-688.

This work was performed in collaboration with Dr. Dewakar Sangaraju, Yesha Patel, Dr. Vernon Walker, Dr. Irina Stepanov, Dr. Daniel Stram, and Dr. Dorothy Hatsukami, under the direction of Dr. Natalia Tretyakova. Urine samples were provided by Vernon Walker, Irina Stepanov, and Dorothy Hatsukami. nanoLC/ESI$^+$-HRMS^3 methodology was developed and used by Dewakar Sangaraju for analysis of urinary EB-Guanine II. Analysis of urinary MHBMA and DHBMA was performed by Emily Boldry. Statistical analysis was carried out by Yesha Patel under the supervision of Daniel Stram. Dewakar Sangaraju, Emily Boldry, Yesha Patel, and Natalia Tretyakova wrote the manuscript.

3.1 Introduction

1,3-Butadiene (BD) is a colorless gas classified as a known human carcinogen.[80, 81] It's use in the production of polymers and synthetic rubber represents a route of occupational exposure for individuals working in those industries;[163] its presence in automobile exhaust, urban air, smoke from wood fires, and cigarette smoke represent routes of environmental exposure for others.[80, 81] BD's carcinogenicity is a direct result of its cytochrome P450 2E1 and 2A6 monoxygenase (CYP450)-mediated metabolism to reactive epoxide species including 1,2-epoxy-3-butene (EB) (EB), 1,2,3,4-diepoxybutane (DEB), and 3,4-epoxy-1,2-butanediol (EBD).[82-84, 86, 87] These electrophilic epoxides can be detoxified via epoxide hydrolase (EH)-mediated hydrolysis or conjugation with glutathione (GSH), with the latter reaction catalyzed by glutathione S transferases (GST). The initially formed glutathione conjugates are further enzymatically processed and eventually excreted in urine as mercapturic acids (Figure 3.1).[48, 97, 100, 101] MHBMA is formed from the conjugation of GSH to EB, whereas DHBMA is formed from the conjugation of GSH to hydroxymethyl vinyl ketone (HMVK), another BD metabolite that is formed from EB.[48, 97, 100, 101] If not detoxified via hydrolysis or conjugation with glutathione, BD derived epoxides have the ability to react with DNA and form nucleobase adducts, leading to the mutagenicity and toxicity seen in BD treated cells[85] and in BD exposed laboratory animals.[70-73] EB forms regioisomeric N7-(2-hydroxy-3-buten-1-yl)guanine (EBGI) and N7-(1-hydroxy-3-buten-2-yl)guanine (EBGII) adducts through reaction at the N7 position of G (Figure 3.2). [112, 164, 165]

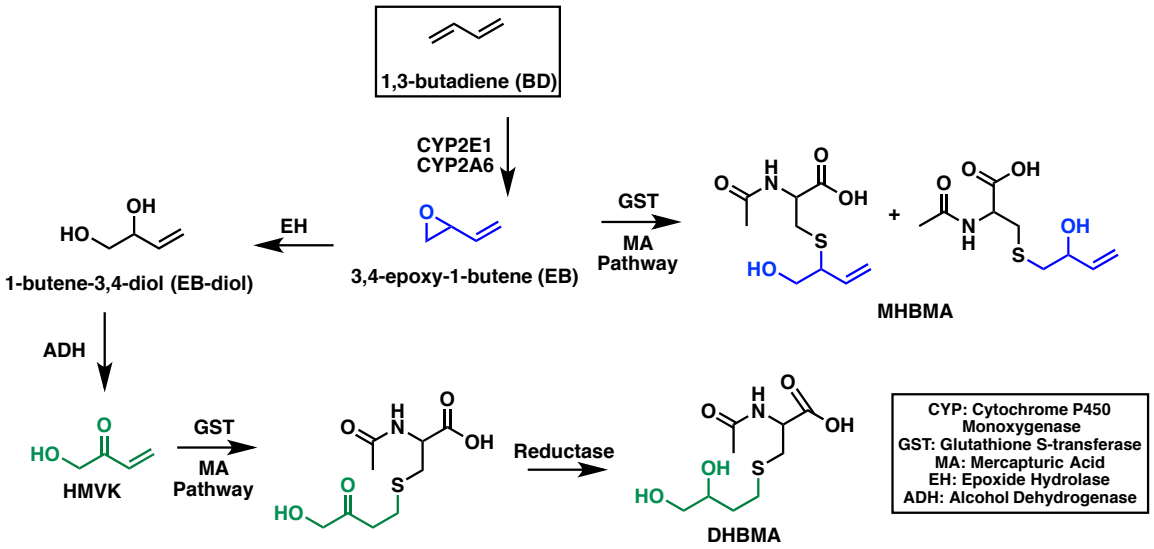


Figure 3.1 Formation of mercapturic acids MHBMA and DHBMA from respective BD metabolites EB and HMVK

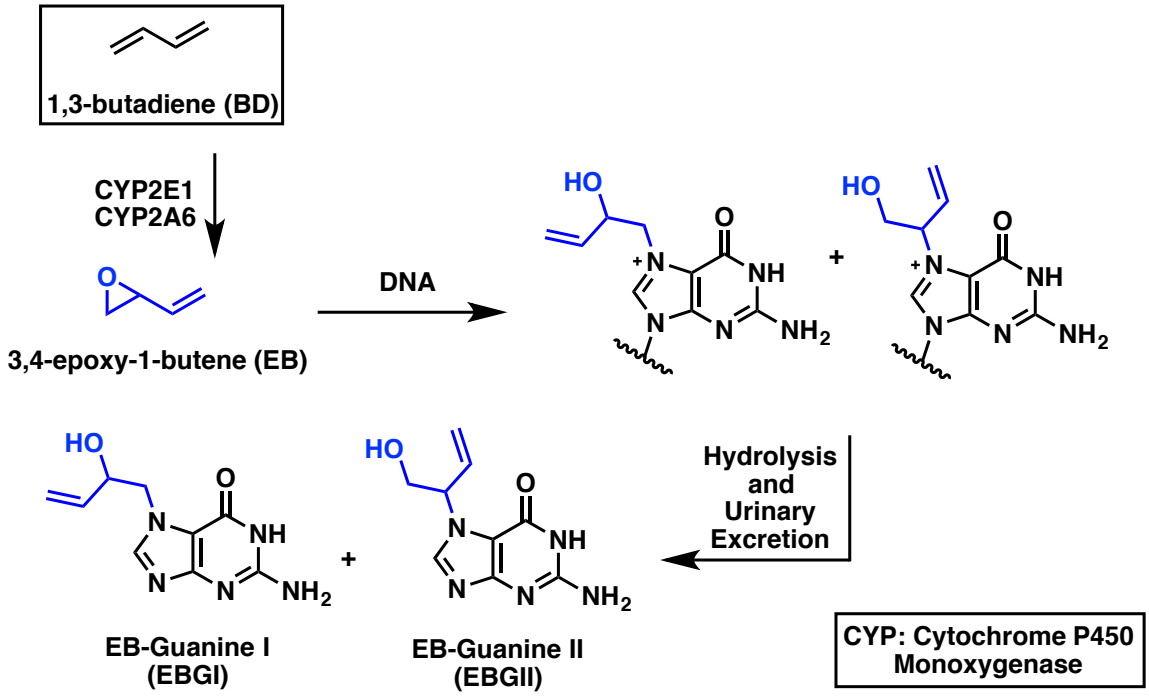


Figure 3.2 Formation of EB-Guanine (EBG) adducts from BD metabolite EB

Citti *et. al.* were the first to identify EBGI and EBGII through reaction of EB with dG in glacial acetic acid;[166] since then, multiple studies have reported their formation in BD-exposed animals and humans. EBGI and EBGII have been detected in EB-treated calf thymus DNA,[109] EB-treated human TK6 cells,[120] and in tissues of laboratory rodents exposed to BD via inhalation.[112, 167] In 1999, Koc *et. al.* quantified EBGI and EBGII in tissues of female F344 rats and B6C3F1 mice exposed to 0-625 ppm BD for six hours a day, five days a week for four weeks.[112] EBGI and EBGII were detected in DNA isolated from liver, lung, and kidney tissues of both species, with mice containing higher amounts of both adducts as compared to rats (EBGI: 0.2-3.0 and 0.1-1.8 adducts/10^6 guanine bases in mice and rats, respectively; EBGII: 0.1-2.4 and 0.1-1.2 adducts/10^6 guanine bases in mice and rats, respectively).[112] These differences in adduct formation were attributed to more efficient detoxification of butadiene-derived epoxides in rats[112] and correlated with interspecies differences in sensitivity to BD.[71, 73]

Our laboratory has developed an ultrasensitive nanoLC –high resolution ESI^+-MS^3 method for accurate detection of EBG II adducts *in vivo*.[167] This methodology was applied to quantify EBG II in DNA extracted from the liver tissue of male and female F344 rats exposed to low levels of BD by inhalation (0, 0.5, 1.0 or 1.5 ppm BD for six hours a day, five days a week for two weeks), as well as in blood leukocyte DNA of occupationally exposed workers and current smokers.[167] In animal exposure studies, a linear relationship was observed between BD exposure and EBGII adducts formed.[167] However, the concentrations of EBGII in leukocyte DNA isolated from current smokers were below the method's limit of quantitation (0.2 fmol in 150 μg of DNA).[167] This can be potentially explained by limited hydrolytic stability of EBGII adducts, which undergo

spontaneous depurination ($t_{1/2}$: 2.20 ± 0.12 days), followed by excretion in urine.[168, 169] Because of this, EBGII was further explored as a potential urinary biomarker of BD exposure.

Using new nanoLC-ESI^+-MS^3 methodology, EBGII was quantified in urine from BD exposed F344 rats, occupationally exposed workers, and White and African American smokers.[170] Urinary EBGII amounts increased in a dose-dependent manner in rats exposed to BD via inhalation.[170] Furthermore, urinary EBGII levels were significantly increased in workers occupationally exposed to 0.1–2.2 ppm BD (1.08 ± 3.54 pg/mg creatinine (Cr)) as compared to administrative controls exposed to < 0.01 ppm BD (0.24 ± 1.85 pg/mg Cr; p = 0.0051), supporting its use as a biomarker of exposure to BD.[170] EBGII was also detected in smokers' urine, with White smokers excreting significantly higher amounts of EBGII than African American smokers (0.48 ± 0.09 vs. 0.12 ± 0.02 pg/mg Cr, p = 3.1 x 10^{-7}) (Table 3.1).[170]

Table 3.1 The geometric means for urinary EBGII levels (pg/ mg creatinine) in White and African American smokers

EBGII (pg/mg creatinine)			
	N	**Geometric Mean ± SD***	**Adjusted p-value***
African Americans	74	0.12 ± 0.02	3.1×10^{-7}
White	75	0.48 ± 0.09	

*The geometric means on the log transformed variables and the p-values are adjusted for age, sex and total nicotine equivalents.

One possible explanation for the observed ethnic differences in urinary EBGII excretion (Table 3.1) is that White and African American smokers metabolize BD differently due to genetic polymorphisms in xenobiotic metabolism genes such as *CYP2E1*, *EPHX1*, and *GSTT1*, which would lead to different amounts of EB available for binding to DNA. Examination of a variety of SNPs on BD metabolizing genes revealed several variants whose frequencies differed considerably between the two ethnic groups (summarized in Table 3.5),[171-173] offering support for this hypothesis.

In order to test this hypothesis, the same urine samples from White and African American smokers (N = 151) were analyzed for 2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene) (together referred to as MHBMA) and *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine (DHBMA).[48, 97, 100, 101] These same individuals were genotyped for single nucleotide polymorphisms (SNPs) in BD metabolizing genes (*CYP2E1*, *EPHX1*, *GSTT1*) and as well as a gene potentially involved in repair of BD-DNA adducts (*FANCE*). By identifying correlations between butadiene metabolism, DNA adduct levels, and specific SNPs, we can begin to build a more complete picture of the contribution of ethnic differences in BD metabolism to lung cancer risk in smokers.

3.2 Experimental

3.2.1 Materials

LC-MS grade water, methanol, and acetonitrile were acquired from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO). 2-(*N*-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(*N*-acetyl-L-cystein-S-yl)-2-hydroxy-but-3-ene (MHBMA), DHBMA, $^{2}H_{6}$-MHBMA, and

$^{2}H_{7}$-DHBMA were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis HLB 96 well plates were procured from Waters Corp. (Milford, MA). Solutions for DNA extraction from blood leukocytes were purchased from Qiagen (Germantown, MD).

3.2.2 Human Study Subjects

Urine samples were collected from male (N = 76) and female (N = 73) smokers of African American (N = 74) and European American (N = 75) ancestry. Male (age: 42 ± 11) and female (age: 44 ± 11) subjects who smoked an average of 17 cigarettes per day for at least one year. Overall, mean CO levels in male and female smokers were 16 ± 7 and 20 ± 10 ppm, respectively. Subjects were asked to collect a 24-hour urine sample and bring it to a research clinic at the University of Minnesota. Urine samples were stored at -20 °C prior to analysis. This study (ID #: 1007M85757) was approved by the University of Minnesota Institutional Review Board.

3.2.3 HPLC-ESI^{-}-MS/MS Analysis of Urinary MHBMA and DHBMA

Urinary concentrations of MHBMA and DHBMA were determined using previously published methods.[105, 156, 174] Briefly, human urine (200 μL) was mixed with 200 μL of water, 20 μL of 1 N HCl, and 60 ng each of $^{2}H_{6}$-MHBMA and $^{2}H_{7}$-DHBMA (internal standards for mass spectrometry). Samples were subjected to solid phase extraction on a Waters Oasis HLB 96 well plate (1 mL/30 mg, Waters, Corp. Milford, MA). The cartridges were conditioned with 1 mL of methanol, followed by 1 mL of water. Following sample loading, the cartridges were washed with 1 mL of 5% methanol. MHBMA, DHBMA, and their respective internal standards were eluted with 1.2 mL of 75% methanol, dried under vacuum, and reconstituted in 30 µL of 0.1% formic acid.

HPLC-ESI$^-$-MS/MS analyses of MHBMA and DHBMA were performed on an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a 96 well plate autosampler interfaced to a Thermo TSQ Vantage mass spectrometer (Thermo Scientific Corp., Waltham, MA). Samples were loaded onto an Agilent Pursuit 3 Diphenyl column (3 μm, 2.0 x 150 mm, Agilent Technologies, Santa Clara, CA) fitted with an Agilent Metaguard Pursuit 3 DP guard column (Agilent Technologies, Santa Clara, CA). The column was maintained at 5 °C and eluted with a gradient of 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 150 μL/min. The HPLC gradient started at 3% B, was linearly increased to 9% B in 12 minutes, and further increased to 50% B in 2 min. The percentage of solvent B was maintained at 50% B for 2 min, increased to 80% B in 2 min, and held at 80% for another 2 min. Finally, the solvent composition was returned to 3% B in 2 min and equilibrated for 12 min. The following MS/MS transitions were used for selected reaction monitoring: MHBMA, *m/z* 232.11 → 103.11, $^2H_6$-MHBMA, *m/z* 238.15 → 109.15, DHBMA, *m/z* 250.10 → 75.13, and $^2H_7$-DHBMA, *m/z* 257.16 →78.13. Additional details regarding mass spectrometer settings can be found in a prior publication.[105, 156] MHBMA and DHBMA amounts were determined by comparing analyte peak areas with those of their respective internal standards using standard curves.

3.2.4 DNA Isolation

DNA was isolated from human blood leukocytes using commercially available solutions from Qiagen (Germantown, MD) as described below. Samples from each individual contained blood leukocytes, also known as the buffy coat, which were previously extracted from whole blood samples. To 1 mL of buffy coat was added 3 mL

of Red Blood Cell Lysis Solution. The sample was inverted and centrifuged at 2000 x g at 4 °C for 10 min, after which the supernatant was removed. The remaining pellet was then treated with 1.5 mL of Cell Lysis Solution to lyse the remaining white blood cells, and the sample was incubated at room temperature for 30 min. Proteins were digested through the addition of 10 μL of Puregene Proteinase K solution (20 mg/mL), followed by incubation overnight at room temperature. The following day, RNA was digested through the addition of 10 μL of RNase A (10 mg/mL); incubation continued at room temperature for an additional 30 min. Proteins were precipitated using Protein Precipitation Solution (700 μL). Samples were vigorously vortexed prior to centrifugation for 15 min at 2000 x g at 4 °C. At this point, the pellet in the sample was completely dissolved, rendering the sample transparent but reddish in color. Lastly, the supernatant was poured into a clean polypropylene tube to which 3 mL of ice cold isopropyl alcohol (IPA) was added. The sample was gently swirled to precipitate DNA, which was removed and washed several times with 1 mL aliquots of ice cold ethanol. After washing, DNA was left for approximately 1 h to dry and subsequently redissolved in 200 µL of Milli Q Millipore water. DNA concentrations were determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA).

3.2.5 Genotyping

The genetic variants in BD metabolizing genes were identified at the University of Hawaii Cancer Center Genomics Shared Resource. *CYP2E1* SNPs rs2070676 and rs6413432 were genotyped with a predesigned and custom TaqMan assays, respectively. Both of the *EPHX1* SNPs, rs1051740 and rs2234922, were genotyped with predesigned

TaqMan assays, as was the rs9462088 (*FANCE*). The *GSTT1* copy number assay was run using TaqMan copy number assay Hs00010004_cn.

All assays were run on the 7900HT FAST Real-Time System (Life Technologies, Carlsbad, CA). SNPs were called using the TaqMan Genotyper software, and copy number calls were determined using the CopyCaller v2.0 software (Life Technologies, Carlsbad, CA). Approximately 5% blind duplicate samples were included for quality control.

3.2.6 Statistical Analyses

Least-square means (or geometric means) of EB-G II, MHBMA and DHBMA were calculated for each ethnic group. Several candidate SNPs in *CYP2E1, EPHX1* and *FANCE* genes, along with a GSTT1 copy number genotype were also analyzed and correlated with geometric mean levels of urinary EB-GII, MHBMA and DHBMA. All associations were evaluated using linear regression models, with adjustment for age, sex, total nicotine equivalents (and race), with a significance threshold at $p < 0.05$. To examine correlations between urinary EB-GII levels and concentrations of BD-mercapturic acids, Pearson's partial correlations (r) were reported and adjusted for age, sex, total nicotine equivalents, and race.

3.3 Results

In order to evaluate ethnic differences in metabolism of BD by African American and White smokers, BD mercapturic acids were quantified in urine of subjects belonging to the two ethnic groups (N = 151) using isotope dilution HPLC-ESI-MS/MS method previously developed in our laboratory (Table 3.2).

Table 3.2 The geometric means for urinary MHBMA and DHBMA levels (ng/ mg creatinine) in White and African American smokers.

MHBMA (ng/mg creatinine)			
	N	**Geometric Mean ± SD***	**Adjusted p-value***
African Americans	75	7.39 ± 0.82	3.6×10^{-6}
White	76	15.70 ± 1.71	
DHBMA (ng/mg creatinine)			
	N	**Geometric Mean ± SD***	**Adjusted p-value***
African Americans	74	236.98 ± 15.30	0.051
White	75	283.71 ± 18.07	

*The geometric means on the log transformed variables and the p-values are adjusted for age, sex and total nicotine equivalents.

With regard to MHBMA, White smokers were found to have statistically significantly higher urinary levels as compared to African Americans (15.70 ± 1.71 vs. 7.39 ± 0.82 pg/mg Cr, $p = 3.6 \times 10^{-6}$; Table 3.2). White smokers also excreted slightly higher levels of DHBMA as compared to African Americans, but the difference was not statistically significant (283.71 ± 18.07 vs. 236.98 ± 15.30 pg/mg Cr, p = 0.051; Table 3.2). Additional statistical analyses have revealed only a weak association between urinary EBGII adduct levels and the concentrations of BD-mercapturic acids (EB-GII with MHBMA, r = 0.035; EB-GII with DHBMA, r = 0.028; Table 3.3). The lack of correlation between MHBMA and EBGII could be due to the fact that both compounds are formed from EB and therefore might be equally likely to form and/or get excreted.

Table 3.3 Partial correlation between EBGII, MHBMA, and DHBMA for all smokers (N = 151)	
	r Value
EBGII with MHBMA	0.035
EBGII with DHBMA	0.028

To determine whether the inter-individual differences in urinary EBGII concentrations were caused by genetic polymorphisms, the same smokers were genotyped for GSTT1 copy number and common SNPs in *CYP2E1* (rs2070676, rs6413432), microsomal epoxide hydrolase, *EPHX1* (rs2234922, rs1051740), and the DNA repair gene *FANCE* (rs9462088). After adjusting for age, gender, race, and total nicotine equivalents (TNE), we identified a significant association between urinary MHBMA levels and GSTT1 copy number ($p = 6.97 \times 10^{-10}$, Table 3.4). No other significant correlations were found between urinary excretion of EB-GII, MHBMA, or DHBMA and polymorphisms in *CYP2E1, EPHX1,* or *FANCE* genes.

Table 3.4 Geometric mean values of urinary EBGII, MHBMA, and DHBMA stratified by GSTT1 copy number genotype and candidate SNPs in *CYP2E1, EPHX1,* and *FANCE*.

N = 151	MHBMA (ng/mg creatinine)			DHBMA (ng/mg creatinine)			EB-Guanine II (pg/mg creatinine)		
	N	Geometric Mean [Std Err]*	p value*	N	Geometric Mean [Std Err]*	p value*	N	Geometric Mean [Std Err]*	p value *
rs1051740									
0	99	11.09 [1.06]	0.564	99	248.86 [14.03]	0.496	97	0.23 [0.04]	0.613
1	41	11.41 [1.72]		41	278.52 [24.69]		41	0.29 [0.07]	
2	8	7.52 [2.74]		8	287.63 [61.70]		8	0.16 [0.10]	
rs2234922									
0	84	11.72 [1.24]	0.631	84	263.45 [16.39]	0.543	83	0.22 [0.04]	0.188
1	55	9.99 [1.30]		55	247.88 [18.90]		54	0.23 [0.05]	
2	11	10.15 [2.95]		11	301.23 [51.54]		11	0.56 [0.26]	
rs2070676									
0	62	9.45 [1.41]	0.443	62	252.82 [22.81]	0.378	61	0.37 [0.09]	0.085
1	50	11.10 [1.49]		50	284.11 [23.11]		50	0.17 [0.04]	
2	37	13.37 [2.44]		37	242.72 [26.78]		36	0.19 [0.06]	
rs9462088									
0	103	11.14 [1.07]		103	272.82 [15.46]		10	0.27 [0.04]	
1	37	10.90 [1.76]	0.379	37	242.56 [23.24]	0.274	2	0.17 [0.04]	0.277
2	10	7.06 [2.16]		10	206.07 [37.36]		37 9	0.31 [0.16]	
rs6413432									
0	128	10.58 [0.90]		128	258.00 [12.81]		12	0.27 [0.04]	
1	19	11.73 [2.58]	0.663	19	285.76 [36.67]	0.477	6	0.20 [0.07]	0.412
2	-	-		-	-		19 -	-	
GSTT1_CN									
0	36	4.95 [0.68]	**6.97 x 10^{-10}**	36	232.44 [22.12]		35	0.29 [0.07]	
1	54	9.98 [1.12]		54	270.58 [20.94]	0.571	53	0.22 [0.05]	0.552
2	51	18.55 [2.14]		51	270.82 [21.62]		51	0.25 [0.05]	
3	5	15.46 [5.71]		5	284.15 [72.53]		5	0.57 [0.39]	

*The geometric means and p-values are adjusted for age, gender, race, and total nicotine equivalents

3.4 Discussion

If not repaired, DNA adducts can cause heritable mutations, potentially leading to the development of cancer.[175-178] Therefore, DNA adducts represent biologically relevant biomarkers of carcinogen exposure and potential indicators of cancer risk.[20] Identifying and measuring such adducts in smokers can provide useful information regarding the metabolic formation of electrophilic species from specific tobacco carcinogens, as well as assessing the role these metabolites play in cancer risk.[20, 179, 180] Smokers' exposure to BD is significant due to its high concentrations in tobacco smoke, therefore the role of BD metabolizing genes in lung cancer risk in humans must be explored.[80, 81]

Initial research by our laboratory focused on DNA adducts formed from the most abundant BD metabolite *in vivo*, 3,4-epoxy-1,2-butandiol (EBD).[165] EBD alkylates the N7 position of G to form the corresponding DNA adduct, N7-(2,3,4-trihydroxybut-1-yl)-guanine (N7-THBG).[112, 181] Though this adduct was successfully detected in leukocyte DNA of occupationally exposed workers smokers, and nonsmokers, it was also present in measureable amounts in the DNA of control individuals with no known exposure to BD.[181] We concluded that N7-THBG is formed endogenously and thus cannot be used as a biomarker of BD exposure in humans.

The second most abundant BD metabolite *in vivo*, EB,[165] similarly reacts with the N7 position of G of DNA to form regioisomeric guanine adducts, EBGI and EBGII (Figure 3.1).[112, 164] We have developed a sensitive nano HPLC -ESI-MS-MS methodology for EBGII employing an Orbitrap mass spectrometer.[167] EBGII was detected in leukocyte DNA from occupationally exposed workers, smokers, and nonsmokers but its amounts were below the method's limit of quantitation (LOQ: 0.2

fmol in 150 μg of DNA).[167] Because alkylation at the N7 position of guanine renders this position positively charged, adducts like EBGI and EBGII are hydrolytically unstable.[168, 169] It was hypothesized that the low EBGII levels in blood DNA could be due to its spontaneous release from the DNA backbone and subsequent urinary excretion. Therefore, we evaluated the possibility of detecting EBGII excreted in urine.[170] When exposed to increasing amounts of BD via inhalation, urinary levels of EBGII in F344 rats were found to increase in a dose dependent manner, and workers occupationally exposed to BD were found to have statistically significantly increased urinary EBGII concentrations as compared to their administrative (control) counterparts (1.08 ± 3.54 vs. 0.24 ± 1.85 pg/mg Cr; $p = 0.0051$).[170] Smokers were also found to have increased urinary EBGII concentrations as compared to nonsmokers (0.71 ± 2.81 vs. 0.32 ± 2.31 pg/mg Cr; $p = 0.08$).[170]

With EBGII readily detectable in urine, attention turned towards comparison of urinary EBGII in smokers from different ethnic groups. Epidemiological studies have shown that smokers belonging to different ethnic groups have different risks of developing lung cancer.[63] Native Hawaiian and African American smokers have a higher risk as compared to White smokers, whereas Latino and Japanese American smokers have a lower risk as compared to White smokers.[63] It is thought that ethnic differences in metabolism of the carcinogens present in cigarette smoke could play a role in this phenomenon.[154, 162, 182] Therefore, we analyzed urine belonging to current smokers of two ethnic groups with differing lung cancer risks: African Americans (high risk) and Whites (intermediate risk). Urinary EBGII concentrations in White smokers were found to be

approximately four times as high as urinary EBGII concentrations in African American smokers ($p = 3.1 \times 10^{-7}$; Table 3.1).[170]

The same urine samples were also analyzed for additional biomarkers of BD exposure, MHBMA and DHBMA. MHBMA and DHBMA represent detoxification products formed from the conjugation of GSH to BD metabolites EB and HMVK, respectively (Figure 3.2).[48, 97, 100, 101] White smokers were found to have statistically significantly higher urinary MHBMA levels and increased urinary DHBMA levels as compared to African Americans (MHBMA: $p = 3.6 \times 10^{-6}$, Table 3.1B; DHBMA: $p = 0.051$, Table 3.2). These results are similar to previous data for BD metabolites: White smokers were found to have significantly higher urinary concentrations of MHBMA as compared to Native Hawaiian and Japanese American smokers, whereas urinary DHBMA concentrations did not significantly differ between these groups.[105]

Although the same trend was observed for BD metabolites and urinary DNA adducts among White and African American smokers, only a weak association between urinary EBGII and MHBMA or DHBMA levels was found (Table 3.3). We hypothesize that the increased urinary EBGII excretion by White smokers represents a more efficient removal of EBGII adducts by White smokers as compared to African Americans.

Table 3.5 Frequencies of chosen SNPs in White (CEU) and African American (YRI) populations. For each SNP, the ancestral allele is listed first, with the mutated allele listed underneath it.

SNP	Gene	Frequency	
		CEU	YRI
rs9462088	*FANCE*	G: 0.955 C: 0.045	G: 0.542 C: 0.458
rs2234922	*EPHX1*	A: 0.792 G: 0.208	A: 0.599 G: 0.401
rs1051740	*EPHX1*	T: 0.668 C: 0.332	T: 0.874 C: 0.126
rs6413432	*CYP2E1*	T: 0.858 A: 0.142	T: 0.915 A: 0.085
rs2070676	*CYP2E1*	G: 0.128 C: 0.872	G: 0.680 C: 0.320
Null	*GSTT1*	0.147	0.218

Study subjects were also genotyped to determine whether ethnic differences in EBGII, MHBMA, and DHBMA excretion were caused by SNPs present in BD metabolizing genes (*CYP2E1* and *EPHX1*), DNA repair genes (*FANCE*), or GSTT1 copy number. Specific SNPs were chosen based on frequency within White and African American populations as well as whether or not there were distinct differences in these frequencies between the two populations (Table 3.5).[171-173] Genotyping revealed a strong association between urinary MHBMA and GSTT1 copy number ($p = 6.97 \times 10^{-10}$; Table 3.4).

GSTT1 is a major enzyme in BD metabolism and is responsible for detoxification of BD epoxides (Figures 1.6, 3.1).[145, 146] The strong relationship between urinary concentrations of MHBMA and *GSTT1* copy number has been demonstrated previously, including in the work presented in Chapter II.[145, 146, 174, 183] However, there were no associations found between urinary EBGII, MHBMA, or DHBMA levels and any of the SNPs on the *CYP2E1*, *EPHX1*, and *FANCE* genes. Interestingly, there was no association between urinary EBGII and *GSTT1* copy number. Though GSTT1 is not directly involved in the formation and/or removal of EBGII, it is possible that *GSTT1* copy number could influence EBGII levels by changing the amount of free EB available to form adducts. These observations are likely due to the small size of our study population (N = 151) and the limited number of variants considered in the analysis.

In summary, methodologies previously developed in our laboratory[105, 156, 170] were used to analyze EBGII, MHBMA, and DHBMA in urine from smokers belonging to two different ethnic groups: White and African American. Our results indicate large ethnic differences in urinary concentrations of EBGII and MHBMA, which can be explained

GSTT1 copy number. Further studies including additional ethnic groups, larger sample sizes, and a larger SNP pool should be conducted to test for potential ethnic differences in the repair of EBGII.

IV. EFFECTS OF 2-PHENETHYL ISOTHIOCYANATE ON THE METABOLISM OF 1,3-BUTADIENE IN HUMANS

Adapted from:

Boldry, E. J., Tessier, K., Yuan, J-M., Carmella, S. G., Wang, R., Hatsukami, D., Hecht, S. S., and Tretyakova, N.; Effects of 2-Phenethyl Isothiocyanate on the Metabolism of 1,3-Butadiene in Humans. *Manuscript in publication*.

This work was performed in collaboration with Katelyn Tessier, Dr. Jian-Min Yuan, Steven Carmella, Dr. Renwei Wang, Dr. Dorothy Hatsukami, and Dr. Stephen Hecht under the direction of Dr. Natalia Tretyakova. Urine samples analyzed for this work were part of a study that was originally conceived and carried out by Jian-Min Yuan, Steven Carmella, Renwei Wang, Dorothy Hatsukami, and Stephen Hecht. Analysis for urinary MHBMA and DHBMA was performed by Emily Boldry. Statistical analyses were performed by Katelyn Tessier. Emily Boldry, Katelyn Tessier, and Natalia Tretyakova wrote the manuscript.

4.1 Introduction

Isothiocyanates are a class of naturally occurring compounds found in fruits and vegetables that have been shown to have chemopreventative effects through multiple mechanisms of action both *in vitro* and *in vivo*.[184, 185] One such compound, 2-phenethyl isothiocyanate (PEITC), found in watercress, garden cress, radishes, and turnips,[186] modulates multiple cancer-associated pathways, such as cell cycle arrest,[187-191] NFκB,[192, 193] and apoptosis.[191, 194] PEITC can inhibit cytochrome P450 monoxygenases (CYP450s) involved in carcinogenic activation[195] as well as induce detoxifying enzymes such as glutathione-*S*-transferases (GSTs) involved in protection against carcinogenesis.[196] More specifically, treatment with PEITC inhibited lung carcinogenesis in both mice and rats exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK), a potent tobacco-specific carcinogen.[197-199] PEITC ingestion by current smokers has also been shown to decrease the metabolic activation of NNK.[200]

Though the link between smoking and lung cancer has long been established, smoking remains the most preventable cause of death in the world.[54] Worldwide, there are approximately 1.1 billion smokers,[201] with cigarettes attributing to 80% and 50% of lung cancer deaths in men and women, respectively.[202] Among the 40 million smokers in the United States, cigarette smoking causes 83% of total lung cancer deaths in men and 50% of all lung cancer deaths in women.[54] While smoking cessation is the best method of reducing smoking related lung cancer deaths, inhibition of the carcinogenic and genotoxic effects of cigarette smoke remains an important alternative route in the fight against smoking related deaths. The use of chemopreventative compounds naturally occurring in various food sources could provide a widely available and relatively

inexpensive method of cancer prevention.

Recently, the effects of PEITC on the metabolic processing of cigarette smoke carcinogens benzene, acrolein, and crotonaldehyde were investigated by measuring their respective mercapturic acids, detoxification products formed through glutathione (GSH) conjugation via GSTs.[203] A statistically significant increase in urinary concentrations of mercapturic acids formed from benzene and acrolein was observed when smokers were given PEITC, while no such increase was seen in urinary concentrations of the respective mercapturic acid formed from crotonaldehyde.[203] The effects of PEITC were more substantial in individuals null for GSTs mu 1 and theta 1 (*GSTM1* and *GSTT1*); for these subjects, PEITC treatment significantly increased urinary concentrations of the mercapturic acids of benzene, acrolein, and crotonaldehyde, indicating that use of isothiocyanates could provide enhanced protection against lung carcinogenesis in individuals lacking both genes.[203]

In the present study, we investigated the effects of PEITC treatment on metabolism 1,3-butadiene (BD) in smokers. BD is among one of the most abundant carcinogens present in cigarette smoke, with concentrations of 20-75 μg and 205-360 μg per cigarette in mainstream and side stream smoke, respectively.[14, 68] It is classified as a known human carcinogen by the National Toxicology Program (NTP) and a Group 1 agent by the International Agency for the Research on Cancer (IARC),[80, 81] and occupational exposure to BD is associated with the development of leukemia, lymphoma, and, more weakly, with lung cancer in women.[74-79, 160, 161] GSTs play an important role in the detoxification of BD epoxides.[105, 145, 146, 174] Quantitative HPLC-ESI$^-$-MS/MS methods previously developed in our laboratory were used to measure urinary concentrations of

BD-mercapturic acids 1- and 2-(*N*-acetyl-L-cysteine-S-yl)-1-hydroxybut-3-ene (MHBMA) and *N*-acetyl-*S*-(3,4-dihydroxybutyl)-L-cysteine (DHBMA).[100] PEITC induced statistically significant increase in urinary MHBMA concentrations in smokers lacking *GSTT1* gene or both *GSTT1* and *GSTM1*, suggesting that it facilitates BD detoxification and potentially decreases it carcinogenicity.

4.2 Experimental

4.2.1 Materials

LC-MS grade water, methanol, and acetonitrile were acquired from Fisher Scientific (Pittsburgh, PA). All other chemicals and solvents were obtained from Sigma-Aldrich (St. Louis, MO). MHBMA, DHBMA, $^{2}H_{6}$-MHBMA, and $^{2}H_{7}$-DHBMA were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis HLB 96 well plates were procured from Waters Corp. (Milford, MA).

4.2.2 Study Design

The study was a randomized, placebo-controlled, double-blind, phase II clinical trial with a crossover study design. Details of the study design can be found in prior publications.[200, 203] Over a duration of five weeks, qualified participants were asked to smoke cigarettes containing [pyridine-D_4]NNK (to allow for measurement of NNK metabolism). After an adjustment period of two weeks, individuals were placed into one of two treatment groups: those receiving PEITC before receiving a placebo or those receiving a placebo before receiving PEITC. PEITC (10 mg in 1 mL olive oil) was administered 4 times per day every 4 hours for 5 days during weeks three or five; the placebo (1 mL olive oil) was administered on the same schedule, also during weeks three or five. Week four consisted of a washout period, where participants did not receive

PEITC or the placebo. This study was approved by the Institutional Review Boards of the University of Minnesota (0712M22651) and the University of Pittsburgh (PRO11110669).

Twenty-four hour urine samples were collected at the end of weeks two and four and on days three, four, and five of weeks three and five. For the purposes of this investigation, urine samples used for analysis were from weeks two and four and day five of weeks three and five. Blood and buccal cell samples were collected at the end of each week. DNA from blood lymphocytes collected during week one was used to genotype *GSTM1* and *GSTT1*. Details regarding genotyping methods can be found in prior publications.[200, 203, 204]

4.2.3 HPLC-ESI$^-$-MS/MS Analysis of Urinary MHBMA and DHBMA

Urinary concentrations of BD-mercapturic acids (MHBMA and DHBMA) were determined by isotope dilution HPLC-ESI$^-$-MS/MS as described in earlier publications.[105, 156, 174] The limits of detection (LOD) for this assay were 0.2 ng/mL urine and 5 ng/mL urine for MHBMA and DHBMA, respectively. One sample was discarded for having DHBMA values below the LOD of the method. A positive control was analyzed (three times per batch, fifteen times total) for the purposes of quality control and used to account for inter-batch variation. The mean coefficient of variation for these replicates was 10.99% and 11.77% for MHBMA and DHBMA, respectively.

4.2.4 Statistical Analyses

Urinary MHBMA and DHBMA concentrations were adjusted to creatinine by dividing each value by the appropriate creatinine value and by batch. The average value of the creatinine-adjusted outcomes of each batch were taken to get $a_1, \ldots, a_5$ and calculated $\bar{a} = \frac{a1+\cdots+a5}{5}$. To adjust for those in batch one, each MHBMA and DHBMA value was multiplied by $\bar{a}$ and divided it by a_1. Similar processes were done for subjects in batches two through five. Therefore, final outcomes were defined as below:

$$MHBMA\ outcome\ for\ set\ i = \frac{MHBMA}{Cr}\frac{\bar{a}}{a_i}$$

These outcomes were log-transformed and back-transformed and presented as geometric means. In addition to adjusting for creatinine and batch, the models were adjusted by log-transformed creatinine-adjusted TNE. Eight subjects were removed from all analyses due to missing outcomes at baseline. One subject was removed from all analyses because the urinary DHBMA concentration was below the LOD of the method.

A table of baseline demographics and urinary biomarkers was obtained. Means and standard deviations are presented for continuous variables, N (%) are presented for categorical variables, and geometric means and 95% confidence intervals are presented for urinary biomarkers. Chi-square or Fisher's exact tests were used for categorical variables and Student's t-tests were used for continuous variables to determine if there were differences between the two treatment sequences at baseline.

To determine if there was an effect of PEITC on the urinary MHBMA or DHBMA concentrations, linear mixed models with random effects that also take into

account period and sequence were used. Similar models were used to determine if there was an effect of PEITC treatment when stratified by GST genotype. These models also included an interaction term between treatment and GST genotype to look at the effect of GST genotype and treatment.

To compare PEITC to baseline and placebo to baseline, the outcomes were defined by taking the urinary biomarker for each respective baseline and subtract them from the urinary biomarkers for PEITC or placebo, depending on the sequence. A linear regression model was then performed on these outcomes, adjusting for sequence and creatinine-adjusted TNE, to test the difference between PEITC and baseline, and placebo and baseline.

To investigate the relationship between GST genotype and the outcomes at baseline, a linear regression model was used with a covariate for GST genotype, and adjustment for creatinine-adjusted TNE. For all analyses, a significance level of 0.05 was used. Statistical analyses were performed using SAS 9.4.

4.3 Results

Characteristics of the study participants are detailed in Table 4.1. Between the two randomly assigned treatment groups, e.g. PEITC-Placebo and Placebo-PEITC, there was no significant difference in age, body mass index (BMI), gender, race, level of education, amount of cigarettes smoked per day (CPD), or alcohol consumption. Of the 79 total participants, 53% were men, 67% were white, 22% were black, and the overall mean age was 41.2 years, with an average CPD smoked of 21.7. There were 7.6% of study participants with the *GSTT1* null genotype, 30.4% with the *GSTM1* null genotype, and 15.2% with the double null genotype. Overall baseline levels of urinary MHBMA and

DHBMA were not statistically significant between the two treatment groups; there was a significant difference found between the two sequences for TNE ($p = 0.04$), which was adjusted for in the later analyses.

Table 4.1 Summary of study demographics and urinary biomarkers at baseline.

Characteristics or Biomarkers	Treatment Sequence Assignment		
	PEITC-Placebo	Placebo-PEITC	P-value[1]
Number of subjects[2]	39	40	
Age (years), mean (SD)	41.5 (10.5)	40.8 (9.6)	0.74
Body mass index (kg/m^2), mean (SD)[4]	28.2 (4.8)	28.2 (6.3)	0.97
Gender, n (%)			0.31
Male	23 (59.0)	19 (47.5)	
Female	16 (41.0)	21 (52.5)	
Race, n (%)			~1.0
Black	8 (20.5)	9 (22.5)	
White	26 (66.7)	27 (67.5)	
Other	5 (12.8)	4 (10.0)	
Level of education, n (%)			0.20
High school or lower	13 (33.3)	19 (47.5)	
College or higher	26 (66.7)	21 (52.5)	
Cigarettes per day, mean (SD)[5]	22.2 (9.4)	21.1 (7.1)	0.57
Alcohol drinking, n (%)[4]			0.65
Never	13 (34.2)	17 (43.6)	
Monthly or less	13 (34.2)	12 (30.8)	
Weekly	12 (31.6)	10 (25.6)	
GSTM1 and *GSTT1* genotypes, n (%)			0.67
Present and present	19 (48.7)	18 (45.0)	
Present and null	3 (7.7)	3 (7.5)	
Null and present	13 (33.3)	11 (27.5)	
Null and null	4 (10.3)	8 (20.0)	
Urinary biomarkers[3], geometric mean (95% CI)			
Total nicotine equivalents (nmol/mg Cr)	45.7 (38.1, 54.8)	59.3 (48.9, 71.8)	0.04
MHBMA (ng/mg Cr)	15.2 (10.5, 22.1)	13.4 (9.9, 18.3)	0.60
DHBMA (ng/mg Cr)	753.3 (601.2, 943.9)	642.7 (442.2, 934.1)	0.46

[1]P-value is for Student's t-test for continuous variables and Chi-Square test or Fisher's exact test for categorical variables.

[2]There were 88 subjects, but nine were removed from all analyses: eight due to missing outcomes at baseline, and one due to values of DHBMA and LOD.

[3]Urinary biomarkers are adjusted for creatinine and log-transformed. MHBMA and DHBMA were also adjusted for batch.

[4]Two subjects were excluded from this analysis due to missing data.

[5]Five subjects were excluded from this analysis due to missing data.

Though previous studies have focused on the effect of treatment group/order on urinary concentrations of other mercapturic acids derived from carcinogens present in cigarette smoke,[200, 203] our work looked at effects of treatment in individuals regardless of treatment group or order. Comparison of PEITC treatment effect versus that of the placebo on urinary concentrations of MHBMA and DHBMA is examined in Table 4.2. When compared to the placebo, urinary concentrations of both MHBMA and DHBMA increased with PEITC treatment (12.27 vs. 11.04 ng MHBMA/mg Cr and 593.81 vs. 572.91 ng DHBMA/mg Cr), though this is increase was not statistically significant ($p = 0.17$ and 0.64, respectively). Table 4.3 further examines the effect of PEITC or placebo treatment on urinary MHBMA and DHBMA as compared to appropriate baseline levels (e.g. weeks two or four in the duration of the study). Weeks during which a placebo was administered showed no significant difference in urinary concentrations of MHBMA and DHBMA when compared to the appropriate baseline week ($p = 0.93$ and 0.54, respectively). Urinary DHBMA concentrations did significantly differ between weeks during which PEITC was administered and the appropriate baseline week ($p = 0.01$), but surprisingly, DHBMA levels were higher at baseline as compared to PEITC treatment. There was no such effect seen for urinary MHBMA concentrations ($p = 0.21$).

Table 4.2 Effect of PEITC compared to placebo on urinary MHBMA and DHBMA.

	Geometric means			
Urinary biomarkers[1]	**Placebo**	**PEITC**	**% Difference (95% CI)**	**P-value[2]**
MHBMA (ng/mg Cr)	11.04	12.27	11.13 (-4.21, 28.92)	0.17
DHBMA (ng/mg Cr)	572.91	593.81	3.65 (-10.90, 20.57)	0.64

[1]Urinary biomarkers were adjusted for creatinine and batch, and log-transformed. [2]Two-sided p-values were from mixed models that test the PEITC treatment effect, after adjusting for creatinine-adjusted TNE

Table 4.3 Effect of PEITC compared to baseline and placebo compared to baseline on urinary MHBMA and DHBMA.

Urinary biomarkers[1]	Geometric Mean (95% CI)	P-value[2]
PEITC vs. baseline		
MHBMA (ng/mg Cr)	0.69 (0.39, 1.23)	0.21
DHBMA (ng/mg Cr)	0.53 (0.33, 0.84)	0.01
Placebo vs. baseline		
MHBMA (ng/mg Cr)	0.97 (0.53, 1.80)	0.93
DHBMA (ng/mg Cr)	1.24 (0.62, 2.49)	0.54

[1]Urinary biomarkers were adjusted for creatinine and batch, and log-transformed. To define the outcome, the urinary biomarkers for each respective baseline were subtracted from the urinary biomarkers for PEITC or placebo, depending on the sequence.

[2]Two sided p-values were from linear regression models that test this difference, after adjusting for sequence and creatinine-adjusted TNE.

However, when stratified by *GSTM1* and *GSTT1* genotype, there was a significant effect of PEITC treatment on urinary MHBMA (Table 4.4). In participants null for *GSTT1*, PEITC increased urinary MHBMA levels by 58.68% as compared to the placebo (6.93 vs. 4.37 ng/mg Cr; p = 0.004). Similar results were seen in participants null for both *GSTT1* and *GSTM1*: PEITC increased urinary MHBMA levels by 89.97% as compared to the placebo (5.03 vs. 2.65 ng/mg Cr; p = 0.001). PEITC treatment also resulted in an increase in urinary MHBMA regardless of the presence or absence of *GSTM1* (null: 19.5% increase, p = 0.12; present: 5.2% increase, p = 0.62). Though these differences were not statistically significant, individuals with the *GSTM1* null genotype did show a larger percent increase in urinary MHBMA as compared to individuals with the gene present. GST genotype did not appear to significantly influence PEITC treatment effect on urinary DHBMA, with the interaction terms for *GSTT1*, *GSTM1*, and both *GSTT1*/*GSTM1* being p = 0.67, 0.82, and 0.33, respectively.

Table 4.4 Effect of PEITC compared to placebo on urinary MHBMA and DHBMA, stratified by GST genotype.

GST genotype	N[4]	Geometric mean		% Difference (95% CI)	P-value[2]	P-value[3] interaction
		Placebo	PEITC			
MHBMA (ng/mg Cr)[1]						
GSTT1						
Null	18	4.37	6.93	58.68 (17.10, 115.02)	0.004	0.01
Present	61	14.49	14.43	-0.38 (-15.24, 17.08)	0.96	
GSTM1						
Null	36	11.38	13.60	19.50 (-4.41, 49.39)	0.12	0.40
Present	43	10.73	11.28	5.20 (-13.70, 28.23)	0.62	
GSTT1 & GSTM1						
Both null	12	2.65	5.03	89.97 (30.31, 176.95)	0.001	0.01
One present	30	19.34	19.08	-1.34 (-21.64, 24.20)	0.91	
Both present	37	11.0	11.29	2.67 (-16.36, 26.04)	0.80	
DHBMA (ng/mg Cr)[1]						
GSTT1						
Null	18	713.14	696.21	-2.37 (-29.28, 34.78)	0.88	0.67
Present	61	536.57	567.92	5.84 (-10.87, 25.68)	0.52	
GSTM1						
Null	36	596.65	631.41	5.82 (-15.75, 32.93)	0.63	0.82
Present	43	553.07	565.11	2.18 (-16.66, 25.27)	0.84	
GSTT1 & GSTM1						
Both null	12	716.72	613.72	-14.37 (-42.47, 27.46)	0.45	0.33
One present	30	580.17	688.85	18.73 (-6.95, 51.49)	0.17	
Both present	37	528.01	523.07	-0.94 (-20.31, 23.15)	0.93	

[1]Urinary biomarkers were adjusted for creatinine and batch, and log-transformed.
[2]Two-sided p-values were from the mixed models that test PEITC effect on the change of MHBMA and DHBMA within each specific GST genotypes before and after PEITC intake, after adjusting for creatinine-adjusted TNE.
[3]Two sided p-values were from the mixed models that test the interaction term between PEITC intake and GST genotype on the levels of MHBMA and DHBMA, after adjusting for creatinine-adjusted TNE.
[4]Value of N varies based on missing outcomes or adjusting variables.

Urinary MHBMA concentrations among individuals with at least one copy of *GSTT1* were 219.5% higher than in those with null *GSTT1* genotype (p = 0.01, Table 4.5). In contrast, *GSTM1* genotype did not have a significant effect on urinary MHBMA concentrations in smokers. These data support results from our previous publications show the influence of *GSTT1* genotype on BD metabolism to MHBMA.[105, 156, 174] Furthermore, neither *GSTT1* nor *GSTM1* genotype appeared to have a significant effect on baselines levels of urinary DHBMA (p = 0.22 and 0.24, respectively, Table 4.5), though concentrations were higher in individuals null for either gene as compared to individuals with at least one copy. Participants null for *GSTT1* excreted 891.34 ng DHBMA/mg Cr versus 645.92 ng/mg Cr in individuals with the gene present (Table 4.5); participants null for *GSTM1* excreted 803.35 ng DHBMA/mg Cr versus 615.77 ng/mg Cr in individuals with the gene present (Table 4.5).

Table 4.5 Effects of GST genotype on urinary MHBMA and DHBMA concentrations at week two baseline.

GST genotype	Geometric mean (95% CI)	P-value[2]
MHBMA (ng/mg Cr)[1]		
GSTT1		
Null (N=18)	7.79 (4.87, 12.46)	*
Present (N=61)	17.10 (13.29, 22.01)	*
% Difference	219.5 (28.25, 375.61)	0.01
GSTM1		
Null (N=36)	15.14 (10.67, 21.49)	*
Present (N=43)	13.62 (9.90, 18.74)	*
% Difference	90.0 (-44.41, 45.54)	0.67
DHBMA (ng/mg Cr)[1]		
GSTT1		
Null (N=18)	891.34 (568.78, 1396.81)	*
Present (N=61)	645.92 (507.48, 822.12)	*
% Difference	72.5 (-56.64, 21.12)	0.22
GSTM1		
Null (N=36)	803.35 (584.34, 1104.45)	*
Present (N=43)	615.77 (460.57, 823.27)	*
% Difference	76.7 (-50.52, 18.75)	0.24

[1]Urinary biomarkers were adjusted for creatinine and batch, and log-transformed.
[2]Two-sided p-values were from linear regression models that test the GST genotype effect on week 2 baseline values, after adjusting for creatinine-adjusted TNE.

4.4 Discussion

The chemopreventative properties of isothiocyanates are well documented, showing inhibition of carcinogenesis at multiple sites in rodents including mammary gland,[205, 206] lung,[199, 207-212] pancreatic,[213] colon,[214, 215] skin,[216] and liver tissues.[217] In particular, PEITC has been found to attenuate cancer progression and development caused by a variety of chemical carcinogens including 7,12-dimethylbenz[a]anthracene (DBMA),[205] *N*-nitrosobenzyl-methylamine (NBMA),[218, 219] azoxymethane (AOM),[214, 215] benzo[a]pyrene (BaP),[208-210] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK).[199, 207, 208] However, to our knowledge, the effects of PEITC on metabolism of 1,3-butadiene (BD) have not been previously investigated. BD is classified as a known human carcinogen,[81] and occupational exposure to BD has been linked to leukemia, lymphoma, and lung cancer.[74-79, 160, 161] BD is among the most abundant carcinogens in tobacco smoke likely to contribute to the etiology of lung cancer.[69] Investigation into the effects of PEITC on BD metabolism could provide a greater understanding into the mechanistic effects by which PEITC exerts its chemopreventive effects.

The mercapturic acids MHBMA and DHBMA are detoxification products of different BD metabolites (1,2-epoxybutene (EB) and hydroxymethylvinyl ketone (HMVK), respectively), and are formed through conjugation to glutathione (GSH), either non-enzymatically or through a reaction catalyzed by GSTs.[48, 97, 100, 101] Though treatment with PEITC increased urinary concentrations of both MHBMA and DHBMA in smokers by 11.13% and 3.65% respectively, these increases were not statistically significant (Table 4.2). Further differentiation of the data by genotype, however, revealed that PEITC treatment significantly increased urinary MHBMA levels by 56.68% in

individuals null for *GSTT1* (Table 4.4). Given that individuals null for *GSTT1* had the lowest baseline levels of urinary MHBMA (7.79 ng/mg Cr, Table 4.5), the results indicate that treatment with PEITC could provide a protective effect in those null for this genotype, possibly inducing other GSTs to catalyze detoxification of BD derived epoxides. Our previous work has shown that GSTT2 is able to catalyze the formation of an EB-GSH conjugate, the precursor to MHBMA, but further studies are warranted to determine the importance of this particular protein in the detoxification of BD metabolites.[174] These findings provide additional support to prior work that shows the marked effect of *GSTT1*-null status on urinary MHBMA levels,[105, 156, 174] and are similar to a recent study examining the effects of *GSTT1*-null status on mercapturic acids formed from metabolites of other chemical carcinogens, such as benzene, acrolein, and crotonaldehyde.[203]

The effect of *GSTM1* genotype on urinary MHBMA however, is less distinct. PEITC treatment increased MHBMA levels independent of *GSTM1* genotype, and although individuals null for the gene saw a greater increase (19.5%) than those containing the gene (5.2%), the difference between treatment with PEITC and the placebo was not statistically significant (Table 4.4). Furthermore, there was no significant difference in baseline levels of MHBMA between individuals null for *GSTM1* versus those where the gene was present ($p = 0.67$, Table 4.5); however, this could be due to a smaller sample size, as our previous study involving a much larger number of subjects (n = 1, 068 versus 79) did show significant differences among smokers when stratified by *GSTM1* genotype.[174] In that work, individuals with two, one, or no copies of the gene excreted urinary MHBMA concentrations of 5.5, 5.3, and 4.4 ng/mL urine,

respectively ($p < 0.0001$).[174] Interestingly, the largest increase in urinary MHBMA by PEITC treatment was seen in individuals null for both *GSTT1* and *GSTM1* (89.97%, Table 4.4), still potentially indicating a protective effect, albeit small, in *GSTM1*-null individuals.

Unlike MHBMA, PEITC treatment did not have a significant effect on urinary DHBMA concentrations, even when stratified by GST genotype. However, prior work also indicates that GST genotype does not necessarily seem to influence excretion of this particular mercapturic acid. In the same aforementioned study containing 1,068 subjects, statistical analysis showed no significant effect on urinary DHBMA in smokers when data was stratified by *GSTT1* or *GSTM1* copy number ($p = 0.226$ and 0.94, respectively);[174] in an additional study containing 584 subjects, analyses also showed no significant effect on urinary DHBMA in smokers ($p = 0.181$).[105] These results could likely be due to the fact that DHBMA is typically a less sensitive biomarker of BD exposure from smoking as compared to MHBMA. In a smoking cessation study, urinary levels of MHBMA decreased 92% three days post cessation, whereas DHBMA levels only decreased 16% in the same amount of time; this disparity remained throughout the study.[48] Additionally, urinary DHBMA in smokers is only about 35% higher than in nonsmokers, suggesting potential DHBMA formation from sources other than BD.[48, 104]

Overall, this study is the first of its kind to examine the effect of PEITC treatment on BD metabolism in smokers. Our results indicate that ingestion of PEITC could provide a strongly protective effect against BD-mediated carcinogenesis in smokers null for *GSTT1* or both *GSTT1* and *GSTM1*. More broadly, these results support other work investigating the anti-cancer properties of dietary isothiocyanates and provide additional

evidence that consumption of these compounds could provide a wide-reaching and cost effective method of cancer prevention.

V. DEVELOPMENT OF N^E, N^E-(2,3-DIHYDROXYBUTAN-1,4-DIYL)-L-LYSINE AS A URINARY BIOMARKER OF EXPOSURE TO 1,2,3,4-DIEPOXYBUTANE

5.1 Introduction

1,3-Butadiene (BD) is an important industrial chemical widely used in the production of synthetic rubber and plastics.[64] Global demand for BD is high: in 2004, worldwide production of BD reached approximately 9 million metric tons.[64] Occupational exposure of worked to BD in monomer and polymer industries range from 0.005 – 378 ppm, with typical exposure levels at 2 ppm[65] In addition, humans can be exposed to BD through various other routes[220] including cigarette smoke (25 – 75 ug in mainstream smoke),[14, 68] motor vehicle exhaust (20 – 60 ppb),[67, 221] urban air (1 – 10 ppb),[67] and smoke from wood burning fires (up to 15 ppb).[66] This is a cause for concern due to its adverse health effects. BD has been shown to be a multi-site carcinogen in laboratory rodents,[70-73] and epidemiological studies have revealed an association between occupational exposure to BD and the development of lymphatic and hematopoietic cancer in humans.[74-79] Taken together, the results of these studies have led to the classification of BD by the International Agency for the Research on Cancer as a group 1 agent and by the National Toxicology Program as a known human carcinogen.[80, 81]

BD undergoes metabolic activation to form 3,4-epoxy-1-butene (EB) by cytochrome P450 monooxygenases 2E1 and 2A6 (CYP2E1 and 2A6).[82, 83] The epoxide ring on EB can be hydrolyzed by epoxide hydrolase (EH), forming 1-butene-3,4-diol (EB-diol).[85] EB-diol can either be converted to hydroxymethylvinyl ketone (HMVK) by alcohol dehydrogenase (ADH) or to 3,4-epoxy-1,2-butanediol (EBD) by CYP2E1.[89] EB can also undergo further oxidation by CYP2E1 to form 1,2,3,4-diepoxybutane (DEB).[84]

BD-derived mercapturic acids, DNA adducts, and hemoglobin adducts can be used a biomarkers of BD exposure in humans.[48, 98, 101, 220, 222]

Among all BD metabolites, DEB is considered to be the ultimate carcinogenic species. DEB shows mutagenic activity at concentrations 40- to 100- fold lower than EB or EBD in treated TK6 human lymphoblastoid cells.[88] Because of this, there is a great need to develop a DEB-specific biomarker. Currently, the only DEB specific biomarker is *N*,*N*-(2,3,-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val), a hemoglobin adduct formed from the reaction of DEB at the N-terminus of valine.[135] However, using *pyr*-Val as a DEB-specific biomarker in humans necessitates the acquisition and use of blood samples; by contrast, urine samples are much easier to obtain. Therefore, the development of a DEB-specific urinary biomarker remains an avenue to be further explored. In 2014, Kotapati et al. showed that DEB undergoes conjugation with two molecules of GSH and can be excreted in urine as 1,4-bis-(*N*-acetyl-L-cystein-S-yl)butane-2,3-diol (*bis*-BDMA).[99] Concentration-dependent excretion of *bis*-BDMA was observed in laboratory rats exposed to 0-200 ppm BD.[99] However, *bis*-BDMA was not detected in urine of occupationally exposed humans, even when sensitive methods (LOD 0.1 ng/mL urine) and large amounts of urine (1 mL) were employed in the analysis.[99] Thus, there are still currently no DEB-specific urinary biomarkers to evaluate exposure to BD in humans.

Given the evidence for the formation of exocyclic DEB-protein adducts such as *pyr*-Val[124, 133, 135] we hypothesized that DEB similarly *bis*-alkylates the N^{ε} nitrogen of lysine in proteins and can be excreted in urine as N^{ε}, N^{ε}-(2,3-dihydroxybutan-1,4-diyl)-L-lysine (DHB-Lys) following proteolytic degradation (Figure 5.1). The following work describes the initial attempts at developing isotope dilution HPLC-ESI^{+}-MS/MS

methodology for the detection and quantitation of a novel DEB-specific urinary biomarker.

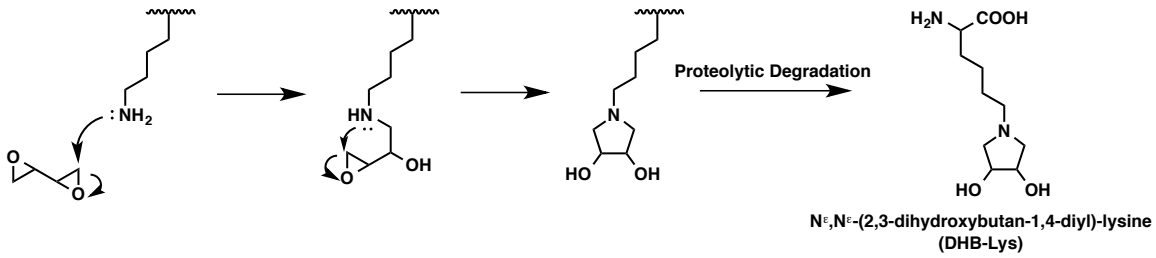


Figure 5.1 Hypothesized formation of DHB-Lys, a novel DEB-specific urinary biomarker

5.2 Experimental

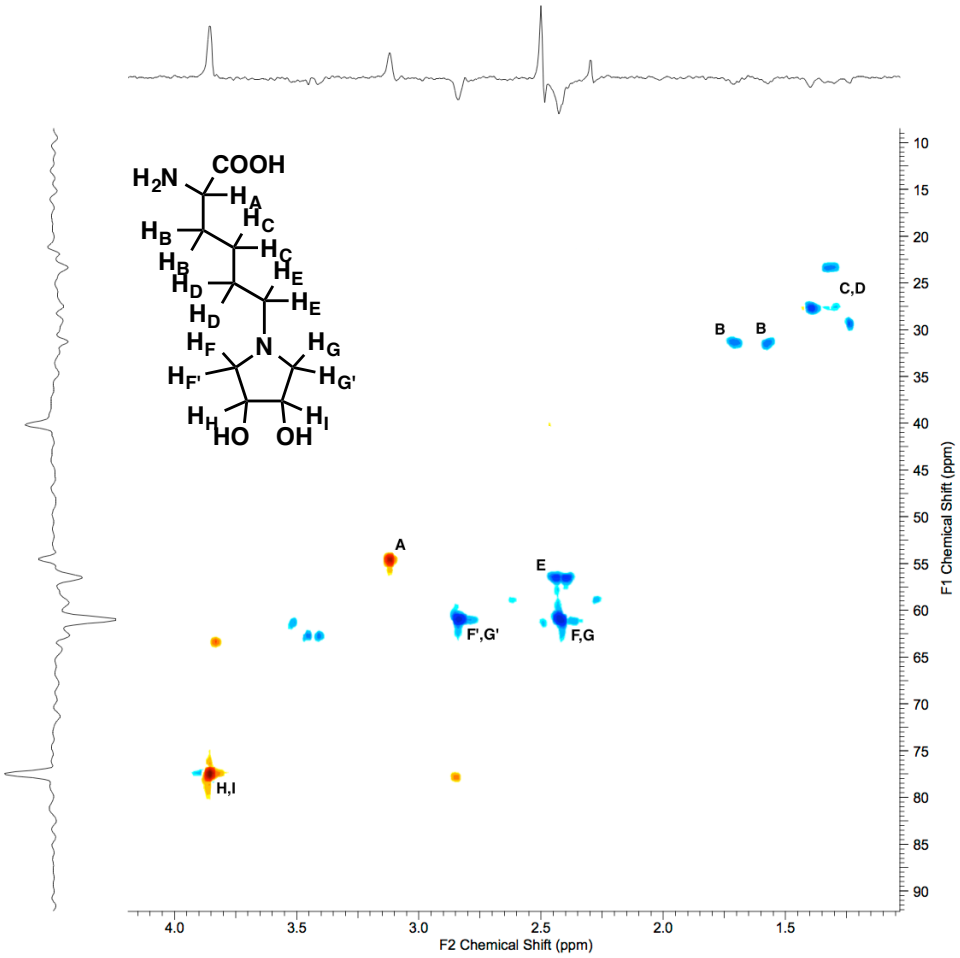
5.2.1 Materials

DHB-Lys, $^{2}H_{6}$-DHB-Lys, and other common chemicals were purchased from Sigma Aldrich (St. Louis, MO). LC/MS grade ammonium acetate was obtained from Sigma Aldrich (St. Louis, MO), and LC/MS grade water and methanol were acquired from Fisher Scientific (Pittsburgh, PA). Oasis HLB and MCX cartridges were purchased from Waters Corporation (Milford, MA).

5.2.2 Synthesis and Characterization of N^{ε}, N^{ε}-(2,3-dihydroxybutan-1,4-diyl)-L-lysine (DHB-Lys)

DHB-Lys was prepared using a modified version of a published procedure.[223] A stoppered 50 mL round bottomed flask containing water (2 mL), triethylamine (0.2 mL), glacial acetic acid (60 μL), DEB (77.56 μL), and N^{α}-Boc-L-lysine (246.30 mg) was stirred at 50 °C for 7 days. The reaction mixture was cooled to room temperature, neutralized with dilute ammonium hydroxide, and extracted with chloroform. DHB-Lys was purified using preparative TLC on a silica plate developed with 9:1 chloroform:methanol, The product was isolated as a yellow oil (est. yield, 5%). The deuterated internal standard, $^{2}H_{6}$-DHB-Lys, was prepared analogously starting with $^{2}H_{6}$-DEB (Sigma Aldrich, St. Louis, MO).

Structural characterization of DHB-Lys and $^{2}H_{6}$-DHB-Lys was carried out using NMR (Figure 5.2) and high-resolution mass spectrometry (Figure 5.3). Stock solutions of DHB-Lys and $^{2}H_{6}$-DHB-Lys were prepared in water and stored at -20 °C.



Assigned Position	δ_H
A	3.10
B	1.58, 1.70
C, D	1.23, 1.31, 1.40
E, F, G	2.39, 2.42
F', G'	2.81
H, I	3.84

Figure 5.2 ^{1}H-^{13}C HSQC spectra of DHB-Lys

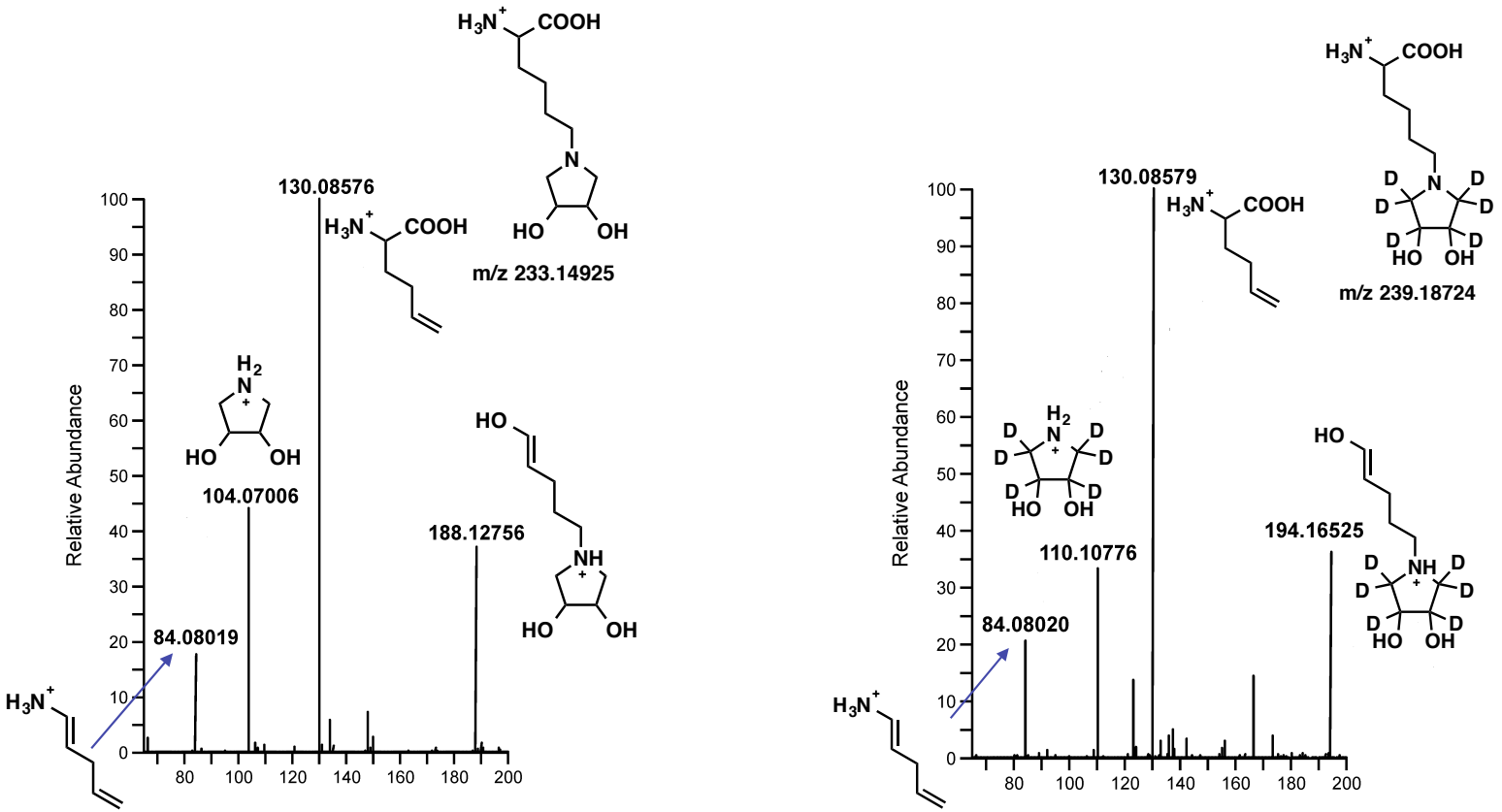


Figure 5.3 HRMS^2 of DHB-Lys (Left) and $^2H_6$-DHB-Lys (Right)

5.2.3 HPLC-ESI$^+$-MS/MS Method Development Using an Ion Pairing Agent

5.2.3.1 Sample Preparation

Solid phase extraction (SPE) was performed using Oasis MCX cartridges (1 mL/30 mg; Waters Corporation, Milford, MA). Blank urine samples (200 μL) were diluted with water (200 μL) followed by the addition of $^2H_6$-DHB-Lys internal standard (500 fmol), DHB-Lys (500 fmol), and 0.1% formic acid (150 μL). The samples were loaded onto Oasis MCX cartridges conditioned with 1 mL of methanol followed by 1 mL of water. After loading, the cartridges were washed with 1 mL 2% formic acid followed by 1 mL of methanol. DHB-Lys and $^2H_6$-DHB-Lys were eluted with 1 mL of 5% ammonium hydroxide in methanol, dried under vacuum, and reconstituted in 30 μL of 5 mM perfluoroheptanoic acid (PFHA) in water for analysis by HPLC-ESI$^+$-MS/MS.

5.2.3.2 HPLC-ESI$^+$-MS/MS Analysis

HPLC-ESI$^+$-MS/MS was conducted using an Agilent 1100 HPLC coupled to a Thermo Finnigan TSQ Quantum Discovery Max mass spectrometer. Samples were run at room temperature on a Phenomenex Synergi 4u Hydro-RP 80A column (100 x 2 mm, 4 um) equipped with a guard column of the same packing using a solvent system of A: 0.5 mM PFHA and B: acetonitrile at a flow rate of 200 μL/min. B was increased linearly from 2% to 42% over forty minutes, then decreased to 2% over two minutes, and finally held at 2% for eight minutes for a total time of fifty minutes. Using this gradient, DHB-Lys and $^2H_6$-DHB-Lys eluted at 30.4 minutes (Figure 5.4).

The mass spectrometer was operated in positive mode with the following settings: spray voltage, 3500 V; sheath gas pressure, 50 psi; capillary temperature, 270 °C; source CID, 8 V; collision gas pressure, 1.0 mTorr; Q1(fwhm), 0.3; Q3 (fwhm), 0.7; scan width, 0.4 m/z; scan time, 0.4 s. Quantification of DHB-Lys was performed using selected reaction monitoring (SRM) with $^{2}H_{6}$-DHB-Lys as an internal standard. The following transitions were used for DHB-Lys and $^{2}H_{6}$-DHB-Lys, respectively: m/z 233.1 → 84.1 and m/z 239.1 → 84.1. Additional transitions were monitored for conformational purposes: m/z 233.1 → 130.1, 188.1, and 104.1 for DHB-Lys and m/z 239.1 → 130.1, 194.1, and 110.1 for $^{2}H_{6}$-DHB-Lys.

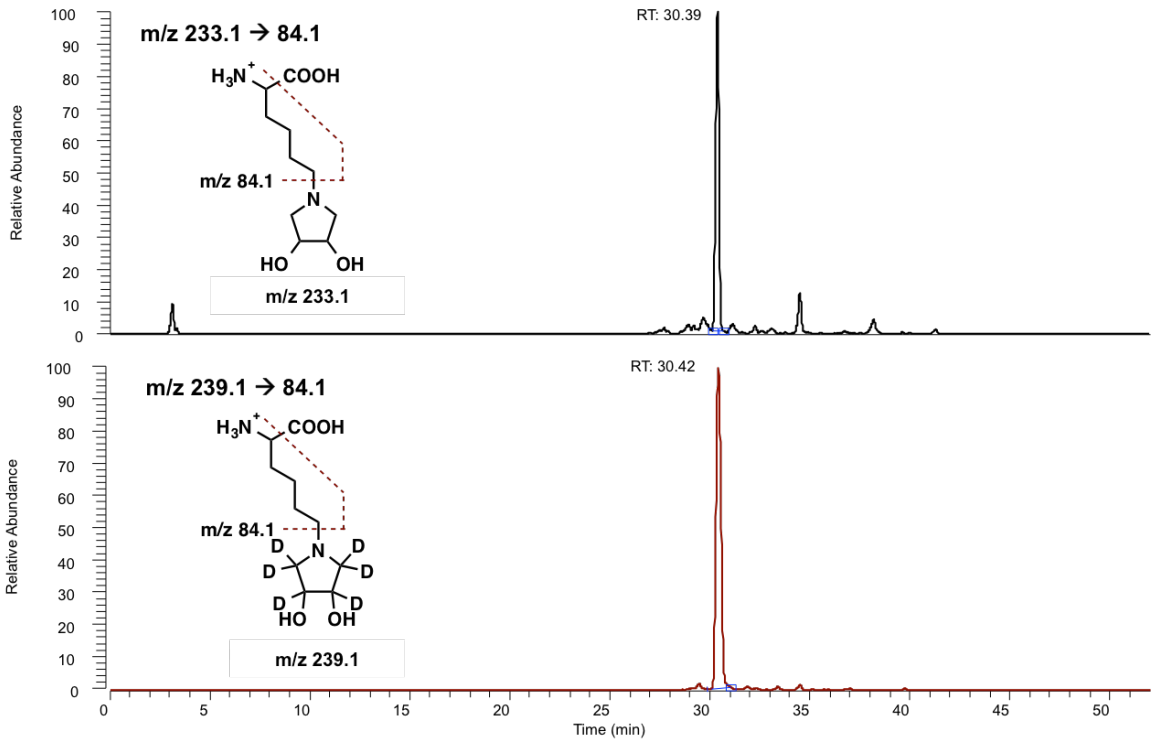


Figure 5.4 HPLC-ESI$^+$-MS/MS of DHB-Lys and $^2H_6$-DHB-Lys spiked in synthetic urine (1 pmol each)

5.2.3.3 HPLC-ESI$^+$-MS/MS Method Validation

A method calibration curve was constructed by analyzing solutions of increasing amounts of DHB-Lys (0 – 0.4 pmol) and a fixed amount of $^2H_6$-DHB-Lys (1 pmol) in water, followed by regression analysis of the observed and added amounts of DHB-Lys. A validation curve was constructed in a similar manner. Synthetic urine (200 μL) was spiked with increasing amounts of DHB-Lys (0 – 0.2 pmol) and a fixed amount of $^2H_6$-DHB-Lys (1 pmol), and underwent sample processing and HPLC-ESI$^+$-MS/MS analysis as described above. Regression analysis was performed on the observed versus added amounts of DHB-Lys.

SPE recovery was determined by spiking synthetic urine (200 μL) with $^2H_6$-DHB-Lys (1 pmol), which was subject to sample processing as described above. Prior to HPLC-ESI$^+$-MS/MS analysis, DHB-Lys (0.5 pmol) was added, and recovery was determined by comparing added and observed peak area ratios between $^2H_6$-DHB-Lys and DHB-Lys.

5.2.4 Method Development Using AccQ-Tag Ultra Derivatization

5.2.4.1 Derivatization of DHB-Lys

Derivatization was performed using an AccQ-Tag Ultra Derivatization Kit (Waters, Medford, MA), which uses the derivatizing agent, 6-aminoquinolyl-N-hydroxysuccinimdyl carbamate (AQC). AQC reacts at the backbone nitrogen (N^α) of DHB-Lys, forming a more hydrophobic compound (6-AQ-DHB-Lys) through the addition of an aminoquinolyl group at that position (Figure 5.5). This kit included pre-

measured portions of the AQC, as well as all other solvents needed for the reaction. A solution of AQC in acetonitrile was prepared following the manufacturer's instructions. In a microcentrifuge tube, the prepared AQC soltution (20 μL) and either DHB-Lys or $^{2}H_{6}$-DHB-Lys (10 μL aliquot, various concentrations used) were added a sodium tetraborate buffer (70 μL). The tube was capped, covered with Parafilm, and vortexed for several seconds before being left at room temperature for one minute. Afterwards, the tube was transferred to a heating block, and the reaction continued for an additional ten minutes at 55 °C. After removal from the heating block, the derivatized standard solution can be stored in a parafilmed tube at room temperature for up to one week or at -20 °C for up to one month. Product formation was confirmed using mass spectrometry (Figure 5.6).

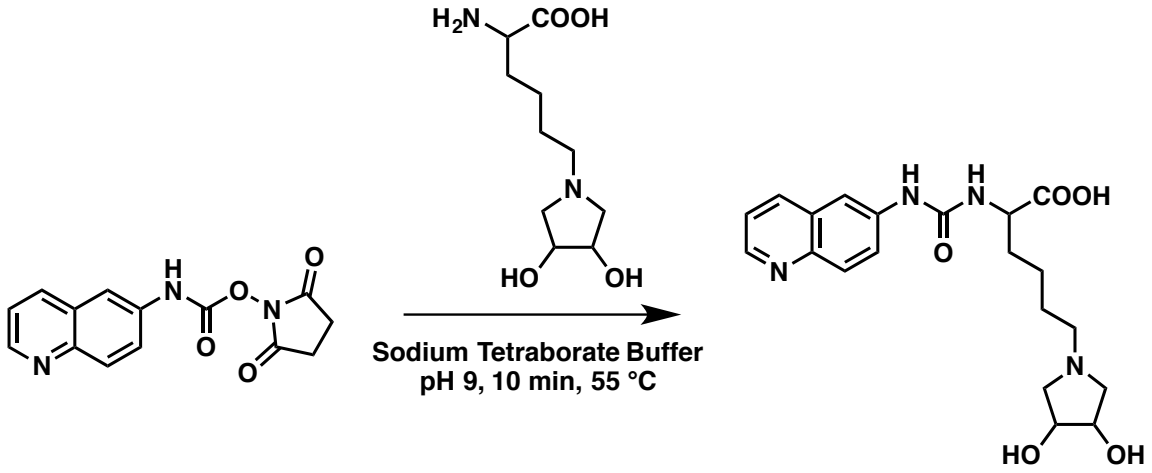


Figure 5.5 Reaction scheme for the derivatization of DHB-Lys

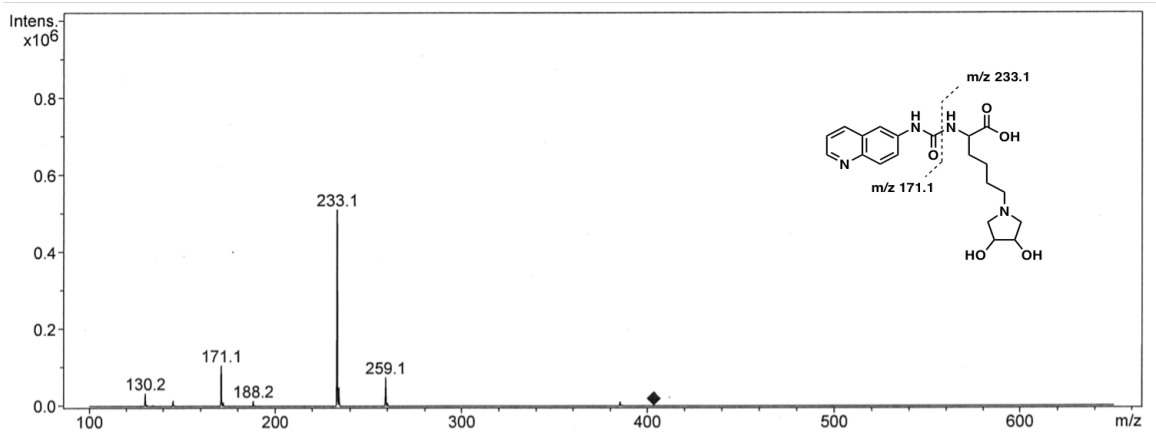


Figure 5.6. MS^2 Spectrum of 6-AQ-DHB-Lys

5.2.4.2 Sample Preparation via Solid Phase Extraction and AccQ-Tag Derivatization

Urine samples (200 μL) were diluted with water (200 μL), spiked with 500 fmol each DHB-Lys and $^{2}H_{6}$-DHB-Lys, and subjected to SPE purification as described in Section 5.2.3.2 using Oasis MCX cartridges (1 mL/30 mg, Waters Corporation, Milford MA). The resulting eluent was dried down under reduced pressure, and samples were reconstituted in 70 μL of the sodium tetraborate buffer. The AccQ-Taq derivatization reaction was performed as described above, and the derivatives were purified using Oasis HLB cartridges (1 mL/30 mg Waters Corporation, Milford MA). The derivatized samples were loaded onto cartridges conditioned with 1 mL of methanol followed by 1 mL of water. After loading, the cartridges were washed with 1 mL water followed by 1 mL of 10% methanol. DHB-Lys and $^{2}H_{6}$-DHB-Lys were eluted with 1 mL of 40% methanol, dried under vacuum, and reconstituted in 30 µL of 10 mM ammonium acetate for analysis by HPLC-ESI^{+}-MS/MS.

SPE recovery for the Oasis HLB was determined by spiking synthetic urine (100 µL) with 6-AQ-$^{2}H_{6}$-DHB-Lys (250 fmol), which was subject to sample processing as described above. Prior to HPLC-ESI^{+}-MS/MS analysis, 6-AQ-DHB-Lys (250 fmol) was added, and recovery was determined by comparing added and observed peak area ratios between 6-AQ-$^{2}H_{6}$-DHB-Lys and 6-AQ-DHB-Lys.

5.2.4.3 HPLC-ESI$^+$-MS/MS Analysis of 6-AQ-HB-Lys

HPLC-ESI$^+$-MS/MS of 6-AQ-DHB-Lys was conducted using an Eksigent nanoLC Ultra 2D HPLC coupled to a Thermo Vantage triple quadrupole mass spectrometer. Samples were loaded on a Waters NanoEase BEH C18 column (150 x 0.3 mm, 3.5 μm) eluted maintained at 55 °C and eluted at a flow rate of 8 μL/min using a solvent system of 10 mM ammonium acetate (A) and methanol (B). Solvent composition was changed linearly from 8% to 16.2% over 9 minutes, increased again to 75% over one minute, held at 75% for another minute, and then decreased to 8% over one minute before being held at 8% for an additional eight minutes (total run time of 20 minutes). Using this gradient, 6-AQ-DHB-Lys eluted at 10.1 minutes and 6-AQ-$^2H_6$-DHB-Lys at 9.4 minutes (Figure 5.7).

The mass spectrometer was operated in positive mode with the following settings: spray voltage, 3400 V; sheath gas pressure, 45 psi; capillary temperature, 260 °C; source CID, 7 V; collision gas pressure,1.5 mTorr; Q1 (fwhm), 0.4; Q3 (fwhm), 0.4; scan width, 0.2 m/z; scan time, 0.25 s. HPLC-ESI$^+$-MS/MS quantification of 6-AQ-DHB-Lys was performed using selected reaction monitoring (SRM) with 6-AQ-$^2H_6$-DHB-Lys as an internal standard. The following transitions were used for 6-AQ-DHB-Lys: *m/z* 403.2 → 233.1 and *m/z* 403.2 → 171.1; the following transitions were used for $^2H_6$-6-AQ-DHB-Lys: *m/z* 409.2 → 239.1 and *m/z* 409.2 → 171.1

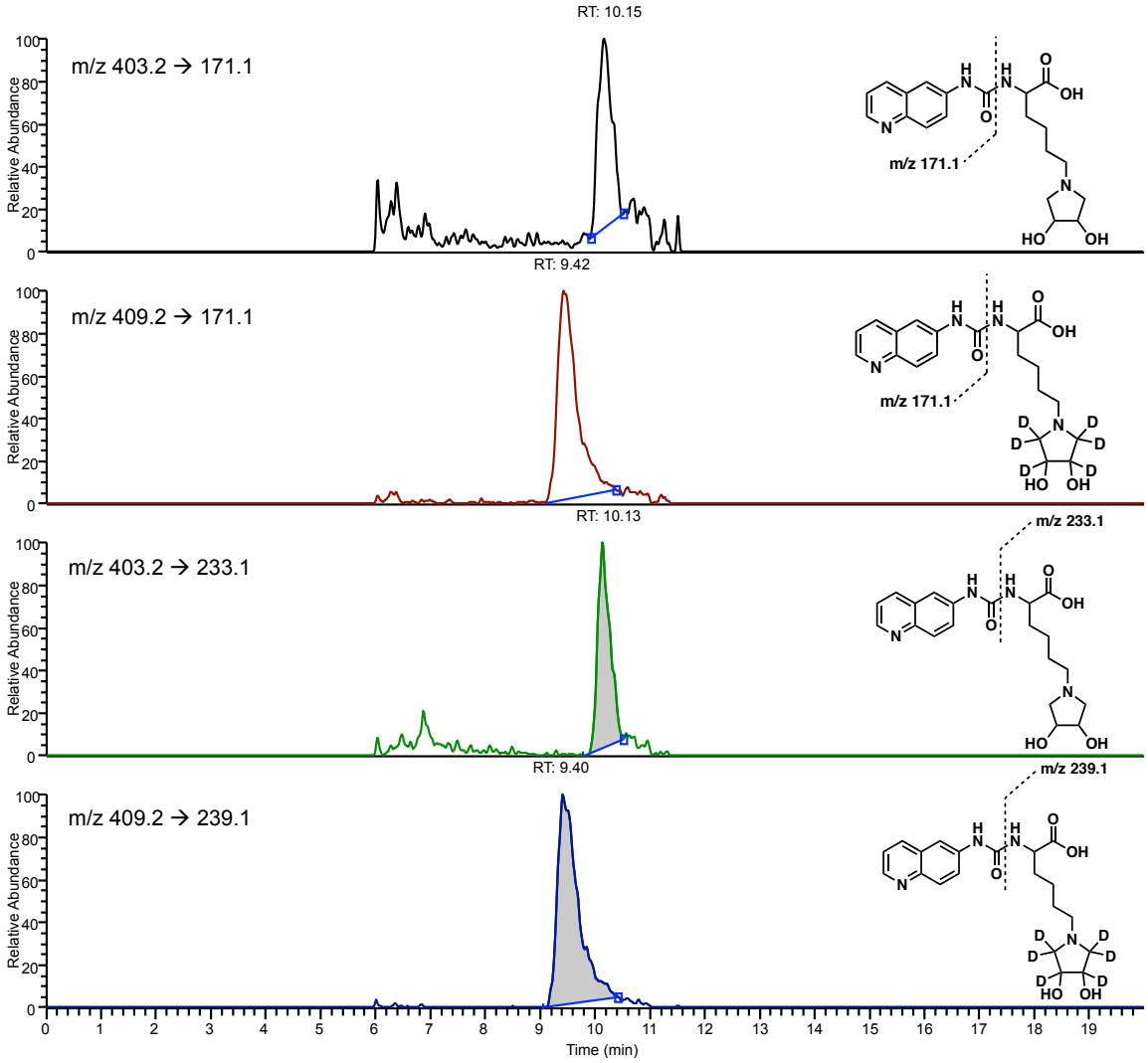


Figure 5.7 HPLC-ESI^{+}-MS/MS of 50 fmol each 6-AQ-DHB-Lys and 6-AQ-$^{2}H_{6}$-DHB-Lys

5.2.4.4 DEB Treatment of Model Protein (AGT)

O^6-alkylguanine DNA alkyltransferase (AGT) protein (2 nmol) was dissolved in 10 mM Tris-HCl buffer (pH 7.5) (50 µL) and treated with 0, 15, 25, or 50 molar equivalents of DEB for 2 h at 37 °C. Samples were processed using Bio-Spin 6 columns (Bio-Rad Laboratories, Hercules, CA) to remove excess DEB. The pH of the eluent was adjusted with 25 mM ammonium bicarbonate (final pH 7.5); the solution was digested to peptides using 1 µg trypsin at 37 °C overnight, and further digested to amino acids using 10 µg proteinase K at 37 °C overnight. Solutions were acidified to a pH of 1 with 2% formic acid, spiked with 500 fmol of $^2H_6$-DHB-Lys, and processed by Oasis MCX SPE, Acc-Taq derivatization, and Oasis HLB SPE as described above, followed by HPLC-ESI-MS/MS analysis.

5.3 Results and Discussion

5.3.1 HPLC-ESI$^+$-MS/MS Method Using Perfluoroheptanoic Acid

Development of urinary biomarkers presents inherent challenges, as urine is a complex and variable matrix containing high concentrations of salts, polar molecules, and other small compounds that can lead to ESI signal suppression.[224, 225] Therefore, we employed solid phase extraction (SPE) in order to remove potentially interfering impurities[226] and enrich DHB-Lys. DHB-Lys is a bisubstituted amino acid that exists as a zwitterion under physiological conditions due to the presence of amino group (pK_a = 8.9) and carboxylic acid functionalities (pK_a = 2.2) in its structure. When considering potential SPE solid phases, ion exchange sorbents were chosen to take advantage of the fact that DHB-Lys was a charged species. Strong cation- and anion-exchange (SCX,

SAX) as well as mixed mode cation- and anion-exchange (MCX, MAX) phases were initially tested with standards spiked into synthetic urine; of these, MCX showed the best analyte recovery (70.2%), and thus was selected for use in sample preparation for DHB-Lys.

The highly charged nature of DHB-Lys presented inherent challenges for HPLC method development because of its poor retention on common HPLC stationary phases A variety of columns, including Hypercarb (Thermo Fisher Scientific, Waltham, MA), Beta Max Acid (Thermo Fisher Scientific, Waltham, MA), Diphenyl (Agilent Technologies, Santa Clara, CA), and Synergi Hydro-RP (Phenomenex, Torrance, CA), were tested, however, under all conditions tested DHB-Lys eluted within the first five minutes of the gradient and did not afford the necessary separation from interfering endogenous molecules. We also investigated the use of hydrophilic interaction liquid chromatography (HILIC) methodology, which works through the use of hydrophilic stationary phases with traditional reverse phase eluents to provide separation of polar compounds.[227] Although HILIC column afforded good retention of the DHB-Lys standard, spiked urine samples showed poor analyte signal under the variety of conditions (mobile phases, column temperatures) tested

We next investigated the use of an ion pairing agent, perfluoroheptanoic acid (PFHA), in an attempt to improve DHB-Lys retention on HPLC columns.[228] Under acidic conditions, PFHA is negatively charged and can form an ion pair with DHB-Lys. PFHA's long alkyl chain can then interact with more conventional solid phases used in

reverse phase HPLC, providing retention, and therefore better separation, of DHB-Lys. HPLC conditions using PFHA were further optimized, and then used to create calibration and validation curves (Figure 5.8), both of which showed a linear correlation between the spiked and observed amounts of DHB-Lys ($y = 0.9955x - 0.0033$, $R^2 = 0.99935$ for water and $y = 0.9579x - 0.0005$, $R^2 = 0.99891$ for synthetic urine).

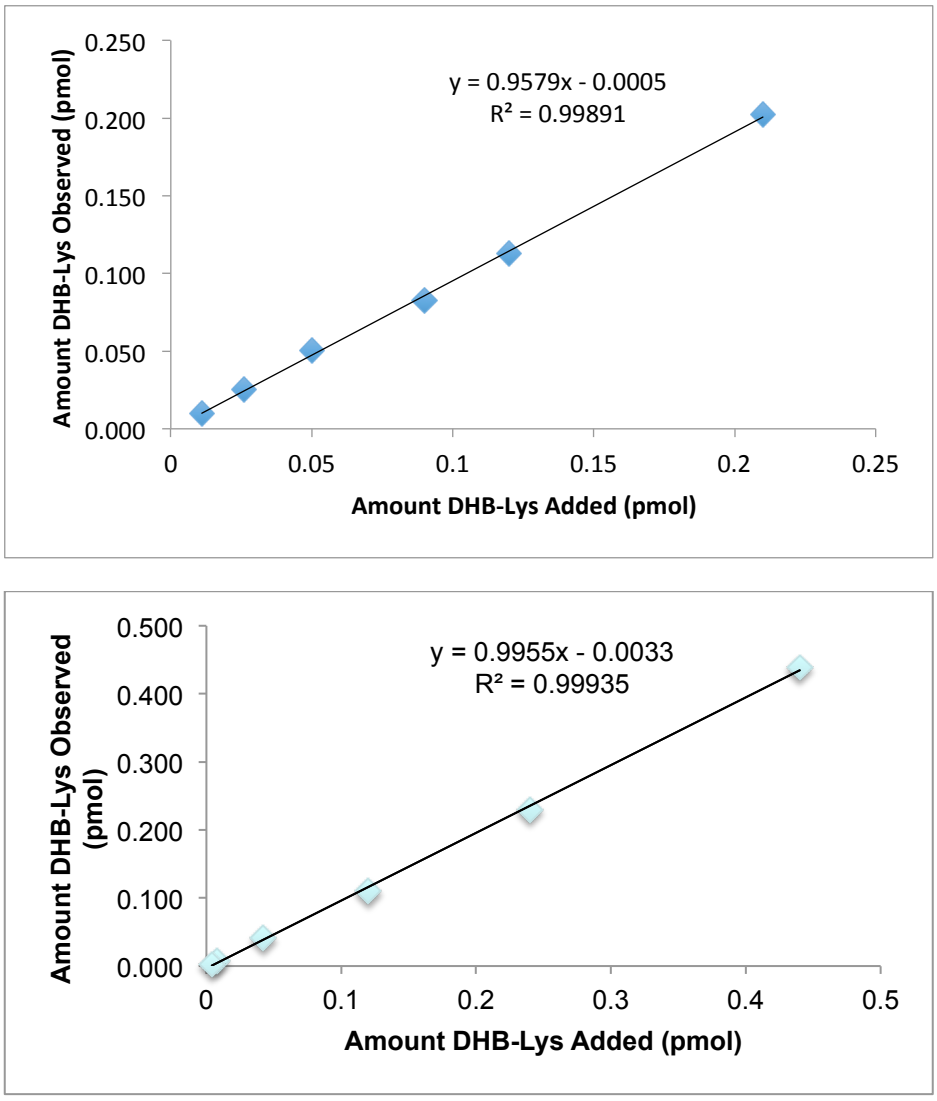


Figure 5.8 Correlation between the spiked and observed amounts of DHB-Lys spiked into water (top) or synthetic urine (bottom). Samples were spiked with 0, 0.005, 0.05, 0.1, 0.25, or 0.4 pmol DHB-Lys (in water, top) or with 0, 0.025, 0.05, 0.1, 0.15, or 0.2 pmol DHB-Lys (in synthetic urine, bottom) and 1 pmol of $^{2}H_{6}$-DHB-Lys.

Although these preliminary method validation results were promising, we were not successful in applying the methodology to human and rat urine samples spiked with internal standard. This could be attributed to an increased chemical complexity of these samples, resulting in co-elution with endogenous molecules and ion suppression. Furthermore, the use of PFHA presented additional problems including analyte carryover and persistence of PFHA in the HPLC-MS/MS system. Although extensive wash methods were developed to remove excess PFHA from the instrumentation, time and cost needed to implement these wash runs outweighed the benefits of PFHA use. Ultimately, it was decided to abandon the use of PFHA in the methodology, and explore an alternative approach.

5.3.2 AccQ-Tag Derivatization of DHB-Lys

AccQ-Tag Ultra Derivatization reagent available from Waters employs 6-aminoquinolyl-N-hydroxysuccinimdyl carbamate (AQC) as the derivatizing agent, which increases hydrophobicity of DHB-Lys through the addition of the aminoquinolyl group at the alpha-nitrogen (Figure 5.5). Initial derivatization reactions with this reagent proved successful, and method development regarding further sample preparation by SPE and HPLC-ESI$^+$-MS/MS analysis continued. Derivatization of DHB-Lys with AccQ-Tag forms a hydrophobic derivative (6-AQ-DHB-Lys), which can be purified using HLB SPE cartridges with good analyte recovery (93.7%). HPLC-ESI$^+$-MS/MS method was developed for 6-AQ-DHB-Lys, which showed a good retention on a Waters NanoEase BEH C18 column using a gradient of methanol in ammonium acetate buffer (Figure 5.7).

In order to test the applicability of this to DHB-Lys detection in samples, a model protein (AGT, 2 nmol) was treated with 0, 15, 25, or 50 molar excess DEB and proteolytically digested to amino acids, which were subjected to derivatization and SPE to look for evidence of DHB-Lys formation. Results from this experiment showed a dose-dependent increase in DHB-Lys formation (Figures 5.9 and 5.10); however, the analyte was also observed in the control sample. We speculate that the appearance of this background signal in untreated is due to analyte carryover and/or sample contamination.

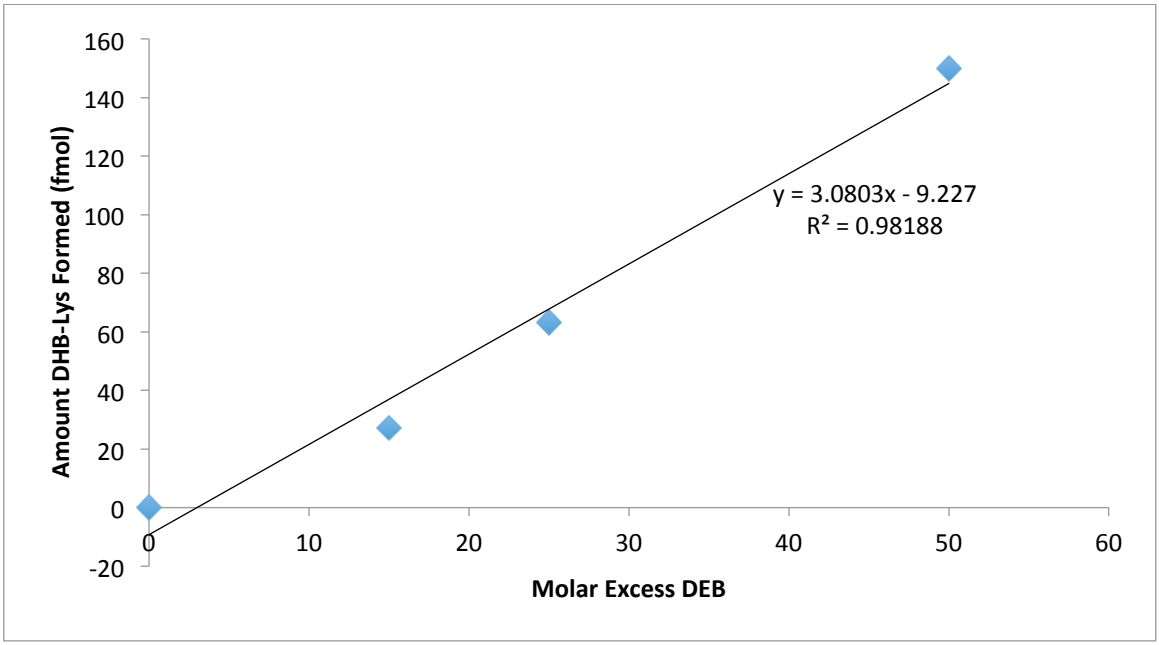


Figure 5.9 Dose dependent increase in DHB-Lys formation in DEB-treated AGT . Background amounts present in control sample were subtracted.

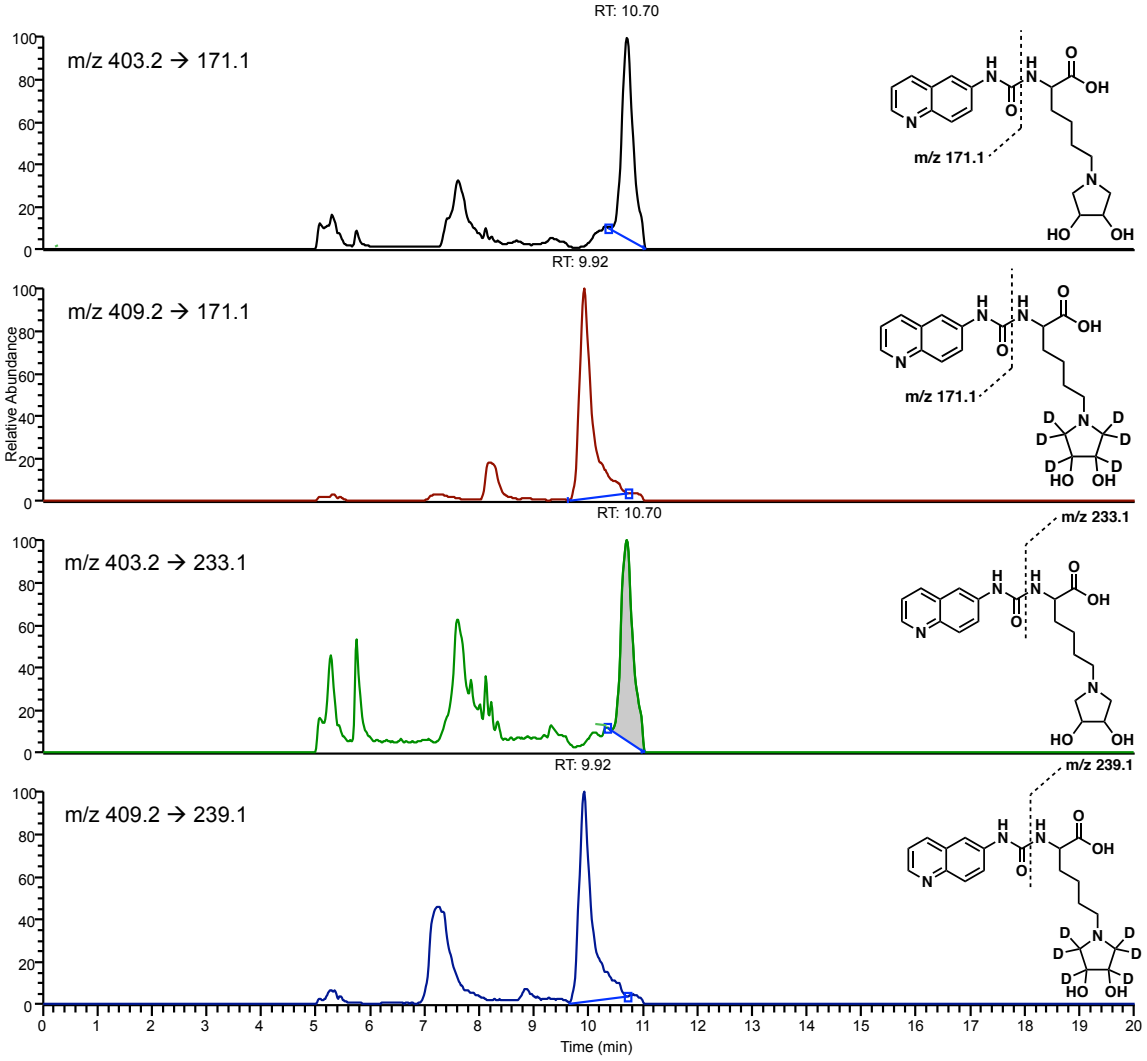


Figure 5.10 HPLC-ESI$^+$-MS/MS trace depicting DHB-Lys formation in AGT protein treated with 25 fold molar excess of DEB

In summary, the AccQ tag derivatization method is a promising approach for detection of DHB-Lys in biological samples. Future experiments should include alternative methods of sample preparation to better isolate and enrich DHB-Lys. Method development using a mixture of derivatized amino acid standards might give greater insight into where DHB-Lys elutes using our current methods relative to other amino acids that likely remain in the sample due to similarities in structure. Being able to visually confirm separation of DHB-Lys from these other potential co-elutes could be useful in improving the chromatography of the current HPLC-ESI^+-MS/MS method or in developing an offline HPLC method for enrichment of DHB-Lys from these samples prior to HPLC-ESI^+-MS/MS analysis.

VI. CONCLUSIONS

Though the link between smoking cigarettes and its harmful health effects has long been established, smoking remains the most preventable cause of death in the world.[56] Smoking is the leading cause of lung cancer, causing 83% and 76% of lung cancer deaths in men and women in the United States, respectively.[54] Furthermore, there are inherent differences in lung cancer risk among smokers of different ethnic groups; compared to European American smokers, African American and Native Hawaiian smokers have an increased lung cancer risk, whereas lung cancer risk decreases for Japanese American and Latino smokers.[63] These variances in risk are thought to be a result of ethnic differences in the metabolism of the carcinogens present in cigarette smoke;[63] the work presented here investigates this possibility with respect to the metabolism of known human carcinogen, 1,3-butadiene (BD).

Among the 60+ carcinogens present in cigarette smoke,[14] BD was investigated because it is one of the most abundant (approximately 20-75 μg in main-stream smoke and 205-260 μg in side-stream smoke)[68] and was shown to have a high cancer risk index;[69] a risk analysis suggests that BD contributes the most to potential health risks out of all the carcinogenic constituents in cigarette smoke. [69] BD is metabolically activated by CYP450s 2E1 and 2A6[83] to form the chemically reactive species 3,4-epoxy-1-butene (EB),[82] 1,2,3,4-diepoxybutane (DEB),[84, 229] 3,4-epoxy-1,2-butanediol (EBD)[89] and hydroxymethyl vinyl ketone (HMVK)[89] (Figure 1.7). If not detoxified, these metabolites can alkylate DNA at nucleophilic positions, leading to the formation of DNA adducts

(Figure 1.10), which can result in mutations potentially contributing to cancer development.[57, 176] EB and HMVK can be detoxified through glutathione conjugation and excreted in urine as the mercapturic acids MHBMA[101] and DHBMA,[48] respectively (Figure 1.9), and have been used as biomarkers of BD exposure. Therefore, differences in BD metabolism between individuals or ethnic groups could result in different urinary concentrations of BD metabolites MHBMA and DHBMA. HPLC-ESI$^-$-MS/MS methodology developed in our laboratory for the simultaneous quantitation of MHBMA and DHBMA proved to be highly applicable and was used in multiple projects (Chapters II-IV) that focused on the role of BD metabolism in cancer risk.

In Chapter II, ethnic differences in BD metabolism among African American, European American, and Japanese American smokers were investigated through quantitation of urinary MHBMA and DHBMA in 1,072 urine samples. HPLC-ESI-MS/MS analyses revealed significantly different MHBMA and DHBMA concentrations between all three ethnic groups that was consistent with their lung cancer risk (Table 2.2).[63] African American smokers excreted the highest amounts of urinary MHBMA and DHBMA, followed by European American and Japanese American smokers (MHBMA: 6.4, 5.7, and 3.3 ng/mL urine, respectively, $p = 4.0 \times 10^{-25}$; DHBMA: 362.0, 294.6, and 292.7 ng/mL urine, respectively, $p = 3.3 \times 10^{-4}$). A similar trend was seen upon comparison of the metabolic ratio—defined as total urinary MHBMA divided by the total urinary MHBMA plus the total urinary DHBMA [MHBMA / (MHBMA + DHBMA)]—among the groups: European American smokers showed the highest ratio, followed by

African American and Japanese American smokers (0.020, 0.018, and 0.012 ng/mL urine, respectively, $p = 1.7 \times 10^{-14}$; Table 2.2). *GSTT1* and *GSTM1* copy number were both significantly associated with urinary MHBMA levels and metabolic ratio ($p < 0.0001$ for all comparisons; Table 2.3), but not with urinary DHBMA levels (GSTT1: $p = 0.226$; GSTM1: $p = 0.94$; Table 2.3).

The large study size afforded the opportunity to perform a genome wide association study (GWAS) to look for associations between excreted MHBMA and DHBMA and single nucleotide polymorphisms (SNPs) present in the genomes of the study subjects. With respect to MHBMA, GWAS analyses confirmed the importance of GSTT1 in BD metabolism: associations with 136 SNPs were detected, and all of them were located between 24.2—24.4 Mb near the *GSTT1* gene on chromosome 22q11. Though MHBMA is formed from conjugation of GSH to EB,[101] EB had never been confirmed as a substrate for GSTT1. Incubation of EB and GSH in the presence or absence of recombinant GSTT1 or GSTT2 verified EB as a substrate for both enzymes, with increased levels of the EB-GSH conjugate detected in the presence of either GSTT1 or 2 as compared to controls. GWAS analyses performed with respect to metabolic ratio yielded similar results to those seen with MHBMA, but no significant genome-wide associations were observed with respect to DHBMA levels. Overall, this work highlighted the strong effect *GSTT1* gene deletion has on urinary MHBMA and showed that the inter-ethnic differences in MHBMA levels, and therefore BD metabolism, between African American, European American, and Japanese American smokers can be

partially explained by *GSTT1* genotype and contribute to the ethnic differences seen in smoking-induced lung cancer risk.

Ethnic differences in BD metabolism were further explored in Chapter III through quantitation of MHBMA and DHBMA in urine samples from a total of 151 European American and African American smokers previously analyzed for urinary N7-(1-hydroxy-3-buten-2-yl) guanine (EBGII), a DNA adduct formed from the metabolite EB.[109, 164, 166] Both urinary MHBMA and DHBMA levels were higher in European American smokers as compared to African American smokers, though the difference was only statistically significant with respect to MHBMA (MHBMA: 15.70 ± 1.71 vs. 7.39 ± 0.82 ng/mg creatinine (Cr), respectively, $p = 3.6 \times 10^{-6}$; DHBMA: 283.71 ± 18.07 vs. 236.98 ± 15.30 ng/mg Cr, respectively, p = 0.051; Table 3.2). It should be noted that these values do not correlate to lung cancer risk for these groups, as European American smokers have a reduced lung cancer risk relative to African American smokers;[63] however, the results are similar to those reported previously by our laboratory in which Native Hawaiian smokers excreted significantly less MHBMA than European American smokers despite having a relative higher lung cancer risk.[105] Furthermore, overall urinary MHBMA and DHBMA levels did not correlate with urinary EBGII in these same subjects (with MHBMA: r = 0.035, with DHBMA, r = 0.028; Table 3.3). Because EB is required for the formation of both MHBMA and EBGII, a lack of any correlation between the two biomarkers could mean that they are equally likely to form and/or get excreted. Statistical analyses looking for an association between urinary EBGII,

MHBMA, or DHBMA and variety of specific SNPs from BD-metabolizing genes (*EPHX1* and *CYP2E1*) and DNA repair genes (*FANCE*) or *GSTT1* copy number revealed only a significant association between urinary MHBMA and *GSTT1* copy number ($p = 6.97 \times 10^{-10}$, Table 3.4). The absence of any association between urinary EBGII and *GSTT1* copy number was surprising. Though GSTT1 is not directly involved in the formation and/or removal of EBGII, it is possible that *GSTT1* copy number could influence EBGII levels by changing the amount of free EB available to form adducts. Given the small size of our study population (N = 151) as well as the limited number of variants considered for the analyses, application of this methodology to larger sample sizes and a larger SNP pool is warranted to confirm these results.

The project depicted in Chapter IV employed similar methods as those used in chapters II and III, but focused on the effect of 2-phenethyl isothiocyanate (PEITC), a chemopreventative agent,[184, 185] on urinary levels of MHBMA and DHBMA in smokers. For this work, MHBMA and DHBMA levels were quantified in a total of 316 urine samples from 79 smokers (4 samples per smoker) participating in a randomized, double-blind, multi-week long clinical trial with a crossover study design.[200, 203] As a part of this study, individuals were given a placebo (olive oil) during one week and then treated with PEITC (10 mg in olive oil per day, 4 times per day for five days) during another week or vice versa. PEITC partially exerts its anticancer properties by inducing GSTs involved in the detoxification of carcinogens and/or their reactive metabolites;[196] therefore, treatment with PEITC was expected to increase urinary levels of MHBMA and DHBMA when

compared to levels during the week in which the placebo was administered. Overall, PEITC treatment resulted in slight increases in urinary MHBMA and DHBMA when compared to treatment with the placebo, but these differences were not statistically significant (MHBMA: 12.27 vs. 11.04 ng/mg Cr, $p = 0.17$; DHBMA: 593.81 vs. 572.91 ng/mg Cr, $p = 0.64$; Table 4.2). Stratification of the data by *GSTT1* and *GSTM1* genotype, however, revealed significant effects of PEITC treatment on MHBMA levels: in individuals null for *GSTT1*, MHBMA levels increased 58.85% as compared to the placebo (6.93 vs. 4.37 ng/mg Cr, $p = 0.004$; Table 4.4), and in individuals null for both *GSTT1* and *GSTM1*, MHBMA levels increased 89.97% as compared to the placebo (5.03 vs. 2.65 ng/mg Cr, $p = 0.001$; Table 4.4). Conversely, individuals null for only *GSTM1* did not see a significant increase in urinary MHBMA as a result of treatment with PEITC, and urinary DHBMA appeared to be unaffected by PEITC treatment regardless of GST genotype (Table 4.4). This study was the first of its kind to examine the effect of PEITC treatment on BD metabolism in smokers. The results further emphasize the importance of *GSTT1* genotype in the formation of MHBMA, ultimately indicating that ingestion of PEITC could provide a strongly protective effect against BD-mediated carcinogenesis in smokers null for *GSTT1* or both *GSTT1* and *GSTM1*.

The continued use of MHBMA and DHBMA as urinary biomarkers of BD is pertinent in investigating the role of BD metabolism in smoking induced lung cancer. However, there still remains a need to develop new biomarkers of BD exposure. Of the BD metabolites, the *bis*-electrophile DEB is the most mutagenic, and therefore

quantitation of a DEB-specific biomarker is necessary in order to measure its formation. Though DEB-specific biomarkers exist, only one—*pyr*-Val, a hemoglobin adduct (Figure 1.11)—has ever been quantitated in humans occupationally exposed to BD,[135] and the methodology requires blood samples for analysis. The work presented in Chapter V focuses on the initial steps taken towards the development of a novel urinary DEB-specific biomarker, N^{ε}, N^{ε}-(2,3-dihydroxybutan-1,4-diyl)-L-lysine (DHB-Lys), which is hypothesized to form from the bis-alkylation of the lysine side chain by DEB (Figure 5.1).

Method development presented many challenges, particularly with the HPLC retention, as DHB-Lys is an inherently polar analyte. Initially, the addition of the ion pairing agent perfluoroheptanoic acid (PFHA) to the aqueous mobile phase during HPLC-ESI^+-MS/MS analysis was chosen to surmount this obstacle, but its use also lead to analyte carryover, sample contamination, and ion suppression. Therefore, alternative methods to increase retention were explored, and success was found via derivatization of DHB-Lys through the addition of a 6-aminoquinolyl group (6-AQ) at the alpha nitrogen of DHB-Lys to form 6-AQ-DHB-Lys (Figure 5.5). Using a solvent system of 10 mM ammonium acetate and methanol, 6-AQ-DHB-Lys eluted at 7.4 minutes and the respective internal standard, 6-AQ-$^2H_6$-DHB-Lys, eluted at 8.0 minutes (Figure 5.7) on a Waters NanoEase BEH C18 column (150 x 0.3 mm, 3.5 μm) maintained at 55 °C and eluted at a flow rate of 8 μL/min. Sample preparation and analyte enrichment were achieved using two rounds of solid phase extraction (SPE): prior to derivatization,

samples underwent SPE using Waters Oasis MCX cartridges, which provided 70.2% analyte recovery, and post-derivatization, samples were further processed using Waters Oasis HLB cartridges, which provided 93.7% analyte recovery. The described methodology was used in initial experiments that involved the treatment of O^6-alkylguanine DNA alkyltransferase (AGT) protein with increasing amounts of DEB (0, 15, 25, 50 molar excess). Results showed a dose-dependent increase in the formation of DHB-Lys (Figure 5.9), but also showed presence of the analyte in the control sample, most likely explained through cross-contamination. Despite this, derivatization remains the most encouraging approach in the detection of DHB-Lys in biological samples.

Overall, the work presented in this thesis described new applications of an existing HPLC-ESI$^-$-MS/MS methodology for the simultaneous quantitation of urinary MHBMA and DHBMA to generate new information regarding ethnic differences in BD metabolism, to compare the formation of BD-detoxification products versus BD-DNA adducts, and to evaluate the ability of a potential chemopreventative agent in mitigating the effects of BD exposure. Preliminary method development of the novel DEB-specific urinary biomarker DHB-Lys was also established and used to detect DHB-Lys in a model protein treated with DEB.

VII. FUTURE DIRECTIONS

7.1 Expansion of Multi-Ethnic Studies to Additional Ethnic Groups or Correlation Studies

In Chapter II, urinary concentrations MHBMA and DHBMA were measured in African American, European American, and Japanese American smokers (N = 1,072); MHBMA levels were significantly different among these groups, and a GWAS analysis showed an association between urinary MHBMA and several SNPs corresponding to *GSTT1*. It would be interesting to expand this study to include Native Hawaiian (N = 350) and Latino smokers (N=350), ethnic groups with high and low smoking induced lung cancer risks, respectively.[63] Including data from these new groups would allow for an even larger pool of subjects (N = 1700) on which to perform a GWAS, which could potentially uncover variants on new genes involved in BD metabolism or provide additional insight regarding variants on genes previously identified by these types of analyses.

A similar expansion could be applied to the project in Chapter III, in which urinary concentrations of MHBMA and DHBMA were measured in African American and European American smokers (N = 151) and compared to urinary EBGII levels from the same individuals. Generally, it would be beneficial to expand the total number of samples from both African American and European American smokers to be analyzed for MHBMA, DHBMA, and EBGII. Larger overall sample size allows for a better representation of the populations being studied, and would serve to provide even more

accurate information regarding ethnic differences in BD metabolism and greater understanding between the balance of detoxification versus bioactivation of BD metabolites. Furthermore, urinary EBGII was only measured in African American and European American smokers, groups that have high and intermediate levels of smoking induced lung cancer risk.[63] However, the addition of an ethnic group at a low risk of smoking induced lung cancer, such as Japanese Americans or Latinos,[63] could provide interesting information regarding trends in EBGII levels among these groups.

Though Chapter IV did not focus on ethnic differences in urinary MHBMA or DHBMA, the ethnicity of the individuals is known as part of the study, and additional statistical analyses comparing the effect of PEITC treatment when stratified by ethnic group could potentially reveal ethnic differences in the efficacy of PEITC as a "protective agent" against BD-mediated cancer.

7.2 Kinetics of EB-GSH Formation Using Human Recombinant GSTT1 and GSTT2

Incubation experiments described in Chapter II confirmed EB as a substrate for both human recombinant GSTT1 and GSTT2 for the first time by measuring formation of an EB-GSH conjugate in the presence and absence of the enzymes. With this established method, kinetics experiments could be performed to determine the catalytic activity of these enzymes with respect to EB as a substrate. Use of a rapid quench flow instrument would allow for short incubation times of the appropriate enzyme, GSH, and increasing amounts of EB. HPLC-ESI$^-$-MS/MS methodology would need to be developed for quantitation of the amount of EB-GSH conjugate formed, but could be based upon

methodology for the detection of MHBMA since they share structural similarities. For similar reasons, EB-GSH formation could be quantified using $^{2}H_{6}$-MHBMA as the internal standard with the use of a calibration curve. Alternatively, isotopically labeled GSH also could be used in the synthesis of a labeled EB-GSH internal standard for absolute quantitation.

7.3 Inclusion of *GSTT2* Genotype in Statistical Analyses Comparing Urinary MHBMA and DHBMA

Individuals who participated in the studies examined in chapters II-IV were all genotyped for both *GSTT1* and *GSTM1*, and that information was able to be used in statistical analyses comparing both MHBMA and DHBMA levels by copy number for each gene. In each of the aforementioned chapters, differences in urinary MHBMA were statistically significant when stratified by *GSTT1* copy number, highlighting the importance of GSTT1 in the detoxification of EB through the formation of MHBMA. Because EB has been confirmed as a substrate for GSTT2, being able to determine the *GSTT2* copy number and its potential effects on MHBMA or DHBMA levels could shed an important light on the involvement of another GST in the detoxification of BD metabolites.

7.4 Further Development of DHB-Lys as a Biomarker of DEB

Though the creation and validation of a method to detect DHB-Lys is still in the development phase, use of 6-aminoquinolyl-N-hydroxysuccinimdyl carbamate (AQC) as a derivatizing agent has produced the most promising results so far with regard to DHB-

Lys detection. However, given that this derivatizing agent has the ability to react with any amino acid, it's important that future method development efforts include alternative methods of sample preparation to better isolate and enrich DHB-Lys. As suggested in Chapter V, using a mixture of derivatized amino acid could give visual information with respect to the elution of DHB-Lys elutes using our current methods relative to other amino acids that remain in the sample due to similar size and structure. Improving the chromatographic separation of DHB-Lys from other interfering compounds combats potential ion suppression from co-elutes, or could be applied to the development of an offline HPLC method for enrichment of DHB-Lys prior to HPLC-ESI^+-MS/MS analysis. With a working method, the potential applications are many, but initial experiments could include re-testing the formation of DHB-Lys in DEB treated AGT, as well as sample processing of urine from rats exposed to high levels of BD (> 62.5 ppm) or humans occupationally exposed to BD.

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