GAPS IN BUTADIENE BIOMARKERS OF EXPOSURE AND EFFECT

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Sujey Carro-Mojica: Gaps in Butadiene Biomarkers of Exposure and Effect
(Under the direction of James A. Swenberg)

1,3-Butadiene (BD) is a well-characterized carcinogen that is both an occupational and an environmental hazard. BD is metabolized mainly by CYP4502E1 into epoxides, 1,2-epoxybutene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butandiol (EB-diol). These reactive epoxides can react with and form adducts with both hemoglobin (Hb) and DNA. Tumor formation in rodents shows species and gender differences, with mice being much more sensitive than rats, and female mice being more sensitive than male mice. The weight of evidence presented across rodent species suggests there are sex specific differences in BD-DNA crosslinks, tumor formation, and mutagenicity and supports the hypothesis that DNA repair processes rather than differences in BD metabolism drive tumor formation in a sex dependent manner. Combining metabolic biomarkers data with tumor formation presented interspecies differences that were difficult to understand. Some of the questions that remained unclear were how BD was acting as a carcinogen, how we could use this data, and what these findings meant for human risk assessment. To help answer these questions, we looked at BD metabolic activation in male and female mice, rats and humans and BD-epoxide induced DNA repair.
ACKNOWLEDGEMENTS

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Damage Response portion of this work. Fifth, I want to extend my warmest thanks to Esra Mutlu, April Luke, Jack Ridpath, Irene Abraham, Mitch Troutman, Ben Moeller and Kun Lu. Spending so much time together made us family. My eternal love and thanks to both my parents: Carmen and Roberto, whom have never given up on me, my ideas or my projects. My greatest thanks to Arayoán Vergara-Mojica and Stephanie Vergara-Mojica. They are my brick wall of love, support, and inspiration. During this project, they have reminded me every day, with their own accomplishments, that we have exerted a positive influence on each other in both our careers and lives. Ultimately, I want to dedicate this thesis to my grandfather Feliciano Mojica-Oferral; this one is for you. I’m sure you will have plenty of time to read it in heaven, just like we use to read the newspapers together, every morning, with a really good cup coffee.
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<th>Definition</th>
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<tbody>
<tr>
<td>Ade</td>
<td>Adenine</td>
</tr>
<tr>
<td>ADH</td>
<td>Alcoholdehydrogenase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGT</td>
<td>O$^6$-alkylguanine DNA alkyltransferase</td>
</tr>
<tr>
<td>AP-site</td>
<td>apurinic/apyrimidinic site or abasic site.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BD</td>
<td>1,3-Butadiene</td>
</tr>
<tr>
<td>BDM</td>
<td>Butadiene monomer</td>
</tr>
<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom Syndrome RecQ Like Helicase</td>
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<tr>
<td>BRCA</td>
<td>Breast Cancer Protein:</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast Cancer 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast Cancer 2</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelocytic leukemia</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CSA &amp; CSB</td>
<td>Cockayne syndrome factors A and B</td>
</tr>
<tr>
<td>CtIP</td>
<td>C terminal interacting protein</td>
</tr>
<tr>
<td>DBS</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>DDB</td>
<td>DNA Damage Binding Protein:</td>
</tr>
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<td>DDB1</td>
<td>Component 1</td>
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<td>-------------</td>
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<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
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<td>1,2:3,4-diepoxybutane</td>
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<tr>
<td>DNA2</td>
<td>DNA 2 Nuclease</td>
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<tr>
<td>DPC</td>
<td>DNA Protein-crosslink</td>
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<td>DT40</td>
<td>Chicken B-lymphocyte cell line</td>
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<td>EB</td>
<td>1,2-epoxybutene</td>
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<td>GADPH</td>
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<td>Guanine</td>
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<td>HB-Val</td>
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<td>HB-Ade I</td>
<td>N-1-(2-hydroxy-3-buten-1-yl) adenine</td>
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<td>HB-Ade II</td>
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<td>N-3-(2-hydroxy-3-buten-1-yl) adenine</td>
</tr>
<tr>
<td>HB-Ade IV</td>
<td>N-3-(1-hydroxy-3-buten-2-yl) adenine</td>
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HB-Gua:

HB-Gua I  N-7-(2-hydroxy-3-buten-1-yl)guanine
HB-Gua II N-7-(1-hydroxy-3-buten-2-yl)guanine
HMVK     Hydroxymethylvinylketone
HPLC     High pressure liquid chromatography
HR       Homologous Recombination
hHR23B   Human Homolog Rad23 B
IA       Immunoaffinity
IARC     International Agency for Research on Cancer
IBD      Internal Blood Dose
ICL      Interstrand crosslinks
IDLs     Insertion/deletion loops
LC-MS/MS Liquid chromatography tandem mass spectrometry
LD50     Lethal Dose 50%
LOD      Limit of Detection
LOQ      Limit of Quantitation
M1       1,2-dihydroxy-4-(N-acetylcysteinyl)-butane
M2       1-(N-acetylcysteinyl)-2-hydroxy-3-butene
M3       1,3,4-trihydroxy-2-(N-acetylcysteinyl)-butane
MMR      Mismatch Repair
MF       Mutant frequencies
MOA      Mode of Action
MPG      Methyl purine glycosylase
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>MRN complex</td>
<td>Mre11/Rad50/Nbs1:</td>
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<tr>
<td>Mre11</td>
<td>Miotic Recombination 11 Protein</td>
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<tr>
<td>Nbs1</td>
<td>Nijmegen Breakage Syndrome-1 Protein</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MSH</td>
<td>Mut S Homolog</td>
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<tr>
<td>N1-THB-A</td>
<td>N1-(2,3,4-trihydroxybut-1-yl)adenine</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non Homologous End Joining</td>
</tr>
<tr>
<td>NIOSH</td>
<td>National Institute for Occupational Safety and Health</td>
</tr>
<tr>
<td>NTP</td>
<td>National Toxicology Program</td>
</tr>
<tr>
<td>NTV</td>
<td>N terminal Valine</td>
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<tr>
<td>OSHA</td>
<td>United States Occupational Safety and Health Administration</td>
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<tr>
<td>PARP-1</td>
<td>Poly [ADP-ribose] polymerase 1</td>
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<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PEL</td>
<td>Permissible exposure limit</td>
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<tr>
<td>PFPTH</td>
<td>Pentafluorophenylthiohydantoin</td>
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<tr>
<td>Pol</td>
<td>Polymerase</td>
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<td>pyr-Val</td>
<td>N, N-(2, 3-dihydroxy-1, 4-butadiyl)-valine</td>
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<td>RFC</td>
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<tr>
<td>SBR</td>
<td>Styrene-butadiene rubber</td>
</tr>
<tr>
<td>STEL</td>
<td>Short-term exposure limit</td>
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</table>
TC-NER  Transcription Coupled Nucleotide Excision Repair

TFIIH  Transcription Factor II Human

THB-Ade:
N3 THB-Ade  N-3-(2′,3′,4′-trihydroxybut-1′-yl)-adenine
N6 THB-Ade  N-6-(2′,3′,4′-trihydroxybut-1′-yl)-adenine
THB-Gua  N-7-(2′,3′,4′-trihydroxybut-1′-yl)guanine
THB-Val  N-(2,3,4-trihydroxybutyl) valine

TLS  Translesion Synthesis

TWA  Time-weighted average

U.S. EPA  U.S. Environmental Protection Agency

UV-DDB  Ultra-Violet Damage Deoxyribonucleic Acid Binding Protein B

WHO  World Health Organization

XPC  Xeroderma pigmentosum Complementation Group

XPG  Xeroderma pigmentosum Group

XRCC  X-Ray Repair Cross Complementing Protein

XTT  sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt
CHAPTER I: BUTADIENE LITERATURE REVIEW

1.0 BACKGROUND

1.1 History Sources and Exposure

1,3 Butadiene (BD) was first isolated in 1863 by the French chemist Caventou, but not identified until 1886, by Armstrong, and finally polymerized by Lebedev in 1910. Initially, the industry of BD was mainly controlled by the British government and primarily derived from natural rubber. BD production dependence on its natural source became apparent, propelling its synthetic production worldwide. In 1930, the Soviet Union had the first pilot BD production plant, using ethanol from potatoes. In 1936, its manufacture evolved, using petroleum as a source, and producing poly-butadiene (PBD). It was not until 1940, that the Soviet Union turned into the largest producer of BD, followed by Germany and the US. As of today, BD continues its production for several uses that include car and truck tires in its majority, as well as an additive in the manufacture of plastics, synthetic rubbers, resins, and a small portion designated to the production of nylon. BD is also indirectly generated as a by-product of ethylene manufacture, as a component of cigarette smoke, and also found as part of vehicle exhaust emissions. Alternatively, in nature, BD may also be released from the burning of natural wood in forest fires and or homes that depend on wood stove burners.

BD is a volatile organic compound (VOC), whether the source is natural or anthropogenic, its properties are the same. BD is a flammable gas with gasoline like odor,
composed of 4 carbons in the form of \([\text{CH}_2=\text{CH}-\text{CH}=\text{CH}_2]\). It has a molecular mass of 54.09 and Chemical Abstracts Service (CAS) number. 106-99-0. Because BD is a gas, the primary route of exposure is via inhalation with the general population exposed to it, at different levels. For instance, BD has been identified to be present at 0.001-0.01 ppm in urban air, 0.02-0.06 ppm from motor vehicle emissions, and ~2 ppm BD in occupational settings. Also, BD is a major component of cigarette smoke with 0.4mg per cigarette and with the highest cancer risk index. The two largest groups of anthropogenic BD exposure are occupationally exposed workers, and cigarette smokers.

1.2 Butadiene Metabolism and Epoxide Generation

BD is inhaled into the lungs and enters the bloodstream through passive diffusion. Those organs actively involved in BD metabolism include liver, lungs, and kidneys. BD absorption, distribution, and metabolic activation have been shown to lead to the generation of three reactive epoxides, which can ultimately covalently bind and modify both proteins and DNA or be excreted in urine.

The first step of BD activation is the oxidation of BD into the first monoepoxide, 1, 2-epoxy-3-butene (EB) by enzymes CYP2E1 and CYP2A6. The second step is further oxidation of EB, into the diepoxide: 1,2;3,4 diepoxybutene (DEB) by enzymes CYP2E1 or CYP3A4. Both EB and DEB can undergo hydrolysis and form butene-diol (BD-diol) from EB or lead to the formation of the second mono epoxide, 1,2-epoxy-3,4-butanediol (EB-diol) from DEB, by the enzyme epoxyhydrolase (EH). Ultimately BD-diol can be further oxidized into more EB-diol.

[Figure 1.1]
1.2.1 Mutagenicity of Butadiene Epoxides

In human lymphoblastoid Tk6 cells, and rats and mice T-cells individual mutagenic potency for each of these BD-epoxides can vary up to 200-fold (Walker, 2009, Meng, 2007, Meng, 2007b). Results from T-cloning assay from mice and rats exposed to BD showed that BD epoxides were most to least mutagenic, in the following order DEB > EB > EB-diol (Walker, 2009). There is also an inversely proportional relationship across BD-epoxides with respect to mutagenicity and their abundance of generation. The most mutagenic epoxides are the least abundant (DEB > EB > EB-diol) and the least mutagenic epoxides are the most abundant (EB-diol > EB > DEB) both in vitro and in vivo (Swenberg 2011, Meng, 2010). When looking closer at gender and species, Meng has shown that the mutagenic response in T-cells to BD is highest in female followed by male mice then female rats and male rats (Meng 2007b) supporting BD as a stronger carcinogen for mice than rats. These observations are consistent with EB, DEB and EB-diol alkylations in both DNA, and more specifically, the N-terminal Valine (NTV) of hemoglobin (Hb) upon BD exposure (Moll 1999). When looking at biomarkers of exposure and effect, earlier reports have identified species differences in the formation of DNA and Hb adducts endpoints; ultimately indicating that BD metabolic activation is species dependent as evidenced by biomarkers of exposure. When considering biomarkers of effect, the biggest difference in mutagenicity lies between DEB and EB-diol with DEB being almost 200 times more mutagenic.

1.3 Butadiene as a Carcinogen

BD has been classified as “known human carcinogen” by IARC, NTP and EPA. Exposure to BD results in multi-site carcinogenesis in rodents (Melnick, 1993, Owen, 1987) and an increased incidence of leukemia and lymphoma in occupationally exposed workers (IARC,

1.3.1 Rodent Carcinogen

1.3.1.1 Acute Studies

One of the earlier studies was performed by Carpenter in 1944. Here, he examined the lethality of BD. Acute exposure data in humans was limited, however, men exposed for 6-8 hours at 8,000 ppm presented no effects. Shugaev performed experiments in laboratory animals and humans in 1968 and 1969. Humans exposed to 10,000 ppm BD for 5 minutes presented effects that ranged from irritation and dryness of the upper respiratory tract to increased heart rate without affecting blood pressure of respiration (Shugaev, 1968). In rabbits exposed to 250,000 ppm BD via inhalation death was noted after 30 minutes. At almost half the exposure concentration used on rabbits, (122,000 and 129,000 ppm), BD caused 50% lethality (LC50) in rats and mice exposed for 4 and 2 hours respectively (Shugaev, 1969). Acute exposures in the literature are observed to drop from being performed above 10,000 ppm, to become below this concentration.

Several experiments followed these acute exposures, particularly in rodents, and these studies were important in identifying organ targets of toxicity. However, the historical acute exposure data indicated that dose-response assessment for inhalation exposures needed to be considered, placing particular importance on long term exposure at even lower levels. Most of these acute experiments did not provide evidence that the observed differences were attributable
to species differences in the metabolism of BD. Therefore subsequent studies focused on the evaluation of chronic BD-exposures at lower levels.

1.3.1.2 Chronic Studies

NTP and EPA presented three different long-term exposure BD inhalation rodent studies that have served as proof that BD is a rodent carcinogen (NTP, 1984, Melnick, 1990, NTP, 1993, U.S. EPA, 1985, Owen, 1990). Levels of BD exposure via inhalation in B6C3F1 mice studies include the following concentrations (1st study): 62.5ppm, 625ppm and 1,250ppm (NTP, 1984), and (2nd study): 6.25ppm, 20ppm, 200ppm and 625ppm (NTP, 1993). F344 rats have only one study to which exposure was at high concentrations (3rd study) at 1,000ppm and 8,000pppm (U.S. EPA, 1985). The first study with BD-exposed mice had to be terminated early due to observed tumor formations at higher exposure levels (625ppm and 1250ppm). Accounting for survival adjusted tumor rates of neoplasms, results indicated that the organ with the higher tumor rates was the lungs. However, when comparing lung to liver tumor rate, there was a significant difference in those BD levels of exposure that induced tumors. The lowest levels that presented increased tumors rates of neoplasms at more than one site were between BD exposures of 6.25ppm for female mice and 20ppm for male mice. Particularly, female mice had tumors in lung and livers at both 6.25ppm and 20ppm respectively, whereas, in male mice, the same tumors were formed but at 62.5 ppm and 200ppm. Overall, this represents a 10-fold sex-related difference amongst tumor formation on the same organs for the same species. The only other parallel 2 year long-term study in rodent exposure tested BD via inhalation in rats but did not consider those lower range levels of exposure as those performed for mice. In rats, the increase in tumor formation was only present when exposed to BD at 1,000 ppm. The first general observation from these 2 year rodent bioassays for carcinogenicity was that BD demonstrated to
be a tumorigen of weak influence in rats and strong influence in mice. [Figure 1.2]

**1.3.2 Human Carcinogen**

BD classification as a carcinogen is extensively documented by epidemiologic studies of workers in the BD rubber industry exposed to both the BD monomer (BMD) or co-exposed to styrene (styrene butadiene rubber; SBR). The following discussion summarizes those key findings as well as any limitations in studies that need to be considered when interpreting the data.

The first study that considered BD co-exposure was performed in Texas (Meinhardt 1982). Two SBR plants A and B, measured exposure levels at 1.2ppm and 13.5ppm respectively. The ratio of mortality amongst workers to the average US value (standardized mortality ratios or SMR) was the highest for Lymphosarcoma (1.7 SMR) but the mortality rate from all cancers was not elevated. Because there were two leukemia deaths reported from employees of these plants, BD and mortality from lymphohematopoietic cancers (LHC) became a highlight then. Results, however, showed that LHC was only elevated for plant A, which had the lowest BD exposure. In addition, the period from which these workers were employed until they developed Leukemia (3-4 years) was too short to be solely attributed to BD exposure.

In 1987, Downs et al. recognized that in previous studies, those workers from SBR had multisite cancer excess, but it was still inconclusive if BD was the etiologic culprit; giving rise to the first BMD specific cohort. Here, cause-specific mortality was examined across 2,586 male workers employed for at least six months between 1943 and 1979. Findings suggested that BD was associated but not solely responsible for the increases in the SMRs, for Lymphosarcoma and Reticulum cell sarcoma (SMR 2.4). One limitation merits mentioning however, subjects were divided into routine, non-routine and low-exposure groups, the SMRs were inconsistent when
compared amongst each other. The authors couldn’t conclude that BD exposure was directly responsible for this increase in deaths (Downs, 1987).

Divine and Hartman examined the same BMD facility that now employed 2,800 male workers, again for a minimum of 6 months in an aging cohort that extended from 1943 until 1996, with a final follow up in 2001. A total of three analysis of this cohort (Divine, 1990, Divine, 1993 and Divine & Hartman, 1996) showed no increase in most cancers (SMR 0.9). The most striking difference was a significant elevation for deaths from LHC’s (SMR 1.5) due to an increase in deaths from Lymphosarcoma (SMR 1.9), Leukemia (SMR 1.1) and Non-Hodgkin’s Lymphoma (SMR 1.7). The last and fourth study published by the same authors in 2001 included an additional five-year follow-up after the plant closed due to its sale in 1996 (Divine & Hartman, 2001). Similar results were found confirming no increase in most cancers (SMR 0.9). A similar observation found an increase in deaths from LHC (SMR 1.4), Leukemia (SMR 1.3) and Non-Hodgkin’s Lymphoma (SMR 1.5). In this final follow-up they concluded that for the totality of this cohort, LHC were elevated specifically in short-term workers employed before 1950 (Divine & Hartman, 2001). These findings coincide with observations from all three previous cohorts where both SMRs for Leukemia and Lymphosarcomas were specifically found to be higher in workers that were employed for less than ten years when compared to those that were employed for more than ten years for Leukemia (1.6 fold and 3.3-fold respectively). Despite this, the overall Relative Risks (RR) for LHC were 1.0, representing no increase in risk with increased BD exposure.

Epidemiological data on BD co-exposure presented a robust dataset with 12,110 employees from 8 plants (7 in North America and 1 in Canada) that included subjects from both SBR and BMD industries with one or more year of employment. Significant observations for this
cohort are here reviewed using a total of four publications: Matanoski & Schwartz, 1987, Matanoski, 1989, Matanoski, 1990, and Santos-Burgoa, 1992. It’s important to note most exposure data was obtained from personal monitors. However, some of this exposure data had no values and was therefore estimated from previous exposures obtained from representatives of the plant, making some of this exposures relative and not actual.

Matanoski found that mortality rates for all cancers (SMR 0.8) and LHCs (SMR 1.0) were not significantly elevated. Duration of employment and or exposure category subdivision by production process did not affect mortality rates. However, when workers were subdivided by race, there was a distinct 4.9-fold difference in mortality for leukemia for blacks (SMR 6.6) when compared to whites (SMR 1.3). Both Matanoski and Santos-Burgoa then proceeded to incorporate the use of a substantial control group from all plants to match them to those exposed workers using specific variables. Santos-Burgoa identified risks depending on the intensity of the exposure (ranks 0-10) and the duration of employment in an exposed job. It’s noteworthy to consider that the cases and controls were not perfectly matched on duration of employment, but the periods were similar. The excess risk of Leukemia was associated with BD, even when correcting for styrene exposure. Using odds ratio (OR) to compare controls with cancer development, they found that the highest correlation between exposure and disease for Leukemia was BD (OR 7.6) with a 2.6-fold higher OR than styrene (OR 2.9). Styrene exposure was associated with Lymphosarcoma and Myeloma, whereas BD exposure was associated with Leukemia and Hodgkin’s disease.

Delzell and colleagues compiled data from a total of 15,649 workers who were studied in 1995 and followed up in 1996 in facilities located both in US and Canada (Delzell, 1995, Delzell, 1996). To date, this study ranks as the second most comprehensive dataset of
occupationally exposed workers to SBR that extended from 1943 to 1992. The cohort includes data from previously discussed facilities and from Meindhart, Matanoski, and Santos-Burgoa et al. The limitations of the study were incomplete identification of workers who terminated their employment before 1950, and lack of medical records that could confirm if the death that was certified due to leukemia and/or other lymphopoietic cancers. Mortality rates for all death causes and cancers had SMR’s similar to controls irrespective of race (whites or blacks), hourly/salary status, duration of employment and/or period since hire. However, when focusing on certain parameters of the cohort groups, they found a strong overall positive relationship between employment in these SBR plants and leukemia mortality (SMR 1.3). The subgroup with most significantly elevated SMR’s were hourly workers with long duration of employment (10 years worked and age 20+ years at the time of hire). Lymphosarcoma was elevated in white males who died after 1985 (SMR 2.8) whereas other LHC’s were prominent in blacks (SMR 1.4). When looking at Leukemia only, the subgroup of hourly black workers had mortality ratios (SMR 2.3) 1.8-fold greater than that of whites (SMR 1.3). The overall association for Leukemia was even more underlined when they worked for 10+ years and had 20 years or more since hire as hourly workers (SMR 2.2) and it was here where there was a 2.3 fold difference amongst races (SMR’s whites 1.9 and blacks 4.4). The largest Leukemia excess was primarily found in those deaths after 1985 (SMR 1.9), in subjects that were 65 years old or younger (SMR 1.8), and in subjects that were hired between 1950-1959 (SMR 2.0). Moreover, the greatest increase in Leukemia mortality was identified in the group of workers that were 20-29 years since hire and that worked for 10+ years (SMR 3.2). This study presented that the strongest association between BD exposure and Leukemia was in hourly subjects with long duration of employment and with 20+ years when hired, specifically for subjects in process groups of polymerization (SMR 2.5),
coagulation (SMR 2.5), labor (SMR 2.2), maintenance labor (SMR 2.7) and laboratory (SMR 4.3) jobs.

In 2015 Sathiakumar presented the latest and most updated cohort with 11 years of mortality data through 2009 that now includes an update that now includes 16,411 BD exposed workers that worked for at least one year from 1944 (Sathiakumar, 2015). Results indicated a positive exposure-response relationship between BD cumulative exposure and Leukemia. Using Cox regression analyses, 10 models were employed, and for all, the exposure-response trend for BD-ppm-years and Leukemia was not only positive but also statistically significant. Also, models adjusted only for age had a better fit than models that adjusted for age, year of birth, race and plant. When closely looking at the subset group by year of hire, only subjects that were employed before 1960 had both positive and statistically significant values in the exposure-response trend between BD-ppm-years and leukemia. Within the same group, all models also identified Non-Hodgkin’s Lymphoma to be positively associated with no statistical significance and no association with Multiple Myeloma, however Lymphoma/Lymphosarcoma was not evaluated.

Despite the number of epidemiological exposure studies, the one that presents the most consistent data to date, with the largest population of exposed workers reviewed and longer terms of BD exposure, was the latter: University of Alabama at Birmingham cohort. When summarizing all cohort findings, we agree on the following consistent observations. BD-exposed workers present a deficit in overall cancer mortality. In most of the cohorts that analyzed exposures before 2000, there was an excess of LHC’s mortality when compared with the average population in long term exposed workers. Length of employment and the era in which the workers were employed had a strong influence in LHC’s as an outcome, particularly for exposed
workers hired at ages that ranged between 20-29 years old that worked around 1950-1960 and died after 1985. This suggests that the approximate time for the induction of LHC’s upon exposure to BD via monomer or both exposed the BD-styrene is ~35-40 years.

The observation of increased risk for Leukemia cannot exclude styrene as a confounding factor, since SBR plant employees are exposed to both BD and styrene. In this respect, we must consider that although the models that have been applied to those cohorts with workers employed in SBR industries presenting a high risk of Leukemia, the exposure to the BD and styrene in SBR facilities is not mutually exclusive and the combination does play an influential role in the increase of risk for this category. Moreover, we want to reiterate that Lymphoma/Lymphosarcoma risk in BDM industries is an exclusive hallmark when looking at exposure to the BD monomer.

1.3.3 Exposure Limits

All previously discussed epidemiological studies that have analyzed the risk of BD-exposure in humans as a carcinogen have served as means to shape industrial limits to which BD-workers can perform their tasks in these plants. In the publication of Sathiakumar et al. in 2015 they state that the historical time-weighted averages (TWA) were around 10 ppm in the 1940s to the early 1970s and declined to about 2 ppm in the 1980s. Currently, there are four different set exposure levels for BD-workers and three organizations that regulate them. Levels established by OSHA include the Permissible Exposure Limit (PEL) and 15 minute Short-Term Exposure Limit (STEL) which are 1ppm and 5ppm respectively. The other two organizations are the National Institute for Occupational Safety and Health (NIOSH) and American Conference of Governmental and Industrial Hygienist (ACGIH). NIOSH recommends an exposure limit for an 8 hour shift (TWA) of 1ppm. ACGIH has set a Threshold Limit Value (TLV), or a maximum
1.4 Butadiene Biomarkers

Occupational exposure limits and regulations for BD have been shaped by results obtained from historical-occupational cohorts. However, one of the main data gaps that remain unresolved is the accuracy of retrospective estimation of occupational exposure levels for BD-workers. Alternatively, biomarkers have proven to be a useful approach not only to document exposure-effect relationships but also to internally estimate actual BD-exposure and address this gap.

First, let’s define biomarkers as an objectively measured characteristic on a subject (animal or human), as means to evaluate a process or response upon a specific stimulus. A thorough review on biomarkers as a risk assessment tool in dose-response relationships was presented by Swenberg and colleagues in 2008. They delineated two main types of biomarkers for risk assessment: (a) Biomarkers of Exposure and (b) Biomarkers of Effect. Here, we will review studies and their key findings from the standpoint of those BD biomarkers of exposure, how they relate to biomarkers of effect, how they have improved our understanding on dose-response relationships, and ultimately how they have allowed us to estimate the “internal-dose” in BD-exposed workers. For practical purposes, the first focus will be placed on Biomarkers of Exposure including: urinary metabolites and enzymes, and both DNA adducts and hemoglobin adducts. The second focus will be directed towards Biomarkers of Effect including: DNA Damage response pathways.
1.4.1 Butadiene Biomarkers of Exposure

1.4.1.1 Butadiene Urine Metabolites and Metabolic Enzymes

BD is metabolized in the human body into its reactive intermediates (EB, EB-diol and DEB) and later detoxified via oxidation by CYP’s or aldehyde dehydrogenase (AHD), conjugation by glutathione-s-transferase (GST) or hydrolysis via EH. The final result is creation of four different BD mercapturic acids (MAs) that are excreted in urine. Initial formation of the MAs starts with EB conjugation by GST and excretion as 2-(N-acetyl-L-cystein-S-yl)-1-hydroxybut-3-ene and 1-(N-acetyl-L-cystein-S-yl)-2-hydroxybut-3-ene (MHBMA or M2). The second MA arises from EB hydrolysis by EH into BD-diol that can be oxidized into hydroxymethylvinylketone (HMVK) via ADH. Subsequently, HMVK is conjugated by GST and excreted as 4-(N-acetyl-L-cystein-S-yl)-1,2-dihydroxybutane (DHBMA or M1). The third MA springs from the further oxidation of EB into DEB via CYP oxidation and later GST conjugation of DEB into 1,4-bis-(N-acetyl-L-cystein-S-yl)butane-2,3-diol (bis-BDMA or M3). BD-diol oxidation by CYP or DEB hydrolysis by EH both generate EB-diol, which ultimately is conjugated by GST into 4-(N-acetyl-L-cystein-S-yl)-1,2,3-trihydroxy-butane (THBMA). Of all four MA monitored, M1 and M2 have been the most predominantly discussed in the literature across species in earlier studies, and have contributed to our understanding of those underlining similarities and differences in the metabolism of all 3 specific BD-epoxides.

The most predominant BD specific biomarker in urine for occupationally exposed workers is M1. This is consistent with findings of the higher activity of EH activity in human liver when compared to that of rodents. The two most commonly used formulas to evaluate BD detoxification are the relative activity of hydrolysis defined as the ratio of MA = M1/(M1 + M2) and/or the relative activity of conjugation of MA = M2/(M1 + M2).
One of the earlier studies of urinary biomarkers across species was in 1993 by Bechtold, who analyzed two BD-specific MA; M1 and M2. He incorporated Csanady’s kinetic constants for EH and GST and calculated an enzymatic ratio rate constant [EH/GST] for comparison with the relative activity of hydrolysis [M1/(M1 + M2)]. First, it’s very important to recognize that the original rate constant of the hepatic clearance of EB via EH hydrolysis as reported by Csanady in 1992 goes from higher to lower for Humans > Rats > Mice whereas conjugation of EB via GST follows the order Rats > Mice > Humans (Csanady, 1992). A higher enzymatic ratio rate constant suggests that EB is hydrolyzed into BD-diol in the liver, and conversely, a smaller value indicates a predominant conjugation of EB by GST. The difference in this enzymatic ratio rate is 45-fold in Humans vs Mice, 23-fold in Humans vs Rats and 2-fold in Rats vs Mice. Csanady with Bechtold integrated results, found a higher the enzymatic ratio rate, the greater the proportion of M1 with respect with M1 + M2. Across species, the order from higher to lower ratio of MA was Humans > Rats > Mice. The differences in relative activity of hydrolysis was 4.9-fold in Humans vs Mice, 1.9-fold in Humans vs Rats and 2.6-fold in Rats vs Mice.

Two hallmark studies have been performed in occupationally exposed workers in 2001 (Albertini I) with all male subjects (Albertini, 2001), and 2007 (Albertini II) for both male and females subjects employed at a facility in the Czech Republic (Albertini, 2007). In the first study, Albertini and colleagues determined MA concentrations in urine for 86 male occupationally exposed workers. Air exposures in 8-hour shifts were approximately 0.290 ppm and 0.812 ppm in BDM and SBR facilities respectively; with a control group with background concentrations of 0.010 ppm.

Net concentrations of BD-specific MA after the work shift resulted in an M1 40-fold higher than M2 in BDM workers. In SBR workers, M1 was 32-fold higher than M2. On average,
M1 was ~36 times more abundant than M2 in exposed workers to BD, independent of exposure to the monomer or polymer [Table 1.1]. They also estimated BD detoxification as the relative activity of conjugation = M2/(M1 + M2). Mean values for measurements performed after work resulted in BD exposure stimulated conjugation. As BD exposure increased, so did the relative activity of conjugation. However, the authors underlined that conjugation accounts for approximately 1-2% of the urinary excretion for both MA. In addition, all workers were genotyped for a homozygous null GST phenotype (GSTT1). Workers with this phenotype had a reduced production of the M2, reflecting a lower ratio activity of conjugation when compared to those with GST positive phenotype.

In the second study (Albertini II) published in 2007, they incorporated some changes to the original study. They re-tested males from Albertini I and included 18 females (for a total of 104 subjects), double-blinded all samples, and monitored exposure as an average of ten 8-hour shifts per subject over the course of 4 months. BD-air exposure levels were 0.004 ppm and 0.003 ppm for control females and males, and 0.180 ppm 0.367 ppm for exposed females and males respectively. When examining the average MA, females excreted lower levels than males for both M1 and M2. After-work BD-specific MA’s resulted in an M1 22.2-fold higher than M2 for all workers. Individually, females exhibited a difference of M1 being 26.5-fold higher than M2 whereas in males this difference was 17.8-fold higher. Estimated BD detoxification, defined as the relative activity of conjugation = M2/(M1 + M2) versus hydrolysis was also calculated. Mean values for measurements performed after-work resulted in BD exposure also stimulating overall conjugation in females and males when compared to that of controls. The relative activity of conjugation was specifically increased by 1.68 fold with BD exposure in all subjects; specifically 1.54 fold for females and 1.79 fold for males [Table 1.2]. This ratio was maintained considering
the units of BD to which they were exposed, suggesting sex-related differences in metabolism but not in the pathway, which once again, reflected that hydrolysis is favored over conjugation. In his studies, Albertini has clearly shown the overall dominance of hydrolytic detoxification of BD epoxides via EH with a distribution of 99% in Humans > 51% in Rats > 23% in Mice. Additional endpoints for biomarkers of effect were measured for both Albertini studies (Albertini, 2001, Albertini, 2007). These additional endpoints were sister-chromatid exchange frequencies, chromosomal aberrations and hprt mutations. None of these endpoints demonstrated a significant difference for either study.

Some studies in occupationally exposed workers have focused on analyzing these key enzymes and their genetic sensitivity. For instance, in 2001 Abdel-Rahman and colleagues studied 49 non-smoking workers exposed to > 0.15 ppm or < 0.15 ppm in two SBR plants in Texas (Abdel-Rahman, 2001). All subjects were genotyped for polymorphic variants in both GST (GSTM1 and GSTT1), and microsomal epoxy hydrolase (mEH) and tested for genetic sensitivity as measured by hprt Vf. A particular polymorphic variant in mEH is Tyr113His, where tyrosine has been substituted by histidine at residue 113. This polymorphism in mEH has been shown to reduce its enzymatic activity by 40%, making this variant an attractive marker for human sensitivity. Overall results indicated that exposed workers in the high exposure group > 0.15 ppm BD had a 2-fold increase in the mutation frequency when compared to those that were in the low exposure group < 0.15 ppm BD. Moreover, mEH played a significant role in the sensitivity of BD exposure to genetic damage using the HPRT gene mutation assay. Those individuals exposed to > 0.15 ppm, and with at least one His allele (His/Tyr or His/His), had 3-fold higher mutation frequencies (Vf) than individuals with a normal phenotype for mEH. When combining both GST and mEH polymorphisms, they found similar results in the high exposure
group > 0.15 ppm BD. The level of genetic damage was highest in individuals with one mEH His allele and either GSTM1 and/or GSTT1 null genotypes. Perhaps this implies that exposure levels of BD > 0.015ppm represent a significant turning point in occupational settings particularly for individuals with sensitive enzymatic polymorphic variants, such as mEH, that can ultimately lead to genetic sensitivity.

The latest publication of BD-MA was released in 2015 by Kotapati and colleagues. A total of 72 workers employed at a BDM and SBR production facility near Prague, Czech Republic were analyzed for all four BD-MA in their urine (Kotapati, 2015). This study included both females and males in both controls (BD at 0.0136 ppm) and exposed (BD at 0.023-0.680 ppm) workers. Results confirmed that BD metabolism in humans is predominantly via the hydrolytic pathway. Concentrations of MHBMA (M2), DHBMA (M1) and THBMA were determined in after-work urine samples. The order of predominance of MA metabolites in all groups, for both sexes was DHMBA > THBMA > MHBMA. When looking at all MA; MHBMA, DHBMA and THBMA levels were greater in both male and female exposed workers compared to controls. Only. Females formed less MHBMA per unit dose of BD than males, but this sex-specific difference was not significant for DHBMA or THBMA. As a reminder, MHBMA is formed by EB conjugation via GST, whereas DHBMA corresponds to EB hydrolysis via EH. Using the metabolic ratios as determined by the formula \[ \frac{\text{MHBMA}}{\text{MHBMA} + \text{DHBMA} + \text{THBMA}} \], the authors concluded that in humans, less than ~ 3% of EB is detoxified by conjugation via GST, and ~ 97% of EB formed by hydrolysis by EH to EB-diol. In addition, HPRT mutation frequencies (Vf) and BD-hemoglobin adducts from Albertini II were evaluated for correlations with BD-MA, but no statistical association was found. In contrast, they
did find that all three BD-MA were also strongly associated BD-hemoglobin adducts with some limitations.

We therefore summarize all the previous data with the statement that humans metabolically favor the hydrolysis of EB and later oxidation into the EB-diol and to a minor extent oxidation into DEB. Results in BD-MA and BD-metabolic enzymes on all previously presented studies support this statement. M1 is the most predominant biomarker of exposure in urine for all species and polymorphic variants in both BD-metabolic enzymes (mEH and GST) play an important role in the genetic sensitivity of occupationally exposed subjects. Despite its conjugation being the minor pathways in humans, BD has been shown to stimulate the relative activity of conjugation in these exposed subjects. Moreover, it has been shown that females metabolize less BD than males per unit of exposure (Vacek, 2010). Other biomarkers, such as BD-hemoglobin adducts have been included in these studies and will be discussed in the next section.

1.4.1.2 Butadiene Hemoglobin Adducts: pyr-Val, HB-Val, and THB-Val

Each BD-epoxide induces a distinctive alkylation at the N-terminal Valine of Hb upon exposure (Moll 1999) generating three adducts: N,N-(2,3 dihydroxy-1,4 butadiyl)-valine (pyr-Val); N-(2-hydroxy-3-butenyl)-valine (HB-Val); and N (2,3,4 trihydroxybutyl)-valine (THB-Val) (Swenberg, 2000) [Figure 1.3]. The most abundant adduct across species is THB-Val, followed by HB-Val and pyr-Val. The THB-Val adducts differ from the other two adducts in that they are also formed endogenously from reactions with sugars erythrose and threose (Ryderberg, 1996). HB-Val and pyr-Val have no endogenous sources, but other exogenous sources have been implicated to contribute in their formation. Occupationally exposed workers employed in SBR
facilities have increased HB-Val adducts due to co-exposure to styrene (Albertini, 2001). Smoking status has notably shown to contribute in the formation of both pyr-Val and THB-Val adducts.

Vacek and others reported as evidence when measuring THB-Val adducts amongst BD exposed workers. Occupational levels presented a marked difference when considering smoking habits in the group. Smokers, had 502 pmol/g globin versus 179 pmol/g globin in non-smokers (Vacek, 2010). The significant difference reported on smokers, presents that smoking is a cofounding factor in the generation of THB-Val adducts. However, the difference was independent from sex, reinforcing that metabolic activation of BD, from sources such as cigarette smoking, is not a driving factor that comes into play when looking for sex specific differences.

All three BD-Hb adducts serve as surrogate markers of BD exposure, and they have been used to identify underlying differences in metabolism/metabolic activation through the estimation of internal dose in blood (IBD) (Fred 2008, Swenberg, 2011). Different from DNA adducts, Hb adducts are attractive endpoints due to their abundance in blood, non invasive access sampling, chemical stability and persistence due to lack of repair under biological conditions (Albertini 2001, Ospina 2005, Tornqvist 1986, Boysen 2004). These adduct levels are useful in molecular dosimetry comparisons and correlation analysis with other biomarkers (Booth 2004) such as DNA adducts and induced Mutation Frequency (MF) (Georgieva 2010, Swenberg 2011) and when comparing the formation of electrophilic intermediates across species in blood, by using the Internal Blood Dose (IBD) (Swenberg 2011, Fred 2008). Perhaps the major advantage of the measurement of Hb adducts is that they represent the cumulative exposure from 0 to 45, 60 and 120 days for mice, rat and humans.
The structure of hemoglobin is composed of two alpha chains (141 aminoacids) and two beta chains (146 aminoacids). Several residues of these sequences have been positively identified as locations for Hb adduct formation when incubated with BD. They include Histidine, Lysine, Serine, Methionine and Valine (Moll, 2000). Distinctively, the gold standard for the measurement of Hb adducts has favored the use of residue 1, the N-terminal valine (NTV). The NTV is conserved across species and present in the same position in both alpha and beta chains. Therefore, this common residue which is modified as a result of BD exposure is targeted in the process of developing methods to isolate and quantify BD-hemoglobin adducts in blood samples.

1.4.1.3 Quantitation of Butadiene-Hemoglobin Adducts

1.4.1.3.1 Early Studies

One of the earlier studies in BD-Hb adducts was performed by Sun in 1989 (Sun, 1989). Here, male B6C3F1 mice and Sprague-Dawley rats were injected intraperitoneally with 1, 10, 100, or 1,000 μmol of radiolabeled $^{14}$C-BD and sacrificed 24 hours later. All BD-Hb adducts were measured by liquid scintillation counting of radioactivity. Results showed a linearity with increasing doses up to 100 μmol per kilogram body weight for rodents. Adducts were formed more efficiently by rats than mice (expressed as pmol of $^{14}$C-adducts/mg globin)/(pmol of retained $^{14}$C-BD/kg body weight). For the first time, they determined the lifetime of BD-Hb adducts to be 24 days for mice and 65 days for rats. They also concluded that BD-Hb adducts were formed from metabolites of BD rather than the parent compound. However, they weren’t able to distinguish what proportion of those adducts corresponded to which BD-specific metabolite. Due to the use of radioactive $^{14}$C-BD for exposures, this assay couldn’t be used in the future to evaluate BD-Hb adducts from human occupationally exposed samples.
In 1991, Osterman-Golkar exposed Wistar rats to BD via inhalation at: 0, 250, 500 and 1000 ppm (6 h/day, 5 days/week, 2 weeks) (Osterman-Golkar, 1991). Samples of blood were pooled from 8 rats per exposure level. These authors used different analytical techniques for each individual endpoint. HPLC for urine BD-MA and GC-MS for BD-Hb adducts. Both endpoints were found to linearly increase in proportion to exposure concentrations. When correlating rats BD-Hb adducts to other endpoints, such as the induced SCE frequency it presented to be at its highest at 250 ppm. However, between 250 ppm-1000 ppm where BD-Hb’s continued to linearly increase, SCE’s decreased. Overall, they found that the current HPLC method used wasn’t sensitive enough for the quantitation BD-MA at human occupational levels of exposure, since these adducts are considerably lower than that of rats exposed at higher levels. In addition, they found two stereo-isomeric derivatives were produced in the analysis HB-Val adducts. Authors suggested that in vivo formation of other reactive intermediates needed to be considered, such as DEB, due it being a more effective mutagen.

Two years later, in 1993, the same authors proceeded to specifically analyze HB-Val which is formed by the reaction of EB with Hb. Only male rodents (B6C3F1 mice and Sprague-Dawley rats) were exposed to BD via inhalation (0, 2, 10 and 100 ppm for 6 h/day, 5 days/week, 4 weeks) (Osterman-Golkar, 1993). HB-Val increased linearly with BD concentration in mice, whereas with rats there was a deviation from linearity. Specifically, for concentrations < 100 ppm, HB-Val presented no significant difference between rodents however at the 100 ppm level, HB-Val adducts were 4-fold greater in mice > than rats. In a preliminary assessment of HB-Val in occupationally exposed (~1 ppm BD) non-smokers HB-Val ranged between 1-3 pmol/g of globin with smokers having increased HB-Val levels. The authors concluded for the first time
that HB-Val was simultaneously detected across species, with humans having the lowest levels when compared to rodents.

The same authors subsequently included additional samples to the previously presented human BD-Hb levels in 1993 (Osterman-Golkar, 1993). Subjects were non-smoking workers at a SBR plant in the US. TWA 8-hr area samples averaged a BD exposure level of 3.5 ppm, however, most samples contained < 1 ppm and thus the authors agreed that average BD exposures were < 3.5 ppm. HB-Val was found in exposed workers at adduct levels that ranged between 1.1-2.6 pmol/g globin in BD production areas. Controls from non-production areas, and external controls exposed to ~ 0.03 ppm BD were evaluated for HB-Val levels, however, samples were below the detection limit of 0.5 pmol/g globin. Authors suggested that additional research was needed to define the relationship between BD exposure and BD-Hb levels.

Albrecht and colleagues in 1993 analyzed HB-Val, but this time, in female rodents (Albrecht, 1993). Both CB6F1 mice and Wistar rats were exposed to BD (0, 50, 200, 500 or 1300 ppm, 6h/d, 5d) via inhalation. BD-Hb adducts were analyzed by derivatization of the N-terminal valine via modified Edman degradation procedure. HB-Val presented to be 5-fold higher in mice than in rats (17 and 3.5 nmol/g globin) at 500 ppm. Adducts increased linearly with BD exposure in rats, however in mice there was a decrease in formation at 500ppm. At higher levels, there was still an increase in HB-Val, but at a slower rate (less steep slope). The authors concluded that this sharp decrease in the formation of HB-Val adducts could explain in part why in female mice has a greater susceptibility to tumor formation when exposed to BD.

Authors Van Sittert & Van Vliet, and Richardson measured HB-Val in control and BD-exposed workers at a plant that produces BD by a naptha cracking process in a chemical plant in the Netherlands (Van Sittert & Van Vliet, 1994, Richardson, 1996). The method employed the
quantitation of derivatized N-terminal valine using the Edman degradation and later analysis by GC-MS. The 1994 study determined by air samples that BD-exposures had a TWA 8h of < 1 ppm. HB-Val was detected in both smokers and nonsmoking subjects. In the BD-exposed group, levels of HB-Val for both smokers and non-smokers had an average of less than < 2 pmol/g hemoglobin. Similarly, controls had averages of HB-Val with less than < 4 pmol/g hemoglobin in non-smokers and < 3 pmol/g hemoglobin in smokers. There was no apparent increase in HB-Val adducts with increase BD-exposure. Considering that the detection limit for HB-Val in this study was between 1-8 pmol/g hemoglobin, the authors concluded that the method was not sensitive enough for the detection of HB-Val adducts at BD-exposures of < 1 ppm. For the Richardson 1996 study, they incorporated the use of in-vitro multispecies erythrocytes as well as in-vivo exposures for rodents (B6C3F1 mice and Sprague-Dawley rats). In-vitro rat, mice and human washed erythrocytes were treated with EB (0.7 mg/mL for 2h). Rodents were exposed to EB at different ranges via intraperitoneal injection (0, 10, 20, 40 and 60mg/kg of body weight for 6h). They encountered numerous difficulties during their experiments that included: lack of an appropriate/corresponding internal standard, an LOD of 10-20 pmol/g hemoglobin in vitro, and low adduct levels with a high background signals for in-vivo samples. Authors didn’t discuss the data any further due to these innumerable limitations (Richardson, 1996).

In 1997, we started seeing more progress with studies being performed to measure 2 out of the 3 BD-Hb adducts (Perez, 1997). Here, they measured both HB-Val and THB-Val across species via derivatization of the N-terminal valine using Edman degradation and later analysis via GC-MS/MS. Male Sprague Dawley rats were directly exposed to all BD-epoxides [EB (78.3 mg/kg), DEB (16.7 and 33.4 mg/kg) and EB-diol (30 and 60 mg/kg)] via intraperitoneal injection. Male Wistar rats were exposed to BD via inhalation (0, 50, 200, or 500 ppm, 6 h/day
for 5 days). A few BD occupationally exposed workers (~ 1 ppm) from a Portuguese petrochemical plant and controls were also examined (n = 4; two exposed and two controls). HB-Val and THB-Val adducts were also quantified to determine the Hb binding index (HBI) and this numerical value indicated that in rats, EB-diol has an HBI of 10, whereas in DEB is 8. Authors explained that the closeness in the value of the HBI demonstrates that both EB-diol and DEB are similarly capable in alkylating nucleophilic sites in vivo. For BD-Hb adducts, THB-Val was not linearly related to exposed rats. The highest formed adduct was THB-Val followed by HB-Val in both rats and humans. Authors concluded that now in addition to HB-Val, THB-Val was also a useful biomarker of BD, specifically for BD-exposure for humans due to its abundance at low levels of exposure.

In 1998, Osterman-Golkar and others measured HB-Val adducts in rodents, estimated adduct stability in vivo and the corresponding EB concentration in blood using the area under the concentration curve (AUC) (Osterman-Golkar, 1998). Here, male rodents (B6C3F1 mice and Sprague Dawley rats) were exposed to low BD concentrations (0, 2, 10, or 100 ppm of 1,3-butadiene, 6 h/day, 5 days/week for 1, 2, 3, or 4 weeks). HB-Val adducts were found to be chemically stable in vivo and were detected as early as one week of exposure for all BD concentrations (following the turnover of red blood cells). Also, HB-Val adducts increased linearly with BD exposure in mice within these ranges. In rats, the same pattern followed up to 10 ppm, but from 10 to 100 ppm the increase of HB-Val was a lesser rate, indicated by a shallower slope. Comparing both species at 100 ppm BD, HB-Val adducts were 4-fold greater in mice than rats, but this difference was less evident at lower levels of exposure. Estimated blood concentrations of EB from HB-Val were similar to those previously published in rodents exposed to BD at 62.5 ppm. Authors successfully showed that HB-Val adducts were useful in the
prediction of blood concentration of EB in exposed rodents, despite their low levels at the ranges of BD-exposure that were considered.

Begemann presented the analysis of THB-Val adducts in 2001 for humans occupationally exposed to low levels of BD from two sites: subjects of a BDM plant in Italy, and diesel-exposed subjects from a Potassium Mine (PoM) in Germany (Begemann, 2001a). THB-Val adducts were analyzed by derivatization of the N-terminal valine via modified Edman degradation with post procedure acetylation, and final quantitation of derivatives by GC-MS. BD air exposures were measured by personal sampling after one 8h shift [average of 0.014 ppm BD (31ug/m3)] for BDM workers. Diesel-particulate measurements were available for the PoM workers. THB-Val in BDM subgroups from highest to lowest in polymerization (44.8 pmol/g globin) > co-polymerization (33.0 pmol/g globin) > and controls (35.3 pmol/g globin). Since THB-Val is known to be endogenously formed, as well as through low levels of BD-exposure, the authors couldn’t show a dose-response correlation or distinction between sub-groups. For the PoM workers in Germany, average THB-Val levels (43.5 pmol/g globin) were similar to those found in BDM polymerization. Here, THB-Val increase was associated with increasing diesel particulate exposure. Different from BDM-workers, PoM-workers had both smokers and non-smokers. Smoking status showed a positive contribution to these THB-Val adducts (42.3 pmol/g globin), when compared to non-smokers (35.1 pmol/g globin). The authors concluded that smoking was an important confounding variable in the formation of THB-Val adducts.

The same year, Begemann et al. presented a second publication for another BD-exposed worker population in a BDM plant in the Czech Republic following a similar procedure with similar results (Begemann, 2001b). This time, they analyzed HB-Val instead of THB-Val. BD-workers were exposed to 0.200 ppm BD, whereas controls had background concentrations of BD
at 0.0027 ppm. Significant sample clean-up was developed in the method, until they finally achieved an LOD of 0.2 pmol/g globin prior to its analysis via GC/MS. Average HB-Val adducts were 0.7 pmol/g globin for BD-exposed, whereas controls had 0.2 pmol/g globin. Smoking status was found to have significantly higher levels of HB-Val in control group, indicating the contribution of this lifestyle factor to BD exposure.

1.4.1.3.2 Recent Studies

In the last 15 years, several methods have been developed and employed for the detection and quantitation of BD-NTV Hb adducts (Fred 2004, Boysen 2004, Georgieva 2007, Georgieva 2010, Stednick 2010). Initially, detection of pyr-Val, HB-Val and THB-Val from both Hb-chains (H-α and H-β) were attempted by employing the N-alkyl Edman method with subsequent analysis via gas chromatography-mass spectrometry (GC-MS and GC-MS/MS). Although this approach worked for specific BD-adducts HB-Val and THB-Val, it presents limitations. First, this method cannot distinguish if those adducts are from the α-Hb or β-Hb chains, second it is unsuitable for pyr-Val, and third it had limited sensitivity on samples at low levels of BD exposure (Törnqvist 1986, Booth 2004, Powley 2005). The N-terminal pyr-Val adduct cannot be detected by the Edman degradation because it blocks coupling of phenylisothiocyanate and the subsequent cleavage required to generate the phenylthiohydantoin derivates used in the quantitation step (Törnqvist 1986). Also, earlier work performed in our laboratory using the N-alkyl Edman method, resulted in poor recovery of THB-Val analyte and internal standard (Powley 2005).

These particular challenges led to method improvement. Alternatively, tryptic digestion of Hb and an LC-MS/MS method incorporating the use of N-terminal modified pyr-Val-[\textsuperscript{3}H\textsubscript{8}] heptapeptide standards (Fred, 2004a) were used for quantitation of pyr-Val adducts, while the
use of the N-alkyl Edman method was employed for both HB-Val and THB-Val adducts (Fred, 2004b). Using two methods for the detection of all three adducts is labor intensive and costly because each sample has to be processed twice (once for pyr-Val adducts and separately for HB-Val and THB-Val adducts). Also, most of these in-vivo samples from rodents, as well as the human samples are limited (valuable and irreplaceable). It became apparent that further method development was needed in order to improve the following: (1) reduce the amount of globin used per sample; (2) reduce the cost of materials for sample processing plus cost of analysis; (3) reduce the sample processing time; (4) increase the sensitivity for more accurate quantitation in both animal and human samples at low levels of exposures; and (5) uniformly process samples for all adducts and all species simultaneously using a single method.

1.5 Single Immunoaffinity Enrichment Method

Georgieva and Boysen with collaborators from our group generated a novel alternative technique that incorporated the use of N-terminal Valine (NTV) modified (1-11) pyr-Val [\(^2\)H\(_3\)] and (1-11) HB-Val-[\(^{13}\)C\(_5\) \(^{15}\)N] peptide standards for rat, mouse and human α Hb chain sequences. The method opened the possibility of not only analyzing the pyr-Val adduct but also HB-Val at low levels of exposure. In brief, rabbit polyclonal antibodies recognizing these modified peptides were generated and the globin tryptic digest was enriched via immunoaffinity (IA) and coupled to analysis by nano-UPLC-ESI+ MS/MS (Nanoflow Ultra Performance Liquid Chromatography Positive Electrospray Ionization Tandem Mass Spectrometry). This method proved to be successful for pyr-Val and HB-Val quantitation and estimation of multispecies DEB and EB internal epoxide formation (Boysen, 2004, Georgieva, 2007, Georgieva, 2010, Boysen, 2012). IA enrichment is more attractive alternative to just tryptic digest analysis because it reduces the complexity of the mixture down to the fraction of interest. The initial amount of globin required
for the analysis is species and exposure dependent ranging from 10-50mg and has an LOD/LOQ of 1 & 4 fmol injected on column (nanoLC). These single antibody IA columns have proven to be a suitable method for the measurement of both pyr-Val and HB-Val using N-terminal modified (1-11) peptide standards.

Recently the method has been adjusted in our laboratory to perform simultaneous adduct enrichment and analysis. These improvements significantly reduce the use of laboratory supplies, labor time, amount of globin required as well as the number of samples to be analyzed and overall sample processing expenses over previously existing methods. Another advantage of using IA enrichment was that it enabled quantitation of pyr-Val which has been unsuitable for N-alkyl Edman method. Results for single IA columns have been published for both pyr-Val and HB-Val but not for THB-Val using this method (Boysen, 2004, Georgieva, 2007, Georgieva, 2010). In order to complete the evolution from single IA enrichment (just pyr-Val or just HB-Val) to simultaneous IA enrichment, it required the generation of unique alpha N-terminal modified (1-11) peptide standards for THB-Val. These standards were readily available for pyr-Val and HB-Val peptides, but not for THB-Val.

1.6 Simultaneous Immunoaffinity Enrichment Method

Initial production of N-terminal Val (1-11) THB-Val peptides of alpha hemoglobin was developed and optimized by our group using the human sequence. This synthetic peptide was used to first, optimize the synthesis direct alkylation conditions, and second to generate antibodies for later use in the construction of IA enrichment columns. In addition, the human sequence required substitution of a Cysteine at the C terminus, instead of the normal Lysine at

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1 Human 1-11 sequence (VLSPADKTNVC)
the 11th position, to enhance antibody conjugation. THB-Val peptide standards were not only necessary for polyclonal antibody generation and construction of IA columns, but also needed for calculation of IA columns recovery, instrument tuning and optimization, method validation, and evaluation of calibration curves and LOD’s. The overall goal was to integrate the THB-Val peptide standards into the IA method to facilitate the future analysis of all three BD adducts (pyr-Val and HB-Val, and THB-Val) simultaneously, across our comprehensive sample sets of mice, rat and human species. The N-terminal modified (1-11) THB-Val peptide standards were critical for the simultaneous quantitation of pyr-Val, HB-Val and THB-Val adducts across species under a simultaneous IA enrichment method.

1.6.1 THB-Val Peptide Standards

The generation of THB-Val peptide standards entailed direct alkylation of species specific NTV (1-11) peptide sequences for: rat\(^2\), mouse\(^3\) and human\(^4\) and their corresponding \([^{15}\text{N}\,^{13}\text{C}_5\text{Val}]\) stable isotope labeled peptide sequences by reaction with EB-diol. THB-Val peptide standards were purified by separation of the mono-alkylated fraction of the reaction mixture by HPLC. Characterization of NTV alkylation was confirmed through the analysis of b and y peptide fragment ions generated by collision induced dissociation (MS/MS) (Roepstorff and Fohlman, 1984). Each peptide standard was quantified by performing acid hydrolysis followed by UPLC-MS/MS analysis for amino acid quantitation (as previously described by Bordeerat, 2009). After the combined IA columns were built, they underwent a process of validation, in which the THB-Val was incorporated with both pyr-Val and HB-Val, by using known concentrations of both analyte and internal peptide standards. Simultaneous IA

\(^2\) Rat 1-11 sequence (VLSADDKTNIK)
\(^3\) Mouse 1-11 sequence (VLSGEDKSNIK)
\(^4\) Human 1-11 sequence (VLSPADKTNVK)
enrichment was successfully validated, and adducts from mouse samples were quantitated, including samples at low levels of BD-exposure. In contrast, human samples proved difficult to measure.

1.6.2 Quantitative Analysis of N-terminal Valine Adducts in Mice Exposed to 1,3-Butadiene, Internal Blood Dose Formation, and EB-equivalency

Overall, THB-Val is the major adduct formed in Hb across species after BD exposure. The development and validation of this method provided additional tools on the role of the EB-diol epoxide formation and its contribution to mutagenesis and carcinogenesis across species. There was an existing gap in our comprehensive BD inhalation dataset for this particular adduct, under the same method for the subset of mice samples. Species dependent differences in the metabolic activation of BD into EB-diol can be better understood through the use of the specific hemoglobin adduct, THB-Val, as a surrogate marker of exposure.

Specific Aims 1 and 2 (Chapter 2 and 3) included the preparation of THB-Val peptide standards followed by a validation of the triple IA columns and completion of simultaneous quantitation of our samples by nano-UPLC MS/MS. It was important to develop a method sensitive enough that could simultaneously analyze all BD-Hb adducts, including now THB-Val from our comprehensive rodent data set exposed to BD at low levels of exposure. The specific focus of this work was to first generate multispecies NTV analyte and internal (1-11) THB-Val-$[^{13}\text{C}_5^{15}\text{N}]$ peptide standards in order to perform method validations and THB-Val adduct measurements in rodents; including now the mice dataset. Ultimately, multispecies comparisons, now incorporating these THB-Val adducts, allowed rats and mice species comparisons. These
values were transformed into the internal blood dose of EB-diol to determine the epoxide influence in the overall contribution to the EB-Equivalency.

1.7 Butadiene DNA Adducts

1.7.1 DNA Adducts Introduction

As reviewed in section 1.2; BD is metabolized mainly by Cytochrome P450 enzyme CYP2E1 into three epoxides; EB\textsuperscript{5}, DEB\textsuperscript{6} and EB-diol\textsuperscript{7}. DEB has shown to be the minor metabolite formed with the highest mutagenicity, whereas EB-diol is the mayor metabolite with the lowest mutagenicity. There is a 200-fold difference among BD-epoxides mutagenicity, with DEB being 50 times more mutagenic than EB, and 200 times more mutagenic than EB-diol.

All three butadiene epoxides (EB, DEB, EB-diol) have the ability to react DNA and form covalent adducts. These BD-DNA adducts can be subdivided into three categories: (1) DNA-monoadducts, (2) DPCs\textsuperscript{8} or (3) DNA-DNA crosslinks. In vitro, all BD-epoxides have demonstrated the capacity to alkylate all DNA bases. However, only two DNA bases have been found to be alkylated in vivo: with the most predominant being with Guanine (Gua), and to a lesser extent Adenine (Ade). Therefore, it is important to recognize BD-Gua adducts as relevant to study due to their higher abundance. In the literature, it has been reviewed that there are three key aspects of BD-DNA adducts: (i) the distinction of the N\textsuperscript{7} position of Gua as main site of alkylation from all DNA bases, (ii) EB-diol adducts are the multispecies predominant BD-DNA adducts in all tissues, (iii) mice are the species with the highest number of BD-DNA adducts formed, and (iv) liver is the target tissue for overall highest BD-DNA adduct formation.

\textsuperscript{5} 1,2-epoxybutene
\textsuperscript{6} 1,2:3,4-diepoxybutane
\textsuperscript{7} 3,4-epoxy-1,2-butanediol
\textsuperscript{8} DNA-protein crosslinks
1.7.2 Butadiene Epoxide Specific Guanine Adducts

1.7.2.1 Mono-adducts

The most metabolically abundant epoxide formed from BD is EB-diol, and consists of four stereoisomers (2R,3R; 2R,3S; 2S,3R; and 2S,3S) that react with Gua and form THB-Gua\(^9\). Followed in abundance is EB, which consists of two enantiomers (2S; 2R) that react with Gua generating HB-Gua\(^{10}\). Alternatively, some of these HB-Gua adducts can be transformed back into THB-Gua, if the remaining unbound portion becomes hydrolyzed. The third and least abundant epoxide, DEB, consists of three stereoisomers (2R,3R; 2S,3S and meso-S,R/R,S) that react with Gua and form monoadduct DEB-Gua.

1.7.2.2 DNA Crosslinks

There are two main BD-Gua monoadducts that are considered to be the precursors of those DNA-DNA crosslinks or DNA-Protein crosslinks: HB-Gua and DEB-Gua. The only difference between HB-Gua and DEB-Gua is the opposite side of the chemical that remains unbound. Once HB-Gua is formed, the unbound portion can become hydrolyzed and turn into more THB-Gua, or get oxidized and turn into DEB-Gua. Now, the DEB-Gua unbound epoxy-ring remains available to react and produce different crosslinks depending on the target: (i) if it reacts with another Gua at the 7\(^{th}\) position or an (ii) Ade at the 1\(^{st}\) position they become DNA-DNA crosslinks. If it reacts with (iii) a protein it becomes a DPC. The first DNA-DNA crosslink is N7Gua-N7Gua-BD\(^{11}\) adduct, whereas the second is the N7Gua-N1Ade-BD\(^{12}\) adduct. The latter is rearranged into N7Gua-N6Ade-BD (Goggin, 2009). In addition, it has been reported that the DEB-Gua unbound epoxy-ring target certain proteins, leading to DPC’s formation with:

\(^9\) N-7-(2,3,4-trihydroxybut-1-yl)guanine  
\(^{10}\) HB-Gua I & II: N-7-(2-hydroxy-3-buten-1-yl)guanine & N-7-(1-hydroxy-3-buten-2-yl)guanine  
\(^{11}\) 1,4-bis-(guan-7-yI)-2,3-butanediol  
\(^{12}\) 1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol
AGT\textsuperscript{13}, GADPH\textsuperscript{14}, Histone 3, and PARP-1\textsuperscript{15} (Loeber, 2006, Loecken, 2007 & 2009, Tretyakova, 2010).

\textbf{1.7.3 Integration of Hemoglobin and DNA Adducts}

Similar to BD-Hb adducts, BD-DNA adducts are considered biomarkers of exposure, are indicators of internal dose, and tissue distribution to ultimately provide insights on metabolic activation differences across species. When integrating molecular dosimetry results, BD-Hb adducts have shown sex-independent but multispecies dependent differences in metabolic epoxide formation: mice (~200 fold) > rat (~40 fold) > human (1 fold), and BD-epoxide abundance of EB-diol > EB > DEB. These metabolic species differences are parallel to the formation of BD-DNA adducts in rodents: EB-diol specific THB-Gua (10-20 fold) > EB specific HB-Gua ≈ followed by DEB specific crosslinks N7Gua-N7Gua-BD (~100 fold) > N7Gua-N6Ade-BD. However, striking evidence has shown gender differences are only highlighted in DNA crosslinks, mutations, and tumor formation. DNA cross-links, from DEB specific N7Gua-N7Gua BD crosslink, were higher in females (2-3 fold) than males in liver tissue of exposed mice at 625ppm. Furthermore, rodents exposed to BD, presented additional sex-specific differences where females had: higher mutagenicity (2.5-3 fold) at the HPRT gene, greater number of MFs (2.3 fold) in mice exposed to 1250 ppm, mice tumor formation (10 fold) in lung and livers at 6.25 and 20 ppm, and rats IBD from EB-diol (6 fold) 6 hour inhalation. The weight of evidence presented here suggest that the sex specific differences seen in DNA crosslinks, along with tumor formation and mutagenicity, is more attributable to sex-dependent DNA repair efficiency rather than metabolism itself as shown by our results from Hb adducts.

\textsuperscript{13} O\textsuperscript{6}-alkylguanine DNA alkyltransferase
\textsuperscript{14} glyceraldehyde 3-phosphate dehydrogenase
\textsuperscript{15} Poly [ADP-ribose] polymerase 1
1.8 Biomarkers of Effect

1.8.1 DNA Adducts and DNA Repair

DNA adducts are considered biomarkers of exposure, and DNA repair is considered a biomarker of effect, and both have been recognized as non-mutational manifestations for BD-carcinogenic MOA (Albertini, 2010). In the absence of DNA repair, or if DNA replication occurs before DNA adducts are repaired, point mutations, single and double stand breaks and deletions might occur, leading to mutations, and consequently tumor formations. Previously, the literature has presented how other biomarkers of effect such as mutagenesis, and gene expression, have provided information on translational animal and human carcinogenic potential for BD.

1.8.2 Damage Induced by DNA Adducts

In contrast to globin adducts, DNA adducts undergo DNA repair and loss from chemical depurination, so there are less consistent half-lives to model exposure. In addition, all BD DNA mono-adducts adducts have been classified as non-promutagenic due to their chemical instability and the fact that the N7 position has no participation in the Watson Crick base pairing (Boysen, 2009). These adducts are lost from DNA primarily by chemical depurination, with a half-life of \(\sim 4\) days. This results in the formation of AP-sites\(^{16}\), which if not repaired, can result in mutations. However, the 2R,3S isomer is 30-fold more cytotoxic and mutagenic than the other three forms of EB-diol (Meng, 2007). In the case of DNA-DNA crosslinks, it is important to recognize that there are differences in their half-lives. For example, the half-life of intrastrand crosslink N7Gua-BD is 35h while interstrand bis- N7Gua-BD is 147h. Interstrand cross-links are more toxic because they affect both DNA strands, preventing DNA transcription, replication, and repair. Still, these BD adducts can alter DNA bases by forming AP-sites due to their chemical

\(^{16}\) apurinic/apyrimidinic site or abasic site
instability and can either further be repaired, cause point mutations, or cause DNA strand breaks (Goggin, 2009). Recent investigations presented in this dissertation have integrated how DNA repair is modulated by BD-epoxides into the description of BD MOA.

1.8.3 DNA Damage Response Pathways and Butadiene DNA Adducts

All cells are equipped with DNA-repair components that are capable of reducing the number of mutations that might arise. These components can work in two ways. (1) Some will execute its function before replication, and identify unusual modifications to these DNA nucleotides by adducts, resulting in bypass, or repair before replication occurs. (2) Other components will operate after replication to survey newly synthesized DNA for mistakes, to make corrections of such errors. When a mutation arises, it indicates that repair has been deficient in the removal of the direct modification or the indirect damage caused by it. Therefore, DNA-repair is an orchestrated network of sensors, transducers, and effectors organized in interactive repair pathways that execute a response upon DNA damage. Several DNA repair pathways have evolved to guard cells against the DNA damage induced from endogenous sources and by alkylating agents such as BD-epoxides. These pathways include: direct repair, mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end-joining (NHEJ) and translesion synthesis (TLS). Moreover, the specific response of one or more of these will vary depending on the types of DNA adduct formed.

1.8.3.1 Base Excision Repair

In base excision repair (BER), the enzyme involved in removing adducted nucleobases from the sugar-phosphate back-bone is a glycosylase, leading to an AP-site (Robertson, 2009). The site is incised by an AP-endonuclease, and the single nucleotide gap is ultimately filled with a
new unmodified nucleotide by BER-specific DNA Pol β\textsuperscript{17}, successively the nick is sealed with ligase complex XRCC1\textsuperscript{18}/Ligase III. Alternatively, BER also repairs any single strand breaks. Here, PARP1\textsuperscript{19} senses the damage and gets activated via auto-poly-ADP-ribosylation\textsuperscript{20}, recruiting the ligase complex to close the gap.

### 1.8.3.2 Nucleotide Excision Repair

Nucleotide Excision Repair (NER), has two different ways to detect DNA damage: TC-NER\textsuperscript{21}, and GG-NER\textsuperscript{22}. In TC-NER, transcription is allowed to resume after it stalls due to a lesion. First, damage is sensed anywhere in the actively transcribed genome by stalled polymerase RNAP2\textsuperscript{23}, followed by its stabilization via interaction with intermediates CSB with subsequent recruitment of CSA\textsuperscript{24} to modify and reposition the stalled RNAP2. However, in GG-NER, the damage is recognized and discriminated via two intermediate complexes: (1) XPC\textsuperscript{25}/hHR23B\textsuperscript{26} and (2) UV-DDB\textsuperscript{27} (DDB1\textsuperscript{28} and DDB2\textsuperscript{29}/XPE\textsuperscript{30}). Both TC-NER and GG-NER pathways have their previously respective intermediates converge in the recruitment of NER helicase TFIIH\textsuperscript{31} along with endonuclease XPG\textsuperscript{32}. This bi-directional helicase (TFIIH) opens the damaged DNA segment of ~30 nucleotides, and the helix is stabilized by the assembly

\[\text{Polymerase} \beta\]
\[\text{X-Ray Repair Cross Complementing Protein 1}\]
\[\text{Poly [ADP-ribose] polymerase 1}\]
\[\text{Auto-poly-Adenosine diphosphate ribose ribosylation}\]
\[\text{Transcription Coupled Nucleotide Excision Repair}\]
\[\text{Global Genome Nucleotide Excision Repair}\]
\[\text{Ribonucleic Acid Polymerase II}\]
\[\text{Cockayne syndrome factors A and B}\]
\[\text{Xeroderma pigmentosum Complementation Group C}\]
\[\text{Human Homolog Rad23 B}\]
\[\text{Ultra-Violet Damage Deoxyribonucleic Acid Binding Protein B}\]
\[\text{DNA Damage Binding Protein Component 1}\]
\[\text{DNA Damage Binding Protein Component 2}\]
\[\text{Xeroderma pigmentosum Group E}\]
\[\text{Transcription Factor II Human}\]
\[\text{Xeroderma pigmentosum Group G}\]
of XPA\textsuperscript{33} and RPA\textsuperscript{34}, both of which also further orientate any of the two endonucleases that cuts the damaged strand (~30-50 nucleotides) with respect to the lesion. These endonucleases cut 5’ from the lesion by XPG and 3’ from the lesion by the ERCC1\textsuperscript{35}-XPF\textsuperscript{36} complex. The single strand gap is filled by DNA replication proteins (RFC\textsuperscript{37}, PCNA\textsuperscript{38}, RPA, and DNA polymerases δ, ε, or κ). The nicks are then sealed by DNA ligases I or III (Giglia-Mari, 2011).

1.8.3.3 Homologous Recombination

Homologous recombination (HR) requires a homologous sister chromatid to perform repair, and thus takes place when cells are in the S and G2 phases of the cell cycle. HR initiates repair of double strand breaks (DSB) via sensor PARP-1, which competitively binds against Ku70\textsuperscript{39} to damaged DNA, leading to the recruitment of the MRN\textsuperscript{40} complex (3 proteins: Nbs1\textsuperscript{41} brings MRE11\textsuperscript{42} and RAD50 into the nucleus). In turn, the MRN complex binds to broken strand ends and holds them, while leading to a secondary successive recruitment with nuclease CtIP\textsuperscript{43}. This generates complex MRN-CtIP that together with complex BRCA1/BARD1 initiate resection of un-even DSB ends. Resections are processed in tandem by now endo/exo-nuclease active* MRN-CtIP complex (*MRE11 in active contact with DNA). Two exonucleases: EXO1\textsuperscript{44}, and DNA2\textsuperscript{45}, along with helicase BLM\textsuperscript{46} extend the resection to several 1,000 base pairs. Double strands with 3’ ends are degraded towards the 5’ end, producing 3’ single strand tails (or

\begin{itemize}
    \item Xeroderma pigmentosum Group A
    \item Replication Protein A
    \item Excision Repair Cross Complementation Group 1
    \item Xeroderma pigmentosum Group F
    \item Replication Factor C
    \item Proliferating Cell Nuclear Antigen
    \item Protein Ku70 Recognizes free ends from DNA double breaks
    \item Mre11/Rad50/Nbs1
    \item Nijmegen Breakage Syndrome-1 Protein
    \item Meiotic Recombination 11 Protein
    \item C terminal interacting protein
    \item Exonuclease 1
    \item DNA2 nuclease
    \item Bloom Syndrome RecQ Like Helicase
\end{itemize}
overhangs). MRN complexes detach, and RPA proteins instantly coat these exposed 3’ single strand tail regions. BRCA1/BRCA2/PALB2 assist RAD51 in replacing RPA proteins from the 3’ single strand regions by forming RAD51 single stranded nucleofilaments. This step allows homologous search and invasion of the single stranded RAD51 coated portions with its homologous sequence from a sister chromatid. Strand exchange occurs, and complementary DNA is synthesized by polymerase using the sister chromatid as a template. Once the process is completed, the ends are sealed by a DNA ligase (Chen, 2017, De Lorenzo, 2013, Giglia-Mari, 2011).

### 1.8.3.4 Non-Homologous End-Joining

The Non-Homologous End-Joining (NHEJ) pathway trims and ligates un-even DNA DSB to keep helix integrity. Different from HR, NHEJ: (1) does not require a sister chromatid as a template, (2) is more prone to generate downstream replication errors, and (3) it operates at different phases of the cell cycle. NHEJ initiates DSB repair after the break is recognized by the Ku70/Ku80 complex, which binds to DSB ends, recruiting intermediate DNA-PKcs. In turn, DNA bound DNA-PKcs undergoes both auto-phosphorylation and dissociates from the DSB ends. The ends are now free for binding, and complex XRCC4/Ligase IV (known as XL) ligates the ends. However, if the ends have been damaged by 3’ phosphate or 5’ hydroxyl groups, XRCC4 proceeds to recruit PNKP to repair the ends. DNA-PKcs further phosphorylates other downstream factors (nuclease Artemis, PNKP and DNA polymerases). The Artemis nuclease then recognizes and cleaves any overhanging DNA tails. The ends are then aligned by XRCC4/XLF into a protein-DNA filament that allows ligation. The final step is carried by other DNA polymerases (Pol μ and Pol λ) to process the ends (Chu, 2014, Goodarzi et. al., 2006).

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47 DNA-dependent protein kinase catalytic subunit
48 Polynucleotide kinase/phosphatase
1.8.3.5 Mismatch Repair

Mismatch repair (MMR) is a post-replicative pathway that corrects mismatched base-base nucleotides, or insertion/deletion loops (IDLs). Replication errors by synthesis or proof-reading executed via low-fidelity polymerases can lead to DNA mismatches. The most abundant mismatch binding factor is heterodimer MSH2\textsuperscript{49}/MSH6\textsuperscript{50} (or MutSα), and it repairs base-base mismatches and 1-3 IDLs. Larger sequences of IDLs (2-10) are repaired by heterodimer MSH2/MSH3\textsuperscript{51} (or MutSβ). In absence of MSH2, MMR becomes completely impaired. Therefore, the initial proteins implicated in MMR will vary depending on the type of damage. In addition, in MMR, protein-protein and DNA protein interactions are modulated by ATP\textsuperscript{52}/ADP\textsuperscript{53}. First, damage is recognized: (1) for mismatched bases, helix distortion is recognized via MutSα complex. (2) Alternatively, recognition of IDLs are via MutSβ complex. MutS (α or β) complex binds where DNA has been damaged and recruit their corresponding MutL (α or β) as well as MutH (MLH1\textsuperscript{54}/PMS2\textsuperscript{55}). This generates a ternary complex (MutS/MutL/MutH). In base-base-MMR, the ternary complex is (1) [MutSα/MutLα/yPMS1], and for IDL-MMR the ternary complex is (2) [MutSβ/MutLβ/hPMS1]. The PMS2 protein present on the ternary complex has endonuclease activity that’s stimulated by ATP/ADP, leading to the introduction on nicks in the discontinuous strand of DNA. A secondary recruitment takes place where nuclease EXO1 allows the mismatch containing strand to be cleaved in a 5′ to 3′ direction. The PCNA clamp is then loaded with polymerase Pol δ at the 3′ terminus. Both PCNA and Pol δ fill the gap finalized by sealing the nick via DNA ligase I (Hsieh, 2008, Jiricny, 2006).

\textsuperscript{49} MutS Homolog 2
\textsuperscript{50} MutS Homolog 6
\textsuperscript{51} MutS Homolog 3
\textsuperscript{52} Adenosine triphosphate
\textsuperscript{53} Adenosine diphosphate
\textsuperscript{54} MutL Homolog 1
\textsuperscript{55} Mismatch Repair Endonuclease PMS2
1.8.3.6 Translesion Synthesis (bypass)

Translesion synthesis is not a repair pathway but a damage tolerance pathway. TLS can take place during replication or post replication. For example, during replication, a high-fidelity polymerase may encounter DNA damage and stalls at the replication fork. The now exposed single stranded DNA gets coated by protein RPA. This stimulates ubiquitin ligase-RAD18 (along with enzyme RAD6) to form complex RAD18/RAD6, and this complex monoubiquitylates the PCNA polymerase clamp. PCNA monoubiquitylation enhances its interaction with other translesion polymerases. This step allows the replicative polymerase to be replaced by another translesion polymerase. Depending on the specific damage, any of these TLS polymerases are then recruited to resume replication. This allows the damage to be tolerated via lesion bypass. TLS polymerases belong to the y-family polymerases and they could insert complementary correct or incorrect bases. The TLS replication extends for a short tract and then replaced by the original replicative polymerase.

1.8.4 DNA Damage Response and Butadiene Epoxides

One of the main gaps of BD-carcinogenesis MOA that remains unanswered is what specific DNA repair pathways are involved in the repair of each BD-epoxide. Upon BD-induced DNA damage, we hypothesize that several lesions can arise. Let’s consider first that the most prevalent lesions from N7Gua monoadducts (HB-GuaI, HB-GuaII, and THB-Gua) will be chemically unstable and thus spontaneously depurinate, leaving an abasic site.

Taking all this information into account the identity of each one of these DNA lesions will ultimately influence a corresponding biological response. We have a clear understanding of what specific DNA-epoxide lesions are formed, but as for what DNA damage response is summoned upon the damage caused by each individual epoxide remains unclear. To gain
knowledge on BD-induced DNA repair, we proposed as the third Aim of this project (Chapter 4) to identify those relevant DNA repair pathways involved in the response of DNA damage induced by BD-epoxides. Ultimately, the assessment and elucidation of DDR (DNA damage response) by BD-epoxides will provide valuable information on DNA repair as a non-mutational event to the BD-carcinogenic MOA. The determination of BD epoxides DDR pathway can be assessed by using the DT40/XTT DNA damage response system. This high throughput multi-well plate approach employs the use of normal and DNA repair and cell cycle pathway-deficient mutant DT40 chicken lymphocytes. These cells are treated with chemicals such as BD-epoxides and later stained with XTT dye to quantitatively determine the LD50 by absorbance with an automatic absorbance well plate reader. Survival data is statistically analyzed to compare wild type and mutant DT40 cells, identifying those pathways involved in BD-epoxide specific DNA repair. To better understand the effects on the genotoxicity of BD-epoxides, we screened several DNA-repair-deficient DT40 mutants [Table 1.3] and compared results with the parental DT40 cell line. The genotoxicity profiles were calculated as the relative LD50. This calculation requires the LD50 of the parental DT40 vs the mutant DT40 LD50. Ultimately, results from this assay will provide information of what potential mechanisms of repair might be involved by BD-epoxides.

1.8.4.1 EB and EB-diol Epoxides Potentially Repaired by Base Excision Repair

BER could be the DNA Damage Response (DDR) pathway to repair EB and EB-diol (and its isomers) epoxide damage using their BD-monoadducts (both HB-Gua and THB-Gua) as reference. Since these N7 adducts have half-lives of ~4 days, both N7 adducts and resulting AP-
sites from spontaneous depurination will arise. The BER specific protein MPG\textsuperscript{56}, flips the damaged base out of the double helix and excises the N7 monoadducts leaving an AP-site. AP-sites generated upon N\textsuperscript{7} monoadducts removal or their spontaneous depurination would be repaired by filling the gap via Pol β with a new unmodified Gua, and repair is finished by closing the gap with the ligase complex. Therefore, we hypothesize that the DDR induced from EB, and EB-diol (and its isomers) that repairs their corresponding HB-Gua, THB-Gua adducts, or its AP-sites lesions would require the involvement of the BER pathway.

1.8.4.2 DEB Epoxide Potentially Repaired by

Homologous Recombination or Non-Homologous

End-Joining

HR or NHEJ could be the DNA Damage Response (DDR) pathway to repair DEB epoxide damage using their crosslinks as reference. Therefore, we hypothesize that the DDR induced from DEB that repairs their corresponding crosslinks, AP-sites or double strand breaks would require the involvement of the HR and or NHEJ pathway.

1.8.5 DNA Damage Response and DT40/XTT System

Dr. Jun Nakamura and colleagues at UNC-Chapel Hill, developed a novel assay employing a reverse genetic approach with chicken DT40 B-lymphocyte cell lines that are DNA-repair-deficient for numerous DDR pathways. The assay facilitates the process of narrowing specific DDR pathways involved when exposing them to an agonist (i.e.: BD). In addition to being specific for elucidating the mechanism of DDR that’s involved against the agonist, it provides sensitive quantitation per each of DDR pathways (Ridpath, 2007, Ridpath, 2011). Therefore, this method is an excellent tool to attain our goal; to elucidate those DDR pathways

\textsuperscript{56} Methyl purine glycosylase
involved in each specific BD-epoxides. The DT40/XTT assay will identify those critical DDR pathways summoned by each individual BD-epoxide

1.9  Research goal and specific aims

The overall goal of this project was to address dataset gaps in BD-epoxide metabolic activation and DNA repair and improve our understanding of BD’s MOA, and its relationship to BD-carcinogenesis. Our hypothesis was that through the development and combination of new and improved analytical methods, incorporation of comprehensive data sets, and in vitro techniques, we would identify multispecies similarities and differences in BD-metabolic activation. Also, the integration of DNA-damage response would serve as an endpoint to strengthen BD carcinogenic MOA and identify relevant epoxide-specific differences in repair. Results contributed in the improvement of scientific risk assessment, public health, and further employed as an application to other known multispecies carcinogens.

This dissertation, integrated and applied previously developed methods from our laboratory. Aim 1 was the generation, puriification, and quantitation of EB-diol epoxide-specific THB-Val peptide standards. In Chapter 2, methods are described for the direct alkylation, purification by LC/MS, characterization by MS/MS, and quantitation by UPLC/MS/MS-based amino acid analysis of BD specific α (1-11) NTV peptide standards containing the EB-diol-specific adduct THB-Val. Three analyte, and three internal THB-Val peptide standards were successfully prepared and used in several downstream analytical applications. THB-Val peptide standards were later integrated in the validation of a simultaneous IA-enrichment method used in the analysis and completion of a unique multispecies data-set exposed to BD. The second Aim was to further develop an improved method for simultaneous analysis of all three adducts (HB-Val,
pyr-Val, THB-Val) formed by butadiene epoxides at the NTV of Hb α chain in mice exposed to butadiene via inhalation.

This completed the quantitation of BD Hb adducts in our comprehensive rodent dataset. Chapter 3 integrates the use of species specific mice (1-11) peptide standards with a recently improved IA coupled UPLC-positive mode electrospray ionization-tandem mass spectrometry (UPLC-ESI\textsuperscript{+} -MS/MS) method for the simultaneous quantitative analysis of all three BD-Hb adducts from globin of mice exposed to BD. Tryptic digests of isolated globin cleaved the protein into peptides containing the BD-Hb adducts. The digests were processed via triple antibody IA enrichment. Samples were quantitated with nano-UPLC-MS/MS analysis using \textsuperscript{13}C\textsubscript{15}N\textsubscript{5}-labeled internal standard peptides. BD-Hb adducts results were integrated in the calculation of the IBD of BD-epoxides, and transformed into the EB-equivalency for further comparisons. The third Aim was to evaluate DNA damage response pathways induced by selected BD-epoxides. Chapter 4 incorporates the use of a high-throughput in vitro vertebrate cell assay. DT40 wild type cells, and an extensive collection of DNA repair mutant clones, were used to assess BD-epoxides DNA Damage Response pathways. Effects were expressed as the Relative LD50, using DT40 parental cells and DT40\textsuperscript{-/−} mutants. These mutants were deficient in seven DNA repair pathways and include mutants in cell cycle checkpoint, BER, NER, HR, NHEJ, MMR, TLS, DNA damage sensors and Helipses.
### Table [1.1]: Albertini et al 2001 (Albertini I): Analysis for urine metabolites by group in occupationally exposed workers to BD. (a) n = 34

<table>
<thead>
<tr>
<th>Net Work Group</th>
<th>n</th>
<th>BD (ppm)</th>
<th>M1 (ug/l)</th>
<th>SD</th>
<th>M2 (ug/l)</th>
<th>SD</th>
<th>Fold Difference (M1/M2)</th>
<th>Activity of Conjugation ( M2/(M1 + M2) )</th>
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<tbody>
<tr>
<td>Control</td>
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<td>0.290</td>
<td>−219</td>
<td>274</td>
<td>−0.50</td>
<td>1.22</td>
<td>4.4</td>
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<td>Monomer</td>
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<td>213</td>
<td>565</td>
<td>5.28</td>
<td>8.54</td>
<td>40.3</td>
<td>0.0115</td>
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<td>5868</td>
<td>84.26</td>
<td>225.95</td>
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\(^a\)
<table>
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<tr>
<th>After Work Group</th>
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<th>M1</th>
<th>SD</th>
<th>M2</th>
<th>SD</th>
<th>Fold Difference (M1/M2)</th>
<th>After-Work Ratio Activity of Conjugation M2/(M1 + M2)</th>
<th>After-Work Ratio Activity of Hydrolysis M1/(M1 + M2)</th>
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<tr>
<td></td>
<td>ppm (ug/l)</td>
<td>(ug/l)</td>
<td>±</td>
<td></td>
<td></td>
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<td><strong>Females</strong></td>
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<td>0.004</td>
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<td>8.3</td>
<td>10.1</td>
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<td>0.964 (0.98 fold) (^a)</td>
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<td>272.1</td>
<td>14.9</td>
<td>10.3</td>
<td>34.4</td>
<td>0.029</td>
<td>0.052 (1.79 fold) (^a)</td>
<td>0.947 (0.97 fold) (^a)</td>
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<tr>
<td><strong>Exposed</strong></td>
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<td>508.1</td>
<td>19.2</td>
<td>27.5</td>
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<td><strong>Males</strong></td>
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<td>0.027</td>
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<td>0.973</td>
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<td><strong>All Exposed</strong></td>
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Table [1.2]: Albertini et al 2007 (Albertini II): Urine metabolites by group in occupationally exposed workers to BD. (a) Fold differences calculated from [Ratio of Activity Exposed/Ratio of Activity Control]
<table>
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<tr>
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<td>Wild-Type</td>
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<td>Sensor</td>
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Table [1.3]: DT40 wt and DT40 mutant cells
1.11 FIGURES:

Figure [1.1]: Butadiene Metabolism and Fate
Figure [1.2]: Survival Adjusted Tumor Rates of Neoplasms in Female and Male B6C3F1 Mice Exposed to 1,3 Butadiene via Inhalation (2 years)
Figure [1.3]: Butadiene Hemoglobin Adducts; Metabolism and NTV Adducts
REFERENCES


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2.1 OVERVIEW

1,3-Butadiene (BD) generates three reactive epoxides that can form covalent bonds forming protein adducts at the N terminal Valine (NTV) of Hemoglobin (Hb). Of all three epoxides formed from BD, the most abundant across species is 1,2-epoxy-3,4-butenediol (EB-diol), and when bound to Hb it forms BD-Hb adduct N-(2,3,4-trihydroxybutyl)-valine (THB-Val). These adducts can be measured and used as biomarkers of exposure to estimate the internal dose of the epoxides that arose from BD exposure. Recent methods for the detection of all three Hb-adducts of BD were adjusted for simultaneous Immunoaffinity (IA) enrichment. Part of this development required the use of THB-Val analyte and internal peptide standards that were not previously available. Here we describe the methods for the direct alkylation, purification by LC/MS, characterization by MS/MS, and quantitation by UPLC/MS/MS-based amino acid analysis of BD specific α (1-11) NTV peptide standards containing the EB-diol-specific adduct THB-Val. Three analyte, and three internal THB-Val peptide standards were successfully prepared and used in several analytical applications. THB-Val peptide standards were later

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1 A portion of this Chapter previously appeared as part of a major article in Elsevier: Journal of Chemico-Biological Interactions. The original citation is as follows: J.A. Swenberg et al “1,3-Butadiene: Biomarkers and Application to Risk Assessment”, Chemico-Biological Interactions 192 (2011): 150-54.
integrated in the validation of a simultaneous IA-enrichment method used in the analysis and completion of a unique multispecies data-set exposed to BD.

2.2 INTRODUCTION

Exposure to Butadiene (BD) has been associated with multi-site carcinogenesis in rodents (Swenberg 2000, Booth 2004) and with an increased incidence of leukemia and lymphoma in humans in occupationally exposed workers (Ospina 2005). BD is relatively non-reactive, until absorbed, distributed and metabolically activated, leading to the generation of reactive epoxides that ultimately generate covalent binding with proteins, such as Hemoglobin (Hb) and DNA.

The first step of BD activation [Figure 2.1] is the oxidation of BD into the first monoepoxide, 1, 2-epoxy-3-butene (EB) by enzymes CYP2E1 and CYP2A6. The second step is further oxidation of EB, into the diepoxide: 1,2;3,4 diepoxybutene (DEB) by enzymes CYP2E1 or CYP3A4. Both EB and DEB can undergo hydrolysis and form Butene-diol (BD-diol) from EB or the formation of the second mono epoxide, 1,2-epoxy-3,4-butanediol (EB-diol) from DEB, by the enzyme epoxy hydrolase (EH). Ultimately BD-diol can be oxidized again into more EB-diol. Of all three epoxides, EB-diol is the most abundantly produced across species.

There is an inversely proportional relationship across BD-epoxides; whereas the most mutagenic epoxides are the least abundantly generated (DEB > EB > EB-diol) and the least mutagenic epoxides are most abundantly generated (EB-diol > EB > DEB) (Swenberg 2011, Meng 2010). The individual mutagenic potency of these BD-epoxides can vary up to 200 fold (Walker 2009, Meng 2007); going from most to least mutagenic in the following order DEB > EB > EB-diol (Walker 2009). When looking closer at gender and species; the mutagenic response to BD has shown to be higher in female mice > male mice > female rats > male rats.
(Meng 2007b). In part, this has presented as to why BD is a potent carcinogen for mice but weak in rats.

The epoxy terminus of EB, DEB and EB-diol, reacts with the N-terminal Valine (NTV) of Hb upon BD exposure (Moll 1999) forming three epoxide specific adducts. The most abundant epoxide formed across species upon BD activation is EB-diol and produces epoxide specific Hb-adduct (N-(2,3,4-trihydroxybutyl)-valine (THB-Val). Epoxide EB and DEB also bind to the NTV of Hb forming adducts N-(2-hydroxy-3-butenyl)valine (HB-Val) and N,N-(2,3-dihydroxy-1,4-butadiyl)valine (pyr-Val) respectively.

All three Hb-adducts can be measured as biomarkers of exposure, and are used to calculate the internal dose resulting from exposure to BD. Earlier reports have identified species differences in the formation of both endpoints; ultimately indicating BD has a species dependent metabolic activation. In the last 15 years, several methods were developed and employed for the measurement and quantitation of BD-NTV Hb adducts (Fred 2004a, Fred 2004b, Boysen 2004, Georgieva 2007, Georgieva 2010, Stednigk 2010). Initially, detection of pyr-Val, HB-Val and THB-Val from both Hb-chains (α-Hb and β-Hb) were attempted by employing the N-alkyl Edman method (Edman 1950) with subsequent analysis via gas chromatography-mass spectrometry (GC-MS and GC-MS/MS). Although this approach worked for specific BD-adducts HB-Val and THB-Val, it presents limitations. First, this method cannot distinguish if those adducts are from the α-Hb or β-Hb chains, second it is unsuitable for pyr-Val measurement, and third it had limited sensitivity on samples at low levels of BD exposure (Törnqvist 1986, Booth 2004, Powley 2005). Particularly, pyr-Val adduct detection was not possible because its cyclic structure blocks coupling and cleavage required for the N-alkyl Edman reaction reagents to generate the derivatives which are the products used in the
quantitation step (Törnqvist 1986). Also, earlier work performed in our laboratory using the N-alkyl Edman method, resulted in poor recovery of analyte and internal THB-Val standard (Powley 2005).

Earlier work from our laboratory presented a method that opened the possibility of analyzing both the pyr-Val and HB-Val adducts at low levels of exposure. The alternative technique incorporated the use of NTV modified (1-11) pyr-Val [$^2$H$_3$] and (1-11) HB-Val-$[^{13}$C$_5^{15}$N] peptide standards for rat, mice and human α Hb chain sequences. First, globin (10-50mg) undergoes tryptic digest and it’s later enriched via immunoaffinity (IA). Elutes obtained from IA undergo analysis of the fraction of interest using a nano-UPLC-ESI+ MS/MS. The method was sensitive enough to quantitate both pyr-Val and HB-Val adducts with a LOD/LOQ of 1 & 4 fmol on column. (Boysen 2004, Georgieva 2007, Georgieva 2010, Boysen, 2012). Results from these single antibody IA columns proved to be a suitable method for measuring both pyr-Val and HB-Val using N-terminal modified (1-11) peptide standards.

Recently, the method has been adjusted to perform simultaneous adduct enrichment and analysis by our laboratory. These improvements significantly reduce the use of laboratory supplies, labor time, amount of globin required as well as the number of samples to be analyzed and overall sample processing expenses over previously existing methods. Another advantage of using IA enrichment was that it was suitable for pyr-Val quantitation, overcoming the limitation of N-alkyl Edman method. Results for single IA columns have been published for both pyr-Val and HB-Val but not for THB-Val (Boysen, 2004, Georgieva, 2007, Georgieva, 2010). To complete the evolution from single IA enrichment (just pyr-Val or just HB-Val) to simultaneous IA enrichment, first required the generation of unique N-terminal modified α (1-11) THB-Val
peptide standards for mouse, rats and human. These were readily available for pyr-Val and HB-Val, but not for THB-Val.

2.3 METHODS

2.3.1 Materials:

All reagents and solvents were ACS grade or higher. NTV (1-11) peptides [Rat (VLSADDKTNIK & [15N13C5V]-LSADDKTNIK; MW 1203/1209), Mouse (VLSGEDKSNIK & [15N13C5V]-LSGEDKSNIK; MW 1189/1195) and Human (VLSPADKTNVK & [15N13C5V]-LSPADKTNVK; MW 1172/1178)] were purchased from Anaspec (Fremont, CA). Microspin filter tubes (regenerated cellulose, 0.2 um) were from Alltech Associates Inc. (Deerfield, IL). Jupiter Proteo 4μ, 90A, 250 x 4.60 mm Chromatographic Column was purchased from Phenomenex (Torrance, CA). The UPLC™ BEH SHIELD RP18 2.1 mm × 150 mm, 1.7 μm column was purchased from Waters Corp. (Milford, MA). EB-diol (MW 104) was synthesized and purified by the UNC Chapel Hill Chemistry Core in the Department of Environmental Sciences and Engineering.

2.3.2 Methods:

2.3.2.1 Hemoglobin a (1-11)-THB-Val peptide standards

2.3.2.1.1 Direct alkylation of peptide standards

Generation of THB-Val analyte and internal [13C515N] peptide standards were achieved by direct alkylation of the NTV with EB-diol as previously described (Fred 2004a and Fred 2004b) with modifications. All peptides were weighed and diluted in 18 Mega Ohm deionized water to a final concentration of 10mM. Small test reaction mixture solutions were mixed with
10mM analyte peptide at both 1:10 and 1:20 ratios peptide: EB-diol in 0.1M Ammonium Bicarbonate at pH 4, pH 6.8, and pH 8. Reaction mixtures were incubated at 37.5°C with shaking and monitored every 24 hours for three days (data not shown). The final optimized conditions for direct alkylation reaction were performed at 1:20 molar ratio of peptide: EB-diol in 0.1M Ammonium Bicarbonate buffer (pH 6.8) with shaking at 37.5°C for 72 hours [Figure 2.2]. Aliquots of diluted reaction mixtures were analyzed by direct (loop) injection in a Thermo Finnigan LCQ DECA Ion Trap Mass Analyzer (Thermo Fisher Scientific, Waltham, MA), to characterize reaction products by setting the MS to do a Full Scan (range 200-2000 m/z) in positive ion mode [Table 2.1]. Once monoalkylation was achieved, formic acid was added to reduce the pH of the reaction mixtures down to pH2. This destroyed any excess of EB-diol, preventing any further unwanted alkylation at other positions other than at the NTV. Reaction mixtures were kept at -20°C until purified.

2.3.2.1.2 Purification by LC-ESI+ MS/MS

Analyte and internal THB-Val peptide standards were purified from reaction mixtures by monitoring and separating the monoalkylated products via LC-ESI+ MS/MS. Five channels were monitored to observe ion Chromatograms for (1) the Full Scan Spectra, and the MS/MS of the (2) unalkylated, (3) monoalkylated, (4) dialkylated and (5) trialkylated reaction mixture products [Table 2.1]. Different mobile phases and gradients were tested until appropriate conditions were met. Reverse phase chromatographic separation of each reaction products was achieved by monitoring the monoalkylated fraction [(1-11 THB-Val)] by MS/MS m/z in each reaction mixtures, using a Jupiter 4u Proteo 90A 250 x 4.60 mm column in a DECA Ion Trap Turbo analyzer. Mobile phases were tested to monitor retention times for the (1-11) THB-Val peptide standards [Table 2.2]. Purification of peptide standards was completed once there was separation.
of the chromatographic peaks of the reaction products. By manually diverting the valve to waste, the corresponding (1-11) THB-Val peptide standards were collected into Eppendorf tubes. These purified fractions were concentrated by vacuum centrifugation in a Savant speed-vac (Thermo Scientific, Waltham, MA) and reconstituted with 500uL of 18 Mega Ohm deionized water. Micro-spin filters were pre-rinsed with water and purified fractions were filtered, followed by an additional rinse. The purified (1-11) THB-Val fractions were stored at -20°C until characterized.

2.3.2.1.3 Characterization by MS/MS (b and y fragments)

The THB-Val adducted peptides were characterized using MS/MS comparing the expected and obtained THB-Val specific b and y ion fragments.

Analyte and stable isotope labeled internal (1-11) THB-Val peptide standards were re-analyzed on column using the same chromatographic conditions as described above for isolation [Figure 2.3]. In addition, aliquots of 10-20uL were also assessed by loop injection to confirming collection of the monoalkylated fraction by Full Scan 100→1500 m/z [Table 2.1]. Both on column injections, and loop injections were used to perform an MS/MS fragmentation analysis (collision energy of 35) that provided the spectrum of THB-specific b fragments, confirming the specific alkylation at the NTV [Figure 2.5a]. Theoretical b and y fragments of each peptide standard were calculated using the free online proteomic tool MS-Product Peptide Sequence, from the Protein Prospector Database v 5.10.1 (University of California, San Francisco, CA), available online [Tables 2.3, 2.4 and 2.5]. Experimental b and y fragmentation patterns obtained from the MS/MS fragmentation analysis were compared to those theoretical fragments, and THB-specific fragments and NTV alkylation was confirmed [Figure 2.5b]. Un-quantitated
purified and characterized THB-Val peptide standards were prepared in aliquots and stored at -80°C until further analysis.

2.3.2.1.4 Quantitation of 1-11-THB-Val peptide standards by UPLC MS/MS

Peptide standards were acid hydrolyzed and analyzed by UPLC MS/MS to perform amino acid quantitation based on a procedure previously described (Bordeerat 2009). In brief, duplicate 50uL aliquots of each peptide standard was acid hydrolyzed with HCl for 4 hours at 150°C. Peptide standard samples were quantitated based on the concentration of at least 3 amino acids from the peptide standard sample sequence and using Norleucine as internal standard in both samples and calibration peptide standard amino acid mix [Figure 2.6a and 2.6b]. Samples were analyzed in SRM mode using a 2.1 mm × 150 mm UPLC™ BEH SHIELD RP18 1.7 μm column in a linear gradient from 20% methanol-0.1% formic acid to 80% methanol-0.1% formic acid for 7 minutes at a flow rate of 200 μL/min. Amino acids from the (1-11) peptide sequence were monitored using their individual MS/MS transitions [Table 2.6 and 2.7]. Peptide standard amino acids with the best reference factors were obtained and used for the quantization and the peptide final concentrations [Table 2.8]. Quantitated (1-11) THB-Val peptide standards were prepared in aliquots and stored at -80°C until used for validation, instrument tuning and sample analysis.

2.4 RESULTS

Here we present the methods for the direct alkylation, purification, characterization and quantitation of BD specific (1-11) NTV peptide standards containing the EB-diol-specific adduct
N-(2,3,4-trihydroxybutyl)-valine (THB-Val). Direct alkylation of α (1-11) peptide sequences by EB-diol, and the resulting mono-alkylated adducts (1-11 THB-Val) were purified by HPLC, characterized by LC-ESI+ MS and LC/MS/MS peptide fragmentation, and finally quantitated by UPLC/MS/MS-based amino acid quantitation after acid-hydrolysis.

2.4.1 Direct alkylation of Hb α (1-11) peptide sequences for (1-11)

**THB-Val peptide standards**

Generation of THB-Val analyte and internal \[^{13}\text{C}_5\ ^{15}\text{N}]\ peptide standards were achieved by direct alkylation of the NTV with EB-diol. The direct alkylation approach allowed us to identify three main direct alkylation reaction products in the reaction mixtures; unalkylated, monoalkylated [1-11-THB-Val], dialkylated and in some trialkylated. Details of reaction mixture products by species are illustrated in [Table 2.1]. At 72 hours we achieved a high proportion of monoalkylation in all peptides reaction mixtures. The most critical variable that affected reaction mixture products was pH. When reaction mixtures were above 6.8 pH, the indiscriminate alkylation of Lysines was favored. Since the goal was to alkylate the NTV, we had to monitor pH through the course of the incubation to ensure we avoided unwanted Lysine alkylations at the 7th and 11th position of the (1-11) peptide sequence. Optimal direct alkylation conditions were obtained at 1:20 molar ratio of peptide-EB-diol in 0.1M Ammonium Bicarbonate buffer (pH 6.8) incubated-shaking at 37.5°C for 72 hours.

2.4.2 Purification of (1-11) THB-Val by LC-ESI+ MS/MS

Analyte and internal (1-11)-THB-Val peptide standards were purified from reaction mixtures by monitoring and separating the monoalkylated products via LC-ESI+ MS/MS, and using a single reverse phase chromatographic column Jupiter 4u Proteo 90A 250 x 4.60 mm. Both rat and human (1-11) THB-Val peptide standards were successfully separated with similar
solvent conditions, and by adjusting the pH we were able to separate the human reaction mixture [Table 2.2]. We were not able to separate the mouse reaction mixture with the same solvents used as we did for the rat and human reaction mixtures. We overcame this obstacle by switching both solvents and only using TFA as a mobile phase modifier [Table 2.2].

2.4.3 Characterization of NTV Alkylation by MS/MS (b and y fragments)

The (1-11) THB-Val peptide standards were positively collected by Full Scan MS and confirmed that EB-diol alkylated the NTV by THB-specific b fragments using MS/MS. The most common THB-Val specific fragments were b10, and b9. Also, fragments y5 and y6 served as confirmation that the Lysines were not alkylated. Since all three species have Lysines in positions 7th and 11th, this was a critical step to ensure we directly alkylated and purified the correct (1-11)-THB-Val peptides standards. In addition, all (1-11) peptide sequences had Serine in the 3rd position, and their corresponding fragments b3-b10 and y10-y9 were also clear from EB-diol alkylation [Figure 2.5a and 2.5b]. Previous reports have indicated the alkylation of Lysine, Histidine, Serine, and Methionine residues in both α-Hb and β-Hb chains by EB and DEB (Moll 2000, Basile 2001, Miraglia 2002, Badghisi 2002).

2.4.4 Quantitation of 1-11-THB-Val peptide standards by UPLC MS/MS

Amino acid quantitation for all (1-11)-THB-Val peptide standards was obtained by using 3 or more amino acids as reference. All (1-11) THB-Val peptide standards shared 3 common reference amino acids were Threonine, Leucine and Aspartic Acid. The specific amino acids
used as reference in the peptide standard quantitation were as follows: (1) Proline, Threonine, Leucine and Aspartic Acid for Human-THB-Val; (2) Alanine, Threonine, Isoleucine, Leucine and Aspartic Acid for Rat-THB-Val; (3) and Alanine, Threonine, Isoleucine, Leucine and Aspartic Acid for Mice-THB-Val [Table 2.6].

2.5 CONCLUSIONS

2.5.1 THB-Val peptide standards

The combined methods here presented the direct alkylation of Hb α (1-11) NTV peptides, chromatographic purification and characterization by LC-ESI+ MS/MS prior to amino acid quantization by UPLC MS/MS. These combine methods successfully generated both analyte and internal peptide standards for Hb α (1-11) Human THB-Val, Rat THB-Val, and Mouse THB-Val are here described.

Optimal direct alkylation conditions were obtained at 1:20 molar ratio of peptide-EB-diol in 0.1M Ammonium Bicarbonate buffer (pH 6.8) incubated-shaking at 37.5°C for 72 hours. Direct alkylation presents a more straightforward peptide standards synthesis than the alkylation of single valine- followed by its derivatization as previously reported (Stedingk 2010). As an advantage, we separated monoalkylated products from their reaction mixtures by using a single reverse phase chromatographic column (Jupiter 4u Proteo 90A 250 x 4.60 mm). The only difference was the type of mobile phase solvents used. The (1-11) peptide sequences are very similar, between the Human and the Rat THB-Val peptide standards, sharing a 73% sequence homology. However, we speculate that differences in the purification conditions were different in the Mouse THB-Val peptide standard because it has a 64% sequence homology when compared to that of Human and Rat. All (1-11) THB-Val peptide standards shared 3 common
reference amino acids for their quantitation (Threonine, Leucine and Aspartic Acid). Final THB-Val peptide standards concentrations obtained through this method were sufficient to generate enough material for future method development and validation, instrument tuning and sample processing.

2.5.2 Incorporation and Application of peptide standards

Several downstream analytical applications incorporated the use of THB-Val peptide standards. These included: tryptic digest optimization and nano-UPLC-ESI+ MS/MS instrument tuning. Of most importance, our goal was to incorporate the use of these standards to test recovery in our combined antibody IA columns. We were able to validate these columns with (1-11) pyr-Val, (1-11) HB-Val and now including (1-11) THB-Val. Once validation of the columns was completed, we measured all three BD Hb-adducts and compared them across species from our comprehensive data set.

2.5.3 Achievements

Generating sensitive and accurate methods for the quantitation BD-Hb adducts provides the opportunity to determine the role on the formation of the EB, DEB and EB-diol reactive epoxides and their contribution to mutagenesis and carcinogenesis after exposure to BD. The goal of this report was to generate α (1-11) THB-Val peptide standards and employ them in the detection, quantitation, and comparison of THB-Val adducts across species. This initial step was required to complete our ultimate goal the detection and quantization of BD-specific Hb-adducts, including pyr-Val, HB-Val and THB-Val, using simultaneous IA enrichment coupled to nano-UPLC-ESI+ MS/MS (Chapter 3).
THB-Val continues to be the major adduct formed in Hb across species, and also serves as a surrogate marker of EB-diol formation in blood. Being the greatest contributor in the internal blood dose, compared to EB and DEB, EB-diol poses the greatest risk to humans (Swenberg, 2011). The group’s overall final goal was to improve our understanding in the role of BD reactive epoxides and their toxicity across species to ultimately determine which of the two rodent species was the most appropriate for human risk assessment.

2.6 ACKNOWLEDGEMENTS

I would like to thank Narisa Bordeerat for her training in both direct alkylation of peptide standards, and amino acid quantitation techniques.
### 2.7 TABLES:

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Table [2.1]: Observed EB-diol + (1-11) Peptide reaction mixture products for all three species and their m/z: (a) α (1-11) THB-Val analyte and (b) α (1-11) THB-Val-[^{13}C_5 ^{15}N] internal peptide standards with other reaction products in and their corresponding MW.
### Table [2.2]: (1-11) THB-Val Peptide Standards Chromatographic Purification Conditions

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Table [2.3]: Protein Prospector b and y fragments for Mouse α (1-11) peptide and Mouse α (1-11) THB-Val peptide standard.
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<td>8</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1057.5524</td>
<td>10</td>
<td>K</td>
<td>1</td>
<td>147.1128</td>
<td>10</td>
<td>I</td>
<td>2</td>
<td>260.1969</td>
<td></td>
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<td></td>
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</tr>
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</table>

Table [2.4]: Protein Prospector b and y fragments for Rat α (1-11) peptide and Rat α (1-11) THB-Val peptide standard.
<table>
<thead>
<tr>
<th>Fragments b</th>
<th>Position</th>
<th>AminoAcid</th>
<th>Position</th>
<th>Fragments y</th>
<th>Position</th>
<th>AminoAcid</th>
<th>Position</th>
<th>Fragments b</th>
<th>Position</th>
<th>AminoAcid</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>1</td>
<td>V</td>
<td>11</td>
<td>---</td>
<td>1</td>
<td>V</td>
<td>11</td>
<td>---</td>
<td>1</td>
<td>V</td>
<td>11</td>
</tr>
<tr>
<td>213.16</td>
<td>2</td>
<td>L</td>
<td>10</td>
<td>1072.60</td>
<td>2</td>
<td>L</td>
<td>10</td>
<td>1072.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300.19</td>
<td>3</td>
<td>S</td>
<td>9</td>
<td>959.52</td>
<td>3</td>
<td>S</td>
<td>9</td>
<td>959.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>397.24</td>
<td>4</td>
<td>P</td>
<td>8</td>
<td>872.48</td>
<td>4</td>
<td>P</td>
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<td>872.48</td>
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<td>A</td>
<td>7</td>
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<td>5</td>
<td>A</td>
<td>7</td>
<td>775.43</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>583.31</td>
<td>6</td>
<td>D</td>
<td>6</td>
<td>704.39</td>
<td>6</td>
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<td>589.37</td>
<td>7</td>
<td>K</td>
<td>5</td>
<td>589.37</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>812.45</td>
<td>8</td>
<td>T</td>
<td>4</td>
<td>461.27</td>
<td>8</td>
<td>T</td>
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<tr>
<td>1025.56</td>
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<td>246.18</td>
<td>10</td>
<td>V</td>
<td>2</td>
<td>246.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>11</td>
<td>K</td>
<td>1</td>
<td>147.11</td>
<td>---</td>
<td></td>
<td>1</td>
<td>147.11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table [2.5]: Protein Prospector b and y fragments for Human α (1-11) peptide and Human α (1-11) THB-Val peptide standard.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molecular Weight</th>
<th>M/Z</th>
<th>Human</th>
<th>Rat</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine (G)</td>
<td>76.1</td>
<td>30.1</td>
<td>x</td>
<td>x</td>
<td>xx*</td>
</tr>
<tr>
<td>Alanine (A)</td>
<td>90.1</td>
<td>44.1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Serine (S)</td>
<td>106.1</td>
<td>60.1</td>
<td>x</td>
<td>x</td>
<td>xx*</td>
</tr>
<tr>
<td>Proline (P)</td>
<td>116.1</td>
<td>70.1</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine (V)</td>
<td>118.1</td>
<td>72.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine (T)</td>
<td>120.1</td>
<td>74.1</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Isoleucine (I), Leucine (L) and Norleucine</td>
<td>132.1</td>
<td>86.1</td>
<td>(I) x</td>
<td>(I) x</td>
<td></td>
</tr>
<tr>
<td>Asparagine (N)</td>
<td>133.1</td>
<td>87.1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Aspartic Acid (D)</td>
<td>134.1</td>
<td>88.1</td>
<td>x</td>
<td>xx*</td>
<td>x</td>
</tr>
<tr>
<td>Lysine (K)</td>
<td>147.1</td>
<td>84.1</td>
<td>(Could be adducted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine (Q)</td>
<td>147.1</td>
<td>84.1</td>
<td>xx*</td>
<td>xx*</td>
<td>xx*</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>148.1</td>
<td>89.1</td>
<td>x</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table [2.6]: Amino acids Used for Peptide Standard Quantitation (as described in Bordeerat et al. 2009). The (x) marks selected amino acids for quantitation of the standard peptide. * Indicates that there are two of the same amino acids in the specific (1-11)
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Position from Valine</th>
<th>CID(^1) energy [eV]</th>
<th>MS/MS transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine (L)</td>
<td>2</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Proline (P)</td>
<td>4</td>
<td>10</td>
<td>116 → 70</td>
</tr>
<tr>
<td>Aspartic Acid (D)</td>
<td>6</td>
<td>10</td>
<td>134 → 88</td>
</tr>
<tr>
<td>Threonine (T)</td>
<td>8</td>
<td>10</td>
<td>120 → 74</td>
</tr>
<tr>
<td>Norleucine</td>
<td>Int. Standard</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine (A)</td>
<td>4</td>
<td>10</td>
<td>90 → 44</td>
</tr>
<tr>
<td>Threonine (T)</td>
<td>8</td>
<td>10</td>
<td>120 → 74</td>
</tr>
<tr>
<td>Isoleucine (I)</td>
<td>10</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Leucine (L)</td>
<td>2</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Aspartic Acid (D)</td>
<td>5 and 6</td>
<td>10</td>
<td>134 → 88</td>
</tr>
<tr>
<td>Norleucine</td>
<td>Int. Standard</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine (S)</td>
<td>3 and 8</td>
<td>10</td>
<td>106 → 60</td>
</tr>
<tr>
<td>Isoleucine (I)</td>
<td>10</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Leucine (L)</td>
<td>2</td>
<td>10</td>
<td>132 → 86</td>
</tr>
<tr>
<td>Aspartic Acid (D)</td>
<td>4</td>
<td>10</td>
<td>134 → 88</td>
</tr>
<tr>
<td>Glutamic Acid (E)</td>
<td>6</td>
<td>10</td>
<td>148 → 102</td>
</tr>
<tr>
<td>Norleucine</td>
<td>Int. Standard</td>
<td>10</td>
<td>132 → 86</td>
</tr>
</tbody>
</table>

Table [2.7]: UPLC-MS/MS Reference Table for CID energy [eV] and MS/MS transitions of selected amino acids used for peptide standard quantitation of Human, Mice, and Rat Hb α (1-11) THB-Val peptide standards using Norleucine as Internal Standard.

\(^1\) Collision Induced Dissociation
<table>
<thead>
<tr>
<th>Species</th>
<th>THB-Val Quantitation concentrations pmol/uL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF Amino acids</td>
</tr>
<tr>
<td>Human</td>
<td>P T L D</td>
</tr>
<tr>
<td>VLSPADKTNVK</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>S I L D E</td>
</tr>
<tr>
<td>VLSGEDKSNIK</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>A T I L D</td>
</tr>
<tr>
<td>VLSADDKTNIK</td>
<td></td>
</tr>
</tbody>
</table>

Table [2.8]: (1-11) THB-Val peptide standards Amino acids Reference Factors (RF) used for Quantitation and Final Measured Concentrations
2.8 FIGURES:

Figure [2.1]: BD metabolism and formation of N-terminal valine adducts
Figure [2.2]: Schematic representation of 1-11 THB-Val standard peptides. (1-11) NTV Peptides sequences were mixed with EB-diol in a 1:20 ratio. Optimal conditions for direct mono-alkylation of (1-11) NTV peptides by EB-diol were achieved in 0.1M Ammonium Bicarbonate buffer, pH 6.8, incubated at 37.5°C for 72 hours. Final products of mono-alkylation were identified as 1-11 THB-Val standard peptides: [Human (THB-Val-VLPADKTNVK; MW 1282/1288*), Mouse (THB-Val-VLSGEDKSNIK 1293/1299*), and Rat (THB-Val-VLSADDDKTNIK 1308/1314*).]

* = Labeled Valine in internal standard peptides. (Increases the MW +6).
Figure [2.3]: MS/MS of Purified (1-11) NTV Mouse labeled peptide from Reaction Mixture. Mouse (1-11) THB-Val-[\textsuperscript{13}C\textsubscript{5}\textsuperscript{15}N] labeled peptide standard (MW 1299.86) was purified by from unwanted reaction mixture products and collected. The Mouse internal peptide standard fraction was verified by LC-ESI+ MS/MS in a DECA Ion Trap Turbo analyzer.
Figure [2.4]: Full Scan Spectrum of (1-11) NTV Mouse labeled peptide by EB-diol in a 1:20 ratio. Two other reaction mixture products were identified with the Mouse (1-11) THB-Val-[13C, 15N] labeled peptide standard (MW 1299.86). Purification of the standard peptide was performed by chromatographic separation and collection of the peptide standard fraction by LC-ESI+ MS/MS in a DECA Ion Trap Turbo analyzer.
Figure [2.5]: Schemes of \(b\) and \(y\) fragments and Fragmentation Spectrum of Rat THB-Val Internal Standard: (a) General schematic representation of theoretical B and Y fragments of THB-Val peptide standards. (b) Full Scan MS/MS in positive ion mode of purified Rat (1-11) THB-Val-[\(^{13}\text{C}_{5}\text{N}_{5}\)] labeled peptide standard (MW 1314). The most commonly abundant ions were \(y_5\), \(y_6\), \(b_9\), and \(b_{10}\). The Full Scan MS/MS of the purified peptide standard was performed in a DECA Ion Trap Turbo analyzer.
a  AMINOACID QUANTITATION METHOD

Aliquots Un-quantitated/Purified THB-Val peptide standards
↓
Add Norleucine as Internal Standard (20pmol)
↓
HCl hydrolysis
150°C incubation 4h
↓
Filtration (2 µm)
↓
UPLC-ESI-MS/MS
↓
Calibration of 20 purified amino acids
0.1N HCl
Norleucine

b  THB-VAL REFERENCE AMINO ACIDS

HUMAN


MOUSE


RAT


Figure [2.6]: Schematic Representations of Amino-acid Quantitation. (a) General schematic representation of the amino acid quantitation method used for THB-Val peptide standards. (b) Schematic representation of specific amino acids used to calculate the RF for each THB-Val peptide standard.
REFERENCES


12. Georgieva et al. (2010). Exposure Response of 1,2:3,4-Diepoxybutene Specific N-terminal Valine Adducts in Mice and Rats after Inhalation Exposure to 1,3-Butadiene. Toxicological Sciences. 115 (2): 322-329.


21. Powley et al. (2005). Quantification of DNA and hemoglobin adducts of 3,4-epoxy-1,2-butanediol in rodents exposed to 3-butene-1,2-diol. Carcinogenesis. 26 (9): 1573-1580.


CHAPTER III: SIMULTANEOUS QUANTITATIVE ANALYSIS OF ALL BUTADIENE N-TERMINAL VALINE ADDUCTS IN MICE EXPOSED TO 1,3-BUTADIENE (10 DAYS) WITH A RECENTLY IMPROVED METHOD\(^2,^3\)

3.1 OVERVIEW

1,3-Butadiene (BD) is a well-characterized carcinogen that is both an occupational and environmental hazard. It is an important industrial chemical widely used in the production of rubber and plastic and is also present in automobile exhaust and cigarette smoke. BD is metabolized mainly by P450 2E1 to three epoxides, 1,2-epoxybutene (EB), 1,2:3,4-diepoxybutane (DEB) and 3,4-epoxy-1,2-butanediol (EB-diol). They have the ability to react with hemoglobin (Hb) in blood. The globin adducts formed by EB, DEB, and EB-diol are \(N\)-(2-hydroxy-3-buten-1-yl)-valine (HB-Val), \(N,N\)-(2,3-dihydroxy-1,4-butadiyl)-valine (pyr-Val) and 1,2,3-trihydroxybutyl-valine (THB-Val), respectively. The goal of this research was to integrate the use of species specific mice (1-11) N-terminal Valine (NTV) peptide standards with a recently improved immunoaffinity (IA) coupled UPLC-positive mode electrospray ionization-tandem mass spectrometry (UPLC-ESI\(^+\)-MS/MS) method for the simultaneous quantitative analysis of all three BD-Hemoglobin (BD-Hb) adducts from globin of mice exposed to BD. Using the tryptic digest approach, BD-Hb adducts are cleaved from globin proteins. Digests are

\(^2\) This Chapter previously appeared as part of a major article in Elsevier: Journal of Chemico-Biological Interactions. The original citation is as follows: J.A, Swenberg et al “1-3, Butadiene: Biomarkers and Application to Risk Assessment”, Chemico-Biological Interactions 192 (2011): 150-54.

\(^3\) Chapter III of this Thesis is limited to the discussion of Butadiene Hemoglobin Adducts in Mice.
processed by triple antibody IA enrichment followed by sample quantitation by nano-UPLC-MS/MS analysis using $^{13}$C$^{15}$N$_5$-labeled internal peptides standard. The detection limit of the method is 5-10 fmol in globin (10 mg). We previously used this method for the analysis of BD-Hb adducts in rats and is now used for the quantitation in mice samples exposed to BD, as low as 0.5 ppm via inhalation. This method successfully allowed the simultaneous quantitation of mice BD-Hb adducts, including those at low levels of exposure. The formation of BD-epoxide adducts increased with exposure, and the highest formation ratio of all three adducts per ppm BD was observed at low exposures (0.5-1.5 ppm). The amounts of pyr-Val, HB-Val and THB-Val formation showed no signs of saturation at exposures up to 625 ppm for 10 days. Previous results obtained by this method in BD-exposed rats were integrated for comparisons, and we found that formation ratios for all BD-Hb adducts were higher in mice than in rats but continued to be more efficiently formed at lower levels of exposure; making this relevant due to similar levels of exposure in occupational settings. BD-Hb adducts results were integrated in the calculation of the Internal Blood Dose (IBD) of Epoxides and transformed into the EB-equivalency for further comparisons.

### 3.2 INTRODUCTION

1,3-Butadiene (BD) is an important industrial chemical used primarily in the production of synthetic rubbers and plastics. The general population is exposed to BD from cigarette smoke, burning of wood and automotive exhaust (Brunnemann, 1990; Gustafson, 2007, Pelz, 1990). Epidemiologic studies have linked occupational BD exposure to increased mortality from lymphatic and hematopoietic cancers (Santos-Burgoa, 1992). In long-term (6 h/day, 5 days/week, 2 years) carcinogenicity studies, inhaled BD was weakly tumorigenic in rats exposed to 1,000 or 8,000 ppm (Owen, 1990) but was highly effective in mice exposed to BD.
concentrations ranging from 6.25 to 625 ppm (Melnick, 1990). Furthermore, rodents exposed to BD, presented additional sex-specific differences in rodents where females had: (1) higher mutagenicity (2.5-3 fold) at the HPRT gene (Walker, 2009), greater number of mutation frequencies (MFs) (2.3 fold) in mice exposed to 1250 ppm (Meng, 2007), and tumor formation (10 fold) in mice lung and livers exposed at 6.25 and 20 ppm (Melnick, 1993). Therefore, the International Agency for Research on Cancer (IARC) has classified BD as group 1, a human carcinogen via inhalation (IARC, 2008) using epidemiologic studies and laboratory animal data. BD is a potent carcinogen in mice and considerably weaker in rats. In addition, species and gender differences related to BD metabolism and DNA repair can be examined by measuring suitable biomarkers in order to elucidate the formation of individual epoxides and assign their importance in mutagenesis and carcinogenesis.

Differences are generally related to species-specific metabolic activation to reactive epoxy metabolites, mainly 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB) and 1,2-epoxy-3,4-butanediol (EB-diol). The primary pathways in BD metabolism are shown in Figure 3.1. Oxidation of BD by cytochrome P450 results in the formation of EB, which can be further oxidized by P450 to DEB. EB and DEB can be conjugated with glutathione (GSH) in presence of GSH transferase (GST) and can also be hydrolyzed by epoxide hydrolase (EH) to the detoxication pathways forming 3,4-dihydroxy-1-butene (BD-diol) from EB and EB-diol from DEB. BD-diol can be oxidized by CYP450 to EB-diol and by alcohol dehydrogenase to 1-hydroxy-2-butanone, 1-hydroxy-3-butene-2-one and 2-hydroxy-3-butenal.

Globin adducts have been widely used as surrogate biomarkers for the formation of each epoxide after exposure to chemicals. The globin adducts formed by EB, DEB, and EB-diol are \(\text{N-}(2\text{-hydroxy-3-butene-1-yl})\text{-valine (HB-Val)}\), \(\text{N,N-}(2,3\text{-dihydroxy-1,4-butadiyl})\text{-valine (pyr-Val)}\).
and 1,2,3-trihydroxybutyl-valine (THB-Val), respectively [Figure 3.1]. Our laboratory has previously developed quantitative immunoaffinity (IA) LC-ESI$^+$-MS/MS method for two adducts: the EB-specific adduct, HB-Val, and the DEB-specific adduct, \textit{pyr}-Val, (Boysen, 2004, Georgieva, 2007). Both types of adducts were observed in globin extracted from laboratory animals that were exposed to BD by inhalation (Georgieva, 2007, Georgieva, 2010). We have recently used an IA ultra-pressure liquid chromatography-tandem mass spectrometry (UPLC-ESI$^+$-MS/MS) method for analysis of \textit{pyr}-Val (Georgieva, 2010). This method allowed the measurement of \textit{pyr}-Val in a wide range of BD exposed samples from mice and rats, ranging from concentrations as low as 0.1 ppm BD to 1250 ppm BD. However, this current method was not available for analysis of THB-Val specific adduct of EB-diol. Furthermore, the application of the method is labor intensive requiring extensive sample processing time since the analysis of all three adducts cannot be measured in a single run due to the single IA enrichment. We recently developed a method with triple IA enrichment that allows simultaneous quantitative analysis of all three BD derived NTV adducts in the globin. Previously, data was generated for the rodent database for female and male rats, but not mice. The goal of this research was to integrate the use of species specific mice (1-11) N-terminal Valine (NTV) peptide standards with an improved immunoaffinity (IA) coupled UPLC-positive mode electrospray ionization-tandem mass spectrometry (UPLC-ESI$^+$-MS/MS) method for the simultaneous quantitative analysis of all three BD-Hemoglobin (BD-Hb) adducts from globin of mice exposed to BD. This will complete our data set of rodents exposed to BD, that include low levels of exposures (0.5ppm-1.5ppm), which are relevant due to its similarity to at which human occupational exposures occur.
3.3 MATERIALS AND METHODS

3.3.1 Materials:

Chemicals for standard peptides synthesis, Immunoaffinity (IA) enrichment, and UPLC-MS/MS analysis.

3,4-epoxy-1-butene (98%) and 1,2,3,4 diepoxybutane (97%) were purchased from Sigma-Aldrich (St. Louis, MO). 3,4-epoxy-1,2-butanediol was generously provided by Dr. Karupiah Jayraj, Dr. Avram Gold and Dr. Louise M. Ball from the Department of Environmental Science and Engineering, University of North Carolina at Chapel Hill. The N-terminal alpha chain peptides sequences were as follows: Rat VLSADDKTNIK; analyte and internal standards $[^2H_3]pyr$-Val, HB-Val-$[^{13}C_5^{15}N]$ and THB-Val-$[^{13}C_5^{15}N]$. (2) Mice VLSGEDKSNIK; analyte and internal standards $[^2H_3]pyr$-Val, HB-Val-$[^{13}C_5^{15}N]$ and THB-Val-$[^{13}C_5^{15}N]$. (3) Human VLSPADKTNVK; analyte and internal standards $[^2H_3]pyr$-V-11, HB-Val-$[^{13}C_5^{15}N]$ and THB-Val-$[^{13}C_5^{15}N]$ [Table 3.1]. These peptides were either synthesized by the peptide synthesis facility core at the University of North Carolina at Chapel Hill or purchased from Anaspec. Trypsin (biotin-agarose from bovine pancreas) was purchased from Sigma-Aldrich. All reagents and solvents were ACS grade or higher. Amicon ultra-4 filters were obtained from Amicon Inc. (Deerfield, IL). Polyclonal antibodies against all three adducts (HB-Val, $pyr$-Val, and THB-Val) were produced by Anaspec (San Jose, CA). The N-terminal alpha chain analyte and internal standard peptides for all three adducts were synthesized based on the procedure described below and used for antibody production.
3.3.2 Methods

3.3.2.1 Synthesis of standard peptides

Analyte standard peptides, internal standard peptides, and calibration standards for tuning the analytical equipment were synthesized as described below.

3.3.2.1.1 pyr-Val (analyte) and $[^2H_3]$ (internal standard)

The N-terminal alpha chain standard peptide for pyr-Val in rat, mice and human stable isotope (Leu-$d_3$) labeled rat, mice and human standard peptide were synthesized as described previously (Jayaraj, 2003).

3.3.2.1.2 HB-Val (analyte) and $[^{13}C^{15}N_5]$ (internal standard)

The analyte and stable isotope-labeled rat, mice and human, internal standard peptides were synthesized for accurate quantitation by direct alkylation method. The in vitro alkylation was done by addition of EB to an aqueous solution of (1-11) peptide at molar ratio of 1:100, in 0.1 M ammonium bicarbonate ($\text{NH}_4\text{HCO}_3$) buffer pH 6.5. The concentration of the peptides and the pH of the reaction solution were optimized in order to increase the reactivity of valine, to minimize the alkylation of lysine, and to minimize the cross-linking of peptides. The reaction was incubated at 37ºC for 72 h. After incubation, the reaction was stopped with 20% formic acid and adjusted to pH 2. The yield of reaction products was characterized and profiled by LC-ESI$^+$-MS/MS.
3.3.2.1.3 **THB-Val (analyte) and I\(^{13}\)C\(^{15}\)N\(_{5}\) (internal standard)**

The analyte and stable isotope-labeled rat, mice and human internal standard peptides were synthesized by the direct alkylation method, similar to HB-Val standard peptides. The *in vitro* alkylation was done by addition of EB-diol to an aqueous solution of (1-11)-NTV-peptide at a molar ratio of 1:20, in 0.1 M ammonium bicarbonate buffer pH 6.8. The concentration of the peptides and the pH of the reaction solution have been re-optimized. The reaction was incubated at 37°C for 72 h. After incubation, the reaction was stopped with 20% formic acid and pH was adjusted to strong acid condition (pH 2). The yield of reaction products were characterized and profiled by LC-ESI\(^+\)-MS/MS.

### 3.3.3 Animal and Exposures

Male and female F344 rats and B6C3F1 mice were exposed by inhalation to 0.1, 0.5, 1, 1.5, 6.25, 62.5, 200, and 625 ppm BD for 10 days (2 weeks, 5 days/week, and 6 hours/day). All exposures were performed at the Lovelace Respiratory Research Institute (LRRI, Albuquerque, NM) as described previously in (Georgieva, 2010). Animal procedures were approved by the LRRI Institute Animal Care and Use committee. Within 2 hours of the last exposure, animals were killed by exsanguination under CO\(_2\) anesthesia. Blood samples were collected by cardiac puncture. Red blood cells were isolated, washed twice with 0.9% saline, diluted in distilled water, and stored at -80°C before globin extraction (Georgieva, 2010).
3.3.4 Globin extraction and trypsin hydrolysis

Globin was isolated according the protocol of (Mowrer, 1986). Globin was precipitated by adding 6 volumes of 50 mM of hydrochloric acid in isopropanol, followed by centrifugation for 45 min at 3,000 g at 4 °C. The supernatant was washed three times with cold ethyl acetate to precipitate globin. The precipitate was washed with pentane and dried overnight under a gentle stream of nitrogen gas in vacuum oven. The globin was stored at -70°C until use. In sample processing, globin samples were digested with trypsin as described previously (Georgieva, 2007). All globin samples (5-50 mg) were dissolved in 1-2 ml of 0.1 M NH₄HCO₃, pH 8 containing 2 pmol of [²H₃]pyr-Val (1-11) peptide, 2 pmol of [¹³C¹⁵N₅]HB-Val, and 10pmol of [¹³C¹⁵N₅]THB-Val as internal standard peptides. Ten µl of 10% SDS were added and the globin was digested with 50-100 µl of trypsin-biotin agarose enzyme suspension at 37°C overnight. Samples were filtered through Centricon-3 filters and dried by centrifugal lyophilization.

3.3.5 Immunoaffinity (IA) enrichment

After trypsin hydrolysis, the samples were dried and redissolved in 600 µL PBS buffer and loaded on immunoaffinity columns that had been pre-conditioned twice with PBS. The IA columns were built specifically to retain all three N-terminal peptides (HB-Val, pyr-Val, and THB-Val). The columns were capped and left for 4 h, washed 5 times with 7 ml of water (5×7 ml), eluted in 5% formic acid (3ml), followed by drying under reduced pressure, filtration on microspin filters, and final drying. Samples were stored at -20°C until analysis and dissolved in 20 µl of water prior to nano-UPLC-MS/MS analysis.
3.3.6 Liquid chromatography mass spectrometry

A Thermo-Finnigan TSQ Quantum Ultra-triple quadrupole mass analyzer (ThermoFinnigan, San Jose, CA) interfaced to Waters Acuity nano-UPLC system (Waters Corporation, Milford, MA) was used in all analyses. The system utilized a 2.0 × 20 mm Symmetry C18, 5 μm column (Waters) as a “trap column” for samples loading at 15 μl/min 15 mM ammonium formate-0.7% formic acid for 1 min. After sample loading, the flow rate was reduced to 1.2 μl/min and the column exit flow was directed to a 100 μm × 100–mm BEH C18 UPLC column (Waters) for analysis. The LC solvents were 15 mM ammonium formate in 0.7% aqueous formic acid (A) and acetonitrile in 0.1% aqueous formic acid (B). A linear gradient was run from 5% B to 70% B for 15 min, at a flow rate of 1.2 μl/min. After elution, the column was washed at 90% B for 10 minutes and equilibrated at 5% B for 5 min. Under these conditions, all three adducts and internal standard eluted within the range 5.9 - 6.1 min\(^4\). The MS was operated in the positive ion mode with nitrogen used as a sheath gas. Electrospray ionization was performed at a spay voltage of 1800 V and capillary temperature of 240 °C. Collision induced dissociation (CID) energy was achieved at 28 V. The MS parameters were optimized during the infusion of standard peptides solution and sample analysis was performed in the selected reaction monitoring (SRM) mode by monitoring either the singly or doubly charged molecular ions for rodents analyte and internal (1-7) peptide standards, depending on the adduct [Table 3.1].

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\(^4\) Adducts and internal standard eluted 9-16 minutes.
3.3.7 Method validation

3.3.7.1 Neat Standards

A total of 32 combined BD-Hb adduct columns were built and tested for neat standard recovery using pyr-Val, HB-Val and THB-Val mice standard peptide. Both pyr-Val and HB-Val, were tested with 0.5, 1, 2.5 and 5 pmol of analyte to 5 pmol of internal standard peptide. THB-Val was tested for 0.5, 2.5, 5 and 10 pmol of analyte to 10 pmol of internal standard peptide (n = 3 per concentration). Internal standard peptide and blanks were included in the recovery test (n = 2 per group). Relative recoveries for all 32 combined BD-Hb adduct columns were calculated as the response for all three adducts divided by the actual spiked concentration.

3.3.7.2 Matrix Spike

After neat standard recovery test was completed, we selected 16 BD-Hb adduct columns with the highest peptide standards recovery. We proceeded to then use these prescreened columns to test control globin for validation and to assess matrix effects. The original matrix test was done in our laboratory using control globin from unexposed mice. A three-point validation was run on three days (n=3 for each validation point for one day, n=9 for each validation point for three days). Validation samples were prepared by spiking control globin with standard peptides. Relative recoveries in matrix effects were calculated as the response for all three adducts in a biological matrix spike, divided by the actual spiked concentration. Results were reported in % recovery for each standard peptide. The limits of detection (LOD) were determined by signal to noise ration 3:1 based on 5 µl injection volume of calibration standard. The lower limit of quantitation (LOQ) was defined as the lowest concentration to be detectable from diluted spiked control globin. The interday and intraday precisions were expressed as %
relative standard deviation (%RSD) and accuracies were expressed as % relative error (%RE)

### 3.3.7.3 Statistical analysis

All statistical analyses were performed using Microsoft Excel spreadsheet analysis tools. Classified precision and accuracy were estimated by simple statistics of mean and standard deviation of the estimated concentration of the determined peptide standards.

### 3.3.8 Mice Internal Blood Dose Formation and EB-Equivalency

#### 3.3.8.1 Mice Internal Blood Dose

The internal dose in blood (IBD) of the BD-epoxides (EB, DEB, and EB-diol) was calculated from those results obtained from BD-Hb adducts of mice exposed to BD for 10 days. The formation of BD-Hb adducts work as an indirect internal dosimeter in blood per BD-epoxide. The IBD is obtained with two values: (1) quantitated BD-Hb adduct values ($A$, and expressed in mol/grams of globin), (2) divided by the reaction rate of the BD-epoxide to react with the NTV (or $K_{val}$, and expressed in L/grams of globin/hours of exposure). We used Fred’s previously reported $K_{val}$ rate constants values (Fred, 2008) for our calculations. Mouse BD-epoxides $K_{val}$ values were:

- **Mouse-EB $K_{val}$** = $2.9 \times 10^{-5}$ L·g⁻¹·h⁻¹
- **Mouse-DEB $K_{val}$** = $4.7 \times 10^{-5}$ L·g⁻¹·h⁻¹
- **Mouse-EB-diol $K_{val}$** = $2.1 \times 10^{-5}$ L·g⁻¹·h⁻¹
This Dose (or $D$ expressed in mM per hour/ppm per hour) and was individually calculated for each individual epoxide with the equation considering that mice total exposures were 60 hours:

$$D = A / K_{val}$$

$$D = \text{Mice-EB-Internal Blood Dose} = \frac{HB-Val\ Adducts\ (mol/g)}{2.9 \times 10^{-5}\ L\cdot g^{-1}\cdot h^{-1}}$$

$$D = \text{Mice-DEB-Internal Blood Dose} = \frac{pyr-Val\ Adducts\ (mol/g)}{4.7 \times 10^{-5}\ L\cdot g^{-1}\cdot h^{-1}}$$

$$D = \text{Mice-EB-diol-Internal Blood Dose} = \frac{THB-Val\ Adducts\ (mol/g)}{2.1 \times 10^{-5}\ L\cdot g^{-1}\cdot h^{-1}}$$

### 3.3.8.2 Mice EB-Equivalency

The EB-equivalency reflects the total genotoxic dose by considering the sum of each individual BD-epoxide genotoxic dose that’s adjusted by their genotoxic potency. The Mice EB Dose Equivalency was calculated by first multiplying each epoxides’ IBD ($D$), with its corresponding epoxide mutagenic potency. The mutagenic potency is a numerical value that was assigned by the efficiency of each epoxide to induce mutations in the hprt loci in human TK6 cells as previously reported by Meng in 2010. These individually adjusted IBDs genotoxic doses are summed to generate the total genotoxic dose in terms of EB in mice.

$$EB-IBD \times 1$$

$$DEB-IBD \times 100$$

$$EB-diol-IBD \times 0.2$$

**Mice EB-Dose Equivalency**
3.4 RESULTS AND DISCUSSION

3.4.1 A simultaneous IA-UPLC-MS/MS method

It is well known that N-terminal globin adducts are well suited to study BD carcinogen metabolism across species (Törnqvist, 2002). Specific globin adducts formed by each BD metabolite need to be identified and quantified in order to reveal important insight into species, gender, and exposure concentration differences in BD metabolism. We have recently demonstrated DEB specific adduct, pyr-Val, in mice and rats exposed to BD at different concentrations (Georgieva, 2010). Since our previous analyses of pyr-Val adducts was labor intensive and time consuming, the objective of this study was to improve the IA UPLC-MS/MS method and simultaneously detect all three globin adducts specific for BD metabolites in a single run. We employed the same concept as the method for pyr-Val and were able to adapt the method for the analysis of HB-Val and THB-Val in the concurrent sample.

IA columns were produced by combining antibodies raised against all three alkylated standard peptides and used for sample purification. Control globin was spiked with different amounts of analyte and internal standard peptides to determine the reproducibility of IA column. The results showed that the percentage of recovery was lower compared to IA columns prepared with individual antibodies. To overcome this problem, we adjusted the incubation time on IA columns from 1 h to 4 h at room temperature. Using this approach, better recoveries (>70%) were obtained for all three adducts (data not shown).

The MS was performed in the selected reaction monitoring (SRM) mode by monitoring of the singly charged molecular ion (m/z 817→141) for HB-Val, (m/z 851→175) for THB-Val, and the corresponding transition of the [\(^{13}\text{C}_{15}\text{N}_5\)]-label internal standard peptide (m/z 823→147) for HB-Val, (m/z 857→181) for THB-Val. At the same time, we monitored the doubly charged
molecular ion (m/z 417→158) for pyr-Val and the corresponding transition of the [\(^3\)H\(_3\)]-labeled internal standard peptide (m/z 418→158), since this transition, presented better sensitivity compared to the singly charged molecular ion.

Quantitative measurement was based on the ratio of area under the peak in the selected ion chromatogram corresponding to the analyte and internal standard peptides. Calibration curves were constructed by analyzing the solutions containing all three adducts (0.050 – 0.5 pmol) and internal standard peptides (0.5 pmol), followed by regression analysis [Figure 3.3]. All curves showed a linear response over the range of concentrations with a high coefficient of determination (R\(^2\) = 0.992, R\(^2\) = 0.998, and R\(^2\) = 0.999 for HB-Val, pyr-Val and THB-Val, respectively).

3.4.2 Method validation

3.4.2.1 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD was 1 fmol for all three adducts based on 5 µL of injection volume and a signal to noise ratio of 3:1, while the LOQ was 5 fmol on column for HB-Val and pyr-Val and 10 fmol on column for THB-Val. The lower LOQ for THB-Val adducts compared to the other two adducts was attributed to the lower signal on ESI\(^+-\)MS/MS. This LODs and LOQs allowed detection of all three adducts in mice exposed to BD at 0.5 ppm for 10 days.

3.4.2.2 Simultaneous Immunoaffinity Recovery Tests

3.4.2.2.1 Neat Standards

The pre-screened neat standard test helped us select a set of 16 out of 32 combined BD-Hb adduct columns with the highest relative recovery values. Results for the top 16 columns with the highest recoveries were evaluated as % recovery for each standard peptide. Average recovery
was above 80% for all neat peptide standards, and the internal standard contribution was less than 2% [Table 3.2].

### 3.4.2.2 Matrix Spike

Relative recovery values for all three adducts in matrix were as follows; 92-96% for HB-Val, 90-95% for \textit{pyr}-Val, and 63-73% for THB-Val (data not shown). The relatively low yield of THB-Val was likely due to the low titer of the THB-Val antibody, which may have cause less retention during the immunoaffinity purification step. However, despite the low yield, our method produced a highly linear calibration curve with acceptable precision and accuracy.

### 3.4.2.3 Accuracy and Precision

The method accuracy and precision were determined for three replicates of three concentrations of all three adducts. The validated samples were analyzed on three separate days, the interday and intraday precision and accuracy. The RSD of both interday and intraday for all three adducts on the nano-UPLC-ESI$^+$-MS/MS were below 7% (1.1%-6.6%). The accuracies calculated as relative error (RE) were within the range of 2%-6%. The improved quantitation method in this study is highly sensitive, accurate, and selective for all three BD adducts, allowing the detection of low amounts of adducts in 10-15 mg globin in mice from exposures as low as 0.5 ppm BD. However, the detection of adducts at 0.1 ppm BD exposure for 10 days was under the LOQ due to the presence of very small amounts of adducts.
3.4.3 Simultaneous Butadiene Hemoglobin Adducts in Samples

3.4.3.1 B6C3F1 Mice

Here, we completed our comprehensive exposure-response for BD reactive epoxides in both female and male rodents by incorporating samples from B6C3F1 mice exposed to BD by inhalation to various concentrations of BD, ranging from 0.1 to 625 ppm for 10 days. Previous reports have shown occupational exposures of BD at concentrations ranges from 0.3 to 3 ppm (Albertini, 2003; Hayes, 2002; Sram, 1998; Tates, 1996; Ward, 2001). Therefore, this previously improved quantitative assay allowed analysis of NTV-adducts after BD exposures for mice at low levels, similar to those found in BD-exposed workers. This is the first time that all three BD-epoxide Hb adducts in B6C3F1 mice have been simultaneously quantitated under one method. Our results are will ultimately help understand important aspects of BD metabolism, carcinogenesis and mutagenicity. Using this validated quantitative immunoaffinity nano-UPLC-MS/MS method will enhance the accurate measurement of BD-Hb adduct formation in mice, at low levels of BD-exposure, and complete the rodent dataset.

To test the method, background levels of HB-Val, pyr-Val, and THB-Val adducts were determined in each of the control globin samples (n = 5/group/per exposure). The control animals did not contain detectable amounts of HB-Val or pyr-Val, suggesting that these adducts are not formed endogenously. However, THB-Val was detected in both control female mice (35 ± 8 pmol/g globin) and male mice (38 ± 5 pmol/g globin) without any exposure to BD. Moreover, endogenous formation of THB-Val has been previously reported as result of NTV alkylation by erythrose and threose (Rohlfing, 2002). Even though pyr-Val is the least abundant of the three BD adducts quantified, it was higher than the method’s LOQ, ensuring accurate quantification and presenting excellent signal to noise ratio for those samples exposed at lower
levels (0.5 ppm). Finally, we were able to quantitate the formation of the three BD adducts in mice exposed at ranges of exposure from 0.5-625 ppm BD for 10 days, and acquired data on exposure-response relationships [Table 3.3].

Overall results showed that at low levels of BD exposures (< 6.25 ppm), HB-Val was 10-15 fold lower than THB-Val. However, both pyr-Val and HB-Val continue to increase at 200 ppm or less, that until it reaches the 625 ppm BD-exposure, where we see HB-Val being 2 fold that of pyr-Val in both female and male mice. This indicates that mice are capable of efficiently metabolize all three BD-epoxides without saturation when exposed to BD up to 625ppm. The exposure response curves are shown in [Figures 3.5 and 3.7] and individual results are discussed below.

3.4.3.1.1 Mice Exposure Response to HB-Val

The exposure response curve for the HB-Val adduct is illustrated in Figure 3.8 and Table 3.3. We observed a linear exposure-response curve for the HB-Val adducts. The levels of HB-Val in mice increased with exposure and did not show any signs of metabolic saturation at the levels studied (0-625 ppm). When comparing adduct formation ratio as (pmol/g/ppm BD), mice HB-Val adducts showed a higher formation ratio at lower levels of BD exposure (0.5 ppm, 1.5 ppm) than at higher levels (200 ppm-625 ppm) [Figure 3.11 and Table 3.4]. In comparison to previous results, the overall HB-Val fold difference in formation ratio for mice was higher than rats at lower levels of BD-exposure. The formation ratio of HB-Val adducts indicate that EB is most efficiently formed at low BD-exposures (0.5 ppm, 1.5 ppm) but does not saturate at high exposures (200 ppm, 625ppm) in mice. This parallels previous findings in rats, where HB-Val formation continues to increase at all levels of exposure (up to 625ppm), indicating that EB
metabolic activation via CYP2E1/CYP2A6 does not saturate in rodents. Similar results have reported this linear dose-response increase in HB-Gua adducts for rodents exposed to BD at low levels (20ppm), and high levels where it has shown tumor formation (Koc, 1999, Swenberg, 2001).

### 3.4.3.1.2 Mice Exposure response of pyr-Val

The exposure response curve for the pyr-Val adduct is illustrated in Figure 3.8 and Table 3.3. The formation of pyr-Val increased with BD-exposures in a linear manner. Different from rats, mice pyr-Val formation does not plateau at any of the exposure levels (up to 625 ppm). When comparing adduct formation ratio (pmol/g/ppm BD), pyr-Val adducts also showed a higher formation rate at lower levels of BD exposure (0.5 ppm, 1.5 ppm) than at higher levels (200 ppm-625 ppm) [Figures 3.11 and Table 3.4]. Comparing our previous results in rats, the overall pyr-Val fold difference in adduct formation ratio for mice was higher in both females and males [Tables 3.4, 3.5, 3.6 and 3.8]. This indicates that DEB is metabolized more efficiently by CYP2E1/CYP3A4 at lower levels of exposures (0.5 ppm, 1.5 ppm) but does not saturate at high exposures (200 ppm, 625ppm) in mice. This contrasts previous findings in rats, where pyr-Val formation plateaus, as a sign of saturation of DEB metabolic activation via CYP2E1/CYP3A4.

### 3.4.3.1.3 Mice Exposure response of THB-Val

The exposure response curve for the THB-Val adducts are illustrated in Figures 3.5, 3.7 and 3.8. Formation of THB-Val (pmol/g) showed a linear dose-response at all levels of exposure. Not only it presented to be the most predominant adduct for mice exposed to BD, but also had no evident saturation in its formation, even at 625 ppm. When comparing THB-Val with
other BD-Hb adducts, we noticed that at low BD exposures (< 6.25 ppm), THB-Val was 10 to 15-fold higher than HB-Val, and at higher levels (> 62.5 ppm), THB-Val is 11-fold higher than HB-Val in male, but only 3-fold higher in females [Tables 3.3 and 3.7]. In contrast, previous results have shown rats THB-Val formation being supralinear; increasing at low levels, followed by a decrease in its formation (1.5ppm-6.25ppm), until saturation is reached (200ppm-625ppm) [Figures 3.6, 3.7, and 3.9]. Values of THB-Val adducts formation ratio (pmol/g/ppm BD), were higher at lower levels of BD exposure (0.5 ppm, 1.5 ppm) than at higher levels (200 ppm, 625 ppm) [Figures 3.10 and 3.11, and Table 3.4]. In comparison with previous results for THB-Val in rats, the overall formation ratio was higher in mice at all BD-exposures [Table 3.8]. These species difference indicates that metabolic activation of EB-diol by EH is more efficient at low levels without saturation at high levels BD-exposures, whereas in rats, EB-diol metabolic activation reaches saturation by EH.

Integrating our results with BD-DNA adducts, we compared THB-Val to THB-Gua formation for those animals from the same exposure study. THB-Gua formation rate remains consistent to THB-Val in both female and male mice, with a reduction in its formation as exposure concentration increases. However, at higher concentrations (200ppm-625ppm), there are signs of saturation only for female mice (Troutman, 2011). This confirms that both biomarkers are not evidencing saturation from BD to EB-diol at any low levels of exposure (< 200 ppm) in blood or tissue.
3.4.3.1.4  Mice Internal Blood Dose Formation and EB-Equivalency

The IBD of BD-epoxides, and their corresponding EB-equivalencies for mice, were calculated and presented in [Table 3.9]. Quantitated BD-Hb adducts were first converted into IBDs and represent circulating BD-epoxides in blood. The EB-equivalency was the sum of all three BD-epoxides, which were first adjusted by their individual genotoxic potency to convert both EB-diol and DEB into EB equivalents.

There is an undeniable species differences in the formation of both the BD-epoxides as evidenced by both the IBD and the EB-equivalency. Here we present that mice are more effective in metabolizing BD-epoxides at lower levels of exposure. First, as exposure increases, the IBD decreased for all epoxides. The highest values of IBDs were found at the lowest levels of exposures (0.5 ppm). Mice had a similar contribution from both DEB and EB from their IBD values. Furthermore, the IBD of DEB is lower in both rats and humans. The greatest fold difference is the IBD equivalency was Mice vs. Humans (~200-fold), whereas the lowest was Rats vs. Humans (~5-fold).

It’s important to understand that the attributable carcinogenic potential of BD in mice is directly influenced a greater contribution of DEB in their IBDs. DEB has been identified as the epoxide with the greatest genotoxic potential, and has shown to be the most influential epoxide in tumor formation in mice. However, the contribution in rats seems to be driven by EB and EB-diol, but not by DEB which reaches saturation around 200ppm. These differences across rodents explain why there is greater susceptibility in mice due to a greater contribution of DEB in blood and lack of saturation in the metabolic activation of BD.

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5 Additional calculations were performed to adjust for the half-life of adducts.
3.5 CONCLUSION

We successfully employed the use of (1-11) THB-Val peptide standards into the development, validation, and analysis of samples exposed to BD. The recently improved and adjusted immunoaffinity enrichment nano-UPLC-ESI\(^{+}\)-MS/MS method allowed the simultaneous quantitation of all three BD-derived epoxide NTV globin adducts in mice. These results were critical for the completion of our comprehensive rodent dataset exposed to BD by inhalation (10 days), by including data for both female and male mice, and using as little as 10 mg of globin. Employing this method proved to reduce processing time, materials and experimental analysis costs by two thirds when compared to the original single immunoaffinity enrichment.

BD-Hb adducts formation in mice was dominated by the THB-Val adduct, followed by HB-Val, and lastly pyr-Val as the minor adduct. We observed no indications of metabolic saturation in the formation BD-Hb adducts, even at exposures of 625 ppm. The formation of all BD-Hb adducts are species dependent with mice generating more overall adducts than those previously reported for rats (Bordeerat, 2011). Integrating our results with rats, we found that mice shows no saturation in the metabolic activation of BD into any of the BD-epoxides, including DEB and past exposures above 200 ppm. However in rats, both pyr-Val and HB-Val presented saturation. No sex specific differences were identified in mice at any of the exposure concentrations. Similar than previous results in rats, this confirms that BD-Hb adducts formation was species depended but sex independent.

Mice had the highest formation ratio for all adducts (pmol/g/ppm), at all exposures (0-625 ppm), and with the greatest difference being at low levels (0.5 ppm and 1.5 ppm). Also, BD-Hb adduct formation ratio was greater in mice than that of rats across all adducts and concentrations. It is of key importance to consider that where rodents have shown to have highest
BD-Hb formation ratio is at the same levels where occupationally exposed subjects are involved in the industrial production of BD.

The IBD of BD-epoxides, and their corresponding EB-equivalencies, were calculated for mice using Fred’s 2008 Kval constants for BD-epoxides and mutagenic potency from Meng’s 2010 publication. The IBD is highest at lower levels of exposure (0.5 ppm) and decreases with increasing concentration. A previous collaborator of this project presented her results for the same dataset but in rats (Bordeerat, 2011). There is an undeniable species differences in the formation of both the BD-epoxides and the EB-equivalency. Mice are more efficient in BD-epoxides metabolism at lower levels of exposure than at higher levels. No sex specific differences were noted. The attributable carcinogenic potential of BD in Mice is directly influenced by the greater contribution of DEB, rather than EB and EB-diol as shown by the EB-equivalency. DEB has been identified as the epoxide with the greatest genotoxic potential, it’s the most influential epoxide in tumor formation in mice.

These findings provide important information relevant to why mice are the most susceptible species from the rodent models, and accounts for the specific species differences in BD metabolic activation as estimated by BD-Hb adducts. The current investigation has provided insights of our knowledge on BD risk assessment. Finally, mice’s greater DEB contribution in blood, and their corresponding biomarker of exposure pyr-Val does point out the reason for this species susceptibility to BD and tumor formation. However, mice don’t represent to be the most appropriate animal model for BD risk assessment in human, rat does.
3.6 ADDITIONAL NOTES

Part of our initial goal was the development and integration of this method to quantitate BD-Hb adduct formation in our occupationally exposed human dataset. This data set has been previously processed by a combination of two different methods, and results for \( \text{pyr-Val} \), HB-Val and THB-Val have been acquired. In attempts to process the same human samples under this method, we encountered at early stages that it proved to be difficult for its application, specifically for the quantitation of THB-Val. Specifically, human samples presented successful in the recovery of \( \text{pyr-Val} \), and HB-Val but not for THB-Val. Although attempts to achieve this goal were made through the development of two other alternative methods\(^6\), a number of limitations hindered the re-evaluation of human samples under this simultaneous method.

3.7 ACKNOWLEDGEMENTS

Our work was performed with the assistance of the Biomarker Mass Spectrometry facility and their staff, all their recommendations using all instruments, including the UPLC and nano-ESI\(^+\)-MS/MS as well as essential ideas during the method development and sample analysis. We would like to thank Valeriy Afonin for isolating all the globin samples. This work was financially supported by grants from the NIH (1 R01 ES012689, 5 P30-ES10126), the Health Effects Institute (agreements 99-5 and 05-12) and the American Chemistry Council.

\(^6\) Derivatization using PITC, PFPI TC, tryptic digest followed by fraction collection and the FIRE Method (data not shown).
### 3.8 TABLES:

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<td>(1-11)</td>
<td></td>
<td>1172</td>
<td>1178</td>
<td>1282</td>
<td>1288</td>
</tr>
<tr>
<td></td>
<td>(1-7)</td>
<td></td>
<td>813</td>
<td>815</td>
<td>833</td>
<td>839</td>
</tr>
<tr>
<td></td>
<td>a1</td>
<td></td>
<td>155</td>
<td>158</td>
<td>141</td>
<td>147</td>
</tr>
</tbody>
</table>

Table [3.1]: Molecular weights and m/z (1-11) BD-NTV peptide standards: pyr-Val, HB-Val and THB-Val.
<table>
<thead>
<tr>
<th>N</th>
<th>Ratio AS/IS</th>
<th>pyr-Val</th>
<th>HB-Val</th>
<th>THB-Val</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Recovery</td>
<td>SD</td>
<td>Recovery</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>97.7%</td>
<td>7.8%</td>
<td>116.6%</td>
</tr>
<tr>
<td>3</td>
<td>0.20</td>
<td>96.6%</td>
<td>7.2%</td>
<td>102.6%</td>
</tr>
<tr>
<td>3</td>
<td>0.50</td>
<td>92.4%</td>
<td>7.4%</td>
<td>91.7%</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>92.4%</td>
<td>8.7%</td>
<td>83.5%</td>
</tr>
<tr>
<td>2</td>
<td>IS Blank</td>
<td>0.5%</td>
<td>0.3%</td>
<td>1.6%</td>
</tr>
<tr>
<td></td>
<td>Recovery</td>
<td>94.8%</td>
<td>2.8%</td>
<td>98.6%</td>
</tr>
</tbody>
</table>

Table [3.2]: Mice NTV Neat Peptide Standard Recovery on BD Triple IA Columns.
<table>
<thead>
<tr>
<th>B6C3F1 Mice (N = 5)</th>
<th>BD ppm</th>
<th>Adducts (pmol/g)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb-Val (±/−)</td>
<td>pyr-Val (±/−)</td>
<td>THB-Val (±/−)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0 ND</td>
<td>ND 35</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 2.3</td>
<td>0.8 2.1</td>
<td>1.4</td>
<td>15 8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 11</td>
<td>1 11</td>
<td>3</td>
<td>152 43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 16</td>
<td>1.2 20</td>
<td>2</td>
<td>352 34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 30</td>
<td>5 38</td>
<td>2</td>
<td>525 160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.25 59</td>
<td>9 76</td>
<td>10</td>
<td>1008 235</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62.5 364</td>
<td>55 419</td>
<td>63</td>
<td>1448 445</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 751</td>
<td>23 859</td>
<td>62</td>
<td>2587 238</td>
<td></td>
</tr>
<tr>
<td></td>
<td>625 3644</td>
<td>534 1532</td>
<td>170</td>
<td>4490 467</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0 ND</td>
<td>ND 38</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 2.3</td>
<td>0.5 3 1</td>
<td>1 19 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 11</td>
<td>1 15</td>
<td>2</td>
<td>142 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 32</td>
<td>3 50</td>
<td>9</td>
<td>260 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 1165</td>
<td>112 859</td>
<td>126</td>
<td>2324 212</td>
<td></td>
</tr>
<tr>
<td></td>
<td>625 3644</td>
<td>520 1980</td>
<td>195</td>
<td>4383 373</td>
<td></td>
</tr>
</tbody>
</table>

Table [3.3]: Hemoglobin Adducts (pmol/g) of B6C3F1 Mice Exposed to Butadiene for 10 days. ND: not detected, Endogenous THB-Val was subtracted in all groups using data from control mice.
<table>
<thead>
<tr>
<th>B6C3F1 Mice</th>
<th></th>
<th>Adducts per ppm of BD</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 5</td>
<td>BD ppm</td>
<td>Hb-Val</td>
<td>pyr-Val</td>
<td>THB-Val</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>23</td>
<td>21</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.1</td>
<td>22</td>
<td>22</td>
<td>304</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.5</td>
<td>26</td>
<td>20</td>
<td>352</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>20</td>
<td>25</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.5</td>
<td>9</td>
<td>12</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6.25</td>
<td>6</td>
<td>7</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>62.5</td>
<td>4</td>
<td>4</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>200</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>625</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
<td>23</td>
<td>30</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.1</td>
<td>22</td>
<td>30</td>
<td>284</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.5</td>
<td>21</td>
<td>33</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>200</td>
<td>6</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>625</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Table [3.4]: Hemoglobin Adduct Formation Ratio (Adducts/ppmBD) in B6C3F1 Mice Exposed to Butadiene for 10 days.
<table>
<thead>
<tr>
<th>F344 Rats</th>
<th>BD ppm</th>
<th>Adducts (pmol/g)</th>
<th>Hb-Val (+/-)</th>
<th>pyr-Val (+/-)</th>
<th>THB-Val (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>36.8</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>NQ</td>
<td>NQ</td>
<td></td>
<td>NQ</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.9</td>
<td>0.1</td>
<td>0.8</td>
<td>0.1</td>
<td>2.4</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>0.5</td>
<td>1.4</td>
<td>0.2</td>
<td>29.0</td>
</tr>
<tr>
<td>1.5</td>
<td>2.5</td>
<td>0.4</td>
<td>2.0</td>
<td>0.5</td>
<td>59.0</td>
</tr>
<tr>
<td>6.25</td>
<td>8.1</td>
<td>0.7</td>
<td>6.7</td>
<td>0.7</td>
<td>220.0</td>
</tr>
<tr>
<td>62.5</td>
<td>60.0</td>
<td>3.0</td>
<td>46.5</td>
<td>8.3</td>
<td>481.0</td>
</tr>
<tr>
<td>200</td>
<td>148.0</td>
<td>19.0</td>
<td>123.0</td>
<td>8.0</td>
<td>606.0</td>
</tr>
<tr>
<td>625</td>
<td>410</td>
<td>15</td>
<td>124.0</td>
<td>11.0</td>
<td>737</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>34</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.8</td>
<td>0.1</td>
<td>0.7</td>
<td>0.2</td>
<td>1.1</td>
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<tr>
<td>1.5</td>
<td>2.3</td>
<td>0.4</td>
<td>2.00</td>
<td>0.40</td>
<td>70</td>
</tr>
<tr>
<td>200</td>
<td>171</td>
<td>0.4</td>
<td>120</td>
<td>11</td>
<td>585</td>
</tr>
<tr>
<td>625</td>
<td>394</td>
<td>8</td>
<td>117</td>
<td>12</td>
<td>796</td>
</tr>
</tbody>
</table>

Table [3.5]: Hemoglobin Adducts (pmol/g) of F344 Rats Exposed to Butadiene for 10 days.*

*Data from this table was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
<table>
<thead>
<tr>
<th>F344 Rats</th>
<th>BD ppm</th>
<th>Adducts per ppm of BD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hb-Val</td>
<td>pyr-Val</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>1.7</td>
<td>1.30</td>
</tr>
<tr>
<td>6.25</td>
<td>6.25</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>62.5</td>
<td>62.5</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>625</td>
<td>625</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>1.5</td>
<td>1.30</td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.30</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>625</td>
<td>625</td>
<td>0.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table [3.6]: Hemoglobin Adduct Formation Ratio (Adducts/ppmBD) in F344 Rats Exposed to Butadiene for 10 days.*

*Our results were calculated using data presented in N.K. Bordeerat’s dissertation. Results were included for comparisons with her permission.
**Table [3.7]: Hemoglobin Adducts (pmol/g) and Fold Differences in BD-adducts in B6C3F1 Mice Exposed to Butadiene for 10 days.** Females and males have been divided as low (0-6.25ppm) and high (62.5-625ppm) BD exposures. Mean fold differences in both groups for both sexes have been calculated as fold differences in Hb-adducts for: (THB/HB), (THB/pyr) and (HB/pyr). The THB/HB and THB/pyr fold difference in Hb-adducts decrease with increasing concentration of exposures in both female and male mice. However, in HB/pyr the fold difference remains similar.

<table>
<thead>
<tr>
<th>B6C3F1 Mice N = 5</th>
<th><strong>BD</strong></th>
<th><strong>Adducts (pmol/g)</strong></th>
<th><strong>Fold Differences [Adducts (pmol/g)]</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>Hb-Val (+/-)</td>
<td>pyr-Val (+/-)</td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.3 0.8</td>
<td>2.1 1.4</td>
</tr>
<tr>
<td></td>
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<td>11 1</td>
<td>11 3</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>16 1.2</td>
<td>20 2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>30 5</td>
<td>38 2</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>59 9</td>
<td>76 10</td>
</tr>
<tr>
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<td>62.5</td>
<td>364 55</td>
<td>419 63</td>
</tr>
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<td>751 23</td>
<td>859 62</td>
</tr>
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<td>625</td>
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<td>1532 170</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.3 0.5</td>
<td>3 1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>11 1</td>
<td>15 2</td>
</tr>
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<td>32 3</td>
<td>50 9</td>
</tr>
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<td>200</td>
<td>1165 112</td>
<td>859 126</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>3644 520</td>
<td>1980 195</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
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<td>23</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>22</td>
<td>1.8</td>
<td>12</td>
</tr>
<tr>
<td>0.5</td>
<td>26</td>
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<td>16</td>
</tr>
<tr>
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<td>20</td>
<td>1.7</td>
<td>12</td>
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<tr>
<td>1.5</td>
<td>9</td>
<td>1.3</td>
<td>7</td>
</tr>
<tr>
<td>6.25</td>
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<td></td>
<td></td>
</tr>
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<td>6</td>
</tr>
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<td>200</td>
<td>4</td>
<td>0.7</td>
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</tr>
<tr>
<td>625</td>
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</tr>
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<td>23</td>
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</tr>
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<td>1.5</td>
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<td>0.6</td>
<td>10</td>
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</table>

Table [3.8]: Hemoglobin Adducts Formation Ratio (Adducts/ppmBD) and Fold Difference in B6C3F1 Mice vs F344 Rats Exposed to Butadiene for 10 days. Females and males have been divided as low (0-6.25ppm) and high (62.5-625ppm) BD
<table>
<thead>
<tr>
<th>Species/Sex</th>
<th>BD Exposure</th>
<th>Doses in Blood nMh/ppmh</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>ppmh</td>
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<tr>
<td>Female Mice B6C3F1</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>0.1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>62.5</td>
<td>3,750</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>37,500</td>
</tr>
<tr>
<td>Male Mice B6C3F1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>625</td>
<td>37,500</td>
</tr>
</tbody>
</table>

Table [3.9]: Individual estimated Butadiene-epoxides doses in blood [nMh/ppmh] and the EB-equivalency for Female and Male B6C3F1 Mice exposed to Butadiene (10 days).
Figure [3.1]: BD metabolic activation and formation of BD reactive epoxides.
Figure [3.2]: BD metabolism and formation of N-terminal valine adducts
Figure [3.3]: Calibration Curve for N-terminal valine adduct THB-Val. Concentrations ranged between 50-500 fmol of analyte and 500 fmol of internal peptide standard. Limit of detection was 1 fmol and limit of quantitation was 10 fmol on column with a 5 uL injection.
Figure [3.4]: Ion chromatogram of THB-Val adduct in B6C3F1 Mice exposed to BD and its corresponding internal standard. Analyzed in selected reaction monitoring mode (SRM) and obtained the best sensitivity with the singly charged molecular ion. Mice samples were analyzed (m/z 851→175) for THB-Val analyte and (m/z 857→181) for the THB-Val $^{13}$C$^{15}$N$_5$-internal peptide standard.
Figure [3.5]: Exposure response curves of THB-Val adducts formation (pmol/g) in B6C3F1 mice exposed to BD for 10 days.
Figure [3.6]: Exposure response curves of THB-Val adducts formation (pmol/g) in F344 rats exposed to BD for 10 days.

*Data to make this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.*
Figure [3.7]: Exposure response curves of THB-Val adducts formation (pmol/g) in Rodents exposed to BD for 10 days.

*Partial data from this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
Three Hemoglobin Adducts in B6C3F1 Mice Exposed To Butadiene (10-days)

Figure [3.8]: Exposure response curves of HB-Val, pyr-Val, and THB-Val adducts formation (pmol/g) in B6C3F1 mice exposed to BD for 10 days.
Figure [3.9]: Exposure response curves of HB-Val, pyr-Val, and THB-Val adducts formation (pmol/g) in F344 rats exposed to BD for 10 days.

*Data to make this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
Figure [3.10]: THB-Val Adduct Formation Ratio ([pmol/g]/[ppmBD]) in Rodents Exposed to BD for 10 days.

*Partial data use to make this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
Figure [3.11]: Hemoglobin Adducts Formation Ratio ([pmol/g]/[ppmBD]) in Rodents Exposed to BD (10 days)

*Partial data to make this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
Figure [3.12]: IBD: Internal Blood Doses Formation [nMh/ppmh] in Female and Male Mice Exposed to Butadiene by Inhalation (10 days).

*Partial data to make this figure was presented in N.K. Bordeerat’s dissertation. Results for were included for comparisons with her permission.
Figure [3.13]: EB-equivalency of Male and Female Mice Exposed to Butadiene (10 Days)
REFERENCES


CHAPTER IV: *IN VITRO ASSESSMENT OF DNA DAMAGE RESPONSE TO BUTADIENE EPOXIDES*

4.1 OVERVIEW

All three Butadiene (BD) epoxides (EB, DEB, EB-diol) have the ability to react DNA and form covalent adducts. Limited information is available regarding those DNA damage response (DDR) pathways involved in counteracting each specific BD-epoxide insult. Therefore, we have identified that BD-DDR is a gap in knowledge in BDs current biomarkers of exposure-effect database. DT40 cells provide the most extensive collection of DNA repair mutant clones in vertebrate cells to assess DDR using a high-throughput in vitro assay. BD-epoxides DDR were assessed with the DT40/XTT assay, and results were expressed as the Relative LD50, using both wild type and several DT40 mutants cells. Results showed that EB’s DDR was primarily TLS, EB-diol’s DDR was FA-TLS-HR, and DEB’s DDR was FA-TLS. Our results unraveled that EB-diol induced a DDR that correspond to the repair of a crosslink (DNA-DNA or DNA-protein crosslink). In humans, metabolism favor the hydrolytic pathway towards the formation of EB-diol. Our results offer a new perspective on how BD-epoxides DDR plays a role in improving our understanding in the importance of EB-diol in human risk assessment.

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1 Manuscript 3: This manuscript will be incorporated elsewhere for publication.
4.2 INTRODUCTION

4.2.1 Butadiene Epoxides

Epoxides that arise from Butadiene (BD) metabolism, their corresponding mutagenicity, and DNA adducts, have been extensively studied in vitro and in vivo: in rodents and humans. Overall, BD-epoxides have shown an inversely proportional relationship; whereas the most mutagenic epoxides are the least abundantly generated (DEB > EB > EB-diol) and the least mutagenic epoxides are the most abundantly generated (EB-diol > EB > DEB) (Meng, 2010, Swenberg 2011). To this day, DEB remains to be BDs minor metabolite formed across species, and has shown to be the most mutagenic and genotoxic of all three BD-epoxides. The overall individual epoxide mutagenicity varies greatly, with up to a ~200 fold difference in the following order: DEB > EB > EB-diol (Swenberg, 2011, Meng, 2007a, Meng, 2007b, Meng, 2010). An interesting observation was noted in EB-diol epoxide mutagenicity. EB-diol is composed of 4 different isomers, and it was observed that in vitro, the EB-diol 2R3S isomer was the most mutagenic when compared to the other three (2R,3R, 2S,3S, and 2S,3R) (Meng, 2010). When integrating these mutagenicity results with other BD-epoxides, the EB-diol isomer 2R,3S was 5-10 fold less potent than DEB, and 10-20 fold more potent than EB in both HPRT and TK locus (Meng, 2010, Swenberg, 2011).

4.2.2 BD-Epoxide DNA-Adducts

All three butadiene epoxides (EB, DEB, EB-diol) have the ability to react with DNA and form covalent adducts. These BD-DNA adducts can be subdivided into three categories: (1) DNA-monoadducts, (2) DNA-Protein crosslinks (DPC) or (3) DNA-DNA crosslinks (Swenberg,
In vitro, all BD-epoxides have demonstrated the capacity to alkylate all DNA bases. However, only two DNA bases have been found to be alkylated in vivo: with the most predominant being with Guanine (Gua), and to a lesser extent Adenine (Ade) (Tretyakova, 1998). Therefore, it is important to recognize BD-Gua adducts are relevant due to their higher abundance when compared to the BD-Ade adducts. In the literature, it has been reviewed that there are three key aspects of BD-DNA adducts: (i) the distinction of the N7 position of Gua as main site of alkylation from all DNA bases, (ii) EB-diol derived adducts are the multispecies predominant BD-DNA adducts in all tissues, (iii) mice are the species with the highest number of BD-DNA adducts formed, and (iv) liver is the target tissue for overall highest BD-DNA adduct formation.

The most metabolically abundant epoxide formed from BD is EB-diol, and consists of four stereoisomers (2R,3R; 2R,3S; 2S,3R; and 2S,3S) that react with Gua and form THB-Gua\(^2\). Followed in abundance is EB, which consists of two enantiomers (2S; 2R) that react with Gua generating HB-Gua\(^3\). Alternatively, some of these HB-Gua adducts can be transformed back into THB-Gua, if the remaining unbound portion becomes hydrolyzed by EH. The third and least abundant epoxide, DEB, consists of three stereoisomers (2R,3R; 2S,3S and meso-S,R/R,S) that react with Gua and form monoadduct DEB-Gua.

From all BD-Gua monoadducts adducts, two are considered to be the precursors of DNA-DNA crosslinks, or DNA-Protein crosslinks (DPC); HB-Gua and DEB-Gua. The only difference between HB-Gua and DEB-Gua is the opposite side of the chemical that remains unbound. Once HB-Gua is formed, the unbound portion can become hydrolized by EH and turn into more THB-Gua, or get oxidized by CYP450 and turn into DEB-Gua. Now, the DEB-Gua unbound epoxy-

\(^2\) N-7-(2,3,4-trihydroxybut-1-y1)guanine
\(^3\) HB-Gua I & II: N-7-(2-hydroxy-3-buten-1-yl)guanine & N-7-(1-hydroxy-3-buten-2-yl)guanine
ring remains available to react and produce different crosslinks depending on what the epoxide targets: (i) if it reacts with another Gua at the 7th position or an (ii) Ade at the 1st position they become DNA-DNA crosslinks. If it reacts with (iii) a protein it becomes a DPC. The most abundant BD DNA-DNA crosslinks are N7-Gua-N7-Gua\(^4\), followed by N7-Gua-N1-Ade\(^5\). The latter is rearranged into N7-Gua-N6-Ade (Goggin et al, 2009). In addition, it has been reported that the DEB-Gua unbound epoxy-ring target certain proteins, leading to DPC’s formation with: AGT\(^6\), GADPH\(^7\), Histone 3, and PARP1\(^8\) (Loeber, 2006, Loecken, 2007, Loecken, 2009 and Michaelson, 2010, and Gherezghiher, 2013)

### 4.2.3 BD-Epoxides Genotoxicity

Information on BD’s animal and human carcinogenic potential has been well documented in previous studies where they have examined, toxicity, DNA adduct formation, mutagenesis, and gene expression. BD requires metabolic activation to induce genotoxic damage through its reactive metabolites. These are capable of producing epoxide-specific genotoxic insults by directly alkylating DNA, and ultimately, generating several adducts that include: DNA-monoadducts, DNA-DNA adducts (DNA-DNA), and DNA-Protein cross links (DPC) (Swenberg et al 2001).

Extensive studies on BD-biomarkers of effect done in mice and rats, exposed to various concentrations of BD by inhalation have been previously reported. Results showed the induction of point mutations, large deletions, and chromosomal aberrations. For instance, in transgenic

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\(^4\) 1,4-bis-(guan-7-y1)-2,3-butanediol  
\(^5\) 1-(guan-7-y1)- 4-(aden-1-y1)-2,3-butanediol  
\(^6\) O\(^6\)-alkylguanine DNA alkyltransferase  
\(^7\) glyceraldehyde 3-phosphate dehydrogenase  
\(^8\) Poly [ADP-ribose] polymerase 1
mice (B6C3F1 laci) exposed to BD there was a statistically significant increase in A:T $\rightarrow$ G:C and AT: $\rightarrow$ T:A (traversions) mutations (Recio, 2001). Particularly, EB induces point mutations: where A:T $\rightarrow$ T:A (traversions) with small deletions, whereas DEB results in large deletions. However, no significant differences in sister-chromatid exchange frequencies, chromosomal aberrations or hprt mutations were found in studies of human occupationally exposed to BD (Albertini, 2001 and Albertini, 2007).

4.2.4 Gaps in Knowledge in DNA Repair

In Chapter1 and 3, we discussed how BD-epoxides molecular dose and tissue distribution have shown that metabolic activation of BD has species-dependent differences by using BD-Hb adducts. However, a clear distinction has to be made in the use of DNA vs Hb adducts. The lack of repair mechanisms in Hb-adducts are ideal to make inferences in metabolic activation contribution by each epoxide. However, DNA adducts can be more informative about potential differences in how DNA repair is modulated by each epoxide. Moreover, the formation of BD-DNA adducts provides indication(s) of one or more genotoxic event(s) that leads to non-mutational manifestations, contributing in the initiation of mutagenesis, and thus considered a relevant event to BD-carcinogenic mode of action (MOA). The formation of these BD-DNA-adducts across species have been previously studied, as well as their importance with regard to the genotoxicity of BD. Limited information is available regarding those DNA damage response (DDR) pathways involved in counteracting each specific BD-epoxide insult. Therefore, we have identified that BD-DDR is a gap in knowledge in BDs current biomarkers of exposure-effect database. Of particular interest, all EB-diol isomers will be pre-evaluated for cytotoxicity, to test reproducibility from that obtained by Meng’s in 2010, and if positive results are obtained, then
be included as part of the elucidation of what DDR pathways are involved in their repair.

4.2.5 DNA Damage Response XTT-DT40 System

Several in vitro cell-based assays have been developed to assess toxicity of chemicals, but none have presented to be as versatile, quick, reproducible and as cost effective as the DT40/XTT system. This application has been developed, validated as an integrated system for the assessment of DNA damage response with other chemicals (Ridpath 2007 & 2011). In addition, DT40 cells present many positive advantages that make them an ideal choice for this aim. DT40 cells were originally generated in 1985 from a chicken B lymphocyte cell line derived from an avian leucosis virus induced bursal lymphoma (Baba, 1985). These cells have provided a stable karyotype/phenotype, quick doubling time (8-10 hours) (Yamazoe, 2004) and lack functional p53 genes, to ensure the event of cell death to be a cause of defective repair upon DNA damage (Ulrich, 1992). In addition, these cells are not adherent and do not require trypsinization, reducing hands-on time and cell stress.

Adjustments have been made to perform experiments in both 24 and 96 well plates to include the use of multiple concentrations (6 concentrations in 24 well plates, and 9 concentrations in 96 well plates), with their corresponding controls and blanks. Following this plate format, exposures were performed in replicates, with controls and blanks; easing the process or reproducibility between experiments. DT40 cells and their multiple DNA-repair deficient mutants come from the same single parent cell (isogenic) and allowing direct multiple mutant comparisons. This system covers DNA repair pathway and cell cycle checkpoint mutants in base excision repair (BER), nucleotide excision repair (NER), homologous recombination
(HR), non-homologous end-joining (NHEJ), mismatch repair (MMR), translesion synthesis (TLS), DNA damage sensors and Helipses.

4.3 MATERIALS AND METHODS

4.3.1 Materials:

4.3.1.1 Chemicals for exposures and XTT

High purity EB and DEB were purchased from Sigma. Stereoisomers of EB-diol and racemic EB-diol were synthesized by Dr. Zhenfa Zhang (UNC-Chapel Hill Chemistry Core). All BD-epoxides were kept in aliquots and stored at -80°C.

4.3.1.2 Chemicals for cell culture

Fetal Bovine Serum and Chicken Serum, RPMI-1640 cell culture medium, penicillin, streptomycin, trypan blue dye, and PBS were purchased from Invitrogen (Grand Island, NY).

4.3.1.3 Cell Lines

4.3.1.3.1 DT40 and Mutants

DT40 normal and 28 different DT40 mutants [see Table 1] were cultured and maintained in RPMI-1640 cell culture medium, supplemented with 10% fetal bovine serum (heat inactivated), 1% chicken serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin and kept in humidified 5% CO₂ at 39.5°C.

4.3.1.3.2 TK6 cells

TK6 cells were a gift from Dr. Rebecca Fry, and were cultured in RPMI-1640 cell culture medium, supplemented with 10% fetal bovine serum (heat activated), 100 μg/ml penicillin, and 100 μg/ml streptomycin, and kept in humidified 5% CO₂ at 37.0°C.
4.3.2 Methods

4.3.2.1 Exposures

Before exposures, cells were counted using an hemocytometer and Trypan blue dye, to estimate required cell aliquot volumes required for 104,000 cells; in order to seed ~4,000 cells x 250 μL/well. A total of 7 mL of supplemented media was pre-warmed at the incubator and mixed with the cells aliquot, capped and gently tilted 10-times to ensure equal distribution of cells across the well plates. Plates were seeded and returned to incubation for ~2h to allow recovery from cell passage and to for cells to acclimate to incubation conditions.

Frozen stocks of 100mM of the BD-epoxides were left to thaw and serial dilutions were freshly prepared, Wells were distributed to accommodate from 9 to 20 concentrations of exposure in replicates, media blanks and vehicle controls [Figure 4.1].

a) EB-diol: (1,000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 0 μM).

b) EB: (500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95 and 0 μM).

c) DEB: (500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98, 0.49, 0.24 μM);&
(122.07, 61.04, 30.52, 15.26, 7.63, 3.81, 1.91, 0.95 and 0 nM)

Dilutions were done in PBS. Cells were exposed to for a period of ~48 hours, and monitored by microscope to verify that control wells reached desired confluence by visual inspection.

4.3.2.2 DT40/XTT Absorbance

At the end of the exposures, cells were dyed with an XTT cocktail returned to incubation at a lower temperature (33°C) to allow metabolic reduction of the dye by living cells without overgrowing them for ~8-12 hours (Ridpath, 2007 & 2011). The XTT dye developed from a
colorless yellow dye into a bright orange formazan product. The amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. The absorbance of the dye was quantitated with spectrophotometer Tecan Safire multi-well plate well reader (Tecan Systems San Jose, CA) and analyzed with Magellen6 software (Tecan version 6.4). Software was set to quantitate formazan dye at 450 nm with a 650 nm reference. Absorbance data was background corrected from each plate blanks and percentage of living cells was calculated using individual controls per plate.

### 4.3.2.2.1 Relative LD50 Calculation

Concentrations of exposures were converted into log values, and standard deviations were calculated using Excel. This data was further analyzed using GraphPad Prism 5 software for LD50 estimation using a log-linear regression and basic statistical analysis.

### 4.3.2.2 DNA Damage Response Categories

Our research group assessed the DNA Damage Response of BD-epoxides. Here we report quantitative data for EB-diol and qualitative data for EB and DEB, as EB-diol was the original objective of this project without excluding results of the other two epoxides and identify pathways similarities and differences. The dose required to induce the LD50 was first calculated for DT40<sup>wt</sup> and each DT40 mutant. These values were transformed into the Relative LD50 to generate an equivalent endpoint comparison across BD-epoxides. The relative LD50 was calculated by using the following formula:

\[
\text{Relative LD50} = \frac{\text{DT40 mutant LD50}}{\text{DT40wt LD50}}
\]
Results for all DT40<sub>wt</sub> LD50s were expressed as 1, and categories of response were created with cut-off lines to distinguish DT40 mutants degree of sensitivities and determine their significance. A Relative LD50 = or > than 1 was as sensitive as DT40<sub>wt</sub>, and therefore not a significant pathway involved whereas Relative LD50s < 0.50 were considered as required. Also, four subcategories of Relative LD50 sensitivities were created to describe; low sensitivity (0.76-0.99), moderate sensitivity (0.50-0.75), sensitivity (0.26-0.50) and high sensitivity (< 0.25) [Table 4.1]. A total of 28 cell lines were tested, including DT40<sub>wt</sub> and 26 DT40 mutants.

4.4 RESULTS AND DISCUSSION:

4.4.1 EB-diol Isomers [Limitations]

Initially, Dr. Zhenfa Zhang (UNC Chemistry Core), synthesized for us racemic EB-diol and each one of its stereoisomers (2R,3R, 2R,3S, 2S,3S, 2S,3R). Originally, we planned to test DNA Damage response pathways on every EB-diol isomer; of particular interest, EB-diol 2R,3S. The goal was to better understand if DNA repair was modulated differently, and be able to further explain how this was a driving factor in differential cytotoxicity and mutagenicity observed in isomer EB-diol 2R,3S (Meng, 2010). Cytotoxicity was extensively tested for all isomers, to verify reproducibility, to that previously reported. Cell lines Tk6 and DT40<sub>wt</sub> were simultaneously tested at concentration ranges (1,000, 500, 250, 125, 62.5, 31.25, and 0 μM) that included those previously tested.

Results in TK6 cells showed no significant difference in cytotoxicity amongst EB-diol or any of the individual isomers at all concentrations tested. Also, results with DT40<sub>wt</sub> confirmed similar results [Figure 4.2]. Combinations of paired isomers were also tested but in Tk6 cells only. No differences in cytotoxicity were observed (data not shown). The last effort was made to
acquire aliquots of those EB-diol isomers synthesized from the original experiments, to test them directly the DT40/XTT system, and analyze the purity of the material. No additional material from Meng’s experiments was available to perform additional tests or analysis. With this at hand, we had to redirect our efforts into testing all three BD-epoxides.

4.4.2 Butadiene Epoxides DNA Damage Response

The most sensitive DT40-/- mutant cell line for all three BD-epoxides was $REV1^{-/-}$ cells [Figure 4.11]. Interestingly, our findings not only connected REV1 with the TLS pathway, but also describes how the FA pathway is involved in the repair of EB-diol. This plays an new and important role in BD human risk assessment.

4.4.2.1 DEB DNA Damage Response

DEB DDR does not involve BER or MMR pathways due to the lack of sensitivity on any of the MMR or BER mutants treated with DEB [Table 4.3a and 4.4a]. The only DT40 mutant that exhibited any sensitivity in NER, and it was moderate in $XPA^{-/-}$ cells [Table 4.3b]. XPA has been implicated in the repair of crosslinks (Wood, 2010), maintaining helix stabilization when helicase TFIIH opens the strands in damaged areas (Fadda, 2016), and regulating cell cycle checkpoint kinase ATR (Chk1) (Martejin, 2014). The XPA protein seems to have a role in DEB DDR, but NER does not seem to be a primary pathway for repairing DEB damage.

TLS mutants with high sensitivity to DEB were $REV1^{-/-}$ cells, followed by $RAD18^{-/-}$ cells, sensitivity in $Pol \eta^{-/-}$ cells, and low to no sensitivity for $Pol \kappa^{-/-} > Pol \theta^{-/-}$ cells respectively [Table 4.4b]. Sensor RAD18 functions as a ubiquitin ligase in complex RAD18/RAD6, which mono-ubiquitylates polymerase clamp PCNA. This enhances the clamp interaction with adaptor REV1
and facilitates the switch from a replication polymerases, to a TSL polymerase to perform lesion bypass. REV1’s function in TLS is to incorporates dCTP\(^9\) opposite to AP-sites and adducted Gua (Nair, 2005). REV1 also participates in the FA pathway. When a replication fork encounters an ICL, the FA core complex recruits a secondary complex formed by both REV1 and Polymerase \(\zeta\) (Pol zeta) (Budzowska, 2015), to promote the extension step during ICL repair (Räschle et al, 2008). It our understanding then that DEB DDR primarily involves the bypass of mono adducts and crosslinks via TLS, in particular via polymerase Pol \(\eta\). TLS polymerase Pol \(\eta\) has been found to specialize in the bypass intrastrand T-T dimers (Rey, 2009). In parallel, DEB can not only intrastrand crosslinks, but also intrastrand crosslinks, making Pol \(\eta\) an important player in the bypass of these lesions. We are not excluding the participation of other specialized polymerases in the repair of DEB induced damage, with the exception of Pol \(\theta\) due to its lack of sensitivity in this assay.

In both FA and HR pathways, DEB induced the highest sensitivity in mutant cells \(\textit{FANCD2}^{-/-}\) > followed by \(\textit{BRCA2}^{-/-}\) > and moderate sensitivity in \(\textit{XRCC2}^{-/-}\) and \(\textit{XRCC3}^{-/-}\) [Table 4.5a and 4.5b]. However, the lack of sensitivity in specific HR mutant cells \(\textit{BRCA1}^{-/-}, \textit{RAD54}^{-/-}, \textit{RAD51c}^{-/-}, \textit{RAD51d}^{-/-}, \textit{BLM}^{-/-},\) and \(\textit{WER}^{-/-}\) clearly indicates that FA repair does require the direct involvement of the HR pathway. Here, DEB DDR primarily requires FA proteins FANCD2 and BRCA2, and HR proteins XRCC2 and XRCC3 but only moderately. The FA pathway repairs ICL (Ceccaldi, 2016, Giglia, 2011) such as DEB-crosslinks. Both FANCD2 and BRCA2 proteins participate in the initial steps of unhooking the ICL from the DNA double strand (Ceccaldi, 2016). Alternatively, proteins XRCC2, and XRCC3 are part of the group that carries out the invasion-extension in HR (Sung, 2006). Since DEB induced moderate sensitivity in \(\textit{XRCC2}^{-/-}\)

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\(^9\) dCTP: Deoxycytidine triphosphate (Cytosine nucleotide)
and XRCC3−/− we understand that both XRCC2 and XRCC3 proteins participate in DEB DDR, but independently from HR related repair.

Mutant cells from the NHEJ pathway showed that DEB induces moderate sensitivity in Ku70−/− LIGIV−/− cells, whereas the sensitivity in DNAPKcs−/− cells was low [Table 4.6a]. Protein Ku70 forms part of the Ku70/Ku80 recognition complex that binds to DSB-DNA, and recruits other NHEJ intermediates to trim (Burma, 2006) and ligate then un-even DNA ends via Ligase IV [complex XRCC4/Ligase IV] (Lieber, 2008). DEB DDR in NHEJ presents that proteins Ku70 and LIGIV have a modest participation in the recognition and sealing of the broken ends of DSB without the specific involvement of intermediate DNA-PKcs. This suggests that NHEJ plays an alternative role in DEB DDR.

The DNA Damage Sensor mutant RAD17−/− was moderately sensitive to DEB. Cell cycle checkpoint mutant ATM−/− sensitivity was low. [Table 4.6b]. DEB crosslinks can lead to stalled replication forks. The exposed single-stranded DNA gets coated by Replication protein A (RPA), leading to the recruitment and binding of DNA Damage Sensor RAD17 and ATR kinase complex [ATR-ATRIP] stimulating the activation of Chk1, to hold cells at the G2 phase (Marechal, 2013, Zhang, 2014). Sensor RAD17 also recruits the clamp-shaped 9-1-1 complex [RAD9/HUS1/RAD1] around damaged DNA (Parrilla, 2004) to function as a checkpoint coordination, activation of DNA repair (Delacroix, 2007) and to facilitates ATR-phosphorylation of Chk1, regulating cell cycle and slowing down replication. At this point, the replication fork stabilizes to preventing stalling that could lead to DNA strand breaks. DEB DDR partially requires sensor RAD17 signaling for the recruitment of other downstream proteins involving repair, fork stalling prevention. However, the lack of sensitivity in ATM−/− cells suggests that DEB DDR responds independently of cell-cycle checkpoint ATM.
4.4.2.2 DEB DNA Damage Response Pathway

4.4.2.2.1 DEB Monoadducts

We hypothesize that the primary DEB DDR will be to bypass monoadducts via TLS. DEB predominantly generates monoadducts THB-Gua and THB-Ade. Whereas THB-Ade adducts may persist, THB-Gua adducts will undergo spontaneous depurination, creating AP-sites. A high-fidelity replicative polymerase will recognize both DEB monoadducts and AP-sites as DNA damage at the replication fork. Exposed single-stranded DNA gets coated by protein RPA. This triggers the recruitment and DNA binding of sensor RAD17 and ATR kinase complex [ATR-ATRIP]. This is followed by the activation of Chk1, arresting cells at the G2/S phase. RAD17 recruits the clamp-shaped 9-1-1 complex [RAD9/HUS1/RAD1] near damaged DNA to activate its repair. Simultaneously, RPA coated DNA stimulates ubiquitin ligase-RAD18 to form complex RAD18/RAD6. The complex mono-ubiquitylates the PCNA polymerase clamp, enhancing its interaction with REV1, and recruiting TLS polymerase Pol η. Replication is resumed by Pol η to bypass the DEB monoadduct or AP-sites and extend replication for a short track. The final step is the replacement of the TLS polymerase by the original replicative polymerase. The strands are then ligated to close the gap when it reaches an Okazaki Fragment.

4.4.2.2.2 DEB Crosslinks

We hypothesize that a mixture of all variations of DEB-crosslink will have a DDR that involves FA-TLS coupled repair. A replicative polymerase encounters the DEB-crosslink at the replisome/replication fork. Exposed single-stranded DNA gets coated by protein RPA, stimulating the recruitment of DNA damage sensors RAD17 and RAD18. Sensor RAD17 and
ATR kinase complex [ATR-ATRIP], to activate Chk1, and arrest cells at the G2/S phase. The 9-1-1 DNA clamp assembles near damaged DNA site to activate its repair.

First, the FA pathway will stabilize the replication fork. The DEB-crosslink is recognized by FANCM with FAAP24 by associating with the DNA-bound MHF complex (MHF1 and MFH2). The FANCM-FAAP-24 serves as a platform to recruit and assemble the rest of the FA core complex, ultimately acting as a ubiquitinating ligase. Sensor RAD18 recruits PCNA via mono-ubiquitilation, initiating the convergence of TLS pathway and the FA pathway. Ubiquitilated PCNA also promotes the recruitment of both FANCL and FANCD2 subunits. Subunits FANCL and FANCT of the DNA-bound FA-core complex proceeds to mono-ubiquitinates FANC-ID [FANCD2-FANCI], loading onto DNA, and initiating the ICL-unhooking process. The FANCD2 protein allows FANC-ID to control the incision of the DEB-crosslink from one of the sides of the DNA strand near the replication fork by two effector proteins: nucleases FANCP and FANCQ. The DEB-crosslink is cut out from one side of the parental strand, resulting in (1) a strand with an anti-space (or DSB), and (2) the opposite strand with the one-sided left-over DEB-crosslink.

The second step is processing the strand that has the leftover DEB-crosslink via TLS bypass. First, protein FAAP20 binds to the FA core complex forming FAAP20-FA core complex. The RAD18 ubiquitinated PCNA clamp, in conjunction with the FAAP20-FA core complex, assembles TLS related complex PCNA-REV1 via ubiquitin-binding with REV1 (Mirchandani, 2008, Kim, 2012, Kim & D’Andrea, 2012). This interaction switches the replicative polymerase for REV1 and Pol η. Replication is resumed by Pol η, bypassing the leftover DEB-crosslink, and extending replication for a short track. Once the extension is completed beyond the lesion (Räschle, 2008), Pol η is switched back to the replicative
polymerase. The final product is ligated to close the gap when it reaches an Okazaki Fragment (Buzowska, 2015).

DEB generates six predominant DNA-crosslinks, favoring the formation of inter-strand over intra-strand DNA crosslinks (Millard 1993, Sawyer, 2004, Parker, 2005). Inter-stand DEB-crosslinks include: (1) N7-Gua-DEB-N7-Gua, (2) N7-Gua-DEB-N6-Ade, (3) N7-Gua-DEB-N3-Ade, and (4) N6-Ade-DEB-N6-Ade. Intra-strand DEB-crosslinks are: (5) N2-Gua-DEB-N2-Gua and (6) N7-Gua-DEB-N7-Gua. REV1 in TLS to bypass these DEB-crosslinks by directly incorporating a dCTP opposite to the crosslink. This could potentially create post-repair mismatches on DEB-crosslinks that have adducted Ade by incorporating a Cyt via REV1.

4.4.2.3 EB DNA Damage Response

All mutant cells in BER (PARP1−/−, Pol β−/−, and FEN1−/− cells), NER (XPA−/−, and XPG−/− cells), had no sensitivity to EB [Table 4.3a and 4.3b]. MMR mutant (MSH3−/− cells) had low sensitivity to EB [Tables 4.4a]. EB DDR does not depend on BER or NER repair of due to the lack of sensitivity in the panel of mutant cell lines for those pathways. MSH3−/− low sensitivity denotes that if the MSH3 protein participates in EB monoadducts repair, it could be MMR independent.

In the TLS pathway, EB induced sensitivity in mutant cell line REV1−/−, moderate sensitivity in RAD18−/− cells, and similar sensitivity to that of wt in Pol κ−/− and Pol θ−/− cells [Table 4.4b]. EB DDR is via TLS bypass of EB monoadducts but without requiring the involvement of polymerases Pol κ, Pol θ. However, what it will require is the stimulation of ubiquitin ligase-RAD18 to activate REV1 and perform repair.
All FA mutant cells (\(FANCD2^{+/−}\), \(BRCA2^{−/−}\), \(BRCA1^{−/−}\), and \(RAD51c^{+/−}\)), showed that EB sensitivity was close to that of wt [Table 4.5a]. HR mutant cells EB sensitivities were in the following order: moderate sensitivity in \(RAD51d^{+/−}\), > \(RAD54^{+/−}\) = \(XRCC2^{−/−}\) and low sensitivity in \(XRCC3^{−/−}\) [Table 4.5b]. The remaining HR mutant cell lines (\(BRCA2^{+/−}\), \(BRCA1^{−/−}\), \(RAD51c^{−/−}\) and \(BLM^{−/−}\)), were close to that of wt. Proteins RAD51d, RAD54, XRCC2 and XRCC3 are implicated in the invasion-extension step of HR (Sung, 2006). EB DDR is primarily carried via other pathways, however, the moderate/low EB sensitivity suggests that proteins RAD51d, RAD54, XRCC2 and XRCC3 could participate in the repair of EB monoadducts, but independently from HR pathway.

NHEJ deficient cell lines, those that showed sensitivity to EB were in the following order: moderate sensitivity in \(Ku70^{+/−}\) > \(LIGIV^{+/−}\) >, and low sensitivity in \(DNAPKcs^{−/−}\) [Table 4.6a]. EB DDR is potentially modulates repair by recognizing broken ends via Ku70, and sealing the ends by LIGIV. The low sensitivity in DNAPKcs suggests that NHEJ may be an alternative pathway for the repair of EB monoadducts.

DNA Damage Sensor and Cell cycle checkpoint EB sensitivities were moderate in \(RAD17^{−/−}\), but > \(ATM^{−/−}\) [Table 4.6b]. The DNA Damage Sensor RAD17 recruitment of the 9-1-1 complex, and DNA binding of ATR kinase complex [ATR-ATRIP] to activate Chk1 to arrest cells at the G2/S phase to initiate DNA repair.

EB DDR moderately requires RAD17 signaling for the recruitment of other downstream proteins involving repair and fork stalling prevention but its ATM independent.
We hypothesize that primary EB DDR will be to bypass monoadducts HB-Gua, HB-Ade and HB-Ura via TLS [Figure 4.13]. During replication, a high-fidelity polymerase can encounter EB monoadducts and stall at the replication fork. Exposed single-stranded DNA gets coated by protein RPA stimulating ubiquitin ligase-RAD18 to form complex RAD18/RAD6. This mono-ubiquitylates polymerase clamp PCNA, enhancing its interaction with adaptor REV1 to facilitate the switch from a replication polymerases, to a TLS polymerases and bypass these lesions and AP-sites. REV1 is known to incorporates dCTP $^{10}$ opposite to AP-sites and adducted Gua (Nair, 2005).

EB predominantly generates mono-adducts; with Guanine (HB-Gua) at N2 and N7 positions, with Adenine (HB-Ade) at the N3 and N6 positions, and one with Uracil (HB-Ura) at the N3 position. Adducts of HB-Gua at the N7 position are more abundant than those at the N2 position, but the latter are more mutagenic$^{11}$ and hydrolytically stable. N3-HB-Ura are exceptionally unstable and depurinate a lot quicker than the N7-HB-Gua. Adducts N2-HB-Gua and N3-HB-Ura have shown partial or complete block of Pol η (Chang, 2017). After 48 hours of EB treatment, at least half of the original N7-HB-Gua adducts (half-life of 50 hours) will spontaneously depurinate, generating AP-sites (Boysen, 2009). Due to the unstable nature of the N3-HB-Ura we would also expect them to undergo depurination, leaving AP-sites. The resulting adducts and its AP-sites will mainly be processed by TLS. Here, REV1 incorporates dCTP without potentially creating mismatches, since Gua pairs with Cyt. Also, REV-1, would

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$^{10}$ dCTP: Deoxycytidine triphosphate (Cytosine nucleotide)

$^{11}$ N2-HB-Gua is more mutagenic for the S isomer.
incorporate dCTP opposite to N2-HB-Gua, and N6-HB-Ade to bypass these adducts by inserting a Cyt. This step won’t create a mismatch for adduct N2-HB-Gua, however it could generate a mismatch for adduct N6-HB-Ade. In, addition, REV1 incorporation of dCTP opposite to AP-sites from N3-HB-Ura, will also generate a mismatch. This confirms that the primary DDR in the repair of EB DNA damage is via TLS bypass by REV1 of adducts N7-HB-Gua, N2-HB-Gua, N3-HB-Ade and N6-HB-Ade adducts and AP-sites.

4.4.2.5 EB-diol DNA Damage Response

BER deficient PARP1−/− cells were sensitive, Pol β−/− cells were moderately sensitive, and FEN-1−/− cells had low sensitivity [Table 4.3a]. EB-diol DDR requires BER via PARP1 and Pol β. PARP1 seems essential in the recognition and repair of strand breaks, and AP-sites generated from the spontaneous depurination of THB-Gua. However, it can’t be excluded that this unique sensitivity in PARP1−/− mutant cells could reflect cellular stress in response to EB-diol exposure by depleting glutathione, leading to an indirect increase in reactive oxygen species. The depletion of glutathione for all BD epoxide isomers, including EB-diol isomers have been previously evaluated in vitro. In liver cytosol fractions from rat hepatocytes, EB-diol cytosol mediated removal of glutathione was 10-35%, without any differences in reactivity amongst isomers (Niesuma, 1998). Pol β is moderately involved in filling-in those AP-sites generated from removed bases by a glycosylase, or perhaps lost via spontaneous depurination of THB-Gua. Pol β participates in removing adducted nucleobases from the sugar-phosphate backbone, repairs AP-sites, and single strand breaks (Kim, 2012a). FEN1 typically plays an essential role in long patch BER repair by cleaving displaced oligonucleotides before the nick is sealed with a ligase (Robertson, 2009). The lack of sensitivity in our experiments for FEN1 confirms that EB-diol
DDR responds preferentially via short patch, rather than long patch BER. EB-diol treatment seems to primarily induce DDR via short-patch BER to repair adducts THB-Gua or THB-Ade, AP-sites, any EB-diol induced strand breaks as sensed by PAPR1, as well as potential oxidative damage.

NER deficient cell lines $XPA^{-/-}$ and $XPG^{-/-}$ presented moderate sensitivities [Table 4.3b]. EB-diol DDR in NER moderately requires both XPA and XPG. Our results suggest that post-EB-diol treatment, (1) a DNA adduct is causing some helix distortion, perhaps a DNA-Protein crosslink (DPC) or (2) an intra-strand crosslink. The NER pathway readily removes intra-strand crosslinks (O'Donovan, 1994, Huang, 2013). The most predominant EB-diol monoadducts formed in vivo are N7 THB-Gua, N2 THB-Gua, and N6 THB-Ade. Although EB-diol monoadducts might not distort the DNA helix, they could affect its flexibility if the unbound trihydroxy group that’s available reacts with other proteins, resulting in a DPC. Overall, the moderate sensitivity of NER related proteins to EB-diol indicated that this pathway is an alternate pathway of repair. MMR cell line $MSH3^{-/-}$ sensitivity was similar to that of wt [Table 4.4a], and thus EB-diol DDR does not involve MMR to recognize and repair mismatched bases via complex MutSβ.

TLS mutant cell line $REV1^{-/-}$ had the highest sensitivity to EB-diol, followed by sensitivity to $RAD18^{-/-}$, moderate sensitivities in both $Pol \kappa^{-/-} > Pol \theta^{-/-}$, and $Pol \eta^{-/-}$ was as similar as wt [Table 4.4b]. EB-diol will trigger a DDR via TLS, relying on REV1 to bypass the DNA damage, initially via stimulation of ubiquitin ligase-RAD18. REV1 incorporates dCTP nucleotides opposite to AP-sites and/or THB-Gua adducts. In addition, TLS polymerases: Pol κ may also participate in the bypass over Pol θ, but not Pol η.
Sensitivities in FA and HR pathway mutant cells against EB-diol were in the following order: highly sensitive $\text{RAD54}^{+/}$ > $\text{BLM}^{+/}$, sensitive $\text{FANCD2}^{+/}$ > $\text{XRCC2}^{+/}$ > $\text{BRCA2}^{+/}$ > $\text{BRCA1}^{+/}$, moderately sensitive $\text{WER}^{+/}$ > $\text{XRCC3}^{+/}$ = $\text{RAD51c}^{+/}$, and low sensitivity $\text{RAD51d}^{+/}$ [Table 4.5a and 4.5b]. EB-diol DDR requires these proteins to stabilize replication forks and repair an ICL and/or double strand break by a combination of pathways that includes the FA/BRCA as a primary pathway. In addition, EB-diol induces “BRCAness” as marked in both $\text{BRCA1}^{+/}$ and $\text{BRCA2}^{+/}$ mutant cells sensitivities. When combining results from previous EB-diol DDR sections, we see both HR and TLS pathways functioning downstream the FA pathway. It is strongly implied that EB-diol treatment triggers a DDR that responds to the repair of an unidentified ICL and or DSB breaks. This kind of DDR was unexpected for EB-diol using the existent BD DNA-adduct data.

EB-diol DDR does not seem to require NHEJ for DNA repair. $\text{Ku70}^{+/}$ > $\text{LIGIV}^{+/}$ cells had moderate sensitivities, and $\text{DNAPKcs}^{+/}$ cells had low sensitivity [Table 4.6a]. Its implied then that EB-diol modulates DSB repair (if any) by recognition of the broken ends via Ku70 competitive binding against PARP1. Our previous results in PARP1 sensitivity for EB-diol were greater than the sensitivity of Ku70. The low sensitivity in $\text{LIGIV}^{+/}$, $\text{DNAPKcs}^{+/}$ suggests that NHEJ could be an alternative pathway.

EB-diol sensitivities are in the following order: $\text{RAD17}^{+/}$ > $\text{RAD9}^{+/}$ > $\text{ATM}^{+/}$ [Table 4.6b]. EB-diol DDR relies heavily on RAD17 signaling for the recruitment of RAD9 as part of the 9-1-1 complex, which activates DNA repair, and we speculate it potentially facilitate ATR-phosphorylation of Chk1 to prevent fork stalling by inducing G2/S arrest.
Our results for lack of ATM sensitivity in EB-diol, suggests that ATR signaling is favored. EB-diol treatment is likely to induce stalled replication forks due to a possible EB-diol-ICL.

4.4.2.6 EB-diol DNA Damage Response Pathway

4.4.2.6.1 EB-diol Monoadducts

EB-diol generates monoadducts; at the N2 and N7 positions of Guanine (THB-Gua) and at the N6 position of Adenine (THB-Ade). Adducts of THB-Gua at the N7 position are more abundant than those at the N2 position, but the latter are more mutagenic\(^{12}\) and hydrolytically stable. In addition, N2-THB-Gua lesions have previously shown to partially block Pol \(\eta\) (Chang, 2017). In our results, after 48 hours of EB-diol treatment, we expect that at least half of the original N7-THB-Gua to remain while the other half will leave behind AP-sites due to spontaneous depurination. Both the N7-THB-Gua adduct and the AP-site will rely on REV1 for the incorporation of dCTP without potentially creating mismatches, since G pairs with C. REV1, unlike any other polymerases, highly specializes in the incorporation of dCTP over any other nucleotide in adducted Guanines, particularly in N2-adducted-Gua, which have shown to obstruct replication (Nair, 2008, Choi, 2008). This also clarifies that those N2-THB-Gua adducts will also depend on REV1 for bypass via incorporation of Cyt opposite to the adducted-Gua without creating mismatches. However, if regular REV1 activity incorporates a dCTP opposite to an N6-THB-Ade adduct, a mismatch is expected.

REV1 also participates with other DNA repair pathways. In stalled replication forks, REV1 participates to resolve ICL lesions as part of the Fanconi Anemia (FA) pathway (Ceccaldi, 2016). Initially, REV1 forms a complex with B-family polymerase Pol \(\zeta\) to perform the

\(^{12}\) For the S,S isomer
extension step during ICL repair. We did not perform tests for Pol ζ. Moreover, a specific component of the FA core complex (FAAP20), interacts and recruits REV1, in collaboration with ubiquitylated PCNA via RAD18, to promote TLS (Mirchandani, 2008, Kim, 2012, Kim & D’Andrea, 2012), without involving the FANC-ID complex (Budzowska, 2015). It has not yet been documented that EB-diol can induce an ICL, however the spectrum of DDR presented here strongly suggests that possibility (see FA/HR Pathway).

In addition to REV1’s involvement in the repair of EB-diol related adducts, we also found that TLS polymerase Pol κ and Pol θ (polymerase also involved in alternative end-joining), were sensitive and therefore part of the repair “team” but to a lesser extent than REV1 and RAD18. Some of the THB monoadducts could be detected as bulky adducts, and will partially require to be repaired/bypassed during replication via Pol κ. Previous studies have noted the recruitment of Pol κ upon DNA damage from benzo[a]pyrene diol epoxide (BPDE) as essential in the repair of bulky adduct benzpyrene diol epoxide-Gua (Suzuki, 2002, Avkin 2004). Pol κ can also accurately bypass benzopyrene-N2-Gua adducts via TLS (Ohashi 2000, Makridakis, 2012). Pol κ has demonstrated to accurately bypass a N2-Gua-N2-Gua ICL in vitro following mitomycin C exposure (Minko, 2008). In the event that a N2-Gua-N2-Gua ICL from EB-diol is formed, Pol κ could potentially ensue in its repair. Therefore, we speculate that Pol κ is cooperating with REV1 in the bypass of N2-THB-Gua, N6-THB-Ade, N7-THB-Gua, including a possible not-yet-identified EB-diol crosslink via TLS with or without involving the FA pathway.

The moderate sensitivity of Pol θ−/− cells may suggest the repair of DSB via alt-EJ as playing as an important role in EB-diol repair. Pol θ efficiently incorporates an Ade opposite to AP-sites and extend past them (Seki, 2004, Knobel, 2011). Most AP-sites we expect to be generated consequently from N7-THB-Gua spontaneous depurination. It is therefore that this
could lead to mismatches due to mis-incorporation of Ade at the AP-site, instead of the corresponding Cyt. The lack of sensitivity in Pol η−/− cells to EB-diol points out that the generation of intrastrand-crosslinks (such as T-T dimers) are unlikely or that Pol η does not play an important role in EB-diol repair.

Overall, we haven’t excluded the possibility of THB-monoadducts as precursors of DPCs via reaction of the unbound tri-hydroxy group. DPCs are considered bulky adducts, and future experiments should consider to test N2-Gua-THB, N7-Gua-THB and N6-Ade-THB as well as EB-diol with the hydroxy groups in distinct positions as precursors of these unidentified DPCs. Recent methods have been developed and applied for the isolation and analysis of DPCs from BD-epoxide DEB in vitro using HeLa cells (Tretyakova, 2015), however EB-diol was not included in their experiments. A new research project should considered future experiments that identify and characterize these target proteins from nuclear protein extracts of DT40 cells followed by proteomic analysis of DPCs.

4.4.2.6.2 EB-diol ICL

We hypothesize, that EB-diol is generating an un-identified ICL. The induced sensitivity in our panel of mutant cells have identified a combination of different DDR pathways that combine FA-TLS-HR.

First, the “EB-diol ICL” is generated, leading to a replication forks stall. ATR is initially activated and recruited to DNA through RPA bound to single stranded DNA as a result of stalled replication forks, or from exposed single stranded 3’ overhangs after HR DNA resection. ATRIP13 then recruits ATR to single stranded DNA and activated by topoisomerase binding partner 1 (TOBP1), and into a complex with clamp loader Rad17-Rfc2-514, clamp formed by the

13 ATR-interacting protein
14 Replication Factor C Subunit 2
9-1-1 complex, Claspin and RHINO\textsuperscript{15} (Brown, 2016). The adapter protein Claspin binds to BRCA1 and kinase Chk1 to facilitate the ATR phosphorylation of both, whereas RHINO binds to the 9-1-1 clamp and TOPBP1 to stabilize them (Lindsey-Boltz, 2015). CHK1 is then activated by ATR, inhibiting cyclin-dependent kinase (CDK) activity via phosphorylation of CDC25A to regulate the G2/S cell cycle arrest and preventing the replication fork to collapse.

The sensitivity in and \textit{FANCD2\textsuperscript{−/−}} and \textit{RAD18\textsuperscript{−/−}} mutants denote how their corresponding proteins are important in the process of recognition of the ICL via FA as well as initiation of TLS [\textbf{Figure 4.14 and 4.15}]. First, the ICL is recognized by FANCM with FAAP24 by associating with the DNA-bound MHF complex (MHF1 and MHF2) to commence the assembly of the FA core complex. FANCM-FAAP-24 acts as a platform to recruit the rest of the core complex (10 remaining proteins). Once assembled, it acts as a ubiquiting ligase. In parallel, sensor RAD18 recruits PCNA via mono-ubiquitilation, initiating the convergence of TLS pathway and the FA pathway. Ubiquitilated PCNA promotes the recruitment of both FANCL and FANCD2. Here, phosphorylation of FANC-ID [FANCD2+FANCI] activates the complex and leads it to interact with the DNA-bound FA-core complex.

The FA-core complex subunits FANCL and FANCT mono-ubiquitinates FANC-ID [FANCD2-FANCI] leading to its loading onto DNA and initiating the ICL-unhooking process. The FANCD2 protein allows FANC-ID to control the incision near the replication fork by its effector proteins. Recruited nucleases FANCP and FANCQ cut and release the ICL from one of the two parental strands, leaving (1) a strand with an anti-space (or DSB), and (2) another strand with the left-over lesion [\textbf{Figure 4.15}].

\textsuperscript{15} \textit{Rad9, Rad1, Hus1 interacting nuclear orphan}
The bypass of the one-sided-left-over lesion will be carried on via TLS [Figure 4.16]. When the high-fidelity replication polymerase encounters this DNA damage, it stalls at the replication fork. Exposed single-stranded DNA gets coated by RPA, stimulating ubiquitin ligase-RAD18 to form complex RAD18/RAD6. The complex mono-ubiquitylates PCNA polymerase clamp, to enhance its interaction with REV1, and recruiting TLS polymerases. Simultaneously, protein FAAP20 binds to the FA core complex forming FAAP20-FA core complex. Here, both RAD18 ubiquitinated PCNA, and now the FAAP20-FA core complex assembles the TLS related complex PCNA-REV1 via ubiquitin-binding with REV1 (Mirchandani, 2008, Kim, 2012, Kim & D’Andrea, 2012). This interaction allows direct lesion bypass on the strand with the ICL leftover via TLS recruitment of REV1, and forming a complex with polymerase Pol ζ, or Pol θ at the replication fork to perform the extension step. Once the extension is completed beyond the ICL (Räschle, 2008), the TLS polymerase is switched back to the replicative polymerase. The final product is one of the two strands being ligated to close the gap when it reaches an Okazaki Fragment (Buzowska, 2015).

The bypassed ICL left over is excised by the NER pathway during either transcription or replication [Figure 4.17]. During transcription, the ICL leftover could lead polymerase RNAP2 to stall (in TC-NER). Intermediate CSB recruits CSA and stabilizes the replication block by repositioning RNAP2 past the lesion. Alternatively, the damage is recognized upon binding by intermediate complexes, XPC/hHR23B, with or without DDB/XPE (in GG-NER). Either pathway will recruit helicase TFIIH to unwind the DNA helix, XPA and RPA to stabilize the helix opening, recruit endonuclease XPG and ERCC1-XPF and make incisions 5’ and 3’ respectively from the lesion. DNA replication proteins fill the single strand gap (RFC, PCNA,
RPA, and DNA polymerases δ, ε, or κ) and nicks are then sealed by DNA ligases I or III (Giglia, 2011).

The induced sensitivity of EB-diol in PARP1−/− cells, and moderate sensitivity in Pol θ−/− cells suggests the alt-EJ pathway as minor contributor in DDR. Others have identified that Pol θ as a multifunctional polymerase/helicase that promotes alt-EJ (Wood, 2016), and PARP1 has been suggested as a facilitator protein in the recruitment such alt-EJ repair factors, including TLS polymerases (i.e. Pol θ) (Black, 2016, Ceccaldi, 2016). Both alt-EJ and HR share the initial DSB resection. Specifically, in HR, resection is extended and RAD51 proteins help in forming filaments on the 3’ overhangs, driving sister chromatid exchange, while in alt-EJ is thought that PARP1 mediates annealing using micro-homologies. Therefore, we are inclined to imply that the combination of FA-TLS pathways will distribute the next stage of repair between two sub-pathways. Each pathway will be dependent on the TLS polymerase that performed the bypass. If Pol κ performs the bypass then the following step will be HR and will be less likely to introduce errors. However if Pol θ performs the bypass then alt-EJ will follow but will be more likely to introduce errors. Our results for EB-diol showed Pol κ−/− and Pol θ−/− cells as sensitive, however, the sensitivity in Pol κ−/− cells was greater than that of Pol θ−/− cells.

Our previously repaired one-sided ICL will now serve as template for repairing the opposite strand that has the unrepaired anti-space via HR [Figure 4.18]. Because an incision was made at the replication fork, it created a DSB as a by-product of DNA repair. First, HR initiates the repair of the DSB via DNA-binding of sensor PARP1. This leads to the recruitment of the nuclease active MRN-CtIP complex and the BRCA1/BARD1 for the resection of DSB ends. Exonucleases: EXO1, and DNA2, associate with DNA unwinding helicases BLM and WER to extend the resections several 1,000 base pairs. The product of the resected double strands are 3’
single strand tails (or overhangs). In-tandem, RPA proteins coat these exposed 3′ single strand tail regions. Here, BRCA1/BRCA2/PALB2 assist RAD51 in replacing RPA proteins from the 3′ single strand tail regions by forming RAD51 single stranded nucleofilaments through its RAD51 paralogues RAD51c, RAD51d, XRCC2, and XRCC3. These pre-synaptic filaments, binds duplex DNA to form the synaptic complex and “searches” for DNA homology in a duplex-sister-chromatid obtained from the previously repaired one-sided ICL. This process is facilitated by several proteins that include Rad54, allowing single stranded DNA invasion of the homologous region in the duplex and forming a displacement loop (D-loop). Strand exchange occurs, and complementary DNA is synthesized by polymerases from the 3′ to 5′ using the sister chromatid as a template. We propose that the previous intermediate products from strand exchange, result in a double Holliday Junction that needs to be resolved by resolvase enzymes. These resolvases include both the activity of a complex formed in part by BLM helicase and Topoisomerase III α [BTR complex], and are arranged in a complex that promotes the dissolution of the double Holliday junction, resulting in the exclusive formation of non-crossover products (Kitano, 2014). Once the process is completed, FANCD-ID is de-ubiquitilated, the ends are sealed by a DNA ligase completing the EB-diol ICL repair (Chu, 2014, Sung, 2006, Chen, 2017, De Lorenzo, 2013, Giglia, 2011).

4.5 CONCLUSIONS

Understanding the importance of BD-DNA adducts has been discussed regarding the genotoxicity of BD. However, limited information was available to date as to which DNA damage response pathways were essential for cells to counteract BD-epoxides damage. To this date, DT40 cells provide the most extensive collection of DNA repair mutant clones in vertebrate cells to assess DNA Damage Response using a high-throughput in vitro assay. The biological
significance of these DNA Damage Responses was established with by employing the DT40/XTT method to assess BD-epoxides, and their sensitivities. Effects were expressed by the Relative LD50, using DT40 parental cells and twenty seven DT40−/− mutants. These mutants were deficient in seven DNA repair pathways, including: BER, NER, MMR, TLS, FA, HR, NHEJ, with Damage Sensors, and Cell Cycle Checkpoints.

Overall results showed that REV1−/− was the most sensitive mutant cell line across BD-epoxides. REV1 is not only an pivotal protein in TLS, but also represents the common link between DNA Repair pathway for FA and TLS. Irrespectively of differences on the corresponding DNA adducts formed by each epoxide, the primary form of response upon DNA damage is via bypass via TLS with the difference being the specific TLS bypass polymerases involved. The second common observation was that epoxides DEB and EB-diol had similar DDR signatures for certain pathways. For instance, in the FA pathway both DEB and EB-diol overlapped with sensitivity for mutant cells FANCD2−/− and BRCA2−/−. This was perhaps one of the most striking and unexpected results to this date for EB-diol. There is extensive evidence that supports the generation of DEB crosslinks, but no documentation of EB-diol crosslinks (ICL) has been presented to this date. Also, both FA mutant cells BRCA1−/− and BRCA2−/− had induced sensitivity by EB-diol, and different from DEB where no sensitivity was seen in BRCA1−/− mutants. This indicates that the recognition and excision to unhook the ICL was similar for both epoxides. However, for EB-diol, sensitive BRCA1−/− cells signifies that the DSB needs to be processed via resection as part of the HR pathway. In contrast, EB did not induced FA or HR mutants sensitivity and DEB did not induced the latter. One of the highlight findings was EB-diol’s unique DDR signature through six DT40 both highly sensitive and sensitive mutants, that were not sensitive for EB nor DEB. These unique EB-diol mutant cells were BLM−/−, RAD17−/−,
PARP1<sup>−/−</sup> and the combined sensitivity in both BRCA1<sup>−/−</sup> and BRCA2<sup>−/−</sup>. PARP1 is uniquely required for EB-diol DNA damage. In addition, BLM not only identified that EB-diol required HR as part of its DDR but also that strand exchange was mediated post resection, perhaps forming a dHJ that was resolved also by BLM to promote its dissolution; producing non-crossover products.

The DT40/XTT allowed the elucidation of DDR for all three epoxides derived from BD. Results showed coupled DNA repair pathways as follows: EB’s DDR was primarily centered by TLS, EB-diol’s DDR is FA-TLS-HR, and DEB’s DDR is FA-TLS. Most of the Dt40 mutant cells results suggest that EB-diol treatment is inducing an ICL. This response to DNA damage was unaccounted for since we originally hypothesized to see EB-diol induced sensitivity in BER mutants as DDR. We cannot yet conclude or assume if the nature of the ICL is a DNA-DNA crosslink or a DNA-protein crosslink (DPC), or a combination of the two, and we think future work should be done to clarify this.

Finally, the implication in the different DDR responses induced by BD-epoxides suggests that EB-diol is indeed a very important chemical that should continue to be examined, and not be underestimated in BD human risk assessment. EB-diol is the “Titan” of the BD-epoxides, due to its abundance across species. Our previous results in Chapter 3 solidifies this statement. Moreover, our measurements across species for BD-Hb adducts, IBD, and EB-Equivalency have proven that EB-diol is the most significant BD-epoxide across species, and particularly, the most influential in humans (Swenberg, 2011). The attributable carcinogenic potential of BD, particularly in mice has been directly influenced by the greater contribution of DEB, rather than EB and EB-diol as shown by the EB-equivalency (Swenberg, 2011). No doubt that DEB has been the BD-epoxide with the greatest genotoxic potential, because it has demonstrated to be
linked to induce tumor formation in mice. The formation of DEB is species dependent, where mice are more metabolically apt to favor the oxidation of EB into DEB. However, this just clarifies why there is a species differences in tumor formation between rats and mice, but does not reflect the importance of EB-diol in human risk assessment. In humans, DEB is a minor metabolite, since metabolism favor the hydrolytic pathway towards the formation of EB-diol. These observations have been clearly demonstrated with EB-diol as greatest contributor of THB-Val Hb-adducts, THB-Gua DNA adducts, highest internal blood dose, and the greatest contributor in the EB-equivalency across species. In this report, we have strengthened the premise that DEB serves to understand its importance as a strong influence in mice carcinogenesis, but it is truly EB-diol that’s relevant for human risk assessment. We understand these results are contributing in our understanding and completion of existent data gaps for BD-epoxides repair in BD.

4.6 ACKNOWLEDGEMENTS

We want to wholeheartedly thank Dr. Jun Nakamura for allowing us to perform our experiments using his developed methods, and extensive knowledge with the DT40/XTT system. His help was critical in completing solubility issues with EB, more than half of the DEB DNA Damage response, and all the additional replicates for all experiments here presented. We also want to acknowledge, Dr. Zhang Zenfha for the intial synthesis and discussions regarding for EB-diol and EB-diol isomers. We would like to include Dr. Rebecca Fry for her kind gift of Tk6 cells to perform our preliminary cytotoxicity testing of the EB-diol isomers.
### Table [4.1]: Relative LD50 of EB-diol in DT40 wt and DT40 mutants

<table>
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<th>Pathway</th>
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<th>Relative LD50 (EB-diol)</th>
<th>Mean</th>
<th>SD</th>
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<td>-</td>
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<tr>
<td></td>
<td>BRCA1$^{-/-}$</td>
<td>0.49</td>
<td>0.09</td>
<td>6</td>
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<tr>
<td></td>
<td>BRCA2$^{-/-}$</td>
<td>0.34</td>
<td>0.08</td>
<td>6</td>
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<tr>
<td></td>
<td>FANCD2$^{-/-}$</td>
<td>0.32</td>
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<td>HEL</td>
<td>BLOOM$^{-/-}$</td>
<td>0.26</td>
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<td>WERNER$^{-/-}$</td>
<td>0.59</td>
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<td>NHEJ</td>
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<td></td>
<td>DNAPKcs$^{-/-}$</td>
<td>0.83</td>
<td>0.05</td>
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<tr>
<td></td>
<td>LIGIV$^{-/-}$</td>
<td>0.76</td>
<td>0.06</td>
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<tr>
<td>TLS</td>
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<td>0.34</td>
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<td>POLQ$^{-/-}$</td>
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<tr>
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<td>POLB$^{-/-}$</td>
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<td></td>
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<td>0.77</td>
<td>0.19</td>
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<td>PARP1$^{-/-}$</td>
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<td>0.02</td>
<td>6</td>
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<td>NER</td>
<td>XPA$^{-/-}$</td>
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<td>0.08</td>
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<tr>
<td></td>
<td>XPG$^{-/-}$</td>
<td>0.66</td>
<td>0.06</td>
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<td>MMR</td>
<td>MSH3$^{-/-}$</td>
<td>0.96</td>
<td>0.13</td>
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<td>Sensitivity</td>
<td>Relative LD50 Categories</td>
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<tr>
<td>----------------------</td>
<td>--------------------------</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Sensitivity</td>
<td>&lt; 0.25</td>
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<tr>
<td>Sensitivity</td>
<td>0.26 - 0.50</td>
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<tr>
<td>Moderate Sensitivity</td>
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<tr>
<td>Low Sensitivity</td>
<td>0.75 - 0.99</td>
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<td></td>
</tr>
<tr>
<td>No Sensitivity</td>
<td>1 and &gt;</td>
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Table [4.2] Relative LD50 Sensitivity Ranges: The LD50 of DT40 mutants divided by the LD50 DT40wt were used to determine the Relative LD50 to allow comparisons. We have subcategorized degrees of sensitivity by assigning categories and ranges to the Relative LD50. The five categories are: High Sensitivity (0.01-0.25), Sensitivity (0.26-0.50), Moderate Sensitivity (0.51-0.75), Low Sensitivity (0.75-0.99) and No Sensitivity (1.00 and above).
Table [4.3] Relative Sensitivity of (a) Base Excision Repair and (b) Nucleotide Excision Repair to Butadiene Epoxides.
### Table [4.4] Relative Sensitivity of (a) Mismatch Repair and (b) Translesion Synthesis to Butadiene Epoxide

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<thead>
<tr>
<th>Butadiene Epoxides</th>
<th>Mismatch Repair</th>
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<tr>
<td></td>
<td>MSH3&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<tr>
<td>Epoxybutene (EB)</td>
<td>Low</td>
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<tr>
<td>Diepoxybutene (DEB)</td>
<td>None</td>
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<tr>
<td>Epoxybutene-diol (EB-diol)</td>
<td>Low</td>
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</table>

<table>
<thead>
<tr>
<th>Butadiene Epoxides</th>
<th>Translesion Synthesis</th>
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<tbody>
<tr>
<td></td>
<td>PCNA RELATED</td>
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<tr>
<td></td>
<td>REV1&lt;sup&gt;-/-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epoxybutene (EB)</td>
<td>Sen</td>
</tr>
<tr>
<td>Diepoxybutene (DEB)</td>
<td>High</td>
</tr>
<tr>
<td>Epoxybutene-diol (EB-diol)</td>
<td>High</td>
</tr>
</tbody>
</table>

Table [4.4] Relative Sensitivity of (a) Mismatch Repair and (b) Translesion Synthesis to Butadiene Epoxide
Table [4.5] Relative Sensitivity of (a) Fanconi Anemia and (b) Homologous Recombination to Butadiene Epoxides. *N.T. means not tested.
### Table [4.6] Relative Sensitivity of (a) Non-Homologous End Joining and (b) Sensors and Cell Cycle Check Points to Butadiene Epoxides.

#### (a)

<table>
<thead>
<tr>
<th>Butadiene Epoxides</th>
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<tr>
<td></td>
<td>KU70&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epoxybutene (EB)</td>
<td>Mod</td>
</tr>
<tr>
<td>Diepoxybutene (DEB)</td>
<td>Mod</td>
</tr>
<tr>
<td>Epoxybutene-diol (EB-diol)</td>
<td>Mod</td>
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</table>

#### (b)

<table>
<thead>
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<th>Butadiene Epoxides</th>
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<th>Cell Cycle Checkpoint</th>
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<tr>
<td></td>
<td>Sensor</td>
<td>Sensor</td>
</tr>
<tr>
<td></td>
<td>RAD17&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>RAD9&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epoxybutene (EB)</td>
<td>Mod</td>
<td>N.T.</td>
</tr>
<tr>
<td>Diepoxybutene (DEB)</td>
<td>Mod</td>
<td>N.T.</td>
</tr>
<tr>
<td>Epoxybutene-diol (EB-diol)</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>DT40 DNA Repair Pathways</td>
<td>EB</td>
<td>DEB</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>BER</strong></td>
<td></td>
<td>REV1, RAD18, Pol H</td>
</tr>
<tr>
<td><strong>TLS</strong></td>
<td>REV1</td>
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<tr>
<td><strong>FA-BRCA</strong></td>
<td>FANCD2, BRCA2</td>
<td>FANCD2, BRCA1 and BRCA2</td>
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<td><strong>HR</strong></td>
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<td>DNA Damage Response</td>
<td>TLS</td>
<td>FA-TLS</td>
</tr>
</tbody>
</table>

Table [4.7] DNA Damage Response in DT40 Cells with a Relative LD50 < 0.5
4.8 FIGURES:

(a)

![Diagram of 24-well plate format for doses, controls and blanks.](image)

(b)

![Diagram of general method: exposures of DT40wt and DT40 mutants, and XTT dye incubation/quantitation.](image)

XTT $\rightarrow$ Formazan Product $\rightarrow$ Quantifiable by Absorbance (450nm-650nm)

Develop XTT - 8h

XTT = sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt

Figure [4.1] Schematic Representations of the DT40/XTT Method: (a) Schematic representation of 24-well plate format for doses, controls and blanks. (b) Schematic representation of the general method: exposures of DT40wt and DT40 mutants, and XTT dye incubation/quantitation.
Figure [4.2] Cytotoxicity of Butadiene epoxide EB-diol isomers (2R,3S, 2S,3R, 2S, 3S and 2R,3R) in both (a) DT40wt cells and (b) TK6 cells.
Figure [4.3] Sample Graphs from Dose-Response Curves for EB-diol exposures of DT40wt against DT40 mutants: (a) Sensors: R17⁻/⁻ and R9⁻/⁻ (b) Translesion Synthesis: Polη⁻/⁻, Polκ⁻/⁻, and REV1⁻/⁻ (c) BRCA proteins: BRCA1⁻/⁻ and BRCA2⁻/⁻.
Figure [4.4]: EB-diol Relative LD50 in BER Repair DT40 mutants.
Figure [4.5]: EB-diol Relative LD50 in NER Repair DT40 mutants.
Figure [4.6]: EB-diol Relative LD50 in MMR Repair DT40 mutants.
Figure [4.7]: EB-diol Relative LD50 in TLS Repair DT40 mutants.
Figure [4.8]: EB-diol Relative LD50 in HR Repair DT40 mutants.
Figure [4.9]: EB-diol Relative LD50 in FA Repair DT40 mutants.
**NON-HOMOLOGOUS END-JOINING PATHWAY**

<table>
<thead>
<tr>
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<th>RELATIVE LC50</th>
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<tr>
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<td>DNAPKcs -/-</td>
<td>0.83</td>
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<td>LIGIV -/-</td>
<td>0.76</td>
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Figure [4.10]: EB-diol Relative LD50 in NHEJ Repair DT40 mutants.
Figure [4.11]: EB-diol Relative LD50 in Cells and Cell Cycle Checkpoints and Sensor DT40 mutants
Figure [4.12]: EB-diol DNA Damage Response as the Relative LD50 of DT40 mutants to DT40 wt.
Figure [4.13]: EB, DEB and EB-diol monoadducts or AP-sites DDR via TLS bypass. REV1 inserts a Cystosine opposite to adducted bases and or AP sites in order to bypass the damage and continue replication. Pink outline and white center ovals were DT40 mutants that were tested.
Figure [4.14]: DEB and EB-diol crosslink DDR of FA ICL recognition, and ICL un-hooking. The FA core complex assembles and activates FANCID [FANCD2 and FANCI] to remove the ICL via nuclease FNACP and FANCQ. Pink outline and white center ovals were DT40 mutants that were tested.
Figure [4.15]: DEB and EB-diol crosslink DDR of FA and TLS proteins convergence into FA-TLS coupled repair. Pink outline and white center ovals were DT40 mutants that were tested.
Figure [4.16]: DEB and EB-diol crosslink DDR of TLS proteins from FA-TLS coupled repair bypassing the ICL left over lesion. Pink outline and white center ovals were DT40 mutants that were tested.
Figure [4.17]: EB-diol crosslink DDR of NER proteins participating in the excision of the ICL leftover. Pink outline and white center ovals were DT40 mutants that were tested.
Figure [4.18] Diagram of HR in the Repair of DSB created by FA repair in EB-diol crosslinks. Pink outline and white center ovals were DT40 mutants that were tested.
REFERENCES


CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

5.1.1 Introduction


These epoxides have the capacity to bind to both DNA, and hemoglobin (Hb), forming epoxide-specific adducts at both endpoints. Earlier reports with rodents exposed to BD have identified species differences in the formation of BD-specific DNA and Hb adducts, ultimately indicating that metabolic activation of BD is species dependent. Mice generated greater amounts of the most mutagenic BD-epoxide (DEB) in blood, Hb adducts, and DNA adducts in rodents. However, these DEB epoxides in blood and Hb adducts are more similar in proportion in both rats and humans, than in mice. Understanding this difference was fundamental to explain why mice have been the most susceptible species, even more so than rats, as shown by tumor development at much lower levels of BD exposure. In addition to species differences, gender differences in
tumor formation for female mice have been observed at lower concentrations than that of male mice.

The weight of evidence presented across rodent species has suggested sex specific differences in BD-DNA crosslinks, tumor formation, and mutagenicity. Therefore DNA repair efficiency rather than metabolism can be attributed to the observed sex-dependence. Initial data interpretation in metabolic biomarkers and tumor formation presented species differences that were difficult to understand. Some of the questions that remained unclear were how BD was acting as a carcinogen, how we could use this data, and what these findings meant for human risk assessment.

5.1.2 Contribution of this Thesis

The goal of this project was to complete our dataset gaps in BD-epoxide metabolic activation and DNA repair. This will further improve our understanding of BD’s Mode of Action (MOA), and its relationship to BD-carcinogenesis. We hypothesized that through the development of new methods, incorporation of comprehensive data sets, and in vitro techniques, we would identify multispecies similarities and differences in BD-metabolic activation. Also, the integration of DNA-damage response as an endpoint would serve to strengthen BD carcinogenic MOA and identify relevant epoxide-specific differences in repair. Results are expected to contribute in the improvement of scientific risk assessment, public health, and further employed as an application to other known multispecies carcinogens.

This dissertation integrates and applies previously developed methods from our laboratory: (1) for the development and completion the set of BD Hb adducts peptide
standards, (2) to complete the quantitation of BD Hb adducts formation in our mice dataset, and (3) to evaluate DNA damage response pathways induced by selected BD-epoxides.

5.1.3 Thesis Chapters

This dissertation was presented in three inter-related chapters (Chapters 2, 3 and 4). Chapter 2 describes a combination of methods that successfully generated a total of six unique EB-diol specific (1-11) THB-Val peptide standards. These included both analyte and internal peptide standards for Hb α (1-11) Human, Rat, and Mouse THB-Val. We employed direct alkylation of the Hb α (1-11) NTV peptide sequences with EB-diol. This was followed by chromatographic purification and characterization by LC-ESI+ MS/MS prior to amino acid quantitation by UPLC MS/MS-based amino acid analysis. THB-Val peptide standards were not previously available for the quantitation of EB-diol-specific Hb-adducts. Now, we were able to employ them in several downstream analytical applications. This included their integration in the validation of a simultaneous IA-enrichment method prior to the analysis of a unique sample-set of rodents exposed to BD.

All BD-epoxides react with the NTV of Hb upon BD exposure (Moll 1999) forming three epoxide specific adducts. The most abundant epoxide formed across species upon BD activation is EB-diol producing epoxide-specific Hb-adduct (N-(2,3,4-trihydroxybutyl)-valine (THB-Val). Epoxide EB and DEB also bind to the NTV of Hb forming adducts N-(2-hydroxy-3-butenyl)valine (HB-Val) and N,N-(2,3-dihydroxy-1,4-butadiyl)valine (pyr-Val) respectively. All three BD-Hb adducts have been used as surrogate markers of BD exposure, and helped in the identification of underlying differences in metabolism/metabolic activation through the estimation of their internal
dose in blood (IBD) (Fred 2008, Swenberg, 2011). In addition, species and sex-related differences in the formation of each individual epoxide were quantitatively assessed by the use of these biomarkers of exposure. Our final goal was to better understand the role of BD reactive epoxides and the metabolic species differences in toxicity, to ultimately determine which of the two rodent species is the most appropriate for human risk assessment.

Chapter 3 describes quantitation all three BD Hb-adducts formed by epoxides EB, DEB, and EB-diol in B6C3F1 mice exposed to BD via inhalation for 10 days. Our goal was to integrate the use of species-specific (mouse) NTV(1-11) peptide standards with a recently improved immnoaffinity (IA) method coupled to UPLC-positive mode electrospray ionization-tandem mass spectrometry (UPLC-ESI⁺-MS/MS). This approach allowed the simultaneous quantitative analysis of all three BD-Hb adducts from hemoglobin of mice exposed to BD. Proteolytic digestion with trypsin cleaved BD-Hb adducted peptides from globin proteins. Digests were processed by triple antibody IA enrichment and the adducted peptides eluted under acidic conditions were collected and quantitated by nano-UPLC-MS/MS analysis. The $^{13}$C$^{15}$N$_2$-labeled internal standard peptides facilitated the accurate quantitation, with a limit of detection as low as 5-10 fmol, and with little globin (10 mg).

Previously, this method was used for the analysis of BD-Hb adducts in rats (Bordeerat 2012). We now used it in the quantitation of samples from mice exposed via inhalation to BD, at concentrations that are relevant to human occupational exposures (0.5 ppm). The formation of BD-epoxide adducts increased with exposure, and the highest formation ratio of all three adducts per ppm BD was observed at lower levels of
exposures (0.5-1.5 ppm). The amounts of pyr-Val, HB-Val and THB-Val formation showed no signs of saturation at exposures up to 625 ppm for 10 days. Previous results obtained by this method in BD-exposed rats were integrated for comparisons, and we found that formation ratios for all BD-Hb adducts were higher in mice than in rats. Interestingly both species were more efficient in the formation of these adducts at lower levels of exposure, making this relevant due to similar levels of exposure in human occupational settings. However, results confirmed that there were no sex-specific differences in the formation of BD-Hb adducts.

BD-Hb adducts results were integrated in the calculation of the Internal Blood Dose (IBD) of epoxides and transformed into the EB-equivalency for further comparisons. The IBD of BD-epoxides, and their corresponding EB-equivalencies, were calculated for mice using Fred’s 2008 Kval constants for BD-epoxides and mutagenic potency from Meng’s 2010 publication. The IBD was found to be highest at lower levels of exposure (0.5 ppm) and decreased with increasing concentrations. A collaborator of this project presented similar results but for rats (Bordeerat, 2012). There are undeniable species differences as reflected in the formation of BD-epoxides and their contribution to the EB-equivalency. Mice were more efficient in metabolic activation of BD-epoxides at lower levels of exposure than at higher levels, however no sex specific differences were noted. The carcinogenic potential of BD in mice is directly influenced by the greater contribution of DEB, rather than EB and EB-diol as shown by the EB-equivalency. DEB has been identified as the epoxide with the greatest genotoxic potential, and thus the most influential epoxide in tumor formation in mice.
These findings provide important information relevant to why mouse is the most susceptible species of the rodent models, and account for the specific species differences in BD metabolic activation as estimated by BD-Hb adducts. The current investigation has provided insights to our knowledge for BD risk assessment. Finally, in mice the greater DEB contribution in blood and corresponding biomarker of exposure pyr-Val does point out the reason for this species susceptibility to BD and tumor formation. Therefore, the rodent species that most appropriately represented the best animal model for BD risk assessment in human was rat, not mouse. This was accounted by the sharp contrast amongst species in the EB-equivalency per ppm, where mice had the greatest difference in the equivalency being ~50-fold greater than Rats and ~200-fold greater than Humans (Swenberg 2011).

In Chapter 4, we identified the biological significance of DNA Damage Responses (DDR) by employing the DT40/XTT method to assess BD-epoxides, and their sensitivities. All three butadiene epoxides (EB, DEB, EB-diol) have the ability to react with DNA, and form covalent adducts. The formation of BD-DNA adducts are indicators of one or more genotoxic event(s) that leads to a non-mutational event, contributing in the initiation of mutagenesis. Therefore, BD-DNA adducts are considered a relevant event to BD-carcinogenic mode of action (MOA). Limited information was available regarding those DDR pathways involved in counteracting each specific BD-epoxide insults. Therefore, we identified that BD-DDR was a gap in knowledge in BDs current biomarkers of exposure-effect database.

The DT40 cells used in this research have an extensive collection of DNA repair-deficient mutant clones to assess in vitro DDR using a high-throughput assay. The
biological significance of these responses was established by employing the DT40/XTT system to assess BD-epoxides, and their sensitivities. Effects were expressed by the Relative LD50, using DT40 parental cells and DT40 mutants. These mutants were grouped by deficiencies in seven DNA repair pathways. These pathways included mutants in cell cycle checkpoint, base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end-joining (NHEJ), mismatch repair (MMR), translesion synthesis (TLS), DNA damage sensors and Cell Cycle Checkpoints.

Overall results showed that \( REV1^{-/-} \) was the most sensitive mutant cell line across BD-epoxides. REV1 is not only an pivotal protein in TLS, but also represents the common link between FA and TLS DNA Repair pathways. Irrespective of differences on the corresponding DNA adducts formed by each epoxide, the primary form of response upon BD DNA damage is via bypass by TLS polymerases employed. The second common observation was that epoxides DEB and EB-diol had similar DDR signatures for certain pathways. In the FA pathway, both DEB and EB-diol overlapped with sensitivity for mutants \( FANCD2^{-/-} \) and \( BRCA2^{-/-} \). This was perhaps one of the most striking and unexpected results to this date for EB-diol. There is extensive evidence that supports the generation of DEB crosslinks, but no documentation of EB-diol crosslinks (ICL) has been presented to this date. However, only EB-diol induced sensitivity in both DT40 mutants \( BRCA1^{-/-} \) and \( BRCA2^{-/-} \). This indicates that the recognition and excision to unhook the ICL was similar for both epoxides. However, for EB-diol, \( BRCA1^{-/-} \) signifies that the DSB needs to be processed via resection as part of the HR pathway. In contrast, EB did not induced sensitivity in FA or HR mutants, and DEB did not induced the latter.
One of the highlight findings was EB-diol’s uniques DDR signature through six DT40 both highly sensitive and sensitive mutants, that were not sensitive for EB nor DEB. These unique EB-diol mutants were $BLM^{−/−}$, $RAD17^{−/−}$, $RAD9^{−/−}$, $PARP1^{−/−}$ and the combined sensitivity in both BRCA’s $BRCA1^{−/−}$ and $BRCA2^{−/−}$. EB-diol induces a strong G2/S arrest in a ATR dependent manner as indicated by both $RAD17^{−/−}$ and $RAD9^{−/−}$ sensitivities, whereas EB and DEB were ATM independent. The unique sensitivity in $PARP1^{−/−}$ mutant cells to EB-diol suggests that cellular stress as response to exposure due to glutathione depletion, leading to indirectly increasing reactive oxygen species. In addition, sensitivity in $BLM^{−/−}$ mutant cells not only identified that EB-diol required HR as part of its DDR, but also that strand exchange was mediated post resection, perhaps forming a dHJ that was resolved also by BLM to promote its dissolution; producing non-crossover products.

When looking at overall results, we noticed that DNA repair pathways were coupled to promote repair. For instance, results showed that EB’s DDR was primarily TLS, EB-diol’s DDR was FA-TLS-HR, and DEB’s DDR was FA-TLS. Our results unraveled that EB-diol induced a DDR that correspond to the repair of a crosslink (DNA-DNA or DNA-protein crosslink). The most striking finding in this project was in EB-diol DDR. The FA pathway mainly coordinates the protection of replication forks by “unhooking ICL’s”. Also, the FA pathway coupled with other secondary pathways that included TLS, and HR to mediate repair. Overall, results from the DT40 mutant cells panel suggests that EB-diol treatment is inducing an ICL, posing a threat to the stability of replication forks. This response to DNA damage was unaccounted for, since we originally hypothesized EB-diol to induce sensitivity in BER-related mutants as DDR.
Finally, the implication in the different DDR responses induced by BD-epoxides suggests that EB-diol is indeed a very important chemical that should continue to be examined, and not be underestimated in BD human risk assessment.

**Overall**, the research presented here completes selected gaps in knowledge of BD biomarkers of exposure (BD-Hb adducts) and biomarkers of effect (BD DDR). Our goal was to contribute to better understanding of BD exposure-response and improve risk assessment. DEB has been the BD-epoxide with the greatest genotoxic potential, because it has demonstrated to be linked to induce tumor formation in mice. The formation of DEB is species dependent, where mice are more metabolically apt to favor the oxidation of EB into DEB. The species differences in mice and rats tumor formation, does not reflect the importance of EB-diol in human risk assessment. In both rats and humans, DEB is a minor metabolite, since metabolism favors the hydrolytic pathway towards the formation of EB-diol. These observations clearly demonstrated that EB-diol is the “Titan” of BD-epoxides. It is also, the greatest contributor of THB-Val Hb-adducts, THB-Gua DNA adducts, highest IBD epoxide, and the most influential factor in the EB-equivacency across species. In this report, we have strengthened the premise that DEB serves to understand its importance as a strong influence in mice carcinogenesis, but it is truly EB-diol that’s relevant for human risk assessment. These results have completed existent data gaps for BD-epoxides metabolic activation, and BD induced DNA repair as means to improve overall risk assessment.
5.2 FUTURE DIRECTIONS

We believe that BD biomarkers of exposure, as represented by BD-Hb adducts won’t hold much potential for any additional future experiments with respect to BD. However, the methods here presented do provide an excellent platform to investigate other chemicals, pharmaceuticals, and even drugs that might present discrepancies in the effects of their bio activated reactive metabolites, using BD as a model.

Where this project does promise potential for future experiments is in respect to our findings in relation to biomarkers of effect, as suggested by EB-diol’s DDR. Our results from the DT40 mutant cells panel suggests that EB-diol treatment is inducing Interstrand crosslinks (ICL). The nature of this DNA lesion has not been reported to date, and presents a new exciting opportunity to perform a study that identifies and characterizes the ICL. EB-diol stereoisomers with distinct positions of their hydroxy groups are currently being investigated by our group. These can be precursors of the unidentified lesion. In addition, an alternative could include the analysis of target proteins from nuclear protein extracts of DT40 cells followed by proteomic analysis of DPCs.

Recent methods have been developed and applied for the isolation and analysis of DPCs from BD-epoxide DEB in vitro using HeLa cells (Tretyakova, 2015), however EB-diol was not included in their experiments. Additional research will be required to identify the possibility of THB-monoadducts as precursors of DNA protein crosslinks (DPC’s) via reaction of the unbound trihydroxy group. DPCs are considered bulky adducts, and future experiments should consider the in vitro formation of DPC’s using N2-Gua-THB, N7-Gua-THB and N6-Ade-THB adducts as precursors.
REFERENCES


