CHAPTER I

INTRODUCTION

Mutagenesis by Bis-Electrophiles

DNA is a critical cellular component that allows for the propagation of life by serving as a genetic blueprint (Friedberg *et al.*, 1995).   Despite its importance, the structure of DNA was only elucidated in the 1950s after its critical role in the transmission of genetic material was discovered (Avery *et al.*, 1944). DNA consists of only four nucleotide bases; adenine (A), guanine (G), thymine (T) and cytosine (C), which are linked together via a sugar-phosphate backbone. The double helical structure is formed by hydrogen bonds between purine and pyrimidine bases to yield A:T and G:C base pairs (Watson and Crick, 1953). The specific sequence of these bases is what determines the identity of genes, which are the inherited units first discovered by Gregor Mendel in the 1800's (Pearson, 2006). The opposing directionality of each strand preserves the DNA sequence via the mechanism of semi-conservative DNA replication, allowing for the propagation of life (Meselson and Stahl, 1958) (Figure 1).

Due to its importance, the stability of DNA is expected to be quite high; however, both the sugar-phosphate backbone and nucleotide bases are susceptible to damage. Spontaneous depurination and deamination events alone are expected to occur in the DNA of a single cell at a rate of over 10,000 per day (Lindahl and Nyberg 1972, 1974). In addition, endogenous events, such as oxidation, nitrosylation, methylation, and

modification by lipid peroxidation products alter the structure of DNA (Loeb, 1989; Wood *et al.*, 2001; Halliwell and Aruoma, 1991). Cells are also exposed to exogenous
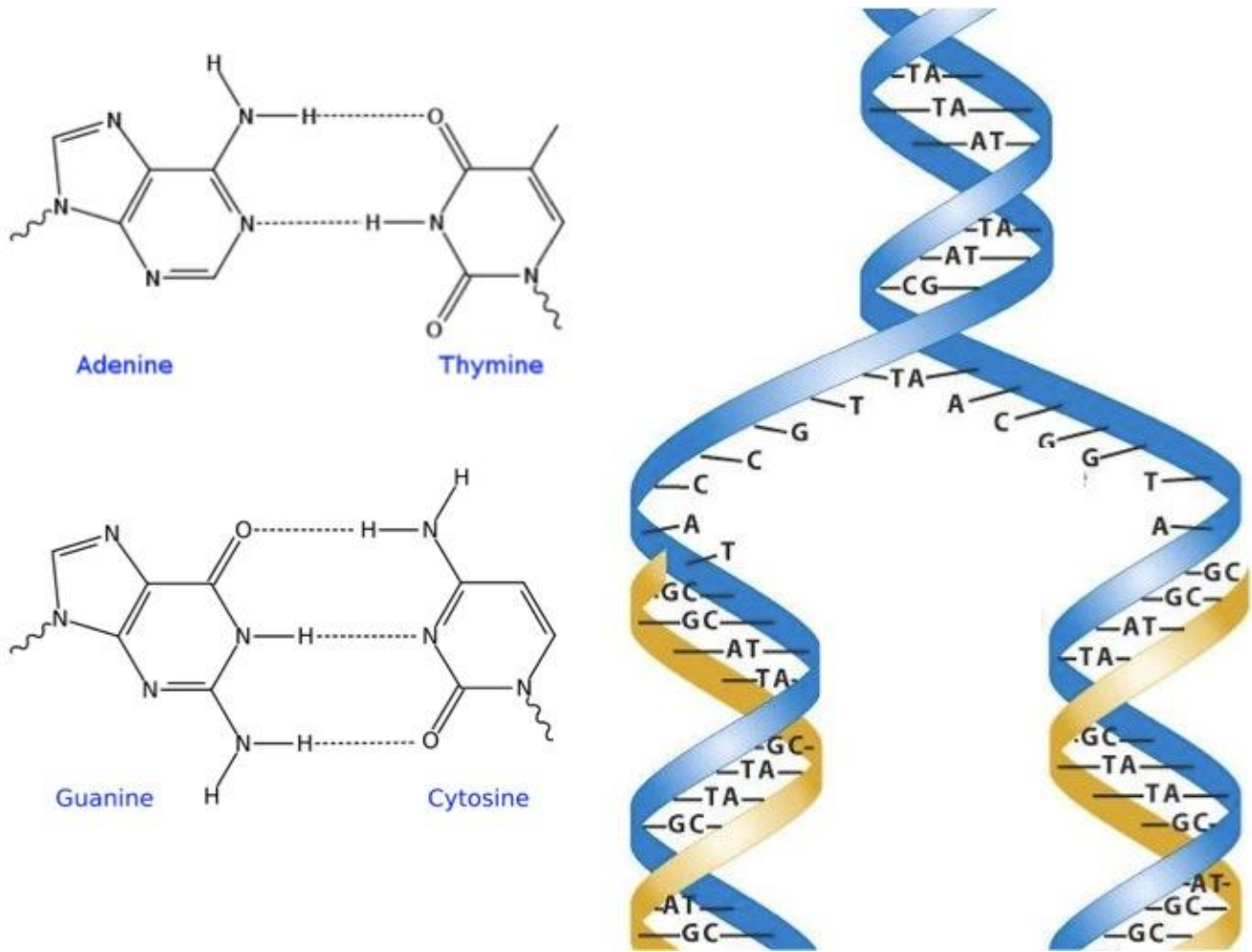

Figure 1. Structure of base pairing and mechanism of semi-conservative replication.

chemicals, which also have the potential to damage DNA (Ames *et al.*, 1973; Ames, 1973). Indeed, a diversity of DNA lesions are known to exist, including nucleotide adducts, basic sites, cross-links, and strand breaks that a cell must contend with in order to allow for normal DNA metabolism, including genomic replication and transcription (Loeb, 1985).

In order to combat this damage, several DNA repair mechanisms maintain genomic integrity despite the physical and chemical disparity of DNA lesions. Base-excision repair (BER) involves the removal of a single base damaged by oxidation or alkylation (Roth and Samson, 2002; Duncan *et al.*, 1976), while nucleotide-excision repair (NER) employs a network of enzymes to extricate a small (~30 nucleotides) oligonucleotide surrounding larger DNA adducts (Setlow *et al.*, 1963; Setlow and Carrier 2003). Mechanisms also exist to repair mismatched base pairs created by the infidelity of DNA polymerases or oxidative demethylation of certain bases (Modrich, 1991; Sedgwick, 2004). Still other DNA lesions, e.g. double strand breaks and DNA cross-links, are known to be repaired by mechanisms that are not yet fully understood (Friedberg *et al.,* 1995; Zheng *et al.,* 2006; Kuraoka *et al.,* 2000). In addition, alkylated bases can be repaired via a direct reversal mechanism that involves the transfer of the aberrant alkyl group to a repair protein, thus restoring the canonical sequence (Gerson, 2002).

While cellular mechanisms exist to reverse or repair DNA damage, not all lesions are effectively processed. DNA lesions can block replication forks if left unrepaired, resulting in cytotoxicity via the triggering of pathways that eventually lead to cell death (Wright and Shay, 2001). DNA damage can also result in mutations when the lesion is

not properly repaired or when low-fidelity polymerases bypass aberrant DNA structures in an error-prone manner (Masutani *et al.*, 2000; Sweasy *et al.,* 2006). These changes are essentially permanent and are transmitted to all daughter cells (Loeb and Harris, 2008).

The alteration of DNA sequences can be beneficial when considering long-term adaptation of an organism to an environment, but it can also have dire effects. While some mutations are silent, the process of mutagenesis is thought to be an initial step in the path to cancer formation (Alberts *et al.,* 2002). Various lines of evidence exist in support of this theory, including the finding that cancerous cells harbor genetic abnormalities, which distinguish them from normal cells. In addition, studies of certain genetic diseases known to predispose people to cancer show that the underlying defect occurs in a DNA repair mechanism responsible for maintaining the integrity of the genome. Finally, agents that alter the structure of DNA can also induce the formation of cancer (Friedberg *et al.*, 1995). Chemical carcinogens are one class of these agents and have been extensively studied due to their exposure to humans, and many have provided key insights into the mechanisms of tumor initiation (Figure 2).

The association between carcinogenesis and exposure to specific compounds was realized over two centuries ago. Hill (1761) was the first to suggest a link between snuff use and cancer formation, followed by the observation that chimney sweeps were at an increased risk of cancer formation (Pott, 1775). The studies that eventually emerged from these initial observations were critical for the understanding of cancer initiation and
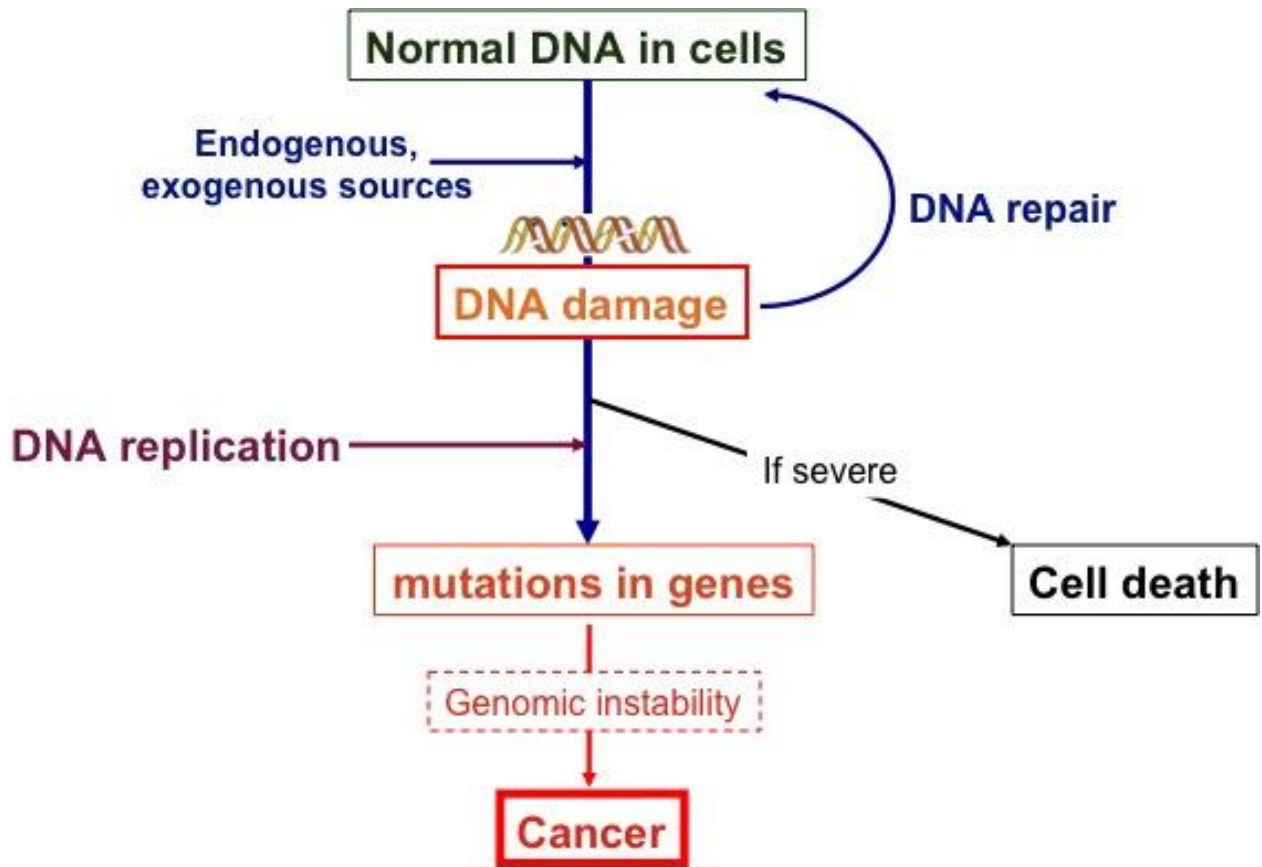
Figure 2. Cellular responses and potential outcomes of DNA damage.

provided insights to fundamental cellular mechanisms. The link between exposure to tar and soot with cancer formation was further supported when the carcinogenic potential of these chemical mixtures was observed in animal models (Yamagiwa, 1918). One of the most critical aspects of delineating the exact mode of action by chemical carcinogens was the purification of the specific carcinogenic species, such as benzo[a]pyrene (B[*a*]P) in the case of tar and soot, from a complex mixture and much effort went toward this goal (Kennaway, 1930; Cook *et al.,* 1933).

Another critical discovery prompted by the studies of chemical carcinogens was the role of metabolic activation of chemicals into reactive compounds capable of covalent binding to cellular macromolecules, such as DNA (Price *et al.,* 1948; Miller *et al.,* 1952). The cytochrome P450 enzymes implicated in the activation of B[*a*]P where also later shown to be critical for the activation and normal metabolism of many endogenous and exogenous chemicals. The activation of B[*a*]P leads to direct alkylation of DNA and with a preference for specific sequences, such as those found in the in tumor suppressor p53 (Sims *et al.,* 1974; Denissenko *et al.,* 1996). The most convincing evidence for the chemical induction of carcinogenesis was provided by the discovery that key alkylation types and patterns known to be induced by B[*a*]P in cell cultures were closely correlated with those found in tumors (Pfeifer *et al.,* 2002).

The work with B[*a*]P and other compounds established the utility of biomarkers, assessed the risk of exposure to human and established guidelines for exposure helped push the limits of technology to establish a causal relationship. While much work has been done, there are many chemicals that have been implicated in mutagenesis but their direct role in carcinogenesis has yet to be delineated. One such chemical is 1,2,3,4-

diepoxybutane (DEB), a metabolite of 1,3-butadiene, an important industrial monomer used in the synthesis of rubber and plastics (Morrow, 1990).

Interest in 1,3-butadiene was initiated by observation of increased cancer risk in rubber industry workers (IARC rubber industry 1982 p21-454). Later it was found that 1,3-butadiene was not only an occupational risk, but it also exists as an environmental contaminant due to its presence in automobile exhaust and cigarette smoke (Pelz *et al.*, 1990; Hecht, 1999). Early epidemiology studies were unable to confirm a causal relationship between 1,3-butadiene exposure and increased rate of cancer formation (Divine *et al.,* 1993; Downs *et al.,* 1987; Matanoski and Schwartz, 1987; Matanoski *et al.,* 1990; Santos-Burgoa *et al.*, 1992). As with most epideological studies, difficulties lie in determining the contribution of other chemicals that workers could be concurrently exposed to. However, follow-up analyses of these epidemiological studies do support the role of 1,3-butadiene as a leukemogen in industry workers (Delzell *et al*., 1996; Macaluso *et al*., 1996, Graff *et al.*, 2005; Alder *et al.,* 2006).

The carcinogenic potential of 1,3-butadiene inhalation was confirmed with animal models, where it was found to induce neoplasms at multiple sites in rodents although differences in species susceptibility were significant. In comparison with rats, mice display enhanced sensitivity to the carcinogenic effects of 1,3-butadiene along with differences in the location of tumor formation (Himmelstein *et al.,* 1996; Melnick *et al.,* 1993; Owen *et al.,* 1987; Huff *et al.,* 1985). These findings led to further investigations of species-specific metabolic pathways responsible for 1,3-butadiene activation that could explain these discrepancies.

1,3-Butadiene by itself is relatively unreactive, but when introduced to cells it is readily oxidized by cytochrome P450 monooxygenases to several reactive epoxides. Oxidation of 1,3-butadiene occurs primarily by P450 2E1 and P450 2A6 and yields either 1,2-epoxybutene or DEB, both of which can be further metabolized to 3,4-epoxy-1,2-butanediol (Figure 3) (Boogaard *et al.,* 1996; Cheng *et al.,* 1993; Malvoisin *et al.,* 1982). The epoxide metabolites are also subject to detoxication pathways, such as those catalyzed by epoxide hydrolases and glutathione transferases (GST) (Himmelstein *et al.,* 1997; Jackson *et al.,* 2000). 1,2-Epoxybutene, 3,4-epoxy-1,2-butanediol, and DEB can all react with cellular macromolecules, such as DNA and proteins, and are expected to be responsible for the mutagenic properties of 1,3-butadiene (Jackson *et al.,* 2000; Bolt *et al.,* 1983; Csanady *et al.,* 1992; Malvoisin *et al.,* 1979). However, the individual contribution of each metabolite to carcinogenicity is still under investigation (Hurst *et al.,* 2007; Swenburg *et al.,* 2007) (Figure 3).

Studies suggest that the metabolite most likely responsible for the carcinogenic effects of 1,3-butadiene is DEB, despite that fact that it is a minor metabolite. DEB, 1,2-epoxybutene, and 3,4-epoxy-1,2-butanediol are all direct-acting mutagens in Ames tests and mutagenic in cultured human cells (Henderson *et al.,* 2000; Meng *et al.,* 2000). However, DEB is the most efficient at inducing micronuclei, sister chromatid exchanges (SCEs), and chromosomal abnormalities in animals and human cells (Jelitto *et al.,* 1987; Henderson *et al.,* 1996). Indeed, the enhanced genotoxic and mutagenic potential of DEB was supported by the finding that DEB induced SCEs and chromosomal abnormalities in cultured human lymphocytes at a concentration of 0.5 μM, while the concentration required for 1,2-epoxybutene was 25 μM (Sasiadek *et al.,* 1991).
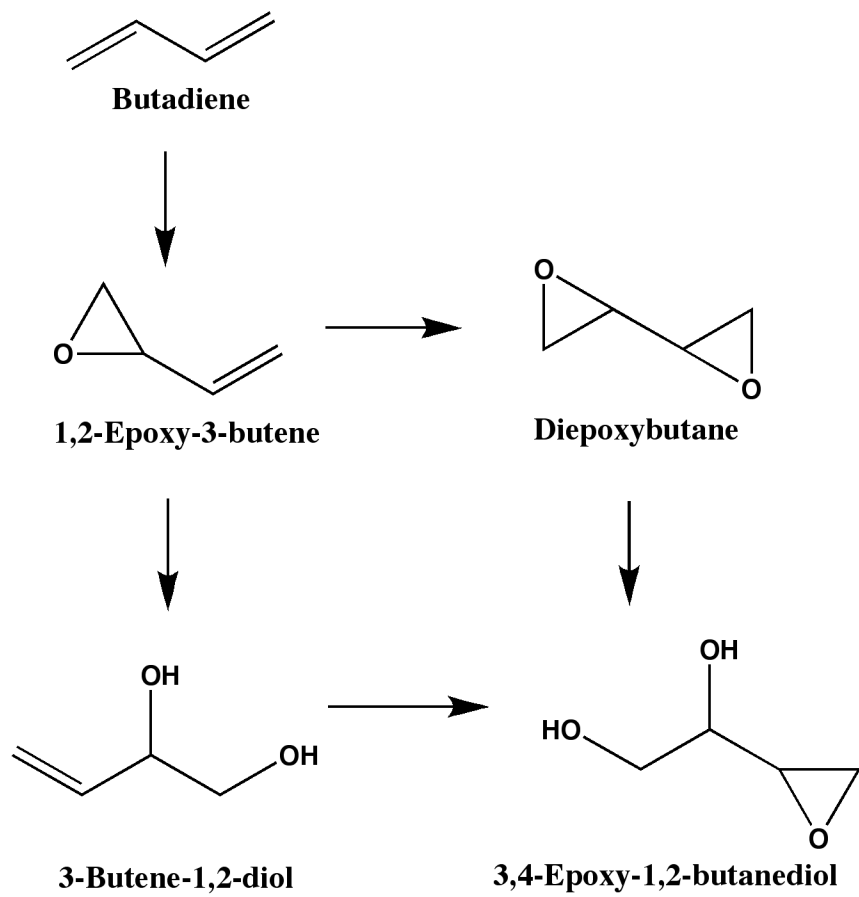
Figure 3. Metabolism of 1,3-butadiene.

Mutations in lymphoblastoid cells were observed at a concentration of 1 µM of DEB, while 1,2-epoxybutene and 3,4-epoxy-1,2-butanediol required 100 µM and 350 µM, respectively (Cochrane and Skopek, 1994a,b). The data lead to the ranking of potency for genotoxicity by 1,3-butadiene-derived epoxides, with DEB being 100 times more potent than 1,2-epoxybutene and 300 times more potent than 3,4-epoxy-1,2-butanediol (Adler *et al.,* 1995, 1997).

The importance of DEB as an ultimate carcinogen is also supported by the finding that mice oxidize 1,3-butadiene to a greater extent than rats, leading to higher levels of DEB in circulation, which could explain their susceptibility to the effects of 1,3-butadiene (Himmelstein *et al.,* 1996; Thornton-Manning *et al.,* 1997). In addition, mouse, human, and rat cells are equally sensitive to DEB in genotoxicity assays in which the metabolite is directly introduced to lymphocytes (Kligerman *et al.,* 1999; Kligerman *et al.,* 2006). These and other studies support the claim that only DEB could effectively induce the types and levels of mutations observed with 1,3-butadiene (Recio *et al.,* 2001).

While all the epoxide metabolites of 1,3-butadiene can alkylate cellular macromolecules, the bi-functional electrophilic nature of DEB is suggested to be responsible for its potency (Boysen *et al.,* 2007; Lawley *et al.,* 1967). DEB preferentially reacts at the N7 atom of guanine to give N7-2-hydroxyl-3,4-epoxybutane adducts, which can either be hydrolyzed to 2,3,4-trihydroxybutane products or less frequently form cross-links with other nucleophiles (Tretyakova *et al.,* 1997). In comparison with the mono adducts, cross-links involving DEB are more thermodynamically and biochemically stable, possibly leading to their accumulation (Park *et al.,* 2004). In addition to exocyclic DNA adducts, DEB can also induce DNA-DNA inter- and

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intrastrand cross-links and DNA-protein cross-links. (Goggin *et al.,* 2009; Zhang *et al.,* 2005; Cochrane and Skopek*,* 1994a,b).   Cross-links, specifically DNA-protein cross-links, can be deleterious to cells because they present major obstacles to normal DNA metabolism (Barker *et al.,* 2005b) (Figure 4).

DNA-protein cross-links are induced by a wide variety of both exogenous and endogenous agents, including UV and ionizing radiation, transition metals, and some chemotherapeutic agents (Lai *et al.,* 1987; Dizdaroglu *et al.,* 1989; Toyokuni *et al.,* 1995; Covey *et al.,* 1987; Loeber *et al.,* 2009).   It has been known for some time that bifunctional chemicals can also induce DNA-protein cross-links, with the majority of effort going towards the study of bifunctional aldehydes (Ohba *et al.,* 1979; Heck *et al.,* 1990).   In addition, normal cellular processes — including DNA replication and DNA repair — form DNA-protein cross-links as intermediates (Reardon *et al.,* 2006).   While the exact consequences of these diverse DNA lesions are unknown, the formation of DNA-protein cross-links by both carcinogenic and chemotherapeutic agents highlights the need for more studies.

DNA-protein cross-links are generally longer lived than other types of DNA damage and have been found to persist through replication cycles or are only partially repaired (Tsapako *et al.,* 1983; Cupo *et al.,* 1985; Sygiyama *et al.,* 1986).   In mice, DNA-protein cross-links have been found to accumulate with age (Izzoti *et al.,* 1999) and some have been suggested to be permanent (Oleinick *et al.,* 1987).   The fate of these large, bulky DNA lesions is still under investigation, but evidence suggests that most cells are
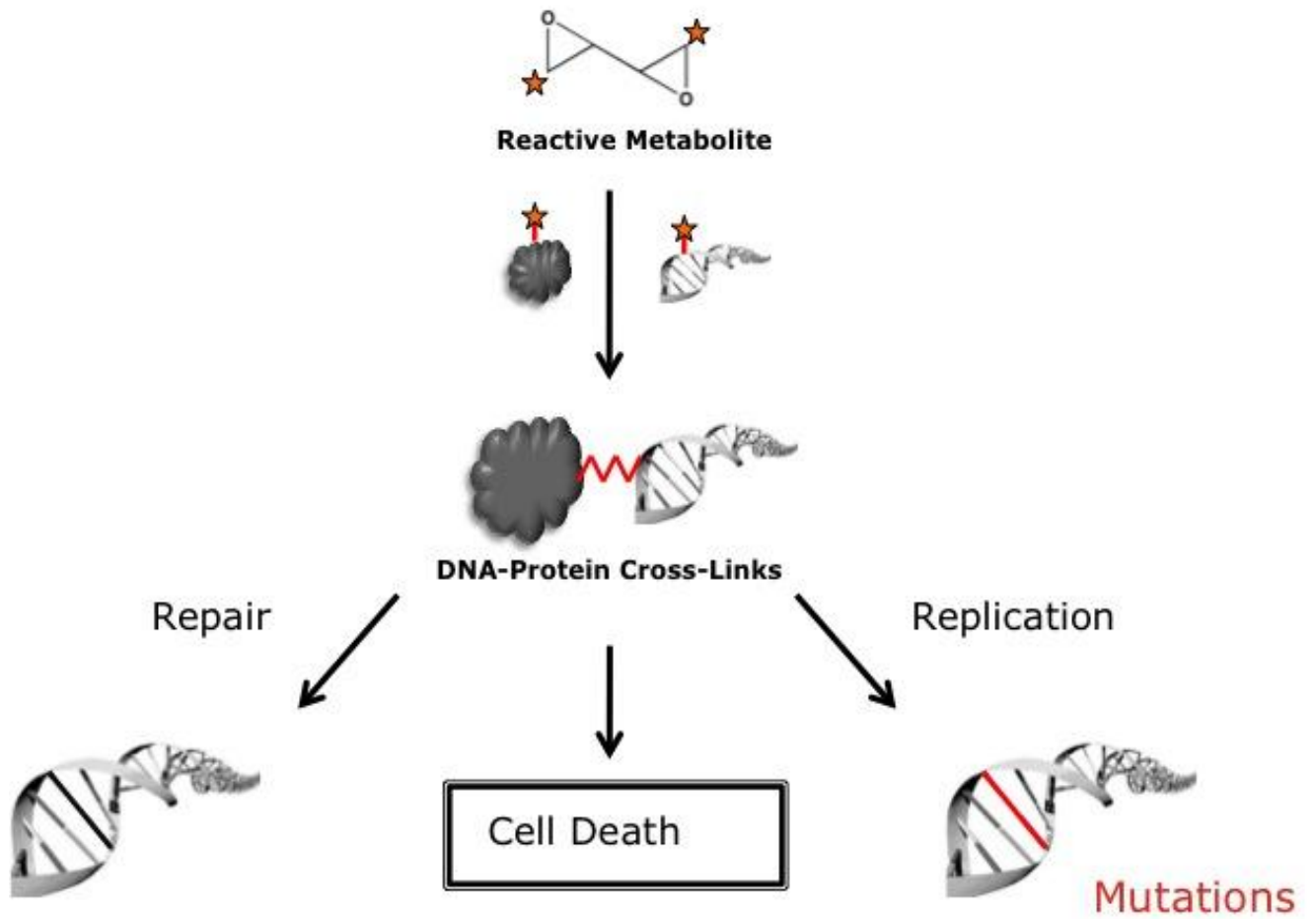
Figure 4. Mechanism and cellular comsequences of DNA-protein cross-linking by DEB.

capable of repairing this damage (*vide infra*). Factors such as the diversity of DNA-protein cross-link chemical composition, spontaneous hydrolysis events, contribution of DNA-DNA cross-links, and the limitations pertaining to DNA-protein cross-link isolation have made studying these species difficult (Barker *et al.,* 2005a).

Early studies suggested that repair of DNA-protein cross-links does occur in mammalian cells and that it most likely involves multiple pathways (Fornace *et al.,* 1979; Grafstrom *et al.,* 1984; Gantt *et al.,* 1987). Evidence such as the active removal of DNA-protein cross-links in cells continually undergoing treatment (Toyokuni *et al.,* 1995) supports the initial findings and points to NER as playing a critical role in repair of DNA-protein cross-links. NER-deficient lymphocyte lines have longer-lived DNA-protein cross-link lesions than NER-proficient cells lines (Barret *et al.,* 1995) and synthetic DNA-protein cross-links are acted on by NER components in *Escherichia coli* (Minko *et al.,* 2005). Other evidence suggests that mammalian cells utilize NER in combination with homologous recombination repair proteins to repair these lesions (Thompson *et al.,* 1996; Legerski *et al.,* 2002), similar to a mechanism used in bacteria to restart stalled replication forks (Rocca and Cox*,* 1997). Proteolysis has also been implicated in repair of DNA-protein cross-links, and may be important in reducing steric hindrance imposed by these bulky lesions (Quievryn and Zhitkovich. 2000; Desai *et al.,* 1997). Indeed, DNA-peptide cross-links are better substrates for NER in both human (Reardon and Sancar, 2006; Baker *et al.,* 2007) and bacterial cells than DNA-protein cross-links (Minko *et al.,* 2005).

Elucidating the exact biological consequences of these lesions is confounded by the fact that all agents know to induce DNA-protein cross-links also cause other forms of

DNA damage. These bulky lesions are expected to significantly distort the DNA helix leading to replication fork blockage and disruption of other normal DNA processes such as transcription and regulatory DNA binding (Barker *et al.,* 2005a,b). The deleterious consequences of such lesions is supported by the finding that many agents known to induce DNA-protein cross-links cause SCEs, cytotoxicity, and cellular transformation (Bradley *et al.,* 1979; Bradley and Kohn*,* 1979; Fornace*,* 1982; Merk and Speit, 1998). Associations between human cancer and DNA-protein cross-link accumulation have also been postulated (Wu *et al.,* 2002).

As mentioned earlier, biological consequences of a particular DNA-protein cross-link are highly dependent on its chemical composition, including both the cross-linking agent and the identity of the protein. Immunochemical methods have detected numerous proteins involved in DNA-protein cross-links, but those techniques require prior knowledge of cross-linking candidates (Barker *et al.,* 2005b). Early efforts to identify previously unknown DNA-protein cross-link proteins focused primarily on quantifying cross-link induction using the comet assay, which utilizes the reduced migration of cross-linked DNA fragments and does not allow for isolation of lesions (Roti Roti *et al.,* 1998; Merk *et al.,* 2000). Other methods, including gradient separation and filter binding, also suffer from issues such as nonspecific binding and incomplete fractionation, making DNA-protein cross-link isolation and identification especially difficult (Moss *et al.,* 1997; Cress *et al.,* 1990; Costa *et al.,* 1996). More recent studies have utilized mass spectrometry (MS) in combination with other isolation techniques to identify DNA-protein cross-links; however, proteins that tightly bind DNA are difficult to control for

and MS may be biased towards high-abundance proteins (Loeber *et al.,* 2009; Barker *et al.,* 2005a,b).

Many early studies involving DNA-protein cross-links focused on histone proteins because of their direct interactions with DNA (Barker *et al.,* 2005b). However, more recent experiments involving cross-linking agents, such as ionizing radiation and the nitrogen mustard mechloethamine, have identified cross-linked proteins with known nuclear functions (Barker *et al.,* 2005b; Mattagajasight *et al.,* 1999). Several groups of proteins are represented in these findings, including those that function in cell motility and structure, chromosomal remodeling components, and DNA repair (Loeber *et al.,* 2009). Further studies are required to verify *in vivo* cross-links formation and establish the biological effects of individual lesions.

Much effort has since gone into the study of different compounds that induce DNA-protein cross-links and new techniques are continuously improving their detection due to the deleterious effects of their formation. However, the proteins found to cross-link to DNA by DEB were discovered serendipitously. It began with investigations involving the conjugation of another *bis*-electrophile, 1,2-dibromoethane, to glutathione (GSH) (Rannug *et al.,* 1978; van Bladeren *et al.,* 1979). The cytoprotective effects of GSH have been known for some time due to its central role as a substrate to many detoxicating enzymes, e.g. GSH transferases, as well its ability to detoxicate free radicals and certain electrophiles through non-enzymatic reactions (Habig *et al.,* 1974; Anders, 2004).

GSH transferases are divided into several families depending on cellular location, but all function by catalyzing the conjugation of nonpolar compounds to GSH by

facilitating nucleophilic attack of the chemical by the cysteine residue of the GSH tripeptide. This conjugation usually leads to detoxication of reactive chemicals, such as cancer chemotherapeutic agents, environmental pollutants, and endogenous intermediates. Following conjugation, the GSH tripeptide usually undergoes further enzymatic processing that eventually leads to the cellular clearance of reactive compounds. While most GSH conjugation leads to the formation of less reactive compounds, some reactions actually lead to further activation. Other chemicals with GSH-conjugated metabolites that are more reactive than the parent compound include 1,2-dihaloethanes (Hayes *et al.,* 2005).

Bifunctional dihalomethanes (e.g. dichloromethane) and dihaloethanes (e.g. 1,2-dibromoethane) are of interest because of ubiquitous use in industry (ATSDR 1989, 1993 Methlene Chloride). 1,2-Dibromoethane was widely used as a gasoline additive and fumigant before it was discovered to be mutagenic (Rannug *et al.,* 1980; Letz *et al.,* 1984) and carcinogenic in animal models (Olsen *et al.,* 1973). Vicinal dihaloalkanes undergo transformation via cytochrome P450-mediated oxidation to haloactetaldehydes (Guengerich *et al.,* 1994), but the mutagenicity of these chemicals is dependent on its GSH-mediated activation (Rannug *et al.,* 1979; van Bladeren *et al.,* 1979).

Reactions between vicinal dihaloaklanes and GSH result in the nucleophilic displacement of one halide atom to yield a half-mustard at the sulfur atom of the GSH cysteine residue, which then undergoes cyclization to form an episulfonium ion. Episulfonium ions are highly reactive and can alkylate nucleophilic protein residues, as well as DNA. The metabolism of dihaloalkanes by GST is known to yield DNA adducts that effectively cross-link GSH primarily to the N^7 atom of guanine (Peterson *et al.,* 1988;

Cmarik *et al.,* 1992; Kim *et al.,* 1990). These findings were unexpected because reactions involving the conjugation of electrophiles with GSH normally act to reduce the reactivity of these chemicals (Figure 5).

In the 1990s, studies revealed that dihaloethanes also displayed enhanced mutagenic activity in systems overexpressing the repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) (Abril *et al.,* 1997; Abril *et al.,* 1999a; Abril *et al.,* 1999b). AGT is most noted for its cytoprotective activity resulting from its ability to directly reverse DNA damage caused by many common alkylating agents. AGT acts through a base-flipping mechanism that positions O^6-alkylated guanines in the vicinity of a nucleophilic cysteine residue. A catalytic triad consisting of His146, Arg147, and Glu172 reduces the pK_a of the active site cysteine (Cys152) to ~ 5 (Guengerich *et al.,* 2003). This activation allows for nucleophilic attack and transfer of the alkyl adduct to AGT, leading to its eventual degradation. Along with protecting the genome from the damage induced by many carcinogenic and mutagenic agents, AGT can also reduce the effectiveness of some chemotherapeutic drugs that alkylate DNA (Gerson *et al.,* 2002; Pegg, 2000) (Figure 6).

The mechanism of mutagenic enhancement by bifunctional electrophiles in systems overexpressing AGT is similar to that observed with GSH. The reaction of AGT with vicinal dihaloethanes results in the formation of a reactive episulfonium ion at the active site cysteine residue that can cross-link AGT to DNA (Liu *et al.,* 2002). As in the case of GSH, DNA-protein cross-links formed by these chemicals result primarily in N^7-guanine adducts (Liu *et al.*, 2004a,b). The elucidation of the mechanism of enhanced mutagenesis by *bis*-electrophiles in cells overexpressing AGT mostly utilized 1,2-
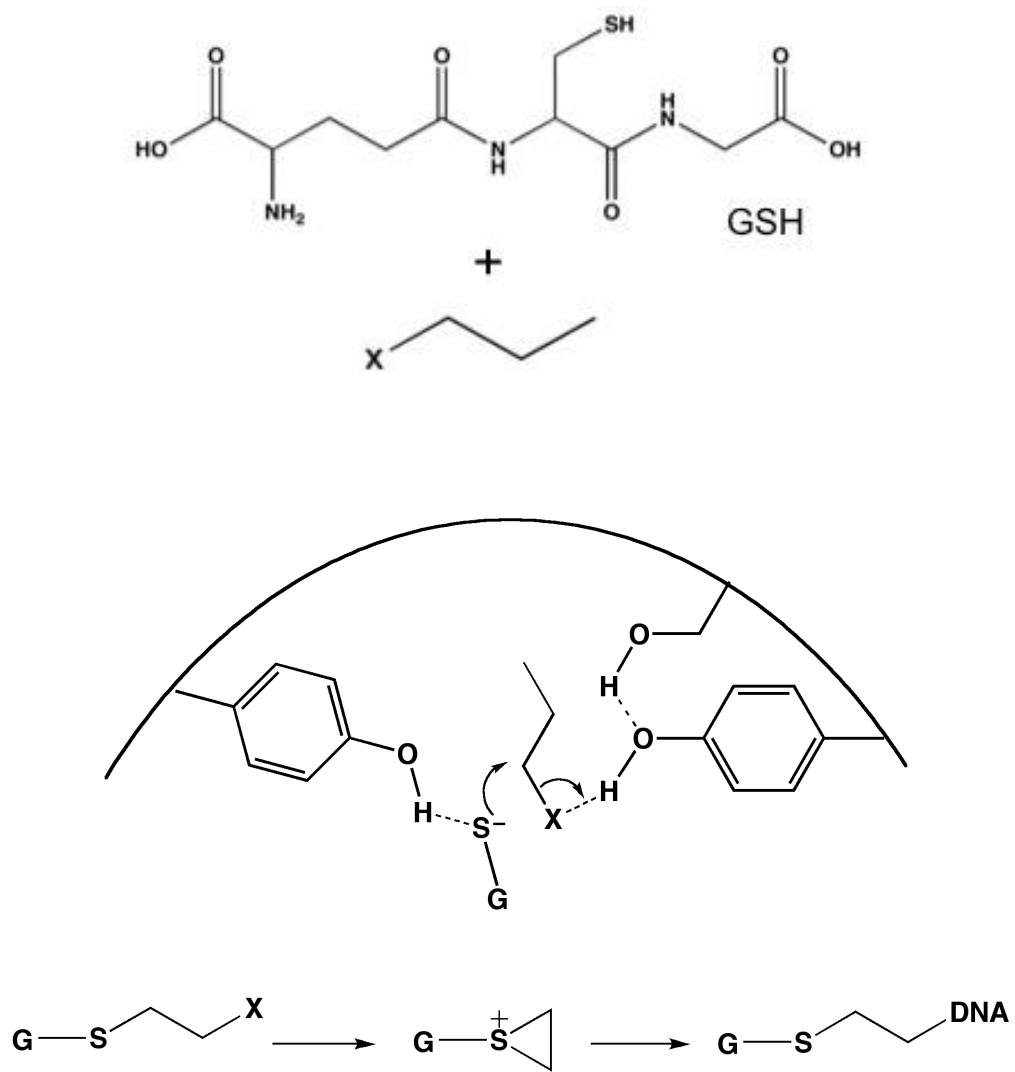
Figure 5. Mechanism of electrophile conjugation to GSH by GST (adapted from Hayes e*t al.*, 2005).
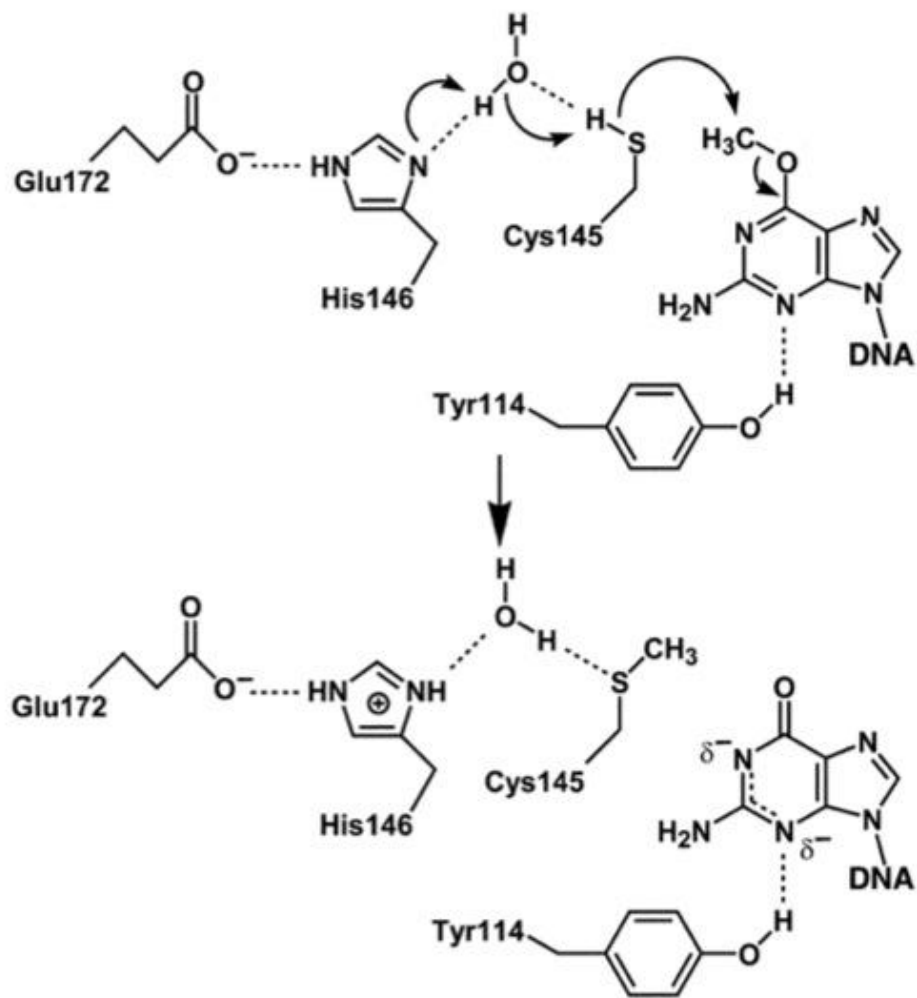
Figure 6. Mechanism of DNA alkylation reversal by AGT (Daniels *et al.*, 2004).

dibromoethane as a model electrophile; however, this phenomenon was also observed and characterized with DEB (Valadez *et al.*, 2004; Loeber *et al.,* 2006) (Figure 7).

The fate of these cross-links is still unknown, although the large lesions produced are expected to inhibit normal DNA metabolism if not properly repaired.   The contribution of *bis*-electrophile-induced DNA-protein cross-links to the mutagenic or carcinogenic properties of these chemicals is also unclear. Discovering the identity of proteins prone to forming cross-links may be an important step to answering these questions.

Research Aims

The overall goal of this research is to understand the mechanism of genotoxicity by *bis*-electrophiles (e.g. 1,2-dibromoethane and DEB). Our central hypothesis is that DNA-protein cross-link formation contributes to mutagenesis by *bis*-electrophiles and that other proteins, beside AGT and GST, can contribute to enhancement of mutagenesis by these chemicals. Interest in these chemicals stems from their ubiquitous use and carcinogenic properties.   The mutagenic potential of *bis*-electrophiles is thought to be partly dependent on reactions with cellular nucleophiles to form reactive intermediates, which then react with DNA to form DNA-protein cross-links. Much of what is known about the process of mutagenesis by *bis*-electrophiles is the result of studies involving the cellular nucleophiles GSH and AGT. The assays developed in this laboratory to study cross-linking by GSH and AGT were used in order to discover other protein systems that undergo similar chemistry (Figure 8).

GAPDH was identified as a candidate protein for DNA-cross-linking because of the nucleophilic character of its active-site residue cysteine 246 (Cys^{246}) (Dennehy *et al.*,
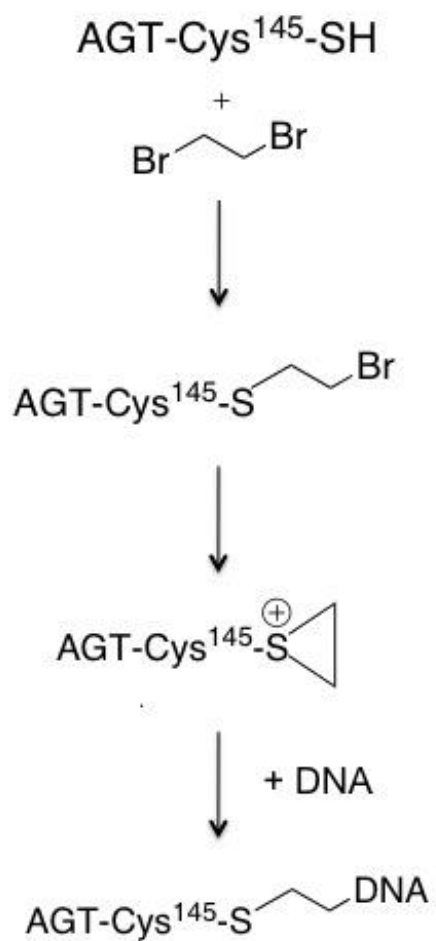

Figure 7. Mechanism of mutagenic enhancement of 1,2-dibromoethane by AGT.

Bis-Electrophile-Induced Result	AGT
Modified cysteine residue	✔
Inhibition of activity	✔
Cross-linking to DNA	✔
Enhanced mutagenesis	✔

Figure 8. Approach to investigating DNA-protein cross-linking candidates by using the characteristics of reactivity observed with AGT.

2006). The ability of *bis*-electrophiles to inhibit the activity of GAPDH was assessed using activity assays and alkylation of reactive residues was verified with mass

spectrometry. Heterologous expression of wild type and mutant protein was performed in bacterial cells in order to determine if expression of GAPDH in the presence of *bis*-functional electrophiles enhances mutagenicity and cytotoxicity.

In order to identify DNA-protein cross-links specifically formed by *bis*-electrophiles, a screen was performed using human liver nuclei and bacterial cells. DNA-binding protein isolated and enriched before incubating them with DNA-cellulose beads in the presence of 1,2-dibromoethane. MS was utilized to identify candidate proteins, while gel-shift assays verified their ability to cross-links to DNA. Tandem mass spectrometry was also used to identify modified residue(s) on candidate proteins found to form cross-links. Finally, heterologous expression of the candidate protein in bacterial systems was used to examine the effect of *bis*-electrophile-induced mutagenicity and cytoxicity (Figure 9).

The enhancement of mutagenesis by DEB has been thoroughly investigated in systems expressing GST; however, the reactive intermediate has not been fully investigated. Initial studies suggested the products formed by reaction between DEB and GST still retained epoxide activity. Cross-links between GSH, DEB, and DNA were also identified; however, further studies are needed to confirm the exact structure and mutagenicity of this intermediate.

CHAPTER II

REACTIONS OF *BIS*-ELECTROPHILES WITH GLYCERALDEHYDE 3-

PHOSPHATE DEHYDROGENASE

Introduction

The hypothesis that DNA-protein cross-link formation is a general mechanism for genotoxicity by *bis*-electrophiles has yet to be fully investigated. The studies involving cross-links induced by *bis*-electrophiles 1,2-dibromoethane and DEB result from interest in the carcinogenic properties of these chemicals, as well as the potential for human exposure. 1,2-Dibromoethane was formerly used as a fuel additive and fumigant, and is an environmental contaminant, although industrial use of this chemical has been drastically reduced (National Toxicology Porgram, 2002a). DEB is an oxidation product of the major industrial chemical 1,3-butadiene, which is used in the synthesis of plastics and rubber and found in automobile exhaust and cigarette smoke (National Toxicology Program, 2002b). 1,2-Dibromoethane and DEB are both carcinogens in laboratory animals (Wong *et al.*, 1982; Huff *et al.*, 1983; Henderson *et al.*, 1999; Bird *et al.*, 2001; Hughes *et al.*, 2003) and induce mutagenicity and toxicity in *in vitro* systems (van Bladeren *et al.*, 1980; Rannug *et al.*, 1980; Sasiadek *et al.*, 1998).

The ability of these chemicals to cross-link proteins or peptides to DNA was discovered in systems overexpressing O^6-alkylguanine-DNA alkyltransferase (AGT) (Abril *et al.*, 1995; Abril *et al.*, 1997; Liu *et al.*, 2000) and GSH transferase (Rannug *et al.*, 1978). The mechanism of cross-link formation between AGT and DNA has been

extensively investigated for 1,2-dibromoethane (Liu *et al.*, 2002; Liu *et al.*, 2004a,b; Valadez *et al.*, 2004) and was found to involve the formation of half-mustards at the active site cysteine, which can cyclize into unstable episulfonium ions (Peterson *et al.*, 1988). Reaction between highly electrophilic episulfonium ions and DNA results in the formation of DNA-protein or DNA-peptide cross-links. As a consequence of this reaction, *bis*-electrophiles also inhibit the activity of this DNA repair protein (Liu *et al.*, 2002).

DNA-protein cross-links are formed with many endogenous and exogenous chemicals, leading to highly persistent DNA lesions whose repair is poorly characterized (Bjorklund *et al.*, 2007; Voitkun *et al.*, 1999; Minko *et al.*, 2005). These large, bulky adducts are thought to disrupt normal DNA replication, producing genotoxic responses to the cross-links (Costa *et al.*, 1993; Barker *et al.*, 2005a,b). Many chemotherapeutic agents induce genotoxicity by forming DNA-protein cross-links. Identification of the specific proteins cross-linked to DNA by *bis*-electrophiles may lead to biomarker development for occupational and environmental exposure (Barker *et al.*, 2005a,b).

While mechanisms of cross-linking and misincorporation by *bis*-electrophiles have been extensively studied with GSH transferase/GSH and AGT (Guengerich, 2003), the formation of cross-links involving other proteins has not been investigated. We sought to determine whether or not other proteins and peptides could enhance mutagenesis of *bis*-electrophiles through an AGT-like mechanism. In order to address this question, isolated human nuclear proteins reactive toward model electrophiles were identified in a screen, along with their nucleophilic sites (Dennehy *et al.*, 2006). One protein found to be reactive with monofunctional electrophiles was glyceraldehyde 3-

phosphate dehydrogenase (GAPDH). GAPDH is a glycolytic enzyme responsible for catalyzing the oxidative phosphorylation of glyceraldehyde 3-phosphate (G3P) to 1,3-
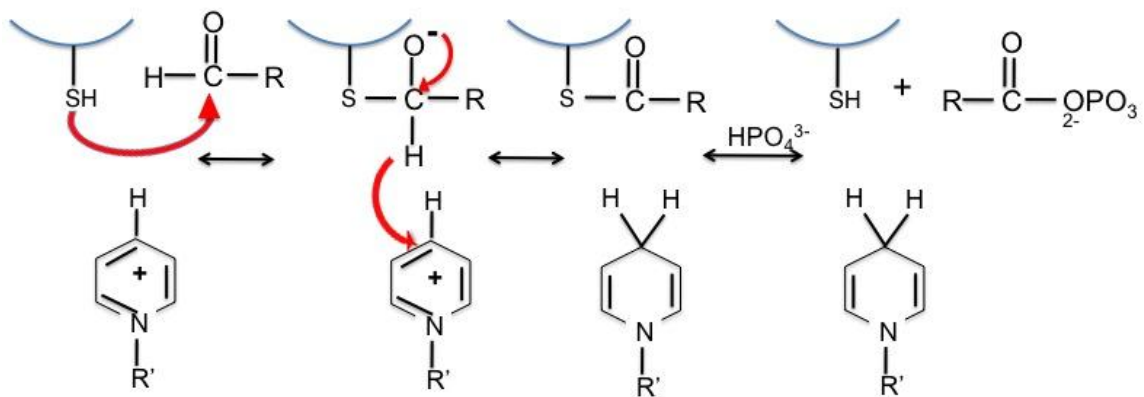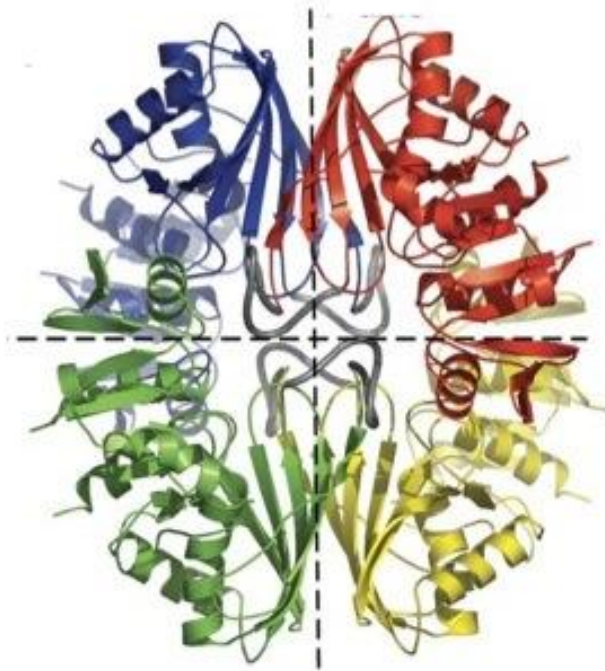

Figure 9. Crystal structure of homotetramer GAPDH and mechanism of oxidative phosphorylization of glyceraldehyde 3-phosphate to 1,3-bisphosphoglyerate (Ferreira-da-Silva *et al.*, 2006).

bisphosphoglycerate via its active site cysteine residue (Cys^{152}) (Figure 9). Recent studies provide evidence that GAPDH also functions within the nucleus during DNA replication and repair (Sirover, 2005; Hara, 2006). Cys^{156}, located near the active site and a peripheral cysteine (Cys^{246}) were identified as reactive nucleophiles with the monofunctional reagents (Jenkins *et al.*, 2006; Dennehy *et al.*, 2006) (Figure 10).

Using the AGT mechanism as a model, reactivity and cross-linking ability of GAPDH were investigated upon treatment with *bis*-electrophiles. Although GAPDH displayed several of the characteristics of AGT (Liu *et al.*, 2002), enhancement of mutagenesis was not observed in *E. coli* cells. Our results show that reactivity toward *bis*-electrophiles does not necessarily predict the ability of a protein to enhance mutagenesis by these compounds.

Materials and Methods

Materials. 1,2-Dibromoethane, DEB (racemic mixture containing meso isomers), and CH_2Br_2 were purchased from Aldrich Chemical Co. (Milwaukee, WI). The oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' was synthesized by Midland Certified (Midland, TX) and purified by denaturing gel electrophoresis. Purified human erythrocyte GAPDH was purchased from Sigma-Alrich (St. Louis, MO) and *Escherichia coli* recombinant human AGT was a gift of A. E. Pegg (Pennsylvania State, Hershey, PA).

GAPDH Activity Assays. Activity assays were performed as previously described (Worthington, 1993) using GAPDH. GAPDH was diluted to a concentration of
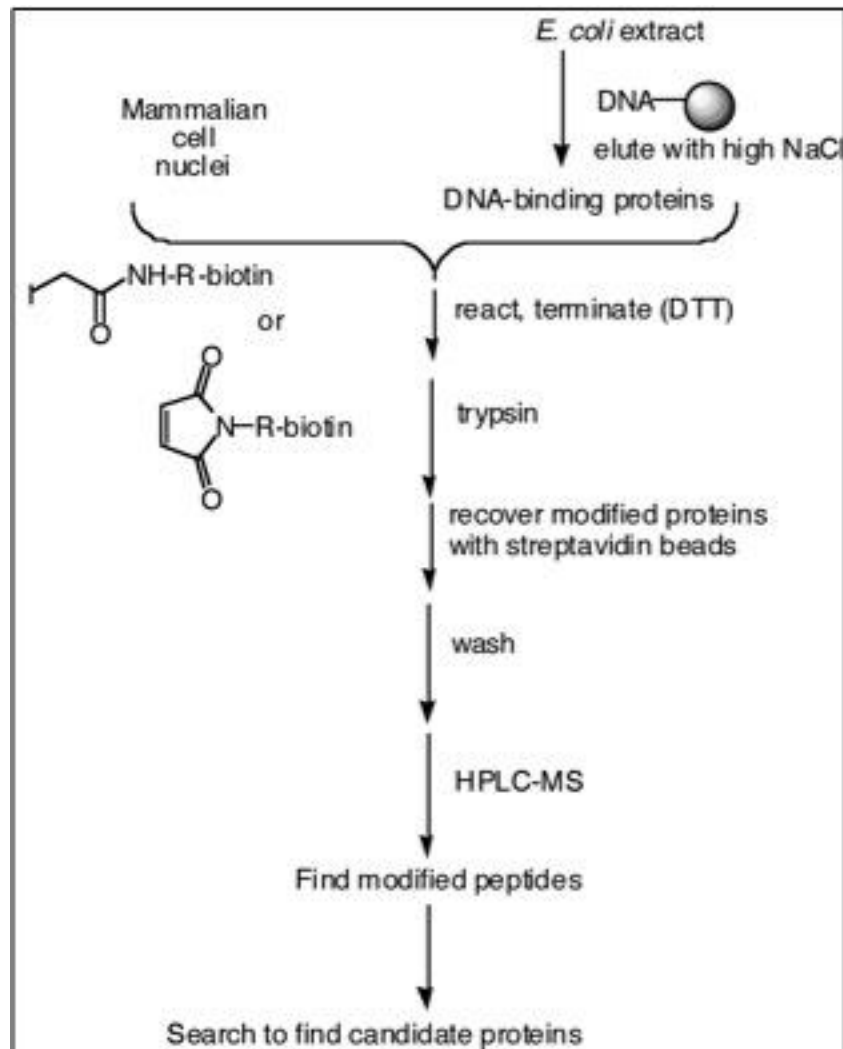
Figure 10. Mass spectrometry-based screen for nuclear proteins with nucleophilic sites (Dennehy *et al.*, 2006).

30 µg/mL with the reaction buffer prior to incubation. Reactions (1.0 mL) containing 0.25 mM NAD^+, 3.3 mM DTT, and 1 µg/mL GAPDH in 15 mM sodium pyrophosphate buffer (pH 8.5) containing 30 mM sodium arsenate were treated with each *bis*-electrophile dissolved in DMSO for 30 min at 37 ºC in a shaking water bath (the concentration of DMSO was <1%, v/v). Reactions were cooled to room temperature for 5 min before the addition of G3P to a final concentration of 0.5 mM. Activity was determined as the change in A_{340} observed for 30 s immediately following the addition of G3P, using a continuous assay in a Cary 14-OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA).

GAPDH activity of cell lysates was assessed by inducing 5 mL cultures in LB at an OD_{600} of 0.5 for 3 h. Cultures were briefly centrifuged and the pellets were resuspended in 1 mL of 15 mM sodium pyrophosphate buffer (pH 8.5) containing 30 mM sodium arsenate and sonicated for 15 s. Lysate supernatant (33 µL) was incubated with the same sodium pyrophosphate/arsenate buffer containing 0.25 mM NAD^+ and 3.3 mM DTT for 30 min at 37 ºC while shaking. Samples were analyzed for activity as described above.

Gel Mobility Shift Assays. Gel mobility shift assays were performed as previously described using a 12% SDS (w/v) polyacrylamide gel and the 5'-end ^{32}P-labeded oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' (Liu *et al.*, 2002). Reactions (10 µL) contained 2 µg GAPDH and were incubated for 1 h at 37 °C with CH_2Br_2, 1,2-dibromoethane, or DEB prior to analysis.

Mass Spectral Analysis. GAPDH (1 µg) was incubated with 10 mM 1,2-dibromoethane or DEB in 50 µL of 50 mM Tris-HCl (pH 7.6) buffer containing 0.1 mM

EDTA for 1 h at 37 °C. Samples were reduced, alkylated, and digested with trypsin as previously described (Dennehy *et al.*, 2006). Sample peptide solutions were desalted with P10 C_{18} ZipTips (Millipore, Billerica, MA) according to the manufacturer's instructions (www.millipore.com/userguides.nsf/docs/pr02358). Mass spectral analysis was performed in the positive ion mode by electrospray LC-MS/MS using a TSQ Quantum HPLC-ESI-MS instrument coupled to a Surveyor autosampler and Surveyor MS pump (ThermoElectron, San Jose, CA). Samples were separated as described elsewhere (Liu *et al.*, 2007) with an ODS-AQ column (5 μm, 2.0 mm × 150 mm, YMC, Kyoto) with a 98:2:0.05:0.4 (v/v/v/v) mixture of H_2O, CH_3OH, CF_3CO_2H, and CH_3CO_2H. The Quantum mass spectrometer was equipped with a standard electrospray ionization source and fused silica capillary. Source CID was used at 10 V. SRM mode analysis was performed for adducted peptides containing Cys^{246} (*m/z* = 759.9, 789.4) and the unmodified peptide (*m/z* = 737.4). Mass spectral resolution was set to a peak width of 1.0 u for both precursor and product ions. Mass transitions at the specified collision energy (*m/z* 759.9, 737.4, and 789.4; -26, -24, and -28 eV, respectively) were monitored for adducted peptides. For the peptides containing the active site Cys^{152} residue were also monitored with similar parimeters for adducted peptides (*m/z* 883.4, 912.5) and unmodified adducts (*m/z* 860.5) (Table 1). Data were acquired in the profile mode. Xcalibur Software, version 1.3 (ThermoElectron), was used on a Dell Optiplex GX240 computer (Dell Computer, Round Rock, TX) running a Microsoft Windows 2000 operating system (Microsoft, Redmond, WA) to control all instruments and process data.

GAPDH cDNA Synthesis and Expression. The amino acid sequence and native cDNA sequence for human GAPDH was obtained via GenBank (NM_002046). Forty

overlapping 40- to 50-mer oligonucleotides were synthesized (Operon, Huntsville, AL) based on DNAWorks results (http://helixweb.nih.gov/dnaworks/) (Wu *et al.*, 2006). The codons were optimized automatically for *E. coli* codon preferences. Overlapping melting temperatures were designed to be 65 ± 3 °C. The 5′- and 3′-flanking sequences were 5′-CCGAATT-3′ (sense) and 5′-GACCCCTGGATCCCGC-3′ (sense) respectively. Flanking sequences were engineered to contain an *Eco*RI restriction site on the 5'-end and a *Bam*HI restriction site on the 3'-end of the gene. Polymerase cycling assembly and polymerase chain reaction (PCR) amplification of the gene was performed as described elsewhere (Wu *et al.*, 2006). The PCR product was digested with *Bam*HI and *Eco*RI restriction enzymes (New England Biolabs, Ipswich, MA) and purified by agarose gel electrophoresis. The resulting fragment was ligated into a pINIII-A3(lpp^{p-5}) vector, which was also digested with *Eco*RI and *Bam*HI restriction enzymes. The ligation mixture was transformed into DH5α *E. coli* cells and selected for ampicillin resistance on LB plates containing 50 μg/mL ampicillin. Vectors were purified from surviving colonies with a QIAprep Miniprep kit (Qiagen, Valencia, CA), and restriction digestion with *Eco*RI and *Bam*HI was used to screen for ligated vectors. The GAPDH insert of a plasmid yielding approximately 7 and 1 kb DNA fragments upon restriction digestion was analyzed in the Vanderbilt DNA Sequencing facility using an Applied Biosystems Model 3700 fluorescence sequencing unit with a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA). The sequenced plasmid was amplified and isolated from DH5α cells and transformed into TRG8 *E. coli* cells, which were selected for with LB plates containing both ampicillin (50 μg/mL) and kanamycin (50 μg/mL). GAPDH expression was verified in cells induced with IPTG (0.2 mM) by immunoblot analysis

using polyclonal human anti-GAPDH antibodies (Promega, Madison, WI) according to the manufacturer's instructions. Enhanced chemiluminescense (GE, Buckinghamshire, UK) was used to detect protein bands and expression was quantified with an Alpha Imager 3400 (Alpha Innotech, San Leandro, CA).

Cell Survival and Mutagenicity. TRG8 cells expressing AGT or containing the empty pINIII vector served as positive and negative controls, respectively. All strains were induced at an OD_{600} of 0.6 and incubated at 37 °C while shaking for 3 h prior to *bis*-electrophile treatment. The toxic and mutagenic effects of treatment were assessed as described previously (Liu *et al.*, 2000), but with culture dilutions of $1:10^6$ on *his*[+] plates.

Electrophoretic Mobility Shift Assays. The DNA binding ability of GAPDH and purified human AGT was analyzed essentially as previously described (Rasimas *et al.*, 2007) but using the ^{32}P-end labeled oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' and 10% (w/v) PAGE native gel. Running buffer containing 10 mM Tris acetate (pH 7.6) and 100 mM NaCl was circulated frequently to prevent buffer exhaustion while samples were run at 8 V/cm following a 30 min pre-run.

Results and Discussion

GAPDH Inhibition. Inhibition of GAPDH activity by sulfhydryl-reactive chemicals, e.g. iodoacetamide (IOA), has been recognized for some time and is known to be the result of alkylation at the active site cysteine (Cys[152]) (Worthington, 1993). *bis*-Electrophiles are also known to selectively alkylate sulfhydryls, making Cys[156] (located near the active site) a potential target for *bis*-electrophile reactivity. The activity of GAPDH was assessed by monitoring changes in A_{340} in the presence of G3P. Human
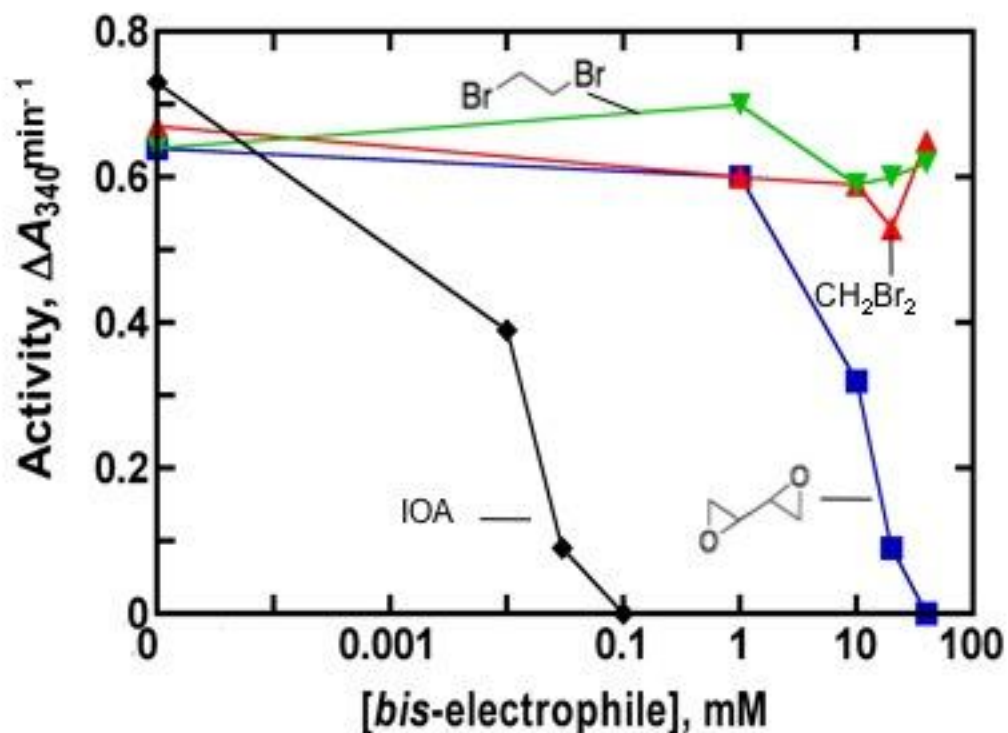
Figure 11. Inhibition of GAPDH activity by DEB. Reactions (1.0 mL) containing 30 μM GAPDH were incubated with varying concentrations of the *bis*-electrophiles CH_2Br_2 (▲), 1,2-dibromoethane (▼), or DEB (■) in sodium pyrophosphate (15 mM) buffer containing 30 mM arsenic pyrophosphate, 250 μM NAD^+, and 3 mM DTT for 30 min at 37 °C in a shaking water bath. The change in A_{340} was monitored for 30 s following the addition of 0.25 μmol G3P and inhibition was compared with IOA (◆) treatment.

erythrocyte GAPDH was incubated with varying concentrations of CH_2Br_2, 1,2-dibromoethane, or DEB. A reduction in GAPDH activity was only observed for samples treated with high concentrations of DEB (Figure 11). The addition of 1,2-dibromoethane, CH_2Br_2, or only DMSO did not affect the ability of GAPDH to reduce NAD^+. Inhibition was observed with DEB, but the concentration required for IC_{50} was 10^3-fold higher than the concentration of IOA needed for the same effect. AGT activity is also known to be more sensitive to treatment with DEB, with an IC_{50} of 1.0 mM (Valadez *et al.*, 2004). The assays used to monitor GAPDH and AGT activity differ, but comparison of the DEB concentrations required at IC_{50} for AGT (Valadez *et al.*, 2004) and GAPDH show a 10-fold difference.

GAPDH-DNA Crosslinking Assays. The mutagenic properties of *bis*-electrophiles were first shown to be enhanced in systems overexpressing GSH transferases in 1978 (Rannug *et al.*, 1978). Subsequently this phenomenon was also identified in systems overexpressing AGTs (Abril *et al.*, 1995). *bis*-Electrophiles induced more mutations in these systems through induction of DNA-GSH and DNA-AGT cross-links by reaction between nucleophilic sulfhydryls, *bis*-electrophiles, and DNA (Peterson *et al.*, 1988; Liu *et al.*, 2002). In order to assess the ability of *bis*-electrophiles to form cross-links between DNA and GAPDH, reactions containing ^{32}P-labeled 16mer oligonucleotides, GAPDH, and *bis*-electrophile were incubated at 37 °C for 1 h and analyzed for cross-linking using SDS-polyacrylamide gel electrophoresis. The only significant cross-link formation between GAPDH and DNA was observed with DEB (Figure 12) and was dependent on incubation time and *bis*-electrophile concentration (data not shown). Treatment of cross-link reactions with hot piperdine resulted in the
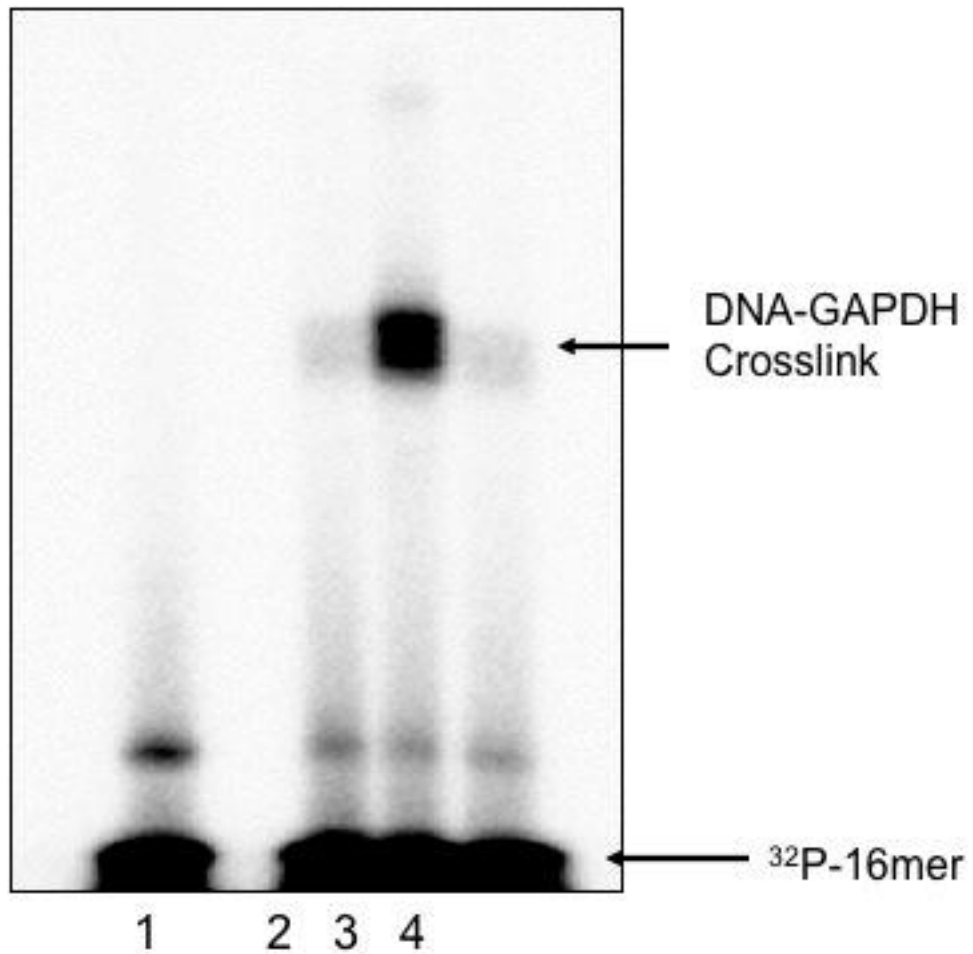
Figure 12. *In vitro* formation of GAPDH-DNA cross-links by DEB. Human GAPDH (2 μg) was incubated with ^{32}P-labeled 16mer oligonucleotide in AGT buffer (50 mM Tris-HCl (pH 7.4), 0.1 mM DTT, and 1 mM EDTA) containing DMSO vehicle (<1%, v/v) (lane 1), 20 mM CH_2Br_2 (lane 2), 20 mM DEB (lane 3), or 20 mM 1,2-dibromoethane (lane 4) at 37 °C for 1 h. Samples were separated on a 12% (w/v) SDS-polyacrylamide gel, resulting in the formation of a distinct, slowly migrating band corresponding to a DNA-protein cross-link present in only DEB -treated sample.

disappearance of the slower migrating band, consistent with the N^7-guanine or N^3-adenine cross-links formed between AGT and DNA (Liu *et al.*, 2004a,b). As in the activity assays, very high concentrations (20 mM) of DEB were required to induce *in vitro* cross-link formation.

MS Analysis of *bis*-Electrophile-Reactive Cysteine Residues. Cross-link formation between DNA and AGT was previously shown to be dependent on the reaction between the active site cysteine (Cys^{145}) and *bis*-electrophiles (Liu *et al.*, 2002), and demonstrated using site-directed mutagenesis with C145A and C145S mutants. The nucleophilic active site cysteine (Cys^{152}) of GAPDH, also known to be nucleophilic and reactive toward monofunctional electrophiles, made this protein a potential candidate for reacting with *bis*-functional electrophiles (Worthington, 1993). In order to identify *bis*-electrophile-reactive residues of GAPDH, MS analysis was used to probe treated protein for hydrolyzed 1,2-dibromoethane and DEB adducts. Analysis was focused on two tryptic peptides identified in the monofunctional electrophile screen (Dennehy *et al.*, 2006), each containing a nucleophilic cysteine residue (Cys^{152} and Cys^{246}) (Table 1). One peptide contained Cys^{156} as well as the active site cysteine Cys^{152}, while the second peptide contained the peripheral Cys^{246}, which maps to the interface of two monomers in the functional homotetramer (Ferreira-da-Silva *et al.*, 2006). A 1,2-dibromoethane-induced hydroxyethyl adduct and DEB-induced trihydroxybutyl adduct (*m/z* = 759.9 and 789.4, respectively) were observed only for the peptides containing Cys^{246} (Figures 13A, B). The ethylene elimination product from 1,2-dibromoethane was also only observed with the Cys^{246} peptide, while spectra corresponding to unmodified and carboxymethylated cysteines were observed for both peptides, confirming ionization

Table 1. GAPDH peptides (*m/z*) analyzed for detection of *bis*-electrophile adducts.
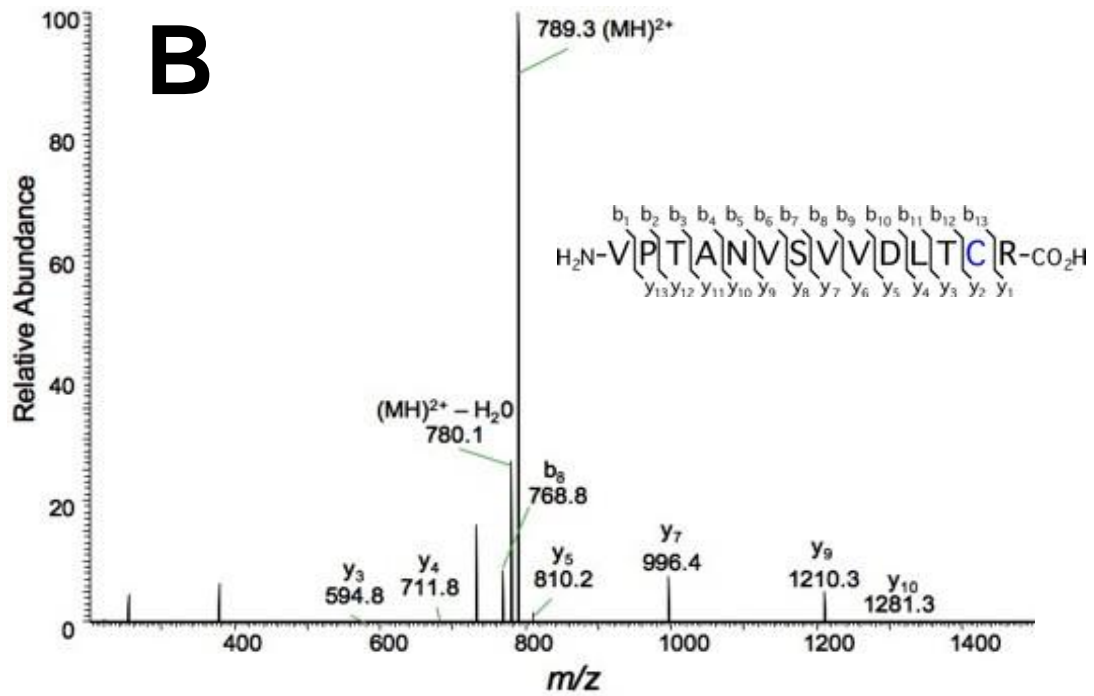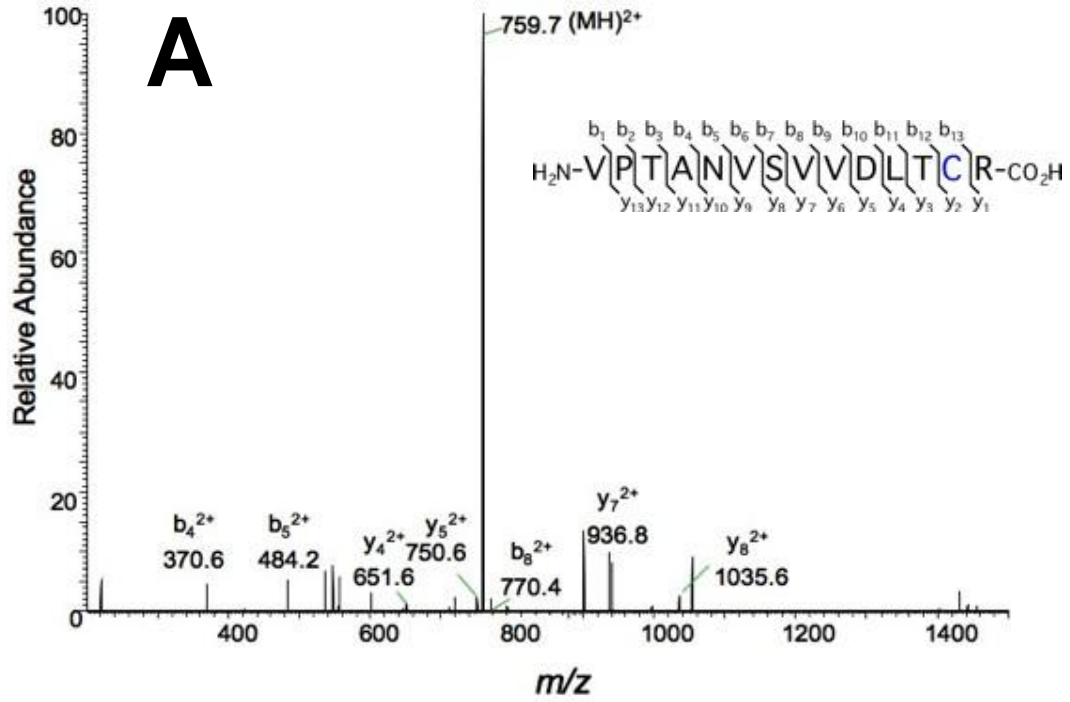
Peptide	IISNASC^{152}TTNC^{156}LAPLAK	VPTANVSVVDLTC^{246}R
Unmodified	860.5	737.4
Dibromoethane	883.5	759.9
DEB	903.5, 912.5	780.4, 789.4

* All *m/z* values represent (M+2H)$^{2+}$ ions.

(data not shown). Selective reaction monitoring (SRM) was used to verify that 1,2-dibromoethane and DEB hydrolysis adducts are distinguishable due to the differential elution and reaction specificity for each *bis*-electrophile (Figure 13C).

In contrast to the expected reactions at Cys^{152}, *bis*-electrophile adducts were only observed with Cys^{246}. Indeed, reactions between Cys^{246} and other cellular electrophiles have been previously reported (Alderson *et al.*, 2006). The lack of reactivity at Cys^{152} seemed inconsistent with the inhibition assay results. The possibility exists that reactions at the peripheral Cys^{246} result in an inhibitory structural change or protein-protein cross-link formation, which has been previously documented (Shaltiel *et al.*, 1971). Supporting this potential mechanism, silver staining of a polyacrylamide gel containing *bis*-electrophile-treated GAPDH indicated the presence of a high M_r band consistent with that of a GAPDH dimer (data not shown).

Overexpression of GAPDH in *E. coli* Cells and *bis*-Electrophile-Induced Mutagenesis. Based on the precedent of AGT work (Liu *et al.*, 2002), human GAPDH was heterologously expressed in *E. coli* TRG8 cells engineered to lack endogenous O^6-alkylguanine transferase background (*ogt⁻, ada⁻*). Immunoblot analysis verified overexpression of GAPDH in pINIII-hGAPDH transformed cells through comparison with cells containing the empty pINIII vector (Figure 14A). Quantification of AGT expression by immunoblot analysis showed values (17 nM) similar to that of GAPDH expression (14 nM) 3 h after induction, showing that lack of mutagenesis was not due to lower protein levels. The lysate from cells overexpressing recombinant GAPDH also displayed 2-fold higher total GAPDH activity (*E. coli* and human) in comparison with control cells (0.17 $\pm$ 0.026 versus 0.086 $\pm$ 0.016 ΔA_{340} min^{-1}) providing evidence that an
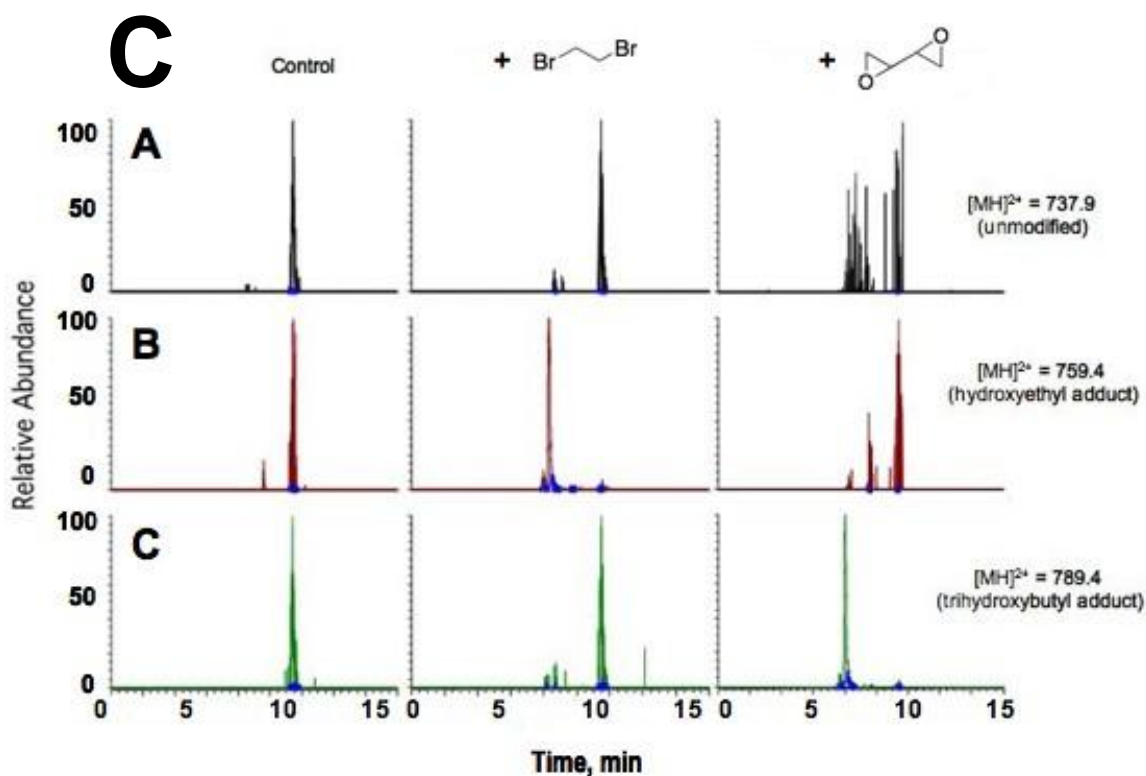
38

A

759.7 (MH)$^{2+}$

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13}
H$_2$N-VPTANVSVVDLTCR-CO$_2$H
y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

b_4^{2+}
370.6

b_5^{2+}
484.2

y_4^{2+} y_5^{2+}
651.6 750.6

b_8^{2+}
770.4

y_7^{2+}
936.8

y_8^{2+}
1035.6

B

789.3 (MH)$^{2+}$

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10} b_{11} b_{12} b_{13}
H$_2$N-VPTANVSVVDLTCR-CO$_2$H
y_{13} y_{12} y_{11} y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

(MH)$^{2+}$ – H$_2$0
780.1

b_8
768.8

y_3
594.8

y_4
711.8

y_5
810.2

y_7
996.4

y_9
1210.3

y_{10}
1281.3

Figure 13. Mass spectral analysis of Cys^{246} adducts formed from 1,2-dibromoethane or DEB -treated GAPDH. GAPDH (1 µg) was incubated in AGT buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 mM DTT) with 10 mM 1,2-dibromoethane or DEB for 1 h at 37 °C and reduced, alkylated, and digested with trypsin. Tryptic peptides were separated and analyzed by mass spectrometry. The hydrolyzed adducts resulting from incubation with (A) 1,2-dibromoethane or (B) DEB were identified as conjugated only to Cys^{246}, resulting in peptides displaying *m/z* 759.4 and 789.9, respectively. (C) SRM analysis was used to confirm that the specific adducts were distinct and present in the samples treated with the corresponding *bis*-electrophile.

active form of this enzyme was being produced. Toxicity and mutagenicity of DEB treatment utilized the pINIII-containing strain as controls due to some bleed-through expression of GAPDH in uninduced TRG8 cells (without IPTG). GAPDH over-expression did not significantly affect the growth rate of these cells (results not shown). Treatment of cells expressing AGT or GAPDH or containing empty pINIII vectors with DEB resulted in reduced survival for all strains, consistent with previous AGT studies (Valadez *et al.*, 2004) (Figure 14B).

In order to assess the effect of GAPDH overexpression on mutagenesis by diepoxybutane, treated cells were plated on restrictive plates lacking histidine and grown for several days, allowing selection of cells containing reversion mutations in the *hisG* gene. As previously observed, AGT expression in DEB -treated *E. coli* resulted in more his^+ reversion mutants (Valadez *et al.*, 2004); however, GAPDH expression did not significantly affect the mutation rate (Figure 14C).

Reduced DNA-binding Ability of GAPDH in Comparison with AGT.
Incubation of GAPDH with high concentrations of diepoxybutane was found to inhibit activity and induce *in vitro* DNA-protein cross-links (Figures 11, 12) and MS analysis also confirmed the reactivity of nucleophilic Cys^{246} with *bis*-electrophiles (Figure 13). However, overexpression of GAPDH in cells treated with DEB did not enhance mutagenesis (Figure 14). GAPDH and AGT are both reactive with *bis*-electrophiles *in vitro*, but overexpression of these proteins in treated cells had very different consequences.   In order to understand this apparent discrepancy, we considered differences between the inherent characteristics of each protein. One of the most obvious
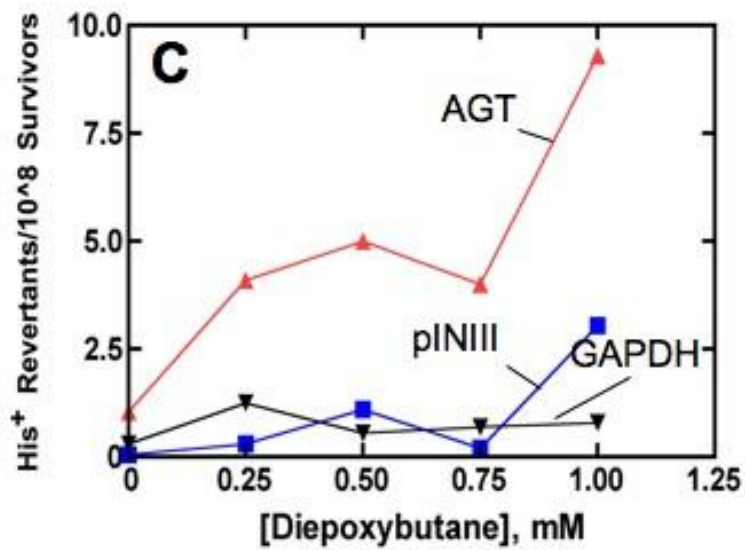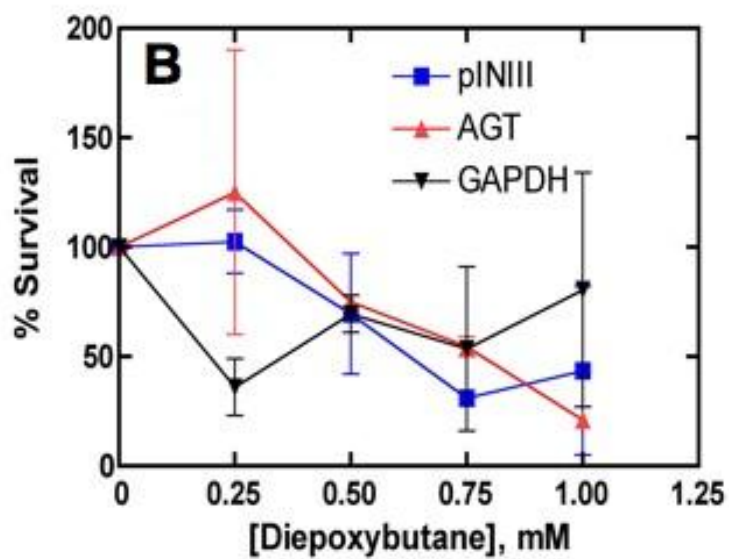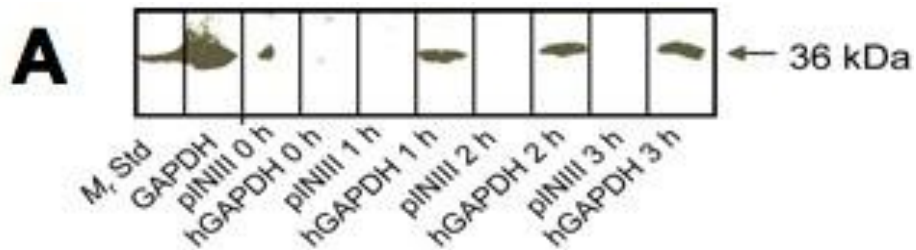
Figure 14. Lack of enhanced mutagenesis by DEB in cells overexpressing GAPDH. (A) Immunoblot analysis of GAPDH expression induction by TRG8 *E. coli* cells containing pINIII-hGAPDH vectors in comparison with those transformed with the empty pINIII vector showed significant production of hGAPDH. Expression of GAPDH (14 nM) was similar to that of AGT (17 nM) 3 h after induction. (B) TRG8 *E. coli* cells expressing GAPDH (▼) or AGT (▲) or containing the empty pINIII vectors (■) were treated with varying concentrations of DEB for 30 min at 37 °C prior to plating. The toxicity of DEB was shown to reduce the percent survivorship for all three strains. (C) Mutational frequency was scored as the number of *his*$^+$ revertant colonies on histidine deficient plates per 10^8 survivors.

is that AGT is a nuclear protein known to bind and repair DNA (Daniels *et al.*, 2004; Rasimas *et al.*, 2007). Conversely, GAPDH is predominantly cytosolic although some nuclear functions have been proposed (Shirover, 2005) and DNA-binding ability has been documented (Arutyunova *et al.*, 2003). One possibility is that the lack of mutation enhancement in treated cells overexpressing GAPDH is due to differing nuclear locations with respect to DNA during reactions with *bis*-electrophiles. Assessment of the differences in DNA binding ability between AGT and GAPDH indicated that AGT associates with DNA to a much greater extent than GAPDH (Figure 16), which may explain the lack of mutagenesis observed for GAPDH.
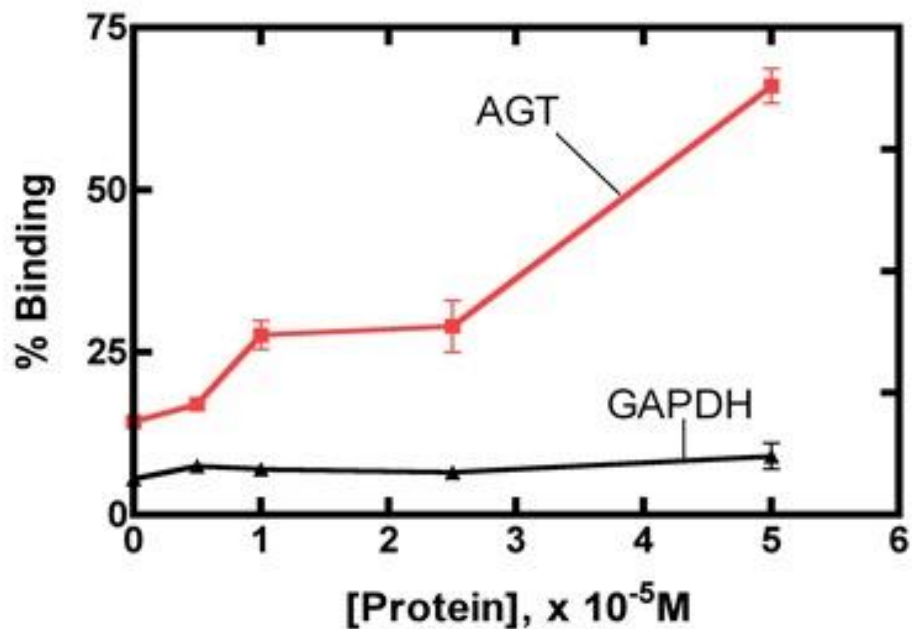
Figure 15. DNA-binding ability of GAPDH and AGT. Human GAPDH or human AGT (0 - 50 μM) was incubated with 6 μM ^{32}P-16mer in binding buffer (10 mM Tris (pH 7.6), 100 mM NaCl, 1 mM DTT, and 10 μg/mL bovine serum albumin) for 30 min at 23 °C. Samples were separated on a native 10% (w/v) polyacrylamide gel at 8 V/cm and protein-DNA complexes were quantified using autoradiography.

Conclusions

As in the case of AGT, treatment of GAPDH with DEB results in inhibition of activity and DNA-protein cross-link formation in *in vitro* assays in response to treatment with DEB. Although the reactivity of GAPDH with both DEB and 1,2-dibromoethane was validated with MS results, the overexpression of this protein in treated *E. coli* cells did not result in enhanced mutagenesis, in contrast with AGT (Liu *et al.*, 2002). The lack of mutational enhancement may be due to the inherently lower DNA binding ability of GAPDH (Figure 15), which could reduce the efficiency of cross-link formation. In addition, the high concentration of DEB required to inhibit GAPDH activity (Figure 11) and induce cross-link formation (Figure 12) suggest that GAPDH is also inherently less reactive toward *bis*-electrophiles than AGT. These findings provide evidence that not all *bis*-electrophile-reactive proteins are capable of enhancing mutagenesis and suggest that *in vivo* cross-linking depend in part on the inherent characteristics of a protein. These studies also provide evidence that proteins display varying degrees of reactivity toward different *bis*-electrophiles, with AGT being more reactive toward 1,2-dibromoethane (Valadez *et al.*, 2004) and GAPDH selectively reacting with DEB. Other protein candidates are currently being investigated for their ability to reactive with and enhance mutagenicity of *bis*-electrophiles.

CHAPTER III

THE *BIS*-ELECTROPHILE DIEPOXYBUTANE CROSS-LINKS DNA TO HUMAN HISTONES BUT DOES NOT RESULT IN ENHANCED MUTAGENESIS IN RECOMBINANT SYSTEMS

Introduction

Efforts to fully understand the mechanisms by which bis-electrophiles exert their toxic effects are ongoing, but their importance is highlighted by the fact that 1,2-dibromoethane and 1,3-butadiene are both mutagens and potential human hazards due to environmental contamination and occupational exposure (NTP, 2002; NTP, 2003). 1,2-Dibromoethane was widely used as an anti-knock additive in leaded gasoline and as a pesticide before its mutagenic and carcinogenic properties were discovered (Olson *et al.,* 1973; Letz *et al.,* 1984; Hill *et al.,* 1978). 1,3-Butadiene is an important industrial chemical widely used in the production of rubber and plastic and is also present in automobile exhaust and cigarette smoke (Himmelstein *et al.,* 1997). DEB is a minor but highly mutagenic product resulting from epoxidation of 1,3-butadiene by P450 enzymes (Guengerich, 2005). The oxidation products resulting from 1,3-butadiene are thought to be responsible for the mutagenic and carcinogenic properties observed in experimental animals treated with 1,3-butadiene (Melnick and Sills, 1990; Henderson *et al.,* 1996; Rice and Boffetta, 2001). The direct alkylation and modification of DNA by these molecules is thought to contribute to their detrimental effects. Indeed, we know modification of DNA is a major pathway by which carcinogens can exert their harmful effects

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(Hemminki *et al*., 1997). The resulting DNA adducts can prevent accurate replication of the genome, leading to the accumulation of mutations, which can give rise to cancer (Poirier and Beland, 1992). Understanding the mechanisms by which DNA adducts contribute to mutagenesis is critical for developing potential chemoprevention strategies and discovering biomarkers.

In addition to mono-alkylating biological molecules, 1,2-dibromoethane and DEB also cross-link biological molecules through reactions at the two electrophilic centers. This phenomenon was initially observed in systems overexpressing GST (Rannug *et al.,* 1978) or AGT (Abril *et al.,* 1995, 1997; Liu *et al.,* 2000), where an increase in mutagenesis was observed with treatment by *bis*-electrophiles. Isolated DNA-protein cross-links provided evidence for the tethering of reactive cysteine residues to nucleophilic sites on DNA by *bis*-electrophiles (Liu *et al.,* 2002; Guengerich *et al.,* 2003). These lesions are known to be mutagenic, which may be a consequence of their bulk and DNA-distorting behavior that can block replicative polymerases, disrupting normal DNA processing (Costa *et al.,* 1993; Barker *et al.,* 2005a,b). DNA-protein cross-links have also been shown to be relatively stable in comparison with other adducts, but the comprehensive characterization of these DNA lesions has not been achieved in part due to difficulties isolating these complexes (Bjorkland and Davis, 2007; Voitkum and Zhitkovich, 1999; Minko *et al.,* 2005).

In order to determine if the formation of DNA-protein cross-links is a general mechanism of mutagenesis by *bis*-electrophiles, we employed a mass spectrometry-based search of DNA-binding proteins (Figure 16). From the candidate proteins identified, human histones H2b and H3 were examined in detail because of their DNA-binding

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ability and basic nature (Peterson and Laniel, 2004) (Figure 17). Although reactions
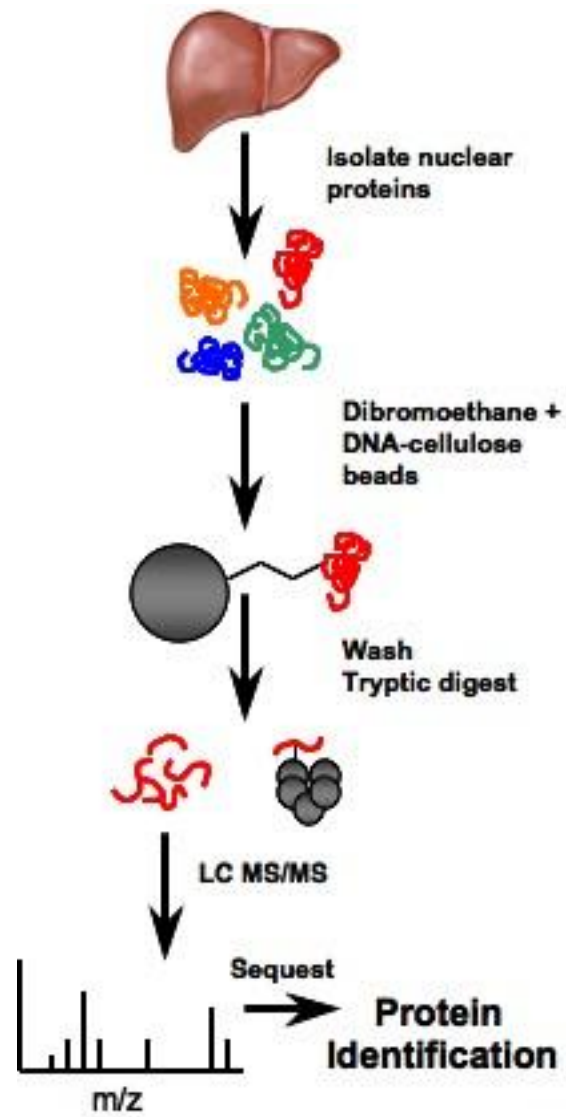

Figure 16. Mass spectrometry-based screen for DNA cross-linked proteins induced by
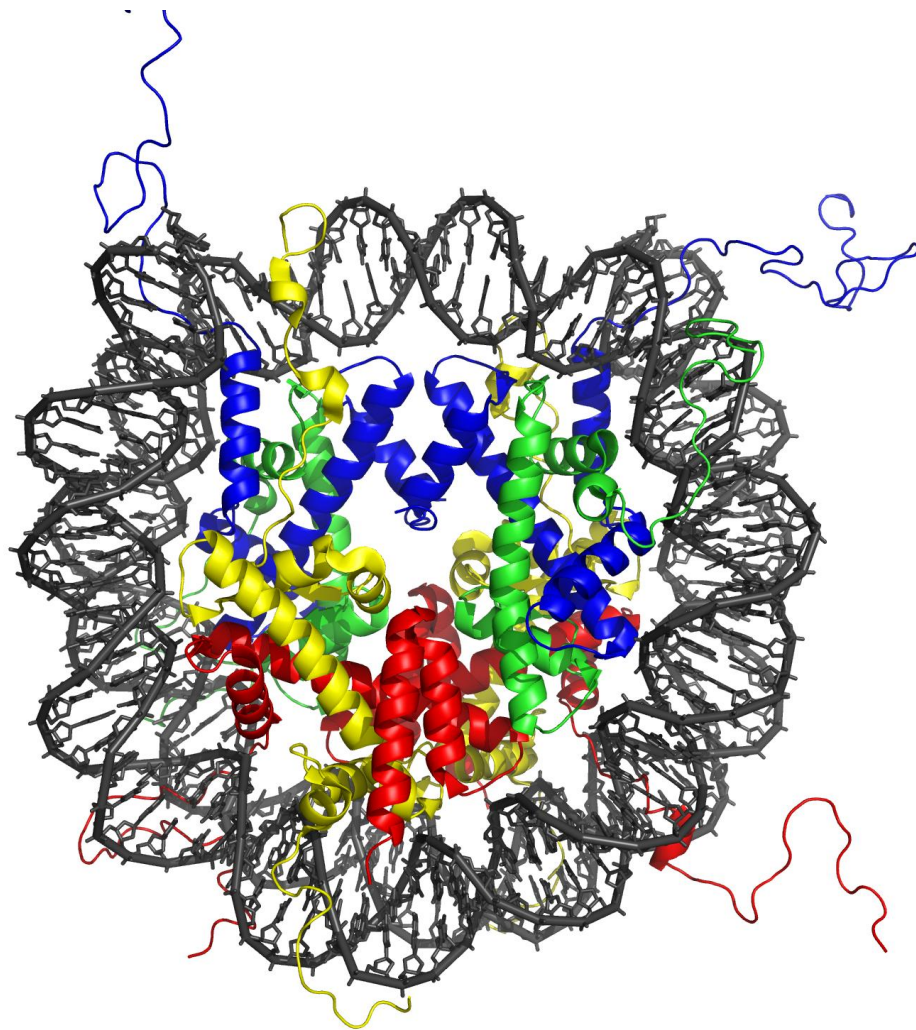
1,2-dibromoethane.

Figure 17. Crystal structure of core nucleosome structure at 2.8 Å resolution (Luger *et al.*, 1997).

were observed between isolated histones and diepoxybutane, heterologous expression of histone H2b in treated *E. coli* cells did not lead to mutagenic enhancement in contrast to AGT, although *in vivo* DNA-histone H2b cross-links were observed. These findings are similar to those observed with GAPDH (Loecken and Guengerich, 2008) and provide some insight into the molecular properties required for proteins to enhance *bis*-electrophile-induced mutagenesis.

Materials and Methods

Materials. 1,2-Dibromoethane, DEB (racemic mixture containing meso isomers), and CH_2Br_2 were purchased from Aldrich Chemical Co. (Milwaukee, WI). The oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' was synthesized by Midland Certified Reagent Co. (Midland, TX) and purified by denaturing gel electrophoresis. Purified human histone H2b was purchased from New England Biolabs (Ipswich, MA). Bovine DNase I and single-stranded calf thymus DNA-cellulose was purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of Nuclear Proteins from Human Liver. Liver samples were from six individuals (Tennessee Donor Services, Nashville, TN). All donors were males between the age of 8 and 56, with various causes of death (motor vehicle accident [4], respiratory arrest [1], and gun shot wound to the head [1]). The frozen samples (10 g each sample) were thawed on ice, pooled, and homogenized in four volumes of buffer (0.10 M Tris-acetate (pH 7.4) containing 0.10 M KCl, 1.0 mM EDTA, and 25 μM butylated hydroxytoluene) using a Potter-Elvehjem homogenizer, filtered through cheesecloth, and

centrifuged at $10^3 \times g$ for 10 min. The nuclei pellet was resuspended in 300 mL of 2.2 M sucrose containing 5 mM MgCl$_2$ and centrifuged at $7.5 \times 10^4 \times g$ for 1 h. The nuclei pellet was resuspended in 300 mL of 0.32 M sucrose containing 5 mM MgCl$_2$ and centrifuged again at $10^3 \times g$ for 10 min. The resulting pellet was resuspended in 60 mL of 20 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl$_2$, 1 mM EDTA, 2 mM CaCl$_2$, and 1 mM β-mercaptoethanol and sonicated for 90 s. DNase I was added to a final concentration of 100 μg/mL and the mixture was incubated at 15 °C for 45 min. EDTA was added to a final concentration of 20 mM before freezing the samples overnight at -20 °C. The nuclear homogenate was thawed and dialyzed overnight in 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 1 mM β-mercaptoethanol, and 2 mM EDTA at 4 °C. DNA-binding nuclear proteins were enriched using a column (2×20 cm) containing 2 g DNA-cellulose, which was prewashed with 200 mL of Buffer A (20 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 10% glycerol (v/v)). The dialyzed extract was centrifuged for 20 min at $10^3 \times g$ to remove cellular debris, and glycerol was added to 10% (v/v). Fifteen-mL aliquots were added to the column, which was then eluted sequentially with 20 mL solutions of Buffer A containing 0.1 M, 0.5 M, 1.0 M, and 2.2 M NaCl. Fractions from the 2.2 M NaCl wash were collected (~ 23 mL) and dialyzed overnight in 50 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA at 4 °C. The samples were concentrated using Amicon 5,000 MWCO spin filters (Millipore, Billerica, MA) at $4 \times 10^3 \times g$ for 45 min at 4 °C. Protein concentrations were determined using a bicinchoninic acid (BCA) assay according to the manufacturers' protocol (ThermoFisher, Rockford, IL).

Screening for Dibromoethane-Induced Cross-links. Reactions containing 100

μL of concentrated nuclear protein solution (77 μg/mL), 5 mg DNA-cellulose, and 20 mM 1,2-dibromoethane or dimethyl sulfoxide (DMSO) were incubated at 37 °C for 1 h, with gentle vortex mixing every 5 to 10 min. Samples were pelleted by centrifugation and the beads were washed four times with 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, followed by two washes with 0.10 M NH_4HCO_3, three washes with 2.2 M NH_4HCO_3, and finally three washes with 0.10 M NH_4HCO_3 (Alberts and Herrick, 1971; Sjastad *et al.*, 1983). Reduction, alkylation, and digestion of the mixtures with trypsin were performed as previously described (Dennehy et al., 2006), with the exception of frequent gentle mixing. After pelleting the beads by centrifugation, the peptide solution was concentrated and desalted with P10 C_{18} ZipTips (Millipore) according to the manufacturer's instructions (www.millipore.com/userguides.nsf/docs/pr02358).

Analysis of Human Liver Screens by LC-MS/MS. LC-MS/MS analysis of the peptides was performed using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler and Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur 2.0 SR2 instrument control. Peptides were resolved on a fused silica capillary column, 100 μm × 11 cm, packed with C18 resin (Jupiter C_{18}, 5 μm, 300 Å, Phenomonex, Torrance, CA) using an inline solid phase extraction column (100 μm × 4 cm) packed with the same C18 resin (using a frit generated with liquid silicate Kasil 1 (Cortes *et al.,* 1987)) and utilizing a "vented column" setup similar to that previously described (Licklider *et al.,* 2002) except that the flow from the HPLC pump was split prior to the injection valve. The flow rate during the loading and desalting phase of the gradient was 1 μL/min and was 700 nL/min during the separation phase. Mobile phase A was 0.1% HCO_2H in H_2O and mobile phase B was 0.1% HCO_2H in CH_3CN (v/v). A 95

min LC separation was performed with a 15 min washing period diverted to waste after the pre-column (100% A for the first 10 min, followed by a gradient to 98% A at 15 min) to allow for the removal of any residual salts. After the initial washing period, a 60 min gradient was applied in which the first 35 min was a slow, linear gradient from 98% A to 75% A (v/v), followed by a faster gradient to 10% A (v/v) (at 65 min) and an isocratic phase (at 10% A, v/v) to 75 min. MS/MS scans were acquired using an isolation width of 2 *m/z*, an activation time of 30 ms, and 30% normalized collision energy using 1 microscan. The MS/MS spectra of the peptides were acquired using data-dependent scanning in which one full MS spectra, using a full mass range of 400–2000 a.m.u., was followed by three MS/MS spectra. Dynamic exclusion was enabled for 60 s with an exclusion list size of 50 and a repeat count of one.

Database Searching, Filtering, and False-Discovery Rate Determination. The "ScanDenser" algorithm was used to extract tandem mass spectra from Thermo RAW files and transcode them to DTA files. Spectra containing fewer than 25 peaks or had less than 2E1 measured total ion current were not extracted. Singly-charged DTA files were created if 90% of the total ion current occurred below the precursor ion, and all other spectra were processed as both doubly and triply charged DTA files. Proteins were identified using the TurboSEQUEST v.27 (rev. 12) algorithm (Thermo Electron, San Jose, CA) searching against the unihuman2-1205_rev database (96858) sequences with an appended reversed version of the database for a total of 193,716 sequences. Searches were performed allowing the following differential modifications: +57 on cysteine (for carboxyimidomethylation from iodoacetamide), +16 on methionine (oxidation). Peptide and fragment ion tolerances were set to 2.5 and 1.0 Da, respectively. Protein matches

were preliminarily filtered using the following criteria, based on the parent charge state of the peptide: minimum cross correlation scores (Xcorr) of 1.0, 1.8, and 2.5 were required for charge states 1, 2, and 3, respectively. Additionally, a minimum preliminary score (Sp) of 350 was required, as well as placement within the top 5 (Sp) scores for that search. After application of filtering criteria, false-discovery rates were estimated from peptide matches to the reverse database, in which the total number of reverse peptides was multiplied by two and divided by the total number of peptide hits. These filtering criteria achieved a false positive rate of ≤1% in all datasets.

Gel Shift Assays. Gel mobility shift assays were performed as previously described in order to detect cross-links, with the exception of using larger 20 × 20 cm 15% SDS (w/v) polyacrylamide gels (Liu *et al.,* 2002). Reactions (10 μL) containing 1 μg recombinant purified histone H2b or H3 and 5'-end ^{32}P-labeled oligonucleotide 5'-GGAGGAGGAGGAGGAG-3' were incubated overnight at 37 °C with *bis*-electrophiles prior to analysis. *In vitro* cross-linking assays using double-stranded DNA utilized the same 16-mer oligonucleotide annealed to a complimentary 16-mer oligonucleotide.

Sample Preparation for Mass Spectral Analysis of Histone H2b and H3 Adducts. Samples (10 μL) consisting of 1 μg purified recombinant histone H2b protein or 0.8 μg histone H3 in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA were treated in order to identify protein adducts resulting from reactions with *bis*-electrophiles. Samples were incubated with 10 nM, 100 μM, or 20 mM of each *bis*-electrophile and incubated for 2 h at 37 °C. The reduction, alkylation, and tryptic digestion of the samples were carried out as previously described (Dennehy *et al.,* 2006). Likewise, the sites directly involved in DNA cross-linking were analyzed by adding 16-mer oligonucleotides

(5'-GGAGGAGGAGGAGGAG-3') to the reactions. After incubation these samples were dried *in vacuo* and reconstituted in a solution containing 100 mM NaCl and 15 mM sodium citrate (pH 7.0). The labile adducts were then subject to neutral thermal hydrolysis by heating the samples to 100 °C for 30 min and peptides were digested as above.

Analysis of Histone Peptides by LC-MS/MS. LC-MS/MS analysis of the peptides was performed using an LTQ-Orbitrap mass spectrometer equipped with an Eksigent NanoLC-AS1 Autosampler 2.08 and the Eksigent NanoLC-1D plus HPLC pump nanospray source. The peptides were resolved on a packed fused silica capillary column, 100 μm × 15 cm, packed with C18 resin (Jupiter C_{18}, 5 μm, 300 Å, Phenomonex, Torrance, CA) and coupled with an inline trapping column that was 100 μm × 4 cm packed with the same C18 resin (using a frit generated with liquid silicate Kasil 1 (Licklider *et al., 2002)*). Liquid chromatography was carried out at ambient temperature with a flow rate of 0.5 μL/min using a gradient mixture of 0.1% (v/v) HCO_2H in H_2O (solvent A) and 0.1% (v/v) HCO_2H in CH_3CN (solvent B). The flow rate during the loading and desalting phase of the gradient was 1.5 µL/min and during separation phase was 500 nL/min. A 95 min gradient was performed with a 10 min washing period diverted to waste after the precolumn (98% A for the first 10 min followed by a gradient to 98% A at 15 min, all v/v) to allow for removal of any residual salts. After the initial washing period, a 60 min gradient was performed where the first 35 min was a slow, linear gradient from 98% A to 75% A (v/v), followed by a faster gradient to 10% A at 65 min and an isocratic phase at 10% A to 75 min. Centroided MS/MS scans were acquired on the LTQ-Orbitrap using an isolation width of 2 *m/z*, an

activation time of 30 ms, an activation q of 0.25, and 30% normalized collision energy using 1 microscan with a maximum injection time of 0.1 s for each MS/MS scan and 1 microscan with a maximum injection time of 1 s for each full orbitrap MS scan. The tune parameters were as follows: spray voltage of 1.9 kV, a capillary temperature of 160 °C, a capillary voltage of 48 V, and tube lens of 125 V. The AGC target values were set at 10^6 for full MS and 10^4 for MS/MS spectra. A full scan obtained for eluting peptides in the range of 400-2000 a.m.u. was collected on the Orbitrap at a resolution of 6×10^4, followed by five data-dependent MS/MS scans on the LTQ portion of the instrument with a minimum threshold of 10^3 set to trigger MS/MS spectra. A dynamic exclusion list of the 50 previously analyzed precursors was maintained for 60 s in which time MS/MS was not performed on those masses.

Database Search Pipeline for Protein Adducts. Tandem mass spectra were converted from Thermo RAW format to the mzML format by the msconvert tool of ProteoWizard (Kessner *et al.,* 2008). Peptides were identified against a database containing the two histone sequences along with 19 other proteins identified in the mixture (mostly keratins and *E. coli* proteins). The full database was then doubled in size by adding the reversed version of each sequence. The MyriMatch database search algorithm version 1.5.2 (Tabb *et al.,* 2007) identified tandem mass spectra to peptide sequences. Semi-tryptic peptide candidates were included as possible matches. Potential modifications included formation of *N*-terminal pyroglutamine, and the following additions to peptide *N*-termini, lysine, arginine, or cysteine: 44, 86, 104, 178, 238, or 220 a.m.u. (Figure 2). Precursors were required to be within 0.1 *m/z* of the peptide monoisotopic mass, or of the monoisotopic mass plus or minus one neutron. Fragment

ions were uniformly required to fall within 0.5 *m/z* of the monoisotope. IDPicker v2.2.4 (Zhang *et al.,* 2007) filtered peptide matches to a 0 % FDR (including only those matches that outscored the best-scoring reversed match) and applied parsimony to the protein lists, requiring all proteins to match at least two distinct peptides. The summation of two independent searches of the data differing only in the presence of + 238 a.m.u. of the search criterial were used to report the findings.

Human Histone H2b cDNA Synthesis and Expression. The amino acid sequences and native cDNA sequences for human histones H2b and H3 were obtained from GenBank (NM_003528 and NM_003532, respectively) (Figure 18). Sixteen overlapping oligonucleotides (30- to 40-mers) were synthesized (Operon, Huntsville, AL) according to DNAWorks results (http://helixweb.nih.gov/dnaworks/) (Wu *et al.,* 2006), with codons optimized automatically for *E. coli* type B. Melting temperatures of the designed oligonucleotides were selected to be 62 ± 3 °C. The 5′- and 3′-flanking sequences were 5′-CCGAATT-3′ (sense) and 5′-GACCCCTGGATCCCGC-3′ (sense) respectively. Flanking sequences were engineered to contain an *Eco*RI restriction site on the 5'-end and a *Bam*HI restriction site on the 3'-end of the cDNA. Polymerase cycling assembly and polymerase chain reaction (PCR) amplification of the gene was performed as described elsewhere (Wu *et al.,* 2006). The PCR product was digested with *Bam*HI and *Eco*RI restriction enzymes (New England Biolabs) and purified by agarose gel electrophoresis. The resulting fragment was ligated into a pINIII-A3(lpp^{p-5}) vector, which was also digested with *Eco*RI and *Bam*HI restriction enzymes. One Shot Top10 *E. coli* cells (Invitrogen, Carlsbad, CA) were transformed with the ligation mixture and selected for with Luria-Bertani medium plates containing 50 µg/mL ampicillin.

H2b: PEPA**K** SAPAP **KK**GS**K** **K**AVT**K** AQ**K**KD G**KK**R**K** RSR**K**E

SYSIY VY**K**VL **K**QVHP DTGIS S**K**AMG IMNSF VNDIF ERIAG

EASRL AHYN**K** RSTIT SREIQ TAVRL LLPGE LA**K**HA VSEGT

KAVT**K** YTSS**K**

H3.1: ART**K**Q TAR**K**S TGG**K**A PR**K**QL AT**K**AA R**K**SAP ATGGV

KKPHR YRPGT VALRE IRRYQ **K**STEL LIR**K**L PFQRL VREIA

QDF**K**T DLRFQ SSAVM ALQEA CEAYL VGLFE DTNLC AIHA**K**

RVTIM P**K**DIQ LARRI RGERA

Figure 18. Sequence of human histone H2b and H3.1 with nucleophilic lysines highlighted.

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Purification of vectors from surviving colonies was performed with a QIAprep Miniprep kit (Qiagen, Valencia, CA), and digested with *Eco*RI and *Bam*HI restriction enzymes in order to screen for ligated vectors. Digested plasmids (from selected colonies that yielded 0.5 and 7 kb DNA fragments upon restriction digestion) were analyzed in the Vanderbilt DNA Sequencing facility using an Applied Biosystems Model 3700 fluorescence sequencing unit with a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA). The sequenced plasmid was amplified in One Shot Top10 cells (Invitrogen) and isolated before transformation of TRG8 *E. coli* cells. Luria-Bertani plates containing both ampicillin (50 μg/mL) and kanamycin (50 μg/mL) were used to select colonies for both cell type and pINIII vector. Histone H2b expression was verified in *E. coli* TRG8 cells induced with 0.2 mM isopropyl β-D-thiogalactopyranoside by immunoblot analysis using polyclonal human anti-histone H2b antibodies (New England Biolabs) according to the manufacturer's instructions. Secondary antibodies with infrared fluorescence tags were used for detection on a LI-COR Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Mutagenesis and Survival Assays. Overnight cultures of TRG8 cells containing AGT, histone H2b, or empty pINIII vector were used to inoculate 5 mL of Luria-Bertani media containing ampicillin (50 μg/mL) and kanamycin (50 μg/mL). Cultures were grown at 37 °C while shaking at 250 rpm until an OD_{600} of ~ 0.4 was reached. Expression was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside for 90 min prior to treatment. Mutagenesis and survival assays were carried out as previously described (Liu *et al.,* 2000), but with 60 min reaction time and *his*[+] plate dilutions of $1:10^6$.

DNA Binding Assays. Electrophoretic mobility shift assays were carried out as previously described (Loecken and Guengerich, 2008; Rasimas *et al.,* 2007), but using a 15% (w/v) native polyacrylamide gel. Briefly, various concentrations of recombinant AGT or histone H2b were added to ^{32}P-labeled 16-mer oligonucleotides (5'-GGAGGAGGAGGAGGAG-3') in 10 mM Tris-acetate buffer (pH 7.4) containing 100 mM NaCl. Mixtures were incubated at 23 °C for 30 min and glycerol was added to 10% (v/v) glycerol before loading on a 20 cm gel, which was run at 8 V/cm.

In Vivo **Cross-link Detection.** Identification of *in vivo* cross-links was performed as previously described with some modifications (Liu *et al.,* 2004a,b). Cultures of *E. coli* TRG8 cells containing pINIII-histone H2b or the empty pINIII vector were grown in Luria-Bertani medium containing ampicillin (50 μg/mL) and kanamycin (50 μg/mL) to an OD_{600} of ~ 0.5 and induced with 0.2 mM isopropyl β-D-thiogalactopyranoside for 1 h at 37 °C while shaking at 250 rpm. Cultures were then treated for 90 min with 0.032 or 0.2 mM diepoxybutane dissolved in DMSO (< 1% v/v) or a DMSO vehicle control. One-mL aliquots were centrifuged and washed with 1 mL of 1X M9 salt solution before isolating genomic DNA with Promega Wizard Genomic DNA purification kit (Promega, Madison, WI) according to manufacturer's protocol for Gram-negative bacteria. The isolated DNA was washed with 2 M NaCl for 30 min at 37 °C and precipitated with 2.5 volumes of isopropanol. Pellets containing the DNA were suspended in 0.5 mL of 50 mM Tris-HCl (pH 7.4) buffer containing 10 mM $MgCl_2$ and 20 μg/mL DNase I and digested for 30 min at 37 °C. The solutions were then dried *in vacuo* prior to reconstitution with 25 μL H_2O and 5 μL 5X SDS-PAGE loading buffer. Samples (20 μL) were heated at 95 °C for 10 min and loaded onto a 15% SDS-PAGE gel

and immunoblotting as described above for expression analysis. Quantification was performed using secondary antibodies with infrared fluorescence tags that were detected with a LI-COR Odyssey Infrared Imaging System.

Results and Discussion

Screening for Crosslink Candidates. The ability of DNA-protein cross-links to contribute to *bis*-electrophile-mediated mutagenesis prompted our examination of other proteins that might form mutagenic cross-links (Liu *et al., 2002, 2004).* In order to identify other proteins cross-linked to DNA by 1,2-dibromoethane, human liver nuclear extract was enriched for DNA binding proteins by collecting the high-salt eluting fractions from a DNA-cellulose column. The protein mixture was then incubated with DNA-cellulose and 20 mM 1,2-dibromoethane. The proteins bound to the beads were then digested with trypsin and the resulting peptides were analyzed by multidimensional LC-MS/MS, using a Sequest software search. In two independent screens, variants of human histones were identified from multiple peptides and from these we selected human histone H2b as a candidate (Table 2). The analyzed peptides should represent covalently bound proteins, but it is impossible to rule out false positives because the cross-linked peptides remain bound to the beads. This type of analysis is also biased toward high abundance proteins and those that can efficiently ionize in MS (Smith and Denu, 2009). AGT was only identified in one of the screens, presumably due to its low abundance (O'Connor and Laval, 1989).

Cross-linking of Histones H2b and H3 to DNA by *bis*-Electrophiles. *In vitro*

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cross-linking of AGT to oligonucleotides was observed in the presence of *bis-*

Table 2. Histone proteins identified in two independent screens for cross-link candidates.

Histone Protein	Uniref100 ID	% Coverage
Histone H2A.m	P04908	41
Histone H3/b	Q93081	13
Histone H4	Q6FGB8	17
Histone Protein	Q5R2W0	27
Histone 1, H2aj	Q5JXQ5	14
Histone 4	Q4A487	22
Core histone macro-H2A.1	O75367	11
Histone Protein	Q5R2W0	38
Histone H3/b	Q5R2W0	20
Histone H4	Q6FGB8	50
Histone H2A.m	P04908	36

electrophiles and shown be involved in mutagenesis (Liu *et al.*, 2002; Liu *et al*., 2004a,b).

Accordingly, histones H2b and H3 were incubated with varying concentrations of *bis-*

electrophiles and radiolabeled oligonucleotide. Gel mobility shift assays revealed that

both histones H2b and H3 cross-link to DNA with DEB in a concentration-dependent

manner. In comparison with AGT (Liu and Guttman, 2004), histone H2b and H3 cross-

links appeared to require moderately higher concentrations of *bis*-electrophile (Figure 19). Comparisons between DNA-protein cross-link formation involving double-stranded or single-stranded DNA oligonucleotides revealed that histone H2b is only slightly more reactive toward single-stranded DNA (Figure 20). The reactivity bias towards DEB was unexpected because 1,2-dibromoethane was used in the initial screen. One possible explanation is carryover of non-covalently bound histones on the DNA-cellulose beads. However, the only cross-link identified between DNA and peptide was the result of a reaction with 1,2-dibromoethane (*vide infra*), possibly as a result of different reaction conditions that lead to biased reactivity towards *bis*-electrophiles. These results are similar to previous findings with GAPDH (Loecken and Guengerich, 2008).

MS Analysis of *bis*-Electrophile-Treated Histones H2b and H3. Recombinant protein was incubated with *bis*-electrophiles and subjected to tryptic digestion before analysis by high resolution LC-MS/MS. Data collected were analyzed with IDPicker software in targeted searches for hydrolyzed histone H2b and H3 protein adducts formed by 1,2-dibromoethane (+44 a.m.u.) or diepoxybutane (+86 and +104 a.m.u.). DNA-protein cross-links induced by *bis*-electrophiles were also analyzed in samples containing oligonucleotides that were digested with trypsin after neutral thermal hydrolysis (Figure 21). This procedure yielded labile N^7-guanyl cross-links between histone amino acids
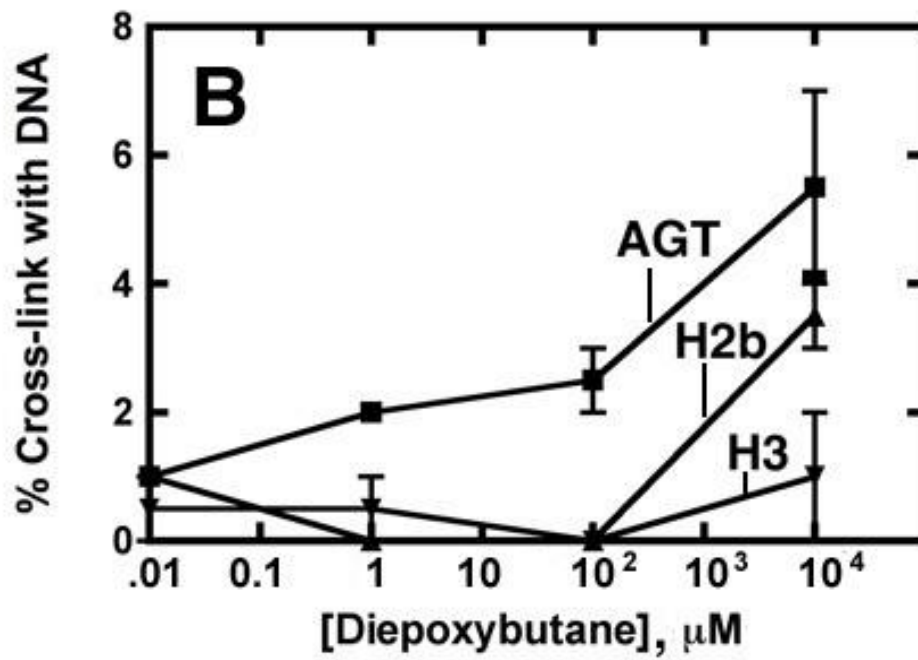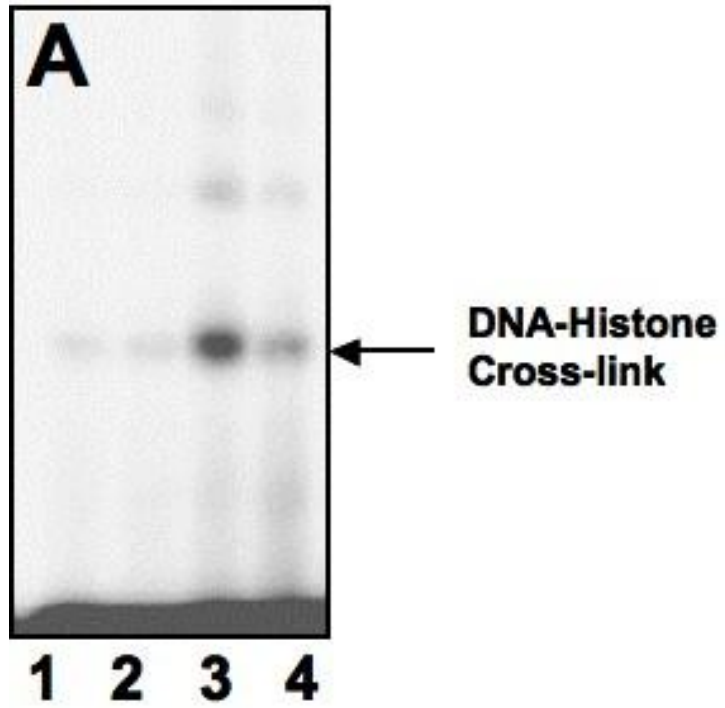
A

DNA-Histone
Cross-link

1 2 3 4

B

% Cross-link with DNA

AGT

H2b

H3

[Diepoxybutane], μM

Figure 19. Cross-linking of purified histone H2b to oligonucleotides by DEB. (A) Gel shift assays were performed by incubating histone H2b (1 µg) and ^{32}P-5'-endlabeled 16-mer oligonucleotide in reactions containing DMSO (<1%, v/v) (lane 1), 20 mM CH_2Br_2 (lane 2), 20 mM DEB (lane 3), or 20 mM 1,2-dibromoethane (lane 4) for 1 h at 37 °C. Samples were separated by SDS-polyacrylamide gel (15% w/v) electrophoresis. (B) DNA-protein cross-links were detected via autoradiography and quantified using Quantity One software (BioRad).
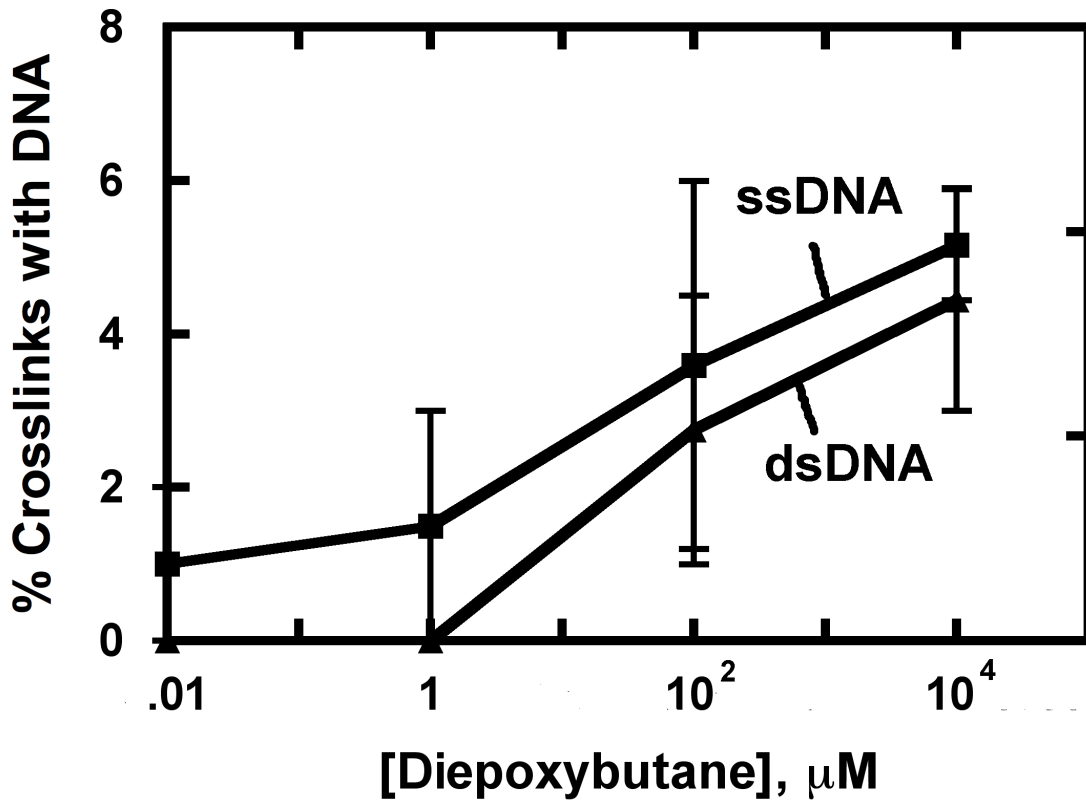
Figure 20. Cross-linking of purified histone H2b to single-stranded or double-stranded oligonucleotides by DEB. Gel shift assays were performed by incubating histone H2b (1 µg) with ^{32}P-5'-endlabeled 16-mer single-stranded or double-stranded oligonucleotides in reactions containing various concentrations of DEB overnight at 37 °C. Samples were separated by SDS-polyacrylamide gel (15% w/v) electrophoresis and DNA-protein cross-links were detected via autoradiography and quantified using Quantity One software (BioRad).
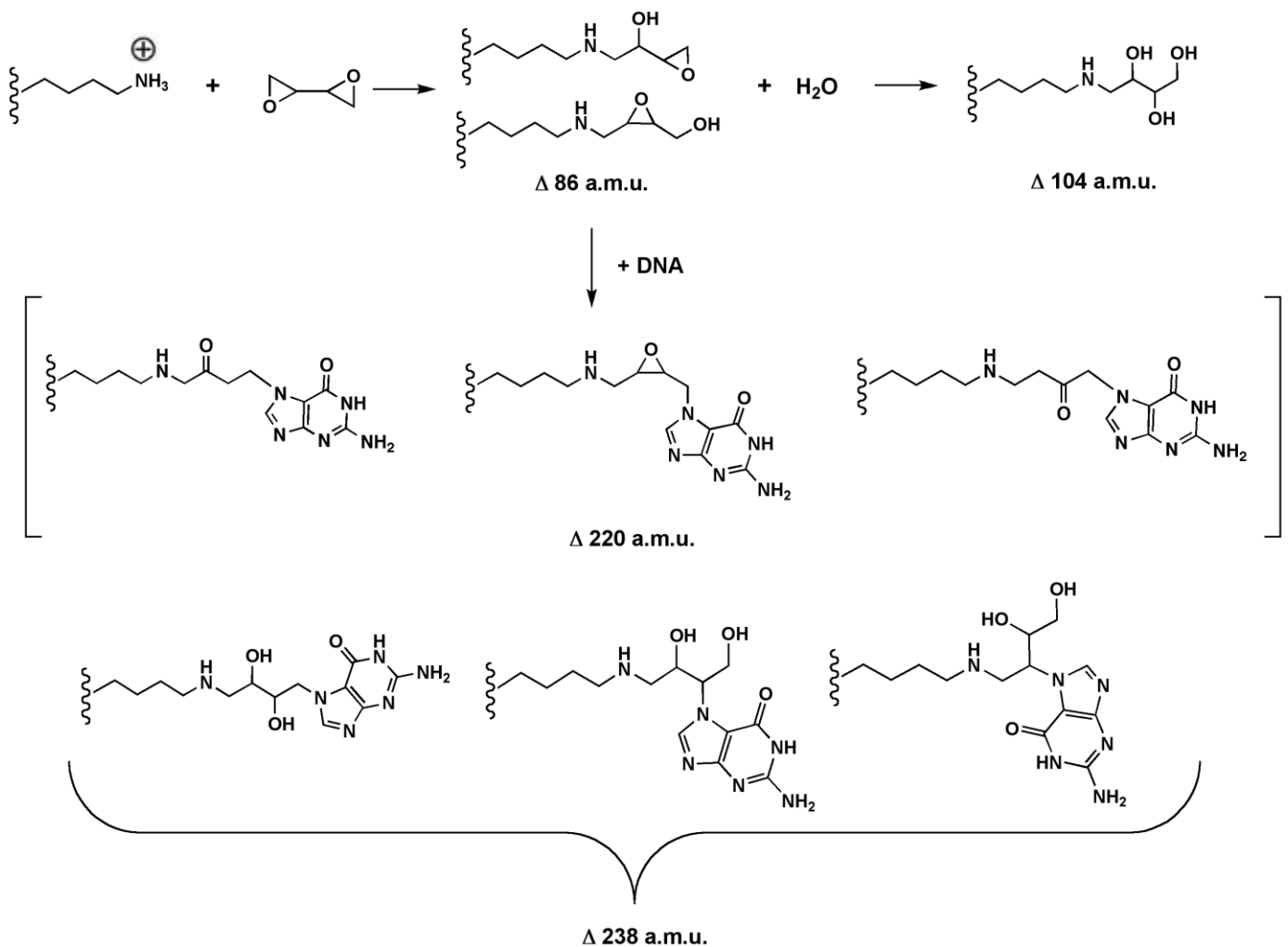
Figure 21. Proposed products of reactions between lysine, DEB, and guanine. A targeted MS search for protein adducts was performed on the tryptic peptides from samples of purified histone H2b incubated with DEB. Reactions containing histone H2b, DEB, and 16-mer oligonucleotides from the *in vitro* cross-linking assays were subjected to neutral thermal hydrolysis prior to digestion. In addition to lysine residues, changes in a.m.u. corresponding to the DEB adducts and guanine cross-links were monitored on arginines, cysteines, and N-termini.

and DNA corresponding to +220 and +238 a.m.u. (DEB) as well as +178 a.m.u. (1,2-dibromoethane) adducts (Liu *et al.,* 2004). Although precedence exists for these particular adducts being formed (Kaina *et al.,* 2007), it is possible that other adducts are produced that the targeted search would not identify, such as those reacting at sites other than the guanine N7 and adenine N3 atoms (Liu *et al.,* 2004a,b). Analysis of digested purified protein yielded ~ 90% protein coverage of histone H2b and ~ 80% histone H3 protein coverage. Of the 14 residues not observed in the MS data from histone H2b, six were lysines and two were arginines. There were two lysines, six arginines, and one cysteine in the 29 residues of histone H3 not identified in the samples (Figure 22).

Treatment of histone H2b with DEB resulted in nine adducted lysine residues, with the relative amount of +86 a.m.u. adducts approximately equaling that of the +104 a.m.u. protein adducts. Nearly all ions containing these modifications were found in samples treated with the highest concentration of DEB (20 mM). The most commonly modified residue on histone H2b was Lys[6] with a total of 24 ions observed in the 20 mM

DEB-treated samples, which represented a total of ~ 7 % of all Lys^6 ions identified. Treatment of histone H2b with diepoxybutane yielded numerous ions corresponding to N-terminus modifications, as well as adduction on Arg^{73}. DEB-induced histone H2b-guanine cross-links where not observed on lysine, arginine, or the N-terminus proline residues.

Treatment of histone H3 with DEB yielded protein adducts on five lysine residues, with approximately twice as many epoxide (+86 a.m.u.) as triol (+104 a.m.u.) adducts observed. Two-thirds of all DEB adducts corresponded to samples treated with 20 mM *bis*-electrophile. Lys^{28} was found to be the major target for modification by
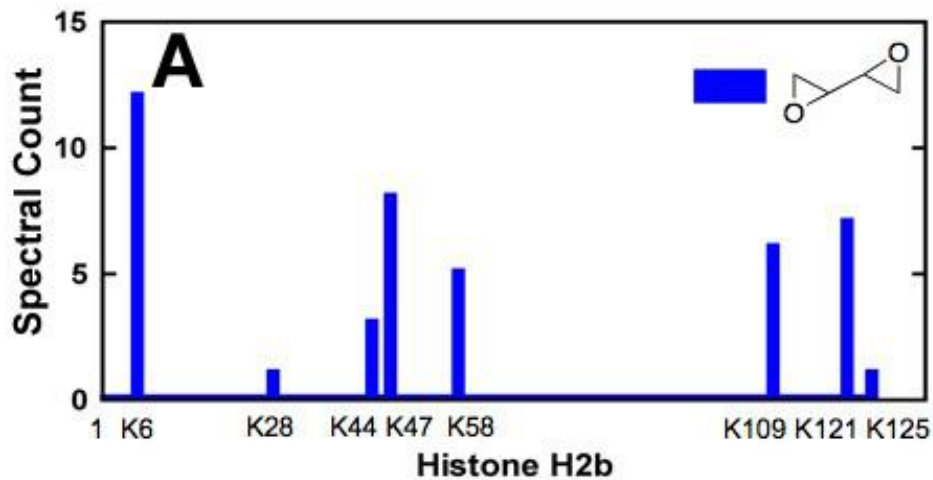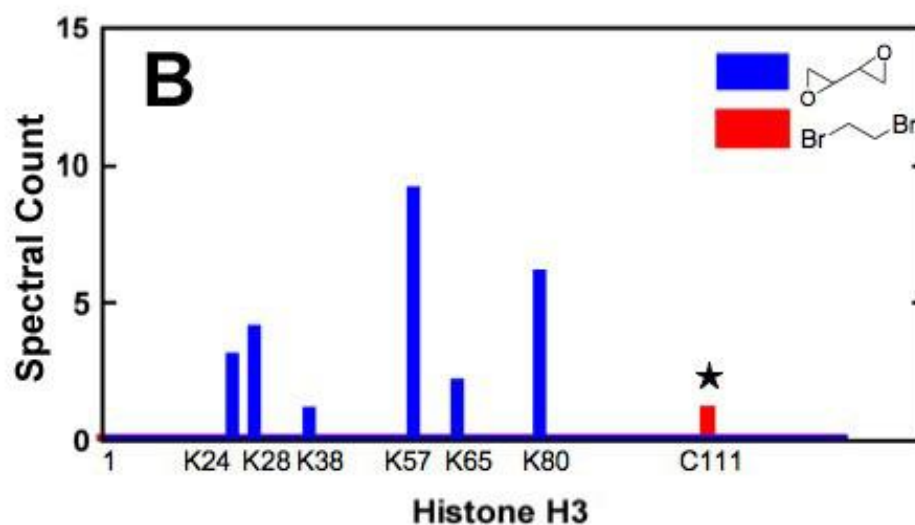
Figure 22. MS analysis of reactions between *bis*-electrophiles and histones H2b and H3. (A) Purified histone H2b (1 μg) or (B) histone H3 (1 μg) was incubated with 10 nM to 10 mM 1,2-dibromoethane or DEB for 1 h at 37 °C and digested with trypsin. Peptides were analyzed with a high-resolution mass spectrometer (Thermo Orbitrap) and the data were mined for adducts using Myrimatch and IDPicker software. The single peptide-DNA cross-link to be identified (☆) formed between DNA and a histone H3 cysteine with 1,2-dibromoethane treatment.

DEB with ~16% of all observed ions being adducted. Modification of 3 arginine residues (Arg[27], Arg[43], Arg[127]) was observed, as well as 11 N-terminal adducts in samples treated with DEB. While arginine residues were not anticipated to be major targets for alkylation, there is precedence for post-translational modification of these sites in histone (Valadez *et al.,* 2004), and evidence exists that arginine undergoes carcinogen-induced alkylation in treated proteins (Goggin *et al.,* 2008). Although no modifications corresponding to DEB protein adducts (+86, +104 a.m.u.) were observed on Cys[111], two

ions indicate that this residue cross-links to DNA (+220 a.m.u.) in 20 mM DEB.

Treatment of H2b with 1,2-dibromoethane resulted in identification of only one +44 a.m.u.-modified ion at Lys^{109}, which was also the only lysine found to be cross-linked to DNA by 1,2-dibromoethane (+178 a.m.u.). The only histone H3 residue found to be adducted by 1,2-dibromoethane (+44 a.m.u.) was Cys^{111}, which yielded one ion. Two lysines (Lys^{57}, Lys^{80}) were found to cross-link to DNA with 1,2-dibromoethane although protein adducts were not observed with 1,2-dibromoethane treatment alone (+44 a.m.u.). The Lys^{57} and Lys^{80} residues, however, did form protein adducts with DEB.

Expression of Human Histone H2b in *E. coli* and Mutagenesis Assays with *bis*-Electrophiles. Overexpression of the DNA repair protein AGT has been shown to paradoxically enhance mutagenesis by *bis*-electrophiles (Liu *et al.,* 2002*;* Lambert *et al.,* 2007). The identification of human histones in both liver screens (Table 2) suggested that DNA-protein cross-links may form in part because of their basic charge and DNA binding ability. Following demonstration that histones H2b and H3 were reactive with DEB *in vitro*, the ability of H2b to enhance *in vivo* mutagenesis via heterologous expression in *E. coli* cells was assessed (Figure 23A).
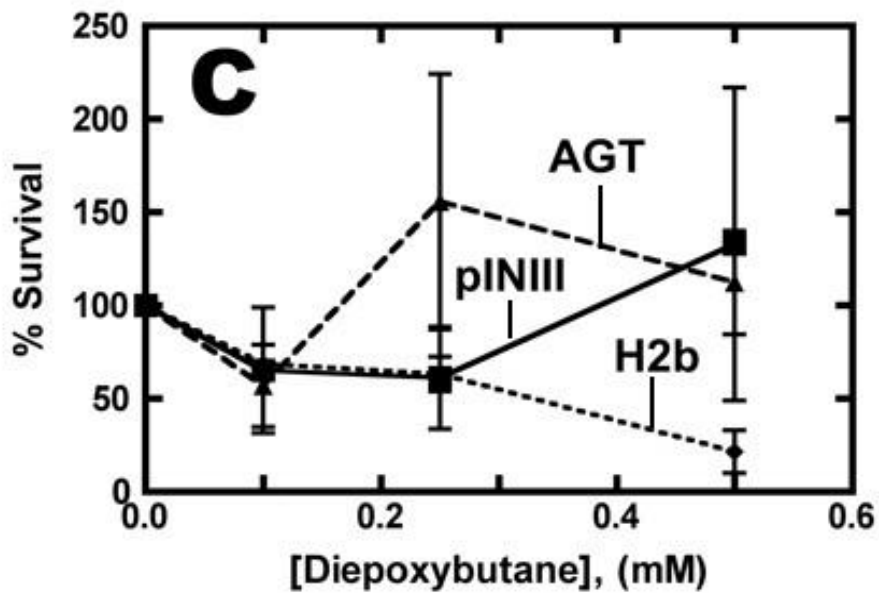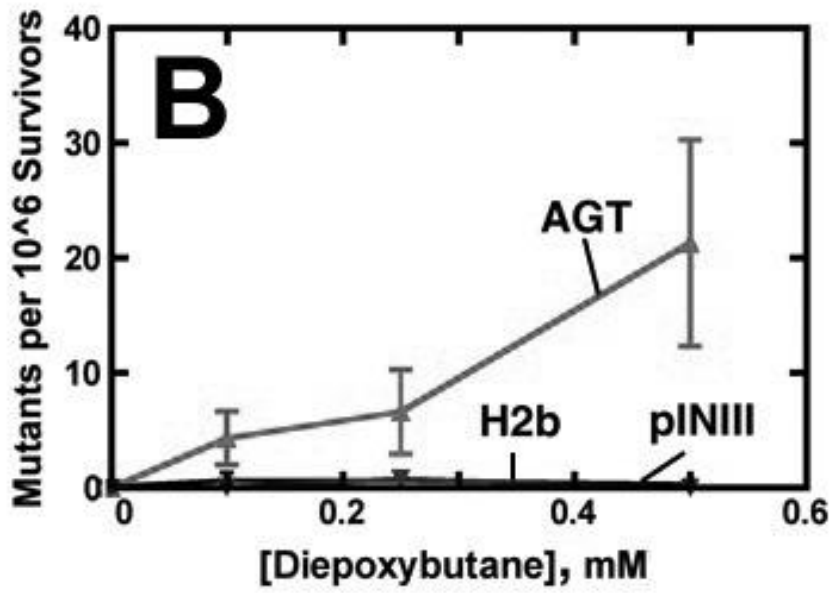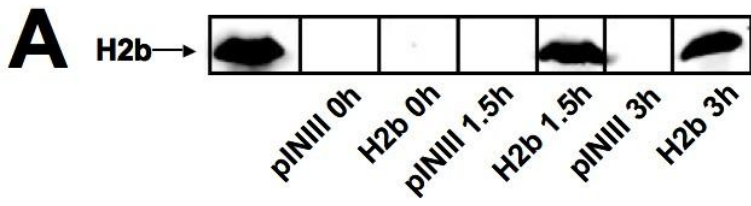
A

H2b→

pINIII 0h   H2b 0h   pINIII 1.5h   H2b 1.5h   pINIII 3h   H2b 3h

B

Mutants per 10^6 Survivors

AGT

H2b   pINIII

[Diepoxybutane], mM

C

% Survival

AGT

pINIII

H2b

[Diepoxybutane], (mM)

71

Figure 23. Histone H2b expression in *E. coli* does not enhance mutagenesis by *bis*-electrophiles. (A) Recombinant expression of histone H2b in TRG8 cells was quantified at various time points after induction by immunoblotting. Expression of histone H2b (100 nM) was compared to the extract of TRG8 cells containing pINIII empty vector, which contains no detectable histone protein. (B) TRG8 cells expressing AGT or histone H2b or containing the control pINIII vector were treated with varying concentrations of DEB for 30 min at 37 °C. Mutant colonies that grew on *his*⁻ plates were quantified visually, and mutagenesis was assessed after determining the quantity of viable cells grown on *his*⁺ plates. (C) AGT, H2b, and pINIII TRG8 cells were treated with DEB on *his*⁺ plates. Colonies were quantified and compared with untreated plates to determine survivorship.

Heterologous expression of histone H2b in *E. coli* TRG8 cells was quantified by immunoblotting and showed ~ 5-fold higher protein expression for histone H2b (100 nM) compared to AGT (17 nM) (data not shown). Histones are not found in prokaryotes, so the observation that no histone H2b protein was detected in blots of TRG8 extract containing empty pINIII vector was expected (Peterson *et al.*, 1988). Attempts to express histone H3 cDNA were unsuccessful for reasons that are unclear.

Cells induced to express histone H2b were assessed for survival and mutagenesis upon treatment with varying concentrations of *bis*-electrophiles. TRG8 cells containing an empty pINIII vector or expressing human AGT served as the negative and positive controls, respectively. Treated cells were plated on agar lacking histidine (*his*⁻) to select for cells with reversion mutations in the *hisG* gene. As expected (Liu *et al.*, 2002), AGT

expression resulted in more mutant colonies forming than in untreated controls. Neither the pINIII-containing cells nor those expressing histone H2b displayed enhanced mutagenesis (Figure 23B). Survival of DEB-treated cells was not affected in either pINIII- or histone-H2b containing cells, while cells expressing AGT displayed increased sensitivity to *bis*-electrophiles (Figure 23C). Similar results were observed following treatment with 1,2-dibromoethane (Figure 24).

DNA Binding Ability of Histone H2b and AGT. Previous work with the cross-link candidate GAPDH showed that reactivity toward DEB *in vitro* is not necessarily an indication of the ability of a protein to enhance mutagenesis *in vivo* (Loecken and Guengerich, 2008). We proposed that the difference in DNA-binding ability between AGT and GAPDH could contribute to the lack of mutagenic enhancement by DEB. In contrast, histone H2b is a critical component of chromatin and is known to bind DNA with high affinity (Peterson and Laniel, 2004). We demonstrated that, unlike GAPDH, histone H2b binds DNA with much higher affinity than AGT (Figure 25). While histones are primarily basic proteins containing a high proportion of lysine residues, they lack a highly nucleophilic active site such as the reactive cysteine residue necessary to enhance mutagenesis by AGT (Guengerich *et al.,* 2003).
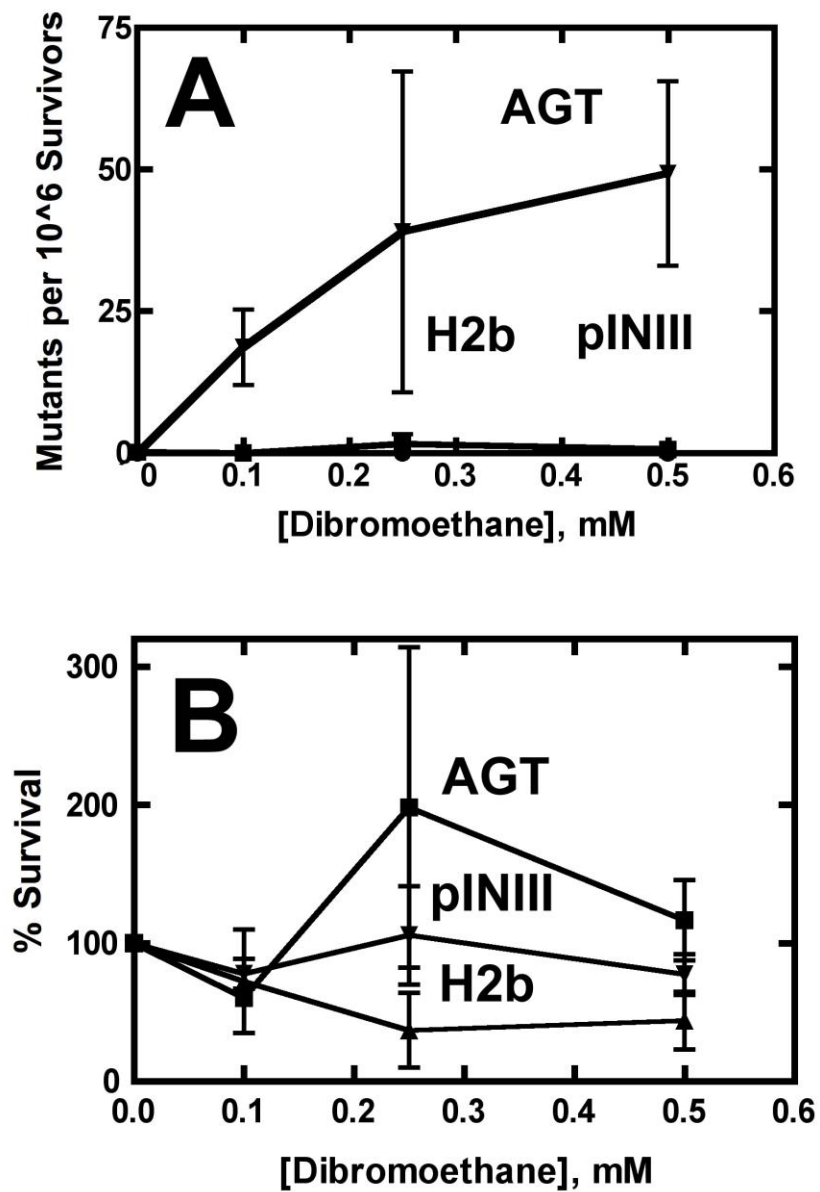
Figure 24. Lack of mutagenic enhancement in cells expressing histone H2b by 1,2-dibromoethane. Mutagenicity and survivorship in TRG8 cells (expressing AGT or histone H2b or containing pINIII vector treated) with 1,2-dibromoethane for 30 min at 37 °C before growing the cells on his^+ and his^- plates.

Detection of *In Vivo* DNA-Histone H2B Cross-links. The ability of AGT to enhance mutagenesis of *bis*-electrophiles by cross-linking to DNA has been demonstrated *in vivo* (Liu *et al.,* 2004a,b*).* GAPDH was also found to react with DEB in a manner similar to that of AGT; however, *in vivo* mutagenic enhancement was not observed *(*Loecken and Guengerich, 2008). This finding was partly attributed to the reduced DNA-binding ability of GAPDH in comparison with AGT, which could potentially prevent the formation of DNA-GAPDH cross-links *in vivo*. Like GAPDH, purified human histone H2b cross-linked to DNA and yielded protein adducts with DEB treatment (Figures 19, 22), but expression of histone H2b in treated *E. coli* did not enhance mutagenesis by *bis*-electrophiles (Figure 23). However, treatment of TRG8 cells expressing histone H2b with DEB yielded detectable DNA-protein cross-links upon analysis of genomic DNA (Figure 26). These findings suggest the cellular processing of these lesions differs from that of AGT-DNA cross-links in *E. coli* cells. Identifying the exact mechanism responsible for this disparity is complicated by the fact that information is limited about the processing, repair, and bypass of specific DNA-protein cross-links *(*Barker *et al.,* 2005a*).*

Conclusion

Characterization of DNA-protein cross-links formed by *bis*-electrophiles is

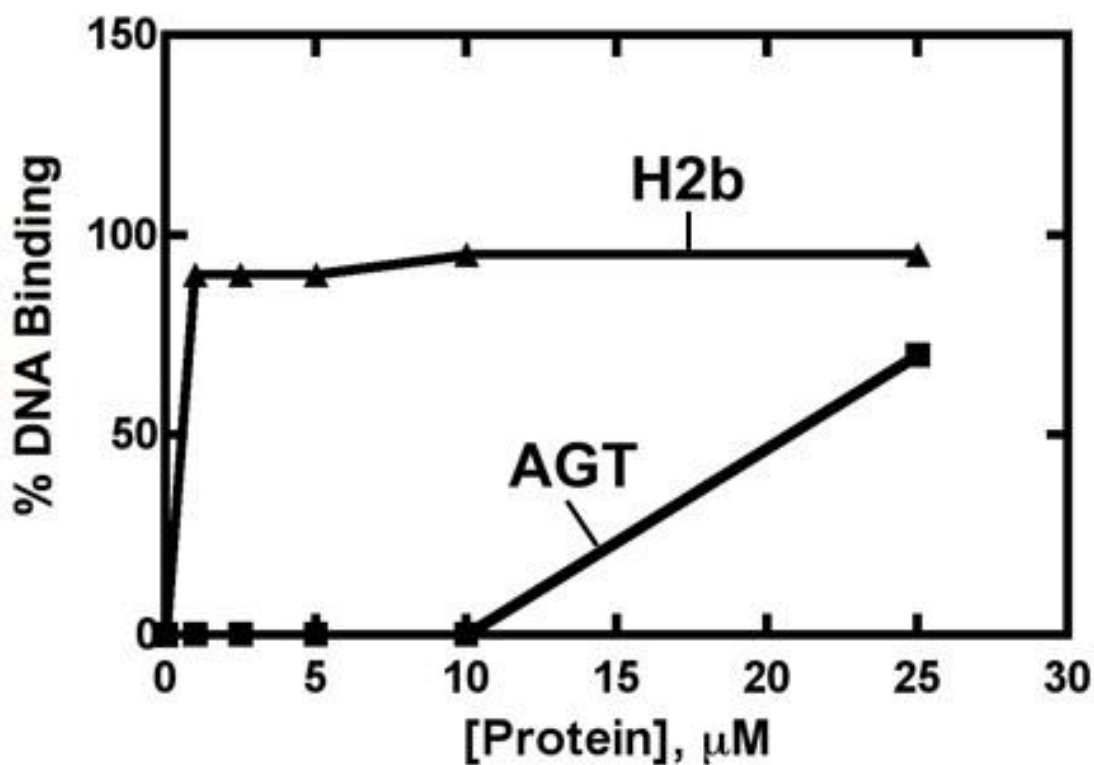**Figure 25.** DNA binding assays with AGT and histone H2b. Samples containing purified histone H2b or AGT were incubated with ^{32}P-5'-end-labeled 16-mer at 23 °C for 30 min. A 15% (w/v) native polyacrylamide gel electrophoresis system was used to separate protein-DNA complexes from unmodified oligonucleotides. A voltage of 8 V/cm was used to separate DNA-protein cross-links, which were then quantified with autoradiography.
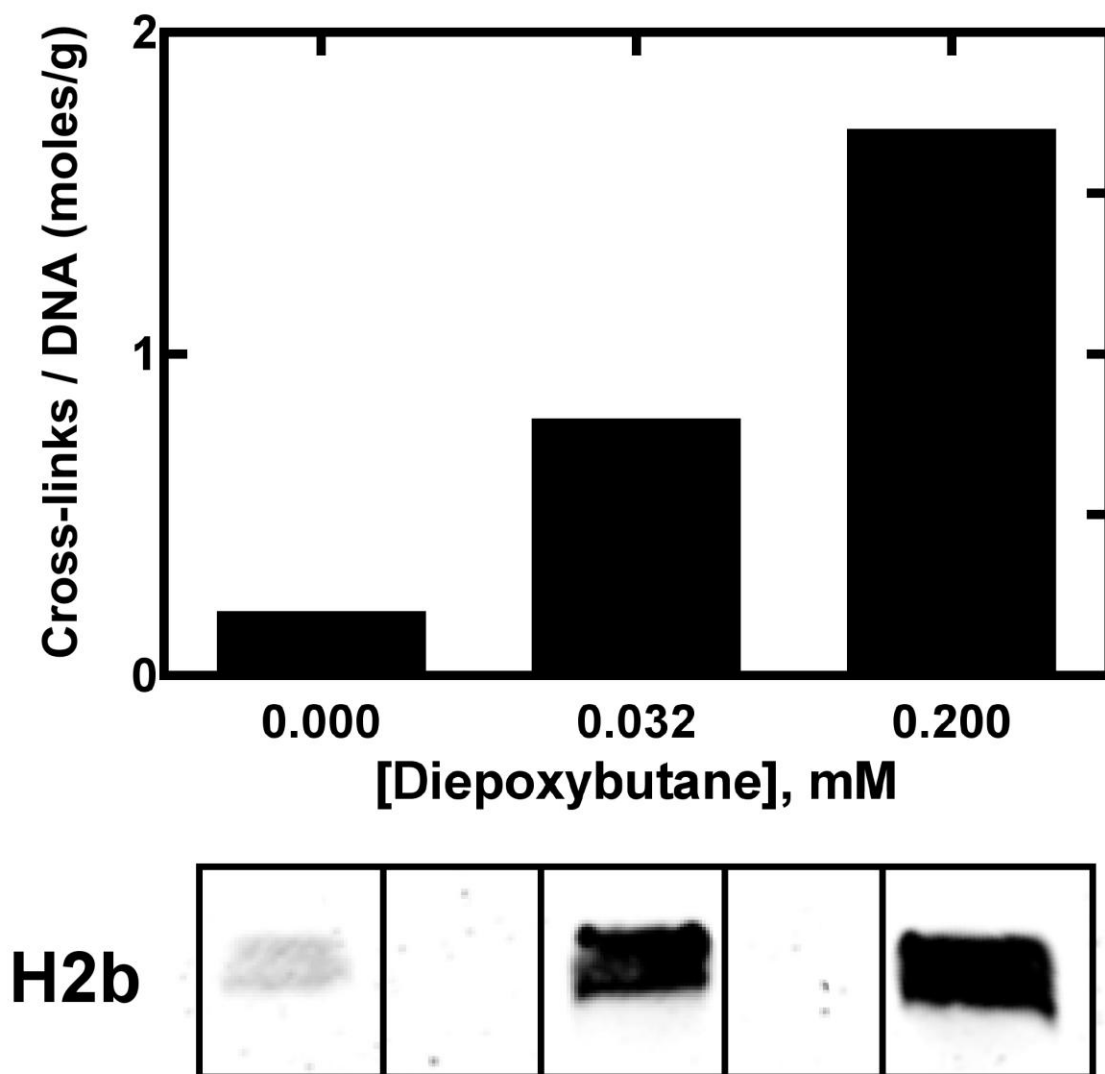
Figure 26. Detection of *in vivo* DNA-histone H2b cross-links. *E. coli* TRG8 cells expressing histone H2b or containing an empty pINIII vector were treated with 0, 0.032, or 0.2 mM DEB for 90 min at 37 °C. The genomic DNA from 1 mL cultures was isolated, washed, and digested with DNase I prior to separation on a 15% (w/v) SDS-polyacrylamide gel and immunoblot analysis. Samples were quantified using a LI-COR Odyssey Infrared Imaging System.

required to understand the mechanism of mutagenesis and is especially critical because of the potential for human exposure to these chemicals. The ability of other proteins to behave in a similar manner toward *bis*-electrophiles was addressed in our human liver screen. We established that purified histones H2b and H3 were able to cross-link with DNA using *in vitro* gel shift assays, using AGT as a positive control (Figure 19). In accord with these findings, DNA adducts resulting from protein alkylation and protein-DNA cross-links were identified (Figure 22). However, treatment of histone H2b-overexpressing *E. coli* cells with *bis*-electrophiles did not elevate mutation levels (Figure 23). The higher DNA binding ability of histone H2b in comparison with AGT (Figure 25) suggests that the absence of enhanced mutagenesis was not the result of reduced protein-DNA interactions. However, it is reasonable to at least partially attribute differences in mutagenic enhancement to the innate differences in reactivity of histone H2b and AGT with *bis*-electrophiles (Figure 19B), including the ability of AGT to flip out damaged bases from the DNA duplex (Sandman *et al.,* 1998). The identification of *in vivo* histone H2b-DNA cross-links supports the hypothesis that lack of mutagenesis in *E. coli* cells is not due to the absence of DNA-protein cross-links (Figure 26). This may be the result of differential processing of DNA-protein cross-links. The mechanisms by which cells deal with these large lesions are poorly understood but are thought to involve proteolysis, nucleotide excision repair, and homologous recombination (Barker *et al.,* 2005a). The processing of cross-linked proteins may begin with the proteolytic cleavage of cross-linked proteins to peptides, which could go on to elicit DNA polymerase bypass, possibly resulting in mutations. The biochemical differences between histone and AGT cross-links may lead to differential processing and repair. Indeed, DNA-peptide cross-

links that vary in their amino acid sequences have distinctly different mutagenic potentials (Tubbs *et al.,* 2007*,* Minko *et al.,* 2008a,b). To date AGT remains unique in its ability to enhance *bis*-electrophile-induced mutagenesis, most likely due to the reactivity of its active site residue and distinct DNA-binding capabilities (Guengerich, 2005) and possibly to post-cross-linking phenomena.

CHAPTER IV

CHARCTERIZATION OF DIEPOXYBUTANE REACTIONS WITH GLUTATHIONE

Introduction

Butadiene is an important industrial chemical used primarily in the synthesis of plastics and rubber and it is also present in small quantities in cigarette smoke and automobile exhaust (Melnick and Kohn, 1995).   Studies in laboratory animals have shown the mutagenic and carcinogenic potential of butadiene. These findings, along with epidemiology studies of industrial workers lead to the classification of butadiene as a probable human carcinogen (Rice and Boffetta, 2001).

Butadiene is not reactive by itself, but is converted in cells by cytochrome P450 enzymes to three reactive epoxides that are capable of alkylating DNA (Figure 3). Of these, DEB is considered an ultimate carcinogen because of its enhanced reactivity with DNA and bifunctional alkylating potential. It is known that bifunctional alkylating agents not only form DNA-DNA cross-links, but they can also create linkages between DNA and proteins (Cochrane and Skopek, 1994a,b). The large and bulky lesions formed by DNA-protein cross-links are thought to significantly distort the helical structure of DNA and inhibit normal DNA metabolism or function (Barker *et al.*, 2005a,b).

Much of the interest in the cross-linking potential of industrial bifunctional electrophiles stem from early discoveries involving GST-expressing systems. The treatment of cells that overexpress these detoxicating enzymes with *bis*-electrophiles

paradoxically displayed an enhancement of mutagenesis (Thier *et al.,* 1995; Thier *et al.,* 1996). This observation was attributed to the formation of cross-links between GSH and DNA. This theory was confirmed using the ubiquitous *bis*-electrophile 1,2-dibromoethane, which reacts initially with GSH to form a reactive half-mustard that undergoes cyclization to an episulfonium ion before cross-linking to DNA (Peterson *et al.*, 1988).

The ability of DEB to form DNA-protein cross-links was characterized using the repair protein AGT. These reactions were found to involve the active cysteine residue of AGT and primarily the N7 atom of guanine residues of DNA. Nucleophilic attacks occurred on the C1 and C4 position of DEB to form a dihydroxybutyl linkage (Loeber *et al.,* 2006). While the ability of GSTs to enhance mutagenesis by DEB was realized over a decade ago, the chemical mechanism responsible for this observation has not been investigated (Rannug *et al.,* 1979; van Bladeren *et al.,* 1979). The goal of this project is to characterize the chemical cross-link between GSH and DNA induced by DEB. While initial results suggest reactions occur at the active cysteine residue of GSH, difficulties with purification of the complex prevented complete characterization.

Materials and Methods

Materials. DEB (racemic mixture containing meso isomers) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Purified equine liver GST, deoxyguanosine, and single-stranded calf thymus DNA-cellulose was purchased from Sigma-Aldrich (St. Louis, MO). Reduced GSH was also purchased from Sigma-Aldrich (St. Louis, MO), used without further purification, and dissolved immediately prior to use. Rat GST μ and

5-5 were expressed and purified as previously described (Cmarik *et al.*, 1990; Their *et al.,* 1993)

Synthesis of GSH-DEB conjugate. GSH (25 mg, 0.08 mmole) was dissolved in a sodium solution (7.5 mg, 0.33 mmol) in 2.5 mL dry methanol, before the drop wise addition of DEB (0.1 mmole). The reaction was stirred overnight at room temperature. Peptides were precipitated with the addition of 3 volumes of ethyl ether and the residue was dried under nitrogen.

The purification of GSH-DEB conjugates was attempted on a Phenomenex Prodigy (250×10 mm, 5μ, ODS(3), 100Å) column was used for the separation of the GSH-DEB conjugate from the reaction mixture. Mobile solvents (A) H_2O contained 0.01% acetic acid and (B) acetonitrile with 0.01% acetic acid. HPLC analysis utilized a UV detector set at 215 nm and a flow rate of 2 mL/min after a 50-100 μL injection of crude reaction.

The presence of epoxides was confirmed in HPLC peaks by adding the isolated peak to an equal volume of a solution containing 0.35 M 4-nitrobenzyl-pyridine in acetone, 0.1 M K^+ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), and ethylene glycol in a ratio of 1:2:4 (v/v/v). The solution was then heated at 95 °C for 10 min, allowed to cool to room temperature before adding a solution of 1:1 $(C_2H_5)_3N/(CH_3)_2CO$ (v/v) of equal volume. The colorimetric reaction was then assessed with a spectrophotometer at 562 nm (Peterson *et al.*, 1988) (Figure 27).

Reactions of purified GST with DEB. One mL reactions containing 0.1 mg/mL of purified rat GST μ or 5-5 protein were incubated with 2.0 mM DEB and 5.0 mM GSH in a buffered solution containing 0.1 M Tris-HCl (pH 7.7) for 1 h at 37 °C (Cmarik *et al.,*

1990). Reactions (0.5 mL) utilizing equine liver GST contained 75 ug enzyme, 10 mM

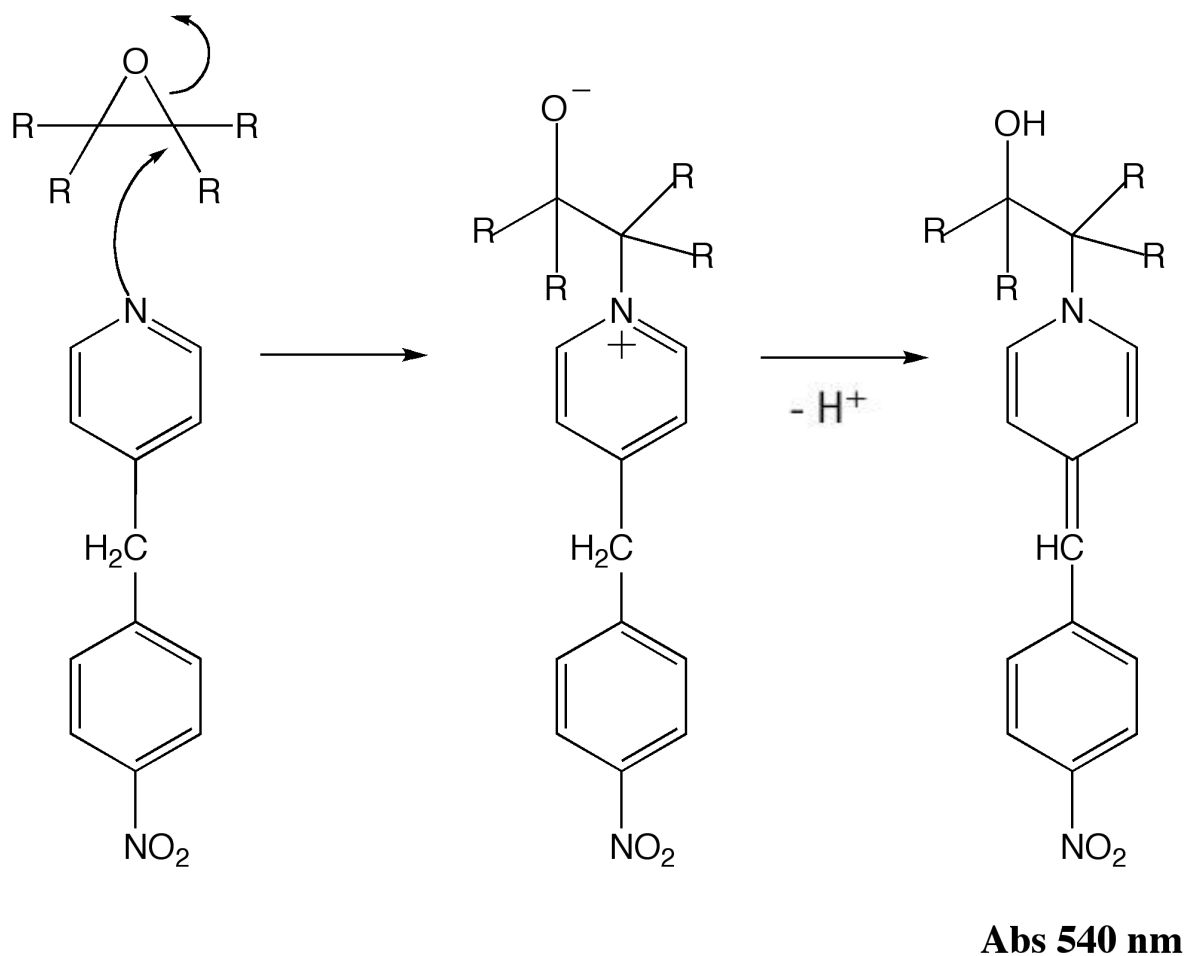GSH, and 5 mM DEB in a buffer solution containing 0.1 M Tris-HCl (pH 7.4) for 1 h at

Abs 540 nm

Figure 27. Mechanism of colorimentric detection of epoxides by 4-nitrobenzyl-pyridine.

37 °C. Reactions were extracted with 3 volumes of ethyl ether before analysis by mass spectrometry or HPLC.

Mass spectral analysis was performed in the positive ion mode by electrospray LC-MS/MS using a TSQ Quantum HPLC-ESI-MS instrument coupled to a Surveyor autosampler and Surveyor MS pump (ThermoElectron, San Jose, CA). Crude and purified solutions were analyzed using a ModMac Precision column (100 x 2.1mm, C18, 5um) using solvents (A) H_2O (0.1% acetic acid) and (B) acetonitrile (0.1% acetic acid) at a flow rate of 300 µL/min. MS/MS results were obtained using CID = 20 V with a 10 µL injection of crude sample.

Reactions of synthesized GSH-DEB with DNA. One mL reactions containing 200 mg/mL of GSH conjugates from unpurified synthetic reactions was added to an aqueous solution containing 20 mM deoxyguanosine and incubated at 37 °C for 24 h. Reactions were heated under neutral conditions, but were not further purified before analysis with mass spectrometry or HPLC.

Reactions (1.0 mL) containing 2.8 mg/mL of the unpurified synthetic solution and 2.0 mg/mL calf thymus DNA were incubated at 37 °C for 30 m in a buffered solution containing 500 mM Tris-HCl (pH 7.4) and 10% CH_3OH. The reactions were then heated under neutral conditions to release labile DNA adducts, followed by precipitation of DNA with cold ethanol. The resulting supernatant was added directly for analysis by HPLC and mass spectrometry.
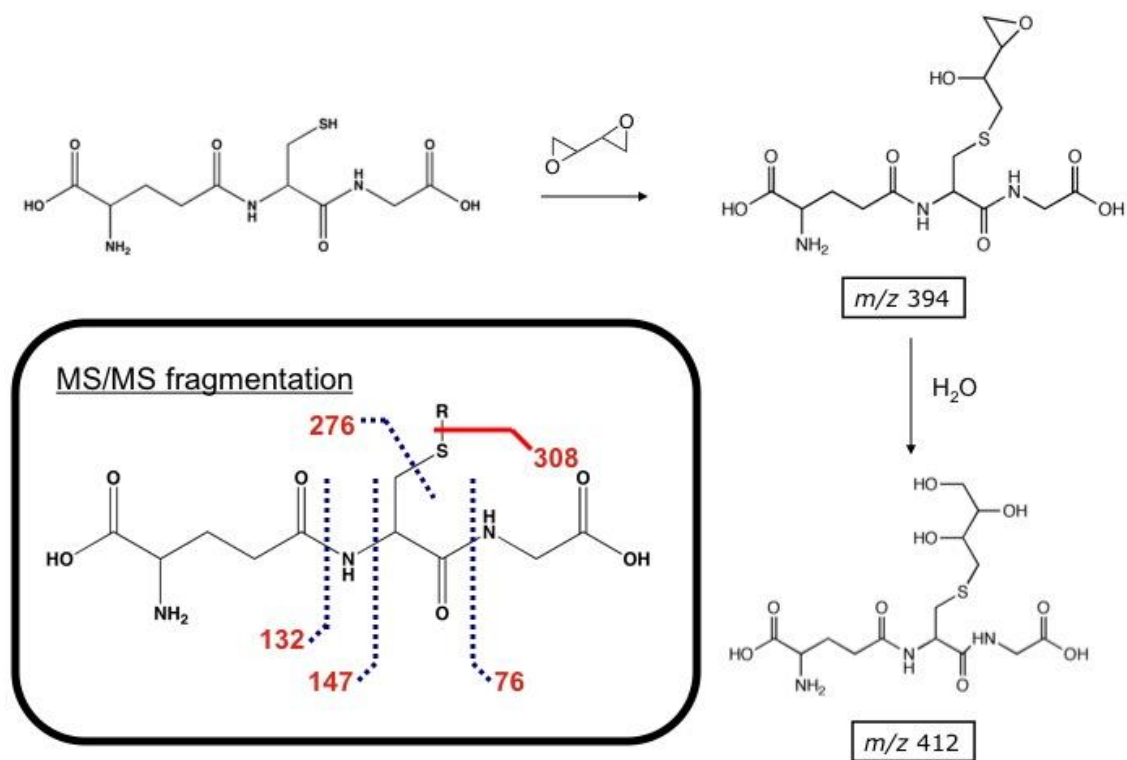
Figure 28. Predicted reactions between GSH and DEB and MS/MS fragmentation expected for GSH conjugates.

Results and Discussion

Formation of GSH-DEB conjugates. Reactions between DEB and GSH are expected to occur on the sulfur atom of the cysteine residue of GSH (Figure 28). Ions corresponding to mono-adducts were observed for both the enzymatic and non-enzymatic reactions, although other species where also formed, including di-adducted DEB (data not shown). The quantity of DEB was altered during the chemical synthesis of GSH-DEB in order to reduce the presence of multi-adducted species, but the mixture of compounds resulting from the synthesis was too complex for the direct purification of the GSH-DEB adduct (Figure 29).

Previous studies have shown that there are differences in DEB reactivity between GST of different classes (Wheeler *et al.*, 2001). In our studies we found that both rat GSTs of the theta and mu class are effective at conjugating DEB to GSH (Figure 30), along with purified equine liver GST (data not shown). Mass spectral analysis of the conjugate is consistent with known fragmentation patterns of GSH adducts, including the presence of *m/z* 308 and 265 (GSH and MH$^+$-129 a.m.u.).

Reactions of synthesized GSH-DEB with DNA. Previous studies of DNA-protein cross-links induced by DEB showed AGT was preferentially cross-linked on the N7 atom of guanine (Loeber *et al.,* 2006). The fragmentation pattern of the parent ion corresponding to a cross-link between guanosine and the GSH-DEB conjugate present in the synthetic reaction mixture was consistent with previous findings (Figure 31).

In order to verify the ability of DEB to cross-link GSH to DNA, reactions between the crude synthetic mixture and calf thymus DNA were conducted. Surprisingly, no cross-links were observed between guanine and GSH, however, cross-

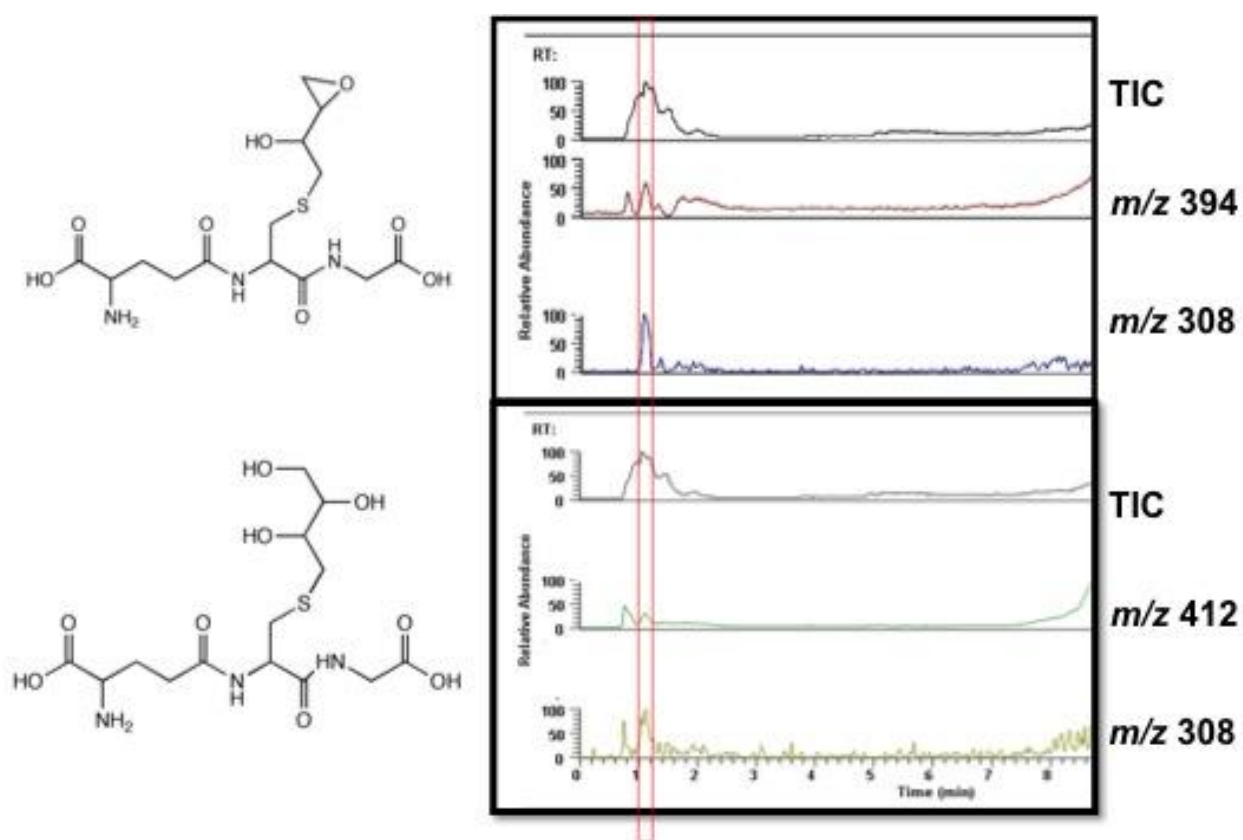**Figure 29**. Chromatographic results of synthetic reactions between GSH and DEB.
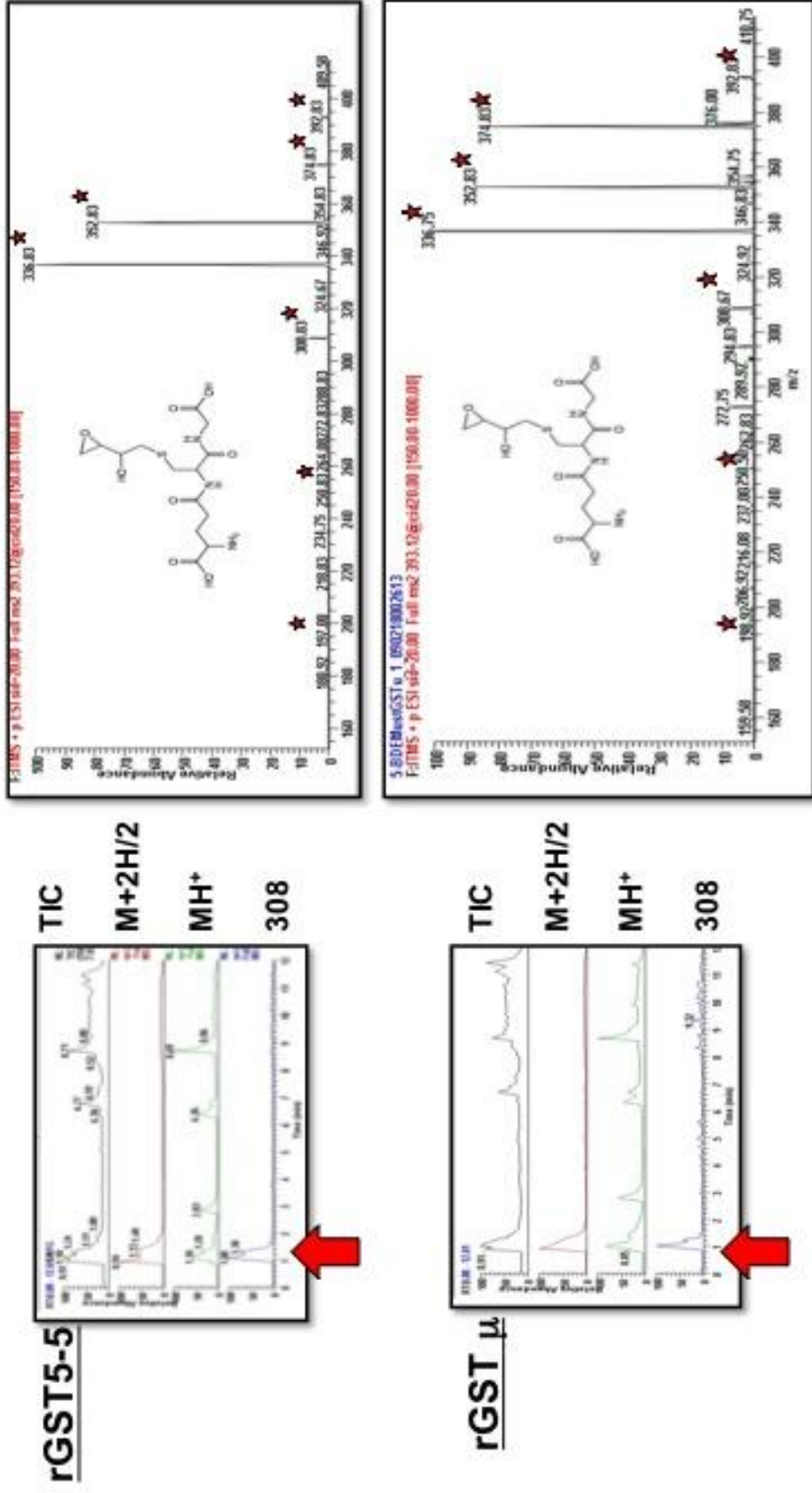
Figure 30. Mass spectral fragmentation of GSH-DEB adducts resulting from conjugation by rat GST mu and theta.
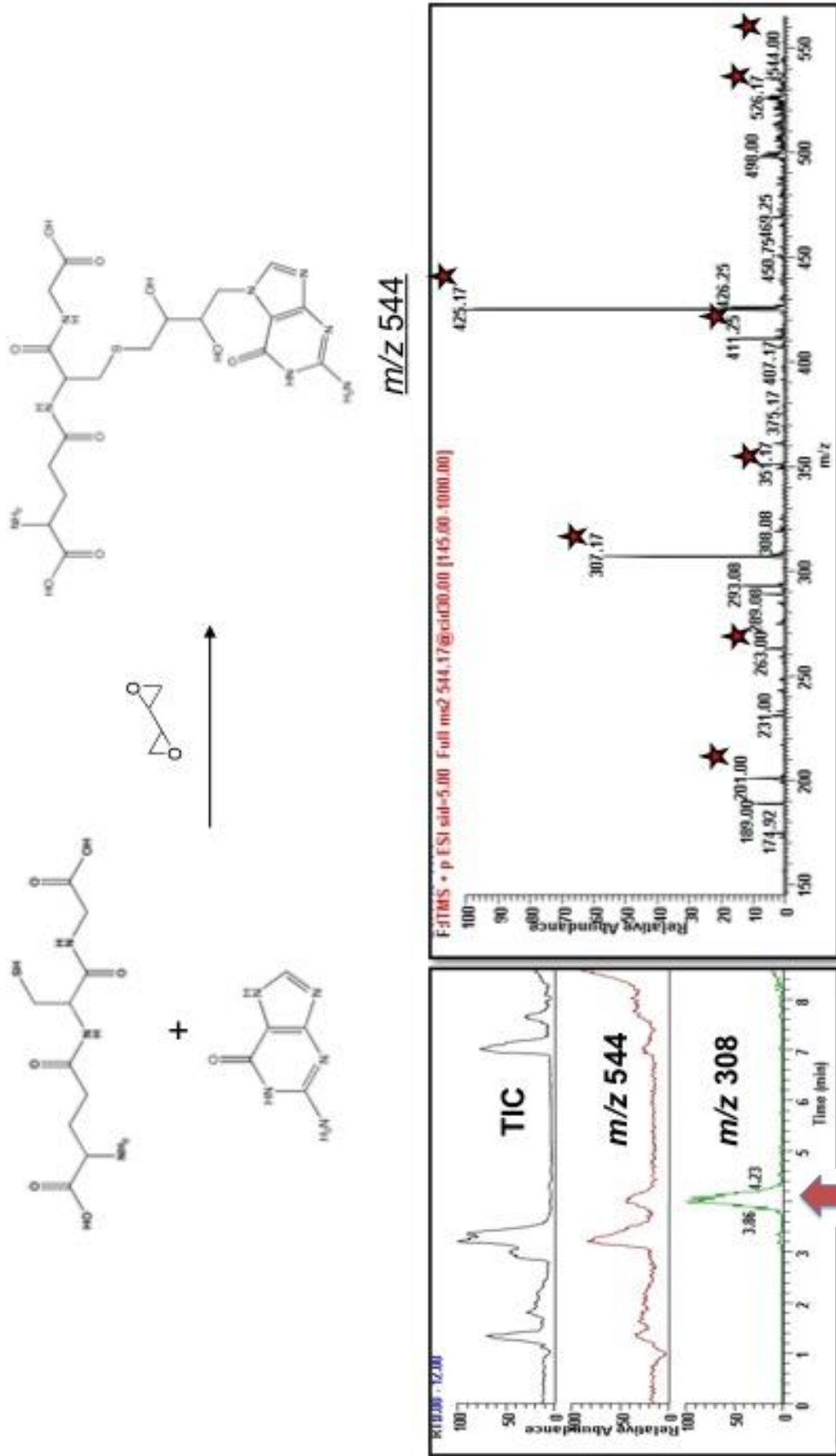
Figure 31. Mass spectral fragmentation of cross-links resulting from reactions between GSH-DEB conjugates and guanine.
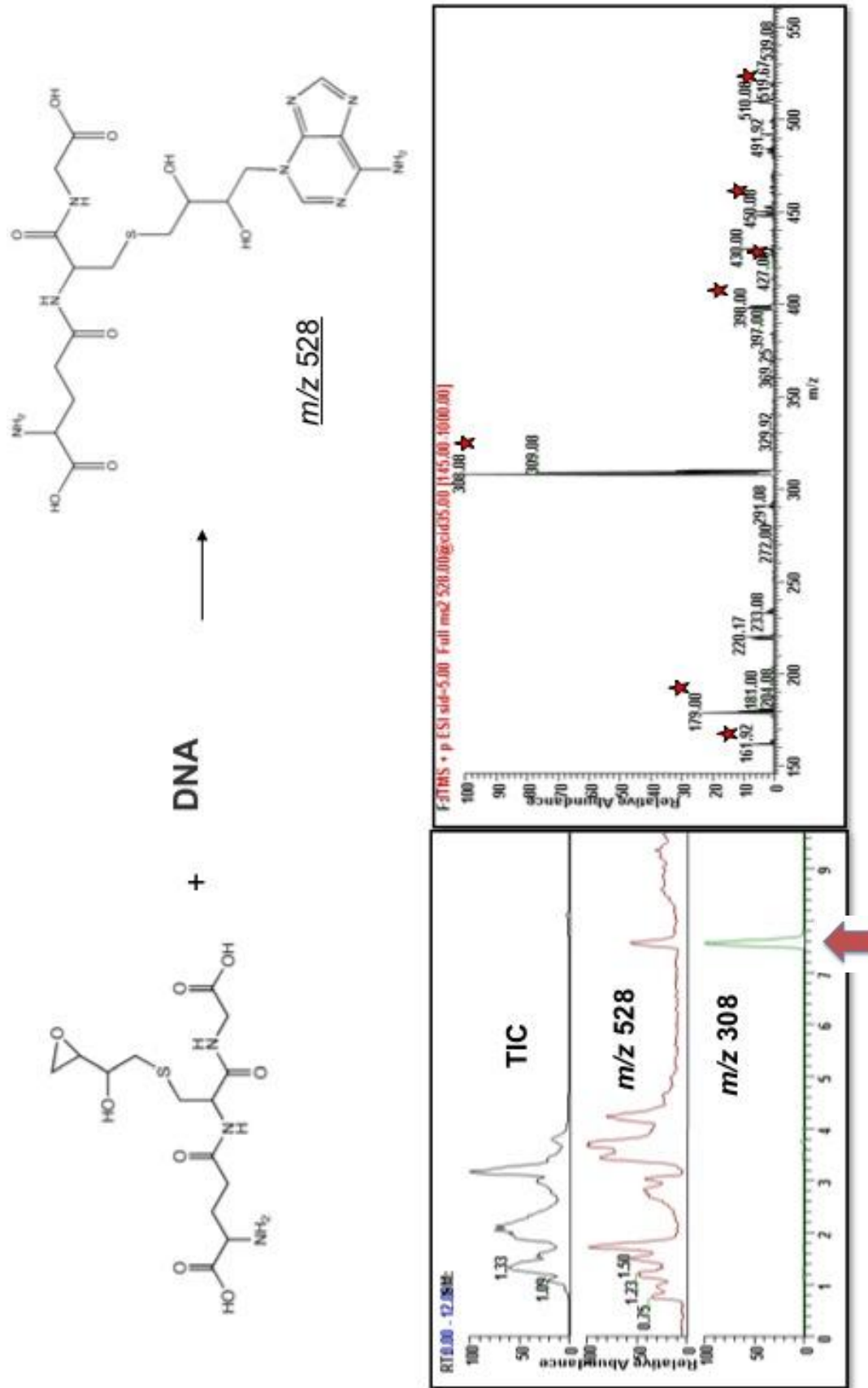
Figure 32. Mass spectral fragmentation of an adenine-GSH cross-link formed by reactions with DEB.

links involving adenine bases were observed (Figure 32). While the cross-linking of AGT to adenine by DEB was not observed, DEB is known to form DNA adducts on adenine (Kim *et al.*, 2007; Tretyakova *et al.*, 1997; Loeber *et al.*, 2007).

Conclusion

Understanding the mechanism by which DEB exerts harmful effects is important because of its ubiquitous use. The results of our preliminary studies suggest that enhancement of mutagenesis by DEB in systems overexpressing GST may be the result of cross-linking between GSH and DNA. Our mass spectral results show that ions corresponding to GSH conjugates with mono-adducts of DEB covalently bound are present in both synthetic and GST-catalyzed reactions. The resulting fragmentation ions are also consistent with GSH conjugates present on the sulfur atoms on the cysteine residues (Figure 30).

Reactions of the conjugates with isolated bases and DNA were also consistent with the formation of DNA-peptide cross-link formation. While cross-links were observed with guanine using the purified base, no guanine cross-links were observed resulting from incubations with calf thymus DNA (Figure 31). Reactions of the crude conjugate with single stranded DNA did provide evidence for the formation of adenine cross-links (Figure 32). Further studies with all DNA bases, along with efforts to characterize all adducts, not just those resulting from neutral thermal hydrolysis, will aid in the understanding of mutagenesis induced by DEB.

CHAPTER V

SUMMARY AND CONCLUSIONS

The importance of studying bifunctional electrophiles is underscored by the classification of many of these compounds as probable human carcinogens. Indeed, studies in animal models have proven the mutagenic potential of 1,3-butadiene, which has led to the introduction of guidelines in an attempt to limit occupational exposure. The ability of its metabolite DEB to alkylate cellulate macromolecules, such as DNA and proteins, has been well established, the mechanisms by which these chemicals exert their effects is still not known.

Studies in the 1970s were the first to suggest the ability of *bis*-electrophiles to induce DNA-peptide cross-links and the ability of these chemicals to induce cross-links were extensively studied in systems expressing the normally protective proteins AGT and GST. While technical difficulties limit our knowledge of DNA-protein cross-links, there is mounting evidence that these large and bulky lesions are detrimental to cells due to their mutagenic and carcinogenic properties.

The goal of this project was to establish if other proteins cross-linked to DNA through reactions with *bis*-electrophiles. This work emerges from a central hypothesis that DNA-protein cross-links formed by *bis*-electrophiles contribute to the deleterious effects of these chemicals. As a model for these studies, we used the findings observed with AGT in order to establish reactivity. The pool of candidate proteins used to start of investigation bis-electrophile-induced DNA-protein cross-links was obtained from a

screen preformed in the laboratory of Dr. Daniel C. Liebler in which isolated human nuclear proteins were investigated for their reactivity toward model electrophiles (Dennehy *et al.*, 2006) (Figure 10).

One protein found to be reactive with monofunctional electrophiles was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As in the case of AGT, treatment of GAPDH with DEB results in inhibition of activity and DNA-protein cross-link formation in *in vitro* assays in response to treatment with DEB. Although the reactivity of GAPDH with both DEB and 1,2-dibromoethane was validated with MS results, the overexpression of this protein in treated *E. coli* cells did not result in enhanced mutagenesis, in contrast with AGT (Liu *et al.*, 2002). The lack of mutational enhancement may be due to the inherently lower DNA binding ability of GAPDH (Figure 15), which could reduce the efficiency of cross-link formation.

In addition, the high concentration of DEB required to inhibit GAPDH activity (Figure 11) and induce cross-link formation (Figure 12) suggest that GAPDH is also inherently less reactive toward *bis*-electrophiles than AGT. These findings provide evidence that not all *bis*-electrophile-reactive proteins are capable of enhancing mutagenesis and suggest that *in vivo* cross-linking depend in part on the inherent characteristics of a protein. It also appears that different proteins display varying degrees of reactivity toward different *bis*-electrophiles, with AGT being more reactive toward 1,2-dibromoethane (Valadez *et al.*, 2004) and GAPDH selectively reacting with DEB.

The ability of other proteins to behave in a similar manner toward *bis*-electrophiles was addressed in our human liver screen, which employed a mass spectrometry-based search of DNA-binding proteins. From the candidate proteins

identified, human histones H2b and H3 were examined in detail because of their DNA-binding ability and basic nature (Peterson and Laniel, 2004). We established that purified histones H2b and H3 were able to cross-link with DNA using *in vitro* gel shift assays, using AGT as a positive control (Figure 19). In accord with these findings, DNA adducts resulting from protein alkylation and protein-DNA cross-links were identified (Figure 22). However, treatment of histone H2b-overexpressing *E. coli* cells with *bis*-electrophiles did not elevate mutation levels (Figure 23). The higher DNA binding ability of histone H2b in comparison with AGT (Figure 25) suggests that the absence of enhanced mutagenesis was not the result of reduced protein-DNA interactions. However, it is reasonable to at least partially attribute differences in mutagenic enhancement to the innate differences in reactivity of histone H2b and AGT with *bis*-electrophiles (Figure 19B), including the ability of AGT to flip out damaged bases from the DNA duplex (Sandman *et al.,* 1998).

The identification of *in vivo* histone H2b-DNA cross-links supports the hypothesis that lack of mutagenesis in *E. coli* cells is not due to the absence of DNA-protein cross-links (Figure 26). The biochemical differences between histone and AGT cross-links may lead to differential processing and repair. Indeed, DNA-peptide cross-links that vary in their amino acid sequences have distinctly different mutagenic potentials (Tubbs *et al.,* 2007*,* Minko *et al.,* 2008a,b). To date AGT remains unique in its ability to enhance *bis*-electrophile-induced mutagenesis, most likely due to the reactivity of its active site residue and distinct DNA-binding capabilities (Guengerich, 2005) and possibly to post-cross-linking phenomena (Figure 33).

Understanding the mechanism by which DEB exerts harmful effects is important because of its industrial use and the potential for human exposure. The results of our preliminary studies suggest that enhancement of mutagenesis by DEB in systems

Bis-Electrophile-Induced Result	AGT	GAPDH	H2b
Modified cysteine residue	✓	✓	✓ ← Lys Arg
Inhibition of activity	✓	✓	☐
Crosslinking to DNA	✓	✓	✓
Enhanced mutagenesis	✓	✗	✗

Figure 33. Results from analysis of candidate proteins for the cross-linking of proteins to DNA.

overexpressing GST may be the result of cross-linking between GSH and DNA. Our mass spectral results show that ions corresponding to GSH conjugates consisting of DEB mono-adducts are present in both synthetic and GST-catalyzed reactions. The resulting fragmentation ions are also consistent with GSH conjugates present on the sulfur atoms on the cysteine residues (Figure 30).

Reactions of the conjugates with isolated bases and DNA were also consistent with the formation of DNA-peptide cross-link formation. While cross-links were observed with guanine using the purified base, no guanine cross-links were observed resulting from incubations with calf thymus DNA (Figure 31). Reactions of the crude conjugate with single stranded DNA did provide evidence for the formation of adenine cross-links (Figure 32). Further studies with all DNA bases, along with efforts to characterize all adducts, not just those resulting from neutral thermal hydrolysis, will aid in the understanding of mutagenesis induced by DEB. Finally, purification of the GSH-DEB is necessary for the chemical characterization of the mutagenic species.

This research highlights the complexity of reactions involving *bis*-electrophiles. Both GAPDH and histone H2b possessed characteristics thought to be necessary to contribute to mutagenesis by bifunctional electrophiles, including presence of nucleophilic residues and access to DNA. Neither protein displayed the enhancement of mutagenesis by *bis*-electrophiles observed with AGT in the bacterial system used. However, some reactions observed with AGT did occur with both GAPDH and histone H2b. These reactions include *in vitro* cross-linking and direct alkylation by *bis*-electrophiles; however, all results required high concentrations of the diepoxybutane.

While the bacterial systems used in these studies did not enhance the deleterious

effects of DEB or dibromothane, the ability of these proteins to contribute to *bis*-electrophile-induced mutagenesis may exist in other systems. It's also possible that the alkylation reactions observed for both GAPDH and histone H2b can disrupt your normal function in cells, which would also not be evident in the bacterial systems. This research proves that not all nucleophilic proteins in the nucleus are capable of contributing to mutagenesis by *bis*-electrophiles. Future studies will hopefully use this information to directly focus on candidates that, like AGT, primarily function in DNA maintenance through the use of a highly nucleophilic residue.

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