ANALYSIS OF PROTEIN ADDUCTION KINETICS AND THE EFFECTS OF PROTEIN ADDUCTION ON C-JUN N-TERMINAL KINASE SIGNALING

by

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SIGNED: Christopher Reed Orton
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DEDICATION

I wish to dedicate this dissertation work to my father, Errol M. Orton. Dad lost his battle with cancer in June of this year prior to seeing me finish my graduate work. He is the bravest man I know and has endured so much during these last five years. I am so proud to have him as my father just as I know he is proud of all I have accomplished. I love you Dad and will see you in eternity.
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LIST OF ABREVIATIONS

ACN  acetonitrile
AMBIC ammonium bicarbonate
ALD  alcohol liver damage
AP-1 activator protein 1
APP  amyloid precursor protein
AQUA absolute quantification
ARE  antioxidant response element
ASK1 apoptosis signal-related kinase 1
ATF  activating transcription factor
BMCC 1-Biotinamido-4-(4’-[maleimidoethyl-cyclohexane]-carboxamido)butane
CDNB 1-chloro 2,4-dinitro benzene
CHCA α−cyano-4-hydroxycinnamic acid
CID collision induced dissociation
Cys cysteine
DDT dichloro-diphenyl-trichloroethane
DLK dual leucine zipper kinase
DMEM Dulbecco’s modified eagle medium
DMSO dimethyl sulfoxide
DNA deoxyribonucleic acid
DTT dithiotheritol
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>EDTA</td>
<td>ethylenedinitrillo tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis[β-aminoethyl ether]-N,N,N'N'-tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extra cellular-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>fumarylacetone</td>
</tr>
<tr>
<td>FAA</td>
<td>fumarylacetoacetone</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293 cell line</td>
</tr>
<tr>
<td>His</td>
<td>histadine</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IAB</td>
<td>N-iodoacetyl-N-biotinylhexylenediamine</td>
</tr>
<tr>
<td>IAM</td>
<td>iodoacetamide</td>
</tr>
<tr>
<td>ICAT</td>
<td>isotope-coded affinity tag</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>JIP</td>
<td>JNK interacting protein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>JSAP1</td>
<td>JNK/stress-activated protein kinase-associated protein 1</td>
</tr>
<tr>
<td>$k$</td>
<td>reactivity constant</td>
</tr>
</tbody>
</table>
Keap1  Kelch-like ECH-associated protein 1
LC-MS-MS  liquid chromatography tandem mass spectrometry
LDL  low-density lipoproteins
LDS  lithium dodecyl sulfate
Lys  lysine
LZK  leucine zipper-bearing kinase
MA  maleylacetone
MAA  maleylacetoacetone
MAF  musculoaponeurotic fibrosarcoma
MALDI-TOF  matrix assisted laser desorption ionization time-of-flight
MAPK  mitogen-activated protein kinase
MAPKK  MAPK kinase
MAPKKK  MAPKK kinase
MDA  malondialdehyde
MEF3T3  mouse embryo fibroblast cell line
M KK4  MAP kinase kinase 4
MKK7  MAP kinase kinase 7
MKP  MAP kinase phosphatase
MLK  mixed linkage kinase
MMP  matrix metalloproteinases
MMS  methyl methanesulfonate
MRM  multiple reaction monitoring
<table>
<thead>
<tr>
<th>Term</th>
<th>Full Form</th>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS-MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetylbenzoquinoneimmine</td>
</tr>
<tr>
<td>NBSCI</td>
<td>2-nitrobenzenesulfenyl chloride</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>nuclear factor-E2-related factor 2</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>O-GlcNAc</td>
<td>O-linked N-acetylglucosamine</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>PARP</td>
<td>poly-ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEO-IAB</td>
<td>(+)-Biotinyl-iodoacetamidyl-3, 6-dioxaoctanediamine</td>
</tr>
<tr>
<td>PGA2</td>
<td>prostaglandin A2</td>
</tr>
<tr>
<td>PGJ2</td>
<td>prostaglandin J2</td>
</tr>
<tr>
<td>PHS</td>
<td>prostaglandin H synthase</td>
</tr>
<tr>
<td>PIC</td>
<td>phenyl isocyanate</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RelEx</td>
<td>relative expression</td>
</tr>
</tbody>
</table>
ROS  reactive oxygen species
rt-PCR  real time polymerase chain reaction
SALSA  scoring algorithm for spectral analysis
SAPK  stress-activated protein kinase
SCX  strong cation exchange
SDS-PAGE  sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Ser  serine
SIM  selective ion monitoring
SUMO  small ubiquitin-like modifier
TAK  transforming growth factor-β activated kinase
TBS  tris-buffered saline
TBST  tris-buffered saline – tween-20
TCEP  tris-carboxyethyl phosphine
TFA  trifluoro acetic acid
Thr  threonine
TNF-α  tumor necrosis factor alpha
TPL2  tumor progression locus 2
Trx  thioredoxin
Tyr  tyrosine
UV  ultraviolet
ABSTRACT

Defining the mechanics and consequences of protein adduction is crucial to understanding the toxicity of reactive electrophiles. Application of tandem mass spectrometry and data analysis algorithms enables detection and mapping of chemical adducts at the level of amino acid sequence. Nevertheless, detection of adducts does not indicate relative reactivity of different sites. In this dissertation I describe a method to measure the kinetics of competing adduction reactions at different sites on the same protein using quantitative mass spectrometry. Adducts are formed by electrophiles at Cys-14 and Cys-47 on the metabolic enzyme glutathione-S-transferase P1-1 and accompanied by a loss of enzymatic activity. Relative quantitation of protein adducts was done by tagging N-termini of peptide digests with isotopically labeled phenyl isocyanate and tracking the ratio of light-tagged peptide adducts to heavy-tagged reference samples. This method was used to measure rate constants for adduction at both positions with two different model electrophiles, IAB and BMCC. The results indicate that Cys-47 was approximately 2-3-fold more reactive toward both electrophiles than was Cys-14. This result was consistent with the relative reactivity of these electrophiles in a complex proteome system. Quantitative analyses of protein modifications provide a means of determining the reactivity and selectivity of damaging protein modifications in chemical toxicity.

Another area of study explored in this dissertation is looking at the effects of protein alkylation on activating cellular signaling pathways, specifically the JNK
signaling pathway. Protein adduction has been shown to be selective between different alkylation agents. It would then be reasonable to think this selectivity of adduction translates to selectivity of downstream consequences or cellular events directly tied to specific adductions. My work will show how treatment of HEK293 cells with either IAB or BMCC leads to differences in activation of JNK signaling. In addition, I’ve been able to show a difference in selectivity of a number of adducted targets by each alkylation agent, which are directly involved in regulation of the JNK signaling pathway. These studies illustrate not only the significance of protein adduction, but the importance for continual research to better understand their behavior in living systems.
CHAPTER 1 – INTRODUCTION

Significance of protein adduction

Protein modification plays an important role in cell biology. Following synthesis, proteins can undergo several possible modifications. The most common of these are structural modifications, such as folding or formation of disulfide bonds, which give proteins their secondary structures and provide the structural basis for their functions. Other modifications that occur early in the life of a protein include deletion of N-terminal methionine, carboxylation of glutamate residues, glycosylation, or deletion of leader peptides, which direct a newly formed protein to its proper location within the cell (1). Proteins also are subject to additional regulatory modifications. Two of the most common are phosphorylation and ubiquitination. Phosphorylation is a critical modification that regulates enzymatic activity, protein-protein interactions and stability and occurs on serine, threonine, or tyrosine residues. Reversible protein phosphorylation plays an important role in cellular signaling and communication. Ubiquitination occurs on lysine residues and can tag proteins for degradation (2). This process regulates protein turnover and can also be important in regulating a protein’s activity. In some cases ubiquitin modifications do not lead to proteasomal degradation, but instead regulate protein interactions and functions (3-5). Endogenous modifications such as these are tightly regulated by cells for proper function and development. Disruption of these mechanisms could result in toxicity, disease, and even death.
Besides modifications just mentioned, proteins can potentially be modified by other factors that are not regulated by the cell and are introduced by the external environment. Modifications of this kind are generally referred to as *adducts* and result from proteins coming into contact with reactive, electrophilic compounds that covalently bind nucleophilic residues. Sources of these reactive compounds include environmental pollutants, industrial chemicals, drugs, and even certain components of our diet. Many of these compounds themselves are relatively harmless in small amounts, but can become harmful in larger doses and through metabolism that generates electrophilic intermediates. Protein adduction can cause harmful and potentially fatal consequences to the cell as a result of disruption of cellular structure and function. For this reason, it is critical to understand protein adduction, from identification of potentially reactive intermediates capable of protein adduction, to identification of the protein targets within cells that are most susceptible to damage and the consequences of protein damage. Vital then, is the further development of methods to characterize and compare adduction at multiple protein targets in an effort to better understand specific effects of different alkylating agents. It is also essential to understand the effects of adduction at different protein targets, and their resulting consequences within cellular signaling networks and regulatory systems.

**A Brief History of Protein Adduction**

The study of protein adduct formation can be traced to the late 1940s, when the Millers showed that the carcinogen N-methylaminoazobenzene could covalently bind to
liver proteins in vivo (6). One of the earliest and most insightful studies on protein adduction and its effects was reported in 1973, by Jollow, Gillette, Mitchell, Brodie and colleagues, who published a series of papers on the effects of feeding high doses of acetaminophen to mice and/or rats (7-10). Acetaminophen (ie., Tylenol®) is a common analgesic sold over-the-counter as well as a component of several prescription drugs used for analgesia. Acetaminophen toxicity serves as a perfect example of the basic principles of metabolic activation and protein adduction. At therapeutic doses the drug is effective and harmless. It is metabolized primarily in the liver, where it is conjugated to either glucuronic acid or sulfate, both of which can be easily excreted. An alternate metabolic path involves activation by cytochrome P450 (P450) or prostaglandin H synthase (PHS) into the N-acetylbenzoquinoneimmine (NAPQI) intermediate (Figure 1-1). This highly reactive intermediate can be inactivated through conjugation with glutathione (GSH), or can easily bind covalently to nucleophilic residues on proteins, leading to cellular toxicity (11).

This series of papers reported several key findings. First, high doses of acetaminophen given to mice resulted in a severe dose-dependent hepatic necrosis, whereas rats were barely susceptible to necrosis at weight-normalized doses more than twice those administered to mice. Second, pretreatment with phenobarbital, an inducer of drug-metabolizing enzymes, intensified both the incidence and severity of necrosis in mice and rats. In contrast, pre-administration of piperonyl butoxide, an inhibitor of drug-metabolizing enzymes decreased the incidence and severity of necrosis in both species (9). Third, experiments using 3H- or 14C-acetaminophen indicated that conditions of (12)
Figure 1-1: Depiction of the metabolism and resulting bioactivation of acetaminophen by P450 and PHS to the reactive intermediate NAPQI.
hepatic necrosis correlated with covalent binding of radiolabeled compound to hepatic proteins (13). These results showed that hepatic necrosis is directly related to covalent modification of hepatic proteins by an acetaminophen metabolite and not by acetaminophen itself. Because this pathway is metabolism-dependent, it can differ between species based on their metabolic capability for different chemicals.

A fourth important finding from this group was that covalent binding was reproduced in liver microsomes incubated with acetaminophen in vitro and that covalent binding is mediated by microsomal enzymes that require molecular oxygen and NADPH, which provided evidence of the cytochrome P450 family of enzymes (10). Finally, they were able to show covalent binding of acetaminophen to hepatic proteins was accompanied by a decrease in cellular GSH, and covalent binding only occurred at doses of acetaminophen that caused a depletion of GSH $\geq 70\%$. These results revealed that GSH serves a protecting role for proteins against electrophilic adduction (8).

These results defined essential elements of protein adduction in chemical toxicity. First, protein adduction is associated with severe tissue toxicity and even death. Next, a parent compound may be transformed into a reactive intermediate capable of protein binding. Finally, protein adduction can deplete a cell of GSH stores, which serve to protect the cell from adduction as well as oxidative stress.

**Biological Consequences of Protein Adduction**

For years now the vast majority of studies related to reactive intermediates and their resulting cellular toxicity focused on adduction of DNA and resulting effects on the
genome and carcinogenesis. While this work has been beneficial, there has been increasing interest in exploring the possibility of proteins being targets for these reactive compounds. Since proteins express the functions of genes, it would certainly be important to consider the consequences of adduction on these cellular functions. Since the discoveries described above regarding acetaminophen toxicity and protein binding, there has been considerable work directed at discovery of reactive intermediates resulting from xenobiotic metabolism, their respective protein targets and target cells, and cellular consequences related to adduction of these targets. Studies throughout the 1980s and early 1990s established adduction as a selective process and not a random event (14).

Protein adduction appears to be dependent on several factors such as the chemistry of the alkylating agent, concentration and dose of the agent, cell type targeted, and duration of exposure, all of which play key roles in determining resulting effects of protein alkylation (15).

While much of the research on protein adduction has focused on their toxicological significance (including the work described in this dissertation), there remain relevant examples of compounds that bind to cellular proteins yet are not linked to or induce cellular toxicity. One example is in rats administered either bromobenzene or one of its metabolites, \( p \)-bromophenol. Studies have shown both compounds covalently bind to proteins in the liver, kidney, and small intestines of these animals but only bromobenzene caused hepatotoxicity (16;17). Another example suggests covalent binding of paracetamol in rat hepatocytes leads to slight GSH depletion but is insufficient to cause cell death (18). This result suggests protein adduction itself is not the cause of
cell death, but may act as a trigger to additional events causing toxicity and/or cell death. This study also showed how these secondary events can be blocked by addition of a protecting agent. A third example of protein adduction not being linked to cellular toxicity is in mice administered either acetaminophen or 3-hydroxyacetanalide, a positional isomer of acetaminophen (19;20). These studies show how administration with both compounds resulted in significant covalent binding of hepatocyte proteins (including mitochondrial proteins) and GSH depletion, only cells treated with acetaminophen exhibited toxic effects. Furthermore, covalent binding of acetaminophen to liver proteins caused inhibition of Ca\(^{+2}\)-ATPase activity, leading alterations of calcium homeostasis, an event linked to cell toxicity and death, while covalent binding following 3-hydroxyacetanalide administration did not affect Ca\(^{+2}\)-ATPase activity or calcium homeostasis.

Toxicological consequences of protein adduction encompass many cellular mechanisms, beginning with inhibition of enzymatic activity (21) to cell death (22). Enzyme active sites often contain nucleophilic amino acid side chains that participate in catalysis. Adduction of any of these sites could result in a loss of enzymatic activity, the results of which could be deleterious to cell function. Evidence linking protein adducts to loss of function has been shown in several families of enzymes including cytochrome P450 (21;23), quinone oxidoreductase (24), and phase II metabolizing enzymes, such as glutathione S-transferase P1-1 (25). In addition to inhibiting enzymatic function, protein adduction can compromise the structural integrity of proteins. This has been shown to initiate autoimmune responses in alcohol liver damage (ALD) (26). When proteins are
adducted, their secondary structures can change such that they become recognized by the organism as foreign. This may then lead to the generation of antibodies specific to the protein target and initiate an immune response. One example has been reported that shows how metabolites of ethanol can form adducts in the liver, inducing antibody production and increasing T-cell proliferation (27).

Recent discoveries have shown how certain targets of adduction act as triggers, or sensors of specific cellular signals. In many cases, these targets are cysteine residues. These residues play key roles in protein secondary structure through the formation of disulfide bonds as well as acting as redox sensors for oxidative stress in cellular systems. One example is adduction of cysteine residues on the sensor protein Kelch-like ECH-associated protein 1 (Keap1), which induces antioxidant/electrophile response element (ARE)-regulated phase 2 enzyme and antioxidant genes through activation of the transcription factor nuclear factor-E2-related factor 2 (Nrf2). Studies show specific patterns of adduction of certain cysteine residues on Keap1 act as sensor triggers to activate this response (28-31). Another example is regulation of apoptosis signal regulated kinase 1 (ASK1) signaling by thioredoxin (Trx). Studies by Wang Min and colleagues have shown Trx-1 and Trx-2 negatively regulates ASK1 activation in the cytosol and mitochondria, respectively through interaction via disulfide linkage (32;33).

Protein ubiquitination plays a key role in protein turnover and regulation of protein expression in cellular systems. Ubiquitination occurs on lysine residues and acts as a signal for protein degradation via the proteosome (2). A similar modification by the
small ubiquitin-like modifier (SUMO) ubiquitin family protein has also been shown to be perturbed in response to protein adduction by reactive alkylation agents (34).

Protein adduction has also been implicated as a mechanism of development of disease states in several organ systems. Some examples include alcoholic liver disease, which has been related to formation of adducted liver proteins by reactive metabolites of ethanol such as acetaldehyde (35), as well as metabolites of lipid peroxidation induced by oxidative stress that include malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (26). Protein adduction by common neurotoxicants 2,5-hexanedione and acrylamide has also been linked to neurotoxicity (36). These and other lipid-derived carbonyls have also been linked to the development of Alzheimer’s disease (37). In addition to these, adducts formed by the endogenously produced HNE in modified low-density lipoproteins (LDLs) have been reported as markers for atherosclerosis (38). These examples clearly show the importance of gaining an understanding of the protein targets, as well as their specific consequences of adduction in cellular systems.

**Early studies of protein adduction**

Precise mechanisms of cellular consequences resulting from protein adduction are still largely unknown. However, techniques for characterizing protein binding in cells and tissues have improved at a rapid pace in recent years. Whereas early studies in the 1970s used radiolabeling to simply show the existence of protein binding by reactive electrophiles, mass spectrometry (MS) analysis used today can identify hundreds of
potential cellular protein targets and map amino acid locations on those proteins where adduction occurs (39).

Early detection of protein adduction relied on treatment of cells or animals with radio-labeled chemicals, where protein adduction could be observed and measured by analysis of covalently bound radiolabel to tissue proteins. This technique was used in studies mentioned earlier regarding acetaminophen toxicity (8;10;13). This technique, although useful for detecting bulk protein binding by reactive intermediates, provided no information as to the identities of the protein targets. Advances occurred later during the 1980s, when work in the Pohl laboratory discovered that antibodies could be developed to detect specific protein adducts (40;41). The toxicity of several chemicals has since been characterized along with immunochemical studies to identify their specific protein targets. Examples of chemicals whose binding was studied in this way include nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetaminophen (42), diclofenac, sulindac, and ibuprofen (43), the halocarbon trichloroethylene (44), and the lipid oxidation product HNE (45). Techniques such as these proved useful not only in identifying protein targets but also in gaining a better understanding of cellular toxicity by protein adduction that can result in immune reactions and hypersensitivity (46). Work by the Pohl group utilized this technique to explore mechanisms of halothane-induced hepatotoxicity (47). Immunochemical techniques provided the next step as far as identifying specific protein targets of specific toxicants. However, these methods still were not able to unambiguously identify specific protein targets or localize the site of adduction on target proteins. In addition, development of specific antibodies for so many
specific adducts can be quite costly and labor intensive. Detection of adducts by antibodies also raises questions regarding the specificity of these antibodies directed against relatively small epitopes and the possibilities they may not be able to identify every protein target, as well as the possibility of nonspecific binding, which could produce false-positive identifications.

**Analytical Proteomics Methods to Characterize Protein Modifications**

Many of these problems have since been resolved during the latter portion of the 1990s with the advancement of MS approaches for both protein identification and mapping of protein adducts. The age of MS truly advanced with development of algorithms designed to correlate tandem mass spectral (MS-MS) data of peptides with amino acid sequences in a protein database. The two most commonly used today include Sequest (48;49) and MASCOT (50). Techniques such as these have allowed for analysis and identification of proteins from complex mixtures that are digested into even more complex mixtures of peptides and analyzed by MS, generally referred to as ‘shotgun’ proteomics (51-53). These advances, along with improving separation methods have revolutionized the identification of proteins with MS.

Shotgun MS analysis of proteins generally follows a basic procedural flow that begins with collection of protein sample (Figure 1-2). This could be from cell or tissue extracts, which are either test (treated, disease, etc.) or control (normal, untreated) samples. At this point proteins may be separated by techniques such as sodium dodecyl sulfide- polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF).
Protein Sample Collection

Separation “Pre-digestion” proteins

Affinity Tags
Biotin, His6-, GST

IEF

Digestion

Protease – Trypsin, Chymotrypsin, Pepsin, Glu C, Lys C, Asp N

Separation “Post-digestion” peptides

Chromatography
Affinity, reverse phase, strong cation/anion, size exclusion

MS-MS Analysis
Data Collection

LC-MS-MS
MALDI-TOF

Data Analysis
Identification

Database Search Algorithms
SEQUEST, Mascot

Figure 1-2: General workflow of a typical “shotgun” proteomic analysis from sample collection to protein identification.
Next, would involve digestion of the protein mixture with a protease such as trypsin or chymotrypsin to yield an even more complex mixture of peptides followed by additional separation by chromatographic techniques such as strong cation exchange (SCX), reverse phase, or size exclusion. Finally, fractionated samples are analyzed by LC-MS-MS to obtain MS-MS spectra of peptides in the digest mixture. This has been facilitated by the interface of high performance liquid chromatography (HPLC) with MS, which allows for separation of complex peptide mixtures, followed by direct infusion into the mass spectrometer. The combination of SCX or IEF and reverse phase LC-MS-MS allows for multi-dimensional separation of complex mixtures, which reduces complexity and increases peptide and protein identification (54;55). Recent advances have continued to provide additional resources and methods to aid in these processes that include comparing protein identifications between two samples (56), quantitative analysis (57), and even statistical models to identify peptides from MS-MS data and estimate the accuracy of results (58;59).

MS-MS spectra for peptides are generated by collision induced dissociation (CID) of an intact peptide to generate fragments detected by the mass spectrometer that encode the peptide sequence. More specifically, when peptide ions collide with neutral gas atoms in a mass spectrometer, they undergo fragmentation. Fragmentation generally occurs along the peptide backbone, between the carbonyl oxygen and amide nitrogen. The result is smaller fragments that are either classified as “b-ions” or “y-ions”. Fragment ions that retain a positive charge on the N-terminus are b-ions, while ions retaining a positive charge on the C-terminus are referred to as y-ions. Fragmentation of
a doubly charged peptide (a peptide with two charges, usually on each terminus) would yield two charged fragments detectable by the mass spectrometer, whereas a singly charged peptide (contains only one charge) would yield one charged fragment, and a neutral fragment that would not be detected by the mass spectrometer. Therefore, doubly charged peptides yield approximately twice as much information in MS-MS spectra that can be utilized for identifying the sequence of the parent peptide.

Fragmentation of an example peptide will better illustrate this point. For example, the peptide sequence AVAGCAGAR (Figure 1-3), bond breakage along the peptide backbone generates both b- and y-ion series, which are indicated. A modification such as methylation on the thiol group of the cysteine residue affects both b- and y-ion series. Modifications will cause the product ion series to shift by the amount of the added modification present on each b- or y-ion. Therefore, diagnostic fragmentations in MS-MS spectra to identify unadducted peptides as well as map adduction to specific residues on adducted peptides.

Shotgun analysis of complex mixtures yields MS-MS spectra not only of unmodified peptide sequences, but also of peptides bearing endogenous modifications and adducts. While most proteomics techniques geared toward analyzing protein modification are focused on endogenous modifications such as ubiquitination, phosphorylation, glycation and other regulatory modifications, these tools can be applied to analyze protein adduction by exogenous xenobiotics and endogenous electrophiles. MS-MS spectra encode not only identities of protein targets, but also specific sites of adduction.
Figure 1-3: Theoretical fragmentation of model peptide TpepC (AVAGCAGAR). Spectra shows fragmentation of all identified b- and y-ions non adducted and showing theoretical mass shifts with a methylation adduction on the cysteine (C) residue (red). This modification results in mass shifts of b- and y-ions in their series > 4.
A major challenge is identifying MS-MS spectra that arise from adducted peptides. One such tool that can be applied to identify protein modifications is an algorithm called SALSA (Scoring ALgorithm for Spectral Analysis) (60;61). Modified peptides contain features in their MS-MS spectra that distinguish them from their unmodified counterparts. SALSA enables the user to identify spectra that display certain features, in MS-MS spectra and score the spectra based on appearance of these features and their intensities in the spectrum. SALSA can detect product ions, which appear at particular m/z values in MS-MS spectra. The algorithm detects charged losses, where multiply charged precursors fragment into charged fragments as well as neutral losses where precursors generate a charged fragment equal to the mass of some neutral fragment undetected by the mass spectrometer. Adducts may display characteristic neutral or charged losses in MS-MS fragmentation (62). SALSA is also able to detect ion pairs and series, which refers to signals separated by specific m/z values anywhere in the MS-MS spectrum. These can be useful in identifying components of a peptide sequence, such as a particular amino acid residue, where the m/z difference between the pair would correlate with the mass of that amino acid. Likewise, multiple ion pairs constitute ion series, which denote peptide sequences and may arise from both adducted and unadducted peptides. This algorithm has proven to be a valuable tool in mapping protein adductions (63) and has been used to identify epoxide adducts in human hemoglobin (64). One drawback to SALSA is the algorithm only ranks spectra based on correspondence to search characteristic(s) used, but does not interpret or annotate spectra. Thus, the user must evaluate all spectra manually for quality assurance and validation.
P-Mod is a scoring algorithm designed to match peptide sequences to MS-MS spectra (65). P-Mod matches peptide sequences, as designated by the user, to MS-MS spectra by doing the following: First, it estimates the mass difference between a peptide sequence and the precursor ion for each MS-MS spectrum. Next, it generates an array of all possible $b$- and $y$- fragment ions for the peptide(s) entered by the user with mass shifts localized at each possible position. P-Mod then compares spectra to each array element and calculates a raw score for all spectra matched with search sequences. This score is based on criteria such as mass accuracy, correspondence of spectral signals to predicted $b/y$ ions, and geometric mean of intensities of matched ions. Then P-Mod assigns a mass and sequence position of modification. Finally, P-Mod reports a p-value to estimate the probability of a false-positive match. This feature along with links to the original spectrum from which a match is found allows users to quickly verify all reported matches. This particular tool has greatly expedited the process of mapping protein adducts in our laboratory and was used extensively in these studies.

**Quantitative Mass Spectrometry**

Discovery of protein modification leads immediately to questions regarding the extent of modification and its relationship to a specific consequence. These questions are essential to consider when addressing rate of protein adduction causing deviations from normal cell states. Thus, quantification of certain modified and unmodified cellular components in various cell states is essential to describe effects. In the case of protein adduction, one could use this information to address questions such as *how much*
adduction is required to cause significant changes in a cell’s phenotype. One may also wish to measure differences in protein expression in a stressed condition relative to a normal condition? Methodology for quantitative measurements has been developed for gene expression analysis with the use of microarray technology (66) and rt-PCR (67) to quantify messenger ribonucleic acid (mRNA) at any given “test” state (disease, treatment, etc.) relative to control. Similarly, MS analysis provides standard methods for quantification that have been used for small molecule analysis for over 25 years. These approaches involve isotope labeling and have been adapted to analysis of proteins and proteomes, the end products of mRNA translation. Quantifying proteomes provides a compliment to transcriptome profiles.

Quantification in proteomics is generally carried out through the use of stable isotope tagging (68). This methodology uses small molecules to label or “tag” proteins or peptides for analysis. These tags usually come in native (“light”) and labeled (“heavy”) forms via labeling with isotopically enriched reagents (Table1-1). Some of the most commonly used isotopes include $^2$H, $^{13}$C, $^{15}$N, and $^{18}$O, which can be incorporated onto a protein before digestion or onto peptides post-digestion. They may also be incorporated into proteins metabolically in cell cultures using media enriched with isotope-labeled amino acids or by carrying out a protein digestion in H$_2^{18}$O. An advantage to incorporating labels metabolically is the ability to label virtually every protein in a cell system. A limitation of metabolic labeling is that specific labeled amino acids such as leucine or isoleucine, though common, may not appear in every peptide following digestion. The solution to this could be carrying out protein digestion in $^{18}$O-
<table>
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<th>Formula</th>
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<td>N-terminal</td>
<td>Phenyl Isocyanate</td>
<td>C&lt;sub&gt;7&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;NO</td>
<td>13C&lt;sub&gt;6&lt;/sub&gt; or 2H&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>13C&lt;sub&gt;6&lt;/sub&gt; and 15N</td>
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<td>Cysteine</td>
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<td>C&lt;sub&gt;22&lt;/sub&gt;H&lt;sub&gt;39&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;SI</td>
<td>2H&lt;sub&gt;8&lt;/sub&gt;</td>
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<td>Tryptophan</td>
<td>2,4-dinitrobenzene-sulfenyl chloride</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;NO&lt;sub&gt;2&lt;/sub&gt;SCl</td>
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<td>Methanol</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;OH</td>
<td>2H&lt;sub&gt;3&lt;/sub&gt;</td>
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**Table 1-1:** Examples of commonly used small molecule, isotope-labeled tags for protein and/or peptide quantification, indicating residues specific for each tag, isotopes used, and its mode of incorporation. *Tags used for labeling of aspartate are also label the C-terminal of proteins/peptides.
labeled water. This technique incorporates one or two labeled oxygens at the C-terminus of each product peptide during the digestion reaction. Another disadvantage of metabolic labeling is the high costs of isotope-labeled compounds. Finally, metabolic labeling is restricted to cell models and *in vivo* studies with simple model organisms.

Small molecule isotope tags can be incorporated into proteins and peptides non-metabolically via simple chemical reactions. In a majority of cases these labeling procedures are designed to target specific amino acids and post-translational modifications and can be used to exploit specific residues on proteins or to isolate specific modifications for quantitation. These small molecule tags are used in both isotope-labeled and unlabeled forms for labeling separate samples such as test and control. This way samples can be combined and easily distinguished via mass spectrometry and relative quantitation can be carried out. Other examples of selective tags used in protein quantification include labeling of the N-terminus with phenyl isocyanate (PIC) (69), esterification of aspartate, glutamate, and the C-terminus with methanol (70), labeling of cysteine residues with either alkyl halides or N-alkylmaleamide compounds (71), lysine tagging with O-methylisourea and imidazole derivatives (72;73), and even targeting tryptophan residues with 2-nitrobenzenesulfenyl chloride (NBSCI) (74). The major drawback of reagents targeted to specific amino acids is they may not be present in all proteins or peptides.

Tagging by this method has proven to be useful in studying the functionality of many of these important amino acid residues in relation to protein function, as well as significance of several post-translational modifications. One example is analysis of
function of cysteine residues, which tend to be quite abundant in many proteins and serve important functions in protein structure and activity. Oxidized cysteine residues can form disulfide bonds, which are crucial elements in a protein’s secondary structure. Disulfide bonds can serve to join two subunits or monomers of a large protein complex or connect different domains of the same protein, thus contributing to three-dimensional structure. Reduced cysteines are also found in many proteins and essential for catalytic or regulatory function. These residues are susceptible to oxidation or alkylation and therefore serve as signaling residues to increase or decrease protein activity. A recent study used isotope-coded affinity tags (ICAT) to distinguish and quantify oxidant-sensitive thiols in complex mixtures \(75;76\) from samples treated with oxidants such as hydrogen peroxide.

Another recent study demonstrated how this tagging technique can be designed to quantify a specific post-translational modification, O-linked N-acetylglucosamine (O-GlcNAc) \(77\). This modification occurs abundantly on serine and threonine residues in cytosolic and nuclear proteins, but whose function remains largely unknown. An azide analog of O-GlcNAc was used to metabolically label and chemoselectively conjugate with a biotinylated phosphate reagent. Addition of a biotin tag allowed for enrichment of modified proteins via affinity capture with immobilized avidin, followed by LC-MS-MS analysis. Modified proteins were detected and quantified.

The quantification methods described above have proven to be effective for relative quantification, in which levels of one species are compared to levels of some standard or control. However, some studies require absolute quantification, to accurately
measure the amount or concentration of a particular protein. Absolute quantification is achieved by generating calibration curves for target peptides at different concentrations compared to a constant amount of labeled standard spiked into each sample. MS analysis provides signals from which integrated peak area ratios of test peptides to the corresponding standard is obtained, which can be compared to the calibration curve to determine the absolute amount of the peptide of interest. A recent study using this technique for absolute quantification (termed AQUA) demonstrated that proteins can be quantified using stable isotope-labeled synthetic peptides as internal standards (78) (Figure 1-4). These synthetic standards can be synthesized with covalent modifications, such as phosphorylation in order to quantify post-translational modified proteins. Sensitive, selective detection of the peptides and standards is done with selected ion monitoring (SIM) and multiple reaction monitoring (MRM) in LC-MS-MS. These methods use MS instruments to selectively scan for specific precursor ions or fragment ions based on specific m/z values. This AQUA strategy has been used in a study to quantify biomarkers in patients with rheumatoid arthritis (79).

Software tools have been developed to make data acquisition and analysis for this purpose more efficient. Several groups in the area of proteomics have developed software tools for quantitative analysis from MS-MS data. One example is work from the group of Ruedi Abersold, who developed the software programs XPRESS and INTERACT to quantify and organize peptides identified by the SEQUEST sequence database searching software (80). The first of these tools was designed to isolate deuterium-labeled and unlabeled peptide elution profiles (peptides labeled with the
Figure 1-4: AQUA strategy for absolute quantification of proteins (and in this scheme phosphoproteins). In stage 1, peptides are selected and synthesized for use as internal standards. Standards may be modified or unmodified, and during synthesis, stable isotopes are incorporated ($^{13}$C, $^{15}$N, etc.) at a single amino acid such as leucine shown above with *. Standards are analyzed by MS-MS to examine fragmentation patterns of labeled and unlabeled peptides in order to create a SRM method for analysis of test samples. Stage 2 shows how proteins are harvested from a biological sample, followed by incorporation of the synthesized internal standards. LC-MS-MS is then performed where test peptides are differentiated from internal standards and quantified from their chromatographic peaks (Gerber, S., et al, (2003) PNAS, 100(12); 6940-6945).
group’s published ICAT reagents (71), determine the area of each peptide peak and calculate the abundance ratio based on these areas. The INTERACT software then allows the researcher to sort the data based on user defined criteria (identity, quantity, localization, function, etc.). Another tool from the group of John Yates, is RelEx (Relative Expression) which automatically performs function of peak detection peptide ratio calculations, and estimates protein ratios (57) based on corresponding isotope-labeled and unlabeled peptides. A major advantage of this program is that it has been refined to generate chromatograms directly from MS-MS spectra rather than MS scans (81), which provides higher signal-to-noise peptide and protein quantification.

Comparing Multiple Protein Adduction Targets on the Same Protein

A recent study in our laboratory identified nuclear and cytosolic protein targets of thiol-reactive electrophiles (39). In this study, both nuclear and cytosolic fractions of HEK293 cell lysates were reacted with either of two thiol-specific model electrophiles in an attempt to identify potential targets of these compounds. The study identified and mapped 897 adducts to specific cysteine residues on 539 proteins. Adduction was shown to be selective and reproducible, with >90% of all adducted proteins being modified at only one or two sites (Figure 1-5). While this percentage is high, a significant number of proteins nevertheless are modified at multiple sites. These could potentially represent protein targets that play significant roles in important cellular functions. When proteins are adducted at multiple sites, new questions arise regarding consequences of adduction on the protein in question. What are the relative reactivities of multiple target sites on the
Figure 1-5: Results of a study showing (A) the selectivity of protein adduction between two model electrophiles in protein sample taken from HEK293 cellular extracts, and (B) how a large majority of adducted proteins are adducted at only one or two sites (Dennehy, M., et al, (2006) Chem. Res. Tox., 19(1); 20-29).
same protein? Is one of the modified sites more critical to a biological effect than the other(s)? What factors make certain sites more susceptible to adduction than others? Do differences hold for different electrophiles? Adduction at multiple sites on the same protein potentially extends the significance of adduction from one adduction target equals one downstream effect to multiple adduction targets equal multiple downstream effects.

An example of this dynamic is protein phosphorylation. Many proteins whose activity is regulated by phosphorylation are modified at multiple sites. In many of these cases, multiple phosphorylations are not only possible, but are necessary for complete activation of that protein. This is most common in activation of tyrosine kinases including activation of the insulin receptor (82) and also applies to activation of the mitogen activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) (83). Although phosphorylation and many other post-translational modifications are regulated in the cell, adduction by electrophiles is governed by chemistry. Nevertheless, the same questions apply and there is a need to understand consequences of protein adduction at multiple sights on the same protein.

**GST Family of Enzymes**

The glutathione S-transferases (GST) comprise a large family of multifunctional phase II metabolic enzymes that play important roles in xenobiotic metabolism. The family of mammalian, soluble GSTs is further subdivided into at least eight distinct isoforms or classes designated alpha, mu, pi, theta, sigma, kappa, zeta, and omega with an additional microsomal form. These enzymes are cytosolic and widely distributed
throughout all tissues, with the liver having the highest concentration and pi being the most abundant extra-hepatic isoform. Cytosolic forms exist as either homo- or heterodimeric species. Heterodimers form only between species of the same class. For example, members of the alpha class may form dimers with identical or different subunits of the alpha class but not with members of the mu or pi classes. This provides the basis for GST nomenclature where GSTP1-1 would designate a homodimer of type 1 subunits of the pi class, where a heterodimer of type 1 and type 2 subunits of the pi class would be designated GSTP1-2 (84). Sequence comparison of the different classes of GSTs show subunits within the same class are as much as 70% homologous, whereas subunits across different classes are only up to 40% homologous, which would explain the formation of dimers between members of the same class. In addition, three dimensional crystal structures have been determined for members of each of the eight mammalian classes (85-92).

The phase II metabolic role of GST enzymes is fundamentally different than those of other phase II enzymes. The main activity of GSTs is catalyzing conjugation of glutathione (GSH), a tripeptide consisting of γ-glutamylcysteinylglycine, to xenobiotics. Substrates for GSH conjugation include a huge array of electrophilic xenobiotics, or xenobiotics that can be biotransformed to electrophilic species (Figure 1-6). This is in contrast to other phase II metabolism (eg., conjugation with amino acids, acetylation, glucuronidation, and sulfation) where conjugation occurs on a nucleophilic site either present in the parent molecule or exposed during phase I metabolism. Many substrates for GSH conjugation are hydrophobic, contain an electrophilic site, and are able to react
Figure 1-6: Examples of GSH conjugation reactions catalyzed by GST. These include (top) direct conjugation by displacement of an electron withdrawing group (alkyl halides), (middle) direct conjugation by addition of GSH (alkenes, quinones, and a,b-unsaturated carbonyls), and (bottom) conjugation of a strained ring system (epoxides, epi-sulfonium ions).
nonenzymatically with GSH at some measurable rate (11). Some endogenous substrates metabolized by GSTs include α,β-unsaturated keto prostaglandins and products of fatty acid oxidation (PGA2, PGJ2, HNE), cholesterol and cholesterol derivatives, quinones of catecholamines, catechol estrogens, and maleylacetoacetate. A large array of exogenous substrates for GST enzymes include drugs (cisplatin, nitroglycerine, and ethacrynic acid), pesticides (DDT, methyl parathion, and lindane), and other environmental toxicants/carcinogens (styrene oxide, acrolein, trichloroethylene) (93). Examples such as these help illustrate the important role of GSH conjugation preventing these compounds, or their reactive intermediates, from adducting major cellular macromolecules, such as protein and DNA, which could lead to serious consequences.

In addition to its well-established enzymatic functions, the GST family of enzymes has been linked to additional regulatory functions independent of its metabolic activity. These represent novel functions for what was once thought to be a relatively simple enzyme that catalyzed a simple reaction. One of the most studied of these regulatory functions is regulation of mitogen- or stress-activated protein kinases (MAPK/SAPK). Two classes of the GST family have been shown to act as key regulators of a signal cascade in this group of kinases. The first is the pi class, where GSTP1-1 has been shown to bind and negatively regulate c-Jun N-terminal kinase 1 (JNK1) (94). Similarly, GSTM1-1 has been shown to bind and repress activity of apoptosis signal-regulating kinase 1 (ASK1) (95). These proposed mechanisms represent interesting, yet unproved functions for these proteins. The significance of these actions of GSTs remains a subject of active investigation. Previous work on the GSTP1-1-JNK
interaction has suggested that GST binds to JNK, but is dissociated upon stress, resulting in JNK activation (94;96). Additional evidence continues to be brought forth illustrating mechanisms of regulation of JNK signaling by GSTP1-1 (97;98).

Besides regulation of stress kinases, GST enzymes have been shown to play key roles in additional cellular functions. Evidence has been presented identifying GST O1-1 as a target of cytokine release inhibitory drugs and this interaction may be significant in the ability of these drugs to arrest stimulus-induced interleukin-1β posttranslational processing (99). Isoenzymes of the GST alpha class have been shown to exhibit additional glutathione peroxidase activity that plays a protective role against lipid peroxidation (100). GSTs of the zeta and sigma classes show additional activities in endogenous metabolic pathways. The zeta class of GST enzymes are also isomerases that catalyze the cis-trans isomerization of maleylacetocaceton (MAA) or maleylacetone (MA) to fumarylacetocaceton (FAA) or fumarylacetone (FA), respectively in the tyrosine degradation pathway (101;102). Sigma class GSTs are well known for their role in prostaglandin metabolism as a GSH-dependent prostaglandin D2 synthase (103). GSTP1-1 has also recently been shown to possibly have an interesting role as a nitric oxide (NO) carrier through its interactions with S-nitrosoglutathione and the dinitrosyl-diglutathionyl complex, two compounds involved in the storage and transport of NO in biological systems (104). In addition, GSTP1-1 has been shown to be overexpressed in several different cancers (105) and has been thought to play a major part in drug resistance to chemotherapy in cancer patients (106;107).
Characterization of adduction of the GST family of enzymes to this point has remained a rather small area of interest. Work by the M.W. Anders group has mapped target sites and characterized their effects on human GSTZ1-1 (108;109). Besides these studies, the pi class of GSTs has been by far the most extensively studied with respect to protein adduction. This could be mainly due to this class as being the most abundant extrahepatic GST enzymes, as well as its diversity of functions in physiological systems besides catalyzing GSH conjugation reactions. GSTP1-1 has been shown be a target of a variety of alkylating agents in their native form or through their metabolic activation to reactive electrophilic species. Some of these include flavenoids (110) and other quinone compounds (111), α,β-unsaturated aldehydes and ketones (112), and estrogens and estrogen metabolites (113;114). All of these studies indicated four cysteine residues in the GSTP1-1 sequence (Cys-14, 47, 101, and 169) (Figure 1-7) as sites susceptible to adduction by these alkylating agents and ranked their relative reactivities as 47>14≈101>169. Furthermore, all results lead to the conclusion that Cys-47 is a key residue in GSTP1-1 activity and that alkylation of this residue results in inhibition of enzymatic activity, while alkylation at Cys-14 appeared to inhibit activity even further. These results were all verified using site-directed mutagenesis of all four cysteine residues demonstrating the potential consequences of alkylation of this enzyme as well as demonstrating the benefits of better understanding the dynamics of protein adduction to identifying and mapping protein targets with multiple alkylating agents and comparing multiple sites of adduction on the same protein.
Figure 1-7: Three-dimensional structure of the GSTP1-1 dimer, showing the four cysteine residues that have been shown to be susceptible to adduction by various alkylating agents.
The MAPK Signaling Pathways

Mitogen-activated protein kinases (MAPK) are a family of enzymes that play critical roles in regulating cell responses to changes in their environment. Some of these changes include growth factors, cytokines, and changes in vital nutrients. Additional environmental factors that perturb cell function include changes in temperature, pH, redox state, and osmolarity as well as exposure to radiation and xenobiotics. Many of these exposures lead to stress which triggers either defensive adaptations to promote survival or toxic injury. These responses are regulated by signaling networks that include the families of MAPK enzymes.

MAP kinase signaling has been characterized in simple eukaryotic cells such as the yeast *Saccharomyces cerevisiae* and in mammalian cells with parallels being drawn between the two systems (115). Like many kinase enzymes, MAPKs are regulated through phosphorylation by one or more upstream kinases. MAPKs are phosphorylated by MAPK kinases (MAPKK), which themselves are phosphorylated by MAPKK kinases (MAPKKK). These organizational patterns are referred to as a kinase signaling cascades whose structural organization is carried out by scaffold proteins (116), whose functions are to bind members of kinase modules and co-localize them within cells (Figure 1-8). These scaffolds provide spatial and stimulus-specific regulation of MAPK function. Activation of all MAPKs occurs via dual phosphorylation of a conserved tripeptide motif (Thr-Xaa-Tyr) located in a region of the enzyme known as the activation loop or T-loop. Kinase activity is then down-regulated by dephosphorylation by a variety of phosphatase enzymes, which not only inactivate kinases following responses to stimuli, but also insure
Figure 1-8: Example showing organization of MAPK cascades by scaffold proteins. These examples show organization of JNK cascades by JNK-interacting protein (JIP), filamin, β-arrestin, JNK/stress-activated protein kinase-associated protein 1 (JSAP1), and p130Cas. These proteins provide organization to individual scaffolds to ensure proper propagation of signals (Manning, A. and Davis, R., (2003) Nature Rev. Drug Disc., 2; 554-565).
minimal levels of basal kinase activity in unstimulated cells and are specific for the kinase families they inactivate. MAP kinases are inactivated by phosphatases referred to simply as MAP kinase phosphatases (MKP) \((117)\). Three major groups of MAPKs identified in mammalian systems include p38, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinases (JNKs) or stress-activated protein kinase (SAPK) \((118)\). It is the JNKs that will be the main focus of my work described here.

**The JNK Signaling Pathway**

The JNK family of MAP kinases comprises a group of enzymes that have been shown to play several important and diverse roles in regulating diverse cellular functions. Currently, ten different JNK isoforms are known to be derived from alternative splicing of mRNA transcripts from three distinct genes: JNK1, JNK2, and JNK3 \((119)\). JNK1 and 2 isoforms are ubiquitously expressed in virtually all tissues, whereas JNK3 isoforms appear to have a limited expression largely restricted to the brain, heart, and testes. Activation of JNK occurs in cells treated with inflammatory cytokines (TNF and IL-1) as well as through cellular exposure to environmental stressors including heat and osmotic shock, redox stress, and radiation \((120)\). Once activated, JNK primarily phosphorylates the AP-1 transcription factor c-Jun on two serine residues (Ser-63 and Ser-73). It also phosphorylates other members of the AP-1 group (JunB, JunD, and ATF2) as well as additional transcription factors that include p53, ELK-1 and c-Myc \((121)\) (Figure 1-9). In addition, JNK also phosphorylates non-transcription factors of the Bcl-2 family (Bax, Bak) \((122;123)\). This diversity of targets helps explain how JNK activity influences key
Figure 1-9: Schematic of the JNK signaling cascade. As one can see, JNK activation can be initiated by different stressors and a variety of upstream MAPKKK. Activation of JNK then leads to phosphorylation of an equal diversity of downstream targets, the primary being c-Jun, a component of the AP-1 transcription factor (*Manning, A. and Davis, R.,* (2003) *Nature Rev. Drug Disc.*, 2: 554-565).
cellular functions such as growth, transformation, and apoptosis while playing key roles in different disease states, that include inflammatory (rheumatoid arthritis), neurodegenerative (Alzheimer’s), metabolic (diabetes) and even many types of cancer (124).

Mechanistically, JNK is activated in the same fashion as other MAP kinases, through dual phosphorylation of the conserved tripeptide motif on its activation or T-loop. In JNK, this tripeptide Thr183-Pro-Tyr185 is phosphorylated by two upstream MAPK kinases (MAPKK), specifically MKK4 (125) and MKK7 (126). An interesting study showed how these two kinases show selectivity as to which residue they phosphorylate, with MKK4 being selective for the tyrosine residue and MKK7 being selective for the threonine residue leading to the conclusion that MKK4 and MKK7 produce a synergistic increase in JNK activity far greater than monophosphorylation at either site by itself (83). A closer comparison of these two MAPKKs shows additional differences in their behavior. While both are activated in response to environmental stressors, only MKK7 is activated by tumor necrosis factor-alpha (TNF-\(\alpha\)) (126). Additionally, MKK7 is selective for activation of JNK enzymes, whereas MKK4 can activate both JNK and p38 kinases of the MAPK superfamily (121). Activation of JNK kinases is mediated by several different MAPKK kinases (MAPKKK) that initiate these JNK signaling cascades. There are known to be several different MAPKKKs expressed in various tissues, which can eventually lead to JNK activation. These include members of the MEKK group (MEKK1 through 4), the mixed-lineage protein kinase group (MLK1 through 3, DLK, and LZK), the ASK group (ASK1 and
ASK2), TAK1, and TPL2 (121). As is the case with other MAPK cascades, JNK signaling modules are organized by scaffold proteins. Five groups of potential scaffolds for JNK signaling have been reported: JNK-interacting protein (JIP), filamin, β-arrestin, JNK/stress-activated protein kinase associated protein 1 (JSAP1), CrkII, and p130Cas (124;127). Termination of JNK activation is achieved via dephosphorylation catalyzed by members of a large family of dual specificity protein phosphatases (117). This family of MKPs forms a negative feedback loop in the regulation of MAPKs and these enzymes are tightly regulated by substrate-induced activation and auto-inhibition mechanisms (128). All of these factors taken together allow for multiple mechanisms of extracellular activation and multiple effects of JNK activation.

Kinetics of JNK activation through these cascades has been shown to be a biphasic process, where initially JNK is activated quickly (within 30 min) with a large transient increase in activity followed by a more sustained phase of activation (1-6 hrs following exposure) that can last for up to several hours (Figure 1-10). This time course of activation also contributes to JNK functions governing cell survival and apoptosis (129). This information collectively indicates JNK activation and downstream effects depend on three main factors: 1) the nature of the stimulus, 2) the cell or tissue type, which determines which members of the JNK cascade are expressed, and 3) the duration of the stimulus. These facts show the overall complexity of JNK activation as well as the evidence for diverse and in some cases contrasting functions and cellular responses to JNK activation. In a way one might say JNK does not tell cells what to do but rather cells are interpreting JNK signals based on several contributing factors.
Figure 1-10: Western blot and corresponding graph depicting the biphasic activation of JNK following treatment with TNF-α. The plot shows two distinct phases of JNK activation, each thought to play different roles in JNK-regulated apoptosis (Ventura, J. et al., (2006) Molecular Cell, 21; 701-710).
Significance of JNK Signaling

JNK signaling plays important roles in several different cellular functions and has been shown to have significant functions in many disease states. Because of these roles, JNK and other JNK related proteins have been given close scrutiny in recent years as potential drug targets. Examples include autoimmune and inflammatory diseases such as arthritis, multiple sclerosis, asthma, inflammatory bowel disease, and psoriasis. These diseases involve improper activation of the immune system that leads to overproduction of immune cells (monocytes, mast cells, and T-cells), which express genes such as inflammatory cytokines (TNFα, IL-2), and tissue destructive enzymes (matrix metalloproteinases (MMPs), and collagenases). These genes are regulated through JNK signaling by the activation of transcription factors AP-1 and ATF-2 (130).

JNK activity also is dysregulated in neurodegerative diseases, which include Alzheimer’s and Parkinson’s disease, as well as metabolic diseases such as obesity and diabetes. An altered distribution and activation of the three JNK genes has been observed in post-mortem brain sections of patients with Alzheimer’s disease, suggesting a significant role with pathogenesis (131). Additionally amyloid precursor protein (APP) is a target of phosphorylation by JNK3, demonstrating possible regulation of this protein implicated in Alzheimer’s development (132). Recent studies have also shown that JNK activity is abnormally elevated in obesity, can interfere with insulin action, and is activated by inflammatory cytokines and free fatty acids, molecules that have been implicated in development of type-2 diabetes (12). These studies also show that in
different models of mouse obesity, absence of JNK1 results in decreased adiposity, improved insulin sensitivity, and enhanced insulin receptor signaling capacity (12).

Another disease state where JNK signaling has been shown to play a significant role is cancer. Evidence suggests that JNK activation and c-Jun phosphorylation are required for transformation induced by Ras, an oncogene mutationally activated in almost 30% of all cancers (133). In addition, several tumor cell lines have been reported to possess constitutively active JNK, suggesting a role in tumor development (120). These data, together with a study in mouse fibroblasts possessing a mutated c-Jun, which lacks the two JNK phosphorylation sites are resistant to transformation by active Ras and Fos (134) confirm the necessity of JNK activity in cancer development. However, the role of JNK in cancer development is not that simple. Fibroblasts isolated from mice lacking JNK expression were transformed by Ras and showed increased size and numbers of tumors and tumor modules in mice injected with these cells (135). These results suggest enhanced tumor formation due to the absence of JNK induced apoptosis. Apoptosis is a major cellular event that contributes to tumor suppression, thus suggesting that JNK can induce apoptosis through phosphorylation of pro-apoptotic members of the Bcl-2 family (Bax and Bak). This leads to cytochrome c release from the mitochondria, apoptosis and could account for actions of JNK as a tumor suppressor (136). Therefore, activity could promote or suppress tumor development in different settings or different tumor types.

**JNK Signaling and Apoptosis**
Probably the most complex issue surrounding JNK activity is its role in apoptosis, which has been a topic of great interest in the area of MAPK signaling. There have been many reports of JNK playing a role in both pro- and anti-apoptotic events. Results such as these have led to questions of whether JNK is actually an intrinsic component or just a modulator of apoptosis. As mentioned earlier, these questions are made more complex by the fact that JNK activation kinetics seems to have a role in determining its apoptotic functionality, where early phase transient activation being involved in cell survival functions, while a late, more suspended activation phase being implicated in apoptosis signaling (129). Again, multiple factors such as stimulus, cell type, which components of the JNK cascade are activated, and whether other MAPK cascades contribute to determine if JNK activity is destined to act as a pro-apoptotic modulator, or contribute to a cell’s survival response.

**Pro-Apoptotic Effects**

Initial reports showing JNK as being involved in apoptosis came from studies in neuronal cells. Apoptosis in cultured sympathetic neurons, induced by nerve growth factor (NGF) withdrawal was blocked following injection of neutralizing antibodies against c-Jun or overexpression of a c-Jun mutant lacking its N-terminal transactivation domain (137). Overexpression of wild-type c-Jun in these cultured cells induced apoptosis (137). Since this study, the JNK pathway has been implicated in apoptosis of other cell types. Most convincing is evidence from mouse embryonic fibroblasts (MEFs) deficient in both JNK1 and JNK2 isoforms (138). These cells were shown to be resistant to apoptosis induced by UV irradiation, which suggested JNK involvement in this
process. In contrast to observations in neurons, the pro-apoptotic role of JNK in UV-induced MEF apoptosis appeared to be independent of c-Jun-mediated transcription, since it was not affected by the protein synthesis inhibitor cycloheximide or the mRNA synthesis inhibitor actinomycin D. However, in a separate study, MEFs isolated from mice in which c-Jun is replaced by a non-phosphorylatable c-Jun$^{AA63/73}$ mutant were shown to be insensitive to UV-induced cell death (139), suggesting c-Jun phosphorylation and activation by JNK are in fact required for UV-irradiation-induced cell death.

**Anti-Apoptotic Effects**

Evidence supporting an anti-apoptotic role of JNK is just as compelling as that supporting its pro-apoptotic roles. Mice lacking c-Jun showed no defect in neuronal apoptosis and many stimuli that activate c-Jun did not induce apoptosis in neurons (140). This latter finding is also true of stimuli that have been shown to activate JNK, they don’t all induce apoptosis. Other studies have shown that apoptosis is increased in hindbrain and forebrain regions of JNK1/2 knockout mice (141), suggesting a requirement for JNK in cell survival in these brain regions during development. In certain tumor cells, JNK also functions as an anti-apoptotic module. Probably the most interesting observation of the anti-apoptotic activity of JNK in tumor cells is its relationship with the tumor suppressor p53. Inhibition of JNK by specific antisense oligonucleotides inhibits cell growth in certain p53-deficient, but not p53-positive tumor cells (142). It has since been reported that activation of the JNK pathway can inhibit p53 induced cell cycle arrest and promote p53 induced apoptosis (143). This would explain why JNK only exerts its anti-
apoptotic functions in p53 deficient tumor cells. Again, these results indicating dual pro- or anti-apoptotic roles suggest that JNK is more of a modulator rather than an intrinsic component of apoptosis.

What exactly then is the role of JNK in apoptosis? A recent review by Anning Lin takes a close look at the role of JNK in apoptosis (144). Lin also carefully considers many of the determining factors involved in JNK activation and its apoptosis-related roles described above. First, components of the JNK signaling pathway can have opposing effects on the apoptotic process. Previously mentioned were studies indicating that JNK1 and JNK2 deficient mice showed defects in neuronal apoptosis during development (138), while mice lacking c-Jun did not exhibit this phenotype (140). This was interpreted as suggesting that the pro-apoptotic roles of JNK1 and JNK2 in neurogenesis are mediated by a downstream effector other than c-Jun. These observations lead to assumptions that individual components of the JNK pathway could affect apoptosis through interaction with other cellular signaling effectors. Along these lines is the possibility of different JNK isoforms playing distinct roles in apoptosis. Mice deficient in JNK1 and JNK2 had defective apoptosis in certain brain regions during development (141) while a separate study showed mice lacking JNK3 exhibited normal development (145).

Other key factors in determining the role of JNK in apoptosis are the nature of the death signal and the cell type. Again, JNK1 and JNK2 deficient mice were resistant to apoptosis induced by UV irradiation, anisomycin, or MMS, but were still susceptible to
anti-Fas antibody (138). This is also observed in JNK1 deficient thymocytes and peripheral T cells, which were very susceptible to apoptosis mediated by Fas/CD95 and CD3, but not to other stressors such as UV and γ-radiation, dexamethasone, anisomycin, and heat shock (146). So while JNK can be activated by a variety of death signals, only certain signals utilize the JNK pathway to induce apoptosis in a cell type-dependent manner.

The remaining factors that appear to play critical roles in determining the role of JNK in apoptosis are the duration of the death signal and the interaction of JNK with other signaling pathways. These factors were analyzed together in a previously mentioned study of JNK activation kinetics following cellular treatment with TNF-α (129). This study demonstrated a biphasic increase in JNK activity, with contrasting functions of each phase. First, the early transient phase appeared to be involved in cell survival signaling, and is dependent on JunD and cooperation with NF-κB and Akt signal transduction pathways. These investigators proposed that JNK-induced AP-1 activity provides a cooperative effect to increase expression of anti-apoptotic genes by the NF-κB and Akt pathways, which needs to occur early in the stimulus response. The second, late-phase of JNK activation appears to mediate pro-apoptosis signaling and requires inhibition of the NF-κB pathway. This process is thought to involve JNK as a positive regulator of TNF-α-induced production of reactive oxygen species (ROS) sufficient to overcome a threshold needed to induce cell death. In addition, sustained activation of JNK may also be required as a consequence of the expression, phosphorylation, and
complex interactions of pro- and anti-apoptotic members of the Bcl-2 family of proteins, whose expression and activation states (via phosphorylation) are regulated by JNK (147-150). These studies provide some useful explanations and hypotheses for how JNK can act as both a pro- and anti-apoptotic regulator.

**Definition of the Problem and Specific Aims of this Research**

Protein adduction is a significant contributing factor to mechanisms of toxicity. Further study in identifying relevant alkylating agents and susceptible protein targets will provide a clearer understanding of cellular consequences of protein damage. The ability of new analytical methods to pinpoint sites of adduction and quantify specific adducts have become valuable tools for evaluating selectivity of adduction as well as understanding specific downstream effects of change to identified protein targets. My work encompasses many of these aspects of studying protein adduction in cellular systems and attempts to build on current knowledge through method development and evaluation of the effects of protein adduction on an important cellular signaling pathway.

This dissertation research addresses two problems. First, the ability to simultaneously compare and contrast multiple sites of adduction on the same protein can provide identification of key residues that may serve as sensor triggers in activating specific responses, such as protein degradation and turnover, gene expression, or any other type of cellular signaling mechanisms including protein and cell functionality and
even death. A recent study from our laboratory identified potential nuclear and cytosolic targets of thiol-reactive electrophiles (39). Although a majority of identified proteins were found to be adducted on only one site, many identified target proteins contained multiple sites of adduction. The first part of my project addresses the development of quantitative MS methods enabling the simultaneous analysis of the reactivities of multiple targets on the same protein. This new methodology involves the use of a small molecule, isotope-coded tag for labeling the N-terminal of all peptides in a complex mixture following enzymatic digestion, previously developed for protein quantification in our laboratory (69). The initial goal of my work was to adapt stable isotope tags to determine rate constants \((k)\) for different alkylating reactions on multiple protein sites. This can provide means to better understand the nature of protein adduction as well as a better understanding of specific cellular effects of adduction. We believe protein adduction is selective for specific proteins and specific sites on protein targets, and this selectivity is directly related to specific downstream consequences. GSTP1-1 was selected for study both because it has been well-established as a target of electrophile adduction at multiple sites as well as because of its potential relevance to MAPK signaling in stress.

The second problem to be addressed is to understand mechanisms of activation of cellular signaling systems in response to protein alkylation. This work focuses on the JNK signaling pathway. The role of JNK in apoptosis is of intense interest to researchers in the field of JNK signaling. A fundamental, unanswered question is how protein adduction activates a signaling cascade, particularly JNK. This project proposes to
identify which, if any of these targets are susceptible to protein adduction by different alkylating agents, and attempt to understand if alkylation of these targets leads to JNK activation. This work also addresses how alkylation of JNK related targets contributes to its balance of pro-and anti-apoptotic roles. We believe the selective nature of protein adduction translates into specific cellular responses that include activation of the JNK signaling pathway.

In order to gain a more concise understanding of the kinetic nature of protein adduction and to gain an understanding of specific responses following protein adduction in cells, the specific aims of my dissertation work are as follows:

1. Develop new techniques to compare protein adduction kinetics on multiple sites on the same protein using quantitative mass spectrometry. This methodology will provide means of determining a rate constant ($k$) to compare not only reactivities between different sites on the same protein but also compare differences in reactivity between different alkylating agents.

2. Identify potential protein targets for alkylation related to regulation of the JNK signaling pathway and determine if these correspond to JNK activation in HEK293 cells and compare these results between two model electrophiles. These results can help in understanding how protein adduction selectivity leads to specific effects that include JNK activation and cell death.
CHAPTER 2 – ANALYSIS OF PROTEIN ADDUCTION KINETICS BY QUANTITATIVE MASS SPECTROMETRY. COMPETING ADDUCTION REACTIONS OF GLUTATHIONE S-TRANSFERASEP1-1 WITH ELECTROPHILES

Introduction

GST enzymes play a critical role in xenobiotic metabolism. GSTs function by catalyzing the conjugation of glutathione with electrophilic substrates, increasing their water solubility and allowing for their excretion. Cytosolic GSTs include at least six individual isoforms (alpha, mu, pi, theta, kappa, and sigma) in mammals. This family of abundant enzymes is widely distributed in virtually all tissues, which demonstrates their importance in chemical detoxification (84). These enzymes also are over expressed in several different cancers and are believed to play a role in tumor resistance to chemotherapy (105).

In addition to their roles in xenobiotic metabolism, recent studies suggest important secondary functions of GST enzymes. These functions derive from the interaction of GSTs with other proteins. Murine GSTp and GSTm interact with c-Jun N-terminal Kinase-1 (JNK1) (94) and apoptosis signal regulating kinase-1 (ASK1) (95), respectively. These interactions inhibit the activity of these mitogen activated protein kinases and dissociation of GST-kinase interactions activate kinase-regulated transcription factors (c-Jun, ATF2, p53, ELK-1, and c-Myc) and non-transcription factors
of the Bcl-2 family (94). This activity of GST enzymes is hypothesized to regulate important cellular functions such as growth, transformation, and apoptosis.

Detoxification of reactive electrophiles by GST prevents adduction of nucleophilic protein residues (84;151). Formation of adducts results in damage that affects protein structure, function, and turnover. These changes contribute to chemical toxicity and disease processes. Protein adduction may also perturb protein-protein interactions and elicit significant changes in cellular signaling that contribute to toxicity. An understanding of the mechanics and consequences of protein adduction is crucial to understanding the role of reactive intermediates in toxicology.

Proteomics approaches employing LC-MS-MS together with data analysis algorithms such as Sequest, SALSA and P-Mod have enabled identification of protein adducts (1;39;60;61;65). MS-MS spectra not only identify the adducted proteins, but also pinpoint the amino acid residue where adduction occurs (60;61;65). LC-MS methods are also readily used in quantitation of proteins and protein adducts. Relative quantitation is done by incorporating stable isotope labels (2H, 13C, and 15N are most common) (68) into peptide sequences either metabolically (152) or by labeling with small molecule tags (71), which can bind covalently to specific amino acid residues or to peptide N- or C-termini. Our laboratory has described a method for relative quantitation using isotope-labeled PIC, that labels peptides at the N-terminus (69).

A major problem in the analysis of protein adduction is that multiple sites on the same protein could be susceptible to adduction by electrophilic species. Which sites are most reactive? We have adapted a stable isotope tagging method employing N-terminal
labeling of peptides with PIC and selective quantiation of the adducted, PIC-labeled peptides by LC-MS-MS. This approach allows the simultaneous measurement of kinetic constants for multiple competing adduction reactions in the same protein. This approach will be useful for understanding the selectivity and biological consequences of protein modifications involved in chemical toxicity and disease.
EXPERIMENTAL PROCEDURES

Purification and characterization of His$_6$-GSTP1-1 in vitro

Recombinant His$_6$-GSTP1-1 was expressed and purified from *E. coli* strain BL21 (DE3) (Novagen, Madison, WI). Cells were transformed with pET-His$_6$-GSTP1-1-15b plasmid generously provided by Dr. Judy Bolton at the University of Illinois at Chicago and expressed as described previously (153). Expressed protein was purified by immobilized metal affinity chromatography (IMAC) on His-select Nickel coated 96-well plates (Sigma, St. Louis, MO) using the manufacturer’s protocol. Final protein concentration was determined by BCA assay kit (Pierce, Rockford, IL). His$_6$-GSTP1-1 was identified by SDS-PAGE and Western blot techniques, using antibodies for GST (DAKO, Carpinteria, CA) and the His$_6$-tag (Quiagen, Valencia, CA).

Following elution from Ni$^{2+}$-coated 96-well plates, proteins were subjected to SDS-PAGE on NuPAGE® Novex Bis-Tris Gels (Invitrogen, Carlsbad, CA). Bands corresponding to His$_6$-GSTP1-1 were excised, and the proteins were reduced, alkylated and digested with trypsin as described previously (154). Peptides were re-suspended in 0.1% TFA for analysis by liquid chromatography-tandem mass spectrometry (LC-MS-MS). Protein digests were also carried out in the Ni$^{2+}$-coated plates. For in-plate digestions, 30 µL of a digest buffer containing 0.1 M ammonium bicarbonate (Sigma), 4 mM tris-carboxyethyl phosphine (TCEP, Pierce, Rockford, IL), and 10mM DTT (Sigma, St. Louis, MO) was added to each well and incubated for 30 min at 50° C. Then 5 µL of 200 mM IAM (Sigma, St. Louis, MO) was then added to the samples and incubated for 15 min at room temperature. For tryptic digestions prior to PIC-labeling (see below),
DTT was omitted. Trypsin Gold (Promega, Madison, WI) or chymotrypsin (Sigma, St. Louis, MO) was then added in a 1:50 w/w ratio and samples were incubated for 18-24 hours at 37° C. Following digestion, samples were analyzed by LC-MS-MS. Protein identification was done from MS-MS data using the Sequest algorithm (48) with filtering of matches based on criteria described previously (155).

GST activity was determined as previously described (112;156). The assay was carried out in the 96-well plates by diluting the protein in 230 µL of assay buffer containing 0.2 M potassium phosphate, pH 6.5, 0.2 mM EDTA, and 5 mM GSH. After incubation at room temperature for 2 min, CDNB was added to a final concentration of 1 mM and absorbance was monitored at 340 nm on a Dynex (Worthing, West Sussex, UK) 96-well plate reader over a five minute time course.

**Adduction of GSTP with electrophiles**

To study kinetics of electrophile adduction, immobilized GST proteins were incubated with various concentrations of electrophilic compounds in PBS solution pH 7.2 at 37° C. Electrophiles used for these experiments included IAB and BMCC (Figure 2-1) (Pierce, Rockford, IL) prepared in 0.1% DMSO. Each well on the 96-well plate corresponded to one replicate of a time point. Adduction with BMCC was carried out at concentrations of 5, 12.5, 16, and 25 µM for five hours with time points taken at 0, 10, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 240, and 300 min. Adduction with IAB was carried out in the dark at concentrations of 25, 50, 100, 250, and 500 µM for eight hours with time points taken at 0, 20, 40, 60, 80, 100, 120, 150, 180, 240, 300, 360, 420, and
Figure 2-1: Structures of BMCC (top) and IAB (bottom) as well as examples of how the chemistries of these two compounds are analogous with those of other toxicologically relevant compounds.
480 min. Reactions were stopped at the specified time points by removing the solution from assigned wells and washing the well quickly with 250 µL PBS. MS-MS spectra of adducted peptides were identified by analysis with Sequest (with IAB and BMCC adducts specified as variable modifications on cysteines) and P-Mod (65).

**PIC-labeling of tryptic peptides**

PIC-labeling of peptide N-termini was carried out as described previously (69) with slight modifications. Digested samples for each time point were pooled and reacted with either $^{12}$C$_6$-PIC or a heavy isotope labeled ($^{13}$C$_6$-PIC or $^2$H$_5$-PIC) (Isotec, St. Louis, MO) at a PIC concentration of 10 mM (from 0.1 M PIC stocks prepared in acetonitrile) at pH 8.0. For kinetic analyses, samples from the longest incubation time, representing maximum adduction ($t_{\text{max}}$), were labeled with the heavy PIC, while all other samples ($t_0, t_1, t_2, \ldots t_{\text{max}}$) were labeled with the light PIC (Figure 2-2). After addition of PIC, each sample was mixed for 5 sec and then allowed to stand for at least 30 sec at room temperature. Reaction was stopped by addition of concentrated formic acid to 1% (v/v). Aliquots of each time point (labeled with light PIC) were mixed with an equivalent aliquot of the $t_{\text{max}}$ (heavy PIC-labeled) sample prior to LC-MS-MS or MALDI-TOF-MS analysis.

**MALDI-TOF-MS and LC-MS/MS analysis**

MALDI-TOF-MS analysis was done on a Voyager 4700 instrument (Applied Biosystems, Foster City, CA). Samples were evaporated to near-dryness and
Figure 2-2: Reaction of PIC with peptide N-termini and an outline of the PIC-labeling procedure used for protein adduction kinetic analysis and comparison.
resuspended in 60% ACN containing 0.1% TFA. A 0.3 µL aliquot of sample was spotted on a MALDI sample target and mixed with a 0.3 µL aliquot of CHCA (Sigma, St. Louis, MO) matrix solution, consisting of 30 mM CHCA and 4 mM ammonium citrate (Fluka, St. Louis, MO) in 60% ACN containing 0.1% TFA and allowed to dry prior to analysis. Peptide spectra were acquired and processed in reflector positive mode for MS with laser settings being varied between 5100 and 6500. Spectral analysis was done with Data Explorer software.

LC-MS-MS analysis was done on a Thermo LTQ ion trap instrument equipped with a Thermo Surveyor HPLC pump and microelectrospray source and operated with Thermo Excaibur software (Thermo Electron, San Jose, CA). Peptides were resolved with an 11 cm, 100 µm ID, Monitor C18 (Column Engineering, Ontario, CA) microcapillary column with a 5-15 µm tip opening. The flow rate from the HPLC pump was adjusted to achieve 750 nL min⁻¹. The gradient rose to 5% ACN after 3 min, which increased to 50% at 36 min, 80% at 38 min, then to 90% at 40 min before decreasing to 1% at 44 min. The total analysis time for this method was 50 minutes.

Selective quantitation of PIC-labeled peptides was done by targeted MS-MS of m/z values corresponding to light and heavy PIC-labeled Cys-14 adducts (C_{BMCC/1AB}AALR) and Cys-47 adducts (ASC_{BMCC/1AB}LYGQLPK) in both doubly and triply charged forms. Thus, 4 different precursor ions were analyzed for each peptide adduct studied. LC-MS-MS quantitation was done by integrating selected ion chromatogram peak areas with Thermo Xcalibur software. Chromatograms were
generated for each peptide form by plotting the sum of ion currents for the three most abundant product ions from each of the selected precursors. For example, to generate chromatograms for the light PIC-labeled form of the IAB modified Cys-47 peptide, ion current for the \( b_8^+ \) (\( m/z \) 1337.6), \( b_7^+ \) (\( m/z \) 1224.5), and \( b_5^+ \) (\( m/z \) 1039.3) were summed and plotted, these being consistently the most intense product ions from MS-MS fragmentation of the doubly charged precursor ion at \( m/z \) 790.9. For the corresponding heavy PIC-labeled peptide (\( m/z \) 794.0), the sum of the ion currents for the \( b_8^+ \) (\( m/z \) 1343.7), \( b_7^+ \) (\( m/z \) 1230.5), and \( b_5^+ \) (\( m/z \) 1045.3) were plotted and integrated. The integrated peak areas then were used to generate a light:heavy ratio for the target peptide. This analysis was done for each peptide adduct at each time point in the analysis. Relative quantitation of adduct formation at each time point was done by determining ratios of light:heavy PIC-labeled peptides. Kinetic curves were generated by plotting of light:heavy ratios against time and rate constants (\( k_{obs} \)) were calculated by fitting the data to a one-phase exponential association with Prism 4.02 software for windows (GraphPad, San Diego, CA).
RESULTS

Inhibition of GSTP1-1 activity and adduction of GSTP1-1 protein by BMCC and LC-IAB

Recombinant His₆-GSTP1-1 was expressed in *E. coli* and immobilized on Ni²⁺-coated 96-well plates. Analysis of the immobilized protein by SDS-PAGE with Comassie staining showed a distinct band at 23 kDa, corresponding to the molecular weight of GSTP1-1 monomer *(Figure 2-3)*. Digests of proteins from the plate wells with either trypsin or chymotrypsin followed by LC-MS-MS resulted in identification of human GSTP1-1 peptides by database searching with Sequest and corresponding to overall protein sequence coverage of 99% (trypsin) and 80% (chymotrypsin). Western blotting with both anti-GST and anti-His₆ antibodies of elution fractions purified on plates also detected the GSTP band near 23kDa *(Figure 2-3)*. GST activity assays with the immobilized protein on 96-well plates indicated GSTP1-1 activities of 0.464 mmol mg⁻¹ min⁻¹ for conjugation of CDNB with GSH. Thus, our *in vitro* system provided GSTP1-1 of high purity and activity for parallel studies of adduction and inhibition by electrophiles.

Treatment of immobilized GSTP1-1 with BMCC over a two hour time course resulted in an inhibition of enzymatic activity toward CDNB that was both concentration and time dependent *(Figure 2-4)*. BMCC concentrations from 8-25 µM eliminated activity after two hours, whereas 4 µM BMCC reduced activity to 40% of control.

LC-MS-MS analysis of His₆-GSTP1-1 following reaction with BMCC or IAB identified adducts on Cys-14 and Cys-47. The MS-MS spectra for the adducted tryptic
Figure 2-3: Identification of purified His$_6$-GSTP1-1 by Ni$^{2+}$ affinity chromatography on Ni$^{2+}$-coated 96-well plates by SDS-PAGE visible gel (top) and by western blotting (bottom) with antibodies selective for both GST and His$_6$-tag.
Figure 2-4: Time and concentration-dependent inhibition of GSTP1-1 enzymatic activity following treatment with 8 (■), 16 (▲), and 25µM (▼) BMCC.
peptides C$_{14}$AALR and ASC$_{47}$LYGQLPK exhibited b- and y-ion signals consistent with alkylation of cysteine by either BMCC (+533 amu) or IAB (+382 amu) (Appendix A).

**Analysis of kinetics of adduction at Cys-14 and Cys-47 of GSTP1-1 by MALDI-TOF-MS**

We initially explored the application of MALDI-MS to analyze PIC-labeled peptide adducts because of the relative simplicity and speed of this analytical platform. Samples from reaction of GSTP1-1 with 12.5, 16, 20, and 25 µM BMCC were taken over a two hour time course, digested with trypsin and derivatized with light or heavy ($^2$H$_5$) PIC as described under Experimental Procedures. The 120 min sample was labeled with the heavy PIC, whereas samples taken at the other time points were labeled with the light PIC. Each light-PIC labeled sample was combined with an equimolar portion of the heavy-PIC-labeled digest and the mixed samples then were subjected to MALDI-MS (Figure 2-5). A peak at $m/z$ 1190.7 corresponds to the Cys-14 tryptic peptide BMCC adduct with $^2$H$_5$-PIC at the N-terminus. Analysis of samples from each time point indicated a peak at $m/z$ 1185.7, which corresponds to the same peptide labeled with light PIC. Peak area ratios of light:heavy labeled peptides were calculated for each time point and plotted against time for a reaction with 12.5 and 25 µM BMCC (Figure 2-6). This ratio increased over the time course, eventually approaching unity. The slopes of this graph gave estimates of the apparent rate constant $k_{obs}$ for the reaction at each specific concentration of BMCC. Attempts to perform this analysis with the Cys-47 adduct were
Figure 2-5: MALDI-TOF analysis of BMCC adduction at Cys-14 of GSTP1-1. The spectra show the increasing formation of the d0-PIC-labeled peptide (m/z 1185.7) with time compared to the reference d5-PIC-labeled peptide (m/z 1190.7). The ratio of the signals approached unity at approximately 105 minutes.
Figure 2-6: Plots of MALDI-TOF signal ratios for the BMCC-adducted, Cys-14 GSTP1-1 tryptic peptide CAALR at 12.5 (■) and 25 µM (▲) BMCC. Observed reaction rates ($k_{obs}$) were determined from the slopes of the respective plots; at 12.5 µM, $k_{obs} = 0.12 \mu M^{-1} s^{-1}$ ($R^2 = 0.9755$) and at 25 µM, $k_{obs} = 0.20 \mu M^{-1} s^{-1}$ ($R^2 = 0.9733$).
unsuccessful because the adducted, PIC-labeled peptide did not ionize under the analysis conditions used and was not observed in any of the spectra.

**Analysis of kinetics of adduction at Cys-14 and Cys-47 of GSTP1-1 by LC-MS-MS**

To better analyze the kinetics of GSTP1-1 adduction at Cys-14 and Cys-47 simultaneously, LC-MS-MS analysis was used. Both the Cys-14 and Cys-47 peptide adducts ionize well in electrospray and are easily detected. A complication of stable isotope tagging strategies employing LC-MS-MS analyses is the partial chromatographic resolution of deuterated isotopomers from the unlabeled derivatives (69;157), which complicates quantitation of the light and heavy forms. For this reason, we used $^{13}$C$_6$-PIC as the heavy isotope label for LC-MS-MS studies.

The simplest approach to adduct quantitation is to detect the ions corresponding to the intact, PIC-labeled peptide adducts by SIM. A sample of IAB-adducted GSTP1-1 was digested and analyzed by LC-MS-MS with SIM of the precursor peptides at $m/z$ 527.7 and 529.7, which correspond to the doubly charged ions of the light and heavy PIC-labeled Cys-14 adducts, respectively. Similarly, SIM of $m/z$ 791.0 and 794.0 detects the doubly charged ions of the light or heavy PIC-labeled Cys-47 adducts. **Figure 2-7** depicts signals detected by SIM of the precursor ion at $m/z$ 794.0 with a signal to noise ratio of 11. A disadvantage of this mode of analysis is that other species of similar $m/z$ that co-elute with the target peptide will bias quantitation.

More sensitive, specific peptide quantitation can be achieved using MRM with a triple quadrupole instrument, which acquires signal from specific product ions resulting
Figure 2-7: Comparison of signal-to-noise for SIM and MRM analyses of the $^{13}\text{C}_6$- PIC-ASC$_{47}$(IAB)LYGQLPK adduct. The upper trace depicts SIM of the precursor ion. The lower traces depict MRM for the $b_8^+$ ($m/z$ 1343.68), $b_7^+$ ($m/z$ 1230.52), and $b_5^+$ ($m/z$ 1045.34) product ions used for quantitation.
from fragmentation of a selected precursor (78). Similarly, Venable et al. recently demonstrated improved signal to noise for quantitation with a linear ion trap by fragmentation of all species in a \( m/z \) window containing a precursor (81). This approach generally approves signal to noise ratio for quantitative analyses. Accordingly, we also performed LC-MS-MS analyses of the same sample by targeting the doubly-charged precursor ions corresponding to the light and heavy PIC-labeled Cys-14 and Cys-47 adducts. In these analyses, the only MS scans acquired were the MS-MS spectra of these selected precursor ions. We then plotted the combined ion current for the three most abundant product ions in each spectrum. The \( m/z \) values of the precursors and the corresponding product ions used for quantitation are listed in Table 2-1 and MS-MS spectrum of Cys-47 peptide adducted with IAB showing these product ions is also shown (Figure 2-8; other spectra for C14 adduct and corresponding PIC-labeled BMCC adducted peptides are found in appendix A). Comparison of the SIM and MRM approaches indicates an increase in signal to noise of up to 6-fold (Figure 2-8). We used the MRM approach for analyses of PIC-labeled Cys-14 and Cys-47 peptide adducts for all of the kinetic studies.

GSTP1-1 was incubated with several different concentrations of IAB or BMCC for either an eight or five hour time course respectively. Tryptic peptides from treated samples then were PIC-labeled and analyzed by MRM LC-MS-MS as described above. Chromatographic peaks corresponding to the light and heavy PIC-labeled adducts were generated from selected product ions by MRM and ratios of peak areas for light and heavy forms were plotted against reaction time.
<table>
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<th>Product</th>
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Table 2-1: m/z values of all precursors and corresponding product ions used in SIM and SRM quantitation analysis
Figure 2-8: MS-MS spectra of ASC47(IAB)LYGQLPK peptide adducts from GSTP1-1 adducted with IAB and labeled with either $^{12}$C$_6$-PIC (top), or $^{13}$C$_6$-PIC (bottom). Spectra show b- and y-ions and those product ion peaks chosen for quantitation.
Plots of light:heavy peptide adduct ratios vs. time were fitted to first-order rate equations with Prism software for treatments of GSTP1-1 with both IAB (Figure 2-9) and BMCC (Figure 2-10). Data were plotted to a one-phase exponential association equation, \( Y = Y_{\text{max}} \cdot (1-e^{-kX}) \), which yielded the observed rate constant \( (k_{\text{obs}}) \) for each electrophile concentration. In these experiments, \([\text{electrophile}]>>>[\text{GSTP1-1}]\) and thus \( k \) is a pseudo-first order rate constant. Measurements of \( k_{\text{obs}} \) were made at several concentrations of electrophile. The value of the pseudo-first order rate constant \( k \) was obtained from the slope of plots of \( k_{\text{obs}} \) against electrophile concentration for both IAB (Figure 2-9) and BMCC (Figure 2-10).

These analyses indicated a significant difference between the \( k \) values measured for adduction of Cys-14 and Cys-47 and for the relative reactivity of the two electrophiles. The \( k \) values measured at both Cys-14 (2 x 10^{-4} \text{ min}^{-1}) and Cys-47 (7 x 10^{-4} \text{ min}^{-1}) for BMCC exceeded those measured for IAB by at least 10-fold at Cys-14 (2 x 10^{-5} \text{ min}^{-1}) and Cys-47 (4 x 10^{-5} \text{ min}^{-1}), respectively. For both IAB and BMCC, \( k \) values for adduction at Cys-47 were approximately 2-3 times that for adduction at Cys-14. These data suggest that, regardless of other competing reactions of the electrophiles, Cys-47 adducts will be formed at levels approximately 2-3-fold higher than Cys-14 adducts. Complete lists of \( k_{\text{obs}} \) values are shown in Table 2-2.
Figure 2-9: Kinetics of formation of adducts on Cys-14 (■) and Cys-47 (▲) of GSTP1-1 during treatment with 50 μM IAB (top). Ratios of $^{12}$C$_6$/$^{13}$C$_6$-PIC-labeled peptide adducts approached unity over time. Values of $k_{obs}$ were plotted against [IAB] to derive pseudo-first order rate constants ($k$) from resulting slopes of these plots for alkylation of GSTP1-1 at Cys-14 (2 x 10$^{-5}$ min$^{-1}$) and at Cys-47 (4 x 10$^{-5}$ min$^{-1}$) (bottom).
Figure 2-10: Kinetics of formation of adducts on Cys-14 (■) and Cys-47 (▲) of GSTP1-1 during treatment with 25 µM BMCC (top). Ratios of $^{12}$C$_6$/$^{13}$C$_6$-PIC-labeled peptide adducts approached unity over time. Values of $k_{\text{obs}}$ were plotted against [BMCC] to derive pseudo-first order rate constants ($k$) from resulting slopes of these plots for alkylation of GSTP1-1 at Cys-14 ($2 \times 10^{-4}$ min$^{-1}$) and at Cys-47 ($7 \times 10^{-4}$ min$^{-1}$) (bottom).
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**Table 2-2:** Values of observed reaction rate constants (k<sub>obs</sub>) for adduction of GSTP1-1 by IAB and BMCC.
DISCUSSION

The analysis of protein posttranslational modifications and xenobiotic adducts is essential to understanding the effects of toxicants on proteins and proteomes. Proteomics approaches to analyze modifications to date have identified modification sites, but have not addressed the kinetics of modifications. Protein damage by reactive electrophiles is a dynamic process that reflects rates of generation of electrophiles, rates of competing reactions with targets, rates of adduct decomposition and rates of turnover of adducted proteins. To address this problem, we have adapted a stable isotope tagging approach that we introduced for relative quantitation of protein adducts (69). In contrast to the thiol-reactive ICAT reagents (71), PIC can label peptides and peptide adducts regardless of their amino acid composition. Because the tagging reagent PIC reacts with peptide N-termini, essentially any peptide can be analyzed in light and heavy PIC-labeled forms. Our approach to kinetic analyses employs comparative quantitation of heavy and light PIC-labeled forms over time, which makes absolute quantitation of the adducts unnecessary. We have used this approach to analyze the relative reactivities of two electrophile target sites in GSTP1-1. The measured rate constants reflect the observed reactivity of the protein toward similar electrophiles in a complex proteome mixture.

Our results clearly show the differences in reactivity of two model electrophiles. The N-alkylmaleimide BMCC was at least 10-fold more reactive than the alkyl iodide IAB at both Cys-14 and Cys-47 of GSTP1-1. This suggests that the nucleophilic cysteines on GSTP1-1 react more readily with Michael acceptors such as α,β-unsaturated carbonyls (from fatty acid oxidation) or reactive quinones (xenobiotic metabolites) than
with electrophiles that react through an S\textsubscript{N}2 nucleophilic substitution mechanism. In this context, measurements of adduction kinetics for model compounds on selected proteins could prove useful in predicting and comparing relative reactivities of known toxicants. For example, Michael acceptor compounds could be compared with S\textsubscript{N}2 alkylators would include alkyl halides, aliphatic epoxides, and episulfonium ions. It is important to point out our experiment involved a relative comparison of a maleimide group with a primary alkyl halide and did not take into account critical factors such as steric effects. In fact, the structures of the two compounds used in this study (Figure 2-1) differ not only in the electrophile moiety, but also in the linker structure. The cyclohexyl ring in the linker makes the BMCC molecule somewhat more bulky, rigid and hydrophobic than to IAB. These factors should be considered in interpreting reactivities based on alkylation kinetics.

Our kinetic data clearly indicate that Cys-47 is the more reactive of the two alkylation sites we observed. This supports previous work on the structure of GSTP1-1 showing Cys-47 to be solvent accessible, thus allowing it to be highly reactive toward alkylating agents (25). Additional studies have shown an additional influence on the reactivity of Cys-47 to be its interaction with Lys-54, which is positioned in such a way to facilitate deprotonation of the thiol group of Cys-47 (158,159). This interaction results in a shift in the pKa value of Cys-47 from 9.5 to 4.2, so at physiological pH, Cys-47 exists as a thiolate ion (R—S\textsuperscript{-}) stabilized by the interaction with Lys-54 and accounts for its higher reactivity compared with the other cysteine residues of GSTP1-1 (Figure 2-11).
**Figure 2-11:** Three dimensional structure of GSTP1-1 highlighting the interaction between Cys-47 and Lys-54 that accounts for the formation of a thiolate anion and corresponding drop in pKa on the cysteine residue. These factors account for its higher reactivity in comparison to Cys-14.
Using the PROPKA web interface software (http://propka.chem.uiowa.edu/), the pKa of Cys-14 was estimated to be 10.22, while pKa values for Cys-101 and Cys-169 were estimated to be 12.69 and 9.10 respectively (160). Similar values were also obtained with the PDB2PQR feature with the web software(161).

We have identified adducts on two of four Cysteine residues of GSTP1-1 and shown that alkylation of these residues inhibits its enzymatic activity. Cys-47 is located near the glutathione binding site and alkylation of this residue inhibits activity by lowering the enzyme’s affinity to bind glutathione (25). It is unclear whether the alkylation of Cys-14 has any effect on this inhibition or if it is simply alkylated as a result of a conformational change following Cys-47 adduction. Other studies with mutant Cys-Ser enzymes have suggested alkylation of Cys-101 and Cys-169, based which could also have affects on the enzyme function, particularly Cys-101, which is thought to play a role in GSTP1-1 homodimerization (112;114). These adducts were not observed in our LC-MS-MS analyses.

Our study describes a method that can simultaneously compare the kinetics of competing protein adduction reactions using quantitative mass spectrometry. The measured rate constants should predict the relative amounts of adducts in complex proteomes. We recently have reported a proteome-scale inventory of targets of BMCC and the IAB analog (+)-biotinyl-iodoacetamidyl-3, 6-dioxaoctanediamine and found that GSTP1-1 was alkylated in cytoplasmic proteome mixtures (39). However, the only adduct detected was at Cys-47. One interpretation of this result is that multiple alkylation reactions will only be observed if the rate constants differ by less than 3-fold. It is also
possible that interaction of GSTP1-1 with another protein masked the Cys-14 site and prevented alkylation. Thus, the order of rate constants for alkylation of a purified protein in vitro may not necessarily predict the sites of alkylation observed in vivo.

Another area of interest regarding GSTP1-1 is its proposed role as a negative regulator of JNK1 (94). Decreased levels of GSTP1-1 results in increased activity of JNK1 (98). Additional studies show this function to be independent of the glutathione transferase catalytic function (94;162). An important question is whether alkylation of either GSTP1-1 or JNK1 could cause perturbation of this complex and result in activation of JNK1 signaling events that regulate important cellular functions, including growth, differentiation, and apoptosis. Alkylation of GSTP1-1 could trigger JNK activation and it would be of interest to determine whether Cys-47 or Cys-14 alkylation contributed to any affects observed.

We have demonstrated a generally applicable method to determine rate constants for the formation of protein adducts using stable isotope PIC tags and quantitative mass spectrometry. Although we have applied the method to analyze protein adduction kinetics, the method is applicable to a broad range of problems involving the kinetics of protein modification, including changes in endogenous posttranslational modifications. The experimental design is certainly not limited to PIC tags and can be employed with other commonly used derivatives, including iTRAQ™ reagents (163) and other N-terminal reactive labels (68).
CHAPTER 3 – THE EFFECTS OF PROTEIN ALKYLATION ON C-JUN N-TERMINAL KINASE (JNK) SIGNALING IN HUMAN EMBRYONIC KIDNEY (HEK293) CELLS

Introduction

c-Jun N-terminal kinase (JNK) is a member of the mitogen activated protein kinases (MAPK) family of enzymes, also known as the stress activated protein kinases (SAPK) (118). This family of enzymes plays roles in important cellular functions such as growth, differentiation, and apoptosis (121;127) and has been shown to have significant functions in many disease states including arthritis, Alzheimer’s disease, diabetes, and cancer, thus making JNK and other MAPKs attractive targets of interest in new drug development (124). These enzymes function by regulating cellular activity in response to various external stimuli, including growth factors and cytokines, as well as changes in temperature, pH, redox state, osmolarity, and exposure to stressors such as radiation or reactive chemicals introduced from the cell’s external environment (120).

JNK is a member of a MAPK cascade and is activated by two upstream kinases; MAPK kinase 4 (125) and MAPK kinase 7 (126) (M KK4, M KK7), which cooperatively activate JNK by phosphorylation of JNK selectively on Tyr-185 and Thr-183 respectively (83). These enzymes are themselves activated by different MAPK kinase kinases that include apoptosis signal-regulating kinase 1 (ASK1) (121). MAPK cascades such as these are grouped together and additionally regulated by structural proteins known as scaffolds such as JNK interacting protein (JIP) (116). Once activated, the primary
function of JNK is to phosphorylate and activate transcription factors such as members of the AP-1 family (c-Jun, JunB, JunD, and ATF2), ELK-1 and c-Myc (121). JNK signaling is then terminated via dephosphorylation--primarily by a group of the dual specificity protein phosphatase family known as MAPK phosphatase (MKP) (117). JNK signaling has also been shown to be regulated by additional enzymes that include glutathione S-transferase (GST) and thioredoxin (Trx). GST isoforms pi and mu are known to bind and negatively regulate JNK and ASK1 respectively (94;95), whereas Trx-1 and 2 are known to act in the same way to regulate ASK1 activation in both the cytosol and mitochondria, respectively (32;33).

A growing area in toxicological research is the identification and characterization of the effects of protein adduction on cellular systems. Proteins are adducted by reactive, electrophilic alkylating agents introduced to the cell from a variety of sources, such as environmental pollutants and diet, and which may also be produced endogenously as byproducts of important metabolic pathways (164). Many xenobiotic compounds are not themselves toxic, but can be converted to electrophilic intermediates through phase I metabolic pathways such as oxidation by cytochrome P450.

Protein adduction by electrophiles can have significant impacts on protein and cell functions (165). Adduction can certainly alter protein structural integrity, which may ultimately translate into altered structural, enzymatic, or regulatory functions. Protein adduction is also selective and has been linked to target organ toxicity leading to disease states and even cell and tissue death (42). Cell signaling pathways such as MAPK pathways certainly could be affected by toxic insults such as these.
Proteomics technologies have enabled scientists to identify not only targets of protein alkylation in cellular systems, but also to pinpoint the location of adduction to specific residues in targeted protein sequences. New techniques in protein and peptide separation coupled with mass spectrometry provide the means to analyze proteomes and more accurately identify protein adduction targets in even large-scale analyses (54). Developing alongside these methodologies are software programs and search algorithms that allow investigators to characterize protein adduction at an incredibly rapid pace (60;65), thus reducing the need to manually review MS and MS-MS spectra. In some cases, these algorithms allow for identification of protein adducts without any prior knowledge of potential adduction (65). Tools such as these have enabled investigation into the questions of specific cellular effects of protein adduction and how protein adduction selectivity leads also to selective downstream cellular effects.

Our laboratory has studied the effects of protein adduction and has identified targets of different reactive electrophiles in vitro (39). This work indicates that protein adduction is highly selective and is based on chemical properties of both alkylating agent and protein target. This selectivity translates into specific downstream cellular effects directly related to a specific protein adduction. As mentioned above, JNK activation is a complex process tightly regulated by a variety of scaffold proteins and enzymes that include upstream kinases and protein regulators. One may hypothesize that adduction at one or more of these sites could lead directly to JNK activation.

Our goal in this work is to identify targets of electrophile adduction that are directly related to JNK regulation and evaluate the relationship of these modifications to
JNK activation as a result of selective protein damage in the mammalian human embryonic kidney (HEK293) cell line.
EXPERIMENTAL PROCEDURES

Cell culture treatments and preparation of cell lysates

HEK293 cells were grown in Dulbecco’s Modified Eagle Media (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and L-glutamine to a final concentration of 2 mM at 37°C and 5% CO₂. The HEK293 cell line was chosen for this study because of its high levels of GSTP1-1 expression, an enzyme directly related to JNK signaling. Cells were regularly split at approximately 60-70% confluent at dilutions from 1:5-1:10. Cell treatments were done in DMEM without L-glutamine and supplemented with only 5% FBS and varying concentrations of the following compounds: TNF-α (10 ng/mL) (R&D Systems, Minneapolis, MN) dissolved in phosphate buffered saline (PBS), anisomycin (10 µg/mL) (Sigma Aldrich, St. Louis, MO), IAB (100µM), or BMCC (100 and 400 µM) (both from Pierce Biotechnology, Rockford, IL) dissolved in DMSO. The total concentration of DMSO vehicle per culture was ≤ 0.3% (v/v) with the exception of incubations with 400 µM BMCC, in which the DMSO concentration was increased to 1.2%. All cell treatments were carried out over a 12 hour time course. Cells were approximately 50-70% confluent at the time of treatment.

Following treatments, cells were washed with ice cold PBS, cell lysis buffer was added, and cells were scraped off and collected in eppendorf tubes. Cell lysis buffer was prepared from a 10X stock (Cell Signaling, Danvers, MA) and had a final composition of 20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1µg/mL
leupeptin, and supplemented with a mammalian protease inhibitor cocktail (Sigma Aldrich). Samples were sonicated in a water bath for 30 min and centrifuged at 14,000g for 10 min at 4°C. Supernatant was collected and stored at -80°C until use.

**JNK activity assay**

JNK activity was measured with a kit purchased from Cell Signaling Technologies (Danvers, MA, Catalog #9810) and the assay was carried out according to the manufacturer’s protocol. Briefly, JNK was purified from whole cell lysates using a GST-c-Jun fusion protein immobilized on agarose beads (10µL bead slurry/100µL lysate). Following overnight incubation at 4°C, the beads were collected by centrifugation at 14,000g for 30 sec at 4°C, washed twice with each of cell lysis buffer and a kinase buffer composed of 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na$_3$VO$_4$, and 10 mM MgCl$_2$. The agarose pellet was then resuspended in 50 µL of kinase buffer supplemented with 200 µM ATP and incubated for 30 minutes at 30°C. Reaction was terminated with addition of 25 µL 4x NuPAGE® LDS sample buffer (Invitrogen). JNK activity was determined by western blotting (see protocol below) using antibody specific for phosphor-c-Jun (Ser-63).

**Purification of biotinylated proteins**

Biotinylated proteins were purified by affinity capture with immobilized streptavidin (Amersham Biosciences, Piscataway, NJ). One mL of whole cell lysate (diluted to a concentration of no more than 2 mg mL$^{-1}$) was added to prepared
streptavidin beads of packed volume of \( \approx 300 \, \mu\text{L} \) and incubated with gentle rocking at room temperature for 1 hr. Samples were centrifuged at 6500g for 3 min and unbound fractions were collected. Remaining streptavidin beads then were subjected to washes with (1 mL/wash) of: 1) 0.1 M ammonium bicarbonate (AMBIC) (Sigma-Aldrich, St. Louis, MO) (one wash), 2) 1.0 M sodium chloride (NaCl) and 0.1% Igepal (three washes), 3) 0.1 M AMBIC (two washes), and 4) deionized water (two washes). Biotinylated proteins were eluted from beads with three elution steps. First, beads were incubated with 0.5 M formic acid in 25% acetonitrile with gentle rocking overnight at 4°C. Then, bound proteins were eluted with two portions of 5% formic acid in 70% acetonitrile for one hour each; the first elution was at room temperature and the second at 70°C. Eluted fractions were analyzed by SDS-PAGE and western blotting described below.

**Western blot analysis**

Samples were prepared for electrophoresis by adding an appropriate volume of 4x NuPAGE® LDS sample buffer (Invitrogen, Carlsbad, CA) to samples to a final 1x concentration. Samples were then heated to 70°C for 10 min. Appropriate volumes containing 10-20 \( \mu\text{g} \) total protein were loaded onto NuPAGE® 4-12% Bis-Tris gels and ran at 200V for 60 min. Visible gels were stained for protein detection using colloidal blue staining kit (Invitrogen) using the manufacturer’s protocol. For western blotting, protein was transferred to 0.2 \( \mu\text{m} \) PVDF membranes at 30V for 80 min.
Following transfer, membranes were blocked with a 1:1 solution of tris-buffered saline (TBS) and a blocking buffer for near infra red fluorescent western blotting (Rockland Immunochemicals, Gilbertsville, PA) for 1-3 hours. Primary antibodies were added in a 1:1000 dilution in 1:1 blocking buffer and TBS with 0.1% Tween-20 (TBST) and incubated overnight at 4°C. After washes with TBST, fluorescent dye-labeled secondary antibodies were added to membranes at room temperature for 45 min. Membranes were then scanned and analyzed using a LI-COR Odyssey® (LI-COR Biosciences, Lincoln, NB) infrared imager and Odyssey® v2.0 software. All primary antibodies for JNK signaling proteins (ASK1, MKK4, MKK7, JNK1, JNK2, and p-c-Jun) were purchased from Cell Signaling Technologies (Danvers, MA). All other antibodies were purchased from Abcam (Cambridge, MA). All secondary antibodies are from the Alexa Fluor® 680 line of secondary antibodies from Invitrogen. Western blotting was also used to determine overall biotinylation of proteins following treatments. Aliquots containing 10µg of total protein/sample were tested with biotinylation being detected by an Alexa Fluor® 680 fluorescently labeled streptavidin probe also purchased from Invitrogen.
RESULTS

Induction of apoptosis by IAB/BMCC in HEK293 cells

In addition to identifying activation of JNK as previously mentioned, experiments by Dr. Hansen Wong in the Liebler lab have shown differences in induction of apoptosis-related events following treatment of HEK293 cells with IAB or BMCC. These experiments looked for activation of caspase-3 and subsequent cleavage of one downstream target of the caspase activation cascade, poly-ADP ribose polymerase (PARP). Treatment of HEK293 cells for 24 hr with IAB and BMCC revealed different responses to the two model alkylating agents (Figure 3-1). Analyses of caspase-3 activation with the colorimetric CaspACE™ assay system (Promega, Madison, WI) indicated that, at concentrations up to 100 µM, IAB induced a nearly 2-fold increase in caspase-3 activity compared to control cells; this increase was similar to that induced in cells treated with staurosporine, a positive control for apoptosis induction. The opposite result was observed in cells treated with BMCC, where no induction of caspase 3 activity was observed, even at concentrations up to four times greater than those of IAB. The same contrast was observed in western blot analyses to detect cleavage of PARP. IAB treated samples showed the presence of bands at both 116 and 89 kDa, which correspond to the full length PARP and large cleavage fragment, which was also produced by staurosporine treatment. BMCC treatment did not yield a band corresponding to the cleaved PARP fragment.

Patterns of protein biotinylation between IAB and BMCC
Figure 3-1: Graph depicting activation of caspase-3 in HEK293 cells following treatments with IAB (■) or BMCC (■), relative to control (■ ■) and compared to Staurosporine treatment (■) (top) and western blot analysis showing cleavage of PARP in 293 cells following the same treatment conditions with control being labeled as a vehicle control showing percentages of DMSO used in treatments (bottom).
Two biotin-tagged electrophiles, IAB and BMCC, were used in this study to adduct proteins at cysteine residue. The biotin tag facilitates separation of adducted from unadducted proteins for MS analyses (39), as well as detection of adducted proteins by western blotting with streptavidin. The structures of these two agents are similar with respect to biotin and linker chemical structure. The chemistry of the electrophilic reactive groups responsible for protein alkylation represents two broad categories of reactive intermediates commonly encountered in biology (see Figure 2-1). IAB is an alkyl halide that reacts via an S_N2 mechanism, whereas BMCC contains a reactive maleimide group, which alkylates via Michael addition chemistry.

Despite their general reactivity towards thiols, IAB and BMCC display striking differences in patterns of protein biotinylation in 293 cells (Figure 3-2). IAB was significantly more potent and efficient at adducting proteins in this cell line. Treatment of 293 cells with 100 µM IAB over a three hour time course induced time dependent biotinylation. In contrast, alkylation with 100 µM BMCC did not appear to increase over time and was significantly less than produced by IAB treatment. Approximately four-fold higher concentration of BMCC (400 µM) was required over this same time period to produce an overall level of biotinylation equivalent to that produced by 100 µM IAB, when examined by western blotting. These results were the same in cell treatments with or without FBS present in media preparations (Figure 3-2). The experiments without FBS in the medium were done to assess the possible contribution of electrophile binding to serum proteins in modulating cellular protein alkylation.
Figure 3-2: Western blot detection of protein biotinylation in HEK293 whole cell lysate following treatment with either IAB or BMCC over a three hour time course with (top) and without (bottom) FBS in media preparations. Fluorescently-labeled streptavidin was used as probe for protein biotinylation.
Repeating these treatments over a twelve hour time course intensified the contrasts in biotinylation patterns between these two agents. Biotinylation following treatment with 100 µM IAB remained consistent with a time-dependent increase and reached a stable maximum at twelve hours. Treatments with 100 and 400 µM BMCC produced early increases in biotinylation (though not as high as IAB), followed by a gradual decrease in biotinylated protein levels by as much as one-third the maximum from earlier time points after twelve hr (Figure 3-3).

**JNK activation in 293 cells**

To evaluate the activation of JNK by IAB and BMCC, cells were treated with the electrophiles and JNK activity was measured by analyzing the phosphorylation of a c-jun-GST fusion substrate in cell extracts. Effects of IAB and BMCC on JNK activity was compared to the effects of two positive controls, tumor necrosis factor alpha (TNF-α) and anisomycin. These two compounds are prototypical activators of JNK that act by different mechanisms \(166;167\). Treatment with TNF-α induced JNK activation in 293 cells. JNK activation displayed a biphasic response with respect to time, wherein a transient increase was observed at early time points, followed by a more sustained increase at later time points (Figure 3-4). Treatment with anisomycin yielded a rapid and strong increase in JNK activity, followed by a gradual decrease over the remainder of the time course studied. This temporal pattern of JNK activation was distinctly different than that produced by TNF-α (Figure 3-4).
Figure 3-3: Western blots and corresponding graphs illustrating trends in biotinylation in whole cell HEK293 lysates over a 12 hour time course. Fluorescently-labeled streptavidin was used as probe for detecting protein biotinylation.
Figure 3-4: Western blot analysis showing activation of JNK in HEK293 cells following treatment with TNF-α and Anisomycin (control samples) over a twelve hour time course. Activity was observed probing for phospho-c-Jun on GST-c-Jun fusion protein.
Treatment with 100 µM IAB generated a sustained, time-dependent increase in JNK activity up to 12 hours, which reached levels similar to those produced by TNF-α and anisomycin (Figure 3-5). In contrast to the responses produced by TNF-α and anisomycin, JNK activation by 100 µM IAB was monophasic, but sustained. Treatment with BMCC, on the other hand, did not yield a significant increase in JNK activity even at a concentration of 400 µM, which produced covalent binding equivalent to 100 µM IAB (Figure 3-5).

**Identification of JNK signaling-related proteins adducted by IAB and BMCC**

The selective activation of JNK by IAB, but not BMCC led to the hypothesis that certain proteins that regulate JNK activation may be differentially adducted by the two electrophiles. Following treatment of 293 cells with IAB and BMCC, biotinylated proteins were purified from lysates by avidin affinity chromatography. Samples eluted from the column (biotinylated proteins) were subjected to western blot analysis. Presence of biotinylated proteins in these fractions was confirmed by probing with a fluorescently labeled streptavidin probe to determine overall biotinylation in whole cell lysates. These purifications confirm previously observed patterns of protein biotinylation between IAB and BMCC (Figure 3-6).

To identify specific proteins adducted by the biotinylating electrophiles, 293 cells were treated with IAB and BMCC and cell extracts were applied to streptavidin bead columns. Both the flow-through and elution fractions collected and subjected to western
Figure 3-5: Western blot analysis showing activation of JNK in HEK293 cells following treatment with 100 µM IAB, 100 and 400 µM BMCC (test samples) over a twelve hour time course. Activity was observed probing for phospho-c-Jun on GST-c-Jun fusion protein.
Figure 3-6: Elution fractions following purification of biotinylated proteins via streptavidin affinity chromatography from HEK293 cells following treatments with either IAB or BMCC.
 blot analysis with antibodies directed against proteins that regulate JNK activity. Blots were probed for ASK1, MKK4, MKK7, JNK1, JNK2, Trx-1, Trx-2, and GST.

Results of these blots clearly show differences in the selectivity of protein alkylation between IAB and BMCC (Figure 3-7). Proteins that were adducted by the electrophiles were detected by western blotting in the elution fractions, which contain adducted proteins. Confirmed targets of adduction by IAB include MKK4, MKK7, JNK1, JNK2 GST, Trx-1 and Trx-2 (Figure 3-7,8). At both 100 and 400 µM BMCC, the only confirmed targets shared with those identified for IAB treatment were Trx-1 and 2. BMCC did not adduct any of the other proteins studied that regulate JNK activity targets. Furthermore, closer inspection of adduction of Trx proteins with time confirmed the time-dependent differences in IAB and BMCC adduction observed in whole cell protein biotinylation experiments (see above). Whereas the level of IAB-adducted Trx-1 and Trx-2 increased between 1 hr and 8 hr of incubation, BMCC adduction of these proteins declined (Figure 3-8).
Figure 3-7: Western blot detection showing flow-through and elution fractions of biotinylated targets from HEK293 cells following purification by streptavidin affinity capture. Cells were treated with either 100 µM IAB or 400 µM BMCC for 8 hrs.
Figure 3-8: Western blot detection of Trx-1 and Trx-2 in elution fractions of streptavidin affinity purifications of treated HEK293 cell lysate at specific time points.
DISCUSSION

JNK activation by stress associated with chemical toxicity is thought to be a critical determinant of the balance between cell survival and cell death. However, most work on JNK activation has employed agents with mechanisms of action distinct from those of chemical toxicants and, thus little is known about JNK activation by reactive electrophiles. This work has shown distinctly different patterns of protein adduction between the two model electrophiles IAB and BMCC. These different patterns of adduction correlate with different effects of these compounds on JNK signaling. The induction of JNK activity by IAB is temporally distinct from either of two positive controls for JNK activation, TNF-α and anisomycin. Treatments of HEK293 cells with 100 µM IAB induced JNK activity in a time dependant manner, with increasing adduction and JNK activation over a 12 hour time course. This contrasts with activation by TNF-α, which induces JNK activation in a biphasic manner (see Figure 1-10 and (129)). These patterns of activation differ from that of anisomycin, a classic JNK activator that dramatically activates JNK at early time points (up to 2 hrs) followed by a steady reduction in activity returning to near basal levels over a twelve hour period. These examples suggest the existence of multiple avenues of activation of the JNK signaling pathway that now includes activation as a result of protein adduction.

While treatment with IAB induced JNK activity, treatment with BMCC did not significantly induce activity. These two agents alkylate protein thiols via different chemistries; IAB via Sβ2 mechanism and BMCC via a Michael addition. Our laboratory
has previously reported the selective nature of these compounds in alkylating proteins in vitro (39) consistent with other previous work demonstrating the selective nature of protein adduction (42). The observations described in this chapter suggest that JNK activation is highly dependent on the extent and duration of protein adduction by alkylating electrophiles. Alkylation by IAB was more potent and efficient than alkylation by BMCC. First, levels of alkylation (biotinylation) were significantly higher with IAB when compared with equal doses of BMCC. A 4-fold higher concentration of BMCC was required to achieve biotinylation equivalent to that produced by 100 µM IAB in HEK293 cells. In addition, whereas IAB yielded a time dependent increase in protein biotinylation, treatment BMCC at both equal and higher concentrations yielded a rapid increase up to one hour, followed by a steady decrease in biotinylation over the remainder of the twelve hour time course, resulting in biotinylation levels of no more than half the observed maximum. The apparent instability of BMCC binding in vivo is interesting, but unexplained at present. Other studies in the Liebler laboratory are addressing this problem. Nevertheless, these observations suggest that protein adduction must be sustained over longer periods of time in order to activate the JNK pathway.

Stress-related changes in proteins that directly or indirectly regulate JNK have been implicated as elements of JNK regulation in vivo (33;166;168;169) Thus, it seemed reasonable to hypothesize that adduction of JNK-related proteins may contribute to JNK activation. For this study we chose to look at several potential protein targets directly related to regulation of JNK activity in some fashion (Figure 3-9). Our screening for adducts following treatment with IAB or BMCC showed that MKK4, MKK7, JNK1, and
Figure 3-9: Schematic of the JNK signaling pathway showing enzymes looked at in this study and their relationship to JNK activation. Also shown are the targets we’ve identified as targets of either IAB or BMCC or both in HEK293 cell treatment.
JNK2, are targets of adduction by IAB but not BMCC at either 100 or 400 µM. These results suggest that JNK activation is simply the result of overall cellular protein adduction, but may be due to selective adduction of JNK regulators by alkylating agents.

However, the only identified target of alkylation common to both agents provides a different insight into the issue of selectivity and suggests adduction kinetics as the driving force of JNK activation. Trx-1 and Trx-2 have been reported as acting as negative regulators of ASK1, an upstream MAPKKK that signals JNK activation (32;33). Trx-1 and Trx-2 interact with and inhibit ASK1 activity in both the cytosol and mitochondria respectively, where activation in each cellular compartment leads to apoptotic signals via a JNK-dependent pathway in the cytosol and a JNK-independent pathway in the mitochondria. With this in mind, it seems logical to postulate that adduction of Trx-1 specifically leads to dissociation of the complex, followed by activation of ASK1, which leads to activation of JNK. With only one common target identified between the two alkylating agents and their resulting JNK activation patterns, it would also be possible to postulate that activation of JNK (or other cellular signaling events) as a result of protein alkylation is more than adduction of a single target but rather the result of collective adduction of a specific group of targets.

Although this work has identified several targets of alkylation in 293 cells and has also shown corresponding JNK activation by IAB, it is not yet clear whether the two findings are mechanistically linked. Does JNK activation occur as a direct result of adduction of these JNK regulating proteins or is activation indirectly the result of overall cell damage caused by protein adduction? Studies have shown how certain protein
adductions may act as sensor triggers of specific cellular signals, particularly cysteine residues as has been shown in the case of the sensor protein Kelch-like ECH-associated protein 1 (Keap1) (28-31) and as previously mentioned in the case of Trx (32;33). These proteins contain redox- and alkylation-sensitive thiols, which can act as redox and electrophile sensors. Perhaps one or more of the identified IAB protein targets is adducted on a redox sensitive cysteine residue that triggers JNK activation. This is indeed the proposed mechanism of ASK1 activation via redox modification of Trx-1 and 2.

This study has focused rather narrowly on the issue of activation of JNK by electrophilic protein adduction. However, it is important to note that JNK signaling is an important pathway in cellular systems that is activated by a variety of stimuli and regulates important cellular functions depending on cell type and the type and duration of stimulus. Understanding the consequences of JNK activation by electrophiles requires consideration of the diversity of JNK downstream targets, each with its own cellular role. Our studies have focused on c-Jun, a well established target of phosphorylation by JNK that is a member of one of four known families of proteins (Jun) that comprises the activator protein 1 (AP-1) transcription factor together with the Fos, activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma (MAF) protein families (170), another of which (ATF) is also a target of phosphorylation of JNK. Members of each of these families form both homo and heterodimers to form the AP-1 complex, which has been reported to mediate both cell survival and death depending on which
components of AP-1 have been activated and dimerized to activate gene transcription \((170;171)\).

The principal outcome of IAB treatment of HEK 293 cells is the induction of apoptosis. Other downstream targets of JNK that could be significant would include members of the Bcl-2 family, which are key regulators of apoptosis \((147-150)\). JNK has been shown to play roles in the expression, activation and inactivation, and complex interactions regulating the balance of both pro- and anti-apoptotic members of this family of proteins. One issue yet to be explored is which, if any, of these other targets of JNK phosphorylation are affected in response to treatment with alkylating agents. This would certainly provide additional perspective on the mechanisms by which JNK regulates both pro- and anti-apoptotic outcomes and would provide context for interpreting endpoints of toxicity for different alkylating agents.

The results reported here describe how alkylating agents differ in their effects on JNK signaling, which correlates with distinct patterns of alkylation in HEK 293 cells. While these results leave many unanswered questions regarding an exact mechanism of activation of cell signaling pathways in response to alkylation, they nevertheless advance our understanding of the relationship between targets of alkylation in cells and activation of stress signaling pathways.
CHAPTER 4 – DISCUSSION

Protein adduction by reactive electrophiles has been a longstanding issue of interest in toxicology. Damage to proteins is associated with toxic responses from inhibition of enzymatic activity (21) to cell death (22). For years, closer study into the effects of protein adduction was hampered by the lack of methods to accurately identify protein targets of adduction to link specific damage with cellular effects. The advance of MS-based proteomics technology during the late 1990s provided new means to not only identify protein targets susceptible to adduction, but to also map the location of modifications to specific amino acid residues in the protein structures. These advances now allow researchers to take the next steps in dissecting the consequences of damage, which focus on understanding specific downstream consequences of identified adductions. Our research group has been focused on developing new technology tools to accurately and efficiently identify and map protein adducts in recent years (60;65) and is currently extending those goals to identify specific cellular effects directly linked to adducted proteins in cellular systems. This dissertation has described methodology using quantitative mass spectrometry to analyze kinetics of adduction in order to more accurately compare multiple targets on the same protein. I have also described the effects of protein adduction by two model electrophiles on the activation of JNK signaling in the mammalian HEK293 cell line.

Protein adduction kinetics
Previously, our lab developed a method for quantifying proteins using a new stable isotopic label, phenyl isocyanate (PIC) \( (69) \). PIC-labeling was shown to be a useful stable isotope tag for relative quantitation of proteins and specific protein forms. The advantage of quantitatively labeling N-termini of all peptides in complex mixtures is that it allows for relative quantitation of any peptide in paired samples. We have now applied this quantitative method for comparison of multiple adduction sites on the same protein. Work in our laboratory has shown close to 95% of all identified modified proteins in a sample are modified at only one or two sites \( (39) \). There still remain several proteins where adduction occurs at multiple sites. Moreover, defined multiprotein complexes constitute functional targets of electrophiles, in which competing alkylation reactions may dictate the course of toxicity. A means to compare the reactivities of these targets is essential in order to compare the significance of damage at one target \textit{versus} another.

In Chapter 2, I have presented a method to determine rate constants \( (k) \) for reactions of electrophiles at different residues using PIC-labeling. In contrast to conventional quantitative methods, this approach does not require synthesis of stable isotope labeled standards for absolute quantitation of adducts. The synthesis of specifically modified, isotope-labeled standards would be a large barrier to characterizing protein site reactivity. The method described here uses isotope ratio measurements to derive kinetic constants. In addition, this method allows for simultaneous comparisons of these targets.
Several factors are taken into account when comparing reactivities of these targets. These include the chemistry of the electrophile and the nucleophile (amino acid side chains), relative pKa and ionization state of side chain nucleophile, and the three-dimensional protein structure and environment of the target amino acid location within that structure. A method that provides a measure of site-specific reactivity that encompasses all these factors can be powerfully combined with protein structure information to explore the basis of site-selective damage.

Identifying the sites most susceptible to adduction on proteins can also provide valuable insights into issues such as which sites of adduction may have the greatest impact on protein functions or perhaps even which modifications are most likely to trigger other downstream cellular effects such as dissociation of protein complexes, activation of signaling pathways, and initiating protein degradation or signaling changes in gene expression.

Application of PIC-labeling

A recent study in our laboratory used PIC-labeling to compare reactivities of sites on human serum albumin (HSA) toward alkylation by 4-hydroxynonenal (HNE). This study identified six different alkylation targets (H67, K233, H242, H247, H367, and H510) on HSA that are adducted at different rates. Of these residues, H242 was identified as the most reactive with the highest observed rate constant ($k_{obs}$), thus making it a potential “hot spot” to look for biomarkers of oxidative stress and disease. This residue resides in a hydrophobic pocket that facilitates binding of lipophilic molecules.
and also confers unusually high reactivity on the histidine imidazole (172). These results demonstrate the power of combining PIC-labeling to compare reactivities of multiple alkylation sites and analysis of the structural features of the corresponding protein sites.

The effects of protein alkylation on JNK signaling in HEK293 cells

The second half of the work described in this dissertation has dealt with effects of protein adduction on JNK signaling, a common MAPK pathway that responds to certain cellular stressors including UV, osmotic, and oxidative stress (121). Until now, studies have not shown whether protein alkylation can also trigger responses through this pathway. These studies employed HEK293 cells and treatment with two model electrophiles, IAB and BMCC and have shown that IAB induces significant activation of JNK signaling while BMCC does not, even at concentrations four times that of IAB. Activation by IAB was time dependent, increasing gradually over a twelve-hour time course, and was temporally distinct from activation patterns observed with TNF-α (129) and anisomycin. These results show not only that protein alkylation activates cell stress signaling responses, but their mechanisms of activation differ from activation through receptor signaling (i.e., TNF-α). This difference in activation between IAB and BMCC also correlates with the differences in their kinetics of covalent binding to cellular proteins in HEK293 cells. This presents strong evidence supporting the hypothesis that protein binding is directly related to JNK activation, and could be the case in other signaling pathways.
A major contribution of this work is the identification of several protein targets of alkylation by IAB, which are all associated with the JNK cascade and whose modification could be directly linked to JNK activation. Supporting this argument is the fact that most of these proteins were not identified as targets of alkylation by BMCC. This demonstrates not only the selectivity of targets by electrophilic compounds but the differences in cellular responses as a result of alkylation by different electrophilic species.

Both model electrophiles reacted with the Trx proteins Trx-1 and Trx-2. These proteins have been reported to act as negative regulators of JNK through interaction with ASK1 in the cytosol and mitochondria, respectively (32;33). Regulation of ASK1 by Trx enzymes was dependent on the redox state of the Trx internal dithiol/disulfide redox couple. Oxidized Trx dissociated from ASK1, thus allowing ASK1 activation. Alkylation of Trx at cysteine residues involved in these interactions could possibly cause dissociation from ASK1 and activation of its kinase activity, leading to downstream activation of JNK via MKK4. These data suggest an explanation of the small, yet not significant increases in JNK activity following treatment with BMCC. While this mechanism of ASK1 activation could induce JNK activation via MKK4 reports show that complete JNK activation is achieved by activation by both upstream JNK kinases MKK4 and MKK7 (83). Our observations would then suggest IAB is a significant activator of JNK through its effects on both MKK4 and MKK7 activity. Besides this, alkylation of Trx-1 and 2 by both IAB and BMCC appears to be time dependent; alkylation by IAB increases over time, whereas alkylation by BMCC decreases over time. This suggests
that detectable cellular effects of protein adduction present themselves only following a more sustained adduction over time.

**Future efforts in studying effects of protein alkylation in biological systems**

While the results I have presented in this work regarding protein adduction and JNK signaling have provided some significant conclusions regarding selectivity of downstream effects following alkylation by different electrophiles, they have brought up new topics of interest for further consideration. First is the question of whether activation of JNK is directly related to alkylation of the identified targets or the more indirect result of a secondary mechanism such as oxidative stress as a result of overall cellular damage. If JNK activation is a direct consequence of alkylation of the identified targets, is there a single target that triggers the JNK activation response, or does activation result from alkylation of all these targets collectively? Studies into issues such as these could shed valuable light on understanding mechanisms of toxicity elicited by protein alkylation.

One approach that could be valuable to answering some of these questions is the mapping of adducts on our identified targets. Localizing adducts within three dimensional structures of these targets would allow for determination of how alkylation might be affecting protein activity, structure, and interactions with other proteins. Another strategy would be to evaluate effects of alkylation on each target individually to determine which targets are functionally affected and thus may play significant roles in JNK activation. Tools such as mutant cell lines with knockouts for targeted proteins, siRNA inhibitors of genes of interest, small molecule inhibitors, and *in vitro* kinase
assays would all allow for observation of alkylation effects on each target individually to
better understand effects collectively.

While the results described in this dissertation have shed light on the importance
of understanding effects of protein alkylation, particularly with respect to cell signaling
mechanisms (JNK signaling) there remain ongoing efforts in our laboratory to compare
and contrast protein alkylation effects with respect to additional cellular processes
including ubiquitination and apoptosis. It is clear that protein adduction has significant
consequences to biological systems and these effects vary between alkylating agents,
whether they are from endogenous or environmental sources. Our results emphasize the
need for further study of protein adduction and its effects on cellular systems.
APPENDIX

Index of GSTP1-1 peptides identified and used in PIC-labeling analysis

a. C$_{IAM}$AALR
b. ASC$_{IAM}$LYGQLPK
c. C$_{BMCC}$AALR
d. ASC$_{BMCC}$LYGQLPK
e. C$_{IABA}$AALR
f. ASC$_{IAB}$LYGQLPK
g. Chromatograms of PIC-labeled, IAB-adducted peptides
h. PIC- C$_{IABA}$AALR
i. $^{13}$C$_{6}$- PIC- C$_{IABA}$AALR
j. Chromatograms of PIC-labeled, BMCC-adducted peptides
k. PIC- C$_{BMCC}$AALR
l. $^{13}$C$_{6}$- PIC- C$_{BMCC}$AALR
m. PIC-ASC$_{BMCC}$LYGQLPK
n. $^{13}$C$_{6}$-PIC-ASC$_{BMCC}$LYGQLPK
$C_{IAM}$AALR
MH$^+$ 589.35
ASC_{IAM}LYGQLPK
MH^{+2} 568.18

y_2 = 244.20
y_3 = 319.15
y_4 = 432.03
y_5 = 542.35
y_6 = 705.39
y_7 = 818.43
y_8 = 818.43
y_9 = 978.55
b_3 = 357.29
b_4 = 485.32
b_5 = 595.17
b_6 = 652.30
b_7 = 780.39
b_8 = 893.43
b_9 = 990.45
b_{10} = 1065.36
$C_{BMCC}AALR$

MH$^+ 1066.41$
ASC$_{BMCC}$LYGQLPK
MH$^{+2}$ 807.16
ASC, LYGQLPK

MH+2 731.38

652.54 - Y8 2+
705.51 - Y6
542.46 - Y5
244.23 - Y2

1218.73 - b8
920.63 - b7
818.55 - b6
757.55 - b5
542.46 - b4
357.31 - b3
244.23 - b2

644.38 - Y7
757.55 - Y4
485.51 - Y3
357.31 - Y2
244.23 - Y1
1105.66 - b7
977.62 - b6
757.55 - b5
542.46 - b4
357.31 - b3
244.23 - b2

Relative Abundance
PIC-C$_{1AB}$AALR

MH$^{+2}$ 517.66

y$_1$ 175.16
y$_2$ 288.26
y$_3$ 359.39
y$_4$ 430.39
b$_5$ 509.01
b$_1$ 605.30
b$_2$ 676.43
b$_3$ 747.41
b$_4$ 860.44
$^{13}$C$_6$-PIC-C$_{AB}$-AALR

$\text{MH}^+ 520.68$

$m/z$

Relative Abundance

$430.44$

$611.28$

$866.51$

$288.31$

$753.35$

$175.20$

$y_1$

$b_1$

$b_2$

$b_3$

$b_4$

$y_2$

$y_3$

$y_4$
PIC-CBMCC_AALR
MH+² 593.24

m/z

Relative Abundance

y1 175.19
y2 288.25
y3 359.30
y4 430.35
a1 727.95
b1 756.29
b2 870.44
b3 898.34
b4 1011.35
a3 1011.35

m/z range: 0 to 1200

y1, y2, y3, y4, a1, b1, b2, b3, b4, a3 are labeled features of the mass spectrum.
$^{13}\text{C}_6\text{-}\text{PIC-ASC}_{\text{LAB}}\text{LYGQLPK}$

$\text{MH}^+ 869.58$

$y_9(MH^+)$

$y_2, y_3, y_4, y_5, y_6, y_7, b_4, b_5, b_6, b_7, b_8, b_9$
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