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Reactive Intermediates: Molecular and MS-Based Approaches to Assess the Functional Significance of Chemical:Protein Adducts1

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Abstract

Biologically reactive intermediates formed as endogenous products of various metabolic processes are considered important factors in a variety of human diseases, including Parkinson's disease and other neurological disorders, diabetes and complications thereof, and other inflammatory-associated diseases. Chemical-induced toxicities are also frequently mediated via the bioactivation of relatively stable organic molecules to reactive electrophilic metabolites. Indeed, chemical-induced toxicities have long been known to be associated with the ability of electrophilic metabolites to react with a variety of targets within the cell, including their covalent adduction to nucleophilic residues in proteins, and nucleotides within DNA. Although we possess considerable knowledge of the various biochemical mechanisms by which chemicals undergo metabolic bioactivation, we understand far less about the processes that couple bioactivation to toxicity. Identifying specific sites within a protein that are targets for adduction can provide the initial information necessary to determine whether such adventitious post-translational modifications significantly alter either protein structure and/or function. To address this problem we have developed MS-based approaches to identify specific amino acid targets of electrophile adduction (electrophile-binding motifs), coupled with molecular modeling of such adducts, to determine the potential structural and functional consequences. Where appropriate, functional assays are subsequently conducted to assess protein function.

Keywords

Bioactivation; chemical-induced toxicities; covalent binding; drug metabolism; protein adducts; proteomics; reactive intermediates

Introduction

Biologically reactive intermediates (BRI) are formed as endogenous products from various metabolic processes, and are thought to play an important role in various human diseases.

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For example, a number of reactive glucose metabolites such, as the α,β-dicarbonyls glyoxal and 3-methyglyoxal, have been implicated in a variety of complications associated with diabetes (Rabbani and Thornalley, 2011), including nephropathies (Dunlop, 2000; Thornalley and Rabbani 2009), retinopathies (Stitt et al., 2004), neuropathies (Jack and Wright, 2012), and cardiovascular disorders (Beisswenger, 2012). Reactive metabolites of dopamine (Sidell et al., 2001; Galvin, 2006) and reactive products of lipid peroxidation (Ruiperez et al., 2010) have been implicated in Parkinson's disease. Indeed, reactive electrophilic aldehydes generated during lipid peroxidation, such as 4-hydroxy-2-nonenal (4-HNE) are considered to contribute to a number of neurodegenerative conditions associated with protein dysfunction (Perluigi et al., 2012) and in easrly alcoholic liver disease (Galligan et al., 2012). In particular, protein modification by 4-HNE adducts is considered to play a key role in the adverse effects of this lipid-derived reactive intermediate (Fritz and Petersen, 2011).

Toxicities associated with exposure to drugs or environmental chemicals are also frequently mediated not by the parent compound, but via metabolic conversion (bioactivation) to BRIs; the overlap between mechanism of chemical-induced toxicities, and diseases in which endogenous electrophiles might play a causative role, has been succinctly summarized by Dan Liebler (Liebler, 2006). BRIs are typically electrophilic in nature, and as such possess the ability to react with nucleophilic sites within tissue macromolecules, including DNA (see Preston, this issue) and proteins. The resulting adventitious post-translational modification (PTM; or DNA adduct) typically alters the function of the affected macromolecule. Indeed, the pharmaceutical industry has taken advantage of this process to develop effective drugs that bind and irreversibly inhibit their desired target; the proton-pump inhibitors being a classic example of this strategy. Proton-pump inhibitors are prodrugs that are converted to their active form within the acidic environment of the gastric antrum. For example, omeprazole is a weak base that specifically concentrates in the acidic secretory canaliculi of the parietal cell, within the cleft of the oxyntic gland, where it is activated in a proton-catalysed process to generate a reactive sulphenamide. The thiophilic sulphenamide subsequently forms covalent adducts with cysteinyl sulphydryl residues located in the extracellular domain of the H^+K^+-ATPase, particularly C813, thereby inhibiting its activity. Other examples of drugs working via the irreversible modification of a therapeutic target exist. The reader is referred to recent excellent reviews on this topic, and on the subject of reactive metabolites in drug development (Park *et al.*, 2011; Singh *et al.*, 2011; Stepan et al., 2011).

The role of covalent binding of reactive electrophilic drug/chemical metabolites to proteins in the development of toxicity has been the subject of investigations for more than 60 years. In particular, initial studies on the role of such covalent binding in the hepatotoxicity of acetaminophen and bromobenzene, and on the myelotoxicity of benzene, utilized radiolabelled substrates that revealed a correlation between the extent of covalent binding of radiolabel to protein and the degree of cytotoxicity; manipulations that increased metabolism increased covalent binding and toxicity; manipulations that decreased metabolism decreased covalent binding and toxicity. Other manipulations that either increased or decreased covalent binding to protein (such as altering hepatic glutathione [GSH] levels), had

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corresponding effects on toxicity. There were exceptions to these generalities; with investigators demonstrating certain chemicals could give rise to reactive metabolites that covalently bound to protein in the absence of any apparent untoward effects. This dichotomy eventually fixed the discussion on the qualitative nature of the target protein(s) rather than on the quantitative aspects.

Ensuing attempts to couple the initial covalent binding event with the subsequent development of toxicity focused primarily on measuring downstream biochemical changes, such as perturbations in calcium and thiol homeostasis, and alterations in energy stores (ATP and NAD[P]H:NAD[P] concentrations). Whether or not such changes were coupled to the ability of a reactive intermediate to covalently bind to, and alter the function of a target protein(s) was not known, primarily because the technologies required to address such a question were simply not available. Moreover, even just the identification of target proteins was a Herculean task, requiring the linking of a reactive metabolite to an appropriate carrier protein to create an immunogen that, when inoculated into an appropriate animal, was capable of generating antibodies that recognized the original hapten, attached to what would be immunoreactive proteins following drug/chemical exposure. This approach, pioneered by Lance Pohl and colleagues at the NIH (Satoh et al., 1985, 1989; Pohl, 1993), led to the identification of a plethora of target proteins, and to the eventual creation of the “Target Protein Database” by Bob Hanzlik and colleagues (Hanzlik et al., 2009) at the University of Kansas (http://tpdb.medchem.ku.edu:8080/protein_database/). The ability to identify protein targets of reactive metabolites was the first step in the quest to ascertain the extent to which such adducted proteins contribute to the toxicity observed following compound exposure. With subsequent advances in mass spectroscopy, coupled to powerful computing capabilities (software and hardware) it is now possible to not only identify proteins that are targets for reactive electrophiles, but also to identify the specific residues within proteins that become adducted. Such information, coupled to molecular modeling, permits initial interpretation of the potential structural and functional changes that are likely to occur to the adducted protein. This paper summarizes some of our laboratory’s own efforts in assessing the consequences of adventitious post-translational modifications, and concludes with a discussion of the extent to which such consequences may (or may not) contribute to chemical-induced toxicities.

Results and Discussion

As a model for the interaction between intact native proteins and electrophilic chemicals, we selected cytochrome *c* as a target substrate. Cytochrome *c* is well-characterized structurally by X-ray crystallography and NMR (Banci et al., 1997). It is particularly relevant for the study of interactions of electrophilic chemicals with non-thiol nucleophiles, having no free sulfhydryls.

Adventitious Post-Translational Modifications and Post-adduction chemistry

The detection of adventitious PTMs requires knowledge of the bioactivation pathway that yields the ultimate electrophilic binding species. This is necessary to facilitate the process of searching through mass spectral data files to identify modified electrophile-modified

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peptides from their unmodified equivalents, the former usually being far less abundant than the latter. However, the search for modified peptides can be complicated by reactions occurring subsequent to formation of the initial adducts. Such post-adduction chemistry renders the search for adventitious PTMs even more challenging. As an example, our studies on 1,4-benzoquinone (BQ)-mediated adduction of cytochrome c revealed the dominant adduct produced a +194 Da mass addition (Person *et al.*, 2003). BQ has an average mass of 106.1 Da and will add 106.1 in the oxidized form or 108.1 in the reduced hydroquinone form when covalently bound to a protein. The preference for the +194 Da mass addition was first noted in the whole protein MALDI-TOF spectrum, which revealed adducts at +199 ± 6 Da, somewhat less than the expected +212 Da from the addition of two single BQ adducts. The MALDI-MS and targeted MS/MS further identified the adduct as a +194 Da cyclized product, found at two separate locations on the protein, representing the addition of two BQ molecules followed by the loss of water. Both locations where the 194 Da adduct were found contained two lysines in close proximity, either KK or KHK. The final adduct formed as a consequence of the addition of two BQ adducts to two separate lysine residues, followed by a cyclization reaction. One lysine adds to the *ortho* position of BQ, and the second lysine forms a Schiff base with a second molecule of BQ. The two lysine-adducted quinones then form a cyclic product and oxidize to form the final stable ring structure with mass addition of +194 Da.

A metabolite of BQ, 2-(glutathion-S-yl)-1,4-BQ also forms covalent adducts with cytochrome *c*, targeting several histidine and lysine residues (Person et al., 2005). The initial reaction of 2-(glutathion-S-yl)-1,4-BQ with cytochrome *c* at pH 6 results in Michael addition of lysine nitrogens, preferentially at K25 to K27 and K86 to K87, to the BQ ring to yield the reduced, quinol adduct. Under acidic conditions, the quinol adduct is stabilized against rapid oxidation and the protonation of lysine R-amine groups retards addition to any quinone that is formed. At higher pH, oxidation of the quinol adduct to a quinone, and deprotonation of the adjacent lysine R-amine group, together facilitate a Michael addition of the second lysine onto the quinone ring. This is followed by β-*elimination* of the GSH cysteinyl thiol to afford the final product (Person et al., 2005).

The extent to which post-adduction chemistry occurs subsequent to the initial reactive intermediate:protein interaction is likely determined by the microenvironment where adduction takes place, and the chemical nature of the interacting electrophile. For example, in our studies on cytochrome c adduction with 2-(N-acetylcystein-S-yl)-1,4-BQ, we identified a peptide (^{92}EDLIAYLKK^{100}) with a 268 Da addition at E92 (Fisher et al., 2007). The glutamic acid residue represents a novel site of adduction and was specific for the interaction between 2-(Nacetylcystein- S-yl)-1,4-BQ and cytochrome c at pH 6. Although manual validation specified that the modification could occur on either E61 or E62, X! Tandem (open source software that can match tandem mass spectra with peptide sequences) and P-Mod (an algorithm and software to map modifications to peptide sequences using tandem MS data; Hansen et al., 2005) both found the modification on E62. In this case, the low pKa of this protein region most likely allows for the stability of the 268 Da adduct, without loss of the thiol moeity. The glutamic acid residue is able to adduct 2-(N-acetylcystein-S-yl)-1,4-BQ via two possible mechanisms. One possibility is through

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carboxylate anion formation and stabilization at pH 6. This allows for Michael addition of the *γ-carbon* to the ring of the BQ. An additional mechanism of 2-(N-acetylcystein-S-yl)-1,4-BQ adduction is via deprotonation of the R-hydrogen, followed by resonance structure formation and stabilization at pH 6 via protonation of the backbone carbonyl. The R-carbon is then able to adduct the BQ ring of 2-(N-acetylcystein-S-yl)-1,4-BQ via Michael addition. Additional evidence supporting the adduction of glutamic acid by 2-(N-acetylcystein-S-yl)-1,4-BQ was provided in studies with malonic acid, a structural analog of glutamic acid. MS analysis of incubations of malonic acid with 2-(N-acetylcystein-S-yl)-1,4-BQ at pH 6, 7, and 8 revealed that malonic acid was adducted by 2-(N-acetylcystein-S-yl)-1,4-BQ via similar chemistry at pH 6 and 7, but not at pH 8 (Fisher et al., 2007).

Electrophile-binding Motifs

The sites of 2-(glutathion-S-yl)-1,4-BQ adduction to cytochrome c were also identified as targets of BQ adduct formation (Person et al., 2003), and in both cases cyclic reaction products were preferentially formed at these sites, presumably because of the presence of multiple basic residues present in a conformationally flexible region. Moreover, when cytochrome *c* was reacted with 2-(N-acetylcystein-S-yl)-1,4-BQ, an additional six peptides were identified as containing lysine adducts residing on very basic portions of the protein, generally immediately adjacent to another basic residue (Fisher et al., 2007). We subsequently proposed that reactive electrophiles might preferentially adduct specific motifs within proteins (electrophile binding motifs [EBMs]) that facilitate the chemical adduction reaction (Fisher et al., 2007). We address this idea we used an established Eker rat (Tsc-$2^{EK/-}$) model of chemical-induced nephrotoxicity and nephrocarcinogenicity (Lau et al., 2001) to isolate adducted proteins, and to ascertain features that predispose the identified proteins to chemical adduction. Following identification of ~30 adducted proteins (Labenski et al., 2009) we searched for common binding motifs within 2-(glutathion-S-yl)-1,4-BQ-adducted proteins (Person et al., 2003, 2005; Fisher et al., 2007). The cytochrome *c* sequences ^{23}GGKHKTG^{29} and ^{84}GIKKK^{88} were identified by LC/ MS/MS as binding sites for BQ (Person et al., 2003), MGHQ (Person et al., 2005), and 2-(N-acetylcystein-Syl)-HQ (Fisher et al., 2007). We subsequently searched the primary sequences (obtained via the Swiss-Prot data- base) of the proteins identified in the in vivo study (Labenski et al., 2009) for the presence of basic amino acid run-ons. Interestingly, all 30 adducted proteins possessed lysine-rich sequences. Moreover, 2-(glutathion-S-yl)-1,4-BQ -adducted proteins contained either lysine residues flanking a potentially nucleophilic amino acid (KXK), or two lysine residues preceded, or followed, by a nucleophilic amino acid (XKK or KKX) (Labenski et al., 2009). The presence of 1, 2, 3, 4 to 6, 7 to 9, 10 to 20, and >20 lysine-rich sequences were found in one, five, four, eight, seven, three, and two of the 2-(glutathion-S-yl)-1,4-BQ-adducted proteins, respectively. Adducted proteins that contained more than nine XKK or KKX sequences included Hsp90α, Hsp90 β, moesin, radixin, clathrin heavy chain, and ezrin (Labenski et al., 2009). These proteins possessed an elevated lysine content (9–16%) compared with the average protein lysine content within the entire proteome (5.5%). Knowledge of such preferential EBMs is a prerequisite for determining the potential biological/toxicological consequences of chemical adduction to proteins.

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Structural and Functional Consequences of the Adventitious PTM of Cytochrome c

The preferential sites for BQ adduction on cytochrome *c* were intriguing, and the selectivity of adduction was considered to influence the biological function of cytochrome *c*. Cytochrome *c* plays an important role in apoptosis. The onset of apoptosis in vertebrates is initiated by cytochrome *c* release from mitochondria (Liu et al., 1996; Zou et al., 1997), and cytochrome *c* subsequently binds to apoptosis protease activation factor-1 (Apaf-1) (Yu et al., 2001). A number of cytochrome *c* residues, including residues 7, 25, 39, 62- 65, and 72, are involved in the critical cytochrome *c* Apaf-1 interaction (Yu et al., 2001). Whether BQ alkylation of K25 and K27 on cytochrome *c* influences the cytochrome *c/Apaf-1* interaction, thereby altering the ability of cytochrome *c* to initiate apoptosis, was determined. A 3D model generated from the Swiss-Model program revealed the location of the two adduction sites. The model also revealed that both sites were solvent-exposed. In addition, these reactive sites were located in conformationally flexible loop regions so that the residues may change orientation to form the cyclic product. BQ-adducted cytochrome c failed to activate caspase-3 (Fisher et al., 2007,2011), with increasing numbers of BQ-lysine adducts on cytochrome c corresponding to a greater inability to activate the apoptosome (Fisher et al., 2011). The inability of BQ-adducted cytochrome c to activate cytochrome is likely a direct consequence of its inability to promote the oligomerization of Apaf-1 (Fisher et al., 2007). The structural adjustments within cytochrome c subsequent to adduction induce changes in the spatial orientation of additional residues within cytochrome c, particularly those residues that facilitate the cytochrome *c-Apaf-1* protein-protein interactions.

The above studies illustrate the impact of adventitious PTMs on critical protein:protein interactions. Although reactive electrophile-mediated disruption of protein:protein interactions plays an important role in the adaptive response to electrophile stress (cf Nrf2/Keap-1, NF-κB/IκB, HSF1/Hsp70–90, see below) interference with protein:protein interactions that reside outside of the adaptive response might be expected to have unpredictable effects that contribute to cytotoxicity. Again, our studies with cytochrome c have proved enlightening in this regard. Thus, although the circular dichroism (CD) spectra of BQ-adducted cytochrome c appear similar to those of native cytochrome c with respect to an intact secondary structure, there appears to be more α-helical structure associated with BQ-adducted cytochrome c, as indicated by increased CD signals at 208 nm, a characteristic of α-helices (Fisher et al., 2011). In particular, cytochrome c molecules with either three or four BQ-lysine adducts exhibited a more disrupted CD spectra than those cytochrome c molecules containing only two or three BQ-lysine adducts. Moreover, since the most predominant BQ adduct on cytochrome c is the 196-Da diquinone adduct, which represents an intramolecular cross-link between adjacent or neighboring lysines, this likely contributes significantly to the resulting structural rigidity. Thus, BQ-induced PTMs likely induce a more rigid cytochrome c structure, with a higher population of α-helical structures, which inhibits the protein flexibility necessary for critical protein-protein interactions. A similar observation was made with respect to an increase in the molecular rigidity of brain Na^+-K^+-ATPase upon modification by 4-HNE (Miyake et al 2003). Bioinformatic analysis of the protein partners of electrophile-adducted proteins may therefore be beneficial in further elucidating the wave of events that ripple through the cell subsequent to the initial splash of electrophile hitting its target(s).

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Endogenous Electrophiles and Diabetes

Metabolism of glucose under conditions of oxidative stress can give rise to reactive dicarbonyl metabolites, including methylglyoxal (MG), an α-oxo aldehyde generated by the spontaneous degradation of triose phosphates (glyceraldehyde-3-phosphate and dihydroxyacetone phosphate) and autoxidation of Amadori products (Thornalley, 1996). Similar products are formed under conditions of high glucose concentrations (e.g. diabetes), when the glycolytic pathway becomes overwhelmed and glucose metabolism is shunted down the polyol pathway, generating sorbitol and subsequently fructose phosphates. MG and other dicarbonyls generated via “glyco-oxidation” such as glucosone and 3-deoxyglucosone, are electrophiles that covalently adduct to proteins, reactions which may induce structural and functional changes at critical amino acid residues. Such protein modifications may represent an important mechanistic link between glucose metabolism and vascular complications. Since MG is a highly reactive intermediate, the major fraction in blood does not exist free in solution, with some estimates suggesting that as much as 99% of MG is protein bound (Chaplen et al., 1998). Depending on the analytical platform, measurements of unbound plasma MG range from 40 nM to 4.5 μM (Khuhawar et al., 2008; Dhar et al., 2009). At physiological concentrations of free MG (<5 μM), the primary amino acid residue target for protein adduction is arginine, and the major adduct formed is the MG-H1 hydroimidazolone (Ahmed et al., 2002). The reaction produces a net loss of positive charge, with potential functional consequences for arginine residues located at key sites for protein function. For example, a hotspot for MG adduction on human serum albumin (HSA) at R410 influences ketoprofen binding and esterase activity in drug binding site II (Ahmed et al., 2005). Although R410 is a major site for MG modification *in vitro*, studies with human albumin mutants revealed that this modification results in the rapid clearance of HSA in mice (Iwao et al., 2006). Thus, MG modification at R410 creates a modified protein that appears to be efficiently recognized and degraded, and is thus unlikely to accumulate to appreciable levels *in vivo*.

Our own MS-based studies revealed seven new sites for MG modification in HAS (Kimzey et al., 2011). At low MG concentration (100 μM) the preferential sites, in decreasing order of affinity, were R257 > R209 > R222 > R81 > R485 > R472 > R10. The study verified four sites described previously (R410, R186, R218, and R428) (Ahmed et al., 2005). All of the arginine binding sites exhibit varying degrees of solvent exposure (Ahmed et al., 2005), although there is no apparent correlation between solvent exposure and degree of modification. Hydroimidazolone formation is likely governed by the arginine microenvironment where neighboring residues facilitate the condensation of MG on arginine. MG modification of HSA on R257 alters the warfarin binding conformation in drug site I. Structure coordinates from PDB 2BXD were used as a starting model for molecular dynamics and energy minimization. Changes in the drug site I pocket of unmodified R257 to MG-modified R257, and the energy-minimized conformations of warfarin were determined by molecular dynamics simulation based on the available crystal structures. In its unmodified form, R257 participates in hydrogen bonding with the aliphatic ketone of warfarin. Another important interaction of warfarin and the drug site I pocket is between R222 and the lactone carbonyl. These two key arginine interactions (R257 and R222) help stabilize and orient warfarin in this drugbinding site. MG-modified R257 is not

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able to form hydrogen-bonding interactions with warfarin, and this causes a shift that frees warfarin to rotate and undergo additional interactions with H242 and K199. These two residues (H242, K199) do not form hydrogen-bonding interactions with warfarin when R257 is in its unmodified, positively charged form. MG modification at R257 essentially pulls K199 into the binding pocket to replace this lost interaction, which is between the ketone of warfarin and the ε-amino side chain of K199. H242 also picks up an interaction, and forms a hydrogen bond between the imidazole nitrogen of the histidine side chain and the ketoenol oxygen of warfarin. The overall result of MG modification at R257 is that warfarin loses this arginine interaction, and this effect is compensated by a change in pocket conformation that pulls in H242 and K199, thus creating a tighter pocket. One interaction that remained unchanged, however, was between R222 and the lactone ketone of warfarin. Following identification of the R257 MG adduct we subsequently examined the effects of this modification in an assay sensitive to R257 function. Site-directed mutagenesis revealed that R257 is critical for HSA- mediated prostaglandin catabolism (Yang et al., 2002). MG inhibited the HSA-mediated conversion of 15-keto PGE2 to 15-keto PGA2 (Kimzey et al., 2011). MG modification at R257 likely decreases the base-catalyzed elimination reaction, as the neutral hydroimidazolone is not able to abstract a proton from carbon 10 of PGE2. The potential link between MG adduction and altered PG metabolism warrants further investigation.

Summary & Conclusions

The ability to demonstrate that the covalent adduction of a reactive intermediate derived from a therapeutic agent or an environmental chemical can alter protein structure and function is insufficient to determine whether or not such functional changes contribute to the observed adverse response. Indeed, intuitively it seems unlikely that alterations in the function of a single protein will be sufficient to elicit a toxic response. This seems especially true since our ability to detect perturbations in the transcriptome following chemical exposure reveals that many biological networks are impacted by chemical stress. Indeed, adaptive responses to stress require the engagement of multiple biological networks, tailored to the particular nature of the stress. With respect to the covalent modification of proteins by reactive metabolites, one would anticipate engagement of the endoplasmic reticulum “unfolded protein” stress response, as misfolded or denatured/damaged proteins are detected and processed. Complementary biological networks would similarly be engaged. Given such complexity in the response to chemical stress, how can one envision that loss of protein function, as a consequence of adventitious PTMs, is the critical event sufficient to cause the observed phenotypic response?

Experience from genetic knock-outs inform us that loss of a single protein can indeed have catastrophic consequences, especially during critical stages of development. Moreover, mechanisms of venom toxicity tend to be relatively specific; cardiotoxins depolarize cardiac cell membranes, leading to systolic arrest, venoms containing neurotoxins similarly depolarize axonal membranes for extended periods of time, other neurotoxins antagonize acetyl choline and block neuromuscular transmission. Specificity in these instances, and with other natural products, is likely a function of the structural complexity of these toxins, each of which interact with a target protein that contains a complementary (hand-in-glove)

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and accessible toxin binding site. Perhaps of more relevance to the present discussion, reactive aldehydes generated during lipid peroxidation, also inhibit membrane transporters, including glucose transporters and the Na^+-K^+-ATPase, the effects of which might contribute to neurodegeneration (Miyake et al., 2003; Mark et al., 1997). In addition, several small molecules target oxidative phosphorylation and the mitochondrial electron transport chain (rotenone, carbon monoxide, cyanide, oligomycin, 2,4-dinitrophenol, etc), causing a collapse in energy supply and rapid cell death. In these instances it is clear that interaction with a critical cellular target is sufficient to determine cell fate. A major challenge therefore in attempts to elucidate mechanisms of toxicity is in sifting through those responses that represent an adaptation to the chemical exposure, from those that directly lead to cell and tissue damage; with the caveat that an unsuccessful adaptive response can also facilitate overt toxicity. In the latter case however, whether toxicity is a direct consequence of the failure of the adaptive responses, or a consequence of the initial interaction of the reactive electrophile with one or more targets that can no longer be tolerated, is open for debate.

For obvious reasons the majority of studies on chemical-induced toxicities focus on the loss of protein function. Much harder to assess are those chemical-induced effects that might result in an *adverse* gain-of-function. Protein function is exquisitely regulated by phosphorylation/dephosphorylation, methylation/demethylation, acetylation/deacetylation and similar cycles. Might similar gain-of, or change-of-function contribute to reactive intermediate mediated toxicities? For example, modification of myoglobin by hydrogen peroxide converts it to an oxidase capable of catalyzing the formation of reactive oxygen species (Osawa and Korzekwa, 1991). In this context, the activation of the Nrf-2 (*N*F-E2-*r*elated *f*actor *2*) stress response pathway by protein carbonylation, and other oxidants/electrophiles, should be considered less as a gain-of-function response (Grimsrud *et al.*, 2008), and more appropriately as an adaptive response. Thus, the *function* of Nrf-2 is not altered by the electrophile-mediated alkylation of Keap 1(*K*elch-like *E*CH-*a*ssociated *p*rotein *1*), itself an electrophile sensor, but rather Nrf-2 function is *engaged* by this protein modification. In an identical fashion, 4-HNE-mediated adduction of Hsp70 and Hsp90 releases HSF-1 (heat-shock factor 1) to engage it's downstream transcriptional targets (Jacobs and Marnett 2011).

In summary, the initial interaction between electrophilic BRIs, whether they are endogenous BRIs or those derived from the bioactivation of drugs and other chemicals, and their protein target(s) triggers a pleiotropic response that includes engagement of an adaptive response. Although the adaptive response *per s*e can, on occasion, contribute to cytotoxicity (as in the case of the overactivation of poly(ADP-ribose) polymerase 1 [Luo and Kraus, 2012]), it is more frequently the failure of the adaptive response that *facilitates*, rather than *causes*, cytotoxicity. Under these circumstances, cytotoxicity is likely the consequence of the disruption of biological networks or pathways (for example a disruption in calcium and/or redox homeostasis) caused by the loss (or perhaps gain) of one or more critical functions triggered by the alteration(s) in protein function mediated by the adventitious, reactive intermediate induced PTMs.

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Abbreviations

Apaf-1	apoptosis protease activation factor-1
BRI	Biological reactive intermediate
CD	Circular dichroism
EBMs	Electrophile-binding motifs.
GSH	Glutathione
HSA	Human serum albumin
LC-MS/MS	Liquid Chromatography tandem mass spectrometry
Keap1	Kelch-like ECH-associated protein 1
MALDI-TOF	Matrix-assisted laser desorption ionization – time of flight
Methylglyoxal	MG
Nrf-2	Nuclear factor (erythroid-derived 2)-related factor 2

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