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Genomics and proteomics in chemical warfare agent research: Recent studies and future applications

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Mini review

Genomics and proteomics in chemical warfare agent research: Recent studies and future applications

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ABSTRACT

Medical research on the effects of chemical warfare agents (CWAs) has been ongoing for nearly 100 years, yet these agents continue to pose a serious threat to deployed military forces and civilian populations. CWAs are extremely toxic, relatively inexpensive, and easy to produce, making them a legitimate weapon of choice for terrorist organizations. While the mechanisms of action for many CWAs have been known for years, questions about their molecular effects following acute and chronic exposure remain largely unanswered. Global approaches that can pinpoint which cellular pathways are altered in response to CWAs and characterize long-term toxicity have not been widely used. Fortunately, innovations in genomics and proteomics technologies now allow for thousands of genes and proteins to be identified and subsequently quantified in a single experiment. Advanced bioinformatics software can also help decipher large-scale changes observed, leading to mapping of signaling pathways, functional characterization, and identification of potential therapeutic targets. Here we present an overview of how genomics and proteomics technologies have been applied to CWA research and also provide a series of questions focused on how these techniques could further our understanding of CWA toxicity.

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1. Introduction

Many questions about the medical effects of chemical warfare agents (CWAs) remain unanswered even though research on CWAs has been ongoing since World War I. As noted by a simple PubMed search (keywords “genomics” or “proteomics”), the relatively recent development of genomics and proteomics techniques has resulted in over 50,000 research studies since the 1990 s using these technologies to understand various biological processes. The development of microarrays and advances in mass spectrometry instrumentation now allow for thousands of genes or proteins to be quantified in a single experiment (Schena et al., 1998; Aebersold and Mann, 2003). Likewise, these developments also provide alternative methods of studying CWAs. However, an exhaustive database search revealed a limited number of large-scale genomics- and proteomics-related research publications focused on CWAs (Table 1).

Abbreviations: CWA, chemical warfare agent; SILAC, stable isotope-labeling with amino acids in cell culture; ICAT, isotope-coded affinity tags; MS, mass spectrometry; GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; HD, sulfur mustard; CN, cyanide; BoNT, botulinum neurotoxin; TICs, toxic industrial chemicals; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Table 1
List of systems biology-related genomics and proteomics studies used in CWA research.

CWA	Genomics/proteomics	Study
GB	Genomics	Pachiappan et al. (2009)
		Damodaran et al. (2006a)
		Damodaran et al. (2006b)
GD	Genomics	Dillman et al. (2009)
VX	Genomics	Blanton et al. (2004)
HD	Genomics	Price et al. (2009)
		Gerecke et al. (2009)
		Rogers et al. (2008)
		Yu et al. (2006)
		Dillman et al. (2006)
		Dillman et al. (2005)
		Rogers et al. (2004)
		Shahin et al. (2001)
		Lakshmana Rao et al. (1999)
		Meier and Millard (1998)
	Proteomics	Everley and Dillman (2010)
		Mehrani et al. (2009)
		Mol et al. (2008)
		An et al. (2006)
		Dillman et al. (2003)
		Dillman and Schlager (2003)
Phosgene	Genomics	Sciuto et al. (2005)

MEDLINE, ToxFile, Biosis Previews, EMBASE and CA SEARCH databases were searched for articles containing "genomic" or "proteomic" terms using the following keywords: sarin (GB), soman (GD), VX, VR, sulfur mustard (HD), chlorine, cyanide, cyclosarin (GF), and phosgene. Articles returned from the database search were filtered for CWA relevance. PubMed was further examined for relevant genomic and proteomic articles that may have been missed by the original database search.

Given the variety of unanswered questions in the chemical defense field and the recent use and promise of these technologies toward other biological applications, we expect a surge in the use of genomics and proteomics techniques to help us better understand the molecular toxicology of chemical threat agents. The more we understand about the effects of CWAs on molecular signaling, cellular function, and organ homeostasis, the better we can advance the development of medical countermeasures in the event of battlefield injuries and terrorist attacks. This review article will focus on the recent use of genomics and proteomics techniques in CWA research as well as provide discussion and additional questions that these technologies may help us answer.

2. Genomics

Genomics, transcriptomics, and gene expression profiling are focused on assaying the expression of thousands of genes simultaneously in a single biological sample by quantifying the levels of individual mRNA transcripts. The microarray is the primary tool used for these studies, with the oligonucleotide microarray (Fodor et al., 1993) and the cDNA microarray (Schena et al., 1995) being the most common formats. For oligonucleotide microarrays, the mRNA extracted from the test tissue is typically amplified using two *in vitro* reactions. The mRNA is first reverse transcribed into cDNA and then transcribed into cRNA. This second reaction utilizes labeled nucleotides (commonly biotinylated nucleotides), which are incorporated into the cRNA. The labeled cRNA is then hybridized to the oligonucleotides on the microarray and stained with a fluorescent marker. In the case where the nucleotides are labeled with biotin, the stain is comprised of fluorescently labeled streptavidin, which binds tightly to the biotin-tagged nucleotides. The fluorescent signal is detected by means of a laser array scanner. The levels of fluorescence of the experimental sample on one microarray are compared to those of the control sample on another microarray to determine which genes are up- and down-regulated.

For cDNA microarrays, the control sample and the experimental sample are labeled separately with different fluorescent dyes (e.g., Cy3 and Cy5), pooled together in equal amounts, and hybridized to the same microarray. The fluorescent signal is detected using a confocal laser scanner with dual fluorescence capability. The ratio of the two fluorescent dyes at each cDNA spot on the microarray is used to calculate the level of expression of each gene in the study and determine which genes are up- and down-regulated.

The field of toxicology has exploited the potential of microarrays to help elucidate molecular mechanisms of chemical toxicity. Microarrays are used to characterize changes in gene expression due to a toxicant of interest, identify genes up- or down-regulated, map regulatory pathways modulated by the toxicant, and, in the case of CWAs, identify potential therapeutic targets in these pathways (Thomas et al., 2001; Hamadeh et al., 2002). In addition, microarrays can be used to examine the role of microRNA (miRNA) in chemical toxicity. miRNAs are short, single-stranded RNA sequences that regulate gene expression by binding to the regulatory regions of mRNA and preventing translation, representing another mechanism of regulating gene expression besides up- or down-regulation of transcription (Fabian et al., 2010). The potential role of miRNA in CWA toxicity has not been explored.

3. Genomics applications to CWAs

Early work by Damodaran et al. suggested differential distribution of acetylcholinesterase (Damodaran et al., 2003) and alpha tubulin (Damodaran et al., 2002) mRNA expression levels in different regions of the rat brain following exposure to the nerve agent sarin (GB). While these targeted mRNA studies were restricted to only a limited set of genes, they paved the way for additional large-scale genomics analyses of GB-induced toxicity.

Consequently, Damodaran and coworkers used genomic microarrays to explore both early (15 min, 0.5× LD_{50}) and late (3 month, 1× LD_{50}) time points following GB exposure in the rat brain (Damodaran et al., 2006b). While this work identified many genomic alterations unique to each time point, seven genes were identified that were consistently altered in both time points, including Ania-9, Arrb-1, CX-3C, Gabab-1d, Nos-2a, Nrxn-1b, and PDE2. Further genomic studies by their group showed that following acute GB exposure, several gene categories – most notably those of ion channels and those of calcium channel and binding proteins – were identified in the rat brain as significantly altered (Damodaran et al., 2006a). This work went on to provide additional evidence that both degenerative and regenerative pathways are activated early after GB exposure. More recently, Pachiappan and colleagues used human neuronal cells (SH-SY5Y) exposed to low-dose GB to further explore neurodegeneration signaling (Pachiappan et al., 2009). Using microarrays, they identified over 200 genes significantly dysregulated following GB administration, and subsequent bioinformatics analysis characterized the ETS2-regulated mitochondrial death pathway as the main neurodegenerative signaling pathway activated in response to GB. It should be noted that this study was performed using a cell line and thus the gene expression profiles represent the response of a single cell type. This is in contrast to the study using brain tissue, which represents multiple cell types and thus the gene expression profiles represent a tissue response rather than the response of a single cell type. This is often the case in *in vivo* microarray studies in which an organ or tissue is isolated for microarray analysis.

A comparison of these studies reveals several similarities. Both Damodaran studies showed significant alterations in the following gene categories: those of calcium channel and binding proteins and those of cytoskeletal and cell adhesion molecules (Damodaran et al., 2006a,b). Furthermore, these two studies along with the Pachiappan study (Pachiappan et al., 2009) all showed dysregulation of mitochondrial-associated genes, including several Bcl-2-related

genes. Future genomics methods could be employed to identify additional mitochondrial genes involved, expand on any parallel pathways, and to further delineate the roles these genes play in GB-induced neuronal toxicity.

Soman (GD) is another extremely toxic nerve agent that inhibits acetylcholinesterase. While the mechanism of action is similar to other nerve agents, GD presents a unique problem in that interventional treatment must occur immediately after exposure due to a secondary reaction that results in dealkylation of the nerve agent adduct ("aging") (Loomis and Johnson, 1966). Accordingly, are there downstream signaling changes and long-term effects of this agent following acute exposure that may differ from those of GB? Is the mechanism of neurodegeneration similar to that caused by other nerve agents? Work by our group and coworkers profiled gene expression changes in rat hippocampus following GD exposure to gain insight into the molecular pathogenesis of GD-induced neurodegeneration (Dillman et al., 2009). Results showed numerous signaling mechanisms – particularly inflammatory pathways – perturbed following exposure. Many of these perturbations were still present one week post-exposure, indicating several mechanisms for future follow-up to determine their role in GD-induced neurodegeneration.

While the genomics studies performed to date have provided a wealth of information on nerve agent-induced toxicity, an accurate comparison of agents to draw valuable conclusions is difficult due to differences in exposure paradigms between the studies. Is it possible that the differences observed between GB and GD at the genomic level are due to variations in the doses of agents used in each study? Can pretreatments and therapeutics impact the long-term effects of agent exposure on gene expression? To compare and contrast differences between the long-term effects of agent neurotoxicity, future work should focus on using the same exposure paradigm for each agent. For example, an attempt should be made to provide standardization of agent exposure route (e.g. inhalation as well as intramuscular, intraperitoneal, percutaneous, and subcutaneous injections), dose, exposure time points, and similar types of prophylactics and therapeutics. Only under these controlled conditions can we accurately draw comparisons between large-scale signaling changes observed at the genomic level for different nerve agents.

Genomics may also shed light on some additional poorly understood toxic effects of nerve agents. For instance, it has been noted that civilians exposed to industrial organophosphates such as pesticides have a higher incidence of Parkinson's disease (Hatcher et al., 2008; Manthripragada et al., 2010), but it is unknown if nerve agents lead to the same neurodegenerative effects. Acute effects of nerve agents include neuroinflammation (Svensson et al., 2001; Williams et al., 2003; Chapman et al., 2006; Dhote et al., 2007; Dillman et al., 2009), but does this neuroinflammation diminish across time? Are long-term regional differences observed in the brain following nerve agent exposure reflected in large-scale gene expression changes, and can this information be used to correlate gene expression profiles with altered behavioral patterns? Genomics studies can be expanded to answer these questions and provide additional information as we come to understand the myriad of chronic effects of nerve agent exposure.

Large-scale genomics have also been applied to blistering agents such as sulfur mustard (HD). HD causes DNA damage and activates numerous signaling cascades upon exposure, most of which are poorly understood with respect to their role in HD-induced vesication. Thus, one clear benefit of microarray analysis is the potential to identify thousands of genes dysregulated in response to HD and, using mapping software, to characterize significantly altered pathways. Early microarray studies identified pathways such as inflammation, apoptosis, protein catabolism, and cell cycle as involved in HD-induced toxicity (Rogers et al., 2004; Dillman et al., 2005). Likewise, genomics efforts from our group are now focused on assessing how HD exposure dose and time affect these signaling pathways.

HD is known to cause serious cutaneous damage, but the mechanism by which this occurs at the molecular level is poorly understood. Since similar therapies have historically been used to treat thermal and HD burns, recent microarray work assessed similarities and differences between thermal- and HD-induced skin injuries (Rogers et al., 2008; Price et al., 2009). Gene expression patterns identified potential therapeutic targets for wound healing and a significant overlap in inflammatory mechanisms. For example, IL-6, IL-10, and p38 MAPK canonical signaling pathways increased in both HD and thermal burn models (Price et al., 2009), suggesting that pharmacologic and biologic modulators that target these pathways and that are currently in development for treating thermal burns may also be used to treat HD injuries. However, are there any etiological differences between thermal and HD burns such that treatment regimens for these two classes of injury may not overlap? Differences observed between thermal- and HD-induced injuries may point toward additional therapeutic targets following HD exposure.

4. Proteomics

Despite the potential of microarray approaches to assist in understanding toxicant mechanisms, their primary limitation lies in the fact that they can only assess gene expression changes. Effects due to changes in protein expression, modification, or function can only be inferred from this approach and not directly observed or measured. To assess protein expression status, techniques from the field of proteomics must be used.

Two-dimensional SDS-PAGE (2D-PAGE) has been vital to early proteomics studies. Using gels, proteins collected from cell lysates or tissues can be resolved in a two-dimensional matrix and, following staining, their intensities can be compared to determine relative abundance between samples. Due to the high variation between gels, 2D difference gel electrophoresis (2D-DIGE) has emerged to allow for different protein samples to be resolved and quantified on a single gel (Alban et al., 2003). Alternatively, protein arrays (e.g. ligand- and antibody-based arrays) also offer a means for large-scale differential analysis of proteins from complex mixtures and have already been used in protein expression monitoring, drug target discovery, and biomarker identification (Tao et al., 2007). Regardless of gel or array analysis used, qualitative information for proteins (i.e. identification) is most easily provided by mass spectrometry (MS) to further characterize changes in abundance.

The introduction of faster and more sensitive MS instrumentation has led to advances in protein identification technologies. Early protein identification methods such as Edman degradation took up to 10 hours to sequence a single peptide. With MS, high-quality sequence information for that same peptide can be obtained in less than a second. Combining liquid chromatography with MS-based analyses of complex mixtures also overcomes many of the drawbacks to gel-based methods, such as limited dynamic range and sensitivity, as well as low throughput with respect to protein identification (Peng and Gygi, 2001). The addition of stable isotopes to the proteome via metabolic and chemical labeling strategies has further empowered MS by allowing for simultaneous protein identification and highly accurate quantitation.

Metabolic and chemical labeling of the proteome with stable isotopes are most commonly used in conjunction with MS (Fig. 1). A popular and effective method of metabolic labeling is stable isotope-labeling with amino acids in cell culture (SILAC) (Ong et al., 2002). Briefly, amino acids containing either $^{12}C^{14}N$ or $^{13}C^{15}N$ are added to the growth medium for cultured cells. Several population

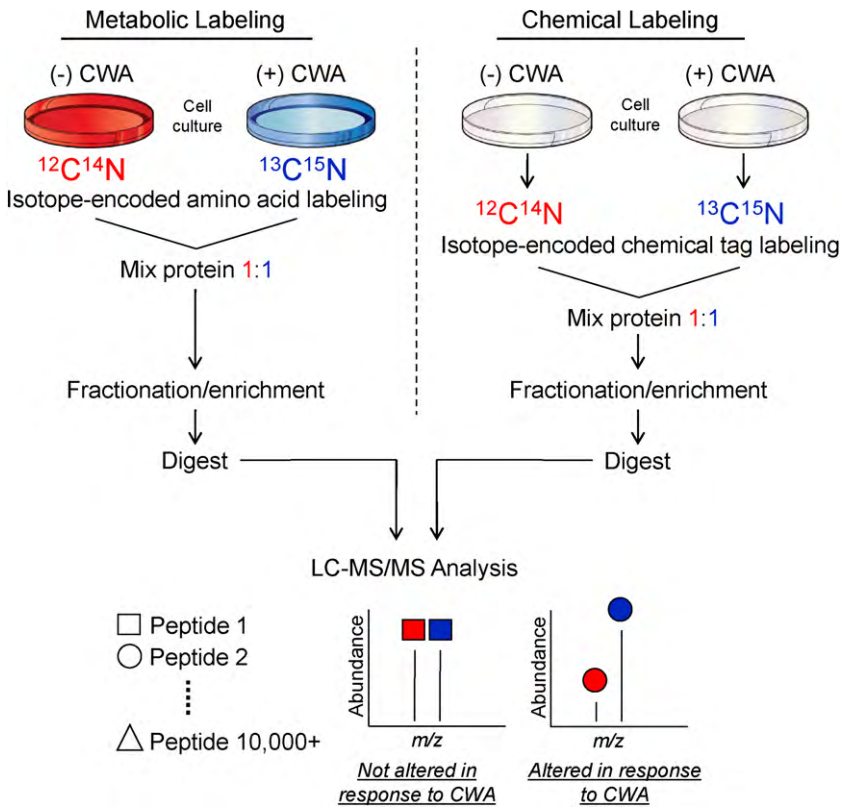

Fig. 1. Examples of common isotopic labeling strategies for use in quantitative proteomics studies. The main difference between metabolic and chemical labeling studies is the time at which labeling occurs. In metabolic labeling experiments, stable isotope-containing amino acids are added during cell culture and therefore incorporated directly into the proteome. In chemical labeling experiments, stable isotope-containing chemical tags are added after protein samples are collected. Regardless of labeling strategy, samples are combined after the labeling step at equal ratios and processed as a single sample, thus reducing variations in sample handling. After additional fractionation/enrichments steps to reduce mixture complexity and increase sensitivity, samples are proteolytically digested and processed for mass spectrometry (LC–MS/MS), which results in protein identification and quantitation. Red and blue denote isotopically "light" and "heavy" samples/peptides, respectively. Note: depending on the type of enrichment strategy used (peptide vs. protein), the fractionation/enrichment step may occur after sample digestion.

doublings allow full proteome incorporation, giving rise to isotopically "light" and "heavy" cellular proteomes. In contrast, chemical labeling strategies such as isotope-coded affinity tags (ICAT) (Gygi et al., 1999a) are performed after protein is collected. The basic concept is the same in that two distinct cell populations are differentially labeled with $^{12}C^{14}N$ and $^{13}C^{15}N$ isotopes (in this case, affinity tags that covalently bind to cysteine residues), again giving rise to isotopically light and heavy cellular proteomes. Regardless of the type of isotopic labeling strategy used, proteins are then combined at a 1:1 ratio and processed together so that any variability in sample processing will be drastically minimized. Samples can be further fractionated prior to proteolytic digestion to reduce mixture complexity and increase sensitivity in protein identification. Depending on the type of analysis, additional enrichment strategies can be used, such as those that isolate post-translational modifications. Finally, samples are analyzed using MS and bioinformatics programs that will yield protein identification and quantitation. See review article by Ong and Mann for additional information on quantitative MS (Ong and Mann, 2005).

5. Proteomics applications to CWAs

As shown in Table 1, very few studies have used proteomics approaches in CWA research. These techniques represent an untapped resource for the field since the majority of cellular functions are carried out at the protein level. Some studies have suggested that mRNA and protein levels do not always correlate (Gygi et al., 1999b; Ahram et al., 2002), further strengthening the argument for using proteomics. In fact, this RNA/protein disconnect could be relevant in CWA research. For example, Blanton and colleagues suggested that their inability to discern any permanent gene expression changes following two weeks of low-dose VX exposure could have been due to more sustained up-regulation of protein levels (Blanton et al., 2004). In addition, post-translational modifications such as phosphorylation are important in various cellular processes, with several MS-based techniques uniquely suited to carry out this analysis.

Early work focused on the use of gel-based methods to identify proteins involved in HD toxicity. Cultured keratinocytes were used as a model system to identify keratin aggregates found to crosslink with each other following HD exposure, with high-molecular weight aggregates forming in as little as 15 min (Dillman et al., 2003). Additional studies used 2D-PAGE and MS to investigate large-scale protein changes after HD exposure (Dillman and Schlager, 2003; An et al., 2006; Mol et al., 2008). Specifically, Mol and colleagues exposed human keratinocytes to ^{14}C-labeled HD (100 μM), resolved and visualized protein lysates with 2D gels and autoradiography, and used MS to identify proteins that formed adducts with HD (Mol et al., 2008). The identification of numerous cytokeratins, actin, stratifin, and galectin-7 in their study indicated that HD may alter cellular architecture, suggesting a possible role of the cytoskeleton for the onset of vesication. To further determine the role of these cytoskeletal proteins in vesication, future quantitative proteomics studies could focus on whether any of these are up- or down-regulated following HD exposure.

An and coworkers also used 2D-PAGE to analyze differential protein expression from control vs. HD-exposed (5 μL, 160 g/L) mouse ears (An et al., 2006). Complementing the Mol et al. study noted above, their results showed dysregulated expression of several cytoskeletal proteins, such as actin, destrin, and profilin-1. Furthermore, their results showed dysregulated expression of proteins involved in oxidative stress responses, apoptosis, and energy metabolism, suggesting additional pathways for future follow-up.

More recent studies by our group have incorporated the use of SILAC and phosphorylation enrichment, leading to the identification and quantitation of over 2300 phosphopeptides from HaCaT cells exposed to HD (200 μM) (Everley and Dillman, 2010). The NFκB and caspase pathways were identified in this study and have already been shown to play a role in HD toxicity (Ruff and Dillman, 2007), but many additional proteins not implicated in previous HD studies were also identified. These include DPF2 (a predicted transcription factor involved in apoptosis), ZMYND8 (a protein known to be phosphorylated upon DNA damage), and BCLAF1 (a Bcl-2 transcription factor involved in apoptosis), among others (Everley and Dillman, 2010). Determining the extent that these proteins play in HD-induced vesication and their impacts on signaling will be the topic of future work.

Even with recent proteomics work on HD, many of the molecular changes following exposure are still poorly understood. We observe numerous protein changes following agent exposure, but future studies should determine how many of these proteins have a direct role in vesication and how many are simply altered as downstream effects. We have shown that protein phosphorylation is altered on a large-scale early after exposure, but can we mine our current proteomics datasets and expand the scope of these studies to identify additional kinases activated in response to HD that may serve as effective therapeutic targets? Bioinformatics software programs such as Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA) are vital to mining large-scale datasets and will prove to be even more valuable with the continued expansion of MS-based technologies, with a goal of providing a more global characterization of the proteome.

Multiplex-labeling proteomics strategies such as iTRAQ (Ross et al., 2004) have also gained prominence in recent years and now allow up to eight different samples to be simultaneously characterized using MS. Instead of using Western blots to analyze a series of pre-selected proteins for time-course studies, iTRAQ and MS could be used to assess hundreds or even thousands of proteins and how their levels change following different HD exposure times, all in a single experiment. iTRAQ and ICAT can also be applied to the analysis of tissues from HD-exposed animal models to discover and understand *in vivo* changes on a large-scale. We are confident that further insight will be gained as proteomics technologies mature, giving researchers the potential to characterize additional proteins and piece together signaling pathways that play a role in HD-induced toxicity.

6. Other agents of interest

This review has focused on nerve and blister agents, but there are numerous other classes of chemical threats for which genomics and proteomics analyses could be helpful. For example, toxic industrial chemicals (TICs) pose a unique risk due to their ease of production and acquisition, and their acute toxicity. This class includes bromine (Sagi et al., 1985), chlorine (Squadrito et al., 2010), cyanide (Baskin and Rockwood, 2002), carbonyl chloride (phosgene) (Borak and Diller, 2000), and pesticides such as parathion and chlorpyrifos (Ballantyne and Marrs, 1992).

One TIC of particular concern is cyanide (CN), an extremely lethal blood agent that is easy and inexpensive to produce (Baskin and Rockwood, 2002). The effects of CN can appear within seconds of exposure and particularly affect the heart and brain. Early treatments with nitrites are effective but can produce central nervous system side effects, such as altered motor function and learning behaviors. However, an attempt to understand these long-term side effects at the molecular level in different regions of the brain has not been undertaken. This knowledge gap could be addressed by the use of genomics and/or proteomics technologies to determine the molecular effects of various CN exposure therapies, which could in turn shed light on which therapies are most effective with the fewest long-term side effects.

Another TIC of concern is phosgene, which is widely used in industry for the production of synthetic products and which, if inhaled, can cause pulmonary edema and irreversible lung injury (Sciuto et al., 2005). Microarray analysis showed that one of the most significantly altered genes is glutamate cysteine ligase (GCL) catalytic subunit, the rate-limiting enzyme in the synthesis of glutathione. These results were cross-validated with biochemical data from a previous study (Sciuto et al., 2003), confirming the biological significance of this result. Furthermore, other genes involved in glutathione synthesis and maintenance of cellular redox status were significantly increased, indicating a molecular response consistent with oxidative stress. These results provide the basis for pursuing therapeutic strategies that target redox systems in the lung as a medical countermeasure for chemically induced lung injury.

Aside from TICs, other chemicals of biological origin pose similar threats as CWAs. Among these is botulinum neurotoxin (BoNT), a 150 kDa protein that blocks acetylcholine release from neurons and can eventually cause severe paralysis and death if introduced at high enough doses (Simpson, 2004). BoNT intoxication renders neurons incapable of communication with muscle cells, eventually resulting in changes in cell morphology (Meunier et al., 2003). Since this in turn suggests changes in cell signaling, what are the transcriptional and translational pathways activated in response to this blockade? Do they influence long-term resumption of synaptic activity? Current genomic and proteomic studies are ongoing at USAMRICD to help answer these questions and others. The fact that BoNT is one of the most lethal substances known – the LD_{50} (ng/kg) is over 1000-fold lower than that of VX – underscores the importance of understanding both its short- and long-term effects.

7. Conclusions

Transcriptomics-related CWA research over the past several years has spanned a variety of chemical threats. This work has provided insight into mechanisms of acute vesicant toxicity and also mechanisms of nerve agent-induced brain injury. In addition, work with phosgene has revealed potential therapeutic strategies to treat chemically induced lung injury.

Proteomics-related CWA research has been predominantly focused on HD. Surprisingly, large-scale proteomics studies using nerve agents have been lacking. Just as microarrays have been used to map signaling networks for GB and GD, proteomics studies could also complement and expand on that work to identify additional signaling pathways impacting agent toxicity. Furthermore, genomics and proteomics could be applied to the remaining nerve agents, such as cyclosarin (GF) and VR, to determine if these agents have similar long-term effects at the molecular level as other agents.

CWAs have been a threat since WWI and continue to be a threat today. Medical research on these agents has led to treatments to improve survivability, but many questions remain with respect to mechanisms of toxicity at the molecular level. We are confident that as technologies improve and techniques become more commonplace, the use of genomics and proteomics will further accelerate

our understanding of CWA toxicity and ultimately lead to the identification of candidate pathways for better targeted therapeutic intervention.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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