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**Optimization of Quantitative
Proteomics Using 2-Dimensional
Difference Gel Electrophoresis to
Characterize Molecular Mechanisms
of Chemical Warfare Nerve Agent
Exposure in the Rat Brain**

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Summary

Rational development of medical countermeasures for chemical warfare nerve agent (CWNA) exposure requires a thorough understanding of the pathological, biochemical and molecular alterations that occur in those tissues affected by such exposure. Although pathological outcomes following soman exposure have been extensively studied, molecular consequences of CWNA exposure are not well understood. Proteomic approaches utilizing traditional two-dimensional (2-D) gel electrophoresis have been used to study the effects of sulfur mustard. However, there are no publications using proteomic approaches to study effects of CWNAs. This report describes the optimization and use of 2-dimensional difference gel electrophoresis (2-D DIGE) to investigate the proteomic changes induced by CWNA in whole cell homogenates and isolated mitochondria from rat brain regions. Rats were pretreated with the oxime HI-6, exposed to a seizurogenic dose of the CWNA soman, and then administered atropine methyl nitrate (AMN). Control animals were given HI-6, an equivalent volume of agent vehicle (saline), and AMN. Brain tissue was dissected into anatomical regions and either homogenized or processed for mitochondrial isolation. Proteins were fluorescently labeled, separated in two dimensions, and analyzed to quantify changes in protein abundance. Increases and decreases in protein abundance, as well as changes in protein isomer form, were observed in response to soman exposure, HI-6/AMN treatment, and/or soman exposure in the presence of HI-6/AMN treatment. The proteomics approach described in this report in combination with mass spectrometry has recently been used to identify proteins and pathways altered by exposure to CWNA.

Introduction

Chemical weapon nerve agents (CWNAs) are organophosphorus (OP) cholinesterase inhibitors that cause a progression of symptoms including hypersecretions, loss of consciousness, flaccid paralysis, and seizures [1]. Exposure to CWNAs causes inhibition of acetylcholinesterase and an ensuing increase of acetylcholine (ACh) concentration, resulting in seizure activity in susceptible brain regions. Further seizure activity causes the release of excess glutamate, leading to glutamate-induced excitotoxicity and neuronal death. Brain areas found to sustain considerable damage after acute soman (GD) exposure include the hippocampus, piriform cortex, septum, entorhinal cortex, dentate gyrus, amygdala, and thalamus [2-5]. Brain damage from nerve agent exposure includes necrotic and apoptotic cell death, gliosis, cytoplasmic vacuolization, and mitochondrial swelling with vacuolization ([6-7] and Kan *et al.*, unpublished data). CWNA-induced brain damage can cause profound cognitive and behavioral changes that can drastically reduce quality of life for CWNA exposure survivors [8].

Potential exposure to CWNAs exists both on the battlefield and in civilian populations through terrorist attacks. There is an urgent need to develop medical countermeasures to protect both the soldier and civilian from the damaging effects of nerve agents. Currently, one prophylactic (pyridostigmine) and three therapeutic drugs (atropine, pralidoxime chloride, and diazepam) are used to counteract nerve agent exposure. However, these drugs do not completely prevent the neuronal and cardiac damage induced by nerve agent exposure [9]. To efficiently develop medical countermeasures to CWNAs, a thorough understanding of the pathological, biochemical, and molecular alterations in the brain after nerve agent exposure is needed. Although pathological outcomes following soman (GD) exposure have been extensively studied, molecular consequences of CWNA exposure are not well understood. Identification of the molecular changes occurring in response to CWNA exposure provides novel targets for the protection and/or restoration of neuronal function.

Modern genomic and proteomic techniques allow large-scale identification of molecular changes induced by disease or insult. Genomics is the study of changes in messenger RNA (mRNA) transcript level due to changes in gene regulation or transcript stability. Proteomics is the study of the entire protein complement in a cell or organelle and includes changes in protein expression as well as post-translational modifications (e.g., phosphorylation, glycosylation, etc.). Although the genomic approach is higher throughput, proteomics has the advantage of better predicting functional changes since mRNA levels do not necessarily reflect changes in protein expression and cannot account for post-translational modification of proteins [10]. There are 3 quantitative methods frequently used for proteomic analysis: 2-dimensional (2-D) difference gel electrophoresis (DIGE), isotope-coded affinity tag (iCAT), and isobaric tags for relative and absolute quantification (iTRAQ; [11]). The iCAT and iTRAQ methods are liquid chromatography-based, whereas 2-D DIGE is gel-based. For 2-D DIGE, the free amino group of lysine residues in proteins is labeled with fluorescent dyes (Cy2, Cy3, and Cy5). Unlike traditional 2-D gel electrophoresis, 3 samples are run on each gel (2 experimental samples and an internal control). The fluorescently labeled internal standard increases gel alignment confidence for improved spot matching and minimization of gel-to-gel variation for quantitation.

Proteomic approaches utilizing traditional 2-D gel electrophoresis have been used to study the effects of the chemical warfare agent sulfur mustard on human epidermal keratinocytes (HEKs; [12-14]) and on mouse ears [15]. In addition, a mass spectrometry-based proteomic

approach using stable isotope labeling with amino acids in cell culture (SILAC) has also been used to study sulfur mustard exposure of HEK cells [16]. However, there are no publications for the use of proteomics to examine the effects of CWNA such as soman, sarin, tabun, cyclosarin, VX or VR *in vivo* or *in vitro*. In this report, the optimization and application of 2-D DIGE for identification of rat cellular and mitochondrial proteins and pathways altered by exposure to CWNA in anatomical brain regions will be presented.

Materials and Methods

Soman (GD) Administration

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; CRL: CD [SD]-BR, 250 - 350 g) were used in this study. GD (GD-U-2323-CTF-N, purity 98.8 wt%) was obtained and diluted in saline at the United States Army Medical Research Institute of Chemical Defense (USAMRICD, Aberdeen Proving Ground, MD). Thirty minutes prior to GD challenge, rats were pretreated with the oxime HI-6 (Sigma-Aldrich, St. Louis, MO; 125 mg/kg, i.p.). A convulsive dose of soman (1.6 LD₅₀; 180 µg/kg, s.c.) was used to induce seizures in 100% of exposed animals. One minute after GD challenge, animals were treated with atropine methyl nitrate (AMN; Sigma-Aldrich, St. Louis, MO; 2.0 mg/kg, i.m.). Vehicle control animals received an equivalent volume of agent vehicle (saline), HI-6 and AMN. Naïve control animals did not receive any injections. Rats were observed for convulsive activity to determine time of seizure onset.

Tissue Procurement

Animals were deeply anesthetized with sodium pentobarbital (200 mg/kg, i.p.) at 0.5, 1, 3, 6, 12, 24, or 48 hours after seizure onset. Vehicle controls were also taken at these time points, whereas naïve controls were taken at any time point. Brain tissue was dissected to isolate anatomical regions of the hippocampus, thalamus, and piriform cortex. Brain regions were immediately snap-frozen in liquid nitrogen with the exception of the hippocampus, thalamus, and piriform cortex tissues that were designated for mitochondrial isolation. Those were processed immediately after dissection.

Mitochondrial Isolation

Mitochondria were isolated by homogenization and differential centrifugation using a protocol modified from C.P. Lee [17]. All steps were completed at 4°C. Brain tissue was bathed in 1.5 mL of isolation buffer (150 mM sucrose, 10 mM HEPES, 1 mg/ml bovine serum albumin fraction V, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.3), minced finely with scissors, and transferred to a pre-cooled hand-held glass dounce homogenizer. The pestle was passed through the dounce until the solution was uniform. The solution was transferred to microcentrifuge tubes and centrifuged at 2,000 x g for 3 minutes. The supernatant was placed into a new microcentrifuge tube and centrifuged at 12,000 x g for 8 minutes. After removing the supernatant, the pellet was resuspended in 1 mL of isolation buffer and centrifuged at 12,000 x g for 10 minutes. The resulting pellet was resuspended in 1 mL of 250 mM sucrose and was centrifuged at 12,000 x g for 10 minutes. The final pellet containing the isolated mitochondria was resuspended in 100 µl 250 mM sucrose and 10% (v/v) DMSO and slowly frozen to -80°C.

Transmission Electron Microscopy for Isolated Mitochondria

Isolated mitochondria were fixed in 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide/0.1M sodium cacodylate buffer, dehydrated in graded ethanol, and embedded in PolyBed 812® epoxy resin (Polysciences, Inc., Warrington, PA). Sections were imaged using a JEOL 1230 transmission electron microscope (JEOL USA, Peabody, MA).

Brain Region Homogenization

Hippocampus and piriform cortex brain regions were homogenized in 15 µl of lysis buffer (20 mM HEPES, 1 mM EDTA, 2 mM EGTA, 150 mM NaCl, 0.1% SDS, 1% IGEPAL, 0.5% deoxycholic acid, 1 mM DTT, pH 3.5, supplemented with cOmplete™ protease inhibitor cocktail tablet from Roche Diagnostics Corp. [Indianapolis, IN]) for each milligram of tissue. Steel beads were loaded into screw-cap tubes (1-4 for 2 mL tubes with at least 0.5 mL of sample, 4-7 for 0.5 mL tubes), and the brain region and appropriate volume of lysis buffer were added. The samples were homogenized using the Mini Beadbeater™ (BioSpec Products, Inc., Bartlesville, OK) in a cooled block with two 30-second pulses with a 15-minute pause between pulses. The samples were incubated on ice for at least 30 minutes and centrifuged at 2,000 x g for 3 minutes, and the supernatant was transferred to a new tube. Protein concentrations were determined using the EZQ™ Protein Quantitation Kit following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Samples were stored at -80°C until further processing.

2-Dimensional Gel Electrophoresis Sample Preparation

For 2-D gel electrophoresis, 25 µl of mitochondrial sample was transferred to a microcentrifuge tube and centrifuged at 15,000 x g for 10 minutes. The mitochondrial pellet was resuspended in 25 µl of the appropriate lysis buffer and incubated at room temperature for 30 minutes to fully solubilize the proteins. For some experiments, we used destreak lysis buffer consisting of Destreak™ Rehydration Solution with 0.5 to 2% IPG Buffer (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ). For difference gel electrophoresis (DIGE) and other experiments, we used DIGE lysis buffer, which consisted of 30 mM Tris-HCl, 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS, pH 8.5.

For homogenized brain regions, approximately 100 µg total protein was desalted using the 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. Pelleted protein samples were resuspended in 50 µl of DIGE lysis buffer and incubated at room temperature for at least 30 minutes to fully solubilize the proteins.

After proteins were solubilized in DIGE lysis buffer, the pH of the solution was checked by spotting 0.5 µl onto a Hydrion® Brilliant pH dip stick (pH 5.0-9.0, Sigma-Aldrich) to verify that it was pH 8.5. If the pH was too low (< pH 8.0), DIGE lysis buffer (pH 9.5) was added to raise the pH. Protein concentrations were determined using the EZQ™ Protein Quantitation Kit following the manufacturer's instructions (Invitrogen Corp.). IPG buffer (0.5 or 1.0% (v/v) final concentration) and Destreak™ Reagent (2% (v/v) final concentration; GE Healthcare) were added to the DIGE lysis buffer prior to electrophoresis.

Labeling Protein with the CyDyes for DIGE

The proteins for DIGE experiments were labeled using the CyDye™ DIGE Fluor minimal labeling kit (GE Healthcare). Recommended protein concentration for labeling is 5-10 µg/µl, although concentrations of 1-20 µg/µl have been successfully labeled. The CyDyes were reconstituted in fresh dimethylformamide (DMF, Sigma-Aldrich) according to the instructions provided with the labeling kit. To label 50 µg of protein, 1 µl of 400 µM CyDye working solution is required. As little as 5 µg of protein can be labeled (0.1 µl CyDye working solution). For analytical purposes, only 1 to 5 µg of labeled protein per CyDye should be loaded when focusing samples on 11-cm IEF gels. Half of the samples from each group were labeled with Cy3, and the remaining half was labeled with Cy5. Routinely, 15 µg of protein was labeled with 0.3 µl CyDye working solution. The Cy2 dye was used to label the internal standard, which consisted of equal amounts of all samples in the experiment. The following calculations were used to determine the amount of each sample to use for the pooled sample:

$$(15 \mu\text{g pooled sample/gel}) \times (\text{number of gels in the experiment}) = Y \mu\text{g pooled sample} \\ \text{number of gels in the experiment} = (\text{number of samples in experiment}) \div 2$$

To account for pipetting error (10%):

$$Y \mu\text{g pooled sample} + (0.1 \times Y) = Z \mu\text{g pooled sample total}$$

$$\text{Protein amount } (\mu\text{g}) \text{ of each sample in the experiment to add to the pooled sample tube} = \\ Z \mu\text{g pooled sample total} \div \text{number of samples in experiment}$$

To label the internal standard, 0.02 µl CyDye working solution was used for each microgram of total protein in the pooled sample.

The protein samples were mixed with the appropriate amount of CyDye and incubated on ice in the dark for 30 minutes. The labeling reaction was stopped using a CyDye volume equivalent of 10 mM lysine in water. After addition of lysine, the samples were incubated on ice in the dark for 10 minutes. The labeled protein was either used immediately for isoelectric focusing or stored at -80°C for up to 3 months.

Prior to loading onto the IEF gel, the volume of the labeled proteins was adjusted to a concentration of 1 µg/µl using DIGE lysis buffer. Cy3- and Cy5-labeled samples were randomly paired, and 5 µg of each labeled sample was combined with 5 µg of Cy2-labeled internal standard. The volume was adjusted to 200 µl by addition of Destreak™ Rehydration Solution and the appropriate amount of IPG buffer for the desired final concentration of ampholytes.

The First Dimension: Isoelectric focusing (IEF)

First dimension isoelectric focusing (IEF) was carried out on an Ettan™ IPGphor™ IEF system (GE Healthcare) at 20°C and 50 µA per strip. The 200 µl samples were loaded onto 11-cm Immobiline™ DryStrip™ gels pH 3-10 (GE Healthcare) by active rehydration at 30 V for 10 hours. For mitochondrial studies, the IEF program consisted of a hold at 300 V for 1 minute, a 300-3500 V gradient for 1.5 hours, and a hold at 3500 V for 4.5 hours [18]. For brain region homogenates, the IEF program consisted of a hold at 500 V for 1 hour, a 500-1000 V gradient for 1 hour, a 1000-6000 V gradient for 2.5 hours, and a hold at 6000 V for 50 minutes ([12] and personal communication). The strips were equilibrated in SDS equilibration buffer (50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, pH 8.8) containing 1% (w/v) DTT by rocking at room

temperature in the dark for 15 minutes. This equilibration step was repeated with SDS equilibration buffer containing 2.5% (w/v) iodoacetamide. After the equilibration steps, any strips to be stored at -80°C were washed in gel electrophoresis running buffer and placed plastic side down in a foil-covered glass dish. Prior to running the second dimension, frozen strips were brought to room temperature.

The Second Dimension: SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Second dimension SDS-PAGE was performed using 12% XT Bis-Tris Criterion precast gels with the IPG + 1 well comb and XT MOPS buffer (Bio-Rad Laboratories, Inc., Hercules, CA). Precision Plus unstained protein standards (Bio-Rad) were used to avoid potential fluorescence interference from stained protein standards. After removal of the comb, the well for the Immobiline™ DryStrip™ was filled half way with running buffer and any visible bubbles were removed using gel loading pipet tips. The Immobiline™ DryStrip™ was carefully loaded into the well using the Wonder Wedge separation tool (Hoefer, Inc., San Francisco, CA), and any bubbles present between the drystrip and the well bottom were removed. The first dimension strips were sealed into the second dimension gel using 0.5% agarose containing 1% bromophenol blue. The gels were run at 150 V (constant voltage) for 2 hours and 15 minutes.

Gel Imaging and Analysis

Deep Purple™ Total Protein Stain

The Deep Purple™ total protein stain (GE Healthcare) was used as described in the manufacturer's instructions to visualize proteomic maps during optimization of 2-D gel electrophoresis conditions. Deep Purple™ is an environmentally friendly fluorescent stain with high sensitivity, low background fluorescence without speckling, and mass spectrometry compatibility. Gels were fixed in 7.5% (v/v) acetic acid, 10% (v/v) methanol with gentle rocking at room temperature for at least 1 hour. For maximum sensitivity, the fixation step was carried out overnight. The gels were washed with 200 mM sodium carbonate in a foil-covered dish at room temperature for 30 minutes. The wash solution was removed, and 0.5% (v/v) Deep Purple™ total protein stain in water was added. Gels were incubated at room temperature with gentle agitation for 1 hour and washed twice with 7.5% (v/v) acetic acid at room temperature for 15 minutes. Proteins were visualized on a Typhoon Trio Plus imager (GE Healthcare) using the 532 nm laser and 610 BP 30 emission filter. Final images were scanned at a 100-micron resolution with the photomultiplier tube (PMT) set for maximum pixel intensity between 35,000 and 60,000 to avoid saturation.

DIGE

After 2-D gel electrophoresis, the gels were rinsed in deionized water and transferred to the Typhoon Trio Plus imager. The Cy2-labeled pooled sample proteins were detected using the 488 nm laser and 520 BP 40 emission filter. Cy3- and Cy5-labeled proteins were detected using the 532 nm and 633 nm lasers and 580 BP 30 and 670 BP 30 emission filters respectively. The PMT setting was optimized for each CyDye for maximum pixel intensity between 35,000 and 60,000 at 1000 micron resolution to avoid saturation. For DIGE experiments that continued for more than one day, scanning parameters were optimized using the first gel of the day. For final images, the automated file naming for DIGE option was chosen in the scanning software and gels were scanned at a 100-micron resolution.

The DIGE gel scans were analyzed using DeCyder™ 2-D differential analysis software v6.5 (GE Healthcare) in the batch processing mode. This software automates spot detection and matching across samples and gels and measures protein differences with statistical confidence. Proteins were identified that exhibited increases or decreases in abundance of 1.2-fold or greater with student's t-test p-values ≤ 0.05 . To determine the proteomic changes induced by GD, the proteomic maps from animals that received HI-6, GD, and AMN were compared to proteomic maps from time-matched animals that received HI-6, saline, and AMN (vehicle control animals). To identify proteomic changes induced by HI-6 and AMN, proteomic maps from animals that received HI-6, saline, and AMN were compared to proteomic maps from animals that did not receive any injections (naïve control animals). To determine the combined effects of GD in the presence of HI-6/AMN treatment, proteomic maps from animals that received HI-6, GD, and AMN were compared to proteomic maps from naïve control animals.

Colloidal Coomassie Brilliant Blue

Visualization of proteins after 2-D gel electrophoresis by colloidal coomassie brilliant blue staining was used for both analysis and spot picking. Gels were incubated in 20% methanol, 8% ammonium sulfate, 1.6% phosphoric acid, 0.08% coomassie brilliant blue G-250 at room temperature with gentle agitation for at least 3 hours. The stained gels were washed several times in deionized water at room temperature and incubated overnight in deionized water to eliminate background staining. For some experiments, the stained gels were dried using the Invitrogen large gel drying kit (Invitrogen Corp.) according to the manufacturer's instructions. Coomassie brilliant blue stained gels were imaged with the Typhoon Trio Plus imager using the 633 nm laser without a filter. Final images were scanned at a 100- or 200-micron resolution with the PMT set for maximum pixel intensity between 35,000 and 60,000 to avoid saturation.

Results and Discussion

Isolation of Mitochondria

Mitochondria were isolated from whole brain and brain regions using a protocol modified from C.P. Lee and colleagues [17]. The published protocol was optimized for whole rat brain, and purity was assessed using various biochemical assays. For isolation of mitochondria from brain regions, the homogenization and final resuspension volumes were adjusted from the published protocol as described in the materials and methods section. To assess mitochondrial purity and integrity, isolated mitochondria were processed for transmission electron microscopy (TEM) and analyzed for ultrastructure. The highest purity of intact mitochondria obtainable was required for our proteomic studies. In an attempt to increase the purity of mitochondria, two discontinuous gradients (percoll and sucrose) were tried after the differential centrifugation isolation. For both gradients, two distinct bands were observed (Figure 1A and 1B). The brown bands were collected and processed for TEM. Electron micrographs after either gradient revealed intact, but swollen mitochondria (Figure 1C and data not shown). Two commercially available mitochondrial isolation kits (MitoSciences, Inc., Eugene, OR and Pierce subsidiary of Thermo Fisher Scientific, Inc., Rockford, IL) were also compared to our differential centrifugation protocol using TEM. Mitochondria isolated with either kit were extremely swollen with all cristae structure absent, and membrane integrity was often compromised (data not shown).

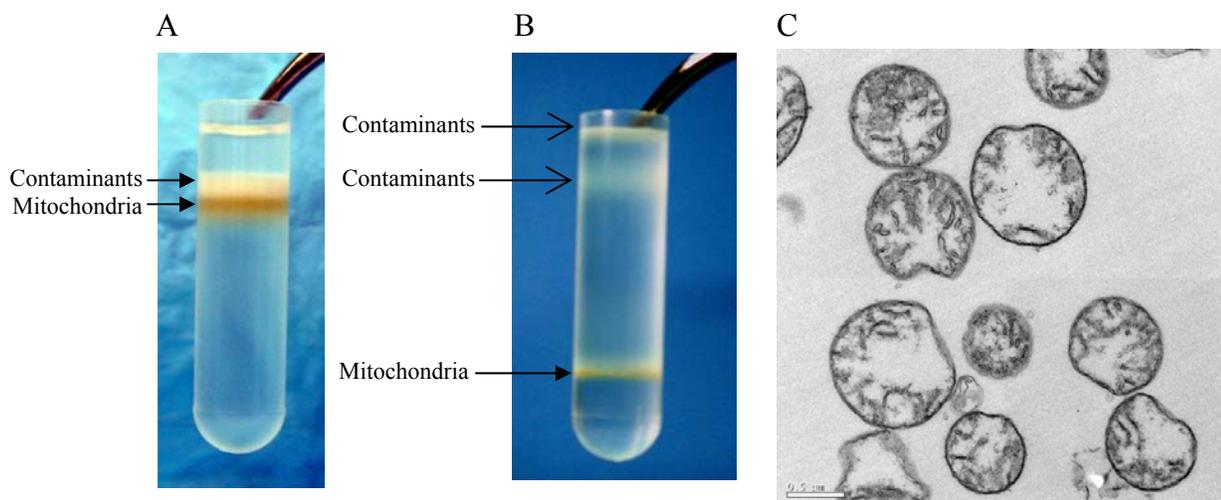


Figure 1: Discontinuous gradient purification of mitochondria causes mitochondrial swelling. A) Percoll gradient after ultracentrifugation. The arrows point to the contaminating fraction (top) and the mitochondrial fraction (bottom). B) Sucrose gradient after ultracentrifugation. Arrows with open heads indicate the contaminating fractions. The arrow with the filled head indicates the mitochondrial fraction. C) TEM micrograph of percoll purified mitochondria.

After thorough evaluation of TEM micrographs, mitochondria isolated using our modified differential centrifugation procedure were of high purity and maintained membrane integrity. Therefore, all mitochondria used in the proteomic studies were isolated by differential centrifugation as described in the materials and methods section. Interestingly, four conformations of mitochondrial morphology were observed: an *in vivo* conformation with distinct cristae structure, a conformation indicative of state 3 respiration, a conformation indicative of state 4 respiration, and a condensed form of mitochondria (Figure 2). The mitochondrial morphology conformations observed have been previously described for isolated mitochondria from rat liver [19].

2-D Gel Electrophoresis of Mitochondrial Proteins

The protocol used to separate the mitochondrial proteins by 2-D gel electrophoresis was based on the published work by A. Venkatraman and colleagues [18]. Although this protocol resulted in resolved protein spots, other conditions were tried to optimize resolution of the mitochondrial proteome. The first optimization experiment compared Tris-HCl SDS-PAGE with Bis-Tris SDS-PAGE criterion gels. The Bis-Tris gels provided better protein spot resolution than the Tris-HCl gels (Figure 3) and were therefore used for all subsequent experiments.

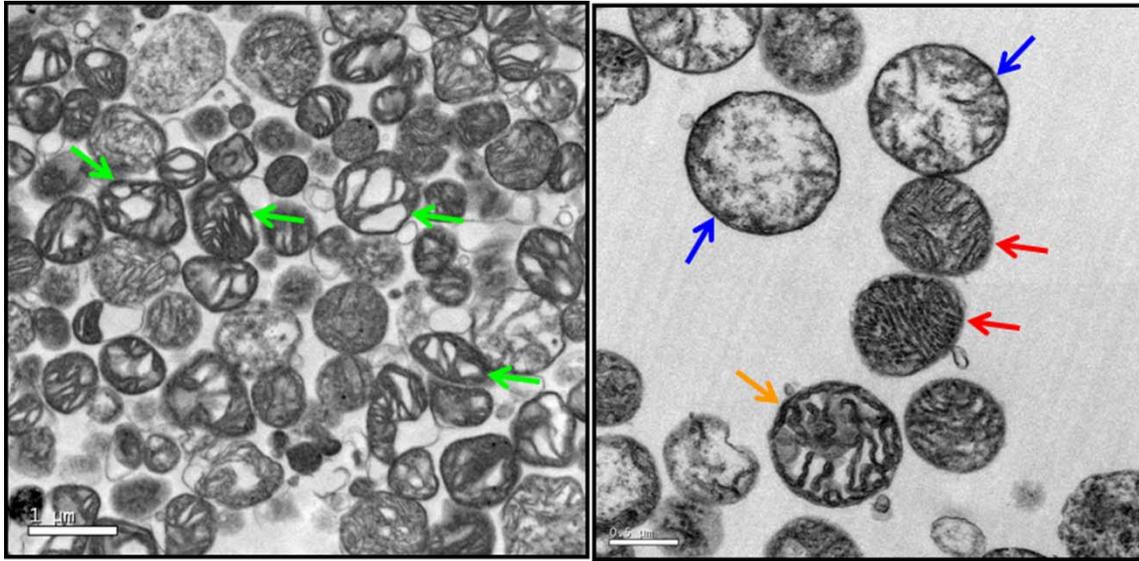


Figure 2: Mitochondria isolated using differential centrifugation remain intact. Four conformations are observed in the electron micrographs of isolated mitochondria. The red arrows depict mitochondria in an *in vivo* conformation with distinct cristae structure. Mitochondria in state 3 (green arrow) and in state 4 (blue arrow) respiration are also present. Occasionally, a condensed form of mitochondria (orange arrow) is also observed.

The second optimization experiment for mitochondrial samples compared concentrations of IPG buffer and also tested gel-to-gel reproducibility. IPG buffer concentrations from 0.5% to 2% were tested to determine which concentration provided the best resolution. Concentration of IPG buffer at either 0.5% or 2% reduced resolution of the proteomic map, whereas 1% and 1.5% IPG buffer resulted in comparable resolution (Figure 4). At 1% IPG buffer, the 2D gels were very reproducible, with identical protein spot patterns (data not shown). Based on these results, all mitochondrial samples were prepared in 1% IPG buffer.

The final optimization experiment investigated the different first dimension Immobiline™ DryStrip™ gel conditions. Separation of the mitochondrial proteins on pH 3-10, pH 3-11 non-linear, pH 6-11, and pH 4-7 was tested to determine which pH range was optimal for the mitochondrial proteome. The pH 3-11 non-linear strip spaced the majority of the spots closer to one another and therefore did not provide an increase in resolution over the pH 3-10 strip (data not shown). Basic mitochondrial protein spot resolution was lost on the pH 4-7 strip and acidic mitochondrial proteins lost resolution on the pH 6-11 strip (data not shown). The only strip that provided proper resolution of the mitochondrial spots was the pH 3-10 DryStrip™, and that strip was used for all subsequent experiments.

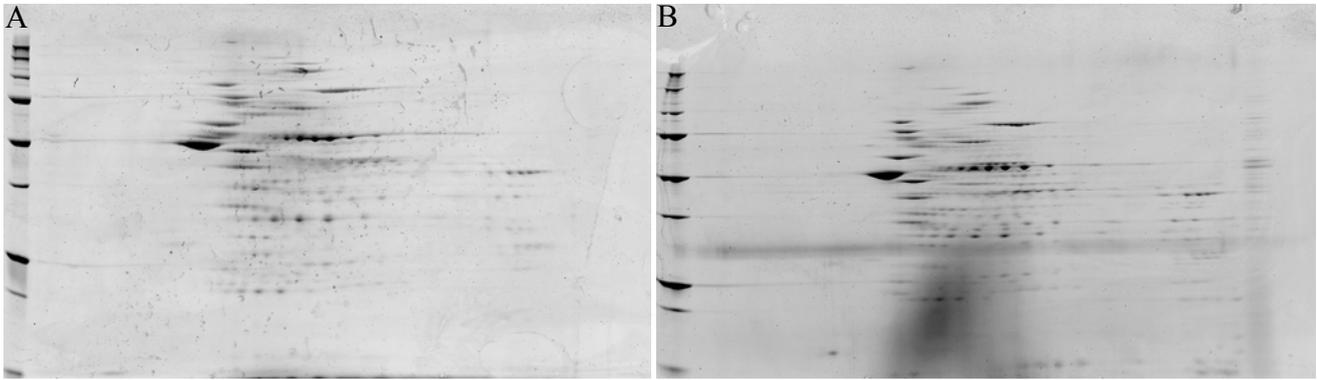


Figure 3: Bis-Tris Criterion gels provide better resolution than Tris-HCl Criterion gels. Both (A) Tris-HCl and (B) Bis-Tris Criterion gels were run with the same mitochondrial protein sample (50 μ g) and visualized with Deep Purple™ total protein stain as described in the materials and methods.

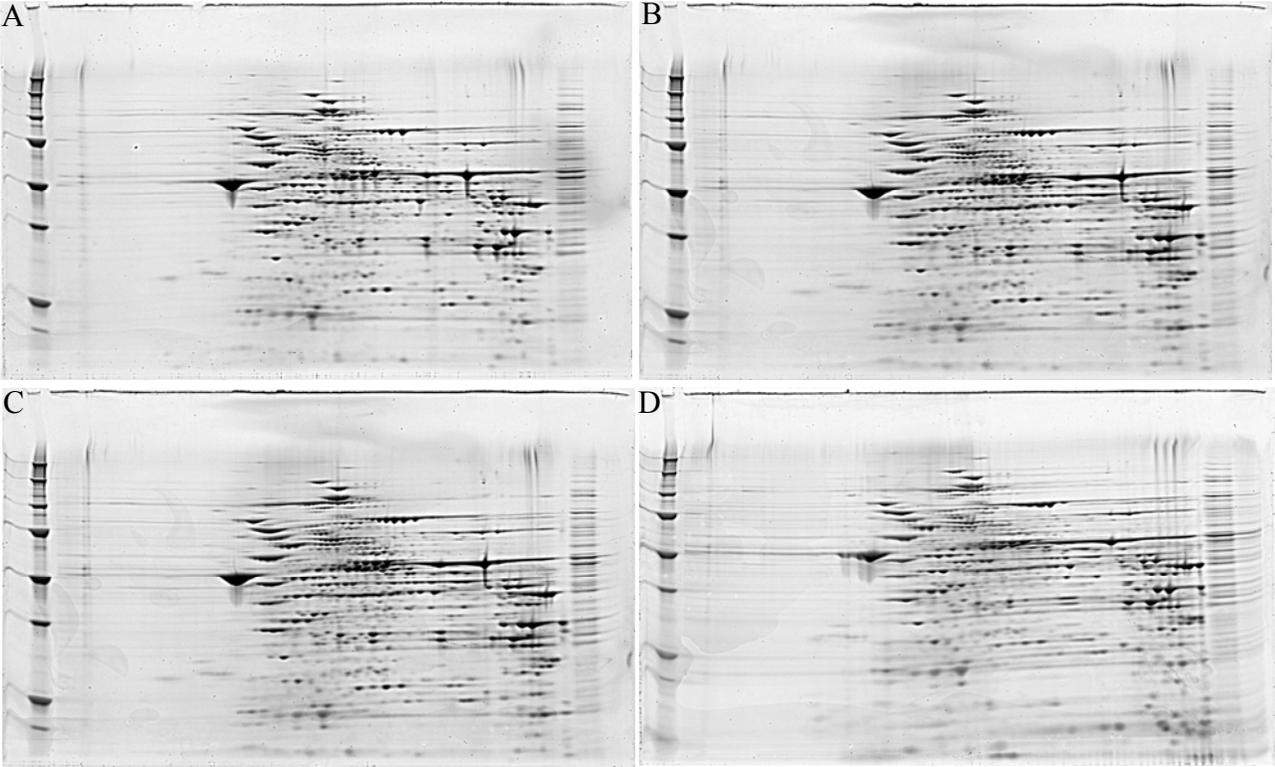


Figure 4: Optimal resolution for mitochondrial proteins is achieved with 1% IPG buffer. All gels were run with the same mitochondrial protein sample (75 μ g) and visualized with Deep Purple™ total protein stain as described in the materials and methods. A) 0.5% IPG buffer. B) 1% IPG buffer. C) 1.5% IPG buffer. D) 2% IPG buffer.

2-D Gel Electrophoresis of Total Cellular Proteins

The 2-D gel electrophoresis procedure used to separate cellular proteins from brain region homogenates was based on the work of Dillman and colleagues ([12-13] and personal communication). The first optimization experiment verified the proper pH range for the IEF gels. The pH 3-10 Immobiline™ DryStrip™ (GE Healthcare) is the commercially available IEF gel that covers the widest linear pH range. Cellular proteins from brain region homogenates separated on this first dimension gel reveal protein spots between approximately pH 4 and pH 9 (Figure 5). This pH range is similar to that observed for the mitochondrial proteins. Selection of the pH range for the subsequent 2-D gel electrophoresis experiments was limited to those pH gradients that were commercially available to ensure reproducibility within an experiment. Based on the mitochondrial results with the pH 3-11 non-linear strip and the pH range needed to resolve the brain region homogenate proteins, the linear pH 3-10 Immobiline™ DryStrip™ (GE Healthcare) was used for all 2-D gel electrophoresis experiments.

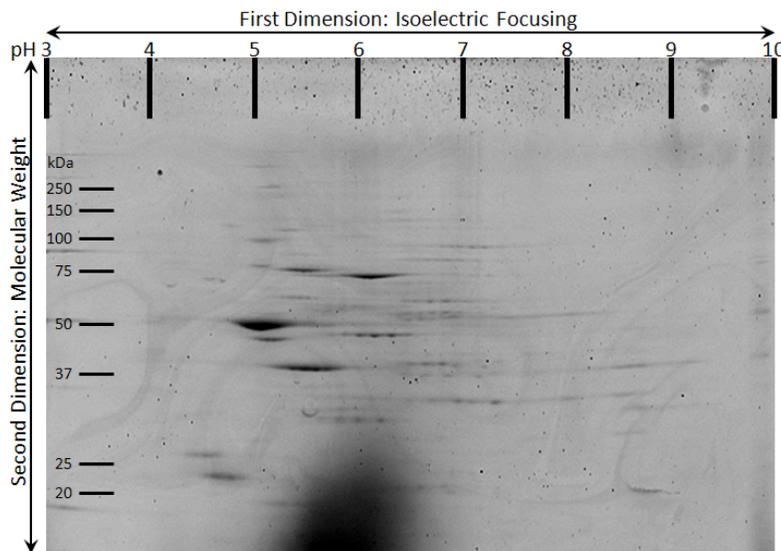


Figure 5: Brain region homogenate proteins are optimally resolved using linear pH 3-10 IEF gels. Piriform cortex homogenate proteins (35 µg) separated in the first dimension by isoelectric point between pH 3 and 10 and in the second dimension based on molecular weight were visualized using coomassie brilliant blue as described in the materials and methods.

The second optimization experiment compared IPG buffer concentration as well as the addition of paraffin oil to the protein sample prior to IEF. The IPG buffer concentrations chosen for the optimization experiment were the concentration previously used by the laboratory of Dr. James F. Dillman, III (USAMRICD, Aberdeen Proving Ground, MD), for rat hippocampus homogenates and HEK homogenates (0.5%; personal communication and [13]) and the concentration used for the mitochondrial experiments presented in this report (1%). Addition of 100 µl of paraffin oil was also tested for improvement of strip rehydration and resolution of the protein samples in the first dimension. Paraffin oil is routinely used to cover the first dimension strip during IEF to prevent evaporation of the sample. Accidental inclusion of paraffin oil during a previous experiment suggested an increased absorption of the protein sample into the IEF strip as well as improvement in the resolution of the protein spots (L.N. Bottalico and J.F. Dillman,

personal communication). For the brain region homogenates, 0.5% IPG provided the best resolution (Figure 6). Although the addition of the paraffin oil appeared to increase the amount of protein into the IEF gel (Figure 6B and 6D), it reduced the resolution of the protein spots. Based on these results, 0.5% IPG buffer was used for all subsequent brain region homogenate experiments.

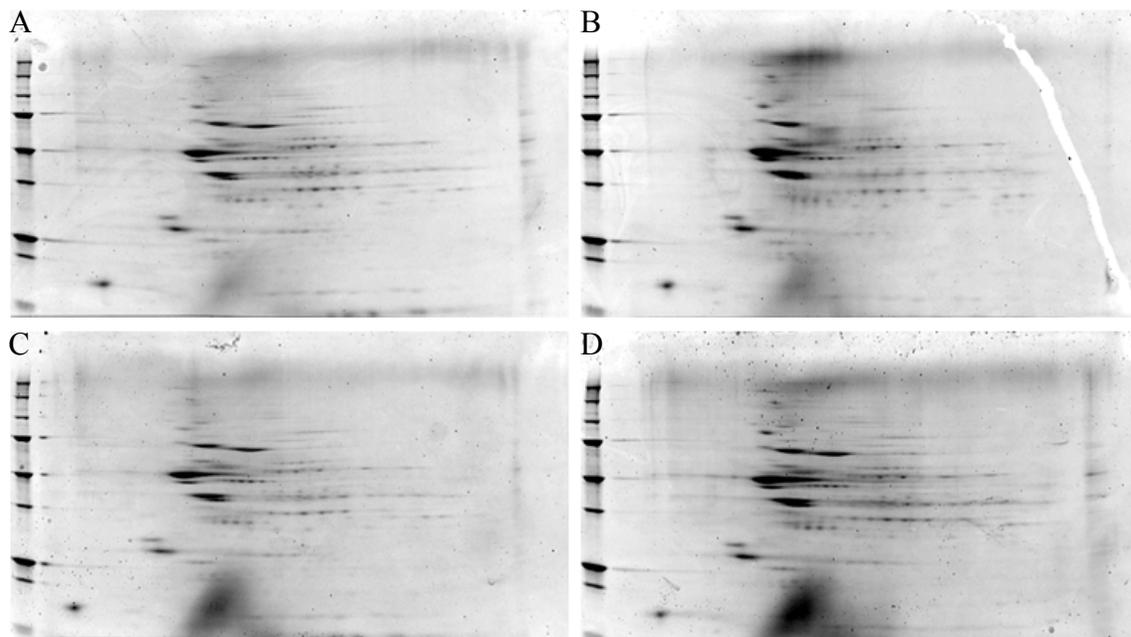


Figure 6: Addition of paraffin oil to protein samples increases absorption into the IEF gel, but does not improve resolution. The same piriform cortex homogenate sample (100 μ g) was run in the first dimension in the presence of (A) 0.5% IPG buffer, (B) 0.5% IPG buffer with paraffin oil, (C) 1% IPG buffer, or (D) 1% IPG buffer with paraffin oil.

2-D DIGE for Cellular and Mitochondrial Proteomes

Following optimization of the 2-D gel electrophoresis conditions, the cellular and mitochondrial proteomes from rat brain regions were analyzed by 2-D DIGE. Both cellular and mitochondrial proteomes were resolved, and CyDye fluorescence was detected using the Typhoon Trio Plus imager (Figure 7). All data analyses were performed using the DeCyder™ 2-D differential analysis software (v6.5; GE Healthcare). In this software, samples are grouped by condition, and the internal standard is used to statistically derive data within and across the gels in the experimental set. The inclusion of this internal standard and simultaneous analysis of multiple samples minimizes system variability, allowing accurate assessment of protein expression changes that can be related to induced biological change with confidence. For our proteomic analysis, GD-exposed samples were compared to the corresponding time-matched vehicle controls to determine the protein spots that were altered in response to GD. The vehicle control samples were compared to naïve control samples to determine the time course of protein changes in response to HI-6/AMN treatment. Combined effects of GD in the presence of HI-6/AMN were determined by comparing the GD-exposed samples to naïve control.

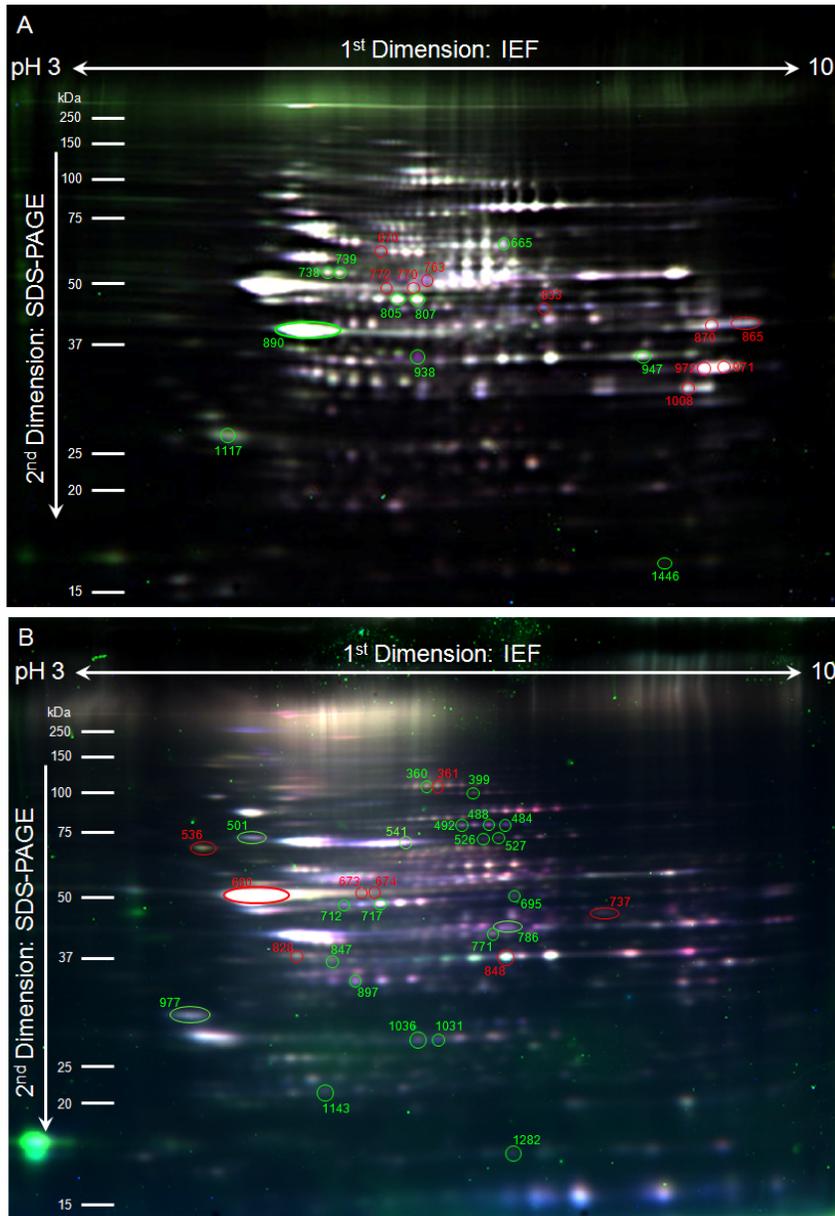


Figure 7: CyDye-labeled cellular and mitochondrial proteins were fluorescently detected for 2-D DIGE analysis. Experimental samples and the internal standard were detected using the Typhoon Trio Plus imager. The overlays of the Cy2-, Cy3-, and Cy5-labeled proteins (5 μ g protein/CyDye) are shown for the hippocampus mitochondrial (A) and cellular (B) proteomes. Proteins visible on Coomassie brilliant blue stained gels for excision and mass spectrometry analysis are circled. Red ovals depict proteins that decrease and green ovals depict proteins that increase in response to GD exposure.

Mitochondrial proteomes from the rat hippocampus, piriform cortex, and thalamus were analyzed to examine the protein changes in response to GD exposure, HI-6/AMN treatment in the absence of GD, and GD with HI-6/AMN treatment. Comparison of the spot changes in the three brain regions revealed no differences in naïve mitochondrial proteomes. In response to GD exposure, 25, 28, and 13 protein spots displayed significant changes in abundance in the hippocampus (Figure 7A), piriform cortex, and thalamus mitochondria, respectively. Mitochondrial proteomic changes were also observed in response to HI-6/AMN treatment in the hippocampus (38 spots), piriform cortex (13 spots), and thalamus (11 spots). The combined effects of GD and HI-6/AMN caused 34, 23, and 12 protein abundance changes in the hippocampus, piriform cortex, and thalamus, respectively. Analysis of naïve cellular proteomes showed only 3 protein spot differences between the hippocampus and piriform cortex brain regions. In response to GD exposure, 51 and 44 protein spots significantly changed in abundance in the hippocampus (Figure 7B) and piriform cortex, respectively. Cellular proteomic changes were also observed in response to HI-6/AMN treatment in the hippocampus (39 spots) and piriform cortex (20 spots). Combined effects of GD and HI-6/AMN resulted in 33 and 29 protein abundance changes in the hippocampus and piriform cortex, respectively. The identification of the cellular and mitochondrial proteins and pathways affected by GD exposure and HI-6/AMN treatment will be the topic of future manuscripts.

Conclusions

The conditions for quantitative proteomic experiments using 2-D DIGE have been optimized for studying the molecular effects of CWNA exposure in the rat brain. Coupling this optimized 2-D DIGE procedure with mass spectrometry has now been used to identify protein and pathway changes induced by exposure to GD, administration of HI-6/AMN in the absence of GD, and produced by GD in the presence of HI-6/AMN treatment. The identification of these proteins and pathways provides new insight into the molecular mechanisms of GD exposure and of the current therapeutic regimen. Additionally, this approach can be expanded to include other CWNAs to identify common and unique proteins and pathways affected by the various agents. Finally, these studies provide potential novel pathways for future therapeutic intervention following CWNA exposure.

References

1. Shih, T.-M., T.A. Koviak, and B.R. Capacio, Anticonvulsants for poisoning by the organophosphorus compound soman: pharmacological mechanisms. *Neuroscience and Biobehavioral Reviews*, 1991. **15**(3): p. 349-362.
2. Britt, J.O.J., et al., Histopathologic changes in the brain, heart, and skeletal muscle of Rhesus Macaques, ten days after exposure to soman (an organophosphorus nerve agent). *Comparative Medicine*, 2000. **50**(2): p. 133-139.
3. Carpentier, P., M. Lambrinidis, and G. Blanchet, Early dendritic changes in hippocampal pyramidal neurones (field CA1) of rats subjected to acute soman intoxication: a light microscopic study. *Brain Research*, 1991. **541**(2): p. 293-299.
4. McDonough, J.H.J., C.G.J. McLeod, and M.T. Nipwoda, Direct microinjection of soman or VX into the amygdala produces repetitive limbic convulsions and neuropathology. *Brain Research*, 1987. **435**(1-2): p. 123-137.
5. McLeod, C.G.J., Pathology of nerve agents: perspectives on medical management. *Fundamental and Applied Toxicology*, 1985. **5**(6): p. S10-S16.
6. Baille, V., et al., Soman-induced convulsions: the neuropathology revisited. *Toxicology*, 2005. **215**(1-2): p. 1-24.
7. Zimmer, L.A., et al., Nerve gas-induced seizures: role of acetylcholine in the rapid induction of fos and glial fibrillary acidic protein in piriform cortex. *Journal of Neuroscience*, 1998. **18**(10): p. 3897-3908.
8. Yanagisawa, N., H. Morita, and T. Nakajima, Sarin experiences in Japan: acute toxicity and long-term effects. *Journal of the Neurological Sciences*, 2006. **249**(1): p. 76-85.
9. Shih, T.-M. and J.H. McDonough, Efficacy of biperiden and atropine as anticonvulsant treatment for organophosphorus nerve agent intoxication. *Archives of Toxicology*, 2000. **74**(3): p. 165-172.
10. Barlow, T., et al., Report of a symposium on the use of genomics and proteomics in toxicology. *Mutagenesis*, 2003. **18**(3): p. 311-7.
11. Wu, W.W., et al., Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D gel- or LC-MALDI TOF/TOF. *Journal of Proteome Research*, 2006. **5**(3): p. 651-8.
12. Dillman, J.F., 3rd, K.L. McGary, and J.J. Schlager, Sulfur mustard induces the formation of keratin aggregates in human epidermal keratinocytes. *Toxicology and Applied Pharmacology*, 2003. **193**(2): p. 228-36.
13. Dillman, J.F., 3rd and J.J. Schlager, Application of proteomics to elucidate the mechanisms of toxicity of the chemical warfare agent sulfur mustard. 2003, USAMRICD-TR-03-06, U.S. Army Medical Research Institute of Chemical Defense: Aberdeen Proving Ground, MD. AD#A418243.
14. Mol, M.A., R.M. van den Berg, and H.P. Benschop, Proteomic assessment of sulfur mustard-induced protein adducts and other protein modifications in human epidermal keratinocytes. *Toxicology and Applied Pharmacology*, 2008. **230**(1): p. 97-108.
15. An, W.-H., et al., Identification of differential proteins of pinnal skin of mice poisoned by sulfur mustard. *Chinese Journal of Pharmacology and Toxicology*, 2006. **20**(2): p. 152-156.

16. Everley, P.A. and J.F. Dillman, 3rd, A large-scale quantitative proteomic approach to identifying sulfur mustard-induced protein phosphorylation cascades. *Chemical Research in Toxicology*, 2010. **23**: p. 20-25.
17. Lee, C.P., M. Sciamanna, and P.L. Peterson, Intact rat brain mitochondria from a single animal: preparation and properties. *Methods in Toxicology*, 1993. **2**: p. 41-50.
18. Venkatraman, A., et al., Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *Journal of Biological Chemistry*, 2004. **279**(21): p. 22092-22101.
19. Mintz, H.A., et al., Morphological and biochemical studies of isolated mitochondria from fetal, neonatal, and adult liver and from neoplastic tissues. *Journal of Cell Biology*, 1967. **34**(2): p. 513-523.