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Mitochondrial Protein Targets of Thiol-Reactive Electrophiles

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Abstract

Mitochondria serve a pivotal role in the regulation of apoptosis or programmed cell death. Recent studies have demonstrated that reactive electrophiles induce mitochondrion-dependent apoptosis. We hypothesize that covalent modification of specific mitochondrial proteins by reactive electrophiles serves as a trigger leading to the initiation of apoptosis. In this study, we identified protein targets of the model biotin-tagged electrophile probes *N*-iodoacetyl-*N*-biotinylhexylene-diamine (IAB) and 1-biotinamido-4-(4′-[maleimidoethylcyclohexane]carboxamido)butane (BMCC) in HEK293 cell mitochondrial fractions by liquid chromatography-tandem mass spectrometry (LC-MS-MS). These electrophiles reproducibly adducted a total of 1693 cysteine residues that mapped to 809 proteins. Protein modifications were selective in that only 438 cysteine sites in 1255 cysteinyl peptide adducts (35%) and 362 of the 809 identified protein targets (45%) were adducted by both electrophiles. Of these, approximately one-third were annotated to the mitochondria following protein database analysis. IAB initiated apoptotic events including cytochrome c release, caspase-3 activation, and poly(ADP-ribose)polymerase (PARP) cleavage, whereas BMCC did not. Of the identified targets of IAB and BMCC, 44 were apoptosis-related proteins, and adduction site specificity on these targets differed between the two probes. Differences in sites of modification between these two electrophiles may reveal alkylation sites whose modification triggers apoptosis.

Introduction

Mitochondria govern the balance between life and death in eukaryotic cells. Through respiration, electron transport, and oxidative phosphorylation, mitochondria supply most of the bioenergetic requirements for life. Mitochondria also serve as integrators for apoptosis, in which cellular stresses and receptor-mediated death-signaling pathways cause the release of mitochondrial apoptogenic factors, including cytochrome c, apoptosis-inducing factor (AIF),[1] and secondary mitochondrial activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO) into the cytosol. These factors trigger downstream events such as caspase activation, DNA fragmentation, inhibition of DNA repair proteins, membrane blebbing, and cell fragmentation (1). It is believed that mitochondria release these factors through the opening of the permeability transition pore (PTP) channel, although other pathways may also contribute (2, 3).

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Supporting Information Available: Western blots comparing the purity of the whole cell homogenate and crude and purified mitochondrial fractions (Figure S1); comparison between streptavidin elution methods from a previous study by Dennehy and co-workers (*30*) and the present study on a number of protein targets identified by LC-MS-MS (Figure S2); distribution of the number of cysteine adducts on identified protein targets of BMCC and IAB (Figure S3); induction of caspase-3 activity by IAB but not BMCC by the colorimetric CaspACE assay system (Figure S4); several representative MS-MS spectra of protein targets of IAB and BMCC (Figures S5–S14); and tables showing the protein targets (Table S1) and protein motifs and features (Table S2) of IAB and BMCC. This material is available free of charge via the Internet at http://pubs.acs.org.

Apoptosis can be triggered by electrophilic xenobiotic metabolites and electrophilic products of oxidative stress that covalently modify nucleophilic sites in DNA and proteins to form adducts (4–6). Although the initiation of apoptosis by DNA damage has been extensively studied (7), proteins also are major targets of electrophiles. The characteristics of protein damage associated with apoptosis have been explored only to a limited extent. Cysteine thiols are thought to be particularly important targets for pro-apoptotic damage, as these nucleophilic residues are easily modified by oxidants and electrophiles and are critical in regulating certain aspects of cellular signaling (8–10). Protein covalent binding can trigger apoptosis, at least in part through modification of critical cellular protein thiols (11–14). Other studies indicate that thiol-modifying reagents can induce the mitochondrial permeability transition (15–20), thus providing a possible mechanistic basis for the induction of apoptosis by reactive electrophiles.

Mitochondria generate reactive oxygen species, especially under conditions of cellular stress, and this enhanced production of reactive oxygen species may lead to oxidative stress (21). Oxidative stress also leads to the generation of reactive electrophilic products of lipid oxidation. 4-Hydroxy-2-nonenal (HNE) and related aldehydic products of lipid peroxidation induced apoptosis in human colorectal carcinoma and endothelial cells (5, 22, 23). The electrophilic lipid 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 induced apoptosis in a human neuroblastoma cell line (24) and in cultured hepatocytes harvested from Sprague—Dawley rat livers (25). Apoptosis was induced in human cell lines by the anticarcinogen isothiocyanates sulforaphane and phenethyl isothiocyanate (26, 27) and in animal models by electrophilic metabolites of the carcinogens acrylonitrile and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (28, 29). Although it is clear that electrophiles induce apoptosis, the mechanism is poorly understood. Given that pro-apoptitic electrophiles can covalently modify proteins and that the mitochondrial permeability transition plays a key role in apoptosis, it seems probable that alkylation of mitochondrial protein targets contributes to apoptosis. However, any test of this hypothesis will require identification of mitochondrial protein targets of pro-apoptotic electrophiles.

We have recently used biotinylated electrophile probes to investigate the scope and characteristics of protein covalent binding to subcellular proteomes (30, 31). The iodoacetamido probe *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB) and *N*-alkylmaleimido probe 1-biotinamido-4-(4′-[maleimidoethylcy-clo-hexane]carboxamido)butane (BMCC) (Figure 1) display reaction chemistries analogous to many electrophilic metabolites and endogenous electrophiles. Adducts formed with these probes have been mapped to over a thousand specific cysteines in cytoplasmic, nuclear, and microsomal proteins (30, 31). Moreover, adduction appears to be associated with different biological effects, as IAB, but not BMCC-induced endoplasmic reticulum (ER) stress in cells exposed to these probes (31).

Here, we have extended this approach to characterize alkylation-induced apoptosis in human embryonic kidney 293 (HEK293) cells. Although both compounds form protein adducts, IAB induces apoptosis, whereas BMCC does not. We identified IAB and BMCC adducts formed in isolated HEK293 mitochondrial fractions that were treated with both probes. These analyses mapped over 1200 cysteine thiol targets of IAB and BMCC, a significant

[1]Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; IAB, *N*-iodoacetyl-*N*-biotinylhexylenediamine; BMCC, 1-biotinamido-4-(4′-[maleimidoethylcyclo-hexane]-carboxamido)-butane; Cyto Ox 4, cytochrome oxidase 4; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; HEK293, human embryonic kidney 293 cells; HNE, 4-hydroxy-2-nonenal; HPLC, high-performance liquid chromatography; LC-MS-MS, liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; PARP, poly(ADP-ribose)polymerase; PBS, phosphate-buffered saline; PEO-IAB, (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine; PTP, permeability transition pore; SDS-PAGE, sodium dodecyl sulfate—polyacrylamide gel electrophoresis; SMAC/DIABLO, secondary mitochondrial activator of caspases/direct IAP binding protein with low pI; TIM, translocase of inner membrane; TOM, translocase of outer membrane; UniProt, Universal Protein Resource human database; VDAC, voltage-dependent anion channel protein.

fraction of which are on mitochondrial proteins. IAB and BMCC displayed distinct differences in the alkylation of mitochondrial protein targets. These differences in site-specific modification between the two electrophiles may govern the relationship between protein damage and apoptosis.

Experimental Procedures

Caution

IAB and BMCC are hazardous alkylating agents and should be handled with care.

Chemicals and Reagents

IAB, BMCC, and the bicinchoninic assay kit were purchased from Pierce Biochemicals (Rockford, IL). Streptavidin Sepharose high-performance beads were obtained from GE Healthcare (Uppsala, Sweden). Trypsin Gold was purchased from Promega (Madison, WI). Complete Mini protease inhibitor cocktail was obtained from Roche Diagnostics (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), 0.5% trypsin in PBS, Novex Colloidal blue stain kit, and trypan blue were purchased from Invitrogen (Carlsbad, CA). Sources of primary antibodies were as follows: antibodies against cytochrome c (raised in rabbit, catalog no. 630105, BD Clontech, Palo Alto, CA), cytochrome oxidase 4 (Cyto Ox 4) (mouse, catalog no. 630105, BD Clontech), caspase-3, (mouse, catalog no. ab7850, Abcam, Cambridge, MA), poly(ADP-ribose)-polymerase (PARP) (rabbit, catalog no. 9542, Cell Signaling, Beverly, MA), actin (mouse, catalog no. 8226, Abcam), glutathione *S*-transferase (rabbit, catalog no. ab14502, Abcam), histone 2A (rabbit, catalog no. 4271, Abcam), Grp78/Bip (rabbit, catalog no. 610979, Abcam), and biotin (mouse, catalog no. 1297597, Roche, Indianapolis, IN). IRDye 700 conjugated affinity goat antimouse and antirabbit secondary antibodies were purchased from Rockland Immunochemicals (catalog no. A21076 and A21058, respectively, Gilbertsville, PA). The corresponding IRDye 800 sary antibodies were also obtained from Rockland (catalog no. 610-432-020 and 611-132-122, respectively). Formic acid (98–100%) was acquired from EM Science (Darmstadt, Germany). All other chemicals were obtained from Sigma (St. Louis, MO) and Fisher Chemical Co. (Fair Lawn, NJ).

Cell Culture

HEK293 cells were purchased from American Type Culture Corporation (ATCC, Manassas, VA). Cells were seeded on plates in DMEM supplemented with 10% fetal bovine serum and antibiotic/antimycotic at 10 mL/L and grown to 80% confluency at 37 °C and 95% air/5% CO_2.

Lactate Dehydrogenase (LDH) Leakage Assay

Cells were washed once with PBS and then treated with IAB (50–200 μM), BMCC (100–400 μM), staurosporine (2 μM), or DMSO vehicle delivered in DMEM without phenol red for 6–24 h. The maximum DMSO concentration in the medium was 1.2% (v/v). LDH leakage was determined as described in the LDH-based in vitro toxicology assay kit (Sigma).

Preparation and Immunoblot Analyses of Subcellular Fractions

Cells were treated for 24 h with IAB, BMCC, staurosporine, or vehicle at the concentrations indicated above for the LDH leakage assay. Three different types of preparations were carried out for immunoblot analysis: cytosolic fractions to detect cytochrome c, whole cell lysates to detect PARP cleavage, and whole cell lysates to monitor caspase-3 activation. Cytosolic fractions were isolated by digitonin treatment as described previously (32) with

the following modifications. Harvested cells were resuspended in ice-cold 70 mM Tris buffer, pH 7.5, containing 250 mM sucrose and protease inhibitors (Sigma, catalog no. P4830). For detection of PARP cleavage, whole cell lysates were prepared as described previously (5). For caspase-3 activation, cells were scraped into the media, washed twice with ice-cold PBS, and then resuspended in 312.5 mM HEPES, pH 7.5, containing 31.25% sucrose (w/v) and 0.3125% CHAPS (w/v) at a concentration of 10^8 cells/mL. Cells were lysed by freeze–thawing and then incubation on ice for 15 min. Following centrifugation at 15000*g* for 20 min, supernatant (whole cell lysate) was collected.

Mitochondria of untreated HEK293 cells were purified by Percoll gradient centrifugation as described previously (33) with the following modifications. After gradient centrifugation, the protein band (approximately 30% down the Percoll gradient) was collected with a Pasteur pipet, diluted with 0.25 M mannitol, 25 mM HEPES-NaOH, pH 7.5, 0.5 mM EGTA, and mammalian protease inhibitor cocktail (Sigma) (buffer A), and then centrifuged at 10000*g* for 15 min to remove the Percoll. The pellet was washed with buffer A, and the purified mitochondrial pellet was resuspended in 10 mM HEPES buffer, pH 7.5. Protein concentrations for cellular fractions were determined with the bicinchoninic acid assay kit (Pierce).

Proteins (20–50 μg per lane) were resolved by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) using 10% NuPAGE Novex Bis-Tris precast gels (Invitrogen) in either NuPAGE MOPS SDS or MES SDS running buffer (Invitrogen). They were transferred to polyvinylidene difluoride membranes (Invitrogen), blocked with 1:1 TBS: blocking buffer for near-infrared fluorescence Western blotting (Rockland, Gilbertsville, PA), and then probed with antibodies against cytochrome c, Cyto Ox 4, biotin, caspase-3, PARP, actin, glutathione *S*-transferase, histone 2A, or Grp78/Bip. Membranes were incubated with IRDye700 or IRDye800 conjugated affinity goat antimouse or antirabbit secondary antibodies and then analyzed on a LI-COR Odyssey Imaging system (Lincoln, NE) with a preset setting “membrane” and resolution of 169 μm.

Caspase Activation Colorimetric Assay

Cells were treated for 24 h with IAB, BMCC, staurosporine, or vehicle at the concentrations indicated above for the LDH leakage assay. Cell treatment was done for four replicate experiments. For each experiment, whole cell lysates were prepared as described above for immunoblot detection of caspase-3. Caspase-3 activities in lysates (45 μg protein per reaction) were measured with the CaspACE Colorimetic Assay System kit (Promega, Madison, WI). The assay reaction mixtures were incubated for 24 h at 37 °C. Reactions were carried out in triplicate and were measured spectroscopically at 405 nm using a 96 well plate reader (Dynex Technologies, Chantilly, VA).

Incubation of HEK293 Cell Mitochondria with Electrophile Probes and Analysis of Adducted Proteins

Mitochondria (4 mg mL^{-1} protein) were incubated with 100 μM IAB or BMCC in 10 mM HEPES buffer, pH 7.5, at 37 °C for 30 min. The reaction was quenched with 10 mM dithiothreitol and placed on ice. NuPAGE LDS sample buffer (Invitrogen) was added to quenched protein mixtures in a ratio of 1:3 (v:v) and incubated at 70 °C for 10 min. SDS-PAGE separation of proteins (100 μg protein per gel lane; four lanes total), in-gel digestion, and peptide extraction were carried out as described previously (31) with the following modifications. SDS-PAGE gel lanes were cut into 20 slices (2–6 mm lengthwise per slice) comprising the entire molecular weight range prior to cutting of individual slices into 1 mm cubes. Following the drying of gel cubes in vacuo, they were rehydrated with trypsin (1:25

approximate trypsin/protein mass ratio) in 25 mM ammonium bicarbonate for 10 min at room temperature and then incubated for 12–16 h at 37 °C.

Streptavidin Capture of Biotinylated (Adducted) Peptides

Streptavidin sepharose beads were prewashed three times with 100 mM ammonium bicarbonate and then diluted with ammonium bicarbonate to achieve a 50:50 (v/v) bead/buffer slurry. The peptide digests in 200 μL of ammonium bicarbonate were mixed with 450 μL of the slurry, and the suspension was mixed with rotation at room temperature for 2 h. The mixture was centrifuged at 8000*g* for 2 min, and the liquid supernatant was removed. The beads containing the biotinylated peptides were washed in the following sequence: twice with 1 mL of 100 mM ammonium bicarbonate, three times with 1 mL of 1 M NaCl, twice with 1 mL of 100 mM ammonium bicarbonate, and twice with 1 mL of H_2O. For each wash, beads were resuspended in the wash solution and then centrifuged at 8000*g* for 2 min, and the supernatant was discarded. Biotinylated peptides were then eluted by washing the beads with 70:25:5 acetonitrile/H_2O/formic acid (300 μL per elution). The first elution was carried out at 4 °C overnight with mixing by rotation. The second and third elutions were at 70 °C for 30 min. Following each elution, beads were centrifuged at 8000*g* for 2 min, and the supernatant was collected. The three elution supernatants were combined. Prior to liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis, samples were concentrated in vacuo in a SpeedVac vacuum centrifuge (Thermo-Fisher Scientific, Waltham, MA) to 10 μL and then diluted with 60 μL of 0.1% formic acid.

LC-MS-MS Analyses and Identification of Electrophile-Adducted Peptides

Peptide digests were analyzed by reverse-phase LC-MS-MS as described previously (31). IAB- and BMCC-adducted peptide sequences were determined by search of MS-MS spectra against the UniRef 100 database (downloaded June 2005) with TurboSequest (ThermoElectron, San Jose, CA) (34). Sequence-spectrum matches were filtered through a custom-designed software and database system as described previously (31) with the following modifications: *S*-Carboxamidomethylation of Cys (+57 amu), oxidation of Met (+16 amu), IAB adduction at Cys [+382.2 and +398.2 amu (oxidized adduct)], and BMCC adduction at Cys [+533.7 and +549.7 amu (oxidized adduct)] were specified as dynamic modifications. Criteria for acceptance of MS-MS spectrum to database sequence matches for adducted peptides have been described previously (30). Sequest outputs were analyzed with a custom-designed software and database system called CHIPS (Complete Hierarchical Integration of Protein Searches), which enables filtering of Sequest output files based on Sequest output parameters for sequence-spectrum matches and other criteria. Sequence-spectrum assignments were accepted based on the following filtering criteria: (i) All peptide sequence assignments were required to result from fully tryptic cleavages; (ii) all peptides possessed the appropriate reactive electrophile adduct mass; (iii) adducted peptides needed to be present in at least three out of five sample sets in the IAB and BMCC experiments to be accepted; (iv) singly, doubly, and triply charged ions were accepted if their XCorr scores were greater than 2, 2.5, and 3, respectively; and (v) all putative matches were confirmed by visual inspection of the spectra. Peptide identifications from all 20 SDS-PAGE gel slice fractions were combined in the database system. Identifications were accepted when the same adducted peptide was found in at least three out of five replicate experiments.

Results and Discussion

Toxicity and Induction of Apoptosis End Points by IAB and BMCC

We chose IAB and BMCC as model electrophile probes because these two compounds display chemistries that are commonly observed in biologically relevant electrophiles generated by metabolism of xenobiotics and by endogenous oxidation of lipids. IAB

undergoes S_N2 nucleophilic displacement reactions with cellular nucleophiles and mimics aliphatic epoxides, alkyl halides, and episulfonium ions. BMCC is a Michael acceptor whose chemistry mimics that of α,β-unsaturated carbonyl metabolites and lipid oxidation products, as well as quinones and quinone methides. Both compounds are highly reactive toward protein cysteine thiols but also may modify other nucleophiles.

Our previous studies with IAB [or its congener (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine, (PEO-IAB)] and BMCC indicated that both the iodoacetamido and the *N*-alkylmaleimido probes alkylate comparable numbers of protein targets, as determined by LC-MS-MS analyses (30, 31). These studies also indicated that the overlap in targets alkylated by the two electrophiles is about 20%, which indicates different selectivities. These probes display notable differences in toxicity and induction of stress responses. IAB induced ER stress in HEK293 cells, whereas BMCC did not (31). IAB also induced activation of the stress-activated transcription factor Nrf2 in HepG2 cells and HEK293 cells, whereas BMCC did not (35).

We began these studies with a focus on the toxicity differences between these compounds. We first measured the leakage of LDH from HEK293 cells, which is due to lysis of the cell membrane due either to primary necrosis or to necrosis secondary to apoptosis, following electrophile treatment for 6, 16, or 24 h. (Figure 2). Cytotoxicity of IAB increased with treatment time, and treatments with 50 and 100 μM IAB increased LDH leakage up to 19 and 39%, respectively, at 24 h. The cytotoxic response was similar to that of staurosporine, a potent inducer of apoptosis (36). In contrast, little cytotoxicity was caused by BMCC, which produced only 1% LDH leakage (100 μM, 24 h).

We next tested the ability of IAB and BMCC to induce apoptosis in HEK293 cells. IAB (50–200 μM) caused the release of cytochrome c from the mitochondria to the cytosol after 24 h of treatment (Figure 3A). This event is characteristic of mitochondrion-dependent apoptosis (1). In contrast, BMCC (100–400 μM) did not induce cytochrome c release at concentrations up to 400 μM (Figure 3A). PARP cleavage by caspase 3 is a hallmark biochemical characteristic of apoptosis (37), and caspase-3 activation was induced by IAB to a similar extent to that caused by staurosporine (Figure 3B). IAB (50–200 μM) induced cleavage of the DNA repair protein PARP from a full length 116 kDa form to a cleaved (89 kDa) form, whereas BMCC (up to 400 μM) did not induce PARP cleavage after 24 h of treatment (Figure 3C). Overall, these results demonstrate that IAB induces mitochondrion-dependent apoptosis, but BMCC does not, even at concentrations up to 400 μM, which is the limit of BMCC aqueous solubility.

Identification of Protein Cysteinyl Adducts of IAB and BMCC

The major objective of the analyses described below was to identify mitochondrial proteins with a high reactivity toward the electrophile probes. As in our previous studies (30, 31), we exposed isolated mitochondrial fractions rather than intact cells to IAB and BMCC. This makes intrinsic reactivity toward electrophiles the predominant factor determining adduction, rather than the ability of the electrophiles to penetrate cells and reach mitochondrial targets. Indeed, physiologically relevant electrophiles may arise from both inside and outside cells or within mitochondria. The major analytical challenge that we faced in the analysis of mitochondrial protein targets is the high proportion of transmembrane, membrane-associated, and other hydrophobic proteins in mitochondrial preparations exposed to the biotin electrophile probes. We employed an analytical approach similar to what we described previously (31). The major elements of the approach are a 1D SDS-PAGE separation to resolve mitochondrial proteins, including hydrophobic proteins by molecular weight, in-gel digestion to generate tryptic peptides, streptavidin capture of

biotinylated (adducted) peptides from the digests, and LC-MS-MS analysis of captured peptides.

Mitochondrial fractions were purified by Percoll gradient centrifugation of the crude mitochondrial fraction (27000*g* pellet). The purity of these fractions was determined by Western blot detection of representative organelle markers as shown in the Supporting Information. Percoll gradient centrifugation enriched the mitochondria relative to the crude mitochondria and the whole cell lysate based on band intensities of cytochrome oxidase IV band (mitochondrial membrane-bound protein). Glutathione-*S*-transferase was not present in the Percoll-purified fractions, which suggested that the purified mitochondrial fraction was minimally contaminated by cytosolic proteins. Detectable amounts of actin (cytoskeleton marker), Grp78/Bip (ER), and PARP (nucleus) were present in the purifed fractions. Our protein adduct inventories suggested that these traditionally employed markers provide an inadequate index of mitochondrial purity (see below).

Mitochondria were incubated with IAB and BMCC in 10 mM HEPES buffer at pH 7.4. This hypotonic buffer maintained near-physiological pH for mitochondrial proteins but would cause swelling and rupture of inner mitochondrial membranes, which enabled comparable exposure of mitochondrial outer membrane, intermembrane, and matrix proteins to the electrophile probes.

Incubations of mitochondrial preparations with IAB and BMCC were terminated by the addition of excess dithiothreitol, which readily reacts with and neutralizes the excess electrophiles. The proteins then were resolved by SDS-PAGE, reduced with dithiothreitol, and then alkylated with iodoacetamide. Following in-gel tryptic digestion, the adducted peptides were captured with immobilized streptavidin. Following high-salt washes to remove nonbiotinylated peptides, the biotinylated peptide adducts were eluted with 70:25:5 acetonitrile/H_2O/formic acid at temperatures up to 70 °C. This elution method was found to elute a greater number of adducted peptides from streptavidin than than the room temperature elution that we described previously (30, 31). To compare the two methods, tryptic digests of seven different gel fractions from IAB-treated purified mitochondria were loaded onto streptavidin sepharose beads, washed, and then eluted by each of the methods. The high-temperature method consistently identified at least 2.7-fold more protein targets as shown in Supporting Information Figure S2. One complication of the high-temperature elution is the elution of some streptavidin protein monomer with the adducted peptides. Consequently, the enriched adduct mixture was refrigerated at 4 °C overnight to precipitate the contaminating streptavidin. The supernatant was isolated from the precipitate by centrifugation and was then analyzed by LC-MS-MS.

Peptide adduct sequence matches were assigned based on Universal Protein (UniProt) human database matches by the Sequest algorithm (34); this program accounts for the expected mass change from cysteine modifications by IAB or BMCC. All identified peptide adducts contained cysteine modifications of +382.2 Da for IAB, +533.7 Da for BMCC, or the corresponding S-oxidized products (+398.2 and +549.7 Da, respectively). These adducts are the S_N2 reaction product of the cysteine thiol and the iodoacetamido group of IAB or Michael addition product of the cysteine thiol and the *N*-ethylmaleimido group of BMCC. Sequence-spectrum matches that met the minimum requirements as outlined in the Experimental Procedures were accepted. Several representative MS-MS spectra-sequence assignments are shown in Supporting Information Figures S5–S14.

Mitochondrial Protein Targets

The complete list of the protein targets from the in vitro reaction of purified mitochondria with the two biotin-tagged electrophiles is given in Supporting Information Table 1. A Venn

diagram summarizing the overlap of cysteine adducts of IAB and BMCC is shown in Figure 4A. There were a total of 1693 IAB or BMCC adducts identified at 1255 cysteine sites. IAB modified 820 of these cysteine adducts, which were mapped to 571 proteins. Similarly, BMCC adducted 873 cysteines, which mapped to 600 proteins. One striking feature of the data set is the difference in cysteine thiol reactivity between the two electrophiles. Only a modest fraction of the identified targets (438 peptides or 35% of the total identified adduct sites; 362 proteins or 45% of the total identified protein targets) was adducted by both IAB and BMCC. This result is similar to our observations with adduction of cytosolic and nuclear proteins (30) and of microsomal proteins (31). However, 209 proteins were selectively adducted by IAB, whereas 238 proteins were selectively adducted by BMCC. Of the proteins adducted by both electrophiles, different cysteine residues were targeted in some cases. An example is adenine nucleotide translocase 3 (ANT3), a mitochondrial inner membrane protein reportedly involved in apoptosis. BMCC covalently modifies Cys159 and Cys256, whereas IAB only reacts with Cys256 (Table 1).

Approximately one-third of all of the identified protein targets were annotated as having mitochondrial localizations in the (http://www.pir.uniprot.org) or Bioinfomatic Harvester (http://harvester.embl.de) protein databases. Our finding that about two-thirds of the identified protein targets were not annotated as having mitochondrial localization is perhaps surprising. Two factors may contribute to this finding. First, despite our use of a widely accepted method for isolation of mitochondria, our mitochondrial preparations were not rigorously purified and were inevitably contaminated with proteins considered to be markers for other subcellular organelles (Supporting Information Figure S1). Second, some of the proteins that we identified may have multiple subcellular localizations, some of which are not represented in current databases. Indeed, it has been demonstrated previously that proteins can translocate from one organelle to another. Two protein targets, hexokinase (38) and cofilin (39), have been shown to translocate from the cytoplasm and nucleus, respectively, to the mitochondria. Thus, cross-contamination and multiorganelle distribution of some proteins presumably account for our observation. Similar findings have been reported by others who have done shotgun proteomic analyses on subcellular fractions (40, 41).

The human genome encodes an estimated 1000–2000 proteins in mitochondria (42, 43). In this study, IAB or BMCC covalently modified a total of 809 proteins in purified mitochondrial fractions (Figure 4). Previously, the two largest proteomic studies of human mitochondria identified 615 proteins from purified heart mitochondria and 680 proteins from mitochondria of T leukemia cells (44, 45). The relatively higher number of identifications in our study may reflect selective enrichment of cysteine-adducted peptides by streptavidin capture. This enables a broader sampling of proteins, although only electrophile targets are captured.

The total of over 800 protein targets identified in this study is dramatically higher than results from previous studies identifying protein targets of reactive electrophiles and oxidants. Marley and co-workers identified 51 mitochondrial protein targets of thiol-reactive (4-iodobutyl)triphenylphosphonium from rat heart mitochondria by LC-MS-MS analysis (46). In a separate study, 29 protein targets were identified from reaction of HEK293 whole cell lysate and the electrophilic lipid 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 by two-dimensional isoelectric focusing, SDS-PAGE, and silver staining (47). Suh and co-workers employed a differential labeling approach, a thiol-reactive biotin-maleimide (structurally similar to BMCC) and 2D SDS-PAGE, to identify 10 mitochondrial proteins that underewent thiol oxidation in alcohol-exposed human hepatoma cells (48). The identified targets included heat shock protein 60, protein disulfide isomerase, protein disulfide isomerase A3, and mitochondrial aldehyde dehydrogenase, all of which were identified in our present study.

Our data confirm previous work but provide a greatly expanded list of mitochondrial proteins that are targeted by reactive electrophiles.

We hypothesize that protein modifications by these electrophiles could act as sensors to trigger events of apoptosis. Although our identification of mitochondrial protein targets per se does not provide a test of this hypothesis, consideration of the identities of the identified targets and their known involvement in apoptotic mechanisms is worth examining and could provide the basis for new mechanistic studies. The differential induction of apoptosis by our probes (IAB induces apoptosis, and BMCC does not) and differential labeling of protein targets provide an interesting framework for consideration. A listing of IAB and BMCC protein targets associated with apoptosis is compiled in Table 1. The PubMed references offer representative evidence for the role of protein targets in apoptosis. (The literature citations can be accessed by clicking the weblinks.)

The three major classes that emerged from this compilation were pore channel proteins (PTP channel, preprotein translocation), thioredoxin family proteins, and heat shock proteins. Adduction of proteins in these classes may offer new insights on mechanisms of electrophile-induced apoptosis.

One proposed mechanism of cytochrome c release is the opening of the PTP channel resulting in the mitochondrial permeability transition, loss of transmembrane potential ($\Delta\psi_m$), mitochondrial swelling, and rupture of the outer membrane (3). Covalent modification of cysteine thiols of ANT, a putative member of the PTP channel, coincided with membrane permeabilization (15). Modification of the ANT and other PTP channel members identified in this study may induce pore opening and cytochrome c release.

The adduction of several apoptosis-related proteins differed for the two electrophiles that differed in inducing apoptosis (Table 1 and Figure 3). IAB reproducibly adducted mitochondrial thioredoxin at Cys 90 whereas BMCC adducts were not detected. This protein is vital in redox regulation, defense against oxidative stress, refolding of disulfide-containing proteins, and regulation of transcription factors (49). Another protein target of note is the C terminus of Hsp70-interacting protein (CHIP), which is also known as STIP1 homology and U box-containing protein 1. It regulates the activation of the stress response of heat shock proteins and protects against stress-induced apoptosis in mice (50). IAB only modified Cys199, whereas BMCC did not modify this residue but instead modified Cys48 and Cys83.

IAB and BMCC adducted several apoptosis-related protein targets at the same cysteine residue. For example, both electrophiles modified voltage-dependent anion-selective channel protein 1 at Cys126 and Cys231 (Table 1). However, this does not mean that adduction by the two probes was equivalent. The adduct data were qualitative (i.e., whether cysteine adducts were detected or not). A quantitative analysis of adduction by IAB or BMCC could reveal differences in adduction kinetics and degrees of modification that correlate with biological effect.

Cysteine residues that are specifically modified by IAB could be triggers for IAB-induced apoptosis, although we have no direct evidence to support this hypothesis. It is nevertheless reasonable to postulate that our IAB target list contains one or more triggers for apoptosis. We note in this context that we have previously demonstrated that adduction selectivity of these probes accounts for their selectivity in stimulating activation of the transcription factor Nrf2 (35) and in mediating inhibition of protein phosphatase 2A (51). Similarly, previous reports have demonstrated that iodoacetamide and *N*-ethylmaleimide, respective analogues of IAB and BMCC, differentially inactivated bovine ferrochelatase (52), probably due to the modification of different cysteine residues. As we have noted previously, IAB and BMCC also contain slightly different linker structures that may contribute to the alkylation

differences that we observed (30, 31), although we cannot estimate the magnitude of these effects. We note also that our electrophile incubations were done in the absence of glutathione, a critical protectant against protein alkylation. Although the presence of glutatione could possibly alter specific protein—electrophile interactions (due perhaps to allosteric effects), the major effect of glutathione would be to increase the electrophile concentration required to alkylate the same targets. Thus, the protein targets identified here would be expected to be most reactive in intact cells.

In summary, our work demonstrates that two different thiol-reactive electrophile probes alkylate mitochondrial proteins with different selectivities. Furthermore, among the identified protein targets involved in apoptosis, these probes have distinct adduction profiles that may explain their differing pro-apoptotic activities. These observations demonstrate that adduction specificity reflects different biological effects. Accordingly, analyses of site-specific protein alkylation by electrophilic xenobiotic metabolites and endogenous electrophiles generated from oxidative stress and inflammation could predict mechanisms of toxicity, including apoptosis. Further work is necessary to test this hypothesis, but our observations reduce the problem to evaluation of the roles of specific protein targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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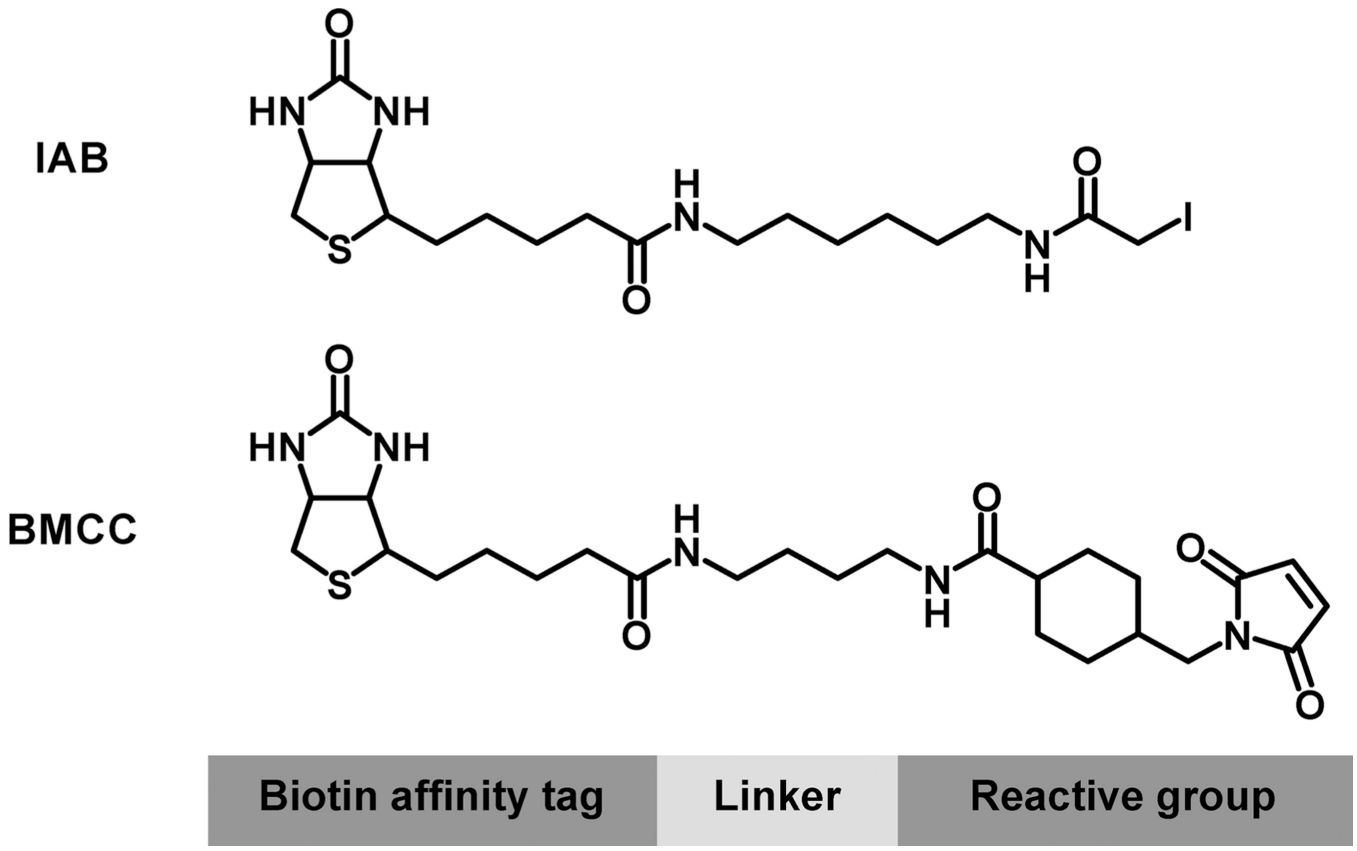


Figure 1.
Structures of reactive electrophile probes used in this study.

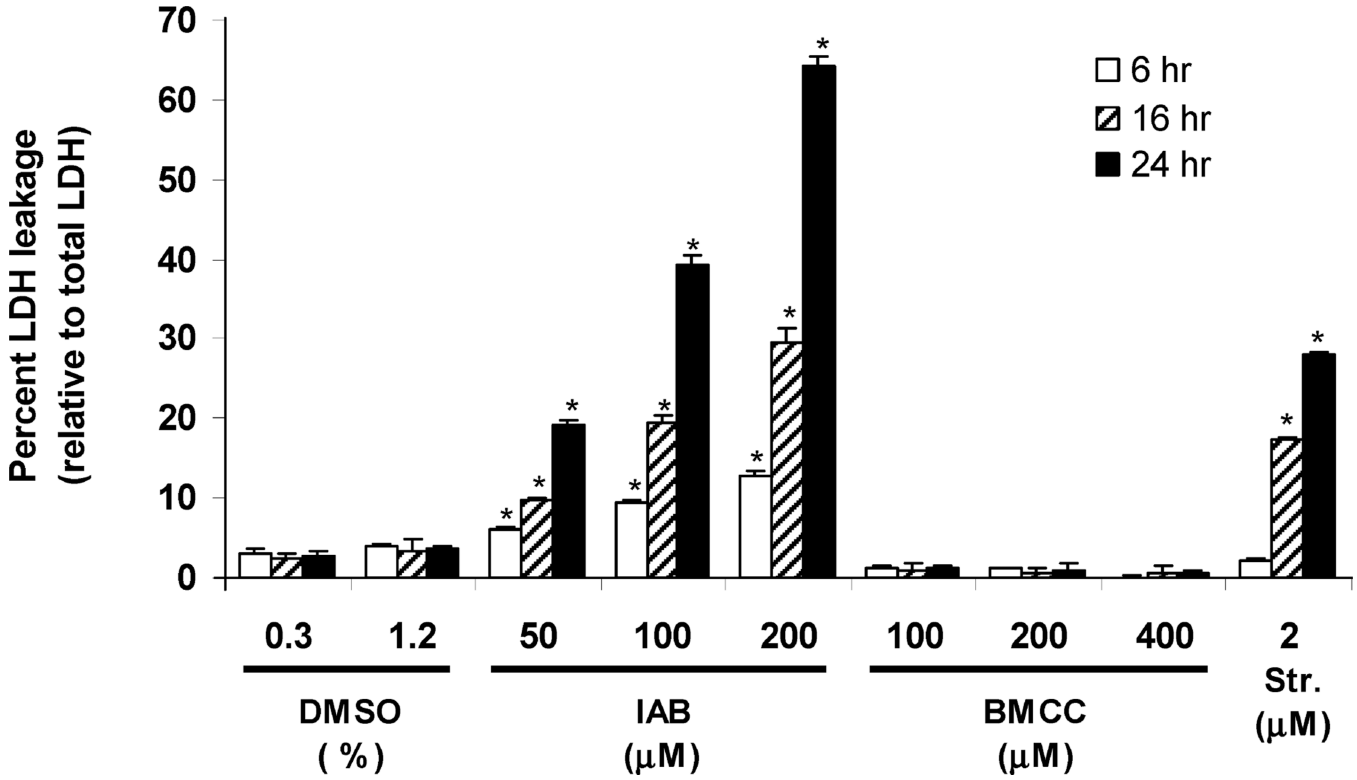


Figure 2.

Cytotoxicity of IAB and BMCC in HEK293 cells. Cells were treated with indicated concentrations of IAB, BMCC, and staurosporine (positive control) for 6 (empty bars), 12 (hatched bars), or 24 h (filled bars). Cytotoxicity was measured as LDH leakage from the cells and is reported as a percent of total LDH in the cells. Results were determined from three independent experiments ($n = 3$). *$p < 0.001$ as compared to respective DMSO controls.

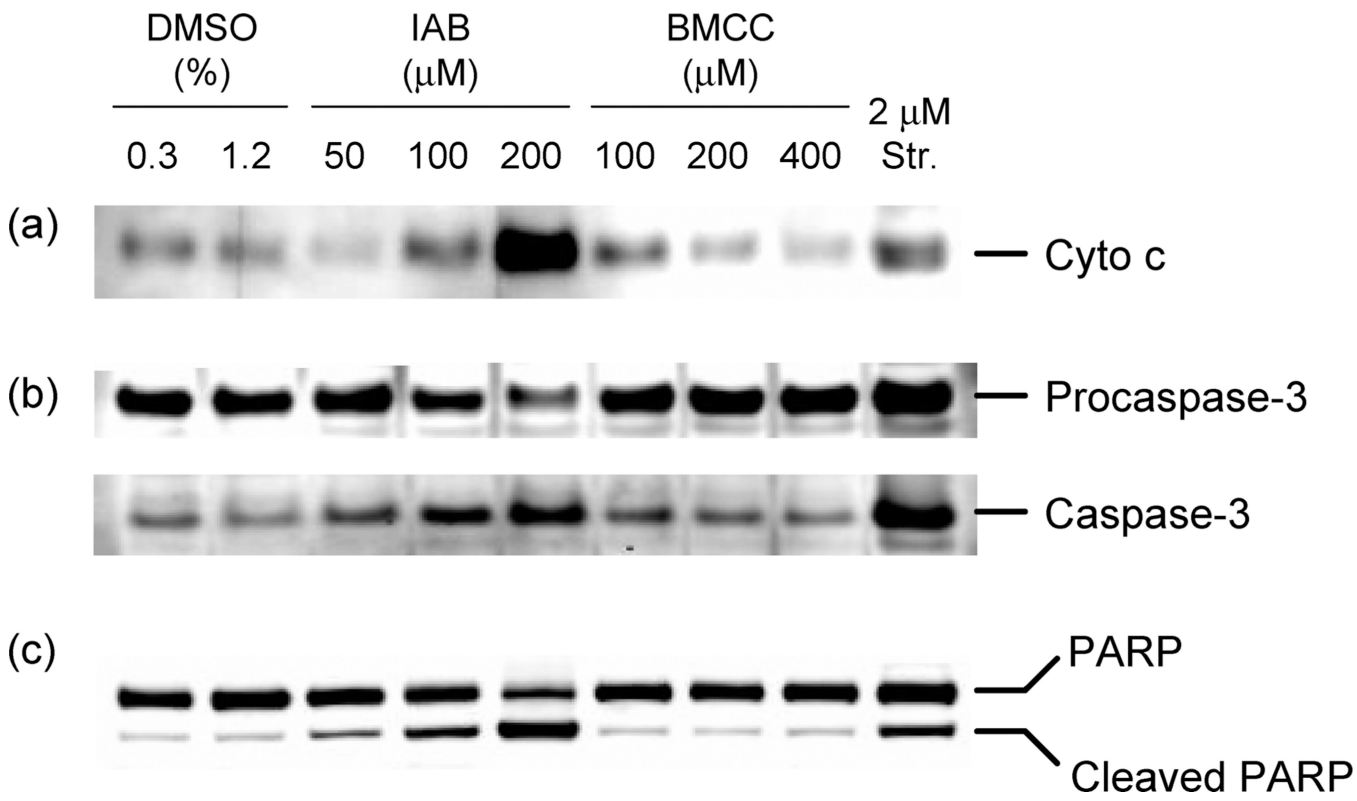
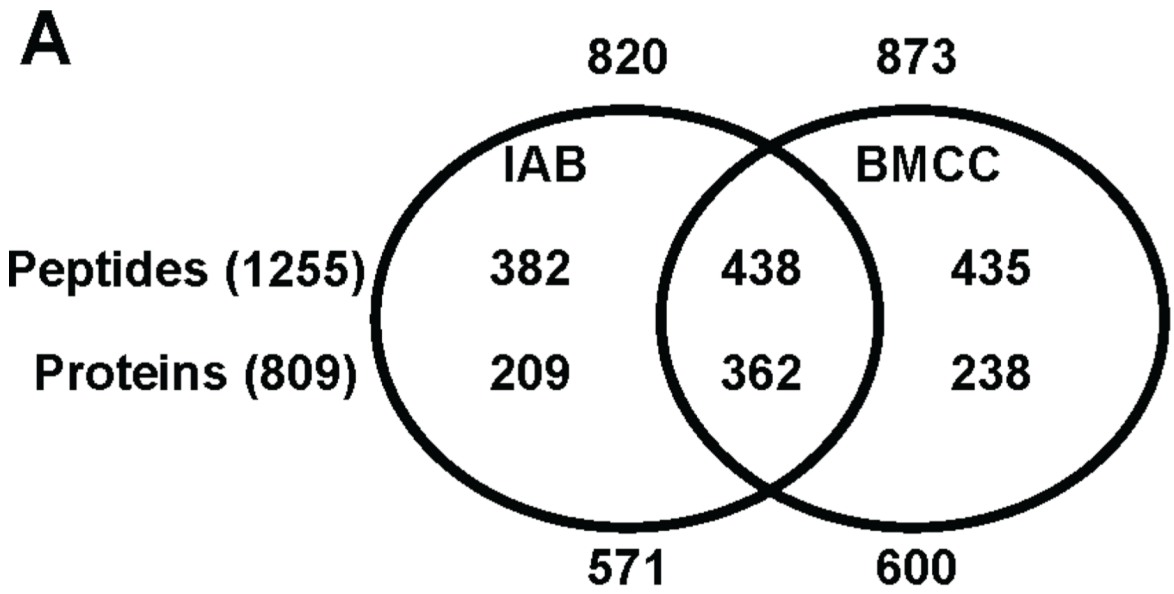


Figure 3.

Apoptosis was induced by IAB but not by BMCC in HEK293 cells. Cells were treated with indicated concentrations of IAB or BMCC for 24 h. (a) Mitochondrial cytochrome c release was measured in cytosolic fractions. (b) Caspase-3 activation and (c) PARP cleavage were measured in whole cell lysates.



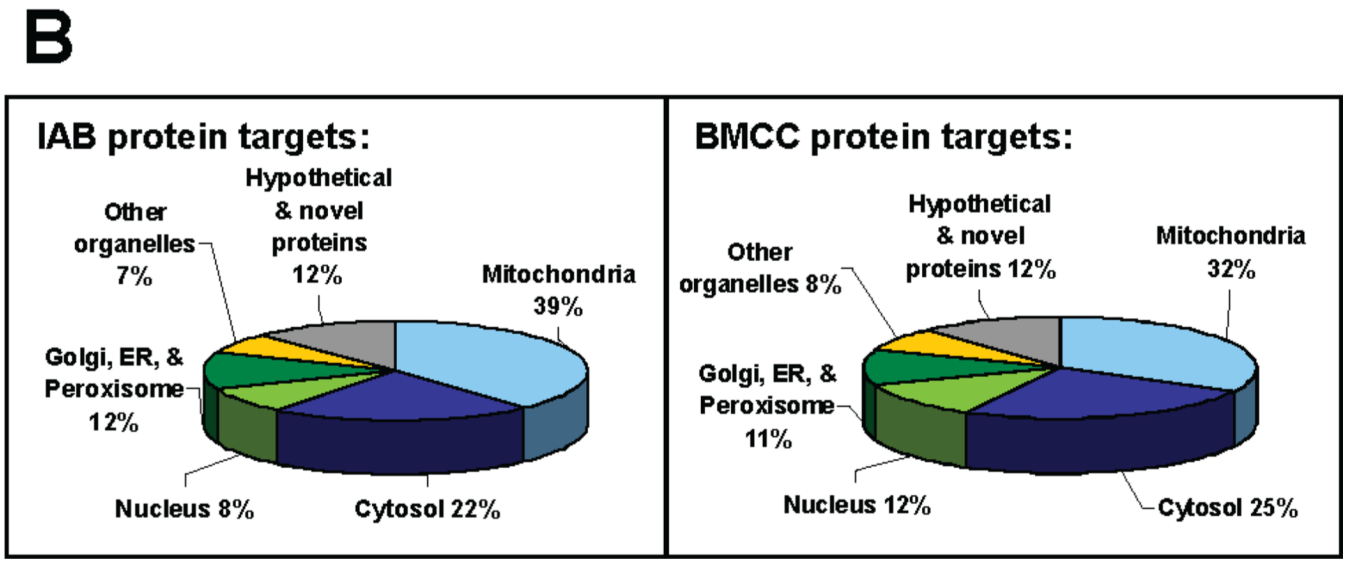


Figure 4.

(a) Venn diagram of the overlap of protein and cysteine adduct targets of IAB and BMCC. (b) Distribution of primary subcellular location for IAB and BMCC protein targets annotated from the UniProt database (http://www.pir.uniprot.org/).

Table 1

Protein Targets of IAB and BMCC Related to Apoptosis

			position of adducted Cys		
protein type	**protein name**	**UniProt KB prot ID**	**IAB**	**BMCC**	**refs**
permeability transition pore complex	ADP/ATP translocase 2	P05141	256	256	53
	ADP/ATP translocase 3	P12236	256	159, 256	54
	creatine kinase, sarcomeric mitochondrial precursor	P17540	317	317	55
	hexokinase, type I	P19367	628	628	38
	splice isoform 3 of VDAC2	P45880-3	13	13	56
	VDAC1	P21796	126, 231	126, 231	57
	VDAC2	P45880	62, 91	62, 91, 242	56
	VDAC3	Q9Y277	36, 65, 229	36, 65	58
preprotein import translocation	import inner membrane translocase subunit TIM44, mitochondrial precursor	O43615		343	59
	import inner membrane translocase subunit TIM50, mitochondrial precursor	Q3ZCQ8		236	60
	probable mitochondrial import receptor subunit TOM40 homologue	O96008	74	74, 76, 86	61
thioredoxin	peroxiredoxin 1	Q06830	83	173	62
	peroxiredoxin 4	Q13162	51, 245	51, 245	63
	peroxiredoxin 5, mitochondrial precursor	P30044	100		64
	thioredoxin	P10599		72	65
	thioredoxin domain containing protein 5 precursor	Q8NBS9	121, 217, 247, 350	121, 128, 247, 254, 381	66
	thioredoxin reductase 2, mitochondrial precursor	Q9NNW7		168	67, 68
	thioredoxin, mitochondrial precursor	Q99757	90		32, 69
	thioredoxin-dependent peroxide reductase, mitochondrial precursor	P30048	229	229	70

protein type	protein name	UniProt KB prot ID	position of adducted Cys: IAB	position of adducted Cys: BMCC	refs
	thioredoxin-like 5	Q9BRA2	43		71
heat shock proteins	60 kDa heat shock protein, mitochondrial precursor	P10809	237, 442, 447	237, 442, 447	72
	heat shock 70 kDa protein 1	P08107	306	17, 306	73
	heat shock 70 kDa protein 4	P34932		34	73
	heat shock cognate 71 kDa protein	P11142	17	17, 63	73
	heat shock protein 75 kDa, mitochondrial precursor	Q12931	573	501, 573	74, 75
	STIP1 homology and U box-containing protein 1	Q9UNE7	199	48, 83	50
	stress-70 protein, mitochondrial precursor	P38646	317, 366, 608	317, 608	76, 77
assorted	38 kDa FK-506 binding protein homologue	Q14318	170, 217, 238	138, 170, 217, 238	78
	aconitate hydratase, mitochondrial precursor	Q99798	385		79
	caspase-3 precursor	P42574	163		80
	caspase-6 precursor	P55212	163		80
	catalase	P04040	376	376	81
	cofilin 1	P23528	138	138	39
	ETHE1 protein, mitochondrial precursor	O95571	170		82
	FEM-1-like death receptor binding protein	Q9UK73	186		83
	FK506-binding protein 8 variant	Q53GU3	137		78
	macrophage migration inhibitory factor	P14174		80	84
	oxidoreductase HTATIP2	Q9BUP3	172	172	85
	peptidylprolyl isomerase A, isoform 2	P62937	101	101	86
	programmed cell death 6-interacting protein	Q8WUM4	250		87
	programmed cell death protein 8, mitochondrial precursor	O95831	256		88
	sphingosine-1-phosphate lyase 1	O95470	285	285	89

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protein type	protein name	UniProt KB prot ID	position of adducted Cys		refs
			IAB	BMCC	
	tax1-binding protein 1	Q86VP1		675	90
	transmembrane GTPase MFN2	O95140		348	91