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Mass Spectrometric Investigation of Liver Proteins Isolated from 2-Aminoanthracene Exposed Fisher-344 Rats

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ABSTRACT

The toxicity associated with 2-aminoanthracene (2-AA) exposure in Fisher-344 (F-344) rats was investigated using proteomics approach. Twenty four post-weaning 3-4 week old F-344 male rats were fed diets of 0 mg kg^{-1} diet (control), 50 mg kg^{-1} diet (low dose), 75 mg kg^{-1} diet (medium dose) and 100 mg kg^{-1} diet (high dose) 2-AA for 14 and 28 days. This was followed by total protein extraction from liver tissues. Protein samples were separated using one dimensional gel electrophoresis. The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system. Generated data were searched using Mascot and quantified via spectral counting. There seem to be more proteins expressed in the short-term study than the long-term experimental group. Employing DAVID analytical tool showed that proteins expressed seem be involved in carbohydrate, lipid and protein metabolic processes. Some of these processes include; galactose metabolism, fatty acid metabolism, cysteine and methionine metabolism, protein complex biogenesis and mitochondrial structural proteins. Using relationship-bioinformatics analytical tool, SLC2A4 and MAPK3 were found to interact with several proteins identified from the current study. The nature of this interaction is unknown at this point. Understanding the role of SLC2A4 in regulating other proteins as observed in the study will be pursued in future investigations. Combining the current data with other toxicogenomic data from the liver and pancreas will be useful in developing biomarkers due to arylamine toxicity.

Key words: Mass spectrometry, 2-aminoanthracene (2-AA), rats liver, SLC2A4 (glucose transporter type 4), DAVID, metabolic processes

INTRODUCTION

Exposure to environmental contaminants such as Polycyclic Aromatic Hydrocarbons (PAH) including arylamines may increase the risk of developing diseases such as diabetes and cancer. Aromatic amines or arylamines, belong to a class of polycyclic aromatic hydrocarbons (ATSDR, 1995; Boudreau *et al*., 2001). They are considered occupational carcinogens that are synthesized for a variety of industrial purposes. For instance, they are used in the manufacturing of agricultural chemicals, drugs, dyes, road tars, synthetic fuels, inks, plastics, as rubber antioxidants, curing agents in the synthesis of epoxy resins and of polyurethanes (Boudreau *et al*., 2001; Snyderwine *et al*., 1992; Zhu *et al*., 1995). Arylamines such as 2-Aminoanthracene, is a by-product of burning of tobacco and the broiling of meat (Baker *et al*., 2001).

In order to express its toxicity effects, arylamine compounds like 2-AA are known to undergo metabolic activation through two separate biotransformation pathways primarily in the liver. These involve the use of drug metabolizing enzymes such as N-acetyltransferases (NAT) and cytochrome P450 (P450). The pathways involve N-hydroxylation as well as N-acetylation (Hatch *et al*., 2001; Serafimova *et al*., 2007; So *et al*., 2008).

It has been previously demonstrated that dietary ingestion of 2-AA induced diabetic-like symptomology. The report showed significantly elevated plasma glucose and glycated hemoglobins and reduced serum protein levels (Boudreau *et al*., 2001). Recently, a follow-up study seems to suggest that 2-AA is involved in the dysregulation of several pancreatic genes that regulate lipid and protein metabolism. This might include feedback mechanism which may ultimately lead to insulin resistance and tissue autolysis (Gato and Means, 2011b) and also reported that 2-AA directly suppresses Ins1 and Ins2 gene expression with prolonged exposure leading to symptomology associated with insulin-dependent diabetes.

In the present study, the effect of 2-AA on the liver of Fisher-344 rats was examined via using proteomic techniques. There has been an increasing interest in employing proteomics to study protein structure and function. Because this technique provides the opportunity to directly quantify the effect of toxicants directly on proteins. The process typically involves isolating total proteins from tissues, purifying them, running either 1D or 2D gels followed by digestion of protein spots with trypsin and analyzing them using mass spectrometry after which peptides will be identified.

MATERIALS AND METHODS

Experimental design: To measure the response of F-344 rats to 2-AA intoxication, the effects of 2-AA exposure on the liver tissues was investigated. Twenty four post-weaning 3-4 week old Fisher 344 rats were purchased from Harlan Laboratories and randomly assigned to one of four dietary dose regimens of 0 mg kg^{-1} diet (control), 50 mg kg^{-1} diet (low dose), 75 mg kg^{-1} diet (medium dose) and 100 mg kg^{-1} diet (high dose) 2-aminoanthracene (2-AA) for either 14 or 28 days Rats were provided distilled water *ad libitum*. Rats were housed at the Southern Illinois University Animal Facility. The animals were treated according to the principles outlined in the NIH and Southern Illinois University Guide for the Care and Use of Laboratory Animals. At the end of each exposure period (14 or 28 days), rats were euthanized with CO_2 and blood was collected by cardiac puncture.

Diet preparation: 2-AA (CAS# 613-13-8) [98+% Pure] was obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification. A kilogram of rat diet supplied by PMI Nutrition International, LLC (Brentwood, MO) was immersed in 1 L molecular grade ethyl alcohol. This contained the mass of 2-AA necessary to yield the target dose concentrations in the diets. The ethyl alcohol was evaporated under the hood with periodic thorough mixing to assure homogeneity. The diet was stored in the freezer and protected from light until given to the animals.

Total protein extraction: Total protein was extracted from liver tissues using Qproteome Mammalian Protein Prep Kit. Approximately 40 mg of tissues were lysed in 1 mL mammalian cell lysis buffer including 10 μL protease inhibitor and 1 U benzonase nuclease. This was followed by tissues disruption for 30 sec at medium speed in 15 mL propylene centrifuge tube. Samples were then transferred into 2 mL precooled microcentrifuge tubes and centrifuged at 7800 rpm for 10 min. The supernatant was filtered through Whatman 0.2 μm PVDF Filter Media and concentrated via 3 K Amicon centrifugal filter device for 20 min. Samples were then aliquoted and stored at -20°C.

One-dimensional gel electrophoresis: Protein samples were separated using one dimensional gel electrophoresis. Protein samples were diluted with Laemmli sample buffer in a ratio of 1:1. The Laemmli buffer was prepared by adding 25 µL β-mercaptoethanol to 475 µL Laemmli sample buffer. The protein-Laemmli mixture was vortexed briefly and heated for 5 minutes at 95°C. Thirty microliters of the mixture along with a molecular weight marker were loaded onto mini-protean TGX precast gel (Bio-Rad, Hercules, CA). The gel was run at constant 200 V and 50 mA using 1XTris/glycine/SDS gel running buffer for 35 min. Then, gel was pulled of the cassette and rinsed three times for 5 min each with high purity MilliQ water. After this, the gel was stained with 50 mL of Bio-Safe Coomassie G-250 stain (Bio-RAD, Hercules, CA) for 1 h with gentle shaking. Finally, the gels were washed again with high purity MilliQ for 30 min and imaged.

Gel sample digestion: Gel bands were excised from control and high dose treatment groups with approximate molecular weights of 50 and 100 kDa and mailed to MSBioworks LLC for analysis. The gel bands were processed using a robot (ProGest, DigiLab) with the following protocol: washed with 25 mM ammonium bicarbonate followed by acetonitrile. This was followed by reduction with 10 mM dithiothreitol at 60°C followed by alkylation with 50 mM iodoacetamide at RT. Then digested with trypsin (Promega, Madison WI) at 37°C for 4 h. And finally quenched with formic acid and the supernatant was analyzed directly without further processing.

Mass spectrometry: The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher LTQ OrbitrapVelos. Peptides were loaded on a trapping column and eluted over a 75 µm analytical column at 350 nL min^{-1}; both columns were packed with Jupiter Proteo resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with MS performed in the Orbitrap at 60,000 FWHM resolution and MS/MS performed in the LTQ. The fifteen most abundant ions were selected for MS/MS.

Data analysis: Data were searched using a local copy of Mascot with the following parameters: Enzyme-Trypsin; Database-IPI Rat v3.75 (combined forward and reverse plus common contaminants); Fixed modification-Carbamidomethyl (C); Variable modifications-Oxidation (M), Acetyl (N-term), Pyro-Glu (N-term Q), Deamidation (N,Q); Mass values-Monoisotopic; Peptide Mass Tolerance-10 ppm; Fragment Mass Tolerance-0.8 Da; Max Missed Cleavages-2. Mascot DAT files were parsed into the Scaffold software for validation, filtering and to create a non-redundant list per sample. Data were filtered using a minimum protein value of 90%, a minimum peptide value of 50% (Prophet Scores) and requiring at least two unique peptides per protein. Data was presented using spectral counts.

Gene ontology: The Gene Ontology (GO) comparison tool provides the opportunity to show the relationship between and the association of gene transcripts with respect to each other in function and biochemical pathways. This tool organizes genes into hierarchical categories via biological process, molecular function and cellular components. Peptides identified from the current experiment were implemented in DAVID (database for annotation, visualization and integrated discovery) (Huang *et al*., 2009).

Network analysis by VisANT: VisANT, a web-based biological network modeling software (Hu *et al*., 2004) was utilized to generate connections of annotated proteins identified via mass spectrometry.

Table 1: An overview of protein and peptide totals

Parameter	2 C	2 HD	4 C	4 HD
Total No. of proteins	44	90	44	41.0
Total No. of spectra matching	861	1283	1216	1218.0
Total No. of unique peptides	318	698	414	301.0
False discovery rats (%)	0	0	0	2.4

RESULTS

To determine the proteins that might mediate 2-AA toxicity, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to analyze protein samples extracted from liver tissues. The samples included control (0 mg kg^{-1} 2-AA), low dose (50 mg kg^{-1} 2-AA), medium dose (75 mg kg^{-1} 2-AA) and high dose (100 mg kg^{-1} 2-AA)-diet. A total of 44 proteins were identified from the 2 week control 50 kDa band. Similarly, 90 proteins were found in the 2HD 50 kDa band. The 4C and 4HD 100 kDa bands showed 44 and 41 proteins, respectively (Table 1). There were more than 300 unique peptides identified in each band analyzed. The total number of spectra matching ranged from 860 to slightly over 1200, with a false discovery rate of 0-2.4%.

Table 2 and 3 illustrate selected proteins identified via mass spectrometry. The molecular weight of the proteins identified ranged from as small as 14 kDa (Fabp1 Fatty acid-binding protein, liver) and 334 kDa (Celsr1 cadherin, EGF LAG seven-pass G-type receptor 1) with respect to the 4 weeks band analyzed. For the two weeks gel bands analyzed, molecular size of identified peptides appears to be closer in value. The smallest protein within this group had a size of 22 kDa (Prdx1 Peroxiredoxin-1) and 103 kDa in the case of the bigger protein (Acat2 Ab2-076). Hendrickson *et al.* (2006) noted that spectral counting is a useful technique for quantifying relative changes in protein abundance. Spectra counts for the control group were between 0 and 60 while that of the treated group ranged from 0 to 49. Some of the proteins which exhibited differences in their spectra counts included; transketolase, trypsin, catalase, beta-galactosidase, hemopexin and glucokinase regulatory protein (Table 2). Similarly, some proteins were observed to show different spectra counts in the 14 day treated group. These were: Long-chain specific acyl-CoA dehydrogenase, mitochondrial; Phosphoglycerate kinase 1; Arginase-1; glutamine synthetase, catalase, hemopexin and Peroxiredoxin-1 (Table 3).

To determine how protein modifications impact on the identified peptides, artifacts such as oxidation, deamidation and N-acetylation were applied in database search. The term Oxidation refers to the addition of 16 Da to Methionine while pyro-Glu converts N-terminal glutamine to pyro glutamic acid (-17 Da). Deamidation on the other hand is the conversion of Asparagine (Asn) to aspartic acid (Asp) or Glutamine (Gln) to Glutamate (Glu). Demonstrated in Fig. 1 was a plot of the number of proteins associated with deamidation, N-acetyl, pyro-Glu and oxidation modifications. With respect to the 2 weeks group, there were 6, 40, 53 and 3 proteins in N-acetyl, deamidation, oxidation and pyro-Glu modifications respectively (Fig. 1a). Similarly, N-acetyl, deamidation, oxidation and pyro-Glu modifications produced 5, 17, 27 and 1 proteins, respectively, for the 4 weeks study group (Fig. 1b).

A protein Venn diagram of control and treated identified proteins was plotted in Fig. 2a and b. The short term treatment group had 46 proteins in common between the control and high dose. Similarly, the four week control and high dose groups had 29 identified proteins in common.In the case of the two weeks study, there were 51 more proteins than the control animals. In contrast, the

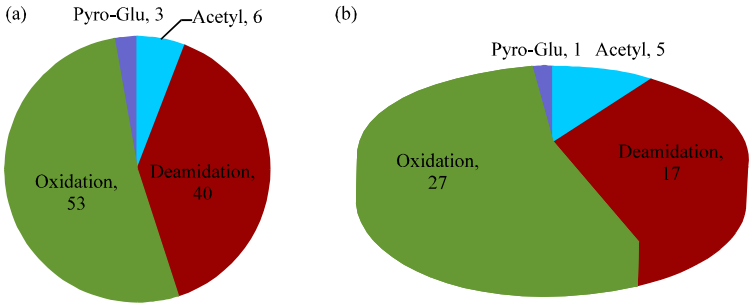

Fig. 1(a-b): N-acetyi, deamidation, Pyro-Glu and oxidation modified proteins. (a) shows proteins identified from 2 weeks treatment group. (b) refers to proteins determined from 4 weeks experimental group

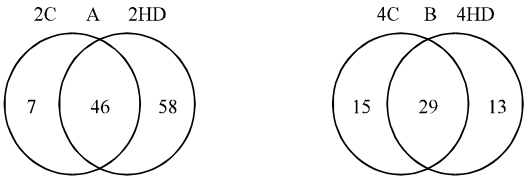

Fig. 2(a-b): Protein Venn diagram of identified number of protein from the different samples. (a) control (2C) versus high dose (2HD) for two weeks exposure period and (b) control (4C) and high dose (4HD) for 4 weeks treatment period

Table 2: Selected proteins identified from 4 weeks treated 2-AA rats. Gel bands were excised from control and high dose treatment groups with approximate molecular weights of 100 kDa. The 4C and 4HD table columns were spectra counts of control (0 mg kg^{-1} 2-AA diet) and high dose (100 mg kg^{-1} 2-AA diet) dose regimens

Identified proteins	Accession No.	Molecular Wt. (kDa)	4C	4HD
AhcyAdenosylhomocysteinase	IPI00476295	48	0	2
Ahsg Alpha-2-HS-glycoprotein	IPI00327469	38	2	3
Anxa6 Annexin A6	IPI00421888 (+2)	76	27	11
ApohApolipoprotein H	IPI00778633	38	4	4
Basp1 Brain acid soluble protein 1	IPI00231651 (+1)	22	0	3
BhmtBetaine--homocysteine S-methyltransferase 1	IPI00332027	45	4	2
CalrCalreticulin	IPI00191728	48	7	2
Car3 Carbonic anhydrase 3	IPI00230788	29	2	0
Cat Catalase	IPI00231742	60	36	23
Cbs Isoform I of Cystathionine beta-synthase	IPI00214299 (+3)	61	0	2
Celsr1 cadherin, EGF LAG seven-pass G-type receptor 1	IPI00361486	334	2	0
CON_Trypsin	CON_Trypsin	24	26	49
Fabp1 Fatty acid-binding protein, liver	IPI00190790	14	0	2
FetubFetub protein	IPI00212708 (+1)	43	2	0
Gc Vitamin D-binding protein	IPI00194097	54	3	0
GckrGlucokinase regulatory protein	IPI00231727	69	6	2
Glb1 Beta-galactosidase	IPI00371132	73	0	4
Glud1 Glutamate dehydrogenase 1, mitochondrial	IPI00324633	61	6	4

Table 2: Continued

Identified proteins	Accession No.	Molecular Wt. (kDa)	4C	4HD
Gusb Ac2-223	IPI00382289 (+1)	93	2	0
Hacl1 2-hydroxyacyl-CoA lyase 1	IPI00193153	64	3	0
HpxHemopexin	IPI00195516	51	41	28
HrgHistidine-rich glycoprotein	IPI00191789 (+4)	58	2	0
Hrsp12 Ribonuclease UK114	IPI00231292	14	0	2
Hspa8 Heat shock cognate 71 kDa protein	IPI00208205 (+2)	71	5	0
Kng1 Kininogen 1	IPI00515829 (+1)	48	7	6
Kng1l1 T-kininogen 2	IPI00679245	48	0	4
LOC259246 Major urinary protein	IPI00191711 (+2)	21	0	4
LOC299282 Serine protease inhibitor A3L	IPI00200591 (+1)	46	11	8
NclNucleolin	IPI00231827 (+2)	77	3	5
Nucb1 Nucleobindin-1	IPI00205022 (+1)	54	0	2
Nucb2 Nucleobindin-2	IPI00200070	50	0	2
Orm1 Alpha-1-acid glycoprotein	IPI00191715	24	2	4
Pdzk1 Na(+)/H(+) exchange regulatory cofactor NHE-RF3	IPI00200998 (+1)	57	6	2
Pgm1 Phosphoglucomutase-1	IPI00231641 (+1)	61	10	5
Sdha Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	IPI00200659 (+1)	72	4	0
Selenbp1 Selenium-binding protein 1	IPI00208026	53	2	2
Serpina1 Alpha-1-antiproteinase	IPI00324019	46	0	2
Serpina3k Serine protease inhibitor A3K	IPI00200593	47	19	19
Serping1 Plasma protease C1 inhibitor	IPI00372792	56	0	2
Sod1 Superoxide dismutase [Cu-Zn]	IPI00231643	16	7	9
Srprb Ba1-667	IPI00196656 (+3)	107	7	3
Stip1 Stress-induced-phosphoprotein 1	IPI00213013	63	15	10
Tkttransketolase	IPI00231139	71	60	48

Table 3: Selected proteins identified from 2 weeks treated 2-AA rats. Gel bands were excised from control and high dose treatment groups with approximate molecular weights of 50 kDa. The 2C and 2HD table columns were spectra counts of control (0 mg kg^{-1} 2-AA diet) and high dose (100 mg kg^{-1} 2-AA diet) treatment groups

Identified proteins	Accession No.	Molecular Wt. (kDa)	2C	2HD
Acaa2 3-ketoacyl-CoA thiolase, mitochondrial	IPI00201413 (+1)	42	0	2
Acadl Long-chain specific acyl-CoA dehydrogenase, mitochondrial	IPI00211225	48	8	48
Acadm Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	IPI00212015	47	0	5
Acads Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	IPI00231359	45	0	13
Acat1 Acetyl-CoA acetyltransferase, mitochondrial	IPI00324302	45	2	25
Acat2 Ab2-076	IPI00382233 (+1)	103	0	3
Acp6 Acid phosphatase 6, lysophosphatidic	IPI00372429	47	9	10
Acy1 Aminoacylase-1A	IPI00464791	46	0	7
Adk Adenosine kinase	IPI00214456	40	0	5
AfmAfamin	IPI00207668	69	0	2
AhcyAdenosylhomocysteinase	IPI00476295	48	10	21
ALB Isoform 1 of Serum albumin precursor	CON_00745872 (+1)	69	0	19
Aldob Fructose-bisphosphatealdolase B	IPI00471911 (+1)	40	0	12
Amacr Alpha-methylacyl-CoA racemase	IPI00188858	42	0	8
Ambp Protein AMBP	IPI00210900	39	10	7

Table 3: Continued

Identified proteins	Accession No.	Molecular Wt. (kDa)	2C	2HD
AmtAminomethyltransferase	IPI00358872	44	0	5
Arg1 Arginase-1	IPI00327518	35	7	28
ArsbArylsulfatase B	IPI00198405	59	2	3
As3mt Arsenitemethyltransferase	IPI00207328	41	0	6
Asrgl1 L-asparaginase	IPI00207252	34	0	2
Azgp1 Zinc-alpha-2-glycoprotein	IPI00211103	34	6	4
Baat Bile acid-CoA:amino acid N-acyltransferase	IPI00207010	46	2	5
Bbox1 Gamma-butyrobetainedioxygenase	IPI00215321	45	0	5
BhmtBetaine--homocysteine S-methyltransferase 1	IPI00332027	45	10	13
Cald1 Non-muscle caldesmon	IPI00208118	61	0	5
CalrCalreticulin	IPI00191728	48	7	5
Cat Catalase	IPI00231742	60	4	6
CON_Trypsin	CON_Trypsin	24	34	32
Creld2 Cysteine-rich with EGF-like domain protein 2	IPI00366452	38	9	10
Ctbs Di-N-acetylchitobiase	IPI00203152	42	27	27
Cth Putative uncharacterized protein Cth	IPI00949840	44	7	34
Ctsd Putative uncharacterized protein Ctsd	IPI00389830 (+1)	48	15	25
Ech1 Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	IPI00326561	36	0	3
Eef1a1 Elongation factor 1-alpha 1	IPI00195372 (+4)	50	2	0
FahFumarylacetoacetase	IPI00214480	46	21	28
Fbp1 Fructose-1,6-bisphosphatase 1	IPI00231745	40	0	14
FetubFetub protein	IPI00212708 (+1)	43	7	0
Gc Vitamin D-binding protein	IPI00194097	54	0	2
Gla Putative uncharacterized protein Gla	IPI00367674	48	7	9
Glul Glutamine synthetase	IPI00324020	42	3	9
Got1 Aspartate aminotransferase, cytoplasmic	IPI00421513	46	0	17
Got2 Aspartate aminotransferase, mitochondrial	IPI00210920	47	0	8
Gsta2;Gsta1;Gsta3 Glutathione S-transferase alpha-1	IPI00231638 (+1)	26	0	2
Hao1 Hydroxyacid oxidase 1	IPI00207601	41	0	10
HdgfHepatoma-derived growth factor	IPI00326566	26	0	3
Hnrnpab Nucleic acid binding factor pRM10	IPI00208193 (+3)	34	2	14
Hnrpd Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0	IPI00201032 (+3)	38	2	3
Hpd 4-hydroxyphenylpyruvate dioxygenase	IPI00211507	45	16	29
HpxHemopexin	IPI00195516	51	3	2
Idh1 Isocitrate dehydrogenase [NADP] cytoplasmic	IPI00194045 (+1)	47	0	5
IvdIsovaleryl-CoA dehydrogenase, mitochondrial	IPI00193716	46	0	5
Kat3 Kynurenine--oxoglutarate transaminase 3	IPI00564133	51	0	9
KynuKynureninase	IPI00191521	52	17	11
MvdDiphosphomevalonate decarboxylase	IPI00210317	44	0	2
Nucb2 Nucleobindin-2	IPI00200070	50	0	6
Nudc Nuclear migration protein nudC	IPI00210009	38	2	2
Orm1 Alpha-1-acid glycoprotein	IPI00191715	24	2	3
Otc Ornithine carbamoyltransferase, mitochondrial	IPI00210139 (+1)	40	0	4
PeciPeroxisomal 3,2-trans-enoyl-CoA isomerase	IPI00208203	43	0	2
PecrPeroxisomal trans-2-enoyl-CoA reductase	IPI00326195	32	0	4
Pgk1 Phosphoglycerate kinase 1	IPI00231426	45	13	25
PipoxPipecolic acid oxidase	IPI00369954	44	0	3
Plbd1 Putative phospholipase B-like 1	IPI00364591	63	2	6

Table 3: Continued

Identified proteins	Accession No.	Molecular Wt. (kDa)	2C	2HD
PpidPeptidyl-prolylcis-trans isomerase D	IPI00373218	41	0	2
Prdx1 Peroxiredoxin-1	IPI00211779 (+1)	22	3	6
RGD1306809 UPF0465 protein C5orf33 homolog	IPI00766238 (+2)	48	26	24
RGD1562373 3-ketoacyl-CoA thiolase B, peroxisomal	IPI00370596 (+1)	44	0	10
Sae1 SUMO-activating enzyme subunit 1	IPI00366795	39	0	7
Sephs1 selenophosphatesynthetase 1	IPI00363160	43	0	2
Serpina1 Alpha-1-antiproteinase	IPI00324019	46	5	6
Serpina3k Serine protease inhibitor A3K	IPI00200593	47	3	0
Serpinb1a Leukocyte elastase inhibitor A	IPI00779473	43	5	11
Serpinf2 Serine (Or cysteine) peptidase inhibitor, clade F, member 2	IPI00199695	55	4	7
Set Isoform 1 of Protein SET	IPI00213688 (+4)	33	0	4
Sord Sorbitol dehydrogenase	IPI00760137	38	0	21
St13 Hsc70-interacting protein	IPI00199273 (+1)	41	0	2
Tcn2 Transcobalamin-2	IPI00206173	47	2	0
Tsta3 tissue specific transplantation antigen P35B	IPI00201528 (+1)	36	0	2
Upb1 Beta-ureidopropionase	IPI00208970	44	22	21
Uqcrc2 Cytochrome b-c1 complex subunit 2, mitochondrial	IPI00188924	48	0	2

Table 4: Pathways determined to be significantly ($p<0.05$) altered by 2-AA dietary exposure using KEGG (Kyoto Encyclopedia of Genes and Genomes). Protein transcripts involved in each pathway range from 2 through 8

Pathways annotation tem	No. of proteins	Enrichment p-value
A		
Fatty acid metabolism	8	7.3 E-8
Valine, leucine and Isoleucine degradation	7	3.0 E-6
Arginine and proline metabolism	6	1.1 E-4
Cysteine and methionine metabolism	5	2.6 E-4
Glycine, serine and threonine	4	2.9 E-3
Fructose and mannose metabolism	4	2.9 E-3
Tyrosine metabolism	4	3.5 E-3
Lysine degradation	4	6.0 E-3
Tryptophan metabolism	4	6.8 E-3
Terpenoid backbone biosynthesis	3	6.9 E-3
Phenylalanine metabolism	3	9.0 E-3
Selenoamino acid metabolism	3	1.5 E-2
Nitrogen metabolism	3	1.8 E-2
Biosynthesis of unsaturated fatty acids	3	2.0 E-2
Alanine, aspartate and glutamate metabolism	3	3.0 E-2
Butanoate metabolism	3	3.6 E-2
Propanoate metabolism	3	3.6 E-2
Phenylalanine, tyrosine and tryptophan biosynthesis	2	4.5 E-2
Glutathione metabolism	3	7.6 E-2
Synthesis and degradation of ketone bodies	2	7.9 E-2
B		
Cysteine and methionine metabolism	3	6.1 E-3
Complement and coagulation cascades	3	2.3 E-2
Selenoamino acid metabolism	2	6.9 E-2
Glycosaminoglycan degradation	2	7.2 E-2
Nitrogen metabolism	2	7.5 E-2
Pentose phosphate pathway	2	7.9 E-2

A: Represents 14-days exposure and B: Shows 28 days treatment period

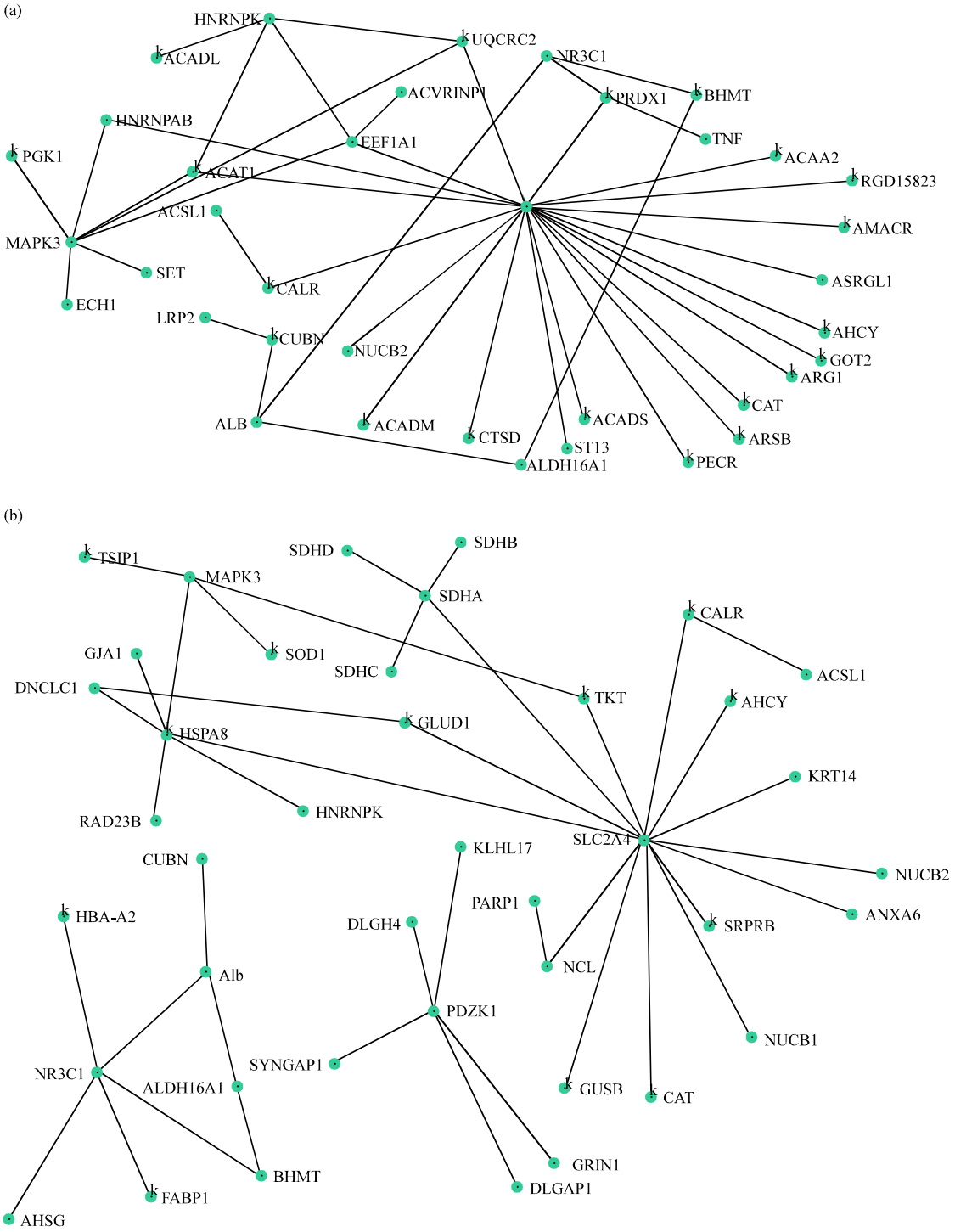

Fig. 3(a-b): Network displaying connections between proteins from dietary exposure of F-344 rats to 2-AA for 14 and 28-days. The links were generated using VisANT, an internet based tool that enables integration of biomolecular interactions data into graphical and cohesive display. (a) Peptides identified from 2 weeks samples and (b) All proteins that were identified from the 4 weeks experiment

high dose group revealed two less proteins than the control, with respect to the four week study. Obviously, more proteins were identified in the two week treated sample than the four week adulterated diet sample.

To explore the relationships between identified proteins of the F-344 exposed to 2-AA via diet, VisANT, interactive software platform that enables biological network modeling was employed to further analyze the dataset. Figure 3 showed network displaying links between genes annotated from dietary exposure of F-344 rats to 2-AA for 14 and 28-days. An "-" sign indicates fully expanded nodes while "+" shows some connections that have not yet been displayed.

DAVID bioinformatics tool was employed to analyze identified protein list. DAVID is useful in the application of biological meaning from gene and protein list. DAVID was employed to glean extra information from the identified rats liver proteins. The findings were presented as pathways affected (Table 4), summary of gene ontology categories (Fig. 4) and selected gene ontology categories (Table 5 and 6). More GO categories we noted for the short-term study than the chronic exposure period (Fig. 4). Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) were 112, 35 and 46 categories, respectively for the 14-day study. Similarly, BP, CC and MF categories for the 28 day experiment were 85, 25 and 19 correspondingly. Selected pathway annotation terms include; fatty acid metabolism, cysteine and methionine metabolism, nitrogen

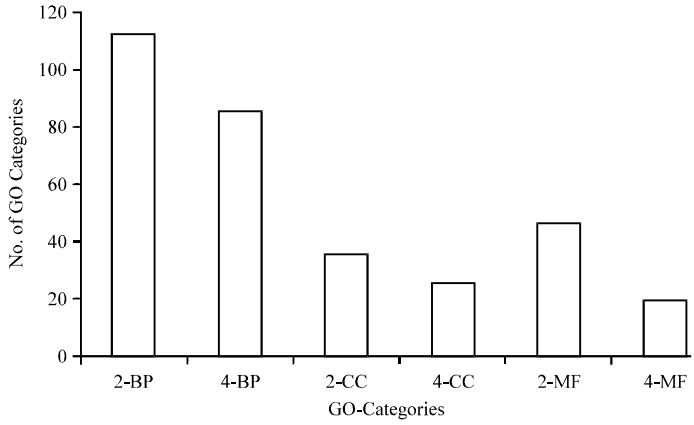

Fig. 4: Gene ontology category distribution of hepatic protein expression analysis of 2-AA exposed male Fisher 344 rats for 14- and 28-days. The categories include Biological Process (BP), Cellular Component (CC) and Molecular Function (MF)

Table 5: Selected Gene Ontology categories of proteins identified from livers of F-344 rats. Proteins were isolated from animals exposed to 2-AA for 28 days

GO Categories	GO term	Protein count	p-value
BP	Acute-phase response	6	2.1 E-8
	Acute inflammatory response	7	1.1 E-7
	Response to wounding	10	1.0 E-6
	Inflammatory response	8	1.3 E-6
	Defense response	8	6.7 E-5
	Regulation of response to external stimulus	5	8.1 E-4
	Positive regulation of multicellular organismal process	5	4.0 E-3
	Regulation of cellular protein metabolic process	6	4.4 E-3
	Cellular chemical homeostasis	5	1.6 E-2

Table 5: Continued

GO Categories	GO term	Protein count	p-value
	Regulation of phosphorylation	5	2.0 E-2
	Regulation of phosphorus metabolic process	5	2.3 E-2
	Regulation of phosphate metabolic process	5	2.3 E-2
	Cellular homeostasis	5	3.1 E-2
CC	Extracellular region	19	6.5 E-8
	Extracellular space	13	6.6 E-8
	Extracellular region part	13	2.6 E-6
	Cytoplasmic vesicle	9	9.2 E-4
	Cytosol	12	1.2 E-3
	Vesicle	9	1.4 E-3
	Cytoplasmic membrane-bounded vesicle	8	2.0 E-3
	Membrane-bounded vesicle	8	2.6 E-3
	Soluble fraction	6	3.7 E-3
	Secretory granule	5	5.8 E-3
	Membrane-enclosed lumen	11	9.8 E-3
MF	Endopeptidase inhibitor activity	10	1.0 E-9
	Peptidase inhibitor activity	10	2.0 E-9
	Enzyme inhibitor activity	11	4.0 E-9
	Cysteine-type endopeptidase inhibitor activity	5	9.0 E-6
	Serine-type endopeptidase inhibitor activity	5	2.4 E-4
	Cofactor binding	7	4.5 E-4
	Coenzyme binding	6	8.7 E-4
	Carbohydrate binding	6	5.0 E-3
	Sugar binding	5	5.6 E-3
	Cation binding	18	8.1 E-3
	Ion binding	18	9.7 E-3
	Metal ion binding	17	1.7 E-2
	Calcium ion binding	7	2.3 E-2

The categories included biological process (BP), cellular component (CC) and molecular function (MF) $p<0.05$

Table 6: Selected Gene Ontology (GO) categories of proteins identified from livers of F-344 rats. Proteins were isolated from animals exposed to 2-AA for 14 days

GO categories	GO term	Protein count	p-value
BP	Carboxylic acid catabolic process	12	4.4 E-12
	Cellular amino acid catabolic process	8	2.8 E-8
	Amine catabolic process	8	7.0 E-8
	Carboxylic acid biosynthetic process	10	1.3 E-7
	Glutamine family amino acid metabolic process	7	1.6 E-7
	Cellular amino acid biosynthetic process	7	2.7 E-7
	Amine biosynthetic process	8	4.1 E-7
	Oxidation reduction	15	5.1 E-6
	Fatty acid metabolic process	9	8.8 E-6
	Nitrogen compound biosynthetic process	11	1.2 E-5
	Response to nutrient levels	10	2.3 E-5
	Response to extracellular stimulus	10	4.0 E-5
	Cofactor metabolic process	8	1.2 E-4
	Response to organic substance	16	1.3 E-4
	Coenzyme metabolic process	7	2.5 E-4

Table 6: Continued

GO categories	GO term	Protein count	p-value
	Cellular amino acid derivative metabolic process	7	3.3 E-4
	Cellular carbohydrate biosynthetic process	5	4.5 E-4
	Response to hormone stimulus	11	4.6 E-4
	Cellular lipid catabolic process	5	6.7 E-4
	Response to inorganic substance	8	6.8 E-4
	Lipid catabolic process	6	8.6 E-4
	Response to steroid hormone stimulus	8	1.1 E-3
	Response to endogenous stimulus	11	1.1 E-3
	Carbohydrate biosynthetic process	5	2.0 E-3
	Response to drug	7	9.3 E-3
	Generation of precursor metabolites and energy	6	1.0 E-2
	Defense response	6	7.0 E-2
	Protein complex biogenesis	6	8.0 E-2
CC	Microbody	12	8.0 E-12
	Peroxisome	12	8.0 E-12
	Soluble fraction	14	4.1 E-8
	Mitochondrion	26	8.4 E-8
	Cytosol	21	1.4 E-5
	Mitochondrial matrix	9	1.5 E-5
	Mitochondrial lumen	9	1.5 E-5
	Cell fraction	18	9.3 E-5
	Lysosome	7	5.8 E-4
	Lytic vacuole	7	5.8 E-4
	Membrane-enclosed lumen	19	7.1 E-4
	Extracellular region	19	9.0 E-4
	Extracellular space	11	1.1 E-3
	Organelle lumen	18	1.4 E-3
	Mitochondrial part	11	1.5 E-3
	Vacuole	7	1.6 E-3
	Extracellular region part	11	1.2 E-2
	Intracellular organelle lumen	15	1.6 E-2
	Organelle envelope	9	2.3 E-2
	Envelope	9	2.4 E-2
MF	Cofactor binding	17	2.5 E-11
	Coenzyme binding	11	1.0 E-6
	Vitamin binding	8	1.4 E-5
	C-acyltransferase activity	4	7.6 E-5
	Acyl-CoA dehydrogenase activity	4	9.4 E-5
	Identical protein binding	13	2.1 E-4
	Carboxylic acid binding	7	5.0 E-4
	Transferase activity, transferring nitrogenous groups	4	8.7 E-4
	Endopeptidase inhibitor activity	6	2.1 E-3
	Serine-type endopeptidase inhibitor activity	5	2.4 E-3
	Peptidase inhibitor activity	6	2.8 E-3
	Ion binding	30	3.0 E-3
	Cation binding	29	4.8 E-3
	Iron ion binding	7	8.3 E-3
	FAD binding	4	1.0 E-2

Table 6: Continued

GO categories	GO term	Protein count	p-value
	Electron carrier activity	6	1.1 E-2
	Enzyme inhibitor activity	6	1.5 E-2
	Transition metal ion binding	20	1.5 E-2
	Metal ion binding	27	1.6 E-2
	Lipid binding	7	2.3 E-2
	Protein dimerization activity	8	4.2 E-2
	Protein complex binding	5	4.7 E-2

The categories included biological process (BP), cellular component (CC) and molecular function (MF) $p<0.05$

metabolism and galactose metabolism. Some GO categories observed include regulation of homeostasis, carbohydrate binding, mitochondrion, lipid binding and protein complex biogenesis and protein complex assembly. The derived GO categories for both the short and long term treatment groups show similar terms.

DISCUSSION

The current study was undertaken to examine the response of Fisher-344 rats to 2-AA diet. Protein expression levels in liver tissues were analyzed following 2-AA exposure. Proteins are direct targets of drugs and xenobiotics. Besides, mRNA levels might not correlate with protein expression levels. Pandey and Mann (2000) noted that verification of gene product by proteomics could be the first step in the annotation of the genome. Proteomics also provides the opportunity to study post-translational modifications. Identified peptides seem to show proteins that are involved in metabolism. For instance, trypsin-a serine protease, phosphoglucokinase-a phosphorus transferring protein, transketolase-a pentose phosphate pathway enzyme and ornithine carbamoyltransferase, mitochondrial-an enzyme that is important in the synthesis of pyrimidine nucleotides were found in this investigation and long-chain specific acyl-CoA dehydrogenase, mitochondrial-an important intermediate in the biosynthesis of lipids and fatty acid degradation (Faergeman and Knudsen, 1997). The GO terms showed a large number of proteins to belong to metabolic process similar to a recent report by Gato and Means (2011a). Some of these metabolic processes were; carbohydrate biosynthetic process, lipid biosynthetic process, fatty acid metabolic process and nitrogen compound metabolic process. In a recent report, Gato and Means (2011b) made similar observations, when they investigated the pancreatic response of F-344 rats to 2-AA via global gene expression analysis. The authors noted that, most of the mRNA transcripts that were differentially expressed are involved in energy metabolism in the pancreas and protein digestion.

Using artifacts to examine different protein modifications, oxidation and deamidation modifications were more than the acetyl and pyro-Glu artifactual protein modifications in both treatment groups. Oxidation modification was typically achieved via the addition of 16 kDa to methionine. Methionine is sulfur rich and a major target for chemical oxidation. Weissbach *et al.* (2002) reported that chemical oxidation contributes to Reactive Oxygen Species generation (ROS). Methionine in proteins can be converted to methionine sulfoxide and methionine sulfone. ROS has been implicated in various diseases including cancers and diabetes (Waris and Ahsan, 2006). Wright *et al.* (2006) suggests reactive oxygen species as the primary reason underlying the development of insulin resistance, beta-cell dysfunction and type 2 diabetes mellitus. Some of the oxidation-related proteins identified from the current study include; apoliprotein H, superoxide dismutase, glutamine synthetase, ornithine carbamoyltransferase and adenosine kinase. The

present findings may indicate the importance of oxidative stress as one of the ways of 2-AA induced toxicity. Deamidation (Asn to Asp) on the other hand, alters peptide and protein structures via essential structural and biological modifications (Robinson and Robinson, 2001). This can reduce protein activity as well as regulate time-dependent biological processes. Some identified deamidated proteins were fumarylacetoacetase, arginase-1, fructose-bisphosphatealdolase B and sorbitol dehydrogenase.

Though the 14 day exposure group showed, greater level of proteins expressed than the 28 day treatment animals, these were not analyzed from the similar size bands. Some proteins that were found in both exposure times included; catalase, trypsin, hemopexin, Gc vitamin D-binding protein and Fetub protein. Fetub protein is normal plasma protein suggested to be translational protein and gene biomarker for the prediction of fibrosis and tumors in multiple species (Mendrick and Daniels, 2007). Wang *et al.* (2010) also reported that Fetub protein could be a potential biomarker for a synthetic anti-angiogenic compound induced liver hepatotoxicity. For the current study, Fetub protein seems to disappear from the treated samples. Similar to Fetub protein, Hemopexin (HPX) is a plasma protein which is highly expressed in the liver. HPX protein has strong binding affinity for heme. Binding of HPX to heme to form heme-HPX complex is believed to induce intracellular antioxidant activities. This may reduce heme-mediated oxidative stress (Tolosano and Altruda, 2002). The expression of HPX was lower in the high dose group than the control group during the 4 weeks of exposure. This trend is similar with respect to the two weeks exposure, though the values are much smaller.

Diabetes is reported to affect many organs including the liver (Sundsten and Ortsater, 2009). Because diabetes mellitus is a heterogeneous disease, this disease alters many genes and their products. The response of the liver to 2-AA adulterated diet consumption was examined via mass spectrometry. Quantification of mRNA via quantitative polymerase chain reaction (Q-PCR) or microarray methodologies is specific and provides immediate identification of differentially expressed genes. However, it is lacking in its ability to measure protein modifications such as post-translational alteration that might affect protein activity (Ohtsubo *et al.*, 2005). Some correlation between mRNA and protein levels has been reported, although that relationship is fairly weak (Nie *et al.*, 2007; Orntoft *et al.*, 2002). Hence, it is necessary to measure protein expression levels in order to fully comprehend cellular function due to 2-AA intoxication. The findings from the present investigation showed proteins that relate to protein and fatty acid metabolism. This is supported by pathway and GO analysis that showed similar annotation terms to be important in the study of 2-aminoanthracene toxicity. The exact proteins were quite different from those observed using mRNA quantification in the pancreas in response to 2AA exposure.

Nevertheless, an interesting trend in the relationships of the current proteins and previous quantified mRNA levels to SLC2A4 and MAPK3 were observed. SLC2A4 and MAPK3 were not expressed as a protein or gene in either study. It appears these proteins were central to the function of the others reported in the current study. In the previous experiment, VisANT was employed to explore the relationships between differentially expressed genes of animals intoxicated with 2-AA. Transcripts SLC2A4 and MAPK3 were found to have at least 50 links with other genes from the study (Gato and Means, 2011a). In the present study, SLC2A4 seems to be linked to such proteins as ACAT1, CAT, ARG1, EEF1A1, TKT, ALDH16A1, HSPA8, GLUD1 and NR3C1. SLC2A4 links up with MAPK3 via TKT and HSPA8 in the 2 weeks experimental group, while its connection was through ACAT1 and EEF1A1 in the 28 day treatment group. These links will be explored in a future experiment.

SLC2A4, solute carrier family 2 (facilitated glucose transporter), also referred to as glucose transporter type 4 or insulin-responsive plays a critical role in glucose homeostasis and diabetes pathogenesis (Tarazona-Santos *et al*., 2010). Glucose transporters (GLUT-4) respond to insulin stimulation by transporting glucose from the cell surface into the cell thereby reducing blood glucose and making it possible for the synthesis of glycogen and triglycerides. It is also reported that the trafficking pathway of GLUT4 involves multiple insulin regulation (Tarazona-Santos *et al*., 2010; Huang and Czech, 2007). Mitogen Activated Protein Kinase (MAPK) on the other hand are involved in signal transduction pathways that regulate gene expression and other important cellular processes (Cuadrado and Nebreda, 2010) in response to extracellular stimuli. Altered MAPK3 gene expression is reported to be implicated in various cancers including melanoma, breast cancer and myeloid leukemias (Yang *et al*., 2004; Gout *et al*., 2006). When mitogens, hormones or neurotransmitters bind to receptor tyrosine kinases, MAP kinases signaling cascade is triggered which consequently activates oncogenic RAS (Inamdar *et al*., 2010).

CONCLUSIONS

The effect of 2-AA on F-344 rats analyzed via proteomics approach show proteins that seem to be involved in either carbohydrate or protein or lipid metabolism. By using a variety of artifactual examination, oxidation and deamidation modifications were greater than acetyl and pyro-Glu protein modifications in both treatment groups. The 14-day exposure group showed, greater level of proteins expressed and subsequent pathways and GO categories than the 28 day treatment animals. Some proteins that were found in both exposure times included; catalase, trypsin, hemopexin, Adenosylhomocysteinase, Calreticulin, Gc vitamin D-binding protein and Fetub protein. Using relationship-bioinformatics analytical tool, SLC2A4 and MAPK3 were found to interact with several proteins identified from the current study. SLC2A4 and MAPK3 proteins were not present in the current study. SLC2A4 also referred to as glucose transporting protein plays a crucial role in glucose homeostasis and diabetes pathogenesis. Understanding the role of SLC2A4 in regulating other proteins as observed will be pursued in future investigations. Combining the current data with other toxicogenomic data from the liver and pancreas will be useful in developing biomarkers due to arylamine toxicity.

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