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A Proteomics Approach to the Study of Absorption, Distribution, Metabolism, Excretion, and Toxicity

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A proteomics approach was used to identify liver proteins that displayed altered levels in mice following treatment with a candidate drug. Samples from livers of mice treated with candidate drug or untreated were prepared, quantified, labeled with CyDye DIGE Fluors, and subjected to two-dimensional electrophoresis. Following scanning and imaging of gels from three different isoelectric focusing intervals (3–10, 7–11, 6.2–7.5), automated spot handling was performed on a large number of gel spots including those found to differ more than 20% between the treated and untreated condition. Subsequently, differentially regulated proteins were subjected to a three-step approach of mass spectrometry using (a) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry peptide mass fingerprinting, (b) post-source decay utilizing chemically assisted fragmentation, and (c) liquid chromatography–tandem mass spectrometry. Using this approach we have so far resolved 121 differentially regulated proteins following treatment of mice with the candidate drug and identified 110 of these using mass spectrometry. Such data can potentially give improved molecular insight into the metabolism of drugs as well as the proteins involved in potential toxicity following the treatment. The differentially regulated proteins could be used as targets for metabolic studies or as markers for toxicity.

KEY WORDS: ADME/Tox, proteomics, 2D gel electrophoresis, DIGE, mass spectrometry

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It is estimated that about 50% of drugs in development fail during clinical trials because of deficiencies in their absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties.[1] The cost of these failures is naturally very high. In addition, 6.7% of hospitalized patients still suffer serious adverse reactions to drugs that have successfully completed development and have been introduced to the market.[1] Improved means of gathering ADME/Tox information earlier in drug development should thus benefit pharmaceutical manufacturers and, of course, patients.

The goal of this study was to evaluate whether a proteomics approach could provide greater molecular insight into the metabolism and toxicity in the livers of animals treated with a candidate drug. Usually, biased experimental ADME/Tox approaches are used to study the level or activity of certain enzymes such as Cytochrome P450s already known to be involved in xenobiotic metabolism. The toxicological studies used in many laboratories are also biased and focus on certain predetermined markers for toxicity or, alternatively, on more general cellular parameters such as membrane permeability. The identification of further proteins involved in ADME/Tox may open possibilities for the development of new complementary tests for ADME/Tox properties.

In this study we tested whether an unbiased proteomics approach would identify differential levels of liver proteins following treatment of mice with a candidate drug. Information from such an approach could help elucidate which proteins are involved in metabolism and toxicity and thereby increase the value of ADME/Tox studies in drug development.

MATERIALS AND METHODS

Treatment of Mice with Candidate Drug

A selected candidate drug was administered orally to C57BL/6 mice over a period of 5 consecutive days. Livers from treated mice and untreated littermates were surgically removed, snap frozen in liquid nitrogen, and stored at −70°C.

Sample Preparation and Quantification

A 0.5-g sample of each liver (three treated livers and one pooled control consisting of three untreated livers) were rinsed in phosphate-buffered saline and homogenized in 5 mL lysis buffer (10 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, 5 mM magnesium acetate, 4% CHAPS).

To remove interfering nonprotein material and concentrate the sample 10 × 100-μL homogenate was subjected to treatment with the 2-D Clean Up kit according to the kit instructions (Amersham Biosciences, Uppsala, Sweden) and resuspended in 10 mM Tris-HCl, pH 8.3, 7 M urea, 2 M thiourea, 5 mM magnesium acetate, 4% CHAPS. For quantification of the samples the 2-D Quant Kit was used according to the kit instructions (Amersham Biosciences).

Sample Labeling

The concentration of each protein sample was adjusted to 10 mg/mL and 100 μg of each sample was labeled with CyDye DIGE Fluor according to the kit instructions.[2,3] Each sample was run in duplicate, and each duplicate stained with both CyDye DIGE Fluor Cy5 minimal dye and CyDye DIGE Fluor Cy3 minimal dye. In accordance with the DIGE (difference gel electrophoresis) recommended experimental design, a pooled internal standard containing all samples included in the experiment was prepared and labeled with CyDye DIGE Fluor Cy2 minimal dye. Two different labeled samples and one pooled standard were mixed prior to electrophoresis and separated on a single two-dimensional (2D) gel.[2,3]

2D Gel Electrophoresis

First Dimension

Cup loading was used for all analytical gels. One hundred fifty micrograms of the mixed sample (50 μg each of the Cy2-, Cy3-, and Cy5-labeled sample on each gel) was applied to each 24-cm Immobiline DryStrip (Amersham Biosciences). Proteins from three different pH intervals were analyzed by running 24-cm Immobiline DryStrips 3–10 NL, 7–11 NL, and 6.2–7.5, respectively. After image analysis (see below) of all analytical gels, preparative gels containing 1 mg of sample were run. For preparative electrophoresis, in-gel rehydration of 1 mg of unlabeled pooled standard mix of all samples was performed in DeStreak Rehydration solution[4] with 2% immobilized pH gradient buffer according to the user manual (Amersham Biosciences).

Second Dimension

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed overnight using lab-cast 12.5% Laemmli gels on LF glass plates run on the Ettan DALTtwelve electrophoresis system according to the manufacturer's protocol (Amersham Biosciences; see also refs. 5 and 6). Deep Purple (Amersham Biosciences) was used for poststaining of the gels.

Scanning

Scanning was performed on Typhoon 9410 Variable Mode Imager using 520BP40 (Cy2), 580BP30 (Cy3), 670BP30 (Cy5), and 560LP (Deep Purple) emission filters. The resolution was set to 100 μm.

Image Analysis

Images were analyzed using DeCyder Differential Analysis Software v5.0 in both the "differential in-gel analysis" module and the "biological variation analysis" module. For statistical analysis the Student's *t*-test *p* value was set to 0.01 and with an average ratio of spots greater than or equal to 1.5 times (for 3–10 NL gels) or 1.2 times (for 7–11 and 6.2–7.5 gels) intensity. Individual images were created for the different Cy2-, Cy3-, and Cy5-labeled gels and spots detected using DeCyder differential in-gel analysis module. The spot maps were then imported to DeCyder biological variation analysis module where gels were matched.

One preparative 3–10 NL, 7–11 NL, and 6.2–7.5 gel of each type was added to each of the experimental studies and pick lists that included all spots of interest (i.e., those differentially regulated following treatment with the candidate drug) were created for automated spot handling and matrix-assisted laser

desorption/ionization mass spectrometry (MALDI-MS) analyses [peptide mass fingerprinting (PMF) and chemically assisted fragmentation (CAF)]. Concerning pH 3–10 NL, two parallel preparative gels were added to the study, one used for protein identification using MALDI-PMF and MALDI-CAF, and one gel for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Spot Handling

Selected proteins were subjected to fully automated spot handling in the Ettan Spot Handling Workstation (Amersham Biosciences). The method selected included spot picking, digestion, extraction of tryptic peptides, and spotting on Ettan MALDI target slides which were automatically run over night.

In the automated procedure gel plugs were cut by a 2-mm picking head, and washed twice in 50% methanol/50 mM ammonium bicarbonate and once in 75% acetonitrile before drying. For digestion, 10 μL trypsin solution (0.02 μg/mL; sequencing grade, Ettan Chemicals) was added before incubation at 37°C for 2 h. Extraction was performed in two steps by addition of 50% acetonitrile and 0.1% trifluoroacetic acid (Ettan Chemicals). The pooled extract was finally dried prior to a two-step spotting procedure in matrix (5 mg/mL recrystallized α-cyano-4-hydroxy-cinnamic acid, LaserBio Labs, Sophia Antipolis Cedex, France). In the final step before MALDI-TOF (time-of-flight) analysis, a tenth of the dissolved sample was mixed with the matrix layer on the target, saving the remaining part of the sample for CAF-MS or LC-MS/MS analyses.

Protein Identification

Peptide Mass Fingerprinting

PMF was performed on Ettan MALDI-ToF Pro[7] (Amersham Biosciences). Using ProFound[8] data acquisition, spectrum processing and database searches were performed in automatic mode with internal calibration using trypsin autolysis peaks.[9]

Chemically Assisted Fragmentation

To further improve the identification rate, Ettan CAF MALDI Sequencing Kit was used on proteins not successfully identified by PMF according to the instructions from the manufacturer (Amersham Biosciences). This technique in conjugation with Sonar[10] enables peptide sequence data to be acquired by easy fragmentation of the CAF-labeled tryptic peptides using MALDI-PSD.[11]

Liquid Chromatography–Tandem Mass Spectrometry

The few spots from the 3–10 NL DryStrip run that were still not identified using MALDI-MS (PMF and CAF) were subjected to LC-MS/MS analysis. The tandem mass spectrometric analysis was performed on Finnigan LTQ linear ion trap mass spectrometer fitted with a BioBasic C18 column (100 × 0.1 mm; Thermo Electron, San Jose, CA) running at 1 μL/min flow rate. A fast gradient profile enabled a total analysis time of 20 min during which approximately 5500 scans were acquired per sample using data-dependent mode. The spectra were then processed automatically by SEQUEST to get unambiguous identification based on peptide sequence contained in the product ion spectrum.[12]

RESULTS AND DISCUSSION

Quantification of samples that had been subjected to 2D Clean Up Kit showed protein concentrations of 10.02 mg/mL for treated sample 1; 9.67 mg/mL for treated sample 2; 10.23 mg/mL for treated sample 3; and 10.40 mg/mL for pooled untreated control, i.e., 1/100 of the prepared sample was protein. Excellent recovery was achieved because this sample was diluted 10 times during the experimental procedure, and an organ such as the liver should contain about 10% protein. Following sample preparation, the samples were labeled with CyDye DIGE Fluor minimal dyes. The complete workflow is outlined in Figure 1.

Cy2 was used throughout the study for the pooled internal standard whereas treated and untreated samples were labeled with Cy3 and Cy5. A duplicate of each sample was labeled using both Cy3 and Cy5 minimal dyes.

After analytical 2D gel electrophoresis and image analysis, over 2500 gel spots were shown to be resolved on each gel (Fig. 2). DeCyder software was used to match the different gels to each other and identify gel spots that were up- or down-regulated more than 20%. From the 3–10 NL, 7–11 NL, and 6.2–7.5 gels, 30, 30, and 61 spots, respectively, were found to be differentially regulated (Table 1). Thus, in total, 121 proteins were found to be up-or down-regulated more than 20%.

Comparison of Analytical 3–10 NL and 7–11 NL Immobiline DryStrips

When the 3–10 NL gels were compared with the 7–11 NL gels it was noticed that a number of additional regulated spots (12 spots, 10 of them identified using MS) were resolved on the 7–11 NL gel. By visual inspection of the gels it was also obvious that the

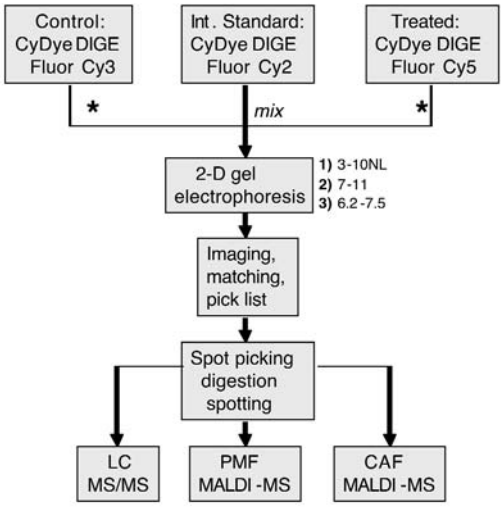


FIGURE 1

The experimental workflow. *In the experimental workflow, a second gel was always run where the labeling was reversed, i.e., treated sample was labeled with Cy3 and the untreated sample labeled with Cy5 CyDye DIGE Fluor minimal dye, respectively.

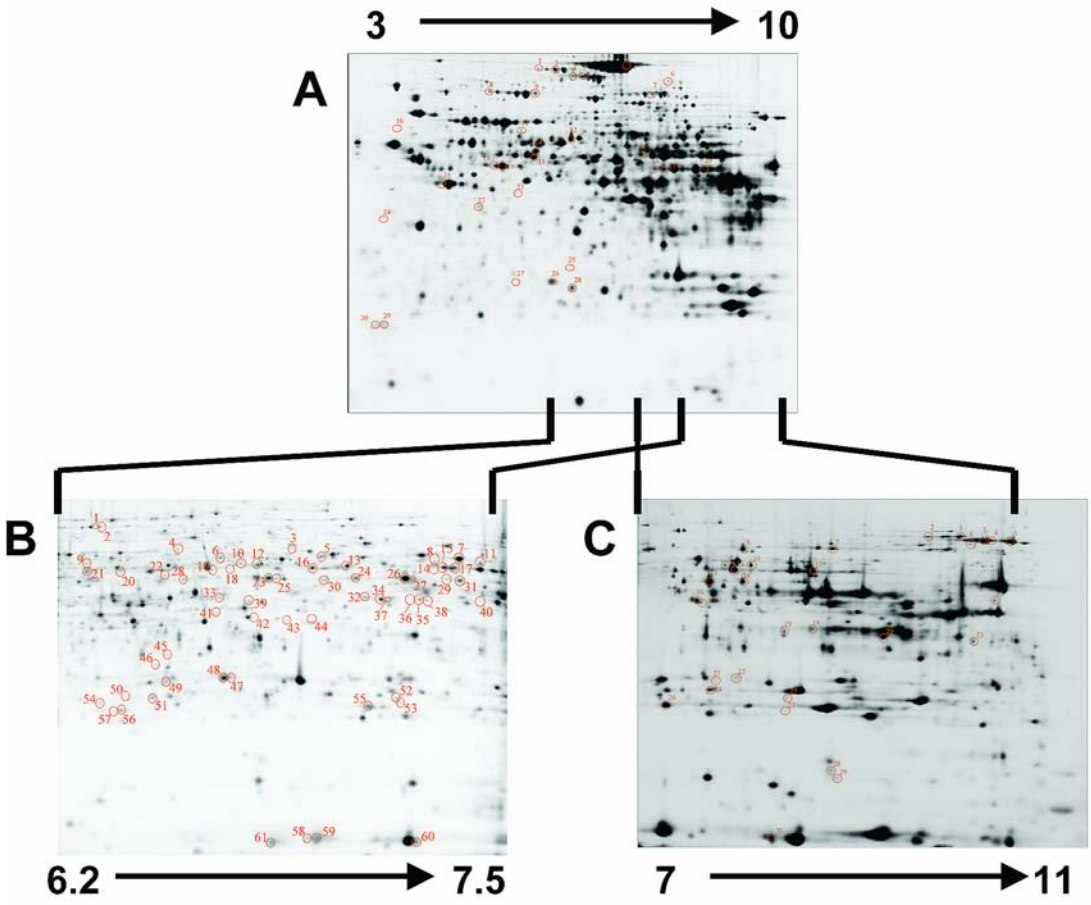


FIGURE 2

2D Gel electrophoresis. **A:** DryStrip 3–10 NL resolved 30 protein spots changed more than ± 1.5 following treatment with candidate drug *(circled spots in red)*. **C:** DryStrip 7–11 NL resolved another 30 spots regulated more than ± 1.2 among which a few overlapped with the proteins identified from the 3–10NL run. **B:** DryStrip 6.2–7.5 resolved yet another 61 proteins regulated more than ± 1.2 following drug treatment.

TABLE 1

Overview of Workflow Showing Total Number of Spots Resolved on Gels and the Number of Protein Spots Found To Be Significantly Up- or Down-Regulated Following Treatment with Candidate Drug

	3–10 NL	7–11 NL	6.2–7.5
Total number of spots on 2D gel	>2500	>2500	>2500
Proteins found to be at least 20% up/down	30	30	61
Regulated proteins identified by PMF	24/30	24/30	52/61
Regulated proteins identified by CAF	4/6	3/6	1/9
Regulated proteins identified by LC-MS/MS	2/2 (5/5)	n.d.	n.d.
Total number identified regulated proteins	30/30 (100%)	27/30 (90%)	53/61 (87%)

Also shown are the number of the protein spots identified using the three different mass spectrometry approaches. The total number of identified regulated proteins in this study was 110 out of 121 spots (91%).

7–11 NL gel resolved the basic part of the 3–10 NL gel to a higher degree. In contrast, the 3–10 NL gel covers a broader part of the pI range. The aforementioned 10 identified regulated spots on the 7–11 NL gel represented 6 different gene products/proteins. Out of these, hydroxymethylglutaryl-CoA synthase was also identified from the 3–10 NL gel; although theoretically based on location on the 3–10 NL gel, 1–3 additional proteins in the 3–10 NL gel could possibly also have been resolved on the 7–11 NL gel.

Comparison of Analytical 3–10 NL and 6.2–7.5 Immobiline DryStrips

In the range 6–7.5 on the 3–10 NL gel, approximately 5–10 spots were found to be differentially regulated (see Figs. 2A and C). When the 6.2–7.5 gel was analyzed, however, 61 protein spots were found to be differentially regulated following treatment with the candidate drug. Thus, the narrow-range gel proved to be a very successful tool in further identifying differentially regulated proteins. Since an equal amount of sample was loaded on all three types of gels, the improved result is most likely due to the outstanding resolution of protein spots on a 6.2–7.5 Immobiline DryStrip gel. Although the narrow-range gels resolved a significantly higher number of proteins spots compared with the 3–10 NL gels, it should be noted that only two spots of hydroxymethylglutaryl-CoA synthase overlapped between these pH ranges. One might have expected that 3–8 more spots from the 3–10 NL gel would also have been resolved on the 6.2–7.5 gel. It is unclear why these additional spots were not resolved.

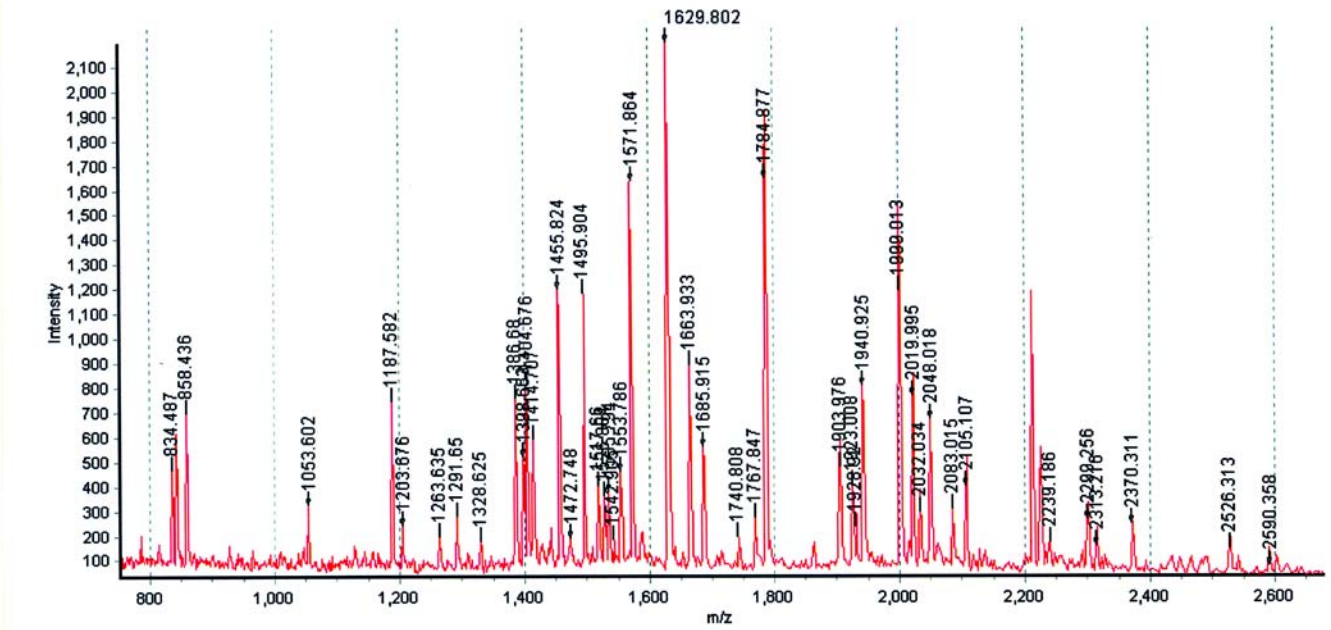
Comparison of Analytical 6.2–7.5 and 7–11 NL Immobiline DryStrips

These gels overlap in the pI range 7.0–7.5. A high degree of overlap was, as expected, observed between spots from the basic part of the 6.2–7.5 gel and the acidic part of the 7–11 NL gel; about 10 spots from the 7–11 NL gel could have been expected to be resolved on the 6.2–7.5 gel. Out of these 10 spots (6 distinct gene products), 7 (4 distinct gene products) were found to be identified also on the 6.2–7.5 gel. In addition, the 6.2–7.5 gels resolved a number of additional proteins in the overlapping region, again indicating the power of using overlapping narrow-range Immobiline DryStrips.

These results indicate that for a "complete" view of the proteome, a range of different strips that resolve proteins within different pI ranges and that include high-resolution narrow-range Immobiline DryStrips should be utilized. Such an approach will, to a large extent, facilitate the unambiguous identification of proteins with changed abundance using DeCyder software.

Preparative 2D Gel Electrophoresis, Spot Handling, and Mass Spectrometry

Preparative gels were run and matched to the analytical gels using DeCyder Differential Analysis software. The pick list was thereby generated and then transfered to the spot handling workstation which was used to pick, digest, and spot all 121 differentially regulated proteins. In addition, 532 and 40 protein spots shown not to be differentially regulated following drug treatment from two types of these gels (3–10 NL and 7–11 NL, respectively) were also processed by the automated spot handling procedure. We focused our



Protein candidates | Candidate details | Candidate sequence | Sequencing result

	Rank	Expectation	Protein information	Coverage %	pI	kDa
	1	0.000	gi|16877282|gb|AAH16899.1 - RIKEN cDNA 1300002P22 gene [Mus musculus]	36.1	9.5	79.04
	1 *	-	gi|17366737|sp|Q9DBM2|ECHP_MOUSE - Peroxisomal bifunctional enzyme (PBE) (PBFE) (Includes: Enoyl-CoA hydratase	36.1	9.5	78.99
	1 *	-	gi|12963805|ref|NP_076226.1 - L-specific multifunctional beta-oxdiation protein [Mus musculus] gi|5830360|emb|CAA0985	30.3	9.3	32.26
	2	0.339	gi|25053918|ref|XP_148584.3 - expressed sequence AW548146 [Mus musculus]	11.3	5.6	123.33
	3	0.393	gi|6753562|ref|NP_034120.1 - cytoplasmic linker 2; cytoplasmic linker protein 1, 115 kDa [Mus musculus] gi|11360356|pir|	11.5	6.0	116.16
▶	3 *	-	gi|24657655|gb|AAH39162.1 - Similar to cytoplasmic linker 2 [Mus musculus]	11.0	6.1	112.54
	3 *	-	gi|9800516|gb|AAF99333.1|AF289664_4 - CYLN2 [Mus musculus] gi|9800526|gb|AAF99340.1|AF289667_2 CYLN2 [Mus r	10.6	6.1	116.58
	3 *	-	gi|20342376|ref|XP_109449.1 - similar to CYLN2 [Mus musculus] gi|25049856|ref|XP_194251.1| similar to CYLN2 [Mus mus	11.2	5.7	94.72

FIGURE 3

MALDI ToF reflectron spectrum of tryptic peptides from spot 5 (see Fig. 2B) and the unambigous protein identification result using ProFound.

attention, however, on the identification of the 30+30+61 differentially regulated spots using a combination of PMF, CAF derivatization, and LC-MS/MS. Table 1 illustrates the workflow and shows the methods used and outcome of protein identification for the 3–10 NL, 7–11 NL and 6.2–7.5 experiments.

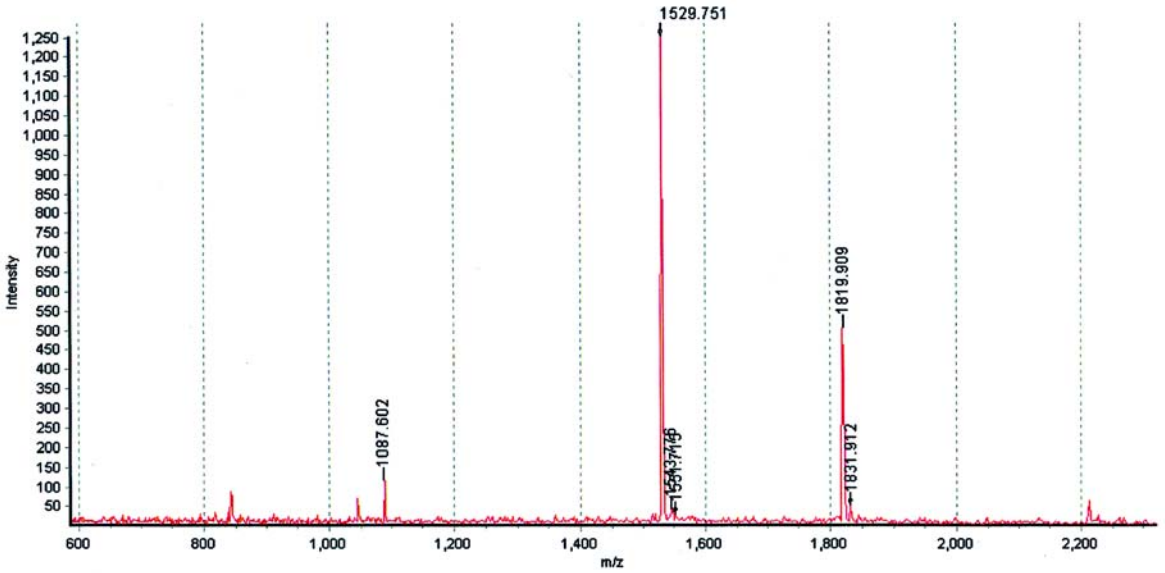
MALDI-TOF analysis identified 24/30 (75%) of the differentially regulated proteins from the 3–10 NL and 7–11 NL gels and 52/61 (85%) proteins from the 6.2–7.5 gel, giving a total 100/121 (83%) of the proteins (see Figs. 3 and 4 for representative mass spectra).

By employing CAF-derivatization and MALDI-PSD analysis, another 4 out of 6 proteins from the 3–10 NL gel, 3 out of 6 proteins from the 7–11 gel, and 1 out of 9 proteins from the 6.2–7.5 gel could be successfully identified. Thus, out of the remaining 21 proteins, 8 (38%) were successfully identified using CAF. Figure 5 shows a representative sequence obtained by CAF.

Finally, from the spots picked from the 3–10 NL gel, LC-MS/MS analysis was performed on the two remaining proteins not identified by PMF or CAF. Both proteins could be identified, providing a 100% success rate for the 3–10 NL experiment (see Fig. 6 for sequence information for one of these proteins). In the 7–11 NL and 6.2–7.5 experiments, LC-MS/MS analysis was not performed. In summary, 110 out of the 121 regulated spots were identified (Tables 2–4), representing 91% of the spots.

CONCLUSION

Using a highly automated proteomics approach, 121 differentially regulated proteins were identified following treatment with the candidate drug. The information that can be obtained with this approach should lead to the discovery of useable biomarkers

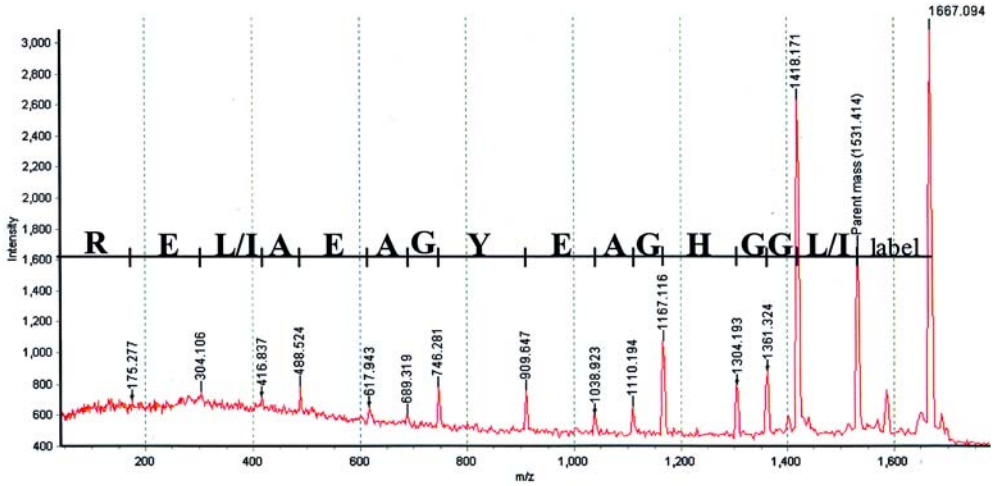


Protein candidates | Candidate details | Candidate sequence | Sequencing result

	Rank	Expectation	Protein information	Coverage %	pI	kDa
▶	1	0.491	gi|553919|gb|AAA37700.1 - alpha-1-globin	32.8	6.8	12.89
	1 *	-	gi|122440|sp|P01945|HBA_MESAU - Hemoglobin alpha chain	28.4	8.1	15.23
	1 *	-	gi|122385|sp|P19014|HBA_CRIGA - Hemoglobin alpha chain gi|109487|pir||A37171 hemoglobin alpha	28.4	8.1	15.03
	1 *	-	gi|122483|sp|P01943|HBA_SPAEH - Hemoglobin alpha chain gi|70181|pir||HAOL hemoglobin alpha c	28.4	7.4	15.24
	1 *	-	gi|12833511|dbj|BAB22552.1 - unnamed protein product [Mus musculus] gi|12837980|dbj|BAB24028	28.2	8.0	15.09
	1 *	-	gi|6680175|ref|NP_032244.1 - hemoglobin alpha, adult chain 1; alpha 1 globin [Mus musculus] gi|122	28.2	8.0	15.07
	1 *	-	gi|12846963|dbj|BAB27381.1 - unnamed protein product [Mus musculus]	28.2	8.0	15.09
	1 *	-	gi|12846939|dbj|BAB27370.1 - unnamed protein product [Mus musculus]	28.2	9.3	15.18
	1 *	-	gi|26345020|dbj|BAC36159.1 - unnamed protein product [Mus musculus]	28.2	8.7	15.16

FIGURE 4

Ettan MALDI ToF Pro reflectron spectrum of peptides from spot 30 (see Fig. 2B) and the resulting unambigous identification.

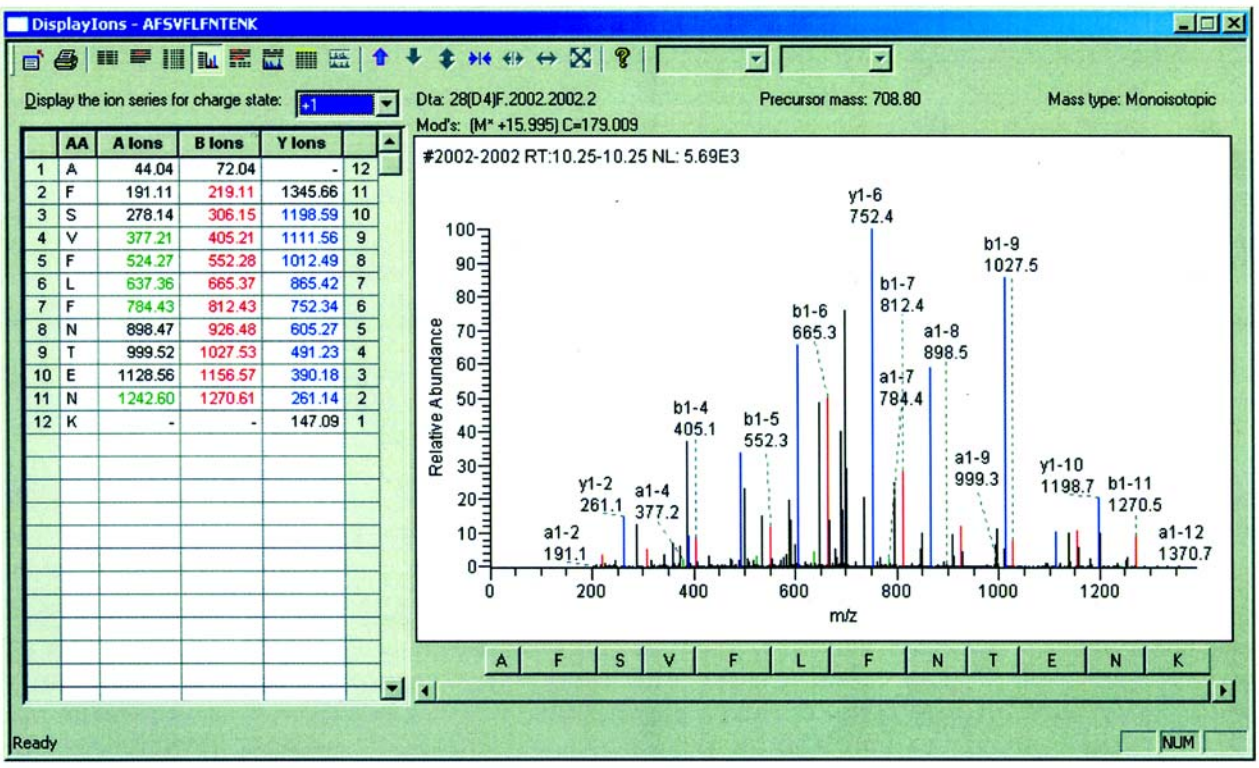


Protein candidates | Candidate details | Candidate sequence | Sequencing result

	Rank	Expectation	Protein information	Coverage %	pI	kDa
▶	1	0.000	gi|12833511|dbj|BAB22552.1| - unnamed protein product [Mus musculus]	10.6	8.0	15.48
	1 *	-	gi|553919|gb|AAA37700.1| - alpha-1-globin	12.3	6.8	13.19
	1 *	-	gi|193761|gb|AAA37783.1| - alpha-globin	25.9	6.8	6.41
	1 *	-	gi|6680175|ref|NP_032244.1| - hemoglobin alpha, adult chain 1; alpha 1 globin [Mus musculus]	10.6	8.0	15.45
	1 *	-	gi|26345020|dbj|BAC36159.1| - unnamed protein product [Mus musculus]	10.6	8.7	15.55
	1 *	-	gi|12846939|dbj|BAB27370.1| - unnamed protein product [Mus musculus]	10.6	9.3	15.45
	1 *	-	gi|12846963|dbj|BAB27381.1| - unnamed protein product [Mus musculus]	10.6	8.0	15.48

FIGURE 5

MALDI-PSD spectrum of the CAF-derivatized digest from spot 30 (see Fig. 2B) and the resulting unambigous identification using Sonar. This spot is identical to the spot identified in Figure 4.



	AA	A Ions	B Ions	Y Ions	
1	A	44.04	72.04	-	12
2	F	191.11	219.11	1345.66	11
3	S	278.14	306.15	1198.59	10
4	V	377.21	405.21	1111.56	9
5	F	524.27	552.28	1012.49	8
6	L	637.36	665.37	865.42	7
7	F	784.43	812.43	752.34	6
8	N	898.47	926.48	605.27	5
9	T	999.52	1027.53	491.23	4
10	E	1128.56	1156.57	390.18	3
11	N	1242.60	1270.61	261.14	2
12	K	-	-	147.09	1

FIGURE 6

Sequence information obtained using LTQ LC-MS/MS of material from spot 27 (see Fig. 2A). The peptide sequence AFSVFLFNTENK was identified as part of isopentyl-diphosphate delta-isomerase (IPP isomerase 1) using SEQUEST.

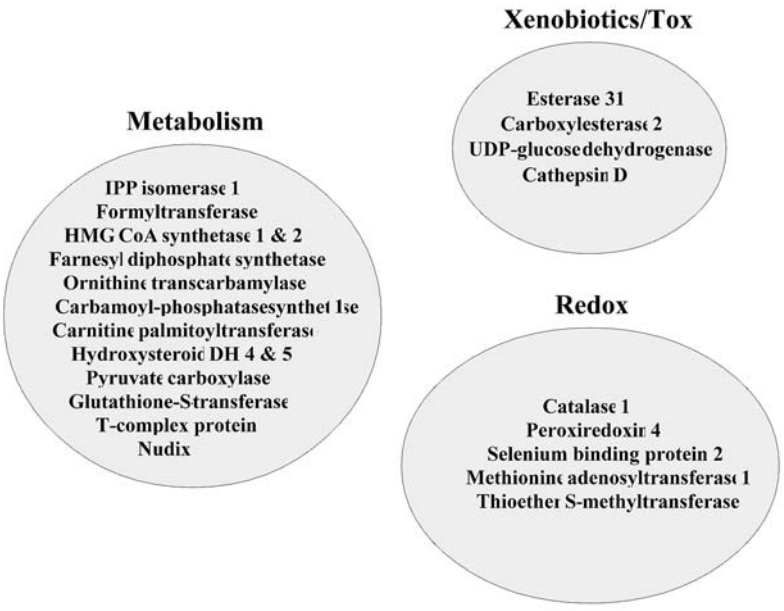


FIGURE 7

Of the 121 differentially regulated protein spots, those listed here are examples of proteins that may be involved in ADME/Tox processes such as metabolism, xenobiotics, and responses to oxidative stress.

TABLE 2

Identification of Differentially Regulated Proteins from DryStrip 3–10 NL (Fig. 2A) Using MALDI-TOF, CAF-MALDI, and/or LC-MS/MS

ID method	Spot No.	Av. Ratio	Name	Protein ID NCBI nr db	T-test Drug +/-	pI	Mw (kDa) theoretical
PMF	1	-1,59	carbamoyl-phosphate synthetase 1	8393186	3,70E-07	6,3	164,58
PMF	2	2,85	carbamoyl-phosphate synthetase 1	23621369	9,00E-08	6,0	116,82
PMF	3	2,65	carbamoyl-phosphate synthetase 1	8393186	1,30E-08	6,2	116,27
PMF	4	1,55	pyruvate carboxylase	7438124	1,10E-08	6,3	129,78
PMF	5	1,74	pyruvate carboxylase	7438124	1,40E-06	6,3	129,78
MS/MS	6	-1,54	ornithine transcarbamylase	762985	9,50E-08	7,3	108,60
PMF	7	-1,69	similar to elongation factor 2	26328763	1,20E-05	6,3	95,26
PMF	8	1,81	Formyl transferase	25050159	9,40E-06	5,6	99,06
PMF	9	-1,61	Formyl transferase	25050159	2,50E-10	5,6	99,06
PMF/MSMS	10	2,75	ubiquilin 1/ serine proteinase inhibitor	20072434/15079234	0,0073	4,7	74,81
CAF + PMF	11	-1,84	esterase 31 + carboxylesterase 2	20886287/19527178	6,90E-10	5,9	72,64
PMF	12	-1,68	Liver carboxylesterase precursor	2494382	3,10E-08	5,9	60,41
PMF	13	1,9	T-complex protein 1	22654291	3,40E-06	6,0	57,48
PMF	14	1,56	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	20988709	4,70E-05	5,7	57,57
PMF	15	-2,25	selenium binding protein 2	9507079	3,40E-10	5,8	52,63
PMF	16	1,59	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	12836439	0,0025	8,7	56,82
PMF	17	1,66	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	20965433	5,30E-06	8,7	56,82
PMF	18	1,61	methionine adenosyltransferase I	19526790	1,70E-06	5,5	43,51
PMF	19	2,02	methionine adenosyltransferase I	19526790	8,10E-10	5,5	43,51
CAF	20	2,06	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	12836371	2,80E-06	7,6	56,22
CAF	21	1,87	gamma-actin	20885782	0,064	5,1	50,04
PMF	22	1,68	cathepsin D	26354406	3,80E-06	6,9	48,37
PMF	23	1,64	farnesyl diphosphate synthetase	19882207	3,00E-06	5,5	40,58
MS/MS	24	2,59	hepatoma derived growth factor	31560691	0,1	4,5	40,20
PMF	25	-2,22	nudix	12847124	9,80E-09	6,0	24,60
PMF	26	-2,02	thioether S-methyltransferase	6678281	1,60E-10	6,0	29,46
PMF/MS/MS	27	-1,75	thioether S-methyltransferase/ IPP isomerase 1	6678281/13878548	2,40E-08	5,8	23,24
CAF/ MS/MS	28	2,21	peroxiredoxin 4/ IPP isomerase 1	7948999/13878548	7,00E-09	6,3	22,08
PMF	29	-1,58	Major urinary proteins 11 and 8	127531	5,40E-07	4,9	17,56
PMF	30	-1,54	Major urinary proteins 11 and 8	127531	0,0022	4,9	17,56

For spots on this broad gel to be considered differentially regulated, Student's *t*-test was set to 0.05 and the average ratio to greater than ±1.5.

TABLE 3

Identification of Differentially Regulated Proteins from DryStrip 7–11 (Fig. 2B) Using MALDI-TOF and CAF-MALDI

ID method	Spot No.	Av. Ratio	Name	Protein ID NCBI nr db	T-test Drug +/-	pI	Mw (kDa) theoretical
PMF	1	1,43	hydroxysteroid dehydrogenase 4	Q9DBM3	1,50E-04	9,0	80,19
PMF	2	1,38	hydroxysteroid dehydrogenase 4	Q9DBM3	7,10E-04	9,0	80,19
PMF	3	1,23	trifunctional enzyme	Q64428	7,80E-05	9,4	83,48
PMF	4	1,24	trifunctional enzyme	Q64428	4,10E-03	9,4	83,48
PMF	5	3,01	bifunctional enzyme	Q9DBM2	4,80E-06	9,5	78,99
PMF	6	1,37	hypothetical Ras GTPase-activating protein	Q8C9I3	3,30E-03	8,9	103,92
PMF	7	1,49	carnitine palmitoyltransferase 2	P52825	4,00E-04	8,8	74,75
PMF	8	1,53	acyl-CoA dehydrogenase	P50544	7,60E-05	7,1	62,35
PMF	9	1,98	UDP-glucose dehydrogenase	O70475	7,20E-03	7,8	55,73
PMF	10	2,48	UDP-glucose dehydrogenase	O70475	1,40E-02	7,8	55,73
PMF	11	1,53	catalase 1	Q8C6E3	3,50E-02	7,8	60,20
PMF	12	1,62	acetaldehyde dehydrogenase	P24549	2,70E-04	8,3	55,26
PMF	13	1,24	hydroxymethylglutaryl-CoA synthase	Q9DBM4	2,00E-03	7,7	55,30
PMF	14	6,73	hydroxymethylglutaryl-CoA synthase	Q9DBM4	3,80E-05	7,7	53,30
PMF	15	-1,45	quinone oxidoreductase	Q8BW20	2,80E-03	9,5	50,87
PMF	16	1,36	acetyl-CoA dehydrogenase	Q91WS8	9,80E-05	9,0	47,07
PMF	17	1,53	acetyl-CoA dehydrogenase	Q91WS8	4,70E-05	8,9	47,07
PMF	18	-1,6	hydroxysteroid dehydrogenase 5	13487925	1,70E-03	8,8	37,56
PMF	19	-1,65	thiosulfate sulfurtransferase	P52196	4,50E-02	7,9	33,75
PMF	20	-1,51	channel protein 1 (VDAC-1)	Q60932	4,10E-04	8,7	32,56
PMF	21	-1,43	malate dehydrogenase	P08249	1,90E-04	9,4	36,20
PMF	22	1,29	-	-	4,80E-03	-	-
PMF	23	1,32	-	-	6,40E-03	-	-
PMF	24	1,61	glutathione S-transferase, mu 1	P10649	1,20E-02	7,8	26,10
PMF	25	1,66	glutathione S-transferase, theta 3	Q99I20	2,80E-03	7,9	27,84
PMF	26	-2,13	glutathione S-transferase, Yfyf	P46425	2,20E-03	8,3	23,69
PMF	27	1,43	-	-	1,10E-03	-	-
CAF	28	-1,21	destrin	Q9R0P5	6,40E-04	8,6	18,92
CAF	29	-1,21	destrin	Q9R0P5	1,30E-04	8,6	18,92
CAF	30	-1,31	hemoglobin	Q8BPF4	8,50E-03	8,7	15,09

For spots on this more basic gel to be considered differentially regulated, Student's *t*-test was set to 0.01 and average ratio to greater than ± 1.2.

TABLE 4

Identification of Differentially Regulated Proteins from DryStrip 6.2–7.5 (Fig. 2C) Using MALDI-TOF and CAF-MALDI

ID method	Spot No.	Av. Ratio	Name	Protein ID NCBI nr db	T-test Drug +/-	pI	Mw (kDa) theoretical
PMF	1	1,27	dimethylglycine dehydrogenase precursor	21311901	8,10E-03	7,8	97,54
PMF	2	2,88	dimethylglycine dehydrogenase precursor	21311901	7,50E-04	7,8	97,54
PMF	3	-1,25	probable urocanate hydratase	Q8VC12	3,30E-02	7,4	75,49
PMF	4	-1,24	SA rat hypertension-associated homolog	Q8BWS7	2,10E-03	7,2	65,36
PMF	5	-1,38	-	-	1,50E-05	-	-
PMF	6	-1,39	catalase 1	Q8C6E3	3,80E-03	7,8	60,2
PMF	7	1,24	catalase 1	Q8C6E3	5,90E-03	7,8	60,2
PMF	8	2,05	catalase 1	Q8C6E3	5,40E-03	7,8	60,2
PMF	9	1,36	cDNA sequence BC021917	21703976	2,10E-03	6,4	60,05
PMF	10	1,21	glutamate dehydrogenase	6680027	1,80E-04	8,3	61,78
PMF	11	1,72	ATP synthase, alpha subunit	6680748	1,20E-03	9,3	59,89
PMF	12	-1,28	Ugp2 protein	19343890	2,30E-03	6,9	55,93
PMF	13	-1,22	-	-	7,00E-03	-	-
PMF	14	1,36	similar to aldehyde dehydrogenase family 1	28386049	1,40E-05	8,6	56,29
PMF	15	1,51	ATP synthase, alpha subunit	6680748	5,60E-05	9,3	59,89
PMF	16	-1,34	Aldh6a1 protein	21410418	6,80E-03	7	50,19
PMF	17	1,55	ATP synthase, alpha subunit	6680748	6,10E-03	9,3	59,89
PMF	18	1,4	glutamate dehydrogenase	6680027	8,60E-03	8,3	61,78
PMF	19	-1,23	glutamate dehydrogenase	6680027	9,00E-03	8,3	61,78
PMF	20	1,4	dihydrolipoamide dehydrogenase	31982856	3,50E-05	8,3	54,94
PMF	21	2,42	-	-	3,40E-06	-	-
PMF	22	1,21	glutamate dehydrogenase	6680027	4,20E-03	8,3	61,78
PMF	23	1,3	RF-C/activator 1 homolog	3641291	8,20E-04	5,8	78,13
PMF	24	1,84	gob-5 protein	7513665	4,00E-03	5,7	101,12
PMF	25	1,46	hydroxymethylglutaryl-CoA synthase	1083370	1,60E-04	7,7	53,3
PMF	26	1,28	hydroxymethylglutaryl-CoA synthase	1083370	2,50E-04	7,7	53,3
PMF	27	1,61	hydroxymethylglutaryl-CoA synthase	1083370	9,90E-03	7,7	53,3
PMF	28	-1,41	G protein-coupled receptor 8	17978288	5,40E-05	9,6	84,75
PMF	29	1,37	-	-	2,30E-03	-	-
PMF	30	3,69	fumarate hydratase 1	33859554	3,00E-03	9,3	54,66
PMF	31	6,69	fumarate hydratase 1	33859554	3,50E-04	9,3	54,66
PMF	32	-1,46	phosphoglycerate kinase 1	6679291	2,20E-03	7,7	45,05
PMF	33	1,21	acetyl-Coenzyme A dehydrogenase	6680618	1,90E-03	9	47,07
PMF	34	1,47	acetyl-Coenzyme A dehydrogenase	15488707	1,70E-04	8,9	47,1
PMF	35	1,56	acetyl-Coenzyme A dehydrogenase	15488707	5,20E-04	8,9	47,1
PMF	36	1,63	acetyl-Coenzyme A dehydrogenase	15488707	3,90E-04	8,9	47,1
PMF	37	1,38	acetyl-Coenzyme A dehydrogenase	15488707	4,80E-04	8,9	47,1
PMF	38	1,54	-	-	7,20E-05	-	-
PMF	39	1,29	nicein	833772	1,80E-03	5,2	80,5
PMF	40	1,46	RIKEN cDNA 1200015K23	25051552	6,20E-03	6,4	22,14
PMF	41	1,25	arginase 1	7106255	2,50E-02	6,5	35,02
PMF	42	1,24	alcohol dehydrogenase (NADP+) (aldehyde reductase)	Q9JII6	2,10E-03	6,7	36,83
PMF	43	1,31	-	-	3,10E-03	-	-
PMF	44	-2,03	Otc protein	19353187	5,40E-03	8,9	39,58
PMF	45	-1,25	actin related protein 2/3 complex, subunit 2	23621467	4,00E-03	6,8	34,49
PMF	46	-1,41	-	-	5,60E-03	-	-
PMF	47	-1,56	-	-	1,50E-03	-	-
CAF	48	-1,26	*carbonic anhydrase 3*	10717134	3,20E-06	6,9	29,35
PMF	49	-1,25	carbonic anhydrase 3	31982861	1,30E-04	6,9	29,73
PMF	50	1,33	RIKEN cDNA 2310016A09	31712014	2,60E-04	6,7	28,34
PMF	51	1,36	biliverdin reductase B (flavin reductase (NADPH))	21450325	3,20E-05	6,5	22,33
PMF	52	1,43	biliverdin reductase B (flavin reductase (NADPH))	21450325	1,80E-04	6,5	22,33
PMF	53	-1,28	glutathione S-Transferase Yfyf, chain A	576133	4,40E-03	8,3	23,69
PMF	54	-1,25	biliverdin reductase B (flavin reductase (NADPH))	21450325	2,20E-04	6,5	22,33
PMF	55	-1,96	glutathione S-transferase, Yfyf, chain A	576133	2,10E-03	8,3	23,69
PMF	56	1,34	glutathione S-transferase, Yfyf Cys 47, chain A	2624495	7,50E-03	8,3	23,58
PMF	57	-1,75	glutathione S-transferase, Yfyf, chain A	576133	1,50E-03	8,3	23,69
PMF	58	-1,55	13 days embryo liver cDNA	Q9CRZ2	2,00E-03	8,1	14,45
PMF	59	-1,53	13 days embryo liver cDNA + haemoglobin beta-2 chain	Q9CRZ2/P02089	7,10E-05	8,1/8,9	14,45/16,47
PMF	60	-1,58	alpha-1-globin	553919	9,40E-06	6,8	12,96
PMF	61	-1,87	13 days embryo liver cDNA + beta-1-globin	Q9CRZ2/4760586	3,80E-07	8,1/7,3	14,45/15,77

For spots on this narrow range gel to be considered differentially regulated, Student's *t*-test was set to 0.01 and average ratio to greater than $\pm$ 1.2.

that signal drug toxicity and/or altered metabolism, which, in turn, can be used to identify candidate drugs that should be excluded early in drug development campaigns.

The study shows the value of combining different pH-range Immobiline DryStrips to separate CyDye DIGE Fluor-labeled proteins, since every new strip included in the study enabled the discovery of additional differentially regulated proteins. By adding a narrow-range (pH 6.2–7.5 and 7–11) strip, many more differentially regulated proteins were resolved compared with the previous 3–10 NL analysis, indicating the power of adding narrow-range strips to the 2D DIGE study. The 3–10 NL Immobiline DryStrip, however, is more suitable for obtaining an overview of the complete pI range, and by using the new 3–11 NL Immobiline DryStrip an even wider overview should be obtained. The study also shows the power of combining different mass spectrometry approaches in the effort to reach a very high success rate.

The identified proteins can be grouped in different functional categories that may be involved in the xenobiotic metabolism of drugs and in toxicity (Fig. 7). Concerning future analyses, the function of the 110 identified proteins will be studied in order to understand more about their biological roles. Analyses will also be conducted using additional narrow-range strips to resolve even more proteins. It should be noted that phase I xenobiotic metabolism of drugs takes place primarily in the endoplasmic reticulum and that many of the responsible proteins (such as Cytochrome P450s) are membrane bound. Therefore subcellular fractionation, and for example the addition of further detergents during 2D gel electrophoresis, might be added to this study to identify such proteins. However, the present study indicates that the 2D-DIGE approach will prove very useful for discovery of new markers signaling toxicity and also for proteins involved in xenobiotic metabolism.

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