

## Differential Protein Expression in the Cytosol Fraction of an MCF-7 Breast Cancer Cell Line Selected for Resistance toward Melphalan

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Analysis of differential protein expression in the cytosol of melphalan-resistant and -susceptible MCF-7 cell lines has been carried out using a combination of two-dimensional gel electrophoresis, mass spectrometry, and bioinformatics. Comparison of multiple digitized gel arrays detected several spots as candidates for differentially expressed proteins in melphalan-resistant MCF-7 cells. The up-regulated proteins included retinoic acid binding protein II, an isoform of the macrophage migration inhibition factor, and other unidentified proteins. The down-regulated proteins included calreticulin, cyclophilin A, and an isoform of the 27 kD heat shock protein. Correlation of the differential expression of some of the proteins with acquired resistance of MCF7 cells to melphalan is discussed.

**Keywords:** proteomics • MCF-7 • melphalan • chemoresistance • post-translational modification • cyclophilin • cofilin • calreticulin • 27 kD heat shock protein • macrophage migration inhibition factor

### 1. Introduction

In the past few years, proteomics strategies have been widely adopted in cancer research.<sup>1</sup> With the improvement of existing analytical methods such as two-dimensional gel electrophoresis, protein microchip arrays, liquid chromatography, and mass spectrometry, proteome analysis has rapidly evolved. Databases related to the proteomes of different cancer tissues are being assembled (see world 2-DE PAGE under <http://www.expasy.ch>). These growing databases and protein identifications are expected to lead to better understanding of tumor biology and behavior at the molecular level.

Two-dimensional gel electrophoresis (2-DE) remains the technique of choice for proteomics studies in a given biological material.<sup>2</sup> It can resolve and provide a global view of many proteins in a single sample. It is appropriate for comparative studies, since it permits proteins of interest to be detected and images to be archived for subsequent comparison to other samples and to other published 2-DE maps. The challenge of this technique is the meticulous and laborious effort required to obtain reproducible data, which are essential for comparative studies. Bridging 2-DE to mass spectrometry and bioinformatics has become a routine and robust strategy for identification of proteins.<sup>3,4</sup>

Often in proteomics studies of cancer, samples of normal and cancerous tissue are compared.<sup>5,6</sup> Several proteins have been found markedly elevated in breast cancer tissue versus normal tissue, while others have been found decreased. The elevated proteins included glycolytic enzymes such as fructose biphosphate aldolase A, glyceraldehyde 3-phosphate dehy-

drogenase, some chaperone and stress proteins such as cyclophilin A, heat shock proteins (HSP-27 kDa, HSP 90- $\alpha$ ), and many others. The decreased proteins consisted mainly of keratin isoforms.<sup>6</sup> Differential protein expression has also been studied in normal and cancerous cultured cell lines.<sup>7,8</sup>

Historically, comparative studies of drug-resistant cancer cells to their nonresistant counterparts have focused on single specific proteins, such as P-glycoprotein involved in drug efflux,<sup>9</sup> glutathione-S-transferases involved in drug detoxification,<sup>10–12</sup> metallothionein involved in drug sequestration,<sup>13</sup> and topoisomerase II involved in DNA metabolism.<sup>14</sup> However, the mechanism by which cancer cells develop resistance toward an anticancer drug is complex and quite probably involves a combination of several factors. Thus, the global protein analysis in resistant cancer cells is of interest. Only a few laboratories have investigated the protein profiles of drug-resistant and nonresistant cancer cells using the 2-DE approach.<sup>15–20</sup> These published studies were carried out with the emphasis toward proteins that are up-regulated in drug resistant cancer cells.

In the present work, 2-DE has been used to compare proteins in the cytosol fraction of melphalan-resistant and -susceptible human breast cancer MCF-7 cell lines to detect and identify proteins that are differentially expressed (up or down regulated) in acquired resistance. Such altered proteins might be directly or indirectly correlated to the resistance of the cancer cells to the drug. In this initial study, the cytosol compartment was chosen to simplify the complexity of the sample and also because this fraction contains important functional proteins.

### 2. Material and Methods

**2.1. Materials.** The Protean IEF cell, Protean II xi cell, IPG strips (17 cm, pH 3–10), Protean II ready gels (8–16% Tris-

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HCl gel, 1 mm thick, 16 × 16 cm IPG well), microbiospin 6 columns in Tris·HCl buffer and Biosafe Coomassie stain were from Bio-Rad (Hercules, CA). Urea, thiourea, CHAPS, DTT, iodoacetamide, TrisBase, SDS, glycerol were electrophoresis grade (Sigma Co., St. Louis, MO). Tributylphosphine (TBP) was purchased from Aldrich (Milwaukee, WI). Modified porcine trypsin (sequencing grad) was purchased from Promega (Madison, WI)

**2.2. Cell Culture.** Human breast cancer cell line MCF-7 (MCF-7) was a gift from K. H. Cowan (Eppley Institute, University of Nebraska Medical Center, Omaha, NE), and melphalan-resistant MCF-7 cells derived from that same line (MeLR MCF-7) were obtained from J. A. Moscow (Department of Pediatrics, University of Kentucky College of Medicine, Lexington, KY). These two cell lines have been maintained in culture in our laboratory in Improved Minimal Essential Medium (ATCC, Manassas, VA) containing 5% fetal calf serum (Sigma Co., St. Louis, MO) and antibiotics at 37 °C under 5% CO<sub>2</sub>–95% air atmosphere. Every 6 months, the MeLR MCF-7 cell line was subjected to a reselection cycle of three passages with culture medium containing increasing amounts of melphalan, from 0.6 to 6 μM.<sup>21</sup>

**2.3. Preparation of the Cytosolic Fraction.** The cell lines MCF-7 and MeLR MCF-7 were grown to synchronous confluence in 150 cm<sup>2</sup> flasks (Corning, Inc., Corning, NY). The cytosolic fractions from MCF-7 and MeLR MCF-7 were prepared in parallel from three different cell culture batches. The cells were gently washed twice with 15 mL of 10 mM phosphate buffer saline solution (PBS). Then 5 mL of trypsin (cell culture grade, Sigma Co., St. Louis, MO) was added to each flask. After 2–3 min at 37 °C, 15 mL of serum-supplemented medium was added and the cells were pelleted by centrifugation at 500g for 10 min. The cell pellets were washed twice with 5 volumes of ice-cold 10 mM PBS, followed by one wash with 5 volumes of ice-cold 100 mM NaCl solution. Extraction of the cytosol fraction was performed as previously described,<sup>22</sup> using digitonin, EDTA solution containing protease inhibitors. The cytosolic fraction was further centrifuged at 140 000g to obtain only soluble proteins. Protein assays were performed using the Bio-Rad protein assay dye reagent following the manufacturer's instructions. The sample was then stored in aliquots of 500 μL in microfuge tubes at –80 °C.

**2.4. Two-Dimensional Gel Electrophoresis.** Prior to the first-dimension gel electrophoresis, the samples were desalted using micro Bio-Spin P-6 columns containing Tris·HCl 10 mM buffer (BioRad, Hercules, CA) following the manufacturer's instructions. The desalted samples containing ~200 μg of cytosolic proteins were dried using a vacuum centrifuge and redissolved in 300 μL of rehydration buffer, consisting of 7 M urea, 2 M thiourea, 4% CHAPS (w/v), 5 mM TBP or 50 mM DTT, and 0.5% IPG buffer 3–10. Dry IPG strips (17 cm, pH 3–10) were rehydrated for 12 h with 300 μL of sample in the focusing tray under a layer of mineral oil. The rehydrated strips were then run at 20 °C with voltages increasing stepwise as follows: 250 V for 15 min, 5000 V for 2 h 30 min, and 8000 V for 5 h. At the end of the run, the total V h reached 65 000. The focused strips were equilibrated for 30 min in a solution containing 6 M urea, 30% glycerol, 2% SDS, 2% DTT, 50 mM Tris·HCl pH 8.8, and traces of bromophenol blue. Alkylation of free cysteine residues was carried out for 30 min in the same solution containing 2.5% iodoacetamide instead of DTT.

For the second-dimension gel electrophoresis, the equilibrated strips were aligned on the top of SDS precast gels (8–

16% Tris·HCl gels, 16 × 16 cm) and sealed with 0.5% molten agarose prepared in SDS-PAGE running buffer containing traces of bromophenol blue. Protein separation was performed in a Protean XI cell (BioRad, Hercules, CA) at 16 mA/gel for 30 min, followed by 24 mA/gel for 5 h at 15 °C. At the end of the run, the gels were removed and fixed for 30 min in a solution containing methanol/water/acetic acid (45:50:5, v/v/v) followed by three washes for 5 min in deionized water. Staining was performed with Bio-Safe colloidal Coomassie blue G-250 (Bio-Rad, Hercules, CA) for 1 h followed by ample rinsing with deionized water until the desired contrast was achieved.

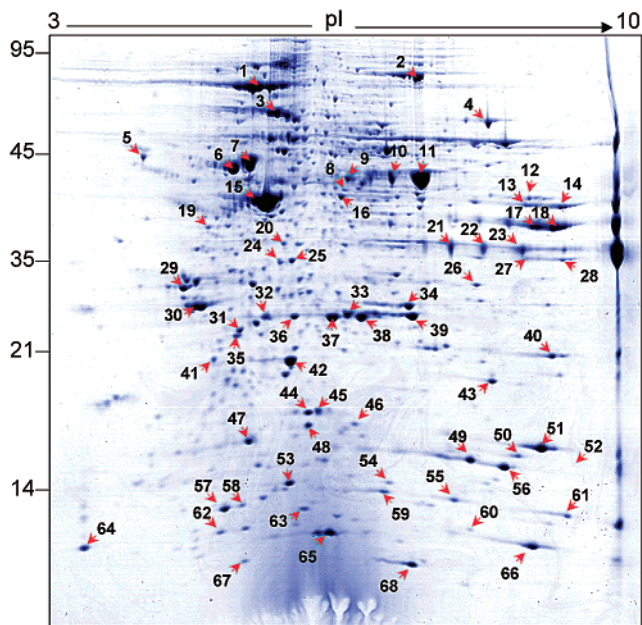
**2.5. Analysis of Gel Images.** The stained gels were scanned with a GS-800 densitometer (BioRad, Hercules, CA), and the images were saved as TIFF files. The 2-DE images of MCF-7 and MeLR MCF-7 were compared using Z3 software (Compugen Ltd., Tel Aviv, Israel). In each gel, the relative intensity of a spot is measured and represents the sum of all pixels in the spot boundary after having subtracted the background level values. For comparative analysis, the relative intensity of each pair of matching spots are measured and recorded and the data are displayed as relative expression and differential expression values. The data are checked manually by the operator to exclude any error in matching pairs and to judge the significance of the differential analysis by zooming in the spot of interest and checking its incidence in another set of gels.

**2.6. Mass Spectrometry Analysis and Protein Identification.** Spots were excised with the tip of clean propylene pipets and transferred into microfuge tubes containing 100 μL of deionized water. Tryptic digestion was performed as described in detail by Jensen et al.<sup>23</sup> The generated peptides were extracted as described<sup>23</sup> and stored at –80 °C for analysis.

Peptide extracts were dried by vacuum centrifugation, redissolved in 10 μL of 0.1% TFA, and desalted using selfmade Poros 50 R2 loaded tips.<sup>4</sup> About 3 μL of a slurry suspension of Poros 50 R2 (PerSeptive Biosystems, Framingham, MA) in methanol (1:2, v/v) was loaded into an Eppendorf gel loader tip (Brinkman Instruments, Inc., Westbury, NY). To retain the Poros material, the end of the tip was squeezed with a pair of tweezers. The solid-phase packed at the end of the tip was then washed once with 20 μL of methanol, followed by 20 μL of 0.1% TFA. The 10 μL of sample was then loaded on the top of the column and pushed through slowly with a 10 mL syringe. The retained peptides were washed twice with 20 μL of 0.1% TFA, eluted with 10 μL of acetonitrile/0.1% TFA (70:30, v/v), and stored at –80 °C.

**2.6.1. Mass Spectrometry Analysis.** Routinely, a MALDI-TOF-MS (Kompact MALDI 4, Kratos Analytical, Chesnut Ridge, NY) was used to check the peptides generated from in-gel digestion. The instrument, equipped with nitrogen laser and pulsed extraction, was operated either in reflectron-positive or linear-positive mode. The extraction voltage was set at 20 kV. In general, the peaks at *m/z* 843.01 and 2212.42, corresponding to the pig trypsin autolysis products, were used to internally calibrate each spectrum. In cases where these autolysis peaks were not detected, mellitin and angiotensin II were used as external calibrants. An aliquot of 0.3 μL was mixed with 0.3 μL of 50 mM α-hydroxycinnamic acid and dried. Spectra were recorded by accumulating 50–100 shots, depending on the signal-to-noise ratio obtained for each sample.

Tandem mass spectra (MS/MS) of the peptides were obtained on a hybrid quadrupole time-of-flight instrument (QStar/Pulsar, Applied Biosystems, Foster City, CA). Typically, 1–2 μL of the peptide solution in methanol/water/acetic acid (50:50:



**Figure 1.** Two-dimensional gel electrophoresis map (Coomassie stained) of cytosolic proteins from MCF-7 breast cancer cells. The numbered spots have been identified by mass spectrometry and database search and are listed in Table 1.

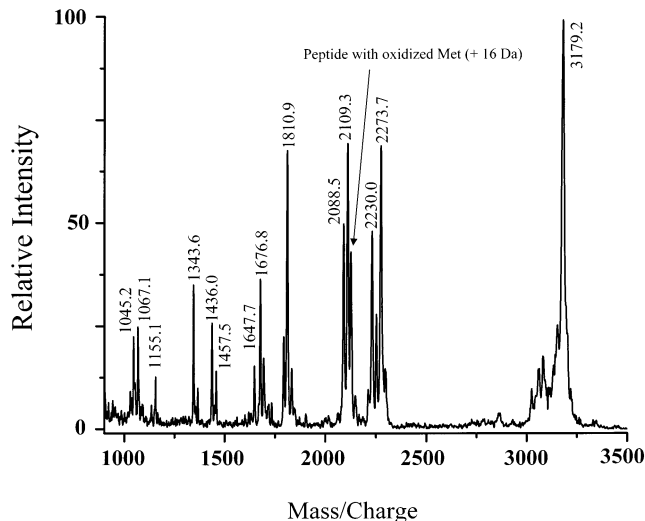
2, v/v/v) was loaded into a capillary nanospray tip (Protana, Odense, Denmark) and mounted into the nanospray ion source. Spray voltage was 0.9 kV, and collision energy was ramped from 15 to 50 eV, depending on the nature of the selected ion. Typically, doubly and sometimes triply charged ions were selected for MS/MS analysis.

**2.6.2. Database Search and Protein Identification.** Protein identification was performed by entering the mass list obtained by MALDI-TOF-MS into the Mascot search form (<http://www.expasy.ch> under proteomics tools). If no conclusive identification could be obtained, the peptides were fragmented by nanospray tandem mass spectrometry to generate sequence tags. These were used to search protein databases with the integrated Qstar software (BioExplore tool). Further database search tools such as TagIdent (<http://www.expasy.ch> under proteomics tools) and MS-Blast (<http://www.dove.embl-heidelberg.de/Blast2/msblast.html>) were used as necessary.

### 3. Results and Discussion

**3.1. Protein Identification.** Although the aim of this study was to analyze differential protein expression between MCF-7 and MeIR MCF-7, identification of nondifferentially expressed proteins was carried out in order to compare our array to published 2-DE maps of proteins from breast cancer cells. Some of these 2-DE maps are available on the web site (<http://www.expasy.ch>) under World 2-DE PAGE databases. A two-dimensional gel electrophoresis map of the cytosolic fraction from MCF-7 control cells studied here is shown in Figure 1. With the amount of protein loaded (200  $\mu$ g) and the Coomassie stain used, 728 significant spots were counted using Z3 software. The detection limit was estimated at 8 ng per spot, based on manufacturer's specifications for the colloidal Coomassie stain.

A number of spots were excised, digested with trypsin, and their peptides analyzed by mass spectrometry as described in the Methods. Figure 2 shows a strong MALDI-MS spectrum of



**Figure 2.** MALDI mass spectrum of a protein (spot 18) digested in-gel with trypsin. This protein was identified as fructose biphosphate aldolase A.

peptides from one of these spots (spot 18). These peptides were entered in the Mascot search form and provided identification of human fructose biphosphate aldolase A. In cases where no conclusive identification could be obtained, especially for low molecular mass proteins and some faint spots, nanospray-MS/MS was used to provide a sequence tag for database searching. Thus, proteins in 68 spots were successfully identified and are summarized with their theoretical *pI*s, molecular masses, and accession numbers in Table 1. All identified proteins were of cytosolic origin, as expected. This validates the experimental method used here for the extraction of cytosolic proteins from the cells. Identification of the proteins was further refined by comparing the theoretical *pI*s and molecular mass values from the SWISS-PROT database to those estimated from the 2-DE array. Additional evaluation was carried out by checking as yet unconsidered low intensity ions in the MALDI-MS spectra against the masses of trypsin digestion products generated in silico from the candidate protein. In addition, peptides that contain methionine can often be recognized, as Met tends to oxidize during processing, generating pairs of peptides 16 Da apart in mass. The 2-DE location of most of these proteins agreed with those in previously published 2-DE maps of proteins from breast cancer cells.<sup>5, 6</sup>

**3.2. Detection of Post-Translational Modification of Some Proteins.** The MALDI-TOF spectra of spots 49 and 56 are shown in Figure 3 (left). Database search using the observed peptides did not provide confident hits for these two spots. However, using sequence tags obtained by nanospray-MS/MS analysis permitted the identification of these two spots as being the same protein (peptidyl-prolyl cis-trans isomerase A, also named cyclophilin A). The MALDI peptide profile was the same for the two spots, except that the N-terminal tryptic fragment at *m/z* 1946.7 detected in spot 56 was not present in spot 49. A peak at *m/z* 1989.0 was detected instead. The difference in mass between these two peaks is 42.3 Da and is proposed to reveal N-terminal acetylation of the protein in spot 49. This was further confirmed by nanospray-MS/MS analysis of the two peptides (Figure 3, right). Indeed, all the observed "y" ion series have the same masses in the two spectra while the "b" ions were shifted by +42 Da in the spectrum of the modified peptide. Previously published 2-DE maps also detected two

**Table 1.** Proteins Identified in the 2-DE Array of the Cytosolic Fraction from Breast Cancer MCF-7 Cells

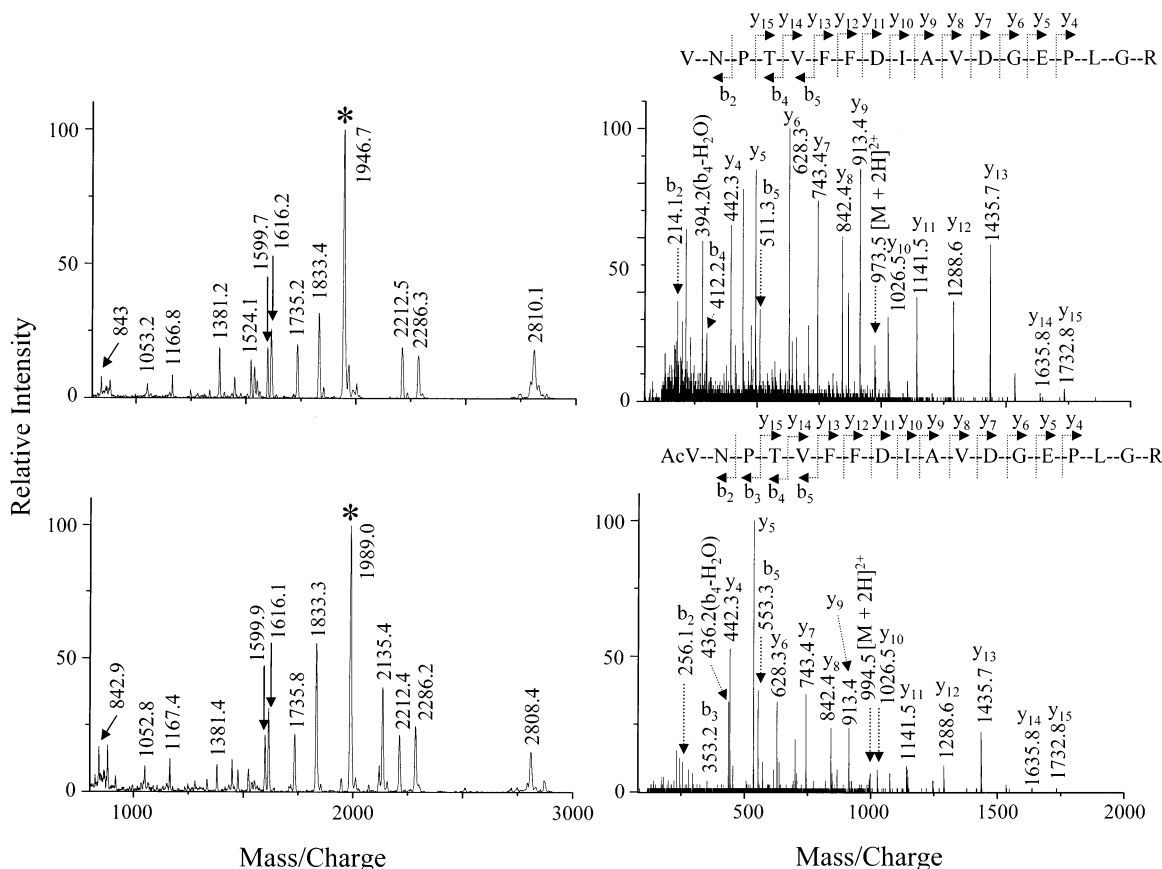
no.	pI	mass (Da)	accession no.	protein name	no.	pI	mass (Da)	accession no.	protein name
1	4.97	83133	P08238	heat shock protein HSP 90- $\beta$ (HSP 90)	35	5.03	23207	P52565	$\rho$ GDP-dissociation inhibitor-1
2	6.42	95207	P13639	elongation factor-2	36	5.98	22782	P04792	phosphorylated heat shock 27 kDa protein
3	5.37	70898	P11142	heat shock cognate 71 kDa protein	37	5.98	22782	P04792	heat shock 27 kDa protein
4	7.58	67878	P29401	transketolase	38	6.02	24904	P30041	heat shock 27 kDa protein
5	4.29	48141	P27797	calreticulin	39	6.51	26538	P00938	triosephosphate isomerase
6	4.75	49759	P07437	tubulin $\beta$ -1 chain	40	8.27	22110	Q06830	peroxiredoxin 1 (thioredoxin peroxidase 2)
7	5.02	50158	P05687	tubulin $\alpha$ -1 chain	41	4.84	19595	P13693	translationally controlled tumor protein
8	6.25	50315	P20073	annexin A7	42	5.66	21892	P32119	peroxiredoxin 2 (thioredoxin peroxidase 1)
9	6.11	50663	P50395	GDP dissociation inhibitor $\beta$ (GDI-2)	43	7.43	20925	P30086	phosphatidylethanolamine-binding protein
10	6.99	47038	P06733	$\alpha$ enolase (phosphopyruvate hydratase)	44	5.75	22848	17454582	similar to phosphoglycerate mutase 1
11	6.99	47038	P06733	$\alpha$ enolase (phosphopyruvate hydratase)	45	5.83	17149	P15531	metastasis inhibition factor nm23
12	8.36	46426	P00966	argininosuccinate synthase	46	nd <sup>a</sup>	nd <sup>a</sup>	P23528	cofilin, nonmuscle isoform phosphorylated at Ser-3
13	8.30	44596	P00558	phosphoglycerate kinase-1	47	5.08	16701	P10159	initiation factor 5A
14	8.30	44596	P00558	phosphoglycerate kinase-1	48	5.77	17171	P16949	stathmin
15	5.29	41737	P02570	actin cytoplasmic-1 ( $\beta$ -actin)	49	nd <sup>a</sup>	17922	P05092	peptidylprolyl isomerase A (cyclophilin A) N-actyl form
16	5.92	47716	P23526	adenosylhomocysteinase	50	8.06	18505	P18282	destrin (actin-depolymerizing factor)
17	8.39	39289	P04075	fructose biphosphate aldolase A	51	8.22	18502	P23528	cofilin, nonmuscle isoform
18	8.39	39289	P04075	fructose biphosphate aldolase A	52	8.52	17298	P22392	nucleoside diphosphate kinase B
19	4.78	32854	P08865	40S ribosomal protein SA	53	5.43	15562	P29373	retinoic acid-binding protein II
20	5.45	32923	P52907	F-actin capping protein $\alpha$ -1 subunit	54	6.36	14395	P25398	40S ribosomal protein S12
21	8.58	35922	P04406	glyceraldehyde 3-phosphate dehydrogenase	55	8.47	14923	P07737	profilin I
22	8.58	35922	P04406	glyceraldehyde 3-phosphate dehydrogenase	56	7.82	17880	P05092	petidylprolyl isomerase A (cyclophilin A)
23	8.58	35922	P04406	glyceraldehyde 3-phosphate dehydrogenase	57	4.82	11606	P10599	thioredoxin
24	5.40	333215	Q96B89	hypothetical 33.2 kDa protein	58	5.35	14584	P09382	galectin-1
25	5.54	32660	Q15181	inorganic pyrophosphatase	59	6.46	13670	P49773	histidine triad nucleotide-binding protein
26	7.60	35077	P25388	guanine nucleotide-binding protein $\beta$	60	7.25	12580	P30046	D-dopachrome tautomerase
27	8.46	36557	P00338	L-lactate dehydrogenase A chain	61	8.47	14923	P07737	profilin I
28	8.46	36557	P00338	L-lactate dehydrogenase A chain	62	5.10	14478	P13662	nuclear transport factor
29	4.63	29174	P42655	14-3-3 protein $\epsilon$	63	5.90	11471	Q99584	S100 calcium binding protein A13
30	4.73	27745	P29312	14-3-3 protein $\zeta/\delta$	64	4.09	16706	P02593	calmodulin
31	5.15	24318	P21964	catechol- <i>o</i> -methyltransferase, soluble form	65	6.56	11740	P31949	calgizzarin (S100C protein)
32	5.37	26889	P21266	glutathione S-transferase Mu 3	66	8.24	12345	P14174	macrophage migration inhibitory factor
33	6.02	24904	P30041	antioxidant protein 2	67	5.32	10180	P06703	calyculin
34	6.75	28672	Q9WCO	phosphoglycerate mutase 1	68	6.56	8565	P02248	ubiquitin

<sup>a</sup> nd: not determined.

spots for cyclophilin A<sup>5,6</sup> in breast cancer cells and even in noncancerous cells,<sup>6,24</sup> but no structural modification was reported. To our knowledge, this is the first time that cyclophilin A is reported with an N-terminally acetylated Val residue. The amount of the acetylated form represents half of the total cytosolic cyclophilin A as judged from the spot intensities in the gel. The significance of the N-terminally acetylated cyclophilin A remains to be investigated.

A protein identified as cofilin (nonmuscle isoform) appeared as two spots with different pI's and different relative intensities (see spots 46 and 51 in Figure 1). Mass spectrometric analysis of the peptides from the most intense spot 51 revealed that cofilin is lacking the N-terminal Met residue and starts with an N-terminal acetylalanine, while in the database cofilin-1 is reported without possible acetylation. In addition, comparative

analysis of the peptides generated from the two spots revealed that the N-terminally acetylated peptide was detected in spot 51 as a doubly charged ion at  $m/z$  572.8, but not in spot 46. In contrast, a peptide at  $m/z$  612.8 for  $[M + 2H]^{2+}$  was detected in spot 46. The difference in mass between these two peptides is 80 Da, suggesting possible phosphorylation. Figure 4 shows the MS/MS spectra of the N-terminal peptide peak at  $m/z$  572.8 and the peptide peak at  $m/z$  612.8. These two peptides have the same sequence except that b ion series were 80 Da higher in mass in the peptide with  $m/z$  612.8 than in the peptide with  $m/z$  572.8. The b<sub>2</sub> ion at  $m/z$  281.05 identifies the phosphorylation site at Ser-2. The "y" ion series that do not contain the modified amino acid residue have the same masses excluding the possibility of phosphorylation at Ser-7. In the SwissProt database, human cofilin-1 is reported with possible phospho-



**Figure 3.** (Left) MALDI mass spectra of an in-gel trypsin digested spots 56 and 49 (cyclophilin A and N-terminal acetylated cyclophilin A, respectively). (Right) ESI-MS/MS spectra of doubly charged ions of peaks with  $m/z$  1947.5 and 1989.1. The sequence of the unmodified peptide is as follows: VNPTVFFDIAVDGEPLGR.

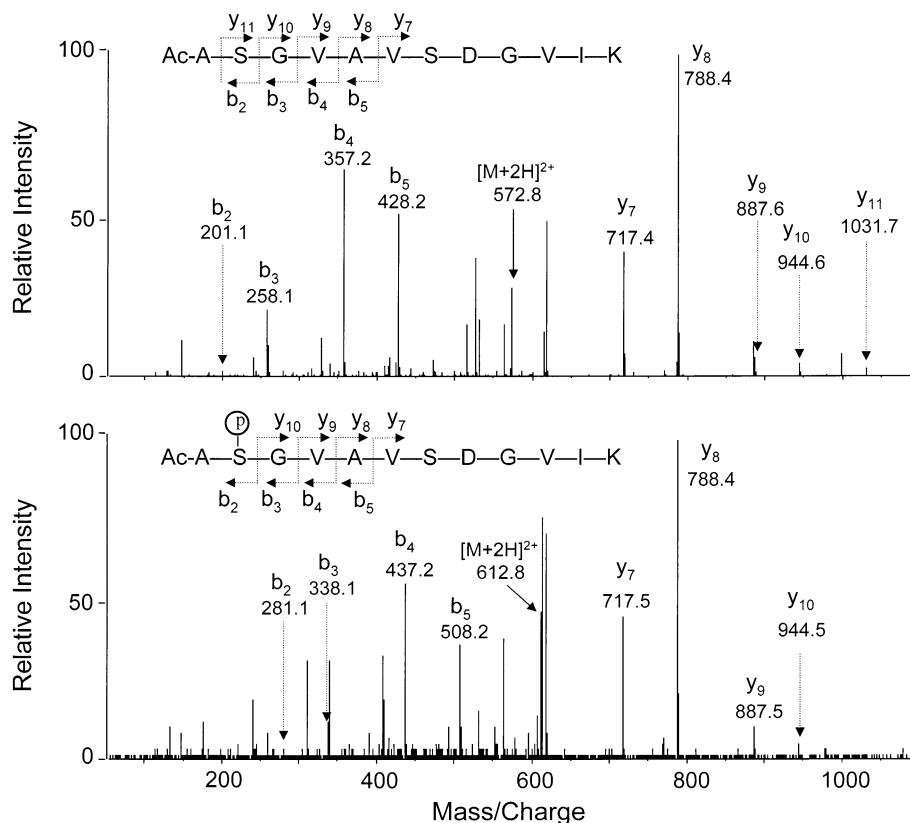
rylation at Ser-24 in an isoform starting with a Met residue. The peptide containing this Ser residue, with or without phosphorylation, was not detected in our case. Because of its short sequence [SSTPEEVK] and its hydrophilic nature, this peptide might have been lost during the desalting process. However, most literature reports agree on phosphorylation of cofilin at Ser-3 (present study Ser-2) rather than Ser-24.<sup>25–27</sup>

Other proteins were detected as multiple spots with similar molecular masses, especially at the basic side of the gel, but did not seem to have any variations in primary structure. For example, spots 17 and 18 are both identified as aldolase A (fructose biphosphate aldolase A). Identical sequence coverage was obtained for greater than 80% for the two protein spots. The phenomenon of charge isoforms has been observed for aldolase in several 2-DE maps of eukaryotic cells.<sup>5,6,24</sup> The ratios of the two spots were variable from run to run and the multiplicity may result from incomplete denaturation of the protein forming populations that migrate at slightly different  $pI$ 's in the first-dimension gel electrophoresis.

**3.3. Comparative Protein Map of Cytosolic Proteins.** Comparative proteome analysis using 2-DE technique is quite tedious, and it is not exempt from errors and variations even when analyzing duplicate arrays of the same sample. To ensure reproducibility of the results, cytosolic fractions of MCF-7 and MelR MCF-7 were prepared in parallel from three different cell batches and two or three gels were run in parallel for each sample. The obtained pairs of gel images were analyzed by Compugen Z3 software. Proteins that were consistently found differentially expressed in different sample preparations and

in different gels are denoted and reported in Figure 5. The proteins that are down or up-regulated in MelR MCF-7 cells by at least a factor of 2 are indicated with red and black arrows, respectively. These differences were not observed when multiple gels of the same sample were compared. Five out of the nine differentially expressed proteins found, were identified, and these are reported in Table 2, with their differential expression values between the two cell lines. Several of these proteins have important cellular functions and their alterations might be directly or indirectly correlated to the development of chemoresistance of MCF-7 toward mephalan.

Calreticulin (spot 5), for instance, was detected as faint spot in MCF-7 and was found significantly less abundant in the MelR MCF-7 cell line. This protein is normally abundant in the reticulum lumen and plays a key role in  $Ca^{2+}$  storage and signaling.<sup>28</sup> Its presence in the cytosol extract of MCF-7 was initially thought to have been due to leakage during the preparation of the cytosolic fraction. However, in three separate preparations, calreticulin was always found in the cytosol of MCF-7 and it was consistently more abundant there than in the cytosol of MelR MCF-7 cells (Figure 6). In a recent study, it has been demonstrated that calreticulin is present in the cytosol compartment of mammalian cells and acts as a carrier for the nuclear-export of protein kinase inhibitor and glucocorticoid receptor.<sup>29</sup> Beside its major function as a  $Ca^{2+}$  storage protein in the ER, calreticulin has been found to be involved in a variety of cellular functions.<sup>30</sup> In the nucleus, this protein was found to regulate nuclear hormone receptors, which are known to modulate gene expression.<sup>31</sup> In a recent study, it has



**Figure 4.** ESI-MS/MS spectrum of the peak at  $m/z$  572.8 (top) and the peak at  $m/z$  612.8 (bottom) found in tryptic digest products from spots 51 and 46. The sequence of the unmodified peptide is as follows: (Ac)ASGVAVSDGVK.

**Table 2.** Candidate Proteins That Were Found Differentially Expressed in the Cytosol Fraction of MelR MCF-7

protein name	spot <sup>a</sup>	differential expression <sup>b</sup> (MelR MCF-7)/(MCF-7)
calreticulin	5	0.4 ± 0.1
phosphorylated HSP27	36	0.3 ± 0.1
retinoic acid binding protein II	53	2.3 ± 0.2
cyclophilin A	49, 56	0.6 ± 0.2
isoform of macrophage migration inhibition factor	69	11.6 ± 3.6

<sup>a</sup> See Figure 5 for spot numbers. <sup>b</sup> Differential expression: The ratio of the spot expression in a comparative image (MelR MCF-7) to the expression of a similar spot in a reference image (MCF-7). The values represent the average and the standard deviation of three experiments.

been shown that calreticulin-deficient cells were significantly resistant to apoptosis induced by staurosporine.<sup>32</sup> Further studies on the differential expression and translocation of calreticulin might be of interest in understanding drug resistance.

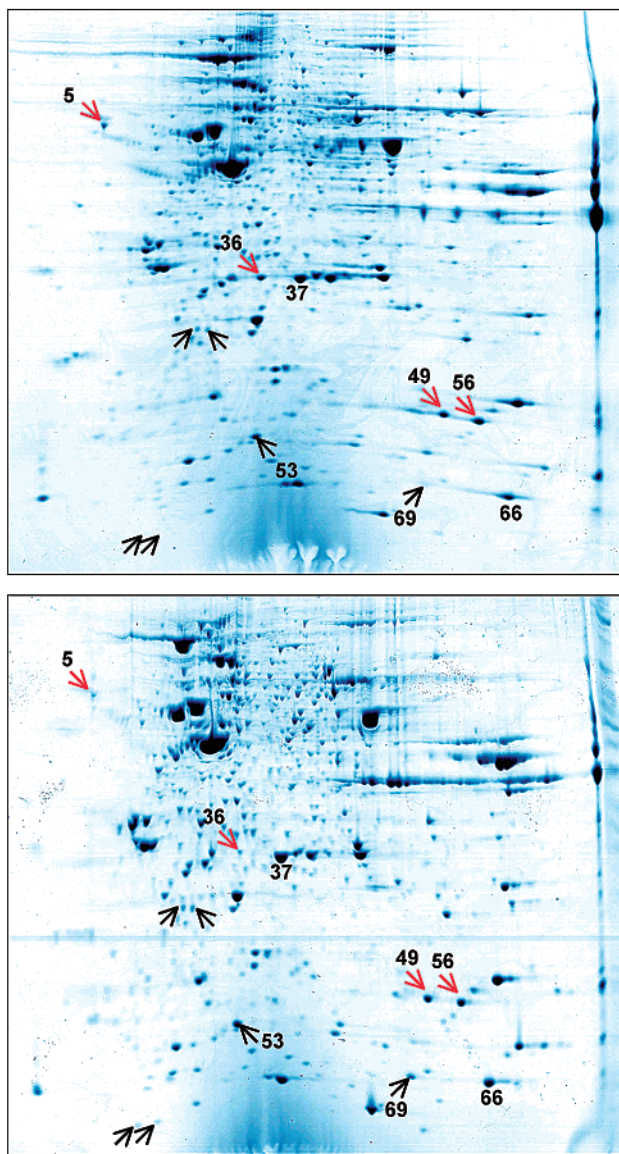
Cyclophilin A (spots 49 and 56) was found down-regulated in MelR MCF7 cells by a factor of 1.6, when compared to MCF-7. This differential expression was observed in two separate experiments, while the ratio was 1.3 in a third experiment. This change is small but consistently observed. Cyclophilin A (Cyp-A), also known as peptidyl-prolyl cis-trans isomerase or rotamase, is involved in protein folding and catalyzes the cis-trans isomerization of peptide bonds preceding a proline residue. Other important functions have also been attributed to this protein.<sup>33</sup> Indeed, in a recent study, it has been demonstrated that Cyp-A inhibits the activity of interleukin-2 tyrosine kinase<sup>34</sup> via formation of stable complex between the two proteins and that this inhibition is abolished by cyclosporine A (an immunosuppressive natural product that binds

specifically to Cyps). It has also been demonstrated that decreasing the level of Cyp-A in neuronal B50 cells by an antisense oligodeoxynucleotide strategy inhibits the activation of caspases that are involved in triggering apoptosis.<sup>35</sup> It has been proposed that melphalan kills cells by triggering apoptosis.<sup>36</sup> The down-regulation of Cyp-A in MelR MCF-7 could render these cells resistant to apoptosis.

Spot 36 was identified as a phosphorylated form of the 27 kD heat shock protein (HSP27). This phosphorylated HSP27 is clearly more abundant in the MCF-7 control than in MelR-MCF-7, while the inverse was found for unphosphorylated HSP27 (spot 37). In previous studies, it has been demonstrated that increased expression of HSP27 prevented apoptosis in mammalian cells treated by staurosporin, doxorubicin or etoposide.<sup>37–39</sup> However, phosphorylation status of HSP27 in these studies was not reported. Close examination of the expression and phosphorylation status of this protein may be of interest in future studies.

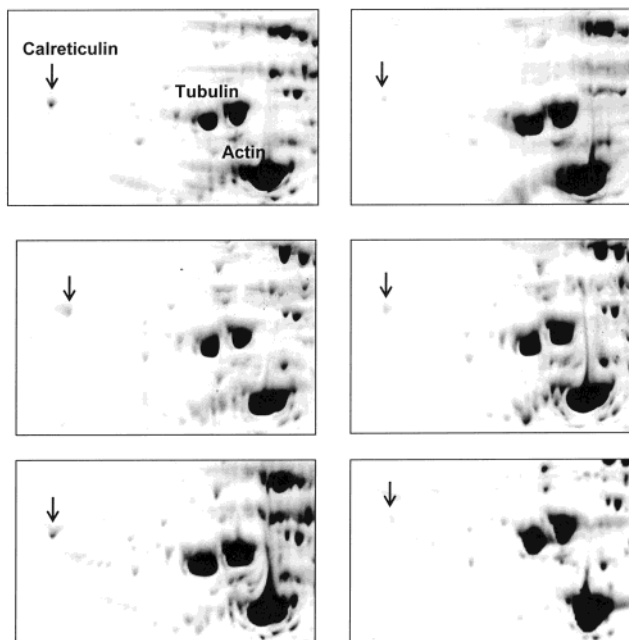
Retinoic acid binding protein II (spot 53) was found up-regulated in MelR-MCF7 by a factor of 2.3. This protein normally binds retinoic acid and is involved in the cellular signaling pathway of this compound, which inhibits cell growth.<sup>40</sup> The up-regulation of this protein in MelR MCF-7 is intriguing and remains to be verified and clarified.

Spot 69 (see Figure 5) was identified as an isoform of macrophage migration inhibition factor (MIF). The peptide maps generated by trypsin from spots 69 and 66 were similar, except for the N-terminal peptide in spot 69, which was 154 Da higher in mass than the N-terminal peptide in spot 66. The structure of this modification is under investigation. Preliminary data obtained by tandem mass spectrometry supports modi-



**Figure 5.** Two-dimensional arrays of cytosolic fractions from MCF-7 (top) and MeIR MCF-7 cells (bottom). Red arrows indicate the proteins that are down-regulated in MeIR MCF-7, and black arrows the up-regulated proteins in MeIR MCF-7 cells. Numbers code the proteins that were identified.

fication at the N-terminal Pro residue of the MIF isoform. This isoform was found to be more abundant in the cytosol of MeIR MCF7 than in the MCF-7 control (Table 2). Recently, MIF was found to be a most interesting factor.<sup>41</sup> Indeed, this protein, which was first assigned as a macrophage migration inhibition factor, was found to have an interesting intracellular function.<sup>42</sup> In the cytosol, MIF can bind to Jab1, an activator of cell growth, and negatively regulate its activity. Thus, the modified MIF found in high abundance in the cytosol of MeIR MCF-7 may be an inactive form of MIF that could be correlated with the rescue of cells from growth arrest. This would mean that MeIR MCF-7 cells may modify the cytosolic MIF to reduce its binding to Jab1. However, this speculation needs to be further investigated. One could expect that unmodified MIF in MeIR MCF-7 should be decreased. However, the relative expression of unmodified MIF (spot 66, Figure 5) was observed to be unchanged between the two cell lines.



**Figure 6.** Zoomed-in images from the 2-DE gels of three different cytosolic preparations from MCF-7 (left) and MeIR MCF-7 (right). The spot corresponding to calreticulin is indicated with an arrow.

In the limited number of already published comparative studies using 2-D gel electrophoresis, the focus has been on proteins that are up-regulated in drug-resistant cancer cells.<sup>17–20</sup> In the present study, we demonstrate that proteins with important functions, such as calreticulin and cyclophilin, are also found down-regulated in such cells.

The aim of the present study was to detect and identify proteins whose expression is altered in the cytosol of an MCF-7 cell subline selected for resistance toward melphalan. While the 2-DE technique has a high resolving power in separating complex mixtures of proteins, it suffers little when studying differential protein expression between control and treated sample. Thus, multiple sample preparations and multiple gels for each preparation were analyzed and a consensus pattern of differential protein expression between MCF-7 and MeIR MCF-7 was extracted. In this preliminary study, several proteins were found to be differentially expressed in MeIR MCF-7. The major change was the reduced level of calreticulin in the cytosol of MeIR MCF7 (Figure 6). Most of these differentially expressed proteins are directly or indirectly involved in apoptosis and might thereby be correlated to the development of resistance of MCF-7 to melphalan. However, other experimental procedures in addition to 2-DE techniques are needed to validate or confirm these results. This is just the beginning of using proteomics strategies to studies development of chemoresistance. It is possible that someday a consensus pattern of differential protein expression will be established across different cancer tissues that are resistant to a particular drug. Identification of such proteins will help us to better understand the complex factors responsible for the development of chemoresistance in cancer cells and suggest new approaches to controlling resistance, thus improving treatment.

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