

## Proteomic Analysis to Dissect Mitoxantrone Resistance-associated Proteins in a Squamous Lung Carcinoma

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**Abstract.** *Background:* Mitoxantrone resistance has been related to the expression of a drug efflux pump breast cancer resistance pump (*BCRP*) but little is known of the intracellular protein changes. In this work, differential protein expression in a squamous lung carcinoma cell line, DLKP, and its mitoxantrone-resistant variant (DLKP-Mitox) was investigated to elucidate other changes associated with mitoxantrone resistance. *Materials and Methods:* Differential protein expression between DLKP and DLKP-Mitox was investigated using 2D-DIGE technology. Proteins of interest were identified by MALDI-ToF mass spectrometry. Western blotting was used to confirm and validate some of these changes. *Results:* Biological variation analysis in Decyder™ software revealed a total of 343 proteins to be differentially regulated with  $p < 0.05$ . Identification of 61 proteins of interest by mass spectrometry revealed changes in proteins involved in many cellular processes including apoptosis and differentiation. *Conclusion:* Alterations in these cellular processes and proteins present alternative sites to circumvent resistance to mitoxantrone.

Lung cancer accounts for more deaths than breast, prostate and colon cancers combined and presents a significant health issue. The lethality of lung cancer is related to the late stage of presentation, the formation of metastases from local and distant tumours and the occurrence of multi-drug resistance (MDR). MDR is the biggest cause of chemotherapy failure and in lung cancer may be inherent or acquired. Mechanisms of resistance include drug efflux via membrane-bound pumps (e.g. P-glycoprotein (Pgp) and multidrug resistance protein (MRP)), alterations in detoxification, enhanced DNA repair and apoptosis (1). Despite increased understanding of MDR, progress in the clinic to date has been poor as in the case of Pgp inhibitors

(2). A greater knowledge of the proteins and pathways involved in MDR may offer new possible targets for therapeutic intervention.

Mitoxantrone is a DNA intercalator and topoisomerase II poison. It has been used to treat hematological (AML, ALL), prostate, breast and other malignancies (3). Resistance to mitoxantrone has been related to overexpression of *BCRP1*, the breast cancer resistance half transporter protein (4) and to a lesser extent, decreases in target proteins topoisomerase II (5). *BCRP* expression in normal lung tissue is low (4) but Western blotting of lung carcinoma, colon cancers, esophageal cancer, myeloma, GIT adenocarcinomas and endometrial carcinomas showed increased expression levels (6).

Previous work in this lab identified *BCRP* as a mechanism of resistance in a mitoxantrone-selected variant of a poorly differentiated squamous lung carcinoma, DLKP (7). As resistance to mitoxantrone is considered to be multifactorial (8), proteomic strategies may provide insights into the global changes in protein expression. Here, we investigate the altered protein expression of DLKP and its mitoxantrone-resistant variant to look for such pathways.

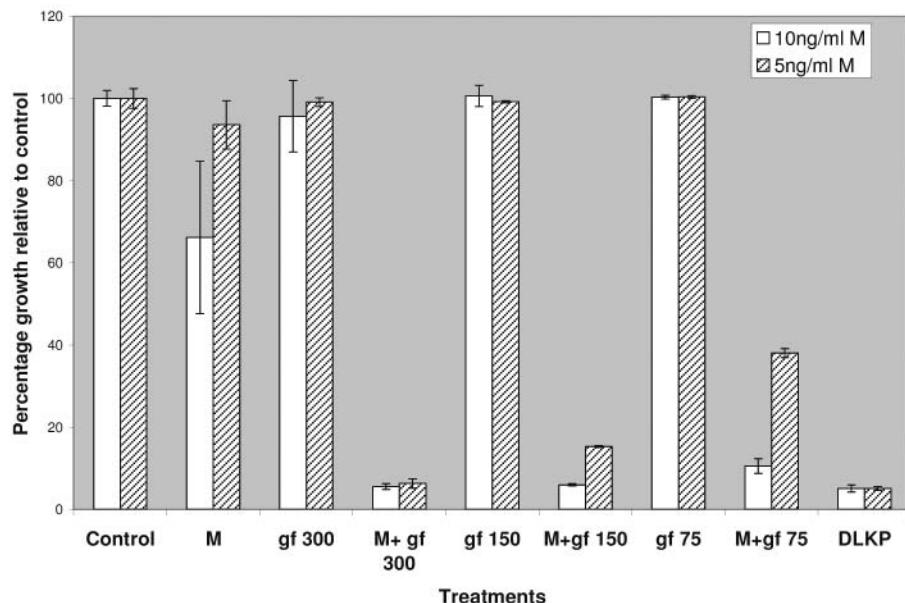
### Materials and Methods

All chemicals (unless otherwise stated), FBS, glutamine and cell culture media were obtained from Sigma (Poole, UK). Mitoxantrone resistance was developed in DLKP, a poorly differentiated human squamous lung carcinoma cell line (9), by pulsing 5 times with 60 ng/ml mitoxantrone (7). Cells were maintained in DMEM/Hams F12 (1:1) with 5% FBS. GF120918 was obtained from GlaxoSmithKline (Middlesex, UK) and Mitoxantrone from Cynamid GB Ltd. (Gosport, UK).

*Two-dimensional-difference in gel electrophoresis (2D-DIGE).* Lysates of exponentially growing cells (8M urea, 4% CHAPS, 30 mM Tris/HCl, pH 8.5, 1X DNase and RNase) were minimally labeled with 200 pmol/50 µg protein Cy2 (DLKP and Mitox: internal control), Cy3 (DLKP) and Cy5 (DLKP-Mitox) according to manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). Six biological repeats were used. Isoelectric focusing was performed using immobilized pH gradient (IPG) strips (pH4-7) equilibrated overnight (Amersham Biosciences, Buckinghamshire, UK). Samples were loaded via cup-loading (50 µg each of Cy2, Cy3 and Cy5) and run in

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**Figure 1.** Combination assay of Mitoxantrone and GF120918 in DLKP-Mitox. Results are the average of two separate assays ( $n=8$ ) each at two concentrations of mitoxantrone (M at 5 or 10 ng/ml) and GF120918 at 75, 150 and 300 ng/ml. The growth inhibition in DLKP-Mitox caused by mitoxantrone with GF120918 at 300 ng/ml corresponds to the growth inhibition seen in the parent DLKP with mitoxantrone alone at the same concentration i.e. at 300 ng/ml GF120918, the resistance conferred by BCRP is abrogated in DLKP-Mitox.

a step and gradient with holds up to a maximum 8000V for 4 h. After second dimension SDS-PAGE (12.5%), gels were scanned using the Typhoon 9400 variable mode imager (Amersham Biosciences, Buckinghamshire, UK). Image analysis was performed using the DeCyder™ Software version 6.2 (Amersham Biosciences, Buckinghamshire, UK). Statistical analysis and quantitation of protein abundance were determined using the biological variation analysis module (BVA) of DeCyder™. Proteins were defined as differentially regulated if the observed fold change was greater than 1.2 with  $p$ -values less than 0.05 (Student's  $t$ -test) between protein spots of control and drug-resistant variant.

**Protein identification by mass spectrometry.** Differentially expressed proteins were identified from preparative colloidal coomassie stained gels (400  $\mu$ g protein) and picked using an Ettan Spot Picker (Amersham Biosciences, Buckinghamshire, UK). In-gel digestion with modified porcine trypsin (Promega, Southampton, UK) was carried out using a microtiter plate format on an Ettan Digestor (Amersham Biosciences, Buckinghamshire, UK) and vacuum-dried in a Maxi Dry Plus (MSC, Dublin, Ireland). Using the Ettan Spotter (Amersham Biosciences, Buckinghamshire, UK), peptides were resuspended (0.5% trifluoroacetic acid in 50% acetonitrile) and spotted onto the target plate after which matrix solution (7.5 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid (LaserBio labs, Cedex, France) in 0.1% trifluoroacetic acid in 50% acetonitrile) was added. PepMix4 (LaserBio labs, Cedex, France) was added as an external calibrant (one to each target plate). A MALDI-ToF (Amersham, Biosciences, Buckinghamshire, UK) instrument was used to detect the mass/Z ratio in positive reflector mode. Each plate was calibrated with the PepMix 4 and internal trypsin peaks were also used to check calibration. Spectra were submitted to the Pro-Found search engine for protein mass fingerprint identification. Gene symbol (GS) for

bioinformatics analysis was determined from the "gi number" conversion software package, DAVID (<http://david.abcc.ncifcrf.gov>) and ontology analysis was performed in PubMed Entrez gene (<http://www.ncbi.nlm.nih.gov>). Bioinformatic analysis was performed with Pathway Studio™ (Ariadne Genomics, Rockville MD, USA) on differentially expressed proteins.

**Western blotting.** Western blotting was performed on cell lysates prepared for DIGE. Samples were separated on a 15% SDS gel (10) with 30  $\mu$ g loaded per well. After Western blotting (11), blots with primary antibodies (Stathmin and NDPK (Calbiochem and AbCam respectively, Merck KGaA, Darmstadt, Germany)) were incubated overnight at 4 °C. Secondary antibodies conjugated to horse-radish peroxidase (Sigma, Poole, UK) were detected by enhanced chemiluminescence (Luminol, Santa Cruz, CA, USA).

**Combination assays.** Combination assays were carried out as performed previously (12). Briefly 10<sup>3</sup> cells in 96-well plate format were incubated overnight at 37 °C and 5% CO<sub>2</sub>. The cells were then exposed to mitoxantrone (5 or 10 ng/ml) or to the Pgp inhibitor GF120918 (75, 150 or 300 ng/ml) or a combination of both. The cells were allowed to grow for a further 6 days until confluence was approached. Acid phosphatase was used as the end point.

## Results

Mitoxantrone resistance in DLKP-Mitox was confirmed as 5.8-fold as previously determined (7). Combination assays with the Pgp and BCRP inhibitor GF120918, showed most, if not all, the resistance could be overcome by inactivating BCRP in DLKP-Mitox (Figure 1).

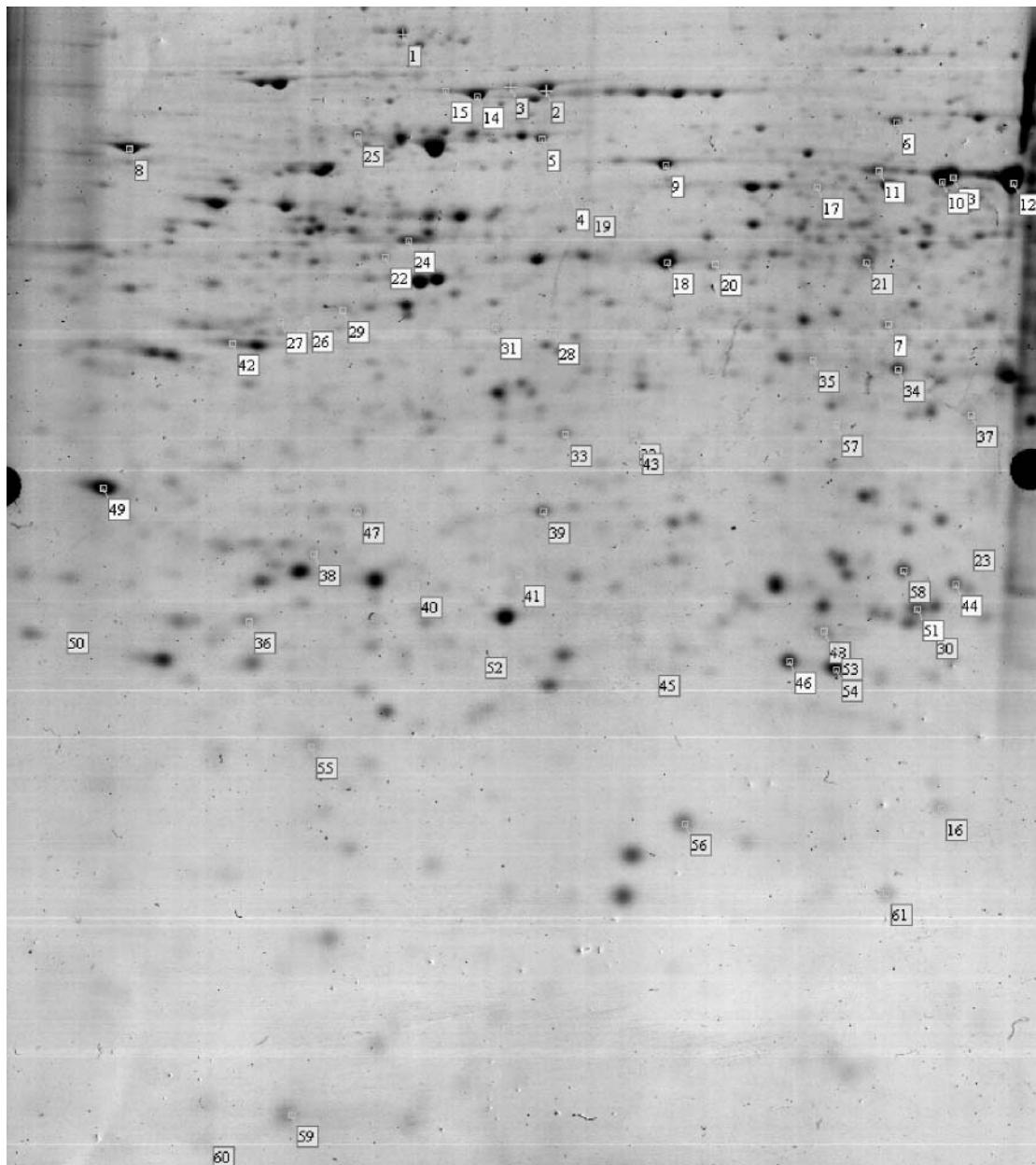


Figure 2. Differentially expressed proteins in DLKP-Mitox. Preparative DLKP-Mitox gel showing 61 differentially expressed proteins identified by MALDI-ToF ( $p \leq 0.05$ , protein fold difference  $\geq 1.2$ ). Proteins were labelled numerically for visual clarity and are outlined in Table I and II.

Analysis using Decyder™ in biological variation analysis revealed a total of 343 proteins to be differentially regulated with  $p < 0.05$  and fold differences ranging from 3.85-fold up-regulation to 3.96-fold down-regulation. Of the 61 proteins identified by MALDI-ToF mass spectrometry (Figure 2), 31 were found to be up-regulated and 30 down-regulated. Sorcin, tropomyosin and actin G were over 3-fold higher in the mitoxantrone-resistant variant (Table I) while phosphoglycerate mutase and cofilin were over 3-fold down-regulated (Table II).

Ontology analysis obtained through PubMed searches, identified many cellular processes with cytoskeleton (19%) and protein turnover (13%) constituting the greatest number of protein expression changes (Figure 3). Moderate changes in apoptosis/redox regulation, ion binding/transport and stress response account for 11% each. Glycolysis and RNA processing proteins account for 10% each. Western blot analysis confirmed the alterations in NDPK (Figure 4). While NDPK appeared as a single protein spot in DIGE, Western blotting showed NDPK as a doublet; perhaps

Table I. Up-regulated proteins expressed in DLKP-Mitox.

Spot No.	Protein ID	gi no	GS	Mw	pI	Fold increase	Function
8	P4H beta subunit	gi 48735337	PRDX3	57.5	4.8	1.28	protein turnover
10	ALDH 1A1	gi 2183299	ALDH1A1	55.44	6.3	1.64	glycolysis
11	ALDH 1 A1	gi 2183299	ALDH1A1	55.44	6.3	1.61	glycolysis
12	ALDH 1A1	gi 2183299	ALDH1A1	55.4	6.3	1.57	glycolysis
13	ALDH 1A1	gi 2183299	ALDH1A1	55.44	6.3	1.32	glycolysis
16	HSP 70 kDa	gi 62896815	HSPA8	53.6	5.6	1.79	stress
17	alpha tubulin 6	gi 62897609	TUBA6	50.49	5	1.39	cytoskeleton
21	OAT mutant y85	gi 78101704		48.81	6.6	1.96	metabolism
23	PDI-related protein 5	gi 1710248	p5	46.52	5	2.82	protein turnover
26	Beta Actin	gi 15277503	ACTB	41.54	5.6	2.97	cytoskeleton
27	Beta Actin	gi 15277503	ACTB	40.54	5.6	2.53	cytoskeleton
29	Beta Actin	gi 15277503	ACTB	40.54	5.6	2.01	cytoskeleton
30	Human Hgprt	gi 47115227	HPRT	39.3	5.5	2.25	RNA process
32	Annexin A4	gi 1703319	ANXA4	36.09	5.8	1.51	ion binding
34	Annexin A1	gi 442631	ANXA1	35.25	7	1.89	ion binding
35	Annexin A1	gi 442631	ANXA1	35.25	7	1.2	ion binding
36	Annexin A1	gi 442631	ANXA1	35.25	5.4	2.21	ion binding
37	PNP	gi 387033	PNP	32.23	6.5	1.38	protein turnover
38	EEF1D	gi 15215451	EEF1D	31.22	4.9	2.04	translation
40	ACTG1	gi 40226101	ACTG1	29.68	5.5	2.45	cytoskeleton
41	ActG	gi 40226101	ACTG1	29.68	5.5	3.19	cytoskeleton
45	Peroxiredoxin	gi 62896877	PRDX3	27.95	7.1	2.2	apoptosis
46	Peroxiredixin 3a	gi 62896877	PRDX3	27.95	8	1.61	apoptosis/redox
49	Tropomyosin	gi 825723	TPM1	26.62	4.8	3.1	cytoskeleton
52	GST-pi chain B	gi 23200511	GSTP1	23.43	5.4	1.8	apoptosis/redox
53	Proteasome beta 3 subunit	gi 15278174	PSMB3	23.22	6.1	1.45	protein turnover
54	K130r Mutant	gi 33358056	PARK7	21.14	6.5	1.25	apoptosis/redox
55	Sorcinc	gi 38679884	SRI	20.61	5.1	3.85	ion binding
57	26S Proteasome Pad1	gi 62088020	PSMD14	18.99	6.5	1.44	protein turnover
58	High mobility Group box 1	gi 55958717	HMGB1	18.32	5.8	2.4	transcript/reg
60	Thioredoxin delta 3	gi 1827674	TXN	11.86	4.8	1.98	apoptosis

Ion binding and transcript/reg refer to ion binding and transport and to transcription/transcription regulation respectively; PDI: protein disulphide isomerase; PNP: purine nucleotide protein; OAT: ornithine aminotransferase; v refers to variant isoforms appearing on gels. Note: For OAT, the Gene symbol was discontinued in Entrez gene.

separation on a 15% gel allowed for better resolution of the doublet. Densitometry analysis (using stathmin as an unchanged internal control from the DIGE experiments) showed 1.6-fold down-regulation of the main NDKP band in DLKP-Mitox, corresponding well to the 1.45-fold down-regulation seen in DIGE.

The gene symbols for the differentially-expressed proteins were submitted to Pathway Studio (permits identification of biological interactions among genes and proteins of interest from published literature) to find common pathways. The common pathways displayed a complex interaction affecting up to 15 different cellular processes including apoptosis, differentiation and synthesis to be important in the development of increased resistance in DLKP-Mitox (image not shown). Amongst the pathways, apoptosis and differentiation showed most changes (Figure 5). Many stimulators of apoptosis (including *NME1*, *LMNB1*, *EEF2*, and *NPM1*) were down-regulated in DLKP-Mitox while

inhibitors of apoptosis (including *HSPA8*, *PRDX3* and *ALDH1A1*) were up-regulated. Stimulators of differentiation include *BAT1*, *TUBB*, *TXN*, *ALHIA1*, *HPRT1*, *PHB*, *GSTP1* and *HMGB1*. The majority of these are increased in DLKP-Mitox. *NME1* inhibits differentiation and is decreased.

## Discussion

The use of DIGE technology has allowed us to study differential protein expression in DLKP and its mitoxantrone-resistant variant. Resistance to mitoxantrone has been associated with changes in ABC membrane pumps, *Pgp*, *MRP-1* and *BCRP* (13), alterations in topoisomerase activity (14) and apoptosis induction (15). This work has yielded a total of 50 differentially expressed proteins (61 including all isoforms) that may present targets for drug resistance intervention. Although *BCRP* is up-regulated (7)

Table II. Down-regulated proteins expressed in DLKP-Mitox.

Spot No.	Protein ID	gi no	GS	Mw	pI	Fold decrease	Function
1	HSP 70kDa 9B	gi 292059	HSPA9B	74.05	6	2.53	stress
2	VCP protein	gi 48257098	VCP	71.56	4.9	2	cell signalling
3	lamin B1	gi 576840	LMNB1	67.79	5.3	2.1	cytoskeleton
4	CGI-46 Protein	gi 4929561	CGI-46	63.25	6.4	1.35	stress
5	KIAA0098	gi 58257644	CCT5	61.49	5.5	1.73	stress
6	CCTCT subunit 3	gi 54696794	CCT3	61.02	6.1	2.02	stress
7	Eif2b	gi 19353009	EEF2	58.17	6.5	1.32	translation
9	ER-60	gi 1208427	PDIA3	57.16	5.9	1.57	RNA process
14	hsp 70kDa	gi 62896815	HSPA8	53.6	5.6	2.99	stress
15	HSP 70kDa	gi 62896815	HSPA8	53.6	5.6	2.3	stress
18	Beta Tubulin	gi 18088719	TUBB	50.11	4.7	1.48	cytoskeleton
19	HLA-B associated trans.	gi 62897383	BAT1	49.56	5.5	2.05	tumor immunity
20	NBLa10058 protein	gi 76879893	PPA1	49	5.9	1.25	protein turnover
22	M-6-P receptor bp	gi 16306789	M6PRBP1	47.2	5.3	1.26	RNA process
24	HNRPF	gi 16876910	HNRPF	46.02	5.4	1.71	RNA process
25	HNRPK	gi 55958547	HNRPK	42.02	5.4	2.69	RNA process
28	Beta Actin	gi 15277503	ACTB	40.54	5.6	1.37	cytoskeleton
31	PKCq-interacting protein	gi 6840947	TXNL2	37.7	5.4	1.58	protein turnover
33	PP protein	gi 33875891	PP	35.97	6	2.88	metabolism
39	Prohibitin	gi 46360168	PHB	29.86	5.6	1.36	transcript/reg
42	Nucleophosmin	gi 13536991	NPM1	29.62	4.5	1.37	ion binding
43	Proteasome activator sub 3	gi 47523754	PSME3	29.6	5.7	1.71	protein turnover
44	Phosphoglycerate mutase	gi 62897753	PGAM1	28.93	6.7	3.34	glycolysis
47	CLIC 1	gi 62898319	CLIC1	27.34	5.1	1.34	ion binding
48	TPI	gi 66360366	TPI	26.95	6.5	1.25	glycolysis
50	Proteasome sub alpha(5)	gi 54696300	PSMA5	26.58	4.7	1.28	protein turnover
51	Peroxidase	gi 3318842	Hprt	24.9	6	1.62	apoptosis
56	NDPK 1	gi 38045913	NME1	19.86	5.4	1.45	transcript/reg
59	Galectin-1	gi 42542977	L-Gals1	14.75	5.3	1.4	apoptosis/redox
61	Cofilin	gi 5031635	CFL1	24.46	6.5	3.77	cytoskeleton

Ion binding and transcript/reg refer to ion binding and transport and transcription/transcription regulation; M6P: mannose-6-phosphate; bp: binding protein; CLIC: chloride intracellular channel; TPI: triosephosphate isomerase; VCP: vasolin containing protein; v refers to variant isoforms appearing on gels; RNA process: RNA processing. Note: HSP 70 kDa refers to HSP 70 kDa protein 8 isoform 2 above.

and active in DLKP-Mitox (as shown by inhibition of *BCRP* with GF120918, Figure 1), it was not identified in DIGE. This is not surprising given the limitations of detecting high molecular weight and hydrophobic proteins on two-dimensional gel electrophoresis.

Previous work revealed thioredoxin, stratafin, annexin 1, cofilin, Rho-GDP inhibitor, *FaBP* and *APRT* to be differentially regulated in mitoxantrone-resistant variants of colon, fibrosarcoma and pancreatic adenocarcinoma (16–18). Further studies in mitoxantrone-resistant MCF-7 breast cells showed tropomyosin, *HMGB1*, prohibitin (*PHB*), *HSP 70*, heterogeneous nuclear ribonucleoproteins (*HNRPH* and *HNRPK*) and nucleophosmin (*NPM*) to be differentially regulated (19, 20).

*HMGB1*, the high mobility group 1 box protein was up-regulated in MCF-7/MX and DLKP-Mitox. *HMGB1* is involved in nuclear complex formation and DNA repair, inflammation, apoptosis and differentiation and may

contribute to drug resistance by enhancing these activities. Significantly, *HMGB1* was found to elicit activation of metalloproteinases MMP-2 and MMP-9 (21), both of which were previously found to be expressed in DLKP and DLKP-Mitox (7). Protein disulphide isomerase (*PDI*) and triose phosphate isomerase (*TPI*) are commonly expressed in a variety of cancers and may contribute to drug resistance (22). *Gst M4* and prolyl 4-hydroxylase B (*P4HB*) were significantly increased in lung adenocarcinomas (23). Many of these proteins have been differentially regulated in DLKP-Mitox, consistent with previous reports (*PDI*, annexin I, *P4HB*, thioredoxin, *HMGB1*, *NDPK* and *NPM*).

Cofilin, tropomyosin, prohibitin, *ER-60*, *HNRPF* and *TPI* expression are not consistent with previous studies. Tropomyosin acts as an anti-oncogene and tumor suppressor with lower expression observed in many transformed cells (24) and the metastatic phenotype (25). Down-regulated in MCF-7/MX and drug resistant gastric cancers (26),

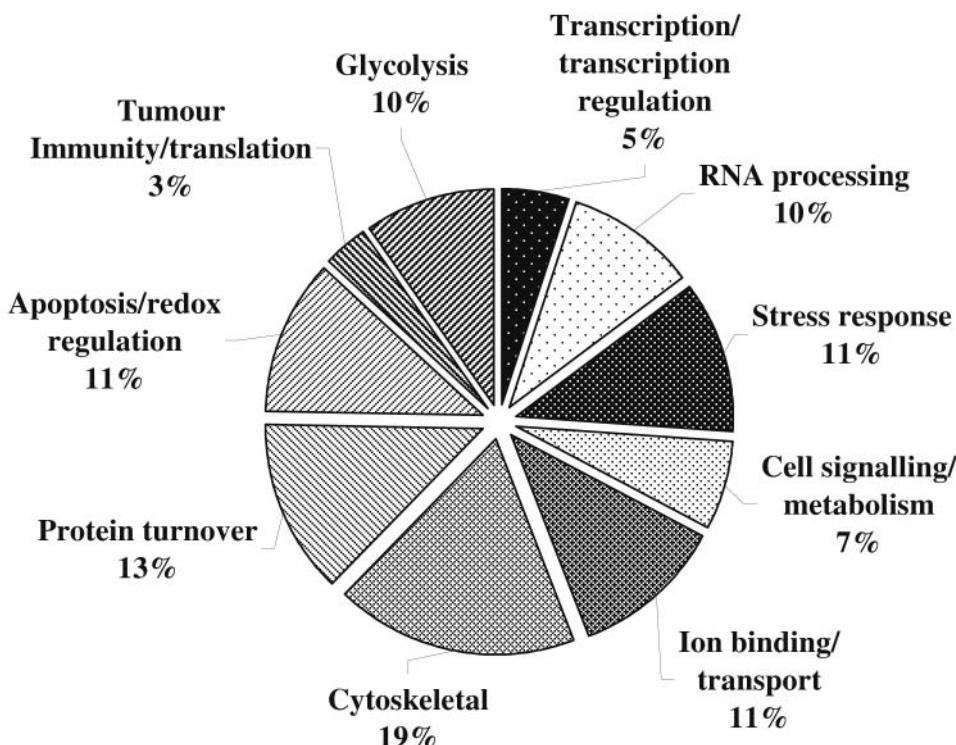


Figure 3. Ontology analysis of differentially-regulated proteins. The pie chart shows the percentage contribution according to function of differentially regulated proteins – a qualitative change not a quantitative change.

tropomyosin was up-regulated in DLKP-Mitox. Prohibitin (*PHB*) is a mitochondrial chaperone involved in cell cycle control, cellular immortalisation, and apoptosis (27), is up-regulated in MCF-7/MX and cisplatin-resistant head and neck tumors (28). *PHB* was down-regulated in DLKP-Mitox. Cofilins are actin binding proteins that regulate actin assembly and are implicated in apoptosis (29).

Sinha and colleagues suggested a mechanism of mitoxantrone resistance involving apoptosis (*via* Rho-GDP dissociation inhibitors and thioredoxin) and concerted actions on PKC activity (downstream effector of *GST*, *Topo II* and *Pgp*) (19). Consistent with this, apoptosis in DLKP-Mitox may become more resistant to apoptotic signals but through multiple apoptotic signals. Seventeen of the differentially-expressed proteins have a role in apoptosis as shown in Pathway Studio with 8 of 14 stimulators reduced and two of the three inhibitors (*ALDH* and *PRDX3*) up-regulated. In addition, many of the proteins showing the greatest changes in expression are related to apoptosis, including cytoskeletal proteins (tropomyosin, actin G and B), sorcin and cofilin. Besides Rho-GDP dissociation inhibitors and thioredoxin mediated apoptosis, other apoptotic pathways may be relevant in DLKP-Mitox. Control of calcium levels *via* increased expression of sorcin and annexins may reduce susceptibility

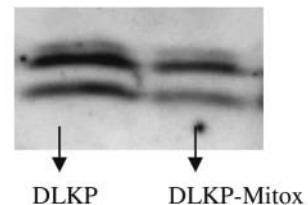


Figure 4. Confirmation of results with Western blotting of NDKP. Samples of DLKP and DLKP-Mitox were immunoblotted with anti-NDKP to confirm down-regulation as seen in DIGE experiments.

to apoptosis. Resveratrol induced apoptosis through phosphoglycerate mutase in LnCAP proteins (30) and phosphoglycerate mutase was 3.34-fold decreased in DLKP-Mitox. ER-stress induced apoptosis may be modified by changes of proteins involved in protein turnover including *PDI*, *P4HB*, *ER-60* and *EIF2B*. Many changes observed in cytoskeletal proteins, may also modify response to apoptotic stimuli.

Our objective in the present research was to investigate factors involved in the development of resistance to mitoxantrone distinct from *BCRP*. These results confirm the importance of apoptosis and the multifactorial nature of apoptotic signaling pathways involved in mitoxantrone

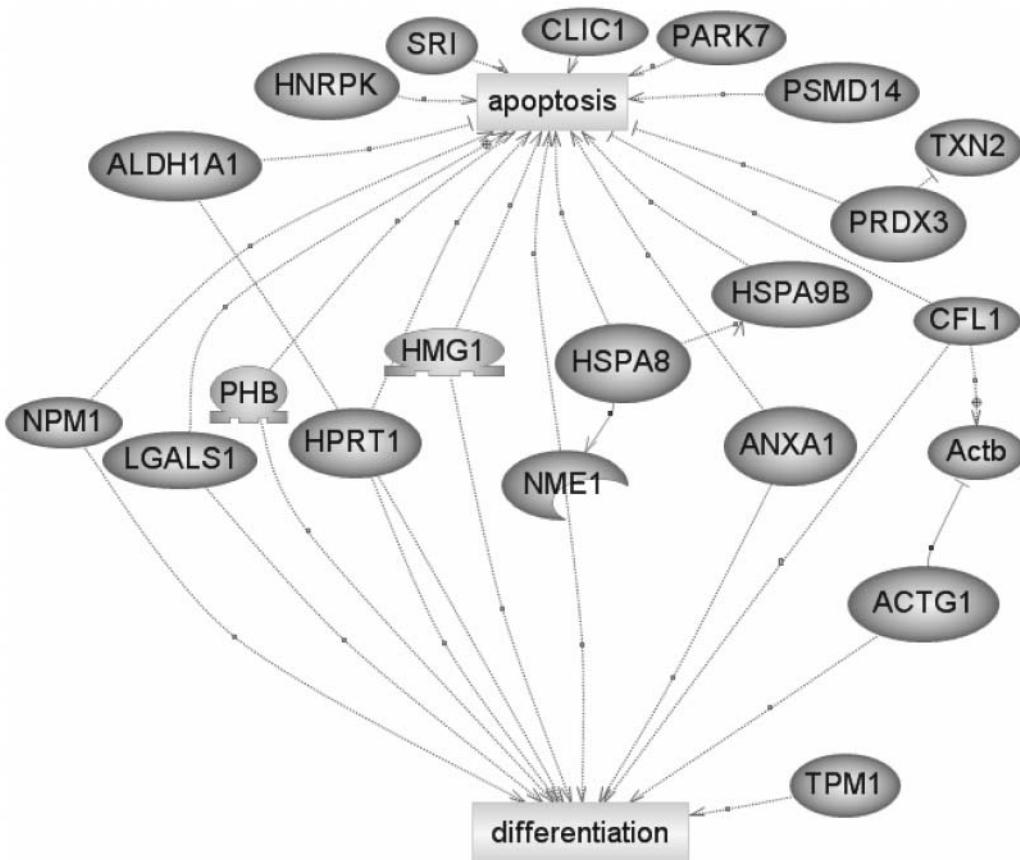


Figure 5. Pathway Studio analysis of common interactions between proteins involved in apoptosis and differentiation. Differentially expressed proteins are shown in oval. PHB and HMGB1 are transcription factors and NME1 is a kinase. Cellular processes are in rectangles.

resistance and may provide biomarkers for future intervention of mitoxantrone-induced resistance.

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