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Pathway-centric analysis of the DNA damage response to chemotherapeutic agents in two breast cell lines

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ABSTRACT

The response to DNA damage by alkylation and DNA topoisomerase inhibition was studied in two breast cancer cells lines. We present data from both a shotgun and a targeted, pathway-centric approach to highlight the different DNA repair pathway modulation in the cell lines and the correlation with viability and DNA damage assays. This type of focussed profiling may be of utility in rapidly defining non-responders undergoing systemic neoadjuvant therapy.

1. Introduction

Each cell in the body is constantly exposed to DNA damaging agents, such as UV light or industrial chemicals, generating thousands of lesions a day in cellular DNA. In order to secure the accurate passing of genetic information to the next generation, the cell has evolved an intricate surveillance system to find and correct this damage. This surveillance system consists of several cell-cycle checkpoints, which ensure DNA repair occurs before replication of the DNA and cell division. The main DNA repair pathways are: mismatch repair (MMR) detects and deals with dNTP misincorporation and insertions/deletions; nucleotide excision repair (NER) recognise helix-distorting base lesions from, for example UV damage while oxidative lesions and alkylation products are repaired through base excision repair (BER) and there are two different mechanisms dealing with double strand breaks (DSB)

Abbreviations: MMR, mismatch repair; dNTP, deoxynucleotide triphosphate; BER, base excision repair; DSB, double strand break; HR, homologous recombination; NEHJ, non-homologous end-joining; NER, nucleotide excision repair; DDR, DNA damage repair; DDS, DNA damage signalling; MMS, methyl methanesulphonate; DR, doxorubicin.

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[1]. These are homologous recombination (HRR) which is restricted to S and G2 phase since it is using sister-chromatid sequences as templates [2] and the more error-prone (NHEJ) that is occurring throughout the cell cycle [3–6].

Cancer cells often increase the rate of mutation during their development in order to enable the acquisition of the other cancer hallmarks. This is achieved by disabling one or more components of the DNA damage surveillance system [7]. As a consequence, mutations in various DNA repair pathways are commonly seen in different cancers, e.g. BRCA1 and 2 mutations giving rise to an aberrant HRR are found in more than 50% of patients with hereditary breast cancer and hereditary defects in NER cause UV sensitivity and skin cancer development [8]. The standard cancer treatment today consists of surgery in combination with chemotherapy or radiotherapy that induce DNA damage. Cancer cells that are rapidly dividing are more sensitive to the DNA damage than normal cells and defects in their DNA repair mechanisms also make them more vulnerable to acquiring too much DNA damage and entering apoptosis. The standard chemotherapeutic agents are divided into three groups depending on their mechanism of action; drugs inducing covalent crosslinks between DNA bases (cisplatin, carboplatin), drugs attaching alkyl groups to bases (methyl methanesulphonate (MMS), temoxolomide) and drugs causing DSBs by trapping topoisomerase I or II (camptothecin, etoposide, DR) [9,10]. Depending on the mutation pattern in the DNA damage repair pathways (DDR) in each tumour they are differently sensitive to the chemotherapeutic agents. Tumours with defects in NER are, for example more sensitive to cisplatin, reflecting their reduced capacity to repair crosslinks [11].

The DDR defects offer an opportunity for more targeted therapies. By inhibiting an additional pathway in a cancer with pre-existing DNA repair defects a synergistic lethal effect is created, exemplified by for instance PARP inhibitors [12]. In normal cells, the effects of PARP inhibition can be buffered by the compensatory pathway HRR, but in BRCA mutants the DSBs are left unpaired and can cause cell death [13,14]. Several PARP inhibitors are now in Phase I and Phase II clinical studies as well as other DDR inhibitors such as inhibitors of CHK1 and APE1 [9,10]. The sensitivity to PARP inhibition is more dependent on the BRCA genotype than the tissue origin, which is shown by their effectiveness in different BRCA deficient cancers such as breast, ovarian and prostate cancer [15,16]. This opens up a new concept of cancer research where patients are selected for therapy based on their DDR landscape rather than the tissue of origin. However, the exact cancer killing mechanism of PARP inhibitors [17] as well as many of the conventional cancer drugs is poorly understood [18] and the design of optimal treatment regimes for cancer patients would benefit from extending our knowledge in this area.

In this study, the aim was to investigate the response of breast cancer cells to treatment with two different types of chemotherapeutic drugs, the alkylation agent MMS [19] and the topoisomerase II inhibitor, doxorubicin (DR) [18]. To this end, the two breast cancer cell lines MCF-7 and MDA-MB-231 were used that have differing chemo-sensitivities [20]. We present a shotgun proteomic analysis together with an SRM analysis of the main DNA repair pathways to investigate the feasibility of developing a method for predicting or following cellular responses to different DNA damage treatments.

2. Materials and methods

2.1. Materials

Acrylamide, urea, Tris, magnesium acetate, DTT, iodoacetamide and methyl methanesulphonate were from Sigma–Aldrich (Stockholm, Sweden). Sequencing-grade modified trypsin was purchased from Pierce (SDS diagnostics, Falkenberg, Sweden). The Micro-Lowry Protein Assay Kit was from Sigma Diagnostics (Stockholm, Sweden). The WST-1 Cell Proliferation Reagent was from Roche Diagnostics (Basel, Switzerland) and the OxiSelect Comet Assay Kit was from Cell BioLabs (San Diego, CA, USA). All HPLC solvents were from Fluka (Sigma–Aldrich, Stockholm, Sweden). Doxorubicin was from Meda (Solna, Sweden).

2.2. Cell culture and viability

The human breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (FBS) and 1% L-glutamine (Invitrogen, Carlsbad, CA) and 10 μg/mL of insulin was added to the MCF-7 cell line. The cells were maintained as monolayer cultures at 37 °C in a humidified atmosphere at 5% CO_2. The cell cultures were exposed to MMS and DR for the various times and concentrations indicated. Cell viability was measured in duplicate by Trypan blue exclusion after 1, 6 and 24 h of treatment with DR or MMS. Cells excluding Trypan blue dye were counted as viable in Cell Countess.

2.3. COMET assay for DNA damage assessment

The amount of DNA damage was assessed with OxiSelect Comet Assay Kit following the manufacturer's protocol for alkaline electrophoresis. Briefly, cells were grown in 6-well plates and treated with the indicated concentrations of DR (2.5 and 25 μg/mL) or MMS (4 and 20 μg/mL). After 1 and 24 h of exposure, cells were trypsinised and washed, then resuspended in PBS and then mixed with agarose at 37 °C in a 1:10 ratio. The suspension was immediately transferred to the COMET slide and incubated for 15 min at 4 °C in the dark. Slides were immersed in lysis buffer for 60 min at 4 °C in the dark and subsequently incubated in alkaline solution for 30 min at 4 °C in the dark. Electrophoresis was performed at 15 V for 30 min and the slides were then washed with deionized water and dried with ethanol before staining 15 min with Vista Green. COMETS were observed by fluorescent microscopy (Nikon) with 20× magnification and the images were analysed using the COMET Score software.

2.4. Nuclear extraction

Cells were cultured as described above and treated with MMS or DR. After 1 or 24 h approximately 10 million cells were washed twice with PBS and then resuspended in a hypotonic homogenisation buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, Complete Mini EDTA-free protease

inhibitor). The MDA-MB-231 cells were incubated on ice for 15 min while the MCF-7 cells were kept in 5 mM HEPES pH 7.9, 5 mM KCl, 0.75 mM $MgCl_2$, 0.25 mM DTT, Complete Mini EDTA-free protease inhibitor) for 20 min on ice. The cells were then disrupted using 20–40 strokes with a tight pestle in a 7 mL Dounce homogeniser until 90% of the cells were broken as determined by microscopy. Subsequently, nuclei were pelleted and stored in the freezer.

2.5. Separation of chromatin-associated and nucleoplasm fractions

Nuclear pellets were thawed on ice and washed once with ice-cold PBS and once with extraction buffer (15 mM Tris–HCl pH 7.4, 1 mM EDTA, 400 mM $MgCl_2$, 10% glycerol, 10 mM β-mercaptoethanol) before resuspension in 500 μL extraction buffer and incubation for 1 h on ice. Samples were then centrifuged for 30 min at 16,000 × *g* and the supernatant was diluted in hypotonic buffer (10 mM Tris–HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM β-mercaptoethanol) and saved as the nucleoplasm fraction. Subsequently, pellets were washed and resuspended in micrococcal nuclease buffer (20 mM Tris–HCl, 10 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 300 mM sucrose, protease inhibitor cocktail). Six units of S7 nuclease were added and incubated with the samples for 30 min on ice with mixing every 4 min. The nuclease was inactivated by the addition of 10 μL 0.5 M EDTA and the samples were centrifuged for 3 min at 1500 × *g*. The supernatant was diluted in hypotonic buffer and saved as chromatin-associated fraction.

Nucleoplasm and chromatin-associated fractions were precipitated by adding DOC to a concentration of 0.08% (w/v) and incubating 30 min on ice, followed by an addition of TCA to a concentration of 2.5% (w/v) and 20 min incubation on ice. The precipitated proteins were collected by centrifugation at 3500 × *g* for 30 min.

2.6. Protein separation and digestion

The protein samples were mixed with sample buffer (0.05 M Tris–Cl, 0.05 M SDS, 5% v/v glycerol, 0.1% DTT) and heated at 98 °C for 90 s before separation on a 12.5% SDS–PAGE gel with a 4% stacking gel at 25 °C. Eighty micrograms of protein were loaded per lane and the gel was run with 25 amp/gel until the bromophenol blue dye front had run off the base of the gel. The gel was stained using Gel Code Blue Stain Reagent (Pierce). Each of the lanes was cut into 10 slices that were then destained in 50% acetonitrile and 25 mM NH_4HCO_3. The gel slices were reduced with 10 mM DTT in 100 mM NH_4HCO_3 at 56 °C for 1 h. Alkylation was performed by adding 55 mM iodoacetamide acid in 100 mM NH_4HCO_3 and incubating for 45 min at RT in the dark. The slices were then washed once with 100 mM NH_4HCO_3 and several times using ACN. Trypsin (25 μL of 12.5 μg/mL trypsin in 50 mM NH_4HCO_3) was added to the dehydrated gel slices. The samples were left for 30 min at 4 °C prior to incubation at 37 °C overnight. The peptides were extracted from the gel by adding 5% TFA in 75% ACN and incubating at RT for 30 min. This was repeated once and the two extractions were pooled. Subsequently, the volume was reduced using a Speed vac and the samples were then dissolved in 10 μL 0.1% formic acid. The 10 fractions were subsequently analysed by RP-HPLC–MS/MS. Samples were run in biological triplicate and technical duplicate.

2.7. LC–MS/MS analysis

The fractions were separated and analysed on an Eksigent 2D NanoLC system (Eksigent Technologies, Dublin, CA, USA) coupled to an LTQ OrbitrapXL (ThermoFisher, Bremen, Germany). Peptides were loaded with a constant flow of 10 mL/min onto a pre-column (Zorbax 300SB-C18 5 mm × 0.3 mm, 5 mm, Agilent Technologies, Wilmington, DE, USA) and subsequently separated on a RP-LC analytical column (Zorbax 300SB-C18 150 μm × 75 μm, 3.5 mm) at a flow rate of 400 nL/min using a standard nanospray interface from Thermo. The peptides were eluted with a 60 min linear gradient of 3–35% ACN (0.1% FA) followed by a 3 min linear gradient from 35 to 90% ACN (0.1% FA). The ion trap loading was set to 30,000 with an MS/MS threshold of 500 counts. The seven most intense ions were selected for fragmentation with the dynamic exclusion duration of 2 min.

2.8. Data analysis

Raw data files were imported in profile mode into Progenesis LC-MS version 3.1 (Nonlinear Dynamics, Newcastle, UK) and aligned automatically. Visual inspection showed that manual adjustment was not necessary. All peptide features with MS/MS data were selected for the quantitation and identification. Searches were done against the human part of SwissProt 2011-08-17 with separate isoform entries and concatenated with a reverse sequence database of equal size, totalling 71,324 entries, using Mascot version 2.3. The mass tolerance was set to 3 ppm for parent ions and 0.6 Da for fragment ions and one missed protease cleavage was allowed. Cys carbamidomethylation was set as fixed modification, and Met oxidation as variable. The FDR was set to <0.01.

2.9. Pathway analysis

Pathway analysis was carried out in Metacore™ (version 6.13 from Thomson Reuters, New York, USA) using both differential pathway enrichment and experimental comparison modes. A threshold of 1.2 up or down-fold regulation with an FDR filter of 0.05 was implemented.

2.10. Specific reaction monitoring

2.10.1. Sample preparation

The nucleoplasm and chromatin-associated protein extracts from each treatment were mixed and samples were run on an SDS–PAGE as previously described. Each sample was cut in three slices and reduced, alkylated and digested as previously described. Extracted peptides from the three slices were pooled together, sample volume was reduced in a Speedvac and the peptides were resuspended in 30 μL of 0.1% FA.

2.10.2. Design of SRM assay

SRM assays for 81 proteins using synthetic peptides. The synthetic peptides (JPT peptides, Berlin, Germany) were mixed and diluted in 5% ACN, 0.1% FA to 50 pmol/μL. The peptide mixture was analysed on a TSQ Quantum Vantage equipped

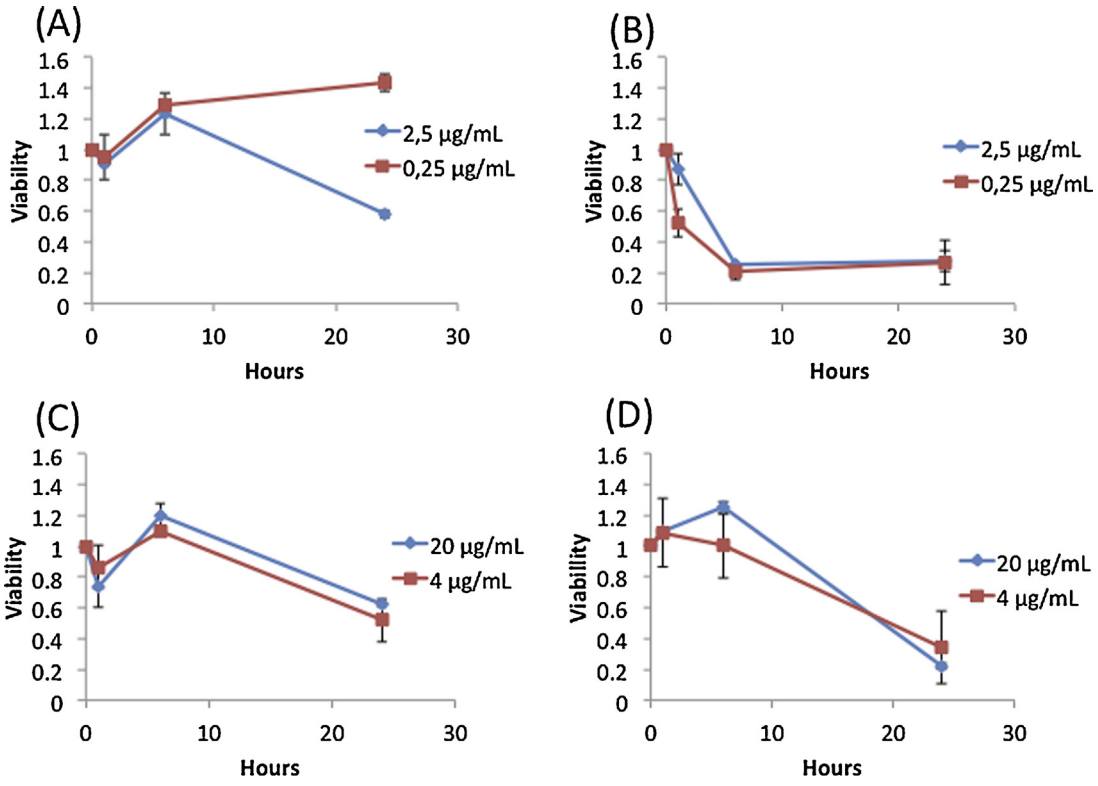

Fig. 1 – Viability of MDA-MB-231 and MCF-7 cells treated with DR or MMS, each at two different concentrations. The viability is calculated as a ratio of the mean compared to the mean of the control cells. (A) DR-treated MDA-MB-231 cells. (B) DR-treated MCF-7 cells. (C) MMS-treated MDA-MB-231 cells. (D) MMS-treated MCF-7 cells.

with a nanoelectrospray ion source (both from Thermo-Fisher Scientific, Waltham, MA). Chromatographic peptide separation was performed on an Eksigent NanoLC 1D plus LC system (Eksigent Technologies, Dublin, CA). Peptides were eluted by a linear gradient of 5–60% ACN in 0.1% FA over 60 min. The dwell time was set to 10 ms and the scan width to 0.01 *m/z*. A scheduled SRM transition list (Supplementary Table S1) was created by running the synthetic peptides in a 1:1 mixture with the samples from the treated cell lines. Samples were run in biological duplicates.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2015.05.002.

2.10.3. *SRM data analysis*

The SRM data was analysed using the Anubis software package version 1.2.1 [21]. The reference library was created from the runs of synthetic peptides. The *q*-value cut-off was set to 0.05; the null distribution to 100 and a limit of 6 transitions was used. Peak areas were normalised by total ion current of a duplicate run in MS-only mode. Protein intensities were obtained by averaging intensities from all peptides from the protein in question.

3. Results

In this study, we describe the proteomic response in a shotgun type study of two breast cancer cell lines to treatment with DR (double stranded breaks) or MMS (alkylation) and then focus on a pathway centric analysis of the DNA repair response based on SRM data.

3.1. *Cell viability after DR or MMS treatment*

The influence of DR and MMS on the viability of breast cancer cells was evaluated after 1, 6 and 24 h of treatment by Trypan blue counting. The percentage of viable cells compared to the control was calculated and is shown in Fig. 1. Both drugs have after 24 h of treatment a cytostatic effect on the two cell lines, with the exception of the lower concentration of DR in the MDA-MB-231, which shows a contrary response. In general, the MDA-MB-231 cell line shows a higher resistance to both of the drugs as compared to MCF-7, with approximately 60% viability compared to approximately 30% after 24 h of treatment.

3.2. *DNA damage assessment*

Since both of the two drugs (DR and MMS) act through the induction of DNA damage, we assessed the amount of DNA breaks in the cells using the COMET assay [22] as shown in Table 1. The percentage of DNA in the COMET tails is proportional to the amount of DNA breaks in the cells. A substantial amount of DNA damage can be detected already after 1 h in the MDA-MB-231 cell line, even though there is no effect on the cell viability. After 24 h of exposure to DR, the amount of DNA damage is lower compared to 1-h exposure, indicating

Table 1 – DR- and MMS-induced DNA damage in MDA-MB-231 and MCF-7 measured by the comet assay. %DNA was calculated as a mean ± standard deviation ($n = 30$).

Cell line	Drug	Time (h)	Dose	%DNA in tail ± SD
MDA-MB-231	Control	1	None	6.4 ± 3.9
	DR		Low	22.3 ± 10.6
			High	31.5 ± 7.1
	MMS		Low	5.1 ± 2.9
			High	17.2 ± 6.7
	Control	24	None	7.8 ± 6.4
	DR		Low	17.1 ± 10.3
			High	14.2 ± 15.6
	MMS		Low	29.1 ± 14.4
			High	16.4 ± 10.5
MCF-7	Control	1	None	3.1 ± 3.3
	DR		Low	8.9 ± 6.5
			High	27.8 ± 10.3
	MMS		Low	3.8 ± 4.0
			High	n/a[a]
	Control	24	None	15.2 ± 15.1
	DR		Low	51.5 ± 16.1
			High	43.7 ± 14.9
	MMS		Low	21.1 ± 14.6
			High	48.3 ± 20.3

[a] Missing value since the cells were too large to measure using this method. The low and high concentrations of DR correspond to 2.5 and 25 μg/mL, respectively, and for MMS to 4 and 20 μg/mL, respectively.

that the DNA damage machinery is efficiently repairing the double-strand breaks. The amount of strand breaks in the cells after 24 h of drug exposure is much higher in the MCF-7 cell line (44 and 52%) than in the MDA-MB-231 cells (14 and 17%).

3.3. *Label-free analysis of protein expression changes*

In order to study the proteomic response in the breast cancer cell lines after drug treatment, the same concentrations of DR and MMS as for the viability assay were used except for the DR concentration that was 10-fold higher for COMET assay. Cells were exposed to the drugs for either 1 or 24 h and the nucleoplasm and chromatin associated nuclear fractions were analysed by HPLC–MS/MS using label-free quantification. The MS runs were aligned and features were detected in Progenesis LC–MS software. From those features, a total of 8618 proteins were identified (Table 2) and 2752 were quantified. Those

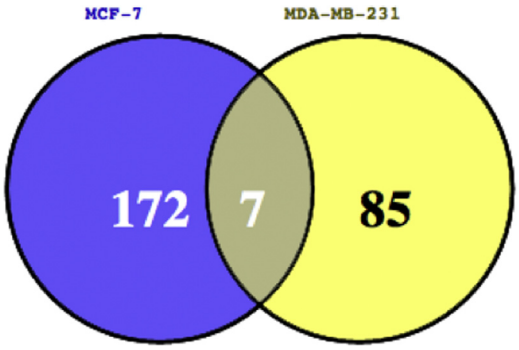

Fig. 2 – Venn diagram of the significantly differentially regulated proteins after MMS-treatment of MDA-MB-231 and MCF-7 cells.

proteins were representing a diverse set of pathways. Only 48% of the quantified proteins were shared between both the cell lines. Among the top 10 statistically significant pathway maps in MetaCore for both cell lines were cell cycle processes, apoptosis, cytoskeleton remodelling and translational events.

The number of proteins that were differentially regulated with DR or MMS treatment was assessed by Student's *t*-test. More proteins were significantly regulated after MMS-treatment than after DR-treatment (Table 2). No proteins that were significantly regulated (p-value < 0.05) after both MMS-treatment and DR-treatment were shared between the cell lines. Only seven differentially regulated proteins were shared between both cell lines as a result of MMS treatment, including aspartate aminotransferase, epiplakin, myosin-4, proteasome subunit alpha type-2, 60S ribosomal proteins and FACT complex subunit SP16 (Fig. 2).

There are substantial response differences between the cell lines and treatments. At 24 h MDA-MB-231 cells show a large up-regulation of oxidative phosphorylation whereas MMS treatment causes down regulation and up-regulation of cytokeratin remodelling and DNA damage checkpoints. MCF-7 does not show these effects and is down regulating cell adhesion via integrins and down regulation of tetraspanins like CD151 and CD82 that can act as migration suppressors. However differential pathway analysis does not show significant differences in DNA repair response although there is overall good coverage of the pathways, there is little overlap between the cell lines and treatments.

Table 2 – Summary of all proteins found and proteins differentially regulated following treatment with DR or MMS.

	MCF-7 chromatin		MCF-7 nucleoplasm		MDA-MB-231 chromatin		MDA-MB-231 nucleoplasm		Total
All proteins identified	4095		4844		4488		3959		8618
All proteins quantified	1352		1415		1377		1210		2752
DNA repair proteins quantified[a]	40		78		39		68		104
	DR	MMS	DR	MMS	DR	MMS	DR	MMS	
All regulated proteins ($p < 0.2$)	216	376	167	224	187	230	111	153	
All regulated proteins ($p < 0.05$)	25	126	23	53	37	56	18	36	

[a] DNA repair proteins, as defined by annotations in Uniprot.

Table 3 – Pathway coverage of the DNA repair pathways. Pathways are those defined by REACTOME and the pathway coverage is calculated from the number of quantified proteins from both breast cancer cell lines.

Pathway name	Proteins in pathway	Proteins in dataset	% Pathway coverage
Nucleotide excision repair	49	27	55
Double-strand break repair	20	10	50
DNA repair	108	43	39
DNA damage reversal	3	1	33
Base excision repair	19	6	31
Telomere maintenance	32	9	28
Fanconi anaemia pathway	25	5	20

3.4. *DNA repair pathway analysis of shotgun data*

The results from the viability studies and the COMET assay demonstrate a higher resistance to DR and MMS for the MDA-MB-231 cell line, which is indicative of a more efficient DNA repair system. The drugs were chosen to typify two different kinds of DNA damage, MMS being an alkylating reagent that introduces a methyl group primarily on N7-deoxyguanine and DR that inhibits topoisomerase II enzyme and thereby stalls the replication fork. Consequently, an up-regulation of different pathways in the DNA damage response depending on the type of damage induced was expected. However, in our label-free shotgun experiment we identify many DNA repair proteins that are responding, covering all of the main DNA repair pathways (Table 3). The values are given in Supplementary Table S2. Most proteins are identified in the nucleotide excision repair, double-strand break repair and base excision repair. When looking at the total intensities of the proteins found in the different pathways an up-regulation of those pathways and the DNA damage checkpoint proteins are seen for the MDA-MB-231 cell line, and to a lesser extent for the MCF-7 cell line (Table 4). It can also be seen that the intensity of the DNA repair proteins in the HR pathway goes up for DR-treated MDA cells, supporting the COMET assay findings that MDA appears to be able to repair the double-strand breaks.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2015.05.002.

3.5. *DNA repair pathway analysis of SRM data*

The result from the SRM analysis targeting of 75 DNA repair enzymes covering all the main pathways is much richer. A pathway-centric comparison of the regulation of each DNA repair pathway as a whole is given in Fig. 3 for MDA-MB-231 cells responding to MMS and DR and in Fig. 4 for the response of MCF-7 cells. The data for the individual proteins is given in Supplementary Table S3. The MDA-MB-231 cells shows the strongest response overall. Thirteen BER and 14 DDS enzymes are seen to be up-regulated in an almost identical manner at both DR treatment time and dosage points in line with the

Table 4 – DNA repair pathway intensities for MDA-MB-231 and MCF-7. Pathway intensity is calculated as the average between the treatments with DR and MMS of the sum of the normalised intensities from Progenesis for all the proteins quantified in each pathway. DNA repair pathway proteins are those defined by Repairtoire. DDS, DNA damage signalling; BER, base excision repair; NER, nucleotide excision repair; MRR, mismatch repair; HRR, homologous recombination repair; NHEJ, non-homologous recombination repair.

	DRR	BER	NER	MRR	HRR	NHEJ
MCF-7 chromatin fraction						
Proteins found	2	3	4	0	0	2
Control	733,752	358,075	487,067	–	–	124,834
DR-treated	623,224	273,464	477,280	–	–	152,819
MMS-treated	868,029	503,711	546,890	–	–	199,650
MCF-7 nucleoplasm fraction						
Proteins found	6	7	8	4	4	3
Control	185,331	1,296,803	754,281	52,973	808,021	972,581
DR-treated	174,031	1,313,898	607,942	72,405	1,000,469	1,391,589
MMS-treated	166,908	1,284,484	613,821	38,664	462,671	783,200
MDA-MB-231 chromatin fraction						
Proteins found	1	2	4	1	1	2
Control	256	340,752	243,268	128,868	128,868	647,357
DR-treated	9085	469,826	271,032	83,370	83,370	716,597
MMS-treated	19,067	596,883	213,906	67,154	67,154	1,044,625
MDA-MB-231 nucleoplasm fraction						
Proteins found	7	6	9	4	3	4
Control	1,644,246	10,117,828	1,155,448	13,045,216	200,593	10,796,633
DR-treated	2,319,296	8,640,002	2,394,286	2,505,779	391,986	11,088,164
MMS-treated	1,821,684	7,405,693	1,613,818	4,978,137	224,691	9,257,219

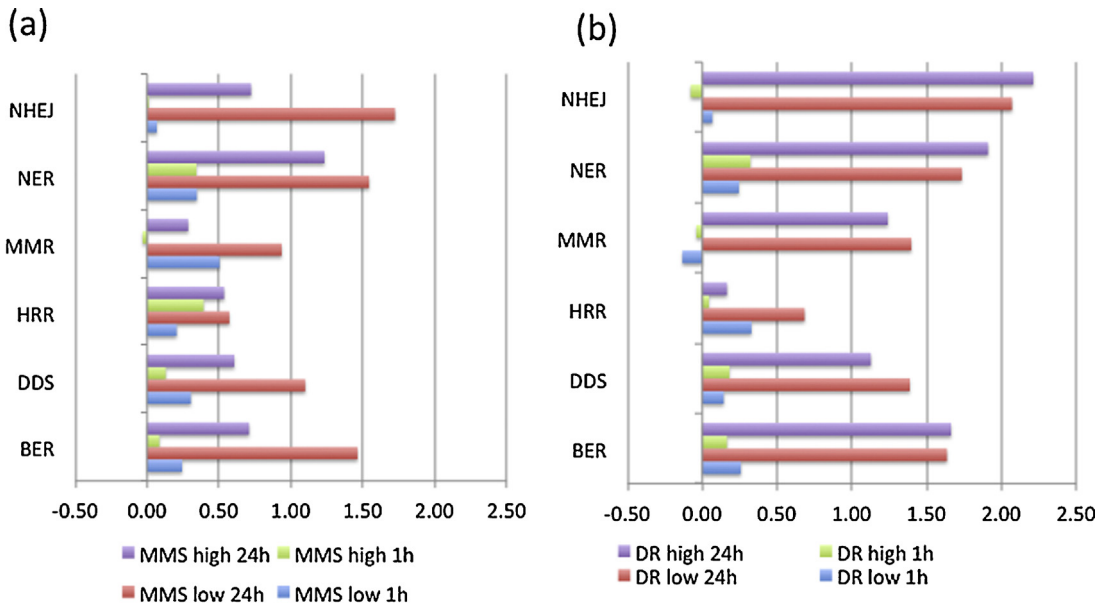

Fig. 3 – Pathway-centric comparison of DNA repair pathway responses by SRM of selected DNA repair proteins in MDA-MB-231 (same as for MCF-7). (A) Response to methyl methanesulphonate. (B) Response to doxorubicin. The response values are log ratios of the SRM response averaged over all peptides used for a protein and then over all proteins assayed in the respective pathway.

viability results shown in Fig. 1. All 16 NER enzymes are also up regulated in a dose independent manner, as are the NHEJ and MMR enzymes. Overall it is clear that the response to DR is found in all pathways in a time dependent fashion. The effect of MMS on the MDA-MB-231 cell line is much more variable with a clear time dependent response for BER, NER, HRR and DDS but with a weak dosage effect. The overall response is weaker and is reflected in both the viability and COMET responses.

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The DR response of MCF-7 cells is rather heterogeneous with only five BER enzymes showing up-regulation at 1 h and all pathways showing down regulation at 24 h. The magnitude of the response is dose dependent at 1 h but all are down at 24 h at high dose. There is virtually no response from the DDS, NER and NHEJ pathways at either time or dosage whereas MMR

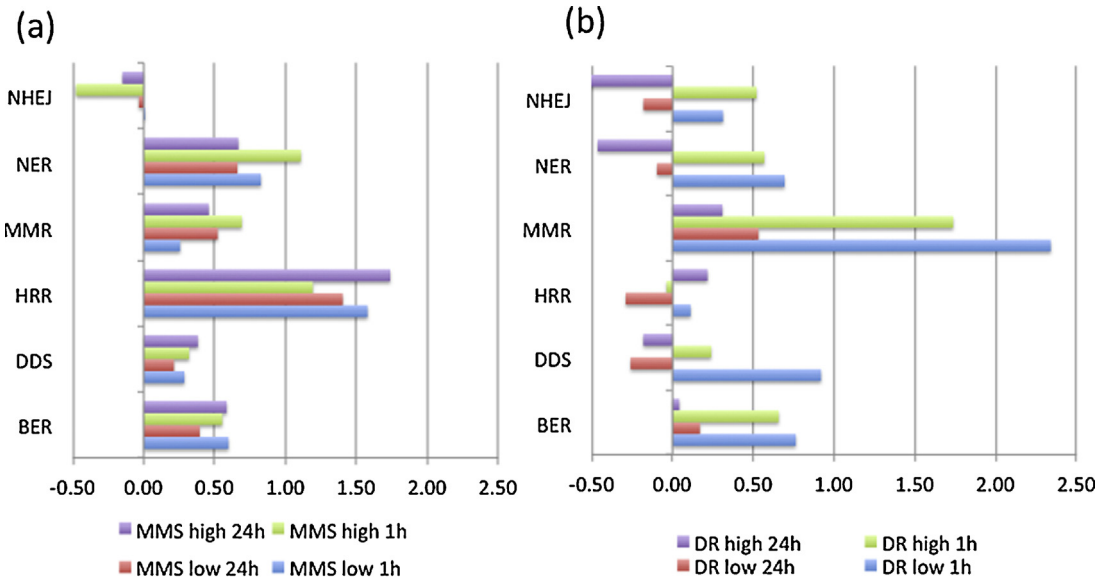

Fig. 4 – Pathway-centric comparison of DNA repair pathway responses by SRM of selected DNA repair proteins in MCF-7 cells. (A) Response to methyl methanesulphonate. (B) Response to doxorubicin.

shows an initial strong response followed by a large decrease. The response to MMS is also highly heterogeneous and generally rather weak. All the observations are in line with the greater sensitivity of the MCF-7 line to DNA damage as compared to MDA-MB-231.

4. Discussion

Defects in DNA repair systems are common in different types of cancers and may contribute to tumour development and therapy resistance. Aberrant DNA damage response has been correlated to lower survival rates for breast cancer patients [23] and it has been proposed that response to chemotherapy can be predicted by analysing DNA damage repair protein expression by immunohistochemistry [24]. We have investigated the response of two breast cancer cell lines, MCF-7 and MDA-MB-231, to chemotherapeutics. MCF-7 is a luminal-type, ER positive cell line while MDA-MB-231 is basal-like, ER negative and p53 is mutated. Cancer cells expressing only mutated p53 are associated with a decreased sensitivity to DNA damage inducing cancer therapies [25]. Higher doses of etoposide, cisplatin and nitrogen mustard were reported to be required for inhibition of cell survival in p53 mutant cells as compared to wild-type cells [26]. p53 has more specifically been shown to be important for the sensitivity to the drug epirubicin [27], which is derived from DR and has a similar mechanism of action [28]. The differences in the molecular subtype of the two breast cancer cell lines in our study can thus explain the higher viability and lower amount of DNA damage after drug treatment in MDA-MB-231 compared to MCF-7 (Fig. 1 and Table 1). Our results are in agreement with previous findings that MDA-MB-231 is more resistant than MCF-7 to DR and retain a higher viability after drug exposure. The heterogeneity in the drug response of the cell lines is also reflected in the low number of differentially expressed proteins in common between the cell lines. However, among the differentially expressed proteins in each cell line, several were reported in a previous study identifying 11 biomarkers of apoptosis following chemotherapy [29] (KRT18, EIF5 and several ribosome 60S proteins).

In contrast, at the proteomic level, the general pathways identified in our datasets were quite similar between the cell lines. A diverse set of pathways was identified, including processes involved in the cell cycle, apoptosis, cytoskeleton remodelling and translation.

To explain the decreased sensitivity at a pathway level we focused on the DNA repair processes by SRM since the drugs are known to induce different kinds of DNA damage. DR is acting by inhibiting topoisomerase II and stalling the replication fork with strand breaks as a consequence. There are reports that the drug concentration used can influence the mode of action of this drug and Gewirtz [18] reported protein-associated strand breaks (presumably from inhibition of topoisomerase II) at lower drug concentrations while non-protein-associated DNA strand cleavage was found as a consequence of free radicals induced at higher concentrations. In our experiment, topoisomerase II was expressed at lower concentration following DR treatment than MMS treatment supporting the topoisomerase II mode of action of DR. The fact that we only identified topoisomerase II in the MCF-7 cell line but not in MDA-MB-231 is in line with the hypothesis that DR-resistant cells have less of this protein. MMS is an alkylating agent adding a methyl group primarily on N7-deoxyguanine and should therefore trigger the base excision pathway for repair [19]. MMS has been reported as a potent inducer of BER in mouse [30]. Thus, we were expecting an increase in the DNA repair proteins associated with BER after MMS treatment. We observed, however, an up-regulation of proteins in several of the DNA damage pathways.

5. Conclusions

The DNA repair pathway response after chemotherapeutic treatment of breast cancer cell lines is not clearly indicating the type of damage induced/the drug used, indicating that a high degree of crosstalk between the pathways occurs. In order to understand the mechanisms of drug sensitivity and resistance, a deeper knowledge of the protein regulation in DNA repair pathways is desirable. A shotgun approach yielded a broad overview of the cellular processes taking place but to a large degree masked the changes in DNA repair pathway level changes occurring in response to DNA damage. SRM analysis could rectify this shortcoming. When overlap occurred between shotgun and targeted SRM there was good agreement on the protein level but on the peptide level some outliers were observed possible due to isoforms or post-translational modifications. We believe that these assays that we have developed cover enough of the DNA repair response that a prospective profiling of patients, before, during and after neoadjuvant chemo- or radiotherapy may be a useful tool to help quantify treatment response at early times before tumour shrinkage is visible.

Author contributions

L.A. and P.C. carried out the experiments, F.L. the MS data analysis and P.J. initiated and designed the study. The manuscript was written through contributions of all authors and all have given approval to the final version of the manuscript.

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Competing interests statement

The authors declare that they have no competing financial interests.

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