Published in final edited form as:

Metabolomics. 2015 December ; 11(6): 1779–1791. doi:10.1007/s11306-015-0831-6.

Metabolic responses induced by DNA damage and poly (ADP-ribose) polymerase (PARP) inhibition in MCF-7 cells

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Abstract

Genomic instability is one of the hallmarks of cancer. Several chemotherapeutic drugs and radiotherapy induce DNA damage to prevent cancer cell replication. Cells in turn activate different DNA damage response (DDR) pathways to either repair the damage or induce cell death. These DDR pathways also elicit metabolic alterations which can play a significant role in the proper functioning of the cells. The understanding of these metabolic effects resulting from different types of DNA damage and repair mechanisms is currently lacking. In this study, we used NMR metabolomics to identify metabolic pathways which are altered in response to different DNA damaging agents. By comparing the metabolic responses in MCF-7 cells, we identified the activation of poly (ADP-ribose) polymerase (PARP) in methyl methanesulfonate (MMS)-induced DNA damage. PARP activation led to a significant depletion of NAD⁺. PARP inhibition using veliparib (ABT-888) was able to successfully restore the NAD⁺ levels in MMS-treated cells. In addition, double strand break induction by MMS and veliparib exhibited similar metabolic responses as zeocin, suggesting an application of metabolomics to classify the types of DNA damage responses. This prediction was validated by studying the metabolic responses elicited by radiation. Our findings indicate that cancer cell metabolic responses depend on the type of DNA damage responses and can also be used to classify the type of DNA damage.

Keywords

DNA damage; PARP; metabolomics; NMR; MCF-7

1. Introduction

DNA damaging agents have been extensively used in anti-cancer therapies (Lord and Ashworth 2012). DNA damage can occur in the form of single strand breaks (SSBs), double strand breaks (DSBs), base mismatch, and DNA modifications by external molecules like alkylating agents or cross-linking agents (Friedberg 2003; Lord and Ashworth 2012). Some types of DNA damage, including double strand breaks (DSBs), are more difficult to repair

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Conflict of Interest

Authors declare no competing financial interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

as compared to single strand breaks (SSBs) (Friedberg 2003; Lord and Ashworth 2012). Cells have evolved several mechanisms, termed DNA damage responses (DDR), which aim to repair the damage or induce cell death in cases of irreparable damage (Surova and Zhivotovsky 2013). Cancer cells use these DDRs to overcome genomic instability by upregulating the activity of DNA repair proteins and/or suppressing activity of tumor suppressor proteins to evade apoptosis (or programmed cell death) (Hanahan and Weinberg 2011). Heightened DNA repair activity can induce resistance against the anti-cancer therapies targeting DNA damage in cancer cells. There are several other diverse factors which may affect the tumor response to any therapy such as intra-tumor heterogeneity (Gerlinder et al. 2012), existence of resistant cancer stem cells (Bao et al. 2006), enhanced DNA repair capacity (Zeng-rong et al. 1995), and metabolic reprogramming (DeBerardinis et al. 2008; Zhao et al. 2013).

Effectiveness of a DNA damaging agent as a cancer therapeutic depends in part on the ability of the cells to repair the damage. Single strand breaks (SSBs) are repaired predominantly by base excision repair (BER) while double strand breaks (DSBs) are repaired either by homologous recombination (HR) or non-homologous end joining (NHEJ) (Supplemental Fig. 1, Online Resource 2). Nucleotide excision repair (NER) is used to repair multiple types of DNA damage, including UV-induced thymine dimers and chemical adducts (Lord and Ashworth 2012).

These repair mechanisms involve recruitment of several proteins to the damage site followed by localization of DNA repair machinery and activation of cell-cycle arrest programs, depending on the type and extent of DNA damage (Surova and Zhivotovsky 2013). DNA repair processes can induce extensive metabolic changes in cells, including decreased purine synthesis, enhanced protein synthesis and redistribution of fluxes across numerous metabolic pathways. Several proteins involved in DDR, including tumor suppressor p53 (Kastan et al. 1991), and poly (ADP-ribose) polymerase-1 (PARP-1) (Bryant et al. 2005; Curtin 2012; Farmer et al. 2005), also play important roles in regulation of metabolic pathways such as the tricarboxylic acid (TCA) cycle, glycolysis and the pentose phosphate pathway (Bai and Cantó 2012; Vousden and Ryan 2009). PARP-1, for example, regulates DNA repair and cell death (Curtin 2012). This enzyme undergoes auto-polymerization in addition to poly (ADP ribosylation) of other proteins, using NAD^+ as its substrate, at the DNA damage site and recruits other proteins which are necessary for DNA repair (Curtin 2012). As NAD^+ is a major cofactor for several reactions and also a major substrate of NAD^+ dependent deacetylases (Sirtuins) (Bao et al. 2006), PARP activation leads to depletion of the NAD^+ pool and affects several metabolic pathways. Beyond the critical role of PARP in DNA repair, there are several DNA independent mechanisms by which PARP activation affects cell survival and signaling pathways (Rouleau et al. 2010; Weaver and Yang 2013) whose effects can be observed on cancer metabolism. Understanding these specific interactions between different DDR pathways and their impact on cancer metabolism may aid in the discovery of new drug targets that enhance the effectiveness of chemotherapeutics and/or radiotherapy (Farmer et al. 2005).

Metabolism in cancer, due to reprogramming of metabolic pathways and genetic mutations, is different from metabolism in normal cells and hence, can be used to selectively target

cancer cells (Dang et al. 2009; DeBerardinis et al. 2008; Figueroa et al. 2010; Vander Heiden et al. 2009; Warburg 1956; Xu et al. 2011; Zhao et al. 2013). One of the earliest metabolic differences observed between cancer cells and normal cells was the increased utilization of glucose for the production of lactate via aerobic glycolysis, also known as the Warburg effect (Warburg 1956). Increased glycolytic flux also generates precursors for biosynthetic pathways including nucleotide, amino acids and lipid synthesis which further help in growth and proliferation of cancer cells (Vander Heiden et al. 2009). Since cancer cells undergo extensive reprogramming for survival and proliferation (DeBerardinis et al. 2008; Zhao et al. 2013), maintaining DNA integrity for cell survival is one of the hallmarks of cancer (Hanahan and Weinberg 2011).

Metabolomics has been extensively used in identifying biomarkers and drug targets, and studying metabolism in different types of cancers (Armitage and Barbas 2014; Dang et al. 2009; Jain et al. 2012; Labuschagne et al. 2014). Several recent studies have used metabolomics to assess the effects of drugs on cancer cells (Bayet-Robert and Morvan 2013; Duarte et al. 2013; Lefort et al. 2014; Morvan and Demidem 2007). In this study, we employed a NMR-based targeted profiling approach to identify metabolic changes associated with different types of DDRs. We used MCF-7, a non-aggressive, estrogen receptor (ER) positive mammary adenocarcinoma cell line, one of the most widely used model cell line in breast cancer studies, because it exhibits all of the components of different DDRs. We observed numerous metabolite differences resulting from DDRs activated by different types of DNA damaging agents and identified features which separate different types of DDRs. Several metabolic pathways including glycolysis, the TCA cycle, protein synthesis, nucleotide synthesis, glutathione metabolism and the hexosamine biosynthesis pathway (HBP) were found to be differentially enriched depending on the type of DNA damaging agent.

We further assessed the metabolic responses of MCF-7 cells upon PARP-1 inhibition, both as a single agent and in combination with DNA damaging agents. We observed that PARP inhibition in combination with an alkylating agent was able to restore NAD^+ levels depleted by the alkylating agent. In addition, the combination of PARP inhibition and an alkylating agent also elicited a similar subset of metabolic responses as zeocin treated cells. PARP inhibition in combination with alkylating agents has been shown to induce double strand breaks (Heacock et al. 2010). Both zeocin and this combination treatment induce double strand breaks suggesting an application of metabolomics to identify signatures of different DDRs in cancer cells. To further validate this hypothesis, we studied the response of radiation treatment on MCF7 cells. Radiation treatment also lead to a similar changes in the subset of metabolites which further supported our hypothesis. Together, these results provide insight into metabolic responses induced by distinct classes of DNA damaging agents and illustrate the value of metabolomics in classifying and potentially characterizing cancer cell DDRs.

2. Materials and Methods

2.1. Cell culture and anticancer drugs

MCF-7 cells were obtained from ATCC and cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (P/S, Invitrogen) in T-75 flasks until they reached sub-confluence (~90% or ~10 million cells). Cells were treated with DNA damaging agents: cisplatin (Biotang Inc.), methylmethane sulfonate (Acros Organics), zeocin (Invivogen) or a phosphate buffered saline (PBS, Invitrogen) control for 24 hr (or 1 hr in the case of MMS followed by 23 hr of recovery due to rapid DNA damage induction in the case of MMS due to a higher rate of interaction with the DNA as compared to other drugs) before harvesting. Cells were treated with the PARP inhibitor veliparib (ABT-888) (Selleckchem) or PBS containing DMSO (1 µg/ml) 24 hr prior to other drug treatments. Stock solution for all drugs, except for veliparib which was in DMSO, were prepared in PBS. All experiments were performed in three independent, biological replicates.

2.2. Radiation treatment

To identify appropriate dosage of radiation treatment, MCF-7 cells were seeded in 6 well plates and after reaching subconfluency cells were irradiated with ^{137}Cs source (J. L. Sheppard Mark I unit) at a dose rate of 221 rad/min with a total dose of 4, 8 or 12 Gy and the cell growth over the next two days was calculated using hemocytometer (Supplemental fig. 2, Online Resource 2). 8 Gy treatment for 48 hours led to a ~80% survival and the samples were collected for NMR analysis.

2.3. NMR sample preparation

The cells were quenched using 3 ml methanol using the protocol described by Teng et al. (Teng et al. 2008). Briefly, the culture medium was aspirated and the cells were immediately rinsed twice with ice-cold PBS (pH 7.4). The residual PBS was removed using vacuum aspiration and the cells were quenched using 3 ml HPLC grade methanol (Sigma-Aldrich). The cells were then detached using a cell scraper and pipetted into 50 ml centrifuge tube. A dual phase extraction procedure adopted from Martineau et al. (Martineau et al. 2011) was used to extract the intracellular metabolites. Briefly, the quenched cells suspended in a mixture of chloroform, methanol and water in the ratio of 6:6:5.4 to make the final volume of 17.4 ml. The aqueous layer (top) contains the water-soluble low-molecular weight metabolites while the organic layer (bottom) contains non-polar metabolites. Proteins and other macromolecules are precipitated by addition of methanol and chloroform and trapped between the aqueous and organic layer. After centrifugation for 15 min at 3600 rpm, only the upper aqueous layer was used for further analysis. The samples were dried at 30°C using a centrifugal vacuum concentrator and stored in -80°C until NMR analysis.

Prior to NMR analysis, the samples were reconstituted in 0.6 ml of 0.1M phosphate buffered D₂O (pH=7.0) solution containing 0.5 mM 3-trimethylsilyl-propionate-2, 2, 3, 3, -d₄ (TMSP, δ =0.0 ppm) as an internal standard. The samples were centrifuged at 18,000g for 10 min and 550 µL of the supernatant was transferred to 5 mm NMR tubes (Norell Inc.).

2.4. NMR acquisition

¹H NMR spectra were recorded at 298 K on Bruker Avance III equipped with a 5 mm cryogenic probe operating at 500 MHz (11.74 T). 1D spectra were acquired using standard NOESYPR1D pulse sequence (RD-90°-t1-90°-tm-90°-acquire) with a relaxation delay of 1 s, a mixing time of 100 ms and a pre-scan delay of 30 μs. Each spectrum consisted of 128 transients or free induction decays (FIDs) collected into 48 K complex data points with a spectral width of 12 ppm and an acquisition time of 4 s. Prior to Fourier transformation, the FIDs were zero-filled to 128k data points and multiplied by an exponential window function (LB=0.5Hz). The chemical shifts were referenced to the TMSP peak (δ=0 ppm), using TopSpin™ software (version 3.1, Bruker).

2.5. Data processing

Spectra were exported to ACD/1D NMR Processor (Advanced Chemistry Development) for phasing, baseline correction and solvent region removal (water: 4.7–5.1 ppm and DMSO: 2.72–2.75 ppm). The peaks were annotated through the HMDB (Wishart et al. 2013) and Metabohunter (Tulpan et al. 2011) and the targeted profiling (Weljie et al. 2006) was accomplished using ChenomX NMR Suite Profiler (version 7.7, ChenomX Inc.) and the concentrations were referenced to a TMSP concentration of 0.5 mM. Over 95% of the peaks were assigned to metabolites available in the ChenomX library and the quantified metabolite concentrations were exported to an Excel file and the metabolites which were found to be unique to a certain treatment method are discussed in detail in the results, but were excluded from the statistical analysis as principal component analysis (PCA) is sensitive to outliers and these metabolites could potentially bias the clustering. Metabolites with high sample to sample variation (within replicates) which had a coefficient of variance higher than 0.3 were also excluded from the analysis. These included citrate, formate, guanidoacetate, isobutyrate, malate, proline, acetate and glucose, majority of which were mainly low confidence metabolites generally with a very low concentration and in the regions with high overlap of peaks. The concentration data matrix (excluding unique metabolites) was organized such that each row represented a biological sample and columns represented the metabolites. The metabolite concentrations from each biological sample were further normalized by the total concentration of that sample, which was determined by summing all non-unique metabolite concentrations to account for the differences in cell count and efficiencies of extraction. This method of row normalization showed least variance among the biological replicates when compared to probabilistic quotient normalization (PQN) (Dieterle et al. 2006) or total spectral area based normalization, and was preferred over spectral area based normalization due to the presence of unique metabolites and high variability of glucose concentration within the replicates. The data was scaled using an auto scaling method (mean centering followed by dividing by the standard deviation) prior to statistical analysis. All the raw data and NMR spectra are available on the MetaboLights database (Haug et al. 2013).

2.6. Statistical Analysis

The concentration data table was processed for statistical analysis using either MetaboAnalyst 2.0(Xia et al. 2012) or PLS toolbox (Eigenvector Technologies).

Unsupervised pattern recognition was performed using principal component analysis (PCA) to identify patterns in the data and to study the similarities and differences among the different treatment conditions by reducing the dimensionality of the data to fewer dimensions, principal components. Hierarchical clustering was performed with Pearson's correlation as the similarity measure and Ward's linkage as the clustering algorithm. Student's t-test was performed to identify significant differences between the two classes with a critical $\alpha=0.05$. One way Analysis of variance (ANOVA) was used with Tukey's HSD (honest significant difference) test for multiple hypothesis testing to identify significant features when comparing multiple conditions ($FDR<0.05$). Pathway analysis module was used to perform Pathway topology analysis on all the annotated metabolites using relative betweenness centrality measure to assess metabolite importance and global test to identify enriched pathways between two classes, adjusted for multiple testings since we are comparing multiple pathways. The *Homo Sapiens* pathway library was used for the analysis and the metabolic pathways with a) more than one hit, b) non-zero impact score and b) $FDR < 0.05$ were considered to be significantly enriched in our analysis. The raw data of the pathway analysis are available in online resource 1.

2.7. PARP activity assay

Activity of poly (ADP-ribose) polymerase was measured using the Universal Chemiluminescent PARP assay Kit (Trevigen Inc.) according to manufacturer's instructions. Cells were grown in 6 well plates with medium alone or medium containing different concentrations of ABT-888 for 24 hr. Cells were then washed twice with PBS and trypsinized to detach from the surface. Medium was added to the cells after detachment and centrifuged to form cell pellets. Medium was aspirated and the cells were rinsed with PBS and centrifuged again. The cell pellet was lysed on ice for 15 min with occasional vortexing in ice cold MPER reagent containing Complete Protease inhibitor (Roche) and 150 mM NaCl. The lysates were centrifuged at 18000g at 4°C for 10 minutes and stored at -80°C until further analysis. Total protein content was calculated using Bradford protein dye reagent (Bio-Rad, Pierce) and the activity of PARP enzyme normalized to the total protein content.

2.8. Neutral comet assay

Cells were treated with cisplatin or zeocin for 6 hr or MMS for 1 hr. ABT-888 was added 1 hr prior to the treatment with either cisplatin or MMS. PBS with 1 μ l/ml of DMSO was used as control. Cells were trypsinized and 10^5 cells/ml in PBS were processed for double strand break formation using the Trevigen's CometAssay® kit (Catalog No. 4250-050-K) and imaged using a Zeiss fluorescence microscope. 150 random comets from 3 biological replicates were analyzed using CASP (Ko et al. 2003).

3. Results and Discussion

In this study we used NMR metabolomics to identify the metabolic responses induced by different types of DNA repair mechanisms triggered by different classes of DNA damage. We employed DNA damaging agents at sub-lethal concentrations, doses corresponding to ~80% cell viability, to trigger DNA repair. We chose cisplatin (a DNA cross-linking agent),

methyl methanesulfonate (MMS) (an alkylating agent) and zeocin (a radiomimetic drug) for our study.

A targeted profiling approach (Weljie et al. 2006) was used to quantify intracellular metabolite concentrations. This approach has several advantages over conventional spectral binning as it is relatively insensitive to changes in pH or water suppression and baseline distortions. Although spectral binning provides an easy and rapid tool to identify biomarkers; subtle changes in peak positions or line width differences can affect the analysis. In targeted profiling, a library of NMR spectra of pure compounds is used to identify and quantify the metabolite concentrations in the NMR spectrum. The TMSP peak was used as a reference signal to determine the concentrations of individual metabolites. For profiling, ChenomX Profiler module was used to superimpose each pure spectrum over the NMR spectrum of each sample. Metabolite identifiers for the metabolites used in this study and the raw concentrations of each metabolite are provided as an Excel spreadsheet in the Supplementary Table (Online Resource 1).

The concentration data were subjected to principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA) and other statistical analysis tools including hierarchical clustering, t-tests, analysis of variance (ANOVA) and pathway topological analysis using MetaboAnalyst 2.0 (Xia et al. 2012) and PLS toolbox. PCA was used to identify patterns in the data by reducing the number of dimensions and highlighting similarities and differences among the samples. PLS-DA was used to further sharpen the separation between the groups. Both PCA (Fig. 1a) and PLS-DA ($R^2Y=99.8\%$ and $Q^2=78\%$, supplementary Fig. 3, Online Resource 2) showed distinct clustering patterns between samples treated with different DNA damaging agents. This was also evident from hierarchical clustering using Pearson's distance measure and Ward's clustering (Fig. 1b). Cisplatin-treated samples clustered closer to MMS-treated samples and zeocin-treated samples clustered closer to control samples in the hierarchical clustering (Fig. 1b).

3.1. Metabolomics of different DNA damaging agents reveals numerous metabolic changes

The significant metabolic changes due to various DNA damaging agents identified by one way ANOVA with Tukey's HSD test as a post hoc method are shown in Table 1 and Fig. 1b. MMS-treated samples exhibited the most extensive metabolic responses as compared to the control samples. Several essential amino acids including phenylalanine, methionine and valine, and other amino acid derivatives such as creatine and glutathione were significantly enriched while NAD^+ and ATP were significantly depleted in MMS-treated samples as compared to the control samples (Fig. 1b and Table 1). There was a significant increase in serine concentrations in both cisplatin and MMS-treated samples as compared to the control (Table 1). Phosphocreatine levels were significantly increased as compared to control in both zeocin and cisplatin treated-samples. We performed pathway topological analysis using MetaboAnalyst 2.0, shown in Figure 2, to highlight the metabolic pathways which were enriched due to different DNA damaging agents. The pathways with more than one hit, a non-zero impact score and a $FDR < 0.05$ were considered to be significantly enriched. The pathways which were common to at least two DNA damaging conditions are indicated at the top followed by the pathways which were significantly enriched only in one of the

conditions. Glutathione metabolism was found to be enriched in all the three DNA damaging conditions.

3.2. Metabolic profile induced by cisplatin triggered DNA damage responses

We observed significantly decreased concentrations of glycine and increased concentrations of serine in cisplatin-treated cells as compared to control cells. Glycine and serine metabolism play crucial roles in cancer including purine synthesis necessary for proliferation and maintaining low levels of reactive oxygen species (ROS) (Amelio et al. 2014). A recent study showed that serine supports nucleotide synthesis by feeding into the folate metabolic pathway (Labuschagne et al. 2014). Also, UDP-GlycNac concentration was significantly increased following cisplatin treatment. UDP-GlycNac acts as an important donor molecule for glycosylation of proteins and lipids, a modification which is significant in a number of biological processes and diseases, including cancer (Ohtsubo and Marth 2006). UDP-GlycNac is synthesized via HBP which also plays an important role in pyrimidine synthesis (Huang and Graves 2003). UDP-GlycNac and other nucleotide sugars have been reported as early markers of cisplatin treatment in brain tumor cells, but concentrations of these sugars remain unchanged in cisplatin-resistant cells (Pan et al. 2011). A metabolomics study on lung cancer cells also suggested UDP-GlycNac as a potential marker for cisplatin treatment response (Duarte et al. 2013). Our results further extend their result of accumulation of UDP-GlycNac due to cisplatin treatment in MCF-7 cells suggesting that this observed effect could be a biomarker for cisplatin treatment across different types of cancer cells.

Pathway topological analysis showed that pathways associated with nucleotide synthesis (e.g. purine metabolism), methane (or one carbon) metabolism and amino sugar and nucleotide sugar metabolism were enriched due to cisplatin treatment in MCF-7 cells (Fig. 2).

3.3. Zeocin-induced DNA damage leads to accumulation of osmolytes and a reduction in several essential amino acids

Myo-inositol and sorbitol concentrations were significantly increased in zeocin-treated samples as compared to control cells. Myo-inositol plays an important role in cellular signal transduction and also acts as an osmoregulator (Holub 1986). Accumulation of sorbitol, which is a precursor of fructose and also an osmoregulator (Burg and Kador 1988), was observed exclusively in zeocin-treated samples (Supplemental Fig. 4, Online Resource 2). Together, these results suggest an increased osmotic stress induced as a result of zeocin treatment in MCF-7 cells. Several essential amino acids including phenylalanine, valine, methionine, isoleucine and leucine were significantly depleted in zeocin-treated samples as compared to control cells. Other changes due to zeocin treatment involved elevated levels of glutamine and correspondingly, depleted levels of glutamate concentration as compared to control. Pathway topological analysis illustrated several pathways associated with amino acid metabolism to be enriched (Fig. 2). In addition to these pathways, several other pathways including the citric acid cycle and inositol phosphate metabolism were also identified to be enriched. Overall, zeocin, which predominantly induces double strand

breaks, led to accumulation of osmolytes (myo-inositol and sorbitol) and reduction in the essential amino acids pool in MCF-7 cells.

3.4. MMS-induced DNA damage leads to PARP activation and extensive metabolic changes

DNA repair following MMS-mediated methylation is proposed to occur via base excision repair and single strand break repair mechanisms (Friedberg 2003; Lord and Ashworth 2012). Our study provides evidence for the first few steps in DDR induced by methylation. First, MMS predominantly methylates the N7-deoxyguanosine which is removed by BER. Methylguanidine, a methylated derivative of guanosine, accumulated only in MMS-treated samples (Supplemental Fig. 5, Online Resource 2). This leaves an apurine site in the DNA, causing a single strand break which is recognized predominantly by PARP. PARP undergoes poly (ADP ribosylation) using NAD^+ as a substrate, leading to a significant depletion of NAD^+ in the cells. NAD^+ depletion reduces the flux through glycolysis and also reduces the activity of NAD^+ dependent acetylases (e.g. Sirtuin 1 or SIRT1) which control the energy homeostasis of the cells (Canto and Auwerx 2012), further reducing the cellular ATP levels. Indeed NAD^+ and ATP were significantly depleted in MMS-treated samples as compared to control cells (Table 1). Intracellular NAD^+ levels also reflect the energy levels of the cells which are sensed by SIRT1, a NAD^+ -dependent deacetylase, and correspondingly adapt the cellular energy output to match the energy requirements (Pan et al. 2011). NAD^+ depletion may therefore affect metabolic regulation by SIRT1 and affect several processes including lipid anabolism, mitochondrial respiration and adaptation to oxidative and osmotic stress (Canto and Auwerx 2012). Pyruvate and lactate concentrations were also significantly depleted while fumarate was significantly accumulated in response to MMS treatment as compared to control.

Creatine concentration was significantly increased in MMS-treated cells as compared to control cells suggesting an increased shuttling of ATP by creatine kinase, most likely to delay ATP depletion (Bessman and Carpenter 1985). Phosphocreatine concentration, however, was unchanged. Creatine accumulation can also be a result of increased creatine synthesis. Other metabolic changes exclusive to MMS treatment included significant increased levels of glutathione and significantly lower levels of glutamine as compared to control. Depletion of glutamine concentration is consistent with an increased utilization of glutamine as a carbon source for energy production or increased glutathione synthesis by conversion to glutamate, a precursor for glutathione synthesis (DeBerardinis et al. 2008; Zhao et al. 2013). Accumulation of glutathione in MMS-treated samples could be attributed to either increased glutathione synthesis or increased anti-oxidation capacity due to recycling. Activation of the Ataxia telangiectasia mutated (ATM) pathway during DNA repair can upregulate the activity of glucose-6-phosphate dehydrogenase (G6PDH), generating NADPH and providing increased antioxidation capacity (Bryant and Helleday 2006).

Several amino acids, including phenylalanine, serine, valine, and methionine, were also found to be enriched in MMS-treated cells. Correspondingly several pathways related to amino acid metabolism were found to be enriched in MMS-treated MCF7 cells. Other pathways including nicotinate and nicotinamide metabolism, pyruvate metabolism and

glycolysis were also found to be enriched exclusively in MMS-treated MCF7 cells. Overall, these results indicate a widespread impact on central metabolism and redox status of cells due to MMS treatment. We hypothesize that most of the energy-associated metabolic changes including NAD^+ and ATP depletion could be attributed to PARP activation.

3.5. Effect of PARP inhibition on MCF-7 cells as a single agent and in combination with other DNA damaging agents

NAD^+ concentration significantly decreased in MMS-treated samples as compared to control (Fig. 1b). Depletion of NAD^+ levels has been associated with PARP activation and thus, we next tested whether Veliparib (ABT-888 or for PARP 1 is 5.2 nM, for PARP2 is 2.9 nM), could restore NAD^+ PI, a potent PARP1 and PARP2 inhibitor (IC_{50} levels in the cells when treated in combination with MMS. We observed a 70% inhibition in PARP activity in MCF-7 cells after 24 hours of treatment with 50 μM ABT-888 (Supplemental Fig. 6, Online Resource 2). We compared the metabolic response of MMS-treated samples in the presence and absence of PARP inhibition. Also, to show that PARP inhibition in combination with other DNA damaging agents which do not necessarily activate PARP may not affect the original metabolic response, we assessed the metabolic response of cells treated with cisplatin in combination with the PARP inhibitor. Cisplatin was chosen to compare against MMS since cisplatin induced a metabolic response which had some similarities with the MMS-induced response, based on hierarchical clustering (Fig. 1b), and the NAD^+ concentration in cisplatin treated cells was similar to that in control cells, suggesting that PARP may not be recruited at the DNA damage sites in cisplatin treated cells.

Fig. 3 shows the results from PCA and hierarchical clustering of metabolic responses showing the effect of PI on MMS-induced metabolic responses. The first principal component was able to successfully distinguish between MMS and MMS+PI-induced metabolic responses (Fig. 3a). It can also be seen from Fig. 3b that PI leads to significant changes in the metabolic response induced by MMS treatment in MCF-7 cells. On the other hand, it was observed that PI doesn't significantly impact the metabolic responses induced by cisplatin treatment (Supplemental Figure 7, Online Resource 2). These results support the involvement of PARP in MMS induced DDR and therefore, we discuss the metabolic responses of PI and combination of PI and MMS in detail in the following sections.

3.6. PARP inhibition restored the NAD^+ levels in the MMS-treated samples

Depletion in ATP and NAD^+ concentrations in MMS-treated samples was reversed by PARP inhibition (Fig. 3b, Supplemental fig. 8a, Online Resource 2). While the creatine concentration was significantly increased in MMS-treated samples as compared to control, its concentration decreased significantly upon PARP inhibition in combination with MMS (MMS+PI) as compared to MMS treatment alone. Correspondingly, phosphocreatine levels were significantly increased due to MMS+PI treatment, suggesting an increased conversion of creatine to phosphocreatine in MMS+PI treated cells. Fig. 4 shows the loading plots from the PCA. Loadings 1 indicates the metabolites affected due to PI in combination with MMS as we observed that the 1st PC could separate these two classes. Loadings 2 indicates the metabolites affected due to MMS treatment in MCF-7 cells.

There was no significant effect of PARP inhibition on several other metabolites including glutamine and glutathione (Fig. 3b). Increased glutathione synthesis or recycle could lead to the glutathione accumulation observed after MMS treatment. These metabolic responses induced by MMS are more likely independent of PARP activation and hence were not affected by PARP inhibition.

3.7. PARP inhibition increases osmolyte levels

PARP inhibition, both as a single agent and in combination, led to a significant increase in several osmolytes including taurine, myo-inositol, and GPC (Supplemental Fig. 8b, Online Resource 2). Other metabolites which were also significantly affected due to PARP inhibition relative to control included glutamine, alanine, phosphocreatine and various metabolites in the glycolytic pathway, such as pyruvate, lactate, fumarate and NAD^+ . NAD^+ levels were significantly increased in PARP inhibitor-treated samples and lactate concentration was significantly decreased as compared to control. Overall, these results suggest that PARP inhibition can induce changes in osmolyte (myo-inositol, taurine and GPC) concentrations and also affect the NAD^+ levels in the cells.

3.8. PARP inhibition in combination with MMS elicits a similar metabolic response to that induced by zeocin

Some metabolic responses were found to be exclusive to MMS treatment in the presence of PI. These included a significant decrease in concentrations of amino acids including alanine, glycine, phenylalanine, threonine, methionine and valine as compared to control (Fig. 4a and Supplementary Fig. 8b, Online Resource 2). Also, there was significant accumulation of several osmolytes including GPC, myo-inositol and taurine in MMS+PI as compared to control. These metabolic responses are similar to the metabolic response observed in zeocin-treated samples. PARP inhibition in combination with alkylating agents has been shown to induce double strand break formation by stalling replication forks (Heacock et al. 2010). Using the neutral comet assay, we also found a similar extent of double strand break formation in these two conditions (Supplementary Figure 9, Online Resource 2). Both zeocin and MMS+PI are, therefore, expected to activate similar DDRs. We predicted that one of the possible reasons for the similarity of a subset of metabolic responses induced by zeocin and MMS+PI treatment could be due to the similarity in the type of DDRs. To test the hypothesis that these metabolic responses are specific to DDRs activated by double strand break, we studied the metabolic responses due to radiation treatment in MCF-7 cells. Several amino acids including alanine, glycine, phenylalanine, threonine, valine, and lysine were significantly depleted in radiation treated cells (Supplementary Figure 10, Online Resource 2; and Supplementary Table, Online Resource 1). Also, taurine and phosphocholine were significantly enriched due to radiation treatment. These responses share high similarity with MMS+PI treatment. All the amino acids depleted in radiation treatment, except for lysine, were also significantly depleted in MMS+PI treatment. At least six amino acids were found to be significantly depleted in zeocin, MMS+PI and radiation and different osmolytes were found to be significantly enriched in these treatment conditions. This further supports our hypothesis these metabolic responses are specific to DDRs activated by double strand break inducing agents.

4. Concluding Remarks

Pathway topological analysis identified several metabolic pathways which were uniquely responsive to different DNA damaging agents. Several other metabolic responses were found to be common in cells exposed to different DNA damaging agents. These pathways (e.g., glutathione metabolism) provide potential drug targets to modulate cell responses to or enhance the efficacy of DNA damaging agents including radiation and chemotherapeutics. Also, we have shown that PI in combination with MMS can restore NAD⁺ levels in the cells and identified the metabolites which were not affected due to PARP activation in MMS-treated MCF-7 cells. This information is useful to decouple the effects of PARP activation on metabolism and highlight that several metabolites are affected due to DDR triggered by MMS in MCF-7 cells. Finally, multiple DNA damaging agents that result in double strand breaks elicited similar metabolic responses in cells, suggesting that metabolomics can be potentially used as a tool to identify biomarkers of DNA damaging agents and DDRs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources: This study was supported by the National Institutes of Health grant R01CA164492. This study made use of the National Magnetic Resonance Facility at Madison, which is supported by National Institutes of Health grants P41GM103399 (National Institutes of General Medical Sciences) and P41GM66326 (National Institute of General Medical Sciences). Equipment in the facility was purchased with funds from the University of Wisconsin, the National Institutes of Health (P41GM66326, P41RR02301, P41GM103399, RR02781, RR08438), the National Science Foundation (DMB-8415048, OIA-9977486, BIR-9214394), and the U.S. Department of Agriculture.

Authors would like to thank National Institutes of Health for supporting this research. Authors would like to thank the staff of Nuclear Magnetic Resonance facility of Madison for providing guidance and training for using NMR spectrometers.

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Figure 1.

Principal component analysis (PCA) and hierarchical clustering of MCF-7 cell metabolic responses to different DNA damaging agents. a) Scores plot between 1st and 2nd principal component (PC) with 95% confidence region (represented by the dashed ellipses). The corresponding variance explained in each PC is shown in the bracket. b) Heat map showing the hierarchical clustering of metabolic concentrations (top 25 ranked according to ANOVA) using Pearson's correlation as the similarity measure and Ward's linkage as the clustering algorithm. Abbr. CNT: control, CSP: cisplatin, MMS: methyl methanesulfonate and ZEO: zeocin. The index for abbreviations used in heat map is given in Table 1 and Supplementary table 1 (Online Resource 1).

Figure 2. Pathway topological analysis of metabolic responses relative to control in MCF-7 cells. The pathways which are found to be significantly enriched (number of hits >1, FDR <0.05 and a non-zero impact score) in different DNA damaging agents-treated samples are shown. Metabolite importance was assessed using relative betweenness centrality measure and a global test was used to identify enriched pathways in treatment relative to control (adjusted for multiple testings). Abbreviation: CSP: cisplatin, MMS: methyl methanesulfonate and ZEO: zeocin. Grey color represent that the pathways is not significantly enriched.

Figure 3.

Principal component analysis (PCA) of metabolic responses to MMS in the presence or absence of PARP inhibition. a) Scores plot between 1st and 2nd principal components (PC) with 95% confidence region (represented by the dashed ellipse). The cells were treated with MMS in presence or absence of ABT-888. The corresponding variance explained in each PC is shown in the bracket. b) Heat map showing the hierarchical clustering of metabolic concentrations (top 25 ranked according to ANOVA) using Pearson's correlation as the similarity measure and Ward's linkage as the clustering algorithm. Abbr. CNT: control, MMS: methyl methanesulfonate PI: PARP inhibitor and MMS+PI: combination of MMS and PI. The index for abbreviations used in heat map is given in Table 1 and Supplementary table 1 (Online Resource 1).

Figure 4.

Effect of PARP inhibition as a single agent and in combination with MMS. Loading plots for principal component 1 (a) and principal component 2 (b) are shown. a) Loadings 1 represent the relationship between the metabolites which are mainly responsible for the differences between MMS and MMS+PI and b) Loadings 2 represents the metabolites which are mainly responsible for the differences due to addition of MMS. The index of abbreviations is given in the Supplementary table 1 (Online Resource 1).

Table 1

Comparison of metabolic responses induced by different DNA damaging agents on MCF-7 cells.

Metabolite	ChEBI ID	Abbr.	p-value	FDR	Tukey's HSD
NAD+	15846	NAD	5.04E-07	1.71E-05	MMS-CNT
Creatine	16919	Cr	2.81E-06	4.77E-05	MMS-CNT
L-Glutamate	16015	Glu	1.26E-05	0.000143	ZEO-CNT
Glutathione	16856	GSH	1.89E-05	0.00016	MMS-CNT
L-Serine	17115	Ser	3.41E-05	0.000232	CSP-CNT; MMS-CNT
L-Glutamine	18050	Gln	0.00032	0.001813	MMS-CNT; ZEO-CNT
L-Phenylalanine	17295	Phe	0.000412	0.002002	CSP-CNT; MMS-CNT; ZEO-CNT
Valine	16414	Val	0.000556	0.002362	MMS-CNT; ZEO-CNT
Glycine	15428	Gly	0.000955	0.003609	CSP-CNT
Methionine	16643	Met	0.001226	0.004169	MMS-CNT; ZEO-CNT
Isoleucine	17191	Ileu	0.001598	0.00494	ZEO-CNT
myo-Inositol	17268	myoI	0.001785	0.005058	ZEO-CNT
Tyrosine	17895	Tyr	0.002156	0.005164	ZEO-CNT
sn-Glycero-3-Phosphocholine	16870	GPC	0.00217	0.005164	MMS-CNT
Leucine	15603	Leu	0.002278	0.005164	ZEO-CNT
UDP-N-Acetylglucosamine	16264	UDP	0.008496	0.018055	CSP-CNT

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Metabolite	ChEBI ID	Abbr.	p-value	FDR	Tukey's HSD
ATP	15422	ATP	0.012005	0.022386	MMS-CNT
Creatine phosphate	17287	PCr	0.01251	0.022386	CSP-CNT; ZEO-CNT
Lactate	24996	Lac	0.013753	0.02338	MMS-CNT
Pyruvate	15361	Pyr	0.022788	0.036895	MMS-CNT
Sorbitol*	17924	Sor	-	-	ZEO-CNT
Methylguanidine**	16628	MG	-	-	MMS-CNT

Raw p value and FDR values calculated from two way ANOVA with Tukey's HSD test as post hoc method are shown for different metabolites. Metabolites with FDR <0.05 were considered to be significantly affected. Green: depleted as compared to control, red: enriched as compared to control.

* Sorbitol peaks were found exclusively in zeocin treated samples and

** methylguanidine peak was observed exclusively in MMS treated samples.

Abbreviations: CNT: control, MMS: methylmethane sulfonate, CSP: cisplatin, ZEO: zeocin