Using quantitative phosphoproteomics to understand key phosphorylation signaling pathways in HCT116 cells after chemotherapy drug treatment

Todd Mullis[1*], Andrew Lee[2], Rebekah Woolsey[3], David Quilici[3], Qian Wang[1]

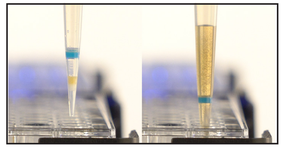
[1]University of South Carolina, Department of Chemistry and Biochemistry, Columbia, SC; [2]Integrated Micro-Chromatography Systems, Inc, Irmo, SC

[3]Mitch Hitchcock, Ph.D. Nevada Proteomics Center, 1664 N Virginia Street MS 0330, Reno, NV 89557

integrated micro-chromatography systems

INTRODUCTION

We previously established an automated phosphopeptide enrichment method using an automated liquid handler and IMCStips®. Using this method, we generated nearly 10,000 phosphopeptide IDs without prior fractionation from cell digest. We applied this automated method to probe the effect of a thymidylate synthase inhibitor, 5-fluorouracil (5-FU), on a common colorectal cancer (CRC) cell line, HCT116. 5-FU is a well-documented chemotherapeutic agent that is commonly used as a first-line treatment of many cancers, including CRC. However, innate and acquired resistance to the drug is common [1,2]. There is a need for new drug targets that increase the drug's efficacy and prognostic markers that can predict the patient's sensitivity to the drug. In this study, comparative phosphoproteomic analysis using automated sample preparations is presented as an initial proof of concept to dissect the differential phosphorylation events in HCT116 cells treated with 5-FU. The resulting data allows for the mapping of signaling cascades and monitoring of cellular events which may be useful in identifying new drug targets and predicting patient's sensitivity to the drug treatment.

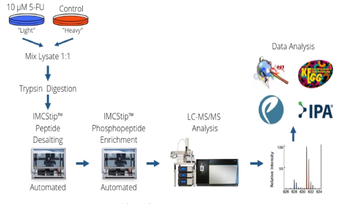


MATERIALS AND METHODS

HCT116 cells were grown in Gibco™ DMEM/F-12 containing 15 mM of HEPES and 10% fetal bovine serum. Freshly thawed cells were passaged by trypsin/EDTA treatment at 80-90% confluence. Once the cells were passaged five times, they were treated with various concentrations of 5-FU for 48 hours. The treated cells were assayed to determine cell viability using the Promega™ CellTiter-Blue™ Cell Viability Assay. Stable isotope labelling with amino acids in cell culture (SILAC) was used to isotopically label HCT116 cells. They were grown in Thermo Scientific™ DMEM:F-12 Media containing 10% dialyzed FBS. Mass spectrometry confirmed that incorporation of the heavy labeled amino acids ($^{13}C_6$ L-Lysine and $^{13}C_6{}^{15}N_4$ L-Arginine) was above 95%. Upon complete labelling, "light" cells were treated with 10 µM 5-FU and "heavy" cells were treated with 0.05% DMSO for 48 hours. The cells were lysed with RIPA buffer containing a protease and phosphatase inhibitor cocktail. Based on protein concentrations determined using the bicinchoninic acid (BCA) assay, the protein lysates were combined 1:1. The proteins were reduced in 10 mM TCEP at 56°C for 30 minutes and were then alkylated in 25 mM iodoacetamide at room temperature in the dark for 30 minutes. Following reduction and alkylation, the proteins were trypsin digested (1:50 enzyme to protein ratio) overnight at 37°C. For automated sample processing we used the Microlab® STAR™ from Hamilton Robotics. Five mg RP 300 µL IMCStips® were used for desalting prior to phosphopeptide enrichment with 10 mg PolyTi or 5 mg ZrO_2 300 µL IMCStips®.

- Mass spectrometer.....Orbitrap Fusion, Thermo Scientific
- Liquid chromatography.....Ultimate 3000 RSLCnano, Thermo Scientific
- Mobile phase B.....0.1% formic acid in acetonitrile
- LC gradient.....2% - 90% B for 180 min
- Analytical column.....40-cm in-house packed column with 1.8 µm Sepax GP-C18 (120Å, Sepax Technologies, Newark, DE).
- Column oven temperature...50°C

For global phosphopeptide identification, we used an Orbitrap Fusion mass spectrometer coupled to an UltiMate 3000 RSLCnano system. The mass spectrometer was operated in data-dependent mode at top speed. The dynamic exclusion duration was set to 60 s with a 10 ppm mass tolerance. Spectra were searched using the Sequest HT algorithm within Proteome Discoverer v2.2 (Thermo Scientific) in combination with the human UniProt protein FASTA database (2017 March, 20,119 entries). Search parameters were as follows: FT-trap instrument, parent mass error tolerance of 10 ppm, fragment mass error tolerance of 0.6 Da (monoisotopic), variable modifications of 15.995 Da (oxidation) on methionine and 79.966 Da (phosphorylation) on serine, threonine and tyrosine, fixed modification of 57.021 Da (carbamidomethylation) on cysteine. For SILAC quantitation, the heavy labelled cells were used as the control. The resulting data was analyzed using Ingenuity Pathway Analysis software. The overall scheme of the sample preparation and analysis process can be seen in **Scheme 1**.



Scheme 1. *Experimental workflow of SILAC-based proteomic and phosphoproteomic analysis of 5-FU treated HCT116 cells. The workflow incorporates an automated desalting and phosphopeptide enrichment step that significantly reduces the hands-on time required for sample generation.*

RESULTS

We established an in-house 5-FU treatment of HCT116 cells by determining the 50% growth inhibition concentration (GI_{50}) for a 48-hour treatment. HCT116 cells were treated with various concentrations of 5-FU including: 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µM for 48 hours. Using the CTB assay, the relative cell viability was determined for each 5-FU concentration (**Table 1**). A Monod equation was generated to calculate the GI_{50} based on the data points from the CTB assay (**Figure 1**). Based on this equation, the GI_{50} for our HCT116 cell line was 42 µM. Previous studies have shown the GI_{50} 5-FU concentration for the HCT116 cell line to be between 0.5-77 µM [3,4]. Using these results, 10 µM 5-FU was selected as the concentration to be used for the cell treatment with a calculated theoretical growth inhibition of 35%. This established 5-FU treatment of HCT116 cells was then used to perform the SILAC-based quantitative phosphoproteomic analysis.

$$RFU_{[5\text{-}FU]} = RFUo \quad X \quad \frac{1.064^{0.187}}{[5\text{-}FU]}$$

Table 1

[5-FU], µM	590 nm, RFU	Percent Inhibition, %
0	3470.7	0%
1.5625	3379.3	3%
3.125	2689.8	22%
6.25	2488.2	28%
12.5	2012.6	42%
25	1660.7	52%
50	1586.9	54%
100	1434.2	59%
200	1469.0	58%
400	1491.6	57%
800	885.9	74%

The relative growth inhibition percentage of HCT116 after 48-hour treatment with specified concentrations of 5-FU. The growth inhibition was calculated based the CTB assay. Percent inhibition was calculated using the control (0 µM 5-FU) as the relative value for 0% inhibition.

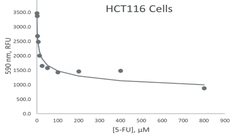


Figure 1. *Cell viability plot of HCT116 cells after 5-FU treatment at each concentration. The cell viability was quantified by 590 nm RFU values after incubation with CTB reagent. The displayed trendline is based on the generated Monod equation using the 590 nm RFU values from the CTB assay.*

In previous studies, we established a fully automated desalting and phosphopeptide enrichment method using IMCStips® on a Hamilton Microlab® STAR™, which allowed us to identify over 10,000 phosphopeptides from 200 µg of HCT116 cell lysate without fractionation. Here, as a proof of concept study, we used the established automated method to desalt and enrich for phosphopeptides from 200 µg of 1:1 mixed Heavy:Light cell lysate to quantitate protein fold changes in 5-FU treated HCT116 cells. For the analysis, 8 samples were desalted using RP IMCStips®. Two of the desalted samples were collected and vacuum dried without further enrichment. Six samples were further enriched using phosphopeptide enrichment IMCStips®, 3 using PolyTi IMCStips® and 3 using ZrO_2 IMCStips®. Our previous studies showed a slight differential enrichment between the two resin types, so both PolyTi and ZrO_2 resin types were used for enrichment to obtain a comprehensive phosphorylation profile.

From the analysis, a total of 5,985 unique proteins and 67,250 unique peptides were identified from our analyzed samples. For the phosphopeptide enriched samples, the PolyTi IMCStips® samples averaged a total of 10,465 phosphopeptides identified and an average specificity of 85%. ZrO_2 IMCStips® samples averaged a total of 8,351 phosphopeptides identified with an average specificity of 90% (**Figure 2**). The unenriched samples processed using RP IMCStips® samples show very low phosphopeptide identifications, which highlights the need for the phosphopeptide enrichment.

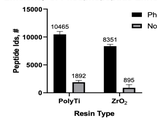


Figure 2. *A comparison of the total amount of phosphopeptides and non-phosphopeptides identified by either PolyTi or ZrO_2 resin. All samples were processed with IMCStips® using our fully automated phosphopeptide enrichment method (n=3).*

Table 2

Sample	Resin	Protein Ids, #	Peptide Ids, #	Phosphopeptide Ids, #	Phosphopeptide Specificty, %	Quantified Proteins, #	Quantified Peptides, #
1	RP	4200	38423	260	1%	3490	19785
2	RP	4132	36539	234	1%	2838	18911
3	PolyTi	2886	12598	11156	89%	2458	6788
4	PolyTi	2879	12388	10365	84%	2195	6678
5	PolyTi	2835	12085	9874	82%	2221	6422
6	ZrO_2	2395	9012	8489	94%	1842	4919
7	ZrO_2	2483	9589	7895	82%	1835	5175
8	ZrO_2	2407	9138	8670	95%	1862	4921

Summary of generated MS data from 8 samples analyzed. 8.200 µg 1:1 Heavy:Light HCT116 cell digest samples were analyzed. Samples prepared with RP resin were not enriched for phosphopeptides, whereas samples prepared with PolyTi or ZrO_2 were enriched for phosphopeptides.

Further, the reproducibility of the quantitation based on peptide fold change ratios were compared for both unenriched and enriched samples by plotting the log_2 fold change value for biochemical replicates (**Figure 3**). By Pearson correlation, the r-values for the comparison of calculated fold change ratios for two replicates replicates from RP, PolyTi and ZrO_2 were 0.88, 0.80 and 0.78 respectively. Based on these high r-values, the sample preparation and analysis process is able to generate reproducible quantitative proteomic and phosphoproteomic data.

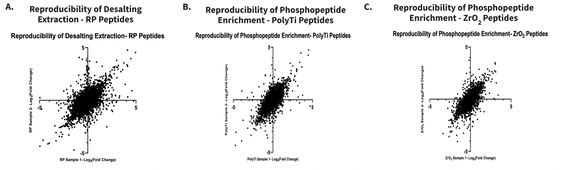


Figure 3. *A comparison peptide fold change ratios (log2) between two biochemical replicates of 200 µg of 1:1 mixed SILAC samples that were prepared using (A) the desalting extraction using RP IMCStips®, (B) the desalting extraction using RP IMCStips® followed by the phosphopeptide enrichment using PolyTi IMCStips® or (C) the desalting extraction using RP IMCStips® followed by the phosphopeptide enrichment using ZrO_2 IMCStips®. R-values equal to 0.88, 0.80 and 0.78 respectively by Pearson correlation (P-Values < 0.00001).*

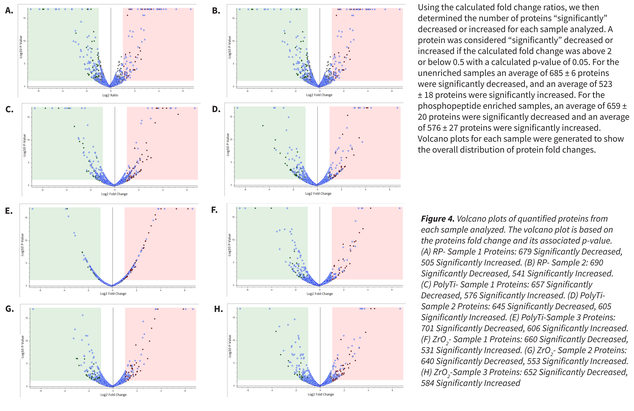


Figure 4. *Volcano plots of quantified proteins from each sample analyzed. The volcano plot is based on the proteins fold change and its associated p-value. (A) RP- Sample 1 Proteins: 679 Significantly Decreased, 505 Significantly Increased. (B) RP- Sample 2: 690 Significantly Decreased, 541 Significantly Increased. (C) PolyTi- Sample 1 Proteins: 657 Significantly Decreased, 576 Significantly Increased. (D) PolyTi- Sample 2 Proteins: 645 Significantly Decreased, 605 Significantly Increased. (E) PolyTi-Sample 3 Proteins: 701 Significantly Decreased, 606 Significantly Increased. (F) ZrO_2 - Sample 1 Proteins: 660 Significantly Decreased, 531 Significantly Increased. (G) ZrO_2 - Sample 2 Proteins: 640 Significantly Decreased, 553 Significantly Increased. (H) ZrO_2-Sample 3 Proteins: 652 Significantly Decreased, 584 Significantly Increased*

Using the calculated fold change ratios, we then determined the number of proteins "significantly" decreased or increased for each sample analyzed. A protein was considered "significantly" decreased or increased if the calculated fold change was above 2 or below 0.5 with a calculated p-value of 0.05. For the unenriched samples an average of 685 ± 6 proteins were significantly decreased, and an average of 523 ± 18 proteins were significantly increased. For the phosphopeptide enriched samples, an average of 659 ± 20 proteins were significantly decreased and an average of 576 ± 27 proteins were significantly increased. Volcano plots for each sample were generated to show the overall distribution of protein fold changes.

To further determine the biological relevance of the data, Ingenuity Pathway Analysis (IPA) software was used. For this proof-of-concept study, we were primarily interested in analyzing the phosphopeptide enriched data set to understand the phosphorylation signaling pathways affected by the 5-FU treatment. The expression fold change data from the phosphopeptide enriched samples was analyzed using IPA to determine the affected signaling pathways (**Figure 5**).

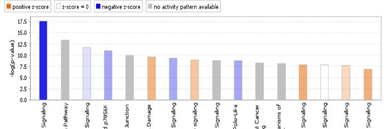


Figure 5. *Canonical pathways identified using the proteins from the phosphopeptide enriched data set. The p-value represents the probability of random association of molecules with the identified pathway. A -log(P-value) threshold of 5 for this dataset. A positive z-score indicates an increase of the pathway, while a negative z-score indicates an inhibition of the pathway.*

From this analysis, the EIF2 signaling pathway was shown to be significantly inhibited. The EIF2 signaling pathway is generally associated with cellular growth and proliferation. It is known that in response to stress and amino acid starvation, phosphorylation of eIF2a can inhibit translational initiation and by association cellular growth [5,6,7]. In our data set, we observed a collective decrease in kinases within the EIF2 pathway, which are generally associated with growth factors such as PI3K, AKT, c-RAF, potentially in response cell stress (**Figure 6**).

Further, a tox list was generated to understand the pathways associated with toxicity that were enriched in the analyzed dataset (**Figure 7**). The NRF2-Mediated Oxidative stress response pathway was shown to be increased within our dataset. This pathway has been implicated as a primary response pathway to 5-FU treatment due to an increase in reactive oxygen species[7]. In previous studies, it has been shown that upregulation of NRF2 can lead to resistance to 5-FU treatment. Additionally, phosphorylation is thought to play a key role in the upregulation of NRF2 and NRF2-associated proteins[8]. The p53 signaling pathway was also shown to be affected in our dataset. It has recently been reported that p53 signaling pathway is crucial for initiating cell death in response to 5-FU treatment. In cells resistant to 5-FU treatment, the WNT-signaling pathway can inactivate the p53 signaling pathway cell death response[2].

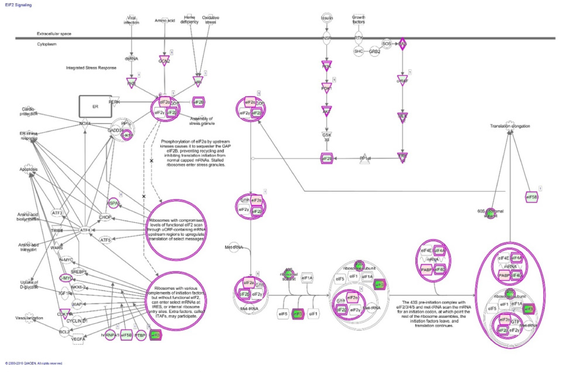


Figure 6. *EIF2 signaling pathway with proteins identified and quantified within the phosphopeptide enriched sample set. The z-score for this pathway was calculated to be -2.46 indicating inhibition of the EIF2 signaling pathway. Molecules with purple bands indicate identification of the protein within the dataset.*

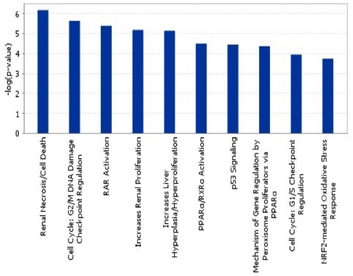


Figure 7. *Tox list generated by IPA software based on molecules from the phosphopeptide enriched dataset. The p-value represents the probability of random association of molecules with the identified pathway. A -log(P-value) threshold of 3 for this dataset.*

The phosphopeptide enriched data set was further filtered by selecting proteins that were identified across all analyzed phosphopeptide samples, and were calculated to have a fold change of greater than 2 or less than 0.5. In total 22 proteins were identified, 11 proteins with a fold change greater than 2 and 11 proteins with a fold change less than 0.5 (**Table 3**). Lamin-B1 is of particular interest as it was recently reported that the overexpression of the protein leads to worse clinical outcomes in patients with colon cancer due to an increase in mitotic catastrophe allowing cancers cells to avoid apoptosis[9]. Additionally, upregulation of deoxycytidine kinase, an important enzyme in DNA synthesis, has been shown to correlate well with 5-FU sensitivity and has been highlighted as a potential prognostic marker for the drug treatment [10].

Table 3

Uniprot Id	Protein Description	Average Fold Change	Phospho-Site(s)
Q8IV50	LysM and putative peptidoglycan-binding domain-containg protein 2	100.00	S24
Q9Y285	Phenylalanine--tRNA ligase alpha subunit	100.00	S301
Q8N6N3	UPF0690 protein C1orf52	11.91	T155
Q92878	DNA repair protein RAD50	9.77	S635; T690
Q92547	DNA topoisomerase 2-binding protein 1	5.46	S350; S888
P27707	Deoxycytidine kinase	4.35	S74
Q8N450	Coiled-coil domain-containing protein 82	4.03	S282
O14545	TRAF-type zinc finger domain-containing protein 1	3.50	S327; S415
Q8WWA1	Transmembrane protein 40	2.88	S141
Q08174	Protocadherin-1	2.78	S962; S984
Q9Y6W5	Wiskott-Aldrich syndrome protein family member 2	2.35	S293
Q5VTL8	Pre-mRNA-splicing factor 38B	0.44	S529
Q5JVS0	Intracellular hyaluronan-binding protein 4	0.36	S108
P22234	Multifunctional protein 4	0.31	S27
O43583	Density-regulated protein	0.30	S73
Q6AI08	HEAT repeat-containing protein 6	0.30	S337; S643
Q15717	ELAV-like protein 1	0.29	S202
Q9UKX7	Nuclear pore complex protein Nup50	0.28	S296
P20700	Lamin-B1	0.26	S23; S391; T575
Q9H910	Hematological and neurological expressed 1-like protein	0.24	S97
Q9UN79	Transcription factor SOX-13	0.01	S334
Q86U06	Portable RNA-binding protein 23	0.01	S149

Based on these results, this study shows the usefulness of SILAC-based phosphoproteomic studies for understanding drug treatments affects, and for identifying phosphorylated proteins that could be further studied to determine if they could be used as prognostic markers for drug sensitivity or resistance. In future studies we plan to expand upon our current dataset by performing a phospho-tyrosine enrichment in addition to the global phosphopeptide enrichment as our current dataset is primarily enriched for serine- and threonine-phosphorylated peptides.

CONCLUSION

In this proof of concept study, we used an established, fully automated desalting and phosphopeptide enrichment method to conduct a SILAC-based phosphoproteomic study of a 5-FU chemotherapy treatment of HCT116 cells. In this study we were able to identify 5,985 unique proteins and 67,250 unique peptides. From the mass spectrometry analyses, we quantified fold changes of phosphoproteins in response to the chemotherapy treatment and correlated those changes with changes in biological signaling pathways. Additionally, we identified 22 phosphoproteins that were significantly increased or decreased in all phosphopeptide enriched samples in response to the 5-FU treatment. These proteins could be explored further as potential drug targets or prognostic markers for 5-FU treatments.

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**Contact: Todd Mullis – mullist@email.sc.edu*

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