# Automated sample preparations to analyze time treatments of thymidylate inhibitors on colon cancer cell lines

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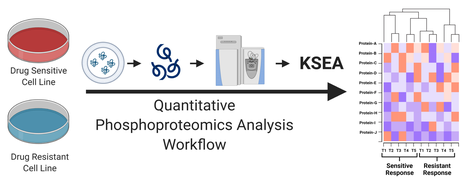
## INTRODUCTION

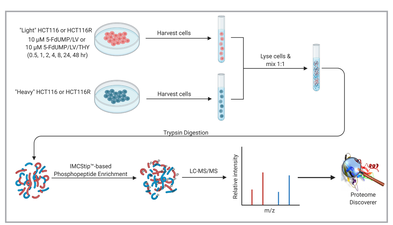



Chemotherapy drugs, like thymidylate synthase (TS) inhibitors, are used as first line treatments of colorectal cancer (CRC). However, chemoresistance is a major problem in CRC treatment. There are efforts to provide predictive markers of chemo resistance and to identify additional targets for combination therapies that would improve overall outcomes. The aim of this work is to use our previously established phosphopeptide enrichment protocol and quantitative MS-based analysis methods to probe the phosphoproteomic changes in TS-sensitive cells as well as in TS-resistant cells when treated with 5-fluoro-2'-deoxyuridine monophosphate (5-FdUMP) and folinic acid (drug name leucovorin or LV). We find that early changes are observed within half hour of treatment in a reproducible manner using an automated phosphopeptide extraction method.

## METHODS

HCT116 and HCT116/200 cells were grown in Gibco™ DMEM/F-12, containing 15 mM of HEPES and 10% FBS. Cells were passaged by trypsin/EDTA treatment at 80-90% confluence. The cells were passaged at least 5 times prior to treatment with 10 µM 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP) and 10 µM folinic acid (LV) or 10 µM 5-FdUMP, 10 µM LV, and 10 µM Thymidine (Thy) for 24 hrs. Cell viability was determined using the Promega™ CellTiter-Blue™ Cell Viability Assay after 24 hours of treatment.

Stable isotope labelling with amino acids in cell culture (SILAC) was used to label HCT116 and HCT116/200 cells. They were grown in Thermo Scientific™ DMEM/F-12 Media containing 10% dialyzed FBS. Incorporation of $^{13}C_6$ L-Lysine and $^{13}C_6{}^{15}N_4$ L-Arginine was ≥ 95% and confirmed by mass spectrometry. Unlabeled cells were treated with 10 µM 5-FdUMP and 10 µM LV, or 10 µM 5-FdUMP, 10 µM LV, and 10 µM Thy in 0.05% DMSO and isotopically labeled cells were treated with 0.05% DMSO for the specified amount of time. After treatment, the cells were washed 3 times with cold 1x PBS. The cells were collected by scraping from the dish and pelleted by centrifuging for 5 min at 500 × g. Cell pellets were stored at -80°C until further processing. Cells were lysed with RIPA buffer containing a protease and phosphatase inhibitor cocktail. Protein concentrations were determined using the bicinchoninic acid (BCA) assay. The lysates were combined 1:1. The proteins were reduced in 10 mM TCEP at 56°C for 30 minutes and were alkylated with 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. RP IMCStips® (cat. # 04T-H3R05-1-5-8) were used for desalting prior to phosphopeptide enrichment with PolyTi IMCStips® (cat. # 04T-H3R73-1-10-8). The sample preparation and analysis scheme are detailed in Scheme 1.




***Scheme 1.*** *SILAC-based time course thymidylate synthase inhibitor treatment experiment. The workflow incorporates an automated desalting and phosphopeptide enrichment step that significantly reduces the hands-on time required for sample generation.*

Thermo Orbitrap Fusion mass spectrometer coupled to an UltiMate 3000 RSLCnano was used for global phosphopeptide identification. Mass spectrometer was operated in data-dependent mode at top speed. 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 fold change between the light and heavy (treated and untreated) phosphoproteins was calculated for each of the samples for each time point using Proteome Discoverer. The cutoff for the calculated fold change was set at ≥ 2 and ≤ 0.5. For each time point and each treatment type, the proteins within this cutoff were identified. KSEAapp was implemented as an R package. The source code and documentation are freely available from GitHub (https://github.com/casecpb/KSEAapp).[1] KSEA was used to determine which kinases were most likely responsible for this early response to the drug treatment. KSEA is a software tool that draws on publicly available phosphosite databases such as PhosphoSitePlus and NetworKin to calculate kinase activity scores (z-scores) from quantitative phosphoproteomic data.[1] KSEA outputs a bar plot of kinases based on their calculated z-score and their associated p-values as well as the substrate links the software was able to be determined within the dataset. For the KSEA analysis, both the PhosphoSitePlus database and the NetworKin database were used. The minimum number of substrates per kinase required ("m.cutoff") was set to 5 and the p-value cutoff was set to 0.05. GraphPad Prism 8 was used for graph generation, statistical analysis, and heat map generation. Biorender was used to generate schematics and graphical workflows.

## RESULTS AND DISCUSSION

Two cell lines, HCT116 and HCT116/200, were treated with 10 µM 5-FdUMP and 10 µM LV. HCT116/200 cells overexpress TS and are resistant to conventional TS inhibitor treatments while HCT116 cells are sensitive to the treatment.[2] TS inhibition is confirmed when supplementing thymidine bypasses the thymidineless stress response, avoiding cell death, as shown in the cell viability plot for HCT116 (Figure 1A) whereas HCT116/200 cells are unaffected by treatment. Treated HCT116 cells decreased viability to 57.2 ± 1.0 %.

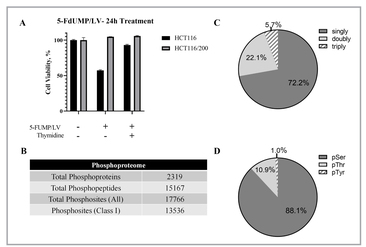



***Figure 1.*** *Treatment for two cell lines and subsequent MS profiling.* ***A)*** *Cell viability for HCT116 and HCT116/200 cells untreated and treated for 24 hours with 10 µM 5-FdUMP/LV or 10 µM 5-FdUMP/LV/THY (n = 3, data are mean ± s.d.).* ***B)*** *Numbers of phosphoproteins, phosphopeptides, total phosphosites, and phosphosites (Class 1) from the MS analysis.* ***C)*** *The phosphopeptide profile of the percentage of singly, doubly, and triply phosphorylated peptides and* ***D)*** *the percentage of phosphoserines, phosphothreonines, and phosphotyrosines from all 18 samples. Samples were collected from each different time point (0.5-, 1-, 2-, 8-, 24-, 48-hour) and from each different sample type (5-FdUMP/LV treated HCT116 cells, 5-FdUMP/LV treated HCT116/200 cells, and 5-FdUMP/LV/THY treated HCT116 cells) for a total of 18 different samples.*

For the SILAC-based phosphoproteomic analysis of the time course samples the total number of phosphoproteins identified was 2,319 and total number of phosphopeptides was 15,167 with specificity of 85% (Figure 1B). The total number of Class I phosphosites, defined as phosphosites that were localized to an amino acid with a probability of at least 75% and a probability localization score difference greater than or equal to 5, was 13,536. 10,944 (72.2%) were singly phosphorylated, 3,351 (22.1%) were doubly phosphorylated, and 872 (5.7%) were triply phosphorylated (Figure 1C). This is in line with our previously published phosphoproteomic data for sample enriched with PolyTi IMCStips.[3] The number of phospho-serines, phospho-threonines, and phospho-tyrosines were 11,919 (88%), 1,478 (11%), and 139 (1.0%), respectively (Figure 1D). This ratio of phospho-serines to phospho-threonines to phospho-tyrosines is in line with reported values and most likely represents the overall naturally occurring abundance of each phosphosite, but a bias to the purification method.[4]

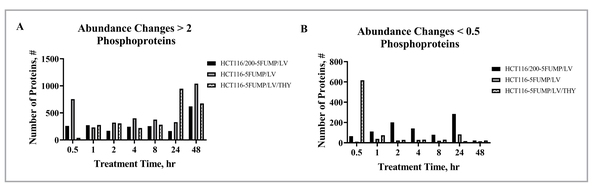



***Figure 2.*** *SILAC-based quantitative data from the TS inhibitor time course samples.* ***A)*** *Bar graph of the number of phosphoproteins with abundance ratios greater than 2 or* ***B)*** *less than 0.5 for each of the different treated sample types and at different treatment times. Samples from each different time point (0.5-, 1-, 2-, 8-, 24-, 48-hour) and from each different sample type (5-FdUMP/LV treated HCT116 cells, 5-FdUMP/LV treated HCT116/200 cells, and 5-FdUMP/LV/THY treated HCT116 cells) for a total of 18 different samples*

At 0.5-hour time point, we identified more proteins with a ≥ 2-fold change in 5-FdUMP/LV treated HCT116 sample (754) in comparison to the 5-FdUMP/LV treated HCT116/200 cells (259) and to the 5-FdUMP/LV/THY treated HCT116 cells (38) (Figure 2A, B). An early response toward 5-FdUMP/LV treatment is unexpected, and the differences in cell response appears to be rapid, and such response is avoided in presence of thymidine. TS-inhibitor resistant cells also exhibit an early response, but the phosphoproteome profile is less significantly altered than TS sensitive cells.

HCT116 cell treatment with 5-FdUMP/LV for 0.5 hour was repeated in triplicate and the average number of proteins identified from each sample was 2,018 ± 26, the average number of phosphopeptides was 5334 ± 249 with an average phosphopeptide specificity of 85 ± 2% (Figure 3A). Based on this replicate dataset, phosphoproteins with a fold change ratio ≥ 2 (394) and a low number of phosphoproteins with a fold change ratio less than or equal to 0.5 (5) were identified (Figure 3B).

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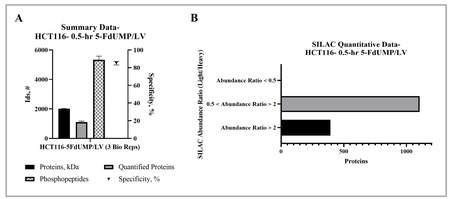


***Figure 3.*** *Triplicate samples of 0.5-hour 5-FdUMP/LV treated HCT116 samples.* ***A)*** *Summary of phosphoproteomic data from 0.5-hour 5-FdUMP/LV treated HCT116 cell samples (n = 3) including number of protein identifications, phosphopeptide identifications, quantified proteins, and phosphopeptide specificity. The average number of proteins identified from each sample was 2,018 ± 26 and the average number of phosphopeptides was 5334 ± 249 with an average phosphopeptide specificity of 85 ± 2%.* ***B)*** *Abundance ratios calculated from 0.5-hour 5-FdUMP/LV treated HCT116 cell samples (n = 3, mean ± s.d.).*

KSEA analysis of the 0.5-hour 5-FdUMP/LV HCT116 cell sample triplicate dataset, resulted in CDK2, CDK1, CDK4, and Mitogen-activated protein kinase 8 (MAPK8) having the highest calculated z-scores of 5.00, 3.84, 3.05, and 3.03, respectively, with associated p-values less than 0.05. Ribosomal Protein S6 Kinase B1 (RPS6KB1) and Ribosomal Protein S6 Kinase A1 (RPS6KA1) had the lowest calculated z-scores of -2.52 and -3.23, respectively, with associated p-value less than 0.05 (Figure 4A). Of note, the KSEA software normalizes the fold change data across the data set so that the resulting kinase z-scores are relative to the dataset. This is shown when plotting the substrate groups for the kinases with the highest and lowest calculated z-scores, where the average $\log_2$FC of substrates within the CDK2 and CDK1 substrate group is 1.14 and the average $\log_2$FC of substrates with the RPS6KB1 and RPS6KA1 substrate group is 0.13 (Figure 4B).

Based on heatmaps of the $\log_2$FC of the specified substrates within different kinase substrate groups (Figure 5), CDK1 substrate group, CDK2 substrate group, and MAPK family substrate group were the top 3 in the 0.5-hour 5-FdUMP/LV treated HCT116 cells.

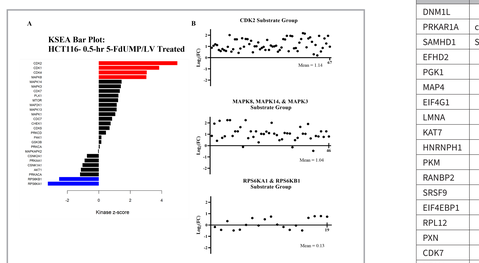


***Figure 4.*** *KSEA analysis of data from 0.5-hour 5-FdUMP/LV treated HCT116 samples (n = 3). This dataset only includes class 1 phosphosites.* ***A)*** *KSEA bar plot from phosphoproteomic data from 0.5-hour 5-FdUMP/LV treated HCT116 cell samples (n = 3). Kinases with red or blue bars have at least 5 linked substrates found within the dataset and the calculated z-score has a p-value that is less than 0.05.* ***B)*** *Dot plots of substrates' $\log_2$ fold changes associated with the indicated kinase(s). The average $\log_2$ fold change is indicated for each substrate group.*

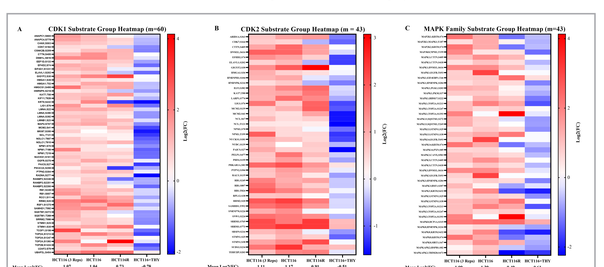


***Figure 5.*** *Heatmaps generated using data from 0.5-hour 5-FdUMP/LV treated HCT116, HCT116/200, and HCT116+THY samples.* ***A)*** *Heatmap of CDK1 substrate groups from each of the sample groups at the 0.5-hour time point. The mean $\log_2$(FC) for the substrate group is calculated for each sample group.* ***B)*** *Heatmap of CDK2 substrate groups from each of the sample groups at the 0.5-hour time point. The mean $\log_2$(FC) for the substrate group is calculated for each sample group.* ***C)*** *Heatmap of MAPK family (MAP2K1, MAP2K2, MAP3K8, MAPK1, MAPK13, MAPK14, MAPK3, MAPK8, MAPKAPK2) substrate groups from each of the sample groups at the 0.5-hour time point. The mean $\log_2$(FC) for the substrate group is calculated for each sample group.*

Comparing the 0.5-hour 5-FdUMP/LV treated HCT116 cell sample heat maps to the 0.5-hour 5-FdUMP/LV treated HCT116/200 cell sample shows aslight decrease in the fold change averages across each substrate group and the 0.5-hour 5-FdUMP/LV/THY treated HCT116 cell sample heatmaps show a decrease in average fold change averages across each substrate group (Figure 5).

Previous reports have shown that CDK1, CDK2, and various kinases within the MAPK family are involved in response to TS inhibition, however generally these responses are shown in cells that have been subjected to the treatment for 24-72 hours rather than 0.5 hour. To our knowledge this is the first report of the early phosphorylation response to TS inhibition.

Select proteins from these substrate groups could be used to establish a quantitative assay for the specific phosphopeptides to probe TS inhibitor sensitivity (Table 1). This "top 20" list of phosphorylated proteins could provide a tool to screen patients for sensitivity to TS inhibitor treatment prior to treatment to determine if TS inhibitor treatment will be effective or if alternative treatments should be pursued.

**Table 1.** Top 20 phosphorylated proteins from CDK1, CDK2, or MAPK family substrate groups that show differential responses to the 5-FdUMP/LV treatment at the 0.5-hour time point.

| | | | $\log_2$(Fold Change) Data | | | |
|---|---|---|---|---|---|---|
| **Protein** | **Description** | **Phosphosite** | **HCT116 (3 Reps)** | **HCT116** | **HCT116R** | **HCT116+THY** |
| DNM1L | Dynamin-1-like protein | S616 | 1.910 | 1.868 | 1.122 | -0.236 |
| PRKAR1A | cAMP-dependent protein kinase type I-alpha regulatory subunit | S83 | 1.812 | 2.096 | 1.267 | 0.343 |
| SAMHD1 | SAM And HD Domain Containing Deoxynucleoside Triphosphate | T592 | 1.712 | 1.922 | 1.234 | -0.249 |
| EFHD2 | EF-hand domain-containing protein D2 | S74 | 1.334 | 1.125 | 0.568 | -1.204 |
| PGK1 | Phosphoglycerate kinase 1 | S203 | 1.256 | 1.159 | 0.148 | -0.527 |
| MAP4 | Microtubule-associated protein 4 | S787 | 1.176 | 1.405 | 0.737 | -0.949 |
| EIF4G1 | Eukaryotic translation initiation factor 4 gamma 1 | S1231 | 1.063 | 1.267 | -0.146 | -0.241 |
| LMNA | Prelamin-A/C | S392 | 1.009 | 1.018 | 0.324 | -0.869 |
| KAT7 | Histone acetyltransferase KAT7 | T88 | 0.976 | 1.051 | 1.642 | -0.295 |
| HNRNPH1 | Heterogeneous nuclear ribonucleoprotein H | S104 | 0.883 | 1.044 | -0.160 | -0.468 |
| PKM | Pyruvate kinase PKM | S37 | 0.869 | 0.888 | 0.305 | -0.730 |
| RANBP2 | E3 SUMO-protein ligase RanBP2 | S2280 | 0.833 | 0.809 | 0.200 | -1.228 |
| SRSF9 | Serine/arginine-rich splicing factor 9 | S216 | 0.816 | 1.038 | -0.108 | -0.667 |
| EIF4EBP1 | Eukaryotic translation initiation factor 4E-binding protein 1 | T46 | 0.807 | 0.923 | 2.314 | 0.543 |
| RPL12 | 60S ribosomal protein L12 | S38 | 0.691 | 0.759 | 0.088 | -0.390 |
| PXN | Paxillin | S85 | 0.660 | 0.707 | -0.037 | -0.935 |
| CDK7 | Cyclin-dependent kinase 7 | S164 | 0.596 | 0.509 | -0.480 | -0.969 |
| NPM1 | Nucleophosmin | S70 | 0.581 | 0.447 | -0.063 | -0.911 |
| MCM2 | DNA replication licensing factor MCM2 | S41 | 0.530 | 0.377 | 1.092 | -1.510 |
| HNRNPK | Heterogeneous nuclear ribonucleoprotein K | S216 | 0.425 | 0.642 | -0.476 | -0.824 |

## CONCLUSION

TS inhibitor resistance is a major problem in cancer treatment. To understand why innate or acquired resistance occurs, this exploratory study examined the early phosphorylation events in response to 5-FdUMP/LV treatment. Using automated phospho-enrichment with IMCStips®, reproducible data was generated to advance our current understanding of how colorectal cancer cells respond to TS inhibitor treatments. Changes in the phosphoproteome was observed within half hour of treatment and replicate samples confirmed these changes. CDK1, CDK2, and MAPK family substrate groups were different between TS inhibitor sensitive, resistant, and thymidine rescued cells.

### CONFLICT OF INTEREST

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## REFERENCES

1. Casado, P.; Rodriguez-Prados, J.-C.; Cosulich, S. C.; Guichard, S.; Vanhaesebroeck, B.; Joel, S.; Cutillas, P. R., Kinase-substrate enrichment analysis provides insights into the heterogeneity of signaling pathway activation in leukemia cells. *Sci. Signal* **2013,** *6* (268), rs6.
2. Ozer, U.; Barbour, K. W.; Clinton, S. A.; Berger, F. G., Oxidative Stress and Response to Thymidylate Synthase-Targeted Antimetabolites. *Mol. Pharmacol.* **2015,** *88* (6), 970-81.
3. Mullis, B. T.; Hwang, S.; Lee, L. A.; Iliuk, A.; Woolsey, R.; Quilici, D.; Wang, Q., Automating Complex, Multistep Processes on a Single Robotic Platform to Generate Reproducible Phosphoproteomic Data. *SLAS Discov.* **2020,** *25* (3), 277-286.
4. Riley, N. M.; Coon, J. J., Phosphoproteomics in the Age of Rapid and Deep Proteome Profiling. *Analytical Chemistry* **2016,** *88* (1), 74-94.
5. Du, Z.; Lovly, C. M., Mechanisms of receptor tyrosine kinase activation in cancer. *Mol. Cancer* **2018,** *17* (1), 58.

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