Triple SILAC Phosphoproteomics Reveals Drug Targets in Multiple Myeloma Cells: a Case for CID over HCD using the Orbitrap Elite & Metabolomics

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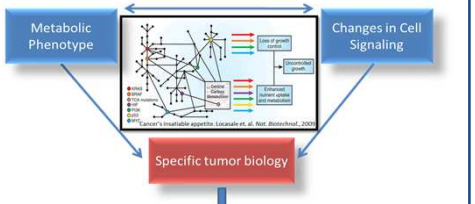
INTRODUCTION

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) is widely used for accurate relative protein and peptide quantification to compare phosphorylation states which regulate signaling pathway activity and cellular functions including cell proliferation, growth and survival, especially in cancers. We used *triple SILAC* labeling (1) of H929 multiple myeloma cells for global scale phosphoproteomics to better understand the signaling drivers of growth and proliferation when inhibited with Imatinib or Bortezomib. We compared the ion trap-based collision-induced dissociation (CID) with the higher energy collisional dissociation (HCD) fragmentation in the HCD collision cell of the Orbitrap Elite. CID and HCD runs were performed separately, combined in one MaxQuant run to analyze the phosphoproteomics data set of ~ 12,000 unique pSTY sites. We also performed SRM based polar metabolomics (2) in H929 cells and found by both technologies that Imatinib not only abrogates BCR-ABL signaling but also inhibits mRNA processing.

METHODS

- For phosphoproteomics, SILAC labeled human H929 cell lysates treated with or without inhibitors were digested with trypsin and peptides separated into 12 SCX fractions. Each fraction was enriched by Fe^{3+} IMAC. IMAC fractions were run by RP-LC-MS/MS on the **Orbitrap Elite** in either HCD or CID fragmentation mode over a 120 min. gradient. Data were run in triplicate (2 HCD, 1 CID). SILAC ratios and HCD/CID comparisons were analyzed using MaxQuant v. 1.3.05 against the Human database (UniProt) and DAVID software was used to interrogate affected pathways.
  - **CID:** Top 20 DDA; Max Ion Time 100 msec; Ion Trap AGC Target 10,000
  - **HCD:** Top 12 DDA; Max Ion Time 300 msec; FTMS AGC Target 50,000
- For polar metabolomics, H929 cells were incubated with 80% methanol for polar metabolite extraction. Metabolite samples were run by HILIC-LC-MS/MS in targeted mode via SRM using a **5500 QTRAP** mass spectrometer over a 15 min. gradient. Q3 peaks were integrated with MultiQuant 2.0 and data analysis was performed using MetaboAnalyst 2.0 software.
  - **SRM:** 287 Q1/Q3 transitions; Dwell time 3 msec; 10-14 Data points per peak

The phenotype of a cancer cell is based on alterations in *both* the proteome and metabolome

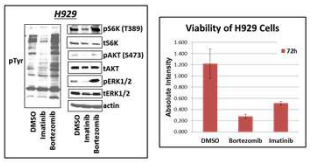


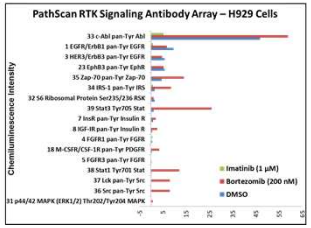
Increase in cell growth and proliferation

Metabolic reprogramming can be linked to specific alterations in genes *and* altered metabolism can create a survival benefit for onco-proteins. The identification of phosphoproteomic and metabolic profiles can lead to biomarkers and cancer therapies using a combination of metabolic inhibitors with existing kinase inhibitors.

RESULTS

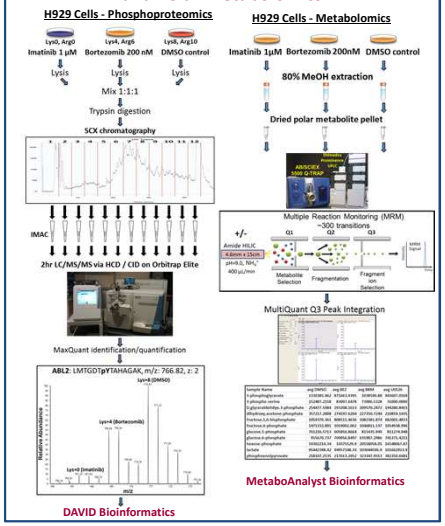
BCR-ABL positive H929 multiple myeloma cells are responsive to the inhibitors Imatinib and Bortezomib





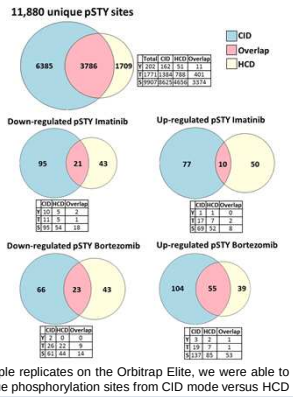
H929 cells drive growth and proliferation via BCR-ABL fusion and Imatinib down-regulates phosphorylation of key signaling events and cause an inhibition of cell proliferation (3). Bortezomib (proteasome inhibitor) also leads to a decrease in viability. We decided to use both quantitative phosphoproteomics and metabolomics to investigate the mechanism of these cell death consequences.

Workflow for Triple SILAC Phosphoproteomics and Polar Metabolomics



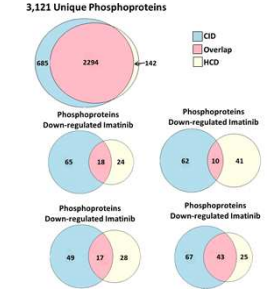
PHOSPHOPROTEOMICS RESULTS

Phospho-sites from CID and HCD modes



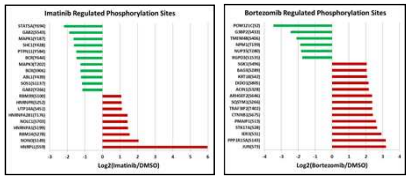
From multiple replicates on the Orbitrap Elite, we were able to identify more unique phosphorylation sites from CID mode versus HCD mode.

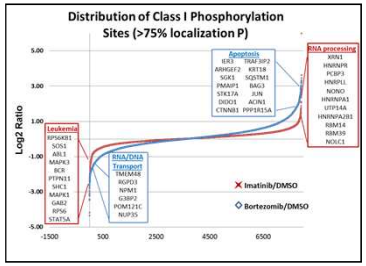
Phospho-proteins from CID and HCD modes

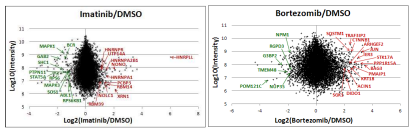


The discrepancy between CID and HCD was less apparent when analyzing the unique number of phosphorylated proteins.

Distribution of SILAC Phosphorylation Sites

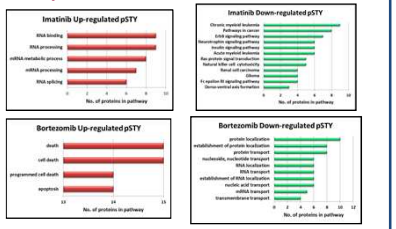






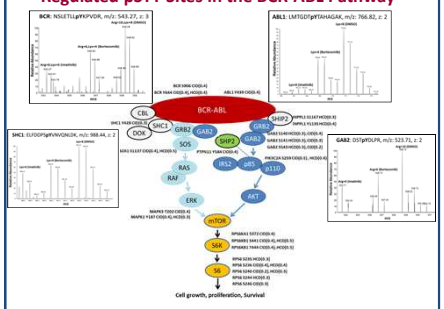
We analyzed the regulated pSTY sites across all experiments using triple SILAC ratios and plotted bar plots, S-curves and scatter plots of relative intensities (ratios). We used the regulated protein/gene names for further bioinformatics analyses to identify regulated biological pathways.

Pathways affected with Imatinib and Bortezomib



We used DAVID to identify the significant (P-value) biological pathways associated with inhibitor treatment. Imatinib inhibits the pSTY sites in the BCR-ABL pathway and increases pSTY sites involved in the mRNA processing. Bortezomib inhibits sites that are involved in the RNA/DNA transport and increases pSTY site levels of the apoptosis pathway.

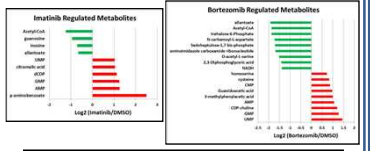
Regulated pSTY Sites in the BCR-ABL Pathway

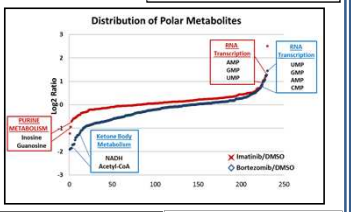


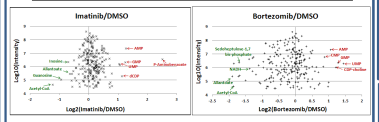
Imatinib regulated pSTY sites by both HCD and CID modes in the BCR-ABL pathway and some examples of supporting SILAC FT-MS spectra.

METABOLOMICS RESULTS

Distribution of Unlabeled Polar Metabolites by Targeted LC-MS/MS

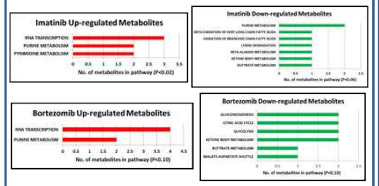






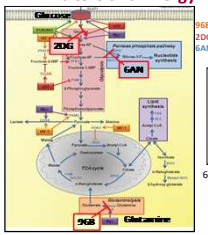
We analyzed the regulated polar metabolites using bar plots, S-curves and scatter plots of relative intensities (ratios). We used the regulated metabolite names for further bioinformatics analyses to identify regulated metabolic pathways.

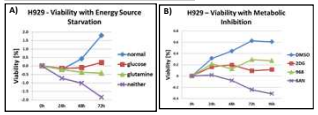
Metabolic Pathways affected with Imatinib and Bortezomib



When treated with Imatinib or Bortezomib, the significant up-regulated metabolites are part of the RNA transcription process and purine metabolism. Down-regulated metabolites by Imatinib also lead to alteration in purine metabolism and additional affected pathways. Bortezomib also down-regulates glucose metabolism pathways as the cells begins to cease proliferation.

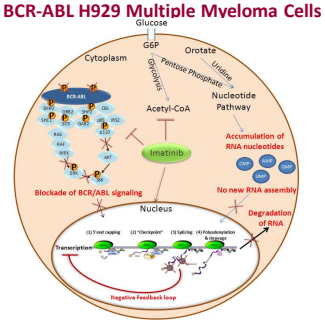
H929 Cells Treated with Metabolic Inhibitors and Energy Deprivation





A) H929 cells fed only with Glucose have a higher viability than H929 cells fed only with Glutamine. **B)** Cells treated with the PPP inhibitor 6AN are least viable which proves the dependence on the PPP, responsible for nucleotide synthesis. These data support the lethality of RNA processing inhibition in H929 cells.

Biological Model for Imatinib Treated BCR-ABL H929 Multiple Myeloma Cells



Upon Imatinib treatment, BCR-ABL signaling is down-regulated to inhibit growth and proliferation of H929 cells. Additionally, phosphoproteins regulating the spliceosome are up-regulated, inhibiting RNA transcription in the nucleus. The generated RNA nucleotides by the highly active pentose phosphate pathway do not polymerize and mRNA degrades to accumulate nucleotides in the cytoplasm.

CONCLUSIONS - Proteomics

- ***More unique and total pSTY sites were identified in CID mode on the Orbitrap Velos Elite***
- **Both HCD and CID identify unique pSTY sites and both fragmentation modes is necessary for maximum coverage**
- ***Triple SILAC phosphoproteomics successfully identified phospho sites related to BCR-ABL fusion in H929 cells***
- **We show that these cells can be effectively treated with the kinase inhibitor Imatinib and the proteasome inhibitor Bortezomib**
- ***Imatinib treatment increases the phosphorylation levels of proteins involved in mRNA processing, inhibiting its function while also down-regulating phosphorylation levels in the BCR-ABL pathway***

CONCLUSIONS - Metabolomics

- **H929 MM cells are more highly dependent upon glucose than glutamine for growth and proliferation**
- **H929 cells have a higher glucose flux through the pentose phosphate pathway than glycolysis versus control multiple myeloma cells and BCR-ABL positive leukemia cells**
- ***Imatinib treatment results in accumulation of RNA nucleotides and correlates with RNA transcription inhibition found by triple SILAC phosphoproteomics in addition to abrogating signaling through BCR-ABL***

REFERENCES

1. Oppermann FS et. al., Mol Cell Proteomics. 2009;8(7):1751-64.
2. Yuan M et. al. , Nat Protoc. 2012;7(5):872-81.
3. Breitkopf SB et. al., Proc Natl Acad Sci U S A. 2012;109(40):16190-5.

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