PTM- and protein-level proteome profiling of drug response in human gastric carcinoma cells using antibody and metal affinity phosphopeptide enrichment.

Matthew P. Stokes[1], Charles L. Farnsworth[1], Hongbo Gu[1], Jian Min Ren[1], Vicky Yang[1], Camilla R. Worsfold[2], Kimberly A. Lee[1], Jeffrey C. Silva[1]

(1) Cell Signaling Technology, Inc., Danvers MA 01923, (2) Emory University, Atlanta GA 30322

INTRODUCTION

Post-translational modification (PTMs) of proteins, including phosphorylation, acetylation, methylation, and ubiquitination, are critical events in all aspects of cellular signaling. Antibody-based and metal affinity-based enrichments of post-translationally modified peptides combined with LC-MS have proven to be powerful methods for the study of PTMs in a wide variety of cells and tissues, and in profiling various disease states (1-4). PTM profiling studies alone do not provide the corresponding quantitative information to describe the relative changes occurring at the protein level. Here, both antibody-based and metal affinity-based enrichments, along with total protein-based proteome profiling, are used to study the response of the human gastric carcinoma cell line MKN-45 to the Met inhibitor SU11274 and the PKC inhibitor Staurosporine.

METHODS

Human MKN-45 cells were treated with DMSO, SU11274, or Staurosporine, lysed, digested with trypsin, and desalted over C18 columns. Phosphopeptides were enriched using both IMAC and combinations of anti-phospho motif antibodies. Prior to enrichment, a portion of each sample was reserved for C18 purification on StageTips (5) and used in total protein analysis. Peptide eluates from the phosphopeptide enrichment were purified using StageTips. Immunoprecipitated peptides were run in LC-MS/MS on an Orbitrap™ Elite mass spectrometer using a top 20 DDA method. MS/MS spectra were assigned to peptide sequences using SEQUEST® (6), and label-free quantification was performed using Progenesis® with manual review of selected peptides using XCalibur™ software. Cell signaling network analysis was performed using Ingenuity Pathway Analysis (IPA) software.

REFERENCES

(1) Rush, J. et. al. (2005) *Nat. Biotechnol.* 23, 94–101. **(2)** Villen, J. et. al. (2007) *Proc. Natl. Acad. Sci.* 104, 1488–93. **(3)** Rikova, K. et. al. (2007) *Cell* 131, 1190–203. **(4)** Stokes, M.P. et. al. (2012) *Mol. Cell Proteomics* 11, 187–201. **(5)** Rappsilber, J. et. al. (2003) *Anal. Chem.* 75, 663–70. **(6)** Eng, J.K. et. al. (1994) *J. Am. Soc. Mass. Spectrom.* 5, 976–89.

CONCLUSIONS

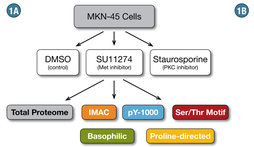
The analysis of MKN-45 cells treated with SU11274 or Staurosporine resulted in the identification of over 20,000 sites of phosphorylation across all samples. There was minimal overlap (~ 10%) between phosphopeptides identified using IMAC enrichment versus the antibody-based enrichments, emphasizing the complementarity of the two separate enrichment strategies. Thousands of phosphopeptides changed in relative abundance upon treatment with SU11274 or Staurosporine, relative to the DMSO control sample, including known targets of SU11274 such as c-terminal phosphopeptides from the Met receptor. Many changes were confirmed by western blot using total and site-specific primary antibodies (including c-Met, Erk1/2, STAT1, and Cdk1). Although thousands of changes were observed from phosphorylated peptides, there were almost no changes in protein levels between samples, indicating the drug effects at this time point (2 hours post-drug addition) are virtually all at the level of post-translational modification. Network analysis of identified proteins/sites allowed for a more complete picture of drug-induced changes in MKN-45 signaling including profound effects on canonical signaling pathways. Together, this work outlines an optimized workflow for generating the best possible data from proteomic studies.

Cell Signaling TECHNOLOGY

Matthew P. Stokes
Cell Signaling Technology
email: mstokes@cellsignal.com

www.cellsignal.com/cstposters

Figure 1: Experimental Design

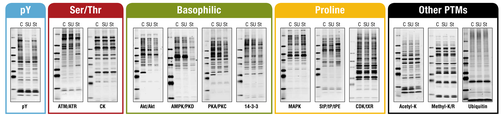


1B

Antibody	Motif	pY	Baso	Pro	Ser/Thr
Phosphotyrosine	y	●			
Akt Substrate	RXX(s/t)		●		●
Akt Substrate	RXRXX(s/t)		●		●
AMPK/PKD Substrate	LXRXX(s/t)		●		●
Cdk Substrate	(K/R)(s/t)P(X)(K/R)		●		●
PKA Substrate	(K/R)(K/R)X(s/t)		●		●
PKC Substrate	(K/R)(s/t)(K/R)		●		●
MAPK Substrate	PX(s/t)P			●	●
PLK Binding Motif	S(s/t)P			●	●
tP Motif	(s/t)P			●	●
tPE Motif	(s/t)PE			●	●
tXR/tPR Motif	(s/t)(K/P)R			●	●
14-3-3 Binding Motif	(R/K)XX(s/t)XP			●	●
ATM/ATR Substrate	(s/t)Q				●
ATM/ATR Substrate	(s/t)QG				●
CK Substrate	(s/t)(D/E)X(D/E)				●

MKN-45 cells were treated and profiled as outlined **(1A)**. Combinations of motif antibodies were used to enrich for phosphopeptides as indicated **(1B)**.

Figure 2: Western Blot Prescreen



Lysates from MKN-45 cells treated with DMSO **(C)**, SU11274 **(SU)**, or Staurosporine **(St)** were analyzed by western blot using the motif antibodies **(1B)** as well as antibodies against other post-translational modifications. Blots are organized by motif antibody mix in which they were included.

Figure 3: Overlap between antibody and metal affinity enrichments

Overlap Between Antibody and Metal Affinity Enrichments. Area proportional Venn diagram of the overlap between IMAC enrichment and motif antibody enrichment of phosphopeptides from MKN-45 cells. Comparisons were made based on unique protein/sites. Percent overlap is indicated with arrows. Antibody mixes were prepared as outlined **(1B)**.

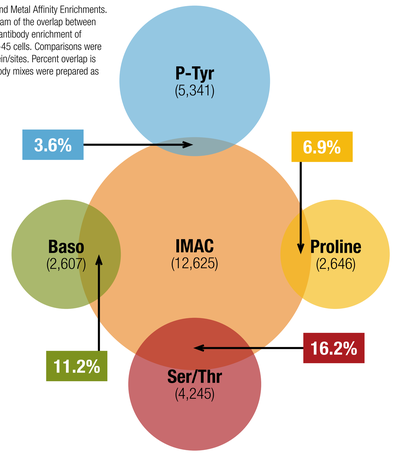
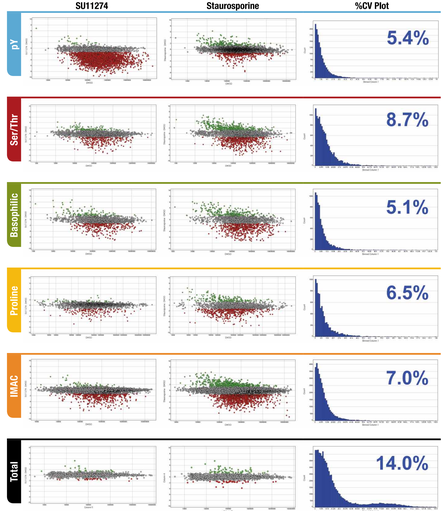
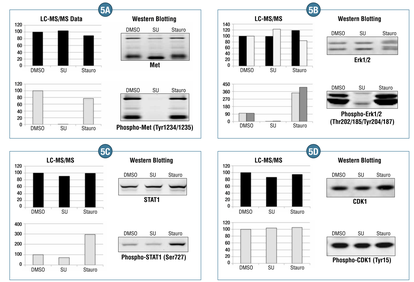


Figure 4: Quantitative Analysis



Peptides from MKN-45 cells treated with DMSO (control), SU11274, or Staurosporine were enriched using the indicated antibody or by IMAC. In parallel, total protein levels were profiled by running unenriched material in LC-MS/MS. Peptides that decreased in abundance with SU11274 or Staurosporine are indicated in red, peptides that increased with treatment in green. A % CV histogram of analytical replicates for each enrichment is shown on the right with the median % CV indicated in blue.

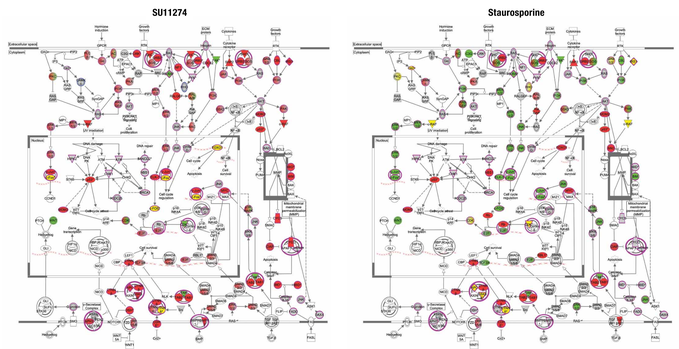
Figure 5: Western Blot Validation



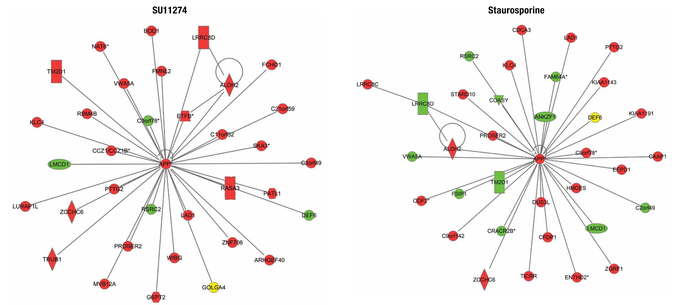
The same samples used in LC-MS/MS analysis were analyzed by western blot using antibodies against c-Met **(5A)**, Erk1/2 **(5B)**, STAT1 **(5C)**, and Cdk1 **(5D)**. Relative intensity values from LC-MS/MS analysis are shown on the left of each panel, western blots on the right. Black bars = total protein levels, grey bars = phosphopeptide levels.

Figure 6: Pathway Analysis

6A Canonical Pathway: Molecular Mechanisms of Cancer

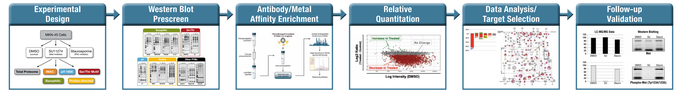


6B IPA Generated Networks: Changes Only



Data from all phosphopeptide enrichments was imported into Ingenuity Pathway Analysis (IPA) software using only direct connections with high confidence. Protein nodes were color-coded by fold change values with Red indicating a decrease in phosphopeptide abundance with treatment, green indicating an increase, and yellow indicating proteins with peptides that both increased and decreased. Both IPA Canonical Networks **(6A)** and *de novo* generated networks **(6B)** were exported from IPA.

Figure 7: Proteomic Analysis Workflow



The steps taken for experimental design, prescreening, LC-MS/MS analysis, data analysis, and follow-up are shown. Together, these steps comprise an optimized workflow for start to finish analysis of research samples.