KLARISA RIKOVA, **BENJAMIN HALL**, ANTHONY POSSEMATO, KERI MROSZCZYK, KIMBERLY A. LEE, JOAN MACNEILL, JIAN MIN REN, AILAN GUO, DANIEL MULHERN, YI WANG, SEAN A. BEAUSOLEIL, MICHAEL J. COMB

Understanding of Differing Sensitivity in EML4-ALK NSCLC Patients to Crizotinib and Geldanamycin

Abstract

Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, afflicting nearly 200,000 people in the United States each year. Abnormal ALK is found in about 5% of NSCLC cases, meaning more than 5,000 new patients could benefit from the tyrosine kinase inhibitor (TKI) crizotinib. However, not all patients benefit from such treatment, with the clinical response to crizotinib differing among patients who harbor the same molecular abnormality. Similarly, patients with ALK fusion proteins have shown varying sensitivity to the HSP90 inhibitor geldanamycin in preclinical studies.

We have chosen two NSCLC cell lines, H3122 and H2228, as a model to address these questions. Both cell lines harbor EML4-ALK fusions with differing sensitivity to the inhibitors crizotinib and geldanamycin. We used the established method of TMT peptide labeling coupled with serial peptide immunoprecipitation. To observe the effects of crizotinib and geldanamycin on ALK sensitive H3122 and non-sensitive H2228 NSCLC cell lines quantitative analysis was performed. Phosphotyrosine, acetylation, methylation, ATM/ATR substrate (s/tQ), Akt/AMPK subs AGC/CAMK/STE, and MAPK family kinase motif antibodies were used for immunoprecipitation allowing us to characterize and quantify post translational modifications before and after treatment.

In this study, we identify extensive signaling networks downstream of ALK across multiple spaces, including phosphorylation, acetylation, and methylation. These differences may be clinically significant and highlight the possibility that ALK inhibitors alone may only be effective in a subset of NSCLC ALK-positive patients with EML4-ALK inversion.

Sample	Channel
H3255 Control	126
H3255 Control	127
H3255 1 hr Iressa	128
H3255 3 hr Iressa	129
H3255 6 hr Iressa	130
H3255 24 hr Iressa	131
MKN 45 Control Starved	126
MKN 45 Control Starved	127
MKN 45 1 hr Crizotinib	128
MKN 45 3 hr Crizotinib	129
MKN 45 6 hr Crizotinib	130
MKN 45 24 hr Crizotinib	131

Sample	Channel
H2228 Control Starved	126
H2228 Control Unstarved	127
H2228 1hr Crizotinib	128
H2228 3hr Crizotinib	129
H2228 15hr Geldanamycin	130
H2228 24hr Geldanamycin	131
H3122 Control Starved	126
H3122 Control Unstarved	127
H3122 1hr Crizotinib	128
H3122 3hr Crizotinib	129
H3122 15hr Geldanamycin	130
H3122 24hr Geldanamycin	131

Table 1. 2mg of each sample was labeled using TMT reagents with 100ug used for total proteome analysis and the rest used to profile PTM spaces. Labeled samples were then combined and subjected to sequential immunoprecipitations using motif antibodies for PTMs and in parallel basic reverse phase (bRP) chromatography was performed to profile the full proteome. Samples were purified over Sep-Pak® C18 columns before being processed by LC-MS/MS/MS analysis. Data generated from the MS analysis were processed through several modules in CORE, ending with quantitated data.

Ab Target	Clone #	Motif
pY-1000	D1G10/D2D1	Phospho Tyr
AGC/PSD	D3E5/D8D9/D4E2/D8B11	AGC/CAMK/STE kinase acitivation loop
Akt substrate	100B7	RXX(s/t)
AMPK	D72H3/D78G9	LXRXXT(S)
ATM/ATR substrate	D23/D69	(s/t)Q
ATM/ATR substrate	D14/D86	(s/t)Q
Ac-K	D10G3/11D/16E	Acetyl lysine
K-Me	D4P3J, D3Z9J, D8R1C	K-Me
R-Me	Me-R4-100	R-Me
Epigenetic Regulator Mix	-	-
Ubiquitin Library	D4A7A10	kGG

Table 2. Motif antibodies chosen for PhosphoScan® profile (MS/MS).

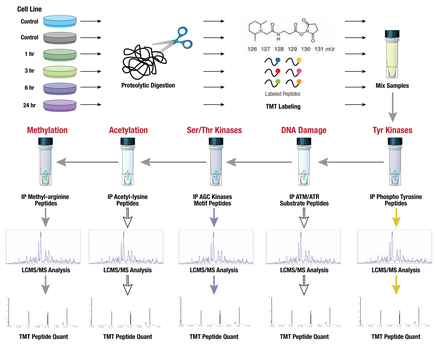

Figure 1. Experimental flowchart. Cell lysates were prepared from RTK inhibitor treated and untreated cell lines, proteins were digested with trypsin, peptides were TMT-labeled, and samples were mixed for 6-plex analysis. The peptide mixtures were serially fractionated through immunoprecipitation with antibodies specific for phosphotyrosine, ATM/ATR substrate motif, AGC/CAMK/STE family kinase motif, acetyl-lysine, and methyl-arginine, though any motif-recognizing antibody could be used with this protocol. The enriched peptides from each immunoprecipitation were analyzed by LCMS/MS using an LTQ® Orbitrap® Elite System, with quantification of the TMT labels enabled by HCD fragmentation.

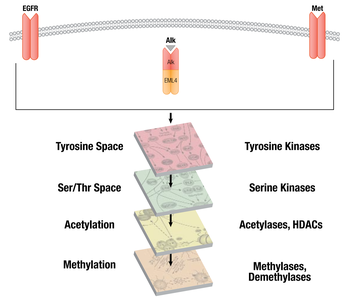

Figure 2. RTK driven cell lines profiled through the following spaces: phosphotyrosine, phosphoserine/phosphothreonine, acetylation, methylation.

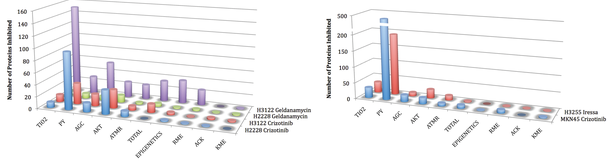

Figure 3. Number of proteins inhibited in different PTM classes (phosphotyrosine, ATM/ ATR substrate phosphorylation, AGC/CAMK/STE kinase family motif phosphorylation, AKT/AMPK, lysine acetylation, arginine methylation, lysine methylation) by greater than 3.8 fold upon TKI treatment.

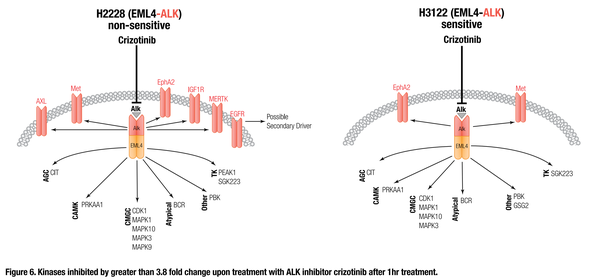

Figure 6. Kinases inhibited by greater than 3.8 fold change upon treatment with ALK inhibitor crizotinib after 1hr treatment.

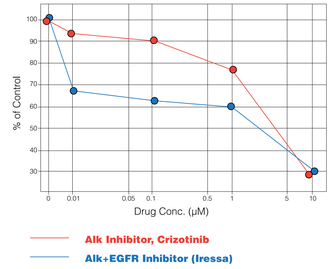

Figure 5. MTT assay showing sensitivity of H2228 to crizotinib vs. crizotinib combined with iressa.

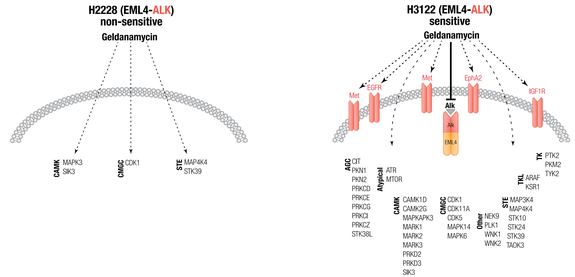

Figure 4. Kinases inhibited by greater than 3.8 fold change upon treatment with HSP90 inhibitor geldanamycin after 24hr treatment.

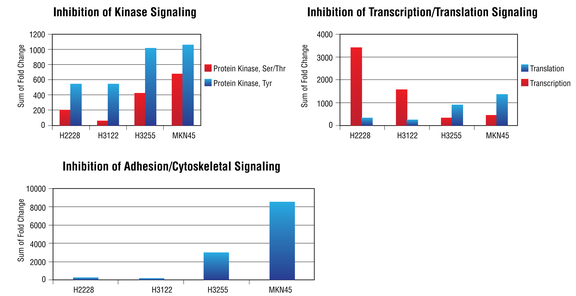

Figure 7. Sum of fold change above 3.8 in RTK driven cell lines upon treatment with TKIs by protein type (transcriptional regulation, translation, protein kinsases, cytoskeletal/adhesion).

Summary

- We have developed a unique proteomic approach to elucidate changes in signaling pathways upon drug treatment in RTK driven cell lines.
- Our data suggest that ALK activates different pathways in ALK driven cell lines in comparison to cell lines driven by EGFR/Met.
- The presence of EGFR as a parallel pathway in H2228 cells could explain the differing sensitivity observed to the ALK inhibitor crizotinib.
- Geldanamycin inhibited ALK and other RTKs in a crizotinib-sensitive cell line (H3122) and had no effects on RTKs in a crizotinib-insensitive cell line (H2228).
- Therapy with ALK inhibitors alone is only effective in a subset of NSCLC ALK-positive patients with EML4-ALK fusions. In cases of resistance to ALK inhibitors, combination therapy may be desirable.

Contact Information

Klarisa Rikova
Cell Signaling Technology, Inc. **Tel:** (978) 867-2330 **Email:** krikova@cellsignal.com

Cell Signaling TECHNOLOGY®