Kinase Inhibition Studies Using High Resolution/Accurate Mass MS Targeted Approach and Nucleotide Probes

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Overview

Purpose: To develop an integrated strategy for detection, quantitation, and verification of global kinase profiling for drug inhibition studies.

Methods: Desthiobiotin-ATP and -ADP probes were used to selectively label kinase active-sites in K562 cell lysates before enrichment of active-site, enzymatic peptides. Labeled-active-site peptides were identified, characterized and quantified using orbitrap mass spectrometry.

Results: A total of 200 kinases were identified and quantified for the K562 cell line using desthiobiotin-ATP and -ADP probes. Inhibitor titration studies using HR/AM MS level quantitation showed that approximately 75% of the kinases are partially or completely inhibited following staurosporine treatment.

Introduction

Development of novel protein kinase inhibitors remains a strong focus of the pharmaceutical industry. Performing multiplexed protein kinase detection and activity assessment in complex mammalian proteomes presents many challenges due to the large number of kinases, substrate overlap of related kinases, and wide dynamic range of kinase expression. Recently, ATP and ADP nucleotide derivatives have been used as ATPase active-site probes to enrich kinases, as well as assess global kinase inhibition using tandem mass spectrometry (Figure 1).[1] Although considered essential for discovery-based experiments, the application of high resolution/accurate mass (HR/AM) MS to targeted peptide analyses has been less explored until recently.[2] Here, we present a HR/AM MS targeted approach for kinase inhibitor screening with an integrated sample preparation, data acquisition and data processing workflow.

Methods

Sample Preparation

K562 cell lines were grown in RPMI media supplemented with 10% FBS. Cell lysates (1 mg) were desalted using 7K Thermo Scientific Zeba Spin Desalting Columns and labeled with 5 µM of desthiobiotin-ATP or -ADP for 10 minutes. For inhibitor profiling, cell lysates were pretreated with 0-10 µM of staurosporine before labeling with desthiobiotin nucleotide probes. Labeled proteins were digested with a proteolytic enzyme and captured with Thermo Scientific High Capacity Streptavidin Agarose Resin for 2 hours. Bound peptides were washed and eluted using 50% acetonitrile/0.1% TFA. For Western blot analysis, labeled proteins were captured on streptavidin agarose beads and eluted with SDS-PAGE sample buffer before SDS-PAGE separation and Western blotting with specific antibodies.

LC/MS

A Thermo Scientific EASY-nLC nano-HPLC system and Magic™ C18 spray tip (15 cm x 75 µm i.d. column, Michrom Bioresources) were used to separate peptides with a 5-45% acetonitrile gradient in 0.1% formic acid over 70 min. at a flow rate of 300 nL/min. The samples were analyzed with a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer.

Data Analysis

Thermo Scientific Proteome Discoverer 1.3 software was used to search MS/MS spectra against the International Protein Index (IPI) human database using both SEQUEST® (University of Washington) and Mascot™ (Matrix Science, Ltd.) search engines. Static modifications included carbamidomethyl (57.02 Da) with desthiobiotin (196.12 Da) and methionine oxidation used as dynamic modifications. Database search results were imported into Thermo Scientific Pinpoint 1.1 software to perform HR/AM MS-level quantitation. Data extraction was based on the four most abundant isotopes per charge state per targeted peptide. The area under the curve (AUC) values were summed for total AUC values. The relative AUC values for each of the isotopes were compared against the theoretical isotopic distribution for confirmation.

FIGURE 1. Mechanism of desthiobiotin-ATP and -ADP labeling of kinase active-sites.

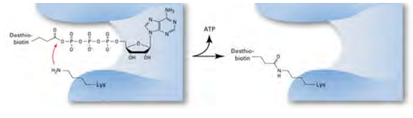
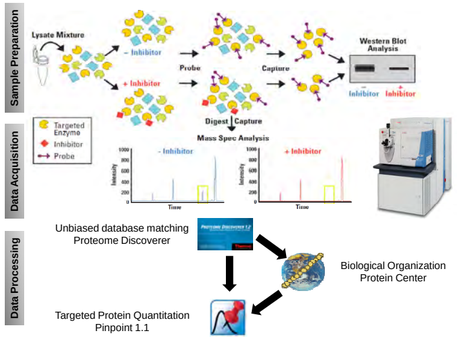


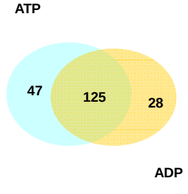
FIGURE 2. Protein and peptide workflows for the profiling, capture, and detection of ATPases including kinases, chaperones, and metabolic enzymes with active-site probes. Pre-incubation of enzymes with inhibitors allows for the determination of binding affinity by Western blot and mass spectrometry.



Results

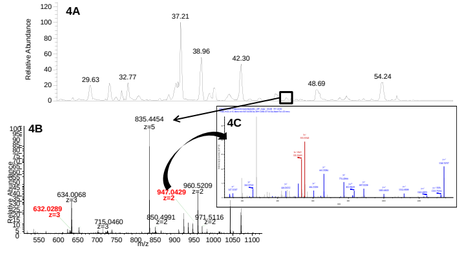
The ActivX™ Desthiobiotin-ATP and -ADP probes (ActivX Biosciences) were used in an integrated workflow for global kinase identification and inhibition analysis (Figure 2). In the first step, kinase spectra libraries were created through data-dependent MS/MS acquisition of enriched kinase active-site peptides. Thermo Scientific Proteome Discoverer software spectral library searches identified, in total, 200 kinases using ATP or ADP nucleotide probes (Figure 3). Spectral libraries generated by Proteome Discoverer were then imported into Pinpoint 1.1 software for quantification. The Proteome Discoverer spectral libraries contain peptide sequences, precursor charge state distribution, retention times, and product ion distribution. An example of the analysis is presented in Figure 4. The high resolution and mass accuracy of the LTQ Orbitrap Velos™ mass spectrometer, as well as the wide dynamic range afforded by the high charge density in the trap, facilitated the global kinase active-site peptide identification. Precursor charge states were detected at 15 and 4% relative abundance in a complex background. Despite low levels of some precursors, unbiased data-dependent acquisition resulted in a high confidence verification of target peptides. The high resolution detection capabilities of the LTQ Orbitrap Velos mass spectrometer enabled the isotopic profile to be detected, even at levels less than 2% relative abundance and matched to theoretical values.

FIGURE 3. Number of protein kinases identified by LTQ Orbitrap Velos using ActivX Destiobiotin-ATP or -ADP probes.



To confirm peptide precursor identity, we employed an analysis which extracts the four most abundant isotopes and generates extracted ion chromatographic (XIC) profiles (Figure 5). The XICs are overlaid to confirm the retention time. Each XIC is integrated and summed to calculate the response for each sample. In addition to measuring response for the active-site peptide, the relative abundance profile for the isotopic distribution is compared against the theoretical distribution for correlation analysis. The resulting coefficient enables background interference analysis. The area-under-the-curve (AUC) values for each isotope per charge state were used to determine the relative amount for each targeted peptide. Due to the high resolution, the background interference is almost completely eliminated enabling accurate quantitation.

FIGURE 4. Data processing strategies used to identify, characterize, and quantitate the active site peptides tagged with the biotin modification. Figure 4A shows the base peak chromatogram for kinase extraction following 0.01 µM staurosporin addition. The full scan shows the RT point for the targeted peptide DIK*PENLLLGSAGELK from AurA (Figure 4B), and the full scan MS/MS spectrum acquired in the ion trap mass spectrometer for sequence verification. The +2 precursor charge state triggered the MS/MS data acquisition.



AUC values are summed, increasing the signal-to-noise value that increases detection limits. The relative abundance from the four isotopic XICs are compared to the theoretical isotopic distribution as a function of each sequence by a Pearson's correlation at 0.95 confidence.

The summed AUC values were compared as a function of staurosporine concentration to determine % kinase inhibition. Figure 6 shows the results for Aurora A kinase (AurA, O14965, Kinexus, CA) K2 active site peptide quantitation across the staurosporine dose range. A rapid decrease in the measured AUC values for the targeted peptide resulted in a low IC_{50} value, consistent with this kinase being inhibited by low concentrations of staurosporine treatment as shown by Western blot (Figure 7A). The reproducibility of the measurements were determined using the technical replicates across staurosporin titration levels, resulting in over a 10-fold AUC value until it is completely inhibited. Confirmation of AurA active-site peptide detection and integration was evaluated across all samples and injections using the strategy shown in Figure 5. The overlap analysis for all samples and injections is shown in Figure 6B. The isotopic distribution for the targeted peptide across all injections shows excellent overlap despite the low-level signal resulting from inhibition. The correlation coefficients per sample are also listed.

FIGURE 5. HR/AM data extraction and correlation method for targeted acquisition and quantitation. A theoretical mass spectrum is used to determine the four most abundant isotopes per targeted peptide for data extraction as a function of mass tolerance (±5 ppm). The relative abundance values generated from the XICs are used for the correlation coefficient analysis to determine overlap.

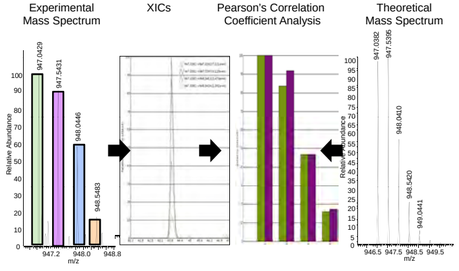
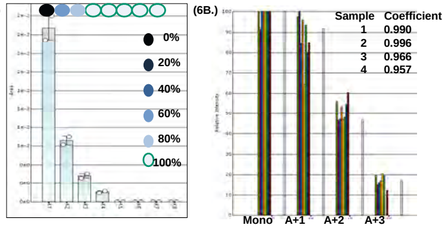


FIGURE 6. Histogram for the AUC values of the targeted peptide DIK*PENLLLGSAGELK from AurA kinase across all of the staurosporine titration samples. Decreased AUC values are used to determine drug inhibition effects. Colored spots represent Western blot analysis. Figure 5B shows the isotopic correlation between the experimental and theoretical distribution used for peptide identity confirmation. Each color represents a separate injection.



Differential expression analysis is used to determine the IC_{50} values for each kinase and identify on- and off-target effects of inhibitors across ATPase family groups. Figure 7 shows the comparison of Western blot (WB) to targeted mass spectrometry results for selected kinases. The WB results shows that AurA is inhibited at lower levels than Rsk2 and Cdk5. The corresponding targeted mass spectral analysis shows similar response. Figure 7B shows the comparative inhibition analysis for a series of kinases, including the three in the WB analysis. Additional targeted kinase responses are also presented to demonstrate global quantitation for different responding targeted peptides. Some show little inhibition (Akt1), while others are effected at low staurosporine levels. Overall, 105 kinases were quantified, and 75% of quantified proteins were sensitive to staurosporine treatment (Table 1).

FIGURE 7. (A.) Western blot analysis for three representative kinases as a function of staurosporine (stauro) titration amounts. (B.) Inhibition curves generated from MS analysis of kinase active site peptides.

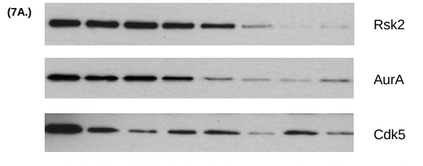


TABLE 1. Relative quantitation of 105 kinase with and without 10 µM staurosporine treatment enriched with desthiobiotin-ATP or ADP from K562 cell lysates.

% Inhibition: > 90%, 60-89%, 30-59%, < 30%

Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition
ABL1/2	75%	IRE1	90.00%	p70S6K	70.00%
ACK	100.00%	IRAK4	100.00%	PAN3	0.00%
AMPKa1/2	100.00%	KHS1/2	100.00%	PDK1	0.00%
ATR	50.00%	LATS1	100.00%	PHKg2	100.00%
AurA/B/C	100.00%	LKB1	100.00%	PIP5K2c	0.00%
AurA	100.00%	LOK	100.00%	PITSLRE	0.00%
AurB	100.00%	LYN	100.00%	PKCi	80.00%
BRAF	0.00%	MAP2K1/2	90.00%	PKD2	100.00%
CaMK2g	100.00%	MAP2K3	100.00%	PKD3	100.00%
CAMK2	0.00%	MAP2K4	95.00%	PKR	30.00%
CDC2	50.00%	MAP3K1	80.00%	PLK1	0.00%
CDK5	100.00%	MAP3K2	100.00%	PKN1	100.00%
CDK6	65.00%	MAP3K4	0.00%	PRPK	0.00%
CDK7	100.00%	MAP3K5	100.00%	PRP4	40.00%
CDK8/11	100.00%	MARK1/2	100.00%	PRKDC	0.00%
CDK9	0.00%	MARK2	100.00%	PYK2	0.00%
CDK10	100.00%	MARK3	100.00%	ROCK1/2	100.00%
CDK12	0.00%	MARKY	100.00%	RSK1	95.00%
CHK1	95.00%	MAST3	65.00%	RSK1/2/3	100.00%
CHK2	97.00%	MASTL	0.00%	RSK2(1)	100.00%
CRK7	100.00%	MINK	100.00%	RSK2(2)	0.00%
CSK	85.00%	MLK3	100.00%	SGK3	100.00%
EF2K	0.00%	MST1	100.00%	SRPK1/2	50.00%
EphA1	66.00%	MST2	100.00%	STLK6	0.00%
EphA7	33.00%	MST3	93.00%	SYK	100.00%
EphB2	88.00%	MST4	100.00%	TAO1/3	100.00%
Erk1/2	0.00%	NDR1	95.00%	TAO2	100.00%
FAK	100.00%	NDR2	0.00%	TEC	100.00%
FER	100.00%	NEK1	20.00%	TLK1/2	100.00%
GCK	100.00%	NEK3	0.00%	TYR03	100.00%
GCN2	75.00%	NEK7	0.00%	ULK3	100.00%
IKKa	0.00%	NEK9	40.00%	Wnk1/2/4	0.00%
IKKb	0.00%	p38a	0.00%	YES	100.00%
ILK	10.00%	p70S6Kb	0.00%	ZAK	0.00%

Conclusion

The integrated workflow provides a means to perform global kinase profiling for drug inhibition studies as a function of tissues and cell lines. Additional biotinylated probes are under evaluation to increase kinase coverage.

- Desthiobiotin-ATP and –ADP probes provide an effective method for tagging and enriching for active-site kinase peptides.
- The probes enabled a direct means of evaluating drug inhibition studies for global kinases.
- Targeted quantitation using HR/AM MS on the LTQ Orbitrap Velos mass spectrometer provided the necessary capabilities to simultaneously quantitate large numbers of kinases covering large dynamic ranges.
- Pinpoint software provides automated data analysis, confirmation, and quantitation for rapid data processing for kinase inhibition studies.

References

1. Patricelli, M.P., *et al.* (2007). Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* **46:**350-8.
2. Jaffe, J.D., *et al.* (2008). Accurate inclusion mass screening: a bridge from unbiased discovery to targeted assay development for biomarker verification. *Mol. Cell. Proteomics* **7:**1952-62.