Profiling Kinase Inhibition Using Active-Site Probes and Targeted High-Resolution, Accurate Mass Spectrometry

P1409

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Overview

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

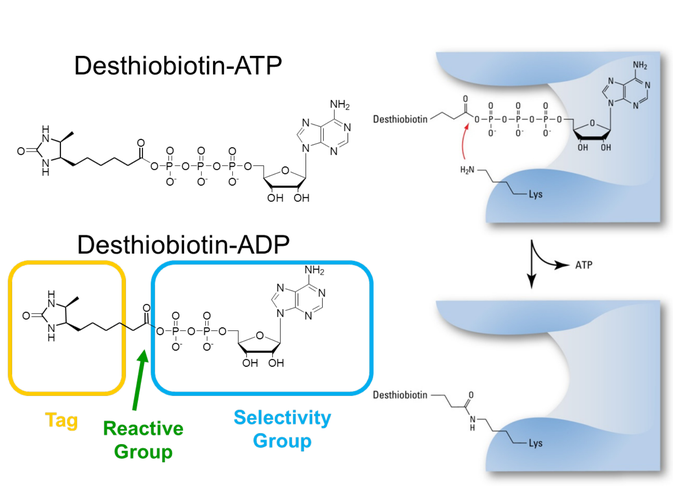
Methods: K562 cell lysates were treated with different concentrations of staurosporine. Each sample was reacted with desthiobiotin-ATP or -ADP probes and then processed to enrich labeled kinase active-site peptides. Labeled-active-site peptides were identified, characterized and quantified using Orbitrap 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 high-resolution, accurate mass 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 an HR/AM MS targeted approach for kinase inhibitor screening with an integrated sample preparation, data acquisition and data processing workflow.

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



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 minutes 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 software version 1.3 was used to search MS/MS spectra against the Swiss-Prot 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 software version 1.2 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.

Results

The Thermo Scientific ActivX Desthiobiotin-ATP and -ADP probes 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. 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 software were then imported into Pinpoint™ software for quantification.

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.

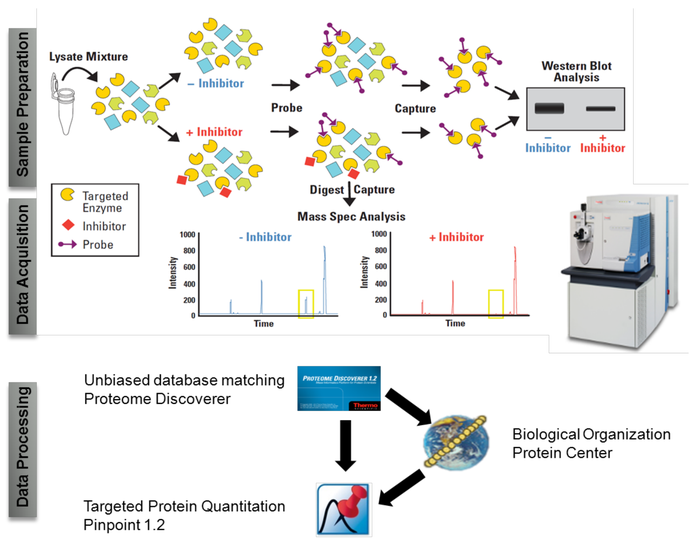


FIGURE 3. Number of protein kinases identified by LTQ Orbitrap Velos™ MS using ActivX™ Destiobiotin-ATP or -ADP probes

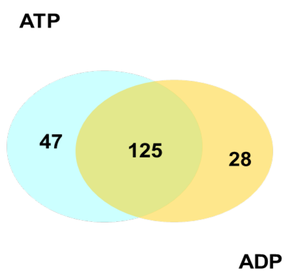


Figure 4 shows the results for Tao 1/3 kinase (Q9H2K8) K2 active site peptide quantitation across the staurosporine dose range. Precursor charge states were detected at 4% relative abundance in a complex background (Figure 4A). To confirm peptide precursor identity, we employed an analysis that extracts the four most abundant isotopes and generates extracted ion chromatographic (XIC) profiles. The XICs were overlaid to confirm the retention time (Figure 4B).

FIGURE 4. Mass spectrometry analysis of Tao 1/3 K2 site (DIkAGNILLTEPGQVK) staurosporine inhibition

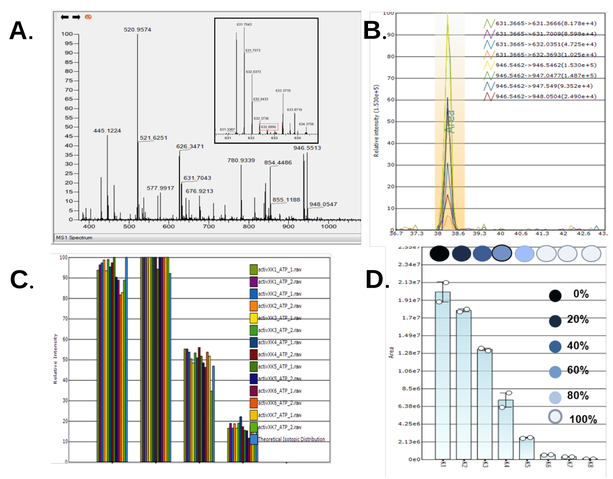
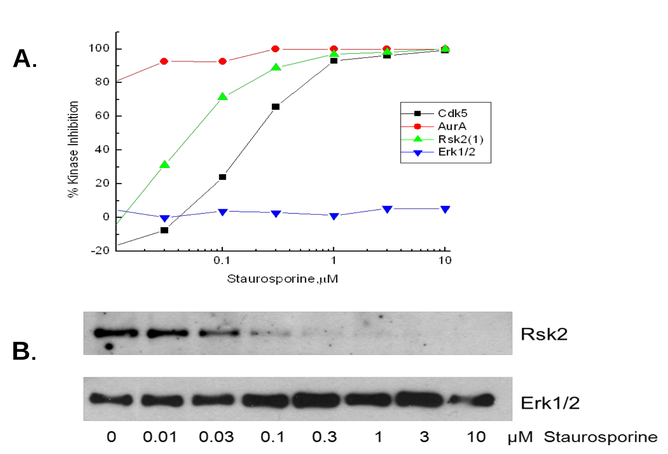


FIGURE 5. (A.) Inhibition curves generated from MS analysis of kinase active site peptides. (B.) Western blot analysis for two representative kinases as a function of staurosporine (stauro) titration amounts.



Each XIC was 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 was compared against the theoretical distribution for correlation analysis (Figure 4C). The area-under-the-curve (AUC) values for each isotope per charge state were used to determine the relative amount for each targeted peptide. AUC values were summed, increasing the signal-to-noise value that increases detection limits. The relative abundance from the four isotopic XICs were 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 4D). The reproducibility of the measurements was determined using the technical replicates across staurosporine titration levels.

To validate our targeted mass spectrometry results, we performed Western blot analysis for selected kinases. Figure 5A shows the inhibition curves for four kinases (Cdk5, AurA, Rsk2, and Erk1/2). Figure 5B is a Western blot for two kinases showing Erk1/2 is not sensitive to staurosporine treatment where Rsk2 is inhibited at concentrations above 30 nM. Additional kinase active-site peptides were targeted for global quantitation of staurosporine kinase inhibition. Differential expression analysis was used to determine the IC_{50} values for each kinase[3] and identify on- and off-target effects of inhibitors across ATPase family groups. Overall, 105 kinases were quantified, and 75% of quantified proteins were sensitive to staurosporine treatment (Figure 6 and Table 1).

FIGURE 6. Kinase inhibition response distribution to staurosporine in K562 cell lysates

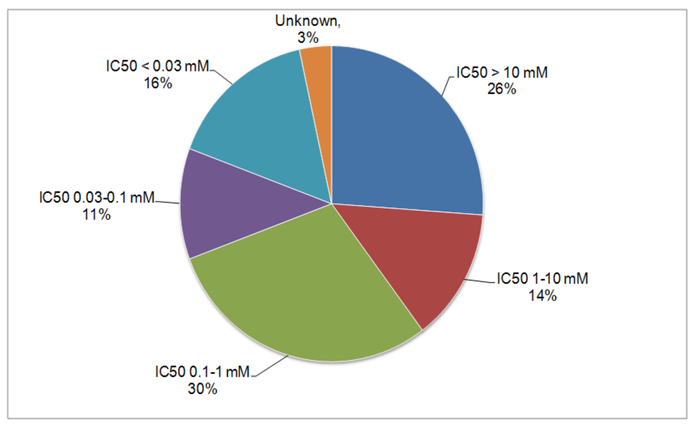


TABLE 1. Relative quantitation of 105 kinases with and without 10 µM staurosporine treatment enriched with desthiobiotin-ATP or -ADP from K562 cell lysates

Kinase	% Inhibition	Kinase	% Inhibition
ABL1/2	75%	MARKY	100.00%
ACK	100.00%	MAST3	65.00%
AMPKa1/2	100.00%	MASTL	0.00%
ATR	50.00%	MINK	100.00%
AurA/B/C	100.00%	MLK3	100.00%
AurA	100.00%	MST1	100.00%
AurB	100.00%	MST2	100.00%
BRAF	0.00%	MST3	93.00%
CaMK2g	100.00%	MST4	100.00%
CAMKK2	0.00%	NDR1	95.00%
CDC2	50.00%	NDR2	0.00%
CDK5	100.00%	NEK1	20.00%
CDK6	65.00%	NEK3	0.00%
CDK7	100.00%	NEK7	0.00%
CDK8/11	100.00%	NEK9	40.00%
CDK9	0.00%	p38a	0.00%
CDK10	100.00%	p70S6Kb	0.00%
CDK12	0.00%	p70S6K	70.00%
CHK1	95.00%	PAN3	0.00%
CHK2	97.00%	PDK1	0.00%
CRK7	100.00%	PHKg2	100.00%
CSK	85.00%	PIP5K2c	0.00%
EF2K	0.00%	PITSLRE	0.00%
EphA1	66.00%	PKCi	80.00%
EphA7	33.00%	PKD2	100.00%
EphB2	88.00%	PKD3	100.00%
Erk1/2	0.00%	PKR	30.00%
FAK	100.00%	PLK1	0.00%
FER	100.00%	PKN1	100.00%
GCK	100.00%	PRPK	0.00%
GCN2	75.00%	PRP4	40.00%
IKKa	0.00%	PRKDC	0.00%
IKKb	0.00%	PYK2	0.00%
ILK	10.00%	ROCK1/2	100.00%
IRE1	90.00%	RSK1	95.00%
IRAK4	100.00%	RSK1/2/3	100.00%
KHS1/2	100.00%	RSK2(1)	100.00%
LATS1	100.00%	RSK2(2)	0.00%
LKB1	100.00%	SGK3	100.00%
LOK	100.00%	SRPK1/2	50.00%
LYN	100.00%	STLK6	0.00%
MAP2K1/2	90.00%	SYK	100.00%
MAP2K3	100.00%	TAO1/3	100.00%
MAP2K4	95.00%	TAO2	100.00%
MAP3K1	80.00%	TEC	100.00%
MAP3K2	100.00%	TLK1/2	100.00%
MAP3K4	0.00%	TYR03	100.00%
MAP3K5	100.00%	ULK3	100.00%
MARK1/2	100.00%	Wnk1/2/4	0.00%
MARK2	100.00%	YES	100.00%
MARK3	100.00%	ZAC	0.00%
MARKY	100.00%		

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

Conclusion

The integrated workflow provides a means to perform global kinase profiling for drug inhibition studies in tissue and cell lysates.

- 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 instrument 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

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