Relative Quantitation of Kinase Inhibition in Neuroblastoma Cells Using Active-Site Probes and Tandem Mass Tags

Ryan D. Bomgarden[1], Chris L. Etienne[1], Michael M. Rosenblatt[1], Eugene Cichon[1], Rosa I. Viner [2], Matthew P. Patricelli[3], John C. Rogers[1]
[1]Thermo Fisher Scientific; Rockford, IL; [2]Thermo Fisher Scientific, San Jose, CA; [3]ActivX Biosciences Inc, La Jolla, CA

Overview

Purpose: To determine relative kinase expression and inhibition in TrkA- and TrkB-expressing neuroblastoma cells.

Methods: Active-site probes were used to enrich and profile kinase activity. Kinase abundance was determined by mass spectrometry (MS) of active-site peptides labeled with isobaric Thermo Scientific Tandem Mass Tag (TMT) reagents.

Results: Combining active-site probe labeling with TMT® reagents resulted in identification and relative quantitation of over 150 kinases with more than 20 showing > 50% inhibition by staurosporine and/or lestaurtinib.

Introduction

Neuroblastoma is one of the most common and deadly tumors in children. Clinical behavior of this disease is variable; some cases spontaneously regress, and others are untreatable despite maximally tolerable chemotherapy. Although much genetic data has been collected on these tumors, the biology of this disease is not well understood. Current evidence suggests that the tropomyosin-receptor kinase (Trk) family of neurotrophin receptors is critical in neuroblastoma phenotypes and prognosis. Tumors expressing TrkA are usually benign and prone to spontaneous regression; however, tumors expressing TrkB have poor prognosis and are associated with MYCN amplification.[1]

To gain more insight into this pathology, we examined the relative expression of kinases in parental SH-SY5Y cells or cells stably expressing TrkA or TrkB. We used a proteomic approach with desthiobiotin nucleotide probes to specifically capture and profile the kinome of each cell line using mass spectrometry to identify labeled kinase active-site peptides.[2] Differential expression of protein kinases between cell lines was assessed by relative quantitation of labeled kinase active-site peptides and validated using a parallel Western blot workflow. We also used kinase inhibitors staurosporine and lestaurtinib to profile kinase targets using TMT reagents. Combining active-site probe labeling with TMT reagents enabled concurrent identification and multiplexed quantitation of kinases in TrkA and TrkB cells.

Methods

Sample Preparation

Cell culture and kinase labeling: TrkA and TrkB stable SH-SY5Y cell lines were grown in RPMI with 10% FBS and 0.3 mg/mL G418 (Sigma). Cell lysates (1 mg) were desalted using 7K Thermo Scientific Zeba Spin Desalting Columns and labeled with 5 µM of desthiobiotin-ATP and -ADP for 10 minutes. Labeled proteins were denatured with 6 M urea and captured with Thermo Scientific High Capacity Streptavidin Agarose Resin. Bound proteins were washed and eluted by boiling in sample buffer before SDS-PAGE separation and Western blotting using specific antibodies.

Active-site peptide capture and TMT reagent labeling: Desthiobiotin-ATP and -ADP labeled proteins were reduced and alkylated before buffer exchange into digestion buffer (20 mM Tris pH 8.0, 2 M urea). Each sample was enzymatically digested for 2 hr before capture with streptavidin agarose resin and elution using 50% acetonitrile/0.1% TFA. For TMT reagent labeling, active site-labeled proteins were enzymatically digested after streptavidin agarose resin capture and eluted using 50% acetonitrile/0.1% TFA. Enriched peptides were labeled with TMTsixplex™ reagent (126-131) for 2 hr at room temperature, quenched with hydroxylamine, desalted using C18 resin, and combined before LC-MS/MS analysis.

LC/MS

A NanoLC-2D™ High-Pressure Liquid Chromatograph (HPLC) (Eksigent) with a Magic C18 column 75-µm ID x 20 cm (Michrom) was used to separate peptides using a 5-35% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 250 nL/min for 90 min. A Thermo Scientific LTQ Orbitrap XL hybrid mass spectrometer with equipped with electron transfer dissociation (ETD) was used to detect peptides using a top 3 x 3 experiment consisting of single-stage MS followed by acquisition of three MS/MS spectra with higher-energy collisional dissociation (HCD) fragmentation. Finally, three MS/MS with collision-induced dissociation (CID) was used for protein identification.

Data Analysis

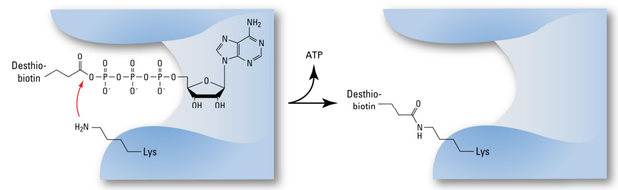
Using the Thermo Scientific Proteome Discoverer 1.2 software, CID and HCD data were processed through a branched workflow that implemented a reporter ion quantizer (20 ppm mass tolerance of fragment ions) to quantify ratios. Data were searched using both SEQUEST® and Mascot™ search engines against the IPI human database. Static modifications included carbamidomethyl (57.02 Da), N-terminal TMTsixplex (229.16 Da). Desthiobiotin (196.12 Da), TMT K and methionine oxidation were used for dynamic modifications.

Results

Thermo Scientific ActivX Desthiobiotin-ATP and -ADP Probes are nucleotide derivatives that covalently modify the active site of enzymes at conserved lysine residues in the nucleotide binding site (Figure 1).[2,3] The structure of these probes consists of a modified biotin (desthiobiotin) attached to the nucleotide through a labile acyl-phosphate bond. Desthiobiotin is a biotin analog that binds less tightly to biotin-binding proteins, resulting in binding that is easily reversed by biotin displacement, low pH or heat denaturation. Depending on the position of the lysine within the enzyme active site, either desthiobiotin-ATP or -ADP may be better for labeling specific ATPases.

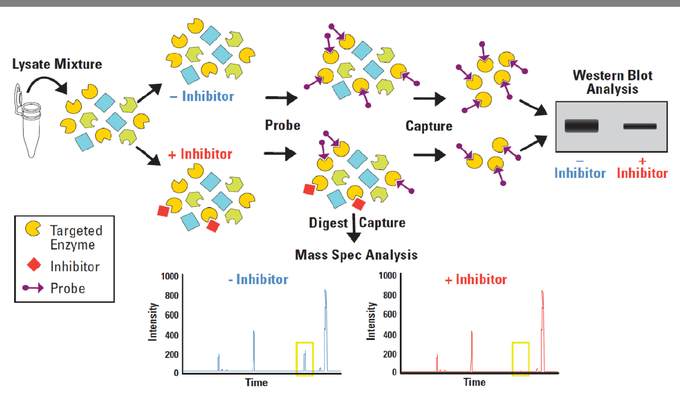
Both desthiobiotin-ATP and -ADP can be used to selectively enrich, identify and profile target enzyme classes in samples by Western blot or MS (Figure 2). Because many ATPases and other nucleotide-binding proteins bind nucleotides or inhibitors even when they are enzymatically inactive, the desthiobiotin probes allow profiling of both inactive and active enzymes in a complex sample. Pre-incubation of samples with small-molecule inhibitors that compete for active-site probes can be used to determine inhibitor binding affinity and target specificity.

FIGURE 1. Active-site probe structure and mechanism.



Desthiobition-ATP and -ADP bind to the active sites of ATPases where the desthiobiotin affinity tag is irreversibly transferred to highly conserved lysine residues in the active site.

FIGURE 2. Schematic of Western blot and mass spectrometry workflows.



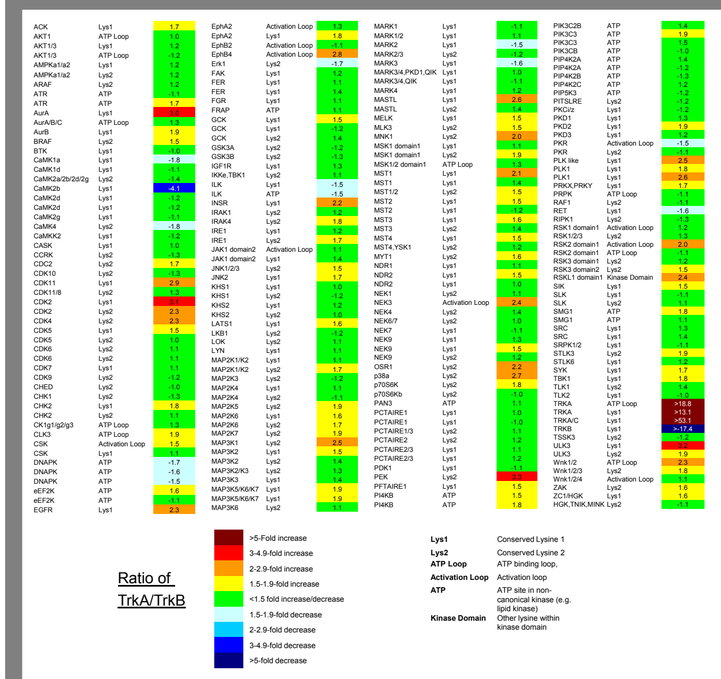
Protein and peptide workflows for the profiling, capture, and detection of ATPases including kinases, chaperones and metabolic enzymes with active-site probes. Preincubation of enzymes with inhibitors enables determination of binding affinity by Western blot or MS.

Using the active-site probe MS workflow, we identified and quantified over 150 kinases in TrkA- and TrkB-expressing cells. Twelve kinases resulted in greater than two-fold increased expression in TrkA- versus TrkB-expressing cells (Figure 3).

We also used these probes in combination with TMT Reagents quantitation to measure multiplexed kinase expression and inhibition in TrkA- and TrkB-expressing neuroblastoma cells. TMT reagents are isobaric MS quantification reagents that produce a unique reporter ion during MS/MS analysis of peptides. The TMTsixplex reagent workflow for peptide labeling after sample treatment and digestion is shown (Figure 4).

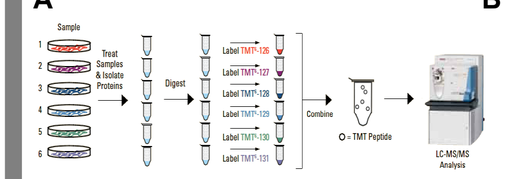
The Western blot of TrkA vs TrkB cell lysates after kinase enrichment using desthiobiotin-ATP probe shows differential kinase expression (Figure 5A). Both RSK2 and AurA resulted in higher expression in TrkB cells compared to TrkA cells. Cdk5 had over two-fold lower expression in TrkB-expressing cells and correlated well with TMT reagent quantitation by MS (Figure 5B).

FIGURE 3. Profile of Trk cell kinase expression



Relative quantitation of 154 kinases (219 peptides) in TrkA SH-SY5Y-stable cells compared to TrkB-expressing cells using desthiobiotin-ADP in the MS workflow.

FIGURE 4. TMT reagent workflow and labeling scheme.

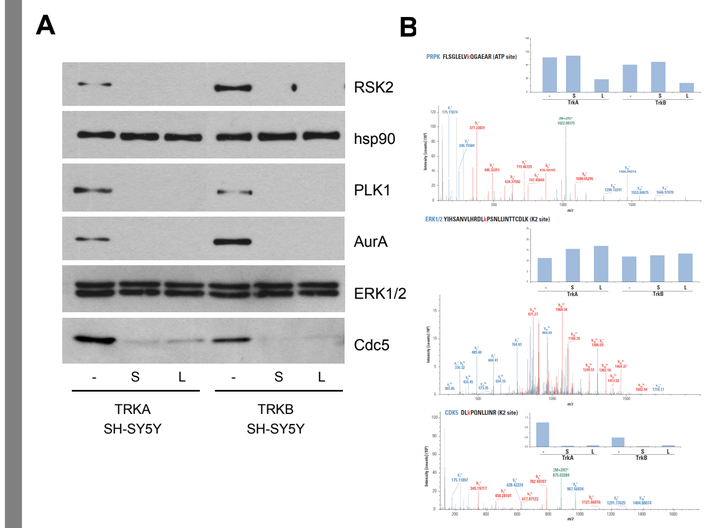


B

Inhibitor	Cell line	TMT Reagent
No treatment	TrkA	126
Staurosporine	TrkA	127
Lestaurtinib	TrkA	128
No treatment	TrkB	129
Staurosporine	TrkB	130
Lestaurtinib	TrkB	131

(A) Sixplex TMT reagent workflow for peptide labeling after sample treatment and digestion. (B) Table of different cells and inhibitor treatments labeled with TMTsixplex reagents.

FIGURE 5. Kinase inhibitor assessment by Western blot and mass spectrometry.



A) TrkA or TrkB cell lysates were pretreated with 100 µM of staurosporine (S) or lestaurtinib (L) before labeling with 5 µM of desthiobiotin-ATP probe and enriched using streptavidin agarose before Western blotting. B) Mass spectra of TMT-labeled kinase peptides showing changes in relative peptide abundance after each drug treatment as described in Figure 4B.

Treatment of cell lysates with staurosporine or lestaurtinib before labeling with desthiobiotin-ATP probe resulted in significant reduction in RSK2, PLK1, AurA and Cdk5 kinase enrichment for both inhibitors. In contrast, we observed no differences in expression or inhibition for mitogen-activated protein kinase 3 (ERK 1/2) and the chaperone hsp90. These data correlated well with changes in relative peptide abundance after each drug treatment. We were also able to identify proteins that showed specific inhibition in response to each drug, such as PRPK (TP53 kinase) for lestaurtinib and AMPK2 for staurosporine (Figure 5B and data not shown).

Conclusion

- Active-site kinase labeling and enrichment resulted in identification and quantification of over 150 human kinases in TrkA- and TrkB-expressing cell lines.
- More than 18 kinases resulted in > two-fold increased expression in TrkA- versus TrkB-expressing cells.
- Mass spectrometry of active-site-labeled peptides correlated with Western blot analysis of labeled proteins.
- Combining active-site probe labeling with TMT reagents resulted in identification and relative quantitation of more than 50 kinases, some of which were inhibited by staurosporine and lestaurtinib.

References

1. Brodeur, G.M., *et al.* (2009). Trk receptor expression and inhibition in neuroblastomas. *Clin Cancer Res* **15(10)**:3244-50.
2. Patricelli, M.P., *et al.* (2007). Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* **46:**350-8.
3. Okerberg, E.S., *et al.* (2005). High-resolution functional proteomics by active-site peptide profiling. *Proc Natl Acad Sci USA* **102(14)**:4996-5001.

Acknowledgements

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