A Novel Fragment Ion Tag Approach to the Measurement of Tyrosine Kinase Signaling Pathways

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ABSTRACT

• Anti-phosphotyrosine affinity chromatography coupled with multiplex isobaric peptide labeling was used to monitor inhibition of c-kit, a receptor tyrosine kinase, by a selective thiophene inhibitor of kit and KDR kinase activity.
• Protein measurement was achieved using a multiplexed set of isobaric reagents which yield amine-derivatized peptides that are chromatographically identical and indistinguishable in MS, but which produce strong low-mass MS/MS signature ions following CID that permit quantitation. The complexity of MS and MS/MS data is not increased by mixing multiple proteome samples together.
• We have incorporated this quantitation technology into a simple workflow consisting of parallel protein extraction, digestion and peptide labeling followed by simple cation exchange fractionation to remove interfering sample and buffer artifacts and nano RP-HPLC of the combined mixture. The use of these labels therefore permits simultaneous measurement of relative and/or absolute protein abundance of multiple, complex samples.

INTRODUCTION

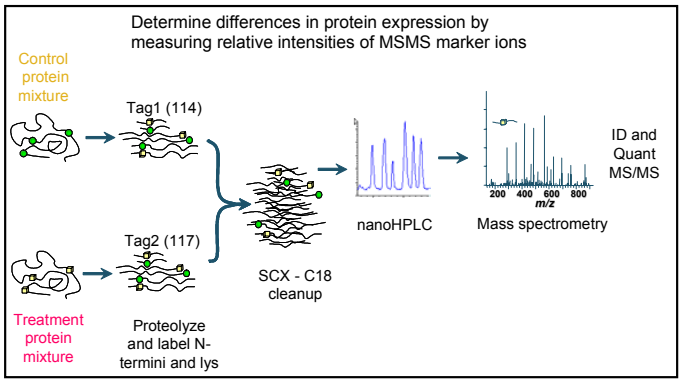
Disregulation of tyrosine kinases are frequent events in human cancers. Previously we have used anti-phosphotyrosine affinity chromatography coupled to multi-dimensional LC/MS/MS to identify signaling networks associated with receptor tyrosine kinase activation and pharmacological blockade. Current methods for measuring phosphorylation changes in response to neoplastic transformation or to kinase inhibitors have used ICAT® reagents and stable isotope labeling approaches. In an effort to improve sensitivity and dynamic range of these measurements, we have used a novel tagging method with improved peptide fragment ion generation and quantitation.

MATERIALS AND METHODS

• Anti-phosphotyrosine antibodies PY20 and PY100 were covalently linked to Protein G resin using disuccinimidyl suberate at a 1:10 ratio and washed with citrate buffer pH2.8 to remove non-specifically bound antibody. HMC1 cells, a mast cell leukemia, were exposed to thiophene inihibitor (0.5μM for 0, 1, 4 or 24 hours), lyzed in cold isotonic buffer containing 1% Triton X100, protease and phosphatase inhibitors and incubated with blank Protein G resin for 30 minutes. pY containing proteins immunoprecipitated for 2 – 4 hours at 4°C, washed with >200 column volumes and eluted with 0.1% TFA, 5% MeOH.
• Proteins were denatured, reduced and alkylated prior to digestion with trypsin. Peptides were labeled on free α and ε amino groups using isobaric multiplex labels (Applied Biosystems) for 1 hour, subjected to SCX and C18 microbore steps prior to LC/MS/MS. On-line C18 chromatography was performed using a 0.1 x 150 mm column packed with C18 resin (MagicC18, Michrom Bioresources, Auburn, CA) and developed using a 2 – 70% acetonitrile, 0.1% formic acid gradient with a flow rate of ~800 nl/minute (~1200psi). The electrospray ionization source was fitted with an uncoated tapered fused silica tip (15μm ID; New Objective, Cambridge, MA) to which a voltage of 3.0 kV was applied with nebulizing nitrogen gas. Information-dependent MS and MS/MS acquisitions were made on an orthogonal quadrapole-TOF (QqTOF) instrument (AB/SCIEX, Toronto, Canada) using a 0.8 second survey scan followed by 3 consecutive 2 second product ion scans of 2+, 3+ and 4+ parent ions (m/z 400 – 1200) where ions were stored and released from Q2. Identification and quantitation was performed using Pro QUANT software (AB, Foster City, CA) using both SwissProt and Celera Discovery System™ protein databases, with an MS and MS/MS mass tolerance of 0.15Da. Protein identifications with confidence scores of >90% were considered.

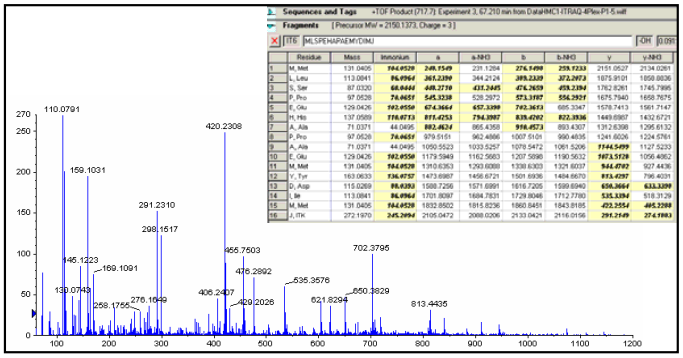
RESULTS

Figure 1. Isobaric Labeling Workflow



Peptides are labeled on free α and ε amino groups, subjected to SCX and C18 steps, followed by on-line LC/MS/MS.

Figure 2. No Peak Splitting in 4-plex Labeled kit Peptide

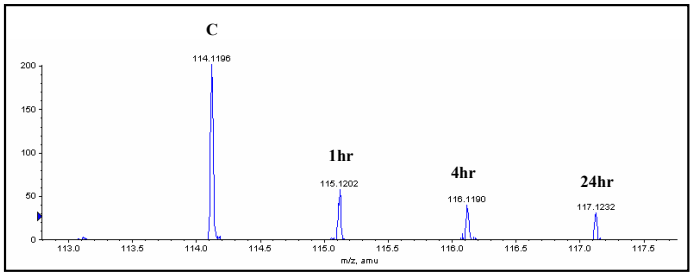


Kit peptide identified from anti-pY selection and LC/MS/MS is a mixture of 4 peptides from 4 time points labeled with isobaric iTRAQ™ reagent labels. The labels are isobaric and peak splitting is difficult to observe.

Results Summary

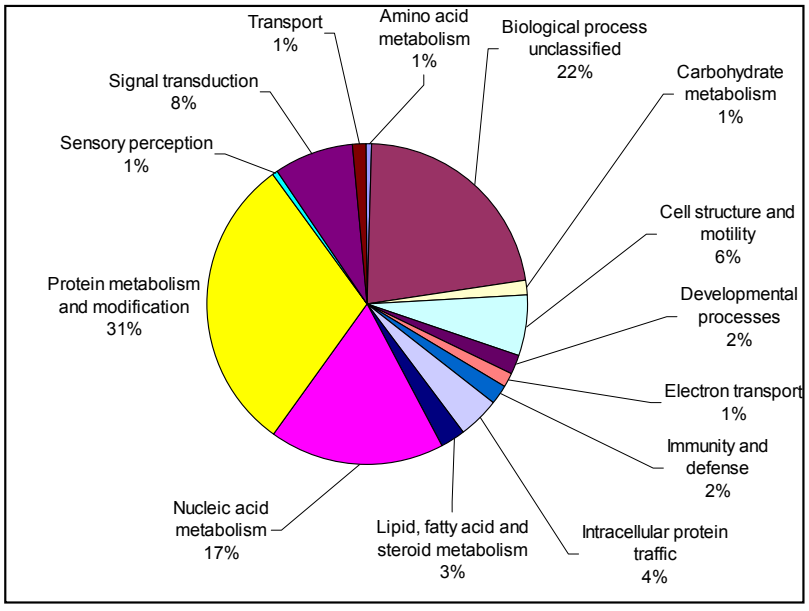
- 150 proteins identified with two or more peptides at 99% confidence.
- c-kit was the major anti-pY affinity selection protein identified from HMC1.
- Phosphorylation of kit and PTP1C peptide directly measured.
- Dynamic range in complex mixtures estimated to be ~3 logs.
- Greatly enhanced fragment ion spectra observed by labeling in terms of counts.

Figure 3. Quantitation of pY Selected kit Peptide using Encoded MS/MS Tags



MS/MS region revealing iTRAQ™ reagent quantitation tags from the kit peptide fragmentation spectra shown in Figure 2. Fragment ion spectra of kit peptide from 4-plex labeled anti-pY sample following 1, 4 or 24 hours exposure to Kit/KDR inhibitor or control (C).

Figure 4. CDS Panther Protein Classification



150 proteins identified by anti-pY selection with 2 or more peptides identified with 99% confidence were classified by parsed CDS Panther bioprocess terms

Table 1. Kit Coverage from Anti-pY Selected HMC1 Cell Proteins, showing Time-dependent Reduction in Phospho-kit after Treatment with kit/KDR Inhibitor (0.5 μM) for 1, 4 or 24 hours.

Unique Peptide	Δ Mass	Mass	Conf. (%)	Score	1 Hour	4 Hours	24 Hours
AVPVVSVSJ	0.04	1172.8	99	45	0.09	0.05	0.02
AYNDVGJ	0.03	1053.6	99	31	0.16	0.06	0.05
DSFICSJ	0.05	1132.6	99	30	0.11	0.06	0.03
EALMSELJ	0.05	1207.7	98	33	0.11	0.03	0.03
HGLSNSIYVFVR	0.04	1534.9	99	29	0.36	0.19	0.12
ICDFGLAR	0.05	1083.6	99	36	0.09	0.08	0.03
LCLHCSVDQEGJ	0.08	1710.9	99	23	0.61	0.59	0.27
LLCTDPGFVJ	0.04	1425.8	99	41	0.06	0.03	0.02
MLSPEHAPAEMYDIMJ	0.10	2150.1	99	49	0.19	0.19	0.13
NDSNYVVJ	0.05	1225.7	99	39	0.12	0.04	0.02
QEDHAEAALYJ	0.04	1561.8	99	46	0.18	0.10	0.06
QEDHAEAALYJ	79.98	1641.8	99	38	0.06	0.02	0.02
QIVQLIEJ	0.05	1257.9	99	35	0.12	0.04	0.03
QJPVVDHSVR	0.02	1451.9	99	38	0.06	0.03	0.02
QNEWITEJ	0.03	1334.8	99	34	0.08	0.03	0.02
SDAAMTVAVJ	0.04	1279.7	99	47	0.16	0.06	0.04
TCWDADPLJ	0.04	1381.7	99	41	0.07	0.03	0.03
TFTDJWEDYPJ	0.10	1861.1	99	30	0.22	0.11	0.09
WTFEILDETNENJ	0.04	1926.0	99	46	0.05	0.03	0.03
YNSWHHGDFNYER	0.11	1867.9	99	28	0.59	0.35	0.18
YTCTNJ	0.03	1062.5	95	24	0.04	0.03	0.01
YVSELHLTR	0.07	1260.8	99	38	0.13	0.12	0.06

Individual unique kit peptides and phosphopeptide observed, showing quantitation over time. The phosphopeptide derives from position 681, within the kinase domain of kit.

Table 2. Quantitation of kit Phosphotyrosine Signaling Proteins following Inhibition of kit Kinase Activity for 1, 4 or 24 hours

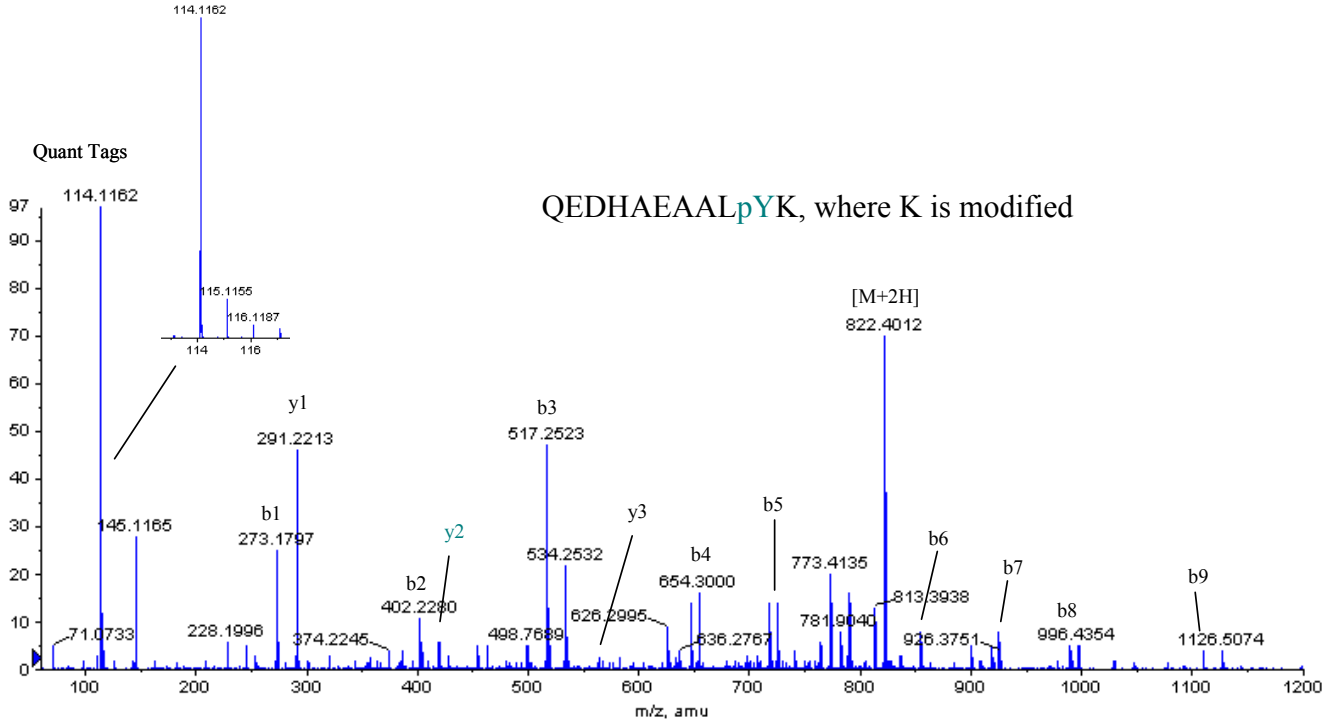
Name	Peptides	Ratio: OSI930 vs Control		
		1 hour	4 hours	24 hours
c-Kit	69	0.18	0.15	0.13
c-Fes/c-Fps	40	1.10	0.52	0.53
Erk	5	0.14	0.13	0.08
c-Cbl	3	0.39	0.14	0.21
Ubiquitin	6	0.51	0.46	1.10
Stat5B	1	0.61	0.14	0.35
PI3-kinase	4	0.65	0.43	1.10
WASP3	4	0.70	0.26	0.40
Btk	12	1.20	0.50	0.45
c-Src	2	1.40	0.51	1.00
SYK	7	4.11	0.54	0.30
Trypsin autoD	6	1.06	0.98	1.06

Selected proteins involved in Kit signal transduction and the number of peptides from multiple LC/MS/MS runs are shown

Table 3. Reproducible Time -dependent Quantitation of Phosphopeptides from c-kit.

Protein Name	Phosphopeptide	Δ Mass	% Conf.	Score	Kit kinase inhibition		
					1 Hour	4 Hours	24 Hours
KIT protein	QEDHAEAALYJ	80.022	95	28	0.057	0.010	0.013
	QEDHAEAALYJ	79.982	94	24	0.058	0.026	0.014
	QEDHAEAALYJ	79.982	99	38	0.058	0.023	0.019
	Mean	-	-	-	0.057	0.019	0.015

Figure 5. 2s Fragment Ion Spectra of 821.9m/z kit Phosphopeptide from Information Dependent Acquisition (IDA) based LC/MS/MS. Fragment ion y2 is pY and quantitation following kit kinase inhibition is shown. Mock treated control is labeled with 114 and 1, 4 and 24 hour kit inhibition samples are labeled with 115, 116 and 117 respectively.



CONCLUSIONS

Multiple tyrosine phosphorylated and associated proteins were identified and relative abundance measured by anti-pY selection and isobaric multiplex labeling. Proteins identified based on peptide fragment ion spectra were readily quantitated. The relative abundance of proteins over multiple time points could be inferred from peptide quantitation within a single LC/MS/MS experiment. The low noise in MS/MS spectral space where the 114, 115, 116 and 117Da ions released from the multiplex tags reside contributes to the improved dynamic range when compared to other labeling methods (data not shown). The mean % error for measurement of kit abundance within the anti-pY fraction was ~22%. This method provides a rapid means to identify and measure proteins associated with phosphotyrosine signaling pathways with respect to time after stimulation or blockade of kinase activity.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING