Supporting Information for

"Imatinib-induced Changes in Protein Expression and ATP-binding Affinities of Kinases in Chronic Myelocytic Leukemia Cells"

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Experimental Section

Cell culture

K-562 cells (ATCC) were cultured in Iscove's modified Dulbecco's medium (IMDM). GM-00637 (obtained from Prof. Gerd P. Pfeifer), GM15876A (provided by Prof. Karlene Cimprich),[1] HCT-116, HEK293T (ATCC), and MDA-MB-231 (provided by Prof. Jian-Jian Li from UC Davis) cells were cultured in DMEM medium. DU-145, WM-115 and WM-266-4 cells (ATCC) were cultured in Eagle's minimum essential medium (EMEM). HL-60, Jurkat-T and CEM cells (ATCC) were cultured in RPMI 1640 medium. All culture media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin (100 IU/mL). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Approximately 2×10^7 cells were harvested, washed with ice-cold PBS for three times, and lysed by incubating on ice for 30 min with CelLytic M (Sigma) cell lysis reagent containing 1% protease inhibitor cocktail. The cell lysates were centrifuged at 9,000g at 4°C for 30 min, and the resulting supernatants collected. For SILAC experiments,[2] K-562 cells were cultured in medium containing [$^{13}C_6$,$^{15}N_2$]-lysine and [$^{13}C_6$]-arginine for at least 2 weeks to promote complete incorporation of the stable isotope-labeled amino acids.

Preparation of the desthiobiotinylated nucleotide affinity probe, desthiobiotin labeling and affinity purification of ATP-binding proteins

The desthiobiotinylated nucleotide affinity probes were prepared following previously published procedures.[3-4] Approximately 2×10^7 cells were harvested, washed with ice-cold PBS for three times, and lysed in a 1-mL lysis buffer, which contained 0.7% CHAPS, 50 mM HEPES (pH 7.4), 0.5 mM EDTA, 100 mM NaCl, and 10 μL (1:100) protease inhibitor cocktail on ice for 30

min. The cell lysates were centrifuged at 16,000g at 4°C for 30 min and the supernatants collected. Endogenous nucleotides in the resultant protein extract were removed by gel filtration using a NAP-25 column (Amersham Biosciences). Cell lysates were subsequently eluted into a buffer containing 50 mM HEPES (pH 7.4), 75 mM NaCl, and 5% glycerol. The amounts of proteins in the lysates were quantified using Quick Start Bradford Protein Assay (Bio-Rad). Prior to the labeling reaction, stock solutions of $MgCl_2$, $MnCl_2$, and $CaCl_2$ were added to the concentrated cell lysate until their final concentrations reached 50, 5 and 5 mM, respectively.

For pulling down kinases and other ATP-binding proteins, approximately 1 mg cell lysate in a 1-mL solution was incubated, at room temperature with gentle shaking for 2.5 h, with the light desthiobiotin-C3-ATP affinity probe at a final concentration of 100 μM.[4] To the resulting mixture was subsequently added 300 μL avidin-agarose resin (Sigma-Aldrich), and the mixture was then incubated at room temperature with gentle shaking for 1 h. The agarose resin was washed sequentially with 3 mL PBS buffer and 3 mL H_2O to remove unbound proteins, and the desthiobiotin-conjugated proteins were subsequently eluted with 1% TFA in CH_3CN/H_2O (7:3, v/v) at 75°C.

Tryptic digestion of enriched ATP-binding proteins and whole-cell protein lysates, and LC-MS/MS analyses in the data-dependent acquisition (DDA) mode

The above enriched ATP-binding proteins from the lysates of eight human cell lines (i.e. CEM, Du-145, GM00637, GM15876A, HCT-116, HEK293T, HL-60, and Jurkat-T) were washed with 8 M urea for protein denaturation, and then treated with dithiothreitol and iodoacetamide for cysteine reduction and alkylation, respectively. The proteins were subsequently digested with modified MS-grade trypsin (Pierce) at an enzyme/substrate ratio of 1:100 in 50 mM NH_4HCO_3 (pH 8.5) at 37°C overnight. The peptide mixture was subsequently dried in a Speed-vac, desalted

with OMIX C18 pipette tips (Agilent Technologies), and analyzed by LC-MS and MS/MS on a Q Exactive Plus quadruple-Orbitrap mass spectrometer (Thermo Fisher Scientific) in the DDA mode.

The mass spectrometer was coupled with an EASY-nLC 1200 system, and the samples were automatically loaded onto a 4-cm trapping column (150 μm i.d.) packed with ReproSil-Pur 120 C18-AQ resin (5 μm in particle size and 120 Å in pore size, Dr. Maisch GmbH HPLC) at a flow rate of 3 μL/min. The trapping column was coupled to a 20-cm fused silica analytical column (PicoTip Emitter, New Objective, 75 μm i.d.) packed with ReproSil-Pur 120 C18-AQ resin (3 μm in particle size and 120 Å in pore size, Dr. Maisch GmbH HPLC). The peptides were then resolved using a 140-min linear gradient of 9-38% acetonitrile in 0.1% formic acid and at a flow rate of 300 nL/min. The spray voltage was 1.8 kV. Full-scan mass spectra were acquired in the range of *m/z* 350-1500 in the Orbitrap mass analyzer at a resolution of 35000 with an automated gain control (AGC) target of 1×10^6. Up to 25 most abundant ions found in MS with a charge state of 2 or above were sequentially isolated and collisionally activated in the HCD cell at a collision energy of 29 to yield MS/MS. MS/MS were acquired in the Orbitrap analyzer at a resolution of 17500 with an AGC target of 1×10^5.

The whole cell lysates prepared from three human cell lines (i.e. WM-115, WM-266-4 and HEK293T) were again denatured with urea, followed by cysteine reduction/alkylation, and tryptic digestion, as described above. The resulting peptide mixtures were subsequently dried in a Speed-vac, desalted using OMIX C18 pipette tips (Agilent Technologies), and subjected to LC-MS and MS/MS analyses on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) in the DDA mode. Up to 20 most abundant ions found in MS were sequentially isolated and collisionally activated in the linear ion trap at a normalized collision energy of 35 to yield MS/MS.

Database search

Maxquant, Version 1.5.2.8, was used to analyze the LC-MS and MS/MS data for protein identification.[5] The database we used for search was human IPI database, version 3.68, which contained 87061 protein entries. The maximum number of miss-cleavages for trypsin was two per peptide. Cysteine carbamidomethylation was set as a fixed modification. Methionine oxidation and serine, threonine and tyrosine phosphorylation were set as variable modifications. The tolerances in mass accuracy were 20 ppm for MS, and 20 ppm and 0.5 Da for MS/MS acquired on the Q Exactive Plus and LTQ Orbitrap Velos, respectively. The maximum false discovery rates (FDRs) were set at 0.01 at both peptide and protein levels, and the minimum required peptide length was 6 amino acids. The total identified proteins were then filtered by the DAVID (version 6.7) bioinformatic tool with the Gene Ontology (GO) term of kinase.[6]

Sample preparation, MRM and PRM analyses

For assessing the alterations in ATP-binding affinities of kinases modulated by imatinib treatment, we treated K-562 cells with 1 μM imatinib for 24 hr. The cells were subsequently harvested by centrifugation and lysed following the aforementioned procedures. The removal of endogenous nucleotides from protein lysate, labeling with desthiobiotin-conjugated ATP-affinity probe, tryptic digestion, and affinity enrichment of desthiobiotin-labeled peptides were performed following previously published procedures.[7] The resultant peptide mixture was subjected to LC-MS/MS analysis on a TSQ Vantage triple-quadrupole mass spectrometer coupled with an Easy-nLC II system (Thermo Fishier), where the mass spectrometer was operated in the scheduled MRM mode with a retention time window of 8-min. The peptides were separated using a 130-min linear gradient of 2-35% acetonitrile in 0.1% formic acid and at a flow rate of 230 nL/min. The spray voltage was 1.9 kV. Q1 and Q3 resolutions were 0.7 Da and the cycle time was 5 s.

To assess the differential expression of kinases in inhibitor-treated cells, both forward and reverse SILAC labeling experiments were conducted. In this context, the lysates of light-labeled, inhibitor-treated cells and heavy-labeled, mock-treated cells (with DMSO) were combined at 1:1 ratio (by mass) in the forward SILAC experiments, whereas the reverse SILAC experiments were performed in the opposite way. After cysteine reduction and alkylation, the whole cell lysates were digested with modified MS-grade trypsin (Pierce) at an enzyme/substrate ratio of 1:100 in 50 mM NH_4HCO_3 (pH 8.5) at 37°C overnight. The ensuing peptide mixture was subjected to LC-MS/MS analysis on a Q Exactive Plus quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) in the scheduled PRM-mode with the parameters described above.

All raw data acquired from LC-MRM and LC-PRM analyses were processed using Skyline (version 3.5[8]) for the generation of extracted-ion chromatograms and peak integration. The targeted peptides were first manually inspected to ensure the overlaid chromatographic profiles of multiple fragment ions derived from light and heavy forms of the same peptide. The data were then processed while ensuring that the distribution of the relative intensities of multiple transitions associated with the same precursor ion correlates with the theoretical distribution in the kinome MS/MS spectral library. The sum of peak areas from all transitions of light or heavy forms of peptides was used for quantification.

Western blot

K-562 cells were cultured in a 6-well plate and the cells were lysed at 40-50% confluency following the above-described procedures. The concentrations of proteins in the resulting lysates were determined by using Bradford Assay (Bio-Rad), and 10 μg protein lysate was denatured by boiling in Laemmli loading buffer and resolved by SDS-PAGE. The proteins were subsequently transferred onto a nitrocellulose membrane at 4°C overnight. The resulting membrane was

blocked with PBS-T (PBS with 0.1% Tween 20) containing 5% milk (Bio-Rad) at 4°C for 6 h. The membrane was then incubated with primary antibody at 4°C overnight and subsequently with secondary antibody at room temperature for 1 h. After thorough washing with PBS-T, the HRP signal was detected with Pierce ECL Western Blotting Substrate (Thermo).

Antibodies recognizing human AK1 (Santa Cruz Biotechnology, sc-165981, 1:1000 dilution), CCND3 (Santa Cruz Biotechnology, sc-453, 1:2000 dilution), CHK1 (Cell Signaling Technology, 2360S, 1:2000 dilution), p-CHK1 S296 (Abcam, ab79758, 1:1000 dilution), SCYL3 (Santa Cruz Biotechnology, sc-398328, 1:2000 dilution) were employed as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG and IRDye® 680LT Goat anti-Mouse IgG were used as secondary antibodies. Membranes were also probed with β-actin antibody (Cell Signaling #4967, 1:10000 dilution) to confirm equal protein loading.

Clonogenic survival assay

MDA-MB-231 cells were seeded in six-well plates at densities of 100-300 cells per well. The cells were exposed to various doses of neocarzinostatin (NCS, 0-100 ng/mL) and a fixed dose of imatinib (1 μM) in DMEM medium and cultured for 10 days. The colonies were then fixed with 6% glutaraldehyde and stained with 0.5% crystal violet. Colonies with more than 50 cells were counted under a microscope.[9]

References:

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Table S1 (in Excel**).** List of kinases included in the Skyline human PRM kinome library.

Table S2 (in Excel)**.** Expression levels and ATP probe labeling efficiency of kinases after imatinib treatment. All the ratios listed in the table represents for imatinib/DMSO.

Figure S1. Experimental strategy for PRM- (left) and MRM-based (right) targeted proteomic approaches.

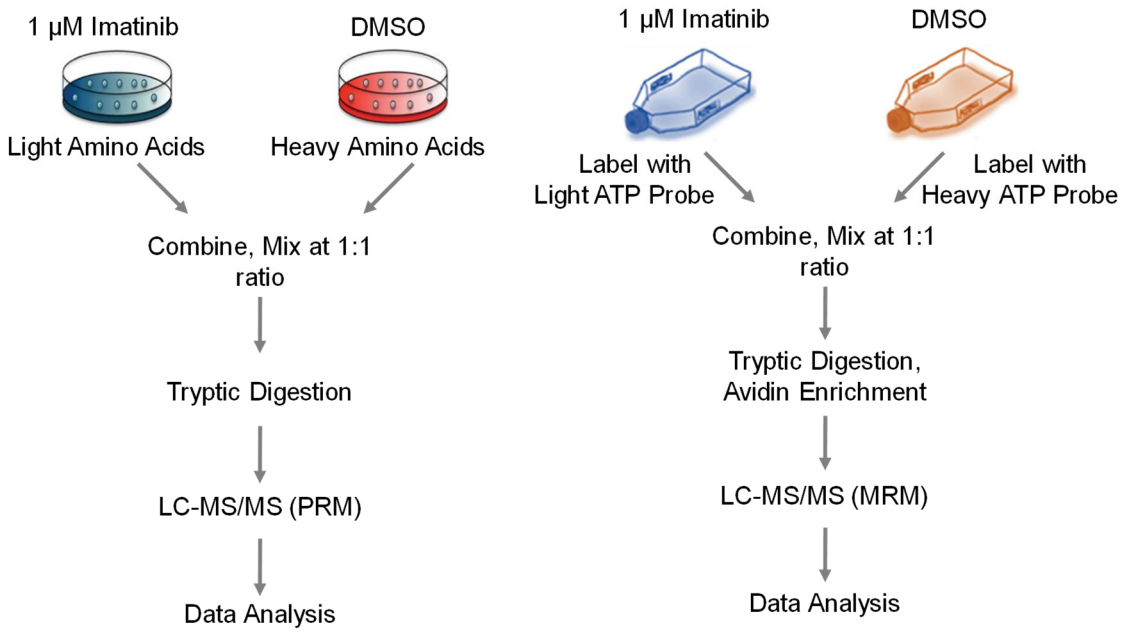

Figure S2. Extracted-ion chromatograms for representative kinases (AK1, CCND3) obtained from PRM-based method. The peptide sequences and the transitions employed for plotting the ion chromatograms are listed in the figure.

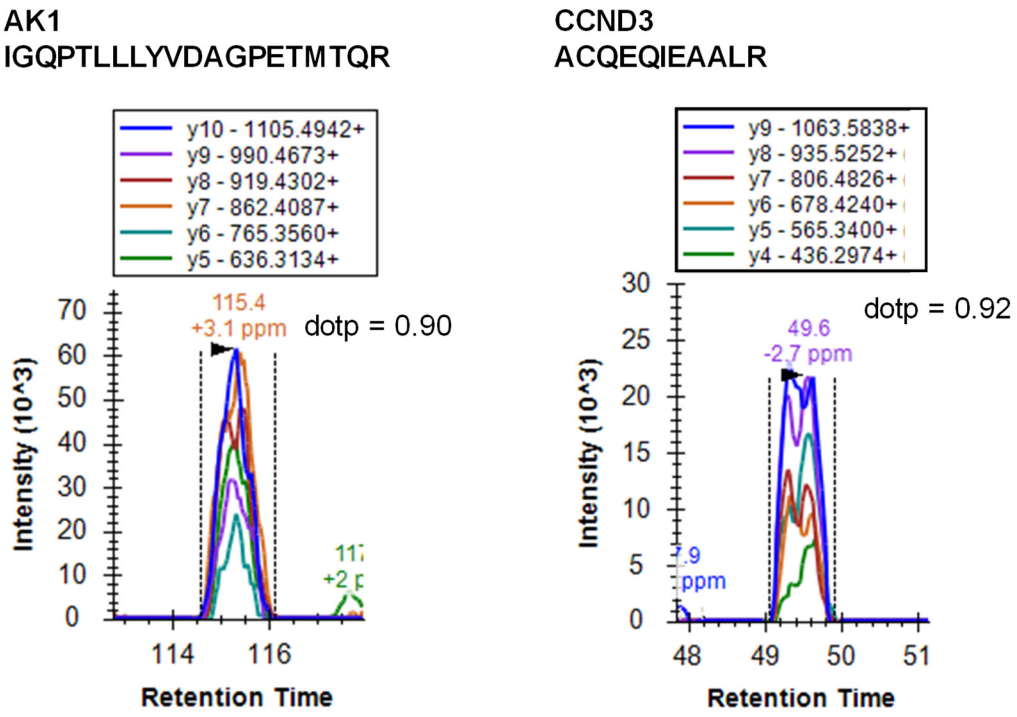

Figure S3. Performances of the PRM method. (a) A Venn diagram displaying the overlap between quantified kinases from the forward and reverse experiments of K562 cells with or without imatinib treatment acquired from PRM (left) and MRM (right) (Table S2). (b) A scatter plot displaying the correlation between the ratios obtained from forward and reverse experiments acquired from PRM (left) and MRM (right). The number represents for $\log_{10}$ ratio of imatinib/DMSO.

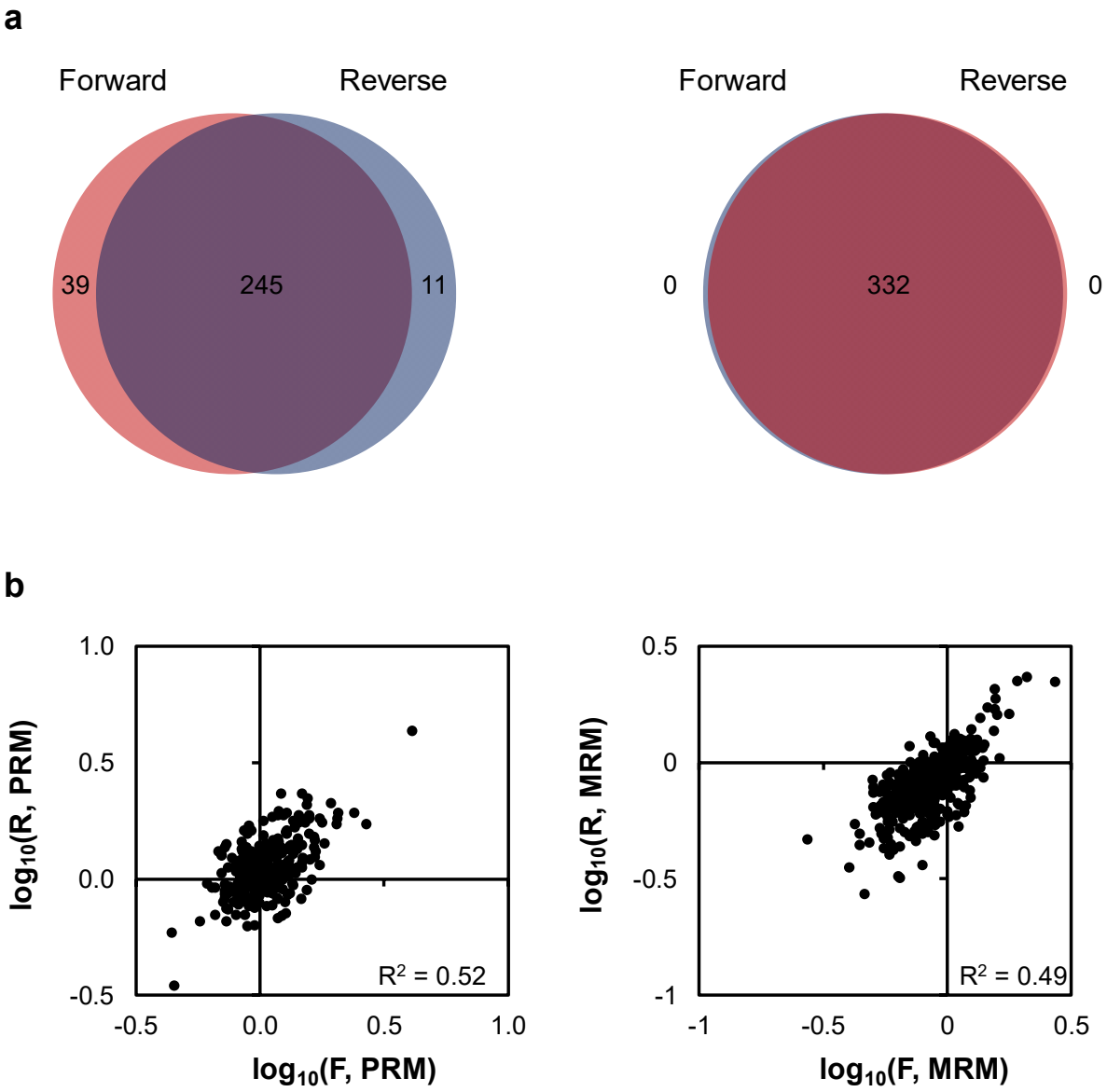

Figure S4. Kinome map of the quantified kinases by ATP probe pull down method. Figure was generated using online tool (https://peptracker.com/epd/analytics/?show_plot).