Supporting Information

Intracellular Protein–Drug Interactions Probed by Direct Mass Spectrometry of Cell Lysates

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Author Contributions

R.R. Conceptualization: Equal; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Writing – original draft: Lead
A.R. Investigation: Supporting
I.B. Resources: Supporting
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N.L. Methodology: Supporting; Resources: Supporting
M.S. Conceptualization: Lead; Funding acquisition: Lead; Supervision: Lead; Writing – original draft: Equal.
1. Experimental Methods

*Plasmids and Cloning*

The pHLsec plasmid encoding SOD1 was a gift from Lucia Banci’s lab (University of Florence, Italy). The gene coding for the kinase domain of Bruton’s tyrosine kinase (residues 386-659), here termed BTK_KD, was PCR-amplified from pFastBac-1, a gift from Tamar Unger (Weizmann Institute, Israel), and cloned into pHLsec between the EcoRI and XhoI sites using the InFusion kit (Takara Bio). A Kozak sequence and a single Gly residue were added before residue 386 to facilitate expression and cloning, respectively.

*Cell Culture and Transient Transfection*

HEK293T cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin-100 μg/mL streptomycin (Biological Industries), 0.1 mM sodium pyruvate (Biological Industries), MEM-Eagle non-essential amino acids (Biological Industries) and MycoZap Prophylactic (Lonza). Cells were split the day before transfection and plates at a confluence of 80-90% were typically used. For transfection of SOD1 plasmid, we followed the previously published protocol\(^{17}\), although we found that excellent expression was obtained at a lower DNA concentration of 5 μg per 100 mm diameter plate. For transfection of BTK_KD plasmid, we used the JetPEI reagent (PolyPlus) and 15 μg of DNA per 100 mm plate. The DNA was added to 250 μL of manufacturer-provided NaCl together with 37.5 μL of JetPEI reagent, and the two solutions were gently mixed and allowed to incubate at room temperature for 20 minutes. Plates were washed with 3 mL of DMEM, and then 5 mL of DMEM was added. The transfection mixture was gently added to the side of the plate and plates were rocked to distribute mixture. Four to five hours after transfection, media was replaced with 8 mL DMEM containing MEM-Eagle non-essential amino acids.

SOD1-expressing cells were harvested 3 days after transfection and BTK_KD-expressing cells 2 days after transfection. Cells were harvested by trypsinization, washed once with PBS, and then again with cold 150 mM
ammonium acetate. Cell pellets were flash-frozen in liquid nitrogen and stored at -80°C.

**SDS-PAGE and Western Blot Analysis**

Lysate was mixed with Laemmli buffer and heated to 95 °C for 5 minutes. Proteins were separated on 12% SDS-PAGE gels and stained with InstaStain Coomassie to visualize them. Alternatively, proteins were transferred to 0.45 µm Immobilon-P PVDF membranes (Millipore) pre-activated in methanol, in Tris-Glycine transfer buffer (pH 8.3) supplemented with 20 % methanol. Blots were run at 4 °C either for 2 hours at 400 mA or overnight at 20 V. Membranes were blocked with 5 % skim milk powder in TBS-T for 1 hour, followed by incubation with appropriate primary antibodies on an orbital shaker at 4 °C overnight. Membranes were rinsed thoroughly in TBS-T, followed by incubation with appropriate secondary HRP conjugated antibodies for 1 hour on an orbital shaker at room temperature. Membranes were rinsed thoroughly and developed using WesternBright ECL (Advantasta) in a MyECL Imager (Thermo Scientific) according to the manufacturer’s instructions. The primary antibody used for detecting BTK_KD was #56044 from Cell Signaling Technologies.

**Cellular Measurement of Ibrutinib-NH2 Activity**

Mino cells were treated with either 0.1% DMSO or the indicated concentrations of Ibrutinib-NH2 for 1 hour. The cells were then incubated with 10 µg/ml anti-human IgM (Jackson ImmunoResearch, 109-006-129) for 10 min at 37 °C and harvested. Pellets were washed x1 with ice-cold PBS and lysed using RIPA-buffer (Sigma, R0278). Lysates were clarified at 14,000 rpm for 15 minutes at 4 °C and protein concentration was determined using BCA protein assay (Thermo Fisher Scientific, 23225). 50 µg of lysate were then loaded on a 4-20% bis-tris gel (GeneScript SurePAGE, M00657), proteins were separated by electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, 1704158) using Trans-Blot Turbo system (Bio-Rad). The membrane was blocked using 5% BSA in TBS-T (w/v) for 1 hour at room temperature, washed 3 times for 5 minutes with TBS-T and incubated with the following primary antibodies: rabbit anti phospho-BTK (#87141s, cell-signaling, 1:1000, over-night at 4°C), mouse anti BTK (#56044s, cell-signaling, 1:1000, 1 hour at room-temperature), mouse anti β-actin (#3700, cell-signaling, 1:1000, 1 hour at room-temperature). The membrane was
washed 3 times for 5 minutes with TBS-T and incubated with the corresponding HRP-linked secondary antibody (Mouse #7076 / Rabbit #7074, cell-signaling) for 1 hour at room-temperature. EZ-ECL Kit (Biological Industries, 20-500-1000) was used to detect HRP-activity. The membrane was stripped using Restore stripping buffer (Thermo Fisher Scientific, 21059) after each primary antibody before blotting with the next one. Gel bands corresponding to phosphorylated BTK and total BTK were quantified using ImageJ software. Phospho-BTK (p-BTK) signal was normalized to total-BTK for each sample and the DMSO treated sample was defined as 100% activity. LogC (µM) of Ibrutinib-NH₂ concentrated was plotted against % activity and the data were fitted to a Sigmoidal 4PL model using GraphPad Prism 8 to extract the cellular IC₅₀.

**In-Cell Drug Binding Experiments**

Drug binding experiments were performed following the protocol shown in Fig S4. BTK binding drugs used are Ibrutinib, Ibrutinib-NH₂, the L1, L5, and L6 compounds, pluripotin, PP-121, LY2409881, and vemurafenib. Pluripotin, PP-121, LY2409881 and vemurafenib were provided by Haim Barr, supported by a grant from the Nancy and Stephen Grand Israel National Center for Personalized Medicine. Cells were harvested by Trysinization, re-suspended in PBS, and a cell count was performed using a Burker hemocytometer. Two million cells were re-suspended in 1 mL of PBS and incubated at 37 °C for varying time points with varying concentrations of Ibrutinib, Ibrutinib-NH₂, or L6 from ref. For multiplexing experiments with pluripotin and other drugs, 2 million cells were re-suspended in a reduced volume of 500 µL.

This was followed by rapid centrifugation (1 min), rinsing with 600 µL of ice-cold 150 mM ammonium acetate, and flash freezing of cell pellets. For experiments with the covalent inhibitor Ibrutinib, an additional wash step with 1 mL of PBS was added before the ammonium acetate wash. Time frames given in the Results section include the one minute of centrifugation before the drug containing solution was removed. To test that our rinsing protocol did not lead to dissociation of the drug, we prepared samples incubated with 100 nM Ibrutinib-NH₂ that were either not rinsed or rinsed once or twice with 600 µL ammonium acetate.
The Trypan Blue exclusion assay of viability\textsuperscript{19} was used to verify that cell viability was not affected by incubation at 37 °C in PBS. After 1 hour of incubation, cell viability remained 95 ±2 \% (3 biological repeats), similar to its value before incubation.

A saturated solution of L6 was prepared by taking PBS, adding L6 to a final concentration of 500 µM, and repeatedly sonicking and vortexing for 20 minutes. The insoluble drug was collected by centrifugation and the supernatant was removed and added to the cells.

\textit{MS Sample Preparations}

Flash-frozen pellets were re-suspended in 150-300 mM ammonium acetate and protease inhibitors (1 mM PMSF, 5 µg/mL pepstatin A, and 2.5 mM benzamidine) to a final density of \(~ 1\) million cells / 25 µL. Two mM DTT was also added for BTK_KD containing samples. Cells were lysed by 5 freeze/thaw cycles in liquid Nitrogen including 2 rounds of 1 minute gentle bath sonication. Cell debris was pelleted by centrifugation at 16,000 g at 4 °C and supernatant was collected. Total protein concentration was measured by the BioRad protein assay, with typical protein concentrations ranging from 2-4 mg/mL. This supernatant was then typically diluted 10-50 fold into 300-500 mM ammonium acetate for MS analysis.

\textit{In-Vitro Determination of Drug Affinity}

For analysis of drug binding, lysates prepared as above were incubated with a range of drug concentrations for 30 minutes on ice before analysis.

\textit{Native Mass Spectrometry Analysis}

Nanoflow electrospray ionization MS and tandem MS experiments were conducted under non- denaturing conditions on a Q-Exactex Plus Orbitrap EMR (ThermoFisher Scientific). Four µl of sample was electrosprayed from gold-coated borosilicate capillaries prepared in-house. Assays were performed in positive ion mode and conditions were optimized to enable the ionization and removal of adducts. In MS/MS experiments, the relevant m/z values were isolated and argon gas was admitted to the collision cell. Spectra are shown without smoothing or background subtraction. The following range of experimental conditions were used: capillary voltage 1.7 kV, resolution of
At trapping gas pressure setting of 1-2. For MS/MS analyses, an isolation window of ± 5 m/z the selected charge state was set in the quadrupole. Transmitted ions were subjected to collision induced dissociation in the HCD cell at a range of collision voltages.

Analysis of Drug Bound MS-Data

Spectra of BTK_KD with noncovalent drugs, namely Ibrutinib-NH$_2$, L1, L5 and L6, were collected without HCD voltage, since we observed that the HCD cell caused the non-covalent drugs to dissociate from BTK_KD. To correct for background and drug dissociation, the most intense 10$^+$ charge state (for Ibrutinib-NH$_2$) and the 9+ charge state (for L6) corresponding to both bound and unbound were fitted to a Gaussian in PeakFit 4.2. Peak areas were normalized by dividing by the total intensity for all BTK peaks. The normalized intensity of the bound peaks was then background corrected by subtracting the normalized intensity of the bound peaks in a spectrum obtained when no drug was added and dividing by the normalized intensity of the bound peaks in a spectrum obtained with a saturating excess of drug (20-100 µM) according to the formula $I - I_0$ 

$\frac{I_{max} - I_0}{I_{max} - I_0}$

where $I$ represents normalized intensity of bound peaks, $I_0$ normalized intensity for a sample with no drug added, and $I_{max}$ normalized intensity for saturated sample. The percent of protein bound was then plotted as a function of drug concentration (see Fig. 4 and Fig. S6). Data represent at least 3 separate biological repeats per data point with error represented as standard deviation.

For analysis of experiments in which we varied the number of times that the samples were rinsed, keeping the concentration of Ibrutinib-NH$_2$ constant, we integrated only the 10$^+$ charge states in PeakFit V 4.2 and took the integrated intensities for comparison without background subtraction.

To calculate the affinity of L6 for BTK_KD in the lysate, we fitted the data to a quadratic model accounting for free drug and protein depletion upon binding:

$$PD = \frac{D_0 + P_0 + K - \sqrt{(D_0 + P_0 + K)^2 - 4D_0P_0}}{2}$$

(Equation 1)

where PD is the concentration of the protein-drug complex, $D_0$ is the total drug concentration and $P_0$ is the total protein concentration. Since in the
direct-MS experiments we do not know the total protein concentration, equation 1 is converted to:

\[ y = \frac{ax+1+ b-\sqrt{(ax+1+ b)^2-4ax}}{2} \] (Equation 2)

where \( y \) is the percent of bound protein, \( x \) equals to \( D_0 \), \( a = \frac{1}{P_0} \) and \( b = \frac{K}{P_0} \). Data were fitted to Equation 2 using Origin 2021.
2. Supplementary Figures

Figure S1. SOD1 is overexpressed in HEK293T cells.
SDS-PAGE gel of lysate from control cells and cells overexpressing SOD1. The first three lanes show pellet, pellet wash, and supernatant respectively from control cells; the next three lanes show the same for transfected cells. A dominant band corresponding to SOD1 is clearly seen in the supernatant of the transfected cells (marked with box).
Figure S2. The dominant cellular SOD1 form is a dimer bound to two zinc ions.

The top panel shows the full MS spectrum of SOD1 lysate from cells grown with zinc in the media. MS/MS analysis through isolation of the 11+ charge state and gentle HCD activation reveals that it is composed of two monomers, each bound to one zinc ion (middle panel). Upon increased activation in the HCD cell by raising the collision voltage to 90 V, the dimer dissociates into two monomers, one stripped of metal and one bearing two metals (bottom panel). These monomers are likely unfolded at the high HCD values used and therefore the doubly bound monomer may not have both zinc ions bound specifically.
Figure S3. Overexpression of the BtK kinase domain (BtK_KD).
A- SDS-PAGE gel of supernatant from HEK293 control cells and cells overexpressing BtK_KD, with the BtK_KD band marked with a square (predicted MW of 32 kD). As expected, the unphosphorylated BtK_KD is detected in HEK293 cells.
B- To roughly assess the amount of BtK_KD present in the direct-MS samples, Western blots of a BtK_KD standard as well as defined amounts of direct-MS lysate from two different biological samples were quantified using ImageJ. Using a standard curve composed of the BtK_KD standards, the amount of BtK_KD in the samples was defined. The BtK_KD was estimated to be approximately 4 ± 1 % w/w of the lysate. This corresponds to approximately 8 µg/mL (250 nM) BtK_KD in the final MS sample. Shown is one representative measurement; quantification was performed for three separate Western blots with a total of nine samples.
Figure S4. Protocol for assessing drug uptake via direct-MS.
HEK-293T cells were transiently transfected with the pHLsec plasmid using jetPEI. Cells were harvested via trypsinization, washed once in PBS, and then drugs were added to the media and allowed to incubate for selected periods of time at 37 °C. Cells were then collected by gentle centrifugation, rinsed with PBS and/or ammonium acetate, and then centrifuged. After cell lysis via freeze/thaw, direct-MS spectra were collected directly from the lysate.
Figure S5. Rinsing does not lead to significant drug washout.

To confirm that our rinsing protocol (see Materials and Methods) did not lead to drug washing out of the cells, we prepared samples of BtK_KD expressing cells and incubated them with 100 nM Ibrutinib-NH$_2$. We directly compared samples that were not rinsed to samples that were rinsed once and twice, with four biological repeats for each condition. From integration of the 10$^+$ charge state, the three conditions yielded an essentially identical amount of bound drug, with the un-rinsed samples having 44% ± 5% and the samples rinsed once and twice having 43% ± 5% and 43% ± 4% bound, respectively. The spectrum above shows representative overlays of the 10$^+$ charge state of BtK-KD from the three conditions, demonstrating the similarity between relative intensity of the bound and unbound peaks.

These results indicate that the rinsing protocol does not lead to significant drug leakage under the conditions and time frame chosen. We chose to continue with rinsing the cells once, both to remove adduct ions and also to ensure that any extracellular drug was removed.
Figure S6. Dose response curve of ibrutinib uptake.

A- The percentage of BtK_KD bound to the non-covalent Ibrutinib-NH$_2$ is plotted as a function of drug concentration in the media. Each point is an average of at least three experimental measurements and error represents standard deviation. The data were fitted in Python to a saturation binding equation of the form $y = \frac{xA}{x+B}$ where A represents the maximal % saturation and B is the K$_D$, for A = 0.94 and B = 0.16 µM. As mentioned in the main text, we do not know the % of total Ibrutinib-NH$_2$ in the MS samples and therefore this fit is for the purpose of determining the Kd, or concentration needed to achieve 50% binding.

B- Representative overlays of the 10$^+$ charge state of BtK_KD from cell lysates incubated with the varying concentrations of Ibrutinib-NH$_2$ shown in A. As can be seen, the bound peak increases as a function of drug added, concomitant with a decrease in the unbound peak.
Figure S7: Cellular BtK activity assay to assess the cellular potency of ibrutinib-NH₂.

Mino cells, which natively express full length BtK under regulation of the B-cell receptor, were used to measure the cellular activity of Ibrutinib-NH₂. Cells were incubated with either DMSO or ibrutinib-NH₂ for 1 hour, followed by IgM stimulation for 10 minutes. Cells were harvested and levels of phospho-BtK (p-BtK) and total BtK were measured via Western blotting (see Materials and Methods for details).

A- Western blot of Mino cells with staining for p-BtK, total BtK, and β-Actin (used as a loading control).

B- The extent of activity was plotted as a function of the log₁₀ of ibrutinib-NH₂ concentration and fitted to the Sigmoidal 4PL function in GraphPad Prism to extract a cellular IC₅₀ of 0.4 µM for Ibrutinib-NH₂.
Figure S8. Direct-MS demonstrates cellular uptake of L6. MS spectrum of cellular lysate incubated with a saturating L6 solution. The major charge series corresponds to the bound protein (> 80%), with a minor contribution from the free protein.
Table S1. Theoretical masses of dimeric and monomeric SOD1 species. When Zn$^{2+}$ and Cu$^{2+}$ bind to SOD1, they are each chelated by 3 histidines – His63, His71 and His80 for Zn$^{2+}$ and His 46, His48, and His120 for Cu$^{2+}$. Therefore, metal binding to the protein causes displacement of histidine protons so that masses need to be calculated accordingly.

| Species                      | Mass, Da  | Notes                                                   |
|------------------------------|-----------|---------------------------------------------------------|
| hSOD1$_{dimer}$, apo         | 31,693.1  | Includes removal of N-terminal Met and N-terminal acetylation |
| Zn$^{2+}$- hSOD1$_{dimer}$   | 31,817.7  | H$^+$ displacement as a result of Zn$^{2+}$ binding is   |
| Zn$^{2+}$, Cu$_{1}$- hSOD1$_{dimer}$ | 31,878.4  | H$^+$ displacement as a result of Cu$^{2+}$ binding is    |
| Zn$^{2+}$, Cu$_{2}$- hSOD1$_{dimer}$ | 31,939.0  | H$^+$ displacement as a result of Cu$^{2+}$ binding is    |

Table S2. Masses of drugs and theoretical m/z of BtK_KD/drug complexes
We tested a mix of drugs multiplexed with BtK_KD (see Figure 3, main text). Shown here are the masses of the drugs added along with the theoretical m/z of the 10$^+$ charge state for each protein/drug complex.

| Condition     | MW of drug | Theoretical m/z of 10$^+$ state |
|---------------|------------|---------------------------------|
| Apo           | --         | 3192.8                          |
| Pluripotin    | 550.5      | 3247.9                          |
| PP-121        | 319.4      | 3224.7                          |
| Vemurafenib   | 489.9      | 3241.8                          |
| LY2409881     | 594.4      | 3252.2                          |
| Ibrutinib-NH$_2$ | 386.5    | 3231.4                          |