Supporting Information

Kinetic Optimization of Lysine-Targeting Covalent Inhibitors of HSP72

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**Computational Chemistry Methods**

**ROCS Ligand Based Alignments and Mogul Torsion Analysis**

The two query structures used for this analysis were derived from the bound ligands of PDB 5MKR, PDB 5MK2 and PDB 4I08. The ligands extracted, standardized and protonated using the implementation of MMFF94\(^1\) within QUAPAC. A combination of default shape and pharmacophore queries were generated using ROCS.\(^2\) These queries were used to search a conformational database of in-house HSP72 ligands. 500 conformations were generated for each HSP72 compound in our database using the MMFF94 implementation in OMEGA.\(^3\) The ROCS Tanimoto Combo score was used to rank the top scoring alignments to our queries. These alignments were then subjected to a Mogul\(^4\) based torsion analysis to assess the likelihood that a given torsional angle was within the optimal distribution of those observed experimentally within organic molecules within the Cambridge Structural Database (CSD).\(^5\) From this final alignments were selected and used to infer the possible binding modes of TCI’s to HSP72.
Derivation of percentage occupancy equations for Figure 1

For TCIs that follow a two-step mechanism of inhibition, the observed rate constant of inactivation at a given inhibitor concentrations ($k_{\text{obs}}$) can be expressed in terms of the maximum rate constant of inactivation ($k_{\text{inact}}$), the concentration of inhibitor that gives a half-maximal rate of inactivation ($K_i$) and the inhibitor concentration ([I], Equation 1):

$$k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_i + [I]} \quad (1)$$

Equation 1 Covalent kinetics equation.

The percentage covalent occupancy of a protein (%CO) over time (t) can be calculated from $k_{\text{obs}}$ (Equation 2):

$$\%\text{CO} = 100(1 - \exp(-k_{\text{obs}}t)) \quad (2)$$

Equation 2 %Covalent occupancy calculation.

The reversibly bound fractional occupancy (FO) of a target can be derived from a reversible equilibrium binding equation:

$$K_D = \frac{[E][I]}{[EI]} \quad \text{rearranged for } [E]: \quad [E] = \frac{K_D[EI]}{[I]} \quad (3)$$

$$FO = \frac{[EI]}{[E]_{\text{tot}}} \quad \text{where } [E]_{\text{tot}} = [E] + [EI]$$

$$\text{so } \quad FO = \frac{[EI]}{[EI] + [E]} \quad (4)$$

Because it is difficult to measure the free and bound enzyme concentrations in equation 4, FO can be expressed in terms of [I] and $K_D$ using the rearranged form of the dissociation equilibrium constant, equation 3:

$$FO = \frac{[EI]}{[EI] + (K_D[EI]) / [I]}$$

so:

$$FO = \frac{[EI][I]}{[EI][I] + K_D[EI]}$$

Therefore:

$$FO = \frac{[I]}{[I] + K_D}$$
With FO expressed in terms of [I] and $K_D$, equation 5 can be combined with the %CO equation 2 to take into account the initial reversible binding of the TCI. The resulting equation 5 describes the percentage total occupancy (%TO):

$$%TO = 100(1 - (1 - FO)(\exp (- k_{obs}t)))$$

(6)

**Equation 3** Percentage total occupancy of protein expressed in terms of reversible fractional occupancy (FO), $k_{obs}$ and time (t).
**Assay Experimental**

**Fluorescence polarisation assay**

**General experimental**
The aqueous buffer contained 50 mM Tris base pH 7.4, 150 mM KCl, 5 mM CaCl$_2$ and 0.1% (w/w) CHAPS. The assay was conducted using 384 Plus F ProxiPlates (PerkinElmer) with a final assay volume of 10 μL. Plates were centrifuged at 1000 rpm for 1 minute prior to incubation and read using a 2103 Envision Multilable Plate Reader. Excitation and emission wavelengths used were 480 nm and 535 nm, respectively. Fluorescence polarisation was measured in units of millipolarisation (mP). All experiments were performed in triplicate. Data were plotted and analysed using GraphPad Prism 6, graphical data represent the arithmetic mean ± curve fitting standard error of the mean for a single representative experiment.

**K$_D$ determination**
To each well, 5 μL of fluorescent probe molecule (N$^6$-(6-aminohexyl)-ATP ATTO-488, Jena Bioscience; 20 nM in assay buffer, 10 nM final concentration) and increasing concentrations of HSP72-NBD protein (5 μL, two-fold dilution series) were added. Fluorescence polarisation values for tracer control wells (10 nM probe in assay buffer only) were subtracted from each data point prior to data analysis. K$_D$ determination was performed using non-linear regression analysis (GraphPad Prism 6, one site-specific binding model). pK$_D$ values are quoted as geometric mean ± standard error of the mean from 3 independent experiments.

**Competitive binding experiments**
Compounds (0.2 μL at 50 x screening concentration in DMSO) were dispensed using an ECHO 550 Liquid Handler (Labcyte Inc.). To the corresponding wells was added, 5 μL of probe molecule (20 nM in assay buffer, 10 nM final concentration) and 5 μL of protein (twice their final concentration in assay buffer) to give a 50% bound fraction. Tracer controls (10 nM probe molecule only) and bound tracer controls (10 nM probe in presence of appropriate protein concentration) were included on each assay plate. IC$_{50}$ determination was performed using non-linear least squares curve fitting (GraphPad Prism 6, log(inhibitor) vs. response – variable slope (four parameters)). K$_i$ values were calculated using the method described by Huang. pK$_i$ values are quoted as geometric mean ± standard error of the mean from 3 independent experiments.
**Kinetic experiments**

Compounds (0.2 μL at 50 x screening concentration in DMSO) were dispensed using an ECHO 550 Liquid Handler (Labcyte Inc.). 5 μL of probe molecule (20 nM in assay buffer) and 5 μL of protein (twice their final concentration in assay buffer) were pre-incubated in the dark for two hours and then added to the corresponding wells. Tracer controls (10 nM probe molecule only) and bound tracer controls (10 nM probe in presence of appropriate protein concentration) were included on each assay plate. Plates were then read every 30 seconds using the described protocol to give a time course.

**Protein mass spectrometry**

**LC-MS for intact proteins**

HSP72-NBD (2.3 μM) in buffer (50 mM Tris base pH 7.4, 150 mM KCl, 5 mM CaCl₂) was mixed with compound (200 μM from 10 mM DMSO stock) and incubated at 21 °C prior to analysis. The total reaction volume was 100 μL. The reaction was stopped by taking sample using auto-sampler. 1 μL injections of the sample were made onto a Security Guard C8 column cartridge (4 x 3 mm, AJO-4290, Phenomenex). Samples were incubated at 21 °C in a G1367B auto-sampler (Agilent) with G1330B thermostat module prior to injection. Chromatographic separation at 21 °C was carried out using an Agilent 1200 Series HPLC over a 1 minute gradient elution of 95:5 to 5:95 water and acetonitrile (both modified with 0.1% formic acid) at a flow rate of 0.5 mL/min. The post column eluent flow was infused into an Agilent 6520 Series qToF mass spectrometer fitted with a dual ESI ionisation source. An initial divert to waste was used to aid desalting. LC eluent and nebulising gas was introduced into the grounded nebuliser with spray direction orthogonal to the capillary axis. The aerosol was dried by heated gas (10 L/min of nitrogen at 350 °C, 50 psi), producing ions by ESI. Ions entered the transfer capillary along which a potential difference of 4 kV was applied. The fragmentor voltage was set at 190 V and skimmer at 65 V. Signal was optimised by AutoTune.m. Profile mass spectrometry data were acquired in positive ionisation mode over a scan range of m/z 650-2000 (scan rate 1.0) with reference mass correction at m/z 922.009798 hexakis(1H,1H,3H-perfluoropropoxy)phosphazene. Raw data were processed using Agilent MassHunter Qualitative Analysis B.06.00 and graphs made using GraphPad Prism 6.
Worked example of kinetic FP assay

The output of the FP assay is in millipolarisation (mP) units, a measurement of the degree of polarization that the light retains after interacting with the rapidly tumbling fluorescent probe. When the probe is bound to HSP72-NBD, it tumbles at a slower rate compared to when the probe is not bound. The greater the tumbling rate, the more the degree of polarisation is reduced. When the probe is bound to the protein, it tumbles slowly, leading to a high mP value. When the probe is free, it tumble more quickly, leading to a lower mP value. Therefore, the degree of polarization of fluorescent light directly describes bound fraction of the protein. Because the bound fraction of the fluorescent probe is proportional to the concentration of free protein, time-dependent changes in mP are interpreted as time-dependent changes in the bound fraction. Changes in the bound fraction could occur either from changing the concentration of a reversible inhibitor, which effectively reduces the free total concentration of the protein, or from the formation of a covalent adduct. Assuming there is no depletion of the covalent inhibitor, initial reversible occupancy will reduce the effective free concentration of the protein, dependent on the free concentration of the inhibitor and its reversible affinity, leading to a reduction in bound fraction. As the covalent bond begins to form, the covalent occupancy of the protein, coupled with the reversible occupancy, will reduce the effective protein concentration in a time-dependent manner, leading to a time-dependent decrease in the bound fraction.

Huang’s Equation describes the relationship between IC\textsubscript{50} and \(K_i\):\(^8\)

Huang’s equation was used to calculate \(K_i\) values from the measured IC\textsubscript{50}s. See equation below:

\[
IC_{50} = \left( \frac{f_0K_d}{(1-f_0)(2-f_0)} + \frac{f_0L_0}{2} \right) \left( \frac{K_i(2-f_0)}{K_df_0} + 1 \right)
\]

The equation states that the IC\textsubscript{50} for a ligand that is competitive for binding with the assay probe is related to the binding affinity of the ligand (\(K_i\)), the bound fraction of the probe (\(f_0\)), the binding affinity of the probe (\(K_d\)) and the concentration of the probe (\(L_0\)). For competition experiments, it is recommended that a protein concentration giving a bound fraction between 0.5 and 0.8 be selected. A bound fraction below 0.5 will often result in an assay that is not statistically robust due to the decreased size of the binding window, however as the bound fraction approaches 1 the relationship between \(K_i\) and IC\textsubscript{50} deviates from linear and the resolvable range of the assay decreases. For these reasons, a bound fraction of 0.5 was used for all assays.
Based on the analysis by Huang,\textsuperscript{8} the free unbound protein concentration displays a non-linear relationship with the bound fraction. To carry out the kinetic FP-assay, an initial protein concentration that would lead to a high bound fraction ($F_b = 0.8$) was selected as the reversible occupancy of the protein would rapidly displace the probe and reduce $F_b$. At low bound fractions of probe ($F_b < 0.4$), the assay is insensitive (Figure 1c), as large changes in $F_b$ are required for a small change in protein concentration. At high bound fraction of the probe ($F_b > 0.8$), the relationship between $F_b$ and protein concentration is essentially non-linear and small changes in $F_b$ result in large changes in protein concentration (Figure 1b). Therefore, selection of an initial bound fraction of 0.8 ensures that the working range of the assay remains between these limits ($0.4 < F_b < 0.8$, Figure 1d), where changes in the free protein concentration ($< 30\%$) displaying an approximately linear relationship with changes in $F_b$ (red dashed line, Figure 1d).

\begin{equation}
[HSP72] = \left(\frac{K_D F_b}{1-F_b}\right) + F_b[\text{probe}] \quad (7)
\end{equation}

\textbf{Figure 1} a) equation linking protein concentration with the bound fraction of the FP probe, the $K_D$ of the probe and the concentration of the probe; b) graphical representation of the relationship between protein concentration and $F_b$, using $K_D = 92$ nM, [probe] = 10 nM; c)
zoom of $0.1 < F_b < 0.4$ – assay is insensitive to small changes in [HSP72]; d) zoom of $0.55 < F_b < 0.8$ – 30% drop in protein concentration gives an approximately linear change in $F_b$ (red dashed line).

The measured mP values are first converted into units of anisotropy, $A$ (Figure 2a, equation 8). Anisotropy and polarisation are mathematically related and are often used interchangeably when taking measurements in FP assays. The bound fraction of the fluorescent probe ($F_b$) is directly proportional to the anisotropy ($A$) and is calculated from the anisotropy value of the fluorescent probe in the absence of protein ($A_f$) and the anisotropy value of the fluorescent probe when fully bound to protein ($A_b$) (Figure 2b, equation 9).

$$A = \frac{2P}{3-P} \quad (8) \quad F_b = \frac{A_f - A}{A_f - A_b} \quad (9)$$

Figure 2 a) Equation 4 showing the relationship between anisotropy (A) and polarisation (P); b) equation 5 showing the relationship between the measured anisotropy (A), the free and bound anisotropy values for the fluorescent probe ($A_f$ and $A_b$ respectively), and the bound fraction of the fluorescent probe ($F_b$).

The time-dependent FP assay was performed with a focus on early time points, taking a measurement every 2 minutes from 2 minutes, to allow for mixing and the establishment of reversible equilibria (Figure 3a). All compounds are assumed to display fast-on/fast-off reversible kinetics, relative to the kinetics of the covalent bond formation. The calculated $F_b$ values were then plotted against time and extrapolated back to $t=0$ using linear regression to estimate the initial $F_b$ (Figure 3b) at different concentrations of the inhibitor, which was used to calculate an initial IC$_{50}$ using non-linear regressions and subsequent initial K$_i$ for TCI 14 (Figure 3c).
Figure 3 See Supplementary data excel file for raw data; a) Time-dependent inhibition of HSP72-NBD with SF TCI 14 assessed by displacement of ATP-ATTO488 probe over time. HSP72-NBD concentration selected so $F_b = 0.8$. Measurements taken every 2 minutes over a 20-minute period and plotted on same axes; b) calculated bound fraction values plotted over time. Different colour lines represent different concentrations of SF TCI 14 as shown in legend. Y-Intercepts calculated using linear regression (GraphPad Prism 6); c) plot of initial $F_b$ against [I]. IC$_{50}$ determined using non-linear least squares curve fitting (GraphPad Prism 6, log(inhibitor) vs. response – variable slope (four parameters)).

The initial $K_i$ value was then used to focus a subsequent FP titration at TCI concentrations below the initial $K_i$. A 1.5-fold dilution series from initial $K_i$ concentrations of TCI 14 was used to measure the time-dependence in mP values (Figure 4a).
Figure 4 See Supplementary data excel file for raw data; a) Time-dependent inhibition of HSP72-NBD with SF TCI 14 assessed by displacement of ATP-ATTO488 probe 64 over time. Measurements taken every 2 minutes over a 30-minute period and plotted on same axes, [SF TCI 14] < initial $K_i$; b) calculated bound fraction values plotted over time. Different colour lines represent different concentrations of SF TCI 14 as shown in legend.

Converting the time-dependent mP values into $F_b$ and plotting this value over time, rates of change of bound fractions at different inhibitor concentrations can be determined (Figure 4b). Linear regression on the linear region of this graph gave the rate of change of bound fraction in units of “bound fraction per minute”, which was converted into the rate constant, $k_{obs}$ (units of min$^{-1}$), by dividing by the $F_b$ values at t=0. Finally, a plot of $k_{obs}$ against [TCI 14] allowed us to calculate $k_{inact}/K_i$ from the gradient of the linear region of the graph (Figure 5).

For TCI 14, the overall second-order rate constant for covalent bond formation with HSP72-NBD was $k_{inact}/K_i = 35 \text{ M}^{-1}\text{s}^{-1}$. The $k_{inact}$ value was estimated using the initial $K_i$ value calculated from the t=0 extrapolation, which assumed that $K_i = K_f$. By multiplying $k_{inact}/K_i$ by the initial $K_i$ value, $k_{inact}$ was calculated as $3.6x10^{-4} \text{ s}^{-1}$. This is equal to a half-life at a theoretical 100% occupancy of 32 minutes ($t_{1/2} = \ln2/k_{inact}$).
**Figure 5** See Supplementary data excel file for raw data; Calculated $k_{obs}$ values plotted against [SF TCI 14]. Linear regression performed on the linear region of the graph (data points that deviate from linearity are shown in red). *a* $k_{\text{inact}}/K_i$ values are quoted as mean ± SEM of three independent experiments.

| TCI | $k_{\text{inact}}/K_i$ ($M^{-1}s^{-1}$)* | Initial $K_i$ (µM) | $k_{\text{inact}}$ (s$^{-1}$) | $t_{1/2}^\infty$ (min) |
|-----|-----------------------------------------|---------------------|-----------------------------|-----------------------|
| 14  | 35 ± 1.7                                 | 10                  | 3.6x10$^{-4}$               | 32                    |
Kinetic FP and Intact- protein MS figures

**Compound 2**

**Figure 6**

a) structure of TCI 2; b) time-dependent FP assay for TCI 2 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) intact protein mass spectrometry for HSP72-NBD [2.3 µM] and TCI 2 [200 µM]; d) $k_{\text{inac}}/K_{I}$ plot from kinetic FP assay for TCI 2. The gradient of the slope was calculated from the linear regression, representative example of N = 3 independent biological repeats.
**Compound 8**

a) structure of TCI 8; b) time-dependent FP assay for TCI 8 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) intact protein mass spectrometry for HSP72-NBD [2.0 μM] and TCI 2 [20 μM].

**Compound 9**

a) structure of 9; b) FP assay for 9 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM.

**Figure 7** a) structure of TCI 8; b) time-dependent FP assay for TCI 8 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) intact protein mass spectrometry for HSP72-NBD [2.0 μM] and TCI 2 [20 μM].

**Figure 8** a) structure of 9; b) FP assay for 9 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM.
**Compound 13**

a)

![Structure of Compound 13](image)

b)

![Time-dependent FP assay](image)

**Figure 9** a) structure of control TCI 13; b) time-dependent FP assay for control TCI 13 with HSP72-NBD WT. Each concentration represents an $n=3$ statistical repeat, arithmetic mean ± SEM; c) intact protein mass spectrometry for HSP72-NBD [2.0 μM] and control TCI 13 [20 μM].
Compound 14

Figure 10 a) structure of TCI 14; b) time-dependent FP assay for TCI 14 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) intact protein mass spectrometry for HSP72-NBD [2.0 µM] and TCI 14 [20 µM]; d) $k_{inac}/K_I$ plot from kinetic FP assay for TCI 14. The gradient of the slope was calculated from the linear regression, representative example of N = 3 independent biological repeats; e) time-dependent FP assay for TCI 14 with HSP72-NBD K56A. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM.
**Compound 15**

![Structure of Compound 15](image)

**Figure 11** a) structure of 15; b) FP assay for 15 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM.

**Compound 16**

![Structure of Compound 16](image)

**Figure 12** a) structure of control TCI 16; b) time-dependent FP assay for control TCI 16 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) $k_{inact}/K_i$ plot from kinetic FP assay for TCI 16. The gradient of the slope was calculated from the linear regression, representative example of N = 3 independent biological repeats.
Figure 13  a) structure of control TCI 17; b) time-dependent FP assay for control TCI 17 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) $k_{\text{inact}}/K_i$ plot from kinetic FP assay for TCI 17. The gradient of the slope was calculated from the linear regression, representative example of N = 3 independent biological repeats; d) intact protein mass spectrometry for HSP72-NBD [2.0 µM] and TCI 17 [20 µM].
**Figure 14** a) structure of control TCI 18; b) time-dependent FP assay for control TCI 18 with HSP72-NBD WT. Each concentration represents an n=3 statistical repeat, arithmetic mean ± SEM; c) $k_{inact}/K_i$ plot from kinetic FP assay for TCI 18. The gradient of the slope was calculated from the linear regression, representative example of N = 3 independent biological repeats; d) intact protein mass spectrometry for HSP72-NBD [2.0 µM] and TCI 18 [20 µM].
NMR Spectra of Final Compounds

4-fluorophenyl 4-(((2R,3S,4R,5R)-5-(6-amino-8-((4-chlorobenzyl)amino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)methyl)benzoate (8)
4-(((2R,3S,4R,5R)-5-(6-amino-8-((4-chlorobenzyl)amino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)methyl)benzonitrile (9)
((3aR,4R,6R,6aR)-6-(6-amino-8-((4-chlorobenzyl)amino)-9H-purin-9-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)methyl 4-(fluorosulfonyl)benzoate (13)
((2R,3S,4R,5R)-5-(6-amino-8-((4-chlorobenzyl)amino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl 4-(fluorosulfonyl)benzoate (14)
((2R,3S,4R,5R)-5-(6-amino-8-((4-chlorobenzyl)amino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl 4-cyanobenzoate (15)
((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl 4-(fluorosulfonyl)benzoate (16)
((2R,3S,4R,5R)-5-(6-amino-8-(methylamino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl 4-(fluorosulfonyl)benzoate (17)
((2R,3S,4R,5R)-5-((6-amino-8-((quinolin-6-yl)methyl)amino)-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl 4-(fluorosulfonyl)benzoate (18)
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