Fragment Screening Yields a Small-Molecule Stabilizer of 14-3-3 Dimers That Modulates Client Protein Interactions

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Experimental methods

Fluorescent Polarization

N-terminal fluorescein (FAM) labelled and phosphorylated estrogen receptor alpha (ERα) peptide had the sequence FAM – G-E-A-E-G-F-P-A-pT-V– COOH (see table S1). The FAM - ERα peptide was dissolved in phosphate saline buffer (PBS) with a pH of 7.5. Concentration curves of recombinant GST-14-3-3η were produced by mixing various concentrations of the protein diluted in assay buffer (PBS, pH=7.5) with a fixed 100 nM of the FAM labelled peptide and either a DMSO vehicle control or a fixed 100 μM Fusicoccin – A (FC-A) or 1 mM of fragment 2. FC-A and fragment 2 concentration curves were generated by mixing various concentrations of FC-A diluted in PBS with a fixed 100 nM of FAM - ERα and a fixed 1.5 μM of GST-14-3-3η. Samples were added to sterile black 384 wells plate and incubated at room temperature (RT) for 30 minutes before polarization measurements were performed on the PHERAsystem FS (BMG labtech GmbH, Ortenberg, Germany) using the FP 480/520 nm fixed polarization filter set.

Homogenous Time Resolved Fluorescence

Homogenous time resolved fluorescence (HTRF) measurements were performed using recombinant GST-14-3-3η and N-terminally biotinylated ERα peptides (see table S1). Peptide concentration curves were made by diluting various concentrations of ERα peptides in HTRF buffer (NaCl 50 mM, Tris-HCL 20 mM, NP40 0.01%, BSA 0.5%, pH=7.0) before mixing 1:1 with 100 mM KF, a fixed 20 nM of GST-14-3-3η, 10 nM of an anti-GST europium (Eu) cryptate labelled monoclonal antibody (Cis Bio; Cat# 61GSTKLA) and 40 nM of streptavidin conjugated to XL665 (Cis Bio; Cat# 610SAXLA). Compound concentration curves were made by diluting various concentrations of compounds in HTRF buffer before mixing 1:1 with a fixed 50 nM of ERα peptide, 100 mM KF, a fixed 20 nM of GST-14-3-3η, 10 nM of an anti-GST europium (Eu) cryptate labelled monoclonal antibody and 40 nM of streptavidin conjugated to XL665. The modified HTRF assay for detecting 14-3-3/14-3-3 protein interactions was performed by diluting various concentrations of compounds in HTRF buffer before mixing 1:1 with a fixed 50 nM of ERα peptide, 100 mM KF, a fixed 10 nM of GST-14-3-3η, 1 nM of an anti-GST europium (Eu) cryptate labelled monoclonal antibody and 1 nM of an anti-His XL665 labelled monoclonal antibody (Cis Bio;Cat# 61HISXLA). Samples were subsequently incubated for 120 minutes at RT in a sterile white 384 wells plate before HTRF measurements were performed using the PHERAsystem FS (BMG labtech GmbH, Ortenberg, Germany) using the HTRF filter set (337/620/665 nm).

Z’ factors were calculated using the following formula: Z’ factor = 1-(3(SDp+SDn))/(µp-µn) wherein SDp=standard deviation of the positive control consisting of the complete HTRF mix, GST14-3-3η, ERα-pTV peptide and FC-A, SDn= standard deviation of the negative control consisting of the complete HTRF mix, GST14-3-3η and ERα-pTV peptide, µp= mean of the positive control consisting of the complete HTRF mix, GST14-3-3η, ERα-pTV peptide and FC-A, µn= mean of the negative control consisting of the complete HTRF mix, GST14-3-3η and ERα-pTV peptide.

Supporting info table 1: Peptide information

| Peptide name:       | N-terminal modification | Binding site: | Sequence:          | Supplier   |
|---------------------|-------------------------|---------------|--------------------|------------|
| ERα-pTV             | Biotinylation           | pT<sup>594</sup> | KYYITGEAEGFPApTV   | GL Biochem |
| ERα-pTF             | Biotinylation           | pT<sup>594</sup> | KYYITGEAEGFPApTF   | GL Biochem |
| ERα-pTVF            | Biotinylation           | pT<sup>594</sup> | KYYITGEAEGFPApTVF  | GL Biochem |
| ERα-P592N           | Biotinylation           | pT<sup>594</sup> | KYYITGEAEGFNApTV   | GL Biochem |
| ERα-Condor          | Biotinylation           | pT<sup>585</sup> | FYINSKEEEMQNpTI    | GL Biochem |
| FAM-ERα-pTV         | Fluorescein             | pT<sup>594</sup> | GEAEFGPApTV        | GL Biochem |
14-3-3 protein production and purification

The expression vector pGex-2T-Hs 14-3-3ƞ was a kind gift from Dr. J. D. Martinez, Arizona Cancer Center, Department of Cell Biology and Anatomy, The University of Arizona, Tucson, AZ, USA. Rosetta 2 (DE3)pLysS E. coli transformed with the pGex-2T-Hs 14-3-3ƞ plasmid or pPROEX HTB vector containing His14-3-3ƞ was grown in TB medium with 50 µg/mL of Ampicillin at 37°C until a OD₆₀₀ of 0.6 was reached, protein expression was then induced with 0.4 mM of IPTG for 16 hours at 24°C shaking at 220 rpm. Bacteria were pelleted and subsequently resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl, 2 mM Dithiothreitol, 1 mg/mL Lysozyme, 10 µg/mL DNAsel, Glycerol 10% and complete protease inhibitor cocktail Roche; Cat# 11697498001). Cells were lysed by passage through an emulsifier and cell debris was removed by ultra-centrifugation at 100 000 g for 30 minutes. Protein purification was performed on an AKTA FPLC using GSTrap HP columns (GE Healthcare; Cat#GE17-5281-01) or HisTrap HP columns (GE Healthcare; Cat# GE17-5248-02).

Paraformaldehyde cross-linking

Paraformaldehyde (PFA) cross-linking was performed by first mixing 1 µg of His14-3-3ƞ with 1 mM fragment 2 or fragment 3 diluted in PBS and subsequently incubating samples for 5 minutes at RT. Afterwards cross-linking was initiated by the addition of 0.2% PFA, after 10 minutes the reaction was quenched by the addition of 750 mM of ice-cold glycine. Samples were then boiled for 10 minutes at 96°C in Laemmli’s sample buffer. Proteins were visualized by running samples on a 10% SDS-PAGE gel and subsequent 1 hour staining with instant blue protein stain (Abcam; Cat# ab119211).

Nitrate reductase extraction

Barley (Hordeum vulgare seeds) plants were grown for 7 days in soil and subsequently treated with 100 mM KNO₃ 24 hours before harvesting. Plants were either left in the light or placed in the dark 30 minutes before harvesting. 5 g of leaf tops were ground in liquid nitrogen, followed by the addition of 10 mL extraction buffer (100 mM Hepes/KOH pH 7.5, 10 % (v/v) glycerol, 20 mM NaF, 10 mM EDTA, 20 µM flavin adenine dinucleotide (FAD), 5 µM Na-MoO₄, 0.1 % (v/v) Triton X-100, 6 mM dithiothreitol (DTT), 5 µM cantharidin, 2 mM PMSF, protease inhibitor cocktail EDTA free 3 tablets per 500 ml (Roche; Cat# 11873580001) and 1 % (w/v) polyvinylpolypyrrolidone (PVPP)). The suspension was filtered through four layers of Miracloth (Merckmillipore; Cat# 475855) and the filtrate was centrifuged at 100 000 g for 1 hour at 4°C. The supernatant was filtered through a 0.45-µM filter, the filtrate was applied to a HiTrap Q anion exchange column (GE healthcare; Cat# 17-5053-01) and subjected to a 0 to 500 mM NaCl gradient in extraction buffer (without PVPP). Nitrate reductase (NR) activity was localized as previously described¹.

Nitrate reductase assay

Concentration curves were made by mixing various concentrations of recombinant GST-14-3-3ƞ diluted in PBS with 40 uL of NR extract diluted in NR-assay buffer (50 mM Hepes/KOH pH 7.5, 5 mM EDTA, 10 mM KNO₃ and 0.5 mM NADH). Samples were subsequently incubated for 30 minutes at 30°C. NR enzyme reactions were quenched by the addition of 142 mM of zinc acetate. Samples were subsequently centrifuged at 13 000 g for 5 minutes after which 100 µL of the supernatant was mixed with 100 µL of 0.01% NAP and 0.5% Sulfanilamide in 0.75 M HCl. Absorbance measurements were subsequently measured in the CLARIOstar (BMG labtech; Germany) platereader at 540 nm in an F-bottom 96 wells plate.

Quantification and statistical analysis

Statistical tests were performed using Prism 6.0 (Graphpad, Cat#RRID:SCR_002798). The following statistical tests, as indicated in the figure legends, were used: an unpaired t-test corrected for multiple comparisons with the Holm-Sidak method, one way ANOVA with a Dunnett’s multiple comparison test. Sample sizes are indicated in figure legends and significance was defined as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. NS=not significant.
Anhydrous toluene was obtained by elution through an activated alumina column prior to use. Bicyclo[2.2.2]octan-2-one was purchased from Enamine. All other solvents and chemicals were acquired from commercial suppliers and were used as received. ChemBioDraw Ultra 20 was used to generate systematic names for all molecules.

NMR spectra (1H, 13C, and 2D) were recorded on a Bruker 300 (300 MHz), Bruker 500 (500 MHz) or a Bruker 600 (600 MHz) spectrometer. Data are reported as follows: chemical shift (integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad signal, m = multiplet), and coupling constants (Hz)). A Bruker microTOF mass spectrometer using ESI in positive ion mode was used to record HRMS spectra.

Elemental analysis was carried out at Mikroanalytisches Labor Pascher (Remagen, Germany). A Shimadzu LC-20AD liquid chromatograph pump system linked to a Shimadzu SPD-M20A diode array detector with MS detection using a Shimadzu LC-MS-2010EV mass spectrometer was used to perform LC-MS analyses. An Xbridge (C18) 5 µm column (50 mm, 4.6 mm) was used. The solvents that were used were the following: solvent B (acetonitrile with 0.1% formic acid) and solvent A (water with 0.1% formic acid), flow rate of 1.0 mL/min, start 5% B, linear gradient to 90% B in 4.5 min, then 1.5 min at 90% B, then linear gradient to 5% B in 0.5 min, then 1.5 min at 5% B; total run time of 8 min.

**Di(quinuclidin-3-yl)amine (2, VUF15640)**

Racemic quinuclidin-3-amino dihydrochloride (2.50 g, 13.0 mmol) and quinuclidin-3-one hydrochloride (2.00 g, 13.0 mmol) were mixed with MeOH until full dissolution. NaOH (1.50 g, 37.5 mmol) in H2O was added to liberate the free bases. The solvents of the resulting mixture were evaporated and the residue was mixed with toluene (120 mL). The reaction mixture was heated to reflux in a Dean-Stark set up for 16 hr. The volume of toluene was reduced by draining the Dean-Stark apparatus to approximately 30 mL. The mixture was cooled to room temperature. Dry MeOH (30 mL) was added to the reaction mixture to dissolve the formed precipitate. The reaction mixture was cooled with an ice bath. NaBH4 (0.48 g, 13 mmol) was added. After 30 min, the ice bath was removed and the reaction mixture was allowed to react overnight at rt. The reaction mixture was quenched with water (100 mL) and made basic with 2.5 N aq. NaOH. The water layer was extracted with DCM (3 x 100 mL). The combined organic layers were dried (Na2SO4), filtered and evaporated. The hydrobromide salt was converted to the free base by basic extraction of the salt using 2.5 N aq. NaOH/DCM to give 2 as an off-white solid. 1H NMR (500 MHz, CD3OD) δ 3.26 – 3.16 (m, 2H), 3.02 – 2.75 (m, 10H), 2.57 – 2.47 (m, 2H), 2.03 – 1.86 (m, 4H), 1.84 – 1.74 (m, 2H), 1.69 – 1.56 (m, 2H), 1.56 – 1.44 (m, 2H). 13C NMR (126 MHz, CD3OD) δ 56.5, 56.3, 52.5, 52.1, 48.0, 47.4, 26.1, 25.9, 25.8, 25.4, 19.8, 19.7. Two overlapping signals. High-Resolution MS (ESI): calculated for C14H26N3 (M+H)+ 236.2121, found 236.2126. The crystalline HBr salt was used for an AgNO3 assay and elemental analysis to further confirm the skeleton structure. NMR analysis showed a trace of iPrOH to be present in this salt and AgNO3 analysis indicated ca. 2.7 eq Br counterion to be present per salt. This was further corroborated by elemental analysis (Supporting Information Table 2), which also revealed a minor percentage of chloride counterions in the sample.

**Supporting info table 2: Elemental analysis results for fragment 2.**

| Atom | Calculated | Experimental | Margin | Compound | Mass % |
|------|------------|--------------|--------|----------|--------|
| C    | 34.62      | 34.36        | 0.26   | Product  | 48.25  |
| H    | 6.44       | 6.30         | 0.14   | IPA      | 0.25   |
| N    | 8.61       | 8.61         | 0.00   | H2O      | 5.9    |
| O    | 5.31       | 5.04         | 0.27   | HBr      | 45     |
| Br   | 44.44      | 44.40        | 0.04   | HCl      | 0.6    |
| Cl   | 0.58       | 0.58         | 0.00   | Total    | 100    |
| Total| 100        | 99.29        |        |          |        |
N-(bicyclo[2.2.2]octan-2-yl)quinuclidin-3-amine dihydrobromide (3.2HBr, VUF25305)

This compound was prepared analogously to 2 from racemic quinuclidin-3-amine dihydrochloride (330 mg, 1.66 mmol), but with bicyclo[2.2.2]octan-2-one (226 mg, 1.82 mmol) as the ketone reagent. The title compound 3 was isolated as the dihydrobromide salt after recrystallisation from iPrOH/MeOH, affording 3.2HBr as a white solid (70 mg, 11 %).

\[
\begin{align*}
\text{N} & \quad \text{H}_2 \text{N} & \quad \text{O} \\
\text{2HCl} & \quad \text{H}_2 \text{O} & \quad \text{HBr}
\end{align*}
\]

1H NMR (500 MHz, DMSO-d6) δ 9.74 (s, 1H), 8.74 (d, J = 22.5 Hz, 2H), 3.80 – 3.65 (m, 2H), 3.41 – 3.35 (m, 1H), 3.31 – 3.22 (m, 2H), 3.22 – 3.12 (m, 1H), 2.50 – 2.46 (m, 4H), 2.23 – 2.12 (m, 1H), 2.05 – 1.96 (m, 1H), 1.94 – 1.75 (m, 5H), 1.69 – 1.65 (m, 1H), 1.65 – 1.54 (m, 3H), 1.54 – 1.37 (m, 4H).

13C NMR (126 MHz, DMSO-d6) δ 54.5, 49.7, 48.3, 45.7, 45.2, 39.9, 39.7, 39.5, 39.4, 39.2, 39.0, 30.2, 25.5, 24.4, 24.1, 23.7, 23.3, 21.7, 21.4, 18.7, 16.6.

High-Resolution MS (ESI): calculated for C15H27N2 (M+H)+ 235.2169, found 235.2172.

AgNO3 analysis indicated ca. 2.05 eq Br counterion to be present per salt. This was further corroborated by elemental analysis: calculated for C15H26N2·2.05HBr·0.8H2O: C: 43.45%, H: 7.21%, N: 6.76%, Br: 39.50%, O: 3.09%, found: C: 43.26%, H: 7.06%, N: 7.69%, Br: 39.80%, O: 2.80%.
### Supporting info Pharmacological characteristics

#### Supporting info table 3 HTRF assay

| Peptides       | FC-A \( \text{pEC}_{50} \pm \text{SEM} \) | Fragment 2 \( \text{pEC}_{50} \pm \text{SEM} \) |
|----------------|------------------------------------------|-----------------------------------------------|
| ER\(\alpha\)-pTV | 5.82 ± 0.03                             | 3.55 ± 0.05                                   |
| ER\(\alpha\)-pTF | N.D.                                    | 3.5 ± 0.03                                    |
| ER\(\alpha\)-pTFV | N.D.                                    | 3.43 ± 0.03                                   |
| ER\(\alpha\)-P592N | 5.52 ± 0.11                             | 3.40 ± 0.01                                   |
| ER\(\alpha\)-Condor | 5.55 ± 0.04                             | 3.39 ± 0.03                                   |

#### Supporting info table 4 FP assay

| Peptides/protein | FC-A \( \text{pEC}_{50} \pm \text{SEM} \) | Fragment 2 \( \text{pEC}_{50} \pm \text{SEM} \) | Fragment 2 + 100 µM FC-A \( \text{pEC}_{50} \pm \text{SEM} \) |
|-----------------|------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| FAM-ER\(\alpha\)-pTV | 5.23 ± 0.05                             | 3.21 ± 0.05                                   | 3.47 ± 0.05                                     |
| GST14-3-3\(\eta\) titration | 6.16 ± 0.01                             | 5.92 ± 0.02                                   | N.D.                                            |

#### Supporting info table 5 Nitrate Reductase assay

| Nitrate reductase assay | Fragment 2 \( \text{pIC}_{50} \pm \text{SEM} \) |
|-------------------------|-----------------------------------------------|
| Potency                 | 5.74 ± 0.11                                  |
Supporting info figure 1 Overview of the in-house fragment library screen for PPI stabilizers of the 14-3-3/ERα-pTV interaction complex.

A-E) Overview of the Z-scores obtained for library fragments tested at 200 μM in our HTRF assay divided over five plates. F-J) HTRF ratios obtained for controls per fragment library plate. The background signal is shown in purple in which no interacting peptide is present. The basal signal for the interaction between GST14-3-3η and the ERα-pTV peptide is shown in blue. Finally in red is shown the effect of 10 μM of FC-A on the GST14-3-3η/ERα-pTV peptide interaction. Data is shown as mean ± SD. K-O) FC-A concentration-response curves taken along per plate targeting the GST14-3-3η/ERα-pTV peptide interaction complex. Data is shown as mean ± SD.
Supporting info figure 2 Fragments selected for deselection and validation.

XY plots of selected fragments per plate. Effects of fragments at 200 μM on the ERα-pTV peptide A-E) or ERα-pTF F-J) peptide is plotted against their effects on the HTRF probes in absence of any peptide. FC-A at 10 μM is taken along as a positive control shown in red. The hit fragment 2 is shown in purple C & H).
Supporting info Figure 3 crosslinking of His14-3-3γ

A) Paraformaldehyde (PFA) crosslinking of His14-3-3γ in the presence of either buffer control (CTL) or 1 mM of fragment 2 for 10 minutes at 0.2% PFA. Samples visualized by running on a SDS-PAGE gel followed by staining with instant blue. A representative SDS-PAGE gel is shown of three independent experiments. B) Quantification of crosslinking band intensities plotted as 65/30 kDa band ratios. All data points of three independent experiments are shown.
Supporting info Spectral analyses

Supporting info Figure 4: $^1$H NMR spectrum of fragment 2 in CD$_3$OD
Supporting info Figure 5: $^{13}$C NMR spectrum of fragment 2 in CD$_3$OD
Supporting info Figure 6: HRMS analysis of fragment 2
Supporting info Figure 7: $^1$H NMR spectrum of fragment 3.2HBr in DMSO-$d_6$
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