Supporting Information for:

Structural elucidation of a small molecule inhibitor of protein disulfide isomerase

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1) Supporting Figure

**Figure S1.** Chemical stability of IBS141. (a) Viability of PC12 mHTT-Q103 cells after 48 hour treatment with IBS141. IBS141 was pre-incubated at 37°C for 0, 1, 2, or 3 days in complete PC12 growth medium before addition to the cells. Data represent the average of triplicates ± SD and plotted as percentage of uninduced PC12 mHTT-Q103 cells. (b) LC/MS spectrum of IBS141 before incubation in complete PC12 growth medium.
2) Supporting Tables

Table S1. Intrinsic clearance of securinine in mouse liver microsomes.

| Compound          | Elimination rate constant (k) | Half-life (t_{1/2}), min | Intrinsic Clearance (CL_{int}), mL/min/g liver |
|-------------------|-------------------------------|--------------------------|-----------------------------------------------|
| Securinine        | 0.0791                        | 8.8                      | 8.30                                          |
| 7-Ethoxycoumarin  | 0.2341                        | 3.0                      | 24.58                                         |

Compound concentration was 0.5 µM. 7-Ethoxycoumarin, a substrate of cytochrome P450 enzymes, was used as a control.
Table S2. Stability of securinine in mouse plasma.

| Compound   | Elimination rate constant (k) | Half-life ($t_{1/2}$), hours | % Remaining after 2-hr incubation |
|------------|-------------------------------|-------------------------------|----------------------------------|
| Securinine | 0.0071                        | 1.6                           | 38.9                             |
| Enalapril  | 0.0201                        | 0.6                           | 4.3                              |

Compound concentration was 1.0 µM enalapril, which undergoes degradation in plasma, was used as a control compound.
Table S3. Securinine plasma protein binding.

| Compound | % Bound  | % Recovery |
|----------|----------|------------|
| Securinine | 48.96 ± 2.93 | 123.75 ± 3.53 |
| Warfarin  | 94.21 ± 0.10  | 109.50 ± 3.54  |

Compound concentration was 2000 ng/ml. Warfarin, an anticoagulant, was used as a control compound. Data are shown as mean ± SD (n=3).
3) Analytical chemistry procedures

Elemental Analysis

Elemental analysis was performed by Schwarzkopf Microanalytical Laboratory.

NMR Spectroscopy

All 1D $^1$H-NMR experiments were carried out on Bruker Avance III 300 MHz or 400 MHz spectrometer at 298 K and analyzed with Topspin 3.0 software (Bruker). The 1D $^{13}$C-NMR experiments were performed on Bruker DRX 300 MHz spectrometer at 298 K.

Infrared Spectroscopy (IR)

IR analysis of the solid compound sample was performed on Thermo Nicolet FTIR equipped with Zinc Selenide (ZnSe) Attenuated Total Reflectance (ATR) crystal accessory.

Liquid chromatography/Mass Spectrometry (LCMS)

LC/MS analysis was performed using Agilent 1100 series preparative HPLC (Agilent Technologies) coupled online to an Esquire 3000 Plus mass spectrometer (Bruker Daltonics), which consisted of nano-electrospray ionization source (ESI) and a quadrupole ion trap mass analyzer.

Compound Characterization

**IBS141**: $^1$H-NMR (400 MHz, CDCl$_3$): δ 6.60 (d, J= 9.1Hz, 1H), 6.41 (dd, J= 5.3Hz, J=9.2Hz, 1H), 5.55 (s, 1H), 3.82 (t, J= 4.7Hz, 1H), 2.97 (dt, J= 3.8Hz, J=10.5Hz, 1H), 2.51 (dd, J= 4.1Hz, J=9.3Hz, 1H), 2.42 (m, 1H), 2.10 (dd, J= 2.4Hz, J=11.3Hz, 1H), 1.88 (m, 1H), 1.78 (d, J=9.2Hz, 1H), 1.59 (m, 6H), 1.23 (m, 2H); $^{13}$C-NMR (75MHz, CDCl$_3$): δ 173.83, 170.24, 140.34, 121.58, 105.27, 89.67, 63.18, 58.96, 48.93, 42.46, 27.43, 26.07, 24.68; $^{13}$C-DEPT NMR (75MHz, CDCl$_3$) CH: δ 140.64, 121.89, 105.54, 63.43, 59.22 CH$_2$: δ 49.20, 42.71, 27.66, 26.29, 24.92 CH$_3$ δ: none; IR (Attenuated Total Reflection): 1738.21 cm$^{-1}$, 1626.49 cm$^{-1}$, 1250.87 cm$^{-1}$; UV/vis: $\lambda_{max}$ 272nm; elemental analysis (% calculated, % found for C$_{13}$H$_{15}$O$_2$N): C (71.87, 71.91), H (6.96, 7.05), N (6.45, 6.52), B (0,
0.24), F (0, <0.1), P (0, <0.05), S (0, 0.40), Cl (0, <0.1); HRMS (m/z): [M]+ calculated for C_{12}H_{15}O_{2}N, 217.11028; found, 217.110279.

**Securinine**: ¹H-NMR (400 MHz, CDCl₃): δ 6.61 (d, J= 9.1Hz, 1H), 6.42 (m, 1H), 5.55 (s, 1H), 3.82 (s, 1H), 2.97 (d, J= 10.6Hz, 1H), 2.46 (m, 2H), 2.10 (m, 12H), 1.88 (d, J= 13.4Hz, 1H), 1.78 (d, J= 9.3Hz, 1H), 1.58 (m, 4H), 1.24 (s, 1H); ¹³C-NMR (75MHz, CDCl₃): δ 173.76, 170.16, 140.24, 121.46, 105.09, 89.54, 63.04, 58.79, 48.80, 42.34, 27.29, 25.92, 24.56. IR (Attenuated Total Reflection): 1737.34 cm⁻¹, 1626.59 cm⁻¹, 1250.43 cm⁻¹; HRMS (m/z): [M]+ calculated for C_{12}H_{15}O_{2}N, 217.11; found, 217.11.
6) Biological Assays

Compound Libraries

Small molecules totaling 68,887 compounds were acquired from the following commercial sources: Asinex, ChemBridge, Comgenex, InterBioScreen, Life Chemicals, MicroSource Discovery Systems, Sigma-Aldrich, and TimTec. The compounds were stored in 384-well clear, round bottom, polypropylene plates (Greiner: 781280) at a concentration of 4 mg/mL in dimethylsulfoxide (DMSO). The plates were stored frozen at -80°C until the day of the assay.

Cell Culture

PC12 mHTT-Q103 cells, kindly provided by Erik S. Schweitzer (David Geffen School of Medicine, University of California, Los Angeles), were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, 25 mM HEPES and sodium pyruvate (Mediatech cat. # 15-018-CV), supplemented with 10% Cosmic calf serum (Thermo Fisher Scientific cat. # SH30087), 2 mM L-glutamine (Life Technologies cat. # 25030-081), 100 U/ml of penicillin-streptomycin (Life Technologies cat. #15140-122), and 0.5 mg/ml geneticin (Life Technologies cat# 11811-031). Cells were grown at 37°C, 9.5% CO₂, and medium was replaced with fresh medium every 2-3 days. Tebufenozide, 200 nM final concentration from 1 mM stock in 85% ethanol (kindly provided by Lynne Moore and Fred H. Gage, The Salk Institute for Biological Studies, La Jolla, CA) was added to medium to induce mutant HTT-Q103 (mHTT-Q103) expression.

High-Throughput Screening

The high-throughput screening assay has been previously described. Briefly, replica daughter plates were prepared with Biomek FX (Beckman Coulter) robotic liquid dispenser by transferring 2 µl of compounds from the stock DMSO plates into 384-deep-well clear, round bottom, polypropylene plates (Greiner cat. # 781270) containing 98 µl of PC12 medium without selective agent Geneticin to obtain compound concentration of 80 µl/ml in 2% DMSO. Assay plates were set up by seeding tebufenozide-induced or uninduced mHTT-Q103 cells into 384-well black, clear-bottom plates (Corning Inc cat. # 3712) at a density of 7,500 cells per well in 57 µl PC12 medium without Geneticin. Three microliters of compound from the daughter plate were added to the assay plate for a final compound concentration of 4 µl/ml in 0.1% DMSO. The assay plates were incubated at 37°C, 9.5% CO₂ for 48
hours. 20 microliters of 40% Alamar blue (Life Technologies cat. # DAL1100) solution in PC12 medium was added to each well (1:10 final dilution) and the plates were incubated for an additional 12-24 hours at 37°C, 9.5% CO₂. Alamar blue fluorescence was read on a fluorescence plate reader (PerkinElmer Victor3) with 530 nm excitation filter and 590 nm emission filter. Both tebufenozide-induced and uninduced cells were tested in triplicate.

**Molecular Cloning, Protein Expression and Purification of PDIa.** Cloning, PDI protein expression and purification has been performed as previously described². PDIa is the catalytic a domain of human PDI A1 and was referred to as PDIa throughout the paper.

**Protein NMR spectroscopy.** The residue numbering in all HSQC spectra are based on the sequence of the mature PDI protein i.e., residue 1 of the mature PDI corresponds to residue 18 in the full length PDI. The first 17 amino acids in full length PDI are the signal sequence that is processed out to generate the mature PDI.

The ¹H⁻¹⁵N HSQC spectra were performed on Bruker Avance III 500 Ascend (500 MHz) spectrometers at 300 K. Uniformly ¹⁵N-labeled PDIa was dissolved at 50 µM in 90% H₂O/10% D₂O (v/v), pH 5.1. The ¹H carrier frequency was positioned at the water resonance. The ¹⁵N carrier frequency was positioned at 115 ppm. The spectral width in the ¹H dimension was 7500 Hz and the width in ω₁ (¹⁵N) dimension was 1824.6 Hz. Suppression of water signal was accomplished using the WATERGATE sequence. Heteronuclear decoupling was accomplished using GARP decoupling scheme. The ¹H⁻¹⁵N HSQC spectrum shown contained 50 µM PDIa and 500 µM of securinine (10 molar excess over protein). The resonance assignments of reduced PDIa have been previously published².

**Intrinsic Tryptophan fluorescence assay.** All fluorescence reading were carried out on a Tecan Infinity M200 microplate reader in a 384-well low volume, black bottom plates. Securinine was dissolved in 100% DMSO at 300 mM concentration and then diluted into Assay Buffer A (100 mM sodium phosphate buffer, pH 7, 2 mM EDTA) to make working stocks at 2X the needed concentrations (from 9000 µM to 70 µM). Each reaction well contained the indicated concentrations of securinine (from 4500 µM to 35 µM) or DMSO (1.5%) and 10 µM final concentration of PDIa in Assay Buffer A. The samples were incubated at 37 °C for 1 hr. and then emission spectra recorded from 300- to 400-nm wavelength with excitation at 295 nm. The maximum PDIa Trp fluorescence intensity was at 330 nm wavelength (λmax). Securinine did not shift the λmax. As a control emission spectra was
also recorded for compounds alone without PDIa (excitation of 295 nm). Securinine and PAO did not show any fluorescence between 300 and 400 nm on their own. All emission spectra are normalized by the maximum fluorescence intensity of PDIa only sample. Emission spectra were collected in triplicate and plotted as mean ± SEM and fitted to a log normal distribution equation in GraphPad Prism. ∆F was calculated by subtracting the Trp fluorescence intensity (at 330 nm wavelength) of the securinine treated samples from the Trp fluorescence intensity (at 330 nm) of the DMSO treated control. ∆Fmax is the maximum difference in Trp fluorescence between securinine and DMSO treated samples. ∆F/∆Fmax was calculated by diving ∆F of each securinine treated PDIa samples by the maximum ∆F (∆Fmax) of the series. The K_d in Figure 5b was determined by fitting the data at each securinine concentration to the one-site total with non-specific binding equation in GraphPad Prism.

**Reversibility Assay using Jump-Dilution Method.** Securinine (10 mM), Phenylarsine oxide (3 mM), or DMSO only (3%), were incubated with 1 mM PDIa for 2 hr. at 37 °C in Assay Buffer A. After the incubation, the samples were diluted 1:100 with Assay Buffer A (now concentration is 10 µM PDIa with either 100 µM securinine, or 30 µM PAO, or 0.03% DMSO). 40 µl/well of the sample was added to the assay plate and emission spectra recorded from 300- to 400-nm wavelength with excitation at 295 nm. All emission spectra are normalized by the maximum fluorescence intensity of PDIa only sample. Emission spectra were collected in triplicate and plotted as mean ± SEM and fitted to a log normal distribution equation in GraphPad Prism.

**Mouse microsome stability assay.** Securinine (0.5 µM) was incubated at 37ºC for up to 45 minutes in 100 mM of potassium phosphate buffer pH 7.4 containing microsomal protein (0.5 mg/mL) and an NADPH generating system (0.34 mg/mL β-nicotinamide adenine dinucleotide phosphate (NADP), 1.56 mg/mL glucose-6-phosphate, 1.2 units/mL glucose-6-phosphate dehydrogenase). At 0, 5, 15, 30 and 45 min intervals an aliquot was taken and quenched with acetonitrile (ACN) containing internal standard. No-cofactor controls at 45 min were prepared. Following completion of the experimentation, the samples were analyzed by LC-MS/MS. The half-life (t_1/2) is calculated using the following equation: 
\[ t_{1/2} = \frac{0.693}{k} \]  
Where, k is the elimination rate constant of test compounds obtained by fitting the data to the equation:  
\[ C = \text{initial} \times \exp (-k \times t) \]  
Intrinsic clearance (CL_int) is calculated as liver clearance from the half-life using the following equation:  
\[ \text{CL}_{\text{int}} = k \times (\text{ml incubation/ 0.5 mg protein}) \times (52.5 \text{ mg} \text{ protein}) \]
protein/g liver). Results were reported as peak area ratios of analyte to internal standard. The intrinsic clearance (CL_{int}) was determined from the first order elimination constant by non-linear regression.

**In Vitro Drug Metabolism Studies: Mouse plasma stability assay.** Securinine (1 µM) was incubated at 37°C for 120 minutes in mouse plasma. At 0, 15, 30, 60, and 120 min intervals an 100 µL aliquot was taken and quenched with 200 µL acetonitrile (ACN) containing internal standard and analyzed by LC-MS/MS. The half-life (t_{1/2}) was calculated using the following equation: t_{1/2} = 0.693 / k Where, k is the elimination rate constant of test compounds obtained by fitting the data to the equation: C = initial \times exp (-k \times t). Results were reported as peak area ratios of analyte to internal standard.

**In Vitro Drug Metabolism Studies: Mouse plasma protein binding assay.** Securinine at 2000 ng/mL in plasma was added into the sample chamber, Phosphate-buffered saline (PBS) was added into the buffer chamber, and incubated for 4 hours at 37°C on an orbital shaker. Samples were then taken from both plasma and buffer chamber and analyzed by LC-MS/MS. Protein binding and free fraction percent was determined using peak area ratio of analyte to internal standard. Fraction bound percent was calculated as: % Bound = 100 \times \frac{C_{\text{plasma}} - C_{\text{PBS}}}{C_{\text{plasma}}} . The fraction recovered percent was calculated as: % Recovery = \frac{V_{\text{PBS}} \times C_{\text{PBS}} + V_{\text{plasma}} \times C_{\text{plasma}}}{V_{\text{plasma}} \times C_{\text{spike}}} where V_{\text{PBS}} is Volume of PBS, V_{\text{plasma}} is Volume of Plasma, C_{\text{PBS}} is Drug concentration in PBS (Analyte/IS peak area ratio), C_{\text{plasma}} is Drug concentration in plasma (Analyte/IS peak area ratio), C_{\text{spike}} is Drug concentration in spiked plasma (Analyte/IS peak area ratio).

**In Vitro Drug Metabolism Studies.** The microsome stability, plasma stability, and plasma protein binding assays were performed by Alliance Pharma, Inc. (Malvern, PA).
References:

(1) Hoffstrom, B. G.; Kaplan, A.; Letso, R.; Schmid, R. S.; Turmel, G. J.; Lo, D. C.; Stockwell, B. R., Inhibitors of protein disulfide isomerase suppress apoptosis induced by misfolded proteins. Nat. Chem. Biol. 2010, 6, 900-6.

(2) Kaplan, A.; Gaschler, M.M.; Dunn, D.E.; Colligan, R.; Brown, L.M.; Palmer, A.G.; Lo, D.C.; Stockwell, B.R., Small molecule-induced oxidation of protein disulfide isomerase is neuroprotective. Proc Natl Acad Sci USA. 2015, 112, E2245-52.