Supporting Material

Drug Target Engagement using Tandem Cellular Thermal Shift Assay- acoustic Reverse Phase Protein Array

Adrien Herledan [a], Marine Andres [a,c], Aurore Lejeune-Dodge [b], Florence Leroux [a,c], Alexandre Biela [a], Catherine Piveteau [a], Sandrine Warenghem [a], Cyril Couturier [a], Benoit Deprez [a,c], Rebecca Deprez-Poulain [a,c,d]

[a] *INSERM "Drugs and Molecules for Living Systems", Univ. Lille, and Institut Pasteur de Lille, 59000 Lille, France*

[b] *LABCYTE INC,* 170 Rose Orchard Way, San Jose, CA 95134, USA

[c] *European Genomic Institute for Diabetes, EGID, University of Lille, F-59000, France.*

[d] *Institut Universitaire de France (IUF), 75231 Paris, France*

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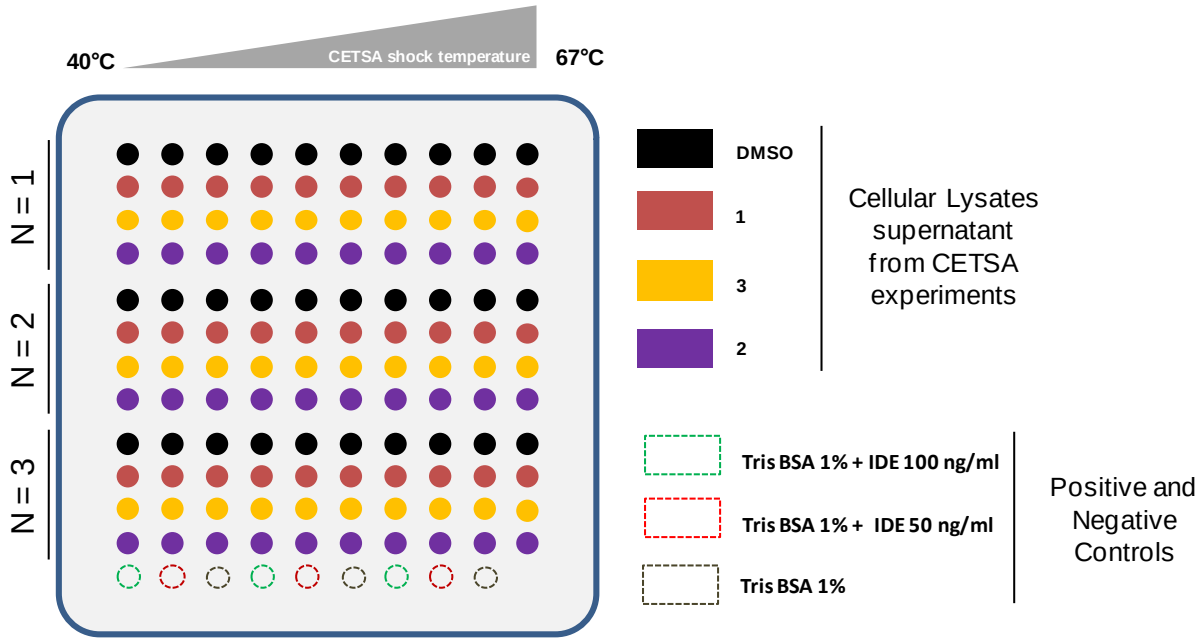
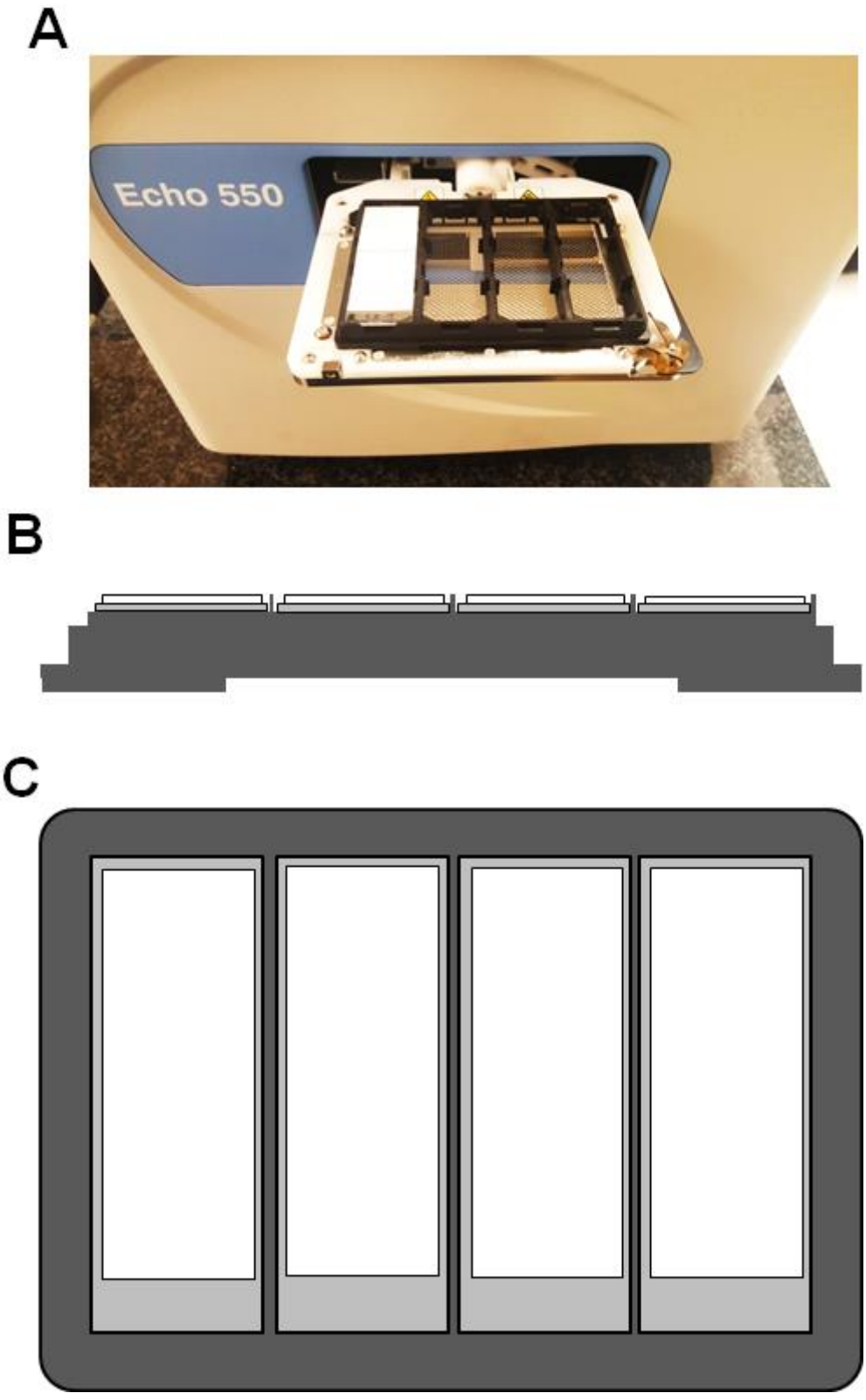
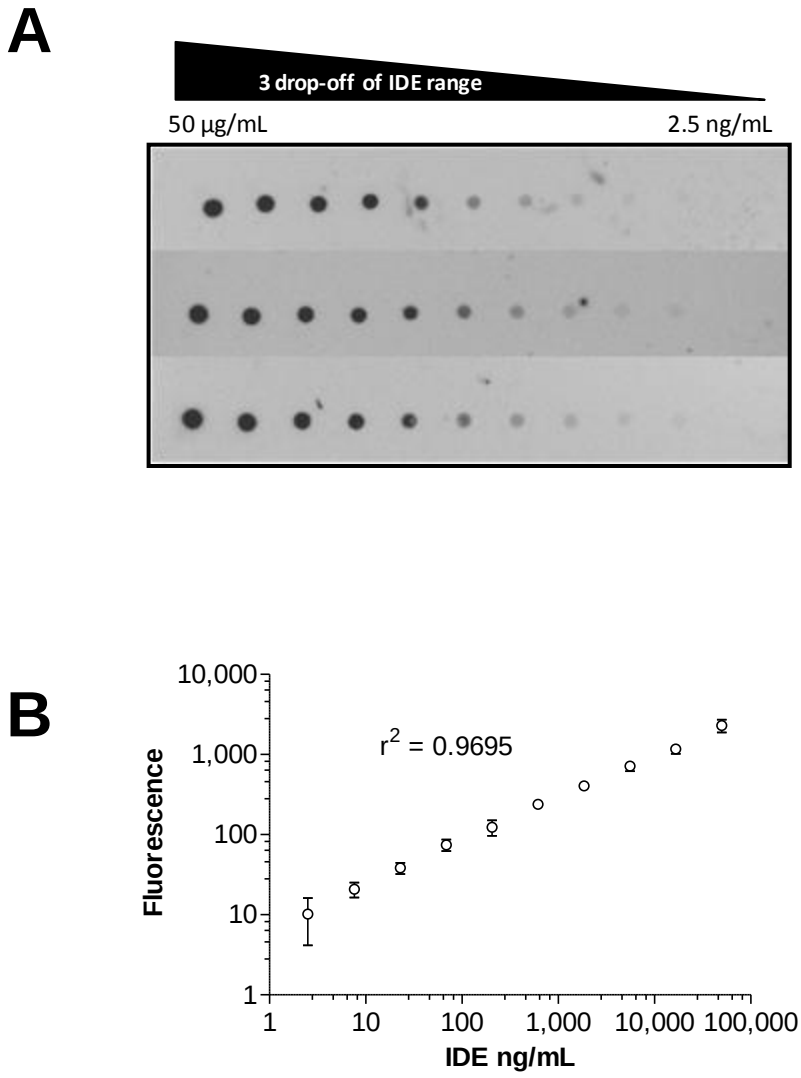


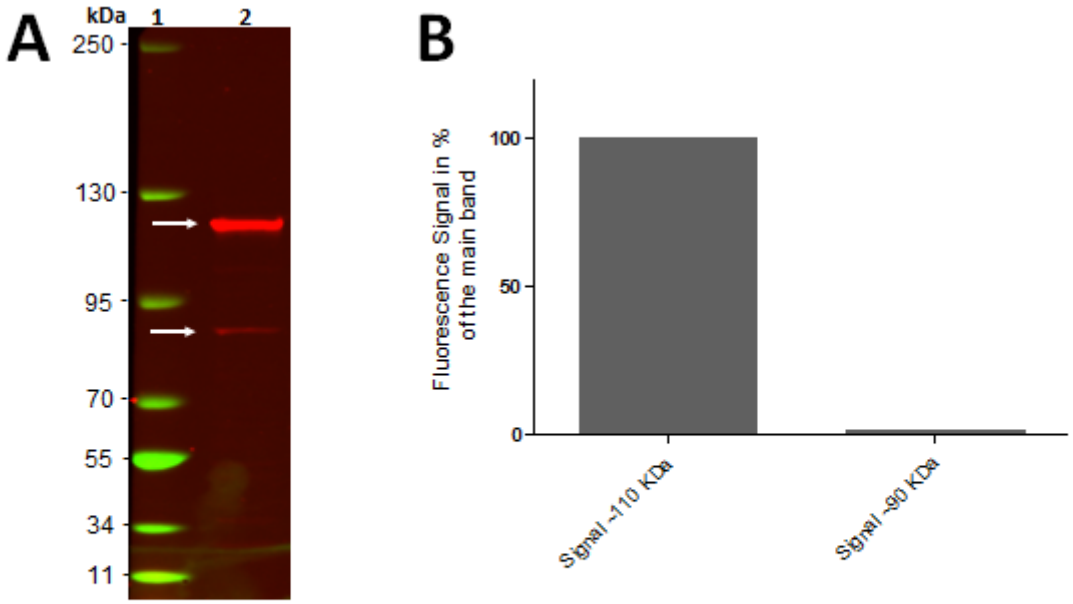
Supplementary Figure S1. Design of the CETSA Protein Microarray pattern. Cellular lysates from CETSA experiments (1: BDM44768, 2:analog of BDM44768, 3: 6bK) corresponding to three CETSA independent experiments (N = 1-3) and controls were spotted (20 nL) with Echo 550 device (384_LDV_AQ_P2 calibration) on nitrocellulose membrane. Positive and negative controls (Tris BSA 1% with or without purified IDE) are used to validate the specificity of Insulin Degrading Enzyme detection.



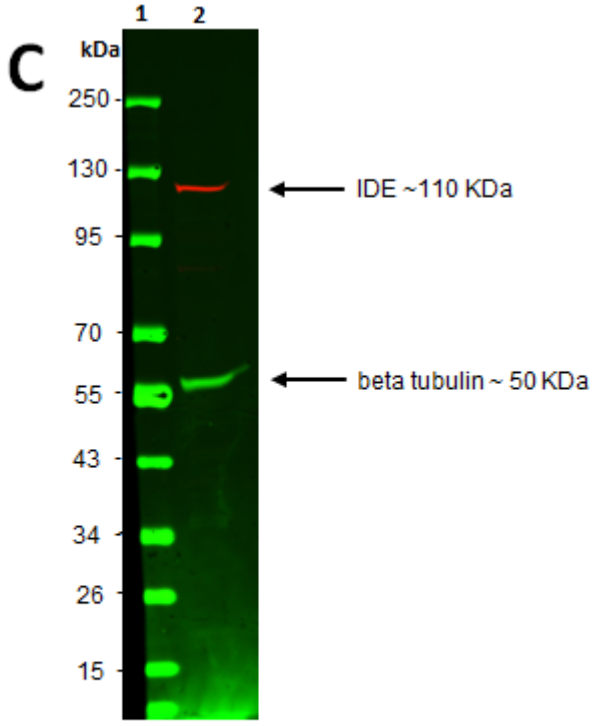
Supplementary Figure S2. Set-up Nitrocellulose membranes fixed on glass slides and supported on a slide holder. Picture of the assembly on the carrier destination of the Echo 550 (A) and plan of the mounting system in lateral (B) and top view (C).



Supplementary Figure S3. Acquired images of purified IDE : between 50 µg/mL and 2.5 ng/mL printed (20 nL) on 3 nitrocellulose membranes. (A) Intensity of fluorescence plotted against concentration in the solution (B).

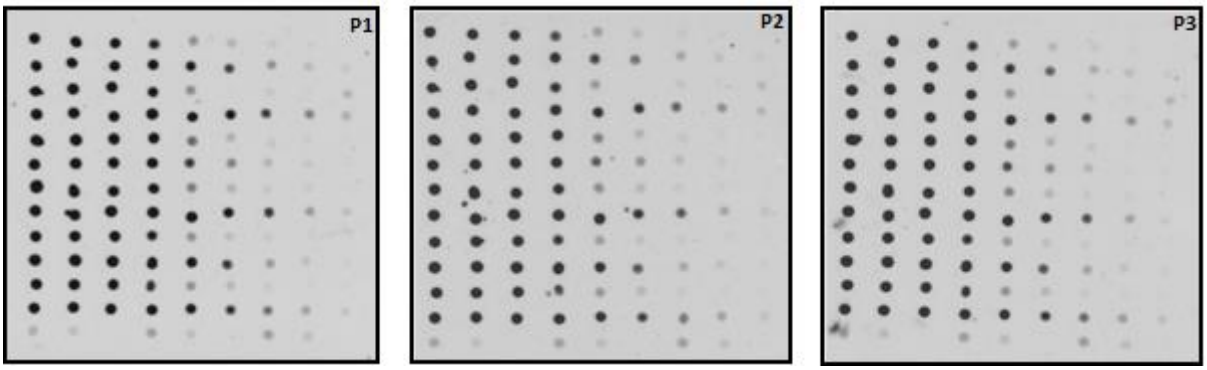


1: Prestained NIR Protein Ladder
2: HepG2 cells protein extract

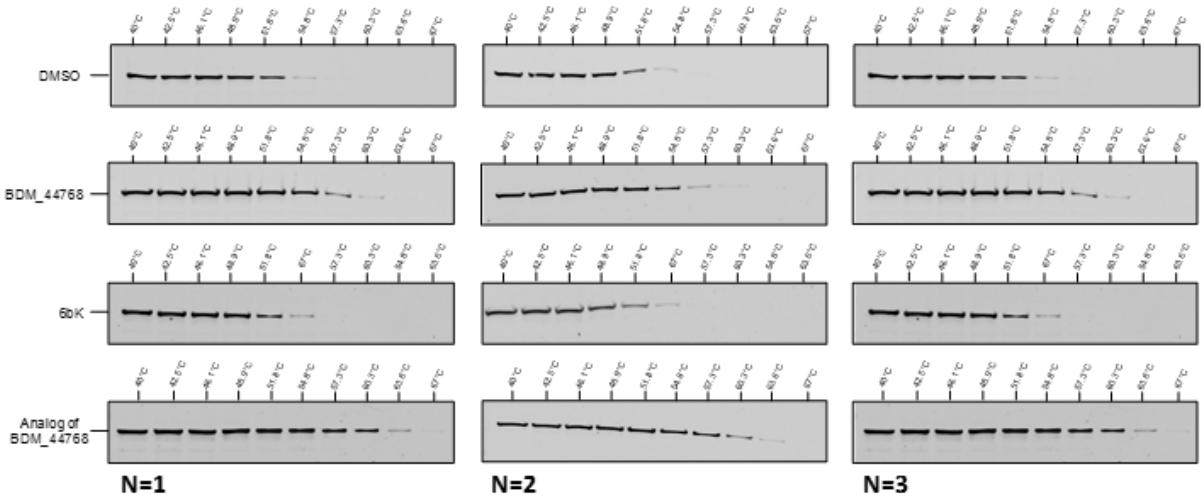


1: Prestained NIR Protein Ladder
2: HepG2 cells protein extract

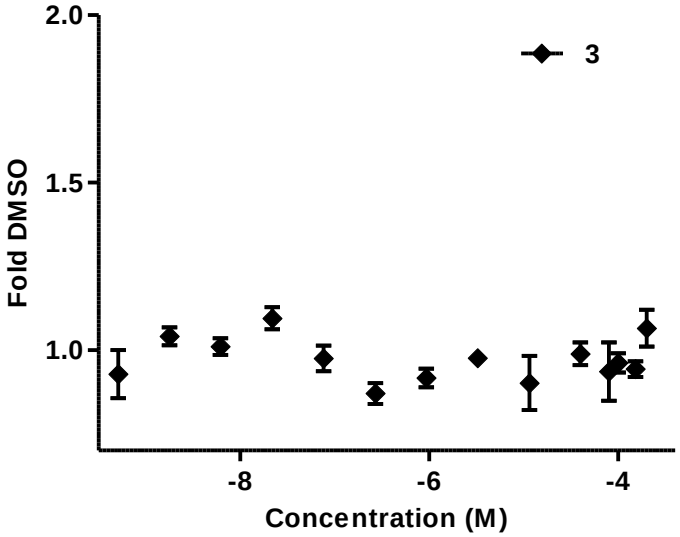
Supplementary Figure S4. Specificity of the F-9 anti-IDE antibody in WB. F-9 anti-IDE antibody (Santa Cruz, sc-393887) in (A) WB (B) quantification of signals at 110kDa (IDE) and 90kDa (non-specific binding), (C) WB with double detection of IDE (F-9 antibody) and β-tubulin (10068-1-AP) for used in CETSA-ITDRF-aRPPA.



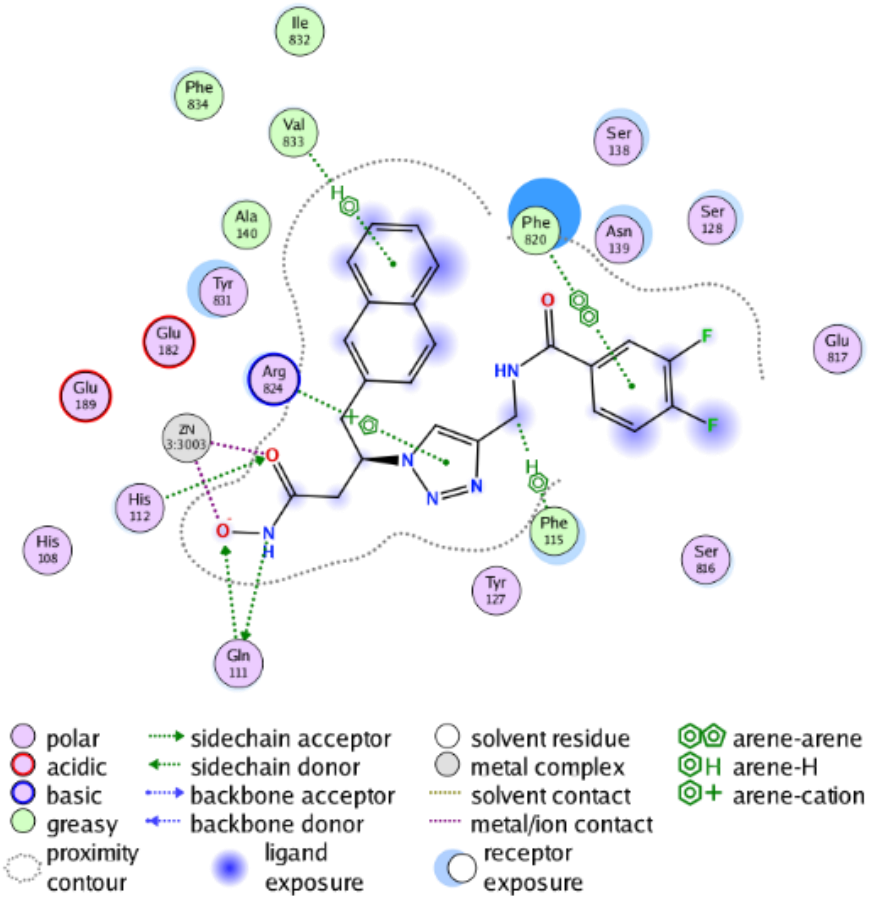
Supplementary Figure S5. Acquired images of CETSA Protein Microarray patterns. CETSA samples were printed in triplicate on 3 nitrocellulose membranes (P1, P2, P3), for precision measurement.



Supplementary Figure S6. Acquired images of CETSA Protein Western Blot. DMSO, BDM44768, 6bK and analog of BDM44768 corresponding to the three independent experiments (N=1, N=2, N=3).



Supplementary Figure S7. ITDRF of 3 with IDE. in HepG2 cells.



Supplementary Figure S8. Interactions of 2 with IDE. (PDB: 4NXO) using MOE 2018.0101.

Supplementary Tables

	aRPPA			WB		
Cpd	**T_{Agg} (°C)**	**Δ_{Agg} (°C)**	**R^2 value**	**T_{Agg} (°C)**	**Δ_{Agg} (°C)**	**R^2 value**
DMSO	49.25	NA	0.996	48.61	NA	0.951
1	52.31	3.06	0.982	53.05	4.44	0.961
2	54.88	5.63	0.980	56.34	7.73	0.974
3	48.89	-0.36	0.962	48.76	0.15	0.984

Supplementary Table S1. Tagg of hIDE. in the presence of inhibitors or DMSO by aRPPA or Western Blot.

Cpd	MW (g/mol)	Mode	Capillary Voltage (kV)	Parent Ion	Cone Voltage (V)	Product Ion	Collision Energy (eV)	Retention time (min)
1	447.5	ES+	1.5	**448.1**	44	**123.0**	38	1.32
2	465.5	ES+	1.5	**466.2**	44	**195.1**	26	1.36
3	757.9	ES+	3.0	**758.4**	58	**84.0**	76	1.24

Supplementary Table S2. MS/MS parameters: for compounds **1-3**, for permeability studies.

Supplementary Methods.

LC-MS/MS analysis of CETSA samples

To correlate CETSA results with cell permeability of compounds, the incorporated fraction of compounds in cells was measured. CETSA samples (10 million cells per mL in Tris-buffered saline) and culture medium containing drugs at 30 µM or vehicle were prepared in a 1 to 20 ratio in an acetonitrile/methanol solution (5:5, v/v). Then, the samples were vigorously mixed with a vortex and centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatants were transferred into tubes for liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Chromatography of 1 µL of the sample was performed on an Acquity BEH C18 column (50*2,1mm 1,7µm, Waters) by elution with a gradient from Ammonium acetate 10 mM (spontaneous pH) (A) to acetonitrile 0,1 % formic acid (B). The gradient was controlled as follows: 0-0.2 min, 2% B, 0.2-2 min, 2 to 98% B, 2-2.5 min, 98% B, 5.5-2.6 min, 98 to 2% B, 2.6-4 min, 2%B. Flow rate was 0.6 mL/min. Column oven and autosampler temperatures were maintained at 40°C and 10°C respectively. The column outlet of the LC was either connected to Xevo TQD triple Quadrupole Mass Spectrometry (Waters) for MS/MS detection in positive electrospray ionization (ESI) mode. The source temperature was 150 °C, desolvation temperature 600 °C, cone gas flow 50 L/h and desolvation gas flow 1200 L/h. The analytes were monitored in multireaction monitoring mode (MRM). Analyses were processed using MassLynx software (Waters). The ratio between cellular and medium fraction peak areas permitted to compare the membrane-crossing ability of compounds.

Solubility and Log D measurements

10µL of a 10 mM solution in DMSO of the compound are diluted either in 490µL of PBS pH 7.4 or in organic solvent MeOH in a 700µL-microtube (in triplicate). The tubes are gently shaken 24 h at room temperature, then centrifuged for 5 minutes at 4000 rpm. The mixtures are filtered over 0.45 µm filters (Millex-LH Millipore). 20 µL of sample are diluted in 180 µL of MeOH. The solubility is determined by the ratio of mass signal area PBS/ organic solvent. 40µL of a 10 mM solution in DMSO of the compound were diluted in 1.960 mL of a 1/1 octanol /PBS at pH 7.4 mixture. The mixture was gently shaken 2 h at room temperature. 20 µL of each phase was diluted in 480 µL of MeOH and analyzed by LC-MS. Each compound is tested in triplicate. Log D was determined as the logarithm of the ratio of concentration of product in octanol and PBS respectively, determined by mass signals.

Docking of compound 2 in IDE PDB:4NXO

Modeling and simulations were performed using MOE 2018.0101 (Chemical Computing Group, Inc.) using non-aged X-ray crystal structure of *h*IDE PDB code 4NXO with **1** (BDM44768). A monomer including solvent was selected and compound **2** was docked. Before analysis, ligands, solvents and proteins were protonated at 300 °K, pH 7.0 with a salt concentration equal to 0.1 mol/L for the generalized Born / Volume integral (GB/VI) with a cutoff value of 15. The dielectric constants of solute and solvent are set to 1 and 80, respectively. The method 800R3 was applied to simulate the repulsive part from the van der Waals energy. Then for each compound the industry standard molecular mechanics force fields MMFF94(s) was used in association with a

MOPAC quantum code. The structure was rendered in PyMOL (Delano, W. L. *The PyMOL Molecular Graphics System.* DeLano Scientific LLC: San Carlos, CA, 2002).

Isothermal dose response fingerprint experiments (ITDRFCETSA-aRPPA)

HepG2 (2 mL) cells were dispensed at 150 000 cells per mL in 6 well cell culture Microplates (Corning Costar® TC-Treated Multiple Well Plates, 3516) previously coated with gelatin and were maintained at 37°C in humidified 5% CO_2 atmosphere for 72 hours until confluence. $ITDRF_{CETSA}$ were performed replacing culture medium by 1 mL of fresh medium and by addition of 10 µL of compounds corresponding to final concentrations (200 µM to 14.5 pM) or vehicle (1% DMSO). After 2 hours of incubation (37°C in humidified 5% CO_2), HepG2 cells were washed with 2 mL of PBS and detached using 250 µL of trypsin/EDTA. 1 mL of medium was added and cells were transferred in 1.5 mL Protein LoBind Tubes (Eppendorf, 0030108116) for centrifugation at 300 g (4 min). The medium was discarded and cells pullets were homogenised in 60 µL of TBS 1X. PCR 0.2 mL microtubes with HepG2 cells (50 µL) were heatshocked at 50.5°C for 3 minutes with a SureCycler 8800 Thermal Cycler and left 3 minutes at room temperature before protein extraction as previously described for classical CETSA. Supernatants (35 µL) were stored for analysis in Reverse Phase Protein Array with three biological replicates (independent experiments).

Immunoblotting proceeds like for CETSA-aRPPA . An extra signal corresponding to an housekeeping protein was measured incubating an anti-Tubulin Beta polyclonal antibody (ProteinTech, 10068-1-AP) diluted at 1:2000 in the primary antibody solution (with anti-IDE antibody) and a Donkey anti-Rabbit IgG Cross-Adsorbed Secondary Antibody conjugued to DyLight 680 (Invitrogen, SA5-10042) diluted at 1:20000 in the secondary antibody solution (with Anti-Mouse IRDye 800 CW). The simultaneous detection of IDE and a constitutively expressed housekeeping protein beta-tubulin was

operated by quantifying the intensities (corresponding to sample fluorescence minus background fluorescence) of each spot with Image Studio™ Lite Analysis Software (Licor) into each channel (700 and 800 nm). These normalised signals (*ratio* between *800 nm* and *700 nm) were expressed in fold of DMSO condition* and plotted against concentration in Graphpad (Prism) software following the nonlinear regression analysis to the 4 parameter logistic equation: Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)). The EC_{50} (Half maximal effective concentration) of each compound were determined from dose response curves expressed as means of three experiments (n = 3) +/- SEM.