A real-time cellular thermal shift assay (RT-CETSA) to monitor target engagement

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Figure S1. RT-CETSA prototype device.

Figure S2. Measuring thermal stability of ThermLuc constructs and furimazine substrate.

Figure S3. Comparisons with RT-CETSA thermal melt and analysis of a subset of LDHAi.

Figure S4. RT-CETSA thermal profiles of additional targets of interest (TOI).

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Supplementary Movie S1. Monitoring thermal destabilization of LDHA-ThermLuc in HEK293T cells using real-time CETSA (RT-CETSA).

Supplementary Methods

References
Figure S1. RT-CETSA prototype device. (A) OEM camera on the Roche LC480 II Real-Time PCR instrument. Dimensions of the stock CCD lens, which are held within a metal bracket in the machine, are shown. (B) Schematic of RT-CETSA components. Emission filters were removed and the stock camera was replaced with a water-chilled Orca R2 CCD camera for an unfiltered light path and improved luminescence detection. The 35mm f/1.6 lens was a direct replacement for the original stock CCD lens aperture and focal length.
Supplementary Figure S2

Figure S2. Measuring thermal stability of ThermLuc constructs and furimazine substrate. (A) Thermal melt profile of NLuc variants with different Gly-Ser linker lengths between the 11S and HiBiT fragments, as measured using RT-CETSA device (mean ± SD, n=64 wells) and a commercially available furimazine (NanoGlo, Promega). Right panel depicts data normalized to starting luminescence for each individual well. (B) Thermal stability of furimazine (NanoGlo, Promega) and LiveGlo (Promega) substrates were assessed by first heating 0.2X substrate to the indicated temperature for 3.5min in the presence of HEK293 cells (untransfected), cooling to room temperature, and then adding recombinant 11S and HiBiT peptide. Luminescence was measured (mean ± SD, n=4). (C) Thermal stability of furimazine synthesized in-house was examined in the RT-CETSA device, either with temperature ramp or using a 37 °C hold, for identical timeframes (n=64 wells). (D) Melting profile of LDHA-ThermLuc was examined using different temperature ramping, either holding at each temperature integer for 4 sec or 20 sec (mean ± SD, n=96 wells). (E) Cell viability after 48h incubation with furimazine, diluted from Promega 50X stock or DMSO solution (mean ± SD, n=4). *p<0.05, **p<0.01, ***p<0.001. (F) Systemmetric CellHealth screening performed using furimazine formulated in DMSO. CM= cell morphology; CMI=cell membrane integrity; ROS = reactive oxygen species; GSH = glutathione; NMI1/2 = nuclear membrane integrity metric 1 and 2; CC = cell cycle; MMD = mitochondrial membrane depolarization. Overall score of 0.4 is threshold for high cell stress. (G) Target engagement and thermal shift of LDHA-ThermLuc was examined using endpoint CETSA analysis, where furimazine was excluded (left) or included (right) during the heating step. Fresh substrate was added to all samples before measuring luminescence (mean ± SD, n=2). (H) Thermal shift was examined for LHDA-ThermLuc using the endpoint method after treatment with 10 µM LDHAi 1. Samples were heated for 30 sec (left) or 3.5 min (right) before lysis. Furimazine was added and luminescence was measured (mean ± SD, n=2). (I) Rapid aggregation of LDHA-ThermLuc after transitioning to 72 °C. Luminescence was monitored using the RT-CETSA device. (J) RT-CETSA analysis of LDHA-ThermLuc thermal shift for cells pre-treated with 10 µM LDHAi 1 for 15, 30, or 60 min.
Figure S3. Comparisons with RT-CETSA thermal melt and analysis of a subset of LDHAI. (A) Log EC_{50} values for highlighted prior art compounds when analyzed with RT-CETSA methods, acoustic CETSA, SplitLuc CETSA, lactate assay, biochemical assay and SPR. Compounds with no detected binding are annotated as “0”, and compounds with no annotated data for a particular method are blank squares. (B) Thermal Dose-Response Curves for highlighted compounds from the LDHAI set. Curves shown are baseline-corrected data from single experiments. (C) Concentration-Response curves for highlighted compounds using RT-CETSA and analyzed by T_{agg}, AUC, and NPARC. Data shown are from n=3 biological replicates (mean ± SD). (D) Correlation between target engagement potency values calculated using SplitLuc CETSA or RT-CETSA (NPARC method), for twenty-nine LDHAI inhibitors. Dotted line represents equipotency in the two assays. (E) Testing of the minimum significant ratio (MSR) and related parameters for the AUC metric. The mean ratio (MR) is shown as a solid blue line, Limits of Agreement (LsA) in dashed red lines, and ratio limits (RL) in dashed green lines.
**Supplementary Figure S4**

**Figure S4.** RT-CETSA thermal profiles of additional targets of interest (TOI). (A) RT-CETSA simultaneous thermal profile of eight TOI-ThermLuc fusions (mean ± SD, n=32 wells). The secreted fraction (labeled ‘sup’) was examined for several proteins by transferring culture medium to a PCR plate before beginning the assay. (B) Target engagements of 10 µM Dasatinib (orthosteric inhibitor) or 40 µM GNF-2 (allosteric inhibitor) with cAbl-ThermLuc (ABL1, kinase-only domain) was examined by RT-CETSA (Mean ± SD, n=5). (C) Thermal melt profile of DHFR fusions (SplitLuc, NanoLuc, or ThermLuc), for vehicle or methotrexate (MTX) treated cells, examined using the endpoint CETSA method (mean, n=2). (D) Seventeen different linkers were inserted between DHFR and ThermLuc to examine effects on thermal stability in RT-CETSA (mean ± SD, n=3). Rank-ordering of melting temperatures is indicated in the legend. (E) Methotrexate target engagement was examined for DHFR fusions containing an A[EAAAK]₃ linker between the target and NanoLuc or ThermLuc (mean ± SD, n=2).
Figure S5. Analysis of cell membrane integrity through RT-CETSA thermal gradient. Propidium iodide signal measured in HEK293T cells subject to RT-CETSA experimental conditions. The purple dashed line denotes the temperature at which there is a significant increase in fluorescent signal in the cells-only group (mean ± SD, n=5).
Supplementary Movie 1: Monitoring thermal destabilization of LDHA-ThermLuc in HEK293T cells using real-time CETSA (RT-CETSA). Cells were treated with vehicle control, LDHAI 1 or LDHA inhibitors (in dose-response format), as indicated.
SUPPLEMENTARY METHODS

**Molecular biology.** ThermLuc plasmids were created using a pcDNA3.1(+) backbone and cloning into the Nhel and EcoRI sites using Genestrands (Eurofins) encoding LgBiT-[1/3/6/9/12/15 GlySer]-HiBiT-GlySer. The gene strand was inserted into the linearized backbone using InFusion (Takara) following the manufacturer’s instructions. For TOI-ThermLuc fusions, the sequence ggatccGGCGGTGGTGGCTCT (GlySerGlyGlyGlyGlySer) was placed immediately upstream of ThermLuc, to create a BamHI (in lowercase above) site such that various targets could be cloned as N-terminal fusions using Nhel and BamHI sites and InFusion reagents. For transmembrane and secreted targets (CD19, CD20, NGF, PCSK9), fusions were created in TOI-ThermLuc orientation to maintain signal sequences and partitioning to the secretory pathway. An acceptor plasmid containing ThermLuc in the N-terminal orientation was created by cloning the ThermLuc gene strand into pcDNA3.1(+) using the NheI and EcoRI sites, where the sequence GGCGGTGGTggatcc (GlyGlyGlyGlySer) was placed immediately downstream of ThermLuc to create a BamHI site (in lowercase above) such that various targets could be cloned as C-terminal fusions using BamHI and EcoRI sites and InFusion reagents. To create plasmids encoding DHFR-ThermLuc with different intervening linkers, we first created a pcDNA3.1(+) construct in which the nucleotide sequence

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ggatccGCTGAAGCCGCGGCTAAAGAGGCTGCCGCGAAAGAAGCTGCAGCTAAGGAGGCTGCAGCGAAAGAGGCAGCGGCAAAGGAGGCTGCCGCGAAGGCTaagctt
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inserted between the final amino acid of DHFR and the start codon of ThermLuc. This represented DHFR-A[EAAAK]6A and allowed subsequent digestion with BamHI and HindIII (sites in lowercase in sequence above) and insertion of linker sequences using InFusion PCR. Linker sequences were synthesized as two complementary oligonucleotides (Supplementary Table 1) that were annealed in a 50 μL solution containing 10 μM forward oligo, 10 μM reverse oligo, 10 mM TrisHCl (pH 8.0), 50 mM NaCl and 1 mM EDTA. The solution was placed in a beaker of boiling water, beaker was removed from heat, and tube remained in the water to cool to room temperature without intervention. The resulting duplex was used for InFusion PCR following the manufacturer’s instructions.

**Cell culture.** HEK293T cells were grown in a high-glucose DMEM with sodium pyruvate (Gibco) plus 10% (v/v) FBS (Hyclone), 1X GlutaMax (Gibco), 100U/ml penicillin, and 100U/ml streptomycin. Cells were incubated at 37 °C with 5% CO₂ and 95% humidity. Cells were routinely tested for mycoplasma using a Lonza MycoAlert kit.

**Prototype RT-CETSA hardware.** All emission filters were removed from the light path in the Roche LightCycler 480 II for RT-CETSA experiments. After instrument warmup and pre-run checks with the stock camera in place to allow the machine to initialize, the xenon bulb in the light cycler was removed and covered to shield the plate from excess light and the original LightCycler camera was swapped with a
Hamamatsu Orca II camera. A 35 mm Navitar high resolution c-mount fixed focal length lens was added to the Orca II camera. The outer casing of the lens (32 mm diameter) was slightly filed to allow it to snugly fit into the 32 mm diameter mount present in the LightCycler. It was important that the instrument be left in its stock configuration upon power-up so that pre-run checks could proceed without error. The LightCycler control software (Roche) was set to run a protocol including a 2 min hold at 37 °C to allow for camera equipment swaps and enclosure reset after the instrument had completed pre-run checks with the stock camera in place. Once the equipment changes were made and the 2 min pre-run hold ended, LabView Software interfaced with the Orca II camera took exposures at user-defined intervals until the operator stopped operation at the end of the heating cycle. The temperature ramp was set so that the time to ramp and the time held at each step/°C in the thermal ramp was equal to the shutter speed of the camera (in testing, was either 2, 4, or 8 sec).

**Melting curve analysis.** The automated MATLAB workflow is proceeded as follows: for time-series analysis of RT-CETSA images, the calibration steps (details below) are required only on the initial time point. All subsequent time-series images were automatically processed in batch mode, and results for each sample were exported as time-indexed series tables. Prior to running the analysis, the user defines the script parameters to account for the size of the square bounding box region surrounding each sample in the image (estimated number of pixels, width, and height). When executed in the MATLAB computational environment (MATLAB version 2021a) the script functions allow the user to select the first image file in the time series for display, and a graphical user interface prompts the user to locate the positions of the upper left sample, the upper right sample, and the lower left sample. The user also enters the total number of sample rows and columns that are in the selected contiguous block of samples. Based on the above calibration information, the script functions adjust the image to correct for any rotation of the samples and applies an evenly spaced analysis grid. Each grid region contains luminescence information from a single sample. An automatic threshold is applied to each region to segment the signal region from the local background region within each grid region. The size (in pixels) of the signal region is reported and can be automatically gated to eliminate irregular signals (for example, small false positive speckle noise regions or large false positive regions when no true signal region is present in the grid). $T_{agg}$, AUC, and NPARC analyses were performed as described in the Methods.

**Endpoint CETSA protocol to test ThermLuc fusions.** HEK293T cells were transiently transfected with plasmids containing TOI-luciferase variants using a reverse transfection procedure. Briefly, 3 µg of the plasmid and 6.25 µl of Lipofectamine 2000 was combined in 1.25 mL Opti-MEM (Gibco) to create the transfection complex. Following a 20-min incubation, the transfection complex was added to 1.25 x 10^6 cells suspended in 1.25 mL of a phenol-free high glucose DMEM with 10% (v/v) FBS and 1X GlutaMax in a six-well plate. After 24 or 48 hours, cells were lifted with 0.25% (v/v) trypsin and resuspended in a phenol-free high glucose DMEM with 1X GlutaMax without FBS. Next, 10 µl of cells per well were dispensed into white 384-well PCR plates (Roche) using a Multidrop Combi (ThermoFisher
Next, an Echo 550 was used to acoustically transfer DMSO or compound to the cells, which were then incubated for an hour at 37 °C with CO₂ and 95% humidity. Following the incubation, cells were heated for 3.5 min using a 384-well thermal cycler; then 10 µL of 2X furimazine (Promega 50X stock) solution diluted in phenol-free high glucose DMEM with GlutaMax was added to each well.

For a 96-well plate, cells were treated in bulk with DMSO or compound then transferred to PCR tubes (30 µL/tube) for subsequent incubation and heating. Once cooled to room temperature, 10 µL of cells from each tube was transferred to a white 384 well plate then 10 µL of 2X furimazine was added to each well. Luminescence was detected using a PerkinElmer ViewLux microplate reader equipped with clear filters.

**SplitLuc CETSA.** Cells transfected with LDHA fused to an 86b peptide were aliquoted for CETSA experiments as previously described. Briefly, after 24h transfection cells were harvested by trypsinization, resuspended at 1 million cells per mL in CETSA buffer and dispensed (10 µL per well) into 384-well PCR plates (Roche) using a Multidrop Combi (ThermoFisher). 20 nL of compounds or DMSO vehicle controls were acoustically transferred using an Echo 550 and incubated for 1 hour at 37 °C. Plates were sealed and heated at 37, 61, 65 and 69 °C for 3.5min and cooled to 25 °C using qPCR machine (Applied Biosystems) using ramp speed of 1.5 °C/sec for heating phase and max ramp rate for the cooling phase. 2 µL of 6% (v/v) NP40 were added per well and incubated at room temperature for 30 min to allow cell lysis, followed by the addition of 11S and furimazine substrate at final concentrations of 100nM and 0.5X, respectively. Samples were analyzed for luminescence intensity using a ViewLux reader equipped with clear filters (Perkin Elmer).

**Endogenous CETSA.** Acoustic reverse phase protein array CETSA (HT-CETSA-aRPPA) for LDHA was previously described. HEK293 cells were trypsinized and resuspended in phenol-free DMEM + Glutamax at 1 x 10⁶ cells per mL. 20 µL of cells were plated in 384-well PCR plates (Roche LightCycler 480 Multiwell plate, white, Cat #04729749001). DMSO or compound was acoustically transferred to the 384-well PCR plates using an Echo 555 prior to adding the cells. Cells were then incubated at 37 °C for 1 h. Following compound incubation, cells were heated at 71 °C for 3.5 min using a 384-well thermocycler. Once the cells cooled to room temperature, they were lysed for 30 min with 0.3% (v/v) NP-40 and 1× protease inhibitor (final concentrations), with mixing performed by an Apricot Personal Pipettor. After lysing, plates were centrifuged at 2000g for 30 min; then, the top 10.5 µL of lysate was transferred to an Echo LDV source plate using an Apricot Personal Pipettor. Next, 50 nL of lysate was transferred from the source plate to a nitrocellulose membrane using an Echo 525 (1536 samples total on a nitrocellulose membrane). After drying for at least 10 min, the membranes were hydrated with 1× TBS before immunoblotting with anti-LDHA (Cell Signaling Technology, Cat #3582T, used at 1:300). Quantification was performed using a MATLAB script, which can be accessed at https://github.com/ncats/HT-CETSA-aRPPA-Analysis.
NanoDSF. The interaction between LDHA and a small molecule was evaluated using a label-free approach. Specifically, 30 µL of 0.1 mg mol⁻¹ recombinant LDHA in assay buffer [25 mM Tris, 100 mM NaCl, pH 7.5] was incubated with 50 µM compound for 10 min at room temperature, and then loaded into 3 standard capillaries for triplicate readings in a Prometheus NT.48 instrument (Nanotemper Technologies, Munich, Germany). Experiments were run using 40% excitation power, and a 1 °C/min temperature ramp. Analysis was performed on the instrument using manufacturer's software to derive the temperature of melting and first derivatives for each sample. The experiments determining the thermal stabilization between LgBiT and HiBiT were performed as described above. LargeBiT (11S) large fragment and HiBiT peptide were synthesized as previously described and diluted to 14 µM and 30 µM in PBS, respectively. 1 The 156 and NP (native peptide) proteins consist of the first 156 amino acids and final 13 amino acids, respectively, of unmodified NanoLuc 3 (Genscript).

Cellular lactate production assay. HEK293T cells were cultured as described above. Cells were trypsinized and resuspended in phenol red free DMEM (Life Technologies) without supplements. Cells were immediately plated to 1536-well black clear bottom plates (Corning) at 250 cells per well in 4 µL volume. Compound or vehicle control was added to wells via pin tool transfer and cells were incubated at 37 °C for 1 h. Two µL of lactate reaction mixture (Biovision K607-100) was added to each well and plates were covered and incubated at room temperature for 30 min. Fluorescence was measured using a ViewLux microplate imager equipped with Ex/Em 528/598nm filters.

Cellular viability assay using Cell Titer glo. CellTiter-Glo (Promega) experiments were conducted according to the manufacturer's protocol. Briefly, 5000 cells were dispensed into 384-well plates (10 µL per well) and treated with 20 nL of LDHA inhibitors and DMSO controls for 1 hour at 37 °C. 10 µL of CellTiter-Glo reagent was then added and the plate was incubated at room temperature for 30 min with continuous shaking. Luminescence was detected using a PerkinElmer ViewLux microplate reader equipped with clear filters.

Cell membrane permeability assay. HEK293T cells were cultured as described above. Cells were lifted and resuspended in phenol-free high glucose DMEM with 10% (v/v) FBS and 1X GlutaMax at a concentration of 5 x 10⁵ cells/mL and 5 µL was immediately plated into Roche 384-well PCR plates (2500 cells per well in 10 µL final volume). Next, 2.5 µL of a 20 µM propidium iodide solution (5 µM final) was added to each well, as well as 2.5 µL of media or 4% (v/v) NP-40 (1% final) to appropriate control wells, sealed, and incubated with mild agitation for 15 minutes at 25 °C. The plate was spun down at 200 x g for 1 minute and immediately ran as described for RT-CETSA experiments.

SYSTEMETRIC Cell Health Screen. The Cell Health Screen (AsedaSciences) is a multiparametric acute cell stress assay using a panel of fluorescent physiological reporting dyes on an automated flow cytometry platform with a supervised machine learning (ML) classifier using a multiparametric logistic model 4. The final probability score, or "Cell Health Index", is a quantitative assessment of a multiparametric phenotype's similarity to a diverse set of known bad actors. In a 384-
well platform, HL60 cells (100,000 cells in 40 µL volume) were exposed to a 10-step, 3X dilution series of each test compound (5nM – 100 µM) for 4 hours. After compound exposure, cells were stained with a panel of fluorescent dyes that report physiological signatures of both mitochondrial dysfunction and gross cell stress. Fluorescence data were collected using automated flow cytometry, still in the original 384-well plate, with no gating. In addition, forward scatter and side scatter at 488nm were acquired for conversion into a cell morphology parameter. For each test compound, ungated detection parameters were converted to a tensor of values based upon QF distances between each step in the dilution series and both the positive and negative control wells in each row of the assay plate. This tensor becomes the input used for supervised machine learning classification relative to the training set of 300 known compound drawn from on-market pharmaceuticals, withdrawn drugs, research compounds, and a few industrial/agricultural compounds. First, all training set compounds were assigned to either the "positive" or "negative" training class based upon external information from the scientific literature, clinical trial reports, and/or known commercial histories. The classifier was trained by first dividing the training set into the two training classes, based upon external information, and then optimizing the fit of its multiparametric logistic model on the empirical screening data for all training compounds. The training set contained an approximate 1:3 ratio of "high cell stress" to "low cell stress" screen phenotypes, defined by an arbitrary cutoff at a probability of 0.5 that the classifier can assign any individual compound to the "high cell stress" class based upon the similarity of its screen phenotype to the rest of that class. For each test compound, ungated detection parameters are converted to a feature vector as follows. For each concentration step in a test compound dilution series, quadratic form (QF) distance is calculated between the empirical distribution of a flow cytometry parameter and that same parameter in the negative-control. All QF distance values for the dilution series then form a dose-response distance curve for that FC parameter. The same process is executed for all FC parameters, after which each of these curves is further reduced to two values: the point of the maximum rate of change and the range within which change occurs. These two values for each FC parameter are assembled into a feature vector representing all FC parameters. This vector serves as the quantitative digital phenotype for the test compound, to be used in subsequent ML classification relative to the training set of 300 known compounds drawn from on-market pharmaceuticals, withdrawn drugs, research compounds, and a few industrial/agricultural compounds.

Chemistry. All compounds were dissolved in DMSO. LDHA inhibitors were titrated in two-fold serial dilutions and 20 nL were transferred using acoustic dispenser Echo 550 (Labcyte). DMSO concentrations were maintained at less than 0.5% (v/v) in total volume. Furimazine was synthesized in-house as previously reported and stored at -20 °C as a solid powder.

Statistics. Statistical analyses were performed using GraphPad Prism (GraphPad Software). A p value of < 0.05 was considered statistically significant.
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