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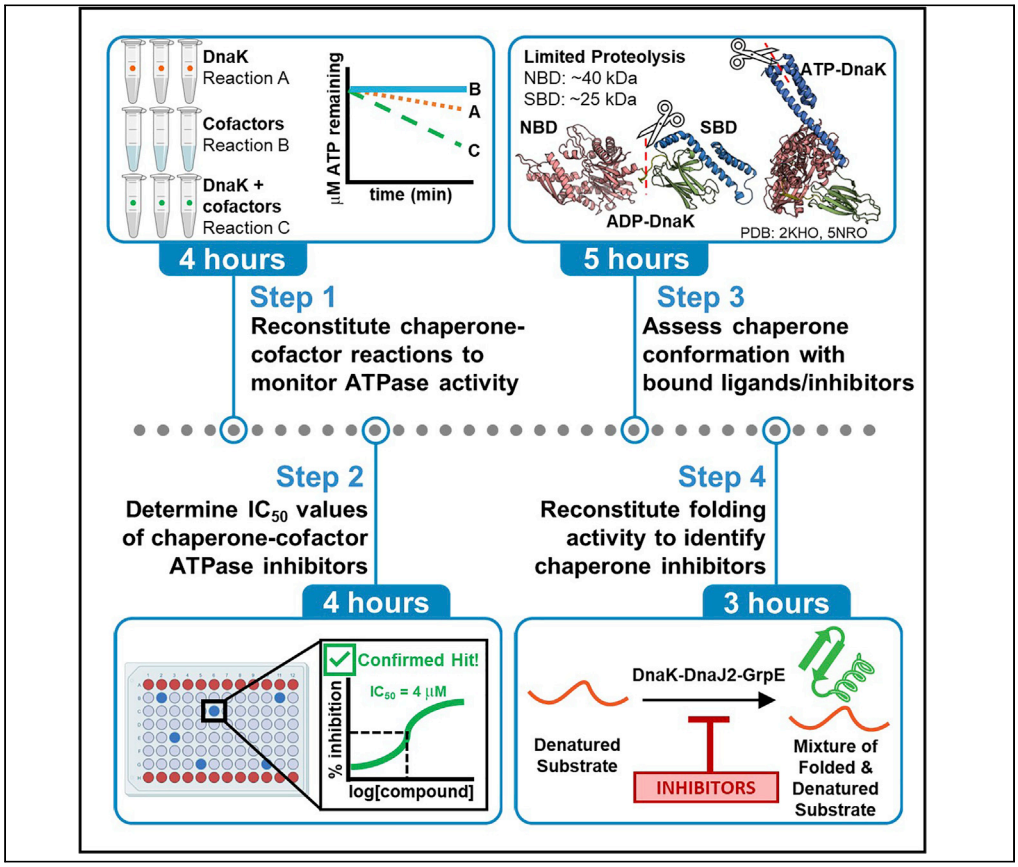
Complementary protocols to evaluate inhibitors against the DnaK chaperone network



Bacterial DnaK belongs to the Hsp70 chaperone family, which plays a critical role in maintaining proteostasis by catalyzing protein folding, and is a proposed antibacterial target in the pathogen *Mycobacterium tuberculosis*. Here, we describe an experimental toolbox for evaluating inhibitors against the mycobacterial DnaK chaperone network: a coupled-enzymatic assay to monitor ATPase activity, a proteolytic cleavage assay to study DnaK conformational changes upon ligand addition, as well as a protein renaturation assay to assess chaperone function.

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Highlights

Measurement of ATPase activation of mycobacterial DnaK by cofactors DnaJ2 and GrpE

Evaluation of compound inhibition of the chaperone network using IC50 values

Using SDS-PAGE to detect conformational changes of DnaK in the presence of ligands

Assay of protein folding activity in response to inhibitors

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Complementary protocols to evaluate inhibitors against the DnaK chaperone network

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SUMMARY

Bacterial DnaK belongs to the Hsp70 chaperone family, which plays a critical role in maintaining proteostasis by catalyzing protein folding, and is a proposed antibacterial target in the pathogen *Mycobacterium tuberculosis*. Here, we describe an experimental toolbox for evaluating inhibitors against the mycobacterial DnaK chaperone network: a coupled-enzymatic assay to monitor ATPase activity, a proteolytic cleavage assay to study DnaK conformational changes upon ligand addition, as well as a protein renaturation assay to assess chaperone function. For complete details on the use and execution of this protocol, please refer to Hosfelt et al. (2021).

BEFORE YOU BEGIN

Assay protocol list

Protocol 1: Overexpression and purification of *M. tuberculosis* (Mtb) chaperones and cofactors (steps 2–4).

Protocol 2: Measurement of DnaK ATPase stimulation by chaperone-cofactors (steps 1–8).

Protocol 3: Determination of the half-maximal inhibitory concentration (IC_{50}) of compounds against cofactor-mediated chaperone ATPase activation (steps 9–17).

Protocol 4: Assessment of global chaperone conformational changes in the presence of inhibitors/ligands (steps 18–21).

Protocol 5: Reconstitution of DnaK-cofactor folding activity to identify chaperone inhibitors (steps 22 and 23).

Preparation of buffers for chaperone purification

Timing: 1 h

1. Prepare the TBS buffer and purification Buffers A, B, and C as described in materials and equipment.

Protocol 1: Overexpression and purification of *M. tuberculosis* (Mtb) chaperones and cofactors

Timing: 5 days

Note: For complete details on the source of plasmids, as well as the expression and purification of Mtb chaperones in this protocol, please refer to Hosfelt et al. (2021), Lupoli et al. (2016).

2. Perform the following steps for the bacterial transformation of overexpression plasmids:
   a. Incubate 25 μL of competent *E. coli* Rosetta2 (DE3) cells with 10 ng of the corresponding plasmid DNA encoding His_6-SUMO fusion Mtb chaperones (DnaK and DnaK T175A) and co-factors (DnaJ2 and GrpE) in a microcentrifuge tube on ice for at least 10 min.
   b. Heat shock cells for 45 s at 42°C.
   c. Incubate cells on ice for 2 min.
   d. Add 80 μL of Super Optimal broth with Catabolite repression (SOC) medium.
   e. Incubate at 37°C for 30 min with shaking (200 RPM).
   f. Add transformed cells to 5 mL of LB liquid media supplemented with carbenicillin (50 μg/mL) and 0.1% glucose (v/v).

   Note: While transformed Rosetta2 cells can be plated to obtain a single colony followed by inoculation of a single cell into liquid media, we found that the degree of growth of overnight cultures varied from single colony inoculations.

   g. Incubate at 37°C for 18 h with shaking at 200 RPM.
3. Perform the following steps for overexpression of mycobacterial chaperone and cofactors:
   a. Add the 5 mL overnight culture from step 2g (above) to an Erlenmeyer flask containing 500 mL of LB supplemented with carbenicillin (50 μg/mL).
   b. Incubate the flask at 37°C with shaking (200 RPM) until the culture reaches log-phase as indicated by OD600 (optical density at 600 nm) values of 0.4–0.6.
   c. Incubate the flask at 25°C for 30 min with shaking (200 RPM) followed by induction with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside).
   d. Induce cells for 3–4 h at 25°C with shaking (200 RPM).
   e. Harvest cells by centrifugation at 3,724 × *g*, 30 min, 4°C.
   f. Resuspend pellets using 15 mL of TBS buffer and transfer into conical tubes.

   Note: TBS buffer solution should be filtered and stored at 25°C for 1 year.

   g. Pellet cells by centrifugation at 3,724 × *g*, 10 min, 4°C and remove supernatant.
   h. Store cells at −80°C until further use.

   ⏸ **Pause point:** Pellets can be stored at −80°C for over a year.

4. Perform the following steps for purification of mycobacterial chaperones and cofactors:

   ⚠ CRITICAL: All purification steps should be performed at 4°C.

   a. Resuspend His_6-SUMO-DnaK and His_6-SUMO-GrpE cell pellets (each from a 500 mL culture) in 15 mL of Buffer A. Resuspend His_6-SUMO-DnaJ2 cell pellet in 15 mL of Buffer A containing SIGMAFAST EDTA-free protease inhibitor cocktail (1 tablet per 100 mL buffer).

   Note: Buffer A should be filtered and stored at 4°C for 1 month. Buffer A containing SIGMAFAST EDTA-free protease inhibitor cocktail should be stored at 4°C for a maximum of 2 weeks.

      i. Supplement resuspended cells with lysozyme (0.1 mg/mL) and DNaseI (6.6 μg/mL).
      ii. Incubate cells with rocking for 1 h at 4°C.
   b. Lyse cells using a sonicator (5 min, 30 s on/30 s off, 50% amplitude, with cooling between runs) or a homogenizer (15K psi).
   c. Clear cellular debris and insoluble fraction by centrifugation (10,976 × *g*, 30 min, 4°C).

Note: Prepare samples for SDS-PAGE analysis by adding supernatant (20 μL) and a small sample of pellet to 20 μL and 200 μL of 2× Laemmli buffer, respectively. Samples can be stored at −20°C for several days.

Note: 2× Laemmli buffer can be stored at 25°C for 1 year.

d. Add the supernatant to a column containing 1 mL pre-washed Ni-NTA Superflow resin (equilibrated with Buffer A) supplemented with 2 mM imidazole. For His_6-SUMO-DnaJ2, incubate the supernatant with 0.75 mL His-Pur Cobalt resin.
   i. Incubate for 40 min to 1 h at 4°C with rocking.
e. Wash affinity columns with Buffer A containing 30 mM imidazole (at least 20 column volumes). Elute with Buffer A containing 200 mM imidazole (at least 5 column volumes).

Note: For His_6-SUMO-DnaJ2, wash the resin with a step gradient of imidazole in Buffer A (5 column volumes each of 10–30 mM imidazole in Buffer A). Elute with an increased concentration of imidazole over a step gradient in Buffer A (5 column volumes each of 50–200 mM imidazole in Buffer A).

Note: Prepare samples of each fraction for SDS-PAGE analysis by mixing 20 μL of sample with 20 μL of 2× Laemmli buffer . Samples can be stored at −20°C for several days.

   i. Add SUMO protease His-Ulp1 (Uehara et al., 2010; Muona et al., 2008) to the eluate fractions of each protein (about 700 μg of protease per each 500 mL of culture purified).
   ii. Separately dialyze the protease-eluate samples of each protein for 18 h using SnakeSkin Dialysis Tubing (Thermo Scientific, 10K MWCO, 35 mm) in 2 L of Buffer A at 4°C with stirring.
f. The next day, incubate dialyzed protein samples on a column containing 0.75 mL–2 mL Ni-NTA resin (equilibrated with buffer A) for 30 min to 1 h at 4°C with rocking.
   i. Collect the flow-through.
   ii. Wash the affinity column with 1–2 column volumes of Buffer A.
   iii. Combine the flow-through fraction and Buffer A eluate.
   iv. Concentrate cleaved protein sample using an Amicon centrifugal unit (MilliporeSigma, 10K MWCO) by centrifugation (3,724 × *g*, 4°C) to < 1 mL.
   v. Prepare a final protein sample for SDS-PAGE analysis by mixing 20 μL of sample with 20 μL of 2× Laemmli buffer.
   vi. Heat all collected protein samples in sealed microcentrifuge tubes at 95°C for 30 min.
   vii. Spin down the samples for 2 min and use standard protocols to analyze protein samples by SDS-PAGE with a 12 or 15% acrylamide gel. After Coomassie staining, assess the cleavage of the His_6-SUMO tag (Figure 1A, compare lanes 5 and 6) and the purity of the protein sample (Figure 1B).
g. Follow these additional steps to obtain the apo-state of the catalytically dead mutant of Mtb DnaK (T175A):
   i. Express and purify the Mtb DnaK T175A construct as described for wild-type Mtb DnaK (steps 2–4f).
   ii. Transfer the protein sample from step 4f, part iii to fresh dialysis tubing and perform an 18 h dialysis at 4°C in 2 L of Buffer B.

Note: Buffer B can be stored at 4°C for 1 week.

   iii. On the following day, transfer the protein sample in dialysis tubing to 2 L of Buffer C and let dialyze for 18 h at 4°C.

Note: Buffer C can be stored at 4°C for 1 week.

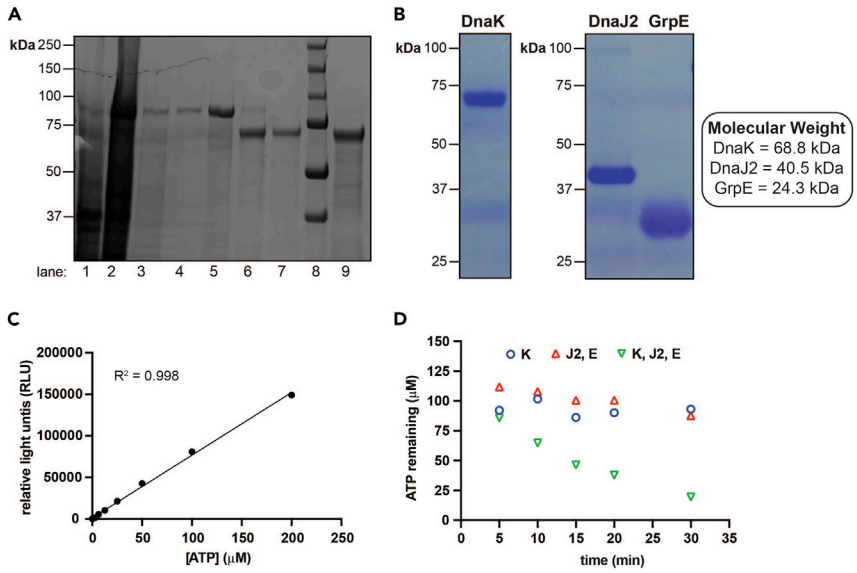

Figure 1. Purified mycobacterial cofactors stimulate the ATPase activity of DnaK

(A) SDS-PAGE analysis of Mtb DnaK for all purification steps. Lanes: (1) pellet, (2) flow-through, (3) 30 mM imidazole wash, (4) 30 mM imidazole wash, (5) 200 mM imidazole eluate, (6) post-cleavage dialysis sample, (7) post-dialysis flow-through collection, (8) Precision Plus Protein Ladder (Bio-Rad), and (9) 5 μg Mtb DnaK.

(B) SDS-PAGE analysis of purified Mtb DnaK, DnaJ2, and GrpE (5 μg each). Note that GrpE resolves as an oligomer via SDS-PAGE, resulting in a higher-than-predicted molecular weight.

(C) ATP standard curve generated using coupled-enzymatic assay reagents (Promega Kinase-Glo Max, simple linear regression shown).

(D) Time course of ATPase activity of Mtb DnaK (4 μM) +/- cofactors DnaJ2 and GrpE (0.4 μM each) demonstrates that functional cofactors activate the rate of ATP hydrolysis by DnaK. Instrumental error causes the calculated μM ATP remaining per reaction to exceed 100 μM in some cases. Representative singleton experiment shown.

iv. On the following day, concentrate the protein using an Amicon centrifugal unit by centrifugation (3,724 × *g*, 4°C) to <1 mL.

v. Analyze purified protein sample by SDS-PAGE as described in step 4f, parts v–vii.

h. Determine protein concentrations using the DC Protein Assay (Bio-Rad) with BSA (bovine serum albumin) as the standard following the manufacturer's instructions.

i. Immediately following protein purification, aliquot protein samples and flash freeze in $N_{2(l)}$ for storage at −80°C until use in assays.

Pause point: Protein samples are stable for several months at −80°C. Protein aliquots should not undergo multiple freeze/thaw cycles prior to use in enzymatic assays.

Note: All proteins were stored with 10% glycerol except for apo-DnaK T175A. All proteins containing 10% glycerol are stable for several months at −80°C. For the apo-DnaK T175A, protein was stored at −80°C for a maximum of 6 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli Rosetta2 (DE3)	Millipore Sigma/Novagen	Cat # 71400-3
Escherichia coli strains for overexpression of His-SUMO fusions of Mtb DnaK, DnaJ2, and GrpE	Lupoli et al. (2016)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Escherichia coli strain for overexpression of His-SUMO fusion of Mtb DnaK T175A	Hosfelt et al. (2021)	N/A
Chemicals, peptides, and recombinant proteins		
Luria-Bertani Broth (LB or Miller)	BD Difco/Fisher Scientific	Cat # DF0446-07-5
Carbenicillin, Disodium Salt	Teknova	CAS: C2113
Glucose	Sigma-Aldrich	CAS: 50-99-7
Isopropyl β-D-thiogalactopyranoside (IPTG)	Chem-Impex Int'l Inc.	Cat # 00194
Trizma Base	Sigma-Aldrich	CAS: 77-86-1
Lysozyme from chicken egg white	Sigma-Aldrich	CAS: 12650-88-3
Deoxyribonuclease I (DNaseI) from bovine pancreas	Sigma-Aldrich	CAS: 9003-98-9
NaCl	Sigma-Aldrich	CAS: 7647-1405
Glycerol	Sigma-Aldrich	CAS: 56-81-5
SIGMAFAST Protease Inhibitor Cocktail Tablets, EDTA-free	Sigma-Aldrich	Cat #S8830
Imidazole	Sigma-Aldrich	CAS: 288-32-4
Nickel-NTA Superflow	QIAGEN	Cat # 30450
His-Pur Cobalt resin	Thermo Fisher Scientific	Cat # 89964
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	CAS: 60-00-4
$MgCl_2$	Sigma-Aldrich	CAS: 7786-30-3
KCl	Sigma-Aldrich	CAS: 7447-40-7
PBS pH 7.4 (10×)	Gibco	Cat # 70011-044
Tween-20	Sigma-Aldrich	CAS: 9005-64-5
Urea, 98+%	Sigma-Aldrich	CAS: 57-13-6
KH_2PO_4, monobasic	Sigma-Aldrich	CAS: 7778-77-0
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Fisher Scientific	Cat # BP310-5
$CaCl_2$	Sigma-Aldrich	CAS: **10043-52-4**
Dithiothreitol (DTT)	Fisher Bioreagents	CAS: 3483-12-3
Sodium dodecyl sulfate (SDS)	Fisher Scientific	CAS: 151-21-3
Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)	Sigma-Aldrich	CAS: 1185-53-1
β-mercaptoethanol	Sigma-Aldrich	CAS:60-24-2
Coomassie Brilliant Blue R	Sigma-Aldrich	CAS: 6104-59-2
Proteinase K	New England Biolabs	Cat # P8107S
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	CAS: 329-98-6
$Mg(OAc)_2 \cdot 4H_2O$	Sigma-Aldrich	CAS: 16674-78-5
Formic acid	Sigma-Aldrich	CAS: 64-18-6
Bovine serum albumin (BSA) Fraction V	Sigma-Aldrich	Cat # 10735078001
Telaprevir	Cayman Chemical	CAS: 402957-28-2
Firefly Luciferase: QuantiLum Recombinant Luciferase	Promega	Cat # E1701
ATP (≥99% HPLC)	Sigma-Aldrich	CAS: 24369-07-8
ADP (Ultrapure)	VMR Life Science	CAS: 16178-48-6
Hemoglobin from Bovine Blood	Sigma-Aldrich	CAS: 9008-02-0
Critical commercial assays		
Kinase-Glo Max Luminescent Kinase Assay Kit	Promega	Cat # V6711
Luciferase Assay System	Promega	Cat # E1500
DC Protein Assay Kit	Bio-Rad Laboratories	Cat # 5000112
Recombinant DNA		
Plasmid: pET-HisSUMO	Gift from Scott Gradia, University of California, Berkeley	Addgene #29711
Plasmid: pHYR52: overexpression vector for Ulp1	Gift from Hideo Iwai, University of Helsinki; Muona et al. (2008)	Addgene #31122

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid: pETHisSUMO-DnaJ2: for overexpression of Mtb DnaJ2 in *E. coli*	Lupoli et al. (2016)	N/A
Plasmid: pETHisSUMO-GrpE: for overexpression of Mtb GrpE in *E. coli*	Lupoli et al. (2016)	N/A
Plasmid: pETHisSUMO-DnaK: for overexpression of Mtb DnaK in *E. coli*	Lupoli et al. (2016)	N/A
Plasmid: pETHisSUMO-DnaK T175A: for overexpression of Mtb DnaK T175A in *E. coli*	Hosfelt et al. (2021)	N/A
Software and algorithms		
Image Lab	Bio-Rad Laboratories	https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z
ImageJ (Image Processing and Analysis in Java)	Schneider et al. (2012)	https://imagej.nih.gov/ij/
GraphPad Prism version 9.0	GraphPad software	https://www.graphpad.com/
PyMOL 2.0	Schrodinger	https://pymol.org/edu/
Phyre2 (Protein Homology/analogY Recognition Engine V 2.0)	Kelley et al. (2015)	http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index
Excel	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
Other		
Allegra X-I15R centrifuge	Beckman Coulter	N/A
SpectraMax iD5 plate reader	Molecular Devices	N/A
FlexStation 3 plate reader	Molecular Devices	N/A
Sonicator Q500	Fisherbrand	N/A
EmulsiFlex-C5 High Pressure Homogenizer	Avestin	N/A
96-well half-area black plates	Greiner	Cat # 5667-5077
96-well polypropylene V-bottom plate	Greiner	Cat # 651261

MATERIALS AND EQUIPMENT

TBS Buffer (50 mM Tris (pH 8.0), 150 mM NaCl)		
Reagent	Final concentration	Amount
Trizma Base, pH 8.0 (1 M)	50 mM	50 mL
NaCl (5 M)	150 mM	30 mL
ddH_2O	n/a	Adjust to 1 L
Total	**n/a**	**1 L**

Cell Lysis Buffer (Buffer A: 25 mM Tris (pH 8.0), 400 mM NaCl, 10% glycerol)		
Reagent	Final concentration	Amount
Trizma Base, pH 8.0 (1 M)	25 mM	50 mL
NaCl	400 mM	46.75 g
Glycerol (100%)	10%	200 mL
ddH_2O	n/a	Adjust to 2 L
Total	**n/a**	**2 L**

Chelating Buffer (Buffer B: 20 mM imidazole (pH 7.2), 2 mM ethylenediaminetetraacetic acid (EDTA), and 10% glycerol)		
Reagent	Final concentration	Amount
Imidazole, pH 8.0 (2 M)	20 mM	20 mL
Ethylenediaminetetraacetic acid (EDTA), pH 8.0 (0.5 M)	2 mM	8 mL
Glycerol (100%)	10%	200 mL
ddH_2O	n/a	Adjust to 2 L
Total	**n/a**	**2 L**
Adjust the pH to 7.2 with 1 N HCl, filter solution, and store at 4°C for 1 week.		

Storage Buffer (Buffer C: 40 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$)		
Reagent	Final concentration	Amount
Tris-HCl, pH 7.4 (1 M)	40 mM	80 mL
KCl (2.5 M)	50 mM	40 mL
$MgCl_2$ (1 M)	10 mM	20 mL
ddH_2O	n/a	Adjust to 2 L
Total	**n/a**	**2 L**

Proteolysis Buffer (10× Buffer D: 200 mM HEPES (pH 7.5), 100 mM $MgCl_2$, 50 mM $CaCl_2$, 1 M KCl, 50 mM DTT)		
Reagent	Final concentration	Amount
HEPES, pH 7.5 (1 M)	200 mM	1.0 mL
$MgCl_2$ (1 M)	100 mM	0.5 mL
$CaCl_2$	50 mM	0.0277 g
KCl	1 M	0.3728 g
Dithiothreitol (DTT) (0.5 M)	50 mM	0.5 mL
ddH_2O	n/a	Adjust to 5 mL
Total	**n/a**	**5.0 mL**

10× PBS Buffer (pH 7.4), 20 mM $MgCl_2$, 0.1% Tween-20		
Reagent	Final concentration	Amount
10× PBS, pH 7.4	~10×	4.8 mL
$MgCl_2$ (1 M)	20 mM	0.1 mL
Tween-20 (5% in ddH_2O)	0.1%	0.1 mL
Total	**n/a**	**5.0 mL**

1× PBS Buffer (pH 7.4), 2 mM $MgCl_2$, 0.01% Tween-20		
Reagent	Final concentration	Amount
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20)	1×	0.5 mL
ddH_2O	n/a	4.5 mL
Total	**n/a**	**5.0 mL**

ATP Standard Buffer (1× PBS (pH 7.4), 2 mM $MgCl_2$, 0.01% Tween-20, 10% DMSO)		
Reagent	Final concentration	Amount
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20)	1×	0.5 mL
100% DMSO	10%	0.5 mL
ddH_2O	n/a	4.0 mL
Total	**n/a**	**5.0 mL**

Hemoglobin Buffer (100 mM potassium phosphate (pH 7.5), 2% hemoglobin, 6 M urea)		
Reagent	Final concentration	Amount
Hemoglobin	2% (w/v)	0.100 grams
Urea	6 M	1.8 grams
Potassium phosphate, pH 7.5 (1 M)	100 mM	0.5 mL
ddH_2O	n/a	Adjust to 5 mL
Total	**n/a**	**5.0 mL**

Following the manufacturer's instructions from Sigma-Aldrich, heat solution for 1 h at 37°C to dissolve components. Prepare fresh for each experiment.

2× Laemmli Buffer		
Reagent	Final concentration	Amount
Sodium dodecyl sulfate (SDS) (10%)	4%	20 mL
Glycerol (100%)	20%	10 mL
Tris-HCl, pH 6.8 (0.5 M)	120 mM	12 mL
β-mercaptoethanol	5% (v/v)	2.5 mL
Coomassie Brilliant Blue R	0.01% (w/v)	0.005 grams
ddH_2O	n/a	5.5 mL
Total	**n/a**	**50 mL**

100 mM potassium phosphate, 3 M urea buffer		
Reagent	Final concentration	Amount
Potassium phosphate (1 M)	100 mM	0.5 mL
Urea (6 M)	3 M	2.5 mL
ddH_2O	n/a	2 mL
Total	**n/a**	**5 mL**

2× Renaturation Buffer (2× Buffer R: 40 mM HEPES (pH 7.5), 200 mM KCl, 4 mM $Mg(OAc)_2$)		
Reagent	Final concentration	Amount
HEPES, pH 7.5 (1 M)	40 mM	8 mL
KCl (2.5 M)	200 mM	16 mL
$Mg(OAc)_2$ (1 M)	4 mM	0.8 mL
ddH_2O	n/a	175.2 mL
Total	**n/a**	**200 mL**

1× Renaturation Buffer (1× Buffer R: 20 mM HEPES (pH 7.5), 100 mM KCl, 2 mM $Mg(OAc)_2$)		
Reagent	Final concentration	Amount
2× Buffer R	1× Buffer R	50
ddH_2O	n/a	50
Total	**n/a**	**100 mL**

STEP-BY-STEP METHOD DETAILS

Protocol 2: Measurement of DnaK ATPase stimulation by chaperone-cofactors

Timing: 4 h

This protocol details how to monitor the ATP hydrolysis (ATPase) activity of mycobacterial chaperone DnaK and protein cofactors DnaJ2 and GrpE over a time course following purification. Activity of reactions containing (A) DnaK alone, (B) DnaJ2 and GrpE combined, and (C) DnaK with DnaJ2 and GrpE will be evaluated.

1. Perform the following steps for the preparation of an ATP standard curve using Kinase-Glo Max (Promega) reagents for ATP detection:

⚠ CRITICAL: Make sure to thaw the Kinase-Glo Max reagent prior to starting and let it warm to 25°C before use.

   a. Prepare a stock of 100 mM ATP (UltraPure) in ddH_2O.
   b. Prepare 200 µL of 200 µM ATP using ATP Standard buffer (described in materials and equipment).

Table 1. Preparation of ATP standards for calibration curve

Microcentrifuge tube (#s)	1	2	3	4	5	6	7	8	9	10
[Ultrapure ATP, μM]	200	100	50	25	12.5	6.25	3.12	1.56	0.78	0

Prepared in ATP Standard buffer. Aliquoted and stored at −20°C for single use.

Note: ATP Standard buffer can be stored at 25°C for 1 month.

c. Follow Table 1 to label ten 0.65 mL microcentrifuge tubes (#1–10) for serial dilution. Perform a two-fold serial dilution using ATP Standard buffer in a total volume of 50 μL per tube. Vortex tubes and spin down briefly on a table-top centrifuge to mix.
d. Label another set of ten 0.65 mL microcentrifuge tubes (#1–10) and add 15 μL of 0.05% formic acid to each tube. Transfer 15 μL of each ATP dilution from step 1c to each tube. Vortex to mix.

Note: ATP standard samples will be transferred to a microplate at the end of the experiment.

Note: ATP standard samples prepared in steps 1b and c can be stored at −20°C for several months. ATP standard samples prepared with 0.05% formic acid cannot be stored at −20°C.

2. Dilute DnaK, DnaJ2, and GrpE using 1× PBS buffer, 2 mM $MgCl_2$, 0.01% Tween-20 in at least 50 μL final volume to initial concentrations of 40 μM, 4 μM, and 4 μM, respectively. Keep on ice.

Note: 1× PBS buffer, 2 mM $MgCl_2$, 0.01% Tween-20 can be stored at 25°C for 1 month.

3. Prepare master mixes for each reaction. To do so, mix components in Tables 2, 3, and 4 in separate 1.5 mL microcentrifuge tubes. Each reaction should be performed in triplicate and tubes can be labeled as A1, A2, A3, B1, B2, etc.

Note: 10× PBS (pH 7.4), 20 mM $MgCl_2$, 0.1% Tween-20 can be stored at 25°C for 2 weeks.

Note: Master mixes **A** and **C** contain DnaK because the chaperone is pre-warmed at 37°C prior to the addition of cofactors DnaJ2 and GrpE in a subsequent step. While five time points will be measured, the master mix volumes are calculated for 6 reactions to account for loss in volume due to aliquoting. DMSO is added to master mixes because compounds dissolved in DMSO will be tested in subsequent steps.

a. Agitate each microcentrifuge tube and spin down briefly using a table-top centrifuge to mix.
b. Incubate reactions A1-3, B1-3 and C1-3 on a heating mantle for 15 min at 37°C.

Table 2. Master mix for reaction A (reaction mixtures for the basal ATPase activity of DnaK)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$	Volume (μL) for 6 reactions
DnaK (40 μM)	1.5	4 μM	9
100% DMSO	1.5	10% DMSO	9
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	9
ddH_2O	9	n/a	54
Total	**13.5**[a]	**n/a**	**81**

[a]Note: The volume per reaction will be brought to 15 μL when ATP is added at a later step to initiate reactions.

Table 3. Master mix for reaction B (reaction mixtures for the background ATPase activity of cofactors DnaJ2 and GrpE)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$	Volume (μL) for 6 reactions
100% DMSO	1.5	10% DMSO	9
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	9
ddH_2O	7.5	n/a	45
Total	**10.5**[a]	**n/a**	**63**

[a]Note: The volume per reaction will be brought to 15 μL when DnaJ2, GrpE and ATP are added at later steps to initiate the reactions.

Note: During this incubation time, prepare the tubes that will be used to quench five time points per reaction by adding 15 μL of 0.05% formic acid to five 0.65 mL microcentrifuge tubes for each replicate. For this experiment, a total of 45 microcentrifuge tubes will be required for quenching the reactions.

c. Add 9 μL of 4 μM DnaJ2 and 9 μL of 4 μM GrpE to reactions B and C for a final concentration of 0.4 μM of each (Tables 3 and 4). Agitate tubes and spin down briefly using a table-top centrifuge to mix.
d. Incubate reactions A1-3, B1-3, and C1-3 on a heating mantle for an additional 15 min at 37°C.

4. Initiate reactions by adding 9 μL of 1 mM ATP to each for a final concentration of 100 μM, starting with reaction A1, in 30-s intervals (see Table 5, line 1). Agitate each tube and spin down briefly using a table-top centrifuge to mix.

Note: The indicated volumes of ATP and cofactors added to the master mix will bring the final volume per reaction to 15 μL (See Note under each table).

Note: Staggering reaction initiation times as shown in Table 5 will allow you to run multiple time course experiments simultaneously.

5. At 5 min, quench the first reaction (A1) by removing 15 μL from microcentrifuge tube A1 and transferring it to a 0.65 mL microcentrifuge tube containing 15 μL of 0.05% formic acid.
   a. Vortex the quenched reaction and spin down briefly using a table-top centrifuge.
   b. Thirty seconds later, remove 15 μL of the next reaction (A2) and transfer it to a 0.65 mL microcentrifuge tube containing 15 μL of 0.05% formic acid. Vortex the quenched reaction and spin down briefly using a table-top centrifuge.
   c. Repeat step 5b to terminate each subsequent reaction at 30-s intervals (see Table 5, line 2) to achieve a 5-min time point for each reaction.

Table 4. Master mix for reaction C (reaction mixtures for the ATPase activity of DnaK, DnaJ2 and GrpE)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$	Volume (μL) for 6 reactions
DnaK (40 μM)	1.5	4 μM	9
100% DMSO	1.5	10% DMSO	9
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	9
ddH_2O	6	n/a	36
Total	**10.5**[a]	**n/a**	**63**

[a]Note: The volume per reaction will be brought to 15 μL when DnaJ2, GrpE and ATP are added at later steps to initiate the reactions.

Table 5. Example of a time course set-up to analyze multiple chaperone-cofactor ATPase reactions

	Reactions (in triplicate)	A1	A2	A3	B1	B2	B3	C1	C2	C3
1	Initiate Reactions	0	0:30	1:00	1:30	2:00	2:30	3:00	3:30	4:00
2	5-min time point	5:00	5:30	6:00	6:30	7:00	7:30	8:00	8:30	9:00
3	10-min time point	10:00	10:30	11:00	11:30	12:00	12:30	13:00	13:30	14:00
4	15-min time point	15:00	15:30	16:00	16:30	17:00	17:30	18:00	18:30	19:00
5	20-min time point	20:00	20:30	21:00	21:30	22:00	22:30	23:00	23:30	24:00
6	30-min time point	30:00	30:30	31:00	31:30	32:00	32:30	33:00	33:30	34:00

6. For each remaining time point, terminate the reactions as described in step 5 in 30-s intervals following the times shown in Table 5, lines 3–6.
7. Once the time course is complete:
   a. Transfer 15 μL of the ATP standards from step 1d into one row of a 96-well half-area black microplate.
   b. Transfer 15 μL of each quenched time point to remaining wells in the same 96-well half-area black microplate.
   c. Add 15 μL of Kinase Glo Max Reagent to each reaction well (including the ATP standard wells).
   d. Spin down the microplate at 233 × *g* for 1 min.
   e. Cover plate with foil and rock at 200 RPM for 10 min at 25°C.
   f. Spin down the microplate at 233 × *g* for 1 min.
   g. Perform a luminescence measurement on a plate reader (1s integration, 25°C).

⚠ CRITICAL: Standards and reactions containing 0.05% formic acid should not be stored at −20°C; hence, all steps after formic acid addition should be performed in sequence on the same day.

8. Data Analysis of the time course experiment:
   a. Generate a linear calibration curve by plotting the relative light units (RLUs) of the ATP standards against the concentration of ATP (μM) (Figure 1C). Data can be analyzed in Excel using the simple linear regression equation y = mx + b.
   b. Quantify the [ATP] (μM) in each reaction time point using the linear calibration curve in Excel.
   c. Transfer data to Graphpad Prism and plot as "remaining ATP (μM)" vs. "time (min)" for each reaction (Figure 1D). Data can be plotted as average values with standard deviation (SD) indicated.

Alternatives: This assay can be modified to test potential inhibitors by diluting a 10× concentration of compound in DMSO in place of the DMSO component in Tables 2, 3, and 4.

Protocol 3: Determination of the half-maximal inhibitory concentration (IC_{50}) of compounds against cofactor-mediated chaperone ATPase activation

Timing: 4 h

This section details how to determine the IC_{50} for a potential inhibitor (compound) against the Mtb DnaK-DnaJ2-GrpE co-chaperone network. Here, we use the model compound telaprevir, known to inhibit the activation of Mtb DnaK's ATPase activity by cofactors (Hosfelt et al., 2021). The last step of this section details how to perform a necessary control experiment to rule out false-positive hits that may result from inhibition of coupled assay reagents.

⚠ CRITICAL: Make sure to thaw the Kinase-Glo Max reagent prior to starting and let it warm to 25°C before use.

Table 6. Example of a two-fold serial dilution plate set-up for a compound of interest

	1	2	3	4	5	6	7	8	9	10	11	12
[Compound, mM]$_{INITIAL}$	2.5	1.25	.625	.312	.156	.781	.0391	.0195	.00977	.00488	.00244	0
[Compound, μM]$_{FINAL}$ in reaction wells	250	125	62.5	31.2	15.6	7.81	3.91	1.95	0.977	0.488	0.244	0

9. Prepare ATP standards as described in steps 1b and c above (Note that formic acid is not used as a quench buffer in this protocol).

10. Prepare compound dilutions, DMSO, and ATP stocks in a single V-bottom plate as follows:
    a. In the first row of a V-bottom plate, perform a two-fold serial dilution of the desired compound, with an initial concentration of 2.5 mM in the first well (Table 6). If possible, dilute the desired compound in 100% DMSO and perform the serial-dilution in DMSO in a final volume of at least 50 μL per well.

⚠ CRITICAL: It is important to check the solubility of the compound being tested. In this protocol, the model compound, telaprevir, can be dissolved in 100% DMSO. Please refer to the manufacturer's site for the solubility of commercial compounds in common solvents. Note that if the reaction conditions are changed due to the addition of a different solvent, the buffers used to prepare ATP standards must be adjusted to match the reaction buffers, and enzyme activity must be tested in the desired solvent concentration as detailed in steps 1–8.

    b. In the following row of the V-bottom plate, add 50 μL of DMSO to each well.
    c. In the next row of the V-bottom plate, add 50 μL of 1 mM ATP in ddH_2O to each well.

Note: Reagents are added to the V-bottom plate to facilitate the use of a multichannel pipette for reagent addition in subsequent steps. A prepared V-bottom plate can be sealed and stored at −20°C for 2 months (unless different compound storage conditions are suggested).

11. Dilute DnaK, DnaJ2, and GrpE using 1× PBS (pH 7.4), 2 mM $MgCl_2$, 0.01% Tween-20 in a final volume of 100 μL each as 40 μM, 4 μM, and 4 μM stocks, respectively. Keep on ice.
12. Add 15 μL of each ATP standard mixture (from step 9) to **row A** of a 96-well half-area black microplate (Figure 2A).
13. Prepare reactions to determine IC_{50} values of a given compound against DnaK and cofactors as follows:

Note: DnaK is pre-incubated in reaction mixtures with or without compound prior to adding cofactors to pre-warm the chaperone mixture and facilitate compound binding.

    a. Follow Table 7 to prepare the DnaK-DnaJ2-GrpE master mix reaction that will have varying concentration of added compound.
        i. In a 1.5 mL microcentrifuge tube, add the indicated reagents and agitate the tube to mix.
        ii. Briefly spin down using a table-top centrifuge.
        iii. To perform technical triplicates, pipette 9 μL of the master mix to **rows B-D** of a 96-well half-area black microplate (Figure 2A).

Note: Master mixes shown here are calculated for an excess number of reactions to account for volume loss due to aliquoting reactions and for the analysis of 12 compound concentrations in triplicate.

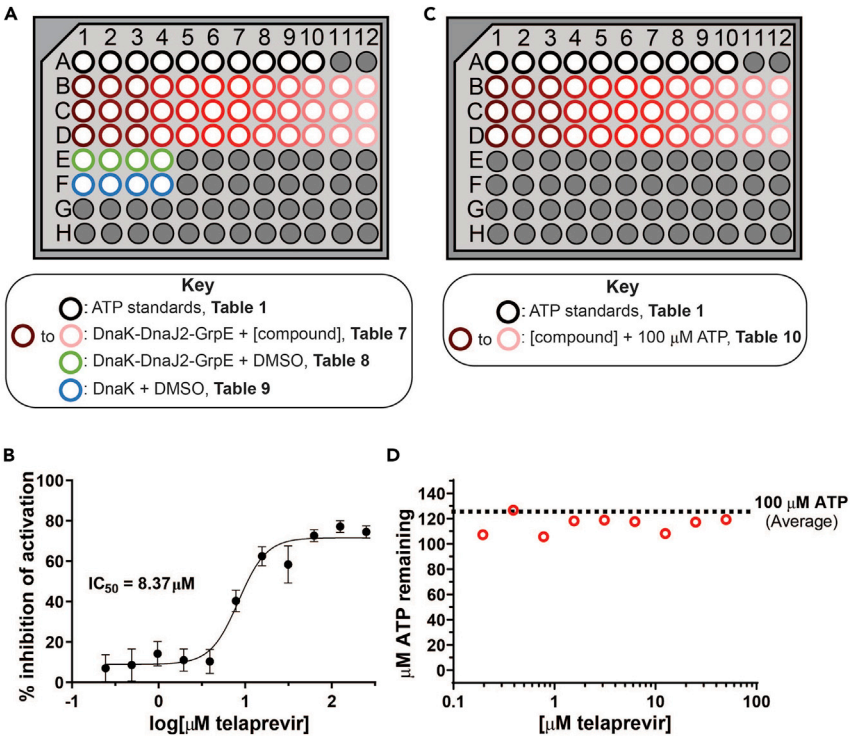

Figure 2. Small molecule inhibitors can prevent ATPase stimulation of Mtb DnaK by cofactors

(A) Representative plate map for an IC_{50} experiment of the model inhibitor telaprevir against mycobacterial DnaK (4 μM), DnaJ2 and GrpE (0.4 μM each).

(B) Dose-response curve of telaprevir against DnaK ATPase activation by DnaJ2 and GrpE. [ATP] measured using Kinase-Glo Max reagent (n=3, error bars represent standard deviation (SD)).

(C) Representative plate map for assessment of false positives that inhibit coupled-enzymatic assay reagent (Kinase-Glo Max).

(D) Representative analysis of a range of [telaprevir] incubated with Kinase-Glo Max confirms that it does not interfere with ATP detection. Instrumental error causes the calculated μM ATP remaining per reaction to exceed 100 μM in some cases.

b. Follow Table 8 to prepare the master mix for the negative control (DnaK, DnaJ2 and GrpE with vehicle only).
   i. In a 1.5 mL microcentrifuge tube, add the indicated reagents and agitate the tube to mix.
   ii. Briefly spin down using a table-top centrifuge.
   iii. To perform technical replicates, pipette 9 μL of the master mix to **wells E1-4** of a 96-well half-area black microplate (Figure 2A).
c. Follow Table 9 to prepare the master mix for the positive control (DnaK alone).
   i. In a 1.5 mL microcentrifuge tube, add the indicated reagents and agitate the tube to mix.
   ii. Briefly spin down using a table-top centrifuge.
   iii. To perform technical replicates, pipette 12 μL of the master mix to **wells F1-4** in a 96-well half-area black microplate (Figure 2A).
d. Spin down the 96-well half-area black microplate at 233 × *g* for 1 min.
e. Use a p10-μL multichannel pipette to add 1.5 μL of 100% DMSO from the V-bottom plate (from step 10b) to **wells E1-E4** and **F1-F4** of the 96-well half-area black microplate (Figure 2A).
f. Use a p10-μL multichannel pipette to add the serially-diluted compounds from the V-bottom plate (from step 10a) to wells in **rows B-D** of the 96-well half-area black microplate (Figure 2A).
g. Cover the microplate with adhesive foil and rock plate at 200 RPM for 2 min at 25°C.

Table 7. Master mix for DnaK, DnaJ2, GrpE reactions with varying [compound] of interest

$[Components]_{INITIAL}$	Volume (µL) per reaction	$[Components]_{FINAL}$	Volume (µL) for 40 reactions
DnaK (40 µM)	1.5	4 µM	60
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	60
ddH_2O	6	n/a	240
Total	**9**[a]	**n/a**	**360**

[a]Note: The volume per reaction will be brought to 15 µL when compound, DnaJ2, GrpE and ATP are added at later steps to initiate reactions.

h. Spin the plate down at 233 × *g* for 1 min.
i. Pre-incubate the plate in a 37°C incubator for 20 min.
j. Remove the plate from the incubator and spin at 233 × *g* for 1 min.
k. Remove the adhesive foil from the plate and add 1.5 µL of 4 µM DnaJ2 and 1.5 µL of 4 µM GrpE to **rows B-D** and **E1-E4** of the 96-well half-area black microplate (Figure 2A) to obtain a final concentration of 0.4 µM of each cofactor in the indicated wells.
l. Cover the plate with adhesive foil, rock the plate at 200 RPM for 2 min at 25°C, and then spin the plate down at 233 × *g* for 1 min.
m. Pre-incubate the plate in a 37°C incubator for 20 min.

14. Initiate each reaction with ATP and quench as follows:
    a. Remove the plate from the incubator and spin down at 233 × *g* for 1 min.
    b. Use a p10-µL multichannel pipette to add 1.5 µL of 1 mM ATP from the V-bottom plate prepared in step 10c to all of the filled wells of the 96-well half-area black microplate (Figure 2A).
    c. Cover the plate with adhesive foil, rock for 2 min at 25°C, spin down plate at 233 × *g* for 1 min, and place in a 37°C incubator for 30 min.
    d. After removal from the incubator, spin down the plate at 233 × *g* for 1 min.

    Note: The time point (t = 30 min) was selected because it is within the linear phase of the reaction.

    e. To quench reactions, add 15 µL of Kinase-Glo Max reagent to each well (including the ATP standards) using an automatic pipette.
    f. Immediately spin down the plate at 233 × *g* for 1 min.
    g. Cover plate with foil and rock for 10 min at 25°C.

    ⚠ CRITICAL: It is important to be consistent with incubation times prior to measuring luminescence on a plate reader.

    h. Spin down the plate at 233 × *g* for 1 min and measure luminescence using a plate reader (1s integration, 25°C).

Table 8. Master mix for DnaK, DnaJ2, and GrpE reactions with vehicle (negative control)

$[Components]_{INITIAL}$	Volume (µL) per reaction	$[Components]_{FINAL}$	Volume (µL) for 5 reactions
DnaK (40 µM)	1.5	4 µM	7.5
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	7.5
ddH_2O	6	n/a	30
Total	**9**[a]	**n/a**	**45**

[a]Note: The volume per reaction will be brought to 15 µL when DMSO, DnaJ2, GrpE and ATP are added at later steps to initiate reactions.

Table 9. Master mix for reactions with DnaK only with vehicle (positive control)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$	Volume (μL) for 5 reactions
DnaK (40 μM)	1.5	4 μM	7.5
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	7.5
ddH_2O	9	n/a	45
Total	**12**[a]	**n/a**	**60**

[a]Note: The volume per reaction will be brought to 15 μL when DMSO and ATP are added at later steps to initiate reactions.

i. Proceed to step 16 for data analysis.

15. Perform the following steps to evaluate if the compound of interest inhibits Kinase-Glo Max reagents.
    a. Add 15 μL of ATP Standards to **row A** of a 96-well half-area black microplate (Figure 2C).
    b. Follow Table 10 for the preparation of the reaction mixture that will contain serially-diluted compound.
        i. In a 1.5 mL microcentrifuge tube, add the indicated reagents and agitate the microcentrifuge tube to mix.
        ii. Briefly spin down tube using a table-top centrifuge.
        iii. To perform technical triplicates, pipette 13.5 μL of the mixture to **rows B-D** of a 96-well half-area black microplate (Figure 2C).
        iv. Using a p10-μL multichannel pipette, add 1.5 μL of serially-diluted compound (prepared in V-bottom plate from step 10a) to **rows B-D** of a 96-well half-area black microplate (Figure 2C).
    c. Follow steps 14e–h and proceed to step 17 for data analysis.
16. Perform the following steps for data analysis of IC_{50} experiments (from steps 13 and 14).
    a. Generate a linear calibration curve by plotting the relative light units (RLUs) of the ATP standards against the concentration of ATP (μM). The data can be analyzed in Excel using the simple linear regression equation y = mx + b.
    b. Convert the RLU data of the plate in Figure 2A to [ATP] (μM) using the linear calibration curve in Excel.
    c. Export the data into GraphPad Prism. Plot the "remaining ATP (μM)" from each reaction (analyzed from **rows B-D**) versus the "$\log_{10}$([compound (μM)])". The data can be plotted as average values with the standard deviation (SD) indicated.
    d. Normalize the data by setting the "remaining ATP (μM)" of the positive control (DnaK only + DMSO) as 100% and the "remaining ATP (μM)" of the negative control (DnaK + cofactors + DMSO) to 0%. This will adjust the y-axis from "remaining ATP (μM)" to "percent inhibition of ATPase activation".
    e. Using GraphPad Prism, calculate the half-maximal inhibitory concentration (IC_{50}) of the compound using the nonlinear equation logEC50 = logECF – (1/HillSlope)*log(F/(100-F)) and Y = Bottom + (Top-Bottom)/(1+10^((logEC50-X)*HillSlope) wherein F is set to 50 to calculate 50% inhibition values (see Figure 2B).

Table 10. Master mix for range of [compound] in reaction buffer lacking chaperones to evaluate false positives

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$	Volume (μL) for 40 reactions
ATP (1 mM, in ddH_2O)	1.5	100 μM	60
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20	60
ddH_2O	10.5	n/a	420
Total	**13.5**[a]	**n/a**	**540**

[a]Note: The volume per reaction will be brought to 15 μL when compound is added to each well.

Note: EC50 is the effective concentration (EC) of compound necessary for producing 50% of the maximal response. See the manufacturer's instructions for a detailed description of the equation used, wherein F is defined as the percentage of the maximal response. Since the response is inhibition of DnaK ATPase activation, this equation provides the IC50 value, which is the half-maximal inhibitory concentration of the compound.

17. Perform the following steps for data analysis of possible false-positives (from step 15).
    a. In Graphpad Prism, plot the data from step 15 as averaged RLU values versus [compound] (μM). The averaged RLU can also be converted to "remaining ATP (μM)" (following steps 16a and b).
    b. Compare results from 'vehicle-only' reactions (B12, C12 and D12 from Figure 2C), which represents 100 μM ATP only, and 'compound' reactions (**rows B-C**, wells 1–11) (see Figure 2D).

Protocol 4: Assessment of global chaperone conformational changes in the presence of inhibitors/ligands

Timing: 5 h

Partial proteolysis experiments are used to help deduce the conformational state of DnaK in the presence of ligands or inhibitors of interest (Meng et al., 2018; Kassenbrock and Kelly, 1989; Wei et al., 1995; Kamath-Loeb et al., 1995; Buchberger et al., 1995; Preissler et al., 2017). A catalytically dead mutant of the Mtb DnaK, T175A, is used to prevent ATP turnover during analysis. In the first step, a control experiment is performed to ensure that the compound of interest does not inhibit the protease at the concentrations tested (steps 18 and 19). In the second step, Mtb DnaK conformation is compared in the apo-, ATP-, and ADP-bound states with the ligand NRLLLTG (Meng et al., 2018) or the model inhibitor telaprevir (steps 20 and 21).

18. Perform the control proteolysis experiment using denatured hemoglobin monomer as a model substrate, as follows:
    a. Prepare Hemoglobin buffer as described in materials and equipment.

    Note: Hemoglobin buffer must be prepared fresh before each experiment.

    b. Prepare compound dilutions in a V-bottom plate according to Table 11, with the compound dissolved in DMSO at an initial concentration of 10 mM and two-fold serially diluted in DMSO across 7 wells in a final volume of at least 50 μL per well.
    c. Dilute proteinase K to 1 μM in 50 μL of ddH_2O prior to each experiment. Keep on ice.
    d. Follow Table 12 to prepare the hemoglobin control that lacks added protease in a 0.65 mL low-affinity microcentrifuge tube (VWR). Pipette to mix components.

    Note: 100 mM potassium phosphate (pH 7.5), 3 M urea buffer must be prepared fresh before each experiment.

    Note: 3 M urea is added to the potassium phosphate buffer to assist in solubilizing the hemoglobin already dissolved in buffer.

    e. Follow Table 13 to prepare seven hemoglobin digestion reactions in seven 0.65 mL low-affinity microcentrifuge tubes. Pipette to mix components.
        i. Add 2 μL of either compound (#1–6) or vehicle only (#7) (DMSO) (Table 11) to the seven reaction mixtures and pre-incubate at 25°C for 30 min.

    Note: To ensure consistent temperature in all reactions, incubate reactions at 25°C using a heat block.

Table 11. Example of two-fold serial dilution of compound of interest

Experimental reactions	1	2	3	4	5	6	7
[Compound, mM]$_{INITIAL}$	10.0	5.00	2.50	1.25	0.625	0.312	0
[Compound, μM]$_{FINAL}$ in reaction wells	500	250	125	62.5	31.2	15.6	0

ii. To initiate proteolysis reactions, add 2 μL of 1 μM proteinase K for a final concentration of 0.050 μM to each mixture in 30-s intervals.

iii. Agitate microcentrifuge tubes and briefly spin-down using a table-top centrifuge to mix.

f. After a t=15 min incubation for each tube, quench each reaction by transferring 20 μL of the mixture to a microcentrifuge tube with 4 μL of 100 mM PMSF and 10 μL of 2× Laemmli buffer.

g. For each sample, analyze 3 μg of hemoglobin (calculated based on amount of protein in quenched samples) by SDS-PAGE (15% acrylamide gel) using standard protocols. Stain with Coomassie Brilliant Blue (see Figure 3A).

19. For data analysis of the control proteolysis experiment, upload an image of the stained acrylamide gel to Bio-Rad Image Lab.

a. Follow the manufacturer's instructions to calculate the percent band intensities in each lane (see Figure 3B).

Note: Based on the results, choose a concentration of compound for the next steps that does not inhibit the protease, but is predicted to bind DnaK according to the IC_{50} value calculated above (step 16). For telaprevir (TP), a final concentration of 50 μM was chosen based on hemoglobin proteolysis results and its IC_{50} value (4–8 μM).

20. Perform the following steps for limited proteolysis analysis of Mtb DnaK T175A (the catalytically dead mutant) in the presence of a compound of interest or a known ligand.

a. Prepare Proteolysis buffer (10× Buffer D) as stated in materials and equipment.

Note: Proteolysis buffer (10× Buffer D) must be prepared fresh before each experiment.

b. Dilute proteinase K to 1 μM in 50 μL total volume using ddH_2O prior to each experiment.

c. Dilute DnaK T175A to an initial concentration of 200 μM using 1× PBS (pH 7.4), 2 mM $MgCl_2$, 0.01% Tween-20 in a final volume of at least 15 μL.

Note: DnaK T175A was dialyzed to remove bound nucleotides prior to starting the experiment. See Protocol 1, step 4g.

d. To prepare nucleotide-bound DnaK with and without ligand/inhibitor, follow Table 14.

i. Prepare six 0.65 mL low-affinity microcentrifuge tubes and label according to the components (see ***Note*** below).

Table 12. Mixture for Hemoglobin control sample (no protease added)

[Components]$_{INITIAL}$	Volume (μL) per reaction	[Components]$_{FINAL}$
Hemoglobin buffer	10	n/a
100% DMSO	2	5% DMSO
100 mM potassium phosphate, pH 7.5, 3 M urea	28	n/a
Total	40	n/a

Table 13. Reaction mixture for partial digestion of Hemoglobin

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
Hemoglobin Buffer	10	n/a
100 mM potassium phosphate, pH 7.5, 3 M urea	26	n/a
Total	36[a]	n/a

[a]Note: The volume per reaction will be brought to 40 μL when compound/DMSO and protease is added to each tube.

ii. Add indicated components (ddH2O, 10× Buffer D, DnaK T175A, and appropriate nucleotide).
iii. Agitate tubes and briefly spin down using a table-top centrifuge to mix.
iv. Incubate at 25°C for 1 h.

Note: Six tubes are suggested because each ATP- and ADP-bound DnaK will be incubated with (1) vehicle, (2) the known ligand NRLLLTG and (3) the model inhibitor telaprevir. Tubes should be labeled according to the components (*e.g.*, ADP, ADP + inhibitor, ADP + ligand, ATP, ATP + inhibitor, ATP + ligand).

e. To prepare apo-DnaK, follow Table 15.
   i. Prepare three 0.65 mL low-affinity microcentrifuge tubes and label according to the components (apo, apo + ligand, and apo + inhibitor).
   ii. Add indicated components (ddH_2O, 10× Buffer D, and DnaK T175A).
   iii. Agitate tubes and briefly spin down using a table-top centrifuge to mix.
   iv. Incubate at 25°C for 1 h.
f. To prepare the control lacking protease, follow Table 16.
   i. Prepare one 0.65 mL low-affinity microcentrifuge tube.
   ii. Add indicated components (ddH_2O, 10× Buffer D, and DnaK T175A). Agitate tube and briefly spin down using a table-top centrifuge to mix.
   iii. Incubate at 25°C for 1 h.
g. After 1 h of pre-incubation for all microcentrifuge tubes, add 1 μL of vehicle (DMSO) or 20× [compound/ligand] to the appropriate reactions. Agitate tubes and briefly spin down using a table-top centrifuge to mix. Pre-incubate at 25°C for 30 min.

Note: Final concentrations of telaprevir and NRLLLTG (Pellecchia et al., 2000; Stevens et al., 2003) in the reactions described here are 50 and 500 μM, respectively. Hence, initial concentrations were 1 and 10 mM, respectively.

h. After the second pre-incubation step, initiate proteolysis reactions by adding 1 μL of 1 μM proteinase K to each reaction in 30-s increments. Agitate tubes and briefly spin down using a table-top centrifuge to mix.

Table 14. Reaction mixture for partial digestion of ADP- and ATP-bound DnaK

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
[a]DnaK T175A (200 μM)	1	10 μM
[a]Nucleotides ADP/ATP (10 mM)	4	2 mM
100% DMSO or 20× compound/ligand	1	5% DMSO or 1× compound/ligand
Proteinase K (1 μM)	1	0.050 μM
PMSF (100 mM)	0.2	1 mM
[a]10× Buffer D	2	1× Buffer D
ddH_2O	10.8	n/a
Total	20	n/a

[a]Note: These components will be added first and pre-incubated for t=1 h at 25°C.

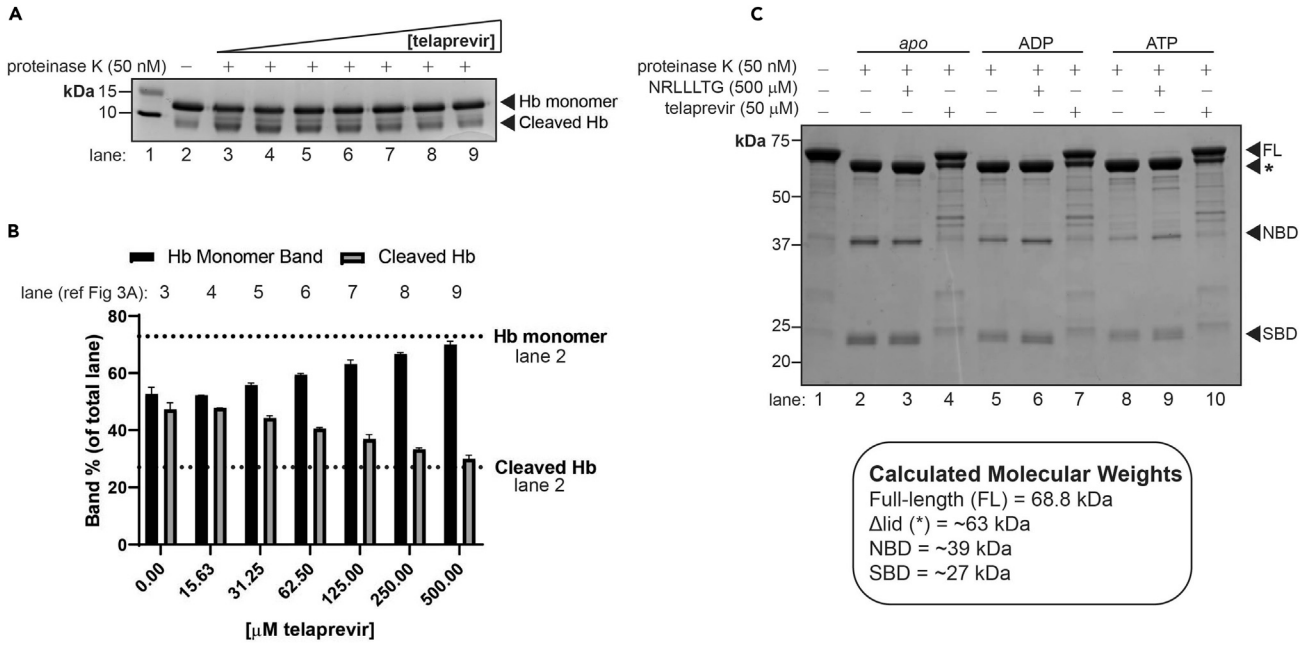

Figure 3. Limited proteolysis experiments deduce global conformational changes in Mtb DnaK in the presence of a ligand/inhibitor

(A) Representative SDS-PAGE analysis of limited proteolysis of denatured hemoglobin (Hb) monomer with increasing concentrations of telaprevir as a control experiment to evaluate protease inhibition by compound of interest.

(B) Quantification of the SDS-PAGE result from part A. Hb cleavage is not prevented by 50 μM telaprevir; hence, this concentration was used for conformational analysis experiments. Dashed lines represent the indicated band percentages from lane 2 (no protease added) (n = 3, error bars indicate SD).

(C) Representative SDS-PAGE analysis of limited proteolysis of Mtb DnaK +/− excess nucleotides +/- ligand/inhibitor shows that samples with telaprevir retained the greatest amount of full-length (FL) DnaK compared with the apo and NRLLLTG samples. NBD: nucleotide binding domain; SBD: substrate binding domain; * indicates partial cleavage of the C-terminal α-helical lid. A portion of this figure is reprinted with permission from Hosfelt et al., 2021.

i. Quench each reaction after 15 min by adding 0.2 μL of 100 mM PMSF and 20 μL of 2× Laemmli buffer. Vortex to mix and spin down using a table-top centrifuge.

j. Heat samples for 5 min at 95°C and load 1 μg of DnaK (calculated based on the amount of protein in the quenched samples) on a 12% acrylamide gel (Invitrogen) for SDS-PAGE analysis with a protein molecular weight ladder. Stain with Coomassie Brilliant Blue using standard protocols.

Note: Percentage of acrylamide gels should be adjusted based on the desired molecular weights that will be analyzed.

k. Experiments should be run in triplicate prior to data analysis.

21. For data analysis of partial proteolysis of Mtb DnaK, upload an image of the stained acrylamide gel to Bio-Rad Image Lab.

a. Follow the manufacturer's instructions to calculate the predicted molecular weights of the proteolysis fragments using a standard molecular weight ladder (such as Bio-Rad Precision Plus).

b. Compare the distribution of truncations for each state of DnaK with vehicle only, known ligand and compound of interest (Figure 3C, box).

Alternatives: The molecular weight of proteolysis bands can be determined using Image Processing and Analysis in Java (ImageJ), which is available for free download. Bio-Rad Image Lab requires an account to log-in.

Table 15. Reaction mixture for partial digestion of apo-DnaK

[Components]$_{INITIAL}$	Volume (μL) per reaction	[Components]$_{FINAL}$
[a]DnaK T175A (200 μM)	1	10 μM
100% DMSO or 20× compound/ligand	1	5% DMSO or 1× compound/ligand
Proteinase K (1 μM)	1	0.050 μM
PMSF (100 mM)	0.2	1 mM
[a]10× Buffer D	2	1× Buffer D
[a]ddH_2O	14.8	n/a
Total	**20**	**n/a**

[a]Note: These components will be added first and pre-incubated for t=1 h at 25°C.

Protocol 5: Reconstitution of DnaK-cofactor folding activity to identify chaperone inhibitors

Timing: 3 h

This experiment is performed to evaluate the ability of inhibitors to disrupt the chaperone-mediated folding activity of a model substrate, denatured luciferase, over a time course. An overview of the experimental scheme is presented in Figure 4A. Four mixtures will be set up as replicates: reaction D: chaperone and cofactors with denatured luciferase and vehicle; reaction E: chaperone and cofactors with denatured luciferase and added compound; reaction F: denatured luciferase; reaction G: native luciferase.

⚠ CRITICAL: Use 0.65 mL low-affinity microcentrifuge tubes (VWR) for all preparations in the assay to prevent the denatured substrate luciferase from sticking to tubes.

⚠ CRITICAL: Make sure to thaw the Luciferase Assay reagent (Promega) prior to starting and let it warm to 25°C before use.

⚠ CRITICAL: Perform this experiment near a plate reader with settings preset for the luminescence measurements (1 s integration) or use an autoinjector to add luciferase reagent in the plate reader.

⚠ CRITICAL: Ensure to add bovine serum albumin (BSA) to buffers containing luciferase samples to stabilize luciferase, and to prevent adhesion to tubes/plate wells.

Note: This protocol is written so that components are prepared during the incubation steps.

22. Perform reconstitution of DnaK and cofactor chaperone activity by carrying out the following steps:
    a. Prepare both the 2× and 1× Buffer R as described in materials and equipment. Filter solutions and store at 25°C.

Table 16. Mixture for DnaK control sample (no protease added)

[Components]$_{INITIAL}$	Volume (μL) per reaction	[Components]$_{FINAL}$
[a]DnaK T175A (200 μM)	1	10 μM
100% DMSO	1	5% DMSO
PMSF (100 mM)	0.2	1 mM
[a]10× Buffer D	2	1× Buffer D
[a]ddH_2O	15.8	n/a
Total	**20**	**n/a**

[a]Note: These components will be added first and pre-incubated for t=1 h at 25°C.

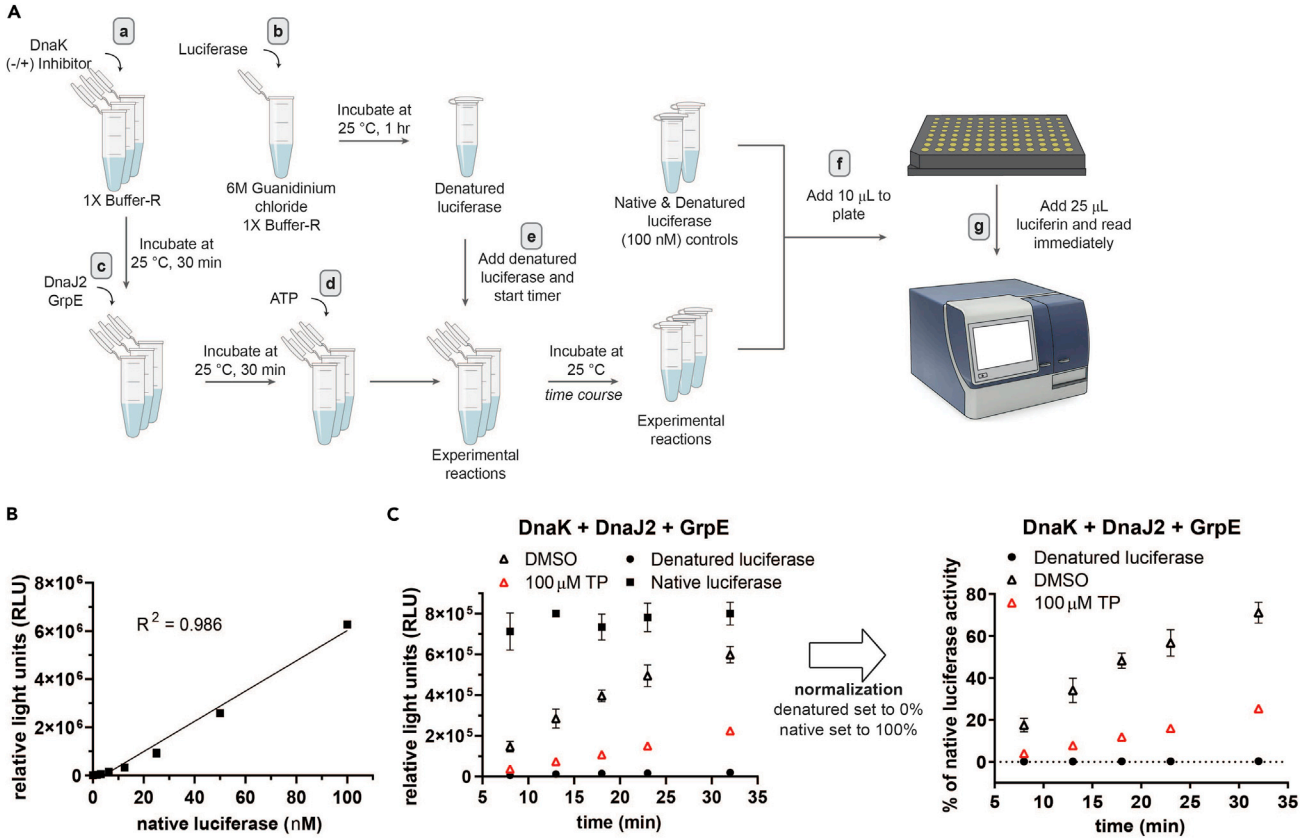

Figure 4. Reconstitution of protein folding by Mtb DnaK and cofactors can be used to evaluate inhibitors of chaperone activity

(A) Schematic of DnaK-DnaJ2-GrpE-mediated protein folding assay of a model substrate, denatured luciferase, performed in triplicate.

(B) Standard curve of luminescence (in RLU) versus varying concentration of native luciferase to determine appropriate concentration of luciferase for given plate reader settings.

(C) Denatured luciferase reactivation by Mtb DnaK (5 µM), DnaJ2 (1 µM), GrpE (2.5 µM) +/- excess telaprevir (100 µM) over a time course with the data represented as relative light units (RLU) (left) or normalized compared to the native and denatured luciferase controls (right) indicates telaprevir inhibits protein folding (n=3, error bars indicate SD). A portion of this figure is reprinted with permission from Hosfelt et al., 2021.

b. Prepare protein stocks and 40 mM ATP as follows:
   i. Prepare 5 mL of 40 mM ATP in ddH_2O.
   ii. Dilute chaperones DnaK, DnaJ2, and GrpE to initial concentrations of 100 µM, 40 µM, and 100 µM, respectively, using 1× PBS buffer in at least 50 µL total volume. Agitate tubes and briefly spin down using a table-top centrifuge to mix. Keep on ice.
   iii. Dilute luciferase to 100 µM using 1× Buffer R in a final volume of 20 µL. Agitate the tube and briefly spin down using a table-top centrifuge to mix. Keep on ice.

Note: A standard curve should be generated to determine how much luciferase to use in the experiment by plotting the relative light units (RLU) against the concentration of luciferase (Figure 4B). Select a concentration of luciferase that is within the linear range of your plate reader settings.

c. Label twelve 0.65 mL low-affinity microcentrifuge tubes for the four reactions (D-G) that will be set up in triplicate. Tubes can be labeled as D1, D2, D3, E1, E2, E3, etc.
d. Follow Table 17 for the preparation of reactions D and E: initially add respective volumes of indicated components (2× Buffer R, ddH_2O, and DTT) to each tube.

Table 17. Reaction mixtures for protein folding by DnaK, DnaJ2, and GrpE in the presence of vehicle (reaction D) or compound (reaction E)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
[a]2× Buffer R	50	1× Buffer R
[a]ddH_2O	30.5	n/a
[a]Dithiothreitol (DTT) (500 mM)	1	5 mM
100% DMSO or 40× compound	2.5	2.5% DMSO or 1× compound
DnaK (100 μM)	5	5 μM
DnaJ2 (40 μM)	2.5	1 μM
GrpE (100 μM)	2.5	2.5 μM
ATP (40 mM)	5	2 mM
Denatured luciferase (10 μM)	1	100 nM
Total	**100**	**n/a**

[a]Note: These components will be added first.

Note: 2× and 1× Buffer R must be filtered and can be stored at 25°C for 1 year.

i. For reaction D, add 2.5 μL of 100% DMSO to each tube.
ii. For reaction E, add 2.5 μL of 40× compound to each tube.

Note: The desired final concentration of telaprevir is 100 μM, thus the initial stock concentration used is 4 mM.

iii. For both reactions, add 5 μL of DnaK. Agitate tubes to mix and briefly spin down using a table-top centrifuge. Incubate reactions at 25°C for 30 min.

Note: To ensure a consistent temperature in all reactions, incubate reactions at 25°C using a heat block. DnaK is added prior to cofactors to facilitate compound binding.

e. Follow Table 18 to prepare reactions F and G. Pipette appropriate volumes of indicated components (2× Buffer R, ddH_2O, DTT and 100% DMSO) into each tube.
   i. Agitate tubes and briefly spin down using a table-top centrifuge to mix. Incubate reactions at 25°C for 1 h.
f. Prepare the denatured luciferase stock solution by following Table 19 in a low-affinity microcentrifuge tube. Vortex to mix all components and briefly spin down using a table-top centrifuge. Incubate at 25°C for 1 h.
g. After 30 min of incubation at 25°C, add 2.5 μL of DnaJ2 and GrpE to Reactions D and E (Table 17). Agitate tubes and briefly spin down using a table-top centrifuge to mix. Incubate reactions at 25°C for 30 min.
h. Prepare the native-luciferase stock solution following Table 20 in a low-affinity microcentrifuge tube. Agitate tube and briefly spin down using a table-top centrifuge to mix. Place on ice.

Table 18. Mixtures for control samples: denatured (reaction F) and native luciferase (reaction G)

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
[a]2× Buffer R	50	1× Buffer R
[a]ddH_2O	40.5	n/a
[a]Dithiothreitol (DTT) (500 mM)	1	5 mM
[a]100% DMSO	2.5	2.5% DMSO
ATP (40 mM)	5	2 mM
Denatured luciferase (10 μM) or native luciferase (10 μM)	1	100 nM
Total	**100**	**n/a**

[a]Note: These components will be added first and pre-incubated at 25°C for 1 h.

Table 19. Mixture for denatured luciferase stock

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
Guanidinium chloride (6 M)	44.5	5.34 M
Dithiothreitol (DTT) (500 mM)	0.5	5 mM
Luciferase (100 μM)	5	10 μM
Total	**50**	n/a

i. To initiate reactions and start time course:
   i. Add 5 μL of 40 mM ATP to all of the tubes (Reactions D-G).
   ii. Add 1 μL of denatured luciferase to reaction D1 to initiate the reaction. Agitate tube, spin down using a table-top centrifuge, and incubate at 25°C.
   iii. Proceed to add 1 μL of denatured luciferase to Reactions D2 to F3 following the instructions in step ii above.
   iv. Add 1 μL of native luciferase to Reactions G1 to G3. Agitate each tube, spin down using a table-top centrifuge, and incubate at 25°C.

⚠ CRITICAL: Additions of 1 μL of denatured and native luciferase should be done as quickly as possible. Upon addition of denatured luciferase to each reaction, the time course starts.

j. For the 5-min time point: Approximately 45 s prior to the time point, transfer 10 μL of triplicate reactions (D1-3, E1-3, F1-3 and G1-3) into separate wells of a 96-well half-area black plate.
   i. At t = 5 min, immediately add 25 μL of Luciferase Assay reagent using an automatic pipette and perform a luminescence measurement on a plate reader (1s integration, 25°C). Repeat for each replicate.

k. Follow the same process for conducting the t = 10-, 15-, 20-, and 30-min time points.

23. Data Analysis of the chaperone folding experiment:
   a. Export luminescence data to Prism. Plot as the average relative light units (RLU) versus time (min) for each reaction (D-G).
   b. Normalize data by setting the native luciferase control (reaction G) to 100%, and the denatured luciferase control (reaction F) to 0%. This will adjust the y-axis from being relative light units (RLU) to percentage (%) of native luciferase activity (see Figure 4C). Data can be plotted as average values with standard deviation (SD) indicated.

EXPECTED OUTCOMES

Overexpression and purification of mycobacterial chaperones and cofactors described in this protocol is expected to provide protein in sufficient purity for all assays. Typically, expression levels yield approximately 3–4 mg/L of protein.

In the ATP hydrolysis time course measurement, DnaK has low basal ATPase activity. The addition of cofactors DnaJ2 and GrpE should cause stimulation, resulting in lower remaining ATP throughout the time course. The rate of DnaK's ATPase activity should increase by a factor of ~3–5 in the presence of cofactors DnaJ2 and GrpE (Lupoli et al., 2016). Because DnaJ2 and GrpE are not ATPases,

Table 20. Mixture for native luciferase stock

$[Components]_{INITIAL}$	Volume (μL) per reaction	$[Components]_{FINAL}$
1× Buffer R	44	n/a
Dithiothreitol (DTT) (500 mM)	0.5	5 mM
Bovine serum albumin (BSA) (10 mg/mL)	0.5	0.1 mg/mL
Luciferase (100 μM)	5	10 μM
Total	**50**	n/a

the protein cofactor mixture should have no background activity. Any background ATPase activity observed for the cofactors indicates that the protein stocks should be purified further.

Cofactor-mediated stimulation of the ATPase activity of Mtb DnaK is used as a readout for IC_{50} analysis, which allows for the evaluation of potential chaperone or cofactor inhibitors obtained from high-throughput screens or computational approaches. Achieving a high "percent inhibition of ATPase activation" (Figure 2B) reflects the ability of a compound to inhibit activation of DnaK by cofactors. Lower IC_{50} values indicate more potent inhibitors. Compounds that show greater than 100% inhibition of activation likely inhibit the basal ATPase activity of DnaK. Verified chaperone/cofactor inhibitors will not affect the activity of luciferase-based coupled assay reagents (Kinase-Glo); hence, the ATP concentration values will be similar between reactions with vehicle only (DMSO) and each concentration of compound (Figure 2D) in the presence of luciferase reagent only. If the compound does affect the readout of Kinase-Glo reagent, RLU values will change with varying [compound]. If the latter is true, alternate modes of inhibitory analysis should be used.

Partial proteolysis of DnaK will provide insight into the overall conformational state of DnaK in the presence of ligands or inhibitors. In the apo, ADP- and ATP- bound states, Mtb DnaK is susceptible to cleavage of a portion of the C-terminal α-helical lid, as well as the linker, to form truncated NBD (approximately 40 kDa) and SBD (approximately 25 kDa) fragments, even in the presence of the ligand NRLLLTG (Figure 3C). In the case of telaprevir, binding to the SBD of DnaK protects the α-helical lid and linker from cleavage, resulting in the presence of a greater amount of full-length DnaK (approximately 70 kDa), suggesting a more compact state is present. Depending on the inhibitor, distinct overall conformational states may be observed. It should be noted that prokaryotic DnaKs and eukaryotic Hsp70s can produce different cleavage patterns upon treatment with protease (Meng et al., 2018).

Addition of DnaK and cofactors, DnaJ2 and GrpE, to denatured luciferase is sufficient to restore luciferase activity via protein folding over a short time course (t = 30 min, Figure 4C). For each experiment, luciferase activity is normalized to the native luciferase used, since the activity of each stock varies. Chaperone reactions typically achieve about 80 percent native luciferase activity in one hour. Compounds that inhibit this reaction will reduce the percentage of luciferase activity that is regained in chaperone/cofactor reactions. Validated compounds will not directly inhibit luciferase activity. Some compounds, such as telaprevir, will inhibit DnaK/cofactor ATPase and chaperone activity, while other compounds may inhibit only chaperone activity.

LIMITATIONS

While the protocol has been written for the analysis of the mycobacterial chaperone network, the assays can be applied to other chaperone and cofactor systems but will require optimization described by others (Chang et al., 2008; Miyata et al., 2010; Wisén and Gestwicki, 2008; Rauch and Gestwicki, 2014). The described protocol focuses primarily on utilizing coupled luciferase-based reporter assays to analyze the ATPase and refolding capabilities of the DnaK chaperone system. This detection method facilitates robust analyses; however, the downside to this approach is that some compounds may interact with luciferase and give a false positive result. In particular, both DnaK and luciferase bind to nucleotides; therefore, nucleoside analogs may not easily be evaluated using the described protocol. As an alternative, direct assays for measuring the amount of ATP and ADP can be used, such as our reported HPLC-based analysis method (Hosfelt et al., 2021). To measure the extent of chaperone refolding activity without a luciferase reporter, alternative assays based on measuring the activity of dehydrogenase enzymes have been published (Hristozova et al., 2016; Diamant and Goloubinoff, 1998; De Los Rios et al., 2006). Finally, the limited proteolysis experiment only provides a qualitative measurement of protein conformational changes upon addition of a ligand, and so higher resolution methods, such as structural analysis, may be pursued.

Table 21. Example of modified reaction using different compound vehicle

$[Components]_{INITIAL}$	Volume (µL) per reaction	$[Components]_{FINAL}$
DnaK (40 µM)	1.5	4 µM
DnaJ2 (4 µM)	1.5	0.4 µM
GrpE (4 µM)	1.5	0.4 µM
10× Compound (dissolved in ddH_2O or buffer)	1.5	1× Compound
ATP (1 mM, in ddH_2O)	1.5	100 µM
10× PBS, pH 7.4, 20 mM $MgCl_2$, 0.1% Tween-20)	1.5	1× PBS, pH 7.4, 2 mM $MgCl_2$, 0.01% Tween-20)
ddH_2O	6	n/a
Total	15	n/a

TROUBLESHOOTING

Problem 1

DnaK does not exhibit stimulation of ATPase activity upon addition of cofactors (Protocol 2, step 8).

Potential solution

DnaK and/or cofactors might require further purification due to background ATPase activity in the preparation. Protein can be further purified by FPLC using manufacturer's protocols or a previously described method for cofactor purification (Lupoli et al., 2016).

Problem 2

Compound of interest may not be soluble in 100% DMSO (see CRITICAL after Protocol 3, step 10a).

Potential solution

Commercial compounds should be dissolved according to the manufacturer's instructions. If the compound can be dissolved in ddH_2O or 1× PBS buffer, the reaction conditions can be adjusted as shown in Table 21. Note that the final buffer composition must match in all reactions, as well as the buffer used to dilute ATP standards. Enzyme activity should be tested using the ATPase time course protocol (Protocol 2, steps 1–8) to ensure it is still active in the relevant buffer system.

Problem 3

The compound inhibits Kinase-Glo Max reagent or affects the readout (Protocol 3, step 15).

Potential solution

Use an HPLC-based assay (Hosfelt et al., 2021) to measure ATP hydrolysis, as this assay does not rely on coupled-reporter enzymes for readout. Alternatively, a malachite green-based coupled assay can be used (Rauch and Gestwicki, 2014).

Problem 4

Over- and under-digestion of DnaK is observed in the proteolysis experiment in Protocol 4 (steps 18–21).

Potential solution

There are several potential solutions to these problems:

Perform a time course proteolysis experiment with *apo*-DnaK to find the optimal time point for formation of sufficient cleavage bands.
Prepare a new stock of 1 µM proteinase K (do not freeze/thaw for use in multiple experiments).
Troubleshoot with a different protease, such as trypsin.

Note: Trypsin cleaves at peptide bonds C-terminal to Arg and Lys residues whereas proteinase K cleaves at peptide bonds C-terminal to aliphatic and aromatic amino acids. Since the linker region of DnaKs and Hsp70s are hydrophobic and exposed to solvent in the *apo*- and ADP-state, resolution of cleaved NBD and SBD bands is more consistent with proteinase K (Freeman et al., 1995; Ivey et al., 2000; DeLuca-Flaherty et al., 1990; Liberek et al., 1991; Taylor et al., 2018).

Problem 5

Reactivation of luciferase activity is not observed upon adding chaperone and cofactors in Protocol 5 (steps 22 and 23).

Potential solution

Native luciferase (Table 20) does not store well on ice and should be diluted at the intended start time of the experiment. Restart the protocol at step 22 using a freshly diluted aliquot of luciferase.

Problem 6

Inconsistent luciferase refolding percentage is observed over multiple experiments after Data Analysis (Protocol 5, step 23).

Potential solution

To obtain a consistent percentage of folding of denatured luciferase, vary the cofactor concentrations to determine the optimal amounts for the reaction. Equal volume of titrants must be added to the reaction from different stock concentrations of protein.

Problem 7

Inconsistent IC_{50} values are measured over replicates, or no inhibitory activity of compounds is observed after Data Analysis of Protocol 3 (step 16).

Potential solution

It is important to read the plate at a consistent time point (t = 10 min) after addition of Kinase-Glo Max reagent. If the IC50 values are still inconsistent with each replicate, quench reactions by adding an equal volume of 0.05% formic acid and Kinase-Glo Max reagent. If no inhibition is observed, a higher concentration range of compound may be needed to achieve inhibition.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Tania Lupoli (tjl229@nyu.edu).

Materials availability

Plasmids used in this study are available from the lead contact upon request and upon completion of an MTA.

Data and code availability

This study did not generate code or new datasets.

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AUTHOR CONTRIBUTIONS

Conception and design, T.L., A.R., G.Y., and B.N.; investigation, A.R., G.Y., and B.N.; analysis and interpretation of data, T.L., A.R., G.Y., and B.N.; writing of the manuscript, A.R., T.L., G.Y., and B.N.; administrative, technical, or material support, T.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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