GroEL/ES inhibitors as potential antibiotics

Supporting Information

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Table of Contents:

Page

Table S1: Amino acid sequence conservation for ESKAPE pathogen and human chaperonins and co-chaperonins. Values represent % identical amino conservation compared to *E. coli* GroEL and GroES.

	GroEL (HSP60)	GroES (HSP10)
E. coli	**100%**	**100%**
E. faecium	**56.5%**	**47.3%**
S. aureus	**57.1%**	**44.1%**
K. pneumoniae	**96.5%**	**93.8%**
A. baumannii	**75.5%**	**62.1%**
P. aeruginosa	**80.0%**	**60.4%**
E. cloacae	**96.3%**	**93.8%**
H. sapiens	**48.0%**	**35.4%**

Table S2: Biochemical inhibition results for *E. coli* GroEL/ES inhibitors. Statistical analyses (two-tailed t-tests) were performed for compound log(IC_{50}) values determined from the GroEL/ES-dRho and GroEL/ES-dMDH refolding assays. Compounds for which there is a statistically significant difference between inhibition results have been marked with a "*" between the two assay results being compared ($p < 0.05$). P-values could not be calculated for compounds marked with a "#"as one IC_{50} is greater than the maximum compound concentration tested. For most compounds, IC_{50} values are not statistically different (17/22 compounds). IC_{50} correlations are represented graphically in **Figure 3** in the main text.

	Biochemical Assay log(IC_{50} /µM) Values ± SD					
#	Native Rho Reporter Activity	Native MDH Reporter Activity	GroEL/ES-dRho Refolding		GroEL/ES-dMDH Refolding	GroEL/ES-dMDH ATPase
1	>2	>1.8	1.47 ± 0.35	*	0.88 ± 0.61	2.08 ± 0.07
5	1.16 ± 0.28	>1.8	-0.23 ± 0.12		-0.16 ± 0.35	>2.4
8	>2	0.85 ± 0.33	0.16 ± 0.06		0.15 ± 0.20	>2.4
9	>2	>1.8	0.14 ± 0.11		-0.03 ± 0.27	1.90 ± 0.07
10	-0.60 ± 0.03	1.73 ± 0.08	-0.33 ± 0.19		-0.10 ± 0.21	2.24 ± 0.05
11	1.06 ± 0.17	>1.8	-0.08 ± 0.04		0.09 ± 0.50	2.34 ± 0.13
14	0.40 ± 0.32	>1.8	0.40 ± 0.31		0.48 ± 0.57	>2.4
15	1.36 ± 0.12	>1.8	0.24 ± 0.14		0.44 ± 0.52	>2.4
18	>2	>1.8	0.83 ± 0.31		0.76 ± 0.29	>2.4
19	0.91 ± 0.30	>1.8	0.48 ± 0.22		0.68 ± 0.48	>2.4
20	>2	>1.8	1.34 ± 0.10	*	0.73 ± 0.26	>2.4
23	0.31 ± 0.17	>1.8	0.38 ± 0.11		0.67 ± 0.82	>2.4
24	>2	>1.8	0.37 ± 0.12		0.56 ± 0.45	>2.4
25	0.16 ± 0.14	>1.8	0.41 ± 0.16		0.81 ± 0.46	>2.4
27	1.09 ± 0.07	>1.8	0.98 ± 0.09	*	0.67 ± 0.30	>2.4
28	0.73 ± 0.54	>1.8	-0.05 ± 0.15		0.42 ± 0.62	>2.4
29	>2	>1.8	1.44 ± 0.13		1.38 ± 0.07	2.27 ± 0.06
31	1.72 ± 0.21	>1.8	1.27 ± 0.14		1.49 ± 0.61	>2.4
32	1.00 ± 0.55	>1.8	1.04 ± 0.32	*	1.62 ± 0.16	2.34 ± 0.04
33	>2	>1.8	>2.4		>2	>2.4
34	>2	>1.8	1.40 ± 0.10		1.38 ± 0.37	>2.4
35	>2	>1.8	1.90 ± 0.34	#	>2	2.03 ± 0.04

IC_{50} = Inhibitor Concentration resulting in 50% reduction of biochemical activity

Table S3: *E. coli* and *B. subtilis* bacterial proliferation inhibition results for GroEL/ES inhibitors. Results are presented as log(EC_{50} /μM) values ± their standard deviations (SD).

	Bacterial Proliferation log(EC_{50} /μM) Values			
#	DH5α *E. coli*	MC4100 *ΔAcrB E. coli*	SM101 *E. coli*	*B. subtilis*
1	>2	>2	>2	>2
5	>2	1.96 ± 0.10	1.42 ± 0.16	1.40 ± 0.23
8	>2	0.37 ± 0.38	-0.48 ± 0.78	-0.99 ± 0.25
9	>2	>2	1.88 ± 0.12	>2
10	>2	>2	>2	>2
11	>2	>2	1.68 ± 0.31	>2
14	>2	>2	>2	>2
15	>2	>2	1.92 ± 0.09	>2
18	>2	1.32 ± 0.37	0.51 ± 0.25	-0.32 ± 0.34
19	>2	>2	0.88 ± 0.49	1.21 ± 0.46
20	>2	>2	>2	0.45 ± 0.33
23	>2	>2	>2	>2
24	>2	>2	>2	>2
25	>2	>2	>2	>2
27	>2	>2	>2	>2
28	>2	>2	>2	1.63 ± 0.32
29	>2	>2	>2	>2
31	>2	>2	1.27 ± 0.58	1.92 ± 0.09
32	>2	>2	1.83 ± 0.18	1.86 ± 0.07
33	>2	>2	>2	>2
34	>2	>2	1.40 ± 0.23	1.10 ± 0.06
35	>2	>2	>2	>2

EC_{50} = Effective Concentration of compound resulting in 50% reduction of bacterial proliferation

Table S4: Inhibition results for GroEL/ES inhibitors against the *ESKAPE* pathogens.

	Bacterial Proliferation log(EC_{50} /μM) Values ± SD						
#	*E. faecium*	*S. aureus*	*MRSA*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*E. cloacae*
1	>2	>2	>2	>2	>100	>2	>2
5	1.96 ± 0.04	1.13 ± 0.24	0.96 ± 0.13	>2	1.76 ± 0.16	>2	>2
8	-0.82 ± 0.10	-0.69 ± 0.20	-0.88 ± 0.13	>2	1.47 ± 0.50	>2	>2
9	>2	>2	>2	>2	>100	>2	>2
10	>2	1.91 ± 0.16	>2	>2	>100	>2	>2
11	>2	1.36 ± 0.29	1.60 ± 0.30	>2	>100	>2	>2
14	>2	>2	1.97 ± 0.06	>2	>100	>2	>2
15	>2	>2	>2	>2	1.91 ± 0.11	>2	>2
18	>2	0.25 ± 0.25	0.12 ± 0.28	>2	>100	>2	>2
19	>2	1.12 ± 0.30	0.17 ± 0.31	>2	>100	>2	>2
20	>2	>2	1.33 ± 0.12	>2	>100	>2	>2
23	>2	>2	>2	>2	>100	>2	>2
24	>2	>2	1.79 ± 0.08	>2	>100	>2	>2
25	>2	>2	>2	>2	>100	>2	>2
27	>2	1.90 ± 0.08	>2	>2	>100	>2	>2
28	>2	1.65 ± 0.13	1.87 ± 0.05	>2	>100	>2	>2
29	>2	>2	1.19 ± 0.15	>2	>100	1.93 ± 0.09	>2
31	>2	>2	1.75 ± 0.20	1.98 ± 0.03	1.50 ± 0.28	>2	>2
32	1.16 ± 0.09	1.92 ± 0.13	1.73 ± 0.11	>2	>100	>2	>2
33	>2	>2	>2	>2	>100	>2	>2
34	>2	>2	>2	>2	>100	>2	>2
35	>2	>2	>2	>2	>100	>2	>2
Ampicillin	-0.20 ± 0.06	-1.23 ± 0.25	1.88 ± 0.08	1.95 ± 0.01	0.41 ± 0.37	1.44 ± 0.01	>2
Minocycline	<-1.3	<-1.3	-0.46 ± 0.06	-0.15 ± 0.25	<-1.3	0.38 ± 0.22	0.17 ± 0.29
Rifampicin	0.08 ± 0.13	<-1.3	-0.81 ± 0.09	0.73 ± 0.08	-0.33 ± 0.26	0.71 ± 0.15	0.79 ± 0.08
Chloramphenicol	0.32 ± 0.03	0.35 ± 0.15	0.37 ± 0.09	0.13 ± 0.12	1.81 ± 0.17	1.21 ± 0.06	0.34 ± 0.08
Kanamycin	>2	0.91 ± 0.18	>2	1.65 ± 0.15	1.20 ± 0.41	1.39 ± 0.24	1.56 ± 0.06
Streptomycin	1.70 ± 0.22	0.56 ± 0.40	>2	0.53 ± 0.58	>2	0.47 ± 0.31	>2
Vancomycin	-0.52 ± 0.07	-0.69 ± 0.15	-0.76 ± 0.05	>2	1.23 ± 0.18	1.87 ± 0.19	>2
Daptomycin	1.51 ± 0.01	0.76 ± 0.28	0.76 ± 0.27	>2	>2	>2	>2

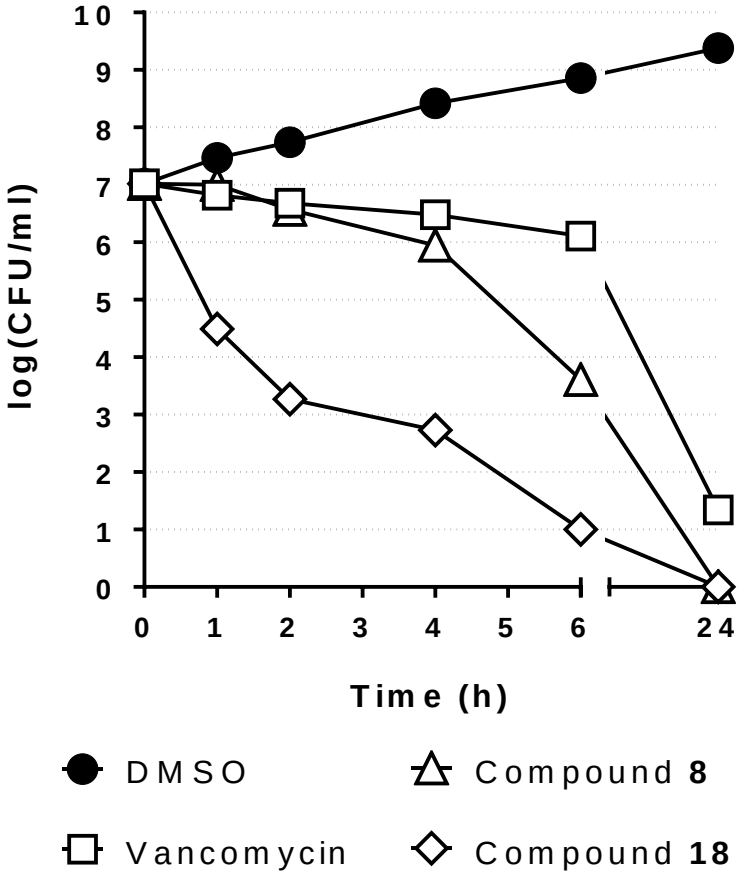
EC_{50} = Effective Concentration of compound resulting in 50% reduction of bacterial proliferation

Table S5: Human HSP60/10 biochemical inhibition and liver and kidney cytotoxicity results for the GroEL/ES inhibitors. Statistical analyses (two-tailed t-tests) were performed for compound log(IC_{50}) values determined from the GroEL/ES-dMDH and HSP60/10-dMDH refolding assays. Compounds for which there is a statistically significant difference between inhibition results have been marked with a "*****" between the two assay results being compared ($p < 0.05$). P-values could not be calculated for compounds marked with a "#"as one IC_{50} is greater than the maximum compound concentration tested. IC_{50} correlations are represented graphically in **Figure 5A** in the main text.

	Biochemical Assay log(IC_{50} /μM) Values ± SD				Human Cell Viability Assay log(EC_{50} /μM) Values ± SD	
#	GroEL/ES-dMDH Refolding		HSP60/10-dMDH Refolding	HSP60/10-dMDH ATPase	THLE-3 (Liver)	HEK 293 (Kidney)
1	0.88 ± 0.61	*	1.95 ± 0.08	2.02 ± 0.16	1.47 ± 0.25	1.53 ± 0.24
5	-0.16 ± 0.35	*	0.69 ± 0.59	>2.4	1.20 ± 0.06	1.35 ± 0.27
8	0.15 ± 0.20	*	0.74 ± 0.44	0.55 ± 0.09	1.08 ± 0.09	1.89 ± 0.14
9	-0.03 ± 0.27		0.20 ± 0.22	>2.4	>2	>2
10	-0.10 ± 0.21	*	0.52 ± 0.04	2.15 ± 0.28	>2	>2
11	0.09 ± 0.50	*	0.71 ± 0.15	>2.4	0.99 ± 0.02	1.10 ± 0.10
14	0.48 ± 0.57		0.87 ± 0.15	>2.4	>2	>2
15	0.44 ± 0.52		1.01 ± 0.22	>2.4	>2	1.99 ± 0.03
18	0.76 ± 0.29	#	>2	>2.4	>2	>2
19	0.68 ± 0.48		1.20 ± 0.27	>2.4	1.62 ± 0.10	1.64 ± 0.03
20	0.73 ± 0.26		0.45 ± 0.54	>2.4	0.56 ± 0.31	1.23 ± 0.48
23	0.67 ± 0.82		1.12 ± 0.15	>2.4	>2	>2
24	0.56 ± 0.45		0.91 ± 0.25	>2.4	1.53 ± 0.05	1.74 ± 0.17
25	0.81 ± 0.46		1.19 ± 0.11	>2.4	>2	>2
27	0.67 ± 0.30	*	1.12 ± 0.17	>2.4	>2	>2
28	0.42 ± 0.62		0.26 ± 0.31	>2.4	>2	>2
29	1.38 ± 0.07	*	1.58 ± 0.18	>2.4	>2	>2
31	1.49 ± 0.61	#	>2	>2.4	1.17 ± 0.17	0.89 ± 0.39
32	1.62 ± 0.16	#	>2	>2.4	1.78 ± 0.11	1.79 ± 0.16
33	>2		>2	>2.4	>2	>2
34	1.38 ± 0.37		1.41 ± 0.53	>2.4	>2	>2
35	>2		>2	>2.4	>2	>2

IC_{50} = Inhibitor Concen IC_{50} = Inhibitor Concentration resulting in 50% reduction of biochemical activity
EC_{50} = Effective Conce EC_{50} = Effective Concentration of compound resulting in 50% reduction in cell viability

Figure S1: Evaluation of bacteriostatic *vs.* bactericidal mechanisms of action. Compounds **8**, **18**, and vancomycin were tested at concentrations of 4x their MICs against *S. aureus* (ATCC 25923). MIC values were determined to be 0.60 μM (0.34 μg/mL) for compound **8**, 8.0 μM (2.4 μg/mL) for compound **18**, and 1.6 μM (2.3 μg/mL) for vancomycin under these experimental conditions. All compounds appear to be bactericidal. Data presented are the averages of triplicate experiments.



General Materials and Methods.

DH5α and BL21 (DE3) *E. coli* cells were purchased from New England Biolabs, and Rosetta™ 2 (DE3) *E. coli* cells from EMD Millipore. MC4100 Δ*acrB E. coli* cells were provided by Prof. Eli Chapman, University of Arizona, College of Pharmacy. *E. coli* SM101 *lpxA2* cells were obtained from the *Coli* Genetic Stock Center at Yale University (CGSC #7255). *B. subtilis 168* cells were provided by Dr. Leendert Hamoen from the University of Amsterdam, Swammerdam Institute for Life Sciences (SILS). The *ESKAPE* pathogens were purchased from the American Type Culture Collection (ATCC): *E. faecium* (Orla-Jensen) Schleifer and Kilpper-Balz strain NCTC 7171 (ATCC 19434); *S. aureus* subsp. *aureus* Rosenbach strain Seattle 1945 (ATCC 25923); Methicillin-resistant *S. aureus* (MRSA) subsp. *aureus* Rosenbach strain HPV107 (ATCC BAA-44); *K. pneumonia*, subsp. *pneumoniae* (Schroeter) Trevisan strain NCTC 9633 (ATCC 13883); *A. baumannii* Bouvet and Grimont strain 2208 (ATCC 19606); *P. aeruginosa* (Schroeter) Migula strain NCTC 10332 (ATCC 10145); *E. cloacae*, subsp. *cloacae* (Jordan) Hormaeche and Edwards strain CDC 442-68 (ATCC 13047). HEK 293 kidney cells (ATCC CRL-1573) and THLE-3 liver cells (ATCC CRL-11233) were used for compound toxicity assays. Antibiotics were used in following concentrations when appropriate: Kanamycin (34 μg/mL), Ampicillin (50 μg/mL), Chloramphenicol (30 μg/mL) and Streptomycin (100 μg/mL). Test compounds were purchased from commercial suppliers where available (Chembridge, ChemDiv, Ambinter, Aldrich, Asinex, and Ryan Scientific), or synthesized according to literature procedures (compounds **10**, **15**, **23**, **24**, and **25** – synthetic protocols are presented in the Supplementary Data below). Compounds **2-4**, **6**, **7**, **12**, **13**, **16**, **17**, **21**, **22**, **26**, **30**, and **36** were omitted from evaluation as they were either not commercially available, or purchased compounds were not readily identified by LC-MS and/or did not have acceptable purities confirmed by HPLC. For ease of comparison, compound numbering from **1**-**36** was maintained as presented in our previous high-throughput screening study.[1]

Statistical considerations.

All IC_{50} (or EC_{50}) values reported are averages of IC_{50} (or EC_{50}) values determined from individual dose-response curves in replicate assays as follows: 1) Individual IC_{50} values from replicate assays were first log-transformed and the average log(I/EC_{50}) values and standard deviations (SD) calculated; 2) Replicate log(I/EC_{50}) values were evaluated for outliers using the ROUT method in GraphPad Prism 6 (Q of 10%); 3) Average IC_{50} (or EC_{50}) values were then back-calculated from the average log(I/EC_{50}) values. To compare statistical differences between log(IC_{50}) values, two-tailed, unpaired t-tests were performed using GraphPad Prism 6 (0.05 alpha level).

Protein expression and purification.

E. coli GroEL was expressed from a *trc*-promoted plasmid in DH5α *E. coli* cells and purified as previously reported.[1] *E. coli* GroES was expressed from a *T7*-promoted plasmid in *E. coli* BL21 (DE3) cells and purified as previously reported.[1] Human HSP60 was expressed from a *T7*-promoted plasmid in Rosetta™ 2 (DE3) *E. coli* cells and purified as previously reported.[2] For human HSP10 purification, pET30-*HSP10* was transformed into Rosetta™ 2 (DE3) *E. coli* cells for over-expression. Cells were grown at 37°C in LB/Kanamycin/Chloramphenicol medium until an OD_{600} of 0.5 was reached, then were induced with 0.5 mM IPTG and continued to grow for 2-3 h at 37°C. The culture was centrifuged at 14,000 rpm, and the cell pellet was re-suspended in Buffer A (50 mM sodium acetate, pH 4.5, and 0.5 mM EDTA), supplemented with EDTA-free complete protease inhibitor cocktail (Roche), 100 μg/ml lysozyme, 10 μL (1000 u/ml) DNAase, and lysed by sonication. Clarified cell lysate was loaded on a cation exchange column (SP Sepharose fast flow resin, GE) and eluted with linear NaCl gradient with Buffer B (sodium acetate, pH 4.5, 0.5 mM EDTA, and 1 M NaCl). Fractions containing HSP10 were concentrated, dialyzed with storage buffer (50 mM Tris-HCl, pH 7.4, and 300 mM NaCl) using 10 kDa SnakeSkin™ dialysis tubing (Thermo Scientific) and re-purified on a Superdex 200 column (HiLoad 26/600, GE) in storage buffer. The concentration of protein was determined by Coomassie Protein

Assay Kit (Thermo Scientific). Protein was stored at 4°C in 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, and 1 mM DTT.

Evaluation of compounds in GroEL/ES and HSP60/10-mediated dMDH refolding assays.
Reagent preparation: For these assays, four primary reagent stocks were prepared: 1) GroEL/ES-dMDH or HSP60/10-dMDH binary complex stock; 2) ATP initiation stock; 3) EDTA quench stock; 4) MDH enzymatic assay stock. Denatured MDH (dMDH) was prepared by 2-fold dilution of MDH (5 mg/ml, soluble pig heart MDH from Roche, product #10127248001) with denaturant buffer (7 M guanidine-HCl, 200 mM Tris, pH 7.4, and 50 mM DTT). MDH was completely denatured by incubating at room temperature for 30 min. The binary complex solutions were prepared by slowly adding the dMDH stock to a stirring stock with GroEL (or HSP60) in folding buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT), followed by addition of GroES (or HSP10). The binary complex stocks were prepared immediately prior to use and had final protein concentrations of 83.3 nM GroEL (*Mr* 800 kDa) or HSP60 (*Mr* 400 kDa), 100 nM GroES or HSP10 (*Mr* 70 kDa), and 20 nM dMDH in folding buffer. For the ATP initiation stock, ATP solid was diluted into folding buffer to a final concentration of 2.5 mM. Quench solution contained 600 mM EDTA (pH 8.0). The MDH enzymatic assay stock consisted of 20 mM sodium mesoxalate and 2.4 mM NADH in reaction buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, and 1 mM DTT).

Assay Protocol*:* First, 30 μL aliquots of the GroEL/ES-dMDH or HSP60/10-dMDH binary complex stocks were dispensed into clear, 384-well polystyrene plates. Next, 0.5 μL of the compound stocks (10 mM to 4.6 μM, 3-fold dilutions in DMSO) were added by pin-transfer (V&P Scientific). The chaperonin-mediated refolding cycles were initiated by addition of 20 μL of ATP stock (reagent concentrations during refolding cycle: 50 nM GroEL or HSP60, 60 nM GroES or HSP10, 12 nM dMDH, 1 mM ATP, and compounds of 100 μM to 46 nM, 3-fold dilution series). After incubation for 60 minutes at 37°C, the assays were quenched by addition of 10 μL of the EDTA to final concentration of 100 mM. Enzymatic activity of the refolded MDH was initiated by addition of 20 μL MDH enzymatic assay stock (20 mM sodium mesoxalate and 2.4 mM NADH in reaction buffer, 50 mM Tris pH 7.4, 50 mM KCl, 1 mM DTT), and followed by measuring the NADH absorbance in each well at 340 nm using a Molecular Devices, SpectraMax Plus384 microplate reader (NADH absorbs at 340 nm, while NAD^+ does not). A_{340} measurements recorded at 0.5 minutes (start point) and at successive time points until the amount of NADH consumed reached ~90% (end point, generally between 30-60 minutes). The differences between the start and end point A_{340} values were used to calculate the % inhibition of the GroEL/ES or HSP60/10 machinery by the compounds. IC_{50} values for the test compounds were obtained by plotting the % inhibition results in GraphPad Prism 6 and analyzing by non-linear regression using the log (inhibitor) vs. response (variable slope) equation. Results presented represent the averages of IC_{50} values obtained from at least triplicate experiments.

Counter-screening compounds for inhibition of native MDH enzymatic activity.
Reagent Preparations & Assay Protocol: Reagents were identical to those used in the GroEL/ES-dMDH refolding assay described above; however, the assay protocol differed in the sequence of compound addition to the wells. Compounds were pin-transferred after the EDTA quenching step, but prior to the addition of the enzymatic reporter reagents. Thus, the refolding reactions were allowed to proceed for 60 min at 37°C in the absence of test compounds, but the enzymatic activity of the refolded MDH reporter enzyme was monitored in the presence of test compounds (inhibitor concentration range during the enzymatic reporter reaction is 83.3 μM to 38 nM – 3-fold dilutions). IC_{50} values for the MDH reporter enzyme were determined as described above. Results presented represent the averages of IC_{50} values obtained from at least triplicate experiments.

Evaluation of compounds for inhibition of chaperonin-dependent ATPase activity.
Reagent preparation: For these assays, four primary reagent stocks were prepared: 1) GroEL/ES-dMDH or human HSP60/10-dMDH binary complex stock; 2) ATP initiation stock; 3) EDTA quench stock; 4) malachite green reporter stock. The dMDH stock was prepared as in the above refolding assays. Binary complex solutions were immediately prepared prior to use, with final concentrations of 100 nM GroEL or HSP60, 120 nM GroES or HSP10, and 250 nM dMDH in folding buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT). For the ATP initiation stock, ATP solid was diluted into folding buffer to a final concentration of 2 mM. Quench solution contained 300 mM EDTA (pH 8.0). The malachite green reporter stock consisted of 0.034% malachite green and 1.04% ammonium molybdate tetrahydrate in 1 M HCl with 0.02% Tween-20.

Assay protocol: First, 10 μL aliquots of binary stock were dispensed into clear, flat-bottom, 384-well polystyrene plates. Next, 0.5 μL of the compound stocks (10 mM to 4.6 μM, 3-fold dilutions in DMSO) were added by pin-transfer. The ATP-dependent chaperonin refolding cycles were initiated by addition of 10 μL ATP stock (reagent concentrations during the assay; 50 nM GroEL or HSP60, 60 nM GroES or HSP10, 125 nM dMDH, 1 mM ATP, and compounds from 250 μM to 114 nM, 3-fold dilution series). The reactions were incubated at 37°C for 60 minutes, then were quenched by addition of 10 μL of the EDTA solution. After quenching, 60 μL of the malachite green reporter stock was added and incubated at room temperature for 15 min, then the absorption was measured at 600 nm. A second set of baseline control plates were prepared analogously, but without binary solution, to correct for possible interference from compound absorbance or turbidity. IC_{50} values for the test compounds were obtained by plotting the OD_{600} results in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of IC_{50} values obtained from at least triplicate experiments.

Evaluation of compounds in GroEL/ES in denatured Rhodanese refolding assay.
Reagent preparation: For this assay, five primary reagent stocks were prepared: 1) GroEL/ES-dRho binary complex stock; 2) ATP initiation stock; 3) thiocyanate enzymatic assay stock; 4) formaldehyde quench stock; 5) ferric nitrate reporter stock. Denatured Rhodanese (dRho) was prepared by 3-fold dilution of Rhodanese (Roche product #R1756, diluted to 10 mg/mL with H_2O) with denaturant buffer (12 M Urea, 50 mM Tris-HCl, pH 7.4, and 10 mM DTT). Rhodanese was completely denatured by incubating at room temperature for 30 min. The binary complex solution was prepared by slowly adding the dRho stock to a stirring stock of concentrated GroEL in modified folding buffer (50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 5 mM $Na_2S_2O_3$ and 1 mM DTT). The solution was centrifuged at 16,000 x g for 5 minutes, and the supernatant was collected and added to a solution of GroES in modified folding buffer to give final protein concentrations of 100 nM GroEL, 120 nM GroES, and 80 nM dRho in modified folding buffer. The binary complex stock was prepared immediately prior to use. For the ATP initiation stock, ATP solid was diluted into modified folding buffer to a final concentration of 2.0 mM. The thiocyanate enzymatic assay stock was prepared to contain 70 mM KH_2PO_4, 80 mM KCN, and 80 mM $Na_2S_2O_3$ in water. The formaldehyde quench solution contained 30% formaldehyde in water. The ferric nitrate reporter stock contained 8.5% w/v $Fe(NO_3)_3$ and 11.3% v/v HNO_3 in water.

Assay Protocol*:* First, 10 μL aliquots of the GroEL/ES-dRho complex stock was dispensed into clear, 384-well polystyrene plates. Next, 0.5 μL of the compound stocks (10 mM to 4.6 μM, 3-fold dilutions in DMSO) were added by pin-transfer. The chaperonin-mediated refolding cycle was initiated by addition of 10 μL of ATP stock (reagent concentrations during refolding cycle: 50 nM GroEL, 60 nM GroES, 40 nM dRho, 1 mM ATP, and compounds of 250 μM to 114 nM, 3-fold dilution series). After incubating for 60 minutes at 37°C for the refolding cycle, 30 μL of the thiocyanate enzymatic assay

stock was added and incubated for 60 min at R.T. for the refolded Rhodenase enzymatic reporter reaction. The reporter reaction was quenched by adding 10 μL of the formaldehyde quench stock, and then 40 μL of the ferric nitrate reporter stock was added to quantify the amount of thiocyanate produced during the enzymatic reporter reaction, which is proportional to the amount of dRho refolded by GroEL/ES. After incubating at R.T. for 15 min, the absorbance by $Fe(SCN)_3$ was measured at 460 nm using a Molecular Devices, SpectraMax Plus384 microplate reader. A second set of baseline control plates were prepared analogously, but without binary solution, to correct for possible interference from compound absorbance or turbidity. IC_{50} values for the test compounds were obtained by plotting the A_{460} results in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of IC_{50} values obtained from at least triplicate experiments.

Counter-screening compounds for inhibition of native Rhodanese enzymatic activity.
Reagent Preparations & Assay Protocol: Reagents were identical to those used in the GroEL/ES-dRho refolding assay described above; however, the assay protocol differed in the sequence of compound addition to the wells. Compounds were pin-transferred after the incubation for 60 minutes at 37°C for the refolding cycle, but prior to the addition of the thiocyanate enzymatic assay stock. Thus, the refolding reactions were allowed to proceed for 60 min at 37°C in the absence of test compounds, but the enzymatic activity of the refolded Rhodanese reporter enzyme was monitored in the presence of test compounds (inhibitor concentration range during the enzymatic reporter reaction is 100 μM to 46 nM – 3-fold dilutions). IC_{50} values for the Rhodenase reporter enzyme were determined as described above. Results presented represent the averages of IC_{50} values obtained from at least triplicate experiments.

Evaluation of compounds for inhibition of bacterial cell proliferation.

All *E.coli* strains and *B. subtilis* 168 were grown using Luria-Bertani (LB) media broth/agar, unless specified otherwise. *S. aureus* and MRSA were grown using ATCC Medium 18 (Tryptic Soy Broth). All other *ESKAPE* pathogens (*E. faecium*, *K. pneumonia*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae*), were grown using Brain Heart Infusion media broth/agar (Becton, Dickinson and Company).

General Assay Protocol: Stock bacterial cultures were streaked onto agar plates and grown overnight at 37°C. Fresh aliquots of broth were inoculated with single bacterial colonies and the cultures were grown overnight at 37°C with shaking (250 rpm). The following morning, the overnight cultures were sub-cultured (1:5 dilution) into fresh aliquots of media and grown at 37°C for 2 h with shaking. After 2 h, cultures were diluted into fresh media to achieve final OD_{600} readings of 0.0125. Aliquots of these diluted cultures (80 μL) were added to clear, flat-bottom, 384-well polystyrene plates that were stamped with 1.0 μL of test compounds in 20 μL media (8x, 3-fold dilution series stocks ranging from 10 mM to 4.6 μM in DMSO). Plates were sealed with "Breathe Easy" oxygen permeable membranes (Diversified Biotech) and left to incubate at 37°C without shaking (stagnant assay). OD_{600} readings were taken at the 6-8 h time points, depending on the time for each bacterial strain to reach mid-log phase growth. A second set of baseline control plates were prepared analogously, but without any bacteria added, to correct for possible compound absorbance and/or precipitation. EC_{50} values for the test compounds were obtained by plotting the OD_{600} results in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of EC_{50} values obtained from at least triplicate experiments.

Evaluation of compounds for inhibition of MC4100 ΔacrB E. coli cell growth: MC4100 Δ*acrB E. coli* were streaked onto an LB agar plate containing 50 μg/mL kanamycin and grown overnight at 37°C. The following morning, the overnight culture was sub-cultured (1:5 dilution) into a fresh aliquot of media, without addition of antibiotic, and grown at 37°C for 2 h with shaking. After 2 h, the culture was diluted

into fresh media to achieve a final OD_{600} reading of 0.0125. All the other steps were followed identically as described above.

Evaluation of compounds for inhibition of SM101 lpxA2 E. coli cell growth:[3, 4] SM101 *lpxA2 E. coli* were streaked onto an LB agar plate containing 50 μg/mL streptomycin and grown at 31°C. A fresh aliquot of LB media (without any NaCl but with streptomycin added) was inoculated with a single bacterial colony and the culture was split into two, with one half grown overnight at 31°C and the other at 37°C (no growth control), with shaking at 250 rpm. The following morning, the culture grown at 31°C was diluted into fresh LB media to achieve a final OD_{600} reading of 0.0125 (if the culture incubated at 37°C grew, new cultures were prepared). Aliquots of this diluted culture (80 μL) were added to 384-well clear bottom polystyrene plates that had been previously stamped with 1.0 μL of test compounds in 20 μL media (8x, 3-fold dilution series stocks ranging from 10 mM to 4.6 μM in DMSO; compounds were added by pin transfer). The plates were sealed with "Breathe Easy" oxygen permeable membranes and left to incubate at 31°C for 12 h without shaking (stagnant assay). At the 12 h time point, the plates were removed and the OD_{600} read using a SpectraMax Plus 384 UV-Vis spectrophotometer to follow cell growth. A second set of baseline control plates were prepared analogously, but without any bacteria added, to correct for possible compound absorbance and/or precipitation. Separate control cultures were prepared from the diluted SM101 *lpxA2* bacteria and grown at 31°C and 37°C, with shaking at 250 rpm, to verify growth in the 31°C culture and no growth in the 37°C culture. If bacteria grew in the 37°C no growth control, then the assay was repeated. EC_{50} values were determined as described above. Results presented represent the averages of EC_{50} values obtained from at least triplicate experiments.

Evaluation of bacteriostatic vs. bactericidal mechanisms of action.

Minimal inhibitory concentration (MIC) determination.

A fresh aliquot of ATCC Medium 18 was inoculated with a single *S. aureus* (ATCC 25923) bacterial colony and the culture was grown overnight at 37°C with shaking (250 rpm). The following morning, the overnight culture was sub-cultured (1:5 dilution) into fresh media and grown at 37°C for 1 h with shaking. After 1 h, the sub-culture was diluted to an OD_{600} of 0.01 (~10^7 CFU/ml) in fresh media. 200 μL aliquots were added to clear, flat-bottom, 96-well polystyrene plates. 2 μL of compounds **8**, **18,** and vancomycin in DMSO were then added to the wells (final concentrations of compounds were: **8** = 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 μM; **18** = 2, 4, 6, 8, 10, and 12 μM; vancomycin = 1.2, 1.4, 1.6, 1.8, 2.0, and 2.2 μM). The plates were then sealed with "Breathe Easy" oxygen permeable membranes (Diversified Biotech) and incubated at 37°C without shaking (stagnant assay) for 24 h, after which the MICs were identified visually (MICs: **8** = 0.6 μM; **18** = 8 μM; Vancomycin = 1.6 μM).

Bactericidal activity of compounds.

A fresh aliquot of ATCC Medium 18 was inoculated with a single *S. aureus* (ATCC 25923) bacterial colony and the culture was grown overnight at 37°C with shaking (250 rpm). The following morning, the overnight culture was sub-cultured (1:5 dilution) into fresh media and grown at 37°C for 1 h with shaking. After 1 h, the sub-culture was diluted to optical density OD_{600} of 0.01 (~10^7 CFU/ml) in fresh media. Aliquots of these diluted cultures (6 mL) were treated with DMSO (no compound control) or 4x MIC of compounds **8**, **18**, and vancomycin (60 μL of each compound stocks were added to achieve respective 4x MIC: final DMSO concentration in all cultures were maintained at 1%). All cultures were incubated at 37°C with shaking (250 rpm) and viable cell counts were determined at 0, 1, 2, 4, 6, and 24 h time intervals by dilution and plating on ATCC Medium 18 agar. Time-kill curves presented in **Figure S1** are averaged values from triplicate experiments.

Evaluation of compounds for cytotoxicity to HEK 293 and THLE-3 cells.

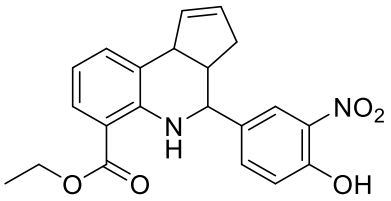
Cell cytotoxicity assays were performed using the Alamar Blue reporter reagents as previously described.[5, 6] HEK 293 cells were maintained in MEM medium (Corning Cellgro, 10-009 CV) supplemented with 10% FBS (Sigma, F2242). THLE-3 cells were maintained in Clonetics BEBM medium (Lonza, CC-3171) supplemented with the BEGM bullet kit (Lonza, CC-3170) and 10% FBS. All assays were carried out in 384-well plates (BRAND cell culture grade plates, 781980). Briefly, cells at 80% confluence were harvested and diluted in growth medium, then 50 μL of the HEK 293 cells (15,000 cells/well) or THLE-3 cells (5,000 cells/well) were plated and incubated at 37°C with 5% CO_2 for 24 h. Compound stocks (0.5 μL of 10 mM to 4.6 μM, 3-fold dilutions in DMSO) were added by pin-transfer and the plates were incubated for an additional 48 h at 37°C with 5% CO_2. The Alamar Blue reporter reagents were then added to a final concentration of 10%. The plates were incubated for 4 h (HEK 293) or 6 h (THLE-3), then sample fluorescence (535 nm excitation, 590 nm emission) was read using a Molecular Devices FlexStation II 384-well plate reader. Cell viability was calculated as per vendor instructions. EC_{50} values for the test compounds were obtained by plotting the % Alamar Blue reduction results in GraphPad Prism 6 and analyzing by non-linear regression using the log(inhibitor) vs. response (variable slope) equation. Results presented represent the averages of EC_{50} values obtained from at least triplicate experiments.

General Synthetic Methods.

Test compounds were purchased from commercial suppliers where available (Chembridge, ChemDiv, Ambinter, Aldrich, Asinex, and Ryan Scientific), or synthesized as indicated below for compounds **10**, **15**, **23**, **24**, and **25**. Compounds **2-4**, **6**, **7**, **12**, **13**, **16**, **17**, **21**, **22**, **26**, **30**, and **36** were omitted from evaluation as they were either not commercially available, or purchased compounds were not readily identified by LC-MS and/or acceptable purities confirmed by HPLC. Reaction progress was monitored by thin-layer chromatography on silica gel 60 F254 coated glass plates (EM Sciences). Flash chromatography was performed using a Biotage Isolera One flash chromatography system and eluting through Biotage KP-Sil Zip or Snap silica gel columns. Reverse phase high performance liquid chromatography (RP-HPLC) was performed using a Waters 1525 binary pump, 2489 tunable UV/Vis detector (254 and 280 nm detection), and 2707 autosampler. For analytical HPLC evaluation, samples were chromatographically separated using a Waters XSelect CSH C18 column (part number 186005282, 130 Å pore size, 5 μm particle size, 3.0x150 mm), eluting with a H_2O:CH_3CN gradient solvent system. Linear gradients were run from either 100:0, 80:20, or 60:40 A:B to 0:100 A:B (A = 95:5 H_2O:CH_3CN, 0.05% TFA; B = 5:95 H_2O:CH_3CN, 0.05% TFA. All test compounds were found to be >95% purity, with exception of the following: **14** = 85%, **19** = 79%, **20** = 92%, **27** = 94%, **28** = 78%, and **34** = 84%. For preparatory HPLC purification, samples were chromatographically separated using a Waters XSelect CSH C18 OBD prep column (part number 186005422, 130 Å pore size, 5 μm particle size, 19x150 mm) employing the above H_2O:CH_3CN solvent systems. All compounds (purchased or synthesized) were confirmed by mass spectrometry, with data collected using an Agilent analytical LC-MS at the IU Chemical Genomics Core Facility (CGCF). ^{1}H-NMR spectra were recorded on either a Bruker 300 MHz or Bruker 500 MHz spectrometer. Chemical shifts are reported in parts per million and calibrated to the d_6-DMSO solvent peaks at 2.50 ppm.

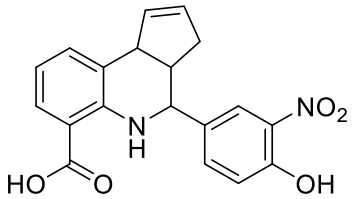
Compound Syntheses.

Ethyl-4-(4-hydroxy-3-nitrophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-6-carboxylate (37).[7-9]



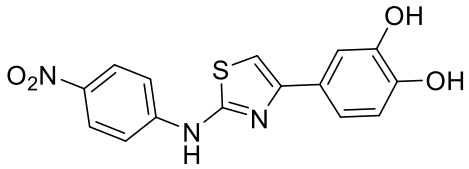
To a stirring mixture of 4-hydroxy-3-nitrobenzaldehyde (289 mg, 1.73 mmol) and ethyl-2-aminobenzoate (0.25 mL, 1.7 mmol) in anhydrous acetonitrile (20 mL) was added TFA (0.12 mL, 1.6 mmol), and the reaction was left to stir for 1 h at R.T. (under Ar). Then, cyclopentadiene was added (0.75 mL, 18 mmol, freshly distilled from dicyclopentadiene) and the reaction was left to stir for 18 h. The reaction was then concentrated and flash chromatographic purification over silica (hexanes:DCM gradient) afforded ethyl-4-(4-hydroxy-3-nitrophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-6-carboxylate (**37**) as a yellow solid (530 mg, 82%). ^{1}H-NMR (500 MHz, d_6-DMSO) δ 7.92 (d, *J*=2.2 Hz, 1H), 7.66 (dd, *J*=1.1, 8.0 Hz, 1H), 7.57-7.63 (m, 2H), 7.29 (d, *J*=7.3 Hz, 1H), 7.15 (d, *J*=8.8 Hz, 1H), 6.68 (t, *J*=7.7 Hz, 1H), 5.85-5.89 (m, 1H), 5.61 (d, *J*=5.0 Hz, 1H), 4.69 (d, *J*=3.5 Hz, 1H), 4.25 (q, *J*=6.9 Hz, 2H), 4.12 (d, *J*=9.1 Hz, 1H), 2.97-3.05 (m, 1H), 2.33-2.40 (m, 1H), 1.72-1.80 (m, 1H), 1.29 (t, J=7.3 Hz, 3H); LC-MS $[MH]^+$ expected = 381.2 ($C_{21}H_{21}N_2O_5$), observed = 381.1; HPLC: 98% pure.

4-(4-Hydroxy-3-nitrophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-6-carboxylic acid (10).



Compound **37** (153 mg, 0.402 mmol) was stirred with LiOH•H_2O (167 mg, 3.98 mmol) in H_2O/MeOH/THF (1/1/3 mL) at 60°C. After 1 h, the reaction was acidified with 1 M HCl and extracted into EtOAc. The organics were washed with brine, dried over Na_2SO_4, filtered, and concentrated. Prep-HPLC purification afforded 4-(4-hydroxy-3-nitrophenyl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-6-carboxylic acid (**10**) as a yellow solid (119 mg, 84%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 7.86-7.963 (m, 2H), 7.64 (d, *J*=6.6 Hz, 2H), 7.25 (d, *J*=6.9 Hz, 1H), 7.19 (d, *J*=8.8 Hz, 1H), 6.64 (t, *J*=7.6 Hz, 1H), 5.87 (br s, 1H), 5.6 (d, *J*=5.4 Hz, 1H), 4.69 (br s, 1H), 4.11 (d, *J*=9.1 Hz, 1H), 2.95-3.05 (m, 1H), 2.33-2.41 (m, 1H); LC-MS $[MH]^+$ expected = 353.1 ($C_{19}H_{17}N_2O_5$), observed = 353.1; HPLC: >99% pure.

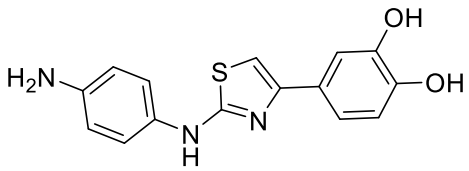
4-(2-((4-Nitrophenyl)amino)thiazol-4-yl)benzene-1,2-diol (38).[10, 11]



2-Chloro-3',4'-dihydroxyacetophenone (567 mg, 3.04 mmol) and *N*-(4-nitrophenyl)thiourea (555 mg, 2.81 mmol) were refluxed in ethanol (20 mL) for 4 h, then cooled on ice. The precipitate was filtered, rinsed with ice cold EtOH, and dried to afford 4-(2-((4-nitrophenyl)amino)thiazol-4-yl)benzene-1,2-diol

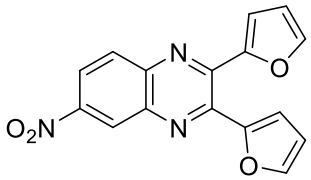
(**38**) as a yellow powder (750 mg, 81%). ^{1}H-NMR (500 MHz, d_6-DMSO) δ 11.04 (s, 1H), 8.25 (d, *J*=9 Hz, 2H), 7.94 (d, *J*=9.1 Hz, 2H), 7.35 (d, *J*=2.2 Hz, 1H), 7.24 (dd, *J*=2.0, 8.0 Hz, 1H), 7.19 (s, 1H), 6.79 (d, *J*=8.2 Hz, 1H); LC-MS [M-H]$^-$ expected = 328.0 ($C_{15}H_{10}N_3O_4S$), observed = 327.9; HPLC: 98% pure.

4-(2-((4-Aminophenyl)amino)thiazol-4-yl)benzene-1,2-diol (15).



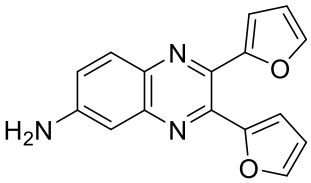
Tin powder (884 mg, 7.45 mmol) was added portion-wise to a stirring mixture of compound **38** (721 mg, 2.41 mmol) in 10% HCl in AcOH (11 mL) and the reaction was stirred at 100°C for 2 h, then at R.T. overnight. The reaction was then diluted into 50% EtOAc in water and neutralized with solid $NaHCO_3$. The slurry was filtered and the organics collected, dried over Na_2SO_4, filtered, and concentrated. Flash chromatographic purification over silica (hexanes:EtOAc gradient), followed by prep-HPLC purification afforded 4-(2-((4-aminophenyl)amino)thiazol-4-yl)benzene-1,2-diol (**15**) as an off-white solid (21.3 mg, 3%). ^{1}H-NMR (500 MHz, d_6-DMSO) δ 10.31 (s, 1H), 9.06 (br s, 1H), 8.92 (br s, 1H), 7.78 (d, *J*=8.8 Hz, 2H), 7.31 (d, *J*=1.9 Hz, 1H), 7.26 (d, *J*=8.2 Hz, 2H), 7.20 (dd, *J*=2.2, 8.2 Hz, 1H), 7.00 (s, 1H), 6.77 (d, *J*=8.2 Hz, 1H); LC-MS [MH]$^+$ expected = 300.1 ($C_{15}H_{14}N_3O_2S$), observed = 300.0; HPLC: 99% pure.

2,3-Di(furan-2-yl)-6-nitroquinoxaline (39).[12-14]



1,2-Diamino-4-nitrobenzene (1.57 g, 10.3 mmol) and 2,2'-furil (1.93 g, 10.2 mmol) were refluxed together in EtOH (40 mL) for 4 h. The reaction was then cooled over ice and the precipitate filtered, rinsed with ice-cold EtOH and hexanes, collected, and dried to afford 2,3-di(furan-2-yl)-6-nitroquinoxaline (**39**) as an orange solid (2.54 g, 81%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 8.84 (d, *J*=2.2 Hz,, 1H), 8.51 (dd, *J*=2.6, 9.2 Hz, 1H), 8.28 (d, *J*=9.2 Hz, 1H), 7.97-8.02 (m, 2H) 6.92 (dd, *J*=0.7, 3.4 Hz, 1H), 6.87 (dd, *J*=0.7, 3.5 Hz, 1H), 6.77 (dt, *J*=1.6, 3.4 Hz, 2H); LC-MS [MH]$^+$ expected = 308.1 ($C_{16}H_{10}N_3O_4$), observed = 308.0; HPLC: 96% pure.

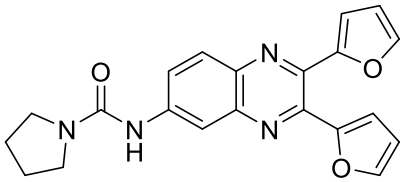
2,3-Di(furan-2-yl)quinoxalin-6-amine (40).



Tin powder (2.83 g, 23.8 mmol) was added portion-wise to a stirring mixture of compound **39** (2.40 g, 7.81 mmol) in 10% HCl in AcOH (22 mL) and the reaction was stirred at R.T. overnight. The reaction was then diluted into 50% EtOAc in water and neutralized with solid $NaHCO_3$. The slurry was filtered and the organics collected, dried over Na_2SO_4, filtered, and concentrated. Flash chromatographic purification over silica (hexanes:EtOAc gradient) afforded 2,3-di(furan-2-yl)quinoxalin-6-amine (**40**) as

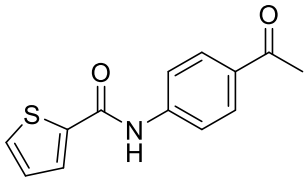
a brown-orange solid (996 mg, 46%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 7.78-7.85 (m, 2H), 7.75 (d, *J*=9.0 Hz, 1H), 7.26 (dd, *J*=2.4, 9.0 Hz, 1H), 6.91 (d, *J*=2.3 Hz, 1H), 6.64 (ddd, *J*=1.2, 1.8, 3.4 Hz, 2H), 6.53 (dd, *J*=0.8, 3.4 Hz, 1H), 6. 50 (dd, *J*=0.7, 3.4 Hz, 1H), 6.29 (s, 2H); LC-MS $[MH]^+$ expected = 278.1 ($C_{16}H_{12}N_3O_2$), observed = 278.1; HPLC: 88% pure.

_N_-(2,3-Di(furan-2-yl)quinoxalin-6-yl)pyrrolidine-1-carboxamide (23).[12-14]



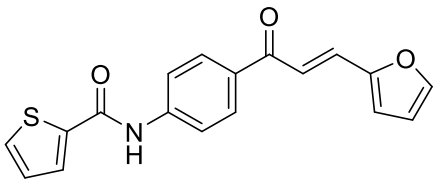
Triethylamine (0.11 mL, 0.79 mmol) was added to a stirring mixture of compound **40** (72.2 mg, 0.261 mmol) and triphosgene (29.1 mg, 0.0981 mmol) in anhydrous DCM (5 mL), and the reaction was stirred at R.T. (under Ar). After 2 h, pyrrolidine (65.0 μL, 0.79 mmol) was added and the reaction stirred for an additional 4 h. Flash chromatographic purification over silica (hexanes:EtOAc gradient), followed by prep-HPLC purification afforded *N*-(2,3-di(furan-2-yl)quinoxalin-6-yl)pyrrolidine-1-carboxamide (**23**) as an orange solid (34.2 mg, 93%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 8.72 (s, 1H), 8.36 (d, *J*=2.0 Hz, 1H), 8.02 (dd, *J*=2.3, 9.2 Hz, 1H), 7.95 (d, *J*=9.0 Hz, 1H), 7.85-7.89 (m, 2H), 6.62-6.72 (m, 4H), 3.42-3.50 (m, 4H), 1.86-1.93 (m, 4H); LC-MS $[MH]^+$ expected = 375.2 ($C_{21}H_{19}N_4O_3$), observed = 375.1; HPLC: 98% pure.

_N_-(4-Acetylphenyl)thiophene-2-carboxamide (41).



Pyridine (0.39 mL, 4.8 mmol) was added to a stirring mixture of 2-thiophenecarbonyl chloride (0.43 mL, 4.0 mmol) and 2-aminoacetophenone (537 mg, 3.97 mmol) in anhydrous DCM (8 mL) and the reaction was left to stir at R.T. (under Ar). After 4 h, the reaction was extracted into EtOAc and the organics were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated. Flash chromatographic purification over silica (hexanes:EtOAc gradient) afforded *N*-(4-acetylphenyl)thiophene-2-carboxamide (**41**) as a pale yellow solid (784 mg, 81%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 10.54 (s, 1H), 8.08 (dd, *J*=1.1, 3.7 Hz, 1H), 7.95-8.01 (m, 2H), 7.86-7.93 (m, 3H), 7.25 (dd, *J*=3.8, 5.0 Hz, 1H), 2.55 (s, 3H); LC-MS $[MH]^+$ expected = 246.1 ($C_{13}H_{12}NO_2S$), observed = 246.0; HPLC: 99% pure.

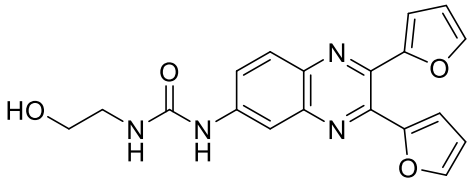
(_E_)-_N_-(4-(3-(Furan-2-yl)acryloyl)phenyl)thiophene-2-carboxamide (24).[15, 16]



Compound **41** (0.145 mg, 0.591 mmol) and 2-furaldehyde (54.0 μL, 0.652 mmol) were refluxed in a mixture of EtOH (8 mL) in 1 M NaOH (12 mL). After 4 h, the reaction was cooled on ice and the precipitate filtered, rinsed with ice cold EtOH, collected, and dried. Flash chromatographic purification

over silica (hexanes:EtOAc gradient) afforded (*E*)-*N*-(4-(3-(furan-2-yl)acryloyl)phenyl)thiophene-2-carboxamide (**24**) as a yellow solid (140 mg, 73%). ^{1}H-NMR (500 MHz, d_6-DMSO) δ 10.54 (s, 1H), 8.07-8.14 (m, 3H), 7.90-7.97 (m, 4H), 7.53-7.62 (m, 2H), 7.26 (dd, *J*=3.8, 5.0 Hz, 1H), 7.10 (d, *J*=3.5 Hz, 1H), 6.70 (dd, *J*=1.7, 3.5 Hz, 1H), 3.86 (s, 3H); LC-MS $[MH]^+$ expected = 324.1 ($C_{18}H_{14}NO_3S$), observed = 324.0; HPLC: 96% pure.

1-(2,3-Di(furan-2-yl)quinoxalin-6-yl)-3-(2-hydroxyethyl)urea (25).[12-14]



Triethylamine (0.29 mL, 2.1 mmol) was added to a stirring mixture of compound **40** (191 mg, 0.691 mmol) and triphosgene (88.8 mg, 0.299 mmol) in anhydrous DCM (7 mL), and the reaction was stirred at R.T. (under Ar). After 2 h, ethanolamine (0.21 mL, 3.5 mmol) was added and the reaction stirred for an additional 4 h. Flash chromatographic purification over silica (hexanes:EtOAc gradient), followed by prep-HPLC purification, afforded 1-(2,3-di(furan-2-yl)quinoxalin-6-yl)-3-(2-hydroxyethyl)urea (**25**) as a red-orange powder (74.5 mg, 68%). ^{1}H-NMR (300 MHz, d_6-DMSO) δ 9.26 (s, 1H), 8.26 (d, *J*=2.2 Hz, 1H), 7.96 (d, *J*=9.1 Hz, 1H), 7.84-7.89 (m, 2H), 7.72 (dd, *J*=2.3, 9.1 Hz, 1H), 6.67-6.71 (m, 2H), 6.63-6.67 (m, 2H), 6.47 (t, *J*=5.6 Hz, 1H), 4.81 (br s, 1H), 3.45-3.53 (m, 2H), 3.18-3.26 (m, 2H); LC-MS $[MH]^+$ expected = 365.1 ($C_{19}H_{17}N_4O_4$), observed = 365.1; HPLC: 98% pure.

Table S6: Compound characterization data.

#	HPLC Purity	MS Formula	MS Expected	MS Observed
1	99%	$C_{27}H_{25}N_4O_4S_2$ $[MH]^+$	533.1	533.0
5	99%	$C_{16}H_{10}NO_4S_2$ $[M-H]^-$	344.0	343.9
8	99%	$C_{20}H_{10}Br_2ClN_2O_2S_2$ $[M-H]^-$	566.8	566.6
9	99%	$C_{20}H_{15}N_2O_5$ $[MH]^+$	363.1	363.0
10	99%	$C_{19}H_{15}N_2O_5$ $[M-H]^-$	351.1	351.0
11	99%	$C_{15}H_{10}NO_5$ $[M-H]^-$	284.1	284.0
14	85%	$C_{18}H_{15}N_4O_3$ $[MH]^+$	335.1	335.0
15	99%	$C_{15}H_{14}N_3O_2S$ $[MH]^+$	300.1	300.0
18	99%	$C_{16}H_{14}N_3O_3$ $[MH]^+$	296.1	296.1
19	79%	$C_{16}H_{10}NO_4S_2$ $[M-H]^-$	344.0	343.9
20	92%	$C_{16}H_{12}ClN_2O_3$ $[MH]^+$	315.1	315.0
23	98%	$C_{21}H_{19}N_4O_3$ $[MH]^+$	375.2	375.1
24	96%	$C_{18}H_{13}NO_3S$ $[M-H]^-$	324.1	324.0
25	98%	$C_{19}H_{17}N_4O_4$ $[MH]^+$	365.1	365.1
27	94%	$C_{20}H_{19}N_2O_4S_2$ $[MH]^+$	415.1	415.0
28	78%	$C_7HBr_2O_3S_2$ $[M-H]^-$ carbamate fragmented	322.8	322.6
29	99%	$C_{22}H_{14}N_3O_6$ $[M-H]^-$	416.1	416.0
31	99%	$C_{18}H_{16}N_3O_3$ $[MH]^+$	322.1	322.1
32	99%	$C_{20}H_{17}N_4O_4$ $[MH]^+$	377.1	377.0
33	98%	$C_{17}H_{15}BrNO_4$ $[MH]^+$	376.0	375.9
34	84%	$C_{24}H_{22}FN_4O_4S$ $[MH]^+$	481.1	481.0
35	97%	$C_{16}H_{14}N_3OS_2$ $[MH]^+$	328.1	328.0

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