Supporting Information

Novel Inhibitors of Toxin HipA Reduce Multidrug Tolerant Persisters

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Content:

Supplementary Experimental Materials and Methods

Experimental Materials

Phusion high-fidelity DNA polymerase, NcoI and NotI restriction endonucleases, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Mutations were constructed using the QuikChange site-directed mutagenesis kit (SBS Genetech, Beijing, China). Ampicillin, kanamycin, isopropyl-thiogalactoside (IPTG), phenylmethanesulfonyl fluoride (PMSF), and dimethyl sulfoxide (DMSO) were purchased from Amresco (Solon, OH). Penicillinase was purchased from Sigma−Aldrich (St. Louis, MO). Compounds selected for experiments were obtained from the SPECS and ChemDiv compound databases. Compound purity was >90%, confirmed by the supplier using NMR, LSMS, or both (data available on the supplier websites). Consumables for surface plasmon resonance (SPR) assays were purchased from GE Healthcare Biacore (Uppsala, Sweden). All water used in this study was purified using a Millipore Milli-Q Reagent Water System (Billerica, MA).

Molecular Cloning, Protein Expression, and Purification

As HipA is toxic to *E. coli* and can't be expressed without HipB,[1] we constructed its mutant D309Q which exhibit no toxicity and still binds ATP with comparable affinity as the wild-type.[2] The primers for HipA amplified from *E. coli* genome were HipA-NcoI-F and HipA-NotI-R (Supplementary Table S1). The amplicon was double-enzyme-digested with restriction endonucleases NcoI and NotI. The resulting fragments were ligated into the pET28a(+) vector using T4 DNA ligase to construct the pET28a-HipA plasmid. The pET28a-HipA plasmid was then introduced with D309Q mutant using the primers HipA(D309Q)-F and HipA(D309Q)-R by use of QuikChange site-directed mutagenesis kit, which produced the pET28a-HipA(D309Q) plasmid. Each plasmid was verified by DNA sequencing (Biomed, Beijing, China).

Supplementary Table S1. Primers used to amplify wild type and mutant *lon* domains and *hipA* for molecular cloning.

Name	Sequence
HipA-NcoI-F	5' – CATG***CCATGG***[a]CGATGCCTAAACTTGTCAC − 3'
HipA-NotI-R	5' − ATAAGAAT***GCGGCCGC***CTTACTACCG − 3'
HipA(D309Q)-F	5' − GGTTGATTGGCGCAACGCAGGGTCATGCAAAAAACTTCTCC − 3'
HipA(D309Q)-R	5' − GGAGAAGTTTTTTGCATGACCCTGCGTTGCGCCAATCAACC − 3'

[a]The cleavage sites of restriction endonucleases are in bold-italic font.

The plasmids pET28a-HipA(D309Q) was transformed into *E. coli* strain BL21 <DE3> for protein expression. Overnight cultures were inoculated 1:100 into 1 L Luria−Bertani (LB) medium containing 50 μg/mL kanamycin. Cells were incubated at 37 ℃ until the optical density at 600 nm (OD_{600}) reached 0.6−0.8, at which point 0.5 mM IPTG was added to induce protein expression. Cells were then incubated for 6 hours at 30 ℃, subsequently harvested by centrifugation at 6000 rpm for 10 minutes at 4 ℃, and lysed by sonication in buffer A (50 mM Tris−HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole) with 1 mM PMSF. The supernatant was collected by centrifugation at 17,000 rpm for 30 minutes at 4 °C, and was then applied to a nickel−nitrilotriacetic column (HisTrap HP; GE Healthcare Biacore, Uppsala, Sweden). After the column was equilibrated with buffer A, the proteins were eluted with a 0−100% gradient of buffer B (50 mM Tris−HCl, pH 7.5, 300 mM NaCl, 500 mM imidazole). The target protein, confirmed by sodium dodecylsulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and western blot analyses, was concentrated and loaded onto a gel-filtration column (Sephacryl S-200 HR, GE Healthcare) pre-equilibrated with buffer C (buffer A without imidazole). Protein concentrations were measured with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA).

Persister Assays and the EC_{50}s of the active compounds

Persister assays were performed adapted from Korch *et al*[3] with the following modifications: *E. coli* strain K-12 MG1655 stored at -80 ℃ was inoculated 1:1000 into LB medium until the OD_{600} reached 0.50 to 0.55. Then, 78 μL of culture was transferred into a 2 mL EP tube and 2 μL DMSO stock solution was added. Three replicate cultures were prepared. After 8 hours of incubation (when the cultures entered the stationary phase), cells were diluted $1:10^3$ in sterile phosphate-buffered saline (PBS, 20 mM phosphate buffer, 137 mM NaCl, and 2.7 mM KCl; pH 7.4) and spread onto LB plates containing 100 μg/mL ampicillin. After cultivated for 16 hours at 37 ℃, 20 U penicillinase (Sigma−Aldrich, St. Louis, MO) was sprayed onto the plate to hydrolyze the ampicillin. After an additional 24 hours, the recovered colonies, defined as persister cells, were counted. The number of colony-forming units per milliliter (CFUs/ml) of persister cells out of the initial cell population was calculated as the persister fraction. The percentage survival was calculated by normalizing to the control culture, which was set at 100%. The half-effective concentration (EC_{50}) was obtained by fitting the percentage survival to the Hill model using Origin 8.0.

Persister screened with kanamycin was as follows, according to the results of Keren *et al*[4]: 2 μL different concentrations of DMSO stocked compounds were added to 78 μL cultures once the OD_{600} reached 0.50 to 0.55. Kanamycin was added to the final concentration of 50 μg/mL at the same time to kill non-persister cells. After 8 hours of incubation at 37 ℃, putative persisters were harvested and diluted $1:10^3$ and spread onto LB plates. Percentage survival was calculated as described above.

Structural based Virtual Screening

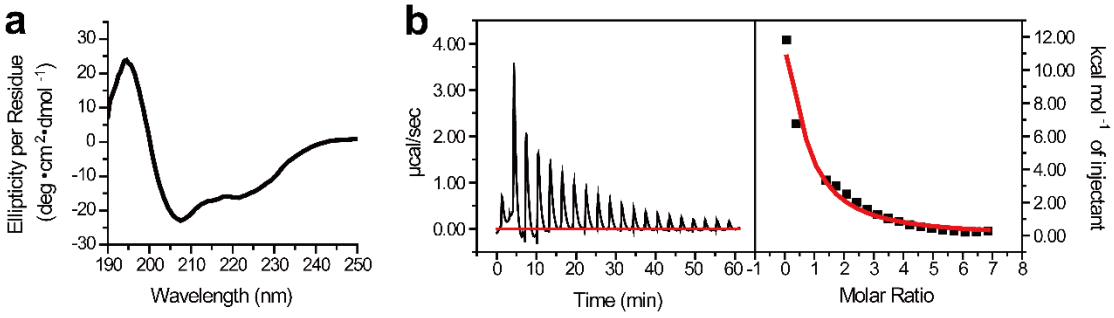
The crystal structure of *E. coli* HipA D309Q mutant (PDB code: 3FBR)[2] was used for potential inhibitor discovery. First, using Pymol (Schrödinger LLC, New York, NY), residue Q309 in 3FBR was mutated back to aspartate. Then, using the program Glide (Schrödinger, New York, NY), the ATP analogue was selected as the center of a grid generated using the default parameters, followed by standard precision ligand-docking, also performed using default parameters. Two compound libraries, the Chemdiv kinase library (26,478 kinase inhibitors and analogues) and the SPECS compounds library (November 2009 version, 201,007 compounds) were used to screen for potential inhibitors. After standard precision-mode docking, the top 2,000 compounds from Chemdiv and the top 20,000 from SPECS with the lowest docking scores were selected for extra-precision ligand docking. The top 20% from each library were then manually evaluated according to the following criteria: (a) molecular weight >300 Da; (b) at least three hydrogen bonds between the small molecule and HipA; and (c) π-π stacking or hydrophobic interaction between the molecule and Phe236/Tyr331 of HipA. At last, we purchased 30 compounds from Chemdiv kinase library and 160 compounds from SPECS compounds library for experimental tests.

Surface Plasmon Resonance (SPR) Assays

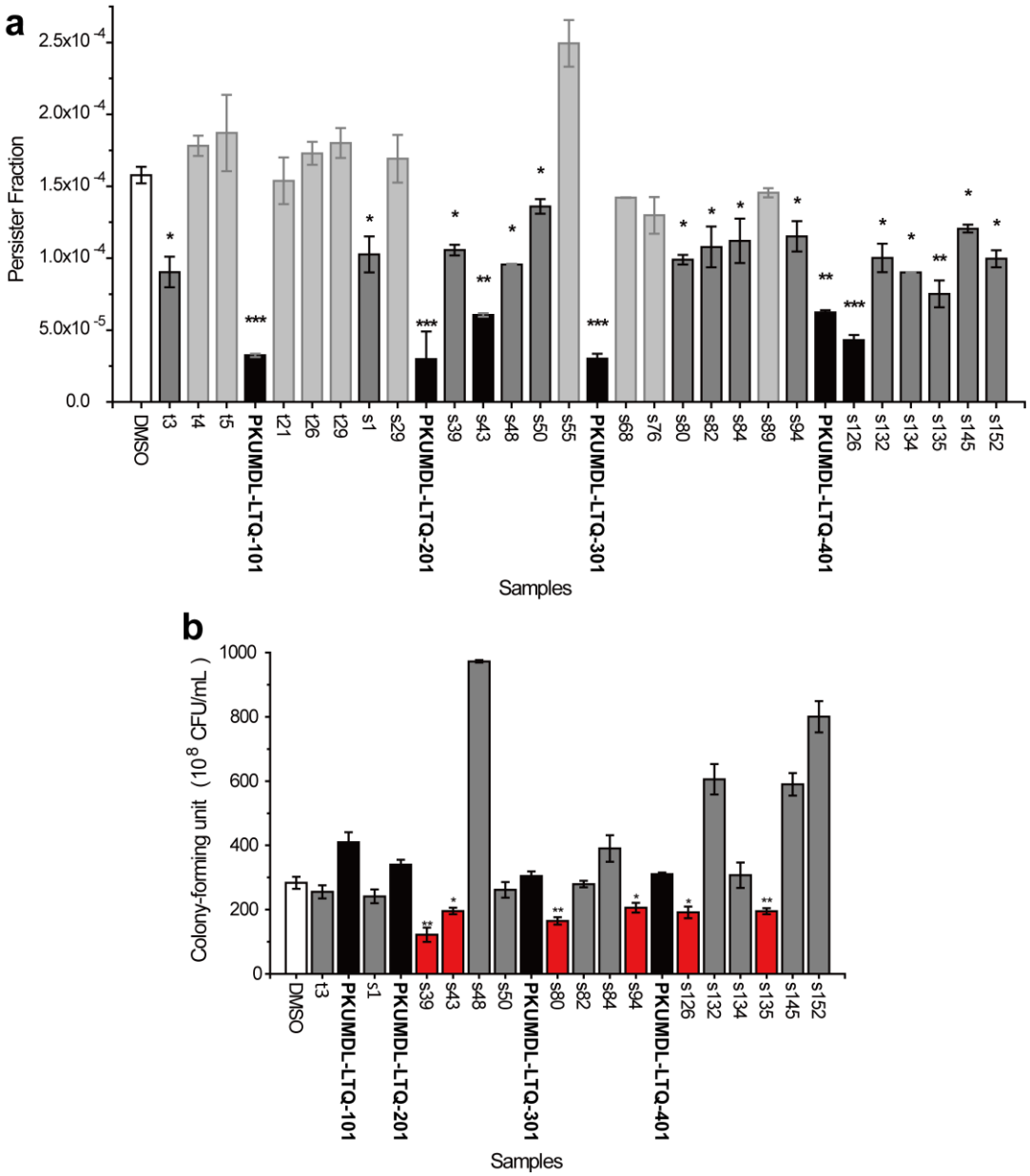
The SPR-based Biacore T200 instrument was used to exclude false-negatives. First, HipA(D309Q) was diluted to 10 ng/μL in 10 mM filtered sodium acetate buffer (pH 4.5). Then, the protein was immobilized on a CM5 sensor chip using the standard amine-coupling methods with running buffer HBS-P (20 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, and 0.05% surfactant P-20; pH 7.4). The immobilization level for false-negative exclusion was fixed at 4000 response units (RUs). The 190 selected compounds stored in DMSO were diluted in 1.05 × HBS-P buffer to a final DMSO concentration of 5%. Then the compounds were diluted in HBS-P buffer containing 5% DMSO to the final concentration of 100 μM. The samples were subsequently injected into the channel serially. The results were evaluated using Biacore T200 Evaluation Software. Molecules with RUs >5 after molecular weight (MW)-normalization were selected for further evaluation.

Using the SPR-based Biacore T200 instrument for quantitative binding-affinity experiments, we set the immobilization level of HipA(D309Q) protein at 800 RUs for most tests (i.e. compounds **2** and **4**), and at 250 RUs for the compounds with high nonspecific binding or low solubility (i.e. compounds **1** and **3**). The concentrations of the compounds varied from 0.15 nM to 100 μM depending on the binding affinity. Different concentrations of compounds were serially injected into the channel with the running buffer HBS-P containing 5% DMSO. The results were evaluated using the Biacore T200 Evaluation Software. Data at equilibrium were fitted with the Hill model.

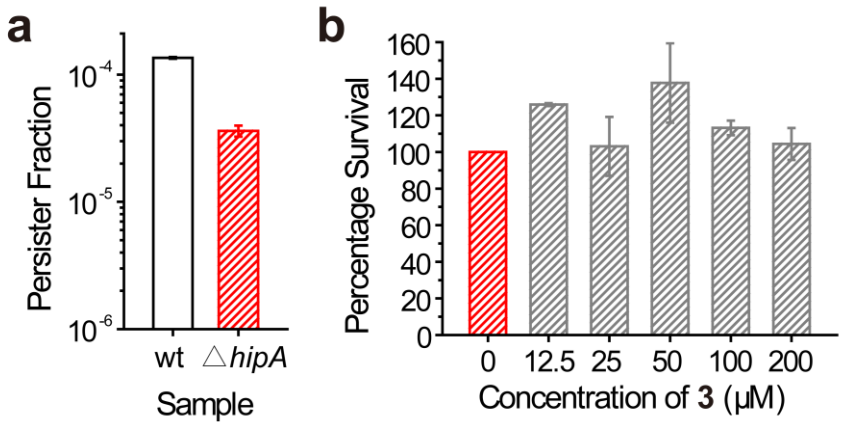
Supplementary Results



Supplementary Figure S1. HipA(D309Q) is well folded and can bind ATP. (a) CD spectrum of HipA exhibits the characteristic peak at 195 nm, 208 nm and 222 nm which confirmed that HipA was folded. (b) Raw titration calorimetry data (left) and integrated binding isotherm (right) was analyzed. The fitted thermogram of ATP binding to HipA gave a K_D of 43 ± 2 µM.



Supplementary Figure S2. The antipersistence effects of the 30 candidate HipA inhibitors. (a) The fraction of *E. coli* persisters associated with each compound. (b) The cytotoxicities of 20 candidate HipA inhibitors which reduced *E. coli* persisters in (a). White bars, sample with DMSO but no candidate inhibitors; light gray bars, sample with candidate inhibitors that did not reduce persisters; dark gray bars, sample with candidate inhibitors that reduced persisters ($p < 0.5$); black bars, sample with candidate inhibitors that reduced persisters ($p < 0.01$); red bars, sample plus candidate inhibitors that were cytotoxic at the given concentration. The concentration of all candidate inhibitors was 250 μM. **P* <0.5, ***P* <0.01, ****P* <0.001. Data are presented as the mean ± S.E.M. of three replicates.



Supplementary Figure S3. Persister assays on △*hipA* strain. (a) White bar, wild-type strain (wt) that treated with DMSO; diagonal red bar, *hipA* knockout strain (△*hipA*) that treated with DMSO. (b) The effects of different concentrations of compound **3** on △*hipA* strain. The persister fraction of △*hipA* strain treated with DMSO but no compound was set as 100% (diagonal red bar). Data are presented as the mean ± S.E.M. of three replicates.

Supplementary References

(1) Rotem, E.; Loinger, A.; Ronin, I.; Levin-Reisman, I.; Gabay, C.; Shoresh, N.; Biham, O.; Balaban, N. Q., Regulation of Phenotypic Variability by a Threshold-Based Mechanism Underlies Bacterial Persistence. *Proc. Natl. Acad. Sci. U. S. A.* **2010,** *107*, 12541-12546.

(2) Schumacher, M. A.; Piro, K. M.; Xu, W.; Hansen, S.; Lewis, K.; Brennan, R. G., Molecular Mechanisms of Hipa-Mediated Multidrug Tolerance and Its Neutralization by Hipb. *Science* **2009,** *323*, 396-401.

(3) Korch, S. B.; Hill, T. M., Ectopic Overexpression of Wild-Type and Mutant Hipa Genes in Escherichia Coli: Effects on Macromolecular Synthesis and Persister Formation. *J. Bacteriol.* **2006,** *188*, 3826-3836.

(4) Keren, I.; Shah, D.; Spoering, A.; Kaldalu, N.; Lewis, K., Specialized Persister Cells and the Mechanism of Multidrug Tolerance in Escherichia Coli. *J. Bacteriol.* **2004,** *186*, 8172-8180.