Supplemental methods

Supplemental method M1: Purification of proteins

Large-scale expression cultures of *E. coli* cells carrying expression plasmids of recombinant full-length *E. coli* GyrA, GyrB, ParC and ParE (T7 promoter, C-terminal 6xHis tag) were grown separately using standard procedures (1-6 L). When cultures reached late log-phase, the incubation temperature was lowered to 18°C and protein expression was induced by adding IPTG to 1 mM, with growth continuing overnight. Cells were collected by centrifugation, resuspended in 5X volume of IMAC buffer A [50 mM Tris-HCl, pH 7.5; 300 mM NaCl; 2 mM $MgCl_2$; 10% (v/v) glycerol; 1 mM TCEP] containing 15 mM imidazole, Benzonase® endonuclease (EMD Millipore) and protease inhibitors (cOmplete™, EDTA-free; Roche), and lysed by sonication. The total lysate was centrifuged to obtain the soluble lysate, which was applied to a column of Ni Sepharose FF (GE Healthcare Life Sciences) equilibrated with 6% (v/v) IMAC buffer B [50 mM Tris-HCl, pH 7.5; 300 mM NaCl; 2 mM $MgCl_2$; 10% (v/v) glycerol; 1 mM TCEP; 250 mM imidazole]. The column was washed with the same buffer and developed with a linear gradient to 100% (v/v) IMAC buffer B over 20 column volumes. Fractions were analyzed by SDS-PAGE and pooled accordingly, concentrated, and applied to a Superdex 200 (GE Healthcare Life Sciences) size-exclusion column equilibrated with 50mM Tris-HCl, pH 7.5; 50 mM KCl; 2 mM $MgCl_2$; 10% (v/v) glycerol; 1 mM TCEP. Fractions were pooled based on analysis by SDS-PAGE, made to 2 mM spermidine, quantified by Bradford protein assay, aliquoted, flash-frozen in liquid nitrogen, and stored at -80°C for reconstitution prior to biochemical assay. *S. aureus* DNA gyrase enzyme and human

23 topisomerase II alpha were purchased from TopoGen (Buena Vista, CO; TG2000H-2 and
24 TG2000GSA-5).
25
26 **Supplemental method M2: Biochemical assays**
27 The *E. coli* and *S. aureus* GyrAB assay buffer contained 50 mM Tris HCl pH7.5, 10 mM
28 $MgCl_2$, 30 mM KCl, 10% glycerol, 0.005% BSA, 0.005% CHAPS, 75 mM ammonium
29 acetate, 0.5 mM EDTA and 4 mM DTT. Tetramers of *E. coli* GyrA and GyrB for use in
30 the assay were prepared by incubating 10 µM of each subunit in assay buffer for 60 minutes
31 at room temperature.
32 The *E. coli* topoisomerase IV (ParCE) assay buffer contained 50 mM Tris HCl pH7.5, 10
33 mM $MgCl_2$, 50 mM KCl, 100 mM K-Glu, 1 mM EDTA, 0.01% Tween 20, 4 mM DTT and
34 0.01% BSA. Tetramers of ParC and ParE for use in the assay were prepared by incubating
35 10 µM of each subunit in assay buffer for 60 minutes at room temperature.
36 The human topoisomerase II alpha assay buffer contained 50 mM Tris HCl pH8, 10 mM
37 $MgCl_2$, 150 mM NaCl, 0.5 mM DTT and 0.003% BSA.
38 Compound stock solutions were diluted by 3.16-fold 8-point serial dilutions into a 384-
39 well low dead volume microplate using the Bravo Liquid Handler with VWorks software
40 (Agilent). A volume of 0.25 µL of each dilution was aliquoted onto 384-well
41 polypropylene assay plates using the Echo 555 liquid handler with Tempo software
42 (Labcyte). Using a Multidrop™ Combi (Thermo Fisher Scientific), 5 µL of enzyme and
43 DNA substrate (see **Table S1**) were added to a 384-well assay plate containing 0.25 µL
44 compound dilution or controls. Enzyme, DNA, and compound were pre-incubated for 30
45 minutes at room temperature. Using a Multidrop™ Combi, 5 µL of ATP substrate (**Table**

46 **S1**) was added to initiate the reactions which were incubated at room temperature according
47 to the reaction times in **Table S1**. Using the Multidrop™ Combi, the reaction was quenched
48 with 50 µL per well 0.12% SDS (final concentration of SDS was 0.1%). The quenched
49 plate was sealed on the Agilent PlateLoc and the product was analyzed using size exclusion
50 chromatography.

51
52 **Table S1**. Integration parameters for supercoiled and relaxed DNA and decatenated DNA
53 SEC separation

Assay	[Enzyme] (final)	[DNA substrate] (final)	[ATP] (final)	Reaction time (hours)
E. coli GyrAB	10 nM tetramer	40[a]	1	2
E. coli ParCE	1.25 nM tetramer	10[b]	1	1
S. aureus GyrAB	100 nM tetramer	40[a]	2	1
Human topo II alpha	1.3 nM	10[b]	1	2

54 [a]Relaxed DNA (TopoGEN TG2035-3)
55 [b]Kinetoplast DNA (TopoGEN TG2013-3)
56

57 DNA supercoiling mediated by GyrAB was measured by size exclusion chromotography
58 using an Agilent HPLC containing an 1100 well-plate autosampler (G1367A), 1100
59 autosampler chiller (G1330B), 1100 binary pump (G1312A), and a 1100 diode array
60 detector (G1315B) to acquire data. The instrument was controlled using the Agilent
61 ChemStation software (Revision B.0.03.02-SR2). A Waters Acquity BEH200 SEC column
62 (1.7 µm particle, 4.6 x 300 mm) was used to develop separation of supercoiled and relaxed
63 DNA with an isocratic flow using a mobile phase of 100 mM sodium phosphate, pH 6.8.
64 Injection volume was 10 µL and the autosampler temperature was held at 4ºC (column
65 temperature was uncontrolled). The diode detector was set for 260 nm. This yielded good
66 resolution of supercoiled and relaxed DNA (30 second shift). The peak height was used
67 to calculate percent inhibition and IC_{50} values by Helios software (1) according to a
68 previously described method (2) Guidelines for accurate EC_{50}/IC_{50} estimation.

69 Pharmaceutical Statistics; 10:128-134.). Geometric mean ± standard error of the mean
70 (SEM) were calculated using Excel software.
71 Decatenation mediated by *E. coli* ParCE or human topoisomerase II apha was measured
72 using an Agilent capillary HPLC containing a 1200 capillary pump (G1367A), 1260 micro
73 autosampler (G1377A), 1200 autosampler chiller (G1330B), and 1200 variable
74 wavelength detector (G1314B) to acquire data. The instrument was controlled using the
75 Agilent MassHunter Data Acquisition software (B.06.00, Build 6.0.6025.0). A Sepax
76 Zenix SEC guard column (3 µm particle, 4.6 x 50 mm) was used with a mobile phase of
77 100 mM sodium phosphate, pH 6.8. The separation was developed with an isocratic flow
78 of at 600 µL/min for 7 minutes per sample. The injection volume was 8 µL and the
79 autosampler temperature was held at 4ºC (column temperature uncontrolled) and the diode
80 detector was set at 260 nm. Data were analyzed and integrated using Agilent MassHunter
81 Qualitative Analysis software (B.06.00, build 6.0.633.0). UV chromatograms were
82 extracted and only the decatenated kDNA peak was integrated using the parameters shown
83 in **Table S2**. Manual integration was used to adjust integration in the event of additional
84 peak integration. The area under the curve of decantenated kDNA was used to calculate
85 the percent inhibition and the IC_{50} values by Helios software (1). Geometric mean ±
86 standard error of the mean (SEM) were calculated using Excel software.

87 **Table S2**. Integration parameters for concatenated and decatenated DNA SEC separation

Time (min)	Event	Value
Initial	Slope sensitivity	0.1
Initial	Peak width	0.07
Initial	Area reject	0
Initial	Height reject	0
Initial	Shoulders	Off
0	Integration	Off

Time (min)	Event	Value
0.4	Integration	On
0.75	Integration	Off

88

89 **Supplemental method M3: Preparation of *S. aureus* ATCC29213 isogenic mutants**

90 *Selection of mutants encoding GyrA (S84L) in S. aureus ATCC29213:* Mueller Hinton agar
91 (MHA) was melted then cooled to 50-60°C, and nadifloxain was added to 50 mL of media
92 to concentrations of 0.0625-0.125 µg/mL. Media containing compound was then poured
93 into large petri plates (Corning 430599, 150 mm x 25 mm) and allowed to solidify for at
94 least one hour before plating of cells. *S. aureus* strain ATCC 29213 was streaked for
95 isolation on MHA plates and incubated overnight at 37°C. The following day, cells were
96 suspended in 5 mL of cation-adjusted Mueller Hinton broth (MHIIB) and this suspension
97 was used to establish a 250 mL culture at 0.05 OD600 in a 1 L flask. The culture was then
98 shaken at 220 rpm, 37°C, until it reached log phase at 0.5-0.6 OD600. The culture was split
99 into four 50 mL conical tubes (Corning, 352070) and centrifuged at 6000 x *g* (4000 rpm,
100 Sorvall) for 15 min at 4°C. The supernatant was removed, and each pellet was suspended
101 in 1.15 mL of fresh MHIIB and combined into one 50 mL conical tube. Four hundred
102 microliters of the suspension was spread onto each compound plate and allowed to dry.
103 The plates were incubated at 37°C for up to 3 days to allow for the growth of resistant
104 colonies

105 *Selection of mutants encoding GrlA (S80Y, S80F, and E84L) in S. aureus ATCC29213:*
106 MHA was melted, cooled (50 -60°C), and norfloxacin was added to 50 mL of media to
107 concentrations of 2-16 µg/mL. Media containing compound was then poured into large
108 petri plates (Corning 430599, 150 mm x 25 mm) and allowed to solidify for at least one
109 hour before plating of cells. *S. aureus* strain ATCC29213 was streaked for isolation on

110 MHIIB plates and incubated overnight at 37°C. The following day, cells were suspended
111 in 5 mL of MHIIB; this suspension was used to establish a 250 mL culture at 0.05 OD600
112 in a 1 L flask. The culture was shaken at 220 rpm, 37°C, until it reached log phase at 0.5-
113 0.6 OD600. The culture was split into four 50 mL conical tubes (Corning, 352070) and
114 centrifuged at 6000 x *g* (4000 rpm, Sorvall) for 15 min at 4°C. The supernatant was
115 removed, and each pellet was suspended in 1.15 mL of fresh MHIIB and combined into
116 one 50 mL conical tube. Four hundred microliters of the suspension was spread onto each
117 compound plate and allowed to dry. The plates were incubated at 37°C for up to 3 days to
118 allow for the growth of resistant colonies.

119 *Selection of mutants encoding GrlA (S80Y, S80F, E84L) in S. aureus NB01001-DRL0024:*
120 MHA was melted, cooled (50-60 °C), and norfloxacin was added to 50 mL of media to
121 concentrations of 2-16 µg/m. Media containing compound was then poured into large petri
122 plates (Corning 430599, 150 mm x 25 mm) and allowed to solidify for at least one hour
123 before plating of cells. *S. aureus* strain NB01001-DLR0024 was streaked for isolation on
124 MHIIB plates and incubated overnight at 37°C. The following day, cells were suspended
125 in 5 mL of MHIIB; this suspension was used to establish a 250 mL culture at 0.05 OD600
126 in a 1 L flask. The culture was shaken at 220 rpm, 37°C, until it reached log phase at 0.5-
127 0.6 OD600. The culture was split into four 50 mL conical tubes (Corning, 352070) and
128 centrifuged at 6000 x *g* (4000 rpm, Sorvall) for 15 min at 4°C. The supernatant was
129 removed, and each pellet was suspended in 1.15 mL of fresh MHIIB and combined into
130 one 50 mL conical tube. Four hundred microliters of the suspension was spread onto each
131 compound plate and allowed to dry. The plates were incubated at 37°C for up to 3 days to
132 allow for the growth of resistant colonies.

133 *PCR amplification and sequencing of gyrA and grlA QRDRs:* The gyrA and grlA QRDR
134 regions were individually PCR amplified from spontaneous resistant mutants using the
135 primers listed in **Table S3**. The basic PCR parameters were 1 cycle at 95°C for 2 min, 55°C
136 for 30 sec, and 72°C for 2 min, followed by 35 cycles at 95°C for 10 sec, 55°C for 30 sec,
137 and 72°C for 1 min, and finishing with 1 cycle of 95°C for 10 sec, 55 °C for 30 sec, and 72
138 °C for 10 min. The PCR products were submitted for sequencing to Elim
139 Biopharmaceuticals, Incorporated (Hayward, CA) and sequencing analysis was performed
140 using Sequencher 5.0 software.

141 **Table S3**. Oligonucleotide primers

Primer/Probe Name	Sequence 5' - 3'	Sequence
S. aureus GyrA Ser84		
Forward	AGC ACG TAT CGT TGG TGA CG	*gyrA*
Reverse	CCA TCT CCA TCC ATT GAA CC	*gyrA*
S. aureus GrlA Ser80		
Forward	TCA AGT GGT AAT ACA CAC GA	*grlA*
Reverse	GCG GAT CAT TAT CGA TAC TA	*grlA*
gyrA		
Forward	GAG TGT TAT CGT TGC TCG TG	QRDR of *gyrA*
Reverse	CCA TCT CCA TCC ATT GAA CC	QRDR of *gyrA*
grlA 12-Forward	GAG TTT GGT ATG CAA GAG GA	QRDR of *grlA*
grlA 751-Reverse	ACC AGT TGG AAA ATC AGG AC	QRDR of *grlA*

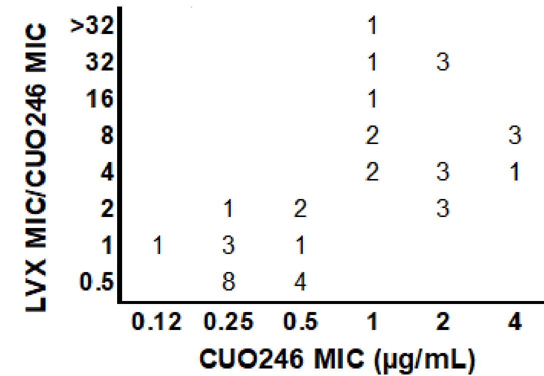
142

143 **Supplemental method M4: Antibiotic susceptibility testing for *Chlamydophila*
144 *pneunoniae***

145 *C. pneumoniae* testing was performed on HEp-2 monolayers seeded on flat glass-bottom,
146 96-well plates. HEp-2 cells infected with 10 µL of *C. pneumoniae* (ATCC 53592) EB stock
147 to obtain approximately 40% infection. Infected cells were treated with the test compounds
148 dissolved in assay medium consisted of Eagle's Minimum Essential Medium (Corning;

149 Manassas, VA) with 1.5 g/L sodium bicarbonate, nonessential amino acids, L-glutamine,
150 and sodium pyruvate supplemented with 10% fetal bovine serum (Corning) and 10 µg/mL
151 cycloheximide (Sigma-Aldrich), containing 2-fold serial dilution of antibiotics, at 35°C in
152 a humidified atmosphere with 5% CO_2 for 72 hours. *C. pneumoniae* inclusions were then
153 fixes with 100% methanol for 10 minutes before staining. A commercially available kit,
154 containing FITC-Chlamydia LPS antibody and Evan's Blue protein staining dye
155 (Pathfinder Chlamydia Culture Confirmation System, Bio-Rad, Redmond, WA) was used
156 for staining *C. pneumoniae* inclusions following the manufacturer directions. Hoechst
157 33342 DNA staining dye (Thermo Fisher Scientific) was added to each well and plates
158 were incubated for an additional 30 minutes at room temperature. Fluorescence microscopy
159 image acquisition was performed on an ImageXpress Micro XLS widefield high-content
160 analysis system (139130, Molecular Devices) using MetaXpress High Content Image
161 Acquisition & Analysis Software (Version 5.0). Images were captured at 40X
162 magnification, using the transmitted light, FITC, DAPI, and Texas Red channel settings to
163 obtain images. The total number of HEp-2 cells, infected and uninfected, was measured
164 using the image acquired on the DAPI channel. HEp-2 nuclei, stained with Hoechst DNA
165 staining dye (excitation wavelength, 350 nm; emission wavelength, 461 nm), possessing a
166 diameter between 10 to 20 µm, were counted as a cell. The total number of *C. pneumoniae*
167 inclusions was measured using the image acquired on the FITC channel. *C. pneumoniae*
168 inclusions, labeled with FITC-Chlamydia LPS antibody (excitation wavelength, 495 nm;
169 emission wavelength, 519 nm), possessing a diameter between 3 and 15 µm were counted
170 as an inclusion. Infection forming particles can form multiple inclusions in a cell. Percent
171 infection was calculated as the number of *C. pneumoniae* inclusions per one hundred HEp-

172 2 cells. This counting method did not distinguish between a single inclusion per HEp-2
173 cell, or multiple inclusions in a single cell. Specified coordinate fields were sampled, and
174 counts of HEp-2 cells and *C. pneumoniae* inclusions were used to calculate percent
175 infection per field. MIC value was defined as the lowest concentration of antibiotic that
176 yielded a sample mean of less than 10% infected cells, within a 95% confidence interval.
177
178 **Supplemental results**
179
180 **Figure R1. Scattergram of levofloxavin MIC and CUO246 MIC against *S. aureus***
181 **(n=40)**
182



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186 **Table R1**. MIC (µg/mL) of CUO246 against *S. aureus* with variations to *in vitro* testing
187 parameters
188

Test variable	CUO246 MIC (µg/mL)			
	ATCC 29213	ATCC 33591	NB01058	BAA-1717
Reference	0.25-0.5	0.25-0.5	2-4	0.25-0.5
10% human serum	0.12	0.12	1	0.12
50% human serum	0.25	0.25	2	0.25
1% surfactant	0.25	0.25	4	0.25
5% surfactant	0.25	0.25	2	0.25
Unadjusted MHB	0.5	0.25	4	0.5
CAMHB, fresh	0.12	0.12	2	0.12
5% LHB supplement	1	1	2	0.25
pH 6.4	0.5	0.5	2	0.25
pH 8.4	0.25	0.5	4	0.5
50 mg/L Ca^{2+}	0.25	0.25	4	0.5

Test variable	CUO246 MIC (µg/mL)			
	ATCC 29213	ATCC 33591	NB01058	BAA-1717
5% NaCl supplement	1	0.5	4	0.5
48 hr incubation	**2**	1	8	**2**
5% CO_2	0.25	0.25	2	0.25
Microaerobic	0.25	0.25	2	0.25
Anaerobic	0.25	0.5	2	0.25
Low inoculum	0.5	0.5	4	1
High inoculum	0.5	0.5	4	1
Aged plate inoculum	0.5	0.5	4	1
Log-phase inoculum	0.5	1	4	1

189 Bold values indicate a ≥4-fold from reference MIC values

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