

1 **Developing Cyclic Peptomers as Broad-Spectrum Gram negative Bacterial Type III**
2 **Secretion System Inhibitors**

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5 Running title: Broad-spectrum inhibitors of the Type III secretion system

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22 word count for abstract, and importance: 246 and 150, respectively

23 word count for the text: 3,384

24 **ABSTRACT**

25 Antibiotic resistant bacteria are an emerging global health threat. New antimicrobials are
26 urgently needed. The injectisome type III secretion system (T3SS), required by dozens of
27 Gram-negative bacteria for virulence but largely absent from non-pathogenic bacteria, is
28 an attractive antimicrobial target. We previously identified synthetic cyclic peptomers,
29 inspired by the natural product phepropeptin D, that inhibit protein secretion through the
30 *Yersinia* Ysc and *Pseudomonas aeruginosa* Psc T3SSs, but do not inhibit bacterial
31 growth. Here we describe identification of an isomer, 4EpDN, that is two-fold more
32 potent (IC₅₀ 4 μM) than its parental compound. Furthermore, 4EpDN inhibited the
33 *Yersinia* Ysa and the *Salmonella* SPI-1 T3SSs, suggesting that this cyclic peptomer has
34 broad efficacy against evolutionarily distant injectisome T3SSs. Indeed, 4EpDN strongly
35 inhibited intracellular growth of *Chlamydia trachomatis* in HeLa cells, which requires
36 the T3SS. 4EpDN did not inhibit the unrelated Twin arginine translocation (Tat) system,
37 nor did it impact T3SS gene transcription. Moreover, although the injectisome and
38 flagellar T3SSs are evolutionarily and structurally related, the 4EpDN cyclic peptomer
39 did not inhibit secretion of substrates through the *Salmonella* flagellar T3SS, indicating
40 that cyclic peptomers broadly but specifically target the injectisome T3SS. 4EpDN
41 reduced the number of T3SS basal bodies detected on the surface of *Y. enterocolitica*, as
42 visualized using a fluorescent derivative of YscD, an inner membrane ring with low
43 homology to flagellar protein FliG. Collectively, these data suggest that cyclic peptomers
44 specifically inhibit the injectisome T3SS from a variety of Gram-negative bacteria,
45 possibly by preventing complete T3SS assembly.

46 **IMPORTANCE**

47

48 Traditional antibiotics target both pathogenic and commensal bacteria, resulting in
49 a disruption of the microbiota, which in turn is tied to a number of acute and chronic
50 diseases. The bacterial type III secretion system (T3SS) is an appendage used by
51 many bacterial pathogens to establish infection, but is largely absent from
52 commensal members of the microbiota. In this study, we identify a new derivative of
53 the cyclic peptomer class of T3SS inhibitors. These compounds inhibit the T3SS of
54 the nosocomial ESKAPE pathogen *Pseudomonas aeruginosa* and enteropathogenic
55 *Yersinia* and *Salmonella*. The impact of cyclic peptomers is specific to the T3SS, as
56 other bacterial secretory systems are unaffected. Importantly, cyclic peptomers
57 completely block replication of *Chlamydia trachomatis*, the causative agent of
58 genital, eye, and lung infections, in human cells, a process that requires the T3SS.
59 Therefore, cyclic peptomers represent promising virulence blockers that can
60 specifically disarm a broad spectrum of Gram-negative pathogens.

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63 INTRODUCTION

64

65 Antibiotic resistance is of great concern to global public health. Bacterial pathogens have
66 evolved numerous mechanisms to survive treatment with clinically-available antibiotics
67 (1). Alternative therapies against multidrug-resistant strains of so-called ESKAPE
68 pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*
69 *pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*,
70 and *Enterobacter* species) are urgently needed. Various strategies have been explored to
71 avoid the antimicrobial apocalypse (2). One promising approach is to inhibit bacterial
72 virulence mechanisms to disarm pathogens without affecting non-pathogenic members of
73 the microbiota or environmental bacteria (3, 4). This approach has the potential to not
74 only control infection but to do so in a way that preserves the integrity of the
75 microbiome, which is beneficial for human health and is often the source of antibiotic
76 resistance genes (5, 6).

77

78 The type III secretion system (T3SS), a needle-like injectisome apparatus, is required for
79 virulence in many Gram-negative pathogens including *Salmonella*, *Yersinia*, *Chlamydia*
80 and the ESKAPE pathogen, *P. aeruginosa*. The T3SS is largely absent from commensal
81 bacteria, making it a good target for virulence blocker antimicrobials. Phylogenetic
82 analysis suggests that T3SSs evolved from the flagellar system (7, 8). Indeed, the
83 flagellar basal body is a secretion system, referred to as the flagellar T3SS, that secretes
84 flagellin and other structural components into the extracellular space in order to build the
85 flagellar filament to power motility. The flagellar and injectisome T3SS share a number

86 of conserved basal body and export apparatus components (9). However, the injectisome
87 T3SS does not mediate motility, but instead delivers effector proteins into target host
88 cells.

89

90 A number of small molecules, antibodies, and vaccines have been studied for T3SS
91 targeted therapies (10). Despite showing promising effects on the T3SS *in vitro* and in
92 animal models, only one antibody-based therapy has entered clinical trials. A bispecific
93 antibody, MEDI3902, against the *P. aeruginosa* T3SS needle tip protein PcrV and the Psl
94 exopolysaccharide is effective against both acute and chronic infection models and is in
95 phase II clinical trials for prevention of ventilator-associated pneumonia (11, 12).

96

97 As narrow-spectrum antimicrobials require more precise diagnostics, broad-spectrum
98 T3SS inhibitors would be more valuable clinically than those only able to target one
99 bacterial species. In addition, most mammalian pathogens that utilize a T3SS only require
100 their T3SS during growth within, but not outside, the host animal. However, *Chlamydiae*,
101 which cause lung, genital, and eye infections, are obligate intracellular bacteria and their
102 T3SS is strictly required for their growth (13). Interestingly, the *Chlamydia* T3SS belongs
103 to its own T3SS family (7, 8). Here we identify a derivative of a synthetic cyclic
104 peptomer family of T3SS inhibitors (14) that can inhibit the T3SS machinery of three
105 evolutionarily distinct T3SS families used by five different bacterial species to cause
106 human disease, including *Chlamydia trachomatis*.

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110 RESULTS

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112 Structure-activity relationship study of cyclic peptomers.

113 Previously we identified a group of cyclic peptomers that inhibited secretion of substrates
114 from *Y. pseudotuberculosis* and *P. aeruginosa* T3SSs, but did not inhibit bacterial
115 growth, motility, or HeLa cell metabolism (14). The results suggested a potential for
116 development of these cyclic peptomers as pathogen-specific virulence blockers. Based on
117 dose response curves and concentration of half maximal inhibition (IC₅₀) of the *P.*
118 *aeruginosa* T3SS, 1EpDN (previously named as EpD1,2N) was chosen for structure-
119 activity relationship (SAR) analysis. Compounds used in SAR analysis are listed in Table
120 1.

121

122 We first assessed the effect of alanine replacement at each of the six positions of the
123 parent scaffold, 1EpDN. Note that because peptoids have side chains appended to a
124 nitrogen atom rather than carbon as in amino acids, positions 1 and 2 were synthesized
125 with N-methylglycine, also known as sarcosine (Sar), as the peptoid equivalent of alanine
126 (Ala). Ala or Sar replacement at any of the six positions resulted in significant loss of
127 activity, suggesting that all side chains contribute to the activity (Fig. S1). Next, we
128 carried out a stereochemistry scan, in which different combinations of L- and D-amino
129 acids at positions 3 to 6 were generated. The parent compound, 1EpDN, has
130 **P**ropylamine, and **B**enzylamine at positions 1 and 2, and **D**-Leu, **L**-Ile, **L**-Leu, and **D**-Phe
131 at positions 3-6. For the stereochemistry scan, we will refer to 1EpDN as PBDLLD.

132 While most stereoisomers had the same or reduced T3SS inhibitory activity, 4EpDN
133 (PBLDD) showed improved activity, with an IC₅₀ of ~4μM compared to the parent
134 compound IC₅₀ of ~8μM (Fig. 1A-B). Replacement of position 1 (4EpDN 1Sar) or
135 position 2 (4EpDN 2Sar) with Sar significantly reduced activity of 4EpDN (Fig. 2A-B).
136 4EpDN and 4EpDN 2Sar were used as an active compound and a negative control,
137 respectively, in most follow-up experiments.

138

139 **Cyclic peptomers inhibit secretion of T3SS substrates from the Inv-Mxi-Spa T3SS** 140 **family, but does not inhibit secretion through the flagellar T3SS.**

141 Based on phylogenetic analysis of core T3SS proteins, T3SSs were classified into seven
142 families (7, 8). However, T3SSs have many highly conserved structural components (9).
143 T3SS genes are typically encoded on virulence plasmids or pathogenicity islands,
144 indicative of horizontal gene transfer (15); therefore, phylogeny of T3SSs does not follow
145 organismal phylogeny. We previously showed that cyclic peptomers inhibited the Ysc
146 T3SS family found in *P. aeruginosa* and *Yersinia* (Fig. 1-2) (14). In order to test whether
147 cyclic peptomers are active against other T3SS families, we evaluated the effect of cyclic
148 peptomers on the Inv-Mxi-Spa T3SS in *Y. enterocolitica* and *Salmonella enterica* serovar
149 Typhimurium.

150

151 The *Y. enterocolitica* Ysa system, a chromosomally encoded T3SS, is distinct from the
152 *Yersinia* Ysc T3SS and contributes to *Y. enterocolitica* colonization of the terminal ileum
153 and gastrointestinal system associated tissues (16, 17). A *Y. enterocolitica* mutant that
154 lacks expression of the Ysa T3SS ($\Delta ysaT$) was used as a negative control, while a mutant

155 lacking the Ysc T3SS ($\Delta yscL$) (18) was used to evaluate the effect of compounds
156 specifically on the Ysa system. Secretion of the Ysa effector protein YspF was
157 quantified. 4EpDN inhibited secretion of YspF in a dose dependent manner, while
158 4EpDN 2Sar did not affect its secretion (Fig. 3). Together, these results suggest that
159 cyclic peptomers are active against both the Ysc and Ysa T3SSs in *Yersinia*.

160

161 In order to evaluate whether the cyclic peptomers are active against T3SSs distinct from
162 the Ysc T3SS outside the *Yersinia* genus, we tested cyclic peptomer efficacy in
163 *Salmonella*. *Salmonella* employs two T3SSs during infection, with the SPI-1 T3SS
164 belonging to the Inv-Mxi-Spa T3SS family (7, 8). Inhibition of SPI-1 T3SS effector
165 protein SipC and SipA (19-21) secretion by 4EpDN was observed at $\sim 1 \mu\text{M}$ and ~ 1.4
166 μM , respectively, while 4EpDN 2Sar showed inhibition of SipC and SipA secretion only
167 at concentrations greater than $30 \mu\text{M}$ (Fig. 4). In order to test whether inhibition of
168 secretion by the cyclic peptomers in *Salmonella* could be affected by aggregation of the
169 compounds, we also evaluated secretion in the presence of detergent. The presence of
170 Tween-20 (0.003%, Fig. S2) did not reduce activity of 4EpDN (Fig. 4), suggesting that
171 aggregation of cyclic peptomers does not affect its activity.

172

173 As 4EpDN inhibited both the Ysc and Inv-Mxi-Spa T3SS families, we tested whether this
174 cyclic peptomer could inhibit the flagellar T3SS, which is the most distantly related T3SS
175 based on previous phylogenetic analysis (7). Conveniently, *Salmonella* expresses the SPI-
176 1 and its flagellar system under the same conditions *in vitro* (rich media). This allowed us
177 to investigate effects of cyclic peptomers on both the SPI-1 T3SS and flagellar systems

178 under the same culture conditions. Because of the conservation between the injectisome
179 and flagellar T3SSs, flagellar substrates can be secreted through both systems. Therefore,
180 secretion of flagellar substrates (FliC and FliD) was quantified in both WT and Δ SPI-1
181 strains to distinguish secretion through both the SPI-1 T3SS and flagellar system (WT
182 strain) or only through the flagellar system (Δ SPI-1 strain). 4EpDN inhibited FliC and
183 FliD secretion in WT *Salmonella* at concentrations of $\geq 60 \mu\text{M}$ and $\geq 3.75 \mu\text{M}$,
184 respectively (Fig. S3), consistent with the ability of the SPI-1 T3SS being able to secrete
185 flagellar substrates. However, 4EpDN only inhibited FliC and FliD secretion at high
186 concentrations ($\geq 60 \mu\text{M}$) in the Δ SPI-1 mutant, with unfavorable dose response curves
187 compared to WT *Salmonella*. This suggests that the inhibitory effect of 4EpDN on FliD
188 secretion in the WT strain was mainly through inhibition of its secretion through the SPI-
189 1 T3SS. 4EpDN 2Sar had no significant effect on FliC secretion or FliD secretion. These
190 data suggest that the cyclic peptomer 4EpDN does not significantly inhibit substrate
191 secretion through the flagellar T3SS in *Salmonella* but strongly inhibits the SPI-1 T3SS
192 under the same conditions.

193

194 **Cyclic peptomers affect the T3SS basal body.**

195 The T3SS basal body must be assembled prior to T3SS substrate secretion (22-24). In
196 *Yersinia*, the T3SS basal body component YscD is an inner ring protein that is conserved
197 among injectisome T3SSs, but has low sequence homology with the flagellar ortholog
198 FliG (9). Absence of YscD at the inner membrane prevents assembly of other T3SS
199 machinery (YscL, YscK, YscQ) (23, 25), and secretion of T3SS substrates (26). We used
200 a *Y. enterocolitica* strain expressing a YscD allele translationally fused with EGFP to

201 visualize the effect of compounds on YscD assembly (23). 4EpDN caused reduction in
202 the number of YscD puncta, although not to the levels seen under non-T3SS inducing
203 conditions (high Ca^{2+}), while the inactive isomer 4EpDN 2Sar had no effect (Fig. 5).
204 These data suggest that cyclic peptomers affect the assembly or stability of the T3SS
205 basal body, ultimately dampening secretion of effector proteins.

206

207 **Cyclic peptomers do not inhibit T3SS gene expression.**

208 As cyclic peptomers inhibit substrate secretion and basal body assembly on the bacterial
209 membrane, we evaluated the effect of cyclic peptomers on T3SS gene expression, as this
210 step occurs prior to T3SS assembly. Interestingly, 1EpDN did not inhibit transcription of
211 *exoT* while phenoxyacetamide MBX1641 did (Fig. 6A). Expression of the *dsbA* gene,
212 whose product was shown to be required for expression of the T3SS (27), was unchanged
213 following either cyclic peptomer or the phenoxyacetamide treatment (Fig. 6A). Similarly,
214 4EpDN did not affect expression of the effectors *exoS* and *exoT* in a strain of *P.*
215 *aeruginosa* lacking all known efflux pumps (28) (Fig. 6B). In contrast, MBX1641
216 strongly reduced expression of both *exoS* and *exoT* in this strain. These data suggest that
217 cyclic peptomers do not block T3SS activity by blocking T3SS expression.

218

219 In *P. aeruginosa*, secretion and transcription are coupled through a negative regulator
220 ExsE (29). Secretion of ExsE allows transcription of T3SS genes by the *Pseudomonas*
221 regulator ExsA (29). We evaluated the impact of cyclic peptomers on secretion of ExsE
222 and the effector ExoS by Western Blot. Both 1EpDN and 4EpDN significantly reduced
223 the amount of secreted ExoS, with a concomitant accumulation of ExoS in the bacterial

224 cytosol (Fig. 7). In contrast, we did not observe any decrease in secreted ExsE and could
225 not detect any cytosolic ExsE (data not shown). These data suggest that cyclic peptomers
226 block effector protein secretion but not secretion of T3SS regulators.

227

228 **Cyclic peptomers do not inhibit secretion through the Twin-arginine translocation**
229 **(Tat) system.**

230 In order to determine if cyclic peptomers inhibit the activity of secretion systems
231 completely unrelated to the T3SS, we sought to assess the impact of cyclic peptomers on
232 the Twin arginine translocation (Tat) system in *Y. pseudotuberculosis*. The Tat system
233 translocates fully folded substrates across the inner membrane, while the T3SS
234 translocates partially unfolded substrates across the inner, outer, and target host cell
235 membranes (30). To monitor Tat secretion system activity, a reporter strain expressing an
236 IPTG-inducible β -lactamase TEM-1 domain fused to the signal peptide of the SufI Tat
237 substrate (31) was constructed. Following IPTG induction, β -lactamase confers resistance
238 to the β -lactam peptidoglycan-targeting antibiotic penicillin G when the SufI- β -lactamase
239 reporter has successfully translocated into the periplasm (Fig. 8A). The presence of
240 known Tat inhibitors, Bay 11-7082 or N-phenylmaleimide (32), strongly reduced growth
241 of bacteria after four and six hours, while growth of bacterial cultures treated with cyclic
242 peptomers were similar to the DMSO control (Fig. 8B,C). These results suggested that
243 4EpDN does not inhibit the Tat secretion system.

244

245 **Cyclic peptomers block *Chlamydia* infection.**

246 Our data point to the cyclic peptomer 4EpDN being able to inhibit multiple T3SS
247 injectisome families but not other secretion systems, indicating a broad yet specific
248 activity. In order to evaluate whether this cyclic peptomer can disarm virulence, we chose
249 to examine the effect of this compound on the *Chlamydia* infection, as this pathogen
250 requires the T3SS for infection and growth within human cells. The *Chlamydial* life cycle
251 involves two major bacterial forms: the extracellular infectious Elementary Bodies (EBs),
252 and the intracellular replicative Reticulate Bodies (RBs). Upon entry, EBs discharge
253 preloaded T3SS effectors and are taken up into a membrane-bound compartment (the
254 inclusion) where they differentiate into RBs, secrete additional T3SS effectors and
255 replicate, and then re-differentiate into EBs. Initial stages of infection were assessed by
256 quantifying the number of inclusions/cell at 24 hpi in the presence of 9 μ M cyclic
257 peptomers or DMSO; a decrease in inclusion number or size suggests inhibition of
258 binding, entry, EB-RB differentiation, or replication. Production of infectious progeny,
259 which assays RB-EB re-differentiation (including production of pre-packaged effectors)
260 and/or release of EBs, was assayed by collecting EBs at 48 hpi, and infecting fresh
261 monolayers for 24 hpi, and then quantifying inclusion formation. INP0400, a known
262 T3SS inhibitor was used as a control (33). 4EpDN but not 4EpDN 2Sar decreased
263 primary inclusion formation ~50% but inhibited formation of infectious progeny ~98%,
264 without affecting host cell viability as assayed by LDH release (Fig. 9). Together, these
265 results suggest that 4EpDN may predominantly inhibit production or secretion of pre-
266 packaged *C. trachomatis* effectors rather than those made during RB replication.

267

268 **DISCUSSION**

269 In this study we further developed cyclic peptomers as T3SS inhibitors and investigated
270 their effects on various virulence mechanisms and pathogens. A newly synthesized cyclic
271 peptomer derivative, 4EpDN, exhibited an IC_{50} in the low μM range. 4EpDN inhibits
272 secretion through the T3SS of a number of pathogens including the nosocomial ESKAPE
273 pathogen *Pseudomonas aeruginosa*, enteropathogenic *Yersinia*, *Salmonella* and the
274 obligate intracellular pathogen *Chlamydia trachomatis*. 4EpDN does not inhibit secretion
275 from two other secretion systems – the flagellar T3SS and the Tat secretion system.
276 Cyclic peptomers do not inhibit T3SS gene expression, but affect localization of the
277 T3SS basal body component YscD, indicating disruption of normal T3SS assembly.
278 These data suggest that cyclic peptomers specifically inhibit the injectisome T3SS in a
279 broad spectrum of pathogens.

280

281 **Structure activity relationship analysis resulted in a T3SS inhibitor with an IC_{50} in**
282 **the low μM range.** Through alanine and stereochemistry scans, we identified 4EpDN, a
283 cyclic peptomer with an IC_{50} of 4 μM , as inhibiting secretion of T3SS effector proteins in
284 *P. aeruginosa* and 1 μM in inhibiting the *Salmonella* SPI-1 T3SS. Compared to
285 previously published T3SS inhibitors (Table 2), this low μM activity is encouraging. The
286 only published T3SS inhibitors with comparable IC_{50} are the phenoxyacetamides (MBX
287 2359 and its optimized derivatives), which inhibit *P. aeruginosa* T3SS secretion at 1-3
288 μM (28). Stereoisomers of 1EpDN showed a wide range of potencies, suggesting that
289 differences in their three dimensional structures affect their biological activity. 9EpDN is
290 a true enantiomer of 1EpDN with an IC_{50} of $\sim 13 \mu M$, a lower potency than the 1EpDN
291 parental compound of $\sim 8 \mu M$. Importantly, the activity of these isomers do not positively

292 correlate with solubility (Fig. S4), indicating that the observed activity is due to a specific
293 molecular reaction rather than a non-specific biophysical effect due to aggregation.
294 Furthermore, the presence of nonionic detergent did not affect activity of compounds
295 (Fig. 4). Moreover, 4EpDN is potent at concentrations significantly lower than its
296 solubility (Table S1). These data suggest that 4EpDN is an active cyclic peptomer with
297 specific T3SS inhibitory activity.

298

299 **Cyclic peptomers act as broad-spectrum, but specific, inhibitors of the injectisome**
300 **T3SS.** Secretion of protein substrates through the injectisome T3SS, the flagellar system,
301 and the Tat secretion system require the proton motive force (34-36). Although cyclic
302 peptomers inhibited secretion from the injectisome T3SS, they did not inhibit the Tat
303 system and only weakly inhibited flagellar substrate secretion, suggesting that the proton
304 motive force is unaffected, as we previously suggested (14), and that the cyclic
305 peptomers do not inhibit bacterial secretion in general.

306

307 The 4EpDN cyclic peptomer demonstrated efficacy against the T3SSs of *P. aeruginosa*,
308 *Y. pseudotuberculosis*, *Y. enterocolitica*, *Salmonella enterica* Typhimurium, and
309 *Chlamydia trachomatis*, with an IC₅₀ in the range of 1 μM (for the *Salmonella* SPI-1
310 T3SS) to ~16 μM (for the *Y. pseudotuberculosis* Ysa T3SS) (Table 2). Based on
311 phylogenetic analysis of core T3SS proteins, T3SSs group into seven T3SS families, five
312 of which contain T3SSs from human pathogens (37). 4EpDN has efficacy against T3SSs
313 from three of these T3SS families: the Ysc (Ysc and Psc), Inv-Mxi-Spa (SPI-1 and Ysa),
314 and *Chlamydiales*. Interestingly, the flagellar ATPase from *E. coli* falls at the root of the

315 phylogenetic tree (38), distinct from other T3SS families. As 4EpDN impacted secretion
316 through the flagellar T3SS significantly less than through the injectisome T3SS in the
317 same bacterial species and under the same culture and experimental conditions, we
318 reason that the pathway targeted by cyclic peptomers is common to all injectisome T3SSs
319 but absent from the flagellar system.

320

321 **Cyclic peptomers decrease cell envelope localization of the T3SS basal body protein**
322 **YscD and inhibit secretion of T3SS effector proteins, but do not inhibit T3SS gene**
323 **expression.** The T3SS is a complex system made of ~20 different proteins and is
324 assembled in a hierarchical manner prior to secretion of effector proteins (22, 39). Our
325 study showed that 4EpDN inhibited secretion of T3SS effector proteins in *Yersinia*,
326 *Pseudomonas*, and *Salmonella* and blocked *Chlamydial* growth, which requires
327 translocation of T3SS effector proteins in human cells. Importantly, 4EpDN did not
328 decrease expression of T3SS genes in *Pseudomonas* or *Salmonella* (Fig. 6, Fig. S5),
329 suggesting that cyclic peptomers do not act at the level of T3SS gene expression.
330 However, the T3SS of *Yersinia*, *Pseudomonas*, *Salmonella*, and *Chlamydia* share a
331 number of orthologous basal body components that, if targeted, could lead to disruption
332 of effector protein secretion. Interestingly, 4EpDN reduced localization of the inner
333 membrane ring protein YscD to the *Yersinia* cell envelope (Fig 3), indicating a
334 perturbation to T3SS assembly or stability.

335

336 Surprisingly, the effect of 4EpDN on the T3SS did not significantly impact secretion of
337 the *Pseudomonas* regulator ExsE. In *P. aeruginosa*, ExsA is the key positive

338 transcriptional regulator of genes encoding the T3SS machinery and substrates. ExsE
339 leads to sequestration of ExsA, preventing it from inducing T3SS gene expression
340 through a complex partner switching pathway (29, 40, 41). Under secreting conditions,
341 ExsE, a T3SS substrate, is secreted, enabling release of ExsA and thereby allowing
342 increased transcription of T3SS genes (29). However, we did not detect a decrease in
343 ExsE secretion in the presence of cyclic peptomers, in contrast to the marked decrease in
344 effector protein secretion. These results are consistent with the data showing that 4EpDN
345 did not inhibit T3SS gene expression in *P. aeruginosa*, as ExsE mediates elevation of
346 gene expression in response to secretion. It is not completely clear why the cyclic
347 peptomers inhibited secretion of effector substrates and ExsE differently, although the
348 small size of ExsE compared to other effectors such as ExoU (~8.7 kDa versus 74 kDa)
349 may be a factor.

350

351 **Cyclic peptomers strongly inhibit *Chlamydia* primary and secondary infection.**
352 4EpDN strongly inhibited *Chlamydia* from infecting HeLa cells during primary infection,
353 and furthermore prevented *Chlamydia* from infecting subsequent host cells (Fig. 9). This
354 highlights the potential of cyclic peptomers to prevent the spread of *Chlamydia* infection.
355 *Chlamydia* relies on its T3SS effector proteins to interact with host factors, such as the
356 actin cytoskeleton, Golgi network, endoplasmic reticulum, and microtubule network, to
357 mediate invasion and intracellular growth (42). It is possible that compounds that inhibit
358 these host pathways could interfere with *Chlamydial* growth (43-45). However,
359 microscopic analysis of many cellular structures in HeLa cells in the presence of 4EpDN
360 did not show any gross changes to the actin cytoskeleton, Golgi network, endoplasmic

361 reticulum, or microtubule network at the concentration used in our *Chlamydia* infection,
362 9 μ M (Fig. S6). *C. trachomatis* infection may cause infertility in female patients and eye
363 damage, in addition to lung infections (13). Antibiotics, such as β -lactam antibiotics, are
364 a common way to treat *Chlamydia* infection, but the chance of recurrence is high (46,
365 47). Current vaccine development efforts are underway but multiple challenges remain
366 (48). There is increased demand for drugs against *Chlamydia* due to antibiotic resistance
367 (49). The strong efficacy of cyclic peptomers highlights their potential for development
368 as an anti-*Chlamydial* drug.

369

370 MATERIALS AND METHODS

371 **Bacterial strains and culture conditions.** The bacterial strains and cell lines used in this
372 study are listed in Table 3. All cultures were grown with shaking at 250 rpm unless
373 otherwise noted. *Y. pseudotuberculosis* was grown in 2xYT (2x yeast extract and
374 tryptone) at 26°C overnight. To induce the T3SS, the cultures were subcultured to an
375 optical density at 600 nm (OD₆₀₀) of 0.2 into low-calcium medium (2xYT with 20 mM
376 sodium oxalate and 20 mM MgCl₂). *Y. enterocolitica* was grown in BHI (brain heart
377 infusion) medium at 26°C overnight. The Ysc T3SS in *Y. enterocolitica* was induced
378 using low-calcium BHI (BHI with 20 mM sodium oxalate and 20 mM MgCl₂). The Ysa
379 T3SS was induced as described previously (50) using ‘L media’ (1% Tryptone, 0.5%
380 Yeast extract) with 290 mM NaCl at 26°C. *P. aeruginosa* and *S. enterica* were grown in
381 Luria-Bertani (LB) medium overnight at 37°C. For *P. aeruginosa*, the T3SS was induced
382 using low-calcium medium (LB with 5 mM EGTA and 20 mM MgCl₂). SPI-1 T3SS
383 secretion was assessed after subculturing into fresh LB at 37°C unless noted otherwise.

384 HeLa cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM)
385 with 10% fetal bovine serum (FBS). All cell lines were incubated at 37°C with 5% CO₂.

386

387 **Preparation of bacteria for T3SS induction.** Visualization of secreted proteins was
388 carried out as described previously (25). Briefly, *Y. pseudotuberculosis*, *P. aeruginosa*, or
389 *S. enterica* was grown in T3SS-inducing medium (as described above) in the presence of
390 cyclic peptomers or an equivalent volume of DMSO at 37°C, 2 hrs for *Y.*
391 *pseudotuberculosis* Ysc T3SS, 3 hrs for *P. aeruginosa*, 4 hrs for *S. enterica*, or at 26°C
392 for 6 hrs for the *Y. enterocolitica* Ysa T3SS. The cultures were normalized to bacterial
393 density (OD₆₀₀) and then centrifuged for 15 min at 14,800 rpm. The supernatants were
394 transferred to new tubes and mixed with trichloroacetic acid (TCA) to a final volume of
395 10% by vortexing vigorously for 30 s. Samples were incubated on ice for 1 hr and then
396 spun down at 4°C for 15 min at 13,200 rpm. The supernatants were carefully removed,
397 and pelleted proteins washed with acetone and spun down at 4°C for 15 min at 13,200
398 rpm for a total of three washes. The pellet was then resuspended in final sample buffer
399 (FSB) and 20% dithiothreitol (DTT) and boiled for 15 min prior to SDS-PAGE. Tween-
400 20 was added to the bacterial culture at the same time as the compounds in *S. enterica*
401 secretion assays at 0.003% (v/v).

402

403 **T3SS secretion cargo quantification.** Image Lab software (Bio-Rad) was used to
404 quantify T3SS cargo protein bands relative to those of DMSO-treated controls. The WT
405 *Y. pseudotuberculosis* YopE, *P. aeruginosa* ExoU, or *S. enterica* SipA, SipC, FliC and
406 FliD bands in DMSO control samples were set to 1.00. To evaluate type III secretion of

407 ExoS and ExsE in *P. aeruginosa*, Western blots against T3SS cargo were carried out,
408 using a PVDF membrane (Millipore), blocking in 5% non-fat milk for 2 hrs at room
409 temperature, and incubated at 4°C overnight with gentle shaking. Blots were washed
410 three times for 5 minutes each in Phosphate-Buffered Saline with 0.1% Tween® 20
411 (PBST). Horseradish Peroxidase conjugated secondary antibody was then incubated for 1
412 hr at room temperature. Signals were detected with Luminol Kit after washing. ExoS-Bla,
413 ExsE and SipC was visualized using β -lactamase (MA120370, Fisher Scientific) (7.5%
414 gel), anti-ExsE antibody courtesy of Timothy Yahr (20% gel) and anti-SipC
415 (ABIN335178, antibodies-online Inc.)(10% gel), respectively.

416

417 **YscD visualization assay.** *Y. enterocolitica* expressing YscD-EGFP was cultured
418 overnight in BHI supplemented with nalidixic acid (35 μ g/mL) and diaminopimelic acid
419 (50 μ g/mL), at 26°C with shaking (23), followed by subculture into low calcium BHI
420 medium (20mM NaOX, 20mM MgCl₂, 0.4% Glycerol) with nalidixic acid and
421 diaminopimelic acid to OD₆₀₀ of 0.2 for 1.5 hrs. Compounds or an equivalent volume of
422 DMSO was added prior to inducing the T3SS. After 3 hrs at 37°C with shaking, cells
423 were pelleted and resuspended in PBS, spotted onto a 0.1% agarose pad, and imaged live
424 at 63X/1.4 oil magnification using a Zeiss AxioImager widefield microscope. Analysis of
425 YscD puncta was carried out in Imaris 8 using spot tracking analysis with the same
426 arbitrary threshold to call bacterial cell and puncta for all samples.

427

428 **mRNA quantification.** Overnight *P. aeruginosa* (PA103 or PA01) cultures were
429 subcultured and shifted to T3SS inducing conditions (see above) in the presence of 60

430 μM 1EpDN, 60 μM 1EpDN 2Sar, or 50 μM MBX1641. Samples were taken after 3 hrs of
431 induction. Overnight *Salmonella* cultures were subcultured into fresh LB with 0.3 M
432 NaCl at 37°C in the presence of 9 μM 4EpDN, 4EpDN 2Sar, or equivalent DMSO.
433 Samples were taken after 2 hrs and 4 hrs of induction. Samples were stored in
434 RNAprotect reagent (Qiagen) and processed within a week. Total RNA was isolated
435 using an RNAeasy Kit (Qiagen), according to the manufacturer's instructions, followed
436 by two rounds of Turbo DNase (ThermoFisher scientific) treatment. A total of 2 μg of
437 RNA was used to make cDNA and qPCR reactions were run with SYBR Green PCR
438 master mix (Applied Biosystems). DNA helicase (*dnaB*) and 16S rRNA genes were used
439 as a reference for *P. aeruginosa* and *Salmonella* samples, respectively. Two to three
440 technical replicates were averaged for each sample. Primers used are listed in Table S2.
441 Results were analyzed using Bio-Rad CFX software.

442

443 **Tat translocation assay.** To make Tat targeting constructs, plasmid pMMB67EH
444 (ATCC 37622) was digested with KpnI. TEM1 of β -lactamase was PCR amplified from
445 *yopH*-Bla (courtesy of Melanie Marketon) using primers oHL210 and oHL217 (Table
446 S2) and *sufI* signal peptide DNA was PCR amplified from genomic DNA of *Y.*
447 *pseudotuberculosis* with primers oHL218 and oHL219 (Table S2). The digested
448 pMMB67EH, TEM1 and *sufI* signal peptide DNA were assembled into a plasmid (*sufI*-
449 Bla) using Gibson assembly.

450

451 WT *Yersinia* or *tatB::Tn* carrying *sufI*-Bla was grown in 2 x YT supplemented with
452 15 $\mu\text{g}/\text{mL}$ gentamicin at 26°C with shaking. Overnight cultures were subcultured to OD₆₀₀

453 of 0.1 and grown for 1.5hr at 26°C with shaking. 5 mM IPTG was added to the culture for
454 0.5 hrs to allow for expression and translocation of SufI-Bla. Penicillin G (25µg/mL) was
455 added to the cultures. Cultures were then treated with cyclic peptomers or DMSO and
456 OD₆₀₀ measured every hour up to 8hrs.

457

458 ***Chlamydia* infection and imaging.** Primary Infections: HeLa cell monolayers were
459 infected with *C. trachomatis*, Serovar L2 at an MOI of one in the presence of one of the
460 following compounds at 9 µM: (a) DMSO, (b) 4EpDN, or (c) 4EpDN 2Sar. Cells were
461 incubated for 24 hrs in the presence of the above compounds at 37°C, and then fixed with
462 4% Paraformaldehyde (PFA). Cells were stained for IncA (*Chlamydia* inclusion
463 membrane marker), DNA, and MOMP (*Chlamydia* major outer membrane protein). The
464 percentage of cells infected (i.e. stained positively for the listed *Chlamydia* markers) in
465 the presence of the compounds was quantified using confocal microscopy. 10 randomly
466 selected fields of view were measured per experiment. Data represents three biological
467 replicates.

468

469 Secondary Infections: HeLa cell monolayers were infected with *C. trachomatis*, Serovar
470 L2 at an MOI of 1.0 in the presence of one of the following compounds at 9 µM: (a)
471 DMSO, (b) 4EpDN, or (c) 4EpDN 2Sar. Cells were incubated for 48 hrs in the presence
472 of the above compounds at 37°C. Infected cells were then lysed, and the lysate was
473 applied to fresh HeLa monolayers to enumerate infectious particles. These secondary
474 infections were fixed in 4% PFA at 24 hpi, and were stained against MOMP and DNA.
475 Infectious units per mL (IFU/mL) were calculated by averaging the number of infected

476 cells in each of 10 randomly selected fields of view at 60X magnification on a confocal
477 microscope, and multiplying this by the appropriate dilution and area factors. Data
478 represents four biological replicates.

479

480 **Cytological Profiling (CP).** Briefly, HeLa cells were cultured and seeded into 384-well
481 at 2,500 cells/well. After 48 hrs, compounds were added using a Janus MDT robot
482 (PerkinElmer). Two stain sets were used; Stain set 1: Hoechst, EdUrhodamine, anti-
483 Phosphohistone H3, and GM130, Stain Set 2: Hoechst, FITC-alpha tubulin, rhodamine-
484 phalloidin, and Calnexin. For stain set 1, cells were incubated with 20 μ M
485 EdUrhodamine for 1 hr prior to fixing in 4% formaldehyde solution in PBS for 20 min.
486 Cells were then washed with PBS and permeabilized with 0.5 % Triton-X in PBS for 10
487 min before blocking with 2 % BSA in PBS solution for at least 1 hr. Following this, cells
488 were incubated with primary antibodies overnight at 4°C. The following day, excess
489 primary antibody was washed off with PBS and Alexa-488 and Alexa-647 secondary
490 antibodies and Hoechst solution were incubated for 1 hr. Plates were washed with PBS
491 and preserved with 0.1% sodium azide in PBS solution prior to imaging. For stain set 2,
492 cells were fixed with a 4 % formaldehyde solution in PBS for 20 min. Cells were then
493 washed with PBS and permeabilized with 0.5 % Triton-X in PBS for 10 min before
494 blocking with 2 % BSA in PBS solution for at least 1 hr. Following this, cells were
495 incubated with primary antibodies overnight at 4°C. After blocking, the cells were
496 washed, and incubated with FITC conjugated anti-alpha tubulin antibody and rhodamine-
497 phalloidin overnight at 4°C. The following day the cells were washed and then incubated
498 with secondary Alexa-647 and Hoechst stain for 1 hr. Plates were washed with PBS and

499 preserved with 0.1% sodium azide in PBS solution prior to imaging.

500

501 Two images per well were captured with an ImageXpress Micro XLS automated
502 epifluorescent microscope (Molecular Devices, Sunnyvale). Images were then processed
503 as described (51). Briefly, initial image processing was performed using MetaXpress
504 image analysis software, using built-in morphometry metrics, the multiwavelength cell
505 scoring, transfluor, and micronuclei modules. Custom written scripts were used to
506 compare the treated samples with the DMSO control wells, and then to convert each
507 feature to a “histogram difference” (HD) score. This produced a 452-feature vector CP
508 fingerprint. Compound treatment wells were labeled as dead if the cell count for the
509 treatment well was < 10% of the median cell count in the treatment plate. In addition to
510 the CP fingerprint, feature cell counts (nuclei, EdU S-phase, and phospho-histone H3)
511 were used to determine effects of compounds on HeLa cell replication.

512

513 **Synthesis of cyclic peptomers**

514 **Cyclic peptide synthesis.** Peptides were synthesized using standard Fmoc solid-phase
515 peptide synthesis, utilizing the submonomer approach for peptoid synthesis (52), either at
516 room temperature or with microwave assistance. Cyclization was done in solution at a
517 high dilution. Fmoc-Xaa (10 mmol) was added to a flame-dried round-bottomed flask
518 and dried in a vacuum desiccator with phosphorous pentoxide overnight. 50 ml of dry
519 dichloromethane (DCM) was cannula transferred into the flask, followed by 2.5 ml of
520 N,N-diisopropylethylamine (DIPEA) transferred via syringe. After sonication for 10 min,
521 5g of 2-chlorotrityl resin was added under a stream of nitrogen and allowed to shake for 4

522 hrs. The resin was capped with a 15 ml solution of 1:2:17 methanol
523 (MeOH):DIPEA:dimethylformamide (DMF) (3 times for 15 min each). The resin was
524 washed with DMF (3 times with 15 ml each) followed by DCM (3 times with 15 ml
525 each). The loading value was calculated by determining the mass increase of dried,
526 loaded resin.

527

528 **Amino acid coupling at room temperature.** Four equivalents (eq) of Fmoc-Xaa, 8 eq of
529 DIPEA, and 4 eq of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-
530 b]pyridinium 3-oxide
531 hexafluorophosphate, Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
532 (HATU) were added to the resin in DMF. The reaction mixture was agitated via shaking
533 for 45 min and then drained. The resin was washed with DMF (3 times with 3 ml each)
534 and DCM (3 times with 3 ml each). The reaction was monitored by liquid
535 chromatography-mass spectrometry (LC-MS) and repeated until the starting material was
536 no longer observed. For microwave conditions, a solution of 4 eq of Fmoc-Xaa, 4 eq of
537 HATU, and 6 eq of DIPEA in DMF was allowed to prereact for 5 min. This solution was
538 added to the deprotected peptide on-resin and allowed to react for 10 min at 50°C under
539 microwave heating. The solution was drained, and the resin was washed with DMF (3
540 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by
541 LC-MS and repeated until the starting material was no longer observed.

542

543 **Coupling of BrAcOH at room temperature.** A solution of 10 eq of bromoacetic acid
544 (BrAcOH) and 5 eq of N,N'-diisopropylcarbodiimide (DIC) in DMF was allowed to

545 prereact for 10 min. This solution was added to the deprotected peptide on-resin. The
546 reaction mixture was agitated via shaking for 45 min and then drained. The resin was
547 washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The
548 reaction was monitored by LC-MS and repeated until the starting material was no longer
549 observed. The reaction was monitored by LC-MS and repeated until the starting material
550 was no longer observed.

551

552 **Peptoid side chain addition.** A solution of 5 eq of the desired amine was prepared in a
553 minimum volume of DMF. The resin containing the BrAc-peptide was swollen with
554 DCM for 5 min prior to reaction. The amine was added, and the reaction mixture was
555 agitated via shaking for 3 to 20 hrs. The solution was drained, and the resin was washed
556 with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was
557 monitored by LC-MS and repeated until the starting material was no longer observed.

558

559 **Removal of the N-Fmoc protection group at room temperature.** A solution of 2%
560 piperidine and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF was added to the
561 resin. The reaction mixture was agitated via shaking for 20 min and then drained. The
562 resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each).
563 For microwave conditions, a solution of 2% piperidine and 2% DBU in DMF was added
564 to the resin. The reaction mixture was allowed to react for 5 min at 50°C under
565 microwave heating and then drained. The resin was washed with DMF (3 times with 3 ml
566 each) and DCM (3 times with 3 ml each).

567

568 **Peptide cleavage.** Complete linear peptides were cleaved off the resin in 5 resin volumes
569 of 2.5% trifluoroacetic acid (TFA) in DCM for 4 min, three times, with a 5-resin-volume
570 DCM wash between steps. Solvent was removed under N₂, followed by dissolution in
571 acetone or DCM and evaporation under reduced pressure. Residual TFA was removed in
572 vacuo overnight.

573

574 **Cyclization with COMU.** Linear peptides were dissolved in 20 ml of dry acetonitrile
575 (ACN) with 4 eq of DIPEA and added dropwise (final concentration, 1 mg crude peptide
576 per ml) to a solution of 1:1 tetrahydrofuran (THF)-ACN containing 2 eq of (1-cyano-2-
577 ethoxy-2-oxoethylideneaminoxy) dimethylamino-morpholinocarbenium
578 hexafluorophosphate (COMU). Reaction mixtures were stirred for 0.5 to 24 hrs, until
579 complete cyclization was achieved as monitored by LC-MS. The reaction mixture was
580 reduced in vacuo for purification via high pressure liquid chromatography (HPLC).

581

582 **Purification of peptides.** COMU by-products were removed after solution-phase
583 cyclization on a Biotage Isolera Prime system equipped with a SNAP Ultra-C18 30g
584 column eluting with H₂O-acetonitrile modified with 0.1% TFA. The mass spectra of all
585 peptides are shown in Fig. S7 in the supplemental material.

586

587 **Proton NMR of peptides.** Peptides were analyzed through nuclear magnetic resonance
588 (NMR) spectroscopy measured in ppm and were obtained on a 500 MHz spectrometer
589 using CDCl₃ ($\delta=7.26$) as an internal standard for ¹H NMR. Identity of compounds for
590 SAR study was confirmed by LCMS and ¹H-NMR (Fig. S7).

591

592 **Kinetic Solubility.** A 15 mM stock of the compounds in DMSO was prepared. 125 μ L of
593 M9 and DMEM (no antibiotics) was dispensed into 96 v-bottom plate. One microliter of
594 15mM stock compound was added to make a solution of 120 μ M final concentration with
595 0.8% DMSO. The solution was shaken at 37°C for ~2 hrs. The solution was passed over a
596 0.7 μ M glass fiber filter. Then the solution was diluted 1:4 in acetonitrile to crash out any
597 proteins. The solution was centrifuged at 500 x g for 10 minutes. Avoiding the pellet, 10
598 μ L of supernatant was added to a fresh plate with 90 μ L of acetonitrile. The final dilution
599 is 40 times lower. 10 μ L of 40x dilution of solution was injected on the Orbi-trap. A 1 μ M
600 standard was used for the ratiometric comparison and the assay was done in triplicate.

601

602 **Cyclic peptide manipulation.** Stock peptides were stored at 15 mM at -70°C and were
603 prediluted in DMSO prior to experiments. All treatment and control pairs, in all assays,
604 had the same DMSO volumes.

605

606 **Statistical analysis.** GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) was
607 used to calculate the mean, standard error of the mean, median, standard error of median,
608 and one-way ANOVA values shown.

609

610 **ACKNOWLEDGEMENTS**

611 The authors acknowledge the National Institutes of Health grant R01AI141511 (to V.A.
612 and R.S.L.) and K99AI139281 (to H.L.) for support. We thank Benjamin Abrams

613 (University of California, Santa Cruz) for technical support on the YscD spot tracking
614 analysis. We thank Timothy Yahr for the anti-ExsE antibody.

615

616

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- 952
- 953

954 **Table 1: Compounds synthesized and used in this study**

955

Simplified Name	Full Name/ side chain identity (1-6)	Exact Mass	Reference
1EpDN	EpD-1,2N / Propylamine, Benzylamine, D-Leu, L-Ile, L-Leu, and D-Phe (PBDLLD)	732.46	(14)
1EpDN 1Sar	EpD1,2N 1Sar / Sarcosine, Benzylamine, D-Leu, L-Ile, L-Leu, and D-Phe (SarBDLLD)	704.43	This study
1EpDN 2Sar	EpD1,2N 2Sar / Propylamine, Sarcosine, D-Leu, L-Ile, L-Leu, and D-Phe (PSarDLLD)	656.43	This study
1EpDN 3Ala	EpD-1,2N 3Ala / Propylamine, Benzylamine, D-Ala, L-Ile, L-Leu, and D-Phe (PB,D-Ala,LLD)	690.41	This study
1EpDN 4Ala	EpD-1,2N 4Ala / Propylamine, Benzylamine, D-Leu, L-Ala, L-Leu, and D-Phe (PBD,L-Ala,LD)	690.41	This study
1EpDN 5Ala	EpD-1,2N 5Ala / Propylamine, Benzylamine, D-Leu, L-Ile, L-Ala, and D-Phe (PBDL,L-Ala,D)	690.41	This study
1EpDN 6Ala	EpD-1,2N 6Ala / Propylamine, Benzylamine, D-Leu, L-Ile, L-Leu, and D-Ala (PBDL,L,D-Ala)	656.43	This study
2EpDN	2-EpD1,2N / Propylamine, Benzylamine, L-Leu, L-Ile, L-Leu, and D-Phe (PBLLLD)	732.46	This study
3EpDN	3-EpD1,2N / Propylamine, Benzylamine, D-Leu, L-Ile, D-Leu, and D-Phe (PBDLDD)	732.46	This study
4EpDN	4-EpD1,2N / Propylamine, Benzylamine, L-Leu, L-Ile, D-Leu, and D-Phe (PBLDD)	732.46	This study
5EpDN	5-EpD1,2N / Propylamine, Benzylamine, L-Leu, L-Ile, D-Leu, and L-Phe (PBLDDL)	732.46	This study
6EpDN	6-EpD1,2N / Propylamine, Benzylamine, D-Leu, L-Ile, D-Leu, and L-Phe (PBDLDDL)	732.46	This study
7EpDN	7-EpD1,2N / Propylamine, Benzylamine, L-Leu, L-Ile, L-Leu, and L-Phe (PBLLLL)	732.46	This study
8EpDN	8-EpD1,2N / Propylamine, Benzylamine, D-Leu, L-Ile, L-Leu, and L-Phe (PBDLLL)	732.46	This study
9EpDN	9-EpD1,2N / Propylamine, Benzylamine, L-Leu, D-Ile, D-Leu, and L-Phe (PBLDDL) (Enantiomer of 1EpDN)	732.46	This study
4EpDN 1Sar	4-EpD1,2N 1Sar / Sarcosine, Benzylamine, L-Leu, L-Ile, D-Leu, and D-Phe (SarBLLDD)	704.43	This study
4EpDN 2Sar	4-EpD1,2N 2Sar / Propylamine, Sarcosine, L-Leu, L-Ile, D-Leu, and D-Phe (PSarLLDD)	656.43	This study

956

957

958

959 **Table 2: Efficacy of cyclic peptomers and other type III secretion system inhibitors.**

960

Species/T3SS family ^a	PA	Ysc	Ysa	SPI-1	EPEC /EHEC	<i>Chlamydia</i>	PS	fliC	Ref.
	Psc/Ysc		Inv-Mxi-Spa		Ssa-Esc	Chlamydiales	Hrc1	fla	
4EpDN	3.9 ExoU ^b	~7.5 YopE ^b	16.1 YspF ^b	1 SipAC ^b		~9 ^c		NE ^d	This study
4EpDN 2Sar	139.5 ExoU ^b			~30 SipAC ^b		X		NE ^d	This study
1EpDN	8.2 ExoU ^b	14.3 YopE ^b							(14)
MBX1641 Phe [*]	10 ExoS ^b					~10 ^c			(53)
MBX2359 Phe	2.5 ExoS ^b								(28)
MBX2401 Phe	1.2 ExoS ^b								(54)
Hydroxybenzimidazoles	~3.5 ExsA ^e	~3.9 LcrF ^c							(54)
INP1750 HQ [*]	~80 ^c	12.4 YopEe EC ₅₀				25 MIC ^c		~80	(55, 56)
INP1767 HQ		14.6 YopE ^c EC ₅₀				12.5 MIC ^c			(55)
INP1855 HQ	~60 ^c	6.3 YopE ^c EC ₅₀				3.13 MIC ^c		~30	(55, 57)
INP0341 SAH [*]	~80 ^c					~20 ^c			(56, 58)
INP0400 SAH						~20 ^c			(58)
INP0403 SAH				~100 SipAC ^b					(59, 60)
INP0007 SAH		~50 YopE ^b		~100 SipAC ^b					(60, 61)
C2, C4 SAH		~20; ~5 Yops ^b							(62)
ME0052 SAH		~20 Yops ^b							(63)
INP0010 SAH C1 SAH		~50 YopE ^b ~50 Yops ^b							(64)
ME0055 SAH (INP0031)					~20 LEE genes ^e				(65)
RCZ12 SAH RCZ20 SAH					~25 EspD ^b				(66)
INP0401 SAH 5277768 SAH							~50 hrp ^e		(67)
Compound 3	13 ExoS ^b	6 YopE ^b							(53)
C20	~60 ^c	~60 YopE ^f							(68)
Compound D	~60 ExoU ^c	~60 YopE ^b							(69)
Salicylideneanilide					15 EspB ^b				(70)

Piercidin A1, Mer-A 2026B		~36; ~9 YopM ^f						(71)
N-arylbenzylamines						~50 IncA ^f		(72)
Baicalein Flavonoid				3.6 SopE2 ^b		0.5mM ^c		(73, 74)
Licoflavonol				~50 SipC ^b				(75)
Epigallocatechin gallate		~16µg/ml 1 Yops ^b		~12µg/ml Sips ^b	~16µg/ml EspB ^b			(76)
Sanguinarine chloride				~5 SipA ^{bf}				(77)
Obovatol				19.8 ^c				(78)
Thymol				~0.2 mM SipA ^f				(79)
TTS29 thiazolidinone		~380 Yops ^b	~380 Ysps ^b	~100 Sips ^b		~380 ^c		(80)
WEN05-03					~100 ^c			(81)
Fluorothiazinon (also CL-55)	~20µg/ml ExoT ^b ExoY ^b			~10mg/kg ^c		~25 ^c		(82-84)
(-)-Hopeaphenol	~50 ExoS ^b	3.3 YopD ^b				~25 ^c		(85)
Resveratrol oligomers						~100 hrpA ^e		(86)
Paeonol				~95 Sips ^b				(87)
Syringaldehyde				~180 Sips ^b				(88)
Fusaric acid				53.5 SipC ^b				(89)
Cytosporone B				6.25 SipC ^b			NE ^d	(90)
Aurodox					0.5 ug/mL EspABCD _b			(91)
W1227933, W1774182						25 IncA ^f		(72)
BCD03						67.3 ^b		(92)
α-tocopherol	~10 ^c ExoY ^f							(93)
Cinnamaldehyde				~100 ^c SipAB ^e				(94)

961 ^a Species/T3SS family: PA: *Pseudomonas aeruginosa*, Ysc: *Yersinia pseudotuberculosis*

962 Ysc, Ysa: *Yersinia enterocolitica* Ysa, SPI-1: *Salmonella enterica* Typhimurium SPI-1,

963 SPI-II: *Salmonella enterica* Typhimurium SPI-II, EPEC/EHEC: Enteropathogenic *E.*

964 *coli*/Enterohemorrhagic *E.coli*, PS: *Pseudomonas syringae*, Fla: flagella. Empty square

965 denotes activity not tested.

966 ^b IC₅₀ (in µM, unless otherwise indicated) measured using the indicated organism/T3SS

967 family/effector protein in a culture-based secretion assay. If IC₅₀ data is not available,
968 either the lowest known inhibitory concentration (indicated by “~”), EC₅₀ (half maximal
969 effective concentration), or MIC (minimal inhibitory concentration) are shown.

970 ^c IC₅₀ (in μM) measured using cell-based, infection assays.

971 ^d No effect observed

972 ^e IC₅₀ (in μM) measured using a biochemical assay (i.e.-binding assay, enzymatic assay,
973 qPCR).

974 ^f IC₅₀ (in μM) measured using translocation assay.

975 * Phe: Phenoxyacetamide. HQ: hydroxyquinoline. SAH: Salicylidene acylhydrazides.

976

977

978 **Table 3: Bacterial strains used in this study**
979

Strain	Description	References
<i>Y. pseudotuberculosis</i> strains		
Wild type	<i>Y. pseudotuberculosis</i> IP2666	(95)
<i>tatB::Tn</i> - Bla	IP2666 $\Delta YopHEMOJ$ <i>tatB::TnHimarI</i> insertion; carrying 30aa _{suff} :: β -lactamase TEM1	This study
Wild type - Bla	IP2666 carrying 30aa _{suff} :: β -lactamase TEM1	This study
<i>Pseudomonas aeruginosa</i> strains		
Wild type	<i>P. aeruginosa</i> PA103	(96)
$\Delta exoUT$	PA103 $\Delta exoU/\Delta exoT$	(97)
PAO1 efflux pump mutant	PAO1 $\Delta(mexAB-oprM)$ nfxB $\Delta(mexCD-oprJ)$ $\Delta(mexEF-oprN)$ $\Delta(mexJKL)$ $\Delta(mexXY)$ $\Delta opmH362::pGSV3$ -Spr -exoT'- aacC1::miniCTXexoS(E379A/E381A)- blaM	(28)
<i>Yersinia enterocolitica</i>		
Wild type	<i>Y. enterocolitica</i> 8081 serotype O:8	(98)
pYV40-EGFP-yscD	<i>Y. enterocolitica</i> serotype O9 strain E40	(23)

carrying EGFP-yscD

Salmonella enterica Typhimurium strains

WT *S. enterica* Typhimurium SL1344 (99)

Δ *fliC* SL1344 Δ *fliC* (99)

Escherichia coli

E. coli DH5 α *E. coli* DH5 α carrying 30aa_{suff}:: β - This study

lactamase TEM1

Chlamydia trachomatis *C. trachomatis* serovar L2 (Joanne Engle)

980

981

982 **Table S1: Solubility of cyclic peptomers**

983

Compounds	Solubility (μM)			IC ₅₀
	PBS	DMEM	M9	
1EpDN 3Ala	180	ND ^a	ND ^a	>1000*
1EpDN 4Ala	75	ND ^a	ND ^a	>1000*
1EpDN 5Ala	127	ND ^a	ND ^a	>1000*
1EpDN 6Ala	125	ND ^a	ND ^a	>1000*
1EpDN	14-17	ND ^a	ND ^a	8.2
2EpDN	12	ND ^a	ND ^a	15.3
3EpDN	18	ND ^a	ND ^a	16
4EpDN	13	12	10	3.9
5EpDN	13	ND ^a	ND ^a	6.3
6EpDN	1	ND ^a	ND ^a	>1000*
7EpDN	26	ND ^a	ND ^a	6.7
8EpDN	1	ND ^a	ND ^a	41.5
9EpDN	10	ND ^a	ND ^a	12.8
4EpDN 1Sar	ND ^a	6	4	19
4EpDN 2Sar	ND ^a	61	61	~139.5

984

985 * >1000 indicates IC₅₀ could not be calculated

986 ^a: Not Determined (ND)

987

988

989

990 **Table S2. Primers used in this study**
991

Name	Sequence	Description
oHL364	TACTGGAAACGGTGGCTAATAC	16S Forward for <i>Salmonella</i> qPCR
oHL365	TACCTCACCAACAAGCTAATCC	16S Reverse for <i>Salmonella</i> qPCR
oHL362	GCCAACGACGGTGAAACTA	fliC Forward for <i>Salmonella</i> qPCR
oHL363	GCCGTATCGCTGACCTTATATT	fliC Reverse for <i>Salmonella</i> qPCR
oHL346	ACGACTCATAATTGGCGATAC	hilA Forward for <i>Salmonella</i> qPCR
oHL347	CTGCGATAATCCCTTCACGATAG	hilA Reverse for <i>Salmonella</i> qPCR
oHL348	GGCGTCTCTATGCACTTATC	hilD Forward for <i>Salmonella</i> qPCR
oHL349	GCAGGAAAGTCAGGCGTATAG	hilD Reverse for <i>Salmonella</i> qPCR
oHL350	GCAGCAAATTATTACGCCTTCTC	invF Forward for <i>Salmonella</i> qPCR
oHL351	CTGGTTGACTGAGCGAGTAAAT	invF Reverse for <i>Salmonella</i> qPCR
oHL352	ATGCGTTGTCCGGTAGTATTT	sipC Forward for <i>Salmonella</i> qPCR
oHL353	TTAAGCGCGCCTCTTTCA	sipC Reverse for <i>Salmonella</i> qPCR
oHL354	TCTTGTTATGCAGGAGGTGATG	sipA Forward for <i>Salmonella</i> qPCR
oHL355	GTCAACAAGGTGCGTAAGATTG	sipA Reverse for <i>Salmonella</i> qPCR
BQ89	CATGACCATCGCCTGATCTT	dnaB Forward for <i>P. aeruginosa</i> qPCR
BQ90	GTTGTCCTTCCTTCTCCAAC	dnaB Reverse for <i>P. aeruginosa</i> qPCR

oHL258	ATGCGGTAATGGACAAGGTC	exoT Forward for <i>P. aeruginosa</i> qPCR
oHL259	ACTCGCCGTTGGTATAGAGA	exoT Reverse for <i>P. aeruginosa</i> qPCR
oHL282	CCGGCAGATGTCCATTTC	DsbA Forward for <i>P. aeruginosa</i> qPCR
oHL283	CTCGACACCCATGCTTTC	DsbA Reverse for <i>P. aeruginosa</i> qPCR
BQ91	CTCTACACCGGCATTCACTAC	exoS Forward for <i>P. aeruginosa</i> qPCR
BQ92	CATACCTTGGTCGATCAGCTT	exoS Reverse for <i>P. aeruginosa</i> qPCR
oHL210	agcgaattcgagctcggtaccATGTCACTCAGTC GTCGC	Forward primer for 33 amino acid of <i>sufI</i> N terminus
oHL217	tttctgggtgAGGTTGCTGAGTACTACTAG	Reverse primer for 33 amino acid of <i>sufI</i> N terminus
oHL218	tcagcaacctCACCCAGAAACGCTGGTG	Forward primer for β -lactamase gene
oHL219	tctagaggatccccgggtaccTTACCAATGCTTA ATCAGTGAGG	Reverse primer for β -lactamase gene

992

993

994

995 **Figure legends**

996

997 **Figure 1: Stereochemistry scan of cyclic peptomers results in a more potent**

998 **derivative, 4EpDN. (A)** Structures of 1EpDN stereoisomers. Isomers were generated

999 from different combination of four side chains at position 3 to 6. Numbers preceding

1000 compounds were used to distinguish the different isomers and the conformation of the

1001 four side chains. D-amino acid side chain is shown in red. **(B)** WT *P. aeruginosa* PA103

1002 was grown under T3SS-inducing conditions with increasing concentrations of cyclic

1003 peptomer isomers. Secretion of T3SS cargo into the culture supernatant was assessed by

1004 precipitating secreted proteins and visualizing them with Coomassie blue. ExoU band

1005 intensities were quantified and normalized to that of the DMSO control. The results are

1006 from at least two independent experiments. Nonlinear curve fitting is shown to depict the

1007 trend of inhibition.

1008

1009 **Figure 2: Sarcosine replacement of 4EpDN at position 1 or 2 eliminates activity. (A)**

1010 Structures of 4EpDN and its derivatives, 4EpDN 1Sar and 4EpDN 2Sar. D-amino acid

1011 side chain is shown in red. **(B)** WT *P. aeruginosa* PA103 was grown under T3SS-

1012 inducing conditions with increasing concentrations of compounds. Secretion of T3SS

1013 cargo into the culture supernatant was assessed on SDS-PAGE gel. ExoU band intensities

1014 were visualized with Coomassie blue, quantified and normalized to that of the DMSO

1015 control. The results are from at least two independent experiments.

1016

1017 **Figure 3: Effect of cyclic peptomers on secretion of *Yersinia* Ysa T3SS substrates.** *Y.*
1018 *enterocolitica* serotype O:8 was grown under T3SS-inducing conditions with increasing
1019 concentrations of cyclic peptomer isomers, 4EpDN (A) and 4EpDN 2Sar (B). Secretion
1020 of T3SS cargo into the culture supernatant was assessed by precipitating secreted proteins
1021 and visualizing them with Coomassie blue. YspF band intensities were quantified and
1022 normalized to that of the DMSO control. Representative gel images and quantification of
1023 YspF are shown. The results are from two independent experiments.

1024

1025 **Figure 4: Cyclic peptomers inhibit the *Salmonella* SPI-1 T3SS.** *Salmonella enterica*
1026 Typhimurium was grown with increasing concentrations of cyclic peptomer isomers.
1027 Secretion of SPI-1 T3SS cargo into the culture supernatant was assessed by precipitating
1028 secreted proteins and visualizing them with Coomassie blue. SipA and SipC band
1029 intensities were quantified and normalized to that of the DMSO control. The experiments
1030 were carried out without the detergent Tween 20 (A), or with Tween 20 (B). A Δ SPI-1
1031 *Salmonella* mutant and INP0007, a known SPI-1 inhibitor (60), were used as controls.
1032 The results are from at least two independent experiments.

1033

1034 **Figure 5: The cyclic peptomer 4EpDN disrupts localization of the *Yersinia* T3SS**
1035 **basal body component YscD.** *Y. enterocolitica* expressing YscD-EGFP was grown
1036 under T3SS inducing condition (low Ca^{2+}) in the presence of 9 μM cyclic peptomers, 50
1037 μM INP007, or DMSO. High Ca^{2+} media was used as a non-secreting control. (A)
1038 Histogram showing the frequency of YscD puncta/cell. (B) Average number of
1039 puncta/cell after treatment \pm standard error of the mean. Data represents three

1040 independent experiments. One-way ANOVA with Dunnett's multiple-comparison test
1041 was used. ****, $P < 0.0001$; ns: not significant.

1042

1043 **Figure 6: Cyclic peptomers do not affect transcription of T3SS genes in *P.***

1044 *aeruginosa*. *P. aeruginosa* PA103 (A) or PA01 (B) was grown in low calcium media in
1045 the presence of 60 μ M cyclic peptomers or DMSO. Samples were collected 3 hrs after
1046 induction for qPCR analysis. The phenoxyacetamide MBX1641 (53), a known T3SS
1047 inhibitor predicted to inhibit type III secretion by binding to the T3SS needle subunit
1048 (28), was used as a control. Data were from three replicates, analyzed by one-way
1049 ANOVA with Dunnett's multiple-comparison test. **, $P < 0.01$.

1050

1051 **Figure 7: The cyclic peptomer 4EpDN inhibits secretion of the effector protein**

1052 **ExoS, but not the regulator ExsE.** PA01 carrying ExoS-Bla was grown in T3S
1053 inducing condition on the presence of 60 μ M cyclic peptomers or DMSO. A. Secretion of
1054 ExoS into the culture supernatant and synthesis of ExoS in the cell pellets were assessed
1055 by Western Blot using a β -lactamase antibody. B. In the same samples, Western blot was
1056 carried out for ExsE. ExsE in the supernatant was observed and quantified while ExsE in
1057 the cell pellets was undetectable. Data were from at least two independent experiments.
1058 One-way ANOVA with Dunnett's multiple-comparison test was used. *, $P < 0.05$; ns: not
1059 significant, compared to DMSO.

1060

1061 **Figure 8: Cyclic peptomers do not affect the twin arginine translocation (Tat)**

1062 **system.** (A) *Y. pseudotuberculosis* expressing a SufI- β -lactamase Tat secretion reporter

1063 incubated in the presence of penicillin G will only grow if the Tat secretion system
1064 remains functional. **(B)** *Y. pseudotuberculosis* SufI- β -lactamase reporters were treated
1065 with the Tat inhibitors Bay 11-7082, N-Phenyl maleimide, or DMSO, and culture optical
1066 density was measured. WT refers to bacteria expressing a functional Tat secretion
1067 system. A mutant strain with a transposon insertion in the *tatB* gene serves as a control.
1068 **(C)** The same assay as in (B) was repeated in the presence of cyclic peptomers or DMSO.
1069 The result was from two independent replicates.

1070

1071 **Figure 9. The cyclic peptomer 4EpDN inhibits *Chlamydia* infection.** **(A)** HeLa cells
1072 were infected with *C. trachomatis* L2 at a multiplicity of infection (MOI) of three (left
1073 hand panel) or one (right hand panel) in the presence of 9 μ M cyclic peptomers, 30
1074 μ M INP0400, or DMSO. Cells were stained for the *Chlamydia* major outer membrane
1075 protein (MOMP) and nucleic acids (DAPI), and imaged after 24 hrs of infection to
1076 determine the number of infected cells (primary infection). **(B-C)** Infectious elementary
1077 bodies (EB) were harvested after 48hrs of HeLa cell infection in the presence of
1078 inhibitors and used to infect fresh HeLa cells without applying inhibitors (secondary
1079 infection). After 24 hrs, cells were imaged as in **(A)**. Representative images **(B)** and
1080 infectious units/mL **(C)** are shown from three to four independent experiments. One-way
1081 ANOVA with Dunnett's multiple-comparison test was used. **, $P < 0.01$, ****, P
1082 < 0.0001 , ns: not significant.

1083

1084 **Figure S1: 1EpDN alanine/Sarcosine scan suggests peptoid sidechains are important**
1085 **for biological activity.** **(A)** Structures of 1EpDN alanine derivatives. D-form of side

1086 chain is shown in red. **(B)** WT *P. aeruginosa* PA103 was grown under T3SS-inducing
1087 conditions with increasing concentrations of cyclic peptomers. Secretion of T3SS cargo
1088 into the culture supernatant was assessed by precipitating secreted proteins and
1089 visualizing them with Coomassie blue. ExoU band intensities were quantified and
1090 normalized to that of the DMSO control. The results are from at least two independent
1091 experiments. Nonlinear curve fitting is shown to depict the trend of inhibition.

1092

1093 **Figure S2: Secretion of *Salmonella* T3SS substrate in the presence of non-ionic**
1094 **detergents.** **(A)** *Salmonella enterica* Typhimurium was grown in LB with increasing
1095 concentrations of NP-40, Tween 20, or Triton X-100. Secretion of SPI-1 T3SS effector
1096 SipC into the culture supernatant was assessed by precipitating secreted proteins and
1097 visualizing them with Coomassie blue. **(B)** Secretion of SipC in the presence of
1098 increasing concentrations of Tween 20 or Triton X-100 was detected by Western Blot.
1099 0.003% Tween-20 is the highest concentration of Tween-20 that resulted in little effect
1100 on secretion of SipC.

1101

1102 **Figure S3: Cyclic peptomers do not affect secretion of flagellar proteins.** *Salmonella*
1103 *enterica* Typhimurium was grown in LB with increasing concentrations of cyclic
1104 peptomers. Secretion of flagellar structural proteins FliC and FliD were assessed by
1105 precipitating the secreted proteins and visualizing them with Coomassie blue. A Δ *fliC*
1106 mutant and azithromycin (100), which inhibits flagellin secretion, were used as controls.
1107 The SPI-1 mutant and WT *Salmonella* were both tested, as flagella substrates can be
1108 secreted through both flagellar and SPI-1 T3SS systems.

1109

1110 **Figure S4: Relationship between solubility and activity of cyclic peptomers.** IC₅₀ of
1111 stereoisomers and their solubility (table S1) were plotted on a log₁₀ scale. Average
1112 solubility was used when the solubility was measured in different conditions.

1113

1114 **Figure S5: Cyclic peptomers do not affect transcription of T3SS genes in**
1115 *Salmonella*. *Salmonella enterica* Typhimurium was grown in LB with 300 mM NaCl in
1116 the presence of 9 μM cyclic peptomers or DMSO. Samples were taken 2 hrs (A) and 4
1117 hrs (B) after addition of compounds at 37°C and expression of flagellar (*fliC*) and
1118 injectisome T3SS (*hilA*, *hilD*, *invF*, *sipC*, *sipA*) genes were assessed using qPCR. Data
1119 are from two replicates, analyzed by one-way ANOVA showing no significant difference
1120 between control and treatments.

1121

1122

1123 **Figure S6: Effect of cyclic peptomers on HeLa cells.** HeLa cells were incubated with
1124 compounds for 48 hrs. Cells were then stained with: (A) Stain Set 1-Hoechst, FITC-alpha
1125 tubulin, rhodamine-phalloidin (actin), and Calnexin (ER induced protein); or (B) Stain set
1126 2-Hoechst, EdU-rhodamine (S-phase detection), anti-Phosphohistone H3 (mitosis
1127 marker), and GM130 (Golgi matrix protein). Representative images of cells treated with
1128 different concentrations of 4EpDN or DMSO are shown. (C) Quantification of all cell
1129 features for 4EpDN-treated cells. The total CP score is the square root of sum of square
1130 of the difference between treatment and DMSO for all measured features.

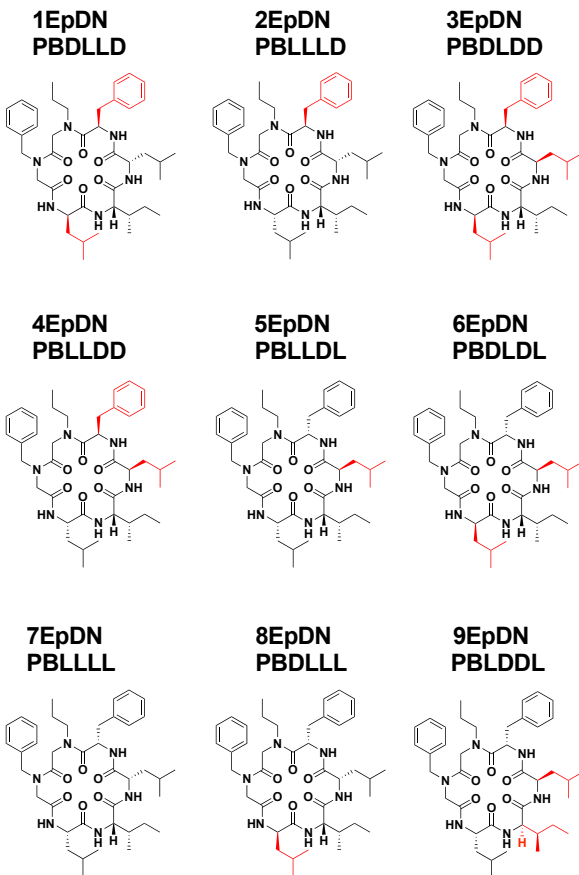
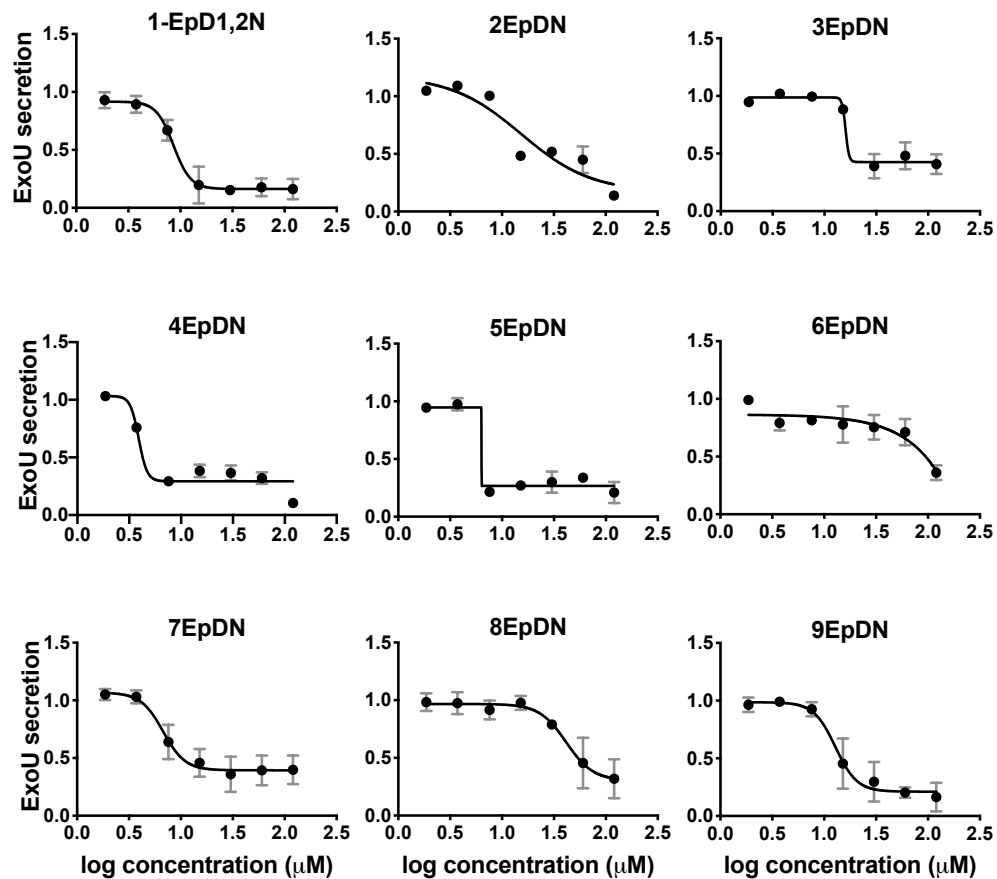
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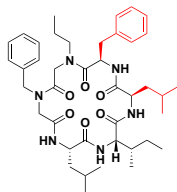
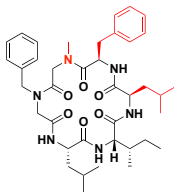
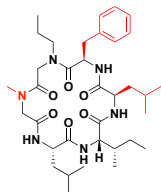
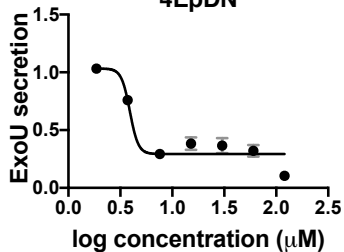
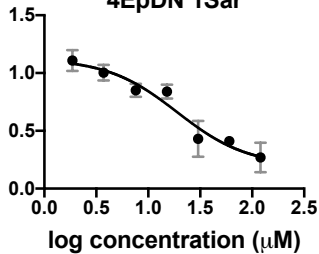
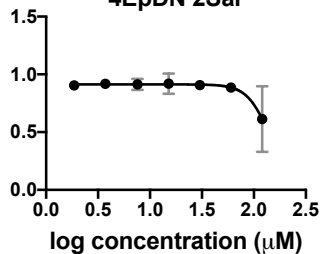
1132 **Figure S7:** Characterization of cyclic peptomers. Drawn structures, SMILE structures,

1133 molecular weight, LCMS Spectra, and $^1\text{H-NMR}$ Spectra are shown.

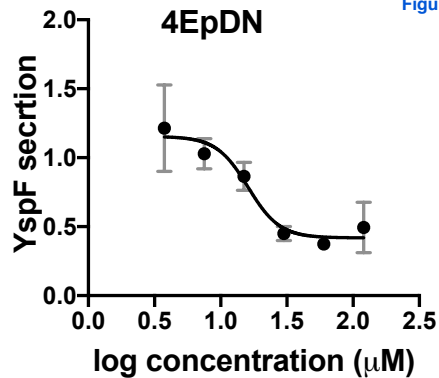
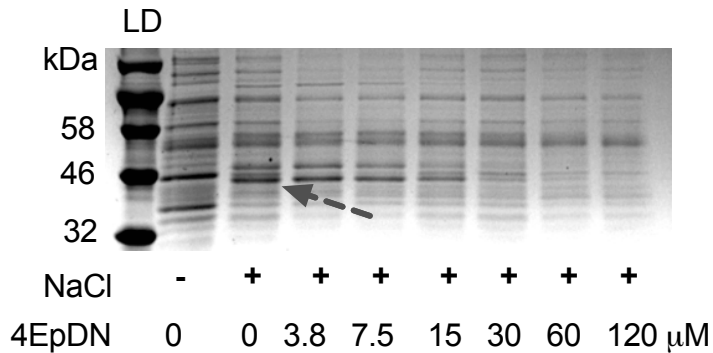
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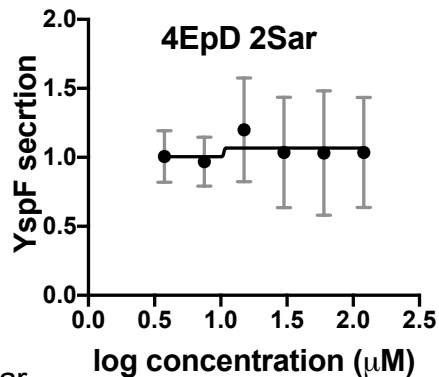
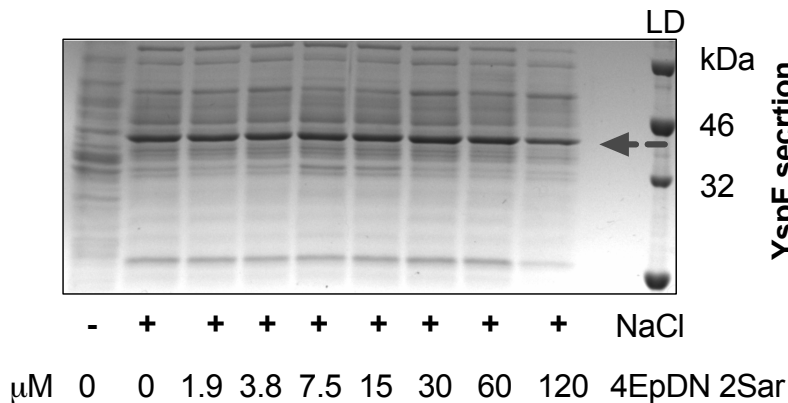
A**B**

A**4EpDN****4EpDN 1Sar****4EpDN 2Sar****B****4EpDN****4EpDN 1Sar****4EpDN 2Sar**

A



B

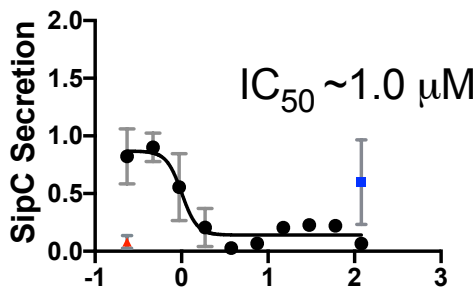


■ INP0007 100 μ M ◆ Δ SPI-1

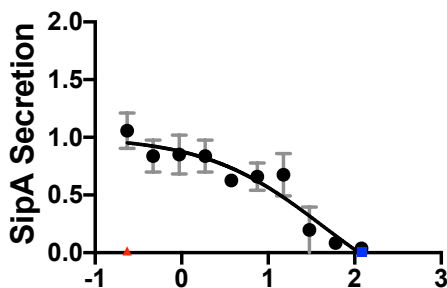
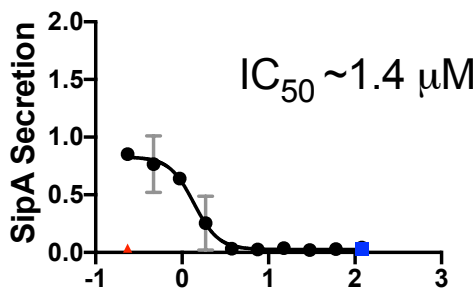
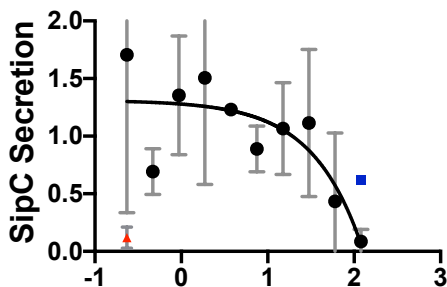
No Tween 20

A

4EpDN



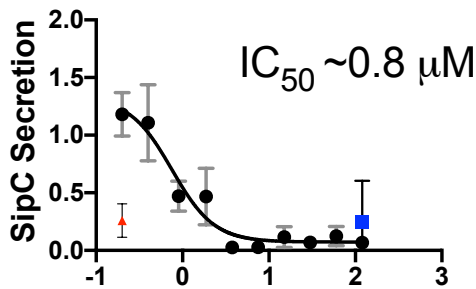
4EpDN 2Sar



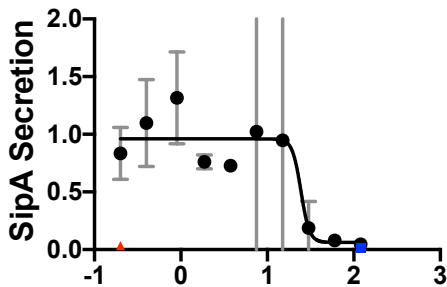
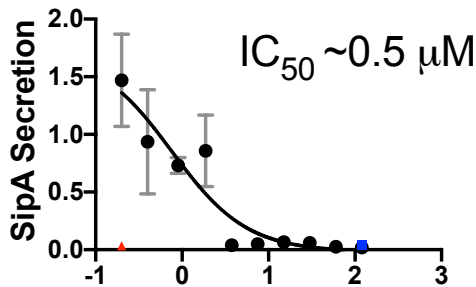
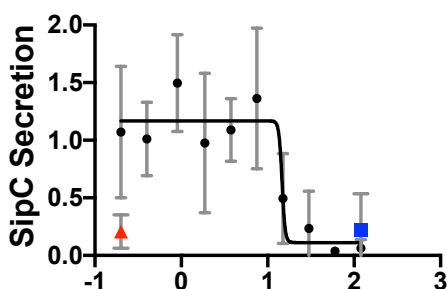
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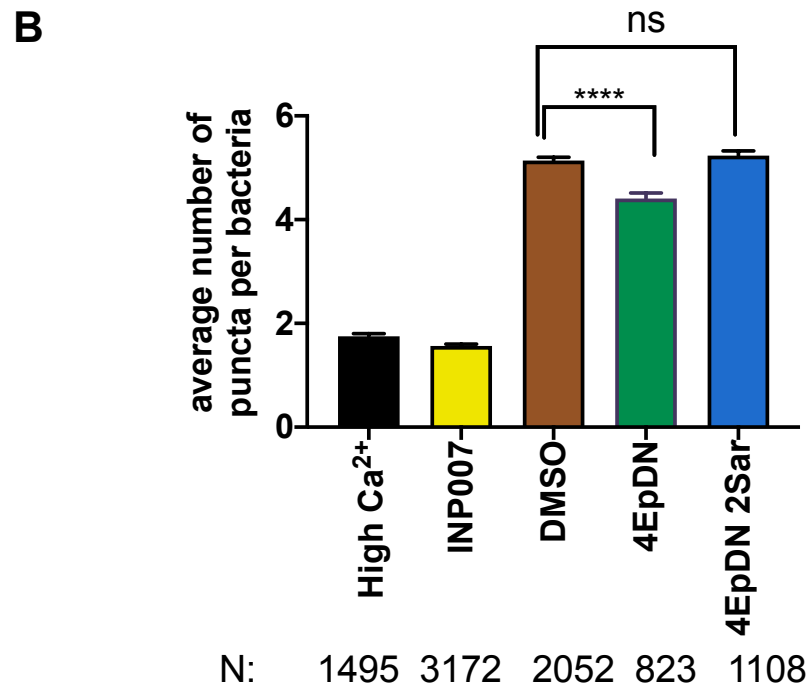
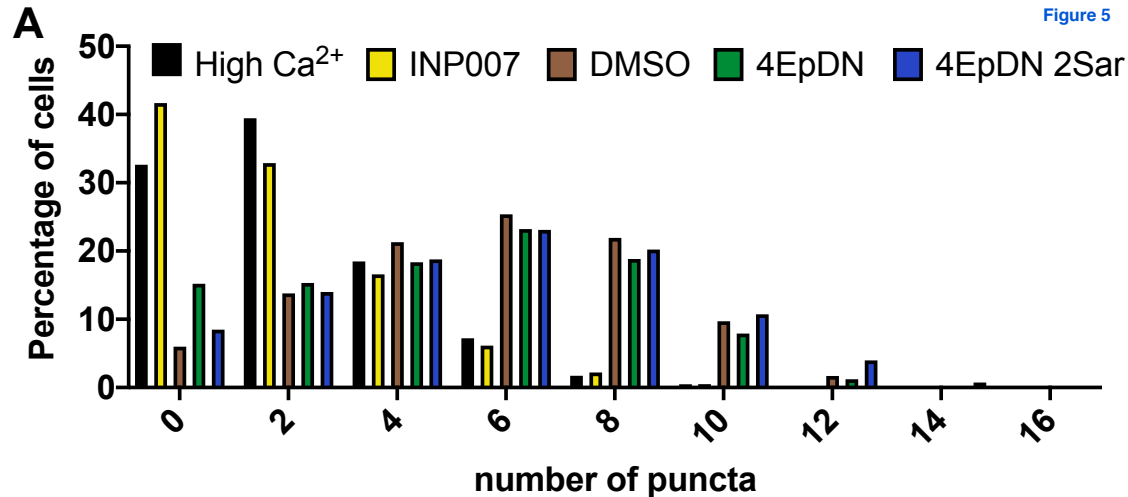
With Tween 20 (0.003%)

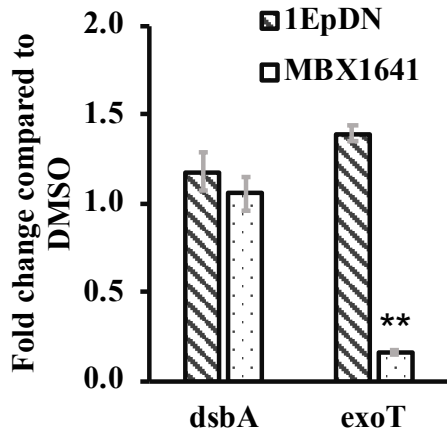
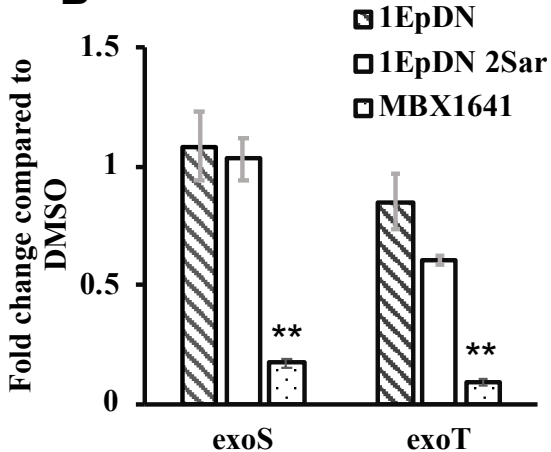
4EpDN



4EpDN 2Sar





A.**B.**

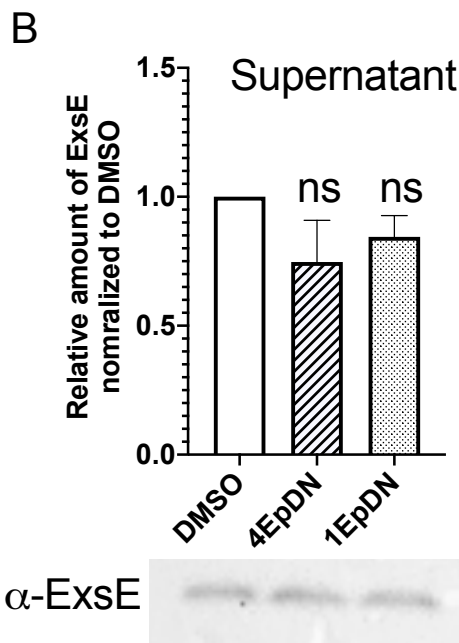
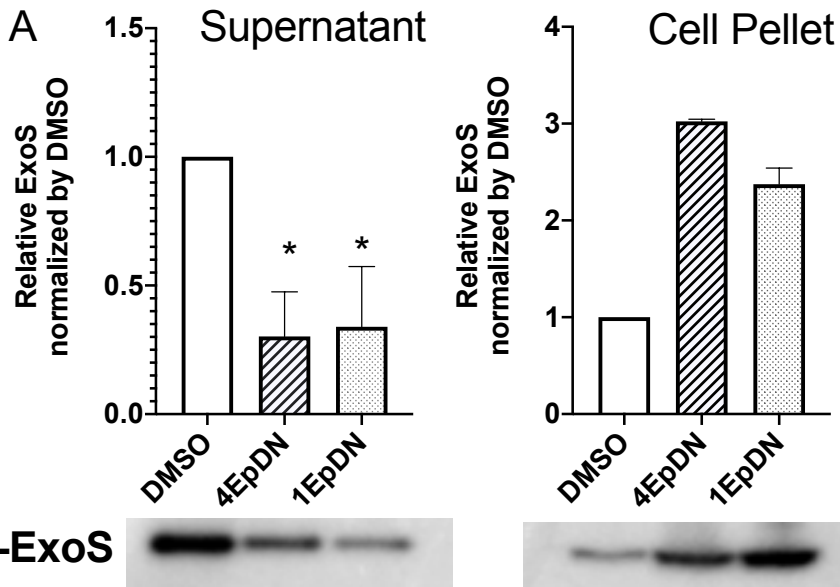
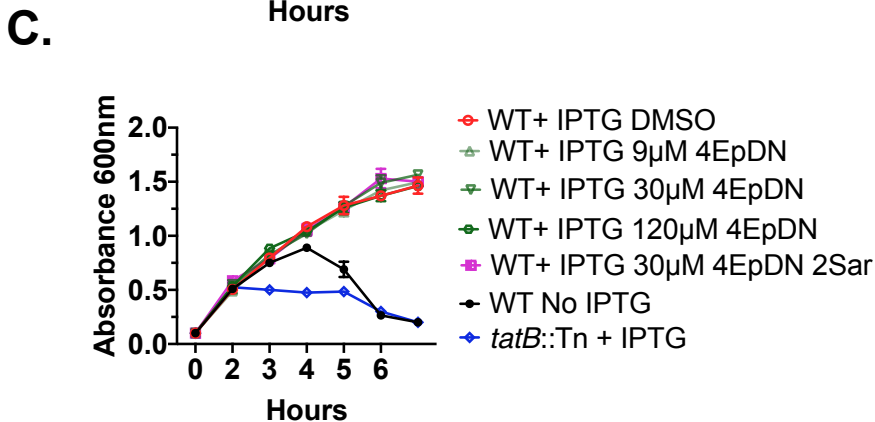
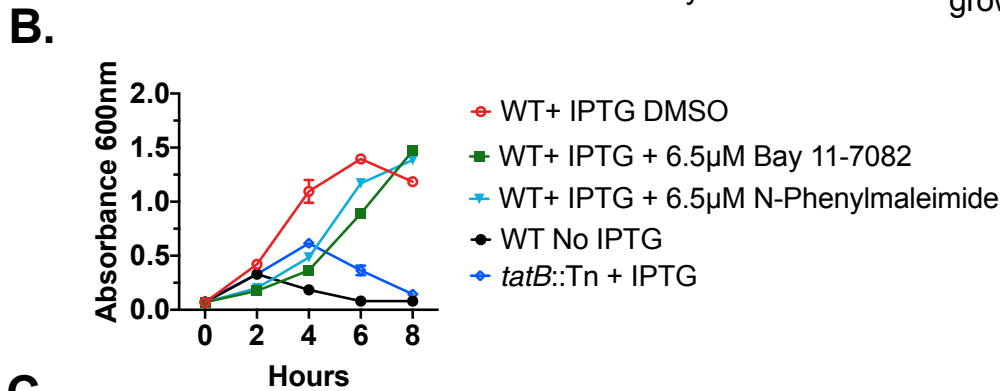
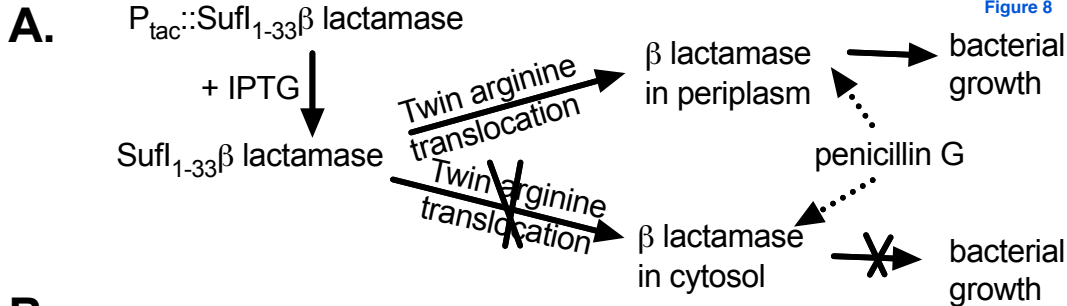
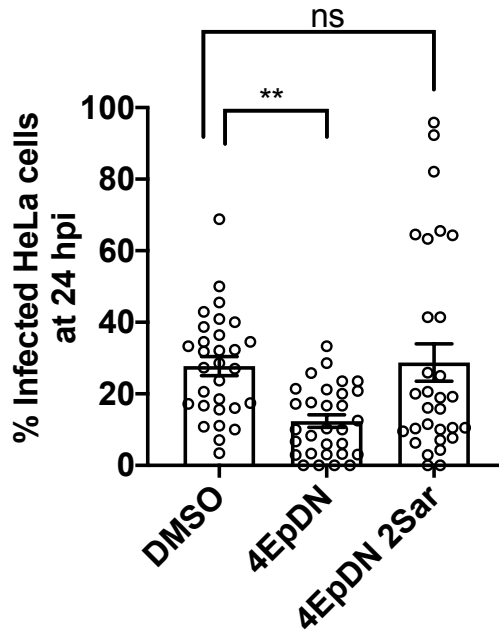
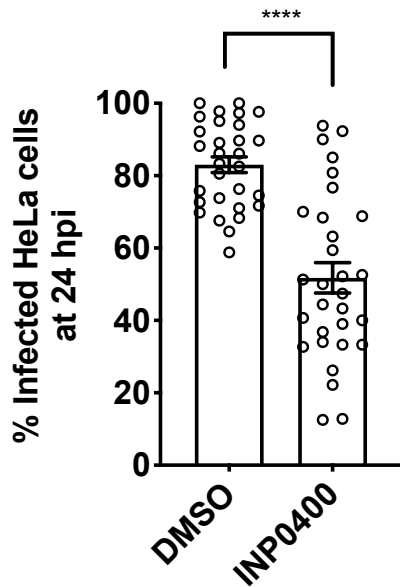


Figure 7

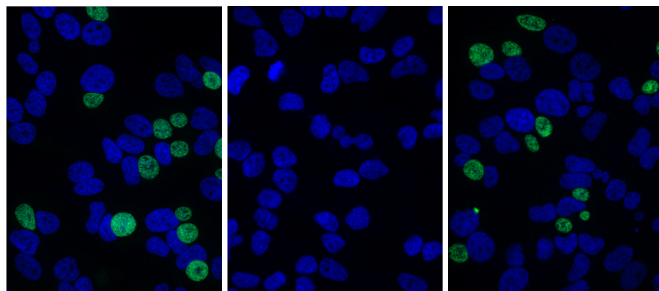


A



B

Chlamydia MOMP
DAPI



DMSO

4EpDN

4EpDN 2Sar

C

