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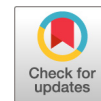
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# Synthetic Cyclic Peptomers as Type III Secretion System Inhibitors

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**ABSTRACT** Antibiotic-resistant bacteria are an emerging threat to global public health. New classes of antibiotics and tools for antimicrobial discovery are urgently needed. Type III secretion systems (T3SS), which are required by dozens of Gram-negative bacteria for virulence but largely absent from nonpathogenic bacteria, are promising virulence blocker targets. The ability of mammalian cells to recognize the presence of a functional T3SS and trigger NF- $\kappa$ B activation provides a rapid and sensitive method for identifying chemical inhibitors of T3SS activity. In this study, we generated a HEK293 stable cell line expressing green fluorescent protein (GFP) driven by a promoter containing NF- $\kappa$ B enhancer elements to serve as a readout of T3SS function. We identified a family of synthetic cyclic peptide-peptoid hybrid molecules (peptomers) that exhibited dose-dependent inhibition of T3SS effector secretion in *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa* without affecting bacterial growth or motility. Among these inhibitors, EpD-3'N, EpD-1,2N, EpD-1,3'N, EpD-1,2,3'N, and EpD-1,2,4'N exhibited strong inhibitory effects on translocation of the *Yersinia* YopM effector protein into mammalian cells (>40% translocation inhibition at 7.5  $\mu$ M) and showed no toxicity to mammalian cells at 240  $\mu$ M. In addition, EpD-3'N and EpD-1,2,4'N reduced the rounding of HeLa cells caused by the activity of *Yersinia* effector proteins that target the actin cytoskeleton. In summary, we have discovered a family of novel cyclic peptomers that inhibit the injectisome T3SS but not the flagellar T3SS.

**KEYWORDS** *Yersinia*, type III secretion system, T3SS, cyclic peptides, peptoids, peptomers, virulence blocker, *Pseudomonas aeruginosa*

As currently available antibiotics become ineffective due to the rise in antibiotic resistance among pathogenic bacteria, development of completely new classes of antibiotics is critical (1). Classic antibiotics target pathogens and commensal bacteria indiscriminately; therefore, their use puts selective pressure on both populations. Because of the abundance of commensals within a mammalian host ( $10^{13}$  to  $10^{14}$ ), antibiotic resistance is thought to arise more frequently in commensal bacteria and is horizontally transferred to pathogens (2–4). In contrast to classic antibiotics, virulence blockers are compounds that selectively inhibit the expression or function of a virulence factor in a pathogen or group of pathogens (5). Advantages of virulence blockers are twofold. For one, selective pressure on a limited number of microbes, i.e., only pathogens expressing the molecular target of the virulence blocker, should limit the evolution of resistance (6). Second, the decreased commensal killing by virulence blockers has the potential to preserve a healthy microbiota, which is critical for maintaining gut homeostasis and defending against opportunistic pathogens (7, 8).

Type III secretion systems (T3SS) are bacterial appendages required by dozens of pathogens to cause disease, including *Salmonella*, enteropathogenic *Escherichia coli* (EPEC), *Shigella*, *Pseudomonas*, and *Yersinia*, but they are largely absent in nonpatho-

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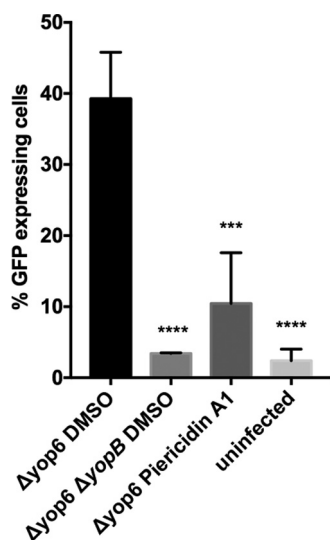
genic bacteria (9). Bacteria use T3SS to inject bacterial effector proteins into target host cells to manipulate host processes for the benefit of the pathogen. Seven T3SS injectisome families have been identified (9) and share a number of homologous membrane-associated components with the flagellar basal body (10). While many aspects of T3SS structure are similar among different T3SS families, the repertoire of encoded T3SS effector proteins is unique for each genus or even species (11, 12). For example, pathogenic *Yersinia* species, which include the plague agent *Yersinia pestis* and the enteropathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, and the opportunistic pathogen *Pseudomonas aeruginosa* encode a Ysc family T3SS that contributes to virulence. However, the *Yersinia* T3SS translocates the *Yersinia* outer proteins (Yops) YopH, YopE, YopM, YopO, YopJ, and YopT, whereas *Pseudomonas* secretes the proteins ExoS, ExoU, and ExoT (13, 14). *Yersinia* avoids phagocytosis by using effectors targeting the actin polymerization process and signaling pathways. YopE has RhoGAP activity and leads to depolymerization of actin stress fibers in eukaryotic cells (15, 16). YopH, a protein tyrosine phosphatase, antagonizes several signaling pathways associated with focal adhesions (17, 18). YopO, also known as YpK, binds to actin monomers, blocking actin filament polymerization, and sequesters actin polymerization regulators, which disrupts phagocytosis (19).

*P. aeruginosa* is a major cause of hospital-acquired infections (20, 21), and its T3SS is important in infection establishment, dissemination, and survival in animals (14, 22). *P. aeruginosa* is resistant to a number of antibiotics because its outer membrane is highly restrictive (20, 21, 23); therefore, new drugs to treat *P. aeruginosa* are of high interest. The widespread importance and structural conservation of T3SS among bacterial pathogens make T3SS optimal targets for antimicrobial agents.

NF- $\kappa$ B (nuclear factor kappa light chain-enhancer of activated B cells) is a family of mammalian transcription factors involved in inflammation and development (24). *Yersinia* lacking the YopHEMOJT T3SS effector proteins but expressing an otherwise functional Ysc T3SS triggers NF- $\kappa$ B activation in HEK293T cells, while *Yersinia* lacking the YopB translocator protein essential for translocation of T3SS cargo inside host cells does not activate NF- $\kappa$ B (25). Previously, we used host NF- $\kappa$ B-driven luciferase in HEK293T cells as a readout for *Yersinia* T3SS activity in a pilot T3SS inhibitor screen and identified a family of T3SS inhibitors called piericidins (26). In order to improve our screening strategy, we generated a HEK293 stable cell line expressing an NF- $\kappa$ B-driven green fluorescent protein (GFP) reporter gene. Using this cell line, we identified a group of eight cyclic peptomers that inhibit type III secretion in *Yersinia* and *Pseudomonas aeruginosa*.

## RESULTS

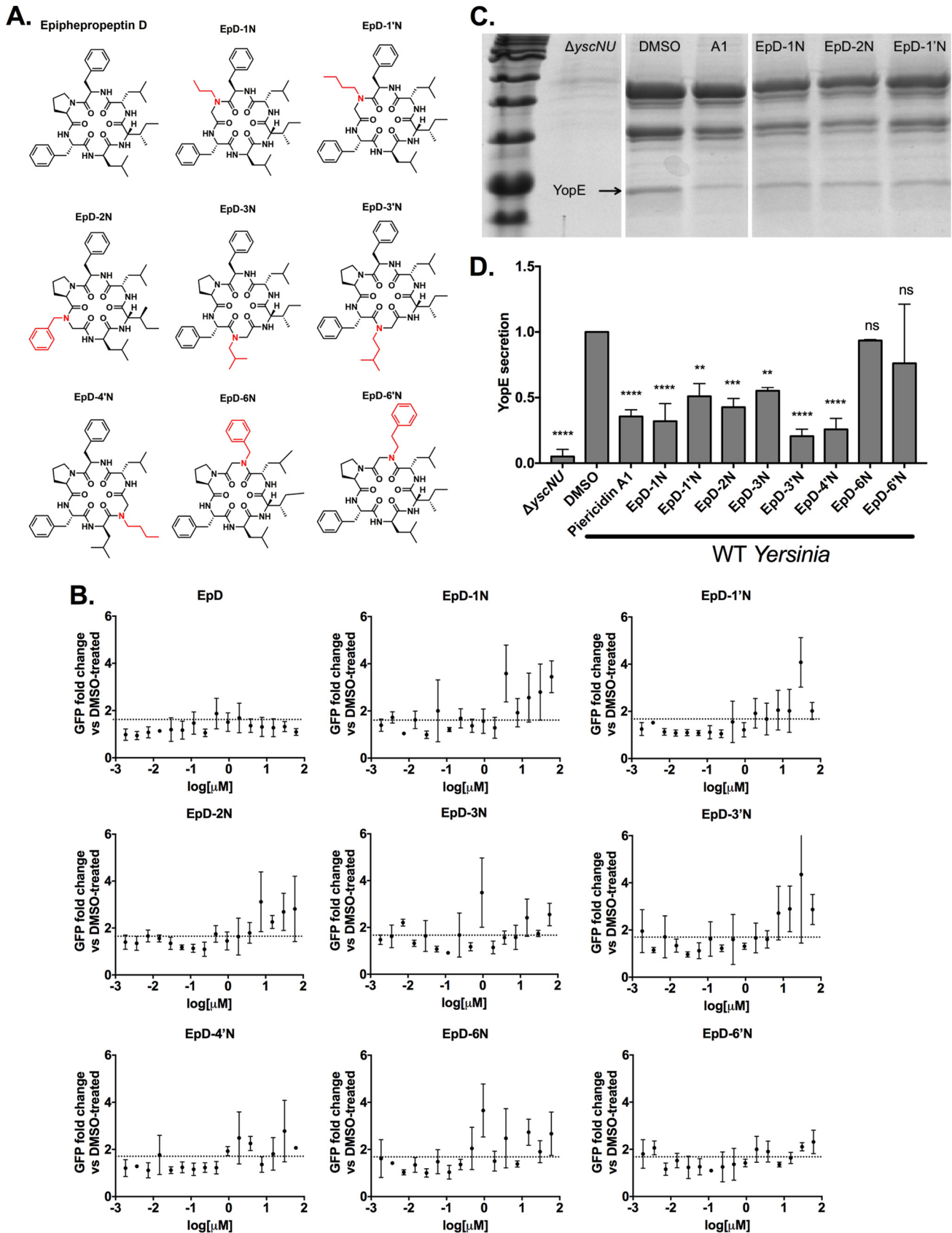
**Development and use of an NF- $\kappa$ B-GFP stable cell line for identification of T3SS inhibitors.** To reduce the variability and batch effects from transient transfection of an NF- $\kappa$ B reporter, we improved upon our previous NF- $\kappa$ B-based screen for T3SS inhibitors by generating an NF- $\kappa$ B-driven GFP-expressing HEK293 stable cell line (HEK293-GFP). To validate this cell line, we infected HEK293-GFP cells with *Y. pseudotuberculosis* lacking the *Yersinia* YopHEMOJT effector proteins ( $\Delta$ yopHEMOJ; this strain is referred to as the  $\Delta$ yop6 strain from this point forward), as several Yops modulate NF- $\kappa$ B signaling (13). Approximately 40% of the cells were positive for GFP after 5 h of infection with the  $\Delta$ yop6 strain at a multiplicity of infection (MOI) of 7 (Fig. 1). In contrast, *Y. pseudotuberculosis* carrying a nonfunctional T3SS ( $\Delta$ yop6  $\Delta$ yopB) induced low levels of GFP expression similar to those of uninfected samples. The known T3SS inhibitor piericidin A1 significantly reduced the percentage of GFP-expressing cells in response to the  $\Delta$ yop6 strain (Fig. 1). A Z-score, described in detail previously (27), is a single numerical value that accurately validates the reproducibility of high-throughput assays. Our stable cell line generated data with Z-scores ranging from 0.4 to 0.8, which is in the reliable range. These data suggest that our stable cell line can be used for high-throughput screens for T3SS inhibitors.



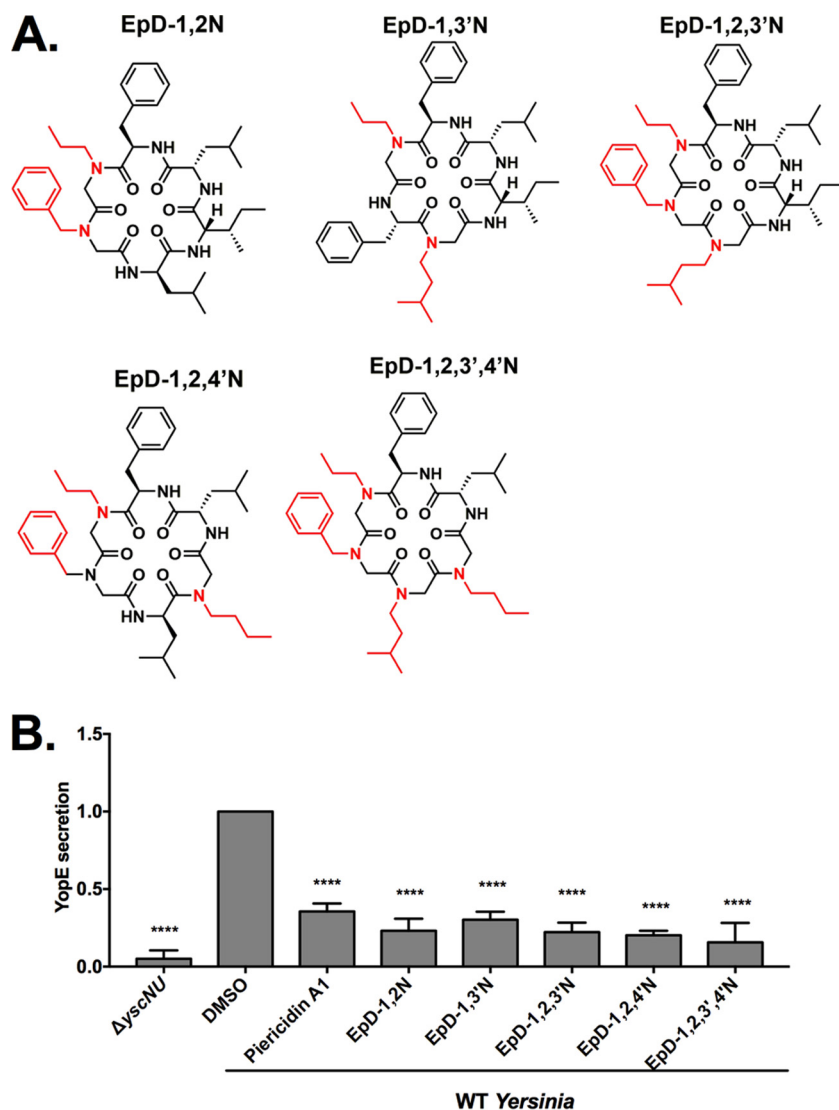
**FIG 1** A newly developed NF- $\kappa$ B-GFP reporter stable cell line can be used to identify chemical inhibitors of the *Yersinia* T3SS. A HEK293-based stable cell line developed for this study was infected with *Y. pseudotuberculosis*  $\Delta$ yop6 (T3SS<sup>+</sup>) or  $\Delta$ yop6  $\Delta$ yopB (T3SS<sup>-</sup>), and GFP fluorescence was quantified as a readout of T3SS-induced NF- $\kappa$ B activation in the presence or absence of a known T3SS inhibitor, piericidin A1. The results are representative of three independent replicates. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ .

As part of a previous study (28), a library of 20 synthetic peptides and peptomers based on the structure of a natural product, phepropeptin D (29), were synthesized and placed in an internal screening library. These peptomers were not previously screened for any bioactivity and may have a high intrinsic potential to disrupt protein-protein interactions (30), such as those critical for assembly of a large proteinaceous structure such as the T3SS. The compounds were screened for the ability to inhibit NF- $\kappa$ B activation during *Y. pseudotuberculosis*  $\Delta$ yop6 infection of HEK293-GFP cells. We used 2 standard deviations below the mean for the dimethyl sulfoxide (DMSO) control as the threshold for hit calling (26). The following 8 of the 20 peptomers reduced NF- $\kappa$ B-driven GFP expression at the two highest concentrations tested: EpD-1N, EpD-1'N, EpD-2N, EpD-3N, EpD-3'N, EpD-4'N, EpD-6N, and EpD-6'N (Fig. 2A and B) (Z-score,  $\sim 0.5$ ). While the "hit rate" is high, each of these eight peptomers has a single peptide-to-peptoid substitution compared to the parent compound, epiphepropeptin D (EpD), a phepropeptin D derivative containing a stereo-inversion at the proline position, and are thus structurally similar to each other (Fig. 2A).

**Cyclic peptomers inhibit type III secretion in *Yersinia pseudotuberculosis*.** In order to validate whether the cyclic peptomers we identified in our primary screen were indeed T3SS inhibitors, we evaluated the effects of our hits on secretion of *Y. pseudotuberculosis* effectors under T3SS-inducing conditions *in vitro*. The previously identified T3SS inhibitor piericidin A1 (26) was used as a positive control. Wild-type (WT) *Y. pseudotuberculosis* was grown in low-calcium medium in the presence or absence of one of the compounds at 60  $\mu$ M, an equivalent volume of DMSO, or 71  $\mu$ M piericidin A1, and secretion of the YopE effector protein was analyzed as a measure of general type III secretion. YopE was selected because it can easily be distinguished from other secreted proteins visible upon Coomassie blue staining (Fig. 2C; see Fig. S1 in the supplemental material) (26). *Y. pseudotuberculosis* lacking one of the T3SS structural operons ( $\Delta$ yscNU) was used as a negative control. YopE secretion was reduced  $\geq 70\%$  by EpD-4'N and EpD-3'N treatments ( $P < 0.0001$ ) and  $\geq 45\%$  by EpD-1N, EpD-1'N, EpD-2N, and EpD-3N treatments ( $P < 0.001$ ) (Fig. 2D). Furthermore, the abundances of the other Yops visible upon Coomassie blue staining were also reduced upon compound treatment (Fig. 2C; Fig. S1). Given that each of the eight peptomers carries only one peptoid substitution compared to the parent compound, we hypothesized that a



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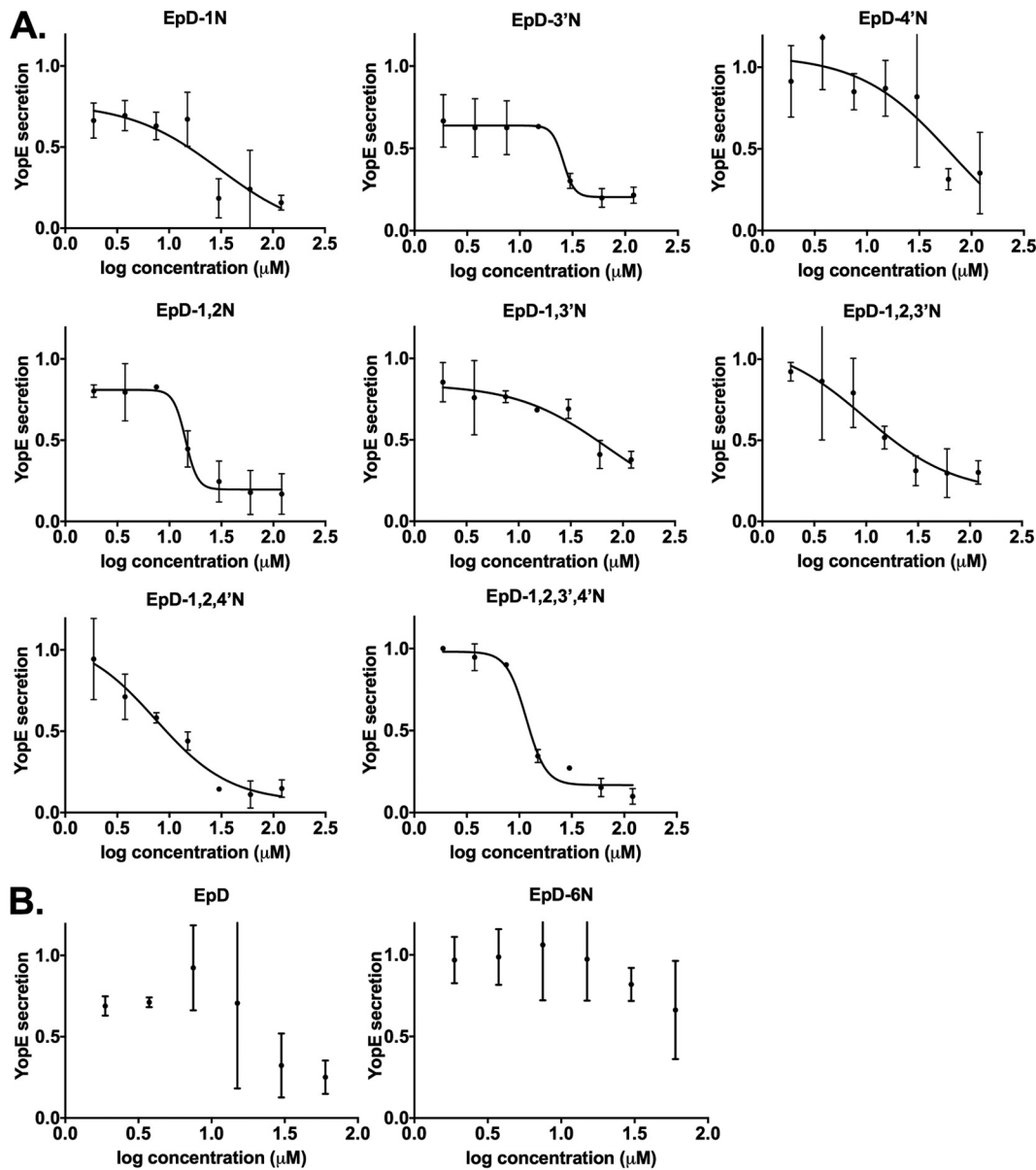
**FIG 3** Second-generation cyclic peptomers inhibit secretion of *Yersinia* T3SS effector proteins. (A) Structures of the second-generation cyclic peptomers, each of which carries two, three, or four side chain modifications compared to EpD. (B) WT *Y. pseudotuberculosis* was grown under T3SS-inducing conditions with 60  $\mu\text{M}$  cyclic peptomer, 71  $\mu\text{M}$  piericidin A1, or an equivalent volume of DMSO, and secretion of T3SS cargo into the culture supernatant was assessed by precipitating secreted proteins and visualizing with Coomassie blue. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. \*\*\*\*,  $P < 0.0001$ .

combination of peptoid substitutions may further improve the compounds' bioactivity. We synthesized second-generation peptomers with combinatorial changes at positions 1, 2, 3, and 4 on the cyclic peptide backbone (Fig. 3A). All five new compounds led to >70% reductions in secretion ( $P < 0.0001$ ) (Fig. 3B).

To further validate that the candidate T3SS inhibitors are active in a dose-dependent manner, the eight compounds showing the greatest inhibition at 60  $\mu\text{M}$  were chosen

#### FIG 2 Legend (Continued)

was quantified. The fold reductions of the GFP signals in the presence of cyclic peptomers compared to the signal with DMSO are shown as averages for three independent experiments. The dotted lines represent 2 standard deviations above the DMSO control level. (C and D) WT *Y. pseudotuberculosis* was grown under T3SS-inducing conditions with 60  $\mu\text{M}$  cyclic peptomer, 71  $\mu\text{M}$  piericidin A1, or an equivalent volume of DMSO, and secretion of T3SS cargo into the culture supernatant was assessed by precipitating the secreted proteins, visualizing them with Coomassie blue (C), and quantifying the YopE effector band intensity (D). The data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; ns, not significant. For panel C, the lanes containing the ladder and samples shown were separated by unrelated samples in the intervening lanes of the same gel and are therefore shown divided by white borders.



**FIG 4** Cyclic peptomers inhibit type III secretion in *Yersinia pseudotuberculosis* in a dose-dependent manner. WT *Y. pseudotuberculosis* was grown under T3SS-inducing conditions with increasing concentrations of peptomers. Secretion of T3SS cargo into the culture supernatant was assessed by precipitating secreted proteins and visualizing them with Coomassie blue. YopE band intensities were quantified and normalized to that of the DMSO control. The results are from two independent experiments. Nonlinear curve fitting is shown to depict the trend of inhibition. (A) EpD-1N, EpD-3'N, EpD-4'N, EpD-1,2N, EpD-1,3'N, EpD-1,2,3'N, EpD-1,2,4'N, and EpD-1,2,3',4'N. (B) EpD and EpD-6N.

for testing at 2-fold increasing concentrations from 1.875  $\mu\text{M}$  to 120  $\mu\text{M}$  (Fig. 4; Table 1). EpD-1N, EpD-3'N, EpD-1,2N, EpD-1,2,3'N, EpD-1,2,4'N, and EpD-1,2,3',4'N showed a dose-response relationship and reduced secretion of YopE, to less than 40% of normal DMSO control levels, at 30  $\mu\text{M}$ . Surprisingly, although the parental compound EpD did not decrease NF- $\kappa\text{B}$  activation in our primary screen (Fig. 2B), EpD did inhibit YopE secretion in a dose-dependent manner (Fig. 4B). However, the EpD-6N peptomer did not show dose-dependent inhibition of YopE secretion (Fig. 4B), consistent with the inability of this compound to inhibit YopE secretion at 60  $\mu\text{M}$  (Fig. 2D). These data suggest that specific peptoid substitutions in the epiphepropeptin scaffold improve the bioactivity of peptomers as T3SS inhibitors.

We observed that several of the cyclic peptomers formed aggregates over time when they were added to culture media. We set out to measure the amounts of

**TABLE 1** Cyclic peptomer data summary<sup>h</sup>

Compound	YopE secretion (%) <sup>a</sup>		ExoU secretion (%) <sup>b</sup>		YopM-Bla translocation (%) <sup>c</sup>	Concn ( $\mu$ M) with cell rounding <sup>d</sup>	Concn ( $\mu$ M) with cell toxicity <sup>e</sup>	% reduction in motility <sup>f</sup>	Growth <sup>g</sup>
	60 $\mu$ M	30 $\mu$ M	60 $\mu$ M	30 $\mu$ M					
EpD-1N	30	18	17	21	62	NE	All	NE	NE
EpD-1'N	51	ND	ND	ND	ND	ND	ND	ND	ND
EpD-2N	43	ND	ND	ND	ND	ND	ND	ND	ND
EpD-3N	55	ND	ND	ND	ND	ND	ND	ND	ND
EpD-3'N	21	30	11	17	59	4	NE	NE	NE
EpD-4'N	28	82	13	84	75	2	>102	NE	NE
EpD-1,2N	21	25	19	15	54	NE	NE	NE	NE
EpD-1,3'N	36	69	21	21	51	2	NE	NE	NE
EpD-1,2,3'N	26	31	18	14	51	2	NE	20	NE
EpD-1,2,4'N	17	14	18	12	56	2	NE	NE	NE
EpD-1,2,3',4'N	16	27	14	12	65	2	NE	NE	NE
EpD-6N	94	ND	ND	ND	ND	ND	ND	ND	ND
EpD-6'N	76	ND	ND	ND	ND	ND	ND	ND	ND

<sup>a</sup>*Yersinia* YopE secretion with 60 or 30  $\mu$ M peptomers compared to that with DMSO (100%).

<sup>b</sup>*Pseudomonas* ExoU secretion with 60 or 30  $\mu$ M peptomers compared to that with DMSO (100%).

<sup>c</sup>YopM-Bla translocation into CHO-K1 cells treated with 7.5  $\mu$ M peptomers, normalized to that with DMSO (100%).

<sup>d</sup>Lowest concentration of peptomers at which cell rounding in HeLa cells was statistically significantly reversed.

<sup>e</sup>Concentration of compound showing cytotoxicity in HeLa cells by the MTT assay.

<sup>f</sup>Percent reduction in *Yersinia* motility compared to that with DMSO (100%).

<sup>g</sup>*Yersinia* pYV<sup>-</sup> growth at 37°C over 8 h compared to that of the DMSO control.

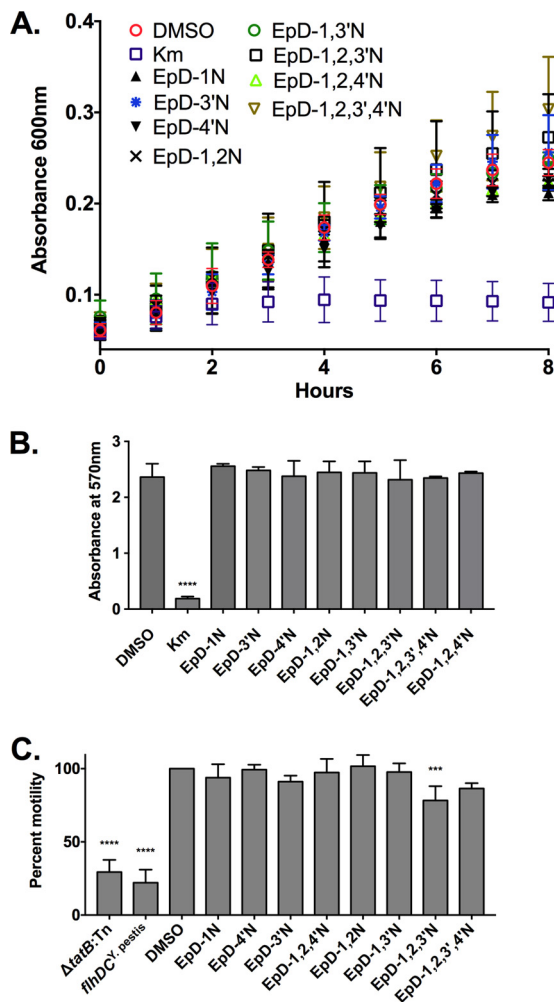
<sup>h</sup>NE, no effect (not significantly different from the DMSO control); ND, not determined.

monomeric peptomers in bacteriological and cell culture media. The solubility of peptomers able to inhibit type III secretion ranged from 1 to 84  $\mu$ M (Fig. S4). To determine whether cyclic peptomer aggregates were responsible for the observed T3SS-inhibitory activity, we sought to disaggregate the compounds and test their activity by adding the detergent Triton X-100 to bacterial cultures during type III secretion. Concentrations of Triton X-100 of >0.005% (vol/vol), in the absence of cyclic peptomers, led to enhanced Yop release into the culture media (Fig. S5A), perhaps as a result of cell envelope leakiness or perturbations to normal T3SS regulation with high levels of detergent. Therefore, we chose to carry out our disaggregation experiment with 0.001% (vol/vol) Triton X-100, as this concentration of detergent did not cause enhanced Yop release but has been shown to be effective at inhibiting nonspecific activity due to aggregate formation (31) and to give a decreased absorbance at 600 nm when added to cyclic peptomers (data not shown). In the presence of 0.001% Triton X-100, EpD-1,2,4'N showed activity comparable to that of the no-detergent control (Fig. 4; Fig. S5B), suggesting that peptomer aggregates that can be disrupted by 0.001% Triton X-100 are not required for T3SS-inhibitory activity.

In order to rule out nonspecific interaction with protein as the cause of the T3SS-inhibitory activity observed for the cyclic peptomers, we carried out the Yop secretion assay in the presence of increasing concentrations of bovine serum albumin (BSA). We observed no effect of BSA on secretion inhibition by EpD-1,2N, and the presence of BSA did not shift the dose-response curve for EpD-1,2N (Fig. S6). These data indicate that nonspecific protein binding does not underlie the T3SS-inhibitory activity of the cyclic peptomers.

**Cyclic peptomers do not affect *Yersinia* growth or flagellar motility.** To determine whether the peptomers affect type III secretion specifically or have a broader impact on *Yersinia* physiology, we monitored bacterial growth and metabolic activity following drug treatment. Active type III secretion, induced under low-calcium conditions at 37°C *in vitro*, causes growth arrest, complicating analysis of bacterial growth. Therefore, we used a strain of *Y. pseudotuberculosis* lacking the T3SS-encoding virulence plasmid pYV (*Yersinia* pYV<sup>-</sup>). Kanamycin was used as a positive control because it is known to inhibit *Yersinia* growth. In the presence of 60  $\mu$ M cyclic peptomers, *Yersinia* pYV<sup>-</sup> grew similarly to the DMSO-treated control, while kanamycin inhibited growth (Fig. 5A). Furthermore, in the presence of a high concentration of cyclic peptomers (120





**FIG 5** Cyclic peptomers do not affect *Yersinia* growth or motility. (A) *Yersinia* pYV<sup>-</sup> was grown in low-calcium medium at 37°C in the presence of 60  $\mu$ M cyclic peptomer, an equivalent volume of DMSO, or kanamycin. The bacterial density over time was measured by determining the absorbance at 600 nm. (B) *Yersinia* pYV<sup>-</sup> was grown in low-calcium medium at 37°C in the presence of 120  $\mu$ M cyclic peptomer, an equivalent volume of DMSO, or kanamycin for 24 h. MTT was added, and the absorbance at 570 nm was used as a readout of bacterial metabolic activity. (C) Cyclic peptomers were added to motility agar plates at 60  $\mu$ M, and the diameter of WT *Yersinia* colonies was measured as a readout of flagellar motility. Averages for three independent experiments are shown. Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ .

$\mu$ M), no significant difference in metabolic activity was detected compared to that of the DMSO-treated control, while kanamycin induced a significant reduction in metabolic activity (Fig. 5B). These results suggest that, unlike conventional antibiotics, peptomers do not inhibit bacterial replication or metabolic activity, yet they inhibit type III secretion in *Yersinia*.

The flagellar basal body, which is a T3SS, is structurally related to the injectisome T3SS (32). For *Y. enterocolitica*, the proton motive force was shown to be required for both type III secretion and motility (33). In addition, the twin-arginine translocation (Tat) secretion system is required for motility in *Y. pseudotuberculosis* and requires the proton motive force (34, 35). Therefore, compounds that affect the proton motive force or the Tat system should affect motility. To determine if the cyclic peptomers affect either of these processes, WT *Y. pseudotuberculosis* was spotted on soft motility agar with 60  $\mu$ M cyclic peptomers or an equivalent volume of DMSO. A *Y. pseudotuberculosis* mutant carrying the inactive *Y. pestis* allele of the *flhDC* flagellar master regulator genes (*flhDC<sup>Y. pestis</sup>*) and a Tat mutant (*tatB::Tn*) were used as negative controls. Colony diameters were measured and

normalized to that of the DMSO control (100%). At 60  $\mu\text{M}$ , the cyclic peptomers did not affect *Yersinia* motility compared to that with DMSO, except for EpD-1,2,3'N, which reduced motility by 20% ( $P = 0.0005$ ) (Fig. 5C). These results suggest that cyclic peptomers do not inhibit type III secretion by targeting the Tat secretion system, the flagellar T3SS, or the proton motive force.

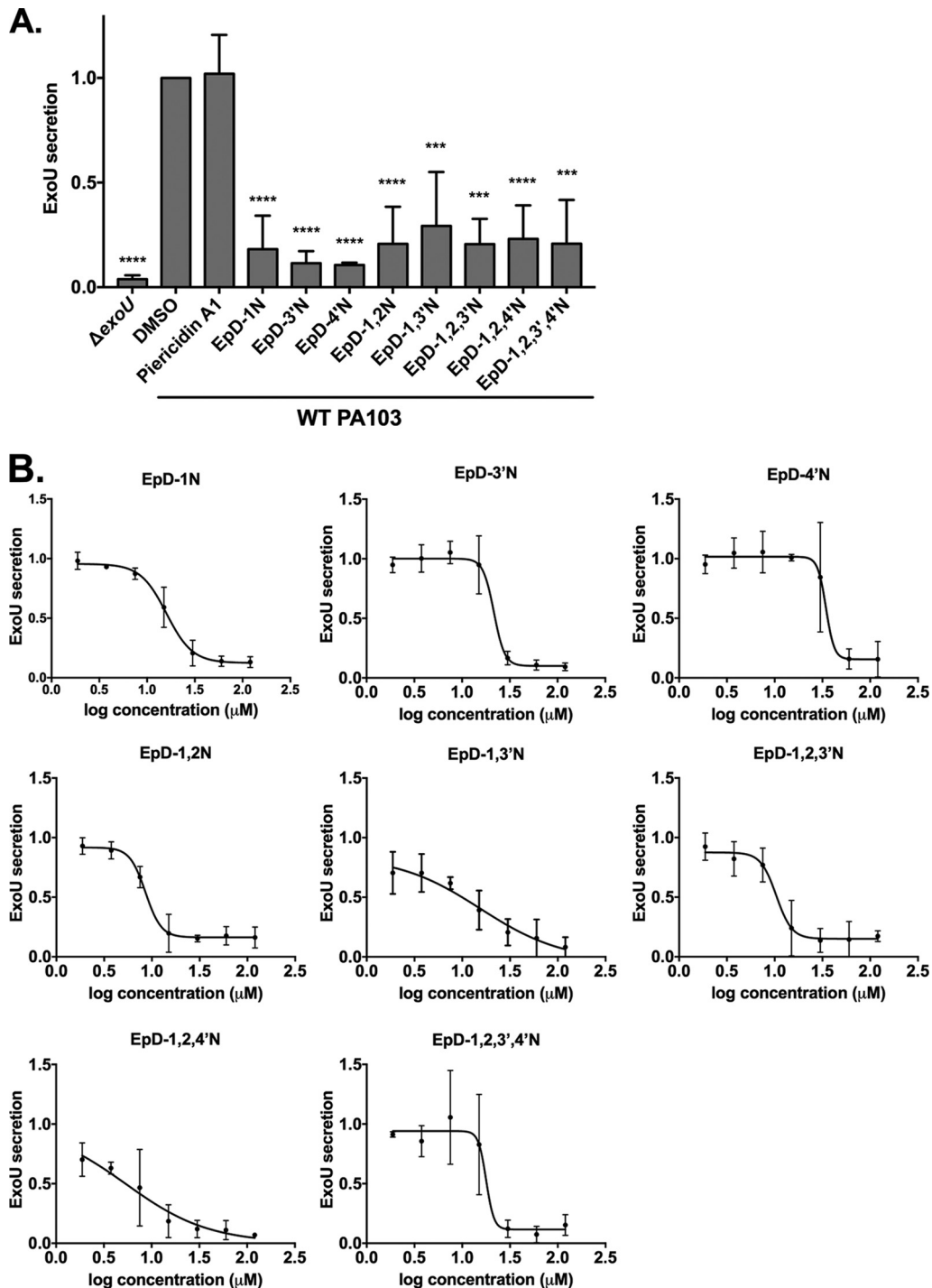
In order to assess the ability of the cyclic peptomers to disrupt flagellar T3SS activity in liquid culture over a shorter period more comparable to that of the T3SS secretion conditions, we assessed the motility of *Salmonella* and *Pseudomonas* in liquid culture after 2 to 3 h of growth in the presence or absence of cyclic peptomers. These bacteria were chosen because, unlike *Yersinia*, they express flagella at 37°C (36, 37). As the bacterial culture increased in density 32-fold during this time, new flagellar synthesis should have occurred in the presence of the cyclic peptomers. However, we observed no difference in swimming distance, speed, or percentage of motile bacteria (Fig. S7). These data suggest that cyclic peptomers do not nonspecifically block the assembly or activity of the flagellar T3SS or, more broadly, the functions of proteinaceous appendages in bacteria, other than the injectisome T3SS.

**Cyclic peptomers inhibit type III secretion in *Pseudomonas aeruginosa*.** To determine whether the cyclic peptomers specifically blocked type III secretion in *Yersinia* or were active against the T3SS of other pathogens, we tested their bioactivity in *P. aeruginosa*. We found that the cyclic peptomers at 60  $\mu\text{M}$  significantly reduced secretion of the effector protein ExoU compared to the DMSO control (Fig. 6A). In addition, these compounds showed a dose-response inhibitory effect (Fig. 6B; Table 1). At 30  $\mu\text{M}$ , EpD-3'N, EpD-1,2N, EpD-1,2,3'N, EpD-1,2,4'N, and EpD-1,2,3',4'N reduced secretion of ExoU to less than 20% of that with DMSO, which was normalized to 100%. These data suggest that five cyclic peptomers, EpD-3'N, EpD-1,2N, EpD-1,2,3'N, EpD-1,2,4'N, and EpD-1,2,3',4'N, strongly inhibit type III secretion in both *Yersinia* and *Pseudomonas*.

**Cyclic peptomers inhibit T3SS effector protein translocation and activity but are not toxic to host cells.** To test whether the cyclic peptomers affect mammalian cell physiology, we evaluated HeLa cell metabolic activity following exposure to the compounds (Fig. 7). TPEN, a known cytotoxic compound (38–40), was used as a positive control and showed toxicity at concentrations of  $>3.75 \mu\text{M}$ , in agreement with previous findings (38, 39). Most cyclic peptomers were not cytotoxic to HeLa cells, except for EpD-4'N, which showed toxicity at concentrations of  $>102 \mu\text{M}$ . In addition, EpD-1N-treated cells displayed low absorbance in the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay at all concentrations, for reasons that are unclear but may reflect the compound's physical properties under these conditions. These data suggest that the majority of the cyclic peptomers are not toxic to mammalian cells.

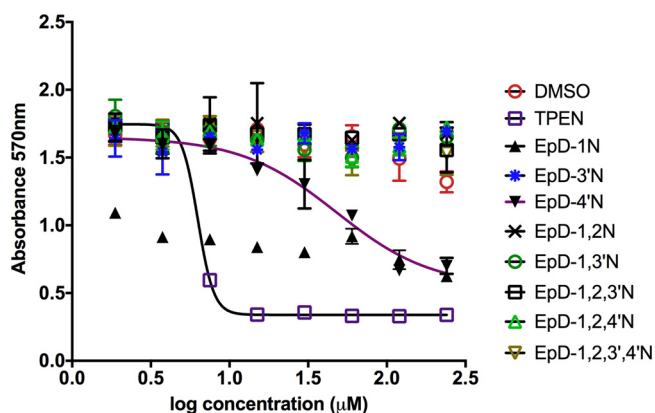
We tested the ability of the cyclic peptomers to inhibit translocation of the *Y. pseudotuberculosis* effector YopM, translationally fused to a reporter  $\beta$ -lactamase (YopM-Bla), into CHO-K1 cells. Infected cells were loaded with the fluorescent  $\beta$ -lactamase substrate CCF2-AM, and green (uncleaved) versus blue (cleaved) fluorescence was monitored. All eight peptomers significantly reduced YopM-Bla translocation into CHO-K1 cells at 7.5  $\mu\text{M}$  (Fig. 8A). DMSO affected the percentage of blue cells at high concentrations (Fig. S2). Thus, we chose 7.5  $\mu\text{M}$  because the volume of DMSO necessary to achieve this final concentration does not exceed the maximum tolerable dose of DMSO for this assay.

We next tested the ability of the cyclic peptomers to inhibit the ability of translocated *Yersinia* effector proteins to target the host actin cytoskeleton. As a result of YopEHO activity on the actin cytoskeleton, HeLa cells become rounded following *Yersinia* infection and YopEHO translocation. HeLa cells were treated with increasing concentrations of cyclic peptomers during WT *Y. pseudotuberculosis* infection at an MOI of 40, and the total cellular area was quantified as a measure of cell rounding (Fig. 8B). EpD-3'N and EpD-1,2,4'N reversed cell rounding induced by WT *Yersinia* with increasing



**FIG 6** Cyclic peptomers inhibit type III secretion in *Pseudomonas aeruginosa*. WT *Pseudomonas* PA103 was grown under T3SS-inducing conditions with 60  $\mu\text{M}$  cyclic peptomers (A), cyclic peptomers at concentrations ranging from 1.9  $\mu\text{M}$  to 120  $\mu\text{M}$  (B), or an equivalent volume of DMSO. Secretion of T3SS cargo into the culture supernatant was assessed by precipitating secreted proteins and visualizing them with Coomassie blue. ExoU band intensities were quantified and normalized to that of the DMSO control. Piericidin A1, which does not inhibit T3SS secretion in *P. aeruginosa*, was used as a negative control. (A) Data were analyzed by one-way ANOVA with Dunnett's multiple comparison test. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ . (B) The results are averages for at least two independent experiments. Nonlinear curve fitting is shown to depict the trend of inhibition.

concentrations of these compounds, starting at 3.75  $\mu\text{M}$  and 1.875  $\mu\text{M}$ , respectively (Fig. 8B and C). EpD-4'N, EpD-1,3'N, EpD-1,2,3'N, and EpD-1,2,3,4'N reversed cell rounding to some degree at lower concentrations but enhanced rounding at higher concentrations, indicating a possible effect on the actin cytoskeleton at the higher



**FIG 7** The majority of cyclic peptomers are not toxic to mammalian cells. HeLa cells were treated with increasing concentrations of cyclic peptomers or an equivalent volume of DMSO for 24 h. MTT was added to the culture, and the absorbance at 570 nm was used as a readout of cellular metabolism. TPEN, a known cytotoxic compound, was used as a control. Data shown are the averages for two independent biological replicates.

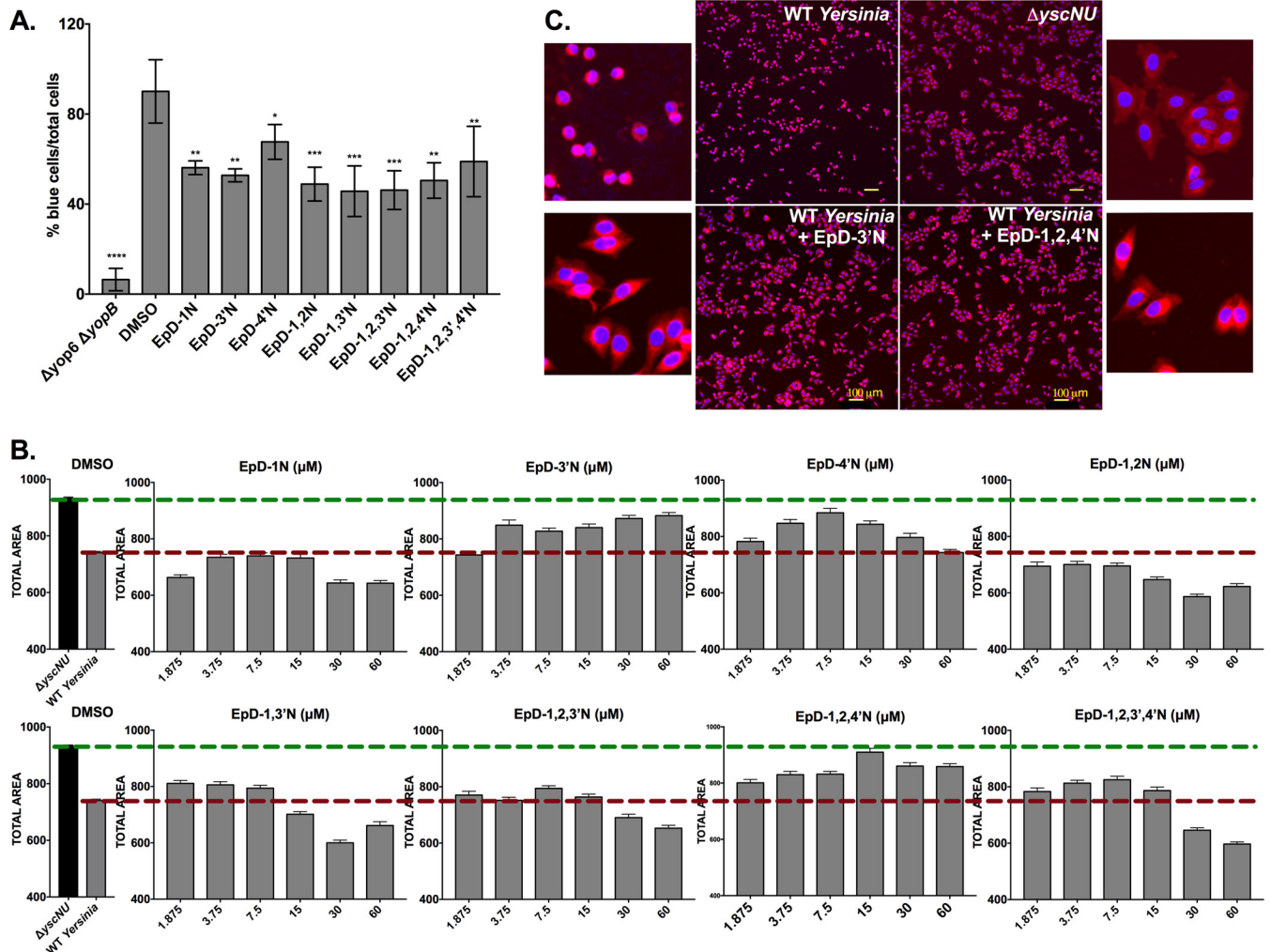
concentrations. EpD-1N and EpD-1,2N enhanced rounding at almost all concentrations tested, indicating an even greater off-target effect. These data suggest that while some of the cyclic peptomers may affect mammalian cell physiology, EpD-3'N and EpD-1,2,4'N inhibit the activity of Yop effector proteins on the actin cytoskeleton. This is especially important because the activity of these effectors is critical for *Yersinia* to avoid important host defense mechanisms and cause disseminated disease (41).

## DISCUSSION

Here we present a HEK293 stable cell line expressing an NF- $\kappa$ B-GFP reporter that enables high-throughput screening for inhibitors of the bacterial T3SS. We used this cell line to identify a novel set of T3SS inhibitors, i.e., synthetic peptomer analogs of the natural product phepropeptin. We found that a subset of these compounds inhibit type III secretion in both *Yersinia* and *Pseudomonas* as well as limit translocation of *Yersinia* effector proteins inside target host cells and, consequently, their activity on host cell processes. Importantly, the majority of these compounds do not affect bacterial growth, mammalian cell metabolism, or flagellar motility, indicating a specific effect on the T3SS.

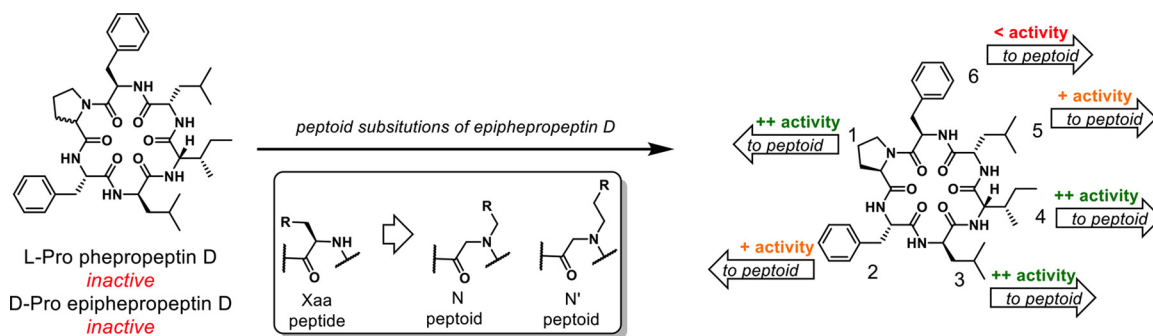
Cyclic peptides may be particularly well suited for novel therapeutic strategies, including the targeting of virulence factors. The rich structural diversity of cyclic peptides and their larger size than that of traditional drugs may allow greater potency and specificity than those of typical small molecules (42). Oligomers of N-substituted glycine units ("peptoids") were originally conceived as synthetic peptide derivatives that possess the modularity and protein binding characteristics of peptides but avoid the pitfalls associated with the notoriously poor metabolic stability of peptides (43, 44). Peptide-to-peptoid substitutions can increase or maintain the ability of cyclic peptides to permeate eukaryotic cells while enabling greater side chain diversity than that in the pool of commercially available amino acids (45). The phepropeptins are cyclic hexapeptides isolated from *Streptomyces* sp. and previously characterized for their activity as weak proteasome inhibitors (29). It was shown previously that stereo-inversion of the Pro residue to form epiphepropeptin (EpD) significantly altered the compound conformation as determined by nuclear magnetic resonance (NMR) analysis (28). Thus, it is unlikely that the compounds described here as possessing T3SS-inhibitory activity would retain the proteasomal inhibition of the natural product. In addition, *Yersinia* and *Pseudomonas* lack a proteasome. This suggests that phepropeptin compared to EpD and its cyclic peptomer analogs has distinct bioactivities and that proteasome inhibition does not underlie the T3SS-inhibitory activity of the EpD cyclic peptomers.

Peptides as a class have rich antimicrobial activities, such as that of antimicrobial



**FIG 8** Cyclic peptomers block translocation of a T3SS effector protein into host cells and protect host cells from T3SS effector activity. (A) *Yersinia* carrying a Yop effector- $\beta$ -lactamase fusion ( $\Delta$ yop6/YopM-Bla) was used to infect CHO-K1 cells at an MOI of 5 in the presence of 7.5  $\mu$ M peptomer or an equivalent volume of DMSO. CCF2-AM was added, and the percentage of cells with cleaved CCF2-AM (blue) among the total number of cells (green) was quantified. Data shown are averages for 3 independent experiments. (B) HeLa cells were infected with WT *Yersinia* at an MOI of 40 in the presence of increasing concentrations of peptomers for 3.5 h. *Yersinia* lacking a T3SS ( $\Delta$ yscNU) was used as a negative control. Total cell area was quantified as a measure of cell rounding, which is a readout of host actin cytoskeleton modulation by the *Yersinia* T3SS YopEHO effector proteins. Median total area values for all imaged cells were plotted. Data shown are averages for two independent replicates with three technical replicates each. (C) Micrographs of HeLa cells infected with the  $\Delta$ yscNU mutant or WT *Yersinia* and treated with EpD-3'N, EpD-1,2,4'N, or DMSO.

peptides (AMPs), which are naturally produced by host cells to protect themselves from pathogens (46). AMPs kill a wide range of species, including fungi and viruses (46–48) as well as Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Pseudomonas aeruginosa* (49). However, AMPs, which are ribosomally produced, exhibit unfavorable properties for use as antimicrobial therapies, such as susceptibility to degradation, cytotoxicity to host cells, instability with heat or enzymes, and a high cost of production (50, 51). It might be worth noting that most nonribosomal antimicrobial peptides, such as polymyxin, fusaricidin, and daptomycin, are “lipopeptides” which contain a mixture of charged residues and aliphatic tails and act by disrupting cell membrane or cell wall function (52, 53). In contrast, our peptides contain only aliphatic groups and are reminiscent of natural products that inhibit intracellular targets, such as griselimycin, which targets DnaN of *Mycobacterium tuberculosis* (54). Whether the peptomers can cross the bacterial membrane remains to be tested. Peptide analogues of a native autoinducing peptide signal in *Staphylococcus aureus* were shown to be potent at inhibiting quorum sensing and attenuating the virulence of the bacterium



**Generation One**

Compound	Position						YopE Secretion 60 μM	
	1	2	3	4	5	6	N Peptoid	N' Peptoid
EpD-1N or 1'N	Naa*	AA	AA	AA	AA	AA	0.32	0.51
EpD-2N or 2'N	AA	Naa*	AA	AA	AA	AA	0.43	0.70
EpD-3N or 3'N	AA	AA	Naa*	AA	AA	AA	0.55	0.21
EpD-4'N	AA	AA	AA	Naa'	AA	AA	not tested	0.26
EpD-5'N	AA	AA	AA	AA	Naa'	AA	not tested	0.48
EpD-6N or 6'N	AA	AA	AA	AA	AA	Naa*	0.94	0.76

Naa\*= N peptoid or N' peptoid, Naa' = N' peptoid, AA=amino acid

**Generation Two**

Position	Position						YopE Secretion 15 μM
	1	2	3	4	5	6	
EpD-1,2N	Naa	Naa	AA	AA	AA	AA	45%
EpD-1,3'N	Naa	AA	Naa'	AA	AA	AA	68%
EpD-1,2,3'N	Naa	Naa	Naa'	AA	AA	AA	52%
EpD-1,2,4'N	Naa	Naa	AA	Naa'	AA	AA	44%
EpD-1,2,3',4'N	Naa	Naa	Naa'	Naa'	AA	AA	35%

Naa= N peptoid, Naa' = N' peptoid, AA=amino acid

**FIG 9** Structure-activity relationships and structural descriptions of peptoid analogs of epiphepropeptin D. N and N' peptoids differ by a single -CH<sub>2</sub> group in the peptoid chain. The YopE secretion assay was used to evaluate potency, with the first- and second-generation sets analyzed at 60 μM and 15 μM, respectively. Secretion inhibition was colored to highlight potency differences between compounds.

(55). Amide-bridged peptide analogues were more enzymatically stable than the peptides while still showing moderate quorum sensing inhibition (56). Our finding provides the first evidence suggesting that peptide-peptoid derivatives (peptomers) can act as T3SS inhibitors. Furthermore, our compounds contain peptoid positions that allow for facile diversification, making further optimization of these compounds synthetically tractable.

Our results suggest that the assay for T3SS inhibition with the largest dynamic range is the secretion assay, for both *Yersinia* and *Pseudomonas* T3SS effector proteins. In the absence of a standard measurement of compound activity, such as the MIC typically determined for classical antibiotics that inhibit bacterial growth or viability, we relied mainly on the secretion assay to determine the relative potencies of cyclic peptomers. When the single peptoid substitutions are compared to the parent compound, it appears that positions 1, 3, and 4 are the most important peptoid positions for activity. Although not every peptoid substitution was tested as both the N-peptoid and the chain-elongated N'-peptoid (Fig. 9), there appears to be a preference for one side chain over the other in all matched pairs, especially for position 3, with EpD-3'N inhibiting 79% of secretion while EpD-3N inhibits only 45% of secretion at 60 μM (Fig. 2 and Table 1),

hinting at some specificity of interaction. While the peptoids with single substitutions at positions 1, 3, and 4 display high levels of YopE secretion inhibition at 60  $\mu\text{M}$ , none of them show >50% inhibition when diluted to 15  $\mu\text{M}$ . When both positions 1 and 2 are replaced with a peptoid, the potency is increased compared to those for both individual substitutions and the 1,3 combination (Fig. 4 and Table 1). The peptomers containing substitutions at both positions 1 and 2 all inhibit secretion >50% at 15  $\mu\text{M}$ , with EpD-1,2,4'N and EpD-1,2,3',4'N showing the most inhibition at this concentration. As several cyclic peptomers were found to perturb the host actin cytoskeleton and a subset of these disrupted mammalian cell metabolism, further derivatization is warranted to mitigate such off-target effects while improving activity. Importantly, the activities displayed by the cyclic peptomers are comparable to those of other known T3SS inhibitors, such as piericidin A1 (active at 71  $\mu\text{M}$ ) (26), *N*-hydroxybenzimidazole derivatives (50% inhibitory concentration [ $\text{IC}_{50}$ ] in the range of 3 to 70  $\mu\text{M}$ ) (57), various T3SS ATPase inhibitors ( $\text{IC}_{50}$  in the range of 17 to 70  $\mu\text{M}$ ) (58), and the salicylidene acylhydrazide INP0010 (active at 40  $\mu\text{M}$ ), for which three molecular targets have been identified (59).

Aggregation effects have been attributed to nonspecific or promiscuous inhibitor activity (60). Most of the active cyclic peptomers have solubility values of <30  $\mu\text{M}$ , while secretion inhibition was observed at higher concentrations. However, detergent disaggregation of compounds did not ablate T3SS-inhibitory activity, indicating that aggregate formation is not essential for T3SS inhibition. In addition, adding BSA to bacteria undergoing type III secretion in the presence of compounds did not abolish the compounds' activity, indicating that secretion inhibition was not due to nonspecific protein binding. Taken together, our data show that it is possible that both monomeric cyclic peptomers and peptomer aggregates are able to inhibit secretion. Alternatively, the active moiety, perhaps of a specific aggregate size, may be present at a range of cyclic peptomer concentrations as well as in the presence of Triton X-100. Without further testing (e.g., using dynamic light scattering), the observed T3SS inhibition in the presence of detergent and BSA does not formally rule out the possibility that some degree of aggregation plays a role in these compounds' activity. Future medicinal chemistry optimization will be aimed at improving both inhibitory potency and solubility, for example, by replacing one or more of the peptoid side chains with more polar groups.

The proton motive force is important for the *Yersinia* flagellar T3SS, as the proton motive force inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) prevents *Yersinia* motility (33, 61). Yet the majority of our compounds do not affect flagellar motility. This suggests that the cyclic peptomers do not inhibit type III secretion by perturbing the proton motive force. Since the Tat secretion system has been shown to be essential for *Yersinia* type III secretion (34), our data also indicate that the cyclic peptomers do not generally block bacterial secretion systems. Furthermore, the cyclic peptomers inhibit type III secretion in both *Yersinia* and *Pseudomonas* but do not block flagellar motility in *Yersinia*, *Pseudomonas*, or *Salmonella*. The flagellar and injectisome T3SS share eight conserved components, including an ATPase and several proteins that make up basal body membrane rings (62). However, over a dozen other T3SS components are conserved between *Yersinia* and *Pseudomonas* but are divergent or absent from the flagellar T3SS. Our data predict that the cyclic peptomers act on such a target, such as the SctF needle subunit. The activity of the cyclic peptomers is in contrast to that of the T3SS inhibitor piericidin A1, which inhibits the *Yersinia* Ysc T3SS but is inactive against the *Pseudomonas* T3SS (61). The *Pseudomonas* cell envelope is particularly exclusive to chemicals (63); the activity of the cyclic peptomers on the *Pseudomonas* T3SS suggests that these compounds either act on an extracellular T3SS component or can gain access to the *Pseudomonas* periplasm or cytoplasm. The specific mechanism of action of the cyclic peptomers will be the subject of another study.

In summary, the HEK293-GFP stable cell line and high-throughput screen reported here provide a rapid and reproducible platform for T3SS inhibitor discovery. Using this strategy, we have identified a new class of T3SS inhibitors, synthetic derivatives of the

**TABLE 2** Bacterial strains used in this study

Strain	Description	Reference
<i>Y. pseudotuberculosis</i> strains		
Wild type (WT)	Strain IP2666	68
$\Delta yop6$	$\Delta yopHEMOJ$ mutant	25
$\Delta yop6 \Delta yopB$	$\Delta yopHEMOJB$ mutant	25
$\Delta yop6/pYopM-Bla$	$\Delta yopHEMOJ$ mutant carrying pYopM-Bla	64
$\Delta yop6 \Delta yopB/pYopM-Bla$	$\Delta yopHEMOJB$ mutant carrying pYopM-Bla	64
$\Delta yscNU$	Deletion of <i>yscNU</i> operon	69
pYV <sup>-</sup>	IP2666 cured of its virulence plasmid	69
<i>tatB::Tn</i>	$\Delta yop6$ with a <i>tatB::TnHimar1</i> insertion	This study
<i>flhDC<sup>Y. pestis</sup></i>	$\Delta yop6$ with inactive <i>Y. pestis flhDC</i>	64
<i>Pseudomonas aeruginosa</i> strains		
Wild type	Strain PA103	70
$\Delta exoUT$	$\Delta exoU \Delta exoT$ mutant	71
PAO1	PAO1	72
<i>Salmonella</i> Typhimurium strains		
Wild type	Strain LT2	73
$\Delta flhDC$	TH5736 ( $\Delta flhDC-motAB-cheAW-tar$ )	

natural product phepropeptin, that significantly decrease type III secretion in *Y. pseudotuberculosis* and *P. aeruginosa* without affecting bacterial survival, motility, or other secretion systems, and without host cell toxicity. Future studies will focus on compound EpD-1,2,4'N, as this compound inhibited type III secretion in both *Yersinia* and *Pseudomonas* and blocked the native activity of *Yersinia* T3SS effector proteins on target host cells.

## MATERIALS AND METHODS

**Bacterial strains and mammalian cells used in this study.** The bacterial strains and cell lines used in this study are listed in Table 2. *Y. pseudotuberculosis* was grown in 2× YT (2× yeast extract and tryptone) at 26°C with shaking at 250 rpm overnight, unless otherwise noted. The cultures were back diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.2 in low-calcium medium (2× YT with 20 mM sodium oxalate and 20 mM MgCl<sub>2</sub>) or M9 medium supplemented with Casamino Acids to induce the T3SS (64). Cultures were grown for 1.5 h at 26°C with shaking at 250 rpm. Compounds or DMSO was added to bacterial cultures prior to shifting to 37°C to induce T3SS synthesis. *Pseudomonas aeruginosa* was grown in Luria-Bertani medium (LB) at 37°C with shaking at 250 rpm overnight. Low-calcium medium (LB with 20 mM EGTA and 20 mM MgCl<sub>2</sub>) was used to induce the T3SS.

HeLa (ATCC), HEK293 (ATCC), and HEK293-GFP (described below) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). CHO-K1 cells (ATCC) were cultured in F-12K medium with 10% FBS. All cell lines were incubated at 37°C with 5% CO<sub>2</sub>.

**Stable cell line generation.** A promoter containing five repeats of an NF-κB enhancer element (5'-TGGGGACTTCCGC-3') (26) was cloned upstream of the GFP gene, followed by integration of the construct into the HEK293 chromosome. The primers 5'-ATTAGAGCTCTGCAATTGTTGTTAACTTGTTT ATT-3' (forward primer; SacI restriction site is underlined) and 5'-ATTAAGCTTTATATACCCTCTAGAGTCTCCGCG-3' (reverse primer; HindIII restriction site is underlined) were used to clone the NF-κB reporter construct (26) into the multicloning site of pTurboGFP-PRL-dest1, containing a gene encoding a destabilized variant of green fluorescent protein (TurboGFP; Evrogen, Russia). The plasmid was linearized with BsaI and transfected into HEK293 cells by use of Lipofectamine 2000 (Invitrogen). Neomycin was used to select for cells carrying the plasmid, as previously described (65). Single cells were grown in DMEM supplemented with fresh neomycin every 2 weeks until they reached confluence in 96-well plates. Monoclonal NF-κB-driven GFP-expressing HEK293 cells were tested for their NF-κB response to the *Yersinia* T3SS, and positive clones were transferred to 25-cm<sup>2</sup> and then 75-cm<sup>2</sup> flasks and cryotubes. One clone, HEK293-GFP, was selected and used for the screen described below.

**NF-κB screen for T3SS inhibitors.** HEK293-GFP cells were plated on poly-L-lysine-coated 384-well plates at a density of  $1.2 \times 10^4$  cells/well in 25 μl FluoroBrite DMEM (Thermo Fisher Scientific). *Y. pseudotuberculosis*  $\Delta yop6$  or  $\Delta yop6 \Delta yopB$  (Table 2) overnight cultures were back diluted to an OD<sub>600</sub> of 0.2 in M9 medium supplemented with Casamino Acids and grown for 1.5 h with shaking at 250 rpm and 26°C. Bacteria were transferred to 384-well plates. Compounds were added to bacteria prior to shifting the plate to 37°C, as well as to cell monolayers prior to infection by use of a pinning robot (Janus MDT robot; PerkinElmer). After 2 h of incubation at 37°C, bacteria were added to cell monolayers at an MOI of 7 (Janus MDT robot), and the infected cell plates were incubated for 5 h at 37°C and 5% CO<sub>2</sub>. Cells were stained with the nucleic acid dye Hoechst 33342 (Thermo Fisher Scientific) and fixed with 1% paraformaldehyde (PFA; Sigma-Aldrich) on ice for 10 min. Plates were washed with phosphate-buffered saline (PBS), and residual PFA was quenched by incubation with 0.1 M glycine for 30 min. Plates were washed



in PBS four times and imaged with a Molecular Devices Image Express instrument within 24 h. T3SS function was quantified as the percentage of GFP-expressing cells among the total number of cells (total nuclei).

**Cyclic peptide synthesis.** Peptides were synthesized using standard Fmoc solid-phase peptide synthesis, utilizing the submonomer approach for peptoid synthesis (66), either at room temperature or with microwave assistance. Cyclization was done in solution at a high dilution.

**Loading of 2-chlorotrityl resin.** Fmoc-Xaa (10 mmol) was added to a flame-dried round-bottomed flask and dried in a vacuum desiccator with phosphorous pentoxide overnight. Fifty milliliters of dry dichloromethane (DCM) was cannula transferred into the flask, followed by 2.5 ml of *N,N*-diisopropylethylamine (DIPEA) transferred via syringe. After sonication for 10 min, 5 g of 2-chlorotrityl resin was added under a stream of N<sub>2</sub> and allowed to shake for 4 h. The resin was capped with a 15-ml solution of 1:2:17 methanol (MeOH):DIPEA:dimethylformamide (DMF) (3 times for 15 min each). The resin was washed with DMF (3 times with 15 ml each) followed by DCM (3 times with 15 ml each). The loading value was calculated by determining the mass increase of dried, loaded resin.

**Amino acid coupling at room temperature.** Four equivalents of Fmoc-Xaa, 5 eq of DIPEA, and 4 eq of *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) were added to the resin in DMF. The reaction mixture was agitated via shaking for 45 min and then drained. The resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by liquid chromatography-mass spectrometry (LC-MS) and repeated until the starting material was no longer observed. For microwave conditions, a solution of 4 eq of Fmoc-Xaa, 4 eq of HBTU, and 6 eq of DIPEA in DMF was allowed to prereact for 5 min. This solution was added to the deprotected peptide on-resin and allowed to react for 10 min at 50°C under microwave heating. The solution was drained, and the resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by LC-MS and repeated until the starting material was no longer observed.

**Coupling of BrAcOH at room temperature.** A solution of 5 eq of bromoacetic acid (BrAcOH) and 5 eq of *N,N'*-diisopropylcarbodiimide (DIC) in DMF was allowed to prereact for 10 min. This solution was added to the deprotected peptide on-resin. The reaction mixture was agitated via shaking for 45 min and then drained. The resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by LC-MS and repeated until the starting material was no longer observed. For microwave conditions, a solution of 5 eq of BrAcOH and 5 eq of DIC in DMF was allowed to prereact for 10 min. This solution was added to the deprotected peptide on-resin and allowed to react for 10 min at 50°C under microwave heating. The solution was drained, and the resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by LC-MS and repeated until the starting material was no longer observed.

**Installation of peptoid side chain.** A solution of 5 to 10 eq of the desired amine was prepared in a minimum volume of DMF. The resin containing the BrAc-peptide was swollen with DCM for 5 min prior to reaction. The amine was added, and the reaction mixture was agitated vigorously for 45 min. The solution was drained, and the resin was washed with DMF (3 times with 3 ml each) and DCM (3 times with 3 ml each). The reaction was monitored by LC-MS and repeated until the starting material was no longer observed.

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

**Peptide cleavage.** Complete linear peptides were cleaved off the resin in 5 resin volumes of 2.5% trifluoroacetic acid (TFA) in DCM for 4 min three times, with a 5-resin-volume DCM wash between steps. Solvent was removed under N<sub>2</sub>, followed by dissolution in acetone or DCM and evaporation under reduced pressure. Residual TFA was removed *in vacuo* overnight.

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

**Purification of peptides.** COMU by-products were removed after solution-phase cyclization on a Biotage Isolera Prime system equipped with a KP-C<sub>18</sub>-HS 12g column eluting with H<sub>2</sub>O-acetonitrile modified with 0.1% TFA. Peptides of insufficient purity were subsequently purified on a Waters mass-directed AutoPure system equipped with an X-Bridge BEH130 5- $\mu$ m 19  $\times$  150 C<sub>18</sub> column eluting with H<sub>2</sub>O-acetonitrile, modified with 0.1% formic acid or 0.1% TFA. The mass spectra of all peptides are shown in Fig. S3 in the supplemental material.

**Cyclic peptide manipulation.** Stock peptides were kept at 15 mM and were prediluted in DMSO prior to experiments. All the treatment and control pairs had the same DMSO volumes in all assays.

**Secretion assay.** Visualization of proteins secreted into the culture medium was carried out as described previously (25). Briefly, *Y. pseudotuberculosis* or *Pseudomonas aeruginosa* was grown in T3SS-inducing medium (as described above) at 37°C in the presence of peptomers or an equivalent volume of DMSO carrier for 2 h. The cultures were normalized based on bacterial density (OD<sub>600</sub>) and then centrifuged at 13,200 rpm for 15 min. The supernatants were transferred to a new tube, mixed with

6.1 N trichloroacetic acid (TCA) to a final volume of 10%, and vortexed vigorously for 30 s. Samples were incubated on ice for 30 min and then spun down at 13,200 rpm for 15 min at 4°C. The pellet was resuspended in final sample buffer (FSB) plus 20% dithiothreitol (DTT) and boiled for 5 min prior to running in a 12.5% or 7.5% SDS-PAGE gel for *Y. pseudotuberculosis* or *Pseudomonas aeruginosa*, respectively. Quantification of the bands relative to those of DMSO-treated controls was carried out using Image Lab software (Bio-Rad). The WT *Y. pseudotuberculosis* YopE or *Pseudomonas aeruginosa* ExoU bands in DMSO control samples were set to 1.00. Bovine serum albumin (BSA) or Triton X-100 (Sigma-Aldrich) was added to the bacterial culture at the same time as the compounds in a subset of secretion assays, as indicated in Results. Note that the amount of BSA used in the assays (32 µg/ml) approached the maximum amount of BSA possible, as more BSA resulted in BSA aggregation following TCA addition (data not shown).

**Translocation assay.** Translocation of T3SS effector proteins into mammalian cells was assayed as previously described (26). Briefly, 6,000 CHO-K1 cells were plated in each well of a 384-well plate in 50 µl of F-12K medium plus 10% FBS and incubated overnight at 37°C with 5% CO<sub>2</sub>. Overnight cultures of *Y. pseudotuberculosis* encoding a T3SS effector protein translocation reporter, YopM translationally fused with a β-lactamase (Bla), were back diluted to an OD<sub>600</sub> of 0.2 and grown at 26°C for 1.5 h with shaking. The cultures were diluted to obtain a multiplicity of infection (MOI) of 5 and then transferred to a 384-well plate. Peptomers or DMSO alone was added to the plates containing the bacterial cultures and incubated for 1.5 h at 37°C. Immediately prior to infection, the peptomers or DMSO was added to the CHO-K1 cells. Bacteria were then transferred to cells by use of a pinning robot (Janus MDT; PerkinElmer) and incubated for 1 h at 37°C and 5% CO<sub>2</sub>. The β-lactamase substrate CCF2-AM (Invitrogen) was added to each well, and the plate was incubated in the dark for 30 min at room temperature. Cells were fixed with 4% PFA for 20 min and then stained with the DNA dye DRAQ5 (Cell Signaling Technology) in PBS for 10 min. Plates were washed once with PBS and imaged using an ImageXpress automated microscope, and the images were analyzed using MetaXpress analysis software (Molecular Devices). Translocation was quantified as the number of YopM-Bla-positive cells (blue cells containing cleaved CCF2) divided by the total number of cells (green cells containing uncleaved CCF2).

**Growth curves.** Overnight cultures of *Y. pseudotuberculosis* pYV<sup>-</sup> (lacking the virulence plasmid that encodes the Ysc T3SS) were back diluted to an OD<sub>600</sub> of 0.1 in low-calcium medium, and 100 µl was added to each well of 96-well plates. This T3SS-deficient strain was used because active type III secretion induces growth arrest in *Yersinia*. Peptomers were added to each well, to a final concentration of 60 µM, in three technical replicates. Equivalent volumes of DMSO were added to the positive-control wells, and kanamycin was used as a negative control (50 µg/ml). Bacteria were maintained at 37°C, and the OD<sub>600</sub> values of the cultures were measured every hour for 8 h in a plate reader (VersaMax tunable microplate reader; Molecular Devices).

**Motility agar assay.** Motility agar plates (1% tryptone, 0.25% agar) supplemented with peptomers at a final concentration of 60 µM or with an equivalent volume of DMSO were prepared in triplicate. Motility was analyzed by spotting 1-µl aliquots of either nonmotile *Y. pseudotuberculosis* (*flhDC*<sup>Y. pestis</sup> or *tatB*::Tn) or motile bacteria (WT) onto the motility agar from overnight cultures standardized to an OD<sub>600</sub> of 2.5 in PBS. Plates were incubated at room temperature for 1 day. Colonies were imaged by use of a Chemidoc system (Bio-Rad), and the diameters of the colonies were determined in Image Lab (Bio-Rad) and used to calculate the percent motility relative to the motility with DMSO, which was set to 100%.

**Liquid motility assay.** *Salmonella* Typhimurium or *Pseudomonas aeruginosa* PAO1 was cultured in LB at 37°C with shaking at 250 rpm overnight. The cultures were then diluted to an OD of 0.025 in LB, and 30 µM peptomers or an equivalent volume of DMSO was added. An *S. Typhimurium* flagellar mutant ( $\Delta$ *flhDC*) (Table 2) was used as a negative control for motility. *S. Typhimurium* and PAO1 were filmed by use of a Hamamatsu C4742-95 digital camera and a Nikon Eclipse E600 microscope after 2 and 3 h of incubation, respectively. Ten-microliter aliquots of culture were spotted onto coverslips, and videos were taken every 1 s for 60 frames. Five videos, from the four corners and the center of the slide, were recorded for each sample. The movies were analyzed in Imaris v.8 and subsequently processed in Matlab2016a and GraphPad Prism 7. The movement observed for nonmotile bacteria was due to drift of the culture on the slide during the course of the movie, as all bacteria in the negative-control culture showed drift in the same direction. Bacteria were considered motile if they traveled a distance 2 standard deviations greater than the average nonmotile bacterial drifting distance.

**Cell viability.** The yellow tetrazolium MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] is reduced by metabolically active cells by the action of dehydrogenase enzymes, resulting in intracellular purple formazan. The MTT assay measures the cell proliferation rate, which correlates with formazan signals. To measure mammalian cell cytotoxicity in the presence of peptomers, HeLa cells were placed in 384-well plates at a density of 2,000 cells/well in triplicate and incubated at 37°C and 5% CO<sub>2</sub> for 24 h. Compounds were added to each well in a 2-fold dilution series, starting at 240 µM, and the cultures were incubated at 37°C and 5% CO<sub>2</sub> for 20 h. Ten microliters of MTT was added to each well, and plates were returned to the incubator for 4 h. Plates were washed once with PBS. Fifty microliters of ammonia-DMSO (67) was added to each well to solubilize the formazan crystals. Plates were incubated in the dark for 30 min at room temperature, and the absorbance at 570 nm was read in a plate reader (VersaMax tunable microplate reader; Molecular Devices).

To measure bacterial cell cytotoxicity in the presence of peptomers, *Y. pseudotuberculosis* pYV<sup>-</sup> overnight cultures were back diluted as described above for growth curves and then treated with compounds at a final concentration of 120 µM. The cultures were incubated at 37°C for 24 h. Ten microliters of MTT reagent was added to each well, and the plate was incubated for 2 h in the dark. One hundred microliters of 0.04 N HCl-DMSO (67) was added to each well and mixed thoroughly to solubilize

the formazan crystals. Plates were read at 570 nm in a plate reader (VersaMax tunable microplate reader; Molecular Devices) within a few minutes.

**Cell rounding.** HeLa cells were placed in 384-well plates at a density of 2,000 cells/well, centrifuged at 1,000 rpm for 5 min, and maintained at 37°C with 5% CO<sub>2</sub>. WT *Yersinia pseudotuberculosis*, which encodes the actin-targeting YopEHO effectors that cause cell rounding in host cells, was treated with peptomers in a 2-fold dilution series, starting at 120 μM, in low-calcium medium at 37°C for 2 h. HeLa cells were infected with WT *Yersinia* at an MOI of 40, and plates were spun at 750 rpm for 5 min and maintained at 37°C for 3 h. WT *Yersinia* treated with an equivalent amount of DMSO was used as a positive control, and the T3SS-deficient *ΔyscNU* mutant was used as a negative control for induction of cell rounding. Cells were stained with the plasma membrane stain CellMask deep red (Thermo Fisher Scientific) and the nuclear stain Hoechst 33342 (Thermo Fisher Scientific) for 20 min, fixed with 4% PFA (Sigma-Aldrich) for 10 min, washed with PBS, and imaged with a Molecular Devices Image Express system.

**Thermodynamic solubility assay.** Stocks (15 mM) of the compounds were prepared in DMSO. Each stock solution was dispensed into a 96-well plate and evaporated overnight under reduced pressure with heat (50°C). To the evaporated film/solid, 125 μl of DMEM was added to make a 2 mM solution of the target compound. This solution was sonicated for 1 h and then shaken at 37°C for 16 h. The turbid solution was passed over a 0.7-μm glass-fiber filter. The solution was diluted 1:4 in acetonitrile. The solution was centrifuged at 1,000 × *g* for 10 min. The supernatant (10 μl) was injected onto a Thermo Fisher Orbitrap Velos Pro instrument. A 10 μM standard was used for comparison, and the assay was done in triplicate.

**Kinetic solubility assay.** The kinetic solubility assay was done similarly to the thermodynamic solubility assay, except that the compounds were shaken for 2 h in the presence of 0.8% DMSO to mimic the conditions in most of our assays. The kinetic solubility assay mimics many of our assay conditions, in that compounds are predissolved in DMSO and tested after a short incubation time.

**Statistical analysis.** GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) was used to calculate the means, standard errors of the means, medians, standard errors of the medians, and one-way analysis of variance (ANOVA) values shown in the figures.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00060-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 5.1 MB.

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