**Small-Molecule Inhibitors of toxT Expression in Vibrio cholerae**

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**ABSTRACT** *Vibrio cholerae*, a Gram-negative bacterium, infects humans and causes cholera, a severe disease characterized by vomiting and diarrhea. These symptoms are primarily caused by cholera toxin (CT), whose production by *V. cholerae* is tightly regulated by the virulence cascade. In this study, we designed and carried out a high-throughput chemical genetic screen to identify inhibitors of the virulence cascade. We identified three compounds, which we named toxtazin A and toxtazin B and B’, representing two novel classes of toxT transcription inhibitors. All three compounds reduce production of both CT and the toxin-coregulated pilus (TCP), an important colonization factor. We present evidence that toxTazin A works at the level of the toxT promoter and that toxTazins B and B’ work at the level of the tcpP promoter. Treatment with toxTazin B results in a 100-fold reduction in colonization in an infant mouse model of infection, though toxTazin A did not reduce colonization at the concentrations tested. These results add to the growing body of literature indicating that small-molecule inhibitors of virulence genes could be developed to treat infections, as alternatives to antibiotics become increasingly needed.

**IMPORTANCE** *V. cholerae* caused more than 580,000 infections worldwide in 2011 alone (WHO, Wkly. Epidemiol. Rec. 87:289-304, 2012). Cholera is treated with an oral rehydration therapy consisting of water, glucose, and electrolytes. However, as *V. cholerae* is transmitted via contaminated water, treatment can be difficult for communities whose water source is contaminated. In this study, we address the need for new therapeutic approaches by targeting the production of the main virulence factor, cholera toxin (CT). The high-throughput screen presented here led to the identification of two novel classes of inhibitors of the virulence cascade in *V. cholerae*, toxTazin A and toxTazins B and B’. We demonstrate that (i) small-molecule inhibitors of virulence gene production can be identified in a high-throughput screen, (ii) targeting virulence gene production is an effective therapeutic strategy, and (iii) small-molecule inhibitors can uncover unknown layers of gene regulation, even in well-studied regulatory cascades.

*Vibrio cholerae*, a Gram-negative pathogen, colonizes the human intestine and causes cholera, an acute disease characterized by vomiting, profuse watery diarrhea, and severe dehydration. The symptoms are caused by the secreted cholera toxin (CT) (1), which binds and enter intestinal epithelial cells and increases cyclic AMP (cAMP) production. This leads to a decrease in sodium uptake and a concomitant increase in chloride extrusion into the lumen of the intestine, resulting in water secretion and thus causing diarrhea and dehydration (2).

Because CT is the major virulence factor produced by *V. cholerae*, much research has gone into understanding how its expression is regulated. Epidemic strains of *V. cholerae* are divided into two biotypes, classical and El Tor, both of which regulate the virulence cascade via the master virulence regulator, ToxT (see Fig. 6). Transcription of the ctxAB operon, which encodes the two CT subunits, and the tcp operon, which contains the genes for the toxin-coregulated pilus (TCP), is activated by ToxT (3, 4). Transcription of toxT is activated by a protein complex comprised of four inner membrane proteins, ToxRS and TcpPH (5). The transcription of tcpPH is activated by two transcription activators, AphA and AphB, which respond to cell density, anaerobiosis, and other factors (6–8).

Currently, cholera is treated with oral rehydration therapy (ORT), which restores fluids to the patient and allows the immune system to clear the infection (9, 10). Antibiotics are sometimes administered as a second line of treatment, as they can reduce vomiting and diarrheal volume by 50% and shorten illness duration by 50%. However, antibiotics are not effective alone because patients are still at risk of severe dehydration caused by CT (9, 11). Because of this, other treatment modalities could improve the treatment of cholera, and this is an area of active research (12–14). Previous work identified an inhibitor called virstatin, which inhibits ToxT dimerization and thereby alters its activity. Further experiments using virstatin revealed that ToxT dimerization affects its activity at various promoters (15). When ToxT was crystallized, it was bound to a sixteen-carbon fatty acid, cis-palmitoleate. This and other similar fatty acid ligands were shown to hold ToxT in a closed conformation, inhibiting its ability to bind and activate the tcp and ctx promoters (16).

Antivirulence drugs are becoming an increasingly popular strategy in combating diseases. Unlike antibiotics, antivirulence drugs aim to disarm a pathogen by eliminating its virulence potential and allowing the immune system to clear the infection, providing several advantages. First, targeting virulence genes im-
poses a weaker selective pressure than targeting growth, decreasing the potential for the emergence of resistant strains (17, 18). In fact, under some conditions, spontaneous nontoxicogenic mutants of V. cholerae outcompete the pathogenic wild-type parental strains (19). Additionally, targeting pathogens with antivirulence drugs has minimal effects on the endogenous microbiota, diminishing the risk of dysbiosis caused by antibiotics, which can lead to acute and chronic intestinal problems (20, 21).

In addition to the potential therapeutic advantages of antivirulence compounds, small molecules have significant value as molecular probes for investigating the basic biology governing virulence. Because such compounds do not kill bacteria, they can be used experimentally to probe virulence traits without genetic manipulation of the organism. Compounds used as molecular probes act quickly and are often reversible, and their effects can be enhanced or diminished simply by changing the concentration.

In this study, we performed a high-throughput screen to identify small molecules that inhibit the expression of toxT. We identified three compounds, which we named toxtazin A and toxtazin B and B’; the latter two are structural analogs of each other. All three compounds decrease CT and TCP levels. We determined that toxtazin A does not affect the protein or transcript levels of the ToxR, TcpP, AphA, or AphB, which are encoded by the genes upstream of toxT in the virulence cascade, indicating that it prevents toxT transcription. Toxtazins B and B’ both inhibit virulence by decreasing TcpP protein and tcpP transcript levels but not AphA or AphB protein or transcript levels, indicating that these compounds function by inhibiting tcpP transcriptional activation.

**RESULTS**

A GFP-based high-throughput screen identifies two novel classes of toxT expression inhibitors. To identify small-molecule inhibitors of toxT transcription that do not affect general growth, a reporter strain was constructed consisting of wild-type V. cholerae harboring a plasmid on which the toxT promoter drives the expression of green fluorescent protein (GFP). Culturing the toxT::gfp reporter strain NB39 under toxin-inducing conditions resulted in high numbers of fluorescence intensity units in a wild-type background and relatively low numbers of fluorescence intensity units in the isogenic ΔtoxR strain NB40 (Fig. 1A). Molecules that decrease GFP expression in a wild-type cell could inhibit any event in the virulence cascade prior to toxT transcription.

Using NB39, approximately 63,000 diverse compounds were screened at the University of Michigan Center for Chemical Genomics. A compound was scored as active if it reduced GFP fluorescence to below six standard deviations from the mean for the untreated controls, without decreasing growth (measured as changes in optical density at 600 nm [OD_{600}] >10% relative to the untreated controls, indicating that the compound is not toxic at that dose. Of the 1,411 compounds that met these requirements, 175 exhibited dose-dependent inhibition of GFP fluorescence (Fig. 1B). We prioritized these based on potency and purchased the top 50 compounds for further characterization. We measured the levels of CT produced in cultures treated with the top 50 compounds by ELISA and selected the three compounds that resulted in the lowest level of toxin production for further characterization. We named these compounds toxtazins A, B, and B’, and their chemical structures are shown in Fig. 1C.

**Toxtazin A, B, and B’ inhibit the virulence cascade.** To determine an optimal concentration for our experiments, cultures of NB39 were inoculated overnight under toxin-inducing conditions, with various concentrations of compounds or the equivalent volume of dimethyl sulfoxide (DMSO). Addition of compounds had significant and dose-dependent effects on GFP fluorescence (Fig. 2A), and the half-maximal effective concentrations (EC_{50}) of toxtazins A, B, and B’ were 24.5 μM, 2.7 μM, and 7.2 μM, respectively. All three compounds had a statistically significant effect on growth at concentrations of 25 μM and higher (Fig. 2B) and a statistically significant effect on GFP fluorescence at concentrations of 5 μM and higher. Thus, subsequent in vitro experiments were performed at 10 μM unless otherwise indicated.

If these compounds indeed inhibit toxT expression as opposed to altering GFP function, compound treatment should lead to decreased expression of ToxT-activated genes, such as those encoding CT and TCP. Additionally, for these compounds to have therapeutic potential, they should work in both the classical and El Tor biotypes of V. cholerae and should work under various toxin-inducing growth conditions. CT levels were determined in both classical and El Tor biotypes under various in vitro conditions known to induce the ToxT system for each of the two biotypes. For the classical strain O395, cultures were incubated overnight with 10 μM concentrations of each compound either in LB (pH 6.5) at 30°C or in minimal medium supplemented with amino acids asparagine, glutamate, arginine, and serine (NERS). For the El Tor strain E7946, cultures were incubated with a 10 μM concentration of each compound under AKI conditions (Fig. 2C). Toxtazins A, B, and B’ significantly inhibited CT production in both biotypes and under all three tested growth conditions, validating the target of the compounds as toxT transcription and ruling out trivial effects, such as inhibition of GFP activity. These results also indicate that the compounds are not biotype- or condition-specific inhibitors, though toxtazin B is more effective against the classical biotype. We note that toxtazin A (but not toxtazin B or B’) completely inhibited growth in M9+NERS (data not shown).

![FIG 1](https://example.com/fig1.png)

**FIG 1 Identification of inhibitors of the virulence cascade in V. cholerae.** (A) The wild-type reporter strain NB39 has a high GFP/OD_{600} ratio when grown overnight under toxin-inducing conditions, while the isogenic ΔtoxR strain NB39 does not. (B) Funnel figure illustrating the triage process used in the screen. (C) Structures of the toxtazins.
indicating that toxtazin A may affect a pathway required for growth under these conditions and that toxtazin A and B/B' work by different mechanisms.

O395 cultures grown overnight in LB (pH 6.5) at 30°C in the presence or absence of compounds were also analyzed for TcpA expression by immunoblot (Fig. 2D). All three compounds decreased TcpA levels relative to the DMSO-treated control, supporting the conclusion that these compounds affect the virulence cascade by altering toxT expression.

To confirm that the compounds work on the cascade prior to toxT expression, we tested the effects of the toxtazins on bacteria expressing toxT ectopically, under the control of an IPTG (isopropyl-$\beta$-D-thiogalactopyranoside)-inducible promoter. We predicted that if the compounds alter the cascade of gene regulation that leads to toxT expression, then removing toxT control from that cascade would confer resistance to the effects of the compounds. Strain RA286, which is a wild-type V. cholerae classical strain with a plasmid-encoded IPTG-inducible allele of toxT, was cultured overnight under toxin-inducing conditions in either DMSO, toxtazin A, or toxtazin B, and toxT was induced with 100 $\mu$M IPTG. The resulting supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) to quantify CT levels (Fig. 2E).

Toxtazin A and B both led to decreased CT expression in bacteria cultured without IPTG, while CT levels were restored in bacteria cultured with IPTG to induce toxT transcription, indicating that both toxtazins A and B act upstream of toxT in the virulence cascade. We noted that overexpression of ToxT in the DMSO control led to a slight decrease in CT levels. While the mechanism for this is unclear, we have observed that overexpressing components of this regulatory cascade (including ToxR and TcpP) can have a slight inhibitory effect on CT, perhaps due to altered stoichiometry of activator complexes required for gene expression. This observation notwithstanding, the fact that ectopic ToxT expression restores CT expression in the presence of the toxtazins indicates that they inhibit the virulence cascade prior to toxT transcription.

**Toxtazin B, but not toxtazin A, decreases V. cholerae colonization in an infant mouse model.** Host colonization by V. cholerae is dependent on TCP expression. Based on our in vitro findings that the toxtazins decrease TcpA levels, we hypothesized that they could decrease colonization loads of V. cholerae in a mouse model of colonization. To test toxtazin A activity in vivo, 4- to 6-day-old mice were orogastrically inoculated with $10^{6}$ bacteria and either toxtazin A or DMSO and received a booster of compound 3 h postinfection. Because not all the compound administered will reach the desired location within the mouse (i.e., the small intestine), and because some of the compound may be metabolized or otherwise degraded within the mouse, we used higher concentrations of compounds in these experiments. To ensure that any observed decreases in colonization are due to inhibition of TcpA and not toxicity against V. cholerae, 3-ml LB cultures were started using the same inoculum, boosted with compound 3 h later, and then cultured overnight. Administration of either 20 pg, 40 pg, or 60 pg toxtazin A to mice inoculated with V. cholerae did not decrease colonization levels relative to those in the DMSO-treated mice (Fig. 3A). While toxtazin A did not reduce colonization even at the highest level tested, that concentration was toxic to V. cholerae grown in vitro (Fig. 3B). We note that cultures grown in sublethal concentrations of toxtazin A routinely grow better in LB, and this was also seen in the in vitro experiment at 20 pg and 40 pg (Fig. 3B). The mouse data indicated that toxtazin A was not toxic in vivo at 60 pg, but higher doses were not tested because it would be impossible to distinguish whether a decrease in colonization resulted from the ability of toxtazin A to inhibit ToxT activity or from its antibacterial activity. The lack of

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**FIG 2** Toxtazin A, B, and B’ inhibit the virulence cascade in V. cholerae. (A and B) GFP expression (A) and terminal OD$_{600}$ (B) after 16 h in cultures grown overnight under toxin-inducing conditions with 0.16 $\mu$M to 50 $\mu$M compounds. (C) CT expression levels in wild-type cultures grown overnight under various toxin-inducing conditions in the presence or absence of 10 $\mu$M compounds. The dotted line indicates the limit of detection. An isogenic ΔtoxR strain served as a control. (D) Western blot of O395 cultures treated with 10 $\mu$M compounds overnight in LB pH 6.5 at 30°C, using a TcpA antibody. (E) CT levels in cultures of RA286 grown overnight under toxin-inducing conditions in the presence or absence of 10 $\mu$M compounds. Error bars represent the standard deviations for three biological replicates. The dotted line indicates the limit of detection.
in vivo killing by toxazin A at the 60-μg dose may reflect poor bioavailability of the compound in the infant mouse. Because toxazin B and B’ are structural analogs and have behaved similarly in all assays thus far, and to reduce the number of animals used, we focused on toxazin B in the following experiments. The same in vivo experiment performed with toxazin A was performed with toxazin B. Administration of either 100 μg or 200 μg toxazin B to mice inoculated with V. cholerae decreased colonization levels approximately 100-fold relative to those in the DMSO-treated mice (Fig. 3C). This effect was not due to toxazin B toxicity against V. cholerae, since the 3-ml in vitro cultures treated with toxazin B had no growth defect compared to the DMSO-treated cultures (Fig. 3D). These results suggest that toxazin B inhibits expression of the TcpA colonization determinant both in vitro and in vivo.

The toxazins do not affect ToxR protein levels or activity. To determine where in the regulatory cascade leading to toxT expression each compound works, a targeted approach was used. We first tested whether the compounds altered levels or activity of ToxR, one of the direct transcription activators of toxT. ToxR protein levels were unaffected by toxazins A, B, and B’ in cultures grown overnight under toxin-inducing conditions (Fig. 4A). ToxR activity was investigated by measuring levels of OmpU and OmpT, outer membrane proteins whose transcription is activated or repressed, respectively, by ToxR. Inhibiting ToxR activity would decrease OmpU levels and elevate OmpT levels, similar to a toxR mutant. Cell lysates from overnight cultures grown with or without compounds under toxin-inducing conditions were subjected to SDS-PAGE and stained with Coomassie blue to visualize OmpU and OmpT (Fig. 4B). Toxazin-treated cultures have the same OmpU/OmpT profile as the DMSO control, indicating that ToxR activity is not affected by toxazin A, B, or B’.

TcpP protein levels are differentially affected by toxazin A and B/B’. Next, we analyzed the effect on levels of TcpP, the other major transcription activator that regulates toxT. Cultures were grown overnight under toxin-inducing conditions in the presence or absence of 10 μM toxazin A, B, or B’, and cell lysates were analyzed by Western blotting with antiserum directed against TcpP. Toxazin-treated cultures have the same OmpU/OmpT profile as the DMSO-treated wild-type culture, indicating that ToxR activity is not affected by toxazin A, B, or B’.

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FIG 3 In vivo efficacy of toxazins A and B. Asterisks denote the statistical significance of a group relative to the DMSO control. NS, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001. (A) Number of V. cholerae organisms recovered from mice orogastrically inoculated with 10⁶ wild-type V. cholerae organisms and treated with either DMSO or 0 μg, 20 μg, 40 μg, or 60 μg toxazin A. (B) Number of V. cholerae organisms recovered from 3-ml cultures grown overnight with the same inocula and boosters as the mice. (C) Number of V. cholerae recovered from mice orogastrically inoculated as described for panel A and treated with DMSO or 100 μg or 200 μg toxazin B. (D) Number of V. cholerae organisms recovered from 3-ml cultures grown overnight with the same inocula and boosters as the mice.

FIG 4 Effects of toxazin A and B on the virulence cascade. (A) ToxR Western blot of cultures grown overnight under toxin-inducing conditions with or without 10 μM concentrations of compounds. (B) Coomassie stain showing OmpU and OmpT levels in cultures grown as described for panel A. (C) TcpP Western blot of cultures grown as described for panel A. (D) Transcript levels of toxT, tcpP, aphA, and aphB were determined for cultures grown as described for panel A. Significance was calculated by two-way ANOVA. NS, not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
DISCUSSION

Despite the use of antibiotics and oral rehydration therapy (ORT), _V. cholerae_ remains a major public health concern in much of the developing world. The number of yearly cholera cases has steadily increased since 2007, and the number of fatalities has increased in the past year (22). New treatment therapies that target toxin production and colonization by _V. cholerae_, either alone or in combination with current therapies, could be very useful in reducing the global health burden caused by this pathogen.

In this study, a bacterial-cell-based, high-throughput screen was used to identify and characterize three small molecules, toxazin A, B, and B’. All three reduce the virulence potential of _V. cholerae_ in _in vitro_ and toxazin B also reduced colonization _in vivo_. We show that all three compounds reduce CT production in both the classical and El Tor biotypes and reduce TcpA levels relative to a DMSO control.

The two classes of compounds identified here work by different mechanisms. Toxazin A does not alter TcpP or ToxR protein levels, suggesting that it affects toxT transcription via a different mechanism. We have not ruled out the possibility that toxazin A inhibits ToxR or TcpP function, perhaps keeping one of them from physically binding to its site on the toxT promoter. Both ToxR and TcpP may sense environmental signals and transmit the information to the cytoplasm by activation of toxT transcription. Environmental signals such as temperature, pH, osmolarity, bile, oxygen, and amino acids have all been shown to affect toxT activation _in vitro_ (8, 23–25). In fact, when ToxT was crystalized, a _cis_-palmitoleate was discovered in a solvent-inaccessible binding pocket. Oleic acid, another _C-9_ monosaturated fatty acid, may be the natural ligand for this binding pocket, as it also strongly regulates ToxT activity and is more abundant in bile than _cis_-palmitic acid. Other natural compounds have been reported to influence expression of the virulence cascade in _V. cholerae_ (26, 27). The stressed physiological state of the cell grown with toxazin A revealed by proteomic analysis (data not shown) and the growth-inhibitory phenotype we observed in minimal medium containing NERS (Fig. 2C) led us to propose that toxazin A induces a nonpermissive physiological state in the cell which feeds back to shut off toxT transcription, and we are currently exploring this hypothesis. We investigated the potential for the toxazins to act as general redox-active compounds by measuring the activity of alkaline phosphatase, which requires disulfide bond formation. Tested both _in vitro_ and in cells treated with the toxazins (data not shown), alkaline phosphatase activity was unaffected, suggesting that the toxazins do not affect the general redox state of the cell. Furthermore, the compounds do not affect the bicinchoninic acid (BCA) protein assay, which does not work in the presence of a reducing agent, indicating that, at the concentrations used in our experiments, these compounds do not act as general reducing agents.

The mechanism of toxazin B/B’ inhibition of virulence is clearer. Toxazin B and B’ decrease TcpP but not ToxR protein levels, and they decrease the levels of _toxT_ and _tcpP_ transcripts but not those of _aphA_ or _aphB_, nor do they decrease AphA or AphB protein levels. We noticed that toxazin A consistently caused an increase in AphA protein levels, but it is unlikely that this accounts for the diminished _toxT_ transcription levels. Taken together, the data suggest that toxazin B inhibits the virulence cascade at the level of the _tcpP_ promoter—downstream of AphA and AphB protein levels.

![Image](https://mbio.asm.org/content/4/4/e00403-13/Fig5)

**FIG 5** Toxazins A and B do not affect AphA or AphB protein levels. Western blot for AphA and AphB in samples grown overnight under toxin-inducing conditions in the presence or absence of 10 μM concentrations of compounds. Band intensities were quantified with ImageJ and normalized to the wild-type DMSO-treated samples.
tein production but upstream of tcpP transcription. Supporting this, ectopic expression of either AphA or AphB does not restore CT production (Fig. 5) in the presence of toxtazin B.

Toxtazin B inhibits virulence gene expression in the classical biotype more strongly than in the El Tor biotype. It is notable that tcpP transcription, the level at which our data suggest this compound works, is regulated differently in the two biotypes. A single A-to-G base pair difference in the tcpP promoters of the two biotypes disrupts the dyad symmetry of the AphB binding motif (28–30); thus, AphB binds 10 times more strongly to the classical tcpP promoter than to the El Tor tcpP promoter (31, 32). We speculate that the natural differences in tcpP regulation in classical versus El Tor may be responsible for the different effectiveness of toxtazin B seen in the two biotypes in Fig. 2C, but further study must be done to confirm this.

The tcpP promoter is a highly regulated feature of the complex regulatory cascade controlling toxT transcription, assimilating multiple signals, including pH, osmolarity, cAMP levels, and phosphate levels. For example, when cells are grown at the non-permissive pH, a protein called PepA partially inhibits tcpP transcription in the classical biotype (33). The tcpP promoter is also negatively regulated by the cAMP-CRP complex, whose binding site at the promoter overlaps the binding sites of AphA and AphB (30). Finally, the tcpP promoter is negatively regulated by PhoB, which binds at a site distinct from both the AphA and AphB binding sites (34). Toxtazin B may inhibit tcpP expression by altering the binding properties of any these proteins, or of the AphA and/or AphB proteins, at the tcpP promoter. Future work will aim to determine the precise mechanism by which toxtazin B inhibits gene expression required for colonization and pathogenicity.

In this study, the activity of the toxtazins in vivo was also determined using the infant mouse model of colonization. The ability of V. cholerae to colonize and replicate in this mammalian host is in large part dependent on expression of TCP, the major subunit of which is TcpA; wild-type strains are significantly more competitive than tcpA mutants in vivo (35, 36). Our in vitro results (Fig. 2B) demonstrate that toxtazin B leads to reduced TcpA levels, which we propose as the main reason for reduced colonization caused by toxtazin B. We note that a tcpA mutant colonizes more poorly than a toxtazin B-treated wild type, and we take this to mean that the local compound concentration (that is, the amount of toxtazin B that actually reaches V. cholerae in the gut) is lower than what would be required for complete inhibition. Pharmacodynamic and pharmacokinetic studies might optimize the ability of toxtazin B to inhibit colonization.

Unlike toxtazin B, toxtazin A did not decrease colonization level of V. cholerae at the concentrations tested. Perhaps this compound does not inhibit colonization because it does not reduce TcpA levels as efficiently as toxtazin B, or perhaps not enough compound reached the bacteria in the gut. Structure-activity relationship (SAR) studies to identify an analog with increased potency and lower toxicity would be useful in future in vivo studies and for determining whether toxtazin A decreases colonization.

In summary, we identified two novel classes of toxT transcription inhibitors, toxtazin A, B, and B'. They are potent inhibitors of V. cholerae virulence gene expression and function at distinct points in the virulence regulatory cascade (Fig. 6). Toxtazin A inhibits by preventing toxT transcription late in the regulatory cascade, evidenced by the fact that ToxR and TcpP protein levels are equal to those of DMSO-treated cultures and that mRNA levels of tcpP, aphA, and aphB are not affected. Toxtazin B works by altering tcpP transcription, shown by a decrease in both transcript and protein levels of tcpP but no decrease in the protein or transcript levels of AphA or AphB relative to the DMSO-treated controls. Toxtazin B also reduces colonization of V. cholerae in an infant mouse model. We are currently working on better characterizing the mechanism of action of the toxtazins to gain deeper insight into the requirements of V. cholerae pathogenesis. In addition to providing new chemical probes for richer study of the virulence cascade in V. cholerae, the toxtazins add further proof of principle that potent small-molecule inhibitors can be discovered by high throughput screening and can be used both as molecular probes for basic research and as a starting point for therapeutic development.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and chemical inhibitors.** The strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the V. cholerae classical biotype strain O395 was used in these studies. Strains were maintained at −80°C in Luria-Bertani broth (LB) containing 20% glycerol. Overnight cultures were grown in LB medium at 37°C. Growth of the O395 classical strain under toxin-inducing conditions consists of subculturing an overnight culture 1:100 in LB pH 6.5 and growing at 30°C with shaking for 16 to 18 h, or as indicated above. Growth in minimal medium consists of subculturing an overnight culture 1:100 in M9 minimal medium containing NERS (M9 salts, 2 mM MgSO4, 4% glycerol, 0.1 mM CaCl2, and 5 mM [each] asparagine, glutamic acid, ar-
Inhibitors of toxT Expression in *V. cholerae*

**TABLE 1 Strains and plasmids used in this study**

| Strain or plasmid | Relevant genotype and phenotype | Source |
|-------------------|--------------------------------|--------|
| V. cholerae strains |                               |        |
| O395              | Classical Ogawa, Sm<sup>−</sup> | Laboratory collection |
| E7946             | El tor, Sm<sup>−</sup>        | Laboratory collection |
| RA25              | O395 ΔtoxR                     | This work |
| RA6               | E7946 ΔtoxR                     | This work |
| NB39              | O395 + toxT-GFP                 | Laboratory collection |
| NB40              | O395 ΔtoxR + toxT-GFP           | Laboratory collection |
| RA179             | O395 ΔtoxT                     | 43 |
| RA67              | O395 ΔtcpP                     | 44 |
| RA305             | O395 ΔaphA                     | This work |
| RA282             | O395 ΔaphB                     | This work |
| RA286             | O395 + pMT5                    | 45 |
| RA289             | O395 + pMMB66EH-aphA           | This work |
| RA290             | O395 + pMMB66EH-aphB           | This work |
| RA306             | O395 ΔaphA + pMMB66EH           | This work |
| RA307             | O395 ΔaphA + pMMB66EH-aphB     | This work |
| RA308             | O395 ΔaphB + pMMB66EH          | This work |
| RA309             | O395 ΔaphB + pMMB66EH-aphB     | This work |

**Plasmids**

| toxT-GFP         | pBH6119-toxTpro-gfp            | This work |
| pMT5             | pMMB66EH-toxT                  | 45 |
| pWM91-Δapha      | pWM91-ΔaphA                    | 46 |
| pWM91-ΔaphB      | pWM91-ΔaphB                    | 46 |
| pMMB66EH-aphA    | pMMB66EH-aphA (O395)           | This work |
| pMMB66EH-aphB    | pMMB66EH-aphB (O395)           | This work |

**Plasmids**

| toxT-GFP         | pBH6119-toxTpro-gfp            | This work |
| pMT5             | pMMB66EH-toxT                  | 45 |
| pWM91-Δapha      | pWM91-ΔaphA                    | 46 |
| pWM91-ΔaphB      | pWM91-ΔaphB                    | 46 |
| pMMB66EH-aphA    | pMMB66EH-aphA (O395)           | This work |
| pMMB66EH-aphB    | pMMB66EH-aphB (O395)           | This work |

**Strains and plasmids used in this study**

- **O395**
  - Classical Ogawa, Sm<sup>−</sup>
  - Laboratory collection
- **E7946**
  - El tor, Sm<sup>−</sup>
  - Laboratory collection
- **RA25**
  - O395 ΔtoxR
  - This work
- **RA6**
  - E7946 ΔtoxR
  - This work
- **NB39**
  - O395 + toxT-GFP
  - Laboratory collection
- **NB40**
  - O395 ΔtoxR + toxT-GFP
  - Laboratory collection
- **RA179**
  - O395 ΔtoxT
  - 43
- **RA67**
  - O395 ΔtcpP
  - 44
- **RA305**
  - O395 ΔaphA
  - This work
- **RA282**
  - O395 ΔaphB
  - This work
- **RA286**
  - O395 + pMT5
  - 45
- **RA289**
  - O395 + pMMB66EH-aphA
  - This work
- **RA290**
  - O395 + pMMB66EH-aphB
  - This work
- **RA306**
  - O395 ΔaphA + pMMB66EH
  - This work
- **RA307**
  - O395 ΔaphA + pMMB66EH-aphB
  - This work
- **RA308**
  - O395 ΔaphB + pMMB66EH
  - This work
- **RA309**
  - O395 ΔaphB + pMMB66EH-aphB
  - This work

**Detectors of cholera toxin by ELISA**

*V. cholerae* were grown under toxin-inducing conditions for 16 to 18 h with 10 μM compound or DMSO. GM1 ganglioside enzyme-linked immunosorbent CT assays were performed as previously described (42) on equal volumes of the resulting supernatants. CT expression values were normalized to the OD<sub>600</sub> and are the averages for samples grown in at least duplicate.

**Western blot analysis of TcpA, ToxR, TcpP, ApdB, and ApfA**

Cells were cultured under toxin-inducing conditions in the presence or absence of 10 μM compounds. Cell extracts were subjected to SDS-PAGE, transferred to a nitrocellulose membrane (GE Water and Processes Technologies, Feasterville-Trevose, PA), probed with the appropriate antibody, and visualized with alkaline phosphatase. The TcpA antibody was used at a 1:1,000 dilution, the ToxR antibody at a 1:1,000 dilution, and the TcpP antibody at a 1:500 dilution. The ApdB antibody was kindly provided by Jun Zhu. The ApfA antibody (kindly provided by Karen Skorupski) was used at a 1:10,000 dilution, the ToxR antibody at a 1:1,000 dilution, and the TcpP antibody at a 1:500 dilution. Band densities were determined with the Imagej software (http://rsb.info.nih.gov/ij/) and normalized to the wild-type DMSO-treated samples.

**qRT-PCR analysis of mRNA expression**

Cells were cultured under toxin-inducing conditions in the presence or absence of 10 μM toxT. RNA was harvested with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions, and DNA was removed using Turbo A template.
and NB40.

| Target | Forward primer | Reverse primer |
|--------|----------------|----------------|
| toxT   | CAGCGATTTCCTTCTTGA | CTCTGAAAACCATTTAC |
| tcpP   | GTTGGTTGCTACGTTGTA | CCGGTAACCTTGCTA |
| aphA   | GAAGGGACACGAGATG | AGAGGCTAAGGTTCAG |
| aphB   | CGGAAAGTTTAC | CGTAGTGA |
| 16S rRNA | GCCAAAACCTGAGAAGA | TTGCTCAGTTCCAGT |
|        | GCAAAGCA |GCCGTAT |

DNase (Amemb, Austin, TX). The qRT-PCR experiments were performed using the QuantiTect SYBR green RT-PCR kit (Qiagen, Valencia, CA) according the manufacturer’s manual. The qRT-PCR primers are shown in Table 2. Expression levels were normalized to 16S rRNA, and fold change was calculated using the 2-ΔΔCt method described in Applied Biosystems user bulletin no. 2 (P/N 4303859). Results are the averages for three biological replicates with three replicates each. Significance was determined by two-way analysis of variance (ANOVA).

**Infant mouse colonization assays.** Four- to six-day-old CD1 mice (Charles River, Wilmington, MA) were orogastrically inoculated with a 30-μl bolus containing 10^6 CFU of V. cholerae O395, Cremaphor EL (20% final volume to solubilize compounds), and either DMSO or compound (doses are given above). An additional 30-μl bolus lacking bacteria was delivered to each mouse 3 h postinoculation, and the mice were incubated at 30°C. Mice were euthanized 18 to 24 h after inoculation, and the intestines were isolated, weighed, and homogenized in phosphate-buffered saline (PBS). Homogenates were serially diluted and plated on LB agar containing X-Gal and streptomycin to determine the number of CFU recovered. CFUs were normalized to the weight of the intestines and to the exact CFU of the initial inoculum. Significance was determined using one-way ANOVA.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00403-13/-/DCSupplemental.

Figure S1, DOCX file, 0.9 MB.

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