

# Assay development and high-throughput screening for small molecule inhibitors of a *Vibrio cholerae* stress response pathway

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**Abstract:** Antibiotics are important adjuncts to oral rehydration therapy in cholera disease management. However, due to the rapid emergence of resistance to the antibiotics used to treat cholera, therapeutic options are becoming limited. Therefore, there is a critical need to develop additional therapeutics to aid in the treatment of cholera. Previous studies showed that the extracytoplasmic stress response ( $\sigma^E$ ) pathway of *Vibrio cholerae* is required for full virulence of the organism. The pathway is also required for bacterial growth in the presence of ethanol. Therefore, we exploited this ethanol sensitivity phenotype in order to develop a screen for inhibitors of the pathway, with the aim of also inhibiting virulence of the pathogen. Here we describe the optimization and implementation of our high-throughput screening strategy. From a primary screen of over 100,000 compounds, we have identified seven compounds that validated the growth phenotypes from the primary and counterscreens. These compounds have the potential to be developed into therapeutic agents for cholera and will also be valuable probes for uncovering basic molecular mechanisms of an important cause of diarrheal disease.

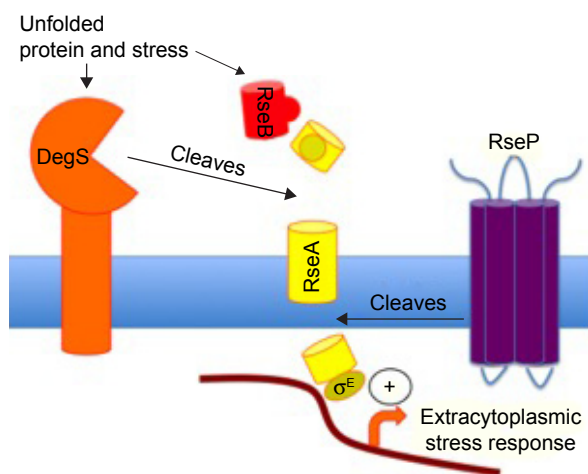
**Keywords:** *Vibrio cholerae*, stress response,  $\sigma^E$ , high-throughput screening

## Introduction

RpoE, encoding  $\sigma^E$ , regulates a response referred to as the extracytoplasmic (or envelope) stress response in that it targets stresses that affect components of the Gram-negative cell envelope, including the periplasmic space and the outer membrane.  $\sigma^E$  is a member of the extracytoplasmic function family of alternative sigma factors, which control the expression of genes whose products function outside the bacterial cytoplasm.<sup>1,2</sup>  $\sigma^E$ -regulated genes encode proteins that are involved in folding of proteins in the periplasm, proteases, other sigma factors, and lipopolysaccharide biogenesis and/or modification factors.<sup>3–6</sup>

$\sigma^E$  has been most thoroughly studied in *Escherichia coli*, where it is an essential sigma factor.<sup>7</sup> The activity of  $\sigma^E$  is negatively regulated when bacteria are not experiencing external stress. This is accomplished through the activity of RseA, a membrane-bound anti-sigma factor that binds  $\sigma^E$  and sequesters it in the inner membrane, rendering it unable to bind RNA polymerase and activate stress-inducible genes.<sup>8,9</sup> In *E. coli*, the  $\sigma^E$  regulon is induced when misfolded outer membrane proteins (OMPs) accumulate in the periplasm (Figure 1; refer Urban<sup>10</sup>). A C-terminal motif of the unfolded OMPs binds to the PDZ domain of the protease DegS and induces a conformational change in the protein, which activates its protease activity.<sup>11</sup> DegS cleaves within the RseA periplasmic domain, creating a substrate for a second protease,

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**Figure 1** Diagram of the  $\sigma^E$  pathway.

**Notes:** In the absence of extracytoplasmic stress,  $\sigma^E$  is sequestered at the bacterial inner membrane through its interaction with the anti-sigma factor RseA. In the presence of unfolded outer membrane proteins or other stress, RseA is degraded in a sequential manner by the proteases DegS and RseP (also known as YaeL), releasing  $\sigma^E$  into the cytoplasm where it directs transcription of stress-inducible genes.

YaeL (RseP). YaeL then cleaves the transmembrane domain of RseA, releasing bound  $\sigma^E$  from the membrane.<sup>12,13</sup> The cytoplasmic ClpXP proteases further degrade RseA, releasing  $\sigma^E$ .<sup>14,15</sup> Unbound  $\sigma^E$  complexes with core RNA polymerase at  $\sigma^E$ -dependent promoters, thus activating the transcription of stress-inducible genes.<sup>16</sup>

$\sigma^E$  plays a role in the survival of many bacterial pathogens within their hosts, making it an excellent target for potential therapeutics. For example, in *Salmonella typhimurium*,  $\sigma^E$  is required for protection against reactive oxygen species and antimicrobial peptides. Consequently, *rpoE* mutants are highly attenuated in a mouse model of infection.<sup>17,18</sup> In *Pseudomonas aeruginosa*, the  $\sigma^E$  ortholog AlgU promotes resistance to killing by phagocytes. In addition, AlgU is responsible for conversion of cells to the mucoid phenotype, rendering them less efficiently cleared from the respiratory tract of infected mice than nonmucoid strains.<sup>19,20</sup> In nontypeable *Haemophilus influenzae*-infected macrophages, *rpoE* is highly expressed and is required for intracellular survival.<sup>21</sup> *Mycobacterium tuberculosis rpoE* mutants are defective in their ability to grow and survive inside macrophages.<sup>22</sup> The  $\sigma^E$  pathway is also important in *Actinobacillus pleuropneumoniae* and uropathogenic *E. coli* infection models.<sup>23,24</sup>

$\sigma^E$  is required for virulence in *Vibrio cholerae*, the causative agent of epidemic cholera. An *rpoE* mutant is highly attenuated in the infant mouse model of infection due to a defect in the ability to colonize the intestinal tract.<sup>25</sup> *rpoE* is not required for the growth of *V. cholerae* at high

temperatures, unlike *E. coli* and *S. typhimurium*. However, *rpoE* is required for the growth and survival of classical *V. cholerae* strains in Luria–Bertani media containing 3% ethanol.<sup>25</sup> It is this phenotype that we targeted in developing a small molecule screen for inhibitors of the  $\sigma^E$  pathway.

Here we describe the optimization and implementation of a high-throughput screen (HTS) designed to identify inhibitors of the  $\sigma^E$  pathway. We performed the primary screen on over 100,000 small molecules, followed by counterscreening, dose–response analysis, and medicinal chemistry. We ultimately identified seven compounds that validated our initial studies. These compounds are of high interest for therapeutic development because they have the potential to inhibit this stress response and virulence in *V. cholerae* and may also target similar responses in other important bacterial pathogens. By targeting mechanisms that inhibit virulence and decrease fitness of these bacteria *in vivo*, such treatment strategies would be advantageous as they allow the host to clear an infection with reduced selective pressure for bacterial resistance than a traditional antibiotic.

## Materials and methods

### Bacterial strains and culture conditions

*V. cholerae* classical strain O395 was used throughout this study. The *rpoE*, *yaeL*, and *degS* deletion strains used in this study were previously described.<sup>26</sup> Strains were maintained at  $-80^{\circ}\text{C}$  in Luria–Bertani (LB) broth containing 20% glycerol. Overnight cultures were grown in LB medium at  $37^{\circ}\text{C}$  with shaking.

### High-throughput screening

For the HTS assay, 30  $\mu\text{L}$  of LB +3% ethanol was added to each well using a Multidrop™ plate dispenser (Thermo LabSystems, Beverly, MA, USA) followed by the addition of compounds by using a Biomek™ FX Liquid Handler (Beckman®, Indianapolis, IN, USA). Ten microliters of classical *V. cholerae* strain O395 (in LB +3% ethanol) was then added to the sample wells by the Multidrop plate dispenser to achieve a final  $\text{OD}_{600}$  of  $\sim 0.01$ . The bacteria were added subsequent to compound addition to prevent the contamination of the liquid handler. All plates contained two columns (32 wells) of each control. The positive control for this assay was the *rpoE* deletion strain, and the negative control was the wild-type strain grown without added compound. Dimethylsulfoxide (DMSO) vehicle was added to all control wells. After 7 hours of static incubation at  $37^{\circ}\text{C}$ , the final  $\text{OD}_{600}$  was read on a PHERAstar® multimode plate reader (BMG Labtech, Worcester, MA, USA).

## Chemical inhibitors

Fresh powders of 60 high priority compounds were purchased from ChemDiv (San Diego, CA, USA). Compound stock solutions were made at 20 mM in deuterated DMSO and stored in the dark at  $-20^{\circ}\text{C}$ . Cultures were treated with  $10\ \mu\text{M}$  compound unless otherwise specified, as this was the concentration of compound used in the primary screen.

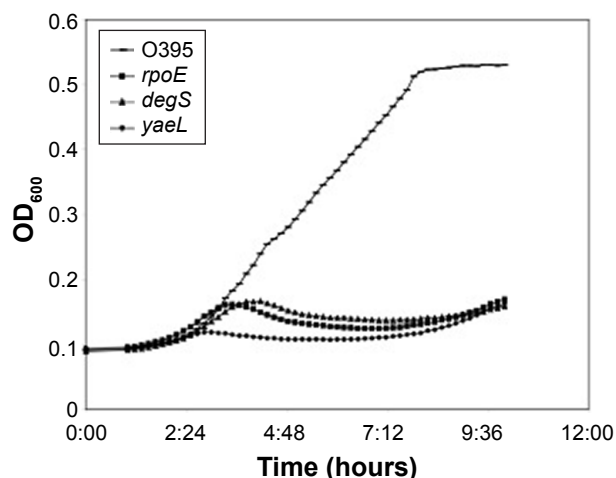
## Low-throughput screening

Low-throughput screening of the 60 candidate compounds was performed in a volume of 1 mL of culture. Overnight cultures were diluted 1:100 into LB medium with and without ethanol. Compounds were initially added to cultures at the highest concentration ( $20\ \mu\text{M}$ ) and then serially diluted by half to the lowest concentration tested ( $2.5\ \mu\text{M}$ ) while keeping the DMSO concentration constant. Three 1 mL replicates of the cultures containing different concentrations of compounds (ie,  $20\ \mu\text{M}$ ,  $10\ \mu\text{M}$ ,  $5\ \mu\text{M}$ , and  $2.5\ \mu\text{M}$ ) were grown for 7 hours with shaking at  $37^{\circ}\text{C}$ . Control (untreated) cultures received an equal volume of DMSO. Following growth,  $100\ \mu\text{L}$  samples of each culture replicate were placed in a 96-well plate, and the final  $\text{OD}_{600}$  was determined by using a CLARIOstar<sup>®</sup> plate reader (BMG Labtech).

## Results

### Rationale for screening strategy

Our screen for small molecule inhibitors of  $\sigma^{\text{E}}$  function was relatively straightforward in design: We exploited the ethanol sensitivity phenotype of  $\sigma^{\text{E}}$ -mutant *V. cholerae* and assayed for compounds that inhibited the growth of a wild-type classical *V. cholerae* strain (O395) in the presence of 3% ethanol. Our findings and those of others demonstrated this phenotype for an *rpoE* mutant (Figure 2; refer Kovacicova and Skorupski<sup>25</sup>). In addition, we demonstrated that mutations in the two proteases that cleave the anti-sigma factor RseA (DegS and YaeL) also showed this phenotype, likely due to constitutive sequestration of  $\sigma^{\text{E}}$  at the bacterial membrane in their absence (Figure 2; Matson and DiRita<sup>26</sup>). Importantly, no other pathways that contribute to ethanol sensitivity/resistance have been identified in *V. cholerae*. Thus, our hypothesis was that small molecules that inhibited  $\sigma^{\text{E}}$  activity would cause the ethanol sensitivity phenotype. Inhibition of activity could happen at several steps in this pathway, including by interfering with the  $\sigma^{\text{E}}$ -RNA polymerase interaction or by causing  $\sigma^{\text{E}}$  to be constitutively sequestered at the membrane (by inhibiting protease activity/efficiency). We reasoned that any of these steps might be inhibited by a set of compounds, resulting in numerous targets/compound



**Figure 2**  $\sigma^{\text{E}}$  pathway mutants of *Vibrio cholerae* cannot grow in media containing ethanol.

**Notes:** O395 (wild type) and deletion strains of *rpoE* ( $\sigma^{\text{E}}$ ), *degS*, and *yaeL* were grown in 96-well plates for 10 hours at  $37^{\circ}\text{C}$  with shaking in Luria-Bertani media containing 3% ethanol.

combinations that could produce the desired outcome. Therefore, we aimed to develop and optimize an HTS to identify small molecules that inhibit the growth of wild-type *V. cholerae* in the presence of 3% ethanol.

### Miniaturization of the assay for screening in 384-well plates

For a screen to be considered “high-throughput,” it should be optimized for the smaller-scale formats used for such assays, typically using 384- or 1,536-well microtiter plates. Since we consistently observed the growth of the wild-type strain and lack of growth for the *rpoE* deletion strain in 96-well plates, we did not anticipate the need for dramatic changes in the miniaturization process. For the 96-well plates,  $100\ \mu\text{L}$  cultures of the wild type and *rpoE* deletion strains were assayed for growth, which was scaled down to  $40\ \mu\text{L}$  cultures in the 384-well format. We initially tested the growth phenotypes of the two strains in 384-well plates in the presence of 3% ethanol and read the  $\text{OD}_{600}$  of the cultures after either 7 hours or overnight growth at  $37^{\circ}\text{C}$ . The plates were incubated without shaking, in contrast to previous assays, as that would be optimal for subsequent HTS. Although the final  $\text{OD}_{600}$  of the wild-type cells was lower in this smaller format, there was still a clear difference between the wild-type strain, which grew to an  $\text{OD}_{600}$  of  $\sim 0.18$  at 7 hours, and the *rpoE* mutant, which remained at the level of detection ( $\sim 0.05$ ). After overnight growth, there was no longer a significant difference in  $\text{OD}_{600}$  between strains. Therefore, we proceeded with the optimization of the assay at the 7-hour time point.

A key parameter for determining whether an assay is robust enough to use in a large-scale screen is the  $Z'$  factor.<sup>27</sup> This statistical parameter is calculated to evaluate the effectiveness of an assay used for HTS. An assay with a very large dynamic range (difference in average signal between the positive and negative controls) and a very small SD has a  $Z'$  factor approaching the theoretically perfect value of 1. An assay with a  $Z'$  factor between 0.5 and 1 is considered an excellent assay; therefore, we sought to optimize our assay to fall within this range before proceeding to screening compounds. After several preliminary experiments, through which we determined that sealing the plates in plastic is necessary to prevent the evaporation of media in wells near the edges of the plates, we obtained a reliable  $Z'$  factor  $>0.7$  for this assay (Figure 3). Therefore, we concluded that the assay was robust enough in the 384-well format to proceed with a small-scale pilot screen.

## Pilot screen

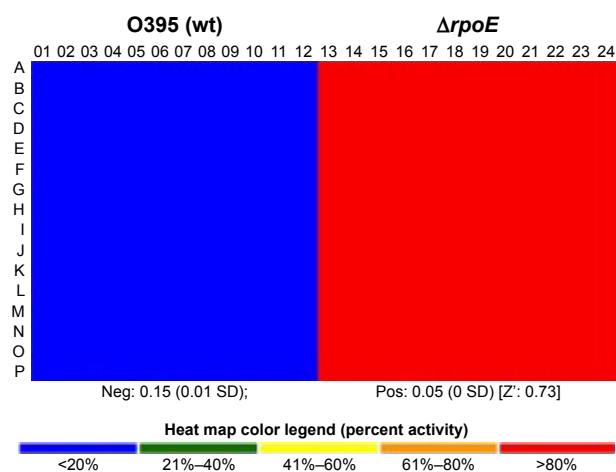
A screen of the 2,000 compounds of Spectrum Library and the 446 compounds of National Institutes of Health (NIH) clinical collection was carried out at the Center for Chemical Genomics (CCG) in the Life Sciences Institute at the University of Michigan. The Spectrum Collection from MicroSource Discovery Systems, Inc. consists of 70 compounds (3.5%) approved for and restricted to

agricultural use, 343 compounds (17.15%) with reported biological activities at the experimental level, 629 natural products and derivatives (31.45%) with undetermined biological activities, and 958 known drugs (47.9%) that have been used in human therapy. The NIH clinical collection consists of 446 compounds that have a history of use in human clinical trials. This screen was accomplished in the 384-well format as described in the “Materials and methods” section. The assay performed well in this pilot assay ( $Z'=0.76$ , signal/noise =3, coefficient of variation [%] =8.3 [negative control]) as illustrated in Figure 4. Based on the commonly used cutoff of three standard deviations from the mean (Figure 4, red line), ~41 compounds (1.7%) answered our pilot screen and would be considered “actives.” Due to the success of this pilot assay, we concluded that the screen was suitable for proceeding with HTS of a larger library.

## Large-scale HTS for candidate $\sigma^E$ inhibitors

The optimized primary screen was used to test an additional 99,680 diverse compounds from the ChemDiv 100K collection at the CCG screening facility. This brought the total number of compounds screened to 102,126 including those from the pilot screen. Compounds were used at a concentration of 10  $\mu\text{M}$  for primary screening. Compounds were initially assessed for their ability to inhibit growth in the presence of 3% ethanol, similar to the *rpoE* mutant (the positive control for this screen). After primary screening, we removed all compounds showing  $\geq 45\%$  of the positive control value (Figure 5). The remaining compounds were confirmed by repeating the assay in triplicate. Any compounds that did not inhibit growth to this cutoff in three of the four repetitions were excluded from further study. At this stage, we also tested the effect of these compounds on *V. cholerae* growth in the absence of ethanol to exclude those that generally inhibited bacterial growth, such as antibiotics. This counterscreen was included to increase the likelihood that we would identify compounds that targeted the  $\sigma^E$  pathway and were not non-specific inhibitors of *V. cholerae* replication. Compounds that inhibited growth in the absence of ethanol ( $>50\%$ ) were removed from further study.

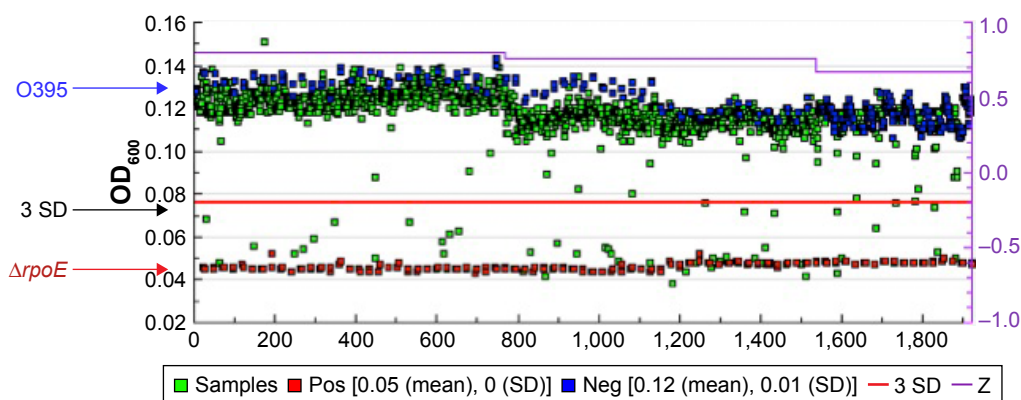
Compounds remaining in the pool at this stage were subsequently tested for their dose–response characteristics. Assays were carried out using a range of concentrations of compound from 1 to 100  $\mu\text{M}$  to determine the concentration required to inhibit the assay to 50% of its maximum value ( $\text{IC}_{50}$ ). Compounds that did not exhibit a good relationship



**Figure 3** Wild-type *Vibrio cholerae* and an *rpoE* deletion strain grow to significantly different densities in the 384-well format.

**Notes:** The wells in the left half of a 384-well plate were inoculated with O395 (ethanol-sensitive negative control), and the wells in the right half were inoculated with the *rpoE* deletion strain (ethanol-sensitive positive control) in media containing 3% ethanol and DMSO vehicle control. The plate was sealed in a plastic bag and incubated at 37°C for 7 hours, when the  $\text{OD}_{600}$  was recorded. O395 (Neg) grew to an average OD of 0.15 and the *rpoE* deletion strain (Pos) grew to an average OD of 0.05. The  $Z'$  factor for this individual experiment was 0.73.

**Abbreviations:** DMSO, dimethylsulfoxide; Neg, negative; Pos, positive; wt, wild type.



**Figure 4** Pilot screen for small molecule inhibitors of *Vibrio cholerae* growth in the presence of 3% ethanol.

**Notes:** Bacteria were seeded into wells containing compound to a final  $OD_{600}$  of  $\sim 0.01$ . Two columns of each control strain with DMSO vehicle were included as controls for each plate. O395 served as the ethanol-sensitive negative control (Neg, blue), and the *rpoE* mutant served as the ethanol-sensitive positive control (Pos, red) for the purpose of this screen. The rest of the plate contained individual compounds in each well. Growth levels in experimental wells are represented as green data points (Samples). The plates were sealed in plastic bags and incubated at 37°C for 7 hours when the  $OD_{600}$  was recorded. Representative view of final  $OD_{600}$  readings is from 384-well plates.

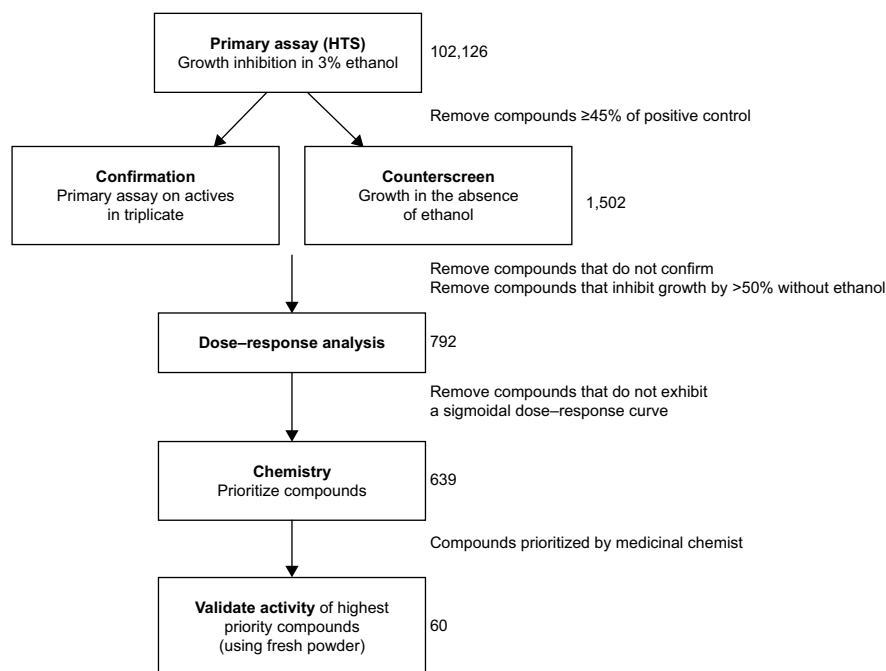
**Abbreviations:** DMSO, dimethylsulfoxide; Neg, negative; Pos, positive.

between the concentration and the level of inhibition were excluded from further study. The remaining compounds were then prioritized for further study by a medicinal chemist based on potency, potential for toxicity/reactivity, and so on. By applying medicinal chemistry analysis, the compounds were categorized into high, medium, and low priority groups for further study. The next step was to establish confidence in the activity of any compounds we were interested in pursuing further. To that end, we purchased 60 of the highest priority

compounds from ChemDiv in order to confirm the activity observed in the primary assay using fresh powders.

## Compound validation

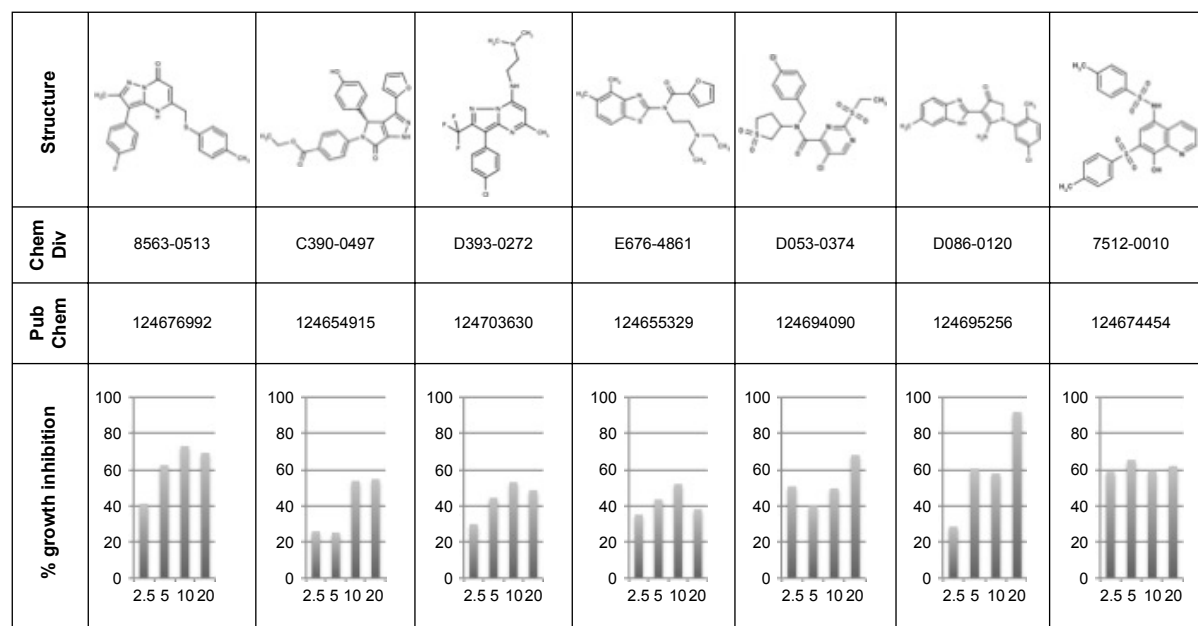
The purchased compounds were retested for growth inhibition in the presence of ethanol in a low-throughput format. Seven of the 60 compounds demonstrated reduced growth ( $\leq 50\%$  of untreated cultures at 10  $\mu\text{M}$ ), validating our previous studies (Figure 6). In addition, all compounds were



**Figure 5** Flowchart of screening and triage strategy.

**Notes:** Criteria for including/excluding compounds at each step are indicated, as well as the number of remaining compounds.

**Abbreviation:** HTS, high-throughput screen.



**Figure 6** Lead compounds.

**Notes:** Of the 60 compounds purchased from ChemDiv for retesting, seven showed reduced growth in the presence of ethanol and limited toxicity in its absence. For each of the seven compounds, the chemical structure, ChemDiv ID, PubChem ID, and percent growth inhibition (relative to untreated cultures) are shown at 2.5  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, and 20  $\mu$ M.

tested for growth inhibition in the absence of ethanol, as was previously performed as a counterscreen. None of these seven compounds significantly reduced growth at a concentration of 10  $\mu$ M, although some showed toxicity at higher concentrations (Figure S1). We consider these seven compounds to be our lead compounds for mechanistic studies and potential therapeutic development. The structures of the seven compounds do not appear to be closely related, which may indicate that different steps in the  $\sigma^E$  pathway are targeted by different compounds or that some of the compounds targeted an unidentified pathway that results in the same growth phenotype. Future studies aim to characterize the mechanism of action of each of the lead compounds and to determine their ability to reduce *V. cholerae* virulence in vivo.

## Discussion

In spite of the use of oral rehydration therapy (ORT), antibiotics, and recently developed vaccines, cholera remains a significant cause of morbidity and mortality in many parts of the world.<sup>28</sup> ORT is the first line of treatment, replacing the fluids lost by the patient and allowing the infection to run its course.<sup>29</sup> ORT is occasionally supplemented by antibiotic treatment, which reduces the symptoms/duration of disease and limits spread to contacts.<sup>30–32</sup> However, as with most bacterial diseases, antibiotic resistance in *V. cholerae* is increasing rapidly. Therefore, we and others are actively

pursuing alternative therapeutic options to improve the treatment of cholera infections.<sup>33–35</sup>

In this study, we used a cell-based, HTS to target a conserved Gram-negative stress response pathway. Although we ultimately identified seven compounds that validated the expected phenotype after purchase and reexamination, most of the purchased compounds did not behave as would be expected. Upon testing the newly purchased compounds, we identified those that did not show any growth inhibition in the presence of ethanol, those that showed significant growth inhibition (toxicity) in the absence of ethanol, and those that inhibited growth in the presence of ethanol, but not at a significant level that would make them candidates for further study. This lack of reproducibility is likely due to the gradual degradation of some compounds in the original screening library. The originally observed phenotypes may have been due to a breakdown product and not the original small molecule. This is why it is critical to validate any phenotypes of interest using freshly purchased compounds.

The strategy of inhibiting a well-characterized stress response mechanism is advantageous for several reasons. Targeting such a pathway is ideal as it does not inhibit virulence per se, but alters a pathway in such a way that treatment with these compounds would decrease the fitness of the organism in the host. Inhibition of bacterial fitness is one of many strategies that are currently being explored

in response to increasing antibiotic resistance in bacterial pathogens.<sup>36</sup> When fitness of a pathogen is decreased in the human host, spread or growth of the organism may be limited, allowing the host microbiota to outcompete the pathogen. In addition, when a therapeutic agent is used that does not kill the pathogen, it is less likely to select for resistance as the selection pressure is less intense. Identification of fitness factors in *V. cholerae* and a range of other bacterial pathogens is underway, due to their potential for therapeutic development.<sup>37–39</sup>

An additional benefit of targeting this specific pathway is the fact that  $\sigma^E$  plays a role in the survival of many other important bacterial pathogens within their hosts. Therefore, it is expected that inhibitors of this conserved pathway could be developed into treatments for other bacterial infections. In addition to the therapeutic potential of such compounds, small molecules have significant value as molecular probes for studying the basic biology of bacterial pathogens. Because these compounds do not inhibit growth or kill the bacteria in the absence of ethanol, they can be used experimentally to study this stress response pathway without genetically altering the pathogen. Due to the conserved nature of this pathway, such probes may be of particular use for studying the  $\sigma^E$  pathway in bacteria where some of these stress response genes are essential for growth and survival.

## Conclusion

We have designed and successfully carried out a novel HTS targeting a stress response pathway that plays a role in the virulence of *V. cholerae*. In addition, we provide confirmatory and counterassays, allowing us to ultimately identify seven lead compounds that are of significant interest for future therapeutic development. Current work focuses on better characterizing the mechanism of action of each of the seven lead compounds, ie, validating that the  $\sigma^E$  pathway is the target of each compound and subsequently determining what step in the  $\sigma^E$  pathway is impacted. Our future goals are to test lead compounds for their toxicity in cultured human cells and then to test any nontoxic compounds for their ability to reduce colonization in an animal model of *V. cholerae* infection.

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## Disclosure

The authors report no conflicts of interest in this work.

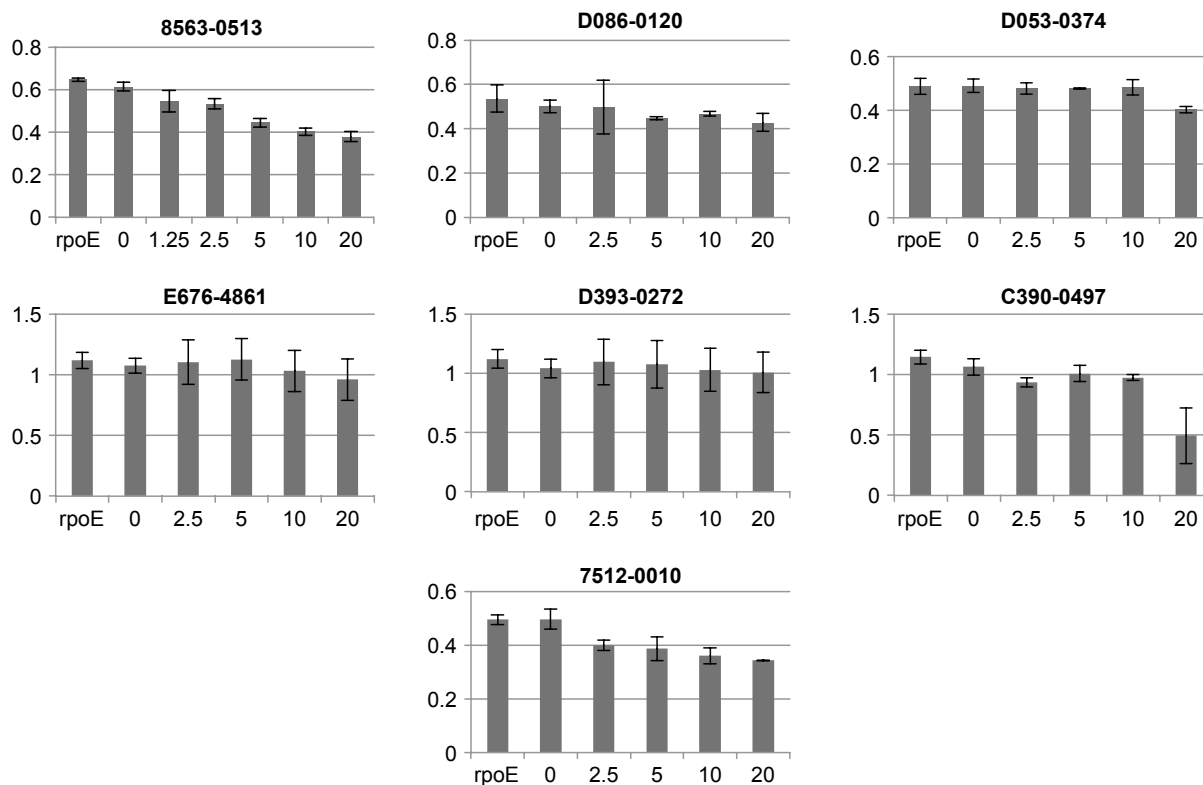
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## Supplementary material



**Figure S1** *Vibrio cholerae* growth in the presence of lead compounds.

**Notes:** The seven lead compounds were assayed for growth inhibition of *V. cholerae* in the absence of ethanol to assess general toxicity of the compounds. The OD<sub>600</sub> of the rpoE mutant (rpoE; positive control) and O395 (wt) grown in the presence of increasing concentrations of compound (μM) are shown.

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