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Amino acid divergence in the ligand binding pocket of *Vibrio* LuxR/HapR proteins determines the efficacy of thiophenesulfonamide inhibitors

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Summary

The quorum sensing signaling systems in *Vibrio* bacteria converge to control levels of the master transcription factors LuxR/HapR, a family of highly conserved proteins that regulate gene expression for bacterial behaviors. A compound library screen identified 2-thiophenesulfonamide compounds that specifically inhibit *Vibrio campbellii* LuxR but do not affect cell growth. We synthesized a panel of 50 thiophenesulfonamide compounds to examine the structure-activity relationship effects on *Vibrio* quorum sensing. The most potent molecule identified, PTSP (3-phenyl-1-(thiophen-2-ylsulfonyl)-1H-pyrazole), inhibits quorum sensing in multiple strains of *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *V. campbellii* at nanomolar concentrations. However, thiophenesulfonamide inhibition efficacy varies significantly among *Vibrio* species: PTSP is most inhibitory against *V. vulnificus* SmcR, but *V. cholerae* HapR is completely resistant to all thiophenesulfonamides tested. Reverse genetics experiments show that PTSP efficacy is dictated by amino acid sequence in the putative ligand binding pocket: F75Y and C170F SmcR substitutions are each sufficient to eliminate PTSP inhibition. Further, *in silico* modeling distinguished the most potent thiophenesulfonamides from less effective derivatives. Our results revealed the previously unknown differences in LuxR/HapR proteins that control quorum sensing in *Vibrio* species and underscore the potential for developing thiophenesulfonamides as specific quorum sensing-directed treatments for *Vibrio* infections.

Introduction

Vibrio species are principal pathogens of marine animals, including fish, shellfish, and coral. Global warming and the concomitant rise of ocean temperatures correlates with increases in *Vibrio* prevalence and spread to regions beyond their typical equatorial habitats (Le Roux *et al.*, 2015, Newton *et al.*, 2012, Vezzulli *et al.*, 2016, Coly *et al.*, 2013, Martinez-Urtaza *et al.*, 2013), consequently harming fish and shellfish aquaculture industries and natural marine ecosystems worldwide. Thus, there is a global need for new treatments for vibriosis in coral reef ecosystems, aquaculture, and in human health, due to consumption of contaminated fish and shellfish. In pathogenic marine *Vibrio* species studied to date, the bacterial cell-cell signaling system called quorum sensing controls biofilm formation, as well as expression and secretion of virulence factors (reviewed in Girard, 2019, Rutherford & Bassler, 2012, Ng & Bassler, 2009). Quorum sensing involves the production and detection of signaling molecules called autoinducers that provide information about the number and type of bacterial cells in the near vicinity. As populations of cells grow denser, autoinducer concentrations increase, and detection of these molecules drives changes in gene expression to alter population-wide behaviors, including those required for pathogenesis.

In vibrios, autoinducers are sensed by membrane-bound histidine kinase receptors that participate in a phosphorylation cascade, ultimately controlling production of the master regulator LuxR (Fig. 1) (reviewed in Ball & van Kessel, 2019, Ng & Bassler, 2009, Rutherford & Bassler, 2012). At low cell densities (LCD), the receptors do not bind autoinducers and function as kinases, shuttling phosphate through LuxU to the Sigma-54-dependent LuxO response regulator. Phosphorylated LuxO activates transcription of the quorum regulatory small RNAs, the Qrrs, which together with the Hfq chaperone repress LuxR translation and activate AphA translation. Thus, at LCD, low levels of LuxR are produced and high levels of AphA are produced, leading to expression of genes for individual behaviors. At high cell densities (HCD), high concentrations of autoinducers results in binding of autoinducers to the receptors, which switches the activity of the receptors to phosphatases. Because LuxO is not phosphorylated, the Qrrs are not transcribed, and thus AphA is not produced and maximal LuxR protein is produced. The general structure of the *V. campbellii* quorum sensing pathway depicted in Figure 1 is conserved in numerous *Vibrio* species, although the autoinducer synthases/receptors and the number of Qrrs vary (reviewed in Girard, 2019). For example, the *luxMN* genes are primarily present only in the Harveyi clade (Simpson *et al.*, 2021), but the vast majority of *Vibrio* species encode LuxO (Girard, 2019). In addition, a nitric oxide sensor pair

H-NOX/HqsK that also feeds phosphate into the system has been identified in some *Vibrio* species (Henares *et al.*, 2013, Hossain *et al.*, 2018, Ueno *et al.*, 2019, Henares *et al.*, 2012).

In each *Vibrio* species in which quorum sensing has been studied, the signaling cascade culminates in the master LuxR-type transcription factor, which activates and represses many genes ranging from ~100 to ~400 genes depending on the species (Fig. 1) (reviewed in Ball *et al.*, 2017, Girard, 2019). The LuxR protein is highly conserved in all pathogenic vibrios studied to date (Rutherford *et al.*, 2011, Kernell Burke *et al.*, 2015, Kim *et al.*, 2013, Lee *et al.*, 2008, Tsou *et al.*, 2009, Ball *et al.*, 2017, Kim *et al.*, 2010, De Silva *et al.*, 2007). Although the naming of the *V. campbellii* protein LuxR causes confusion, this protein does not resemble or function like the LuxR protein that is part of the *Vibrio fischeri* LuxI/LuxR quorum sensing system, which requires binding to the autoinducer molecule made by LuxI for activity (Fuqua *et al.*, 1994). Conversely, the LuxR from *V. campbellii* belongs to the TetR superfamily (Ramos *et al.*, 2005), and these proteins are structurally, genetically, biochemically, and functionally distinct from the *V. fischeri* LuxR. LuxR/TetR homologs in vibrios include SmcR in *V. vulnificus*, HapR in *V. cholerae*, OpaR in *V. parahaemolyticus*, and VcpR in *Vibrio coralliilyticus*, which share 76-96% amino acid identity (Ball *et al.*, 2017). LuxR/TetR proteins in vibrios directly bind to multiple sites in promoter regions and interact with other proteins (e.g., RNA polymerase, IHF) or compete with other proteins (e.g., H-NS) to activate or repress transcription of hundreds of quorum sensing genes (Ball & van Kessel, 2019, Chaparian *et al.*, 2016, Chaparian *et al.*, 2020, van Kessel *et al.*, 2013a, van Kessel *et al.*, 2013b). It is critical to note that these quorum-sensing LuxR/TetR-type proteins do not have a known ligand, although a putative ligand binding pocket has been defined in structures and shown to bind inhibitors (Kim *et al.*, 2018, Kim *et al.*, 2010, De Silva *et al.*, 2007). Thus, if there is a native ligand, it is also unknown how it affects function of the LuxR protein and its impact on the physiology and group behaviors of the cells.

Vibrio LuxR/TetR-type proteins play crucial roles in colonization and infection of hosts through quorum-directed regulation of biofilm formation, type III and type VI secretion systems, motility, and production of proteases, hemolysins, siderophores, and cytotoxins (Girard, 2019). Deletion or inhibition of LuxR proteins in several vibrios reduces or eliminates colonization and toxicity, effectively increasing host survival (Hasegawa & Hase, 2009, Kim *et al.*, 2013, Kim *et al.*, 2018). Thus, LuxR represents a key target for designing therapeutics to block quorum sensing in vibrios. LuxR inhibitors would presumably render *Vibrio* cells unresponsive to quorum sensing signals even at HCD, thus restricting cells to their LCD gene expression program. Indeed, recent studies have

shown that quorum sensing inhibitors are viable alternative approaches to traditional antibiotics in disease treatment with demonstrated efficacy in animal models (Remy *et al.*, 2018, Jiang *et al.*, 2019). Because quorum sensing inhibitors typically do not affect growth of the bacteria but rather inhibit specific pathways (Starkey *et al.*, 2014, Guendouze *et al.*, 2017), these molecules are hypothesized to generate less selective pressure for evolving resistance.

V. campbellii LuxR-specific inhibitors have been identified via a variety of methods, including both *in vitro* and *in silico* screening strategies. These include compounds with varying functional groups such as aromatic enones, sulfonamides, sulfones, cinnamaldehydes, furanones, and brominated thiophenones (Brackman *et al.*, 2011, Defoirdt *et al.*, 2012, Rajamanikandan *et al.*, 2017, Rajamanikandan *et al.*, 2016, Defoirdt *et al.*, 2007, Kim *et al.*, 2020, Brackman *et al.*, 2008), and each was shown to specifically inhibit bioluminescence, biofilm formation, and/or protease activity in *V. campbellii* and in some cases other *Vibrio* species. However, either the maximum inhibition of bioluminescence for some molecules was low (~3-fold) or the inhibitory concentrations required to observe phenotypic effects *in vitro* or *in vivo* were high (>20 μ M). Several of these molecules have shown favorable therapeutic potential because addition of these molecules to brine shrimp larvae infected with *Vibrio* cells increased survival. In particular, the molecule called Qstatin (1-((5-bromothiophen-2-yl)sulfonyl)-1*H*-pyrazole) is a highly promising molecule that was shown to be a specific inhibitor of the LuxR homolog SmcR in *Vibrio vulnificus* both *in vitro* and *in vivo* (Kim *et al.*, 2018).

Here, we used an *E. coli* bioassay to screen chemical libraries to identify specific inhibitors of LuxR that do not affect bacterial cell growth. Our structure-activity relationship data show that multiple thiophenesulfonamide-containing molecules with heterocycle variations are strong inhibitors of LuxR-type proteins in a wide-range of pathogenic *Vibrio* species. Our data also show that LuxR homologs are more functionally distinct from each other than previously hypothesized.

Results

A dual-color bioassay screen identifies LuxR chemical inhibitors

LuxR proteins have been shown to activate and repress gene expression in vibrios through direct binding to specific DNA sequences in promoters (Ball *et al.*, 2017, Ball & van Kessel, 2019, van Kessel *et al.*, 2013b). Previously, we developed a bioassay that consists of a dual-color fluorescent reporter plasmid that reports both LuxR activities: the *luxCDABE* promoter is activated by LuxR and drives expression of *gfp*, and the *VIBHAR_05222* promoter is repressed by LuxR and

drives expression of *mCherry* (van Kessel *et al.*, 2013b) (Fig. 2A). We used this reporter plasmid in an *E. coli* strain that also contains a plasmid expressing LuxR from its native promoter. Thus, in the presence of LuxR, GFP levels increased and mCherry levels decreased compared to the control strain (Fig. 2B). As a positive control, we showed that Qstatin inhibited LuxR activity in this assay (Fig. S1). We screened ~60,000 molecules in the ChemBridge (Fig. S1A, S1C) and Chemdiv (Fig. S1B) libraries and identified nine compounds that inhibited LuxR activation and/or repression but did not affect the final growth yield more than 10%. Four of these compounds contained a sulfonamide or sulfamide core with variable groups on each side similar to Qstatin, whereas the other compounds were structurally dissimilar (Fig. 2C). We synthesized or purchased these molecules and determined the half-maximal inhibitory concentration (IC_{50}) for each in the *E. coli* bioassay strain using titration curves (Table 1, Fig. 2D, 2E). P0053 I18 had the best inhibitory effect on LuxR in the *E. coli* bioassay with an IC_{50} similar to Qstatin (Table 1, Fig. 2D, 2E, S3). P2065 E16 had only a minor inhibition of LuxR, and a variation of this molecule lacking the CF_3 group on the heterocycle had no activity (Fig. 2D, 2E, S3). We also observed consistent inhibition by P0074 H04 and P0053 O05, which also contain sulfamide/sulfonamide cores, though the IC_{50} values for these were higher than Qstatin or P0053 I18 (Fig. 2D, 2E).

Structure-activity relationship study of thiophenesulfonamide panel

To further explore the thiophenesulfonamide class of compounds that includes Qstatin, P0053 O05, and P0074 H4, we performed a structure-activity relationship (SAR) study of Qstatin derivatives. We chose to focus on these thiophenesulfonamide molecules for SAR instead of P0053 I18 because the synthesis of sulfonamides is a single step, and the heterocycle components are readily available commercially. We synthesized a panel of Qstatin derivatives with steric and electronic structural variations in both heterocycles (Fig. 3A). Modifications to the heteroaromatic amine ring (pyrazole in the case of Qstatin) are referred to with number designations for each class and modifications to the thiophene ring with letter designations for each class (Fig. 3A). Assays with these molecules in the *E. coli* bioassay showed that the most active compounds contained a 3-methyl- (class 8) or 3-phenyl-substituted pyrazole (class 10), an unsubstituted pyrazole (class 1), or a pyrrole in place of the pyrazole (class 3) (Fig. 3B, 3C, S2, S3). Compounds containing other heterocycles did not have activity against LuxR (Fig. S2, S3). It is particularly noteworthy that the compounds containing an imidazole ring (class 2) were not active, which was not expected given that the structure is highly similar to pyrroles and pyrazoles (classes 1 and 3). Methyl substitution at the 3

position of the pyrazole did not alter activity compared to Qstatin (class 8), however methyl substitutions at both the 3- and 5- positions of the pyrazole eliminated activity (class 9). In most cases, the presence/absence of Br or Cl atoms on the thiophene ring did not alter activity (Fig. 3). For example, Qstatin, 1B, and 1C had similar activities, and 10A, 10B, and 10C had similar activities. The conformation of the sulfonamide core appeared to be critical because substitution with a carbonyl eliminated activity of that class of compounds (classes F and G). The most potent molecules were compounds 10A, 10B, and 10C, all of which contain a phenyl group on the 3-position of the pyrazole and vary in the presence or absence of bromine or chlorine on the thiophene ring (Fig. 3, S2, S3).

2-thiophenesulfonamide compounds specifically inhibit LuxR proteins

Many sulfonamide-containing compounds are known to target the folate synthesis pathway in bacteria and inhibit growth (bacteriostatic), and thus these “sulfa drugs” have been used as broad-spectrum antibiotics for decades (Hammoudeh *et al.*, 2013, Roland *et al.*, 1979). However, the structurally distinct 2-thiophenesulfonamides generally did not have any bacteriostatic activity; only two compounds (4A and 4C) in this entire panel limited *E. coli* growth yield more than 10% compared to negative controls, and this effect was only observed at concentrations of 100 μ M and higher (Fig. S4A). Addition of high concentrations of 10B or 10C to *V. campbellii* or *V. vulnificus* did not alter growth rate or growth yield (Fig. S4B, 4C), suggesting that the inhibitory activity is specific to LuxR, and these are not general antibacterial compounds. We also tested the stability of the inhibitors in bacterial cultures and the timing of the inhibitory effect. We observed that molecule 10B inhibited LuxR activity rapidly; GFP production was blocked 120 minutes after 10B addition to the culture, and this was maintained through the longest timepoint tested at 16 hours (Fig. S4D).

RNA-seq performed with Qstatin added to *V. vulnificus* cultures showed that Qstatin has a specific effect on the SmcR regulon (Kim *et al.*, 2018). The Qstatin-treated regulon overlapped with the SmcR regulon by more than 50%, with similar gene expression fold-changes. There were no significant off-target effects in the presence of Qstatin in the $\Delta smcR$ strain, indicating that Qstatin specifically targets only SmcR. Finally, addition of Qstatin to wild-type *V. vulnificus* increases *smcR* transcript levels 2.5-fold; however, the authors did not observe a correlating increase in SmcR protein levels (Kim *et al.*, 2018). This is important to note because LuxR and HapR proteins are known to autorepress their own expression (Chatterjee *et al.*, 1996, Lin *et al.*, 2005). To test the effects of adding inhibitors to *V. campbellii* LuxR, we added 10A, 10B, and 10C to wild-type *V.*

campbellii and used qRT-PCR and western blots to examine the effects on *luxR* transcript and protein levels. We also examined the transcript levels for *luxC* and *05222* genes that are directly activated or repressed by LuxR, respectively. We observed a significant 6- to 7-fold increase in *luxR* transcript levels in the presence of each of the 10A-C inhibitors, as well as a visible increase in LuxR protein level (Fig. S5A, S5D). Addition of 10A-C to wild-type cultures significantly decreased *luxC* transcripts to levels in a $\Delta luxR$ strain (Fig. S5B). Similarly, addition of 10A-C to wild-type cultures significantly increased *05222* transcripts (Fig. S5C). Collectively, these data show that 2-thiophenesulfonamide molecules are stable in bacterial culture, specifically inhibit LuxR activity, and do not significantly affect cell growth.

2-thiophenesulfonamides inhibit quorum sensing gene expression in Vibrios

Qstatin has been shown to be an effective inhibitor of SmcR *in vitro* and *in vivo* (Kim *et al.*, 2018). It also inhibits pathogenesis in *V. campbellii*, *V. parahaemolyticus*, and *V. vulnificus* in a shrimp infection assay, presumably through inhibition of the LuxR-type protein in these strains (Kim *et al.*, 2018). To assess the activity of our panel of thiophenesulfonamide inhibitors against other vibrios, we assayed quorum-sensing controlled phenotypes in several strains from five *Vibrio* species: *V. campbellii*, *V. coralliilyticus*, *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. Although most of these vibrios are not bioluminescent, the *luxCDABE* operon from *V. campbellii* BB120 is routinely used to measure quorum sensing and LuxR regulation in other vibrios because LuxR directly binds this promoter and is required for gene expression (Jung *et al.*, 2016, Kim *et al.*, 2018, Lenz *et al.*, 2005, Miller *et al.*, 2002, Simpson *et al.*, 2019). We introduced a plasmid containing the *luxCDABE* promoter driving expression of *gfp* (pCS19 (kanamycin-resistance cassette) or pCS42 (gentamicin-resistance cassette)) into each of the *Vibrio* strains. We focused our assays on the molecules with the most activity determined in Fig. 3, which we refer to as the “top panel”. We observed that 10A, 10B, and 10C molecules were the most inhibitory in *V. campbellii*, *V. vulnificus*, and *V. parahaemolyticus*, and in each, 8A had a similar IC₅₀ to Qstatin (Fig. 4A-E, Table 2). Molecules 10A, 10B, and 10C are so potent in *V. vulnificus* that we performed extended serial dilutions to obtain accurate IC₅₀ data because initial titrations did not yield enough points for a complete curve (Fig. 4E, S6A-C, Table 2). Notably, 10A, 10B, and 10C were much more inhibitory compared to Qstatin, with IC₅₀ values that were 30- to >500 lower than Qstatin in *V. vulnificus* (Table 2). We also observed that few molecules were active in *V. coralliilyticus* OCN008, but 3B had the most noticeable effect (Fig. 4C). Importantly, there was a distinct difference in the IC₅₀ for each

molecule when compared across the five species. *V. vulnificus* exhibited very low IC₅₀ values for all the top panel molecules (Fig. 4E), whereas *V. cholerae* was completely resistant to these molecules even at high concentrations (Fig. 4B). Using 10B as an example, the IC₅₀ values were orders of magnitude different comparing *V. vulnificus* (0.002 μ M) to *V. campbellii* (0.35 μ M) (Fig. 4A, 4E, Table 2). To further examine this observation, we assayed the top panel of molecules against additional strain types for each species (Table 2). We observed that the inhibitory effect of the molecules was similar for each strain within a species (Table 2). For example, the IC₅₀ values for 10B for all three *V. vulnificus* clinical isolates ranged from 2-30 nM. From these data, we conclude that 10B has the highest inhibitory activity in most *Vibrio* species, with variation in the IC₅₀ that is specific to the *Vibrio* species tested.

We next focused on the best inhibitor in the panel, 10B, which is called 3-phenyl-1-(thiophen-2-ylsulfonyl)-1*H*-pyrazole, and thus we refer to 10B as PTSP from here on. We assessed LuxR function in the presence of PTSP by assaying protease production, a key virulence activity in vibrios during pathogenesis and host cell lysis (Fig. 4F, 4G). LuxR proteins have been shown to activate several genes encoding proteases in *Vibrio* species, such as HapA in *V. cholerae*, VvpE in *V. vulnificus*, PrtA in *V. parahaemolyticus*, and VcpA/VcpB in *V. coralliilyticus* (previously called VtpA and VtpB when the strain was misidentified as *Vibrio tubiashii*) (Hasegawa & Hase, 2009, Jeong *et al.*, 2003, Jobling & Holmes, 1997, Chang & Lee, 2018, Shinoda & Miyoshi, 2011). We analyzed protease activity of bacterial supernatants using azocasein as a substrate and compared wild-type strains to their isogenic $\Delta luxR$ counterparts for one representative strain for each species. In each species except *V. parahaemolyticus*, the wild-type strain produced significantly more protease activity than the $\Delta luxR$ strain (Fig. 4F). Addition of PTSP to the wild-type strain significantly reduced protease activity in *V. campbellii*, *V. coralliilyticus*, and *V. vulnificus*, but not in *V. cholerae* (Fig. 4F). *V. parahaemolyticus* exhibited extremely low protease activity in this assay, thus the effect of PTSP on *V. parahaemolyticus* cannot be ascertained in this experiment. We observed that the effect of PTSP on protease activity mirrors that of bioluminescence: PTSP had minimal activity against *V. coralliilyticus* and highest activity against *V. vulnificus*. (Fig. 4F). Although we assessed the effect of PTSP on protease activity in numerous strains for *V. campbellii*, *V. coralliilyticus*, and *V. parahaemolyticus*, the protease activity was so low in many of these isolates that an IC₅₀ could not be reliably calculated for these (Fig. S6). However, for the three *V. vulnificus* strains, we calculated the IC₅₀ values for PTSP inhibition and observed a similar range of protease inhibition as we observed for bioluminescence: ATCC 27562 = 6.8 nM, CMPC6 = 78.1 nM, and YJ016 = 18.3 nM

(Fig. 4G). From the bioluminescence and protease assay data, we conclude that PTSP is a potent inhibitor of *V. vulnificus*, *V. parahaemolyticus*, and *V. campbellii*, with moderate effects on *V. coralliilyticus*. Inhibitor PTSP and derivatives were not active against *V. cholerae* (Fig. 4B, 4F, 4G). Further, we conclude that the use of the *E. coli* bioassay reporter is a valid assay for monitoring endogenous LuxR activity in these *Vibrio* species.

2-thiophenesulfonamide efficacy is driven by amino acid conservation in the ligand binding pocket

We hypothesized that there were several possible reasons for the observed difference in PTSP inhibition in the various *Vibrio* species, including but not limited to: 1) differences in LuxR-inhibitor interaction(s), 2) differences in diffusion of the inhibitor across the membranes, or 3) stability of the inhibitor in the cell and/or cell culture. To examine PTSP activity in a common strain background, we cloned the *luxR* gene from each *Vibrio* species into a plasmid under control of an IPTG-inducible promoter and assayed the effect of PTSP against these proteins in the *E. coli* bioassay. We found that HapR was unresponsive to PTSP in *E. coli*, exhibiting a similar level of GFP expression to the negative control (Fig. 5B). Conversely, SmcR, LuxR, OpaR, and VcpR were each inhibited by PTSP with a trend similar to that observed in their native *Vibrio* cells, in which the order of sensitivity to PTSP inhibition was SmcR>LuxR>OpaR>VcpR (Fig. 5B). We conclude that the differences in PTSP activity in *Vibrio* species is due to differences in interaction between PTSP and the LuxR-type protein in each *Vibrio*.

Using the SmcR-Qstatin X-ray crystal structure as a guide (Kim *et al.*, 2018), alignment of the LuxR-type proteins from each *Vibrio* species showed that there are four residues that interact with Qstatin in SmcR that are variable in HapR and/or VcpR (Fig. 5A). We therefore sought to determine if any of the four residues that differ between HapR and LuxR/SmcR in the putative ligand binding pocket are sufficient to render SmcR insensitive to PTSP. First, we introduced substitutions in SmcR to mimic the amino acid sequence of HapR: F75Y, I96L, V140I, and C170F. We observed that the substitutions of F75Y and C170F in SmcR abolished PTSP inhibition, suggesting that F75 and C170 are both necessary for PTSP inhibition of SmcR transcription regulation *in vivo* (Fig. 5C). However, SmcR I96L and V140I were not significantly different from wild-type SmcR. Next, we introduced single substitutions in HapR to mimic the amino acid present in SmcR: Y76F, L97I, I141V, and F171C. However, none of these substitutions alone were sufficient to make HapR sensitive to PTSP (Fig. 5C). Further, combination of Y76F and F171C did not result in PTSP inhibition. From these

results, we conclude that SmcR F75 and C170 are critical residues necessary for PTSP inhibition of SmcR activity.

Modeling of 2-thiophenesulfonamide inhibitors predicts efficacy in vivo

Previous studies have used molecular docking simulations to predict effective inhibitors of LuxR family proteins (Rajamanikandan *et al.*, 2017). To examine the accuracy of using molecular docking to predict effective inhibitors of LuxR proteins, we used Autodock Vina software (Trott & Olson, 2010) to simulate binding of molecules to the structure of SmcR and calculate the best binding mode and predicted binding energy (kcal/mol). First, to validate this modelling approach, we used Autodock Vina to predict the binding position of Qstatin into the SmcR (apo) X-ray crystal structure and compared it to the solved structure of SmcR-Qstatin (Fig. 6A). Although there was a slight shift (0.6 to 1.4 Å), the position and orientation of Qstatin within the putative ligand binding pocket of SmcR predicted by Autodock Vina was consistent with the SmcR-Qstatin structure (Fig. 6A). We next used Autodock Vina to predict the binding position of PTSP in both SmcR and HapR (Fig. 6B). We observed that PTSP was modeled in the opposite orientation in SmcR compared to HapR. We suspected that the orientation of PTSP was likely driven at least partially by the rotational position of glutamine 137 in SmcR (Q138 in HapR) that clashes with the phenyl ring of PTSP. We also noted that SmcR Q137 had multiple rotamers among the four SmcR chains within the asymmetric unit (Fig. 6C), and Autodock Vina modelling indicated that PTSP was oriented differently in SmcR chain A compared to chain B (Fig. 6D). Thus, the binding orientation and interactions of PTSP with SmcR are likely influenced, at least in part, by the orientation of Q137. The predicted orientation of PTSP was different in chains A and B that have different Q137 rotamers and different predicted binding energies (Fig. S7; -7.5 kcal/mol for chain A compared to -0.2 kcal/mol for chain B). In addition, the predicted binding energies for PTSP in HapR were clearly worse at +1.6 kcal/mol for chain A and +10.4 kcal/mol for chain B (Fig. S7).

Because Autodock Vina accurately predicted the binding position of Qstatin in SmcR, we modelled binding of all the molecules in the sulfonamide panel into SmcR (Table S1). For the molecules that were active *in vivo*, we plotted the predicted binding affinities for each molecule against the calculated IC₅₀ values from the *in vivo* assay in *V. vulnificus* (Fig. 6E). We were unable to plot the remaining molecules because an IC₅₀ value could not be calculated from any *in vivo* data because titrations of these molecules did not produce an inhibition curve (Fig. S2). Molecules 10A, 10B (PTSP), and 10C clustered in a group with the similarly low predicted binding energies and

lowest observed IC₅₀ values (Fig. 6E). Conversely, the molecules with the low-moderate inhibitory activity were predicted to have similar binding energies that were indistinguishable (Fig. 6E). From these data, we conclude that molecular docking simulations can accurately predict sulfonamide molecules with tight binding affinity and strong inhibitory activity against SmcR and LuxR.

In vitro analysis of PTSP binding affinity and effects on DNA binding activity

To test the validity of the modelling predictions for PTSP, we performed isothermal titration calorimetry (ITC) to determine the binding affinity of SmcR and LuxR for PTSP. We found that PTSP had a lower (tighter) dissociation constant (K_d) for SmcR (0.23 μ M) compared to LuxR (0.51 μ M). As a control, we tested the binding affinity of Qstatin for SmcR and observed a dissociation constant of 0.74 μ M (Table 3, Fig. S8C, Dataset S1), which is slightly higher than previously published ITC experiments with Qstatin and SmcR that reported a 0.47 μ M dissociation constant (Kim *et al.*, 2018). We noted that PTSP binding to both LuxR and SmcR had lower entropy and enthalpy compared to SmcR binding to Qstatin. Further, PTSP binding to SmcR had a lower binding energy than Qstatin (Table 3), although the modeling predictions were overall higher binding energies than experimentally observed: -23 kJ/mol for Qstatin and -30 kJ/mol for PTSP determined by modeling compared to -35 kJ/mol for Qstatin and -38 kJ/mol for PTSP determined by ITC. Our collective data show that PTSP had a lower IC₅₀ in *V. vulnificus* than in *V. campbellii*, and PTSP had a tighter binding affinity to *V. vulnificus* SmcR than *V. campbellii* LuxR. Thus, we conclude that the efficacy of PTSP inhibition *in vivo* in these two *Vibrio* species correlates to the binding affinity determined *in vitro* for these two proteins. Further, our data suggest that the increased efficacy of PTSP inhibitory activity against SmcR is due to lower (tighter) binding affinity of PTSP to SmcR compared to Qstatin.

We noted that Qstatin and PTSP are both predicted to bind in the putative ligand binding pocket of SmcR. It is currently unknown how Qstatin allosterically affects DNA binding through its interactions in the ligand binding domain. Kim *et al.* showed that Qstatin does not appreciably alter the DNA binding constant for SmcR, but rather affects the entropy and enthalpy with which it interacts with DNA (Kim *et al.*, 2018). We therefore also assessed the effect of sulfonamide PTSP on the DNA binding activity of LuxR and SmcR *in vitro*. Addition of saturating concentrations of PTSP did not alter DNA binding to LuxR or SmcR (Fig. 6F, Fig. S9). This result is similar to the finding that Qstatin has very little effect on SmcR DNA binding affinity (Fig. 6F) (Kim *et al.*, 2018). Collectively, these data show that thiophenesulfonamides inhibit the function of LuxR proteins to different levels.

In addition, these data suggest that the mechanism of inhibition by thiophenesulfonamides is likely similar in both SmcR and LuxR.

Discussion

This study tested a panel of inhibitors against quorum sensing, a non-essential cell signaling pathway that controls pathogenesis in *Vibrio* species. Using a previously established dual-color bioassay, we screened thousands of compounds and synthesized a broad panel to find potent inhibitors of the quorum sensing master transcription factor LuxR (van Kessel *et al.*, 2013a, van Kessel *et al.*, 2013b). We observed that some of our key candidates contain sulfamide/sulfonamide heterocycles, but there exists a vast range of inhibition between these various compounds. Using the best candidate inhibitor PTSP, we tested its efficacy against five vibrios *in vivo* and found that it is most effective towards *V. vulnificus*, followed by *V. campbellii*, *V. parahaemolyticus*, *V. coralliilyticus*, and not effective against *V. cholerae*. The efficacy observed in inhibition of bioluminescence reporter assays was mimicked in protease assays where measurable. These results show that PTSP blocks bioluminescence and protease activities of LuxR proteins. This result is comparable to what was observed for the thiophenesulfonamide Qstatin, which blocks SmcR regulation of genes across the *V. vulnificus* genome to similar levels as a $\Delta smcR$ strain (Kim *et al.*, 2018). Thus, our results show that thiophenesulfonamides are broadly inhibitory of LuxR activities in multiple *Vibrio* species.

We were intrigued by the finding that the compounds we tested have no effect on HapR from *V. cholerae*. Our data show that HapR resistance is due to amino acid residue differences in the putative ligand binding pocket. We focused on differences in the residues in the Qstatin binding pocket across five *Vibrio* species, though substitution of single amino acids in HapR are not sufficient to render the protein sensitive to PTSP. We note that substitutions Y76F and F171C did not alter sensitivity to the PTSP molecule, but rather affected overall activity of HapR. We presume that multiple, combined substitutions in the HapR ligand binding pocket to change the sequence to match SmcR would likely result in HapR sensitivity to PTSP, though this needs to be formally tested. Interestingly, *V. cholerae* also has a very different pathogenic life cycle compared to other vibrios. Pathogenesis in the human host caused by *V. cholerae* occurs at LCD, where the bacterial cells attach to intestinal epithelial cells through the toxin co-regulated pilus and grow as a biofilm, producing cholera toxin. Growth of the population and accumulation of autoinducers drives inhibition of biofilms through various regulatory mechanisms, cleavage from the host epithelium by the HapA

protease, and the cells are shed back into the marine environment (Hammer & Bassler, 2003, Vance *et al.*, 2003, Waters *et al.*, 2008, Zhu & Mekalanos, 2003, Papenfort *et al.*, 2017). This poses some intriguing evolutionary questions about *V. cholerae* pathogenesis and growth in the environment and the selective pressures that may have driven differences in amino acid conservation between HapR and other LuxR-family proteins. This could underscore the stark contrast in efficacy of inhibitors against HapR and other *Vibrio* LuxR-type regulators that we observed in this study. This difference in inhibition between HapR and the other LuxR-type regulators contradicts the conventional thought in the field that these proteins are similar enough to act interchangeably due to cross-complementation experiments at specific loci (Jobling & Holmes, 1997, Shao & Hor, 2001). We hypothesize that successful HapR inhibitors will require a HapR-specific targeted approach.

In addition to our *in vivo* studies, our *in silico* studies allowed us to model binding affinities of compounds to accurately predict which compounds are the best inhibitors of LuxR proteins. Our modelling experiments generated predicted binding energies that are consistent with the efficacy of the 10A, 10B (PTSP), and 10C molecules, which are the most potent LuxR inhibitors identified thus far. This tool will be very useful for future drug development as more compound classes are examined. However, it is also clear that the modelling could not reliably predict every critical amino acid contact. Although we identified two critical residues in SmcR, F75 and C170, we hypothesize that more than one critical contact exists because these substitutions did not render HapR sensitive to PTSP. Although the structure of PTSP is similar to that of Qstain, we were able to show that PTSP is >500X more inhibitory in some assays. We hypothesize that this molecule would make a better initial candidate for future experiments aimed at developing these molecules as therapeutics. While modeling has shown to be very effective in our experiments thus far, we recognize that a structure of PTSP bound to SmcR would provide the most information to further optimize *Vibrio* infection therapeutics for PTSP and derivatives.

Experimental procedures

Bacterial strains and media

All strains used in this study are listed in Table S2. *E. coli* strains DH10B and S17-1 λ pir were used for cloning, and BL21(DE3) was used for overexpression of LuxR and SmcR proteins. All *E. coli* strains, *V. cholerae* strains, and derivatives were grown in Lysogeny Broth (LB) at 30°C shaking at 275 RPM in LB media with the appropriate antibiotic. *V. campbellii*, *V. parahaemolyticus*, *V. coralliilyticus*, and *V. vulnificus* strains and derivatives were grown shaking at 275 RPM at 30°C in

Luria Marine (LM) medium (LB with 2% NaCl) with appropriate antibiotics. Antibiotics were used at the following concentrations: kanamycin 50 µg/mL or 250 µg/mL (*E. coli* or *Vibrio*, respectively), chloramphenicol 10 µg/mL, ampicillin 100 µg/mL, gentamicin 100 µg/mL, and tetracycline 10 µg/mL.

Molecular methods

All PCR reactions were performed using Phusion HF polymerase (NEB). T4 polynucleotide kinase (T4 PNK) used in EMSAs and all other enzymes were purchased from NEB and used according to manufacturer's instructions. Site-directed mutagenesis for construction of plasmids expressing mutant proteins was carried out using the Agilent QuikChange II XL Site-Directed Mutagenesis Kit. All oligonucleotides were purchased from Integrated DNA Technologies (IDT), and those used in this study are listed (Table S3). All plasmid constructs (Table S4) were confirmed by DNA sequencing (Eurofins). Cloning details for plasmids are available upon request. Western blot analysis was performed as previously described (van Kessel *et al.*, 2013b), except that new anti-LuxR antibodies were generated through Cocalico against purified LuxR protein. In addition, the western blots were probed with anti-RpoB (Neoclone).

RNA analysis by qRT-PCR

To collect RNA samples, cells were grown at 30°C shaking at 275 RPM to an OD600 of approximately 0.2. Then cells were induced with 50 µM IPTG and grown under the same conditions until cells reached an OD600 of approximately 1, at which 5 mL of cells were collected by centrifugation and frozen in liquid N₂. RNA was extracted using a Trizol/chloroform extraction protocol previously described (Rutherford *et al.*, 2011). Quantitative real-time PCR (qRT-PCR) was performed as previously described (Chaparian *et al.*, 2016). Samples were normalized to the internal standard *hfq* gene. The $\Delta\Delta CT$ values were used to analyze data from three independent biological replicates. Symbols on graphs represent the mean values and error bars represent the standard deviations. All statistical analysis was performed with functions from GraphPad Prism version 8 and the primers used are listed in Table S3.

Compound synthesis and purchase

Synthesis and characterization data for 1B-C, 3A-C, 8A-C, 10A-C, P007 H4, P2065 E16, and P2065 E16-CF₃ are provided in the supplementary methods. Compounds P0053 O05 and P1117

F20 were purchased from Lab Network, P1120 D05 and P0074 N08 were purchased from EnamineStore, and P0053 I18 was purchased from ChemDiv.

E. coli bioassay

The dual-promoter fluorescence reporter assays were performed using *E. coli* strain DH10B containing two plasmids: 1) plasmid pJV064 containing the P_{luxC} fused to GFP and P_{05222} fused to mCherry to assess LuxR transcriptional regulation (van Kessel *et al.*, 2013b), and 2) plasmid pKM699 expressing *V. campbellii luxR* under control of its native promoter or empty vector pLAFR2 (Hustmyer *et al.*, 2018). Overnight *E. coli* cultures containing either pKM699 or pLAFR2 and the pJV064 reporter were diluted 1:100 into LB with chloramphenicol and tetracycline and aliquoted into black-welled, clear-bottomed 96-well plates (150 μ l final volume). Compounds were resuspended in DMSO and added to *E. coli* cultures at varying concentrations, or DMSO was added as a negative control at equal volumes. 96-well plates were covered in microporous sealing tape and grown for 16 hours shaking at 275 RPM at 30°C. The OD₆₀₀ and fluorescence (both GFP and mCherry) were measured on a BioTek Cytation plate reader.

In *E. coli* assays expressing the five different LuxR-type proteins (*luxR*, *smcR*, *opaR*, *vcpR*, *hapR*), slight changes to the protocol were implemented. The *luxR* genes were cloned under control of the IPTG-inducible P_{tac} promoter in plasmid pMMB67EH-kanR. *E. coli* DH10B strains containing one of these plasmids and the pJV064 reporter were diluted 1:1,000 into LB with chloramphenicol and kanamycin and aliquoted into black-welled, clear-bottomed 96-well plates (150 μ l final volume). Strains expressing *hapR* or *smcR* required 50 μ M IPTG to observe activation of the GFP reporter; IPTG was not added to strains expressing *luxR*, *vcpR*, or *opaR*. Strains were incubated with either 25 μ M PTSP or DMSO (equal volumes) in 96-well plates covered in microporous sealing tape and grown for 16 hours shaking at 275 RPM at 30°C. The OD₆₀₀ and fluorescence (both GFP and mCherry) were measured on a BioTek Cytation plate reader.

Protein purification, electrophoretic mobility shift assays (EMSAs), and isothermal titration calorimetry (ITC)

SmcR and LuxR were purified as described previously (Newman & van Kessel, 2020). EMSAs were conducted as described previously (Newman *et al.*, 2020, Newman & van Kessel, 2020) using oligonucleotides corresponding to the *luxC* and *vvpE* promoter sequences (Table S3). ITC analysis was conducted using purified LuxR or His-tagged SmcR, each in gel-filtration buffer (25

mM Tris pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 2% DMSO). All molecules analyzed were diluted in the same buffer. The samples were degassed for 10 min by vacuum aspiration. Titrations were performed on a Nano-ITC (TA Instruments). SmcR in the syringe (100 μ M dimer) or LuxR in the syringe (77.4 μ M dimer) was titrated against 20 μ M 10B (PTSP) or Qstatin in the reaction cell with 35x 1 μ l injections for PTSP or 23x 2 μ l for Qstatin, stirring at 250 RPM, 120 seconds between injections, and temperature held at 25°C. Thermograms were analyzed using NanoAnalyze software with the independent binding model.

Assaying compounds in Vibrio cultures

Vibrio strains were inoculated in 5 ml LM (or LB for *V. cholerae*) overnight at 30°C shaking at 275 RPM with kanamycin (100 μ g/ml) or gentamicin (15 μ g/ml) to select for the *PluxC-gfp* reporter plasmids pCS19 or pCS42, respectively. Cultures were back-diluted 1:1,000 in LB or LM with antibiotics, and the cell mixture was aliquoted into black-welled, clear-bottomed 96-well plates. Compounds were titrated into the wells (4-fold dilution series; final volume of 150 μ l). DMSO was added as a negative control at equal volumes into control reactions. 96-well plates were covered in microporous sealing tape and grown for 16 hours shaking at 275 RPM at 30°C. The OD₆₀₀ and GFP fluorescence or bioluminescence were measured on a BioTek Cytation plate reader.

Protease assays

Protease assays were conducted with a modified version of the protocol described previously (Hasegawa & Hase, 2009). *Vibrio* strains were inoculated in 5 ml LM (or LB for *V. cholerae*) overnight at 30°C shaking at 275 RPM. Cultures were back-diluted 1:1,000 in LB or LM, and the cell mixture was aliquoted into black-welled, clear-bottomed 96-well plates. Compounds were either added into the wells to a specific final concentration or a titration series was performed (4-fold dilution series; final volume of 150 μ l; 3 technical replicates per sample). DMSO was added as a negative control at equal volumes into control reactions. 96-well plates were covered in microporous sealing tape and grown for 16 hours shaking at 275 RPM at 30°C. After incubation, the OD₆₀₀ was measured on a BioTek Cytation plate reader. The cultures were pelleted in the 96-well plate by centrifuging at 3700 RPM for 5 min at room temperature. 20 μ l of the supernatant was transferred to a new clear 96-well plate. 80 μ l of 1% azocasein (dissolved in dH₂O) was added to the supernatants and incubated at 37°C for 30 min. 120 μ l of 10% trichloroacetic acid was added to the reaction, and

the plate was incubated on ice for 30 min, then centrifuged at 3700 RPM for 5 min at room temperature. 80 μ l of the protease reaction was transferred to a new clear 96-well plate, and 20 μ l of 1.8N NaOH was added. The OD₄₂₀ was measured on a BioTek Cytation plate reader. Protease activity was calculated by dividing OD₄₂₀ by OD₆₀₀. Each assay was performed in biological triplicates.

Autodock Vina modeling and analyses

All docking experiments were performed using Autodock Vina (Trott & Olson, 2010) or Webina, the web browser-supported version (Kochnev *et al.*, 2020). Xray crystal structures of apo SmcR (PDB ID 3KZ9) and HapR (PDB ID 2PBX) were used for all simulations (De Silva *et al.*, 2007, Kim *et al.*, 2010). Structures were prepared for docking using AutoDockTools-1.5.6 for addition of hydrogen atoms and assignment of partial charge (Morris *et al.*, 2009). Ligand structures were similarly prepared to include using AutoDockTools which was additionally used to define torsional degrees of freedom. An approximately 14 x 14 x 14 Å box was defined surrounding the residues previously reported to form the Qstatin binding site (box size varied slightly with protein) (Kim *et al.*, 2018). Pymol was used visualize the lowest energy solutions. The predicted binding energies from AutoDock Vina and/or Webina modeling were analyzed compared to the observed IC₅₀ values using K-means clustering in R using 3 clusters of sizes 3, 6, and 4.

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Author Contributions

JVK and LCB designed the experiments, JVK, JDN, PS, JC, ES, MEM, and LCB performed experiments, JVK, JDN, RH, JC, ES, MEM, and LCB analyzed experimental results, and JVK, JDN, and LCB wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abbreviated summary to accompany graphical abstract

LuxR/HapR proteins are the central regulators of quorum sensing genes in *Vibrio* species.

Thiophenesulfonamide compounds such as PTSP are specific and potent inhibitors of LuxR/HapR function. The efficacy of thiophenesulfonamide inhibition is driven by amino acid sequence in the putative ligand binding pocket of LuxR/HapR proteins.

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Tables

Table 1. Inhibition (IC₅₀) of GFP production by LuxR in *E. coli* bioassay.

Molecule	IC ₅₀ (μM)	IC ₅₀ 95% confidence interval (μM)
P0053 I18	1.9	1.2 – 3.0
P0053 O05	16.5	12.0 – 22.8
P0074 H04	ND	ND
P0074 N08	14.6	8.1 – 26.9
P1032 E02	ND	ND
P1117 F20	1.1	0.2 – 4.1
P1120 D05	73.7	40.7 – 171.2
P2046 F14	ND	ND
P2065 E16	5.3	2.8 – 9.9
Qstatin	5.0	3.5 – 7.0

ND = a curve could not be fit to these data to determine IC₅₀.

Table 2. Inhibition (IC₅₀) of the P_{luxC-gfp} reporter in *Vibrio* strains.

IC ₅₀ (μM) ^a	Qstatin	1B	1C	1E	3A	3B	3C ^b	8A	8B	8C	10A	10B	10C	P0053 I18
<i>Vcamp</i> BB120	4.0	52.6	3.3	51.4	15.0	18.9	8.3	3.8	32.4	6.7	0.58	0.35	0.71	2.0
<i>Vcamp</i> HY01	1.2	23.2	1.0	14.8	1.6	3.6	ND	1.2	8.7	2.7	0.40	0.10	0.16	4.3
<i>Vcamp</i> ATCC 25920	0.8	12.8	0.5	8.4	1.1	2.0	ND	0.7	3.9	1.5	0.05	0.03	0.09	1.8
<i>Vcoral</i> OCN008	11.6	55.7	9.1	49.2	23.1	39.2	1.3	4.2	26.1	2.7	2.09	1.43	1.07	18.9
<i>Vcoral</i> OCN014	22.2	100.6	9.4	66.2	22.6	95.1	ND	9.6	26.8	8.4	2.01	2.37	2.42	10.4
<i>Vpara</i> RIMD 2210633	2.3	15.9	1.7	11.0	2.6	2.5	2.0	2.0	7.3	3.8	0.63	0.17	0.68	2.8

Vpara D4	3.0	18.9	2.5	12.7	2.8	3.5	ND	2.4	8.4	4.5	0.87	0.27	1.28	6.4
Vpara 12-297/B	1.7	11.8	1.6	8.1	3.0	4.0	ND	2.1	6.7	3.3	1.03	0.32	1.04	8.1
Vvul ATCC 27562	0.5	3.4	0.4	2.7	0.7	0.9	0.5	0.4	1.9	0.7	0.00144	0.00196	0.00075	0.7
Vvul CMPC6	2.1	29.6	2.3	22.0	6.1	5.9	ND	2.1	11.5	4.6	0.03392	0.02949	0.06046	4.9
Vvul YJ016	0.9	6.5	0.8	4.6	1.3	1.5	ND	0.5	3.0	1.0	0.00584	0.00378	0.00546	3.2

a. *Vcamp*, *V. campbellii*; *Vcoral*, *V. coralliilyticus*; *Vpara*, *V. parahaemolyticus*; *Vvul*, *V. vulnificus*

b. ND, not determined.

Table 3. ITC analysis of LuxR/SmcR interaction with inhibitors.

	K_d (M)	ΔH (kJ/mol)	K_a (M⁻¹)	-TΔS (kJ/mol)	ΔG (kJ/mol)	ΔS (J/mol·K)
LuxR 10B	5.13E-07	-136.10	2.30E+06	99.99	-36.09	-335.40
SmcR 10B	2.27E-07	-142.10	4.85E+06	104.05	-38.04	-348.97
SmcR Qstatin	7.38E-07	-81.91	1.36E+06	46.91	-35.00	-157.30

Figure Legends

Figure 1. The quorum sensing pathway in *Vibrio* species. Shown is the general quorum sensing scheme for *Vibrio campbellii*, with a general list of individual and group behaviors observed in *Vibrio* species. Autoinducer molecules (HAI-1, CAI-1, AI-2) are synthesized by LuxM, CqsA, and LuxS, respectively, and bound by membrane-bound histidine kinase receptors LuxN, CqsS, and LuxPQ, respectively. The histidine kinase receptor HqsK senses nitric oxide. At low cell densities, autoinducer concentrations are low, the receptors are unbound, and the receptors act as kinases to phosphorylate LuxU, the phosphotransfer protein, which phosphorylates LuxO, the response regulator. Phosphorylated LuxO and Sigma-54 activate expression of the five Qrrs, which repress LuxR translation and activate AphA translation, leading to expression of genes for individual behaviors. At high cell densities, binding of autoinducers and/or nitric oxide to the receptors changes their activities to phosphatases, resulting in dephosphorylated LuxO and lack of Qrr expression. Thus, the production of the transcription factor LuxR is maximal, and LuxR activates and represses genes encoding proteins with various functions. LuxR protein homologs in various *Vibrio* species are listed.

Figure 2. Screen for LuxR inhibitors in *E. coli* bioassay. (A) Diagram of the two plasmids in the *E. coli* bioassay used to screen for LuxR inhibitors. Plasmid pJV064 contains divergent promoters for *V. campbellii* luxCDABE and 05222 driving expression of *gfp* and *mCherry*, respectively. Plasmid pKM699 contains the *V. campbellii* luxR gene under control of its native promoter. (B) Fluorescence expression (GFP/OD₆₀₀ or mCherry/OD₆₀₀) in *E. coli* bioassay cells (with pJV064) expressing LuxR (pKM699) or the empty vector control (pLAFR2). (C) Structures of LuxR inhibitors identified and verified from the compound library screen. The sulfonamides and sulfamide are highlighted. (D, E) Production of GFP (panel D; GFP/OD₆₀₀) or mCherry (panel E; mCherry/OD₆₀₀) in the presence of LuxR inhibitors titrated into the *E. coli* bioassay strain (pKM699, pJV064). DMSO was titrated as a solvent control with an equal volume to the 100 μ M concentration of compound and compared to cells in which nothing was added (-, plotted at 100 μ M point on x-axis). Data shown represent the mean and standard deviation of at least three biological replicates.

Figure 3. Panel of thiophenesulfonamide molecules that inhibit LuxR. Substrates with modifications to the heteroaromatic ring (pyrazole in the case of Qstatin) have number designations,

and substrates with modifications to the thiophene ring have letter designations. (B, C) Production of GFP (panel B; GFP/OD₆₀₀) or mCherry (panel C; mCherry/OD₆₀₀) in the presence of thiophenesulfonamide compounds titrated into the *E. coli* bioassay strain (pKM699, pJV064). DMSO was titrated as a solvent control with an equal volume to the 100 μ M concentration of compound and compared to cells in which nothing was added (-, plotted at 100 μ M point on x-axis). Data shown represent the mean and standard deviation of at least three biological replicates.

Figure 4. Thiophenesulfonamides have a range of inhibition against *Vibrio* species. (A-E) Titration of molecules from the top panel of thiophenesulfonamides in *Vibrio* strains compared to DMSO solvent control (DMSO was titrated with an equal volume to the 100 μ M concentration of compound). Data shown represent the mean and standard deviation of three biological replicates. (F) Protease activity (final assay OD₄₂₀/initial culture OD₆₀₀) for wild-type and mutant *Vibrio* strains in the presence of 2.5 μ M PTSP or an equal volume of the DMSO solvent. Asterisks indicate significant differences (Unpaired t test, two-stage step-up (Benjamini, Krieger, and Yekutieli), $n = 3$; *, $p = 0.05$; **, $p = 0.01$; ***, $p = 0.001$; ****, $p = 0.0001$; ns, not significant). (G) Protease activity (final assay OD₄₂₀/initial culture OD₆₀₀) for *Vibrio* strains in the presence of PTSP titrated into the cultures. Data shown represent the mean and standard deviation of three biological replicates.

Figure 5. Sulfonamide 10B does not inhibit *V. cholerae* HapR. (A) LuxR sequences from *V. coralliilyticus* OCN008 (accession: ERB64458.1), *V. campbellii* BB120 (accession: ABU72404.1), *V. vulnificus* ATCC 27562 (accession: WP_011079558.1), *V. parahaemolyticus* RIMD 2210633 (accession: WP_005479697.1), and *V. cholerae* C6706 (accession: ACB30340.1) aligned using Clustal Omega and Boxshade. Blue dots indicate the amino acids in SmcR that contact Qstatin in the published SmcR-Qstatin X-ray crystal structure (Kim *et al.*, 2018). Green dots indicate amino acid differences between LuxR proteins in each species among the SmcR-Qstatin contacts. (B) Production of GFP (GFP/OD₆₀₀) in the presence of 25 μ M PTSP or DMSO solvent control in the *E. coli* bioassay strain (pJV064) containing plasmids expressing SmcR (pJN22), HapR (pJV387), LuxR (pJV388), OpaR (pJV389), VcpR (pJV390), or empty vector (pMMB67EH-kanR). SmcR and HapR expression in strains was induced with 50 μ M IPTG; IPTG was not added to cultures of the remaining strains. (C) Production of GFP (GFP/OD₆₀₀) in the presence of 25 μ M PTSP or DMSO solvent control in the *E. coli* bioassay strain (pJV064) containing plasmids expressing SmcR (pJN22), HapR (pJV387), or the listed various amino acid substitution mutants. SmcR and HapR expression was

induced with 50 μ M IPTG. For panels B and C, asterisks indicate significant differences (two-way ANOVA followed by Sidak's multiple comparisons test, $n = 3$ (panel B), $n = 6$ (panel C); ****, $p = 0.0001$; ns, not significant; data are normally distributed (passed Kolmogorov-Smirnov and Shapiro-Wilk normality tests $\alpha = 0.05$).

Figure 6. (A) Autodock Vina modelling of Qstatin into the SmcR apo X-ray crystal structure (3KZ9; cyan) overlaid with the X-ray structure of SmcR complexed with Qstatin (5X3R; green). (B) Autodock Vina modelling of PTSP (10B) in the X-ray crystal structures of SmcR (3KZ9; cyan) and HapR (2PBX; gray). (C) The rotational orientation of Q137 in the four chains of SmcR from the asymmetric unit (3KZ9). (D) Autodock Vina modelling of PTSP into chain A (left) and chain B (right) of SmcR (3KZ9). (E) Binding energies predicted by Autodock Vina or Webina for each molecule modelled into the SmcR X-ray crystal structure (3KZ9) are graphed against the IC_{50} value for each molecule determined by assaying GFP production in *V. vulnificus* ATCC27562 (Table 2). Clusters of the 3 (red), 3 (blue), and 7 (green) data points were generated using K-means clustering analysis in R. (F) Electrophoretic mobility shift assays (EMSAs) of SmcR purified protein in varying concentrations incubated with radiolabeled dsDNA corresponding to the *V. vulnificus* *vvpE* promoter. DNA shifts were quantified using ImageJ, and the graphs show the mean and standard deviation for three biological replicates.

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