A High Throughput Screening Assay for Inhibitors of Bacterial Motility Identifies a Novel inhibitor of the Na⁺-driven Flagellar Motor and Virulence Gene Expression in *Vibrio cholerae*

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ABSTRACT

Numerous bacterial pathogens, particularly those that colonize fast flow areas in the bladder and gastrointestinal tract, require motility to establish infection and spread beyond the initially colonized tissue. Vibrio cholerae of serogroups O1 and O139, the causative agent of the diarrheal illness cholera, expresses a single polar flagellum powered by sodium motive force and requires motility to colonize and spread along the small intestine. Therefore, motility could be an attractive target for small molecules that could prevent and/or block the infective process. In this study we describe a high throughput screening (HTS) assay to identify small molecules that selectively inhibit bacterial motility. The HTS assay was used to screen a ~8,000 compound structurally diverse chemical library for inhibitors of V. cholerae motility. The screen identified a group of quinazoline 2,4 diamino analogs that completely suppressed motility without affecting growth rate in broth. A further study on the effect of one analog, hereafter designated Q24DA, showed that it induces a flagellated but non-motile (Mot-) phenotype and was specific for the Na⁺-driven flagellar motor of pathogenic Vibrios. A mutation conferring phenamil-resistant motility did not eliminate inhibition of motility by Q24DA. Compound Q24DA diminished the expression of cholera toxin, the toxin-coregulated pilus, biofilm formation and fluid secretion in the rabbit ileal loop model. Furthermore, treatment of V. cholerae with Q24DA impacted additional phenotypes linked to Na⁺ bioenergetics such as the function of the primary Na⁺ pump Nqr and susceptibility to fluoroquinolones. The above results clearly show that the described HTS is capable of identifying small molecules that specifically block bacterial motility. New inhibitors like Q24DA can be instrumental in probing the molecular architecture of the Na⁺-driven polar flagellar motor and studying the role of motility in the expression of other virulence factors.
Cholera is an acute water-borne diarrheal disease caused by *Vibrio cholerae* of serogroups O1 and O139. This Gram-negative pathogen continues to be a major public health concern in endemic areas of South Asia and Africa where cholera is estimated to cause 5 million cases and more than 100,000 deaths per year. Cases of severe cholera are commonly treated with antibiotics to diminish the duration of its life-threatening clinical symptoms. In this regard, the emergence of multiple antibiotic resistant *V. cholerae* O1 and O139 strains has been recognized as a major concern. The availability of novel prophylactic measures and/or adjunctive therapies could contribute to diminish the burden of cholera and antibiotic resistances.

*V. cholerae* strains that cause epidemic cholera exhibit three major virulence traits: (i) production of cholera toxin (CT), (ii) expression of the toxin-coregulated pilus (TCP) and (iii) expression of a single fast-rotating sheathed polar flagellum driven by sodium motive force (SMF). CT is an ADP-ribosyl transferase responsible for the profuse rice-watery diarrhea typical of this disease. TCP is a Type IV pilus required for intestinal colonization in humans. Motility is required to establish infection, for colonization of the small intestine, to detach and spread along the GI tract and/or to exit the host and return to the environment. Flagellar motility has also been shown to influence the expression of CT and TCP. Furthermore, motility can influence cholera transmission by enhancing *V. cholerae* biofilm formation. Finally, shedding of *V. cholerae* flagellins has been reported to induce an inflammatory response in the host by interacting with Toll-like receptor V to induce the production of proinflammatory interleukin-8. Taken together, the expression of flagellar motility appears to be an attractive target for small molecules capable of preventing and/or blocking the infective process.
Motility is a highly complex biological process that requires the synthesis and export of the flagellum and its motor, coupling of the flagellar motor to an energy source and controlling the direction of flagellum rotation by chemotaxis. The *V. cholerae* genome encodes multiple flagellin genes but only mutants lacking FlaA are non-flagellated and non-motile (30-32). The expression of motility in the cholera bacterium results from a complex hierarchical gene expression cascade involving alternative RNA polymerase sigma subunits $\sigma^{54}$ and $\sigma^{28}$ and multiple transcriptional regulators (8-11,30-32,42,48,56). The organization of the *V. cholerae* motility genes is almost identical to the previously published Na$^+$-driven polar flagellar gene system of *V. parahaemolyticus* (29). Genes required for flagellum rotation include *pomA* (*motA*), *pomB* (*motB*), *motX*, *motY*, *fliG*, *fliM* and *fliN*. The inactivation of these genes by mutation abolish motility but do not prevent flagellum assembly (6, 29,40,48). MotA and MotB translocate Na$^+$ by forming the Na$^+$ conducting channel while MotX and MotY are required for torque generation (1). The presence of an extended domain that could interact with peptidoglycan suggests that MotY could constitute the stator of the flagellar motor (40). FliG, FliM and FliN form the switch complex at the base of the flagellum basal body (6,48).

Whether the flagellum rotates counterclockwise or clockwise is determined by the activity of the response regulator CheY3 that interacts with the FliM component of the motor (4,5,25). To maintain the Na$^+$ gradient required to drive flagellum rotation, *V. cholerae* expresses multiple Na$^+$ pumps such as the Na$^+$-translocating NADH:quinone oxidoreductase (Nqr) as well as several Na$^+/H^+$ antiporters (21).

Despite significant advances in our understanding of this complex phenotype, much remains to be learned. Of particular interest to cholera pathogenesis is to determine how the expression of motility intersects the regulatory pathways that control the expression of other virulence factors and biofilm formation. Chemical genetics is a novel approach to interrogate
complex biological processes that use small molecules to directly change the way proteins work in real time rather than indirectly by manipulating their genes (54). This approach has not been applied to the investigation of Vibrio motility due to the shortage of specific inhibitors. To date, the only available inhibitors of motility are amiloride, which also inhibits growth and acts competitively with respect to extracellular Na\(^+\) (55), and the amiloride analog phenamil that poisons the Na\(^+\) channel in a non-competitive manner with respect to external Na\(^+\) (2,27,40). The availability of a high throughput screening (HTS) assay to identify additional inhibitors could accelerate the investigation of motility and related phenotypes. In this study we describe the development and validation of a HTS assay for small molecules that selectively inhibit motility. We also describe the properties of a novel inhibitor of the Na\(^+\)-driven flagellar motor identified in this HTS and examine its effect on virulence gene expression, biofilm formation and two additional phenotypes related to Na\(^+\) bioenergetics: Nqr activity and susceptibility to fluoroquinolones.

**MATERIALS AND METHODS**

**Strains, media and plasmids.** Strains used in this study are described in Table 1. C7258 and C7258Mot were used to develop an HTS assay for inhibitors of *V. cholerae* motility. *V. cholerae* and *V. parahaemolyticus* were grown in LB medium at 37\(^\circ\)C while *V. vulnificus* was grown in Marine broth (Difco). To measure the production of CT and TcpA, strain C7258 was grown in AKI medium at 30\(^\circ\)C (26). As a test to determine the function of the Nqr primary Na\(^+\) pump, strain C7258 was grown in LB medium pH 8.6 containing 0.3M NaCl. Culture media were supplemented with streptomycin (Str) (100 \(\mu\)g/mL), tetracycline (Tet) (5 \(\mu\)g/mL), carbonylcyanide m-chlorophenylhydrazone (CCCP) (5\(\mu\)M), 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO) (5\(\mu\)M) and phenamil (50 - 100 \(\mu\)M) as required and described in figure
legends. Susceptibility to norfloxacin and ciprofloxacin was measured in 96-well microtiter plates containing LB medium supplemented with 0.5 to 62 µg/mL of each antibiotic.

**Strain construction.** For assay development a non-motile derivative of C7258 (C7258Mot') was constructed by introducing a ΔmotY mutation as described previously but using the above strain as conjugal receptor (52). A similar procedure was used to construct the flagelin A-defective strain C7258Fla'. To construct isogenic *V. cholerae* strains expressing phenamil-sensitive and phenamil-resistant motility, cosmids pLM2058 and pLM2059 (Tet<sup>R</sup>) encoding wild type and phenamil resistant motor genes (motA and motB), respectively (27), were transferred to strain O395ΔAB (Str<sup>R</sup>) (16) in a tripartite cross using *E. coli* pRK2013 as Tra donor. Exconjugants were selected in LB agar supplemented with Tet and Str.

**Swarm agar test.** Motility was measured by stabbing overnight cultures on LB medium or Marine broth containing 0.3 % agar (swarm agar). The assay was conducted in 6-well tissue culture plates or Petri dishes containing 4 and 30 mL of swarm agar, respectively. Plates were incubated at 30°C for 16 h.

**Motility HTS.** The HTS described in this study was based on a recently described 96-well format assay for *S. thyphimurium* antimicrobial compounds using motility as readout (38). This assay is based on (a) off-center inoculation of a bacterial culture to wells containing soft agar, (b) an incubation period allowing motile bacteria to spread throughout the well and, (c) a diagonal off-center reading of absorbance (OD<sub>615</sub>) to estimate the extent to which motile bacteria spread across the well. We have made three critical improvements to this method consisting of automation, miniaturization to a 384-well format and combining the OD<sub>615</sub> reading with detection of viability using alamarBlue to differentiate between compounds that inhibited motility and compounds that were toxic to the bacteria. To validate the assay for HTS we conducted a pilot screen of ~8,000 structurally diverse commercially available
compounds dissolved in dimethyl sulfoxide (DMSO) with a repeat run on a second day. DMSO was shown to have no effect on either readout at concentrations up to 1%. Compounds identified to specifically inhibit motility were screened in dose response to calculate their IC$_{50}$ and checked for mammalian cell cytotoxicity using THP-1 cells (ATCC TIB-202) and the Cell Titer Glo kit (Promega). Cytotoxic compounds were removed from the primary hit list. A brief description of the HTS assay is shown in Table 2. A more detailed description of the method and required instrumentation is provided as supplementary material.

**Determination of FlaA expression.** To detect FlaA, the flaA gene from strain C7258 was amplified using the Advantage 2 PCR system (Clontech Laboratories Inc.) and oligonucleotide primers FlaAF and FlaA-R3 (Table 1). The PCR product was directionally cloned as a NdeI/SpeI fragment in the vector pTXB1 for expression and purification of FlaA protein using the IMPACT$^\text{TM}$ kit (New England BioLabs). Purified FlaA was dialyzed against phosphate-buffered saline pH 7.4 (PBS) and used to generate the anti-FlaA monoclonal antibody 3E1 at Southern Biotech (Birmingham, AL). Cell pellets corresponding to the same number of cells based on OD$_{600}$ readings were boiled in 100 µL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and proteins were separated in a 12% polyacrylamide gel. Then, gels were transferred to polyvinylidene difluoride (PVDF) membranes and FlaA protein was detected by Western blot using the BM Chemiluminescence western blot kit (Roche Applied Sciences, Indianapolis).

**Transmission electron microscopy.** Flagellum assembly was confirmed by transmission electron microscopy (TEM). Strain C7258 was grown in LB medium for 16 h, the cells pelleted and fixed by reconstitution in 2.5 % glutaraldehyde sodium cacodylate buffer pH 7.3.
Samples were adhered to a carbon-coated grid and stained with 1% uranyl acetate before microscopy.

**Measurement of cholera toxin and toxin co-regulated pilus expression.** Cholera toxin was determined by ganglioside GM₁ enzyme-linked immunosorbent assay (GM₁-ELISA) using a rabbit peroxidase-conjugated anti-cholera toxin B subunit IgG (Pierce/Thermo Fisher Scientific) and a standard curve of pure CT (Sigma Chemical Co.) as described previously (52). TcpA, the major TCP subunit was determined in western blots using a rabbit anti-TcpA serum kindly provided by Biao Kan (CDC Beijing). The pellet corresponding to 0.5 OD₆₀₀ units was boiled in SDS-PAGE loading buffer and TcpA detected using the BM Chemiluminescence western blot kit as described above. A parallel gel was stained with Coomassie dye to confirm equal loading per well.

**Biofilm assay.** Biofilm formation was measured by the crystal violet staining method and the results normalized for growth and expressed as the OD₅₇₀/OD₆₀₀ ratio (61).

**Quantitative reverse transcription PCR (qRT-PCR).** Total RNA was isolated using the RNeasy kit (Qiagen Laboratories). The RNA samples were analyzed by qRT-PCR to determine relative gene expression of target genes using the iScript two-step RT-PCR kit with SYBR green (Bio-Rad Laboratories) as described previously (52). Relative expression values were calculated as $2^{(Ct_{target} - Ct_{reference})}$ where Ct is the fractional threshold cycle. The level of recA mRNA was used as a reference. Internal primer pairs were designed for motA, motB, motX, motY and toxT using the Oligo primer analysis software (Table 1). A control mixture lacking reverse transcriptase was run for each reaction to exclude chromosomal DNA contamination.

**Rabbit ileal loop experiments.** Rabbit ileal loop experiments were conducted as described by De and Chatterjee (13). Briefly, New Zealand White male rabbits (1.5 to 2 kg) were fasted
for 48 h prior to surgery and fed only water ad libitum. Rabbits were anesthetized by intramuscular administration of ketamine (35 mg/kg of body weight) and xylazine (5 mg/kg). A laparotomy was performed, and the ileum was washed and ligated into discrete loops of approximately 7 cm. Each loop was inoculated with $10^8$ CFU of challenge bacterium in PBS. Pure CT (20 µg; Sigma Chemical Co.) and PBS were used as positive and negative controls, respectively. The intestine was returned to the peritoneum, and the animals were sutured and returned to their cages. After 9 h, rabbits were sacrificed by intravenous injection of pentobarbital (150 mg/kg) and the loops were excised. Fluid volume and loop length were measured and secretion was recorded as fluid accumulation (FA) per centimeter of loop length.

**RESULTS**

**Development of a HTS assay for inhibitors of V. cholerae motility.** Numerous bacterial pathogens, particularly those that colonize fast flow areas in the bladder and GI tract, require motility to establish infection and subsequently spread within and beyond the initially colonized tissue (46). Thus, an HTS assay for inhibitors of bacterial motility could be instrumental for the discovery of novel anti-infective drugs. Here we describe the development of an HTS assay for inhibitors of bacterial motility based on a previous method using a soft agar motility assay in 96-well microtiter plates (38). To miniaturize the assay we first confirmed that, in contrast to the motile V. cholerae strain C7258, inoculation of the isogenic non-motile strain C7258Mot’ in 384-well plates did not interfere with the OD$_{615}$ reading even after 24 h of incubation (data not shown). To differentiate between compounds that inhibited motility and compounds that were toxic to the bacteria, a readout using alamarBlue was added to the protocol. As shown in **Fig. 1A**, a bactericidal compound such as tetracycline could be differentiated from a motility inhibitor compound such as phenamil.
The inhibitory effect on motility of phenamil can be seen at 100 µM (Fig. 1A) but has little effect on viability when measured with alamarBlue (Fig. 1B). In contrast, tetracycline kills the bacteria and while they would appear non-motile by the OD615 reading (Fig. 1A), the alamarBlue reading shows that they are non-viable (Fig. 1B). As shown in the sample plate from a pilot screen (Fig. 1C), tetracycline-like toxic compounds can be differentiated visually from phenamil-like compounds that selectively inhibit motility. Four compounds were identified that inhibited motility (IC50 1.9 - 14 µg/mL) and showed no toxicity to the bacterium and mammalian cells.

Inactivation of motility by mutation does not commonly affect growth rate in broth. Therefore, we re-screened the compounds showing no toxicity for their effect on growth rate in broth. Out of these compounds, we selected a quinazoline-2,4-diamino analog, 2-[(2,5-dimethylphenyl)amino]quinazolin-4-yl]amino)ethanol (Fig. 9, structure 1) hereafter referred as Q24DA for further studies because it completely suppressed motility in soft agar plates stabbed with an overnight culture of C7258 containing > 10⁹ bacteria/mL but had no detectable effect on growth rate (Fig, 2). Motility mutants are commonly classified as Fla− when they fail to express flagellum and Mot− when they express a paralyzed flagellum (29).

Thus, we used TEM and western blot to determine if compound Q24DA chemically induced a Fla− or a Mot− phenotype. As shown in Fig. 3, Q24DA had no effect on the expression of FlaA or on the assembly of a normal flagellum indicating that it chemically induces a Mot− phenotype. As in the case of phenamil, the inhibitory effect of Q24DA on motility could not be reversed by increasing the concentration of NaCl in the medium (data not shown).

Inhibition of motility by Q24DA affects virulence gene expression and biofilm formation. Since inhibition of motility by mutation has been reported to affect virulence gene expression and biofilm formation, we hypothesized that inhibition of motility by Q24DA might
also affect these phenotypes. As shown in Fig. 4A, growth of strain C7258 in permissive AKI medium (26) in the presence of Q24DA diminished the expression of CT and TcpA. Expression of ToxT, the transcriptional activator of tcpA and ctxA was determined by qRT-PCR. Relative expression of this activator was 0.2 ± 0.01 in AKI medium and 0.1 ± 0.01 (n = 3) in the same medium supplemented with Q24DA. As expected from the known role of motility in surface attachment, Q24DA also diminished static biofilm formation 1.6- and 1.9-fold at 18 and 30 h, respectively.

Fluid accumulation in the rabbit ileal loop model has been shown to closely reflect the production of CT (13). Since we had no information on the possible fate of Q24DA in a live organism, we selected this model to test the effect of Q24DA on CT production in vivo. We reasoned that a closed system requiring a short observation time would more likely reveal any effect of Q24DA on V. cholerae enterotoxicity. As shown in Fig. 4B, co-inoculation of C7258 with Q24DA significantly diminished fluid secretion.

**Compound Q24DA is a novel inhibitor of the Na⁺-driven flagellar motor.** Different Vibrios differ with regard to the flagellum system they express. For instance, while V. vulnificus expresses a single Na⁺-driven polar flagellum similar to the cholera bacterium, V. parahaemolyticus expresses a polar flagellum driven by SMF (Fla) and a lateral flagellum (Laf) powered by proton motive force (PMF) (39,41). These differences provided a mean to determine if Q24DA specifically inhibits the Na⁺-driven polar flagellum. Compound Q24DA inhibited motility in V. cholerae O395 (classical biotype) and V. vulnificus which express a single polar flagellum and in V. parahaemolyticus strains lacking its lateral flagellum (Fla⁺Laf). Contrastingly, Q24DA had no effect in V. parahaemolyticus strains that express the lateral flagellum (Fla⁺Laf⁺ or Fla⁻Laf⁺) (Fig. 5). These results suggest that Q24DA could be a novel inhibitor of the Na⁺-driven flagellar motor. As expected, compound Q24DA did not affect
motility in *E. coli* and *S. thyphimurium* known to use PMF to power flagellum rotation (data not shown). Q24DA had no effect on the expression of polar flagellum motor genes measured by qRT-PCR (data not shown) suggesting that it inhibits motility by interacting with the motor rather than by affecting its expression. The only known specific inhibitor of the Na\(^+\)-driven flagellar motor is the Na\(^+\) channel blocker and amiloride analog phenamil (2, 27). Thus, we examined the possibility of phenamil and Q24DA affecting motility by interacting with the flagellar motor in a similar manner. To this end we constructed isogenic *V. cholerae* strains expressing wild type (phenamil-sensitive) *motA* and *motB* motor genes and the corresponding phenamil-resistant genes from *V. parahaemolyticus*, respectively. As shown in Fig. 6, the *V. parahaemolyticus* motor genes fully complemented the non-motile phenotype of strain O395ΔAB. Furthermore, the strain receiving the phenamil-resistant *motA* gene exhibited detectable swarming activity in the presence of phenamil. However, resistance to phenamil had no effect on motility inhibition by Q24DA.

**Q24DA impacts additional cellular processes linked to Na\(^+\) bioenergetics.** Flagellar rotation requires the influx of Na\(^+\) through the MotA/MotB channel as well as maintenance of the Na\(^+\) gradient by the activity of several Na\(^+\)/H\(^+\) antiporters and the primary Na\(^+\) pump Nqr (21). We hypothesized that a compound affecting Na\(^+\) flux to the extent of paralyzing the flagellum could have pleiotropic effects on other cellular processes linked to Na\(^+\) bioenergetics. For instance, in alkaline media (pH 8.6; 0.3M NaCl) the activity of Nqr allows *V. cholerae* to make ATP and withstand a collapse of PMF induced by the protonophore CCCP (34). Thus, we examined if Q24DA affected the function of Nqr. As shown in Fig. 7A, Q24DA increased the inhibitory effect of CCCP in alkaline medium but to a lesser extent than HQNO (a specific Nqr inhibitor). Another Na\(^+\)-related phenotype is susceptibility to fluoroquinolones. *V. cholerae* susceptibility to norfloxacin and ciprofloxacin has been shown
to involve Na+-driven efflux pumps (23,24,53). As predicted, Q24DA also enhanced susceptibility to norfloxacin and ciprofloxacin (Fig. 7B). Taken together, the specificity of Q24DA for the Na+-driven flagellum and its pleiotropic effect on two additional phenotypes linked to sodium bioenergetics suggests that Q24DA affects Na+ membrane flux.

**Inhibition of motility by chemical analogs of Q24DA.** Quinazoline-2,4-diamino analogs showed selectivity for motility inhibition in the HTS assay. Motility inhibition data of all the active quinazoline analogs identified the presence of a 2-ethanolamino functionality at the 4-position of the quinazoline ring as a minimum structural feature required for selective inhibition of motility. On this basis, 22 structurally similar analogs to Q24DA possessing 2-ethanolamino or related (tetrahydrofuran-2-yl) methylamino group at the 4-position were purchased and screened for motility and growth inhibition. Six compounds (Fig. 8, structures 2-7) were found to completely suppress motility without affecting growth in broth (IC$_{50}$ 1.9 - 20.1 µM). Four compounds (Fig. S1, structures 8-11) showed only partial motility inhibition while compounds shown in Fig. S2 were inactive.

**DISCUSSION**

We have developed and validated an HTS assay for small molecule inhibitors of bacterial motility based on a previous assay for *S. thyphimurium* antibacterial compounds using a 96-well off-center inoculation method (38). The two major shortcomings of the former method were its low throughput and the inability to distinguish between compounds that inhibit motility or affect viability. Thus, not surprisingly, screening of a 960 structurally diverse compound library using this method did not identify any specific inhibitor of motility (38). To enable the screening of larger chemical libraries and increase the likelihood of identifying compounds that specifically inhibit motility we miniaturized and automated the original method and included a viability reading using alamarBlue. These improvements allowed us to screen a
larger chemical library and identify compounds specifically inhibiting motility. To our knowledge, this is the first HTS assay for small molecule inhibitors of bacterial motility. In principle, the assay can be adapted to other motile bacteria in which motility is detected using the standard swarm agar assay. The assay can also be used for HTS of bacterial mutants exhibiting inhibitor-resistant motility and target identification.

Since non-motile *V. cholerae* mutants of the El Tor biotype are severely attenuated and make altered biofilm (7,37,52,58), we used the HTS assay to identify inhibitors of *V. cholerae* motility. Several hits were identified that inhibited motility without exhibiting toxicity. A quinazoline 2,4 diamino analog was found to completely suppress motility in swarm agar plates without affecting growth rate in broth. This compound chemically induced a Mot- (non-motile flagellated) phenotype. This is the phenotype observed when components of the flagellar motor or chemotaxis are inactivated by mutation or when the MotA/MotB channel is poisoned with the amiloride analog phenamil. Several studies have suggested a complex link between the function of Na⁺-driven polar flagellum and the expression of CT and TCP. For instance, mutating motility genes, inhibiting motility with phenamil or changes in membrane Na⁺ flux have been shown to have variable effects on virulence gene expression (15,18,20,19,52,56). Here we show that inhibition of motility with compound Q24DA diminished CT and TCP expression *in vitro* and reduced enterotoxicity in the rabbit ileal loop model. Furthermore, inhibiting motility with Q24DA had a similar effect on fluid secretion to mutating the motor gene *motY* used as a non-motile control. These findings are consistent with the above evidences linking motility to toxin and TCP production and suggest that motility inhibitors could be useful as chemical probes to examine how motility affects the regulation of other virulence factors. The finding that Q24DA diminished biofilm formation is in agreement with an earlier study showing that deletion of *motA* or *motX* as well as poisoning...
the Na\(^+\) channel with phenamil diminished biofilm formation suggesting that the flagellar motor could act as a mechanosensor that stimulates phosphorylation of the positive biofilm regulator VpsR (36). We later found that deletion of another motor gene \textit{motY} diminished biofilm formation (52). A more recent study also supported a role for the flagellar motor and Na\(^+\) bioenergetics in \textit{V. cholerae} permanent surface attachment (57). Altogether, it is likely that inhibitors of \textit{V. cholerae} motility could serve as a useful source of druggable compounds to inhibit toxin production and biofilm.

We have analyzed the effect of Q24DA on the motility of several members of the \textit{Vibrionaceae} family expressing a Na\(^+\)-driven polar flagellum, a H\(^+\)-driven lateral flagellum or both flagellar systems. Q24DA was found to be specific for the Na\(^+\)-driven polar flagellum. Thus, we suggest that Q24DA targets a common component of the polar flagellar system present in the members of the \textit{Vibrionaceae} family examined. We note that the polar and lateral flagellar motors of \textit{V. parahaemolyticus} are directed by a common chemotactic control pathway and the same CheY molecular specie (33,51). Therefore, the finding that Q24DA does not impair the function of the \textit{V. parahaemolyticus} lateral flagellum suggests that it poisons the polar flagellar motor of this bacterium rather than affecting chemotaxis.

Since both Q24DA and phenamil appear to specifically act on the Na\(^+\)-driven flagellar motor, we examined whether a mutation that confers phenamil-resistant motility affected inhibition by Q24DA. The phenamil-resistant motor used in this study contains a D148Y mutation in \textit{motA} (27). D148 in MotA and P16 in MotB have been suggested to form a phenamil-binding pocket at the inner side of MotA/MotB channel complex (35). Mutating either D148 to Y or P16 to S is sufficient to induce phenamil-resistant motility (27,35). The D184Y mutation had no effect on motility inhibition by Q24DA. Though we have not tested the effect of other mutations that confer phenamil-resistance such as MotB\(^{A23G}\) (27), our
results suggest that inhibition of the Na\(^+\)-driven flagellar motor by phenamil and Q24DA involve different molecular interactions. For instance, Q24DA might interact with additional conserved sites of the MotA/MotB Na\(^+\)-conducting channel, other components of the flagellar motor (i.e. MotX, MotY) or the FliGMN switch complex. It is noteworthy that mutation conferring amiloride-resistant motility did not map to MotA and MotB (27). We do not know if Q24DA penetrates the cell and is capable of interacting with components of the motor located on the cytoplasmic side of the cell membrane. Similar to phenamil, which acts at the inner face of the MotA/MotB channel complex, inhibition by Q24DA could not be counteracted by increasing the extracellular Na\(^+\) concentration (data not shown). Thus, investigation of the mechanism of action of Q24DA could potentially reveal new information on the architecture and function of the polar flagellar motor.

Consistent with the hypothesis that Q24DA is a novel inhibitor of the Na\(^+\)-driven flagellar motor we here show that Q24DA affected other phenotypes linked to Na\(^+\) bioenergetics. For instance, it diminished the ability of *V. cholerae* to withstand a CCCP-induced collapse of PMF in alkaline media. Since this effect was less severe than directly inhibiting Nqr with HQNO, we propose it to be a downstream effect of Q24DA. We note that deletion of Nqr or addition of HQNO to the medium did not significantly affect motility in swarm agar at neutral pH (data not shown). Another interesting property of Q24DA related to Na\(^+\) bioenergetics was to enhance susceptibility to norfloxacin and ciprofloxacin. The *V. cholerae* Na\(^+\)-coupled NorM, VcrM and VcmA efflux pumps belong to the MATE family of multidrug resistance pumps widely distributed among clinically significant bacteria such *Haemophilus influenzae* (HmrM), *Pseudomonas aeruginosa* (pmpM), *Clostridium difficile* (CdeA) and *Staphylococcus aureus* (47). Our results suggest that Q24DA could also affect Na\(^+\) flux through the *V. cholerae* pumps. Therefore, clarification of the mechanism of action of Q24DA could provide
information on the architecture of other Na\(^+\) antiporter systems and lead to compounds with practical applications in antibiotic combination therapy.

The quinazoline-2,4-diamino analog scaffold is well established as a source of antimetabolites exhibiting potent anti-bacterial, anti-parasitic, anti-cancer and anti-inflammatory activities. Here we have identified a group of analogs showing excellent selectivity against *V. cholerae* motility. From the initial motility assay it was found that the presence of a 2-ethanolamino functionality at the 4-position of the quinazoline ring is essential for selective inhibition of motility and we believe that the 2-ethanolamino group could act as a bidentate ligand for Na\(^+\) complexation. Among 22 compounds similar to Q24DA screened, compounds with structures 2-7 (Fig. 8) showed comparable selective anti-motility activity. All active and partially active compounds against *Vibrio* motility were found to possess substituted aminophenyl moiety at the 2-position of quinazoline whereas compounds possessing saturated cyclicamino and benzylamino type substitutions were found to be inactive. Further compound analoging for extensive structure-activity relationship is underway for more potent and selective inhibitors of motility in *V. cholerae*.

In summary, the availability of a HTS assay for inhibitors of bacterial motility makes it now possible to identify a more diverse set of inhibitors to chemically interrogate the pathways leading to the synthesis, assembly and function of the Na\(^+\)-driven polar flagellum and its link to toxin production and biofilm formation. Investigation into the mechanism of action of Q24DA will likely reveal new information on the architecture of the polar flagellar motor and could shed light on the function of other Na\(^+\)-driven membrane transport systems.
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REFERENCES


**Table 1. Strains plasmids and primers**

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<tr>
<td><em>V. cholerae</em> O395ΔAB</td>
<td>O395ΔmotAmotB (16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> B22</td>
<td>Wild type, Fla&quot;Laf&quot; + (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> LM5674</td>
<td>Wild type, Fla&quot;Laf&quot; + (27,29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> LM1017</td>
<td>Fla&quot;Laf&quot; + (27,29)</td>
<td></td>
<td></td>
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<tr>
<td><em>V. parahaemolyticus</em> LM5392</td>
<td>Fla&quot;Laf&quot; + (27,29)</td>
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<tr>
<td><em>V. parahaemolyticus</em> LM7890</td>
<td>Fla&quot;Laf&quot; + (27,29)</td>
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<tr>
<td><em>V. vulnificus</em> LAM624</td>
<td>Wild type (59)</td>
<td></td>
<td></td>
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<tr>
<td>O395ABPheS</td>
<td>O395ΔAB containing pLM2058 This study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O395ABPheR</td>
<td>O395ΔAB containing pLM2059 This study</td>
<td></td>
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</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLM2058</td>
<td>Cosmid containing wild type <em>V. parahaemolyticus</em> motor genes.</td>
</tr>
<tr>
<td>pLM2059</td>
<td>Cosmid containing phenamil-resistant motor genes.</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for mobilization of non-self-transmissible plasmids (KmR)</td>
</tr>
</tbody>
</table>

**Oligonucleotide primers (5'-3')**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>FlaAF</td>
<td>GATCGCATATGACCATTAACGTAATACCAA</td>
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<tr>
<td>FlaA-R3</td>
<td>TACTAGTGATCTCCCGTGATGCACTGAATAACGAGAT</td>
</tr>
<tr>
<td>ToxT189</td>
<td>GCCCCTCTATTCAGCTTTT</td>
</tr>
<tr>
<td>ToxT400</td>
<td>GCCCCTCCATAGCATCAAGATC</td>
</tr>
<tr>
<td>MotA212</td>
<td>ATGCTCCCGAAGATCGTTGTTG</td>
</tr>
<tr>
<td>MotA56</td>
<td>ACCAGTGTCGGATCATCCC</td>
</tr>
<tr>
<td>MotB433</td>
<td>CAACAGCAGCAAGCAAGCAAGC</td>
</tr>
<tr>
<td>MotB651</td>
<td>CgcActCggg gaatgctc</td>
</tr>
<tr>
<td>MotX240</td>
<td>TATGCTGGGTTGGGAGATGTC</td>
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<tr>
<td>MotX476</td>
<td>CATCCTCATAACGACGGACTG</td>
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<tr>
<td>MotY362</td>
<td>CGCGTGGTCTGTAAATAGT</td>
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<tr>
<td>MotY572</td>
<td>GACGCTTTATCAACTCAACACTG</td>
</tr>
<tr>
<td>RecA578</td>
<td>GTGCTGGGATGTACGTTGTC</td>
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<tr>
<td>RecA863</td>
<td>CCACACTTTCTAGCAC</td>
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2
Table 2. Protocol summary and assay statistics

<table>
<thead>
<tr>
<th>Protocol</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Growth media and microtiter plates</td>
<td>384-well clear bottom plates containing 50 µL of LB media with 0.3 % agar was used.</td>
</tr>
<tr>
<td>Compounds</td>
<td>Compounds were dispensed to the top of the soft agar and allowed to diffuse for 2-4 h at room temperature.</td>
</tr>
<tr>
<td>Inoculation</td>
<td>2.5 nL containing approximately 20 CFU of strain C7258 were dispensed into the bottom left corner of each well (Fig. S3).</td>
</tr>
<tr>
<td>Incubation</td>
<td>Plates were incubated 16-24 h at 30°C and high humidity</td>
</tr>
<tr>
<td>Motility reading</td>
<td>Spreading of motile bacteria was measured by reading the OD&lt;sub&gt;615&lt;/sub&gt; above and to the right diagonal to the inoculation site (Fig. S3).</td>
</tr>
<tr>
<td>Viability reading</td>
<td>Viability was estimated by adding 5 µL of 100 % alamarBlue to each well. Plates were incubated 1-1.5 h at 30°C and fluorescence read (excitation 535; emission 595).</td>
</tr>
<tr>
<td>Control drugs</td>
<td>Phenamil (motility) and tetracycline (viability)</td>
</tr>
</tbody>
</table>

**Assay statistics for motility**

<table>
<thead>
<tr>
<th>Z values</th>
<th>0.5 – 0.8 (average 0.7)</th>
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<tbody>
<tr>
<td>Signal to noise</td>
<td>173</td>
</tr>
<tr>
<td>Signal to background</td>
<td>7</td>
</tr>
</tbody>
</table>

**Pilot screen**

<table>
<thead>
<tr>
<th>Compounds screened</th>
<th>8,093 compounds in duplicate on two different days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (OD&lt;sub&gt;615&lt;/sub&gt;)</td>
<td></td>
</tr>
<tr>
<td>Carrier control (DMSO)</td>
<td>Average, 0.73; coefficient of variation, 5.6</td>
</tr>
<tr>
<td>Motility control (phenamil)</td>
<td>Average, 0.10; coefficient of variation, 3.2</td>
</tr>
<tr>
<td>Viability control (tetracycline)</td>
<td>Average, 0.10; coefficient of variation, 3.6</td>
</tr>
<tr>
<td>Hit rate</td>
<td>0.2 %</td>
</tr>
</tbody>
</table>
LEGEND TO FIGURES

Fig. 1. HTS assay for inhibitors of bacterial motility. A. Dose response evaluation of phenamil (■) and tetracycline (▲) for motility inhibition in 384-well plates measured by OD615.

B. Dose response evaluation of phenamil (■) and tetracycline (▲) for toxicity in 384-well plates measured with alamarBlue. In each case the data is expressed as percent inhibition relative to the OD615 reading or alamarBlue fluorescence signal in the absence of compound.

C. Sample plate. Plates were inoculated with the motile strain C7258. Columns 1-2 contain phenamil (rows 1-4) or tetracycline (rows 5-16). Compounds that specifically inhibit motility (phenamil-like) without affecting viability can be distinguished from those with bactericidal activity (Tet-like) in this plate. Compounds like phenamil that inhibit motility but not viability yield a bright (pink) alamarBlue fluorescence signal (see column 8, row 5); compounds that do not affect motility or viability, yield a blurred alamarBlue signal due to bacterial spreading across the well; bactericidal compounds yield a Tet-like (blue) alamarBlue signal (i.e. column 8, row 3).

Fig. 2. Effect of Q24DA on V. cholerae motility and growth rate. Top: confirmation of motility inhibition by Q24DA in swarm agar stabbed with a saturated culture of strain C7258.

Bottom: Growth curve of strain C7258 in LB medium pH 7.4 in the absence (□) and presence (■) of compound Q24DA (10 µg/mL).

Fig. 3. Effect of Q24DA on V. cholerae flagellum expression. Top: strain C7258 was grown in LB medium in the absence (control) and presence (treated) of Q24DA (10 µg/mL) and the cells were examined by TEM for the assembly of a wild type flagellum. Bottom: Strain C7258 was grown in LB medium in the absence (lane a) and presence (lane b) of Q24DA to stationary phase. The cell pellets corresponding to equivalent amount of cells were analyzed by western blot for FlaA expression using monoclonal antibody 3E1 raised against...
pure FlaA protein. Lane c, corresponds to the cell pellet of a flagellin A-deficient mutant (C7258ΔflaA).

**Fig. 4.** Q24DA inhibits *V. cholerae* virulence gene expression. **A.** Strain C7258 was grown in AKI medium (13) (control) and the same medium containing Q24DA (10 µg/mL). CT and the TCP major subunit TcpA were determined by GM₁-ELISA and Western blot, respectively. Abbreviations: BI, band intensity **B.** Three rabbit ileal loops were inoculated with pure CT (20 µg), strain C7258 (WT), C7258 containing 10 µg/mL Q24DA and C7258Mot⁺. Results were expressed as fluid accumulation (FA) in mL per cm of loop. Symbols *, significant p = 0.026; **, significant p = 0.029.

**Fig. 5.** Effect of Q24DA on the function of *Vibrio* polar and lateral flagella. **A.** *V. cholerae* O395; **B.** *V. parahaemolyticus* B22 (Fla⁺Laf⁺); **C.** *V. parahaemolyticus* LM5674 (Fla⁺Laf⁺); **D.** *V. parahaemolyticus* LM1017 (Fla⁺Laf⁺); **E.** *V. parahaemolyticus* LM5392 (Fla⁺Laf⁺); **F.** *V. parahaemolyticus* 7890 (Fla⁺Laf⁺); **G.** *V. vulnificus* LAM624.

**Fig. 6.** Effect of Q24DA on the motility of phenamil resistant mutants. Overnight cultures started from four independent exconjugants of strain O395ΔAB containing cosmids pLM2058 (WT) or pLM2059 (Phe⁺) were stabbed in swarm agar (control), swarm agar plus phenamil and swarm agar with Q24DA. Plates were incubated 16 h at 30°C and photographed.

**Fig. 7.** Phenotypic effects of compound Q24DA linked to Na⁺ bioenergetics. **A:** Strain C7258 was grown in LB medium pH 8.6 containing 0.3 M NaCl. At OD₆₀₀ 0.35, CCCP was added to inhibit PMF. Symbols: (Δ), control; (▲), medium containing Q24DA (10 µg/mL); (●) medium containing HQNO. **B:** C7258 was grown in LB medium in 96-well microtiter plates containing different concentrations of norfloxacin and ciprofloxacin. Plates were incubated at...
37°C for 16 h and growth measured by reading the OD$_{600}$. Symbols: (Δ) norfloxacin; (▲) norfloxacin and Q24DA; (□), ciprofloxacin; (■) ciprofloxacin and Q24DA.

Fig. 8. Inhibition of motility by chemical analogs of Q24DA. A. Structure and IC$_{50}$. The IC$_{50}$ values were determined by measuring the swarm diameter produced by three independent cultures of strain C7258 stabbed in swarm agar supplemented with twofold dilutions of each compound (numbered 1 through 7) starting at 10 µg/mL. B. Swarm agar test and growth in broth. Overnight cultures of strain C7258 containing > 10$^9$ cells/mL were stabbed in 4 mL of soft agar containing 10 µg/mL of compounds 2-7. In parallel, three overnight cultures of strain C7258 were diluted 1:100 in LB broth containing the above concentration of compounds 2-7 in 96-well microtiter plates. Plates were incubated 16 h at 30°C.
Control | Treated
---|---
A | Polar
B | Polar and Lateral
C | Polar and Lateral
D | Polar
E | Lateral
F | Polar
G | Polar

Flagellum type