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# 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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**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* strains of serogroups O1 and O139, the causative agents of the diarrheal illness cholera, express a single polar flagellum powered by sodium motive force and require motility to colonize and spread along the small intestine. Therefore, motility may be an attractive target for small molecules that can 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 an ~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 the growth rate in broth. A further study on the effects of one analog, designated Q24DA, showed that it induces a flagellated but nonmotile ($Mot^-$) phenotype and is specific for the $Na^+$-driven flagellar motor of pathogenic *Vibrio* species. A mutation conferring phenamil-resistant motility did not eliminate inhibition of motility by Q24DA. Q24DA diminished the expression of cholera toxin and toxin-coregulated pilus as well as 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 assay is capable of identifying small molecules that specifically block bacterial motility. New inhibitors such as Q24DA may be instrumental in probing the molecular architecture of the $Na^+$-driven polar flagellar motor and in studying the role of motility in the expression of other virulence factors.**

Cholera is an acute waterborne diarrheal disease caused by *Vibrio cholerae* strains of serogroups O1 and O139. This Gram-negative pathogen continues to be a major public health concern in areas of endemicity in South Asia and Africa, with an estimated 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 (12, 43, 45, 49). The availability of novel prophylactic measures and/or adjunctive therapies could contribute to diminishing the burden of cholera and antibiotic resistance.

*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) (34). CT is an ADP-ribosyl transferase responsible for the profuse rice-watery diarrhea typical of this disease (14, 28). TCP is a type IV pilus required for intestinal colonization in humans (22). Motility is required to establish infection, for colonization of the small intestine, to detach and spread along the gastrointestinal (GI) tract, and/or to exit the host and return to the environment (7, 37, 44, 52). Flagellar motility has also been shown to influence the expression of CT and TCP (15, 18, 20, 52, 56). Furthermore, motility can influence cholera transmission by enhancing *V. cholerae* biofilm formation (52, 58). 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 (17, 50, 60). Taking these observations 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 control of the direction of flagellum rotation by chemotaxis. The *V. chol-*

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TABLE 1. Strains, plasmids, and primers used for this study

| Strain, plasmid, or primer | Relevant description or sequence (5′–3′) | Reference(s) or source |
|---|---|---|
| Strains (pathogenic *Vibrio* strains) | | |
| *V. cholerae* C7258 | Wild type, O1 El Tor biotype, Ogawa | Peru (1991), clinical isolate |
| *V. cholerae* C7258 Mot$^-$ | C7258 Δ*motY* | This study |
| *V. cholerae* C7258 Fla$^-$ | C7258 Δ*flaA* | This study |
| *V. cholerae* O395 | O1, classical biotype, Ogawa | 16 |
| *V. cholerae* O395ΔAB | O395 Δ*motA* Δ*motB* | 16 |
| *V. parahaemolyticus* B22 | Wild type; Fla$^+$ Laf$^+$ | 3 |
| *V. parahaemolyticus* LM5674 | Wild type; Fla$^+$ Laf$^+$ | 27, 29 |
| *V. parahaemolyticus* LM1017 | Fla$^+$ Laf$^-$ | 27, 29 |
| *V. parahaemolyticus* LM5392 | Fla$^-$ Laf$^+$ | 27, 29 |
| *V. parahaemolyticus* LM7890 | Fla$^+$Laf$^-$ | 27, 29 |
| *V. vulnificus* LAM624 | Wild type | 59 |
| O395ΔAB Phe$^s$ | O395ΔAB containing pLM2058 | This study |
| O395ΔAB Phe$^r$ | O395ΔAB containing pLM2059 | This study |
| Plasmids | | |
| pLM2058 | Cosmid containing wild-type *V. parahaemolyticus* motor genes | 27 |
| pLM2059 | Cosmid containing phenamil resistance motor genes | 27 |
| pRK2013 | Helper plasmid for mobilization of non-self-transmissible plasmids (Km$^r$) | |
| Oligonucleotide primers | | |
| FlaAF | GATCGCATATGACCATTAACGTAAATACCAA | |
| FlaA-R3 | TACTAGTGCATCTCCCGTGATGCACTGCAATAACGAGAT | |
| ToxT189 | GCCCTCTATTCAGCGATTTTCTTTG | |
| ToxT400 | GCCCCTCCATAGCATCAAGATCATC | |
| MotA212 | ATGCTCCCGAAGATCTGATTGCCA | |
| MotA56 | ACCAGTGTCCCGATCATCCCCAT | |
| MotB433 | CAACAGCAGCAAGCACAAGCGATG | |
| MotB651 | CGCACTTTACCGGGAATGTCTTTG | |
| MotX240 | TATGCTGGCTTGGGGAGTGTGTGT | |
| MotX476 | CATCTTCATAATCGAGCGGACTGC | |
| MotY362 | CCGCGTGGTCGTTACTCAGTGAA | |
| MotY572 | GACGCTTTATTCAACTCAACACTGG | |
| RecA578 | GTGCTGTGGATGTCATCGTTGTTG | |
| RecA863 | CCACCACTTCTTCGCCTTCTTTGA | |

*erae* genome carries multiple flagellin genes, but only mutants lacking FlaA are nonflagellated and nonmotile (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 that of the previously published $Na^+$-driven polar flagellar gene system of *Vibrio 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 abolishes motility but does not prevent flagellum assembly (6, 29, 40, 48). MotA and MotB translocate $Na^+$ by forming a $Na^+$-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 may 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, which 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) pump 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 by using small molecules to change the way proteins work in real time directly 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, which poisons the $Na^+$ channel in a noncompetitive 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 an 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 assay and examine

TABLE 2. Protocol summary and assay statistics

| Parameter | Description or value |
|---|---|
| Protocol summary | |
| Growth medium and microtiter plates | Clear-bottom plates (384 wells) containing 50 μl of LB medium with 0.3% agar |
| Compound usage | Compounds were dispensed to the top of the soft agar and allowed to diffuse for 2–4 h at room temp |
| Inoculation | A 2.5-nl sample containing ~20 CFU of strain C7258 was dispensed into the bottom left corner of each well (see Fig. S3 in the supplemental material) |
| Incubation | Plates were incubated for 16–24 h at 30°C and high humidity |
| Motility reading | Spreading of motile bacteria was measured by reading the $OD_{615}$ above and to the right diagonal of the inoculation site (see Fig. S3) |
| Viability reading | Viability was estimated by adding 5 μl of 100% alamarBlue to each well; plates were incubated for 1–1.5 h at 30°C, and fluorescence was read (excitation wavelength, 535 nm; emission wavelength, 595 nm) |
| Control drugs | Phenamil (motility) and tetracycline (viability) |
| Assay statistics | |
| Z value (mean) | 0.5–0.8 (0.7) |
| Signal-to-noise ratio | 173 |
| Signal-to-background ratio | 7 |
| Pilot screen | |
| No. of compounds screened | 8,093 (in duplicate on two different days) |
| Control $OD_{615}$ (avg [coefficient of variation]) | |
| Carrier control (DMSO) | 0.73 (5.6) |
| Motility control (phenamil) | 0.10 (3.2) |
| Viability control (tetracycline) | 0.10 (3.6) |
| Hit rate (%) | 0.2 |

its effects 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.** The strains used in this study are described in Table 1. C7258 and C7258 Mot$^-$ 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°C, while *Vibrio vulnificus* was grown in marine broth (Difco). To measure the production of CT and TcpA, strain C7258 was grown in AKI medium (26) at 30°C. As a test to determine the function of the Nqr primary $Na^+$ pump, strain C7258 was grown in LB medium, pH 8.6, containing 0.3 M NaCl. Culture media were supplemented with streptomycin (Str) (100 μg/ml), tetracycline (Tet) (5 μg/ml), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (5 μM), 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (5 μM), and phenamil (50 to 100 μM) as required and as described in the 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 nonmotile derivative of C7258 (C7258 Mot$^-$) was constructed by introducing a Δ*motY* mutation as described previously, but using the above strain as a conjugal receptor (52). A similar procedure was used to construct the flagellin A-defective strain C7258 Fla$^-$. To construct isogenic *V. cholerae* strains expressing phenamil-sensitive and phenamil-resistant motility, cosmids pLM2058 and pLM2059 (Tet$^r$), carrying wild-type and phenamil resistance motor genes (*motA* and *motB*), respectively (27), were transferred to strain O395ΔAB (Str$^r$) (16) in a tripartite cross using *Escherichia coli* pRK2013 as a Tra donor. Exconjugants were selected in LB agar supplemented with Tet and Str.

**Swarm agar test.** Motility was measured by stabbing overnight cultures into 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 or 30 ml of swarm agar, respectively. Plates were incubated at 30°C for 16 h.

**Motility HTS assay.** The HTS assay described in this study was based on a recently described 96-well assay for *Salmonella enterica* serovar Typhimurium antimicrobial compounds that uses motility as a readout (38). This assay is based on (i) off-center inoculation of a bacterial culture into wells containing soft agar, (ii) an incubation period allowing motile bacteria to spread throughout the well, and (iii) a diagonal off-center reading of absorbance (optical density at 615 nm [$OD_{615}$]) 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 $OD_{615}$ reading with detection of viability using alamarBlue to differentiate between compounds that inhibit motility and compounds that are toxic to the bacteria. To validate the HTS assay, 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 of up to 1%. Compounds identified to specifically inhibit motility were screened in dose-response assays to calculate their 50% inhibitory concentrations ($IC_{50}$s) and were checked for mammalian cell cytotoxicity by using THP-1 cells (ATCC TIB-202) and a 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 in the supplemental material.

**Determination of FlaA expression.** To detect FlaA, the *flaA* gene from strain C7258 was amplified using an Advantage 2 PCR system (Clontech Laboratories Inc.) and oligonucleotide primers FlaAF and FlaA-R3 (Table 1). The PCR product was cloned directionally as an NdeI-SpeI fragment into the vector pTXB1 for expression and purification of the FlaA protein by use of the Impact 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. Gels were transferred to polyvinylidene difluoride (PVDF) membranes, and the FlaA protein was detected by Western blotting using a BM chemiluminescence Western blot kit (Roche Applied Sciences, Indianapolis, IN).

**TEM.** Flagellum assembly was confirmed by transmission electron microscopy (TEM). Strain C7258 was grown in LB medium for 16 h, and the cells were pelleted and then 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-coregulated pilus expression.** The amount of cholera toxin was determined by a ganglioside $GM_1$ enzyme-linked immunosorbent assay ($GM_1$-ELISA) using a peroxidase-conjugated rabbit anti-cholera toxin B IgG (Pierce/Thermo Fisher Scientific) and a standard curve for pure CT (Sigma Chemical Co.) as described previously (52). TcpA, the major

TCP subunit, was detected in Western blots by use of a rabbit anti-TcpA serum kindly provided by Biao Kan (CDC Beijing). A pellet corresponding to 0.5 $OD_{600}$ unit was boiled in SDS-PAGE loading buffer, and TcpA was detected using a BM chemiluminescence Western blot kit as described above. A parallel gel was stained with Coomassie blue dye to confirm equal loading in each well.

**Biofilm assay.** Biofilm formation was measured by the crystal violet staining method, and the results were normalized for growth and expressed as the $OD_{570}/OD_{600}$ ratio (61).

**qRT-PCR.** Total RNA was isolated using the RNeasy kit (Qiagen Laboratories). The RNA samples were analyzed by quantitative reverse transcription-PCR (qRT-PCR) to determine relative gene expression of target genes, using an iScript two-step RT-PCR kit with SYBR green (Bio-Rad Laboratories) as described previously (52). Relative expression values were calculated as $2^{-(CT\ \text{target} - CT\ \text{reference})}$, where $C_T$ 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 Oligo primer analysis software (Table 1). A control mixture lacking reverse transcriptase was run for each reaction to exclude the presence of 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. 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 the amount of fluid accumulation (FA) per centimeter of loop length.

## RESULTS

**Development of 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 case for motile *V. cholerae* strain C7258, inoculation of the isogenic nonmotile strain C7258 Mot$^-$ 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. 1, a bactericidal compound such as tetracycline could be differentiated from a motility inhibitor compound such as phenamil. The inhibitory effect of phenamil on motility could be seen at 100 μM (Fig. 1A), but there was little effect on viability measured with alamarBlue (Fig. 1B). In contrast, tetracycline killed the bacteria, and while they appeared nonmotile by the $OD_{615}$ reading (Fig. 1A), the alamarBlue reading showed that they were nonviable (Fig. 1B). As shown in the sample plate from a pilot screen (Fig. 1C), tetracycline-like toxic compounds could be differentiated visually from phenamil-like compounds that selectively inhibit motility. Four compounds were identified that inhibited motility ($IC_{50}$, 1.9 to 14 μg/ml) and showed no toxicity to the bacterium or to mammalian cells.

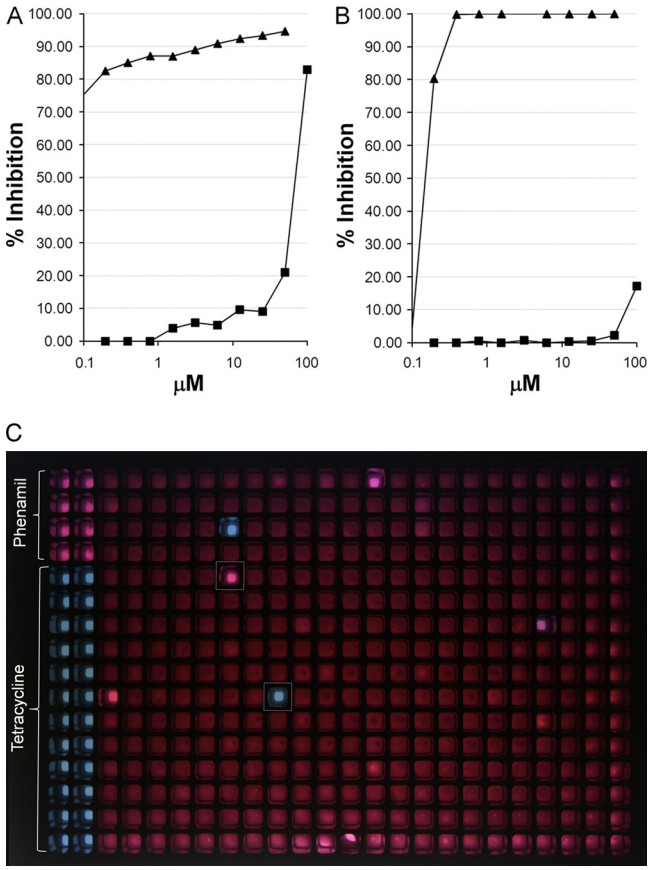


FIG. 1. HTS assay for inhibitors of bacterial motility. (A) Dose-response evaluation of phenamil (■) and tetracycline (▲) for motility inhibition in 384-well plates, measured by the $OD_{615}$. (B) Dose-response evaluation of phenamil (■) and tetracycline (▲) for toxicity in 384-well plates, measured with alamarBlue. In each case, the data are expressed as percent inhibition relative to the $OD_{615}$ reading or alamarBlue fluorescence signal in the absence of compound. (C) Sample plate. Plates were inoculated with the motile strain C7258. Columns 1 and 2 contained phenamil (rows 1 to 4) or tetracycline (rows 5 to 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 such as phenamil, which inhibits motility but not viability, yield a bright (pink) alamarBlue fluorescence signal (column 8, row 5); compounds that do not affect motility or viability yield a blurred alamarBlue signal due to bacterial spreading across the well; and bactericidal compounds yield a Tet-like (blue) alamarBlue signal (column 8, row 3).

Inactivation of motility by mutation does not commonly affect the growth rate in broth. Therefore, we rescreened the compounds showing no toxicity for their effect on the growth rate in broth. Of these compounds, we selected a quinazoline-2,4-diamino analog, 2-({2-[(2,5-dimethylphenyl)amino]quinazolin-4-yl}amino)ethanol (see Fig. 8, structure 1), referred to here as Q24DA, for further studies because it completely suppressed motility in soft agar plates stabbed with an overnight culture of C7258 containing $>10^9$ bacteria/ml but had no detectable effect on growth rate (Fig. 2). Motility mutants are commonly classified as Fla$^-$ when they fail to express a flagellum and as Mot$^-$ when they express a paralyzed flagellum (29). Thus, we used TEM and Western blotting to determine if the Q24DA compound chemically induced a Fla$^-$ or Mot$^-$ phe-

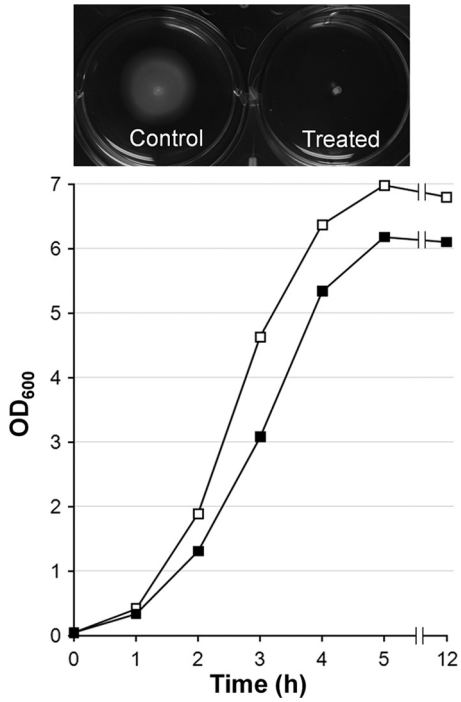


FIG. 2. Effects of Q24DA on *V. cholerae* motility and growth rate. (Top) Confirmation of motility inhibition by Q24DA in swarm agar stabbed with a saturated culture of strain C7258. (Bottom) Growth curve for strain C7258 in LB medium, pH 7.4, in the absence (□) and presence (■) of compound Q24DA (10 μg/ml).

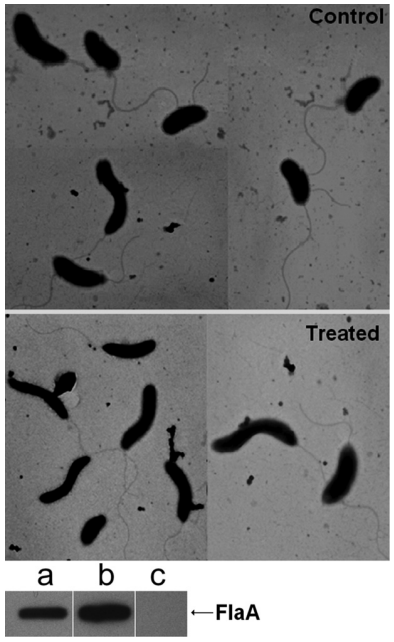


FIG. 3. Effect of Q24DA on *V. cholerae* flagellum expression. (Top) Strain C7258 was grown in LB medium in the absence (control) and presence (treated) of Q24DA (10 μg/ml), and the cells were examined by TEM for the assembly of a wild-type flagellum. (Bottom) Strain C7258 was grown to stationary phase in LB medium in the absence (lane a) and presence (lane b) of Q24DA. The cell pellets corresponding to equivalent amounts of cells were analyzed by Western blotting 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 (C7258 Δ*flaA*).

notype. 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 induced 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 $0.2 \pm 0.01$ in AKI medium and $0.1 \pm 0.01$ ($n = 3$) 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, coinoculation of C7258 with Q24DA significantly diminished fluid secretion.

**Compound Q24DA is a novel inhibitor of the $Na^+$-driven flagellar motor.** Different *Vibrio* species differ with regard to the flagellum system they express. For instance, while *V. vulnificus* expresses a single $Na^+$-driven polar flagellum similar to that of 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 means 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 the lateral flagellum ($Fla^+$ $Laf^-$). Contrastingly, Q24DA had no effect on *V. parahaemolyticus* strains that expressed 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, the Q24DA compound did not affect motility in *E. coli* and *S.* Typhimurium, which are 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 similar ways. To this end, we

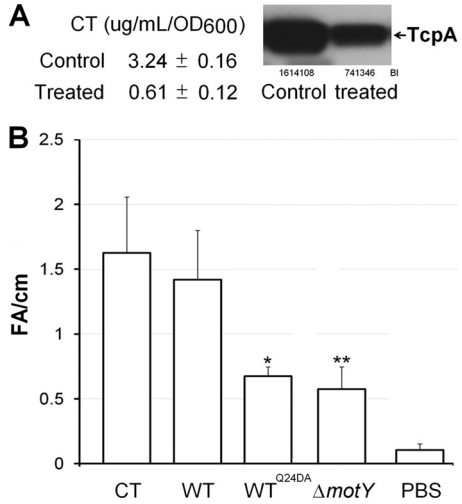


FIG. 4. Q24DA inhibits *V. cholerae* virulence gene expression. (A) Strain C7258 was grown in AKI medium (13) (control) and in the same medium containing Q24DA (10 μg/ml). CT and the TCP major subunit TcpA were detected by $GM_1$-ELISA and Western blotting, respectively. BI, band intensity. (B) Three rabbit ileal loops were inoculated with pure CT (20 μg), strain C7258 (WT), C7258 containing 10 μg/ml Q24DA, or C7258 Mot$^-$. Results are expressed as amounts of fluid accumulation (FA) in ml per cm of loop. *, $P = 0.026$; **, $P = 0.029$.

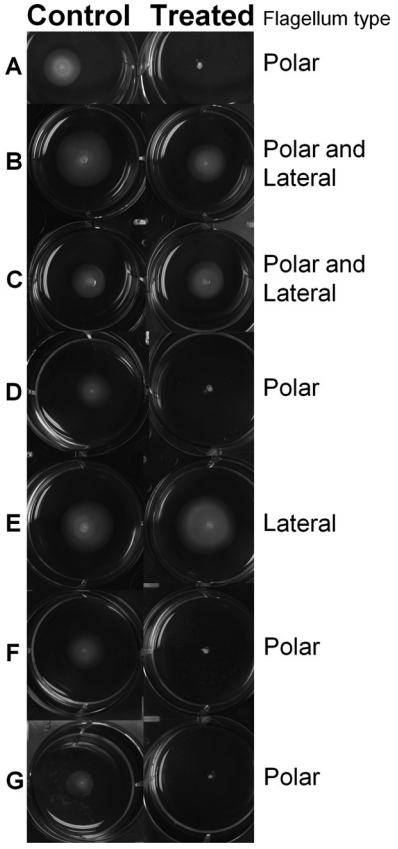


FIG. 5. Effect of Q24DA on function of *Vibrio* polar and lateral flagella. (A) *V. cholerae* O395; (B) *V. parahaemolyticus* B22 (Fla$^+$ Laf$^+$); (C) *V. parahaemolyticus* LM5674 (Fla$^+$ Laf$^+$); (D) *V. parahaemolyticus* LM1017 (Fla$^+$ Laf$^-$); (E) *V. parahaemolyticus* LM5392 (Fla$^-$ Laf$^+$);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

constructed isogenic *V. cholerae* strains expressing wild-type (phenamil-sensitive) *motA* and *motB* motor genes and the corresponding phenamil resistance genes from *V. parahaemolyticus*. As shown in Fig. 6, the *V. parahaemolyticus* motor genes fully complemented the nonmotile phenotype of strain O395ΔAB. Furthermore, the strain receiving the phenamil resistance *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 an influx of $Na^+$ through the MotA/MotB channel as well as maintenance of the $Na^+$ gradient by the activities 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; 0.3 M 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 that by 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 effects on two additional phenotypes linked to sodium bioenergetics suggest 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 for all active quinazoline analogs identified the presence of a 2-ethanolamino group at position 4 of the quinazoline ring as a minimum structural feature required for selective inhibition of motility. On this basis, 22 analogs structurally similar to Q24DA and possessing a 2-ethanolamino or related (tetrahydrofuran-2-yl) methylamino group at position 4 were purchased and screened for motility and growth inhibition. Six compounds (Fig. 8, structures 2 to 7) were found to completely suppress motility without affecting growth in broth ($IC_{50}$, 1.9 to 20.1 μM). Four compounds (see Fig. S1 in the supplemental material [structures 8 to 11]) showed only partial motility inhibition, while the 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.* Typhimurium antibacterial compounds that used 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 and those that affect viability. Thus, not surprisingly, screening of a 960-compound structurally diverse compound

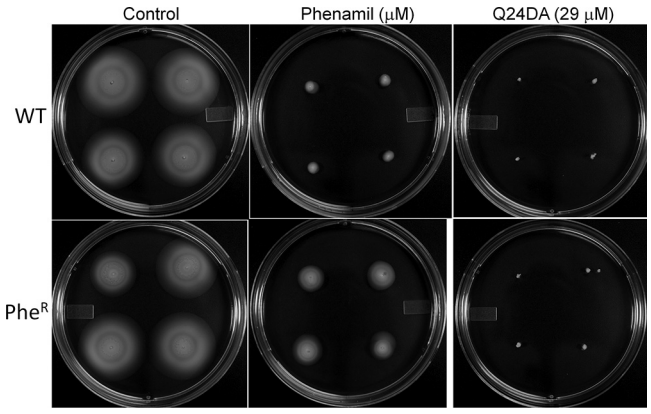


FIG. 6. Effect of Q24DA on motility of phenamil-resistant mutants. Overnight cultures started from four independent exconjugants of strain O395ΔAB containing cosmid pLM2058 (WT) or pLM2059 (Phe$^r$) were stabbed into swarm agar (control), swarm agar plus phenamil, or swarm agar with Q24DA. Plates were incubated for 16 h at 30°C and then photographed.

library by the former method did not identify any specific inhibitors 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 to 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 whose 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 for target identification.

Since nonmotile *V. cholerae* mutants of the El Tor biotype are severely attenuated and make altered biofilms (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 the growth rate in broth. This compound chemically induced a Mot$^-$ (nonmotile flagellated) phenotype. This is the phenotype observed when components of the flagellar motor or chemotaxis machinery 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 the $Na^+$-driven polar flagellum and the expression of CT and TCP. For instance, mutation of motility genes, inhibition of motility with phenamil, and changes in membrane $Na^+$ flux have been shown to have variable effects on virulence gene expression (15, 18–20, 52, 56). Here we showed that inhibition of motility with 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 that of mutating the motor gene *motY*, used as a nonmotile control. These findings are consistent with the above evidence 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 of 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, *motY*, diminished biofilm formation (52). A more recent study also supported a role for the flagellar motor and $Na^+$

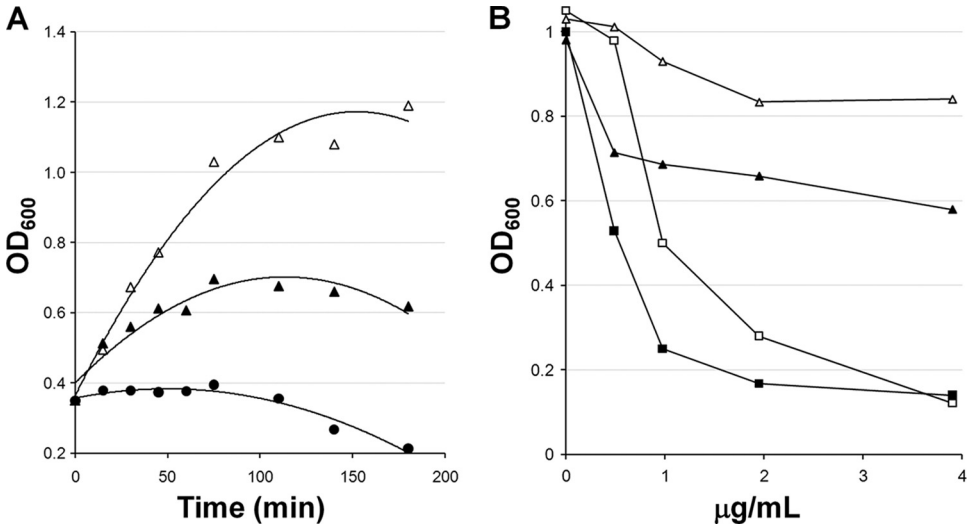


FIG. 7. Phenotypic effects of compound Q24DA linked to $Na^+$ bioenergetics. (A) Strain C7258 was grown in LB medium, pH 8.6, containing 0.3 M NaCl. At an $OD_{600}$ of 0.35, CCCP was added to inhibit PMF. Symbols: △, control; ▲, medium containing Q24DA (10 μg/ml); ●, medium containing HQNO. (B) C7258 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 was measured by reading the $OD_{600}$. Symbols: △, norfloxacin; ▲, norfloxacin and Q24DA; □, ciprofloxacin; ■, ciprofloxacin and Q24DA.

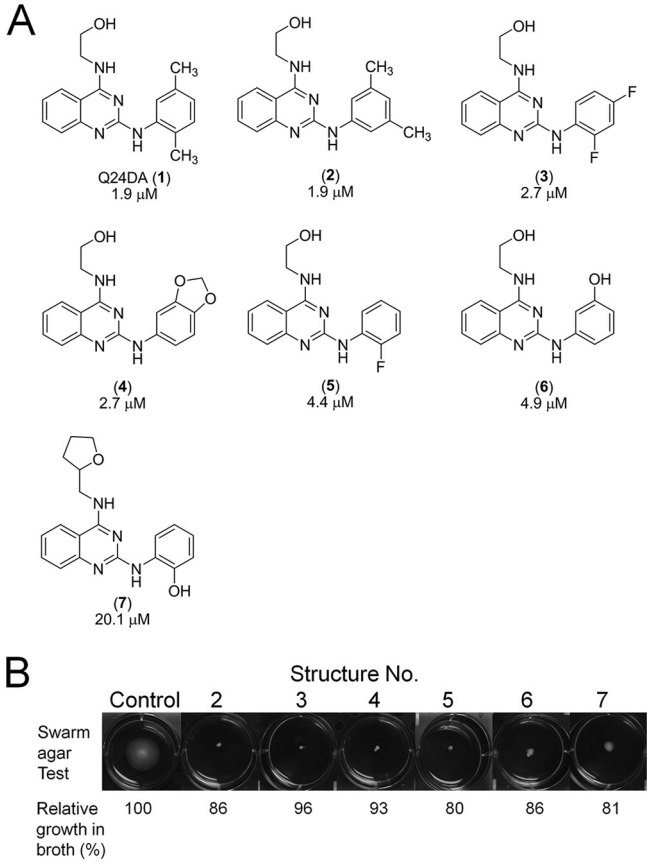


FIG. 8. Inhibition of motility by chemical analogs of Q24DA. (A) Structures and $IC_{50}$s. The $IC_{50}$s were determined by measuring the swarm diameters produced by three independent cultures of strain C7258 stabbed into swarm agar supplemented with 2-fold dilutions of each compound (numbered 1 through 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 into 4 ml of soft agar containing 10 μg/ml of compounds 2 to 7. In parallel, three overnight cultures of strain C7258 were diluted 1:100 in LB broth containing the above concentration of compounds 2 to 7 in 96-well microtiter plates. Plates were incubated for 16 h at 30°C.

bioenergetics in *V. cholerae* permanent surface attachment (57). Altogether, it is likely that inhibitors of *V. cholerae* motility may serve as a useful source of compounds for development as drugs to inhibit toxin production and biofilm formation.

We analyzed the effects of Q24DA on the motility of several members of the *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 *Vibrionaceae* family examined. We note that the polar and lateral flagellar motors of *V. parahaemolyticus* are directed by a common chemotactic control pathway and the same CheY molecular species (33, 51). Therefore, the finding that Q24DA does not impair the function of the *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 MotA (27). D148 in MotA and P16 in MotB have been suggested to form a phenamil-binding pocket at the inner side of the 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 effects of other mutations that confer phenamil resistance, such as the MotB A23G mutation (27), our results suggest that inhibition of the $Na^+$-driven flagellar motor by phenamil and Q24DA involves 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 and MotY), or the FliGMN switch complex. It is noteworthy that a 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 inhibition by 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 showed here 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 medium. Since this effect was less severe than that obtained by 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, which are widely distributed among clinically significant bacteria, such as *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 may 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 antibacterial, antiparasitic, anticancer, 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 group at position 4 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 that we screened, compounds with structures 2 to 7 (Fig. 8) showed comparable selective antimotility activities. All active and partially active compounds against *Vibrio* motility were found to possess a substituted aminophenyl moiety at position 2 of quinazoline, whereas compounds possessing saturated cyclicamino and benzylamino substitutions were found to be inactive. Further compound analog research for extensive structure-activity relationship characterization is under way to detect more potent and selective inhibitors of motility in *V. cholerae*.

In summary, the availability of an HTS assay for inhibitors of bacterial motility now makes it 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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