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3-Amino 1,8-naphthalimide, a structural analog of the anti-cholera drug virstatin inhibits chemically-biased swimming and swarming motility in vibrios

Hongxia Wang[§], Anisia J. Silva, and Jorge A. Benitez^{*}

Morehouse School of Medicine Department of Microbiology, Biochemistry and Immunology, 720 Westview Dr., SW, Atlanta, Georgia 30310

Abstract

A screen for inhibitors of *Vibrio cholerae* motility identified the compound 3-amino 1,8-naphthalimide (3-A18NI), a structural analog of the cholera drug virstatin. Similar to virstatin, 3-A18NI diminished cholera toxin production. In contrast, 3-A18NI impeded swimming and/or swarming motility of *V. cholerae* and *V. parahaemolyticus* suggesting that it could target the chemotaxis pathway shared by the polar and lateral flagellar system of vibrios. 3-A18NI did not inhibit the expression of *V. cholerae* major flagellin FlaA or the assembly of its polar flagellum. Finally, 3-A18NI enhanced *V. cholerae* colonization mimicking the phenotype of chemotaxis mutants that exhibit counterclockwise-biased flagellum rotation.

Keywords

Vibrio; *Vibrio cholerae*; cholera toxin; virstatin; motility; chemotaxis

1. Introduction

Vibrio cholerae of serogroups O1 and O139 is a major human pathogen that causes the diarrheal disease cholera, an illness that disproportionately affects impoverished regions worldwide with prevalence of 2.8 million cases and close to 100,000 deaths, annually. In addition, non-O1/O139 *V. cholerae* strains and other members of the *Vibrionaceae* such as *V. parahaemolyticus* and *V. vulnificus* have been demonstrated to cause infections in humans ranging from mild gastroenteritis to fatal life-threatening septicemia. A distinguishing feature of cholera, however, is its potential for pandemic dissemination. In endemic areas, the occurrence of cholera follows predictable climatic patterns. Introduction of *V. cholerae*

^{*}Corresponding author: jbenitez@msm.edu.

[§]Current address, Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL 35294.

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Disclosure of potential conflicts of interest

The authors declared no conflicts of interest

O1 in non-endemic areas with poor sanitation commonly results in rapidly spreading outbreaks that can overwhelm the response capacity of the public health system. In this scenario, it is critical to diminish the duration of purging of virulent vibrios and hospitalization time. A mean to achieve this goal is the use of antibiotics to treat moderate and severe cholera [1]. A recognized downside of this approach is the development of antibiotic resistance over time [2]. The development of drugs that target virulence factors has been suggested as an alternative approach to antibiotic therapy [3]. An example of this approach is the drug virstatin (4-(N-(1,8-naphthalimide))-n-butyric acid), a compound that prevents *V. cholerae* from expressing its major virulence factor, cholera toxin [4]. Expression of cholera toxin genes (*ctxAB*) is activated by the AraC-family transcription activator ToxT, which binds as a dimer to the *ctxA* promoter to activate its transcription [5]. Virstatin has been shown to inhibit the production of cholera toxin by preventing the dimerization of ToxT [6].

V. cholerae expresses a polar flagellum powered by sodium-motive force [7] that is required for intestinal colonization [8, 9]. We recently reported the development of a high throughput screening assay for inhibitors of *V. cholerae* motility [10]. The screen identified a quinazoline 2,4 diamino analog (Q24DA) that specifically paralyzed the *V. cholerae* polar flagellum by targeting PomB, a component of the inner membrane sodium-conducting channel that energizes flagellum rotation [11]. Here we report on the properties of a second compound identified in the above screen, 3-amino 1,8-naphthalimide (3-A18NI), a structural analog of virstatin. We show that similar to virstatin, 3-A18NI significantly diminished production of cholera toxin without affecting growth. Contrary to virstatin, 3-A18NI (i) suppressed *V. cholerae* swimming motility in swarm agar, (ii) suppressed swimming and swarming motility in *V. parahemolyticus* strains that express polar and/or lateral flagella and (iii) enhanced *V. cholerae* intestinal colonization.

2. Materials and methods

2.1. Strains and media

V. cholerae C7258 (El Tor biotype, Ogawa) and O395 (classical biotype, Ogawa) were routinely grown in Luria-Bertani (LB) medium at 37°C. Specific conditions for expression of cholera toxin and the toxin co-regulated pilus (TCP) were (i) AKI medium (1.5% Bacto peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% NaHCO₃) at 30°C [12] for El Tor biotype *V. cholerae* or (ii) LB at 30°C for classical biotype strains. *V. parahemolyticus* strains expressing polar (Fla) and/or lateral (Laf) flagella LM5674 (Fla⁺Laf⁺), LM1017 (Fla⁺Laf⁻), and LM5392 (Fla⁻Laf⁺) [13] were grown in LB medium at 37°C. Compounds 3-A18NI and virstatin were obtained from ChemBridge (San Diego, California) and Key Organics (Bedford, Massachusetts), respectively. Compounds were dissolved in dimethyl sulfoxide (DMSO) and added to medium at a final concentration of 25 μ M.

2.2. Production of cholera toxin, the toxin-coregulated pilus (TCP) and flagellin A

Production of cholera toxin was determined by a ganglioside GM₁ enzyme-linked immunosorbent assay using a peroxidase-conjugated rabbit anti-cholera toxin B IgG (Pierce/Thermo Fisher Scientific) and a standard curve with varying concentrations of pure cholera

toxin (Sigma Chemical Co.) as described previously [8]. TcpA, the major TCP subunit, was detected in western blots with a rabbit anti-TcpA serum. Cell pellets corresponding to 0.5 optical density units at 600 nm (OD₆₀₀) were boiled in 100 µL of sodium dodecyl sulfate — polyacrylamide gel electrophoresis loading buffer and proteins were separated in a 12 % polyacrylamide gel. Gels were transferred to polyvinylidene difluoride membranes and TcpA was detected by western blotting using a BM chemiluminescence western blot kit (Roche Applied Sciences, Indianapolis, IN). Flagellin FlaA expression was determined using the same procedure and the monoclonal antibody 3E1 raised against pure FlaA protein [10].

2.3. Swarm agar test

Motility was measured by stabbing overnight cultures into LB medium containing 0.3% agar (swarm agar). The assay was conducted in 6-well tissue culture plates or 100 mm petri dishes containing 4 or 30 ml of swarm agar, respectively. Plates were incubated at 30°C for 16 h. The IC₅₀ of 3-A18NI was determined in a dose-response assay by measuring the swarm diameters produced by three independent stationary phase cultures of strain C7258 or O395 stabbed into swarm agar supplemented with a 2-fold dilution series of compound starting at 25 µM.

2.4. Suckling mouse colonization assay

Animal studies were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and a protocol approved by the Morehouse School of Medicine Institutional Animal Care and Use Committee. Five days-old CD-1 mice were starved for 6 h and inoculated orally through a 21-gauge feeding needle with 10⁵ colony forming units (CFU) of challenge strain in 50 µL in phosphate-buffered saline (PBS) pH 7.4 containing 3-A18NI (25 µM) or vehicle. Mice were maintained for 16 h in their cages with bedding at 28–30°C. The animals were sacrificed by decapitation and the entire small intestine was removed, weighed, and homogenized in 5 ml of PBS. The bacterial load was determined by dilution plating on LB agar and normalized by tissue weight.

2.5. Transmission electron microscopy

Flagellum assembly was confirmed by transmission electron microscopy (TEM). Strain O395 was grown in LB medium to mid-exponential phase, 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.

3. Results and discussion

The compound 3-A18NI is a structural analog of the cholera drug virstatin that harbors an amino substitution in position 3 of the naphthalimide ring and lacks the butyric acid side chain present in the virstatin molecule (Pub Chem ID: 145949). In contrast to virstatin, we found that 3-A18NI suppressed *V. cholerae* swimming motility in swarm agar plates (Fig. 1A,B). The swarm agar assays measures the capacity of bacteria to move away from an

inoculation site following a chemical (nutrient) gradient through a combination of straight runs and tumbling. Both non-motile chemotactic and motile non-chemotactic mutants show a negative phenotype in this assay. Dose response analyses conducted in triplicate revealed IC₅₀ values of 12.8 ± 1.3 and 12.6 ± 0.9 for El Tor and classical biotype *V. cholerae*, respectively. TEM examination, however, showed that vibrios treated with 3-A18NI are flagellated (Fig. 1H,I). Further, *V. cholerae* cells treated with 3-A18NI appeared motile when examined by light microscopy exhibiting straight runs. This behavior suggests that 3-A18NI inhibits the outward migration of cells from the inoculation site in swarm agar plates by preventing cell tumbling rather than flagellum rotation. Tumbling occurs when the direction of flagellum rotation is inverted from default counterclockwise to clockwise by the response regulator CheY. Thus, our observation indicates that, at the same concentration 3-A18NI fully suppresses motility in swarm agar, it does not prevent counterclockwise flagellum rotation. We have not been able, however, to quantitate the effect of 3-A18NI on tumbling frequency. Virstatin had no effect on swarm agar motility under equal conditions (Fig. 1F). Identical results were obtained when 3-A18NI was applied to *V. vulnificus* and *V. anguillarum*, which similar to *V. cholerae*, express a single polar flagellum (data not shown). To determine if 3-A18NI is specific for vibrios expressing a polar sodium-driven flagellum, we took advantage of *V. parahaemolyticus* mutants expressing either a sodium-driven polar flagellum (Fla), a proton-driven lateral flagellum (Laf) or both flagellar systems [13]. *V. parahaemolyticus* uses the polar flagellum to swim in liquid and the lateral flagellum to swarm over solid surfaces [13]. We note that in contrast to *V. parahaemolyticus*, swarming motility has not been demonstrated in *V. cholerae* [14]. As shown in Fig. 1C–E, 3-A18NI suppressed motility in swarm agar in *V. parahaemolyticus* expressing both flagellar systems. In Fig. 1G, we show that 3-A18NI did not affect *V. cholerae* growth when cultivated in broth.

Given the structural similarity between 3-A18NI and virstatin, we investigated its effect on the production of cholera toxin and TCP. To this end, strain C7258 and O395 were grown under their respective ToxR-permissive conditions described in materials and methods in the presence of virstatin, 3-A18NI or vehicle. As shown in Fig. 2AB, virstatin and 3-A18NI significantly diminished cholera toxin production in both *V. cholerae* biotypes. Both compounds differed, however, with respect to inhibition of TCP expression. While virstatin strongly inhibited TCP expression in both biotypes, 3-A18NI had little effect on TCP production in the El Tor biotype (Fig. 2C). Compound 3-A18NI diminished TCP expression in classical biotype *V. cholerae* but still showed a weaker inhibitory effect compared to virstatin (Fig. 2C). Stripping of the Fig. 2C blot and re-probing with anti-FlaA mAb showed that 3-A18NI did not affect the production of FlaA (Fig. 2D), the major *V. cholerae* flagellin [15], which therefore served as a loading control.

The report that orogastric administration of virstatin protected infant mice from intestinal colonization by *V. cholerae* [4], prompted us to test if 3-A18NI affects intestinal colonization in the same animal model. As shown in Fig. 3A, 3-A18NI significantly enhanced intestinal colonization in the El Tor biotype strain C7258 ($p < 0.01$, unpaired T test, $n = 10$). A lesser positive effect, though still statistically significant ($p < 0.01$, $n = 8$), was observed in the classical biotype strain O395 (Fig. 3B).

The finding that 3-A18NI diminished cholera toxin production almost to the same extent as virstatin indicates that this compound retains the capacity of interacting with ToxT. This finding is in agreement with structure-activity relationship analysis of virstatin showing that the length of the carboxylate side-chain attached to the imide nitrogen of the naphthalimide scaffold has little effect on the degree of ToxT-dependent transcription inhibition of cholera toxin genes [6]. The effect of 3-A18NI on motility is clearly independent of ToxT, which is encoded within the *V. cholerae* TCP pathogenicity island [16] that is absent in *V. parahemolyticus*. Conversely, 3-A18NI diminished production of cholera toxin in a *pomAB* deletion mutant of strain C7258 [11] (DMSO, $3.7 \pm 0.2 \mu\text{g/mL}/\text{OD}_{600}$; 3-A18NI; $0.4 \pm 0.04 \mu\text{g/mL}/\text{OD}_{600}$) that lacks the stator of the flagellar motor. We conclude that inhibition of cholera toxin production and motility in swarm agar by 3-A18NI result from the interaction of this compound with distinct targets.

The differential effect of virstatin and 3-A18NI on the production of TCP in strain C7258 remains unclear. It is known that ToxT binds to toxboxes at the *tcpA* promoter to prime an auto regulatory loop that results in the expression of more ToxT and subsequent activation of the TCP cluster [17]. However, it has not been established if ToxT transcription activation at the *tcpA* and *ctxA* promoters require the same extent of ToxT dimerization. Virstatin and 3-A18NI may differ in their capacity to prevent ToxT dimerization resulting in differential inhibition of *ctxA* and *tcpA* ToxT-dependent transcription initiation. Consistent with this explanation, ToxT activation of the *ctxAB* promoter occurs at a later time point and a higher concentration of ToxT compared to the *tcpA* promoter *in vitro* and *in vivo* [18, 19]. Thus, the *tcpA* promoter may be more responsive to monomeric ToxT.

Compound 3-A18NI did not affect expression of flagellin FlaA, the assembly of the *V. cholerae* polar flagellum or motility when examined by light microscopy. This rules out the possibility of 3-A18NI acting at a component of the flagellar motor required for the generation of torque. This interpretation is in agreement with reports showing that inhibiting the polar flagellar motor by mutation or by treatment of *V. cholerae* with phenamil or Q24DA enhances cholera toxin production [8, 11, 20], while 3-A18NI exhibited the opposite effect. Distinct from motility mutants, 3-A18NI did not diminish *V. cholerae* surface attachment and quorum sensing-regulated biofilm development (data not shown). It has been demonstrated that the polar and lateral flagellar system of *V. parahemolyticus* and *V. alginolyticus* are controlled by a common CheY response regulator [21, 22]. Therefore, it is possible that 3-A18NI could act by inhibiting chemotaxis to suppress chemically-directed locomotion resulting from both flagellar systems. We note that a flagellum-independent and lipopolysaccharide-dependent motility was described in *V. cholerae* strain O395 [23]. However, this behavior was only observed when dense cell suspensions were spotted onto the surface of semisolid agarose plates and not under the conditions used in this study.

Our finding that 3-A18NI differed from virstatin by significantly enhancing rather than diminishing intestinal colonization in the El Tor biotype strain C7258 is consistent with the observation that it did not prevent the expression of TCP, which is known to be essential for intestinal colonization [24]. The above observation is also in line with the hypothesis that 3-A18NI may act by inhibiting chemotaxis. We note that motility mutants colonize poorly the suckling mouse intestine [8, 9] while chemotaxis mutants of the El Tor biotype bearing

counterclockwise-biased flagellum rotation exhibit enhanced colonization [25]. These mutants aberrantly colonized the entire suckling mouse small intestine compared to wild type strains that are confined to the distal small intestine [25]. Compound 3-A18NI showed a smaller positive effect on intestinal colonization in the classical biotype strain O395. We suggest that in this strain the positive effect of 3-A18NI on intestinal colonization is partially offset by diminished expression of TCP.

4. Concluding remarks

Taken together, the above findings suggest that the virstatin analog 3-A18NI suppresses vibrio chemically-biased swimming and/or swarming motility in the swarm agar test by possibly inhibiting chemotaxis, while retaining its capacity to interact with ToxT to inhibit toxin production in the cholera bacterium. In contrast to virstatin, 3-A18NI enhanced *V. cholerae* colonization of the suckling mouse intestine. Commonly, structure-activity relationship analyses are aimed to diminish the dose of a drug required to produce a specific effect. This report provides an example of how a chemical modification of a preexisting drug could enable it to interact with a distinct target to elicit an unexpected effect. The determination of 3-A18NI's secondary target responsible for suppressing motility in swarm agar plates has remained elusive. Efforts in this direction may have been hampered by the fact that vibrio genomes encode an impressive number of chemotaxis paralogues whose function and interaction with the flagellar motor remains unknown. Further studies are required to determine mechanism of action of 3-A18NI. These studies could provide new chemical tools to study the role of motility and chemotaxis in the pathogenesis of vibrios.

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Fig. 1. Effect of 3-A18NI on vibrios expressing polar and lateral flagella

Overnight cultures of **A**, *V. cholerae* O395; **B**, *V. cholerae* C7258; **C**, *V. parahemolyticus* LM5674 (Fla⁺Laf⁺); **D**, *V. parahemolyticus* LM1017 (Fla⁺Laf⁻); **E**, *V. parahemolyticus* LM5392 (Fla⁻Laf⁺) were stabbed into swarm agar plus 3-A18NI (25 μM) dissolved in DMSO and in the same medium containing an equivalent volume of DMSO alone. In panel **F**, overnight cultures of strain O395 (classical) or C7258 (El Tor) were stabbed into swarm agar plus virstatin (25 μM) dissolved in DMSO and in the same medium containing the same volume of DMSO alone. Plates were incubated for 16 h at 30°C. The results shown are representative of three separate experiments. In panels **G**, *V. cholerae* strain C7258 was grown in LB medium with agitation (250 rpm) at 37°C in triplicate. Each overnight culture was diluted 1:1000 in fresh medium supplemented with 3-A18NI (25 μM) dissolved in DMSO or with an equivalent volume of DMSO alone as a control. Growth was determined by periodically measuring the optical density at 600 nm. In panels **H** and **I**, *V. cholerae* C7258 was grown in LB medium to mid-exponential phase in the presence of 3-A18NI (25 μM) (**H**), or an equivalent volume of DMSO alone (**I**) and the polar flagellum visualized by TEM as described in methods. Flagella are indicated by arrows.

Fig. 2. Comparison of the effect of 3-A18NI and virstatin on the production of cholera toxin, TCP and flagellin FlaA

Production of cholera toxin and expression of TcpA and FlaA was determined as described in materials and methods in the presence of virstatin (25 μM), 3-A18NI (25 μM) or an equivalent volume of DMSO alone. **A.** Production of cholera toxin by strain C7258 (El Tor biotype) in AKI medium. **B.** Production of cholera toxin by strain O395 (classical biotype) in LB medium. **C.** Expression of major TCP subunit TcpA in AKI (El Tor) and LB (classical) medium, respectively. **D.** Detection of FlaA in the samples of *V. cholerae* cells treated with DMSO, 3-A18NI or virstatin used in panel C. The data presented is representative of three independent experiments. Error bars in panels A and B indicate the standard deviation (*, $p < 0.01$, unpaired T test).

Fig. 3. Effect of compound 3-A18NI on *V. cholerae* colonization of the suckling mouse intestine
A. Strain C7258 (El Tor biotype); **B.** strain O395 (classical biotype). Each data point represents the bacterial load of one mouse. The line through each data set indicates the geometric mean. The increased bacterial recovery from the mice population infected in the presence of 3-A18NI (25 μM) was statistically significant in both biotypes ($p < 0.01$, unpaired T-test).