Treatment with Entinostat heals experimental cholera by affecting physical and chemical
barrier functions of intestinal epithelia

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\textbf{Running Title:} Entinostat in treating experimental cholera

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Abstract

We have previously shown that oral treatment of an experimental model of shigellosis with sodium butyrate or phenylbutyrate improves clinical outcome and induces the expression of the antimicrobial peptide CAP-18 in the large intestinal epithelia. In a subsequent study, we have found that Entinostat, an aroylated phenylenediamine compound has similar therapeutic potential against shigellosis. Here, we aim to evaluate Entinostat as a potential candidate for host-directed therapy against cholera in an experimental model. *Vibrio cholerae*-infected rabbits were treated with two different dose regimen of Entinostat i.e. 0.5 mg twice daily for 2 days and 1 mg once daily for 2 days. Treatment effect on clinical outcomes and shedding of *V. cholerae* (CFU count in stool) was observed. Immunohistochemical analysis was carried out to assess CAP-18 expression in ileal and jejunal mucosa. Serum zonulin level was measured by ELISA to evaluate gut permeability. Infection of rabbits with *V. cholerae* down-regulated CAP-18 expression in the ileal epithelium; the expression was replenished by oral treatment with Entinostat at both dose regimens. Level of serum zonulin, a marker of gut permeability was up-regulated after infection, which was counteracted after treatment with Entinostat. Entinostat treatment also led to recovery from cholera and decline in *V. cholerae* count in stool. In conclusion, improved clinical outcome of treatment with Entinostat in cholera is associated with induction of CAP-18 and reduction of gut epithelial permeability.
Introduction

Cholera, an acute secretory diarrhea, caused by infection of the small intestine with the bacterium *Vibrio cholerae* remains one of the major public health concerns in many parts of the world. According to World Health Organization, a total of 172,454 cholera cases with 1304 deaths were reported in 42 countries in 2015, resulting in case fatality rate of 0.8% (1). After entering into the host with contaminated food and water, *V. cholerae* colonizes the small intestine and releases cholera enterotoxin (CT). By activating adenylate cyclase, CT induces the production of cyclic adenosine monophosphate, which in turn signals the opening of chloride ion channels and a subsequent net loss of salt and water from the intestine (2, 3). Loss of water and electrolytes quickly leads to severe dehydration and death if timely treatment is not given. *V. cholerae* secretes additional toxins e.g. zonula occludens toxin, and haemagglutinin/protease that increase the paracellular permeability of epithelial barrier and thus add to the CT-mediated fluid secretion (4, 5).

Patients with mild cholera are managed with compensation of fluid loss through oral or intravenous rehydration therapy, but for patients with moderate to severe cholera, additional treatment with antibiotic is recommended (6). Continued emergence of multidrug-resistant bacteria and adverse effects of antibiotic treatment e.g. disruption of the normal flora, allowing secondary infection and release of toxic microbial components limit the use of antibiotics for the management of infectious diseases (7, 8). Therefore, development of novel antimicrobial therapies is warranted.

Antimicrobial peptides (AMPs) are effectors of the innate immune system, limiting growth and virulence of pathogens in the host-microbe interface (9). Cathelicidins and defensins are two major classes of AMPs in mammals having wide range of antimicrobial activity.
However, several pathogens have evolved strategies to suppress the expression of these AMPs and thereby constitute an immune escape mechanism. Our group previously demonstrated that expression of the sole human cathelicidin LL-37 and human beta defensin (HBD)-1is down-regulated in intestinal epithelial cells of patients, during acute shigellosis and watery diarrhea (10). Besides, in polarized human intestinal cells, *Shigella flexneri* suppressed LL-37 and HBD-3 (11). In an experimental model of enteropathogenic *E. coli* (EPEC)-induced diarrhea, we showed downregulation of CAP-18, a rabbit homologue of LL-37 in intestinal epithelia (12). *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC) were shown to suppress CAP-18 and mouse cathelicidin mCRAMP in ileal mucosa of mouse and rabbit, respectively (13). The suppression was mediated by CT and heat labile toxin (LT) of *V. cholerae* and ETEC, respectively (13). Downregulation of LL-37 in duodenal epithelium was confirmed in patients with cholera and ETEC diarrhea (14). A novel therapeutic approach would be to compensate the loss of AMPs by inducing the production at the local sites of infection. Indeed, we have shown that treatment of *Shigella*- or EPEC-infected rabbits with sodium butyrate and/or its analogue phenylbutyrate induces CAP-18 in the intestinal epithelia, which is associated with improved clinicopathological features (12, 15, 16). We also conducted a clinical trial in *Shigella*-infected patients, where use of butyrate simultaneously enhanced human cathelicidin LL-37 and reduced the local inflammation of the large intestine (17). However, we did not observe therapeutic effect of butyrate or phenylbutyrate in an experimental model of cholera (Raqib R unpublished data).

For large scale screening of novel inducers of LL-37 expression, a reporter cell line (MN8CampLuc) producing a luciferase-LL-37 fusion protein has recently been constructed (18). Using MN8CampLuc, we have discovered a novel class of compounds, aroylated phenylenediamines (APDs) as potent inducer of LL-37 (19). Entinostat was the stronger inducer
of this class and its treatment potential was subsequently tested in a rabbit model of shigellosis.
We observed that oral administration of Entinostat counteracted the downregulation of CAP-18,
which correlated with clinical recovery from shigellosis (19).

Built on these findings, in the present study, we aimed to evaluate the efficiency of
Entinostat in treating acute secretory diarrhea caused by *V. cholerae* infection in a rabbit model.
We investigated whether 2 dose regimens of Entinostat, i.e., 0.5 mg twice a day for 2 days and 1
mg once a day for 2 days can lead to recovery from cholera symptoms, reduce *V. cholerae* count
in stool and upregulate CAP-18 expression in ileal and jejunal epithelia. Additionally, the impact
of Entinostat treatment on gut epithelial barrier function was explored.
Materials and methods

Ethics Statement

The study was approved by the Animal Experimentation Ethics Committee of icddr,b. All experiments conformed to the rules and guidelines of icddr,b, which was developed based on the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

Bacteria

Vibrio cholerae 01 Ogawa was isolated from stool of a cholera patient; the isolate was a kind gift from ‘Clinical Microbiology and Immunology Laboratory’ of icddr,b. The virulence of the clinical isolate was confirmed by rabbit ileal loop assay; ileal segments infected with this isolate resulted in pronounced accumulation of fluid.

Bacteria were maintained in nutritive soft agar (T1N1, 0.75% agar, mineral oil) at room temperature for further use.

Preparation of infectious inoculums

Vibrio cholerae 01 Ogawa from stock culture was streaked on a Taurocholate-Tellurite-Gelatin Agar (TTGA) plates (icddrb media facility) to obtain individual colonies. Two-three isolated colonies were taken from plate in culture flask containing ~20 ml Tryptic soya broth (TSB; Becton Dickinson, NJ, USA) and grown to exponential phase by incubating at 37 °C for 2 hours in a shaker incubator. Bacterial cells were then pelleted by centrifugation at 7000 rpm for 10 min. After 2X wash with sterile normal saline, the concentration of bacteria was adjusted to an
OD$_{595}$ of 0.2, which corresponds to infectious dose of $\sim 1 \times 10^8$ CFU/ml. Two fold dilution was made to prepare the infectious dose of $\sim 5 \times 10^7$ CFU/ml for each rabbit.

The Reversible Intestinal Tie Adult Rabbit Diarrhea (RITARD) model of infection and treatment procedure

Previously established RITARD model for *V. cholerae* and ETEC was adopted with slight modification (20). Briefly, Inbred New Zealand white rabbits (Charles River Laboratories, Wilmington, MA, USA) of either sex, aged between 2.5–3 months and weighing 1.7–1.9 kg were maintained in the animal resource facilities of icddr,b. Healthy rabbits free of enteric pathogens e.g., *Salmonella, Shigella, V.cholerae* and *Coccidia* were starved for 24 hour before inoculation with *V. cholerae*. On the day of inoculation (day 0) rabbits were anesthetized intravenously with 33 mg/kg body weight of sodium pentobarbital (Sigma-Aldrich, MO, USA). The cecum was brought out through a mid-line incision and ligated close to the ileocecal junction. The small intestine was brought out and a slip knot was tied around it close to the mesoappendix. Thereafter, 1 ml of *V. cholerae* Ogawa suspension containing $\sim 5 \times 10^7$ CFU in sterile normal saline was injected into the lumen of the anterior jejunum. The intestine and cecum were returned to the peritoneal cavity, and the incision was closed. The rabbit was kept in a special holding box, and the temporary tie was removed 2 hour after bacterial challenge. The unclosed portion of the skin incision was sutured and the animal was returned to its cage and provided with food and water *ad libitum*.

After the development of diarrheal symptoms (usually within 24 hour of inoculation, day 1), infected rabbits were treated with 0.5 mg Entinostat (LC laboratories, Woburn, MA, USA) twice a day for 2 days (n=5) or 1 mg Entinostat once a day for 2 days (n=5) or left untreated (n =
Entinostat was given in 7-ml of normal saline via sterile orogastric feeding tube. Treatment regime was started on day 1 afternoon and completed on day 3. All treated rabbits were sacrificed on day 4 with an overdose of intravenous sodium pentobarbital (66 mg/kg body weight). Infected but untreated rabbits were sacrificed within 4 hours after development of diarrhea to relieve them from suffering as well as to collect biopsies before they die. Figure 1 depicts the course of inoculation and treatment as well as when the rabbits were sacrificed. Additional five rabbits were taken as healthy controls.

**Specimen collection**

Stool specimens from infected untreated rabbits were collected once after development of diarrhea. From rabbits of the treatment group, stool samples were collected once on day 1 before start of the treatment, and once/twice daily during and after treatment (days 2-4) based on availability. Blood was collected by puncturing the heart immediately after the rabbits were sacrificed. The abdomen of each sacrificed rabbit was opened and tissue samples from ileum and jejunum were collected in 10% buffered formalin for immunohistochemical evaluations. Stool, blood and autopsy samples were also collected from healthy rabbits.

**Vibrio cholerae count in stool**

Number of *V. Cholerae* was counted in stools collected during specified time-point (day 1-4). Stool (100 mg) was mixed well with normal saline to prepare 5 ml suspension, followed by 10 fold serial dilutions up to $10^4$ dilution. Hundred µl of $10^2/10^3/10^4$ dilutions were cultured (spread plate method) on TTGA plates; plating was done in duplicates. Colonies were counted after an overnight incubation at 37 °C, Only plates with 30 - 300 colonies were counted as a rule.
Counts of duplicate plates were averaged and number of colony forming units (CFU) per gram of stool was calculated as below:

\[
\text{CFU/ml of diluted suspension} = \frac{\text{CFU}}{100 \ \mu l \text{ of diluted suspension}} \times 10 \quad [A]
\]

\[
\text{CFU/ml of original suspension} = A \times \text{dilution factor} \quad [B]
\]

\[
\text{CFU/100 mg of stool} = \frac{\text{CFU}}{5 \ \text{ml of original suspension}} = B \times 5 \quad [C]
\]

\[
\text{CFU/g of stool} = C \times 10
\]

When stool was collected twice a day, average count of the two time points was used.

**Antimicrobial peptide CAP-18 and the corresponding specific antibody**

Synthetic CAP-18 peptide (GLRKLRLKFRNKLKEKLLKIGIQGKLAPRTDY) (Innovagen, Lund, Sweden) is the rabbit homologue of the human cathelicidin LL-37. Affinity purified polyclonal chicken antiserum against CAP-18 (Innovagen) recognizes both the proform and the mature CAP-18 peptide. The proform/mature CAP-18 peptide has been designated as CAP-18 protein/peptide in this study.

**In situ Immunohistochemical staining for detection of CAP-18**

Formalin fixed tissue pieces of ileum and jejunum were embedded in paraffin and cut into 3 micron thick sections by utilizing a microtome (Thermo Scientific, Walldorf, Germany). Tissue sections were deparaffinised, microwave-treated in retrieval buffer (Dako, Glostrup, Denmark) for antigen retrieval, followed by quenching of endogenous peroxidase activity by hydrogen peroxide. Sections were then incubated subsequently with CAP-18 antibody (0.5 µg/mL), biotinylated goat anti-chicken IgG (H+L) (Vector Laboratories Inc., CA, USA), and avidin-biotin complex conjugated with peroxidise enzyme (Vector Laboratories Inc.). Colour reaction
was developed by adding hydrogen-peroxide as substrate and 3, 3′-diaminobenzidine (DAB; Sigma-Aldrich) as chromogen; brown colour represents positive staining of CAP-18. Sections were counterstained with hematoxyline (Fisher Scientific, New Jersey, USA) resulting in blue nuclear stain, dehydrated and mounted with permanent mounting medium (Vector Laboratories Inc.).

Quantification of CAP-18 peptide/protein expression in ileal/jejunal mucosa of rabbits

CAP-18 expression in ileal and jejunal tissue was analyzed by using a microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the image analysis system Quantimate Q550 (Leica). CAP-18 staining was quantified in the epithelial area of the whole tissue section. The results were given as ACIA (Acquired Computerized Image Analysis) score, i.e., total positively stained area x total mean intensity (1–256 levels per pixel) of the positive area divided by total cell area (16).

Quantitative determination of zonulin in serum by ELISA

Serum was separated from blood, aliquoted and stored at -80 °C. Zonulin, a marker of gut permeability (21) was quantified in stored serum by using commercially available Rabbit Zonulin (ZON) ELISA kit (MyBioSource, San Diego, CA, USA). The kit applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-ZON antibody and ZON-HRP conjugate.

In Vitro bacterial killing

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Vibrio cholerae 01 Ogawa isolate was grown to exponential phase as described in subsection “Preparation of infectious inoculums”. Different concentrations of synthetic CAP-18 peptide (3.0, 3.5, 4.0, 4.5, 5.0 µg/ml) or Entinostat (0.25, 0.5, 1.0 mg/ml) or combination of both were added to the bacterial suspension (~2 x10⁴ CFU/ml) in Mueller-Hinton broth (MHB; Becton Dickinson, NJ, USA) in wells of microtiter plate (Thermo Fisher Scientific, Roskiide, Denmark) in a final volume of 200 µl. Control wells contained MHB alone (negative control) and bacteria in MHB without CAP-18 or Entinostat (positive control). The plate was incubated at 37°C in a shaker incubator for 2 hours or overnight. Bacterial suspension/media from individual wells were plated on TTGA and CFU were counted after overnight incubation at 37 °C.

Evaluation of toxic effect of Entinostat treatment

For safety evaluation, serum levels of hepatic enzymes e.g. alanine transaminase (ALT), aspartate transaminase (AST) and γ-glutamyl transferase (GGT) and renal markers e.g creatinine and Blood Urea Nitrogen (BUN) in infected and Entinostat treated rabbits were assessed in Clinical Chemistry Analyzer Cobas C311 (Roche Diagnostics GmbH, Mannheim, Germany) and compared to that in untreated healthy and infected rabbits.

Statistical Analyses

Statistical analyses were performed by using IBM SPSS Statistics 20.0. Data are expressed as mean ± standard error of mean (SEM). Data that were not normally distributed and/or failed the equal variance test were log transformed. Difference in outcomes between different groups was estimated by the mixed model ANOVA. Least significant difference (LSD) was used for
multiple comparison test of means of outcomes across the different group. Probabilities were regarded as significant when 
p < 0.05.
Results

Improvement of clinical outcome of cholera with oral Entinostat treatment

Infected rabbits (n=13) developed mild to moderate cholera within 24 hours of infection (on day 1) as apparent by liquid stool mixed with soft stool pellet. One rabbit developed severe watery diarrhea and died within approximately 16 hour of infection; this rabbit was considered as untreated rabbit. Autopsy and stool specimens were collected from this rabbit, but blood could not be collected. One rabbit exhibited diarrheal symptoms on day 2 and treatment was not given to this rabbit. It is worth mentioning that this rabbit did not defecate on day 1. All rabbits became anorexic, lethargic, lost body weight to some extent and a few rabbits (n=4) developed transient fever. Untreated rabbits were sacrificed within 4 hours after development of diarrhea on day 1 (n=3) or day 2 (n=1). In previous studies, rabbits challenged with *V. cholerae* died within 18-25 hours post-challenge (20, 22). Therefore, in the present study no risk was taken to keep them alive as they were weak and might die at night and biopsy collection might be missed from these rabbits. Treatment with Entinostat for 2 days (0.5 mg twice daily or 1 mg once daily) led all infected rabbits to recover from the disease; formed stool reappeared and rabbits revived from lethargy and anorexia. However, body weight remained similar.

Entinostat treatment reduces *Vibrio cholerae* count in the intestine

To investigate if recovery from cholera after Entinostat treatment is linked to the number of pathogens in the gut, shedding of *V. cholerae* in stool was measured over time. Oral treatment of infected rabbits with both dose regimens of Entinostat led to gradual decrease of *V. cholerae* count in stool (Figure 2). On day 4, numbers of bacteria were 3-4 logs lower than that on day 1, i.e, before the start of the treatment.
Downregulation of CAP18 in ileum by *V. cholerae* infection, restored by Entinostat

Immunostaining of ileal and jejunal mucosa of healthy rabbits with CAP-18 specific antibody revealed localization of CAP-18 peptide/protein in the surface epithelia (SE) of the villi (Figure 3A, panel 1). On semi-quantitative image analysis, a significant downregulation of CAP-18 expression was noticed in the SE of the ileum villi after infection with *V. cholerae* in comparison to healthy rabbits (p< 0.001) (Figure 3B). Downregulation of CAP-18 in ileal SE of the infected rabbits was accompanied by partial shedding of the epithelial cells (Figure 3A, panel 2). After treatment of infected rabbits with both dose regimens of Entinostat, CAP-18 expression was enhanced significantly (p= 0.001 for 0.5 mg dose regimen; p= 0.02 for 1 mg dose regimen) (Figure 3B); eroded epithelium also healed after treatment (Figure 3A, panel 3 & 4). In jejunal epithelia, there was no significant change in CAP-18 expression after infection and treatment compared to healthy rabbits.

Entinostat restores epithelial barrier integrity

Increased paracellular permeability of intestinal epithelia may contribute to fluid loss in cholera. Therefore, the effect of *V. cholerae* infection as well as Entinostat treatment on gut permeability was studied in the current experimental model. For this purpose, the level of circulatory Zonulin was measured, which has been identified as a marker of gut permeability in several diseases (21, 23). In infected untreated rabbits, zonulin concentration in serum was significantly higher compared to healthy controls (p= 0.024). Treatment of infected rabbits with Entinostat at both dose regimens resulted in significant decrease of serum zonulin level (p= 0.005 for 0.5 mg dose regimen; p= 0.002 for 1 mg dose regimen) (Figure 4).
In vitro antibacterial activity of Entinostat and CAP-18

To explore if the reduction of *V. cholerae* in stool after treatment is due to the direct inhibitory effect of Entinostat or due to increased CAP-18 level in ileal epithelium, the antibacterial activity of Entinostat or synthetic CAP-18 peptide against *V. cholerae* was assessed in an *in vitro* experiment. Entinostat at a concentration range of 0.25-1.0 mg/ml did not reduce bacterial count when incubated for 2 hours (Figure 5A) or overnight. After 2 hours of incubation, CAP-18 at concentration of 3.0, 3.5, 4.0, 4.5 and 5.0 µg/ml exhibited a dose-dependent vibriocidal effect and a complete clearance of *V. cholerae* was observed with 5 µg/ml concentration (Figure 5B). After overnight incubation, killing of *V. cholerae* by CAP-18 was not dose-specific. In some experiments, 4 µg/ml of CAP-18 killed all bacteria; in others, there was no significant reduction of bacterial count up to concentration of 4.5 µg/ml, but complete killing occurred with 5 µg/ml CAP-18. It is possible that after initial dose-dependent reduction remaining viable bacteria grew overnight to a saturation level in the growth media, leading to similar count at different doses of CAP-18. When used in combination for 2 hours’ Entinostat (1.0 mg/ml) did not display any additive/synergistic effect on CAP-18 mediated killing.

Entinostat has no harmful effect on renal and hepatic functions of rabbits

To exclude any toxic effect of the Entinostat treatment on renal and hepatic functions, concentrations of renal biomarkers (creatinine and BUN) and hepatic enzymes (ALT, AST and GGT) were measured in serum. No significant differences were observed in the serum levels of these biomarkers between healthy untreated rabbits, infected untreated rabbits and infected
rabbits treated with Entinostat (Table 1), indicating that Entinostat was safe for rabbits at the given doses.
Discussion

In an experimental rabbit model, we have demonstrated that oral treatment with Entinostat led to clinical recovery from cholera. Improved clinical outcome was accompanied with a marked reduction of *V. cholerae* count in the stool and restoration of epithelial barrier integrity. *V. cholerae* infection resulted in significant down-regulation of the cathelicidin CAP-18 in the ileal epithelium, which was counteracted by Entinostat therapy.

Downregulation of cathelicidin family of AMP in intestinal epithelial cells by *V. cholerae* as shown previously in rabbit and mouse ileum (13) and in patients’ duodenum (14) has been reproduced here in a RITARD model. The experimental set-up has been established in order to test the treatment approach of compensating the loss of AMPs at the local sites of infection.

In the present study, downregulated CAP-18 in ileal epithelium during *V. cholerae* was restored after treatment with the two different dose regimens of Entinostat (0.5 mg twice daily or 1 mg once daily for 2 days). Importantly, infected rabbits revived from disease symptoms after the treatment; treated rabbits were no more lethargic and anorexic and passed normal stool. Recovery from disease symptoms was accompanied by a reduced number of *V. cholerae* in the gut lumen. Hydrophobic degeneration and subsequent shedding of dead epithelial cells by cholerogenic *V. cholerae* (24) was replaced with new cells after the treatment. Induced CAP-18 could be involved, at least in part in re-epithelialization of intestinal mucosa as shown previously for LL-37 in human skin wounds (25).

In an *in vitro* antibacterial assay, synthetic CAP-18 peptide in a dose dependent manner killed the *V. cholerae* Ogawa isolate that was used to infect the rabbits. Entinostat neither exhibited vibriocidal effect, nor did enhance the killing capacity of the CAP-18 peptide. These findings indicate that Entinostat has no direct involvement in eliminating *V. cholerae* from the
gut lumen, but contributes indirectly by inducing CAP-18 and possibly additional AMPs in intestinal epithelia.

Enhanced acetylation of histones by histone deacetylase inhibitors (HDACi) e.g. butyrate, phenylbutyrate and trichostatin A (TSA) has been proposed to mediate transcriptional induction of LL-37 (26-28). A panel of HDACi that are analogues of butyrate or phenylbutyrate also induced LL-37 in the MN8CampLuc reporter cell line (18). Entinostat is a benzamide histone deacetylase inhibitor and hence this activity could be included in the induction of LL-37. However, the much higher induction by Entinostat and other APDs, compared to more potent HDAC inhibitors (e.g., Vorinostat and Trichostatin A) suggest that HDAC inhibition cannot be the major route (19). Other mechanisms are likely to be responsible for the efficient induction with APDs. One major route seems to be activation of STAT3 which in turn induces HIF-1 that subsequently increases the expression of LL-37 and HBD-1 (29).

The intestinal epithelium forms a physical barrier between gut lumen and underlying tissue compartments, allowing absorption of nutrients and selective passage of molecules, while preventing entry of noxious luminal contents. Paracellular permeability of this barrier is tightly regulated by apical junction complex consisting of tight junction, adherence junction and desmosome (30). Zonula occludens toxin and haemagglutinin/protease produced by *V. cholerae* were shown to modify tight junction and thus increase the paracellular permeability of the epithelial barrier (4, 5, 31, 32). Interestingly, the production of Zonula occludens toxin correlated with diarrheagenicity of *V. cholerae* strains in volunteers (4). Zonulin, a mammalian protein was identified as the analogue of Zonula occludens toxin and till date is the only known physiologic modulator of intercellular tight junction (21, 33). Circulating zonulin level has been established as the marker of gut permeability in several diseases (21, 23). Here, we demonstrated increased
concentration of zonulin in serum of rabbits infected with *V. cholerae*. After treatment with both doses of Entinostat, zonulin levels decreased to the level of healthy controls, indicating the reversal of gut permeability. However, due to the lack of a rabbit specific antibody, we could not detect the expression of tight junction proteins in the intestinal tissue and hence could not identify the tight junction modification after infection and treatment. Interestingly, butyrate and other HDACi have been shown to maintain epithelial barrier integrity by altering tight junction permeability (34-36). It is plausible that Entinostat through HDAC inhibition can recover *V. cholerae*-induced disruption of intestinal tight junction and consequently decrease gut permeability. On the other hand, recent studies have reported emerging roles of host defense peptides in intestinal barrier function by directly regulating mucin and tight junction protein expression (37). Cathelicidin-BF, a peptide purified from the snake venom of *Bungarus fasciatus* has been shown to ameliorate LPS-induced epithelial barrier disruption of rat epithelium via prevention of the down-regulation of tight junction proteins (38). Therefore, it is quite possible that similar modification of tight junction proteins by Entinostat-induced CAP-18 may contribute to re-establishment of the epithelial barrier integrity.

Entinostat at a concentration of 6-8 mg/ m² was reported to be safe and well tolerated in phase I clinical trials in patients with myeloid leukemia, solid tumors and lymphomas (39-41). Entinostat in combination with other drugs have also been found safe and efficacious in phase II trials (42, 43). In rat liver, no significant histopathological changes were shown after administration of up to 49 mg/kg Entinostat for 7 days (44). In the current study, we used much lower concentration of Entinostat to treat infected rabbits based on strong *in vitro* LL-37 inducing capacity of Entinostat. Expectedly, no toxic effect was observed in rabbits treated with Entinostat.
In conclusion, we have demonstrated that Entinostat can be used in treating watery diarrhea caused by *V. cholerae* through its ability to induce epithelial barrier integrity and innate defenses. Induction of AMPs suggest that Entinostat and other APDs are possible candidates against infectious diseases in general, where pathogens down-regulate the mucosal expression of AMPs as a means of evading host immunity as shown, for example, in shigellosis and cholera.


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Figure legends

Figure 1: Schematic diagram showing the infection and treatment schedule of the rabbits as well as when they were sacrificed.

Figure 2: Effect of Entinostat treatment on shedding of *Vibrio cholerae* in stool. Stool samples were collected from *V. cholerae*-infected rabbits treated with Entinostat at doses of 0.5 mg bds for 2 days (n=5) or 1 mg qd for 2 days (n=5) or not given any treatment (n=5). From untreated rabbits, stools were collected once before sacrifice. From treated rabbits, stools were collected before the start of the treatment (day 1) and during or after the treatment (Days 2-4). Serially diluted stool suspensions were plated onto TTGA plates and colonies were counted after an overnight incubation at 37 °C. The results were expressed as CFU per gram of stool. Data are given as mean ± SEM. TTGA: Taurocholate-Tellurite-Gelatin Agar, CFU: colony forming unit; SEM: Standard error of mean; bds: twice daily; qd: once a day.

Figure 3. CAP-18 peptide/protein expression in ileal mucosa of healthy, *Vibrio cholerae*-infected, infected and Entinostat treated rabbits. Mucosal sections of ileum from healthy rabbits (n=5), *V. cholerae*-infected rabbits (n=5), infected rabbits treated with 0.5 mg Entinostat bds for 2 days (n=5) or with 1 mg Entinostat qd for 2 days (n=5) were stained with CAP-18 specific antibody. (A) Representative photomicrographs are shown. CAP-18 positive staining is brown in color; cell nuclei are stained blue with hematoxylin. Prominent expression of CAP-18 (thick arrows) in the SE of healthy ileal mucosa (Panel 1, top left). Downregulation of CAP-18 expression in the SE after *V. cholerae* infection; partial erosion of SE (arrowheads) was also noted (Panel 2, top right). Recovery of epithelial erosion and restoration of CAP-18 expression...
Figure 4: Serum zonulin level in healthy, *Vibrio cholerae*-infected, infected and Entinostat-treated rabbits. Serum was collected from healthy rabbits (n = 5), *V. cholerae*-infected untreated rabbits (n = 4), infected rabbits treated with 0.5 mg Entinostat bds for 2 days (n = 5) or with 1 mg Entinostat qd for 2 days (n = 5). Serum level of zonulin, a marker of gut permeability was quantified by using commercially available Rabbit Zonulin ELISA kit. Data are given as mean ± SEM. Difference in outcomes between different groups was estimated by the mixed model ANOVA. The differences are significant when p<0.05. VC: *Vibrio cholerae*; SEM: Standard error of mean; bds: twice daily; qd: once a day.

Figure 5. *In vitro* inhibitory effects of Entinostat or CAP-18 on the growth of *Vibrio cholerae*. Bacterial suspension of *V. cholerae* Ogawa was incubated with different concentration of (A) Entinostat or (B) synthetic CAP-18 in MHB in wells of microtiter plate at 37 °C for 2 hours. Bacterial suspensions from individual wells were plated on TTGA and CFU were counted after overnight incubation. Data are given as mean ± SEM of 2 and 4 individual experiments for...
Entinostat and CAP-18, respectively. MHB: Muller Hinton Broth, TTGA: Taurocholate-
Tellurite-Gelatin Agar, CFU: colony forming unit; SEM: Standard error of mean.
Table 1: Levels of hepatic and renal biomarkers in serum of healthy, *Vibrio cholerae*-infected and infected rabbits treated with Entinostat.

<table>
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<tr>
<th>Hepatic enzymes and renal markers</th>
<th>Healthy (n=5)</th>
<th>Infected (n=4)</th>
<th>Treated with 0.5 mg Entinostat bds for 2 days (n=5)</th>
<th>Treated with 1 mg Entinostat qd for 2 days (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>55.9 ± 6.2</td>
<td>62.02 ± 16.2</td>
<td>39.91 ± 11.4</td>
<td>49.78 ± 8.2</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>66.8 ± 14.4</td>
<td>30.69 ± 6.1</td>
<td>30.18 ± 6.6</td>
<td>41.6 ± 14.8</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>10.6 ± 1.0</td>
<td>10.1 ± 3.2</td>
<td>11.25 ± 2.8</td>
<td>6.4 ± 1.2</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>105.6 ± 11.9</td>
<td>70.83 ± 16.3</td>
<td>63.42 ± 12.5</td>
<td>77.82 ± 2.3</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>4.08 ± 1.0</td>
<td>17.05 ± 5.8</td>
<td>19.3 ± 6.9</td>
<td>8.2 ± 1.4</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. ALT: alanine transaminase, AST: aspartate transaminase, GGT: γ-glutamyl transferase, BUN: Blood Urea Nitrogen; bds: twice daily; qd: once a day; SEM: standard error of mean.
Inoculation with *Vibrio cholerae* 01 Ogawa

Starvation for 24h

Day 0

Day 1

Day 2

Day 3

Day 4

0.5 mg Entinostat, 2nd and 3rd doses at around 9:00 AM and 4:30 PM, respectively

1.0 mg Entinostat, 2nd dose at around 2:00 PM

Treated rabbits sacrificed

Development of diarrhea

0.5 mg Entinostat 4th dose at around 9:00 AM

Untreated rabbits sacrificed

1st dose of Entinostat (both concentrations) at around 2:00 PM
Figure A: Bars showing the concentration of Entinostat (mg/ml) on the x-axis and the V. cholerae count (CFU/ml) on the y-axis.

Figure B: Bars showing the concentration of CAP-18 (µg/ml) on the x-axis and the V. cholerae count (CFU/ml) on the y-axis.