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A cyclohexadepsipeptide from entomogenous fungi *Metarhizium anisopliae* inhibits the *Helicobacter pylori* induced pathogenesis through attenuation of vacuolating cytotoxin-A activity

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ABSTRACT

Infection with *Helicobacter pylori* in the gastric mucosa is thought to be increased the risk of peptic ulcer disease and gastric cancer. Vacuolating cytotoxin A (VacA) was an important bacterial virulence factor and was closely associated with *H. pylori*-induced pathogenesis. Upon *H. pylori* infection, the VacA can be transferred directly from bacteria into the host cells where can stimulate vacuolar-type ATPase (V-ATPase) proton pump and induce the formation of vacuoles in gastric epithelial cells followed by intoxication of cells. Destruxin B (Dtx-B), a cyclodepsipeptide from the fungus *Metarhizium anisopliae* bearing insecticidal and anticancer effects. In this study, Dtx-B demonstrated inhibitory effects against *H. pylori* VacA-induced vacuolization in gastric epithelial cells. Our data further showed that attenuation of *H. pylori*-induced vacuolization in gastric epithelial cells by Dtx-B is mediated through inhibition of V-ATPase activity rather than VacA expression levels. The results from this study reveal a novel role for Dtx-B in the modulation of *H. pylori* VacA-induced pathogenesis.

1. Introduction

Gram-negative microaerophilic bacterium *Helicobacter pylori* is an important pathogen that colonizes in the stomach and infects approximately half of the human population in the world [1,2]. Persistent infection by *H. pylori* that is usually caused several types of gastrointestinal diseases, including chronic gastritis, peptic ulcer, and gastric adenocarcinoma [3,4]. Previous studies reported that a set of virulence factors that was involved in *H. pylori*-induced pathogenesis and contributed to enable bacterium to survive, multiply, protect from immune attack, and eventually lead to persistent infection in the host [5–7]. *H. pylori* contained two major bacterial cytotoxins: vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA), which were considered to be more virulent and that were closely associated with disease progression [8]. The VacA can be secreted from *H. pylori* in the culture supernatant, but remained a large amount on the bacterial cell wall [9]. Infection of *H. pylori*, the VacA can be transferred directly from bacteria into the host cells where can lead to intoxication of cells [9]. Previous studies indicated that *H. pylori* VacA-induced vacuoles were associated with rab7, which was a small GTPase and localized at the late endosomal compartments [10]. The intracellular compartments endowed with vacuolar-type ATPase (V-ATPase) which is a proton pump can be activated by VacA, resulting in increasing anionic permeability and lead to cell swelling [11,12]. Following two decades of the extensive research

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of VacA, this has been recognized as a multifunctional toxin [12,13]. Therefore, development of an inhibitor against VacA might be an ideal strategy for attenuation of *H. pylori*-related diseases.

Identification of natural constituents as alternative drugs against *H. pylori*-induced pathogenesis has been pursued by many pharmaceutical research groups. Destruxins (Dtx) isolated from the fungus *Metarhizium anisopliae* have been well characterized previously [14]. The broad activities bearing by Dtx have been known as insecticidal effect [15], immunosuppressive [16], and anti-tumoral activity in various types of cancer cells [17–19]. Destruxin B (Dtx-B), a cyclic cyclohexadepsipeptide, which has been shown to contain the inhibitory effect against V-ATPase activity [20]. Therefore, it is reasonable to investigate whether Dtx-B harboring the potent in the inhibition of *H. pylori* VacA-associated pathogenesis. In this study, the biological functions of Dtx-B against *H. pylori* VacA-actions were further explored. Our investigation indicates that Dtx-B attenuates *H. pylori*-induced vacuolization in gastric epithelial cells mediated through inhibition of V-ATPase activity rather than suppression of VacA levels.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies against V-ATPase and *H. pylori* VacA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 568-conjugated goat anti-rabbit IgG, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA). Bafilomycin A1 (Baf A1), neutral red, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals and reagents were of the highest grade commercially available and supplied either by Sigma–Aldrich or Merck (Whitehouse Station, NJ).

2.2. Production and purification of destruxin B (Dtx-B)

The compound destruxin B (Dtx-B) used in this study was obtained from the submerged cultivation of *M. anisopliae* F061 according to the production and purification processes as we described previously [18,19]. The purity of the Dtx-B was >98% by HPLC. Its structure (Fig. 1A) was confirmed by comparison of its mass and nuclear magnetic resonance spectral data with those in the literature [18,19].

2.3. H. pylori strains and bacterial culture

The *H. pylori* reference strain 26,695 (ATCC 700392), and *vacA* isogenic mutant (ΔVacA) strain were obtained as we described previously [21]. *H. pylori* strains were cultured on Brucella agar plates (Becton Dickinson, Franklin Lakes, NJ), supplemented with 10% sheep blood and contained vancomycin (6 μg/mL), amphotericin B (2 μg/mL). The bacterial strains were incubated under microaerophilic condition at 37 °C for 2–3 days prior to infection of epithelial cells [22].

2.4. Cell culture and cytotoxicity assay

The human gastric epithelial cell line, AGS cells, was purchased from the American Type Culture Collection (ATCC CRL 1739, Rockville, MD) and cultured in F12 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT) at 37 °C in 5% CO_2. The MTT assay was employed to determine the cytotoxicity of Dtx-B in AGS cells as described previously [23].

2.5. Neutral red uptake assay

H. pylori-induced vacuolization activity was analyzed by using neutral red uptake (NRU) assay [24]. AGS cells (2×10^4/well) were cultured in 96-well plates for 18 h and then added Dtx-B (0–1000 nM) or Baf A1 (10 nM) prior to infection with *H. pylori* at an MOI of 100 for 12 h. The culture medium was removed and the cells were stained with 0.05% (w/v) neutral red (Sigma–Aldrich). After staining for 10 min, the cells were washed and the neutral red was then extracted using acidified alcohol (1% of 12 N HCl in 75% alcohol) [25]. The level of NRU was measured at OD 570 nm by a Microplate Photometer (Thermo Scientific, Waltham, MA). The control was a population of AGS cells uninfected with *H. pylori* and was used to define 100% cellular vacuolization. The results were expressed as the percentage of relative vacuole formation by comparison with the control group.

2.6. Analysis of reversal of H. pylori-induced vacuolization by Dtx-B

AGS cells (1.5×10^5/well) were seeded in a 6-well plate for 18 h. The cells were then infected with *H. pylori* at an MOI of 100 for 8 h followed by treatment with penicillin (100 units/mL) and streptomycin (100 μg/mL) to inhibit the over-growth of bacteria. Dtx-B (1000 nM) was added at the indicated time points and evaluated the vacuolating activity continually for an additional 14 h. The cell vacuolization was determined from five-independent microphotographs per sample (×200 magnification with a phase-interference inverted microscope) by counting the number of cells having more than five vacuoles per cell [26].

2.7. Immunofluorescence labeling of cells

AGS cells (1.5×10^5/well) were seeded on coverslips in 6-well plates for 18 h. Cells were treated with or without Dtx-B (1000 nM) followed by infection with *H. pylori* at an MOI of 100 for 12 h. The treated cells were washed and then fixed with 4% paraformaldehyde (Sigma–Aldrich). To label VacA and V-ATPase, cells were incubated with anti-VacA and anti-V-ATPase antibodies (Santa Cruz Biotechnology) at 4 °C for 18 h, and then probed with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Invitrigen), respectively. Samples were washed and mounted with Fluoromount-G (Southern Biotechnology Assoc., Birmingham, AL) along with DAPI, and then analyzed under a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Göttingen, Germany) with a 63× oil immersion objective.

2.8. Western blot analysis

AGS cells (1.5×10^5/well) were cultured in 6-well plates for 18 h and then treated with or without Dtx-B (1000 nM) prior to infection with *H. pylori* at an MOI of 100 for 12 h. The cells were washed with phosphate-buffered saline (PBS) and lysed with RIPA (150 mM NaCl, 50 mM Tris base pH 7.4, and 1 mM EDTA, 1% NP-40, 0.25 mM deoxycholate) containing protease inhibitor (Roche, Indianapolis, IN). The samples were boiled in SDS-PAGE sample buffer for 10 min and then resolved by 12% SDS-PAGE followed by transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). The blots were incubated with antibodies against V-ATPase or β-actin at 4 °C for 18 h. The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore). The expression levels of V-ATPase or β-actin were detected using ECL Western blotting

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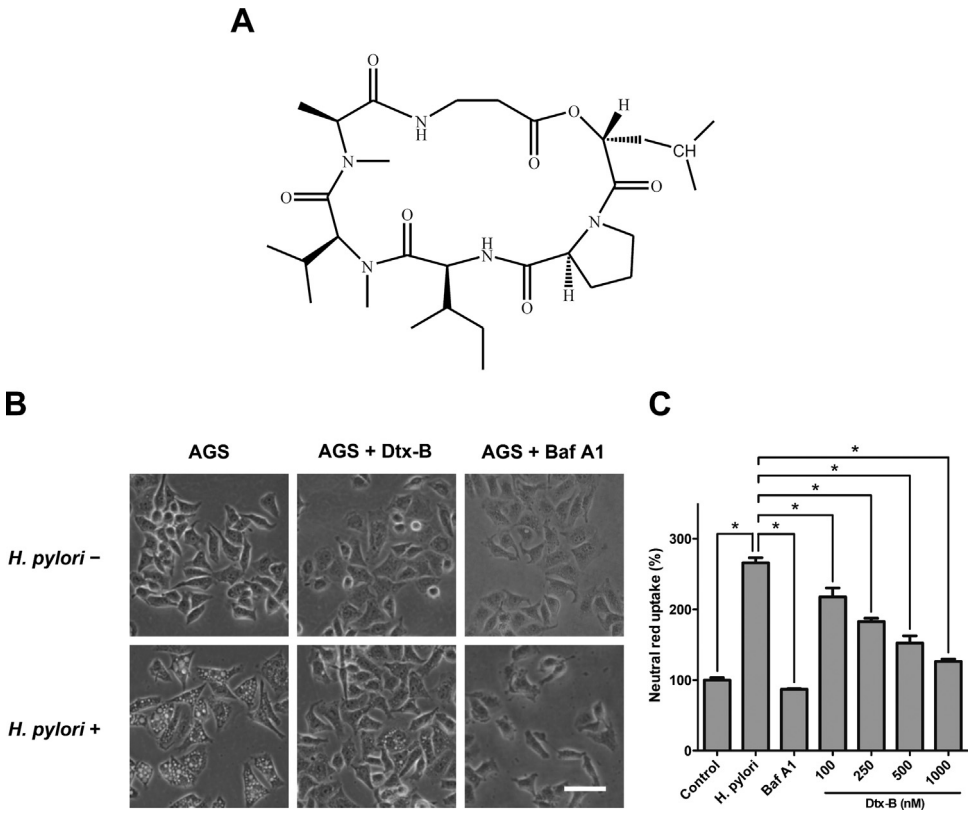

Fig. 1. (A) Chemical structures of destruxin B. (B) Attenuation of *H. pylori*-induced vacuolization by Dtx-B. AGS cells were treated with Dtx-B (0–1000 nM) or Baf A1 (10 nM) followed by infection with *H. pylori* at an MOI of 100 for 12 h. The vacuolization activity was observed using reverse optical microscope. Scale bar, 10 μm. (C) The effect of DtxB inhibits *H. pylori*-induced vacuolization was assessed using neutral red uptake assay. Statistical significance was evaluated using Student's *t*-test (*$P < 0.05$).

detection reagents (GE Healthcare, Piscataway, NJ) and visualized using X-ray film (Kodak, Rochester, NY).

2.9. Statistical analysis

The data are presented as mean ± standard deviation of triplicate experiments. The Student's *t*-test was performed to calculate the statistical significance of experimental results between two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Inhibition of H. pylori-induced vacuolization by Dtx-B

H. pylori vacuolating cytotoxin A (VacA) can stimulate V-ATPase proton pump and induce the formation of vacuoles in gastric epithelial cells [27]. Destruxin B (Dtx-B), one of destruxin constituents which harbor the inhibitory activity of V-ATPase [14]. In this study, we sought to investigate whether Dtx-B can serve as an inhibitor of V-ATPase and therefore prevent the *H. pylori* VacA-induced vacuolization of gastric epithelial cells. We first tested the influence of Dtx-B on the growth of gastric epithelial cells. AGS cells were untreated or treated with Dtx-B at the concentrations of 100−1000 nM for 24 h and assessed the cell viability using the MTT approach. As shown in Supplementary Fig. S1, at the maximum concentration (1000 nM) of the Dtx-B which exhibited barely any effects on AGS cell growth. In parallel, Dtx-B did not show bactericidal activity on the growth of wild-type and ΔVacA isogenic mutant *H. pylori* (Supplementary Fig. S2).

We then explored whether Dtx-B can inhibit *H. pylori*-induced vacuolization of gastric epithelial cells. AGS cells were untreated or treated with Dtx-B (1000 nM) followed by infection with *H. pylori* for 12 h. As shown in Fig. 1B, the cell vacuolization was dramatically inhibited by treatment with Dtx-B compared to that un-treatment control. In this study we used bafilomycin A1 (Baf A1) as a positive control which is reported as a specific inhibitor of V-ATPase, inhibit acidification and prevent the vacuole formation [28]. The neutral red uptake (NRU) assay was then employed for detection of cell vacuolization [25]. Our data showed that AGS cells infected with *H. pylori* accumulate significantly more dye than un-infected cells (Fig. 1C). In the treatment of cells with Baf A1 which exhibited great inhibition of *H. pylori*-induced vacuolization when compared to the control group. Moreover, treatment of cells with various concentrations of Dtx-B, the level of *H. pylori*-induced NRU was inhibited in a concentration-dependent manner. In contrast, the vacuolization was not observed in cells treated with ΔVacA isogenic *H. pylori* mutant (Supplementary Fig. S3). These results indicate that Dtx-B has comparable inhibitory effect with Baf A1 on *H. pylori*-induced vacuolization in gastric epithelial cells.

3.2. Attenuation of H. pylori-induced vacuolization by Dtx-B

Since Dtx-B was able to prevent the formation of vacuoles, we therefore investigated whether Dtx-B can rescue *H. pylori*-induced vacuolization. AGS cells were infected with *H. pylori* followed by treatment with Dtx-B (1000 nM) at the time points of 8 h and 12 h, respectively. As shown in Fig. 2A, Dtx-B effectively reversed *H. pylori*-induced vacuolization. Moreover, the cells can be rescued and recovered to be normal appearance by the culture contained Dtx-B up to 24 h.

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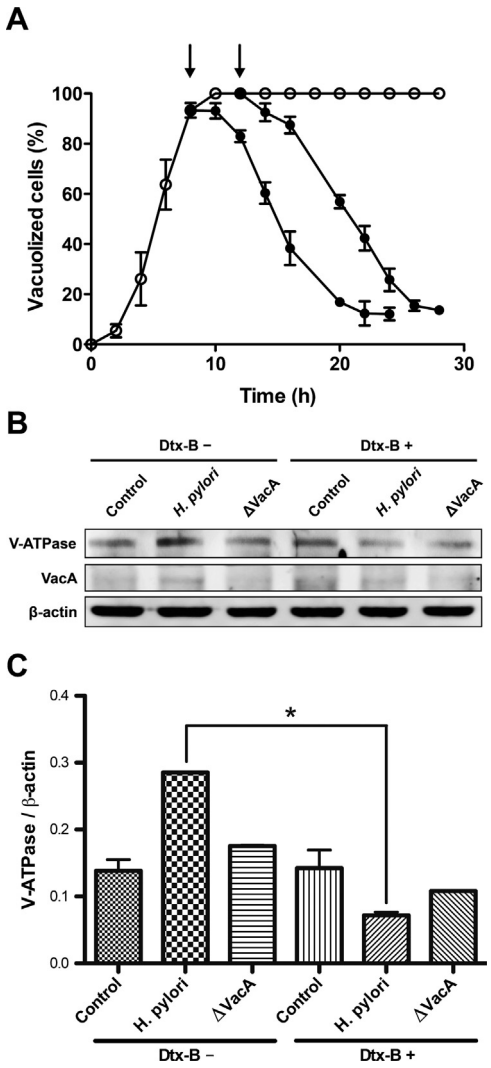

Fig. 2. (A) Reversion of *H. pylori*-induced vacuolization by Dtx-B. AGS cells were infected with *H. pylori* at an MOI of 100 for 8 h and then treated with penicillin (100 units/mL) and streptomycin (100 μg/mL) to inhibit the over-growth of bacteria. Cells were treated with Dtx-B (1000 nM) at the time points of 8 h and 12 h after *H. pylori*-infection of cells. The cell vacuolization was determined as described in Section 2. Open circle, cells were infected with *H. pylori* at the indicated time. Closed circle, *H. pylori*-infected cells were treated with Dtx-B at 8 h and 12 h, respectively (indicated by arrows). The data are the means and standard deviations of three independent experiments. (B) Inhibition of *H. pylori* VacA-induced V-ATPase activity in AGS cells. AGS cells were treated or untreated with Dtx-B (1000 nM) and infected with wild-type or ΔVacA isogenic mutant *H. pylori* strains for 12 h. Whole-cell lysates were analyzed the expression levels of V-ATPase, VacA, and β-actin. (C) The level of V-ATPase was evaluated by densitometric analysis. β-actin was detected to ensure equal loading. The values are means and standard deviations from 3-independent experiments (*$P < 0.05$).

3.3. Dtx-B inhibits V-ATPase activity rather than VacA levels

To understand the role of Dtx-B played in the inhibition of *H. pylori*-induced vacuolization, we next attempted to assess the expression levels of V-ATPase and *H. pylori* VacA. As shown in Fig. 2B, the expression levels of V-ATPase in AGS cells were increased after infection with wild-type strain rather than ΔVacA *H. pylori* strain. After treatment of cells with Dtx-B, the expression level of *H. pylori*-induced V-ATPase was dramatically reduced when compared to Dtx-B untreated group (Fig. 2B and C). However, the protein expression level of *H. pylori* VacA in cells treated with Dtx-B was similar to that in Dtx-B untreated group (Fig. 2B). We then directly observed whether Dtx-B inhibited *H. pylori*-induced V-ATPase using confocal microscopy. The fluorescence intensity signals showed that equal expression of VacA in the *H. pylori*-infected cells upon un-treated or treated with Dtx-B (Fig. 3). Without treatment of Dtx-B in *H. pylori*-infected AGS cells, more apparent V-ATPase was localized around the vacuoles in the cytoplasm. However, remarkably reduced the vacuoles and faint V-ATPase signal was observed in *H. pylori*-infected AGS cells upon treated with Dtx-B. Our data indicate that attenuation of *H. pylori*-induced vacuolization in gastric epithelial cells by Dtx-B might mediate via inhibition of V-ATPase activity rather than VacA expression levels.

4. Discussion

VacA, one of the most important virulence factors which secreted from *H. pylori*, has been extensively studied for over two decades. Infection of *H. pylori* strains bearing toxigenic *vacA* alleles that were highly associated with an increased risk of peptic ulcer disease and gastric adenocarcinoma [29]. Previous studies indicate that VacA has various biological functions on host and has been recognized as a multifunctional toxin [12]. Several characteristic activities were exerted by VacA including induction of cell vacuolization [10], inhibition of T cell proliferation [5], and induction of apoptosis [30]. The mechanism for VacA-actions in vacuolization, apoptosis, and inhibition of lymphocyte proliferation is relied on one event so-called as pore-forming activity which eventually leads to osmotic swelling and vacuolization of cells [12]. Considering the critical role of VacA in *H. pylori*-induced pathogenesis, development of a new strategy to target VacA was thought to be a potential therapeutic modality.

Dtx, cyclodepsipeptides, showed extensive spectrum of bioactive functions that included antifungal, anticancer, and immunosuppressant effects [14]. Several studies have been demonstrated that Dtx-B harbors insecticidal and antiviral effects [31,32]. However, the real actions of Dtx-B relying on anti-bacterial infection have never been explored. In the present study, we showed that Dtx-B has no role on *H. pylori* viability (Supplementary Fig. S1). Additionally, our study demonstrated that Dtx-B has inhibition effect on *H. pylori*-VacA functions. This study firstly demonstrated that Dtx-B attenuates VacA-induced vacuolization in *H. pylori*-infected cells (Fig. 1B and C). Moreover, presence of Dtx-B in the cultured cells for a longer time effectively reversed *H. pylori*-induced vacuolization (Fig. 1C). The molecular mechanism for Dtx-B attenuates *H. pylori*-induced vacuolization in gastric epithelial cells is through inhibition of V-ATPase activity by which the function was similar to Baf A1 [10,28]. It is worth noting that the expression level of VacA did not influent by Dtx-B treatment (Fig. 3), suggesting that the inhibition of cell vacuolization by Dtx-B neither mediated via prevention of VacA binding on cell surface nor internalization into cells (Fig. 4).

Baf A1, a specific inhibitor of V-ATPase, is able to inhibit the growth of human gastric cancer cells [33]. The molecular actions of Baf A1-induced apoptosis of cancer cells were included increasing lysosomal pH and activation of caspase-3 [33]. Additionally, a very recent study reported that treatment of gastric cancer with Baf A1, decreased the lysosomal chloride and therefore causes dysfunction of autophagy [34]. In the study of Dtx-B which harbored the same activity with Baf A1 that have been demonstrated bearing apoptosis effects on several types of cancers including lung cancer [18], colorectal cancer [35], and lymphoma [36]. Moreover, VacA and other virulence factors of *H. pylori* that were associated with increasing the risk of gastric cancer [1]. Our present study demonstrated that Dtx-B, similar to Baf A1, which is a potent inhibitor of V-ATPase and VacA-mediated vacuolization. Therefore, implying that inhibition

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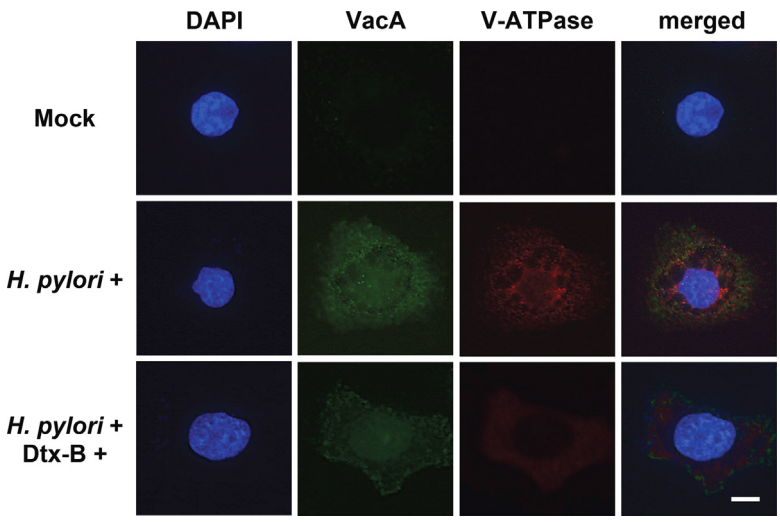

Fig. 3. Dtx-B inhibits *H. pylori* VacA activity is mediated via down-regulation of V-ATPase. AGS cells were treated or untreated with Dtx-B (1000 nM) followed by infection or un-infection with *H. pylori* for 12 h. The cells were fixed and stained with anti-VacA (green), anti-V-ATPase (red), and with DAPI (blue) to visualize cell nucleus. The samples were then observed by confocal fluorescence microscopy as described in Section 2. Region of VacA and V-ATPase co-localization appeared in yellow in the overlay. Scale bar, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

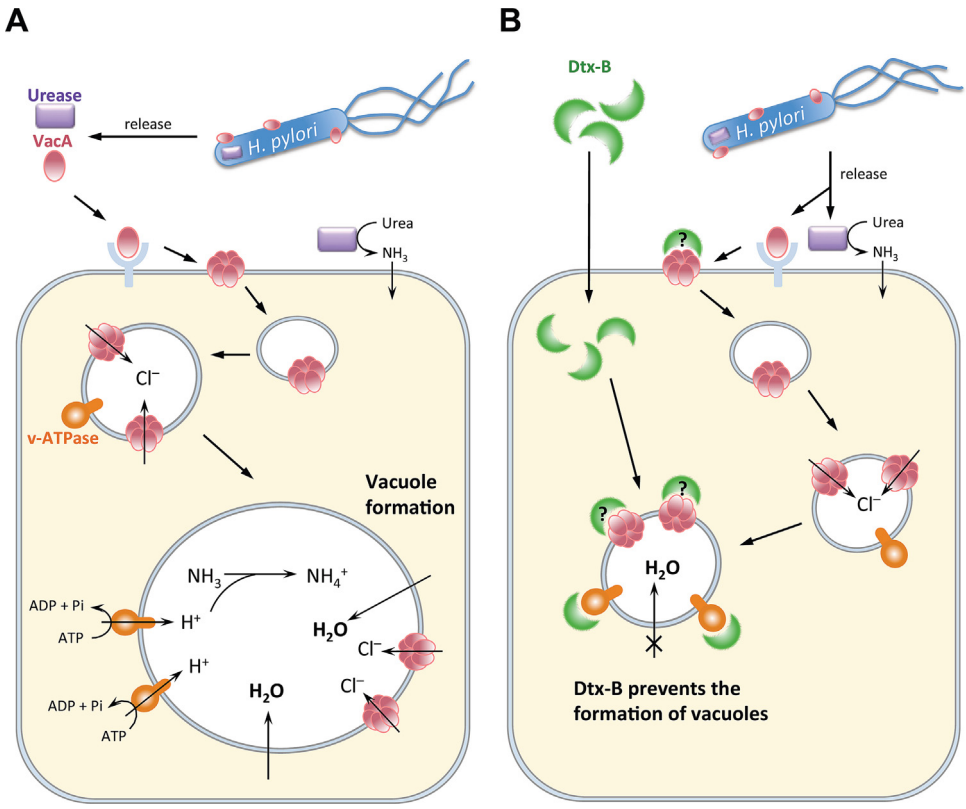

Fig. 4. Model depicting Dtx-B inhibits *H. pylori* VacA-induced vacuolization in gastric epithelial cells. (A) VacA secreted by *H. pylori* binds to receptors followed by delivery into the cytoplasm where can induce V-ATPase activation and enhance vacuole formation. (B) Dtx-B inhibits *H. pylori* VacA-induced vacuolization is mediated through inhibition of V-ATPase.

of VacA-actions by Dtx-B may modulate the development of gastric cancer induced by *H. pylori*.

In conclusion, we have demonstrated for the first time that a cyclodepsipeptide destruxin B from fungus *M. anisopliae* potently reverses *H. pylori* VacA-induced vacuolization of gastric epithelial cells. Furthermore, destruxin B also found has potent effects in inhibiting pathogenesis of cells through attenuation of VacA-elicited ATPase activity. Therefore, suggesting that destruxin B might serve as a potent compound for developing a complementary therapeutic drug in the prevention of *H. pylori*-associated diseases.

Conflicts of interest statement

The authors declare that there are no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.10.017.

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