Everolimus ameliorates Helicobacter pylori infection-induced inflammation in gastric epithelial cells

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ABSTRACT
Helicobacter pylori (H.pylori) infection caused by gastric mucosal inflammation plays a pivotal role in the progression of gastric diseases. The recruitment and attachment of monocytes to the gastric mucosal epithelium are a major event in the early stages of H. pylori-associated gastric diseases. Everolimus is a mechanistic/mammalian target of rapamycin (mTOR) inhibitor used to prevent tumor growth by inhibiting the PI3K signaling pathway. Here, we examined the pharmacological role of Everolimus against H.pylori-induced damage in gastric epithelial cells. Firstly, we found that Everolimus ameliorated H.pylori-induced oxidative stress by reducing reactive oxygen species (ROS) and malondialdehyde (MDA). Secondly, Everolimus significantly reduced the expressions of the pro-inflammatory cytokines interleukin (IL)-6, tumor necrosis factor-α (TNF-α), and IL-8. Moreover, it decreased the production of the pro-inflammatory chemokines C-X-C motif ligand 1 (CXCL1) and macrophage chemoattractant protein-1 (MCP-1). Importantly, Everolimus suppressed the induction of the adhesion molecule intracellular adhesion molecule-1 (ICAM-1) and the attachment of THP-1 monocytes to gastric epithelial AGS cells. Our data also shows that Everolimus inhibited the activation of the NF-kB signaling pathway. Therefore, we conclude that Everolimus could protect gastric epithelial cells by mitigating H.pylori-induced inflammatory response and the attachment of monocytes to epithelial cells.

Introduction
Helicobacter pylori (H. pylori) is a gram-negative pathogenic bacterium that has been designated as a major carcinogen of human gastric cancer [1–5]. It is reported that approximately 70% of registered gastric cancer patients suffer from gastric cancer caused by H. pylori infection [6]. Thus, finding promising therapy for gastric diseases associated with H. pylori infection has increasingly attracted the interest of scientists. H. pylori produces a large amount of lipopolysaccharide (LPS), which is unique to Gram-negative bacteria. LPS stimulates cells to produce pro-inflammatory mediators, inducing oxidative stress and the initiation of an inflammatory response via nuclear factor kappa-B (NF-kB). In addition, activation of gastric epithelial cells caused by H. pylori plays a key role in the initiation and development of chronic gastritis. H. pylori LPS interacts with toll-like receptor (TLR)-4 and triggers the expressions of pro-inflammatory mediators, such as TNF-α, IL-6, IL-8, CXCL1, and MCP-1 [7], as well as adhesion molecules including ICAM-1, leading to the attachment of monocytes to gastric epithelial cells. Oxidative stress has been involved in aberrant epithelial damages, inflammation, and death associated with H. pylori infection [8,9]. H. pylori infection is able to increase the generation of ROS in gastric epithelial cells [10,11].

Everolimus is a mechanistic/mammalian target of rapamycin (mTOR) inhibitor and is thought to inhibit tumor growth directly and indirectly by suppressing the proliferative signals of the PI3K/AKT/mTOR signaling pathway [12]. The molecular structure of Everolimus is shown in Figure 1 (a). mTOR activation is critical for driving inflammatory response in an acute lung injury model [13]. mTOR inhibitors such as sirolimus have also been found to have an inhibitory effect on the expressions of pro-inflammatory markers...
chemokines such as IL-8 and MCP-1 in LPS-stimulated THP-1 and human primary monocytes via suppressing the NF-kB signaling pathway [14]. However, the effect of Everolimus on *H. pylori*-induced damage in gastric epithelial cells is unknown. The aim of our investigation is to test for the potential function of Everolimus on *H. pylori*-induced oxidative stress, inflammation, and attachment of THP-1 monocytes to gastric epithelial cells.

**Material and methods**

**Cell culture and treatment**

AGS cells [15] and human THP-1 cells were obtained from ATCC, USA. The gastric epithelial AGS cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, USA) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1-glutamine 2 mmol/L. The medium was changed every 3 days. Before infection, the antibiotics were removed. For experimental treatment, the cells were stimulated with Helicobacter pylori (#700,392, ATCC, USA) at a bacterium/cell ratio of 500:1, followed by stimulation with Everolimus (purity ≥95%, #159,351-69-6, Sigma-Aldrich, USA) at the concentrations of 25 and 50 nM for 24 hours. THP-1 cells were grown in RPMI1640 medium (Gibco, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin.

**Cell viability**

The effect of Everolimus on the cell viability of AGS cells was assessed using the MTT assay. Cells were plated in a 96-well plate and treated with Everolimus at the concentrations of 0, 0.5 2.5, 5, 25, 50, 250, and 500 nM for 24 hours. 20 μL of 5 mg/ml sterilized MTT (Sigma-Aldrich; USA) was added to each well. Before reading the OD value, the incubation medium was replaced with 150 μl DMSO. The absorbance was then detected with a DynaPro Plate Reader at 490 nm.

**Real-time PCR analysis**

RNA was extracted from AGS cells using Fast Pure Cell RNA Isolation Kit (#RC101, Vazyme). The RNA concentration was quantified using a Nanodrop spectrometer (Thermo Fisher Scientific, USA). 2 μg RNA was transcribed to cDNA using a kit (Thermo Fisher Scientific, USA). The cDNA was subjected to qPCR with SYBR green master mix on a StepOne Plus Real-Time PCR System (Applied Biosystems). The target gene expression was normalized to GAPDH using the $2^{-ΔΔCt}$ method [16].

**ELISA**

AGS cells were treated as described above. The supernatants were collected and stored at −80°C until further analysis. The secretions of IL-6, TNF-α, IL-8, CXCL1, MCP-1, and ICAM-1 were
determined using ELISA following the product instructions. Elisa kits from R&D system were used: IL-6 (#D7050,), TNF-α (#DTA00D), IL-8 (#8000C), CXCL1 (# SGR00B), CCL2 (# SCP00), and ICAM-1 (#SCIM00). First, 96-well cell culture plates were coated with 100 µL coating solution and incubated overnight at 4°C. After 3 washes, the plates were blocked with 200 µL blocking buffer for 1 hour at room temperature. 100 µL of standards and samples were added into designated wells and incubated for 1 hour at room temperature. After 5 washes, 100 µL of the detection antibody solution was added to each well and incubated for 2 hours at room temperature, followed by incubation with horseradish peroxidase (HRP)-linked secondary antibody for 30 minutes. 100 µL of tetramethylbenzidine (TMB) substrate solution was then added to each well and incubated for another 30 minutes. 100 µL of stop solution was then added to each well to stop the reaction. Absorbance at 450 nm was recorded to calculate the concentration of target proteins [17].

**Western blot analysis**

After lysis in the RIPA buffer with protease inhibitor cocktail (Roche Diagnosis, USA) the nuclear protein was isolated using a Qproteome Nuclear Protein Kit (#37,582, QIAGEN). 20 µg protein was separated on a 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (EMD Millipore, USA). After being probed for 1 hour, the blots were incubated with the following primary antibodies from Cell signaling technology: IkBa antibody (Cat#9242), Phosphorylated IkBa antibody (Cat#2859), NF-κB p65 (Cat#3987), GAPDH (Cat#5174S), and lamin B1 (Cat#13,435), membranes were incubated with HRP Goat Anti-Rabbit (IgG) (#7074, Cell signaling Technology, USA) or Goat anti-Mouse (IgG) secondary antibody (#7076, Cell signaling Technology, USA) for 1 hour at room temperature. Blots were developed using enhanced ECL Western blotting Substrate (Thermo Fisher Scientific, USA). The blots were visualized on X-ray film with a film developer machine, and the result was analyzed using Image J software (NIH, USA) [18].

**Calcein-AM staining**

Attachment of THP-1 cells to gastric epithelial AGS cells was measured using calcein-AM (Cat#148,504-34-1, Abcam) staining. In brief, AGS cells were cultured in a 6-well plate until they reached ~90% confluence, and then cells were infected with Helicobacter pylori, followed by stimulation with Everolimus at the concentrations of 25 and 50 nM for 24 hours. The calcein-AM stained THP-1 cells were then added to the AGS cells and incubated for an additional 1 hour. The plates were washed thoroughly to remove the unattached cells and then observed under a fluorescence microscope (Zeiss Autoplan2).

**Dihydroethidium (DHE) assay**

Intracellular ROS in AGS cells was measured using DHE staining. After treatment, cells were incubated with 5 µM DHE and incubated for 15 minutes at 37°C in darkness. Fluorescent signals were visualized using a fluorescent microscope. The level of ROS was quantitated using Image J software.

**Malondialdehyde (MDA) assay**

MDA concentration in AGS cells was examined using a kit (Calbiochem, USA). The cell lysate was mixed with N-methyl-2-phenyldione in acetonitrile in the ratio of 1:3, followed by adding 1% HCl and incubated at 45°C for 1 hour. Absorbance was recorded at 586 to index MDA concentration.

**Luciferase activity of NF-κB**

Cells were co-transfected with luciferase reporter plasmid pNF-κB-Luc (0.1 µg) and pRL-TK plasmid (0.05 µg) (Promega, USA). 12 hours later, cells were infected with Helicobacter pylori, followed by stimulation with Everolimus at the concentrations of 25 and 50 nM. Firefly luciferase and Renilla luciferase activities were determined using the Dual-luciferase reporter assay system (Promega, USA). Renilla luciferase activity was used as a normalization control.
Statistical analysis

All results were displayed as Mean ± standard deviation (S.D.). Statistical analysis was performed by Prism 7.05 (GraphPad, USA). Comparisons were performed using analysis of variance (ANOVA) with Bonferroni’s post-hoc test. A P-value less than 0.05 was considered as a significant difference.

Results

In order to investigate the effects of Everolimus on H. pylori-challenged gastric epithelial AGS cells, we tested several important factors. First, we investigated the potential benefits of Everolimus on oxidative stress and the expression of pro-inflammatory cytokines. Then we investigated the effects of Everolimus on the attachment of THP-1 by inhibiting the expression of adhesion molecules such as ICAM-1. Importantly, we further clarified the involvement of NF-κB in this inflammatory response.

The effects of Everolimus on the cell viability of gastric epithelial AGS cells

First, we assessed the cytotoxicity of Everolimus in AGS cells. When the concentration of Everolimus
was lower than 50 nM, the cell viability was not affected (Figure 1(b)). However, when the concentration of Everolimus was higher than 50 nM, the cell viability of gastric epithelial cells was reduced significantly to 91% (250 nM of Everolimus) and 83% (500 nM of Everolimus). Therefore, 25 and 50 nM Everolimus were used in this study.

**Everolimus ameliorates *H. pylori*-induced oxidative stress**

To clarify the effect of Everolimus on *H. pylori*-induced oxidative stress in gastric epithelial cells, the generation of ROS and production of MDA were measured. As shown in Figure 2(a), the generation of ROS was remarkably elevated to 3.3-fold by *H. pylori* only and decreased to 2.4- and 1.7-fold by 25 and 50 nM of Everolimus, respectively. Similarly, as shown in Figure 2(b), the production of MDA was inhibited to 2.2- and 1.6-fold by the same doses of Everolimus, respectively, compared to a 2.9-fold increase stimulated by *H. pylori*. These results indicate the anti-oxidative effect of Everolimus on *H. pylori*-challenged gastric epithelial cells.

**Everolimus inhibits *H. pylori*-induced expression of pro-inflammatory mediators**

As pro-inflammatory cytokines play a critical role in gastric inflammation, we examined the effect of Everolimus on *H. pylori*-induced expressions of IL-6, TNF-α, and IL-8. The mRNA level of IL-6 increased approximately by 3.9-fold after exposure to *H. pylori* alone, but 25 and 50 nM Everolimus reduced it to approximately 2.7- and 1.8-fold, respectively (Figure 3(a)). The same doses of Everolimus decreased the mRNA level of TNF-α to 2.0- and 1.5-fold, respectively, compared to a 2.6-fold increase from exposure to *H. pylori* (Figure 3(b)). The mRNA level of IL-8 was reduced to 2.4- and 1.6-fold by Everolimus, respectively, compared to a 3.3-fold increase induced by *H. pylori* (Figure 3(c)). Similarly, 25 and 50 nM Everolimus significantly inhibited the secretions of IL-6, TNF-α, and IL-8 at their protein levels (Figure 3(d-f)).

We then tested for the role of Everolimus on the expressions and secretions of CXCL1 and MCP-1, which are important chemokines in gastric epithelial cells. Our results show that 25 and 50 nM Everolimus downregulated the expression of
CXCL1 to 2.9- and 2.1- fold at the mRNA level, respectively, compared to a 4.2- fold increase from exposure to *H. pylori* only (Figure 4(a)). Similarly, *H. pylori*-stimulation increased the mRNA level of MCP-1 to 3.4- fold, which was dose-responsively decreased to 2.5- and 1.6- fold by the two doses of Everolimus (Figure 4(b)). Additionally, Everolimus significantly suppressed the secretions of CXCL1 and MCP-1 at the protein levels (Figure 4(c-d)).

**Everolimus suppresses *H. pylori*-induced expression of ICAM-1 and the attachment of THP-1 cells to gastric epithelial AGS cells.**

Next, we assessed the effects of Everolimus on the expression of ICAM-1, an important adhesion molecule that mediates the attachment of monocytes to gastric epithelial cells. *H. pylori* increased the expression of ICAM-1 to 3.8- fold at the mRNA level (Figure 5(a)), which was downregulated to 2.7- and 2.1- fold by 25 and 50 nM Everolimus, respectively. Similarly, the same doses of Everolimus decreased the protein level of ICAM to 267.6 and 158.7 pg/mL, initially increased to 426.5 pg/mL by exposure to *H. pylori* only (Figure 5(b)).

We then investigated the effect of Everolimus on *H. pylori*-induced attachment of THP-1 cells to gastric epithelial AGS cells. The attachment of THP-1 cells was increased 4.2- fold by *H. pylori*-stimulation but reduced to 2.7- and 1.9- fold by 25 and 50 nM Everolimus, respectively (Figure 6(a)). To clarify whether the effects of Everolimus on *H. pylori*-induced attachment of THP-1 cells to gastric epithelial AGS cells are dependent on or independent of mTOR inhibition, another mTOR inhibitor, Rapamycin was used.
Interestingly, results in Figure 6(b) indicate that treatment with Rapamycin did not have a significant impact on H. pylori-induced attachment of THP-1 cells to gastric epithelial AGS cells, suggesting that the effect of Everolimus might be independent of the inhibition of mTOR.

**Everolimus prevents H. pylori-induced activation of the IκBα/NF-κB signaling pathway**

The results in Figure 7 indicate that H. pylori-stimulation increased the phosphorylation of IκBα to 3.4-fold, which was decreased to 2.4- and 1.6-fold by 25 and 50 nM Everolimus, respectively. Then, we found that the same doses of Everolimus rescued the total IκBα to 73% and 91%, compared to a 53% decrease stimulated by H. pylori. Our data shows that H. pylori-stimulation significantly increased the nuclear levels of p65 to 3.6-fold, which was later downregulated to 2.7- and 1.6-fold by 25 and 50 nM Everolimus, respectively (Figure 8(a)). The luciferase activity of NF-κB was reduced to 271.2- and 193.3-fold by the same doses of Everolimus, compared to a 365.6-fold increase induced by H. pylori (Figure 8(b)).

**Discussion**

Oxidative stress, inflammation, and monocyte adhesion play a pivotal role in the development of H. pylori infection-associated gastric diseases. Here, we examined the protective effects of Everolimus on H. pylori-stimulated gastric epithelial cells. Previous studies have demonstrated that H. pylori infection remarkably reduced the expressions of antioxidant enzymes such as SOD and reduced GSH in human gastric epithelial cells [19,20]. Our results show that H. pylori infection significantly increased the expressions of ROS and MDA. It has been reported that the overproduction of ROS in H. pylori-stimulated gastric epithelial cells depends on NADPH oxidase, which could be directly activated by H. pylori infection [21]. The excessive generation of ROS plays an important role in cellular oxidative stress mechanisms of chronic gastritis through the induction of MDA and cell apoptosis [22,23]. Furthermore, previous research indicates that ROS can regulate the mTOR-related signaling pathway to be involved in cell autophagy [24]. mTOR inhibition has displayed an inhibitory capacity in the generation of ROS [25]. In this study, we show new evidence that Everolimus could alleviate oxidative stress by inhibiting H. pylori-induced generation of ROS and the production of MDA.

Monocyte attachment has been reported to play a vital role in H. pylori-associated gastric diseases and has been considered the main treatment target. As our results have shown that Everolimus significantly suppressed the attachment of THP-1 cells to gastric epithelial cells, we believe that it could be a potential therapeutic agent. Pro-inflammatory cytokines and chemokines are major factors in the progression of H. pylori-associated gastric diseases.
TNF-α is a well-known cytokine for its role in initiating the inflammatory cascade to trigger the production of various other inflammatory factors and induction of cell death [26]. IL-6 and IL-8 are considered key elements involved in the progression of gastritis because they have broad biological effects on mononuclear cells [27]. Furthermore, IL-8, CXCL1, and MCP-1 are responsible for monocyte migration to the sites of infection in H. pylori-associated gastric diseases [28–30]. H. pylori infection highly increased the expressions of IL-8, CXCL1, and MCP-1 in gastric mucosa tissues of patients [31–33]. In our study, we found that Everolimus greatly inhibited the increased levels of these pro-inflammatory regulators induced by H. pylori. ICAM-1 is significantly upregulated at sites of inflammation in H. pylori-stimulated gastric epithelial cells, indicating the important role of ICAM-1 in H. pylori-related gastric diseases. Our findings show that Everolimus robustly reduced the expression of ICAM-1, implying its beneficial effect against H. pylori-induced immune attachment.

Figure 6. Everolimus but not Rapamycin suppresses Helicobacter pylori-induced attachment of THP-1 cells to gastric epithelial AGS cells. (a). Cells were infected with Helicobacter pylori at a bacterium/cell ratio of 500:1, followed by stimulation with Everolimus at the concentrations of 25 and 50 nM for 24 hours. Attachment of THP-1 cells to gastric epithelial AGS cells was measured using calcein-AM staining. (b). Cells were infected with Helicobacter pylori at a bacterium/cell ratio of 500:1, followed by stimulation with Rapamycin at the concentrations of 25 and 50 nM for 24 hours. Attachment of THP-1 cells to gastric epithelial AGS cells was measured using calcein-AM staining. Scale bar, 100 μm (####, P < 0.0001 vs. vehicle group; $, P < 0.01, 0.001 vs. Helicobacter pylori group, N = 5).
to epithelial cells. NF-κB is activated during *H. pylori* infection in various types of cells [34,35]. The expressions of IL-6, TNF-α, IL-8, CXCL1, MCP-1, and ICAM-1 in *H. pylori*-stimulated gastric epithelial cells are mediated by NF-κB [36–38]. *H. pylori* activates NF-κB by phosphorylating and degrading IκBα, promoting NF-κB p65 to translocate into the nucleus and bind to the promoter of DNA sequences. Inhibition of the NF-κB signaling pathway could be impactful for protecting against gastric diseases [32]. Additionally, the NF-κB system has been found to be deregulated in gastric cancer and recommended for therapeutic targeting. Here, we demonstrate that Everolimus prevented the activation of NF-κB by inhibiting the phosphorylation and degradation of IκBα, suggesting a promising therapeutic effect of Everolimus in *H. pylori*-associated gastric diseases. In recent decades, antibiotic treatment is considered a therapeutic strategy against *H. pylori*. Clarithromycin, Metronidazole, and Tetracycline have been widely used for the treatment of *H. pylori*-associated diseases. In fact, these antimicrobials have been shown to exert powerful benefits in *H. pylori*-associated diseases. However, due to the misuse of antibiotics, antibiotic resistance has become an increasing problem [39]. Compared with traditional antibiotic treatment, Everolimus prevents inflammation in *H. pylori*-associated disease by regulating signaling pathways such as NF-κB, thereby avoiding antibiotic resistance.

The mechanism of inflammatory response in gastric diseases is still complex. Previous studies...
have shown that activated mTOR could promote activation of the NF-κB signaling pathway in neuro-inflammation of Parkinson’s disease and in an in vivo obstructive sleep apnea (OSA) model [40,41]. Dan et al. (2008) further clarified the underlying mechanism by which mTOR regulates the activation of NF-κB [42]. These findings indicate that Everolimus, an mTOR inhibitor, may exert its anti-inflammatory benefits through the mTOR/NF-κB signaling pathway. However, it is also possible that Everolimus directly regulates the NF-κB signaling pathway. The pharmacological mechanisms of Everolimus are limited. Interestingly, the activation of extracellular signal-regulated kinase (ERK) by Everolimus has been associated with its limited efficacy in gastric cancer. The combined inhibition with Everolimus and trametinib can overcome resistance by specifically inhibiting ERK and regulating ERK-mediated Bcl-2 family proteins in gastric cancer cells [43]. Our preliminary experiments (Figure 1b) indicate that 25 and 50 nM Everolimus are both optimal concentrations used for the treatment in AGS cells, which is relevant to the doses used in clinics [44]. In our future work, we will further study the effects of Everolimus on H. pylori-associated gastric diseases, to reveal the full picture of the underlying mechanism involved in gastric diseases.

Conclusion
In summary, our study provides new evidence that Everolimus suppresses oxidative stress and monocyte attachment in H. pylori-stimulated gastric epithelial cells, indicating a potential protective effect of Everolimus on H. pylori-induced gastric diseases. However, the mechanisms of H. pylori infection-related gastric diseases are still complicated, more research is required to further elucidate the benefits of Everolimus.

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Author Contribution
Jinglei Liu and Chunning Zheng made a substantial contribution to experimental design and data analysis; Jinglei Liu, Fangxu Zhang, and Zheming Zhang made a substantial contribution to investigation and data collection; Chunning Zheng drafted the manuscript. All authors have read and approved the manuscript.

Availability of Data Materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent to publication
All the authors have read and approved the final submission of this study.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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