

# Rebamipide, a novel antiulcer agent, attenuates *Helicobacter pylori* induced gastric mucosal cell injury associated with neutrophil derived oxidants

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## Abstract

The effect of rebamipide, a novel anti-ulcer compound, on *Helicobacter pylori* activated neutrophil dependent in vitro gastric epithelial cell injury was investigated. Luminol dependent chemiluminescence (ChL), which detects toxic oxidants from neutrophils exhibited a 12-fold increase when the bacterial suspension of *H pylori* was added to the isolated human neutrophils. This change was significantly attenuated by rebamipide at a concentration less than 1 mM, showing that rebamipide may inhibit oxidant production from *H pylori* elicited neutrophils. To assess whether rebamipide attenuates gastric mucosal injury, we tested its inhibitory action on *H pylori* induced gastric mucosal damage associated with neutrophils in vitro. Rabbit gastric mucosal cells were monolayered in culture wells and coincubated with human neutrophils and *H pylori*, and the cytotoxicity index was then calculated. Cultured gastric cells were significantly damaged when they were incubated with human neutrophils activated by *H pylori*. This cellular damage was attenuated by rebamipide in a dose dependent manner. Furthermore, spectrophotometrical measurement showed that rebamipide (1 mM) inhibits urease activity by 21.7%. As monochloramine (an oxidant yielded by reaction of neutrophil derived chlorinated oxidant and ammonia) is proposed as an important toxic molecule in this model, the current findings suggest that the preventive effect of rebamipide on *H pylori* elicited neutrophil induced gastric mucosal injury may result from its inhibitory actions on the neutrophilic oxidative burst as well as *H pylori* derived urease activity.

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*Helicobacter pylori* has been shown to be an important pathogen of gastric disorder including gastritis and gastric ulcer. The pathogenic mechanisms, however, by which *H pylori* causes the gastric mucosal damage still remain obscure. Antibiotics have been, therefore, only recently been used for *H pylori* positive patients for the purpose of bacterial eradication. One of the potential toxic factors involving gastric

mucosal cell injury is reactive oxidants, which are released from activated neutrophils. Several studies have already shown that *H pylori* itself exhibits chemotactic activity for neutrophils.<sup>1-5</sup> We have also reported that *H pylori* elicited neutrophils produce toxic oxidants, which subsequently injure cultured gastric mucosal cells, and that ammonia, which is derived from urea by *H pylori* associated urease enhances the mucosal cytotoxicity, which may be regulated by monochloramine.<sup>6,7</sup> Considering these mechanisms, the agents that can inhibit either neutrophil activation or ammonia production are proposed to be effective for *H pylori* induced gastric mucosal injury. Rebamipide had been reported to promote mucus synthesis,<sup>8</sup> mucosal prostaglandin content, and rapid ulcer healing.<sup>9</sup> Moreover, it has recently been reported to inhibit the production of oxygen derived free radicals from stimulated neutrophils.<sup>10</sup> In this report, we have evaluated whether rebamipide may influence active oxidant production elicited by *H pylori* activated neutrophils and urease activity, and whether it attenuates gastric mucosal cell damage associated with *H pylori* activated neutrophils in vitro.

## Methods

### CHEMICALS

The fluorochrome dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl-ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). Urea was obtained from Sigma Chemicals (St Louis, MO). BCECF-AM solution was prepared in dimethyl sulphoxide (DMSO) at 1 mM. Rebamipide, ( $\pm$ )-2-(4-chlorobenzoylamino)-3[2(1H)-quinolinon-4-yl] propionic acid (Otsuka Pharm Co, Japan) was dissolved in DMSO and was then diluted in phosphate buffered saline (PBS) before experiments. The final concentration of DMSO in each assay was adjusted at 3%. Crystalline urease (Jack beans) was obtained from Sigma.

### MICROBIOLOGY

*H pylori*, strain NCTC11637 (RPH13487) was a generous gift from Dr B J Marshall, Royal Perth Hospital, Australia. The bacteria was inoculated on sheep blood agar plate (Becton Dickinson Microbiology Systems,

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Cockeysville, MD) at 37°C under micro-aerophilic conditions using an anaerobic chamber (Gaspak, Becton Dickinson Microbiology Systems). The bacteria was harvested and then washed with PBS (pH 7.4) for experiments.

#### NEUTROPHILS

Neutrophils were isolated from the human peripheral blood of one healthy volunteer with the use of standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma). This procedure yields a neutrophil population that is 95% viable (trypan blue exclusion) and 98% pure (acetic acid crystal violet staining).

#### CHEMILUMINESCENCE ASSAY

The release of active oxidants from neutrophils stimulated by *H pylori* was determined using a luminol dependent chemiluminescence (ChL) using the modified method as previously reported.<sup>7</sup> Briefly, one millilitre of Dulbecco's modified Eagle's medium containing neutrophils ( $1.5 \times 10^6$ ) and *H pylori* ( $1.5 \times 10^7$ ) was incubated with 20 µg luminol for 60 minutes. The ChL counts were continuously recorded using a computer assisted 6-channel ChL analyser (Biolumat LB9505, Berthold, FRG). Integrated ChL counts (0–60 min) were used for the assessment.

#### CYTOTOXICITY ASSAY

The cytotoxicity measurement of cultured gastric mucosal cells was performed according to the previously reported method.<sup>6</sup> Briefly, the stomachs of rabbit fetuses were separated and cultured. The cells were finally monolayered in a 48-well tissue culture plate at 37°C in 5% carbon dioxide atmosphere. Histochemical analysis was performed by conventional staining with haematoxylin and eosin, periodic acid Schiff (PAS), and alkaline phosphatase. These methods showed that >70% of the cells were identified as mucus producing epithelial cells by PAS staining. No alkaline phosphatase activity was detected, suggesting that the cultures were free of vascular endothelial cells. The monolayered mucous cells were labelled with BCECF-AM (5 µM) for 20 minutes then incubated with modified Eagle's medium (0.5 ml) containing 2 mM urea, neutrophils ( $7.5 \times 10^5$ ), and *H pylori* ( $7.5 \times 10^6$ ) for two hours at 37°C. For BCECF measurements, supernatants were harvested and centrifuged to remove floating leucocytes and bacteria. The supernatant was assayed for fluorescence intensity ( $F_{\text{super}}$ ). The cells in each well were lysed by 0.5% Triton X100 to measure the fluorescence of targets ( $F_{\text{lysate}}$ ). The spontaneous release ( $F_{\text{spont}}$ ) was determined by incubating targets with medium alone. The samples were diluted in bicarbonate buffer (pH 9) and then the fluorescence intensity was measured using a fluorescence spectrophotometer (F-3000, Hitachi). The excitation and emission wavelength was set at

490 nm and 510 nm, respectively. The specific % cytotoxicity was calculated from the equation:

$$\text{Specific \% cytotoxicity} = \frac{F_{\text{super}} - F_{\text{spont}}}{F_{\text{super}} + F_{\text{lysate}} - F_{\text{spont}}} \times 100$$

#### UREASE ACTIVITY MEASUREMENT

The inhibitory action of rebamipide on urease activity was determined spectrophotometrically. A mixture of 10 µl of rebamipide solution was combined with 10 µl of 1.07 mg/ml of urea then preincubated for 10 minutes at 37°C. Thereafter, one ml of phosphate buffer (90 mM, pH 7.0) containing 0.1 U/ml urease, 0.25 M sodium salicylate, and 6.7 mM sodium nitrosylpentacyanoferrate (III) dihydrate was added and incubated for 15 minutes at 37°C. The reaction was completed by adding one ml chromogen reagent (sodium hypochlorite and NaOH), and then the absorbance was measured with a spectrophotometer (UNIDEC, Model 340, Japan) at 570 nm. The inhibition percentage was calculated for the assessment.

#### BACTERIAL GROWTH INHIBITION TEST

The influence of rebamipide on *H pylori* growth was determined by the agar dilution method. The bacterial suspensions (n=5) were applied to the brucella blood agar plates containing rebamipide (0.1, 0.5, and 1 mM). The plates were incubated at 37°C in a microaerobic environment. Readings were performed after three days of incubation.

#### STATISTICAL ANALYSIS

The statistical differences were determined by one way analysis of variance (ANOVA) and Dunnett type multiple comparison. All values are expressed as mean (SEM), and statistical significance was set at  $p < 0.05$ .

## Results

#### CHEMILUMINESCENCE ASSAY (Fig 1)

The ChL value of resting neutrophils (neutrophils only, 3% DMSO included) was 0.42 (0.34) ( $\times 10^8$  counts, n=6) while it increased 12-fold after adding *H pylori* (5.10 (1.49), n=5,  $p < 0.01$ ). This is attenuated by rebamipide at concentrations of 0.1 mM and 0.5 mM (3.60 (1.29), 1.75 (0.63), n=6) while 1 mM rebamipide significantly decreased the ChL value (1.01 (0.38), n=6,  $p < 0.05$ ). These results show that rebamipide attenuates the release of active oxidants from *H pylori* elicited neutrophils in a dose dependent manner.

#### CYTOTOXICITY ASSAY (Fig 2)

*H pylori* activated neutrophils significantly increased specific % cytotoxicity of cultured gastric mucosal cells to 33.9 (4.89) (n=6,  $p < 0.01$ ) compared with the control value (-0.08 (2.41), n=4). This damage was

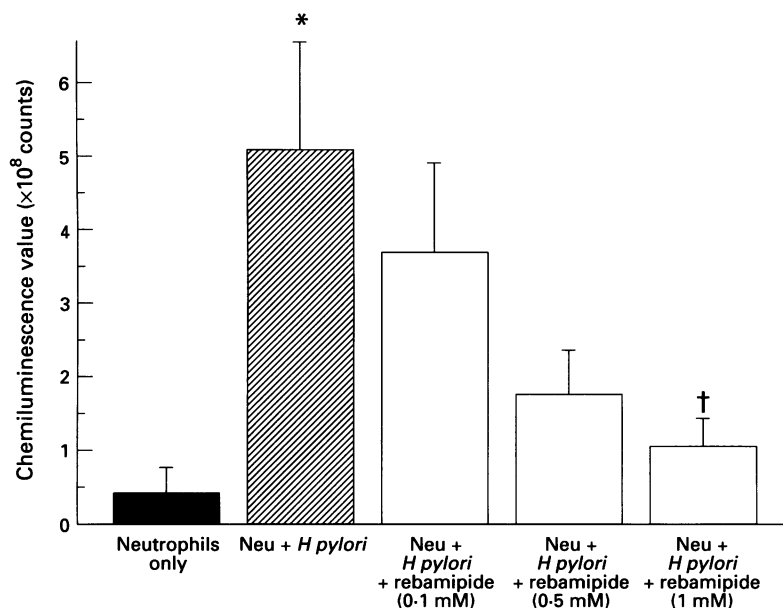


Figure 1: Luminol dependent chemiluminescence of human neutrophils. The application of bacterial suspension of *H pylori* to neutrophils causes a 12-fold increase of the ChL value, indicating the release of neutrophil derived oxidants. Pretreatment of rebamipide significantly attenuated this enhanced ChL value. Bars express standard error. \* $p < 0.01$ , compared with neutrophils only value. † $p < 0.05$ , compared with neutrophils (Neu) + *H pylori* value.

attenuated by 0.1 and 0.5 nM rebamipide (28.3 (4.23) and 21.6 (2.8), respectively,  $n = 6$ ) whereas 1 mM rebamipide significantly inhibited it (17.4 (3.17),  $n = 6$ ,  $p < 0.05$ ), suggesting the protective action of rebamipide on *H pylori* induced gastric mucosal injury associated with activated neutrophils.

UREASE INHIBITION STUDY (Table)

Urease activity was significantly inhibited by rebamipide. Rebamipide (0.1 mM) suppressed enzyme activity by 6.4% while it was further inhibited by doses of 0.5 and 1 mM (14.9% and 21.7%). These data suggest that rebamipide suppresses *H pylori* mediated

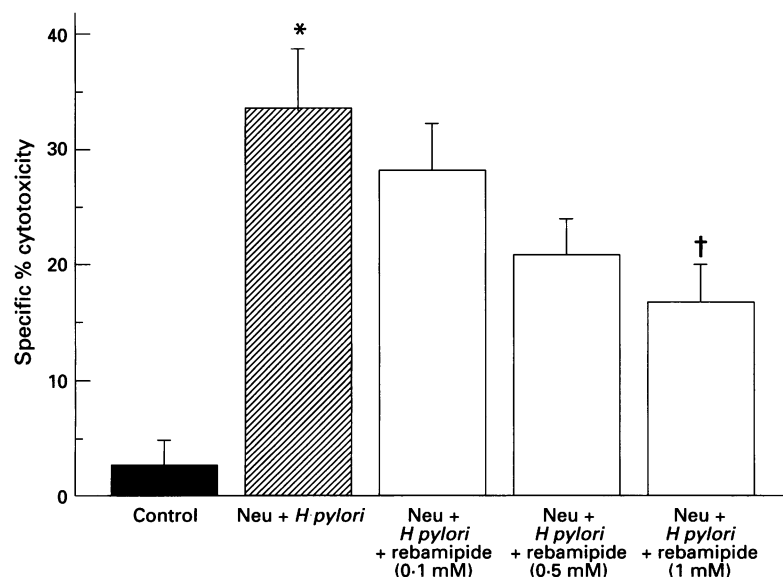


Figure 2: Gastric mucosal cell damage induced by *H pylori* activated neutrophils and the effect of rebamipide. The treatment with rebamipide significantly attenuated gastric mucosal injury induced by *H pylori* elicited neutrophils. Bars express standard error. \* $p < 0.01$ , compared with control (modified Eagle's medium only) value. † $p < 0.05$ , compared with neutrophils + *H pylori* value.

ammonia production by its inhibitory action on urease.

BACTERIAL GROWTH TEST

The growth of *H pylori* was not inhibited by rebamipide at concentrations less than 1 mM. This result suggests that rebamipide may not have any influence on bacterial viability at a concentration of less than 1 mM.

Discussion

Eradication treatment using antibiotics has been generally approved for the treatment of patients with *H pylori* positive gastritis and gastric ulcer. Few therapeutic strategies, however, which can abolish the cytotoxic action of *H pylori* have been described. Among *H pylori* associated cytotoxic factors, chemotaxin, which is detectable in *H pylori* and its culture filtrate is one of the potential determinants of virulence because a significant neutrophil infiltration is characterised in *H pylori* positive gastric mucosa.<sup>11 12</sup> Yoshida *et al* have reported that this active factor elicits the upregulation of leucocyte adhesion molecules causing neutrophil adherence to venular endothelium in rat mesentery and to monolayers of human umbilical vein endothelial cells.<sup>13</sup> Kurose *et al* have also seen that the mesenteric microvascular permeability is dramatically increased by *H pylori* extract, which is neutrophil mediated.<sup>14</sup> We have recently reported that *H pylori* elicits chlorinated toxic oxidant production from neutrophils and that these substances play a critical part in *H pylori* associated gastric mucosal damage.<sup>6 7</sup>

Rebamipide is a quinolinon derivative and its molecular weight is 370.79. This compound has been reported to possess antiulcer properties against ethanol or acid induced gastric mucosal damage. These effects have been interpreted by its pharmacological action on prostaglandin synthesis in gastric epithelium.<sup>9</sup> The local concentration of rebamipide has not been reported in the human stomach while its mucosal concentration is more than 0.2 mM after the oral intake of 10 mg/kg rebamipide in rats.<sup>15</sup> Our experimental result that *H pylori* enhanced ChL release from neutrophils was significantly suppressed by rebamipide shows that rebamipide attenuates the release of oxidants from *H pylori* elicited neutrophils. It is not clear whether this inhibition results from an oxidant scavenging effect or from a direct action for neutrophil activation process. Yoshikawa *et al* reported that rebamipide can only scavenge hydroxyl radical, which is not detectable in the luminol dependent ChL assay

Effect of rebamipide on urease activity in vitro

	Urease activity (% of control)	p Value
Rebamipide 0.1 mM	93.6 (1.9)	NS
0.5 mM	85.1 (2.5)	<0.01
1.0 mM	78.3 (0.8)	<0.01

Values are expressed as mean (SEM) of four findings. NS=non-significant.

that we used.<sup>16 17</sup> It is also known that FMLP (f-methionyl-leucyl-phenylalanine) stimulated neutrophil dependent superoxide production was inhibited by rebamipide.<sup>18</sup> It is, therefore, concluded that rebamipide may suppress active oxidant production through modulating the neutrophil activation process, independently of its radical scavenging properties. Accordingly, the attenuation of rebamipide on toxic oxidant release may be important in preventing gastric mucosal injury associated by *H pylori* elicited neutrophils. This toxic mechanism is supported by our experimental evidence that gastric mucosal cell damage associated with *H pylori* and neutrophils is significantly inhibited by rebamipide.

This study shows that rebamipide significantly inhibits urease activity as well, which implies the possibility that the ammonia concentration can be reduced in gastric mucosa infected by *H pylori*. Although it is not certain how much *H pylori* associated urease can be inhibited by rebamipide at the concentration found in the human stomach, ammonia has been proposed to be an important pathogen to *H pylori* associated gastric mucosal injury. The ammonia concentration of gastric juice is reported as 3.4 mM, which is not cytotoxic.<sup>19 20</sup> This concentration of ammonia, however, has been proposed to be pathogenic in the presence of activated neutrophils because neutrophil derived hypochlorous acid can react with ammonia to produce monochloramine, which is a highly toxic molecule. We have emphasised the unique role of monochloramine in *H pylori* induced gastric mucosal damage.<sup>6</sup> Morishita *et al* have also shown the clinical efficacy of taurine (monochloramine scavenger) for patients with *H pylori* positive gastritis.<sup>21</sup> Although monochloramine concentration was not measured in this in vitro study and the attenuating magnitude of rebamipide on urease activity is comparatively slight (22% at 1 mM), the synchronous inhibition of rebamipide in neutrophilic oxidative bursts and *H pylori* derived urease activity may be advantageous for preventing *H pylori* induced gastric mucosal damage, which is mediated by monochloramine.

The in vitro bacterial growth test shows that bacterial growth of *H pylori* is not influenced by rebamipide at concentrations less than 1 mM. This finding suggests that oral intake of rebamipide in itself cannot clinically eradicate *H pylori* from gastric mucosa.

In conclusion, the results of this in vitro study suggest that rebamipide may be one of the valuable antiulcer compounds that also exhibit an additional efficacy for *H pylori* induced mucosal damage associated with the ammonia monochloramine system.

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