Short Communication

Anti-*Helicobacter pylori* actions of CV-6209, a platelet-activating factor receptor antagonist

Yasuhisa Iwao,¹ Tomomi Takano,¹ Ikue Taneike,¹ Ivan Reva,¹ Hirokazu Isobe,¹
Hui-Min Zhang,² Akihito Nishiyama,¹ and Tatsuo Yamamoto^{1,*}

¹ Division of Bacteriology, Department of Infectious Disease Control and International Medicine,
Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

² Department of Dermatology, Shu-Guang Hospital, Shanghai University, Shanghai 200021, China

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Helicobacter pylori is a spiral-shaped, Gram-negative microaerophilic bacterium that colonizes the gastric mucosa of more than 50% of individuals worldwide. *H. pylori* motility conferred by polar flagella is necessary for its colonization in the gastric mucosa (Eaton et al., 1992). *H. pylori* colonization is closely associated with gastritis and peptic ulcers and is also a bacterial risk factor for gastric cancer (Asaka et al., 2010; Atherton and Blaser, 2009; Malfertheiner et al., 2007). The role of *H. pylori* infections in autoimmune diseases including idiopathic thrombocytopenic purpura (ITP) has also been discussed (Gasbarrini et al., 1998; Malfertheiner et al., 2007). It is suggested that some anti-*H. pylori* antibodies developed during *H. pylori* infections recognize platelets, resulting in disruption of platelets and ITP development (Gasbarrini et al., 1998). Moreover, structural mimics between the O-side chain of *H. pylori* lipopolysaccharide (LPS) and human Lewis-x and -y antigens have been proposed

as a responsible mechanism for autoimmune diseases (Negrini et al., 1996).

For eradication of *H. pylori*, a combination therapy using a proton pump inhibitor (PPI) and two anti-*H. pylori* agents (amoxicillin and clarithromycin or metronidazole) has been recommended (Asaka et al., 2010; Graham and Fischbach, 2010; Malfertheiner et al., 2007). However, increased eradication failure due to resistance to current chemotherapy has become problematic (De Francesco et al., 2010; Gerrits et al., 2006); therefore, further drug development is required to control *H. pylori* infections.

2-[N-Acetyl-N-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl) aminomethyl]-1-ethylpyridinium chloride (CV-6209, Fig. 1A) is a platelet activating factor (PAF) receptor antagonist that antagonizes the PAF receptor signaling, resulting in inhibition of PAF-induced aggregation of human and rabbit platelets (Terasita et al., 1987). Since *H. pylori* contains similar surface antigens to those on human cells including platelets (Gasbarrini et al., 1998; Negrini et al., 1996), we assumed that *H. pylori* may possess the PAF receptor or its structural analogues, and CV-6209, which targets the PAF receptor of platelets, may exhibit anti-*H. pylori* activity. The anti-bacterial activity of CV-6209 has not been investigated. The aim of this study was to investigate the effect of CV-6209 on *H. pylori* growth,

* Corresponding author: Dr. Tatsuo Yamamoto, Division of Bacteriology, Department of Infectious Disease Control and International Medicine, Niigata University Graduate School of Medical and Dental Sciences, 757 Ichibanchou, Asahimachidori, Niigata 951-8510, Japan.

Tel: +81 25 227 2050 Fax: +81 25 227 0762

E-mail: tatsuoym@med.niigata-u.ac.jp

Platelet activating factor (PAF) antagonists

A. CV-6209

B. CV-3988

PAF

C. PAF (C18 and C16)

Fig. 1. Chemical structures of CV-6209 (A), CV-3988 (B), and platelet activating factor (PAF; C).

morphology, and motility, compared with other anti-*H. pylori* agents.

PAF receptor antagonists, CV-6209 and CV-3988, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Two forms of PAF (C16 and C18) were purchased from Cayman Chemical (Ann Arbor, MI). Chemical structures of these compounds are shown in Fig. 1 (A-C; Terashita et al., 1987).

H. pylori strains used (54 strains), including strain C7M, were isolated from gastric biopsy specimens of patients with gastritis, peptic ulcers, and gastric cancer during 1999–2010. The following Gram-negative bacteria were also employed: *Campylobacter jejuni* (25 strains), *C. coli* (two strains), *Vibrio cholerae* O1 (ten strains) and O139 (ten strains), *V. parahaemolyticus* (14 strains), *V. mimicus* (seven strains), *V. alginolyticus* (six strains), *V. fluvialis* (seven strains), *Escherichia coli* (44 strains), *Aeromonas hydrophila* (seven strains), *A. sobria* (seven strains), *A. caviae* (seven strains), *Plesiomonas shigelloides* (seven strains), *Pseudomonas aeruginosa* (seven strains), *Salmonella* sp. (four strains), *S. enterica* serovars Typhi (three strains), Typhimurium (one strain), Enteritidis (one strain), and Paratyphi A (three strains), *Shigella dysenteriae* (three strains), *S. flexneri* (three strains), *S. sonnei* (three strains), *S. boydii* (two strains), and *Yersinia enterocolitica* (two strains); they were our laboratory stock.

Susceptibility testing of bacterial strains was carried

out by the agar dilution method with Mueller-Hinton agar (Difco) supplemented with 5% sheep blood (Nippon Bio-Test, Tokyo) according to previous procedures (CLSI, 2011). The final concentrations of the drugs were from 0.001 to 128 µg/ml.

Morphological changes of *H. pylori* cells were examined by scanning electron microscopy, as described previously (Taneike et al., 2001). *H. pylori* cells were incubated in brain heart infusion (BHI) broth containing 10% fetal bovine serum (FBS) for 5 h at 37°C in a microaerophilic atmosphere in the presence or absence of antimicrobial agents. After washing, *H. pylori* cells were suspended in the original volume of RPMI 1640 medium containing 10% FBS. One milliliter each of these *H. pylori* suspensions was added to plastic coverslips in each well of a 24-well multidish plate, followed by incubation for 3 h at 37°C in a microaerophilic atmosphere. Coverslip samples were fixed and finally analyzed by scanning electron microscopy, as described previously (Taneike et al., 2001).

H. pylori motility was examined under an inverted, phase-contrast microscope with a microwarm plate (Kitazato Co., Tokyo) that regulates the temperatures of specimens, as described previously (Tsutsui et al., 2000). *H. pylori* swimming was carried out at 37°C in a liquid layer of BHI broth containing 10% FBS (10^6 to 10^7 CFU/ml) between an FBS-coated glass slide and an FBS-coated glass cover. Motility was measured using a motion analysis system with the program C-Imaging C-MEN (Complx, Inc., PA). The mean speed of ≥ 4.0 µm/s (speed 10 times higher than that for Brownian motion) was judged as positive motility.

The ATP levels of *H. pylori* cells (2×10^7 /ml) were analyzed using a luciferin-luciferase-based bioluminescence assay kit, Lucifer LU plus (Kikkoman Corporation, Chiba, Japan), essentially according to the manufacturer's instructions. Prior to analysis, *H. pylori* were mixed with a drug for 30 min at 37°C.

Statistical comparison was performed using Student's *t* test. $p < 0.05$ was considered significant.

MICs of CV-6209 for *H. pylori* (including strains resistant to clarithromycin or metronidazole), other spiral bacteria (*Campylobacter*), curved bacteria (*Vibrio*), and straight rod bacteria (such as *E. coli*) are shown in Table 1. Interestingly, CV-6209 exhibited *H. pylori*-specific growth inhibition (MICs: ranges, 0.063–16 µg/ml; MIC₅₀, 2 µg/ml; and MIC₉₀, 4 µg/ml), in contrast to anti-*H. pylori* agents, amoxicillin and clarithromycin (Table 1). Its MIC levels were relatively high, compared with

Table 1. The effect of CV-6209 on the growth of Gram-negative spiral, curved, and straight rods, in comparison with those of lansoprazole, amoxicillin, and clarithromycin.

Bacterial pathogens (n)	MICs ($\mu\text{g/ml}$):											
	CV-6209			Lansoprazole			Amoxicillin			Clarithromycin		
	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀	Range	MIC ₅₀	MIC ₉₀	Range
Spiral rod												
<i>Helicobacter pylori</i> (54)	2	4	0.063-16	1	2	1-2	0.03	0.25	0.004-4	0.03	16	0.004- ≥ 256
<i>Campylobacter</i> spp. ^a (27)	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	2	4	0.125-8	0.5	4	0.125-16
Curved rod												
<i>Vibrio</i> spp. ^b (54)	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	4	32	4-32	4	8	2-8
Straight rod^c												
<i>E. coli</i> (44)	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	≥ 256	4	≥ 256	0.5- ≥ 256	32	≥ 256	32- ≥ 256

^a *Campylobacter* spp. include *C. jejuni* and *C. coli*.^b *Vibrio* spp. include *V. cholerae* O1/O139, *V. parahaemolyticus*, *V. mimicus*, *V. alginolyticus*, and *V. fluvialis*.^c In addition to *E. coli*, other Gram-negative, straight rods including *Aeromonas* spp. (21 strains), *Plesiomonas shigelloides* (seven strains), *Pseudomonas aeruginosa* (seven strains), *Salmonella* spp. (12 strains), *Shigella* spp. (11 strains), and *Yersinia enterocolitica* (two strains) were also tested. For all strains, MICs of CV-6209 or lansoprazole were $\geq 256 \mu\text{g/ml}$.

amoxicillin (e.g., MIC₉₀, 0.25 $\mu\text{g/ml}$) and clarithromycin (e.g., MIC₉₀, 16 $\mu\text{g/ml}$), but were at relatively similar levels to those of a PPI agent, lansoprazole (Table 1).

A PAF receptor antagonist, CV-3988 (Fig. 1B), showed much weaker activities against *H. pylori* growth (MICs: ranges, 64- $\geq 256 \mu\text{g/ml}$; MIC₅₀, 128 $\mu\text{g/ml}$; and MIC₉₀, $\geq 256 \mu\text{g/ml}$). PAF (C16 and C18 forms; Fig. 1C) showed no anti-*H. pylori* activities (all MIC data, $\geq 256 \mu\text{g/ml}$).

Next, morphological changes of *H. pylori* induced by CV-6209 were investigated. CV-6209 caused formation of typical coccoid (with flagella) (Fig. 2A, left) and coccoid aggregation (Fig. 2A, right); normal spiral-shaped *H. pylori* is shown in Fig. 2B. Of anti-*H. pylori* agents, only amoxicillin caused coccoid formation (Fig. 2C to F), but in the case of amoxicillin, a short rod (with flagella) was connected to the *H. pylori* coccoid body (Fig. 2C; indicated by an arrow). Lansoprazole, which also induced *H. pylori*-specific growth inhibition, caused a rod shape with partial cell burst (Fig. 2F), in contrast to coccoid formation by CV-6209. Unlike CV-6209, CV-3988 did not cause coccoid formation of *H. pylori* cells under the same conditions (data not shown).

The effect of CV-6209 was bactericidal (Fig. 3A); its effect was much greater than that of lansoprazole. Lansoprazole is a *H. pylori* respiratory system inhibitor, and reduces cellular ATP levels (Nagata et al., 2001); therefore, the effect of CV-6209 on *H. pylori* cellular ATP levels was examined (Fig. 3B). Its effect was again greater than that of lansoprazole (concentrations to reduce ATP levels by 50% were 6.0 $\mu\text{g/ml}$ and 15.5 $\mu\text{g/ml}$, respectively). This cellular ATP reduction was induced by lower concentrations of CV-6209, compared to its bactericidal effect.

Finally, since CV-6209 induced morphological changes in *H. pylori*, the effect of CV-6209 on *H. pylori* motility was examined (Fig. 4). As expected, CV-6209 inhibited *H. pylori* motility at a relatively similar level to that of lansoprazole.

In addition to inhibitory effect on platelet aggregation, CV-6209 is known to inhibit endotoxin-induced migration of immune cells in vitro and reduce gastric mucosal injury induced by ischemia-reperfusion and hypertension in vivo (Shen et al., 1998; Terashita et al., 1987; Yoshikawa et al., 1992). CV-6209 also reduces Shiga toxin-induced development of hemorrhagic lesions, accumulation of neutrophils, and production of tumor necrosis factor α (Tashiro et al., 1994; Zhang et

Fig. 2. Scanning electron micrographs showing *H. pylori* morphological damage from CV-6209 or anti-*H. pylori* agents.

H. pylori cells (strain C7M) were incubated in BHI broth supplemented with 10% FBS for 5 h at 37°C in the presence or absence of the drug, and then bound to plastic cover slips. Addition of the drugs: A, CV-6209 (8 MIC, 16 μg/ml); B, no drug; C, amoxicillin (1 MIC, 0.015 μg/ml); D, clarithromycin (1 MIC, 0.06 μg/ml); E, metronidazole (1 MIC, 2 μg/ml); F, lansoprazole (1 MIC, 2 μg/ml). Bars represent 2 μm. The arrow in C points to a short rod (with flagella) connected to coccoid body.

Fig. 3. Viable counts of *H. pylori* (A) and reduction of *H. pylori* cellular ATP levels after incubation with CV-6209 or lansoprazole (B).

In A, *H. pylori* strain C7M was incubated with 8 and 16 μg/ml of CV-6209 or 16 μg/ml of lansoprazole at 37°C, and portions of the samples were periodically taken and examined for viable counts. Mean ± SD. Closed triangle (with solid line), 8 μg/ml of CV-6209; closed circle (with solid line), 16 μg/ml of CV-6209; open circle (with solid line), no drug; open triangle (with dashed line), 16 μg/ml of lansoprazole. In B, reduction of *H. pylori* cellular ATP levels by various concentrations of CV-6209 or lansoprazole is shown. Mean ± SD. Closed circle (with solid line), CV-6209; open triangle (with dashed line), lansoprazole.

al., 2001). This study described the first anti-*H. pylori* effect of a PAF receptor antagonist, CV-6209. The direct binding of CV-6209 to *H. pylori* is still under inves-

Fig. 4. Time course of the inhibition of *H. pylori* motility by CV-6209 or lansoprazole.

Time courses of the inhibition of *H. pylori* motility by 1–64 μg/ml of CV-6209 or 16 μg/ml of lansoprazole are shown. Bacterial suspensions (of *H. pylori* strain C7M) were incubated at 37°C in the presence or absence of a drug, and portions of the samples were periodically taken and examined for motility. Mean ± SD. Open circle, no drug; closed diamond, 1 μg/ml of CV-6209; closed square, 8 μg/ml of CV-6209; closed circle, 16 μg/ml of CV-6209; closed triangle, 64 μg/ml of CV-6209; open triangle, 16 μg/ml of lansoprazole.

tigation. However, a previous study suggested that some anti-*H. pylori* antibodies developed during *H. pylori* infections recognize platelets, resulting in disruption of platelets and ITP development (Gasbarrini et al., 1998). Therefore, the presence of the PAF recep-

tor-like structures in *H. pylori* is possible. In contrast, PAF (C16 and C18 forms; Fig. 1C) and PAF receptor antagonist CV-3988 (Fig. 1B) showed no or much lesser effects on *H. pylori*. The target structure on *H. pylori* for CV-6209 remains to be clarified.

The *H. pylori* metabolic systems are also possible targets of CV-6209. CV-6209 caused a drastic morphological change (coccoid formation) of *H. pylori*; the coccoid forms induced by CV-6209 and amoxicillin seemed slightly distinct (the latter induced a coccoid form with a short rod body). Coccoid formation of *H. pylori* is induced not only by chemical factors (e.g., amoxicillin), but also by environmental factors [e.g., long-term culture, anaerobiosis (representing the conditions in the large intestine), and fresh water or seawater (representing the conditions in the river or sea)] (Andersen and Rasmussen, 2009; DeLoney and Schiller, 1999; Mizoguchi et al., 1998; Shirai et al., 2000). A previous report showed the significant reduction of 60-kDa penicillin-binding protein (PBP; a possible target of amoxicillin) and 63-kDa PBP in *H. pylori* coccoid induced by long-term culture (DeLoney and Schiller, 1999). The role of M23B family metallopeptidase in *H. pylori* coccoid formation was also suggested (Bonis et al., 2010). However, the molecular mechanisms which induce coccoid formation are largely unknown. Most probably as a result of coccoid formation, CV-6209 inhibited *H. pylori* motility. Moreover, CV-6209 inhibited *H. pylori* in vitro adherence to MKN74 cells (a human gastric epithelial cell line) (data not shown), consistent with *H. pylori* motility inhibition (motility is required for *H. pylori* adherence) (Eaton et al., 1992).

Lansoprazole is a *H. pylori* respiratory system inhibitor [the possible target of which is NADPH-quinone oxidoreductase system (complex I) of the respiratory chain], containing a pyridine ring and a benzimidazole in its molecular structure. Lansoprazole inhibits *H. pylori* growth and reduces *H. pylori* motility and intracellular ATP level (Nagata et al., 2001; Tsutsui et al., 2000). In this study, the effect of CV-6209 was bactericidal in contrast to that of lansoprazole (Tsutsui et al., 2000), and reduced *H. pylori* cellular ATP levels, to a greater extent than lansoprazole (Nagata et al., 2001). In contrast to lansoprazole, CV-6209 contains 2-(*N*-acetyl-*N*-carbonylaminomethyl)-1-ethylpyridinium in its molecular structure (Fig. 1A). Generally, some compounds containing a quaternary ammonium cation [including an alkyl pyridinium (e.g., cetylpyridinium chloride)] show bactericidal effects. It is also known that one of

the pyridinium metabolites, 1-methyl-4-phenylpyridinium ion (MPP⁺), inhibits the mitochondrial NADPH-quinone oxidoreductase system (complex I) of mammalian nerve cells (Gluck et al., 1994).

Denizot et al. (1990) reported that *H. pylori* enzymatically converted lyso-PAF (1-O-alkyl-*sn*-glycero-3-phosphorylcholine) to PAF (Fig. 1C), suggesting a role in gastric pathogenesis (Denizot et al., 1990). Unlike *H. pylori*-specific activity of CV-6209, PAF synthesis from lyso-PAF was also reported in *E. coli* (Denizot et al., 1989). Furthermore, CV-6209 is not a simple analogue of PAF and lyso-PAF. However, this converting enzyme may also be a possible target of CV-6209.

In conclusion, CV-6209, which is a PAF receptor antagonist acting on platelets, showed *H. pylori*-specific growth inhibition, suggesting the presence of a CV-6209 target structure in *H. pylori*. Although MIC levels of CV-6209 for *H. pylori* were at a similar level to those of a PPI, lansoprazole, in contrast to lansoprazole, CV-6209 action was bactericidal and CV-6209 reduced *H. pylori* cellular ATP levels to a greater extent. CV-6209 induced *H. pylori* coccoid formation and inhibited *H. pylori* motility and adherence. CV-6209 exerts its anti-*H. pylori* action in a novel fashion. Further study of the structure-activity relationship will provide the mechanisms of *H. pylori*-specific inhibitory action of CV-6209 that may contribute to novel anti-*H. pylori* drug development. Study in this field is also important to gain an understanding about mimic structures between human blood cells and *H. pylori* and autoimmune diseases.

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