Chapter

Development of a Novel Antibacterial Medicine that Targets a Characteristic Lipid of the Cell Membranes of *Helicobacter pylori*

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Abstract

*Helicobacter pylori* is one of the most prevalent causes of gastritis. This pathogen colonizes for many years human stomach and asymptotically leads the persons to chronic gastritis. The eradication of *H. pylori* from human stomach is, therefore, important in order to prevent the digestive diseases including peptic ulcers and gastric cancer that develop via chronic atrophic gastritis. Wide-spectrum antibiotics such as amoxicillin and metronidazole are used for the treatment for *H. pylori* infectious diseases. However, the *H. pylori* strains resistant to these antibiotics are increasing year by year around the world. On this basis, we need urgently to develop the antibacterial medicines that act on *H. pylori* with a novel mechanism. Recent studies by our group have demonstrated that *H. pylori* shows susceptibility to the bactericidal action of indene compounds derived from decomposition of vitamin D. The bactericidal action of indene compounds is selective not against commonplace bacteria but against *H. pylori*. The indene compounds turned out to target the *H. pylori*’s phosphatidylethanolamine that retains a myristic acid as the saturated fatty acid side chain. These findings will contribute to the development of new antibacterial medicines specialized to the treatment for *H. pylori* infectious diseases.

Keywords: *Helicobacter pylori*, phosphatidylethanolamine, myristic acid, vitamin D, indene compound

1. Introduction

*Helicobacter pylori* is a Gram-negative microaerophilic helical bacillus equipped with polar flagella as the motility organ. This bacterium colonizes human stomach and causes chronic atrophic gastritis [1]. In addition to gastritis, the patients infected with this pathogen are capable of having various digestive diseases such as gastric ulcer, duodenal ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer [2–7]. Therefore, the eradication of *H. pylori* from human stomach is aggressively carried out around the world. Wide-spectrum
antibiotics such as amoxicillin, metronidazole, and clarithromycin are used for the treatment for *H. pylori* infectious diseases. However, the *H. pylori* strains resistant to the wide-spectrum antibiotics have been increasing year after year [8]. Especially, almost all *H. pylori* strains clinically isolated from African people have been acquiring the resistance to amoxicillin and metronidazole. In addition, wide-spectrum antibiotics act not only on *H. pylori* but also on commonplace bacteria inhabiting the mucosa of mouth and intestines. Therefore, the patients infected with *H. pylori*, who orally take wide-spectrum antibiotics for the treatment, often suffer from side effects such as stomatitis, constipation, and loose bowels resulted from the collapse of the balance of either oral bacterial flora or enterobacterial flora. When the side effects are serious, the patients develop pseudomembranous colitis accompanied with bloody feces and are compelled to discontinue the eradication of *H. pylori* [9]. To solve the difficult problems on the chemotherapy, we have to develop a novel antibacterial medicine that acts on only *H. pylori* without affecting the survival of human mucosal bacterial flora.

The assimilation of exogenous cholesterol into the cell membranes is one of the unique biological features of *H. pylori*. A part of cholesterol assimilated into the cell membranes is modified with a α-glucose at the carbon position-3 of its steroid framework, and the cholesteryl glucosides generated are used as the bacterial cell membrane constituents [10]. A previous study by our group has revealed that *H. pylori* possesses at least three types of cholesteryl glucosides, cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside (CAG), and cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside (CPG) ([Figure 1](#)) [11]. In addition to the three cholesteryl glucosides, other researchers have identified the lyso-type of CPG [12]. CGL is synthesized by the catalytic action of cholesterol α-glucosyltransferase (CGT) that localizes to the cytoplasm-side of the inner membrane of *H. pylori* [13–15]. CGT transfers a glucose derived from a uridine diphosphate glucose (UDP-Glc) to the cholesterol. Although the enzyme proteins involved in the synthesis of either CAG or CPG remain to be clarified, a recent study by other group has demonstrated that the enzymatic activities for the synthesis of CAG and CPG are detected in *H. pylori*’s outer membrane and inner membrane, respectively [16]. In addition, both enzymatic activities for the synthesis of CAG and CPG turned out to use phosphatidylethanolamine (PE) as the substrate [16]. In sum, the CGL acyltransferase (CGLAT) transfers a fatty acid derived from PE to the CGL and thereby synthesizes CAG. Meanwhile, the CGL phosphatidyltransferase (CGLPT) transfers a phosphatidyl group derived from PE to the CGL and thereby synthesizes CPG.

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**Figure 1.**
*Chemical structures of cholesteryl glucosides found in *H. pylori* cell membranes. In addition to the three types of cholesteryl glucosides, *H. pylori* possesses the lyso-type of CPG that dissociated a myristic acid. CGL, cholesteryl-α-D-glucopyranoside; CAG, cholesteryl-6-O-tetradecanoyl-α-D-glucopyranoside; CPG, cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside; C14, myristic acid; C19, phytomonic acid.*
A number of studies, including our own, have revealed that *H. pylori* assimilates exogenous cholesterol in order to acquire the resistance to the antibacterial actions of antibiotics and lipophilic compounds [17–20]. Meanwhile, *H. pylori* glucosylates the assimilated cholesterol to evade the host immune systems and/or to detoxify the toxic steroid compounds with 3β-hydroxyl [12, 21]. The mechanism as for cholesterol uptake of *H. pylori*, however, remained for many years to be clarified. In a study in 2012, it has been revealed that PE of *H. pylori* cell membranes functions as a cholesterol-binding lipid [22]. PE is the most predominant glycerophospholipid component composing Gram-negative bacterial cell membranes. The PE of Gram-negative bacteria such as *Enterobacteriaceae* bacteria and *Pseudomonas aeruginosa* retains a palmitic acid (C\(_{16:0}\)) as the saturated fatty acid side chain, whereas the PE of *H. pylori* retains a myristic acid (C\(_{14:0}\)) as the saturated fatty acid side chain [22–26]. In sum, the PE molecular species composition of *H. pylori* turned out to completely differ from that of typical Gram-negative bacteria. A previous study by our group has demonstrated that the PE accounts for greater than 60% in the total lipids (excluding lipopolysaccharide) of *H. pylori* in the logarithmic growth phase [27]. Moreover, it has been revealed that the PE molecular species (DMPE) with two myristic acids accounts for approximately 30% in the total PE molecular species of *H. pylori* [22]. Intriguingly, DMPE showed higher binding affinity for cholesterol than for cholesteryl ester (Figure 2). In sum, the selective intermolecular interaction was found between the low-molecular-weight hydrophobic compounds.

Based on a number of studies including our own, the overview from the cholesterol assimilation to the cholesterol glucosylation was partially clarified in *H. pylori*: (1) cholesterol binds at least to DMPE of the outer membrane of *H. pylori* and is assimilated into the membranes; (2) a part of cholesterol is glucosylated by the catalytic action of CGT localized to the cytoplasm-side of the inner membrane, and thereby CGL is generated; (3) CGL is next exchanged to CAG and CPG by the enzymatic actions of CGLAT and CGLPT that localize to the outer membrane and the inner membrane, respectively (Figure 3). CGT utilizes an UDP-Glc as the glucose donor of cholesterol. Both enzymes of CGLAT and CGLPT utilize PE (myristoyl-PE) as the acyl group donor and phosphatidyl group donor of CGL.

![Figure 2. Binding affinity of dimyristoyl-phosphatidylethanolamine of *H. pylori* cell membranes for either cholesterol or cholesteryl ester. Dipalmitoyl-PE with two palmitic acids (C\(_{16:0}\)) shows the high binding affinity for both of cholesterol and cholesteryl ester, whereas dimyristoyl-PE (DMPE) with two myristic acids (C\(_{14:0}\)) shows the selective high binding affinity only for cholesterol.](image-url)
respectively. Incidentally, cholesterol assimilated into H. pylori is distributed to both of the inner and outer membranes, whereas cholesteryl glucosides (CGL, CAG, and CPG) synthesized by H. pylori predominantly localize to the outer membrane [17].

As described above, it has been revealed that DMPE is one of the most prevalent lipid components of H. pylori cell membranes and shows the unique interaction not with cholesteryl ester but with cholesterol. Apart from this, previous studies by our group have demonstrated that a steroid hormone, progesterone acts on the cholesterol-binding site in the H. pylori cell membranes and confers the bactericidal action to H. pylori [28, 29]. Although it was unclear as for whether the progesterone shows the selective binding affinity for DMPE, this steroid turned out to destabilize the cell membrane structure of H. pylori and to ultimately induce the bacteriolysis. These findings drove us to the investigations of the low-molecular-weight hydrophobic compounds that induce the serious structure change to the DMPE molecule through the specific interaction. This chapter mentions the bactericidal activity of the indene compounds against H. pylori.

2. Development of new antibacterial medicines for H. pylori

2.1 Finding of an indene compound as the anti-H. pylori substance

The anti-H. pylori activity of various steroidal compounds was investigated. As a consequence, 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ of seco steroids turned out to confer the strong bacteriolytic action to H. pylori [30]. However, these vitamin D₃ derivatives conspicuously attenuated their bactericidal activity by the nonbiological degradation (Table 1). In contrast, H. pylori did not almost
succumb to the bacteriolytic action of vitamin D$_3$. Surprisingly, the nonbiological degradation of vitamin D$_3$ augmented the bactericidal activity of its secosteroid against *H. pylori*. These results indicated that the vitamin D$_3$ derivatives directly act as bactericidal substances on *H. pylori*, and that some decomposition product of vitamin D$_3$ possesses potent bactericidal activity against *H. pylori*. It was, therefore, attempted to extract the anti-*H. pylori* substance from the decomposition products of vitamin D$_3$. As a consequence, the indene compound species (VDP1), otherwise known as Grundmann’s ketone, was successfully obtained [31, 32]. VDP1 is a low-molecular-weight hydrophobic compound in which the indene consisting of 5- and 6-membered rings of hydrocarbons was modified with alkyl, carbonyl, and methyl (Figure 4). Intriguingly, *H. pylori* showed high susceptibility to the bacteriolytic action of VDP1, whereas commonplace bacteria such as *Enterobacteriaceae* bacteria, *P. aeruginosa*, and *Staphylococcus aureus* showed insusceptibility to that of VDP1 [30]. In addition, VDP1 conferred the effective bacteriolytic action to *H. pylori* regardless of the assimilation of cholesterol into the cell membranes. These results indicate the possibility that VDP1 is a beneficial fundamental structure for the development of antibacterial medicines to selectively eradicate *H. pylori* without collapsing the balance of human mucosal bacterial flora.

2.2 Interaction between VDP1 and PE molecular species

The collapse induction activity of VDP1 against lipid vesicles was next examined using the unilamellar vesicles prepared with DMPE, dipalmitoyl-PE (DPPE), and dioleoyl-PE (DOPE). Intriguingly, VDP1 turned out to specifically induce the structure collapse of DMPE unilamellar vesicles without affecting the structural stability of either DPPE unilamellar vesicles or DOPE unilamellar vesicles (Figure 5). The structure collapse induction activity of VDP1 against DMPE unilamellar vesicles completely corresponded to the bactericidal activity of the indene compound.
against *H. pylori* that abundantly contains DMPE in the cell membranes. Based on these results, VDP1 was considered to specifically interact with the DMPE, to induce the serious structure change to the myristic acid side chain in the PE molecule, and to ultimately disrupt the vesicular conformation of DMPE. In addition, these results strongly suggested that VDP1 exerts the bactericidal effect on *H. pylori* by targeting at least DMPE of the cell membranes.

The intermolecular interaction between VDP1, DMPE, and DPPE was, therefore, simulated by the computational chemistry. One of the computer simulations showed that VDP1 induces "the winding-structure change" to a myristic acid side chain in DMPE molecule, while VDP1 induced no structure change to a palmitic acid side chain in DPPE molecule (Figure 5). The alkyl of VDP1 seemed to be crucial conformation for the induction of the structure change of the myristic acid side chain of DMPE. In other words, the slight difference of the length of carbon
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chain composing the fatty acids in PE molecules appeared to play an important role on the specific interaction between VDP1 and PE molecular species.

2.3 Bactericidal mechanism of the indene compound species

As described earlier, VDP1 was considered to confer the bacteriolytic action to *H. pylori* by the disruption of the cell membranes through the induction of the structure change of DMPE that is one of the most prevalent PE molecular species constructing the bacterial cell membranes. To investigate in detail the crucial conformation of VDP1 for exerting the structure collapse effect on DMPE unilamellar vesicles and for exerting the antibacterial effect on *H. pylori*, various VDP1 derivatives were chemically synthesized [33]. The structure collapse induction activity of VDP1 against DMPE unilamellar vesicles was already ascertained to almost completely correspond to the bactericidal activity of the indene compound against *H. pylori*. When the carbonyl of VDP1 was replaced with a hydroxyl, the indene compound (VD3-1) maintained both activities against DMPE unilamellar vesicles and *H. pylori* (Table 2). As seen in VDP1, VD3-1 turned out to confer no structure collapse induction activity against DPPE unilamellar vesicles. In addition, VD3-1 also had no influence on the viability of commonplace bacteria such as *Enterobacteriaceae* bacteria, *P. aeruginosa*, and *S. aureus*. Intriguingly, VD2-2 that lacks the alkyl chain of VD3-1 was ascertained to induce no structure collapses of either DMPE unilamellar vesicles or DPPE unilamellar vesicles and to completely forfeit the effective bactericidal activity against *H. pylori*. In combination with the result of computer simulation as for the intermolecular interaction between VDP1 and DMPE, these results indicate that the alkyl structure in the indene compound

| Indene compound | Collapse induction activity | Bactericidal activity | Summary |
|-----------------|-----------------------------|-----------------------|---------|
|                 | DMPE vesicle | DPPE vesicle | *H. pylori* | Others* |
| VDP1            | Potent       | Lack       | Potent       | Lack     |
| VD3-1           | Potent       | Lack       | Lack         | Lack     |
| VD2-2           | Lack         | Lack       | Lack         | Lack     |

'Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Serratia marcescens, Salmonella sp., Pseudomonas aeruginosa, and Staphylococcus aureus. VD3-1, (1R,3aR,7aR)-7a-methyl-1-((R)-6-methylheptan-2-yl)octahydro-1Hinden-4-ol; VD2-2, (1R,3aR,7aR)-1-((S)-1-hydroxypropan-2-yl)-7a-methyl octahydro-1Hinden-4-ol.

Table 2.
Relationship between indene compounds, PE unilamellar vesicles and *H. pylori."
species plays a crucial role on the induction of the structure change of the myristic acid side chain in DMPE molecule. Moreover, the alkyl structure in the indene compound species turned out to be essential for exerting the effective bacteriolytic action to *H. pylori*. The functional groups such as carbonyl and hydroxyl of the indene compound species seem to be significant conformation for bonding to the phosphate head in the PE molecules with an electrostatic attraction. Meanwhile, the indene-ring structure is guessed to be significant to stabilize the hydrophobic interaction between the indene compound species and PE molecules.

A recent study by our group has demonstrated that VDP1 confers the antibacterial action not only to *H. pylori* but also to other *Helicobacter* species [33]. Especially, *Helicobacter felis* showed high susceptibility to the bacteriolytic action of VDP1, as similar to *H. pylori*. *H. felis* is a Gram-negative microaerophilic spiral bacillus possessing bipolar tufts of flagella. This bacterium is isolated from the gastric mucosa of cats and dogs [34–36]. As seen in *H. pylori*, *H. felis* causes chronic gastritis and gastric MALT lymphoma in mouse when it colonizes the mouse stomach [37, 38]. An earlier study by our group has revealed that a myristic acid accounts for approximately 16% in the fatty acid composition of *H. felis* PE, and that the PE molecular species retaining a myristic acid and a palmitic acid accounts for approximately 37% in total PE molecular species of the bacteria [39]. Though *H. felis* completely succumbs to the bacteriolytic action at the same concentration of VDP1 (less than 3 μg/ml) that eradicates *H. pylori*, this *Helicobacter* species did not possess DMPE. On this basis, VDP1 is considered to interact not only with DMPE but also with myristoyl-PE that retains a myristic acid as one of the two fatty acid side chains. In addition, *H. felis* turned out to possess lauryl-PE as the most prevalent PE molecular species. The PE molecular species retaining a lauric acid (C12:0) and a palmitic acid accounted for approximately 40% in total PE molecular species of *H. felis*. In sum, large parts of PE molecular species of *H. felis* bind a palmitic acid and either a myristic acid or a lauric acid as the fatty acid side chains. Given that a lauric acid is shorter in the length of carbon chain than a myristic acid, we can assume that VDP1 collapses the vesicular conformation consisting not only of myristoyl-PE but also of lauryl-PE. In the future, it will need to elucidate the hydrophobic interaction between VDP1 and lauryl-PE in addition to myristoyl-PE.

In contrast, *Helicobacter cinaedi* showed low susceptibility to the bacteriolytic action of VDP1, even though the PE molecular species composition in *H. cinaedi* is similar to the PE molecular species composition in *H. felis* [39]. *H. cinaedi* is a Gram-negative rod-like bacillus equipped with bipolar flagella and isolated from the intestinal tracts and livers of various mammals such as human, dog, cat, and hamster [40–42]. Therefore, this bacterium is classified into the enterohepatic *Helicobacter* species [43, 44]. Meanwhile, *H. pylori* and *H. felis* are classified into the gastric *Helicobacter* species. Most of the persons infected with *H. cinaedi* have no clinical symptoms, but some persons suffer from systematic inflammations, namely phlegmone, arthritis, and meningitis, due to the bacteremia [45]. Although it is unclear as for why *H. cinaedi* is lower in the VDP1-susceptibility than the two *Helicobacter* species, the involvement of lipopolysaccharide (LPS) is considered as one possibility. LPS is a glycolipid constructed of a long polysaccharide chain and fatty acids and is one of the composition components of the outermost layer of the outer membrane of Gram-negative bacteria [46]. The part of polysaccharide chain in LPS comes into direct contact with the outsides of the bacterial cells and limits the membrane permeability of various lipophilic compounds. The LPS contents in *H. cinaedi* may be higher than those in *H. pylori* and *H. felis*. The membrane permeability of VDP1 through the LPS barrier may be, therefore, stricter in *H. cinaedi* than in *H. pylori* and *H. felis*. Further investigation will be necessary to compare the LPS contents between the *Helicobacter* species.
Based on the current studies, the following bactericidal mechanism of the indene compounds synthetically derived from vitamin D in *H. pylori* was proposed: the indene compounds bind to the myristoyl-PE (including DMPE) of *H. pylori* cell membranes, induce “the winding-structure change” to the myristic acid side chain
in the PE molecules, destabilize the membrane conformation, and ultimately confer the bacteriolytic action to *H. pylori* (Figure 6).

In the case of the cholesterol assimilation in *H. pylori*, cholesterol is distributed to both membranes of the outer membrane and the inner membrane, and a part of the cholesterol is, thereafter, metabolized to cholesteryl glucosides (CGL, CAG, and CPG), and these metabolites localize to the outer membrane (Figure 7). Cholesterol and cholesteryl glucosides have no influence on the stability of the cell membrane conformation of *H. pylori*, and rather these steroidal compounds serve to strengthen the membrane lipid barrier of the bacteria on the limitation of the permeability of lipophilic compounds. A recent study by other group has demonstrated that cholesteryl glucosides are responsible for the morphological maintenance of *H. pylori*, for the acquirement of resistance to antibiotics such as polymyxin B, colistin, and tetracycline, and for the promotion of biofilm formation [47]. This suggests that cholesteryl glucosides of *H. pylori* play an important role to limit the membrane permeability of various low-molecular-weight compounds. However, VDP1 confers the bacteriolytic action even to *H. pylori* retaining cholesterol and cholesteryl glucosides. Further investigation will be necessary to clarify the detailed intermolecular interactions between myristoyl-PE and steroidal compounds.

3. Conclusions

Almost all hydrophobic drugs are pharmacologically designed to inhibit the functions of either protein molecules or nucleic acids in the target creature species. However, no drugs that target a characteristic lipid molecule in the creatures are discovered for many years. In addition, a number of biochemists on lipid research leave great achievements in the analysis of biosynthetic enzymes of various lipophilic compounds such as fatty acids and complex lipids and in the identification of receptors of various hydrophobic ligands such as steroid hormones and eicosanoids. However, these achievements are not as for lipid itself but as for merely proteins. This chapter described the unique interaction between lipids: the indene compound species specifically disrupt the vesicular conformation consisting of DMPE. These findings will bring the new aspects to the drug discovery research and the lipid biochemistry research.

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Conflict of interest

The authors declare no conflict of interest.
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