

moval of the infecting pathogen.

Why did Kindt et al. choose duodenal mucosa over gastric biopsies for the histopathological evaluation in the FD patients? As is well known, FD symptoms are mainly attributable to disturbed gastric function. However, the approach of studying inflammation in the duodenum has been used previously⁵ and in fact, was reported to be more successful for detecting the changes in FD as compared to gastric biopsies.⁶ According to the report by Lee et al.,⁷ since duodenal acidification induces proximal gastric relaxation, increases the sensitivity to gastric distension, and inhibits gastric accommodation to a meal, duodenal mucosa is also an important sensory portion for the pathogenesis of FD. Furthermore, when histological inflammation is assessed by gastric biopsy, we have to bear in mind the possible co-existence of *Helicobacter pylori* (*H. pylori*) infection of the stomach. Even though the Rome III classification does not require ruling out of *H. pylori* infection for diagnosing FD,^{8,9} the *H. pylori*-colonized gastric mucosa exhibits significant levels of inflammatory cell infiltration with CD8⁺-, CD4⁺-T cells and macrophages.¹⁰⁻¹³ Even after the eradication of *H. pylori*, many mononuclear cells as T cells or macrophages persist in the mucosa. Such inflammatory changes present before and even after *H. pylori* eradication could play a significant role in the pathophysiology of this type of dyspepsia. Taken together, functional dyspepsia with a present or even past history of *H. pylori* infection should be considered as a different disease entity from FD, such as *H. pylori*-infectious FD or post-*H. pylori*-infectious FD.

In conclusion, the concept of PI-FD is potentially valid and the causal relationship between remnant inflammatory features and the gastroduodenal motor or sensory machinery should be further investigated. However, the major microorganism infecting the stomach, *H. pylori*, should not be overlooked when considering the pathophysiology of FD, especially in Asia.

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ORIGINAL ARTICLE

Homology model of the DNA gyrase enzyme of *Helicobacter pylori*, a target of quinolone-based eradication therapy

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Conflict of interest

The authors declare no conflicts of interests.

Abstract**Background and Aims:** Resistance of *Helicobacter pylori* to the standard therapeutic antimicrobial agents has been demonstrated. Although quinolones are an alternative candidate for third-line eradication therapy, quinolone resistance of *H. pylori* is also increasing. Quinolone resistance of *H. pylori* is caused by a point mutation of the DNA gyrase subunit A (GyrA) protein, especially on amino acids 87 and 91. The aim of this study is to surmise the structure of *H. pylori* GyrA.**Methods:** The modeling of the 3-D structure of *H. pylori* GyrA was performed by an automated homology modeling program: SWISS-MODEL. The position of amino acids 87 and 91 in *H. pylori* GyrA was plotted on the homology model. To estimate the function of quinolone resistance-determining region (QRDR), the structure of *H. pylori* GyrA was compared with *Escherichia coli* GyrA.**Results:** A molecular model of *H. pylori* GyrA could be predicted using SWISS-MODEL. The GyrA N- and C-terminal domains closely resembled those of *E. coli*. The position of amino acids 87 and 91 in *H. pylori* GyrA was part of the DNA binding region (head dimer interface) on the GyrA N-terminal domain.**Conclusion:** Our homology model of *H. pylori* GyrA suggests that the quinolone resistance-determining region is on the head dimer interface of the GyrA N-terminal domain.**Introduction**

Because *Helicobacter pylori* infection is linked to peptic ulcer disease and gastric cancer,¹⁻⁵ eradication is an important preventive measure. However, increasing bacterial resistance to clarithromycin and metronidazole is compromising the eradication of *H. pylori* and causing failures in therapy.^{6,7} One of the alternative candidates for third-line therapy is quinolones.⁸⁻¹³ Quinolones show marked activity against *H. pylori*, as well as having good oral absorption and no major side-effects.¹⁴ A recent *in vitro* study showed synergistic effects of quinolones and PPI against *H. pylori* strains.¹⁵

Quinolones exert their antimicrobial activity by inhibiting the enzyme, DNA gyrase,¹⁶ which introduces negative supercoils into the DNA (Fig. 1). DNA gyrase is involved in DNA replication, recombination and transcription. The bacterial enzyme, gyrase, is a tetramer consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. The resistance of *H. pylori* to quinolones is caused by point mutations in the so-called quinolone resistance-determining region (QRDR) of the *gyrA* gene,^{17,18} mainly involving amino acid substitutions at amino acid 87 (Asn to Lys) and amino acid 91 (Asp to Gly, Asp to Asn, and Asp to Tyr).^{19,20}

The aim of this study was to predict the structure of *H. pylori* GyrA and the function of the QRDR. This will help to determine the mechanism of the quinolone resistance of *H. pylori*, and to develop new drugs to overcome the resistance.

Methods

The modeling of the 3-D structure of *H. pylori* GyrA was performed by an automated homology modeling program: SWISS-MODEL.²¹⁻²³ SWISS-MODEL workspace²⁴ can be freely accessed at <http://swissmodel.expasy.org/workspace/>. We used the automatic modeling mode, and applied the protein sequence of *H. pylori* GyrA, which is available in GenBank (AAA74376.1). Obtained data of the homology model was visualized using DeepView ver. 4.0.1 (The Swiss Institute of Bioinformatics). The structure was compared with the structure of *Escherichia coli*'s GyrA N-terminal domain²⁵ (Protein Data Bank [PDB] code: 1ab4) and GyrA C-terminal domain²⁶ (PDB code: 1zi0B), which were also visualized using DeepView. The positions of amino acids 87 and 91 of *H. pylori* were plotted on the homology model.

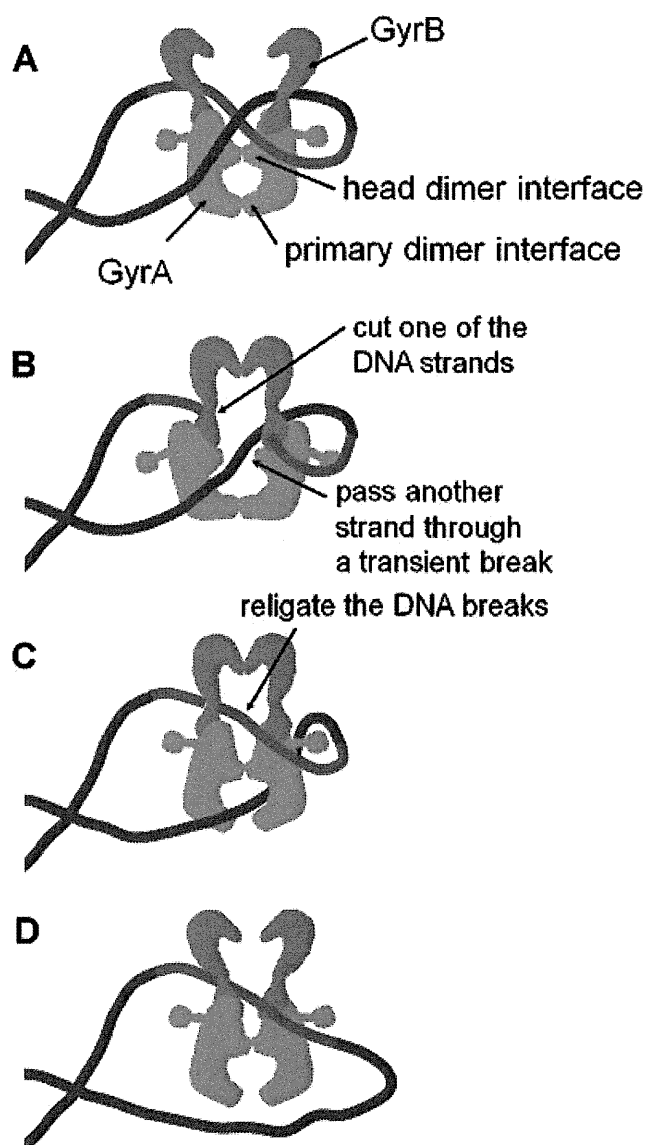


Figure 1 Function of DNA gyrase. (a) DNA gyrase is composed of two GyrA and two GyrB subunits. The two GyrA can bind at each primary dimer interface and head dimer interface. (b) DNA gyrase introduces a double-stranded break in the DNA. Another segment of DNA passes through the break to the opposite side of DNA gyrase. (c) The break in the strands is repaired. (d) One positive supercoil of DNA molecule is disentangled.

Results

According to GenBank (AAA74376.1), *H. pylori* GyrA consisted of 826 amino acids. Amino acids 37–497, which correspond to the *H. pylori* GyrA N-terminal domain, were based on the crystal structure of the topoisomerase IV subunit A (GrlA) from *Staphylococcus aureus* (PDB code: 2inrA). Amino acids 504–800, which correspond to the *H. pylori* GyrA C-terminal domain, were based on X-ray crystallography of the GyrA C-terminal domain from *E. coli* (PDB code: 1zi0B). The homology model of the *H. pylori* GyrA N-terminal domain closely resembled the crystal structure of

the *E. coli* GyrA N-terminal domain (Fig. 2). The positions of amino acids 87 and 91 on *H. pylori* GyrA corresponded to part of the head dimer interface of the *E. coli* GyrA N-terminal domain. The homology model of the *H. pylori* GyrA C-terminal domain also resembled the structure of the *E. coli* GyrA C-terminal domain (Fig. 3).

Discussion

Alignment of the *H. pylori* *gyrA* nucleotide sequence with that of *E. coli* also revealed that amino acids 87 and 91 in *H. pylori* correspond to amino acids 83 and 87 in *E. coli*.^{14,18,20,27–30} Point mutations of amino acids 83 and 87 are known hot spots for quinolone resistance in *E. coli*.^{31–33} It is proposed that the head dimer interface binds a segment of DNA, performs a double-stranded cleavage, and then pulls the broken ends of the DNA apart.³⁴ On the other hand, quinolones bind to a specific site on DNA in the DNA-enzyme complex.¹⁶ Quinolones do not prevent the cleavage of the DNA but they interfere with the strand passage and re-ligation by stabilizing an intermediate of the catalytic cycle.³⁵ The head dimer interface is known to bind both DNA and quinolones.³⁶ Taken together, a mutation on the head dimer interface would have either a lower affinity for quinolones or a higher affinity for DNA, and prevent quinolones from binding to the DNA-enzyme complex.

We previously demonstrated a high resistance rate (47.9%) to gatifloxacin after eradication failure in Japan, and a significant association between gatifloxacin resistance and *gyrA* mutations in *H. pylori*.³⁰ On the other hand, we also demonstrated that the minimum inhibitory concentration (MIC) of garenoxacin was fourfold lower and that of sitafloxacin was 16-fold lower than that of gatifloxacin against the *H. pylori* strains with *gyrA* mutations.³⁷ These results suggest that new generation quinolones, such as sitafloxacin and garenoxacin, may overcome the resistance of *H. pylori* with *gyrA* mutations. The mechanism by which new generation quinolones would be able to overcome the resistance is still unknown. New generation quinolones may be able to bind mutant GyrA and inhibit the function of DNA gyrase. It is reported that sitafloxacin has a stronger activity to interact with the so-called quinolone pocket in GyrB.³⁸ However, *gyrB* mutation has not been described as involved in the quinolone resistance of *H. pylori*.²⁸ This suggests that new generation quinolones may also be able to bind *H. pylori* GyrB stronger than other quinolones. Switching to these new generation quinolones may improve the efficacy of quinolone-based *H. pylori* eradication therapy.

The target of quinolones is not only DNA gyrase, but also topoisomerase IV, in several bacteria.^{39–42} Genome sequencing has revealed that *H. pylori* lacks the topoisomerase IV *parC* and *parE* genes.^{43,44} In addition, multidrug efflux pumps do not yet appear to mediate quinolone resistance,⁴⁵ which suggests that DNA gyrase in a unique target of quinolones in *H. pylori*. Therefore, it is important to investigate the structure of DNA gyrase in *H. pylori* in order to overcome the mechanism of quinolone resistance.

In conclusion, the structure of *H. pylori* GyrA resembles that of *E. coli* GyrA. The unique target of quinolones is supposed to be the head dimer interface of GyrA in *H. pylori*. Garenoxacin and sitafloxacin are likely to act differently to gatifloxacin against *H. pylori* GyrA.

Figure 2 Comparison between *Helicobacter pylori* GyrA N-terminal domain and *Escherichia coli* GyrA N-terminal domain. (a) Crystal structure of *E. coli* GyrA N-terminal domain (Protein Data Bank code: 1ab4). (b) Homology model of *H. pylori* GyrA N-terminal domain generated by SWISS-MODEL. The mutations of amino acids 87 and 91 on *H. pylori* GyrA (green region) cause quinolone resistance.

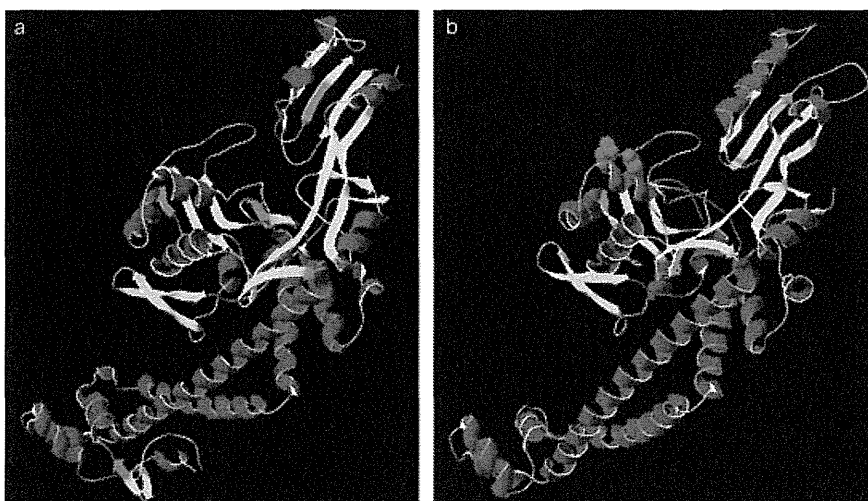
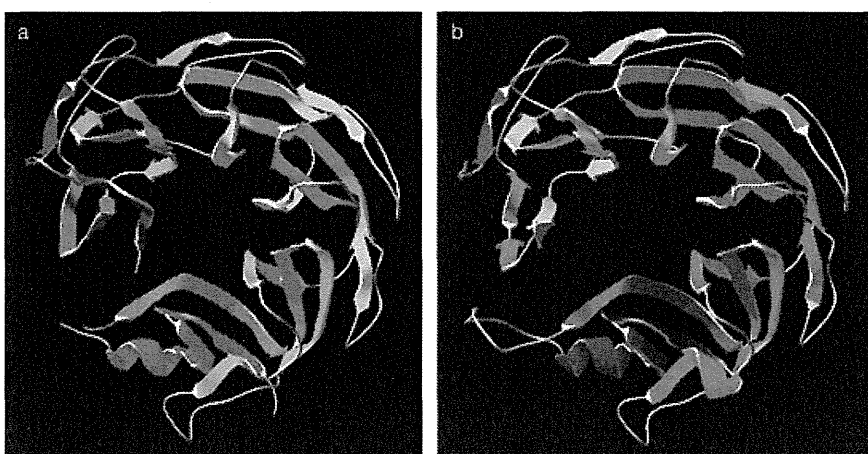


Figure 3 Comparison between *Helicobacter pylori* GyrA C-terminal domain and *Escherichia coli* GyrA C-terminal domain. (a) Structure of *E. coli* GyrA C-terminal domain (Protein Data Bank code: 1zi0B). (b) Homology model of *H. pylori* GyrA C-terminal domain generated by SWISS-MODEL.



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ORIGINAL ARTICLE

Contribution of efflux pumps to clarithromycin resistance in *Helicobacter pylori*Kenro Hirata,* Hidekazu Suzuki,* Toshihiro Nishizawa,[†] Hitoshi Tsugawa,* Hiroe Muraoka,[‡] Yoshimasa Saito,* Juntaro Matsuzaki* and Toshifumi Hibi*

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Key words

clarithromycin, efflux pump, *Helicobacter pylori*, Phe-Arg- β -naphthylamide.

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Conflict of interest

No conflict of interest has been declared by the authors.

Abstract

Background and Aims: Although clarithromycin (CLR) is one of the most commonly recommended component drugs of *Helicobacter pylori* eradication regimens, the prevalence of CLR-resistant *H. pylori* has been increasing. It is well known that CLR resistance is associated with point mutations in 23S rRNA, but an active multidrug efflux mechanism of *H. pylori* may also play a role in its drug resistance. At least four gene clusters have been identified as efflux pump systems in *H. pylori* and the present study was designed to investigate their role in the CLR resistance of clinical isolates of *H. pylori*.

Methods: Fifteen CLR-resistant *H. pylori* strains (minimal inhibitory concentration [MIC] $\geq 1 \mu\text{g/mL}$) isolated from patients at Keio University Hospital were examined for expression of efflux pump mRNA by real-time polymerase chain reaction. In addition, the MIC of CLR in the presence or absence of Phe-Arg- β -naphthylamide (PA β N), an efflux pump inhibitor (EPI), were determined.

Results: In all 15 strains, efflux pump mRNA was expressed, and the MIC of CLR were decreased by using EPI, despite possessing 23s rRNA point mutations. In addition, the MIC of CLR was decreased by the EPI in a concentration-dependent fashion.

Conclusion: The efflux pump of *H. pylori* is associated with the development of resistance to CLR, in addition to 23S rRNA point mutations. Efflux pumps could be a novel target for reversing drug resistance in *H. pylori*.

Introduction

For *Helicobacter pylori* infection, clarithromycin (CLR) is one of the most common drugs recommended as first-line eradication therapy and has been often used in combination with a proton pump inhibitor and amoxicillin.¹ CLR is a macrolide antibiotic that binds to the subunit 50S of the bacterial ribosome and inhibits the translation of peptides, thus preventing the bacteria from growing by interfering with their protein synthesis. However, the prevalence of CLR-resistant *H. pylori* has been gradually increasing in Japan.²

In pathogenic bacterium, the suspected mechanisms of acquired drug resistance include acquiring the ability to resolve drugs, changing the target region of the drug or permeability for the drug and having an active drug efflux mechanism. Versalovic *et al.* reported that CLR-resistant *H. pylori* strains often have 23S rRNA mutations (A2143G or A2144G)³ that change the target region of CLR and make it difficult for CLR to bind to 23S rRNA (which constitutes the subunit 50S of the ribosome). We also reported a significant correlation between the results of drug susceptibility testing and the incidence of 23S rRNA mutations.⁴

Having a drug efflux mechanism is also important for acquiring drug resistance and at least five conserved families of efflux systems associated with bacterial resistance to antibiotics have been identified.⁵ One of these, which is widespread among Gram-negative bacteria only, is the resistance-nodulation-cell division (RND) family.⁶ These efflux systems, including the ArcAB-TolC system in *Escherichia coli*⁷ and the MexAB-OprM system in *Pseudomonas aeruginosa*,⁸ are responsible for the intrinsic resistance of those bacteria to a wide variety of antimicrobial compounds. Recently, the role of the RND-type multidrug efflux pump in CLR resistance was reported for *Campylobacter jejuni*,⁹ and also that *H. pylori* contains an active RND efflux mechanism.¹⁰ Karin *et al.* reported four gene clusters (HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, HP1489–HP1487) as candidates for the efflux pump in *H. pylori*.¹¹

A few putative bacterial efflux pump inhibitors (EPI) have been described¹² and one of them, Phe-Arg- β -naphthylamide (PA β N), is active against RND efflux systems such as the AcrAB-TolC system in *E. coli*⁷ and the MexAB-OprM system in *P. aeruginosa*.¹³ In the presence of PA β N, a significant reduction in the minimal inhibitory concentration (MIC) for ciprofloxacin was reported for

clinical isolates of *E. coli* and *P. aeruginosa*, and this effect might be attributed to efflux pump inhibition.¹⁴ Pharmacological inhibition of efflux pumps might be an attractive strategy for reversing drug resistance in *H. pylori* and broadening the therapeutic options, so the present study was designed to investigate the contribution of efflux systems in CLR-resistant *H. pylori* strains by testing the efficacy of the EPI.

Methods

Clinical isolation of *H. pylori*

All of 18 CLR-resistant clinical isolates from patients at Keio University Hospital (16 men, two women; aged 57.2 ± 13.1 years, mean \pm standard deviation; September 2004 to June 2005) were examined. All strains were isolated after failure of the eradication therapy by the regimen including CLR. All patients provided informed consent before examination.

Culture conditions

The strain was maintained at -80°C in *Brucella* broth (Becton-Dickinson, Cockeysville, MD, USA) containing 25% (v/v) glycerol. The bacterium was cultured on Columbia HP agar (Becton-Dickinson, Cockeysville, MD, USA) for 2–5 days at 37°C under microaerobic conditions maintained with AnaeroPack MicroAero (Mitsubishi Gas, Tokyo, Japan).

Construction of structural model

Homology modeling of predicted amino acid sequence (HP0605, HP0606, HP0607, HP0971, HP0970, HP0969, HP1327, HP1328, HP1329, HP1489, HP1488 and HP1487) was performed in the SWISS-MODEL server (<http://swissmodel.expasy.org>)¹⁵ and handled by DeepView/Swiss PDB-viewer ver. 4.0.1 (<http://spdbv.vital-it.ch/>).¹⁵

Preparation of total RNA and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using a Promega SV Total RNA Isolation Kit (Promega, Madison, USA). The RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan) and used for RT-PCR analysis with a Dice system (Takara) using SYBR Premix Ex TaqII (Takara). The primer sequences were as follows: HP0605–HP0607 mRNA, forward primer 5'-AGC GCA AGA ACT CAG TGT CA-3' and reverse primer 5'-GCT TGG AGT TGT TGG GTG TT-3'; HP0971–HP0969 mRNA, forward primer 5'-TTA CCG GCA AAG GGA TAC G-3' and reverse primer 5'-AAA TTG GAT CGC TCG TTG TAT G-3'; HP1327–HP1329 mRNA, forward primer 5'-GCC AGG CTT GAT GAA GAA AA-3' and reverse primer 5'-TTA GCC TGC TTG CCG TAA AT-3'; and HP1489–HP1487 mRNA, forward primer 5'-TAG GCG CTC AAG TGG CTT AT-3' and reverse primer 5'-TCA GAT CGG GCA GAT TTT TC-3'. The PCR products were analyzed by agarose gel electrophoresis.

Examination of 23S rRNA point mutation

Total DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN Sciences, Germantown, MD, USA). We amplified it by PCR and direct-sequenced the 163-bp coding sequence of the region surrounding residue 2143 within the conserved loop of the 23S rRNA gene using the BigDye Terminator ver. 1.1 Cycle Sequencing Kit (Applied Biosystems, Scoresby, Victoria, Australia). The 23S rRNA gene primers were as follows: forward primer 5'-TCA ACC AGA GAT TCA GT-3' and reverse primer 5'-TCC ATA AGA GCC AAA GC-3'.¹⁶

Determination of MIC

The susceptibility of the *H. pylori* isolates to CLR was determined by the agar dilution method according to the guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS).¹⁷ Isolates were considered resistant to CLR if the MIC of the drug was $1 \mu\text{g}/\text{mL}$ or more. PA β N was purchased from Sigma-Aldrich (Steinheim, Germany). We determined the MIC of CLR in the presence or absence of, which was incorporated in the agar plates at a fixed concentration of 10, 20, 40, 60 or 120 mg/L in the EPI dose–response test, and 40 mg/L in the EPI alone test.

Results

Of the candidate 12 proteins (HP0605, HP0606, HP0607, HP0971, HP0970, HP0969, HP1327, HP1328, HP1329, HP1489, HP1488 and HP1487), three models (HP0605, HP0606, HP0970) were constructed by the SWISS-MODEL server (Fig. 1).¹⁵ The HP0605 protein had a TolC-like structure,¹⁸ and the HP0606 and HP0970

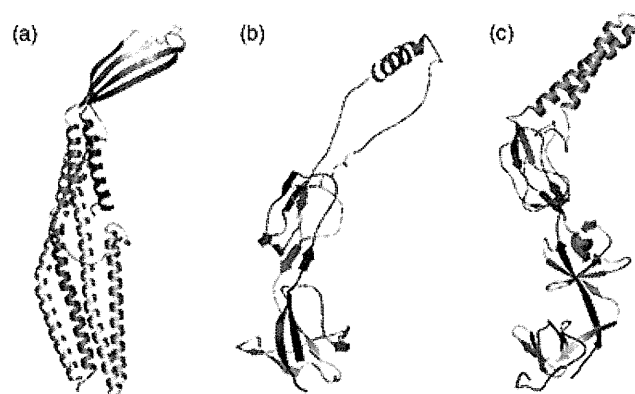


Figure 1 Homology modeling of the HP0605, HP0606 and HP0970 in the SWISS-MODEL server (<http://swissmodel.expasy.org>) and handled by DeepView/Swiss PDB-viewer ver. 4.0.1 (<http://spdbv.vital-it.ch/>).¹⁵ SWISS-MODEL structural protein of HP0605 (a), HP0606 (b) and HP0970 (c). The HP0605 protein has six α -helices and four β -sheets, a structure that is very similar to that of *Escherichia coli* TolC.¹⁸ The structure of HP0606 and HP0970 was also similar to that of *Pseudomonas aeruginosa* MexA.^{19,20} It is quite possible that HP0605, HP0606 and HP0970 have a role in the resistance-nodulation-cell division-type efflux system.

proteins had a MexA-like structure.^{19,20} The genetic homology data were able to confirm that they had the structure of an RND-type multidrug efflux pump.

The results for the MIC of CLR under changing concentrations of PAβN (10, 20, 40, 60 or 120 mg/L) are shown in Figure 2. The MIC of CLR decreased dose-dependently in response to the EPI, suggesting that RND-type efflux pumps contribute to CLR resistance in *H. pylori*. No MIC reduction occurred with PAβN alone at 40 mg/L without CLR (data not shown).

Using rt-PCR for CLR-resistant strains, we identified the expression of mRNA for each efflux pump (HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, HP1489–HP1487). HP0605–HP0607 mRNA was expressed in all 15 strains (100%). HP0971–HP0969 mRNA was expressed in 14 strains (93.3%) without KS0187, HP1327–HP1329 mRNA was expressed in 12 strains (80.0%) without KS0152, KS0187 and KS0188, and HP1489–HP1487 mRNA was expressed in 13 strains (86.7%)

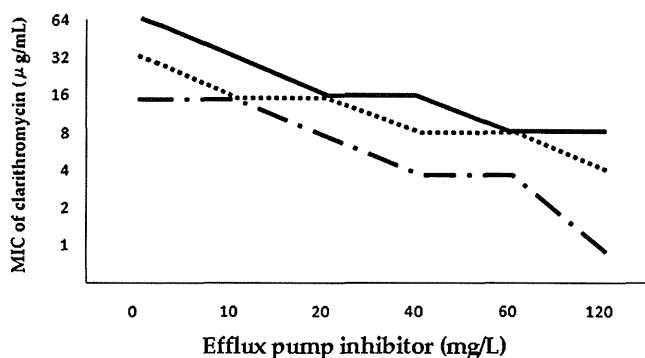


Figure 2 Minimal inhibitory concentration (MIC) of clarithromycin in the presence of various concentrations of the efflux pump inhibitor (Phe-Arg-β-naphthylamide). Each line indicates each of three clarithromycin (CLR)-resistant *Helicobacter pylori* strains: (.....) KS0143 (—) KS0147 (---) KS0157. The MIC of CLR in the presence of various concentrations of Phe-Arg-β-naphthylamide (0, 10, 20, 40, 60 or 120 mg/L) is shown.

without KS0142 and KS0158. Moreover, 10 strains (KS0145, KS0146, KS0149, KS0151, KS0154, KS0155, KS0162, KS0172, KS0178, KS0188) (66.7%) expressed all the efflux pump genes (Fig. 3).

The MIC of CLR in the presence or absence of 40 mg/L PAβN are shown in Figure 4 and Table 1. Remarkably, the MIC of CLR decreased in all 15 strains (100%). A fourfold or greater reduction in the MIC of CLR was observed for 14 of the 15 strains particularly (93.3%). In examining the 23S rRNA point mutation, we identified that KS0145 had the A2143G mutation, and 14 strains without KS0145 had A2144G mutations. Therefore, all strains showed an MIC reduction in the presence of PAβN, even though they had 23S rRNA mutations (A2143G or A2144G).

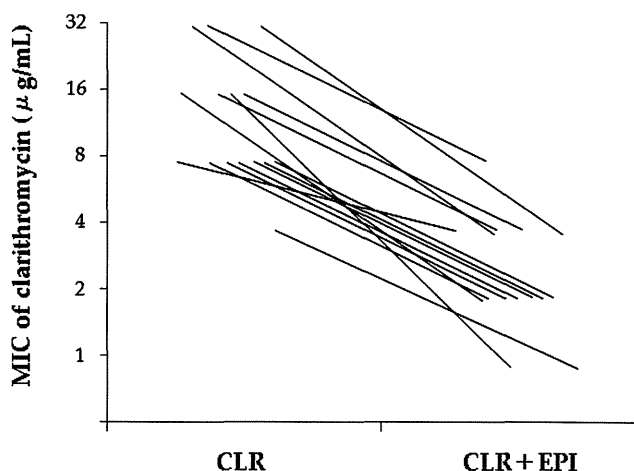


Figure 4 Alteration in the minimal inhibitory concentration (MIC) of clarithromycin (CLR) in the presence or absence of Phe-Arg-β-naphthylamide in 15 CLR-resistant *Helicobacter pylori* strains. The MIC decreased in all 15 strains (100.0%) and a fourfold or greater reduction was observed for 14 of the 15 strains particularly (93.3%). EPI, efflux pump inhibitor.

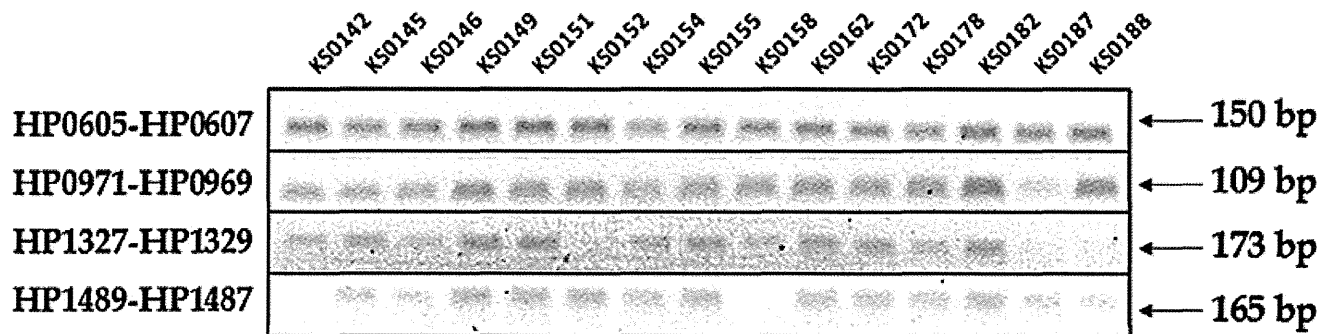


Figure 3 Efflux pump mRNA expression of 15 clarithromycin-resistant clinical isolates. Four efflux pump gene clusters (HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, HP1489–HP1487) mRNA expression were examined by real-time polymerase chain reaction. HP0605–HP0607 cDNA was 150 bp, HP0971–HP0969 cDNA was 109 bp, HP1327–HP1329 cDNA was 173 bp and HP1489–HP1487 cDNA was 165 bp. HP0605–HP0607 mRNA was expressed in all 15 strains (100%). HP0971–HP0969 mRNA was expressed in 14 strains (93.3%) without KS0187, HP1327–HP1329 mRNA was expressed in 12 strains (80.0%) without KS0152, KS0187 and KS0188, and HP1489–HP1487 mRNA was expressed in 13 strains (86.7%) without KS0142 and KS0158. Moreover, 10 strains (KS0145, KS0146, KS0149, KS0151, KS0154, KS0155, KS0162, KS0172, KS0178, KS0188) (66.7%) expressed all four efflux pump gene clusters.

Table 1 23S rRNA mutation and the minimal inhibitory concentration (MIC) of clarithromycin (CLR) in the presence or absence of an efflux pump inhibitor (EPI) for 15 *Helicobacter pylori* strains

Strain number	CLR MIC ($\mu\text{g}/\text{mL}$)		23S rRNA mutation
	CLR	CLR + EPI	
KS0142	8	2	A2144G
KS0145	16	4	A2143G
KS0146	8	2	A2144G
KS0149	8	2	A2144G
KS0151	32	4	A2144G
KS0152	4	1	A2144G
KS0154	8	2	A2144G
KS0155	32	4	A2144G
KS0158	16	4	A2144G
KS0162	16	1	A2144G
KS0172	32	8	A2144G
KS0178	8	4	A2144G
KS0182	8	2	A2144G
KS0187	8	2	A2144G
KS0188	16	2	A2144G

Discussion

Because PA β N is a broad-spectrum EPI capable of reversing the multidrug resistance phenotype of several Gram-negative bacteria,^{13,16} it is unknown which among four candidate efflux pumps (HP0605–HP0607, HP0971–HP0969, HP1327–HP1329, HP1489–HP1487) were inhibited by PA β N in *H. pylori*. Van Amsterdam *et al.* reported that the HP0605-knockout mutant appeared to be more susceptible to novobiocin than the wild type, whereas the HP1489-knockout mutant was more susceptible to ethidium bromide than the wild type.¹¹ Our data show that all of the 15 strains of *H. pylori* that we examined in the present study expressed HP0605–HP0607 mRNA, so this gene cluster may confer resistance to CLR.

In CLR-resistant strains, our data shows that all 15 strains express efflux pump mRNA (Fig. 3) and MIC of CLR was decreased in the presence of EPI (Fig. 4). On the other hand, efflux pump mRNA was also expressed in the CLR-susceptible strain.¹⁰ However, Kutschke *et al.* reported that there was no significant change of the MIC of CLR among a wild-type strain of *H. pylori* (ARHp80) and its three isogenic mutants that had the putative RND cytoplasmic membrane protein, HP0607, HP0969 and HP1329, genetically inactivated.²¹

One potential explanation for these data is that CLR is easier to bind to the bacterial ribosome than efflux pump in CLR-susceptible strains because they have no mutation of 23S rRNA. Therefore, in CLR-susceptible strains, no significant change of MIC was detected even after efflux pump interference. On the other hand, in CLR-resistant strains, because it is difficult for CLR to bind to bacterial ribosome because of 23S rRNA mutations, it becomes easy to bind to efflux pump relatively, resulting in the increase of MIC for CLR. In the presence of EPI, it appears that the ability to discharge CLR from the cell is suppressed, and then the CLR concentration inside the cell remains higher. Therefore, CLR binds to bacterial ribosome even with 23S rRNA mutation. Thus, the MIC of CLR was decreased in the presence of EPI. 23S rRNA mutation is thought to be more dominant in acquiring

resistance to CLR than the efflux pump mechanism. It is necessary to examine some CLR-resistant strains without 23S rRNA mutation. However, all of the CLR-resistant strains in the present study have 23S rRNA mutation.

Although our data show that the MIC of CLR was decreased to 1–8 $\mu\text{g}/\text{mL}$ in combination with EPI, these MIC still remained in the range of resistant values. Therefore, after EPI treatment, combination therapy including CLR may be more effective clinically. The reason for this effectiveness is considered to be the preventive effect of efflux pump systems. Namely, *H. pylori* may be exposed to higher concentration of CLR inside the cell after EPI treatment. Zullo *et al.* reported an alternative eradication regimen consisting of PPI and amoxicillin for 5 days followed by that consisting of PPI, CLR and 5-nitroimidazole for a further 5 days.²² This sequential therapy has been reported as being more effective than standard triple therapy for 7 days.²³ For this reason, amoxicillin may weaken bacterial cell walls in the initial phase of treatment. To take these ideas one step further, by adding EPI in the first 5 days of treatment, sequential therapy might be a more potent treatment for CLR-resistant strains.

The present data show that the efflux pump could be a novel target for restoring the CLR susceptibility of *H. pylori*. Furthermore, because the RND-type efflux pump is uniquely expressed in Gram-negative bacteria only, inhibition of the pump may be a safe treatment for humans.

In conclusion, not only 23S rRNA mutations but also expression of an efflux pump plays an important role in the acquiring of CLR resistance.

Acknowledgments

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VIEWPOINT

Acotiamide (Z-338) as a possible candidate for the treatment of functional dyspepsia

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Abstract

Acotiamide hydrochloride is a novel upper gastrointestinal (GI) motility modulator and stress regulator currently being developed for the treatment of functional dyspepsia (FD). The mechanism underlying the enhancement of GI motility by this agent has been proposed to be based on its muscarinic antagonism and inhibitory effects on acetylcholinesterase activity. Pathophysiological studies showed that acotiamide significantly improved both delayed gastric emptying and feeding inhibition in restraint stress-induced model, but did not affect both normal gastric emptying and feeding in intact animals, indicating that acotiamide exerted effects only on the impaired gastric emptying and feeding behavior. According to the clinical pilot study in Europe, acotiamide, at the dose of 100 mg t.i.d., showed to improve the symptoms and quality of life of patients with FD, indicating the need for larger scale symptomatic studies on the efficacy of acotiamide in patients with FD. The recent phase II studies conducted in Japan presented in this issue of the journal also confirmed that acotiamide, at the optimal dose of 100 mg, has potential therapeutic efficacy, especially for meal-related FD symptoms. Although a phase III study is on going, acotiamide is now expected as a novel treatment option for FD.

Keywords acetylcholine, acetylcholinesterase, muscarinic, prokinetics, Rome III, symptom.

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INTRODUCTION

Functional dyspepsia (FD) is a clinical syndrome characterized by symptoms originating from the region of the stomach and duodenum, in the absence of explainable organic disease.^{1,2} According to the Rome III criteria, the major symptoms of FD consist of bothersome postprandial fullness, early satiety, epigastralgia, and epigastric burning.^{1,2} The symptom pattern and the underlying pathophysiology of FD are heterogeneous. Visceral hypersensitivity in response to distension,³ impaired meal accommodation,⁴ and delayed gastric emptying^{5,6} have frequently been demonstrated in patients with FD. Furthermore, the involvement of several other mechanisms has also been suggested, including duodenal hypersensitivity to the luminal contents, small bowel dysmotility, *Helicobacter pylori* infection,^{7,8} psychological disturbances, and central nervous system (CNS) disorders.⁹

There are several therapeutic options, including herbal medicines,¹⁰ for patients with FD,² however, proven and satisfactory treatment strategies are still limited. As one of the possible strategies, treatment based on the underlying pathophysiological disorders mentioned above might seem to be a logical option. Unlike its analogs, the recently introduced acotiamide hydrochloride trihydrate (Z-338), [N-(N9,N9-diisopropylaminoethyl)-[2-(2-hydroxy-4,5-dimethoxy-benzoylamino)-1,3-thiazole-4-yl] carboxamide monohydrochloride trihydrate] has little affinity for serotonin 5-HT₂, 5-HT₃ and 5-HT₄ receptors, and weak affinity for dopamine D₂ receptors, whereas having a strong affinity for muscarinic M₁/M₂ receptors. Acotiamide exerts gastroprokinetic activity, partly by enhancement of acetylcholine (ACh) release via its antagonistic actions on the M₁, M₂ muscarinic

receptors, and partly by inhibiting acetylcholinesterase (AChE) activity. In addition, acotiamide may also act directly on the gut and indirectly on the CNS through the brain gut axis. Acotiamide is now expected as a novel treatment option for FD, especially the meal-related syndrome.

PHARMACOLOGICAL DATA OF ACOTIAMIDE

Although acotiamide enhances the gastrointestinal (GI) motility in conscious dogs, unlike conventional prokinetic agents such as itopride^{11,12} or mosapride,¹³ it has only weak affinity for the dopamine D₂ receptor and no affinity for the serotonin 5-HT₂, 5-HT₃, and 5-HT₄ receptors. It has been suggested that the drug enhances GI motility via facilitation of acetylcholine release from the enteric cholinergic nerve terminals.^{14,15}

According to a basic study,¹⁵ acotiamide inhibits the activity of AChE derived from human erythrocyte membranes and produces contraction of antrum preparations from the guinea pig stomach *in vitro*, and enhances the GI motility in conscious dogs and gastric emptying in rats and dogs *in vivo*. Among the muscarinic M₁, M₂, and M₃ receptors present in the neurons and/or smooth muscle cells of the GI tract, acotiamide acts as an antagonist on the M₁ and M₂ receptors.¹⁵ These muscarinic receptors appear to be located on the cholinergic nerve terminals and to operate as autoreceptors. The release of ACh from cholinergic nerves is modulated by a negative feedback mechanism that is triggered by the stimulation of presynaptic muscarinic receptors. In the enteric nervous system, the release of ACh from the guinea pig myenteric and submucosal plexus neurons is inhibited by the presynaptic M₁ receptor^{16,17} and M₂ receptor in the rat antral mucosal or submucosal neurons.¹⁸ Acotiamide facilitates ACh release from the cholinergic nerve terminals by blocking muscarinic M₁ and M₂ autoreceptors which regulate the release of ACh.^{15,19} In *Xenopus* oocytes expressing M₁ and M₂ muscarinic receptors, acotiamide did not produce any response, but inhibited ACh-induced outward currents, indicating that the drug acts as an antagonist on the M₁ and M₂ muscarinic receptors.¹⁵

According to a report by Ogishima *et al.*,¹⁵ contractions of strips of the guinea pig stomach were sensitive to tetrodotoxin and atropine, and were enhanced by acotiamide, probably due to the facilitation of ACh release by the drug. They also showed that acotiamide enhanced the electrically stimulated outflow of tritium as a measure of ³H ACh release from antral strips.²⁰

In the guinea pig ileum, scopolamine has been shown to greatly facilitate the stimulated release of

ACh in the presence of a cholinesterase inhibitor.²¹ Thus, ACh release is clearly enhanced by inhibition of muscarinic autoreceptors by muscarinic antagonists when the cholinesterase activity is inhibited. The facilitation of ACh release in the presence of an M₁ receptor antagonist (pirenzepine) or M₂ receptor antagonist¹⁵ could be attributed to blockade of the presynaptic M₁ and M₂ autoreceptors that are activated by ACh released from the nerve terminals. These findings suggest that acotiamide apparently does not increase ACh release simply by inhibiting acetylcholinesterase.

IN VIVO STUDIES

In an animal model, acotiamide reversed stress-induced delayed gastric emptying and feeding inhibition through inhibition of the expression of stress-induced genes in the hypothalamus and medulla oblongata.²² Although the delayed gastric emptying was reversed by acotiamide in several animal models, normal or non-dysfunctional gastric emptying was unaffected by acotiamide, suggesting that acotiamide may alter the regulation of stress responses, to specifically improve the symptoms of FD caused or aggravated by stress.

The hypothalamic paraventricular nucleus (PVN) is a major site of the production and release of corticotropin-releasing factor (CRF). Released CRF activates the hypothalamic–pituitary–adrenal (HPA) axis, and also affects motility and sensitivity of the gut.²³ DNA microarray analysis of the hypothalamus and medulla oblongata in rats²² showed that acotiamide exerted an impact on the expression of genes related to the expression of neuromedin U (NmU), known as a stress-related neuropeptide²⁴ that suppresses food intake and induces delayed gastric emptying,^{25,26} and to GABA/glutamate, which are major excitatory and inhibitory neurotransmitters within the CNS,²⁷ and mediates restraint stress-induced delayed gastric emptying.^{28,29} Following administration of acotiamide, although the expression of NmU mRNA in the hypothalamus and the increase of its expression induced by restraint stress were suppressed, the delayed gastric emptying induced by administration of exogenous NmU or CRF was not reversed,²² suggesting that while acotiamide may have a partial effect on the stress response, it does not reduce stress.

Taken together with the results of these animal studies, it is concluded that acotiamide is most probably to reverse delayed gastric emptying, feeding inhibition, and altered expression of stress-related genes in the brain stem that are induced by restraint stress.²²

CLINICAL STUDIES OF ACOTIAMIDE

Previously, a double-blind, parallel-group trial of 12 weeks duration (after a 2 weeks PPI run-in and 2 weeks washout) randomized patients with FD (based on Rome II criteria) to placebo, 300 mg acotiamide, 600 mg acotiamide and 900 mg acotiamide, all three times daily.³⁰ According to this study, therapeutic gain was highest in the first month. In terms of the influence of PPI run-in, pH monitoring and nutrient tolerance on efficacy outcomes of acotiamide, the yield of a PPI run-in was low, and a nutrient challenge test, but not pH monitoring, would predict responsiveness of acotiamide.³¹ Among dose ranges, acotiamide 300 mg significantly improved postprandial epigastric pain and nausea at 4 weeks, and significantly improving 3 of 5 quality of life Nepean Dyspepsia Index scales. According to their report, adverse events were minor and similar across all arms.³⁰

Then, Tack *et al.* conducted a randomized, double-blind, placebo-controlled, parallel-group study on 71 patients from eight European centres (62 evaluable) with lower doses of acotiamide (50, 100, and 300 mg). In their pilot study, although there was no effect of the drug on gastric emptying as evaluated by the ¹³C octanoic acid breath test and sensitivity to distension based on gastric barostat studies, acotiamide exhibited the potential to improve FD symptoms as assessed by the overall symptom scores based on the severity and frequency of nine dyspeptic symptoms, and also the quality of life (QOL) as assessed by the SF-36 questionnaire. In that study, 300 mg was better than placebo for meal accommodation and 100 mg acotiamide was better than placebo at week 2 for upper abdominal bloating and the overall symptom score, and at week 3 for bloating and heartburn.³² Moreover, 100 mg acotiamide was also better than placebo for the QOL (physical function). This pilot study concluded that at the dose of 100 mg t.i.d., acotiamide showed the potential to improve the symptoms and QOL of patients with FD, indicating the need for larger scale symptomatic studies on the efficacy of acotiamide in patients with FD.

Although the optimal endpoint to be used in FD clinical trials has not yet been established, 'the subjects' global assessment of overall treatment efficacy (OTE)', recommended for use as the primary endpoint by the Rome group, evaluates improvement and worsening by means of a 7-point symmetrical Likert scale, it overcomes some of the limitations of a binary endpoint. Matsueda *et al.* also chose the improvement rate of 'the subjects' global assessment of OTE' at the final evaluation as the primary endo-

point in their study conducted to determine the dose-dependent therapeutic efficacy of acotiamide in patients with FD. They reported the results of two randomized, double blind, placebo controlled, parallel-group, comparative studies of acotiamide in Japanese FD patients in the present issue of *Neurogastroenterology and Motility*.³³ Both studies consisted of a baseline period of 8 days, followed by a randomized controlled treatment period of 28 days, during which the patients were allocated to placebo or different doses of acotiamide (50, 100 or 300 mg t.i.d.). In their study 1, the OTE was 41.7% in the placebo group, 51.5% in the 100-mg cohort, and 49.5% in the 300-mg cohort, while those in their study 2, the values were 49.1% in the placebo group, 48.7% in the 50-mg cohort, 58.3% in the 100-mg cohort, and 56.9% in the 300-mg cohort, suggesting that the improvement rates were approximately 10% higher in the 100-mg acotiamide cohorts than in the placebo groups, with good reproducibility. The elimination rate of postprandial fullness in the 100-mg acotiamide cohort was significantly higher than that in the placebo group. In the absence of effective treatment options for FD, the 10% gain over placebo may be considered as clinically and scientifically significant. The phase II studies conducted by the same group confirmed that acotiamide, at the optimal dose of 100 mg, has potential therapeutic efficacy, especially for meal-related FD symptoms. At present the results of phase III studies on the efficacy of 100 mg acotiamide in FD patients are awaited.

SUMMARY

Functional dyspepsia is a highly prevalent condition with few efficacious treatments. Although GI prokinetics have long been considered the therapeutic options of choice for the treatment of FD, on the basis of the assumption that delayed gastric emptying or poorly coordinated antro-pyloro-duodenal coordination was present in a majority of these patients, as there are poor correlation between delayed emptying and symptoms and the frequent co-existence of other sensorimotor disorders, the efficacy of prokinetics is usually limited. Acotiamide is a novel class of prokinetic drugs, which enhance acetylcholine release, and would be efficacious for the FD therapy both by central and by peripheral way. As was shown in the results of recent phase II trials, acotiamide has been shown to have a symptomatic benefit for the treatment of FD, especially for meal-related syndrome, without major adverse effects. As generally known, management of patients with functional GI disorders has become more difficult because several

candidate drugs were associated with significant adverse events leading to the withdrawal of medication such as cisapride³⁴ and tegaserod.³⁵ For this

reason, we need to be very careful for its safety and dose selection, based on the further results of well-organized clinical trials.

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Acid Suppression by Proton Pump Inhibitors Enhances Aquaporin-4 and KCNQ1 Expression in Gastric Fundic Parietal Cells in Mouse

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Abstract

Background The widespread use of proton pump inhibitors (PPIs) is known to cause sporadic gastric fundic gland polyps (FGPs). Altered expression and localization of the water or ion transport proteins might contribute to the excess fluid secretion into the cystic lumen for the development of FGPs.

Aims We investigated the alteration of the murine gastric fundic mucosa after PPI treatment, and examined the expression of water channel aquaporin-4 (AQP4) and potassium channel KCNQ1, which are expressed only in the parietal cells in the gastric mucosa.

Methods Male 5-week-old C57BL/6J mice were administered lansoprazole (LPZ) by subcutaneous injection for 8 weeks. The expression of AQP4 and KCNQ1 were investigated by Western blotting, quantitative RT-PCR, and immunohistochemistry. The expression of mucin-6 (Muc6), pepsinogen, and sonic hedgehog (Shh) were also investigated as mucosal cell lineage markers.

Results Gastric mucosal hyperplasia with multiple cystic dilatations, exhibiting similar histological findings to the FGPs, was observed in the LPZ-treated mice. An increase in

the number of AQP4-positive parietal cells and KCNQ1-positive parietal cells was observed. The extension of the distribution of AQP4-positive cells toward the surface of the fundic glands was also observed. The expression levels of AQP4 mRNA and protein were significantly enhanced. The expression of KCNQ1 mRNA was correlated with that of AQP4 mRNA in the LPZ-treated mice. Mucous neck-to-zymogenic cell lineage differentiation was delayed in association with decreased expression of Shh in the LPZ-treated mice.

Conclusions PPI administration increased the number of parietal cells with enhanced expression of AQP4 and KCNQ1.

Keywords Proton pump inhibitors · Aquaporins · Potassium channels · Fundic gland polyps

Introduction

Proton pump inhibitors (PPIs) strongly inhibit the function of H^+/K^+ -ATPase in gastric parietal cells, causing profound suppression of acid secretion. The use of PPIs has become widespread for the treatment of peptic ulcer disease and gastroesophageal reflux disease [1]. Such widespread use of PPIs has recently come to be known to be associated with the formation of gastric sporadic fundic gland polyps (FGPs), particularly in patients with *Helicobacter pylori*-free stomachs [2, 3]. FGPs are the most common gastric polyps [4]. A recent study showed that the prevalence of gastric polyps in the esophagogastroduodenoscopy population was 6.4% in the USA, and 77% of these lesions were FGPs [5]. FGPs are defined as cystic dilatations of the oxyntic glands lined by variably flattened parietal and chief cells with or without foveolar

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cells [6–8]. Although the mechanism of the development of the cystic dilatations in the fundic glands is not fully understood, increased fluid secretion into the cystic lumen is believed to play an important role.

The aquaporins (AQPs) are a family of small integral plasma membrane proteins that primarily transport water across the plasma membrane driven by osmotic gradients [9–11]. Although aquaporin-4 (AQP4) is expressed predominantly on the basolateral membrane of the parietal cells, the function of AQP4 in the stomach still remains unknown. In AQP4 knockout mice, a tendency towards decrease in fluid secretion in the fundic glands has been observed [12]. A recent study suggested that the expression of AQP4 played key roles in the proliferation of gastric epithelial cells and tumor biology [13].

KCNQ1 and its subunit KCNE2 are also expressed predominantly in the parietal cells [14], and both proteins unite to form a voltage-gated potassium channel [15–17]. KCNQ1 channel blockers inhibit acid secretion [14, 16, 17], and both KCNQ1-deficient and KCNE2-deficient mice exhibit loss of acid secretion [18–21], suggesting an essential role of KCNQ1 in acid secretion.

Since PPIs exert histamine-, gastrin- and acetylcholine-independent inhibition of acid secretion, chronic PPI usage is known to induce hypergastrinemia [22, 23]. In gastrin knockout mice, in which the parietal cells are reduced in number and the mRNA expression of the H^+/K^+ -ATPase β subunit is reduced [24], the expressions of AQP4 and KCNQ1 were decreased and was reversed by gastrin supplementation [25]. Therefore, we hypothesized that the expression of AQP4 and KCNQ1 may be altered by PPI administration, and that the altered expression and localization of these transport proteins might contribute to the development of FGPs associated with PPI administration.

High-dose and long-term PPI treatment has been reported to cause various morphological changes in the gastric fundic glands of rats, such as increase in the number of small mucous neck cells, of cystic degenerative cells with amorphous eosinophilic contents and of acinar-cell-like cells with red granules, and decrease in the number of chief cells [26]. In addition, we previously reported that gastric hypochlorhydria caused impaired mucous neck-to-zymogenic cell lineage differentiation in the fundic glands of *H. pylori*-colonized Mongolian gerbils [27, 28] and histamine H_2 receptor (H_2R)-null mice, with suppressed sonic hedgehog (Shh) expression [22, 29, 30]. Shh is believed to regulate epithelial cell differentiation in the adult stomach [28, 31, 32]. All of these results of previous studies prompted us to examine whether gastric acid suppression by PPI administration might influence Shh expression and the homeostasis of the gastric mucosa.

Interestingly, AQP4-positive parietal cells are localized principally in the lower part of the gastric fundic glands

[33]. In addition, KCNQ1 expression is stronger in the lower parietal cells than in the upper parietal cells, although KCNQ1-positive parietal cells are widely observed in the isthmus and neck region of the fundic glands [17, 34]. These findings suggest that the functions of parietal cells may differ depending on their vertical localization in the gastric fundic glands, and that AQP4 and KCNQ1 could be useful markers to investigate impaired parietal cell differentiation.

In the present study, we showed the disturbance of parietal cell differentiation and mucous neck-to-zymogenic cell lineage differentiation with enhanced expression of AQP4 and KCNQ1 in the parietal cells.

Methods

Animals

Male 5-week-old C57BL/6J mice were purchased from Sankyo Labo (Tokyo, Japan). All the mice were used for the study after acclimatization for 1 week. The proton pump inhibitor, lansoprazole (LPZ), was gifted from Takeda Pharm. Co., Ltd. (Tokyo, Japan). The mice in the LPZ-treated group ($n = 10$) were administered LPZ at the dose of 30 mg/kg, 1 x/day, by subcutaneous injection for 8 weeks. The mice in the control group ($n = 11$) were administered vehicle (0.5% CMC solution) by subcutaneous injection for 8 weeks. The experimental mice were killed after 24 h of food deprivation, although access to water was not restricted, and the stomachs were resected and blood samples obtained. The gastric surface pH was measured with a pH meter using a flat probe (D-51, Horiba Ltd, Kyoto, Japan). The blood samples were withdrawn from the orbital plexus of the experimental mice with a capillary tube. The samples were centrifuged at $6,000 \times g$ for 15 min to separate the serum. Gastrin concentrations were determined by radioimmunoassay using human gastrin-17 as the standard (Mitsubishi Chemical Medience, Tokyo, Japan). All the experiments and procedures in the present study were approved by the Keio University Animal Research Committee.

Histology, Immunohistochemistry, and Immunofluorescence

The lesser curvature of the stomach tissue specimens from the experimental mice were fixed in 10% neutralized buffered formalin, embedded in paraffin, placed on poly-L-lysine-pretreated slides, and then stained with hematoxylin and eosin (H&E) for histological examination.

The antibodies used for the immunohistochemistry are listed below: rat monoclonal anti-Ki67 antibody (TEC-3,

1:25, Dako Japan, Tokyo, Japan), rabbit polyclonal anti-AQP4 antibody (AB3068, 1:100, Chemicon, CA, USA), goat polyclonal anti-KCNQ1 antibody (sc-10646, 1:100, Santa Cruz, CA, USA), mouse monoclonal anti-H⁺/K⁺-ATPase α subunit antibody (clone 1H9, 1:300, Research Diagnostics, NJ, USA), rabbit anti-mouse pepsinogen C antibody (mPep; a kind gift from Dr. Yasushi Fukushima), and mouse monoclonal anti-Muc6 antibody (HIK1083, 1:30, Kanto Chemical, Tokyo, Japan).

For immunohistochemistry, after deparaffinization and rehydration, the antigens were retrieved by heating in citrate buffer (10 mM, pH 6.0) for anti-mouse pepsinogen C antibody (121°C, 15 min), anti-AQP4 antibody (105°C, 10 min), and anti-KCNQ1 antibody (105°C, 10 min). For anti-Ki67 antibody, anti-H⁺/K⁺-ATPase and anti-Muc6 antibodies, the sections were digested with proteinase K solution (Dako Japan, Tokyo, Japan) for 4 min at room temperature. After the antigen retrieval, endogenous non-specific peroxidases were quenched with 0.3% hydrogen peroxide. Nonspecific binding was blocked by a blocking reagent (Protein Block, Dako Japan). For H⁺/K⁺-ATPase and Muc6 staining, the Nichirei Histofine Mouse Staining Kit (Nichirei, Tokyo, Japan) was used in order to eliminate endogenous mouse Ig cross-staining. Sections were incubated overnight with each of the primary antibodies at 4°C. After rinsing in TBS-T, the slides were incubated with HRP-labeled anti-rat IgG, anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG (HISTOFINE, Simple stain MAX-PO (Rat/Goat/Rabbit/Mouse; Nichirei)) for 30 min at room temperature. Thereafter, staining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution. Counterstaining was performed with Gill's hematoxylin.

For immunofluorescent double staining for H⁺/K⁺-ATPase and AQP4, primary antibody reaction was performed in a way similar to that described above. Immunoreactivity was detected using Alexa Fluor 568 goat anti-mouse IgG and 488 goat anti-rabbit IgG (1:1000, Molecular Probes, OR, USA).

For morphometric analysis of H⁺/K⁺-ATPase, AQP4, or KCNQ1-positive cells, ten randomly chosen micrographs of the gastric fundic glands (50 μ m wide from the bottom of the glands to the mucosal surface) were obtained from each immunostained specimen, and the mean numbers of H⁺/K⁺-ATPase-, AQP4-, or KCNQ1-positive cells on the micrographs were compared between the LPZ-treated and the control mice.

Western Blotting

Liquid nitrogen frozen specimens of the stomach were homogenized in ice-cold RIPA Buffer (Upstate, Temecula, CA, USA) containing protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO), incubated on ice for 30 min,

and centrifuged at 10,000 \times *g* for 15 min; supernatant was used for the experiments. The total protein concentration was measured with the BCATM Protein Assay kit (PIERCE, Rockford, IL, USA). Proteins were separated by 12.5% NuPAGE Bis-Tris gel (Invitrogen, CA, USA) electrophoresis using MOPS running buffer (Invitrogen) in the presence of an antioxidant reagent (Invitrogen). Proteins were then transferred onto polyvinylidene difluoride membranes (Invitrogen). Blots were blocked with a solution containing Block Ace (Dainippon Sumitomo pharma Co., Osaka, Japan) for 1 h at room temperature and probed with anti-AQP4 antibody (AB3068, 1:1000, Chemicon, CA, USA) followed by reprobing with anti- β -actin antibody (1:20000, clone: AC-74, Sigma) as the loading control. Signal detection of the positive bands was facilitated by enhanced chemiluminescence assay using ECL plus (GE Healthcare, Uppsala, Sweden). Band quantitation was performed using the ImageJ program (National Institutes of Health, Maryland, USA).

RNA Purification and Quantitative RT-PCR Analysis

Total RNA was extracted from each stomach tissue specimen using the RNeasy Mini Kit (Qiagen, CA, USA) and DNase treatment was performed with an RNase-free DNase kit (Qiagen). RNA was converted into cDNA using the PrimeScript RT reagent kit (Takara, Ohtsu, Japan). The cDNA was diluted ten-fold and used for quantitative PCR analysis with Dice (Takara) using SYBR Premix Ex TaqII (Takara). The mRNA expressions of mouse AQP4, KCNQ1, H⁺/K⁺-ATPase α subunit (ATP4a), Shh, Muc6, pepsinogen C, and β -actin were measured. The primer sequences are shown in Supplemental Table S1. Data for each gene were normalized to the expression level of β -actin.

Statistical Analysis

All the data were expressed as mean \pm standard error. Statistical significance of the differences between two groups was evaluated using unpaired Student's *t* test. All the statistical analyses were performed using SPSS software for Windows, version 17.0J (SPSS Japan, Tokyo, Japan). A two-sided *p* value of <0.05 was considered as denoting statistical significance.

Results

PPI Administration Induced Mucosal Hyperplasia with Multiple Cystic Dilatations

The gastric mucosal surface pH was higher in the LPZ-treated mice (*n* = 10) than in the control mice (*n* = 11)

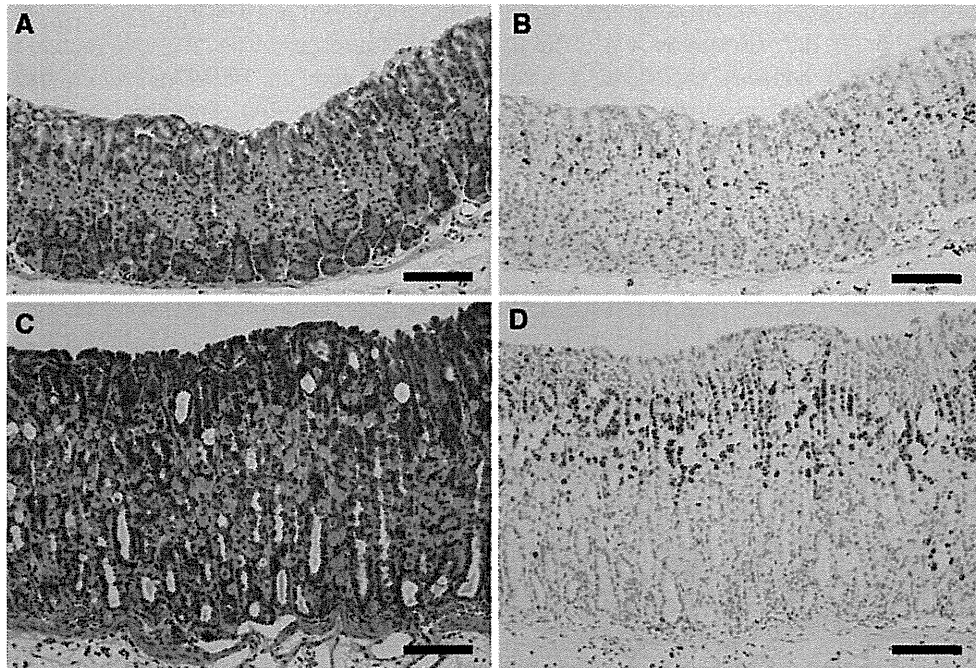


Fig. 1 Hyperplasia with multiple cystic dilatations after PPI administration. **a** Representative histology (H&E stain) in the control mouse. **b** Representative immunohistochemistry for Ki67 in the control mouse; Ki67-positive cells are localized in the proliferative zone at the neck region of the fundic glands. **c** Representative

histology (H&E stain) in the LPZ-treated mouse; gastric mucosal hyperplasia with multiple cystic dilatations are observed. **d** Representative immunohistochemistry for Ki67 in the LPZ-treated mouse; Ki67-positive cells are observed widely in the isthmus and neck region of the fundic glands ($\times 200$, bars = 100 μm)

(LPZ 6.7 ± 0.1 ; Cont. 3.4 ± 1.7 ; $p = 0.002$). Serum gastrin levels were also higher in the LPZ-treated mice than in the control mice (LPZ 285 ± 40 pg/ml; Cont. 184 ± 26 pg/ml; $p = 0.049$).

The results of the histological analysis revealed gastric mucosal hyperplasia with multiple cystic dilatations in eight of 10 LPZ-treated mice, although no polyp formation could be observed (Fig. 1c). Inflammatory cell infiltrations in the submucosa and lamina propria were not observed. The number of Ki67-positive cells was significantly increased in the LPZ-treated mice compared to that in the control mice (Fig. 1b, d), suggesting that LPZ administration induced gastric mucosal hyperproliferation.

Disturbed Parietal Cell Differentiation After PPI Administration

The results of immunohistochemical analysis revealed that, in the control mice, AQP4-positive parietal cells were localized in the basal region (Fig. 2b). The KCNQ1-positive parietal cells were distributed at a slightly lower level than the H^+/K^+ -ATPase-positive parietal cells in the fundic glands (Fig. 2a, c). This finding is consistent with the results of the previous study, which showed that H^+/K^+ -ATPase-specific signal was stronger in parietal cells from the upper part of the fundic glands, and KCNQ1 expression

is stronger in the basal parietal cells [34]. On the other hand, in the LPZ-treated mice, the distribution of AQP4-positive parietal cells extended upward to the neck region of the fundic glands (Fig. 2e). A significant increase in the number of KCNQ1-positive parietal cells was observed in the LPZ-treated mice (Fig. 2f). The results of morphometric analysis also supported the findings of the significant increase in the number of AQP4-positive parietal cells and KCNQ1-positive parietal cells (Fig. 2h, i), while the number of H^+/K^+ -ATPase-positive parietal cells was not significantly different between the LPZ-treated mice and the control mice (Fig. 2g).

The results of immunofluorescent double staining for AQP4 and H^+/K^+ -ATPase revealed that AQP4 was expressed predominantly on the basolateral membrane of the parietal cells in the lower part of the fundic glands (Fig. 3a). In the LPZ-treated mice, the extension of AQP4-positive parietal cells towards the neck region of the fundic glands is clearly observed. H^+/K^+ -ATPase-positive parietal cells became smaller in size after LPZ treatment.

Taken together, PPI administration induced the alteration of the distribution of AQP4-positive parietal cells, and increased the number of both AQP4-positive cells and KCNQ1-positive cells in the fundic glands. These results suggest the disturbance of parietal cell differentiation.

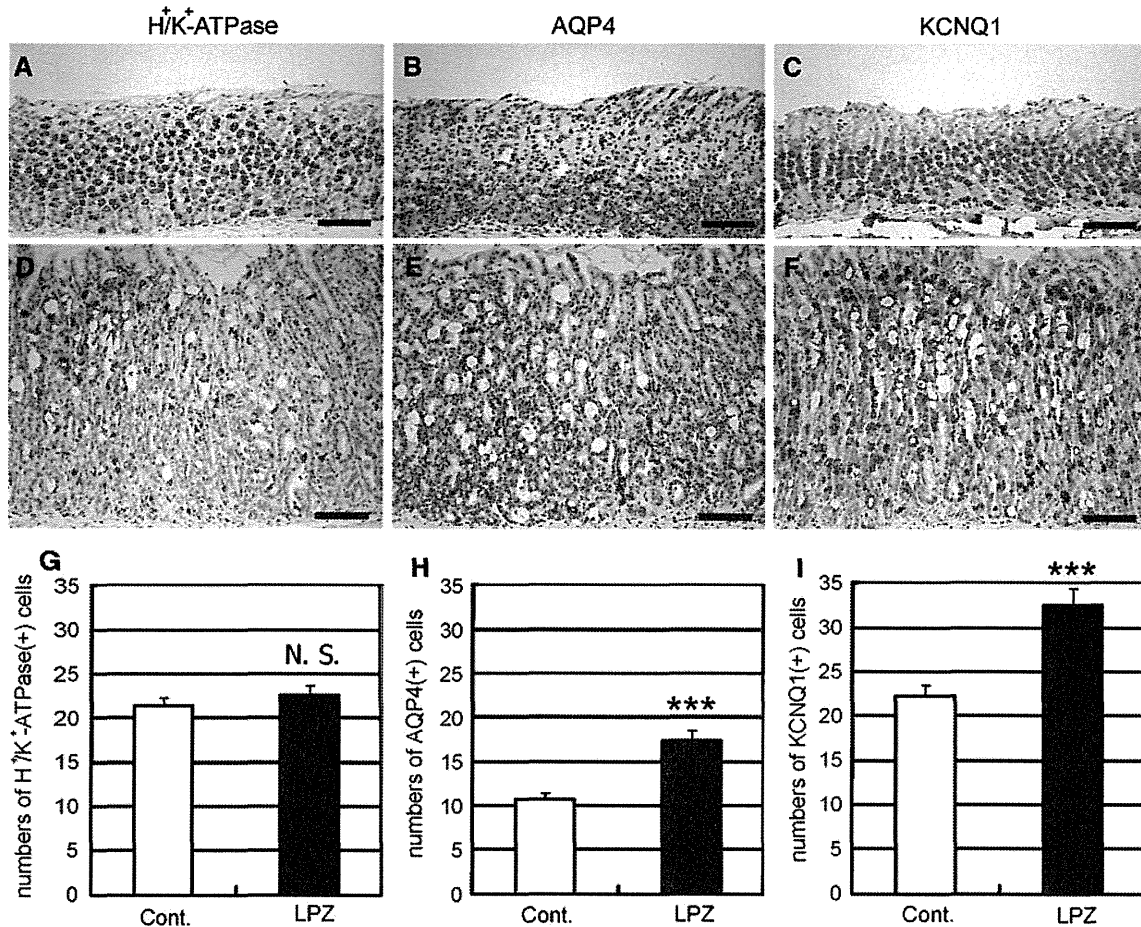


Fig. 2 Impaired parietal cell differentiation after PPI administration. **a** Representative immunohistochemistry for H⁺/K⁺-ATPase in the control mouse; H⁺/K⁺-ATPase-positive parietal cells are observed in the isthmus and neck region of the fundic glands. **b** Representative immunohistochemistry for AQP4 in the control mouse; AQP4-positive parietal cells are mainly localized in the lower part of the fundic glands. **c** Representative immunohistochemistry for KCNQ1 in the control mouse; KCNQ1-positive parietal cells are observed in the isthmus and neck region of the fundic glands. The KCNQ1-positive parietal cells were distributed at a slightly lower level than the H⁺/K⁺-ATPase-positive parietal cells in the fundic glands. **d** Representative immunohistochemistry for H⁺/K⁺-ATPase in the LPZ-treated mouse; the distribution of H⁺/K⁺-ATPase-positive parietal cells extend to the entire length of the fundic glands. **e** Representative

immunohistochemistry for AQP4 in the LPZ-treated mouse; the distribution of AQP4-positive parietal cells extends towards the neck region of the fundic glands are observed. **f** Representative immunohistochemistry for KCNQ1 in the LPZ-treated mouse; the distribution of KCNQ1-positive parietal cells extend to the entire length of the fundic glands (×200, bars = 100 μm). **g–i** Morphometric analysis of H⁺/K⁺-ATPase, AQP4 or KCNQ1-positive parietal cells; significant increase in the mean numbers of both AQP4-positive parietal cells and KCNQ1-positive parietal cells are observed in the LPZ-treated mice, while the mean number of H⁺/K⁺-ATPase-positive parietal cells is not different between in the LPZ-treated mice and in the control mice (means ± SE, *** *p* < 0.001, *n* = 10 LPZ-treated mice, *n* = 11 control mice)

Increased Expression of AQP4 and KCNQ1 and Decreased Expression of Sonic Hedgehog in the Gastric Parietal Cells After PPI Administration

The result of Western blotting revealed that the gastric mucosa of the LPZ-treated mice exhibited a higher expression level of the AQP4 protein than that of the control mice (Fig. 4). The expression level of the AQP4 mRNA also increased in the LPZ-treated mice (Fig. 5a). In addition, the expression of KCNQ1 mRNA tended to

be increased in the LPZ-treated mice (*p* < 0.1; Fig. 5b). The expression of KCNQ1 mRNA was correlated with that of AQP4 mRNA in the LPZ-treated mice, but not in the controls (Fig. 5c, d). Moreover, the mRNA expression of Shh, which is also expressed in the parietal cells, was decreased in the LPZ-treated mice as compared with that in the control mice (Fig. 5f). However, no significant difference in the expression of H⁺/K⁺-ATPase mRNA was noted between the LPZ-treated and control mice (Fig. 5e).