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Effect of mupirocin in *Helicobacter pylori in vitro*

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Abstract

Mupirocin (MUP) is an effective antibiotic against MRSA. Its bactericidal effect is stable under acid condition. By validating its antibacterial effect of *Helicobacter pylori*, we try to clarify MUP effect on *H. pylori*. The present study was conducted to investigate the effect of MUP on clarithromycin (CLR) / metronidazole (MNZ) -resistant and -susceptible strains of *H. pylori*, the time-kill effect of MUP, and the post antibiotic effect (PAE). We investigated the minimal inhibitory concentration (MIC) and the minimal bactericidal effect (MBC) of MUP against 140 *H. pylori*, which include clinical strains, ATCC43504, 26695 and J99. Ten of them were CLR -resistant strains and 3 were MNZ-resistant strains. The MIC_{90} and MBC of MUP on all 140 strains is 0.064 μg / ml, and 0.1 μg / ml, respectively. There were no differences of MUP effect between susceptible and resistant strains either for CLR or MNZ. Time-kill curve test and PAE test of MUP on ATCC43504 were performed. By adding MUP, time-kill curve showed that bacterial quantities decreased in dose and time-dependent manner. No viable colony was found after 12-hour culture with 0.1 μg / ml MUP. The value of PAE is 12. MUP is a potential effective antibiotic for *H. pylori* even those for CLR / MNZ -resistant strains.

Keywords: *Helicobacter pylori*; Mupirocin; Time-kill curve; Electron microscopy; Postantibiotic effect

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that infects the human gastric mucosa [1]. It infects approximately 80% of the population in many developing countries and about 30% in developed countries [2],[3]. This bacterium plays an important role in the pathogenesis of chronic gastritis, peptic ulcer diseases, gastric carcinomas, and gastric marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue type [4, 5, 6, 7]. The National Institutes of Health Consensus Development Conference has recommended the addition of antimicrobial agents to antisecretory drugs for the treatment of patients with metronidazole (MNZ), are most often used [8]. As antimicrobial agents, including amoxicillin, clarithromycin (CLR), and MNZ, are most often used, therapy with two of the three antibiotics and an antisecretory drug usually achieves an eradication rate above 80% [9][10]. However, resistance to these antibiotics in *H. pylori* is increasing and becoming a major problem in eradication treatment of *H. pylori* [11].

In Japan, resistance to CLR and MNZ was detected in 20.0% and 15.0% of the patients, respectively and dual resistance to CLR and MNZ was detected in 8.3% patients [12]. Given that CLR and MNZ have a stronger antibacterial effect on *H. pylori* than other agents, the presence of resistant microbes may result in eradication failure. The identification of new antibacterial agents is therefore strongly desirable.

MUP is a topical antibiotic that is used for the treatment of impetigo due to *staphylococci* and *streptococci* [13]. It is a naturally occurring agent produced by *Pseudomonas fluorescens* and has successfully been used to reduce substantially

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the nasal and hand carriage of MRSA. MUP binds to the isoleucyl-tRNA synthetase (IIeS) target in the vicinity of an ATP-binding subsite and is a bifunctional inhibitor with characteristics of both isoleucine and ATP. It was thought that the unique mode of action and the low incidence of purely low-level resistance from early studies would have made higher degrees of resistance a rather remote possibly.

MUP has properties that make it a good candidate for use in gastric *H. pylori* infections [14]. Because its unique chemical structure, mode of action, lack of cross-resistance to existing antimicrobial agents, stability, and higher activity at acid pH against bacteria included in its spectrum, water solubility. However, there was no further study of MUP against *H. pylori*.

The present study was conducted to investigate the effect of MUP on CLR/MNZ -resistant and -susceptible strains of *H. pylori*, the time-kill effect of MUP, and the postantibiotic effect (PAE).

2. Material and methods

2.1. Antibiotics

Mupirocin was obtained from the United States Pharmacopeial Convention, Inc. CLR was kindly provided from Sankyo Co. ltd. MNZ was purchased from Sigma Aldrich Co.

2.2. Bacterial strains

One hundred forty *H. pylori* strains including a quality control strain (ATCC43504), two standard strains, (26695, J99), and clinical isolates were measured [15]. The clinical isolates were from lesion biopsy specimens obtained by endoscopy from different patients of gastroenterological disease at Nagoya University Hospital and a regional health-care center. At first time, stock cultures of *H. pylori* were grown for 4 days on brucella broth agar plates (BB agar) (Difco Inc., Detroit, MI, USA) supplemented with 7% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Rockville, MD, USA) at 37 °C. in a microaerophilic atmosphere. Broth cultures of *H. pylori* were prepared by sub cultural colonies from freshly cultured agar plates into brucella broth (BB) (Difco Inc.) supplemented with 7% FCS for 48 hours at 37 °C. in a microaerophilic atmosphere. The identification of *H. pylori* was confirmed by characteristic colony morphology, Gram's stain and positive reactions in urease, catalase, and oxidase tests.

2.3. Susceptibility testing

The minimal inhibitory concentration (MIC) for *H. pylori* were determined by an agar dilution method with minor modification [16]. Briefly, Müeller-Hinton agar (M-H agar) (Difco Inc.) plates (20 ml/each) were prepared containing 7% horse blood and twofold serial dilutions of the antibiotics. They were inoculated with 5 µl of each bacterial suspension (10^7 CFU / mL) and incubated at 37 °C for 3 days in a microaerobic atmosphere. An antibiotics-free plate and plates with corresponding dilution were used as negative controls to ensure bacteria viability and no contaminants in the inoculums.

Susceptibility of CLR and MNZ -resistant strains was determined by the agar dilution method. The MIC of more than 1 µg/mL and more than 8 µg/mL were considered to show CLR and MNZ resistance, respectively. The MIC of MUP was defined as the lowest concentration agent that inhibited visible growth of isolates completely by the agar dilution method on M-H agar (Difco Inc.) plates supplemented with 7% FCS. Briefly, all isolates were incubated for 4 days on BB agar at the first time. After this incubation, inocula were prepared by suspending growth from BB agar plates with agents in PBS equivalent to a 2.0 McFarland standard. Final inoculums of 10^5 CFU / spot were applied to M-H agar. All plates were incubated for 3 days at 37 °C in a microaerophilic atmosphere. In a second method, the minimal bactericidal concentrations (MBC), defined as the lowest concentration of MUP that killed isolates completely, were determined using liquid culture with agents. In general, bacteria were inoculated with both medium and respective compound in 25 mL tissue culture flasks (Becton, Dickinson and Company Ltd, Franklin Lakes, NJ, USA), and an equal number was retained as control. Subculturing from freshly cultured BB agar plates was carried out in the BB liquid culture. After 2 days, samples of liquid culture with bacteria were removed, plated on BB agar, incubated for 3 days, and the number of colonies forming unit (CFU) was counted.

2.4. Time kills curve experiment

The time course of antibacterial effects was determined using strain ATCC43504. Briefly, bacteria cultured for 4 days on BB agar were diluted with fresh BB to ~10^8 CFU/mL. A final inoculum of 10^6 CFU/mL was applied into a 25 mL tissue culture flask containing 10 mL of the same medium with MUP. Broth cultures were incubated for 2 days at 37 °C. in a

microaerophilic atmosphere. At 0, 12, 24, 36 and 48 hours, samples were removed and 0.1 mL of 10-fold serial dilutions was plated onto BB agar plates. The number of colonies growing on the plates after 3 days' incubation was counted.

2.5. Electron microscopy analysis

The approximately one drop of the organisms from control and treated cultures was applied to a Formvar/carbon-coated 300-mesh copper grid (Nisshin EM, Tokyo, Japan), the excess solution was removed, and 2% phosphotungstic acid (PTA) (Fujifilm wako pure chemical co., Osaka, Japan) was added for negative staining. The samples were then observed under a transmission electron microscope (JEM2000EX; JEOL, Tokyo, Japan) and digital images were taken with a MegaView Slow-scan camera (JEOL).

2.6. Determination of PAE

PAE in bacteria was measured with some modification [17][18]. After four days' growth of strain ATCC43504 on BB agar, the bacteria were harvested into PBS and diluted with BB to ~10^8 CFU/mL, and a final inoculum of 10^5 CFU/mL was applied to BB agar. MUP was added into the broth contained in a 25 mL tissue culture flask at a concentration of 1 μg / mL. After incubation for 8 hours, the antibiotics were removed by diluting 1:10^3 into BB and incubated for 36 hours. Samples were collected for viable counts (CFU/mL) every 4 hours, plated onto BB agar plates, and incubated for 3 days. Viability curves were then determined. The control culture, which was not exposed to any antimicrobial agents, was treated similarly. For quantitation of the PAE, viable counts were determined before and after antibiotic exposure. The values of T and C were the time required for the CFU count in the MUP treated and an untreated culture to increase 10-fold above the count observed immediately after antibiotic removal, respectively. As a, the PAE value was calculated in T-C.

2.7. Statistical analysis

The degree of significance between means was determined using the paired *t*-test. A *p*-value of < 0.05 was regarded as significant. These studies were repeated at least 5 times to confirm the reliability of the data.

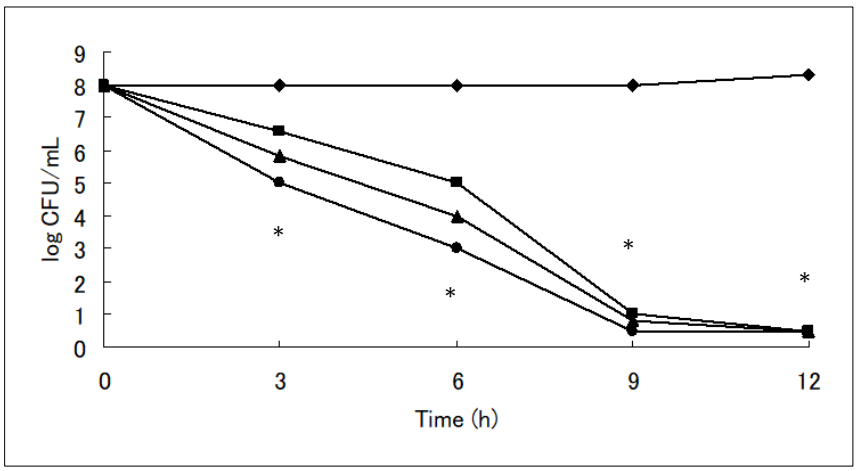
3. Results

3.1. MIC and MBC of MUP

We checked 140 *H. pylori* strains including a quality control strain (ATCC43504), two standard strains, (26695, J99). Our study showed that ten of them were CLR-resistant strains and 3 were MNZ-resistant strains. MIC_{90} and MBC of MUP on all 140 strains is 0.064 μg / ml and 0.1 μg / ml, respectively. There were no differences of MUP effect between susceptible and resistant strains either for CLR or MNZ.

3.2. Time kill curve experiment with MUP

The results of time-kill curve experiments with MUP alone at a concentration of 0 to 10 μg/mL are shown in Figure 1.



♦:untreatment, ■:MUP 0.1 μg / mL, ▲:MUP 1 μg / mL, ●:MUP 10 μg / mL. *: $p < 0.05$ compared to untreatment

Figure 1 Time-kill curve test of MUP

The number of CFU increased according to time course when no MUP was present in the culture medium. When MUP was present, however, a significant decrease in CFU was seen at 12 hours at all concentrations ($p < 0.05$). These results show that the effect of MUP was bactericidal, because the CFU was not stable but was decreased by MUP administration.

3.3. Morphological changes of *H. pylori* exposed to MUP in a liquid medium

The morphological changes of *H. pylori* cells exposed to 0.1 μg/mL of MUP for both 0, 1 and 3 hours were examined by electron microscopy in comparison with the morphology of untreated control cells (Figure 2). The control cells appeared as slightly curved bacilliforms with bluntly rounded ends, and had two or more unipolar, sheathed flagella. (Figure 2(A)). In contrast to the untreated control cells, most of the MUP-treated cells appeared as atypical bacilliforms were also observed. The form of *H. pylori* turned to coccoid after 1 hour. (Figure 2(B)) After three hours, electron microscopy confirmed the development of bubble like-formation of bacterial cells (Figure 2(C)).

3.4. PAE of MUP

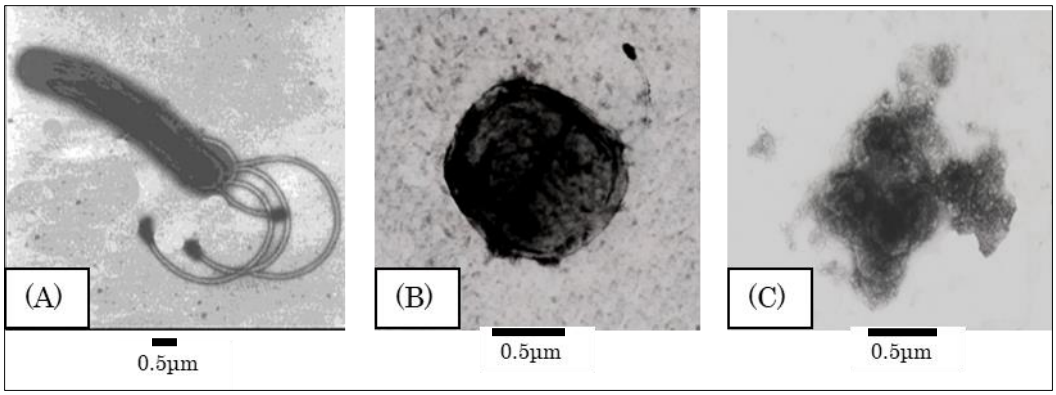
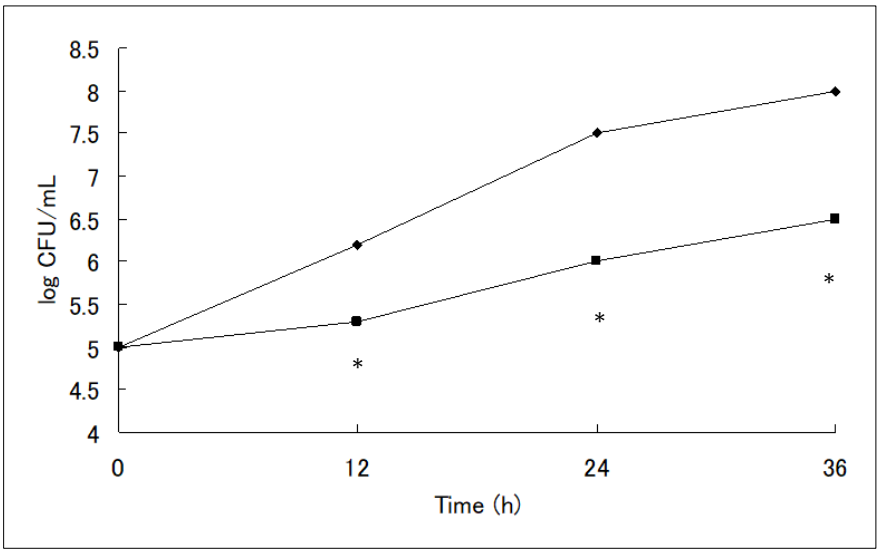


Figure 2 Morphological change among MUP-treated *H. pylori* (A): untreatment, (B) MUP (0.1 μg/mL) treatment (1 hour), (C) MUP (0.1 μg/mL) treatment (3 hour)

MUP showed good PAE on all the strain tested. Bacteria were cultured for 8 hours after adding MUP, and then the medium was diluted to eliminate the antibacterial.



♦:untreatment, ■:MUP 1 μg / mL , *: $p < 0.05$ compared to untreatment

Figure 3 PAE of MUP

Effect of MUP on the bacteria. After that, the culture was continued, and the number of bacteria was counted at every 12 hours. After first 12 hours, the MUP-treated group had 1/10 the number of bacteria in the untreated group. Moreover, this difference increased significantly as the culture time passed (Figure 3). PAE duration for MUP was significantly

longer in MUP treated cells than untreated cells ($p < 0.05$). The value of PAE is 12 and killing effect on *H. pylori* remained after cessation of MUP exposure.

4. Discussion

In the present study, we evaluated the effect of MUP on *H. pylori* biologically and morphologically. None of the strains tested exhibited resistance to MUP, in contrast to that observed with CLR and MNZ, and there was no cross-resistance with CLR or MNZ. The absence of cross-resistance between MUP and other groups of antibiotics is not altogether unexpected in view of the unique chemical structure and inhibition of isoleucyl-tRNA synthetase, which is different from that of any available antibiotics. It is unlikely given the unique mode of action of MUP; resistance may be selected for by other agents since co-transfer of other resistances with *mupA* has been described.

MUP was very active against *H. pylori* at both acid and neutral pH values, and it would be especially suitable for use in the acidic environment of the stomach. Associated with lethal effect of MUP, some morphological abnormalities were observed by electron microscopy; most cells became irregularly constricted forms. The exposure to MUP resulted in morphological changes of cell shape and cell lysis. From this result, it was morphologically clarified that MUP damages bacteria.

The PAE is defined as the period of persistent suppression of bacterial agents [18].

The clinical efficacy of drugs is also clearly linked to their pharmacokinetic properties, such as intracellular concentrations and half-life. The high values obtained with MUP could be due in part to its persistence above the MIC intracellular. We suggest that the PAE induced by MUP may represent the time required for it to dissociate from the receptor binding sites and to diffuse out of the bacterium because MUP bind bacterial isoleusin transferase. However, we cannot exclude that other mechanism such as a slow recovery from non-lethal cellular damage, and a lag time for the synthesis of new proteins and/or enzymes may be involved.

Our results are useful in planning future clinical investigations to clarify the possible implications of PAE in drug schedule and dosage, a line of information that is urgently needed to guide drug administration in *H. pylori* infected patients. Previous report showed that MUP was highly active at pH 7.4 and 5.4 (MIC_{90}; 0.25 and 0.12 μg / mL, respectively) against 57 strains of *H. pylori* [14]. This report is in good agreement with our results. However, the previous report does not show changes in bacterial morphology due to MUP. Our findings complement this point.

We describe the limitation of this study here. Once MUP enters the blood it is rapidly eliminated by hydrolysis of the ester link by non-specific esterase [19]. Its plasma half-life in man is less than 30 minutes. Thus, MUP is unsuitable for systemic use. But this problem seems to be few if limited as a treatment for bacteria present in the stomach.

5. Conclusion

In this study, our results suggest that MUP shows a crucial antibacterial effect against *H. pylori*. Even though the mechanisms of MUP on *H. pylori* remain obscure, this approach may be a suitable alternative to eradication treatment, at least in the subgroup of CLR and MNZ - resistant *H. pylori*. Although further investigations *in vivo* will be required, MUP may be useful in inhibiting this pathogenic bacterial growth.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors declare no conflict of interest of regarding the publication of this paper.

Statement of informed consent

Informed consent was obtained from individual participants when collecting bacteria.

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