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ORIGINAL ARTICLE

Synergistic effects of vancomycin and β-lactams against vancomycin highly resistant *Staphylococcus aureus*

Fumiaki Tabuchi[1], Yasuhiko Matsumoto[1,2], Masaki Ishii[1], Keita Tatsuno[3], Mitsuhiro Okazaki[4], Tomoaki Sato[3], Kyoji Moriya[3] and Kazuhisa Sekimizu[1,2]

We previously reported isolating vancomycin (VAN) highly resistant *Staphylococcus aureus* (VRSA) strains from clinical methicillin-resistant *S. aureus* strains by repeating steps of *in vitro* mutagenesis and VAN selection. Here we describe that the *in vitro* susceptibility of these VRSA strains to VAN was markedly increased by combined treatment with β-lactams such as ceftriaxone and oxacillin. Furthermore, in an *in vivo* silkworm infection model with VRSA, a combination of VAN and ceftriaxone exhibited therapeutic effects, whereas a combination of VAN and oxacillin did not. These findings suggest that combining VAN with an appropriate β-lactam, such as ceftriaxone, is therapeutically effective against infectious diseases caused by VRSA.
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INTRODUCTION

Vancomycin (VAN) is widely applied clinically to treat infection by methicillin-resistant *Staphylococcus aureus* (MRSA). The emergence of VAN-intermediate-resistant *S. aureus* (VISA) strains that exhibit weak tolerance against VAN (MIC: 4–8 μg ml^{-1}) was reported recently.[1] VRSA, which is highly resistant to VAN because of an exogenous gene, such as *vanA*, was also reported.[2,3] Teicoplanin, arbekacin, linezolid and daptomycin are considered to be therapeutically effective against VAN-resistant bacteria. Strains resistant to these antibiotics have already manifested,[4–7] however, and thus there is an urgent need to establish novel treatment strategies effective against infection by VAN-resistant strains.

Recently, we established a method to obtain MRSA strains that are highly resistant to VAN from clinical isolates of MRSA strains by repeating steps of mutagenesis and VAN selection.[8] VRSA strains isolated using this procedure accumulated multiple genetic mutations that led to increased cell wall thickness and tolerance against high concentrations of VAN (⩾16 μg ml^{-1}).[8] VR7, which is a VRSA strain, also showed daptomycin resistance.[8] We consider that these VRSA strains will be useful for establishing effective treatment strategies against emerging VAN-resistant bacteria.

Some reports suggested that combinations of VAN and β-lactams are effective against VISA.[9,10] The MIC values of VAN against VISA used in these studies, however, were relatively low (MIC: 4–8 μg ml^{-1}),[9] and thus an increased susceptibility of VISA to VAN by β-lactam was not clearly demonstrated. We considered that VRSA isolated by our method[8] would be a useful tool for testing the effectiveness of a combination of VAN and β-lactams. We previously reported the synergistic effects of VAN and cefazolin or oxacillin (OXA) against VRSA strains *in vitro*.[8] In this paper, we describe the synergistic effects of VAN and other β-lactams.

Animal models of disease are used to evaluate the therapeutic effects of drug candidates. We established an infection model using silkworms to evaluate the therapeutic effects of drug candidates against bacteria or true fungi pathogenic to humans.[11–14] The silkworm model is expected to be superior as an *in vivo* evaluation system for drug candidates to examine their therapeutic effectiveness against infectious diseases.[15–21] In this paper, we describe our *in vivo* evaluation of the therapeutic effects of the combination of VAN and β-lactams against VRSA using the silkworm infection model.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 1. Eight MRSA strains (MR1–8) were isolated from human patients at the University of Tokyo Hospital.[8] VAN-resistant strains (VR1–8) isolated from MRSA strains (MR1–8) by repeating steps of mutagenesis and antibiotic selection[8] were used in the experiments (Table 1). Mu50 is a VISA.[22] Bacteria were cultured at 37 °C in Tryptic soy broth according to the previously described method.[8]

Measurement of anti-bacterial activity

The MIC values of various antibiotics were determined by the microdilution method. Colonies of each strain cultured on an agar plate were scraped, suspended in sterilized saline and the OD_{600} was adjusted to 0.5. The samples were diluted to 1/400 in Mueller–Hinton broth supplemented with Ca^{2+} and

[1]Laboratory of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan; [2]Institute of Medical Mycology, Teikyo University, Tokyo, Japan; [3]Department of Infection Control and Prevention, Faculty of Medicine, The University of Tokyo, Tokyo, Japan and [4]Department of Medical Technology, School of Health Sciences, Tokyo University of Technology, Tokyo, Japan
Correspondence: Dr Y Matsumoto or Dr K Sekimizu, Institute of Medical Mycology, Teikyo University, 359 Otsuka, Hachioji, Tokyo 192-0395, Japan.
E-mail: ymatsumoto@main.teikyo-u.ac.jp or sekimizu@main.teikyo-u.ac.jp
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Table 1 Bacterial strains used in this study

Strains	*Description*	*Reference*
MR7	Clinically isolated MRSA from the University of Tokyo hospital	Ishii *et al.*[8]
VR1–8	VRSA obtained artificially from MR1–8	Ishii *et al.*[8]
Mu50	VISA, clinical isolate	Hiramatsu *et al.*[22]

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VISA, vancomycin-intermediate-resistant *Staphylococcus aureus*; VRSA, VAN highly resistant *Staphylococcus aureus*.

Mg^{2+}; a 190 μl bacterial suspension was dispensed in the top well of a 96-well round bottom plate and 100 μl suspensions were dispensed in the other wells. Solutions (10 μl) containing test samples were added to the top well, mixed and serially diluted twofold. After 48 h incubation, bacterial growth in each well was measured as described previously.[8] A more than fourfold difference in the MIC values was judged to be significant.

Silkworm rearing

Silkworm larvae were bred according to Kaito *et al.*[11] Briefly, eggs were purchased from Ehime Sanshu (Honai, Ehime, Japan), hatched in an incubator at 27 °C and bred. Artificial feed, Silkmate 2S, was purchased from Nihon Nosan (Yokohama, Kanagawa, Japan). Fifth instar larvae were used in the infection experiments.

Evaluation of the therapeutic effects of antibiotics using the silkworm infection model

Evaluation of the therapeutic effects of antibiotics using the silkworm infection model was performed according to Hamamoto *et al.*[13] with some modification. Fifth instar larvae were fed artificial food (1.1–1.2 g, antibiotic free; Nihon Nosan) and bred for 20 to 24 h at 27 °C. Fifty microliters of a twofold diluted overnight culture of *S. aureus* (injected bacterial numbers were 1.1×10^8 per larva) was injected into the silkworm hemolymph, followed by injection of 50 μl of sterilized saline or various concentrations of antibiotics. The concentration of the injected antibiotics was as follows: VAN 100 μg ml^{-1}, ceftriaxone (CTRX) 800 μg ml^{-1} and OXA 800 μg ml^{-1}. After being injected with the bacterial culture and antibiotics, the silkworms were reared at 37 °C, and silkworm survival was monitored. The log-rank test was performed for statistical processing using the Prism software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Synergistic effects of VAN and β-lactams on the growth inhibition of VRSA

We previously reported that OXA decreased the MIC values of VAN against VRSA (VR3 and VR7).[8] The concentration of OXA needed for the effect was much lower than the MIC value of OXA against the VRSA strains, indicating that the actions of OXA and VAN are synergistic.[8] In the present study, we first examined whether the synergistic effects of OXA and VAN were also observed for six other VAN-resistant strains. The MIC values of OXA against these VAN-resistant strains were higher than 128 μg ml^{-1}. In the presence of OXA 2 μg ml^{-1} (1/50 MIC), the MIC of VAN against the VAN-resistant strains was reduced to ⩽1/8 (Table 2). We next tested whether β-lactams other than OXA exhibited synergistic effects against VRSA (VR7). The results indicated that all nine tested β-lactams, including cefazolin and OXA, markedly decreased the MIC values of VAN against VR7 (MIC = 32 μg ml^{-1} → 2–4 μg ml^{-1}; Table 3). We checked that the concentrations (2 μg ml^{-1}) of the nine tested β-lactams were lower than their MIC values against VR7 (Table 4). The results suggested that the synergistic effect with VAN is a general characteristic of β-lactams.

Table 2 Effect of OXA on the susceptibility of VRSA strains and Mu50 strain to VAN

Strains	*OXA*	*VAN*	*VAN+OXA*
VR1	>128	16	2
VR2	>128	16	2
VR3	>128[a]	32[a]	2[a]
VR4	>128	32	4
VR5	>128	16	2
VR6	>128	32	2
VR7	>128[a]	32[a]	2[a]
VR8	>128	16	2
Mu50	>128	16	4

Abbreviations: OXA, oxacillin; VAN, vancomycin; VRSA, VAN highly resistant *Staphylococcus aureus*.
MIC values (μg ml^{-1}) of OXA and VAN in the absence or presence of 2 μg ml^{-1} OXA were determined by the microdilution method (37 °C for 48 h).
[a]Values reported in Ishii *et al.*[8]

To obtain synergistic effectiveness by combined therapy with two drugs, the pharmacokinetic parameters of the two compounds, such as the half-life in blood, should be consistent.[23–25] The half-lives of β-lactams including OXA are relatively short (0.4–2 h),[26,27] whereas the half-life of VAN (4–8 h)[28] is much longer. On the other hand, the half-life of CTRX in human blood is relatively long (6–9 h).[29] Therefore, we next examined the synergistic effects of CTRX and VAN against the VRSA strains. Our findings indicated that the MIC values of VAN against the VRSA strains were reduced by 4 μg ml^{-1} CTRX (Table 5). CTRX concentrations higher than 1 μg ml^{-1} markedly decreased the MIC value of VAN against VR7 (Figure 1). All eight VAN-resistant strains were resistant to CTRX (MIC > 128 μg ml^{-1}), suggesting that CTRX and VAN have synergistic activities.

Therapeutic effect of VAN combined with a β-lactam in the silkworm infection model with VRSA

Experiments with animal models are important for evaluating the therapeutic effects of antibiotics. We tested whether the combination of VAN and β-lactams exhibited therapeutic effects against the VRSA strains in the silkworm infection model. VRSA strains were injected into the silkworm hemolymph followed by injection of VAN and β-lactam, and survival of the animals was monitored. Survival was not extended in silkworms injected with VAN, OXA or CTRX alone (Figure 2). Silkworms injected with a combination of VAN and CTRX survived much longer than those injected with either VAN or CTRX alone ($P < 0.0001$) (Figure 2). On the other hand, the combination of VAN and OXA did not extend the survival of the silkworms (Figure 2). In this experiment, we observed that the injection of saline or antibiotic did not cause death in non-infected silkworms.

DISCUSSION

In this paper, we revealed that β-lactams (OXA, CTRX, cefazolin, methicillin, ampicillin, benzylpenicillin, carbenicillin, cephalexin and cloxacillin) increased the susceptibility of VRSA strains to VAN. We also demonstrated that VAN combined with CTRX exhibited therapeutic effects in the silkworm infection model with VRSA. The combination of VAN and OXA, however, exhibited no therapeutic effects in the silkworm infection model. These results suggest that combined treatment with VAN and an appropriate β-lactam is therapeutically effective against VRSA, which is expected to be a serious threat to humans.

Table 3 Effects of various β-lactams on the susceptibility of VR7 and MR7 to VAN

Strains	*VAN*	*VAN+CFZ*	*VAN+OXA*	*VAN+DMPPC*	*VAN+ABPC*	*VAN+PCG*	*VAN+CBPC*	*VAN+CTRX*	*VAN+CEX*	*VAN+MCIPC*
VR7	32	2	2	2	2	2	2	4	4	2
MR7	2	1	1	1	1	1	1	2	1	1

Abbreviations: ABPC, ampicillin; CBPC, carbenicillin; CEX, cephalexin; CFZ, cefazolin; CTRX, ceftriaxone; DMPPC, methicillin; MCIPC, cloxacillin; OXA, oxacillin; PCG, benzylpenicillin; VAN, vancomycin.
MIC values (μg ml^{-1}) of VAN in the absence or presence of 2 μg ml^{-1} of each β-lactam against VR7 and MR7 were determined by the microdilution method (37 °C for 48 h).

Table 4 MIC values of various β-lactams against VR7 and MR7

Strains	*CFZ*	*OXA*	*DMPPC*	*ABPC*	*PCG*	*CBPC*	*CTRX*	*CEX*	*MCIPC*
VR7	128	128	>256	8	16	64	>256	128	>128
MR7	256	256	>256	16	32	64	>256	>128	64

Abbreviations: ABPC, ampicillin; CBPC, carbenicillin; CEX, cephalexin; CFZ, cefazolin; CTRX, ceftriaxone; DMPPC, methicillin; MCIPC, cloxacillin; OXA, oxacillin; PCG, benzylpenicillin.

Table 5 Effect of CTRX on the susceptibility of VRSA strains and Mu50 strain to VAN

Strains	*CTRX*	*VAN*	*VAN+CTRX*
VR1	>128	16	2
VR2	>128	16	2
VR3	>128	32	4
VR4	>128	32	8
VR5	>128	16	4
VR6	>128	32	4
VR7	>128	32	4
VR8	>128	16	8
Mu50	>128	16	8

Abbreviations: CTRX, ceftriaxone; VAN, vancomycin; VRSA, VAN highly resistant *Staphylococcus aureus*.
MIC values (μg ml^{-1}) of CTRX and VAN in the absence or presence of 4 μg ml^{-1} CTRX were determined by the microdilution method (37 °C for 48 h).

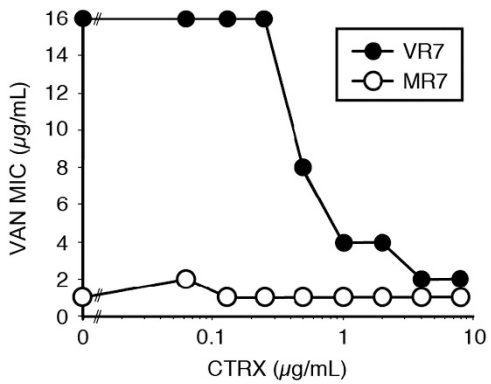

Figure 1 Effect of ceftriaxone (CTRX) on the susceptibility of VAN highly resistant *Staphylococcus aureus* (VRSA) to vancomycin (VAN). MIC values of VAN against VR7 and MR7 in the presence of various concentrations of CTRX were determined.

β-Lactam antibiotics have anti-bacterial activity by associating with the penicillin-binding proteins (PBPs) of *S. aureus* and inhibiting the formation of the crosslinked structure between sugar chains, resulting in the inhibition of peptidoglycan biosynthesis.[30] MRSA strains contain SCC*mec* regions that have the *mecA* gene coding the PBP2′, whose affinity for β-lactam is much lower than that of other PBPs.[31–33] By expressing PBP2′, MRSA strains become resistant to β-lactams. Both OXA and CTRX, at concentrations at which they exhibited

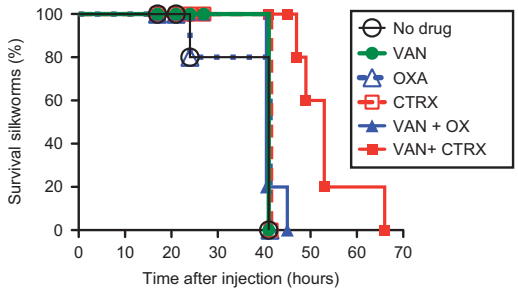

Figure 2 Therapeutic effects of combined vancomycin (VAN) and ceftriaxone (CTRX) in the silkworm infection model with VAN highly resistant *Staphylococcus aureus* (VRSA). Survival of silkworms infected with VR7 after injection of VAN, OXA or CTRX alone or with the combinations of VAN and OXA or VAN and CTRX is shown. The number of injected bacteria was 1.1×10^8 colony-forming unit (CFU) per larva; $n = 5$ per group.

no anti-bacterial effects alone, 0.125 μg ml^{-1} (ref. 8) and 1 μg ml^{-1}, respectively, markedly decreased the MIC values of VAN against VRSA strains. The precise mechanism underlying the synergistic effects of VAN and β-lactams is unclear. We assume that β-lactams affect cell wall synthesis at low concentrations by binding to PBPs other than PBP2′ of VRSA. VAN inhibits the peptide crosslinking reaction by binding to the D-alanine-D-alanine of the GlcNAc-MurNAc unit end.[30] Therefore, we consider that a change in the cell wall structure of VRSA caused by the binding of β-lactams to PBPs could induce efficient actions of VAN, resulting in the inhibition of VRSA growth. Further studies will be needed to elucidate the detailed molecular mechanisms of the effects of β-lactams against VRSA.

We found that the MIC value of VAN against Mu50 strain, which is a clinical strain of VISA, was decreased by the addition of OXA (Tables 2 and 5). The genetic background of Mu50 and VRSA strains used in this study is different from the clinically isolated VRSA strains, which have *vanA* gene. Further studies will be needed to elucidate the synergistic effects of VAN and β-lactams against the clinically isolated VRSA.

Here we revealed that combinations of all nine β-lactams examined, including OXA, exhibited clear synergistic effects with VAN against the *in vitro* growth of VRSA (Table 1). On the other hand, in the *in vivo* evaluation of drug efficacy in silkworms, the combination of OXA and VAN exhibited no therapeutic effects (Figure 2). In humans, the half-life of OXA in the blood is very short, and binding rates to serum proteins are very high, which complicates the pharmacokinetics.[27] We consider that the half-life of OXA may be also short in the silkworm hemolymph, resulting in a loss of therapeutic effectiveness in combination with VAN. Further studies using mouse infection models treated by humanized doses of antibiotics will be needed, to evaluate the combination taking into account the respective pharmacokinetic profile of VAN and β-lactams.

Several reports have suggested the effectiveness of the combination of VAN and β-lactams against MRSA.[34–36] Because the MIC values of VAN against MRSA are relatively low (≤2 μg ml^{-1}), it is unclear

whether the MIC values of MRSA are decreased by β-lactams. In this study, we used VRSA that is highly resistant to VAN, and demonstrated that β-lactams decreased the MIC value of VAN against these VRSA strains. In the experiments we performed at the same time with VRSA, most of the combinations of VAN and β-lactams were effective against MRSA. Recently, a phenomenon called 'MIC creep', that is, an increase in the MIC value of VAN against MRSA, was reported in patients.[37] Our results support the notion that combined treatment with VAN and β-lactams might be an effective strategy against the MIC creep.

The concept of 'drug repositioning' in which the application of existing drugs is expanded to treat various diseases has been proposed.[38,39] Extending the idea of drug repositioning, we propose a new concept of 'drug reuse'. Key to this concept is the combined use of antibiotics that are judged to be unsuitable by themselves due to the emergence of drug-resistant bacteria. Because the safety of individual antibiotics has already been established, combinations of these drugs with sufficient evidence for their clinical efficacy have attracted attention as novel treatments against drug-resistant bacteria. For example, the combination of sulfametoxazol with trimethoprim, two types of folic acid metabolism antagonists, exhibits clinical effects.[40] The development of new therapies by combined treatment with antibiotics, such as VAN and β-lactams, whose safety and pharmacokinetics are well established, will be useful to overcome the problems associated with the emergence of drug-resistant bacteria.

CONFLICT OF INTEREST

KS has an advisory role at Genome Pharmaceuticals Institute (Tokyo, Japan). The other authors declare no conflict of interest.

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