Original Article

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In vitro and *in vivo* anti-MRSA activities of nosokomycins

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Summary **The anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of nosokomycins A to D discovered in the silkworm-MRSA infection screening was investigated. The minimum inhibitory concentration (MIC) values of nosokomycins for authentic MRSA and *S. aureus* strains were calculated to be 0.06 to 2.0 μg/mL. They also showed potent inhibitory activity against 54 clinically isolated MRSA strains. Furthermore, nosokomycin A proved effective in the mouse-MRSA infection model.**

Keywords: Nosokomycins, silkworm infection assay, anti-MRSA antibiotic, *Streptomyces cyslabdanicus* K04-0144, therapeutic efficacy

1. Introduction

Drug candidates discovered from *in vitro* screening systems sometimes show no therapeutic effects in *in vivo* models using mice, rats, rabbits and so on as a host animal. There are gaps between *in vitro* and *in vivo* assay systems due to the following reasons: membrane permeability of drugs, metabolism of drugs and involvement of host immune systems; therefore, the introduction of *in vivo* assay systems at the early stage of screening programs is effective to fill the gaps, although *in vivo* screening is a time- and cost-consuming method and is therefore unrealistic. On the other hand, there is increased public concern towards eradicating animal experiments from the perspective of animal protection. Furthermore, the implementation of animal experiments is stringently regulated worldwide. Particularly in the European Union, widespread administration of all new drug candidates to healthy animals was forbidden in 1998.

To overcome these problems, researchers have focused on non-mammalian animals such as Zebrafish (*1*), *Caenorhabditis elegans* (*2*), *Drosophila melanogaster* (*3*), and silkworms (*4-8*) as alternative hosts for *in vivo* screening systems. On the discovery of antibiotics active against methicillin-resistant *Staphylococcus aureus* (MRSA) from microbial metabolites, we established an *in vivo*-mimic MRSA infection assay using silkworm larvae as a host animal and started to apply this assay to the primary screening for new anti-MRSA antibiotics of microbial origin. In this assay, MRSA-infected silkworm larvae die within 3 day. If drug candidates are effective, silkworm larvae can survive. We predicted that drug candidates discovered by the silkworm infection assay would have higher potential effectiveness in *in vivo* systems than those discovered by *in vitro* assays using paper disks.

During the course of this screening program, nosokomycins A to D (Figure 1) were isolated as potent antibiotics from the culture broth of *Streptomyces cyslabdanicas* K04-0144 (*8,9*). In this study, *in vitro* and *in vivo* anti-MRSA activities of nosokomycins are described.

2. Materials and Methods

2.1. *Materials*

Nosokomycins A to D were purified from a culture broth of *Streptomyces cyslabdanicas* K04-0144, as reported (*8,*9). Vancomycin and linezolid were obtained

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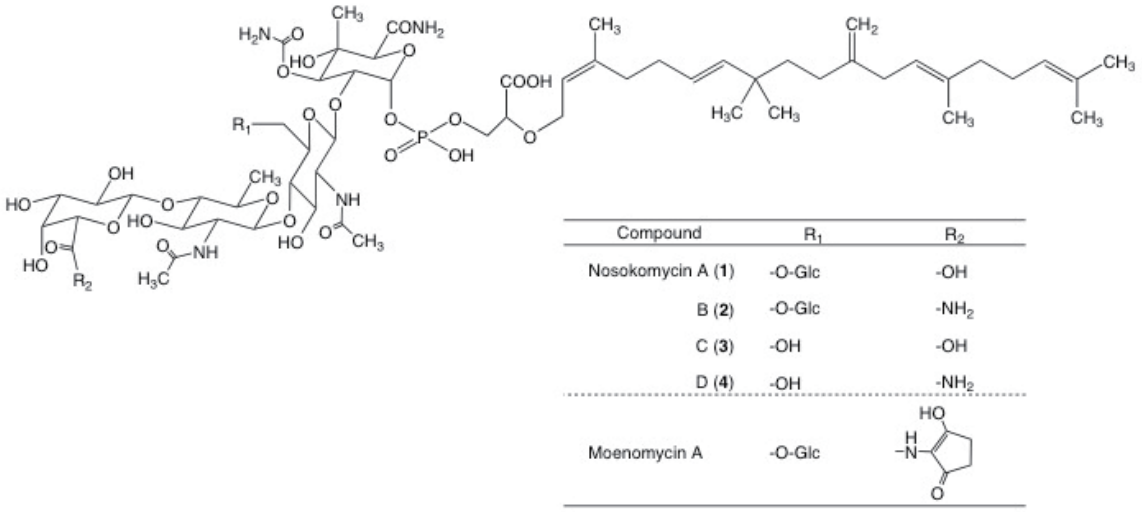

Compound	R_1	R_2
Nosokomycin A (**1**)	-O-Glc	-OH
B (**2**)	-O-Glc	$-NH_2$
C (**3**)	-OH	-OH
D (**4**)	-OH	$-NH_2$
Moenomycin A	-O-Glc	

Figure 1. Structures of nosokomycins A to D.

from Wako Pure Chemical Industries (Osaka, Japan). Arbekacin was purchased from Meiji Seika Pharma (Tokyo, Japan). Unless otherwise stated, all other reagents were reagent-grade commercial products.

2.2. *Animals*

Fertilized silkworm eggs, Bombyx mori (Hu•Yo × Tukuba•Ne), were purchased from Ehime Sansyu (Ehime, Japan) and fed artificial food (Silkmate 2S; Nihon Nosan Kogyo; Silkmate; Katakura Industries, Tokyo, Japan) until the fourth-instar larval stage. Female ICR mice (18-20 g, 4 weeks old) were obtained from Charles River (Kanagawa, Japan).

2.3. *Microorganisms*

Fifty-four MRSA strains, including N315 IR94, N315 IR94 HR-1 and K24, were clinically collected in Japan (*10*). The origin of other test microorganisms was as follows: *Staphylococcus aureus* FDA209P, *S. aureus* ISP447, *S. aureus* 8325 pEP2104 (partial macrolide and streptogramin B-resistant strain), *S. epidermidis* IFO12648, *Micrococcus luteus* ATCC9341, *Enterococcus faecalis* ATCC21212, *E. faecalis* NTCT12201 (*vanA*-type vancomycin-resistant strain), *Escherichia coli* NIHJ JC-2, *Citrobacter freundii* ATCC8090, *Klebsiella pneumoniae* NCTN9632, *Proteus mirabilis* IFO3849, *P. vulgaris* OX-19, *Morganella morganii* IID Kono, *Serratia marcescens* IFO12648, *Enterobacter cloacae* IFO13535, *E. aerogenes* NCTC10006, *Pseudomonas aeruginosa* 46001, *P. aeruginosa* E-2 (ceftazidime-sensitive strain), and *Acinetobacter calcoaceticus* IFO2552.

2.4. *Preparation of microorganism suspension*

All microorganisms except *Staphylococcus* sp. were grown overnight at 37°C in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, MD, USA). The cultures were diluted with the same broth and adjusted to an optical density at 600 nm of 0.3 (about 10^8 CFU/mL). *Staphylococcus* sp. was grown overnight at 37°C on Mueller-Hinton agar (MHA; Becton Dickinson, San Jose, CA, USA) containing chocolate horse blood at a final concentration of 10% (v/v), and the colonies were then suspended in a sufficient amount of TSB to make a cell suspension with an optical density at 600 nm of 0.3.

2.5. *Determination of minimum inhibitory concentration (MIC) values*

MICs of nosokomycins and authentic antibiotics (vancomycin, arbekacin and linezolid) were measured according to the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (*11*). For the MIC assay, MHA was used as a medium for test microorganisms except *Staphylococcus* sp., and MHA supplemented with 5% horse blood was used for *Staphylococcus* sp. The bacterial suspensions were then diluted 100-fold with the same fresh broth (about 10^6 CFU/mL). One loopful (5 μL) of the cell suspension was inoculated onto agar plates containing various concentrations of nosokomycin and authentic antibiotics using an inoculator (Microplanter; Sakuma Seisakusho, Tokyo, Japan). Growth of bacteria was evaluated after 18-h incubation at 37°C. The MIC was defined as the lowest drug concentration that showed 95% growth inhibition of bacteria.

2.6. *Population analysis of nosokomycins against clinically isolated MRSA*

Resistant subpopulations of 54 clinically isolated MRSA strains (population analysis) were analyzed by the established method (*12,13*). MRSA culture suspension (50 μL, overnight MRSA culture diluted

to an optical density at 550 nm of 0.3) was spread on MHA plates containing various concentrations of nosokomycins or authentic antibiotics. The plates were incubated at 37°C at 48-h and the number of growth strains was counted.

2.7. *In vivo-mimic MRSA infection assay using silkworm larvae*

An *in vivo*-mimic MRSA infection assay using silkworm larvae was carried out by the established method with some modification (*7,8*). Hatched silkworm larvae were raised by feeding an artificial diet containing antibiotics (Silk Mate 2S, Nihon Nosan Kogyo, Kanagawa, Japan) in an incubator at 27°C until the fourth molting stage. On the first day of fifth-instar larvae, silkworms were fed an antibiotic-free artificial diet (Silk Mate, Katakura Industries, Saitama, Japan) for 24 h. On the second day, MRSA K-24 (2.5×10^7 CFU in 50 µL, LB medium containing 10% NaCl) was injected into the hemolymph through the dorsal surface of the silkworms using a disposable 1-mL syringe with a 27G needle (TERUMO, Tokyo, Japan). Immediately (within one hour) after MRSA K-24 injection, a test sample (50 µL in 10% DMSO) was injected into the hemolymph. When they were maintained in an incubator at 27°C, all the MRSA-infected silkworm larvae without a sample died within 3 days. Under these conditions, when vancomycin (50 µg per larva) was injected, all silkworms survived, even on day 3.

2.8. *In vivo MRSA infection assay using mice*

The *in vivo* effect of nosokomycin A on systemic MRSA infection was studied using female ICR mice (18-20 g, 4 weeks old) (*10*). *S. aureus* 92-1191 (highly drug-resistant MRSA; MIC value of methicillin: > 100 µg/mL) was routinely grown in brain-heart infusion broth (BHI; Becton Dickinson) at 35°C overnight with agitation on a rotary shaker at 45 rpm. Mice were intraperitoneally infected with 1×10^9 CFU of MRSA 92-1191 in 0.1 mL phosphate-buffered saline (pH 7.4) containing 0.01% (w/v) gelatin and 10% (w/v) mucin from swine stomach (Wako Pure Chemical Industries). One hour later, nosokomycin A suspended in saline was subcutaneously injected into the back of mice (*s.c.*) or intravenously administrated (*i.v.*) at doses of 3.13, 6.25, 12.5 and 25 mg/kg (five mice per each dose), and the survival rates were recorded for five days. Vancomycin was evaluated under the same conditions.

3. Results

3.1. *Antibacterial activity of nosokomycins A to D against pathogenic microorganisms*

The MIC values of nosokomycins against various pathogenic bacteria including MRSA are shown in Table 1. Under the same conditions, clinically used antibacterial agents, vancomycin, arbekacin and linezolid, were tested (Table 1). Nosokomycins were found to be as active

Table 1. MIC values of nosokomycins against various pathogenic bacteria including MRSA.

Microorganism strain	Nosokomycin				Vancomycin	Arbekacin	Linezolid
	A	B	C	D			
Gram positive bacteria							
S. aureus FDA209P	1.0	≤ 0.25	2.0	1.0	1.0	≤ 0.25	1.0
MRSA N315 IR94	0.06	≤ 0.25	0.125	≤ 0.25	0.50	0.50	1.0
MRSA N315 IR94 HR-1	0.125	≤ 0.25	0.125	≤ 0.25	0.50	1.0	2.0
MRSA K24	0.125	0.125	0.125	0.125	NT	NT	NT
S. aureus ISP447	0.25	≤ 0.25	0.50	≤ 0.25	1.0	≤ 0.25	2.0
S. aureus 8325 (pEP2104)	0.06	≤ 0.25	0.06	0.50	2.0	≤ 0.25	2.0
S. epidermidis IFO12648	4.0	≤ 0.25	8.0	1.0	≤ 0.25	≤ 0.25	2.0
M. luteus ATCC9341	> 16	>128	> 16	> 128	1.0	≤ 0.25	2.0
E. faecalis ATCC21212	1.0	≤ 0.25	2.0	0.50	4.0	> 32	2.0
E. facecalis NTCT12201 (VanA)	1.0	≤ 0.25	2.0	0.50	> 32	> 32	2.0
Gram negative bacteria							
E. coli NIHJ JC-2	> 16	8.0	> 16	8.0	> 32	> 32	> 32
C. freundii ATCC8090	> 16	64	> 16	64	> 32	0.50	> 32
K. pneumoniae NCTN9632	> 16	8.0	> 16	8.0	> 32	≤ 0.25	> 32
P. mirabilis IFO3849	> 16	8.0	16	8.0	> 32	2.0	> 32
P. vulgaris OX-19	> 16	8.0	4.0	4.0	> 32	2.0	8.0
M. morganii IID Kono	> 16	32	> 16	32	> 32	0.50	> 32
S. marcescens IFO12648	> 16	32	> 16	32	> 32	1.0	> 32
E. cloacae IFO13535	> 16	32	> 16	32	> 32	0.50	> 32
E. aerogenes NCTC10006	> 16	32	> 16	32	> 32	≤ 0.25	> 32
P. aeruginosa 46001	> 16	32	> 16	32	> 32	1.0	> 32
P. aeruginosa E-2	> 16	32	> 16	32	> 32	4.0	> 32
A. calcoaceticus IFO2552	16	8.0	8.0	8.0	>32	≤ 0.25	> 32

NT; Not tested

as or more active than the three agents against most Gram-positive bacteria. For example, the MIC values of nosokomycins against multidrug-resistant MRSA N315 IR94 HR-1 (resistant to methicillin, imipenem, ciprofloxacin and tobramycin) were 0.125-0.25 μg/mL, while the three clinically used agents had high MIC values (0.5-2 μg/mL). Among them, nosokomycins B and D also showed moderate activity against Gram-negative bacteria. Although it is difficult to determine the potency order of nosokomycins as antimicrobial agents because of the subtle difference in MIC values, nosokomycin B appeared to be the most potent.

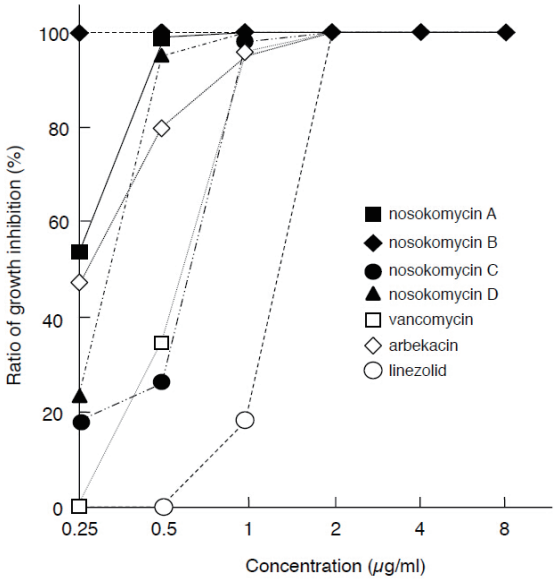

Figure 2. Antibacterial activity of nosokomycins against 54 clinical isolated MRSA strains.

3.2. *Population analysis of nosokomycins A to D*

Since nosokomycins were found to show potent activity against most Gram-positive bacteria, anti-MRSA activity was investigated in more detail by population analysis using 54 clinically isolated MRSA strains. As shown in Figure 2, the growth of all 54 MRSA strains (100%) was inhibited by 0.25 μg/mL nosokomycin B, while the growth of 55%, 22%, and 18% MRSA strains was inhibited by 0.25 μg/mL nosokomycins A, D and C, respectively. At 0.5-2 μg/mL, nosokomycins A, D, and C also showed 100% inhibition of those MRSA strains. From this population analysis, nosokomycin B showed the most potent anti-MRSA activity, followed by nosokomycins A, D, and C. Arbekacin was as potent as nosokomycin D, and vancomycin was as potent as nosokomycin C. Linezolide was the least among the drugs tested in this analysis.

3.3. *Therapeutic efficacy of nosokomycin A in MRSA-infected silkworm larvae*

Nosokomycins were evaluated in the *in vivo*-mimic MRSA infection assay using silkworm larvae. As shown in Figure 3, when nosokomycin A (50 μg per larva) was injected into MRSA-infected silkworm larvae,

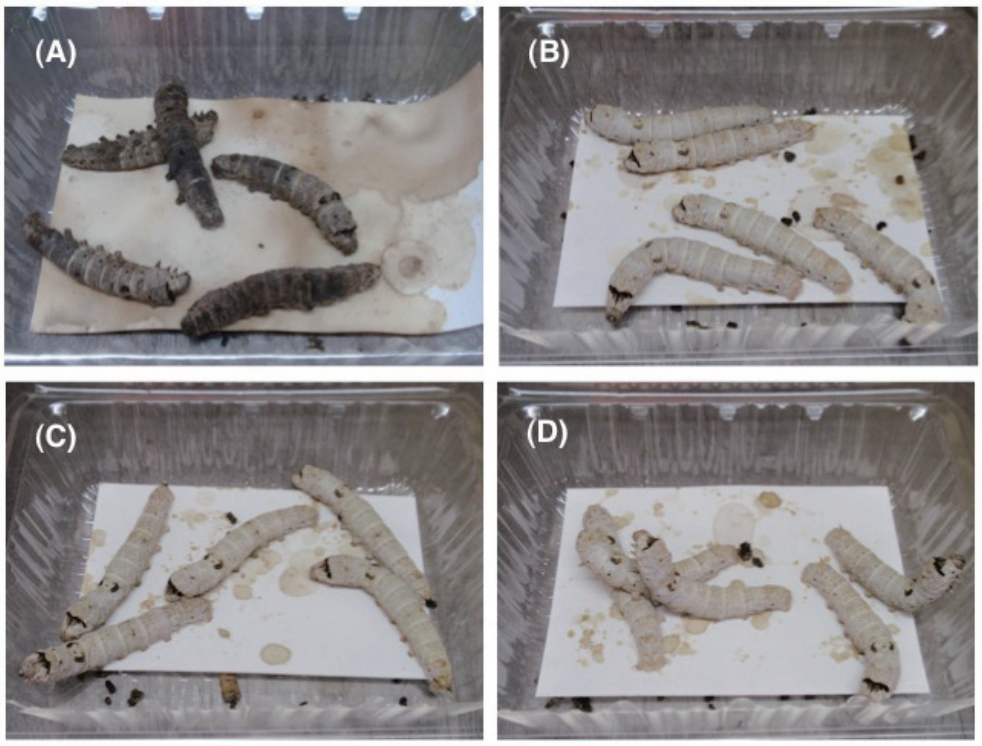

Figure 3. *In vivo* efficacy of nosokomycin A in silkworm infected with MRSA. (A) MRSA suspension. **(B)** Nosokomycin A (25 μg g^{-1}•larvae). **(C)** MRAS suspension + nosokomycin A (25 μg g^{-1}•larvae). **(D)** MRSA suspension + vancomycin (25 μg g^{-1}•larvae).

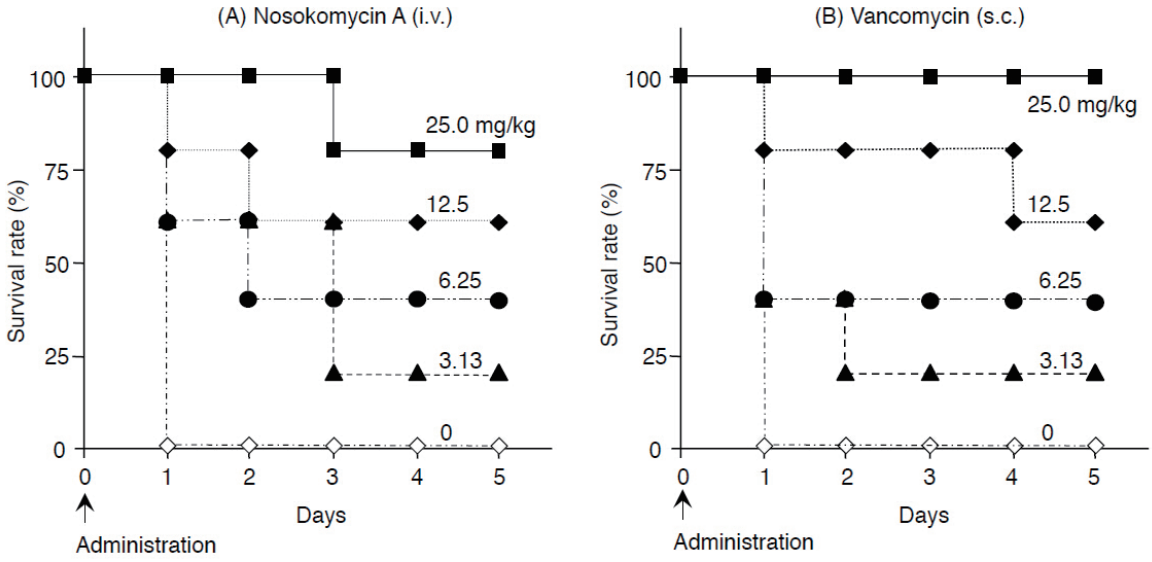

Figure 4. Therapeutic effects of nosokomycin A and vancomycin in mice infected with MRSA. (A) Intravenous administration of nosokomycin A. **(B)** Subcutaneous administration of vancomycin. Drugs were administrated once on day 0.

larvae all survived to at least day 3 (Figure 3C), while untreated larvae became black and died (Figure 3A). Nosokomycin A alone (50 µg per larva) had no toxic effect on uninfected larvae at least for 3 days (Figure 3B), indicating that the compound showed no toxicity to silkworm larvae. Nosokomycin B (50 µg per larva) also showed similar therapeutic efficacy for MRSA infected silkworm larvae (data not shown). Under the same conditions, vancomycin (50 µg per larva) showed the same therapeutic efficacy (Figure 3D).

3.4. *Therapeutic efficacy of nosokomycin A in MRSA-infected mice*

To confirm its *in vivo* efficacy, nosokomycin A was evaluated in an MRSA-infected mouse assay (*7*). When MRSA was intraperitoneally infected to mice, all the mice died on day 1 (next day) (Figures 4A and 4B); however, intravenous administration of nosokomycin A (3.12-25 mg/kg, on day 0) to MRSA-infected mice resulted in the dose-dependent survival of mice from MRSA infection (Figure 4A). At 25 mg/kg, 75% mice could survive MRSA infection. Vancomycin also showed *in vivo* efficacy by both subcutaneous (Figure 4B) and intravenous (data not shown) administration to MRSA-infected mice; however, subcutaneous administration of nosokomycin A did not show efficacy even at 25 mg/kg dose (data not shown).

4. Discussion

In this study, *in vitro* and *in vivo* anti-MRSA activities of nosokomycins A to D were investigated. As reported previously, nosokomycins were discovered in the screening using the silkworm-MRSA infection assay (*8,9*). All nosokomycins showed potent activity against most Gram-positive pathogenic bacteria with analogous MIC values, and nosokomycins B and D also showed moderate activity against Gram-negative bacteria (Table 1). From MIC data, nosokomycin B appeared to show the most potent anti-microbial activity among the four nosokomycins. From population analysis using 54 clinically isolated MRSA strains (Figure 2), it became clear that nosokomycin B is the most potent, followed by nosokomycins A, D, and C. Thus, it was suggested that the presence of a glucose residue at R1 and an amino residue at R2 in the structure is important for potent anti-MRSA activity. Nosokomycins belong to the phosphoglycolipid moenomycin family. Moenomycin A possesses a glucose residue at R1 and a chromophoric cyclopentenone residue via an amide bond at R2 (Figure 1). To understand, in particular, the importance of the cyclopentenone residue, it will be intriguing to compare anti-MRSA activity between nosokomycin B and moenomycin A, although we could not obtain moenomycin A, because nosokomycin-producing *Streptomyces cyslabdanicus* K04-0144 strain did not produce moenomycins. However, it was reported that the anti-*S. aureuse* activity of moenomycin A lacking the cyclopentenone moiety decreased tenfold compared with that of moenomycin A (*14*).

We applied the silkworm-MRSA infection assay to the primary screening method, and discovered nosokomycins from the culture broth of *Streptomyces cyslabdanicus* K04-0144 (*8,9*). This study demonstrated that nosokomycin A proved intravenously active in an *in vivo* MRSA-infected mouse model (Figure 4) (*7*). Unfortunately, nosokomycin A was inactive by subcutaneous administration in the mouse infection assay, while vancomycin proved active in both administration methods, which might have been due to drug permeability into the bloodstream.

All results showed the usefulness of *in vivo*-mimic MRSA infection assay using silkworm larvae to discover anti-MRSA agents effective in *in vivo* using mammalian animals. We hope this methodology accelerates the discover of anti-infective agents, overcoming many problems such as gaps between *in vitro* and *in vivo* model, animal protection and so on.

Acknowledgements

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References

1. Zon LI, Peterson RT. *In vivo* drug discovery in the zebrafish. Nat Rev Drug Discov. 2005; 4:35-44.
2. Kwok TCY, Ricker N, Fraser R, Chan AW, Burns A, Stanley EF, McCourt P, Cutler SR, Roy PJ. A small-molecule screen in *C. elegans* yields a new calcium channel antagonist. Nature. 2006; 441:91-95.
3. Needham AJ, Kibart M, Crossley H, Ingham PW, Foster SJ. *Drosophila melanogster* as a model host for *Staphylococcus aureus* infection. Microbiology. 2004; 150:2347-2355.
4. Asami Y, Horie R, Hamamoto H, Sekimizu K. Use of silkworms for identification of drug candidates having appropriate pharmacokinetics from plant sources. BMC Pharmacol. 2010; 10:7.
5. Kaito C, Akimitsu N, Watanabe H, Sekimizu K. Silkworm larvae as an animal model of bacterial infection pathogenic to humans. Microb Pathog. 2002; 32:183-190.
6. Hamamoto H, Kurokawa K, Kaito C, Kamura K, Razanajatovo IM, Kusuhara H, Santa T, Sekimizu K. Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. Antimicrob. Agents Chemother. 2004; 48:774-779.
7. Hamamoto H, Urai M, Ishii K, *et al*. Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat Chem Biol. 2014. doi: 10.1038/nchembio.1710
8. Uchida R, Iwatsuki M, Kim YP, Ohte S, Ōmura S, Tomoda H. Nosokomycins, new antibiotics, discovered in an *in vivo*-mimic infection model using silkworm larvae. I. Fermentation, isolation and biological properties. J Antibiot. 2010; 63:151-155.
9. Uchida R, Iwatsuki M, Kim YP, Ōmura S, Tomoda H. Nosokomycins, new antibiotics, discovered in an *in vivo*-mimic infection model using silkworm larvae. II. Structure elucidation. J Antibiot. 2010; 63:157-163.
10. Hanaki H, Akagi H, Yasui M, Otani T, Hyodo A, Hiramatsu K. TOC-39, a novel parenteral broad-spectrum cephalosporin with excellent activity against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother. 1995; 39:1120-1126.
11. National Committee for Clinical Laboratory Standards. In: Reference method for performance standards for antimicrobial disk susceptibility tests. Approved Standard M2-A8. National Committee for Clinical Laboratory Standards. 8th ed., Wayne, PA, USA, 2003.
12. Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, Fukuchi Y, Kobayashi I. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet. 1997; 350:670-673.
13. Takayama Y, Hanaki H, Irinoda I, Kokubun H, Yoshida K, Sunagawa K. Investigation of methicillinresistant *Staphylococcus aureus* showing diminished susceptibility isolated from a patient with infective endocarditis. Int J Antimicrob Agents. 2004; 22:567-573.
14. Rühl T, Daghish M, Buchynsky A, Barche K, Volke D, Stembera K, Kempin U, Knoll D, Hennig L, Findeisen M, Oehme R, Giesa S, Ayala J, Welzel P. Studies on the interaction of the antibiotic moenomycin A with the enzyme penicillin-binding protein 1b. Bioorg Med Chem. 2003; 11:2965-2981.

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