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Cyslabdan, a New Potentiator of Imipenem Activity against Methicillin-resistant *Staphylococcus aureus*, Produced by *Streptomyces* sp. K04-0144

II. Biological Activities

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Abstract Cyslabdan produced by *Streptomyces* sp. K04-0144 was found to potentiate imipenem activity against methicillin-resistant *Staphylococcus aureus* (MRSA). The MIC value of imipenem against MRSA was reduced from 16 to 0.015 μg/ml in combination with cyslabdan. Study on anti-MRSA activity of other typical antibiotics in combination with cyslabdan showed that the potentiating activity was limited to β-lactam antibiotics. Furthermore, among β-lactam antibiotics, the activity of carbapenems was most remarkably poteintiated by cyslabdan.

Keywords cyslabdan, imipenem potentiator, methicillin-resistant *Staphylococcus aureus*, MRSA, *Streptomyces* sp. K04-0144, β-lactam

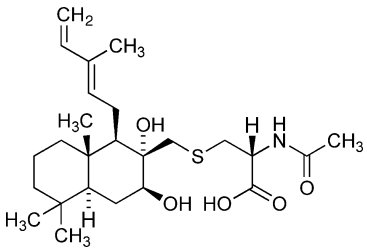

Fig. 1 Structure of cyslabdan.

Introduction

During our screening for potentiators of imipenem activity against methicillin-resistant *Staphylococcus aureus* (MRSA) from microbial metabolites, cyslabdan (Fig. 1) was isolated from the culture broth of *Streptomyces* sp. K04-0144. The compound has a labdane-type diterpene skeleton connecting with an *N*-acetylcysteine *via* thioether linkage [1]. In this study, the biological activities of cyslabdan including potentiation of imipenem activity against MRSA are described.

Materials and Methods

Materials

The following materials were purchased from commercial sources: Cloxacillin (ICN Biomedicals), ampicillin (Wako), cefalexin (Wako), cefazolin (Wako), ciprofloxacin (Wako), vancomycin (Wako), tetracycline (Wako), biapenem (Meiji Seika), streptomycin (Meiji Seika), meropenem (Sumitomo Pharmaceuticals), panipenem (Sankyo), penicillin G (Sigma), cefotaxime (Sigma), cefmetazole (Sigma), and

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imipenem (Banyu Pharmaceutical).

Microorganisms

The following microorganisms were used for antimicrobial tests: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FDA 209P (MSSA), *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Escherichia coli* NIHJ JC-2 IFO 12734, *Pseudomonas aeruginosa* IFO 3080, *Xanthomonas campestris* pv. *oryzae*, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG 8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* KF 1 and *Saccharomyces cerevisiae* ATCC 9763. MRSA (23 strains) including K24 strain and MSSA (5 strains) were clinically isolated in Japan.

Assay for Potentiating Activity of Typical Antibiotics against MRSA by Cyslabdan

Potentiation of anti-MRSA activity of imipenem or other antibiotics in combination with cyslabdan was studied by two methods. 1) Paper disk method: MRSA K24 was cultured in Mueller-Hinton broth (MHB, Difco) at 37°C for 20 hours and adjusted to 1×10^8 CFU/ml. The inoculum (100 μl) was spread on the Mueller-Hinton agar (MHA, Difco) in a plate (10×14 cm, Eiken Kizai) containing MHB and 1.5% of agar (Shimizu Shokuhin) with or without imipenem (10 μg/ml), whose concentration has no effect on growth of MRSA. Paper disks (6 mm i.d., Advantec) containing various amounts of a sample were placed on the MHA plate and incubated at 37°C for 20 hours. Anti-MRSA activity was expressed as the diameter (mm) of the inhibitory zone on the MHA plate. 2) Liquid microdilution method [2]; After MHB (85 μl) was added to each well of a 96-well microplate (Corning), cyslabdan dissolved in H_2O (5.0 μl) was added to the final concentration of 10 μg/ml. Then imipenem, or other antibiotics dissolved in distilled water (5.0 μl) were added to each well to a final concentration of 0.15 to 1,024 μg/ml. Finally, MRSA (5.0 μl) was added at a concentration of 1×10^7 CFU/ml. Microplates were incubated at 37°C for 20 hours without shaking. MIC was defined as the lowest concentration of an antibiotic where MRSA cannot grow.

Population Analysis

Population analysis of clinically isolated MRSA (22 strains) and MSSA (5 strains) was done by the established method [2]. An overnight culture of MRSA and MSSA strains was diluted with fresh medium to the appropriate bacterial density and spread onto a MHA plate containing serial twofold dilutions of imipenem with or without cyslabdan (20 μg/ml). The plates were incubated at 37°C for 20 hours. MIC_{50} and MIC_{90} were defined as the concentration at which 50 and 90% of the strains could not grow, respectively.

β-Lactamase Test

For β-lactamase activity test, MRSA K24 was cultured in MHB at 37°C for 20 hours without shaking and adjusted to 1×10^8 CFU/ml. After MHB was added to each well of a 96-well microplate in the absence or presence of cyslabdan (final concentration of 10 μg/ml), the inoculum was added at a concentration of 1×10^7 CFU/ml. Nitrocefin (Carbiochem, final 25 mg/ml) was then added to the cultures. Microplates were incubated at 37°C for 120 minutes without shaking. β-Lactamase inactivated nitrocefin by the ring cleavage, resulting in a color change from yellow to red.

Other Antimicrobial Assays

Antimicrobial activity against 15 species of microorganisms was measured by the paper disk method. Media for microorganisms were as follows: GAM agar (Nissui Seiyaku) for *B. fragilis*; Bacto PPLO agar (Difco) supplemented with horse serum 15%, glucose 0.1%, phenol red 0.25% and agar 1.5% for *A. laidlawii*; nutrient agar (Difco) for the other bacteria; a medium composed of glucose 1.0%, yeast extract 0.5% and agar 0.8% for fungi and yeasts. A paper disk (6 mm i.d., Advantec) containing 10 μg of sample was placed on an agar plate. Bacteria except *X. oryzae* were incubated at 37°C for 24 hours. Yeasts and *X. oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone.

Results

Antimicrobial Activity of Cyslabdan

First, antimicrobial activity of cyslabdan was tested by the paper disk method. At the concentration of 10 μg/disk, cyslabdan showed no inhibition against 15 microorganisms including MSSA as described in Materials and Methods. Furthermore, the compound did not inhibit the growth of MRSA K24 at the same condition.

Next, anti-MRSA K24 strain activity of cyslabdan was tested by the liquid microdilution method [2]. Cyslabdan showed weak anti-MRSA activity with an MIC of 64 μg/ml.

Potentiation of Imipenem Activity against MRSA by Cyslabdan

By the paper disk assay, cyslabdan alone showed no anti-MRSA activity at 10 μg/disk disk, but showed potent anti-MRSA activity with inhibition zone of 27 mm on MHA plates containing imipenem (10 μg/ml). Then, the potentiating effect of cyslabdan on the activity of imipenem and other typical antibiotics against MRSA was investigated by the liquid microdilution method. The concentration of cyslabdan was set up at 10 μg/ml for these experiments. As summarized in Table 1, cyslabdan markedly reduced the MIC value of imipenem from 16 to 0.015 μg/ml, yielding over 1000-fold potentiation of the activity. However, anti-MRSA activity of other typical antibiotics such as streptomycin, vancomycin, tetracyclin and ciplofloxacin was not enhanced by cyslabdan (Table 1).

Population Analysis

To further substantiate the effect of cyslabdan, population analysis was also performed by using clinically isolated MRSA strains and MSSA strains with imipenem in the presence and absence of cyslabdan (20 μg/ml). The MIC range, MIC_{50} and MIC_{90} values are shown in Table 2. The MIC_{50} of imipenem against MRSA strains decreased from 32 to 0.25 μg/ml in combination with cyslabdan, indicating most clinically isolated MRSA become 128-fold sensitive to imipenem with cyslabdan. whereas, the MIC_{50} of imipenem against MSSA strains showed no change even in the presence of cyslabdan. Thus, cyslabdan was found to affect only MRSA strains.

Potentiation of β-Lactam Activity against MRSA by Cyslabdan

Cyslabdan was found to potentiate imipenem activity against MRSA. Therefore, it was tested whether or not the activity of other β-lactams including penams (penicillin G, ampicillin, and cloxacillin), cephems (cefalexin, cefmetazole and cefotaxime), and carbapenems (imipenem, panipenem and meropenem) was potentiated by the compound. As shown in Table 3, cyslabdan potentiated the

Table 1 Potentiation of anitimicrobial activity against MRSA K24 by cyslabdan

Antibiotic	MIC (μg/ml)		Ratio (−/+)
	Cyslabdan		
	−	+	
Imipenem	16	0.015	1070
Streptomycin	2	2	1
Vancomycin	0.5	0.5	1
Tetracycline	64	64	1
Ciplofloxacin	64	64	1

MRSA K24 strain was used as a test microorganism. The concentration of cyslabdan was set up at 10 μg/ml.

Table 3 MIC values of various β-lactams against MRSA K24 in the presence or absence of cyslabdan

β-Lactam	MIC (μg/ml)		Ratio (−/+)
	Cyslabdan		
	−	+	
Penam			
Ampicillin	>1024	64	16
Penicillin G	512	64	8
Cloxacillin	512	16	32
Cephem			
Cefazolin	512	64	8
Cefalexin	1024	256	4
Cefotaxime	1024	64	16
Cefmetazole	128	4	32
Carbapenem			
Imipenem	16	0.015	1070
Biapenem	16	0.032	500
Panipenem	32	0.032	1000
Meropenem	16	0.125	128

MRSA K24 strain was used as a test microorganism. The concentration of cyslabdan was set up at 10 μg/ml.

Table 2 MIC of imipenem against clinical isolated MSSA and MRSA strains in the presence or absence of cyslabdan

	Cyslabdan (20 μg/ml)	Range of MIC (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
MSSA (5 strains)	−	0.03~0.03	0.03	0.03
	+	0.03~0.03	0.03	0.03
MRSA (22 strains)	−	16~64	32	64
	+	0.125~64	0.25	32

activity of all the β-lactams tested. In particular, carbapenems are most strongly potentiated (128～1024-fold) by cyslabdan among the β-lactams.

Effect of Cyslabdan on Staphylococcal β-Lactamase Activities

MRSA K24 was found to produce β-lactamase, because the color of nitrocefin changed from yellow to red by incubation with the strain. First, it was tested whether or not cyslabdan was degraded by β-lactamase of the MRSA K24. By the HPLC analysis cyslabdan was found to be stable by HPLC after the compound was incubated with MRSA K24. When MRSA K24 was incubated with nitrocefin and cyslabdan, the color was changed from yellow to red. Therefore, cyslabdan showed no effect on β-lactamase activity or β-lactamase production of MRSA K24.

Discussion

Cyslabdan, a new actinomycete metabolite, was found to potentiate imipenem activity against MRSA K24. Although cyslabdan itself showed very weak anti-MRSA activity with an MIC value of 64 μg/ml, the compound (10 μg/ml, no effect on MRSA) synergistically enhanced the imipenem activity against MRSA, decreasing the MIC value from 16 to 0.015 μg in combination with cyslabdan (Table 1). Furthermore, the imipenem potentiating activity by cyslabdan was not limited to MRSA K24 strain, and effective to most MRSA strains clinically isolated (Table 2). However, the activity of other typical antibiotics against MRSA was not enhanced by cyslabdan (Table 1). Cyslabdan potentiated carbapenem activity against MRSA K24 most strongly among β-lactams (Table 3). MRSA K24 strain produced β-lactamase. Cyslabdan did not inhibit the β-lactamase and the β-lactamase could not hydrolyse imipenem. Therefore, carbapenem activity against MRSA was more effectively enhanced by cyslabdan than other β-lactams (Table 3). Anyway, carbapenem is the strongest antibiotic in the field of β-lactams, but the cost of the production is still expensive. Accordingly, our idea of drug discovery in this study is important because such a potentiator would be applied for the decrease of the dose of these types of antibiotics.

Several compounds have been reported to potentiate β-lactam activity against MRSA; polyoxotungstates [3], polyphenols such as epigallocatechin gallate isolated from tea [4], corilagin from *Arctostaphylos uva-urs* [5] and tellimagrandin I from rose red (*Rosa canina*) [5] and diterpenes such as totarol isolated from totara tree [6], and synthetic MC-200,613 [7]. Among these compounds, the structures of totarol, MC-200,616 and a part of cyslabdan are labdane-type diterpenes. Therefore, the labdane part is responsible for the activity.

MC-200,616 was reported to attack the penicillin binding protein 2′ (PBP2′) expressed by MRSA, a strain insensitive to β-lactams and producing β-lactamase [7]. Our preliminary study on the mechanism of action suggested that cyslabdan showed no binding to PBP2′ (data not shown) and showed no effect on PBP2′ expression (data not shown) and β-lactamase activity, although cyslabdan shares a similar labdan skeleton with MC-200,616. Further studies on the mechanism of action are now in progress.

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