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Use of a Sensitive Chemiluminescence-Based Assay to Evaluate the Metabolic Suppression Activity of Linezolid on Methicillin-Resistant *Staphylococcus aureus* Showing Reduced Susceptibility to Vancomycin

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Recently, strains of methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to vancomycin (VCM) have been clinically isolated. The antibacterial activity of a new drug, linezolid (LZD), in such a strain was evaluated by measuring bacterial metabolic activity. A total of 73 MRSA strains having various susceptibilities to VCM were subjected to a novel and highly sensitive chemiluminescence-based assay. LZD MIC in the tested strains, measured by the microbroth dilution method, was within the range 1–4 mg/l (mostly ≤2 mg/l), except for one LZD-resistant strain (NRS127; MIC=7 mg/l), and showed no correlation with VCM resistance. The chemiluminescence assay demonstrated that bacterial metabolic activity was strongly suppressed with increasing LZD concentration. The chemiluminescence intensity curve had a low baseline activity without tailing in most strains. The present results suggest that LZD has strong antibacterial activity against MRSA strains, and would be effective for treatment of infections that are poorly responsive to VCM. The chemiluminescence assay facilitated sensitive and discriminative susceptibility testing within a relatively short time.

Keywords: Linezolid, chemiluminescence, susceptibility test, vancomycin-intermediate *Staphylococcus aureus*

Generally, recent clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) are highly resistant to many kinds of antimicrobials. Drugs of choice for treatment of MRSA infections are becoming limited, and only a few antimicrobials effective for MRSA are now available. Vancomycin (VCM) is virtually one of the last resorts for treatment of MRSA infections, but MRSA strains showing reduced susceptibility to VCM have been isolated worldwide [7, 8, 14, 16], alerting physicians to the need for careful monitoring of VCM therapy [6]. A second (alternative) drug of choice is therefore clearly desirable.

Linezolid (LZD) is a novel and fully synthetic antimicrobial agent belonging to a new class, the oxazolidinones [4, 18, 22]. Although LZD was initially approved for use against infections caused by VCM-resistant enterococci (VRE), LZD is known to be highly effective against many Gram-positive bacteria [4, 18]. Some investigators have reported that LZD gives good results for treatment of MRSA infections [11, 17], and LZD was recently approved for use as an anti-MRSA drug. Interestingly, the clinical outcome of LZD treatment is almost equivalent to that of VCM therapy, even though LZD is merely a bacteriostatic agent [11, 17]. Although the reason for this is unclear, it is possible that a certain pharmacological advantage of LZD has not been properly evaluated by current susceptibility testing.

There are several methods for the evaluation of antibacterial activities *in vitro*, and usually MIC is measured to clarify the extent of drug resistance. However, such methods employed in clinical laboratories (*e.g.*, the microbroth dilution method) have a theoretical limitation. Since bacterial growth is judged visually, any growth below the visual level cannot be evaluated. For example, it is known that VCM-intermediate *S. aureus* (VISA) strains tend to be overlooked by routine susceptibility testing performed in clinical laboratories [19]. In addition, current susceptibility tests fail to show how strongly bacterial viability is suppressed by bacteriostatic agents such as LZD.

However, recent advances in cell biology and biochemistry have allowed the development of easy and sensitive methods for direct determination of cell viability, and the chemiluminescence-based assay seems to be a highly sensitive and promising method [19]. In the present study, using a chemiluminescence-

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based assay method, we evaluated the antibacterial effect of LZD on MRSA strains having various degrees of VCM resistance.

MATERIALS AND METHODS

Culture Media, Antimicrobials, and Reagents

Culture media such as Müller-Hinton (MH) broth were from Difco (MI, U.S.A.). A specially designed medium for chemiluminescence measurement (Rapid-Lumi) was obtained from Eiken Chemicals (Tokyo, Japan) [10, 12, 19, 20]. LZD (obtained from Pfizer, Tokyo branch) and other antimicrobials were of reagent grade. A colorimetric probe, alamarBlue (AlaB) [15], was purchased from Serotec (Kidlington, Oxford, U.K.).

Bacterial Strains

A total of 25 VISA strains (clinical isolates) isolated from 8 countries were tested in comparison with 34 VCM-susceptible *S. aureus* (VSSA) strains and 6 hetero-VISA (hVISA) strains represented by Mu3 [19]. Two VCM-resistant *S. aureus* (VRSA) strains with the *vanA* gene were also used in the experiments, and the sources of the bacterial strains are listed in the legend of Table 1. In addition, 5 MRSA strains having relatively lower susceptibility to LZD (tentatively termed LLSA; MIC(48 h) = ~4 mg/l) were selected from our stock collection (JCSC; Japan Collection of *Staphylococcus* Cultures, Tokyo, Japan). NRS127, a LZD-resistant strain (LRSA), was obtained from NARSA (Network on Antimicrobial Resistance in *Staphylococcus aureus*; Herndon, U.S.A.).

Definition

Although the Clinical and Laboratory Standards Institute (CLSI; formerly known as NCCLS) has recently proposed a new breakpoint for VCM resistance in staphylococci (the M100-S16 version [2]), the *S. aureus* strains used in this study were tentatively classified into the following categories in accordance with our previous report [19] (principally based on the M100-S15 version of the CLSI [1]):

VSSA: MIC(24 h) was ≤2 mg/l

hVISA: MIC(24 h) was ≤2 mg/l, but each strain formed colonies on a brain-heart infusion agar plate containing 4 mg/l VCM at greater frequencies than one in 10^6 CFU (confirmed by population analysis) [8].

Group I VISA: Once identified as "VISA" with MIC ≥8 mg/l, but their current MIC is ≤4 mg/l (although their resistance levels were still greater than that of Mu3) [3, 19].

Group II VISA: Their current VCM resistance levels were high enough to be classified as "VISA" (MIC >4 mg/l).

VRSA: MIC (24 h) was ≥16 mg/l.

Although there is currently no definition of the LZD-intermediate *S. aureus* strain, the following definition was tentatively used in this study:

LLSA (*S. aureus* with lower susceptibility to LZD): MIC (48 h) was ≥4 mg/l (see Table 1).

LRSA (LZD-resistant *S. aureus*): MIC(24 h) was >4 mg/l (CLSI's breakpoint for LZD = 4 mg/l [2]).

Microbroth Dilution Method

MIC was measured (at both 24 h and 48 h) by the microbroth dilution method according to the procedure of the CLSI with slight modifications [13]. Inoculation size was 5×10^4 CFU/well, and antimicrobial concentrations were set to 0, 0.5, 1, 2, 3…9, and 10 mg/l. For MIC measurements under hyperosmolar conditions, 1.2 M sucrose was added to MH broth (1.2 M Suc+MHB), and cell growth was checked at both 48 and 72 h because of bacterial growth retardation [19].

Colorimetric Assay

This method is based on the color change of a redox-sensitive dye (AlaB), which is reduced in viable cells [15]. Details of the protocol were given in our recent report [9].

Chemiluminescence Assay

This chemiluminescence assay is based on generation of hydrogen peroxide by viable cells linking with the bacterial electron transfer system [20], as detailed in our recent report [19]. Briefly, a bacterial suspension (1.1×10^9 CFU/l) was made in Rapid-Lumi medium. Then, 45 µl of this suspension was dispensed into each well of a 96-well microtiter plate containing 5 µl of various concentrations of LZD solution (final concentration = 0, 0.5, 1, 2, 3…9, and 10 mg/l). After 3 h of incubation at 37°C, 5 µl of menadione solution (50 mg/l) was added. The mixture was incubated further for 1 h, and then chemiluminescence intensity (CLI) was measured by the alkaline-Lucigenin method with a luminescence reader (Lucy 2; Rosys-Anthos, Hombrechtikon, Switzerland). The CLI directly reflects the metabolic activity of the tested strain [10, 12, 19, 20].

To evaluate the extent of bacterial drug resistance more accurately, the following three parameters were calculated from the chemiluminescence graphs [19]: (1) ED_{50}: Drug concentration (**e**ffective **d**ose) that produced **50%** CLI relative to the control activity; (2) ED_{25}: Drug concentration that produced **25%** CLI relative to the control activity (*i.e.*, 75% suppression); and (3) AUC: **A**rea **u**nder the chemiluminescence **c**urve.

Comparison of Antibacterial Activities

In order to compare the antibacterial activity of LZD with those of other antimicrobials (especially protein synthesis inhibitors), a chemiluminescence assay of ABK, EM, and MINO was performed on susceptible strains of *S. aureus* (FDA209P and NCTC8325). Since most clinical isolates of *S. aureus* are currently resistant to EM and MINO, a comparison may be possible only among susceptible strains isolated at a much earlier time. The measurement procedure was the same as that for LZD.

RESULTS

LZD MIC of MRSA Strains Having Reduced Susceptibility to VCM

The results are summarized in Table 1. MIC(24 h) of LZD in VSSA, hVISA, VISA, and VRSA strains was 1–3 mg/l (more than 90% of strains showed MIC ≤2 mg/l), although that of one LRSA strain (NRS127) was 7 mg/l. There was no correlation between susceptibilities to VCM and LZD. When the incubation time was prolonged to 48 h, LZD MIC in most MRSA strains was still low, and only 5 strains gave a slightly higher MIC (~4 mg/l), except for NRS127. In contrast, VCM MIC in VISA and hVISA strains was generally increased (going up 2–3 mg/l) by further incubation [19].

Under hyperosmolar conditions using 1.2 M Suc+MH, where β-lactams completely lost their bactericidal activities [19], LZD was still active and its MIC did not increase to higher levels (Table 1).

Table 1. Comparison of MIC (24 h)[c] and effective dose (ED) values for *S. aureus* strains with various levels of susceptibility to LZD and VCM.

	VCM		LZD		LZD (+1.2 M SUC)		LZD			
Strain[a]	MIC(24 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(24 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(72 h)[c] (mg/l)	ED_{50}[d] (mg/l)	ED_{25}[d] (mg/l)	AUC[d,e]	Source[f]
VSSA										
FDA 209P*	0.5	0.5	1	2	0.5	0.5	0.42	0.88	1.08	A
H1	1	1	2	2	2	2	0.66	1.01	1.00	A
N 315	0.5	1	1	2	2	2	0.40	0.99	1.03	A
NCTC 8325*	1	2	2	2	1	2	0.66	1.42	1.29	A
JCSC 108	1	1	2	3	2	2	0.40	0.82	0.74	B
JCSC 119	1	1	2	2	2	2	0.50	0.94	0.84	B
JCSC 134	1	1	2	2	2	2	0.61	1.55	1.49	B
JCSC 170	1	2	2	3	2	2	0.47	0.96	0.91	B
JCSC 199	1	1	2	2	2	2	0.36	0.51	0.64	B
JCSC 221	1	1	2	3	2	2	0.45	0.92	0.96	B
JCSC 227	1	1	2	2	2	2	0.41	0.82	0.75	B
JCSC 248	1	1	1	2	2	2	0.33	0.50	0.68	B
JCSC 276	1	1	3	3	2	2	0.79	1.47	1.26	B
JCSC 311	1	1	2	2	2	2	0.41	0.97	0.89	B
JCSC 343	1	1	2	2	2	2	0.36	0.64	0.72	B
JCSC 431	1	2	2	3	2	2	0.40	0.89	0.95	B
JCSC 1168	1	2	2	2	2	2	0.38	0.71	0.80	B
JCSC 1452	1	1	3	3	2	2	0.64	1.27	1.16	B
JCSC 1453	0.5	1	2	2	2	2	0.45	0.93	0.91	B
JCSC 1454	0.5	1	2	2	2	2	0.61	1.04	1.09	B
JCSC 1455	1	1	2	2	2	2	0.71	1.27	1.21	B
JCSC 1462	1	2	2	2	2	2	0.46	0.85	0.85	B
JCSC 1463	1	1	2	2	2	2	0.70	1.34	1.21	B
JCSC 1470	1	1	2	2	2	2	0.64	1.39	1.24	B
JCSC 1572	2	2	2	2	1	2	0.63	1.14	1.03	B
JCSC 1715	1	1	2	3	2	2	0.61	1.19	1.07	B
JCSC 1718	1	1	3	3	2	2	0.71	1.47	1.35	B
JCSC 2153	0.5	1	2	2	2	2	0.66	1.19	1.20	B
JCSC 2167	1	1	2	2	1	2	0.68	1.49	1.27	B
JCSC 2172	0.5	1	2	2	2	2	0.74	1.47	1.30	B
JCSC 3613	0.5	1	1	1	2	2	0.83	1.65	1.42	B
JCSC 3624	1	2	2	2	2	2	0.87	1.62	1.45	B
JCSC 4409	1	1	2	2	2	2	0.74	1.32	1.22	B
JCSC 4541	2	2	2	3	2	2	0.40	0.84	0.99	B
hVISA										
Mu3	2	3	2	2	2	2	0.46	0.82	0.68	A
JCSC 157	1	2	2	2	2	2	0.35	0.69	0.62	B
JCSC 165	2	2	2	2	2	2	0.37	0.74	0.62	B
JCSC 226	2	2	1	1	2	2	0.38	0.87	0.83	B
JCSC 237	2	2	1	2	2	2	0.46	0.87	0.86	B
JCSC 238	2	3	1	1	2	2	0.28	0.41	0.43	B
VISA (Group I)										
AMC 11094	4	6	1	1	1	1	0.46	0.79	0.64	A
BR 1	4	6	2	2	1	1	0.41	0.75	0.73	A
BR 2	4	6	1	1	2	2	0.41	0.80	0.76	A
BR 3	6	8	1	1	1	1	0.42	0.79	0.72	A
BR 4	3	4	2	2	2	2	0.44	0.84	0.84	A

Table 1. Continued.

Strain[a]	VCM		LZD		LZD (+1.2 M SUC)		LZD			
	MIC(24 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(24 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(48 h)[c] (mg/l)	MIC(72 h)[c] (mg/l)	ED_{50}[d] (mg/l)	ED_{25}[d] (mg/l)	AUC[d,e]	Source[f]
99/3759-V	3	3	1	1	1	1	0.28	0.46	0.57	A
99/3700-W	3	3	1	1	2	2	0.36	0.71	0.66	A
26160	2	3	1	2	2	2	0.41	0.80	0.80	A
98141	2	2	1	1	2	2	0.36	0.66	0.68	A
1834	4	7	2	2	2	2	0.41	0.79	0.83	C
4264	4	4	1	2	1	1	0.54	0.88	0.91	D
LIM 2	3	6	1	1	1	1	0.35	0.59	0.58	A
NRS 65	2	3	0.5	1	0.5	0.5	0.28	0.42	0.55	E
NRS 119	2	2	2	2	2	2	0.41	0.87	0.79	E
VISA (Group II)										
Mu50	6	7	1	1	2	2	0.50	0.84	0.70	A
BR 5	5	6	2	2	2	2	0.36	0.69	0.73	A
IL	5	8	1	1	2	2	0.70	1.14	1.01	A
MI	8	9	0.5	1	2	2	0.33	0.66	0.77	A
NJ	6	8	1	1	1	1	0.36	0.71	0.73	A
PC	5	5	2	2	2	2	0.49	0.97	0.99	A
NRS 14	5	6	2	2	2	2	0.45	0.82	0.78	E
NRS 17	5	6	1	2	2	2	0.44	0.82	0.73	E
NRS 73	5	8	2	2	2	2	0.36	0.61	0.67	E
NRS 74	5	5	2	3	2	2	0.69	1.19	0.96	E
NRS 118	6	7	1	1	2	2	0.36	0.54	0.72	E
VRSA										
VR1	>10	>10	2	2	2	2	0.52	1.06	1.05	C
VR2	>10	>10	2	2	2	2	0.46	1.04	1.08	C
LLSA[b]										
ATCC 29213*	0.5	1	3	4	2	2	0.47	1.27	1.01	A
JCSC 378	0.5	1	3	4	2	3	0.70	1.42	1.19	B
JCSC 1591	2	2	3	4	3	3	1.01	1.70	1.42	B
JCSC 3615	1	1	3	4	3	3	1.04	1.98	1.80	B
JCSC 3794	0.5	1	2	4	3	3	0.98	1.82	1.56	B
LRSA[b]										
NRS 127	1	1	7	8	8	8	1.32	3.37	2.35	E

[a]Strains marked with an asterisk* are methicillin-susceptible *S. aureus* (MSSA), and all the others are MRSA.
[b]These are all VSSA strains (VCM MIC was ≤2 mg/l).
[c]Results of duplicate determinations.
[d]Results of quadruplicate determinations.
[e]AUC is presented as a value relative to the area of the whole graph rectangle sheet (see Fig. 1), which is tentatively set to 10.
[f]Sources of bacterial strains: A=listed in our previous report [19], B=JCSC, C=Center for Disease Control and Prevention (CDC; Atlanta, U.S.A.), D=the National Taiwan University Hospital (Taipei, Republic of China), E=NARSA.

Colorimetric Assay

Typical examples have been presented in our recent report [9]. When AlaB was used as an indicator of bacterial metabolism, the slope of the LZD plot fell to a relatively higher suppression point (~50%), and did not fall further thereafter. Compared with LZD, the VCM slope also fell (~50% suppression), but still declined gradually with time thereafter (~10% decrease/3 h) [9].

Chemiluminescence Assay

Figs. 1A and 1B show the results of the chemiluminescence assay of LZD for representative MRSA strains, with the

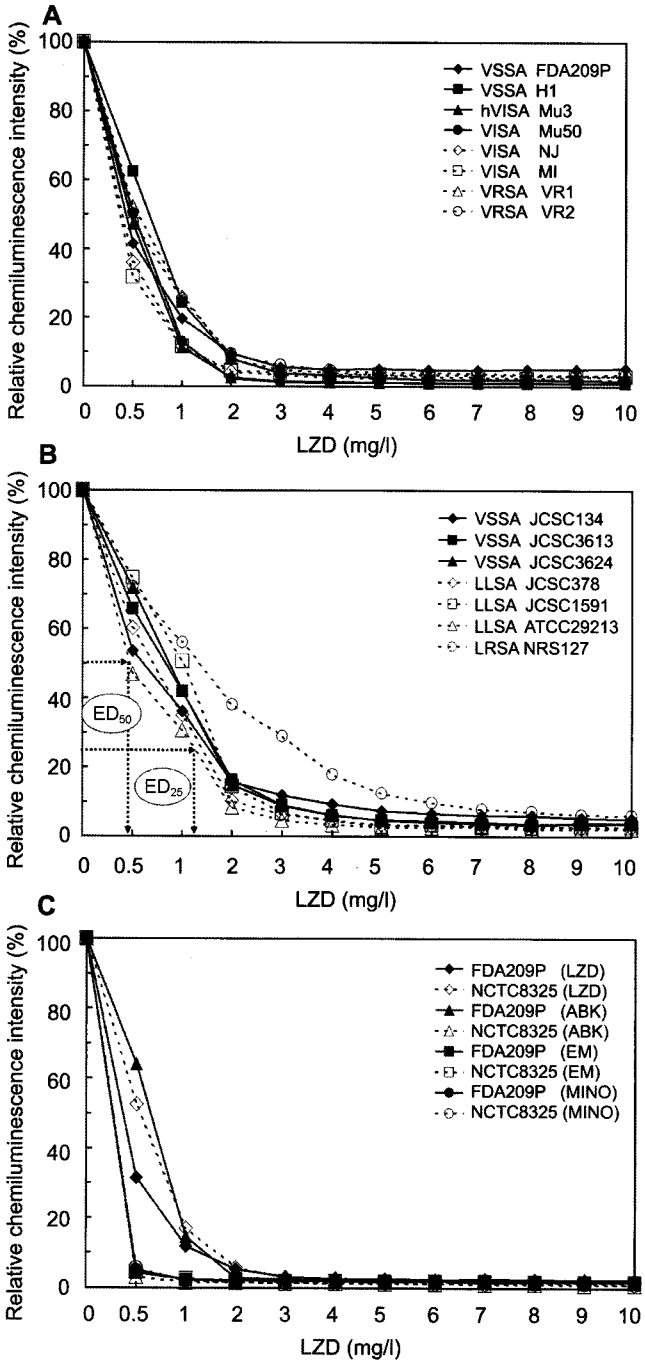

Fig. 1. Chemiluminescence-based linezolid susceptibility test for *S. aureus* strains.

Chemiluminescence intensity measured in a drug-free medium was used as a control (100%). Each curve is the mean for quadruplicate measurements, and the standard deviation was around ≤5–10% [19]. The method used to find ED_{25} and ED_{50} is illustrated by arrows and dotted lines. AUC was calculated from this graph by planimetry (trapezoid method [19]). **A.** Results of representative strains are shown. **B.** Data for strains with reduced susceptibility to LZD are shown. **C.** Antibacterial activity of LZD was compared with that of ABK, EM, and MINO (all of which are protein synthesis inhibitors similar to LZD) in susceptible strains of *S. aureus*. Note that this comparison was made by weight concentration. On a molar-basis comparison, MINO and EM were about ≥10 times more potent than LZD.

ordinate of the graph indicating metabolic activity of the bacterial cells (LZD and other agents did not directly interfere with the measurement system). Except for one LRSA strain (NRS127), the CLI of every strain declined sharply (without tailing or increased baseline activity) as the LZD concentration increased. Fig. 1B demonstrates that the strong metabolic suppression activity of LZD was still retained in *S. aureus* strains having slightly reduced susceptibility to LZD.

Comparison of Antibacterial Activities

Fig. 1C shows the results of a chemiluminescence assay in which the antibacterial activities of LZD and ABK were compared with those of EM and MINO in type strains. Surprisingly, in susceptible strains, it was found that the antibacterial activities of EM and MINO were stronger than those of LZD and ABK, which were developed recently.

Sensitivity and Specificity

The sensitivity and specificity of the chemiluminescence assay for identifying an LRSA strain were 100% in all cases when the cut-off values of ED_{50}, ED_{25}, and AUC were set at 1.05–1.32, 1.99–1.32, and 1.81–2.35, respectively (Table 2).

The sensitivity and specificity for identifying an LLSA strain were relatively low. When MIC (48 h) was chosen as the "gold standard," the sensitivity and specificity of MIC (24 h) were 83% and 96%, respectively. As shown in Table 2, ED_{25} was sensitive (100%), but less specific (87%). In contrast, AUC was quite insensitive (67%), but very specific (97%). Therefore, the best discrimination power was obtained when sensitivity and specificity were judged using a combination of ED_{25} and AUC, respectively. The correlation coefficients between MIC(24 h) and ED_{50}, ED_{25}, and AUC were 0.65, 0.75, and 0.68, respectively, and ED_{25} was best correlated with MIC(24 h).

Discussion

Antibacterial Activity of LZD in *S. aureus* Strains Having Reduced Susceptibility to VCM

As shown in Table 1, LZD was confirmed to have good antibacterial activity against most of the *S. aureus* strains (including 2 *vanA*-positive VRSA strains), even though they had reduced susceptibility to VCM. Although a number of studies have investigated the LZD susceptibility of MRSA strains, they included only a limited number of VISA and/or VRSA strains [4, 18]. Since our VISA collection was exhaustive (*i.e.*, we included most of the clinical isolates of VISA available worldwide), we considered that LZD could be used as a second drug of choice for MRSA infections, especially if VCM therapy has been prolonged and/or failed. This is further supported by several clinical observations describing that clinical outcomes of cases treated with LZD have been fair [11, 17].

Table 2. Sensitivity and specificity of various diagnostic parameters.

	LLSA[a]+LRSA			LRSA		
	Sensitivity (%)	Specificity	Optimal cut-off value[b]	Sensitivity (%)	Specificity	Optimal cut-off value[b]
Microbroth dilution method						
MIC(24 h)	83	96	3	100	100	4–7
MIC(48 h)	100	100	4	100	100	5–8
Chemiluminescence analysis						
ED_{50}	83	87	0.7	100	100	1.05–1.32
ED_{25}	100	81	1.27	100	100	1.99–3.37
AUC	67	97	1.42	100	100	1.81–2.35

Sensitivity and specificity of various diagnostic parameters for detection of LRSA and/or LLSA are shown. Data were calculated from the results obtained using the 67 strains listed in Table 1.

[a]Although there is currently no definition of a LZD-intermediate *S. aureus* strain, a strain with MIC(48 h) ≥4 mg/l was tentatively termed "LLSA."

[b]Optimal cut-off values giving the best discrimination power (see Table 2 of Ref. 19).

Considering that LZD is an inhibitor of bacterial protein synthesis [4, 18, 22], it seems reasonable that LZD did not lose its antibacterial activity under hyperosmolar conditions. This suggests that LZD could be used in a hyperosmolar CAPD (continuous ambulatory peritoneal dialysis) solution.

In addition, LZD MIC for VISA (including hVISA) strains was slightly but significantly lower than that for VSSA strains (p <0.05). This tendency was also confirmed when MIC was measured by the agar plate dilution method, which allows drug-susceptibility to be compared more accurately (data not shown). Chemiluminescence parameters (ED_{50}, ED_{25}, AUC) also showed similar results (Table 1). VSSA strains used in the experiment were chosen randomly to avoid genetic bias, and they had no history of selection and/or passage by LZD prior to the experiments. Therefore, this slightly higher LZD susceptibility in VISA strains is probably significant, although the reason is still unclear.

Shortcomings of Current Drug Susceptibility Tests

As shown in Table 1, VCM MIC(24 h) in some VISA strains (Group I) was apparently low (and thus perhaps underestimated as VSSA). However, the value increased when the incubation time was prolonged to 48 h, and more sensitive and discriminative methods were able to identify these strains as VISA [19]. This means that the microbroth dilution method currently used in clinical laboratories does not have enough sensitivity to detect strains with subtly increased resistance (such as VISA) [19]. Some investigators have stated that such strains, though apparently having lower susceptibility to VCM, should ideally be detected in clinical laboratories, even if they do not meet the extant criteria for resistance [21]. This is because such strains may be associated with unfavorable clinical outcomes [6].

However, current drug-susceptibility tests cannot identify some resistant strains such as VISA, as described in our recent report [19]. This is because such tests cannot demonstrate bacterial growth below the visually detectable level. When incubation time was prolonged, some VISA strains were identified as being resistant to VCM. This seems to be one solution for detecting such strains with subtly increased resistance, although it is rather time-consuming and still incomplete: that is, the sensitivity is increased only slightly [19]. Therefore, another more sensitive and/or discriminative method such as the chemiluminescence assay is clearly desirable as a "golden alternative" test for evaluation of drug susceptibility [19].

When the sensitive chemiluminescence assay was used, most VISA and hVISA strains were successfully distinguished from VSSA strains (sensitivity and specificity >95%), even when MIC measurement gave false-negative results [19]. In this sense, the antibacterial activity of LZD should be evaluated initially by such a highly sensitive and discriminative assay method, even in cases where LZD MIC appears to be sufficiently low.

Time-Suppression Test Using a Colorimetric Probe, AlaB

Besides MIC measurement, the time-killing assay is often used in order to evaluate the antibacterial activity of an agent quantitatively. However, this assay is unsuitable for evaluation of bacteriostatic agents such as LZD, since the antibacterial activity of the agent is expressed as the number of living (surviving) cell colonies; that is, any antibacterial activity that does not cause bacterial cell killing (or growth retardation) will not be reflected in the final result. For example, such a colony-counting method cannot evaluate how strongly the bacterial metabolic activity is suppressed by bacteriostatic agents. To overcome this drawback, a time-suppression test using a colorimetric probe (AlaB) was performed.

When the results for LZD and VCM were compared [9], it was found that the initial antibacterial activity of the two agents was almost equivalent in terms of the period before the antibacterial effect became evident. It appeared that both LZD and VCM exerted equal antibacterial effects within ~2 h (also confirmed by colony counting; data not shown).

Since the AlaB method gave a time course curve similar to that of the classical colony counting method (data not shown), this allowed the time course of the antibacterial agent to be estimated easily and quickly. However, the baseline activity was very high (~50%) [9], and this made it difficult to evaluate how strongly bacterial metabolic activity was suppressed. Although the reason for the high baseline activity was unclear, it is possible that non-specific reduction of the dye molecules (perhaps due to reductants accumulated in the cells) might have occurred even after the cells had lost their viability. It seems that a more sensitive and discriminative assay method is needed to evaluate the antibacterial activity of LZD.

Chemiluminescence Assay

As described above, both simple MIC measurement and colorimetric assay might have failed to evaluate the antibacterial activity of LZD accurately. Therefore, a chemiluminescence assay was applied in order to study susceptibility to LZD. This revealed that LZD had strong suppressive activity on bacterial metabolism, since CLI (directly linked with the bacterial electron transfer system) of most strains declined steeply as the LZD concentration increased.

In contrast to VCM, LZD MIC measured by the microbroth dilution method did not increase markedly when incubation time was prolonged (Table 1). This means that these strains were completely susceptible to LZD (*i.e.*, unlike the case of VCM, there is currently no "hidden" resistance to LZD), and the chemiluminescence assay successfully revealed this feature. When compared with VCM (see Fig. 2 of Ref. 19), the plots for LZD fell to almost "zero" activity, although the CLI plots for VCM tended to show tailing and a raised nadir in the case of VISA strains [19]. This may offer a possible explanation for the fact that the clinical effect of LZD was fair, even though it is merely a bacteriostatic agent (an inhibitor of bacterial protein synthesis) [11, 17]. For instance, it is known that LZD suppresses exotoxin production by *S. aureus* to a greater extent than other classes of antibiotics, and this is probably one of the reasons why LZD provides good clinical outcomes [5]. Judging from Fig. 1, it can be considered that such an effect may result from the strong metabolic suppression activity of LZD.

In addition, the CLI curve of strain NRS127 appeared to be only slightly different from that of susceptible strains, although it is classified as LRSA. This means that the mechanism of resistance in NRS127 may not be far advanced, and that this is still controllable by a therapeutic concentration of LZD (6–20 mg/l according to the manufacturer's technical insert). Furthermore, 5 strains were found to have relatively lower susceptibility to LZD (still susceptible, but tentatively classified as LLSA with MIC(48 h) = ~4 mg/l) in this study. When sensitivity and specificity were judged from a combination of ED_{25} and AUC, respectively, such a strain having slightly reduced susceptibility was successfully identified among other susceptible strains. One merit of the chemiluminescence assay is that such findings can be obtained more discriminatively and quickly (within about 7–8 h from colony isolation) in comparison with culture-based methods (*e.g.*, the microbroth dilution method).

Among the parameters calculated from the chemiluminescence assay, ED_{25} was best correlated with MIC(24 h), but the coefficient was not as high as that for VCM [19]. This may mean that chemiluminescence parameters such as ED_{25} are independent barometers of drug susceptibility.

A Lesson from History

Fig. 1C compares the antibacterial activities of LZD and ABK with those of EM and MINO in susceptible type strains. EM and MINO are now considered to be old and historical drugs, and are probably clinically useful in relatively limited cases. However, it was surprising that EM and MINO had stronger antibacterial effects than the newer drugs, LZD and ABK. This means the sole merit of LZD is that only a few strains are currently resistant to it. Fig. 1C suggests that LZD is only a second-choice drug: that is, in their "heyday," EM and MINO would have had a potency against susceptible *S. aureus* strains about $\geq$10 times greater than that of LZD on a molar basis. Fig. 1C is also informative with regard to how antimicrobial agents should be used, and what therapeutic strategy should be adopted (*i.e.*, with very great care). Otherwise, the effectiveness and value of any promising new drug such as LZD will be rapidly eroded because of the rise of resistant strains, as was the case for EM and MINO.

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