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EXPERIMENTAL THERAPEUTICS

In Vitro and *In Vivo* Activities of DS-2969b, a Novel GyrB Inhibitor, and Its Water-Soluble Prodrug, DS11960558, against Methicillin-Resistant *Staphylococcus aureus*

Tarani Kanta Barman,[a] Manoj Kumar,[a] Tarun Mathur,[a] Eiko Namba,[c] Diksha Singh,[a] Tridib Chaira,[b] Yuichi Kurosaka,[c] Makiko Yamada,[c] Dilip Jatashankar Upadhyay,[a] Nobuhisa Masuda[a,c]*

[a]Department of Microbiology, Daiichi Sankyo India Pharma Private Limited, Gurgaon, Haryana, India

[b]Department of Pharmacokinetics and Metabolism, Daiichi Sankyo India Pharma Private Limited, Gurgaon, Haryana, India

[c]Daiichi Sankyo Co., Ltd., Tokyo, Japan

ABSTRACT DS-2969b is a novel GyrB inhibitor under clinical development. In this study, the *in vitro* activity of DS-2969b and the *in vivo* activities of DS-2969b and its water-soluble prodrug, DS11960558, against methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated. DS-2969b inhibited the supercoiling activity of *S. aureus* DNA gyrase and the decatenation activity of its topoisomerase IV. DS-2969b showed antibacterial activity against Gram-positive aerobes but not against Gram-negative aerobes, except for *Moraxella catarrhalis* and *Haemophilus influenzae*. DS-2969b was active against MRSA with an MIC_{90} of 0.25 μg/ml, which was 8-fold lower than that of linezolid. The presence of a pulmonary surfactant did not affect the MIC of DS-2969b. DS-2969b showed time-dependent slow killing against MRSA. The frequency of spontaneous resistance development was less than 6.2×10^{-10} in all four *S. aureus* isolates at 4× MIC of DS-2969b. In a neutropenic MRSA-induced murine muscle infection model, DS-2969b was more efficacious than linezolid by both the subcutaneous and oral routes. DS-2969b and DS11960558 showed efficacy in a neutropenic murine MRSA lung infection model. The pharmacokinetics and pharmacodynamics of DS-2969b and DS11960558 against MRSA were characterized in a neutropenic murine thigh infection model; the percentage of time during the dosing period in which the free drug concentration exceeded the MIC (fT_{MIC}) correlated best with *in vivo* efficacy, and the static percent fT_{MIC} was 43 to 49%. A sufficient fT_{MIC} was observed in a phase 1 multiple-ascending-dose study of DS-2969b given orally at 400 mg once a day. These results suggest that DS11960558 and DS-2969b have potential for use as intravenous-to-oral step-down therapy for treating MRSA infections with a higher efficacy than linezolid.

KEYWORDS DNA gyrase, *Staphylococcus aureus*, animal models, methicillin resistance, pharmacodynamics, pharmacokinetics

The discovery of penicillin and sulfa drugs in the 1930s significantly improved antibacterial therapy. Numerous antibiotics were discovered in the subsequent 60 years; however, bacteria have constantly evolved and developed resistance to almost all such antibiotics (1–3). Among the Gram-positive bacteria, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and enterococci present global challenges of antibiotic resistance, causing significant public health concern and adding to the cost of health care. In 2014, bacterial infections due to methicillin-resistant *S. aureus* (MRSA) were responsible for 23,000 deaths in the United States alone, as reported by the Agency for Healthcare Research and Quality (4). Gram-positive nosocomial MRSA has become increasingly resistant to multiple antibiotics, such as vancomycin (VAN), trimethoprim-

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Address correspondence to Nobuhisa Masuda, masuda.nobuhisa.up@daiichisankyo.co.jp.

* Present address: Nobuhisa Masuda, Global Project Management Department, Daiichi Sankyo Co., Ltd., Tokyo, Japan.

T.K.B., M.K., and T.M. contributed equally to this article.

sulfamethoxazole, β-lactams, tetracycline, clindamycin, quinolones, and aminoglycosides (5, 6). Approximately 700,000 antibiotic resistance-related deaths are documented yearly, and this number is estimated to increase to 10 million by 2050, surpassing cancer as the leading cause of death globally (7, 8).

The emergence of multidrug-resistant pathogens as well as their increasing contribution to nosocomial infections is a growing medical concern. In U.S. hospitals, MRSA accounted for 59.2, 55.0, and 47.9% of *S. aureus* isolates from non-intensive care unit (non-ICU) inpatients, ICU patients, and outpatients, respectively (9). The ongoing threats of VAN-resistant enterococci and VAN-intermediate-susceptible and VAN-resistant *S. aureus* further underscore the need for novel therapies active against resistant Gram-positive pathogens. The emergence of multidrug resistance in Gram-positive pathogens has significantly limited the therapeutic options for nosocomial and community-acquired infections. Despite extensive research in the past, drugs for the treatment of nosocomial infections are still limited (10–12).

Linezolid (LZD) retains activity against resistant Gram-positive strains and is available as both intravenous and oral formulations, which makes step-down therapy possible. However, LZD may cause myelosuppression when used for a period longer than 14 days. In a phase 3 study performed in Japan, the total success rate of LZD against MRSA infections was more than 60% at the end of therapy, whereas it dropped to less than 40% at 7 to 14 days after therapy (13). This indicates that the dose and therapeutic duration of LZD were not enough to eradicate MRSA in patients. Although the incidence of LZD-resistant MRSA is low, LZD resistance may be transferred from resistant to nonresistant cells via the ribosomal methyltransferase gene, *cfr* (14). Tedizolid, another anti-MRSA antibacterial that is available as both intravenous and oral formulations, is used for the treatment of acute bacterial skin and skin structure infections (ABSSSIs). However, there are safety and efficacy concerns over the use of tedizolid in patients with neutropenia (15). In an animal model of infection, tedizolid showed reduced efficacy in the absence of granulocytes (16). Alternative therapies are recommended when treating ABSSSIs in patients with neutropenia (15, 16). Furthermore, tedizolid is still not indicated for the treatment of nosocomial pneumonia (17). Similarly, although daptomycin is highly active against Gram-positive bacterial infections, including those caused by MRSA, it is not indicated for the treatment of pneumonia because its antibacterial activity is impaired in the presence of pulmonary surfactants. In addition, daptomycin is not indicated for left-sided infective endocarditis due to *S. aureus* because of its poor clinical efficacy. Daptomycin is also associated with myopathy and peripheral neuropathy (18).

Therefore, to meet the inadequacies of current anti-MRSA therapies, the demand for a new class of anti-MRSA drugs is strong. Bacterial DNA gyrase and topoisomerase IV are highly conserved type II topoisomerases that play essential roles in DNA replication and transcription. They are established antibacterial drug targets (19, 20) and have been used for the discovery of inhibitors with a novel scaffold (21, 22, 23). DNA gyrase is a heterotetramer comprising two GyrA and two GyrB subunits, whereas topoisomerase IV is a heterotetramer comprising two ParC and two ParE subunits. The ATPase activities of the GyrB and ParE subunits are essential for the strand-passage reaction and are thought to be attractive targets for antibacterial drug discovery (24, 25). Dual-target inhibitors pose lower risks of resistance development than single-target inhibitors, which show large shifts in their MICs due to single-step mutations in bacteria.

DS-2969b (Fig. 1A) is a new investigational GyrB inhibitor currently under clinical development (26) for the treatment of *Clostridium difficile* infections. DS11960558 (Fig. 1B) is a water-soluble phosphonoxymethyl prodrug of DS-2969b. The water solubilities of DS-2969b and DS11960558 are 0.4 and >100 mg/ml, respectively (27). DS11960558 is inactive in its native form and requires enzymatic conversion to DS-2969b to exert antibacterial activity (27). In this study, the *in vitro* activities and *in vivo* activities of DS-2969b and DS11960558 against MRSA were evaluated.

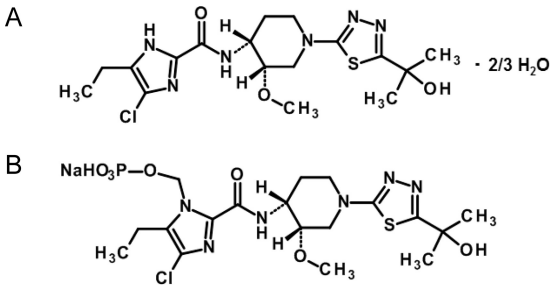

FIG 1 Chemical structures of DS-2969b (A) and DS11960558 (B).

RESULTS

Mechanism of action. To investigate the mechanism of action of DS-2969b, DNA gyrase supercoiling and topoisomerase IV decatenation assays were performed using *S. aureus* enzymes. The 50% inhibitory concentrations (IC_{50}s) of DS-2969b for *S. aureus* DNA gyrase and topoisomerase IV are shown in Table 1. DS-2969b inhibited both the supercoiling activity of DNA gyrase and the decatenation activity of topoisomerase IV. DS-2969b was 371- and 77-fold more potent in inhibiting *S. aureus* DNA gyrase and topoisomerase IV, respectively, than levofloxacin (LVX). DS-2969b did not inhibit human topoisomerase II (Table 1), indicating that DS-2969b selectively inhibits the activity of bacterial topoisomerases.

Antibacterial activity. Table 2 shows the antibacterial activity of DS-2969b against aerobic bacteria. DS-2969b showed potent antibacterial activity (MICs $\leq$ 2 μg/ml) against Gram-positive aerobic bacteria. However, it showed poor activity (MICs $>$ 8 μg/ml) against Gram-negative aerobic bacteria, except for *Moraxella catarrhalis* and *Haemophilus influenzae* (MICs = 0.25 and 0.5 μg/ml, respectively). The MIC_{90} (0.25 μg/ml) of DS-2969b against 67 MRSA clinical isolates was 8-fold lower than the MIC_{90}s of LZD and VAN, and the MIC range of DS-2969b was 0.03 to 0.5 μg/ml (Table 3). The MICs against *S. aureus* ATCC 29213 and two MRSA strains were determined in the presence of 2.5 or 5% pulmonary surfactant (Table 4). The presence of pulmonary surfactant did not affect the activity of DS-2969b. The time-kill curves of DS-2969b and LZD against MRSA 02541, a clinical isolate, are shown in Fig. 2. DS-2969b and LZD showed time-dependent slow killing with exposures at 1×, 4×, and 16× MIC.

Propensity for *in vitro* resistance development. To assess the potential for resistance development, the frequencies of spontaneous resistance development against DS-2969b and LZD were determined in four *S. aureus* isolates, including three MRSA strains, in triplicate (Table 5). The frequency of resistance development at 4× MICs of DS-2969b and LZD was less than 6.2×10^{-10} in all four *S. aureus* isolates. However, DS-2969b showed an 8-fold higher MIC against a VRT-752586-resistant mutant having the T173I mutation in GyrB (24).

Murine muscle and lung infection models. The efficacy of DS-2969b was investigated in a neutropenic murine muscle infection model (Fig. 3) in which the infection was caused by MRSA 02541. DS-2969b at a dose of 50 mg/kg of body weight showed bactericidal potential by both the subcutaneous and the oral routes, whereas LZD showed a static effect at the same dose and by the same routes of administration. At lower doses, DS-2969b reduced the bacterial count in a dose-dependent manner

TABLE 1 IC_{50}s of DS-2969b and levofloxacin for *S. aureus* and human topoisomerases

	IC_{50} (μg/ml)		
	S. aureus		
Compound	DNA gyrase	Topoisomerase IV	Human topoisomerase II
DS-2969b	0.14	0.84	>200
Levofloxacin	52	6.5	>1,024

TABLE 2 Antibacterial activities of DS-2969b and linezolid against aerobic bacteria

Strain	MIC (μg/ml)	
	DS-2969b	Linezolid
Staphylococcus aureus ATCC 6538P	0.25	2
Staphylococcus epidermidis ATCC 14990	0.5	1
Streptococcus pneumoniae ATCC 49619	0.25	1
Streptococcus pyogenes ATCC 12344	≤0.125	1
Enterococcus faecalis ATCC 29212	0.25	2
Enterococcus faecium ATCC 19434	2	4
Moraxella catarrhalis ATCC 25238	0.25	8
Haemophilus influenzae ATCC 49247	0.5	8
Klebsiella pneumoniae ATCC 13883	64	>128
Enterobacter cloacae ATCC 13047	>128	>128
Serratia marcescens ATCC 13880	32	>128
Proteus vulgaris ATCC 13315	16	16
Pseudomonas aeruginosa ATCC 15692	128	>128
Stenotrophomonas maltophilia ATCC 13637	64	>128
Acinetobacter baumannii ATCC 19606	64	>128

compared to the bacterial count in the controls before treatment (precontrol); however, at the same doses of LZD, there was an increase in the bacterial count compared to the bacterial count in the controls before treatment. In a neutropenic murine lung infection model using MRSA 562, another clinical isolate, DS-2969b exhibited a bactericidal effect at 50 mg/kg once a day (QD) and at 6.25 and 12.5 mg/kg/dose four times a day (QID) (Fig. 4A). However, LZD exerted a similar bactericidal effect at a 4-fold higher dose. In the neutropenic murine lung infection model, subcutaneously administered DS11960558 exerted bactericidal effects at 50 and 100 mg/kg/day and via fractionated dosing regimens (Fig. 4B). VAN showed a similar bactericidal potential at a higher dose of 220 mg/kg/day. Overall, DS-2969b and DS11960558 showed bactericidal activities in a neutropenic murine MRSA lung infection model at lower doses than LZD and VAN, respectively.

PK-PD analysis. Pharmacokinetic (PK) studies of DS-2969b and DS11960558 were conducted for PK-pharmacodynamic (PD) analysis in mice. Both drugs showed a dose-dependent increase in exposure from 3.125 to 200 mg/kg/day (as the active free forms) after subcutaneous administration. PK-PD studies were carried out in the neutropenic murine thigh infection model using MRSA 02541. For each PK-PD index, nonlinear regression analysis was done, and the coefficient of determination (R^2) was calculated for both DS-2969b and DS11960558 (Fig. 5). The percentage of time during the dosing period in which the free drug concentration exceeded the MIC (percent fT_{MIC}) correlated best with *in vivo* efficacy. R^2 values between efficacy and the percent fT_{MIC} for DS-2969b and DS11960558 were 0.65 and 0.80, respectively. R^2 values between efficacy and the area under the concentration-time curve (AUC)/MIC (or the area under the concentration-time curve for the free drug [*f*AUC]/MIC) for DS-2969b and DS11960558 were 0.62 and 0.65, respectively. The correlation between efficacy and the maximum concentration (C_{max})/MIC (or the maximum concentration of the free drug [fC_{max}]/MIC or percent T_{MIC}) for DS-2969b and DS11960558 was poor. Static percent fT_{MIC} values for DS-2969b and DS11960558 were 48.9 and 43.0%, respectively.

TABLE 3 Antibacterial activities of DS-2969b and reference compounds against MRSA clinical isolates[a]

Compound	MIC (μg/ml)		
	Range	50%	90%
DS-2969b	0.03–0.5	0.125	0.25
Linezolid	0.5–2	1	2
Vancomycin	0.03–2	1	2
Levofloxacin	8–32	32	32

[a]A total of 67 isolates were tested.

TABLE 4 Effect of pulmonary surfactant on antibacterial activities of DS-2969b and comparator antibacterial agents

	MIC (μg/ml) in the presence of the indicated amt (%) of pulmonary surfactant											
	DS-2969b			Daptomycin[a]			Linezolid			Vancomycin		
S. aureus strain	0	2.5	5	0	2.5	5	0	2.5	5	0	2.5	5
ATCC 29213	0.125	0.125	0.125	1	>16	>16	2	2	2	1	1	1
MRSA 562	0.125	0.125	0.125	0.5	>16	>16	2	2	2	1	1	1
MRSA 02541	0.125	0.125	0.125	2	>16	>16	2	2	2	1	1	1

[a]In MHB with Ca^{2+} (50 μg/ml).

Rat thigh infection model. The efficacy of DS-2969b and DS11960558 was investigated in a neutropenic rat thigh infection model (Fig. 6) in which the infection was caused by MRSA 02541. Both DS-2969b and DS11960558 showed a static effect at 12.5 mg/kg QID.

DISCUSSION

The *in vitro* results indicated that DS-2969b is a novel potent inhibitor of *S. aureus* DNA gyrase and topoisomerase IV. It showed markedly improved activity against *S. aureus* target enzymes compared with LVX (Table 1). It inhibited *S. aureus* DNA gyrase more effectively than its topoisomerase IV, whereas fluoroquinolones are usually more active against *S. aureus* topoisomerase IV than its DNA gyrase (28). DS-2969b showed an 8-fold higher MIC against a VRT-752586-resistant mutant having the T173I mutation in GyrB. This result suggests that DS-2969b shows antibacterial activity primarily via GyrB inhibition in *S. aureus*. In addition, DS-2969b inhibited the ATPase activities of the GyrB and ParE subunits derived from *S. pneumoniae* (E. Namba, N. Masuda, and Y. Kurosaka, unpublished data), and the binding of another derivative which has a structure very similar to that of DS-2969b to the ATPase pocket of *S. aureus* ParE has been confirmed by cocrystallization (E. Imai and H. Hanzawa, unpublished data). These results suggest that DS-2969b shows antibacterial activity via inhibition of the ATPase activity of GyrB/ParE. Most MRSA strains are resistant to fluoroquinolones, thereby limiting the clinical utility of fluoroquinolones (28, 29). DS-2969b had a narrow MIC range against MRSA clinical isolates, suggesting that it does not show cross-resistance to fluoroquinolones (Table 3). It exhibited potent *in vitro* activity against Gram-positive aerobic bacteria, whereas it showed high MICs against Gram-negative aerobic bacteria (Table 2), indicating its specific activity against Gram-positive bacteria. The presence of

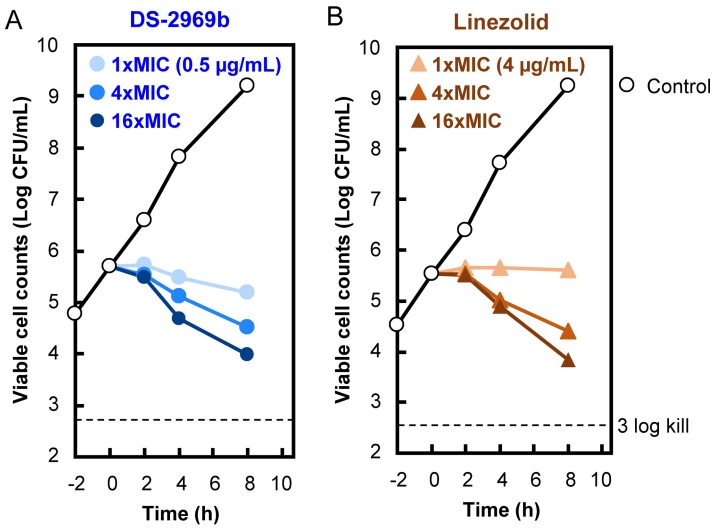

FIG 2 Time-kill curves of DS-2969b (A) and linezolid (B) against MRSA 02541.

TABLE 5 *In vitro* frequency of resistance development against DS-2969b and linezolid in different *S. aureus* strains

S. aureus strain	Frequency of resistance at 4× MIC	
	DS-2969b	Linezolid
ATCC 29213 (MSSA[a])	$<2.5 \times 10^{-10}$	$<2.5 \times 10^{-10}$
IHMA 1170860 (MRSA)	$<4.3 \times 10^{-10}$	$<4.3 \times 10^{-10}$
IHMA 1170865 (MRSA)	$<6.2 \times 10^{-10}$	$<6.2 \times 10^{-10}$
IHMA 966834 (MRSA)	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-10}$

[a]MSSA, methicillin-susceptible *S. aureus*.

a pulmonary surfactant significantly impairs the antibacterial activities of many standard antibiotics, such as moxifloxacin, colistin, and daptomycin (30, 31). Our findings on daptomycin (Table 4) also showed reduced antibacterial activity in the presence of a pulmonary surfactant. However, the activity of DS-2969b was not affected in the presence of a pulmonary surfactant, indicating its potential utility in respiratory tract infections. The low frequency of spontaneous resistance development (Table 5) suggests that DS-2969b may have a low propensity to induce resistance in clinical situations, which was evident in previous LZD surveillance studies (32, 33). We evaluated the *in vivo* efficacy of DS-2969b in neutropenic murine muscle and lung infection models in which the infections were caused by MRSA; these models resemble human ABSSSIs and pneumonia, respectively. Overall DS-2969b and DS11960558 displayed better *in vivo* efficacies than LZD and VAN, respectively, against MRSA after oral and subcutaneous administrations (Fig. 3 and 4). Intravenous therapy for a few days followed by oral therapy is found to be beneficial in many patients; this is called step-down therapy. DS-2969b and DS11960558 were also found to be efficacious against neutropenic rat thigh infections when they were administered by the intravenous route. O'Dowd et al. identified a bacterial topoisomerase inhibitor that exhibited bactericidal potential at 60 mg/kg/day in a murine pneumonia model (34). DS-2969b and DS11960558 showed bactericidal effects at doses lower than 60 mg/kg/day; however, the animal model was different. DS-2969b was efficacious after both parenteral and oral administrations, suggesting that it could be amenable for use in step-down therapy.

PK-PD analysis is a useful tool for predicting the clinical efficacy of an antimicrobial agent (35). In our PK-PD studies (Fig. 5), fT_{MIC} correlated the best with the *in vivo* efficacies of DS-2969b ($R^2 = 0.65$) and DS11960558 ($R^2 = 0.80$), and the static percent fT_{MIC} values for DS-2969b and DS11960558 were 48.9 and 43.0%, respectively. After a single oral administration of DS-2969b at 400 mg to humans (36), the percent fT_{MIC}

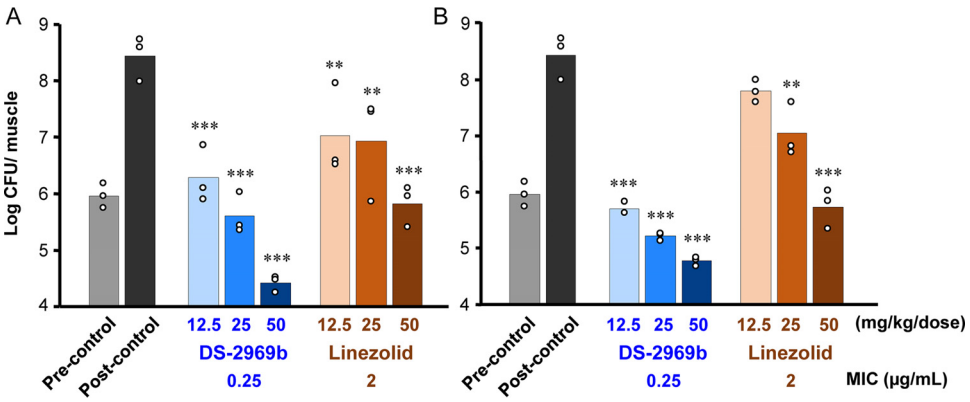

FIG 3 Efficacy of DS-2969b and linezolid after subcutaneous (A) and oral (B) administration in a neutropenic murine muscle infection model in which the infection was caused by MRSA 02541. Each circle represents the number of CFU derived from an individual animal, and each column represents the mean ($n = 3$). The numbers of CFU in each treated group were compared with those in the control group after treatment (postcontrol). Asterisks indicate a significant difference compared with the result for the control group after treatment (**, $P < 0.01$; ***, $P < 0.001$).

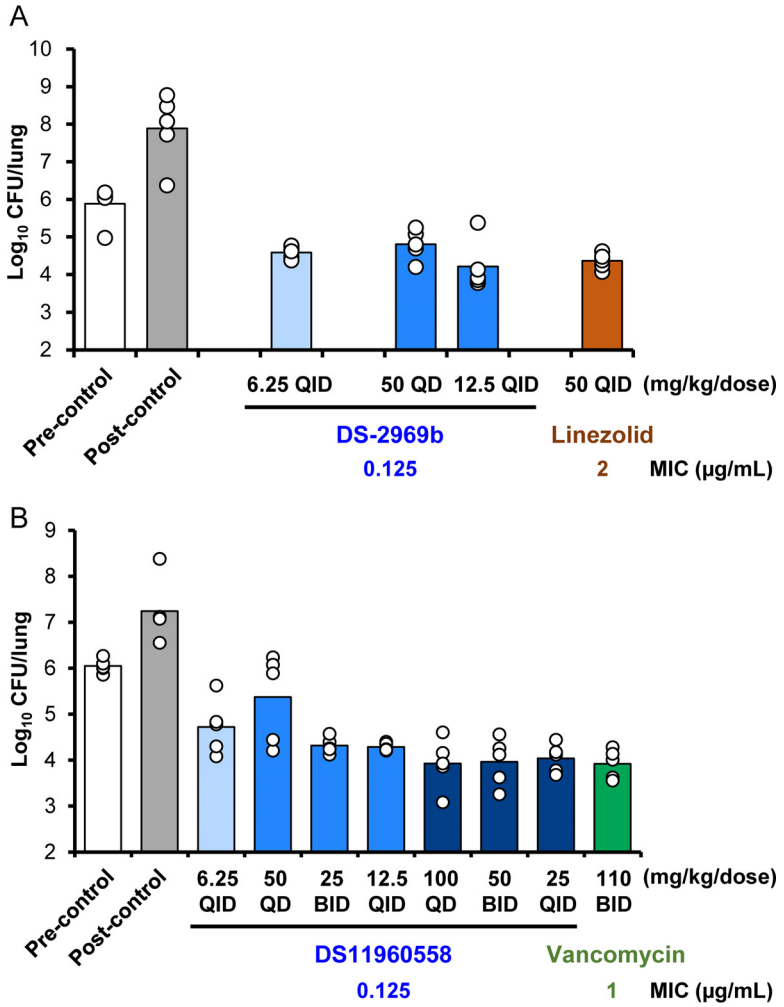

FIG 4 Efficacies of DS-2969b and linezolid (A) and DS11960558 and vancomycin (B) after subcutaneous administration in a neutropenic murine lung infection model in which the infection was caused by MRSA 562. Each circle represents the number of CFU derived from an individual animal, and each column represents the mean ($n = 5$). The numbers of CFU in each treated group were compared with those in the control group after treatment. The numbers of CFU in all treated groups were significantly different from those in the control group after treatment ($P < 0.01$).

against strains with an MIC of 0.25 μg/ml was more than the static percent fT_{MIC} values in murine PK-PD studies. After multiple oral administrations (36), the percent fT_{MIC} in humans increased with repeated administration and reached almost 100% at steady state. These data suggest that 400 mg QD could be sufficient for the treatment of MRSA infections; however, further studies are required for predicting the pharmacologically active dose for MRSA infections. DS-2969b was safe and well tolerated when it was given at a dose of up to 400 mg QD in the multiple-ascending-dose study (36).

DS-2969b displayed potent *in vitro* and *in vivo* activities against MRSA. The results of this investigational study support the possibility that DS11960558 and DS-2969b could have the potential for use as intravenous-to-oral step-down therapy for the treatment of MRSA infections.

MATERIALS AND METHODS

Antimicrobial agents. DS-2969b and VRT-752586 were synthesized at Daiichi Sankyo Co., Ltd., as described previously (37, 38). DS11960558 was synthesized at Daiichi Sankyo India Pharma Private Limited (Gurgaon, India) as described in a filed patent (M. K. Khera, N. Dumbre, and P. Khan, India patent application number 201711008754, 2017). LVX and LZD were synthesized at Daiichi Sankyo Propharma Co., Ltd., and Daiichi Sankyo RD Associe Co., Ltd., respectively. The other antimicrobial agents used in this study were obtained from Sigma-Aldrich Co. LLC.

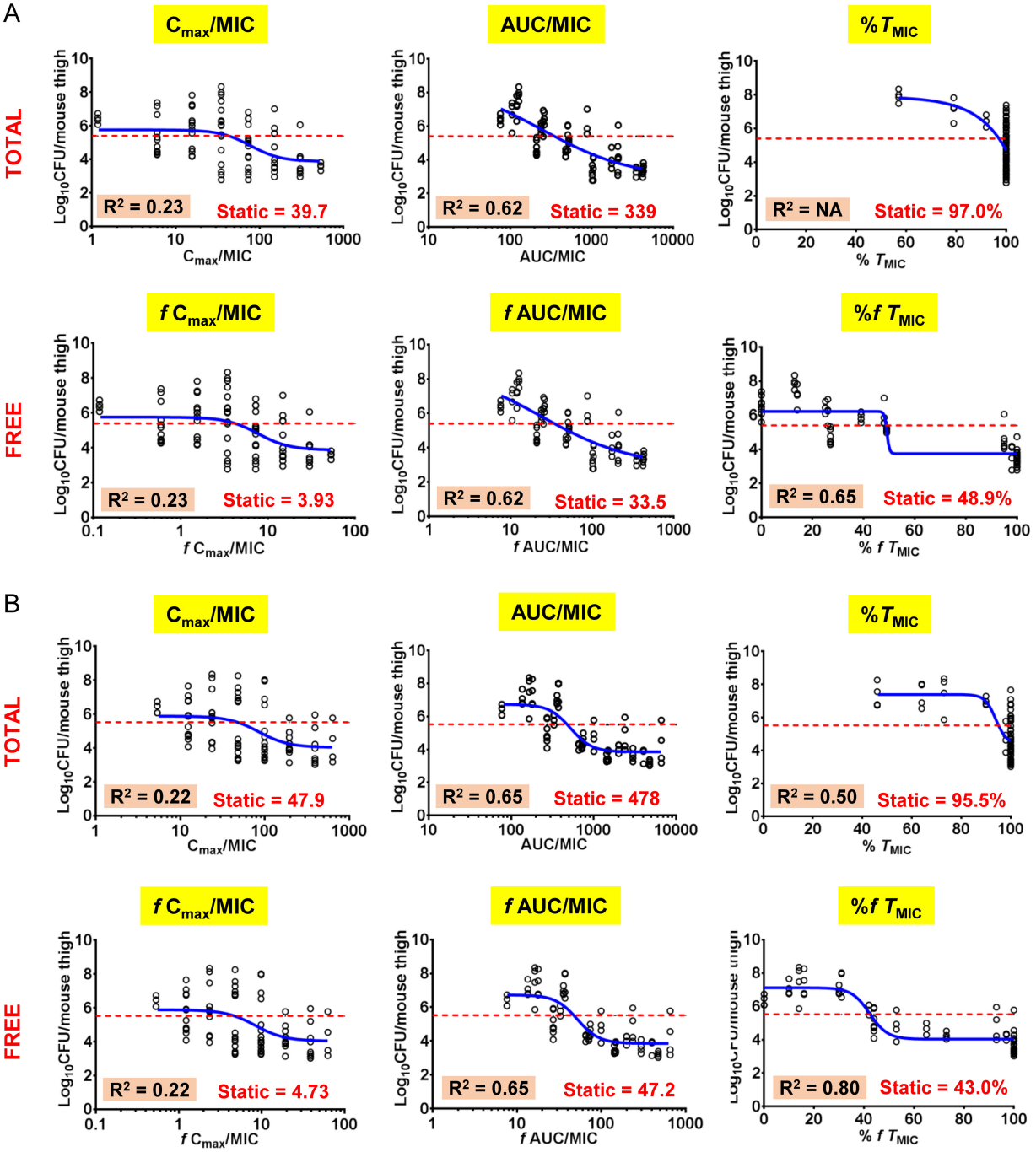

FIG 5 Impact of PD regression in the PK-PD studies with DS-2969b (A) and DS11960558 (B) against MRSA 02541. Individual data points are shown. The dose data are expressed as either C_{max}/MIC, AUC/MIC, the percentage of time during the dosing period in which the drug concentrations exceeded the MIC (%T_{MIC}), fC_{max}/MIC, fAUC/MIC, or %fT_{MIC}.

Bacterial strains. Standard strains were obtained from the American Type Culture Collection. Sixty-seven fresh MRSA clinical isolates were obtained from International Health Management Associates, Inc. (IHMA). MRSA 02541 was a clinical isolate collected by the Levofloxacin Surveillance Group in 2007. MRSA 562 was obtained from Ranbaxy Laboratories Limited. A VRT-752586-resistant mutant having the T173I mutation in GyrB was isolated by plating *S. aureus* ATCC 6538P on Mueller-Hinton agar (MHA; Becton, Dickinson and Company) plates containing 0.12 μg/ml of VRT-752586.

DNA gyrase supercoiling assay. Subunits A and B of *S. aureus* DNA gyrase were purified as fusion proteins with maltose-binding protein in *Escherichia coli*. The assays were carried out in a reaction mixture containing 4.94 ng/ml relaxed pBR322 plasmid (TopoGEN, Inc.) as a DNA substrate, 40 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 1 mM spermidine, 1.5 mM ATP, 1 mM dithiothreitol (DTT), 20

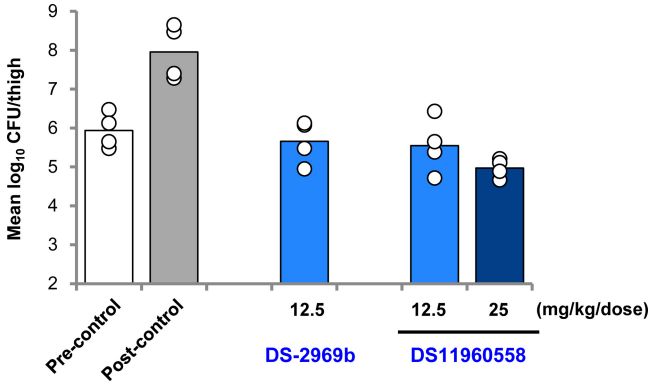

FIG 6 Efficacies of DS-2969b and DS11960558 after intravenous administration in a neutropenic rat thigh infection model in which the infection was caused by MRSA 02541. Each circle represents the number of CFU derived from an individual animal, and each column represents the mean ($n = 4$). The numbers of CFU in each treated group were compared with those in the control group after treatment. The numbers of CFU in all treated groups were significantly different from those in the control group after treatment ($P < 0.05$).

μg/ml bovine serum albumin (BSA), and 10 μg/ml factor Xa. Various concentrations (5 μl) of DS-2969b and LVX, the reaction mixture (10 μl), and 1 unit of *S. aureus* DNA gyrase (5 μl) were mixed to a total volume of 20 μl. After incubation at 37°C for 1 h, the reaction was terminated by adding 1.2 μl of stop solution (5% Sarkosyl, 0.025% bromophenol blue, 25% glycerol). The samples were electrophoresed in a 0.7% (wt/vol) agarose (Wako Pure Chemical Industries, Ltd.) gel at 60 V for 1.5 h. The gel was stained in ethidium bromide solution for 30 min, destained with water, and then photographed under UV illumination by an imaging analysis system (Gel Print 2000i/VGA; RelyOn Ltd.). The amount of the supercoiled DNA band was quantified by densitometric analysis using Intelligent Quantifier software (Bio Image Systems Inc.). Compound-free samples were tested in duplicate in each assay. The percentage of supercoiling activity observed in the presence of a test compound relative to that observed in the absence of the compound (100%) was determined. For each compound, the IC_{50} was estimated by connecting two data points encompassing 50% inhibition. The two relative inhibition values and the corresponding log-transformed concentrations were used for calculation. This analysis was performed using Microsoft Office Excel 2003 software (Microsoft Corporation). The IC_{50}s are rounded to two significant figures. The experiments were repeated twice, and the values were similar. Representative results are presented.

Topoisomerase IV decatenation assay. Subunits A and B of *S. aureus* topoisomerase IV were purified as fusion proteins with maltose-binding protein in *E. coli*. Decatenation assays were carried out in 20 μl of a reaction mixture containing 40 mM Tris-HCl (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 50 μg/ml BSA, 10 μg/ml factor Xa, 0.4 μg catenated kinetoplast DNA (TopoGEN, Inc.) as a DNA substrate, 1 unit of *S. aureus* topoisomerase IV, and various concentrations of DS-2969b and LVX. After incubation at 37°C for 1 h, the reaction was terminated by adding 1.2 μl of the stop solution. The samples were electrophoresed in a 0.7% (wt/vol) agarose gel at 60 V for 1.5 h. The gel was stained in ethidium bromide solution for 30 min, destained with water, and then photographed under UV illumination by the imaging analysis system Gel Print 2000i/VGA. The amount of the decatenated form of kinetoplast DNA was quantified by densitometric analysis using Intelligent Quantifier software. Compound-free samples were tested in duplicate in assays for each compound. The percentage of decatenation activity observed in the presence of the compound was determined when the amount of decatenated kinetoplast DNA in the absence of compound was set to 100%. The IC_{50}s were determined as described for the DNA gyrase supercoiling assay.

Human topoisomerase IIα decatenation assay. Human topoisomerase IIα was prepared as a histidine-tagged protein in *E. coli*. Decatenation assays were carried out in 20 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM DTT, 30 μg/ml BSA, 0.4 μg catenated kinetoplast DNA as a DNA substrate, human topoisomerase IIα enzyme, and various concentrations of DS-2969b and LVX. After incubation at 37°C for 1 h, the reaction was terminated by adding 1.2 μl of the stop solution. The samples were electrophoresed in a 0.7% (wt/vol) agarose gel at 60 V for 2 h. Gel staining and analysis were performed as described for the topoisomerase IV decatenation assay.

Antibiotic susceptibility studies. MICs against aerobic bacteria were determined using the broth microdilution method described in Clinical and Laboratory Standards Institute document M07-A8 (39). Pulmonary surfactant (Survanta; AbbVie Inc.) or calcium chloride was added to Mueller-Hinton broth (MHB; Becton, Dickinson and Company), as required. The experiments were repeated twice or three times, and the values were similar. Representative results are presented.

Time-kill assay. The activities of DS-2969b and LZD against MRSA were determined by the use of time-kill curves as described earlier (40). Solutions of the test compounds at 100 times the final concentration (1×, 4×, and 16× MIC) were prepared in dimethyl sulfoxide. MRSA 02541 was cultured at 35°C overnight on MHA plates. Bacterial colonies grown on the agar plates were suspended in

cation-adjusted MHB, dispensed (9.9 ml) into separate flasks, and incubated at 35°C for 3 h under constant shaking (120 rpm) to obtain cultures containing approximately 10^6 CFU/ml of bacteria. To 9.9 ml of each bacterial culture was added 0.1 ml of each test compound solution to make final concentrations of 1×, 4×, and 16× MIC or 0.1 ml of dimethyl sulfoxide as a control, and the cultures were incubated further. Aliquots were withdrawn after 0, 2, 4, and 8 h of exposure and diluted with saline, and 0.1 ml of the diluted samples was spread onto agar plates for overnight incubation at 35°C. Further, the number of colonies was counted.

Frequency of resistance. A previously described method for determination of the frequency of resistance was followed (40). Bacterial suspensions of each *S. aureus* strain were prepared by suspending the bacterial colonies in tubes containing MHB, and 100 μl of each suspension was plated onto the surface of MHA containing 4× MIC of the test compounds. After incubating for about 48 h at 37°C, the number of colonies was counted and divided by the number of bacteria inoculated to determine the frequency of resistance. MICs determined by the agar dilution method (39) were used for the calculation of 4× MIC.

Experimental animals. Five-week-old female Crl:CD1 (ICR) mice were used for the muscle infection model. Mice were purchased from Charles River Laboratories Japan, Inc., and housed in rooms under controlled conditions (temperature, 23 ± 2°C; relative humidity, 55% ± 20%; artificial light, 12-h light/12-h dark cycle with lights on at 8:00 a.m.). All experimental procedures were performed in accordance with the in-house guidelines of the IACUC of Daiichi Sankyo Co., Ltd.

Other animal studies were carried out at the Daiichi Sankyo India facility. Specific-pathogen-free female Swiss Webster mice (4 to 6 weeks old) and Sprague Dawley (SD) rats (4 to 6 weeks old) were purchased from Vivo Bio Tech Ltd., Hyderabad, India. The mice and rats were used after an acclimation period of 8 days in an animal quarantine facility. The experimental environment was controlled (temperature, 22 ± 2°C; relative humidity, 40 to 60%; 12-h light/12-h dark cycle). Feed and water were provided *ad libitum* during the whole study. All animal protocols were approved by the Institutional Animal Ethics Committee of Daiichi Sankyo India Pharma Private Limited, Gurgaon, India. The study was conducted under the strict guidelines set out by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests and Climate Change, Government of India; the facility registration number is 1390/PO/R/S/2010/CPCSEA, and the approved protocol application numbers were DS-2014/043 and DS-2014/004E1 for the studies with mice and rats, respectively.

Murine muscle infection model. The murine muscle infection model was established as described earlier (41). Colonies growing on Trypticase soy agar (TSA) were inoculated into MHB and incubated overnight at 35°C. After 10-fold dilution with fresh MHB, the bacterial suspension was used as the inoculum. DS-2969b and LZD were administered subcutaneously or orally QID. A mixture of ketamine hydrochloride (Daiichi Sankyo Propharma Co. Ltd.), xylazine hydrochloride (Bayer Medical Ltd.), and water for injection (Fuso Pharmaceutical Industries, Ltd.) in a ratio of 2:1:4 was used for anesthesia. The mice were injected with the anesthetic solution intramuscularly (0.05 to 0.07 ml/mouse). At 4 days and 1 day before MRSA inoculation, the mice were intraperitoneally injected with 150 and 100 mg/kg of cyclophosphamide (Endoxan, Shionogi & Co., Ltd.), respectively, for causing neutropenia. MRSA 02541 (40 μl) was inoculated into the right calf muscle of the anesthetized mice. DS-2969b and LZD solutions were injected subcutaneously or orally 2, 8, 14, and 20 h after the inoculation ($n = 3$). The number of bacteria in the calf muscle was determined at 2 h (at the onset of treatment) and 26 h (at 24 h after treatment) after the inoculation. The anesthetized mice were exsanguinated by severing the axillary artery and vein, and the right calf muscle of each mouse was removed aseptically for the determination of the number of bacteria. The calf muscles were homogenized in 3 ml of phosphate-buffered saline (PBS; TaKaRa Bio Inc., Shiga, Japan) by an autohomogenizer (AH-30; Dainippon Seiki). The homogenates were diluted with PBS and inoculated on agar plates at a volume of 0.1 ml/plate using an autodilutor and -inoculator (DD-700; Dainippon Seiki). The homogenates of the calf muscle were then inoculated on TSA. The inoculated agar plates were incubated overnight at 35°C, and the number of colonies growing on the agar plates was counted. Bacterial densities were expressed as the number of CFU per muscle. When no bacterial colony in the muscle was observed, the value of the detection limit, 30 CFU per muscle, was substituted for descriptive purposes.

Murine lung infection model. The murine lung infection model was established using a clinical isolate, MRSA 562, as described earlier (42) with slight modifications. Briefly, 3 to 5 mice were utilized for each treatment and control group. Mice were rendered neutropenic by intraperitoneally injecting cyclophosphamide (Getwell Pharmaceuticals, India) at 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to MRSA infection. MRSA 562 that had been grown overnight in brain heart infusion (Becton, Dickinson and Company) at 36°C was used for inoculum preparation. The overnight culture was diluted 10-fold with MHB and mixed with an equal volume of 5% mucin (Becton, Dickinson and Company). After anesthetizing the mice with isoflurane (Baxter, India), they were held upright and 50 μl of the inoculum was administered into the nares, which was inhaled by the mice into the lungs. Treatment was initiated 1 h after infection. Mice were subcutaneously or orally administered DS-2969b, LZD, DS11960558, or VAN. Further, they were euthanized after 24 h, and the lungs were harvested and homogenized in 3 ml of sterile PBS. The homogenates were used for the determination of the viable bacterial count after inoculation on TSA plates.

Mouse PK-PD study. A previously described method was followed for the mouse PK-PD study (43). The PK study was carried out after the subcutaneous administration of 3.12, 25, 100, and 200 mg/kg of DS-2969b and molar equivalent doses of DS11960558 at 3.906, 31.25, 125, and 250 mg/kg in healthy mice. Following the administration, blood samples were collected at different time points until 24 h postdosing. Plasma samples were obtained after centrifugation and analyzed using liquid chromatog-

raphy (LC)-tandem mass spectrometry (MS/MS). DS-2969b and DS11960558 were extracted from plasma samples by acetonitrile precipitation. Briefly, 50 μl of water was added to 20 μl of plasma samples. To this, 50 μl of acetonitrile and 350 μl of an internal standard (1 μM niflumic acid in acetonitrile) were added. The contents were mixed on 96-well shakers and filtered through Captiva 96-well polypropylene filter plates. Supernatants (8 μl) were injected into an LC-MS/MS system (AB Sciex API-4000 with an electrospray ionization-MS probe kept at 450°C) coupled with an Acquity ultraperformance liquid chromatography (Waters) system under gradient mobile phase flow. A Sunniest HT C_{18} column (50 by 2.1 mm; particle size, 2 μm) was used, and the flow rate was set at 0.8 ml/min. The mobile phase consisted of 100 mM ammonium acetate-acetonitrile-formic acid (50:950:2, vol/vol/vol) (mobile phase A) and 100 mM ammonium acetate-water-acetonitrile-formic acid (50:900:50:2, vol/vol/vol/vol) (mobile phase B). The selected precursor and product ion were *m/z* 429.2 and 249, respectively, for DS-2969b and, and 539.2 and 411.1, respectively, for DS11960558. Analyst software (version 1.4) was used to process the sample in quantitation mode using (1/*x*) weighted linear regression analysis. PK parameters were calculated using Phoenix WinNonlin software (version 6.3; Certara, L.P.).

A neutropenic murine thigh infection model in which the infection was caused by MRSA 02541 was used for PD evaluation. Mice were rendered neutropenic by intraperitoneally injecting cyclophosphamide (Getwell Pharmaceuticals) 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to MRSA infection. All mice were weighed and randomized into different treatment groups 1 day before the study. The mice were infected by injecting 2×10^5 to 3×10^5 CFU of MRSA 02541 (MIC of DS-2969b = 0.25 μg/ml) in the right thigh. Dose fractionation studies (every 24 h, 12 h, 6 h, and 3 h) were performed after the subcutaneous administration of 25, 50, 100, and 200 mg/kg/day; the first dose was administered 2 h after infection. Thighs were removed 24 h after dosing and processed for determination of the number of CFU. The level of plasma protein binding in mice used for the analysis was 90.1%, which was evaluated using the ultracentrifugation method and was similar to that in humans. For each PK-PD parameter, nonlinear regression analysis was performed, and R^2 values were calculated using GraphPad Prism software (version 5.03; GraphPad Software).

Rat thigh infection model. Rats were rendered neutropenic by a single intraperitoneal injection of cyclophosphamide (100 mg/kg) 4 days prior to MRSA infection. An overnight-grown culture of MRSA 02541 in MHB was adjusted to a 0.5 McFarland standard. The suspension was then diluted 1:10 in fresh MHB, and 200 μl was injected intramuscularly in the right thigh muscle of the rats. The bacterial inocula were confirmed by quantitative culture analyses and found to be 6×10^6 CFU per rat thigh muscle. Treatment started at 2 h postinfection. DS11960558 was intravenously administered at 12.5 and 25 mg/kg QID, whereas DS-2969b was intravenously administered at 12.5 mg/kg QID. Rats were euthanized after 24 h, and the thigh muscles were harvested and homogenized in 3 ml of sterile PBS. The homogenates were used for the determination of the viable bacterial count after inoculation on TSA plates.

Statistical analysis. The statistical significance of the difference between the viable bacterial counts recovered in the muscles of each treated group and those recovered in the muscles of the untreated control group was evaluated by one-way analysis of variance followed by Dunnett's multiple-comparison test. The statistical significance of the difference between the viable bacterial counts in the lungs of each treated group and those in the lungs of the untreated control group was evaluated by nonparametric Mann-Whitney analysis. The difference between the treated and the untreated control groups was considered to be statistically significant if the *P* value was <0.05.

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REFERENCES

1. Williams RE. 1959. Epidemic staphylococci. Lancet 273:190–195. https://doi.org/10.1016/S0140-6736(59)90383-6.
2. Bérdy J. 2012. Thoughts and facts about antibiotics: where we are now and where we are heading. J Antibiot 65:385–395. https://doi.org/10.1038/ja.2012.27.
3. Infectious Diseases Society of America, Spellberg B, Blaser M, Guidos RJ, Boucher HW, Bradley JS, Eisenstein BI, Gerding D, Lynfield R, Reller LB, Rex J, Schwartz D, Septimus E, Tenover FC, Gilbert DN. 2011. Combating antimicrobial resistance: policy recommendations to save lives. Clin Infect Dis 52:S397–S428. https://doi.org/10.1093/cid/cir153.
4. Kavanagh KT, Calderon LE, Saman DM, Abusalem SK. 2014. The use of surveillance and preventative measures for methicillin-resistant *Staphylococcus aureus* infections in surgical patients. Antimicrob Resist Infect Control 3:18. https://doi.org/10.1186/2047-2994-3-18.
5. Bozdogan B, Esel D, Whitener C, Browne FA, Appelbaum PC. 2003. Antibacterial susceptibility of a vancomycin-resistant *Staphylococcus aureus* strain isolated at the Hershey Medical Center. J Antimicrob Chemother 52:864–868. https://doi.org/10.1093/jac/dkg457.
6. Rice LB. 2006. Antimicrobial resistance in Gram positive bacteria. Am J Med 119:S11–S19. https://doi.org/10.1016/j.amjmed.2006.03.012.
7. Cox JAG, Worthington T. 2017. The 'antibiotic apocalypse'—scaremongering or scientific reporting? Trends Microbiol 25:167–169. https://doi.org/10.1016/j.tim.2016.11.016.
8. O'Neill J. 2014. Review on antimicrobial resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Her Majesty's Government, London, United Kingdom. https://amr-review.org/sites/default/files/AMR%20Review%20Paper%20-%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations_1.pdf. Accessed 9 October 2017.
9. Styers D, Sheehan DJ, Hogan P, Sahm DF. 2006. Laboratory-based surveillance of current antimicrobial resistance patterns and trends among *Staphylococcus aureus*: 2005 status in the United States. Ann Clin Microbiol Antimicrob 5:2. https://doi.org/10.1186/1476-0711-5-2.
10. Miller JR, Waldrop GL. 2010. Discovery of novel antibacterials. Expert Opin Drug Discov 5:145–154. https://doi.org/10.1517/17460440903493449.
11. Genilloud O, Vicente F. 2014. Strategies to discover novel antimicrobials to cope with emerging medical needs. *In* Antimicrobials, p 327–360. Springer, Berlin, Germany.
12. Piddock LJV. 2012. The crisis of no new antibiotics—what is the way forward? Lancet Infect Dis 12:249–253. https://doi.org/10.1016/S1473-3099(11)70316-4.
13. Kohno S, Yamaguchi K, Aikawa N, Sumiyama Y, Odagiri S, Aoki N, Niki Y,

Watanabe S, Furue M, Ito T, Croos-Dabrera R, Tack KJ. 2007. Linezolid versus vancomycin for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* in Japan. J Antimicrob Chemother 60:1361–1369. https://doi.org/10.1093/jac/dkm369.
14. Toh SM, Xiong L, Arias CA, Villegas MV, Lolans K, Quinn J, Mankin AS. 2007. Acquisition of a natural resistance gene renders a clinical strain of methicillin-resistant *Staphylococcus aureus* resistant to the synthetic antibiotic linezolid. Mol Microbiol 64:1506–1514. https://doi.org/10.1111/j.1365-2958.2007.05744.x.
15. Wong E, Rab S. 2014. Tedizolid phosphate (Sivextro) a second generation oxazolidinone to treat acute bacterial skin and skin structure infections. P T 39:555–579.
16. Drusano GL, Liu W, Kulawy R, Louie A. 2011. Impact of granulocytes on the antimicrobial effect of tedizolid in a mouse thigh infection model. Antimicrob Agents Chemother 55:5300–5305. https://doi.org/10.1128/AAC.00502-11.
17. ClinicalTrials.gov. 2017. Tedizolid phosphate (TR-701 FA) vs linezolid for the treatment of nosocomial pneumonia (MK-1986-002). U.S. National Library of Medicine, Bethesda, MD. https://clinicaltrials.gov/ct2/show/NCT02019420?term=tr-701&rank=7. Accessed 25 November 2017.
18. Merck & Co., Inc. 2017. Cubicin® (daptomycin for injection package insert) prescribing information. Merck & Co., Inc., Kenilworth, NJ.
19. Tari LW, Li X, Trzoss M, Bensen DC, Chen Z, Lam T, Zhang J, Joong Lee S, Hough G, Phillipson D, Akers-Rodriguez S, Cunningham ML, Kwan BP, Nelson KJ, Castellano A, Locke JB, Brown-Driver V, Murphy TM, Ong VS, Pillar CM, Shinabarger DL, Nix J, Lightstone FC, Wong SE, Nguyen TB, Shaw KJ, Finn J. 2013. Tricyclic GyrB/ParE (TriBE) inhibitors: a new class of broad-spectrum dual-targeting antibacterial agents. PLoS One 8:e84409. https://doi.org/10.1371/journal.pone.0084409.
20. Chiriac AI, Kloss F, Kramer J, Vuong C, Hertweck C, Sahl HG. 2015. Mode of action of closthioamide: the first member of the polythioamide class of bacterial DNA gyrase inhibitors. J Antimicrob Chemother 70:2576–2588. https://doi.org/10.1093/jac/dkv161.
21. Basarab GS, Brassil P, Doig P, Galullo V, Haimes HB, Kern G, Kutschke A, McNulty J, Schuck VJA, Stone G, Gowravaram M. 2014. Novel DNA gyrase inhibiting spiropyrimidinetriones with a benzisoxazole scaffold: SAR and in vivo characterization. J Med Chem 57:9078-9095. https://doi.org/10.1021/jm501174m.
22. Hameed PS, Solapure S, Mukherjee K, Nandi V, Waterson D, Shandil R Balganesh M, Sambandamurthy VK, Raichurkar AK, Deshpande A, Ghosh A, Awasthy D, Shanbhag G, Sheikh G, McMiken H, Puttur J, Reddy J, Werngren J, Read J, Kumar M, R M, Chinnapattu M, Madhavapeddi P, Manjrekar P, Basu R, Gaonkar S, Sharma S, Hoffner S, Humnabadkar V, Subbulakshmi V, Panduga V. 2014. Optimization of pyrrolamides as mycobacterial GyrB ATPase inhibitors: structure-activity relationship and *in vivo* efficacy in a mouse model of tuberculosis. Antimicrob Agents Chemother 58:61–70. https://doi.org/10.1128/AAC.01751-13.
23. Gjorgjieva M, Tomašič T, Barančokova M, Katsamakas S, Ilaš J, Tammela P, Mašič LP, Kikelj D. 2016. Discovery of benzothiazole scaffold-based DNA gyrase B inhibitors. J Med Chem 59:8941–8954. https://doi.org/10.1021/acs.jmedchem.6b00864.
24. Mani N, Gross CH, Parsons JD, Hanzelka B, Müh U, Mullin S, Liao Y, Grillot AL, Stamos D, Charifson PS, Grossman TH. 2006. *In vitro* characterization of the antibacterial spectrum of novel bacterial type II topoisomerase inhibitors of the aminobenzimidazole class. Antimicrob Agents Chemother 50:1228–1237. https://doi.org/10.1128/AAC.50.4.1228-1237.2006.
25. Bisacchi GS, Manchester JI. 2015. A new-class antibacterial—almost. Lessons in drug discovery and development: a critical analysis of more than 50 years of effort toward ATPase inhibitors of DNA gyrase and topoisomerase IV. ACS Infect Dis 1:4–41. https://doi.org/10.1021/id500013t.
26. Mathur T, Barman TK, Kumar M, Singh D, Kumar R, Khera MK, Yamada M, Inoue S, Upadhyay D, Masuda N. 2018. *In vitro* and *in vivo* activities of DS-2969b, a novel GyrB inhibitor, against *Clostridium difficile*. Antimicrob Agents Chemother 62:e02157-17. https://doi.org/10.1128/AAC.02157-17.
27. Yamada M, Uchiyama M, Inoue S, Deguchi T, Furuta Y, Yabe K, Masuda N. 2017. DS11960558, a water soluble prodrug of DS-2969b, for intravenous treatment of *Clostridium difficile* infection. Open Forum Infect Dis 4(Suppl 1):S473–S474. https://doi.org/10.1093/ofid/ofx163.1212.
28. Bax BD, Chan PF, Eggleston DS. 2010. Type IIA topoisomerase inhibition by a new class of antibacterial agents. Nature 466:935–940. https://doi.org/10.1038/nature09197.
29. Gade ND, Qazi MS. 2013. Fluoroquinolone therapy in *Staphylococcus aureus* infections: where do we stand? J Lab Physicians 5:109–112. https://doi.org/10.4103/0974-2727.119862.
30. Schwameis R, Erdogan-Yildirim Z, Manafi M, Zeitlinger MA, Strommer S, Sauermann R. 2013. Effect of pulmonary surfactant on antimicrobial activity *in vitro*. Antimicrob Agents Chemother 57:5151–5154. https://doi.org/10.1128/AAC.00778-13.
31. Silverman JA, Mortin LA, VanPraagh ADG, Li T, Alder J. 2005. Inhibition of daptomycin by pulmonary surfactant: in vitro modeling and clinical impact. J Infect Dis 191:2149–2152. https://doi.org/10.1086/430352.
32. Jones RN, Fritsche TR, Sader HS, Ross JE. 2007. LEADER surveillance program results for 2006: an activity and spectrum analysis of linezolid using clinical isolates from the United States (50 medical centers). Diagn Microbiol Infect Dis 59:309–317. https://doi.org/10.1016/j.diagmicrobio.2007.06.004.
33. Jones RN, Moet GJ, Sader HS, Mendes RE, Castanheira M. 2009. TR-700 in vitro activity against and resistance mutation frequencies among Gram-positive pathogens. J. Antimicrob Chemother 63:716–720. https://doi.org/10.1093/jac/dkp021.
34. O'Dowd H, Shannon DE, Chandupatla KR, Dixit V, Engtrakul JJ, Ye Z, Jones SM, O'Brien CF, Nicolau DP, Tessier PR, Crandon JL, Song B, Macikenas D, Hanzelka BL, Tiran AL, Bennani YL, Charifson PS, Grillot AL. 2015. Discovery and characterization of a water-soluble prodrug of a dual inhibitor of bacterial DNA gyrase and topoisomerase IV. ACS Med Chem Lett 6:822–826. https://doi.org/10.1021/acsmedchemlett.5b00196.
35. Martinez MN, Papich MG, Drusano GL. 2012. Dosing regimen matters: the importance of early intervention and rapid attainment of the pharmacokinetic/pharmacodynamic target. Antimicrob Agents Chemother 56:2795–2805. https://doi.org/10.1128/AAC.05360-11.
36. Inoue S, Dennie J, Nagasawa Y, Gajee R, Masuda N, Zamora C, Senaldi G. 2017. A phase 1 study in healthy subjects to investigate the safety, pharmacokinetics, and effect on intestinal flora of multiple ascending doses of DS-2969b, a novel oral DNA gyrase B inhibitor for the treatment of Clostridium difficile infection. Open Forum Infect Dis 4(Suppl 1):S526. https://doi.org/10.1093/ofid/ofx163.1371.
37. Khera MK, Mathur T, Sattigeri J, Masuda N, Soneda T, Kagoshima Y, Konosu T, Suzuki T, Yamaoka M, Itooka R. April 2017. Hydroxyalkyl thiadiazole derivatives.PCT patent WO 2017056012.
38. Charifson PS, Deininger DD, Grillot A-L, Liao Y, Ronkin SM, Stamos D, Perola E, Wang T, LeTiran A, Drumm J. November 2004. Gyrase inhibitors and uses thereof.US patent 20,040,235,886.
39. Clinical and Laboratory Standards Institute. 2009. Methods for antimicrobial susceptibility testing for bacteria that grow aerobically; approved standard, 8th ed. M07-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
40. Barman TK, Kumar M, Mathur T, Chaira T, Ramkumar G, Kalia V, Rao M, Pandya M, Yadav AS, Das B, Upadhyay DJ, Hamidullah Konwar R, Raj VS, Singh H. 2016. *In vitro* and *in vivo* activities of a bi-aryl oxazolidinone, RBx 11760, against Gram-positive bacteria. Antimicrob Agents Chemother 60:7134–7145. https://doi.org/10.1128/AAC.00453-16.
41. Hoshino K, Inoue K, Murakami Y, Kurosaka Y, Namba K, Kashimoto Y, Uoyama S, Okumura R, Higuchi S, Otani T. 2008. *in vitro* and *in vivo* antibacterial activities of DC-159a, a new fluoroquinolone. Antimicrob Agents Chemother 52:65–76. https://doi.org/10.1128/AAC.00853-07.
42. Lepak AJ, Marchillo K, Pichereau S, Craig WA, Andes DR. 2012. Comparative pharmacodynamics of the new oxazolidinone tedizolid phosphate and linezolid in a neutropenic murine *Staphylococcus aureus* pneumonia model. Antimicrob Agents Chemother 56:5916–5922. https://doi.org/10.1128/AAC.01303-12.
43. Andes DR, van Ogtrop ML, Peng J, Craig WA. 2002. *In vivo* pharmacodynamics of a new oxazolidinone (linezolid). Antimicrob Agents Chemother 46:3484–3489. https://doi.org/10.1128/AAC.46.11.3484-3489.2002.