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Intra- and extracellular activity of linezolid against *Staphylococcus aureus in vivo* and *in vitro*

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Background and aims: Treatment of *Staphylococcus aureus* infections remains problematic (slow responses and frequent recurrences). Intracellular persistence of the *S. aureus* could explain those difficulties because of impaired intracellular efficacy of antibiotics. Our aim was to study linezolid for its intracellular activity.

Methods: (i) Pharmacodynamic (PD) analysis of intracellular activity using *in vitro* (THP-1 macrophages) and *in vivo* (mouse peritonitis) models with determination of key dose–response parameters [maximal relative efficacy (E_{max}), relative potency (EC_{50}) and static concentration (C_{static})] towards methicillin-susceptible *S. aureus* (ATCC 25923; clinical isolate) with linezolid MICs of 4 mg/L; (ii) pharmacokinetic (PK) analysis in uninfected mice for determination of C_{max}, AUC and half-life for total and free drug; and (iii) determination of the predictive PK/PD parameter ($fT>MIC$, $fAUC_{24}$/MIC or fC_{max}/MIC) for therapeutic outcome.

Results: *In vitro*, linezolid showed an E_{max} of ~1 $\log_{10}$ cfu reduction compared with initial inoculum both intra- and extracellularly and an ~3-fold increased relative potency (lower EC_{50} and C_{static}) intracellularly. *In vivo*, the efficacy of linezolid was impaired (<0.5 $\log_{10}$ reduction extracellularly; failure to reduce the cfu to less than the initial load intracellularly) with, however, an increased intracellular potency (lower EC_{50}). Infection outcome correlated better with the $fAUC_{24}$/MIC ($R^2=55\%$) than with the $fT>MIC$ parameter ($R^2=51\%$) for the extracellular compartment, but no parameter emerged as significant for the intracellular compartment.

Conclusions: Linezolid exerts only a weak intracellular activity against the strains of *S. aureus* tested, even though, in contrast to most other antibiotics, its potency does not appear impaired in comparison with the extracellular activity.

Keywords: intracellular *S. aureus*, intracellular antimicrobial activity, THP-1 cells, protein binding, non-linear pharmacokinetics, pharmacodynamics, mouse peritonitis model

Introduction

Staphylococcus aureus is a major human pathogen that causes both community- and hospital-acquired infections[1,2] ranging from simple and uncomplicated skin and wound infections[3,4] to more serious and life-threatening situations such as pneumonia,[5,6] endocarditis,[7,8] osteomyelitis[9,10] and meningitis.[11] *S. aureus* infections are often difficult to treat, with poor and slow therapy response, leading to extended duration of therapy and infection recurrences, with mortality remaining significant despite antibiotic treatment.[8,12–16]

Several aspects of the pathogenesis of the bacteria may be involved in the persistence of the staphylococcal infections, among them the ability of the bacteria to invade and survive inside cells.[17–22] The presence of intracellular bacteria makes the prediction of optimal chemotherapy complicated and uncertain, because the intracellular activity of antibiotics depends on a series of pharmacokinetic (PK) and pharmacodynamic (PD) factors such as penetration, accumulation and bioavailability of the drugs inside cells, as well as the responsiveness of the bacteria to their action.[23,24] For *S. aureus*, which tends to survive and thrive within the vacuolar compartment, the antimicrobial activity of all agents tested so far is globally impaired in terms of maximal efficacy compared with broth or the extracellular milieu.[25–27] Moreover, there is now direct evidence that intracellular activity and accumulation cannot be directly correlated, not only when comparing drugs of different pharmacological classes (such as macrolides versus β-lactams, e.g.[25]) but also

when comparing drugs within the same class but showing distinct levels of accumulation.[28,29] Direct estimations of the intracellular activity of each antibiotic with suitable models are therefore needed.

Linezolid, belonging to the oxazolidinone class,[30,31] has indications for the treatment of a variety of staphylococcal infections. Although mainly used clinically against methicillin-resistant isolates,[32,33] its MIC and general properties make it equally efficacious against susceptible strains *in vitro* as well as in animal models where extracellular activity is mainly being looked at.[34–36] Yet, its potential for activity against intracellular *S. aureus* remains largely unexplored. Because of the intracellular presence of *S. aureus*, detailed knowledge of the intracellular profile of linezolid is mandatory, as it may help in rationalizing the potential use of this drug in relapsing staphylococcal infections, and provide a baseline against which novel oxazolidinones derivatives may need to be compared in this context.[28]

Several *in vitro* models, using either human or animal cells, have been developed to study the activity of antibiotics against intracellular *S. aureus*,[19,25,37–40] and a corresponding *in vivo* model (murine peritonitis) has recently been described and tested with β-lactams, gentamicin, azithromycin and rifampicin.[26] The present study was designed to characterize the intracellular activity of linezolid both *in vitro* and *in vivo*. We first examined the intra- and extracellular time–kill and concentration–kill relationships for two methicillin-susceptible *S. aureus* (MSSA) strains *in vitro* using human THP-1 (macrophages) cells. We then performed corresponding intra- and extracellular dose–kill studies *in vivo* with the murine peritonitis model. In parallel, the PK profile of linezolid in mouse was determined to estimate which PK/PD parameter[41] [C_{max}/MIC ratio, AUC/MIC ratio or duration of time during which the plasma concentration exceeds the MIC (T>MIC)] best predicts the efficacy of linezolid intra- and extracellularly.

Materials and methods

Bacterial strains and antimicrobial agents

The *S. aureus* strains ATCC 25923 (American Tissue Cell Collection, Manassas, VA, USA) and E19977 (clinical MSSA isolate from the Statens Serum Institut, Copenhagen, Denmark) were used for studies both *in vitro* and *in vivo*. Linezolid was procured as the commercial product registered in Denmark for parenteral use (Zyvoxid®; Pfizer Inc., New York, NY, USA) and complying with the provisions of the European Pharmacopeia. A clinical *S. epidermidis* strain (CNS 10559; Statens Serum Institut) was used for the linezolid bioassay.

In vitro susceptibility studies

MICs were determined in Mueller–Hinton broth (Becton Dickinson, Cockeysville, MD, USA) adjusted to pH 5.4 and 7.4, using a standard microtitre tray method according to recommendations of the CLSI[42] as described previously.[40]

In vitro extracellular time–kill and concentration–kill studies in broth

Extracellular time–kill and dose–kill studies were performed for both *S. aureus* strains (ATCC 25923 and E19977) as described previously.[40] In brief, bacteria exhibiting exponential growth were resuspended in cation-adjusted Mueller–Hinton broth to a density of 10^6 cfu/mL. Linezolid was added at the desired concentrations and samples were incubated at 37°C with shaking. Samples for cfu quantification were withdrawn before the antibiotic admixture (time 0) and after 5 and 24 h of drug exposure (test).

In vitro intracellular time–kill and concentration–kill studies in human THP-1 monocytes

In vitro studies of the intracellular activity of linezolid against both *S. aureus* strains (ATCC 25923 and E19977) were performed with THP-1 myelomonocytic cells[43] as described previously.[40,44,45] In brief: (i) bacteria were opsonized with 10% non-decomplemented human serum (Lonza, Walkersville, MD, USA) for 45 min; (ii) phagocytosis was performed at a 4:1 bacteria/macrophage ratio and non-phagocytosed bacteria were subsequently eliminated by incubating the cells in PBS containing gentamicin (50 mg/L) for 45 min; and (iii) after gentamicin washout, cells were resuspended in RPMI 1640 medium (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, and linezolid was added at the desired concentrations for tests or gentamicin (1× the MIC) for controls (to prevent extracellular growth of the bacteria[25]).

Samples for cfu quantification were withdrawn before the antibiotic admixture (time 0) and after 5 and 24 h of drug exposure. To this end, cells were collected by low speed centrifugation, washed twice in PBS and resuspended in ice-cold sterile water. The cfu and cell protein content of the samples were quantified as described previously[40] (the large dilutions of the cellular material made during its collection and actual spread on plates ensured absence of interference with cfu counts by the presence of carried-over antibiotics). All results from these studies are indicated as cfu per mg of cell protein.

The effect of linezolid (up to 24 h at 400 mg/L) on cell viability was assessed by the Trypan Blue exclusion test.

PK studies

Single-dose plasma PK studies were performed in outbred female NMRI mice (Harland Netherlands, Horst, The Netherlands) given subcutaneous doses of linezolid at three increasing concentrations (20, 40 and 80 mg/kg). Blood was removed from each of three mice for each timepoint and placed in EDTA-coated tubes. The plasma was separated by centrifugation and the linezolid plasma concentrations were measured by a microbiological bioassay using *S. epidermidis* (CNS 10559) as test organism. The concentrations were estimated from the zone diameter using the 'AssayDeterm' program in the PKPDsim software package (http://www.ssi.dk/pkpdsim). The detection limit was 1.5 mg/L, with linearity of the response (inhibition zone diameter versus $\log_{10}$ of the concentration) up to 100 mg/L, with a coefficient of variation of ∼8%. PK parameters (elimination half-life, AUC and C_{max}) were determined by non-compartmental modelling using the 'initESTIM' program in PKPDsim. The half-life was estimated from the last four timepoints by fitting of a monoexponential expression (least-squares regression). The exponential expression was also used to extrapolate the time–concentration curve from the last experimental concentration point and to 24 h for each dose tested. The AUC_{24} was therefore calculated partly from the experimental mean concentration points (trapezoidal rule) and partly from the fitted exponential curve (exact integration).

Protein binding of linezolid

Protein binding in NMRI mice plasma was measured using the ultrafiltration method. Blood was collected in EDTA-coated tubes, the plasma separated by centrifugation and finally spiked with known amounts of linezolid (10–160 mg/L). No pH difference was observed between

linezolid-containing and control plasma samples. Samples with the same concentrations as in the plasma samples were prepared in PBS to measure non-specific binding of linezolid to test tubes and other labware surfaces. The plasma and PBS samples were applied to the filter devices (Filter Centricon YM-30, Millipore, Bedford, MA, USA) after 1 h of incubation at 37°C, and the samples were centrifuged at 3000 **g** for 8 min at 37°C. The linezolid concentrations in the plasma samples (before centrifugation) and in the ultrafiltrate were measured using the above-mentioned microbiological bioassay. A theoretical expression based on binding kinetics was fitted to the free fraction of linezolid as a function of total linezolid concentration in plasma using the 'BinKin' program in PKPDsim (see Appendix 1 for a detailed description of the model).

Mouse peritonitis model

This model has been described and validated previously.[26,46] In brief, outbred female NMRI mice were infected by intraperitoneal injection of $10^{7.4}$ cfu (in a volume of 0.5 mL) and animals were examined at regular intervals for signs of major discomfort and impending death. When sampled, mice were euthanized and the peritoneal fluid obtained by injecting 2.0 mL of Hanks balanced salt solution (HBSS) (H-9269; Sigma-Aldrich Inc., St Louis, MO, USA). The peritoneal fluid, containing murine cells and bacteria, collected in EDTA-coated tubes was immediately processed for the intra- and extracellular separation assay and cfu determination as described below.

Dose–kill studies

Mice were treated with a single subcutaneous dose of linezolid (0.01–80 mg/kg) 2 h after inoculation and sampled after 4 h of drug exposure ($n=3$).

Predictive PK/PD parameter studies

Based on the results of the PK and using the fitted mathematical expression for the variable free fraction of the drug, a series of dosage regimens was modelled. This was carried out by linear scaling and interpolation, followed by truncation and repetition of the curves according to the dose size and dosage interval using the 'studyDESIGN' program in PKPDsim. A subset of 14 regimens was chosen (based on the distribution of the $fT>$MIC, $fAUC_{24}$/MIC and fC_{max}/MIC).[47] Mice achieved the first dose of linezolid 2 h after inoculation and were sampled after 24 h of drug exposure ($n=3$; see the Results section for the applied dosing regimens). A control group ($n=6$) was included for the first 6 h of infection. At this point, the mice met the clinical signs of irreversible sickness and were euthanized.

All animal experiments were approved by the Danish Animal Experimentation Inspectorate (licence no. 2004/561-835).

Separation of intra- and extracellular *S. aureus* in peritoneal fluid

This separation assay was performed as a modified version of the procedure described previously.[26] The collected peritoneal fluid from each mouse was diluted 1:1 with HBSS, and a first aliquot used for total cfu count before any further procedure. The remaining sample was then divided into two equal fractions of ~1.5 mL each (fraction A and B). For extracellular cfu quantification, fraction A was centrifuged at 300 **g** at room temperature for 10 min and the supernatant was used for assay. For intracellular cfu quantification, lysostaphin (L-7386; Sigma Aldrich Inc.) was added to fraction B to a final concentration of 15 mg/L and the faction incubated for 15 min at room temperature. The lysostaphin

Table 1. MIC for the *S. aureus* strains used in this study, and *in vitro* intra- and extracellular regression parameters for concentration–kill studies of linezolid against *S. aureus* (24 h drug exposure protocol)

	MIC (mg/L)		Extracellular data[a] (95% confidence intervals)						Intracellular data[b] (95% confidence intervals)					
				EC_{50}[d]		C_{static}[e]				EC_{50}[d]		C_{static}[e]		
Strain	pH 7.4	pH 5.4	E_{max}[c] [Δlog (cfu)]	mg/L	multiple of MIC	mg/L	multiple of MIC	R^2	E_{max}[c] [Δlog (cfu)]	mg/L	multiple of MIC	mg/L	multiple of MIC	R^2
ATCC 25923	4.0	4.0	−0.82 (−1.14 to −0.50)	3.02 (1.81–5.05)	0.76 (0.45–1.26)	12.16 (7.71–19.01)	3.04 (1.93–4.75)	0.98	−0.82 (−1.26 to −0.39)	1.15 (0.56–2.36)	0.29 (0.14–0.59)	4.95 (2.77–8.53)	1.29 (0.69–2.13)	0.95
E19977	4.0	4.0	−0.63 (−0.93 to −0.33)	2.30 (1.34–3.95)	0.58 (0.33–0.99)	12.36 (7.53–21.04)	3.09 (1.88–5.26)	0.98	−0.83 (−1.47 to −0.18)	0.77 (0.28–2.10)	0.19 (0.07–0.52)	3.00 (1.36–8.04)	0.75 (0.34–2.01)	0.91

[a]Original inoculum (time = 0 h) was $(5.27\pm1.33)\times10^5$ cfu/mL for strain ATCC 25923 and $(9.83\pm1.27)\times10^5$ cfu/mL for strain E19977 ($n=3$).
[b]Original (post-phagocytosis) inoculum (time = 0 h) was $(1.77\pm0.29)\times10^6$ cfu/mg of protein for strain ATCC 25923 and $(5.76\pm1.50)\times10^6$ cfu/mg of protein for strain E19977 ($n=3$).
[c]Decrease in colony units (in $\log_{10}$ cfu) at time 24 h from the corresponding original inoculum as extrapolated for an infinitely large antibiotic concentration and using the Hill equation.
[d]Concentrations causing a reduction of the inoculum half way between the initial (E_0) and the maximal (E_{max}) values of the Hill equation.
[e]Concentrations resulting in no apparent bacterial growth (number of cfu identical to the original inoculum), as determined by graphic intrapolation.

was removed by centrifugation (300 **g**) and the fraction prepared for cfu quantification as described previously.[26]

Data analysis

Concentration–kill and dose–kill studies were analysed by using the sigmoidal dose–response equation using a slope factor of 1 (three-parameter logistic equation), which allowed for calculation of E_{max} (maximal relative efficacy), EC_{50} (relative potency), C_{static} (concentration causing no apparent change in cfu compared with the original inoculum) and goodness of fit (R^2). E_{max} was calculated as the decrease in $\log_{10}$ cfu extrapolated for an infinitely large antibiotic concentration relative to the original inoculum measured just before treatment initiation. EC_{50} indicates the concentration or dose needed to obtain 50% of the maximal reduction in colony counts. C_{static} was defined as the concentration or dose needed for a static effect. The best fit values for the E_{max} and EC_{50} were compared between intra- and extracellular data or between strains (in multiples of the MIC) by using the extra sum-of-squares F test. A P value of <0.05 was considered significant. The correlation between the PK/PD parameter values and the infection outcome was calculated by using non-linear regression using least-squares fit. All curve fittings and statistic analysis were performed by using GraphPad Prism® 5.0 (GraphPad Prism Software, San Diego, CA, USA).

Results

Susceptibility studies

The MICs of linezolid were determined at both pH 7.4 and 5.4 since intracellular staphylococci are found in phagolysosomes where the pH is acidic. The results are given in Table 1. The MICs for both strains (ATCC 25923 and E19977) were at the limit of the susceptibility according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (4 mg/L; http://www.eucast.org) and not influenced by acid pH.

In vitro intra- and extracellular time–kill and concentration–kill studies

Linezolid did not have a toxic effect on uninfected THP-1 cells in the concentration range used in our experiments (<1% dead cells detected after 24 h of incubation).

Intra- and extracellular time–kill studies, using both *S. aureus* strains (ATCC 25923 and E19977), were performed to estimate the time perspective of the intra- and extracellular kill of linezolid

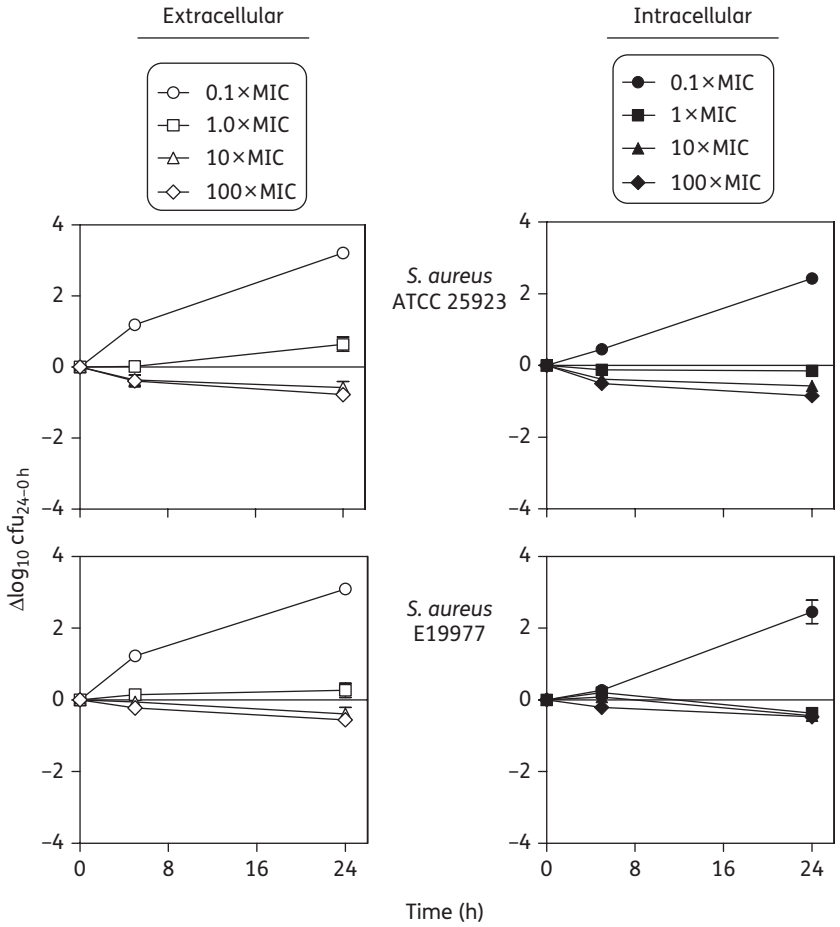

Figure 1. *In vitro* time–kill studies of linezolid against *S. aureus* at different fixed extracellular concentrations. The left-hand panels show the activity against the extracellular form of the bacteria measured in broth and the right-hand panels show the activity against the intracellular forms of the bacteria measured in THP-1 macrophages. The activity was measured against two different MSSA strains (top panels, ATCC 25923; and bottom panels, E19977). The graphs show the activity as changes in cfu ($\Delta\log_{10}$ cfu; means ± SD; $n=3$). The change in cfu is measured as cfu/mL in the extracellular studies and as cfu per mg of cell protein in the intracellular studies.

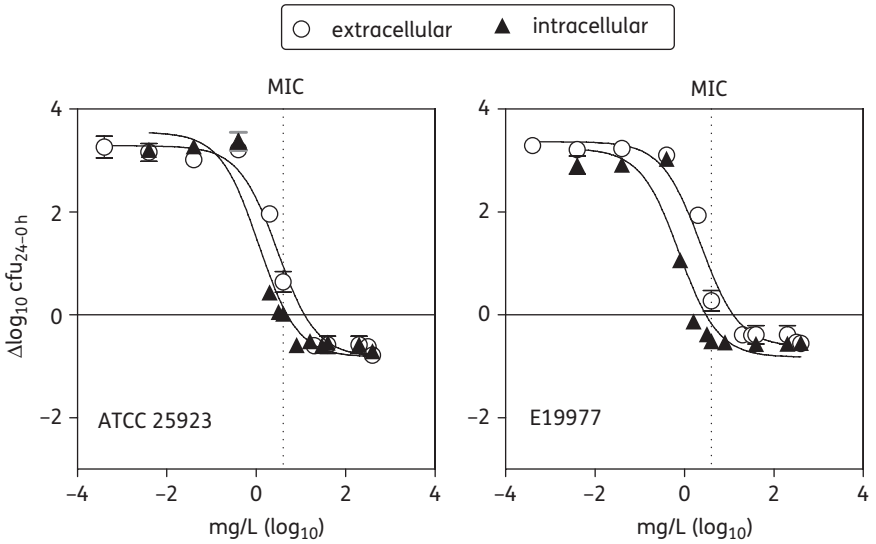

Figure 2. *In vitro* concentration–response curves of linezolid against extracellular (measured in broth) and intracellular (measured in THP-1 cells) *S. aureus*. The activity was measured against two different MSSA strains (ATCC 25923 and E19977). The graphs show the activity as changes in cfu ($\Delta\log_{10}$ cfu; means ± SD; $n=3$), measured as per mL of broth in the extracellular studies and as per mg of cell protein in the intracellular studies. The original inoculum for the extracellular study (time = 0 h) was $(5.27\pm1.33)\times10^5$ cfu/mL for strain ATCC 25923 and $(9.83\pm1.27)\times10^5$ cfu/mL for strain E19977 ($n=3$). The original (post-phagocytosis) inoculum for the intracellular study (time = 0 h) was $(1.77\pm0.29)\times10^6$ cfu/mg of protein for strain ATCC 25923 and $(5.76\pm1.50)\times10^6$ cfu/mg of protein for strain E19977 ($n=3$). The sigmoidal function was used. Goodness of fit and regression parameters are shown in Table 2.

Table 2. Protein binding of linezolid to NMRI mouse plasma

Concentration (mg/L)	Protein binding (%)
160	16.5
80	22.0
40	26.6
20	27.9
10	27.4

in vitro. The results shown in Figure 1 indicate that linezolid had essentially a static effect for both strains (with a maximal cfu decrease of ~0.5 $\log_{10}$ units at 24 h) once the concentration reached or exceeded the MIC, whether considering extracellular or intracellular bacteria. Full concentration–response studies were then performed at 24 h, and the results are shown in Figure 2, with the pertinent regression parameters given in Table 1. The E_{max} of linezolid for both strains was an ~1 $\log_{10}$ cfu decrease compared with the original inoculum, both intra- and extracellularly. The EC_{50} and the C_{static} of linezolid were ~3-fold lower intracellularly for both strains compared with their values extracellularly ($P=0.01$ for ATCC 25923 and $P=0.02$ for E19977), suggesting an enhancement of the activity of the antibiotic in the intracellular milieu. Interestingly enough, these intracellular C_{static} values were close to the MIC values measured in broth (1.29 and 0.75 times the MIC value for strain ATCC 25923 and E19977, respectively).

Protein binding and PK studies

The protein binding was measured in plasma from healthy NMRI mice, and the values are shown in Table 2. Binding was saturable by having the highest percentage value at the lowest concentrations of linezolid, and vice versa. As can be seen from Figure 3, the data from the binding study were well fitted to the theoretical expression for the free fraction as a function of total concentration. The PK of linezolid in mice plasma, as obtained from the three studies carried out at 20, 40 and 80 mg/kg, are shown in Figure 4. Peak levels were observed after 15–45 min. The half-life varied from 45 to

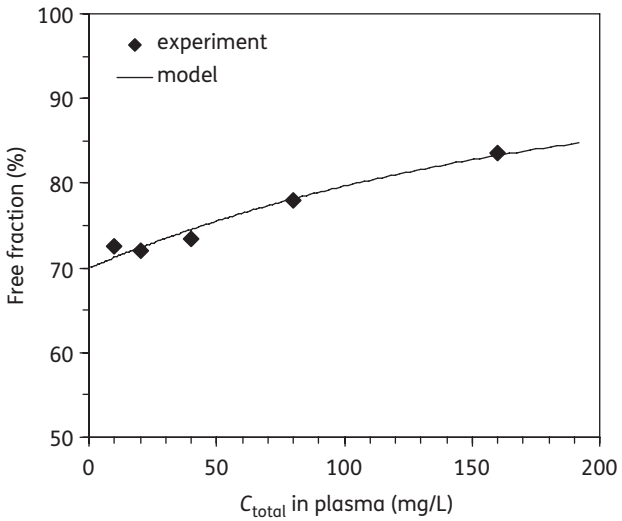

Figure 3. Fitting of a theoretical expression to the experimental data for protein binding of linezolid in plasma from healthy mice. See Appendix 1 for a description of the theoretical model.

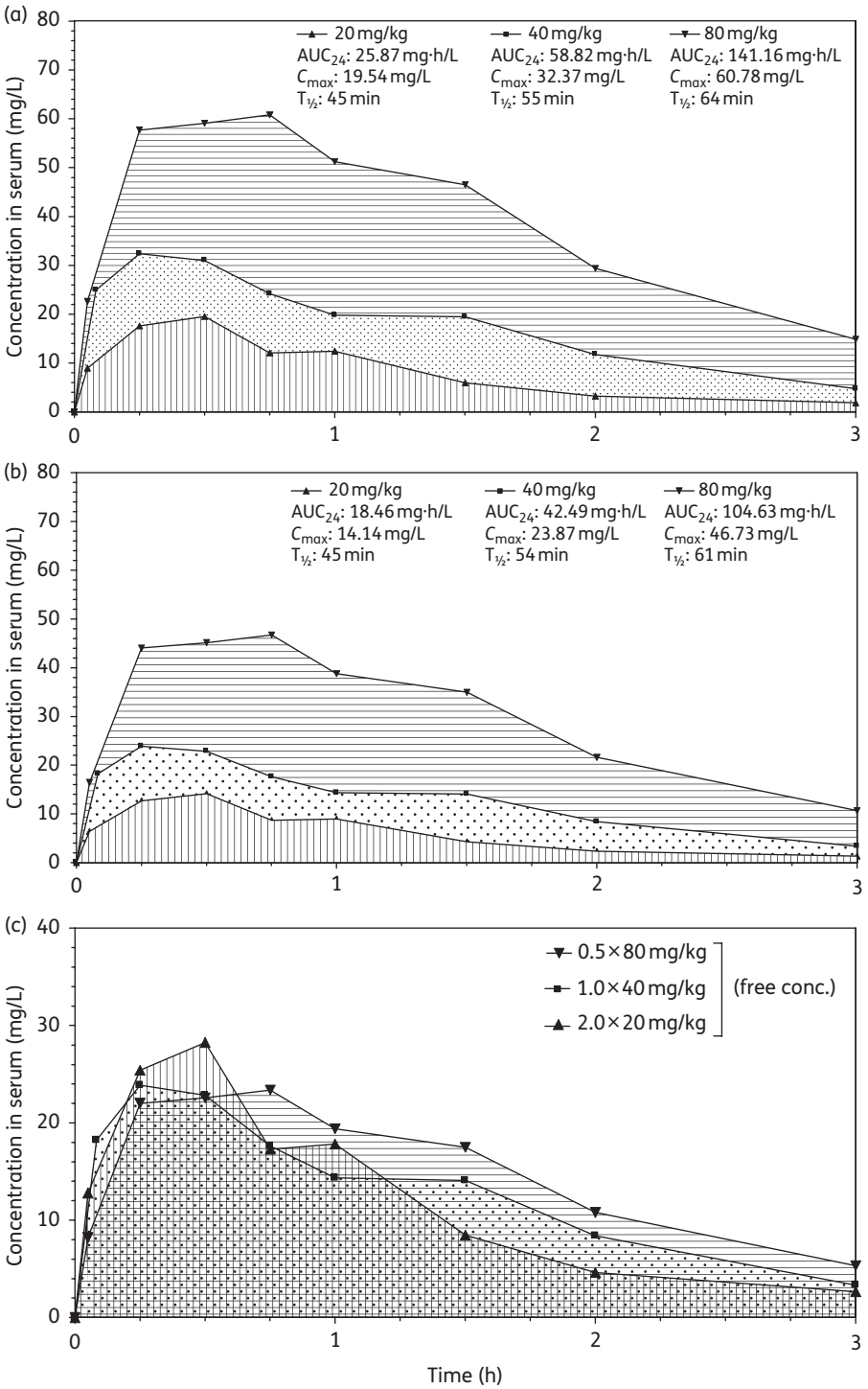

Figure 4. Plasma concentrations of linezolid in NMRI mice following subcutaneous administration of single doses of 80, 40 and 20 mg/kg: (a) total concentration; (b) free concentration; and (c) linearly scaled free concentration simulating the response of a 40 mg/kg dose for all three curves.

64 min. Linezolid exhibited non-linear PK with respect to dose size, as illustrated in Figure 4(c): with full linearity the three curves would have been identical (within the limits of uncertainty).

In vivo intra- and extracellular single dose–kill studies in the mouse peritonitis model

Intra- and extracellular dose–kill studies were first performed for single doses *in vivo* with 4 h drug exposure, using the *S. aureus*

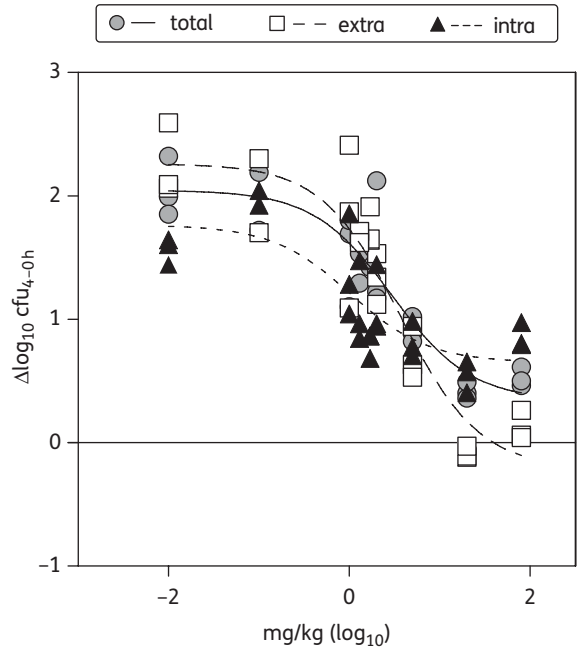

Figure 5. *In vivo* dose–response curves after single doses of linezolid against *S. aureus* as measured in a mouse peritonitis model (4 h drug exposure). The graph displays: (i) the total activity (grey circles and continuous line); (ii) the activity considered to be extracellular (open squares and dashed line); and (iii) the activity considered to be intracellular (filled triangles and dotted line). The activity was measured against MSSA strain E19977. The activity is displayed as changes in cfu ($\Delta\log_{10}$ cfu). The change in cfu is measured as per mL of peritoneal fluid and each datum point represents one mouse. The original inoculum in total (time=0 h) was $(4.87\pm1.70)\times10^5$ cfu/mL ($n=6$). The original inoculum extracellular (time=0 h) was $(2.75\pm0.88)\times10^4$ cfu/mL ($n=6$) and the original inoculum intracellular (time=0 h) was $(3.49\pm1.70)\times10^5$ cfu/mL ($n=6$). Goodness of fit and regression parameters are shown in Table 3.

strain E19977. Results are shown in Figure 5, with the pertinent regression parameters listed in Table 3. While showing globally an essentially bacteriostatic effect, linezolid had a significantly better E_{max} extracellularly than intracellularly ($P<0.0001$), being able to slightly reduce the extracellular bacterial load below the initial inoculum whereas the intracellular load remained consistently larger than this initial inoculum. Conversely, the EC_{50} value for the intracellular activity was slightly lower ($P=0.02$), suggesting a somewhat improved relative potency.

Predictive intra- and extracellular PK/PD parameter determination in the peritonitis model (24 h)

The impact of the three PK/PD parameters on the infection outcome was determined by correlating the number of bacteria in the peritoneum after 24 h of therapy (both as the total bacterial count and as the extra- and intracellular bacterial count) to the $f\text{AUC}_{24}$/MIC ratio, the fC_{max}/MIC ratio and the $fT>\text{MIC}$, respectively, for each of the dosing regimens tested (Table 4). The fitted mathematical expression for the variable free fraction of the drug, measured in the protein binding study (Figure 3), was

Table 3. *In vivo* intra- and extracellular regression parameters for dose–kill studies of linezolid against *S. aureus* (4 h treatment protocol) (Figure 5)

	Total data[a] (95% confidence intervals)				Extracellular data[b] (95% confidence intervals)				Intracellular data[c] (95% confidence intervals)			
Strain	E_{max}[d] [Δlog (cfu)]	EC_{50}[e] (mg/kg)	C_{static}[f] (mg/kg)	R^2	E_{max}[d] [Δlog (cfu)]	EC_{50}[e] (mg/kg)	C_{static}[f] (mg/kg)	R^2	E_{max}[d] [Δlog (cfu)]	EC_{50}[e] (mg/kg)	C_{static}[f] (mg/kg)	R^2
E19977	0.34 (0.08–0.61)	2.95 (1.54–5.65)	–	0.84	−0.21 (−0.56–0.14)	3.69 (2.05–6.64)	40 (17.86–?)	0.88	0.65 (0.41–0.89)	1.02 (0.37–2.81)	–	0.65

[a]Original inoculum in total (time=0 h) was $(4.87\pm1.70)\times10^5$ cfu/mL ($n=6$).
[b]Original inoculum extracellular (time=0 h) was $(2.75\pm0.88)\times10^4$ cfu/mL ($n=6$).
[c]Original inoculum intracellular (time=0 h) was $(3.49\pm1.70)\times10^5$ cfu/mL ($n=6$).
[d]Decrease in colony units (in $\log_{10}$ cfu) at time 24 h from the corresponding original inoculum as extrapolated for an infinitely large dose of antibiotic and using the Hill equation.
[e]Doses causing a reduction of the inoculum half way between the initial (E_0) and the maximal (E_{max}) values, as obtained from the Hill equation.
[f]Doses resulting in no apparent bacterial growth (number of cfu identical to the original inoculum), as determined by graphic intrapolation.

Table 4. Dosing regimens of linezolid applied for the PK/PD study in the mouse peritonitis model

				PK/PD parameter values (free drug)		
Dosing regimen number	Dose (mg/kg)	Dosing interval	Total dose [mg/(24 kg · h)]	$fT>MIC$ (%)	$fAUC_{24}$/MIC (h)	fC_{max}/MIC
1	72	every 24 h	72	17.64	22.69	10.18
2	80	every 12 h	160	37.64	52.43	11.62
3	60	every 12 h	120	31.25	35.55	8.62
4	44	every 12 h	88	25.28	23.91	6.52
5	72	every 8 h	216	52.92	67.69	10.18
6	80	every 6 h	320	75.28	102.58	11.62
7	72	every 6 h	288	70.56	88.91	10.18
8	60	every 6 h	240	62.50	69.88	8.62
9	48	every 6 h	192	54.17	52.60	7.06
10	60	every 4 h	360	93.75	99.35	8.62
11	44	every 4 h	264	75.83	67.72	6.52
12	36	every 4 h	216	67.50	53.19	5.44
13	24	every 4 h	144	45.42	33.11	4.08
14	16	every 4 h	96	34.17	21.47	2.81

used for the calculation. The relationships between the infection outcome (total, extra- and intracellularly) and the corresponding PK/PD parameter values are illustrated in Figure 6. The infection outcome was determined as the decrease in bacterial count at 24 h compared with the bacterial count before treatment initiation. The average colony count for the untreated control group, sampled 6 h after infection initiation, was included in the regression analysis and arbitrarily assigned a value of 1 for the corresponding PK/PD parameter, with values of $(1.37\pm0.24)\times10^7$, $(1.26\pm0.26)\times10^6$ and $(6.56\pm4.92)\times10^6$ cfu/mL for the total, extracellular and intracellular inocula, respectively ($n=8$ for each series). As seen from Figure 6, and considering the extracellular compartment first, a modest correlation between the infection outcome and both the $fT>MIC$ (51%) and the $fAUC_{24}$/MIC parameters (55%) was found, while only a very poor correlation was found between the infection outcome and the C_{max}/MIC ratio. Regarding the intracellular effect, a poor correlation was found for all three parameters (<30%). As also illustrated in Figure 6, modest correlations were observed between the infection outcome and the accumulated total 24 h dose of linezolid [$R^2=49\%$ (total), $R^2=55\%$ (extra) and $R^2=28\%$ (intra), respectively]. It was noteworthy that the E_{max} increased *in vivo* when the drug exposure was increased from 4 to 24 h (2 $\log_{10}$ reduction extracellularly and static effect intracellularly).

Discussion

In the current studies, a detailed characterization of the intra- and extracellular activities of linezolid against *S. aureus* was carried out, using a combination of *in vitro* (cultured macrophages) and *in vivo* (mouse peritonitis) models, and linking the results to the PK parameters of the drug. This, to our knowledge, has never been done with linezolid so far, and paves the way for similar analyses of other antibiotics in the same context.

The *in vitro* THP-1 model offers valuable information concerning the specific intra- and extracellular capacity of the drug, as the model excludes parameters that could affect the antimicrobial activity, e.g. protein binding and the immune system. We found that the intracellular efficacy of linezolid is actually quite similar to, if not slightly better than its extracellular activity, which is in contrast to what we observed for many other antibiotics when comparing their intracellular and extracellular expression of activity.[25] The difference, however, pertains only to a modest increase in the drug relative potency (and corresponding decrease in its static concentration), with no change in its maximal relative efficacy (bacteriostatic in both situations). Thus, even though it does not suffer a loss of activity like other antibiotics, linezolid remains, intracellularly, considerably less active than other antistaphylococcal drugs such as moxifloxacin or the novel lipoglycopeptides telavancin and oritavancin, which all produce a 2–3 $\log_{10}$ cfu decrease of intracellular bacteria when tested in this model.[25,48]

With regard to the *in vivo* situation, we found that linezolid was slightly more potent intracellularly than extracellularly. However, its E_{max} was impaired *in vivo* when compared with the *in vitro* data both intra- and extracellularly. Thus, linezolid could not reduce the intracellular bacterial load below the original inoculum (measured before drug administration), whereas a reduction of 0.2 $\log_{10}$ cfu was obtained extracellularly.

This impaired *in vivo* efficacy of linezolid as compared with the *in vitro* evaluation could be explained by methodical differences. The *in vitro* model offers constant concentrations and no or very limited protein binding in the extracellular milieu because the protein content must be limited to 10% (see discussion in Lemaire *et al.*[44]). Thus, this model may overestimate the true intracellular drug efficacy. However, one should take into account that the *in vivo* dose–kill curve was recorded after 4 h of drug exposure while the *in vitro* concentration–kill curve was made after 24 h of drug exposure. A difference in the cell origin could also affect the results. Yet, even when considering the *in vivo* PK/PD study data (Figure 6), which used determinations made after 24 h of drug exposure, the intracellular efficacy of linezolid remained, on average, lower than its extracellular efficacy. These data also suggest that the E_{max} of linezolid increases with increasing drug exposure *in vivo* both intra- and extracellularly.

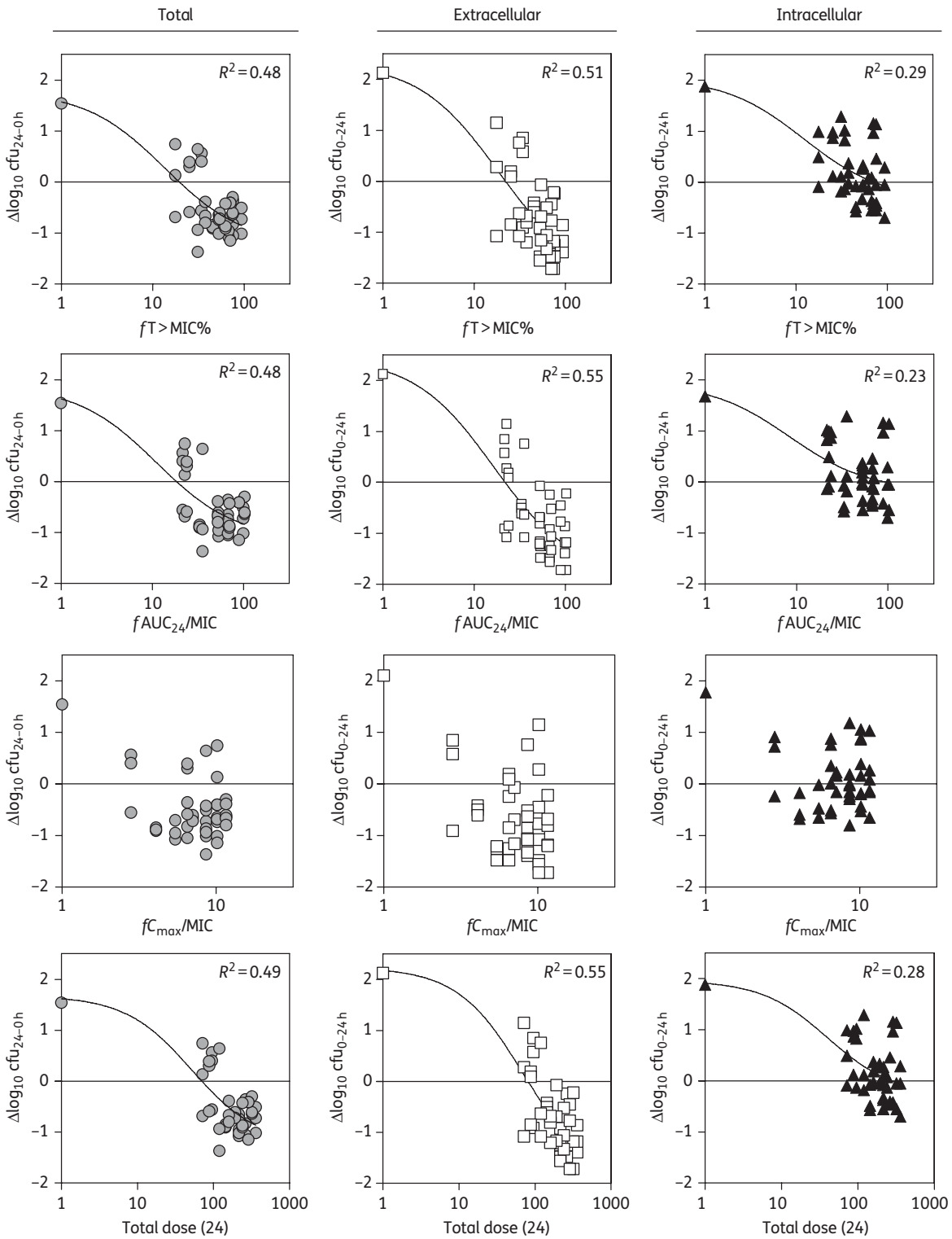

Figure 6. Relationship between the 24 h accumulated free drug (f) fT > MIC, fAUC/MIC, fC$_{max}$/MIC and total 24 h dose and the efficacy of linezolid against MSSA strain E19977 as measured in a mouse peritonitis model (24 h drug exposure). See Table 4 for the applied dosing regimens. The graphs display: (i) the total activity (left-hand panels; grey circles); (ii) the activity considered to by extracellular (middle panels; open squares); and (iii) the activity considered to be intracellular (right panel; filled triangles). The activity is displayed as changes in cfu (Δlog cfu). The change in cfu is measured as cfu per mL of peritoneal fluid and each datum point represents one mouse. The original inoculum in total (time = 0 h) was $(5.46 \pm 1.28) \times 10^5$ cfu/mL and $(1.28 \pm 0.31) \times 10^4$ cfu/mL extracellular, while the original inoculum intracellular was $(1.03 \pm 0.51) \times 10^5$ cfu/mL ($n = 8$). The sigmoidal function was used and R^2 represents goodness of fit.

The drug kinetics in the *in vivo* model enabled us to examine which PK/PD parameter could drive the extra- and intracellular activity of linezolid. Although only modestly correlated, $fT>MIC$ and $fAUC_{24}/MIC$ emerged as the most significant parameters, as was also observed in other animal models. Andes *et al.*[49] performed PK/PD studies for linezolid in a thigh infection model with neutropenic mice, and found, as we did here, that the parameters $fT>MIC$ and $fAUC_{24}/MIC$ correlated almost equally to the infection outcome for *S. aureus* infections (74% and 75%, respectively). These authors pointed out the narrow outcome range between effective and ineffective dosing regimens as the cause of the difficulties in discrimination between the two parameters.[49] We may extend this reflection to also include the following: dissociating PK co-variables such as $T>MIC$ and AUC requires the use of very different dosing regimens, but the slow absorption and elimination in mice together with the poor solubility of linezolid impede this and make it impossible to apply dosing regimens with high accumulative AUC_{24}/MIC values and simultaneously low accumulative $fT>MIC$ values, and vice versa. This explains why the inter-relationship between these parameters is still highly significant for linezolid in our experimental conditions. In spite of these limitations, it is interesting to note that Andes *et al.*[49] found that the AUC_{24}/MIC ratio required to produce a static effect against *S. aureus* was ~80 for linezolid in their model, which quite nicely correlates with our findings for extracellular bacteria, where we indentify a value of 100 for maximal effect (together with a $fT>MIC$ value close to 100%). Correlating these findings to the conventional human linezolid dosing regimen of 600 mg twice daily, this PD goal is achievable for organisms in which the MIC of linezolid is 2–4 mg/L,[49,50] fitting with the clinical susceptibility breakpoint of 4 mg/L set by EUCAST (http://www.eucast.org) and mentioned in the current US labelling (http://media.pfizer.com/files/products/uspi_zyvox.pdf). It is, however, less plausible that this dosing regimen will achieve optimal intracellular activity according to our *in vivo* data, where the results were less promising. The use of linezolid for *S. aureus* infections, where the intracellular compartment plays a major role, is therefore a subject for further investigations. So far, linezolid has shown similar effects to vancomycin in clinical studies concerning skin and soft tissue infections caused by MSSA.[51–53] While vancomycin has not yet been studied in our *in vivo* model, it shows an E_{max} of ~1 $\log_{10}$ cfu in the *in vitro* model,[48] which is similar to what we see here for linezolid, In this respect, the clinical data, therefore, are in line with the results of the present experimental study. Further studies with MRSA on the one hand and more bactericidal antibiotics on the other hand are, however, needed to better establish whether differences in intracellular antistaphylococcal activity are important for the successful treatment of serious *S. aureus* infections.

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Transparency declarations

None to declare.

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Appendix 1

With no influx or removal of antibiotics from the plasma compartment, a general equation for the reversible binding mechanism can be written as:

$$\frac{dC_b}{dt} = -\frac{dC_f}{dt} = k_b C_b (C_{bmax} - C_b) - \frac{C_b}{T_b} \quad (1)$$

where C_b and C_f denote the concentration of protein-bound and free (unbound) antibiotics, respectively, C_{bmax} is the binding capacity, k_b is the binding affinity and T_b is the mean time in the bound state.

By setting the right-hand side in (1) equal to 0, we obtain the relationship between C_b and C_f at equilibrium:

$$C_f = \frac{C_b}{k_b T_b (C_{bmax} - C_b)} \quad (2)$$

Equation (2) can be rewritten to give the free fraction, *ff*, as a function of the total concentration and the binding parameters:

$$\frac{C_f}{C_{total}} = ff\,(C_{total}, k_b T_b, C_{bmax}) \quad (3)$$

where $C_{total} = C_b + C_f$.

Fitting of (3) to the experimental binding data for linezolid in Table 3 gives the following parameter values: $C_{bmax} = 50.4$ mg/L; and $k_b T_b = 0.0085$ L/mg. Figure 3 shows the fitted curve.