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Cellular Pharmacodynamics of the Novel Biaryloxazolidinone Radezolid: Studies with Infected Phagocytic and Nonphagocytic cells, Using *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Legionella pneumophila*▽

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Radezolid is a novel biaryloxazolidinone in clinical development which shows improved activity, including against linezolid-resistant strains. In a companion paper (29), we showed that radezolid accumulates about 11-fold in phagocytic cells, with ~60% of the drug localized in the cytosol and ~40% in the lysosomes of the cells. The present study examines its activity against (i) bacteria infecting human THP-1 macrophages and located in different subcellular compartments (*Listeria monocytogenes*, cytosol; *Legionella pneumophila*, vacuoles; *Staphylococcus aureus* and *Staphylococcus epidermidis*, mainly phagolysosomal), (ii) strains of *S. aureus* with clinically relevant mechanisms of resistance, and (iii) isogenic linezolid-susceptible and -resistant *S. aureus* strains infecting a series of phagocytic and nonphagocytic cells. Radezolid accumulated to similar levels (~10-fold) in all cell types (human keratinocytes, endothelial cells, bronchial epithelial cells, osteoblasts, macrophages, and rat embryo fibroblasts). At equivalent weight concentrations, radezolid proved consistently 10-fold more potent than linezolid in all these models, irrespective of the bacterial species and resistance phenotype or of the cell type infected. This results from its higher intrinsic activity and higher cellular accumulation. Time kill curves showed that radezolid's activity was more rapid than that of linezolid both in broth and in infected macrophages. These data suggest the potential interest of radezolid for recurrent or persistent infections where intracellular foci play a determinant role.

Intracellular infections are difficult to treat because bacteria are shielded from many of the humoral and cellular means of natural defenses while being also partially protected from the action of most antibiotics (7, 12, 47, 58). While intracellular survival is part of the pathogenic cycle of obligatory or facultative intracellular bacteria like *Listeria monocytogenes* or *Legionella pneumophila* (7, 38, 51), it contributes to the recurrent or persistent character of infections caused by opportunistic intracellular bacteria like staphylococci (16). The treatment of such intracellular infections, therefore, requires the use of antibiotics that can express their activity at the site of infection. This, however, cannot be predicted simply on the basis of the ability of drugs to accumulate in cells, as several other factors may play a critical role in enhancing or impeding their local antimicrobial properties (7, 58). For example, previous work in our laboratory using a model of *Staphylococcus aureus*-infected THP-1 cells showed that β-lactams, which do not accumulate in these cells, nevertheless display significant intracellular activity provided their extracellular concentration is brought to sufficiently high but still clinically meaningful levels (31). Conversely, azithromycin, which is known to accumulate in large amounts in cells (6, 18), proves only marginally active against *S. aureus* phagocytosed by macrophages (1, 32). This occurs despite the fact that bacteria persist and thrive for prolonged periods in phagolysosomes after their engulfment by these cells (5, 24, 35), which is also where the bulk of the drug accumulates (6). The difficulty of predicting intracellular activity on the simple basis of pharmacokinetics therefore warrants individual evaluation of new drugs in appropriate models. While animal models are being developed (49), models of cultured cells remain helpful because they offer the possibility of exploring in detail the pharmacological descriptors governing the intracellular activity of antibiotics in the absence of host factors.

Radezolid is a novel oxazolidinone currently in phase II of clinical development (see our companion paper for its structure [29]). In comparison to linezolid, it shows improved activity against a series of bacterial species capable of surviving intracellularly, such as *Staphylococcus*, *Chlamydia*, and *Legionella* species, and remains active against linezolid-resistant strains (25). In the companion paper, we showed that radezolid accumulates to about 12-fold-higher levels than linezolid in human THP-1 cells and localizes in lysosomes for about 40% of the total cell load, while the remainder is found in the cytosol (29). This triggered us to examine the intracellular activity of radezolid using models allowing a quantitative assessment of its pharmacodynamic properties. We selected different types of bacteria with distinct subcellular localizations. We used *L. monocytogenes*, which thrives in the cytosol (15, 21), *L. pneumophila*, which is found in specific replication vacuoles (20), and *S. aureus* and the coagulase-negative *Staph-*

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TABLE 1. Strains and their susceptibilities to linezolid and radezolid

Species and strain	Phenotype[f]	MIC (mg/liter)[g]		Molar MIC ratio of linezolid/radezolid[h]
		Linezolid	Radezolid	
Staphylococcus aureus				
ATCC 25923[a]	MSSA	2	0.25–0.5	5–10
SA040[b]	MSSA	2	1	3
SA040L[b,c]	MSSA, Lzd^r	16	2	10
ATCC 33591[a]	HA-MRSA	1	0.5–1	1–3
SA238[b]	HA-MRSA	2	0.5–1	3–5
SA238L[b,c]	HA-MRSA, Lzd^r	16	2	10
NRS192[d]	CA-MRSA	2	0.5	5
NRS384[d]	CA-MRSA (USA300)	2	0.5	5
NRS52[d]	MSSA and VISA	2	2	1
NRS18[d]	MRSA and VISA	2	0.25	10
VRS1[d]	HA-MRSA and VRSA	1–2	0.5	3–5
VRS2[d]	HA-MRSA and VRSA	1–2	2	1
Staphylococcus epidermidis				
SA362[b]		1	0.25–0.5	5
Listeria monocytogenes				
EGD[e]		1–2	0.03–0.06	43
Legionella pneumophila				
ATCC 33153[a]		4–8	0.5–1	10

[a] From the American Tissue Culture Collection (Manassas, VA).
[b] From P. C. Appelbaum (Hershey Medical Center, Hershey, PA).
[c] Linezolid-resistant clone obtained after multipassage resistance selection studies and subcultures in antibiotic-free medium (23).
[d] From the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program (operated by Eurofins Medinet, Inc., Herndon, VA); further details available from the Website (www.narsa.net).
[e] From P. Berche (Paris, France).
[f] HA, hospital acquired; CA, community acquired; VRSA, vancomycin-resistant *S. aureus*.
[g] MIC determinations were made after 24 h (for *S. aureus*, *S. epidermidis*, and *L. monocytogenes*) or 48 h (for *L. pneumophila*).
[h] Ratio of the MIC of linezolid to that of radezolid calculated in molar concentration (molecular weights: linezolid, 337.35; radezolid, 438.45).

ylococcus epidermidis, which show a phagolysosomal localization in most cell types (1, 3, 13, 40) but may also partially escape in the cytosol of endothelial or epithelial cells (17, 37, 52). We also assessed the intracellular activity of radezolid against different strains of *S. aureus* with various resistance mechanisms, including to linezolid. Finally, we used different cell types as models of territories where *S. aureus* can survive intracellularly (endothelial cells, osteoblasts, respiratory epithelial cells, keratinocytes, and fibroblasts [11, 14, 22, 36, 41]), together with phagocytic cells (macrophages).

Although the maximal effects of both drugs are similar, we found that radezolid acts more rapidly and is consistently more potent than linezolid, mainly due to its higher intrinsic activity and larger cellular accumulation.

(Parts of this study were presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, May 2009, as oral presentations O29 and O30, at the 26th International Conference on Chemotherapy, Toronto, Ontario, Canada, June 2009, and at the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, September 2009 [33, 34].)

MATERIALS AND METHODS

Antibiotics and main reagents. Radezolid (RX-1741, supplied as microbiological standard powder with a potency of 93%) and [^{14}C]RX-1741 (4 μCi/ml, 25 mCi/mmol, labeled on the C of the methylacetamide replacing the oxazolidinone ring) were obtained from Rib-X Pharmaceuticals (New Haven, CT). [^{14}C]RX-1741 was diluted with cold drug to obtain a stock solution at 1 mg/liter (4 μCi/ml). Linezolid was obtained as the corresponding branded product (Zyvoxid) distributed in Belgium for human use by Pfizer SA/NV (Brussels, Belgium). Unless stated otherwise, cell culture media and sera were from Invitrogen Corp. (Carlsbad, CA) and other reagents from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Bacterial strains, susceptibility testing, and extracellular activity. The bacterial strains used in the present study are listed in Table 1. MIC determinations were performed in Mueller-Hinton broth (*S. aureus* or *S. epidermidis*, 24 h, pH 7.3 to 7.4, unless stated otherwise), tryptic soy broth (*L. monocytogenes*, 24 h, pH 7.4), or α-ketoglutarate-buffered yeast extract broth (*L. pneumophila*, 48 h, pH 6.9). For *S. aureus*, time kill curves or concentration response experiments in acellular medium were performed in Mueller-Hinton broth as described previously (1).

Cell lines. Most of the experiments were performed with human THP-1 cells (ATCC TIB-202 [American Tissue Culture Collection, Manassas, VA]), a myelomonocytic cell line displaying macrophage-like activity (55). These cells were maintained in our laboratory as previously described (9). Experiments were also conducted with primary cultures of (i) embryonic rat fibroblasts (isolated as described earlier [56]), (ii) Clonetics normal human osteoblasts (NHOst, cultivated in osteoblast growth medium according to the manufacturer's instructions [Lonza, Inc., Walkersville, MD]), (iii) Clonetics human umbilical vein endothelial cells (HUVEC, cultivated in endothelial cell growth medium and gelatin-treated flasks according to the manufacturer's instructions [Lonza, Inc.]), (iv) immortalized cultures of human bronchial epithelial cell line (Calu-3 [ATCC HBT-55]) maintained in our laboratory as previously described (19) except for the use of uncoated culture flasks, and (v) primary cultures of human epidermidal keratinocytes from neonatal foreskin obtained as described previously (46). These were frozen at passage 7 in Synth-a-Freeze (Cascade Biologics, Portland, OR) and then thawed and seeded on multiwell plates coated with 1 μg/ml collagen type 1 (1 ml/well, coating for 4 h at 37°C) and cultured in EpiLife medium supplemented with supplement S7 (defined growth supplement; Cascade Biologics).

Accumulation and assay of cell-associated radezolid. Antibiotic accumulation was determined following the general procedure used in our previous studies (45, 59), and the cellular content of [^{14}C]radezolid was assayed in cell lysates by liquid

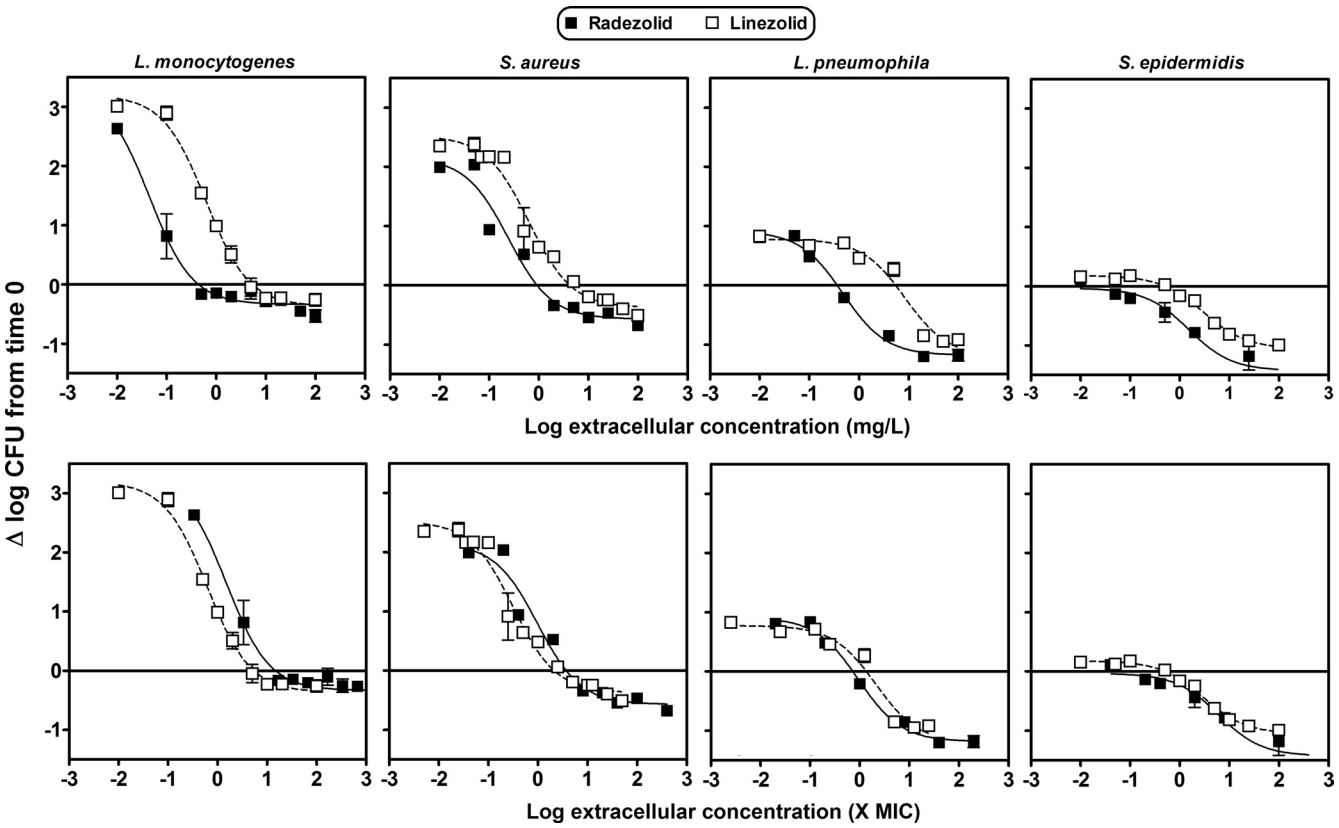

FIG. 1. Dose response curves of linezolid and radezolid toward the intracellular forms of *L. monocytogenes* (strain EGD), *S. aureus* (strain ATCC 25923), *L. pneumophila* (strain ATCC 33153), or *S. epidermidis* (strain CN362) after phagocytosis by human THP-1 cells. Cells were incubated with the antibiotic for 24 h (for *S. aureus*, *S. epidermidis* or *L. monocytogenes*) or 48 h (for *L. pneumophila*) at the concentrations (total drug) indicated on the abscissa, with values expressed in mg/liter or in multiples (X) of the MIC. The ordinate shows the change in the number of CFU per mg of cell protein compared to the postphagocytosis inoculum. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the size of the symbols). The horizontal line corresponds to an apparent static effect. L, liter.

scintillation counting (lowest limit of detection, 0.003 mg/liter; linear response between 0.01 and 0.78 mg/liter; $R^2 = 0.999$; see the companion paper for further details [29]). All cell drug contents were expressed by reference to the total cell protein content (determined using Lowry's method) and converted into apparent total cell concentrations using a conversion factor of 5 μl per mg of cell protein (45, 57).

Cell infection and assessment of antibiotic intracellular activities. Infection of THP-1 cells and assessment of the intracellular activity of antibiotics were performed exactly as described earlier for *L. monocytogenes* (8), for *S. aureus* (1) (the same protocol was used here for *S. epidermidis*), and for *L. pneumophila* (32). For adherent cell lines, we used the general protocol developed previously for J774 macrophages infected by *S. aureus* (50), except that we used an initial inoculum of 5×10^7 to 1×10^8 (2×10^6 for HUVEC) bacteria/ml and a phagocytosis time of 2 h (1 h for HUVEC), as described earlier for infected keratinocytes or Calu-3 cells (28) and for HUVEC (39). This allowed us to reach, for all cell types, a postphagocytosis inoculum of 1.0×10^6 to 4.0×10^6 CFU per mg of cell protein, a value close to that used for THP-1 macrophages.

Statistical analyses. Curve fitting statistical analyses were made with GraphPad Prism, version 4.03, and GraphPad Instat, version 3.06 (GraphPad Software, San Diego, CA).

RESULTS

Antibiotic susceptibilities of *S. aureus*, *S. epidermidis*, *L. monocytogenes*, and *L. pneumophila*. Table 1 shows the susceptibility to linezolid and radezolid of the *S. aureus* strains (with the corresponding relevant resistance mechanisms) and of the other bacterial strains used in the present study. For *S. aureus* and *S. epidermidis*, the linezolid MICs were 1 to 2 mg/liter for all strains, excluding two isolates for which the MICs were 16 mg/liter. These strains will be referred to as linezolid resistant hereinafter. In contrast, the radezolid MICs ranged between 0.25 and 2 mg/liter for all strains. A strain-by-strain comparison shows that radezolid MICs were systematically equal to or lower (up to 3 $\log_2$ dilutions) than those of linezolid for all linezolid-susceptible strains, with an 8-fold difference for the linezolid-resistant strains. For *L. monocytogenes* and *L. pneumophila*, the radezolid MICs were also systematically lower than those of linezolid (3 to 6 $\log_2$ dilutions). When compared on a molar basis, the MICs of radezolid are systematically 1- to 10-fold lower than those of linezolid against *S. aureus* and 5- to 43-fold lower for the other bacteria.

Effect of concentration on oxazolidinone activity against intracellular bacteria in THP-1 cells. In a first set of experiments, we compared the intracellular activity of radezolid to that of linezolid against bacteria showing different subcellular localizations and intracellular growth rates (estimated by the increase in CFU as extrapolated for an infinitely low drug concentration [E_{min}]) in the THP-1 macrophage model. Dose response experiments (at fixed time points [24 h for *S. aureus*, *S. epidermidis*, and *L. monocytogenes* and 48 h for *L. pneumophila*]) were performed to obtain the pertinent pharmacologi-

TABLE 2. Pertinent pharmacological descriptors of antibiotic activity and statistical analysis of the dose response curves illustrated in Fig. 1[a]

Strain	Linezolid						
	E_{min}[b]	E_{max}[c]	EC_{50}[d] expressed in:		C_s[e] expressed in:		R^2
			mg/liter	×MIC	mg/liter	×MIC	
S. aureus	2.52 (2.27 to 2.78) aA	−0.37 (−0.56 to −0.20) aA	0.63 (0.40 to 0.99) aAE	0.32 (0.20 to 0.49) aA	~4.27	~2.2	0.981
S. epidermidis	0.18 (0.12 to 0.24) aB	−1.0 (−1.14 to −0.96) aB	2.99 (2.18 to 4.12) aB	2.99 (2.17 to 4.12) aB	~0.49	~0.5	0.993
L. monocytogenes	3.19 (2.95 to 3.44) aC	−0.37 (−0.54 to −0.19) aA	0.62 (0.45 to 0.84) aCE	0.62 (0.46 to 0.84) aA	~5.47	~5.5	0.994
L. pneumophila	0.78 (0.51 to 1.04) aD	−1.20 (−1.69 to −0.71) aB	8.45 (2.68 to 26.61) aD	2.12 (0.67 to 6.65) aB	~5.14	~1.3	0.966

[a] Calculated from corresponding regression curves (with triplicates for each concentration tested). ×MIC, multiple of the MIC. Statistical analyses: per line analyses (one-way analysis of variance by the Tukey-Kramer test for comparison between each parameters between antibiotics): values with different lowercase letters are significantly different from each other ($P < 0.05$); per column analyses (one-way analysis of variance by the Tukey-Kramer test for multiple comparisons between each parameters for all bacteria): values with different uppercase letters are significantly different from each other ($P < 0.05$).

[b] Increase in CFU (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely low concentration of antibiotics (mean with 95% confidence interval).

[c] Decrease in CFU (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely large concentration of antibiotics (mean with 95% confidence interval).

[d] Concentration (mg/liter) causing a reduction of the inoculum halfway between the minimal (E_{min}) and the maximal (E_{max}) values (mean with 95% confidence interval).

[e] Concentration (mg/liter) resulting in no apparent bacterial growth as determined by graphical interpolation.

cal descriptors of oxazolidinone activity (relative maximal efficacy [E_{max}], relative potency [50% effective concentration {EC_{50}}], and apparent static concentration [C_s]; see reference 1 for a complete description of the models and of these parameters). A graphical representation of the data is presented in Fig. 1, with the numerical values for each pharmacological descriptor shown in Table 2. The activity of both oxazolidinones was concentration dependent within a range extending from roughly 0.1- to 10-fold the MIC, with a plateau reached when exceeding the latter concentration, as indicated by the sigmoidal shape of the curves. Two main observations can be made. First, radezolid shows a greater potency (about 5- to 10-fold lower C_s and EC_{50} values) than linezolid, independent of the bacteria tested, when concentrations are expressed on a weight (mg/liter) basis. When data are expressed as multiples of MICs (extracellular equipotent concentrations), however, the differences between the two molecules are minimized, highlighting the importance of the higher intrinsic activity of radezolid in this context. Second, the maximal relative efficacies (E_{max}) of linezolid and radezolid were similar when comparing the same bacterium. The E_{max} values for both drugs were lower against bacteria that exhibit robust intracellular growth (*L. monocytogenes* and *S. aureus*) than for those showing minimal growth (*L. pneumophila* and *S. epidermidis*). However, the amplitude of the antibacterial response (i.e., by considering the $E_{min} - E_{max}$ difference) was larger for *L. monocytogenes* and *S. aureus* (3 to 2.5 $\log_{10}$ CFU difference) than for *L. pneumophila* or *S. epidermidis* (2 to 1 $\log_{10}$ CFU difference).

Effect of concentration on oxazolidinone activity against intracellular forms of linezolid-susceptible and linezolid-resistant *S. aureus* within THP-1 cells. The activity of radezolid and linezolid was then compared against a series of *S. aureus* of the MSSA (methicillin-susceptible *S. aureus*), MRSA (methicillin-resistant *S. aureus*), or VISA (vancomycin-intermediate *S. aureus*) phenotype and against 2 linezolid-resistant strains selected *in vitro* by exposure to linezolid (SA238L and SA040L, isogenic to SA238 and SA040) (23) (Fig. 2 and Table 3 show regression parameters). For all strains, radezolid shows an improved potency compared to that of linezolid when concen-

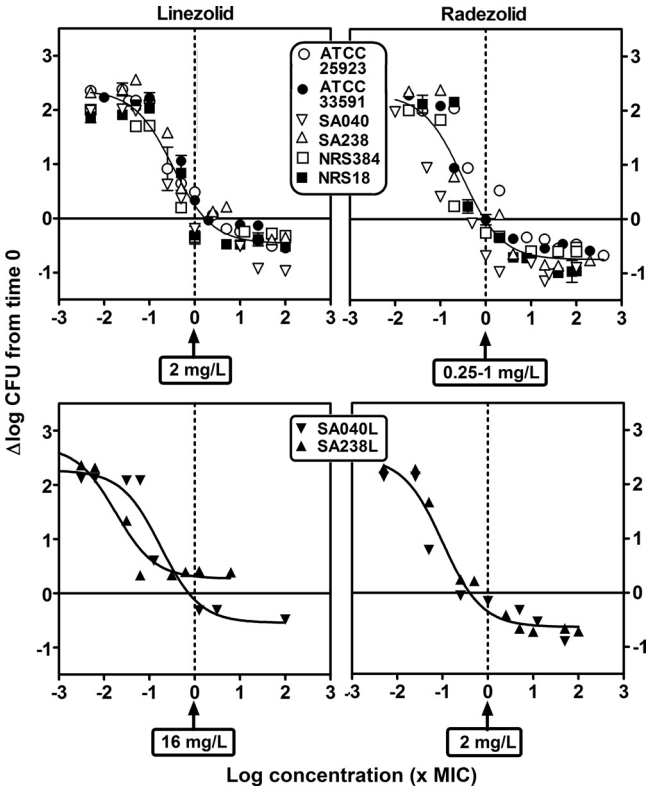

FIG. 2. Dose response curves of linezolid and radezolid toward different strains of *S. aureus* phagocytosed by THP-1 cells (upper panel, linezolid-susceptible strains; lower panel, linezolid-resistant strains). Cells were incubated with the antibiotic for 24 h at the concentrations (total drug) indicated on the abscissa and expressed in multiples (X) of the MIC. The ordinate shows the change in the number of CFU per mg of cell protein compared to the postphagocytosis inoculum. All values are means ± standard deviations (n = 3; when not visible, the standard deviation bars are smaller than the size of the symbols). The horizontal line corresponds to an apparent static effect. The vertical dotted line corresponds to the MIC, the value (or range of values) of which is indicated below each panel. L, liter.

TABLE 2—*Continued*

Radezolid						
E_{min}[b]	E_{max}[c]	EC_{50}[d] expressed in:		C_s[e] expressed in:		R^2
		mg/liter	×MIC	mg/liter	×MIC	
2.16 (1.54 to 2.77) aA	−0.57 (−0.88 to −0.25) aA	0.24 (0.08 to 0.71) bA	0.98 (0.34 to 2.84) aA	~0.91	~3.6	0.961
0.00 (−0.31 to 0.25) aA	−1.43 (−1.77 to −1.09) bB	1.51 (0.39 to 5.88) aB	6.05 (1.56 to 23.51) aB	~0.03	~0.1	0.957
3.30 (2.67 to 3.93) aA	−0.34 (−0.46 to −0.21) aA	0.04 (0.02 to 0.08) bC	1.49 (0.83 to 2.69) aA	~0.43	~14.3	0.985
0.91 (0.68 to 1.14) aA	−1.19 (−1.37 to −0.99) aB	0.47 (0.25 to 0.90) bA	0.94 (0.49 to 1.81) aA	~0.36	~0.7	0.992

trations are expressed on a weight (mg/liter) basis (Table 3). The data were plotted as multiples of the MIC to allow comparison of the activities against the different strains at equipotent concentrations. Interestingly, a single sigmoidal function could be fitted to the whole set of data against linezolid-susceptible strains for both drugs (Fig. 2, upper panel). The C_s values (static concentrations) were close to the respective MIC of each strain (and therefore lower for radezolid than for linezolid). Against linezolid-resistant strains (Fig. 2, lower panel), radezolid's activity was indistinguishable from that observed for linezolid-susceptible strains (similar E_{min}, E_{max}, and EC_{50} values and C_s values slightly lower than the MIC [0.8 mg/liter]). Linezolid showed a C_s value close to its MIC (16 mg/liter) for strain SA040L but was poorly effective against strain SA238L, for which a static effect was never reached.

Concentration-dependent activity of radezolid versus linezolid against intracellular forms of linezolid-susceptible and linezolid-resistant *S. aureus* within nonphagocytic cells. Given that intracellular forms of *S. aureus* can be found in many other cell types than macrophages, we next examined the intracellular activity of radezolid against *S. aureus* strains internalized by human (HUVEC, Calu-3, keratinocytes, and osteoblasts) or animal (fibroblasts) nonphagocytic cells in comparison with its activity against *S. aureus* internalized by THP-1 macrophages (Fig. 3). Radezolid showed concentration-dependent activity that was indistinguishable against both strains and in all cells tested, with C_s values ranging from 0.6 to 3.3 mg/liter (1.2× to 1.5× MIC) and the E_{max} corresponding to CFU reductions of 0.6 to 1.5 $\log_{10}$ compared to the original inoculum. As shown in Fig. 1, larger decreases in CFU (corresponding to more-negative E_{max} values) were observed in cells where bacterial growth was slower, so that the amplitudes of the effects of the antibiotics (difference between E_{min} and E_{max} values) were similar (about 3.5 $\log_{10}$ CFU) in all cases. Linezolid was less potent than radezolid in all models, with C_s values ranging from 2.6 to 9.5 mg/liter (1.3× to 4.8× MIC) for the linezolid-susceptible strain and from 15 (0.9× MIC) to >100 mg/liter for the linezolid-resistant strain. Linezolid's overall activity was also markedly reduced against the linezolid-resistant strain but to various levels in the different cell types.

Cellular accumulation of radezolid in nonphagocytic cells and in infected cells. We showed in the companion paper that radezolid accumulates about 11-fold more in phagocytic cells than extracellularly (29). Therefore, we measured in this work its accumulation in nonphagocytic cells exposed to the drug during 2 or 24 h in comparison to its accumulation in THP-1 cells. At both time points, radezolid reached a cellular concentration of the same order of magnitude in all cell types (Table 4). We also determined the cellular accumulation of radezolid in THP-1 cells infected by *S. aureus* and did not find any difference from what was observed for noninfected cells (data not shown).

Role of intracellular concentration of radezolid in activity. We showed that radezolid accumulates in cells and partially localizes in lysosomes (29). To assess whether this could account for its increased potency in comparison with that of linezolid, we replotted the data for strain SA238 (Lzd^s) as a function of the extracellular concentration expressed (i) as weight values (mg/liter), (ii) as multiples of the MIC at neutral pH or acidic pH (to mimic the conditions prevailing in the extracellular milieu and the phagolysosomes, respectively), and (iii) as a function of the cellular concentration, expressed also as multiples of the MIC at acidic pH (Fig. 4 and Table 5). As a first approximation, and since no data were available regarding the subcellular distribution of linezolid, we used total cellular concentrations for both drugs. Extracellular activity was also determined in parallel. Radezolid proved about 23-fold more potent (lower C_s and EC_{50} values) intracellularly than extracellularly based on these criteria. Interestingly, linezolid showed a similar effect, with a 5-fold-lower C_s value in THP-1 cells than in broth.

While the intracellular relative potency of radezolid was clearly higher than that of linezolid expressed on a weight basis (mg/liter), the two drugs behaved alike when compared on the basis of multiples of their MICs at neutral pH. Interestingly enough, the relative potency of radezolid, which was 10-fold higher (10-fold-lower C_s value) than that of linezolid when expressed in multiples of the MIC at acidic pH, returned to its original value when taking into account its cellular accumulation level. In all cases, the maximal relative efficacies of both oxazolidinones were measurably lower (less-negative E_{max} values) against intracellular bacteria than against bacteria grown in broth.

Time effect on oxazolidinone extracellular and intracellular activities against *S. aureus* (strain ATCC 25923). To further characterize the pharmacodynamic profiles of the oxazolidinones, their activities against *S. aureus* growing in broth or phagocytosed by THP-1 cells were then examined over shorter incubation periods (Fig. 5). Intracellular growth in the absence of antibiotic was minimal over the 5 hours of the experiment. Radezolid exerted a time-dependent effect in both environments, causing a 2 $\log_{10}$ CFU decrease extracellularly and a 0.5 to 1 $\log_{10}$ CFU decrease intracellularly for concentrations as low as 1 mg/liter. The extracellular effect of linezolid was never greater than about 1.3 $\log_{10}$ CFU extracellularly. Linezolid

TABLE 3. Pertinent pharmacological descriptors of antibiotic activity and statistical analysis of the dose-response curves for the individual strains used in Fig. 2[a]

Strains	Linezolid					
	E_{max}[b]	EC_{50}[c] expressed in:		C_s[d] expressed in:		R^2
		mg/liter	×MIC	mg/liter	×MIC	
ATCC 25923	−0.37 (−0.56 to −0.20) aA	0.63 (0.40 to 0.99) aA	0.31 (0.20 to 0.49) aA	~4.27	~2.2	0.981
ATCC 33591	−0.39 (−0.63 to −0.14) aA	0.47 (0.25 to 0.88) aA	0.47 (0.25 to 0.88) aA	~2.95	~2.9	0.975
NRS384	−0.41 (−0.80 to −0.02) aAB	0.40 (0.13 to 1.24) aA	0.20 (0.06 to 0.62) aA	~2.30	~1.16	0.955
NRS18	−0.69 (−1.15 to −0.21) aA	0.84 (0.30 to 2.35) aA	0.42 (0.15 to 1.14) aA	~2.77	~1.52	0.948
SA040	−0.91 (−1.17 to −0.65) aA	0.63 (0.38 to 1.05) aA	0.29 (0.17 to 0.52) aA	~1.59	~0.78	0.986
SA238	−0.42 (−0.87 to 0.02) aAB	0.70 (0.31 to 1.59) aA	0.37 (0.15 to 0.89) aA	~4.55	~3.37	0.964
SA040 L	−0.69 (−1.40 to 0.01) aAB	2.78 (0.85 to 9.03) aA	0.18 (0.05 to 0.57) aAB	~10.15	~0.74	0.938
SA238 L	0.27 (−0.06 to 0.60) aBC	0.29 (0.09 to 0.85) aA	0.02 (0.006 to 0.05) aB	>100	NA	0.952

[a] Calculated from the corresponding regression curves (with triplicates for each concentration tested). × MIC, multiple of the MIC; NA, not applicable. Statistical analyses: per line analyses (one-way analysis of variance by the Tukey-Kramer test for comparison between each parameters between antibiotics), values with different lowercase letters are significantly different from each other ($P < 0.05$); per column analyses (one-way analysis of variance by the Tukey-Kramer test for multiple comparisons between each parameters for all bacteria), values with different uppercase letters are significantly different from each other ($P < 0.05$).

[b] Decrease in CFU (in $\log_{10}$ units) from the corresponding original inoculum as extrapolated for infinitely large concentration of antibiotics (mean with 95% confidence interval).

[c] Concentration (mg/liter) causing a reduction of the inoculum halfway between the minimal (E_{min}) and the maximal (E_{max}) values (mean with 95% confidence interval).

[d] Concentration (mg/liter) resulting in no apparent bacterial growth as determined by graphical interpolation. NA, not applicable.

remained static intracellularly at the highest concentration tested.

DISCUSSION

Using a previously established general experimental design (1), the present study describes the intracellular activity of radezolid in comparison with that of linezolid against 4 bacterial species and in 6 different cell types. This allowed us to show that radezolid is consistently more potent than linezolid in relation to its ability to accumulate to high levels inside those cells.

The models used are representative of true pathological situations seen in the clinics, as they use cell types as models of territories where *S. aureus* can persist in the body, including inside the cells (see references 4 and 10 for examples). At the same time, they permit observation of the characteristics associated with different pathogenic mechanisms at the cellular

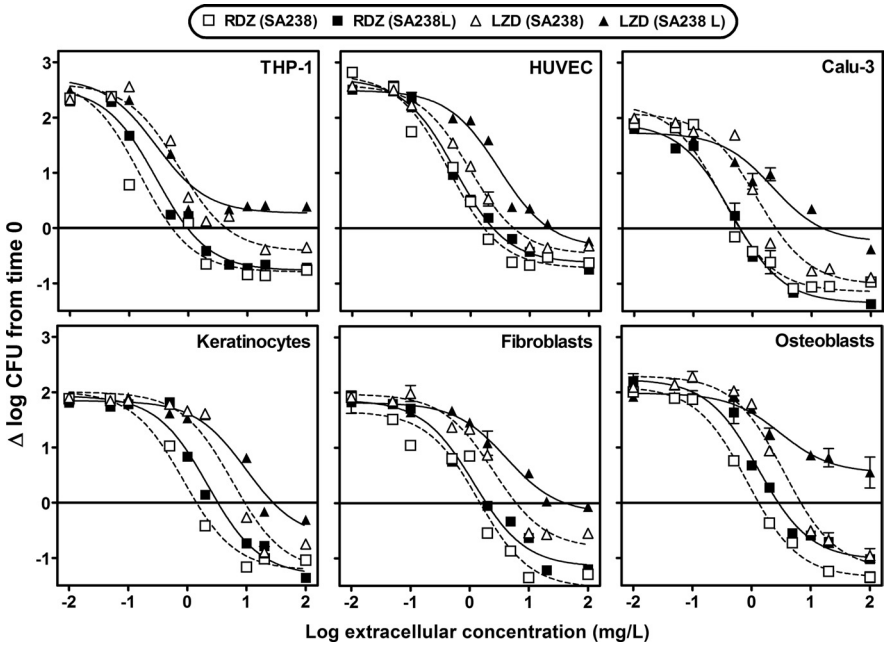

FIG. 3. Dose response curves of linezolid (LZD) and radezolid (RZD) toward two isogenic strains of *S. aureus* that are linezolid susceptible (SA238) or linezolid resistant (SA238L), phagocytosed by different cell types. Cells were incubated with the antibiotic for 24 h at the concentrations (total drug) indicated on the abscissa and expressed in mg/liter. The ordinate shows the change in the number of CFU per mg of cell protein compared to the postphagocytosis inoculum. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the size of the symbols). The horizontal line corresponds to an apparent static effect. L, liter.

TABLE 3—*Continued*

Radezolid					
E_{max}[b]	EC_{50}[c] expressed in:		C_s[d] expressed in:		R^2
	mg/liter	×MIC	mg/liter	×MIC	
−0.57 (−0.880.24 (0.10 to 0.55) −0.25) aA	0.24 (0.08 to 0.71) aA	0.98 (0.34 to 2.84) aA	~0.91	~3.6	0.967
−0.56 (−0.86 to −0.27) aA	0.12 (0.05 to 0.27) aA	0.24 (0.10 to 0.55) aA	~0.56	~1.17	0.976
−0.61 (−0.97 to −0.25) aA	0.07 (0.02 to 0.25) aA	0.15 (0.04 to 0.50) aA	~0.32	~0.62	0.932
−1.00 (−1.48 to −0.52) aA	0.09 (0.03 to 0.37) aA	0.39 (0.11 to 1.45) aA	~0.28	~1.09	0.929
−0.96 (−1.17 to −0.75) aA	0.09 (0.04 to 0.18) aB	0.08 (0.04 to 0.18) aA	~0.21	~0.21	0.971
−0.79 (−1.34 to −0.25) aA	0.16 (0.03 to 0.77) aA	0.31 (0.06 to 1.54) aA	~0.56	~1.1	0.941
−0.66 (−1.06 to −0.26) aA	0.14 (0.04 to 0.49) aB	0.14 (0.04 to 0.49) aA	~0.56	~0.29	0.938
−0.77 (−0.97 to −0.56) aB	0.29 (0.18 to 0.48) aA	0.29 (0.17 to 0.48) bA	~0.97	~0.53	0.987

level, as illustrated from the respective rates of intracellular growth of the different bacteria used in our model (see Table 2). Thus, *L. monocytogenes*, a true facultative intracellular parasite, was found to multiply as efficiently inside cells as in broth (see reference 8 for additional data). This is probably due to its capacity to reach the cytosol (escaping much of the host defense mechanisms) and to modify its metabolism to become fully adapted to this new intracellular environment (21, 42, 60). *L. pneumophila*, which is known more as an intracellular organism in humans, actually shows a slower growth rate than *Listeria* in THP-1 cells, probably because its growth capabilities markedly depend on the culture conditions and environment (48). For staphylococci, which are considered opportunistic pathogens, we observed a fairly robust intracellular growth for *S. aureus* but only after a lag period of about 8 to 10 h, and no apparent intracellular growth of the coagulase-negative *S. epidermidis* (compared to 3 $\log_{10}$ within 24 h in broth [1, 43]). This suggests a reduced capacity of *S. epidermidis* to resist the weak but nevertheless active cell defense mechanisms of THP-1 cells. These defense mechanisms may be somewhat defeated for *S. aureus* by the expression of virulence factors, such as staphyloxanthin, which is under the control of RsbU (39). Within the context of the evaluation of antibiotics, the concomitant use of these models offers us an opportunity to compare antibiotic activities against intracellular bacteria that differ not only by their subcellular localization but also by their multiplication rate.

A critical observation made during this study is that radezolid proves approximately 10-fold more potent than linezolid in all intracellular models when compared at equivalent weight concentrations (this difference being larger for linezolid-resistant strains). This occurs irrespective of the subcellular localization of the bacteria, their intracellular growth rate, the type of cell infected, or the resistance phenotype of the strain. This favorable activity profile of radezolid may result from its higher intrinsic activity (with MICs typically 3 to 6 dilutions lower than those of linezolid) and/or from its higher cellular accumulation. Recent studies with torezolid, another oxazolidinone in development, have suggested that the MIC is the main driver for intracellular potency, as improvement in potency over that of linezolid is normalized when concentrations are expressed in multiples of the MICs (30). The importance of the MIC is also highlighted here, as we see that a single sigmoidal regression can be fitted to the data obtained for all *S. aureus* strains once the linezolid-resistant strains have been excluded. This is also what we observed for ceftobiprole (26), suggesting that this concept can perhaps be generalized. The situation with radezolid, however, is probably more complex. The potencies of radezolid and linezolid are indeed similar when recalculated as a function of cellular concentration expressed in multiples of the MIC at acidic pH. As the activity of radezolid, but not that of linezolid, is reduced at low pH, this recalculation of the data suggests that cellular accumulation is a key property of radezolid's activity, at least against those organisms (staphylococci and *Legionella* spp.) that thrive in acidic compartments. A similar effect has been reported previously for aminoglycosides (2). However, we cannot ascertain that pH exerts similar effects toward bacteria grown in broth and those thriving in cells (where other environmental factors may also influence their susceptibility to antibiotics). Yet, the fact that radezolid is more potent intracellularly than extracellularly against *S. aureus* also lends strong support for a potential role of accumulation. Of interest also is that intracellular activity is observed regardless of the intracellular location of bacteria. This is consistent with radezolid's dual localization in the cytosol and acidic vacuoles (29). In this context, our companion paper (29) showed no association of radezolid with mitochondria, as assessed by cell fractionation studies.

Another important observation is that the activity of radezolid develops rapidly both intra- and extracellularly, as the maximal effect is already reached after 3 to 5 h of incubation even at 1 mg/liter. In contrast, linezolid remains static intra-

TABLE 4. Comparative cellular accumulation of radezolid in phagocytic and nonphagocytic cells

Cell type	Cellular accumulation (cellular to extracellular concn[a] ratio ± SD)	
	2 h	24 h
THP-1 macrophages	9.3 ± 0.4	9.8 ± 0.2
HUVEC	9.5 ± 0.1	10.5 ± 0.5
Calu-3	8.5 ± 0.2	8.3 ± 1.1
Keratinocytes	12.6 ± 0.7	16.1 ± 2.0
Fibroblasts	14.4 ± 1.0	10.9 ± 2.1
Osteoblasts	9.8 ± 2.7	9.7 ± 0.4

[a] Extracellular concentration, 4 mg/liter.

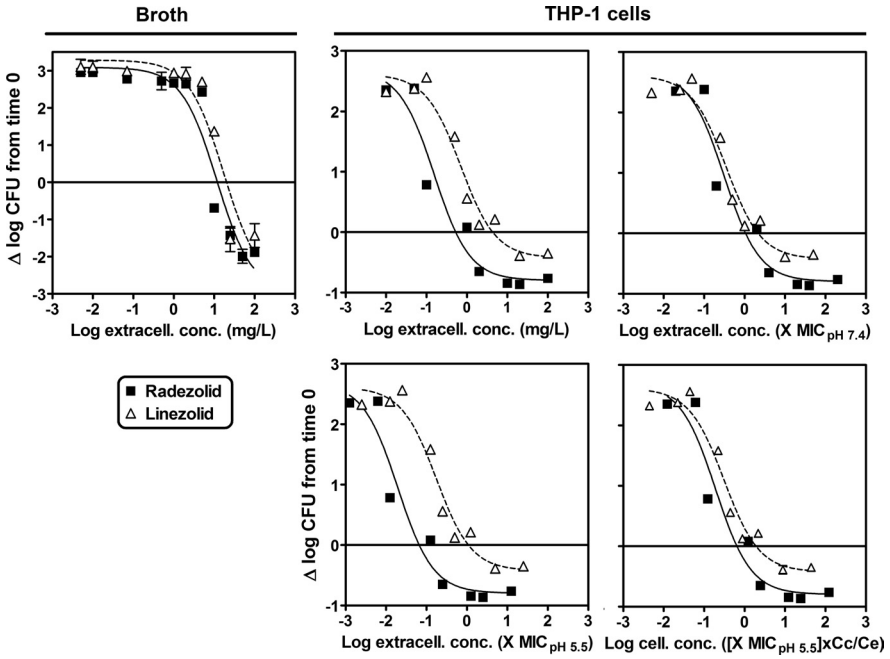

FIG. 4. Dose response curves of linezolid and radezolid toward *S. aureus* SA238 in broth or phagocytosed by THP-1 cells. Activity was determined after 24 h of incubation with an antibiotic at the concentrations (total drug) indicated on the abscissa and expressed as (i) weight concentrations (mg/liter); (ii) multiples of the MIC as determined in broth adjusted to pH 7.4 (linezolid MIC, 2 mg/liter; radezolid MIC, 0.5 mg/liter); (iii) multiples of the MIC as determined in broth adjusted to pH 5.5 (linezolid MIC, 4 mg/liter; radezolid MIC, 8 mg/liter); (iv) multiple of the cellular concentration expressed in multiples of the MIC at pH 5.5, using accumulation factors of 1.7-fold (linezolid) and 9.8-fold (radezolid), respectively. The ordinate shows the change in the number of CFU per mg of cell protein compared to the initial inoculum. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the size of the symbols). The horizontal line corresponds to an apparent static effect. L, liter; X MIC, multiple of the MIC.

cellularly for at least 5 h and shows only a modest drop in CFU in broth at 20 mg/liter. These observations are consistent with the improved interaction of radezolid with its ribosomal target (25, 53, 61). In spite of this, however, the maximal effects reached at 24 h are similar for radezolid and linezolid at the highest concentrations tested (if the comparison is limited to linezolid-susceptible strains). This suggests that in contrast to relative potency, which is markedly influenced by the intrinsic activity of each drug, the maximal relative efficacy should be related to the mode of action and pharmacodynamic profile of the drugs. In a broader context, we noted that bacteriostatic drugs, such as macrolides, also cause only a small reduction in the extracellular and intracellular bacterial counts (<1 $\log_{10}$ CFU). For bactericidal drugs like fluoroquinolones, synergistins, or lipoglycopeptides, the decrease in inoculum reaches the limit of detection extracellularly and 2 to 3 $\log_{10}$ CFU intracellularly (1, 27, 28, 32). It must, however, be pointed out that we deal here with an *in vitro* model where host defenses are minimal and contribute only poorly to the overall antibiotic response. Of interest also, the maximal relative efficacies (E_{max} values) depend on the target bacterial species but not on the strain (if compared in the same cell line) or on the type of cell infected (when comparing different bacterial species). This clearly shows that the maximal relative efficacy of radezolid is driven by species-specific differences that are more probably related to variations in permeability/efflux than in drug-target interactions. Radezolid indeed shows differences in maximal killing rates in broth when different bacterial species are examined (25) but has a very similar capacity to interact with prokaryotic ribosomes (54). On the other hand, differences among the intracellular models for a specified bacterial species may arise from their various rates of multiplication within the cells and/or from cell-related factors like their capacity for defense against bacteria (12, 44).

A third observation is that radezolid fully maintains its intracellular potency against linezolid-resistant strains in all models. This is an important result, as it supports the potential use of radezolid to fight infections with these strains. Of interest, the intracellular activity of linezolid against the two linezolid-resistant strains is not the same, and for SA238L, also varies depending on the cell type infected. The reasons for these differences need to be further investigated but may be underlying resistance mechanisms that are still largely undefined (23).

Altogether, and even with the limitations inherent to our model as discussed in our previous papers (use of static concentrations and fixed serum concentration [1, 27, 30]), the data presented here point to an improvement in intracellular activity for the new oxazolidinone radezolid, probably as a result of the combination of higher intrinsic activity, a higher level of accumulation within eukaryotic cells, and conserved activity against linezolid-resistant strains. The potential clinical impact of these findings will therefore also need to be reexamined in the light of the pharmacokinetic and pharmacodynamic properties of this molecule when administered to humans in order

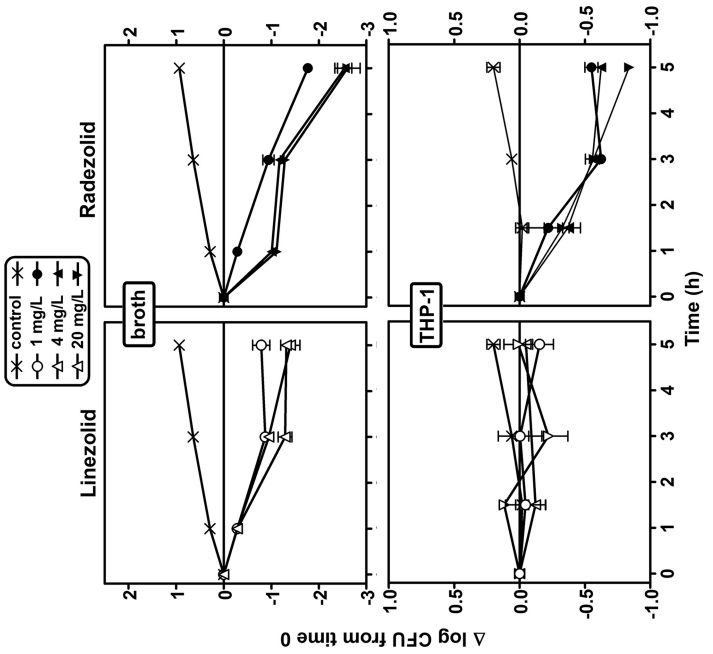

FIG. 5. Influence of time on the extracellular (broth) and intracellular (THP-1 cells) activities of linezolid and radezolid against *S. aureus* ATCC 25923. The ordinate shows the change in CFU compared to the initial inoculum. All values are means ± standard deviations ($n = 3$; when not visible, the standard deviation bars are smaller than the size of the symbols). The horizontal line corresponds to an apparent static effect. L, liter.

to define what advantage can be expected within the range of clinically meaningful concentrations.

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TABLE 5. Pertinent pharmacological descriptors of antibiotic activity and statistical analysis of the dose response curves toward *S. aureus* SA238 illustrated in Fig. 4[a]

Culture conditions[e]	Linezolid				Radezolid			
	E_{max}[b]	EC_{50}[c]	C_s[d]	R^2	E_{max}[b]	EC_{50}[c]	C_s[d]	R^2
Broth (mg/liter)	−2.78 (−5.43 to −0.14) aA	17.13 (4.52 to 65.02) aA	~20.27	0.912	−2.97 (−4.64 to −1.29) aA	11.52 (4.14 to 32.04) aA	~12.74	0.936
THP-1 (mg/liter)	−0.42 (−0.87 to 0.02) aB	0.70 (0.31 to 1.58) aB	~4.55	0.963	−0.79 (−1.34 to −0.26) aB	0.16 (0.03 to 0.76) bB	~0.52	0.941
THP-1 (×MIC at pH 7.4)		0.35 (0.16 to 0.79)	~2.26			0.31 (0.06 to 1.54)	~1.04	
THP-1 (×MIC at pH 5.5)		0.18 (0.08 to 0.39)	~1.09			0.02 (0.004 to 0.09)	~0.06	
THP-1 [(×MIC at pH 5.5) × C_c/C_e]		0.31 (0.14 to 70)	~1.86			0.19 (0.04 to 0.94)	~0.66	

[a] Calculated from corresponding regression curves (with triplicates for each concentration tested).Statistical analyses: per line analyses (one-way analysis of variance by the Tukey-Kramer test for comparison between each parameters between antibiotics), values with different lowercase letters are significantly different from each other ($P < 0.05$); per column analyses (one-way analysis of variance by the Tukey-Kramer test for multiple comparisons between each parameters for all conditions), values with different uppercase letters are significantly different from each other ($P < 0.05$).

[b] CFU decrease (in $\log_{10}$ units) from the corresponding original inoculum, as extrapolated for infinitely large concentration of antibiotics (mean with 95% confidence interval).

[c] Concentration (mg/liter) causing a reduction of the inoculum halfway between the minimal (E_{min}) and the maximal (E_{max}) values (mean with 95% confidence interval).

[d] Concentration (mg/liter) resulting in no apparent bacterial growth, as determined by graphical interpolation.

[e] Data as a function of the extracellular concentration are expressed (i) as weight values (mg/liter), (ii) as multiples of the MIC (×MIC) at neutral pH or acidic pH (to mimic the conditions prevailing in the extracellular milieu and the phagolysosomes, respectively), and (iii) as a function of the cellular concentration, also expressed as the multiple of the MIC at acidic pH. C_c/C_e, cellular to extracellular concentration ratio.

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