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Cellular pharmacokinetics and intracellular activity of the bacterial fatty acid synthesis inhibitor, afabicin desphosphono against different resistance phenotypes of *Staphylococcus aureus* in models of cultured phagocytic cells

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ABSTRACT

Antibiotics with new modes of action that are active against intracellular forms of *Staphylococcus aureus* are sorely needed to fight recalcitrant infections caused by this bacterium. Afabicin desphosphono (Debio 1452, the active form of afabicin [Debio 1450]) is an inhibitor of FabI enoyl-Acyl carrier protein reductase and has specific and extremely potent activity against Staphylococci, including strains resistant to current antistaphylococcal agents. Using mouse J774 macrophages and human THP-1 monocytes, we showed that afabicin desphosphono: (i) accumulates rapidly in cells, reaching stable cellular-to-extracellular concentration ratios of about 30; (ii) is recovered entirely and free in the cell-soluble fraction (no evidence of stable association with proteins or other macromolecules). Afabicin desphosphono caused a maximum cfu decrease of about 2.5 $\log_{10}$ after incubation in broth for 30 h, including against strains resistant to vancomycin, daptomycin, and/or linezolid. Using a pharmacodynamic model of infected THP-1 monocytes (30 h of incubation post-phagocytosis), we showed that afabicin desphosphono is bacteriostatic (maximum cfu decrease: 0.56 to 0.73 $\log_{10}$) towards all strains tested, a behaviour shared with the comparators (vancomycin, daptomycin, and linezolid) when tested against susceptible strains. We conclude that afabicin desphosphono has a similar potential as vancomycin, daptomycin or linezolid to control the intracellular growth and survival of phagocytized *S. aureus* and remains fully active against strains resistant to these comparators.

1. Introduction

Staphylococcus aureus represents a major and recurrent challenge to clinicians due to the combination of bacterial and host factors [1] and is considered by the World Health Organization to be a high priority pathogen for development of novel therapies [2]. *S. aureus* readily adapts to changing environments and acquires antibiotic-resistance genes through several different mechanisms [3]; this has led to an almost constant increase and broadening of resistance that today affects most (if not all) the major classes of clinically-approved antibiotics, including glycopeptides, fluoroquinolones and oxazolidinones [4,5]. *S. aureus* can survive and thrive in professional and non-professional phagocytes, where it evades immune defences and against which antibiotic action is severely limited compared with extracellular forms [6–8]. In this context, while discovery and development of new chemical or biological entities targeting unexploited but essential targets in *S. aureus* is of prime importance to evade existing mechanisms of resistance [9], their activity in difficult environments and intracellular niches must also be carefully assessed to ensure their efficacy against difficult-to-treat *S. aureus* infections.

The present study focuses on the activity of the first-in-class FabI enoyl-Acyl carrier protein reductase inhibitor, afabicin desphosphono (Debio 1452; first described as API 1252 [10] or AFN-1252 [11,12]; see Fig. 1 for structure and main biophysical properties) on intracellular *S. aureus*. Afabicin desphosphono is the active moiety of afabicin (Debio 1450), which has completed Phase II in acute bacterial skin and skin structure infections and is presently being

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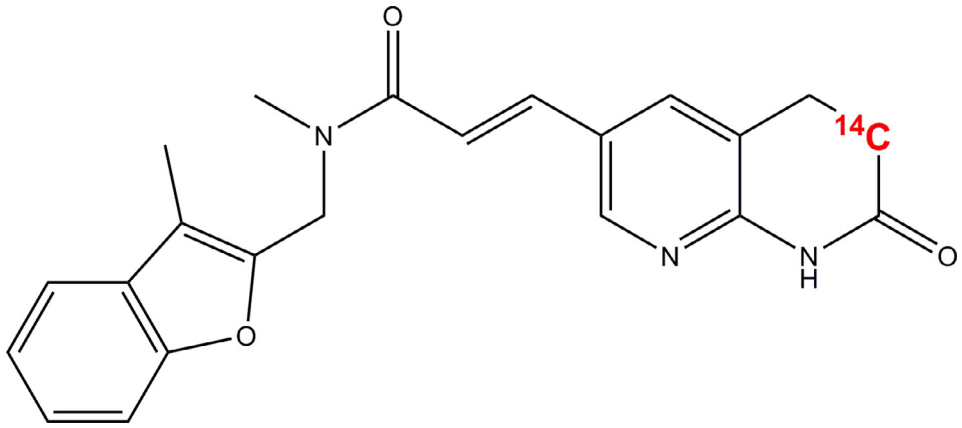

Fig. 1. Structural formula of afabicin desphosphono (Debio 1452 / AFN-1252; free base; preferred IUPAC name: (2E)-N-methyl-N-[(3-methyl-1-benzofuran-2-yl)methyl]-3-(7-oxo-5,6,7,8-tetrahydro-1,8-naphthyridin-3-yl)prop-2-enamide. MW (unlabelled) = 375.42), with the position of the labelled atom in the [^{14}C]-derivative used in this study. The figure shows the calculated predominant microspecies (>94%) at pH 5 to 10 (uncharged). Calculated logP and $logD_{pH7.4}$: 3.01 and 3.01 [calculations made by Marvin Sketch version 18.25 (academic license), Chemaxon (Budapest, Hungary; https://chemaxon.com/)].

investigated for the treatment of bone and joint infections [13]. Of note, afabicin desphosphono displays a selective and highly potent antibacterial activity against Staphylococci, with minimum inhibitory concentrations (MICs) typically ≤0.015 mg/L against contemporary clinical isolates [11,14], and little to no activity against other species, hence causing minimal disturbance to the gut bacterial abundance and composition [15]. Afabicin desphosphono has very limited water solubility, high permeability across the mouse intestinal wall and good distribution in skin structures [16], indicating possible penetration into eukaryotic cells.

In this study, the cellular pharmacokinetics (uptake and release) and subcellular disposition of afabicin desphosphono were examined in cultured mouse macrophages and human monocytes using established techniques developed for other antibiotics [17–19]. Intracellular activity of afabicin desphosphono against phagocytized *S. aureus* with different resistance phenotypes was compared with that of linezolid, daptomycin and vancomycin using a validated pharmacodynamic model of infected human monocytes [20].

2. Materials and Methods

2.1. Materials

Afabicin desphosphono was provided by Debiopharm International (Lausanne, Switzerland) and routinely prepared in dimethyl sulfoxide (DMSO) at concentrations 100-fold higher than the final desired concentrations, then diluted 100-fold in the desired medium. [^{14}]C-labelled afabicin desphosphono (4.77 MBq/mg; label in position 25; see Fig. 1) was provided by Almac Sciences (Craigavon, UK) on order of Debiopharm, and diluted with unlabelled afabicin desphosphono to obtain the desired specific activity. The following antibiotics were obtained as microbiological standards: clarithromycin, from SMB Galephar (Marche-en-Famenne, Belgium); and oxacillin monohydrate and gentamicin sulphate, from Sigma-Aldrich (St. Louis, MO). The other antibiotics were obtained as the corresponding branded products registered for human parenteral use in Belgium and complying with the provisions of the European Pharmacopoeia (vancomycin as Vancomycine Mylan® [Mylan Inc., Canonsburg, PA]; daptomycin as Cubicin® [Novartis, Horsham, United Kingdom]; and linezolid as Zyvoxid® [Pfizer Inc., New York, NY]). Human serum for opsonisation was obtained from Biowest SAS (Nuaillé, France), and cell culture media and sera from Gibco – Thermo Fisher Scientific (Waltham, MA). Unless stated otherwise, all other products were obtained from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

2.2. Cells

Mouse J774 macrophages, originally derived from a mouse reticulosarcoma and obtained from Sandoz Forschung Laboratories (Vienna, Austria), were maintained as monolayers and used at confluency. Human THP-1 monocytes, originally derived from a patient with acute monocytic leukaemia and obtained from the American Culture Collection (ATCC, Manassas, VA) as clone ATCC TIB-202, were propagated in suspension and used at a typical concentration of 0.5×10^6 cells/mL. Both cell lines were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum and 2 mM glutamine (Gibco) in an atmosphere of 95% air–5% CO_2 at 37°C as described previously [21,22].

2.3. Cellular pharmacokinetic and cell fractionation studies

For drug uptake and release studies, J774 macrophages monolayers were quickly washed free of culture medium using phosphate-buffered saline (PBS) and then treated as described previously [23]. For cell fractionation studies, J774 macrophages monolayers were washed with PBS (twice) and then with 0.25 M sucrose-1 mM EDTA-3mM Tris-HCl pH 7.4 (sucrose-EDTA-Tris) twice and collected by scraping with a Teflon® policeman in the same medium. THP-1 monocytes were collected by centrifugation, washed twice in PBS and twice in sucrose-EDTA-Tris and resuspended in the same medium. Cells were then homogenized by 5 to 10 passages of the "tight" pestle of an all-glass Dounce tissue grinder (Thomas Scientific, Swedesboro, NJ) with microscopic (phase contrast) checking for cell disruption. The resulting homogenate was subjected to differential centrifugation as follows: (i) an N fraction (containing unbroken cells and nuclei) was prepared by low-speed centrifugation (1600 revolutions per min [rpm], 10 min, GH-3.8A swinging buckets rotor, Allegra centrifuge X-12R, Beckman-Coulter Life Sciences, Indianapolis, IN) followed by one washing of the pellet with combination of the two supernatants; (ii) the combined supernatants (fraction E [cytoplasmic extract]) were then subjected to high-speed centrifugation (30 000 rpm, 30 min, rotor Ti-50, Beckman-Optima LE-80K ultracentrifuge, Beckman-Coulter) to yield an MLP fraction (containing the bulk of the subcellular organelles and membranes) and an S fraction (soluble material). Isopycnic centrifugation of the whole cytoplasmic extract (fraction E) was made by depositing a sample on top of a linear sucrose gradient (density limits: 1.10 to 1.24) resting on a cushion of sucrose of density 1.34, and centrifuging it at 35 000 rpm for 3 h in a rotor SW 40 Ti (Beckman). In one experiment, the S fraction was deposited on top of the sucrose gradient and cen-

Table 1
Strains used in the study with origin and minimum inhibitory concentration (MIC) in broth

Strain	Origin	MIC (mg/L)[a]						
		Afabicin desphosphono	OXA	CLR	VAN	DAP	LZD	MXF
ATCC 25923	Laboratory standard[b]	0.003906*	0.25	0.25	1	1	4*	0.125*
ATCC 29213	Laboratory standard[c]	0.003906	1	0.25	1	**2***	2	0.0625
SA 040	Clinical isolate[d]	0.003906*	0.25	0.25	1*	**2***	4	0.0625
SA 040 LZD^R	Mutant from clinical isolate[d]	0.003906*	0.25	0.25	2*	**2**	**16**	0.125
SA 312	Clinical isolate[d]	0.003906*	**64**	**64**	1*	**2***	4*	0.0625
NRS 119	Deposited Clinical isolate[f]	0.003906	**>256**	0.5	1	**2**	**128***	**4**
VUB 09	Clinical isolate[g]	0.001953	**64**	**>256**	1*	**2**	2	**4**
MU 50	Deposited clinical isolate[h]	0.003906*	**>256**	**>256**	**8**	**8**	1	**4***

[a] Abbreviations: OXA, oxacillin; CLR, clarithromycin; VAN, vancomycin; DAP, daptomycin; LZD, linezolid; MXF, moxifloxacin. All assays were conducted in triplicate and/or are from previous publications (see [24,25]); values with an asterisk denote assays where a 1 $\log_2$ lower value was occasionally observed. Figures in bold indicate values greater than the EUCAST resistant ("R") clinical breakpoint values for *Staphylococcus* spp. (in mg/L): OXA: >2; CLR: >2; VAN: >2; DAP: >1; LZD: >4; MXF: >0.25; see: The European Committee on Antimicrobial Susceptibility Testing: Breakpoint tables for interpretation of MICs and zone diameters, version 9.0, valid from 2019-01-01- http://www.eucast.org).

[b] Laboratory Standard (American Tissue Culture Collection [ATCC], Manassas, VA).

[c] Laboratory standard (ATCC, Manassas, VA) and EUCAST quality control *Staphylococcus aureus*.

[d] Strain from P. Appelbaum, Hershey Medical Center, Hershey, PA.[e]Respiratory tract infection; strain from P. Appelbaum, Hershey Medical Center, Hershey, PA.

[f] Dialysis-associated peritonitis; strain from M.J. Ferraro, Massachusetts General Hospital, Boston, MA; obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA; presently BEI Resources, Manassas, VA).

[g] Wound infection; strain from D. Pierard, Universitair Ziekenhuis Brussel, Brussels, Belgium.

[h] Wound infection; strain from K. Hiramatsu, Department of Bacteriology, Juntendo University, Tokyo, Japan; also known as NRS1; deposited as *Staphylococcus aureus* Rosenbach and commercially available as strain ATCC 700699 (ATCC, Manassas, VA).

trifuged for 24 h at 35 000 rpm also in rotor SW 40 Ti. After centrifugation, the content of the tube was collected in 12 to 16 fractions of roughly equal volume. All fractions were assayed for radioactivity (when using [^{14}C]-labelled afabicin desphosphono), proteins and marker enzymes (lactate dehydrogenase [LDH; cytosol]; N-acetyl-β-hexosaminidase [NAB; lysosomes], and cytochrome *c*-oxidase [CytOx; mitochondria]). More details about these procedures have been published elsewhere (see [24,25] and the references cited therein).

2.4. Bacterial strains and MIC determinations

The laboratory and clinical strains used in the present study are listed in Table 1 with information on their origins and resistance phenotypes. MICs were determined by microdilution in CA-MHB following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [26]. For afabicin desphosphono testing, we applied the recommendations of CLSI for compounds with limited water solubility, i.e. by preparing stock solutions in DMSO followed by dilution so that the final DMSO concentration was reduced to a constant value of 1% across the whole range of afabicin desphosphono concentrations investigated. For daptomycin, MICs were measured in the presence of 50 mg/L Ca^{++} [26].

2.5. Pharmacodynamic studies

Experiments were conducted essentially as described previously (see [20,25] and the references cited therein) and performed in broth for extracellular bacteria and with infected cells for intracellular bacteria (antibiotics were added to the culture medium immediately after phagocytosis and removal of non-phagocytized bacteria, as per our standard protocol), using a wide range of antibiotic concentrations (typically 0.01-100x MIC) to obtain full concentration-effect relationships. Data were used to fit a sigmoidal function

$$y = E_{max} + \frac{E_{min} - E_{max}}{1 + 10^{((\log EC_{50} - x) * slope)}} \quad (1)$$

(4 coefficients Hill equation), where the dependent variable y is the change in the number of cfu (per mL of medium for extracellular bacteria or per mg of cell protein for intracellular bacteria) from the initial post-phagocytosis inoculum (in $\log_{10}$ units), the independent variable x is the antibiotic extracellular concentration (also in $\log_{10}$ units), and the 4 coefficients (parameters) E_{max}, E_{min}, EC_{50}, and slope are, respectively, (i) the maximal reduction of cfu from the original post-phagocytosis inoculum (in $\log_{10}$ units) as extrapolated for an infinitely large antibiotic concentration (denoting its maximal relative efficacy), (ii) the maximal increase in cfu from the original past-phagocytosis inoculum (in $\log_{10}$ units) as extrapolated for an infinitely low antibiotic concentration(denoting its minimal relative efficacy and corresponding to the maximal bacterial growth that could be observed in the absence of antibiotic), (iii) the antibiotic extracellular concentration giving a change in cfu (in $\log_{10}$ units) halfway between E_{min} and E_{max}, and (iv) the Hill factor describing the steepness of the curves (set always to 1 because (a) there is no theoretical reason nor experimental evidence of positive or negative cooperativity in the responses; (b) attempts to improve the fitting of the function to the data using lower or higher values resulted in divergences between drugs and strains that were without constant emerging trend and, therefore, considered meaningless). The use of logarithmic transformation for concentrations (x) is in line with that commonly used to describe pharmacological dose-responses when doses span several orders of magnitude, as is the case here. The change in cfu (y) also needs to be treated logarithmically because chemotherapeutic responses, unlike enzyme inhibition or proportion of ligand bound to a receptor, for instance, progress by fractional and not constant changes upon finite increases in drug concentration. The fitted functions were used to calculate the parameter C_s (apparent static concentration, i.e. the antibiotic total concentration [in broth or in the cell culture medium] that caused no apparent change in the number of cfu (per mL [broth] or per mg cell protein [THP-1 monocytes]) at the end of the experiment) [20].

2.6. Afabicin desphosphono stability studies

The stability of afabicin desphosphono was assessed by measuring its concentration during and at the end of the experiments using a validated liquid-chromatography-tandem mass spectrometry assay (LC-MS-MS; performed by Atlanbio, Saint-Nazaire, France

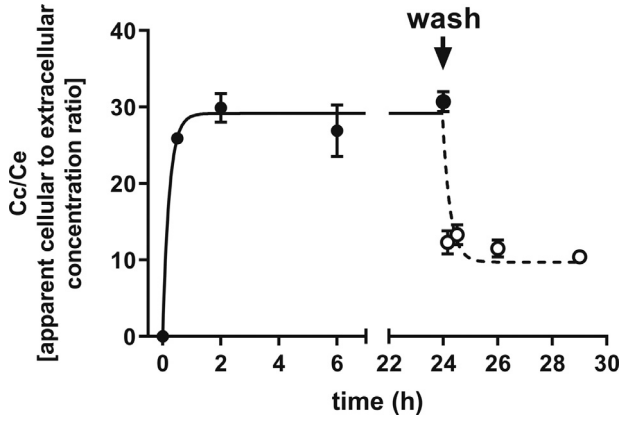

Fig. 2. Accumulation (closed symbols; solid line) and release (open symbols; dotted line) of afabicin desphosphono (Debio 1452) into and out of J774 macrophages. Accumulation: cells were incubated with 1.4 mg/L [^{14}C]-Debio 1452 for up to 24 h and collected. Release: after incubation for 24 h in the presence of 1.4 mg/L [^{14}C]-Debio 1452, cells were quickly washed in situ with PBS (wash) and reincubated in drug-free medium for up to 6 h and collected. Cell-associated drug contents were measured by scintillation counting, expressed in mg/L (based on a cell volume of 5 µL/mg protein) and compared to the extracellular concentration to calculate the apparent cellular-to-extracellular concentration ratio. Data (n=4 for each experimental point) were used for fitting a one-phase association (for accumulation) and a one-phase decay (for release) to estimate the respective rate constants (k_{in} = 4.38 h^{-1}; k_{out} = 3.60 h^{-1}), half-lives (0.15 and 0.19 h) and plateaus (29.2 and 10.4).

under contract with Debiopharm). Nominal mean deviations from known values in the 75 to 750 ng/mL calibration range were 32.8 ± 5.2% (standard error of the mean [SEM]; n=45).

2.7. Curve fitting, calculations, and statistical analyses

Curve fitting (non-linear regression and calculation of the E_{max} and E_{min} function parameters with their standard deviations and confidence intervals [CIs]) was performed with GraphPad Prism (versions 4.03 and 8.2.0) and statistical analyses of the differences with GraphPad InStat (version 3.10) software (GraphPad Software Inc., San Diego, CA). Calculation of the mean C_s parameter (apparent static concentrations) and its CI was made with Excel 2013 (Microsoft Corporation, Redmond, WA) using the functions fitted to each set of replicates (usually 3) as determined by GraphPad.

3. Results

3.1. Cellular pharmacokinetic studies

3.1.1. Uptake and efflux of [^{14}C]-labelled afabicin desphosphono in uninfected J774 macrophages

Fig. 2 shows the accumulation of [^{14}C]-labelled afabicin desphosphono in J774 macrophages incubated with 1.4 mg/L [^{14}C]-Debio 1452 for up to 24 h. Uptake was very rapid, reaching a stable apparent content of about 21 µg/mg of protein, which corresponded to a cellular-to-extracellular concentration ratio of about 30-fold within 2 h (assuming a cell volume to cell protein ratio of 5 µL/mg protein [21]). When cells were transferred to drug-free medium, about two-thirds of the afabicin desphosphono taken up by cells was released as quickly as it had accumulated, while the remaining third remained in an apparently stable fashion for at least 6 h.

3.1.2. Subcellular distribution of [^{14}C]-labelled afabicin desphosphono in uninfected J774 macrophages and THP-1 monocytes

J774 macrophages were exposed to 1 mg/L [^{14}C]-labelled afabicin desphosphono for 1 h before being collected and subjected to controlled disruption, with the resulting homogenate subjected to both differential and isopycnic centrifugation. Table 2 shows that about 70% of the cell-associated radioactivity could be collected in the cytoplasmic extract (obtained after removal of nuclei and unbroken cells [fraction N]), with the same relative specific activity as marker enzymes of mitochondria (cytochrome *c*-oxidase), lysosomes (N-acetyl-β-hexosaminidase), or cytosol (lactate dehydrogenase), thereby ruling out any specific association of afabicin desphosphono to nuclei (almost all if not all collected in the N fraction, based on microscopic [phase contrast] examination). Fig. 3 shows that after subjecting this cytoplasmic extract to isopycnic centrifugation, [^{14}C]-labelled afabicin desphosphono was essentially re-

Table 2
Recovery of radioactivity, marker enzymes and proteins in the cytoplasmic extract prepared from homogenized J774 macrophages incubated for 1 h with 1 mg/L [^{14}C]-labelled afabicin desphosphono.

Constituent	%[1]	SRA[2]
afabicin desphosphono	69.4	0.97
cytochrome *c*-oxidase	70.4	0.98
N-acetyl-β-hexosaminidase	66.0	0.92
lactate dehydrogenase	68.9	0.96
proteins	71.9	

[1] % of the amount present in the original homogenate
[2] Specific Relative Activity (% of each constituent / % of protein)

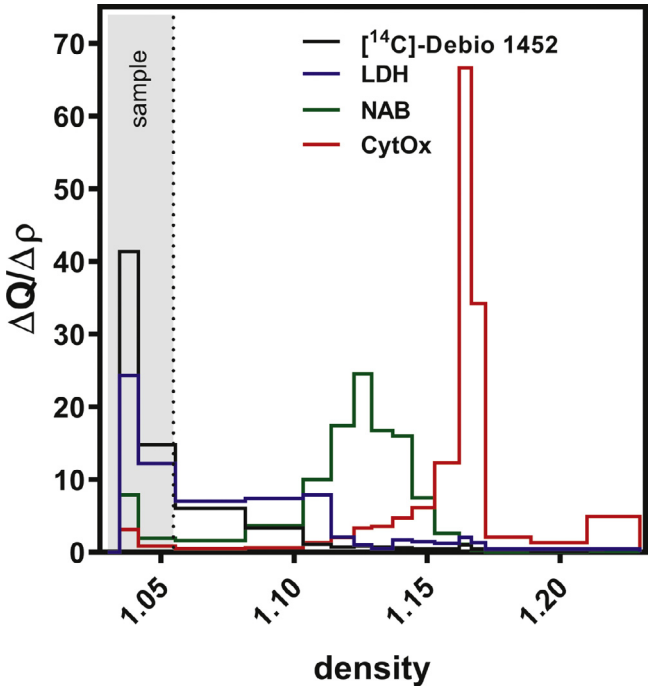

Fig. 3. Fractionation of a cytoplasmic extract of J774 macrophages by isopycnic centrifugation in a linear sucrose gradient. Cells were incubated with 1 mg/L [^{14}C]-labelled afabicin desphosphono (Debio 1452) for 1 h prior to collection. Results are presented as histograms of density distribution of [^{14}C] radioactivity and of the marker enzymes (lactate dehydrogenase (LDH; cytosol), cytochrome *c*-oxidase (CytOx; mitochondria), and N-acetyl-β-hexosaminidase (NAB, lysosomes). The abscissa is the density span of the gradient (with lower and upper limits set arbitrarily at 1.03 and 1.23, respectively [no material was collected below or above these limits]). The ordinate is the distribution frequency defined as the fractional amount of activity recovered in each fraction (ΔQ) divided by the density span ($\Delta\rho$) of that fraction. The surface of each section of each diagram therefore represents the fraction of that constituent recovered in the corresponding fraction and the total area of each diagram is 1 (to enable direct comparison between constituents). The grey area (limited by the vertical dotted line) corresponds to the position of the sample initially deposited on top of the gradient. Data are from a single experiment that was repeated twice with very similar results.

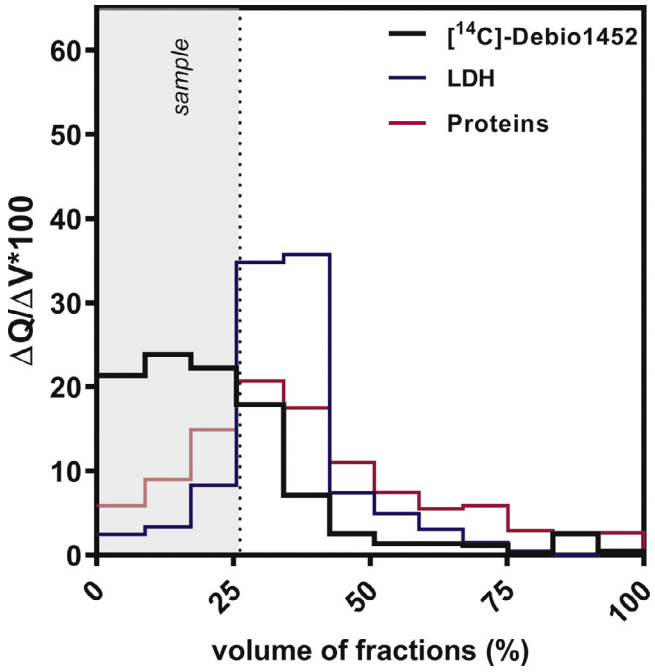

Fig. 4. 24-h high-speed (35 000 rpm) centrifugation of a cell supernatant (fraction S) prepared from THP-1 monocytes incubated with 1 mg/L [^{14}C]-labelled afabicin desphosphono (Debio 1452) for 1.5 h and deposited at the top of a sucrose gradient (density limits 1.10-1.24). Results are presented as histograms of volume distribution of [^{14}C] radioactivity, lactate dehydrogenase (LDH; a soluble protein), and total proteins. The abscissa is the cumulated volume of the fractions (in % of the total volume). The ordinate is the distribution frequency defined as the percent amount of activity or protein recovered in each fraction (ΔQ) divided by the fractional volume (ΔV) of that fraction. The surface of each section of each diagram therefore represents the fraction of activity or protein recovered in the corresponding fraction and the total area of each diagram is 100 (to enable direct comparison between constituents). The grey area (limited by the vertical dotted line) corresponds to the position of the sample initially deposited on top of the gradient.

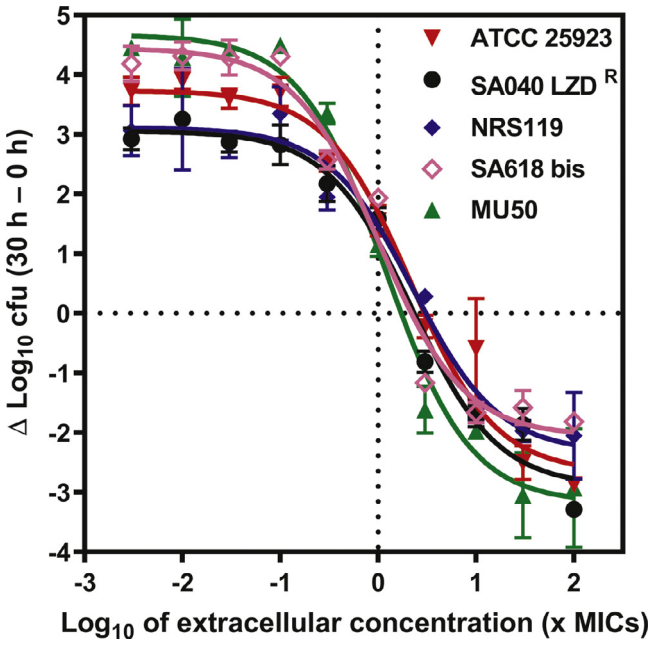

Fig. 5. Concentration-response curves of afabicin desphosphono against extracellular forms (broth) of *S. aureus* strains with different resistance phenotypes (see Table 1). The ordinate shows the change in the number of cfu from the initial inoculum (per mL) after 30 h of incubation. The abscissa shows the extracellular concentrations expressed as multiples of MIC for the corresponding strain (see Table 1). The horizontal dotted line corresponds to the initial inoculum and enables calculation of the apparent static effect (C_s) of each antibiotic. The data were used to fit Hill equations (slope factor = 1). All data are means ± SEM (triplicate experiments with each assay performed in triplicate).

covered in the first two fractions, corresponding to the initial sample, with minimal amounts associated with fractions of higher density. Lactate dehydrogenase (a soluble protein) was also recovered mainly in the top two fractions; however, a small but sizeable fraction was also recovered in denser fractions, indicating partial binding or adsorption to structures migrating into the gradient. This was not observed for [^{14}C]-labelled afabicin desphosphono, which indicates that the drug was essentially soluble. Complete dissociation was obtained from N-acetyl-β-hexosaminidase (lysosomes) and cytochrome *c*-oxidase (mitochondria). A fractionation of control cells (no afabicin desphosphono added) performed in parallel showed similar distributions for the 3 marker enzymes (data not shown), indicating no major effect of afabicin desphosphono on the properties of the corresponding subcellular entities.

Differential centrifugation studies with THP-1 monocytes showed that the bulk of the accumulated [^{14}C]-labelled afabicin desphosphono was collected in the S fraction (soluble material). No attempt was made to subject the fraction E (cytoplasmic extract) of THP-1 monocytes to isopycnic centrifugation because of known poor separation of mitochondria and lysosomes in these cells. To confirm that the predominant recovery of cell-associated afabicin desphosphono in the S fraction corresponded to truly soluble (free) molecule, an experiment was designed in which an S fraction prepared from THP-1 monocytes incubated with 2 mg/L [^{14}C]-labelled afabicin desphosphono (90 min) was deposited on top of a sucrose gradient and subjected to long-term, high-speed centrifugation (enabling partial migration of large molecular weight proteins). Fig. 4 shows that [^{14}C]-labelled afabicin desphosphono was again essentially recovered in the top fractions (accounting for 65.2% of the original sample) and was now largely dissociated from lactate-dehydrogenase and total proteins.

3.2. Microbiological, pharmacodynamic and stability studies

3.2.1. Susceptibility of S. aureus strains of various resistance phenotypes to afabicin desphosphono and comparator antibiotics

Table 1 shows the MICs for afabicin desphosphono and comparator antibiotics (oxacillin, clarithromycin, vancomycin, daptomycin, linezolid, and moxifloxacin) against the laboratory and clinical strains of *S. aureus* used in this study. Afabicin desphosphono consistently showed a very low MIC (0.0039 to 0.0019 mg/L), which was well within the proposed quality control range with *S. aureus* ATCC 29213 reported in an earlier study [27].

3.2.2. Pharmacodynamics of afabicin desphosphono against the extracellular forms of S. aureus strains of various resistance phenotypes

Five *S. aureus* strains were used to explore the concentration-effect relationship of afabicin desphosphono when tested over a wide range of concentrations (0.003-100x MIC) for 30 h in broth (as our previous studies used a standard 24 h incubation time [20,28], we checked with the comparators [historical controls] that prolonging the incubation time to 30 h did not significantly change their maximal relative efficacy or potency). Fig. 5 shows that a Hill function with slope factor set to 1 could be fitted to the data of all strains, with minimal relative efficacies (E_{min}) of +3.1 (strain SA040 LZD^R) to +4.6 (strain MU50) and maximal relative efficacies (E_{max}) of -2.3 (strain NRS119) to -3.2 (strain MU50) $\log_{10}$ cfu compared with the initial inocula, and apparent static concentrations (C_s) ranging from 1.6-fold (strain MU50) to 3.0-fold (strain NRS119) of the corresponding MICs.

3.2.3. Pharmacodynamics of afabicin desphosphono and comparator antibiotics against intracellular forms of S. aureus *of various resistance phenotypes*

Afabicin desphosphono and 3 comparators (vancomycin, daptomycin and linezolid) were used to explore concentration-effect relationships towards the intracellular forms of the fully susceptible ATCC 25923 laboratory strain and towards 3 selected isolates resistant to one or two of the comparators. Fig. 6 illustrates the results obtained after 30 h of incubation (as for our studies of the extracellular forms, the longer incubation time compared with our previous studies did not significantly change the maximal relative efficacy or potency of the comparators [historical controls]), and the corresponding key pharmacodynamic parameters and statistical analysis of the differences are listed in Table 3. A typical sigmoidal concentration-response (Hill function with slope factor set to 1) could be fitted for all strains and all antibiotics as for the extracellular bacteria. E_{min} values (corresponding essentially to the intracellular bacterial growth in the absence of antibiotic) were between +2.8 (strain NRS119) and +4.2 (strain SA040 LZD^{R}) $\log_{10}$ cfu, (i.e., close to E_{min} values for extracellular bacteria). As previously described in this model, E_{max} values (maximal relative efficacies) of afabicin desphosphono and all comparators were lower (less negative) for intracellular bacteria than for extracellular bacteria (with values spanning from -0.56 to only -0.73 $\log_{10}$ cfu; no significant difference between antibiotics). Conspicuously, the apparent intracellular static concentration (C_s) for each antibiotic and each strain was only slightly higher (1.5- to 7-fold) than the corresponding MICs as determined in broth, with no or only small differences across all data.

The last series of experiments examined whether afabicin desphosphono would retain its intracellular activity if added to infected cells 8 h after phagocytosis and removal of the non-phagocytized bacteria. Fig. 7 shows there was no meaningful difference between cells treated according to this protocol and those for which afabicin desphosphono had been added immediately after phagocytosis and removal of the non-phagocytized bacteria.

3.2.4. Stability of afabicin desphosphono in media of infected THP-1 monocytes

To ascertain that afabicin desphosphono was not degraded in the conditions in which it was used to study its intracellular activity, media from infected THP-1 monocytes exposed to afabicin desphosphono at 3 different concentrations (75, 225 and 750 mg/L [about 20, 40 and 200x the MIC, respectively]) for 30 h were collected after 6, 12, 24 and 30 h of incubation and the afabicin desphosphono content compared to that of non-incubated samples (0 h) using an LC-MS-MS assay. Mean recovery of afabicin desphosphono at 30 h was still 76.3% (95%CI: 71.3 to 81.8; n=9) compared with the mean values observed at 0 h (P=0.014 [unpaired t-test; two-tailed]).

4. Discussion

The present study is the first to describe the in vitro cellular pharmacokinetics and disposition of afabicin desphosphono (and, as far as we know, any FabI inhibitor) in murine and human phagocytic cells and to assess its intracellular activity against phagocytized *S. aureus* in a validated pharmacodynamic model of human monocytes.

This study shows that afabicin desphosphono accumulates quickly and markedly in cultured murine macrophages and is released as quickly, although only partially, upon drug removal. Cell-associated afabicin desphosphono is essentially recovered with the soluble cell fraction, with no evidence of significant association with subcellular organelles, and in a form that is unlikely to be

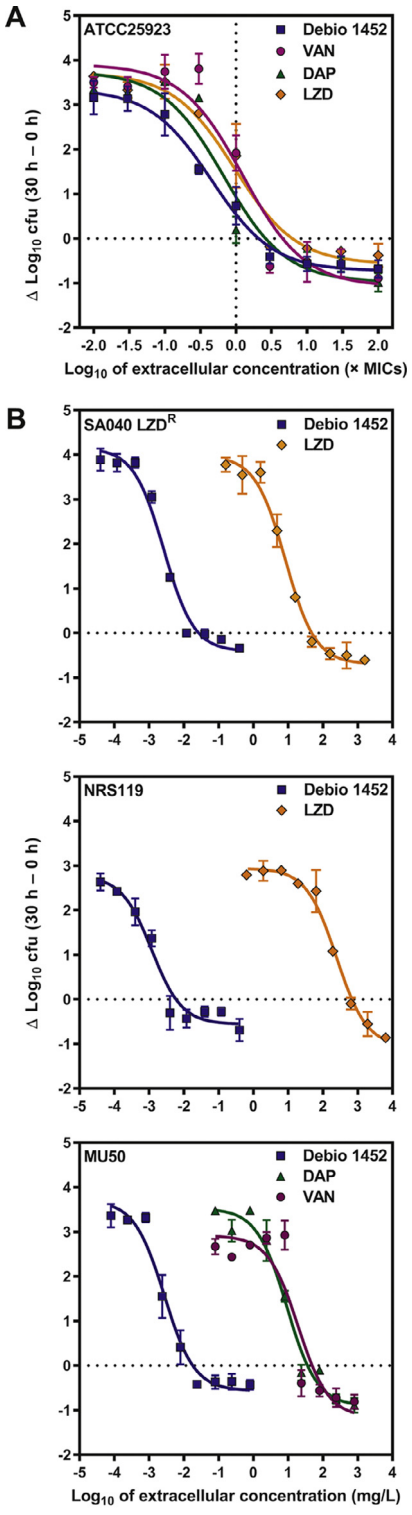

Fig. 6. Concentration-response curves of afabicin desphosphono (Debio 1452), vancomycin (VAN), daptomycin (DAP) and linezolid (LZD) intracellular forms of *S. aureus* strains with different resistance phenotypes (see Table 1). The ordinate of all graphs shows the change in the number of cfu from the initial inoculum per mg of cell protein in THP-1 monocytes after 30 h of incubation. The abscissa shows the extracellular concentrations expressed as follows: **A** (ATCC25923 [categorized as susceptible to VAN, DAP and LZD]) in multiples of MIC for the corresponding antibiotic (the vertical dotted line corresponds to the MIC); **B** (all other strains): in mg/L (to illustrate the difference in susceptibility between afabicin desphosphono and the comparator antibiotic(s) to which the strains is reported to be resistant). The horizontal dotted line in each graph corresponds to the initial inoculum and enables calculation of the apparent static effect (C_s) of each antibiotic. The data were used to fit Hill equations (slope factor = 1). All data are means ± SEM (triplicate experiments for afabicin desphosphono and duplicate experiments for the other antibiotics, with each assay performed in triplicate).

Table 3
Pharmacological parameters and statistical analysis of the dose-response curves of antibiotics against all strains tested in THP-1 monocytes

25923			C_s[c,d]		R^2
Antibiotic and strain	E_{min}[a,d]	E_{max}[b,d]	mg/L	X MIC	
Afabicin desphosphono					
Individual strains (individual curves of Fig 6)					
ATCC25923	3.35 (2.93 to 3.77) A;a	−0.73 (−1.04 to −0.41) A;a	0.009 (0.001 to 0.117) A:a	2.27 (0.58 to 4.21) A;a	0.92
SA040 LZD^R	4.15 (3.89 to 4.42) A;c	−0.41 (−0.64 to −0.18) A;a	0.027 (0.012 to 0.092) A;a	6.97 (2.97 to 23.5) A;a,b	0.97
NRS119	2.77 (2.39 to 3.16) A;b	−0.56 (−0.80 to −0.31) A;a	0.006 (0.002 to 0.022) A;a	1.53 (0.51 to 5.58) A;a,c	0.93
MU50	3.70 (3.26 to 4.15) A;a,c	−0.56 (−0.87 to −0.26) A;a	0.018 (0.006 to 0.074) A;a	4.63 (1.54 to 18.9) A;a	0.94
Pooled data (parameters calculated using one single regression for all strains)					
	3.47 (3.22 to 3.73)	−0.56 (−0.75 to −0.36)	0.011 (0.005 to 0.024)	2.76 (1.36 to 6.32)	0.88
Linezolid					
ATCC25923	3.72 (3.27 to 4.16) A;a	−0.57 (−1.01 to −0.13) A;a	24.8 (6.63 to 226) B;a	6.21(1.66 to 56.5) A;a	0.98
SA040 LZD^R	3.97 (3.61 to 4.33) A;a,b	−0.69 (−0.98 to 0.40) A;a	48.0 (20.3 to 136) B;a	3.00 (1.27 to 8.51) A;a	0.96
NRS119	2.94 (2.72 to 3.16) A;c	−1.06 (−1.42 to −0.69) B;a	683 (303 to 1748) B;b	5.34 (2.37 to 13.7) B;a	0.95
Daptomycin					
ATCC25923	3.75 (3.19 to 4.30) A;a	−0.97 (−1.46 to −0.49) A;a	2.61 (0.76 to11.7) B;a	2.61 (0.76 to 11.7) A;a	0.96
MU50	3.52 (3.16 to 3.88) A,B;a	−0.90 (−1.28 to −0.53) A;a	36.0 (13.8 to 112) B;b	4.50 (1.73 to 14.5) A;a	0.96
Vancomycin					
ATCC25923	3.90 (3.30 to 4.51) A;a	−1.08 (−1.74 to −0.41) A;a	4.53 (1.09 to 29.8) B;a	4.53 (1.09 to 29.8) A;a	0.97
MU50	2.91 (2.33 to 3.50) B;b	−1.15 (−1.96 to −0.34) A;a	49.2 (8.34 to 549) B;a	6.15 (1.04 to 68.7) A;a	0.95

[a] CFU increase (in $\log_{10}$ units, with confidence interval) at 24 h from the corresponding initial inoculum as extrapolated from the Hill equation of the concentration-effect response for an infinitely low antibiotic concentration.
[b] CFU decrease (in $\log_{10}$ units, with confidence interval) at 24 h from the corresponding initial inoculum as extrapolated from the Hill equation of the concentration-effect response for an infinitely large antibiotic concentration.
[c] extracellular antibiotic concentration (with confidence intervals) resulting in no apparent bacterial growth as calculated from the Hill equation of the concentration-response curve.
[d] Statistical analyses: one-way analysis of variance with Tukey-Kramer multiple-comparison *t* test. Values with different upper case letters denote a statistically significant difference for the same strain in cells exposed to different antibiotics (thus, for instance, for E_{max} of strain NRS119 in cells exposed to afabicin desphosphono vs. linezolid [marked A and B, respectively), whereas values with different lower case letters denote statistically significant differences between different strains in cells exposed to the same antibiotic For instance, for C_s (in mg/L) of strain ATCC25923 vs. strain NRS119 in cells exposed to linezolid (marked a and b, respectively). For C_s values that show an asymmetrical confidence interval, statistical analysis used the log transformed data.

bound to proteins or other soluble macromolecules. This behaviour is largely reminiscent of that observed with fluoroquinolones [18] (see also review in [29]) as well as the oxazolidinone, tedizolid [30] and the deformylase inhibitor, GSK1322322 [24]. It differs markedly from that reported for macrolides, the dibasic biaryloxazolidinone radezolid, or the novel triazaacenaphthylene bacterial topoisomerase inhibitor gepotidacin (GSK2140944), all of which accumulate rapidly in cells but become largely associated with lysosomes [17,19,25], probably because of proton-trapping [31]. Afabicin desphosphono may diffuse rapidly through the pericellular membrane, consistent with its non-ionized character and a positive logD value (3.01) at pH 7.4 (two properties shared by tedizolid and GSK1322322, based on biophysical properties calculations [Chemaxon, Budapest, Hungary; https://chemaxon.com/]) and binds reversibly and loosely to still unidentified cellular constituents. Such loose binding, however, is likely to be defeated upon dilution, such as that occuring during the cell fractionation studies, which would explain why the drug is found essentially soluble when applying this technique. If this is the case, the accumulated drug may be able to access various subcellular organelles, such as phagolysosomes. Interestingly, recent studies performed in humans showed an accumulation of afabicin desphosphono in cortical, cancellous, bone marrow and soft tissue and synovial fluid of patients who received the prodrug form, indicating it could act in these niches [13]. Accumulation of afabicin desphosphono into macrophages may also enable increased concentrations at infection sites due to recruitment of leukocytes upon infection and inflammation. Indeed, penetration of afabicin desphosphono in bone tissues of animal models was increased when these were infected [32].

Access to intracellular targets and expression of activity therein was assessed directly in our pharmacodynamic model. Considering extracellular bacteria, this study confirms that afabicin desphosphono has unhindered antibacterial activity against laboratory and clinical *S. aureus* strains, irrespective of their resistance to other

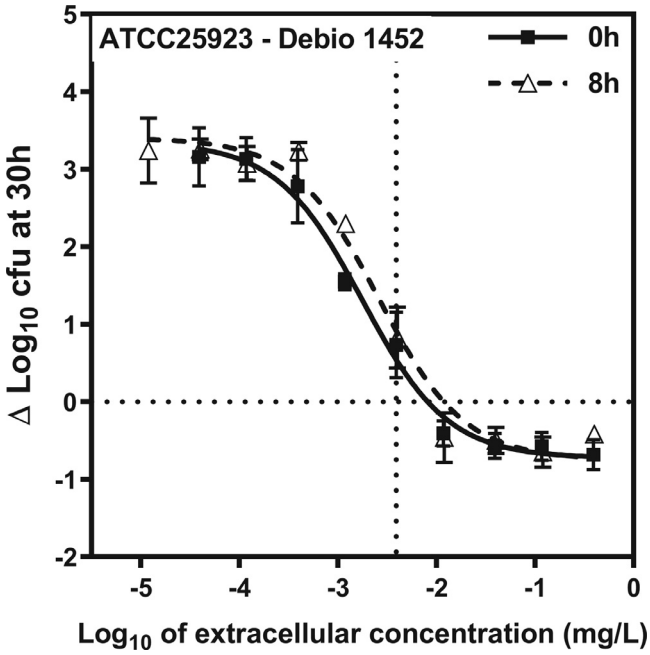

Fig. 7. Concentration-response curves of afabicin desphosphono (Debio 1452) against the intracellular forms of *S. aureus* ATCC25923 in THP-1 monocytes exposed to antibiotics immediately after phagocytosis and removal of the non-phagocytized bacteria (0 h; closed squares and solid line) or added after 8 h of culture of the infected cells in an antibiotic-free medium (8 h, open triangles; dotted line). The ordinate shows the change in the number of cfu from the initial inoculum per mg of cell protein in THP-1 monocytes after 30 h of incubation with the antibiotic. The abscissa shows the extracellular concentrations (in mg/L). The horizontal and vertical dotted lines correspond to the initial inoculum and to the MIC of afabicin desphosphono, respectively. The data were used to fit Hill equations (slope factor = 1). All data are means ± SEM (triplicate experiments).

commonly recommended antistaphylococcal drugs, which is consistent with previous observations [33] and with its specific and distinct mode of action compared with other antibiotics [34]. With regard to intracellular activity, afabicin desphosphono is bacteriostatic against *S. aureus* phagocytized and thriving in human THP-1 monocytes, including strains resistant to other antistaphylococcal antibiotics, with very low C_s values (0.01 to 0.03 mg/L), which is in agreement with its good intracellular penetration. Similar to most antibiotic classes, there was a marked reduction in maximal efficacy of afabicin desphosphono towards intracellular bacteria (with mean E_{max} values never exceeding a 1.1 $\log_{10}$ cfu decrease) compared with extracellular bacteria. Of note, E_{max} is the extrapolated value for an infinitely large extracellular antibiotic concentration, which rules out that its low (less negative) value is related to impairment of penetration into bacteria (pharmacokinetic effect). Instead it indicates a lack of response of the bacteria to the antibiotic action (pharmacodynamic effect). This behaviour was also seen in this study with vancomycin and daptomycin, two antibiotics that have previously been shown to be markedly bactericidal towards extracellular bacteria [25,35,36]. Likewise, other bactericidal antibiotics from different pharmacological classes, such as gepotidacin [25], GSK1322322 [24], ceftaroline [36] and ceftobiprole [37], were shown to be only bacteriostatic against intracellular *S. aureus* in the same model, indicating a global alteration of the bacterial response to antibiotics when sojourning in THP-1 monocytes and disproving a pharmacokinetic or an antibiotic-specific pharmacodynamic effect. Two classes of antibiotics (fluoroquinolones and lipoglycopeptides, with moxifloxacin and oritavancin as typical examples, respectively), however, can have an intracellular bactericidal effect in our model [28], indicating that modulation of the bacterial response is possible, perhaps in relation to the target of the antibiotic under study. It is not known whether this difference in global effects will translate into clinically-demonstrable advantage(s). Plasma concentration at steady state has been presented for the intended therapeutic dosing regimen of 240 mg oral BID [13]. Mean C_{max} was 2.4 mg/L and C_{trough} 1.2 mg/L. These concentrations far exceed the MICs (0.0019 to 0.0039 mg/L [Table 1]) and the extracellular concentrations yielding an intracellular bacteriostatic effect (0.009 to 0.027 mg/L [C_s parameter]; see Table 2) even considering that afabicin desphosphono can be up to 98% protein-bound in human serum. Therefore, afabicin desphosphono could be as effective as the comparators (vancomycin, linezolid, and daptomycin) for controlling the multiplication of intracellular *S. aureus* in vivo.

The present study has limitations that need to be carefully considered. First, the model uses cells that show only very limited defences to infection (see discussion in [20] and the references cited therein). This limitation is by design to provide a simple pharmacological description of the intracellular effects of the antibiotics, but this design ignores how these cell defence mechanisms could improve or hinder the activity of afabicin desphosphono against intracellular *S. aureus* in vivo. Second, afabicin desphosphono is reported to show a marked protein binding (~98%) in human serum, associated with an 8-fold increase in its MIC [38]. The impact of protein binding could not be assessed in the present study model because the serum concentration in the culture medium is low and cannot be varied to the extent required to assess its role on antibiotic intracellular activity. Third, future studies are needed to understand the mechanism(s) of accumulation of afabicin desphosphono in cells (and the reasons for its incomplete release) to establish whether and how the drug redistributes in cells upon homogenization and fractionation, and, most urgently, to find out why bacteria fail to be killed as efficiently in cells compared to in broth. Lastly, we focused our attention on a single time point to be in line with our approach to accurately measure and report pharmacological descriptors of activity, as done previously for many other antibiotics (see e.g. [24,25,28,35–37,39]). Establishing the intracellular pharmacokinetic/pharmacodynamic profile of afabicin desphosphono, including the influence of time on its activity, using dose fractionation studies would require development of an appropriate model and detailed knowledge of the complete human pharmacokinetic profile of afabicin in infected patients to be clinically relevant.

Despite these limitations, the present study shows clearly that afabicin desphosphono is accumulated by macrophages and exerts a bacteriostatic effect against *S. aureus* phagocytized by THP-1 monocytes. Expanding these studies to pertinent in vivo models of intracellular infection is warranted.

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Declarations

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Competing interests: PMT and FVB have received grants and speakers honoraria from various companies involved in the discovery and development of drugs accumulating in cells and/or acting on intracellular bacteria.

Ethical Approval: Not required

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