Cellular pharmacokinetics and intracellular activity of gepotidacin against
Staphylococcus aureus with different resistance phenotypes in models of cultured phagocytic cells.

Frédéric Peyrusson, Paul M. Tulkens, Françoise Van Bambeke

Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium.

Keywords (not in the title): MRSA, THP-1 monocytes, linezolid, vancomycin, moxifloxacin, daptomycin, macrolides

Running title: Gepotidacin cellular pharmacokinetics and activity

$ Corresponding author. Avenue Mounier 73 B1.73.05 – 1200 Brussels – Belgium
Tel: +32-2-7647378; E-mail: francoise.vanbambeke@uclouvain.be

Paper metrics

- Abstract: 250 words (max. 250)
- Figures: 7
- Tables: 2
- Main text length: 4051 words
- References: 45
ABSTRACT

Gepotidacin (GSK2140944), a novel triazaacenaphthylene bacterial topoisomerase inhibitor, is currently in clinical development for the treatment of bacterial infections. This study examines in vitro its activity against intracellular Staphylococcus aureus (involved in the persistent character of skin and skin structure infections) using a pharmacodynamic model and in relation to cellular pharmacokinetics in phagocytic cells. Compared to oxacillin, vancomycin, linezolid, daptomycin, azithromycin, and moxifloxacin, gepotidacin was (i) more potent intracellularly (C_{s} [apparent bacteriostatic effect] reached at an extracellular concentration of about 0.7 x its MIC and not affected by resistance mechanisms to the comparators) and (ii) caused a maximal reduction of the intracellular burden (E_{max}) of about -1.6 log_{10} CFU (better than linezolid, macrolides, and daptomycin; similar to moxifloxacin). After 24 h of incubation of infected cells with antibiotics at 100 X their MIC, the intracellular persisting fraction was < 0.1 % with moxifloxacin, 0.5 % with gepotidacin and > 1 % with other drugs. Accumulation and efflux of gepotidacin in phagocytes was very fast (k_{in} and k_{out} ~0.3 min^{-1}; plateau reached within 15 minutes) but modest (intracellular to extracellular concentration ratio ~1.6). In cell fractionation studies, about 40-60 % of the drug was recovered in the soluble fraction and ~40% associated with lysosomes in uninfected cells. In infected cells, about 20% of cell-associated gepotidacin was recovered in a sedimentable fraction that also contained bacteria. This study highlights the potential for further study of gepotidacin to fight infections where intracellular niches may play a determining role in bacterial persistence and relapses.
INTRODUCTION

In spite of the availability of a large array of both old and newly approved antibiotics active against Gram-positive bacteria, *Staphylococcus aureus* is still considered among the high priority pathogens for research and development of new therapies by the World Health Organization (WHO (1)). Antistaphylococcal antibiotics, indeed, should not only address the challenge of being active against multidrug resistant strains that become increasingly prevalent, but also of showing activity against latent forms of bacterial infection, which are often tolerant to antibiotic treatments. In this context, the capacity of *S. aureus* to survive within the host cells is considered as playing a critical role in the persistence and/or recurrence of infections since intracellular bacteria are largely protected against antimicrobial treatments and host immune defenses (2-4). To act upon intracellular bacteria, antibiotics need to fulfill a series of pharmacokinetic and pharmacodynamic criteria, which globally reflect the intracellular bioavailability of the drug and its capacity to express activity towards bacteria in the specific conditions prevailing in the infected compartment (5). Exploring these properties is thus of high interest for antibiotics acting on new targets.

Gepotidacin (originally known as GSK2140944; see structure and ionization status at physiological pH in Fig. 1) is a novel antimicrobial agent belonging to the triazaacenaphthylene class of novel bacterial topoisomerase inhibitors that are structurally different and present a unique mechanism of action to impair DNA replication compared to fluoroquinolones. Instead of stabilizing DNA double-strand breaks as fluoroquinolones, these antibiotics bind to a distinct site (6) and stabilize the pre-cleavage type II topoisomerase enzyme-DNA complex prior to DNA cleavage, generating single-strand breaks (7, 8).

Gepotidacin has a broad spectrum of activity but with lower MICs and more pronounced bactericidal effects against Gram-positive species (9). It demonstrates robust activity against clinical isolates associated with skin and lower respiratory tract infections, with MIC<sub>90</sub> ≤ 0.5 mg/L, including against methicillin-resistant *S. aureus* (MRSA) and
fluoroquinolone-resistant *S. aureus* or *Streptococcus pneumoniae* (10, 11). Gepotidacin has successfully completed a phase II clinical trial (12) in patients suffering from acute bacterial skin and skin structure infections (ABSSSIs) and also a study for uncomplicated urogenital infections caused by *Neisseria gonorrhoeae*.

The aims of the present study were (a) to determine the intracellular activity of gepotidacin using an *in vitro* pharmacodynamic model of intracellular infection in human THP-1 monocytes by various drug-susceptible and resistant strains of *S. aureus* and to compare it with other antistaphylococcal agents, and (b) to examine in parallel the gepotidacin intracellular pharmacokinetics (influx/efflux; accumulation; and subcellular disposition) in human THP-1 monocytes and murine J774 macrophages. We found that gepotidacin is capable of reducing the intracellular bacterial burden to a larger extent than all comparator antibiotics except moxifloxacin, irrespective to the resistance phenotype of the strain. Gepotidacin showed a fast rate of cellular uptake and efflux but low cellular accumulation levels at equilibrium, with most of the drug localized in the soluble fraction and a smaller proportion in the phagolysosomal compartment where *S. aureus* sojourns.
RESULTS

Susceptibility of *S. aureus* strains to gepotidacin and comparator antibiotics

Table 1 shows the MICs for gepotidacin and comparator antibiotics against all laboratory and clinical strains used in this study. These include strains harboring resistance mechanisms to macrolides, linezolid, daptomycin, vancomycin, and fluoroquinolones (target mutations [SA618b] or efflux transporter NorA [SA1]). Gepotidacin consistently showed low MICs (0.25-1 mg/L) whatever the resistance phenotype of the strain. Based on the physicochemical properties of gepotidacin (see Figure 1) that indicate a more hydrophilic character at acidic pH, MICs were also determined at pH 5.5 and found to be 3-4 log₂ dilutions higher than those measured at neutral pH for most of the strains.

Cellular viability

We first checked for the lack of cytotoxicity of gepotidacin towards eukaryotic cells by measuring the release of the cytosolic enzyme lactate dehydrogenase from THP-1 monocytes in the conditions used for further experiments. This release was lower than 5 % and not significantly different from control values after 24 h of incubation with gepotidacin concentrations up to 50 mg/L (50 x the highest MIC against *S. aureus* in the present study).

Extracellular and intracellular activity of gepotidacin and comparators

**Extracellular activity (CA-MHB)**

The extracellular activity of gepotidacin was evaluated against 6 strains of *S. aureus* that included 5 strains resistant to at least one comparator antibiotic. To this effect, residual CFU were measured after 24 h of incubation with extracellular concentrations ranging from 0.001 to 100 x the MIC of each strain (Fig. 2A, left panel) in order to obtain full
concentration-effect curves (see Table 2 for pharmacological descriptors). Gepotidacin showed a bacteriostatic effect at a concentration close to its MIC and a bactericidal effect (3 log_{10} CFU decrease) at 10 x its MIC for all strains. A single sigmoid function could be satisfactorily fitted to the whole data set when the change in CFU was expressed as a function of equipotent concentrations (multiples of MIC), demonstrating that gepotidacin activity was independent of the resistance phenotype of the strains to the comparators.

Intracellular activity (THP-1 human monocytes)

The same type of experiment was then performed to evaluate the activity of gepotidacin against bacteria phagocytised by THP-1 monocytes (Fig. 2A, right panel and Table 2 for the values of the pharmacological descriptors). Gepotidacin activity developed in a concentration-dependent fashion with similar pharmacodynamic parameters for all strains whatever their resistance phenotype to the other antibiotics. However, the maximal relative efficacy (E_{max}; decrease of CFU compared to the original inoculum for an infinitely large extracellular antibiotic concentration) against intracellular bacteria was considerably lower (less negative) than for extracellular bacteria, with a value of about only -1.2 log_{10} CFU compared to the post-phagocytosis bacterial burden. In contrast, the apparent bacteriostatic concentration (C_s; extracellular concentration of drug [expressed in mg/L or in multiples of MIC] causing no apparent change in CFU) remained close to the MIC as for the extracellular bacteria. In a next step, the intracellular activity of gepotidacin was compared with that of other antibiotics towards the fully susceptible S. aureus strain ATCC25923. Data are shown in Fig. 2B, with extracellular concentrations expressed in mg/L (left) or in x MIC (right) and the corresponding pharmacodynamic parameters presented in Table 2. Moxifloxacin was the most potent (lowest C_s) among the drugs tested owing its low MIC value, but also the most effective, with an E_{max} at -1.7 log_{10} CFU. The other drugs were equi- or less potent but also less effective (less negative E_{max}) compared to gepotidacin. When antibiotics were
compared at equipotent concentrations, their $C_s$ were close (0.7 to 5 times) to their respective MIC.

We then compared the intracellular activity of gepotidacin and other antibiotics against strains harboring resistance mechanisms (Fig. 3 for a direct comparison of gepotidacin and antibiotics affected by resistance and Table 2 for numerical data including against additional susceptible and resistant strains tested with each antibiotic). In all cases, intracellular $C_s$ remained close to the MIC, and was therefore shifted to much larger concentrations for antibiotics affected by resistance but not for gepotidacin. The maximal relative efficacy of each antibiotic ($E_{\text{max}}$) was unchanged except for clarithromycin against the MU50 strain for which a Hill function could not be fitted to the data as we could not expose it to concentrations exceeding its MIC (> 256 mg/L).

Lastly, we compared the persisting bacterial fraction in cells infected by each of the investigated strains and exposed for 24 h to high concentrations of each of the drugs under study (Fig. 4). The highest persisting bacterial fraction (> 1 %) was observed for infected cells exposed to macrolides, oxacillin (β-lactam) or each of the 3 anti-MRSA antibiotics (daptomycin, linezolid and vancomycin from highest to lowest), and the lowest one (< 0.1 %) after incubation with moxifloxacin. With gepotidacin, the persisting bacterial fraction (0.5 %) was slightly higher than that observed with moxifloxacin but significantly lower than that observed with the other drugs.

**Cellular influx, accumulation and efflux of gepotidacin**

The kinetics of accumulation and efflux as well as the level of accumulation at equilibrium of gepotidacin was then determined in uninfected THP-1 monocytes and mouse J774 macrophages exposed to a microbiologically-meaningful and clinically-achievable (12) extracellular concentration of 1 mg/L (Fig. 5A). Gepotidacin uptake proceeded according to a
one-phase exponential association with a constant rate of 0.27 min\(^{-1}\), to reach an apparent stable cellular concentration 1.7-fold higher than the extracellular one after 15 min. Efflux occurred at the same rate (constant: 0.30 min\(^{-1}\)) and was almost complete (residual apparent accumulation of 0.2) after approximately 15 minutes. The accumulation level at equilibrium (after 30 minutes) was similar in THP-1 and J774 cells and not influenced by the extracellular concentration over a broad range (0.1-100 mg/L; Fig. 5B; left panel). In both cells types, accumulation was markedly reduced when cells were incubated at 4°C instead of 37°C. Conversely, efflux was completely abolished when cells loaded at 37°C were reincubated at 4°C in an antibiotic-free medium (Fig. 5B; middle panel). Lastly, the accumulation of gepotidacin was measured in infected vs. non-infected THP-1 cells incubated during 30 min with 1 mg/L gepotidacin, and no major difference was observed (Fig. 5B; right panel). Likewise, residual accumulation was similar in both conditions after 30 min reincubation in gepotidacin-free medium at 37°C.

Subcellular distribution of gepotidacin in uninfected and infected cells

The subcellular distribution of gepotidacin was studied in J774 macrophages incubated during 30 minutes with 1 mg/L gepotidacin. Fig. 6 shows the distribution of the radiotracer and of markers of the cytosol (lactate dehydrogenase), lysosomes (N-acetyl-β-hexosaminidase) and mitochondria (cytochrome c-oxidase) in a sucrose gradient after isopycnic centrifugation of the cell homogenate. Enzymatic markers were distributed in different fractions of the gradient, with lactate dehydrogenase located mainly in the lighter fractions, cytochrome c-oxidase in the heavier fractions, and N-acetyl-β-hexosaminidase, in fractions with a density of around 1.13. Gepotidacin showed a bimodal distribution with 46 % of the radioactivity showing a distribution similar to that of lactate dehydrogenase and 45 %, similar to that of N-acetyl-β-hexosaminidase. This experiment could not be performed with THP-1 cells, since lysosomal and mitochondrial markers equilibrate in the same fractions for
these cells. We therefore also studied the distribution of gepotidacin in parallel in J774 and THP-1 homogenates that had been more grossly fractionated by differential centrifugation in order to separate organelles on the basis of their size (Fig. 7). These experiments were performed in infected cells incubated during 2 h at 37°C after phagocytosis of bacteria to allow for their complete internalization and then 30 min with 0.1 mg/L of gepotidacin (sub-MIC concentration, to maintain bacterial viability). In both cell types, [14C]-gepotidacin was mainly recovered in the final supernatant (60% and 40% in THP-1 and J774, respectively), with smaller amounts in the organelles-containing fraction (20% and 30%, respectively), and the remainder in nuclei/unbroken cells fraction. As previously described, lactate dehydrogenase was mostly recovered in the soluble fraction, and cytochrome c-oxidase and N-acetyl-β-hexosaminidase, in the organelle fraction together with bacteria (13). Cell fractionation experiments performed with uninfected THP-1 and J774 according to the same protocol showed a similar distribution of the drug and of the marker enzymes (data not shown).
DISCUSSION

This study is, to the best of our knowledge, the first one to document the cellular pharmacokinetics and intracellular activity of this new triazaacenaphthylene bacterial topoisomerase inhibitor. Because its mechanism of action is distinct from that of currently approved antibiotics, it is not surprising that gepotidacin shows the same level of activity against fully-susceptible *S. aureus* and against strains resistant to the comparators used in this study as well as to other antistaphylococcal drugs (10, 11). For fluoroquinolones, we show here that gepotidacin activity is maintained not only against strains harboring mutations in fluoroquinolone targets but also those expressing NorA efflux pump that affects ciprofloxacin and other hydrophilic fluoroquinolones (14).

Considering our intracellular pharmacodynamic experiments as a whole, we can apply to gepotidacin the general concepts previously described and discussed at length for other classes of bactericidal antistaphylococcal antibiotics in our previous publications (15, 16), namely that most of them show activity against the intracellular forms of *S. aureus*, but (i) that their maximal relative efficacy ($E_{\text{max}}$) is markedly reduced compared to what is observed against extracellular bacteria (17), while (ii) the extracellular concentration needed to obtain an intracellular static effect ($C_s$) remains close to the MIC as determined in broth, denoting no loss of relative potency. Globally, however, gepotidacin stands as one of the most effective drugs tested in this model so far (see (5) for review) after the lipoglycopeptide oritavancin (18) and the anti-Gram-positive fluoroquinolones moxifloxacin and delafloxacin (19). The lower persisting fraction observed here for bacteria exposed to gepotidacin and moxifloxacin vs. the comparators illustrates the potential interest of targeting topoisomerase enzymes when dealing with intracellular forms of infections by *S. aureus*. Assessing the maximal intracellular relative activity ($E_{\text{max}}$) of antibiotics and measuring the bacterial persisting fraction are probably of high clinical relevance as we know that even low inocula of intracellular *S. aureus* are capable of causing severe infections *in vivo* (20) and that
intracellular survival is associated with the recurrent character of several staphylococcal infections in humans (4, 21, 22). Focusing on gepotidacin, our study shows that this antibiotic consistently decreases the intracellular bacterial burden to a value close to the maximal relative efficacy ($E_{\text{max}}$) when present in the extracellular medium at concentrations that can be reached in human serum based on the results of the recently published clinical trial ($C_{\text{max}}$ ranging between 2.4 and 8.8 mg/L depending on the dose and administration route (12); no $C_{\text{min}}$ values available).

A striking observation, however, is that the intracellular activity of gepotidacin develops without marked accumulation in cells (apparent cellular to extracellular concentration ratio of approx. 1.6), in sharp contrast to fluoroquinolones that accumulate 5 to 20 times (23) or macrolides that accumulate to even much higher values (24). This is actually in accordance with a number of previous observations made with our model where we showed that there is no direct correlation between the global cellular accumulation of antibiotics and the level of their intracellular activity when comparing drugs of different pharmacological classes (see (13, 15, 23, 25) for typical examples). Yet, the molecular reasons for a lack of such correlation remain to be established for most antibiotics. Of note, however, static effects are obtained intracellularly for extracellular concentrations close to the MIC for both gepotidacin and moxifloxacin. This would tend to suggest a higher intracellular bioavailability of gepotidacin compared to moxifloxacin and/or a defeating effect exerted by the intracellular milieu on the potency of moxifloxacin. Of note also, both gepotidacin and moxifloxacin show lower intrinsic activity (higher MIC) at acidic pH, which may contribute to reduce their intracellular potency against $S.\text{ aureus}$ that thrive in acidic intracellular compartments, counteracting the beneficial effect of their accumulation.

Turning our attention to the cellular pharmacokinetics of gepotidacin, our data suggest that it enters inside eukaryotic cells by passive diffusion since (i) rates of influx and efflux are fast and very similar, with no evidence of saturation over the range of
concentrations investigated (that include concentrations up to 12-fold higher than the human C\textsubscript{max} (12) and 100 times the MIC); and (ii) accumulation was almost completely impaired at 4°C (a condition which considerably reduces membrane fluidity). The intracellular disposition of gepotidacin (partly in lysosomes and partly in the cytosol) is also consistent with its character a weak base with pK\textsubscript{a}s close to the range of pH between lysosomes (around 5) and cytosol (around 7), as previously observed for other antibiotics (see (26) for models and (24) and (27) for typical examples). Of interest, this also means that part of the accumulated drug is located in the same compartment as bacteria (see fractionation studies with infected cells as well as data demonstrating a phagolysosomal localization for S. aureus in phagocytic cells (15, 28, 29). While this part seems only minor (about 20 %), we cannot exclude a redistribution of the drug from lysosomes to the cell supernatant during the homogenization and fractionation process that are associated with an extensive dilution of the cellular material.

Gepotidacin has now successfully completed a Phase II clinical trial for the treatment of suspected or confirmed Gram-positive acute bacterial skin and skin structure infections (12). Pharmacodynamic studies in a model of murine lung infection have demonstrated AUC-dependent activity and a pharmacokinetic profile that supports further investigation of this compound for the treatment of infections caused by MRSA (30). The present study adds that gepotidacin may offer a particular interest to act upon intracellular S. aureus, which may play a critical role in persistent or recurrent skin or respiratory tract infections (21, 31, 32).
MATERIALS AND METHODS

Antibiotics and main products. Gepotidacin (see Fig. 1) and $[^{14}C]$-gepotidacin (specific activity, 59.9 mCi/mmol; radiochemical purity, 99.7%) were obtained from GlaxoSmithKline plc (Collegeville, PA). Stock solutions of unlabeled gepotidacin were prepared in dimethyl sulfoxide at a concentration of 50 mg/L and thereafter diluted in water to the desired concentration. The radiolabeled compound was added in a tracing amount to stock solutions of unlabeled gepotidacin in order to obtain appropriate signals under our experimental conditions. The stability of gepotidacin in our experimental conditions was checked by determining MICs of culture media (cation-adjusted Mueller-Hinton broth [CA-MHB; Becton, Dickinson and Company, Franklin Lakes, NJ], RPMI-1640 supplemented or not by 10% fetal bovine serum [FBS]) spiked by gepotidacin, that had been pre-incubated or not during 24 h at 37°C and geometrically diluted in a microtiter plate inoculated with S. aureus SA618 bis. No difference was seen in the concentration of gepotidacin needed to inhibit bacterial growth for samples pre-incubated during 24 h or not, demonstrating its stability in our conditions. The following antibiotics were obtained as microbiological standards: azithromycin and clarithromycin, from SMB-Galephar (Marche-en-Famenne, Belgium); moxifloxacin HCl, from Bayer AG (Wuppertal, Germany); oxacillin monohydrate and gentamicin sulfate, 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 Vancomycin Mylan [Mylan Inc., Canonsburg, PA] and Linezolid as Zyvoxid [Pfizer Inc., New York, NY]). Human serum was obtained from Biowest SAS (Nuaillé, France), and cell culture media and sera, from Gibco/Life Technologies Corporation (Paisley, United Kingdom). Unless stated otherwise, all other products were obtained from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).
Cell lines. Experiments were performed using (i) human THP-1 cells (a myelomonocytic cell line (33)) purchased as clone ATCCTIB-202 from the American Tissue Culture Collection, Manassas, VA, and murine J774 macrophages (derived from a reticulosarcoma (34)) originally obtained from Sandoz Forschung Laboratories, Vienna, Austria. Both cell lines were maintained in our laboratory as previously described, in RPMI-1640 medium supplemented by 10% FBS in a 5% CO₂ atmosphere (35, 36).

Bacterial strains and susceptibility testing. The laboratory and clinical strains used in the present study are listed in Table 1 with information on their origin and resistance phenotypes. MICs were determined by microdilution in CA-MHB following the recommendations of the Clinical and Laboratory Standards Institute (37). In specific experiments, the medium was adjusted to pH 5.5 using HCl.

Assessment of viability of THP-1 monocytes. Cell viability in the presence of increasing concentrations of gepotidacin was evaluated by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture medium after 24 h of incubation using the cytotoxicity Detection KitPLUS [LDH] (Roche Diagnostics GmbH, Manheim, Germany) following the manufacturer’s instructions. The release of LDH was expressed as the percentage of activity detected in the media compared to the total enzymatic activity in the culture.

Determination of extracellular and intracellular activities of antibiotics. For extracellular activity, experiments were performed in CA-MHB with an initial inoculum of 10⁶ CFU/mL. Bacteria were incubated with antibiotics over a broad range of extracellular concentrations during 24 h, after which aliquots were taken, appropriately diluted and plated on agar.
Results expressed as the change (in CFU/mL) from the initial inoculum as assessed by colony counting. Bactericidal activity was defined as a reduction of 99.9% (3 log_{10} CFU/mL decrease) of the total counts. For intracellular activity, cell infection was performed as described previously (15). In brief, bacteria were opsonized with human serum (10% in RPMI-1640) during 30 min at 37°C. Bacteria were then incubated at an inoculum of 4 bacterial per cell during 1 h to allow phagocytosis. After removing of the medium and washing of the cells with PBS, infected cells were incubated during 45 min with gentamicin at 100 X its MIC to eliminate extracellular bacteria, washed with PBS to eliminate gentamicin, and reincubated during 24 h with increasing concentrations of antibiotics. Cells were then washed with PBS and collected in H2O. CFU were determined by plating and proteins were assayed by the Folin-Ciocalteu method. Results were expressed as the change (in CFU/mg cell protein) from the post-phagocytosis inoculum. Data from concentration-response experiments were used to fit a sigmoidal function (Hill equation) and calculate pertinent pharmacodynamic parameters, i.e. E_{max} (maximal relative efficacy; decrease in the number of CFU compared with the original inoculum for an infinitely large extracellular antibiotic concentration [note that this parameter is negative if killing occurs and is more negative for drugs with larger efficacy]), E_{min} (minimal relative efficacy; increase in the number of CFU compared with the original inoculum for an infinitely low extracellular antibiotic concentration [this parameter essentially describes bacterial growth]), and C_s; see (13, 17) for further details). The Hill equation was also used to calculate the intracellular persisting bacterial fraction, which was defined as the ratio between the residual CFU/mg protein after 24h exposure to 100 x MIC of the antibiotic (or the maximal reachable concentration for isolates against which the MIC of the corresponding antibiotic was ≥ 128 mg/L) and after 24 h incubation in the absence of added antibiotic except 1 x MIC gentamicin, to avoid extracellular growth contamination and subsequent cell lysis (15).
Accumulation and release experiments. Gepotidacin accumulation and release were measured using a general protocol developed in our laboratory to study the cellular pharmacokinetics of antibiotics in J774 macrophages and THP-1 monocytes (13, 38). In brief, cells were incubated in the presence of gepotidacin (with a tracing amount of $^{14}$C-labeled drug) for the lengths of time and at the concentrations adapted for the purpose of each experiment. At the end of the incubation period, cells were washed with PBS, lysed by sonication and the antibiotic concentration determined in the lysate by scintillation counting and normalized by reference to the total cell protein content. The apparent cellular accumulation was then calculated using a conversion factor of 3.08 µL of cell volume per mg of cell protein for J774 macrophages (39) and 5 µL of cell volume per mg of cell protein for THP-1 monocytes (15).

Cell fractionation studies. We followed the general protocol developed in our laboratory for studying the subcellular distribution of antibiotics in cells (13, 18, 27, 40). In brief, uninfected cells were incubated with 1 mg/L of gepotidacin (with a tracing amount of $^{14}$C-labeled drug) for 30 min, washed, and collected in ice-cold 0.25 M sucrose–3 mM Na EDTA–3 mM imidazole (pH 7.4) (sucrose-EDTA-imidazole), whereas infected cells were incubated with bacteria to allow phagocytosis as described above, washed, returned to fresh medium for 2 h to allow complete internalization of bacteria, and then incubated for 30 min with gepotidacin at a total concentration of 0.1 mg/L (to avoid killing of the bacteria), washed, and finally collected in sucrose-EDTA-imidazole. Cells were then homogenized in the same medium using a Dounce tissue grinder. Subcellular organelles were separated by differential and isopycnic centrifugation as described previously (13, 35). In brief, for differential centrifugation, homogenates were separated into 3 successive fractions (nuclei and unbroken cells, organelles, and supernate [cytosol]) by successive centrifugations (1,600, 25,000, and 40,000 rpm for 10 min, 6.7 min, and 30 min, respectively; the first one in
a Beckman Allegra X-12R bench-top centrifuge; the latter two in a rotor Ti50 operated in a Beckman Optima LE-80K ultracentrifuge [Beckman Coulter Life Sciences, Indianapolis, IN]).

For isopycnic centrifugation, the cell homogenate was first made free of nuclei and unbroken cells by centrifugation at 1,600 rpm for 10 min, and the resulting cytoplasmic extract deposited on top a linear sucrose gradient with densities spanning from 1.10 to 1.24 and resting on a cushion of sucrose of 1.34 density. After centrifugation at 39,000 rpm for 3 h in a swing-out rotor SWTi50 (Beckman), the gradient was collected into 12 discrete fractions the densities of which were measured by refractometry (ABBE-3L refractometer, Baush and Lomb, Rochester, NY). All fractions were assayed for protein content, radioactivity, activity of marker enzymes (35) and, if infected, viable bacteria (CFU counting).

Curve fitting and statistical analyses. Curve fitting and statistical analyses were performed with GraphPad Prism versions 4.03 and 7.03 and GraphPad InStat version 3.10 (GraphPad Software Inc., San Diego, CA), and JMP Pro version 12.0.1 (SAS Institute Inc., Cary, NC).
Acknowledgments

The authors thank M.C. Cambier, K. Santos Saial and V. Yfantis for expert technical assistance.

Financial support

This work was supported in part by GlaxoSmithKline, Collegeville, PA and has been funded in whole or in part with federal funds from the Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority, under contract HHSO100201300011C. Additional support was obtained from the Belgian Fonds de la Recherche Scientifique (grants T.0189.16 and J.0018.17) and the Interuniversity Attraction Poles Program initiated by the Belgian Science Policy Office (program IAP P7/28).

F.P. is an employee of the Université catholique de Louvain; P.M.T. is an unpaid emeritus Professor from the Université catholique de Louvain; F.V.B. is Research Director of the Belgian Fonds de la Recherche Scientifique (FNRS-FRS).
References

1. Anonymous.  http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/. Accessed August 10, 2017.
2. Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, Chaponnier C, Lew D, Lacroix JS. 2005. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent Staphylococcus aureus rhinosinusitis. J Infect Dis 192:1023-1028. https://doi.org/10.1086/432735
3. Bosse MJ, Gruber HE, Ramp WK. 2005. Internalization of bacteria by osteoblasts in a patient with recurrent, long-term osteomyelitis. A case report. J Bone Joint Surg Am 87:1343-1347. https://doi.org/10.2106/JBJS.D.02649
4. Zautner AE, Krause M, Stropahl G, Holtfreter S, Frickmann H, Maletzki C, Kreikemeyer B, Pau HW, Podbielski A. 2010. Intracellular persisting Staphylococcus aureus is the major pathogen in recurrent tonsillitis. PLoS One 5:e9452. https://doi.org/10.1371/journal.pone.0009452
5. Van Bambeke F, Barcia-Macay M, Lemaire S, Tulkens PM. 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. Curr Opin Drug Discov Devel 9:218-230.
6. Singh SB, Kaelin DE, Wu J, Miesel L, Tan CM, Meinke PT, Olsen D, Lagrutta A, Bradley P, Lu J, Patel S, Rickert KW, Smith RF, Soisson S, Wei C, Fukuda H, Kishii R, Takei M, Fukuda Y. 2014. Oxabicyclooctane-linked novel bacterial topoisomerase inhibitors as broad spectrum antibacterial agents. ACS Med Chem Lett 5:609-614. https://doi.org/10.1021/ml500069w
7. Bax BD, Chan PF, Eggleston DS, Fosberry A, Gentry DR, Gorrec F, Giordano I, Hann MM, Hennessy A, Hibbs M, Huang J, Jones E, Jones J, Brown KK, Lewis CJ, May EW, Saunders MR, Singh O, Spitzfaden CE, Shen C, Shillings A, Theobald AJ, Wohkonig A, Pearson ND, Gwynn MN. 2010. Type IIA topoisomerase inhibition by a new class of antibacterial agents. Nature 466:935-940. https://doi.org/10.1038/nature09197
8. Smart DJ, Lynch AM. 2012. Evaluating the genotoxicity of topoisomerase-targeted antibiotics. Mutagenesis 27:359-365. https://doi.org/10.1039/mutage/ger0089
9. Flamm RK, Farrell DJ, Rhomberg PR, Scangarella-Oman NE, Sader HS. 2017. Gepotidacin (GSK2140944) In Vitro Activity against Gram-Positive and Gram-Negative Bacteria. Antimicrob Agents Chemother 61: pii: e00468-17. https://doi.org/10.1128/AAC.00468-17
10. Biedenbach DJ, Bouchillon SK, Hackel M, Miller LA, Scangarella-Oman NE, Jakielaszek C, Sahm DF. 2016. In Vitro Activity of Gepotidacin, a Novel Triazaacenaphthylene Bacterial Topoisomerase Inhibitor, against a Broad Spectrum of Bacterial Pathogens. Antimicrob Agents Chemother 60:1918-1923. https://doi.org/10.1128/AAC.02820-15
11. Farrell DJ, Sader HS, Rhomberg PR, Scangarella-Oman NE, Flamm RK. 2017. In Vitro Activity of Gepotidacin (GSK2140944) against Neisseria gonorrhoeae. Antimicrob Agents Chemother 61: pii: e02047-16. https://doi.org/10.1128/AAC.02047-16
12. O’Riordan W, Tiffany C, Scangarella-Oman N, Perry C, Hossain M, Ashton T, Dumont E. 2017. Efficacy, Safety, and Tolerability of Gepotidacin (GSK2140944) in the Treatment of Patients with Suspected or Confirmed Gram-Positive Acute Bacterial Skin and Skin Structure Infections. Antimicrob Agents Chemother 61: pii: e02095-16. https://doi.org/10.1128/AAC.02095-16
13. Peyrusson F, Butler D, Tulkens PM, Van Bambeke F. 2015. Cellular pharmacokinetics and intracellular activity of the novel peptide deformylase inhibitor...
various resistance phenotypes: studies with human THP-1 monocytes and J774 murine macrophages. Antimicrob Agents Chemother 59:5747-5760.

14. Yoshida H, Bogaki M, Nakamura S, Ubukata K, Konno M. 1990. Nucleotide sequence and characterization of the Staphylococcus aureus norA gene, which confers resistance to quinolones. J Bacteriol 172:6942-6949.

15. Barcia-Macay M, Lemaire S, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F. 2006. Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant Staphylococcus aureus. J Antimicrob Chemother 58:1177-1184.

16. Lemaire S, Głupczynski Y, Duval V, Joris B, Tulkens PM, Van Bambeke F. 2009. Activities of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages and keratinocytes) forms of methicillin-susceptible and methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother 53:2289-2297. https://doi.org/10.1128/AAC.01135-08

17. Buyck JM, Lemaire S, Seral C, Ananthisrajah A, Peyrusson F, Tulkens PM, Van Bambeke F. 2016. In Vitro Models for the Study of the Intracellular Activity of Antibiotics. Methods Mol Biol 1333:147-157. https://doi.org/10.1007/978-1-4939-2854-5_13

18. Van Bambeke F, Carryn S, Seral C, Chanteux H, Tyteca D, Mingeot-Leclercq MP, Tulkens PM. 2004. Cellular pharmacokinetics and pharmacodynamics of the glycopeptide antibiotic oritavancin (LY333328) in a model of J774 mouse macrophages. Antimicrob Agents Chemother 48:2853-2860. https://doi.org/10.1128/AAC.48.8.2853-2860.2004

19. Lemaire S, Tulkens PM, Van Bambeke F. 2011. Contrasting effects of acidic pH on the extracellular and intracellular activities of the anti-gram-positive fluoroquinolones moxifloxacin and delafloxacin against Staphylococcus aureus. Antimicrob Agents Chemother 55:649-658. https://doi.org/10.1128/AAC.01201-10

20. Hamza T, Dietz M, Pham D, Clovis N, Danley S, Li B. 2013. Intra-cellular Staphylococcus aureus alone causes infection in vivo. Eur Cell Mater 25:341-350; discussion 350.

21. Ou J, Drilling A, Singhal D, Tan NC, Wallis-Hill D, Vreugde S, Psaltis AJ, Wormald PJ. 2016. Association of intracellular Staphylococcus aureus with prognosis in chronic rhinosinusitis. Int Forum Allergy Rhinol 6:792-799. https://doi.org/10.1002/arr.21758

22. Tan NC, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald PJ. 2013. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? Int Forum Allergy Rhinol 3:261-266. https://doi.org/10.1002/arr.21154

23. Vallet CM, Marquez B, Ngabirano E, Lemaire S, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F. 2011. Cellular accumulation of fluoroquinolones is not predictive of their intracellular activity: studies with gemifloxacin, moxifloxacin and ciprofloxacin in a pharmacokinetic/pharmacodynamic model of uninfected and infected macrophages. Int J Antimicrob Agents 38:249-256. https://doi.org/10.1016/j.ijantimicag.2011.05.011

24. Carlberg MB, Garcia-Luque I, Montenez JP, Tulkens PM, Piret J. 1994. Accumulation, release and subcellular localization of azithromycin in phagocytic and non-phagocytic cells in culture. Int J Tissue React 16:211-220.

25. Melard A, Garcia LG, Das D, Rozenberg R, Tulkens PM, Van Bambeke F, Lemaire S. 2013. Activity of ceftaroline against extracellular (broth) and intracellular (THP-1
monocytes) forms of methicillin-resistant Staphylococcus aureus: comparison with vancomycin, linezolid and daptomycin. J Antimicrob Chemother 68:648-658.

https://doi.org/10.1093/jac/dks442

26. de Duve C, de Barys T, Poole B, Trouet A, Tulkens P, Van Hoof F. 1974. Commentary. Lysosomotrophic agents. Biochem Pharmacol 23:2495-2531.

27. Lemaire S, Tulkens PM, Van Bambeke F. 2010. Cellular pharmacokinetics of the novel biaryl oxazolidinone radedorzid in phagocytic cells: studies with macrophages and polymorphonuclear neutrophils. Antimicrob Agents Chemother 54:2540-2548.

https://doi.org/10.1128/AAC.01723-09

28. Lopez de Armentia MM, Amaya C, Colombo Ml. 2016. Rab GTPases and the Autophagy Pathway: Bacterial Targets for a Suitable Biogenesis and Trafficking of Their Own Vacuoles. Cells 5. https://doi.org/10.3390/cells5010011

29. Flannagan RS, Heit B, Heinrichs DE. 2016. Intracellular replication of Staphylococcus aureus in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination. Cell Microbiol 18:514-535.

https://doi.org/10.1111/cmi.12527

30. So W, Crandon JL, Nicolau DP. 2015. Pharmacodynamic Profile of GSK2140944 against Methicillin-Resistant Staphylococcus aureus in a Murine Lung Infection Model. Antimicrob Agents Chemother 59:4956-4961.

https://doi.org/10.1128/AAC.00625-15

31. von Eiff C, Becker K, Metze D, Lubritz G, Hockmann J, Schwarz T, Peters G. 2001. Intracellular persistence of Staphylococcus aureus small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with dairer's disease. Clin Infect Dis 32:1643-1647. https://doi.org/10.1086/320519

32. Stopinska M, Olszewska-Sosinska O, Lau-Dworak M, Zielnik-Jurkiewicz B, Trafny EA. 2014. Identification of intracellular bacteria in adenoid and tonsil tissue specimens: the efficiency of culture versus fluorescent in situ hybridization (FISH).

33. Curr Microbiol 68:21-29. https://doi.org/10.1007/s00284-013-0436-0

34. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 26:171-176.

35. Snyderman R, Pike MC, Fischer DG, Koren HS. 1977. Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. J Immunol 119:2060-2066.

36. Renard C, Vanderhaeghe HJ, Claes Pj, Zenebergh A, Tulkens PM. 1987. Influence of conversion of penicillin G into a basic derivative on its accumulation and subcellular localization in cultured macrophages. Antimicrob Agents Chemother 31:410-416.

37. Scorneaux B, Ouadrhiri Y, Anzalone G, Tulkens PM. 1996. Effect of recombinant human gamma interferon on intracellular activities of antibiotics against Listeria monocytogenes in the human macrophage cell line THP-1. Antimicrob Agents Chemother 40:1225-1230.

38. Anonymous. Clinical and Laboratory Standards Institute. 2017 Performance standards for antimicrobial susceptibility testing; 24th informational supplement (M100-S27). Clinical and Laboratory Standards Institute, Wayne, PA.

39. Lemaire S, Van Bambeke F, Appelbaum PC, Tulkens PM. 2009. Cellular pharmacokinetics and intracellular activity of torezolid (TR-700): studies with human macrophage (THP-1) and endothelial (HUVEC) cell lines. J Antimicrob Chemother 64:1035-1043. https://doi.org/10.1093/jac/dkp267

40. Michot JM, Van Bambeke F, Mingeot-Leclercq MP, Tulkens PM. 2004. Active efflux of ciprofloxacin from J774 macrophages through an MRP-like transporter. Antimicrob Agents Chemother 48:2673-2682. https://doi.org/10.1128/AAC.48.7.2673-2682.2004
40. Carlier MB, Scorneaux B, Zenebergh A, Desnottes JF, Tulkens PM. 1990. Cellular uptake, localization and activity of fluoroquinolones in uninfected and infected macrophages. J Antimicrob Chemother 26 Suppl B:27-39.

41. Kosowska-Shick K, Clark C, Credito K, McGhee P, Dewasse B, Bogdanovich T, Appelbaum PC. 2006. Single- and multistep resistance selection studies on the activity of retapamulin compared to other agents against Staphylococcus aureus and Streptococcus pyogenes. Antimicrob Agents Chemother 50:765-769. https://doi.org/10.1128/AAC.50.2.765-769.2006

42. Kosowska-Shick K, Ednie LM, McGhee P, Smith K, Todd CD, Wehler A, Appelbaum PC. 2008. Incidence and characteristics of vancomycin nonsusceptible strains of meticillin-resistant Staphylococcus aureus at Hershey Medical Center. Antimicrob Agents Chemother 52:4510-4513. https://doi.org/10.1128/AAC.01073-08

43. Tsiodras S, Gold HS, Sakoulas G, Eliopoulos GM, Wenersten C, Venkataraman L, Moellering RC, Ferraro MJ. 2001. Linezolid resistance in a clinical isolate of Staphylococcus aureus. Lancet 358:207-208. https://doi.org/10.1016/S0140-6736(01)05410-1

44. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, Kobayashi I, Cui L, Oguchi A, Aoki K, Nagai Y, Lian J, Ito T, Kanamori M, Matsumaru H, Maruyama A, Murakami H, Hosoyama A, Mizutani-Uy Y, Takahashi NK, Sawano T, Inoue R, Kaito C, Sekimizu K, Hirakawa H, Kuhara S, Goto S, Yabuzaki J, Kaneshita A, Yamashita A, Oshima K, Furuya K, Yoshino C, Shiba T, Hattori M, Ogasawara N, Hayashi H, Hiramatsu K. 2001. Whole genome sequencing of meticillin-resistant Staphylococcus aureus. Lancet 357:1225-1240.

45. Ba BB, Arpin C, Vidaillac C, Chausse A, Saux MC, Quentin C. 2006. Activity of gatifloxacin in an in vitro pharmacokinetic-pharmacodynamic model against Staphylococcus aureus strains either susceptible to ciprofloxacin or exhibiting various levels and mechanisms of ciprofloxacin resistance. Antimicrob Agents Chemother 50:1931-1936. https://doi.org/10.1128/AAC.01586-05
## TABLE 1 Strains used in the study, strain origin, and MIC in broth

| Strain     | Origin          | MIC (mg/L) | GEP | AZM | CLR | OXA | VAN | LZD | DAP | MXF | CIP | GEN |
|------------|-----------------|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| ATCC25923  | Laboratory      | 0.5-1 0.25 | 1   | 1   | 0.25 | 2  | 2   | 1   | 0.03-0.0625 | 0.125-0.25 | 0.5 |
|            | In vitro mutant from clinical isolate | 0.25-0.5 | 2   | 0.25 | 0.25 | 1.2 | 16  | 2   | 0.125 | ND  | 0.5 |
| SA040 LZD<sup>r</sup> | Clinical       | 0.25 | ND  | ND  | 256 | 4   | 2   | 32  | 4   | ND  | 0.125 |
| SA618 bis  | In vitro exposure of a clinical isolate on linezolid increasing concentrations; unknown resistance mechanism. | 0.5-1 4 2 | >256 | 1   | 128 | 2   | 4   | 4   |   | ND  | 64  |
| NRS119     | Clinical<sup>g</sup> | 0.25 | ND  | ND  | >256 | >256 | >256 | 8   | 1   | 8   | 4   | ND  |
| MU50       | Clinical<sup>h</sup> | 0.25 | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | 0.0625 | 4   | ND  |
| SA1        | Laboratory<sup>g</sup> | 0.25 | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | 0.0625 | 4   | ND  |

<sup>a</sup> Figures in bold indicate values greater than the EUCAST resistant ("R") clinical breakpoint values (European Committee on Antimicrobial Susceptibility Testing 2017). Abbreviations: GEP, gepotidacin; AZM, azithromycin; CLR, clarithromycin; OXA, oxacillin; DAP, daptomycin; VAN, vancomycin; LZD, linezolid; MXF, moxifloxacin; CIP, ciprofloxacin; GEN, gentamicin; ND, not determined.

<sup>b</sup> Laboratory Standard (ATCC; Manassas, VA).

<sup>c</sup> From P. Appelbaum, Hershey Med. Center, Hershey, PA (41). Selected by in vitro exposure of a clinical isolate on linezolid increasing concentrations; unknown resistance mechanism.

<sup>d</sup> Respiratory tract infection - from P. Appelbaum, Hershey Med. Center, Hershey, PA. (42) Described as MRSA and hVISA strain.

<sup>e</sup> Peritonitis (43). Described as MRSA and linezolid-resistant with mutated domain V in 23S RNA.

<sup>f</sup> ATCC700699 (Manassas, VA). Surgical wound infection; VISA (44).

<sup>g</sup> In vitro mutant overexpressing NorA; selected by in vitro exposure of ATCC25923 to increasing concentrations of Ethidium bromide (45) – from Claudine Quentin, Université de Bordeaux 2, Bordeaux, France.

---

160730_1_unknown_upload_2898150_p1qf2m.docx – last saved by Françoise Van Bambeke - 29/12/2017 11:36
### TABLE 2 Pharmacological parameters and statistical analysis of the dose-response curves of antibiotics against all strains tested in THP-1 monocytes

| Antibiotic and strain   | E<sub>50</sub> | E<sub>90</sub> | mg/L | X MIC | R<sup>2</sup> |
|------------------------|--------------|--------------|------|-------|----------|
| **Gepotidacin**        |              |              |      |       |          |
| Individual strains (Fig. 2B and 3) |              |              |      |       |          |
| ATCC25923              | 3.29 (3.03 to 3.48) | 0.79 (-0.79 to -0.58) | 6.4 (4.4 to 7.9) | 2.30 (2.20 to 2.40) | 0.98      |
| SA040 LZD<sup>a</sup>  | 3.14 (2.87 to 3.40) | 0.25 (-0.51 to 0.01) | 58.6 (27.8 to 108) | 3.66 (1.74 to 6.79) | 0.96      |
| NRS119                 | 2.69 (2.45 to 2.93) | 1.31 (-1.82 to -0.81) | 69.3 (47.1 to 96) | 1.08 (0.74 to 1.50) | 0.95      |
| **Linezolid**          |              |              |      |       |          |
| ATCC25923              | 3.35 (3.06 to 3.65) | 0.38 (-0.73 to -0.04) | 1.23 (1.10 to 1.34) | 4.90 (4.42 to 5.37) | 0.95      |
| MU50                   | 2.05 (1.93 to 2.17) | 1.98 (1.89 to 2.07) | No convergence | No convergence | 0.08      |
| **Daptomycin**         |              |              |      |       |          |
| ATCC25923              | 3.84 (3.42 to 4.25) | -0.79 (-1.19 to -0.40) | 2.35 (1.03 to 3.67) | 2.53 (1.03 to 3.67) | 0.96      |
| SA040 LZD<sup>a</sup>  | 3.25 (2.91 to 3.59) | -0.35 (-0.62 to -0.08) | 2.36 (1.88 to 2.91) | 1.18 (0.95 to 1.94) | 0.95      |
| SA618bis               | 1.99 (1.70 to 2.29) | -0.76 (-1.08 to -0.42) | 15.7 (14.90 to 16.50) | 0.49 (0.47 to 0.52) | 0.93      |
| NRS119                 | 2.44 (2.20 to 2.68) | -0.44 (-0.67 to -0.20) | 3.85 (3.61 to 4.10) | 1.92 (1.80 to 2.05) | 0.93      |
| MU50                   | 2.07 (1.87 to 2.27) | -0.70 (-0.93 to -0.47) | 16.3 (15.20 to 18) | 2.04 (1.90 to 2.23) | 0.96      |
| **Moxifloxacin**       |              |              |      |       |          |
| ATCC25923              | 3.39 (3.03 to 3.76) | -1.07 (-2.11 to -1.30) | 0.03 (0.03 to 0.04) | 1.10 (0.95 to 1.25) | 0.97      |
| SA040 LZD<sup>a</sup>  | 2.58 (2.29 to 2.88) | -1.14 (-1.46 to -0.81) | 0.54 (0.52 to 0.56) | 4.33 (4.17 to 4.49) | 0.96      |
| SA618bis               | 2.49 (2.20 to 2.79) | -1.23 (-1.61 to -0.84) | 7.10 (4.51 to 9.68) | 1.77 (1.13 to 2.42) | 0.94      |
| NRS119                 | 2.23 (1.91 to 2.54) | -2.11 (-2.54 to -1.68) | 4.50 (3.12 to 5.88) | 1.12 (0.78 to 1.47) | 0.95      |
| MU50                   | 2.13 (1.82 to 2.43) | -2.42 (-2.91 to -1.93) | 3.81 (3.75 to 3.88) | 0.95 (0.94 to 0.97) | 0.95      |
| SA1                    | 2.97 (2.66 to 3.29) | -1.29 (-1.62 to -0.96) | 0.10 (0.08 to 0.11) | 1.52 (1.30 to 1.74) | 0.97      |
| **Ciprofloxacin**      |              |              |      |       |          |
| SA1                    | 3.13 (2.67 to 3.58) | -1.25 (-1.64 to -0.85) | 3.36 (2.97 to 3.76) | 0.84 (0.74 to 0.94) | 0.94      |
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. For E_{max} and C_s, values followed by different uppercase (response of antibiotics to the same strain) or lowercase (response of strains to the same antibiotic) letters are significantly different from each other (p<0.05). For E_{min}, values followed by different uppercase (response of strains irrespective to antibiotics) letters are significantly different from each other (p<0.05).
FIG 1 Structural formula and full IUPAC name of gepotidacin (and position of the $^{14}$C in the labeled compound), with predominant ionized aminofunction(s) at pH 5.4 and at pH 7.4. The calculated pK$_a$ of these amino functions are 8.83 (A) and 6.20 (B), respectively. The calculated logD of the molecule is -0.73 at pH 8, -1.72 at pH 7 and -4.23 at pH 5. The graph shows the proportion of each ionized microspecies over the 5 to 8 pH range, with indication of the protonation status of each of the two aminogroups in these species. The Table shows the MICs as determined at pH 7.4 and 5.5 for the 6 strains under study. All physicochemical parameters were calculated using the Reaxys® software (www.reaxys.com), Elsevier, 2016.
FIG 2 (A) Concentration-response curves of gepotidacin against extracellular (left) and intracellular (right) forms of S. aureus strains with different resistance phenotypes (see Table 1). The graphs show the changes in the number of CFU from the initial inoculum per mL of broth (left) or per mg of cell protein (right) in THP-1 monocytes after 24 h of incubation at increasing extracellular concentrations (expressed in multiple of MIC). (B) Concentration-response curves of the intracellular activity of gepotidacin (GEP) and comparators (CLR, clarithromycin; LZD, DAP, MXF).
linezolid; DAP, daptomycin; MXF, moxifloxacin) against strain ATCC25923 (MSSA). The graphs show the changes in the number of CFU from the initial inoculum per mg of cell protein in THP-1 cells after 24 h of incubation at increasing extracellular concentrations expressed either in total extracellular concentrations in mg/L [left] or in multiples of the corresponding MIC [right]. The horizontal dotted lines highlight a static effect (Cs) and the lowest limit of detection (CFU decrease of 5 log_{10} units compared to the initial inoculum; top panels only), and the vertical dotted line, the MIC, when applicable. All data are means ± SEM (n=3).
FIG 3 Intracellular concentration-response curves of gepotidacin (GEP) against strains with resistance to comparators (CLR, clarithromycin; LZD, linezolid; DAP, daptomycin; MXF, moxifloxacin) and phagocytophized by THP-1 monocytes (only data pertaining to GEP and key comparators for each strain are shown on the graph; see Table 2 for the pharmacological descriptors of the activity of the other drugs). The ordinate shows the changes in the log_{10} CFU per mg of cell protein after 24 h incubation compared to the initial inoculum. The abscissa shows the drug concentration expressed as log_{10} of total extracellular concentrations in mg/L. For all panels, the plain and dotted arrows point to the MIC of GEP and its comparator, respectively, and the horizontal dotted line shows a static effect (C_s). Data are means ± SEM (n=3).
FIG 4 Evaluation of the intracellular persisting fraction: for each tested antibiotic, the bar shows the ratio between the $\log_{10} \text{CFU/mg prot}$ after 24 h incubation with 100 x MIC of antibiotic (or highest value tested if MIC > 256 mg/L) and the $\log_{10} \text{CFU/mg prot}$ in control conditions (24 h of incubation in the presence of gentamicin at its MIC to avoid extracellular contamination (15)).

Data are means ± SEM of the values calculated for the 6 strains investigated in three independent experiments (AZM, azithromycin; CLR, clarithromycin; OXA, oxacillin; DAP, daptomycin; LZD, linezolid; VAN, vancomycin; GEP, gepotidacin; MXF, moxifloxacin; ML, macrolide; FQ, fluoroquinolone). Statistical analysis (one-way ANOVA with Tukey post-hoc test): data sets with different letters are significantly different from one another (p< 0.05).
Peyrusson et al. - Gepotidacin cellular pharmacokinetics and activity

**A**

**THP-1 monocytes**

**Kinetics of accumulation**

**Kinetics of efflux**

**B**

**30 min accumulation**

**30 min accumulation / efflux**

**Influence of extracellular conc.**

**Influence of temperature**

**Influence of infection**

**Extracellular conc. (mg/L)**

**Temperature during incubation**

**Cellular (Cc) to extracellular (Ce) concentration ratio**

*THP-1 monocytes 30 min accumulation / efflux*
FIG 5 Accumulation and release of gepotidacin in human THP-1 monocytes and murine J774 macrophages. (A) THP-1 were incubated with [\(^{14}\)C]-gepotidacin at a fixed concentration (1 mg/L) and collected (accumulation; left panel) or incubated for 30 min and then returned to drug-free medium (efflux; right panel). (B) Left: THP-1 and J774 cells were incubated with [\(^{14}\)C]-gepotidacin at different extracellular concentrations for 30 min. Middle: both cell lines were incubated for 30 min with 1 mg/L [\(^{14}\)C]-gepotidacin and collected (accumulation) or returned to drug-free medium for 30 min at the temperatures indicated (efflux). Right: Same experiment as in middle panel, but comparing uninfected and infected THP-1 cells at 37°C.

For each graph, the ordinate shows the apparent ratio between the cellular and the extracellular concentrations. Data are means ± SD (n=3). Statistical analysis (lower left panel): ANOVA with Tukey-Kramer multiple-comparison t test; (lower middle and right panel): unpaired multiple t test.
FIG 6 Fractionation of cytoplasmic extracts of J774 macrophages by isopycnic centrifugation in a linear sucrose gradient (collected in 12 discrete fractions). Cells were incubated with 1 mg/L [14C]-gepotidacin for 30 min prior to collection. Results are presented as histograms of density distribution of [14C]-gepotidacin and of the marker enzymes (lactate dehydrogenase, cytosol; cytochrome c-oxidase, mitochondria; N-acetyl-β-hexosaminidase, lysosomes). The abscissa is the density span of the gradient. The ordinate is the frequency of distribution defined as the fractional amount of activity recovered in each fraction divided by the density interval of that fraction. The surface of each section of the diagrams therefore represents the fraction of each constituent recovered in the corresponding density span. Data are from a single experiment that was repeated with very similar results.
FIG 7 Subcellular distribution of $[^{14}C]$-gepotidacin, marker enzymes (lactate dehydrogenase [LDH], cytosol; cytochrome c-oxidase [CYTOX], mitochondria; N-acetyl-hexosaminidase [NAB], lysosomes) and bacteria in homogenates of THP-1 (left) or J774 (right), expressed as a percentage of the total recovered amount or activity. Cells were infected 1 h and returned to fresh medium for 2 h to allow complete internalization of bacteria, and exposed to 0.1 mg/L of $[^{14}C]$-gepotidacin for 30 min prior to collection. Homogenates were separated into 3 fractions by centrifugation at increasing centrifugal fields (for the main cytological content: nuclei and unbroken cells; organelles [mitochondria, lysosomes] and final supernatant). Data are the means of 2 experiments with similar results.
