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Activity of three β-lactams (ertapenem, meropenem and ampicillin) against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*

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***Objectives*: Assessment of the activity of three β-lactams [ertapenem (a carbapenem with a prolonged half-life), meropenem and ampicillin] against intraphagocytic *Listeria monocytogenes* and *Staphylococcus aureus*.**

***Methods*: Quantitative measurements of cfu changes in broth and in THP-1 macrophages (post-phagocytosis) over time (5 and 24 h) at concentrations spanning from sub-MICs to C_{max} (maximal concentration typically observed in patients' serum upon administration of conventional doses); morphological studies using an electron microscope; evaluation of drug stability (HPLC), protein binding (equilibrium dialysis) and measurement of drug cellular accumulation (microbiological assay).**

***Results*: Ertapenem was unable to control *L. monocytogenes* growth in THP-1 macrophages at all concentrations and times tested, even under conditions where ampicillin and meropenem were bactericidal. This behaviour could not be ascribed to drug instability, protein binding or lack of cell accumulation in comparison with ampicillin or meropenem. Ertapenem, ampicillin and meropenem were equally effective at reducing the post-phagocytosis inoculum of *S. aureus* (~1 log cfu), and caused conspicuous changes in the morphology of intracellular bacteria consistent with their lysis. These effects were obtained, however, only at large multiples (100-fold or more) of the MIC maintained over 24 h. Because of the high intrinsic antimicrobial potency of the β-lactams studied, these concentrations were below the C_{max}.**

***Conclusions*: Ertapenem will probably be ineffective against intraphagocytic forms of *L. monocytogenes* for reasons that remain to be discovered. Conversely, ertapenem could be an alternative to ampicillin and meropenem against intraphagocytic *S. aureus* since its longer half-life may allow high concentrations to be maintained for more prolonged times.**

Keywords: *L. monocytogenes*, *S. aureus*, THP-1 macrophages, ertapenem, ampicillin, meropenem

Introduction

Treatment of intracellular infections remains a medical challenge, mainly due to the inability of many antibiotics to penetrate and act in the intracellular milieu.[1,2] Infections caused by *Listeria monocytogenes* and *Staphylococcus aureus* are typical in this context since these organisms are difficult to eradicate even after sustained antibiotic therapy, probably because of the persistence of intracellular forms of these bacteria in both phagocytic and non-phagocytic cells.[3,4] Treatment of listeriosis is commonly undertaken with a combination of ampicillin and gentamicin,[5] but meropenem in monotherapy has been found active in experimental meningitis caused by *Listeria*.[6] Treatment of staphylococcal infections mainly relies on a β-lactam resistant to β-lactamase (at least for methicillin-susceptible organisms). In both types of infection, prolonged therapies are often needed to prevent recurrences and/or relapses in case of complicated infections, or in cancer or immunosuppressed patients.[7,8] Ertapenem is a β-lactamase-resistant β-lactam that shares many of the antimicrobial properties of meropenem with respect to

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Gram-positive organisms, but is characterized by a prolonged half-life allowing for a once-daily administration.[9,10] Ertapenem may therefore present a significant clinical advantage over meropenem and most other β-lactams for long-term therapies. As a consequence, we investigated the potential of ertapenem to act upon intraphagocytic *L. monocytogenes* and *S. aureus*. For this purpose, we used THP-1 cells, a model of human macrophages that has been validated for the study of *Listeria* intracellular infection,[11–13] and which we recently adapted for evaluation of the activity of antibiotics against intracellular *S. aureus.* Ertapenem was systematically compared with ampicillin and meropenem, which have both been found active against intracellular *L. monocytogenes* in these cells.[13]

Materials and methods

Bacterial strains, determination of MIC and MBC, and time and dose–kill studies in acellular media

L. monocytogenes. We used a haemolysin-producing strain EGD and followed exactly the methods described previously,[12] except that determination of MBCs used a 10^6 cfu/mL inoculum.

S. aureus. We used a non-β-lactamase-producing strain (ATCC 25923) following the methods described previously.[14]

Cells, cell infection and assessment of intracellular activities of antibiotics

THP-1 myelomonocytic cells[15] were used throughout our experiments. For *L. monocytogenes*, we followed a method described previously[12] with addition of gentamicin (1 mg/L; $\sim 1 \times$ MIC) for control cultures (no β-lactam added) if maintained for more than 5 h.[13] For *S. aureus*, we used an adaptation of the method described previously for J774 macrophages[14] taking into account that THP-1 cells spontaneously grow in suspension. Opsonization was performed with non-decomplemented, freshly thawed human serum diluted 1:10 in serum-free culture medium (RPMI 1640). Phagocytosis was performed at a 4:1 bacteria/macrophage ratio. Elimination of non-phagocytosed bacteria, and collection of cells at the end of the experiment were, therefore, made by centrifugation at room temperature [1300 rpm; 8 min; Eppendorf 5810R Centrifuge equipped with a A-4-62 rotor (Eppendorf Gerätgebau GmbH, Engeldorf, Germany)].

Determination of cellular carbapenem accumulation

Cells were collected and washed free from culture medium by three successive centrifugations in ice-cold phosphate-buffered saline (PBS), resuspended in distilled water and subjected to sonication (10 s at 50 watts; Labsonic L, Braun Biotech International GmbH, Melsungen, Germany). Ertapenem was then assayed by a microbiological method (to avoid the necessity of extraction, and thereby obtaining the needed sensitivity), using *Escherichia coli* as test organism and following the general procedure described previously[16] [lowest limit of detection; 0.25 mg/L for ertapenem and 1 mg/L for meropenem; typical linearity: up to 60–70 mg/L ($R^2 = 0.998$ for ertapenem and 0.989 for meropenem)]. Cell proteins were measured in parallel using the Folin–Ciocalteu/biuret method,[17] and the results used to compute the apparent cellular concentration of antibiotics based on a volume ratio of 5 μL of cell volume per mg protein, as in our previous publications dealing with THP-1 cells.[12,18]

Electron microscopy

Cells were infected as described above, except that the initial inoculum was increased to 20 bacteria per macrophage. This larger inoculum did not modify the intracellular activity of the β-lactams studied. Sample handling was then performed as described previously.[18]

Stability studies of carbapenems

Stability in culture media was evaluated by HPLC to provide us with positive identification of the molecules studied (in comparison with genuine standards) and higher reproducibility compared with bioassays. Assay was based on a published method,[19] but extraction was performed three times in succession with pooling of the upper phases in order to improve drug recovery [typical values: 82.3 ± 0.5% ($n = 3$) for ertapenem; 100.0 ± 0.1% for meropenem; a single extraction of ertapenem yielded only 16.6 ± 0.9% ($n = 3$) recovery]. Chromatography was made through a Lichrosphere 100 RP-18 column (25 cm × 4 mm, 5 μm; Merck AG, Darmstadt, Germany). Elution was made with acetonitrile/25 mM phosphate buffer pH 6.5 (v/v, 7:93) for both ertapenem and meropenem, with typical retention times of 8 and 5 min, respectively [linearity (for standards): 0.09–200 mg/L ($R^2 = 0.999$) and 0.19–200 mg/L ($R^2 = 0.999$)].

Protein binding studies

We used the equilibrium dialysis technique (cut-off, 6000–8000 mol. wt; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) with membranes soaked successively in three baths of water, and three baths of PBS for 15 min each. Preliminary studies showed that equilibration required a minimum of 32 h at 37°C under constant rotation at 8 rpm and without serum. The concentration of antibiotic in the serum-free compartment was measured by HPLC (see method above) at the end of the experiment and compared with the initial concentration to calculate the percentage of bound drug: 100−[(concentration in serum-free cell × 2)/(initial concentration)].

Materials

Ertapenem, meropenem and gentamicin were obtained as Invanz®, Meronem® and Geomycine®, respectively (i.e. the registered commercial products for parenteral administration in Belgium), and supplied by Merck Sharp & Dohme BV (Haarlem, The Netherlands), Astra Pharmaceutical (Brussels, Belgium) and GlaxoSmithKline s.a. [Rixensart, Belgium; on behalf of Schering-Plough Belgium (Brussels, Belgium)]. Ampicillin was purchased as the sodium salt from Sigma–Aldrich (St Louis, MO, USA), and cell culture media and serum were obtained from Gibco Biocult (Paisley, UK). All other reagents were obtained from Merck AG or from Sigma–Aldrich.

Statistical analyses

Curve fitting analyses were made with GraphPad Prism® software (version 4.0) and group comparisons (Student's *t*-test, one-way analyses of variance) with Instat Prism® software (version 3.01), both from GraphPad Prism® software, San Diego, CA, USA.

Table 1. MICs and MBCs of antibiotics for the strains of *L. monocytogenes* and *S. aureus* used in this study

Organism	Antibiotic	pH	MIC (mg/L)[a]	MBC (mg/L)[b]
L. monocytogenes	ampicillin	7.4	0.32 ± 0.10	> 64
	meropenem	7.4	0.05 ± 0.00	> 64
	ertapenem	7.4	0.48 ± 0.03	> 64
S. aureus	ampicillin	5.5	0.03 ± 0.01	
		7.4	0.07 ± 0.01	0.125
	meropenem	5.5	0.06 ± 0.02	
		7.4	0.15 ± 0.01	0.25
	ertapenem	5.5	0.06 ± 0.00	
		7.4	0.11 ± 0.01	0.25

[a]Arithmetic dilutions ($n = 3$).
[b]Geometric dilutions (typical results).

Results

MICs and MBCs

Table 1 shows the MICs and MBCs observed in the present study. With respect to *L. monocytogenes*, ertapenem showed an MIC similar to ampicillin and was thus ~10-fold less potent than meropenem. All three β-lactams were bacteriostatic towards *L. monocytogenes*. For *S. aureus*, MICs were determined both at pH 7.4 and pH 5.5 to mimic the conditions prevailing in the extracellular milieu and in phagolysosomes, respectively. All three β-lactams had low MICs with no significant difference between them, and with slightly lower values at pH 5.5. MBCs were about two-fold larger than the MICs demonstrating the bactericidal activity of these β-lactams against the strain of *S. aureus* used.

Time–kill studies at increasing concentrations

In the first series of experiments, the influence of the incubation time on the activity of antibiotics was evaluated at increasing fractional concentrations observed in the serum of patients receiving conventional doses of the antibiotics tested. The concentrations were chosen as guided by the C_{max} reported for the respective antibiotics in the literature (50, 50 and 155 mg/L for ampicillin, meropenem and ertapenem, respectively[9,20,21]) and using two incubation time periods (5 and 24 h). Results are shown in Figure 1 in a synoptic fashion comparing the activities seen in broth (mimicking the situation of extracellular bacteria) with those in cells (phagocytosed bacteria) for all three antibiotics and for both types of bacteria. Considering the results globally, two main aspects were striking. First, it appears that most of the antibacterial effects (based on the decrease in cfu) were already obtained at the lowest concentration tested [for meropenem towards phagocytosed *L. monocytogenes*, however, a gain of ~1 log cfu decrease could be obtained at 24 h when increasing the concentration from the lowest to the highest value tested (5–50 mg/L)]. Secondly, all effects were also time-dependent, with significant gains of activity in all conditions when incubation was prolonged from 5 to 24 h (the effect of ertapenem, however, was only to slow down bacterial growth). Concentrating now on *L. monocytogenes*, we see that the extracellular activity of all three β-lactams was essentially bacteriostatic at 5 h, and that only a modest decrease in cfu (0.5–0.7 log units) was obtained at 24 h at all concentrations tested. Examination of the results obtained for intracellular activity revealed: (i) that intracellular bacterial growth was essentially similar to that in broth; (ii) that meropenem and ampicillin were bacteriostatic at 5 h at all concentrations tested, but achieved a bactericidal effect at 24 h, as previously described;[13] and (iii) that, in sharp contrast, ertapenem was unable to decrease the post-phagocytosis inoculum, with bacteria actually growing at a multiplication rate about half of that of controls at all concentrations tested. Moving now to *S. aureus*, we first see that all three β-lactams exerted a marked bactericidal effect towards bacteria in broth (~5 log decrease in 24 h) without significant differences between them. Intracellular growth of *S. aureus* in control cells was first delayed, but eventually proceeded at a rate that allowed to reach in 24 h an increase over the original inoculum of about two-thirds of what was seen in broth (as already reported for J774 macrophages[14]). All three β-lactams were able to significantly reduce the number of cell-associated cfu, but this reduction (0.7–1.2 log units) was much less pronounced than for bacteria in broth. No marked difference was seen between the three drugs.

Morphological studies

Because the results shown in Figure 1 suggested that ertapenem was unable to block the intracellular growth of *L. monocytogenes* while being active against intracellular *S. aureus*, we performed electron microscopic studies to directly examine the morphology of the intracellular bacteria after 24 h of incubation in control cells and in cells incubated with this antibiotic. Figure 2 (a–d) shows that both control cells and ertapenem-treated cells contained an abundance of intact *L. monocytogenes* in various stages of phagocytosis (Figure 2a), division (Figure 2b), and clearly present in the cytosol while being surrounded with actin (Figure 2b–d). No apparent differences were seen between control and ertapenem-treated cells. In contrast, *L. monocytogenes*-infected cells that had been incubated with ampicillin or meropenem for 24 h contained no recognizable *Listeria* apart from a few structures that looked like highly damaged bacterial cells (not shown). Cells infected with *S. aureus* and left without antibiotic showed clearly recognizable bacteria, sometimes in a process of division, in what appeared like phagocytic vacuoles (Figure 2e). In contrast to what had been seen with *L. monocytogenes*, the morphology of *S. aureus* was clearly altered in ertapenem-treated cells (Figure 2f–h), where most sections showed bacterial ghosts (Figure 2f and h), bacterial bodies with evidence of partial loss of electron-dense material (Figure 2g), together with apparently intact bacteria (Figure 2f). Similar images were obtained for meropenem- and ampicillin-treated cells (not shown).

Concentration–kill studies

In order to get more insight into the contrasting behaviour of ertapenem towards intracellular *L. monocytogenes* and *S. aureus* on the one hand, and to better analyse the differences in activity seen with ampicillin and meropenem on the other hand, we systematically compared intracellular and extracellular activities

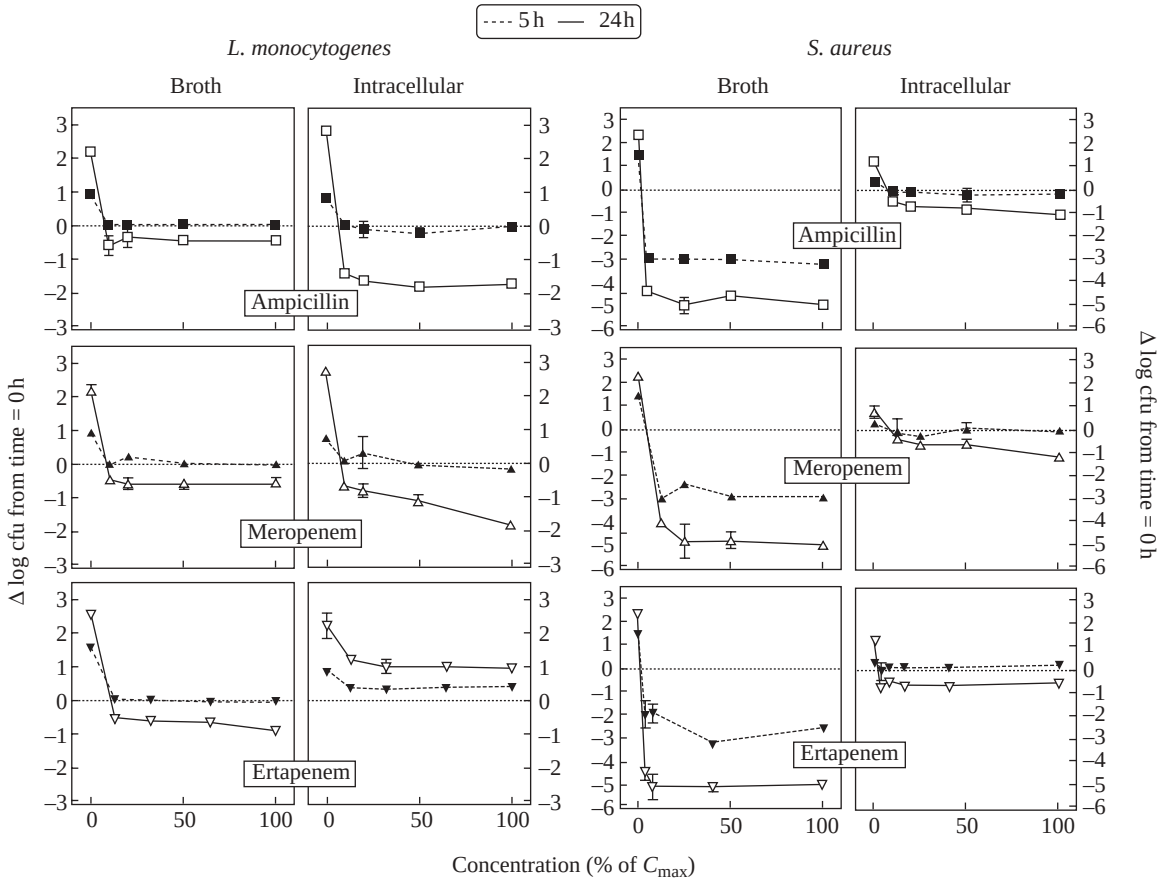

Figure 1. Concentration–killing effects of ampicillin (squares), meropenem (triangles) and ertapenem (inverted triangles) towards *L. monocytogenes* (left-hand panels) and *S. aureus* (right-hand panels) in broth (first and third columns of panels, taken from left to right), or after phagocytosis by THP-1 macrophages (second and fourth columns of panels, taken from left to right). Incubation with antibiotics was for 5 h (closed symbols; broken lines) or 24 h (open symbols, continuous lines) at the concentrations indicated in the abscissa, which are expressed as fractions of the maximal serum concentration (C_{max}) observed in humans after conventional administration of these drugs.[9,20,21] Note that the lowest concentrations tested are always far above the MIC for the corresponding organism (for *L. monocytogenes*, MICs of ampicillin, meropenem and ertapenem are 0.64, 0.1 and 0.31% of C_{max}, respectively; for *S. aureus*, MICs of ampicillin, meropenem and ertapenem are 0.06, 0.12 and 0.04% of C_{max}). All values are means ± SD of three independent determinations (SD bars that are not visible are smaller than the size of the symbols).

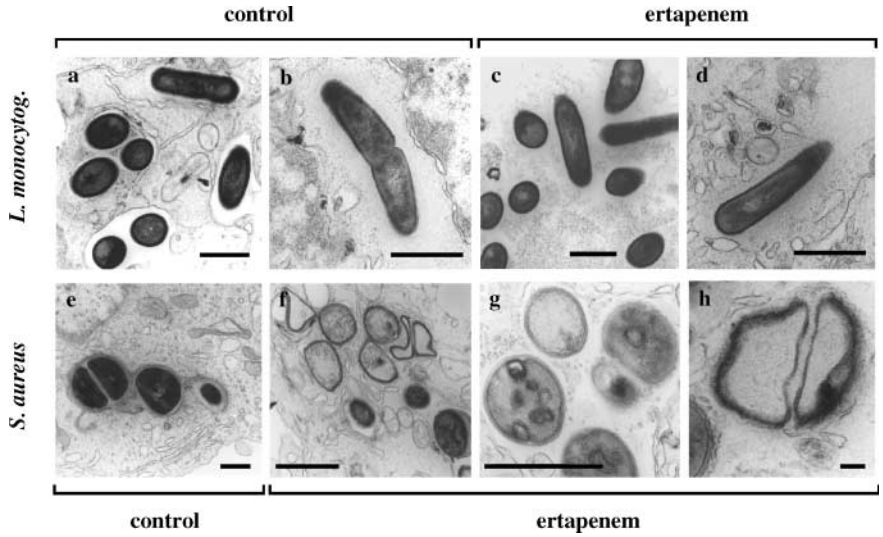

Figure 2. Morphological appearance of *L. monocytogenes* (a–d) and *S. aureus* (e–h) in cells 24 h after infection. Control (a, b and e): cells were incubated with gentamicin (1 × MIC) to prevent the extracellular growth of bacteria and the ensuing cell death due to acidification of the medium; this did not impair the intracellular growth of bacteria.[13,14] Ertapenem (c, d and f–h): cells were incubated in the presence of ertapenem (155 mg/L). Scale bars are 0.5 μm.

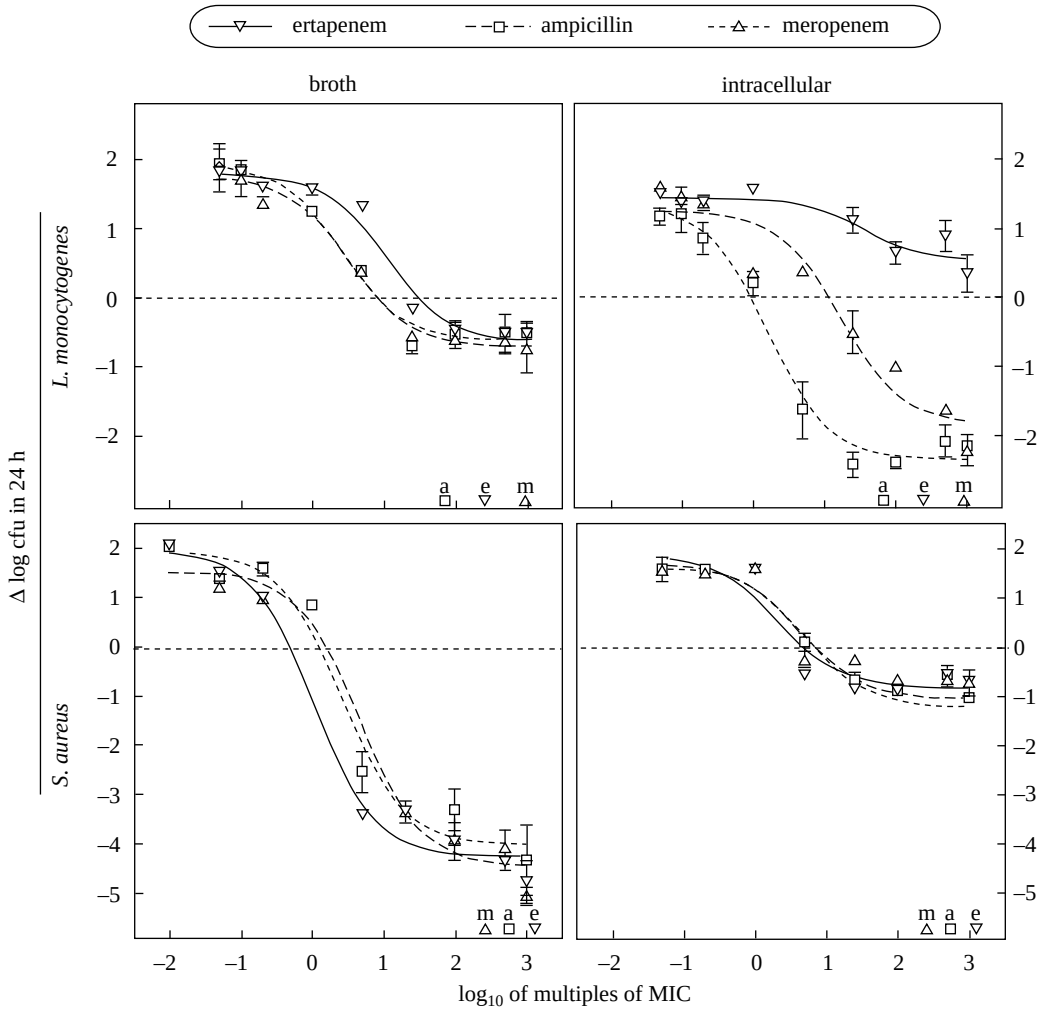

Figure 3. Concentration–killing curves of ampicillin (squares), meropenem (triangles) and ertapenem (inverted triangles) towards *L. monocytogenes* (upper panels) and *S. aureus* (lower panels) in broth (left-hand panels) or in THP-1 macrophages (right-hand panels). The abscissas show the initial concentrations of the antibiotics in multiples of their MIC in broth (measured at pH 7.4 for *L. monocytogenes*; for *S. aureus*, we used the values measured at pH 5.5 for the left-hand panel, and those measured at pH 7.4 for the right-hand panel; see Table 1 for details). Note that the concentration range spans from sub-MIC to concentrations exceeding the MIC by several orders of magnitude. The symbols and letters (e, ertapenem; a, ampicillin; m, meropenem) close to the abscissas in each panel are located at, and indicate the multiples of MIC corresponding to the maximal concentration tested chosen as guided by the C_{max} reported for the respective antibiotics in the literature (50, 50 and 155 mg/L for ampicillin, meropenem and ertapenem, respectively[9,20,21]). The ordinates show the changes in cfu ($\log_{10}$) per mL of broth (left-hand panels) or per mg of cell protein (right-hand panels) as observed after 24 h of incubation in comparison with the original inocula (horizontal broken lines). All values are means ± SD ($n = 3$). Sigmoidal functions were fitted to the data after logarithmic transformation [goodness of fits (R^2): *L. monocytogenes* in broth: ertapenem 0.980, ampicillin 0.982, meropenem 0.987; *L. monocytogenes* in cells: ertapenem 0.858, ampicillin 0.985, meropenem 0.928; *S. aureus* in broth: ertapenem 0986, ampicillin 0.970, meropenem 0.980; *S. aureus* in cells: ertapenem 0.900, ampicillin 0.889, meropenem 0.834].

over a wider range of extracellular concentrations than in the experiments reported in Figure 1. For this purpose, bacteria or cells were exposed to a range of concentrations from sub-MIC to $\sim 1000 \times$ MIC values, allowing for a direct pharmacological comparison between drugs. Results are shown in Figure 3 with activity expressed as a function of multiples of the MIC. This allows for direct comparisons at equipotent concentrations. Considering *L. monocytogenes* first, all three β-lactams showed a concentration-dependent activity in broth in a range of one- to 100-fold their MICs. The bacterial responses to meropenem and ampicillin were undistinguishable whereas ertapenem was about four-fold less potent in that range. For intracellular bacteria, both meropenem and ampicillin showed a concentration-dependent activity that reached a bactericidal effect (~ 2 log decrease in cfu) at high concentrations. Ampicillin was systematically ~ 10-fold more potent than meropenem in the $1-100 \times$ MIC range. In contrast, bacterial growth was seen for ertapenem at all concentrations tested, and the function that could be fitted to the data suggested that ertapenem would be unable to achieve even a bacteriostatic effect whatever its extracellular concentration. Moving now to *S. aureus*, the data show: (i) that a bactericidal effect was obtained against bacteria in broth from low multiples of MIC with four-fold higher potency for ertapenem compared with the two other β-lactams; (ii) that intracellular

activity was, like in broth, concentration-dependent; (iii) but that the reduction of the inoculum was quite limited (~1 log only) in all cases, with no meaningful difference in bacterial response between the three antibiotics.

Stability and protein binding of carbapenems and influence of variation of the serum concentration on activity of ertapenem against L. monocytogenes

The surprising results observed with ertapenem towards intracellular *L. monocytogenes* prompted us to run a series of controls to check for an artefactual cause. We first examined whether the drug would remain sufficiently stable in the culture medium, based on previous experience with meropenem and imipenem that showed a fast degradation of both drugs when incubated as concentrated solutions in aqueous media at 37°C.[22] During incubation in complete culture medium, ertapenem degradation (original concentration 155 mg/L) proceeded according to zero-order kinetics, with ~62% of drug remaining intact after 24 h. For meropenem (original concentration 50 mg/L), degradation reached ~50% over the same period. We also tested whether the high protein binding of ertapenem reported in human serum[23] would play a critical role here. Equilibrium dialysis experiments, showed that, indeed, only 32.1 ± 0.6% ($n = 3$) of total ertapenem was free in complete culture medium (10% fetal calf serum). Reducing the serum concentration to 2% increased this value to 42.6 ± 2.5% ($n = 3$). However, this did not improve the activity against intracellular *L. monocytogenes* [changes in post-phagocytosis inoculum (log cfu) of 0.63 ± 0.09 versus 0.70 ± 0.09 at 5 h and of 1.83 ± 0.12 versus 1.77 ± 0.19 at 24 h for cells incubated with 2% and 10% serum, respectively].

Cellular accumulation of ertapenem

L. monocytogenes-infected and non-infected cells were collected after 5 h and 24 h of incubation with ertapenem (155 mg/L) and subjected to bioassay. The apparent cell antibiotic concentrations (in mg per litre of cell volume) were 83.1 ± 7.1 and 68.6 ± 1.5 at 5 h, and 27.2 ± 9.3 and 37.1 ± 12.2 at 24 h for infected and uninfected cells, respectively.

Discussion

The present study is a pharmacological comparison of the activity of three β-lactams against two kinds of extracellular and intracellular bacteria with different susceptibilities in broth and distinct subcellular localizations in macrophages. Three main limitations prevent extrapolating its results to the *in vivo* situation without caution, namely that: (i) THP-1 cells display only poor intrinsic defences against intracellular infection; (ii) other cells than macrophages may be invaded by the organisms studied; and (iii) only laboratory strains were used. Given these caveats, we, nevertheless, may draw a series of general and specific conclusions as far as antibiotic activity per se is concerned towards both extracellular and intracellular organisms.

Upon testing for activity over a wide range of concentrations covering values below and above the MIC (i.e. when using a pharmacologically oriented design), all three antibiotics showed clear-cut concentration-related effects towards both extracellular and intracellular bacteria. This may come as a surprise, since β-lactams have for long been ranked as concentration-independent antibiotics.[24] However, we show here that the antibacterial concentration effect actually follows a sigmoidal function. Because the MICs of the β-lactams studied here are quite low with respect to the organisms tested, almost maximal effects can be observed at antibiotic concentrations corresponding to low (4–10%) fractional amounts of the maximal concentration tested (chosen based on an estimation of the C_{max} in patients). The clinical implication could be that β-lactam therapy will be optimal, with respect to both extracellular and intracellular bacteria, only if the serum concentration remains at values exceeding the MIC for the whole period of observation (time becoming then the predominant parameter governing activity). Conversely, β-lactams will be expected to show concentration-dependent effects if their concentration falls closer to the MIC, which will be the case *in vivo* upon under-dosing or with organisms with high MIC values.

Quite surprisingly, ertapenem, which shares many of the properties of meropenem against Gram-positive organisms,[9] was inefficient against the intraphagocytic forms of *L. monocytogenes* under conditions in which ampicillin and meropenem are active (as demonstrated here and in our previous publications[12,13]). This could not be ascribed to drug instability (in comparison with meropenem) or lack of cell penetration [the concentrations of ertapenem being higher than those of meropenem or of ampicillin (at extracellular concentrations of 50 mg/L as used here)[12]]. Ertapenem is known to be highly protein bound.[10] While binding to serum proteins present in the culture fluid is probably unimportant in our model (decreasing the protein concentration did not improve the intracellular activity of ertapenem), the situation may be different within the cell where phagocytosed *L. monocytogenes* becomes surrounded by a thick layer of actin.[18]

In contrast, all three β-lactams were active against intracellular *S. aureus*. The morphological studies are of particular interest, since they revealed images of bacterial ghosts quite similar to those seen with extracellular *S. aureus* exposed to penicillin[25] or faropenem,[26] which are directly related to the binding of the β-lactams to their targets. β-Lactams are not expected to accumulate in cells, owing to the presence of a free carboxyl function on all these molecules.[1,2,27] Yet, we observe here that the cellular concentration of ertapenem can reach values far above the MIC for *S. aureus*. When phagocytosed by macrophages, this bacterium is, however, not free in the cytosol but is primarily located in phagolysosomes and related vacuoles,[28] which are acidic,[29] and where the concentration of β-lactams is expected to be much lower.[27] This may explain why many reports have pointed to the apparent inability of β-lactams to act against intraphagosomal organisms in general,[1,2,30] and *S. aureus* in particular.[31,32] In this context, it must be emphasized that a significant reduction in the intracellular inoculum was obtained here only when (i) the extracellular concentration markedly exceeded the MIC for the offending organism (and was actually close to the maximal concentration tested), and (ii) the incubation time was brought to 24 h. This suggests that large extracellular concentrations and prolonged exposure may compensate for the lack of cellular accumulation, as previously proposed based on studies with methicillin,[33] especially since acidic pH does not adversely affect activity. We also cannot exclude the possibility that the activity of β-lactams towards phagosomal organisms is enhanced by cellular factors, as previously suggested.[34,35] However, we have to stress the fact that

eradication of *S. aureus* remained quite limited in our model, as was also noted for fluoroquinolones in similar models.[14,36] Translated to the *in vivo* situation, this may have important consequences in terms of recurrence of the infection and emergence of resistance. These points will need to be specifically addressed in future studies, the aim of which should be to establish what is the true impact or the limitations of the present findings.

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