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Intracellular accumulation and activity of ampicillin used as free drug and as its phthalimidomethyl or pivaloyloxymethyl ester (pivampicillin) against *Listeria monocytogenes* in J774 macrophages

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***Aims*: To determine the intracellular accumulation in a macrophage cell line of ampicillin and ampicillin esters, and to measure their activity against intracellular *Listeria monocytogenes*.**

***Methods*: Quantitative evaluation of the activity of ampicillin, phthalimidomethylampicillin (PIMA) or pivaloyloxymethylampicillin (PIVA) against intracellular *L. monocytogenes*, and direct measurement of cellular ampicillin concentration in J774 macrophages.**

***Results*: Ampicillin, PIMA and PIVA caused a 0.5 log decrease in cell-associated cfu within 5 h when used at an extracellular concentration of 3.6 μM [10 × MIC of ampicillin (1.25 mg/L); 1.83 mg/L for PIMA and 1.67 mg/L for PIVA]. Addition of β-lactamase in the extracellular milieu abolished the activity of ampicillin and of PIMA but not that of PIVA. At low extracellular concentrations [0.5 × MIC ampicillin (62.5 μg/L); equimolar concentrations for PIMA (91.5 μg/L) and PIVA (83.5 μg/L)], ampicillin and PIMA lost all activity (compared with controls), but PIVA remained as active as at the higher concentration. Incubation of cells with PIVA at the low concentration (83.5 μg/L) for 20 h caused a 2 log reduction of cfu if the medium was changed every 5 h (to compensate for the degradation of extracellular PIVA). Incubation of cells with PIVA allowed for a marked (four- to 25-fold) cell accumulation of ampicillin, whereas no ampicillin accumulation was seen for cells incubated with ampicillin or with PIMA.**

***Conclusions*: This is the first demonstration that PIVA (a prodrug of ampicillin) can be used to promote ampicillin cellular accumulation and, thereby to increase ampicillin intracellular activity. PIVA could be useful for control of the intracellular multiplication of *L. monocytogenes*.**

Keywords: prodrugs, β-lactams, β-lactamases, infections

Introduction

β-Lactam antibiotics are of considerable value in the chemotherapy of bacterial infections because of their potency, broad spectrum of activity and low incidence of adverse reactions. Activity against intracellular bacteria, however, has always been perceived as a weakness of all members of this class of antimicrobials, based on the observation of their lack of accumulation in cells and in tissues.[1,2] Previous studies from our laboratory have shown that a basic derivative of penicillin G [*N*-(3-dimethylamino-propyl)benzylpenicillinamide], obtained by substitution of its free carboxyl group, accumulates in macrophages.[3] This behaviour was actually predicted based on general considerations of the distributions of weak organic acids and bases across biological membranes, which predict that weak organic bases will concentrate in acid membrane-bounded compartments such as cells,[4] and within cells, lysosomes and other acidic organelles.[5] Conversely, weak organic acids will be excluded from the same compartments. β-Lactams behave like weak organic acids in this context because they all display a free carboxyl group (or a corresponding proton donor), which is essential for their activity.[6] This means that basic derivatives of β-lactams made by substitution of this acid function to promote their intracellular accumulation must regenerate the free antibiotic intracellularly to be of chemotherapeutic value. Phthalimidomethylampicillin (PIMA) is an example of a novel simple ester of ampicillin tailored for this purpose,[7] since it is

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basic and regenerates free ampicillin *in vitro*.[8] Pivaloyloxymethylampicillin (PIVA; pivampicillin) is a double ester of ampicillin[9] that was originally manufactured to improve the oral absorption of ampicillin,[10] but which also liberates free ampicillin *in vitro*.[8] Both PIMA and PIVA are basic and were therefore expected to accumulate in cells.[5] Experimental studies have shown that PIMA and PIVA are indeed avidly taken up by macrophages.[7] However, more detailed studies concluded that these esters do not truly penetrate cells, but remain largely associated with the pericellular membrane.[11] In the present study, we examined whether PIMA and PIVA could nevertheless deliver ampicillin into cells. We selected a model of *Listeria monocytogenes*-infected macrophages, based on the following considerations: (i) *L. monocytogenes* easily invades macrophages where it actively multiplies in the cytosol,[12] which is the cell compartment to which β-lactams have spontaneous access but in which they do not accumulate;[1] (ii) intracellular *L. monocytogenes* is susceptible to ampicillin, although an effective control of its growth can only be obtained when it is used at an extracellular concentration greater than its MIC to compensate for its lack of accumulation.[13] We therefore compared the activities of PIMA, PIVA and free ampicillin against intracellular *L. monocytogenes* at both high and low concentrations. In parallel, we directly examined whether incubation of macrophages with PIMA and PIVA would allow higher intracellular ampicillin concentrations than can be obtained with the free drug.

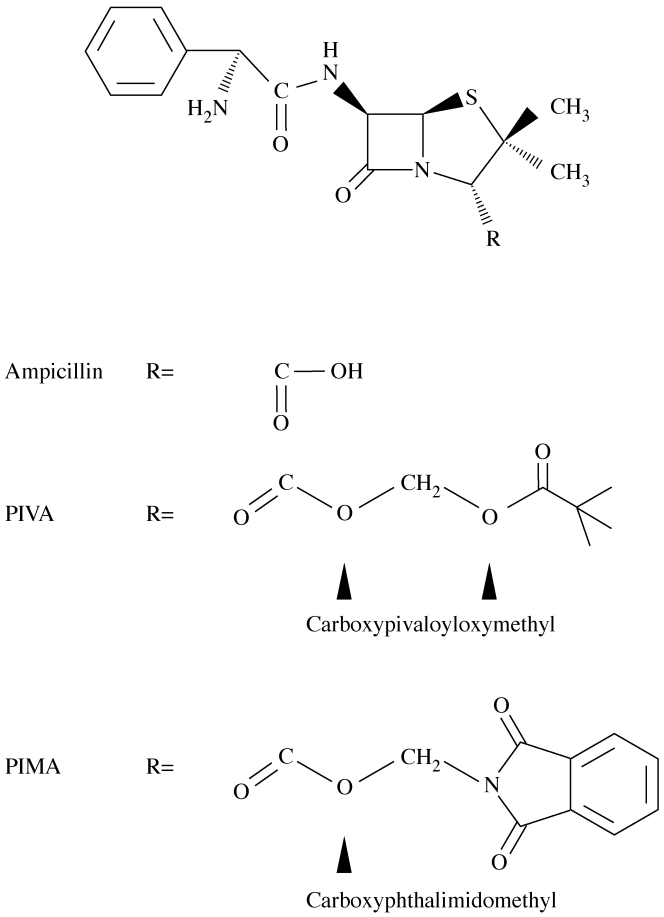

Figure 1. Structural formulae of ampicillin, PIVA and PIMA. Ampicillin possesses a free carboxyl group (R), which is substituted in PIVA and PIMA (the arrowheads in the corresponding substituents indicate the ester bonds; note that PIVA is a double ester).

Materials and methods

Ester prodrugs of ampicillin

Figure 1 shows the structural formulae of ampicillin and of its pivaloyloxymethyl (PIVA) and phthalimidomethyl (PIMA) esters. The synthesis of both esters has been described.[8,9] Their stability in buffered media (pH 7.4) was found to be comparable (half-lives of 92 and 62 min, respectively, at 37°C), with a release of both free ampicillin and degradation products in a molar ratio of 68:32 and 64:36, respectively[8] (as was previously reported for pivampicillin[14]).

Preparation of the products for experiments

Because of intrinsic instability, PIMA and PIVA were kept in their dry state at 4°C. For each experiment, an aliquot was weighed, dissolved in 100% ethanol, and diluted in ice-cold 20 mM sodium acetate buffer (pH 5.4) to prevent degradation. This solution was further diluted in culture medium to the desired final concentration, and used immediately. Ampicillin was prepared as a fresh solution in water for each series of experiments and used within a few hours of suitable dilution.

Bacteria and MIC determination

We used a haemolysin-producing strain of *L. monocytogenes* (serotype 1/2a) obtained, maintained and characterized as described previously.[15] The MICs of ampicillin and gentamicin, as determined in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) by geometric dilution and using standard methods, were 0.125 and 1 mg/L, respectively.

Cell culture, infection and intracellular activities of antibiotics

All experiments were performed with J774 macrophages (a murine continuous reticulosarcoma cell line), which were cultured and maintained as described previously.[3] In brief, cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine, seeded in 6-well dishes at a density of 5×10^4 cells/cm^2 and used upon confluence (2 days of culture). Infection with *L. monocytogenes* was performed as described recently,[16] based on a method previously developed for THP-1 macrophages.[13,15] In brief, infection was obtained with untreated bacteria (at an initial bacteria to macrophage ratio of 5) maintained in contact with cells for 1 h at 37°C. Extracellular bacteria were removed by extensive washing; cells were then returned to fresh medium, and the intracellular growth evaluated after increasing periods of incubation at 37°C. For all incubation periods of more than 5 h, the medium contained gentamicin 2 mg/L (2 × MIC) to prevent the extracellular growth of *Listeria*.[17] For collection, cells were washed with ice-cold PBS, scraped off the culture dish into distilled sterile water with a Teflon spatula, and subjected to vigorous mechanical mixing to achieve homogeneity. Lysates were then used for total cell protein measurement,[18] and, in parallel, for cfu counting by plating on tryptic soy agar after appropriate dilution. Colonies were counted using a Gel Doc 2000 apparatus (Bio-Rad, Hercules, CA, USA) operated with Quantity One software (Bio-Rad). We checked that the carried-over antibiotic (i.e. the amount of ampicillin or gentamicin brought into the dishes with the cell samples) was too low to interfere significantly with *L. monocytogenes* growth in this assay.

Assay of ampicillin

Ampicillin in cell culture media was assayed by high-performance liquid chromatography using the method described previously for PIMA and PIVA,[11] but with a modified mobile phase (acetonitrile:10 mM acetate buffer pH 5, 1:9). Cell-associated ampicillin was assayed using a specific

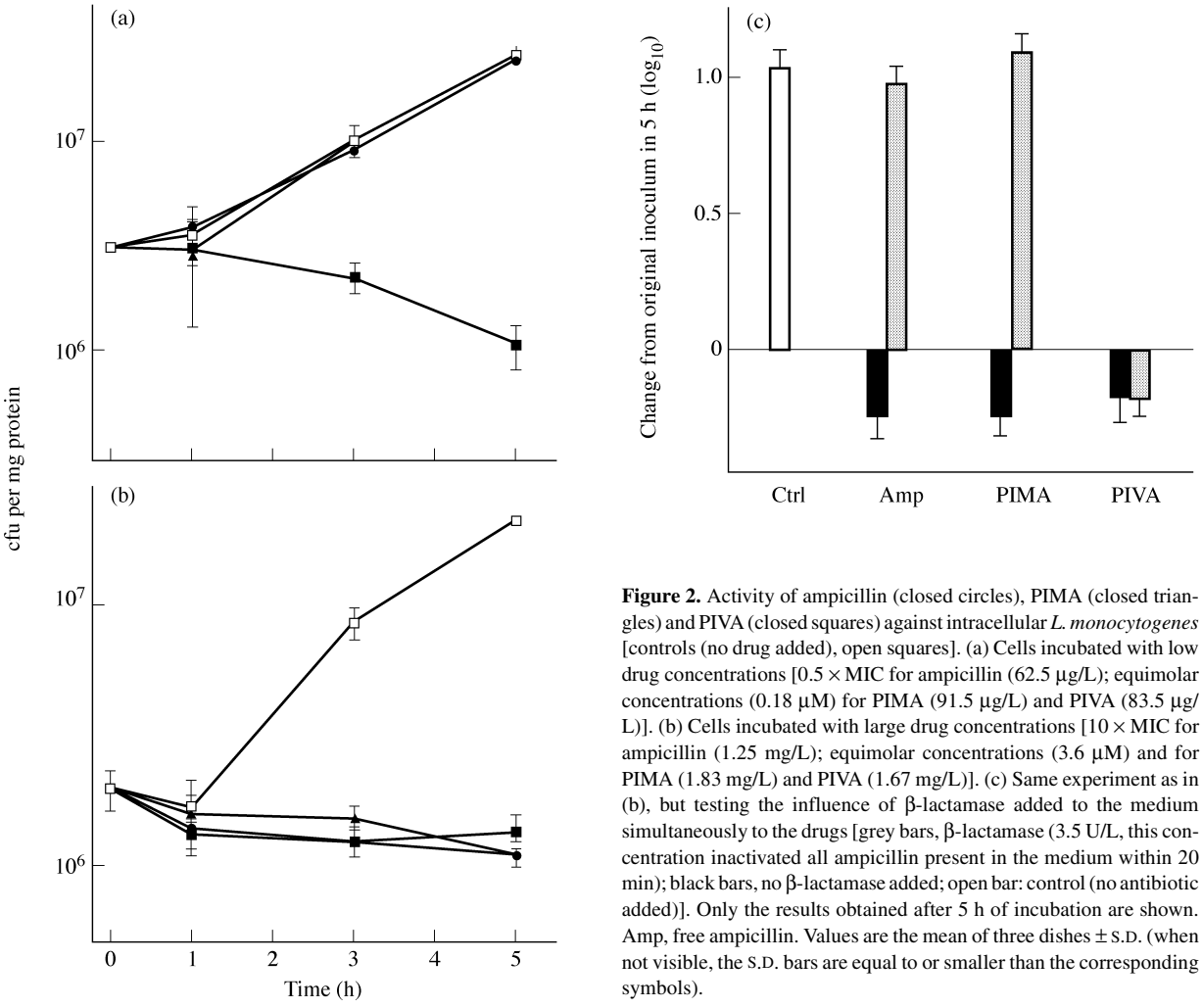

Figure 2. Activity of ampicillin (closed circles), PIMA (closed triangles) and PIVA (closed squares) against intracellular *L. monocytogenes* [controls (no drug added), open squares]. (a) Cells incubated with low drug concentrations [0.5 × MIC for ampicillin (62.5 μg/L); equimolar concentrations (0.18 μM) for PIMA (91.5 μg/L) and PIVA (83.5 μg/L)]. (b) Cells incubated with large drug concentrations [10 × MIC for ampicillin (1.25 mg/L); equimolar concentrations (3.6 μM) and for PIMA (1.83 mg/L) and PIVA (1.67 mg/L)]. (c) Same experiment as in (b), but testing the influence of β-lactamase added to the medium simultaneously to the drugs [grey bars, β-lactamase (3.5 U/L, this concentration inactivated all ampicillin present in the medium within 20 min); black bars, no β-lactamase added; open bar: control (no antibiotic added)]. Only the results obtained after 5 h of incubation are shown. Amp, free ampicillin. Values are the mean of three dishes ± S.D. (when not visible, the S.D. bars are equal to or smaller than the corresponding symbols).

and sensitive enzymatic method based on the ability of β-lactams to bind to and irreversibly inhibit the bacterial DD-carboxypeptidase.[19] In brief, the activity of the enzyme (purified from *Streptomyces* R39) was measured by the release of free D-Ala from *N*,*N*-diacetyl-L-Lys-D-Ala-D-Ala [lowest limit of detection of ampicillin 20 ng/mL; linearity up to 100 ng/mL (R^2 =0.9931)].

Calculation of the apparent cellular concentration of ampicillin

The total cellular ampicillin content was systematically expressed by reference to the protein content of the corresponding samples. The apparent cellular ampicillin concentration was then calculated using a conversion factor of 5 μL of cell volume per milligram of cell protein, as in our previous publications dealing with the cellular accumulation of other antibiotics or drugs.[3,20] We refer to this concentration as an apparent one, since we do not know, from the present experiments, where ampicillin is located within the cell. Previous studies using penicillin G, however, have shown that free β-lactams are distributed in the cytosol.[3]

Materials

PIMA was obtained as the chloride salt with a purity of >95%.[8] PIVA [99.5% purity and complying with the specifications of the *Pharmacopée Européenne*[21] was obtained from Leo Laboratories Ltd (Dublin, Ireland) on behalf of Leo Pharmaceuticals Product Ltd A/S (Ballerup, Denmark). The *N*,*N*-diacetyl-L-Lys-D-Ala-D-Ala (the substrate of the DD-carboxypeptidase) and the purified *Streptomyces* R39 DD-carboxypeptidase were kindly prepared and donated by J.-M. Frère (Centre d'Ingénierie des Protéines, Université de Liège, Liége, Belgium). β-Lactamase [from *Enterobacter cloacae* (0.3 U/mg protein based on benzylpenicillin degradation)] and ampicillin (sodium salt) were purchased from Sigma-Aldrich (St Louis, MO, USA), cell culture media and sera from Life Technologies (Paisley, UK), and all other reagents from E. Merck (Darmstadt, Germany).

Results

Activities of ampicillin, PIMA and PIVA at low and high concentrations, and influence of extracellular β-lactamase

In a series of initial experiments, we examined and compared the activities of free ampicillin, PIMA and PIVA over a 5 h period using a suboptimal concentration of ampicillin (0.5 × MIC in broth; 62.5 μg/L) and equimolar concentrations of PIMA and PIVA [0.18 μM; 91.5 and 83.5 μg/L]. The results are shown in Figure 2(a). As anticipated, ampicillin was inactive compared with controls (no antibiotic added). PIMA did not show any activity either, but PIVA caused a

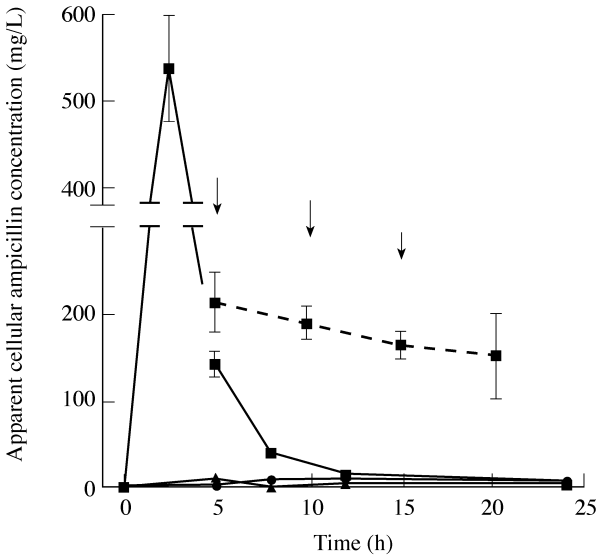

Figure 3. Cellular accumulation of ampicillin in cells incubated with free ampicillin (17.4 mg/L; closed circles) or equimolar concentrations of PIMA (25.4 mg/L; closed triangles) or PIVA (23.2 mg/L; closed squares). For PIVA, the continuous line refers to cultures for which PIVA was added to medium at the beginning of the experiment only; the dotted line refers to cultures to which the medium was changed every 5 h (arrows) by a new, freshly prepared one to reach, upon each addition, a concentration of 23.2 mg/L of PIVA. Values are the mean of three dishes ± S.D. (when not visible, the S.D. bars are equal to or smaller than the corresponding symbols).

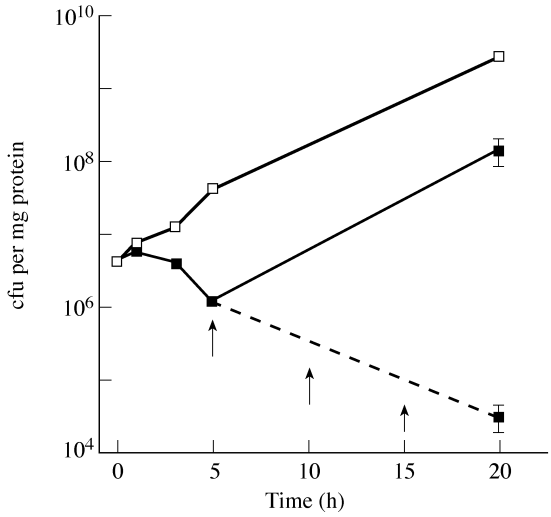

Figure 4. Activity of PIVA (83.5 μg/L; closed squares) against intracellular *L. monocytogenes*. The continuous line refer to cultures for which PIVA was added to medium at the beginning of the experiment only; the dotted line refers to cultures to which the medium was changed every 5 h (arrows) by a new, freshly prepared one to reach, upon each addition, a concentration of 83.5 μg/L of PIVA. Open squares, no drug added. Values are the mean of three dishes ± S.D. (when not visible, the S.D. bars are equal to or smaller than the corresponding symbol).

reduction in cell-associated cfu (0.5 log) that was essentially similar to what we described earlier for *L. monocytogenes*-infected THP-1 macrophages incubated with 50 mg/L ampicillin.[13] Figure 2(b) shows that increasing the extracellular concentration of ampicillin to 10 × MIC, and that of PIMA and PIVA to equimolar concentrations, allowed control of intracellular *L. monocytogenes* growth, with no meaningful difference between each of the three compounds. Since both PIMA and PIVA can regenerate free ampicillin extracellularly, we examined whether inactivating the extracellular drug would modify these results. As shown in Figure 2(c), addition of β-lactamase in the culture medium caused a complete loss of activity of ampicillin and PIMA. In contrast, the activity of PIVA towards intracellular *L. monocytogenes* remained essentially unchanged.

Accumulation of ampicillin in cells incubated with ampicillin, PIMA and PIVA

The extracellular concentration of ampicillin had to be increased to 17.4 mg/L (50 μM) in order to reliably detect ampicillin in cells, and PIMA and PIVA were therefore studied in comparison with ampicillin at an equimolar concentration (PIMA 25.4 mg/L; PIVA 23.2 mg/L). Figure 3 shows that cells incubated with free ampicillin or PIMA accumulated very little ampicillin (the cellular:extracellular ratio of ampicillin remaining close to 1 for up to 24 h in cells incubated with free ampicillin, and with similarly low amounts of ampicillin in cells incubated with PIMA). In sharp contrast, cells incubated with PIVA showed a rapid and extensive accumulation of ampicillin (reaching an apparent cellular concentration of ~500 mg/L (this would correspond to a cellular to extracellular concentration ratio of at least 20:1 if it is assumed that all extracellular PIVA has been quantitatively transformed into free ampicillin). This accumulation was, however, transient, since the cellular ampicillin concentration quickly decreased with a half-life of ~2.5 h. Since the half-life of ampicillin in aqueous media at pH 7.4 (presumably the pH of the cell cytosol) is long (>48 h)[8] and is not reduced by cell extracts (unpublished results), we reasoned that this sharp decrease in the cellular ampicillin concentration could only be due to the fast degradation of extracellular PIVA. This would indeed cause a rapid decrease in the amount of membrane-bound PIVA,[11] thereby decreasing the rate of delivery of ampicillin from PIVA to cells. As a result, cell-associated ampicillin will quickly re-equilibrate with the extracellular ampicillin, thus drastically reducing the intracellular concentrations of the drug (see Figure 3) [detailed studies have shown that J774 macrophages incubated for 5 h with free ampicillin at extracellular concentrations ranging from 10 to 100 mg/L (27–270 μM) achieve an apparent cellular to extracellular drug concentration ratio of only 0.4 ± 0.09 (n = 18)].[11] We therefore undertook to regularly re-expose the cells to the same concentration of PIVA by changing the medium every 5 h. Figure 3 (dotted line) shows that this protocol allowed maintenance of the cellular concentration of ampicillin at an almost constant level of ~200 mg/L (a concentration at least 10-fold larger than the maximum ampicillin concentration that could be present in the extracellular milieu assuming that all extracellular PIVA is converted into free ampicillin).

Activity of PIVA upon prolonged incubation at low concentration (20 h model)

The activity of ampicillin against intracellular *L. monocytogenes* is time dependent, and a 24 h incubation is required to obtain a 2 log decrease in cell-associated cfu in comparison with the original inoculum.[17] In view of the results obtained in the 5 h model with low concentrations of PIVA (Figure 2), and the results of the ampicillin accumulation studies over 20 h (Figure 3), we extended our studies with infected cells to 20 h. As shown in Figure 4, PIVA added at an

initial concentration of 83.5 μg/L with no further medium change afforded only transient control of bacterial growth. Thus, the number of cell-associated cfu declined over the first 5 h, but then rose sharply between 5 h and 20 h. When the medium was changed every 5 h, however, the number of cell-associated cfu declined steadily reaching a 2 log decrease in 20 h. This decline is similar to that observed with ampicillin 50 mg/L in a model of *Listeria*-infected THP-1 macrophages over the same period of time.[17]

Discussion

The data presented in this paper give new insights concerning: (i) the design and evaluation of β-lactam prodrugs in order to confer improved activity against intracellular bacteria susceptible to these molecules; and (ii) the treatment of listeriosis. With respect to the first point, our results provide, to our knowledge, the first unambiguous demonstration that an ester prodrug of ampicillin can be used to increase the cellular accumulation of this antibiotic. Ironically, this property was observed not with PIMA, but with PIVA. PIMA is an original basic ester that was manufactured specifically in the hope of achieving increased intracellular ampicillin concentrations.[7] Conversely, PIVA, a well-known ampicillin prodrug designed to enhance the oral bioavailability of ampicillin,[22] had never been directly examined for intracellular delivery of ampicillin, even though it is also basic. The difference in behaviour between the two esters cannot be related to differences in stability and potential release of free ampicillin in aqueous media, nor to differences in cellular accumulation and subcellular disposition. Both compounds are essentially alike with respect to all these properties.[8,11] We hypothesize that the key difference between PIVA and PIMA actually relates to the fact that PIVA is a double ester, which could make it more susceptible to the activity of cytosolic esterases. Double esters of ampicillin are known to be highly susceptible to serum esterases,[14,23,24] but this property is probably unimportant in our case. Indeed, PIVA remained active against intracellular *L. monocytogenes*, even when β-lactamase was added to the extracellular milieu. This indicates that the conversion of PIVA to ampicillin must take place, at least partially and as far as intracellular activity is concerned, in a place where ampicillin will be shielded from the extracellular milieu. We know that the bulk of cell-associated PIVA is located at the level of the pericellular membrane.[11] A simple explanation could therefore be that membrane-bound PIVA eventually reaches the cytosol but that it is readily hydrolysed therein into ampicillin (this is actually the mechanism proposed for the improved intestinal transport of ampicillin through the intestinal cells in animals receiving pivampicillin).[25] PIMA, which is not a double ester, would be less susceptible to cytosolic esterases, and would therefore be unable to release significant amounts of free ampicillin intracellularly. Alternatively, PIMA may be simply unable to ever penetrate into the cell to any significant extent. These non-mutually exclusive hypotheses will be tested in future experiments. As they stand, however, the data presented here already suggest that double esters of other β-lactams could now be systematically tested for intracellular delivery of the corresponding antibiotics.

Moving now to the treatment of listeriosis, our data also offer a somewhat provocative new insight concerning the optimal choice of agents in this context. A combination of ampicillin and gentamicin is widely considered as a first-line therapy,[26] based primarily on *in vitro* susceptibility data but backed by a limited number of clinical comparative studies. However, we have shown previously that gentamicin is essentially inactive against intracellular *L. monocytogenes* in THP-1 macrophages.[13] This observation is extended here to J774 macrophages, since gentamicin present in the 20 h model studies did not prevent the intracellular multiplication of bacteria. We also showed in the THP-1 model that ampicillin is bacteriostatic against intracellular *L. monocytogenes* in short-term (5 h) incubations, but may become bactericidal if prolonged incubations (24 h) are used.[17] In both cases, however, large concentrations of ampicillin (several times above the MIC) are necessary. Large and sustained concentrations of ampicillin cannot easily be obtained in patients with its conventional mode of administration. We show here that low concentrations of PIVA achieve essentially the same effect against intracellular *L. monocytogenes* as large concentrations of ampicillin, and that a continuous reduction of bacterial counts can be obtained simply by regular restoration of these low concentrations. The data therefore indicate that pivampicillin may provide similar chemotherapeutic effects to ampicillin while not requiring such high serum levels. It must, however, be emphasized that: (i) PIVA will offer such an advantage *in vivo* only if administered intravenously (pivampicillin administered orally will be hydrolysed in the intestinal wall and in the liver[25,27]) and at regular intervals (or even by continuous infusion); and (ii) that the administration of another antibiotic acting upon extracellular organisms will still be necessary. Bearing these caveats in mind, PIVA could be further tested in animal models and carefully designed human studies as a means of better controlling the intracellular survival and spread of *L. monocytogenes* in difficult situations.

Acknowledgements

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