Activity of temocillin in a murine model of urinary tract infection due to Escherichia coli producing or not producing the ESBL CTX-M-15

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Objectives: Temocillin is a 6α-methoxy derivative of ticarcillin that is resilient to ESBLs. Prospective data about its in vivo activity remain scarce. Our aims were: (i) to evaluate the activity of temocillin in a urinary tract infection (UTI) model due to ESBL-producing Escherichia coli and compare it with that of imipenem; and (ii) to define in vivo susceptibility breakpoints.

Methods: Mice were infected with a susceptible E. coli CFT073-RR or its transconjugant (CFT073-RR Tc) harbouring a blaCTX-M-15-carrying plasmid, using an ascending UTI model. Therapeutic regimens were chosen in order to reproduce percentage of time of free drug concentrations above MIC (\( fT_{\text{MIC}} \)) obtained in humans with standard regimens of temocillin (200 mg/kg every 2 h for 2 g every 12 h) or imipenem (100 mg/kg every 2 h for 1 g every 8 h). Additional regimens of temocillin (200 mg/kg every 4 and 6 h) with reduced \( fT_{\text{MIC}} \) were studied.

Results: MICs of temocillin and imipenem were 4/8 and 0.5/0.5 mg/L, for CFT073-RR and CFT073-RR Tc, respectively. In vivo, when given every 2 h (\( fT_{\text{MIC}} = 82\% \) and 70%), temocillin was bactericidal and as effective as imipenem in kidneys against both strains without selecting resistant mutants. Temocillin remained active even when given every 4 h, generating an \( fT_{\text{MIC}} \) of 41% and 35%, which corresponded to a breakpoint of 16 mg/L in humans with the standard regimen.

Conclusions: Our observations support the consideration of a standard regimen of temocillin as an alternative to carbapenems for the treatment of UTI due to CTX-M-producing E. coli strains with an MIC of 16 mg/L or less.

Keywords: imipenem, antibiotic resistance, antibiotic pharmacodynamics

Introduction

Worldwide spread of ESBL-producing Enterobacteriaceae has narrowed therapeutic options for infections due to such organisms.1 In particular, dissemination of multidrug resistance plasmids harbouring blaCTX-M-15 gene in community species such as uropathogenic Escherichia coli represents a major public health problem.2 Indeed, urinary tract infection (UTI) is one of the most common bacterial infections, with a global incidence estimated at nearly 150 million cases worldwide per year, and E. coli is by far the leading bacterial aetiology of UTI.3 Carbapenems are still considered as the first-line therapeutic choice for the treatment of ascending UTI or sepsis due to ESBL-producing Enterobacteriaceae.4 Nevertheless, their increasing consumption has led to the emergence and spread of resistance mechanisms in Gram-negative bacilli, and over the last few years, resistance to carbapenems in Enterobacteriaceae has become more prevalent, reaching endemicity in some regions.5,6 This phenomenon is worrying as the development of new drugs remains slow.7 Thus, alternative therapeutic options should be investigated in order to preserve our therapeutic arsenal and for ecological reasons. Temocillin is a 6α-methoxy derivative of ticarcillin that was commercialized in the early 1980s and eventually abandoned in most countries because of what was regarded as a major drawback, namely its lack of activity against Gram-positive organisms, anaerobes and Pseudomonas aeruginosa.8 What was not taken into account was temocillin’s β-lactamase stability. Indeed, the adjunction of the methoxy radical confers resistance to hydrolysis by serine-protease enzymes such as classes A and C but not by classes B and D of the Ambler β-lactamase classification. However, despite
good in vitro activity against ESBL-producing Enterobacteriaceae. Temocillin is not widely recognized as a potential alternative to carbapenems in clinical practice. Longitudinal studies in countries where it is in use have shown a remarkable stability in MIC distributions for Enterobacteriaceae. However, clinical breakpoints are not universally accepted and thus may vary from one country to another. There is no clinical prospective report on temocillin activity for the treatment of infection due to ESBL producers. Recently, investigators in England reported clinical and microbiological cure rates over 95% in a retrospective evaluation of infections due to ESBL- or AmpC β-lactamase-producing Enterobacteriaceae when the posology of temocillin was optimized to 2 g twice daily.

Therefore, the aim of our study was 2-fold: (i) to evaluate the efficacy of temocillin and compare it with that of imipenem and cefotaxime against strains of uropathogenic E. coli producing or not a CTX-M-15 enzyme in terms of bactericidal activity and selection of resistant mutants in vitro and in an ascending UTI murine model; and (ii) to define in vivo susceptibility breakpoints for temocillin against E. coli in UTI.

Materials and methods

Bacterial strains, resistance mechanisms and plasmid stability

Experiments were performed with E. coli strain CFT073-RR, and its isogenic derivative obtained by conjugation and harbouring a blaCTX-M-15-carrying plasmid: E. coli CFT073-RR Tc (pCFT073-RR CTX-M-15), named E. coli CFT073-RR-CTX-M-15 in this study. Two E. coli strains have been previously used in the murine model of pyelonephritis. Genetic backgrounds for CFT073-RR and its transconjugant were confirmed to be identical by random amplification of polymorphic DNA. For E. coli CFT073-RR CTX-M-15, the multidrug resistance region of the plasmid was investigated by PCR, indicating the presence of genes blaCTX-M-15, blaOXA-1, and aac(6′)-Ib. Plasmid stability of the transconjugant was measured in vitro by subculturing 10 μL of the transconjugant suspension in 10 mL of antibiotic-free trypticase soy broth every 48 h for 3 weeks. Cultures were then plated onto Mueller–Hinton (MH) agar with or without 10 mg/L cefotaxime. No significant plasmid loss was observed (<2.0%).

In vitro antibiotic activities

MICs of temocillin, imipenem, cefotaxime and ticarcillin were determined by the agar dilution method in accordance with CLSI guidelines. MICs of temocillin were also determined by the microdilution and macrodilution broth methods according to CLSI guidelines. In addition, the macrodilution broth method was used to investigate the effect of inoculum size (from 10^3 to 10^7 cfu/mL) and of protein binding (by adding 4% lyophilized human albumin (A931-10G, Laboratoire Sigma-Aldrich, Saint-Quentin, France) to MH broth) on temocillin MIC. Time–kill curve kinetics were performed with temocillin, imipenem and cefotaxime against each strain in 10 mL MH broth glass tubes with antibiotic concentrations equal to 1- and 4-fold the MIC value found by the macrodilution method or with no antibiotic, as previously described.

Antibiotic carry-over did not interfere with bacterial counts at the antibiotic concentrations used.

The frequency of in vitro selection of spontaneous resistant mutants was evaluated for each strain on MH agar plates at 4 times the MIC value for the tested strain as described previously. For each strain, 15 temocillin-resistant mutants were randomly selected and kept in a glycerol stock at –80°C. The MICs of temocillin for the resistant mutants were determined by the agar dilution method. All of the in vitro experiments described above were repeated two to four times.

Drug pharmacokinetics (PK)

Single-dose serum PK studies were performed in mice in order to determine the therapeutic regimen that best reproduced the percentage of time of the dosing interval during which free drug concentrations exceeded the MIC (T>MIC) and the plasma peak concentrations of antibiotics obtained in humans with intravenous standard regimens. Human standard regimens were chosen according to current practice and literature: 2 g every 12 h for temocillin and 1 g every 8 h for imipenem and cefotaxime.

First, we determined the dose of each antibiotic that produced, after a single subcutaneous injection in mice, the same plasma peak concentration as those observed in humans according to the literature (and previous work of our team for imipenem): 200 mg/kg for temocillin, 100 mg/kg for cefotaxime, and 100 mg/kg for imipenem/cilastatin (1:1 ratio). Then PK studies were performed after a single subcutaneous injection in mice of these selected doses. The T>MIC in mice was calculated according to the MIC value of the study strain obtained by agar dilution method. Finally, dosing intervals were chosen in order to achieve values of T>MIC similar to those obtained in humans with standard regimens. For temocillin, additional dosing intervals were investigated in order to achieve various T>MIC for PK/pharmacodynamic (PD) analysis of in vivo efficacy.

Antibiotic assays

Blood samples of at least 800 μL were obtained by intracardiac puncture from anaesthetized mice at different intervals after a single temocillin (200 mg/kg) or cefotaxime (100 mg/kg) subcutaneous injection: four mice for each interval at 15, 30, 60, 120, 240, 360 and 480 min after injection. For imipenem/cilastatin (100 mg/kg), data from a previous work from our laboratory were used. After blood collection, plasma was separated by centrifugation. Concentrations of temocillin and cefotaxime were determined by HPLC with ultraviolet detection at 237 nm. The limit of quantification (LOQ) was 1 μg/L. Intra- and interday precisions were 4.2% and 7.2%, respectively. Free-drug concentrations were determined after ultrafiltration by centrifugation in a Centrifree YM-30 (Millipore SAS, Molsheim, France) at 2000 g and 25°C for 10 min.

Mouse model of pyelonephritis

Animals were cared for in line with French guidelines and experiments were approved by the local Ethics Committee (Departmental Direction of Veterinary Services, Paris, France) (agreement no. 75-861). The ascending unobstructed mouse model of UTI was used in 247 immunocompetent female CBA mice (8 weeks old; weight 20–22 g), as previously described. Previous work from our group has shown that the two strains CFT073-RR and CFT073-RR CTX-M-15 are able to induce stable pyelonephritis in mice with maximal infection between days 5 and 10 without plasmid loss observed at day 10.

Antimicrobial treatment

Antibiotics used in all experiments were provided by the manufacturers: temocillin (Neganob®, Eumedico, Brussels, Belgium), imipenem/cilastatin (in a 1:1 ratio; Tienam®, MSD, Courbevoie, France) and cefotaxime (Mylan S.A.S., Saint-Priest, France). For each therapeutic regimen, ~45 mice were infected. Five days after inoculation, 15 were sacrificed before treatment (start-of-treatment controls), 15 were left untreated (end-of-treatment controls) and 15 were treated over 24 h by subcutaneous injections. End-of-treatment controls and treated mice were sacrificed 24 h after the last dose of antibiotic to avoid carry-over effect. Kidneys were...
PK/PD modelling and the E\text{max} model

The PK data of each antibiotic were analysed by a non-linear regression using ‘nlm’ package of R 3.0.0 software (The R Foundation for Statistical Computing), pooling data for all mice. Data below LOQ (BQL) were omitted. Three basic PK parameters were estimated for each antibiotic: the absorption rate constant ($k_a$), $V$ and $k_e$. The CL was calculated from estimated PK parameters.

The PD endpoint, cumulative percentage of an interval dosing period during which the free drug concentration exceeds the MIC under steady-state PK conditions ($T>MIC$), was determined using the estimated PK parameters and specific MIC for each antibiotic. For temocillin, a sigmoid $E_{\text{max}}$ model was used to describe the relationship between PK/PD endpoint ($T>MIC=4$ mg/L and $T>MIC=8$ mg/L) and temocillin bactericidal activity quantified by log colony counts at the end of treatment ($\log_{10}$ cfu/g of kidney).\textsuperscript{24} The following model was used:

$$\text{Effect} = \frac{E_0 - (E_{\text{max}} \cdot p^\gamma)}{(E_{\text{max}} + p^\gamma)}$$

where $p$ represents the studied PK/PD endpoint, $\gamma$ represents the sigmoid coefficient, $E_{\text{max}}$ represents the value of the PK/PD parameter when the effect is 50% of the $E_{\text{max}}$ and $E_0$ represents the baseline value. All experimental data were analysed from pooled mice data for each strain taking into account the fact that data below the LOQ are left-censored as in a population PK analysis.\textsuperscript{25} A specific R function was used. As only four values of PK/PD parameters were available, we assumed a full inhibition model, where $E_{\text{max}}$ was defined as $E_0$ minus the median of all BQL data.

Statistical analysis

Results are expressed as median (minimum–maximum) for continuous variables. The proportion of spontaneous resistant mutants and the proportion of sterile resistant mutants were compared in the different groups by the Mann–Whitney U test or the Kruskal–Wallis test when appropriate. All statistical analyses were performed using R 3.0.0 software. A $P$ value of <0.05 was considered significant.

Results

MICs and in vitro selection of spontaneous resistant mutants

MICs of temocillin for the $E$. coli strains are presented in Table 1. When the inoculum preparation was increased from $10^3$ to $10^7$ cfu/mL, MICs of temocillin increased in parallel (from 8 to 32 mg/L for CFT073-RR and from 8 to 64 mg/L for the transconjugant). The addition of 4% human albumin to the medium increased the temocillin MICs by two dilutions. The two strains were fully susceptible to imipenem (MICs $= 0.5$ mg/L), whereas susceptibility to ticarcillin and cefotaxime showed a contrast between the susceptible strain (MICs $= 8$ and 0.125 mg/L, respectively) and the transconjugant (MICs $> 1024$ mg/L for both antibiotics). Proportion of spontaneous resistant mutants to temocillin ranged from $7.63 \times 10^{-8}$ to $6.53 \times 10^{-8}$ for CFT073-RR CTX-M-15 to $12.4 \times 10^{-8}$ to $10.2 \times 10^{-8}$ for CFT073-RR. MICs of temocillin for mutants were at least 4 times higher than that of the parental strain, ranging from 32 to 256 mg/L by agar dilution method.

Time–kill curves

For temocillin, at a concentration equal to the MIC determined with the macrolodilution broth method (32 mg/L), an initial killing of 2 $\log_{10}$ cfu/mL at 6 h was followed by a bacterial regrowth at 24 h against the three strains, while a continuous bactericidal activity was similarly achieved against the three strains during 24 h of exposure at a concentration of 4 times the MIC (data not shown). At this concentration, the addition of 4% human albumin to the medium had no impact on temocillin killing rate. For imipenem, a rapid bactericidal activity was achieved against the three strains (data not shown).

PK/PD parameters

Optimal dosing regimens used in mice allowed plasma peak values and $T>MIC$ to be obtained comparable to those achieved in humans with standard therapeutic regimens for the three antibiotics tested (Table 2) despite rapid antibiotic clearance in mice (Figure 1) and low protein binding of temocillin in mice (16%) versus high protein binding (85%) in humans (Table 2). For temocillin, in addition to the optimal dosing regimen of 200 mg/kg every 2 h, two therapeutic regimens were used in mice (200 mg/kg every 4 h

### Table 1. MICs of temocillin for $E$. coli strains according to reference methods, inoculum size and the presence of albumin in the medium

<table>
<thead>
<tr>
<th>$E$. coli strains</th>
<th>MIC (mg/L)</th>
<th>macrodilution broth method$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$10^4$ cfu/mL</td>
</tr>
<tr>
<td>CFT073-RR</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>CFT073-RR CTX-M-15</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$MICs were determined by the macrolodilution method in MH broth with or without 4% human albumin.
Table 2. Optimal dosing regimens used in mice for temocillin, imipenem and cefotaxime and their corresponding PK/PD parameters against susceptible and resistant E. coli strains

<table>
<thead>
<tr>
<th>Optimal dosing regimen in mice</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (mg/L)</th>
<th>Protein binding (%)</th>
<th>f&lt;sub&gt;T&gt;MIC&lt;/sub&gt; (%) for E. coli CFT073-RR (MIC)</th>
<th>f&lt;sub&gt;T&gt;MIC&lt;/sub&gt; (%) for E. coli CFT073-RR CTX-M-15 (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temocillin 200 mg/kg every 2 h</td>
<td>199</td>
<td>16</td>
<td>82% (4 mg/L)</td>
<td>70% (8 mg/L)</td>
</tr>
<tr>
<td>Imipenem 100 mg/kg every 2 h</td>
<td>91</td>
<td>34</td>
<td>87% (0.5 mg/L)</td>
<td>87% (0.5 mg/L)</td>
</tr>
<tr>
<td>Cefotaxime 100 mg/kg every 2 h</td>
<td>128</td>
<td>12</td>
<td>100% (0.125 mg/L)</td>
<td>0% (&gt;1024 mg/L)</td>
</tr>
</tbody>
</table>

C<sub>max</sub>, peak value of total concentration at steady-state obtained from PK model for the three antibiotics; f<sub>T>MIC</sub>, percentage of time of the dosing interval during which free-drug concentrations remained above the MIC for the corresponding strain.

and every 6 h), achieving various values of f<sub>T>MIC</sub> according to the MIC of each strain determined by the agar dilution method (data not shown). In vivo, the three dosing intervals produced f<sub>T>MIC</sub> values ranging from 23% (injection every 6 h against CFT073-RR CTX-M-15) to 82% (injection every 2 h against CFT073-RR). With the optimal dosing regimen of temocillin, values of f<sub>T>MIC</sub> against CFT073-RR and its transconjugant, with MICs of 4 and 8 mg/L, were 82% and 70%, respectively (Table 2).

**Mouse model of pyelonephritis**

During the study period (from days 5 to 7 after inoculation), bacterial counts in kidneys were stable and did not differ between the start-of-treatment control groups [4.89 (1.61 – 6.51) log<sub>10</sub> cfu/mL for CFT073-RR and 4.74 (3.44 – 6.35) for CFT073-RR CTX-M-15] and the end-of-treatment control groups [5.03 (1.55 – 6.21) for CFT073-RR and 5.02 (1.44 – 7.19) for CFT073-RR CTX-M-15]. In addition, no plasmid loss was observed in vivo.

**Efficacy of optimal dosing regimens of antibiotics**

In mice infected with the susceptible strain CFT073-RR, optimal regimens of temocillin, imipenem and cefotaxime produced significant reductions in viable bacterial counts in kidneys as compared with controls (P = 0.002), and sterilized 43%–53% of kidneys (Table 3). No significant differences were observed between the three antibiotics in terms of viable bacterial counts in kidneys from mice infected with the susceptible strain (P = 0.55). In mice infected with CFT073-RR CTX-M-15, temocillin produced significant reductions in viable bacterial counts in kidneys compared with controls (P = 4<sup>×</sup>10<sup>−5</sup>), and was as active as imipenem (P = 0.28; Table 3). When antibiotic activities were analysed according to the infecting strain, there was no statistical difference between the two strains in terms of both bacterial count reduction and rates of sterilization for temocillin and imipenem. In contrast, cefotaxime was significantly less active against the transconjugant than against the susceptible parental strain (P = 0.001) and did not achieve a significant reduction in bacterial counts in kidneys as compared with controls (P = 0.24).

Temocillin dosing regimens of 200 mg/kg every 4 and 6 h remained significantly effective in reducing bacterial titres in kidneys against the two strains, with no significant difference between the parental strain and its transconjugant (Table 3). However, important variability was observed in kidney cfu counts from mice treated with temocillin every 6 h. No resistant mutants were detected in kidneys for any antibiotic regimen against the two study strains.
PK/PD modelling of temocillin in vivo effect

Sigmoid $E_{\text{max}}$ model parameters characterizing the relationship between log$_{10}$ cfu counts in kidneys at the end of treatment and temocillin PK/PD parameters were very similar against both strains (data not shown). Representative dose modelling of the effect of temocillin against both strains in terms of viable bacterial counts in kidneys expressed as log$_{10}$ cfu/g according to $f > \text{MIC}$ are shown in Figure 2. Near maximal bactericidal effect was obtain for values of $f > \text{MIC}$ over 80% against both strains. According to confidence intervals for the PK/PD parameter $f > \text{MIC}$, no significant difference was detected between the two tested strains.

Discussion

Despite good in vitro activity against most prevalent ESBL-producing Enterobacteriaceae and encouraging retrospective clinical studies, temocillin use for infection due to such organisms remains rare. There are two main reasons for this: (i) temocillin is an old compound that has declined into disuse, at least for clinicians; and (ii) it is available only in a few countries. However, its resistance to hydrolysis by most, if not all, ESBLs seems robust and stable over time while its spectrum of activity is focused on Enterobacteriaceae. At a time when CTX-M E. coli clones are disseminating worldwide, mostly causing UTI, alternatives to carbapenems are sought in order to preserve these last-resort molecules.

Our first objective was then to compare temocillin and carbapenem activities against CTX-M-15 uropathogenic E. coli strains in vitro and in vivo. The most important result of our study was the demonstration that, in a murine model of E. coli UTI, optimal dosing of temocillin was not influenced by the acquisition of a bla$_{\text{CTX-M-15}}$-harbouring plasmid by an uropathogenic strain of E. coli, and was as effective as imipenem and more effective than cefotaxime against the CTX-M-15 E. coli strain. Importantly, because major differences exist between the PK characteristics of temocillin in mice and humans, adjustments need to be made in order to draw relevant in vivo conclusions for humans. Specifically, clearance and protein binding are very different for temocillin between mice and humans; the use of very frequent injections on the one hand and the use of a PK/PD parameter integrating only free antibiotic concentrations ($f > \text{MIC}$) on the other hand allowed us to correct for such differences.

The efficacy of temocillin in pyelonephritis was due to the overall interaction of several factors. Two factors appeared to have potential negative impact on temocillin activity: protein binding and the bacterial inoculum effect. Since temocillin protein binding is 85% in humans, we investigated the impact of the presence of albumin on the in vitro activity of temocillin. While the presence of albumin produced a 4-fold increase in MICs, it did not affect the bactericidal rate of temocillin in vitro when tested at a concentration of 4 times the MIC. Similarly, a 4-fold increase in temocillin MICs was observed with an increasing inoculum, suggesting that this could be an issue in vivo. However, since temocillin is eliminated in an active form in the urine (400 to 600 mg/L in humans), high concentrations of temocillin are expected in murine and human kidneys, explaining why temocillin remained perfectly active against both strains in vivo, and sterilized 43%–64% of the kidneys from treated mice (Table 3) without selecting...
resistant mutants. Nevertheless, the relatively low bacterial inoculum in kidneys from control mice is a potential limitation of our study; this implies that our results may not apply to other foci of infection associated with a higher in vivo inoculum.

Our second objective was to help define in vivo susceptibility breakpoints for temocillin in UTI for E. coli. Despite a sparse design, the PK analysis confirmed the chosen model even if the absorption rate constant was rather poorly estimated. In our model, a significant and reproducible activity of temocillin was obtained with the every 4 h regimen, corresponding to an $f_{T>\text{MIC}}$ value of 41\% against the susceptible strain and 35\% against the transconjugant (Table 3). In animal models with Gram-negative bacteria, an $f_{T>\text{MIC}}$ value of 40\% for penicillins was associated with a bacteriostatic effect and survival.\(^\text{24}\) Using Monte Carlo simulation based on PK data provided by intensive care patients receiving the standard regimen of 2 g every 12 h of temocillin, De Jongh et al.\(^\text{21}\) calculated that, with this regimen, an $f_{T>\text{MIC}}$ value of 45\% was achieved for strains with an MIC of 16 mg/L. For an MIC of 32 mg/L, the 2 g every 12 h regimen provided only an $f_{T>\text{MIC}}$ value of 9.5\%, and a 2 g every 8 h regimen was thus necessary to obtain an $f_{T>\text{MIC}}$ value of 27\%. Altogether, these data suggest a breakpoint of 16 mg/L for UTI with a standard regimen of temocillin; for an MIC of 32 mg/L, an increased temocillin dosing regimen would be necessary.

In conclusion, our data suggest that temocillin is an effective alternative to carbapenems for the treatment of UTI due to ESBL-producing E. coli. PK/PD analysis suggests that, for UTI, strains with an MIC of 16 mg/L or less could be treated with a standard temocillin regimen of 2 g every 12 h in humans. Additional studies are necessary for other foci of infection, since specific data obtained in UTI may not apply in other situations with higher bacterial inoculum and lower temocillin concentrations.

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Transparency declarations

None to declare.

Author contributions


References