

## ORIGINAL ARTICLE

# PK-PD modeling of $\beta$ -lactam antibiotics: *In vitro* or *in vivo* models?

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A modified  $E_{\max}$ -pharmacokinetic-pharmacodynamic (PK-PD) model was previously proposed in literature for describing the antimicrobial activity of  $\beta$ -lactam antibiotics based on *in vitro* experiments. However, bacteria behave differently *in vitro* and *in vivo*. Thus, the aims of this study were to model the killing effect of piperacillin (PIP) against *Escherichia coli* on immunocompromised infected rats using this model and to compare the parameters obtained *in vitro* and *in vivo* for the same bacteria/drug combination. The PK-PD parameters determined *in vitro* and *in vivo* were as follows: generation rate constant of  $1.30 \pm 0.10$  and  $0.76 \pm 0.20 \text{ h}^{-1}$ , maximum killing effect of  $3.11 \pm 0.27$  and  $1.38 \pm 0.20 \text{ h}^{-1}$  and concentration to produce 50% of the maximum effect of  $5.44 \pm 0.03$  and  $1.31 \pm 0.27 \mu\text{g ml}^{-1}$ , respectively. The comparison between the *in vitro* and *in vivo* parameters was not straightforward and had to take into consideration the intrinsic differences of the models involved. So far, the main application of the PK-PD model evaluated is for the comparison of different antimicrobial agent's potency and efficacy, under equivalent conditions.

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**Keywords:** *Escherichia coli*; immunocompromised infected rats; *in vitro* model of infection; piperacillin; PK-PD modeling

## INTRODUCTION

The cure of an infection is dependent on factors related to the invasive microorganism such as drug susceptibility, the host physiological and immunological conditions and the pharmacokinetic (PK) and pharmacodynamic (PD) characteristics of the antimicrobial agent used. An important step in addressing this issue is the combination of PK parameters of the antibiotic with its PDs properties against bacteria in a Pharmacokinetic-pharmacodynamic (PK-PD) model. PK-PD modeling links dose/concentration relationship (PK) and concentration/effect relationship (PD), thereby facilitating the description and prediction of the time course of drug effects resulting from a certain dosing regimen.<sup>1,2</sup>

For the PK part of the model, the knowledge of the free interstitial levels of anti-infective agents at the biophase has drawn the attention of researchers, as the free concentrations at the site of infection are responsible for the bacterial killing and the outcome of anti-infective therapy.<sup>3</sup> The feasibility of the prediction of free interstitial levels, determined by microdialysis, based on plasma PK parameters, has been demonstrated in literature for some  $\beta$ -lactam antibiotics such as piperacillin alone<sup>4</sup> or in combination with tazobactam,<sup>5</sup> ceftriaxone,<sup>6</sup> cefaclor<sup>7</sup> and amoxicillin<sup>8</sup> using healthy animals. However, some studies have demonstrated that infection changes the physiological characteristics of the infected tissue due to the inflammatory process developed by host aiming to eliminate the invasive organism, as observed in soft tissue infections, septic shock, pneumonia or vascular

alterations associated to diabetes.<sup>9–12</sup> In this context, it is not always possible to predict the antimicrobial-free fraction at the site of infection, based on the microdialysis data obtained in healthy tissue or unbound plasma levels.

The application of PK-PD modeling was successfully described in literature by Nolting *et al.*<sup>13</sup> for the  $\beta$ -lactam antibiotic piperacillin (PIP) using a modified  $E_{\max}$ -model. This model proved to be useful to describe the association of piperacillin and tazobactam, a  $\beta$ -lactam inhibitor.<sup>14</sup> The development of the model was conceptualized simulating the free concentration profiles of the antimicrobial agent expected at the infection site in humans against bacteria, using an *in vitro* model of infection, as described in majority of studies where antimicrobial PD is evaluated in PK-PD investigation. It has been shown, however, that bacteria have different growth behavior *in vitro* and *in vivo*,<sup>15,16</sup> this event being specially associated to the medium conditions and the source of substrates. Besides, in the *in vitro* model of infection, the bacteria is in suspension, consequently more exposed to the antibiotic than *in vivo*, where they can adhere to cells and fibers present in the matrix making the access of the drug more difficult.<sup>16,17</sup>

In this context, to further explore the applicability of the PK-PD model developed, it is important to challenge the model's ability to describe antimicrobial effect over time in a more realistic scenario, using an experimental infection *in vivo*. Furthermore, the comparison between the parameters generated using an *in vitro* and an *in vivo* infection could be used to evaluate the ability of the *in vitro*

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experiments to forecast the infection's outcome *in vivo* using the same drug/bacteria combination. Therefore, the aims of this study were to model the killing effect of piperacillin against *Escherichia coli* in immunocompromised infected rats using the same modified  $E_{\max}$ -model described previously,<sup>13</sup> and to compare the PK-PD parameters obtained *in vivo* with those determined by simulating *in vitro* against *E. coli* the free tissue levels of piperacillin expected at the infection site in humans, based on the assumption that the correlation between free skeletal muscle and total plasma concentrations established in healthy rodents<sup>4</sup> would hold true in humans. For this study, besides the *in vivo* PD evaluation, the PK data used to carry out the PK-PD modeling was obtained by microdialysis at the infection site of immunocompromised *E. coli*-infected rats.

## MATERIALS AND METHODS

### Animals, drugs and solvents

All animal experiments described below were approved by the UFRGS Ethical in Research Committee (Protocol #01-349).

Piperacillin was purchased from Sigma (St Louis, MO, USA). Analytical grade potassium phosphate monobasic and potassium phosphate dibasic were from Merck (Darmstadt, Germany) and HPLC grade acetonitrile was from Omnisolv (Gibbstown, NJ, USA). HPLC water from Millipore's Milli-Q System (Billerica, MA, USA) was used throughout the analysis.

### PIP quantification in plasma and microdialysate

HPLC method has been adapted from literature<sup>5</sup> for the determination of PIP in rat plasma and microdialysate samples using RP-C18 column (Waters Spherisorb ODS column (Milford, MA, USA)—15 cm × 4.6 mm I.D., 5 μm particle size). The mobile phase (0.1 M phosphate buffer: acetonitrile, 80:20, v/v, pH 7.4) was pumped at a flow rate of 1.5 ml min<sup>-1</sup> and the drug was monitored at 220 nm.

Calibration curves in plasma were prepared in the range of 10–500 μg ml<sup>-1</sup> by spiking rat plasma with PIP. For drug determination in rat plasma, 200 μl samples were precipitated by the same volume of ice-cold acetonitrile. Subsequently, the mixture was centrifuged at 10 621 g (Centrifuge Eppendorf 5417R, rotor f-45-3011) for 15 min at 21 °C. The supernatant (200 μl) was used for injection into the chromatographic system. Tissue samples obtained by microdialysis (20 μl) were injected directly without any sample processing. Calibration curves in Ringer solution in the range of 0.5–200 μg ml<sup>-1</sup> were used to quantify the drug in the microdialysis samples. The method was validated according to FDA guidelines.<sup>18</sup>

### Experimental infection

The infection model adapted for this study was previously described for mice.<sup>19</sup> Neutropenia was induced in male pathogen-free Wistar rats (280–350 g) using cyclophosphamide: 150 mg kg<sup>-1</sup> ip 5 days and 100 mg kg<sup>-1</sup> ip 1 day before the experiment. The immunodepression achieved with cyclophosphamide treatment was evaluated by the total count of leukocytes conducted using the Shilling–Tourgau method in treated and control animals that received saline in a similar dosing regimen ( $n=3$ /group). On the day of the experiment, the left tibial cranial muscle of the animal was inoculated with 0.1 ml of a freshly prepared suspension containing 10<sup>6</sup> CFU per ml of *Escherichia coli* ATCC 25922. The inoculum was prepared from selected colonies incubated overnight on blood-agar plates.

### Pharmacokinetics experiments

The neutropenic and infected animals were divided in three groups: 60 mg kg<sup>-1</sup> (G1), 120 mg kg<sup>-1</sup> (G2) and 240 mg kg<sup>-1</sup> (G3), which received PIP after 2 h of inoculation ( $n=5$ /group). The rats were anesthetized with urethane (1.25 g kg<sup>-1</sup> ip) and the carotid artery was used for blood collection via an indwelling catheter. In the same experiment, the microdialysis probe was inserted in the animals' infected thigh muscle 1 h after inoculation. The microdialysis probe was perfused with Ringer's solution at a flow rate of 1.5 μl min<sup>-1</sup> and it was allowed to equilibrate inside the muscle for 1 h before determination of relative recovery *in vivo* by retrodialysis. The relative recovery

was determined at the infection site for all three groups, previously to the PK experiment, using a PIP solution of 1 μg ml<sup>-1</sup> in Ringer at a flow rate of 1.5 μl min<sup>-1</sup>. Three 20 min samples were collected to determine the recovery. The perfusion solution was switched to plain Ringer and the probes were flushed for 1 h before animals drug dosing. PIP in saline was injected *iv bolus* into the femoral vein (0.5 ml per 100 g) in order to reach 60, 120 and 240 mg kg<sup>-1</sup> doses. Microdialysate samples were collected over 20 min intervals and blood samples (500 μl) were collected immediately before dosing and at 5, 10, 15, 30, 45, 60, 90 and 120 min after drug administration. The animals remained anesthetized for the entire duration of the experiment.

PIP protein binding was determined by microdialysis in rat plasma samples obtained after *iv* administration of 60, 120 and 240 mg kg<sup>-1</sup> ( $n=3$ /group) to immunocompromised animals. Blood samples were collected at 5, 10 and 15 min after dosing, and plasma was separated by centrifugation. A volume of 400 μl of each plasma sample was placed in Eppendorf tubes and the microdialysis probes flushed with Ringer solution 1.5 μl min<sup>-1</sup> were inserted into the system kept at 37 °C. After equilibration of the probes into the media, three samples were collected in 20 min intervals. The microdialysate and the plasma samples were analyzed by HPLC and the free levels of PIP in each sample were determined by the ratio between the microdialysate and total plasma concentration.

Total plasma compartmental analysis was performed using the computer program Scientist v. 2 (MicroMath, St Louis, MO, USA). The relationship between total plasma levels and free tissue levels was evaluated performing a simultaneous fitting of total plasma concentration to equation (1), two compartment model, and free tissue concentrations to equation (2):<sup>5</sup>

$$C_{\text{plasma, total}} = ae^{-\alpha t} + be^{-\beta t} \quad (1)$$

$$C_{\text{tissue, free}} = \frac{f_u f (\beta a + \alpha b)}{(\alpha - \beta)} (e^{-\beta t} - e^{-\alpha t}) \quad (2)$$

where  $C_{\text{plasma, total}}$  is the total plasma concentration and  $C_{\text{tissue, free}}$  is the free muscle concentration at time  $t$ , respectively;  $\alpha$  and  $\beta$  are the hybrid constants for the distribution and elimination phase, respectively, being  $\alpha$  higher than  $\beta$ ;  $a$  and  $b$  as the corresponding zero-time intercepts; and  $f_u$  is the free fraction of piperacillin in plasma and  $f$  is a proportionality factor used to allow the simultaneous fitting of both data sets. A similar weighting factor was used for all the data points. The compartmental PK parameters were determined using classical equations.<sup>20</sup>

Microdialysis probes *in vitro* relative recoveries were determined by retrodialysis using PIP 1 μg ml<sup>-1</sup> in Ringer at a flow rate of 1.5 μl min<sup>-1</sup> as previously described.<sup>4</sup>

### Pharmacodynamics experiments

*In vitro model.* The model used in this work was the same described by Nolting *et al.*,<sup>13</sup> but the concentration time profile simulated *in vitro* was established in order to be equivalent to the free tissue concentrations expected in immunocompromised and infected rats after *iv* administration of a 120 mg kg<sup>-1</sup> dose. To predict this profile, rats PK parameters obtained from this study were used as follows: maximum drug concentration in serum ( $C_{\max}$ ) of 80 μg ml<sup>-1</sup>, half-life ( $t_{1/2}$ ) of 0.5 h and elimination rate constant ( $k_{el}$ ) of 1.38 h<sup>-1</sup>. The free peak concentration was calcd assuming a protein binding of 47%.

*In vivo model.* In a previous pilot study, the treatment of infected animals with PIP 60 mg kg<sup>-1</sup> was not effective, probably because the drug's short half-life in rats (0.5 h) associated with the time-dependent antimicrobial effect of PIP. On the basis of these results, only the higher doses were tested in the PD experiments.

The animals were divided in six groups, which received one of the following treatments: PIP 120 or 240 mg kg<sup>-1</sup> in three different dose regimens q8h, q6h or q4h for 24 h. The control group was immunocompromised and infected in the same manner, but was not treated with the drug.

On the day of the experiment, after 3 h of bacteria inoculation, the first dose of piperacillin was administered in the penile vein and the subsequent doses

were administered in the same manner following the respective dosing regimen. At pre-determined time points, specific for each dosing interval (q4h at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h; q6h at 0, 3, 6, 9, 12, 15, 18, 21 and 24 h; q8h at 0, 4, 8, 12, 16, 20 and 24 h), the animals were euthanized by cervical dislocation. The animals of the control group were killed at 0, 3, 6, 9 and 24 h after inoculation. The tibial cranial muscles were surgically removed and homogenized with the aid of 10 ml of sterile saline (0.9% NaCl) with a tissue homogenizer. Viable counts were determined on sheep blood agar (triptic soy agar and sheep blood 10%) by plating duplicate 100 µl samples of serially 10-fold dilutions of the homogenate. The number of CFU per ml (CFU ml<sup>-1</sup>) was determined after 24 h of incubation at 37 °C.

### PK-PD modeling

A modified E<sub>max</sub>-model, previously described, was used to model the bacterial killing effect of piperacillin *in vitro* and *in vivo* against *E. coli* ATCC 25922.<sup>13</sup>

$$\frac{dN}{dt} = \left( k - \frac{k_{\max} C_t}{EC_{50} + C_t} \right) N \quad (3)$$

where dN/dt is the change in number of bacteria as a function of time (PD effect); k (h<sup>-1</sup>) is the bacteria generation rate constant in the absence of any drug (data from the control group); k<sub>max</sub> (h<sup>-1</sup>) is the maximum killing effect; C<sub>t</sub> (µg ml<sup>-1</sup>) is the free concentration of piperacillin at the infection site, determined by microdialysis; and EC<sub>50</sub> (µg ml<sup>-1</sup>) is PIP concentration required to produce 50% of the maximum killing effect. In this equation, the drug effect is measured by the inhibition of growth or killing (reduction of number of bacteria). In the absence of drug, bacteria growth rate (k) determines the increasing number of bacteria as a function of time. When enough free drug is present in the tissue, actual killing of bacteria is induced and the resultant killing rate constant is k-k<sub>max</sub>. For the fitting, the values of k *in vivo* were fixed as the average value determined after a first fitting of the experimental data.

To model the drug-killing effect after the different dosing regimens investigated, the term related to concentration on equation (2) (C<sub>t</sub>) was replaced by equation (3). The parameters used for equation (2) were determined for piperacillin 60, 120 and 240 mg kg<sup>-1</sup> at the same experimental conditions and are shown in Table 1.

### Statistical analysis

The goodness of the curve fit was evaluated by the model selection criteria (MSC), a modified Akaike function, as well as the correlation coefficient (r) given by the computer program SCIENTIST v.2. (MicroMath). The MSC value is the information content of the fit of the model, and the higher the value, the better is the fit. The PK parameters determined for the three PIP doses were compared by analysis of variance (α=0.05).

**Table 1 Piperacillin compartmental pharmacokinetic parameters determined after simultaneous fitting of plasma and tissue concentration profiles after 60, 120 and 240 mg kg<sup>-1</sup> iv bolus administration to immunocompromised, and 3 h *E. coli*-infected rats (n=5/group)<sup>a</sup>**

PK Parameters	60 mg kg <sup>-1</sup>	120 mg kg <sup>-1</sup>	240 mg kg <sup>-1</sup>
Half-life (min)	27 ± 17	26 ± 13	33 ± 5
Vc (l kg <sup>-1</sup> )	0.09 ± 0.03	0.09 ± 0.02	0.15 ± 0.01 <sup>b</sup>
Vdss (l kg <sup>-1</sup> )	0.17 ± 0.09	0.19 ± 0.08	0.28 ± 0.03 <sup>b</sup>
CL (l h <sup>-1</sup> kg <sup>-1</sup> )	0.29 ± 0.05	0.34 ± 0.10	0.43 ± 0.07
AUC <sub>0-∞</sub> /D (µg min ml <sup>-1</sup> )	214 ± 37	179.6 ± 50.2	141.5 ± 20.1
α (min <sup>-1</sup> )	0.175 ± 0.027	0.190 ± 0.040	0.195 ± 0.050
β (min <sup>-1</sup> )	0.028 ± 0.014	0.030 ± 0.010	0.022 ± 0.003
F	0.35 ± 0.15	0.34 ± 0.09	0.34 ± 0.12
MSC	0.4–3.6	0.9–2.1	1.5–3.2

Abbreviations: AUC, area under the curve; CL, clearance; MSC, model selection criteria; PK, pharmacokinetic; Vc, volume of central compartment; Vdss, volume of distribution at steady state.

<sup>a</sup>Data are expressed as mean ± s.d.

<sup>b</sup>P<0.05, compared with the values at 60 and 120 mg kg<sup>-1</sup>.

## RESULTS

### HPLC method validation

The HPLC method was validated according to FDA guidance.<sup>18</sup> Linear calibration curves could be obtained in investigated ranges with coefficients of correlation of more than 0.993 for plasma and microdialysate. The limits of quantification were 10 µg ml<sup>-1</sup> in plasma and 0.5 µg ml<sup>-1</sup> in microdialysate. Inter- and intraday variability were determined for different quality control concentrations in plasma (10, 150 and 400 µg ml<sup>-1</sup>) and microdialysate samples (0.8, 50, 150 µg ml<sup>-1</sup>) were less than 14.1 and 13.2%, respectively. The method showed an accuracy within 15% for plasma and microdialysate samples (95.4 and 107.4%).

### Experimental infection

The neutropenia induced in the animals using cyclophosphamide was confirmed by counting the total plasma leukocytes in the treated and control groups. The average total count for the treated group was 240 ± 40 cells per ml and for the control group was 11 400 ± 1200 cells per ml.

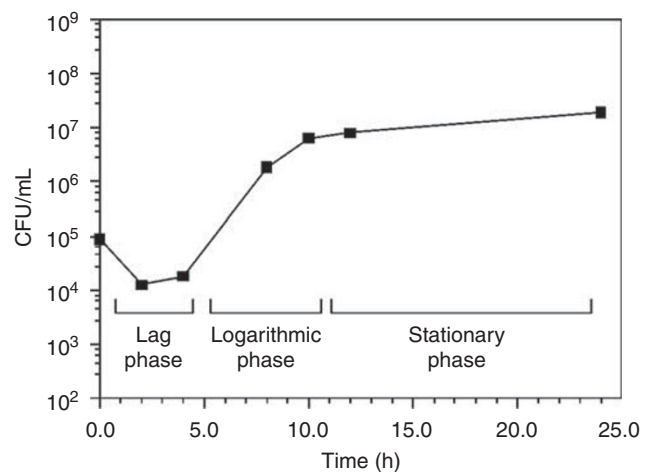
The bacteria generation rate constant was determined in the log-phase of the *in vitro* and *in vivo* curves (Figure 1). The average generation rate constant *in vivo* was 0.76 ± 0.20 h<sup>-1</sup>, and was determined from the data of control group of each experiment. For the modeling, k values were fixed as 0.76 h<sup>-1</sup> for the *in vivo* fitting. The generation rate constant *in vitro* was 1.30 ± 0.02 h<sup>-1</sup>.

### Pharmacokinetics experiments

The *in vitro* recovery was found to be 14.2 ± 3.4% and the *in vivo* recovery determined in infected muscle was 13.5 ± 3.7%, statistically similar to the *in vitro* one (α=0.05), for all groups investigated. The *in vivo* recovery was in the same order of magnitude of the average recovery reported in literature for the same probe and drug determined in healthy rat skeletal muscle (10.1 ± 0.9%) (ref. 4).

The free fractions of PIP determined by microdialysis in rat plasma were found to be 54 ± 27, 53 ± 11 and 69 ± 18%, for the 60, 120 and 240 mg per kg dose, respectively. Although these values did not differ significantly from each other (P<0.05), the value at the highest dose was increased, compared with those at the lower dose. Therefore, each value was applied for the respective dose in the tissue penetration study.

The PK parameters derived from the compartmental analysis of PIP plasma profiles after administration of 60, 120 and 240 mg per kg



**Figure 1** *Escherichia coli* ATCC 25922 growth curve *in vivo*.

doses to immunocompromised and 3 h *E. coli* infected animals are shown in Table 1. Statistical analysis showed differences among the parameters volume of central compartment ( $V_c$ ) and apparent volume of distribution at steady state ( $V_{d_{ss}}$ ) for the higher dose ( $240 \text{ mg kg}^{-1}$ ) in comparison with the lower doses ( $120$  and  $60 \text{ mg kg}^{-1}$ ). No statistical differences were observed among the other PK parameters for the three doses evaluated ( $\alpha=0.05$ ).

Figure 2 shows the average total plasma levels of the drug after the investigated doses. A two-compartment model adequately described the data in all cases. The free interstitial PIP levels determined by microdialysis, as well as the predicted profiles based on plasma data are also shown. As it can be seen, free tissue levels were predicted for all groups of animals using the proportionality factor and the PK parameters derived from fitting the plasma data to a two-compartment model equation. The proportionality factor was determined to be  $0.35 \pm 0.15$ ,  $0.34 \pm 0.09$  and  $0.34 \pm 0.12$  for 60, 120 and 240 mg per kg doses, respectively.

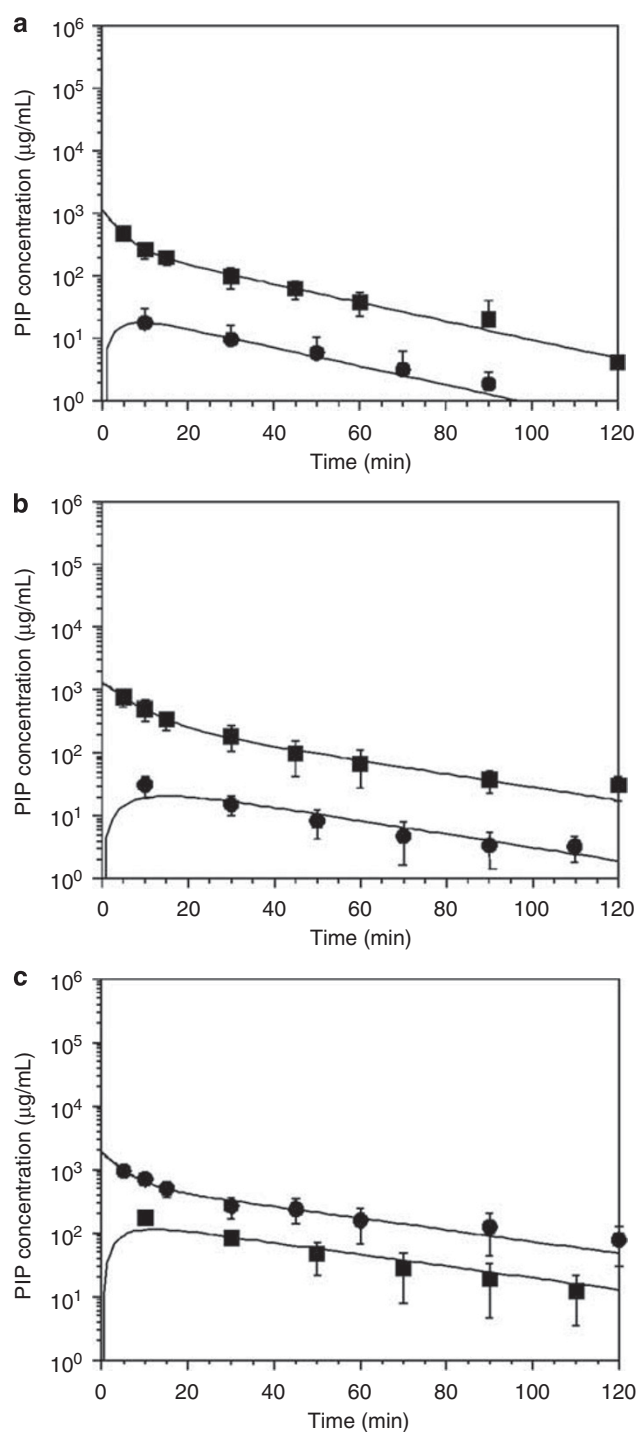
### Pharmacodynamics studies

The curve fittings obtained for PIP  $120 \text{ mg kg}^{-1}$  *in vitro* model are shown in Figure 3 and the PK-PD parameters of these curve fittings are shown in Table 2. The results of *in vivo* studies for 120 and  $240 \text{ mg kg}^{-1}$  administered q4h, q6h and q8h are shown in Figure 4. The values obtained for the PK-PD parameters of these curve fittings are shown in Table 3 and have not shown statistical difference for the different dosing regimens tested ( $\alpha=0.05$ ).

### DISCUSSION

The adequate selection of the animal model to study antimicrobial PK-PD correlation is of fundamental importance for the usefulness of the outcome. The most commonly used animal infection model to investigate PK-PD correlation is the neutropenic mouse thigh model. Some advantages of this model for PK-PD investigations are:<sup>19</sup> the granulocytopenia can be induced in the animals eliminating the influence of neutrophils and other polymorphonuclears on the infection, allowing the determination of the antimicrobial effect; the bacteria counting at the infection site can be easily processed (PD) and the plasma levels of the drug are easily accessed (PK). Finally, because of the fast antimicrobial elimination, the model allows the investigation of several dosing regimens leading to the establishment of PK and PD correlation. Although mice are traditionally used for the thigh infection model, rats present some advantages for PK studies: blood samples can be drawn for longer periods of time, the animal can be kept anesthetized and microdialysis can be carried out concomitantly with blood sampling. Assuming that PKs and PDs should be investigated in the same animal under the same experimental conditions, the mouse thigh infection model was adapted to rats in this work.

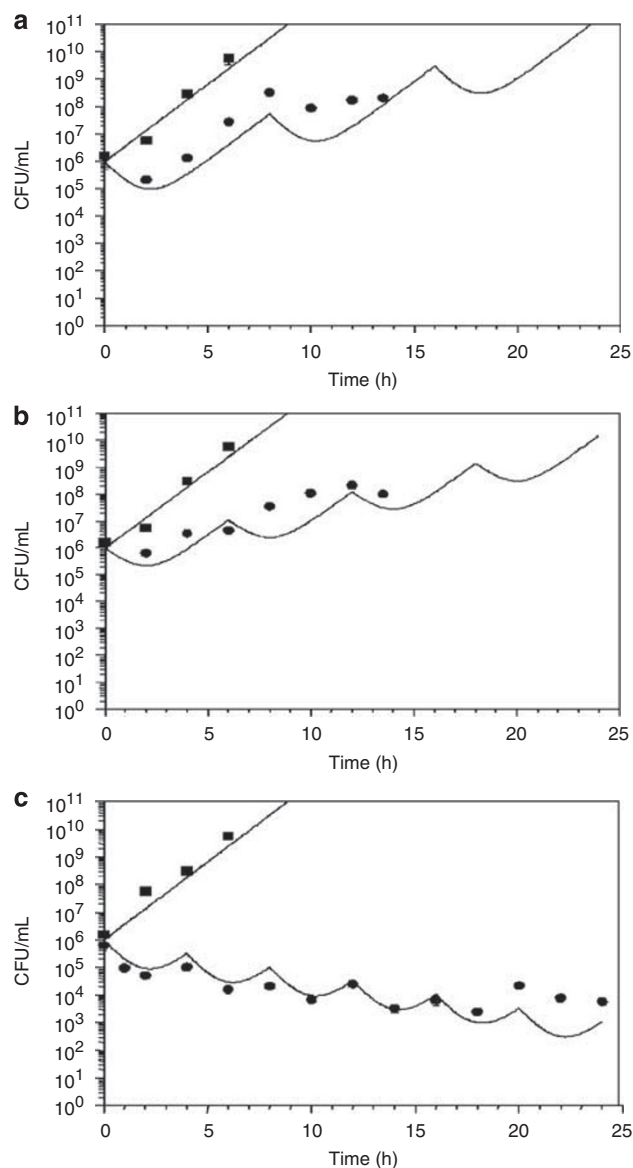
To assure that the infection model was adapted to rats, the total leukocytes count was conducted after the administration of cyclophosphamide. The results showed that the treated animals presented total count lower than 300 leukocytes per ml indicating severe neutropenia.<sup>19</sup> Another experiment conducted to assure that the infection model had been successfully adapted to rats was the determination of the bacterial growth curve (Figure 1). The result showed that it was possible to visualize all the characteristic phases of the bacteria growth:<sup>21</sup> the lag-phase, showing that it took 3 h for the bacteria to adapt to the muscle conditions, the logarithmic-phase, necessary to obtain the effect of  $\beta$ -lactam antibiotics such as PIP, and the achievement of a plateau (stationary phase). These results showed that the adaptation of the infection was successful and that the contribution of



**Figure 2** Concentration-time profiles of PIP in plasma (■) and tissue (●) after iv bolus dosing of  $60 \text{ mg kg}^{-1}$  (a),  $120 \text{ mg kg}^{-1}$  (b) and  $240 \text{ mg kg}^{-1}$  (c) to immunocompromised infected rats ( $n=5/\text{group}$ ). The plasma data were fitted to a two-compartment model (equation (1)). The line for tissue levels is not fitted to the measured microdialysis data, but is predicted based on the plasma data using equation (2) (average  $\pm$  s.d.).

the immune system for the bacteria killing at the infection site was significantly reduced.

Considering the tissue penetration, is known that only the free levels at the biophase are responsible for the pharmacological activity of drugs in general. Although plasma levels have been used to correlate



**Figure 3** Curve fitting of PIP antimicrobial effect *in vitro* against *E. coli*: (■) control group, (●) treated group with PIP 120 mg kg<sup>-1</sup> (a) q4h, (b) q6h and (c) q8h (average ± s.d.).

with PD bacterial killing effect,<sup>22</sup> the free tissue levels of the drug at the infection site are more relevant for the bacterial-killing effect. Nowadays, free interstitial levels of different tissues can be determined using microdialysis.<sup>23</sup>

Piperacillin therapeutic doses for the treatment of human infections are 60 and 120 mg kg<sup>-1</sup> given four times a day.<sup>24</sup> The use of these two doses was initially intended in this work in order to treat the experimental *E. coli* infection produced in the immunocompromised rats. The 60 mg per kg dose, however, presented an insignificant killing effect for the six times a day regimen, resulting in a curve similar to the non-treated control group (data not shown). This result can be attributed to the half-life of piperacillin in rats, which was shown to be around 30 min, whereas in humans it is twice this value. β-lactam antibiotics are classified as time-dependent bacterial-killing drugs meaning that the extent of the microbial killing is primarily dependent on the duration of the exposure to concentrations equivalent to low

**Table 2** Pharmacokinetic-pharmacodynamic parameters of the *in vitro* curve fitting of piperacillin using bacteria rate constant of 1.30 h<sup>-1</sup>

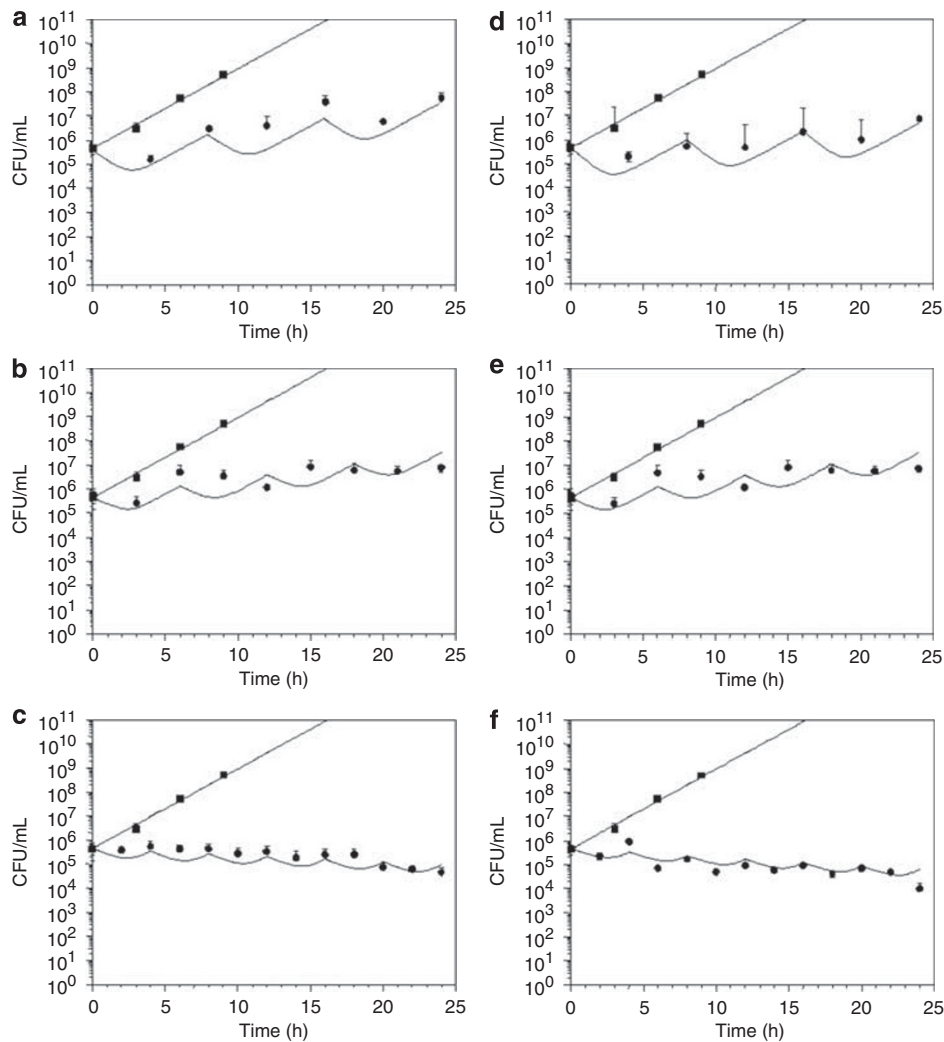
Parameter	120 mg kg <sup>-1</sup>			Average ± s.d.
	q4h	q6h	q8h	
EC <sub>50</sub> (μg ml <sup>-1</sup> )	5.41 ± 0.02	5.46 ± 0.03	5.47 ± 0.03	5.4 ± 0.03
k <sub>max</sub> (h <sup>-1</sup> )	3.3 ± 0.30	2.8 ± 0.25	3.2 ± 0.29	3.1 ± 0.27
MSC	-1.88	0.62	0.60	
r <sup>2</sup>	0.83 ± 0.09	0.99 ± 0.05	0.99 ± 0.07	0.94 ± 0.05

Abbreviation: MSC, model selection criteria

multiples of the MIC.<sup>13</sup> Consequently, the shorter half-life in rats will lead to PIP concentrations below MIC faster than in humans, resulting in lack of bactericidal effect for the smaller dose. Piperacillin MIC for *E. coli* ATCC 25922 used in this work was determined to be 2–4 μg ml<sup>-1</sup>, consistent with literature. To overcome the low concentrations problem, PIP doses investigated in infected animals were 120 and 240 mg kg<sup>-1</sup>. The higher PIP dose, however, led to an increased free fraction in plasma, which was not observed for the 60 and 120 mg per kg doses. This result could be explained by saturation of plasma proteins. To overcome this issue, the free plasma fraction determined for each dose was used for the predictions of piperacillin interstitial unbound levels for the corresponding dose using equation (2).

The tissue penetration study revealed that infection changed the relationship between total plasma and free tissue concentration of PIP. Nolting *et al.*<sup>4</sup> studied this relationship for the same bacteria and drug in healthy Wistar rats and were able to predict the free tissue levels based on total plasma concentration without the need of a proportionality factor, indicating that this drug distribution is governed by passive diffusion process. In this study, however, the proportionality factor was necessary to allow the prediction of free tissue concentrations based on plasma PK parameters, indicating that infection caused physiological changes in muscle such as increase in vascular permeability leading to increase in protein concentration at the infection site, increase in tissue temperature and pH alterations that would ultimately lead to an around 30% decrease in free interstitial levels of PIP. Joukhdar *et al.*<sup>10</sup> studied the tissue penetration of piperacillin in skeletal muscle and s.c. adipose tissue in patients with septic shock, using microdialysis, and observed that the free levels of piperacillin in these situations were 5–10 times lower than the corresponding free plasma concentrations, indicating the infectious process changed the penetration of this antimicrobial, reducing its clinical effectiveness. The difference between this study and that reported by Joukhdar *et al.* is that in the present one the animals had a localized infection instead of a septic shock that definitively alters body homeostasis. A potential explanation for plasma to tissue gradient observed for PIP in infected tissues is that the drug is a zwitterions, and as such, differences in pH between plasma water and interstitial fluid may cause it to be ionized and hinder its diffusion across membranes. Other possible explanations include the presence of peripheral chemical degradation that may occur because of PIP instability in acid pH.

The PD experiments, showed that the *in vivo* bacterial-killing effect of piperacillin obtained for the 120 and 240 mg per kg doses were dependent on the dosing interval investigated: q4h, q6h and q8h (Figure 4). It can also be depicted from this figure that, for all the situations investigated *in vivo*, the PK-PD model used adequately described the experimental data. The goodness of curve fit can be



**Figure 4** Curve fitting of PIP antimicrobial effect *in vivo* against *E. coli* infection in immunocompromised rats. Left panel: (■) control group, (●) treated group with PIP 120 mg kg<sup>-1</sup> (a) q8h, (b) q6h and (c) q4h. Right panel: (■) control group, (●) treated group with PIP 240 mg kg<sup>-1</sup> (d) q8h, (e) q6h and (f) q4h (average ± s.d.).

**Table 3** Pharmacokinetic-pharmacodynamic parameters of the *in vivo* curve fitting of piperacillin using bacteria generation rate constant of 0.76 h<sup>-1</sup>

Parameter	120 mg kg <sup>-1</sup>			240 mg kg <sup>-1</sup>			Average ± s.d.
	q4h	q6h	q8h	q4h	q6h	q8h	
EC <sub>50</sub> (μg ml <sup>-1</sup> )	1.39 ± 0.27	1.59 ± 0.31	1.04 ± 0.19	1.10 ± 0.17	1.31 ± 0.25	1.41 ± 0.26	1.31 ± 0.27
k <sub>max</sub> (h <sup>-1</sup> )	1.31 ± 0.18	1.41 ± 0.19	1.69 ± 0.23	1.20 ± 0.17	1.38 ± 0.19	1.93 ± 0.27	1.38 ± 0.20
MSC	3.03	1.21	0.96	3.07	1.35	1.28	
r <sup>2</sup>	0.99 ± 0.08	0.99 ± 0.10	0.96 ± 0.06	0.99 ± 0.08	0.99 ± 0.07	0.99 ± 0.09	0.99 ± 0.08

Abbreviation: MSC, model selection criteria

confirmed by the values of MSC and correlation coefficient obtained (Table 3).

The comparison of the different dosing intervals for the same total daily dose (Figures 4c and d) showed an increasing magnitude of killing effect for the shorter dosing intervals. As mentioned before, this is consistent with the classification of β-lactam antibiotics as time-dependent killing antimicrobials.<sup>25</sup>

The time dependency of PIP effect can also be observed when comparing the same dosing regimen for the 120 and 240 mg per kg doses (Figures 4a and d for q8h, b and e for q6h, and c and f for q4h). The free interstitial peak concentration observed at the infection site and used to model the PK-PD data was around 40 μg ml<sup>-1</sup> for 120 mg per kg dose and 120 μg ml<sup>-1</sup> for the 240 mg per kg dose. Taking into consideration the MIC of PIP for the *E. coli* used in this study, it can

be expected that the concentrations at the infection site are close to the MIC after 3–6 half-lives for both doses, resulting in bacterial re-growth at approximately the same time. These killing and re-growth profiles can be observed in Figure 4 for both doses.

It can be seen that the model adequately described the experimental data for all dosing regimens investigated. The q6h and q8h regimens were not effective, similar to the results obtained *in vivo* (Figures 4a and d), and bacteria re-growth determined the end of the experiment after 12 h. For q4h (Figure 4a), bacteria killing were observed without infection eradication up to 24 h. The PK/PD parameters values obtained after the *in vitro* data fitting (Table 2) differed from those determined using *in vivo* data, although the PK profile simulated *in vitro* was the same observed in the animals.

The PD parameters obtained by the PK-PD modeling of PIP bacterial killing effect were the maximum killing effect ( $k_{\max}$ ) and the concentration to produce 50% of the maximum effect ( $EC_{50}$ ) (Tables 2 and 3). The  $EC_{50}$  and  $k_{\max}$  were similar for the different dosing regimens investigated *in vivo* as it was previously observed with the results from *in vitro* time-kill curves,<sup>13</sup> indicating that they are characteristics of the bacteria/drug interaction and independent of the doses or dosing regimen investigated.

The comparison between the parameters determined for the same infection model *in vitro* and *in vivo* was one of the goals of this work. The value of the *E. coli* ATCC 25922 generation rate constant obtained *in vitro* was determined to be  $1.30 \pm 0.02 \text{ h}^{-1}$  higher than the value determined *in vivo* ( $0.76 \pm 0.20 \text{ h}^{-1}$ ) considering the control experiments performed concomitantly to the treatments. These results show that the bacterial generation time *in vitro* (approx. 30 min) was faster than *in vivo* (approx. 60 min), which could be foreseen. According to literature, in *in vitro* environment bacteria differ from those cultivated *in vivo* in terms of amino acid composition, synthesis of toxic metabolites and metabolic rate. Although bacteria are in general more metabolically active *in vivo* than *in vitro*, it has been shown that the replication generally occurs faster in an *in vitro* environment.<sup>26</sup>

The maximum killing effect ( $k_{\max}$ ) obtained *in vivo* ( $1.38 \pm 0.20 \text{ h}^{-1}$ ) was two times smaller than the value obtained in the *in vitro* experimental infection ( $3.11 \pm 0.27 \text{ h}^{-1}$ ) demonstrating that it took longer to eradicate the bacteria when the experimental infection took place *in vivo*. The PIP killing rate constant for the two situations can be determined by the difference between the generation rate constant and the maximum killing effect ( $k - k_{\max}$ ) (equation (3)). The killing rate constant *in vitro* ( $1.8 \text{ h}^{-1}$ ) was almost three times bigger than the killing constant *in vivo* ( $0.62 \text{ h}^{-1}$ ). One possible explanation for this phenomenon is the drug mechanism of action.  $\beta$ -lactam antibiotics bactericidal mechanism involves the inhibition of the cell-wall synthesis and the drug is active during the bacteria growth phase, which was shown to be faster *in vitro*, creating better conditions for PIP activity.

In the same manner, the concentration necessary to produce 50% of the maximum effect ( $EC_{50}$ ) *in vivo* ( $1.31 \pm 0.27 \mu\text{g ml}^{-1}$ ) was almost four times smaller than the value obtained *in vitro* ( $5.44 \pm 0.03 \mu\text{g ml}^{-1}$ ). Because the bacteria is more available to antibiotic attack *in vitro* one could expect that the  $EC_{50}$  would be smaller at this situation, assuming the same maximum killing effect *in vitro* and *in vivo*. However, the maximum killing effect obtained for the two situations was not equivalent, thus the values of  $EC_{50}$  can not be compared. One factor that can not be forgotten is that the neutropenia observed confirms that the number of granulocytes was reduced in the immunocompromised animals but the other host defense factors are still present in the animal and could still contribute to the final killing

effect in opposition to the *in vitro* situation where the drug is the only factor responsible for the bactericidal effect. This factor by itself can not account for the  $EC_{50}$  differences observed. The importance of the bacteria different generation rate constant *in vitro* and *in vivo* for the  $\beta$ -lactam antimicrobial killing effect was also pointed out by Bonapace *et al.*<sup>15</sup> when studying ticarcillin against *Pseudomonas sp.*

Another comparison can be made between the *in vitro* parameters determined in this study and the ones previously reported by Nolting *et al.*<sup>13</sup> In the *in vitro* experiment reported in that study, the bacteria was exposed to concentrations above the MIC for shorter periods of time because the immunocompromised rat half-life of 30 min was simulated, whereas piperacillin human half-life simulated in literature was 1 h.<sup>13</sup> The resulting values of  $k_{\max}$  ( $3.11 \pm 0.02 \text{ h}^{-1}$  for rats and  $3.19 \pm 1.39 \text{ h}^{-1}$  for humans) and  $EC_{50}$  ( $5.44 \pm 0.03 \mu\text{g ml}^{-1}$  for rats and  $5.17 \pm 1.81 \mu\text{g ml}^{-1}$  for humans) reported were similar for both experiments. However, the killing curves were different: bacterial eradication was obtained when simulating the humans' free PIP profile expected in the infection site after distinct dosing regimens while only a reduction of the initial inoculum was obtained when the immunocompromised rats' free-tissue levels were simulated. Because the only difference between these two experiments was the drug half-life, one can conclude that, for a fixed bacteria generation rate constant, the drug PKs determines the infection outcome.

Putting together these two comparisons, *in vitro* vs *in vivo* (present study) and *in vitro animals* vs *in vitro humans*,<sup>13</sup> it becomes clear that the generation rate constant affects the parameters of the  $E_{\max}$  PK-PD model to a higher extent than the half-life. The half-life parameter, however, is fundamental in defining the shape and outcome of the killing profiles for the same generation rate constant.

## CONCLUSIONS

The results presented in this work showed that the modified  $E_{\max}$ -model used was adequate to fit the *in vivo* reduction of the number of bacteria at the infection site as a function of time. The comparison between the *in vitro* and *in vivo* PK-PD parameters is not straightforward and several factors related to the intrinsic differences between the experimental infection models involved have to be taken into consideration.

It would be premature, however, to suggest that such PD studies should henceforth be conducted by using only *in vivo* systems instead of *in vitro* infection models. The *in vitro* systems have successfully been used for a long time for comparing antimicrobials potency and for the understanding of the factors that may limit the efficacy of antimicrobials *in vivo*.

The results presented in this paper indicate that the antimicrobial activity evaluated *in vitro* should be followed by the evaluation of the drug in an *in vivo* infection model to ensure the consistency of the results obtained and to approach the results to the clinical outcomes expected. At this moment, the main application of the PK-PD models such as the one evaluated here is for the comparison of different antimicrobial agents' potency and efficacy under equivalent conditions.

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