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Short Communication

Urinary piperacillin/tazobactam pharmacokinetics in vitro to determine the pharmacodynamic breakpoint for resistant Enterobacteriaceae

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ABSTRACT

Urinary tract infections caused by multidrug-resistant Enterobacteriaceae are a growing burden worldwide. Recent studies of urinary pharmacokinetics described high piperacillin/tazobactam (TZP) concentrations in urine, but it is unknown whether this results in treatment efficacy. This study investigated the pharmacodynamics of TZP in a static in vitro model for Enterobacteriaceae to determine the concentration–effect relationship and ultimately the required free (unbound) time above the minimum inhibitory concentration ($fT_{>MIC}$) required for bacterial killing. The static simulation model investigated TZP $fT_{>MIC}$ between 0% and 100%. Resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates with piperacillin/tazobactam MICs of 4096/512, 1024/128 and 128/16 mg/L were investigated; two of the three organisms were carbapenemase-producers. Clinical efficacy was determined as a 3-log reduction over the dosing interval by comparing interval growth with controls. TZP was observed to exhibit time dependence for all organisms. The $fT_{>MIC}$ was determined to be 37.5%, 37.5% and 50% for MICs of 4096/512, 1024/128 and 128/16 mg/L, respectively. Linear regression identified the overall target to be 49.85 ± 16.9% $fT_{>MIC}$. In conclusion, bactericidal activity against TZP-resistant Enterobacteriaceae occurred at 49.85 ± 16.9% $fT_{>MIC}$. This suggests that highly resistant urinary organisms, including carbapenemase-producers, with MICs up to 4096/512 mg/L could be treated with TZP. Further investigations are required to elucidate urinary breakpoints and to explore the impact of different resistance mechanisms.

1. Introduction

Urinary tract infections (UTIs) caused by multidrug-resistant Enterobacteriaceae are a growing burden worldwide [1]. Limited antimicrobial options have increased the cost and decreased the safety of treating this common infection [2]. The search for additional treatment options requires that we renew our understanding of the pharmacokinetics of existing antimicrobial agents.

β-Lactam antibiotics, a commonly prescribed class of antimicrobial agent in the treatment of UTIs [3], have been previously described to exhibit time dependence [4–6]. The concentration–effect plot of time-dependent antimicrobials typically describe a logarithmic curve with a plateau in efficacy above the minimum inhibitory concentration (MIC) [5,6]. The most important pharmacodynamic (PD) parameter when investigating time-dependent antimicrobials is the fraction of time the free (unbound) drug concentration remains above the MIC over the dosing interval ($fT_{>MIC}$). The PD breakpoint for time-dependent killing for most β-lactam antibiotics has been identified to be a 50% $fT_{>MIC}$ [7].

The β-lactam/β-lactamase inhibitor combination piperacillin/tazobactam (TZP) has recently been demonstrated to reach adequate urinary concentrations to meet pre-existing PD targets for Enterobacteriaceae with MICs that would conventionally classify them as being resistant to this drug combination [8]. Tested organisms were predicted to be eradicated by high TZP concentrations achieved in urine using a standard dose of 4.5 g TZP [8]. However, there are limited data combining in vitro MIC data with simulated urinary pharmacokinetics, and it is unclear whether the previously

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established PD parameters for time-dependent killing and the resultant $fT_{>MIC}$ of 50% remain applicable to organisms with high MICs.

This study investigated the pharmacodynamics of TZP in a static in vitro model simulating the urinary concentration–effect profile and determining the required time above the MIC for efficacy against Enterobacteriaceae [7]. It was hypothesised that TZP bactericidal activity against Enterobacteriaceae that are conventionally deemed resistant by Clinical and Laboratory Standards Institute (CLSI)/European Committee on Antimicrobial Susceptibility Testing (EUCAST) (>32/4 mg/L and >16/4 mg/L, respectively) [9,10] would be achieved at 50% $fT_{>MIC}$. Determining this PD breakpoint would provide evidence that conventionally 'resistant' infections could still be treated by high urinary drug concentrations.

2. Materials and methods

2.1. Study design

An in vitro static time–kill descriptive study design was used in all experiments. TZP (Tazopip^{TM}) (4.5 g vial) was used for microdilution methods and all static time–kill experiments. Cation-adjusted Muller–Hinton broth (CAMHB) (Thermo Fisher Scientific, Thebarton, SA, Australia) was used for susceptibility testing and time–kill studies, and cation-adjusted Muller–Hinton agar (CAMHA) was used for viable count assessment.

Enterobacteriaceae were screened from clinical samples received by the microbiology diagnostic laboratory. *Escherichia coli* and *Klebsiella pneumoniae* were chosen because of similar β-lactamase resistance mechanisms. Differential CHROMagar, indole production and disk diffusion antimicrobial susceptibility testing were used to presumptively determine the phenotype of the isolates [9]. PCR was subsequently undertaken to confirm the resistance mechanisms of individual isolates. Bacterial MICs to TZP were determined by microdilution methods in triplicate using CLSI methodology [9], although using variable tazobactam concentrations instead of fixed. Bacteria selected for experiments were *K. pneumoniae* (MIC = 4096/512 mg/L), *K. pneumoniae* (MIC = 1024/128 mg/L) and *E. coli* (MIC = 128/16 mg/L) chosen based on their classification as resistant to TZP according to the CLSI [9]. Organisms were determined by PCR to carry bla_{KPC}, bla_{NDM}/$bla_{CTX\text{-}M}$ and $bla_{CTX\text{-}M}$ genes, respectively.

2.2. Static time–kill experiments

Static simulation pharmacokinetics was conducted by designing various $fT_{>MIC}$ concentration–time profiles, deconstructing the concentration–time of each profile to create static fixed exposure times of 1 h or 2 h, observing growth and then transforming data to reconstruct each $fT_{>MIC}$ profile (see Appendix, examples A–C). Prior to experiments, bacteria were grown on horse blood agar/chromogenic agar (Remel Inc., Lenexa, KS) and were incubated at 37 °C for 20–24 h. A 0.5 McFarland standard (equivalent to 1×10^8 CFU/mL) of a single colony was diluted with CAMHB/TZP to an initial bacterial concentration of 5×10^5 CFU/mL for experiments.

The independent variable in experiments was different $fT_{>MIC}$ profiles that described various concentration–time relationships. TZP concentration–time models were evaluated to provide $fT_{>MIC}$ values of 0% to 100% based on the previously observed ranges [8]. Concentrations used for each time interval are summarised in Supplementary Table S1. Static concentration–time profiles were compared with the dynamic concentration–time curves to determine whether the selected static concentration–time profiles were representative for each $fT_{>MIC}$ profile (refer to Supplementary Equation E1).

All static time–kill studies were conducted using a fixed CFU concentration of bacteria, subjected to different TZP concentrations and exposure times, to determine the concentration–time versus effect (kill rate) relationship. The CFU concentration was controlled between bacteria by maintaining a constant ratio between isolates relative to the MICs (concentration:MIC), allowing a comparison between bacteria. Extraction times reflected time intervals of 0–1 and 1–2 h for an exposure of 1 h, and 2–4, 4–6 and 6–8 h for an exposure of 2 h. CAMHB with isolates and drug remained in a shaking water-bath at 37 °C for the duration of the exposure time until extraction. Positive and negative control plates were used for each respective organism: specifically, organism without antibiotic and the lowest concentration antibiotic with no organism present, respectively. Protocols, organisms and concentrations were randomised and were given codes in order to limit performance bias and were repeated in triplicate for each respective organism.

Centrifugation and re-suspension were performed prior to plating to prevent antibiotic transfer. Two sequential centrifugation and re-suspension steps were used, resulting in an antibiotic dilution factor of 100 ($<0.02 \times$ MIC). Samples were centrifuged at $3220 \times g$ for 10 min at 37 °C and the supernatant was then removed and re-suspended in fresh pre-warmed CAMHB. After two sequential re-suspension processes, 100 μL of diluted or undiluted sample was plated on CAMHA plates. Dilution and wash were carried out using sterile saline. Plates were then incubated at 37 °C for 20 h and a manual count was performed to determine CFU/mL.

2.3. Viable counts

The dependent variable was colony counts, which were performed 20 h post-incubation. Manual counts were performed to determine the CFU and thus the CFU/mL based on previous dilutions. Plates were limited to a count of 1 colony per plate, equivalent to 1.1×10^4 CFU/mL, which was acceptable due to the high expected counts. Plates with >2000 CFU were excluded due to confluence. Plates were assigned codes that corresponded to a known concentration and were blinded to prevent documentation and reporter bias.

2.4. Analysis

The concentration–effect relationship was analysed using GraphPad Prism 7 [11] software to identify the curve of best fit. To determine the concentration–effect relationship, effect was expressed as percentage-kill/h (E_c). Kill percentage was calculated as in Supplementary Equation S2. Mean percentages were calculated among the three repetitions for each organism. Results were expressed graphically as E_c versus concentration:MIC ratio, and a subsequent Akaike's information criteria comparison of fit analysis (AICc) was performed in GraphPad Prism 7 [11].

A log CFU/mL versus time calculation was conducted to determine whether a 3-log reduction [12] was reached to identify the breakpoint for each bacteria. Colony counts of each respective concentration–exposure growth (G_n) were compared with the control growth (Ct_x) to determine the relative reduction. Concentration–time interval growth (CFU_n) was calculated by multiplying the previous CFU/mL interval (CFU_{n-1}) by the relative reduction of antibiotic exposure growth compared with the control (G_n/Ct_x), expressed as in Supplementary Equation S3. Averages for each respective interval growth were taken before analysing the overall profile. This was repeated for each respective $fT_{>MIC}$ profile for each micro-organism. Subsequently, CFU/mL was compared against time by using calculated CFU_0, CFU_{0-1}, CFU_{1-2}, CFU_{2-4}, CFU_{4-6} and CFU_{6-8} consecutive time points. Missing data for excluded plates were averaged among other plates for relative reduction for that interval. The highest $fT_{>MIC}$ to be lower than

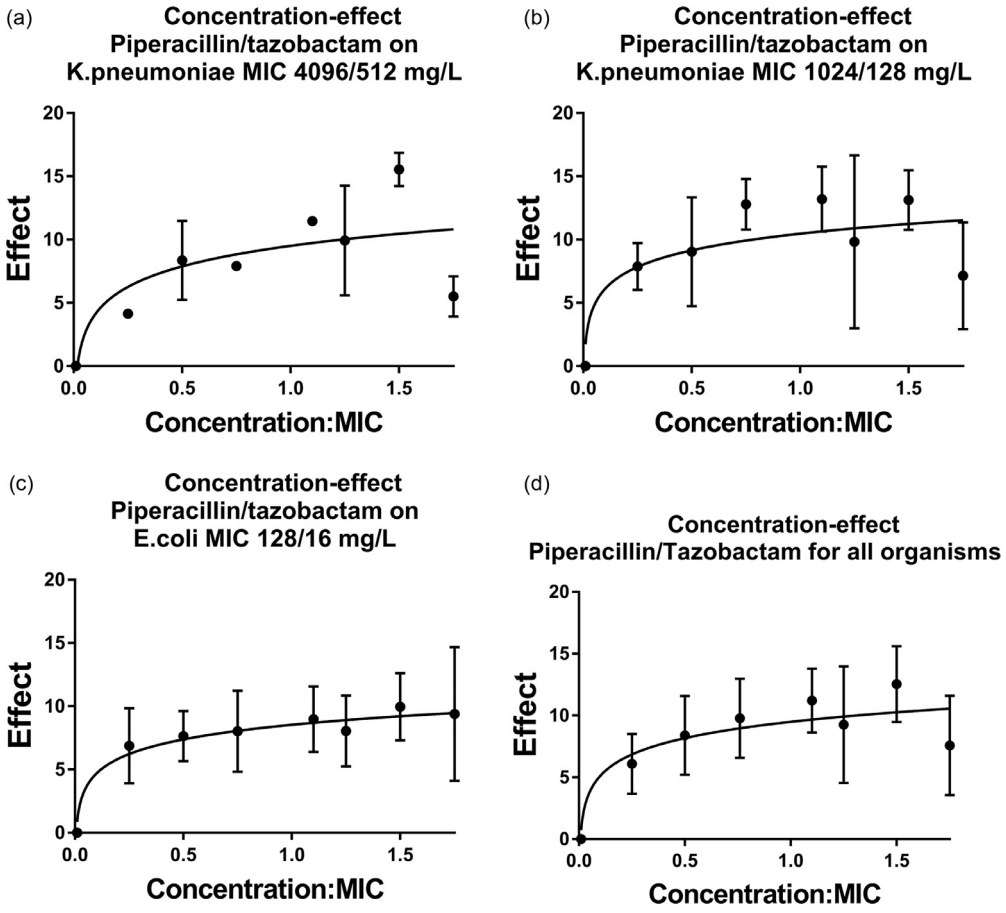

Fig. 1. Concentration–effect of piperacillin/tazobactam (TZP). (a) Concentration–effect of TZP on *Klebsiella pneumoniae* with a minimum inhibitory concentration (MIC) of 4096/512 mg/L. Goodness-of-fit Akaike's information criteria comparison of fit (AICc) analysis, 88.31% probability semi-log line is more predictive than line. Semi-log line [$Y = 5.391\log(X) + 9.506$], degrees of freedom (d.f.) = 23, coefficient of determination (R^2) = 0.3466, standard error of estimate (Sy.x) = 3.505. Line ($Y = 4.911X + 4.06$), d.f. = 23, $R^2 = 0.2319$, Sy.x = 3.8. The mean effect for *K. pneumoniae* 4096/512 mg/L was 4.14%, 7.31%, 7.91%, 11.45%, 11.45%, 12.77% and 15.75% for concentrations of 1024/128, 2048/256, 3072/384, 4505.6/563.2, 5120/640, 6144/768 and 7168/895.25 mg/L, respectively. (b) Concentration–effect of TZP on *K. pneumoniae* (MIC = 1024/128 mg/L). Goodness-of-fit AICc analysis, 86.08% probability semi-log line is more predictive than line. Semi-log line [$Y = 4.371\log(X) + 10.47$], d.f. = 25, $R^2 = 0.1609$, Sy.x = 4.558. Line ($Y = 1.925X + 8.038$), d.f. = 25, $R^2 = 0.03962$, Sy.x = 4.877. *Klebsiella pneumoniae* 1024/128 mg/L was shown to have a mean effect of 5.82%, 6.57%, 9.80%, 9.48%, 10.04%, 9.50% and 7.13% for concentrations of 256/32, 512/64, 768/96, 1126.4/140.8, 1280/160, 1536/192 and 1792/224 mg/L. (c) Concentration–effect of TZP on *Escherichia coli* (MIC = 128/16 mg/L). Goodness-of-fit AICc analysis, 90.74% probability semi-log line is more predictive than line. Semi-log line [$Y = 3.838\log(X) + 8.534$], d.f. = 25, $R^2 = 0.3005$, Sy.x = 2.723. Line ($Y = 2.565X + 5.529$), d.f. =25, $R^2 = 0.1716$, Sy.x = 2.964. *Escherichia coli* 128/16 mg/L was sown to have a mean effect of 6.88%, 6.01%, 8.02%, 8.98%, 9.25%, 9.95% and 9.39% for concentrations of 32/4, 64/8, 96/12, 140.8/17.6, 160/20, 192/24 and 224/28 mg/L, respectively. (d) Concentration–effect of TZP on all organisms. Goodness-of-fit AICc analysis, 95.08% probability semi-log line is more predictive than line. Semi-log line [$Y = 4.38\text{three-log}(X) + 9.489$], d.f. = 75, $R^2 = 0.139$, Sy.x = 3.724. Line ($Y = 2.18X + 6.944$), d.f. =75, $R^2 = 0.07021$, Sy.x = 3.87. Effect is the kill rate in percentage reduction in log CFU/mL/h. Concentration:MIC is the ratio of TZP concentration to organism MIC. •, mean percentage-kill/h; –, line of best fit through points. Effect is in the units of percentage reduction of CFU/mL/h.

the 3-log reduction (CFU_0–3) at $CFU_{6–8}$ was the PD breakpoint for that bacterium.

To determine the overall PD breakpoint among all bacteria and $fT_{>MIC}$ profiles, a linear regression analysis was conducted evaluating $\log_{10}(CFU_0–CFU_8)$ versus $fT_{>MIC}$. The intercept between the 3-$\log_{10}$ reduction line and the linear regression curve enabled calculation of the breakpoint, and statistical significance was subsequently analysed using Fisher's exact test.

3. Results

3.1. Concentration–effect relationship

TZP was shown to maintain time dependence in all experiments. Effect was shown to plateau above the MIC for each organism. Similar trends were noted amongst each bacteria with similar mean effects relative to the concentration:MIC ratios.

A preferred semi-logarithmic concentration–effect relationship was described for all organisms investigated (Fig. 1). AICc comparison of fit analysis showed an 88.31%, 86.08% and 90.74% probability that a semi-logarithmic line fitted the data better than a line for *K. pneumoniae* 4096/512 mg/L, *K. pneumoniae* 1024/128 mg/L and *E. coli* 128/16 mg/L, respectively, showing that time dependence was most likely upheld in these experiments. When all organism data were pooled, the overall model for the sample showed a 95.08% probability that semi-log was a better fit than line, indicating that it is likely that the plateau effect above the MIC was reached, maintaining the concentration–effect paradigm of time dependence. Amongst the equations for each model, there was variability of 0.623 for slope and 0.937 for *Y* intercept, showing that the results were consistent between bacteria.

3.2. Pharmacodynamic breakpoint determination

For $fT_{>MIC}$ profiles of 0%, 12.5%, 25%, 37.5%, 50%, 62.5%, 75%, 87.5% and 100%, the AUC ratio for static compared with dynamic profiles was calculated to be 1.00, 0.86, 0.83, 0.92, 0.89, 0.96, 0.92, 1.00 and 0.96, respectively. Among all profiles, the mean AUC ratio was determined to be 0.93, which was acceptable for experiments.

Table 1
Pharmacodynamic (PD) breakpoints for individual bacteria. The table shows each individual organism with $fT_{>MIC}$ profiles 0–100% with calculated log(CFU_0–CFU_8) values. Values >3-log reduction are coloured in white and values <3-log reduction are bold face. The lowest $fT_{>MIC}$ to be >3 was the PD breakpoint, shown as $fT_{>MIC}$ 37.5%, 37.5% and 50% for *Klebsiella pneumoniae* 4096/512 mg/L, *K. pneumoniae* 1024/128 mg/L and *Escherichia coli* 128/16 mg/L, respectively.

Organism (MIC in mg/L)	$fT_{>MIC}$								
	0%	12.5%	25%	37.5%	50%	62.5%	75%	87.5%	100%
K. pneumoniae 4096/512	**1.87**	**2.62**	**2.62**	3.14	3.14	3.50	3.50	3.93	3.93
K. pneumoniae 1024/128	**1.59**	**2.86**	**2.90**	3.09	3.13	3.10	3.13	3.08	3.11
E.coli 128/16	**2.44**	**2.84**	**2.87**	**2.98**	3.01	3.02	3.30	3.15	3.17

$fT_{>MIC}$, fraction of time the free drug concentration remains above the MIC over the dosing interval; MIC, minimum inhibitory concentration.

TZP was calculated to have a PD breakpoint of 50% $fT_{>MIC}$ for *E. coli* 128/16 mg/L. The average control growth at time zero was determined to be 6.1×10^5 CFU/mL. The $fT_{>MIC}$ profiles of >50% resulted in a >3-log_{10} reduction indicative of a clinical effect (Table 1).

Conversely, *K. pneumoniae* 4096/512 mg/L and *K. pneumoniae* 1024/128 mg/L were calculated to have a PD breakpoint of 37.5% $fT_{>MIC}$. The average control growth at time zero was determined to be 8.2×10^5 CFU/mL and 4.8×10^5 CFU/mL, respectively. For each micro-organism, the $fT_{>MIC}$ profiles >37.5% resulted in a >3-log_{10} reduction indicative of a clinical effect (Table 1).

The overall $fT_{>MIC}$ PD breakpoint was determined to be 49.85 ± 16.9% $fT_{>MIC}$ (Fig. 2). Linear regression analysis of log_{10}(CFU_0–CFU_8) versus $fT_{>MIC}$ described the line to be $Y = 0.01172X + 2.416$. The 3-log reduction line was noted at $Y = 3$. The intercept between the two lines was determined to be the PD breakpoint, which was calculated to be 49.85 ±16.9% $fT_{>MIC}$ ($P < 0.0001$ Fisher's exact test) (Fig. 2). This suggests that over an 8-h dosing interval, the pre-established $fT_{>MIC}$ breakpoint of 50% is likely to be maintained in bacteria with high MICs.

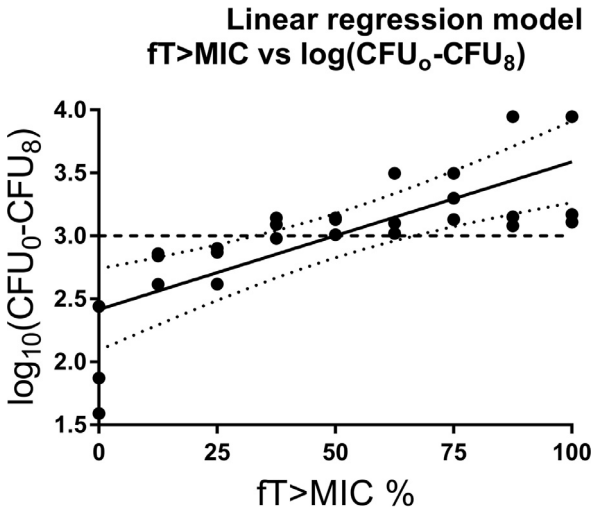

Fig. 2. Piperacillin/tazobactam (TZP) linear regression model of $fT_{>MIC}$ vs. log(CFU_0–CFU_8). Linear regression model for log(CFU_0–CFU_8) versus $fT_{>MIC}$ profiles. Line: $Y = 0.01172 \pm 0.002294X + 2.416 \pm 0.1365$ (—), coefficient of determination (R^2) = 0.7885, standard error of estimate (Sy.x) = 0.221. $Y = 3$ (•••••••), $R^2 = 1$, Sy.x = 0. Intercept of lines is 49.85 ± 16.9% $fT_{>MIC}$ ($P < 0.0001$, Fisher's exact test). Log_{10}(CFU_0–CFU_8) is the difference in log_{10} CFU/mL from time zero (C_0) and time at 8 h post dose (CFU_8). $fT_{>MIC}$, fraction of time the free drug concentration remains above the minimum inhibitory concentration over the dosing interval. •, mean log_{10}(CFU_0–CFU_8) for each organism's respective $fT_{>MIC}$; —, line of best fit through points; •••••••, confidence interval; – – –, 3-log_{10} reduction line.

4. Discussion

4.1. Implications of results

TZP was observed to have a similar concentration–effect relationship in resistant bacteria to that of susceptible organisms. The present study observed a semi-logarithmic concentration–effect relationship for all resistant organisms as anticipated for β-lactam antibiotics. Pooled data from all concentration–effect curves for all organisms achieved a probability of >95% in favour of a semi-logarithmic curve versus a line. This is consistent with other studies where concentration–effect described a maximum efficacy at higher concentration:MIC ratios ranging from 1 to 5 [5,13]. The maximum efficacy in the present study approached a kill rate of 10% log CFU/mL/h (equivalent to 1.6 log CFU/mL/h). This was similar to Hyatt et al. who approached 2 log CFU/mL/h between 2–50 × MIC [13]. The present study only investigated concentration:MIC ratios between 0.25–1.75, which may explain why 2 log CFU/mL/h was not attained. It should be noted that Hyatt et al. investigated the effect of piperacillin on *Staphylococcus aureus* and not Enterobacteriaceae [13]. We believe that these results are representative of what is expected for time-dependent antimicrobials, validating our aim of determining the $fT_{>MIC}$ for efficacy and suggesting that we can treat 'resistant' organisms similarly to susceptible bacteria in the context of high drug concentrations.

The major PD breakpoint in the current study was determined to be an $fT_{>MIC}$ of 49.85 ± 16.9%. Many PD breakpoints have been noted for β-lactam antibiotics over the years, ranging from $fT_{>MIC}$ of 27% to 100% [14]. The most commonly applied $fT_{>MIC}$ used for many years has been a target of 50% over the dosing interval [7,14]. In the present study, the $fT_{>MIC}$ breakpoints remained relatively consistent among repetitions and between bacteria, being 37.5%, 37.5% and 50% for *K. pneumoniae* 4096/512 mg/L, *K. pneumoniae* 1024/128 mg/L and *E. coli* 128/16 mg/L, respectively. The overall $fT_{>MIC}$ target was determined graphically to be 49.85% (confidence interval 38–72%), encompassing the range of targets attained in past studies. Overall, we were able to effectively describe the concentration–effect relationship of TZP on resistant Enterobacteriaceae and ultimately identify the PD breakpoint for efficacy. These results, in combination with pharmacokinetic data, demonstrate that it may be possible to effectively cure TZP-resistant Enterobacteriaceae (by current microbiological classification) UTIs with TZP, therefore providing us with another way to effectively treat resistant UTIs in the context of limited antimicrobial options.

4.2. Methodology and limitations

The methodology in the present study utilised concepts in static time–kill experiments in order to describe a dynamic model. Nielsen et al. suggested that static–time kill experiments predicted results with reasonable accuracy compared with dynamic experiments [15]. The calculated mean AUC ratio, comparing static to dynamic profiles, was determined to be 93% among $fT_{>MIC}$ profiles and was considered acceptable for experiments. Overall, we feel that the static time–kill model is representative of what would be expected of a dynamic model.

Static time–kill studies have limitations in methodology that may have had an impact on the results. Limitations of the study design were the generalisability owing to other bacterial resistance

mechanisms [16] and the physiological conditions of the bladder environment. To make the results more robust, we would have liked to have studied other resistant Enterobacteriaceae with a variety of MICs; however, this study focused on the most common UTI pathogens. Static time–kill experiments do not take into account protein binding [6,12] that may occur in the bladder. However, the extent remains unknown. A limitation in determining the PD target was that time intervals only allowed for profile intervals in increments of 12.5%, which would need to be narrower to identify a more accurate target. This was accounted for in the results by analysing the $\log_{10}(CFU_0–CFU_8)$ of all organisms in order to determine log reduction, similar to Sy et al. [17], and thus the determining the breakpoint. The narrow concentration:MIC ranges used were based on recently described urinary pharmacokinetics [8], thus focusing on concentrations known to be achievable clinically. A major limitation in past in vitro experiments was antibiotic carryover [12]. The present study methods utilised principles and methodologies noted in Rees et al. [18] to overcome antibiotic transfer. Centrifugation and re-suspension processes were used in limiting the antibiotic transfer to $<0.02 \times$ MIC in this study, limiting overestimation of results. Despite some limitations in static–time kill experiments, the current methodology was aimed at optimising determination of the PD target rather than simulating urinary conditions, which would better evaluated in animal models [19].

4.3. Conclusion and future directions

In summary, the targets obtained in the present study offer substantial insight into treating resistant UTIs with TZP. Based on the described time-dependent nature of β-lactam antibiotics, it was observed that bactericidal activity against Enterobacteriaceae that are conventionally deemed resistant was achieved at 49.85 $\pm$ 16.9% $fT_{>MIC}$. This suggests that highly resistant urinary organisms with MICs up to 4096/512 mg/L could be treated with 4.5 g TZP with a 30-min infusion, and furthermore may be made more effective by prolonging the infusion [14]. This calls into question the existing urine microbiological breakpoints of >32/4 mg/L and >16/4 mg/L for CLSI and EUCAST, respectively. The current study reinforces the previous PD target of 50% $fT_{>MIC}$ as we were able to demonstrate a 3-$\log_{10}$ bacterial reduction with this profile. In the context of UTIs, however, future studies are required to elucidate whether different molecular resistance mechanisms require different PD breakpoints. In addition, in vivo animal models are required to determine whether protein binding impacts on efficacy within the bladder environment.

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Competing interests

MG completed this research as part of his MD project at the University of Notre Dame, School of Medicine Sydney. All other authors declare no competing interests.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.05.013.

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