In vitro activity of piperacillin/tazobactam and ertapenem against *Bacteroides fragilis* and *Escherichia coli* in pure and mixed cultures

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Ertapenem and piperacillin/tazobactam are β -lactam antibiotics with a broad spectrum of activity used for the treatment of mixed infections in which *Bacteroides fragilis* and *Escherichia coli* play an important aetiological role. In this study, the activities of piperacillin/tazobactam and ertapenem (MIC and time–kill kinetics) against these bacteria were compared. MICs were determined by the agar dilution method, and the time and slope of time–kill curves were analysed. In the *in vitro* pharmacodynamic assays, pure and mixed cultures of *E. coli* and *B. fragilis* were exposed to peak concentrations of ertapenem ($8.0 \mu\text{g ml}^{-1}$) and piperacillin/tazobactam ($64.0/8.0 \mu\text{g ml}^{-1}$) for 48 h. Treatment with ertapenem reduced the viability of *E. coli* and/or *B. fragilis* by 3 logs in all experiments, whereas piperacillin/tazobactam only affected the viability of *B. fragilis*. Both drugs exhibited their fastest rates of killing when bacteria were grown in mixed cultures. According to the results, ertapenem exhibited activity similar to that of piperacillin/tazobactam against *B. fragilis* alone or in mixed culture. However, ertapenem exhibited a markedly higher activity against *E. coli* alone or in combination with *B. fragilis* relative to piperacillin/tazobactam.

Received 7 December 2006

Accepted 18 February 2007

INTRODUCTION

Complicated intra-abdominal infections require a combination of surgery/drainage and antimicrobial therapy. The choice of antimicrobial therapy must take into account the complex normal aerobic and anaerobic microbiota of the bowel, which reach population levels of up to 10^{11} c.f.u. (g faeces) $^{-1}$ (Solomkin & Konstantin, 2003). *Bacteroides fragilis*, an obligate anaerobe, and *Escherichia coli*, a facultative anaerobe, are the most frequently isolated bacteria from intra-abdominal abscesses (Goldstein & Snyderman, 2004). Thus the presence of mixed infections of anaerobic and facultative bacteria with different susceptibilities often requires the use of either dual therapy, e.g. a penicillin plus an aminoglycoside, or broad-spectrum antibiotics, such as carbapenems or β -lactam/ β -lactamase inhibitor combinations including piperacillin/tazobactam (Perry & Markham, 1999; Solomkin & Konstantin, 2003).

Ertapenem is a new carbapenem with a broad spectrum of activity against Gram-negative pathogens, including extended-spectrum β -lactamase- and AmpC-producing members of the *Enterobacteriaceae*, as well as against

Gram-positive aerobic and anaerobic pathogens (Goldstein & Snyderman, 2004; Wexler, 2004). Clinical trials in complicated intra-abdominal infection have demonstrated that ertapenem has equivalent efficacy and safety to piperacillin/tazobactam (Solomkin *et al.*, 2003; Dela Pena *et al.*, 2006).

The *in vitro* activities of piperacillin/tazobactam and ertapenem have been reported in MIC and time–kill studies using single cultures (Muñoz Bellido *et al.*, 1997; Spangler *et al.*, 1997). However, the killing of bacteria in pure culture may not be identical to the killing in mixed culture (Stearne *et al.*, 2001; Hermesen *et al.*, 2005; Schaumann *et al.*, 2005).

In the present study, an anaerobic time–kill technique was used for a comparative study of the *in vitro* activities of piperacillin/tazobactam and ertapenem against *B. fragilis* and *E. coli* in pure or mixed cultures.

METHODS

Bacterial strains. *B. fragilis* ATCC 25285 and *E. coli* ATCC 25922 were used throughout the experiments. The obligate anaerobe *B. fragilis* was incubated anaerobically at 37 °C in an anaerobic chamber

Abbreviation: LLA, lower limit of accuracy.

(Forma Scientific Company) containing an atmosphere of 85 % N₂, 10 % H₂ and 5 % CO₂, and the facultative anaerobe *E. coli* was incubated aerobically or anaerobically at 37 °C.

Antibiotics. A piperacillin/tazobactam preparation (Tazocin) was purchased commercially (Lederle Piperacillin) and ertapenem sodium was kindly provided as a standard powder by Merck Sharp & Dohme. Antibiotic solutions were prepared according to the instructions of the manufacturer.

Susceptibility testing. The MICs of the test drugs were determined by the agar dilution method using a standard inoculum ($\sim 10^5$ c.f.u.) and a higher inoculum size ($\sim 10^8$ c.f.u.), according to protocols M7-A6 and M11-A6 of the Clinical Laboratory Standards Institute (formerly the NCCLS) (NCCLS, 2003, 2004).

Time–kill curve. Time–kill curves were obtained using peak concentrations of the indicated antibiotics in serum (8.0 $\mu\text{g ml}^{-1}$ and 64.0/8.0 $\mu\text{g ml}^{-1}$ for ertapenem and piperacillin/tazobactam, respectively). Prior to the time–kill curve experiments, several colonies of each bacterial strain were incubated anaerobically overnight in 10 ml Brucella broth. Each overnight culture was then adjusted to an OD₅₅₀ of 0.05 with fresh Brucella broth to yield a starting inoculum of approximately 10^6 c.f.u. ml^{-1} . This concentration was confirmed by 10-fold serial dilution. Cultures were incubated at 37 °C in an anaerobic chamber. When the bacterial population reached the exponential growth phase (approx. 150 min for *E. coli* and 7 h for *B. fragilis*), ertapenem or piperacillin/tazobactam solutions were added to each test vial to produce antibiotic concentrations of 8.0 $\mu\text{g ml}^{-1}$ (ertapenem) or 64.0/8.0 $\mu\text{g ml}^{-1}$ (piperacillin/tazobactam). No antibiotic was added to the control vial. Cultures were then incubated at 37 °C under anaerobic conditions. These procedures were followed for both organisms during the mixed-culture experiments. In this case, when the bacterial population reached the exponential growth phase, an equal volume of each strain was added to a new vial inoculated with antibiotics, as described above. At pre-determined time points of 0, 1, 2, 3, 4, 5, 6, 24 and 48 h following the introduction of antibiotics into the vials, 1 ml samples of the cultures were withdrawn aseptically for bacterial quantification. Bacterial counts were determined by 1 : 10 serial dilution in sterile saline and a 0.1 ml sample of each dilution was plated onto trypticase soy agar for *E. coli* or Brucella agar for *B. fragilis*. In the mixed-culture experiments, MacConkey agar was used to count the number of colonies of *E. coli* after aerobic incubation for 24 h at 37 °C, whilst *Bacteroides* bile aesculin agar was used to count *B. fragilis* colonies after anaerobic incubation for 48 h at 37 °C. Antibiotic carry-over was addressed by using saline dilution techniques. The lower limit of accuracy (LLA) of bacterial counting was 300 c.f.u. ml^{-1} .

Statistical analysis. Colony count data (in log₁₀ c.f.u. ml^{-1}) from duplicate time–kill studies were averaged and plotted as a function of time. The slopes of the time–kill curves were compared for a rate-of-killing analysis using a linear model with ORIGIN software (version 6.1; OriginLab Corporation). Comparison of the mean reduction in bacterial counts of the pure cultures of *B. fragilis* and *E. coli* with that of the corresponding mixed cultures was analysed using a two sample paired *t*-test. A *P* value of <0.05 was considered to be significant.

RESULTS AND DISCUSSION

The MICs of piperacillin/tazobactam have been reported to be strongly affected by an increase in inoculum size (König *et al.*, 1998; Betriu *et al.*, 2006), whilst ertapenem susceptibility with high inoculum did not change (Betriu *et al.*,

2006). We found a moderate effect of the inoculum on the killing of both *B. fragilis* and *E. coli* as indicated by the increase in MICs when the inoculum was increased from 10^5 to 10^8 c.f.u. ml^{-1} . However, these values remained below the susceptible piperacillin/tazobactam breakpoint for both bacteria (Table 1).

Time–kill curve results are presented in Figs 1 and 2. Bacterial interactions in mixed infections can interfere with antimicrobial therapy (Heilmann, 1993; Pendland *et al.*, 2002). In addition to β -lactamase production by bacteria involved in mixed infections, other factors such as bacteriocin production (Jerman *et al.*, 2005) can alter bacterial susceptibility. In the present study, the bactericidal effect of ertapenem and piperacillin/tazobactam in the mixed culture was more evident than in the pure culture (*P* < 0.05). The bactericidal effect against *B. fragilis* was detected at <2 h in mixed culture, but only after 6 h in pure culture. In addition, the number of viable *B. fragilis* cells almost reached the LLA within 6 h of exposure to ertapenem in mixed culture (Fig. 1). Similar data were recently described by Hermesen *et al.* (2005) in assays testing moxifloxacin against the same two bacteria. The time–kill curves showed that the decrease in the count of viable cells occurred more rapidly in the first 2 h, in both pure and mixed cultures (Figs 1 and 2). Therefore, in order to determine the rate of bacterial death in this time interval, we calculated the slope of the time–kill curve by using data for the 0, 1 and 2 h time points. All coefficients of determination in the linear regression analysis were greater than 0.93, with the exception of that for piperacillin/tazobactam against *E. coli* in mixed culture with *B. fragilis* ($r^2=0.82$). This analysis confirmed that a more rapid rate of killing occurred in the mixed culture (Table 2).

The time–kill curve patterns for ertapenem and piperacillin/tazobactam against *B. fragilis* in pure or mixed culture were similar (Fig. 1). In both cultures, ertapenem and piperacillin/tazobactam exhibited a marked bactericidal effect

Table 1. MICs ($\mu\text{g ml}^{-1}$) of ertapenem and piperacillin/tazobactam against *E. coli* ATCC 25922 and *B. fragilis* ATCC 25285

Antibiotic	Bacterial inoculum (c.f.u. ml^{-1})			
	<i>E. coli</i>		<i>B. fragilis</i>	
	10^5	10^8	10^5	10^8
Ertapenem*	0.016	0.016	0.12	0.12
Piperacillin/tazobactam†	2.0/0.25	8.0/1	0.25/0.03	1/0.12

*Breakpoints for ertapenem: *B. fragilis* ≤ 4.0 $\mu\text{g ml}^{-1}$; *E. coli* ≤ 2.0 $\mu\text{g ml}^{-1}$.

†Breakpoints for piperacillin/tazobactam: *B. fragilis* $\leq 32.0/4.0$ $\mu\text{g ml}^{-1}$; *E. coli* $\leq 16.0/4.0$ $\mu\text{g ml}^{-1}$.

Fig. 1. Time-kill curves of ertapenem (●, 8.0 µg ml⁻¹) and piperacillin/tazobactam (▲, 64.0/8.0 µg ml⁻¹) against *B. fragilis* alone (a) and in mixed culture with *E. coli* (b). A growth control (■) and the LLA (dashed line) are shown.

against *B. fragilis*, defined by a ≥ 3 log decrease in the bacterial count relative to the starting inoculum, supporting their clinical use against these bacteria (Sader *et al.*, 1998; Goldstein *et al.*, 2000).

Ertapenem also exhibited a bactericidal effect against *E. coli* in both mixed and pure culture (Fig. 2). A ≥ 3 log decrease in bacterial count was reached with <2 h of drug exposure. In contrast, piperacillin/tazobactam exhibited a bacteriostatic effect against *E. coli* in both mixed and pure culture, as defined by a <2 log decrease from the starting inoculum (Fig. 2). Although the MIC recorded for piperacillin/tazobactam against the *E. coli* strain used in this study was within the sensitivity limits, the results obtained from the bacterial kill curve do not support this susceptibility pattern, as the antimicrobial concentrations used were at least 32-fold higher than the MIC. A similar discrepancy between *in vitro* antimicrobial activity obtained from the bacterial kill curve and susceptibility tests has been described and attributed to the inoculum effect (Klepser *et al.*, 1996; Stearne *et al.*, 2001; Hermesen *et al.*, 2005).

Fig. 2. Time-kill curves of ertapenem (●, 8.0 µg ml⁻¹) and piperacillin/tazobactam (▲, 64.0/8.0 µg ml⁻¹) against *E. coli* alone (a) and in mixed culture with *B. fragilis* (b). A growth control (■) and the LLA (dashed line) are shown.

König *et al.* (1998) demonstrated that the activity of piperacillin was diminished at least 128-fold with high inocula of *E. coli* ATCC 25922, the same strain as used in this study. In addition, Thomson & Moland (2001) demonstrated the *in vitro* sensitivity of piperacillin/tazobactam to the inoculum effect of extended-spectrum β -lactamase-producing bacteria.

Table 2. Killing rates of *E. coli* ATCC 25922 and *B. fragilis* ATCC 25285 in pure or mixed culture during the first 2 h of drug exposure

Antibiotic	Killing rate ($\log_{10}$ c.f.u. h ⁻¹ ml ⁻¹)		
	<i>E. coli</i>	<i>B. fragilis</i>	<i>E. coli/B. fragilis</i>
Ertapenem	-1.800	-0.959	-2.045/-1.751
Piperacillin/tazobactam	-0.386	-1.048	-0.719/-1.562

In the present study, large numbers of actively growing cells ($\sim 10^8$ c.f.u. ml⁻¹) were used to simulate *in vivo* conditions, as abscesses contain high numbers of bacteria in a stationary phase of growth (Goldstein & Snyderman, 2004). These high inocula of *E. coli* might be responsible for the reduced activity of piperacillin/tazobactam, although this antimicrobial agent had been effective against the same high inocula of *B. fragilis*. Soriano *et al.* (1996) demonstrated therapeutic relevance of the inoculum effect using an experimental model of intraperitoneal infection by *E. coli* in non-neutropenic and neutropenic mice. Their results showed a better correlation between *in vitro* activity and efficacy when the MICs considered were those obtained with a large inoculum ($\sim 10^8$ c.f.u. ml⁻¹) instead of the standard inoculum ($\sim 10^5$ c.f.u. ml⁻¹). However, other studies suggest that inoculum effect is an artefact of *in vitro* susceptibility testing and is consequently of little clinical relevance (Craig *et al.*, 2004). Thus the available literature is still controversial and inconclusive about the inoculum effect on the activity of antimicrobial agents. A caveat of the present study is that only a single strain of *B. fragilis* and *E. coli* was examined and it remains to be determined whether the findings of this investigation extend to other strains.

In summary, the results of the present study showed similar activities of ertapenem and piperacillin/tazobactam against *B. fragilis* ATCC 25285, alone or in mixed culture with *E. coli*. However, ertapenem was markedly more efficient than piperacillin/tazobactam against *E. coli* ATCC 25922, in both pure and mixed culture.

ACKNOWLEDGEMENTS

The study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Merck Sharp & Dohme. The authors are grateful to Luzia Rosa Resende and José Sérgio Barros de Souza for technical support. This work was partially presented at the 106th General Meeting of the American Society for Microbiology, Orlando, FL, USA, 21–25 May 2006.

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