

AM-112, a novel oxapenam  $\beta$ -lactamase inhibitor with unexpected synergistic activity with ceftazidime against Enterococci, including some vancomycin-resistant isolatesC E Jamieson<sup>1</sup>, P A Lambert<sup>1</sup>, R Hakenbeck<sup>2</sup> & I N Simpson<sup>3</sup><sup>1</sup>Aston University, Birmingham, B4 7ET, UK; <sup>2</sup>Universitat Kaiserslautern, Kaiserslautern, Germany; <sup>3</sup>Micron Research, Cambridge CB3 7ES, UKP A Lambert  
Aston University  
Birmingham B4 7ET  
Tel. +44 121 359 3611 x4471  
Fax. +44 121 359 0572  
p.a.lambert@aston.ac.uk  
http://www.aston.ac.uk/pharmacy

## INTRODUCTION

Enterococci, usually found in the human colon, are opportunistic pathogens which cause infections of the urinary tract, bloodstream and endocarditis. Enterococcal bacteraemia is associated with high mortality in severely compromised patients and is a challenge to chemotherapy. The emergence of vancomycin-resistant enterococci (VRE) complicates the situation.

Enterococcal species vary in their susceptibility to  $\beta$ -lactam antibiotics. While strains are often sensitive to penicillins, cephalosporins are generally inactive. This lack of cephalosporin activity is due to the poor affinity of cephalosporins for enterococcal penicillin binding proteins (PBPs). AM-112, an oxapenam, is a novel broad-spectrum  $\beta$ -lactamase inhibitor. Although AM-112 has potent activity against staphylococci (MICs 0.5 – 2 mg/L and streptococci (MICs 0.12 – 0.5 mg/L), it is relatively inactive against enterococci (MICs  $\geq$ 32 mg/L). Surprisingly, we have detected synergistic activity between AM-112 and ceftazidime against some enterococci.

The objectives of the study were to:

1. Investigate the mechanism of synergy between AM-112 and ceftazidime against enterococci.
2. Determine the optimum ratio of ceftazidime to AM-112.
3. Investigate the rate of kill of enterococci.

## METHODS

## Strains

Eighteen enterococcal strains were used, including isolates of *E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, *E. gallinarum* and 4 vancomycin resistant isolates (Table 1). All strains were  $\beta$ -lactamase negative in a nitrocefin test.

## Susceptibility Tests

Minimum inhibitory concentrations (MICs) were determined by broth microdilution in accordance with NCCLS guidelines. In addition to single agent tests, ceftazidime MICs were determined in the presence of a fixed 4mg/L concentration of AM-112 or clavulanic acid. Checkerboard studies were also conducted in which the concentration of both ceftazidime and AM-112 were varied.

## Rate of kill studies

Enterococci were adjusted to give a final inoculum of approximately  $10^5$  cfu/ml in shake flasks containing ceftazidime, AM-112 or 2:1 combinations of ceftazidime:AM-112. Samples were removed on an hourly basis, diluted and the viable count determined.

## PBP affinities

Cell membrane extracts of *E. faecalis* SFZ were incubated with AM-112 for 30 minutes at 37°C. <sup>3</sup>H-propionylampicillin (80-100Ci/mmol) was then added and the membranes were further incubated at 37°C for 90 minutes. Labelled proteins were separated by SDS-PAGE using 7.5% polyacrylamide gels. Labelled gels were exposed to X-ray film after being soaked in Enhance (Bio-Rad) and dried. The PBPs were visualised by fluorography.

## RESULTS

## MIC determinations

- Ceftazidime and clavulanic acid were inactive (MICs  $>$ 64 mg/L) against all test strains (Table 1). AM-112 exhibited poor/moderate activity with MICs of 16 –  $>$ 128 mg/L.
- A fixed concentration of 4 mg/L clavulanic acid reduced the ceftazidime MIC for three vancomycin sensitive isolates of *E. faecalis* between 2 and 4-fold, but did not enhance the activity of ceftazidime against any of the other strains.
- At a fixed concentration of 4 mg/L, AM-112 greatly reduced the ceftazidime MIC against all vancomycin susceptible isolates regardless of species, although the most pronounced effect was against *E. faecalis*. In some cases there was a variable endpoint between occasions; generally there was no growth at  $>$ 0.06 mg/L ceftazidime but on some a ceftazidime MIC of 8 mg/L was observed.
- At a fixed concentration of 4 mg/L, AM-112 also reduced the ceftazidime MIC from  $>$ 128 mg/L to  $\leq$ 0.06 mg/L against VRE 300 2043 and from  $>$ 64 mg/L to 16 mg/L against *E. faecalis* 78097, a vanB isolate, but had no effect on the remaining three VRE.

## Checkerboard studies

- Results are summarised in Table 2.
- For three of the four strains, including the vanB strain, the optimal ratio of ceftazidime to AM-112 was 2:1, resulting in MIC ratios of 16:8 or 8:4 mg/L.
- Against the van A strain, a combination of ceftazidime at 0.25 mg/L plus AM-112 at 32 mg/L was also synergistic.

## Rate of kill studies

- At predetermined MIC concentrations, ceftazidime (128 mg/L) and AM-112 (64 mg/L) exhibited a bacteriostatic effect upon vancomycin-susceptible isolates of *E. faecalis* (Figure 1a, b, c).
- However, a combination of ceftazidime at 16 mg/L and AM-112 at 8 mg/L (ie 1/8 MIC of each agent) reduced the viable count of both enterococci by at least 2 log cycles over 8 hours, after which regrowth occurred.
- At predetermined MIC concentrations, AM-112 (64 mg/L) exhibited a slight bacteriocidal effect against *E. faecalis* 78097, a van B isolate (Figure 1c). At 1/8 MIC of both ceftazidime and AM-112, a slightly greater bacteriocidal effect was observed.
- No synergistic effect was observed against *E. faecalis* 56059, a van A strain.

## Affinity for penicillin binding protein affinities

Competition of AM-112 with radiolabelled propionylampicillin for PBPs of *E. faecalis* SFZ

- Imipenem (MIC 0.25 mg/L) inhibited all PBPs at 0.1 – 0.3 mg/L (Figure 2).
- AM-112 (MIC 64 mg/L) inhibited all PBPs, except PBP 3, at 10 mg/L.
- Below 10 mg/L, AM-112 exhibited sequential inhibition of PBPs 7 and 8 at 0.03 mg/L, PBPs 1, 2, at 0.1 mg/L and PBPs 4, 5 and 6 at 2.6 mg/L.

Table 1. Minimum inhibitory concentrations of ceftazidime (CAZ), clavulanic acid (CLAV) and AM-112 alone, and in combination, against a panel of enterococci

Organism	MIC (mg/L)		MIC (mg/L)		MIC (mg/L)
	CAZ	CLAV	CAZ +CLAV at 4 mg/L	CAZ +AM-112 at 4 mg/L	
<b>Vancomycin susceptible isolates</b>					
<i>E. faecalis</i> Phillips	$>$ 128	$>$ 128	32	64	$\leq$ 0.06*
<i>E. faecalis</i> SFZ	$>$ 128	$>$ 128	64	32	$\leq$ 0.06*
<i>E. faecalis</i> NCTC 5957	$>$ 128	$>$ 128	32	64	$\leq$ 0.06*
<i>E. faecalis</i> 24952	$>$ 128	$>$ 128	32	$>$ 128	$\leq$ 0.06*
<i>E. hirae</i> ATCC 10541	$>$ 128	$>$ 128	16	$>$ 128	$\leq$ 0.06*
<i>E. faecium</i> 60060	64	$>$ 64	8	$>$ 64	4
<i>E. faecium</i> NCTC 7171	$>$ 128	$>$ 128	16	$>$ 128	$\leq$ 0.06*
<i>E. faecium</i> 60052	$>$ 64	$>$ 64	8	$>$ 64	4
<i>E. faecium</i> 78090	32	$>$ 64	16	$>$ 64	8
<i>E. gallinarum</i> 56070	$>$ 64	$>$ 64	16	$>$ 64	1
<i>E. gallinarum</i> 56072	$>$ 64	$>$ 64	16	$>$ 64	1
<i>E. casseliflavus</i> (van-I)	$>$ 64	$>$ 64	16	$>$ 64	1
<b>Vancomycin resistant isolates</b>					
<i>E. faecalis</i> 56059 (vanA)	$>$ 64	$>$ 32	$>$ 64	$>$ 64	$>$ 64
<i>E. faecalis</i> 78097 (vanB)	$>$ 64	$>$ 32	$>$ 64	$>$ 64	16
VRE 300 1562	$>$ 128	$>$ 128	128	$>$ 128	$>$ 128
VRE 300 1590	$>$ 128	$>$ 128	$>$ 128	$>$ 128	$>$ 128
VRE 300 1662	$>$ 128	$>$ 128	$>$ 128	$>$ 128	$>$ 128
VRE 300 2043	$>$ 128	$>$ 128	32	$>$ 128	$\leq$ 0.06

\* Trailing endpoint

Figure 1. Rate of kill studies: Bacteriocidal activity of ceftazidime and AM-112 individually and in combination against isolates of *E. faecalis*

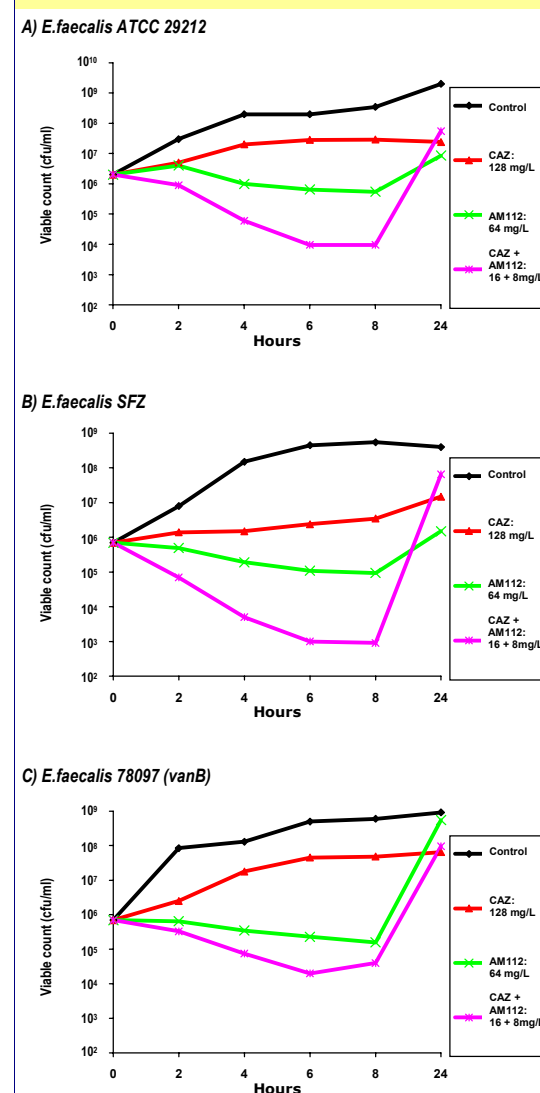


Figure 2. Comparative inhibition of enterococcal PBPs by imipenem and AM-112

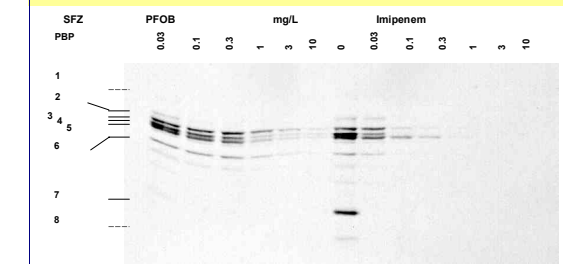


Table 2. Summary of checkerboard results

AM-112 (mg/L)	<i>E. faecalis</i> ATCC 29212	Ceftazidime MIC (mg/L)		<i>E. faecalis</i> 78097 (vanB)
		<i>E. faecium</i> ATCC 10541	<i>E. faecalis</i> 56059 (vanA)	
32	2	$<$ 0.06	0.25	4
16	8	4	$>$ 64	8
8	16	8	$>$ 64	16
4	32	$>$ 64	$>$ 64	16
2	$>$ 64	$>$ 64	$>$ 64	32
1	$>$ 64	$>$ 64	$>$ 64	$>$ 64
0.5	$>$ 64	$>$ 64	$>$ 64	$>$ 64
0	$>$ 64	$>$ 64	$>$ 64	$>$ 64

## CONCLUSIONS

- Individually, ceftazidime, clavulanic acid and AM-112 are essentially inactive against enterococci.
- Combinations of ceftazidime and AM-112, but not ceftazidime and clavulanic acid, act synergistically against enterococci.
- Checkerboard studies indicate that 2:1 is the optimum ceftazidime:AM-112 ratio for synergy.
- Viable count studies confirm that the synergistic effect is cidal for at least 8 hours, after which regrowth occurs. It will be interesting to study the effect of a second ceftazidime/AM-112 dose at eight hours as in the clinical situation.
- AM-112 binds to all eight PBPs of *E. faecalis* with a similar affinity profile to imipenem.
- Seven of the eight PBPs are completely inhibited by AM-112 at 2.6 mg/L; however unlike with imipenem, PBP 3 is not completely inhibited by AM-112 at 9.5 mg/L. These results suggest that PBP 3 is an essential PBP in enterococci.
- The synergy between AM-112 and ceftazidime indicates complementation against the essential PBPs of enterococci. This result offers the potential for extending the antibacterial spectrum of cephalosporins against this difficult pathogen.

Poster prepared by Micron Research