

INTRODUCTION

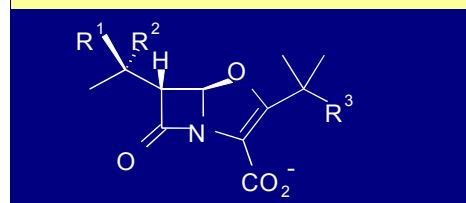
Enterococci are opportunistic pathogens, which can cause infections of the urinary tract, bloodstream and endocarditis. Enterococcal bacteraemia is associated with high mortality in severely compromised patients and is a challenge to chemotherapy. The emergence of vancomycin-resistant enterococci (VRE) was first observed in the UK in 1988 (1). Twenty-four percent of *Enterococcus faecium* isolates in the UK were resistant to vancomycin in 1998 (in 1993 this figure was 6.3 %) (2).

Enterococcal species vary in their susceptibility to β -lactam antibiotics. *E. faecalis* is sensitive to concentrations of ampicillin between 1 and 8 mg/L, while *E. faecium* requires concentrations of 16 to 64 mg/L to inhibit growth (3). Cephalosporins are generally inactive against enterococci, due to the poor affinity of these agents for enterococcal penicillin-binding proteins (PBPs) (4).

The oxapenems are novel broad-spectrum β -lactamase inhibitors, under development by Amura Ltd, Cambridge, UK. While β -lactamase production by enterococci is not normally detectable or inducible (3), we have observed a synergistic effect when ceftazidime was combined with these oxapenem β -lactamase inhibitors against some enterococcal strains. The objectives of this study were to:

1. Investigate the mechanism of synergy between four structurally similar oxapenems (Figure 1) and other β -lactams, particularly cephalosporins, against enterococci.
2. Determine the optimum ratio of ceftazidime to oxapenem AM-112.
3. Investigate the rate of kill of enterococci.

Figure 1. Chemical structures of oxapenems tested



Compound	R ¹	R ²	R ³
AM-112	OH	H	(CH ₂) ₂ NH ⁺
AM-113	OH	H	CH ₃
AM-114	H	OH	CH ₃
AM-115	H	OH	(CH ₂) ₂ NH ⁺

METHODS

Strains
The vancomycin-susceptible (Van-S) and resistant (Van-R) enterococcal strains used included isolates of *E. faecalis*, *E. faecium*, *E. hirae*, *E. gallinarum* and *E. casseliflavus*. Two of the Van-R isolates possessed *vanA* or *vanB* mediated resistance. All strains were negative for β -lactamase when tested with Nitrocefin.

Susceptibility tests
Minimum inhibitory concentrations (MICs) were determined by broth microdilution carried out in accordance with NCCLS guidelines. Combinations of oxapenem and other β -lactams were tested using the oxapenem at a fixed concentration of 4 mg/L, 1:1 ratio, 1:2 ratio or over a wide range of ratios using checkerboards.

Rate of kill studies
Enterococci cultures were adjusted to give a final inoculum of approximately 10⁵ 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 was determined.

PBP affinities
Cell membrane extracts of *E. faecalis* SFZ and *E. faecalis* ATCC 29212 were incubated with AM-112 or imipenem for 30 minutes at 37°C. ³H-propionylampicillin (80-100 Ci/mmol) was then added and the membranes were further incubated for 90 minutes at 37°C. Labelled proteins were then separated by SDS-PAGE using 7.5% polyacrylamide gels. Labelled gels were then exposed to X-ray film after being soaked in Enhance (Bio-Rad) and dried. The PBPs were visualised by fluorography.

RESULTS

Activity of ceftazidime (CAZ) and oxapenems alone and in 1:1 or 2:1 combination against enterococci (Table 1).

- ▶ Alone, CAZ, AM-114 and AM-115 lacked any activity (MICs \geq 32 mg/L) against the enterococci.
- ▶ AM-112 was active against two strains, while AM-113 had a MIC range of 2 to 16 mg/L against all strains tested.
- ▶ Combinations of CAZ and AM-112, at a ratio of 1:1 and 2:1, exhibited marked synergy against all vancomycin susceptible (Van-S) isolates, with MIC reductions of \geq 4 fold. At a ratio of 2:1, the combination also exhibited synergy against the *vanB* isolate.
- ▶ The 2:1 combinations of CAZ and AM-113, exhibited similar results to CAZ and AM-112. The 1:1 ratio was more potent and exhibited synergy against both vancomycin-resistant isolates.
- ▶ AM-114 and AM-115 did not synergise with CAZ.

Activity of ceftazidime (CAZ) alone and in the presence of clavulanic acid or AM-112 at a fixed concentration of 4 mg/L against an extended panel of Enterococcus spp. (Table 2).

- ▶ CAZ and clavulanic acid alone and in combination lacked activity against isolates of *E. faecalis*, *E. faecium*, *E. gallinarum*, *E. hirae* and *E. casseliflavus*.
- ▶ AM-112 had a MIC range of 8 to 64 mg/L against Van-S strains, but little activity against Van-R strains.
- ▶ In the presence of AM-112 at 4 mg/L, CAZ MICs were markedly reduced against Van-S and Van-R isolates of all species and against 2 of the 6 Van-R isolates.

Activity of cephalosporins alone and in the presence of clavulanic acid or AM-112 at a fixed concentration of 4 mg/L against *E. faecalis* SFZ (Table 3).

- ▶ Cefotaxime was the only cephalosporin tested to exhibit activity against *E. faecalis* SFZ (MIC 2 mg/L). The other cephalosporins exhibited MICs of 16 to 64 mg/L.
- ▶ AM-112 at 4 mg/L increased the activity of seven of the eight cephalosporins by at least 4-fold and generally greater than 8-fold.
- ▶ Clavulanic acid at 4 mg/L increased the activity of two cephalosporins by at least 4-fold, but had no marked effect with the other six agents.

Checkerboard studies to investigate a wide range of combinations of ceftazidime (CAZ) and AM-112 or CAZ and clavulanic acid (Figures 2a-d).

- ▶ Checkerboard MICs were determined for combinations of ceftazidime and either AM-112 or clavulanic acid against two Van-S and two Van-R enterococcal strains.
- ▶ AM-112 and CAZ synergised over a wide range of combination ratios against both Van-S isolates and the *vanB* isolate. There was no effect against the *vanA* isolate.
- ▶ The maximal CAZ/AM-112 synergistic effect generally occurred at CAZ:AM-112 ratios of 2:1, 1:1 and 1:2, corresponding to AM-112 concentrations of 4 to 8 mg/L and CAZ concentrations of 8 to 16 mg/L.
- ▶ Combinations of ceftazidime and clavulanic acid failed to show any activity against the enterococcal strains.

PBP affinities of AM-112, clavulanic acid and imipenem (Figure 3).

- ▶ AM-112, imipenem and clavulanic acid exhibited different PBP inhibition profiles.
- ▶ Imipenem (MIC 0.25 mg/L) inhibited all PBPs of *E. faecalis* SFZ at 0.1 mg/L, although PBP3 and PBPs required 0.3 mg/L for marked inhibition.
- ▶ AM-112 (MIC 64 mg/L) exhibited marked inhibition of all PBPs, except PBP3, at 1 mg/L. PBP3 required AM-112 at 3 to 10 mg/L for marked inhibition.
- ▶ Clavulanic acid at <10 mg/L exhibited weak inhibition of all PBPs (not shown).

Rate of kill studies (Figure 4a and 4b).

- ▶ The synergistic effect was investigated using time-kill experiments for *E. faecalis* SFZ, a vancomycin susceptible isolate and two VRE: 56059 (*vanA*) and 78097 (*vanB*).
- ▶ In all experiments, high concentrations of either CAZ (128 mg/L) or AM-112 (64 mg/L) exerted a bacteriostatic effect but did not markedly reduce the viable count over 24 hours.
- ▶ A combination of CAZ and AM-112 at 1/8 of the above concentrations was highly synergistic over 8 hours reducing the viable count of SFZ by 3 logs and 78097 (*vanB*) by almost 2 logs. Both cultures regrew after 8 hours. This may not occur clinically where bid or tid dosing would be an important factor.
- ▶ No synergistic effect was seen against *E. faecalis* 56059, a *vanA* strain.

Figure 2. Checkerboard MIC results for ceftazidime (CAZ) and AM-112 in varying concentrations against enterococci

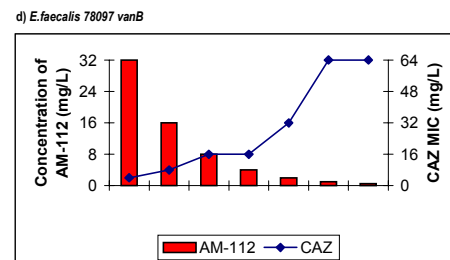
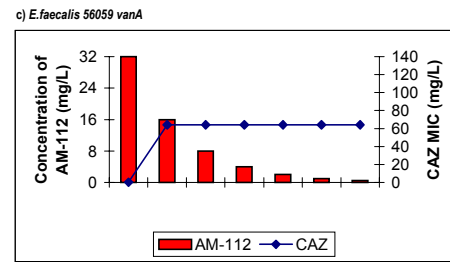
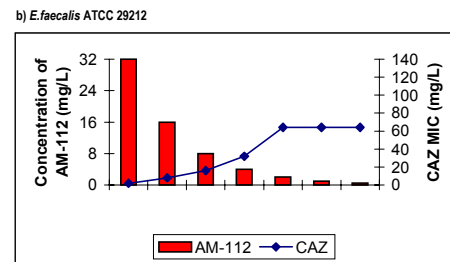
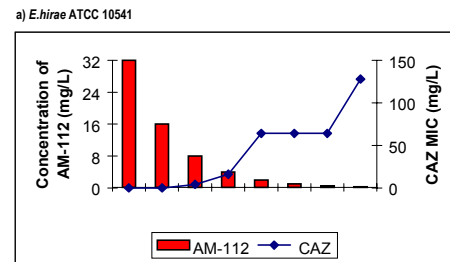


Table 1. Activity of ceftazidime (CAZ) and the oxapenems alone and in 1:1 or 2:1 ratio against a panel of enterococcal strains

Organism	Compounds alone					MIC (mg/L)				CAZ in 1:1 ratio with oxapenems				CAZ in 2:1 ratio with oxapenems			
	CAZ	AM-112	AM-113	AM-114	AM-115	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
<i>E. hirae</i> NCTC 10541	>64	8	2	>64	>64	16	2	>64	>64	16	8	>64	64	16	8	>64	64
<i>E. faecalis</i> NCTC 7171	>64	2	4	>64	>64	16	1	32	>64	32	16	32	>64	32	16	32	>64
<i>E. faecalis</i> NCTC 5957	32	32	8	>64	>64	0.03	0.25	0.03	0.03	8	8	16	16	8	8	16	32
<i>E. faecalis</i> ATCC 29212	32	32	8	>64	>64	8	4	16	16	8	8	16	16	8	8	16	16
<i>E. faecalis</i> 56059 <i>vanA</i>	>64	>64	2	>64	64	64	0.25	>64	>64	32	32	>64	>64	32	32	>64	>64
<i>E. faecalis</i> 78097 <i>vanB</i>	32	>64	16	>64	>64	16	8	16	32	8	8	16	32	8	8	16	32

Key: 4 - fold reduction in the CAZ MIC \geq 8 - fold reduction in CAZ MIC

Figure 3. Comparative inhibition of *E. faecalis* SFZ PBPs by AM-112 and imipenem

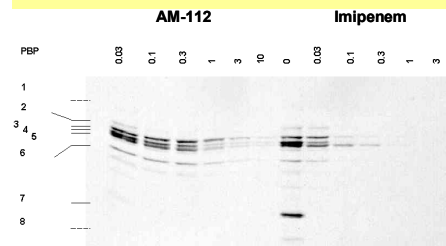


Figure 4. Synergistic activity of ceftazidime (CAZ) and AM-112 against *Enterococcus faecalis* in rate of kill studies

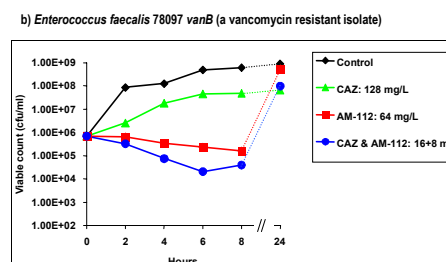
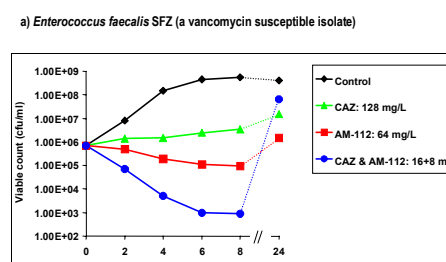


Table 2. Minimum inhibitory concentration (MIC) of ceftazidime, clavulanic acid and AM-112 alone and in combination against an extended panel of enterococci

Organism	MIC (mg/L)				
	CAZ	CLAV	AM-112	CAZ + CLAV (4mg/L)	CAZ + AM-112 (4mg/L)
Vancomycin-sensitive isolates					
<i>E. hirae</i> ATCC 10541	>128	>128	16	>128	<0.06
<i>E. faecalis</i> SFZ	>128	>128	64	32	<0.06
<i>E. faecalis</i> NCTC 5957	>128	>128	32	64	<0.06
<i>E. faecalis</i> ATCC 24952	>128	>128	32	>128	<0.06
<i>E. faecalis</i> NCTC 7171	>128	>128	16	>128	<0.06
<i>E. faecalis</i> Phillips	>128	>128	32	64	<0.06
<i>E. faecium</i> 60060	64	>64	8	>64	4
<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> (<i>vanA</i>)	>64	>64	16	>64	1
Vancomycin-resistant isolates					
<i>E. faecalis</i> 56059 (<i>vanA</i>)	>64	>32	>64	>64	>64
<i>E. faecalis</i> 78097 (<i>vanB</i>)	>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	<0.06

Key: 4 - fold reduction in the CAZ MIC \geq 8 - fold reduction in CAZ MIC

Table 3. Activity of eight cephalosporins alone and combined with a fixed 4mg/L concentration of AM-112 or clavulanic acid against *E. faecalis* SFZ.

Compound	MIC (mg/L)		
	Alone	+ CLAV (4mg/L)	+ AM-112 (4mg/L)
Cefaclor	32	32	16
Cefazolin	32	16	8
Cefuroxime	64	4	0.5
Cefoperazone	32	8	4
Ceftriaxone	64	32	0.5
Cefotaxime	2	1	0.06
Ceftazidime	32	64	1
Cefepime	16	8	4

Key: 4 - fold reduction in the CAZ MIC \geq 8 - fold reduction in CAZ MIC

CONCLUSIONS

- ▶ Individually, cephalosporins, clavulanic acid and oxapenems AM-114 and AM-115 are inactive against enterococci. Oxapenems AM-112 and AM-113 exert moderate activity against some strains.
- ▶ Combinations of cephalosporins and AM-112 or AM-113 act synergistically against enterococci, including some vancomycin-resistant isolates.
- ▶ The activity and synergy summarised in the previous two points demonstrates the importance of the stereochemistry of the hydroxyethyl substituent on the 6-position of the oxapenems. Compounds with a (R)-hydroxyethyl group (AM-112 and AM-113) show activity whereas those with a (S)-hydroxyethyl group (AM-114 and AM-115) do not.
- ▶ Checkerboard studies indicate that the optimum ratio of CAZ:AM-112 ranges from 2:1 to 1:2.
- ▶ Viable count studies confirm that the synergistic effect is bactericidal for at least eight hours, after which regrowth occurs.
- ▶ AM-112 binds to all eight PBPs of *E. faecalis* SFZ, but with different binding affinities to imipenem and clavulanic acid.
- ▶ Seven of the eight enterococcal PBPs are completely inhibited by AM-112 at 3 mg/L. Unlike with imipenem, PBP3 is not completely inhibited by AM-112 at 10 mg/L. These results suggest that PBP3 is an essential PBP in enterococci.
- ▶ The synergy between some oxapenems and cephalosporins indicates complementation against the essential PBPs of enterococci. This result offers the potential of extending the antibacterial spectrum of cephalosporins against this difficult pathogen.

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