

In Vitro Activities of Novel Oxapenems, Alone and in Combination with Ceftazidime, against Gram-Positive and Gram-Negative Organisms

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Received 3 June 2002/Returned for modification 17 February 2003/Accepted 10 May 2003

Four novel oxapenem compounds (i.e., AM-112, AM-113, AM-114, and AM-115) were investigated for their β -lactamase inhibitory activity against a panel of isolated class A, C, and D enzymes, which included expanded-spectrum β -lactamase enzymes (ESBLs). The oxapenems were potent β -lactamase inhibitors. Activity varied within the group, with AM-113 and AM-114 proving to be the most active compounds. The 50% inhibitory concentrations for these agents were up to 100,000-fold lower than that of clavulanic acid against class C and D enzymes. As a group, the oxapenems were more potent than clavulanic acid against enzymes from all classes. The ability of these compounds to protect ceftazidime from hydrolysis by β -lactamase-producing strains was evaluated by MIC tests that combined ceftazidime and each oxapenem in a 1:1 or 2:1 ratio. The oxapenems markedly reduced the MICs for ceftazidime against class C hyperproducing strains and strains producing TEM- and SHV-derived ESBLs. There was little difference between the activity of 1:1 and 2:1 combinations of ceftazidime and oxapenem. The oxapenems failed to enhance the activity of ceftazidime against derepressed AmpC-producing *Pseudomonas aeruginosa* strains.

Group 1 cephalosporinases (also called class C β -lactamases in the Ambler scheme [1]) present a major threat to the continuing effectiveness of cephalosporin antibiotics. They are produced by gram-negative bacteria such as the *Enterobacteriaceae* but also by *Pseudomonas* and *Aeromonas* strains (3, 9). The chromosomal enzyme (AmpC) can be hyperproduced either by reversible induction or stable derepression (8, 15). Approximately 15 to 25% of *Enterobacter cloacae* strains hyperproduce class C enzymes (10). The genes for AmpC enzymes can be plasmid borne and thus have the potential for dissemination between bacterial species. Coupled with the threat of these AmpC enzymes, the evolution of expanded-spectrum β -lactamase (ESBL) enzymes, which are capable of destroying later-generation cephalosporins, serves to further undermine the clinical utility of cephalosporins. Existing inhibitors are active against class A and ESBL enzymes but lack good activity against the class C enzymes (3, 13).

Oxapenems were first described in 1977, and a potent lead compound was discovered by Cherry et al. in 1978 (4). The chemical structure of the oxapenems is illustrated in Table 1. The first compound described inhibited cell-free β -lactamases from gram-negative organisms and was superior in activity to clavulanic acid against staphylococcal β -lactamase and *E. cloacae* AmpC β -lactamase. However, the compound was unstable and lacked activity against intact bacteria. Thus, the oxapenems attracted little interest until the 1990s, when Pfaendler et al. synthesized novel oxapenems possessing bulky substituents at the C₂ position of the five-membered ring. These substituents enhanced the stability of the compounds. Two of these novel compounds—AM-112 and AM-113—were found

to have excellent in vitro activity against penicillin- and methicillin-resistant bacteria, with AM-113 proving more active than AM-112. MICs of AM-113 ranged from <0.78 to 3.12 μ g/ml for methicillin-resistant *Staphylococcus aureus* (MRSA) strains, 2 to 16 μ g/ml for *Enterococcus* spp., 2 to 16 μ g/ml for *Escherichia coli*, 4 to 16 μ g/ml for *Klebsiella*, and 0.5 to 8 μ g/ml for anaerobes including *Bacteroides fragilis* and *Clostridium perfringens* (14). Previously, we reported preliminary results for these compounds (C. E. Jamieson, P. A. Lambert, and I. N. Simpson, Abstr. 41st Intersci. Conf. Antimicrob. Agents Chemother., abstr. F383, 2001). Here, we describe the in vitro activity of four novel oxapenem compounds (i.e., AM-112, AM-113, AM-114, and AM-115) and their β -lactamase inhibitory properties.

MATERIALS AND METHODS

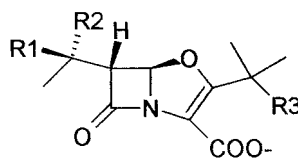
Compounds. Samples of AM-112, AM-113, AM-114, and AM-115 were supplied by Amura Ltd. (Cambridge, United Kingdom). Ceftazidime and clavulanic acid were obtained from commercial sources.

Organisms. The *Escherichia coli* J53 transconjugants (i.e., TEM-1, TEM-3, TEM-6, TEM-9, TEM-10, SHV-1, SHV-2, SHV-3, SHV-4, SHV-5, OXA-1, OXA-2, OXA-3, OXA-5, and PSE-4) listed in Table 2 and the stably derepressed *Enterobacter cloacae* 84-con, *Citrobacter freundii* C2-con, *Serratia marcescens* S2-con, *Moraxella morgani* M1-con, and *Pseudomonas aeruginosa* 1407-con and 2297-con (Table 3) were kindly supplied by D. M. Livermore (Central Public Health Laboratory, Colindale, London, United Kingdom). *Enterobacter cloacae* strain 1051E P99 and *E. cloacae* strain 1194E Hennessey were obtained from the GlaxoSmithKline culture collection. Untyped strains included clinical isolates from Addenbrookes Hospital (Cambridge, United Kingdom); *Enterococcus faecalis* (strains 56059 and 78097), and MRSA Innsbruck, supplied by H. R. Pfaendler (14).

Cell-free β -lactamase assays. Isolated enzyme extracts were prepared from the following organisms: *Escherichia coli* TEM-1, *E. coli* TEM-10, *E. coli* SHV-5, *Enterobacter cloacae* P99, *S. marcescens* S2-con, *P. aeruginosa* S+A, *Escherichia coli* OXA-1, and *E. coli* OXA-5. Overnight cultures of each organism were inoculated into fresh, prewarmed Mueller Hinton broth (MHB) and grown with vigorous shaking at 37°C to exponential-growth phase. Cells were harvested by centrifugation at 12,100 \times g, washed, and resuspended in 10 mM sodium phos-

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TABLE 1. Chemical structure of oxapenems



Oxapenem compound	R1	R2	R3	Mol wt
AM-112	OH	H	(CH ₂) ₃ NH ₃ ⁺	300
AM-113	OH	H	CH ₃	255
AM-114	H	OH	CH ₃	255
AM-115	H	OH	(CH ₂) ₃ NH ₃ ⁺	300

phate buffer (SPB; pH 7.0). Cells were disrupted by six 10-s cycles of sonication by using a MSE Soniprep 150 (MSE Ltd., Crawley, United Kingdom), with constant cooling in an ice bath. Sonicated cells were then centrifuged for 30 min at 15,300 × g at 4°C to remove cell debris. The supernatant was retained and stored at -70°C until required for further use. Purified enzyme extracts were obtained by preparative isoelectric focusing in Sephadex (ampholine; pH range, 3.5 to 10), followed by elution with SPB. The eluate was stored at -70°C in aliquots until required for further use. Enzyme inhibition studies were carried out by using a spectrophotometric assay with nitrocefin as the substrate (50 µg/ml), according to the method of O'Callaghan et al. (12). Enzyme was preincubated with inhibitor in SPB for 15 min at 37°C prior to addition of the substrate. The absorbance at 492 nm was then measured for a 10-min period at 37°C. The initial velocity (V₀) of the reaction was calculated from the slope of the linear portion of a graph of absorbance plotted against time. The 50% inhibitory concentrations (IC₅₀) were determined from a graph of percent inhibition of the enzyme against inhibitor concentration.

In vitro susceptibility tests. MICs were determined for ceftazidime alone and in combination with each oxapenem in a 1:1 and 2:1 combination against a panel of gram-positive and gram-negative strains, some of which produced β-lactamase enzymes. MICs were determined by broth microdilution in MHB, which was carried out in microtiter plates in accordance with NCCLS guidelines. MRSA strains were grown in MHB containing 2% NaCl (wt/vol).

RESULTS

β-Lactamase inhibitory activity. Clavulanic acid is an effective inhibitor of class A enzymes (13). This activity was reflected in the results for clavulanic acid against the three class A enzymes in Table 4, which showed IC₅₀s between 0.008 and 0.12 µM. Clavulanic acid lacked good activity against the class D enzymes OXA-1 and OXA-5, with IC₅₀s of 99 and 202 µM. As expected, the activity against the class C enzymes was poor; clavulanic acid was most active against P99 (IC₅₀, 11 µM) but was poorly active against S2 and S+A (327 and 449 µM, respectively). The IC₅₀s obtained here for TEM-1, TEM-10, P99, and S2 were of the same order of magnitude as those described in a study investigating the interaction between β-lactamase inhibitors and enzymes from each molecular class (2).

AM-112 exhibited a broad spectrum of activity against each of the three classes of enzyme. AM-112 was most active against the ESBL class A enzyme SHV-5, with an IC₅₀ of 0.16 µM. This activity was 100-fold weaker than that of clavulanic acid against this enzyme. AM-112 activity against TEM-1 was approximately 20-fold weaker than clavulanic acid—and against the ESBL TEM-10 enzyme, the activity was 10-fold weaker than clavulanic acid. Nevertheless, the activity against the class A enzymes was good. Class C enzymes proved very susceptible to inhibition by AM-112. IC₅₀s against the three enzymes in the panel were between 1,000- and 100,000-fold lower than those

of clavulanic acid. There was also good activity against the class D enzymes.

A similar profile of activity was seen for AM-113. The activity against class A enzymes was poorest against TEM-1 (IC₅₀, 3.34 µM) and most potent against the ESBL enzyme TEM-10 (IC₅₀, 0.008 µM). Against both the class C and the class D enzymes, AM-113 was very active and (similar to AM-112) had IC₅₀s between 1,000- and 100,000-fold lower than those of clavulanic acid.

AM-114 and AM-115 both displayed potent activity against

TABLE 2. In vitro activities of ceftazidime alone and in combination with oxapenems against a panel of *E. coli* strains producing plasmid-mediated class A or D β-lactamases

Organism	Drug ratio	CAZ ^a MIC (µg/ml)				
		CAZ alone	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
<i>E. coli</i> SHV-1		2				
	1:1 2:1	0.25 0.125	0.25 0.125	0.25 0.25	0.25 0.25	0.25 0.125
<i>E. coli</i> SHV-2		0.25				
	1:1 2:1	0.125 0.125	0.125 0.125	0.125 0.125	0.125 0.125	0.125 0.125
<i>E. coli</i> SHV-3		0.125				
	1:1 2:1	0.03 0.125	0.125 0.03	0.03 0.03	0.03 0.03	0.03 0.03
<i>E. coli</i> SHV-4		>64				
	1:1 2:1	4 8	2 2	2 4	8 0.03	
<i>E. coli</i> SHV-5		16				
	1:1 2:1	8 16	8 4	1 2	8 2	
<i>E. coli</i> TEM-1		0.25				
	1:1 2:1	0.25 0.5	0.25 0.25	0.25 0.25	0.5 0.06	
<i>E. coli</i> TEM-3		16				
	1:1 2:1	4 2	2 2	2 2	4 2	
<i>E. coli</i> TEM-6		>64				
	1:1 2:1	8 4	8 8	2 2	4 2	
<i>E. coli</i> TEM-9		>64				
	1:1 2:1	8 8	4 8	2 4	4 2	
<i>E. coli</i> TEM-10		>64				
	1:1 2:1	8 16	4 8	2 4	4 2	
<i>E. coli</i> PSE-4		0.125				
	1:1 2:1	0.25 0.5	0.25 0.25	0.5 0.25	0.25 0.125	
<i>E. coli</i> ATCC 35218		0.125				
	1:1 2:1	0.125 0.125	0.25 0.125	0.125 0.125	0.125 0.125	
<i>E. coli</i> ATCC 25922		0.25				
	1:1 2:1	0.25 0.25	0.25 0.125	0.25 0.25	0.25 0.03	
<i>E. coli</i> OXA-1		0.25				
	1:1 2:1	0.5 0.25	0.25 0.25	0.25 0.25	0.25 0.25	
<i>E. coli</i> OXA-2		0.25				
	1:1 2:1	0.25 0.25	0.25 0.25	0.25 0.25	0.25 0.125	
<i>E. coli</i> OXA-3		0.5				
	1:1 2:1	0.5 0.5	0.5 0.25	0.5 0.5	0.5 0.25	
<i>E. coli</i> OXA-5		0.5				
	1:1 2:1	0.25 0.25	0.5 0.25	0.25 0.25	0.25 0.03	

^a CAZ, ceftazidime.

TABLE 3. In vitro activities of ceftazidime alone and in combination with oxapenems against gram-negative bacteria producing inducible or derepressed class C β-lactamases

Organism	Drug ratio	CAZ ^a MIC (μg/ml)				
		CAZ alone	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
<i>E. cloacae</i> P99	1:1	32	4	2	4	4
	2:1		4	4	8	8
<i>E. cloacae</i> Hennessey	1:1	>64	4	4	4	15
	2:1		4	8	8	8
<i>E. cloacae</i> 84-con	1:1	>64	4	8	8	8
	2:1		4	16	16	16
<i>C. freundii</i> C2-con	1:1	64	0.03	2	2	4
	2:1		2	4	4	2
<i>S. marcescens</i> S2-con	1:1	1	0.03	0.25	0.5	0.03
	2:1		0.5	0.5	0.5	1
<i>M. morgani</i> M1-con	1:1	8	1	1	0.25	1
	2:1		1	0.25	0.25	1
<i>P. aeruginosa</i> ATCC 27853	1:1	2	4	2	2	2
	2:1		2	4	2	2
<i>P. aeruginosa</i> 1407-con	1:1	>64	64	16	32	64
	2:1		64	32	32	32
<i>P. aeruginosa</i> 2297-con	1:1	>64	32	16	16	32
	2:1		32	32	32	32

^a CAZ, ceftazidime.

each class of enzyme. Activity against the class A enzymes was comparable to that of clavulanic acid and 1,000-fold lower than that of either AM-112 or AM-113 against TEM-1. Activity against the class C and class D enzymes was similar to that of AM-112 and AM-113 and was superior to that of clavulanic acid.

Synergy in combination with ceftazidime. The synergistic activity of ceftazidime at 1:1 and 2:1 ratios with the oxapenems was tested against a panel of β-lactamase-producing *E. coli* (Table 2). Against SHV-1, there were 8- to 16-fold reductions in the MIC, while against SHV-2 and SHV-3 there were between 2- and 4-fold reductions in the MIC. MICs were reduced up to 64-fold against SHV-4 and up to 16-fold against SHV-5.

TABLE 4. IC₅₀s for clavulanic acid and novel oxapenems against a panel of isolated β-lactamases

Enzyme (group/class) ^a	IC ₅₀ (μM)				
	Clavulanic acid	AM-112	AM-113	AM-114	AM-115
TEM-1 (2b/A)	0.12	2.26	3.34	0.002	0.004
TEM-10 (2be/A)	0.03	0.224	0.008	0.063	0.005
SHV-5 (2be/A)	0.008	0.16	0.11	0.012	0.06
P99 (1/C)	11	0.002	0.002	0.001	0.014
S2 (1/C)	327	0.007	0.002	0.005	0.09
S + A (1/C)	449	0.002	0.001	0.0001	0.02
OXA-1 (2d/D)	99	0.005	0.0004	0.003	0.006
OXA-5 (2d/D)	202	0.0007	0.001	0.002	0.005

^a Bush-Jacoby-Medeiros group classification (3) and Ambler class classification (1).

TABLE 5. In vitro activities of ceftazidime alone and in combination with oxapenems against *S. aureus* and *E. faecalis*

Organism	Drug ratio	CAZ ^a MIC (μg/ml)				
		CAZ alone	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
<i>S. aureus</i> NCTC 6571	1:1	4	2	0.5	1	4
	2:1		1	1	2	4
<i>S. aureus</i> ATCC 29213	1:1	8	4	0.5	2	4
	2:1		2	1	2	4
MRSA Innsbruck	1:1	>64	0.03	0.03	0.03	0.03
	2:1		2	0.125	0.06	0.03
<i>E. hirae</i> ATCC 10541	1:1	>64	16	2	>64	>64
	2:1		16	8	>64	64
<i>E. faecalis</i> NCTC 7171	1:1	>64	16	1	32	>64
	2:1		32	16	32	>64
<i>E. faecalis</i> NCTC 5957	1:1	32	0.03	0.25	0.03	0.03
	2:1		8	8	16	32
<i>E. faecalis</i> ATCC 9212	1:1	32	8	4	16	16
	2:1		8	8	16	16
<i>E. faecalis</i> 56059 <i>vanA</i>	1:1	>64	64	0.25	>64	>64
	2:1		32	32	>64	>64
<i>E. faecalis</i> 78097 <i>vanB</i>	1:1	32	16	8	16	32
	2:1		8	8	16	32

^a CAZ, ceftazidime.

Ceftazidime was susceptible to hydrolysis by ESBL TEM enzymes, such as TEM-3 and TEM-10, with MICs between 16 and >64 μg/ml against strains carrying such enzymes. Combination with an oxapenem at 1:1 or 2:1 reduced these MICs by up to 64-fold. Combination of ceftazidime with an oxapenem conferred no additional benefit against PSE-4 or ATCC *E. coli* strains. Against the class D enzyme-producing strains, there was little effect on the MICs of the combinations compared to ceftazidime alone, except the OXA-5 producing strain, for which the MICs of the combinations were twofold lower than that of ceftazidime alone.

Most of the *Enterobacteriaceae* proved highly resistant to ceftazidime, with MICs between 8 and >64 μg/ml (Table 3). These strains had hyperproduced or inducible class C β-lactamases. Two *Pseudomonas* strains also produced derepressed class C enzymes (ceftazidime MIC > 64 μg/ml). For *Enterobacter* strains, the MICs were reduced up to 32-fold by the addition of oxapenems at 2:1 or 1:1 ratios, while MICs against *Serratia*, *Morganella*, and *Citrobacter* were also reduced up to 32-fold. The oxapenems enhanced the activity of ceftazidime against the *Pseudomonas* strains up to eightfold for the derepressed strains but did not enhance the activity against the ATCC 27853 strain.

Alone, ceftazidime was poorly active against the MRSA and enterococcal strains (MIC range, 32 to >64 μg/ml; Table 5). Ceftazidime activity against the staphylococci was enhanced by the addition of oxapenems at 1:1 and 2:1 ratios—MICs were lowered up to 16-fold against methicillin-sensitive strains and up to 2,048-fold against the MRSA strain. Similar to the case of enterococcal strains, ceftazidime lacked activity alone, while

the combination of ceftazidime and oxapenems lowered the MIC compared to ceftazidime alone. AM-112 and AM-113 were the most effective partners for ceftazidime, while AM-114 and AM-115 were less active. Against vancomycin-sensitive enterococci, MICs were reduced between 4- and 512-fold by combinations of AM-112 or AM-113 with ceftazidime. Of the vancomycin-resistant strains, MIC reductions of up to fourfold were observed with combinations of AM-112 or AM-113 with ceftazidime.

DISCUSSION

The oxapenems have potent β -lactamase inhibitory activity. AM-114 and AM-115 are the most potent inhibitors of the class A enzymes (IC_{50} s between 0.002 and 0.063 μ M). AM-114 and its stereoisomer AM-113 are the most active inhibitors of class C enzymes, while AM-113 and AM-112 were the most active oxapenems against class D enzymes (Table 1). As a class, the oxapenems are much more potent inhibitors of class A, C, and D enzymes than clavulanic acid, although this activity varies within the class. The extent to which this β -lactamase inhibitory activity is due to the differing stereochemistry of the inhibitors has not been fully elucidated, although the orientation of the hydroxyethyl group at the C₆ position and the nature of the C₂ side chain appear to be important (data not shown). This stereochemistry also appears to affect the in vitro antibacterial properties of the compounds, as both AM-112 and AM-113 have intrinsic antibacterial activity, while their stereoisomers—AM-115 and AM-114, respectively—lack this activity. The MICs for AM-114 and AM-115 against β -lactamase-producing strains of *Enterobacteriaceae* range from 8 to >64 μ g/ml. IC_{50} s for AM-114 and AM-115 were between 100 and 100,000 times lower than the MICs (C. E. Jamieson et al., 41st ICAAC, abstr. F383).

The antibacterial activity of the oxapenems in combination with ceftazidime was evaluated against gram-negative and gram-positive organisms, including β -lactamase-producing and antibiotic-resistant strains (Tables 2, 3 and 5). In most cases, there was only a twofold difference between values observed for the 1:1 and 2:1 ceftazidime-oxapenem combinations. While a 1:1 combination might be expected to be more potent, it is possible that the concentration of the oxapenem in either combination is at or near the level required for total inhibition of the β -lactamase, thus resulting in the small difference seen between the two combination concentrations. For AM-115, the 2:1 combination with ceftazidime appears to be more active against the panel of enzyme producers than the 1:1 combination. This suggests that the oxapenems have a threshold of potency, above which increases in their concentration do not result in further reductions in the MICs. A similar trend was seen in Tables 3 and 5, although there are some exceptions. For example, against *Enterococcus faecalis* strain NCTC 5957, the higher proportion of oxapenem to ceftazidime (1:1) was much more active for each oxapenem than the corresponding 2:1 combination. Similarly, against MRSA strain Innsbruck, the activity of AM-112 and AM-113 was greater when combined with ceftazidime at a 1:1 ratio, while AM-114 and AM-115 appear to be broadly similar in activity at 1:1 or 2:1. These results are interesting because the gram-positive cocci listed in Table 5 (with the exception of MRSA strain Innsbruck) did not produce a β -lactamase enzyme. As previously discussed, both

AM-114 and AM-115 lack intrinsic antibacterial activity. The results suggest that all of the oxapenems have some affinity for bacterial penicillin-binding proteins (PBPs), and that—while for AM-114 and AM-115 alone this affinity may be insufficient to cause the death of the organism—when combined with the activity of a partner such as ceftazidime, this activity is superior to that of the partner alone. AM-112 has been shown to inhibit the PBPs of *E. coli* strain DC0, with PBP2 being the initial target inhibited by a concentration of 0.1 μ g/ml (7). There is a pressing clinical need for β -lactamase inhibitors that display activity against class C β -lactamases. Existing agents such as clavulanic acid and tazobactam lack sufficient activity against these enzymes (3, 13). The novel penem inhibitor BRL 42715 has potent activity against class A, class C, and class D compounds (5) but proved to be too unstable for further development (6). Syn2190 is a novel monobactam inhibitor that has inhibitory activity against class C compounds but is less potent than tazobactam against class A and ESBL enzymes (11). The results presented in this study confirm that the oxapenems are potent β -lactamase inhibitors, with activity against class A, C, and D enzymes as well as ESBLs. The potential of these compounds to extend the antibacterial spectrum of established compounds such as ceftazidime warrants their further investigation.

ACKNOWLEDGMENT

We thank Amura Ltd. for financial support.

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