

## *In vitro* activity of AVE1330A, an innovative broad-spectrum non- $\beta$ -lactam $\beta$ -lactamase inhibitor

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**Objectives:** Production of  $\beta$ -lactamases is the main mechanism of  $\beta$ -lactam resistance in Gram-negative bacteria. Despite the current use of clavulanic acid, sulbactam and tazobactam, the prevalence of class A and class C enzymes is increasing worldwide, demanding new  $\beta$ -lactamase inhibitors. Here we report the antimicrobial properties of AVE1330A, a representative of a novel class of bridged bicyclico[3.2.1]diazabicyclo-octanones in combination with ceftazidime.

**Materials and methods:** IC<sub>50</sub> and kinetic parameters of the hydrolysis reaction were used to characterize  $\beta$ -lactamase inhibition by AVE1330A. MICs for >600 strains were determined with the combination ceftazidime/AVE1330A at a fixed ratio of 4:1.

**Results:** IC<sub>50</sub>s of AVE1330A for TEM-1 and P99 enzymes were 0.0023 mg/L (8 nM) and 0.023 mg/L (80 nM), compared with 0.027 mg/L (130 nM) and 205.1 mg/L ( $1 \times 10^6$  nM) of clavulanic acid and 0.013 mg/L (40 nM) and 1.6 mg/L (5000 nM) of tazobactam. A highly stable covalent complex led to a low turnover of AVE1330A. MICs of ceftazidime/AVE1330A for Enterobacteriaceae were at least eight-fold lower than those of ceftazidime alone. All of the *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter* and *Proteus mirabilis* strains, including ceftazidime-resistant isolates, were inhibited at 4–8 mg/L. Only 2 mg/L were required to inhibit other Proteaeae, *Enterobacter*, *Salmonella* and *Serratia*.

**Conclusion:** The combination of ceftazidime with AVE1330A exhibited broad-spectrum activity against Ambler class A- and class C-producing Enterobacteriaceae.

Keywords: AmpC, ESBLs, combinations, ceftazidime

### Introduction

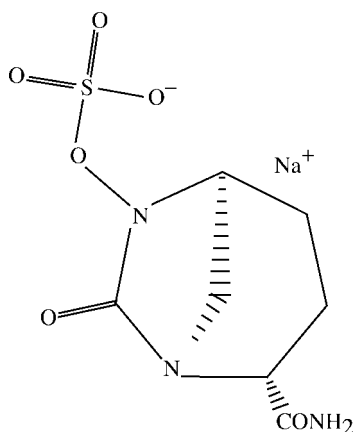
$\beta$ -Lactam antibiotics are one of the most important families of antibacterial agents, about 40 being in clinical use today. However, continuous development of resistance poses some questions about their future, including that of the most recent extended-spectrum cephalosporins.<sup>1</sup> Proliferation of class A extended-spectrum  $\beta$ -lactamases (ESBLs) and class C de-repressed chromosomal cephalosporinases of the Ambler scheme are the major issues in Gram-negative strains, driving the need for new antibacterial agents.<sup>2,3</sup> The widespread use of third-generation cephalosporins, including ceftazidime, has been paralleled by increasing resistance to these agents throughout the world, by early selection of mutant resistant populations among species possessing chromosomally encoded cephalosporinases.<sup>4,5</sup>

Currently, clavulanic acid, sulbactam and tazobactam are marketed  $\beta$ -lactam inhibitors. However, the number of bacteria which produce new ESBLs or  $\beta$ -lactamase-inhibitor-resistant TEM enzymes is escalating alarmingly.<sup>6</sup> Furthermore, plasmid-borne cephalosporinases, which are capable of rapid dissemination, have been described.<sup>7</sup> None of the commercially available inhibitors satisfactorily inhibit class C enzymes.<sup>8</sup> In order to overcome resistance mediated now by more than 400 different enzymes, AVE1330A, a non- $\beta$ -lactam compound, was designed (Figure 1).

We report here the *in vitro* activity of ceftazidime combined with AVE1330A at a fixed 4:1 ratio, in comparison with ceftazidime alone, a ceftazidime/clavulanic acid combination (4:1 ratio), co-amoxiclav and piperacillin-tazobactam.

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## AVE1330A, a novel $\beta$ -lactamase inhibitor



**Figure 1.** Chemical structure of AVE1330A.

### Materials and methods

#### Antibiotics

AVE1330A was prepared by Aventis (Romainville, France).<sup>9</sup> This compound was discovered in the course of a research programme directed toward the identification of  $\beta$ -lactamase inhibitors. Other compounds were obtained or prepared from commercial sources. Ceftazidime (Fortum), co-amoxiclav (Augmentin) and piperacillin–tazobactam (Tazocilline) were used for the MIC testing.

#### Bacterial strains

More than 600 strains were used in this study. Most of the strains listed in Table 4 were kindly provided by P. Nordmann (Service de Bactériologie-Virologie, Hôpital de Bicêtre, Le Kremlin-Bicêtre, France). Other strains were clinical isolates from various European and US hospitals. All isolates were maintained as stab cultures at room temperature. Reference organisms were included for quality control: *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

#### $\beta$ -Lactamase assays

**Isolation of  $\beta$ -lactamases.** Purified TEM-1  $\beta$ -lactamase from *E. coli* was kindly provided by S. Mobashery, Chemistry Department, Wayne State University, Detroit, MI, USA. P99  $\beta$ -lactamase from *Enterobacter cloacae* 293HT6 was prepared from crude extract obtained after lysis by French press, and purified by phenyl boronic acid affinity chromatography, as described previously.<sup>10</sup> Activities of

enzymes were stabilized at 37°C in buffer (50 mM phosphate pH 7.0, 2% glycerol and 0.1 mg/mL bovine serum albumin).

**Inhibition.** Inhibition was determined spectrophotometrically at 37°C after 5 min pre-incubation, in the presence of 100  $\mu$ M nitrocefin as substrate (extinction coefficient: 20 500 M<sup>-1</sup> cm<sup>-1</sup>), and 1 nM TEM-1 or 0.42 nM P99 in a final volume of 0.2 mL. The concentration of inhibitor needed to reduce the initial rate of hydrolysis of substrate by 50% (IC<sub>50</sub>) was recorded as the residual activity of  $\beta$ -lactamase at 485 nm. Data were processed using GraFit (Erithacus Software, Staines, UK).

**Turnover.** The turnover number (Tn) was the number of inhibitor molecules required to inactivate one enzyme molecule. It was determined at 37°C, using different molar enzyme/inhibitor ratios at 10 and 60 min for TEM-1 and P99, respectively.<sup>11</sup> These defined times corresponded to the minimal period of time taken to obtain maximal inhibition. Residual activity was measured with 400  $\mu$ M nitrocefin as substrate. The Tn values were deduced from the extrapolated value for 99% inactivation from the plot of the residual activity versus inhibitor/enzyme ratios.

**Deacylation of the acylenzyme intermediate.** Enzyme (84 nM P99 or 200 nM TEM-1) was saturated by inhibitor at the turnover concentration for 10 min and 60 min in the case of TEM-1 and P99, respectively. Gel filtration using Sephadex G-50 micro-column (Amersham Biosciences, Orsay, France) was used to eliminate free inhibitor. Recovery of  $\beta$ -lactamase activity was measured at 485 nm in the presence of 100  $\mu$ M nitrocefin at 37°C after appropriate enzyme dilutions and expressed as a percentage versus enzyme activity in the absence of inhibitor.

#### *In vitro* antibacterial activity against $\beta$ -lactamase-regulated producers

In order to study the effect of variable amounts of  $\beta$ -lactamase on the inhibitory efficacy of AVE1330A, an isogenic panel of SHV-4- and AmpC-enzyme-producing *E. coli* was constructed in the common host XL-1 Blue strain {from Stratagene, Amsterdam, the Netherlands, ref. Cat. 200236, genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI<sup>q</sup>Z $\Delta$ M15 Tn10* (Tet<sup>r</sup>)]} under an inducible arabinose promoter. The cloning vector was pSTBlue-1 from Novagen (Cat. No. 70184). The expression vector was pBAD<sub>18-kan</sub> with ArabinoseP<sub>BAD</sub> promoter.<sup>12</sup> Overexpression from pBAD was modulated using L-arabinose inducer concentrations ranging from 0.02% to 0.2%. MICs were determined by microdilution in Luria–Bertani broth in the presence of 1–2  $\times$  10<sup>7</sup> bacterial cfu/mL after incubation at 37°C for 18 h.

**Table 1.** Biochemical properties of AVE1330A against isolated  $\beta$ -lactamases

| Enzyme and inhibitor | IC <sub>50</sub> , mg/L (nM)        | Tn  | Recovery of $\beta$ -lactamase activity (%) |
|----------------------|-------------------------------------|-----|---|
| <b>TEM-1</b>         |                                     |     |   |
| AVE1330A             | 0.0023 (8)                          | 2   | 30% after 7 min (50% after 7 days)          |
| clavulanic acid      | 0.027 (130)                         | 214 | 50% after 7 min (75% after 7 days)          |
| tazobactam           | 0.013 (40)                          | ND  | ND  |
| <b>P99</b>           |                                     |     |   |
| AVE1330A             | 0.023 (80)                          | 5   | 50% after 7 days                            |
| clavulanic acid      | 205.1 (1 $\times$ 10 <sup>6</sup> ) | ND  | ND  |
| tazobactam           | 1.6 (5000)                          | 55  | 50% after 290 min (100% after 24 h)         |

ND, not determined.

### Susceptibility testing

Standard MICs were determined by a two-fold agar dilution method in Mueller–Hinton medium. An inoculum of  $10^4$  cfu/spot was used throughout the study. All plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration at which no visible growth could be detected on agar plates. According to the NCCLS breakpoints, MICs for ceftazidime-resistant and -susceptible strains were  $\geq 8$  and  $\leq 8$  mg/L, respectively.

### Effect of inoculum size

A two-fold agar dilution method in Mueller–Hinton medium was used to evaluate the effect of five inoculum sizes ( $\sim 5$ – $9 \log_{10}$  cfu/mL) on the antibacterial activity of the combination ceftazidime/AVE1330A 4:1 against four strains which produced ESBL or AmpC enzymes. Bacterial enumerations were carried out by using a Spiral counter system to ascertain the inoculum sizes, obtained by appropriate dilutions from an overnight culture in Mueller–Hinton broth.

## Results

### $\beta$ -Lactamase assays

Table 1 shows the different properties of enzyme inhibitory activity. Against TEM-1, AVE1330A was five- and 16-fold more active than tazobactam and clavulanic acid in terms of  $IC_{50}$ . Against P99, AVE1330A showed stronger inhibition than tazobactam, with an  $IC_{50}$   $>60$ -fold lower than that of tazobactam; clavulanic acid was inactive.

The Tn for AVE1330A was 2, compared with 214 for clavulanic acid against TEM-1. Maximal TEM-1 inhibition was observed at 10 min for all AVE1330A/enzyme ratios (data not shown). Complete inactivation of P99 by AVE1330A occurred at a ratio of 5, compared with 55 for tazobactam. On the other hand, maximal P99 inhibition occurred after 60 min for AVE1330A and tazobactam (data not shown).

For TEM-1, 50% deacylation was observed over 7 days of incubation with AVE1330A, compared with 7 min with clavulanic acid. In the case of P99, the enzyme recovered 50% of its activity over 7 days, compared with 290 min with tazobactam.

### *In vitro* antibacterial activity against $\beta$ -lactamase overproducers

Table 2 shows the MICs of ceftazidime alone compared with ceftazidime in combination with AVE1330A or clavulanic acid against the three strains tested. MICs of AVE1330A and clavulanic acid alone were  $\geq 16$  mg/L. MICs of ceftazidime for the SHV-4 and AmpC producers clearly increased from 1–2 to  $>32$  mg/L, when  $\beta$ -lactamase production was increased by L-arabinose induction. Unlike clavulanic acid, AVE1330A maintained MICs at  $\leq 1$  mg/L of ceftazidime for all the strains tested, including the AmpC producer, whatever the level of  $\beta$ -lactamase induction.

### Comparative *in vitro* antibacterial activity of ceftazidime combined with AVE1330A at a 4:1 ratio

Comparative MICs of ceftazidime alone, ceftazidime/AVE1330A and ceftazidime/clavulanic acid at a ratio of 4:1 for known  $\beta$ -lactamase-producing strains are reported in Table 3. MICs of AVE1330A and clavulanic acid alone were  $\geq 8$  mg/L

**Table 2.** *In vitro* antibacterial activity of ceftazidime alone, ceftazidime/AVE1330A 4:1 and ceftazidime/clavulanic acid 4:1 against  $\beta$ -lactamase-producing regulated mutants

| Enzyme and drug                    | MIC (mg/L)    |       |       |
|------------------------------------|---------------|-------|-------|
|                                    | % L-arabinose |       |       |
|                                    | 0             | 0.02  | 0.2   |
| <b>Ceftazidime</b>                 |               |       |       |
| SHV-4                              | 1             | 32    | $>32$ |
| AmpC                               | 2             | $>32$ | $>32$ |
| XL-1 Blue                          | 1             | 2     | 1     |
| <b>Ceftazidime/AVE1330A</b>        |               |       |       |
| SHV-4                              | 0.25          | 0.25  | 0.5   |
| AmpC                               | 0.25          | 1     | 1     |
| XL-1 Blue                          | 0.25          | 0.5   | 0.25  |
| <b>Ceftazidime/clavulanic acid</b> |               |       |       |
| SHV-4                              | 0.5           | 0.5   | 0.25  |
| AmpC                               | 0.5           | 8     | 8     |
| XL-1 Blue                          | 0.5           | 0.5   | 0.25  |

for the strains tested. Most of them lacked susceptibility to ceftazidime. For strains producing class A plasmid-encoded enzymes, AVE1330A restored susceptibility to ceftazidime, as did clavulanic acid, both combinations being up to 64-fold more active than ceftazidime alone. Ceftazidime/AVE1330A was also active against class C plasmid-encoded enzymes, MICs ranging from 0.5 to 4 mg/L. Conversely, no synergy was observed with clavulanic acid against ceftazidime-resistant isolates.

More extended data are reported in Table 4. All the *E. coli* isolates were inhibited by 4 mg/L ceftazidime/AVE1330A. No other comparator was as active in terms of reduction in MICs. Against ceftazidime-resistant isolates, the  $MIC_{90}$  of ceftazidime decreased from  $>64$  to 4 and 16 mg/L in the presence of AVE1330A and clavulanic acid, respectively. Both inhibitors had similar behaviour against class A enzyme producers. In contrast to clavulanic acid, AVE1330A restored high ceftazidime activity against class C enzyme producers ( $MIC_{90}$  of 16 or 2 mg/L, respectively). Unlike other species, few *E. coli* isolates were inhibited in the presence of 4 mg/L AVE1330A alone, but the  $MIC_{50}$  and  $MIC_{90}$  still remained  $\geq 8$  mg/L.

For ceftazidime-resistant *Klebsiella*, most of them producing class A enzymes, ceftazidime/AVE1330A lowered the  $MIC_{90}$  from  $>64$  to 2 mg/L, i.e. to values similar to those of ceftazidime/clavulanic acid.

Ceftazidime/AVE1330A was two to four times more potent than ceftazidime alone against ceftazidime-susceptible *Enterobacter* strains. All the ceftazidime-resistant isolates were re-classified as susceptible in the presence of AVE1330A, while ceftazidime/clavulanic acid, co-amoxiclav and piperacillin–tazobactam were inactive.

Against the indole-positive Proteaeae and *Serratia* representatives, the activity of ceftazidime/AVE1330A was most noticeable with regard to maximum MIC, as no ceftazidime-resistant isolate was tested, except *Morganella morganii*, for which the  $MIC_{90}$  was 16 times lower in the presence of AVE1330A (1 mg/L). Combination with clavulanic acid was less active ( $MIC_{90}$  32 mg/L). Ceftazidime/AVE1330A produced the highest

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**Table 3.** Comparative MIC of ceftazidime alone, ceftazidime/AVE1330A 4:1 and ceftazidime/clavulanic acid 4:1 for  $\beta$ -lactamase-producing strains

| Organism                        | Enzyme    | Class | MIC (mg/L)  |                      |                             |
|---------------------------------|-----------|-------|-------------|----------------------|-----------------------------|
|                                 |           |       | ceftazidime | ceftazidime/AVE1330A | ceftazidime/clavulanic acid |
| <i>E. coli</i> GJ2              | PSE-1     | A     | 2           | 1                    | 1                           |
| <i>E. coli</i> GJ3              | PSE-4     | A     | 0.12        | 0.25                 | 0.12                        |
| <i>E. coli</i> GN5482           | TEM-1     | A     | 4           | 0.5                  | 4                           |
| <i>K. pneumoniae</i> IP1        | TEM-2     | A     | 16          | 0.5                  | 0.25                        |
| <i>E. coli</i> HT26             | SHV-1     | A     | 0.5         | 0.25                 | 0.5                         |
| <i>K. pneumoniae</i> 25637      | TEM-4     | A     | 32          | 0.5                  | 0.25                        |
| <i>E. coli</i> CF2              | TEM-5     | A     | 16          | 1                    | 0.5                         |
| <i>E. coli</i> MM1453           | TEM-7     | A     | 16          | 1                    | 1                           |
| <i>E. coli</i> CF3              | TEM-8     | A     | 64          | 2                    | 1                           |
| <i>E. coli</i> CF4              | TEM-12    | A     | 8           | 0.5                  | 0.5                         |
| <i>E. coli</i> CF8              | TEM-16    | A     | >64         | 2                    | 2                           |
| <i>K. pneumoniae</i> IP3        | TEM-20    | A     | 0.5         | 0.06                 | 0.06                        |
| <i>E. coli</i> CF7              | TEM-24    | A     | >64         | 4                    | 4                           |
| <i>E. coli</i> PB1              | TEM-43    | A     | 4           | 0.25                 | 0.25                        |
| <i>K. pneumoniae</i> IP86       | SHV-2     | A     | 64          | 1                    | 1                           |
| <i>E. coli</i> CF6              | SHV-4     | A     | 8           | 0.25                 | 0.25                        |
| <i>E. coli</i> OIP7             | SHV-5     | A     | 32          | 1                    | 1                           |
| <i>K. pneumoniae</i> 3IP7       | SHV-6     | A     | 4           | 1                    | 1                           |
| <i>K. pneumoniae</i> KB3        | SHV-38    | A     | 8           | 2                    | 4                           |
| <i>E. coli</i> KB11             | CTX-M -15 | A     | 64          | 0.5                  | 1                           |
| <i>K. pneumoniae</i> KB1        | TRI-2     | A     | 0.12        | 0.12                 | 0.25                        |
| <i>K. oxytoca</i> MC12          | K1        | A     | 0.25        | 0.12                 | 0.5                         |
| <i>Proteus vulgaris</i> HT9     | K1-like   | A     | 0.12        | 0.06                 | 0.06                        |
| <i>Citrobacter diversus</i> HT1 | K1-like   | A     | 1           | 0.5                  | 0.5                         |
| <i>E. coli</i> KB6              | GES-2     | A     | 8           | 0.5                  | 0.5                         |
| <i>E. coli</i> KB9              | VEB-1     | A     | 2           | 0.5                  | 0.5                         |
| <i>E. coli</i> KB10             | PER-1     | A     | >64         | 4                    | 1                           |
| <i>E. coli</i> KB8              | ACC-1     | C     | >64         | 4                    | 64                          |
| <i>E. coli</i> KB12             | FOX-1     | C     | 32          | 4                    | 16                          |
| <i>K. pneumoniae</i> KB4        | DHA-2     | C     | >64         | 2                    | 64                          |
| <i>K. pneumoniae</i> KB5        | LAT-1     | C     | >64         | 2                    | 64                          |
| <i>K. pneumoniae</i> KB6        | ACT-1     | C     | 2           | 0.5                  | 0.5                         |

activity against *Proteus mirabilis*, all isolates being inhibited at 4 mg/L. As shown by the maximum MICs, clear antagonism (at least an eight-fold increase in the MIC of ceftazidime) was observed with clavulanic acid against several isolates of ceftazidime-susceptible *E. cloacae*, ceftazidime-susceptible *Citrobacter* and *M. morgani*. In contrast, AVE1330A did not produce any such antagonism.

### Effect of inoculum size

As shown in Table 5, the MIC ranges of ceftazidime and ceftazidime/AVE1330A were 0.25–256 mg/L and 0.25–8 mg/L, respectively. For the ceftazidime/AVE1330A combination only, a two- to four-fold increase in MIC was observed when the inoculum size was increased from 5.3 to 9.5 log<sub>10</sub> cfu/mL.

## Discussion

One way to overcome  $\beta$ -lactam resistance is to use  $\beta$ -lactamase inhibitors. This concept has been fully validated by the clinical use of currently available inhibitors.<sup>13</sup> Nevertheless, these

inhibitors cannot encompass both class A penicillinase- and class C cephalosporinase-producing bacteria. Other putative non- $\beta$ -lactam  $\beta$ -lactamase inhibitors have been reported, but none of them has been developed further.<sup>14–16</sup> In contrast, we describe here AVE1330A, a novel bridged bicyclico[3.2.1]diazabicyclo-octanone, capable of inhibiting a broad spectrum of  $\beta$ -lactamases.

When compared with clavulanic acid, tazobactam or sulbactam,<sup>17</sup> AVE1330A remains the best inhibitory compound against both TEM-1 and P99 isolated enzymes. Only AM-112, a recently described oxapenem,<sup>15</sup> has been reported as having lower IC<sub>50</sub>s for P99, but not for TEM-1. Furthermore, two to five AVE1330A molecules only were needed to inactivate one molecule of TEM-1 or P99 enzyme, i.e. far less than reported for clavulanic acid.<sup>17,18</sup>

Classical  $\beta$ -lactam-based  $\beta$ -lactamase inhibitors generally act as substrates of enzymes, giving an unstable acylenzyme intermediate, which is responsible for transient inhibition. This acylenzyme then undergoes a rearrangement, leading to a more stable inhibition. In the case of AVE1330, the half-life of the covalent intermediate was very long, which means efficient irreversible inactivation of both TEM-1 and P99 enzymes.

**Table 4.** Comparative *in vitro* antibacterial activity of ceftazidime alone, ceftazidime/AVE1330A 4:1, ceftazidime/clavulanic acid 4:1, co-amoxiclav and piperacillin–tazobactam

| Organisms (no. of isolates) and drug       | MIC (mg/L) |      |     |
|--|------------|------|-----|
|  | range      | 50%  | 90% |
| <i>E. coli</i> CAZ <sup>R</sup> (20)       |            |      |     |
| ceftazidime                                | 32–>64     | 32   | >64 |
| ceftazidime/AVE1330A 4:1                   | 0.25–4     | 1    | 4   |
| ceftazidime/clavulanic acid 4:1            | 0.25–64    | 2    | 16  |
| co-amoxiclav                               | 4–>64      | 16   | >64 |
| piperacillin–tazobactam                    | 2–>64      | 8    | 64  |
| AVE1330                                    | 4–>16      | 16   | >16 |
| <i>E. coli</i> class A (38)                |            |      |     |
| ceftazidime                                | 0.12–>64   | 8    | >64 |
| ceftazidime/AVE1330A 4:1                   | 0.12–4     | 1    | 2   |
| ceftazidime/clavulanic acid 4:1            | 0.12–8     | 0.5  | 2   |
| co-amoxiclav                               | 4–>64      | 16   | 64  |
| piperacillin–tazobactam                    | 1–>64      | 4    | 64  |
| AVE1330                                    | 4–>16      | 16   | >16 |
| <i>E. coli</i> class C (29)                |            |      |     |
| ceftazidime                                | 0.25–>64   | 8    | 32  |
| ceftazidime/AVE1330A 4:1                   | 0.06–4     | 1    | 2   |
| ceftazidime/clavulanic acid 4:1            | 0.06–64    | 8    | 16  |
| co-amoxiclav                               | 4–>64      | >64  | >64 |
| piperacillin–tazobactam                    | 0.5–>64    | 16   | 64  |
| AVE1330                                    | 4–16       | 8    | 16  |
| <i>K. pneumoniae</i> CAZ <sup>R</sup> (85) |            |      |     |
| ceftazidime                                | 32–>64     | 64   | >64 |
| ceftazidime/AVE1330A 4:1                   | 0.5–8      | 2    | 2   |
| ceftazidime/clavulanic acid 4:1            | 0.25–64    | 1    | 4   |
| co-amoxiclav                               | 2–>64      | 16   | 32  |
| piperacillin–tazobactam                    | 4–>64      | 16   | 64  |
| AVE1330A                                   | 16–>16     | >16  | >16 |
| <i>K. pneumoniae</i> ESBL (108)            |            |      |     |
| ceftazidime                                | 0.06–>64   | 32   | >64 |
| ceftazidime/AVE1330A 4:1                   | 0.06–8     | 1    | 2   |
| ceftazidime/clavulanic acid 4:1            | 0.06–16    | 1    | 2   |
| co-amoxiclav                               | 2–64       | 8    | 32  |
| piperacillin–tazobactam                    | 0.5–>64    | 16   | 64  |
| AVE1330A                                   | 16–>16     | >16  | >16 |
| <i>E. cloacae</i> CAZ <sup>S</sup> (86)    |            |      |     |
| ceftazidime                                | 0.12–8     | 0.5  | 4   |
| ceftazidime/AVE1330A 4:1                   | 0.12–2     | 0.5  | 1   |
| ceftazidime/clavulanic acid 4:1            | 0.06–64    | 0.5  | 4   |
| co-amoxiclav                               | 1–>64      | >64  | >64 |
| piperacillin–tazobactam                    | 0.5–>64    | 4    | 64  |
| AVE1330A                                   | ND         |      |     |
| <i>E. cloacae</i> CAZ <sup>R</sup> (84)    |            |      |     |
| ceftazidime                                | 32–>64     | 64   | >64 |
| ceftazidime/AVE1330A 4:1                   | 0.5–4      | 2    | 4   |
| ceftazidime/clavulanic acid 4:1            | 0.5–>64    | 32   | 64  |
| co-amoxiclav                               | 64–>64     | >64  | >64 |
| piperacillin–tazobactam                    | 4–>64      | 64   | >64 |
| AVE1330A                                   | ND         |      |     |
| <i>Serratia</i> spp. (85)                  |            |      |     |
| ceftazidime                                | 0.06–16    | 0.5  | 1   |
| ceftazidime/AVE1330A 4:1                   | 0.12–2     | 0.5  | 0.5 |
| ceftazidime/clavulanic acid 4:1            | 0.12–4     | 0.25 | 0.5 |
| co-amoxiclav                               | 4–>64      | >64  | >64 |
| piperacillin–tazobactam                    | 0.5–>64    | 4    | 64  |
| AVE1330A                                   | 32–>64     | >64  | >64 |

## AVE1330A, a novel $\beta$ -lactamase inhibitor

**Table 4.** (Continued)

| Organisms (no. of isolates) and drug              | MIC (mg/L) |      |      |
|---|------------|------|------|
|   | range      | 50%  | 90%  |
| <i>Citrobacter freundii</i> CAZ <sup>S</sup> (68) |            |      |      |
| ceftazidime                                       | 0.03–8     | 0.5  | 2    |
| ceftazidime/AVE1330A 4:1                          | 0.06–1     | 0.25 | 0.5  |
| ceftazidime/clavulanic acid 4:1                   | 0.12–64    | 0.5  | 32   |
| co-amoxiclav                                      | 1–>64      | >64  | >64  |
| piperacillin–tazobactam                           | 0.25–64    | 4    | 4    |
| AVE1330A  | 16–>64     | 64   | >64  |
| <i>C. freundii</i> CAZ <sup>R</sup> (14)          |            |      |      |
| ceftazidime                                       | 32–>64     | 64   | >64  |
| ceftazidime/AVE1330A 4:1                          | 0.25–8     | 1    | 2    |
| ceftazidime/clavulanic acid 4:1                   | 32–64      | 32   | 64   |
| co-amoxiclav                                      | >64        | >64  | >64  |
| piperacillin–tazobactam                           | 8–>64      | 32   | 64   |
| AVE1330A  | 32–>64     | 64   | >64  |
| <i>M. morgani</i> (33)                            |            |      |      |
| ceftazidime                                       | 0.03–16    | 1    | 16   |
| ceftazidime/AVE1330A 4:1                          | 0.06–1     | 0.25 | 1    |
| ceftazidime/clavulanic acid 4:1                   | 0.12–64    | 4    | 32   |
| co-amoxiclav                                      | 8–>64      | >64  | >64  |
| piperacillin–tazobactam                           | 0.03–64    | 2    | 16   |
| AVE1330A  | >16        | >16  | >16  |
| Other indole-positive <i>Proteus</i> spp. (106)   |            |      |      |
| ceftazidime                                       | 0.015–4    | 0.12 | 1    |
| ceftazidime/AVE1330A 4:1                          | 0.03–2     | 0.12 | 1    |
| ceftazidime/clavulanic acid 4:1                   | 0.015–8    | 0.12 | 1    |
| co-amoxiclav                                      | 0.25–>64   | 32   | >64  |
| piperacillin–tazobactam                           | 0.12–>64   | 2    | 32   |
| AVE1330A  | 8–>16      | >16  | >16  |
| <i>P. mirabilis</i> (46)                          |            |      |      |
| ceftazidime                                       | 0.06–>64   | 0.12 | 0.25 |
| ceftazidime/AVE1330A 4:1                          | 0.03–4     | 0.12 | 0.25 |
| ceftazidime/clavulanic acid 4:1                   | 0.03–>64   | 0.12 | 64   |
| co-amoxiclav                                      | 0.25–>64   | 2    | 32   |
| piperacillin–tazobactam                           | 0.12–>64   | 0.5  | 8    |
| AVE1330A  | 8–>16      | >16  | >16  |

CAZ<sup>S</sup>, ceftazidime susceptible; CAZ<sup>R</sup>, ceftazidime resistant; ND, not determined.

The efficacy of  $\beta$ -lactamase inhibitors can be reduced when the quantity of enzyme produced is high.<sup>18</sup> Using a panel of isogenic *E. coli* (Table 2), the presence of more enzyme in the arabinose-treated cultures did not alter the MICs of the ceftazidime/AVE1330A combination, reflecting the low Tn for the enzymes. This high efficacy could also explain why AVE1330A protected ceftazidime from the deleterious inoculum effect produced by Gram-negative bacteria, the amount of  $\beta$ -lactamases being parallel to the size of the bacterial population.

Although ceftazidime is one of the most effective cephalosporins, its activity is now jeopardized by both class A (such as TEM-3, TEM-10, SHV-2, SHV-5) and overproduced class C enzymes.<sup>19</sup> While AVE1330A is devoid of any significant intrinsic antimicrobial activity, combination with ceftazidime was synergic against class A enzymes produced by Enterobacteriaceae, irrespective of their phenotype, at a level similar to that of combination with clavulanic acid, but much more active than piperacillin–tazobactam or co-amoxiclav, as already reported for

these species.<sup>20</sup> Against class C enzyme producers, AVE1330A fully restored ceftazidime activity and was more active than piperacillin–tazobactam. AVE1330A tested at a 4:1 ratio was even more effective than reported for AM-112 at a 4 mg/L fixed concentration, in enhancing ceftazidime activity against *Citrobacter*, *Enterobacter*, *Serratia* and *Morganella*.<sup>21</sup> In addition, ceftazidime/AVE1330A was also very effective against new plasmid-mediated class A and class C enzymes, such as CTX-M, GES-2, PER-1, ACC-1, FOX-1, DHA-2, and LAT-1, which are currently expanding over the world, causing nosocomial outbreaks and increasing in prevalence, even in the community.<sup>22,23</sup>

Ceftazidime is documented to induce AmpC cephalosporinases and select stably derepressed bacterial mutants against which it is inactive.<sup>4</sup> Actually, we showed that AVE1330A inhibited enzymes, preventing a sufficient quantity from being induced, and consequently permitted ceftazidime to be active. Furthermore, unlike ceftazidime/clavulanic acid, no antagonism of ceftazidime/AVE1330A was observed in MIC studies for any

**Table 5.** Effect of inoculum size on the *in vitro* antibacterial activity of ceftazidime/AVE1330A 4:1 and ceftazidime alone

| Strains (enzyme)                         | MIC (mg/L)                                  |             |                          |
|--|---|-------------|--------------------------|
|  | inoculum size<br>(log <sub>10</sub> cfu/mL) | ceftazidime | ceftazidime/<br>AVE1330A |
| <i>K. pneumoniae</i><br>IP35 (SHV-2)     | 9.4   | 8           | 2                        |
|  | 8.4   | 8           | 1                        |
|  | 7.4   | 4           | 1                        |
|  | 6.4   | 2           | 0.5                      |
|  | 5.4   | 2           | 0.5                      |
| <i>E. coli</i> SJ4 (SHV-5)               | 9.3   | 256         | 2                        |
|  | 8.3   | 256         | 2                        |
|  | 7.3   | 128         | 2                        |
|  | 6.3   | 128         | 2                        |
|  | 5.3   | 8           | 1                        |
| <i>Serratia marcescens</i><br>UC6 (AmpC) | 9.5   | 2           | 0.5                      |
|  | 8.5   | 2           | 0.5                      |
|  | 7.5   | 2           | 0.5                      |
|  | 6.5   | 0.25        | 0.25                     |
|  | 5.5   | 0.25        | 0.25                     |
| <i>E. cloacae</i><br>HT6 (AmpC)          | 9.1   | 256         | 4                        |
|  | 8.1   | 128         | 2                        |
|  | 7.1   | 64          | 1                        |
|  | 6.1   | 64          | 1                        |
|  | 5.1   | 16          | 1                        |

strain tested, including chromosomally inducible AmpC-producing *Citrobacter*, *Enterobacter* and *Serratia*. This absence of antagonism could be an indirect demonstration of the likely absence of any induction or selection of resistant mutants.<sup>24</sup> This is clearly the result of the non-β-lactam structure of AVE1330A. More specifically designed studies are ongoing to confirm this observation.

No extensive experience in therapy has been published yet with combinations of cephalosporins and β-lactamase inhibitors.<sup>25</sup> However, AVE1330A could have value as an alternative strategy to carbapenems used as single agents.

In conclusion, AVE1330A represents a novel class of β-lactamase inhibitor, devoid of any significant intrinsic antimicrobial activity, effective against both classes A and C β-lactamases, therefore extending the spectrum of ceftazidime to most resistant bacteria. AVE1330A in combination with a β-lactam deserves further investigation as a promising drug in our antibacterial armamentarium.

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