NEW MICROBIOLOGICA, **31**, 37-46, 2008

In vitro interaction between mecillinam and piperacillin-tazobactam in the presence of azithromycin against members of the *Enterobacteriaceae* family and *Pseudomonas aeruginosa*

Elisabetta Maioli, Eugenio A. Debbia, Laura Gualco, Anna Marchese

Section of Microbiology, "C.A. Romanzi" DISCAT, University of Genova, Genova, Italy

SUMMARY

Mecillinam was tested in vitro alone or in combination with piperacillin-tazobactam and azithromycin against representative species of the *Enterobacteriaceae* family and *Pseudomonas aeruginosa* to extend its antibacterial spectrum, and to protect mecillinam from inactivating enzymes taking advantage of the presence of tazobactam. Drug interactions were studied by microdilution method, by selection of spontaneous resistant mutants on agar plates containing the drugs in combination and by time kill experiments. Against *Enterobacteriaceae* mecillinam and piperacillin-tazobactam showed synergistic interaction in 24/60 tests carried out by microdilution technology, in 4/16 by selecting resistant mutants and in 5/9 by time-kill experiments. *P. aeruginosa* reacted indifferently to the drug combinations, with few exceptions, when azithromycin was present a reduction of the MICs were recorded. Mecillinam reacted favourably in vitro in combination with piperacillin-tazobactam against not only strains included in its antibacterial spectrum but also against resistant *Morganella morganii*, *Proteus spp* and *P. aeruginosa*. The addition of azithromycin (8 mg/L) was beneficial for the drug combination increasing the bactericidal effect in the great majority of the cases. Only systematic *in vivo* studies may establish the clinical significance and benefits of the present observations.

KEY WORDS: Mecillinam, Piperacillin-Tazobactam, Azithromycin, In vitro interactions, *P. aeruginosa, Enterobacteriaceae*, Time-kill, Spontaneous-resistant mutants

Received July 17, 2007 *Accepted* September 10, 2007

INTRODUCTION

Mecillinam (amdinocillin) is an oral penicillin that shows antibacterial activity against many gram-negative bacteria with the exception of some members of the *Enterobacteriaceae* group, *Pseudomonas* and non-fermenting gram-negative bacteria, as well as gram-positive organisms (Lund and Tybring, 1972; Neu, 1976a; Fass 1980 and 1982). Mecillinam differs from other compounds of the same class in its ability to bind specifically penicillin binding protein-2 (PBP-2). Generally other β-lactams interact with PBPs-1 and -3 (Spratt, 1977). This unique feature has suggested the combination of this antibiotic with other drugs of the same class.

Marked synergy was in fact reported against many isolates of the *Enterobacteriaceae* family, but not against gram-positive organisms or *Pseudomonas* species (Neu 1976b; Eng *et al.*, 1988).

Although able to hydrolyse mecillinam under some conditions, the β-lactamases present in

Corresponding author
Anna Marchese
Section of Microbiology
"C.A. Romanzi" DISCAT, University of Genova
Largo Rosanna Benzi 10 - 16132 Genova, Italy
E-mail: anna.marchese@unige.it

many Gram-negative species are unlikely to be very effective at protecting the bacteria *in vivo* because of their relatively low affinity for this penicillin.
Organisms which produce high levels of plasmid-mediated beta-lactamase, however, are resistant to the drug (Sougakoff and Jarlier, 2000; Thomas *et al*., 2006). Mecillinam is effective in the treatment of urinary tract infections caused by susceptible strains of *E. coli, Klebsiella* and *Enterobacter* species (Nicolle 2000; Graninger, 2003).
The ability of a β-lactam antibiotic to inhibit the growth of Gram-negative bacteria depends on three main properties, affinity for target sites in the bacterial cell, the ability to penetrate through the outer layers of the bacterial envelope to reach these sites, and the ability to resist β-lactamases hydrolysis in the periplasmic space.
Mecillinam that possesses many of the above characteristics might combine its properties with those of another compound of the same class to complete or maximize its antibacterial spectrum. In this context, piperacillin-tazobactam is an excellent broad spectrum combination between a penicillin and a suicide inhibitor of β-lactamase showing antibacterial activity against a large number of gram-negative isolates (Dela Pena *et al*., 2006; Raveh *et al.,* 2006; Sligl *et al.,* 2006). Therefore, it was hypothesised that piperacillin-tazobactam should have two different effects when used in combination with mecillinam.
First, it should facilitate the entry of mecillinam in bacterial cell.
Furthermore, the concomitant presence of tazobactam should confer protection to mecillinam from the hydrolysing enzymes. Finally if both events occur the interaction of the two drugs which react synergistically should enhance the lethal effect on the bacterial host.
In this study, the activity of mecillinam was tested alone or in combination with piperacillin-tazobactam against representative species of gram-negative bacteria including mecillinam-resistant strains and *P. aeruginosa*.
The tests were also carried out in the presence of azithromycin that it is known to interfere with the synthesis of several metabolic products of the bacterial cell affecting its fitness (Molinari *et al*., 1993; Nalca *et al*., 2006).

MATERIALS AND METHODS

Bacterial strains

60 clinically isolated, members of the *Enterobacteriaceae* family were studied. The complete list of strains is reported in Table 1. They were chosen as representative of either mecillinam - susceptible or - resistant strains such as *M. morganii, Proteus mirabilis*, *Providencia stuartii* and *P. aeruginosa*. The collection also included 3 laboratory strains coding for TEM-2, TEM-3 and TEM-4 previously described (Cagnacci *et al.*, 2005) and various clinically isolated bacteria species producing ESBL not characterised in detail. *E. coli* ATCC25922 and *P. aeruginosa* ATCC 27853 were used as control strains.

Antimicrobial agents

Mecillinam was a generous gift of F. Bjoerkling (Leo-Pharma Ballerup, Denmark), azithromycin and piperacillin-tazobactam were obtained from their respective manufacturers. Sterile stock solutions of the drugs were prepared according the manufacturer's instructions by dissolving the compounds in the specific solvent to obtain a final concentration of 1 mg/ml.

Susceptibility tests

The minimal inhibitory concentrations (MICs) were determined in cation-supplemented Mueller-Hinton (CSMH) broth adopting the microdilution method following the procedure suggested by the Clinical and Laboratory Standards Institute (CLSI, 2005). When time-kill experiments were carried out, MICs were again determined in 250 flasks using an inoculum of about 10^7 CFU/ml. After incubation for 18-24 hours at 37°C in a gyrotory water bath shaker the new MICs were registered.

Interactions between mecillinam and other drugs

Microdilution method

The MIC of mecillinam was determined alone or in the presence of sub-inhibitory concentrations (0.5XMIC) of piperacillin-tazobactam (Eliopoulos and Moellering, 1996) using a micromethod. When azithromycin was included in the test it was used at a fixed concentration of 8 mg/L, a dose which was found to produce physiological perturbations in the strains studied (Molinari *et*

TABLE 1 - *Minimum inhibitory concentrations (MICs) of mecillinam alone or in combination with other drugs against the strains used in this study.*

	MIC (mg/L)			
Strain designation	*Mecillinam*	*Piperacillin/ Tazobactam*	*Mecillinam+ Piperacillin/ Tazobactam*	*Mecillinam+ Piperacillin/ Tazobactam+ Azithromycin*
E. coli 2	1	2	0.125	0.06
E. coli 4	1	2	0.25	0.25
E. coli 5	4	2	0.5	0.125
E. coli 11	0.5	2	0.25	0.25
E. coli 14	0.5	4	0.25	0.25
E. coli 16	1	4	0.5	0.125
E. coli 17	0.5	2	0.25	0.125
E. coli 18	2	2	0.25	0.06
E. coli 20	>256	4	0.5	0.125
E. coli 21	2	2	0.06	0.06
E. coli 24	>256	2	0.25	0.25
E. coli 25	>256	2	0.5	0.125
E. coli 31	>256	4	1	0.25
E. coli TEM-2	>256	64	4	0.25
E. coli TEM-3	256	8	0.5	0.125
E. coli TEM-4	256	8	0.5	0.125
E. coli 507 ESBL	64	8	0.25	0.125
K. pneumoniae 33	>256	4	1	0.5
K. pneumoniae 52 ESBL	>256	128	4	4
K. pneumoniae 53 ESBL	>256	128	4	16
K.oxytoca 7	0.25	2	0.25	0.25
K.oxytoca 34	0.25	1	0.125	0.125
E. aerogenes 002-15	64	4	0.25	0.25
E. aerogenes 002-6	32	2	0.125	0.125
E. aerogenes 035-140	>256	16	0.5	0.125
E. aerogenes 059-47	32	8	0.5	0.125
E. cloacae 002-66	4	64	0.5	0.5
E. cloacae 006-74	4	128	0.25	0.25
E. cloacae 006—84	4	>256	0.5	0.5
E. cloacae 006-86	0.125	64	0.25	0.25
E. cloacae 035-109	4	128	0.25	0.25
E. cloacae 035-130	0.125	128	0.125	0.125
E. cloacae 035-142	1	256	0.5	0.125
E. cloacae 035-151	0.06	16	0.06	0.06
E. cloacae 035-157	0.125	4	4	0.25
S. marcescens 030-1	1	2	0.5	1

→

→ TABLE 1

Strain designation	*MIC (mg/L)*			
	Mecillinam	*Piperacillin/ Tazobactam*	*Mecillinam+ Piperacillin/ Tazobactam*	*Mecillinam+ Piperacillin/ Tazobactam+ Azithromycin*
S. marcescens 030-12	1	2	0.5	0.125
S. marcescens 030-76	1	2	1	0.25
S. marcescens 035-121	> 256	2	0.5	0.25
S. marcescens 020-23	2	0.5	0.125	0.125
S. marcescens 020-70	>256	2	0.25	0.25
S. marcescens 035-13	4	1	0.5	0.25
C. freundii 015-89	>256	64	2	0.25
C. freundii 035-84	>256	128	2	0.125
C. freundii 058-183	128	16	4	0.125
C. koseri 035-111	128	16	4	0.5
M. morganii 15	>256	0.125	0.125	0.125
M. morganii 19	>256	0.5	0.25	0.25
M. morganii 015-120	>256	32	8	0.25
M. morganii 015-77	>256	32	32	0.25
M. morganii 035-149	>256	4	4	0.125
M. morganii 058-139	>256	64	16	2
M. morganii 058-142	>256	32	8	0.25
M. morganii db2	>256	32	0.125	0.125
M. morganii 015-172	>256	16	4	8
M. morganii 015-194	>256	4	0.25	0.125
P. mirabilis 3 ESBL	>256	4	1	4
P. stuartii 031-172	>256	8	4	4
P. stuartii 035-18	>256	4	4	4
P. stuartii 035-29	>256	4	2	8
P. aeruginosa 3	>256	8	8	8
P. aeruginosa 8	>256	128	128	128
P. aeruginosa 9	>256	>256	>256	>256
P. aeruginosa 12	>256	4	4	4
P. aeruginosa 13	>256	4	4	4
P. aeruginosa 22	>256	128	16	32
P. aeruginosa 27	>256	2	16	16
P. aeruginosa 29	>256	8	8	8
P. aeruginosa 30	>256	16	16	16
P. aeruginosa 32	>256	>256	>256	>256
E. coli ATCC25922	2	2	0.03	0.03

al., 1993). For organisms not susceptible to mecillinam a fixed concentration of 128 mg/L was adopted. Synergism was defined as a ≥3-fold dilution decrease in the MIC with the combination in comparison to mecillinam alone. Antagonism was registered if the MIC increased by 1 fold or more. Indifference was defined in all the other cases The presence of azithromycin was considered favourable to the interaction when it caused ≥1 dilution reduction of the MIC values obtained with the drugs in combinations.

Selection of spontaneous mecillinam-resistant strains alone or in combination with other drugs

A bacterial suspension of about 10^9CFU/ml was seeded on Mueller-Hinton agar plates containing increasing doses of mecillinam or piperacillin-tazobactam and a fixed concentration of azithromycin (8 mg/L) when the latter drug was added to the agar plates. For organisms not susceptible to mecillinam a fixed concentration of 128 mg/L was adopted for tests including this drug, as mentioned above. A reduction of 99% of the CFU/ml found in the drugs combination in comparison to the drug alone was defined as synergism, (90%) additivity, and (10%) indifference.

Dynamic bactericidal activity of mecillinam

Time-kill experiments were performed on 12 representative isolates by adding the drugs, at a concentration corresponding to their 0.5XMIC, to log-phase bacterial cultures diluted to 106-107 CFU/ml growing in 250 ml flasks at 37°C. Just before the compounds were added and at 2, 6, and 24 h thereafter, bacterial counts were carried out. Survivors were evaluated by determining CFU on agar plates.

Again when the experiments were carried out with mecillinam-resistant species this drug was included in the medium at a fixed concentration of 128 mg/L.

Azithromycin unless otherwise stated was used at 8 mg/L. Antibiotic interactions were interpreted as synergism, additivity, and indifference or antagonistic if the combinations, compared with the most effective single antibiotic, caused at least a 100-fold reduction or increase, respectively, in the CFU/ml of the survivors at 24 h. Intermediate results were defined as indifference (Eliopoulos and Moellering, 1996).

RESULTS

Minimum inhibitory concentrations of mecillinam alone or in combination with other drugs are reported in Table 1. Mecillinam confirms its antibacterial spectrum inhibiting many members of the *Enterobacteriaceae* family with the exception of *M. morganii*, *P. mirabilis* and *P. stuartii* and the few isolates of *Citrobacter* spp. included in this study. As expected *P. aeruginosa* strains were resistant to mecillinam. The in vitro activity of piperacillin-tazobactam showed a high variability depending on the strains. In this collection 12 organisms out of 70 were found fully resistant to piperacillin-tazobactam. When the activity of mecillinam was tested in combination with the latter compound, a large proportion of the *Enterobacteriaceae* members were inhibited at a concentration of drugs lower than that observed with mecillinam or piperacillin-tazobactam alone. In particular a synergistic reaction was observed in 24 out of 60 cases analysed with *Enterobacteriaceae* species. A ≥3-fold reduction in the MIC values was found. A MIC reduction was recorded with 26 strains and in the remaining 10 tests indifference was the result obtained. Synergism or indifferent reactions were also noted with different isolates of *M. morganii* and *P. mirabilis*, while *P. aeruginosa* isolates demonstrated no difference in the MIC values observed with piperacillin-tazobactam. The addition of azithromycin increased the activity of the combination of mecillinam with piperacillin-tazobactam against many species of the *Enterobacteriaceae* family (32/60 tests), including *M. morganii*. No variation in the MIC values was registered with *P. mirabilis, P. stuartii*, or with *P. aeruginosa*. An increase in the MIC value was found in 5 tests.

The interaction of mecillinam with the other antibiotics was then evaluated in representative isolates, by selecting the spontaneous resistant strains that arose in agar plates containing mecillinam alone or in combinations with the other drugs.

As reported in Table 2, the combination of the two antimicrobials strongly reduced the numbers of survivors registered on selective plates in comparison with those observed employing the drugs alone. In particular, synergism was found in: *E. coli* (2 out of 3 strains), *M. morganii* (1/4), *K. pneu-*

TABLE 2 - *Number of spontaneous mecillinam resistant strains found on agar plates containing the indicated concentrations of mecillinam alone or in combination with other drugs against the strains analysed in this study.*

Strain designation	*Drug Concentration*	*Mecillinam*	*Piperacillin/ Tazobactam*	*Piperacillin/ Tazobactam +Mecillinam*	*Piperacillin/ Tazobactam +Mecillinam + Azithromycin*
E.coli					
2	2xMIC	88	99	0	0
	4xMIC	0	10	0	0
	8xMIC	0	8	0	0
4	2xMIC	cg	716	154	31
	4xMIC	504	195	55	0
	8xMIC	0	68	9	0
16	2xMIC	cg	74	0	0
	4xMIC	142	7	0	0
	8xMIC	3	0	0	0
M. morganii					
15	2xMIC	nd	cg	cg	623
	4xMIC	nd	97	127	89
	8xMIC	nd	73	65	44
19	2xMIC	nd	cg	cg	cg
	4xMIC	nd	507	471	142
	8xMIC	nd	166	181	45
015-77	2xMIC	nd	cg	42	0
	4xMIC	nd	28	0	0
	8xMIC	nd	0	0	0
015-194	2xMIC	nd	cg	362	18
	4xMIC	nd	cg	487	66
	8xMIC	nd	868	735	0
S. marcescens					
030-76	2xMIC	184	63	0	0
	4xMIC	122	14	0	0
	8xMIC	0	0	0	0
030-1	2xMIC	cg	cg	436	355
	4xMIC	cg	1000	771	204
	8xMIC	54	0	0	0
020-23	2xMIC	cg	109	7	0
	4xMIC	67	5	0	0
	8xMIC	0	2	0	0
035-13	2xMIC	112	28	0	0
	4xMIC	5	16	0	0
	8xMIC	0	10	0	0
K. pneumoniae					
33	2xMIC	nd	246	0	0
	4xMIC	nd	19	0	0
	8xMIC	nd	0	0	0
C. freundii					
035-84	2xMIC	nd	cg	180	0
	4xMIC	nd	165	0	0
	8xMIC	nd	0	0	0
E.cloacae					
006-74	2xMIC	cg	cg	26	0
	4xMIC	344	96	0	0
	8xMIC	0	0	0	0

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→ TABLE 2

Strain designation	*Drug Concentration*	*Mecillinam*	*Piperacillin/ Tazobactam*	*Piperacillin/ Tazobactam +Mecillinam*	*Piperacillin/ Tazobactam +Mecillinam + Azithromycin*
E. cloacae					
035-142	2xMIC	cg	cg	80	0
	4xMIC	cg	cg	15	0
	8xMIC	522	387	0	0
P. stuartii					
031-172	2xMIC	nd	175	110	0
	4xMIC	nd	62	33	0
	8xMIC	nd	0	0	0
P. aeruginosa					
3	2xMIC	nd	cg	cg	0
	4xMIC	nd	cg	cg	0
	8xMIC	nd	18	0	0
8	2xMIC	nd	cg	cg	165
	4xMIC	nd	198	55	120
	8xMIC	nd	15	13	17
12	2xMIC	nd	cg	cg	cg
	4xMIC	nd	cg	cg	626
	8xMIC	nd	58	31	56
13	2xMIC	nd	cg	cg	cg
	4xMIC	nd	cg	cg	200
	8xMIC	nd	cg	cg	80
22	2xMIC	nd	cg	cg	0
	4xMIC	nd	11	39	0
	8xMIC	nd	0	8	0
30	2xMIC	nd	cg	cg	0
	4xMIC	nd	54	53	0
	8xMIC	nd	12	28	0

cg, confluent growth; nd, not determined.

moniae (1/1), and *E. cloacae* (1/2). An additive reaction was registered in *E. coli* (1 out of 3 strains), *M. morganii* (2/4), *S. marcescens* (4/4), *C. freundii* (1/1) and *E. cloacae* (1/2). Indifference was the response of the other drug interactions with *M. morganii* (1 /4) and *P. stuartii* (1/1), as well in *P. aeruginosa* (6/6).

The addition of azithromycin caused a further reduction of the number of the CFU/ml found in all the drug combinations studied. Under no circumstances and with no drug combinations was antagonism detected.

Finally, the interaction of mecillinam with other drugs (0.5XMIC) was studied by time-kill experiments. Figure 1 shows the results of some representative isolates. Figures 1A, B and C depict three different *E. coli* strains that were all synergistically inhibited by mecillinam in combination with piperacillin-tazobactam and in the presence of azithromycin. *E. cloacae* (Figure 1D) reacted indifferently to the above drug association and only the addition of azithromycin produced an additive effect.

The single *C. freundii* isolate tested was synergistically killed by mecillinam in association with piperacillin-tazobactam (Figure 1E) irrespective of the presence of azithromycin. The same drugs in combination produced an additive effect against one *S. marcescens* strain (Figure 1F).

With respect to *M. morganii* strains (Figures 1G, H and I) the first isolate analysed was synergistically inhibited by mecillinam in combination with piperacillin-tazobactam, the other two strains studied showed an indifferent response

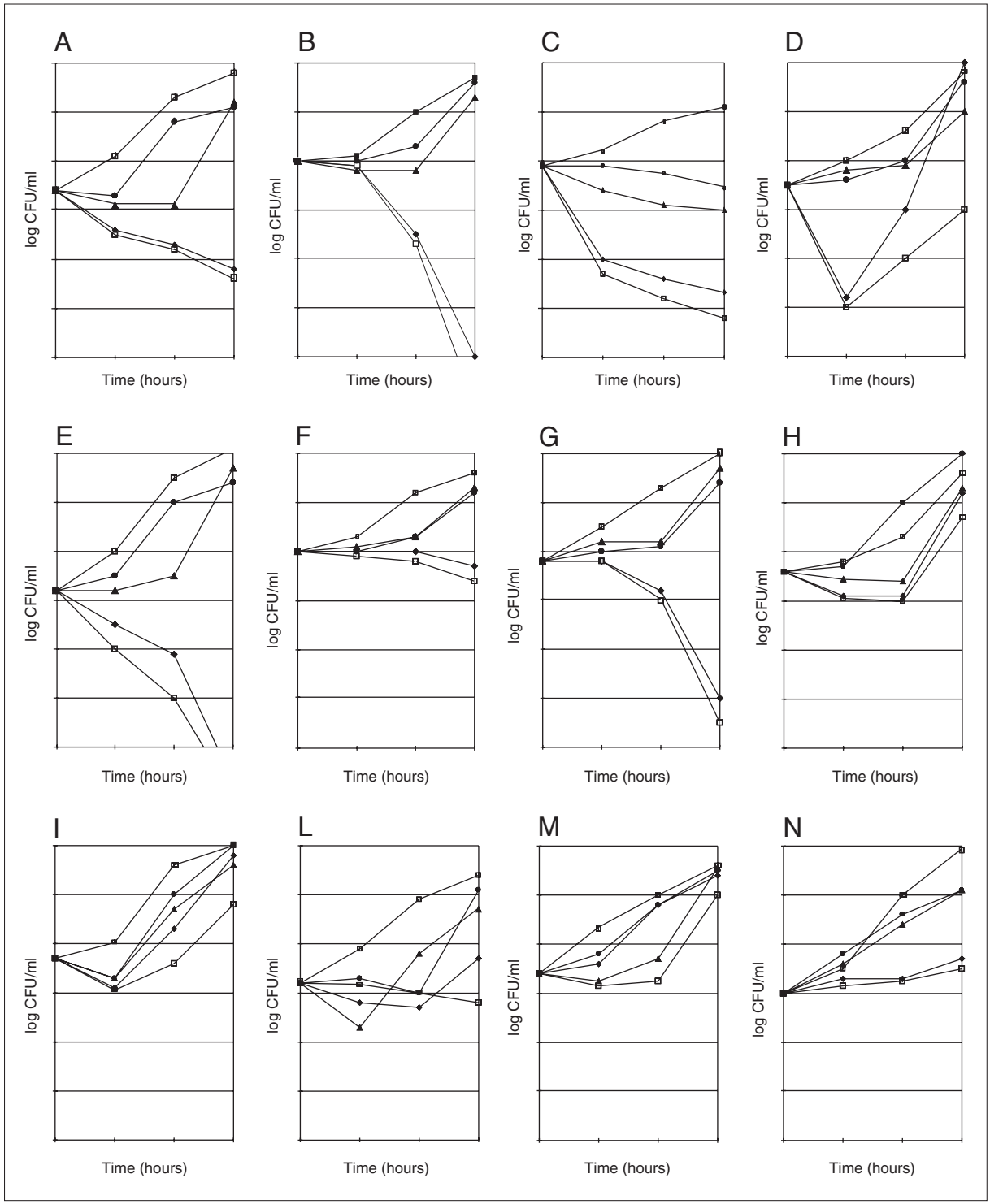

FIGURE 1 - *Bactericidal activity of mecillinam at 0.5XMIC in combination with piperacillin-tazobactam and azithromycin against member of Enterobacteriaceae family and P. aeruginosa.* ■ *Control,* ▲ *mecillinam,* ● *piperacillin-tazobactam,* ◆ *mecillinam+piperacillin-tazobactam,* □ *mecillinam+ piperacillin-tazobactam +azithromycin.* ***A****, E. coli 12;* ***B****, E. coli TEM-3;* ***C****, E. coli 2;* ***D****, E. cloacae 6-84;* ***E****, C. freundii 035-84;* ***F****, S. marcescens 030-1;* ***G****, M. morganii 15-195;* ***H****, M. morganii 15;* ***I****, M. morganii 19;* ***L****, P. aeruginosa 9;* ***M****, P. aeruginosa 176;* ***N****, P. aeruginosa 179.*

after 6 hours of exposure which was confirmed at the end of the experiments (24 hours).
Finally *P. aeruginosa* isolates reacted unpredictably when exposed to the combination of mecillinam and piperacillin-tazobactam. In one case (*P. aeruginosa* 9) (Figure 1L) these drugs interacted indifferently, but the addition of azithromycin gave an additive response. The combination of mecillinam and piperacillin-tazobactam reacted synergistically against *P. aeruginosa* 176 (Figure 1M) irrespective of the presence of azithromycin. The last strain tested (*P. aeruginosa* 179) (Figure 1N) was susceptible to the drugs in combination in the presence or not of azithromycin, an additive effect was in fact found.
In no instance and under no experimental conditions was antagonism encountered.

CONCLUSION

The major findings obtained in this investigation can be summarised as follows. Mecillinam reacted favourably *in vitro* in combination with piperacillin-tazobactam against not only members of the *Enterobacteriaceae* included in its antibacterial spectrum but also against *M. morganii*, *P. mirabilis* and *P. aeruginosa*. The addition of azithromycin (8 mg/L) was beneficial for the drug combination increasing the bactericidal effect in the great majority of the cases. Under no circumstance and with no combination of drugs was an antagonistic effect detected. With respect to the methodology of testing for drug interaction, the data obtained with the selection of spontaneous resistant strains show a good correlation with the time-kill experiments as regards detection of a favourable interaction between mecillinam and piperacillin-tazobactam in the presence of azithromycin.
Interaction between two or more drugs may take place either as a result of a decision to adopt an association of antibiotic or because after a failure of the first molecule the one selected to replace it will certainly interact with a residual level of the previous antimicrobial. Only systematic *in vivo* studies may establish the clinical significance and possible benefits of the combination of the antibiotics analysed in this study. The observations of the present study indicate that in empiric therapy, the combination of these different classes of antibiotics should not give adverse reactions to maintain the original activity or should have a beneficial effect from the presence of each other.
Finally, analysing the mode of action of the above drugs when combined, the data obtained in this study suggest that piperacillin-tazobactam interfering with the cell wall synthesis enables mecillinam to enter into the bacterial cells. This behaviour, that is reminiscent of that of ampicillin and aminoglycosides against enterococci, is also supported by the observation that an increase in the concentration of mecillinam also increases the rate of killing when the drugs are in combination. In this context the role of azithromycin should be that of altering the fitness of the biochemical reactions or some product related to the quorum sensing phenomenon, increasing the magnitude of the synergistic activity of the two drugs or interfering with the bacterial response to the damage caused by the antibiotics.
Furthermore, the addition of piperacillin-tazobactam in the *in vitro* tests of mecillinam showed an enlargement of its antibacterial spectrum and a concomitant protection of mecillinam from inactivating enzymes. The benefits of the presence of a suicide inhibitor have been also reported (Thomas *et al.*, 2006) and the present findings are a further extension of these observations. Together these results suggest that mecillinam in combination with other drugs might offer a new therapeutic option for the treatment of infections other than those affecting the urinary tract, or as alternative compounds where drug resistance to conventional drugs is suspected.

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