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In vitro interaction between ceftazidime and vancomycin/teicoplanin in the presence of azithromycin against *Pseudomonas aeruginosa*

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Abstract - *Pseudomonas aeruginosa* is an opportunistic pathogen, intrinsically resistant to many antibiotics and prone to acquire resistance against many drugs. It is assumed that agents that disorganise the structure of the outer membrane might allow the passage of other drugs into cell. To verify this hypothesis, ceftazidime (CAZ) has been tested in association with glycopeptides (GLYs) and azithromycin (AZI). Time-kill experiments were performed on a representative strain. CAZ in combination with GLYs showed 99, 90 and 10% of CFU/ml reduction in 33.9, 52.5 and 13.6% of the cases, respectively; the addition of AZI increased the incidence of 99% CFU/ml reduction to 42% of the cases. Indifference was the most common finding, and additive/synergism in the other cases. Present findings demonstrated that CAZ favourably reacted with GLYs in the presence of AZI.

Key words: *Pseudomonas aeruginosa,* vancomycin, ceftazidime, azithromycin, drug combination, time-kill tests, selection of resistant strains.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous metabolic versatile common environmental microorganism. Its ability to colonise many different ecological niches and to utilise several compounds as energy source reflects its distribution in many sites including human body. Under favourable circumstances it becomes responsible of several opportunistic infections especially in nosocomial settings (Durack, 1989; Lyczac *et al*., 2000; Kiska and Gilligan, 2003; Rossolini and Mantegoli, 2005). It is intrinsically resistant to many antibiotics and evolution toward resistance occurs very frequently. Multidrug resistance is common, and clinical isolates resistant to virtually all anti-pseudomonal agents are increasingly being reported (Livermore, 2002 and 2003; Juan *et al*., 2005; Rossolini and Mantegoli, 2005; Wang *et al*., 2006). The management of *P. aeruginosa* infections might be very difficult so that surveillance programs are essential for the definition of empirical regimens. Monotherapy is usually adopted for uncomplicated urinary tract infections, while combination therapy is normally recommended for severe infections, such as bacteraemia and pneumonia, although, at least in some cases, the advantage of combination therapy remains a matter of debate (Yanagihara *et al*., 2000; Giamarellou, 2002; Burgess, 2005; Rossolini and Mantegoli, 2005).
Natural resistance can be attributed to the low permeability of the *P. aeruginosa* outer membrane to a variety of antibiotics including glycopeptides (Vaara, 1992; Nikaido, 2003).

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Since glycopeptides are powerful antibiotics against Gram-positive bacteria and resistance towards these drugs very rarely develops, it seemed interesting to evaluate the effect of combining these antimicrobial agents with antibiotics that might disorganise the structure of the outer membrane allowing the entrance of glycopeptides into the Gram-negative cells. In order to verify this hypothesis, ceftazidime has been tested in association with vancomycin or teicoplanin. The same experiments have been carried out also in the presence of azithromycin, which is normally a non anti-pseudomonal agent, but has been shown to interfere with some virulence factors, and to react synergistically in some circumstances, when combined with other antibiotics against *P. aeruginosa* and other non-fermenting bacteria (Molinari *et al*., 1993; Howe and Spencer, 1997; Saiman *et al*., 2002; Nalca *et al*., 2006).

MATERIALS AND METHODS

Bacterial strains. Fifty-nine *P. aeruginosa* strains clinically isolated from different patients and collected from different hospital units were studied. They were chosen on the basis of their susceptibility to ceftazidime. *Pseudomonas aeruginosa* ATCC 27853 was used as control strain.

Antimicrobial agents. Ceftazidime, azithromycin, vancomycin and teicoplanin were purchased from Glaxo Smith Kline SpA (Verona), Pfizer (Rome), Abbott SpA (Latina) and Aventis Pharma (Milan) respectively. Sterile stock solutions of the drugs were prepared according the instructions of the manufacturer by dissolving the compounds in the specific solvent to obtain a final concentration of at least 1 mg/ml.

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

Interactions between ceftazidime and other drugs. The combinations between ceftazidime and the other antibiotics were studied selecting spontaneous resistant strains which appeared in plates contained different concentrations of the drugs. In detail, a bacterial suspension (0.1 ml) of about 10^9 CFU/ml was inoculated on Mueller-Hinton agar plates containing increasing doses of ceftazidime (from 2xMIC to 16xMIC depending on the strain tested) and a fixed concentration of the glycopeptide antibiotics (vancomycin or teicoplanin 300 mg/l) and azithromycin (16 mg/l). After 48 h of incubation at 37° C the plates were analysed and the number of colonies found was determined. Results were interpreted on the basis of the percentage of CFU/ml reduction (≥ 99%, ≥ 90%, and ≥ 10%) found in the drug combinations in comparison to the drug alone.

Dynamic bactericidal activity. Time-kill experiments were also performed on representative isolates by adding the drugs to 10 ml log-phase bacterial cultures in Mueller-Hinton broth diluted to 10^6-10^7 CFU/ml growing in 250 ml flasks at 37 °C. Just before the compounds were added and at 2, 4, and 6 h thereafter, an aliquot of 0.2 ml was collected and serially diluted in saline solution, then a sample of 0.1 ml was seeded in triplicate on Mueller-Hinton agar plates. Survivors were evaluated by determining the average of number of colonies found on the three agar plates, taking into account the dilution factor. The combination was interpreted as synergism or antagonism in the antibiotic association, as compared with the most effective single drug, caused at least a 100-fold reduction or increase, respectively, in the CFU at 24 h. Intermediate results were interpreted as indifference (Eliopoulos and Moellering, 1996). The antibiotic combinations tested by time-kill were CAZ (16) + GLYs (300) + AZI (16), CAZ (32) + GLYs (300) + AZI (16) and CAZ (16) +GLYs (300) +AZI (32 mg/l).

RESULTS

In preliminary experiments ceftazidime in combination with the other drugs was studied on five representative strains, employing concentrations from 2x to 16x MIC. As reported in Fig. 1, on the basis of the CFU/ml detected on the plates containing antibiotics alone or in combinations, at 16x MIC resistant strains were not found under all experimental conditions, while at 8xMIC spontaneous ceftazidime-resistant clones arose in a number that was dependent on the selective plates, prepared with a single drug or containing drugs in combinations. Different concentrations of vancomycin or teicoplanin were also evaluated and the most reproducible results were obtained when the glycopeptides were employed at a dose no lower than 300 mg/l. The other experimental conditions were then not adopted for further tests. When ceftazidime was combined with vancomycin a 99% CFU/ml reduction was registered in 20 cases (Table 1). A 90% decrease was found of the number of CFU/ml in 31 situations and a 10% diminution of CFU/ml was noted in 8 tests out of 59 *P. aeruginosa* strains studied with respected to the tests carried out with the drug alone. The same experiments were repeated in the presence of azithromycin (16 mg/l). As reported in Table 1, the addition of this azalide increased the incidence of 99% CFU/ml reduction to 25 cases. The number of the 90% CFU/ml diminutions did not change, while from 8 to 3 cases were found to show a 10% CFU/ml decrease. When teicoplanin was used in substitution of vancomycin the same results were obtained (data not shown).

The bactericidal activity of ceftazidime, in the presence of vancomycin and azithromycin, was then studied by time-kill experiments against a representative *P. aeruginosa* strain. When the MIC of ceftazidime against this latter isolate was carried out under the same experimental conditions of the time-kill tests, it increased from 4 to 32 mg/l. Figure 2A reports an example of the time-kill curves of ceftazidime (16 mg/l) in combination with vancomycin (300 mg/l). The interaction between the two antibiotics showed an indifferent reaction and the addition of azithromycin at 16 mg/l increased the bactericidal rate by about 1 Log. The same experiment was repeated using ceftazidime at 32 mg/l. As expected the rate of bactericidal activity increased by about 1 Log in comparison with previous test. The addition of vancomycin did modify the rate of killing showed by ceftazidime alone by 50%, while in the presence of azithromycin the lethal effect was enhanced by more than 1 Log (Fig. 2B) In Fig. 2C the bactericidal activity of ceftazidime was analysed in combination with vancomycin and with azithromycin at 32 mg/l. The results obtained were similar to those reported in Fig. 1A with the exception the bactericidal effect of the three antibiotics was more marked by about 2 Log in comparison with that observed with the previous experiments. Under no experimental condition and none of the combinations tested antagonism was detected.

TABLE 1 - *In vitro* interaction between ceftazidime and glycopeptides (vancomycin, 300 mg/l), with and without azithromycin (16 mg/l) against 59 *P. aeruginosa* strains. Number of strains showing CFU/ml reduction (%) found on selective agar plates

		Percentage of reduction		
Drugs	N°	≥ 99	≥ 90	0-10
Without azithromycin	59	20	31	8
With azithromycin	59	25	31	3

Results are expressed as the mean of three separate experiments.

DISCUSSION AND CONCLUSION

The major findings obtained in this investigation can be summarised as follows. Ceftazidime reacted favourably *in vitro* in combination with glycopeptides against *P. aeruginosa* based on the selection of spontaneous resistant colonies on agar plates containing the two antibiotics. A CFU/ml reduction (99, and 90%) was found in 20 and 31 cases respectively accounting for 51/59 events with an inci-

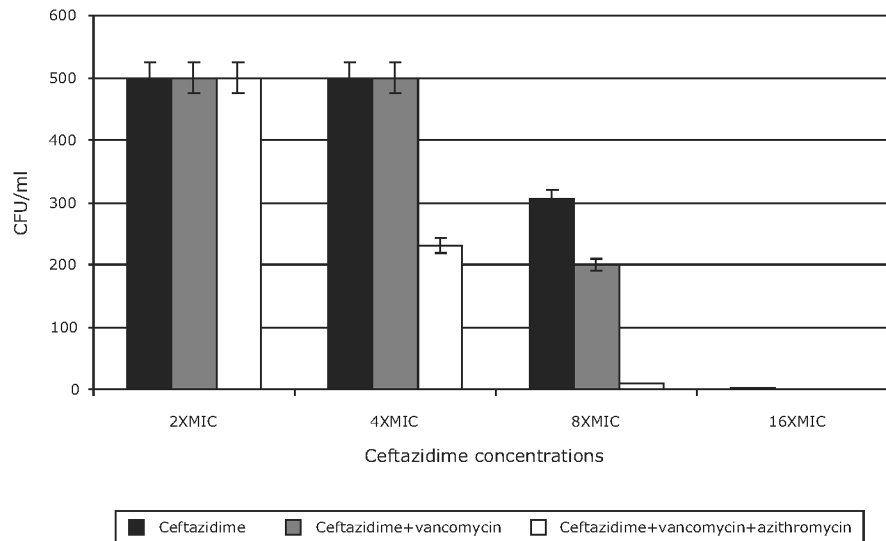

FIG. 1 - Growth (CFU/ml) of five *Pseudomonas aeruginosa* representative strains on plates containing increasing concentration of ceftazidime alone and in combination with vancomycin 300 mg/l and azithromycin16 mg/l. Results are expressed as the mean of three separate experiments.

dence of 86.4% of the total tests. The addition of azithromycin (16-32 mg/l) was beneficial for the drug combinations increasing the favourable bactericidal effect up to 94.9% of the cases. Time-kill experiments confirm the previous results and showed that as the dose of ceftazidime was increased the number of viable cells was also reduced in either in the combination with glycopetides or in that with azithromycin. Finally the increase of the macrolide concentration was related to an increase of the lethal effect of the antibiotic combinations. Under no circumstance and with no combination of drugs an antagonistic effect was 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 far as detection of a favourable interaction between ceftazidime and glycopeptides also in the presence of azithromycin.

Pseudomonas aeruginosa is one of the major opportunistic pathogen responsible for difficult to treat nosocomial infections. Considering that the development of new drugs active against this microorganism appears unlikely to be met in the near future (Livermore, 2004) any suggestion for a new use of our therapeutic armamentarium should be considered, the results reported here indicate a new possible option for the treatment of *P. aeruginosa* infection, where the drugs are able to reach the appropriate concentrations. The concentrations of the glycopeptides employed here seem prohibitive for a systemic use in vivo

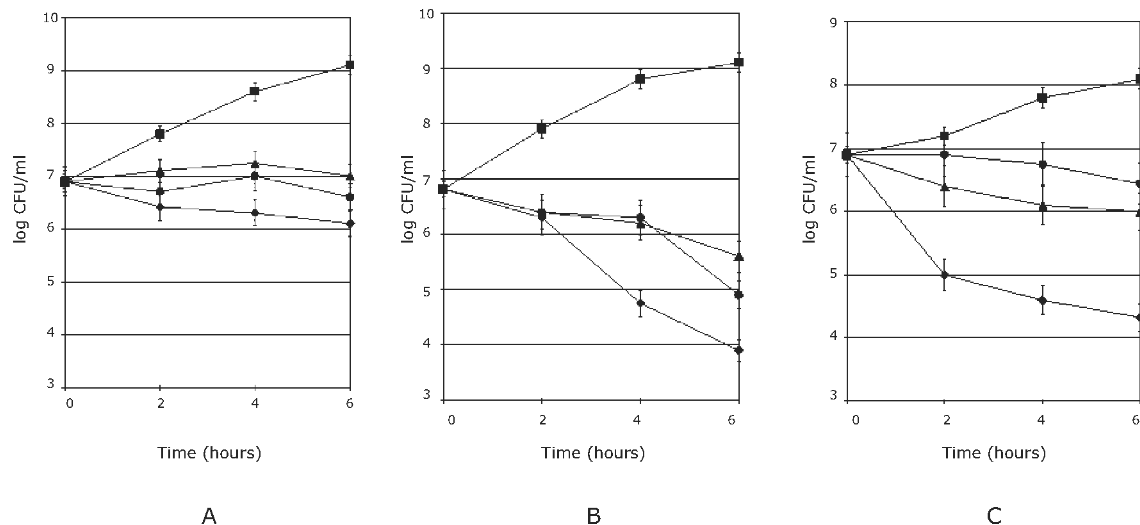

FIG. 2 - Bactericidal activity of ceftazidime at different concentrations (A and C, 16 mg/l; B, 32 mg/l), in combination with vancomycin (300 mg/l) and azithromycin (A and B, 16 mg/l; C, 32 mg/l) against *Pseudomonas aeruginosa* representative strain. The curves of vancomycin and azithromycin alone overlapped the control curve and were not reported. Results for each figure are expressed as the mean of three separate experiments.
■ Control, ▲ Ceftazidime, ● Ceftazidime+ vancomycin, ◆ Ceftazidime+ vancomycin+ azithromycin.

of the above drug combinations; however, for topical applications this approach should be a chance to reduce the incidence of resistant strains and to preserve currently available agents. In the case of cystic fibrosis, for example, inhaled antibiotics reached levels that are more than one order of magnitude than those of MICs and of Mutant Prevention Concentrations (MPCs) for specific antibiotic-pathogen association (Doring *et al*., 2000; Zhao and Drlica, 2002). It is also no rare that therapy is addressed against *P. aeruginosa* and/or *Staphylococcus aureus* using tobramycin, ceftazidime and or vancomycin, with the addition of azithromycin, a situation explored *in vitro* in this study (Doring *et al.*, 2000; Hoiby, 2002; Lyzcaz *et al.* 2002; Fontana *et al*., 2003).

Interaction between two or more drugs represents a situation that may take place either as a result of a decision to adopt an association of antibiotic or because following a failure of the first molecule the one selected to replace it will certainly interact with 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 reaction to maintain the original activity or have beneficial effect from the presence of each other.

Finally, analysing the mode of action of the above drugs when combined each other, the data obtained in this study suggest that ceftazidime interfering with the cell wall synthesis enables the glycopeptide antibiotics to enter into the bacterial cells. This behaviour, that is reminiscent with that of ampicillin and aminoglycosides against enterococci, is also supported by the observation that increasing of the concentration of ceftazidime the rate of killing also increases when the drugs are in combination. In this contest the role of azithromycin should be that to alter the fitness of the biochemical reactions or some product correlated to the quorum sensing phenomenon (Nalca et al., 2006), increasing the magnitude of the synergistic activity of the two drugs or interfering with the bacterial response to the damages caused by the antibiotics.

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