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Investigation of Synergism between Combinations of Ciprofloxacin, Polymyxin, Sulphadiazine and *p*-Aminobenzoic acid

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Abstract—Subinhibitory concentrations of combinations of any two of ciprofloxacin, colistin (or polymyxin B), sodium sulphadiazine and *p*-aminobenzoic acid were shown by checkerboard minimum inhibitory concentration determinations to have synergistic inhibitory activity against *Pseudomonas aeruginosa* and to have either synergistic or additive activity against *Staphylococcus aureus*. In addition, sulphadiazine plus either ciprofloxacin or polymyxin showed markedly enhanced killing activity against both *P. aeruginosa* and *S. aureus*. *p*-Aminobenzoic acid plus either ciprofloxacin or polymyxin also demonstrated enhanced killing activity against *P. aeruginosa* but these combinations were less effective in enhancing activity against *S. aureus*. Ciprofloxacin in combination with polymyxin had a marked synergistic effect against *P. aeruginosa* but only a slight synergistic effect against *S. aureus*. These findings indicate a potential usefulness for the synergistic combinations against *P. aeruginosa* and *S. aureus* in the clinical situation; that is, they indicate an extended role for sulphonamides and support a potential role for *p*-aminobenzoic acid as enhancers of the activity of primary antibacterial agents such as ciprofloxacin and polymyxin. We suggest that for a second antibacterial to enhance the activity of ciprofloxacin, it may be necessary for the second antibacterial to increase cell permeability so increasing bacterial uptake of ciprofloxacin.

The fluoroquinolones have been used for more than five years in many countries and already there is major concern about the development of bacterial resistance to these agents (Neu 1990). Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been reported to develop resistance during ciprofloxacin treatment (Pedersen 1989; Ball 1990). Combinations of quinolones with other classes of antibacterial agent have been used (Eliopoulos & Eliopoulos 1989), to provide activity against bacteria inadequately inhibited by the fluoroquinolones alone (Neu 1991). Sulphonamides, trimethoprim and *p*-aminobenzoic acid can have a synergistic action with primary antibacterials by increasing the bacterial uptake of the primary antibacterials through an effect on the permeability of the bacterial cell envelope (Richards et al 1991a; Richards & Xing 1992a, b).

The present study was undertaken to investigate the antibacterial effect of combinations of two of the following, ciprofloxacin, polymyxins, *p*-aminobenzoic acid, and sodium sulphadiazine against *P. aeruginosa* and *S. aureus*, to explore a possible role for such combinations in treating cystic fibrosis and other conditions involving infection with *P. aeruginosa* or *S. aureus*.

Materials and Methods

Materials

Pseudomonas aeruginosa NCTC 6750 and *Staphylococcus aureus* NCTC 10788 were used as the test organisms and were obtained from the National Collection of Type Cultures, Colindale, London, UK. Isosensitest broth and nutrient broth were obtained from Oxoid, Basingstoke, UK. Inactivating recovery medium was made with either nutrient broth, Tween 80 (ICI, Leatherhead, Surrey, UK) 3·0% w/v and lecithin (BDH, Poole, UK) 0·125% w/v or Isosensitest broth plus lecithin 0·125% w/v.

Ciprofloxacin hydrochloride, colistin methanesulphonate (polymyxin E), polymyxin B sulphate, *p*-aminobenzoic acid and sulphadiazine were all obtained from Sigma, Poole, UK. Sodium sulphadiazine was prepared in the laboratory by the method described previously (Richards et al 1991a).

Checkerboard minimum inhibitory concentration (MIC) determinations

Checkerboard MIC estimates with two chemical combinations were based on the method of Sabath (1968). A 10×10 checkerboard of test-tubes was prepared. Each test-tube contained 9·9 mL Isosensitest broth and was inoculated with 0·1 mL diluted 18 h culture to give approximately 5×10^3 colony-forming units mL^{-1} (CFU mL^{-1}). MICs were determined for each antibacterial combination after 24 h incubation at 37°C and isobolograms plotted. The tests were carried out in duplicate.

Determination of killing times

Killing times were determined by a similar method to that previously described by Richards & Xing (1991). The bacterial cells were grown in Isosensitest broth for 18 h at 37°C and then centrifuged (6000 *g*, 10 min, 4°C). The cell pellets were washed in 0·9% w/v NaCl (saline), recentrifuged and the pellets resuspended in saline. The cell concentration was diluted to approximately 5×10^8 CFU mL^{-1}. Duplicate tubes containing 9·9 mL of the aqueous antibacterial solutions under test were equilibrated in a water bath at 37°C. Then 0·1 mL of the above cell suspension was added to

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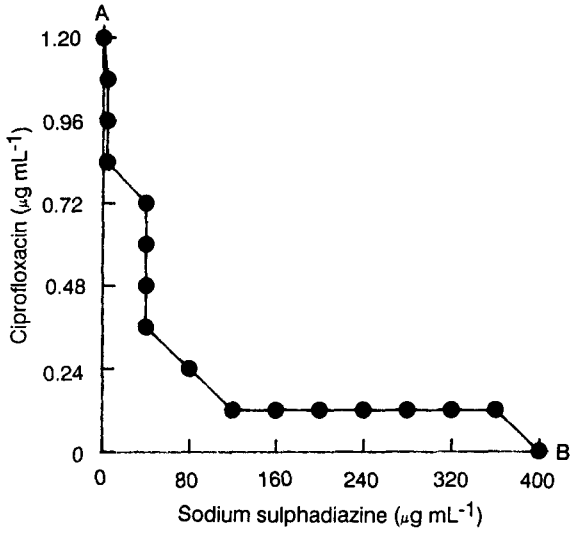

FIG. 1. Isobologram constructed from checkerboard MIC data showing combinations of ciprofloxacin with sodium sulphadiazine against *P. aeruginosa* in Isosensitest broth. MIC of ciprofloxacin, 1·2 μg mL^{-1}; MIC of sodium sulphadiazine, 400 μg mL^{-1}.

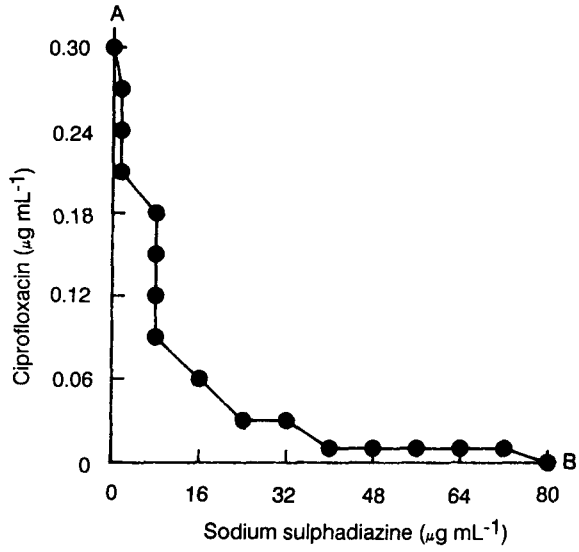

FIG. 2. Isobologram constructed from checkerboard MIC data showing combinations of ciprofloxacin with sodium sulphadiazine against *S. aureus* in Isosensitest broth. MIC of ciprofloxacin, 0·3 μg mL^{-1}; MIC of sodium sulphadiazine, 80 μg mL^{-1}.

give a final inoculum of approximately 5×10^6 cells mL^{-1}. At intervals of 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min after inoculation, 0·5 mL samples were aseptically transferred to 9·5 mL inactivating recovery medium (nutrient broth containing Tween 80 3·0% w/v and lecithin 0·125% w/v) and incubated at 37°C for 72 h. Tween 80 enhances the activity of colistin (Brown & Richards 1964). This necessitated the use of a different inactivating recovery medium for both colistin- and polymyxin B-treated cells. Isosensitest broth plus lecithin 0·125% w/v was found to be suitable. However, the autoclaved broth was cloudy making bacterial growth difficult to detect. In order to overcome this difficulty, 0·5 mL of each inactivated sample was inoculated separately onto agar plates which were incubated for 24 h at 37°C and observed for growth.

The single test antibacterials were as follows: for *P. aeruginosa*, ciprofloxacin hydrochloride 2·0 μg mL^{-1}, colistin methanesulphonate 7·0 μg mL^{-1}, *p*-aminobenzoic acid 400 μg mL^{-1} and sodium sulphadiazine 750 μg mL^{-1}; for *S. aureus*, ciprofloxacin hydrochloride 0·6 μg mL^{-1}, polymyxin B sulphate 1·5 μg mL^{-1}, *p*-aminobenzoic acid 400 μg mL^{-1} and sodium sulphadiazine 400 μg mL^{-1}. The antibacterial combinations were either sulphadiazine or *p*-aminobenzoic acid plus either ciprofloxacin hydrochloride or colistin methanesulphonate (or polymyxin B sulphate). Positive growth controls to demonstrate adequate antibacterial inactivation were prepared by adding approximately 5×10^3 cells from the cell suspension sources of inocula described above to duplicate tubes of inactivator medium containing either ciprofloxacin hydrochloride 5·0 μg mL^{-1}, colistin methanesulphonate 10 μg mL^{-1} (or polymyxin B sulphate 5 μg mL^{-1}), *p*-aminobenzoic acid 1000 μg mL^{-1} or sulphadiazine 900 μg mL^{-1}.

Reduction in bacterial numbers

The reduction in the numbers of CFU mL^{-1} was determined over 5 h for those antibacterial combinations which exhibited markedly enhanced activity in the killing time determinations, compared with the antibacterials used singly.

The experimental procedure was similar to the killing time determinations. However, after 0·5 mL samples had been removed from the antibacterial solutions plus bacterial inoculum reaction mixtures and added to the inactivator solution, the resulting suspension was immediately used to prepare viable counts by an overdried agar plate counting method. When the surviving bacteria in the reaction mixtures were estimated to be less than 3×10^3 CFU mL^{-1}, 0·1 mL quantities were added directly to four nutrient agar plates containing either lecithin 0·125% w/v plus Tween 80 3% w/v or just lecithin 0·125% w/v. CFU were counted after incubation for 18 h at 37°C.

Results

It was found that *S. aureus* was more resistant to colistin than polymyxin B. The MIC for colistin was over 2500 μg mL^{-1} and for polymyxin B was 50 μg mL^{-1}. In the present investigation colistin was used with *P. aeruginosa* and polymyxin B was used with *S. aureus*.

Sample isobolograms constructed from the checkerboard MIC data are shown in Figs 1 and 2.

The fractional inhibitory concentration index (FIC Index) was also calculated using the following equation:

$$\text{FIC index} = \frac{\text{Concentration A in MIC A+B}}{\text{MIC A used alone}} + \frac{\text{Concentration B in MIC A+B}}{\text{MIC B used alone}} \quad (1)$$

The FIC indices for the combinations used are given in Table 1. An FIC index of 0·8 or below indicates a synergistic interaction whereas an FIC index of greater than 1·2

Table 1. FIC indices for the antibacterial combinations determined in Isosensitest broth against *P. aeruginosa* and *S. aureus*.

	FIC indices	
Antibacterial combination	*P. aeruginosa*	*S. aureus*
Ciprofloxacin + sodium sulphadiazine	0·4 Synergism	0·4 Synergism
Ciprofloxacin + colistin	0·3 Synergism	—
Ciprofloxacin + polymyxin B	—	0·7 Synergism
Ciprofloxacin + *p*-aminobenzoic acid	0·5 Synergism	0·7–0·9 Synergism or addition
Colistin + sodium sulphadiazine	0·3 Synergism	—
Polymyxin B + sodium sulphadiazine	—	0·4 Synergism
Colistin + *p*-aminobenzoic acid	0·4 Synergism	—
Polymyxin B + *p*-aminobenzoic acid	—	0·7–1·0 Synergism or addition

indicates an antagonistic interaction. FIC index values between 0·8 and 1·2 are taken to represent addition (Richards & Xing 1991). Therefore, the results listed in Table 1 help quantify the inhibitory antibacterial activity of the antibacterial combinations.

The killing times for ciprofloxacin, colistin, polymyxin B, *p*-aminobenzoic acid and sodium sulphadiazine either alone or in paired combinations against *P. aeruginosa* and *S. aureus* are presented in Table 2. For the combinations of colistin plus either *p*-aminobenzoic acid or sulphadiazine and the combinations of ciprofloxacin plus either colistin or *p*-aminobenzoic acid against *P. aeruginosa* the killing times were greatly reduced when compared with the killing times for the single antibacterials. Sodium sulphadiazine was not effective within 24 h when used alone and *p*-aminobenzoic acid, colistin and ciprofloxacin when used alone were not effective in killing the test inoculum within the 5 h test period. In contrast, all the antibacterial combinations killed the *P. aeruginosa* inoculum within 30–150 min.

The killing times for ciprofloxacin in combination with either polymyxin B or *p*-aminobenzoic acid and polymyxin B in combination with either sulphadiazine or *p*-aminobenzoic acid against *S. aureus* were reduced when compared with the antibacterials used alone. The test inocula of *S. aureus* was not killed within 6 h using the single antibacterials at the test concentrations. All the antibacterial combinations killed the inocula within 3–5 h. However, the reduction in killing times was not as marked as for *P. aeruginosa*.

The test inocula of either organism were not killed within 5 h using the ciprofloxacin plus sulphadiazine combination.

The reduction in the numbers of CFUs with time, for cell suspensions of *P. aeruginosa* and *S. aureus* added to selected single antibacterials and antibacterial combinations, are given in Figs 3 and 4.

Table 2. Killing times against either approximately 5×10^6 *P. aeruginosa* or *S. aureus* cells for ciprofloxacin, colistin, polymyxin B, sodium sulphadiazine and *p*-aminobenzoic acid solutions used either alone or in the combinations indicated.

	Killing times[a] (min) at 37°C	
Chemicals[b]	*P. aeruginosa*	*S. aureus*
Ciprofloxacin	> 360	> 360
Colistin	> 300	—
Polymyxin B	—	> 360
Sodium sulphadiazine	> 24 h	> 24 h
p-Aminobenzoic acid	> 360	> 10 h
Ciprofloxacin + sodium sulphadiazine	> 300	> 300
Ciprofloxacin + colistin	150	—
Ciprofloxacin + polymyxin B	—	180
Ciprofloxacin + *p*-aminobenzoic acid	60	90
Colistin + sodium sulphadiazine	30	—
Polymyxin B + sodium sulphadiazine	—	300
Colistin + *p*-aminobenzoic acid	45	—
Polymyxin B + *p*-aminobenzoic acid	—	300

[a] Duplicate determinations. [b] Chemical concentrations ($\mu g\ mL^{-1}$) used in the tests were: for *P. aeruginosa*, ciprofloxacin 2·0, colistin 7·0, *p*-aminobenzoic acid 400, sodium sulphadiazine 750; for *S. aureus*, ciprofloxacin 0·6, polymyxin B 1·5, *p*-aminobenzoic acid 400, sodium sulphadiazine 400.

Discussion

Previous work has indicated that the antibacterial effectiveness of either sulphadiazine or sulphamerazine in combination with dibromopropamidine is related to a mutual increase in bacterial uptake of the components of the combination, which results from an action of the antibacterials on permeability properties of the cells (Richards & Xing 1991; Richards et al 1991a, b). In the present investigation, sulphadiazine in combination with either ciprofloxacin or colistin (or polymyxin B) caused marked synergism with both *P. aeruginosa* and *S. aureus* (Table 1). Sulphadiazine plus colistin (or polymyxin B) also produced a reduction in killing times against both organisms compared with the single antibacterials (Table 2) and produced a much quicker reduction in the viable count of *P. aeruginosa* compared with the single antibacterials (Fig. 3). Sulphadiazine plus ciprofloxacin did not demonstrate an ability to kill the inocula of test organisms under the conditions of the killing time determinations (Table 2). It is generally considered that sulphadiazine requires dividing cells in order to exert a bacteriostatic effect probably because uptake of sulphadiazine by non-dividing cells is slight. However, Fig. 3 indicates that sulphadiazine can cause a slight reduction in numbers of *P. aeruginosa* over 5 h. On the other hand, ciprofloxacin interferes with DNA synthesis by inhibiting DNA topoisomerase activity (LeBel 1988). Metabolizing cells are thus required for ciprofloxacin activity. In the checkerboard MIC determinations, the cells were in an environment where growth might take place, and thus a markedly enhanced inhibitory effect was observed with sulphadiazine plus ciprofloxacin (Fig. 2). These results are similar to those obtained previously with a combination of sulphonamide and penicillin, which did not show a marked effect on killing times against cell suspensions, but produced markedly increased inhibitory activity against cultures in nutrient

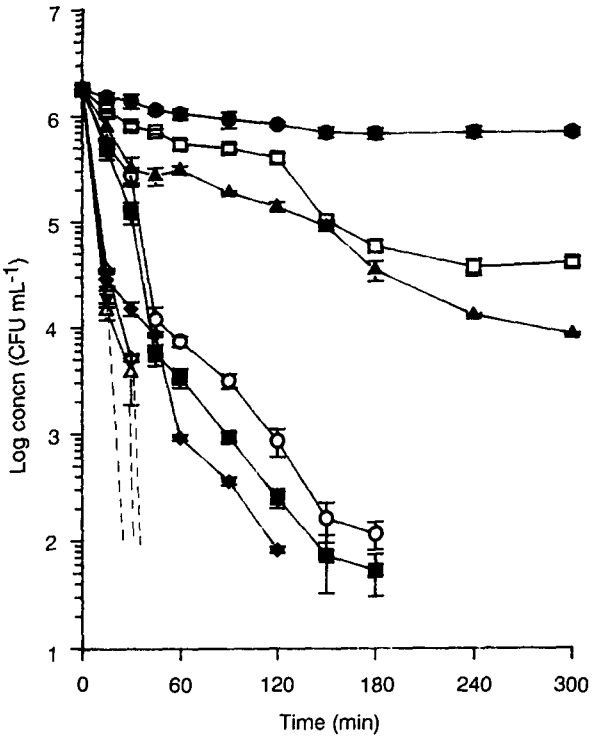

FIG. 3. Reduction in the CFU with time for cell suspensions of *P. aeruginosa* in the presence of selected single antibacterials and antibacterial combinations. (The bars indicate s.d.) ● Control, ○ ciprofloxacin (2·0 μg mL^{-1}), ■ *p*-aminobenzoic acid (400 μg mL^{-1}), □ sodium sulphadiazine (750 μg mL^{-1}), ▲ colistin (7·0 μg mL^{-1}), △ ciprofloxacin + *p*-aminobenzoic acid (2·0 μg mL^{-1} + 400 μg mL^{-1}), ◆ ciprofloxacin + colistin (2·0 μg mL^{-1} + 7·0 μg mL^{-1}), ▼ colistin + sodium sulphadiazine (7·0 μg mL^{-1} + 750 μg mL^{-1}), ◇ colistin + *p*-aminobenzoic acid (7·0 μg mL^{-1} + 400 μg mL^{-1}).

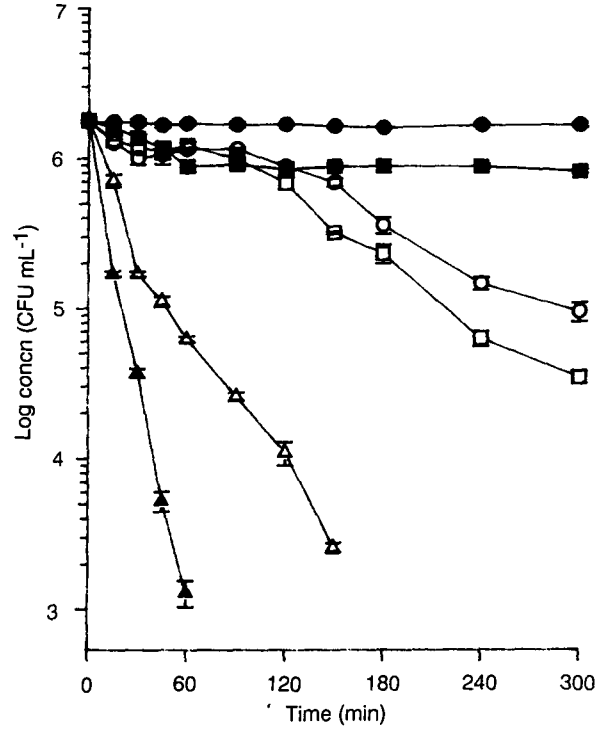

FIG. 4. Reduction in the CFU with time for cell suspension of *S. aureus* in the presence of selected single antibacterials and antibacterial combinations. (The bars indicate s.d.) ● Control, ○ ciprofloxacin (0·6 μg mL^{-1}), ■ *p*-aminobenzoic acid (400 μg mL^{-1}), □ polymyxin B (1·5 μg mL^{-1}), ▲ ciprofloxacin + *p*-aminobenzoic acid (0·6 μg mL^{-1} + 400 μg mL^{-1}), △ ciprofloxacin + polymyxin B (0·6 μg mL^{-1} + 1·5 μg mL^{-1}).

media (Bigger 1944; Weinstein et al 1964; Richards & Xing 1992b).

The synergistic inhibitory effect observed with combinations of sulphadiazine plus either ciprofloxacin or polymyxin (colistin) in Isosensitest broth (Figs 1, 2, Table 1) may be explained in terms of the sulphadiazine modifying cell envelope permeability (Richards et al 1991a) and thereby facilitating uptake of ciprofloxacin and polymyxin. One reported effect of sulphadiazine against *Enterobacter cloacae* appeared to be damage to the peptidoglycan layer of the cell envelope (Richards et al 1993). Similar concentrations of sulphadiazine had already been shown to enhance bacterial uptake of antibacterials and to enhance antibacterial activity (Richards et al 1991a). Polymyxin is known to damage cell envelope structures of both stationary and dividing cells (Newton 1954), and would be expected to have a similar enhancing effect on the uptake of both sulphadiazine and ciprofloxacin.

p-Aminobenzoic acid has been found to inhibit *P. aeruginosa* and *E. coli* (Eagon & McManus 1989, 1990) and to affect the cell permeability of *P. aeruginosa* causing an increased uptake and thus an increased activity of dibromopropamidine isethionate present in the medium (Richards & Xing 1992a). It was also found that *p*-aminobenzoic acid plus carbenicillin at subinhibitory concentrations showed synergism against *P. aeruginosa*, *E. cloacae*, *Proteus mirabilis* and also *S. aureus* (Richards & Xing 1992b). In the present work, the synergistic effect of *p*-aminobenzoic acid in combination with either ciprofloxacin or colistin was demonstrated against *P. aeruginosa* (Fig. 3, Tables 1, 2). This enhanced effectiveness may again be explained in terms of the damage to cell envelope structure and resultant increased antibacterial uptake. This may well be an essential action of a second antibacterial in order to exert synergism with ciprofloxacin. It should be noted that several workers have reported that the combination of ciprofloxacin with other antibacterials, such as fosfomycin, aminoglycosides, β-lactams and imidazoles, only infrequently show synergy against Enterobacteriaceae and Gram-positive bacteria (Haller 1985; Neu 1989, 1990; Chow et al 1989). However, *p*-aminobenzoic acid in combination with either ciprofloxacin or polymyxin B showed less effect against *S. aureus* as determined by killing times, reduction in viable counts and checkerboard MIC determinations. *p*-Aminobenzoic acid 400 μg mL^{-1} is seen (Figs 3, 4) to be much more effective against *P. aeruginosa* than against *S. aureus*. It can also be seen that for the antibacterials and combinations evaluated using viable counts the same overall trends in activity were obtained as with the killing-time determinations. This gives added confidence in the killing-time results. An unexpected result with the viable count determinations was the marked reduction in viable count of *P. aeruginosa* produced by ciprofloxacin 2·0 μg mL^{-1}. The count of *S. aureus* was also reduced by ciprofloxacin 0·6 μg mL^{-1}. This suggests that

cells do not have to be dividing in order for ciprofloxacin to have an effect on cell viability.

The results presented here support a role for sulphonamides to be used clinically to enhance the antibacterial activity of ciprofloxacin and colistin against *P. aeruginosa* and *S. aureus*. The results also support a potential role for *p*-aminobenzoic acid in enhancing the effect of ciprofloxacin and colistin against *P. aeruginosa*.

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