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Investigation of Synergism with Combinations of Dibromopropamidine Isethionate or Propamidine Isethionate and Polymyxin B

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Abstract—Combinations of polymyxin B and dibromopropamidine isethionate exhibited synergistic inhibitory and bactericidal activity against *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Proteus mirabilis*, *Escherichia coli* and *Staphylococcus aureus*. Similar results were obtained with polymyxin B plus propamidine combinations except that propamidine was not as active as dibromopropamidine and the combination of polymyxin B plus propamidine against *S. aureus* only had additive activity. The antibacterial agents were tested in solutions and in a cream formulation. The findings indicate a potential for the use of selected combinations of these antibacterial agents in the treatment of wound and superficial eye infections.

Dibromopropamidine isethionate and propamidine isethionate are diamidine group compounds which have been used as topical antibacterial agents for many years. Dibromopropamidine affects the cell envelope function which enhances the uptake of a second antibacterial when present (Richards et al 1991a) and such affected cells may also have damaged cytoplasmic membranes (Richards et al 1991b, 1993).

Polymyxin B sulphate in combination with sulphonamides has a synergistic effect against *Pseudomonas aeruginosa* but the effect against *Staphylococcus aureus* is less marked (Richards & Xing 1993). The present study was to investigate the antibacterial effect of polymyxin B with combinations of either dibromopropamidine isethionate or propamidine isethionate against clinically significant bacteria in order to assess a possible role for using such combinations in the treatment of wound and eye infections.

Materials and Methods

Materials

Pseudomonas aeruginosa NCTC 6750, *Enterobacter cloacae* NCTC 10005, *Proteus mirabilis* NCTC 60, *Escherichia coli* NCTC 4174 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 Isosensitest agar were obtained from Oxoid Ltd, Basingstoke, UK. Inactivating recovery medium was made with Isosensitest broth plus lecithin 0·125% w/v, BDH, Poole, UK (Richards & Xing 1993).

Dibromopropamidine isethionate (DBPI) and propamidine isethionate were gifts from Rhone-Poulenc Rorer, UK, and polymyxin B sulphate was obtained from Sigma, Poole, UK.

The cream base was cetomacrogol cream formula A of the Pharmaceutical Codex (1979) without the added preservative.

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Determination of killing times

An estimate of the time to kill all of a given inoculum by the different antibacterial agents alone and in combination was determined by a similar method to that previously described by Richards & Xing (1991). In brief, each bacterial strain was grown separately 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 concentrations were adjusted to approximately 5×10^8 colony-forming units (CFU) mL^{-1}. Duplicate tubes containing 9·9 mL of the antibacterial solutions under test in saline were equilibrated in a water bath at 37°C. Then 0·1 mL of the above cell suspensions were added to give final inocula of approximately 5×10^6 CFU mL^{-1}. At intervals of 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min, and for organisms resistant to polymyxin, up to 24 h after inoculation, 0·5 mL samples were aseptically transferred to 9·5 mL inactivating recovery medium. The medium was cloudy. This necessitated that 0·5 mL of each inactivated sample was inoculated separately onto the surface of overdried agar plates, incubated for 24 h at 37°C and observed for growth or no growth.

Polymyxin B was used alone or in combination with either DBPI or propamidine. The test antibacterial concentrations used singly and in combinations were as follows: for *P. aeruginosa*, DBPI 250 μg mL^{-1}, propamidine 250 μg mL^{-1}, polymyxin B 1·5 μg mL^{-1} (12·605 units mL^{-1}); for *Pr. mirabilis*, DBPI 10 μg mL^{-1}, propamidine 15 μg mL^{-1}, polymyxin B 1·5 μg mL^{-1}; for *E. cloacae*, DBPI 10 μg mL^{-1}, propamidine 200 μg mL^{-1}, polymyxin B 1·0 μg mL^{-1} (8·403 units mL^{-1}); for *E. coli*, DBPI 10 μg mL^{-1}, propamidine 15 μg mL^{-1}, polymyxin B 0·3 μg mL^{-1} (2·521 units mL^{-1}); and for *S. aureus*, DBPI 10 μg mL^{-1}, propamidine 25 μg mL^{-1}, polymyxin B 1·5 μg mL^{-1}. Positive growth controls to demonstrate adequate antibacterial inactivation were prepared by adding 5×10^3 CFU mL^{-1} from the sources of inocula described above to duplicate tubes of inactivator medium containing either DBPI 500 μg mL^{-1}, propamidine 500 μg mL^{-1} or polymyxin B 5 μg mL^{-1} singly or as polymyxin combinations. All controls showed growth.

Table 1. Killing times against approximately 5×10^6 mL^{-1} *P. aeruginosa*, *E. cloacae*, *E. coli*, *Pr. mirabilis* or *S. aureus* cells for polymyxin B, dibromopropamidine isethionate (DBPI) and propamidine isethionate solutions, either used alone, or in the combinations indicated.

Chemicals	Killing times (min)† at 37°C*				
	P. aeruginosa	*Pr. mirabilis*	*E. cloacae*	*E. coli*	*S. aureus*
Polymyxin B	300	>24 h	7 h	>300	7 h
DBPI	180	300	240	>300	240
Propamidine	240	>300	240	>300	300
Polymyxin B+DBPI	<15	30	30	60	90
Polymyxin B+propamidine	<15	120	45	90	180

*Duplicate determinations. †The chemical concentrations (μg mL^{-1}) used in the test were as follows: for *P. aeruginosa*, polymyxin B=1·5, DBPI=250, propamidine=250; for *Pr. mirabilis*, polymyxin B=1·5, DBPI=10, propamidine=15; for *E. cloacae*, polymyxin B=1·0, DBPI=10, propamidine=200; for *E. coli*, polymyxin B=0·3, DBPI=10, propamidine=15; for *S. aureus*, polymyxin B=1·5, DBPI=10, propamidine=25.

Reduction in bacterial numbers

The reduction in CFU mL^{-1} was determined at time intervals over a 5 h period for DBPI and polymyxin B used singly and in combination against *Pr. mirabilis* and *S. aureus*. The method was as described by Richards & Xing (1993) and was similar to the killing time determinations except that viable counts were determined at each interval.

Checkerboard minimum inhibitory concentration (MIC) determinations

The checkerboard MIC determinations were carried out for all the test organisms by the method of Sabath (1967). A 10 × 10 checkerboard of test-tubes was prepared. Each test-tube contained Isosensitest broth and was inoculated to give 5×10^3 CFU mL^{-1}; MIC values were determined for each antibacterial combination after 24 h incubation at 37°C and isobolograms plotted. The tests were carried out in duplicate.

Agar-cup diffusion determinations

Agar-cup diffusion determinations were according to the method of Richards et al (1991a). Five replicates were performed at each dose level. Ten millilitre volumes of Isosensitest agar were seeded separately with either 0·1 mL of an 18 h culture or a 1:100 dilution of the culture, to give approximately 5×10^5 or 5×10^3 test organisms per mL and then poured into Petri dishes. Each plate had 4 × 8 mm cups bored with a sterile cork borer and each cup was filled with 0·15 g of a cream containing a known concentration of a single antibacterial or an antibacterial combination. The plates were left at room temperature for 2 h to allow diffusion of the active agent into the agar and then incubated upright for 18 h at 37°C. Zones of inhibition were measured at three diameters and the mean diameters recorded.

Results

The killing times for polymyxin B, DBPI and propamidine either alone or in paired combinations against the test organisms are presented in Table 1. For the combination of DBPI plus polymyxin B, the killing time for each organism was greatly reduced when compared with the killing times for the single antibacterial agents. DBPI alone was not effective against any of the test organisms in less than 3 h. Polymyxin B alone at the concentrations used took a minimum of 5 h to be effective against *P. aeruginosa* and had not killed the inoculum of *Pr. mirabilis* within 24 h. The antibacterial combination killed the inoculum of all four organisms in times varying from less than 15 min (*P. aeruginosa*) to 90 min (*S. aureus*).

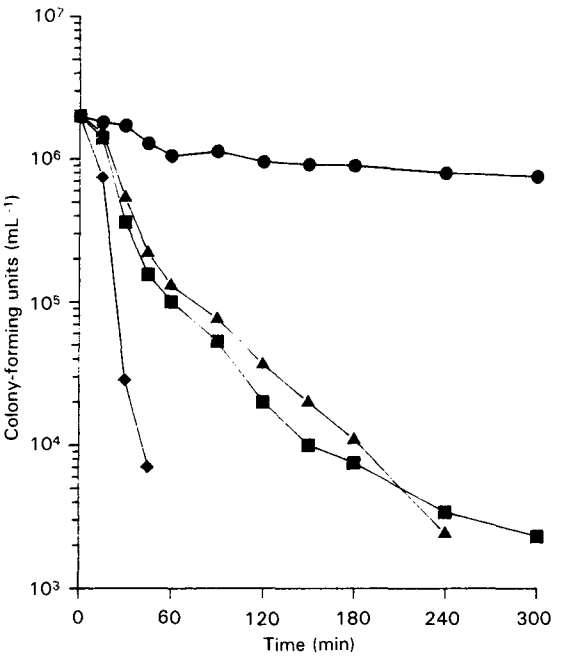

FIG. 1. Reduction in the colony-forming units mL^{-1} with time for cell suspensions of *Pr. mirabilis* in the presence of DBPI and polymyxin B alone and in combination. ● Control, ■ polymyxin B (1·5 μg mL^{-1}), ▲ dibromopropamidine (10 μg mL^{-1}), ◆ polymyxin B plus dibromopropamidine (1·5+10 μg mL^{-1}).

Propamidine was not as effective as DBPI and when it was used alone it was not effective against any of the test organisms in less than 4 h. Polymyxin B alone at the concentrations used took 5 h to be effective against *P. aeruginosa* and was not effective in more than 5 h against *Pr. mirabilis*, *E. cloacae*, *E. coli* and *S. aureus*. However, for the combination of propamidine plus polymyxin B the killing times were greatly reduced. The antibacterial combination killed the inoculum of each test organism in times varying from less than 15 min (*P. aeruginosa*) to 180 min (*S. aureus*).

The killing times confirmed that *Pr. mirabilis* and *S. aureus* were more resistant to polymyxin B than the other organisms tested. Therefore, viable count determinations were per-

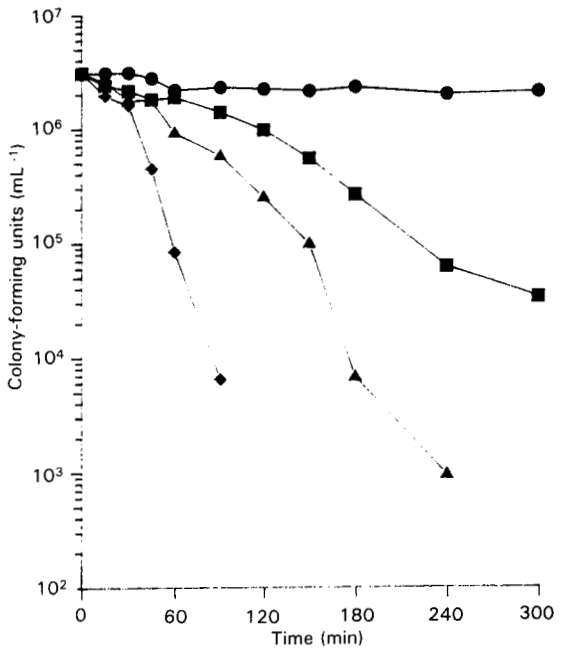

FIG. 2. Reduction in the colony-forming units mL^{-1} with time for cell suspensions of *S. aureus* in the presence of DBPI and polymyxin B alone and in combination. ● Control, ■ polymyxin B (1·5 μg mL^{-1}), ▲ dibromopropamidine (10 μg mL^{-1}), ◆ polymyxin B plus dibromopropamidine (1·5 + 10 μg mL^{-1}).

formed using suspensions of *Pr. mirabilis* and *S. aureus* added to DBPI and polymyxin B alone and in combination. Figs 1 and 2 confirm that the combinations have a much faster rate of kill against both organisms than the antibacterial agents used alone.

Selected isobolograms constructed from the checkerboard MIC data are shown in Fig. 3. The fractional inhibitory concentration index (FIC index) was also calculated using the method described by Richards & Xing (1991) and the values for all combinations are given in Table 2.

DBPI plus polymyxin B at sub-inhibitory concentrations showed synergism against all the test organisms. The FIC index was 0·2 against *P. aeruginosa* and *E. cloacae* which indicates marked synergism and 0·5 against *Pr. mirabilis* and *S. aureus*. Propamidine plus polymyxin B at sub-inhibitory concentrations also showed synergism against all the Gram-negative organisms. The FIC index was 0·4 against *P. aeruginosa*, *Pr. mirabilis* and *E. coli* and 0·5 against *E. cloacae*. The combination of propamidine plus polymyxin B at the subinhibitory concentration tested showed an additive effect against *S. aureus* (FIC index = 0·8).

The diameters of zones of inhibition for DBPI or polymyxin B used alone or in combination in cream formulations against the test organisms are shown in Table 3. Polymyxin B 0·1% w/v showed its greatest activity against *P. aeruginosa*. It was active but less so against *E. cloacae*; only partially active against *Pr. mirabilis* and slightly active against *S. aureus*. DBPI 0·1% w/v alone was active against *E. cloacae* and *S. aureus*, but showed no activity against *P. aeruginosa* and *Pr. mirabilis*. DBPI 0·1% w/v combined with polymyxin B 0·1% w/v exhibited good activity against all the test organisms.

DBPI combined with polymyxin B exhibited a synergistic action against *Pr. mirabilis* although it was resistant to both DBPI and polymyxin B when they were used singly.

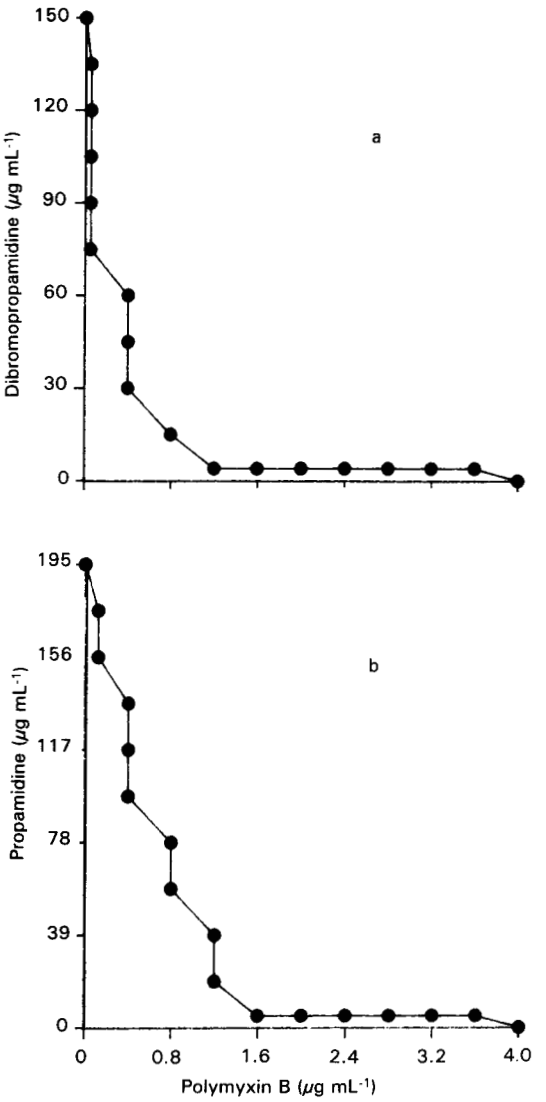

FIG. 3. Isobologram constructed from checkerboard MIC data showing combinations of dibromopropamidine with polymyxin B (a) or propamidine with polymyxin B (b) against *P. aeruginosa* in Isosensitest broth.

Discussion

Previous work has indicated that the antibacterial effective-

Table 2. FIC indices for the antibacterial combinations determined in Isosensitest broth against *P. aeruginosa*, *Pr. mirabilis*, *E. cloacae*, *E. coli* and *S. aureus*.

Organisms	FIC indices	
	Polymyxin B + DBPI	Polymyxin B + propamidine
Organisms		
P. aeruginosa	0·2	0·4
Pr. mirabilis	0·5	0·4
E. cloacae	0·2	0·5
E. coli	0·3	0·4
S. aureus	0·5	0·8

Table 3. Diameters of zones of inhibition for polymyxin B or dibromopropamidine isethionate (DBPI) alone and in combination against either *P. aeruginosa*, *Pr. mirabilis*, *S. aureus* or *E. cloacae* in Isosensitest agar-cup plate determinations.

Cream formulation	Organisms and zone of inhibition (mm)							
	P. aeruginosa		*Pr. mirabilis*		*E. cloacae*		*S. aureus*	
	5×10^3	5×10^5	5×10^3	5×10^5	5×10^3	5×10^5	5×10^3	5×10^5
Polymyxin B alone 0·1%	23·8±0·44	20·4±0·63	0†	0†	19·1±0·48	16·4±0·34	11·07±0·26	0
DBPI alone 0·1%	0	0	0	0	28·6±0·22	13·7±0·44	27·6±0·41	22·5±0·50
Polymyxin B 0·1% + DBPI 0·1%	26·0±0·61	23·0±1·0	28·1±0·37	25·6±0·65	30·3±0·27	25·7±0·44	29·3±0·44	23·9±0·54

Values are mean ± s.d. of five determinations. †An area of less dense growth was observed.

ness of DBPI in combination with sulphonamides is related to a mutual increase in bacterial uptake of the components of the combination, which apparently results from an action of the antibacterial agents in causing changes to the permeability properties of the cells (Richards & Xing 1991; Richards et al 1991a, b). Polymyxin is known to damage cell envelope structures of both stationary and dividing cells (Newton 1954). Recent work has indicated that sub-inhibitory concentrations of combinations of either polymyxin B or colistin plus sulphadiazine have synergistic inhibitory activity against *P. aeruginosa* and *S. aureus* (Richards & Xing 1993). In the present investigation, the combinations of either DBPI or propamidine isethionate plus polymyxin B caused synergism. This can be explained as follows. DBPI (and possibly propamidine) and polymyxin affect cell envelope permeability by affecting both outer and cytoplasmic membranes (Newton 1954; Richards et al 1991a, b, 1993). The difference in structure of Gram-positive cells which lack an outer membrane and *Pr. mirabilis* which has an outer membrane different in structure from most Gram-negative cells accounts for the comparative lack of activity of polymyxin B used alone against *S. aureus* and *Pr. mirabilis*. In combination, it is likely that both antibacterial agents enhance the uptake of each other and thus each may gain access to sites of activity within the cells. Propamidine was not as effective as dibromopropamidine against the test organisms used.

This investigation indicates that DBPI, or propamidine, plus polymyxin B could be used to treat clinical infections. A cream formulation of DBPI with polymyxin B might be an alternative to silver sulphadiazine cream in the treatment of burn wound infections. In addition, polymyxin B plus either DBPI or propamidine have potential for the treatment of superficial infections of the eye.

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