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Evaluation of synergistic effects of combinations of antibacterials having relevance to treatment of burn wound infections

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Summary

Subinhibitory concentrations of combinations of dibromopropamidine isethionate with sodium sulphadiazine, sodium sulphadiazine with silver sulphadiazine, sodium sulphadiazine with silver nitrate, trimethoprim with silver sulphadiazine and dibromopropamidine isethionate with trimethoprim were shown to have synergistic antibacterial activity against cells of *Pseudomonas aeruginosa*. However, dibromopropamidine isethionate with silver sulphadiazine and dibromopropamidine isethionate with silver nitrate could be either antagonistic or synergistic depending on the proportions of dibromopropamidine isethionate to silver salt. The sulphadiazine moiety in silver sulphadiazine was shown to enhance the uptake of Ag^+ from silver sulphadiazine by *P. aeruginosa* cells. All the antibacterials tested at subinhibitory concentrations were shown to have a damaging effect (similar to a post antibiotic effect) on *P. aeruginosa* cells which made the cells more sensitive to the action of benzalkonium chloride and chlorhexidine diacetate. The only exception was with dibromopropamidine isethionate treated cells which were more resistant to the action of benzalkonium chloride than control cells.

Introduction

Recent work indicated that subinhibitory concentrations of any two of sulphadiazine (SD), trimethoprim (TMP) and dibromopropamidine isethionate (DBPI) produced increased uptake of both members of the combination by cells of *Pseudomonas aeruginosa* and *Enterobacter cloacae* (Richards et al., 1991). Decreased bacterial uptake of both DBPI and silver salts could result when certain concentrations of DBPI were used with either silver sulphadiazine (AgSD) or silver nitrate ($AgNO_3$).

The present investigation was undertaken in order to elucidate further the interaction between DBPI and AgSD, DBPI and $AgNO_3$, NaSD and $AgNO_3$, NaSD and AgSD and the effect of the SD moiety in AgSD on the uptake of Ag^+ from AgSD by *P. aeruginosa* cells.

In addition, the subinhibitory effect of combinations of TMP plus AgSD, TMP plus DBPI and NaSD plus DBPI against *P. aeruginosa* were to be evaluated further. These interactions all have relevance to the treatment of burn wound infections.

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Materials and Methods

Materials

P. aeruginosa NCTC 6750 was obtained from the National Collection of Type Cultures, Colindale, London.

Isosensitest broth and bacteriological peptone L37 were obtained from Oxoid, Basingstoke. Silver nitrate ($AgNO_3$) (analar) was obtained from BDH, Poole and sulphadiazine (SD), trimethoprim (TMP), benzalkonium chloride, chlorhexidine diacetate and folinic acid were all obtained from Sigma, Poole. Dibromopropamidine isethionate (DBPI) was a gift from May and Baker, Dagenham.

Silver sulphadiazine (AgSD) was prepared according to the method described previously (Richards et al., 1991).

Checkerboard MIC determinations

Checkerboard MIC estimates with two drug combinations were based on the method of Sabbath (1968). *P. aeruginosa* was the test organism. A 10×10 checkerboard of test-tubes was prepared. Each test-tube contained isosensitest broth and was inoculated to give 5×10^3 cells/ml. MICs were determined for each antibacterial combination after 24 h incubation at 37 °C and isobolograms plotted. The tests were carried out in duplicate.

Determinations of killing times

Estimates of the comparative resistance of cells grown in peptone water (plus 0.89% w/v sodium chloride) alone and in peptone water (plus 0.89% w/v sodium chloride) plus the single antibacterials NaSD (100 μg/ml), DBPI (100 μg/ml) and TMP (100 μg/ml) or combinations of DBPI (100 μg/ml) plus AgSD (0.1 μg/ml), TMP (10 μg/ml) plus AgSD (0.15 μg/ml), DBPI (100 μg/ml) plus NaSD (50 μg/ml), DBPI (100 μg/ml) plus TMP (10 μg/ml) or NaSD (100 μg/ml) plus TMP (10 μg/ml) were determined in a manner similar to that described previously (Richards and McBride, 1973; Richards, 1975). After 72 h incubation at 37 °C, the cultures were centrifuged ($6000 \times g$, 15 min, 4 °C) and the cell pellets washed in 0.9% w/v sodium chloride, recentrifuged and the pellets resuspended in 0.9% w/v sodium chloride. The cell concentration was adjusted to approx. 5×10^8 cells/ml. Duplicate tubes containing 9.9 ml of the 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 give a final inoculum of approx. 5×10^6 cells/ml. 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 of inactivating recovery medium (nutrient broth containing Tween 80, 3.0% w/v and lecithin 0.5% w/v) and incubated at 37 °C for 72 h. The test antibacterial solutions were benzalkonium chloride (25 and 50 μg/ml) and chlorhexidine diacetate (12.5, 25 and 50 μg/ml). Positive controls were prepared by adding 5×10^3 cells from the source of inoculum above to duplicate tubes of recovery medium plus 50 μg/ml of either benzalkonium or chlorhexidine. This demonstrated the effectiveness of the inactivators.

Determination of uptake of silver

The uptake determinations of Ag^+ were carried out as described previously (Richards et al., 1991). 1 ml of 18 h culture of *P. aeruginosa* was inoculated separately into replicate 250 ml conical flasks containing peptone water (plus 0.89% w/v sodium chloride) plus the selected concentrations of $AgNO_3$ or AgSD in the presence or absence of folinic acid, and incubated for 72 h at 37 °C. The bacterial cultures were centrifuged ($6000 \times g$, 15 min, 4 °C) and the supernatants removed; the pellets were washed by resuspending in distilled water and recentrifuged. This treatment of the pellets was repeated. After removing the supernatants the cell pellets were transferred to glass vials and dried at 50–60 °C to constant weight. The calibration was for dry weights over the range 1–5 mg/ml against the absorbance readings of the original suspensions. The uptake of Ag^+ was determined by atomic absorption. This involved concentrating of the silver present in the samples and removing the large concentration of Na^+ by passage through a cation-exchange resin (Zerolit 325). The silver was then eluted with hydrochloric acid (2 M). The first 25 ml of the acidic elute was shown to

contain all the Ag^+ and this was collected and used in the atomic absorption assay. The concentrations of Ag^+ in the bacterial supernatants and the bacterial washings were determined and combined. This concentration subtracted from the original concentration present in the culture flasks gave the concentration of silver taken up by unit mass of cells. All bacterial uptakes were determined in duplicate.

Results and Discussion

The isobolograms constructed from the checkerboard MIC data are shown in Figs 1–7.

DBPI (75–135 μg/ml) was antagonistic to AgSD (0.6–3.6 μg/ml) but DBPI (15–60 μg/ml) was synergistic to AgSD (1.2–4.8 μg/ml) (Fig. 1). Only synergism resulted from the combined antibacterial activity of DBPI and NaSD (Fig. 2). However, antagonism occurred between DBPI (75–135 μg/ml) and $AgNO_3$ (3–6 μg/ml) and slight synergism between DBPI (15–75 μg/ml) and $AgNO_3$ (2.4–5.4 μg/ml) (Fig. 3). Marked synergism and no antagonism resulted from the combined action of NaSD and AgSD (Fig. 4) and NaSD and $AgNO_3$ (Fig. 5). These results indicate that the antagonism between certain concentrations of DBPI and AgSD and DBPI and $AgNO_3$ is due to an interaction between DBP^{2+} and Ag^+. This effect is very likely the result of the two positively charged cations competing for negative sites on the *P. aeruginosa* cell surface (Richards et al., 1991). When DBPI is present at an approximate ratio of DBPI 25 parts to $AgNO_3$ one part then synergism can occur.

TMP plus AgSD and DBPI plus TMP also showed synergism at subinhibitory concentrations (Figs 6 and 7).

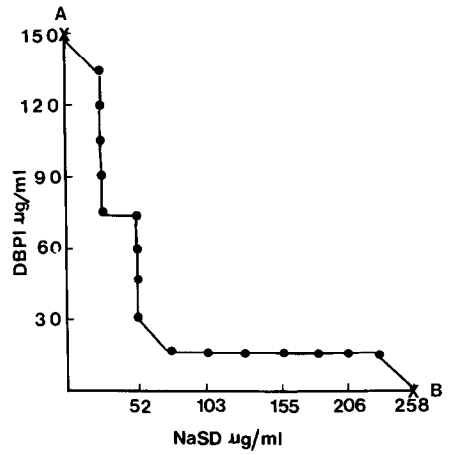

Fig. 2. Isobologram constructed from checkerboard MIC data showing combinations of DBPI with NaSD against *P. aeruginosa* in isosensitest broth. MIC of DBPI = 150 μg/ml; MIC of NaSD = 258 μg/ml.

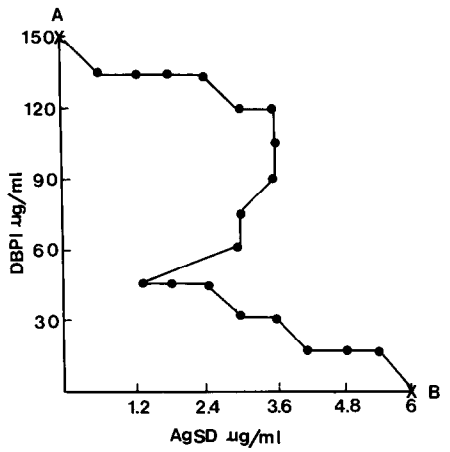

Fig. 1. Isobologram constructed from checkerboard MIC data showing combinations of DBPI with AgSD against *P. aeruginosa* in isosensitest broth. MIC of DBPI = 150 μg/ml; MIC of AgSD = 6 μg/ml.

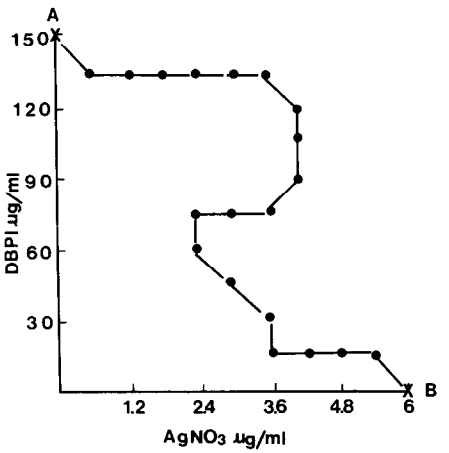

Fig. 3. Isobologram constructed from checkerboard MIC data showing combinations of DBPI with $AgNO_3$ against *P. aeruginosa* in isosensitest broth. MIC of DBPI = 150 μg/ml; MIC of $AgNO_3$ = 6 μg/ml.

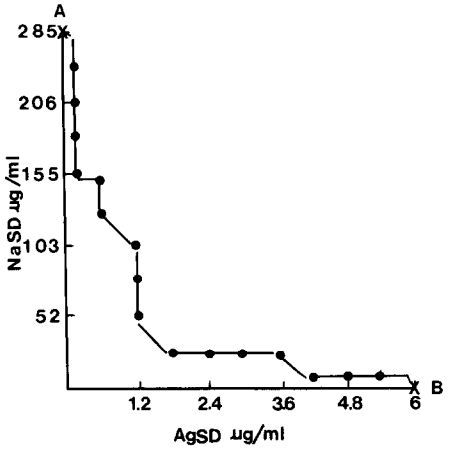

Fig. 4. Isobologram constructed from checkerboard MIC data showing combinations of AgSD with NaSD against *P. aeruginosa* in isosensitest broth. MIC of AgSD = 6 μg/ml; MIC of NaSD = 258 μg/ml.

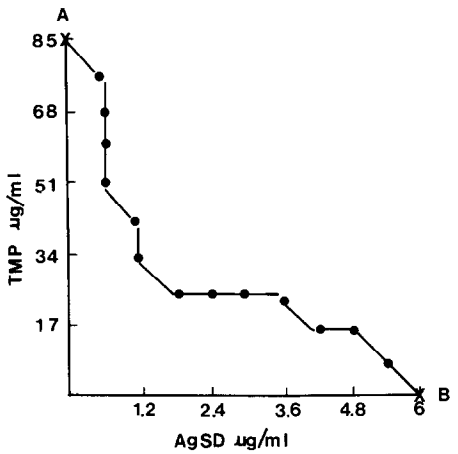

Fig. 6. Isobologram constructed from checkerboard MIC data showing combinations of AgSD with TMP against *P. aeruginosa* in isosensitest broth. MIC of AgSD = 6 μg/ml; MIC of TMP = 85 μg/ml.

When the concentration of each antibacterial present in a combination of antibacterials at the MIC for the combination is calculated as a fraction of the MIC concentrations for the antibacterials used alone and the two fractions obtained are summed, then the fractional inhibitory concentration index (FIC index) for the combination is obtained.

$$\text{FIC index} = \frac{\text{concentration A in MIC A + B}}{\text{MIC A used alone}} + \frac{\text{concentration B in MIC A + B}}{\text{MIC B used alone}}$$

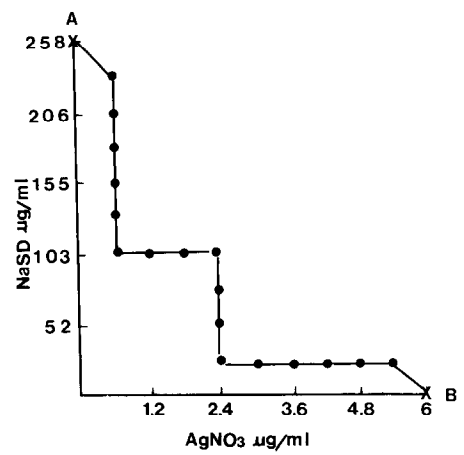

Fig. 5. Isobologram constructed from checkerboard MIC data showing combinations of $AgNO_3$ with NaSD against *P. aeruginosa* in isosensitest broth. MIC of $AgNO_3$ = 6 μg/ml; MIC of NaSD = 258 μg/ml.

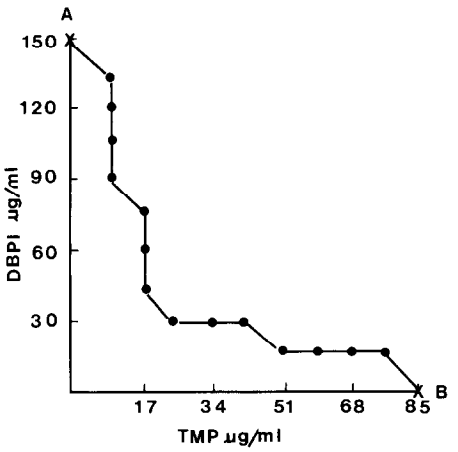

Fig. 7. Isobologram constructed from checkerboard MIC data showing combinations of DBPI with TMP against *P. aeruginosa* in isosensitest broth. MIC of DBPI = 150 μg/ml; MIC of TMP = 85 μg/ml.

TABLE 1

FIC indices for the antibacterial combinations determined in isosensitest broth against P. aeruginosa

Antibacterial combination	FICs	Type of interaction
DBPI + AgSD	0.5 or 1.4	synergism or antagonism
DBPI + NaSD	0.4	synergism
DBPI + $AgNO_3$	0.8 or 1.5	synergism or antagonism
NaSD + AgSD	0.4	synergism
NaSD + $AgNO_3$	0.5	synergism
TMP + AgSD	0.6	synergism
DBPI + TMP	0.5	synergism

The FIC indices for the combinations used are given in Table 1. If the FIC index is 0.8 or below a synergistic interaction is concluded but if the FIC index is greater than 1.2 an antagonistic interaction is concluded. FIC index values between 0.8 and 1.2 are taken to represent addition.

Table 1 summarises the antibacterial effects produced by the antibacterial combinations used. All combinations show marked synergism except DBPI plus either AgSD or $AgNO_3$ which exhibit both synergism and antagonism. The synergism with the AgSD is greater than with the $AgNO_3$ as would be expected, since DBPI can enhance the uptake of both Ag^+ and SD^- and in addition SD^- can enhance the uptake of the DBPI (Richards et al., 1991).

The fact that NaSD was synergistic with AgSD prompted the hypothesis that the SD moiety in AgSD may influence the uptake of Ag^+ from AgSD by *P. aeruginosa* cells. Table 2 indicates that the hypothesis is confirmed, under the conditions of the determination, since there was a greater bacterial uptake of Ag^+ from AgSD compared with the uptake of Ag^+ from the same concentration of Ag^+ supplied by $AgNO_3$. The uptake of Ag^+ from AgSD is seen to be partially blocked by the presence of folinic acid (1 μg/ml). *P. aeruginosa* is known to metabolise folinic acid (Richards et al., 1991). The effect of SD^- in increasing the bacterial uptake of Ag^+ would therefore appear to be the result of the partial blockade of the folinic acid synthetic pathway casued by SD^-. This partial blockade in turn results in cells having an increased permeability to the Ag^+. The synergism between NaSD and AgSD (Fig. 4), NaSD and $AgNO_3$ (Fig. 5) and TMP and AgSD (Fig. 6) may be explained similarly.

It is likely that this increased uptake of Ag^+ from AgSD caused by SD^- could have a significant effect on clinical antibacterial activity in certain situations. That is in those situations where SD^- partially blocks the folate synthetic pathway of an organism such as *P. aeruginosa* and thus influences the permeability properties of the cells.

This effect by any two of DBPI, NaSD and TMP causing an increased uptake of each member of the antibacterial combination by either *P. aeruginosa* or *E. cloacae* cells has been reported (Richards et al., 1991). The increased antibacterial activity of these combinations is illustrated for DBPI plus NaSD and DBPI plus TMP in Figs 2 and 7.

The production of a damaging effect, similar to a post-antibiotic effect (Editorial, 1987), on *P. aeruginosa* cells by subinhibitory concentrations

TABLE 2

Effect of the SD moiety in AgSD on the uptake of silver ion from AgSD by P. aeruginosa grown in peptone water (plus 0.89% w/v NaCl) plus the selected concentrations of AgSD or $AgNO_3$ in the presence or absence of folinic acid

Chemicals (μg/ml)			Ag^+ uptake (μg/mg dry cell weight) [a]
$AgNO_3$	AgSD	FA	
0.112 (0.071 [Ag^+])	–	–	0.00523
0.112	–	1.0	0.00535
–	0.15 (0.071 [Ag^+])	–	0.00725
–	0.15	1.0	0.00607

FA, folinic acid

[a] Mean of two determinations.

TABLE 3

Killing times for benzalkonium chloride and chlorhexidine diacetate solutions against approx. 5×10^6 P. aeruginosa cells which had been grown in peptone water alone or plus either sodium sulphadiazine (NaSD), silver sulphadiazine (AgSD), dibromopropamidine isethionate (DBPI) or trimethoprim (TMP) used alone or in combination, harvested, washed and suspended in 0.9% w/v sodium chloride

Source of inoculum; peptone water (plus 0.89% w/v NaCl) plus antibacterial (μg/ml)	Killing times (min) at 37 °C [a]				
	Benzalkonium chloride (μg/ml)		Chlorhexidine diacetate (μg/ml)		
	25	50	12.5	25	50
Peptone water alone	180–240	60	–	120	60–90
NaSD (100)	60	< 15	–	30	< 15
DBPI (100)	> 300	> 300	–	30	< 15
TMP (100)	180	45	–	90	< 15
AgSD (0.10) + DBPI (100)	> 300	60	–	30	< 15
AgSD (0.15) + TMP (10)	180	< 15	–	90	45
DBPI (100) + NaSD (50)	< 15	< 15	30	< 15	< 15
DBPI (100) + TMP (10)	90	< 15	30	< 15	< 15
NaSD (100) + TMP (10)	60	< 15	60	< 15	< 15

[a] Duplicate determinations.

of NaSD, DBPI and TMP used alone or in combination is indicated by the results presented in Table 3.

Cells grown in the presence of NaSD, DBPI and TMP were all more sensitive to the action of chlorhexidine diacetate than the control cells. Similarly, cells grown in the presence of NaSD and TMP were more sensitive to the action of benzalkonium chloride.

However, the cells grown in the presence of DBPI seem to be cross-resistant to benzalkonium chloride. The killing time for these cells was greater than 300 min, compared with 60 min for the control cells. This supports the finding that *Staphylococcus aureus* (MRSA) resistant to propamidine isethionate was also cross-resistant to the quaternary ammonium compounds (Townsend et al., 1984).

Cells grown in the presence of the antibacterial agents used in combination were all more sensitive to the action of chlorhexidine diacetate. The killing times for cells grown in the presence of these combinations were reduced by between 25 and 75% compared with the control cells.

Similarly, cells grown in the presence of combinations of either DBPI plus TMP, DBPI plus NaSD or NaSD plus TMP were more sensitive to the action of benzalkonium chloride. This occurred even though cross-resistance to benzalkonium chloride was produced when the cells were grown in the presence of DBPI alone and may be explained as follows. When DBPI was used in combination with either NaSD or TMP, the effects of NaSD and TMP on the cell envelope prevented the DBPI from producing resistance to benzalkonium chloride. However, cells grown in the presence of DBPI combined with AgSD show cross-resistance to benzalkonium chloride. In this situation the SD was present at a lower concentration and apparently was unable to prevent the development of the DBPI-initiated resistance to benzalkonium chloride.

The sensitising effect resulting from growth in the presence of the antibacterial agents is thought to result from damage to the permeability control of the cells caused by the action of SD, DBPI or TMP. This sensitising effect has been observed previously with cells grown in the presence of disodium edetate and phenylethanol and subsequently tested against another antibacterial (Richards et al., 1969). Both disodium edetate and phenylethanol are known to affect the *P. aeruginosa* cell envelope permeability and to enhance access of a second antibacterial agent pre-

sent in the medium (Brown and Richards, 1965; Richards et al., 1969; Richards and McBride, 1973; Richards, 1975).

The results of the killing time determinations (Table 3) provide further evidence that SD, DBPI and TMP at subinhibitory concentrations can have a damaging effect, similar to a post-antibiotic effect, on the permeability control of *P. aeruginosa* cells and corroborate previous observations (Richards et al., 1991). The overall findings have potential significance for the use of these antibacterials in the clinical situation. A fuller understanding of the mode of action of AgSD against *P. aeruginosa* is provided.

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