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Investigations of the correlation between bacterial uptakes of trimethoprim and sulphadiazine with antibacterial activities against *Enterococcus faecalis*

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Summary

Bacterial uptake determinations were performed to evaluate the antibacterial effects of trimethoprim, sodium sulphadiazine and dibromopropamidine isethionate against *Enterococcus faecalis* together with checkerboard assays and agar diffusion determinations. The reciprocal effects of trimethoprim (17.5 μg/ml) and sodium sulphadiazine (100 μg/ml) against *E. faecalis* culture produced cell uptakes of 14-times the concentration of sodium sulphadiazine and 5-times the concentration of trimethoprim. Folinic acid partially reversed the bacterial uptakes.

It has been reported that the well known synergism between subinhibitory concentrations of trimethoprim (TMP) and sulphonamides is related to an increased bacterial uptake of both antibacterials by Gram-negative bacteria (Xing, 1990; Richards et al., 1991). It was suggested that the sequential but partial blockade of the folinic acid synthetic pathway resulted in viable cells having impaired permeability properties. This has been substantiated by electron microscope studies of the effect of TMP and sulphadiazine (SD) on the morphology of *Enterobacter cloacae* cells (Richards et al., 1993). These studies indicated that the peptidoglycan layer of *Eb. cloacae* cells was damaged by both antimetabolites but especially by TMP.

This current investigation was to determine whether similar effects are caused by TMP and SD on Gram-positive bacteria. *Enterococcus faecalis*, a common pathogen, was the Gram-positive organism selected. It is especially relevant because there is controversy about the use of either TMP or TMP plus a sulphonamide, against *E. faecalis*. In addition *E. faecalis* has been reported by some authors to utilise folinic acid (FA) (Bushby and Hitchings, 1968; Zernos and Schaberg, 1985), but this has been disputed by others (Greenwood, 1989). It is widely agreed

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that enterococci are intrinsically resistant to sulphonamides but there seems to be much confusion in the literature as to the status of enterococcal sensitivity to TMP, and whether synergy occurs between TMP and sulphonamides for these species, or whether TMP (with or without sulphonamide) should be used for the treatment of *Enterococcus* infections (Zernos and Schaberg, 1985; Hamilton-Miller and Purves, 1986; Hamilton-Miller and Stewart, 1988).

E. faecalis NCTC 775 was obtained from the National Collection of Type Cultures, Colindale, London and *E. faecalis* 463 from The City Hospital, Aberdeen. Iso-Sensitest broth and agar (Oxoid) plus 0.25% w/v glucose were the culture media used. Trimethoprim (TMP), sulphadiazine (SD) and folinic acid (FA) were all obtained from Sigma. Dibromopropamidine isethionate (DBPI) was a gift from May and Baker. The cream base was cetomacrogol cream formula A of the Pharmaceutical Codex, without added preservative.

Minimum inhibitory concentrations, fractional inhibitory concentrations, agar cup diffusion determinations, checkerboard assays and HPLC determinations of antibacterials were carried out as described previously (Richards and Xing, 1991; Richards et al., 1991; Taylor et al., 1990, 1992).

The results presented here agree with those reported by others (Crider and Colby, 1985; Hamilton-Miller and Stewart, 1988) who suggest that in the absence of FA synergy occurs with the combination of TMP plus sulphonamide against *E. faecalis*. The MICs for TMP, SD and DBPI used singly were 70, > 500 and 140 μg//ml, respectively. Checkerboard data showed that combinations of TMP + SD, TMP + DBPI and SD + DBPI had MICs of 35 + 120 (FIC < 0.74), 35 + 28 (FIC 0.70) and 200 + 84 (FIC < 1.00), respectively. These FIC results indicated mild synergy for TMP + SD and TMP + DBPI and the agar diffusion results (Table 1) also indicated synergy for these combinations. There appears to be an additive effect with SD and DBPI when compared with the effect caused by either antibacterial singly.

It can be seen from Fig. 1A that TMP at concentrations of 3.5–17.5 μg/ml markedly increased the uptake of SD from broth containing

TABLE 1

Diameters of zones of inhibition for trimethoprim (TMP), sulphadiazine (SD) and dibromopropamidine isethionate (DBPI) alone and in combination, in the presence and absence of folinic acid 3 μg/ml against E. faecalis NCTC 775 and E. faecalis 463

Cream formulation (%)	Diameters of zone of inhibition (mm) [a]			
	Without folinic acid		With folinic acid	
	5×10^3 (mean ± SD)	5×10^5 (mean ± SD)	5×10^3 (mean ± SD)	5×10^5 (mean ± SD)
E. faecalis NCTC 775				
TMP alone 0.01	34.1 ± 0.40	29.3 ± 0.35	26.8 ± 0.35	23.8 ± 0.50
SD alone 0.2	0	0	0	0
DPBI alone 0.1	19.8 ± 0.75	16.7 ± 0.50	19.7 ± 0.48	17.6 ± 0.35
TMP + SD 0.01 + 0.2	39.5 ± 0.65 [b]	34.8 ± 0.56 [b]	26.9 ± 0.27	23.4 ± 0.20
TMP + DBPI 0.01 ± 0.1	38.1 ± 0.86 [b]	32.6 ± 0.75 [b]	28.5 ± 0.42	24.2 ± 0.28
SD + DBPI 0.2 ± 0.1	20.6 ± 0.24	17.4 ± 0.20	20.0 ± 0.12	18.0 ± 0.22
E. faecalis 463				
TMP alone 0.1	39.4 ± 0.42	30.5 ± 0.46	34.7 ± 0.52	25.9 ± 0.82
SD alone 2	0	0	0	0
DBPI alone 0.1	18.0 + 0.70	14.6 + 0.50	18.0 ± 0.36	14.4 ± 0.42
TMP + SD 0.1 + 2	45.6 ± 0.57 [b]	34.9 ± 0.40 [b]	34.6 ± 0.49	25.8 ± 0.57
TMP + DBPI 0.1 + 0.1	43.2 ± 0.18 [b]	33.4 ± 0.37 [b]	36.5 ± 0.35	27.2 ± 0.56
SD + DBPI 2 + 0.1	18.4 ± 0.42	15.5 ± 0.61	18.2 ± 0.64	15.4 ± 0.48

[a] Mean of five determinations.

[b] *t*-test: significant at $p \leq 0.05$ (comparing means of TMP with means of combinations).

SD (100 μg/ml). The SD uptake was increased by a factor of approx. 14 by TMP (17.5 μg/ml). SD (20–100 μg/ml) also increased the uptake of TMP from cultures containing TMP (17.5 μg/ml) in a stepwise manner up to an approx. 5-fold increase of TMP uptake. Fig. 1B indicates that the highest uptakes of TMP were produced by SD (40–60 μg/ml). This is at an SD:TMP ratio of about 2–3:1. At SD concentrations of 60–100 μg/ml the uptake of TMP decreased steadily. The checkerboard results showed that the greatest synergistic inhibitory effect occurred when the combination of SD:TMP was at a ratio of approx. 3:1. The results in Fig. 1B taken in conjunction with the checkerboard data provide an explanation why the largest synergistic effects occurred at ratios of SD:TMP of approx. 2–3:1 when used against this strain of *E. faecalis*.

From Fig. 1A it can be seen that the uptake of DBPI by *E. faecalis* was increased by a factor of about 7 by the action of 17.5 μg/ml TMP. Fig. 1C shows that DBPI over the range 7–35 μg/ml increasingly enhanced the uptake of TMP from cultures containing TMP 17.5 μg/ml. The highest concentration of DBPI (35 μg/ml) increased the TMP uptake by a factor of approx. 5. The synergy of this combination was indicated both by the checkerboard assay and by the agar diffusion determinations (Table 1).

Although the DBPI uptake was not markedly increased by SD (Fig. 1B, it can be seen from Fig. 1C that DBPI 7–35 μg/ml increased the uptake of SD by a factor of more than 10. This lack of effect of SD on DBPI uptake was different from that obtained with Gram-negative bacteria where an increased uptake of DBPI occurred when it was used in combination with SD (Richards et al., 1991). Checkerboard determinations showed that an additive effect occurs with this combination against *E. faecalis*. It would appear this that the increased uptake of SD does not exert a proportional increase in antibacterial activity.

Previously, FA has been found to reverse the action of TMP against enterococci (Bushby and Hitchings, 1968; Zernos and Schaberg, 1985). This has been disputed by another worker

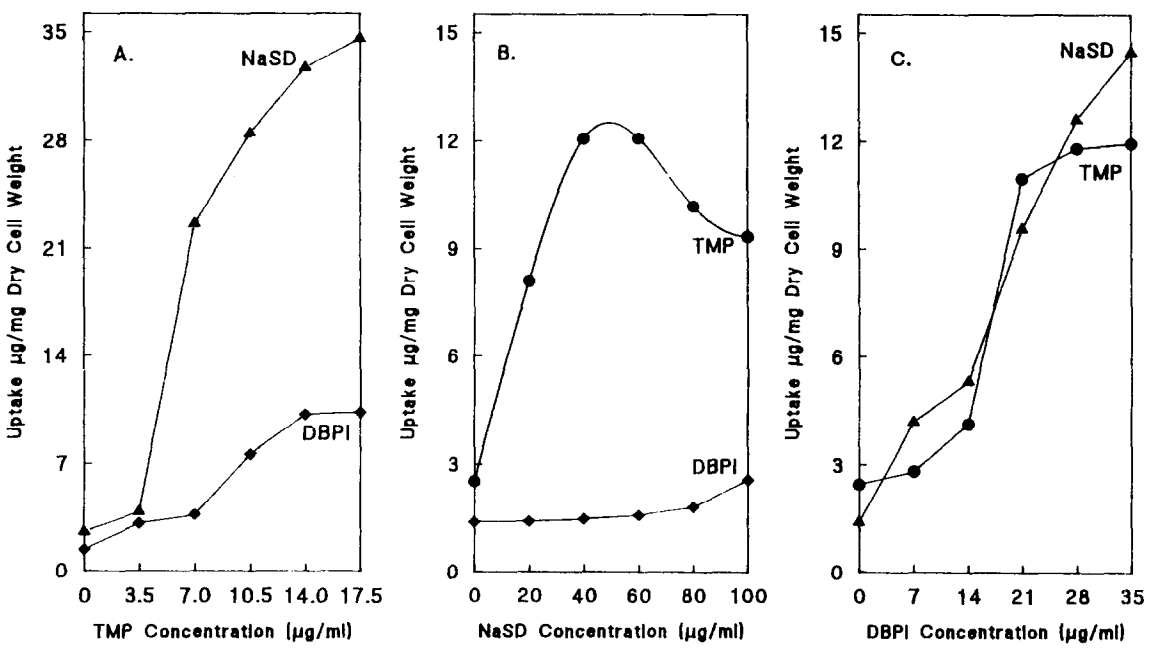

Fig. 1. Uptakes by *E. faecalis* cultures of (A) sodium sulphadiazine (NaSD) 100 μg/ml and dibromopropamidine isethionate (DBPI) 35 μg/ml in the presence of trimethoprim (TMP) 0–17.5 μg/ml, (B) TMP 17.5 μg/ml and DBPI 35 μg/ml in the presence of NaSD 0–100 μg/ml and (C) TMP 17.5 μg/ml and NaSD 100 μg/ml in the presence of DBPI 0–35 μg/ml.

(Greenwood, 1989). Recently, it has been reported that this antagonistic effect of FA on the action of these two antimetabolites also occurred with *P. aeruginosa* (Xing, 1990; Richards et al., 1991). From Table 1, it is seen that folinic acid can partially reverse the activity of TMP against *E. faecalis*. The agar diffusion results indicate that the enhanced activity occurring with combinations of TMP and SD can be completely blocked by FA. This phenomenon provides one explanation why a failure in treatment in vivo may occur with this combination (Grayson et al., 1990). Table 2 shows that FA at concentrations of 0.5 and 3.0 μg/ml markedly reduced the uptake of both components of the combination. This provides a mechanism for the blocking of synergy observed earlier (Table 1). It is seen from Table 2 that FA at concentrations of 0.5 and 3 μg/ml has a blocking effect on the enhancing action of TMP 17.5 μg/ml on the uptake of DBPI by *E. faecalis*. An approx. 7-fold increase in uptake of DBPI is reversed by FA 3 μg/ml to approx. 2.5-times the expected uptake from DBPI 35 μg/ml alone. The uptake of TMP is also reduced in the presence of FA.

Therefore, these results support the hypothesis that the sequential blockade of FA synthesis produced by subinhibitory concentrations of a sulphonamide plus TMP combination produces changes in cell permeability which result in an increased uptake of both antibacterial agents (Richards et al., 1991) and indicate that this occurs not only in Gram-negative but also in Gram-positive bacteria.

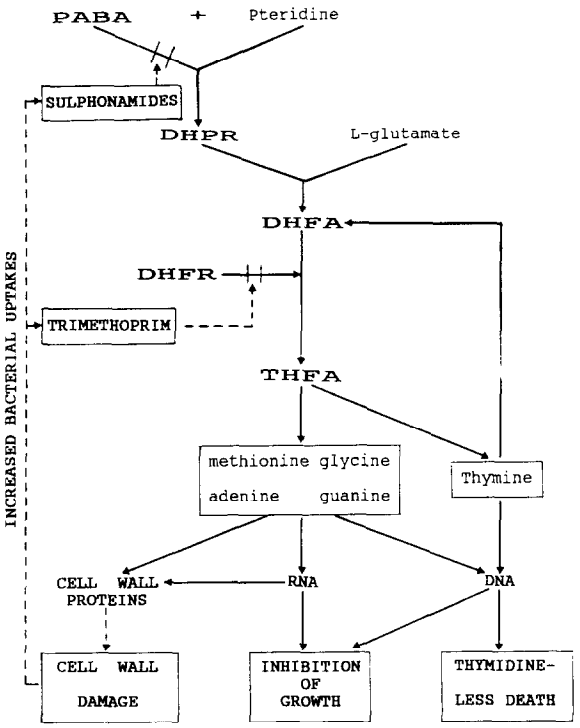

Fig. 2. Mechanisms of action of sulphonamides and trimethoprim on the folic acid pathway. *p*-Aminobenzoic acid (PABA), 7,8-dihydropteroate (DHPA), dihydrofolic acid (DHFA), dihydrofolate reductase (DHFR), tetrahydrofolic acid (THFA). Normal metabolic pathway indicating the generally accepted antibacterial action (continuous line); additional mechanism of action explaining synergy (broken line).

TABLE 2

Bacterial uptake of trimethoprim (TMP), sodium sulphadiazine (NaSD) and dibromopropamidine isethionate (DBPI) by log phase cells of E. faecalis grown in the presence of the antibacterials used singly or in combination in the presence or absence of folinic acid (FA)

	Antibacterial concentration (μg/ml)	Bacterial uptake (μg/mg dry cell weight) (without FA)	Bacterial uptake (μg/mg dry cell weight) (with FA)	
			0.5 μg/ml	3 μg/ml
TMP	17.5	2.60		
NaSD	100	1.93		
DBPI	35	1.35		
TMP + NaSD	17.5 + 100	9.25 + 33.89	3.82 + 13.34	2.76 + 9.55
TMP + DBPI	17.5 + 35	9.18 + 10.46	7.10 + 4.19	4.77 + 3.64
NaSD + DBPI	100 + 35	14.70 + 2.89	14.64 + 1.31	14.58 + 1.26

The evidence of increased bacterial uptake in Gram-negative bacteria (Xing, 1990; Richards et al., 1991) together with the results of this present study with two strains of a Gram-positive bacterium indicate that this increased uptake of antibacterials results from the inability of the FA depleted cells to synthesise certain essential components necessary for the cell envelope permeability control.

The additional aspects of the mode of action of these two antimetabolites can be incorporated into the current generally accepted diagrammatic representation of the mechanism of antibacterial action of sulphonamides and TMP as shown in Fig. 2. It is suggested that this additional information helps provide a more plausible explanation for the mode of action of TMP and sulphonamides used alone. It also explains how synergism occurs with the combination including how synergism is possible against bacterial species which are resistant to either or both members of the antibacterial combination.

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