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Mechanism for Synergism between Sulphonamides and Trimethoprim Clarified

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Abstract

Pseudomonas aeruginosa, *Escherichia coli*, *Pseudomonas cepacia* and *Moraxella catarrhalis* were selected for their markedly different resistance patterns to sulphonamides and trimethoprim. In addition, strains of *E. coli* and *P. cepacia* were selected having different resistance profiles to the inhibition of dihydropteroate synthetase and dihydrofolate reductase.

All inhibitors of dihydropteroate synthetase combined in any combination with inhibitors of dihydrofolate reductase resulted in mutual enhancement of bacterial uptakes of the inhibitors and corresponding increased antibacterial activity of the combinations. High concentrations of sulphonamides or *p*-aminobenzoic acid plus trimethoprim caused a decrease in overall activity of the combination and indicated that both sulphonamides and *p*-aminobenzoic acid at high concentrations can interact with dihydrofolate reductase. The antibacterial activity of *p*-aminobenzoic acid at high concentrations is considered to be a blocking effect on dihydrofolate reductase even though *p*-aminobenzoic acid at low concentrations is an essential part of the synthesis of dihydrofolic acid.

These findings support an alternative hypothesis for the mechanism of antibacterial action of individual antifolates and their mechanism of synergism in combination.

The widely accepted sequential blockade of the bacterial folate synthetic pathway theory for the synergism between sulphonamides and trimethoprim still leaves uncertainties remaining to be clarified. The theory proposes that trimethoprim inhibits dihydrofolate reductase by competing with dihydrofolate and that sulphonamides inhibit the enzyme dihydropteroate synthetase by competing with the substrate *p*-aminobenzoic acid. Poe (1976) made an alternative proposal of a multiple simultaneous inhibition of bacterial dihydrofolate reductase by sulphonamides and trimethoprim acting together. Then (1977) argued against this, particularly because the concentrations of sulphonamides required were too high to be clinically relevant but Lacey (1979) found the sequential blockade theory less convincing than the single site of action proposal. Both Poe and Lacey were particularly concerned with findings that potentiation of trimethoprim by sulphonamides occurs with many sulphonamide-resistant organisms.

Recent observations in this laboratory provide an explanation of how potentiation can occur even when bacteria are resistant to either or both sulphonamides and trimethoprim when used singly. These antibacterials have been shown mutually to enhance their uptakes by the Gram-negative *Pseudomonas aeruginosa* resistant to both antibacterials (Richards et al 1991a), by the Gram-negative *Enterobacter cloacae* which is highly resistant to sulphonamides but sensitive to trimethoprim, and by two strains of the Gram-positive *Enterococcus faecalis*, one highly resistant to sulphonamides and trimethoprim and the other resistant to sulphonamides and sensitive to trimethoprim (Richards et al 1993a, 1995a). Electron microscopy of *E. cloacae* and *E. faecalis* log phase cultures treated with sulphadiazine and trimethoprim alone or in combination at similar concentrations to the uptake studies indicated damage to the bacterial peptidoglycan layer and cytoplasmic membrane and electron-transparent holes in the cytoplasm of sulphadiazine-treated cells. In addition trimethoprim inhibited cell division (Richards et al 1993b, 1995a). Furthermore, analysis of precursors of peptidoglycan synthesis by capillary zone electrophoresis showed that both sulphadiazine and trimethoprim interfered with peptidoglycan synthesis in *E. cloacae* cells (Richards & Xing 1994a, b).

The present work was undertaken to investigate further whether bacteria possessing different patterns of resistance to trimethoprim and sulphonamides responded similarly to those reported previously, and to clarify further the argument for the mechanism of synergism between trimethoprim and sulphadiazine.

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Materials and Methods

Materials

Escherichia coli NCIB 8879, *Pseudomonas aeruginosa* NCTC 6750 and 8626, *Pseudomonas cepacia* NCTC 10743 and *Moraxella catarrhallis* NCTC 11020 were all obtained from the National Collection of Type Cultures, Colindale, London, UK. *E. coli* 326 and *P. cepacia* 42 were obtained from the Aberdeen Royal Infirmary, U.K.

Sulphadiazine, sulphamethoxazole, sulphamerazine, sulphanilamide, dapsone, trimethoprim, pyrimethamine and *p*-aminobenzoic acid were all obtained from Sigma, Poole, UK. Iso-Sensitest broth and nutrient agar were obtained from Oxoid Ltd, Basingstoke, UK.

The high-performance liquid chromatography (HPLC) system consisted of an M6000A pump system (Waters Associates Inc.). Injection was by means of a Rheodyne 7125 valve fitted with a 20-μL fixed-volume loop. The 100-mm long, 4·6 mm i.d. column was slurry-packed with 3 μm ODS-Hypersil. Detection was at 254 nm using a Waters 440 UV-visible detector connected to a potentiometric recorder (BBC SE 120).

Antibacterial activity

Minimum inhibitory concentrations (MICs), checkerboard MIC values and killing curves were determined for single antibacterials and antibacterial combinations as previously described (Richards et al 1991a, b).

Determination of dry cell weight

Ultrafiltration was used to collect the cells for dry cell weight determinations. Bacterial culture (50 mL) was poured onto a cellulose nitrate membrane filter (0·2 μm pore size, 47 mm diameter) which had been dried at 50–60°C to constant weight and fitted onto a filter holder connected to a vacuum for filtration. After the culture had been filtered the membrane was washed with sterile water and subsequently dried at 50–60°C to constant weight. The dry cell weight was obtained from the difference between the filter membrane weights before and after filtration and subsequent drying. Calibration curves of dry cell weight versus absorbance at 600 nm for the suspensions of the test bacteria were determined to check the linearity and validity of the method. The regression coefficient (r) for each of the test bacteria was obtained: *E. coli* NCTC 8879, r = 0·9969; *E. coli* 326, r = 0·9989; *P. aeruginosa* NCTC 8626, r = 0·9970; *P. aeruginosa* NCTC 6750, r = 0·9949; *P. cepacia* NCTC 10743, r = 0·9969; *P. cepacia* 42, r = 0·9975; *M. catarrhalis* NCTC 11020, r = 0·9974.

Determination of uptake of the antibacterials

The bacterial uptake of the antibacterials was determined by an HPLC assay combined with dry cell weight determinations. The precision of the HPLC method for the determination of the various antibacterials was determined as RSD%. This varied between 1·8 and 3·8 for the set. Log phase cultures of each strain were prepared as before (Richards et al 1993a). Each uptake value used in the figures is the mean of three determinations.

Sample pre-treatment

The bacterial culture containing the analytes was filtered through a membrane filter to remove the bacterial cells. A cyclohexyl-bonded silica Bond-Elut cartridge was wetted with 2 mL methanol and conditioned with a further 2 mL pH 2·5 phosphate buffer. A 0·1-mL sample of the filtrate was then added. After adsorption the cartridge was washed with 2 mL pH 2·5 phosphate buffer and a further 1 mL 5% methanol-pH 2·5 phosphate buffer followed with either 1·5 mL pure methanol to elute the analytes or when dapsone was present 1·5 mL 0·1 M HCl in methanol.

Chromatography

The mobile phase system was composed of 5% methanol in phosphate buffer pH 2·5 plus 40 mM tetrabutylammonium bromide (TBA) for the analysis of trimethoprim, for sulphonamides and *p*-aminobenzoic acid or dapsone and pyrimethamine analysis acetonitrile-methanol (5 : 25, v/v) in phosphate buffer pH 2·5 plus 40 mM TBA was used.

Table 1. MIC determinations for trimethoprim, sulphadiazine and sulphamethoxazole alone using 5×10^3 cells mL^{-1} of the selected organisms possessing different resistance patterns incubated at 37°C for 24 h in Iso-Sensitest broth.

Organisms	Antibacterial MICs (μg mL^{-1})		
	Trimethoprim	Sulphadiazine	Sulphamethoxazole
E. coli			
NCIB 8879	0·15	15	15
No.326	0·4	100	80
676	> 400	15	
418	300	> 3000	
314	0·45	20	
P. aeruginosa			
NCTC 8626	240	50	150
NCTC 6750	125	250	500
P. cepacia			
NCTC 10743	0·7	70	150
42	140	180	300
M. catarrhalis			
NCTC 11020	18	2·5	2·5

Table 2. The FIC values and the optimal ratios of trimethoprim to sulphadiazine or sulphamethoxazole for synergism of activity and for maximum bacterial uptakes by bacteria having different resistance patterns.

Organism	FIC (trimethoprim + sulphadiazine)	FIC (trimethoprim + sulphamethoxazole)	Optimal ratio FIC (trimethoprim + sulphadiazine)	Uptake	Optimal ratio FIC (trimethoprim + sulphamethoxazole)	Uptake
E. coli						
NCIB 8879 ST, SS	0·2	0·2	1 : 100	ND	1 : 100	1 : 100
No. 326 ST, RS	0·3	0·15	1 : 125	1 : 100–200	1 : 100	1 : 50
P. aeruginosa						
NCTC 8626 RT, RS	0·25	0·15	1 : 1	1 : 2	1 : 1	1 : 1
NCTC 6750 RT, RS	0·3	0·3	1 : 1	1 : 1	1 : 2	1 : 1
P. cepacia						
NCTC 10743 ST, RS	0·4	0·3	1 : 100	1 : 100	1 : 100	1 : 100
42RT, RS	0·3	0·3	1 : 0·7	1 : 1	1 : 1	1 : 1
M. catarrhalis						
NCTC 11020 RT, SS	0·4	0·3	1 : 0·14	1 : 0·2	1 : 0·28	1 : 4

ST = sensitive to trimethoprim; SS = sensitive to sulphonamides; RT = resistant to trimethoprim; RS = resistant to sulphonamides; ND = not done.

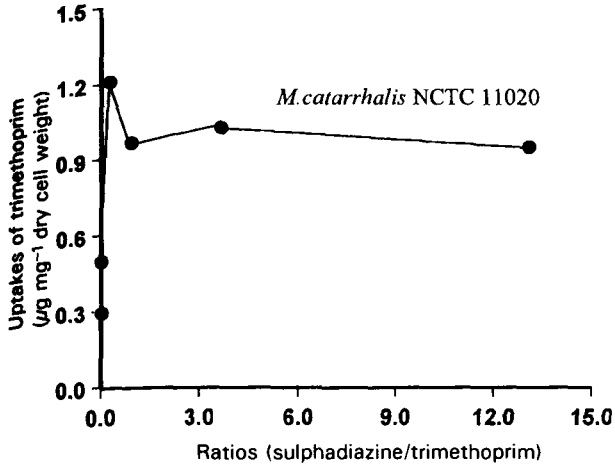

FIG. 1. Plot of bacterial uptakes of trimethoprim 10 μg mL^{-1} in combination with sulphadiazine at different ratios obtained from incubating log phase cultures of *M. catarrhalis* NCTC 11020 in the presence of the two antibacterials in Iso-Sensitest broth at 37°C for 4 h. Each uptake value is the mean of three determinations.

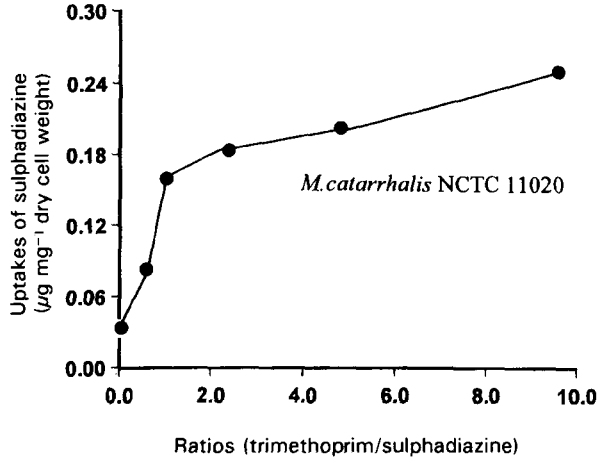

FIG. 2. Plot of bacterial uptakes of sulphadiazine 2·0 μg mL^{-1} in combination with trimethoprim at different ratios obtained from incubating log phase cultures of *M. catarrhalis* NCTC 11020 in the presence of the two antibacterials in Iso-Sensitest broth at 37°C for 4 h. Each uptake value is the mean of three determinations.

Results

P. aeruginosa, *E. coli*, *P. cepacia* and *M. catarrhalis* were selected for their markedly different patterns of resistance to sulphonamides and trimethoprim. The MIC values of trimethoprim, sulphadiazine and sulphamethoxazole against these cultures are given in Table 1. The FIC values are given in Table 2 and are mostly < 0·5 which indicates marked synergism (Richards & Xing 1991b). Mutual enhancement of bacterial uptake was shown to occur with combinations of a range of sulphonamides plus trimethoprim. When the concentration of sulphadiazine was kept constant and the concentration of trimethoprim was increased the uptake of sulphadiazine continued to increase over the concentration range tested except with *E. coli* 326 which reached a plateau of sulphadiazine uptake. However when the concentration of trimethoprim was kept constant and the sulphadiazine concentration was increased there was an increase of trimethoprim uptake to a maximum followed by a decrease in uptake of trimethoprim when the sulphadiazine concentration was increased beyond the optimum ratio. This is illustrated using *M. catarrhalis* NCTC 11020 (Figs 1, 2).

Similar results were obtained when sulphamethoxazole, sulphamerazine, sulphanilamide or dapsone, all inhibitors of dihydropteroate synthetase were used instead of sulphadiazine and when pyrimethamine was used instead of trimethoprim. The latter two are both inhibitors of dihydrofolate reductase. Table 2 compares the optimal ratios determined by FIC value and bacterial uptake of trimethoprim plus either sulphadiazine or sulphamethoxazole. The values determined by each method are seen to be very similar except for *M. catarrhalis* with sulphamethoxazole. However the optimal ratios for trimethoprim plus either sulphamerazine or sulphanilamide did correlate for *M. catarrhalis*.

Discussion

The results presented in Table 2 support and extend previous findings in this laboratory (Richards et al 1991a, 1993a, 1995a) that enhanced antibacterial activity is related to increased uptake of the antimetabolites. Table 2 provides evidence that this phenomenon holds for a range of organisms possessing markedly different resistant patterns to sulphonamides and trimethoprim. The difference between the shape of the plots in Fig. 1 and Fig. 2 supports the finding of Poe (1976) that high concentrations of sulphonamides interact with the bacterial dihydrofolate reductase enzyme and these graphs also indicate that sulphadiazine at high concentrations displaces trimethoprim from that enzyme.

It is observed from Figs 3 and 4 that the bacterial metabolite *p*-aminobenzoic acid not only inhibits the action of sulphonamides at low concentration but also exerts a marked antibacterial action of its own at higher concentrations. Then (1977) indicated that *p*-aminobenzoic acid inhibits the dihydrofolate reductase enzyme and previous findings provided some support for this (Richards & Xing 1995b). The present findings indicate the inhibition of trimethoprim activity that would be expected if trimethoprim was displaced from the dihydrofolate enzyme because *p*-aminobenzoic acid has less antibacterial activity weight for weight than trimethoprim.

When the results in Figs 1, 3 and 4 are compared it can be concluded that both sulphonamides and *p*-aminobenzoic acid influence the folate synthetic pathway at two sites. At low concentrations the interaction is with the dihydropteroate synthetase enzyme and at high concentrations the interaction is with dihydrofolate reductase. In fact, if the sulphonamides interact at both sites it is predictable that *p*-aminobenzoic acid might also act at both sites and vice-versa.

The effect of sulphonamides and trimethoprim on bacterial uptake and permeability described here explains how the antimetabolites are able to enhance the activity of other antibacterial agents in the clinical situation (Rabal et al 1973; Richards & Xing 1991b). This enhancement of other antibacterials by antifolates has been demonstrated frequently in our laboratory (Richards et al 1991a, 1994c; Richards & Xing 1992, 1993c).

The results presented here when combined with previous results (Richards & Xing 1994a; Richards et al 1991a, 1993a,

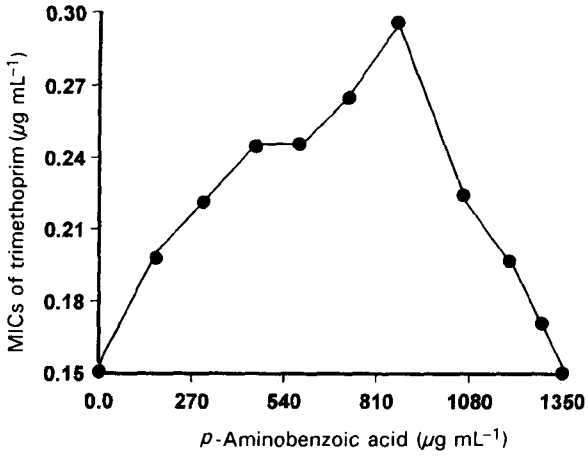

FIG. 3. The effect of *p*-aminobenzoic acid on MICs of trimethoprim against 5×10^3 cells mL^{-1} of *E. coli* NCIB 8879 incubated in Iso-Sensitest broth at 37°C for 24 h.

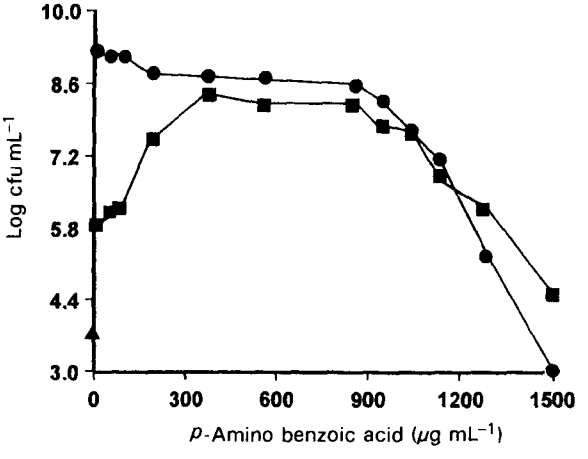

FIG. 4. Viable counts of *E. coli* NCIB 8879 cultures incubated at 37°C for 24 h in Iso-Sensitest broth containing; ▲ trimethoprim 0·15 μg mL^{-1} alone; ■ trimethoprim 0·15 μg mL^{-1} plus increasing concentrations of *p*-aminobenzoic acid; ● increasing concentrations of *p*-aminobenzoic acid alone.

b, 1995a,b) support a modified theory for the action of sulphonamides and trimethoprim and similar antifolates used singly and in combination as follows.

The mechanism of synergy between sulphonamides (inhibitors of dihydropteroate synthetase) and trimethoprim (dihydrofolate reductase inhibitors) is an initial sequential partial blockade of the folate synthetic pathway which results in abnormal protein synthesis, deficient peptidoglycan production and cytoplasmic membrane damage which in turn results in very marked mutual increases in the uptake and thus the activity of the antimetabolites.

The understanding of the mechanism of action of sulphonamides and trimethoprim alone and in combination provided by this hypothesis has value not only for the academic understanding of the synergism of sulphonamide plus trimethoprim combinations but also for understanding their rational use and potential benefits of using either or both agents in combination with other antibacterials or antibiotics to treat clinical infections known to be resistant to single agent therapy.

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