Synergy of gatifloxacin with cefoperazone and cefoperazone–sulbactam against resistant strains of *Pseudomonas aeruginosa*

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Chequerboard and time–kill methods were used to compare the *in vitro* efficacies of the combinations gatifloxacin (GAT) with cefoperazone (CFP) and GAT with cefoperazone–sulbactam (CFP–SUL) against 58 clinical isolates of *Pseudomonas aeruginosa*. The combinations GAT + CFP and GAT + CFP–SUL were shown to be synergistic for 36.2 and 58.6 % of isolates tested, respectively, using the chequerboard method. Time–kill studies with 11 strains showed synergy in 54.5 % for the GAT + CFP combination and 72.7 % for the GAT + CFP–SUL combination. The agreement between these two methods was found to be 72–81 %. There was a significant difference in synergy between the two combinations tested ($P=0.011$).

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INTRODUCTION

Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures, and increased health-care costs. Although defining the precise public-health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that the emergent antibiotic resistance is a serious global problem.

Despite improvements in antimicrobial therapy, *Pseudomonas aeruginosa* remains one of the most prominent Gram-negative bacteria causing nosocomial infections, and comprises 16 % of nosocomial pneumonia cases, 12 % of hospital-acquired urinary tract infections, 8 % of surgical wound infections and 10 % of bloodstream infections (Rossolini & Mantengoli, 2005). This organism is uniquely problematic because of a combination of inherent resistance to many drug classes and its ability to acquire resistance to all relevant treatments (Rossolini & Mantengoli, 2005). Resistance mechanisms include low outer-membrane permeability, multidrug efflux pumps (tetracycline, imipenem, fluoroquinolones and aminoglycosides) and the production of antibiotic-modifying enzymes such as metallo- β -lactamases (aminoglycosides and β -lactams) (Rossolini & Mantengoli, 2005). The development of vaccines and new antimicrobial agents has not kept pace with resistance;

therefore, the search for other methods of therapy, such as synergistic combinations, is necessary.

Combination therapy is used with the aim of expanding the antimicrobial spectrum, minimizing toxicity, preventing the emergence of resistant mutants during therapy and obtaining synergistic antimicrobial activity (Eliopoulos & Moellering, 1991). Many antimicrobial combinations have been studied for synergy *in vitro* and *in vivo* against *P. aeruginosa* (Dawis *et al.*, 2003; Fish *et al.*, 2002; Graderski *et al.*, 2001; Mayer & Nagy, 1999). In this study, we investigated the activity of gatifloxacin (GAT), alone and in combination with cefoperazone (CFP) and cefoperazone–sulbactam (CFP–SUL) (2:1), against 58 clinical isolates of *P. aeruginosa*.

METHODS

Bacterial isolates. A total of 58 non-replicate strains of *P. aeruginosa*, recently isolated from clinical specimens obtained from the Department of Microbiology, Sri Ramakrishna Hospital, Coimbatore, India, were included in the study. Overall, 62 % of the isolates were obtained from the respiratory tract, 17 % from urine and 21 % from a variety of other sources. The isolates were identified by conventional methods and checked for purity by plating on MacConkey agar. *P. aeruginosa* ATCC 27853 was included as a quality-control strain.

Antimicrobial agents. Standard laboratory powders of GAT, CFP and SUL were used in this study.

Abbreviations: CFP, cefoperazone; CFP–SUL, cefoperazone–sulbactam; FIC, fractional inhibitory concentration; GAT, gatifloxacin.

Antibiotic susceptibility testing. Antibiotic susceptibility testing was done on Mueller–Hinton (MH) agar using the Kirby–Bauer disc diffusion method with GAT, CFP and CFP-SUL. The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (CLSI, 2000). The susceptible break points applied were: $\leq 2 \mu\text{g ml}^{-1}$ for GAT, $\leq 16 \mu\text{g ml}^{-1}$ for CFP and $\leq 16 \mu\text{g ml}^{-1}$ for CFP-SUL. Isolates of intermediate sensitivity were categorized as resistant, as the number of intermediate isolates was insignificant when compared with the whole sample. Accordingly, the isolates were categorized as: susceptible (to all agents tested), resistant (to each agent alone) and miscellaneous (resistant to more than one of the agents tested).

MIC determination. The MICs of each agent alone were determined by broth macrodilution using sterile glass test tubes containing MH broth (supplemented with magnesium and calcium cations). The inoculum contained 5×10^5 c.f.u. ml^{-1} . The concentration ranges tested were: $0.125\text{--}16 \mu\text{g GAT ml}^{-1}$, $1\text{--}128 \mu\text{g CFP ml}^{-1}$ and $1\text{--}128 \mu\text{g CFP-SUL ml}^{-1}$. Antimicrobial solutions were prepared and freshly diluted on the day of testing. Each test was performed in duplicate (CLSI, 1999).

Synergy testing

Chequerboard method. Chequerboard synergy testing was performed in sterile glass test tubes using the macrodilution technique. The concentration ranges and inoculum were the same as used for MIC determination. Antimicrobial solutions were prepared and freshly diluted on the day of the test. Each test was performed in duplicate. Fractional inhibitory concentrations (FICs) were calculated as: (MIC of drug A or B in combination)/(MIC of drug A or B alone), and the FIC index was obtained by adding the FIC values. FIC values were interpreted as synergistic if values were ≤ 0.5 , additive or indifferent for values >0.5 to 4.0 and antagonistic for values >4.0 (Eliopoulos & Moellering, 1991; Visalli *et al.*, 1998).

Time–kill method. From each of the above-classified susceptibility groups, three strains (except for CFP-resistant strains, where two strains were used instead of three) were tested by a time–kill method as described by Bajaksouzian *et al.* (1996) and Visalli *et al.* (1998). Antimicrobial solutions were prepared and freshly diluted on the day of testing. All antimicrobial agents were tested alone and in combination. In each case, concentrations ranging from four times above to four times below the MIC were tested. Drug carryover was addressed as described by Bajaksouzian *et al.* (1996). Each test was performed in duplicate. Viability counts were performed in duplicate at 0, 6, 12 and 24 h by plating on MH agar. Synergy was defined as a $\geq 2 \log_{10}$ decrease in the viable count with the combination at 24 h compared with that of the more active of each of the two compounds tested alone. Indifference was defined as a $\geq 2 \log_{10}$ increase or decrease in colony count at 24 h by the combination compared with that by the most active drug alone. Antagonism was defined as a $\geq 2 \log_{10}$ increase in colony count at 24 h by the combination compared with that by the most active drug alone (Bajaksouzian *et al.*, 1996; Visalli *et al.*, 1998).

Statistical determination. Statistical significance was determined using the McNemar test.

RESULTS

In susceptibility testing, 43 % of the isolates were found to be resistant to GAT, 36 % were resistant to CFP and 25 % were resistant to CFP-SUL. The MIC₅₀/MIC₉₀ values of the

agents are presented in Table 1. The MIC ranges of GAT, CFP and CFP-SUL observed were 1–16, 8–128 and 8–64 $\mu\text{g ml}^{-1}$, respectively. The chequerboard results are listed in Table 2. Synergistic FIC indices for the GAT+CFP combination were found in 21/58 strains (36.2 %), which comprised 11/31 strains susceptible to all agents tested, 2/6 strains resistant to GAT alone, 2/2 strains resistant to CFP alone and 6/19 strains classified as miscellaneous. For the GAT+CFP-SUL combination, synergistic FIC indices were found in 34/58 strains (58.6 %), which included 17/31 strains susceptible to all agents tested, 4/6 strains resistant to GAT alone, 2/2 strains resistant to CFP alone and 11/19 strains classified as miscellaneous.

The results of the time–kill synergy tests are listed in Table 3. Time–kill studies revealed synergy in 6/11 strains (54.5 %) for the GAT+CFP combination and 8/11 (72.7 %) strains for the GAT+CFP-SUL combination. In contrast, the chequerboard titrations with these strains showed synergy for three strains (27.3 %) with the GAT+CFP combination and for six strains (54.5 %) with

Table 1. MICs of each agent alone

Group (n)	MIC ($\mu\text{g ml}^{-1}$)					
	GAT		CFP		CFP-SUL	
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Susceptible (31)*	2	2	16	16	8	16
Resistant						
GAT (6)	8	16	16	16	16	16
CFP (2)	2	2	64	64	16	16
Miscellaneous (19)†	8	8	64	128	32	32

*Susceptible to all agents tested.

†Resistant to all agents tested ($n=14$) and resistant to GAT and CFP ($n=5$).

Table 2. Results of chequerboard synergy testing

No antagonistic FIC indices >4 were found. FIC indices ≤ 0.5 were considered synergistic, whilst those $>0.5\text{--}4$ were considered additive or indifferent.

Group (n)	No. of strains with FIC indices of:			
	GAT + CFP		GAT + CFP-SUL	
	≤ 0.5	$>0.5\text{--}4$	≤ 0.5	$>0.5\text{--}4$
Susceptible (31)	11	20	17	14
Resistant				
GAT (6)	2	4	4	2
CFP (2)	2	0	2	0
Miscellaneous (19)	6	13	11	8

Table 3. Results of chequerboard and time-kill methodologies

Values in parentheses indicate the lowest concentration of each agent that yielded bactericidal activity at 24 h compared with that of the more active agent.

Strain	MIC ($\mu\text{g ml}^{-1}$)			Results for drug combination by two methods			
	GAT	CFP	CFP-SUL	GAT + CFP		GAT + CFP-SUL	
				C	T	C	T
Susceptible							
1	2	16	16	Sy	Sy (0.5/4)	Sy	Sy (0.25/4)
2	2	16	8	Ad	Sy (0.5/4)	Ad	Ad
3	2	16	16	Ad	Ad	Sy	Sy (0.25/4)
Resistant to GAT							
4	8	16	16	Ad	Sy (2/4)	Sy	Sy(1/4)
5	8	16	8	Ad	Ad	Ad	Ad
6	8	16	16	Ad	Ad	Ad	Sy(1/4)
Resistant to CEF							
7	2	64	16	Sy	Sy (0.5/16)	Sy	Sy (0.25/4)
8	2	64	32	Sy	Sy (0.5/16)	Sy	Sy (0.25/4)
Miscellaneous							
9	8	128	32	Ad	Sy (2/32)	Sy	Sy (1/8)
10	8	64	16	Ad	Ad	Ad	Sy (2/4)
11	8	128	32	Ad	Ad	Ad	Ad

Ad, Additive or indifferent; C, chequerboard titration; Sy, synergistic; T, time-kill studies.

the GAT + CFP-SUL combination. Concordance of the chequerboard and time-kill testing was demonstrated in 8/11 strains for the GAT + CFP combination and 9/11 strains for the GAT + CFP-SUL combination. Synergy between GAT and CFP-SUL was significantly ($P=0.011$) more common than that between GAT and CFP.

DISCUSSION

Multidrug-resistant *P. aeruginosa* is an important causative agent of nosocomial infections. β -Lactams, aminoglycosides and fluoroquinolones have been the mainstay in the treatment of *P. aeruginosa* infections. Combination treatments are effective choices against nosocomial infections caused by multidrug-resistant isolates (PDR, 2004).

Combinations of fluoroquinolones with other agents have been investigated extensively (Dawis *et al.*, 2003; Fish *et al.*, 2002; Gradelski *et al.*, 2001; Mayer & Nagy, 1999). Most of the studies combining fluoroquinolones with aminoglycosides have shown indifference against members of the *Enterobacteriaceae* and against *P. aeruginosa*, whereas fluoroquinolones with anti-pseudomonal penicillins have been reported to be synergistic against 20–50 % of *P. aeruginosa* isolates (Neu & Chin, 1994).

Gradelski *et al.* (2001) reported that the *in vitro* effect of GAT with CFP was synergistic against 40–60 % of *P. aeruginosa* strains by time-kill methods. Furthermore, it was reported that amikacin in combination with GAT was

synergistic against *P. aeruginosa* but the efficiency was least when compared with a GAT and β -lactam combination.

Two methods to detect *in vitro* synergy – a chequerboard and a time-kill assay – were performed for GAT + CFP and GAT + CFP-SUL combinations against 58 clinical isolates of *P. aeruginosa*. The combinations GAT + CFP and GAT + CFP-SUL were found to be synergistic for 36.2 and 58.6 % of the isolates tested, respectively, using the chequerboard method. The rate of synergy of GAT + CFP correlates with earlier reports using this combination (Gradelski *et al.*, 2001). There was a significant difference in synergy between the two combinations ($P=0.011$). Our study showed that GAT at sub-MIC concentrations of <0.25 – $0.5 \mu\text{g ml}^{-1}$ was synergistic at 24 h when combined with CFP-SUL in 4/11 strains and had comparatively lower synergy rates when combined with CFP alone.

Synergy testing methods are not standardized for reproducibility and interpretation, and therefore it is extremely difficult to compare the results of these methods from different studies. The chequerboard test measures only the inhibitory concentration, and in time-kill studies the concentration is fixed and does not decrease as it would *in vivo*. The time parameter of 24 h can limit or alter the results of an experiment if regrowth occurs with one or both antimicrobial agents (Pankey & Ashcraft, 2005). Although each of these methods uses different conditions and end points, there is frequent agreement between the results of the two methods. In our study, the agreement between these two methods was 72–81 %.

In conclusion, our study demonstrated synergy in the combinations GAT+CFP and GAT+CFP-SUL against clinical isolates of *P. aeruginosa*. Although it is interesting that we could demonstrate *in vitro* synergy against some *P. aeruginosa* isolates, the mechanism of the exhibited synergy is unknown and needs to be explored. Also, no evidence of *in vivo* synergy has been found. Clinical studies are necessary to test the validity of these *in vitro* findings, as well as the significance of regrowth after 24 h.

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