IN VITRO COMBINATION EFFECTS OF NORFLOXACIN, GENTAMICIN, AND β-LACTAMS ON β-LACTAM RESISTANT *PSEUDOMONAS AERUGINOSA*

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In an *in vitro* combination study norfloxacin was compared with gentamicin in combination with four β-lactams (piperacillin, ceftazidime, cefsulodin, and ceftriaxone) against ten strains of clinically isolated *Pseudomonas aeruginosa* by microtitre checkerboard technique. Synergy was found in 30-70% of the tested strains with gentamicin-β-lactam combinations and 0-40% with norfloxacin-β-lactam combinations.

One strain highly resistant to β-lactams was selected for studying the combined effect of three drugs (gentamicin, norfloxacin, β-lactams) in comparison with two drug combinations. Three drug combinations were found to be superior to two-drug as shown by a further reduction in a Fractional Inhibitory Concentration (FIC) index. The best result was obtained from the combination of norfloxacin, gentamicin, and piperacillin. No antagonism was found in any of the combinations tested.

INTRODUCTION

Pseudomonas aeruginosa is notoriously resistant to many antimicrobial agents. Combinations of aminoglycosides and β-lactam antibiotics have been used because of their synergistic action[1-3]. Some strains, however, have proved resistant to both compounds. With the development of many new quinolone compounds which have antipseudomonal activity[4-7], good tissue penetration, and different modes of action from aminoglycosides and β-lactams[8], their combination with aminoglycosides or β-lactams promises a better outcome[9]. With three different mechanisms of action we expected that the combination of three groups of compounds would prove superior to two.

MATERIALS AND METHODS

Antibiotics : Norfloxacin : NFLX (Kyorin) ; gentamicin : GM (Schering) ; piperacillin : PIPC (Toyama) ; ceftazidime : CAZ (Glaxo) ; cefsulodin : CFS (Takeda) ; and ceftriaxone : CTRX (Roche) were used. All drugs supplied were of known potency. Antibiotic solutions were freshly prepared as recommended by the manufacturers.

Antibiotic sensitivity test : Minimal inhibitory concentrations (MICs) were determined by the microdilution method using Mueller-Hinton Broth (MHB ; BBL) and MIC-2000 (Dynatech, U. S. A.). A volume of 0.1 ml of antibiotic solution was put into each well of an MIC-2000 plate using an electronic digital pipette. MIC endpoints were determined as the lowest antibiotic concentrations showing no visible turbidity after 24 hr incubation at 35°C using a Dynatech viewing box. For the testing two-drug combinations, 0.05 ml of each antibiotic at various concentrations was put into each well so that the final concentration of each after mixing would be one half of the original concentration. To test three-drug combinations, the same method was used, but the process was repeated with various concentrations of GM as the third drug. Concentrations of GM at one, one half, one fourth, and one eighth of the MIC were prepared. Each concentration was deposited in 36 wells. Then NFLX was combined with a β-lactam at a concentration of two MICs to one-sixteenth MIC in the presence and absence of GM as a control plate of two-drug combination. Each well had 0.025 ml of GM, 0.025 ml of NFLX,

and 0.025 ml of β-lactam, and 0.025 ml or 0.05 ml of MHB was added to each to make a final volume of 0.1 ml of three or two drugs (without GM) so that the final concentration of each drug after mixing would be one fourth of the original.

Bacteria : Ten strains of *Pseudomonas aeruginosa* isolated from sputum of ten patients were used. All strains were identified by the API 20 NE system (MONTALIEU-VERCIEU, France) and inoculated onto semisolid nutrient agar (containing peptone 5 g/l, beef extract 3 g/l, NaCl 5 g/l and agar 0.7 g/l) at room temperature. For testing, stock strains were subcultured overnight in 4 ml of MHB at 35°C. A bacterial count of 5×10^8-1×10^9 CFU/ml was obtained for all strains tested. These were then diluted with 36 ml of 0.9% normal saline in a flask and poured on to a sterile plate. A volume of 0.001 ml from the plate containing about 1×10^5 CFU, was then inoculated into a well which had 0.1 ml of antibiotic solution using an MIC-2000 inoculator. The final inoculum size of the bacteria in each well was shown to be between 5×10^5-1×10^6 CFU/ml.

Antibiotic synergism : Using the microtitre checkerboard method, synergy was considered present when the combination of antibiotics resulted in at least a four-fold reduction in the MIC of each agent. The FIC was also calculated for each antibiotic combination and synergy was considered present when the FIC was ≦0.5. Additive activity was considered present when the FIC was >0.5 and ≦1. Indifference was found when neither drug exhibited a decrease in the MIC, and an increase in the MIC for either drug was regarded as antagonism. FIC was the sum of the fraction of MIC of each antibiotic in any one combination. The lowest numerical value obtained was chosen to compare the efficacy of different combinations.

RESULTS

Table 1 shows MICs of the ten strains tested. All strains were sensitive to GM, NFLX, CAZ and CFS with MICs ranging from 0.2-6.25 μg/ml. Resistance to PIPC and CTRX was found in strains No. 3, 8, 32, 37 and 3, 8, 37, TU 1, respectively, with MICs ranging from 25-800 μg/ml.

Table 2 summarizes the results of two-drug combinations. The combinations of GM and β-lactams were shown to be more synergistic than the NFLX+β-lactams. Combinations of GM with β-lactams demonstrated synergy in 30-70% of the strains, while NFLX with β-lactams demonstrated synergy in 0-40%.

Table 3 compares the minimal FIC index of two-drug combinations (GM+NFLX ; GM+β-lactams ; and NFLX+β-lactams) with three-drug combinations (GM+NFLX+β-lactams). Except for the combination of GM+NFLX+CFS, the FIC index of the three-drug combinations was less than that of the two-drug combinations. The GM+NFLX+PIPC combination was most effective, as

Table 1 MICs of the strains of *Pseudomonas aeruginosa*

Strain No.	GM	NFLX	PIPC	CTRX	CAZ	CFS
3	0.78	0.39	100	800	6.25	6.25
8	1.56	0.39	25	100	3.13	6.25
16	0.78	0.39	0.78	3.13	0.39	1.56
20	0.78	0.2	1.56	12.5	0.78	1.56
24	0.78	1.56	12.5	3.13	0.78	0.78
32	0.39	1.56	400	6.25	0.78	0.78
37	0.39	0.05	200	50	0.78	0.78
41	1.56	0.2	3.13	6.25	1.56	1.56
TU 1	6.25	1.56	3.13	25	0.78	1.56
AT 2	0.39	0.39	6.25	12.5	0.78	1.56

Table 2 Percentage of strains showing synergy for each combination against ten strains of *Pseudomonas aeruginosa*

	CTRX	PIPC	CAZ	CFS
GM	70%	60%	30%	60%
NFLX	30%	40%	20%	0%

Table 3 Minimal FIC index for two and three-drug combinations on *Pseudomonas aeruginosa* strain No. 3

	NFLX	PIPC	CTRX	CAZ	CFS
GM+	0.75	0.38	0.50	0.50	0.38
NFLX+	—	0.50	0.75	1.00	0.75
GM+NFLX+	—	0.25	0.31	0.41	0.53

Table 4 Minimal FIC index for three-drug combinations at various concentrations of GM on *Pseudomonas aeruginosa* strain No. 3

	with GM at concentration of				
	0	1/4 MIC	1/8 MIC	1/16 MIC	1/32 MIC
NFLX+PIPC	0.50	0.34	0.25	0.38	0.41
NFLX+CTRX	0.75	0.41	0.41	0.34	0.31
NFLX+CAZ	1.00	0.53	0.41	0.69	0.66
NFLX+CFS	0.75	0.53	0.66	0.63	0.66

Fig. 1 The effect of gentamicin at a concentration of 0.1 μg/ml (1/8 of MIC) on NFLX +PIPC combination against *Pseudomonas aeruginosa*

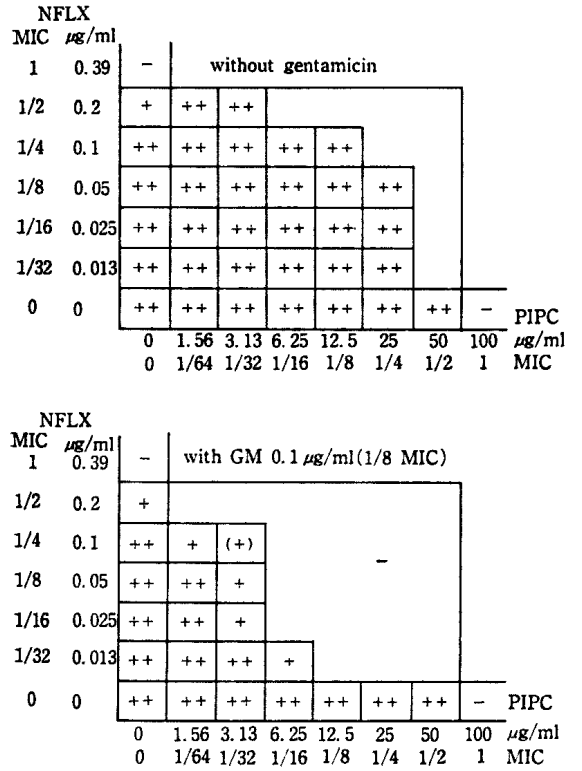
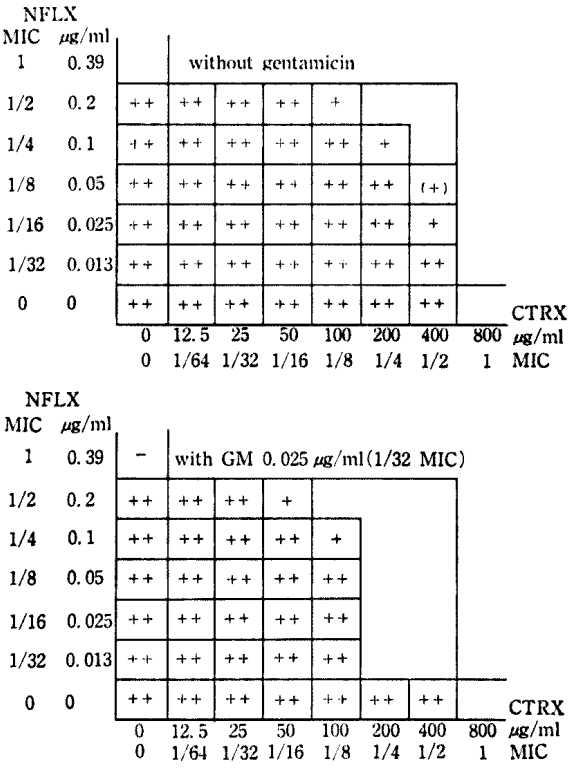


Fig. 2 The effect of gentamicin at a concentration of 0.025 μg/ml (1/32 of MIC) on NFLX+CTRX combination against *Pseudomonas aeruginosa*



shown by the lowest FIC (0.25).

Table 4 shows the effect of GM at different concentrations on NFLX and β-lactam combinations. GM at a concentration of 1/8 MIC was most effective with NFLX+PIPC and NFLX+CAZ, while 1/4 MIC and 1/32 MIC was most

Fig. 3 The effect of gentamicin at a concentration of 0.1 μg/ml (1/8 of MIC) on NFLX+CAZ combination against *Pseudomonas aeruginosa*

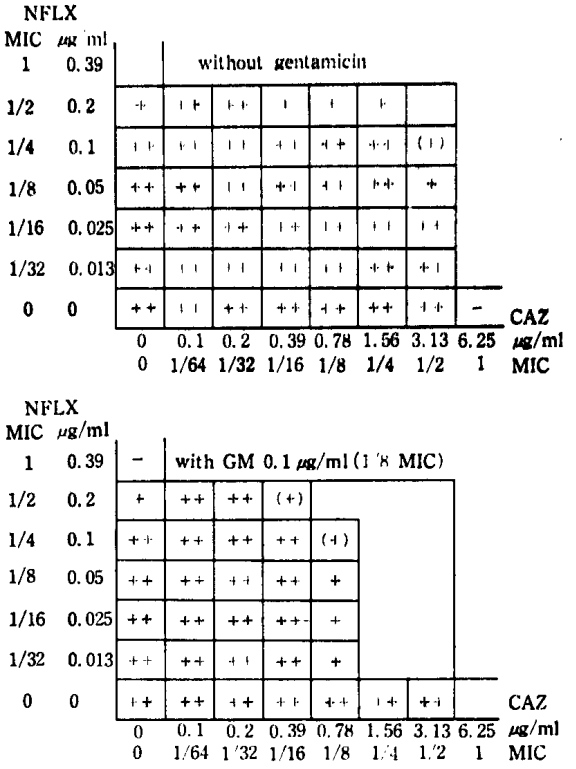
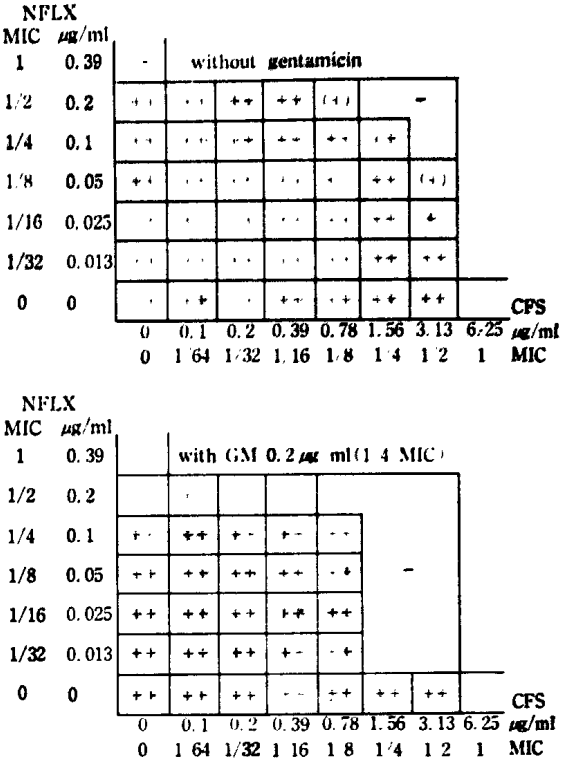


Fig. 4 The effect of gentamicin at a concentration of 0.2 μg/ml (1/4 of MIC) on NFLX+CFS combination against *Pseudomonas aeruginosa*



effective with NFLX+CFS and NFLX+CTRX respectively.

Fig. 1 shows the effect of GM at a concentration of 1/8 MIC on the NFLX+PIPC combination : the MICs of NFLX and PIPC became 1/16 of MIC.

Fig. 2 shows the effect of GM at a concentration of 1/32 MIC on the NFLX+CTRX combination : the MICs of NFLX and CTRX became 1/32 and 1/4 MIC respectively.

Fig. 3 shows the effect of GM at a concentration of 1/8 MIC on the NFLX+CAZ combination : the MICs of NFLX and CAZ became 1/32 and 1/4 MIC respectively.

Fig. 4 shows the effect of GM at a concentration of 1/4 MIC on the NFLX+CFS combination : the MICs of NFLX and CFS became 1/32 and 1/4 MIC respectively.

DISCUSSION

In this study all strains were sensitive to both GM and NFLX, thus providing a reasonable comparison between the two in a combination study with β-lactams. As the percentage of synergy against ten strains was higher for combinations of GM with β-lactams than of NFLX with β-lactams, we concluded that GM was superior to NFLX in this respect. Nevertheless, NFLX is still useful in combination with β-lactams because at least an additive effect was shown and no antagonism found. Synergism was shown against 70%, 60%, 60% and 30% strains for GM+CTRX, GM+PIPC, GM+CFS and GM+CAZ respectively. HALLANDER, et al. have obtained a similar result for GM+CAZ[10]. An other study found synergism against 70% and 30% of strains for amikacin+CTRX and amikacin+CAZ[11]. Synergism has also been shown against 60% of strains for amikacin+PIPC[12]. Our results therefore agree with previous reports[13]. Because of the clinical importance of *Pseudomonas aeruginosa* resistance, especially to β-lactams, we extended our study to three-drug combinations with the expectation of increasing the therapeutic effect. Strain No. 3 was selected,

as it showed remarkable resistance to many β-lactams with high constancy of MICs for the tested drugs throughout the experiment. The best result was, as shown by an FIC of 0.25, a combination of NFLX+GM+PIPC. With the exception of NFLX+GM+CFS, the FICs of three-drug combinations were lower than those of two-drug combinations (Table 3). One reason for this was that NFLX showed synergism with PIPC but not with CFS. If only strains resistant to PIPC were examined (strains No. 3, 8, 32, 37) synergy was found in 75% instead of 40% of the strains for the combination of NFLX+PIPC. We therefore conclude that NFLX should be useful in combination with PIPC and also in three-drug combinations. An other study on three-drug combinations has suggested the combination of tobramycin+β-lactams+fosfomycin against resistant *Pseudomonas aeruginosa*[14]. Although it is not yet known whether the combination of aminoglycoside+β-lactam and a new quinolone will be superior to the combination of aminoglycosides and β-lactams in clinical practice, the results of this study should at least encourage physicians to try these three groups of compounds in combination against clinically resistant *Pseudomonas aeruginosa*.

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References

1) BLASER, J.; B. B. STONE, M. C. GRONER & S. H. ZINNER : Impact of netilmicin regimens on the activity of ceftazidime-netilmicin combinations against *Pseudomonas aeruginosa* in an *in vitro* pharmacokinetic model. Antimicrobial Agents and Chemotherapy 28 : 64～68, 1985
2) LOVE, L. J.; S. C. SCHIMOFF, C. A. SCHIFFER & P. H. WIERNIK : Improved prognosis for granulocytopenic patient with gram-negative bacteria. American Journal of Medicine 68 : 643～648, 1980
3) YOUNG, L. S.: Review of clinical significance of synergy in gram-negative infections at the University of California Los Angeles Hospital. Infection 6 (Suppl. 1) : 47～52, 1978
4) BAUERNFEIND, A. & C. PETERMULER : *In vitro* activity of ciprofloxacin, norfloxacin and nalidixic acid. European Journal of Clinical Microbiology 2 : 111～115, 1983
5) BODY, B. A.; R. A. FROMTLING, S. SHADOMY & H. J. HADOMY : *In vitro* antibacterial activity of norfloxacin compared with eight other antimicrobial agents. European Journal of Clinical Microbiology 2 : 230～234, 1983
6) ITO, A.; K. HIRAI, M. INOUE, H. KOGA S. SUZUE, T. IRIKURA & S. MITSUHASHI : *In vitro* antibacterial activity of AM-715, a new nalidixic acid analog. Antimicrobial agents and Chemotherapy 17 : 103～108, 1980
7) NORRBY, S. R.; M. JONSSON : Antibacterial activity of norfloxacin. Antimicrobial agents and Chemotherapy 23 : 15～18, 1983
8) CRUMPULIN, G. C.; M. KENWRIGHT & T. HIRST : Investigations into the mechanism of action of the antibacterial agent norfloxacin. Journal of Antimicrobial Chemotherapy 13 : 9～23, 1984
9) OGAWA, M.; S. MIYAZAKI & S. GOTO : Influence of dose schedule on efficacies of antibiotic combinations in *Pseudomonas aeruginosa* infection in mice. Chemotherapy 34 : 232～239, 1986
10) HALLANDER, H. O.; K. DORNBUSCH, L. GEZELIUS, K. JACOBSON & I. KARLSSON : Synergism between aminoglycosides and cephalosporins with anti-pseudomonal activity : interaction index and killing-curve method. Antimicrobial Agents and Chemotherapy 22 : 734～752, 1982
11) GOMBERT, M. E. & T. M. AULICINO : Amikacin synergism with beta-lactam antibiotics against selected nosocomial pathogens. Journal of Antimicrobial Chemotherapy 17 : 323～326, 1986
12) KURTS, T. O.; D. J. WINSTON, D. A. BRUCKNER & W. J. MARTIN : Comparative *in vitro* synergistic activity of new beta-lactam antimicrobial agents and amikacin against *Pseudomonas aeruginosa* and *Serratia marcescens*. Antimicrobial agents and Chemotherapy 20 : 239～243, 1981
13) AONUMA, S.; K. ONUMA, K. WATANABE, M. SASAKI, K. OIZUMI & K. KONNO : Studies on combination of antibiotics (I), *In vitro* combined effect of piperacillin, ticarcillin and dibekacin against clinically isolated *Pseudomonas aeruginosa*. Chemotherapy 30: 149～153, 1982
14) TAKAHASHI, K. & H. KANNO : Synergistic activities of combinations of beta-lactams, fosfomycin, and tobramycin against *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy 26 : 789～791, 1984

In vitro におけるノルフロキサシンと β-ラクタム剤およびアミノグリコシド剤の2剤，3剤併用による臨床分離緑膿菌に対する効果

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NFLX と β-ラクタム剤（PIPC, CAZ, CFS, CTRX）の2剤，また GM と β-ラクタム剤（PIPC, CAZ, CFS, CTRX）の2剤の *in vitro* における併用効果を臨床分離の緑膿菌 10 株に対して液体培地希釈法により検討した。相乗効果は GM+β-ラクタム剤で 30～70%，NFLX と β-ラクタム剤では 0～40% に認められた。

次に NFLX+GM+β-ラクタム剤（PIPC, CAZ, CFS, CTRX）3剤による併用効果を調べ，2剤併用時の効果と比較した。3剤併用の効果は2剤併用と比べ FIC (Fractional Inhibitory Concentration) において優れていた。最も優れていたのは，NFLX+GM+PIPC の組み合わせであった。

本実験の中で拮抗作用を示した組み合わせは認められなかった。