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

YONGYUTH JITTAROPAS1), NAOTO RIKITOMI2), and KEIZO MATSUMOTO3)

1) Department of Internal Medicine, Rajavithi Hospital, Bangkok 10400, Thailand,
2) Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, 12-4 Sakamoto machi, Nagasaki 852, Japan.

(Received January 20, 1987)

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 action1-8). Some strains, however, have proved resistant to both compounds. With the development of many new quinolone compounds which have antipseudomonal activity9-70), good tissue penetration, and different modes of action from aminoglycosides and β-lactams9), their combination with aminoglycosides or β-lactams promises a better outcome9). 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 (Tyoama); 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 5×10⁶-1×10⁸ 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 1×10⁵ 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 5×10⁵-1×10⁶ 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>
<thead>
<tr>
<th>Strain No.</th>
<th>GM</th>
<th>NFLX</th>
<th>PIPC</th>
<th>CTRX</th>
<th>CAZ</th>
<th>CFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.78</td>
<td>0.39</td>
<td>100</td>
<td>800</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>8</td>
<td>1.56</td>
<td>0.39</td>
<td>25</td>
<td>100</td>
<td>3.13</td>
<td>6.25</td>
</tr>
<tr>
<td>16</td>
<td>0.78</td>
<td>0.39</td>
<td>0.78</td>
<td>3.13</td>
<td>0.39</td>
<td>1.56</td>
</tr>
<tr>
<td>20</td>
<td>0.78</td>
<td>0.2</td>
<td>1.56</td>
<td>12.5</td>
<td>0.78</td>
<td>1.56</td>
</tr>
<tr>
<td>24</td>
<td>0.78</td>
<td>1.56</td>
<td>12.5</td>
<td>3.13</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>32</td>
<td>0.39</td>
<td>1.56</td>
<td>400</td>
<td>6.25</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>37</td>
<td>0.39</td>
<td>0.05</td>
<td>200</td>
<td>50</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>41</td>
<td>1.56</td>
<td>0.2</td>
<td>3.13</td>
<td>6.25</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>TU 1</td>
<td>6.25</td>
<td>1.56</td>
<td>3.13</td>
<td>25</td>
<td>0.78</td>
<td>1.56</td>
</tr>
<tr>
<td>AT 2</td>
<td>0.39</td>
<td>0.39</td>
<td>6.25</td>
<td>12.5</td>
<td>0.78</td>
<td>1.56</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>CTRX</th>
<th>PIPC</th>
<th>CAZ</th>
<th>CFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM</td>
<td>70%</td>
<td>60%</td>
<td>30%</td>
<td>60%</td>
</tr>
<tr>
<td>NFLX</td>
<td>30%</td>
<td>40%</td>
<td>20%</td>
<td>0%</td>
</tr>
</tbody>
</table>
Table 3 Minimal FIC index for two and three-drug combinations on Pseudomonas aeruginosa strain No. 3

<table>
<thead>
<tr>
<th></th>
<th>NFLX</th>
<th>PIPC</th>
<th>CTRX</th>
<th>CAZ</th>
<th>CFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM+</td>
<td>0.75</td>
<td>0.38</td>
<td>0.50</td>
<td>0.50</td>
<td>0.38</td>
</tr>
<tr>
<td>NFLX+</td>
<td>—</td>
<td>0.50</td>
<td>0.75</td>
<td>1.00</td>
<td>0.75</td>
</tr>
<tr>
<td>GM+NFLX+</td>
<td>—</td>
<td>0.25</td>
<td>0.31</td>
<td>0.41</td>
<td>0.53</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>1/32 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFLX+PIPC</td>
<td>0.50</td>
<td>0.34</td>
<td>0.25</td>
<td>0.38</td>
<td>0.41</td>
</tr>
<tr>
<td>NFLX+CTRX</td>
<td>0.75</td>
<td>0.41</td>
<td>0.41</td>
<td>0.34</td>
<td>0.31</td>
</tr>
<tr>
<td>NFLX+CAZ</td>
<td>1.00</td>
<td>0.53</td>
<td>0.41</td>
<td>0.69</td>
<td>0.66</td>
</tr>
<tr>
<td>NFLX+CFS</td>
<td>0.75</td>
<td>0.53</td>
<td>0.66</td>
<td>0.63</td>
<td>0.66</td>
</tr>
</tbody>
</table>

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 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 effective with NFLX+CTRX and NFLX+CFS combinations.
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. An other study found synergism against 70% and 30% of strains for amikacin+CTRX and amikacin+CAZ. Synergism has also been shown against 60% of strains for amikacin+PIPC. Our results therefore agree with previous reports. 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-lac-
tams 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 combi-
nations were lower than those of two-drug combi-
nations (Table 3). One reason for this was that
NFLX showed synergism with PIPC but not with
CFS. If only strains resistant to PIPC were ex-
amined (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 combina-
tion with PIPC and also in three-drug combina-
tions. An other study on three-drug combinations
has suggested the combination of tobramycin+
β-lactams+fosfomycin against resistant Pseudo-
monas aeruginosa14). 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.

Acknowledgements
We are grateful to Mr. K. Watanabe for his
technical assistance in this study.

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In vitroにおいて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桿菌に対する効果

ジタロッパ コンエト1)・力富 直人2)・松本 達哉3)
1) ラチャピチャ病院（バンコク）、2) 長崎大学熱帯医学研究所内科

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桿菌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。