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Comprehensive Evaluation of Ciprofloxacin-Aminoglycoside Combinations against *Enterobacteriaceae* and *Pseudomonas aeruginosa* Strains

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The in vitro activities of antibiotic combinations containing ciprofloxacin and either gentamicin, sisomicin, netilmicin, amikacin, or tobramycin were evaluated by checkerboard assay (agar dilution method). A total of 220 strains of *Enterobacteriaceae* and *Pseudomonas aeruginosa* (11 species, 20 strains each) were tested. Synergistic or antagonistic effects were observed in less than 1% of the tests performed; they appeared to represent method-dependent fluctuations rather than true antibiotic interactions. No significant differences among the five aminoglycosides tested were seen. Time-kill experiments performed with three representative strains of *Escherichia coli* and *Serratia marcescens* showed additive combination effects with respect to the kill rates and inhibition of bacterial regrowth. Exposure of *Serratia* strains to either ciprofloxacin or gentamicin before the addition of the second drug had little influence on the combination effects observed. No antagonistic drug interactions were seen in vivo when combination therapy with ciprofloxacin and gentamicin was evaluated in a model of *E. coli* thigh muscle infection in neutropenic mice. Comparable therapeutic effects were obtained, regardless of whether the two compounds were administered simultaneously or sequentially at 1- or 2-h intervals.

Ciprofloxacin is highly active against a broad spectrum of pathogenic bacteria (9, 10). However, it may be necessary to use ciprofloxacin in combination with other antibacterial drugs for certain clinical indications. As a prerequisite of combination therapy, extensive in vitro studies are needed to detect potential antibiotic interactions which could impair the therapeutic efficacy in vivo.

Previous reports have indicated that ciprofloxacin-aminoglycoside combinations mostly exhibit additive or indifferent combination effects in vitro against *Enterobacteriaceae* and *Pseudomonas aeruginosa* strains (5, 7; J. A. Moody, L. R. Peterson, and D. N. Gerding, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 393, 1984; R. Wise and J. M. Andrews, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 655, 1983). No antagonistic interactions between ciprofloxacin and aminoglycosides have been seen so far. Combinations of ciprofloxacin and gentamicin or tobramycin appeared to be equivalent or slightly superior to ciprofloxacin alone in protecting neutropenic mice from *P. aeruginosa* (K. Jules and H. C. Neu, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 27, 1984; H. G. Robson and M. Cote, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 25, 1984).

In all of these studies, only a few selected drug-bacteria associations were evaluated. The intention of this study, therefore, was to include significant numbers of bacterial isolates from a broad spectrum of pathogenic species and to compare various commonly used aminoglycoside antibiotics under identical test conditions. Another important question was whether sequential application of the drugs would influence the efficacy of ciprofloxacin-aminoglycoside combinations. A model of *Escherichia coli* thigh muscle infection in neutropenic mice was chosen to confirm the in vitro results in vivo as well.

(A preliminary report of this work has been presented [I. Haller, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A20, p. 4].)

MATERIALS AND METHODS

Antibiotics. The following antibiotic powders were used to prepare drug solutions: ciprofloxacin hydrochloride and sisomicin sulfate (Bayer AG, Leverkusen, Federal Republic of Germany), amikacin sulfate, gentamicin sulfate, netilmicin sulfate, and tobramycin sulfate (Sigma Chemical Co., St. Louis, Mo.).

Bacteria. All test strains were clinical isolates which were stored at −80°C until use. Ten strains of each species were recovered during a collaborative study of the Paul-Ehrlich-Gesellschaft für Chemotherapie. The other half of the test strains was taken from our culture collection. A considerable number of these isolates were resistant to gentamicin, cefamandole, and acylureido penicillins. Susceptibility or resistance to ciprofloxacin was no criterion for selection of the test strains.

Checkerboard assay. The checkerboard assays were performed by the agar dilution method with Iso-Sensitest agar. (Oxoid Ltd., London, England). To prepare the combination plates, two 1-ml drops of the antibiotic solutions were placed on opposite sides of the petri dishes and were thoroughly mixed while adding 18 ml of melted agar. The dried agar plates were inoculated with diluted overnight cultures of the test strains by using a multipoint inoculator (Denly). The inocula were standardized turbidimetrically so that each inoculation plot contained ca. 10^4 bacterial cells. Growth was read after 20 h of incubation at 37°C.

For evaluation of the combination effects, the fractional inhibitory concentration (FIC) (FIC = MIC in the combination/MIC alone) for each component and the sum of the FICs (Σ FIC) of each checkerboard combination were calculated (2, 3). Due to the various antibiotic concentration ratios in the inhibitory combinations, a minimal sum of FICs (Σ FIC_{min}) and a maximal sum of FICs (Σ FIC_{max}) resulted

TABLE 1. Summary of the results of the checkerboard assays

Ciprofloxacin combined with:	No. of test strains against which the antibiotic combination was:				
	Synergistic ($\Sigma FIC_{min} \leq 0.5$)	Additive indifferent			Antagonistic ($\Sigma FIC_{max} \geq 4.0$)
		Two or more ΣFICs >0.5–0.625	Intermediate distribution of ΣFICs	Two or more ΣFICs 2.0–< 4.0	
Gentamicin	1	33	172	14	0
Sisomicin	1	31	177	11	0
Netilmicin	2	33	174	11	0
Amikacin	0	33	177	10	0
Tobramycin	1	32	178	8	1

for each isolate in every checkerboard assay (8). Synergism was assumed when the MIC of each antibiotic in one or more combinations was one-fourth or less of its MIC alone ($\Sigma FIC_{min} \leq 0.5$). Antagonism was assumed when the MIC of either antibiotic was increased fourfold or more over its MIC alone or when the MICs of both antibiotics were increased twofold or more over their respective MICs alone ($\Sigma FIC_{max} \geq 4.0$). All other results were considered additive indifferent.

The MICs of the single antibiotics were determined in duplicate in each experiment. When the two MICs differed by one dilution, the arithmetic means of the two resulting FIC indices were used for evaluation. Experiments with greater discrepancies were repeated.

Time-kill experiments. Conical 100-ml flasks containing 20 ml of test broth (Iso-Sensitest broth; Oxoid), with or without antibiotic, were inoculated with 2×10^7 bacteria of an overnight culture and incubated on a rotary shaker at 37°C. For delayed addition of ciprofloxacin or gentamicin, the required amount of drug was added dissolved in 1 ml of broth. At the intervals indicated, 1-ml samples of the cultures were taken for the determination of viable cell counts by plating on agar. Control experiments were performed initially to exclude the possibility that drug carryover would affect the test results.

Thigh muscle infection model. Male CF1 mice (weight, 23 to 26 g) were rendered neutropenic by two intraperitoneal doses of cyclophosphamide (150 mg/kg 5 days before infection and 100 mg/kg the day before infection). For challenges, 0.05 ml of a diluted overnight culture of *E. coli* (2×10^6 bacteria per ml) was injected into the posterior thigh muscles of the right hind leg. The animals to be treated received 0.1 ml of antibiotic solution subcutaneously at 0.5 and 6.5 h after infection. For combination therapy, the ciprofloxacin and gentamicin solutions were injected separately at various sites on the neck. The infected muscles were removed completely 24 h after infection. Specimens from each group (five animals) were pooled, cut into small pieces, and homogenized in a Potter homogenizer (Braun Melsungen). CFUs were measured by plating diluted samples on agar. Each value shown represents the geometric mean obtained from 4 to 12 groups of mice.

RESULTS

Checkerboard assays. Isolates of the following bacterial species (20 strains each) were tested by checkerboard assay: *E. coli*, *Citrobacter freundii*, *Klebsiella* sp., *Enterobacter cloacae*, *Serratia marcescens*, *Proteus mirabilis*, *Proteus vulgaris*, *Proteus rettgeri*, *Morganella morganii*, *Providencia* sp., *Pseudomonas aeruginosa*. Most of the test strains were susceptible to ciprofloxacin with MICs of 0.25 μg/ml or below. Higher MICs (up to 4 μg/ml) were obtained with a few strains of *S. marcescens*, *P. rettgeri*, and *Providencia*.

The results of the checkerboard assay are summarized in Table 1. Σ FICs indicative of synergistic or antagonistic effects were seen in less than 1% of the tests performed. The Σ FIC_{min} indicative of synergy ranged from 0.375 to 0.5. The single Σ FIC_{max} indicative of antagonism was 4.06. All of these interaction indices were not reproducible when the checkerboard assays were repeated. Thus, they appeared to represent method-dependent fluctuations rather than true antibiotic interactions.

The category of additive-indifferent effects was subdivided into three groups to determine whether there was a predominance of either low or high Σ FICs, which might suggest a tendency towards synergism or antagonism. For this, not only the minimal and maximal Σ FICs but the distribution of all Σ FICs was assessed. Those tests were separated from the intermediate group in which at least two Σ FICs were in the range of >0.5 to 0.625 or 2.0 to < 4.0 No significant predominance of tests with either low or high Σ FICs was revealed by this analysis (Table 1).

Time-kill experiments. The dynamic aspect of the bactericidal activity of ciprofloxacin-gentamicin combinations was assessed by time-kill experiments. The following isolates were chosen as test organisms: *E. coli* 4672 (this strain was also used as the infective organism in the thigh muscle infection experiments) and *S. marcescens* 470 (gentamicin susceptible) and *S. marcescens* 141 (gentamicin resistant) (these strains were selected to compare two isolates of the same species with different susceptibilities to ciprofloxacin). Indifferent checkerboard results had been obtained with all three test strains.

Since the checkboard assays had revealed no differences among the five aminoglycosides tested, only ciprofloxacin and gentamicin were evaluated by time-kill experiments. First assays showed that the rapid killing effects of high concentrations of ciprofloxacin or gentamicin would not be increased by using the compounds in combination. To detect potential synergistic drug interactions, lower concentrations of each compound were tested, which exhibited a moderate initial bactericidal effect when tested alone but did not prevent bacterial regrowth.

Figure 1 shows the kill curves as measured with *S. marcescens* 470 (panel A) and *S. marcescens* 141 (panel B). In both cases, the ciprofloxacin-gentamicin combination led to a marked increase in the kill rates and delayed bacterial regrowth between 10 and 24 h of incubation. These findings are not necessarily indicative of synergistic effects, since a similar enhancement of the bactericidal activity also could be achieved by two- or fourfold concentrations of either ciprofloxacin or gentamicin alone. Against *S. marcescens* 141, for instance, the combination of ciprofloxacin (4 μg/ml)

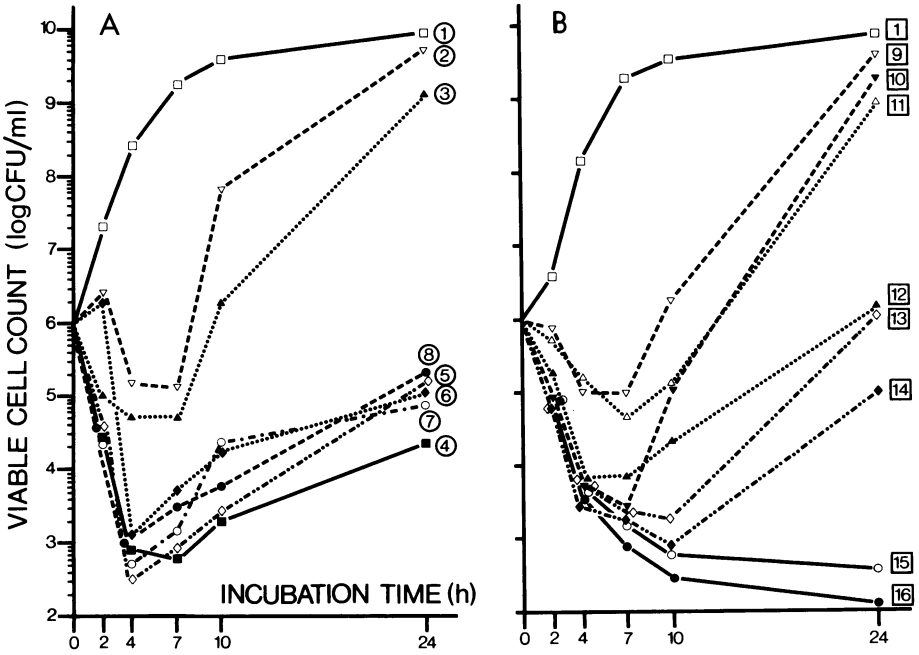

FIG. 1. Results of the time-kill experiments performed with gentamicin (GM)-susceptible *S. marcescens* 470 (A) and GM-resistant *S. marcescens* 141 (B). Curves: 1, growth controls (the antibiotics were added at $t = 0$ h if not indicated otherwise; concentrations of 0.031 μg/ml for ciprofloxacin [CIP] and 0.25 μg/ml for GM were tested with the first strain); 2, GM alone; 3, CIP alone; 4, GM and CIP simultaneously; 5, CIP 1 h after GM; 6, CIP 2 h after GM; 7, GM 1 h after CIP; 8, GM 2 h after CIP. The following antibiotic concentrations were tested with the GM-resistant strain: lane 9, 16 μg/ml (GM); lane 10, 32 μg/ml (GM); lane 11, 4 μg/ml (CIP); lane 12, 8 μg/ml (CIP); lane 13, 4 μg/ml (CIP) + 16 μg/ml (GM); lane 14, 4 μg/ml (CIP) + 32 μg/ml (GM); lane 15, 8 μg/ml (CIP) + 16 μg/ml (GM); lane 16, 8 μg/ml (CIP) + 32 μg/ml (GM). The MICs as determined by the agar dilution method were 0.031 μg/ml (CIP) and 0.5 μg/ml (GM) for *S. marcescens* 470 and 4 μg/ml (CIP) and 8 to 16 μg/ml (GM) for *S. marcescens* 141.

and gentamicin (16 μg/ml) (Fig. 1, curve 13) resulted in effects similar to those of an 8-μg/ml concentration of ciprofloxacin alone (Fig. 1, curve 12). Similar results suggesting additive combination effects between ciprofloxacin and gentamicin were also obtained with *E. coli* 4672 (data not shown).

To simulate the clinical situation, the bactericidal effects of ciprofloxacin-gentamicin combinations were also evaluated when the drugs were applied sequentially at various time intervals. As can be seen from Fig. 1A, sequential application at intervals up to 2 h had little influence on the killing and regrowth of *S. marcescens* 470, regardless of the sequence of application.

TABLE 2. Efficacy of mono- and combination therapy with ciprofloxacin (CIP) and gentamicin (GM) in the model of thigh muscle infection in neutropenic mice

Drug (dose)	Viable cell counts (log CFU ± SEM) after mono- or simultaneous combination therapy with[a]:			
	GM (none)	GM (0.1 mg/kg)	GM (1 mg/kg)	GM (10 mg/kg)
CIP (none)	8.1 ± 0.2	8.0 ± 0.2	5.6 ± 1.3	4.2 ± 0.3
CIP (0.01 mg/kg)	7.9 ± 0.1	8.0 ± 0.5	5.1 ± 0.7	4.4 ± 0.6
CIP (0.1 mg/kg)	5.4 ± 1.2	6.1 ± 1.1	4.2 ± 0.4	3.8 ± 0.4
CIP (1.0 mg/kg)	3.5 ± 0.2	3.5 ± 0.3	3.6 ± 0.4	3.4 ± 0.2

[a] Viable cell counts of *E. coli* 4672 as determined 24 h after infection. Each value represents the geometric mean obtained from 4 to 12 groups of animals.

In vivo experiments. The *E. coli* thigh muscle infection in neutropenic mice was used to assess the therapeutic efficacy of combination therapy with ciprofloxacin and gentamicin. The results obtained in this model are summaried in Table 2. The initial inoculum at the site of infection was about 10^5 cells of *E. coli*. The number of viable cells recovered from the animals treated with ciprofloxacin (0.01 mg/kg) or gentamicin (0.1 mg/kg) were not different from untreated controls. The viable cell counts of animals treated with ciprofloxacin (0.1 mg/kg) or gentamicin (1 mg/kg) were similar to the initial challenge inoculum. The muscles of animals treated with the highest doses of ciprofloxacin or gentamicin contained significantly fewer viable bacteria than did the initial challenge inoculum.

Combination therapy with the low doses of ciprofloxacin and gentamicin did not lead to a therapeutic effect. On the other hand, the efficacy of high doses of ciprofloxacin could not be increased by using the drugs in combination. Slight combination effects were only seen in the intermediate range of antibiotic doses, as an additive effect was achieved when ciprofloxacin (0.1 mg/kg) and gentamicin (1 mg/kg) were combined.

The intermediate doses of ciprofloxacin and gentamicin also were administered sequentially at intervals of 1 and 2 h. For example, in one test gentamicin was administered 1 h after ciprofloxacin, i.e., ciprofloxacin was given 0.5 and 6.5 h after infection while gentamicin was given 1.5 h and 7.5 h after infection. The mean viable cell counts (log CFU ± standard error of the mean) of *E. coli* for each sequential combination therapy test were as follows: for gentamicin (1 mg/kg) administered 1 h after ciprofloxacin (0.1 mg/kg), 4.2 ± 0.8; for gentamicin (1 mg/kg) administered 2 h after

ciprofloxacin (0.1 mg/kg), 4.3 ± 0.3; for ciprofloxacin (0.1 mg/kg) administered 1 h after gentamicin (1 mg/kg), 4.3 ± 1.4; and for ciprofloxacin (0.1 mg/kg) administered 2 h after gentamicin (1 mg/kg), 4.2 ± 1.7. As can be seen, similar therapeutic effects were achieved regardless of whether the two compounds were administered simultaneously or sequentially. The sequence of administration also did not alter the results observed.

DISCUSSION

The results of this study differ from previously reported data insofar as five commonly used aminoglycoside antibiotics were evaluated in combination with ciprofloxacin under identical test conditions. Also, not only a few selected bacteria but a representative number of clinical isolates of *Enterobacteriaceae* and *P. aeruginosa* strains were tested.

The interpretation of the checkerboard titration results deserves some explanations. FIC indices (2) or Σ FICs (3) usually are calculated to characterize the drug interactions observed. Various breakpoints, however, are used to define synergism and especially antagonism. A study of Hallander et al. (3) is cited as a reference method in the instructions to authors of this journal. However, no complete checkerboards of antibiotic combinations but only serial dilutions of distinct combinations containing the MIC of each antibiotic concentrated twofold were evaluated in this paper. When complete checkerboard assays are performed, separate Σ FICs can be calculated for each row of the checkerboard (4), resulting in a minimal and a maximal Σ FIC (8).

The breakpoints used in this study were based on the consideration that a difference of one dilution in the MICs as measured by the agar dilution method is within the range of method-dependent fluctuations and therefore is not significant in determining decreased or increased antibacterial activity. This interpretation is consistent with the criteria used by other authors (1, 6).

Σ FICs indicative of synergistic or antagonistic interactions were seen in less than 1% of the tests performed. Since these combination effects could not be reproduced when the checkerboard assays were repeated, they were considered to reflect method-dependent fluctuations rather than true antibiotic interactions. The distribution of the FIC indices as observed in the tests with indifferent results was interpreted as further confirmation of the additive-indifferent action of ciprofloxacin-aminoglycoside combinations.

The bactericidal activity of ciprofloxacin-aminoglycoside combinations was assessed by time-kill experiments with three representative strains of *S. marcescens* and *E. coli*. As described by Hallander (3), synergism is present if a $2\log_{10}$ decrease in the CFU between the combination and its most active constituent after 24 h is achieved with the amount of antibiotic at least halved in the combination. The combination of ciprofloxacin and gentamicin led to a marked increase in the kill rates and considerably delayed regrowth, but similar effects also were obtained with two- or fourfold concentrations of either compound alone. Thus, the results of the time-kill experiments were in good agreement with the additive-inhibitory effects observed in the checkerboard assays.

When combination therapy with ciprofloxacin and gentamicin was evaluated in the *E. coli* thigh muscle infection model, the bactericidal activity of high doses of ciprofloxacin could not be increased further by using the drugs in combination. The low doses tested probably were too far below the inhibitory level to reveal any combination effects. Additive effects resulted only when suboptimal doses of both drugs were combined. The differences were not sufficient to prove a significant superiority of combination therapy. The data clearly proved, however, that antagonistic drug interactions had not occurred in vivo.

To simulate the clinical situation, ciprofloxacin and gentamicin were administered not only simultaneously but also sequentially in some of the time-kill and animal experiments. The effect of preexposure to gentamicin appeared difficult to anticipate, since some residual protein synthesis may be essential for the bactericidal activity of quinolone derivatives. Sequential application was found to have little influence on the killing and regrowth of the bacteria in vitro, regardless of the sequence of application. The therapeutic efficacy in vivo was not affected at all by the various application schedules tested.

In summary, combination therapy with ciprofloxacin and aminoglycoside antibiotics appears not to implicate any risk of antagonistic drug interactions which could impair the antimicrobial activity against *Enterobacteriaceae* and *P. aeruginosa* strains. Although relevant synergistic combination effects were not observed either, the additive action of ciprofloxacin-aminoglycoside combinations may be clinically useful if sufficient concentrations of drug are difficult to achieve at the site of infection. Combination therapy also may be a possible alternative for the use of lower doses of each drug than those required for monotherapy. In any case, the decision to use ciprofloxacin and aminoglycoside antibiotics in combination should be based on clinical considerations rather than on expected microbiologic combination effects.

LITERATURE CITED

1. **Bertram, M. A., and L. S. Young.** 1984. Imipenem antagonism of the in vitro activity of piperacillin against *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26**:272–274.
2. **Elion, G. B., S. Singer, and G. H. Hitchings.** 1954. Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related metabolites. J. Biol. Chem. **208**:477–488.
3. **Hallander, H. O., K. Dornbusch, L. Gezelius, K. Jacobson, and I. Karlsson.** 1982. Synergism between aminoglycosides and cephalosporins with antipseudomonal activity: interaction index and killing curve method. Antimicrob. Agents Chemother. **22**:743–752.
4. **Krogstad, D. J., and R. C. Moellering, Jr.** 1980. Combinations of antibiotics, mechanisms of interaction against bacteria, p. 298–341. *In* V. Lorian (ed.), Antibiotics in laboratory medicine. The Williams & Wilkins Co., Baltimore.
5. **Overbeek, B. P., M. Rozenberg-Arska, and J. Verhoef.** 1985. Interaction between ciprofloxacin and tobramycin or azlocillin against multiresistant strains of *Acinetobacter anitratum* in vitro. Eur. J. Clin. Microbiol. **4**:140–141.
6. **Peterson, L. R., D. N. Gerding, J. A. Moody, and C. E. Fasching.** 1984. Comparison of azlocillin, ceftizoxime, cefoxitin, and amikacin alone and in combination against *Pseudomonas aeruginosa* in a neutropenic-site rabbit model. Antimicrob. Agents Chemother. **25**:545–552.
7. **Rudin, J. E., C. W. Norden, and E. M. Shinners.** 1984. In vitro activity of ciprofloxacin against aerobic gram-negative bacteria. Antimicrob. Agents Chemother. **26**:597–598.
8. **Verbist, L., and J. Verhaegen.** 1984. Effect of temocillin in combination with other β-lactam antibiotics. Antimicrob. Agents Chemother. **25**:142–144.
9. **Wise, R., J. M. Andrews, and L. J. Edwards.** 1983. In vitro activity of Bay 09867, a new quinoline derivative, compared with those of other antimicrobial agents. Antimicrob. Agents Chemother. **23**:559–564.
10. **Zeiler, H.-J., and K. Grohe.** 1984. The in vitro and in vivo activity of ciprofloxacin. Eur. J. Clin. Microbiol. **3**:339–343.