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Netilmicin: In vitro Activity, Time-Kill Evaluation and Postantibiotic Effect on Microorganisms Isolated from Ocular Infections

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Key Words
Netilmicin · Ocular microorganisms · In vitro activity · Ofloxacin

Abstract
The in vitro activity of netilmicin and other antibiotics against ocular gram-positive and gram-negative microorganisms was evaluated. Netilmicin showed excellent activity against all the tested microorganisms, with more than 90% susceptibility. Many gentamicin- and tobramycin-resistant strains were still susceptible to netilmicin, although the minimum inhibitory concentration values of netilmicin were higher than those for the fully susceptible strains. In time-kill studies, netilmicin showed bactericidal activity within 1 h against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Moreover, netilmicin showed a postantibiotic effect of 2.4 h against *P. aeruginosa* and 1.5 h against *S. aureus*. These values were longer than those showed by ofloxacin, i.e. 2.1 and 1.4 h, respectively.

Introduction

Netilmicin is a derivative of a dehydrogenated C_{1a} gentamicin. Although its activity is similar to that of gentamicin and tobramycin, it also has good activity against many gentamicin- and tobramycin-resistant strains, depending on the N-ethyl substitution of the 2-deoxystreptamine ring [1]. Netilmicin and the other aminoglycosides are bactericidal by binding to intracellular ribosomes and interfering with bacterial protein synthesis [2]. Netilmicin is also available as an ocular formulation. The most frequently isolated microor-

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ganisms from ocular infections are coagulase-negative staphylococci (CNS), *Staphylococcus aureus, Haemophilus influenzae,* Enterobacteriaceae and *Pseudomonas aeruginosa*. However, sometimes it is very difficult to establish the real role of these microorganisms in ocular infections, since these species are often isolated in normal eyes.

In our study, the in vitro activity of netilmicin and other antibiotics used in ophthalmology against gram-positive and gram-negative microorganisms isolated from ocular infections was evaluated. Since the concentration of an antibiotic fluctuates in vivo according to its pharmacokinetic properties, we also evaluated the killing rate and postantibiotic effect (PAE) of netilmicin against microorganisms implicated in ocular infections.

Materials and Methods

Bacterial Strains

One hundred strains – 25 *S. aureus,* 25 CNS, 25 *P. aeruginosa,* 10 *Klebsiella pneumoniae,* 10 *Escherichia coli* and 5 *H. influenzae* – isolated from eye swabs were used in our study. All the microorganisms were identified by standard laboratory methodologies.

S. aureus ATCC 29213 and *P. aeruginosa* ATCC 27853 were included as quality control strains.

Susceptibility Tests

Minimum inhibitory concentration (MIC) was determined by the agar dilution method using supplemented Mueller-Hinton (MH) agar (Oxoid, Milan, Italy). The following antibiotics and susceptibility breakpoints, as suggested by NCCLS [3], were used in this study: netilmicin, ≤ 8 mg/l; gentamicin, ≤ 4 mg/l; tobramycin, ≤ 4 mg/l; ciprofloxacin, ≤ 1 mg/l, and ofloxacin, ≤ 2 mg/l. All the drugs came from commercial sources.

Inoculum of 10^4 colony-forming units (cfu)/spot was delivered onto the surface of agar plates using a multipoint inoculator. The MIC value was defined as the lowest concentration of antibiotic completely suppressing bacterial growth after 18 h of incubation at 37°C.

The minimum bactericidal concentrations (MBCs) of netilmicin and ofloxacin on three *S. aureus* and three *P. aeruginosa* isolates and the control strains were determined by subculturing broth microdilutions. The MBC was defined as the lowest concentration of antimicrobial agent achieving a 99.9% reduction in the original inoculum after 24 h.

Postantibiotic Effect

The PAE of netilmicin and ofloxacin against one *P. aeruginosa* and one *S. aureus* strain was investigated using supplemented MH agar at the antibiotic concentration of 8 mg/l, according to the methodologies of Craig and Gudmundsson [4]. Briefly, antibiotic solution was added to logarithmic-phase cultures (approximately 10^7 cfu/ml) in broth and a growth control containing no antibiotic was also used. After 1 h, the antibiotic concentration was diluted to 1 in 1,000 in prewarmed MH broth and incubated at 37°C for 24 h.

Viable counts were measured on antibiotic-free MH agar before microorganisms were exposed to antibiotic and hourly after neutralization by dilution for 6 h and at 24 h. The number of colonies was determined after cultures had been diluted in phosphate-buffered saline and incubated for 24 h at 37°C.

PAE was determined from the equation PAE = T–C, where T is the time required for the count of cfu in the test culture to increase 1 $\log_{10}$ above the count immediately after dilution, and C is the time needed for a 1 $\log_{10}$ increase in the count for the untreated control compared with the count immediately after dilution.

Time-Kill Determination

Time-kill determinations were performed as previously described [5]. Briefly, three strains of *S. aureus* and three strains of *P. aeruginosa* and the control strains were grown to reach 10^8 cfu/ml on supplemented MH broth and then diluted to 10^6 cfu/ml in the same prewarmed broth containing netilmicin (8 mg/l) or ofloxacin (2 mg/l). These concentrations were selected according to the susceptibility breakpoint values. An antibiotic-free control was similarly inoculated. At 0, 1, 2, 6 and 24 h after drug exposure, 0.1 ml were collected, diluted in phosphate-buffered saline, inoculated onto MH agar plates and then incubated at 37°C for 24 h, to determine viable cfu/ml. All the experiments were performed in duplicate. Killing curves were constructed by plotting the $\log_{10}$ of cfu/ml versus the time. Bactericidal activity was defined as a >3 $\log_{10}$ decrease in the initial inoculum size.

Results

Susceptibility Tests

The in vitro activity of all the antibiotics tested is illustrated in table 1. Netilmicin activity against gram-positive microorganisms was excellent and superior to that of ofloxacin, in particular against CNS. Three gentamicin- and tobramycin-resistant *S. aureus* strains were susceptible to netilmicin, although the MIC values were higher than the fully susceptible strains. Moreover, two of these strains were also oxacillin resistant and were also resistant to quinolones. In general, the activity of gentamicin was similar to that of tobramycin.

The in vitro activity of netilmicin was also excellent against *P. aeruginosa* strains. The activity of netilmicin was similar to that of ciprofloxacin against *P. aeruginosa,* with 80% susceptibility. Only 72% of *P. aeruginosa* strains were susceptible to ofloxacin, gentamicin and tobramycin. In addition, netilmicin showed excellent activity against the other microorganisms. All the *E. coli, H. influenzae* and almost all *K. pneumoniae* strains were fully susceptible to netilmicin.

For all the strains tested, the MBC values of netilmicin were equal to or one dilution above the MIC values, indicating an excellent bactericidal activity (data not shown).

Table 1. In vitro activity of antibiotics against 100 isolates

Antibiotics	Range, mg/l	% Susceptibility
S. aureus (n = 25)		
Netilmicin	0.12–64	84
Gentamicin	0.25–>64	68
Tobramicin	0.25–>64	68
Ofloxacin	0.5–>64	72
Ciprofloxacin	0.12–>32	72
CNS (n = 25)		
Netilmicin	0.06–16	88
Gentamicin	0.25–>64	64
Tobramicin	0.25–>64	64
Ofloxacin	0.5–>64	72
Ciprofloxacin	0.12–>32	72
P. aeruginosa (n = 25)		
Netilmicin	0.25–32	80
Gentamicin	0.5–>64	72
Tobramicin	0.5–>64	72
Ofloxacin	0.12–>64	72
Ciprofloxacin	0.06–>32	80
K. pneumoniae (n = 10)		
Netilmicin	0.03–64	90
Gentamicin	0.03–64	80
Tobramicin	0.03–64	80
Ofloxacin	0.03–64	90
Ciprofloxacin	0.03–32	90
E. coli (n = 10)		
Netilmicin	0.03–0.25	100
Gentamicin	0.03–1	100
Tobramicin	0.03–1	100
Ofloxacin	0.03–2	100
Ciprofloxacin	0.03–0.5	100
H. influenzae (n = 5)		
Netilmicin	≤0.03–0.25	100
Gentamicin	≤0.03–0.5	100
Tobramicin	≤0.03–0.5	100
Ofloxacin	≤0.03–0.5	100
Ciprofloxacin	≤0.03–0.25	100

Postantibiotic Effect

Netilmicin showed a 2.4-hour PAE for *P. aeruginosa* and a PAE of 1.5 h for *S. aureus.* The PAE values of netilmicin were higher than those of ofloxacin, as shown in figures 1a and b.

Time-Kill Studies

The time-kill kinetic data show that netilmicin exhibited a pronounced bactericidal activity against both *P. aeruginosa* ATCC 27853 (fig. 2) and *S. aureus* ATCC 29213 (fig. 3). Netilmicin demonstrated a bactericidal effect (3 $\log_{10}$ decrease in cfu/l) with all the studied strains. The bactericidal activity of netilmicin was compared with that of ofloxacin. The lat-

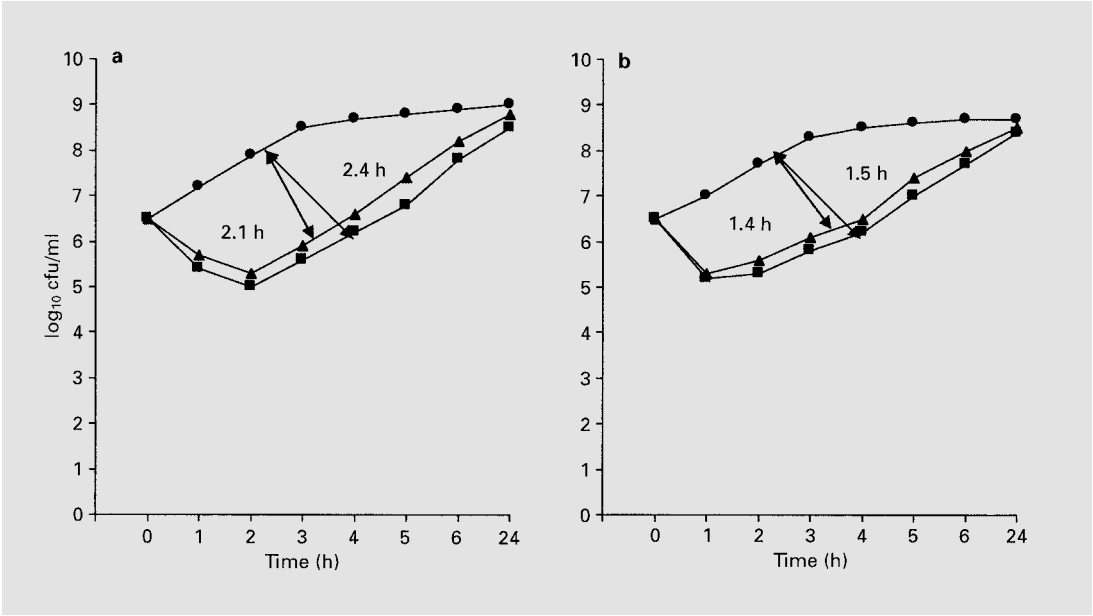


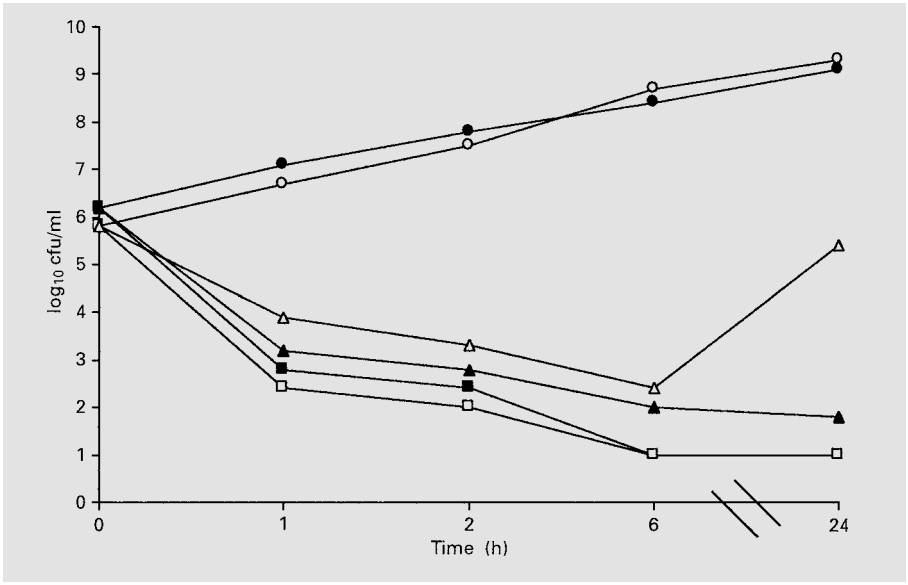
Fig. 1. a PAE of netilmicin (■) and ofloxacin (▲) for *P. aeruginosa.* ● = Control without exposure to antibiotic. **b** PAE of netilmicin (■) and ofloxacin (▲) for *S. aureus.* ● = Control without exposure to antibiotic.

ter showed a less marked bactericidal effect than netilmicin. For both species, ofloxacin exhibited regrowth after 24 h, which was more pronounced for *P. aeruginosa.*

Discussion

The value of an antibiotic in the treatment of ocular infections depends in part on the maintenance of effective concentrations in the eye for periods sufficient to determine microorganism eradication. The most frequently used parameter to describe antibacterial activity is still the MIC. However, other parameters, such as killing rate and PAE, have been recognized as important factors that may influence the optimal dosing regimens for antibiotics. Time-kill kinetics describes the time-dependent reduction in cfu by a fixed concentration of antibiotic, whereas PAE describes continued growth suppression of a microorganism after short-term exposure to an antibiotic. Since, in vivo, the concentration of an antibiotic fluctuates according to its pharmacokinetic proprieties, we studied the rate of killing and PAE of netilmicin against microorganisms responsible for ocular infections.

In our study, the in vitro activity of different antibiotics against microorganisms isolated from ocular swabs was evaluated. In general, the activity of netilmicin was superior to that of ofloxacin against gram-positive and gram-negative bacteria, with more than 90% of the isolated strains being susceptible to netilmicin. Moreover, many gentamicin- and tobramycin-resistant strains were susceptible to netilmicin. In our study, most bacterial isolates were inhibited by a netilmicin



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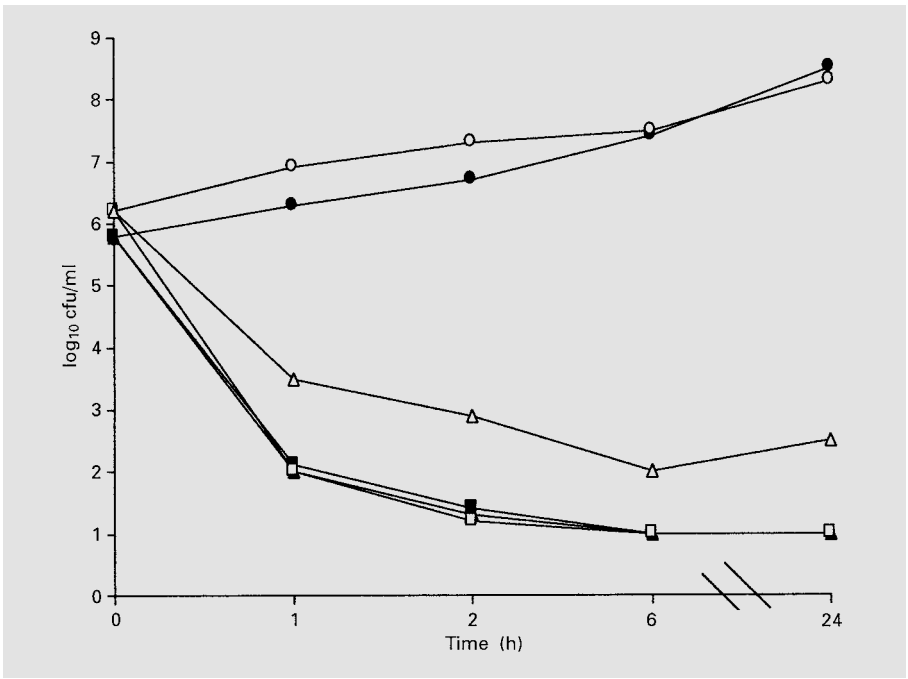

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Fig. 2. Time-kill results for netilmicin (■) and ofloxacin (▲) against *P. aeruginosa* ATCC 27853, and for netilmicin (□) and ofloxacin (△) against *P. aeruginosa* isolates. ●, ○ = Controls without antibiotic.

Fig. 3. Time-kill results for netilmicin (■) and ofloxacin (▲) against *S. aureus* ATCC 29213, and for netilmicin (□) and ofloxacin (△) against *S. aureus* isolates. ●, ○ = Controls without antibiotic.

concentration of less than 8 mg/l. In fact, peak serum levels of netilmicin appear to be 8 mg/l [6, 7].

The time-kill kinetics measured at a constant concentration showed that netilmicin was more bactericidal than ofloxacin against *S. aureus* and *P. aeruginosa* strains under the experimental conditions used in this study.

Netilmicin showed a PAE longer than that shown by ofloxacin. The mechanism and clinical relevance of bacterial growth suppression, denominated PAE, is still not completely known. It is still the subject of speculation; one probable explanation could be nonlethal damage produced by the antimicrobial or limited persistence of the drug at a bacterial binding site. Aminoglycoside antibiotics, such as netilmicin, form a lethal and irreversible bond to ribosome subunits, and this explains the longer PAEs observed. In fact, with netilmicin, the PAE could represent binding of sublethal amounts of drug with subsequent disruption of protein synthesis. The PAE might be clinically relevant to the design of dosing regimens for an antibiotic when drug concentrations between doses decrease below the MIC values of an antibiotic for a microorganism. Thus, drug-organism combinations that do not show a PAE may require more frequent antimicrobial administration than the ones exhibiting a PAE. Moreover, some authors have reported in vivo study PAEs for aminoglycosides of longer than 6 h [8]. The reason for this phenomenon is not clear.

Many experimental and clinical trials have indicated that netilmicin has a lower intrinsic toxicity than currently used aminoglycosides [9]. This characteristic, together with its excellent in vitro activity against many different gram-positive and gram-negative microorganisms, is able to render netilmicin an effective antibacterial drug for the treatment of ocular infections, offering advantages in safety which may indicate its use for patients believed to be at risk of adverse effects.

In conclusion, netilmicin is still an excellent antibiotic against all the gram-positive and gram-negative microorganisms isolated from ocular infections and could be used in the empirical treatment of ocular infections, where the use of an efficient, nontoxic and potent antibiotic is required.

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