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Once- Versus Thrice-Daily Netilmicin Combined with Amoxicillin, Penicillin, or Vancomycin against *Enterococcus faecalis* in a Pharmacodynamic In Vitro Model

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Several in vitro and in vivo studies as well as clinical trials have demonstrated that once-daily aminoglycoside regimens are as effective as or more effective than multiple daily dosings. However, the most favorable aminoglycoside dosing regimen for treating enterococcal endocarditis remains controversial. The same total dose of netilmicin was administered as once-daily (24-μg/ml peaks) and thrice-daily (8 μg/ml) regimens in a pharmacodynamic in vitro model simulating exposure of *Enterococcus faecalis* to human serum kinetics. Netilmicin was administered in combination with continuous infusions of amoxicillin, vancomycin, or penicillin against a bacterial biofilm adhering to glass beads. No significant differences in bacterial killing were found after 24 or 48 h between the once- and thrice-daily regimens. Additional experiments considering animal kinetics (half-life of netilmicin, 20 min) instead of human kinetics (half-life, 2.5 h) in the pharmacodynamic model also revealed similar results. The addition of netilmicin synergistically increased the activity of vancomycin ($P < 0.05$). In contrast, amoxicillin alone was as effective as the combination with netilmicin. Thus, it could not be established in this model that once-daily dosing of aminoglycosides is contraindicated for treating infections caused by *E. faecalis*.

Enterococcal endocarditis requires the administration of a synergistic combination of a cell wall-active agent, usually a penicillin or vancomycin, with an aminoglycoside to achieve maximum rates of cure (11). The evolution in aminoglycoside dosing strategies with a change to larger doses, given less frequently (once daily), follows the conclusions of numerous in vitro and in vivo studies (3, 5–7, 13). Clinical studies support the administration of once-daily dosing regimens for most indications; however, only limited clinical experience with respect to bacterial endocarditis is available. Animal studies considering once-daily dosing of aminoglycosides for the treatment of endocarditis have shown similar efficacies with both regimens (9, 10, 13). However, the most favorable aminoglycoside dosing regimen for treating enterococcal endocarditis remains controversial (8, 9).

The purpose of the pharmacodynamic in vitro study described here was to evaluate the effects of dosage regimens of an aminoglycoside in combination with a penicillin or a glycopeptide against *Enterococcus faecalis*. A laboratory reference strain and a clinical isolate previously used by Fantin and Carbon (8) in an animal model of endocarditis were studied. Adherent pathogens were considered since in vitro treatment of sessile biofilms might better mimic therapy of infectious vegetations in endocarditis. Adherent bacteria have a reduced metabolic activity which might reduce the bactericidal effects of some antibiotics, i.e., cell wall-active drugs (2, 12). In addition, the effect of human versus animal kinetics in the pharmacodynamic model on bactericidal activity was considered since this factor may be critical for extrapolating the results of animal studies to the clinical treatment of patients. Major differences in the half-lives of antibiotics have been observed among elderly patients, healthy volunteers, and small animals frequently used in laboratories. For example, the half-lives of tobramycin are 16 to 22 min in mice, ca. 40 min in rabbits, and 2 to 4 h in human patients (4).

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MATERIALS AND METHODS

Bacteria and drug susceptibility. Two strains of *E. faecalis* were studied: a laboratory reference strain (ATCC 29212) and the clinical isolate previously used in an animal model and referred in this paper as *E. faecalis* FAN (8). The susceptibilities of both strains was determined in tryptic soy broth supplemented (TSB-S) with calcium (50 μg of Ca^{2+} per ml) and magnesium (25 μg of Mg^{2+} per ml). Macrodilution MIC tests were performed with inocula of 10^5 CFU of suspended bacteria per ml. The MICs of netilmicin, vancomycin, amoxicillin, and penicillin for *E. faecalis* ATCC 29212 were 32, 4, 0.5, and 1 μg/ml, respectively. The MICs of netilmicin, vancomycin, and both amoxicillin and penicillin for *E. faecalis* FAN were 32, 2, and 1 μg/ml, respectively. MBC tests were performed with all four drugs and both strains (1). Seven of eight ratios of the MBC to the MIC were either 1 or 2. The ratio was 16 for vancomycin against *E. faecalis* ATCC 29212.

Pharmacodynamic in vitro model. A previously described one-compartment pharmacokinetic model was used in the present study (14). In contrast to the previous study, the dilution reservoirs containing the continuously infused antibiotics had to be kept in a refrigerator, since some experiments were performed with drugs that are not very stable in vitro. The model allows for periodic assessment of the bactericidal effect against both adherent and suspended bacteria. The system was filled with TSB-S, and a peristaltic pump continuously transported sterile broth from the reservoir into the culture compartment. The antibiotic doses administered into the culture compartments were eliminated exponentially because of the continuous perfusion of the compartment.

Inocula. Sterile sintered glass beads containing pores of 60 to 300 μm (Sikug 023/300/A; Schott Schleifer AG, Muttenz, Switzerland) were placed into 25-ml cultures of TSB-S. After inoculation with five bacterial colonies taken from a blood agar plate, the culture was incubated for 20 h (biofilm formation). Subsequently, the overnight broth was separated from the sintered glass beads by putting the beads on filters and rinsing them with 10 ml of a sterile saline solution. The culture compartments of the model were inoculated with 12 sintered glass beads that were placed into the compartments with a sterile surgical forceps.

Antibiotics and dosage regimens. The importance of the aminoglycoside dosing regimen has been studied during treatment of *E. faecalis* infections with antibiotic combinations which are frequently used clinically: (i) penicillin, the standard drug used in combination with aminoglycosides, (ii) amoxicillin, an alternative to ampicillin with better stability in vitro, and (iii) vancomycin, recommended as the drug of choice for patients with allergy to penicillin or for treating infections caused by high-level ampicillin- and penicillin-resistant organisms. Antibiotic doses were chosen to obtain drug concentrations similar to the mean concentrations achieved clinically in patients. Vancomycin (16 μg/ml),

amoxicillin (8 μg/ml), or penicillin (4 μg/ml) was given as a loading dose; this was followed by a continuous infusion of 48 h. Netilmicin doses were administered into the culture compartments as 60-min infusions with a syringe infusion pump and were eliminated exponentially because of the continuous perfusion of the compartment. Concentration-time profiles were defined according to the kinetics in human or animal serum by determining the flow rate of the pump (clearance) as a function of the volume of the culture compartment and the elimination half-life. To investigate the impact of the half-life of netilmicin, a rather wide range of half-lives was simulated: either a very short half-life of 20 min, representative of the kinetics in the serum of mice, or a half-life of 2.5 h, representative of the kinetics in the serum of human patients. The simulation of human kinetics of netilmicin with an elimination half-life of 2.5 h and dosing intervals of 8 and 24 h resulted in peaks of 8 and 24 μg/ml and troughs of 0.89 and 0.03 μg/ml, respectively. Simulation of the animal kinetics of netilmicin with an elimination half-life of 20 min resulted in peaks of 29 and 87 μg/ml and troughs of 2×10^{-6} and 2×10^{-20} μg/ml for the 8- and 24-h dosing intervals, respectively. The presence of these calculated concentration profiles within the culture compartments was confirmed by concentration measurements (on average, 101% ± 11% of the target value) by fluorescence polarization immunoassay (TDx; Abbott Laboratories, Abbott Park, Ill.). The concentrations of vancomycin were also measured by fluorescence polarization immunoassay (TDx; Abbott Laboratories) (on average, 100% ± 5% of the target value). The interday coefficients of variation of the assays of netilmicin and vancomycin were less than 3%. Continuous infusions of vancomycin, amoxicillin, and penicillin were achieved by adding the drug to sterile broth kept at room temperature outside of the 37°C incubator (vancomycin) or at 4°C in a refrigerator (amoxicillin and penicillin). During experiments with amoxicillin or penicillin, the dilution reservoirs were replaced every 12 h by new reservoirs containing a new stock solution of drug diluted in TSB-S. The concentrations of amoxicillin were measured by high-pressure liquid chromatography (HPLC). Samples were diluted with perchloric acid (0.33 M) and centrifuged, and the supernatants were injected into an HPLC cartridge column, Spherisorb 3SODS2 3 μm (Phase Separation Ltd., Clwyd, United Kingdom). KH_2PO_4 buffer (pH 4.6) was used as the mobile phase and was pumped at a rate of 1.2 ml/min. Spectrophotometric detection at 230 nm was performed, and the peaks were quantified by comparing their areas with those obtained with standard solutions of amoxicillin in 0.33 M perchloric acid (coefficient of variation less than 5%) (15). Measurements of amoxicillin sampled from the culture compartment at various times within the 48-h treatment period averaged 104% ± 7% of the target value.

Quantification of bactericidal activity. During the experiments, 0.5-ml specimens of the culture medium and one bead were removed from the culture compartment seven times within the 48-h treatment period (at 0, 2, 8, 24, 26, 32, and 48 h) to document the bactericidal activity over time. Subsequently, each bead was placed into a tube containing 2 ml of sterile broth. The bacteria adhering to the sintered glass beads were removed from the surface by vigorously vortexing the tubes with the beads three times for 15 s each time. It was demonstrated in a previous study that almost all bacteria were removed from the beads by a radiolabeled [^{3}H]thymidine assay (14). Also, longer vortexing or additional treatment in an ultrasonic bath failed to increase the CFU counts by more than 2% (14). Specimens of the broth in which the beads had been vortexed were subsequently processed similarly to the specimens withdrawn directly from the culture compartments. Each sample was diluted 100- to 10,000-fold, and the dilution was subcultured onto tryptic soy agar plates with a spiral plater system (Spiral System Instruments Inc., Bethesda, Md.). In addition, 100 μl of each sample was filtered through a membrane with a pore size of 0.45 μm. Subsequently, the filters were placed on tryptic soy agar and were incubated for 24 h at 37°C. The bacterial colonies growing on the agar were counted with the Laser Bacteria Colony Counter (model 500A; Spiral System Instruments Inc.), and the numbers of CFU per milliliter or per bead were calculated. The absence of any growth on the filter with the 100-μl sample was arbitrarily defined as 10 CFU/ml or 10 CFU per bead for statistical calculations. All experiments were done in triplicate.

Data analysis. Geometric means and standard deviations were used to describe the average number of CFU determined in multiple experiments. Analysis of variance was performed to study the synergistic bactericidal activities of the drug combinations. Student's *t* tests were performed to assess the bactericidal effect after 48 h of netilmicin given once daily versus thrice daily and of antibiotics given alone or in combination and also to compare human versus animal kinetics in the pharmacodynamic model. Probabilities of $P < 0.05$ were considered statistically significant.

RESULTS

Once- versus thrice-daily dosing. No statistically significant differences between the once- and thrice-daily regimens were found following 24 and 48 h of treatment with netilmicin alone or in combination with vancomycin, amoxicillin, or penicillin. This result was observed during treatment of strain ATCC 29212 as well as the clinical isolate (Table 1). Minor differences between the once-daily and the thrice-daily regimens were observed during the initial treatment period. As expected, single drug treatment with netilmicin given once daily was more bactericidal (1 to 2 log CFU per bead) within the first 8 h compared with the bactericidal activity of the thrice-daily regimen against strain ATCC 29212. After 24 and 48 h, however, both netilmicin regimens were ineffective and the CFU counts were identical to the counts for the controls (data not shown). The higher single dose of netilmicin provided by the once-daily regimen resulted in slightly enhanced initial killing of 0.4 to 1.4 log CFU compared with that by the thrice-daily regimen during combined treatment with vancomycin (Fig. 1).

TABLE 1. Bactericidal activity of netilmicin alone or in combination with vancomycin, amoxicillin, or penicillin against *E. faecalis* after 48 h of treatment in a pharmacodynamic in vitro model simulating human kinetics

E. faecalis strain	Drug	Activity of drug regimens[a]		
		Drug alone	Drug combined with netilmicin[b]	
			OD	TD
ATCC 29212	Vancomycin[c]	−2.0 ± 0.1	−4.4 ± 0.4	−4.4 ± 0.5
	Amoxicillin	−4.7 ± 0.1	−4.4 ± 0.4	−5.1 ± 0.3
	Netilmicin		+0.5 ± 0.2	+0.2 ± 0.1
FAN	Vancomycin	−1.0 ± 0.6	−1.3 ± 1.1	−2.3 ± 0.3
	Amoxicillin	<−6.3 ± 0.1[d]	<−6.3 ± 0.2[d]	−5.7 ± 0.7
	Penicillin	−3.5 ± 0.1	−4.4 ± 0.2	−4.6 ± 1.3

[a] Activity is indicated as the change in log CFU per bead (geometric mean ± standard deviation of triplicate tests).
[b] Netilmicin was administered once daily (OD) or thrice daily (TD).
[c] Vancomycin alone was significantly less active than vancomycin in combination with netilmicin ($P < 0.05$).
[d] Eradication of the inoculum below the detection limit.

Impact of dosing on synergy. The netilmicin dosing regimen had no major impact on the synergistic interactions with vancomycin, amoxicillin, or penicillin against either strain of *E. faecalis* (Table 1). Amoxicillin was similarly effective after 24

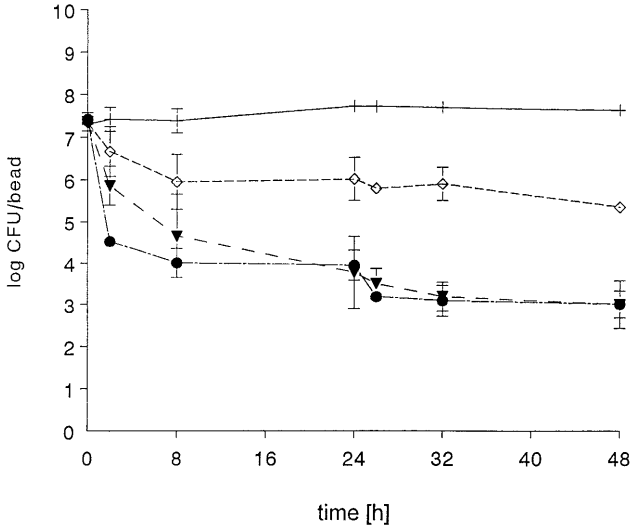

FIG. 1. Efficacy of vancomycin alone or in combination with netilmicin given once daily and thrice daily against adherent *E. faecalis* ATCC 29212. Human kinetics of netilmicin were simulated in an in vitro model. Geometric means of triplicate experiments are shown. Standard deviations are not indicated if they were less than 0.2 log. +, control; ◇, vancomycin; ●, vancomycin plus netilmicin given once daily; ▼, vancomycin plus netilmicin given thrice daily.

TABLE 2. Bactericidal activity of netilmicin in combination with vancomycin, amoxicillin, or penicillin against adherent *E. faecalis* after 48 h of treatment in a pharmacodynamic in vitro model simulating human or animal kinetics

E. faecalis strain	Drug combined with netilmicin	Activity with simulation of the following kinetics[a]:	
		Human	Animal
ATCC 29212	Vancomycin[b]	−4.4 ± 0.4	−2.4 ± 0.9
	Amoxicillin	−4.7 ± 0.4	−4.6 ± 0.4
FAN	Penicillin	−4.7 ± 1.0	−4.7 ± 1.5

[a] Activity is indicated as the change in log CFU per bead (geometric mean ± standard deviation of once-daily and thrice-daily regimens of netilmicin).
[b] Significant difference between human and animal kinetics ($P < 0.001$).

and 48 h when it was given either alone or in combination with netilmicin. In contrast, the addition of netilmicin increased the bactericidal activity of vancomycin ($P < 0.05$) against *E. faecalis* ATCC 29212 in experiments simulating human kinetics (Table 1; Fig. 1). An additional experiment with a reduced single daily dose of netilmicin, providing only one-third of the regular dose, demonstrated a transient enhancement of bactericidal activity compared with that demonstrated by vancomycin alone; however, the synergistic contribution of netilmicin vanished toward the end of the 48-h treatment (data not shown). Against *E. faecalis* FAN, the addition of netilmicin did enhance the bactericidal activities of vancomycin and penicillin by 0.8 to 1.8 log CFU per bead; however, these differences did not reach statistical significance (Table 1) and were also below the minimal difference required for synergy according to the definition of synergism in killing curve tests. Because of the eradication of the inoculum by amoxicillin alone as well as by the combination of amoxicillin and netilmicin after 48 h of treatment, no final assessment of drug interaction can be made for this antibiotic against strain FAN. The bactericidal effect observed during the first 8 h of treatment, however, did show an enhancement of activity by adding netilmicin, particularly with the once-daily regimen (data not shown).

Effect of human versus animal kinetics. Over the 48-h treatment period no difference was observed between experiments simulating human or animal kinetics of netilmicin in combination with amoxicillin against *E. faecalis* ATCC 29212 (Table 2). Similar results were obtained with penicillin plus netilmicin against *E. faecalis* FAN (Table 2). Also, no significant differences in bactericidal activity were noted between the once-daily and the thrice-daily regimens in experiments simulating animal kinetics. The difference in the CFU reduction over the 48-h treatment period between the once-daily and thrice-daily dosing regimens was less than 0.3 log for both netilmicin–beta-lactam combinations.

In contrast, the synergy of netilmicin combined with vancomycin against *E. faecalis* ATCC 29212 was more pronounced in experiments simulating human kinetics than in experiments simulating animal kinetics (Table 2). The difference in the bactericidal activity of once-daily versus thrice-daily combinations with vancomycin was significantly greater in experiments simulating animal kinetics (1.2 ± 0.7 log CFU per bead) than in experiments simulating human kinetics (−0.2 ± 0.0 CFU per bead).

Confirmation experiments. Additional experiments considered the effect of treatment against suspended instead of adherent *E. faecalis* ATCC 29212 and FAN (data not shown). The bactericidal effect of drug treatment against suspended bacteria was enhanced in 13 of the 15 regimens studied ($P < 0.01$). However, the observed differences were minor (an average of 0.9 log better killing), and the aminoglycoside dosing regimen had no significant effect on bactericidal efficacy, which was consistent with the data obtained with adherent bacteria.

DISCUSSION

The importance of the aminoglycoside dosing regimen was studied during treatment of *E. faecalis* infection with antibiotic combinations that are used clinically by simulating human kinetics in an in vitro model. Similar results were obtained with once-daily and thrice-daily netilmicin regimens against a laboratory reference strain after 24 or 48 h of treatment, with the aminoglycoside administered alone or in combination with amoxicillin or vancomycin. Similarly, no differences in bactericidal activity between the once-daily and thrice-daily regimens were found against a clinical isolate with combinations of netilmicin with penicillin, amoxicillin, or vancomycin. The aminoglycoside dosing schedule was of no significance during simulation of human or animal kinetics or during treatment of adherent or suspended pathogens.

These in vitro observations are in agreement with the observations presented in a report by Gavalda et al. (9) on the treatment of experimental enterococcal endocarditis with gentamicin plus ampicillin. In contrast to the results obtained in the present in vitro study and those described in the report of Gavalda et al. (9), Fantin and Carbon (8) have shown with another rabbit model of enterococcal endocarditis better bactericidal efficacies of thrice-daily dosing regimens compared with those of once-daily dosing regimens. However, only a single clinical isolate was tested in their study (8). The identical isolate was also studied in the in vitro system described here to identify strain-specific differences. However, the difference between once-daily and thrice-daily regimens could not be confirmed in vitro after 24 or 48 h of combined treatment with netilmicin and penicillin (Table 1). We were also unable to confirm the hypothesis that the in vivo versus in vitro discrepancy would be due to pharmacokinetic differences. No significant differences in bactericidal activity were observed between thrice-daily and once-daily dosing of netilmicin in combination with a beta-lactam, whatever half-lives were considered.

In conclusion, the bactericidal effects of once-daily and thrice-daily regimens of netilmicin were not significantly different in the in vitro model. The discrepancy in one of the two animal studies considering the effect of scheduling during treatment of enterococcal endocarditis could not be explained by either a strain dependence or the short half-lives of netilmicin observed in animals. Thus, it could not be established or confirmed in the model described in this report that once-daily dosing of aminoglycosides is contraindicated for treating infections caused by *E. faecalis.*

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