Antibacterial Activity of Netilmicin, a New Aminoglycoside Antibiotic, Compared with That of Gentamicin

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Received for publication 13 October 1976

The antibacterial activity of netilmicin (Sch 20569), a new semisynthetic aminoglycoside, was compared with that of gentamicin against a variety of gram-negative bacteria, staphylococci, and streptococci. Both antibiotics had similar activity against most organisms, but netilmicin had appreciably greater activity against gram-negative organisms that were resistant to gentamicin because these species synthesized aminoglycoside 3-*N*-acetyltransferase I or aminoglycoside 2''-*O*-nucleotidyltransferase. Netilmicin was also more active than gentamicin against gentamicin-resistant strains of *Staphylococcus aureus* that produced two enzymes—aminoglycoside-2''-*O*-phosphotransferase and aminoglycoside-6'-*N*-acetyltransferase.

Netilmicin (Sch 20569) (Fig. 1), a new semisynthetic aminoglycoside antibiotic, is a derivative of sisomicin, which itself is a derivative of gentamicin C_{1A}. Although gentamicin and sisomicin have similar activity against most organisms (4, 5), there is evidence that netilmicin is more effective than either gentamicin or sisomicin against certain gentamicin-resistant organisms (6, 9, 11; informational material for SCH 20569, Schering Corp., Bloomfield, N.J., 1975). The purpose of this study was to compare the antibacterial activity of netilmicin with that of gentamicin against a variety of gentamicin-susceptible and gentamicin-resistant bacteria.

MATERIALS AND METHODS

Bacterial strains. The collection consisted of *Acinetobacter anitratus* (7), *A. lwoffii* (9), *Bacteroides fragilis* (19), *Citrobacter freundii* (38), *C. koserii* (30), *Enterobacter aerogenes* (20), *E. cloacae* (29), *Escherichia coli* (41), *Haemophilus influenzae* (16), *Neisseria gonorrhoeae* (39), *Klebsiella aerogenes* (51), *K. ozaenae* (20), *Proteus mirabilis* (31), *P. morgani* (20), *P. rettgeri* (17), *P. vulgaris* (20), *Providencia alkalifaciens* (9), *P. stuartii* (28), *Pseudomonas aeruginosa* (48), *Pseudomonas* spp. (62; *P. acidovorans*, 15; *P. cepacia*, 10; *P. fluorescens*, 3; *P. maltophilia*, 11; *P. pseudoalkaligenes*, 11; *P. putida*, 3; *P. stutzeri*, 7; *P. thomasi*, 5), *Serratia marcescens* (5), *Staphylococcus albus* (41), *S. aureus* (120), *Streptococcus faecalis* (38), *S. pneumoniae* (14), and beta-hemolytic streptococci (56). Most of the strains were isolates from patients in St. Thomas's Hospital, but the collection included the following strains from the National Collection of Type Cultures: *E. aerogenes* (3), *K. ozaenae* (12), *P. rettgeri* (7), *P. Alkalifaciens* (9), *P. Stuartii* (3), *P. Fluorescens* (1), *P. maltophila* (1), *P. pseudoalkaligenes* (1), *P. Stutzeri* (3),

and *S. marcescens* (5). Gentamicin-resistant strains from other sources, mostly in Great Britain, were: *E. cloacae* (1), *E. coli* (1), *K. aerogenes* (1), *P. rettgeri* (1), *P. stuartii* (13), *P. aeruginosa* (14), *P. stutzeri* (3), *S. aureus* (6).

Determination of minimum inhibitory concentrations (MICs). Organisms were grown in nutrient broth (Southern Group Laboratories) at 37°C for 18 h. The medium was supplemented with 10% lysed horse blood for *H. influenzae* and *N. gonorrhoeae* and with 10% horse serum for *S. pneumoniae*. The inoculum of *B. fragilis* was prepared by scraping cells off blood agar and suspending them in nutrient broth. Cultures were suitably diluted and 10³ to 10⁴ colony-forming units inoculated on serial doubling dilutions of the antibiotics in Diagnostic Sensitivity Test agar (Oxoid CM 261). For *B. fragilis*, *N. gonorrhoeae*, and *S. pneumoniae*, 6 to 10% lysed horse blood was added. For *H. influenzae*, Columbia agar base (Oxoid CM 331) supplemented with factor V (2%) and factor X (1%) was used. Incubation was at 37°C aerobically (with 10% CO₂ for *N. gonorrhoeae* and *S. pneumoniae*), except for *B. fragilis*, which was incubated in an atmosphere of 10% H₂, 10% CO₂, and 80% N₂. The MIC was the lowest concentration of antibiotic that resulted in complete suppression of growth after overnight incubation. Linear regression of MICs of gentamicin and netilmicin was performed by the method of least squares after transformation of MICs to log₂ of MICs.

Demonstration of enzyme activity. For investigations of aminoglycoside-modifying enzymes, use was made of the cellulose phosphate paper binding system (2). Suspensions, concentrated 10- to 20-fold, of cells grown at 37°C in Oxoid nutrient broth no. 2 (CM 67) were broken with an ultrasonic disintegrator (MSE Mark II). The resulting preparations were tested for their ability to bind radioactivity from acetyl-coenzyme A or adenosine-5'-triphosphate to aminoglycosides.

For acetylation, each tube contained, in a total

FIG. 1. Structure of netilmicin.

volume of 55 μ l: 360 ng of aminoglycoside, 1.4 nmol of [$1\text{-}^{14}\text{C}$]acetyl-coenzyme A (specific activity, 4.65 Ci/mol), 3 mmol of tris(hydroxymethyl)amino-methane (Tris)-hydrochloride buffer, pH 7.6, 480 nmol of MgCl_2 , 150 nmol of dithiothreitol, and 15 μ l of cell sonicate. For phosphorylation, each tube contained, in a total volume of 55 μ l: 360 ng of aminoglycoside, 7 nmol of [$\gamma\text{-}^{32}\text{P}$]adenosine 5'-triphosphate (1.35 Ci/mol), 3 mmol of Tris-hydrochloride buffer, pH 7.6, 480 nmol of MgCl_2 , 150 nmol of dithiothreitol, and 15 μ l of cell sonicate. For adenylation, each tube contained, in a total volume of 60 μ l: 130 ng of aminoglycoside, 2 nmol of [$8\text{-}^{14}\text{C}$]adenosine 5'-triphosphate (4 Ci/mol), 4 mmol of Tris-hydrochloride buffer, pH 8.1, 640 nmol of MgCl_2 , 285 nmol of dithiothreitol, and 28.5 μ l of cell sonicate.

After incubation at 37°C for 20 to 60 min, 50- μ l amounts of the mixture were pipetted onto squares (20 by 20 mm) of Whatman P81 cellulose phosphate paper. The papers were washed in water, dried, and placed in vials containing scintillation fluid which consisted of toluene containing 2,5-diphenyloxazole (6 g/liter) and 1,4-di-[2]-(5-phenyloxazolyl) benzene (100 mg/liter). Radioactivity was measured in a Beckman liquid scintillation spectrometer. Radioactive materials were obtained from the Radiochemical Centre, Amersham, Bucks, England. Chemicals for scintillation counting were obtained from Koch-Light Laboratories, Colnbrook, Bucks, England.

RESULTS

Figure 2 shows MICs of gentamicin versus MICs of netilmicin for the *Enterobacteriaceae*. Of the 332 strains of species other than *P. stuartii*, 95% were susceptible to both gentamicin and netilmicin (MICs, 4 μ g/ml). No resistant strains were encountered among isolates of *C. freundii*, *C. koserii*, *E. aerogenes*, *K. ozaenae*, *P.morganii*, *P. vulgaris*, and *P. alkalifaciens*. Strains known to produce enzymes that inactivate aminoglycosides (I. Phillips,

FIG. 2. Comparison of MICs of gentamicin and netilmicin for *Enterobacteriaceae*. Symbols: Strains that synthesized AAC(3)I (Δ); strains that synthesized ANT(2'') ($\times$), and the strain that synthesized AAC(6') (∇); *P. stuartii* and the strains of *P. rettgeri* that synthesized AAC(2') ($\circ$); strains that did not synthesize inactivating enzymes ($\square$); for convenience of presentation, where large numbers of such strains shared the same gentamicin and netilmicin MICs, ($\blacksquare$) is used to designate 10 strains. The calculated line of best fit is shown.

King, and K. Shannon, manuscript in preparation) were allocated distinguishing symbols. Seven strains of *K. aerogenes* and one of *E. cloacae* that produced aminoglycoside 3-N-acetyltransferase I [AAC(3)I] were resistant to gentamicin but susceptible to netilmicin, as was the strain of *K. aerogenes* that produced aminoglycoside 2''-O-nucleotidyltransferase [ANT(2'')]. One strain of *S. marcescens* that produced aminoglycoside 6'-N-acetyltransfer-

ase [AAC(6')] was susceptible to gentamicin but resistant to netilmicin. All 28 strains of *P. stuartii* produced aminoglycoside 2'-N-acetyltransferase [AAC(2')], as did three strains of *P. rettgeri*. These strains were generally resistant to both agents.

Results for the genus *Pseudomonas* are shown in Fig. 3. Of the 48 strains of *P. aeruginosa*, 58% were susceptible to gentamicin and 56% were susceptible to netilmicin. Most strains resistant to gentamicin were also resistant to netilmicin, but a few that produced aminoglycoside-inactivating enzymes were susceptible to netilmicin. Fewer strains of *Pseudomonas* spp. (40% of 62 strains) than of *P. aeruginosa* were susceptible to either agent. Only one strain was resistant to gentamicin but susceptible to netilmicin; this strain, of *P. pseudoalkaligenes*, produced AAC(3)I.

Netilmicin was less effective than gentamicin against the genus *Acinetobacter* in which a number of strains produced AAC(6'). All 16 strains were inhibited by gentamicin at a concentration of 2 µg/ml, but five strains were resistant to netilmicin.

H. influenzae had similar susceptibilities to both agents; MICs of 0.5 to 2 µg/ml were found for all strains. *N. gonorrhoeae* was more susceptible to netilmicin (MICs, 2 to 8 µg/ml; geometric mean MIC, 3.3 µg/ml) than to gentamicin (MICs, 4 to 16 µg/ml; geometric mean MIC, 5.6 µg/ml). Neither drug had activity against *B. fragilis* (MICs, 128 µg/ml).

More than 90% of strains of *S. aureus* were susceptible to both agents. The gentamicin-resistant strains were often more susceptible to

FIG. 3. Comparison of MICs of gentamicin and netilmicin for *Pseudomonas*. Symbols: *P. aeruginosa* (□), except for strains that synthesized AAC(3)I (Δ) or ANT(2') (×); other species (○), except for the strain that synthesized AAC(3)I (▽). The calculated line of best fit is shown.

FIG. 4. Comparison of MICs of gentamicin and netilmicin for *S. aureus*. Symbols: Strains that synthesized APT(2'') and AAC(6') (○); other strains (□). The calculated line of best fit is shown.

netilmicin (Fig. 4). Most of these strains were found to produce two enzymes: AAC(6'), which does not produce resistance to gentamicin (1), and an enzyme that phosphorylates gentamicin, tobramycin, and kanamycin but not neomycin or amikacin, and so is probably aminoglycoside 2''-O-phosphotransferase [APT(2'')] (10). A similar enzyme was found in the first gentamicin-resistant strain of *S. aureus* reported in Great Britain (3, 7).

S. albus was very susceptible to both drugs, with more than 90% of strains inhibited by 0.25 µg of gentamicin or netilmicin per ml. One strain was resistant to gentamicin but susceptible to netilmicin; it produced the same enzymes as the gentamicin-resistant strains of *S. aureus*.

Most strains of *S. faecalis* were resistant to both agents (MICs, 8 to 32 µg/ml), although 4 of the 38 strains had netilmicin MICs of 4 µg/ml. *S. pneumoniae* was resistant to both agents (MICs, 32 to 64 µg/ml). For beta-hemolytic streptococci, MICs ranged from 0.5 to 32 µg/ml. At a concentration of 4 µg/ml, 70% of strains were inhibited by gentamicin, and 68% were inhibited by netilmicin.

Table 1 shows in vitro modification of gentamicin, sisomicin, and netilmicin by extracts of representative strains that produce aminoglycoside-inactivating enzymes. Netilmicin was a poorer substrate than gentamicin and sisomicin for AAC(3)I and ANT(2''). Sisomicin was a better substrate than gentamicin for AAC(6'), because this enzyme does not acetylate gentamicin C₁ (1). Netilmicin was acetylated by AAC(6') less well than was sisomicin. For AAC(6') from *S. aureus* netilmicin was a

poorer substrate than gentamicin. There were two types of APT(2''). For the first type, which was more common, sisomicin was a poorer substrate than gentamicin, and netilmicin was phosphorylated only to a small extent. Gentamicin and sisomicin were equally good substrates of the second type and, although it was a poorer substrate, netilmicin was extensively phosphorylated.

DISCUSSION

Despite the general similarity in activity of gentamicin and netilmicin, there was a number of strains against which netilmicin was more active than gentamicin or sisomicin. These strains were mostly ones found to produce enzymes that inactivated gentamicin but for which netilmicin was a poor substrate, although no evidence of inactivating enzymes was detected in one such strain of *E. coli*. Thus, in netilmicin, substitution of an ethyl group on the C1 amino group results in partial protection of the C3 amino and C2'' hydroxyl groups from attack by AAC(3)I, ANT(2''), and APT(2''). This protection usually seems to be sufficient to render producers of AAC(3)I and ANT(2'') fully susceptible to netilmicin. Protection of netilmicin from attack by ANT(2'') has been reported before (6, 11), but for AAC(3)I previous work was contradictory. Soussy and Duval (11) found a strain of *E. coli* that produced AAC(3)I to be susceptible to netilmicin, whereas Kabins et al. (6) found a strain of *P. aeruginosa* to be resistant. Of the organisms that produced this enzyme, we found *Enterobacteriaceae* always to be susceptible to netilmicin; the strain of *P. pseudoalkaligenes* and one of the two strains of *P. aeruginosa* were susceptible and, although the other strain of *P. aeruginosa* was resistant (MIC, 16 $\mu\text{g/ml}$), it was more susceptible to

netilmicin than to gentamicin (MIC, >128 $\mu\text{g/ml}$). We find producers of AAC(3)I and ANT(2'') generally to be susceptible to amikacin (Phillips et al., manuscript in preparation) in agreement with the suggestion that the L- γ -amino- α -hydroxybutyryl group substituted on the C1 amino group in amikacin has a similar role to the ethyl group of netilmicin (8).

The staphylococci that produced APT(2'') always produced AAC(6') as well. One cannot separate the individual contributions of these enzymes to resistance, but the strains were generally more susceptible to netilmicin than to gentamicin. They were, however, sufficiently resistant to netilmicin to rule out treatment with this agent. In this finding, we agree with previous work (11).

The C2' amino group of netilmicin is not protected, since AAC(2') acetylated the drug. Strains of *P. stuartii* and *P. rettgeri* that produced this enzyme tended to be more resistant to netilmicin than to gentamicin, as was found before (6, 11).

Although acetylated less than sisomicin, netilmicin was a good substrate of AAC(6') from gram-negative organisms. The strains that produced this enzyme, mostly *Acinetobacter* spp., were the only ones resistant to netilmicin and sisomicin but susceptible to gentamicin. Netilmicin was a poor substrate of AAC(6') from staphylococci, as is amikacin (3, 10).

For the *Klebsiella-Enterobacter-Serratia* group, in which gentamicin resistance was enzyme mediated, correlation between MICs of gentamicin and netilmicin was poor (correlation coefficient, $r = 0.57$). *P. aeruginosa*, some gentamicin-resistant strains of which produced inactivating enzymes, also had a low correlation coefficient ($r = 0.61$). In contrast, for groups in which there was a sufficiently wide range of MICs there were good correlation coef-

TABLE 1. *In vitro* modification of gentamicin, sisomicin, and netilmicin by inactivating enzymes

Organism	Enzyme	MIC ($\mu\text{g/ml}$)			Incubation time (min)	Background radioactivity (cpm) ^a	Binding of radioactivity to aminoglycosides ^b		
		Gentamicin	Sisomicin	Netilmicin			Gentamicin	Sisomicin	Netilmicin
<i>K. aerogenes</i>	AAC(3)I	8	16	0.5	20	184	14.9	27.0	4.0
<i>P. aeruginosa</i>	AAC(3)I	128	128	16	20	359	3.4	2.0	1.2
<i>P. stuartii</i>	AAC(2')	64	128	128	20	552	6.9	10.2	8.3
<i>S. marcescens</i>	AAC(6')	2	8	32	20	312	1.7	1.7	1.8
<i>A. lwoffii</i>	AAC(6')	2	32	64	60	167	1.6	2.9	2.0
<i>K. aerogenes</i>	ANT(2'')	32	16	1	60	257	1.3	1.2	1.1
<i>P. aeruginosa</i>	ANT(2'')	128	128	8	30	141	7.3	10.0	1.9
<i>S. aureus</i>	APT(2'') type 1	64	64	2	30	1537	1.8	1.2	1.1
	AAC(6')	64	64	2	30	202	3.4	4.9	1.6
<i>S. aureus</i>	APT(2'') type 2	128	NT ^c	128	30	384	7.3	7.3	4.2
	AAC(6')	128	NT	128	30	165	6.6	15.2	4.3

^a Binding of radioactivity to cellulose phosphate paper in the absence of aminoglycosides.

^b Shown as multiples of radioactivity bound to cellulose phosphate paper in the absence of aminoglycosides.

^c NT, Not tested.

ficients. This was true both for groups such as *P. stuartii* ($r = 0.96$) and *S. aureus* ($r = 0.88$), in which both gentamicin and netilmicin were substrates of inactivating enzymes and for groups such as *Pseudomonas* spp. ($r = 0.97$) and beta-hemolytic streptococci ($r = 0.94$), in which resistance was not the result of inactivation but occurred more or less equally to both drugs.

Our studies suggest that, like sisomicin (4, 5), netilmicin does not have appreciably better antibacterial activity than gentamicin against most organisms, but this it is superior against gram-negative organisms whose gentamicin resistance is the result of synthesis of AAC(3)I or ANT(2'') and against staphylococci whose gentamicin resistance is due to the production of APT(2'') and AAC(6').

ACKNOWLEDGMENTS

We are grateful to Schering Corp. (U.S.A.) for financial support and for providing netilmicin and sisomicin, and to British Schering Limited for providing gentamicin. We also wish to thank N. Datta, P. Noone, A. Percival, D. S. Reeves, R. Sutherland, P. Waterworth, R. E. Warren and J. D. Williams who provided gentamicin-resistant organisms.

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