In Vitro Antibacterial Activity of Vertilmicin and Its Susceptibility to Modifications by the Recombinant AAC(6′)-APH(2′′) Enzyme[†]

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Vertilmicin is a new semisynthetic aminoglycoside with a structure similar to that of netilmicin except for a methyl group at the C-6′ position. In the present study, the in vitro antibacterial activity of vertilmicin was studied, and its susceptibility to modifications by the recombinant aminoglycoside bifunctional modifying enzyme AAC(6′)-APH(2′′) was compared with those of verdamycin and netilmicin. A total of 1,185 clinical isolates collected from hospitals in Beijing between 2000 and 2001 were subjected to the in vitro antibacterial activity evaluations, including MIC, minimum bactericidal concentration (MBC), and time-kill curve tests. The MICs were evaluated in non-gentamicin-resistant (gentamicin-susceptible and gentamicin-intermediate) strains and gentamicin-resistant strains, respectively. For most of the non-gentamicin-resistant bacteria (except for the isolates of *Pseudomonas* spp.), the MIC₉₀s of vertilmicin were in the range of 0.5 to 8 µg/ml, comparable to those of the reference aminoglycosides. For the gentamicin-resistant isolates, the three semisynthetic aminoglycosides (vertilmicin, netilmicin, and amikacin) demonstrated low MIC₅₀s and/or MIC₉₀s, as well as high percent susceptibility values. Among the study drugs, vertilmicin showed the lowest MIC₉₀s, 16 µg/ml, for the gram-positive gentamicin-resistant isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Meanwhile, vertilmicin was a potent bactericidal agent, with MBC/MIC ratios in the range of 1 to 2 for *Escherichia coli*, *Klebsiella pneumoniae*, and *S. aureus* and 1 to 4 for *S. epidermidis*. The time-kill curve determination further demonstrated that this effect was rapid and concentration dependent. In evaluations of susceptibility to modifications by the recombinant AAC(6′)-APH(2′′) with maximum rate of metabolism/*K_m* measurements, vertilmicin exhibited susceptibilities to both acetylation and phosphorylation lower than those of netilmicin and verdamycin.

The aminoglycoside antibiotics have been known for more than 60 years, since the discovery of streptomycin in 1944 (1). Aminoglycosides are polycationic broad-spectrum bactericidal antibiotics that are used to treat a number of bacterial infections (5, 21). The primary target of aminoglycosides is the 30S small subunit of ribosomes (2, 5, 21).

However, the clinical use of aminoglycosides is hampered by the emergence of aminoglycoside-modifying enzymes (AMEs) in resistant organisms (3–5). Bacteria become resistant to aminoglycosides, as the modified antibiotics no longer bind with high affinity to their targets due to unfavorable steric and/or electrostatic constraints (3, 5, 17). The AMEs are composed of three families: aminoglycoside nucleotidyltransferase, aminoglycoside acetyltransferase (AAC), and aminoglycoside phosphotransferase (APH) (2–5, 12, 21). The discovery of the semisynthetic aminoglycosides dibekacin, amikacin, and netilmicin

demonstrated the potential for obtaining compounds active against strains that had developed resistance mechanisms against earlier aminoglycosides (21). Vertilmicin, a new semisynthetic aminoglycoside obtained with the same strategy, is a 1-*N*-ethyl derivative of verdamycin, a natural product of *Micromonospora olivoasterospora* subsp. *Wuxiensis* (36). Its structure is similar to that of netilmicin (1-*N*-ethyl-sisomicin) except for a methyl group at the C-6′ position (Fig. 1). In the present study, the in vitro antibacterial activity of vertilmicin was evaluated in clinical isolates collected from hospitals in Beijing, China.

Clinically, the bifunctional modifying enzyme AAC(6′)-APH(2′′) is the most prominent AME in the gram-positive bacterial pathogens, such as *Staphylococcus aureus* and *Enterococcus faecalis* (3, 5, 9). This enzyme is unique in possessing activities of two classes of AMEs, an N-terminal AAC [AAC(6′)-Ie] and a C-terminal APH [(APH(2′′)-Ia) (3–5, 9, 13, 28), and was thought to have resulted from fusion of the *aac* and *aph* genes (5, 13, 28–29). The enzyme has an extraordinary ability to detoxify a wide range of aminoglycosides as a result of its bifunctional nature, as well as the characteristics of each of the activities. The APH domain has an unusually broad regiospecificity, being able to catalyze the phosphorylation of hydroxyls on four different aminoglycoside ring systems; the AAC domain catalyzes O-acetyltransfer, in addition to N-acetyltransfer (3, 5, 10). The two domains do not functionally interact but have intimate structural linkages that are important for con-

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FIG. 1. Chemical structures of netilmicin (1-*N*-ethyl-sisomicin), vertilmicin (1-*N*-ethyl-verdamicin), and verdamicin. The circles indicate the 1-*N* ethylation position and the methyl group at the C-6' position.

formation and stability (5). The *aac*(6')-*aph*(2'') genes can be found in both the R plasmids (6–7, 11, 30–33) and chromosomes (15, 26–27, 34) of aminoglycoside-resistant isolates, with locations on transposons, like Tn4001 (18), Tn4031 (35), and Tn5381 (14). In the present study, we cloned the *aac*(6')-*aph*(2'') genes from *E. faecalis* HH22 (22) and expressed the enzyme in a recombinant strain of *E. coli*. The susceptibilities of vertilmicin, verdamicin, and netilmicin to acetylation and phosphorylation by the recombinant enzyme were analyzed by spectrophotometric coupled assays.

MATERIALS AND METHODS

Antibiotics and reagents. Vertilmicin, verdamicin, and netilmicin were obtained from the Zhejiang Conler Pharmaceutical Co. Ltd., People's Republic of China. Gentamicin and amikacin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, People's Republic of China. Kanamycin was from Amresco, Inc. The pGEM-T vector system (isopropyl-β-D-thiogalactopyranoside) was from Promega. The *Pfu* DNA polymerases and UNIQ-10 spin columns were purchased from the Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., People's Republic of China. The restriction endonucleases *Nde*I and *Xho*I, together with their deoxynucleoside triphosphate mixture, were purchased from Takara, Japan. All other reagents were purchased from Sigma Chemical Co. Oligonucleotide primers were synthesized by the SBS Genetech Co., Ltd., People's Republic of China. Both Mueller-Hinton (MH) agar and MH broth were purchased from Difco. Cation-adjusted MH broth (CAMHB) and *Haemophilus* test medium were prepared according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) (24).

Organisms. A total of 1,185 bacterial isolates were tested in vitro, including *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter diversus*, *Proteus mirabilis*, *Serratia marcescens*, *Morganella morganii*, *Citrobacter freundii*, *Proteus retgeri*, *Pseudomonas aeruginosa* and other *Pseudomonas* spp., *Acinetobacter calcoaceticus*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *S. aureus*, *Staphylococcus epidermidis*, and *E. faecalis*. All bacterial strains used in the study were collected from hospitals in Beijing between 2000 and 2001. *E. faecalis* HH22, which contains the *aac*(6')-*aph*(2'') gene on plasmid pBEM10, was a gift from B. E. Murray of the University of Texas Health Sciences Center. All of the isolates were frozen at –70°C until they were used.

MIC determination. MICs were determined by the agar dilution method recommended by the CLSI (24). Inocula were adjusted to yield approximately 10⁴ CFU/spot using a multipoint inoculator (Denley Instruments, Bolney, Sussex, United Kingdom) and incubated at 35°C for 18 h. The MIC was determined as the lowest concentration of the antibiotic that inhibited the growth of the bacteria on the plate. The MIC₅₀ and MIC₉₀ represented, respectively, the concentrations at which 50% and 90% of the isolates were inhibited. The quality control strains recommended by the CLSI were included as internal controls throughout the study.

MBC determination. Minimum bactericidal concentrations (MBCs) were determined by the macrodilution method using CAMHB according to the CLSI recommendation (23). Briefly, series of broth tubes containing different concentrations of agents were inoculated with the test bacteria (initial inoculum, 5 × 10⁵ CFU/ml) and incubated at 35°C for 18 h; then duplicate 0.1-ml samples of broth from each tube with no growth were taken and transferred onto plain MH agar

plates for an 18-h incubation at 35°C. The mean CFU were calculated. The MBC was the lowest concentration of the antibacterial agent that caused a reduction in the number of colonies by at least 3 log units compared to the initial inoculation.

Time-kill curve study. To further study the bactericidal activity of vertilmicin, the time-kill curves of vertilmicin were determined in four isolates, *E. coli* ATCC 25922, *K. pneumoniae* 01-21, *S. aureus* ATCC 29213, and *S. epidermidis* 01-55, with gentamicin as the reference agent. First, the overnight (35°C; 18-h) cell cultures of the test strains were diluted to 10⁶ CFU/ml in CAMHB and stabilized at 35°C for 30 min. Then, vertilmicin or gentamicin was added to the cultures at various final concentrations (0.25 MIC to 4 MIC), and incubation continued at 35°C. Flasks without antibiotics were used as the controls. Viable cells were counted at 0, 2, 4, and 8 h after incubation with different concentrations of vertilmicin or gentamicin. The drug carryover effect was eliminated by broth and agar dilution. The agents were considered bactericidal at a concentration at which the original inoculum was reduced by over 3 log units (99.9% reduction).

Construction of the plasmid expressing AAC(6')-APH(2''). The genomic DNA of *E. faecalis* HH22 was extracted and used as a template to amplify the open-reading-frame region of the aminoglycoside bifunctional modifying enzyme with *Pfu* DNA polymerase. The primers used were 5'-TCCCATATGAATATAGTTGAAAATG-3' and 5'-CCTCGAGATCTTTATAAGTCCTT-3' (the *Nde*I [CATATG] and *Xho*I [CTCGAG] restriction enzyme sites are underlined, and the start codon [ATG] is in boldface). The stop codon (TGA) was eliminated from the primer in order to add a six-His tag to the C terminus of the protein. The PCR product was cleaned with a UNIQ-10 spin column, digested with *Nde*I and *Xho*I, and ligated first into a pGEM-T vector and then into a pET-30a(+) vector. The ligated DNA in the pET-30a(+) vector was used to transform *E. coli* BL21(DE3). The orientation and DNA sequence of the insert were confirmed by nucleotide sequencing. The pET-30a(+) recombinant plasmid was given the name pET-A6P2.

Expression and partial purification of AAC(6')-APH(2''). For expression of the protein, 3 ml of an overnight culture of *E. coli* BL21(DE3) harboring pET-A6P2 was inoculated into 500 ml of Luria-Bertani medium containing 50 µg/ml of kanamycin, followed by incubation at 37°C with shaking. When the optical density at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM for induction, and the culture grew for an additional 3 h. The cells were harvested by centrifugation, washed twice with 0.85% NaCl, and resuspended in 30 ml of binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride. Then, the cells were disrupted by passage through a French press (Thermo Fisher Scientific) twice at 800 lb/in². After centrifugation at 10,000 × *g* for 20 min at 4°C, the supernatant was loaded onto a HiTrap chelating HP Ni²⁺ affinity column. The protein was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole). Fractions containing the protein were pooled and concentrated using a Millipore Ultrafree-4 centrifugal filter unit, and the buffer was switched to 20 mM Tris, pH 7.6. The purity and size of the protein were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the concentration was determined by the Bradford method with bovine serum albumin as the standard.

Susceptibility of vertilmicin to modifications by recombinant AAC(6')-APH(2''). The susceptibility of vertilmicin to modifications by the recombinant enzyme was examined in comparison with those of netilmicin and verdamicin. Acetylation was determined by coupling the production of coenzyme A to the chemical reaction with 4,4'-dithiodipyridine (16). The reaction progress was monitored continuously at 324 nm with a Cary 3E UV-visible-light spectrophotometer (Varian) for the production of free pyridine-4-thiolate (extinction coef-

TABLE 1. In vitro antibacterial activities of vertilmicin and other aminoglycosides against non-gentamicin-resistant clinical isolates

Organism (no. of strains)	Antibiotic	MIC ($\mu\text{g/ml}$)			% Susceptibility ^a
		Range	50%	90%	
<i>E. coli</i> (54)	Vertilmicin	0.25–8	0.5	0.5	100.0
	Verdamycin	0.25–16	0.5	0.5	98.1
	Gentamicin	0.25–4	0.5	1	100.0
	Amikacin	0.5–4	1	2	100.0
	Netilmicin	0.25–4	0.5	0.5	100.0
<i>K. pneumoniae</i> (83)	Vertilmicin	0.125–16	0.25	0.5	97.6
	Verdamycin	0.125–128	0.25	0.5	98.8
	Gentamicin	0.125–4	0.25	0.5	100.0
	Amikacin	0.5–16	1	1	100.0
	Netilmicin	0.25–128	0.5	0.5	94.0
<i>E. cloacae</i> (55)	Vertilmicin	0.125–8	0.25	0.5	100.0
	Verdamycin	0.25–32	0.25	0.5	98.2
	Gentamicin	0.06–8	0.25	0.5	98.2
	Amikacin	0.25–128	2	4	96.4
	Netilmicin	0.25–128	0.5	2	98.2
<i>E. aerogenes</i> (54)	Vertilmicin	0.25–16	0.5	2	98.1
	Verdamycin	0.25–32	0.5	1	98.1
	Gentamicin	0.125–8	0.25	0.5	98.1
	Amikacin	≤ 0.03 –16	1	2	100.0
	Netilmicin	0.25–64	0.5	1	98.1
<i>C. diversus</i> (23)	Vertilmicin	0.25–2	0.25	0.5	100.0
	Verdamycin	0.25–0.5	0.25	0.5	100.0
	Gentamicin	0.25–1	0.25	0.5	100.0
	Amikacin	0.5–2	1	2	100.0
	Netilmicin	0.25–2	0.5	0.5	100.0
<i>P. mirabilis</i> (63)	Vertilmicin	0.5–16	1	2	98.4
	Verdamycin	0.5–8	1	2	100.0
	Gentamicin	0.25–8	0.5	1	96.8
	Amikacin	0.5–8	2	4	100.0
	Netilmicin	0.5–32	1	2	98.4
<i>S. marcescens</i> (27)	Vertilmicin	0.25–4	0.25	1	100.0
	Verdamycin	0.25–>128	0.25	0.5	96.3
	Gentamicin	0.125–1	0.25	0.5	100.0
	Amikacin	0.5–>128	1	2	96.3
	Netilmicin	0.5–>128	1	4	96.3
<i>M. morganii</i> (25)	Vertilmicin	0.5–4	1	2	100.0
	Verdamycin	0.5–4	1	2	100.0
	Gentamicin	0.5–8	0.5	8	76.0
	Amikacin	0.5–64	1	2	96.0
	Netilmicin	0.5–8	1	2	100.0
<i>C. freundii</i> (18)	Vertilmicin	0.25–32	0.5	8	90.0
	Verdamycin	0.25–128	0.5	64	72.2
	Gentamicin	0.25–2	0.25	1	100.0
	Amikacin	1–8	2	4	100.0
	Netilmicin	0.5–32	1	16	83.3
<i>P. rettgeri</i> (13)	Vertilmicin	0.5–16	2	8	92.3
	Verdamycin	0.5–8	2	8	100.0
	Gentamicin	0.25–8	4	8	84.6
	Amikacin	0.5–32	2	32	84.6
	Netilmicin	0.5–16	2	8	92.3
<i>A. calcoaceticus</i> (49)	Vertilmicin	0.25–32	1	2	93.8
	Verdamycin	0.25–32	0.5	2	98.0
	Gentamicin	0.125–8	0.25	2	98.0
	Amikacin	0.25–64	0.5	2	98.0
	Netilmicin	0.5–>128	1	16	87.8
<i>P. aeruginosa</i> (61)	Vertilmicin	0.25–64	8	32	78.7
	Verdamycin	0.25–>128	8	16	86.9
	Gentamicin	0.06–8	4	8	65.6
	Amikacin	0.06–16	4	16	100.0
	Netilmicin	0.06–64	8	32	63.9
Other <i>Pseudomonas</i> spp. (11)	Vertilmicin	0.25–32	8	16	72.7
	Verdamycin	0.5–8	2	8	100.0
	Gentamicin	0.25–8	2	8	81.8
	Amikacin	0.5–64	4	16	90.9
	Netilmicin	2–128	8	32	72.7

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TABLE 1—Continued

Organism (no. of strains)	Antibiotic	MIC ($\mu\text{g/ml}$)			% Susceptibility ^a
		Range	50%	90%	
<i>H. influenzae</i> ^b (36)	Vertilmicin	0.5–1	1	1	NA
	Verdamicin	0.5–2	1	1	NA
	Gentamicin	0.5–4	2	2	NA
	Amikacin	4–16	8	16	NA
	Netilmicin	0.5–2	1	2	NA
<i>H. parainfluenzae</i> ^b (30)	Vertilmicin	0.5–4	1	2	NA
	Verdamicin	0.25–4	1	2	NA
	Gentamicin	1–8	2	4	NA
	Amikacin	2–16	4	8	NA
	Netilmicin	0.5–4	1	2	NA
<i>S. aureus</i> (77)	Vertilmicin	0.06–32	0.125	2	94.8
	Verdamicin	0.06–32	0.125	8	94.8
	Gentamicin	≤ 0.03 –8	0.125	8	85.7
	Amikacin	0.06–8	1	2	100.0
	Netilmicin	0.06–>128	0.25	16	88.3
<i>S. epidermidis</i> (80)	Vertilmicin	≤ 0.03 –2	0.125	1	100.0
	Verdamicin	≤ 0.03 –32	0.5	32	75.0
	Gentamicin	≤ 0.03 –8	0.06	4	92.5
	Amikacin	≤ 0.03 –64	0.5	2	98.8
	Netilmicin	0.06–4	0.25	1	100.0

^a Susceptibility values were calculated by using the standards of CLSI: $\leq 4 \mu\text{g/ml}$ for gentamicin, $\leq 8 \mu\text{g/ml}$ for netilmicin, and $\leq 16 \mu\text{g/ml}$ for amikacin. For vertilmicin and verdamicin, we used $\leq 8 \mu\text{g/ml}$ as an exploratory standards, considering their structural similarity to netilmicin. NA, not applicable.

^b There are no aminoglycoside standards for these groups of isolates.

ficient, $19,800 \text{ M}^{-1} \text{ cm}^{-1}$). The assay mixture contained 50 mM Tris (pH 7.6), 1 mM EDTA, 2 mM 4,4'-dithiodipyridine, 40 μM acetyl-coenzyme A, 10 μl of the partially purified protein (4.5 mg/ml), various concentrations of aminoglycoside antibiotics (100 μM to 2 mM for verdamicin, 25 μM to 1 mM for netilmicin, and 250 μM to 2 mM for vertilmicin) in a volume of 2 ml. The mixtures without aminoglycosides were stabilized at 37°C for 5 min, and the reactions were initiated by adding 10 μl of aminoglycoside antibiotic stocks of different concentrations. Negative controls were prepared by omitting aminoglycosides, and the readings were subtracted from the test samples.

Phosphorylation of the antibiotics was determined by coupling the release of ADP to the pyruvate kinase/lactate dehydrogenase reaction (19) and monitoring the oxidation of NADH at 340 nm (extinction coefficient, $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) using a Cary 3E UV-visible-light spectrophotometer. The assay mixture contained the following components in a volume of 2 ml: 50 mM Tris (pH 8.0), 40 mM KCl, 10 mM MgCl_2 , 247 μM NADH, 2.5 mM phosphoenolpyruvate, 1 mM ATP, various concentrations of aminoglycosides (5 μM to 1.25 mM for verdamicin, 62.5 μM to 1.25 mM for netilmicin, and 37.5 μM to 1.25 mM for vertilmicin), 5 μl pyruvate kinase/lactate dehydrogenase enzyme solution, and 10 μl of AAC(6')-APH(2''). The mixtures without the AAC(6')-APH(2'') enzyme were preincubated at 37°C for 5 min, and the reactions were initiated by adding 10 μl of the partially purified protein (4.5-mg/ml stock). Negative controls were prepared by omitting aminoglycosides, and the readings were subtracted from the test samples.

The initial velocities were obtained directly from the progress curves and analyzed with a Lineweaver-Burk plot for the maximum rate of metabolism (V_{max}) and K_m values. The physiological efficiency (V_{max}/K_m) was calculated thereafter.

RESULTS

MIC determination. The antibacterial activities of vertilmicin, verdamicin, netilmicin, gentamicin, and amikacin were evaluated with the MIC range, MIC_{50} , MIC_{90} , and percent susceptibility. The results are shown in Table 1 for the non-gentamicin-resistant strains with gentamicin MICs of $\leq 8 \mu\text{g/ml}$ (8) and Table 2 for the gentamicin-resistant strains with gentamicin MICs of $\geq 16 \mu\text{g/ml}$ (8).

For the non-gentamicin-resistant strains (Table 1), the aminoglycosides showed similar activities against *Enterobacteria-*

ceae, *Staphylococcus* spp., and *A. calcoaceticus*, with susceptibility values over 90%. The MIC_{50} s were in the range of 0.125 to 2 $\mu\text{g/ml}$ for vertilmicin and verdamicin, 0.06 to 4 $\mu\text{g/ml}$ for gentamicin, 0.5 to 8 $\mu\text{g/ml}$ for amikacin, and 0.25 to 2 $\mu\text{g/ml}$ for netilmicin. For the isolates of *P. aeruginosa* and other *Pseudomonas* spp., vertilmicin and netilmicin showed lower activities than other aminoglycosides did, with MIC_{50} s of 8 $\mu\text{g/ml}$, MIC_{90} s of 16 to 32 $\mu\text{g/ml}$, and susceptibility values around 70%. For the isolates of *Haemophilus* spp., all the aminoglycosides showed high activities, with MIC_{50} s of 1 to 8 $\mu\text{g/ml}$ and MIC_{90} s of 1 to 16 $\mu\text{g/ml}$; however, as no aminoglycoside standards were available for these groups of isolates, the percent susceptibility was not presented.

Vertilmicin was effective for some of the gentamicin-resistant isolates (Table 2), with susceptibility values over 50% for *E. coli*, *M. morgani*, *C. freundii*, *P. rettgeri*, *S. aureus*, and *S. epidermidis*. For the gram-negative gentamicin-resistant isolates, the susceptibility values of the aminoglycosides were on the order of amikacin > vertilmicin $\approx$ netilmicin > verdamicin in general. Among the study aminoglycosides, vertilmicin was the most active against gram-positive gentamicin-resistant isolates of *S. aureus* and *S. epidermidis*. The MIC_{90} s against *S. aureus* and *S. epidermidis* were 16 $\mu\text{g/ml}$ for vertilmicin, >128 $\mu\text{g/ml}$ for verdamicin, 128 and 64 $\mu\text{g/ml}$ for amikacin, and 128 and 32 $\mu\text{g/ml}$ for netilmicin, respectively. For the isolates of *E. faecalis*, aminoglycosides were inactive in general, but vertilmicin and netilmicin showed activity in some of the strains, with MIC_{50} s of 4 $\mu\text{g/ml}$.

Bactericidal activity. The bactericidal activity tests included MBC and time-kill curve determinations. The MBCs were determined for a total of 69 isolates: *E. coli* (18 strains), *K. pneumoniae* (19 strains), *S. aureus* (19 strains), and *S. epidermidis* (13 strains) (Table 3). For all of the isolates tested, vertilmicin and gentamicin showed high bactericidal activity,

TABLE 2. In vitro antibacterial activities of vertilmicin and other aminoglycosides against gentamicin-resistant clinical isolates

Organism (no. of strains)	Antibiotic	MIC ($\mu\text{g/ml}$)			% Susceptibility ^a
		Range	50%	90%	
<i>E. coli</i> (57)	Vertilmicin	0.5->128	8	64	68.4
	Verdamycin	4->128	32	128	3.5
	Gentamicin	16->128	64	>128	0.0
	Amikacin	0.5->128	1	4	93.0
	Netilmicin	0.5->128	8	64	66.7
<i>K. pneumoniae</i> (28)	Vertilmicin	0.25->128	16	>128	32.1
	Verdamycin	0.25->128	128	>128	10.7
	Gentamicin	16->128	128	>128	0.0
	Amikacin	0.5->128	1	>128	82.1
	Netilmicin	0.5->128	32	>128	17.9
<i>E. cloacae</i> (19)	Vertilmicin	0.25->128	32	>128	26.3
	Verdamycin	0.25->128	128	>128	5.3
	Gentamicin	16->128	>128	>128	0.0
	Amikacin	2->128	16	32	63.2
	Netilmicin	8->128	128	>128	5.3
<i>E. aerogenes</i> (20)	Vertilmicin	0.5->128	16	>128	30.0
	Verdamycin	32->128	>128	>128	0.0
	Gentamicin	32->128	>128	>128	0.0
	Amikacin	1->128	16	64	55.0
	Netilmicin	2->128	32	128	30.0
<i>C. diversus</i> (14)	Vertilmicin	4->128	32	>128	7.1
	Verdamycin	16->128	>128	>128	0.0
	Gentamicin	32->128	>128	>128	0.0
	Amikacin	1-64	32	64	35.7
	Netilmicin	1->128	>128	>128	7.1
<i>P. mirabilis</i> (11)	Vertilmicin	2->128	32	>128	36.4
	Verdamycin	2->128	>128	>128	18.2
	Gentamicin	16->128	128	>128	0.0
	Amikacin	0.5->128	4	>128	63.6
	Netilmicin	1->128	16	>128	36.4
<i>S. marcescens</i> (10)	Vertilmicin	8->128	>128	>128	10.0
	Verdamycin	0.25->128	>128	>128	10.0
	Gentamicin	32->128	>128	>128	0.0
	Amikacin	0.5->128	>128	>128	30.0
	Netilmicin	8->128	>128	>128	10.0
<i>M. morgani</i> (12)	Vertilmicin	1->128	8	32	50.0
	Verdamycin	8->128	64	128	8.3
	Gentamicin	32->128	128	>128	0.0
	Amikacin	1->128	1	4	83.3
	Netilmicin	4->128	8	16	41.7
<i>C. freundii</i> (19)	Vertilmicin	0.5->128	8	32	52.6
	Verdamycin	0.5->128	32	>128	26.3
	Gentamicin	16->128	64	128	0.0
	Amikacin	0.5->128	2	32	31.6
	Netilmicin	0.25->128	8	>128	47.4
<i>P. rettgeri</i> (15)	Vertilmicin	4-128	8	64	53.3
	Verdamycin	4-128	32	128	20.0
	Gentamicin	16->128	64	128	0.0
	Amikacin	0.5-64	1	32	80.0
	Netilmicin	4->128	16	128	40.0
<i>A. calcoaceticus</i> (25)	Vertilmicin	1->128	128	>128	24.0
	Verdamycin	0.5->128	>128	>128	16.0
	Gentamicin	16->128	>128	>128	0.0
	Amikacin	0.5->128	16	>128	48.0
	Netilmicin	1->128	128	>128	20.0
<i>P. aeruginosa</i> (34)	Vertilmicin	4->128	32	>128	17.6
	Verdamycin	8->128	>128	>128	2.9
	Gentamicin	16->128	>128	>128	0.0
	Amikacin	2->128	8	32	85.3
	Netilmicin	8->128	>128	>128	2.9
Other <i>Pseudomonas</i> spp. (26)	Vertilmicin	2->128	128	>128	7.7
	Verdamycin	8->128	128	>128	3.8
	Gentamicin	16->128	128	>128	0.0
	Amikacin	2->128	>128	>128	7.7
	Netilmicin	16->128	>128	>128	0.0

Continued on following page

TABLE 2—Continued

Organism (no. of strains)	Antibiotic	MIC ($\mu\text{g/ml}$)			% Susceptibility ^a
		Range	50%	90%	
<i>S. aureus</i> (36)	Vertilmicin	0.125–32	4	16	83.3
	Verdamycin	8–>128	32	>128	5.6
	Gentamicin	16–>128	32	128	0.0
	Amikacin	1–>128	8	128	80.6
	Netilmicin	0.125–>128	16	128	47.2
<i>S. epidermidis</i> (26)	Vertilmicin	0.125–128	2	16	88.5
	Verdamycin	0.06–>128	8	>128	50.0
	Gentamicin	16–>128	16	>128	0.0
	Amikacin	2–128	4	64	73.1
	Netilmicin	0.5–>128	2	32	84.6
<i>E. faecalis</i> ^b (74)	Vertilmicin	0.25–>128	4	>128	NA
	Verdamycin	1–>128	128	>128	NA
	Gentamicin	2–>128	>128	>128	NA
	Amikacin	32–>128	128	>128	NA
	Netilmicin	0.5–>128	4	>128	NA

^a Susceptibility values were calculated by using the standards of CLSI: $\leq 4 \mu\text{g/ml}$ for gentamicin, $\leq 8 \mu\text{g/ml}$ for netilmicin, and $\leq 16 \mu\text{g/ml}$ for amikacin. For vertilmicin and verdamycin, we used $\leq 8 \mu\text{g/ml}$ as an exploratory standards, considering their structural similarity to netilmicin. NA, not applicable.

^b There are no aminoglycoside standards for these groups of isolates.

with MBC/MIC ratios in the range of 1 to 2 for *E. coli*, *K. pneumoniae*, and *S. aureus* and 1 to 4 for *S. epidermidis*.

In the time-kill curve study (Fig. 2), the bactericidal activities of vertilmicin and gentamicin were determined in *E. coli* ATCC 25922, *K. pneumoniae* 01-21, *S. aureus* ATCC 29213, and *S. epidermidis* 01-55. Vertilmicin and gentamicin showed similar bactericidal effects (over a 3-log-unit decrease in the viable-cell numbers) at concentrations as low as 0.25 or 0.5 MIC in the strains tested. The bactericidal effect of vertilmicin or gentamicin was rapid and concentration dependent and was maintained for up to 8 h when the concentration was equal to or higher than 1 MIC.

Susceptibility of vertilmicin to modifications by recombinant AAC(6')-APH(2''). The susceptibility of vertilmicin to acetylation and phosphorylation by the bifunctional AAC(6')-APH(2'') was compared to those of netilmicin and verdamycin. Taking these aminoglycosides as substrates, kinetic parameters (K_m , $V_{\max}$, and $V_{\max}/K_m$) were determined (Table 4) by coupled enzymatic assay as described in Materials and Methods. A high value of the $V_{\max}/K_m$ ratio (25) indicates a good substrate for modification and a poor antibiotic against aminoglycoside-resistant bacteria containing the modification enzyme. As for acetylation by AAC(6'), the $V_{\max}/K_m$ ratios of vertilmicin and verdamycin were 4.2% and 4.5%, respectively, of that of netilmicin. For phosphorylation by APH(2''), vertilmicin and netilmicin showed $V_{\max}/K_m$ ratios much lower than that of verdamycin (19.9% and 7.4%, respectively). The low $V_{\max}/K_m$ ratios of

vertilmicin for acetylation and phosphorylation strongly suggest that vertilmicin might be a better antibiotic than verdamycin and netilmicin against aminoglycoside-resistant bacteria.

DISCUSSION

As a novel semisynthetic aminoglycoside, the in vitro antibacterial activity of vertilmicin was evaluated by MIC, MBC, and time-kill curve tests. The MIC determination was tested in a total of 1,185 isolates using the agar dilution method with verdamycin, netilmicin, gentamicin, and amikacin as the reference compounds. The 1,185 isolates were divided into two groups, the non-gentamicin-resistant group and the gentamicin-resistant group, according to the CLSI standard. In general, vertilmicin is a broad-spectrum agent with high activity against both gram-negative and gram-positive isolates. Its antibacterial activity was similar to that of netilmicin and higher than those of gentamicin and verdamycin but lower than that of amikacin. The similar activities of vertilmicin and netilmicin reflected the structural similarity of the two antibiotics. The higher activity of vertilmicin than verdamycin, especially against gentamicin-resistant isolates, suggested that 1-N derivation might increase the activity of the antibiotic as a result of the substrate being less vulnerable to the AMEs. Amikacin remained the most potent agent among the five, as was observed by Meyer et al. (20). This might be due to the different structures of the antibiotics, which affect permeability or sus-

TABLE 3. Distribution of MBC/MIC ratios of vertilmicin and gentamicin

Organism	No. of strains	No. (%) of strains with MBC/MIC ratio at:					
		Vertilmicin			Gentamicin		
		1	2	4	1	2	4
<i>E. coli</i>	18	17 (94.4)	1 (5.6)	0 (0.0)	18 (100)	0	0 (0.0)
<i>K. pneumoniae</i>	19	17 (89.5)	2 (10.5)	0 (0.0)	18 (94.7)	1 (5.3)	0 (0.0)
<i>S. aureus</i>	19	17 (89.5)	2 (10.5)	0 (0.0)	17 (89.5)	2 (10.5)	0 (0.0)
<i>S. epidermidis</i>	13	8 (61.5)	3 (23.1)	2 (15.4)	9 (69.2)	2 (15.4)	2 (15.4)

FIG. 2. Time-kill curves of vertilmicin and gentamicin against isolates. (a) Vertilmicin and *E. coli* ATCC 25922 (MIC = 0.25 $\mu\text{g/ml}$). (b) Gentamicin and *E. coli* ATCC 25922 (MIC = 0.5 $\mu\text{g/ml}$). (c) Vertilmicin and *K. pneumoniae* 01-21 (MIC = 0.25 $\mu\text{g/ml}$). (d) Gentamicin and *K. pneumoniae* 01-21 (MIC = 0.25 $\mu\text{g/ml}$). (e) Vertilmicin and *S. aureus* ATCC 29213 (MIC = 0.125 $\mu\text{g/ml}$). (f) Gentamicin and *S. aureus* ATCC 29213 (MIC = 0.125 $\mu\text{g/ml}$). (g) Vertilmicin and *S. epidermidis* 01-55 (MIC = 0.25 $\mu\text{g/ml}$). (h) Gentamicin and *S. epidermidis* 01-55 (MIC = 1 $\mu\text{g/ml}$). The detection limit was 10 CFU/ml. $\diamond$, control; $\blacklozenge$, 0.25 MIC; $\square$, 0.5 MIC; $\blacksquare$, MIC; $\triangle$, 2 MIC; $\blacktriangle$, 4 MIC.

ceptibility to the modifying enzyme, as vertilmicin and netilmicin belong to the gentamicin subgroup while amikacin is from kanamycin.

In the non-gentamicin-resistant isolate group (Table 1), all of the aminoglycosides tested had good activities, with susceptibility values approaching 100%. In the gentamicin-resistant isolate group (Table 2), the susceptibility values were much lower in general and the differences in activities among the aminoglycosides were more dramatic, with amikacin showing the highest susceptibility, followed by vertilmicin, netilmicin, and verdamycin. It should be mentioned here that vertilmicin showed high susceptibility values in gram-positive isolates of *S. aureus* and *S. epidermidis*, even higher than those of amikacin. Netilmicin exhibited high activity against *S. epidermidis*, but not *S. aureus*.

In the MBC test, vertilmicin exhibited potent bactericidal activity against both gram-negative and gram-positive isolates, with MBC/MIC ratios in the range of 1 to 4. The time-kill curve study further demonstrated a rapid, concentration-dependent pattern. Comparison of the time-kill curves of vertilmicin and gentamicin showed similarity in the bactericidal activities of the two agents. Both of them exhibited bactericidal activities at concentrations as low as 0.25 or 0.5 MIC, and when the concentrations went up to 1 MIC, the bactericidal effects could be maintained for up to 8 h.

We also studied the susceptibilities of vertilmicin, netilmicin, and verdamycin to modifications by the recombinant AAC(6')-APH(2''). By comparing the $V_{\max}/K_m$ ratios, vertilmicin proved to be the best antibiotic of the three, showing low efficiency as a substrate to both acetylation and phosphorylation activities of the enzyme. This may explain, to some extent, the high susceptibility of vertilmicin in the gentamicin-resistant *S. aureus*, because this group usually exerts resistance to aminoglycosides by producing an aminoglycoside bifunctional modifying enzyme. In this study, netilmicin was the best substrate for acetylation activity, consistent with previous reports showing that netilmicin could be inactivated by acetyltransferase (6') (20, 21). Verdamycin was the best substrate for phosphorylation activity, and the 1-N derivative of the antibiotic (i.e., vertilmicin) posted a strong inhibition effect on phosphorylation, about fivefold.

The differences in the $V_{\max}/K_m$ ratios of the three aminoglycosides was mainly contributed by the variations in K_m for phosphorylation and $V_{\max}$ for acetylation. In conjunction with the chemical structures, the results suggested that for the phosphorylation reaction, ethylation at the 1-N position caused an increase in K_m (verdamycin versus vertilmicin) and that removal of a methyl group from the 6' position further increased the K_m value (vertilmicin versus netilmicin). None of these structural modifications resulted in a significant change in the $V_{\max}$ for phosphorylation. In contrast, methylation at the 6' position seemed to play a critical role in the $V_{\max}$ for acetylation (vertilmicin and verdamycin versus netilmicin). In conclusion, vertilmicin is a broad-spectrum semisynthetic aminoglycoside antibiotic with high activity against both gram-negative and gram-positive isolates. The activity was similar to that of netilmicin, lower than that of amikacin, and higher than those of verdamycin and gentamicin. Vertilmicin also demonstrated

TABLE 4. Comparison of kinetic modification parameters of vertilmicin, netilmicin, and verdamycin by AAC(6')-APH(2'')

Antibiotic	Acetylation			Phosphorylation		
	K_m^a (μM)	Relative $V_{\max}^b$ (%)	Relative $V_{\max}/K_m^c$ (%)	K_m (μM)	Relative $V_{\max}^b$ (%)	Relative $V_{\max}/K_m^c$ (%)
Vertilmicin	469.4	6.5	4.2	103.6	49.8	19.9
Netilmicin	304.2	100	100	308.2	55.0	7.4
Verdamycin	655.8	9.6	4.5	41.4	100	100

^a K_m was calculated by Lineweaver-Burk plot.

^b $V_{\max}$, the maximal initial rate, was calculated as for K_m ; As the protein was partially purified, the $V_{\max}$ was expressed in percentages compared with netilmicin (100%) in acetylation or verdamycin (100%) in phosphorylation.

^c Physiological efficiency ($V_{\max}/K_m$) is given in percentages, as for $V_{\max}$.

resistance to modifications by AAC(6')-APH(2'') and hence deserves further study with other AMEs.

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