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Susceptibility of Vertilmicin to Modifications by Three Types of Recombinant Aminoglycoside-Modifying Enzymes[▽]

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The susceptibilities of vertilmicin and seven reference aminoglycosides to modifications by six recombinant aminoglycoside-modifying enzymes, AAC(6′)-Ie, APH(2′′)-Ia, AAC(6′)-Ie-APH(2′′)-Ia, ANT(2′′)-Ia, AAC(6′)-Ib, and AAC(6′)-Ib-cr, were studied by coupled spectrophotometric assays in microtiter plates. In comparison to other aminoglycosides, the susceptibility of vertilmicin was 45.8- to 250.0-fold lower for AAC(6′)-Ie acetylation, 39.2- to 116.7-fold lower for AAC(6′)-Ie-APH(2′′)-Ia acetylation, and 1.8- to 7.5-fold lower for ANT(2′′)-Ia adenylation (except that shown by amikacin) while relatively comparable for AAC(6′)-Ib acetylation, AAC(6′)-Ib-cr acetylation, APH(2′′)-Ia phosphorylation, and AAC(6′)-Ie-APH(2′′)-Ia phosphorylation.

The semisynthetic aminoglycosides came into being as an effective way to tackle the problem of bacterial resistance to aminoglycosides mediated by covalent modifications through aminoglycoside-modifying enzymes (AMEs) (8), namely, *N*-acetyltransferase (AAC), *O*-adenylyltransferase (ANT), and *O*-phosphotransferase (APH) (15, 17). The AMEs evolved with antibiotic therapy, and the trend of evolution can be summarized into two mechanisms, gene fusion and gene mutation. Examples of the former are AAC(6′)-Ie-APH(2′′)-Ia, ANT(3′′)-Ii-AAC(6′)-IId, AAC(3)-Ib-AAC(6′)-Ib, and AAC(6′)-30-AAC(6′)-Ib′ (2, 4, 5, 12). One example of the latter is the new variant of AAC(6′)-Ib, termed AAC(6′)-Ib-cr, which can take fluoroquinolones in addition to aminoglycosides as substrates (20).

Vertilmicin (1-*N*-ethyl verdamicin), a new semisynthetic aminoglycoside discovered in 2000, demonstrated increased stability to AAC(6′)-Ie-APH(2′′)-Ia modifications in our initial test (9). In this study, the susceptibility of vertilmicin to three types of AME modifications was further systemically evaluated in comparison with those of seven other aminoglycosides, namely, verdamicin, netilmicin (1-*N*-ethyl sisomicin), sisomicin, amikacin (1-*N*-amino-hydroxybutyryl kanamycin A), kanamycin, etilmicin (1-*N*-ethyl gentamicin C_{1a}), and gentamicin (a mixture of gentamicin C_1 [~28.8%], C_{1a} [~32.9%], C_{2a} [~19.5%], and C_2 [~19.7%]).

The AMEs used were recombinant C-terminal 6-His-tagged AAC(6′)-Ie, APH(2′′)-Ia, AAC(6′)-Ie-APH(2′′)-Ia, ANT(2′′)-Ia, AAC(6′)-Ib, and AAC(6′)-Ib-cr, which were selected based on epidemiologic data and the aminoglycoside resistance profiles of the enzyme. The bifunctional enzyme AAC(6′)-Ie-APH(2′′)-Ia was selected, as it is the most important yet most difficult aminoglycoside resistance protein to overcome (1), which conferred high-level gentamicin resistance in Gram-positive pathogens like *Enterococcus* and *Staphylococcus*. The presence of this enzyme prevents the successful use of most aminoglycosides as therapeutic agents. The truncated AAC(6′)-Ie and APH(2′′)-Ia were used to validate the data with the full-length bifunctional enzyme. AAC(6′)-Ib was selected, as it is probably the most clinically relevant acetyltransferase, present in over 70% of AAC(6′)-I-producing Gram-negative clinical isolates (19). AAC(6′)-Ib-cr, a variant of AAC(6′)-Ib, was selected, considering its significance in extending the resistant spectrum to fluoroquinolones (16, 20). ANT(2′′)-Ia was also selected, as it is the only nucleotidyltransferase which has a resistance profile covering the aminoglycosides we used (gentamicin, sisomicin, kanamycin, etc) and it is widespread among all Gram-negative bacteria (14.9 to 21.1%) (15, 17). An AAC(6′)-Ie-APH(2′′)-Ia overexpression plasmid (pET-A6P2) was constructed previously (9). The plasmids pET-A6, pET-P2, pET-ANT2, pET-AAC6, and pET-AAC6CR for overexpression of AAC(6′)-Ie, APH(2′′)-Ia, ANT(2′′)-Ia, AAC(6′)-Ib, and AAC(6′)-Ib-cr were constructed by cloning the genes from gene-containing strains with the following appropriate primers (in parentheses, with restriction enzyme sites underlined) and ligated into pET29a(+) (*ant(2′′)-Ia*) or pET30a(+) (*aac(6′)-Ie*, *aph(2′′)-Ia*, *aac(6′)-Ib*, and *aac(6′)-Ib-cr*): *aac(6′)-Ie*, *Enterococcus faecalis* HH22 (14) (5′-TCC CAT ATG AAT ATA GTT GAA AAT G-3′ and 5′-TGC CTC GAG CTC AAT TAA ATA T-3′); *aph(2′′)-Ia*, *Enterococcus faecalis* HH22 (5′-GGC ATA TGG AAT ATA GAT ATG ATG AT-3′ and 5′-CCT CGA GAT CTT TAT AAG TCC TTT-3′); *ant(2′′)-Ia*, *Pseudomonas aeruginosa* PA2345 (10) (5′-GGC CAT ATG GAT ACA ACC CAG GTC ACG T-3′ and 5′-ATA AGC GGC CGC ATA TCT CGA CCT GAA AG-3′); *aac(6′)-Ib*, *Acinetobacter baumannii* UAA2544 (13) (5′-AGC CAT ATG ACC AAC AGC AAC GAT TC-3′ and 5′-AGG GTT AAG CTT CAC TGC GTG TTC G-3′); and *aac(6′)-Ib-cr*, *Klebsiella pneumoniae* KP96 (18) (5′-GGC ATA TGA GCA ACG CAA AAA CAA AGT TAG GC-3′

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and 5′-GTT AAG CTT CAC TGC GTG TTC GCT CGC TCG AAT-3′). (CATATG, NdeI; CTCGAG, XhoI; AAGCTT, HindIII; GCGGCCGC, NotI).

For protein overexpression, cultures of *Escherichia coli* BL21(DE3) harboring recombinant plasmids were grown in LB medium with kanamycin (37°C, 200 rpm) until the optical densities at 600 nm (OD_{600}s) reached 0.5 to 0.6, at which point 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction (120 rpm at 30°C for an additional 16 h). Cells were harvested by centrifugation at 5,600 × *g* for 10 min, washed twice with 0.85% NaCl, and resuspended in a buffer containing 25 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.2 mM dithiothreitol (DTT). Cells were disrupted via sonication for 30 min (1 s on, 3 s off pulse) using a sonifier, VC750 (SPRING Scientific). After centrifugation at 21,000 × *g* for 30 min, the supernatants were loaded onto HisTrap chelating HP Ni^+ affinity columns (GE Healthcare, Canada) with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole) with the AKTA purifier (GE Healthcare, Canada). The proteins were eluted with a linear gradient of 20 to 500 mM imidazole over 10 column volumes. Fractions containing the corresponding proteins were collected and loaded onto PD10 desalting columns (GE Healthcare, Canada), and the buffer was switched to 50 mM HEPES (pH 7.5). The purity and sizes of the proteins were confirmed by SDS-PAGE. The concentrations of the proteins were determined by the Lowry method, with bovine serum albumin used as the standard. The proteins were aliquoted and stored at −70°C for later use. All the procedures related to protein purification were done at 4°C.

The susceptibilities of vertilmicin and the reference compounds to modifications by the recombinant enzymes were measured by coupled reactions in microtiter plates with a total volume of 200 μl. Briefly, acetylations by AAC(6′)-Ie, AAC(6′)-Ie-APH(2′′)-Ia, AAC(6′)-Ib, and AAC(6′)-Ib-cr were measured by coupling the production of the sulfhydryl group of coenzyme A to the chemical reaction with 4,4′-dithiodipyridine (DTDP) (7). The assay mixtures contained 50 mM HEPES (pH 7.5), 2 mM DTDP, 1 mM EDTA, and 150 nmol AAC(6′)-Ie, 30 nmol AAC(6′)-Ie-APH(2′′)-Ia, 1.3 μmol AAC(6′)-Ib, or 1.7 μmol AAC(6′)-Ib-cr and variable concentrations of aminoglycosides with a fixed saturating concentration of acetyl coenzyme A (acetyl-CoA). Phosphorylations by APH(2′′)-Ia and AAC(6′)-Ie-APH(2′′)-Ia were determined by coupling the release of ADP to the pyruvate kinase/lactate dehydrogenase (PK/LDH) reaction and monitoring the oxidation of NADH to NAD^+ at 340 nm (extinction coefficient, 6.22 mM^{-1} cm^{-1}) (11). The assay mixtures contained 50 mM HEPES (pH 7.5), 2 μM $MgCl_2$, 0.5 mM NADH, 0.5 U phosphoenolpyruvie acid monopotassium salt (PEP-K), 0.5 U PK/LDH, 80 nmol recombinant enzymes, and variable concentrations of aminoglycosides, with the concentration of ATP fixed at 5 mM. Adenylation by ANT(2′′)-Ia was determined by coupling the enzymatic reaction to the reactions of UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase and monitoring the production of NADPH at 340 nm (6). The reaction mixtures contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.2 mM UDP-glucose, 0.2 mM DTDP, 2 U/ml UDP-glucose pyrophosphorylase, 20 U/ml phosphoglucomutase, 20 U/ml glucose-6-phosphate dehydrogenase, 0.7 μmol recombinant ANT(2′′)-Ia, and variable concentrations of aminoglycosides, with 5 mM ATP. The mixtures without enzymes were preincubated at 37°C for 5 min, and the reactions were initiated by addition of the enzymes.

The kinetics modification data were analyzed using GraFit 7.0 (Erithacus Software, Staines, United Kingdom), the k_{cat} (the number of catalytic turnover events that occur per unit time) and K_m (the substrate concentration that results in half-maximal velocity for an enzymatic reaction, which can be used as a relative measure of substrate binding affinity) values were determined, and the k_{cat}/K_m values, a measure of enzyme efficiency, were calculated thereafter. A high value of k_{cat} (rapid turnover) or a low value of K_m (high affinity for substrate) contributes to an increase in the value of k_{cat}/K_m.

The k_{cat}, K_m, and k_{cat}/K_m values of vertilmicin and the reference compounds to acetylation and/or phosphorylation by AAC(6′)-Ie, APH(2′′)-Ia, and AAC(6′)-Ie-APH(2′′)-Ia are shown in Table 1. Consistent with our previous results (9), vertilmicin was the most stable compound for acetylations by AAC(6′)-Ie and AAC(6′)-Ie-APH(2′′)-Ia, with the lowest k_{cat}/K_m values of $(1.2 \pm 0.1) \times 10^3$ and $(1.2 \pm 0.2) \times 10^5$ M^{-1} s^{-1} for AAC(6′)-Ie and AAC(6′)-Ie-APH(2′′)-Ia acetylation, respectively, which were 45.8- and 39.2-fold lower than those of the parental compound verdamicin and 69.1- to 250.0-fold and 60.0- to 116.7-fold lower than those of the other six aminoglycosides. The lower k_{cat}/K_m values of vertilmicin in comparison to those of the other compounds toward acetylations by AAC(6′)-Ie and AAC(6′)-Ie-APH(2′′)-Ia were contributed to both the higher K_m values (7.4- to 62.2-fold and 7.0- to 47.9-fold higher, respectively) and lower k_{cat} values (2.3- to 12.3-fold and 2.6- to 13.8-fold lower, respectively, except those of verdamicin in AAC(6′)-Ie-APH(2′′)-Ia acetylation). The k_{cat}/K_m values of the aminoglycosides toward phosphorylations by APH(2′′)-Ia or AAC(6′)-Ie-APH(2′′)-Ia were relatively comparable (in the range of 10^4 to 10^5 M^{-1} s^{-1}), with amikacin showing the lowest values of $(1.2 \pm 0.2) \times 10^4$ and $(1.7 \pm 0.1) \times 10^4$ M^{-1} s^{-1} for APH(2′′)-Ia and AAC(6′)-Ie-APH(2′′)-Ia phosphorylation, respectively. The corresponding k_{cat}/K_m values of vertilmicin were $(1.1 \pm 0.3) \times 10^5$ and $(1.1 \pm 0.2) \times 10^5$ M^{-1} s^{-1}, respectively, which were 9.2- and 6.5-fold higher than those of amikacin. Comparison of the acetylation activities of AAC(6′)-Ie and AAC(6′)-Ie-APH(2′′)-Ia demonstrated that gene fusion largely increased (43.7- to 144.6-fold) the acetylation activity of the AAC(6′)-Ie domain, while no significant difference can be found for the phosphorylation activities of APH(2′′)-Ia and AAC(6′)-Ie-APH(2′′)-Ia.

Table 2 presents the kinetic modification parameters of the compounds for ANT(2′′)-Ia adenylation. Amikacin was the most stable compound, as it cannot be modified by ANT(2′′)-Ia under our test condition. This was further confirmed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) with samples incubated at 37°C for 24 h (data not shown). For the other seven aminoglycosides, vertilmicin exhibited the lowest k_{cat}/K_m value, which was 1.8- to 7.5-fold lower than those of the others. The higher stability of vertilmicin toward modification by ANT(2′′)-Ia was contributed mainly to the higher K_m value of vertilmicin than those of the others (269.2 μM versus 22.7 to 69.4 μM).

The kinetic modification parameters for AAC(6′)-Ib and AAC(6′)-Ib-cr acetylations are shown in Table 3. The k_{cat}/K_m values of the compounds varied in a narrow range (10^4 to 10^5

TABLE 1. Comparison of kinetic modification parameters of vertilmicin and reference compounds for AAC(6′)-Ie, APH(2′′)-Ia, and AAC(6′)-Ie-APH(2′′)-Ia activities

Activity [enzyme]	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_{cat}/K_m)^{REF}/(k_{cat}/K_m)^{VTM}$[a]
Acetylation [AAC(6′)-Ie]	Vertilmicin	0.4 ± 0.01	317.2 ± 36.5	(1.2 ± 0.1) × 10^3	
	Verdamicin	0.9 ± 0.04	15.7 ± 2.5	(5.5 ± 0.1) × 10^4	45.8
	Netilmicin	2.0 ± 0.1	10.4 ± 1.4	(1.9 ± 0.3) × 10^5	158.3
	Sisomicin	3.3 ± 0.1	31.6 ± 2.6	(1.0 ± 0.1) × 10^5	83.3
	Amikacin	1.2 ± 0.1	8.8 ± 1.7	(1.3 ± 0.3) × 10^5	108.3
	Kanamycin	4.9 ± 0.4	32.7 ± 7.3	(1.5 ± 0.4) × 10^5	125.0
	Etilmicin	1.6 ± 0.1	5.1 ± 1.2	(3.0 ± 0.7) × 10^5	250.0
	Gentamicin	3.6 ± 0.3	42.6 ± 6.9	(8.3 ± 1.5) × 10^4	69.1
Acetylation [AAC(6′)-Ie-APH(2′′)-Ia]	Vertilmicin	8.4 ± 0.5	71.9 ± 13.0	(1.2 ± 0.2) × 10^5	
	Verdamicin	7.2 ± 0.5	1.5 ± 0.3	(4.7 ± 1.1) × 10^6	39.2
	Netilmicin	45.9 ± 3.4	5.3 ± 0.8	(8.3 ± 1.5) × 10^6	69.2
	Sisomicin	35.4 ± 2.2	2.7 ± 0.5	(1.3 ± 0.5) × 10^7	108.3
	Amikacin	26.0 ± 1.8	3.6 ± 0.6	(7.2 ± 1.2) × 10^6	60.0
	Kanamycin	51.0 ± 3.5	4.9 ± 0.7	(1.0 ± 0.2) × 10^7	83.3
	Etilmicin	21.5 ± 2.6	1.6 ± 0.5	(1.4 ± 0.6) × 10^7	116.7
	Gentamicin	116.0 ± 6.0	10.2 ± 0.9	(1.2 ± 0.7) × 10^7	100.0
Phosphorylation [APH(2′′)-Ia]	Vertilmicin	4.7 ± 0.3	42.7 ± 10.9	(1.1 ± 0.3) × 10^5	
	Verdamicin	3.7 ± 0.2	16.8 ± 3.8	(2.2 ± 0.5) × 10^5	2.0
	Netilmicin	5.3 ± 0.1	52.0 ± 4.0	(1.0 ± 0.1) × 10^5	0.9
	Sisomicin	3.1 ± 0.1	18.3 ± 3.6	(1.7 ± 0.3) × 10^5	1.5
	Amikacin	1.1 ± 0.04	90.4 ± 13.3	(1.2 ± 0.2) × 10^4	0.1
	Kanamycin	4.2 ± 0.2	17.3 ± 3.9	(2.4 ± 0.5) × 10^5	2.2
	Etilmicin	6.3 ± 0.2	50.8 ± 6.3	(1.0 ± 0.2) × 10^5	0.9
	Gentamicin	4.3 ± 0.2	36.8 ± 8.2	(1.2 ± 0.3) × 10^5	1.1
Phosphorylation [AAC(6′)-Ie-APH(2′′)-Ia]	Vertilmicin	4.0 ± 0.2	36.8 ± 8.1	(1.1 ± 0.2) × 10^5	
	Verdamicin	2.9 ± 0.1	5.4 ± 2.1	(5.3 ± 0.2) × 10^5	4.8
	Netilmicin	4.5 ± 0.1	67.2 ± 7.3	(6.7 ± 0.8) × 10^4	0.6
	Sisomicin	4.0 ± 0.2	38.0 ± 9.4	(1.1 ± 0.3) × 10^5	1.0
	Amikacin	1.4 ± 0.01	82.6 ± 3.9	(1.7 ± 0.1) × 10^4	0.2
	Kanamycin	5.3 ± 0.2	69.1 ± 13.6	(8.3 ± 1.8) × 10^4	0.8
	Etilmicin	5.9 ± 0.2	88.8 ± 9.3	(6.7 ± 1.7) × 10^4	0.6
	Gentamicin	3.8 ± 0.1	41.8 ± 8.5	(9.1 ± 1.9) × 10^4	0.8

[a] The k_{cat}/K_m value of the reference compound (REF) divided by the k_{cat}/K_m value of vertilmicin (VTM).

M^{-1} s^{-1}) for both AAC(6′)-Ib and AAC(6′)-Ib-cr modifications. Vertilmicin did not show superiority, with k_{cat}/K_m values of (3.9 ± 0.7) × 10^4 and (4.7 ± 1.0) × 10^4 M^{-1} s^{-1} for AAC(6′)-Ib and AAC(6′)-Ib-cr acetylations, respectively, which were about 1.6- and 2.5-fold higher than the corresponding values of amikacin. The high superiority of vertilmicin in comparison to other compounds in AAC(6′)-Ie and AAC(6′)-Ie-APH(2′′)-Ia acetylations but no superiority in AAC(6′)-Ib and AAC(6′)-Ib-cr modifications may be related to the different protein structures and distributions of the two enzyme groups, as several regions which are conserved in AAC(6′)-Ib are not seen in AAC(6′)-Ie, and AAC(6′)-Ie-APH(2′′)-Ia [AAC(6′)-Ie] is restricted to Gram-positive bacteria, while AAC(6′)-Ib is found only in Gram-negative isolates (17). The acetylation efficiency of AAC(6′)-Ib-cr was comparable to that of AAC(6′)-Ib, demonstrated by similar k_{cat}/K_m values of the aminoglycosides to modifications by the two enzymes. But mutation of AAC(6′)-Ib to AAC(6′)-Ib-cr broadened its substrate profile to fluoroquinolones, even though the acetylation efficiency was not comparable to that of aminoglycosides (data not shown).

Methylation of 16S rRNA is a growing resistant mechanism against aminoglycosides among Gram-negative pathogens like *P.*

TABLE 2. Comparison of kinetic modification parameters of vertilmicin and reference compounds for ANT(2′′)-Ia adenylation

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_{cat}/K_m)^{REF}/(k_{cat}/K_m)^{VTM}$[a]
Vertilmicin	5.3 ± 0.1	269.2 ± 11.8	(2.0 ± 0.1) × 10^4	
Verdamicin	4.9 ± 0.2	47.1 ± 7.5	(1.0 ± 0.2) × 10^5	5.0
Netilmicin	1.6 ± 0.1	37.8 ± 1.7	(4.2 ± 0.2) × 10^4	2.1
Sisomicin	3.3 ± 0.2	22.7 ± 5.1	(1.5 ± 0.3) × 10^5	7.5
Amikacin	NA[b]	NA	NA	NA
Kanamycin	8.4 ± 0.4	63.2 ± 9.1	(1.3 ± 0.2) × 10^5	6.5
Etilmicin	2.4 ± 1.1	69.4 ± 9.4	(3.5 ± 0.5) × 10^4	1.8
Gentamicin	1.1 ± 0.1	26.2 ± 8.5	(4.1 ± 1.3) × 10^4	2.1

[a] The k_{cat}/K_m value of the reference compound (REF) divided by the k_{cat}/K_m value of vertilmicin (VTM).
[b] NA, not applicable.

TABLE 3. Comparison of kinetic modification parameters of vertilmicin and reference compounds for AAC(6′)-Ib and AAC(6′)-Ib-cr acetylations

Acetylation enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	$(k_{cat}/K_m)^{REF}/(k_{cat}/K_m)^{VTM}$[a]
AAC(6′)-Ib	Vertilmicin	$(8.1 \pm 0.1) \times 10^{-2}$	2.1 ± 0.4	$(3.9 \pm 0.7) \times 10^4$	
	Verdamicin	$(11.9 \pm 0.7) \times 10^{-2}$	1.4 ± 0.3	$(7.3 \pm 1.4) \times 10^4$	1.9
	Netilmicin	$(11.0 \pm 0.2) \times 10^{-2}$	3.9 ± 0.7	$(2.8 \pm 0.5) \times 10^4$	0.7
	Sisomicin	$(15.1 \pm 0.3) \times 10^{-2}$	2.6 ± 0.5	$(5.8 \pm 0.6) \times 10^4$	1.5
	Amikacin	$(16.3 \pm 0.4) \times 10^{-2}$	6.7 ± 1.2	$(2.4 \pm 0.4) \times 10^4$	0.6
	Kanamycin	$(11.8 \pm 0.8) \times 10^{-2}$	2.4 ± 0.4	$(4.9 \pm 0.8) \times 10^4$	1.3
	Etilmicin	$(13.8 \pm 0.3) \times 10^{-2}$	4.3 ± 1.4	$(3.2 \pm 1.1) \times 10^4$	0.8
	Gentamicin	$(11.6 \pm 0.1) \times 10^{-2}$	0.9 ± 0.2	$(1.3 \pm 0.3) \times 10^5$	3.3
AAC(6′)-Ib-cr	Vertilmicin	$(9.7 \pm 0.2) \times 10^{-2}$	2.1 ± 0.4	$(4.7 \pm 1.0) \times 10^4$	
	Verdamicin	$(8.7 \pm 0.1) \times 10^{-2}$	0.8 ± 0.1	$(1.1 \pm 0.1) \times 10^5$	2.3
	Netilmicin	$(11.1 \pm 0.1) \times 10^{-2}$	2.5 ± 0.6	$(4.4 \pm 1.1) \times 10^4$	0.9
	Sisomicin	$(11.3 \pm 0.3) \times 10^{-2}$	1.4 ± 0.2	$(7.6 \pm 1.6) \times 10^4$	1.6
	Amikacin	$(8.3 \pm 0.1) \times 10^{-2}$	4.3 ± 0.7	$(1.9 \pm 0.3) \times 10^4$	0.4
	Kanamycin	$(7.9 \pm 0.1) \times 10^{-2}$	0.8 ± 0.1	$(9.9 \pm 1.3) \times 10^4$	2.1
	Etilmicin	$(11.7 \pm 0.4) \times 10^{-2}$	3.4 ± 0.9	$(3.4 \pm 0.9) \times 10^4$	0.7
	Gentamicin	$(7.9 \pm 0.1) \times 10^{-2}$	3.9 ± 0.8	$(2.0 \pm 0.4) \times 10^4$	0.4

[a] The k_{cat}/K_m value of the reference compound (REF) divided by the k_{cat}/K_m value of vertilmicin (VTM).

aeruginosa and *A. baumannii*, which confer high levels of resistance to 4,6-disubstituted deoxystreptamines, including gentamicin, tobramycin, and amikacin (3). Consistent with this point, our initial study demonstrated that the new 4,6-disubstituted deoxystreptamine, vertilmicin, has no superiority in comparison to the reference compounds gentamicin, netilmicin, verdamicin, sisomicin, kanamycin, amikacin, and etilmicin (all aminoglycosides demonstrated MICs of >2,048 μg/ml) against 4 ArmA-producing *A. baumannii* isolates (data not shown).

In conclusion, vertilmicin showed much higher stability to acetylations by AAC(6′)-Ie and AAC(6′)-Ie-APH(2″)-Ia, moderately higher stability to ANT(2″)-Ia adenylation (except that shown by amikacin), and relatively similar stability to APH(2″)-Ia and AAC(6′)-Ie-APH(2″)-Ia phosphorylations and AAC(6′)-Ib and AAC(6′)-Ib-cr acetylations in comparison to those shown by other aminoglycosides. The high stability of vertilmicin to AAC(6′)-Ie-APH(2″)-Ia [AAC(6′)-Ie] acetylation rendered it a useful compound in the treatment of infections caused by Gram-positive bacteria harboring *aac(6′)-Ie-aph(2″)-Ia*. And also, the increase in stability of vertilmicin (which has a methyl group at the 6′ position) versus that of netilmicin toward AAC(6′)-Ie-APH(2″)-Ia [AAC(6′)-Ie] acetylation suggests a possible route for aminoglycoside semisynthetics.

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