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Double Stage Activity in Aminoglycoside Antibiotics

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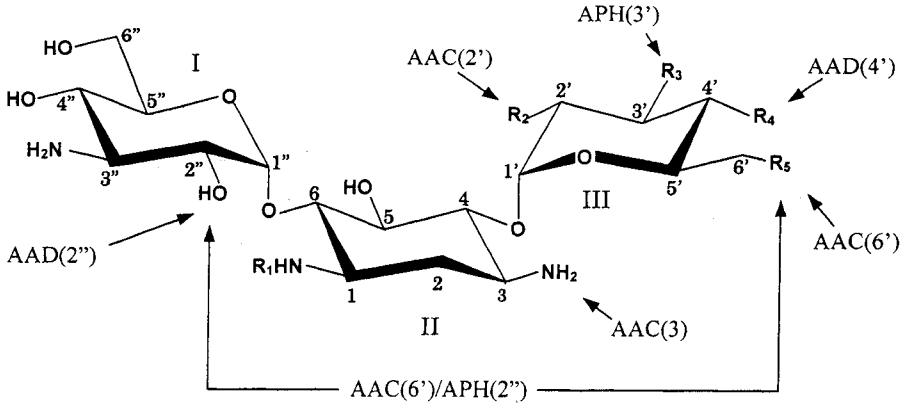
Fourteen different aminoglycoside antibiotics (AGs) were challenged with aminoglycoside acetyltransferases (AACs) of actinomycete origin in order to examine their 'double stage activity' that is arbitrarily defined as antibiotic activity retainable after enzymatic modification. In kanamycin (KM)-group AGs tested [KM, dibekacin (DKB), amikacin and arbekacin (ABK)], ABK retained activity after acetylations by AAC(3), AAC(2′) and AAC(6′). DKB also retained a weak activity after acetylation by AAC(2′). In gentamicin (GM)-group AGs tested [GM, micronomicin, sisomicin (SISO), netilmicin (NTL) and isepamicin], GM, SISO and NTL retained activites after acetylation by AAC(2′). In neomycin (NM)-group AGs tested [ribostamycin, NM, paromomycin], NM retained activity after acetylation by AAC(6′) and AAC(2′). None of astromicin (ASTM)-group AGs tested (ASTM and istamycin B) retained activity after acetylation by AAC(2′) and AAC(6′). The activities of acetylated ABK derivatives by AAC(3) and AAC(2′) were distinctively high, compared to the others. *Streptomyces lividans* TK21 containing the cloned *aac* genes were markedly sensitive to AGs that retained activities after acetylation, indicating the substantial effect of 'double stage activity'.

Aminoglycoside (AG) antibiotics are generally inactivated by acetylation, phosphorylation and adenylylation due to AG acetyltransferases (AACs), AG phosphotransferases (APHs) and AG adenylyltransferases (AADs), respectively[1~3]. A bifunctional modifying enzyme, AAC(6′)/APH(2″), is another critical AG-inactivating enzyme[4~6]. These AG-modifying (or AG-inactivating) enzymes are the resistance bases of most clinically-occurring AG-resistant bacteria. In order to overcome these AG-modifying enzymes, varieties of semisynthetic AGs (Fig. 1) have been developed by chemically modifying kanamycin (KM)- and gentamicin (GM)-group AGs[7]. The first successful development was dibekacin (DKB)[8] of which structure is the 3′,4′-dideoxy derivative of kanamycin B so that DKB is free from the modification by APH(3′) and highly active to APH(3′)-dependent AG-resistant bacteria. Following DKB, amikacin (AMK)[9] with excellent activities against varieties of AG-resistant bacteria was developed by introducing (*S*)-4-amino-2-hydroxybutyryl (AHB) side chain at 1-NH_2 of KM. Subsequently, arbekacin (ABK)[10], netilmicin (NTL)[11] and isepamicin (ISP)[12] were developed by introducing an acyl or alkyl into 1-NH_2 of DKB, sisomicin (SISO) and GM-B, respectively. Thus, it has been generally believed that the activity of semisynthetic AGs against AG-resistant bacteria is due to the removal or absence of target sites of AG-modifying enzymes as well as the introduction of side chains interferring with the access to target sites of AG-modifying enzymes.

The clinical use of these semisynthetic AGs brought the successful control of known AG-resistant bacteria, but sooner or later resulted in the emergence of new AG-resistant bacteria including MRSA (methicillin-resistant *Staphylococcus aureus*) strains with multiple AG-modifying enzymes such as AAC(6′)/APH(2″), AAD(4′) and APH(3′). In such a circumstance, ABK approved as an

Dedicated to the memory of Sir EDWARD ABRAHAM.

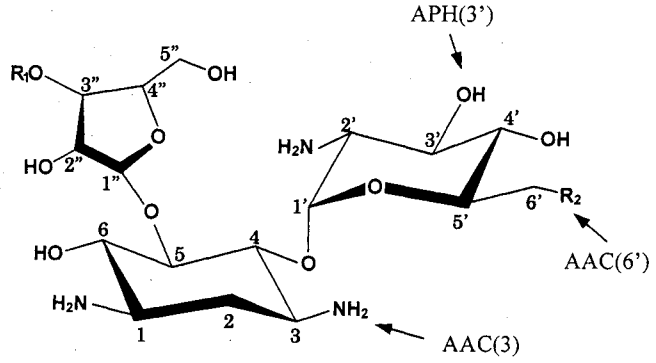
Fig. 1. Structures of aminoglycoside antibiotics and enzymatic modification sites.



Kanamycin- and Gentamicin- group AGs

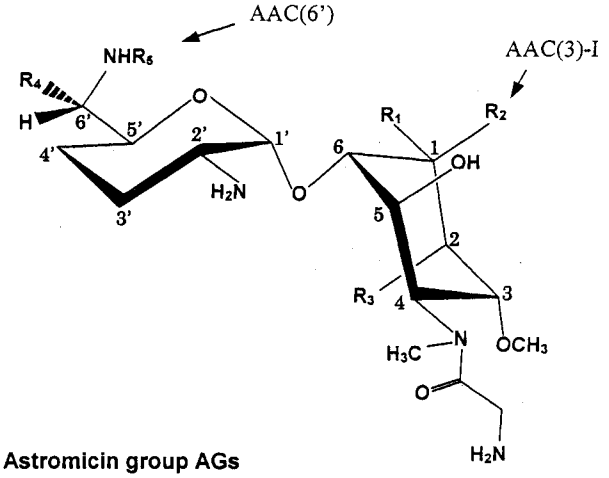
Antibiotic	I	II	III			
		R_1	R_2	R_3	R_4	R_5
Kanamycin (KM)		H	OH	OH	OH	NH_2
Dibekacin (DKB)		H	NH_2	H	H	NH_2
Amikacin (AMK)		AHB*	OH	OH	OH	NH_2
Arbekacin (ABK)		AHB*	NH_2	H	H	NH_2
Gentamicin (GM) -C_1		H				R_6=CH_3 R_7=CH_3
-C_2		H				R_6=CH_3 R_7= H
-C_{1a}		H				R_6= H R_7= H
Micronomicin (MCR)*		H				R_6= H R_7=CH_3
Isepamicin (ISP)		AHP*	OH	OH	OH	NH_2
Sisomicin (SISO)		H				
Netilmicin (NTL)		C_2H_5				

* MCR= GM-C_{2b}, AHB= $COCH(OH)CH_2CH_2NH_2$, AHP= $COCH(OH)CH_2NH_2$



Neomycin group AGs

Antibiotic	R_1	R_2
Neomycin (NM)		NH_2
Paromomycin (PRM)		OH
Ribostamycin (RSM)	H	NH_2



Astromicin group AGs

Antibiotic	R_1	R_2	R_3	R_4	R_5
Astromicin	H	NH_2	OH	CH_3	H
Istamycin B	NH_2	H	H	H	CH_3

anti-MRSA drug in 1990 in Japan showed good activity to the above MRSA strains[13]. This was likely due to that ABK has two advantages (1-*N*-AHB and no 3′,4′-OH) to resist various AG-modifying enzymes. Since then, ABK has been extensively used in clinics, but the emergence of ABK-resistant MRSA strains has so far been restricted to ones with moderate resistance levels (12.5~25 μg/ml) depending on a bifunctional AG-modifying enzyme AAC(6′)/APH(2″)[14,15] at low incidence[16].

In this context, one of our interests was why AAC-dependent ABK resistance has not emerged although ABK retains amino groups as the possible target sites of AAC(3), AAC(2′) and AAC(6′). Then we attempted ABK modifications by using AACs of actinomycete origin in order to probe the possible emergence of AAC-dependent ABK resistance[17~19]. Consequently, it turned out that ABK was rather readily acetylated, but retained substantial antibiotic activity after acetylation by these AACs. Especially, two acetyaled ABK derivatives (3″-*N*-acetylABK and 2′-*N*-acetylABK due to AAC(3) and AAC(2′), respectively) were confirmed to show activities as high as 40~55% of ABK activity[17,18]. These findings led us to raising a concept of 'double stage activity' for antibiotics capable of retaining activities even if they are modified by AG-inactivating enzymes. In additional investigations, neomycin (NM) and paromomycin (PMR) were also confirmed to retain substantial activities after acetylation by AAC(6′)[19] and probably AAC(1)[20], respectively. In this context, it has been known that bacteria with AAC(6′)-dependent resistance to amikacin (AMK) or GM are sensitive to NM[21] and that acetylated derivatives of GM and NM show weak antibiotic activities[22] as well as inhibitory activity against an *in vitro* polypeptide synthesis[23].

Based on these, we reasoned that double stage activity should be taken into account as a novel basis to control AAC-dependent AG-resistant bacteria that have been increasing problems in AG-therapy. In the present study, we examined varieties of AGs for their antibiotic activities after enzymatic acetylation in order to know or compare their double stage activities.

Materials and Methods

Antibiotics

The following 14 aminoglycoside antibiotiocs (AGs) as sulfates were used; kanamycin (KM), dibekacin (DKB), amikacin (AMK) and arbekacin (ABK) of KM-group, gentamicin (GM), micronomicin (MCR), sisomicin (SISO), netilmicin (NTL) and isepamicin (ISP) of GM-group, ribostamycin (RSM), neomycin (NM) and paromomycin (PRM) of NM group, and astromicin (ASTM) and istamycin B (ISMB) of ASTM-group. Their structures were shown in Fig. 1. These AGs were obtained from the antibiotic collections of the Institute of Microbial Chemistry and National Institute of Infectious Diseases.

Acetylation Reaction

Cell free extracts as crude enzyme solutions (S30) were prepared from *S. lividans* strains TK21/pANT3-1[17], TK21/pANT12[18] and TK21/pANT-S2[19] containing AAC(3), AAC(2′) and AAC(6′), respectively, as described previously. Acetylation reactions were carried out at 37°C in a 50 μl reaction mixture with the following composition; 200 μg/ml AG, 0.1 M phosphate buffer (pH 7.0), 10% (v/v) cell free extract and 4 mM acetylCoA (sodium salt; Sigma). Formation of acetylated compounds was monitored by ninhydrin reaction after TLC using a silica gel plate (E. Merk, Art. 5715) and 5% KH_2PO_4 as the developing agent.

Antibiotic Assay

Antibiotic activity of the reaction mixtures after enzymatic acetylation was monitored by regular paper disk assay using Mycin Assay Agar Arei (Mikuni Chemical; Japan) seeded with *Bacillus subtilis* PCI 219.

Antibiotic Resistance Test

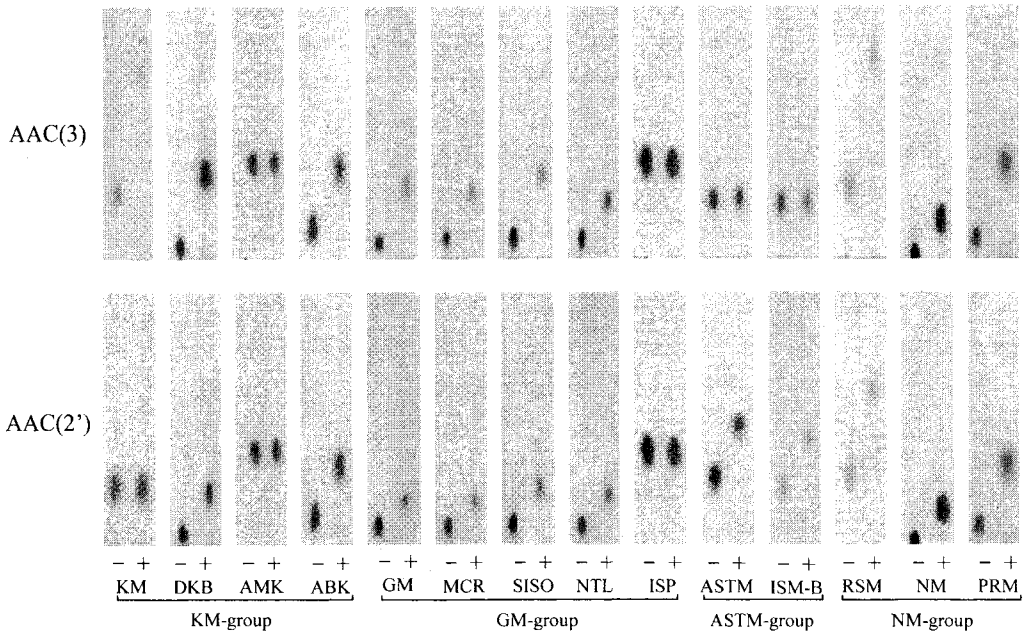
Aerial mycelial suspensions (10 μl) of *S. lividans* strains containing the cloned *aac* genes were spot-inoculated on ISP No. 2 agar plates containing serially diluted concentrations ranging from 2.5 to 100 μg/ml of AGs and incubated at 27°C for 1 week in order to check their growth.

Results

Antibiotic Activity After Enzymatic Acetylation of Reaction Mixtures

Fig. 2 shows the conversion of AGs by cell free extracts containing AAC(3) and AAC(2′). It turned out that AAC(3) relatively readily modified the following 3-NH_2-containing AGs; KM, DKB, GM, MCR, SISO, RSM, NM and PRM. In case of semisynthetic AGs with specific 1-*N*-side chains as well as 3-NH_2, ABK and NTL were completely converted to acetylated derivatives whereas ISP and AMK were refractory to the AAC(3). ASTM and ISMB that do not have 3-NH_2 but 3-OH were free from acetylation by this AAC(3). In case of acetylation by AAC(2′), all the AGs with 2′-NH_2 were completely acetylated, whereas no

Fig. 2. Conversion of aminoglycoside antibiotics by AAC(3) and AAC(2′).



TLC after acetylation in the presence (+) or absence (−) of acetylCoA by cell free extracts prepared from *S. lividans* TK21/pANT3-1 and TK21/pANT12 containing *aac(3)* and *aac(2′)* genes, respectively.

Table 1. Antibiotic activities retained in the reaction mixtures after enzymatic acetylation.

Antibiotics	Activities retained after acetylation*		
	AAC(3)	AAC(2')	AAC(6')
Kanamycin	<1 %	− %	<1 %
Dibekacin	<1	10	<5
Amikacin	ref	−	<1
Arbekacin	100	80	15
Gentamicin	<1	25	25
Micronomicin	<1	<5	<5
Sisomicin	<1	30	<1
Netilmicin	<1	20	1
Isepamicin	ref	−	<1
Ribostamycin	<5	<5	<5
Neomycin	<5	15	50
Paromomycin	<1	<1	−
Astromicin	−	<1	<1
Istamycin B	−	<1	<1

* Relative activities to those of the reaction mixtures with substarte antibiotics and no cell free extracts.
− : no target site. ref: refractory

conversion was observed in the AGs (KM, AMK and ISP) with 2-OH. On the other hand, acetylation by AAC(6′) was observed in the AGs tested except for PRM and a GM component, probably GM-C1, as reported previously[19].

The reaction mixtures after enzymatic acetylation were then examined for their antibiotic activities. As shown in Table 1, the following acetylation reaction mixtures turned out to retain antibiotic activities; the mixture of ABK after acetylation by AAC(3), those of ABK, NM, GM, SISO, NTL and DKB after acetylation by AAC(2′), and those of NM and ABK after acetylation by AAC(6′). It was notable that all of the acetylation mixtures of ABK retained antibiotic activities; especially distinctively high antibiotic activities were observed in those of AAC(3) and AAC(2′).

In cases of KM, AMK, MCR, ISP, ASTM, ISMB and RSM, no substantial antibiotic activity was observed. However, ASTM reaction mixture incubated with AAC(2′) retained antibiotic activity under some assay conditions (data not shown).

Table 2. Resistance and acetylation activity conferred by genes coding for AACs.

Antibiotics	Resistance (μg/ml) conferred by				Acetylation of AG by		
	no *aac*	*aac(3)*	*aac(2')*	*aac(6')*	AAC(3)	AAC(2')	AAC(6')
<KM group>							
KM	<2.5	100	<2.5	100	◎	−	○
DKB	<2.5	50	10	50	◎	○	○
AMK	<2.5	<2.5	<2.5	10	×	−	○
ABK	<2.5	<2.5	<2.5	5	○	○	○
<GM group>							
GM	<2.5	10	<2.5	2.5	○	○	○ *
MCR	<2.5	25	5	10	○	○	○
SISO	<2.5	10	<2.5	25	○	○	○
NTL	<2.5	5	2.5	25	○	○	○
ISP	<2.5	<2.5	<2.5	5	×	−	○
<NM group>							
RSM	<2.5	<2.5	50	100	○	◎	○
NM	<2.5	10	10	2.5	○	○	○
PRM	<2.5	10	10	<2.5	○	○	−
<ASTM group>							
ASTM	5	5	10	100	−	◎	○
ISMB	5	5	50	100	−	◎	○

* Resistance of *S. lividans* TK21/pANT3-1, TK21/pANT12 and TK21/pANT-S2 that contain *aac(3)*, *aac(2')* and *aac(6')*, respectively. ▒ low level resistance in spite of relatively rapid acetylation.

** Acetylation rate: ◎ rapid, ○ moderate, × refractory, − no target site. ○ * GM-C1 is refractory.

AG Resistance Levels of *S. lividans* TK21 with the Cloned *aac* Genes

AG resistance levels of *S. lividans* TK21 with the cloned genes encoding AAC(3), AAC(2′) and AAC(6′) were shown in Table 2. The results indicated that these genes did not confer resistance to the AGs of which acetylation products retained substantial antibiotic activity. Namely, ABK was active to *S. lividans* TK21/pANT3-1, TK21/pANT12 and TK21/pANT-S2 containing *aac(3)*, *aac(2′)* and *aac(6′)* genes, respectively. GM, SISO and NTL were active to *S. lividans* TK21/pANT12 containing *aac(2′)* gene. NM was active to *S. lividans* TK21/pANT-S2 and weakly active to strain TK21/pANT12 containing *aac(6′)* and *aac(2′)*, respectively.

Discussion

We discovered that ABK acetylation products by AAC(3) and AAC(2′) of actinomycete origin exhibit distinctive antibiotic activities[17,18]. These findings brought us to raising a concept of double stage activity for antibiotics capable of retaining activities even if they are modified by AG-inactivating enzymes. In subsequent investigations, NM and PRM were also confirmed to retain substantial activities after acetylation by AAC(6′)[19] and probably AAC(1)[20], respectively. In the present study, we examined varieties of AGs of 4 different groups for antibiotic activities after enzymatic acetylation by AAC(3), AAC(2′) and AAC(6′) of actinomycete origin in order to check additional AGs with double stage activity. Consequently, it turned out that weak but clear antibiotic activities after acetylation were confirmed with acetylation products by

AAC(2′) of DKB, GM, SISO and NTL. In addition, it was also shown that *S. lividans* strains containing *aac* genes were sensitive to AGs with double stage activity. Based on these, we reason that double stage activity should be taken into account as a useful basis to control AAC-dependent AG-resistant bacteria that have been problems in AG-therapy.

Weak antibiotic activities of *N*-acetylated derivatives of GM-C1a and NM had been already reported[22,23] before semisynthetic AGs such as ABK, AMK and ISP became commercially available. However, no continuous attention has been paid by AG researchers except for descriptions such as that *aac(6′)-I* genes did not confer resistance to NM[21]. This must be due to the weak activity of these *N*-acetylated derivatives and the development of semisynthetic AGs such as AMK, NTL and ISP with chemically-introduced 1-*N*-side chains refractory to the action of varieties of AG-modifying enzymes. ABK was also developed in the same line of drug designing so that its novel property of double stage activity had been overlooked until we revealed it.

It is of interest that neither AMK nor ISP that are structurally related to ABK show substantial activity when modified by AAC(6′). Furthermore we demonstrated that 3″-*N*-acetylABK as the product of AAC(3) exhibited high activity whereas 3″-*N*-acetylAMK as the product of the same enzyme was substantially inactive[17]. One of structural differences between ABK and AMK or ISP is the number of amino group in hexose moiety glycosidically linked to the 4-position of 2-deoxystreptamine (see Fig. 1); *i.e.*, ABK has two amino group at 2′- and 6′-positions whereas both AMK and ISP have one amino group at 6′-position. This difference may influence the antibiotic activity of the above acetylated derivatives. NTL, another semisynthetic AG with chemically modified 1-*N*-side chain, retained weak activity after acetylation by AAC(2′) and no substantial activity after acetylation by AAC(6′). Thus ABK is distinctive from the other semisynthetic AGs in terms of double stage activity. Thus double stage activity should be taken into account as a novel basis in designing new semisynthetic AGs.

Acknowledgement

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