Arch. Pharm. Res. 6 (1), 93~102 (1983)

Mechanism of Action of and Resistance to Aminoglycoside Antibiotics

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(Receivld 20 February 1983)

Waksman's group discovered SM in 1944, and opened a new field of antibiotcs: i.e. AGs. A large group of antibiotics containing aminosugar and/or aminocyclitol is called the AGs. A majority of AGs are produced by actinomycetes.

In the first period, AGs effective against tuberculosis were chiefly examined. Following the studies on NM and KM, AGs active against staphyllococci and gram-negative rods were investigated. The discovery of GM and synthesis of DKB and AMK led to the studies on the third generation AGs, which show a broad antimicrobial spectrum including *Pseudomonas aeruginosa* and drug-resistant bacteria. Since opportunistic infection due to *Pseudomonas* and other organisms, and infection caused by drug-resistant bacteria are increasing, the third generation AGs are extensively investigated at present.

Most AGs are bactericidal, exhibiting a broad spectrum; active against mycobateria, staphyllococci and gram-negative bacteria, but practically inactive against streptococci and anerobes. The characteristic antimicrobial spectrum may be attriubted to the transport system of bacteria. Uptake of AGs takes place by an energy-dependent active transport system, operating in aerobic conditions but not in anerobic circumstances. Therefore, AGs are active against aerobes, but not against anaerobes.

The structures of AGs, clinically used in Japan, are presented in Fig. 1.

Structure-Activity Relationship of DOS-containing AGs

A number of AGs were isolated from microorganisms, and their derivatives were chemically synthesized. As a result, the structure-activity relationship of DOS-containing AGs has been elucidated to a certain extent. AGs possess many OH and NH_2 groups in the molecules. For example, KM contains seven OH and four NH_2 groups (Fig. 1). All the seven OH groups can be eliminated by deoxygenation without significantly affecting the antibacterial activity. On the contrary, the NH_2 groups are more important than the OH groups. However, each NH_2 group shows different significance for the biological activity; the 3-NH_2 is essential, but the 1-NH_2 or 2′-NH_2 is not necessary, although

Abbreviations

AG: aminoglycoside, AMK: amikacin, AP: apramycin, BT: butirosin, DKB: dibekacin, DOS: 2-deoxystreptamine, DSM: destomycin, FT: fortimicin, GM: gentamicin, HGM: hygromycin, KM: kanamycin, KM-B: kanamycin B or bekanamycin, KSG: kasugamycin, LVM: lividomycin, NM:neomycin, PRM: paromomycin, RSM: ribostamycin, SM: streptomycin, SPC: spectinomycin, SS: sisomicin, TOB: tobramycin, VM: viomycin.

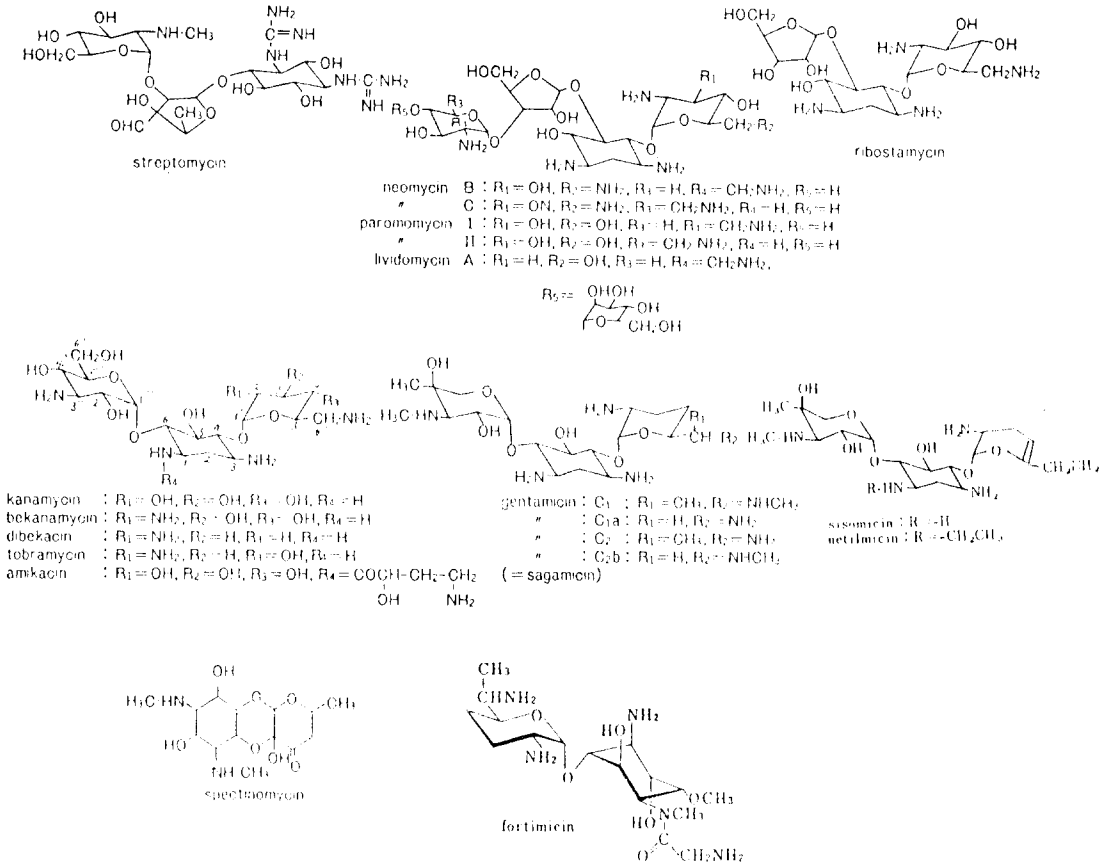

Fig. 1: Aminoglycoside antibiotics clinically used in Japan.

they enhance the activity. KM has OH but KM-B NH_2 at the 2′-position; and KM-B exhibits about four fold higher activity than KM. The antimicrobial activity is well maintained, if the 6′-NH_2 is methylated, although ethylation and other modifications markedly reduce the activity.

The DOS moiety is essential for the codon misreading and lethal effects. All the AGs capable of causing codon misreading, such as SM, KM, GM etc., possess a DOS or streptamine, which is lacking in SPC and KSG. SPC and KSG fail to stimulate misreading. The disaccharide components of KM, 4-O-(6-amino-6-deoxyglucosyl)-DOS and 6-O-(3-amino-3-deoxyglucosyl)-DOS, disturb translation fidelity, although the activity is less than KM. Of the three components of KM, DOS shows a very weak activity of misreading, but 6-amino-6-deoxyglucose and 3-amino-3-deoxyglucose lack the activity. Based on the results, we proposed an assumption that the DOS moiety is responsible for the codon misreading, although the whole structure of KM is required for the full activity.[1]

PRM, HGM-B and DSM exhibit antihelminthic, as well as antibacterial effects. These antibiotics and LVM and KM-C, affecting both prokaryotic and eukaryntic cells, possess 6′-OH instead of 6′-NH_2. However, the relationship

of 6′-OH to the activity against eukaryotes remains obscure.

Mechanism of Action of AGs

1. Target of AGs.

AGs interact with ribosomes, resulting in inhibition of protein synthesis, and also cause membrane damage, inducing leakage of intracellular substances. It remains to be determined which is the target or primary site of action of AGs: ribosome or membrane. However, the resistant mutation of ribosomes, or addition of chloramphenicol or other protein synthesis inhibitors eliminates the membrane damage, suggesting that the target of AGs is the ribosome, and the membrane damage is a secondary effect, resulting from the interaction with ribosomes. However, the mode of action of AGs differs from that of chloramphenicol, tetracyclines, macrolides, lincomycin and other ribosome inhibitors, because a majority of AGs causes different molecular events: codon misreading and membrane damage. Moreover, most AGs are bactericidal but the other antibiotics bacteriostatic. In other words, the effect of AGs is more complicated or pleiotropic than the other ribosome inhibitors.

The selective toxicity of AGs may be attributed to the difference of membrane and ribosome between bacterial and mammalian cells. In general, plasma membrane of mammalian cells do not possess AG transport system. Mammalian ribosomes, particularly cytoplasmic ribosomes, are resistant to AGs.

The ribosome consists of large and small subunits. Each subunit is constituted of RNA and protein. Protein synthesis takes place on the ribosome (Table I). The bacterial ribosome shows a molecular weight of 2.7×10^6 and a sedimentation constant of 70S, but the mammalian ribosome 4.5×10^6 dalton and 80S. The

Table I: Ribosomes of prokaryotes (bacteria) and eukaryotes(mammalia).

Ribosome		Prokaryote	Eukaryote
Molecular weight		ca. 2.7×10^6	ca. 4.5×10^6
Sedimentation constant		70S	80S
Subunits		30S+50S	40S+60S
30S or 40S subunit	RNA	one (16S)	one (18S)
	protein	21 kinds* (S1-S21)	33 kinds**
50S or 60S subunit	RNA	two (23S, 5S)	three (28S, 5.8S, 5S)
	protein	34 kinds* (L1-L34)	48 kinds**

* S20 protein is identical with L26 protein.
** Data of rat liver ribosomes.

Table II: Binding of [^{3}H]kasugamycin to the 30S ribosomal subunit of *E. coli*.

Ribosome		[^{3}H]KSG bound
30S subunit	5 OD_{260} units	0.041 nmoles
50S subunit	10	0.003
70S ribosome	15	0.076

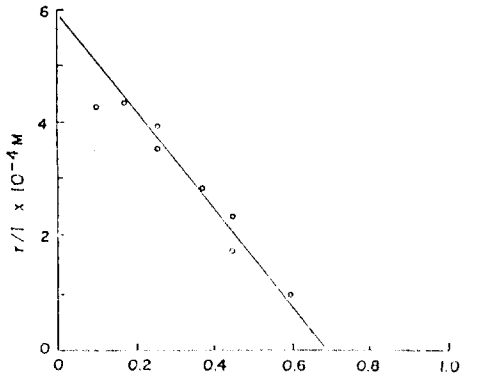

Fig. 2: Scatchard plot for equilibrium binding of [^{3}H] kasugamycin to the ribosome of *E. coli*.
r: $\frac{nmole\ KSG\ bound}{nmole\ ribosome}$
I: molar concentration of free KSG (M)

different sizes indicate different structures. The selective toxicity of AGs may be due to different structures of ribosomes between bacterial and mammalian cells. The mammalian ribosome consists of more proteins and RNAs than the

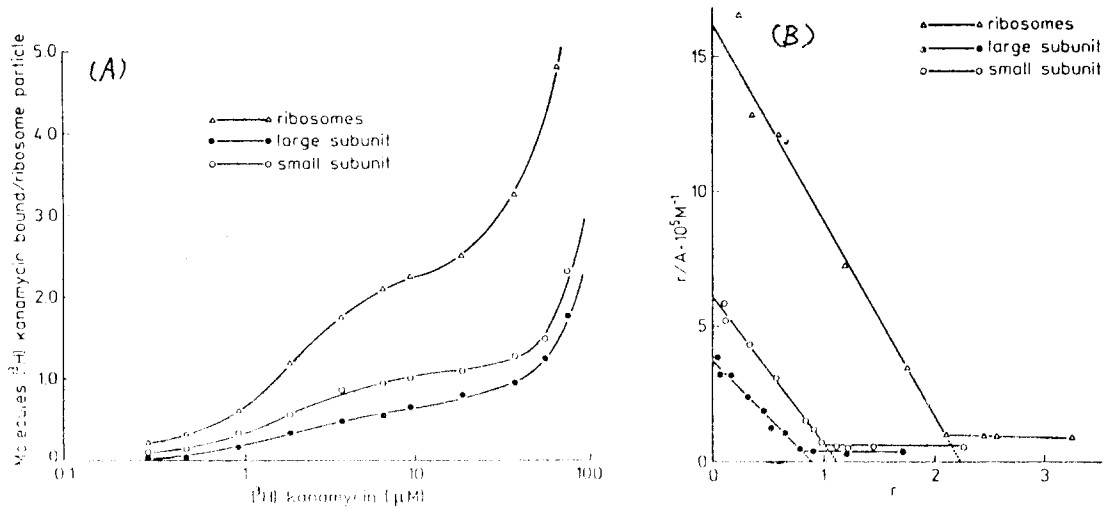

Fig. 3: Binding of kanamycin to ribosome and ribosomal subunits.
(A) The dependence on [^{3}H] kanamycin concentration for its binding to ribosomes and ribosomal subunits.
(B) Scatchard plots of equilibrum binding data for ribosomal subunits.

bacterial ribosome; and each component is larger than bacterial components. The bacterial ribosome is sensitive to SM, KM and GM, but mammalian ribosome is resistant to these AGs. PRM and HGM-B act on both ribosomes.

2. Binding of AGs to Bacterial Ribosomes.

Of AGs, the mechanism of action of SM was most extensively investigated. The target of all the AGs is the ribosome, and DOS-containing AGs cause codon misreading as SM does. Therefore, for a long time, DOS-containing AGs was considered to show the same mechanism of action with that of SM. However, recent studies in our laboratory show that the mechanism of action of KM, GM and other DOS-containing AGs is considerably different from that of SM.[2-5] Dr. E. C. Choi, Seoul National University, performed excellent works in this research.

SM, SPC and KSG act on the 30S subunit of ribosomes, but not on the 50S subunit. On the contrary, DOS-containing AGs interact with both subunits. The different effects of AGs were proved by binding of radioactive AGs to the ribosome and its subunits. For instance, [^{3}H] KSG binds to the 30S subunit but not significantly to the 50S subunit (Table II). As illustrated in Fig. 2, the Scatchard plot for KSG binding shows that the ribosome has a single binding site for KSG.[6] On the contrary, [^{3}H] KM binds to both ribosomal subunits (Fig. 3).[2]

The bacterial ribosome consists of three molecules of RNA and 54 kinds of protein. Extensive investigations have been carried out to elucidate what component of the ribosome participate in binding AGs. However, the precise site of action of AGs on the ribosome still remains to open to discussion. For instance, S12 protein determines sensitivity, resistance and dependency to SM. However, S12 is not the SM-binding site, but controls binding of SM to the ribosome. We found that S9 and L6 proteins participate in binding DKB and KM, although the stereochemical conformation of is essential for bindiog AGs.[7]

3. Ribosomal Resistance to AGs.

In all the SM-resistant mutants, the resistance is attributed to the change of the 30S

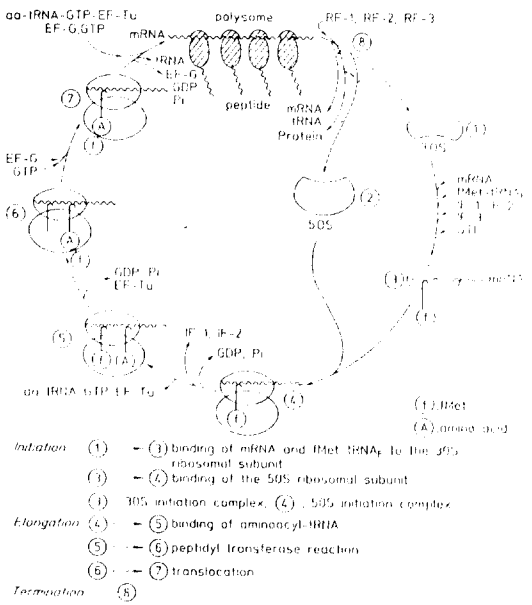

Fig. 4: Scheme of protein synthesis-ribosome cycle.

ribosomal subunit, in which S12 is altered. No SM-resistant mutants due to the alteration of 50S ribosomal subunit has been so far isolated. On the contrary, Dr. E.C. Choi isolated a number of KM-resistant mutants of *E. coli*, carefully examined, and found that the resistance is attributed to the alteration of the 30S subunit in some KM-resistant mutants and to that of the 50S subunit in the others.[4]

Both binding and resistance studies show that SM, SPC and KSG act on the 30S ribosomal subunit, but GM, GM and other DOS-containing AGs on both subunits.

4. Various Steps of Protein Synthesis Affected by AGs.

The function of ribosomes is protein synthesis, which consists of three steps: initiation, elongation and termination(Fig. 4). Protein factors and GTP are required for each process: *i.e.* initiation factors (IF-1, 2 and 3) and GTP for initiation, elongation factors (EF-Tu, -Ts and -G) and GTP for elongation, and releasing factors (RF-1, 2 and 3) for termination. The mRNA, transcripted from the genetic code of DNA, directs the translation as a template, which contains an initiation codon (AUG or GUG), codons corresponding to sequential amino acids, and a termination condon (UAA, UGA or UAG). The process of peptide chain elongation is performed by a cyclic reaction, consisting of (1) aminoacyl-tRNA-GTP-EF-Tu complex binding to the acceptor site (A-site) of ribosomes, (2) peptidyl transferase reaction or peptide bond formation between the C end of peptide moiety of peptidyl-tRNA on the donor site (D-site) and the N end of aminoacyl-tRNA on the A-site, and (3) translocation of peptidyl-tRNA from the A-site to the D-site with one codon movement of mRNA.

The results of effects of AGs on the initiation step are presented in Table Ⅲ. The 30S initiation complex formation or binding of fMet-

Table III: Effects of kasugamycin on binding of fMet-tRNA to ribosomes. (Okuyama *et al.* 1971)

Ribosomes	mRNA	Addition	fMet-RNA bound (pmol/tube)	% Inhibition
70S	f2 RNA	—	4.44	
		ksg $2\times10^{-5}M$	2.01	55
		2×10^{-4}	0.09	98
		str 2×10^{-5}	0	100
70S	AUG	—	6.30	
		ksg $2\times10^{-4}M$	0	100
70S	f2RNA	GMPPCP	1.21	
		+ksg $2\times10^{-4}M$	0	100
		+str 2×10^{-5}	1.11	8
30S	polyAUG	—	4.45	
		ksg $2\times10^{-4}M$	2.14	62
		str 2×10^{-5}	4.42	1
		kan 2×10^{-5}	4.48	—
		gen 2×10^{-5}	4.59	—

Abbreviations: ksg=ksugamycin, str=streptomycin, kan=kanamycin, gen=gentamicin

Table IV: Incorporation of poly[U]-dependent [^{14}C] isoleucine on the ribosomes derived from *E. coli* Q13. Codon misreading.

Antibiotic		Relative uptake
None		1.0*
Kanamycin	3 μM	3.9
	30	10.4
	300	65.8
Dibekacin	30	23.5
	300	62.7
Streptomycin	300	7.9
Neomycin	30	16.5
Gentamicin	30	44.8
Amikacin	3	5.3
	30	10.1
	300	66.4
Fortimicin	3	3.4
	30	5.3
	300	54.3

* 1276 cpm

The reaction mixture in 0.2 ml contained: 50 mM Tris-HCl, pH 7.8, 80 mM NH_4Cl, 8 mM magnesium acetate, 6 mM 2-mercaptoethanol, 2 mM ATP, 5 mM PEP, 4 μg pyruvate kinase, 0.2 mM GTP, 20 μg tRNA (*E.coli*), 51.4 pmoles ribosomes, 160 μg S100 fraction and 0.04 μCi [^{14}C] isoleucine. It was incubated at 37°C for 30 min.

tRNA to the 30S ribosomal subunit is inhibited by KSG but not by SM, or GM. The 70S initiation complex formation is prevented by KSG and SM. The results show that KSG blocks 30S initiation comolex production, and SM causes breakdown of 70S initiation complex. KSG selectively acts on initiation but not on elogation process[8].

In the chain elongation step, codon misreading is caused by SM and DOS-containing AGs, but not by SPC and KSG. As presented in Table IV, the poly [U]-dependent incorporation of [^{14}C] isoleucine into polypeptide is stimulated by KM, DKB, SM NM, GM, AMK and FT.

N-acetylphenylalanyl-puromycin is formed by the ribosome with Ac-Phe-tRNA and puromycin. The reaction can be used as a model for peptidyl transferase reaction. The puromycin reaction itself is not inhibited by AGs. Puromycin reacts with Ac-Phe-tRNA bound to the D-site of ribosomes, but not with Ac-Phe-tRNA bound to the A-site. Therefore, translocation of Ac-Phe-tRNA from the A-site to the D-site can be assayed by the puromycin reaction enhanced by EF-G and GTP. The translocation, observed by this method, is inhibited by DOS-containing AGs, such as KM, GM and NM,

Table V: Effects of aminoglycoside antibiotics on N-acetylphenylalanyl-puromycin synthesis in the absence or presence of EF-G and GTP. (Misumi *et al.* 1978b)

Antibiotic		N-Ac[^{14}C] Phe-puromycn formed	
		Without EF-G and GTP	Enhanced by EF-G and GTP
		(637cpm)	(1714cpm)
None		100%	100%
Kanamycin	0.1μM	97	72
	1	95	32
	10	95	17
	100	85	3
Neomycin	0.1	95	68
	1	93	27
	10	90	12
	100	86	2
Gentamicin	0.1	97	74
	1	95	34
	10	94	15
	100	88	2
Strepmycin	0.1	102	97
	1	97	95
	10	96	92
	100	93	81
BlasticidinS	100	16	6

The assay for peptidyltransferase reaction and translocation of peptidyl-tRNA was carried out by acetylphenylalanyl-puromycin synthesis.

but not significantly by SM (Table V)[2,3].

In the termination process, reading through is caused by misreading of termination codon, and peptide chain longer than normal chain is formed in the presence of SM and DOS-containing AGs.

Mechanism of Resistance of Bacteria to AGs

1. Genetic Mechanism. The drug-resistance is coded by their genes, which are located in bacterial chromosome or extrachromosomal element, plasmid. Plasmid is much smaller than chromosome, and carries various R-determinant genes, resulting in multiple drug resistance. Plasmid is transferred by conjugation or transduction from resistant bacteria to sensitive organisms.

Around a decade ago, transposon was discovered. Transposon has IS (insertion sequence)

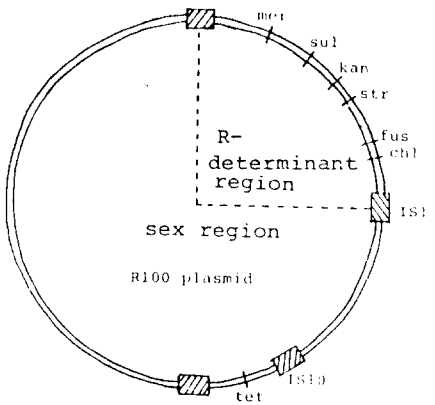

Fig. 5: Plasmid R100.

in both ends. R-determinant genes in transposon move from chromosome to plasmid, from plasmid to chromosomo, or from one plasmid to another. For example, R100 plasmid possesses R-determinant region and sex region (Fig. 5). Most

Table VI: AG-modifying enzymes, derived from *Pseudomonas aeruginosa* and other drug-resistant organisms.

(A) AG	O-phosphotransferase
APH (3′)-Ⅰ	KM, KM-B, NM, PRM, RSM; LVM (5″-OH)
APH (3′)-Ⅱ	KM, KM-B, NM, PRM, RSM, BT, AMK
APH (3′)-Ⅲ	KM, KM-B, NM, PRM, RSM, BT, AMK; LVM (5″-OH)
APH (5″)	RSM
APH (2″)	GM, SS, KM, DKB, TOB; AAC (6′)
APH (3″)	SM
APH (6)	SM
(B) AG	O-adenyltransferase
AAD (2″)	GM, KM, KM-B, DKB
AAD (4′)	TOB, KM, NM, PRM,RSM, BT, AMK; DKB (4″-OH)
AAD (3″)	SM;SPC (9-OH)
AAD (6)	SM
(C) AG	N-acetytransferase
AAC (6′)-1	KM, KK-B, NM
AAC (6′)-2	KM, KM-B, NM, GM
AAC (6′)-3	KM, KM-B, NM, GM, DKB
AAC (6′)-4	KM, KM-B, NM, GM, DKB, AMK
AAC (3)-Ⅰ	GM, SS;FT (1-NH_2)
AAC (3)-Ⅱ	GM, SS, (KM, KM-B, TOB)*
AAC (3)-Ⅲ	GM, SS, KM, KM-B, TOB, NM, PRM
AAC (3)-Ⅳ	GM, SS, KM, KM-B, TOB, NM, PRM, AP
AAC (2′)	GM, DKB, TOB, KM:B, NM, RSM, BT, LVM

* The reaction rates with antibiotics in the bracket are slow.

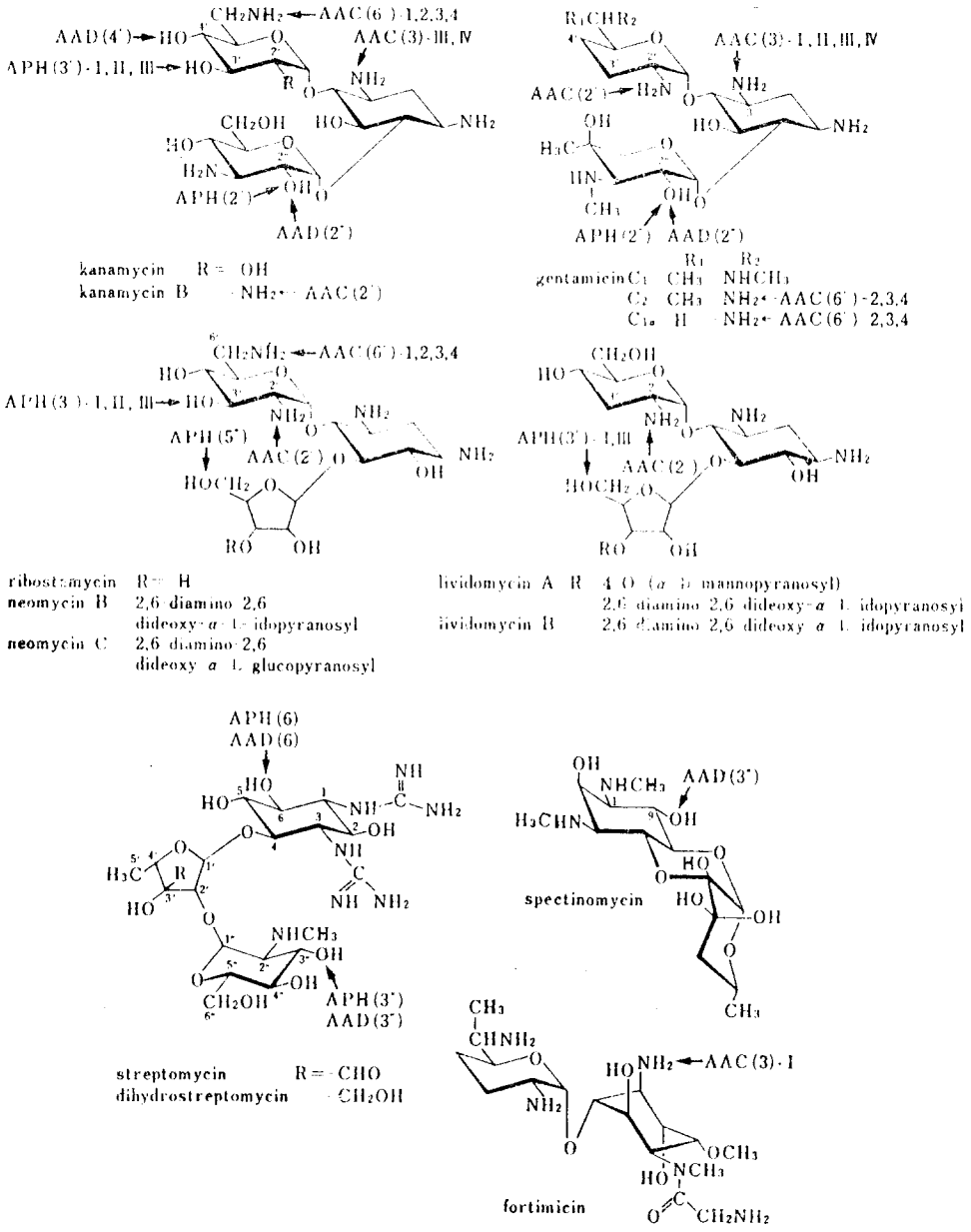

Fig. 6: Sites of action of AG-modifying enzymes in certain AGs.

resistant genes are located in R-determinant region, but tetracycline-resistant gene in sex region. Both are located in transposons, containing IS in both ends.

2. Biochemical mechanism. The products of R-determinant genes are AG-modifying enzymes, drug-resistant ribosomes, or altered transport system (s). A majority of drug-resistant clinical isolates, especially multiple drug-resistant bacteria, and *Pseudomonas aeruginosa*, an opportu-

Table VII: The sites of action of AG-modifying enzymes in the molecules of certain AGs.

Antibiotic	AG-modifying enzyme							Sites of enzyme action
	APH(3′)	APH(2″)	AAC(3)	AAC(2′)	AAC(6′)	AAD(4′)	AAD(2″)	
Kanamycin (A)	+	+	+	—	+	+	+	6
Tobramycin	—	+	+	+	+	+	+	6
Dibekacin	—	+	+	+	+	—	+	5
Propikacin	—	—	—	+	+	+	—	3
1-N-AHB dibekacin	—	—	—	+	+	—	—	2
Amikacin	—	—	—	—	+	+	—	2
6′-N-CH_3 amikacin	—	—	—	—	—	+	—	1
1-N-AHB-6′-N-CH_3 dibekacin	—	—	—	+	—	—	—	1
4′-deoxy-6′-N-CH_3 amikacin	—	—	—	—	—	—	—	0
Gentamicin C_{1a}	—	+	+	+	+	—	+	5
Gentamicin C_1	—	+	+	+	—	—	+	4
Gentamicin C_2	—	+	+	+	+	—	+	5
Micronomicin(gentamicin C_{2b})	—	+	+	+	—	—	+	4
Sisomicin	—	+	+	+	+	—	+	5
Netilmicin	—	—	+	+	+	—	—	3
5-Episisomicin	—	—	+	—	+	—	—	2
Fortimicn	—	—	+	—	—	—	—	1

nistic pathogen, produce AG-modifying enzymes.

Three kinds of AG-modifying enzymes have been isolated from bacteria: one phosphorylates OH group of AGs with ATP, another adenylates OH group of AGs with ATP, and the third one acetylates NH_2 group of AGs with acetyl-CoA. The enzymes are further classified into 12 sorts by the reaction site of AG molecules, and 20 kinds by substrate specificity(TableVI). The mechanism of bacteria to β-lactam antibiotics is rather simple. Bacteria produce a single type of enzyme, β-lactamase. On the contrary, the AG resistance is more complicated, and bacteria synthesize various sorts of enzymes.

AG-3′-phosphotransferase [APH(3′)] phosphorylates 3′-OH of KM and other DOS-containing AGs with ATP. Of various AG-modifying enzymes, APH (3′) is the most important enzyme, because of wide distribution in *P. aeruginosa* and other resistant bacteria. Recently cases of resistant organisms producing 6′-acetyltransferase [AAC (6′)] and 2″-adenyltransferase [AAD (2″)] are increasing (Table VI and Fig. 6)

Based upon the mechanism of resistance, various AGs and their derivatives, resistant to AG-modifying enzymes, have been extensively investigated and proved to be effective against *P.aeruginosa* and other drug-resistant bacteria (Table VII). Since APH (3′) is most widely distributed in *P.aeruginosa* and resistant organisms, 3′-deoxy and 3′, 4′-dideoxy derivatives have been vigorously studied, because of resistance to APH(3′). Of them, GM, sagamicin, SS, netilmicin, TOB and DKB show marked effects on *P. aeruginosa* and other resistant bacteria. 1-N-acyl derivatives, AMK and hab-

ekacin, are not significantly affected by APH (3′)-I, because of steric hindrance of the interaction with the enzyme by 1-N-acyl group; and are effective against *P. aeruginosa* and resistant organisms (Fig. 1).

KM and KM-B are classical AGs and even now widely used. Since both antibiotics are attacked by most AG-modifying enzymes, they are not effective against *P. aeruginosa* and other resistant bacteria. TOB. DKB, GM, sagamicin and SS are not affected by APH (3′); and are effective against *P.aeruginosa* and other resistant organisms. AMK is also resistant to AAC (3). Since AAC (3) is widely distributed in GM-resistant *P.aeruginosa*, AMK is effective against GM-resistant *P. aeruginosa*. Of 20 kinds of AG-modifying enzymes, only one enzyme, AAC (3)-I, can attack FT. Therefore, FT is effective against various resistant bacteria. However, FT is not effective against *P. aeruginosa*, because of transport barrier (Table VII).

The studies on molecular mechanism of AG resistance and on AGs resistant to AG-modifying enzymes have contributed to the problem of AG resistance. The most important problem of AGs at present is their side effects: ototoxcity, nephrotoxicity and neuromuscular blockade. The use of AGs is rather limited by their side effects. The studies on the molecular mechanism of action and side action of AGs are important to elucidate molecular basis for side effects or selective toxicity of AGs.

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