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A proof of the specificity of kanamycin-ribosomal RNA interaction with designed synthetic analogs and the antibacterial activity

Yoshio Nishimura,[a,*] Hayamitsu Adachi,[a] Motoki Kyo,[b] Shoichi Murakami,[c] Seiko Hattori[a] and Keiichi Ajito[c]

[a]*Microbial Chemistry Research Center, 3-14-23, Kamiosaki, Shinagawa-ku, Tokyo, Japan*
[b]*Toyobo Co. Ltd., 10-24, Toyocho, Tsuruga, Fukui, Japan*
[c]*Meiji Seika Kaisha, Ltd., 760, Morooka-cho, Kohoku-ku, Yokohama, Japan*

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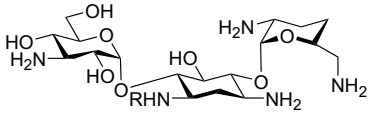
Abstract—The binding specificity of designed synthetic kanamycins with model RNA sequences (wild-type and point-mutated type) derived from the 16S ribosomal A-site was evaluated using surface plasmon resonance imaging. It was observed that kanamycins have nonspecific and multiple interactions with RNA hairpins and that the binding potency is not always proportional to the antimicrobial activity.

Since streptomycin was discovered by Waksman and coworkers in 1944,[1] aminoglycoside antibiotics have been recognized as a clinically important antimicrobial agent with broad antibacterial activities.[2] However, the emergence of resistance[3] and the toxicities such as oto- and nephrotoxicity decrease the usefulness of this class of antibiotics. To overcome resistance as well as side effects, an enormous number of synthetic analogs have empirically been developed for five decades.[4] However, little is understood about the mode of action at the molecular level until Moazed and Noller[5] have recently clarified the principles governing aminoglycoside–RNA recognition. At present it is believed that aminoglycoside antibiotics cause the bactericidal action by interaction with the A-site of the decoding region of ribosomal RNA. Recent crystallographic study has clarified that most aminoglycosides of neomycin and kanamycin families bind to the ribosomal decoding Asite.[6] The enzymatic N-acetylation, O-phosphorylation, and O-nucleotidylation of aminoglycosides by resistant bacteria decrease drastically their affinity to the RNA targets, and cause the inactivation of aminoglycosides.[7] Recently, Wong and Tor groups[8,9] have demonstrated the mode of action of aminoglycosides on a molecular level. They have used surface plasmon resonance (SPR) to study the interactions of a number of structurally diverse aminoglycoside antibiotics by use of commercial ones with RNAs. In contrast to other methods, SPR is able to detect interactions in real time without derivatization of aminoglycosides. It is also well known that the slight structural changes effects antimicrobial activity of aminoglycoside antibiotics. Under



Dibekacin (**1**): R = H

DKB-G (**2**): R = -C(=NH)NH_2

Arbekacin (**3**): R = -C(=O)*C(OH)$CH_2CH_2NH_2$

ABK-G (**4**): R = -C(=O)*C(OH)$CH_2CH_2NHC(=NH)NH_2$

DKB-ButNH_2 (**5**): R = -C(=O)$CH_2CH_2CH_2NH_2$

DKB-ButG (**6**): R = -C(=O)$CH_2CH_2CH_2NHC(=NH)NH_2$

DKB-Arg (**7**): R = -C(=O)*CH(NH_2)$CH_2CH_2CH_2NHC(=NH)NH_2$

DKB-Arg(NO_2) (**8**): R = -C(=O)*CH(NH_2)$CH_2CH_2CH_2NHC(=NH)NHNO_2$

Figure 1. Commercialized dibekacin (**1**) and arbekacin (**3**) and their derivatives (**2**, **4**–**8**).

* Corresponding author. Tel.: +81 3 3441 4173; fax: +81 3 3441 7589; e-mail: nishimuray@bikaken.or.jp

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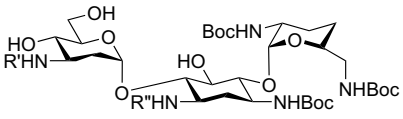
these circumstances, we are interested in the relationship between RNA binding and antimicrobial activity among slightly modified kanamycins to better understand. In this study, an alternative SPR method was applied to examine the binding and the dissociation conditions between aminoglycoside antibiotics (kanamycins) and RNA.[10]



9: R' = -C(=O)CF_3, R" = H

10: R' =Boc, R" = H

11: R' = -C(=O)CF_3, R" = -C(=NBoc)NHBoc

12: R' = Boc, R" = -C(=O)C(S)(OH)CH_2CH_2NHPMZ

13: R' = Boc, R" = -C(=O)C(S)(OH)CH_2CH_2NHPMZ

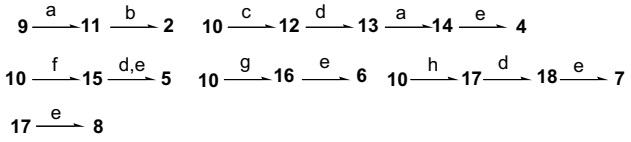
14: R' = Boc, R" = -C(=O)C(S)(OH)CH_2CH_2NHC(=NBoc)NHBoc

15: R' = Boc, R" = -C(=O)$CH_2CH_2CH_2$NHZ

16: R' = Boc, R" = -C(=O)$CH_2CH_2CH_2$NHC(=NBoc)NHBoc

17: R' = Boc, R" = -C(=O)CH(S)(NHBoc)CH_2CH_2NHC(=NH)$NHNO_2$

18: R' = Boc, R" = -C(=O)CH(S)(NHBoc)CH_2CH_2NHC(=NH)NH_2



Scheme 1. Reagents and conditions: (a) $(BocNH)_2CS$, $HgCl_2$, Et_3N, DMF, rt (67%); (b) concd NH_4OH/MeOH, rt; 4 M HCl/dioxane, rt (74%); (c) $HO_2CC(S)OH)CH_2CH_2$NHPMZ, BOP, Et_3N, DMF, rt (54%); (d) H_2, Pd/C, MeOH/EtOAc, rt (81%); (e) 4 M HCl/dioxane (98%); (f) $HO_2CCH_2CH_2CH_2$NHZ, BOP, Et_3N, DMF, rt (53%); (g) $HO_2CCH_2CH_2CH_2$·NHC(=NBoc)NHBoc, BOP, Et_3N, DMF, rt (59%); (h) $HO_2CC(S)H$(NHBoc)$CH_2CH_2CH_2$NHC(=NH)$NHNO_2$, BOP, Et_3N, DMF, rt (70%).

It is well known that the amino groups of aminoglycoside antibiotics play critical role in antibacterial activity,[11] and that acylation or alkylation of the 1-amino group in 2-deoxystreptamine-contaning aminoglycosides as shown in butirosins,[12] amikacin,[13] netilmicin,[14] isepamicin,[15] and arbekacin[16] improve often activity against sensitive and resistant bacteria. On the other hand, the guanidine moiety, a strong basic function is an important feature in streptidinecontaining aminoglycosides such as streptomycins.[1] With the aim of studying the effects of RNA–aminoglycoside interactions on the antibacterial activities in more detail, the only one amino group at C-1 position of dibekacin (DKB, **1**)[17] was replaced by the various types of amino subsituents (Fig. 1). DKB-G (**2**)[18] with a guanidine group was obtained upon treatment of **9** with *N*,*N*′-bis(*tert*-butyloxycarbonyl)thiourea in the presence of $HgCl_2$ in DMF to give **11** which was removed protecting groups (concd NH_4OH; 4 M HCl/dioxane). ABK-G (**4**)[18] was prepared by coupling of **10** with -(*p*-methoxybenzyloxycarbony)

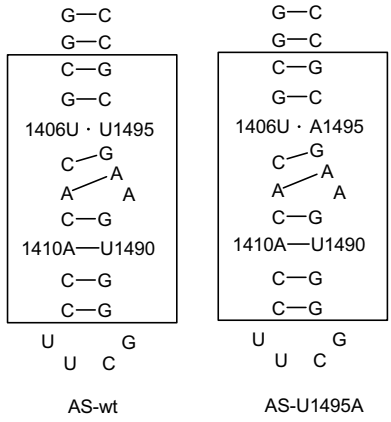


Figure 2. Sequences of the RNA molecules used in this study.

Table 1. Antibacterial activity (MIC μg/ml) of dibekacin (**1**), arbekacin (**3**) and analogs (**2**, **4**–**8**)

Test organisms	1	2	3	4	5	6	7	8
Staphylococcus aureus FDA209P	0.20	0.78	0.05	0.20	1.56	1.56	6.25	12.5
Micrococcus luteus FDA16	6.25	>100	0.39	12.5	100	100	>100	>100
B. subtilis PCI219	0.20	0.78	0.05	0.10	0.78	1.56	3.13	12.5
B. cereus ATCC10702	1.56	25	0.39	0.78	6.25	6.25	25	50
E. coli K-12	0.78	50	0.39	0.78	12.5	12.5	100	50
E. coli K-12 ML1410	0.78	>100	0.78	1.56	25	25	>100	100
E. coli JR66/W677	25	>100	1.56	3.13	25	50	>100	>100
Mycobacterium smegmatis ATCC607[†]	1.56	3.13	0.10	0.39	6.25	6.25	25	100
Klebsiella pneumoniae PCI 602	1.56	>100	0.78	1.56	12.5	12.5	100	50
Serratia marcescens	12.5	>100	6.25	25	>100	>100	>100	>100
Pseudomonas aeruginosa A3	0.39	25	0.20	0.39	3.13	6.25	100	25
P. aeruginosa GN315[a]	>100	>100	6.25	>100	>100	>100	>100	>100
P. aeruginosa 21–75[a]	3.13	>100	6.25	12.5	>100	>100	>100	>100
S. aureus MRSA No.5[a]	50	>100	1.56	1.56	>100	>100	50	>100
S. aureus MRSA No.17[a]	>100	>100	6.25	25	>100	>100	>100	>100
S. aureus MS 16526 (MRSA)[a]	>100	>100	12.5	25	>100	>100	>100	>100

MICs were determined by 2-fold agar dilution streak method at 37 °C for 18 and 42 h.[†]

[a] Clinically isolated resistant strains.

amino-hydroxybutylic acid (BOP, Et_3N, DMF) to give **12**, followed by successive sequences of deprotection of *p*-methoxybenzyloxycarbonyl group (H_2, 10% Pd/C, MeOH/AcOEt = 4:1;**13**), functionalization with a guanidine group [$(BocNH)_2CS$, $HgCl_2$, Et_3N, DMF;**14**], and removal of protecting groups (4 M HCl/dioxane). DKB–$ButNH_2$ (**5**)[18] was generated by coupling of **10** with –(benzyloxycarbonyl)aminobutylic acid to give **15** which was removed protecting groups by the similar methods mentioned above. DKB-ButG (**6**)[18] was similarly prepare via **16** from **10** by coupling with-[*N*,*N*′-bis(*tert*-butyloxycarbonyl)guanidino]butylic acid and removal protecting groups. DKB-Arg (**7**)[18] was obtained by coupling of **10** with -(*tert*-butyloxycarbonylamino) nitro-L-arginine to give **17**, followed by reduction of a nitro group (H_2, 10% Pd/C, AcOH, MeOH/AcOEt = 1:1) to give **18** which was removed Boc groups. DKB-Arg (NO2) (**8**)[18] was also obtained from **17** by removal of protecting groups. On the other hand, DKB (**1**) and arbekacin (ABK, **3**)[16] are clinically useful and commercially available antibiotics (Scheme 1).

Antimicrobial activities of thus obtained compounds (**1**–**8**) against various kinds of bacteria containing resistant ones were evaluated (Table 1). As shown in Table 1, the order of activity is ABK (**3**) > ABK-G (**4**) > DKB (**1**) ≫ DKB-$ButNH_2$ (**5**) = DKB-ButG (**6**) > DKB-G (**2**) ≫ DKB-Arg (**7**) = DKB-$ArgNO_2$ (**8**). Of these compounds, **3** shows the most potent activity against all of the tested bacteria. Activities of **4** and **1** except for those against the specific resistant bacteria are almost 1/2 and 1/2–1/4 of those of **3**, respectively. On the other hand, **5**

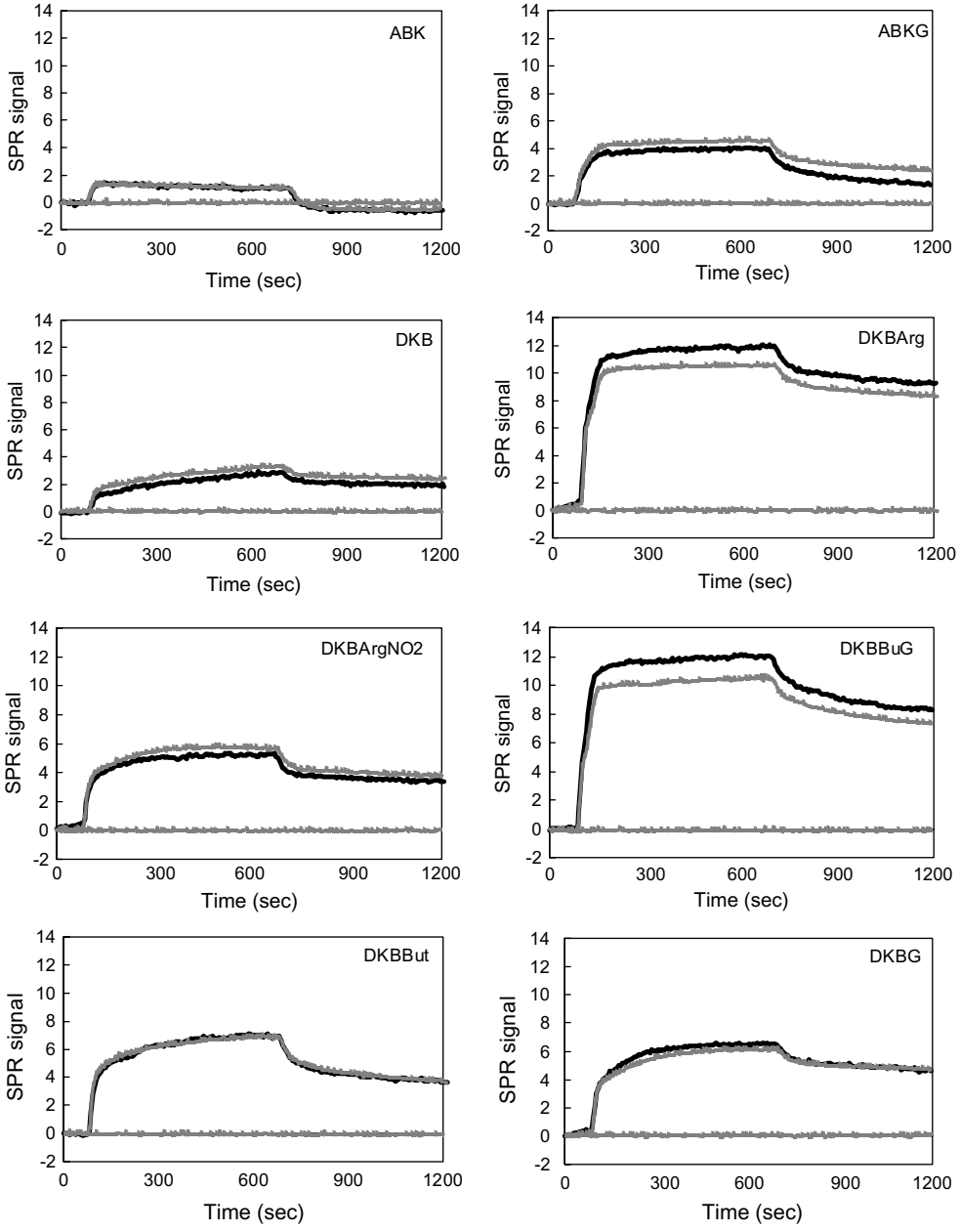


Figure 3. SPR analyses by exposure of aminoglycoside solution on the RNA arrays: SPR signal changes in AS-wt (black line), AS-U1495A (dark gray line) and Blank (light gray line).

and **6** show the very weak activities of 1/10–1/20 of those of **3**, and especially no activities against resistant *Pseudomonas aeruginosa* and MRSA. Compounds **2**, **7**, and **8** lost almost all the bactericidal activities. These results indicate that the slight structural changes lead the big alteration in the activity, and suggest more importantly that the basicity of aminoglycosides is not straightly proportional to the strong activity (see Fig. 1).

Next, we have examined the recognition of RNA kanamycin derivatives (**1**–**8**) and the RNA selectivity of these derivatives. Binding of compounds (**1-8**) to the wild-type (AS-wt)[19] and point-mutated A-site (ASU1495)[19] of the ribosomal decoding region of RNA (Fig. 2) was evaluated using SPR imaging technique.[10,20] As shown in Figure 3, increasing a number of amino groups tends to increase binding. These results indicate that the protonated amino groups at physiological pH play a significant role in RNA-aminoglycoside binding by electrostatic interactions. However, there are no differences between the wildtype and the point-mutated A-site in the binding potency, suggesting that these binding fashion are nonspecific and that aminoglycosides may have multiple interactions with RNA hairpins. The present results show that the increasing amino groups may produce the more nonspecific and multiple bindings to RNA. Although permeability of the compounds into bacterial cell-membrane is not clear at this stage, these bindings seem to reflect not always strong activity. These facts suggest that the antibiotic activity may be affected in cooperation with possible tertiary interaction with ribosomal proteins that can not be accounted in this ribosome-free model system. While the importance of electrostatic interactions of the aminoglycosides with the RNA target is generally recognized,[8,9,21] the observed absence of binding specificity implies that the design of novel aminoglycosides targeted to the defined ribosomal decoding region A-site may need other approaches.

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18. **2**: $[\alpha]_D^{23}$ +57.3 (*c* 0.42, H_2O) **4**: $[\alpha]_D^{21}$ +62.2 (*c* 0.73, H_2O); **5**: $[\alpha]_D^{21}$ +77.7 (*c* 0.76, H_2O); **6**: $[\alpha]_D^{21}$ +74.6 (*c* 0.8, H_2O); **7**: $[\alpha]_D^{21}$ +74.9 (*c* 0.85, H_2O); **8**: $[\alpha]_D^{21}$ +78.8 (*c* 0.85, H_2O).
19. AS-wt and AS = U1495A RNAs were purchased from Intergrated DNA Technologies, Inc., 1710 Commercial Park Coralville, IA, U.S.A.
20. Fabrication of RNA arrays: RNA array was obtained by the modified multi-step procedure, which was reported on DNA array.[9] A gold-coated chip (Toyobo), which has amino groups in 96 areas with 500 μm square and poly ethylene glycol (PEG) background was reacted for 60 min with 300 μL of 10 mg/mL MAL-PEG_{12}-NHS ester (Quanta Biodesign) to create a maleimido-modified surface. 10 nL drops of 10 μM thiol terminated RNA (IDT) were delivered automatically on the maleimido surface using an automated spotter (Toyobo), and the maleimido-thiol reaction was carried out overnight. 300 μL of 10 mg/mL PEG-thiol was reacted on the RNA array for 60 min to block the unreacted maleimido group. Then, the surface was rinsed with phosphate buffer and water. A phosphate buffer (10 mM phosphate: pH 7.2 and 150 mM NaCl) was used for all reactions in array fabrications. SPR imaging analysis: The RNA array was placed into SPR imaging instrument (Toyobo). The aminoglycosides were applied to the array surface with 100 μL/min in the running buffer (10 mM HEPES: pH 7.4, 150 mM NaCl, 3.4 mM EDTA). The array was exposed with 40 μM of aminoglycoside solution for 10 min and rinsed with the running buffer for 10 min. The array was regenerated and used repeatedly. The bound aminoglycosides were desorbed by exposure to 0.2X SSC/0.1% SDS solution for 5 min. The signal data were collected with an analysis program (Toyobo). All SPR experiments were performed at 30 °C.
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