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REVIEW ARTICLE

Kanamycin and its derivative, arbekacin: significance and impact

Kunimoto Hotta[1] · Shinichi Kondo[2]

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Abstract
On the occasion of the 60th anniversary of the discovery (1957) of kanamycin (KM), a series of research achievements on KM and its semisynthetic derivative Arbekacin (ABK) are outlined. KM was first used clinically in 1958 and was appreciated for its remarkable curing effect on various bacterial infections, especially tuberculosis. ABK is a KM derivative rationally semisynthesized to overcome KM resistance due to enzymatic phosphorylation and acetylation. Since its approval in 1990 as an anti-MRSA drug, ABK has been and still is effectively used in chemotherapy because MRSA rarely develops high ABK-resistance. Research that illuminated the unique features of ABK enabling it to resist the development of resistance by MRSA are also described.

Introduction

This year (2017) is the 60th anniversary of the discovery in 1957 of kanamycin (KM) by Prof. Hamao Umezawa and his colleagues. KM is a prestigious antibiotic of Japanese origin that obtained the international reputation for its remarkable activity against a wide variety of pathogenic bacteria, especially streptomycin (SM) resistant *Mycobacterium tuberculosis* and low delayed toxicity. KM cured tremendous numbers of patients suffering from pathogenic bacteria infections and thus brought a remarkably high status to Prof. Umezawa in the antibiotics world. When KM resistant bacteria emerged after about 8 years of clinical use, he immediately began to investigate the mechanism of resistance and demonstrated KM inactivation by phosphorylation and acetylation. In order to develop KM derivatives overcoming the resistance mechanism, a variety of semi-synthetic KM derivatives including dibekacin (DKB; 1971) and arbekacin (ABK; 1973) were rationally designed and synthesized. Both DKB and ABK were introduced to the clinics in 1975 and 1990, respectively. While DKB resistant bacteria emerged before long, ABK approved as an anti-MRSA drug in Japan has been and still is effectively used in chemotherapy since MRSA strains with high ABK resistance are rare.

In order to elucidate the basis for ABK's ability to avoid the development of resistance in MRSA, we characterized over 400 clinical MRSA isolates for their phenotype and underlying genotype of aminoglycoside (AG) resistance, as well as coagulase. This revealed: 1) Both the incidence and level of ABK resistance were markedly low even if the isolates contained *aac(6′)/aph(2″)* known as the critical resistance factor to KM-groups and GM-groups of AG antibiotics. 2) MRSA isolates with a specific coagulase genotype (originally named L21) became overwhelmingly dominant in the 1990s but showed the lowest possessing ratio of *aac(6′)/aph(2″)*. In addition, we discovered another distinctive aspect of ABK; i.e., it retains antibiotic activity even if acetylated by AAC(3), AAC(2′) and AAC(6′). Based on these distinctive features, it was conclusive that it would be difficult for MRSA to develop ABK resistance as long as overwhelming dominance of MRSA with type L21 coagulase continues. Furthermore, AAC-dependent ABK resistance will never emerge even if MRSA acquire any *aac* genes.

In this article, we, former members of Dr. Umezawa's research institute would like to describe an important research series on AG antibiotics with special reference to KM and its derivative, ABK in the memory of Dr. Umezawa who made great contributions to the antibiotic world as the trailblazer, front runner and charismatic leader for both the academic and industrial world in Japan.

This article is written and dedicated to late Prof. Hamao Umezawa on the occasion of the 60th anniversary of kanamycin discovery.

✉ Kunimoto Hotta
khotta@fwf.or.jp

[1] Functional Water Foundation, 2-20-8 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

[2] Bioscience Associates, Tokyo, Japan

Discovery of KM and its impact

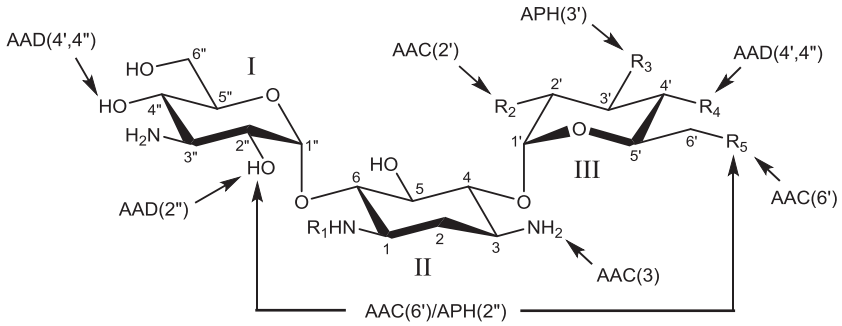
KM should be regarded as a remarkable antibiotic because it had taken only 2 years from its discovery to its clinical use. KM was first reported in Sep. 1957 [1–6]. Because of its antibiotic activity against a wide variety of pathogenic bacteria including SM resistant *M. tuberculosis* in addition to its relatively low delayed toxicity, KM attracted much attention not only in Japan but also in the U.S. [7].

Dr. Umezawa began antibiotic research at the age of 29 in Feb. 1944 during WWII when he was involved in the urgent priority project to establish penicillin production using *Penicillium*. The project proposer, Major Katsuhiko Inagaki at the Japanese Military Medical School organized the penicillin committee consisting of established scientists including Dr. Umezawa from a variety of disciplines. They worked very hard under extremely poor working conditions and realized the project goal by the end of that year, owing to Dr. Umezawa's success in obtaining a brown-colored crude penicillin [8]. This research experience allowed him to receive the most advanced knowledge and technological training from the U.S. after the war. It should be remarked that Japan was the third country in the world able to produce penicillin.

Health conditions of the Japanese people were severe after WWII ended in Aug. 1945. The Allied Occupation Forces substantially governed Japan for several years and encouraged the Ministry of Welfare to produce high-quality penicillin to solve serious infectious disease problems. Based on this policy, Dr. J.W. Foster, a famous penicillin researcher, was invited to Japan to provide technical guidance and lectures on the industrial production of penicillin from Nov. 1946 to Mar. 1947 [9]. In addition, he supplied *P. chrysogenum* Q176, the industrial strain used for penicillin production in the U.S. In May, 1947, the National Institute of Health of Japan (NIHJ) was established by the direction of GHQ (General Headquarter) of the Allied Occupation Forces and Dr. Umezawa became the director of the Department of Antibiotics. During the first 3–5 years, the major goal was the production of penicillin and then SM, for which the producing actinomycete *S. griseus* was supplied through GHQ. It should be noted that when *S. griseus* was brought to Japan, there was no antibiotic scientist acquainted with this group of organisms. Therefore, Dr. Umezawa appointed his colleague, Mr. Okami, to study the taxonomy of *Streptomyces* so that they became capable of handling *Streptomyces*. SM was discovered in 1944 as the water soluble, basic antibiotic with anti-*M. tuberculosis* activity by Drs. Schatz and Waksman [10]. The latter was awarded the Nobel Prize in 1952. As expected, SM greatly contributed to the treatment of tuberculosis which, at that time, was the single greatest cause of infectious mortality in Japan. However, SM resistant *M. tuberculosis* soon began to emerge. In such situation, Dr. Umezawa visited US for the first time in Nov. 1950 to survey industrial production of antibiotics and R&D systems. He returned to Japan in Feb. 1951 bringing the fast growing *Mycobacterium* strain 607 (M607 hereafter). Strain M607 was immediately integrated into his antibiotic screening system in which the major target was water-soluble, basic antibiotics showing anti-*M. tuberculosis* activity and being distinct from SM. Although research conditions in NIHJ at that time were still poor, Dr. Umezawa's research group was successful in obtaining three candidates in cultured broths of soil isolates of *Streptomyces* by early 1956. One of them was the cultured broth of strain K-2j isolated from a soil collected in Nagano prefecture, Japan. Chemical and biological characterization indicated that the active principle produced by the strain K-2j met the above target goals. Therefore, it was regarded as a new compound and named kana (golden)-mycin (KM) after the surface growth color of K-2j on agar media. Later, strain K-2j was taxonomically classified as a new *Streptomyces* species and named *S. kanamyceticus* n. sp.; Okami et al [11]. Since about 20 g of purified KM was obtained from the fermentation broth using a 400 L fermenter, the purified KM was immediately subject to various in vivo investigations including toxicity test using animals and curative effect on experimental animal tuberculosis. It turned out that the KM preparation did not kill the administrated mice but showed efficacy on the experimental tuberculosis in animals such as guinea pigs and mice. Subsequently, dogs and cats were challenged with the purified KM and no delayed toxic effect was observed, while SM caused serious delayed toxic effect such as akinesia. These results were reported at the 106th scientific meeting of Japan Antibiotics Research Association, as well as in Journal of Antibiotics and other journals.

Clinical trials were started in Feb, 1957 by Prof. T. Ichikawa and his colleagues, School of Medicine, University of Tokyo, for urinary infections [12]. They confirmed the efficacy of KM through a 2 months examination and established appropriate administration concentration and quantity. Based on Dr. Ichikawa group's studies, various clinical trials were opened, starting in May that year, mainly at hospitals in Tokyo. Furthermore, systematic clinical studies were started in October by research groups headed by Prof. I. Donomae, University of Osaka, involving several hospitals [13]. More clinical studies on KM were started in the U.S. by the end of 1957. Preparation of the required KM was allocated to Meiji Seika Kaisha, Japan and Bristol Myers Laboratories, U.S. The Japanese and U.S. patents on KM were filed in Sep. 1957 and in Dec. 1957, respectively [14]. Four kinds of KM agents including KM sulfate were approved as drugs by the Japanese government in Mar. 1958.

Two important symposia were held in Japan (May) and in US (July) in 1958 to report, discuss and evaluate the basic and clinical studies of KM. In the symposium at the New York Academy of Sciences, 37 reports were provided and



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=$COCH(OH)CH_2NH_2$

Fig. 1 Structures of KM and GM group AG antibiotics

subsequently compiled as a monograph [7] in Sep. 1958. This monograph was entitled "The Basic and Clinical Research of the New Antibiotic, Kanamycin" and included the following reports; the discovery by Umezawa as the first presenter [15], chemistry by Cron et al. [16], susceptibility and cross resistance by Kunin and Finland [17], pharmacological studies by Tisch et al. [18] and others [19], nephrotoxicity by Winfield et al. [20], and Berman and Katz [21], antituberculosis activity in guinea pigs [14, 22] and humans by a number of groups. Finland provided a summary stressing that KM would be useful to treat a variety of infections by several pathogenic bacteria, especially *Staphylococcus aureus*. Thus, KM began to be used clinically in 1958 and was recognized as the first internationally used antibiotic of Japanese origin. Another KM symposium was held 8 years later in 1966 under the title of "Kanamycin: Appraisal after Eight Years of Clinical Application" [23]. The chemical structure of KM was determined [24–26] as shown in Fig. 1.

Significance of semisynthetic KM derivatives with special reference to ABK

The critical resistance mechanisms of AG antibiotics in bacteria of clinical importance are the enzymatic *N*-acetylation, *O*-phosphorylation, and *O*-adenylylation that generally result in the inactivation of the AG [27, 28].

Eight years after KM was in clinical use, resistant bacteria emerged [29]. Dr. Umezawa immediately began to investigate the resistance mechanism and demonstrated KM inactivation by phosphorylation at the 3′-OH and acetylation at the 6′-NH_2 [30–32]. Based on this resistance mechanism and other information, a variety of semisynthetic KM derivatives including DKB (1971) and ABK (1973) were rationally designed and synthesized in order to overcome KM resistant bacteria [33]. DKB, the first semisynthetic AG, has the structure of 3′, 4′-dideoxyKM-B [34] which is free from the enzymatic 3′-*O*-phosphorylation by APH(3′) and 4′-*O*-adenylylation by AAD(4′,4″). DKB was introduced to the clinics in 1975. However DKB-resistant bacteria emerged rapidly. ABK has the structure of 1-*N*-[(*S*)-4-amino-2-hydroxybutyryl (AHB)]-DKB [35]. The discovery of Butirosin by the Upjohn group had revealed the remarkable protection against several resistance imparted by the 1-*N*-AHB group [36]. Due to the introduction of AHB to 1-*N* position of DKB, the resulting ABK exhibited activity against DKB-resistant bacteria [37]. Other research groups synthesized the following semisynthetic AGs; AMK (Amikacin;1972 [38]), NTL (Netilmicin;1976 [39]) and ISP (Isepamicin;1977 [40]). These semisynthetic AGs were

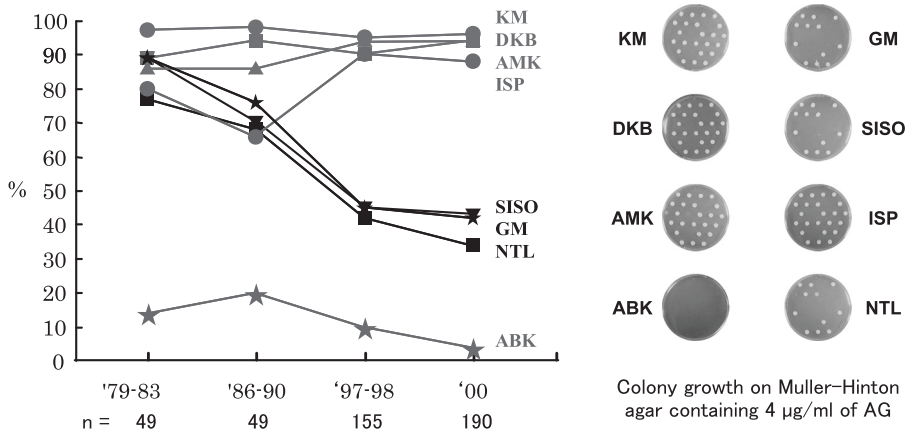


Fig. 2 Transition of AG resistant ratio (left) and AG susceptibility (right) of MRSA isolates (Color figure online)

Table 1 Target sites in KM-group and GM-group AGs of AG-modifying enzymes in MRSA

AG	AAC(x6′)/APH(2″) 6′-NH2 & 2″-OH	AAD(4′,4″) 4′-H & 4″OH	APH(3′) 3′-OH
KM	○	○	○
DKB	○	–	–
AMK	○	○	○
ABK	○	–	–
GM	○	–	–
SISO	○	–	–
NTL	○	–	–
ISP	○	○	–

Existence (○) and absence (–) of enzyme target site

developed by attaching AHB, ethyl, and (*S*)-3-amino-2-hydroxypropionyl groups to the 1-NH_2 of KM, SISO (Sisomicin), and GM-B (Gentamicin B), respectively. The structures of these semisynthetic AG antibiotics were shown in Fig. 1. The side chains introduced into 1-NH_2 were believed to block the access of a variety of AG-modifying enzymes to their target sites (steric hindrance effect). Semisynthetic AGs with these side chains showed remarkable activity against MRSA with AG resistance factors except for *aac(6′)/aph(2″)* coding for AAC(6′)/APH(2″) known as the critical AG resistance factor to KM- and GM-group AG antibiotics [41, 42].

ABK was the latest semisynthetic AG of clinical use in Japan and is still used (over 25 years) since its approval in 1990 as an anti-MRSA-agent. Interestingly, MRSA with ABK resistance already existed at the start of its clinical use and modified ABK by the bifunctional enzyme AAC(6′)/APH (2″) [42]. Fortunately, ABK resistance of clinical MRSA isolates was of low incidence and at a low or moderate level (< 25 μg/ml) [43, 44] and the latest report indicated an MIC_{90} of 1 μg/ml of for ABK against *S. aureus* [45].

In order to understand how ABK avoided increased resistance in MRSA, we surveyed over 400 clinical MRSA isolates during two decades (1979–2000) in terms of transition of phenotype and underlying genotype of AG resistance factors as well as coagulase [46, 47]. As shown in Fig. 2 (left), over 80% of MRSA isolates were constantly resistant to KM, DKB and AMK. The ratios of MRSA isolates with resistance to SISO, GM and NTL were high (75–90%) in the isolates during 1979–1983 and then declined to around 40% in the isolates during the 1990s. By contrast, ABK resistance was substantially and sustainably low throughout the two decades surveyed. Figure 2 (right) represents colony growth of MRSA isolates on Muller–Hinton agar containing AGs at 4 μg/ml. All the isolates were inhibited by ABK, about half by GM, SISO and NTL, and no isolates were inhibited by KM, DKB, AMK, and ISP. Thus, the effect of ABK was distinctive.

In MRSA, five genes, *aph(3′), aad(4′,4″), aac(6′)/aph(2″), aad(6)*, and *aad(9)* encoding AG-modifying enzymes APH(3′), AAD(4′,4″), AAC(6′)/APH(2″), AAD(6) and AAD(9), respectively, have been reported [48]. Among these, the first three were known to be involved in the resistance to KM- and GM-group AGs. We established a colony-direct PCR method for monitoring genes underlying these AG-modifying enzymes [49]. As shown in Table 1 and Fig. 1, all KM-group and GM-group AGs have the target sites (6′-NH_2 and 3″-OH) for the bifunctional enzyme, AAC(6′)/APH(2″). KM, AMK, and ISP have additional target sites for AAD(4′,4″) and KM has APH(3′). Thus, GM and ABK are the same in terms of AG modifying enzyme target sites. Figure 3 (left) shows transition curves of the resistance ratio to KM, GM and ABK and the harboring ratio of *aad(4′,4″)* and *aac(6′)/aph(2″)* in MRSA isolates during 1979 to 2000. Obviously, *aac(6′)/aph(2″)* curve strictly correlated with the GM resistance curve. The same correlation was also seen with resistance curves of SISO and NTL (data not shown). By contrast, the ABK resistance curve was completely independent of the *aac(6′)/aph(2″)* curve. Furthermore, the other remarkable difference was found in the resistance as shown in Fig. 3 (right).

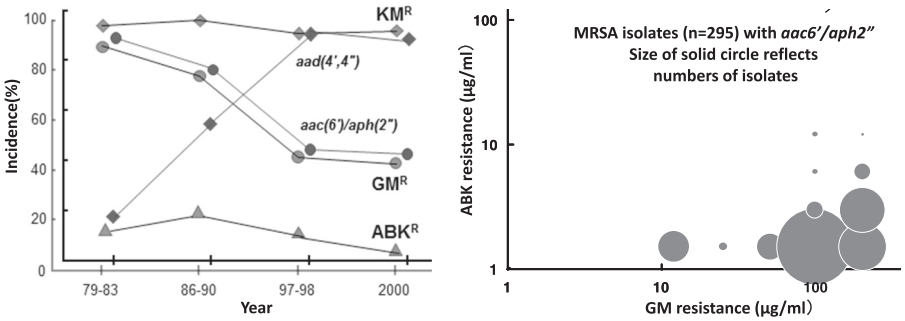


Fig. 3 ABK resistance independence on *aac(6′)/aph(2″)* existence which confers high GM resistance in MRSA (Color figure online)

Table 2 Coagulase genotype based sorting of MRSA isolates with AG-modifying gene

Geno type	Sero type	Total number (%)	Incidence during		Ratio of AG modifying gene		
			'79 ~ 90	'91 ~ 00	*aac/aph*[a]	*aad*[a]	*aph*[a]
L21	II	337 (76.1)	26 (26.5)	307 (89.0)	42%	91%	0%
L22	IV	37 (8.4)	35 (35.7)	2 (0.6)	89	8	49
L31	II	23 (5.1)	20 (20.4)	3 (0.0)	83	9	9
M22	VII	16 (3.6)	12 (12.2)	4 (1.2)	100	75	0
etc	–	30 (6.8)	5 (5.1)	25 (7.2)	77	70	0
		443 (100)	98 (100)	345 (100)			

[a] *aac/aph: aac(6′)/aph(2″), aad: aad(4′,4″), aph: aph(3′)*

i.e., majority of MRSA isolates were scored at the low range (< 10 μg/ml) in ABK resistance but markedly high level range (≥ 100 μg/ml) in GM resistance. In addition, SISO and NTL showed weaker but similar results to that of ABK (data not shown). Therefore, it was indicative that 1-*N*-side chains substantially functioned to block the access of AAC (6′)/APH(2″) to its target sites. These results indicate that ABK must have some means of avoiding the action of AG-modifying enzymes including AAC(6′)/APH(2″).

Moreover, Table 2 shows the coagulase type sorting of MRSA isolates with AG-modifying enzymes. Based on the information that RFLP (restriction fragment length polymorphism) analysis for the coagulase gene (*coa*) of *S. aureus* is useful for typing of MRSA [50, 51], we carried out *coa*-RFLP analysis to classify coagulase types of MRSA isolates together with serotype analysis [46, 47]. Consequently, 16 genotypes were found and L21, L22, L31, and M22 corresponding to serotypes II, IV, II, and VII, respectively, were dominant among MRSA isolates during 1979–1990 (Table 2). However, in the isolates during 1991–2000, type L21 became overwhelmingly dominant (about 90%). This drastic change might be due to an overwhelming use of β-lactams (3rd generation cephems) and quinolones in chemotherapy during the 1980s.

Isolates having coagulase genotypes of L22, L31, and M22 possessed *aac(6′)/aph(2″)* gene at high ratios (83–100%), while L21 type isolates had this gene at a markedly low ratio (42%). In addition, only 5.5% type L21 isolates showed ABK resistance (*i.e.*,: MIC > 4 μg/ml). There were no nucleotide sequence difference in both coding and upstream regions of the *aac(6′)/aph(2″)* gene between ABK-resistant and ABK-susceptible strains with L21 type coagulase (unpublished data). These results make it difficult to correlate ABK resistance with the *aac(6′)/aph(2″)* gene. In the other coagulase genotypes, only M22 showed ABK resistance to a large extent (70%), however, the overall number was small.

Furthermore, we discovered another distinctive feature of ABK; *i.e.*, it retains antibiotic activity even if acetylated by AAC(3), AAC(2′) and AAC(6′). To our knowledge, no MRSA strain containing AAC gene has been confirmed among strains with AG resistance, although the ABK molecule (Fig. 1) has the possible modification sites (3-NH_2, 2′-NH_2 and 6′-NH_2) for AAC(3), AAC(2′) and AAC (6′). Therefore, we could not rule out the possible future emergence of AAC-dependent MRSA strains. To check this possibility, we carried out simulative acetylation of KM-group and GM-group AGs by cell free extracts containing AAC(3), AAC(2′), and AAC(6′) derived from actinomycetes [52–54]. This resulted in ABK and NTL being completely acetylated by AAC(3) whereas ISP and AMK were refractory. In the case of AAC(2′), all the AGs with 2′-NH_2 were completely acetylated whereas no conversion was observed in the AGs with a 2′-OH (KM, AMK and ISP). On the other hand, acetylation by AAC(6′) was observed in all

Table 3 Activity of acetylated AGs and resistance conferred by AAC genes

AG	Activity(%) after acetylation with			Resistance (μg/ml) conferred by		
	AAC(3)	AAC(2′)	AAC(6′)	*aac(3)*[b]	*aac(2′)*[b]	*aac(6′)*[b]
KM	<1	–[a]	<1	100	<2.5	100
DKB	<1	10	<5	50	10	50
AMK	ref[a]	–[a]	<1	<2.5	<2.5	10
ABK	100	80	15	<2.5	<2.5	5
GM	<1	25	25	10	<2.5	2.5
SISO	<1	30	<1	10	<2.5	25
ISP	ref[a]	–[a]	<1	<2.5	<2.5	5
NTL	<1	20	1	5	2.5	25

[a] ref: refractory,–: no 2′–OH

[b] *aac(3), aac(2′), and aac(6′)* genes were cloned from actinomycetes

the AGs examined except for a GM component, probably GM-C1 [54]. Structure determination revealed 3″-*N*-acetylABK, 2′-*N*-acetylABK and 6′-*N*-acetylABK as the acetylation products of ABK. Interestingly, AAC(3) did not acetylate ABK at the 3-NH_2 but rather acetylated 3″-NH_2 [55], although KM was acetylated at 3-NH_2 [56]. Therefore, the ABK acetylation at 3″-NH_2 might be due to the steric hindrance effect of 1-N-AHB side chain.

The reaction mixtures after acetylation were then examined for their antibiotic activities. Surprisingly, the following reaction mixtures retained antibiotic activities; the mixture of ABK after acetylation by AAC(3), those of ABK, GM. SISO, NTL, and DKB after acetylation by AAC(2′), and those of ABK after acetylation by AAC(6′) as shown in Table 3. These findings led us to raising a concept of 'double stage activity' for AG antibiotics capable of retaining activities even if they are modified by AG-inactivating enzymes [53]. By contrast, the other AGs were totally inactivated by acetylation with AAC(3), except for AMK and ISM that are refractory. Therefore, 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 an increasing problem in AG-therapy.

The new findings described above indicate that MRSA strains cannot become ABK-resistant even if they acquired the *aac(3), aac(2″)*, or *aac(6′)* gene. To check this, we examined the AG resistance conferred by the actinomycetes-derived *aac* genes cloned into *Streptomyces lividans* TK21. As shown in Table 3, it turned out that *aac (3)* and *aac(2′)* conferred no ABK resistance and that aac(6′) conferred weak ABK resistance.

Consequently, we predicted that ABK resistance in MRSA would not progress at least for the near future as long as the predominance of MRSA strains with coagulase genotype L21 continues [49]. In fact, MRSA strains with high ABK resistance have rarely emerged [57] and have not spread to date. This situation continues as the latest report [45] indicated that the MIC_{90} of ABK remains at 1 μg/ml for clinical MRSA isolates in Japanese hospitals.

Concluding remarks

We outlined a series of research achievements on KM and ABK. As described, KM was the epoch-making antibiotic which brought the Japanese antibiotics world a breakthrough to the international antibiotic world. Although today KM has only a small contributions to chemotherapy, ABK will keep playing an important therapeutic role in the control of MRSA infections [45]. ABK's features of double stage activity, as well as resistance to the action of AAC(6′)/APH(2″) are also notable.

With patent royalties of KM, Dr. Umezawa established the Microbial Chemistry Research Foundation (1958) and later the Institute of Microbial Chemistry in Tokyo (1962) where he was the Director until his death in Dec. 1986. It was a well-equipped world class R&D facility and also hosted many scientists from outside organizations such as NIHJ, universities and companies. He developed world-leading antibiotic research and opened up new research areas, e.g., anticancer agents, which led to the discovery of sarkomycin and bleomycin; as well as enzyme inhibitors or modifiers of immunological functions, which led to the discovery of leupeptin, bestatin and spergualin [58]. He discovered a total of more than 200 secondary metabolites, many with new structures and functions.

For his achievements in antibiotic research, he was awarded the Japan Academy Prize, the Japan Order of Culture (1962) and many international awards. He was also nominated for the Nobel Prize in Physiology/Medicine during almost 10 years before he passed away in Dec. 1986.

He fostered many men and women of talent and was a charismatic leader. We must keep his legacy alive and further advance science for the social welfare.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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