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NOTE

Aranorosin circumvents arbekacin-resistance in MRSA by inhibiting the bifunctional enzyme AAC(6′)/APH(2″)

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The World Health Organization has recently classified antibiotic resistance as one of the three greatest threats to human health. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) is already a major problem, causing severe, intractable opportunistic infections worldwide.[1] At the end of 1990, arbekacin (ABK) was launched in Japan as a useful chemotherapeutic agent for the treatment of infections caused by MRSA.[2,3] Though a few percent of ABK-resistant strains were found after it was launched, the prevalence of the resistant strains did not increase.[4,5] The main mechanism of ABK resistance is thought to be via inactivation of ABK, caused by a bifunctional enzyme, which catalyzes both phosphorylation and acetylation of aminoglycosides.[6] Consequently, inhibitors of this specific enzyme should prove useful for use with ABK to help maintain its effectiveness.

Natural products produced by microorganisms represent a vast source of potential new antibiotics. During our screening for new anti-MRSA agents, we have already found biverlactones to be circumventors of ABK resistance.[7] Our continuous search for ABK resistance circumventors has led us to find aranorosin (Figure 1).[8] In this paper, activity of aranorosin with respect to circumvention of ABK resistance in MRSA is described.

Using MRSA TH-1466 strain, a clinical ABK-resistant isolate, harboring genes of aminoglycoside-modifying enzyme AAC(6′)/APH(2″), anti-MRSA activity was measured by the paper disc method and agar dilution method. The paper disc method was carried out according to the following protocol; MRSA was cultured in 4 ml of Difco Mueller Hinton broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C for 20 h and adjusted to 1×10^8 CFU ml^{-1}. A 750-μl portion of the culture broth was transferred to a plate (10–14 cm, Eiken Kizai, Tokyo, Japan) containing 20 ml Difco Mueller Hinton agar (MHA; Becton Dickinson), with or without ABK (8 μg ml^{-1}, Meiji Seika Pharma, Tokyo, Japan), whose concentration has no effect on growth of MRSA. Paper discs (6 mm, Advantec Toyo Kaisha, Tokyo, Japan) containing various amounts of a sample (or 7 μg of vancomycin as positive control) were placed on the MHA plate and incubated at 37 °C overnight. Anti-MRSA activity was expressed as a diameter (mm) of inhibition zone. The agar dilution method was performed in accordance with the CLSI method.[9]

Aranorosin was purified from the culture broth of *Gymnascella aurantiaca* FKI-6588, isolated from a soil sample collected in Minato-ku, Tokyo, Japan. The structure of aranorosin was identified by comparison of its spectral data with those reported in literature.[8] Aranorosin showed no anti-MRSA activity at 0.3 μg per 6 mm disc on the MHA plate, but showed anti-MRSA activity (10 mm inhibition zone) on an MHA plate containing ABK (8 μg ml^{-1}). The circumventing effect of aranorosin on the activity of ABK against MRSA was investigated by the agar dilution method (Table 1). The MIC value of aranorosin against MRSA was 2 μg ml^{-1}. Therefore, the concentration of aranorosin for ABK combination was used below 0.5 μg ml^{-1} (one fourth of the MIC value had no effect on growth of MRSA) to investigate the circumventing effect of ABK on MRSA resistance. As shown in Table 1, aranorosin markedly reduced the MIC value of ABK against MRSA, from 16 μg ml^{-1} to 0.25 μg ml^{-1} (64-fold). The circumvention activity of aranorosin was further evaluated by population analysis of ABK MICs using 26 clinically isolated strains of ABK-resistant MRSA. Aranorosin reduced MIC values of ABK against all MRSA strains, and the MIC_{50} was reduced from 8 μg ml^{-1} (without aranorosin) to 0.5 μg ml^{-1} (with aranorosin) as shown in Table 2.

Inhibitory activity against the bifunctional enzyme AAC(6′)/APH(2″) was evaluated using cloned enzyme from the MRSA TH-1466 strain. Details of the assay procedure will be published elsewhere.[10] Briefly, the enzyme (112 μg ml^{-1}) was mixed with kanamycin, and the reaction mixture was applied on TLC and developed with 5% KH_2PO_4. Both acetylated kanamycin and phosphorylated kanamycin were more lipophilic than kanamycin and showed larger R_f values than that of kanamycin in the TLC plate. They were detected by ninhydrin reagent. Although we first tried to use ABK as a substrate for enzyme reaction of recombinant AAC(6′)/APH(2″), no phosphorylated ABK and only a small amount of

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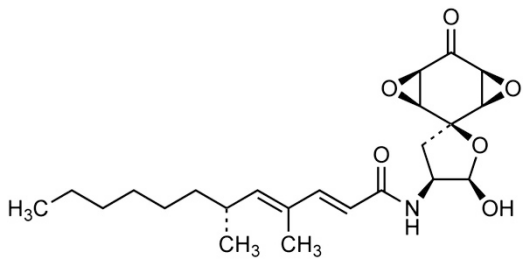

Figure 1 Structure of aranorosin.

Table 1 MIC values of arbekacin against arbekacin-resistant MRSA in the combination of aranorosin

MIC value ($\mu g\,ml^{-1}$) of arbekacin in the combination of aranorosin				
Concentration of aranorosin				
0 $\mu g\,ml^{-1}$	*0.125 $\mu g\,ml^{-1}$*	*0.25 $\mu g\,ml^{-1}$*	*0.5 $\mu g\,ml^{-1}$*	*MIC value ($\mu g\,ml^{-1}$) of aranorosin alone*
16	4	2	0.25	2

Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus*.
Clinically isolated TH-1466 strain was used for ABK-resistant MRSA.

Table 2 MIC population of arbekacin against 26 arbekacin-resistant MRSA strains with or without aranorosin

Dose of aranorosin ($\mu g\,ml^{-1}$)[a]	*Range of MIC ($\mu g\,ml^{-1}$)*	*MIC_{50}*[b] *($\mu g\,ml^{-1}$)*
0	4–32	8
0.5	0.25–2	0.5

Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus*.
[a]MIC values of aranorosin alone against 26 arbekacin-resistant MRSA strains were all $2\,\mu g\,ml^{-1}$.
[b]MIC_{50} shows that 50% of the strains lie below this MIC.

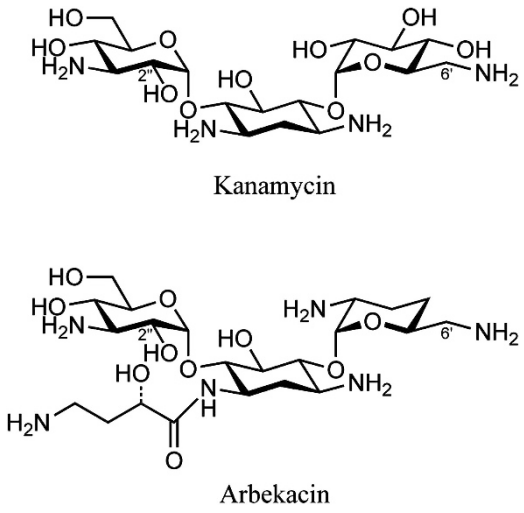

Figure 2 Structures of kanamycin and arbekacin.

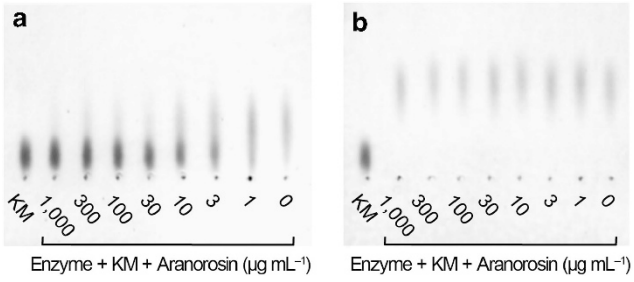

Figure 3 TLC analysis of bifunctional enzyme inhibition by aranorosin. (**a**) Inhibition of phosphorylation. (**b**) Inhibition of acetylation. KM, kanamycin (control). The other lanes are bifunctional enzyme-treated kanamycin in the existence of aranorosin at the described concentration.

acetylated ABK were observed, indicating that ABK is not easily modified by AAC(6′)/APH(2″) due to its 4-amino-2-hydroxybutyryl moiety.[2,11,12] Indeed, ABK resistance is considered to be caused by significant increase in *aac(6′)/aph(2″)* gene or its expression.[13,14] On the contrary, kanamycin is easily phosphorylated or acetylated by the enzyme. Therefore, in this assay, we used kanamycin instead of ABK as a substrate (Figure 2).

As shown in Figure 3, aranorosin only inhibited phosphorylation of the recombinant AAC(6′)/APH(2″) dose dependently. It inhibited kanamycin phosphorylation by half at $1\,\mu g\,ml^{-1}$. These data suggested that aranorosin could circumvent aminoglycoside resistance in MRSA by inhibiting the aminoglycoside-phosphorylation reaction of the bifunctional enzyme AAC(6′)/APH(2″). Aranorosin has an alkyl chain similar to that of biverlactones.[7,8] This suggests that the alkyl chain has an important role in the inhibition activity against AAC(6′)/APH(2″).

It was reported that ABK retained antimicrobial activity after enzymatic acetylation by AAC(3), AAC(2′) and AAC(6′).[15] Kondo *et al*.[6] reported that when ABK was inactivated by crude enzyme prepared from an ABK-resistant MRSA having AAC(6′)/APH(2″), phosphorylated ABK was obtained as a major inactivated product. Therefore, phosphorylation may be much critical than acetylation for the inactivatiton of ABK. Our results that the inhibition of phosphorylation circumvented ABK resistance coincide with this assumption.

ABK is one of the therapeutic agents used for the acute stage of MRSA infection in Japan, and ABK-resistant MRSA is an important problem.[13,16] Although many aminoglycoside-inactivating enzymes have been identified, such as aminoglycoside *N*-acetyltransferases (AACs), aminoglycoside *O*-nucleotidyltransferases (ANTs), aminoglycoside *O*-phosphotransferases (APHs) and aminoglycoside bifunctional modifying enzyme (AAC(6′)/APH(2″)), it is considered that the major mechanism of prevention of ABK activity is enzymatic inactivation by a bifunctional aminoglycoside-modifying enzyme.[6,17,18] Accordingly, circumventors of ABK resistance in MRSA would be very useful to use in combination therapy, allowing for decreases in dose of ABK, thereby helping to abate or remove unwanted side effects.

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