A newly recognized acetylated metabolite of arbekacin in arbekacin-resistant strains of methicillin-resistant Staphylococcus aureus

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Sir, Arbekacin, 1-N-(S)-4-amino-2-hydroxybutyryldibekacin, is a novel, semi-synthetic aminoglycoside which was synthesized in Japan in 1973. It has a broad spectrum of activity, including bacteria resistant to kanamycin, gentamicin and tobramycin, principally because it is not inactivated by aminoglycoside-modifying enzymes. Arbekacin has been used as treatment of patients with infections caused by methicillin-resistant Staphylococcus aureus (MRSA) in Japan since 1990 and the isolation of MRSA strains resistant to this antibiotic was first reported in 1992. The basis of this resistance has been shown to be the bifunctional aminoglycoside-modifying enzyme, aminoglycoside phosphotransferase (APH(2'))–aminoglycoside acetyltransferase (AAC(6')) which simultaneously phosphorylates and acetylates. We describe here a newly recognized metabolite of arbekacin in arbekacin-resistant strains of MRSA.

The six MRSA strains studied were clinical isolates recovered from the wounds of six patients. Four of the strains (1, 3, 4 and 5) were resistant to arbekacin (MICs ≥ 6.25 mg/L) and one (6) was susceptible (MIC = 0.78 mg/L). The sixth strain (2) was initially susceptible to arbekacin, but developed resistance (MIC = 25 mg/L) following serial passage in the presence of the antibiotic at a concentration equivalent to 0.5 × MIC according to a method described previously.

A suspension of each strain in 5 mL of broth containing 5 mg of arbekacin was adjusted to give a turbidity equivalent to that of a no. 1 McFarland standard and incubated for 24 h at 37°C. The suspension was then passed through a 0.22 μm Millipore filter. The metabolites in the cell-free filtrate were separated by Sep-pak (Waters) column chromatography and the eluates were concentrated in vacuo and analysed by various techniques. NMR (1H and 13C) spectra were obtained with a JNS-GSX 400 spectrometer (Jeol, Tokyo, Japan), with sodium 2,2-dimethyl-1,2-silapentane-5-sulphonate (DSS) as an internal standard. Mass spectrometry was performed with a JMS-DX 303/JMA-DA 5000 System high-resolution mass spectrometer (Jeol) and Fourier transform infrared (FT-IR) spectra were obtained with a DR-81 FT-IR spectrometer (Jasco, Tokyo, Japan).

Crude enzyme preparations were obtained by incubating a suspension of each strain in 250 mL of 1% Bacto Tryptone broth (Difco, West Molsey, UK) containing 0.5% NaCl and 0.5% yeast extract at 37°C for 8 h on a rotary shaker. The suspension was centrifuged at 3000 rpm for 20 min at 4°C and the supernatant removed. The cell deposit was then washed with 0.05 M Tris–HCl buffer (pH 7.5) and resuspended in 5 mL of 0.05 M Tris–HCl 10 mM MgCl2 buffer (pH 7.8). Lysostaphin (Sigma, St Louis, MO, USA), at a concentration of 25 mg/L, was added to the suspension which was incubated for 30 min at 37°C. The cells were disrupted by sonication and the suspension was centrifuged at 30,000g for 30 min; the supernatant represented the crude enzyme preparation. The ratio of inactivation of arbekacin was determined by the method of Haas & Dowding. Briefly, a mixture comprising 0.05 mL of arbekacin (at a concentration of 400 mg/L), 0.05 mL of 0.02 M ATP (Sigma), 0.05 mL of 0.5 mM acetyl CoA (Sigma), 0.05 mL

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of 0.02 M magnesium acetate and 0.15 mL of the crude enzyme preparation was made up to 0.5 mL with 0.2 M Tris–maleic acid buffer (pH 7.0). The solution was incubated at 37°C for 3 h. A control solution which contained neither ATP nor acetyl CoA was included. The rate of inactivation of arbekacin was determined by a bioassay method with Bacillus subtilis ATCC 6633 as the test organism and the ratio of inactivation was expressed in terms of the reduction in antimicrobial activity following the period of incubation.

The results of the NMR analyses are shown in the Table. 1H-NMR demonstrated that a chemical shift of 2.21 ppm, accounted for by the acetyl group, had occurred in the medium in which the arbekacin-resistant strains (numbers 1–5) were grown. Proton signal changes were not identified at positions 2\textsuperscript{′} or 6\textsuperscript{′}, but signals at positions 2\textsuperscript{′′}, 3\textsuperscript{′′} and 4\textsuperscript{′′} of the amino-2-hydroxybutyryl (AHB) group shifted towards the lower magnetic field by 0.2–0.3 ppm. 13C-NMR demonstrated that the carbon signals at the same positions of the AHB group in the medium in which the resistant strains were grown shifted towards higher magnetic fields. Mass spectroscopy revealed the presence of an acetylated derivative (mol. wt 595) of arbekacin (mol. wt 553). However, neither phosphorylated derivatives (mol. wt 633), nor derivatives that were both phosphorylated and acetylated (mol. wt 675) were detected. With FT-IR spectrometry, absorption at 1650 cm\textsuperscript{−1} was observed with the eluates of the medium in which the resistant strains were grown (but not with the medium in which the susceptible strain was incubated), thereby confirming the presence of an acetyl group (C=O). For all six strains, there was no absorption at

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Table. 1H-NMR and 13C-NMR spectral data for the metabolites of arbekacin in the medium in which the six MRSA strains were grown

<table>
<thead>
<tr>
<th>NMR</th>
<th>Position</th>
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<th>4</th>
<th>5</th>
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Abbreviations: s, singlet; d, doublet, dd, double doublet; t, triplet; q, quartet; m, multiplet.

a The underlined carbon atom denotes the origin of the NMR signal.

Figure. Chemical structure of arbekacin. The AHB group is boxed and the positions of the carbon atoms affected by the bifunctional aminoglycoside-modifying enzyme, APH(2\textsuperscript{′})–AAC(6\textsuperscript{′}), are noted.
1230–1260 cm$^{-1}$ (absorption indicating the presence of a phosphoric ester).

Arbekacin was inactivated by crude enzyme preparations of all of the resistant strains by acetylation only, the inactivation ratio varying from 20% to 60%, depending on the strain. There was no evidence of inactivation by adenylation or phosphorylation.

Kondo et al.\textsuperscript{3} reported that inactivation of arbekacin relies principally on the phosphorylation of the 2'-OH group and, to a much lesser extent, on the acetylation of the 6'-NH$_2$ group. In the present study, we have identified, for the first time, a metabolite of arbekacin which is mono-acetylated at position 1 of the AHB group. It is of interest that the arbekacin-susceptible strain which developed resistance following incubation in the presence of increasing concentrations of arbekacin had the same acetylating properties as the other resistant strains. This suggests that the acetylating enzyme is encoded in susceptible strains and is induced or modified by in-vitro passage. However, we have demonstrated only that arbekacin-resistant strains have acetylation activities not present in an arbekacin-susceptible strain. It has not been determined whether or not this activity can be attributed to a single enzyme or, indeed, if the enzyme is truly novel. These issues are currently under investigation.

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\textbf{References}