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Apigenin as an anti-quinolone-resistance antibiotic

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ABSTRACT

We previously reported the first 'reverse antibiotic' (RA), nybomycin (NYB), which showed a unique antimicrobial activity against *Staphylococcus aureus* strains. NYB specifically suppressed the growth of quinolone-resistant *S. aureus* strains but was not effective against quinolone-susceptible strains. Although NYB was first reported in 1955, little was known about its unique antimicrobial activity because it was before the synthesis of the first quinolone ('old quinolone'), nalidixic acid, in 1962. Following our re-discovery of NYB, we looked for other RAs among natural substances that act on quinolone-resistant bacteria. Commercially available flavones were screened against *S. aureus*, including quinolone-resistant strains, and their minimum inhibitory concentrations (MICs) were compared using the microbroth dilution method. Some of the flavones screened showed stronger antimicrobial activity against quinolone-resistant strains than against quinolone-susceptible ones. Amongst them, apigenin (API) was the most potent in its RA activity. DNA cleavage assay showed that API inhibited DNA gyrase harbouring the quinolone resistance mutation *gyrA*(Ser84Leu) but did not inhibit 'wild-type' DNA gyrase that is sensitive to levofloxacin. An API-susceptible *S. aureus* strain Mu50 was also selected using agar plates containing 20 mg/L API. Whole-genome sequencing of selected mutant strains was performed and frequent back-mutations (reverse mutations) were found among API-resistant strains derived from the API-susceptible *S. aureus* strains. Here we report that API represents another molecular class of natural antibiotic having RA activity against quinolone-resistant bacteria.

1. Introduction

The emergence of bacteria resistant to antibiotics has become a worldwide concern. Although finding effective treatments for resistant strains is a top priority, few tools are available due to difficulty in the development of new antibiotics by the pharmaceutical industry. The history of antibacterial agents tells us that introduction of a new antibiotic is always followed by the appearance of resistant micro-organisms, with no exceptions [1]. Therefore, development of new drugs based on a completely novel concept is awaited.

We have previously reported nybomycin (NYB) as a candidate for such novel therapeutics [2]. NYB, produced by an actinobacterium, was first reported in 1955 [3]. At that time, quinolone-resistant bacteria had not been detected since clinical introduction of the quinolones did not start until the early 1960s [4]. Therefore, features of NYB with regard to quinolone resistance have not been studied. Our re-discovery of NYB as a 'reverse antibiotic' (RA) was brought about by screening of Actinobacteria cultures using quinolone-resistant meticillin-resistant *Staphylococcus aureus* (MRSA) as well as quinolone-susceptible *S. aureus* clinical strains. We looked for Actinobacteria supernatants that suppressed the growth of MRSA. The screening hit an actinobacterial strain, *Streptomyces hyalinum* MB891-A1, which produced a substance that had strong growth inhibition against MRSA strain Mu50. However, surprisingly the substance exhibited no activity against a meticillin-susceptible *S. aureus* (MSSA) type strain FDA 209P. The finding of this substance, identified as NYB, prompted us to determine the mode of antimicrobial activity of NYB. We noticed that MRSA strain Mu50 was resistant to quinolones but was susceptible to NYB, whereas the MSSA strain FDA 209P was quinolone-susceptible but NYB-resistant. NYB was found to specifically suppress the growth of quinolone-resistant *S. aureus* carrying mutation(s) in the quinolone resistance-determining region (QRDR) of *gyrA*, encoding the A subunit of DNA gyrase. However, NYB was not active against strains carrying intact or wild-type *gyrA* with no QRDR mutations. In addition, *S. aureus* mutant strains that acquired NYB resistance became quinolone-susceptible, and they were found to have reverse mutations to regain the 'wild-type' sequence in the QRDR. Biochemical analysis also revealed that NYB targets and inhibits the function of the mutated GyrA subunit.

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Thus, we found that the antimicrobial activity of NYB is complementary to that of quinolones. We therefore designated such a class of antibiotics represented by NYB as 'reverse antibiotics' (RAs).

During our attempt to screen for new RA candidates, we noticed an old report demonstrating a weak inhibitory effect of quercetin and related flavones on *Escherichia coli* DNA gyrase [5]. Flavones produced by plants are commonly used in folk remedies, thus their antibacterial activity has been studied previously [6]. However, the results were not impressive against the tested bacterial strains, including *S. aureus* [7,8]. Using quinolone-susceptible and -resistant *S. aureus* strains, we found that some flavones do have activity against *S. aureus*, and a few of them inhibited the growth of quinolone-resistant strains much better than that of susceptible ones.

Among such flavones, apigenin (API) was found to be the most conspicuous in its activity. In this report, we describe the mode of action of API and its property as another class of RA.

2. Materials and methods

2.1. Bacterial strains

A total of 36 *S. aureus* strains used in this study were isolated from Japan, the UK and the USA (Table 1). All of the strains have been described previously. Clinical *S. aureus* strains MS5935, MR5867, MS5952 and MR6009 and their stepwise quinolone-resistant mutants have been described previously [9]. *Staphylococcus aureus* NCTC 8325 and FDA 209P are MSSA type strains [2], and *S. aureus* N315 is a quinolone-susceptible MRSA [10]. KSA36, KBSA72 and KBSA56 are clinical MRSA isolated from Japanese hospitals in 2005 [2]. The remaining strains are clinical isolates with either reduced susceptibility [vancomycin-intermediate *S. aureus* (VISA)] ($n = 7$) or resistance [vancomycin-resistant *S. aureus* (VRSA)] ($n = 4$) to vancomycin.

2.2. Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method with an inoculum size of 10^4 CFU/well in a final volume of 100 μL. The procedure was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [11].

API was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was used to prepare a 1024 mg/L API solution. Water containing up to 0.1 M NaOH was used for the preparation of quinolones. All solutions were diluted with cation-adjusted Mueller–Hinton broth (CA-MHB) [BD BBL™ Mueller–Hinton II Broth (Cation-Adjusted); BD Diagnostic Systems, Sparks, MD] for MIC measurement.

2.3. Mutant selection

Staphylococcus aureus strain Mu50 was inoculated in eight tubes containing 4 mL of tryptic soy broth (TSB) (BD Diagnostic Systems). After overnight incubation at 37 °C, the eight full-growth tubes were concentrated by 10-fold and were plated individually on a 150 mm × 15 mm Petri dish (BD Falcon™ 35-1058; BD Biosciences, Franklin Lakes, NJ) containing 75 mL of Mueller–Hinton agar medium (BD Diagnostic Systems) with 20 mg/L API. One colony from each plate was picked and subjected to further investigation.

2.4. PCR amplification and sequencing of the quinolone resistance-determining regions of gyrA and parC

Five sets of forward and reverse primers, respectively, were used: 5′-TTAGGTGATCGCTTTGGAAGATATAG-3′ and 5′-TACCATTGGTTCGAGTGTCG-3′ for *parC*; 5′-GGATTAAATGAACAAGGTATGACACCG-3′ and 5′-TAGTCATACGCGCTTCAGTATAACG-3′ for *gyrA*; 5′-CAGCGTTAGATGTAGCAAGT-3′ and 5′-CAGGACCTTCAATATCCTCC-3′ for *gyrB*; 5′-CGTAAGGACGTCTTGCCTGA-3′ and 5′-GGCTCATGACCAGCTGAACT-3′ for *rsbU*; and 5′-CCAGCAATTGGTAAAATCCAA-3′ and 5′-TGGTTCAAAACCAAGGGATG-3′ for *murC*. Nucleotide sequences were converted to amino acid sequences and were aligned with the amino acid sequences of the type II topoisomerases (ParC, GyrA and GyrB) of the parent strain Mu50 and reference strain FDA 209P to identify non-synonymous (or missense) mutations. Sequencing of the amplified DNA was done using a BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Carlsbad, CA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Life Technologies).

2.5. Whole-genome sequencing and mutation detection

The whole genome sequences of in vitro-selected API-resistant mutants were determined using a MiSeq Genome Analyzer (Illumina, Inc., San Diego, CA). DNA extraction was performed using a QIAGEN Genome-tip System (QIAGEN, Hilden, Germany), and library preparations were performed using Nextera XT DNA sample preparation kits (Illumina, Inc.). Pools of eight samples were sequenced on the Illumina MiSeq platform, and 250-bp end reads were generated. Reads from bacterial strains were then mapped to the reference whole genome sequence of strain Mu50 (GenBank accession no. NC_002758.2) and mutations were identified using Tablet v.1.13.04.22 (The James Hutton Institute, Aberdeen, UK). The identified single nucleotide polymorphism were then verified by re-sequencing using an Applied Biosystems 3730 Capillary Sequencer (Applied Biosystems Ltd., Tokyo, Japan) for each locus.

2.6. Cytokilling assay

Overnight cultures of tested strains in TSB were diluted and ca. 0.5 mL of 10^6 CFU/mL was transferred into 4.5 mL of fresh CA-MHB containing 0, 8, 16, 20, 24 or 32 mg/L API. Test tubes were incubated with gentle shaking and 0.1 mL portions of the culture were harvested to a drug-free plate at 0, 0.5, 1, 3, 5, 7 and 24 h. The grown colonies were counted and plotted on a graph. The cultivation temperature of the test strains was 37 °C for Mu50 and KBSA56. These strains were also tested at a cultivation temperature at 43 °C.

2.7. Demonstration of gyrase inhibition by DNA cleavage assay

The assay was based on an established system as described by Fisher and Pan [12]. Briefly, supercoiled plasmid DNA pTWV228 (a pBR322 derivative) (TaKaRa Bio Inc., Kusatsu, Japan) was pre-incubated in the presence of either API or levofloxacin, followed by addition of GyrB in combination with either the wild-type or mutated GyrA proteins and then incubated at 25 °C for 1 h. The following analyses, including determination of IC_{50} values (50% inhibitory concentration), were carried out as described previously [2].

3. Results

By testing 104 flavones, we found at least three flavones that shared preferential antimicrobial activity against MRSA Mu50 compared with MSSA FDA 209P, including API, myricetin-3,7,3′,4′-tetramethyl ether and isopratol. Their MICs are shown in Table 2. Among the three flavones, API showed the most typical MIC profile of a RA. We therefore decided to study API.

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