Reversing β-Lactam Antibiotic Resistance with Flavonoids in Gram-positive Bacteria

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Keywords: methicillin-resistant S. aureus, traditional herbal remedies, antibacterial agents reverse bacterial resistance, minimum inhibitory concentrations

Abstract
Resistance to β-lactam antibiotics is a global problem. Today over 90% of Staphylococcus aureus strains are β-lactamase positive. This enzyme cleaves the β-lactam ring of the antibiotics and renders it ineffective. In addition, strains of methicillin-resistant S. aureus, which owe their resistance to modification in the penicillin-binding proteins in the cell walls, are usually multiply resistant to many antibiotics and pose life-threatening risks to the hospitalised patients and their care givers. Antibiotics available for the treatment of methicillin-resistant S. aureus infection are fairly toxic and their use is frequently associated with unwanted side effects. The search for new antibacterial agents and compounds that can reverse the resistance to well tried agents which have lost their original effectiveness are research objectives of far reaching importance. Here we show that naturally occurring flavonoids, which are often present in edible plants and/or traditional herbal remedies, have the potential to reverse bacterial resistance to β-lactam antibiotics against some currently almost untreatable organisms.

INTRODUCTION
Bacterial resistance to β-lactam antibiotics is a global problem. Today over 90% of Staphylococcus aureus (S. aureus) strains are β-lactamase positive (O’Brein, 1986). Strains of β-lactam-resistant S. aureus including methicillin-resistant S. aureus (MRSA) now pose serious problem to hospitalized patients, and their care provides (Mulligan et al., 1993). Antibiotics available for the treatment of MRSA infection are fairly toxic and their use is frequently associated with unwanted side-effects (Brumfitt and Hamilton-Miller, 1989). Novel antibiotics and/or new approaches that can reverse the resistance to well tried agents which have lost their original effectiveness are research objectives of far reaching importance (Reading and Cole, 1977). In this study we have investigated the in vitro activity of naturally occurring flavonoids, a major constituent in edible plants and/or traditional herbal remedies (Teubert et al., 1977; H’aznagy et al., 1976), against β-lactam-resistant S. aureus and MRSA when use alone and in combination with β-lactam antibiotics.

MATERIALS AND METHODS

Flavonoids Sources and Structures
Baicalin (the 7-glucuronide of baicalein) was isolated from the Chinese herb Scutellaria amoena C.H. wright and identified by chemical and spectroscopic methods and compared with a reference sample (The Central Drug Control Institute, State Public Health Administration, Beijing). The structure was confirmed by X-ray crystallographic analysis of the methyl ester derived from the isolate. The crystallographic data are filed in the Cambridge Structural Database (CSD) and will be published elsewhere. Other flavonoids were obtained from Sigma-Aldrich (Gillingham-Dorset, UK), Lancaster Synthesis (Morecambe, UK) and Apin (Abingdon, UK).
**MIC Determinations**

MIC determinations were carried out using a microtiter method as described in the literature (American National Standards Institute, 1991) using Iso-sensitest broth (Oxoid). The test strains included *S. aureus* NCTC 11940 (MRSA); 6 fresh Clinical MRSA (from Diagnostic Department, Edinburgh Royal Infirmary) which were also ceftazidime-resistant.

(MICs > 32 µg ml⁻¹); *S. aureus* NCTC 9968 and 11561, both penicillin-resistant; 25 recent clinical strains of penicillin-resistant *S. aureus* (from Microbiology Department, Aberdeen Royal Infirmary) and two recent clinical staphylococci (from Microbiology Department, Aberdeen Royal Infirmary) which were β-lactamase producers and coagulase negative. The bacterial inoculum used in these tests was 2.5 x 10⁵ CFU ml⁻¹ and the concentration of flavonoid 25 µg ml⁻¹ unless otherwise specified. Incubation was at 32°C for 24 hours for MRSA and 37°C for 24 hours for the other strains. Ceftazidime was obtained from Glaxo Wellcome and all other antibiotics from Sigma.

**Viable Counts**

Viable counts were performed using the microtiter method already described (Ricahrd and Xing, 1993).

**Electronmicroscopy**

Subculture of *Staphylococcus aureus* NCTC 11940 was incubated at 37°C in fresh Iso-sensitest broth in 250 ml conical flasks with shaking at 100 oscillation/min for 18 hours. This culture was incubated in fresh Iso-sensitest broth for a further 4 hours, incubation with shaking in a water bath at 100 oscillation/min. Then 40 ml volumes of the log phase culture were removed and inoculated separately into 360 ml of prewarmed Iso-sensitest broth and the same broth containing galangin, benzylpenicillin alone and in combination respectively. After 4 hours incubated with shaken in a water bath at 100 oscillations/min at 37°C, the cell pellets were collected, treated and examined under electronmicroscope as previously detailed (Richards et al., 1995).

**Enzyme Assays**

The β-lactamases of *Bacillus cereus* (*B. cereus*) and *Enterobacter cloacae* (*E. cloacae*) were obtain from Sigma, Poole, England. Enzymes activities were adjusted to concentrations sufficient to hydrolyse 50-60% substrate in 5 minutes. Flavonoids were pre-incubated with enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 5 minute prior to substrate addition. Time-course assays were carried out using methanol/acetic acid (100:1) as stop reagent and analyses of the remaining substrate determined by reverse-phase HPLC (Reading and Farmer, 1983) with acetronitrile/acetate in the mobile phase.

**RESULTS AND DISCUSSION**

**MIC Determinations**

Thirty-six flavonoids were tested for activity and structures of example flavonoids are shown in Fig. 1. All flavonoids tested were detailed in the International Patent Application (PCT/GB98/00512) (Richards et al., 1998). The twenty five fresh clinical isolates of penicillin-resistant *S. aureus*, six isolates of methicillin-resistant *S. aureus* (MRSA) and two clinical isolates of coagulase-negative staphylococci tested were made sensitive to amoxicillin by galangin and had their Minimum Inhibitory Concentrations (MICs) reduced from an initial range of 2- > 250 µg ml⁻¹ to a range of < 0.25-2 µg ml⁻¹ (Table 1). Twentyfour strains had MICs reduced to < 0.25 µg ml⁻¹, six strains to 0.5 µg ml⁻¹ and one strain to 2 µg ml⁻¹. This reversal of resistance was particularly marked when the penicillin was increased more than a thousand fold.

In addition, six clinical isolates of ceftazidime-resistant *S. aureus* strains with MICs 32-250 µg ml⁻¹ had their resistance to ceftazidime reversed by galangin 25 µg ml⁻¹.
to MICs of < 0.25 µg ml⁻¹, while the MICs for galangin alone were > 250 µg ml⁻¹. The highest fractional inhibitory concentration (FIC) for these ceftazidime plus galangin combinations was only marginally over 0.1. An FIC of 0.1 indicates a high level of synergistic activity since values below 0.5 are widely accepted as representing synergism between two antibacterials (Sabath, 1967). A type strain of MRSA (NCTC 1940) also had its resistance to methicillin, cloxacillin, amoxicillin, ampicillin and cefotaxime reversed when any of these β-lactams was combined with baicalein, apigenin, luteolin or galangin (Table 2).

Viable Counts
An example of a typical killing curve obtained with penicillin-resistant S. aureus (NCTC 9968) using viable counts is given in Fig. 2. MICs for benzylpenicillin and baicalin against this strain were 125 and 64 µg ml⁻¹ respectively. The S. aureus strain was tested using the flavonoid alone and in combination. Baicalin at 25 µg ml⁻¹ had little effect on the bacterial growth rate compared with the control. Benzylpenicillin 50 µg ml⁻¹ reduced the viable counts by 1.25 log cycles after just over 2 hours but then the viable counts recovered so that after 24 hours they were 2 log cycles greater than the concentration of cells produced by the initial inoculum. Baicalin 25 µg ml⁻¹ plus either benzylpenicillin 50 or 10 µg ml⁻¹ reduced the viable counts by 3 log cycles within 2 hours and 4 hours respectively and maintained that reduction in over 24 hours (The lower limit of the counting technique was a suspension of 10⁵ CFU ml⁻¹).

Electronmicroscopy
Electronmicroscope investigations clearly showed that the combination of β-lactam with galangin caused damage to the ultrastructures of MRSA cells. Fig. 3 indicates that galangin 25 µg ml⁻¹ reduced the thickness of the cell walls compared with the cell walls of the control cells and also apparently delayed cell division. The galangin treated cells were considerably bigger than the normal cells. Benzylpenicillin 25 µg ml⁻¹ alone apparently had no effect on the cell wall structure but the combination of the antibacterial agents is observed to have affected the integrity of the cell walls and led to an increase in cell size. This latter effect is apparently due to inhibition of cell division.

Enzyme Assays
The ability of flavonoids to inhibit the in vitro activity of β-lactamases varied considerably. Fig. 4 indicates that galangin has an inhibitory activity against β-lactamase I from B. cereus. Baicalin, apigenin, luteolin and scutellarein had less activity but tectochrysin and 6-chloro-7-methylflavone showed greater activity. Against penicillinase type IV from E. cloacae, apigenin showed marked inhibitory activity but none of the other flavonoids tested showed appreciable activity. These results indicate that in addition to the direct effect on cell structure and cell division, the resistance reversing activity of flavonoids against bacteria might also include inhibition of β-lactamase activity.

CONCLUSIONS
The results presented here indicate that flavonoids not only have an activity of their own against β-lactam-resistant staphylococci but they also have the ability to reverse the resistance of such bacterial strains to the activity of the primary antibiotics. This may involve two mechanisms of action by the flavonoids. The first is on the integrity of the cell wall and on septum formation prior to cell division. This implies an effect on protein synthesis including an effect on penicillin-binding proteins. The second mechanism of β-lactam activity is via inhibition of the activity of certain β-lactamase enzymes. The first action could also include an effect on the production and/or release of β-lactamase enzymes within and from the cell walls (Yam et al., 1998). In the last two decades, β-lactamase inhibitors like clavulanic acid have played an important role in fighting β-lactam-resistant bacteria. These inhibitors work as suicide compounds to react with the enzymes since they share the same key structure with β-lactam antibiotics (Coulton and
Francois, 1994). Recent studies demonstrated that clavulanate caused a considerable induction of β-lactamase expression and an increase of clavulanate concentration was followed by an elevation in β-lactamase production (Tzouvelekis et al., 1997; Stapleton et al., 1995). This indicates that the presently available β-lactamase inhibitors can also lose their activity by the same mechanism as the β-lactam antibiotics. Our research provides a unique example that flavonoids without a β-lactam structure can reverse bacterial resistance to β-lactams via multiple mechanisms. Because of this structural dissimilarity these compounds are unlikely to induce β-lactamase production. It should also be remembered that conventional β-lactamase inhibitors, unlike flavonoids, cannot reverse the resistance of MRSA, which is one of the most dangerous bacterial pathogens.

Literature Cited
## Tables

### Table 1. MICs (µg ml⁻¹) of amoxicillin alone and in combination with galangin against clinical isolates of staphylococci.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain Lab. No.</th>
<th>Amoxicillin alone 6.3 µg ml⁻¹</th>
<th>Amoxicillin plus galangin 12.5 µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin-resistant S. aureus</td>
<td>321</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>108</td>
<td>250</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>141</td>
<td>&gt;250</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>296</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>684</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>352</td>
<td>125</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>543</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>975</td>
<td>125</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>593</td>
<td>125</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>718</td>
<td>250</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>349</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>360</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Methicillin-resistant S. aureus (MRSA)</td>
<td>588</td>
<td>32</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>68-15</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>71-16</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>70-15</td>
<td>&gt;250</td>
<td>250</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>428605</td>
<td>16</td>
<td>N/D</td>
</tr>
</tbody>
</table>

* galangin at 25 µg ml⁻¹
N/D: not done

### Table 2. MICs (µg ml⁻¹)* of β-lactams used alone and in combination with flavonoids against S. aureus NCTC11940 (MRSA).

<table>
<thead>
<tr>
<th>Compound</th>
<th>β-lactam alone</th>
<th>β-lactam plus 25 µg ml⁻¹ of the following flavonoids</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>methicillin</td>
<td>210</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>amoxicillin</td>
<td>250</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>ampicillin</td>
<td>350</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>150</td>
<td>0.5</td>
<td>N/D</td>
</tr>
<tr>
<td>cloxacillin †</td>
<td>1000</td>
<td>0.5</td>
<td>N/D</td>
</tr>
</tbody>
</table>

* MIC presented as Geomean of 3-5 observations
† data obtained from cloxacillin-resistant strain induced in this Lab
**Figures**

Fig. 1. Basic structure of flavonoids and structures of example flavonoids tested.  
**a**, flavone;  
**b**, galangin;  
**c**, apigenin;  
**d**, baicalein;  
**e**, luteolin.

Fig. 2. The effect of benzylpenicillin combined with baicalin on the viable counts of penicillin-resistant *Staphylococcus aureus* (NCTC 9968). Open circles, baicalin 25 μg ml⁻¹; open triangles, benzylpenicillin 50 μg ml⁻¹; reversed open triangles, benzylpenicillin 50 μg ml⁻¹ plus baicalin 25 μg ml⁻¹; filled diamonds, benzylpenicillin 10 μg ml⁻¹ plus baicalin 25 μg ml⁻¹; the values plotted are the means of 4 observations, and the vertical bars indicate the standard errors of the means.
Fig. 3. Ultrathin sections of log phase *S. aureus* NCTC 11940 (MRSA) grown in Iso-sensitest broth containing: a, drug-free (control); b, 25 µg ml⁻¹ benzylpenicillin; c, 25 µg ml⁻¹ galangin; d, 25 µg ml⁻¹ benzylpenicillin plus 25 µg ml⁻¹ galangin (a, b, c, d, original magnification, x 17,480; bar, 1 µm; Inset, a, b, d, original magnification, x 42,800; c, x 32,500; bar, 0.25 µm).
Fig. 4. The inhibitory activity of flavonoids against β-lactamase in hydrolyzing benzylpenicillin. a. β-lactamase used from *B. cereus*; symbol represents flavonoids (200 µg ml⁻¹); asterisks, control (without flavonoids); open diamonds, galangin; open triangles, 6-chloro-7-methylflavone; filled squares, tectochrysin. b. β-lactamase used from *E. cloacea*; symbol represent concentrations (µg ml⁻¹) of apigenin; open circles, control (without apigenin); open squares, 20; filled triangles, 40; filled diamonds, 60; filled squares, 80; filled circles, 100.