JPP Journal of Pharmacy and Pharmacology

JPP 2001, 53: 129–132

Received June 8, 2000
Accepted September 19, 2000
ISSN 0022-3573

Vancomycin resistance reversal in enterococci by flavonoids

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Abstract

The development of clinical vancomycin-resistant strains of enterococci (VRE) is a major cause for concern. Here we show that a combination of galangin or 3,7-dihydroxyflavone with vancomycin may be used to sensitize resistant strains of *Enterococcus faecalis* and *Enterococcus faecium* to the level of vancomycin-sensitive strains.

Minimum inhibitory concentrations (MICs) and viable counts were determined in Iso-sensitest broth using a microtitre method. MICs of vancomycin against 67 % of resistant clinical isolates and a type strain of enterococci were lowered from $> 250\ \mu g\ mL^{-1}$ to $< 4\ \mu g\ mL^{-1}$ in the presence of galangin ($12.5\ \mu g\ mL^{-1}$) or 3,7-dihydroxyflavone ($6.25\ \mu g\ mL^{-1}$).

Viable counts for type culture *E. faecalis* ATCC 51299 showed the flavonoids alone significantly lowered numbers of colony forming units (CFUs). CFUs were maintained at low levels ($< 10^3$ CFU mL^{-1}) for 24 h by vancomycin/flavone combinations. This combinational action in reversing vancomycin resistance of enterococci highlights novel drug targets and has importance in the design of new therapeutic regimes against resistant pathogens.

Introduction

The development of resistance of strains of pathogenic bacteria to antibiotics currently in therapeutic use is a problem of continuing concern to public health (Neu 1992). The glycopeptide antibiotic vancomycin has been used in the treatment of severe infections due to Gram-positive bacteria resistant or intolerant to β-lactam antibiotics. However, strains of enterococci have evolved that exhibit multiple antibiotic resistance, including that to both ampicillin and vancomycin.

The mechanism of glycopeptide antibiotic action is postulated to be dependent upon its recognition and binding to the terminal region (ala-ala) of the disaccharide pentapeptide precursor of the peptidoglycan pentapeptide involved in cell wall cross-linking. The resulting complex prevents coupling to penicillin binding protein (PBP) and inhibits subsequent transpeptidase cross-linking to generate a stable cell wall (Figure 3a).

Resistance to vancomycin arises as the result of activation of a cytoplasmic membrane-associated protein VanS and an activator VanR for the *VanHAX* operon, resulting in the production of an alternative disaccharide peptide-producing pathway. Component proteins VanX, VanH and VanA synthesize a disaccharide peptide with terminal region ala-lac, which does not bind vancomycin but couples to PBP, releasing D-lac. The formation of peptidoglycan peptide-PBP intermediates thus restores the route for cell wall cross-linking. The existence of this alternative

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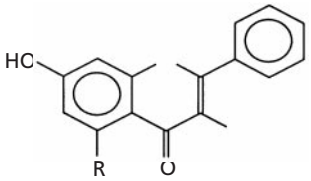

(1) 3,5,7-Trihydroxyflavone R=OH R′=OH

(2) 3,7-Dihydroxyflavone R=H R′=OH

Figure 1 The structure of galangin (**1**) and 3,7-dihydroxyflavone (**2**).

pathway for cell wall precursors provides a mechanism to explain the development of bacterial resistance to vancomycin (Arthur et al 1996).

In this study, we report the sensitization of vancomycin-resistant clinical-isolate strains of *Enterococcus faecalis* and *Enterococcus faecium* to vancomycin in the presence of naturally occurring flavonoids, galangin (3,5,7-trihydroxyflavone) (**1**) and 3,7-dihydroxyflavone (**2**) (Figure 1).

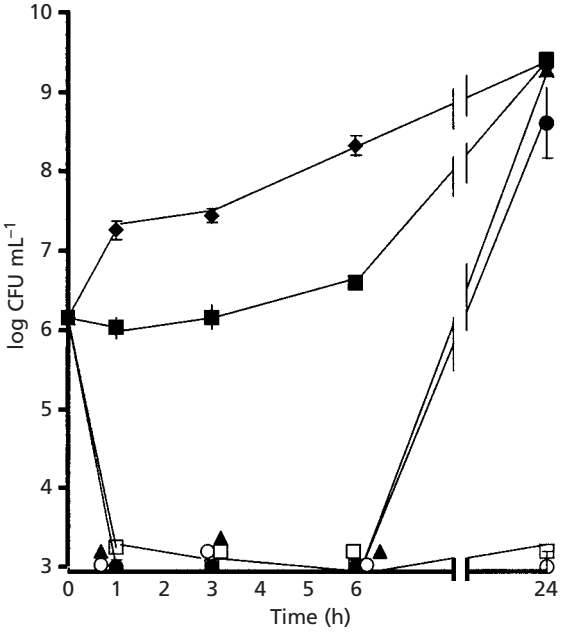

Figure 2 The effect of vancomycin in combination with galangin (**1**) or 3,7-dihydroxyflavone (**2**) on the viable counts of vancomycin-resistant *E. faecalis* ATCC 51299: ◆, control bacterial culture; ■, vancomycin (16 μg mL^{-1}); ▲, **2** (6.25 μg mL^{-1}); ●, **1** (12.5 μg mL^{-1}); ○, vancomycin/**1** (16/12.5 μg mL^{-1}); □, vancomycin/**2** (16/6.25 μg mL^{-1}).

Materials and Methods

Minimum inhibitory concentrations (MICs) were determined using a microtitre method as described in the literature (American National Standards Institute 1991) using an Iso-sensitest broth medium (Oxoid, Basingstoke, UK). Flavonoids were dissolved in 10% sodium carbonate solution with adjustment to give a final concentration of 0.005% sodium carbonate in the assay culture.

Type VRE strain, *E. faecalis* ATCC 51299 (American Type Culture Collection, Rockville, MD) or one of 15

Table 1 MICs (μg mL^{-1}) of vancomycin and in combination with flavonoids against vancomycin-resistant *E. faecalis* and *E. faecium*.

Organism (strain number)	MICs vancomycin	MICs vancomycin in combination with:	
		Galangin (**1**) 12.5 μg mL^{-1}	3,7-Dihydroxyflavone (**2**) 6.25 μg mL^{-1}
E. faecalis ATCC 51299	> 125	8	< 4
Clinical isolates:			
E. faecalis 5542*, 28, 31	125–> 250	< 4	< 4
E. faecalis 24, 33, 47	8–64	< 4	< 4
E. faecalis 30	> 250	32	32
E. faecalis 58	> 250	> 250	> 250
E. faecium 1, 21, 69, 4673*	125–> 250	< 4	< 4
E. faecium 61	64	8	16
E. faecium 4137*	64	64	–
E. faecium 63	> 250	250	> 250

* Not tested against 3,7-dihydroxyflavone.

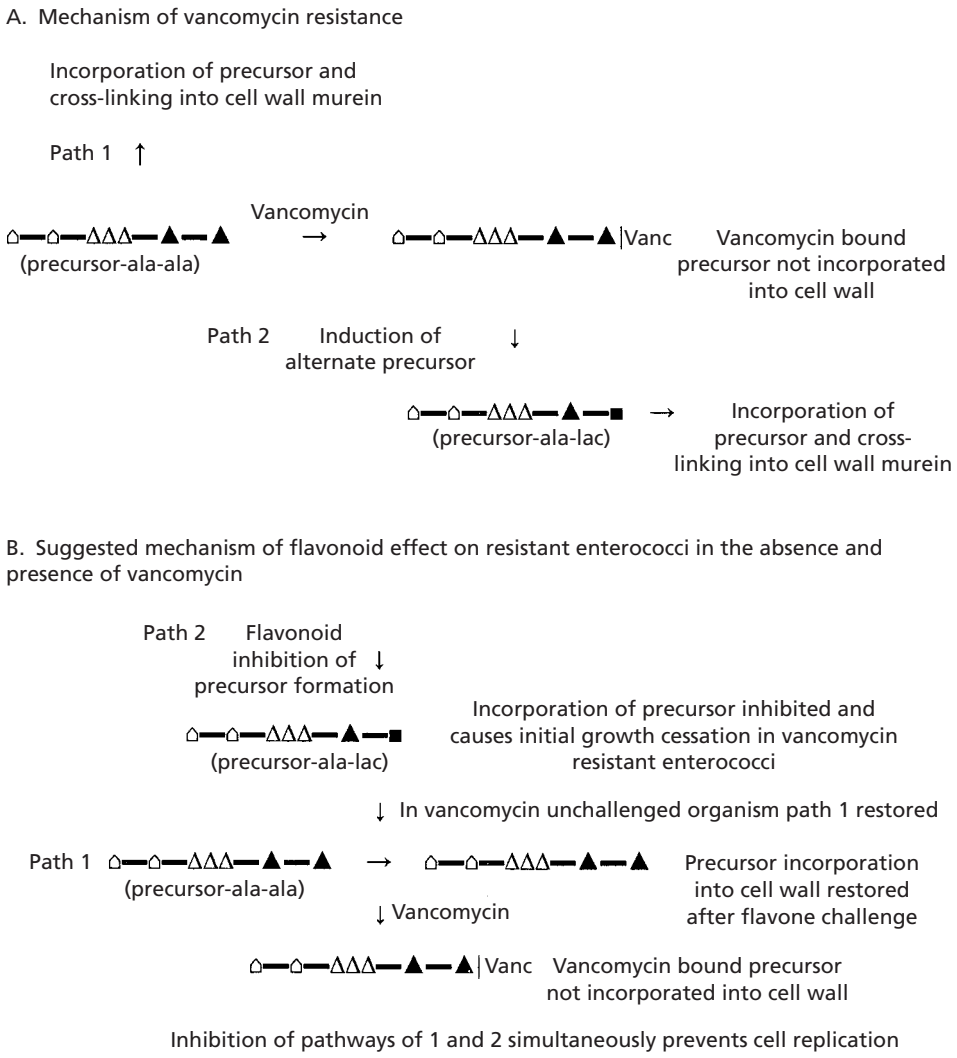

Figure 3 Mechanism of vancomycin/flavone combination inhibition. ⌂—⌂—△△△—▲—▲, Disaccharide-pentapeptide (ala-ala) precursor; ⌂—⌂—△△△—△—■, disaccharide-peptide-lactate (ala-lac) precursor. ⌂, Sugar; △, amino acid: ▲, alanine; ■, lactate.

clinical VRE isolates of *E. faecium* or *E. faecalis* (Diagnostic Department Edinburgh Royal Infirmary, Edinburgh, UK) at an inoculum of 5×10^5 colony forming units (CFU) mL^{-1} were incubated with vancomycin in the presence of either galangin 12.5 μg mL^{-1} (Sigma-Aldrich, UK) or 3,7-dihydroxyflavone 6.25 μg mL^{-1} (Apin Chemicals, Oxford, UK) at 37°C for 24 h. Type vancomycin-sensitive strains, *E. faecalis* NCTC 5957 and NCTC 775 (National Collection of Type Cultures, Colindale, London, UK), type VRE strain ATCC 51299 and clinical isolates of enterococci were incubated in the presence of either vancomycin or the flavones alone to provide controls.

Viable counts were performed using the microtitre method previously described (Richards & Xing 1993; Liu et al 2000), using Iso-sensitest broth containing 0.5% glucose as the growth medium, with incubation at 35°C. Results are for the mean of four sets of replicates.

Results and Discussion

The growth of vancomycin-sensitive type strain cultures of *E. faecalis* NCTC 5957 and NCTC 775 (vancomycin MIC 2.5 μg mL^{-1} or less) were only inhibited at 24 h by flavonoids **1** and **2** alone at concentrations > 250 μg

mL^{-1}. VRE ATCC 51299 and clinical isolates, while varying in their degree of sensitivity to the antibacterial action of vancomycin, were also strongly resistant to inhibition by either flavonoid alone (MIC $> 250\ \mu g\ mL^{-1}$).

The MICs for vancomycin or combinations of flavonoids with vancomycin against enterococcal strains are shown in Table 1. The larger proportion (67%) of the VRE clinical isolates had their vancomycin MICs reduced to $< 4\ \mu g\ mL^{-1}$. Some strains (20%) however maintained their resistance to the combination of agents.

The effects of flavonoids on the growth curve of vancomycin-resistant *E. faecalis* ATCC 51299 are shown in Figure 2. Vancomycin alone at a sub-MIC level, while retarding the initial culture growth-rate, did not reduce the overall level of the CFU values for cultures compared with the levels of the untreated control culture after a 24-h period. The kinetics of disaccharide pentapeptide (ala-lac) production in the resistant strain were initially slow to restore cell growth rate (Figure 3A). Both flavonoids whether or not in combination with vancomycin ($16\ \mu g\ mL^{-1}$) had an effect on bacterial cell growth. Viable counts associated with exposure of the culture to flavonoids over an initial period of up to 6 h showed a significant reduction from a log CFU mL^{-1} value of 6 to 3, whereafter the CFU values for cultures treated by flavonoids alone increased to the levels of the untreated control culture over a 24-h period. This suggested that under these conditions disaccharide peptide (ala-lac) production might be initially inhibited by the presence of the flavonoid, with an extended lag time period before the disaccharide pentapeptide (ala-ala) pathway became compensatory in the absence of vancomycin (Figure 3B). Flavonoids **1** or **2** alone required significantly higher concentrations (MIC $> 250\ \mu g\ mL^{-1}$) to achieve inhibition over a 24-h incubation period. This suggested that differing flavonoid activities might have resulted from multiple target interactions.

A combination of vancomycin with either flavonoid significantly lowered the CFU values for the cultures (log CFU $mL^{-1} = 3$) and maintained that inhibition over a 24-h period. This was consistent with the action of the combination of agents in restoring the MIC of vancomycin against the resistant strains, to the level of that against a sensitive strain. It seems likely that in the presence of both sub-MIC levels of flavonoids and vancomycin that the production of disaccharide peptides ala-ala and ala-lac were inhibited and cell wall biosynthesis interrupted as a result of cessation in peptidoglycan peptide-PBP synthesis. These data clearly demonstrated synergistic effects between combinations of vancomycin and flavonoids.

The molecular mechanisms of the flavonoids, alone or in combination with vancomycin, to affect cell growth remain undefined. Nevertheless the observations substantiate that combinational action of flavones with vancomycin reverses resistance in enterococci, and offers novel drug targets for the design of new therapeutic regimes against resistant strains of pathogens (Richards et al 1998; Liu et al 2000).

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