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Original Article

In vitro synergy of baicalein and gentamicin against vancomycin-resistant *Enterococcus*

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Background and Purpose: Little is known about the possible synergism of baicalein, a bioactive flavone of *Scutellariae radix* (a Chinese herb), when used in conjunction with other antimicrobial agents against vancomycin-resistant *Enterococcus* (VRE). This in vitro study examined the possible synergism of the combination of baicalein and gentamicin against VRE.

Methods: Minimal inhibitory concentrations (MICs) of baicalein as well as gentamicin were determined against 39 clinical isolates of VRE by the agar dilution method. Synergistic activities were determined using the checkerboard method based on the fractional inhibitory concentration indices and also the time-kill method. Further time-kill studies were conducted with these two agents against one randomly chosen clinical isolate, VRE-096.

Results: Minimal concentrations inhibiting 50% (MIC_{50}) and 90% (MIC_{90}) of isolates for baicalein and gentamicin were all >256 μg/mL. Synergism between baicalein and gentamicin was demonstrated against four clinical isolates of VRE (VRE-70, VRE-940, VRE-096 and VRE-721). When approximately 5×10^5 colony-forming units/mL of VRE-096 was incubated with both baicalein at a concentration of 32 μg/mL (1/8 × MIC) and gentamicin at a concentration of 128 μg/mL (1/2 × MIC), there was an inhibitory effect against VRE that persisted for 48 h. At 48 h, the combination of baicalein and gentamicin at these respective concentrations resulted in a reduction of growth by approximately 2 orders of magnitude compared to that for the starting inoculum and by 3 orders of magnitude compared to that for baicalein alone, the more active single agent.

Conclusion: This study demonstrated that baicalein and gentamicin can act synergistically in inhibiting VRE in vitro.

Key words: Baicalein; Drug synergism; *Enterococcus*; Microbial sensitivity tests; Vancomycin resistance

Introduction

The emergence of bacterial resistance to antibiotics is of global concern. Recent multicenter nosocomial surveillance studies indicated that as much as 20% of bloodstream enterococcal isolates in the United States were resistant to vancomycin [1]. Over the past several decades, the pace of discovery of new antibiotic classes has been lagging behind the development of antibiotic resistance. Therefore, the importance of identifying effective antimicrobial agents cannot be overemphasized. Among the potential sources of new agents, medicinal plants have long been investigated.

Scutellariae radix, the root of *Scutellaria baicalensis* (known as *Huang Qin* in China), is one of the most widely used Chinese herbs, prescribed in combination with others in oriental medicines. *Scutellariae radix* has been used systemically or topically for thousands of years to treat a wide range of infectious diseases, such as upper respiratory infections, pneumonia, scarlet fever, jaundice, hepatitis and dysentery [2]. The main bioactive flavone constituents of *Scutellariae radix* are baicalin, baicalein and wogonin. Baicalin and its agylycone, baicalein, were reported to show anti-allergic [3], anti-inflammatory [4,5] as well as antioxidant actions [6], and antibacterial effects against a number

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of pathogens [2,7]. Baicalin was additionally reported to be capable of exerting in vitro synergistic effect against methicillin-resistant *Staphylococcus aureus* when used in combination with beta (β)-lactam agents [8]. Baicalin itself is poorly absorbed in the rat gut, and it is hydrolyzed to baicalein by intestinal bacteria. As a result, when baicalin was administered orally in rats, a considerable amount of baicalein was recovered from the gastrointestinal tract, which was absorbed and then restored to its original form in the body [9]. Promising as the adjunctive role of these flavones are in terms of antimicrobial activity, little is known about whether baicalein can have a synergistic effect when used in combination with other antimicrobial agents against vancomycin-resistant *Enterococcus* (VRE). This in vitro study examined the possible synergism of the combination of baicalein and gentamicin against VRE.

Methods

Bacteria

A total of 39 clinical strains of VRE which included 18 strains of *Enterococcus faecalis* and 21 strains of *Enterococcus faecium* isolated from blood, wound or bullous fluid were collected from Chi Mei Medical Center and National Cheng Kung University Hospital in Taiwan. All isolates were identified by conventional methods [10]. For identification of VRE, enterococci were subjected to antimicrobial disk susceptibility testing in accordance with National Committee for Clinical and Laboratory Standards (NCCLS) guidelines [11]. Briefly, the bacterial suspension was adjusted to 0.5 McFarland standard prepared from a bacterial colony on an agar plate incubated for 18 to 24 h. A sterile cotton swab was dipped into the adjusted suspension. The dried surface of a Muller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface, and a 30-μg vancomycin disk (Becton Dickinson and Company, Sparks, MD, USA) was attached onto the Mueller-Hinton agar plate before it was incubated for a full 24 h in ambient air at 35°C. The interpretation of VRE was in accordance with NCCLS guidelines [11]. Vancomycin-resistant organisms were further confirmed and identified to the species level using the MicroScan Walk-Way system (Dade International, West Sacramento, CA, USA). These VRE strains were stored at –70°C in Protect bacterial preservers (Technical Service Consultants Limited, Lancashire, England) before being cultured on Luria-Bertani agar (Difco Laboratories, Detroit, MI, USA) for conducting experiments.

Determination of minimal inhibitory concentrations

Minimal inhibitory concentration (MIC) values of the following agents were determined by the agar dilution method [12]: vancomycin (Sigma-Aldrich Co., St. Louis, MO, USA), baicalein (Aldrich Chem Co., Milwaukee, WI, USA) and gentamicin (Sigma-Aldrich). Vancomycin and gentamicin were prepared by dissolving in sterile water. Baicalein was dissolved in 1% ammonia solution and diluted with sterile water to the tested concentrations. The drugs were incorporated into the agars in serial two-fold concentrations as follows: vancomycin, 1-128 μg/mL; baicalein, 1-256 μg/mL; gentamicin, 2-256 μg/mL. The bacterial inocula were prepared as previously described [13,14], except that final inocula of approximately 1×10^4 colony-forming units (CFU) per spot of inoculum were applied onto the plates and then incubated at 37°C for 24 h. MICs were the lowest concentrations of antibiotics resulting in complete inhibition of visible growth of the organism. *Escherichia coli* American Type Culture Collection 25922 was used in each run as control for susceptibility testing.

Determination of synergistic activities

Checkerboard determination

Four clinical isolates (VRE-70, VRE-940, VRE-096 and VRE-721) were arbitrarily selected for checkerboard determination of in vitro synergy. Broth microdilution assays were performed at concentrations ranging from 1/32 to 2 times the MIC of baicalein, and from 1/128 to 8 times the MIC of gentamicin. The dilutions were made in 96-well plates (Corning Glass Works, Corning, NY, USA) in a checkerboard fashion, and the inoculum was prepared as aforementioned. The plates were incubated at 37°C in 5% carbon dioxide for 24 h. For evaluation of interactions between antibiotics, we calculated the fractional inhibitory concentration (FIC) and FIC index based on the following formulas: $FIC_A = MIC_A$ in combination/MIC_A alone, $FIC_B = MIC_B$ in combination/MIC_B alone, and the FIC index = $FIC_A + FIC_B$, where FIC_A and FIC_B, and MIC_A and MIC_B are the FICs and MICs for antibiotics A and B, respectively [15]. FIC indices were eventually employed to characterize antibiotic interactions as follows: synergy, FIC index ≤0.5; additivity, FIC index 0.5-<1; indifference, FIC index 1-≤4; antagonism, FIC index >4.

Time-kill method

Bacterial concentrations of the randomly chosen clinical isolate VRE-096 were diluted to about 5.0×10^5 CFU/mL in 25 mL of fresh Mueller-Hinton broth in a 125-mL

Table 1. Susceptibility of 39 isolates of vancomycin-resistant *Enterococcus* (VRE) and the isolate VRE-096 to four antimicrobial agents

Antimicrobial agent	MIC (μg/mL)			
	MIC_{50}	MIC_{90}	Range	VRE-096; *Enterococcus faecalis*
Gentamicin	>256	>256	16->256	>256
Ampicillin	2	64	1-128	2
Vancomycin	256	>256	128->256	256
Baicalein	>256	>256	128->256	>256

Abbreviations: MIC = minimal inhibitory concentration; MIC_{50} = minimal concentration inhibiting 50% of isolates; MIC_{90} = minimal concentration inhibiting 90% of isolates

glass conical flask for each of the concentrations of the drugs tested. Various concentrations of baicalein and gentamicin were prepared and each drug was placed in a separate flask at the indicated concentrations: for baicalein, 16, 32, and 64 μg/mL; for gentamicin 8, 16, 32, and 64 μg/mL. Each flask was incubated under the conditions described above. Duplicate samples were removed for determination of CFUs at specified time intervals as described previously [13], except that Luria-Bertani agar plates were used and incubated at 37°C overnight. Synergism was defined as a ≥2 $\log_{10}$ reduction in the number of CFUs at 48 h with the combined drugs compared to that by the most active single agent, and as a ≥2 $\log_{10}$ reduction compared with the starting inoculum. The lowest detectable limit for counting was 30 CFU/mL. All of the experiments were performed at least twice for confirmation of the results.

Results

MICs

All tested antibiotics showed weak in vitro activities against the 39 clinical isolates of VRE (Table 1). The minimal concentrations inhibiting 90% of isolates of gentamicin, baicalein and vancomycin were each >256 μg/mL. For VRE-096, the MICs of gentamicin and baicalein were each >256 μg/mL, and the MIC of vancomycin was 256 μg/mL.

Table 2. Minimal inhibitory concentrations of baicalein and gentamicin alone and in combination against four clinical isolates of vancomycin-resistant *Enterococcus* (VRE; *Enterococcus faecalis*)

Isolate	Baicalein (μg/mL)	Gentamicin (μg/mL)	Baicalein (μg/mL)/ gentamicin (μg/mL)
VRE-70	>256	1024	16/128
VRE-940	>256	512	2/16
VRE-96	>256	256	8/128
VRE-721	256	1024	16/64

Checkerboard determination

The FIC index of VRE-70, VRE-940, VRE-096 and VRE-721 were 0.189, 0.039, 0.314 and 0.157, respectively. The MICs of baicalein and gentamicin against these 4 VRE isolates are shown in Table 2. Baicalein at concentrations of 2-16 μg/mL, when combined with gentamicin, resulted in significantly reduced MIC values.

Time-kill method

No inhibitory effect was elicited when approximately 5×10^5 CFU/mL of VRE-096 was incubated with baicalein at the concentration of 16 μg/mL. However, when VRE-096 of this inoculum size was incubated with baicalein at a concentration of 32 μg/mL, the bacterial growth was inhibited during the initial 12 h, and thereafter, re-grew and approached the colony count of control at 48 h; when VRE-096 was incubated with baicalein at a concentration of 64 μg/mL, the inhibitory effect persisted for 36 h, with regrowth thereafter (Fig. 1). When VRE-096 of this inoculum size was incubated with the combination of baicalein at a concentration of 32 μg/mL (1/8 × MIC) and gentamicin at 128 μg/mL (1/2 × MIC), the inhibitory effect against VRE persisted for at least 48 h, and at this time point, the colony count was approximately 2 $\log_{10}$ lower than the starting inocula and approximately 3 $\log_{10}$ lower than when baicalein, the more active single agent, was used alone (Fig. 2).

Discussion

This study demonstrated a synergistic effect between baicalein and gentamicin in the inhibition of VRE by both checkerboard and time-kill methods. Previous studies found discordance between checkerboard and time-kill results [16,17]. This is not completely surprising because the two methods measure different phenomena; the checkerboard method, based upon

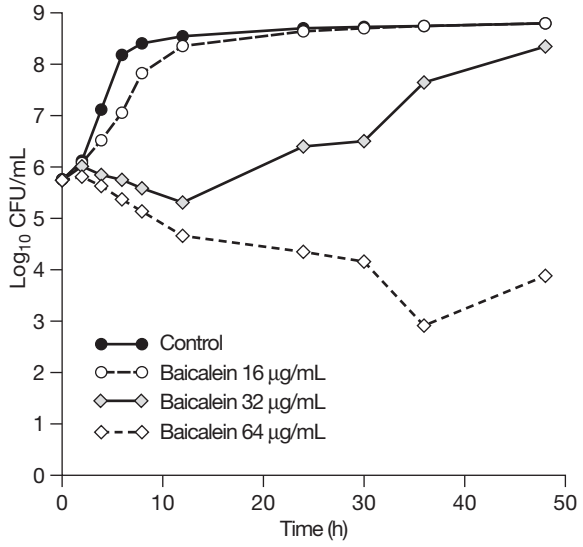

Fig. 1. Inhibition of growth curves for vancomycin-resistant *Enterococcus* (VRE-096; *Enterococcus faecalis*) after incubation with different concentrations of baicalein at a starting inoculum of approximately 5×10^5 colony-forming units (CFU)/mL.

MICs, reflects the inhibition of bacterial growth, whereas the time-kill method measures the extent of killing [15]. In general, synergism was detected at a higher frequency by time-kill experiments. The shortcomings of time-kill study include the potential effect of the inoculum size, and reliance on the readings at arbitrary time points as

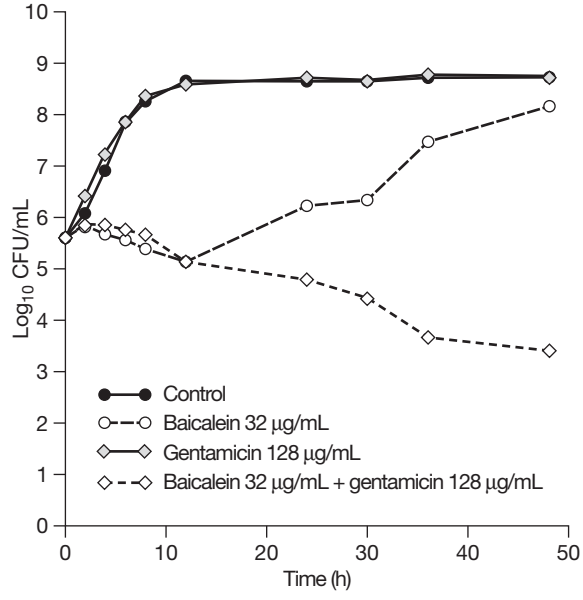

Fig. 2. Inhibition of growth curves for vancomycin-resistant *Enterococcus* (VRE-096; *Enterococcus faecalis*) after incubation with baicalein and gentamicin alone and in combination, at a starting inoculum of approximately 5×10^5 colony-forming units (CFU)/mL.

determinants of the drug interactions. The checkerboard and time-kill methods are not interchangeable in that while all strains affected synergistically based on checkerboard method were also affected synergistically according to time-kill method, the reverse was not true [15]. The consistency of both findings obtained using checkerboard and time-kill methods in this study unequivocally indicates the synergism between both drugs.

Huang Qin, as a result of its two major bioactive components — baicalin and baicalein, posesses a wide variety of pharmacological activities. The structural similarity of baicalin and baicalein suggests that the two substances may exert some equivalent pharmacological activities through similar mechanisms. Liu et al [8] proposed the following three distinct mechanisms in interpretating the synergistic effect of combined baicalin and β-lactam agents against *S. aureus*: 1) a weak to moderate direct antibacterial action of baicalin on cell growth; 2) restoring susceptibility of β-lactam drugs due to inhibition of β-lactamase by baicalin; and 3) in methicillin-resistant *S. aureus*, baicalin inhibits β-lactamase independently of the inhibitory interaction between β-lactams and penicillin-binding protein. Intrinsic resistance of enterococci to aminoglycosides results from a decrease in the ability of aminoglycoside to penetrate the outer cell envelope of bacteria, which can be overcome with the addition of an appropriate cell wall-active agent [18]. Acquired resistance in enterococci is due either to mutations resulting in decreased ribosomal binding of the agent, as occurs in streptomycin only or, more commonly, to the acquisition of new genes that encode enzymes that modify aminoglycosides [19-21].

The high MICs of baicalein in our study indicate that this compound has weak antimicrobial activity against enterococci. However, checkerboard determination indicated that minimal concentrations of 3% to 50% of the MIC of gentamicin against VREs when used alone were required for synergism with baicalein at concentrations of 2 to 16 μg/mL. The results of this study may suggest that baicalein exhibits the characteristics of a cell wall active agent against Gram-positive cocci, and therefore facilitates the action of aminoglycoside. Further studies of the activity of baicalein against enterococci with high-level resistance to aminoglycosides are needed to clarify whether it is able to interfere with other mechanisms involved in aminoglycoside resistance.

Enterococci are capable of evolving a number of remarkably efficient methods of transferring resistance

genes among themselves, and between themselves and other microorganisms, and this greatly facilitates their acquisition of new resistance determinants. One important form of acquired resistance that does not appear to be plasmid- or transposon-mediated is tolerance to cell wall-active agents. A relatively brief exposure to penicillin or other cell wall-active agents may result in a rapid acquisition of tolerance [22]. Because of the intrinsic resistance and tolerance of enterococci to antimicrobial agents that inhibit cell wall synthesis, combination therapy with cell wall-active agents plus aminoglycosides is currently the standard treatment for enterococcal infections such as endocarditis and meningitis, which require bactericidal therapy [22]. This study suggests that baicalein has a potential adjuvant role in clinical bactericidal therapy for severe enterococcal infection.

Baicalein is the major constituent of a Chinese herb *Huang Qin*, which has been used for more than a thousand years in China and Japan [23]. Limited toxicity of baicalein can therefore be expected. Previous study found that oral administration of aqueous extract of *Huang Qin* in dogs at a dosage of 4 to 5 g/kg three times a day for eight weeks did not produce any significant abnormalities in either blood tests or histology of internal organs. Loose bowel movement was observed in the high dosage group, which resolved after discontinuation of the drug [23]. The limited toxicity of baicalein also supports its potential as an adjunctive agent in anti-microbial therapy.

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References

1. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis. 1999; 29:239-44.
2. Wang YS. Pharmacology and applications of Chinese herbs. Beijing: People's Health Publishing House; 1983:1022-7.
3. Koda A, Nagai H, Wada H. Pharmacological actions of baicalin and baicalein. 2. On passive anaphylaxis. Nippon Yakurigaku Zasshi. 1970;66:237-47. [In Japanese].
4. Kubo M, Matsuda H, Tanaka M, Kimura Y, Okuda H, Higashino M, et al. Studies on *Scutellariae radix*. VII. Anti-arthritic and anti-inflammatory actions of methanolic extract and flavonoid components from *Scutellariae radix*. Chem Pharm Bull (Tokyo). 1984;32:2724-9.
5. Wakabayashi I. Inhibitory effects of baicalein and wogonin on lipopolysaccharide-induced nitric oxide production in macrophages. Pharmacol Toxicol. 1999;84:288-91.
6. Kimuya Y, Kubo M, Tani T, Arichi S, Okuda H. Studies on *Scutellariae radix*. IV. Effects on lipid peroxidation in rat liver. Chem Pharm Bull (Tokyo). 1981;29:2610-7.
7. Tsao TF, Newman MG, Kwok YY, Horikoshi AK. Effect of Chinese and western antimicrobial agents on selected oral bacteria. J Dent Res. 1982;61:1103-6.
8. Liu IX, Durham DG, Richards RM. Baicalin synergy with beta-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* and other beta-lactam-resistant strains of *S. aureus*. J Pharm Pharmacol. 2000;52:361-6.
9. Akao T, Kawabata K, Yanagisawa E, Ishihara K, Mizuhara Y, Wakui Y, et al. Baicalin, the predominant flavone glucuronide of scutellariae radix, is absorbed from the rat gastrointestinal tract as the aglycone and restored to its original form. J Pharm Pharmacol. 2000;52:1563-8.
10. Facklam RR, Sahm DF, Teixeira LM. *Enterococcus*. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RT, eds. Manual of clinical microbiology, 7th ed. Washington, DC: ASM Press; 1999:297-305.
11. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptiblity testing. 7th edition. Approved standards. NCCLS document M2-A7. Wayne, Pa: National Committee for Clinical Laboratory Standards; 2000.
12. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 4th edition. Approved standards. NCCLS document M7-A4. Wayne, Pa: National Committee for Clinical Laboratory Standards; 1999.
13. Chuang YC, Liu JW, Ko WC, Lin KY, Wu JJ, Huang KY. In vitro synergism between cefotaxime and minocycline against *Vibrio vulnificus*. Antimicrob Agents Chemother. 1997;41: 2214-7.
14. Chuang YC, Ko WC, Wang ST, Liu JW, Kuo CF, Wu JJ, et al. Minocycline and cefotaxime in the treatment of experimental murine *Vibrio vulnificus* infection. Antimicrob Agents Chemother. 1998;42:1319-22.
15. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother. 1996;40:1914-8.
16. Moody JA, Gerding DN, Peterson LR. Evaluation of ciprofloxacin's synergism with other agents by multiple in vitro methods. Am J Med. 1987;82:44-54.

17. Norden CW, Wentzel H, Keleti E. Comparison of techniques for measurement of in vitro antibiotic synergism. J Infect Dis. 1979;140:629-33.
18. Moellering RC Jr, Weinberg AN. Studies on antibiotic syngerism against enterococci. II. Effect of various antibiotics on the uptake of ^{14}C-labeled streptomycin by enterococci. J Clin Invest. 1971:50:2580-4.
19. Eliopoulos GM, Farber BF, Murray BE, Wennersten C, Moellering RC Jr. Ribosomal resistance in clinical enterococcal to streptomycin isolates. Antimicrob Agents Chemother. 1984; 25:398-9.
20. Leclercq R, Dutka-Malen S, Brisson-Noël A, Molinas C, Derlot E, Arthur M, et al. Resistance of enterococci to aminoglycosides and glycopeptides. Clin Infect Dis. 1992;15:495-510.
21. Murray BE. The life and times of *Enterococcus*. Clin Microbiol Rev. 1990;3:46-65.
22. Moellering RC Jr. *Enterococcus* species, *Streptococcus bovis* and *Leuconostoc* species. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 5th ed. Philadelphia, PA: Churchill Livingstone; 2000:2147-56.
23. Huang HC, Wang HR, Hsieh LM. Antiproliferative effect of baicalein, a flavonoid from a Chinese herb, on vascular smooth muscle cell. Eur J Pharmacol. 1994;251:91-3.

