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Synergistic effects of oxyresveratrol in conjunction with antibiotics against methicillin-resistant *Staphylococcus aureus*

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) infection is a serious clinical problem worldwide. The aim of the present study was to examine the antimicrobial activity of oxyresveratrol (ORV) against MRSA. The antimicrobial activity of ORV was evaluated against three strains of MRSA and one methicillin-susceptible *S. aureus* (MSSA) strain using a minimal inhibitory concentration (MIC) assay, MTT colorimetric assay, checkerboard dilution test and time-kill assay. The MIC of ORV for all strains was moderate at 125 μg/ml. Of note, the antimicrobial activity and fractional inhibitory concentration index values of ORV were markedly increased in the presence of a non-growth inhibitory dose of certain antibiotics. Time-kill curves revealed that a combination of ORV with ciprofloxacin or with gentamicin reduced bacterial counts to below the lowest detectable limit after 24 h. These effective combinations may be used as potential antimicrobial regimens for use in the management of MRSA.

Introduction

Staphylococcus aureus is a bacterium that grows in the human nose and skin and is a major pathogen that causes skin and soft-tissue infections, which have previously been treated with the antibiotic methicillin. Since its detection in 1961, methicillin-resistant *S. aureus* (MRSA) has become the most problematic Gram-positive bacterium in the public health arena (1). This pathogen is associated with a variety of infectious diseases (2) and has an average mortality rate of 36-50% (3). With increasing resistance to various antibiotics, combination therapy is a potential alternative. It may prove particularly useful in developing countries where availability of antibiotics is limited, as it allows for a reduction in the dose of the antibiotic required (4-6). Furthermore, MRSA bacteria are not only resistant to β-lactam antibiotics but also to fluoroquinolones and other families of antibiotics (4).

Oxyresveratrol (ORV) is an antioxidant (7), anthelmintic (8) tyrosinase inhibitor (9) and a cyclooxygenase inhibitor (10,11). Various studies have indicated that oxyresveratrol (Fig. 1) inhibits apoptotic cell death in transient cerebral ischemia (12), is hepatoprotective (13) and is a potent free radical scavenger (7). Oxyresveratrol has been demonstrated to have an inhibitory effect on the herpes simplex and varicella zoster virus (14,15). In addition, the compound has been revealed to have skin-whitening (16) and neuroprotective effects (6,7,13,14). However, the antimicrobial capacity of ORV against *Staphylococcus aureus* remains unknown. Therefore, the antibacterial activity of ORV alone and of ORV in conjunction with commonly-used antibiotics was investigated in the present study.

Materials and methods

Materials and chemicals. Ampicillin (AM), oxacillin (OX), gentamicin (GT), vancomycin (VC), norfloxacin (NR) and ciprofloxacin (CP) (all supplied by Sigma-Aldrich, St. Louis, MO, USA) were used. Oxyresveratrol (>96.32%) was deposited at the Standardized Material Bank for New Botanical Drugs (No NNMBP000018) at Wonkwang University (Iksan, Republic of Korea). The twigs of *Morus alba* were purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2000. A voucher specimen

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Table I. Interpreted FICI response against MRSA and MSSA strains.

A, Response for ORV and AM

	ORV[a]		AM[a]		
Strain	Alone	With AM	Alone	With ORV	FICI
ATCC 33591	125	62.5	1,000	250	0.75
ATCC 25923	125	15.6	31.25	1.95	0.1875
DPS-1	125	31.25	1,000	500	0.75
DPS-2	125	62.5	1,000	500	1.00

B, Response for ORV and OX

	ORV[a]		OX[a]		
Strain	Alone	With OX	Alone	With ORV	FICI
ATCC 33591	125	62.5	500	250	1
ATCC 25923	125	15.6	125	7.81	0.1875
DPS-1	125	62.5	1,000	62.5	0.5625
DPS-2	125	62.5	1,000	250	0.75

C, Response for ORV and GT

	ORV[a]		GT[a]		
Strain	Alone	With GT	Alone	With ORV	FICI
ATCC 33591	125	62.5	31.25	3.9	0.625
ATCC 25923	125	31.25	62.5	7.8	0.375
DPS-1	125	62.5	250	15.6	0.5625
DPS-2	125	62.5	125	31.25	0.75

D, Response for ORV and VC

	ORV[a]		VC[a]		
Strain	Alone	With VC	Alone	With ORV	FICI
ATCC 33591	125	31.25	1.95	0.24	0.375
ATCC 25923	125	62.5	3.9	1.95	1
DPS-1	125	62.5	1.95	0.98	1
DPS-2	125	31.25	3.9	0.98	0.5

E, Response for ORV and CP

	ORV[a]		CP[a]		
Strain	Alone	With CP	Alone	With ORV	FICI
ATCC 33591	125	31.25	500	62.5	0.375
ATCC 25923	125	15.6	31.25	3.9	0.25
DPS-1	125	15.6	125	31.25	0.375
DPS-2	125	31.25	125	31.25	0.5

Table I. Continued.

F, Response for ORV and NR

	ORV[a]		NR[a]		
Strain	Alone	With NR	Alone	With ORV	FICI
ATCC 33591	125	62.5	250	62.5	0.75
ATCC 25923	125	62.5	15.6	3.9	0.75
DPS-1	125	62.5	31.25	15.6	1
DPS-2	125	62.5	31.25	7.8	0.75

[a]Minimun inhibitory concentration (μg/ml). FICI, fractional inhibitory concentration index; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*. ORV, oxyresveratrol; AM, ampicillin; OX, oxacillin; GT, gentamicin; VC, vancomycin; CP, ciprofloxacin; NR, norfloxacin.

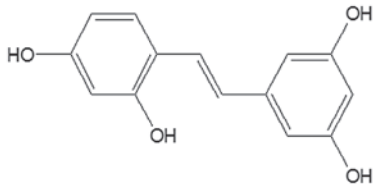

Figure 1. Structure of oxyresveratrol.

(no. WP 217) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Dried twigs of *M. alba* (2 kg) were extracted with EtOH (2 l) for 20 days at room temperature. Dried residue of the EtOH extract (101 g) was dissolved in 40% aqueous MeOH (1 L) and partitioned with n-hexane (800 ml x2), CH_2Cl_2 (800 ml x2) and EtOAc (800 ml x2), successively. The CH_2Cl_2 soluble fraction (8.53 g) was chromatographed on Sephadex LH-20 column (5x 16 cm) using CH_2Cl_2-MeOH (4:1 - 1:1; each volume, 300 ml) to obtain four fractions (Fr. A-D). The EtOAc soluble fraction (4.83 g) was chromatographed on silica gel (250 g) column using CH_2Cl_2-MeOH (8:1 - 4:1; each volume, 600 ml) to obtain three fractions (Fr. D-F). Fr. E (2.77 g) was chromatographed on silica gel (150 g) column (eluent: n-hexane-acetone, 1:1) and further purified by Sephadex LH-20 column (2.5 x 20 cm) chromatography (eluent, CH_2Cl_2-MeOH 4:1) to give oxyresveratrol (1.12 g, 0.056 w/w%). The structure of oxyresveratrol was identified by analysis of nuclear magnetic resonance and mass spectra.

Bacterial strains and growth conditions. Two clinical MRSA isolates were obtained from two patients at Wonkwang University Hospital (Iksan, South Korea). The other two strains were obtained from the American Type Culture Collection (Manassas, VA, USA), and included *S. aureus* (33591; methicillin-resistant strain) and *S. aureus* (25923; methicillin-susceptible strain). Prior to each experiment, all bacteria were stored in 30% glycerol and frozen at -70˚C. The bacteria were cultured in Difco™ Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA) (BD Biosciences, Franklin Lakes, NJ, USA) by incubating at 37˚C for 24 h.

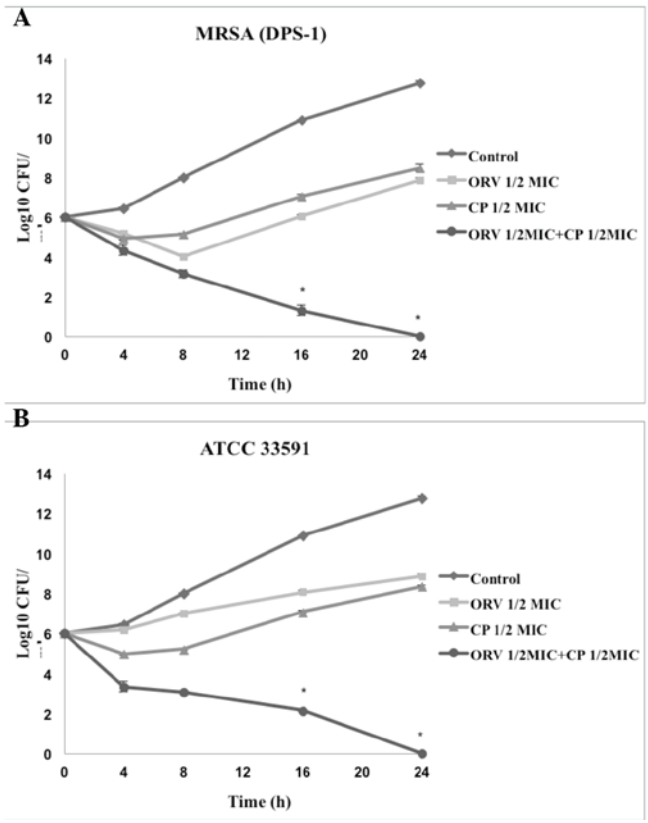

Figure 2. Time-kill curves for combined ORV and CP treatment of the (A) DPS-1 MRSA and (B) standard 33591 MRSA strains. Data represent the average of triple-indipendent experiments. *P<0.001 as compared to CP alone. ORV, oxyresveratrol; CP, ciproflaxin; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *S. aureus*.

Minimum inhibitory concentration (MIC). MICs were determined using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (17). Briefly, a preparation of microorganism suspension was prepared by growing the bacteria in broth for 24 h, which were adjusted to a 0.5 McFarland standard turbidity [~$1.5x10^8$ colony forming units (CFU)/ml]. The final inocu-

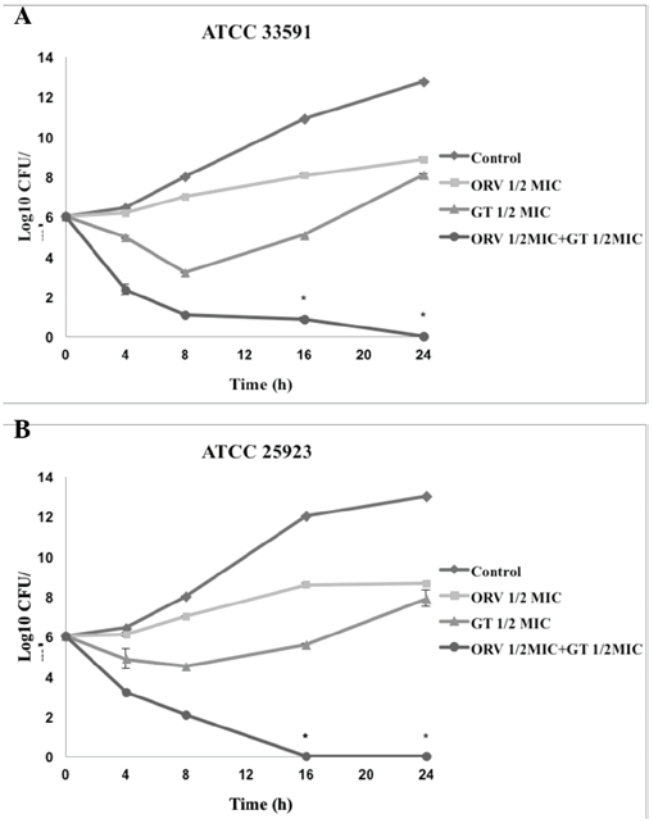

Figure 3. Time-kill curves of ORV and GT against the (A) standard 33591 MSSA and (B) standard 25923 MRSA strains. Data represent the average of triple-indipendent experiments. *P<0.001, as compared with GT alone. ORV, oxyresveratrol; GT, gentamicin; MRSA, methicillin-resistant *S. aureus*; MIC, minimum inhibitory concentration; MSSA, methicillin-susceptible *S. aureus*.

lums were adjusted to $1.5x10^6$ CFU/ml. The serially diluted extracts were then incubated along with the inoculum at 37˚C for 18 h. The MIC was defined as the lowest concentration of antibiotics and ORV that prevented visible growth of the bacteria. At the end of the incubation period, well plates were visually examined for turbidity. Cloudiness indicated that bacterial growth had not been inhibited.

Checkerboard dilution test. Synergistic combinations were investigated using the preliminary checkerboard method according to the published standards (5,18). The MIC was defined as the lowest concentration of drug alone or in combination that inhibited visible growth. *In vitro* interaction was quantified as the fractional inhibitory concentration index (FICI), which was calculated using the following formula: FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FICIs were interpreted as follows: <0.5, synergy; 0.5-0.75, partial synergy; 0.76-1.0, additive effect; 1.0-4.0, indifference; and >4.0, antagonism. The varying levels of synergy between two given agents were determined (19). All experiments were repeated three times.

MTT colorimetric assay. A colorimetric assay based on MTT for rapid detection of the presence of bacteria was performed as previously described (20-22). Briefly, a stock solution of 5 mg/ml MTT (Sigma-Aldrich) was prepared in phosphate-buffered saline (Sigma-Aldrich) and stored at -70˚C. A final concentration of 1 mg/ml MTT was used in the assay. Following 24-h incubation at 37˚C, 20 μl yellow MTT was added to a 96-well microtiter plate and incubated for an additional 20 min. Blue color indicated the presence of bacteria.

Time-kill curve assay. A time-kill curve assay was performed according to a previous method (23) in order to investigate the combined effect of time and antimicrobial agent concentration on bacterial growth. For this assay, standard inoculums of ~10^6 CFU/ml were used. ORV (0.5 MIC) was used with various combinations of antibiotics (0.5 MIC). A test plate containing MHB and inoculum was used as the control. Counts of viable strains were conducted at different intervals up to 24 h at 37˚C. The rate and extent of bacterial death was determined by plotting the viable colony counts (CFU/ml) against the time cultured in MHA. All experiments were repeated three times.

Statistical analysis. All experiments were performed more than three times. Data from the experiments are presented as the mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's t-test (SPSS software, version 19.0; IBM SPSS, Armonk, NY, USA). P<0.001 was considered to indicate a statistically significant difference.

Results

MIC determination and synergic effect. Against all strains, the MIC was 125 μg/ml for ORV. VC presented a range of MICs of 1.95-3.9 μg/ml in the various strains. All strains were resistant to AM, OX, GT, CP and NR, with MIC values ranging from 15.6 to 1,000 μg/ml. ORV + antibiotic combinations all exhibited markedly lower MICs than those when the drugs were used alone. The combined use of ORV and VC or CP against the 33591 MRSA strain resulted in a FICI of 0.375 (synergy) (Table I), indicating that an activity-enhancing effect was present. The combined use of ORV and AM or OX against the 33591 MRSA strain resulted in a FICI of 0.75-1, but against the 25923 MSSA strain resulted in a FICI of 0.1875 (synergy). The combined use of ORV and NR against all of the strains resulted in a FICI of 0.75-1 (partial synergy or additive effect). None of the combinations exhibited an antagonistic effect (FICI >4.0). These results demonstrated that the method of combining ORV with antibiotics has potential to be used to suppress MRSA growth.

The controls displayed no reduction in CFU counts, and the use of ORV or antibiotics alone did not induce cell death at 24 h. When used in combination, ORV and antibiotics led to a marked reduction in bacterial counts. In particular, the combination of ORV + GT and ORV + CP completely inhibited growth of *S. aureus* after 24 h. These were the most effective treatments and thus were selected for further analysis.

Time-kill curve assay. Time-kill tests were performed to investigate the synergistic effects of ORV in combination with antibiotics and the effect of length of treatment on cell viability. The control displayed no reduction in CFU counts,

and the use of ORV or antibiotics alone did not induce cell death at 24 h. When used in combination, ORV and antibiotics caused a marked reduction in bacterial counts. In particular, the combination of ORV + GT and ORV + CP completely inhibited growth of *S. aureus* after 24 h (Figs. 2 and 3).

Discussion

The most effective method to develop antibiotics that produce minimal toxic effects or side effects is to use natural products. Therefore, there is a requirement for the development of alternative antimicrobial drugs against infectious diseases. Combination therapy is the most commonly recommended empirical treatment for bacterial infections in intensive care units where monotherapy may not be effective against all potential pathogens or for the prevention of antibacterial resistance (24). When combined, certain antibiotics are known to markedly increase bactericidal effects (5,24,25).

To the best of our knowledge, the current study was the first to investigate the potentiation of antibiotics by ORV against MRSA. The MIC assay is considered to be the standard method for determining the susceptibility of various microorganisms to antibacterial agents. The *in vitro* results of the present study determined the MIC values of ORV and antibiotics against *S. aureus*.

Synergistic or partially synergistic effects of ORV in combination with the antibiotic agents VC, GT and CP strongly supported this explanation. The time-kill curves and FICI scores confirmed the ability of ORV to synergistically reduce bacterial counts below the lowest detectable limit within 24 h. Therefore, ORV may be a potential antibacterial drug candidate for clinical use against MRSA. The results of the present study are promising and may increase the use of natural products as drugs.

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