Short communication

Antibacterial and synergy of a flavanono-rhamnose with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA)

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**A R T I C L E   I N F O**

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- Anti-MRSA activity
- Taxifolin-7-O-α-L-rhamnopyranoside
- Synergy
- Ceftazidime
- Levofloxacin

**A B S T R A C T**

The in vitro antibacterial activity of taxifolin-7-O-α-L-rhamnopyranoside (TR) and its synergy with four conventional antibiotics (ampicillin (AMP), levofloxacin (LEV), ceftazidime (CAZ) and azithromycin (AZM)) against ten clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated, respectively. Individual MICs and MBCs were determined by microdilution methods following the CLSI guidelines. Anti-MRSA synergy effects were measured using the chequerboard and time–kill curve tests. MICs/MBCs (μg/ml) ranges were 32–64/64–128 for TR alone against all 10 MRSA isolates. Chequerboard method showed that significant synergies were observed for the TR/CAZ and TR/LEV combinations with FICI ranged 0.187–0.375 and 0.25–0.5, respectively. Some synergy and additivity effects were also observed for TR/AMP and TR/AZM combinations. In the time–kill dynamic confirmation test, synergy results kept by the TR/CAZ combination (2.186 log10 cfu/ml increase in killing), but the TR/LEV combination changed to additivity (1.839 log10 cfu/ml increase in killing). These results demonstrated that TR enhanced the efficacy of CAZ and LEV in vitro, which had potential for combinatory therapy of patients infected with MRSA.

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**Introduction**

The first clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA), a so-called “superbug” which was originally termed, was reported in 1961 when only a year after methicillin was introduced for clinical use (Jevons 1961). Presently, the spread of MRSA strains is of great concern in the treatment of *Staphylococcal* infections, since it has quickly acquired resistance to all antibiotics, including even the emergence of glycopeptide resistant strains such as vancomycin resistant *S. aureus* (VRSA) (Chang et al. 2003).

MRSA has become the most common cause of infections among many global pathogenic bacteria, and so many life-threatening diseases such as endocarditis, pneumonia, toxin shock syndrome were attributed to it. In our hospital, MRSA could be examined in over 80 percent sputum samples of pneumonia from severe and elderly patients in intensive care unit (ICU). Therefore, the search for novel anti-MRSA agents with novel mode of action is urgently needed.

Plants have evolved and accumulated an elaborately useful source of anti-infective drugs (Mahady 2005). The therapeutic potential of phytochemicals has been increasingly recognized in the development of anti-MRSA agents (Gibbons 2004, 2008). In recent years, we have been engaged in searching for anti-MRSA compounds from the Chinese herbal medicines (Zuo et al. 2008a,b). The present report deals mainly with the anti-MRSA activity of a flavanono-rhamnose, i.e. taxifolin-7-O-α-L-rhamnopyranoside (TR, 1) isolated from *Hypericum japonicum* Thumb. ex Murray (Guttiferae) and its synergy effects with conventional antibiotics.

**Materials and methods**

**Antibacterial agents**

Four antibiotics represented different conventional types were purchased from the manufacturers, i.e. ampicillin (AMP) (North China Pharmaceutical Co., Ltd., Shijiazhuang, China), ceftazidime (CAZ) (Jida Pharmaceutical Co., Ltd., Kunming, China), azithromycin (AZM) and levofloxacin (LEV) (Yangzhijiang Pharmaceutical Co., Ltd., Taizhou, China), Vancomycin (VAN) (Eli Lilly Japan K.K., Seishin Laboratories) was used as the positive control agent. Cefoxitin disks were purchased from Tianfan biological products Co., Ltd. (Beijing, China). Three flavonoids TR (1), aromadendrin-7-O-α-L-rhamnopyranoside (2) and quercetin-7-O-α-L-rhamnopyranoside (3) were isolated and identified from the aerial parts of *H. japonicum* as described in the previous reports (Awaad et al. 2006; Ishiguro et al. 1991).
Bacterial strains

MRSA strains (ten isolates with SCCmec III genotype) were obtained and characterized from the infectious sputum samples of critically ill patients in Kunming General Hospital (Kloos and Bannerman 1999; CLSI 2006a, 2007). The presence of mecA gene and SCCmec genotypes were determined by multiplex PCR methods at Kunming Institute of Virology, PLA, China, as previously reported (Zhang et al. 2005). ATCC 25923 was used as the control strain.

Media

Standard Mueller-Hinton agar and broth (MHA and MHB, Tianhe Microbial Agents Co., Hangzhou, China) were used as bacterial culture media. MHB was used for all susceptibility testing and time–kill experiments. Colony counts were determined using MHA plates.

Susceptibility testing

MICs/MBCs were determined by standardized broth microdilution techniques with starting inoculums of $5 \times 10^8$ cfu/ml according to CLSI guidelines and incubated at 35°C for 24 h (CLSI 1999, 2006b). They were determined in duplicate, with concentrations ranging up to 2048 µg/ml for AZM.

Synergy testing

Potential anti-MRSA synergy was measured by fractional inhibitory concentration (FIC) indices (FICI) with checkerboard method and by time–kill curves as previously reported (Hu et al., 2002). The FIC of the combination was calculated by dividing the MIC of the MRSA strain by the MIC of the antibiotic alone, and the FICI was obtained by adding the MIC of the MRSA strain and that of the antibiotic. The FICI results were interpreted as follows: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 1, additivity; and 1 < FICI ≤ 2, indifference (no effect) and FICI > 2, antagonism (Hu et al. 2002; Orhan et al. 2005). In the killing curves, synergy was defined as ≥2 log$_{10}$ cfu/ml increase in kill at 24 h with the combination, in comparison with the kill by the most active single drug. Additivity was defined as a 1–2 log$_{10}$ cfu/ml increase in kill with the combination in comparison with the most active single agent. Indifference was defined as ±1 log$_{10}$ cfu/ml killing or growth. Combinations that resulted in >1 log$_{10}$ cfu/ml bacterial growth in comparison with the least active single agent were considered to represent antagonism (Chin et al. 2008). The data from time–kill assays are presented as the means ± standard deviations (Fig. 2).

Results and discussion

Three C-7-rhamnoses of flavanonol TR (1), aromadendrin-7-0-α-L-rhamnopyranoside (2) and quercetin-7-O-α-L-rhamnopyranoside (3) were isolated from the aerial parts of H. japonicum through bioassay-guided fractionation procedure (Zuo et al. 2008b). Their structures (Fig. 1) were identified with spectral analyses (data not shown) and compared with literatures (Awaad et al. 2006; Ishiguro et al. 1991).

Anti-MRSA activities of the three flavonoids and four antibiotics alone against 10 clinical MRSA strains of SCCmec III type were shown in Table 1. The order of potencies follows LEV > TR (1) > AMP > CAZ > 2 > AZM > 3. As the most potent of the three flavonoids, MICs/MBCs (µg/ml) ranges of TR (1) were 32–64/64–128. The potencies of 1–3 were related to both the number of hydroxyl groups (of 1 and 2) and skeleton of the flavonoids (flavanonol of 1 and flavonol of 3). This is the first time report of anti-MRSA properties of the three flavonoid-7-rhamnoses so far to the best of our knowledge (Cushnie and Lamb 2005).

### Table 1

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b: Mx$_{1}$: >2048; Mx$_{2}$: >1024.

c: nt: not determined.
Detailed synergy effects of TR (1) with the four antibiotics against the ten MRSA isolates by chequerboard method and the FICIs were demonstrated in Table 2. Time–killing curves of the synergy combination of TR (1) with AMP, LEV, CAZ and AZM, respectively against MRA 004 (one of the 10 isolates) were shown in Fig. 2.

The chequerboard evaluation was performed with four antibiotics representing four types of antibacterial agents, including AMP (β-lactam), CAZ (cephem), LEV (fluoroquinolone) and AZM (macrolide). TR (1) alone showed only moderate activities, but significant synergies were observed for the combinations of TR (1) with CAZ (FICI = 0.187–0.375) and LEV (FICI = 0.25–0.5) against all the 10 isolates of MRSA, respectively. The effects were exhibited when TR (1)/CAZ combination at concentrations of (1/16–1/4 × MIC) (2–16 μg/ml of TR (1)) and (1/8–1/4 × MIC) (16–128 μg/ml of CAZ), and when TR (1)/LEV combination at concentrations of (1/8 × MIC) (4–8 μg/ml of TR (1)) and (1/4 × MIC) (2–4 μg/ml of LEV), respectively (Table 2). The order of synergy potency (% of MIC reduced; FICI) was CAZ (86.9; 0.275) > LEV (76.5; 0.381) > AMP (55; 0.95) > AZM (−40; 1.25), with the same orders of average percent of MIC values reducing from 86.9% to −40% and corresponding average FICI values mounting up from 0.275 to 1.25, respectively.

![Fig. 2. Time–kill curves of the synergistic effect of the combination at 1 × MIC (alone) concentration of taxofolin-7-O-α-L-rhamnopyranoside (TR) and ampicillin (AMP) (A), levofloxacin (LEV) (B), ceftazidime (CAZ) (C) and azithromycin (AZM) (D), respectively against MRA 004, a clinical MRSA strains of SCCmec III type.](image-url)
In the time–kill analyses, synergy of the combination between TR (1) and CAZ was not fully in agreement with those found in the checkerboard method. Time–kill curves showed the TR (1)/CAZ combination resulted in an increase in killing of $2 \times 10^{10}$ colony counts at 24 h compared with that of CAZ (the most active) alone, and the TR (1)/LEV combination resulted in only the increase of $1.839 \times 10^{10}$ (additivity). The rest increase in killing were $0.548 \times$ and $0.067 \times \log_{10}$ for AMP and AZM (both indifference), respectively (Fig. 2). Hence the order of synergistic combinations was the same as that of in the checkerboard method, though the TR (1)/LEV combination turned out to be only additive effects. It has been confirmed that the overestimate of synergy experienced with the checkerboard test and synergy testing performed by time–kill kinetics was used to confirm the results of checkerboard MIC testing (Petersen et al. 2006). The interactions of TR (1) with different antibiotics might be ascribed to the block of different resistance mechanisms of bacteria (Wagner and Ulrich–Merzenich 2009).

Flavonoids are commonly found in plants and many possessing antibacterial activity (Cushnie and Lamb 2005). It has been reported that taxifolin (= the aglycone moiety of TR (1)) was relatively less cytotoxic against human cells (the 50% lethal concentration of TGF-1 cells and HUV cells were over 300 and 200 μM, respectively; Matsuo et al. 2005). As the clinical MRSA strains has become an increasingly pressing global problem, anti-MRSA synergistic effects between plant natural compounds and conventional antibacterial agents has further been demonstrated here as a promising way of overcoming current antibiotics resistance (Hemaiswarya et al. 2008).

In conclusion, the in vitro antibacterial activities of taxifolin-7-O-α-L-rhamnopyranoside (TR (1)) alone and its synergy with antibiotics demonstrated that TR (1) enhanced the efficacy of cefazidine and levofloxacin, which have the potential for combinatory therapy among patients infected with MRSA and is warranted for in depth pharmacological studies.

Conflict of interest

There was no conflict of interest.

Acknowledgments

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References


