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Short communication

Synergism of prenylflavonoids from *Morus alba* root bark against clinical MRSA isolates

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ABSTRACT

Background: Clinical methicillin-resistant *Staphylococcus aureus* (MRSA) is a thorny problem in current anti-infective therapeutics and a challenge of new drug development. Plant prenylflavonoids possess anti-MRSA activity, but few of the prenylflavonoids have been reported the synergistic anti-MRSA effect when they are used in combination with conventional antibacterial agents.

Purpose: This study deals with anti-MRSA activity of four prenylflavonoids from the root bark of *Morus alba* and their synergism with 11 conventional antibacterial agents.

Methods: Chromatographic methods and spectral analysis were used to isolate and identify the prenylflavonoids. The antibacterial activity and synergism were assessed by the broth microdilution method, checkerboard dilution test, and time-kill curve assay, respectively.

Results: Four prenylflavonoids, i.e., cyclocommunol (Cy, **1**), morusinol (Ml, **2**), morusin (Mi, **3**) and kuwanon E (Ku, **4**), were isolated from *Morus alba* bark ethanol extract. Compounds **1, 3** and **4** showed high antimicrobial activity on both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains with MICs/MBCs at 4–16/32–64 and 4–32/16–128 μg/ml, respectively. Ml (**2**) was not active. Compound **2** showed synergy with amikacin (AK) and streptomycin (SM) against all the ten MRSA isolates. Ml (**2**) and Ku (**4**) also showed synergy with ciprofloxacin (CI), etimicin (EM) and vancomycin (VA) against 7–9 isolates. The fractional inhibitory concentration indices (FICIs) ranged 0.09–1.00 and the dose reduction indices (DRIs) of these antibacterial agents ranged 2–128. Cy (**1**) and Mi (**3**) showed synergy with the tested antibacterial agents against only 1–3 MRSA isolates except VA. Furthermore, the MRSA resistance could be reversed in the combinations of AK with Cy, Ml, Mi and Ku; EM with Mi and Ku; and SM with Ml by the criteria of MIC interpretive standards for *Staphylococcus* spp. of CLSI. All the combinations showed only indifference in the 1 × MIC time-killing experiments. The prenylated substitutions play an important role in the activity of the compounds used alone and combined with the tested antibacterials.

Conclusions: The study revealed for the first time the anti-MRSA synergism of prenylflavonoids **1–4** with eleven antibacterial agents and the reversal of MRSA resistance to aminoglycosides, especially amikacin. The results might be valuable for the development of new antibacterial drugs and synergists against MRSA infection.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common pathogens in both hospital-acquired and community-acquired infections (Hetem et al., 2016). Clinical MRSA strains have developed resistance not only to β-lactams but also to aminoglycosides, quinolones, macrolides, tetracyclines and others (Zarfel et al., 2013). Since antibacterial drugs for MRSA are limited, it is highly necessary to find new leading compounds that alone or in combination with antibacterial drugs, can overpass the resistance issue (Li et al., 2012).

As part of our ongoing studies on the new anti-MRSA compounds from natural sources, we have aimed to the search of compounds which not only have anti-MRSA activity when acting alone, but also could potentiate the anti-MRSA activity of antibacterial drugs.

Abbreviations: AK, amikacin; AM, ampicillin; CFU, colony forming unit; CI, ciprofloxacin; CLSI, Clinical and Laboratory Standards Institute; GN, gentamicin; CX, cefoxitin; Cy, cyclocommunol; DRI, dose reduction index; EM, etimicin; FICI, fractional inhibitory concentration index; I, intermediate; Ku, kuwanon E; LE, levofloxacin; MBC, minimal bactericidal concentration; MH, Mueller–Hinton medium; MIC, minimal inhibitory concentration; Mi, morusin; Ml, morusinol; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; NMR, nuclear magnetic resonance; P/S, piperacillin-sulbactam; PV, penicillin; R, resistant; S, susceptible; SM, streptomycin; TP, teicoplanin; VA, vancomycin

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The root bark of *Morus alba* L. (Moraceae) (also known as white mulberry bark and marketed in China as Sang Bai Pi (SBP)) has long been used in Traditional Chinese Medicine (TCM) for fever asthmatic cough, hemoptysis, edema, beriberi and diuresis (NUTCM, 2005). SBP has showed to possess flavonoids, alkaloids and stilbenoids. Antimicrobial, skin-whitening, cytotoxic, anti-inflammatory and anti-hyperlipidemic properties have been found for this species in previous reports (Chan et al., 2016). The prenylated flavonoids from SBP and other plant resources have shown antimicrobial properties against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) (Sohn et al., 2004; Chen et al., 2005; Ku et al., 2010; Mazimba et al., 2011; Zuo et al., 2012; Mun et al., 2013; Pang et al., 2014). However, only a few of such compounds, e.g. sophoraflavanones B and G from *Sophora* species (Fabaceae) (Mun et al., 2013; Sakagami et al., 1998) and isojacareubin from *Hypericum japonicum* Thunb. ex Murray (Guttiferae) (Zuo et al., 2012), have been reported to possess anti-MRSA synergistic properties.

In this report, we present for the first time the evaluation of anti-MRSA synergism of the four SBP prenylflavonoids (i.e., cyclocommunol (Cy, **1**), morusinol (Ml, **2**), morusin (Mi, **3**) and kuwanon E (Ku, **4**)) by combining them with eleven conventional antibacterial agents, including amikacin (AK), ampicillin (AM), ciprofloxacin (CI), gentamicin (GN), etimicin (EM), levofloxacin (LE), piperacillin-sulbactam (P/S), penicillin (PV), streptomycin (SM), teicoplanin (TP) and vancomycin (VA).

Materials and methods

Plant material

M. alba root bark was bought in August 2010 from Yunnan Lv Sheng Pharmaceutical Co., Ltd (Kunming, China). A voucher specimen (KUN 0515476) is deposited in Herbarium of Kunming Institute of Botany, China.

*Isolation and identification of compounds **1–4***

The dried powder of *M. alba* root bark (5.0 kg) was extracted with 80% ethanol (× 3). The mixtures were filtered and the resulting filtrates were combined. After evaporating the solvent under reduced pressure at 40 °C, the crude methanol extract (635 g) was suspended in 1000 ml deioned water and successively extracted with petroleum ether, ethyl acetate (EtOAc) and butanol to give three main sub-extracts (240 g, EtOAc; 40 g, butanol; 355 g, water). The 200 g sub-extract from EtOAc which showed the highest activity against MRSA by disk diffusion method was subjected to column chromatography with silica gel (200–300 mesh, 1900 g; Qingdao Haiyang Chemical Co., Ltd., Qingdao, China), gradient eluting with petroleum ether: EtOAc (P:E = 10:1–1:1) to give 14 fractions (Mfr-1–14). The fractions were further subjected to activity assay and the resulted active fractions were carried out repeated chromatography to give compounds **1–4**, respectively, i.e., from Mfr-7 with silica gel (300–400 mesh, P:E = 4:1) and Sephadex LH-20 (methanol) to give compound **3**; from Mfr-9 (300–400 mesh, P:E = 4:1, P: chloroform (C) = 30:1 and Sephadex LH-20, $(CH_3)_2CO$) to give compound **1**; from Mfr-12 (300–400 mesh, P:C = 30:1 and 20:1, respectively) to give compound **4**; from Mfr-13 (P:C = 30:1 and P:E = 2:1, successively) to give compound **2**. These compounds were identified through physicochemical and spectral analyses and by comparison with previously reported data as Cy (**1**), (Lin and Shieh, 1992), Ml (**2**), Mi (**3**) and Ku (**4**) (Konno et al., 1977; Kim et al., 2011). The evidence of purity (>95%) of compounds **1–4** was assessed with ^{13}C NMR profile (Supplementary material, S1).

Bacterial strains

Ten MRSA strains with SCCmec III genotype and mecA gene were obtained and characterized from the infectious sputum samples of critically ill patients in Kunming General Hospital as previously reported (Zhang et al., 2005; Zuo et al., 2012). The mecA-mediated oxacillin resistance was confirmed using cefoxitin disk with inhibitory zone ≤ 21 mm (CLSI, 2012). The control strain was *S. aureus* (ATCC25923; MSSA) which was purchased from the Beijing Tiantan Pharmaceutical and Biological Technology Co., Ltd., Beijing, China and were used in this experiment.

Antibacterial agents

The eleven antibacterial agents were purchased from the manufacturers in China, i.e., AK (Jiangsu Wuzhong Pharmaceutical Group Co., Ltd., Suzhou, China); AM and SM (North China Pharmaceutical Co., Ltd., Shijiazhuang, China); CI (Sichuan Kelun Pharmaceutical Co., Ltd., Chengdu, China); GN and LE (Yangtze River Pharmaceutical Group Co., Ltd., Taizhou China); EM (Wuxi Jiming Kexin Shanhe Pharmaceutical Co., Ltd., Wuxi China); PV and P/S (Harbin Pharmaceutical Group Co., Ltd., Harbin, China); TP (Sanofi-Aventis (Beijing) Pharmaceutical Co., Ltd., Beijing China); VA (Eli Lilly Japan K. K., Seishin Laboratories, Japan) was used as the positive control agent. Cefoxitin disks were purchased from Beijing Tiantan biological products Co., Ltd., China. The four prenylflavonoids (**1–4**) were isolated and identified from root bark of *M. alba* with purity >95% which was supported by their ^{13}C NMR spectra (S1).

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 of compounds **1–4** were determined by conventional broth microdilution technique with starting inocula of 5×10^5 CFU/ml according to CLSI guidelines and incubated at 35 °C (CLSI, 2012).

Synergy testing

Anti-MRSA and -MSSA interactions of various antibacterial agents in combination with compounds **1–4** were determined by checkerboard and dynamic time-killing methods (Zuo et al., 2012). Briefly, bacteria were subcultured on MHA plates for 24 h. Colonies were suspended in 0.9% sterile saline and adjusted to a 0.5 McFarland turbidity standard (1.5×10^8 CFU/ml). The suspension was further diluted with MHB to 1×10^6 CFU/ml prior to testing in the 96-well-microtiter-plates. For the checkerboard method, the uppermost row (A_1) of a plate contained an antibacterial agent (A) in a concentration of two times the expected MIC combination. Each following row contained half the concentration of the previous one. The same procedure was carried out along the leftmost column (B_1) with a compound (B)—but not necessarily with the same starting concentration. So, each well contained a unique combination of the two substances (A and B) in 100 µl MHB. At last 100 µl MHB containing 1×10^6 CFU/ml was added to the wells and incubated at 35 °C for 24 h with a starting inoculum of 5×10^5 CFU/ml. The concentrations of the first wells without visible growth along the stepwise boundary between inhibition and growth were used to calculate the FICI values (FICI of A combined with B) = $((MIC_A)_{Combined}/(MIC_A)_{Alone}) + ((MIC_B)_{Combined}/(MIC_B)_{Alone})$ (Iten et al., 2009); For the time-kill testing, the wells containing 1 × MIC of the two substances with a starting inoculum of 5×10^5 CFU/ml was also incubated at 35 °C for the predetermined time points 0, 4, 8, 12, 16, 20, and 24 h. Fifty µl aliquots were removed at each time point from the test solution, diluted and streaked on a MHA plate for colony count determination (Klepser