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Short communication

Synergy of aminoglycoside antibiotics by 3-Benzylchroman derivatives from the Chinese drug *Caesalpinia sappan* against clinical methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

The *in vitro* antimicrobial activities of three 3-Benzylchroman derivatives, *i.e.* Brazilin (1), Brazilein (2) and Sappanone B (3) from *Caesalpinia sappan* L. (Leguminosae) were assayed, which mainly dealt with synergistic evaluation of aminoglycoside and other type of antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) by the three compounds through the Chequerboard and Time-kill curve methods. The results showed that Compounds 1–3 alone exhibited moderate to weak activity against methicillin-susceptible *S. aureus* (MSSA) and other standard strains by MICs/MBCs ranged from 32/64 to >1024/>1024 μg/ml, with the order of activity as 1 > 2 > 3. Chequerboard method showed significant anti-MRSA synergy of 1/Aminoglycosides (Gentamicin, Amikacin, Etimicin and Streptomycin) combinations with $(FICIs)_{50}$ at 0.375–0.5. The combined $(MICs)_{50}$ values (μg/ml) reduced from 32–128/16–64 to 4–8/4–16, respectively. The percent of reduction by MICs ranged from 50% to 87.5%, with a maximum of 93.8% (1/16 of the alone MIC). Combinations of 2 and 3 with Aminoglycosides and the other antibiotics showed less potency of synergy. The dynamic Time-killing experiment further demonstrated that the combinations of 1/aminoglycoside were synergistically bactericidal against MRSA. The anti-MRSA synergy results of the bacteriostatic (Chequerboard method) and bactericidal (time-kill method) efficiencies of 1/Aminoglycoside combinations was in good consistency, which made the resistance reversed by CLSI guidelines. We concluded that the 3-Benzylchroman derivative Brazilin (1) showed *in vitro* synergy of bactericidal activities against MRSA when combined with Aminoglycosides, which might be beneficial for combinatory therapy of MRSA infection.

Introduction

The clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) is a so called "superbug" which was originated date back to 1961 (Jevons 1961). Nowadays the alarm bell of "Entering a post-antibiotic era" is still ringing (Alanis 2005; Kåhrström 2013). MRSA is notorious for not only its multi-drug resistant to conventional antibiotics, but also its global epidemiology of healthcare-acquired/associated (HA), community-acquired/associated (CA) and livestock-associated (LA). It is highly prevalent in hospitals worldwide and the highest rates (>50%) were reported in North and South America, Asia and Malta (Stefani et al. 2012; Dubey et al. 2013). The critical shortage of new antibiotics in development against MRSA and other multidrug-resistant bacteria is of great concern, and new targets and modes of action against MRSA are urgently needed (Freire-Moran et al. 2011).

Medicinal plants have been demonstrated as the potential anti-MRSA resources by researchers worldwide and the therapeutic potential of phytochemicals has been increasingly recognized (Gibbons 2004, 2008; Mahady 2005; Zuo et al. 2008a, 2008b; Zahin et al. 2010; Radulovic et al. 2013). Moreover, special attentions have been paid to the synergy of phytochemicals with conventional antibiotics in reducing or even reversing the drug-resistance (Hemaiswarya et al. 2008). Synergy research has been emphasized as "Approaching a new generation of phytopharmaceuticals" (Wagner and Ulrich-Merzenich 2009). However, we notice that reports of this field are fewer than those of the abundant screening plant extracts and active compounds (Radulovic et al. 2013).

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Table 1
MICs and MBCs of Compounds **1**–**3** against various pathogens (μg/ml).

Strain		MSSA[a]	EC[b]	PA[c]	CA[d]
Brazilin (**1**)	MIC	32	512	512	>1024
	MBC	64	512	512	>1024
Brazilein (**2**)	MIC	128	1024	>1024	>1024
	MBC	256	1024	>1024	>1024
Sappanone B (**3**)	MIC	128	>1024	>1024	>1024
	MBC	512	>1024	>1024	>1024
Vancomycin	MIC	1	–	–	–
	MBC	2	–	–	–

[a] MSSA, methicillin-susceptible *Staphylococcus aureus* (ATCC25923).
[b] EC, *Escherichia coli* (ATCC25922).
[c] PA, *Pseudomonas aeruginosa* (ATCC27853).
[d] CA, *Candida albicans* (ATCCY0109).

Therefore, we have recently focused on searching for new antibiotics' synergistic phytochemicals from the Chinese medicinal plants (An et al. 2011; Zuo et al., 2011, 2012). We herein report the promising anti-MRSA synergy of aminoglycoside and other types of antibiotics combined with three 3-Benzylchroman derivatives, *i.e.* Brazilin (**1**), Brazilein (**2**) and Sappanone B (**3**) from the Chinese drug Sappan Lignum, the heartwood of *Caesalpinia sappan* L. (Leguminosae) (NUTCM 2005).

Materials and methods

Antibacterial agents

Eight antibiotics including four Aminoglycosides were purchased from the manufacturers in China, *i.e.* Gentamicin (GEN) and Ceftazidime (CAZ) (Guangzhou Baiyunshan Tianxin Pharmaceutical Co., Ltd.); Amikacin (AMK) (Jiangsu Wuzhong Pharmaceutical Group Co., Ltd.); Etimicin (ETM) (Wuxi Jimin kexin Shanhe Pharmaceutical Co., Ltd.); Streptomycin (STR) and Penicillin (PEN) (Shandong Lukang Pharmaceutical Co., Ltd.); Cefazolin (CFZ) (Harbin Pharmaceutical Group Co., Ltd.) and Azithromycin (AZM) (Yangtze River Pharmaceutical Group Co., Ltd.). Vancomycin (VAN) (Eli Lilly Japan K. K., Seishin Laboratories) was used as the positive control agent. Cefoxitin disks were purchased from Beijing Tiantan biological products Co., Ltd., China. The three 3-Benzylchroman derivatives Brazilin (**1**), Brazilein (**2**) and Sappanone B (**3**) were isolated from Sappan Lignum, the heartwood of *C. sappan* L. (Leguminosae) (NUTCM 2005). Their structures were identified mainly by spectral analysis and comparison with the data in the literatures (Saitoh et al. 1986; Kim et al. 1997).

Bacterial strains

Ten MRSA strains with SCC*mec* III genotype and mec*A* gene were obtained and characterized from the infectious sputum samples of critically ill patients in Kunming General Hospital as previously reported (An et al. 2011). The control strain was *S. aureus* (ATCC25923; Methicillin-susceptible *S. aureus* (MSSA)). MSSA and other standard strains of *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853) and *Candida albicans* (ATCCY0109) were purchased from the Beijing Tiantan Pharmaceutical and Biological Technology Co., Ltd., China and used in this experiment.

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.

*Bioactivity-guided fractionation, isolation and identification of Compounds **1**–**3***

The Sappan Lignum (5.0 kg; Voucher specimen KUN273 in Herbarium of Kunming Institute of Botany, China) was powdered, macerate and extracted with 80% ethanol for three times at the room temperature (7, 3, 2 days × 40, 20 and 15 l, respectively). The mixtures were filtered and the resulting filtrates were combined. After evaporating the solvent, the crude ethanol extract (685 g, 13.7%) was suspended in 1000 ml deioned water and successively extracted with Petroleum ether, EtOAc and butanol to give four sub-extracts, including the extract from water layer (<1, 400, 42.2, and 24 g, respectively). The 200 g sub-extract from EtOAc which showed the most active against MRSA by disk diffusion method (Zuo et al. 2008b) was subjected to column chromatography with silica gel (200–300 mesh, 4000 g; Qingdao Haiyang Chemical Co., Ltd), gradient eluting with Petroleum ether–EtOAc–MeOH (15:10:0–10:15:2.5) to give 19 fractions (SL-1–19). Further activity tracking of the fractions and repeated chromatography of the active SL-7 (14.85 g) with silica gel (400 mesh; Petroleum ether–EtOAc–MeOH (25:10:1)) and Sephadex LH-20 (Amersham Pharmacia Biotech.; MeOH) to furnish Compounds **1** (858 mg) and **3** (152.2 mg). The similar treatment of SL-12 (10.05 g) with silica gel (400 mesh; petroleum ether–EtOAc–MeOH (20:10:1.5); petroleum ether–$CHCl_3$–MeOH (7:3:2)) and polyamide (petroleum ether–chloroform–MeOH (7:3:3)) to furnish Compound **2** (76.3 mg).

Compound **1**, Pale yellow needles (MeOH), $C_{l6}H_{14}O_5$, ESI-MS *m*/*z*: 309 $[M+Na]^+$. ^{1}H NMR: δ ppm [400 MHz, CD_3OD] δ_H 3.68 (1H, *d*, *J* = 11.3, H-2a); 3.92 (1H, *d*, *J* = 10.2, H-2b), 4.00 (1H, *s*, H-4), 7.18 (1H, *d*, *J* = 8.4, H-5), 6.46 (1H, *dd*, *J* = 8.8, 2.5, H-6), 6.36 (1H, *s*, H-8), 2.77 (1H, *d*, *J* = 15.6, H-9a); 3.02 (1H, *d*, *J* = 15.6, H-9b), 6.59 (1H, s, H-2′), 6.70 (1H, s, H-5′). ^{13}C NMR: δ ppm [100 MHz, CD_3OD] δ_C 70.8 (C-2), 78.1 (C-3), 51.0 (C-4), 115.5 (C-4a), 132.2 (C-5), 109.9 (C-6), 155.7 (C-7), 104.2 (C-8), 157.9 (C-8a), 42.9 (C-9), 131.3 (C-1′), 112.8 (C-2′), 145.6 (C-3′), 145.3 (C-4′), 112.4 (C-5′), 137.4 (C-6′). The data were in agreement with those of Brazilin (Kim et al. 1997).

Compound **2**, Red-brown crystals (MeOH), $C_{l6}H_{12}O_5$, ESI-MS *m*/*z*: 307 $[M+Na]^+$. ^{1}H NMR: δ ppm [400 MHz, CD_3OD] δ_H 4.12 (1H, *d*, *J* = 9.0, H-2a); 4.47 (1H, *d*, *J* = 8.9, H-2b), 8.25 (1H, *d*, *J* = 9.9, H-5), 6.77 (1H, *dd*, *J* = 8.1, 2.8, H-6), 6.54 (1H, *d*, *J* = 2.1, H-8), 3.07 (2H, *s*, H-9), 6.56 (1H, *s*, H-2′), 7.49 (1H, *s*, H-5′). ^{13}C NMR: δ ppm [100 MHz, CD_3OD] δ_C 70.6 (C-2), 78.4 (C-3), 150.9 (C-4), 108.8 (C-4a), 129.9 (C-5), 108.8 (C-6), 164.4 (C-7), 103.3 (C-8), 158.8 (C-8a), 38.9 (C-9), 159.3 (C-1′), 116.0 (C-2′), 182.2 (C-3′), 152.9 (C-4′), 111.2 (C-5′), 124.8 (C-6′). The data were in agreement with those of Brazilein (Kim et al. 1997).

Compound **3**, Yellow needles (MeOH), $C_{l6}H_{14}O_6$, EI-MS *m*/*z*: 302 ($[M]^+$, 17), 284 (8), 180 (87), 137 (72), 123 (100). ^{1}H NMR: δ ppm [400 MHz, CD_3OD] δ_H 3.98 (1H *d*, *J* = 11.2 Hz, H-2a); 4.11 (1H, *d*, *J* = 11.2, H-2b), 7.70 (1H, *d*, *J* = 8.7, H-5), 6.68 (1H, *dd*, *J* = 8.0, 3.9, H-6), 6.52 (1H, *d*, *J* = 2.2, H-8), 2.74 (1H, *d*, *J* = 14.0, H-9a); 2.82 (1H, *d*, *J* = 14.0, H-9b), 6.77 (1H, *d*, *J* = 2.5, H-2′), 6.69 (1H, *d*, *J* = 4.7, H-5′). ^{13}C NMR: δ ppm [100 MHz, CD_3OD] δ_C 73.3 (C-2), 74.1 (C-3), 195.8 (C-4), 113.2 (C-4a), 130.4 (C-5), 112.2 (C-6), 166.7 (C-7), 103.6 (C-8), 164.9 (C-8a), 40.8 (C-9), 127.7 (C-1′), 115.9 (C-2′), 145.8 (C-3′), 145.3 (C-4′), 118.9 (C-5′), 123.2 (C-6′). The data were in agreement with those of Sappanone B (Saitoh et al. 1986).

Susceptibility testing

MICs/MBCs of Compounds **1**–**3** were determined by standardized broth microdilution techniques with starting inoculums of 5×10^5 CFU/ml according to CLSI guidelines and incubated at 35 °C