Phytomedicine 23 (2016) 1814–1820

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Synergism of coumarins from the Chinese drug *Zanthoxylum nitidum* with antibacterial agents against methicillin-resistant *Staphylococcus aureus* (MRSA)

Guo-Ying Zuo[a,*], Chun-Juan Wang[a,b], Jun Han[c,*], Yu-Qing Li[c], Gen-Chun Wang[a]

[a] *Research Center for Natural Medicines, Kunming General Hospital of Chengdu Military Command, Kunming 650032, China*
[b] *School of Pharmacy, Kunming Medical University, Kunming 550004, China*
[c] *School of Basic Medical Sciences, Yunnan Traditional Chinese Medical College, Kunming 650500, China*

ARTICLE INFO

Article history:
Received 2 July 2016
Revised 28 October 2016
Accepted 3 November 2016

Keywords:
Antimicrobial activity
MRSA
Coumarin
Antibacterial agents
Synergy

ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious therapeutic challenge in current clinic and new drug development. Natural coumarins have diverse bioactivities and the potential of resistance modifying effects.

Purpose: This study is to present in-depth evaluations of *in vitro* antimicrobial activities of four natural coumarins 5-geranyloxy-7-methoxycoumarin (Gm, **1**), (5,7-dimethoxy-8-prenyloxycoumarin (artanin, Ar**,** **2**)), isopimpinellin (Is, **3**) and phellopterin (Ph, **4**) from *Zanthoxylum nitidum* (Roxb.) DC. (Rutaceae) extracts, focusing on their potential restoration the activity of conventional antibacterial agents against clinical MRSA strains.

Methods: Bioactivity-guided fractionation and spectral analyses were used to isolate the coumarins and identify the structures, respectively. The double broth microdilution method was used to assay the coumarins' alone activity. The classic checkerboard microdilution and dynamic time-killing methods were used to evaluate combinatory effects.

Results: The four plant coumarins Gm (**1**), Ar (**2**), Is (**3**) and Ph (**4**) were isolated and identified from *Z. nitidum* extracts. Coumarins **1**–**4** displayed promising inhibition against both MSSA and MRSA with minimal inhibitory concentrations (MICs) of 8–64 μg/ml, but very weak against Gram-negative pathogen and yeast with MICs of 256 to $\geq$ 1024 μg/ml. The geranyloxy and prenyloxy substitutions showed to be more active than the methoxy substitution on the coumarin skeletons. **1**–**4** also showing different extent of synergism with a total of eight conventional antibacterial agents, *i.e.* chloramphenicol (CL), gentamicin (CN), fosfomycin (FF), levofloxacin (LE), minocycline (MI), piperacillin/tazobactam (P/T), teicoplanin (TE) and vancomycin (VA) against ten clinical MRSA strains. Four to ten of the tested MRSA strains showed bacteriostatic synergy in the eleven combinations. The anti-MRSA modifying effects were related to different arrangement in the combinations with fractional inhibitory concentration indices (FICIs) from 0.187 to 1.125 and the three combinations CN (Is), CL (Ph) and MI (Gm) were the best ones. The enhancement of activity was also shown by 2–64 of dose reduction indices (DRIs) of the combined MICs, with VA (Ph) combination resulted the biggest DRI. The resistance of MRSA to antibacterial agents could be reversed in the combinations of CL (Gm or Ph), LE (Ph) and MI (Is) following the Clinical and Laboratory Standards Institute (CLSI) criteria. Six combinations P/T (Gm), TE (Ar), CN (Is), VA (Ph) and CL (Gm or Ph) also showed bactericidal synergy with $\Delta \log_{10}$ CFU/ml >2 at 24 h incubation.

Conclusions: The coumarins showed high potentiating effects of the antibacterial agents against multi-drug resistant SA. The resistance reversal effect of CL, LE and MI warrants further pharmacological investigation on combinatory therapy for the sake of fighting against MRSA infections.

Abbreviations: AM, ampicillin; Ar, artanin; AZ, azithromycin; CA, *Candida albicans*; Cd, clindamycin; CH, clarithromycin; CI, ciprofloxacin; CL, chloramphenicol; CLSI, Clinical and Laboratory Standards Institute; CN, gentamicin; CFU, colony forming unit; CPZ/S, cefoperazone/sulbactam; CT, cefathiamidine; CX, cefoxitin; CZ, cefazolin; DRI, dose reduction index; EC, *Escherichia coli*; EM, erythromycin; ESI-MS, electrospray ionization mass spectrometry; FF, fosfomycin; FICI, fractional inhibitory concentration index; GA, gatifloxacin; Gm, 5-geranyloxy-7-methoxycoumarin; I, intermediate; Is, isopimpinellin; LE, levofloxacin; LZ, linezolid; Masc, most active single drug at 24 h incubation; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration; MH, Mueller-Hinton medium; MI, minocycline; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*; NMR, nuclear magnetic resonance; NX, norfloxacin; OX, oxacillin; P/T, piperacillin-tazobactam; PA, *Pseudomonas aeruginosa*; Ph, phellopterin; PV, penicillin; R, resistant; RA, rifampicin; S, susceptible; TE, teicoplanin; VA, vancomycin; XM, cefuroxime.

* Corresponding authors. Fax: +86 871 5414186.
E-mail addresses: zuoguoying@263.net (G.-Y. Zuo), hanzjn@126.com (J. Han).

http://dx.doi.org/10.1016/j.phymed.2016.11.001
0944-7113/

Introduction

Common classes of antibiotics, such as β-lactams, have contributed greatly to the treatment of bacterial infections for decades during the 20th century, but now they are encountering the serious challenges posed by methicillin-resistant *Staphylococcus aureus* (MRSA) (WHO, 2014; Hetem et al., 2016). MRSA is a problematic pathogen in current global clinic. It is a major nosocomial pathogen that causes a broad spectrum of pathologies ranging from skin infections to deep-seated fatal disease. Clinical MRSA strains have developed resistance to not only β-lactams but also to aminoglycosides, quinolones, macrolides, tetracyclines and others (Tomasz, 1994).

Researchers at the pharmaceutical field have made many efforts to cope with MRSA, mainly by looking for natural products with anti-MRSA activity. Plant secondary metabolites have well-known large chemical diversity that have previously demonstrated anti-MRSA activity and resistance modifying effects by many research groups (Hemaiswarya et al., 2008; Wagner and Ulrich-Merzenich, 2009). We have also found several anti-MRSA compounds within the Chinese herbal medicines and studied their synergistic effects when combined with conventional antibacterial agents (Zuo et al., 2015).

Coumarins are an important class of natural polyphenolic compounds which belong to the benzopyrones family. Potential therapeutic uses of coumarin derivatives are very wide, such as antimicrobial, anticancer, anti-inflammatory, anticoagulant, antioxidant, antihypertensive, anticonvulsant, antiadipogenic, antihyperglycemic and neuroprotective properties (Phougat et al., 2016; Barot et al., 2015; Katsori and Dimitra, 2014). Continuing on our projects for searching the plant-derived anti-MRSA synergistic compounds, we are paying special attention to coumarins' effect on MRSA and their interaction with common clinically used antibacterial agents (Yasunaka et al., 2005; Shahverdi et al., 2007; Smyth et al., 2009; Roy et al., 2013; Joshi et al., 2014). Much more in-depth work on the anti-MRSA activities of plant coumarins are highly needed.

From the bioactivity-guided fractionation of the extracts prepared from the Chinese medicinal plant *Zanthoxylum nitidum* (Roxb.) DC. (Rutaceae) (NUTCM, 2005), we found that the coumarin-containing sub-extract showed anti-MRSA activity. Further fractionation of this sub-extract led us to obtain four coumarins, *i.e.* 5-geranyloxy-7-methoxycoumarin (Gm, **1**), (5, 7-dimethoxy-8-prenyloxycoumarin (artanin, Ar, **2**)), isopimpinellin (Is, **3**) and phellopterin (Ph, **4**) (Miyake and Hiramitsu, 2011; Maes, et al. 2008; De Menezes et al., 2014; Bergendorff et al., 1997). We herein report the evaluation of anti-MRSA synergism of the four coumarins (**1**–**4**) by combining them with antibacterial agents, *i.e.* chloramphenicol (CL), gentamicin (CN), fosfomycin (FF), levofloxacin (LE), minocycline (MI), piperacillin/tazobactam (P/T), teicoplanin (TE), vancomycin (VA) for the first time, together with their antimicrobial activity against other clinical infectious pathogens.

Materials and methods

Plant material

The dried roots of *Z. nitidum* was bought in August 2010 from the drug mart in Kunming, China. A voucher specimen (KUN0543426) is deposited in the Herbarium of Kunming Institute of Botany, China.

*Bioactivity-guided fractionation, isolation and identification of compounds **1–4***

The dried powder of Z. *nitidum* roots was extracted with 90% methanol under reflux for three times. The mixtures were cooled and filtered and the resulting filtrates were combined. After evaporating the solvent, the crude methanol extract (1100 g) was suspended in 1300 ml deioned water and successively extracted with petroleum ether, ethyl acetate and butanol. The petroleum ether sub-extract (74.4 g) which showed to be the most active against MRSA by disk diffusion method (Zuo et al., 2008) was subjected to column chromatography with silica gel (200–300 mesh, 1900 g; Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), and eluted with petroleum ether-ethyl acetate (10: 1–6: 1) to give 20 fractions (Zfr-1–20) which were tested for anti-MRSA activity. Repeated chromatography of the active fractions Zfr-5 and Zfr-6 with silica gel (petroleum ether- ethyl acetate (20:1–9:1)) gave compounds **1** (107 mg) and **2** (143 mg). Chromatographic fractionation of fractions Zfr-8 and Zfr-11 [elution solvent: petroleum ether-ethyl acetate (20: 1–4: 1)] allowed the isolation of compounds **3** (502 mg) and **4** (735 mg).

Compound **1** ($C_{20}H_{24}O_4$, Gm) was obtained as colorless needles (MeOH), ESI-MS *m/z*: 351 $[M+Na]^+$. Compound **2** ($C_{16}H_{18}O_5$, Ar) was also obtained as colorless needles (MeOH), ESI-MS *m/z*: 313 $[M+Na]^+$. Compound **3** ($C_{13}H_{10}O_5$, Is) was obtained as light yellow amorphous solid, ESI-MS *m/z*: 269 $[M+Na]^+$. Compound **4** ($C_{17}H_{16}O_5$, Ph) was also obtained as light yellow amorphous solid, ESI-MS *m/z*: 323 $[M+Na]^+$.Their ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data (δ ppm in $CDCl_3$) (Supplementary material, S1) were in agreement with those of reported (Miyake and Hiramitsu, 2011 (**1**), Maes et al., 2008 (**2**), De Menezes et al., 2014 (**3**) and Bergendorff et al., 1997 (**4**)).

Antibacterial agents

The eight antibacterial agents were purchased from the suppliers, *i.e.* CL and P/T (Harbin Pharmaceutical Group Co., Ltd., Harbin, China); FF (North China Pharmaceutical Co., Ltd., Shijiazhuang, China); CN and LE (Yangtze River Pharmaceutical Group Co., Ltd., Taizhou, China); MI (Wyeth Pharmaceuticals, Pennsylvania, U.S.A.); TE (Sanofi-Aventis (Beijing) Pharmaceutical Co., Ltd., Beijing, China) and VA (Eli Lilly Japan K. K., Seishin Laboratories, Nishi-ku, Japan). Cefoxitin disks were purchased from Beijing Tiantan biological products Co., Ltd., Beijing, China. VA was used as the positive control agent. The four coumarins (**1**–**4**) were isolated and identified from *Z. nitidum* with purity >95% by ^{13}C NMR analysis (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 (Zuo et al., 2015). The voucher specimen numbers are provided in Table 1. 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., Beijing, China and were used in this experiment.

Media

Standard Mueller-Hinton agar and broth (MHA and MHB (Sabouraud's media for *C. albicans*), Tianhe Microbial Agents Co., Hangzhou, China) were used as bacterial culture media. MHB was