Med Chem Res (2014) 23:2439–2444
DOI 10.1007/s00044-013-0844-5

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synergistic effects of berberines with antibiotics on clinical multi-drug resistant isolates of methicillin-resistant *Staphylococcus aureus* (MRSA)

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Received: 19 August 2013 / Accepted: 12 October 2013 / Published online: 20 October 2013

Abstract *N*-Methyl-dihydroberberine (M-Ber) was synthesized, and antibacterial activities of Berberine (Ber) and M-Ber alone and combined with antibiotics were studied against ten clinical MRSA isolates. MICs/MBCs (μg/ml, alone) ranges were 32–128/64–256 (Ber) and 64–128/256–1,024 (M-Ber) by a broth microdilution method. Significant synergies of Ber (M-Ber)/Azithromycin and Ber (M-Ber)/Levofloxacin combinations were observed by the chequerboard test. The Ber (M-Ber)/Ampicillin and Ber (M-Ber)/Cefazolin combinations showed indifference. These results demonstrated that Ber and M-Ber enhanced the in vitro inhibitory efficacy of Azithromycin and Levofloxacin, which had potential for combinatory therapy of patients infected with MRSA.

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Keywords Anti-MRSA activity · Berberine · *N*-Methyl-dihydroberberine · Synergy · Azithromycin · Levofloxacin

Introduction

Clinical methicillin-resistant *Staphylococcus aureus* (MRSA) has become the most common cause of infections among many global pathogenic bacteria, a number of life-threatening diseases such as endocarditis, pneumonia, and toxin shock syndrome were ascribed to it. 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 (VAN)-resistant *S. aureus* (Chang *et al*., 2003).

In our hospital, MRSA could be examined in over 80 % sputum samples of pneumonia from sever 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).

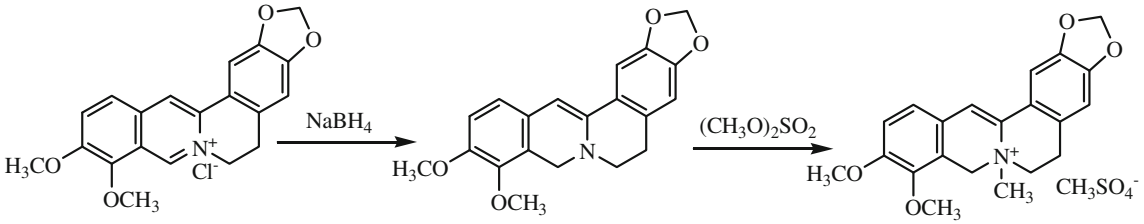
Berberine is an isoquinoline alkaloid from *Coptis chinensis* Franch and *Phellodendron amurense* Ruprecht and a classic plant antimicrobial which has been used in the treatment of gastroenteritis, diarrhea, and cholera diseases (Yu *et al*., 2005). The present report deals with the anti-MRSA activities of Berberine (Ber) and its synthetic derivative *N*-methyl-dihydroberberine (M-Ber) and their

synergistic effects with four conventional antibiotics Ampicillin (AMP), Azithromycin (AZM), Cefazolin (CFZ), and Levofloxacin (LEV).

Results and discussion

Chemistry

N-methyl-dihydroberberine (M-Ber) was synthesized from Ber as its methomethylsulfate following the literature procedure (Onda *et al*., 1973).



Anti-MRSA evaluations

Anti-MRSA activities of the two berberines (Ber and M-Ber) and four antibiotics alone against ten clinical MRSA isolates of SCCmec III type are shown in Table 1. MICs/MBCs (μg/ml) ranges were 32–128/64–256 for Ber and 64–128/256–1,024 for M-Ber alone against all isolates. The $(MICs)_{90}$ of Ber and M-Ber were 128 and 64 μg/ml, respectively. The agents' order of potencies followed LEV > M-Ber ≥ Ber = AMP > CFZ ≫ AZM. This is the first report of anti-MRSA/antibiotic combinatory properties of M-Ber so far to the best of our knowledge (Yu *et al*., 2005). Compared with Ber, M-Ber has higher solubility under physiological conditions, so it showed higher antibacterial potency against MRSA isolates. As mentioned in the introduction part, Ber has been successfully used confined in the treatment of gastrointestinal diseases. The distribution amount of berberine among other tissues and organs will be very low due to its low solubility in water. The increased solubility of M-Ber might be beneficial to its anti-MRSA of systemic infections (Fig. 1).

Synergy effects of the berberines i.e., Ber and M-Ber with the four antibiotics against the ten MRSA isolates by chequerboard method and the FICIs are demonstrated in Table 2. Time-killing curves of the synergy combination of the berberines with the four antibiotics against MRA 004 (one of the ten isolates) are shown in Fig. 2.

Table 1 MICs and MBCs (μg/ml) of Ber and M-Ber and four antibiotics alone against ten clinical MRSA strains of SCCmec III type

Agents	Range of MIC/MBC	$(MIC/MBC)_{50}$	$(MIC/MBC)_{90}$
Ber	32–128/64–256	64/256	128/256
M-Ber	64–128/256–1,024	32/256	64/512
AMP	16–128/64–512	64/512	128/512
AZM	2,000–4,000/nt	4,000/nt	4,000/nt
CFZ	128–256/nt	128/nt	256/nt
LEV	2–16/8–64	16/64	16/64
VAN	1.00/2.00	1.00/2.00	1.00/2.00

The tested maximum concentration of agents was 4,000 μg/ml; 50: values of those 50 % of the tested strains; 90: values of those 90 % of the tested strains

Ber berberine, *M-Ber N*-methyl-dihydroberberine, *AMP* Ampicillin, *CFZ* Cefazolin, *LEV* Levofloxacin, *AZM* Azithromycin, *VAN* vancomycin, *nt* not determined

The chequerboard evaluation was performed with the four antibiotics representing four types of antibacterial agents, including β-lactam (AMP), macrolide (AZM), CFZ (cephem), and LEV (fluoroquinolone). The MIC_{90} of berberines/antibiotics (AZM and LEV) combinations reduced by 50.0–87.5 %, which demonstrated significant antibacterial synergy activities against most of the tested pathogenic strains (FICIs ranged 0.188–0.75) (Tables 1, 2). But all the berberines/(AMP or CFZ) combinations showed indifference (FICIs 1.5–2.0). The order of synergy followed the combinations of Ber/AZM > M-Ber/AZM > Ber/LEV > M-Ber/LEV (Table 2). Therefore, the synergistic effects of Ber are nearly equal to M-Ber when they were combined with the antibiotics.

It is noted that the MICs of Ber alone are consistent with previously reported results, but the indifference effect of Ber/AMP combination in present study is different from the additivity in the literature (Yu *et al*., 2005). This might be due to the different resistance profiles of SCCmec III type MRSA isolates tested in our study, they are the major nosocomical isolates in Asian countries and characteristic for the multi-drug resistant to not only β-lactams but also to other types of antibiotics currently used (McDonald *et al*., 2006).

In the time-kill analyses, synergistic effects of the combinations between the berberines and antibiotics were different from those found in the chequerboard method following the criterion of synergy test (Yu *et al*., 2005), though the overall killing effects of the combination were the best (Fig. 2). Time-kill curves

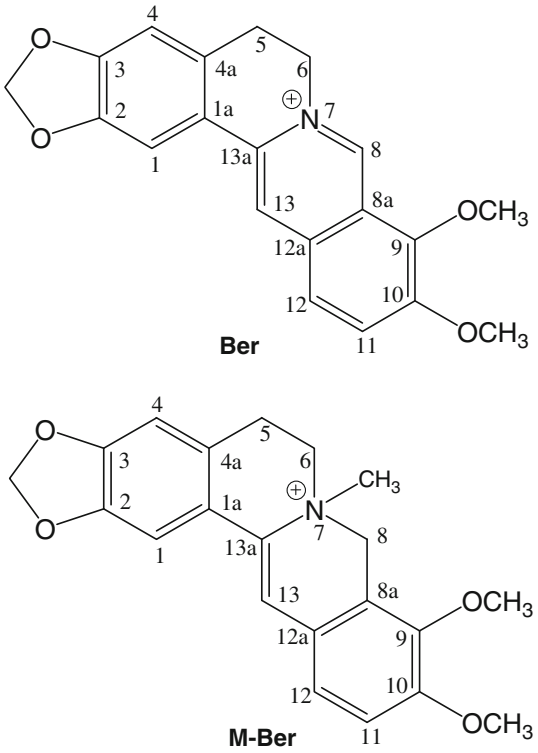


Fig. 1 The structures of compounds Berberine (Ber) and *N*-methyl-dihydroberberine (M-Ber)

showed the berberines were the most active alone, and Ber/AZM and Ber/LEV combinations resulted in an increase in killing of 1.92 (additivity) and 0.92 (indifference) $\log_{10}$ CFU/ml of the colony counts at 24 h in comparison with that of Ber, while the M-Ber/AZM and M-Ber/LEV combinations resulted in much smaller increase of 1.12 (additivity) and 0.64 (indifference), respectively (Fig. 2). Compared with the resulted killing of the antibiotics alone, the increased $\log_{10}$ CFU/ml (combined) values followed the order of 2.95 (M-Ber/LEV) (d) > 2.76 (M-Ber/AZM) (c) > 2.68 (Ber/AZM) (a) > 1.39 (Ber/LEV) (b) (Fig. 2). Hence, bactericidal efficiency of the combinatory schemes was much more potent than those of the antibiotics alone, which is in some agreement with the bacteriostatic results by chequerboard evaluation (Tables 1, 2). It has been confirmed that the overestimate of synergy experienced with the chequerboard test, and synergy testing performed by time-kill kinetics was used to confirm the results of chequerboard MIC testing (Petersen *et al*., 2006). It is noted that the anti-MRSA potentials of M-Ber were similar to that of 8-acetonyl-dihydroberberine (A-Ber) we have reported (Zuo *et al*., 2012).

The varied interactions of the berberines on different antibiotics might be ascribed to their interference with the different resistance mechanisms of bacteria (Wagner and Ulrich-Merzenich, 2009), for example, the efflux pump inhibition (Gibbons, 2008). As the clinical MRSA strains

Table 2 MICs (μg/ml) and FIC indices (FICIs) of Berberines in combination with AZM and LEV against 10 clinical MRSA strains of SCCmec III type

Agent	AMP	AZM	CFZ	LEV
MIC range[a]				
Ber	32–128 + 32–128	2–16 + 250–1,000	32–128 + 64–256	4–32 + 2–8
M-Ber	64–128 + 32–128	8–64 + 125–1,000	64–128 + 64–256	8–32 + 1–8
$MIC_{50/90}$				
Ber	64 + 64/128 + 128	8 + 500/16 + 1,000	64 + 128/128 + 256	16 + 4/16 + 8
M-Ber	64 + 64/128 + 64	8 + 500/32 + 1,000	64 + 128/128 + 256	32 + 4/32 + 4
Rd%[b]				
Ber	0 + 0/0 + 0	87.5 + 87.5/87.5 + 75	0 + 0/0 + 0	75 + 75/87.5 + 50
M-Ber	−100 + 0/−100 + 50	75 + 87.5/50 + 75	−100 + 0/−100 + 0	0 + 75/50 + 75
FICI range				
Ber	1.5–2	0.25–0.5	1.5–2	0.375–0.75
M-Ber	1.5–2	0.188–0.75	1.5–2	0.25–0.5
$FICI_{50/90}$				
Ber	2/2	0.313/0.500	2/2	0.5/0.75
M-Ber	2/2	0.375/0.5	2/2	0.375/0.5
$E^{c}_{50/90}$				
Ber	Ind/ind	Syn/syn	Ind/ind	Syn/add
M-Ber	Ind/ind	Syn/syn	Ind/ind	Syn/syn

[a] Values expressed as those agents of alkaloids + antibiotics

[b] Rd% % of MIC reduced, $Rd\% = (MIC_{alone} - MIC_{combined}) \times 100/MIC_{alone}$

[c] *E* effect, *Syn* synergy (FICI ≤ 0.5), *Add* additivity (0.5 < FICI ≤ 1), *Ind* indifference (1 < FICI ≤ 2)

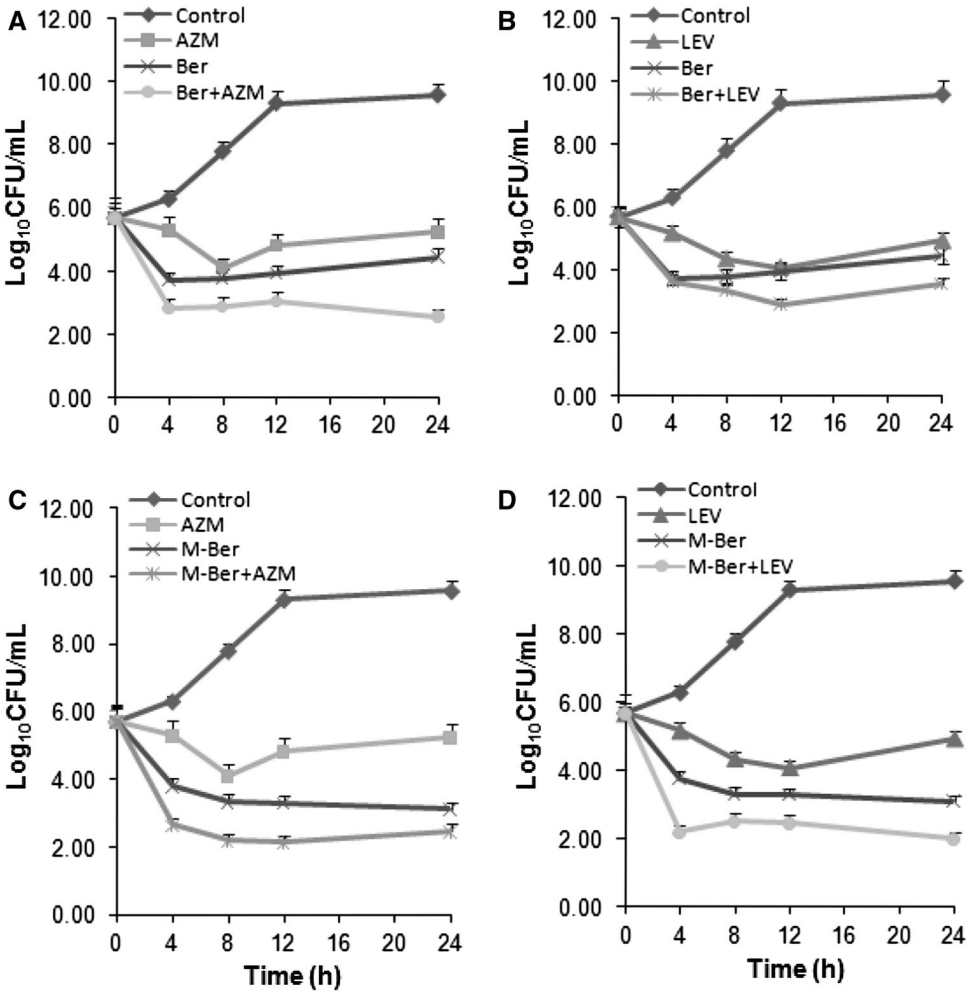


Fig. 2 Time-kill curves of the synergistic effect of the combination at 1 × MIC (alone) concentration of Berberine (Ber) and *N*-methyl-dihydroberberine (M-Ber) with Azithromycin (AZM) (**a**, **c**) and Levofloxacin (LEV) (**b**, **d**), respectively, against MRA 004, a clinical MRSA strains of SCCmec III type. The viable cells counts reduced 1.92 (**a**), 0.92 (**b**), 0.64 (**c**), and 1.12 (**d**), respectively

have become an increasingly pressing global problem, anti-MRSA synergistic effects between plant natural compounds and conventional antibacterial agents have further been demonstrated here as a promising way of overcoming current antibiotics resistance (Hemaiswarya *et al*., 2008).

Conclusion

In conclusions, this study demonstrated that Ber and M-Ber enhanced the in vitro inhibitory efficacy of AZM and LEV, which showed potential for combinatory therapy of patients infected with MRSA and warrant further pharmacological investigation.

Experimental

Chemicals

All the chemicals used were of A. R. Grade. M-Ber was synthesized according to the procedure available in the literature (Onda *et al*., 1973). The solvents were dried according to the standard procedures and distilled before use. ^{1}H NMR spectra were recorded in CD_3OD using TMS as the standard on Bruker AM-400 MHz spectrometer. ^{13}C NMR spectra were recorded in CD_3OD using TMS as the standard on Bruker DRX-500 MHz spectrometer. MS were recorded on API Qstar Pulsar mass spectrometer.

Preparation of dihydroberberine methomethylsulfate

M-Ber was prepared according to the literature procedure (Onda *et al*., 1973) with a slight modification. Dried berberine (5 g) and $NaBH_4$ (0.6 g) were dissolved in 30 ml of anhydrous pyridine in round-bottom flask with continuous stirring for 30 min. Then 0.5 g of $NaBH_4$ was further added and stirred for another 30 min. The reaction mixture was poured into 100 ml ice water, filtered and dried to give 4.1 g yellow solid. The solid was dissolved in a dried 55 ml CH_2Cl_2 by slowly adding dropwise of $(CH_3O)_2SO_2$ (4.1 ml), heated to 40 °C and refluxed for 2 h. The reaction mixture was cooled, filtered and the resulting precipitate

was recrystallized from EtOH and finally a pale yellow powder weighing 4.4 g (yield 70.6 %) was got, i.e., Dihydroberberine methomethylsulfate (M-Ber) (Fig. 1).

M-Ber

$C_{20}H_{18}NO_4$, ESI–MS: *m/z* at 353 $[M+H]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.44 (1H, s, H-13), 7.39 (1H, s, H-4), 7.23 (1H, d, $J = 8.3$, H-12), 7.12 (1H, d, $J = 8.4$, H-11), 6.77 (1H, s, H-l), 5.99 (2H, s, –OCH_2O–), 4.90 (2H, s, H-8), 3.96 (2H, m, H2-6), 3.89 (3H, s, OMe), 3.88 (3H, s, OMe), 3.13 (2H, m, H2-5), 3.08 (3H, s, NMe) ^{13}C-NMR (100 MHz, CD_3OD) δ: 155.8 (C-3), 151.0 (C-10), 150.0 (C-2), 147.4 (C-9), 136.6 (C-12a), 127.1 (C-11), 125.2 (C-12), 123.1 (C-4a), 126.7 (C-9), 123.4 (C-8), 121.8 (C-11a), 120.6 (C-7), 120.2 (C-14a), 119.7 (C-1a), 119.6 (C-8a), 116.8 (C-13a), 114.7 (C-13), 109.4 (C-1), 104.3 (C-4), 103.4 (–OCH2O–), 64.9 (C-6), 62.7 (OMe), 61.7 (OMe), 56.6 (C-8), 46.0 (NMe), 25.1 (C-5).

Antibacterial studies

Antibacterial agents

Four antibiotics represented different conventional types were purchased from the manufacturers, i.e., AMP (North China Pharmaceutical Co., Ltd, Shijiazhuang, China), CFZ (Harbin Pharmaceutical Co., Ltd, Harbin, China), AZM and LEV (Yangzhijiang Pharmaceutical Co., Ltd, Taizhou, China). VAN (Eli Lilly Japan K. K., Seishin Laboratories) was used as the positive control agent. Cefoxitin disks were purchased from Tiantan biological products Co., Ltd (Beijing, China). M-Ber was synthesized from Ber (Changzhou Yabang Pharmaceutical Co., Ltd, Changzhou, China) following the procedure previously reported (Onda *et al*., 1973).

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 (CLSI, 2006a, b, 2007; Kloos and Bannerman, 1999). The presence of mecA gene and SCCmec genotypes was 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., Hang Zhou, 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 5×10^5 CFU/ml according to CLSI guidelines and incubated at 35 °C for 24 h (CLSI, 1999, 2006a, b). They were determined in duplicate, with concentrations ranging up to 4,000 μg/ml for AZM.

Synergy testing

Potential anti-MRSA synergy was measured by fractional inhibitory concentration (FIC) indices (FICI) with chequerboard method and by time-killing curves as previously reported (Yu *et al*., 2005). The FIC of the combination was calculated through dividing the MIC of the berberines/antibiotics combination by the MIC of Berberines or of the antibiotics alone, and the FICI was obtained by adding the FIC of Berberines and that of antibiotics. The FICI results were interpreted as follows: FICI ≤ 0.5, synergy; $0.5 <$ FICI ≤ 1, additivity; and $1 <$ FICI ≤ 2, indifference (or no effect) and FICI > 2, antagonism (Yu *et al*., 2005). In the killing curves, synergy was defined as $\geq$2 $\log_{10}$ CFU/ml increase in killing at 24 h with the combination, in comparison with the killing 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 $\pm$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; Hu *et al*., 2002). All experiments were performed in triplicates.

Acknowledgments This work was supported by the National Natural Science Foundation of China (NSFC 81073126), and the supporting funds of the Department of Health, Chengdu Military Region, PLA (MB09030) and Yunnan Province of China (2008PY001). We are also grateful to the Kunming Institute of Botany (CAS) for spectral analysis.

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