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Original article

Carnosic acid acts synergistically with gentamicin in killing methicillin-resistant *Staphylococcus aureus* clinical isolates

Nicolás M. Vázquez[a], Graciela Fiorilli[b], Paulo A. Cáceres Guido[c,d], Silvia Moreno[a,d,*]

[a] *Leloir Institute and Institute of Biochemistry Research of Buenos Aires-IIBBA-CONICET, Patricias Argentinas 435 (C1405BWE), Ciudad Autónoma de Buenos Aires C1405, Argentina*
[b] *Service of Microbiology, Garrahan Pediatric Hospital, Combate de los Pozos 1881 (1245AAM), Ciudad Autónoma de Buenos Aires, Argentina*
[c] *Integrative Medicine Group, Garrahan Pediatric Hospital, Combate de los Pozos 1881 (1245AAM), Ciudad Autónoma de Buenos Aires, Argentina*
[d] *Maimónides University, Hidalgo 775 (1405BCK), Ciudad Autónoma de Buenos Aires, Argentina*

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is resistant to different commonly used antibiotics, stressing the need for further strategies to treat this human pathogen with worldwide prevalence. The use of phytochemicals within the current pharmacology is a promising approach to enhance the antimicrobial activity of common antibiotics in the battle against these bacteria.
Purpose: The purpose of this study was to determine the antimicrobial effectiveness of carnosic acid, the major constituent of *Rosmarinus officinalis* L. leaves, in combination with gentamicin against multi-drug resistant MRSA clinical isolates obtained from pediatric patients with bacteremia.
Materials and methods: Anti-MRSA activity was studied using the broth microdilution assay and time–kill method. Combinations of subinhibitory concentrations of carnosic acid and gentamicin were examined using the checkerboard method.
Results: Carnosic acid exhibited a good antibacterial activity against all multidrug-resistant MRSA clinical isolates tested, which are resistant to four up to nine antibiotics. In addition, the combination of carnosic acid with gentamicin not only decreased the minimal inhibitory concentration (MIC) of both by 4- to 5-fold, but also improved the bactericidal potency of the common antibiotic by 32- to 40-fold against both gentamicin-susceptible and gentamicin-resistant MRSA clinical isolates. A clear bactericidal synergistic activity between carnosic acid and gentamicin in killing multidrug-resistant MRSA clinical isolates with a fractional bactericidal concentration index (FBCI) of 0.28–0.35 was demonstrated.
Conclusions: Our findings show the potential use of carnosic acid in combination with gentamicin as a promising alternative for the control of healthcare-associated infections caused by multidrug-resistant MRSA.

Introduction

Limited therapeutic options exist to treat patients infected with methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most important sources of antibiotic-resistant healthcare-associated infections resulting in prolonged hospital stays and increased mortality (Chen et al., 2010; WHO, 2014; Yilmaz et al., 2016). This pathogen has acquired resistance to other commonly used classes of antibiotics, such as aminoglycosides, fluoroquinolones, macrolides, and tetracyclines (Vuong et al., 2015).

Gentamicin is habitually used to treat severe infections with *S. aureus, Escherichia coli, Pseudomonas aeruginosa, Enterobacter spp., Klebsiella spp., Serratia spp* and for prophylaxis especially against endocarditis in patients who have different risk factors for harboring multidrug-resistant organisms (McConeghy and La Plante, 2010; Liu et al., 2011). However, there is evidence for nephrotoxicity and ototoxicity associated with high doses of gentamicin in the treatment of *S. aureus* bacteremia (Cosgrove et al., 2009).

Currently, a new generation of phytopharmaceuticals has gained interest within the current pharmacology as a source of new antimicrobials to combat this multidrug-resistant bacterium (Wagner and Ulrich-Merzenich, 2009; Abreu et al., 2012; Worthington and Melander, 2013). Indeed, some polyphenols from medicinal plants

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; MH, Mueller-Hinton medium; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TSB, Trypticase soy broth; FICI, fractional inhibitory concentration index; CA, carnosic acid; GEN, gentamicin; OXA, oxacillin; FOX, cefoxitin; ERY, erythromycin; CLY, clindamycin; CIP, ciprofloxacin; LVX, levofloxacin; LZD, linezolid; Q/D, quinupristin/dalfopristin; RIF, rifampicin; R, resistant; S, susceptible.

* Corresponding author. Fax: +54 11 5238 7501.
E-mail address: smorenocontar@gmail.com, smoreno@leloir.org (S. Moreno).

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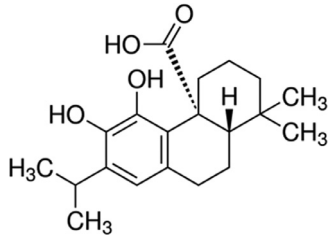

Fig. 1. Structural formula of carnosic acid.

Table 1
Antibiotic resistance profile of MRSA clinical isolates.

Clinical isolates	Bacteria antibiotic susceptibility
MRSA-GM20	$OXA^{R}FOX^{R}ERY^{R}CLY^{R}GEN^{S}CIP^{S}LVX^{S}Q/D^{S}LZD^{S}RIF^{S}$
MRSA-GM31	$OXA^{R}FOX^{R}ERY^{R}CLY^{R}GEN^{R}CIP^{R}LVX^{R}Q/D^{S}LZD^{S}$ RIF^{R}
MRSA-GM34	$OXA^{R}FOX^{R}ERY^{R}CLY^{R}GEN^{R}CIP^{R}LVX^{R}Q/D^{S}LZD^{R}RIF^{R}$

OXA, oxacillin; FOX, cefoxitin; ERY, erytromycin; CLY; clindamycin; GEN, gentamicin; CIP, ciprofloxacin; LVX, levofloxacin; Q/D, quinupristin/dalfopristin; LZD, linezolid; RIF, rifampicin. R: resistant and S: susceptible.

show anti-MRSA activity as well as adjuvant actions on common antibiotics (Zuo et al., 2012; Mun et al., 2013; Bhattia et al., 2014; Boulanger et al., 2015; Rempe et al, 2015). We previously demonstrated that carnosic acid isolated from leaves *of Rosmarinus officinalis* L. (Lamiaceae), commonly known as rosemary, was the major antimicrobial compound against Gram-positive and Gram-negative bacteria and yeasts (Moreno et al., 2006). This abietane-type phenolic diterpene, accounts for many pharmacological activities of *R. officinalis* leaves extracts (Barni et al., 2012; Gaya et al., 2013; Habtemariam, 2016). Earlier, we reported that this natural compound, structurally unrelated to known antibiotics, can function as an efflux pump modulator by dissipation of the membrane potential in Gram-positive cocci (Ojeda-Sana et al., 2013). In addition, a synergistic antibacterial effect in combination with gentamicin, ciprofloxacin, tetracycline, tobramycin and kanamycin against a pan-susceptible *S. aureus* strain was demonstrated by us (Moreno et al., 2012). Nevertheless, little is known about the susceptibility of multi-drug resistant MRSA clinical isolates to carnosic acid in combination with gentamicin. In the search for new strategies to treat staphylococcal infections, the aim of the present study was to explore the *in vitro* activity of carnosic acid alone and in combination with gentamicin against multidrug-resistant MRSA clinical isolates. Here, we report for the first time the *in vitro* synergy between gentamicin and carnosic acid in the killing of MRSA, including gentamicin-resistant MRSA clinical isolates.

Materials and methods

Materials and strain selection

Commercial carnosic acid (Fig. 1) was purchased from Sigma-Aldrich, (USA C0609, carnosic acid from *R. officinalis* ≥91%, powder, Lot #SLBK3241V); stock solutions of 1 mg/ml were prepared in absolute ethanol. Gentamicina Larjan, P.10717 was purchased from Laboratorio Veinfar I.C.S.A, Argentina and ciprofloxacin L6797-4 from Norgreen S.A., Argentina.

All other reagents were of analytical grade. *S. aureus* clinical isolates were obtained from pediatric patients with bacteremia reported as methicillin resistant by the Service of Microbiology at the Garrahan Pediatric Hospital (Argentina). The antibiotic resistance profiles of MRSA clinical isolates used in this study is shown in Table 1. The laboratory strain *S. aureus* ATCC 29213 was used as control.

Antibacterial activity

The microplate assay for assessing the bacterial growth in Mueller-Hinton broth (Difco MD, USA) was performed according to Ojeda-Sana et al. (2013). The antibacterial activity was expressed as percentage of bacterial growth inhibition: % growth inhibition = 100 $(A_{Control} - A_{Sample})/A_{Control.}$ Where $A_{Control}$ is the absorbance measured at 595 nm of bacteria cultured alone**.** The minimal inhibitory concentration (MIC) is defined as the lowest concentration of a compound that inhibits the measurable growth of an organism after overnight incubation according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009–2011). Minimum bactericidal concentrations (MBC) were determined by plating 100 µl from each clear well of the MIC experiment on Trypticase soy broth (TSB) (Britania Lab, Argentina) agar plates in triplicate and enumerating colonies. After incubation for 18 h, the MBC was defined as the lowest concentration that did not permit the growth of 99.9% bacteria (reduction of ≥ 3 $\log_{10}$ ufc/ml) on the agar surface. Each sample was assayed in triplicate and all experiments were performed three independent times.

Antibiotic synergy test

Different doses of the antimicrobial compounds were selected using the checkerboard method (Romano et al., 2009). Compounds were placed into 96-well tissue-culture plates (Greiner Bio-one, USA) to obtain mixtures covering a range of suboptimal concentrations of both compounds, therefore the concentrations chosen were lower than the MIC values of the compounds alone. For each combination (A + B) experimental data were transformed into fractional inhibitory concentrations (FIC) as: MIC of compound A in combination divided by the MIC when used alone. The FIC index (FICI) for the combination of A and B is the sum of their individual FIC values. The FIC values of the most effective combination were used to calculate the FIC index. The fractional bactericidal concentration index (FBCI) was calculated according to Anantharaman et al. (2010). FBCI = (MBC gentamicin combination/MBC gentamicin alone) + (MBC carnosic acid combination/MBC carnosic acid alone). The results were interpreted as a synergistic effect if FICI and FBCI ≤ 0.5 and as an additive effect if 0.5 ≤ FICI and FBCI ≤ 4.

Bacterial time-kill studies were performed using each bacterial strain diluted in MH broth to a working cell density of 10^5 cfu/ml. Compounds tested were added to 100 µl of the diluted culture, and this suspension was incubated at 37 °C. After 6 or 24 h samples were removed, diluted, and plated onto TSB agar plates. The plates were incubated at 37 °C for 20 h and colonies were counted.

MTS and Trypan blue assays

The MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI, USA) was carried out according to the manufacturer's instructions. The murine monocyte-macrophage cell line RAW 264.7 (kindly provided by Dr. Fernando Goldbaum, Fundación Instituto Leloir, Buenos Aires, Argentina) were cultured at 37 °C in 5% CO_2 with 95% air in Dulbecco's modified Eagle's medium containing 10% (v/v) heat inactivated fetal bovine serum (Bioser, Argentina), and 2 mM glutamine. Cells were incubated with 8% MTS and 0.8% phenazine methosulfate for 1 h at 37 °C and the absorbance of the medium was measured at 595 nm in a Beckman coulter. Percentage of metabolically viable (living) cells in the culture (cell viability) was determined as follows: results from nontreated cells were designated 100%, and results from treated cells were divided by results from untreated cells. Percentage of cell viability by the Trypan