Carnosic acid is an efflux pumps modulator by dissipation of the membrane potential in Enterococcus faecalis *and* Staphylococcus aureus

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ORIGINAL PAPER

Carnosic acid is an efflux pumps modulator by dissipation of the membrane potential in *Enterococcus faecalis* and *Staphylococcus aureus*

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Abstract Bacterial resistance to antibiotics has become a serious problem of public health. Along with the controlled permeability by the cell-wall, active efflux systems can provide resistance by extruding antibiotics. Carnosic acid is capable to potentiate the antimicrobial activity of several antibiotics. However, the underlying molecular mechanism governing this effect remains unclear. The present study aims to investigate the effect of carnosic acid on the transport of ethidium bromide, on the permeability or the membrane potential in *Enterococcus faecalis* and *Staphylococcus aureus*. By using fluorimetric assays it was demonstrated that in *E. faecalis*, carnosic acid is a modulator of the uptake and efflux of ethidium bromide which does not induce cell membrane permeabilization phenomena. Such effect was sensitive to the inhibition caused by both the proton-motive force carbonyl cyanide *m*-chlorophenylhydrazone and the calcium antagonist verapamil, but not to vanadate, an ATPase inhibitor. In this work it was demonstrated, for the first time, that the activity of carnosic acid on the uptake/efflux of ethidium bromide is correlated with its capacity to change the membrane potential gradient in *S. aureus* and *E. faecalis*. In conclusion, carnosic acid is a natural compound, structurally unrelated to known antibiotics, which can function as an efflux pump modulator by dissipation of the membrane potential. Therefore, carnosic acid would be a good candidate to be employed as a novel therapeutic agent to be used in combination therapies against drug-resistant enterococci and *S. aureus* infections.

Keywords Carnosic acid · Efflux pumps · Membrane potential · Antibiotic resistance · *Staphylococcus aureus* · *Enterococcus faecalis*

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Introduction

Antibiotic resistance of bacteria is a growing problem worldwide limiting the efficacy of classical antibiotics and narrowing down the current alternatives for the treatment of infectious diseases (Leclercq 2009). The efflux-mediated drug resistance in bacteria is a widespread mechanism which confers bacteria the capacity to expel antibiotics belonging to all the major structural classes. The latter phenomenon has been observed in a large proportion both of hospital and community-acquired infections, in particular, those caused by *Enterococcus faecalis* and *Staphylococcus aureus* (Poole 2005; Piddock 2006). The efflux process is mediated by membrane proteins that are capable of transporting a single class or several structurally different compounds, using either the proton-motive force or the ATP cleavage to provide the required energy (Piddock 2006). It is well established that the multidrug-resistance efflux pumps expressed by bacteria can confer clinically relevant resistance to antibiotics, besides it is now known that these efflux pumps have also a role in the colonization and the persistence of bacteria in the host (Piddock 2006).

Given the clinical significance of drug efflux pumps in the bacterial resistance, the pharmacological inhibition of active efflux appears to be a promising strategy for restoring the activity of existing antibiotics (Zechini and Versace 2009). Indeed, different classes of inhibitors have

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been patented so far (Van Bambeke et al. 2006; Li and Nikaido 2009). A first category of inhibitors are chemotypes of clinically-used antibiotics, with low intrinsic antibacterial effects (Van Bambeke et al. 2006). A second group comprises novel chemical entities, being the most popular one the plant alkaloid reserpine, the calcium antagonist verapamil, or proton pump inhibitors targeting the driving force of the pumps like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Other inhibitors of different natural sources have been reported, such as the diterpenes abietane (carnosic acid, isopimarane and geranylgeranyl diterpenes, ferruginol and 5-epipisiferol) (Van Bambeke et al. 2006).

Carnosic acid is a natural diterpene present in rosemary and sage leaves showing a broad spectrum of pharmacological action, a moderate antimicrobial action against both Gram-positive and Gram-negative bacteria and yeast (Moreno et al. 2006; Barni et al. 2009), anti-inflammatory and anticarcinogenic (Mengoni et al. 2011; Barni et al. 2012). This compound can potentiate the activity of eritromycin and gentamicin against a *S. aureus* eritromycin effluxing strain possessing efflux mechanism of resistance (Oluwatuyi et al. 2004) and in vancomycin-resistant enterococci (Horiuchi et al. 2007). While there is a broad consensus that this compound is capable of potentiating the antimicrobial activity of several antibiotics, the precise mechanism by which this diterpene exerts its effects, remains unclear. Carnosic acid did not affect aminoglycoside-modifying enzymes, which are also known to induce antibiotic resistance (Horiuchi et al. 2007). As intracellular concentration of a given compound is therefore a result of interplay between permeability and efflux, the aim of the present study was to investigate the effect of carnosic acid on cell membrane, assessing whether a membrane permeabilization phenomenon is required for the uptake and efflux of ethidium bromide (EtBr), and to test if carnosic acid can also modulate the membrane potential in *E. faecalis* and *S. aureus*. We report here for the first time that carnosic acid inhibits the drug efflux transport through changes in the membrane potential gradient in *E. faecalis* and *S. aureus*.

Materials and methods

Bacterial strains

Enterococcus faecalis ATCC 29212 and *S. aureus* ATCC 25923 were grown in Mueller–Hinton (MH) broth (Difco Laboratories) at 37 °C using the broth microdilution technique as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines.

EtBr uptake assay

The EtBr uptake was determined by a fluorimetric technique as described (Kaatz et al. 2000). Briefly, overnight cultures of *E. faecalis* ATCC 29212 were diluted in fresh MH medium to a final OD_{625} of 0.4 and 1 ml aliquots were pelleted by centrifugation and then resuspended in MH medium (2.5×10^9 CFU/ml) containing 5 μg/ml EtBr and 48, 96 and 192 μM of carnosic acid concentrations (one-fourth of the MIC, one-half of the MIC and the MIC value, respectively). 0.01 % ethanol was also assayed as control. Suspensions (200 μl) were then immediately dispensed into each well of a black microplate (Costar, Cambridge, Mass). Fluorescence was measured every 10 s during 3 min at excitation and emission wavelengths of 535/595 nm at room temperature. In other experiments, the diluted cultures were pre-incubated for 6 min in the presence of different inhibitors, at optimal concentrations according to previous reports (Jonas et al. 2001; Kaatz et al. 2000; Lee et al. 2003), as the proton motive force CCCP (100 μM), the ATPase inhibitor sodium-*o*-vanadate (10 mM) or the calcium blocker verapamil (1 mM). Carnosic acid was assayed at the same concentration indicated above and the increment in the fluorescence intensity was taken as a measure of EtBr uptake. Carnosic acid purchased from Alexis Co. USA was dissolved in 0.01 % ethanol and diluted to the desired final concentration in MH broth. Verapamil from EBEWE Pharma and all others chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

EtBr efflux assay

The fluorometric assay of EtBr efflux was carried out as previously described (Jonas et al. 2001) with slight modifications. Overnight cultures of *E. faecalis* were washed twice with HEPES buffer (pH 7.0) and bacteria were loaded with EtBr. After cells were loaded, active efflux was allowed to occur energizing the cells with culture media which still contained EtBr to avoid passive efflux by diffusion, and 40 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to dissipate the membrane potential. Cells were incubated for 30 min at room temperature, washed twice with ice cold HEPES buffer containing EtBr (5 μg/ml) and then resuspended to an OD_{625} of 0.4 (1×10^9 CFU/ml). After the final wash, aliquots of 0.5 ml (0.5×10^9 CFU/ml) were collected by centrifugation at 4 °C and kept on ice. To assess efflux, bacteria were resuspended in 200 μl MH plus EtBr (5 μg/ml), carnosic acid (48, 96 and 192 μM), ethanol (0.01 %), or efflux inhibitors (100 μM CCCP, 10 mM sodium-*o*-vanadate, 1 mM verapamil) and dispensed into a black microplate. Fluorescence was measured every 10 s during 14 min at

excitation and emission wavelengths of 535/595 nm at room temperature. Each assay was performed on three separate occasions, with duplicate determinations each time.

SYTOX Green assay

SYTOX Green dye is a nucleic acid probe that does not cross the cell membrane but when the cell plasma membrane barrier is altered this fluorophore enters the cells giving rise to intense green fluorescence upon binding to nucleic acids (Roth 1997). A bacterial culture in logarithmic phase growth of *E. faecalis* was harvested, incubated in fresh MH medium at 26 °C for 1 h in the presence of 48–384 μM of carnosic acid or 0.01 % ethanol as control, washed three times with ice cold sterile 0.9 % NaCl solution. Bacterial suspensions containing 1×10^8 CFU/ml were stained with 5 μM SYTOX Green (Molecular Probes) in a black microplate for 10 min in the dark. The fluorescence of the DNA-bound dye was monitored on a fluorescence multiwell plate reader (DTX 880 Multimode detector, Beckman Coulter) with excitation and emission wavelengths of 504/523 nm, respectively. Maximal permeabilization (100 %) was obtained by using 70 % isopropyl alcohol and the percentage of permeabilized bacteria in relation to that positive permeabilization control was calculated. At least, three independent experiments, each in triplicate were performed. The increment in the fluorescence was normalized to the baseline fluorescence at time 0 and expressed as arbitrary units.

Membrane potential measured by DiSC3(5)

The transmembrane electrical potential (inside negative) was determined by the quenching of the potential-sensitive fluorescent probe DiSC3(5) (3,3′-dipropylthiodicarbocyanine) obtained from Molecular Probes (Eugene, Oreg.) by a method previously reported (Wu and Hancock 1999; Brooijmans et al. 2007). This cationic dye accumulates onto hyperpolarized membranes and is translocated into the lipid bilayer, once it is inside the cells this dye undergoes autoquenching of its own fluorescence, thus resulting in a decrease of the fluorescence signal. Bacterial cells (1×10^9 CFU/ml) were suspended in HEPES buffer (pH 7.0) with 10 mM glucose. A *S. aureus* or *E. faecalis* suspension was incubated with 1.6 μM DiSC3(5) until a stable reduction in fluorescence. A 100 mM KCl solution was then added to equilibrate the cytoplasmic and external K^+ concentration. A blank reaction made up of only cells and the dye was used as control. Carnosic acid was added to the bacterial suspension to a final concentration of 24, 48 and 96 μM. CCCP (50 μM) or polymyxin B (0.4 μg/ml) were assayed in parallel. In another set of experiments, polymyxin B was added before carnosic acid treatment. Changes in fluorescence due to the disruption of the cytoplasmic membrane potential were continuously monitored with a spectrofluorometer Jasco FP-6500 employing an excitation wavelength of 606 nm and an emission wavelength of 670 nm at 37 °C. Each assay was performed on three separate occasions, with triplicate determinations each time.

Results

Effect of carnosic acid on the EtBr uptake and efflux in *E. faecalis*

A synergistic effect of carnosic acid at 16 μg/ml (48 μM) with aminoglycosides in *E. faecalis* ATCC 29212 as well as significant antimicrobial activity at 64 μg/ml (192 μM), has previously been reported (Horiuchi et al. 2007; Moreno et al. 2012). Therefore, these active concentrations were used to analyse the effect of this compound on both uptake and efflux of EtBr under standardized conditions in *E. faecalis* by fluorimetric techniques. The fluorometric determination of the EtBr is an ideal experimental model used to carry out a rapid screening of new drug efflux inhibitors and transport studies across the bacterial cell wall (Paixão et al. 2009).

Figure 1 shows that the presence of carnosic acid rapidly increased the EtBr uptake in a dose-dependent manner, indicating a positive effect on the intracellular accumulation of the drug. Indeed, an increment in the drug uptake of 1.75–2.75 fold was observed after the treatment with 48–192 μM of the diterpene within 1 min. Figure 2 shows experiments in which *E. faecalis* was loaded with EtBr and after, that the active efflux was allowed to occur under conditions that the dye cannot be effluxed from cells (culture medium containing EtBr to avoid passive efflux by diffusion, and 40 μM CCCP to dissipate the membrane potential). Figure 2a showed that a 50 % decrease in the initial intracellular fluorescence was observed in untreated cells, while in the presence of carnosic acid approximately 90 % fluorescence was observed inside cells within the first 10 min, at all concentrations tested. Carnosic acid exhibited a similar effect to that of CCCP at 100 μM and 1 mM verapamil, retaining 80–95 % the initial intracellular fluorescence within the first 10 min, while after the treatment with the ATPase inhibitor vanadate a retention of near 70 % fluorescence intensity inside cells was observed (Fig. 2b).

To determine the influence of different inhibitors of efflux pumps on the modulatory activity of carnosic acid, the general inhibitors of efflux pumps CCCP and verapamil, and the inhibitor of the ATP-Binding Cassette (ABC)

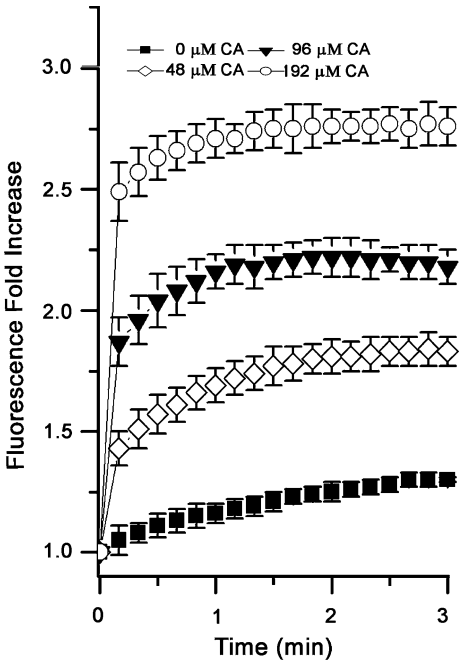

Fig. 1 Enhancement of the EtBr uptake induced by carnosic acid in *E. faecalis*. Bacteria were incubated in the absence (0 μM) or in the presence of the indicated concentrations of carnosic acid (CA). The fluorescence was measured every 10 s and the fluorescence fold increase was normalized to the baseline recorded at time 0. Data represent the mean and standard deviation of three independent performed in triplicates

transporters vanadate, were added to the medium (Fig. 3). Results showed that not only an increase in EtBr accumulation in control cells but also a marked dose-dependent positive effect on EtBr uptake was observed in the presence of the diterpene and vanadate (Fig. 3a). In contrast, accumulation rates of EtBr were affected in the presence of carnosic acid by the addition of verapamil as well as CCCP (Fig. 3b, c). These results suggest the existence of an active efflux activity of carnosic acid.

Effect of carnosic acid on membrane permeability in *E. faecalis*

It is well known that the intrinsic resistance of bacteria to most antimicrobial agents can be attributed to their relatively impermeable cell-wall, which provides a barrier to noxious compounds and limits drug uptake (Nikaido 2001). In order to determine whether carnosic acid may be enhancing drug uptake by disrupting the plasma membrane integrity we used the fluorescent dye SYTOX Green. Cells were treated with the phenolic diterpene for 1 h prior to the assessment of the plasma membrane integrity with the dye. Figure 4 shows that no significant uptake of the dye was observed after the addition of 24–96 μM carnosic acid, while at concentration as high as 384 μM, a slight membrane permeabilization effect (near 20 %) respect to the control cells, was detected. Therefore, carnosic acid was not considered to exert an important cell membrane permeabilization effect at the concentrations that have a synergistic effect with aminoglycosides.

Carnosic acid modulates the cell membrane potential in *E. faecalis* and *S. aureus*

Results suggested that carnosic is capable to induce a pharmacological inhibition of efflux pumps in *E. faecalis*. This effect can be attained through different mechanisms. One the mechanism involved the dissipation of the energy gradient that drives efflux pumps. To investigate this possibility, the fluorescence emitted by the probe DiSC3(5) which can be found either within the cells or the culture

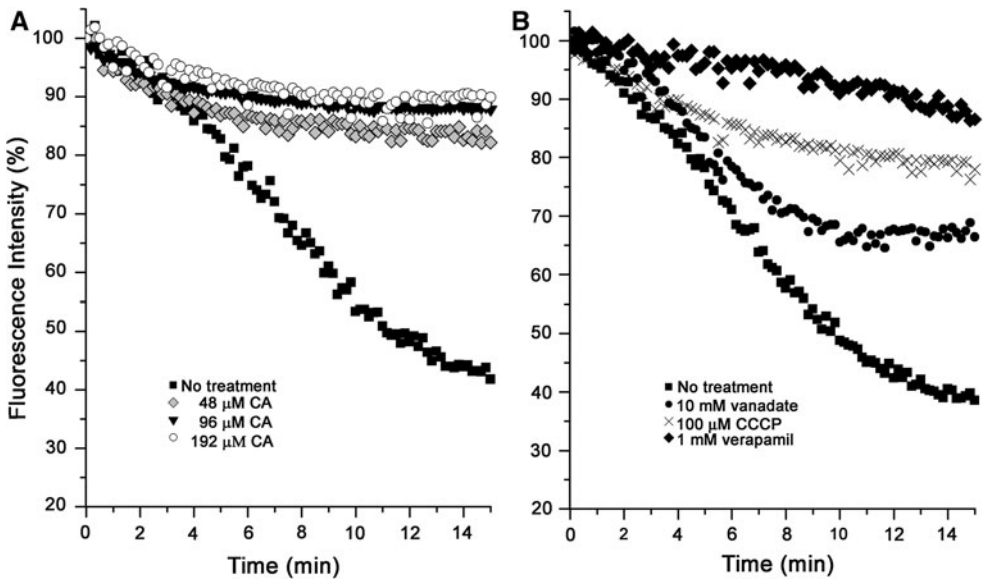

Fig. 2 Inhibition of the EtBr efflux by carnosic acid in *E. faecalis*. Active efflux was allowed to take place in the absence of any treatment or in the presence of indicated concentrations of carnosic acid (CA). Parallel assays were performed using vanadate, CCCP or verapamil (**b**). Results are representative of at least three independent experiments

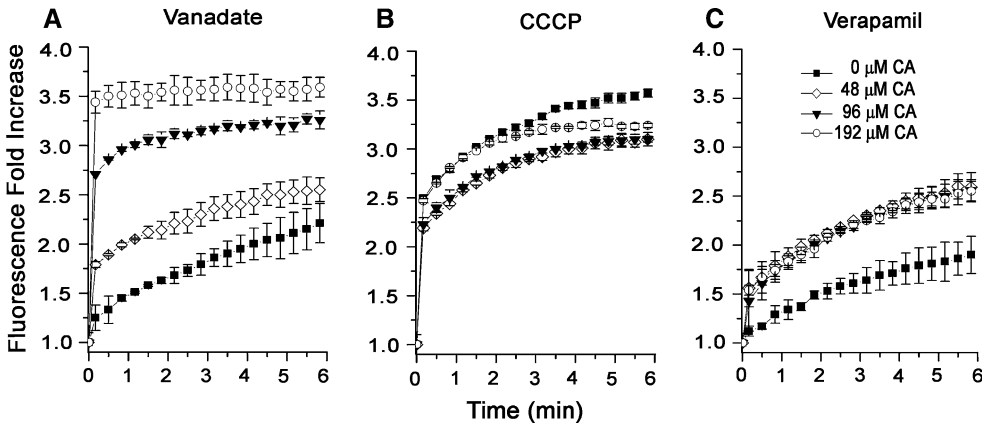

Fig. 3 Time-course uptake of EtBr in the presence of carnosic acid with several efflux pump inhibitors in *E. faecalis*. Bacteria were incubated in the presence of 10 mM vanadate (**a**), 100 µM CCCP (**b**) or 1 mM verapamil (**c**) with the indicated concentrations of carnosic acid (CA). The fluorescence was measured every 10 s and the fluorescence fold increase was normalized to the baseline recorded at time 0. Data are presented as the mean and standard deviation of three independent experiments performed in duplicate

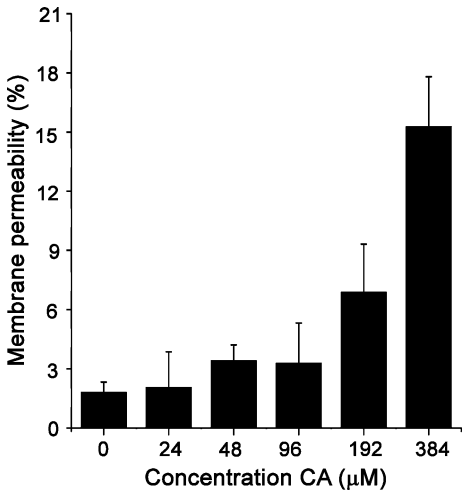

Fig. 4 SYTOX Green uptake assay. *E. faecalis* cells were treated with increasing concentrations of carnosic acid (CA) for 1 h, then SYTOX green was added and fluorescence was determined after 10 min. Values, mean of three independent experiments are given as a percentage of the maximal permeabilization (100 %) which was determined after treating bacteria with 70 % isopropyl alcohol

medium depending on the cytoplasmic membrane potential, was monitored over time in *E. faecalis* suspension. Figure 5a shows that the addition of 48 or 96 µM of carnosic acid led to an immediate decrease in the fluorescence intensity of the probe in *E. faecalis*. A similar effect was observed with 50 µM CCCP, while a rise in the fluorescence was obtained when the antimicrobial cationic peptide polymyxin B was employed in parallel assays. The same decrease in the fluorescence intensity after treatment with carnosic acid was observed following the hyperpolarizing effect of the polymyxin B (Fig. 5b).

Previously, the inhibition of multidrug efflux pump of carnosic acid in *S. aureus* was reported to a concentration of 24 µM, which is equivalent to approximately one-fourth its MIC value (Oluwatuyi et al. 2004; Moreno et al. 2012). Therefore, it is possible that carnosic acid also modulates the membrane potential in *S. aureus*. Figure 5c shows a more accentuated effect on the cell membrane potential in *S. aureus* with respect to *E. faecalis.* The fact that the potential is determined largely by the properties of the excitable membrane (types of voltage-gated channels, channel distributions and ionic concentrations) may explain the differences observed between these both Gram positive bacteria.

Taken together, these results would indicate that the mechanism by which carnosic acid inhibits the efflux pump is related to the alteration of the membrane potential, at least in *S. aureus* and *E. faecalis*.

Discussion

The present study demonstrated, for the first time, that carnosic acid can inhibit the EtBr efflux transport, at concentrations that do not affect significantly the membrane permeability in *E. faecalis.* It was also found that carnosic acid was able to change the membrane potential in *E. faecalis*, thereby suggesting that the modulation of the EtBr efflux and uptake after the exposure to carnosic acid is a consequence of a membrane-potential alteration.

Carnosic acid inhibited the EtBr efflux and uptake in *E. faecalis* with a similar potency to the general efflux pump inhibitor CCCP (Lee et al. 2003; Roth et al. 1997). Accordingly, in the presence of this inhibitor, the effect of carnosic acid on the accumulation rates of the fluorescent probe was abolished. Interestingly, the marked positive effect on the fluorophore uptake induced by carnosic acid remained after the treatment of bacteria with vanadate, a

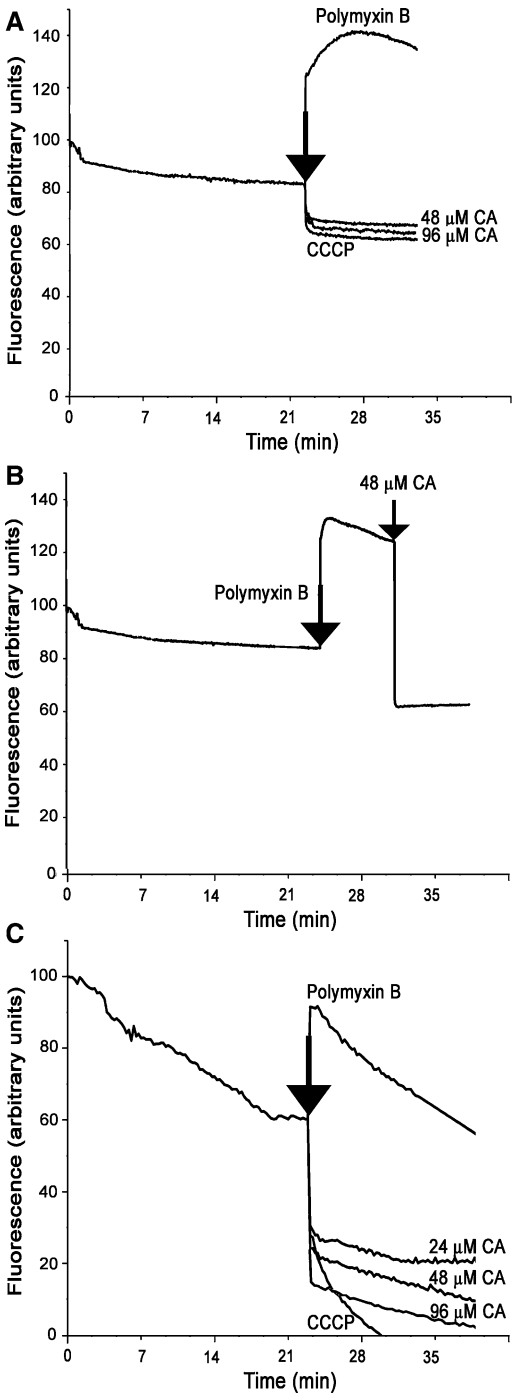

Fig. 5 Changes in the membrane potential in *E. faecalis* and *S. aureus* induced by carnosic acid. Changes in fluorescence of the DiSC3(5) dye were expressed as arbitrary units. The *arrow* indicates the point of compound addition. Different concentrations of carnosic acid (CA) and 50 µM CCCP or 0.4 µg/ml polymyxin B were assayed in parallel in *E. faecalis* (**a**) or *S. aureus* (**c**). Carnosic acid was added after polymyxin B treatment in *E. faecalis* (**b**). Experiments were repeated at least three times

well-known ATP-dependent inhibitor of efflux pumps. These data confirmed the existence of an active efflux inhibition of carnosic acid in *E. faecalis* requiring an active efflux activity of secondary transporters energized by transmembrane electrochemical gradients of either protons or sodium ions and the energy of the membrane potential.

Our data also reported that carnosic acid was able to change the membrane potential in *S. aureus.* This result combined with previous evidence of the inhibitory activity of carnosic on efflux pumps, suggested that this compound works similarly in *E. faecalis* and *S. aureus.*

Inhibition of the efflux-related multidrug resistance pumps can restore the activities of antimicrobial agents that are substrates for these proteins. This fact has become appreciated as a significant factor in the chemotherapy of infections caused by Gram-positive bacteria possessing efflux pumps that confers resistance to antimicrobial agents such as macrolides, tetracyclines, fluoroquinolones, and selected dyes and disinfectants (Van Bambeke et al. 2000). Most of the drug transport pumps in Gram-positive bacteria are members of the major facilitator superfamily, which employ the proton motive force as the energy source for drug translocation of a single class of drug (TetK of *S. aureus*) or multiple drugs (NorA of *S. aureus)*, capable of translocating hydrophilic fluoroquinolones and monocationic dyes and disinfectants (Paulsen et al. 1996). In this sense, carnosic acid can potentiate the activity of erytomycin, tetracycline and aminoglycosides as streptomycin and arbekacin in *E. faecalis* and vancomycin in *E. faecium* (Horiuchi et al. 2007). Carnosic acid also enhanced the activity of eritromycin, causing an eightfold reduction in MIC against the erythromycin-resistant strain RN4220 which expresses the Msr(A) efflux protein (Oluwatuyi et al. 2004). Recently, we reported synergistic interactions of carnosic acid with other aminoglycosides such as tobramycin and kanamycin, in *E. faecalis* at 48 µM (one-fourth its MIC) and with tetracycline, tobramycin, kanamycin, ciprofloxacin and gentamicin at one-fourth to one-eighth its MIC in *S. aureus* (Moreno et al. 2012).

As for the underlying molecular mechanism involved in the potentiation of the antimicrobial activity exerted by carnosic acid, our findings showed a strict correlation between the observed efflux drug inhibition activity of carnosic acid and its capability to modulate the membrane potential gradient in *S. aureus* and *E. faecalis*, since both activities were achieved at concentrations of approximately one-fourth its MIC value in *E. faecalis* and *S. aureus.* Therefore, it is likely that carnosic acid inhibited the efflux of proton motive force-dependent pumps by a mechanism involving a reduction in the transmembrane potential with the consequent dissipation of the membrane potential. A similar effect was observed using 50 µM CCCP, amphipathic molecule which gets dissolved in phospholipid bilayers affecting the energy level of the bacterial membrane, while an opposite effect was found testing polymyxin, amphipathic molecule that disturb the membrane barrier

(Brogden 2005). The mechanism(s) by which carnosic acid exerts its antimicrobial effect and/or potentiates the activity of such a vast array of other antimicrobial agents, may perhaps involve the rates of diffusion through ion channels, the rates of conformational changes that lead to their activation and inactivation, or the rates of the biochemical reactions with which ion channels are modulating and transporting substances into and out of membrane.

Carnosic acid is also known to block eukaryotic ion channels as demonstrated in a model of P-glycoprotein-mediated multidrug resistance displayed by a human leukemic cell line. Thus, this compound could be useful as a resistance-modulating agent to be employed for the treatment of some drug-resistant tumors (Xiao-Ning et al. 2008). According to the classification of efflux pump inhibitors described so far (Van Bambeke et al. 2006), carnosic acid would be a natural product having an inhibitory efflux pump activity structurally unrelated to known antibiotics.

Nowadays, clinical practice for the treatment of infections with antibiotic resistant enterococci and *S. aureus* are restricted to membrane-active agent as daptomycin and polymyxin E (colistin) which main drawback is their high toxicity for host cells (Van Bambeke et al. 2008; Mohr et al. 2009). Likewise, the concentrations required for inhibitors of bacterial efflux pumps as verapamil and reserpine are too high to be clinically relevant (Jonas et al. 2001; Lee et al. 2003). In this regard, experiments performed in our laboratory regarding the toxicological effects of carnosic acid in *Caenorhabditis elegans* demonstrated that this compound does not adversely affect the normal physiology of the nematodes when employed at concentrations of up to 768 μM (250 μg/ml) (Moreno et al. 2012). Moreover, pharmacokinetics studies in Sprague–Dawley rats demonstrated a high bioavailability of the diterpene after intragastric administration (90 mg/kg) (Yan et al. 2009).

Due to the lack of new antibacterial agents, there is considerable interest to improve the efficacy of currently employed antibiotics. A rational approach would be to inhibit the activity of efflux pumps. Among some strategies aimed at overcoming drug resistance due to efflux phenomena, the blocking of either the proteins themselves, using neutralizing antibodies, or the corresponding genes, by means of antisense oligonucleotides or small interfering RNA approaches are not yet applicable for therapeutics (Van Bambeke et al. 2006). Alternatively, pharmacological inhibition of active efflux is a more widely exploited strategy, which is meant to be applied as an adjuvant therapy to be used together with specific antibiotics (Van Bambeke et al. 2006).

In summary, carnosic acid (the main abietane diterpene usually present in *Rosmarinus officinalis* and *Salvia officinalis* phenolic extracts) has a high potential to be used as a resistance-modulating agent for the treatment of some drug-resistant infections. Carnosic acid may be used in combinational therapy with fluoroquinolone, aminoglycosides and tetracycline to treat infections caused by Gram-positive bacteria.

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