Med Chem Res (2018) 27:261–267
DOI 10.1007/s00044-017-2063-y

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Vitamin K enhances the effect of antibiotics inhibiting the efflux pumps of *Staphylococcus aureus* strains

Saulo R. Tintino[1] · Cícera D. M. Oliveira-Tintino[2] · Fábia F. Campina[1] · Paulo Weslley Limaverde[1] · Pedro S. Pereira[2] · José P. Siqueira-Junior[3] · Henrique D. M. Coutinho[1] · Lucindo J. Quintans-Júnior[4] · Teresinha G. da Silva[2] · Teresa C. Leal-Balbino[5] · Valdir Q. Balbino[6]

Received: 13 April 2017 / Accepted: 2 September 2017 / Published online: 14 September 2017

Abstract The strain *Staphylococcus aureus* is commonly cited as being a major hospital-acquired pathogen. The emergence of *S. aureus* strains resistant to a wide distribution of antibiotics. The various antibiotic resistance mechanisms include efflux pumps, are ubiquitous proteins localized in the cytoplasmic membrane of all kind of cells. During the last two decades, numerous structurally diverse compounds have been studied and shown to have efflux-inhibitory activity. These include currently available drugs employed for other indications, as well as natural and synthetic molecules. Menadione (vitamin K3), is a fat-soluble vitamin that has long been recognized for its essential role in coagulation and, more recently, has been proposed as a key nutrient in the regulation of soft tissue calcification. Therefore, the aim of this study is to evaluate the effect of menadione efflux pumps in multidrug resistant strains of *S.aureus*. Were used RN4220 harboring plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein; and IS-58, which possesses the TetK tetracycline efflux protein; 1199B resists hydrophilic fluoroquinolones via a NorA-mediated mechanism and wild strain 1199B. Antibacterial activity test by minimal inhibitory concentration (MIC). Evaluation of possible inhibition of efflux pumps by reduction of MIC of ethidium bromide (EtBr) and antibiotics due the possible inhibitory effect of these substances. Efforts have been directed at identification of EPIs from natural sources. Some of the detrimental effects on bacterial cells can be attributed to the detergent properties of menadione on account of their amphipathic structure. Was observed what in strain 4220 and IS58 it occurred reduction the MIC, indicating possible effect on efflux pump.

Keywords Liposoluble compounds · Efflux pumps · Menadione · *Staphylococcus aureus*

✉ Henrique D. M. Coutinho
hdmcoutinho@gmail.com

[1] Department of Biological Chemistry/CCBS/URCA, Laboratory of Microbiology and Molecular Biology (LMBM), Crato, Brazil

[2] Department of Antibiotics, Laboratory of Farmatoxicological Prospecting of Bioactive Products (BIOFARMATOX), Federal University of Pernambuco (UFPE), Recife, Brazil

[3] Department of Molecular Biology/CCEN/UFPB, Laboratory of Microrganism Genetics (LGM), João Pessoa, Brazil

[4] Physiology Department/UFS, São Cristóvão, SE 49100-000, Brazil

[5] Department of Microbiology, Aggeu Magalhães Research Center, CPqAM/Fiocruz, Recife, Brazil

[6] Department of Genetics | CCB | UFPE, Laboratory of Bioinformatics and Evolutionary Biology (LABBE), Recife, Brazil

Introduction

The strain *Staphylococcus aureus* (*S.aureus*) is commonly cited as being a major hospital-acquired pathogen (Perl 1999). Strains of this species that are resistant to b-lactams, notably methicillin-resistant *S. aureus* have been described from clinical sources for over 40 years (Dos Santos et al. 2007). The emergence of *S. aureus* strains resistant to a wide distribution of antibiotics, including aminoglycosides, macrolides, lincosamides, fluoroquinolones, chloramphenicol, sulfonamides, streptomycin, and tetracycline, has become a pandemic problem owing to limited therapeutic options available (Schito 2006). The ability of these

organisms to resist the action of antiseptics and disinfectants makes environmental eradication extremely challenging. The ability of this Gram-positive organism to acquire resistance to practically all useful antibiotics is cause for great concern.

In the past century, the discovery of antibiotics has been one of the most influential and impressive achievements in science. However, the widespread use of antibiotics created selective pressure for the emergence of strains that would persist despite antibiotic toxicity. Indeed, bacterial drug resistance is an increasing problem in the clinic (Fluman and Bibi 2009). The various antibiotic resistance mechanisms include alteration/modification of the target site, degradation of the antibiotic molecule and reduction of effective intracellular antibiotic concentration as a result of decreased permeability and energy-dependent (or active) efflux (Kumar and Schweizer 2005).

A decrease of the intracellular concentration of an antibiotic can also be due to its extrusion outside the cell via an energy dependant process called active efflux (Van Bambeke et al. 2003a, b), mediated by efflux pumps. Such pumps are ubiquitous proteins localized in the cytoplasmic membrane of all kind of cells, from bacteria to eukaryotes (Van Bambeke et al. 2003a, b; Webber and Piddock 2003). Bacterial efflux systems are responsible for the secretion of toxins or antibiotics produced by the cell itself, efflux of toxic compounds encountered in the bacterial environment such as antibiotics. These systems can confer resistance to a given drug or class of drugs: they are specific drug resistance transporters. But the main problem is caused by the so-called multi drug resistant (MDR) efflux pumps that can handle a wide variety of structurally unrelated compounds (Van Bambeke et al. 2003a, b). Most families of antibiotics are subject to resistance by efflux, with the exception of glycopeptides (Webber and Piddock 2003). MDR efflux pumps have also been suggested to be involved in *S. aureus* virulence (Van Bambeke et al. 2000; Kalia et al. 2012). In these instances, strains expressing an MDR efflux pump had improved fitness and survival in murine abscess models or enhanced invasiveness of macrophages.

In the prokaryotic kingdom there are five major families of efflux transporter: major facilitator, multidrug and toxic efflux, resistance-nodulation-division, small multidrug resistance, and ATP binding cassette. All these systems utilize the proton motive force as an energy source, (Ding et al. 2008) apart from the ABC family, which utilizes ATP hydrolysis to drive the export of substrates. Recent advances in DNA technology and the advent of the genomic era have led to the identification of numerous new members of the above families, and the ubiquitous nature of efflux pumps is remarkable. Analysis of various available bacterial genome sequences has shown that known and putative drug efflux transporters constitute from 6 to 18% of all transporters found in any given bacterial cell (Paulsen et al. 1996).

During the last two decades, numerous structurally diverse compounds have been studied and shown to have efflux-inhibitory activity, including phenylalanyl arginyl-β-naphthylamide, verapamil, phenothiazines (for example: thioridazine, chlorpromazine). These include currently available drugs employed for other indications, as well as natural and synthetic molecules (Stavri et al. 2007).

Menadione (vitamin K3), a naturally occurring metabolite of vitamin K, is also a synthetic form of vitamin K added to animal feed. Menadione (vitamin K3), is a fat-soluble vitamin that has long been recognized for its essential role in coagulation and, more recently, has been proposed as a key nutrient in the regulation of soft tissue calcification. Vitamin K is considered safe at the recommended adequate intake dosages discussed. There is no tolerable upper limit set because there are no known cases of toxicity with vitamin K. Vitamin K deficiency bleeding is a significant public health issue that is of concern in healthy appearing neonates (Truong and Booth 2011).

By this fact, the mandione can be a possible alternative to inhibit one of the most important bacterial mechanisms of resistance against antibiotics, the efflux pumps. So, the aim of this study was evaluate the inhibition of efflux pumps using microdilution assay by the association between menadione and antibiotics in MDR *S. aureus* strains and comparing this effect with the substrate ethidium bromide (EtBr).

Materials and methods

Bacterial strains

The strains of *S. aureus* were used: RN4220 harboring plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein; and IS-58, which possesses the TetK tetracycline efflux protein; 1199B resists hydrophilic fluoroquinolones via a NorA-mediated mechanism and wild strain 1199. The strains, kindly provided by Prof. S. Gibbons (University of London), were maintained on blood agar base (Laboratorios Difco Ltda., Brazil) slants and, prior to use, the cells were grown overnight at 37 °C in Heart Infusion Agar slants (HIA, Difco).

Drugs

The antibiotics were dissolved in dimethylsulfoxide (DMSO) and after sterile water (concentration of 1024 μg/mL). Erythromycin, Norfloxacin and tetracycline were used antibiotics. Menadione was obtained from Sigma Chemical Co., St. Louis, USA. Stock solutions were prepared in 2 mL

Table 1 Antibacterial activity test by MIC of vitamin menadione in strain with pump efflux expression in μg/mL

	Strain	Control DMSO	Menadione
Result of CIM	RN4220	128	64
Result of CIM	IS-58	128	64
Result of CIM	1199B	128	64
Result of CIM	1199	128	64

of DMSO/ Tween 80 at a concentration of 200 mg/ml, after which they were diluted to 1024 μg/mL in distilled water. Was performed the control with DMSO. Ethidium bromide was obtained from Sigma Aldrich Co. Ltd. and dissolved in sterile water (concentration of 1024 μg/mL).

Antibacterial activity test by minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of menadione, were determined in a microdilution assay utilizing 100 μL of each suspended bacterial inoculum in saline solution, corresponding to 0.5 of the McFarland scale, followed by addition of 900 μL of brain heart infusion (BHI) in eppendorfs. These were then transferred to 96-well microtiter plates and serial dilutions of each substance were performed with concentrations ranging from 0.5 to 512 μg/mL (1:1). The plates were incubated at 37 °C for 24 h, and bacterial growth was assessed by the use of resazurin. The MIC was defined as the lowest concentration in which no growth can be observed, according to (CLSI 2008). The antibacterial assays were performed in triplicates and results were expressed as an average of replicates.

Evaluation of efflux pump inhibition by MIC reduction

To observe whether menadione would alter the inhibition of efflux pumps, was performed a comparative study between the capacity to decrease the MIC of EtBr and antibiotics, thus assessing the capacity of both in decreasing the MIC.

Efflux pump inhibition was tested using a sub-inhibitory concentration of menadione (MIC/8). A 100 μL sample of a solution containing inoculum, was suspended in saline solution equivalent to 0.5 of the McFarland scale, and was added to BHI in eppendorfs. After wards, these were transferred to 96-well microtiter plates and 100 μL of antimicrobial drug and EtBr serial dilutions were performed (1:1). The plates were incubated at 37 °C for 24 h, and bacterial growth was assessed by the use of Resazurin. The MIC was defined with antibiotic and EtBr concentrations ranging between 0.5 to 512 μg/mL. Controls were performed using the MIC of antibiotics and EtBr alone.

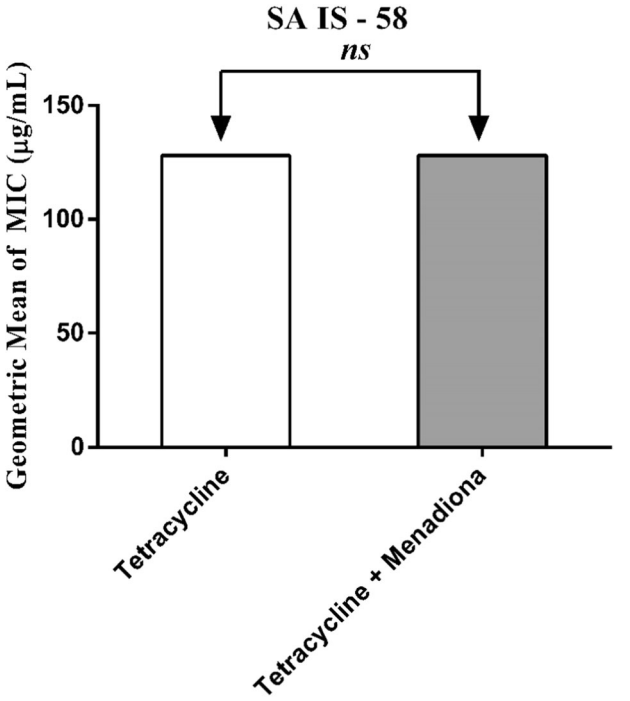

Fig. 1 MIC of Tetracycline alone and in association with the standard vitamins against the strain *S. aureus* IS-58, expressing the efflux system TetK. One Way ANOVA, followed by the test Tukey. *ns* not significant

Antibacterial assays were performed in triplicate and results were expressed as an average of replicates.

Results and discussion

The menadione demonstrated a MIC = 64 μg/mL against RN4220, IS-58 1199B and 1199 being considered a clinically relevant antibacterial activity (Table 1). When the antibiotics and EtBr, were assayed in association with the menadione in sub-inhibitory concentrations (MIC/8), all of them reduced the MIC (Figs. 1–8), indicating an inhibition of the mechanism of resistance to the antibiotic (the efflux pumps). Only when the menadione was associated with the antibiotic against the wild type strain, an antagonistic effect was detected by a reduction of the MIC.

Modifier of antibiotic activity, by reduction of MIC, is a term used for substances that modulate or even reverse bacterial resistance to certain antibiotics, where it can alter the microbial susceptibility to antibiotics by inhibition of efflux pumps (Costa et al. 2008). A MIC reduction ≥ 3 dillutions when combined with the inhibitor is indicative of the resistance mechanism of efflux pump (Davies and Wright 1997). The usage of EtBr as a substrate for the efflux pumps is well described in the literature and as demonstrated in this work (Patel et al. 2010). The MIC of

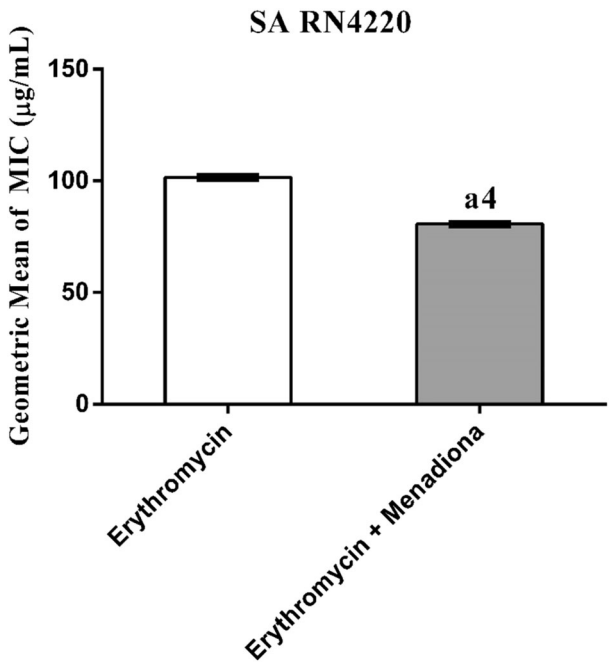

Fig. 2 MIC of Erytromicin alone and in association with the standard vitamins against the strain *S. aureus* RN4220, expressing the efflux system TetK. One Way ANOVA, followed by the test Tukey. a4: $p < 0.0001$ vs. Erytromycin

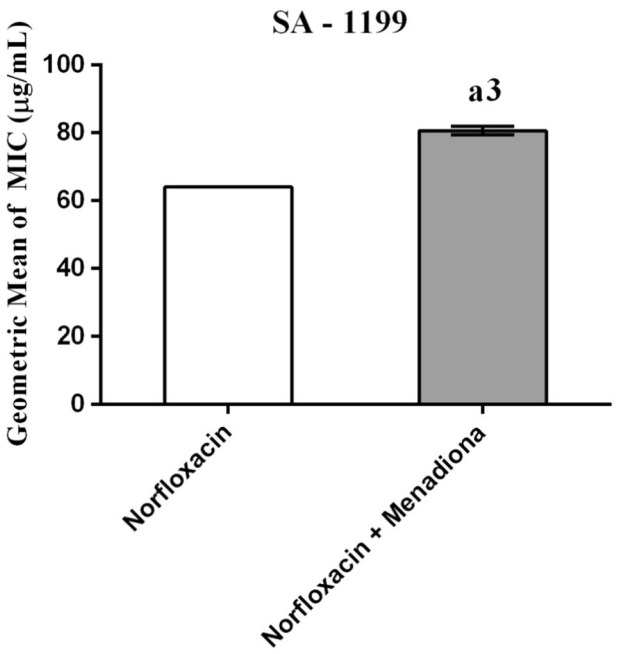

Fig. 4 MIC of Norfloxacin alone and in association with the standard vitamins against the strain *S. aureus* 1199 wild, expressing the efflux system TetK. One Way ANOVA, followed by the test Tukey. a3: $p < 0.001$ vs. Norfloxacin

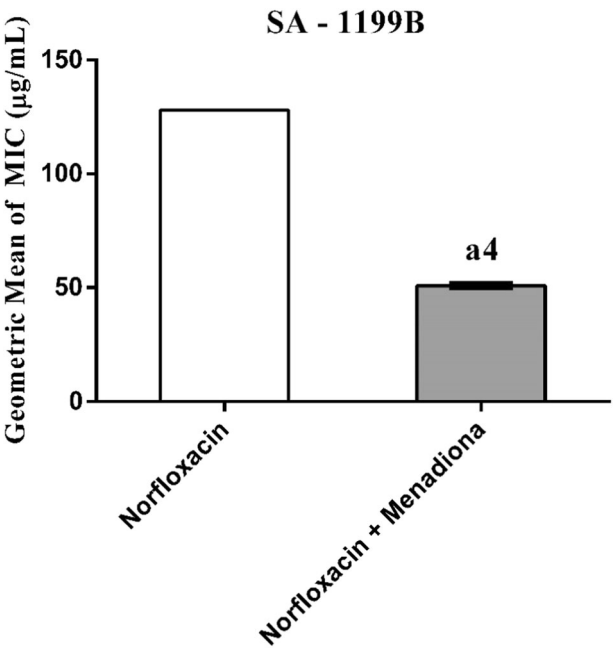

Fig. 3 MIC of Norfloxacin alone and in association with the standard vitamins against the strain *S. aureus* 1199B, expressing the efflux system TetK. One Way ANOVA, followed by the test Tukey. a4: $p < 0.0001$ vs. Norfloxacin

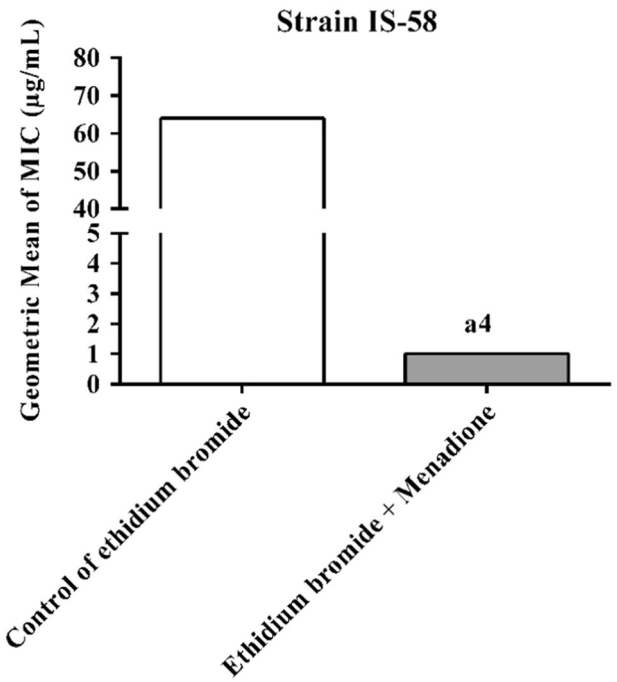

Fig. 5 Effect of menadione on the activity of ethidium bromide (EtBr) *against the strain of Staphylococcus aureus* IS-58. The values represent the geometric mean ± S.E.M. (*Standard Error* of the *Mean*) analyzed through the *T*-Test. a4: $p < 0.0001$ vs. Control of EtBr

ethidium bromide demonstrated similar action to antibiotics, indicating a similar inhibitory mechanism among them against efflux pumps in the assayed strains.

The use of lipossoluble vitamins in association with antibiotics is an interesting alternative to enhance the antibiotic activity of these drugs due their usage in the human

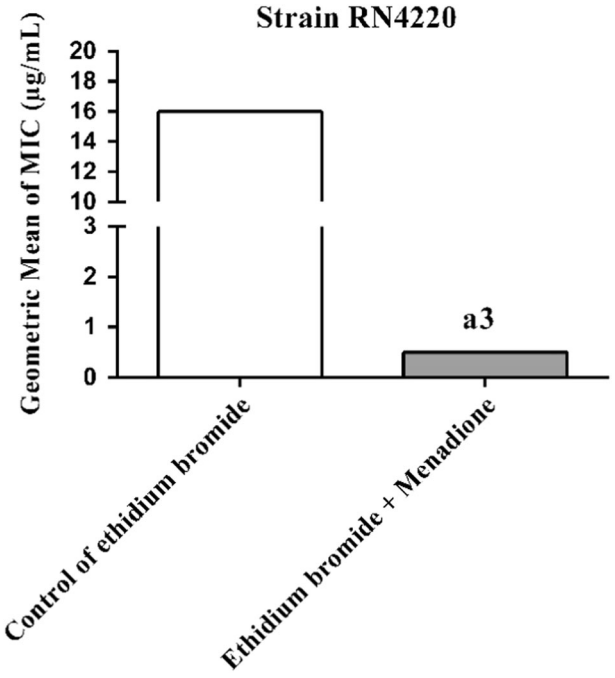

Fig. 6 Effect of menadione on the activity of EtBr against thestrain of *Staphylococcus aureus* RN4220. The values represent the geometric mean ± S.E.M. (standard error of the mean) analyzed through the *T*-Test. a3: $p < 0.001$ vs. Control of EtBr

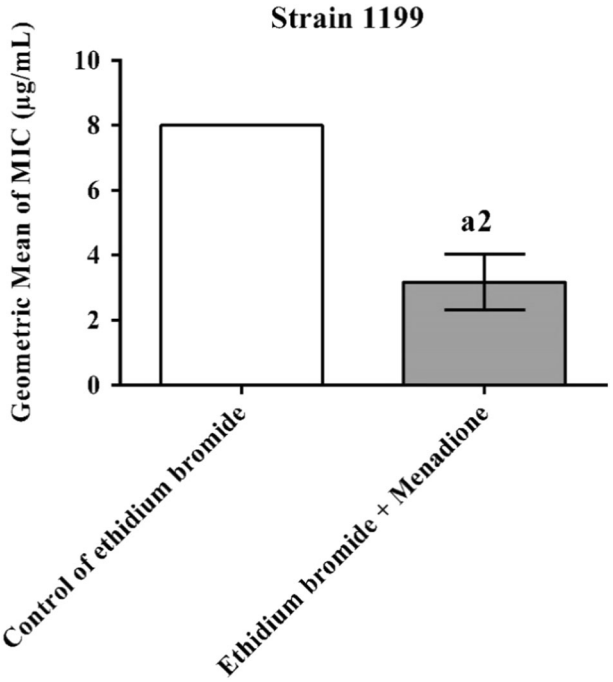

Fig. 8 Effect of menadione on the activity of EtBr against the strain of *Staphylococcus aureus* 1199 wild. The values represent the geometric mean ± S.E.M. (standard error of the mean) analyzed through the *T*-Test. a2: $p < 0.01$ vs. Control of EtBr

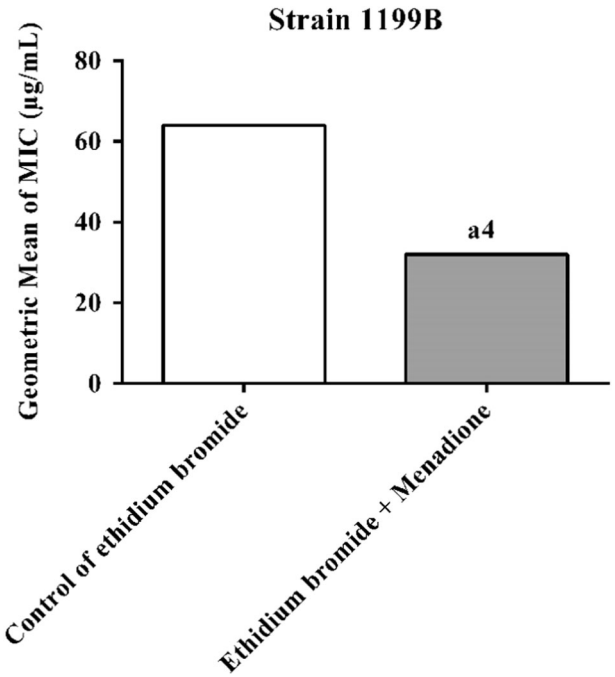

Fig. 7 Effect of menadione on the activity of EtBr against the strain of *Staphylococcus aureus* 1199B. The values represent the geometric mean ± S.E.M. (standard error of the mean) analyzed through the *T*-Test. a4: $p < 0.0001$ vs. Control of EtBr

feed with low toxicity when used in low concentration (DiPalma and Ritchie 1977). Liposoluble compounds are cited as modifiers of plasma membrane permeability in bacteria (Pretto 2004; Nicolson et al. 1999). Thus, menadione is compounds with your liposoluble nature can alter the fluidity of the bacterial membrane, making it more susceptible to penetration by various substances, particularly antibiotics (Andrade et al. 2014).

Beyond that, lipophilic substances and menadione cause disturbances in the bacterial membrane, resulting in damage of the fundamental elements needed for membrane integrity, such as reduced membrane potential and loss of ions, cytochrome C, proteins and radicals, followed by the collapse of the proton pump and ATP depletion (Sikkema et al. 1994; Turina et al. 2006; Hirayama et al. 2006). Lipophilicity is a common feature of several putative efflux pump inhibitors, and this quality, as pointed out by Gibbons (Gibbons 2004), is probably important for its solubility in the bacterial membrane and binding to the efflux proteins, or maybe binding to the pump substrates. This effect may be due the fact of the efflux pumps are transmembrane proteins, having its function associated with cell membrane structure and fluidity (Collnot et al. 2007).

This is the first report about the effect of menadione (vitamin K) against the bacterial efflux systems. In the study performed by Andrade et al. (2014), was observed that menadione enhances the antibiotic activity of drugs by cell membrane permeabilization mechanism. In a similar form, we have observed a MIC reduction when the antibiotic was

associated with the menadione. However, previous studies have demonstrated the effect of lipossoluble vitamins against the cell membrane and against the efflux systems. In this study was performed by Collnot et al (Collnot et al. 2007) which investigated TPGS interactions with P-gp in its membrane environment. Alterations in membrane fluidity by D-α-tocopheryl polyethylene glycol were observed via electron spin resonance spectroscopy. In the absence and presence of P-gp substrates and ATPase activity was measured using an ATPase assay. This study demonstrated that the interaction of this vitamin with the cell membrane affected the cell membrane fluidity, modified the cell membrane permeability and inhibited the efflux system ATP-dependent in cancer cell lines.

Besides the efflux systems studied on this work be bacterial systems, the effect can be very similar the present in eukaryotic and prokaryotic organisms. Other lipophylic substances have been reported to affect bacterial efflux systems by similar mechanisms. Lipophilic and Amphipathic compounds have been reported as putative efflux pump inhibitors against strain SA-1199B, such as a piperidine alkaloid (Pereda-Miranda et al. 2006), acylated oligosaccharides of the orizabin series (Gibbons 2005) of the murucoidin series and stoloniferin I (Chérigo et al. 2008) and the phenolic diterpene totarol (Smith et al. 2007).

In this study, performed by Silva et al (Silva et al. 2009), analyzed liposoluble compounds triterpenes and phenolic isolated from the aerial parts of *Herissantia tiubae* against RN4220 harboring plasmid pUL5054, which carries the gene encoding the MsrA macrolide efflux protein; and IS-58, which possesses the TetK tetracycline efflux protein.

Conclusion

Observed was with the menadione (vitamin k) enhanced the antibiotic activity by the inhibition of the efflux systems. This fact indicates that the menadione (vitamin k) mechanism of action is specific, according the strain and the menadione (vitamin k) molecular structure. In a general manner, the vitamin interacted with the bacterial cell membrane and affected the pump tertiary structure, as observed in studies with other non-polar compounds. However, new studies are necessary to elucidate what the factors that it favors this interaction and how could occur.

Author's contributions SRT, CDMOT, and FFC performed the MIC assays of antibacterial activity of menadione; PWL and PSP performed the efflux Pump Inhibition by MIC Reduction assay; TGS and LJQJ performed the statistical assays; JPSJ, HDMC, TCLB, and VQB supervised the experimental work, wrote and revised the manuscript

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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