Journal of Bioenergetics and Biomembranes (2021) 53:149–156
https://doi.org/10.1007/s10863-021-09885-5

Inhibition of *Staphylococcus aureus* TetK and MsrA efflux pumps by hydroxyamines derived from lapachol and norlachol

Fernando Gomes Figueredo[1,2] · Rodrigo Emmanuel L. T. Parente[2] · Maynara Rodrigues Cavalcante-Figueredo[2] · Jakson Gomes Figueiredo[2] · Raimundo Luiz Pereira da Silva[3] · Edinardo Fagner Ferreira Matias[2] · Tania Maria Sarmento Silva[4] · Celso Amorim Camara[4] · Cícera Datiane de Morais Oliveira-Tintino[3] · Saulo Relison Tintino[3] · Henrique Douglas Melo Coutinho[3] · Marta Maria de Franca Fonteles[1]

Received: 4 October 2020 / Accepted: 18 February 2021 / Published online: 26 February 2021

Abstract
The present study aimed to evaluate the *in vitro* efflux pump inhibitory capacity of hydroxyamines derived from lapachol and norlachol, where compounds 3, 4, and 5 were tested against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58, endowed with the PT181 plasmid. The substances were synthesized from 2-hydroxy-quinones, lapachol and nor-lapachol obtaining the corresponding 2-methoxylated derivatives via dimethyl sulfate alkylation in a basic medium, which then reacted chemoselectively with 2-ethanolamine and 3-propanolamine to form the corresponding amino alcohols. The antibacterial action of the substances was quantified by determining the Minimum Inhibitory Concentration (MIC), while a microdilution assay was carried out to ascertain efflux pump inhibition of *Staphylococcus aureus* strains carrying the MsrA macrolide and the TetK tetracycline efflux pumps with the substances at a sub-inhibitory concentration. The results were subjected to statistical analysis by an ANOVA test and Bonferroni post hoc test. The MIC from the substances exhibited a value ≥ 1024 μg/mL. However, a significant reduction ($p < 0.0001$) of the erythromycin, tetracycline and ethidium bromide MIC was demonstrated when these were in combination with the substances, with this effect being due to a supposed efflux pump inhibition. The tested substances demonstrated effectiveness at decreasing the MIC of erythromycin, tetracycline and ethidium bromide, potentially by inhibiting the MsrA macrolide and the TetK tetracycline efflux pumps present in the tested *S. aureus* strains.

Keywords Efflux pump inhibition · MsrA · TetK · Hydroxyamines · Lapachol · Norlachol

Introduction

Several microorganisms that make up the human bacterial flora exist and are potentially pathogenic, such as the gram-positive *Staphylococcus aureus*, which is carried by roughly 30 % of humans. *S. aureus* is extremely relevant due to its easy adaptation to different environments, its vast spectrum of infectious diseases (such as bacteremia, infectious endocarditis, septic arthritis and pneumonia), its vast incidence and its high lethality, with complication rates from *S. aureus* infections exceeding 25 % (Wertheim et al. 2005; Shurland et al. 2007; Monaco et al. 2017).

In this context, *S. aureus* bacterial resistance to antibiotics has been shown to be an extremely concerning issue, since bacteremia caused by methicillin-resistant *S. aureus* (MRSA) appears to have greater morbidity and mortality compared to bacteremia caused by methicillin sensitive *S. aureus* (MSSA) (Hal et al. 2012; Vance and Holland 2008).

S. aureus has presented multiple strains that are resistant to several antibiotics over several decades due to its great adaptive versatility. Strains resistant to penicillin were identified following approximately a decade of contact with *S. aureus*, presenting the penicillinase gene in their genetic material. Thus, the need to create new antibiotics, such as methicillin,

✉ Henrique Douglas Melo Coutinho
hdmcoutinho@gmail.com

[1] Postgraduate Program in Development and Technological Innovation in Medicines, Federal University of Ceará, Fortaleza CEP 60.430-370, CE, Brazil

[2] Department of Microbiology, Faculdade de Medicina Estácio de Juazeiro do Norte, Juazeiro do Norte- CE, Juazeiro do Norte CEP 63048-080, CE, Brazil

[3] Department of Biological Chemistry, Regional University of Cariri, Crato CEP 63105-000, CE, Brazil

[4] Department of Chemistry, Universidade Federal Rural de Pernambuco, Recife, PE, Brazil

emerged as an option for strains carrying the penicillinase gene. Thereafter, strains integrating the *mecA* gene in their genetic material, a gene that conferred *S. aureus* resistance to methicillin, emerged in 1961, and thus MRSA emerged (Pantosti et al. 2007; Monaco et al. 2017).

In this perspective, the *mecA* gene is essential for resistance not only to methicillin, but to all β-lactams (penicillins, cephalosporins - except ceftaroline - and carbapenems), since this gene encodes the production of Penicillin 2a binding protein (PBP2a), a protein which catalyzes the formation of peptidoglycan, a structural component present in the cellular wall (CW) of *S. aureus* and which has a lower binding affinity to β-lactams. PBP2a catalyzes CW production even when in contact with the antibiotics for which MRSA are resistant to (Pantosti et al. 2007; Taylor and Unaka 2019; Lowy 2019).

In addition, resistance to tetracycline may also occur by two main mechanisms: active transport by efflux pumps (mediated by the protein products from TetK TetL genes) and by protection via ribosomes (through TetM and TetO genes (Pantosti et al. 2007). Resistance to tetracycline is evidenced, for example, in *S. aureus* IS-58 strains that have the TetK efflux pump, tasked with actively extracting tetracycline from the intracellular to the extracellular medium, providing protection to the bacteria (Pantosti et al. 2007; Truong-Bolduc et al. 2005; Limaverde et al. 2017).

It is at this juncture that several therapeutic options for MRSA bacteremia are used, with these ranging from the combination of antibiotics, antibiotics with greater response to a specific species and modification of existing antibiotics, in order to increase treatment effectiveness. While an excellence approach is not yet known, alternatives that demonstrate effectiveness and success exist (Lowy 2019).

The use of vancomycin or daptomycin are good alternatives to monotherapy, where vancomycin is the most commonly used antimicrobial for MRSA bacteremias, since it has an abundance of evidence of success, while daptomycin is a good alternative to vancomycin and has good success rates (Lowy 2019; Murray et al. 2013).

In terms of combined therapy, good results include, for example, the: association between daptomycin and ceftaroline or other β-lactams, association between vancomycin and ceftaroline or other β-lactams, association between daptomycin and trimethoprim-sulfamethoxazole, and association between ceftaroline and trimethoprim-sulfamethoxazole (Lowy 2019; Kullar et al. 2016). Indeed, the mechanisms that provide *S. aureus* with resistance are abundant, diverse and complex, thus urging the need for new therapeutic alternatives, including organically synthesized compounds.

The combination of two or more compounds is generally superior to the use of a single compound, especially for the treatment of serious infectious diseases, caused by bacterial resistance to antibiotics (Hanan et al. 2012). Synergism or additive effect can be obtained by combining antibiotics with extracts or substances at a sub-inhibitory concentration, applied directly to the culture medium, affecting several targets (Wagner and Ulrich-Merzenich 2009; Coutinho et al. 2010; Farias et al. 2015). Between the various targets group, we can cite the efflux pump inhibitors (Van et al. 2006; Piddock 2006).

Thus, plant derived substances and their derivatives have become a viable and efficient alternative (Oliveira et al. 2007, Silva et al. 2007), since the antimicrobial activity of a drug can be amplified or reduced by the action of natural or organically synthesized products (Coutinho et al. 2008; Figueredo et al. 2020a), that hinder antimicrobial resistance mechanisms due to the complexity of their structures, thus avoiding microbial adaptations (Daferera et al. 2003).

In this context, naphthoquinones are important intermediates in the organic synthesis of numerous natural or synthetic compounds (Cavalcanti et al. 2013). Literature reports indicate several biological activities for these compounds, such as antitumor, antifungal, antibiotic, antibacterial, (Powis 1989; Silva et al. 2003) and molluscicide (Barbosa, et al. 2005).

Lapachol is a functionalized naphthoquinone of natural origin, easily obtained by extracting the heartwood from the Bignoniaceae family (Silva et al. 2003; Ferreira et al. 2010). Lapachol has several proven biological activities, such as: an action against esophageal cancer cells (Suthanan et al. 2013), as well as antimicrobial and trypanocide activity (Ferreira et al. 2010). Norlapachol, on the other hand, is a naphthoquinone of synthetic origin, with activity against *Trypanosoma cruzi* (Junior 2007), which can be obtained from a condensation reaction from lausone and isobutyraldehyde, catalyzed by beta-alanine in an acidic medium.

The present study aimed to evaluate the *in vitro* efflux pump inhibitory capacity of the hydroxyamines derived from lapachol and norlachol, 2-(2-hydroxyethylamino)-3-(3-methyl-2-butenyl)-1,4-dihydro-1,4-naphthalenedione, 2-(2-hydroxyethylamino)-3-(2-methyl-propenyl)-[1,4]naphthoquinone and 2-(3-hydroxypropylamino)-3-(3-methyl-2-butenyl)-[1,4]naphthoquinone against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58 endowed with the PT181 plasmid.

Materials and methods

Bacterial material

The *S. aureus* strains used were: RN4220 carrier of the pUL5054 plasmid, which carries the gene coding for the MsrA macrolide efflux protein; IS-58 with the PT181 plasmid, carrying the TetK tetracycline efflux protein gene. The strains were kindly provided by Prof. S. Gibbons (University of London). Prior to the assays, the cells were cultivated for 24 hours at 37°C in heart infusion agar (HIA, Difco Laboratories Ltda.).

Synthesis of substances

General information

Air- and moisture-sensitive reactions were carried out under an argon atmosphere. Reagents were purchased from Sigma-Aldrich, Dinamica or Vetec and distilled or used without further purification. Reactions were monitored by TLC analysis on precoated silica gel plates (Merck, Kieselgel 60 GF_{254}) and the compounds were visualized using UV light. Column chromatography was performed on a silica gel 60 (70–230 mesh, Merck). Melting points were measured in open capillary tubes in a QUIMIS apparatus and are uncorrected. The infrared spectra were recorded on an IFS66 Bruker spectrophotometer using KBr discs or Varian Mercury 640IR with ATR. HRMS analyses were performed on a MALDI-TOF/TOF Autoflex III 10, using positive reflector mode. NMR (^{1}H at 400 MHz and ^{13} C at 100 MHz) spectra were recorded on a Varian Unity Plus-400 spectrometer, 200 MHz Varian Mercury, using $CDCl_3$ or DMSO-d_6 as solvents, and calibrated for the solvent signal. Chemical shifts are expressed in parts per million (ppm) and coupling constants are given in Hz. The compounds lapachol 1 (Camara et al. 2002) and its corresponding 2-methoxy derivative, norlapachol 2 (Barbosa et al. 2005) and its corresponding 2-methoxy derivative, were obtained from previously published procedures (Fig. 1).

Synthesis of 2-aminoalquil derivatives 3–5

1 mmol of the 2-methoxy derivative dissolved in 10 mL of MeOH was slowly added to 1.5 mmol of the appropriate amine (2-aminoethanol or 3-amino-1-propanol), in the same solvent (40 ml), with continuous stirring. After the reaction was completed, as per CCD analysis inspection, the solvent was removed under vacuum and the residue submitted to flash chromatography on a silica gel and ethyl acetate/hexane with increasing polarity.

2-(2-Hydroxyethylamino)-3-(3-methyl-2-butenyl) -1,4-dihydro-1,4-naphthalenedione (3)

This compound was obtained as red crystals with an 88 % yield, mp 80–81°C; ^{1}H NMR (200 MHz, $CDCl_3$) 1.68 (s, 3H), 1.74 (s, 3H), 2.3 (br, 1H), 3.37 (d, 2H, *J* 5.9 Hz), 3.71 (m, 2H), 3.85 (m, 2H), 5.07 (t, 1H, *J* 5.9 Hz), 6.01 (l, 1H), 7.57 (m, 1H), 7.57 (m, 1H), 7.93 (d, 1H, *J* 7.6 Hz), 8.05 (d, 1H, *J* 7.6 Hz); ^{13} C NMR (50 MHz, $CDCl_3$), 18.2, 23.8, 25.8, 47.2, 62.1, 122.7, 1-26.1, 126.3, 130.6, 132.0, 132.8, 133.4, 134.5, 146.2, 183.1, 183.2; IR (KBr) (ν max., cm^{-1}) 3391, 3321, 1678, 1599, 1555, 1513; MS (rel int) m/z 285 (M+, 57), 270 (100), 198 (70). HRMS found: 285.13649. Calcd for $C_{17}H_{19}NO_3$: 285.13649.

2-(2-Hydroxy-ethylamino)-3-(2-methyl-propenyl)-[1,4] naphthoquinone (4) 40

This compound was obtained as red crystals with an 87 % yield, (mp 77–78.5°C) in 80 % yield. ^{1}H NMR ($CDCl_3$, 200 MHz) 1.47 (d, 3H, *J* 1.0 Hz), 1.89 (d, 3H, *J* 1.6 Hz), 2.46 (br s, 1H), 3.37 (q, 2H, *J* 5.4 Hz), 3.73 (t, 2H, *J* 5.4 Hz), 6.06 (dd, 1H, *J* 1.0/1.6 Hz), 6.25 (br t, 1H, *J* 5.4 Hz), 7.51 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.61 (dt, 1H, *J* 1.4/7.6/7.6 Hz), 7.90 (dd, 1H, *J* 1.4/7.6 Hz), 7.99 (dd, 1H, *J* 1.4/7.6 Hz). ^{13} C NMR ($CDCl_3$, 50 MHz) 20.1, 25.4, 46.1, 61.3, 113.6, 117.7, 125.9, 126.1, 130.3, 131.9, 133.3, 134.4, 139.0, 144.8, 182.7, 183.4. IR (KBr) ν max, 3457, 3349, 3268, 2940, 2874, 1675, 1598, 1563, 1511, 1354, 1335 cm^{-1}. HRMS found: 271.1169. Calcd for $C_{16}H_{17}NO_2$: 271.1208.

2-(3-Hydroxy-propylamino)-3-(3-methyl-2-butenyl)-[1,4] naphthoquinone (5) 41

This compound was obtained as red crystals with a 75 % yield, (m.p. 69–70 °C). ^{1}H NMR ($CDCl_3$, 400 MHz) 1.68 (s, 3H), 1.74 (s, 3H). 1.88 (q, 2H, *J* 6.2, 6.16 Hz), 3.39 (d, 2H), 3.69 (t, 2H, *J* 6,6 Hz), 3.80 (t, 2H, *J* 6.2, 5.5 Hz), 5.08 (t, 2H, *J* 5.8), 7.54 (t, 1H, *J* 7.5 Hz), 7.65 (td, 1H, *J* 7.4 Hz), 7.94 (d, 1H), 8.04 (d, 1H); ^{13} C NMR ($CDCl_3$,100 MHz) 17.8,

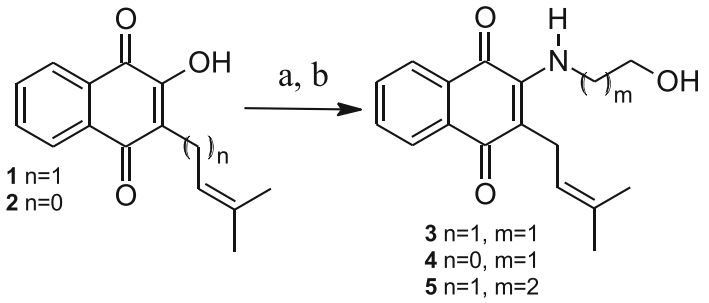

Fig. 1 Synthesis reaction. Figueredo et al. (2020a)

Reagents and conditions: a) Me_2SO_4, K_2CO_3, acetone, r.t.; b) 2-amino-ethanol or 3-amino-1-propanol in MeOH, r.t.

23.3, 23.4, 32.8, 42.4, 60.3, 115.3, 122.7, 125.6, 125.9, 130.1, 131.5, 132.1, 133.1, 134.0, 145.6, 182.74, 182.76; IR (KBr) ν max, 3446, 3334, 1672, 1598, 1557, 1527, 1361, 1276, 1473, 728 cm^{-1}. HRMS found: 299.1501. Calcd for $C_{18}H_{21}NO_3$: 299.1527.

Drugs used

The antibiotics used were specific to the pumps in each bacterium: Erythromycin for the MrsA pump contained in the RN4220 strain; Tetracycline for the TetK pump contained in the IS-58 strain. All antibiotics and compounds were initially dissolved in 10 mg/mL DMSO and subsequently diluted in water, reducing the concentration to 1024 μg/mL. Ethidium bromide was diluted in water to a concentration of 1024 μg/mL. The antibiotics and ethidium bromide were obtained from SIGMA Chemical Co. St. Louis, USA.

Antibacterial activity test - Minimum inhibitory concentration (MIC)

The MIC of the substances was determined with an assay using 100 μL of the bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, followed by the addition of 900 μL of brain heart infusion (BHI) in eppendorfs. The solutions were then transferred to 96-well microdilution plates and a serial dilution of each substance was performed with concentrations ranging from 0.5 to 512 μg/mL. The plates were incubated at 37°C for 24 hours and bacterial growth was evaluated through the use of Resazurin (CLSI 2013). The MIC was defined as the lowest concentration in which growth was not observed, in accordance with CLSI (2013). The antibacterial assays were performed in triplicates and the results were expressed as the mean of the repetitions.

Evaluation of Efflux pump inhibition by MIC reduction

Efflux pump inhibition was tested using subinhibitory concentrations of 128 μg/mL (1/8 MIC) of substances 3, 4 and 5, with the aim of evaluating the capacity of each substance to decrease the MIC of ethidium bromide (EtBr) and of antibiotics, the substrates for the efflux pumps coded for by the genes, present in the pUL5054 and PT181 plasmids from the *S. aureus* strains. 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 1350μL of brain heart infusion (BHI) as a control. For the substance evaluation assays, 150 μL of bacterial inoculum suspended in saline solution, corresponding to 0.5 of the McFarland scale, were added to eppendorfs together with 188 μL (1/8 MIC) of the substances, complemented with 1162 μL of brain heart infusion (BHI). The eppendorf solutions were transferred to 96-well microdilution plates and serial dilutions were performed with 100 μL of the antibacterial drugs (Limaverde et al. 2017).

The plates were incubated at 37°C for 24 hours and bacterial growth was evaluated by using Resazurin. The MIC was defined with the erythromycin, tetracycline and ethidium bromide (EtBr) concentrations, which varied between 0.5 and 512 μg/mL, and were compared to the chlorpromazine and PAβN standards.

Statistical analyses of microbiological results

The results from the assays were performed in triplicates and expressed as geometric means. A one-way ANOVA followed by Bonferroni's post hoc test was used as the statistical analysis test, using the GraphPad Prism 5.0 software.

Results and discussion

The antibacterial action of compounds 3, 4 and 5 was evaluated against the *S. aureus* strains: RN4220 carrying the pUL5054 plasmid; and IS-58 with the PT181 plasmid. The compounds obtained clinically irrelevant results (Houghton et al. 2007), with MIC values ≥ 1024 μg/mL (Table 1), this being the first report of these compounds against these bacterial strains.

These results are in accordance with antibacterial activity tests against standard and multi-drug resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, which did not show an antibacterial activity for the studied compounds (Figueredo et al. 2020a). According to Figueredo et al. (2020b), when assessing the antibacterial potential of these substances against *S. aureus* strains with and without the NorA efflux pump resistance mechanism, clinically irrelevant results were obtained for both strains.

Figueredo et al. (2020a) performed a pharmacological screening showing the possible antibacterial activity of the molecule 2-(2-hydroxyethylamino)-3-(2-methyl-

Table 1 Minimum inhibitory concentration (MIC) values for the antibiotics, ethidium bromide and substances 3, 4 and 5 in isolation, against the *S. aureus* RN4220 and IS-58 multidrug-resistant strains

Compound and Antibiotics	MIC for SA IS-58	MIC for SA RN4420
Ethidium bromide	32 μg/mL	32 μg/mL
Tetracycline	203 μg/mL	-
Erythromycin	-	≥1024 μg/mL
Substance 3	≥1024 μg/mL	≥1024 μg/mL
Substance 4	≥1024 μg/mL	≥1024 μg/mL
Substance 5	≥1024 μg/mL	≥1024 μg/mL

propenyl)-[1,4]naphthoquinone, with replicative DNA helicase and RecA protein being identified as the possible protein targets. In addition to screening, the properties of the three molecules were analyzed, demonstrating that the substances meet some of the molecular requirements for structures with pharmacological activities, such as low molecular weight and a small number of rotating bonds (Figueredo et al. 2020a). A subsequent study performed a structurally based virtual analysis (docking) showing a possible efflux pump inhibitory activity by hydroxyamines derived from lapachol and norlachol, against *Staphylococcus aureus* strains carrying the NorA efflux pump mechanism (Figueredo et al. 2020b).

Table 2 demonstrate a possible efflux pump inhibitory capacity for substances 3, 4 and 5 against the *S. aureus* IS-58 and RN4220 strains, when in association with tetracycline and erythromycin at sub-inhibitory concentrations (1/8 MIC), an increase being observed in the antibiotic activity against the S. aureus strains reducing the MICs of the antibiotics. Relevant results when compared to the standard inhibitor PaβN were also obtained. However, interference of the erythromycin activity when in association with substance 5 was not observed.

The modulatory activity of the substances in this study were superior to that of the chlorpromazine control. Chlorpromazine has been cited as a potentiator of several antibiotics, such as oxacillin, vancomycin and tetracycline against *S. aureus*, with this antibacterial synergism possibly occurring through the inhibition of efflux pumps by chlorpromazine, given the decrease in MIC of several antibiotics (Kaatz et al. 2003; Couto et al. 2008; Barreto et al. 2014). The exact chlorpromazine mechanism of action for inhibiting efflux pumps is not fully understood, however, it has been previously shown that chlorpromazine impairs the flow of K^+ through the *S. aureus* membrane. Additionally, chlorpromazine causes cell wall structural changes and alterations in bacterial cell divisions, with such alterations being capable of potentiating the action of antibacterial agents (Kaatz et al. 2003 and Kristiansen et al. 1992). Another suggested mechanism is based on the inhibition of the NorA transporter in which chlorpromazine induces cellular damage in systems that provide energy for efflux pumps by inhibiting H^+ dependent transporters, which supply energy, causing a collapse in the energy matrix of this transporter (Kaatz et al. 2003 and Lima et al. 2019).

Phe-Arg-β-naphthylamide (PAβN) is another substance used as a control for efflux pump inhibition in bacteria. PaβN has previously had its efflux pump inhibitory activity described in Gram-negative bacteria with resistance nodulation cell division (RND) type carriers - for example AcrB and MexB -, previously shown in agents such as *Escherichia coli* and *Pseudomonas aeruginosa* (Lomovskaya et al. 2001; Schuster et al. 2019). PAβN's action in repairing the sensitivity of certain bacterial strains that are multiresistant to β-lactam antibiotics such as cefepime and ceftazidime has also been previously demonstrated (Laudy et al. 2015). While PaβN's mechanism of decreasing bacterial resistance to drugs is not fully understood, a double action sensitizing these microorganisms to antibacterial agents has been suggested for PAβN: the permeabilization of the external bacterial membrane, which can lead to the extravasation of enzymes that degrade antibiotics (β-lactamases, for example); and an inhibitory activity over efflux pumps, in which predecessor studies have reported a competitive inhibition of RND by PAβN (Lamers et al. 2013; Laudy et al. 2015; Schuster et al. 2019).

These results are in agreement with Figueredo et al. (2020b) who associated substances 3, 4 and 5 with norfloxacin and ethidium bromide showing a significant reduction in the MIC of the antimicrobials, with this effect being attributed to a NorA efflux pump inhibition by the tested compounds. In this same study, a docking tracing a correlation between the interaction of the compounds with the efflux pump was performed and showed a high affinity. A recent study demonstrated antibiotic activity modulation by hydroxyamines derived from lapachol and norlachol against Gram-positive and -negative bacteria, when these were associated with aminoglycosides at subinhibitory concentrations (Figueredo et al. 2020a).

Table 3 shows the potentiation of the ethidium bromide (EtBr) action by substances 3, 4 and 5 at subinhibitory concentrations (1/8 MIC), where a significant synergistic effect against *Staphylococcus aureus* bacteria expressing efflux

Table 2 Efflux pump inhibitory activity of substances 3, 4 and 5 when associated with erythromycin or tha tetracycline, in comparison to the chlorpromazine and PaβN standards, against the *S. aureus* IS-58 and RN442 strains

	MIC of Strain RN4420 μg/mL		MIC of Strain SA IS58 μg/mL
ERY (Alone)	1024	TET (Alone)	203
Chlorpromazine + ERY	1024	Chlorpromazine + TET	203
PAβN + ERY	16	PAβN + TET	128
Substance 3 + ERY	512	Substance 3 + TET	128
Substance 4 + ERY	512	Substance 4 + TET	128
Substance 5 + ERY	1024	Substance 4 + TET	161

ERY: Erythromycin TET: Tetracycline; PAβN: Phenylalanine-arginine β-naphthylamide

Table 3 Efflux pump inhibitory activity of substances 3, 4 and 5 when associated with ethidium bromide, in comparison to the chlorpromazine and PaβN standards, against the S. aureus IS-58 and RN442 strains

	MIC of Strain RN4420		MIC of Strain SA IS58	
	μg/mL	reduction	μg/mL	reduction
Et Br (Alone)	32	-	32	-
Chlorpromazine+Et Br	32	-	32	-
PAβN+Et Br	8	4x	16	2x
Substance 3+Et Br	8	4x	2	16x
Substance 4+Et Br	16	2x	16	2x
Substance 5+Et Br	20.15	1,59	32	0 x

Et Br: Ethidium bromide; PAβN: Phenylalanine-arginine β-naphthylamide

pumps was observed, decreasing the ethidium bromide MIC by up to 16x. According to DeMarco et al. (2007), a 3x reduction in a MIC value is indicative of efflux pump inhibition. Thus, compound 5 had no influence on EtBr activity against the IS-58 strain.

Ethidium bromide is a DNA intercalant which severely damages bacterial DNA (Olmsted and Kearns 1977; Couto et al. 2008). Some multi-resistant strains have efflux pumps that are very effective at expelling ethidium bromide (EtBr) (Costa et al. 2013). In addition, when exposed to EtBr, *S. aureus* (ATCC 25,923) has been shown to present a greater resistance to many compounds, quinolones, tetraphenylphosphonium and dequalinium via efflux pumps. With this in mind, efflux pump inhibitors such as chlorpromazine, have shown a reduction in these bacteria's resistance capacity against EtBr (Costa et al. 2013). Efflux pump inhibitors have also been effective at reducing the resistance to EtBr in multidrug-resistant strains, with the association of efflux pump inhibitors and EtBr being a viable option (Couto et al. 2008; Viveiros et al. 2008; Costa et al. 2013). It should also be noted that EtBr can be used to identify *S. aureus* strains with efflux capacity by determining the EtBr MIC by a simple microdilution, in which strains with a MIC ≥ 25 mg/L present an efflux phenotype (Patel et al. 2010).

Conclusion

The tested substances did not demonstrate a satisfactory antibacterial effect in terms of their MICs. However, the tested substances were effective at decreasing the MIC of erythromycin, tetracycline and ethidium bromide, by potentially inhibiting the MrsA macrolide efflux protein and the TetK tetracycline efflux pump, present in the *S. aureus* strains.

Declarations

Conflict of interest None.

References

Barbosa TP, Camara CA, Silva TMS, Martins RM, Pinto AC, Vargas MD (2005) New 1,2,3,4-tetrahydro-1-aza-anthraquinones and 2-aminoalkyl compounds from nor-lapachol with molluscicidal activity. Bioorg Med Chem 13(2005):6464–6469. https://doi.org/10.1016/j.bmc.2005.06.068

Barreto HM, Fontinele FC, Oliveira AP, Arcanjo DDR, Santos BHC, Abreu APL et al (2014) Phytochemical prospection and modulation of antibiotic activity in vitro by Lippia origanoides H.B.K. In: Methicillin Resistant Staphylococcus aureus. BioMed Res Int 2014:1–7

Camara CA, Pinto AC, Vargas MD, Zukerman-Schpector J (2002) Azepines from the intramolecular prins cyclization of an aminoderivative of lapachol. Tetrahedron 58(30):6135–6140. https://doi.org/10.1016/S0040-4020(02)00581-1

Cavalcanti BC, Cabral IO, Rodrigues FAR, Barros FWA, Rocha DD, Magalhães HIF, Moura DJ, Saffi J, Henriques JAP, Carvalho TSC, Moraes MO, Pessoa C, Melo IMM, Júnior ENS (2013) Potent antileukemic action of naphthoquinoidal compounds: evidence for an intrinsic death mechanism based on oxidative stress and inhibition of DNA repair. J Braz Chem Soc 24(1):145–163. https://doi.org/10.1590/S0103-50532013000100019

CLSI (2013) Performance standards for antimicrobial susceptibility testing; Twentythird informational supplement. CLSI document M100eS23.Publishing PhysicsWeb. https://clsi.org/search/?q=Performance+standards+for+antimicrobial+susceptibility+testing%3B+Twentythird+informational+supplement.+CLSI+document+M100eS23. Accessed 12 May 2019

Costa SS, Viveiros M, Amaral L, Couto I (2013) Multidrug efflux pumps in staphylococcus aureus: an update. Open Microbiol J 7:59–71

Coutinho HDM, Costa JG, Lima EO, Falcão-Silva VS, Siqueira-Júnior JP (2008) Enhancement of the antibiotic activity against a multiresistant Escherichia coli by Mentha arvensis L. and chlorpromazine. Chemotherapy 54:328–330

Coutinho HDM, Costa JGM, Lima EO, Siqueira-Júnior JP (2010) Additive effects of *Hyptis martiusii* Benth with aminoglycosides against *Escherichia coli*. Indian J Med Res 131:106–108

Couto I, Costa SS, Viveiros M, Martins M, Amaral L (2008) Efflux-mediated response of Staphylococcus aureus exposed to ethidium bromide. J Antimicrob Chemother 62(3):504–513

Daferera DJ, Ziogas BN, Polissiou MG (2003) The effectiveness of plant essential oils on the growth of Botrytis cinerea, Fusariumsp. and Clavibactermichiganensissub sp. michiganensis. Crop Prot 22:39–44

DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TAA, Kaatz GW (2007) Efflux-related resistance to norfloxacin, dyes and biocides in bloodstream isolates of Staphylococcus aureus. Antimicrob Agents Chemother 51:3235-3239. https://doi.org/10.1128/AAC.00430-07

Farias PAM, Figueredo FG, Lucas AMB, Moura RB, Coutinho HDM, Silva TMS, Martin ALAR, Fonteles MMF (2015) Antiobiotic-modifying activity of riachin, a non-cyanogenic cyanoglycoside extracted from Bauinia pentandra. Drug Des Devel Ther 9:3067–3072

Ferreira SB, Gonzaga DTG, Santos WC, Araújo KGL, Ferreira VF (2010) β-Lapachona: Sua importância em química medicinal e modificações estruturais. Rev Virtual Quím 2(2):140–160

Figueredo FG, Ramos ITL, Paz JA, Silva, Tania MS, Camara CA, Oliveira-Tintino CDM, Tintino SR, Farias PAM, Coutinho HDM, Fonteles MMF (2020a) In silico evaluation of the antibacterial and modulatory activity of lapachol and nor-lapachol DERIVATES. Microb Pathog 144:104181

Figueredo FG, Ramos IT, Paz JA, Farias PAM, ; SILVA TMS, Camara CA, Tintino SR, Menezes IA, Coutinho HDM, Fonteles MMF (2020) Effect of hydroxyamines derived from lapachol and norlachol against Staphylococcus aureus strains carrying the NorA efflux pump. Infect Genet Evol 84:104370. https://doi.org/10.1016/j.meegid.2020.104370

Hal SJV, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB (2012) Predictors of mortality in Staphylococcus aureus bacteremia. Clin Microbiol Rev 25(2):362–386

Abd El-Kalek HH, Mohamed EA (2012) Synergistic effect of certain medicinal plants and amoxicillin against some clinical isolates of methicillin – resistant Staphylococcus Aureus (MRSA). Int J Pharmaceut Appl 3:387–398

Houghton PJ, Howes MJ, Lee CC, Steventon G (2007) Uses and abuses of in vitro tests in ethnopharmacology: visualizing an elephant. J Ethnopharmacol 110(3):391–400

Júnior ENS (2007) Síntese de novos derivados de lapachonas e nor-laopachonas: Veredas à atividade farmacológica. Dissertation, Universidade Federal do Rio de Janeiro

Kaatz GW, Moudgal VV, Seo SM, Kristiansen JE (2003) Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in Staphylococcus aureus. Antimicrob Agents Chemother 47(2):719–726

Kristiansen J, Mortensen I, Nissen B (1992) Membrane stabilizers inhibit potassium efflux from Staphylococcus aureus strain no. U2275. Biochem Biophys Acta 685:82–379

Kullar R, Sakoulas G, Deresinski S, Hal SJV (2016) When sepsis persists: a review of MRSA bacteraemia salvage therapy. J Antimicrob Chemother 71(3):86–576

Lamers RP, Cavallari JF, Burrows LL (2013) The efflux inhibitor Phenylalanine-Arginine Beta-Naphthylamide (PAβN) permeabilizes the outer membrane of gram-negative bacteria. PLoS One 8(3):1–7

Laudy AE, Osińska P, Namysłowska A, Zając O, Tyski S (2015) Modification of the susceptibility of gram-negative rods producing ESβLS to β-lactams by the efflux phenomenon. PLoS One 10(3):1–14

Lima WG, Ramos-Alves MC, Soares AC (2019) Dos distúrbios psiquiátricos à antibioticoterapia: reposicionamento da clorpromazina como agente antibacteriano. Rev Colomb Cienc Quím Farm 48(1):5–28

Limaverde PW, Campina FF, Cunha FABD, Crispim FD, Figueredo FG, Lima LF, Oliveira-Tintino CD, Matos YMLS, Morais-Braga MFB, Menezes IRA, Balbino VQ, Coutinho HDM, Siqueira-Júnior JP, Almeida JRGS, Tintino SR (2017) Inhibition of the TetK efflux-pump by the essential oil of Chenopodium ambrosioides L. and α-terpinene against Staphylococcus aureus IS-58. Food Chem Toxicol 17:1–18

Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M et al (2001) Identification and characterization of inhibitors of multidrug resistance efflux pumps in pseudomonas aeruginosa: novel agents for combination therapy. Antimicrob Agents Chemother 45(1):105–116

Lowy FD (2019) Methicillin-resistant Staphylococcus aureus (MRSA): Microbiology. IOP Publishing PhysicsWeb. https://www.uptodate.com/contents/methicillin-resistant-staphylococcus-aureus-mrsa-microbiology/print?search=staphylococcus%20aureus&topicRef=3157&source=see_link. Accessed 16 Sep 2019

Monaco M, Araujo FPD, Cruciani M, Coccia EM, Pantosti A (2017) Worldwide epidemiology and antibiotic resistance of Staphylococcus aureus. Curr Top Microbiol Immunol 409:21–56

Murray KP, Zhao J, Davis SL, Kullar R, Kaye K, Lephart P et al (2013) Early use of daptomycin versus vancomycin for methicillin-resistant Staphylococcus aureus bacteremia with vancomycin minimum inhibitory concentration > 1 mg/L: a matched cohort study. Clin Infect Dis 56(11):9–1562

Oliveira FQ, Gobira B, Guimarães C, Batista J, Barreto M, Souza, M2007a. Espécies vegetais indicadas na odontologia. Rev Bras Farmacognosia 17:466–476

Olmsted J III, Kearns DR (1977) Mechanism of ethidium bromide fluorescence enhancement on binding to nucleic acids. Biochemistry 16(16):3647–3654

Pantosti A, Sanchini A, Monaco M (2007) Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol 2(3):323–334

Patel D, Kosmidis C, Seo SM, Kaatz GW (2010) Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in Staphylococcus aureus. Antimicrob Agents Chemother 54(12):73–5070

Piddock LJ (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 19:382–402

Powis G (1989) Free radical formation by antitumor quinones. Free Radic Biol Med 6:63–105. https://doi.org/10.1016/0891-5849(89)90162-7

Schuster S, Bohnert JA, Vavra M, Rossen JW, Kern WV (2019) Proof of an outer membrane target of the efflux inhibitor Phe-Arg-β-Naphthylamide from random mutagenesis. Molecules 24(3):1–12

Shurland S, Zhan M, Bradham DD, Roghmann MC (2007) Comparison of mortality risk associated with bacteremia due to methicillin-resistant and methicillin-susceptible Staphylococcus aureus. Infect Control Hosp Epidemiol 28(3):9–273

Silva MN, Ferreira VF, Souza MCBV (2003) Um panorama atual da química e da farmacologia de naftoquinonas, com ênfase na β-lapachona e derivados. Quím Nova 26:407–416

Silva JG, Souza IA, Higino JS, Siqueira-Junior JP, Pereira JV, Pereira MSV (2007) Atividade antimicrobiana do extrato de AnacardiumoccidentaleLinn. Em amostras multiresistentes de Staphylococcus aureus. Rev Bras Farmacognosia 7:572–577

Suthanan SN, Veale CGL, Gounden NS, Osoniyi O, Hendricks DT, Caira MR, La Mare JA, Edkins AL, Pinto AV, Júnior E N. DA S., Coleman MTD (2013) Cytotoxicity of lapachol, β-lapachone and related synthetic 1,4- naphthoquinones against oesophageal cancer cells. Eur J Med Chem 62:98–100

Taylor TA, Unakal CG. Staphylococcus aureus. StatPearls [Internet]: IOP Publishing PhysicsWeb. https://www.ncbi.nlm.nih.gov/books/NBK441868/. Accessed 16 Sep 2019

Truong-Bolduc Q, Dunman PM, Strahilevitz J, Projan SJ, Hooper DC (2005) MgrA é um regulador múltiplo de duas novas bombas de efluxo em Staphylococcus aureus. J Bacteriol 187(7):2395–2405

Van BF, Pages JM, Lee VJ (2006) Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. Recent Pat Antiinfect Drug Discov 1:157–175

Vance GFJ, Holland TL (2008) Clinical approach to Staphylococcus aureus bacteremia in adults. IOP Publishing PhysicsWeb. https://www.uptodate.com/contents/clinical-approach-to-staphylococcus-aureus-bacteremia-in-adults. Accessed 14 Sep 2019

Viveiros M, Martins A, Paixão L, Rodrigues L, Martins M, Couto I et al (2008) Demonstration of intrinsic efflux activity of escherichia coli K-12 AG100 by an automated ethidium bromide method. Int J Antimicrob Agents 31(5):62–458

Wagner H, Ulrich-Merzenich G (2009) Synergy research: approaching a new generation of phytopharmaceuticals. Phytomedicine 16:97–110

Wertheim HFL, Melles DC, Vos MC, Leeuwen WV, Belkum AV, Verbrugh HA, Nouwen JL (2005) The role of nasal carriage in Staphylococcus aureusinfections. Lancet Infect Dis 5(12):62–751