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ORIGINAL ARTICLE

Inhibition of the NorA efflux pump of *Staphylococcus aureus* by synthetic riparins

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Abstract

Aim: The goal of this study was to increase knowledge about the antimicrobial activity of some synthetic Riparin-derived compounds, alone or in combination with fluoroquinolone antibiotics, against a strain of *Staphylococcus aureus* resistant to fluoroquinolone by way of overexpression of the NorA efflux pump.

Methods and Results: Microdilution tests showed that Riparins A and B did not show any significant antibacterial activity against *Staph. aureus* strains. On the other hand, the intrinsic antibacterial activity increased with increasing lipophilicity of the compounds, in the following order: Riparin-D (MIC 256 μg ml^{-1}; Log P 2·95) < Riparin-C (MIC 102 μg ml^{-1}; Log P 3·22) < Riparin-E (MIC 16 μg ml^{-1}; Log P 3·57). The addition of all riparins to growth media at subinhibitory concentrations caused an increase in the antibacterial activity of antibiotics against the NorA-overexpressing test strain. Riparin-B, which has two methoxyl groups at the phenethyl moiety, showed the best modulatory effect.

Conclusions: Riparin-E is a good anti-staphylococci agent, while Riparin-B functions as a NorA efflux pump inhibitor.

Significance and Impact of the Study: Our data suggest the possibility of using Riparin-B in combination with norfloxacin or ciprofloxacin for therapy of infections caused by multi-drug resistant *Staph. aureus.*

Introduction

Riparins are natural alkamides found in fruits and calyx of *Aniba riparia*, a species of the family Lauraceae (Barbosa-Filho *et al.* 1987; Takaku *et al.* 2007). Several biological activities of these compounds have been reported, in particular in the central nervous system, such as anxiolytic and antidepressive-like activities (Sousa *et al.* 2004, 2005, 2007; Melo *et al.* 2006). Besides their influence on the central nervous system, anti-inflammatory (Carvalho *et al.* 2013) and antinociceptive activities were described for Riparins (Araújo *et al.* 2009). Riparins also exhibit antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* multidrug-resistant strains (Catão *et al.* 2005), as well as the capacity of impairing drug resistance against penicillin (Catão *et al.* 2010).

The extraction and isolation process from source plants has a very low yield. As such, production of synthetic Riparins was adopted. Since the synthetic route became the main way of obtaining molecules from this group of compounds, several synthetic analogues were produced including Riparins A, B, C, D and E (Barbosa-Filho *et al.* 1990).

Some activities of these analogues were already screened, such as cytotoxic and *in vitro* antioxidant activities (Nunes *et al.* 2014). Synthetic salicylamides which are structurally similar compounds to the riparins showed bioactivity against *Plasmodium falciparum* (Fritzson *et al.* 2011).

Methicillin-resistant *Staph. aureus* (MRSA) is a pathogen associated with several infectious diseases acquired in hospitals and communities worldwide (Razera *et al.* 2009; Shittu *et al.* 2009; Sisirak *et al.* 2010). Examples include cutaneous and soft tissue infections, bacteraemia, septic arthritis, osteomyelitis and necrotizing pneumonia (Seymour 2009; Otto 2010). Therapy of these infections may be complicated as many MRSA strains carry multiple antibiotic resistance genes (Otto 2012), including those encoding transmembrane proteins known as efflux pumps, which can extrude antibiotics from the bacterial cell (Kumar and Varela 2012; Andersen *et al.* 2015). Fluoroquinolones have been used in the treatment of MRSA (Bhopale 2014), but resistance to this antibiotic class has become common and widespread (Gade and Qazi 2013; Lastours *et al.* 2014).

The high prevalence of infectious diseases caused by multi-drug resistant bacteria has resulted in a search for new antibiotics (Céliz *et al.* 2011; Duda-Chodak 2012; Kumar and Chopra 2013) or efforts aimed at improving the efficacy of current antibiotics (Laxminarayan 2014). Resistance to fluoroquinolones is frequently mediated by efflux pumps able to pump out these antibiotics from the bacterial cell (Costa *et al.* 2013). Combination therapies with an antibiotic associated with an efflux pump inhibitor (EPI) could be an innovative strategy to reverse bacterial resistance mediated by efflux pumps (Schindler *et al.* 2013; Bharate *et al.* 2015; Chovanová *et al.* 2015).

Previous studies have provided evidence that secondary metabolites derived from plants, including flavonoids (Wang *et al.* 2014), flavones (Chan *et al.* 2011), coumarins (Roy *et al.* 2013; Joshi *et al.* 2014), acylphloroglucinols (Shiu *et al.* 2013), curcuminoids (Joshi *et al.* 2014) and alkaloids (Markham *et al.* 1999) could be potential inhibitors of NorA, a multi-drug transporter of *Staph. aureus*. In this study, Riparin synthetic analogues were tested for their antibacterial activity alone or in combination with fluoroquinolone antibiotics against *Staph. aureus* aiming to evaluate their potential as NorA inhibitors.

Material and methods

Chemicals

Norfloxacin and ciprofloxacin were obtained from Sigma Chemical Co. (St. Louis, MO). Antibiotics were dissolved in sterile water. Stock solutions of *N*-phenethylbenzamide (Riparin-A), *N*-[2-(3,4-dimethoxy-phenyl)ethyl]-benzamide (Riparin-B), 2-hydroxy-*N*-phenethylbenzamide (Riparin-C), 2-hydroxy-*N*-[2-(3,4-dimethoxy-phenyl)ethyl]-benzamide (Riparin-D) and 2,6-dihydroxy-*N*-phenethylbenzamide (Riparin-E) were prepared in dimethyl sulfoxide (DMSO - MERCK) and then diluted with sterile water.

Bacterial strains and growth conditions

All tests were performed with *Staph. aureus* SA-1199B, which over-expresses the *norA* gene encoding NorA. NorA can efflux hydrophilic fluoroquinolones and other drugs such as DNA-intercalating dyes (Kaatz and Seo 1993). The strain SA-1199 which express *norA* at a basal level was used as negative control for modulation and ethidium bromide (EtBr) transport assays (Kaatz and Seo 1995). *Staphylococcus aureus* ATCC 25923 was used as positive control only in the assays for evaluation of intrinsic antibacterial activity. Strains were maintained on Nutrient Agar (Himedia, India, Mumbai) slants at 4°C. Before use in assays cells were grown overnight at 37°C in Brain Heart Infusion (BHI; Himedia).

Evaluation of intrinsic antibacterial activity

Stock solutions of Riparin-A, B, C, D and E (Rip-A through –E respectively), (10 000 μg ml^{-1}) were prepared in DMSO followed by dilution in sterile water to a final concentration of 4096 μg ml^{-1}. Minimal inhibitory concentrations (MICs) were determined using a microdilution assay (CLSI, 2013), employing 10% BHI broth (Barreto *et al.* 2014) and bacterial suspensions of ca. 10^5 CFU ml^{-1} and Riparin solutions ranging from 2048 to 8 μg ml^{-1}. Microtitre plates were incubated at 37°C for 24 h, then 20 μl of resazurin (0·01% w/v in sterile distilled water) was added to each well to detect bacterial growth (Hussain *et al.* 2011) by the change in the colour from blue to pink. MICs were defined as the lowest concentration at which no bacterial growth was observed.

Modulation of the antibiotic activity assay

For evaluation of Riparin-derived compounds as modulators of fluoroquinolone resistance, MICs of norfloxacin and ciprofloxacin were determined in the presence or absence of subinhibitory concentrations (1/8 MIC) of each compound (Coutinho *et al.* 2010). Antibiotics or EtBr concentrations ranged from 128 to 0·125 μg ml^{-1}. Microtitre plates were incubated at 37°C for 24 h and readings were performed with resazurin as previously described.

Ethidium bromide efflux inhibition assay

The potential activity of the Riparins as EPIs was assessed using a semi-automated EtBr method (Kaatz

et al. 2000) with modifications. Strains SA-1199B and SA-1199 were cultured in 2 ml of BHI broth until an optical density at 600 nm of 0·6 was achieved, followed by centrifugation at 5000 G for 5 min. Pellets were re-suspended in 2 ml of normal saline. Bacterial suspensions were vortexed and then transferred to 96 well plates followed by addition of a saline solution containing EtBr (8 μg ml^{-1}) and Riparin-B (128 or 256 μg ml^{-1}). A bacterial suspension containing only EtBr (8 μg ml^{-1}) was used as a positive control. Blank controls for each test were prepared in saline solution. Plates were placed in a StepOnePlus Real-Time PCR System™ Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) and relative fluorescence representing accumulated EtBr by the bacterium was measured after 60 min. Relative Final Fluorescence (RFF) was calculated by subtracting values for the respective blank controls. Differences in RFF between compound-containing and positive control assays was indicative of activity of the compound to inhibit efflux of EtBr.

Species distribution and Log P estimation

Estimation of distribution of the different species in equilibrium of the Riparins under study ($RH_2^+ \rightleftarrows RH \rightleftarrows R^-$) as a function of pH and Log P were performed using the ACD/LabD and ACD/LogP (Advanced Chemistry Development Inc., Toronto, ON, Canada). Log P has been calculated at pH = 7 for the uncharged molecule (RH) due to the typical range of pKas of the aromatic hydroxyls which are around 8·0–10·0. Distribution of species against pH (0–14) was also calculated with the same program for all compounds studied to corroborate that at physiological pH the neutral species predominates.

Structure prediction of NorA and molecular docking

All dockings were carried out using the Schrodinger Suite 2015 molecular modelling software. A two-dimensional structure of Rip-B was built using Maestro. This structure was converted into its three-dimensional form, including various tautomers, conformers and ionization states using LigPrep and ConfGen modules. A three-dimensional model of the NorA was generated using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The best scoring model was used as a receptor for ligand-receptor docking. The receptor was prepared for docking employing Protein Preparation Wizard, and the binding site grid was generated. Ligand-receptor docking was performed using the Glide module, and flexible docking was carried out for all the conformers to determine the ligand binding mode. Extra Precision scoring function of Glide was used.

Statistical analysis

Each experiment was performed three times, and results were normalized by calculation of geometric means. Error deviation and standard deviation of geometric means were revealed. Statistical analyses were performed using GraphPad Prism, ver. 5.02 (GraphPad Software, Inc., La Jolla, CA, USA). Differences between treatment with antibiotics in the absence or presence of Riparin-derived compounds were examined using One-way analysis of variance (ANOVA). The significance of differences was analysed by the Bonferroni post-test, and they were considered statistically significant when $P < 0{\cdot}05$.

Results

Intrinsic antibacterial activity

The intrinsic antibacterial activity of synthetic Riparin-derived compounds was tested by microdilution method against *Staph. aureus* strains, and the results are presented in Table 1. Rip-A, which forms the fundamental core of riparins, as well as Rip-B derivative, showed no intrinsic antibacterial activity against test strains (Holetz *et al.* 2002), while Rip-D showed a moderate inhibitory effect. On the other hand, Rip-C and Rip-E, the more lipophilic synthetic analogues showed a good inhibitory effect (Table 1 and Fig. 1).

Modulation of antibiotic activity

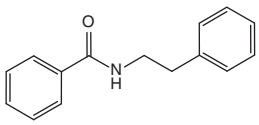
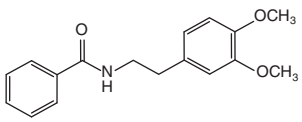
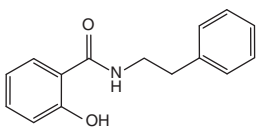
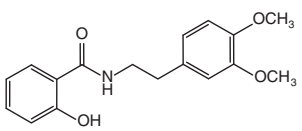
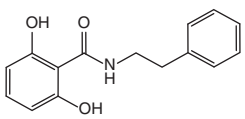
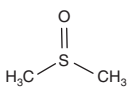
Modulatory activity of compounds on resistance to norfloxacin, ciprofloxacin or EtBr was evaluated using SA-1199 and SA-1199B, which are the wild-type parent and *norA*-overexpressing derivatives respectively. This activity is present when test compounds, added to the growth medium at subinhibitory concentrations, were able to reduce MICs of norfloxacin, ciprofloxacin or EtBr, indicating modulation of resistance to these antibacterial agents.

All Riparins presented modulatory activity towards norfloxacin and ciprofloxacin (Figs 2 and 3), with Rip-B showing the best activity. Only Rip-B showed modulatory activity for EtBr, a known NorA substrate (Fig. 4). On the other hand, no significant change in MICs was verified when antibiotics were tested alone or in combination with Riparins against SA-1199 (the MIC for norfloxacin and ciprofloxacin was 0·5 μg ml^{-1} in the absence of Riparins and ranged from 0·25 to 0·5 μg ml^{-1} in the presence of all Riparins).

Ethidium bromide efflux inhibition by Rip-B

To verify if Rip-B was an EPI for NorA, inhibition of EtBr efflux assays were performed using both SA1199 and

Table 1 Minimal inhibitory concentration (MIC) and theoretical estimated Log P for all Riparin-derived compounds tested against *Staphylococcus aureus* strains

Entry	Riparins	Structure	Log P	MIC (μg ml^{-1})*	
				SA-1199B	ATCC 25923
1	Riparin-A		2·87	≥1024	≥1024
2	Riparin-B		2·61	1024	1024
3	Riparin-C		3·22	102	102
4	Riparin-D		2·95	256	813
5	Riparin-E		3·57	32	25
6	DMSO		–	2048	2048

*Geometric mean of three simultaneous tests.

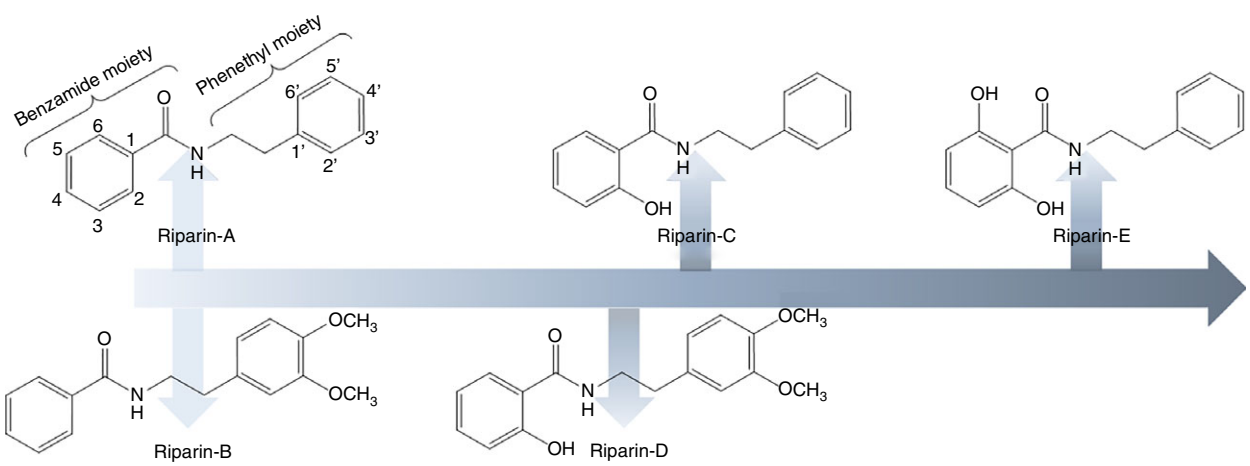


Figure 1 Structural differences and qualitative antibacterial activity of synthetic Riparins against *Staphylococcus aureus* SA-1199B. Colour figure can be viewed at wileyonlinelibrary.com.

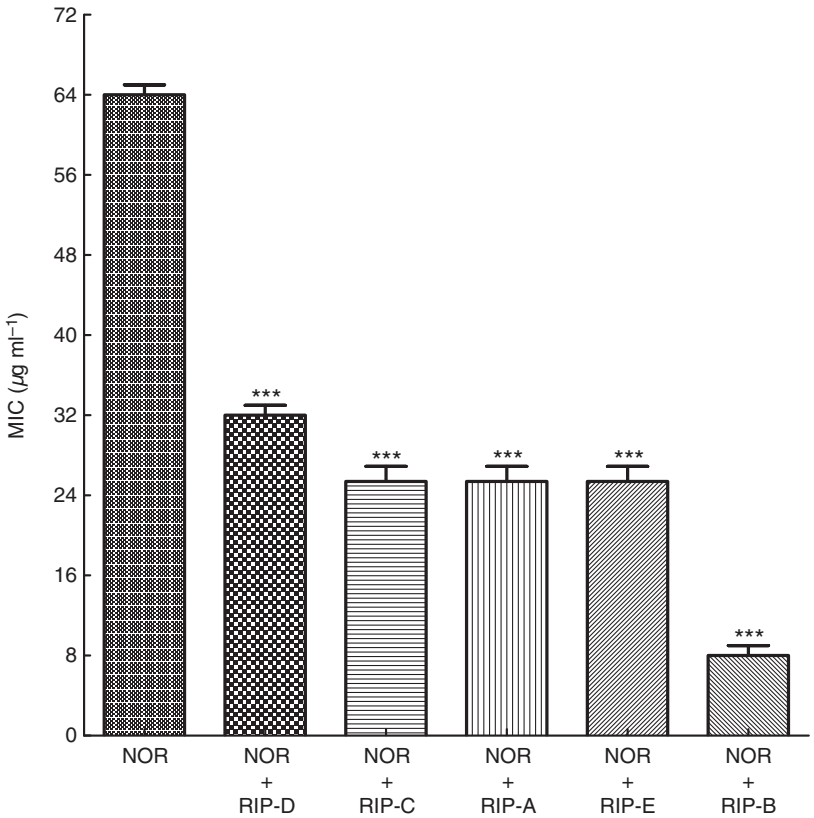


Figure 2 Minimal inhibitory concentration values (geometric means of three simultaneous tests) shown by Norfloxacin in the absence or presence of Riparin-derived compounds for *Staphylococcus aureus* SA-1199B. ***Value statistically significant ($P < 0{\cdot}0001$).

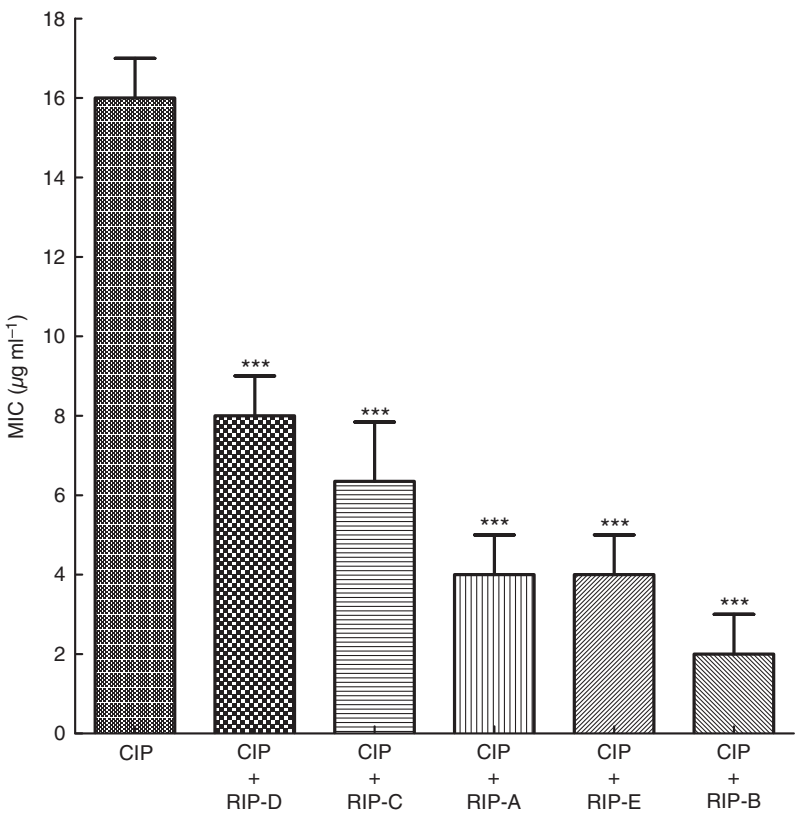


Figure 3 Minimal inhibitory concentration values (geometric means of three simultaneous tests) shown by Ciprofloxacin in the absence or presence of Riparin-derived compounds for *Staphylococcus aureus* SA-1199B. ***Value statistically significant ($P < 0{\cdot}0001$).

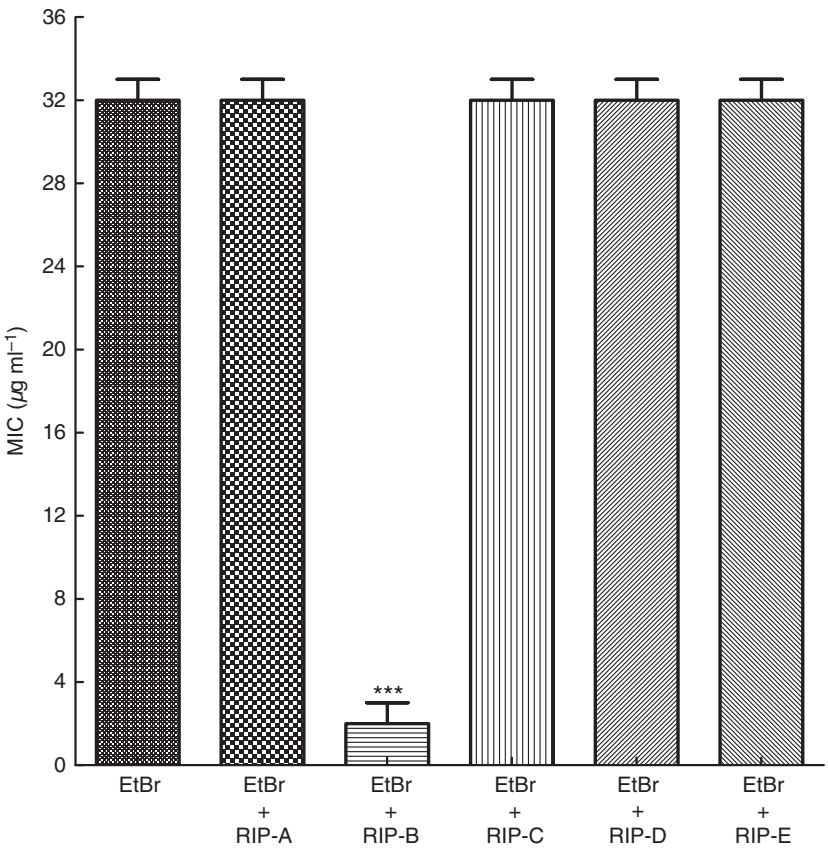


Figure 4 Minimal inhibitory concentration values (geometric means of three simultaneous tests) shown by ethidium bromide in the absence or presence of riparin-derived compounds for *Staphylococcus aureus* SA-1199B. ***Value statistically significant ($P < 0{\cdot}0001$).

SA1199-B (Fig. 5). The addition of Rip-B at subinhibitory concentrations caused an intracellular accumulation of EtBr in the SA1199-B, which can be observed by the increase in the fluorescence signal as a result of intercalation of EtBr into the DNA double helix. On the other hand, the fluorescence signal remained high both in the presence and absence of Rip-B when SA-1199 was analysed.

Molecular docking of Rip-B to the active site of NorA

A molecular docking study of Rip-B with NorA showed that Rip-B fit a pocket formed by ILE19, ILE23, PHE26, PHE47, ALA48, GLN51, MET109, THR113, SER133, ILE136, THR211, ARG310, ILE313, THR314, ASN332, SER333, THR336, SER337, ASN340, PHE341 (Fig. 6). The amide group of Rip-B was also able to form a hydrogen-bond between the side-chain amide oxygen of ASN340 and the amide nitrogen of GLN51.

Discussion

The intrinsic antimicrobial activity of Riparins was described previously against multi-drug resistant *Staph. aureus* and *E. coli* strains (Catão *et al.* 2005). In this study, we verify that this activity is dependent on the chemical structure and lipophilicity of each synthetic analogue.

Riparins with one hydroxyl at C2 (Rip-C) or two hydroxyls at C2 and C6 (Rip-E) had antibacterial activity against *Staph. aureus,* indicating that the addition of hydroxyl groups at the benzamide moiety increases reactivity of these derivatives with bacterial biomolecules thus, improving their activity against *Staph. aureus* (three and five entries in Table 1 and Fig. 1). On the other hand, no inhibitory effect was observed for Rip-B indicating that the presence of two methoxyl groups at the phenethyl moiety (at C3′ and C4′ positions) did not change its intrinsic activity against *Staph. aureus*, (entry 2, Table 1). The presence of methoxyl groups (at C3′ and C4′) at the phenethyl moiety also attenuated the antibacterial activity of Rip-D. These results indicate that methoxyl groups attached at the phenethyl moiety of Riparins are a factor that reduces the intrinsic antibacterial activity of these compounds against *Staph. aureus* (Fig. 1).

The distribution of species of Riparins was calculated at pH values varying from 0 to 14, to know which species (cationic, anionic or neutral) predominate, where these will execute their pharmacological actions, and if they are capable of interaction with the cell membrane. In all

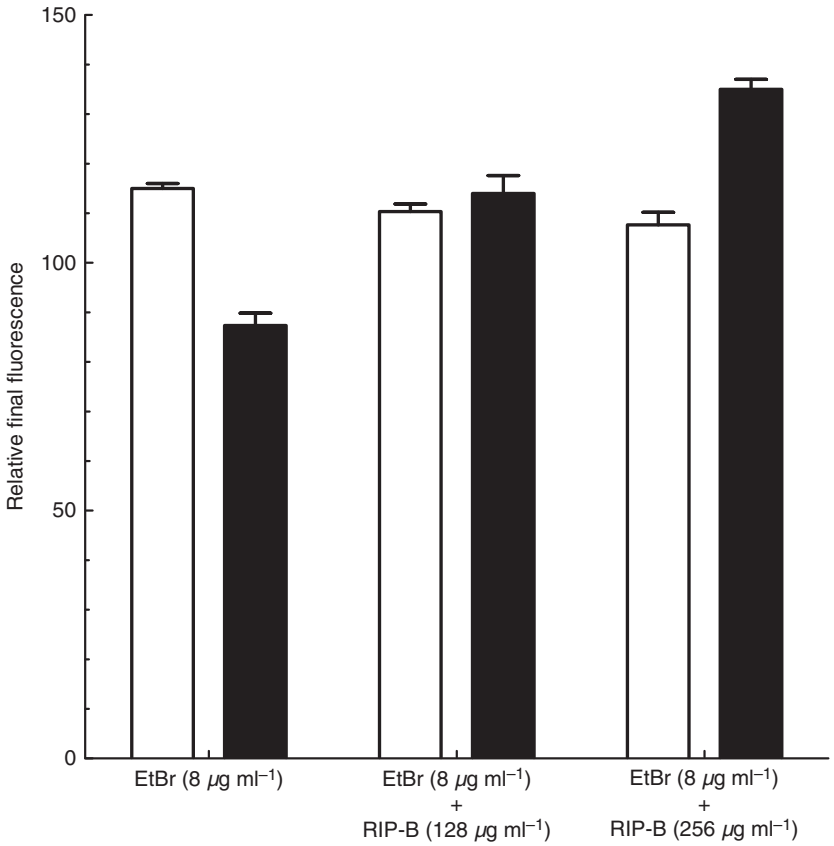


Figure 5 Effect of Rip-B on ethidium bromide (EtBr) accumulation for *Staphylococcus aureus* strains SA1199 (white bars) or SA1199-B (black bars). Bacterial suspension was loaded with EtBr (8 μg ml^{-1}) in the absence or presence of increasing concentrations of Rip-B for 60 min at 37°C. Relative final fluorescence (RFF) was calculated subtracting all points by the respective blank control. Difference in RFF obtained in the absence or presence of Rip-B was indicative of efflux inhibition.

cases, neutral Riparin structures predominate in the range of pH from 6·5 to 7·5. This result is pertinent because the permeability of the bacterial cell membrane is strongly dependent on the polar characteristics of both molecules and medium pH. Only for hydroxylated Riparins (Rip-C, Rip-D, and Rip-E), the mono-ionized anionic species becomes significant at pH values higher than 9·5. This allows us to conclude that if changes in pH (pH 7 ± 1) occur, the predominant species will remain the same.

Lipophilicity studies are relevant to know if a molecule can interact with cell membranes. The Log P value of Metoprolol (Log P = 1·72), which is 95% absorbed in the intestinal tract and possess a high cell membrane permeability, has been used as a reference to estimate the permeability of a vast number of drugs (Kasim *et al.* 2004). Taking this compound as a reference, the Log P of all studied Riparins was calculated (Table 1). All of them presented values between 2·61 and 3·57, showing a very hydrophobic characteristic.

Hydrophobic compounds can intercalate into the plasma membrane, affecting both respiratory chain activity and energy production (Nicolson *et al.* 1999). These compounds can also increase cell membrane permeability leading to leakage of inorganic ions such as K^+ (Souza *et al.* 2013). Interestingly, our results showed that intrinsic antibacterial activity increased with increasing lipophilicity (Table 1). These results indicate that intrinsic antibacterial activity of hydroxylated Riparins tested could be related to cell membrane damage.

A modulatory effect on the activity of fluoroquinolone antibiotics, increasing their activity against SA-1199B was verified for all synthetic analogues of the Riparins. Rip-B significantly decreased the MIC of the test strain for norfloxacin and ciprofloxacin, changing its phenotype from resistant to intermediate resistance. Modulation tests were conducted with EtBr, a known NorA substrate (Markham *et al.* 1999), in the presence or absence of each Riparin to determine if this modulatory effect was a consequence of NorA inhibition. Only Rip-B was able to reduce EtBr MIC against SA-1199B (Fig. 4) suggesting that this compound could be a potential EPI.

To confirm this hypothesis, inhibition of EtBr efflux was determined. EtBr fluoresces only after intercalation into bacterial DNA, and an increase in fluorescence is an indication of intracellular accumulation due to efflux inhibition. The addition of subinhibitory concentrations of Rip-B caused a significant accumulation of EtBr in

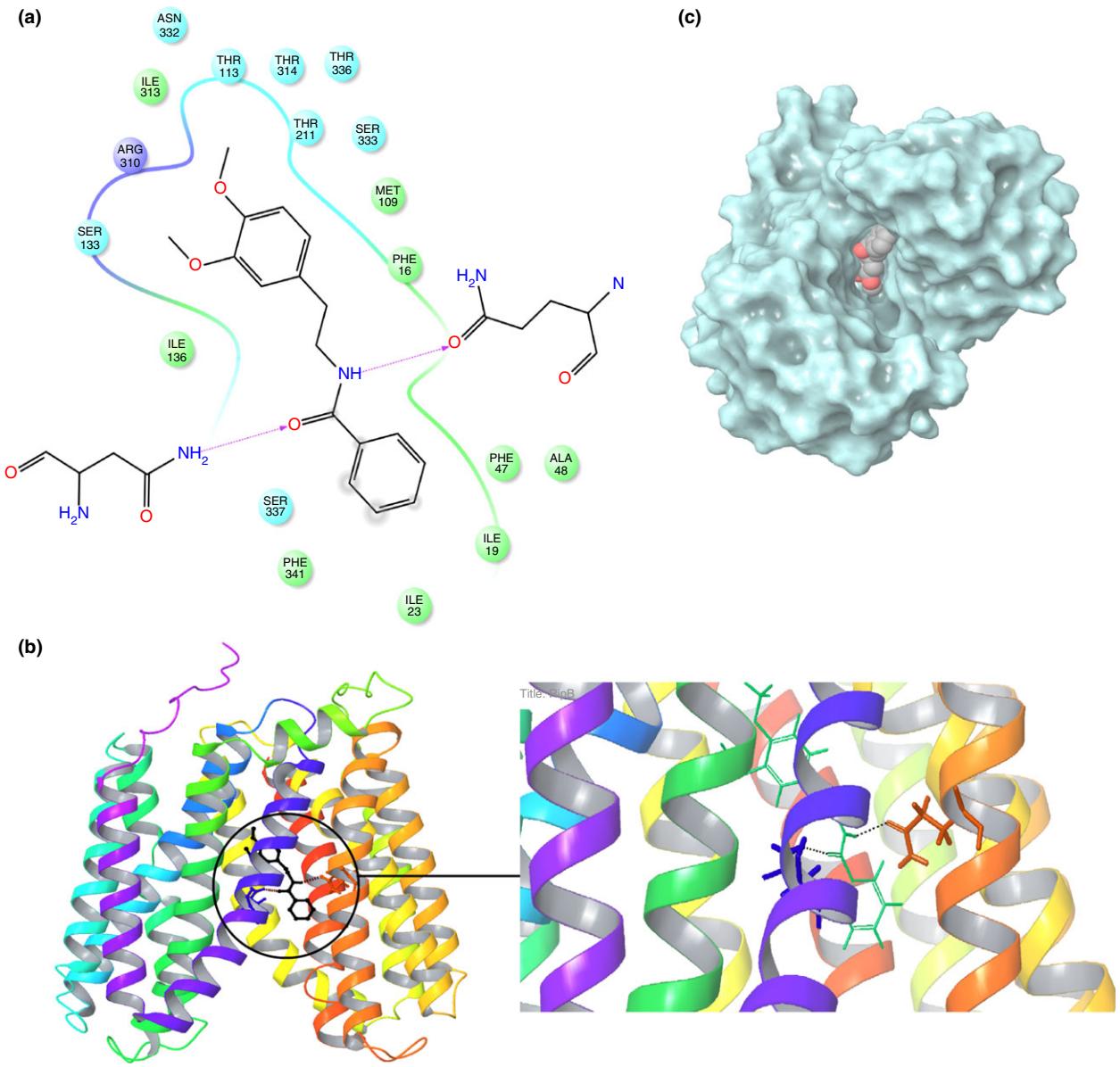


Figure 6 (a) Interaction of Rip-B in the binding site of NorA. H-bonds formation between Rip-B, GLN51 and ASN340 provides stability to the protein/ligand complex. (b) Relative orientation of the binding site residues with respect to the transmembrane helices of NorA predicted three-dimensional form. (c) Orientation of Rip-B within the binding site (rotated view). Rip-B is shown bound to the central cavity of the protein. Colour figure can be viewed at wileyonlinelibrary.com.

SA1199-B strain, as demonstrated by a fluorescence increase (Fig. 5). This inhibitory effect was similar to that reported for reserpine, a known NorA inhibitor (Brincat *et al.* 2011). On the other hand, the intracellular accumulation of EtBr in SA1199 remained unchanged both in the absence or presence of Rip-B.

Thus, the dimethoxylated analogue Rip-B showed the best modulatory effect when associated with fluoroquinolones employed and was able to inhibit efflux of EtBr in a *Staph. aureus* strain overexpressing *norA*. These results allow us to conclude that inhibition of NorA by Rip-B was strongly influenced by dimethoxylation of the phenethyl moiety instead of hydroxylation at the benzamide part in Riparins. In others words, the absence of methoxyl groups (Rip-A, Rip-C and Rip-E) or the presence of hydroxyls (Rip-C, Rip-D and Rip-E) avoid the EPI actions of Riparins (Fig. 5), with Rip-B the only one capable of reducing the MIC of EtBr against SA1199-B.

Methoxylated compounds have been reported as modulators of bacterial drug resistance (Silva *et al.* 2009;

Gomes *et al.* 2011). The addition of methoxyl groups on flavones increased both lipophilicity and modulatory effect on resistance to fluoroquinolones in SA1199-B (Maia *et al.* 2011). The lipophilicity of Rip-B (Log P 2·61) was similar to that reported by these researchers for 4′,5,6,7-tetramethoxyflavone (Log P 2·57). Cell membrane damage caused by intercalation of lipophilic compounds can inhibit efflux systems dependent on the proton-motive force, such as NorA, leading to a higher intracellular accumulation of antibiotics in the bacterial cell (Holler *et al.* 2012). Besides, hydrophobicity is an important requisite to guarantee affinity of a compound with efflux pumps, including NorA (Nora *et al.* 2012).

However, lipophilicity is not the only factor involved in NorA inhibition as some Riparins having higher lipophilicity than Rip-B did not modulate resistance to EtBr (Table 1). Thus, other structural characteristics of Riparins could be involved in the improvement of molecular interactions between Rip-B and cell membrane components, including the NorA protein. Docking results of Rip-B and NorA showed the orientation of Rip-B within the binding site extending into the hydrophobic cleft, which is the same binding site of other NorA inhibitors (Zhang *et al.* 2014). The amide group of Rip-B was also able to form a hydrogen-bond between the side-chain amide oxygen of ASN340 and amide nitrogen of GLN51 (Fig. 6). Multiple sequence alignment of NorA with other proteins belonging to the Major Facilitator Superfamily (MFS) showed that GLN51 and ASN340 are within a conserved domain of the MFS transporter family (Kalia *et al.* 2012). Although there are no site-directed mutation studies of these residues confirming its role in the protein, several docking studies have shown their importance in interactions with diverse ligands (Kalia *et al.* 2012; Thai *et al.* 2015).

Taking all results in mind, the modulatory effect on fluoroquinolone resistance could be achieved through different actions of the Riparins. One of them could be increasing the influx of antibiotics, through the action of these compounds as membrane permeabilizers. It is possible that this mechanism was at play with Riparin-derivatives which were not able to modulate resistance to EtBr, despite modulating resistance to fluoroquinolones. On the other hand, Rip-B showed a relevant modulator effect and significant inhibition of EtBr efflux, both corroborated by docking parameters, supporting the conclusion that Rip-B is an EPI. We believe that this relevant modulating action could be caused by a dynamic competition between Rip-B and antibiotic during transport through the pump or by inhibiting the normal operation of the pump, by bonding the modulator agent inside the efflux pump cavity.

From a molecular point of view, Rip-E and Rip-B appear to be the most complete agents, with one having the very best intrinsic antibacterial activity and the other the best EPI activity. Although Rip-B did not have intrinsic antibacterial activity against *Staph. aureus*, it is a potential adjuvant for fluoroquinolones in the treatment of infections caused by resistant *Staph. aureus* strains overexpressing the NorA efflux pump. However, additional studies will be needed to verify its effectiveness *in vivo* when administrated in association with fluoroquinolones.

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Conflict of Interest

None declared.

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