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Research Article

Modulation of Drug Resistance by Furanochromones in *NorA* Overexpressing *Staphylococcus Aureus*

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Khellin and visnagin are natural furanochromones that photoreact with DNA. Khellin has been used in the treatment of vitiligo and psoriasis, as well as in the treatment of angina pectoris and asthma due to its potent action as a coronary vasodilator and antispasmodic agent. The present study aimed to investigate whether the compounds khellin and visnagin act as inhibitors of NorA protein, an efflux pump overproduced by the strain of *Staphylococcus aureus* SA-1199B that confers resistance to the fluoroquinolones, such as norfloxacin and ciprofloxacin. These substances alone did not show antibacterial activity against the strain tested. On the other hand, when these compounds were added to the culture medium at subinhibitory concentration, they were able to reduce the minimum inhibitory concentration (MIC) of norfloxacin, ethidium bromide, as well as berberine, suggesting that these compounds are modulating agents of norfloxacin resistance, possibly due to NorA inhibition. Molecular docking analysis showed that both khellin and visnagin form hydrogen bonds with Arg310, an important residue in the interaction between NorA and its substrates, supporting the hypothesis that these compounds are NorA inhibitors. These results suggest a possible application of khellin and visnagin as adjuvants to norfloxacin in the treatment of infections caused by strains of *S. aureus* that overproduce NorA.

1. Introduction

Infectious diseases caused by multidrug-resistant bacteria have become a global health problem, leading to high mortality rates [1], and high costs for healthy systems [2]. Bacterial resistance is an adaptive response caused by the intensive use of antibacterial agents in the most diverse areas, including in the human and veterinary medicines, as well as animal feed supplementation, selecting the most adapted strains [3]. Several mechanisms of antibiotic resistance have been demonstrated in bacteria, such as reducing the permeability of bacteria to antimicrobials,

enzymatic modification of the antibiotic target, antibiotic modification, or degradation, as well as drug extrusion by efflux pumps [4].

Efflux pumps are transmembrane proteins that account for much of the bacterial resistance since they pump antimicrobial agents out of the cell [5]. Some of these pumps are specific for a given compound or class of compounds, whereas others remove a variety of structurally unrelated antimicrobial compounds [6, 7]. Several efflux pumps related to drug resistance in *Staphylococcus aureus* have already been identified, including MsrA, MepA, LmrS, MdeA, NorA, NorB, NorC, QacA, and QacC [8]. NorA is a proton-dependent multidrug efflux pump that belongs to Major Facilitator Superfamily (MFS) and it confers resistance to hydrophilic fluoroquinolones, such as norfloxacin and ciprofloxacin, as well as to biocide agents, such as ethidium bromide, acriflavine, and benzalkoniun chloride [9–11].

Resistance-modifying agents/modulators are compounds that potentiate the activity of an antibiotic against resistant strains, and some of these agents may act as efflux pump inhibitors (EPIs), as in the case of several naturally occurring compounds from plants [12–16]. Various phytocompounds have been studied for its ability to inhibit *S. aureus* efflux pumps, including terpenes such as eugenol [17], carvacrol, thymol [18], estragole [19], α-pinene, and limonene [20]. Inhibition of NorA has also been reported for ferulic acid and its esterified derivatives [21], chalcones [22], and vitamin K3 [23]. On the other hand, various synthetic EPIs have been reported as efflux pump inhibitors [24–26].

Chromone (1,4-benzopyrone) is a derivative of benzopyran with a substituted keto group on the pyran ring, being an isomer of coumarin (1,2 benzopyrone). Visnagin and khellin are two naturally occurring furanochromones able to photoreact with DNA [27]. Khellin and visnagin derivatives have anti-inflammatory and analgesic activity [28], epidermal growth factor inhibitory activity [29], as well as light mediated antimicrobial activity against *Escherichia coli* and *Fusarium culmorum* L. [30]. Khellin has been used in the treatment of vitiligo, and psoriasis [31], as well as in the treatment of angina pectoris and asthma due to its potent action as a coronary vasodilator and antispasmodic agent [32, 33]. Furthermore, khellin also has been used in the treatment of kidney stones [34].

Furochromones, as well as furocoumarins, are widely studied for their photoactive properties [27, 35]. The modulating activity of drug resistance by furochromones has not yet been reported, requiring the development of studies with these molecules as potential efflux pump inhibitors.

In an ongoing project to evaluate coumarins as modulators of antibiotic resistance, the modulatory activity of semisynthetic and commercial coumarins [36], as well as of furanocoumarins isolated from Rutaceae species [37] has been demonstrated. Still regarding the furanocoumarins, bergapten, and isopimpinellin do not modulate the resistance to norfloxacin and to ethidium bromide in an effluxing *Staphylococcus aureus* strain. Although furanochromones visnagin and khellin present similar molecular structures to bergapten and isopimpinellin, respectively (Figure 1), these compounds were considered different enough from the furacoumarins to justify the evaluate these homologous furanochromones (visnagin and khellin) as modulators of antibiotic resistance using an effluxing *Staphylococcus aureus* strain.

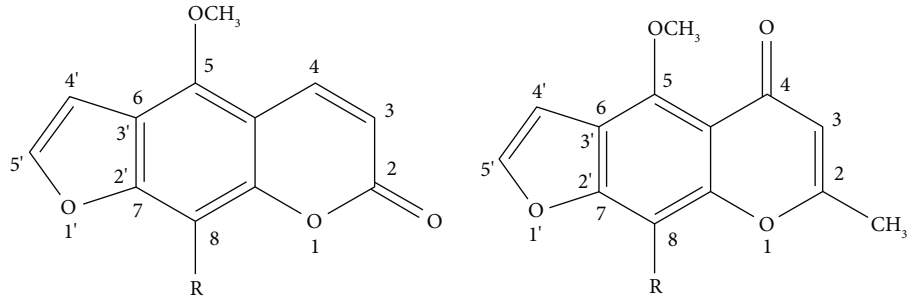
2. Material and Methods

2.1. Chemicals. The stock solution of norfloxacin was prepared in a mixture of 1 M NaOH and sterile distilled water (1 : 9 proportion). The stock solution of ethidium bromide (EtBr) and berberine were prepared in distilled water. The stock solution of the furanochromones—khellin and visnagin, were prepared in DMSO which, at its highest final concentration after dilution in the broth (4%), displayed no inhibition of bacterial growth. Chlorpromazine was prepared in sterile distilled water. All drugs were from Sigma-Aldrich, USA.

2.2. Bacteria. The SA-1199B strain of *S. aureus* was used as it overexpresses the *norA* gene. This gene encodes the NorA efflux protein that extrudes not only norfloxacin but several compounds, such as: hydrophilic fluoroquinolones, quaternary ammonium compounds benzalkonium chloride and cetrimide, intercalating dyes acriflavine and ethidium bromide [10], as well as the alkaloid berberine [38]. The strain, provided by Professor Simon Gibbons (University College London, UK), was maintained in blood agar base (Laboratories Difco Ltda., Brazil) slants, and prior to use, the cells were grown overnight at 37°C in brain heart infusion broth (BHI–Difco Ltda., Brazil).

2.3. Drug Susceptibility Testing and Modulation Assay. The minimum inhibitory concentrations (MICs) of norfloxacin, pefloxacin, ethidium bromide, and furanochromones were determined in BHI by the microdilution assay using a suspension of ca. 10^5 cfu/mL and a drug concentration range of 1024–1 μg/mL (two-fold serial dilutions). The MIC was defined as the lowest concentration at which no growth is observed. The detection was performed after the addition of resazurin at 0.01%. For the evaluation of furanochromones as a modulator of drug resistance, the "modulation assay" was used, a method that has been widely applied to identify potential EPIs [12] i.e. the MICs of norfloxacin, pefloxacin, ethidium bromide, and berberine were determined in the presence of furanochromones at a subinhibitory concentration (MIC 1/4). Chlorpromazine, a known NorA inhibitor [39], was used as a positive control.

2.4. Docking Procedure. The NorA model for the docking procedure was created by retrieving the NorA sequence of *S. aureus* 1199 strain from the Universal Protein Resource database (Uniprot, Entry Q03325). Then, the SWISS-MODEL [40] service was used to build the homology model. Out of the templates generated, the one with the best GMQE (Global Model Quality Estimation) score was the one based on the structure of the *Escherichia coli* YajR transporter



Bergapten, R = H
Isopimpinellin, R = OCH3

Visnagin, R = H
Khellin, R = OCH3

Figure 1: Chemical structure of furanocoumarins (bergapten and isopimpinellin) and furanochromones (visnagin and khellin).

(PDB-ID: 3wdo). The Molprobity [41] service was used for the protonation of the NorA model. For the docking procedure, which was carried out using the Autodock Vina [42] software, the grid box was defined as a 20Åx20Åx20Å box around the geometrical center of the model. Partial Gasteiger charges were added to ligand atoms, nonpolar hydrogen atoms were mixed while all other parameters were kept at their default values. Best results were chosen based on the binding score.

3. Results and Discussion

According to a previous study, the antimicrobial activity of an isolated compound must be considered significant if its MIC value is $\leq 10\,\mu$g/mL [43]. Both furanochromones khellin, and visnagin showed MIC values $\geq 1024\,\mu$g/mL. These results indicate that the tested compounds were inactive against the *S. aureus* strain used.

Despite not showing activity against this specific strain, addition of these compounds to the growth medium at $256\,\mu$g/mL (MIC 1/4) potentiated the antibacterial effect of norfloxacin against the SA-1199B strain, reducing the MIC values for norfloxacin by at least two-fold (Table 1). Modulating effect on the resistance to norfloxacin could be explained by inhibition of NorA efflux pump overproduced by SA-1199B, as already reported for several compound classes, such as alkamides [18], chalcones [44–46], flavonoids [47–50], and lignans [51]. In fact, visnagin, and khellin showed a modulating effect like that exhibited by the known NorA inhibitor chlorpromazine.

To investigate a potential action as NorA inhibitors, assays were performed replacing norfloxacin by two known NorA substrates: EtBr [52] and berberine [53]. EtBr is a well-known substrate for the NorA efflux protein, and active efflux is the only known mechanism of resistance to this DNA-intercalating dye [54]. Therefore, the use of EtBr against SA-1199B is enough to demonstrate that the compounds evaluated here modulated the resistance to norfloxacin by efflux pump inhibition. Results showed that compounds tested also reduced MIC values for EtBr and berberine by at least two-fold. It is worth noting the results obtained with visnagin regarding EtBr (Table 1). All experiments were carried out at least twice with consistent results suggesting that compounds tested could be NorA inhibitors.

Table 1: Minimum inhibitory concentrations (μg/mL) of norfloxacin, ethidium bromide, berberine, and pefloxacin against *Staphylococcus aureus* strain SA-1199B in the absence or presence of khellin, visnagin, and chlorpromazine.

Drugs	Alone	+Khellin	+Visnagin	+Chlorpromazine
Norfloxacin	64	32 (2) ∗	32 (2)	16 (4)
Ethidium bromide	64	32 (2)	16 (4)	8 (8)
Berberine	512	256 (2)	256 (2)	512
Pefloxacin	16	16	16	16

∗ (fold reduction in MIC).

A previous study verified that furanocoumarins bergapten and isopimpinellin did not modulate the antibacterial activity of norfloxacin against the strain evaluated [37]. To understand the differences in efflux pump inhibition (EPI) capabilities of furanocoumarins and furanochromones, a molecular docking study against the NorA efflux pump model was conducted. A comparison of the binding pose of visnagin vs bergapten and khellin vs isopimpinellin is shown in Figures 2 and 3, respectively.

Interestingly, both furanochromones dock in almost the same fashion and interact with the same residues. In particular, both interact through hydrogen bonds with Arg310. There are close contacts with Phe16, Asn340, and Gln51. The binding pocket of NorA is described as being composed by Ile19, Ile23, Gln51, Met109, Ile136, Thr211, Arg310, Ile313, Thr314, Asn332, Ser333, Ser337, Asn340, and Phe341, among others. The furanochromones make close contact with most of these, as can be seen in the protein-ligand interaction diagram in Figure 4. Not only that but a previous study described a chalcone with NorA inhibition properties that interacts with Arg310 through a hydrogen bond [55]. There are also imidazolines EPIs that bind to this same region of the binding site and interact with Arg310 through hydrogen bonds [19]. Interaction through H-bond with Arg310 also was reported to aminoguanidine hydrazones [7, 56]. These results suggest that Arg310 is an important amino acid residue for NorA substrate recognition.

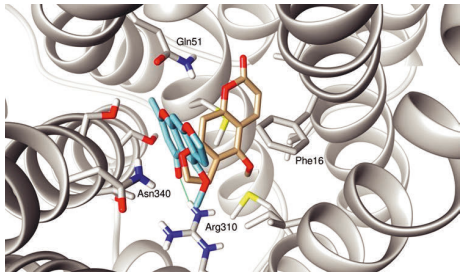


Figure 2: The best poses of visnagin (blue) and bergapten (golden) on the binding site of the NorA model. Hydrogen bond with Arg310 depicted in green.

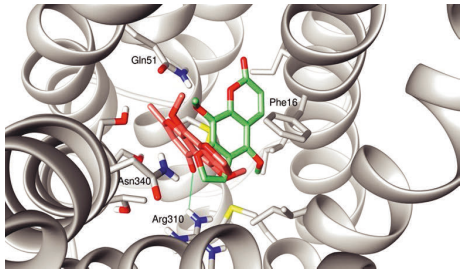


Figure 3: The best poses of khellin (orange) and isopimpinellin (green) on the binding site of the NorA model. Hydrogen bond with Arg310 depicted in green.

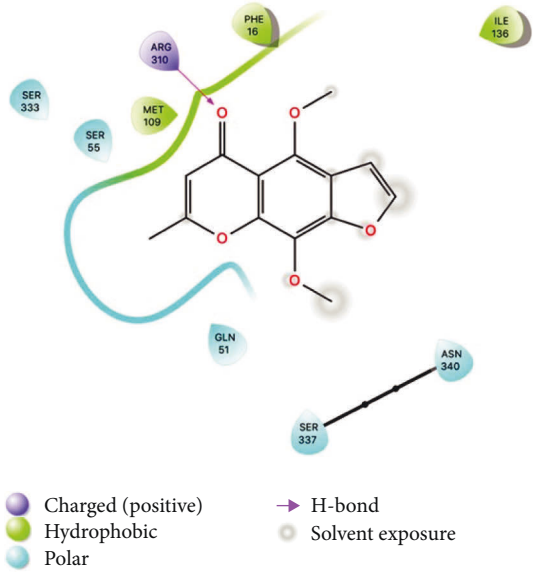


Figure 4: Protein-ligand interaction diagram of khellin on the binding site of the NorA model.

On the other hand, furanocoumarins do not interact with Arg310. Also, the Arg310 hydrogen bond anchors the furanochromones in a way that hinders the binding of EtBr, as can be seen in Figure 5. As such, the binding pose of furanocoumarins shows almost no overlap with the position of EtBr. It could, thus, be argued that the furanochromones could act as efflux pump inhibitors through competition in

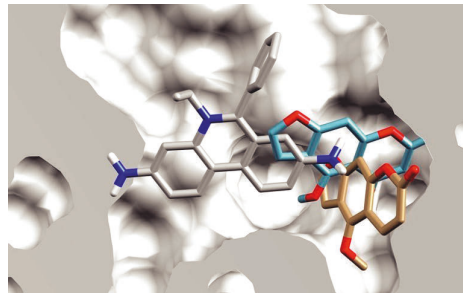


Figure 5: Binding poses of EtBr (white), visnagin (blue), and bergapten (golden) on the binding site of the NorA model.

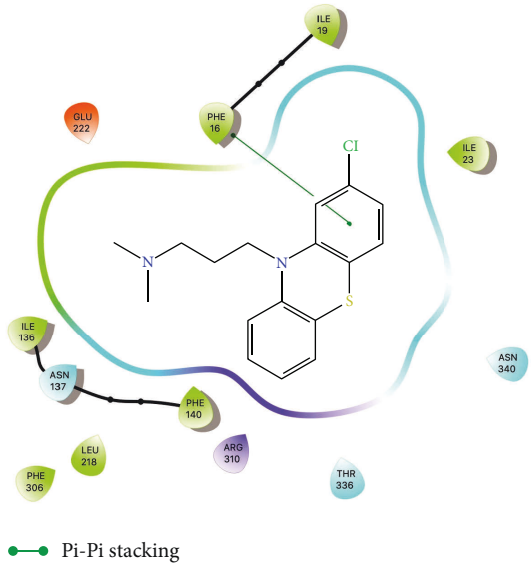


Figure 6: Protein-ligand interaction diagram of chlorpromazine on the binding site of the NorA model.

contrast with furanocoumarins, supporting the experimental results shown before.

We also compared the docked pose of other known efflux pump inhibitors, such as chlorpromazine (Figure 6), as well as piperine (Figure S1), verapamil (Figure S2), and reserpine (Figure S3). Chlorpromazine binds to the same region of the binding site, interacting not only with Arg310, but with Phe16, Asn340, Ile136 and others. More importantly, its best pose overlaps with that of EtBr in a similar fashion as those of furanochromones.

Also, other EPIs such as piperine and verapamil also bind to the same region of the binding site (Figures S1 and S2), as both interact with Arg310, Phe16, Gln51, etc. The binding pose of reserpine, on the other hand, is a bit different. As it is much larger than the previously mentioned EPIs, its interaction site goes from Ile136, Asn340, and Phe16 to other parts of the binding site, such as Ala48 and Met52, its sheer size contributing to inhibition as much as its interactions (Figure S3).

Our results indicate that khellin and visnagin could be applied in combination with norfloxacin against NorA overproducer *S. aureus* strains. Khellin showed a low-level

toxicity in humans, at an average daily dose of 120 mg, administered orally [57]. Liver and dermal histological and pathological analyses demonstrated that hydroxyethyl cellulose hydrogels based on khellin loaded in the ASC10 ascosomes have no toxic effects in rats [58]. On the other hand, both khellin and visnagin showed a strong cytotoxic activity (IC_{50} ranging between 12.54 and 17.53 μg/mL) on breast cancer (MCF-7) and hepatocellular carcinoma (Hep G2) cell lines [59]. Therefore, studies *in vivo* will be necessary to evaluate the safety of using khellin or visnagin combined with norfloxacin.

4. Conclusion

The furanochromones Khellin and Visnagin did not show any antibacterial activity against *S. aureus* resistant to norfloxacin by efflux pump mechanism. However, both compounds reduced the MIC of norfloxacin at subinhibitory concentration. Furthermore, khellin and visnagin modulated the resistance to EtBr and berberine, suggesting a possible inhibition of NorA efflux pump overproduced by *S. aureus* SA-1199B strain. Molecular docking analysis showed that both khellin and visnagin form hydrogen bonds with Arg310, an important residue in the interaction between NorA and its substrates, supporting the hypothesis that these compounds could be NorA inhibitors. Results obtained at this work are quite promising, which may stimulate future studies about the use of natural products concerning the viability of its use against microbial resistance.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request .

Conflicts of Interest

The authors declare that they have no conflicts of interest..

Acknowledgments

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Supplementary Materials

Figure S1: protein-ligand interaction diagram of piperine on the binding site of the NorA model. Figure S2: protein-ligand interaction diagram of verapamil on the binding site of the NorA model. Figure S3: protein-ligand interaction diagram of reserpine on the binding site of the NorA model. (*Supplementary Materials*)

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