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Marcos Vinicius Paranha
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Laís Chaves Guimarães,
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Debora da Silva Pereira,
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Francielle Moreira Sampaio,
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Waldinay Godinho Cordeiro
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Marcus Vinicius Dias-Souza
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Corresponding Author:
Marcus Vinicius Dias-Souza
University Vale do Rio Doce, Governador Valadares, MG, Brazil 6 – Pitagoras College, Ipatinga, MG, Brazil

Repositioning calcium channel antagonists as antimicrobials: *In vitro* study exploring clinical isolates of *Staphylococcus aureus*

Marcos Vinicius Paranha, Laís Chaves Guimarães, Debora da Silva Pereira, Francielle Moreira Sampaio, Waldinay Godinho Cordeiro and Marcus Vinicius Dias-Souza

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Abstract
Staphylococcus aureus is both a commensal and pathogenic bacterial species. Diseases caused by *S. aureus* represent a significant burden on healthcare systems, and over time, the species has developed complex and sophisticated mechanisms of resistance to most of the clinically used antimicrobials, making pharmacological treatments technically difficult. A strategy that has been increasingly explored for this context is drug repositioning, which takes advantage of the established approval of the drug for using it in a new purpose. Here we investigated the antibacterial activity of amlodipine and nifedipine on clinical isolates of *S. aureus.* We performed minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) tests using broth micro dilution. Our findings provide evidence of their antibacterial potential against *S. aureus* strains. Given the scarcity of data in this context, our data becomes even more relevant. Further studies are being conducted to develop pharmaceutical formulations to treat skin infections with these drugs.

Keywords: *Staphylococcus aureus*, nifedipine, amlodipine, antimicrobial

1. Introduction
Staphylococcus aureus is a Gram-positive species that can be naturally present on the skin and mucosas of the nose and oral cavity, colonizing them asymptomatically, whereas pathogenic strains of the species are involved in diseases such as meningitis, endocarditis, septicemia, osteomyelitis, mastitis and furunculosis [1]. Bacteria from this species express various virulence factors, and usually have the ability to form biofilms and to produce proteins that interact with the coagulation system and the extracellular matrix of biological tissues [2]. Due to the growing complexity of resistance mechanisms and the inappropriate use of antimicrobials, several drugs available at the pharmaceutical market have little or no effectiveness to treat staphylococcal and other bacterial diseases [1, 3].

Bacterial resistance to antimicrobials is one of the greatest risks to public health worldwide, and by 2050, the number of deaths caused by resistant bacteria is expected to exceed the number of deaths from malignant neoplasias [4]. The development of new antimicrobial drugs is an increasingly difficult, hybrid, time-consuming, and high-cost task. Pharmaceutical industries lack interest in this research field, due to technical limitations imposed by the quick development of resistance, but mostly due to economic matters. New antimicrobial options are of urgent need, and among the strategies to search for new treatments, drug repositioning has shown promising results.

Repositioning strategies consist on the exploration of a drug - already approved by regulatory agencies and marketed for specific purposes - usually to diagnose or treat diseases for which the drug was not originally designed for [5]. The approach has successful examples such as Zidovudine and Rituximab: both were developed to treat malignant neoplasias, but are used for HIV and rheumatoid arthritis treatments, respectively. As the chemical, pharmacological and toxicological profiles of the drugs are known, the path to launch them with new therapeutic aims can be faster and of lower cost when compared to most drug discovery pipelines currently in execution[6]. Trans membrane calcium influx blocker drugs (i.e. calcium channel antagonists) represent a therapeutic class of interest for repositioning as antimicrobials

They are low-cost options to treat arterial hypertension [7], and there is evidence that they can interfere in the metabolism of different microorganisms [8]. The efficacy of these drugs against pathogens such as *S. aureus*, however, remains poorly explored. Here we investigated the antibacterial activity of largely clinically used calcium channel antagonists against clinical isolates of *S. aureus*, aiming to develop a formulation for the treatment of staphylococcal skin diseases.

Materials and Methods

Preparation of the microorganisms

The microbial inoculum was prepared with 10 isolates in Mueller-Hinton broth (Difco, Becton Dickinson, USA), initially considering the concentration of 1 on Mac Farmland turbidity scale (~$3x10^8$ CFU/mL), to reach half of it on the assays [9]. The optical density was checked by spectrophotometry (1 of absorbance at 600 nm). The clinical isolates are from the collection of the Microbiology Laboratory of UNIVALE, and were obtained from hemodialysis catheter tips. Strains were identified through the Vitek 2 system (version R04.02, bio Meraux, France), using Gram-positive microorganisms identification cards, according to the manufacturer's instructions.

Preparation of drugs

Stock solutions of amlodipine and nifedipine (both from Fargo, Brazil) were prepared at 4 mg/mL as follows: for amlodipine, we used sterile distilled water. For nifedipine, we used a 96 °GL ethanol/sterile injection water in a 6:4 rate. Ethanol was filtered in 0.2 µ m sterile what man cellulose acetate membrane (Sigma Aldrich, St Louis, MO, USA) prior to the preparation of the solution. Previous to the experiments, the lack of antimicrobial activity of this hydro methanolic solution on *S. aureus* isolates was confirmed experimentally. The solutions were prepared at room temperature immediately before use in experiments.

Minimum Inhibitory Concentration (MIC)

We used broth micro dilution methodology for the antimicrobial activity assays, with 96-well untreated flat-bottomed plates [9]. For the MIC assay, the microorganisms were prepared as described above, in a final volume of 100 µL. Then, each drug solution was serially diluted in a two-fold fashion and added to the 96-well plates (volume of 100 µL), reaching a bacterial suspension concentration of 0.5 Mac Farmland scale (~$1.5x10^8$ CFU/mL), and drug concentrations ranging from 1000 to 7.81 µg/mL. Plates were incubated at 35±2°C overnight. On the next day, a total of 20 µL of a 0.1 g/L resazurin solution was pipetted into each well. Plates were kept in the dark for 10 min at 35±2 °C and the result was analyzed as follows: the lowest concentration in which the blue dye (resazurine) was not converted in pink dye (resazurin), was considered the MIC.

Minimum bactericidal concentration (MBC)

For the determination of the MBC, the wells in which there was no color change had their contents suspended by oxygen injection with a micropipette. Spots were made with 10 µL of the contents of each well on Mueller-Hinton agar plates (Difco). A negative control consisting of the sterile drug solution was spotted in Mueller-Hinton plates and broth. The plates were then incubated at 35±2°C overnight. Following, the plates were checked for colony growth. The lowest concentration in which there was no bacterial growth on the plates was considered as the MBC [9].

Results and Discussion

In this study, drugs used in the treatment of hypertension proved to be efficient *in vitro* as antimicrobials against a Gram-positive species of clinical relevance. Nifedipine and amlodipine (figure 1) showed antibacterial activity against the 10 clinical isolates of *S. aureus*, but with different efficiency (table 1). MIC reflects the treatment condition of immunosuppressed patients, and was lower for nifedipine. MBC reflects the treatment condition of immunocompetent patients, and was higher for amlodipine. Due to the high MIC results, it was unlikely that any anti biofilm activity could be detected in the concentration range explored here. Our data is in agreement with the observation of others about the better response of amlodipine when compared to other drugs of the dihydropyridine group [10, 11].

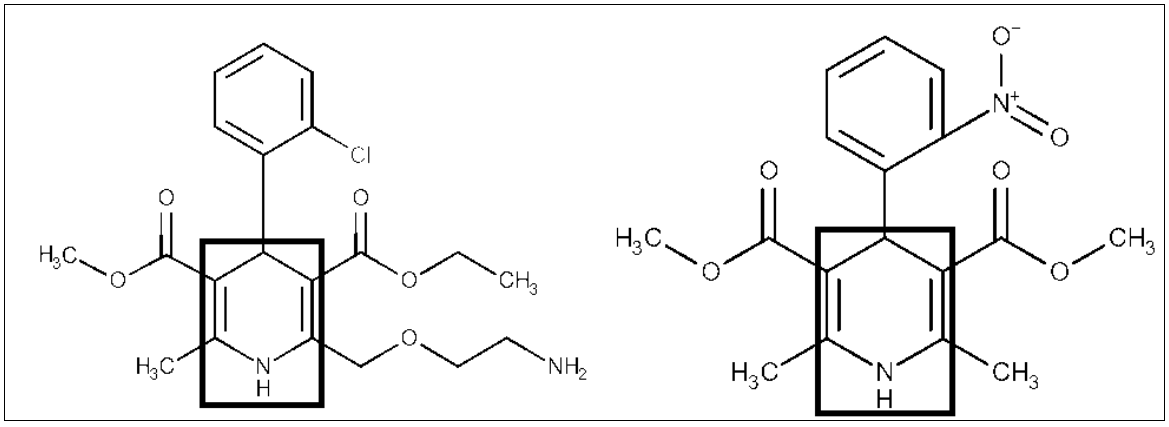

Fig 1: Amlodipine (left) and nifedipine (right). The dihydropyridine ring in both molecules is highlighted inside the rectangle.

Table 1: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Drug	MIC	MBC
Amlodipine	125 µg/mL	1000 µg/mL
Nifedipine	250 µg/mL	500 µg/mL

Results are observations of all strains

The antimicrobial mechanism of amlodipine is believed to involve the presence of benzene rings, which form a very similar basic structure of phenothiazine's tricyclic rings, thus, it is considered an incomplete phenothiazine. The presence of chlorine may also be playing a role in its antibacterial activity, and the drug would also enhance the bactericidal effects of macrophages *in vitro* [11]. A possible mechanism by which nifedipine acts involves decreasing iron intracellular availability via positive regulation of the iron export protein Fpn1, promoting intracellular iron outflow and preventing mobilization of membrane-associated calcium stores[12]. Both drugs could inhibit bacterial resistance mechanisms such as efflux pumps and affect bacterial life by directly inhibiting the activity or expression of bacterial metabolism proteins [10].

A study tested 10 cardiovascular drugs, including nifedipine and amlodipine were tested against 504 clinical isolates and ATCC strains from 13 species. The MIC of amlodipine ranged among clinical isolates from 25 and 400 µg/ml, with most of the bacterial growth inhibited between 25 and 100 µg/ml. Nifedipine inhibited isolates at concentrations of 25 to 200 µg/mL, and *S. aureus* had the highest sensitivity to the drugs [11].

Compounds derived from 1,4-dihydropyridine, such as the drugs tested in the present study, were synthesized by condensing acetoacetic ester, aryl aldehyde and ammonium hydroxide in ethanol, to generate potential antibacterial drugs[12]. A MIC of 400 µg/ml was observed in *in vitro* tests with *Escherichia coli, Klebsiella aerogenes* and *S. aureus*, using the disk diffusion method. The compound 3, 5-Di-(1, 1-dimethylethyl)-2,6-dimethyl-4-(3-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate showed a strong activity against *Klebsiella aerogenes* and *S. aureus*[13]. Synergism has also been explored with calcium channel blockers as a pathway to increase the activity of antimicrobial drugs [14-16]. Clinical isolates of *Acinetobacter baumannii* were more susceptible to impanel when combined to amlodipine, probably due to the inhibition of the Ade ABC efflux pump [14, 15]. Synergy was also detected in this combination in another study, and interestingly, β-lactamase producer strains were not affected [15, 16].

Conclusion

We demonstrated the activity of amlodipine and nifedipine as antibacterial agents. Their mechanisms of action which explain the antimicrobial activity is not related to their ability to block calcium channels. Our study, despite the limitations related to the number of microorganisms used in the tests and molecular approach, opens doors for the development of topical formulations with these drugs for the treatment of dermatological diseases caused by *S. aureus*.

Conflict of Interest

Not available

Financial Support

Not available

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