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Aminoguanidine hydrazones (AGH's) as modulators of norfloxacin resistance in *Staphylococcus aureus* that overexpress NorA efflux pump

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ABSTRACT

One of the promising fields for improving the effectiveness of antimicrobial agents is their combination with efflux pump inhibitors (EPIs), which besides expanding the use of existing antibiotics. The goal of this research was to evaluate a series of aminoguanidine hydrazones (AGH's, **1–19**) as antibacterial agents and NorA efflux pump inhibitors in *Staphylococcus aureus* strain SA-1199B. Molecular modeling and docking studies were also performed in order to explain at the molecular level the interactions of the compounds with the generated NorA efflux pump model. The MICs of the antibiotic and ethidium bromide were determined by microdilution assay in absence or presence of a subinhibitory concentration of aminoguanidine hydrazones and macrophages viability was determined through MTT assay. Bioinformatic software Swiss-Model and AutoDock 4.2 were used to perform modeling and docking studies, respectively. As results, all AGH's were able to potentiate the action for the antibiotic norfloxacin, causing MIC's reduction of 16-fold and 32-fold to ethidium bromide. In the cell viability test, the concentration of 10 μg/mL showed better results than 90% and the concentration of 1000 μg/mL showed the lowest viability, reaching a maximum of 50% for the analyzed aminoguanidine hydrazones. Molecular docking studies showed that both norfloxacin and derivative **13** were recognized by the same binding site of NorA pump, suggesting a competitive mechanism. The present work demonstrated for the first time that AGH derivatives have potential to be putative inhibitors of NorA efflux pump, showing a promising activity as an antibacterial drug development.

1. Introduction

The emergence of drug resistance in different species of bacteria is a growing cause of concern [1]. These bacteria include strains of *Staphylococcus aureus,* which is an opportunistic but potentially serious human pathogen that can be resistant to vancomycin [2], and also to the recently discovered linezolid [3], which is often considered “the last line of defense” against multidrug resistance (MDR) *S. aureus* strains.

Among the MDR efflux pumps present in this species, NorA, which belongs to the Major Facilitator Superfamily (MFS) is considered representative, and the most efficient of the MDR systems in Gram-positive bacteria. In particular, the NorA protein confers resistance to a wide range of structurally unrelated antibiotics and antiseptics such as acridines, ethidium bromide, pentamidine, or more importantly, the hydrophilic fluoroquinolones, an important class of broad-spectrum antimicrobials with potent activity [4–6].

The structural biology data of NorA are still undetermined. However, through sequence homology studies and the sharing of various substrates with other MDR pumps, the hypothesis that NorA may have a large hydrophobic binding site [7] has gained acceptance. This

Abbreviations: EPIs, efflux pump inhibitors; AGH's, aminoguanidine hydrazones; MICs, minimum inhibitory concentrations; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl formazan; MDR, multidrug resistance; MFS, major facilitator superfamily; MRSA, *Staphylococcus aureus* resistant to methicillin; NorA, efflux pump which extrudes norfloxacin and fluoroquinolones; LogP, partition coefficient; BHI, brain heart infusion broth; NCCLS, National Committee for Clinical Laboratory Standards; BrEt, ethidium bromide; NOR, norfloxacin; DMSO, dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle Medium; FBS, foetal bovine serum; PDB, protein database bank; PAβN, dipeptide amide

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peculiarity could explain MDR pumps' broad specificity for substrates.

Even though new methods of treatment have been developed, or are about to be made available, the restoration of clinical antibacterial efficacy (against which resistance has developed), remains an important goal. One of the promising fields for improving the effectiveness of antimicrobial agents is combining them with efflux pump inhibitors (EPIs). This expands the usefulness of existing antibiotics and reduces the emergence of resistant mutant strains [8].

Efforts are underway to generate new antibacterial agents able of circumventing drug efflux by synthesizing compounds that are poor pump substrates, or to identify compounds that reduce or block efflux pump activity [9].

Among these compounds, the aminoguanidine hydrazones (AGH) derivatives may well become a source for new candidates to develop alternatives to antibiotics, and to combat bacterial resistance. AGH represent a class of compounds containing an amidine group (guanyl), connected to a hydrazone moiety [10]. Along with the biological potency of these molecules come; antihypertensive [11,12], antidiabetic [13], antineoplastic [14,15], antitubercular [16], antimalarial, anti-Leishmania, antifungal, antibacterial, and anti-HIV activities [17–19].

In view of these initial considerations, and in order to explore the antibiotic potential of AGHs, which may lead to the synthesis of new structurally related derivatives, the goal of this research was to evaluate a series of AGHs as antibacterial agents and NorA efflux pump inhibitors in *Staphylococcus aureus* resistant to methicillin (MRSA), and their cytotoxicity on macrophages. Molecular modeling and docking studies were also performed and proved necessary in order to explain at the molecular level the interactions of the compounds with the generated NorA efflux pump model.

2. Experimental

2.1. Chemistry

The AGH (**1–19**) involved in this study were previously synthesized by reaction of the appropriate aldehyde with aminoguanidine hydrochloride in refluxing 95% ethanol. The title compounds were isolated after cooling to room temperature, being washed, or recrystallized using an appropriate solvent. Structural characterization of the products is described elsewhere [20,21]. LogP was calculated in the HyperChem™ software Release 8.0.8 Windows.

2.2. Biological assays

2.2.1. Bacterial strains

The *S. aureus* strain used, SA-1199B, over expresses the NorA gene encoding the NorA efflux protein [22], which extrudes hydrophilic fluoroquinolones and other drugs such as DNA-intercalating dyes. The strain, kindly provided by Professor Simon Gibbons (University of London), was maintained on blood agar base slants (Laboratory Difco Ltda., Brazil). Prior to use, the cells were grown overnight at 37 °C in brain heart infusion broth (BHI–Laboratory Difco Ltda., Brazil).

2.2.2. Antibiotics and nucleic-acid binding (NAB) compounds

All antibiotics were prepared according to NCCLS guidelines [23], and were purchased from Sigma-Aldrich Co. The stock solution of ethidium bromide (BrEt), norfloxacin (NOR) and perfloxacin were prepared in distilled water. The stock solutions of the title compounds were prepared in DMSO solutions where, at its highest final concentration after dilution in broth (4%), no bacterial growth inhibition occurred [22].

2.2.3. Susceptibility tests

The minimum inhibitory concentrations (MICs) of the antibiotics, BrEt and AGH derivatives were determined by microdilution assay using a suspension of ca. 10^5 cfu/mL and drug concentrations in the range of 256–0,25 μg/mL (two-fold serial dilutions). MIC is defined as the lowest concentration at which no growth is observed. AGH compounds were evaluated as NorA efflux pump inhibitors based on their effects on MICs of the antibiotic and ethidium bromide, in the presence and absence of AGH's at sub inhibitory concentrations and corresponding to ¼ of their own MICs [24]. All experiments were carried out at least twice with consistent results.

2.2.4. In vitro cytotoxicity on macrophages

Macrophages viability was determined thought the MTT 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl formazan colorimetric assay [25]. Approximately 1,5 × 10^5 macrophages (line J774) per well were seeded into 96-well plate plates and were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM: Life Technologies cat # 11995073) supplemented with 10% foetal bovine serum (FBS: Life Technologies cat # 16000044) in the presence or absence of aminoguanidine hydrazone derivatives at 37 °C and 5% CO_2 for 24 h in three different concentrations (10, 100 e 1000 μg/mL). One hour before addition of MTT, 2 μL of Triton 100X were added to 3 wells for cell death comparison. After incubation, cytotoxicity was determined by adding 100 μL of MTT (500 μg/mL) to each well and further incubated for 2 h. The supernatant was discarded and the precipitate was resuspended in 100 μL of DMSO. Finally, readings were performed at 540 nm on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Optical density data (cell viability) were obtained in quadruplicate and presented as mean ± standard deviation. The MTT reduction activity was determined as % cell viability, calculated by the formula: ([absorbance of treated cells/absorbance of untreated cells] x100). Data were analyzed using Origin (Version 6.1052; Origin Lab Corp Northampton, MA, USA). One-way ANOVA followed by Tukey test was used to compare differences between groups, and data were considered statistically significant for p value ≤ 0,05.

2.3. Molecular modeling studies

2.3.1. NorA modeling

In order to study the structural determinants of the NOR site, it was used *S. aureus* NorA pump primary sequence, taken from the Reference Sequence NP_373905.1 and SWISS-MODEL software [26] to produce a structural model of the protein. The monomer A of the crystal structure of YajR transporter from *E. coli* (PDB id: 3WDO) [27] was selected as template for molecular modeling, according to the HMM-HMM-based lightning-fast iterative sequence search (HHBlits) tool which uses the database SWISS-MODEL (SMTL) from the selected sequence. Best alignments were classified according to the sensitive HMM-HMM-based lightning-fast iterative sequence search (HHBlits) tool parameters. NorA model was constructed with ProMod v.3.7 program [28].

2.3.2. Docking

In order to find structural insights, best modifying activity antibiotic in SA-1199B strains compound **13** (having better antibiotic modifying activity in the SA 1199B strain) and NOR were used to perform Molecular Docking on the generated model of NorA efflux pump using Autodock 4.2 [29]. The structures of both molecules were build using the server PRODGR [30]. Receptor preparation was carried out using model previously generated by SWISS-MODEL [26] software where was add hydrogen atoms and Gasteiger charge to entire molecule. Autogrid was carried out for the preparation of the grid map, where a grid box was 40-40-40 Å npts, spacing was 0.603 Å and grid center was −29.984, 55.831 e 73.869 to x, y, and z-axis. Ligand preparation was set out with flexible bonds at maximum to both compounds. Further, Autodock (protein-rigid and ligand-flexible) were performed using Genetic Algorithm (GA) in standard configuration, with a number of GA runs and a maximum number of individuals survive at 20 and 2, respectively. Best pose of each docking calculation was selected and analyzed using PyMol software [31].