In vitro and *in silico* Approaches to the Identification of New Compounds with Antibacterial Profile

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ABSTRACT

The emergence of multidrug-resistant bacterial strains is a world problem that increases the need for new and more effective antimicrobials. On that purpose, derivatives of cyclic systems may serve as new leads for discovering new active molecules. In this work we evaluated the antibacterial profile of 243 molecules derived from the systems thienopyridine, pyrazolopiridine, quinolone, chalcone, hydrazone and lapachone against Gram-positive and Gram-negative susceptible and multiresistant strains also comparing them with antibiotics of clinical use. Our results showed that among the 243 molecules tested, only eight derivatives were active with promissing MIC values (2-64μg/mL). Our theoretical *in silico* analysis showed that all active compounds fulfilled Lipinski rule of five (molecular weight = 344.37–409.24, clogP = 3.15–4.11, nHBA = 6–7, and nHBD = 2), similarly to commercial drugs as well as presented better druglikeness values (from -3.68 to 0.12) than chloramphenicol (-4.61) and linezolid (-4.08). Most of the active derivatives presented a low *in silico* toxicity risk profile, similar to oxacillin, ampicillin, and penicillin G, and even lower than that observed for chloramphenicol and linezolid. Theoretically HOMO and the electrostatic protential distribution may be contributing for this safer profile. This study used computacional tools and may help to deal with an important world health problem.

Keywords: *In silico*, molecular modeling, bacteria, antibacterial, derivative.

1. INTRODUCTION

Resistance to antibacterials has been a serious world problem since the introduction of antibiotics in the early 1940s [1-2]. This problem is getting worse year after year, due to several factors including the inappropriate use of these drugs [2-3] .

Now this challenge is even more critical, due to the growing emergence of multidrug-resistant bacterial strains and exchange of resistance between different species of bacteria (*i.e. Enterococcus faecalis, Mycobacterium tuberculosis, Neisseria gonorrhoeae, Streptococcus pneumoniae and Staphylococcus aureus)* [3-4].

Since its discovery in the 20th century, antibacterial agents have substantially reduced the threat of infectious diseases. The use of antibiotics combined with improvements in sanitation, housing, food production, and immunization programs have led to a dramatic reduction of morbidity and human mortality [3].

The antimicrobials act by interacting at specific sites of the bacteria mainly through inhibition of different processes including cell wall synthesis, protein synthesis or replication of nucleic acid [2-4]. However, the intensive and inappropriate use of antimicrobials, the natural bacterial resistance and other human inappropriate practices increased the level of bacterial resistance. This event reduces the options of treating bacterial infections and affects medical procedures such as organ transplants and application of prostheses, where the complications are of highest incidence [3 -5]

Antimicrobial resistance is the resistance against the effects of an antimicrobial through different and highly efficient mechanisms. Antibiotic resistance occurs when bacteria genetically change in some way and reduces or eliminates the effectiveness of drugs, chemicals or other agents used to cure or prevent infection. Thus, the bacteria survive and continue to multiply, causing more damage or even death [4-6] .

The emergence of antimicrobial resistant organisms is an important public health problem, especially in hospitals and other health institutions [4-6]. The continuous appearance of multiresistant nosocomial pathogens difficult to treat or cure, like multi-resistant *Klebsiella* (KPC), vancomycin resistant *Enterococci* (VRE) and *S. aureus* with intermediate susceptibility to vancomycin and other glycopeptide antibiotics (GISA) threaten hospitalized individuals and those with chronic conditions whereas it dramatically increases health costs. In addition, community-acquired infections and foodborne infections of humans caused by *S. pneumoniae, S. aureus, Salmonella and Campylobacter species, M. tuberculosis,* and *N. gonorrhoeae* clearly pointed to the continuous increase of resistance to therapies that were efficient ten years ago [3-6]

The increased prevalence of pathogens resistant to drugs comes at a time when the discovery and development of new antimicrobial agents occurs slowly [6]. Accordingly, there is a concern that in a near future it may be a growing number of potentially untreatable infections [5-6].

The emergence of multidrug-resistant strains to antibacterial agents such as carbapenemases-producing bacteria (KPCs) increased the need for searching for new molecules that can allow an improvement in the treatment of infections [7]. Towards this search, derivatives of cyclic systems can serve as new leads for the discovery

of antibacterials. Thus, in this work we performed the synthesis, biological identification and theoretical analysis of 243 molecules derived from the systems thienopyridine, pyrazolopiridine, quinolones, chalcones, hydrazones and lapachones, also comparing with antibiotics of clinical use. All these systems are described in literature by their different biological profiles but the antimicrobial activity was not fully explored [8-11].

To explore the possibility of identifying new antimicrobials, three brazilian groups of organic chemistry synthesized these systems derivatives while one group with expertise in microbiological assays, tested them in antibacterial sensitivity tests (AST) and determined the minimum inhibitory concentration (MIC). Antibiotics of clinical use were used as positive controls, and Gram-positive and Gram-negative susceptible (standards) or multiresistant strains provided by the Antonio Pedro University Hospital (HUAP) were tested for sensitivity. Further the molecular modeling group analyzed the structure-activity relationship of these active systems using *in silico* molecular modeling tools .

2. MATERIAL AND METHODS

Antibacterial susceptibility test (AST)

The assays were performed according to the National Committee for Clinical Laboratory Standards (NCCLS), in Müeller–Hinton medium as described elsewhere. Briefly, the 14 strains were grown at 37 _C in Müeller–Hinton medium, and 1 lL of the stock solution (5 mg/mL) of each derivative in dimethyl sulfoxide (DMSO) was placed in Whatman disks (5 mm diameter). The disks were put on exponentially growing plated cultures with appropriate dilution to 1.0x 10^7 colony forming unit (CFU/mL), which were then incubated for 24 h at 37 ^oC. The inocula used in growth method were those where turbidity was equal to 0.5 McFarland Standard.

The results were verified by measuring the inhibitory zones surrounding the disk. Ciprofloxacin and vancomycin ere used as positive controls, and the halo >15 mm was considered the minimum value for positive antibacterial activity as it generally leads to a minimal inhibitory concentration (MIC) near that observed for the newest antibiotics which are currently present in the market (MIC = 1– 40 μg/mL) using these assays. Vancomycin and ciprofloxacin presented halos 15–17 and 23–25 mm, respectively, in the strains tested herein ($p < 0.005$).

Minimal inhibitory concentration assays (MIC)

MIC was determined only for active compounds on the AST by using the macro-dilution broth method. All MIC were performed in triplicate as described previously.24 Briefly, after 5 h of the bacterial growth, the culture was diluted to obtain 1.0 x 10^5 colony forming unit (CFU/mL). Then each compound was added to reach a final concentration from 0.5 to 1024 lg/mL, and was incubated at 37^oC for 24 h. MIC was defined as the lowest compound concentration preventing visible bacterial growth. All strains were tested at least in duplicate in four separate experiments, and a reference antibiotic (vancomycin) was used as a positive control (MIC = 2 μg / mL).

Molecular modeling and SAR studies

The molecular modeling study was performed using SPARTAN' 08 (Wavefunction Inc. Irvine, CA, 2006) and Osiris programs (http://www.organic-chemistry.org/prog/peo/druglikeness.html) as described elsewhere.9 The conformation analysis was obtained through AM1 and angles of rotation in the range of 30/30. Single Point Calculation in DFT/B3LYP was performed with database 6.31G*. Molecular electrostatic potential maps (MEPs), HOMO, and LUMO eigenvalues and orbital coefficients, and the molecular dipole moments were calculated.

In this work, we also studied the drug-likeness and the drugscore of the compounds, which is based on topological descriptors, fingerprints of molecular druglikeness, structural keys or other properties as clogP and molecular weights. In the case of Osiris Property Explorer (http://www.organic- chemistry.org/), the occurrence frequency of each fragment is determined within the collection of traded drugs and within the supposedly non-drug-like collection of Fluka compounds. Since the compounds are considered for oral delivery, they were also submitted to the analysis of Lipinski Rule of Five, which evaluate some properties of a compound that would make it a likely orally active drug in humans. These structural parameters were performed using Molispiration program (http://www.molinspiration.com/cgi-bin/properties).

3. RESULTS AND DISCUSSION

Our results showed that from the 243 molecules tested, only a few number was active including: a) five derivatives (1a-1c, 1e and 1f) of the system pyrazolopiridine active against *Staphylococcus epidermidis* , b) one hydrazone derivative, active against *S. aureus* and c) two derivatives from lapachone (NORβ and RC-23) active against *Pseudomonas aeruginosa*. The MIC comparison of the active derivatives (2 - 64μg/mL) with antibacterial agents of clinical use (cyprofloxacin, vancomycin, Cefoperazone, Nitrofuratonine, amikacin and Mezlocilins) showed that these derivatives are promising prototypes to be explored in designing new more potent and safe molecules for the treatment of infections caused by multiresistant strains (Table 1).

In this work, the theoretical analysis included the submission of the active compounds to the analysis of Lipinski Rule of Five that indicates if a chemical compound could be an orally active drug in humans. Our results showed that all active compounds (1a, 1b, 1c, 1e, and 1f) fulfilled this rule (molecular weight = 344.37–409.24, clogP = 3.15–4.11, nHBA = 6–7, and nHBD = 2), similarly to commercial drugs (*i.e.* chloramphenicol, oxacill. in, ampicillin, penicillin G, and linezolid) (not shown).

Table 1: Comparison of the antimicrobial profile (Minimum Inhibitory concentration - MIC) of the systems considering the number of the active compounds, suscetible strains and control antibacterials current on the market .

System	Number of Derivatives		Suscetible strains		MIC (µg/mL)	
	Tested	Active	Name	Characteristics	Active derivatives	Control Antibacterials
Pyrazolo pyridine	41	5	*S.epidermidis* 201	β-lactamase-producers, resistant to Penicillin-G, Oxacillin, Gentamicin and Erytromicin	1a,1b,1c ,1e = 16 µg/mL 1f = 64 µg/mL	Vancomycin= 2 µg/mL Amikacin= 16 µg/mL Cefoperazone=16 µg/mL Nitrofurantoine = 32µg/mL
			S.epidermidis 8126		1a,1b,1c ,1e = 16 µg/mL 1f = 64 µg/mL	Vancomycin = 2 µg/mL Amikacyn= 16 µg/mL Cefoperazone=16 µg/mL Nitrofurantoine = 32µg/mL
Lapachone	6	2	*P. aeruginosa 36408*	β-lactamase-producers, resistant to Imipenem, Meropenem and Carbapenens	RC-22 = 2 µg/mL Norbeta = 4 µg/mL	Mezlocylin = 64µg/mL Cefoperazone = 16 µg/mL
Hydrazone	60	1	*S.aureus* ATCC	resistant to Penicillin G	F13 = 64 µg/mL	Vancomycin= 2 µg/mL Amikacin =16 µg/mL Nitrofurantoine =16 µg/mL Cefoperazone =32 µg/mL
			S.aureus 8148	β-lactamase-producers, resistant and Penicillin-G and Gatifloxacin	F13 = 64 µg/mL	Vancomycin= 2 µg/mL Amikacin =16 µg/mL Nitrofurantoine =16 µg/mL Cefoperazone =32 µg/mL
Quinolone	42	0	-	-	-	-
Chalcone	68	0	-	-	-	-
Thiene-pyridine	41	0	-	-	-	-

Currently there are many approaches that assess a compound drug-likeness based on topological descriptors, fingerprints of molecular drug-likeness structure keys or other properties such as clogP and molecular weight. In the Osiris program (http://www.organic-chemistry.org/prog/peo) the occurrence frequency of each fragment is determined within the collection created by shreddering 3300 traded drugs as well as 15,000 commercially available chemicals (Fluka) yielding a complete list of all available fragments. In this case, positive values point out that the molecule contains predominantly the better fragments, which are frequently present in commercial drugs but not in the non-drug-like collection of Fluka compounds.

In this work, we used the Osiris program for calculating the fragment based drug-likeness of the active compounds also comparing them with penicillin G, chloramphenicol, oxacillin, ampicillin, and linezolid. Interestingly, the pyrazolo derivatives (1a, 1b, 1c, 1e, and 1f) presented better drug-likeness values (from -3.68 to 0.12) than chloramphenicol (-4.61) and linezolid (-4.08) (Figure 1).

In this study we also verified the drugscore, which combines drug-likeness, clogP, logS, molecular weight, and toxicity risks in one value and this may be used to judge the compound's overall potential to qualify for a drug (Figure 1). Our theoretical data showed that 1a–f derivatives presented values once again higher than chloramphenicol and linezolid.

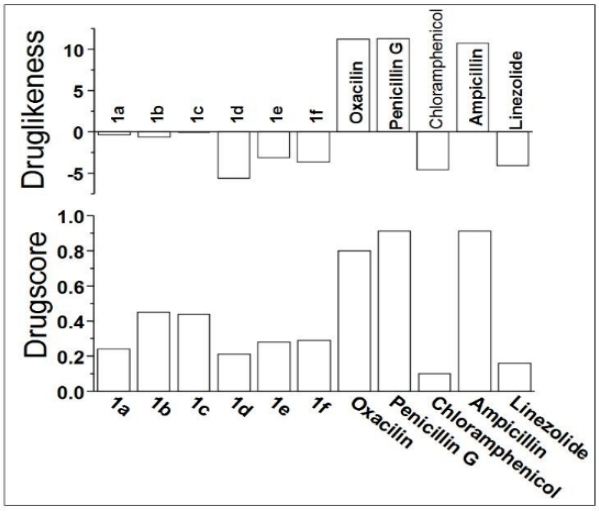

Figure 1: ADMET evaluation of the pyrazolo[3,4-b]pyridine active derivatives with the comparison of the Drugscore and Druglikeness of **1a, 1b, 1c, 1e,** and **1f** and clinic antimicrobials using molecular modeling tools.

Drug toxicity is a factor of great importance for a potential commercial drug, since a significant number of drugs are disapproved in clinical trials based on their high toxicity profile. Herein, we used the Osiris program to predict the overall toxicity of the most active derivatives as it may point to the presence of some fragments generally responsible for the irritant, mutagenic, tumorigenic, or reproductive effects in these molecules.

Interestingly, most of the active derivatives presented a low in silico toxicity risk profile, similar to oxacillin, ampicillin, and penicillin G, and even lower than that observed for chloramphenicol and linezolid. These theoretical data reinforced the cytotoxicity experimental data described in this work pointing these compounds as lead compounds with low cytotoxicity (not shown). The HOMO and electrostatic protential distribution may be contributing for this safer profile (Figure 2).

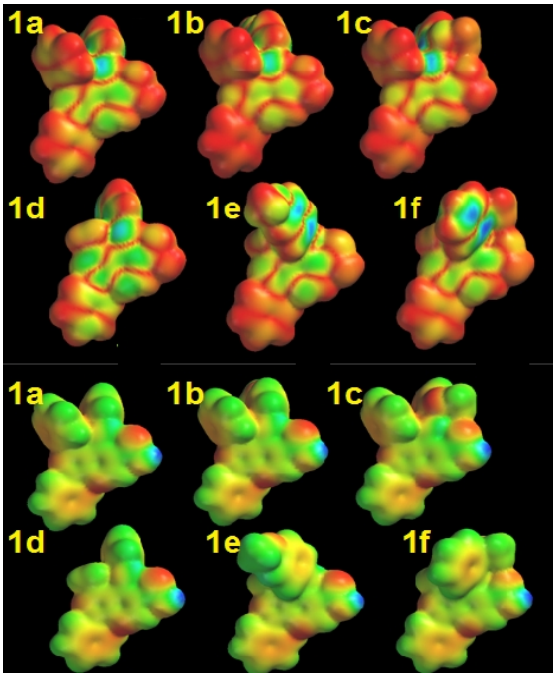

Figure 2: Comparison electronic and structural features of the active series of pyrazolo[3,4-b]pyridine derivatives. HOMO (up) and eletrostatic potential map (down) of the derivatives using molecular modeling tools.

4. CONCLUSION

In this work we evaluated the *in vitro* antimicrobial and *in silico* toxicological profiles of new series of thienopyridine, pyrazolopiridine, quinolones, chalcones, hydrazones and lapachones, Among the 243 products evaluated, the antimicrobial analysis against 14 strains Gram-positive and Gram-negative revealed 8 new active derivatives against different strains. The derivatives had a significant potential profile (MIC = 2-64µg/mL). The pharmacokinetic and toxicological *in silico* analysis of the 8 derivatives suggest a direct relationship between activity and HOMO and electronic distribution. The analysis reinforces the promising potential of these molecules, due to their similary with the antimicrobial agents currently in use in the market with reasonable druglikness drugscore, toxicity and fulfilling the theoretical Lipinski Rule of five.

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