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Original Article

Association study between ceftriaxone and a synthetic amide against strains of *Pseudomonas aeruginosa*

Estudo de associação entre ceftriaxona e uma amida sintética contra cepas de *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a non-lactose fermenting Gram-negative bacteria responsible for causing numerous nosocomial infections. The present research aimed to analyze the anti-Pseudomonas aeruginosa potential of 2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide (A8). The antibacterial potential of A8 was evaluated from the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Association using the checkerboard method. MIC and MBC values were 512 µg/mL for all *P. aeruginosa* strains evaluated, demonstrating predominantly bactericidal activity. Furthermore, when A8 was associated with the drug ceftriaxone, pharmacological additivity and indifference were evidenced. In this sense, the synthetic amide was interesting, since it demonstrates the potential to become a possible candidate for an antimicrobial drug.

Keywords: pharmacology, Gram-negative bacteria, microbiology.

Resumo

Pseudomonas aeruginosa é uma bactéria Gram-negativa não fermentadora de lactose, responsável por causar inúmeras infecções nosocomiais. A presente pesquisa teve como objetivo analisar o potencial anti-*Pseudomonas aeruginosa* da molécula 2-Cloro-N-(4-fluoro-3-nitrofenil)acetamida (A8). O potencial antibacteriano do A8 foi avaliado a partir da Concentração Inibitória Mínima (CIM), Concentração Bactericida Mínima (CBM) e Associação pelo método de *checkboard*. Os valores de CIM e CBM foram de 512 µg/mL para todas as cepas de *P. aeruginosa* avaliadas, demonstrando atividade predominantemente bactericida. Além disso, quando o A8 foi associado ao fármaco ceftriaxona, evidenciou-se aditividade e indiferença farmacológica. Nesse sentido, a amida sintética se mostrou interessante, pois demonstra potencial para se tornar um possível candidato a fármaco antimicrobiano.

Palavras-chave: farmacologia, bactérias Gram-negativas, microbiologia.

1. Introduction

Infections caused by *P. aeruginosa* usually occur in hospitalized people, mainly due to the ability of these microorganisms to persist within many hospital devices. Aside from that, patients with weakened immune systems are also quite vulnerable. Among the main infections caused by these microorganisms are pneumonia, respiratory tract infections, bloodstream infections, surgical site and skin infections (Driscoll et al., 2007; González-Olvera et al., 2019).

The eradication of *P. aeruginosa* has become increasingly difficult due to its remarkable ability to resist antibiotics, limiting treatment options for infections caused by this microorganism. This allows the increasing number of cases of infections to occur. Data show that, per year, around 51,000 healthcare-associated infections (HAIs) caused by *P. aeruginosa* occur in the USA (Bassetti et al., 2018; Tuon et al.,2020). In addition to intrinsic resistance, which includes the low permeability of the outer membrane,

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expression of efflux pumps and the production of enzymes, there are other resistance mechanisms presented by *P. aeruginosa*, such as the acquisition of plasmids that carry resistance genes and mutations (Breidenstein et al., 2011).

Elastase and alkaline protease confer the ability to degrade proteins of the immune system and extracellular matrix, respectively. Pyocyanin favors the increase of oxidative stress in the host (Rada and Leto, 2013; Mulcahy et al., 2014) and the biofilms consisting of sessile cells of the same or different microbial species incorporated into a self-produced matrix of polysaccharides, proteins, lipids and extracellular DNA (eDNA) make it difficult for antimicrobials to penetrate (Flemming and Wingender, 2010).

Currently in clinical practice eight classes of antimicrobials are used in *Pseudomonas aeruginosa* infections. There are aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillins, monobactans, fosfomycin and polymyxin (Ullah et al., 2017). However, the treatment of *P. aeruginosa* infections remains a significant challenge due to this constant increase in antimicrobial resistance. Virulence factors such as bacterial biofilms are a great target for the discovery of new therapies.

Amides are ubiquitous and fundamental components of biological properties (Cordeiro et al., 2020). The high degree of stability of its structure and the conformation of the amides interfere in the structure of peptides and proteins, and consequently in their biological functions, considering that the link between amino acids for the formation of large and complex proteins occurs through amidation modeled from amines and the active esters of amino acid and RNA monomers (Scattolin et al., 2019).

With the growing need for the discovery and development of new therapeutic strategies capable of killing, inhibiting or being used in association with existing antimicrobial therapies in clinical practice for the treatment against *P. aeruginosa*, an interesting alternative is the unprecedented study of the evaluation of the activity antimicrobial activity of Chloro-N-(4-fluor-3-nitrophenyl) acetamide, due to the antimicrobial potential already reported in the literature of chloroacetamides. Several pharmaceutical compounds are formed by amide units, such as ledipasvir and atorvastatin. Carbamates and ureas are compounds related to amides and are of great importance, as they are found in insecticides, polymers, cosmetics, chemotherapy and anti-inflammatory drugs (Scattolin et al., 2019).

Therefore, our objective was to evaluate the biological activity of 2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide against *Pseudomonas aeruginosa*, a new compound belonging to the amide class and with potential antibacterial effect.

2. Materials

2.1. Culture medium

The culture media used were BHI (Brain Heart Infusion) agar and broth, Mueller-Hinton agar and urea agar (DIFCO Laboratories®/USA/France), which were prepared according to the manufacturers' descriptions.

2.2. Strains

For the antibacterial activity assays, 12 strains of *Pseudomonas aeruginosa* were used, identified as: PA-15, PA-19, PA-41, PA-136, PA-163, PA-230, PA-286, PA-356, PA -359, PA362, PA-410, PA411, provided by the biochemical pharmacist Darci de Magalhães Melo, obtained from clinical isolates at the Laboratory of Clinical Pathology HEMATO, located in João Pessoa – PB. Bacterial identification, as well as the analysis of their antibacterial resistance profiles, were performed in the aforementioned laboratory. For control purposes, the standard strain ATCC-27853 (American TypeCultureCollection) was also used, belonging to the MICOTECA collection of the Research Laboratory of Antibacterial and Antifungal Activity of Natural and/or Synthetic Bioactive Products.

2.3. Inoculum

For inoculum preparation, colonies obtained from fresh cultures kept in BHI broth were suspended in sterile 0.85% sodium chloride (NaCl) solution, and adjusted according to McFarland's 0.5 standard, which corresponds to approximately $1.5x10^8$ colony forming units/mL (CFU/mL) (CLSI, 2015; Freire et al., 2014).

2.4. Obtaining 2-Chloro-N-(4-Fluoro-3-nitrophenyl)acetamide

The product 2-Chloro-N-(4-Fluoro-3-nitrophenylacetamide was kindly provided by Prof. Dr. Petrônio Filgueiras de Athayde Filho from the Research Laboratory in Bioenergy and Organic Synthesis of the Center for Exact Sciences and Nature (CCEN) at UFPB and synthesized as described by Cordeiro et al. (2020). In the tests, the substances were weighed and prepared immediately before use by solubilizing in 5% dimethyl sulfoxide (DMSO) and 2% tween 80, completing the final volume with sterilized distilled water in order to obtain a solution final at a concentration of 1024µg/mL (Pereira et al., 2015; Pinheiro et al., 2017).

2.5. Standard antibacterials

The standard antibacterial used in the tests was: Ceftriaxone (TEUTO® Laboratory). For use in the tests, the substance was weighed and properly solubilized in 5% dimethyl sulfoxide (DMSO) and 2% tween 80, completing the final volume with sterilized distilled water in order to obtain a final solution at a concentration of 1024 µg/mL (Pereira et al., 2015; Pinheiro et al., 2017).

3. Methods

3.1. Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the substances was determined using the microdilution technique in a liquid medium in a cell culture plate (TPP/SWITZERLAND/EUROPE) containing 96 wells with a "U" bottom. Initially, 100µL of double-concentrated BHI broth was dispensed into the wells of the microdilution plates. Then, 100µL of the substances were dispensed into the wells of the first row of the plate and, by means of a serial dilution at a ratio of two, concentrations of 1024µg/mL to 0.25µg/mL were obtained.

Finally, 10µL of bacterial strain suspensions (1x10^7 CFU/mL) were added to the wells. Control of strain viability, sterility of the culture medium and diluents was also carried out. The plates were incubated at 35 ± 2 °C for 24 hours (CLSI, 2015).

Then, 20µL of 0.01% resazurin solution (INLAB®), a colorimetric oxide-reduction indicator, was added. After incubation for 1-2h, reading was performed. Viable bacteria reduced the dye, changing the color from blue to pink. The MIC for each product was defined as the lowest concentration capable of visually inhibiting microbial growth and/or verified by the permanence of the indicator dye color (Elshikh et al., 2016).

The assay was performed in triplicate and the results were evaluated according to the analysis performed by Sartoratto et al. (2004) who classify the antimicrobial activity as strong when MIC is less than 600 µg/mL, moderate when the MIC value is between 600 and 1500 µg/mL and weak or inactive when MIC is greater than 1500 µg/mL.

3.2. Determination of the minimum bactericidal concentration (MBC)

After reading the MIC, 10µL aliquots of the supernatants were taken from the wells of the microdilution plates at concentrations corresponding to the MIC, MIC, MIC/2, MIC/4 and MIC/8 of each product for each strain and inoculated into new microdilution plates containing only BHI medium. The assay was performed in triplicate. The plates were incubated at 35 ± 2 °C for 24 hours and then bacterial growth was observed. MBC was defined as the lowest concentration capable of causing complete inhibition of bacterial growth (Pinheiro et al., 2017).

3.3. Association assay of 2-Chloro-N-(4-fluoro-3-nitrophenyl) acetamide with ceftriaxone

The association assay by the checkerboard method is done through microdilution and evaluates the MIC of drugs alone and in combination. From the fractional inhibitory concentration index (IFIC) it is possible to define the nature of the interaction: synergistic, additive, indifferent or antagonistic (EUCAST, 2000; Bonapace et al., 2002).

To perform this assay, 100µL of BHI broth were added to the wells of sterile microplates containing 96 wells, with a "U"-shaped bottom. Then, 100µL of different concentrations (MIC÷8, MIC÷4, MIC÷2, MIC, MIC×2, MIC×4 and MIC×8) da2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide and standard antibacterials were added to the microplate horizontally and vertically, respectively. Finally, 10 µL of bacterial inoculum (1x10^7 CFU/mL) was added to each well. The plates were incubated at 35 ± 2 °C for 24-48 hours and then bacterial growth was observed (Wu et al., 2017).

The checkerboard test results in many different combinations. The value of the most effective combination is determined using the IFIC, which is calculated by adding the fractional inhibitory concentrations (FICs): FICA + FICB, where A represents 2-Chloro-N-(4-fluoro-3-nitrophenyl) acetamide and B the standard antibacterial. The FIC_A, in turn, is calculated through the relationship MIC_A combined/MICA alone, while the FIC_B corresponds to MICB combined/MICB alone (EUCAST, 2000; Bonapace et al., 2002). This index is interpreted as follows: Synergism: IFIC ≤ 0.5; Additivity: 0.5 < IFIC ≤1; Indifference: 1 < IFIC ≤4; Antagonism: IFIC > 4 (WU et al., 2017).

4. Results and Discussion

Assays of antibacterial activity of compound A8 and of the licensed (or standard) antibacterial (ceftriaxone) against the standard strain ATCC 27853; twelve strains of clinical origin of *P. aeruginosa* were evaluated. The results are recorded in Tables 1, 2 and 3.

In Table 1, the results of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of compound A8 are reported.

Table 1. Minimum inhibitory and minimum bactericidal concentration (µg/mL) of 2-Chloro-N(4-fluor-3-nitrophenyl)acetamide (A8) against *P. aeruginosa* strains and classification of the antibacterial effect.

Strains	2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide (A8)			
P.aeruginosa	**MIC (µg/mL)**	**MCB (µg/mL)**	**Ratio MIC/MCB**	**Effect**
ATCC-27853	512	512	01:01	Bactericide
PA-15	512	512	01:01	Bactericide
PA-19	512	512	01:01	Bactericide
PA-41	512	512	01:01	Bactericide
PA-136	512	512	01:01	Bactericide
PA-163	512	512	01:01	Bactericide
PA-230	512	512	01:01	Bactericide
PA-286	512	512	01:01	Bactericide
PA-356	512	512	01:01	Bactericide
PA-359	512	512	01:01	Bactericide
PA-362	512	512	01:01	Bactericide
PA-410	512	512	01:01	Bactericide
PA-411	512	512	01:01	Bactericide

MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration.

It can be seen that the compound A8 at a concentration of 512 µg/mL produced inhibition of the growth of *P.aeruginosa* in all strains used in the microbiological assays, demonstrating a strong antibacterial activity. The MCB of the compound was also 512 µg/mL. Controls for microorganisms and BHI broth were compatible due to the presence and absence of bacterial growth.

When a substance has antibacterial activity, this action can be bacteriostatic or bactericidal in nature, being identified through the MIC/MCB ratio, where a MIC/MCB ratio greater than 1:2 is indicative that the substance acts in a bacteriostatic way. When this ratio is equal to or less than 1:2, the product is considered bactericidal (Flamm et al., 2017). Therefore, the results suggest that starting from the MIC, 2-Chloro-N-(4-fluoro-3-nitrophenyl) acetamide already acts in a bactericidal way.

The MIC and MBC ceftriaxone was determinad. The results obtained are in Table 2.

It is possible to observe that all strains presented the same MIC at 256 µg/mL. Starting from the MIC found and using different concentrations (MIC, MICx2 and MICx4) the MCB for ceftriaxone was performed. The results are also available in Table 2, and it is possible to observe that the MIC/MBC ratio was 1:4, demonstrating that ceftriaxone presented a bacteriostatic action against *P. aeruginosa* strains.

The CLSI (2018) does not establish bacterial growth breakpoints for evaluating the MIC of ceftriaxone against *P. aeruginosa* strains, thus classifying them according to susceptibility. Due to an increased strength profile it is no longer being used, therefore the data has been withdrawn.

For three years in Iran, Karimzadeh et al. (2017) evaluated the pattern of resistance of gram-negative bacteria against various antibiotics, and found that the highest resistance profile of Gram-negative bacteria was against cephalosporins. Corroborating this result, Bhuiya et al. (2018) designed a study to enumerate the comparison of the susceptibility pattern of *P. aeruginosis* isolated from different clinical, environmental and food samples to different antibiotics. As a result, around 70% of the isolates showed intermediate resistance in relation to ceftriaxone and most of them showed a sensitivity profile in relation to carbapenems, with the isolates from environmental and food sources being the ones that had less resistance to antibiotics when compared to the clinical isolates.

In view of the removal of the CLSI (2018) breakpoints for ceftriaxone, reports in the literature evidencing the resistance of clinical isolates of *P. aeruginosa* in relation to ceftriaxone, and observing the high results of MIC and MCB in the present study, one can say that the clinical isolates in the study performed are likely to be resistant to

Table 2. Minimum inhibitory and minimum bactericidal concentration (µg/mL) of ceftriaxone frent strains of *P. aeruginosae* classification of antibacterial effect.

Strains	Ceftriaxone			
P.aeruginosa	**MIC (µg/mL)**	**MBC (µg/mL)**	**Ratio MIC/MBC**	**Effect**
ATCC-27853	256	1,024	01:04	Bacteriostatic
PA-15	256	1,024	01:04	Bacteriostatic
PA-19	256	1,024	01:04	Bacteriostatic
PA-41	256	1,024	01:04	Bacteriostatic
PA-136	256	1,024	01:04	Bacteriostatic
PA-163	256	1,024	01:04	Bacteriostatic
PA-230	256	1,024	01:04	Bacteriostatic
PA-286	256	1,024	01:04	Bacteriostatic
PA-356	256	1,024	01:04	Bacteriostatic
PA-359	256	1,024	01:04	Bacteriostatic
PA-362	256	1,024	01:04	Bacteriostatic
PA-410	256	1,024	01:04	Bacteriostatic
PA-411	256	1,024	01:04	Bacteriostatic

MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration.

Table 3. Fractional inhibitory concentration index (IFIC) of Ceftriaxone in association with 2-Chloro-N-(4-fluor-3-nitrophenyl) acetamide.

P.aeruginosa	***FIC$_A$**	**FIC$_B$**	****IFIC**	**Type of pharmacological association**
	A8	**Ceftriaxone**	**A8+Ceftriaxone**	
ATCC -27853	1	0.125	1.125	Indifference
PA-15	0.5	0.125	0.62	Additivity
PA-136	0.5	0.5	1	Additivity
PA-359	0.5	0.5	1	Additivity
PA-362	0.5	0.5	1	Additivity

*FIC = Fractional Inhibitory Concentration; **IFIC = Fractional Inhibitory Concentration Index.

the cephalosporin tested. It is also observed that a higher percentage of clinical isolates (Table 2) were susceptible to the tested carbapenem - meropenem. Although some strains have shown resistance to it.

Considering the high capacity that *P. aeruginosa* has to adapt to different types of environments, due to hypermutations suffered by it, the presence of virulence factors, such as flagellum, pili, protein, secretion systems, exoenzymes, lectins and mainly formation of bacterial biofilms, the increase in resistance to multiple drugs increases more and more (Al-Wrafy et al., 2017). In addition, Mikhail et al. (2019) observed that the resistance of *P. aeruginosa* isolates may come from the decrease in the outer membrane permeability and/or overexpressed efflux pumps. Although the isolates in the present study are of community origin, the emergence of resistance can be justified based on this strong mutating power of this microorganism, as well as the indiscriminate use of antimicrobials.

Given this global and growing problem of antimicrobial resistance, there is an urgent need to develop new pharmacotherapeutic alternatives to act against these microorganisms. Based on this, 2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide is an interesting substance for investigation of its molecular mechanism of action, since it presented the same minimum inhibitory concentration for all strains, including those that express mechanisms of resistance to conventional antibacterials, and also because there are no reports of its antibacterial activity described in the scientific literature to date, generating the possibility that it acts in such a way as to be classified as a new class of antibacterial agent, in addition to being able to be used as an adjuvant or in association with antibiotic therapy already on the market.

The combination of compounds with intrinsic antibacterial activity or their combination with antibiotics already used in clinical practice may represent a promising alternative for the treatment of infections caused by microorganisms with a resistance profile, enhancing the effectiveness of these antibiotics or even adding benefits such as decreased of adverse reactions, reduction of the drug dose or increase of the spectrum of action (Andrade Júnior et al., 2019).

At the end of the execution and evaluation of the antibacterial activity of 2-Chloro-N-(4-fluor-3-nitrophenyl) acetamide, it was associated with the antimicrobial ceftriaxone. In order to verify whether the sensitivity of the strain to the antimicrobial influenced or not the result of the association. Four different strains were chosen to associate with ceftriaxone. The strains were: PA-15, PA-136, PA-359, PA-362, and ATCC-27853, as can be seen in Table 3.

2-Chloro-N-(4-fluor-3-nitrophenyl)acetamide showed an additive effect when associated with ceftriaxone, with the lowest IFIC ranging between 0.62 and 1, and two strains with indifferent effect, with the lowest IFIC ranging between 1.05 and 1.12. It can be seen, then, that the PA-136 and ATCC-27853 strain were indifferent to the antibacterial tested.

When the result of the association is additivity, it is understood that the sum of the effects of the substances individually occurred. Thus, in additivity, lower concentrations of both molecules are required to obtain the antibacterial effect (EUCAST, 2000) and in the literature there are studies that demonstrate the potential for this effect (Yang et al., 2017). When there is synergism, it is understood that the association results in a greater effect than that observed by each substance individually (EUCAST, 2000), as each one may be contributing to the cell death of microorganisms through mechanisms of different actions (Díaz-Reval et al., 2008).

Combination therapy has proven to be useful against several resistant organisms to achieve maximum antimicrobial effect or even reduce the emergence of resistant microorganisms. The results of this study indicate that A8 in combination with ceftriaxone influenced their effect in an additive or synergistic way, suggesting the use of lower concentrations of the substances and allied to the reduction of their unwanted effects.

5. Conclusion

2-Chloro-N-(4-fluoro-3-nitrophenyl)acetamide has strong antibacterial activity against *P. aeruginosa* strains, including strains resistant to antibacterial already used in clinical practice, with bactericidal action from MIC. The association between ceftriaxone resulted in an additivity action on *P. aeruginosa* strains.

Thus, taking into account the experimental conditions described, A8 showed interesting antibacterial potential, however, more studies need to be carried out to demonstrate the mechanism of action and possible toxicity of this compound in eukaryotic cells.

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