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Artigo

PRELIMINARY INSIGHTS INTO THE ACTION OF NON-ANTIBIOTIC PHARMACEUTICALS IN THE COLONIZATION OF *Pseudomonas aeruginosa* TGC04

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RESUMO

Moléculas com propriedades antimicrobianas, tais como os fármacos não-antibióticos, podem pressionar a adaptação de bactérias no ambiente. Nesse trabalho, cinco fármacos não-antibióticos mais vendidos no Brasil e no Egito em 2020 (losartan 50 mg, ácido acetilsalicílico 100 mg, diclofenaco sódico 50 mg, paracetamol 750 mg e metformina 500 mg e dipirona 100 mg/mL), foram testados contra a linhagem selvagem de *Pseudomonas aeruginosa* TGC04, isolada por nosso grupo e que vem sendo caracterizada ao longo dos anos. Nós avaliamos a inibição da adesão do biofilme durante 48 h, empregando o teste do cristal violeta. A exposição de *P. aeruginosa* TGC04 aos compostos promoveu uma redução moderada da adesão, semelhante, em todas as drogas testadas, perfazendo cerca de 60%. Isto indicou que as células também estavam moderadamente aderentes. Contudo, apesar de todos os fármacos não-antibióticos não terem produzido um efeito biocida à linhagem selvagem, foi observada uma alta atividade antibiofilme, com exceção do paracetamol.

PALAVRAS-CHAVE: Pseudomonadas. Produtos farmacêuticos não-antibióticos. Atividade antibiofilme.

ABSTRACT

Molecules with antimicrobial properties, such as those found in non-antibiotic pharmaceuticals, may pressure bacteria to adapt to the environment. In this work, five of the most sold non-antibiotic pharmaceuticals in Brazil and in Egypt in 2020 (losartan 50 mg, acetylsalicylic acid 100 mg, diclofenac 50 mg, paracetamol 750 mg and metformin 500 mg and dipyrone 100 mg/mL), were tested against the wild strain *Pseudomonas aeruginosa* TGC04, previously isolated by our group and which has been characterized over the years. We evaluated inhibition of biofilm adhesion over 48 h using the crystal violet test. Exposure of *P. aeruginosa* TGC04 to the compounds promoted a similar moderate reduction in adherence for all the pharmaceuticals tested, amounting to about 60%. This indicated that the cells were also moderately adherent. However, although all non-antibiotic pharmaceuticals did not produce a biocidal effect on the wild strain, intense antibiofilm activity was observed, except for paracetamol.

KEYWORDS: Pseudomonads. Non-antibiotic pharmaceuticals. Antibiofilm activity.

INTRODUCTION

Bacteria can grow as free-living and sessile population (1). The preferred bacterial lifestyle choice is complex, strain dependent, and dictated by multiple environmental factors (2). Biofilm lifestyle provides multiple advantages for bacteria and has a relevant virulence mechanism in terms of medically important pathogens (3). In addition, this social lifestyle also favors protection against antimicrobials and other chemical substances, because in addition to the physical barrier, the colony's lifestyle can favor tolerance to different bioactive compounds, through horizontal exchange of resistance genes, magnifying the pathogenicity of certain species (4, 5).

Pseudomonas aeruginosa is a Gram-negative rod widely distributed in nature, being dominant in hostile environments, a factor that promotes it as a leading cause of high mortality rates in the hospital environment (6). Additionally, *P. aeruginosa* is naturally resistant to a variety of compounds and because of this, can adapt and persist in sites with highly selective pressures (7). Thus, this microbe exhibits significant genomic diversity, containing portions of transferable genetic elements greater than 20% of the genome (8), resulting in an indistinguishable difference between clinical isolates and wild isolates (9) as well as damage to clinical isolates when they occur in the natural environment by mechanisms not yet elucidated (10).

In competent cells, natural transformations may occur through acquisition of exogenous DNA. These lead to gene transfer to wild-type strains, which can acquire a certain resistance profile (11). Improper disposal of unused, stored, or expired pharmaceuticals in the environment poses a serious threat to human, animal, and aquatic life (12). Many studies and reviews have linked the inappropriate release of antibiotics in the environment to the emergence of multi- and pan-resistant microbes, commonly called “super bugs”, a popular term that refers to microbes with mutation enhanced morbidity and mortality rates due to critically increased levels of resistance constituting a worldwide concern (13, 14, 15).

The impact of some drugs on the microbiota is not limited to antibiotics (16). This has been known since the 1950s and in recent years has been investigated as a potential chemotherapy alternative using non-conventional drugs, given the concern

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about multidrug-resistant microbes (17). Some non-antibiotic pharmaceuticals have pleiotropic action and, in concentrations found in pharmacokinetic studies in humans, exert antimicrobial and antibiofilm activity against several microbes, especially Gram-positive bacteria and some fungi, by different mechanisms, varying with the species. Among the Gram-negative bacteria, some activity has been registered; however, *P. aeruginosa* seems not to have been much investigated (18).

Non-steroidal anti-inflammatory drugs – NSAIDs (aspirin, diclofenac, and ibuprofen) alter the expression of more than 330 genes or may reduce pyocyanin production in *P. aeruginosa* (19). In addition, a bactericidal effect under high concentrations, caused by the action on the cell wall and membrane, has been observed *in vitro*, with opioids (tramadol) (20), antidepressants (fluoxetine, paroxetine and especially sertraline) (21), beta-blockers and hypolipidemic agents (22). On the other hand, *P. aeruginosa* is more resistant to local anesthetics (23), anticoagulants (24) and antihistamines (25).

Mechanisms against the antimicrobial action of non-antibiotic pharmaceuticals by *P. aeruginosa* are of plasmid origin. The mechanisms are similar to those observed when *P. aeruginosa* is exposed to real antibiotics, such as efflux pumps, expression of enzymes, and alteration of the target protein (26). An understanding of the antimicrobial activity of non-antibiotic pharmaceuticals may help to combat resistant microbes (27), as well as to emphasize the concern about reduced bacterial sensitivity to antibiotics caused by non-specific alterations mediated by non-antibiotic pharmaceuticals (28).

The present work aimed to assess the susceptibility of planktonic cells to non-antibiotic pharmaceuticals. In addition, we researched the reduction of cell adhesion of a wild strain of *P. aeruginosa*, isolated from an environment contaminated by hydrocarbons and high pyocyanin-producer.

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MATERIAL AND METHODS

Strain

The strain *P. aeruginosa* TGC04, isolated from soil contaminated by hydrocarbons (29) and producing up to 685 µg/mL of pyocyanin (30), was used. Maintenance was performed on BHI agar at 4°C. The inoculum was prepared in a 0.85% NaCl solution with standardized turbidity using tube #1 on the MacFarland scale.

Non-antibiotic pharmaceuticals

Based on data from the Brazilian and Egyptian governmental health agencies, the most sold non-antibiotic pharmaceuticals in 2020, common to both countries, were selected, except dipyrone, which is only permitted for use in Brazil (Table 1). The pharmaceuticals used in the study were purchased from drugstores and the standard solutions were prepared by dissolving the tablets or mixing the solutions in sterilized distilled water, without adjusting the pH.

Table 1 – Common medicines most sold in 2020 in Brazil and Egypt and concentrations of standard solutions.

Pharmaceuticals	**Dose**	**Pharmacological Family**	**Concentration (mg/mL)**
Acetylsalicylic acid	100 mg	Non-steroidal anti-inflammatory drug	5.0
Dipyrone	1g/mL	Non-steroidal anti-inflammatory drug	100.0
Losartan	50 mg	Antihypertensive	3.3
Metformin	500 mg	Oral hypoglycemic	50.0
Paracetamol	750 mg	Antipyretic	200.0
Diclofenac	50 mg	Non-steroidal anti-inflammatory drug	2.5

In vitro assay of biofilm formation

The test was carried out by adapting the microdilution test (31) to simulate clinical and environmental concentrations. The wells were filled with 150 µL of Müeller-Hinton

broth, double concentrated; 150 µL of the solution of each drug and 15 µL of the inoculum. The range of concentrations tested were, namely: acetylsalicylic acid (2,500-10 µg/mL), dipyrone (50,000-25 µg/mL), losartan (1,650-103 µg/mL), metformin (25,000-50 µg/mL), paracetamol (100,000-3,125 µg/mL), and diclofenac (1,250-3 µg/mL). The microplates were incubated for 48 hours at 30 °C. Afterwards, the crystal violet test (32) was carried out and the measurement of the absorbance of the crystal violet-ethanol solution was determined at 570 nm (Kasvi, K37-VIS) (33).

Estimation of the microbial population in the biofilm

The relationships between OD_{570} and the mean cell concentration were calculated to verify the effect of non-antibiotic pharmaceuticals on the microbial population of the biofilm. For this, the following premise was used: the number of cells per milliliter equal to $1.9x10^8$, multiplied by the optical density value (570 nm) of the suspension of *P. aeruginosa* TGC04, plus $4x10^6$ (34).

Interpretation and expression of results

All assays were performed in quadruplicate, in two moments, and were expressed as mean ± standard error. The percentage of adhesion reduction was calculated by the ratio between the optical densities obtained in the crystal violet test, treatment, and control. Cells were classified as weakly adhered (if $\leq$ 40.00%), moderately adhered (between 40.01 and 79.99%) or highly adhered (if $\geq$ 80.00%) (35). The degree of biofilm inhibition was classified as strong ($\geq$ 80.00%), moderate (between 40.01 and 79.99%) or weak (if $\leq$ 40.00%) (36). Antibiofilm activity was interpreted as high (between 50 and 500 µg/mL), moderate (between 500 and 1500 µg/mL) or weak (> 1500 µg/mL) (37). The bactericidal or bacteriostatic effect was observed by reducing the number of logarithmic units of the estimated population (31).

RESULTS

Table 2 summarizes the highest percentages of biofilm reduction achieved within the range of concentrations tested for each of the non-antibiotic pharmaceuticals. The Table also presents an interpretation of these findings regarding the type of inhibition,

antibiofilm activity and effect on cells.

Table 2 – Percentage of reduced cell adhesion and classification of activity of non-antibiotic pharmaceuticals tested against *Pseudomonas aeruginosa* TGC04.

Pharmaceuticals (µg/mL)	**Average of highest percentages reduction (±Stand Deviation)**	**Type of inhibition**	**Anti-biofilm activity**	**Effect over cells**
Acetylsalicylic acid (40)	60.88±0.10	Moderate	High	Biostatic
Dipyrone (25)	63.04±0.05	Moderate	High	Biostatic
Losartan (413)	55.72±0.08	Moderate	High	Biostatic
Metformin (200)	58.59±0.09	Moderate	High	Biostatic
Paracetamol (25.000)	63.77±0.09	Moderate	Weak	Biostatic
Sodium diclofenac (20)	56.06±0.08	Moderate	High	Biostatic

All pharmaceuticals exhibited intense antibiofilm activity against *P. aeruginosa* TGC04, except for paracetamol, which showed weak antibiofilm activity. Cells were moderately adhered to the substrate and consequently, the degree of inhibition of biofilm formation was observed to be moderate. Although the percentages of biofilm reduction were all between approximately 55 and 60%, there was a reduction of only one log unit in the relative number of cells, compared to the control (from 10^8 up to 10^7 CFU/mL), revealing a bacteriostatic effect. The bactericidal effect, on the other hand, represented a reduction equal to or greater than three log units. In addition, visually there was no change in the color of the medium in the microplates, presumably due to the absence of pyocyanin.

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DISCUSSION

This study is part of a research project to characterize the strain *P. aeruginosa* TGC04, a wild type hydrocarbonclastic pseudomonad (29), a high pyocyanin producer (38, 39, 40), resistant to exposure to different wavelengths of the visible spectrum (41) and capable of using pesticides (42) and polycyclic aromatic hydrocarbons (43) as a carbon source. In addition, the characterization observed here concerns the susceptibility of *P. aeruginosa* TGC04 surface colonization to non-antibiotic pharmaceuticals.

Due to numerous advantages, biofilm formation is the mode of growth lifestyle of *P. aeruginosa* (44). It is also a virulent factor. For pathogenic bacteria, the formation of biofilm represents a public health problem, since it represents a real resistance to a diverse group of substances, including antibiotics (45). By considering microbial multidrug resistance to antibiotics a worldwide problem, the search for alternative therapies to combat the growth of biofilms opens new horizons in antimicrobial chemotherapy (46).

Wild microbes are constantly exposed to various stressors and environmental selective pressures, regardless of whether they are cosmopolitan or from more distant geographic regions. The ecological niche of these microorganisms influences the composition of their genes more strongly than the habitat. Furthermore, these genes can act as public goods. Additionally, aquatic habitats act as a vehicle in terms of exchange and propagation of antimicrobial resistance genes between microbes that are geographically distant from each other (47). This is true for both fresh water and wastewater (48).

Drugs are bioactive molecules designed to cause a specific effect in certain animal organisms. Daily, large amounts of pharmaceuticals are consumed. Both the active form of a drug and its metabolites are largely discarded in water bodies. As a result, these compounds, entering the public wastewater or drinking water environments, can threaten or cause mutations in non-target organisms, characterizing an important class of aquatic pollutants (49).

Although in this study, the concentrations of non-antibiotic drugs were above the plasma dose or environmental levels needed to be classified as an emerging

contaminant, the lack of changes in the color of the medium, suggestive of the presence of pyocyanin, may indicate that the *P. aeruginosa* TGC04 was neither under oxidative nor nutritional stresses (50). It should be noted, however, that the test in our study lasted 48 hours while the peak of pigment production occurs between 72 and 96 hours (51).

P. aeruginosa exhibits intrinsic resistance to a variety of pharmaceuticals but is also sensitive to lower drug concentrations (52). The outer membrane acts as a barrier against hydrophobic compounds, and this is attributed to LPS. Thus, intact cells, i.e., those without damage to the wall or membrane, are more resistant (53). Losartan (54), dipyrone (55) and diclofenac (56) are hydrophobic, and losartan can also present acidic properties for weeks in aqueous solution. On the other hand, acetylsalicylic acid is amphiphilic (57), while metformin and paracetamol are hydrophilic (58, 59).

The action mechanism of non-antibiotic pharmaceuticals varies from species to species. As an example, it is expected that losartan has no action against *P. aeruginosa* (60), contrary to what was observed in our study. This possibly occurred because different isolates of genetically identical species can exhibit distinct phenotypes due to environmental pressures to which they are exposed (61). Additionally, the exact antimicrobial mechanism has not yet been identified for many non-antibiotic pharmaceuticals, such as NSAIDs, including acetylsalicylic acid (62), dipyrone (63) and paracetamol (64). In the case of dipyrone, as it is banned in many countries, little information is available. NSAIDs are known to act on cyclooxygenase. Thus, we hypothesize that they may have some type of action against oxygenases expressed by *P. aeruginosa*.

In addition, possible activities of diclofenac, such as the inhibition of DNA synthesis or deficiency of membrane activity have been proposed (65). This same mechanism is known to act for metformin in the inner membrane (66), in addition to potentiating the action of antibiotics (67). This result was also observed with acetylsalicylic acid against *P. aeruginosa*. In general, however, NSAIDs do not cause a reduction in antibiotic susceptibility because some NSAIDs are substrates for efflux pumps in Gram-negative bacilli (68). Thus, the risk of non-antibiotic pharmaceuticals promoting cross-resistance is a problem when dealing with pathogens.

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Antimicrobial resistance is believed to occur via horizontal transfer of plasmids through conjugation between intra or intergenerational transfers (22). As a result, there is rapid expression and dissemination of multidrug-resistant phenotypes; it is not clear, how non-antibiotic pharmaceuticals promote this conjugation (69).

Non-antibiotic pharmaceuticals intended for human consumption account for 95% of the pharmaceutical market and their contribution to the spread of microbial resistance to antibiotics is uncertain, but hypothetically clear. Conjugation is correlated with increased reactive oxygen species production and cell membrane permeability, inducing responses similar to those observed in bacteria exposed to antibiotics, for example the efflux mechanism, mutations, abortive mechanisms, changes in external structures, biofilm production, and electrical repulsion (70).

Given this, the risk of inappropriate disposal of pharmaceuticals must be taken seriously, as well as investment in research emphasizing the transfer of resistance genes starting from the contact of the microbiota with non-antibiotic pharmaceuticals that can contribute to the dissemination of multidrug-resistant bacterial phenotypes.

CONCLUSION

The *P. aeruginosa* TGC04 strain formed biofilms but was moderately inhibited by the six non-antibiotic pharmaceuticals tested, in concentrations above those found in the environment, as well as plasma peaks. Even so, the degree of inhibition was considered high, except for paracetamol. However, all compounds produced a bacteriostatic effect.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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