Proceedings Paper

Off-Target Activity of Spiramycin Disarms *Pseudomonas aeruginosa* by Inhibition of Biofilm Formation, Pigment Production and Phenotypic Differentiation [†]

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Abstract: Spiramycin is a 16-membered macrolide used in human medicine as an antibacterial and antiparasitic agent. Spiramycin is effective against bacterial pathogens including Gram-positive and Gram-negative bacteria. In contrast, *Pseudomonas aeruginosa* is intrinsically resistant to macrolides including azithromycin and spiramycin. Despite the results of in vitro susceptibility tests, interest in macrolides in the treatment of some pseudomonal infections arose from both clinical and preclinical studies. For this reason, macrolides have drawn attention as adjunct therapy against chronic and/or biofilm-mediated *P. aeruginosa* infections. While most of the studies on the antivirulence activity of macrolides focus on erythromycin and its derivative azithromycin, there is no information on spiramycin. We used in vitro methods to test the ability of spiramycin to inhibit certain phenotypic features or traits associated with virulence in *P. aeruginosa*. Moreover, we tested spiramycin using *Galleria mellonella* as in vivo model.

Keywords: Spiramycin; *Pseudomonas aeruginosa*; biofilm; antivirulence factor; repurposing

1. Introduction

Macrolides are a class of antibiotics produced by bacteria, such as those of the genus *Streptomyces*. These natural compounds consist of a large macrocyclic lactone ring bound to deoxy sugars. Spiramycin is a 16-membered macrolide used in human medicine as an antibacterial and antiparasitic agent [1–3]. The antibacterial activity of this antibiotic is caused to the ability to bind the 50S ribosomal subunit and block the path through which nascent peptides exit the ribosome [4]. Spiramycin is effective against Gram-positive bacterial pathogens and is also effective against bacteria belonging to the genera *Neisseria*, *Legionella*, *Mycoplasma*, *Chlamydia*, and against *Toxoplasma* spp. [5]. In contrast, *Pseudomonas aeruginosa* is considered intrinsically resistant to macrolides including spiramycin [6].

Macrolides were used in the treatment of infections caused by *P. aeruginosa* in both clinical trials and a mouse model of bacteremia [7,8]. Erythromycin and its derivative azithromycin can inhibit the activity of many virulence factors of *P. aeruginosa* including exotoxin A, protease, elastase, lipase, phospholipase C, lecithinase, gelatinase, DNase, and pyocyanin [8–16]. These findings led to the hypothesis that macrolides may act by downmodulating either the inflammatory response or *P. aeruginosa* virulence [9].

We used several in vitro methods to test the effect of spiramycin on *P. aeruginosa*. Phenotypic traits under study included pigment synthesis, rhamnolipid synthesis, bacterial motility, and biofilm formation. We also used an in vivo infection model, *Galleria mellonella* larvae, to test the effect of spiramycin on *P. aeruginosa* in vivo.

2. Materials and Methods

2.1. Strain and Clulture Conditions

We isolated Pseudomonas aeruginosa GG-7 in a previous study [17]. The medium used for the growth of the bacterium was LB (composition per liter: NaCl 10 g, Tryptone 10 g, Yeast Extract 5 g). This medium was used both as a liquid and solid by adding 15 g per liter of agar. We cultured the bacterium at 37 °C with shaking if necessary (180 rpm).

2.2. Pyocyanin, Pioverdine, Rhamnolipids and Motility Measurement

Pyocyanin production was determined as described previously [18,19] with minor modification. Also, measured the fluoresce intensity to estimate the amount of pyoverdine (Ex: 405 nm, Em 450) [20]. We used the colorimetric analysis of the orcinol reaction to measure the rhamnolipids content [19]. Finally, we performed the motility assay as previously described [19].

2.3. Biofilm Formation and Biomass Estimation

We used hydroxyapatite discs to form the biofilm. We initially grew *P. aeruginosa* in LB liquid overnight (37 °C, 180 rpm). The next morning, we diluted the bacterial suspension 1 in 100. We filled a 24-multiwell (1 mLper well) with the new medium. In each well, we added a hydroxyapatite disk. We incubated the multiwell at 37 °C and 120 rpm for 72 h. After this time, we sampled the hydroxyapatite discs, washed the discs in sterile physiological water three times, and used two biofilm measurement methods. The first measurement we carried out was biomass estimation by counting the CFU/mL with the dilution method. The second measurement we made was the biomass estimation using crystal violet, as previously reported [21].

2.4. In Vivo Expreriment

We used *Galleria mellonella* larvae as an in vivo infection model [22]. We used the procedures previously to carry out the infection of the larvae [23]. We prepared three different samples: (1) control; (2) infected larvae; (3) infected larvae, we injected a suspension of both *P. aeruginosa* and spiramycin (60 μg/mL). After 24 h (at 37 °C) we counted the dead and diseased larvae.

2.5. In Silico Expreriment

We performed docking simulation using SwissDock (http://www.swissdock.ch/). The simulations were performed using spiramycin A as the ligand. We used three protein structures as receptors. These proteins were regulators involved in Quorum Sensing: LasR (4ng2), PqsR (4jvd), and RhlR (AF-P54292-F1).

3. Results

3.1. Influence of Spiramycin on Pigment Production

Initially, we performed MIC (minimal inhibitory concentration) experiments to test the resistance of *P. aeruginosa* to four antibiotics (ampicillin, streptomycin, rifampicin and spiramycin). We inoculated the bacterium in 1 mL of LB and we administered 10 different concentrations of antibiotics:500 μg/mL; 250 μg/mL; 125 μg/mL; 62.5 μg/mL; 31.25 μg/mL; 15.62 μg/mL; 7.8 μg/mL; 3.9 μg/mL; 1.95 μg/mL and 0.98 μg/mL. After 24 h we detected the optical density (O.D.) at 600 nm to estimate the biomass. We noted that spiramycin inhibits the production of pyocyanin, so we measured the absorbance at 520 nm to quantify these green pigments Figure 1. Spiramycin has a small effect on biomass (Figure 1A). There is a marked reduction in pyocyanin (Figure 1B) produced by *P. aeruginosa*. 30 μg/mL of spiramycin already leads to a marked decrease in the pigments produced.
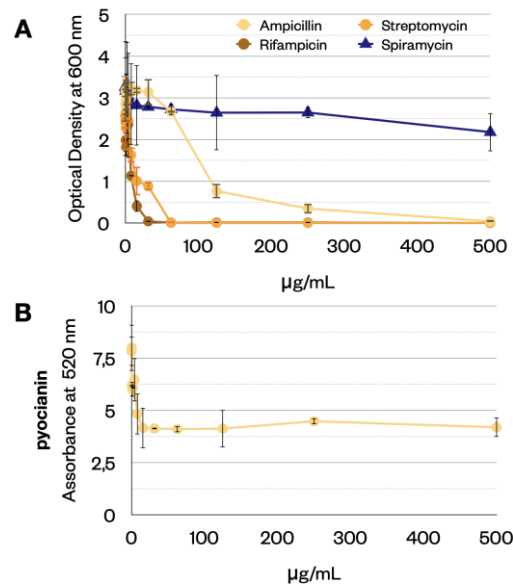
Figure 1. (**A**) Measure of biomass (OD at 600 nm) in the MIC experiment. (**B**) Measure of pyocyanin (absorbance at 520 nm). Error bars show the standard deviation (three replicates).

3.2. Influence of Spiramycin on Biofilm Formation

Some studies have reported that macrolides inhibited the ability of *P. aeruginosa* to form biofilms. We performed biofilm growth experiments to confirm that spiramycin had the same effect. The administration of spiramycin (60 µg/mL) causes a marked reduction in the synthesis of pyocyanin (Table 1) and pyoverdine (Figure 2). Furthermore, the administration of spiramycin (60 µg/mL) inhibits biofilm formation (Table 1) and increases the number of planktonic cells (Table 1).

Table 1. Measurements performed during biofilm growth experiments.

Measure	Control	Spiramycin (60 µg/mL)
Biomass of Biofilm [1]	1×10^6 (±10) [4]	5×10^3(±10) [4]
Planktonic cells [2]	1.5 (±0.37) [4]	2 (±0.21) [4]
Pyocianin [3]	9.22 (±1.54) [4]	4.2 (±0.68) [4]

[1] CFU/support; [2] Total protein content (µg/µL); [3] Absorbance at 520 nm; [4] Standard deviation (three replicates).
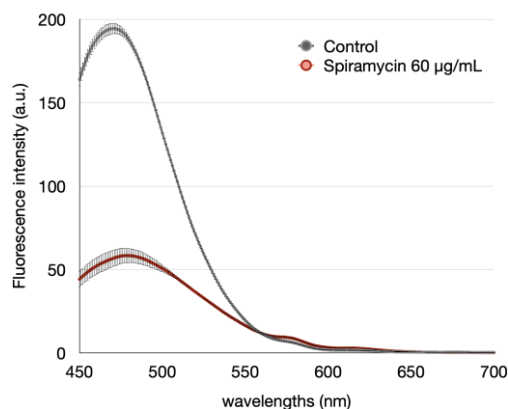

Figure 2. Effect of spiramycin on pyoverdine produced by *P. aeruginosa* in the experiment involving the biofilm formation (Fluorescence intensity, Excitation 405 nm). Error bars show the standard deviation (three replicates).

3.3. Effect of Spiramycin on Motility and Rhamnolipids Production by P. aeruginosa

Some authors had previously shown that azithromycin reduced the motility of *P. aeruginosa* grown on a solid medium [19]. We performed the same experiment using spiramycin to confirm this effect. In the presence of spiramycin (60 µg/mL), the motility of the bacterium is considerably reduced (Figure 3A,B).
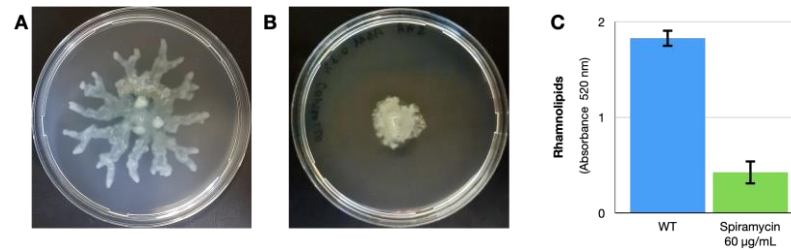

Figure 3. Effect of spiramycin on motility and rhamnolipids production of *P. aeruginosa*. (**A**,**B**) *P. aeruginosa* on the medium described by Gödeke et al., 2013 [19] without (**A**) or with (**B**) spiramycin (60 µg/mL). Error bars show the standard deviation (three replicates).

Rhamnolipids influence swarming motility in *P. aeruginosa* [24], so we measured the rhamnolipids in *P. aeruginosa* cultures. The results confirm that spiramycin (60 µg/mL) also reduces the production of rhamnolipids (Figure 3C).

3.4. Results of Injection of P. aeruginosa in Insect Larvae: Effect of Spiramycin

We observed *G. mellonella* larvae 24 h after infection with *P. aeruginosa*. After 24 h of incubation, we counted the dead or healthy larvae in the three groups: the control group, the group infected with *P. aeruginosa*, and the group infected with *P. aeruginosa* but treated with spiramycin. The results show that spiramycin reduces mortality by about 60%.

3.5. The In-Silico Results Suggest that PqsR Is a New Probable Target for Spiramycin

We hypothesized that possible targets of spiramycin could be the proteins involved in Quorum Sensing. We, therefore, performed some docking simulations to estimate the binding energy between spiramycin and three probable receptors PqsR, RhlR and LasR. SwissDock provides the results as a "*cluster*", a set of positions that the ligand can cover in a specific position on the protein. We analyzed the clusters one by one by selecting only those whose location was compatible with the presence of the binding site. We used all the binding energies calculated for each molecular configuration in the cluster to calculate the mean and standard deviation. The results are reported in Figure 4. As can be seen, the lowest bond energy (and therefore the best bond) is between spiramycin and PqsR.
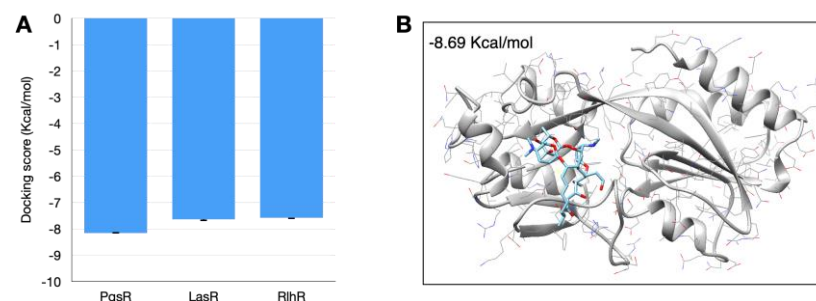

Figure 4. Results of in silico simulation performed with SwissDock. (**A**) binding energy estimation. (**B**) structure of spiramycin (blue) docked with PqsR (gray) showing the docking structure with lower binding energy. Error bars show the standard deviation of the results of the better clusters (lower energy score).

4. Discussion and Conclusions

Our results show as spiramycin inhibits the production of pyocyanin, pyoverdine and rhamnolipids in *P. aeruginosa*. Moreover, the treatment of the bacterium with this antibiotic inhibits biofilm formation in an artificial biomimetic system and blocks the motility on the agar surface. Finally, to test the effect of spiramycin against *P. aeruginosa* in an *in-vivo* system we used the insect *Galleria mellonella* and the preliminary data obtained by this colonization model show a marked reduction in mortality. We explored the probable mechanisms of action of spiramycin against *P. aeruginosa* using computational modeling and docking simulation. Based on the docking data, PqsR could be a target of spiramycin. However, the effects of this molecule on ribosome activity could affect the functionality of the translation causing the observed phenomena. In conclusion, further data are needed to elucidate the mechanism of functioning of spiramycin and other macrolides on *P. aeruginosa*.

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Informed Consent Statement: Not applicable.

Data Availability Statement:

Conflicts of Interest: The authors declare no conflict of interest.

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