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Research Article

Conjugation of LasR Quorum-Sensing Inhibitors with Ciprofloxacin Decreases the Antibiotic Tolerance of *P. aeruginosa* Clinical Strains

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Pseudomonas aeruginosa is a Gram-negative bacterium that commonly infects subjects with weakened immune system causing deadly infections above all at pulmonary level. During infection, *P. aeruginosa* produces a well-organized bacterial structure, called biofilm, activating the quorum-sensing (QS) signaling, a mechanism of gene regulation. In this work, we synthesized already known QS inhibitors (QSi) designed on the scaffold of the N-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) QS molecule and conjugated them with ciprofloxacin to inhibit *P. aeruginosa* biofilm formation and increase the antibiotic susceptibility of clinical strains. We identified, for the first time, a QSi conjugated with ciprofloxacin (**ET37**), that is able to reduce the formation of biofilm and the onset of tolerant clones in *P. aeruginosa* clinical strains. This compound could have a wide application in clinical setting. The possibility to affect biofilm formation in chronically infected patients, such as patients affected by cystic fibrosis, and to reduce the onset of ciprofloxacin resistance would improve patient healing and allow to decrease antibiotic drug dosage.

1. Introduction

Microbial infections can result in complications, such as bacteremia, kidney failure, and toxic shock syndrome. For this, the identification of the systems to counteract bacterial infection is one of the challenges of modern medicine. The absence of novel molecules that control complications due to bacterial infections suggests the defective comprehension of the mechanisms used by bacteria to control host immune response and resist treatment. During host infection, several bacteria organize a bacterial population, and *Pseudomonas aeruginosa* is one of the most commonly studied. *P. aeruginosa* is a Gram-negative bacterium that especially infects subjects with weakened immune system causing deadly

infections above all at pulmonary level. In fact, cystic fibrosis (CF) or HIV patients exhibit increased susceptibility to *P. aeruginosa* lung infections. Since *P. aeruginosa* is a ubiquitous bacterium, exposure to this pathogen in the hospital setting results to be frequent, making it one of the most problematic nosocomial infections.

The antibiotics used to treat *P. aeruginosa* infection include ciprofloxacin, tobramycin, ceftazidime, gentamicin, and imipenem. *P. aeruginosa* bacteria present a high degree of resistance to these antibiotics. Interestingly, the response to ciprofloxacin is very effective at the beginning of the treatment, but high-level resistance is rapidly acquired by *P. aeruginosa*, making the treatment ineffective in the 30% of strains obtained from clinical isolates [1].

The mechanisms that could increase *P. aeruginosa* antibiotic susceptibility still remain unclear [2–4].

P. aeruginosa is characterized by a low permeability of its cell wall that increases its resistance to antibiotics because of a lower drug uptake or higher efflux pumps expression that cause decreased intracellular drug concentrations [5]. Alterations in QRDR of the target sites *DNA gyrase* (*gyrA*) and *topoisomerase IV*, encoded by *parC* and *parE* subunits, are considered the main reason of bacterial resistance to quinolones [6, 7].

The acquisition of resistance to ciprofloxacin is a multistage process in *P. aeruginosa*: in stage I, the exposure to the drug kills the susceptible cells; in stage II, a small population survives antibiotic exposure without increasing the resistance and maintaining a slow growth; in stage III, a the population is reconstituted by a slow-growing population with an increased drug resistance [8]. The appearance of a drug-resistant population limits the possibility to use prolonged therapies with existing or newly developed antibiotic drugs. We suggest the possible use of the inhibitor of quorum-sensing (QS). One of the main defense mechanisms adopted by *P. aeruginosa* is represented by biofilm formation, which allows the bacteria to avoid both host immune system and antibiotics effects [9, 10]. QS is a mechanism of gene regulation sensitive to population density that enables host colonization contrasting the immune surveillance through biofilm formation and the expression of virulence factors [10] via the production of self-generated extracellular signal molecules [11–13]. *P. aeruginosa* is characterized by two QS systems, Las and Rhl, based on acylhomoserine lactone [14] molecules [15]. The las system is controlled by the transcriptional activator LasR and the autoinducer synthase enzyme LasI, which directs the synthesis of N-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL). Similarly, the Rhl system is regulated by the transcriptional activator RhlR and the RhlI AI synthase that synthesizes N-butyryl homoserine lactone (C4-HSL). 3O-C12-HSL binds LasR and activates the LasR/3O-C12-HSL complex; the multimerization will promote the transcription of RhlR, RhlI, LasI genes, and other virulence genes that are connected to the regulon [16–18]. In a similar way, RhlR/C4-HSL complex dimerizes and activates the expression of its own regulon and RhlI [19]. LasR/3O-C12-HSL regulates the quinolone signal by inducing expression of PqsR, as well as PQS [20]. PQS, in turn, increases the transcription of RhlI and the production of C4-HSL [21]. Interestingly, PqsR expression is inhibited by RhlR/C4-HSL [21], suggesting that the ratio between 3O-C12-HSL and C4-HSL concentrations controls the Pqs signaling system.

Since QS molecules are important during infection, the interference on QS signaling represents a potential strategy to contrast bacterial virulence, decreasing antibiotics dosage and facilitating the natural bacterial clearance by host immune response. Brackman et al. have shown that QS inhibitors (QSi) increase the susceptibility of bacterial biofilms to different types of antibiotics [22]. We report the results of our work on the effect of different QSi compounds, designed on the scaffold of QS inducer 3O-C12-HSL, conjugated with ciprofloxacin. These results will set up the initial steps developing new strategies that may subvert the ciprofloxacin resistance of *P. aeruginosa*.

2. Materials and Methods

2.1. Chemical Synthesis. The compounds tested in this work are antagonists of LasR and are designed on 3O-C12-HSL scaffold [23]. Previous evaluations of these derivatives showed that S absolute configuration at the 3-position of the homoserine lactone was important for the activity of the compounds. On the contrary, the R derivative was not effective. For this reason, we synthetized all the compounds in the S absolute configuration. The chemical synthesis was performed as described in Geske et al. [23]. The compounds tested in this study derived from libraries of AHL mimics designed to be capable of intercepting the LasR and RhlR QS system which are specific for *P. aeruginosa*. The LasR antagonists interact with the N-terminal ligand-binding domain of LasR, blocking the binding site for QS molecules [23].

2.2. Samples. Ten sequential *P. aeruginosa* isolates from four patients with CF were chosen from the strains collection of the CF clinic in Hannover. We selected two strains from patients SG (SG1 and SG58, both LasR wild type (wt)), three strains from patients AA (AA2 and AA12 LasR wt and AA11 LasR mutant), three strains from patient TR (TR2 LasR wt and TR1 and TR66 LasR mutants), and two strains from patient KK (KK1 and KK72, both LasR mutants). These strains were characterized in previous work [24]. Patients were checked after the diagnosis of CF, and meanwhile, the respiratory specimens were sampled. The "early" isolates of *P. aeruginosa* strains were collected from the first positive cultures, whereas late isolates were collected 7 to 16 years after colonization or prior to death or lung transplantation. CF isolates behaviour was compared with the laboratory strain PAO1 for their phenotypic diversity [25, 26].

2.3. Phenotypic Analysis for LasR Mutants. Colony surface iridescence and metallic sheen were evaluated and considered as a phenotypic characteristic of LasR mutants [25, 26]. The DNA sequence confirmed the presence of LasR mutations (Table 1).

Table 1: Mutations in LasR in *P. aeruginosa* isolates.

Patients	LasR[a]	*lasR*[b]	LasR[b]
SG1	WT	WT	WT
SG58	WT	WT	WT
AA2	WT	WT	WT
AA12	WT	WT	WT
AA11	MUT	G217A	D73N
TR2	WT	WT	WT
TR1	MUT	C133T	Q45stop
TR66	MUT	C147del	Frameshift
KK1	MUT	T461C	L154P
KK72	MUT	C646T	R216W

[a]LasR status evaluated by phenotypic analysis. [b]Numbering is based on the sequence of LasR gene and LasR protein from PAO1.

2.4. Biofilm Formation Inhibition and Cell Growth Evaluation. *P.aeruginosa* PAO1 or clinical strains were grown in a static culture in M9 medium for liquid culture. Flat-bottomed polystyrene 96-well plates were used for biofilm formation experiments, and optical density measurements were performed in a plate reader at 600 nm. In order to screen compounds for QS inhibition, a microplate-based assay was used. Briefly, *P. aeruginosa* strains were seeded and grown in M9 for 24 hours at 37°C. The optical density (OD600) at inoculation was 0.04. The compounds were dissolved in DMSO added to planktonic cell cultures. Cell growth was evaluated by reading absorbance at 600 nm, while biofilm formation was analysed as previously described [27]. Briefly, after 24 hours of treatment, the planktonic cells were gently removed, and the wells were washed three times with PBS 1x. The plate was let at 60°C for 1 hour in order to dry the biofilm. The biofilm mass was measured by staining with crystal violet 1% w/v and then resuspended in 200 μl of 33% glacial acetic acid and read at 570 nm. 3O-C12-HSL and DMSO were used as positive and negative controls, respectively. Results are reported as mean ± SD and are obtained from three independent experiments.

2.5. Syto9 Assay. *P.aeruginosa* was cultured in 8-chamber slides and treated and left for 24 hours at 37°C. Planktonic cells were removed, and each chamber was washed with PBS 1x. 200 μl of Syto9 working solution prepared by diluting Syto9 stock solution (5 mM in DMSO, Invitrogen) 1 : 1200 was added and kept in dark for 30 minutes at RT. The chambers were then washed two times with PBS 1x, and the biofilm was visualized by fluorescent microscopy (ZOE Fluorescent Cell Imager, Biorad). Pictures of the biofilm were compared to the untreated control.

2.6. Minimal Inhibitory Concentration. The MIC for each strain was determined in triplicate using M.I.C. Evaluator (Oxoid, Basingstoke, Hants, UK) containing ciprofloxacin in final concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015, 0.008, 0.004, 0.002, and 0 μg/ml. The M.I.C. was evaluated after 3 passages in antibiotic-free media [28]. The inoculum was Mueller-Hinton agar inoculated with a standard inoculum (10^5 to 10^6 CFU/ml) according to the European Committee on Antimicrobial Susceptibility Testing guidelines [29].

2.7. Time-Kill Studies. Ciprofloxacin and **ET37** time-kill studies were performed at concentrations equal to the theoretical plasma peak (4 μg/mL), then at concentrations equal to 1/2, 2, 4, 8, X MIC_{50} of the antibiotic used. Bacteria were cultured for 24 h at 37°C on Mueller-Hinton agar (MHB, BioMérieux, France) and used to prepare the exponential growth phase at standard inoculum (10^6 CFU/mL) in Mueller-Hinton broth. The inoculum of 10^6 CFU/ml was supplemented with ciprofloxacin or **ET37** at the different concentrations and cultured for 24 h at 37°C. 100 ml of culture supernatants were collected at 2, 4, 6, and 24 h and plated on agar for colony counts.

2.8. Pyocyanin and Elastase Assay. *P. aeruginosa* strains were cultured overnight at 37°C with shaking. Cultures were back diluted 1 : 1,000 into fresh medium and grown for 18 h. The cells were pelleted by centrifugation, and the culture supernatants were filtered through 0.22 μm filters. The production of pyocyanin was reported as A_{380}/A_{600} ratio [30]. The production of LasB elastase was assessed through the measurement of elastase activity using elastin-Congo red and reported as A_{495}/A_{600} ratio [31].

2.9. GyrA and ParC Amplification and DNA Sequencing. QRDR amplification of gyrA and parC from resistant and intermediate isolates was carried out using specific primers: GyrA-1 (5′-GTGTGCTTTATGCCATGAG-3′) and GyrA-2 (5′-GGTTTCCTTTTCCAGGTC-3′) for the amplification of 287 bp of the fluoroquinolone resistance-determining region of the gyrA gene and ParC-1 (5′-CATCGTCTACGCCATGAG-3′) and ParC-2 (5′-AGCAGCACCTCGGAATAG-3′) were used to amplify 267 bp of the fluoroquinolone resistance-determining region of ParC as previously reported [14]. Amplified products were then separated using 1.5% agarose gels, and PCR products were sequenced, and the sequence of each of the sample was compared with *P. aeruginosa* PAO1 sequence. The sequences were multiple aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw) in order to detect mutations. Nucleotide sequences were translated by Expasy Bioinformatics Resource Portal (http://web.expasy.org/translate/) then compared with *P. aeruginosa* PAO1 protein sequence using ClustalW2 to find changes in amino acid sequences.

2.10. Disk Diffusion Ciprofloxacin Susceptibility Testing. The antibiotic susceptibility of bacterial isolates was determined using the disk diffusion method standardized according to the EUCAST (http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/), with interpretation based on the EUCAST Clinical Breakpoint Tables v. 9.0, valid from 2019 to 01-01, the zone diameter breakpoint >26 for susceptible strains (*S*), and <26 for resistant strains (*R*). The bacterial strains were inoculated onto Mueller-Hinton agar. Antibiotic disks were placed on the

surface, and this was followed by incubation at 35 ± 1°C in air for 18 ± 2 h ours. Inhibition zone diameter values were read by a pair of calipers, in millimeters to one decimal place. Ciprofloxacin disks (5 μg) were obtained from Oxoid Ltd (Oxoid AB, Hampshire, UK).

2.11. Determination of Minimum Biofilm Inhibitory Concentration (MBIC). The MBICs of ciprofloxacin and **ET37** were determined in PAO1 strain, as previously reported [32]. The experiments were done in 96-well polystyrene microtiter plates with round bottoms. An overnight culture with a turbidity equivalent to that of a 0.5 McFarland standard, obtained with TSB, was aliquoted into the wells of microtiter plates. The plates were incubated for 24 h at 37°C. The wells were washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria and dried in an inverted position. Volumes of 100 μL of appropriate two-fold dilutions of the ciprofloxacin or **ET37** in Mueller-Hinton broth were transferred into the dried wells with established biofilms. The microtiter plates were incubated for 18–20 hours at 37°C, and minimum biofilm inhibitory concentration (MBIC) was determined, which corresponds to the lowest concentration of antibiotic which inhibits growth of biofilm cells as indicated by absence of visible growth in the wells. A positive control and a negative control were included in all experiments. The experiment was repeated three times.

2.12. Determination of Minimum Duration for Killing 99% of the Population (MDK_{99}). MDK_{99} was determined by measuring the time to kill 99% of the population [33]. MDK99 was tested in *P. aeruginosa* clinical strains with concentration 1x the MIC of ciprofloxacin or 1x the MIC **ET37** in *P. aeruginosa* wild-type and LasR mutant clinical strains.

3. Results

3.1. Effect of LasR Antagonists on Biofilm Formation in P. aeruginosa PAO1 Laboratory Strain. We selected 4 compounds reported to be active antagonists of LasR of *P. aeruginosa* and designed on 3-O-C12-HLS scaffold (Figure 1, # **1, 2, 3, 4**) [23]. The synthesis was performed as described in Geske et al. and references cited therein.

We evaluated the effect of the selected 4 LasR antagonists on cell growth and biofilm formation of *P. aeruginosa* PAO1 laboratory strain. We treated PAO1 immediately after seeding the planktonic bacteria with LasR antagonists at different concentrations (0.1, 1.0, 10.0, 25.0, and 50.0 μM) for 24 hrs. We evaluated cell growth by reading the optical density at 600 nm, and we observed no effect on this parameter (data not shown). Considering biofilm formation, the treatment with number **3** and number **4** compounds reduced the formation of biofilm of more than 50% (Figure 2(a)) ($p < 0.0001$; Student's t-test). In particular, in the concentration range of 1.0–10.0 μM, we observed the highest effect on biofilm inhibition for both number **3** and number **4** compounds. On the contrary, compounds number 1 and 2 failed to significantly inhibit biofilm formation, also increasing the time of exposure to 48–72 hrs (data not shown). On the basis of these results, we selected number **3** and number **4** compounds and the range of concentrations 1.0–10.0 μM for further investigations on clinical isolates.

3.1.1. Effect of Number 3 and Number 4 Compounds on Biofilm Formation in Clinical Strains. First, we evaluate the efficacy of QSi in acting as LasR antagonists in *P. aeruginosa* clinical strains. We selected two subgroups of *P. aeruginosa* clinical strains: (i) wild type for the expression of 3O-C12-HSL receptor (LasR); (ii) *P. aeruginosa* LasR mutant. LasR mutant clinical strains were phenotypically evidenced as producing iridescent and metallic colonies [34, 35]. We performed our analysis on 10 different isolates from four CF individuals (SG, AA, TR, and KK) wild type or mutant for LasR. In order to evaluate LasR functionality, we treated wild-type ($N = 5$) and LasR mutant ($N = 5$) clinical strains immediately after seeding the planktonic bacteria with LasR agonist (1.0, 5.0, 10.0, 25.0, and 50.0 μM) for 24 hours. We analysed cell growth by reading the optical density at 600 nm, and we observed no effect on this parameter (data not shown). Concerning biofilm formation in wild-type strains, we observed biofilm inhibition of 60% with the compound number **3** at the concentrations of 5.0–10.0 μM (Figure 2(b)) and compound number **4** reduced biofilm formation of the 30–40% (Figure 2(b)). We observed also a reduction in biofilm formation in LasR mutant clinical strains treated with the same two compounds (number **3** and **4**) at the concentrations of 5.0–10.0 μM, reporting a decrease of the 30% and 60–70%, when treated with LasR antagonist number **3** and **4**, respectively, (Figure 2(c)). To confirm the results obtained by crystal violet staining, we performed the Syto9 assay, based on fluorescence staining. We choose to use Syto9 staining to confirm our data and to overcome the variability of the results that could arise by crystal violet staining of *P. aeruginosa* biofilm [36]. Similarly, Syto9 assay showed a clear decrease of biofilm formation after the addition of the compounds number **3** and **4** in both wild-type and mutant strains (Figure 2(d)), with the highest effect observed at the concentration of 10.0 μM.

Since QS inhibition results in a reduced expression of the virulence factors pyocyanin and elastase and in an alteration of biofilm morphology/phenotype [37], the effect of compound numbers **3** and **4** was tested also on the expression of these genes. We observed that both compound numbers **3** and **4** were able to reduce the production of pyocyanin and elastase in wild-type and LasR mutant strains (Figure 3).

3.1.2. ET37 Effect on PAO1 Strain Susceptibility to Ciprofloxacin. Ciprofloxacin belongs to the group of fluoroquinolones, active against bacterial infections. It has demonstrated a high efficacy towards *P. aeruginosa* infection and experienced in clinical practice. Since both compounds **3** and **4** resulted efficient in decreasing biofilm formation, we selected both of them to associate with ciprofloxacin, on the basis of its chemical structure. The chimeric derivatives

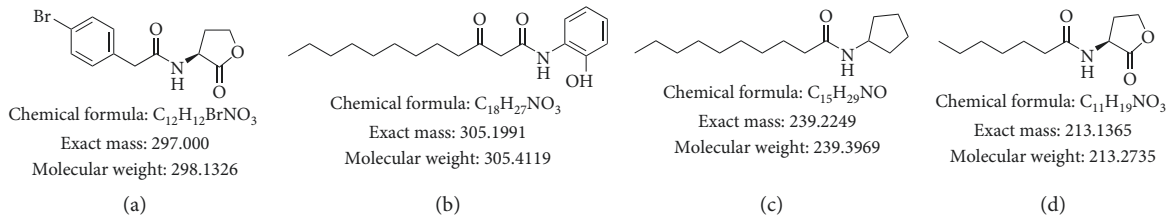

FIGURE 1: Molecular structure of *P. aeruginosa* QS antagonists (#**1, 2, 3, 4**) designed on 3-O-C12-HSL scaffold. (a) LasR antagonist #1. (b) LasR antagonist #2. (c) LasR antagonist #3. (d) LasR antagonist #4.

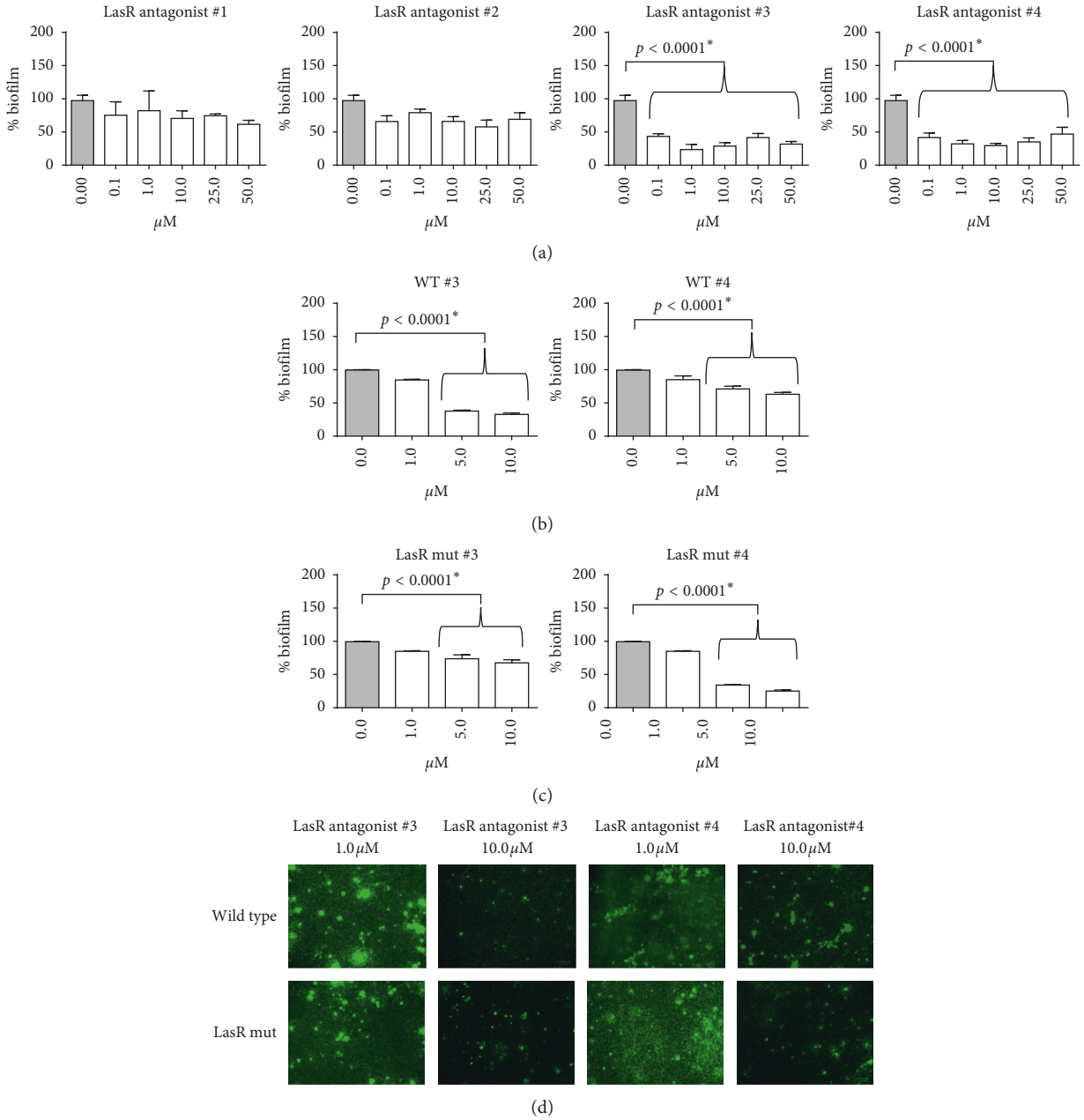

FIGURE 2: Evaluation of biofilm mass after LasR antagonists (#**1, 2, 3, 4**) treatment on (a) *P. aeruginosa* PAO1 strain and after treatment with number **3** and number **4** compounds on (b) wild-type and (c) LasR mutant clinical strains, using crystal violet assay. (d) Evaluation of biofilm mass after treatment with number **3** and number **4** compounds, using Syto9 assay in LasR wild-type (upper line) and mutant clinical (lower line) strains. *Student's *t*-test.

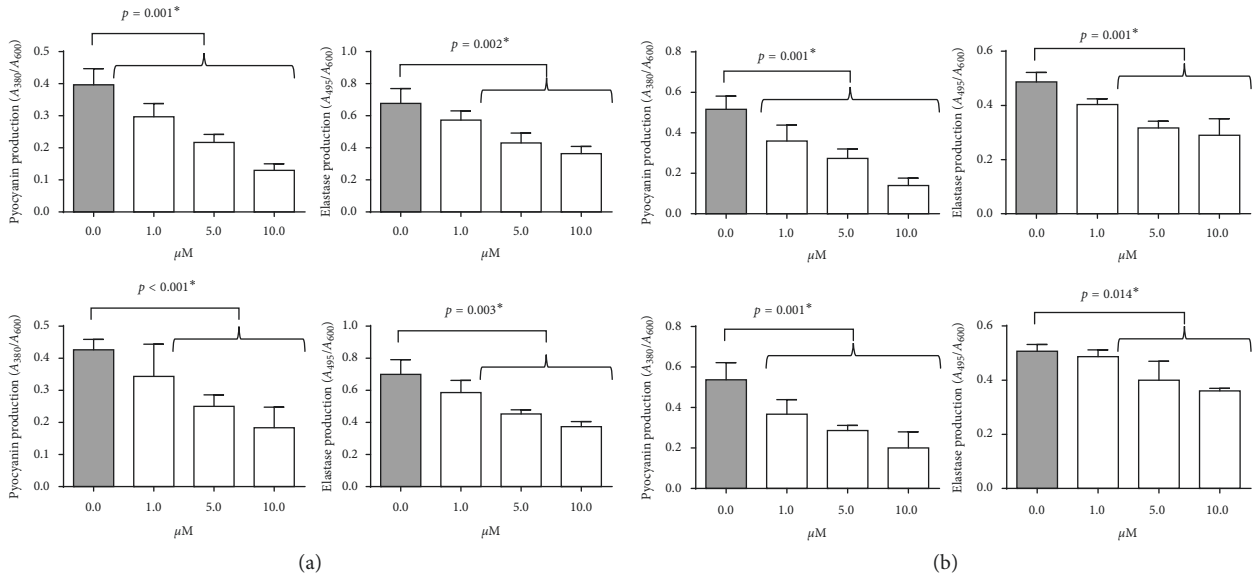

Figure 3: Evaluation of pyocyanin and elastin production after compound number **3** (upper panels) or compound number **4** (lower panels) treatment of (a) wild-type and (b) LasR mutant clinical strains. *Student's *t*-test.

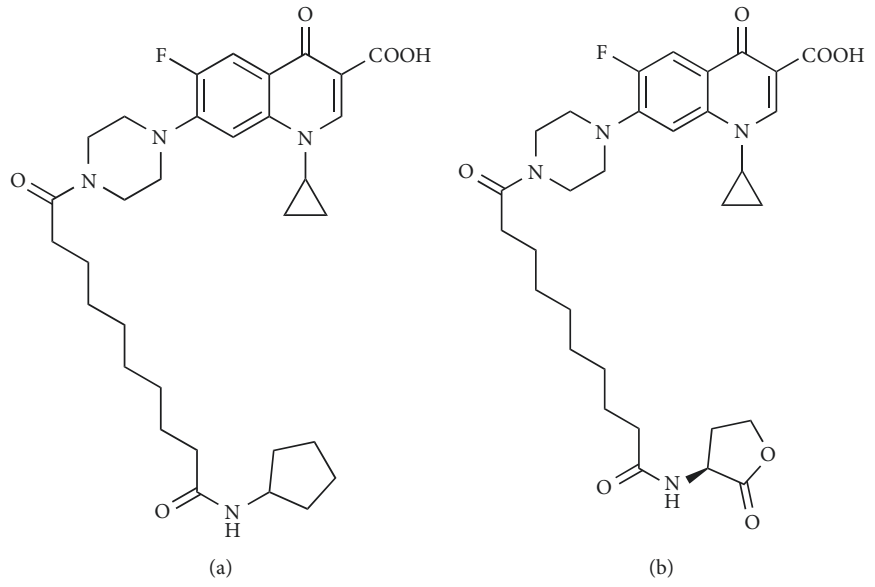

Figure 4: The chimeric derivatives between compounds **3** and **4** and ciprofloxacin. (a) ET-37. (b) ET-39.

between compounds **3** and **4** and ciprofloxacin are depicted in Figure 4.

The molecules **ET37** and **ET39** were synthesized as depicted in Figure 5; the decanedioic acid monoethylester **5** was condensed to the amine moiety of the piperazine ring present in the commercially available ciprofloxacin antibiotics to obtain compound **6** that was subjected to 2N NaOH saponification to achieve acid derivative **7** [38]. Finally, compound **7** was condensed to the cyclopentylamine and L-homoserine lactone using WSC/HOBt as activating agents to obtain, respectively, compound **ET37** and compound **ET39** that were fully characterized by NMR and exact mass spectrometry.

ET37 and **ET39** were preliminarily tested on PAO1 and *P. aeruginosa* clinical strains. **ET37** maintained its ability to decrease biofilm formation in PAO1 (Figure 6(a)), wild-type (Figure 6(b)) and LasR mutant clinical strains (Figure 6(c)). On the contrary, **ET39** lost the efficacy of compound number **4** (Figure 6). Similarly, pyocyanin and elastin were reduced by **ET37** treatment in both wild-type (Figure 7(a)) and LasR mutant clinical strains (Figure 7(b)). Starting with PAO1 strain, we evaluated the minimal inhibitory ciprofloxacin concentration [28] using the Etest to establish a baseline. The MIC_{50} was within the expected ranges, with the PAO1 MIC_{50} at 0.5 μg/ml ciprofloxacin, corroborating previous research [39]

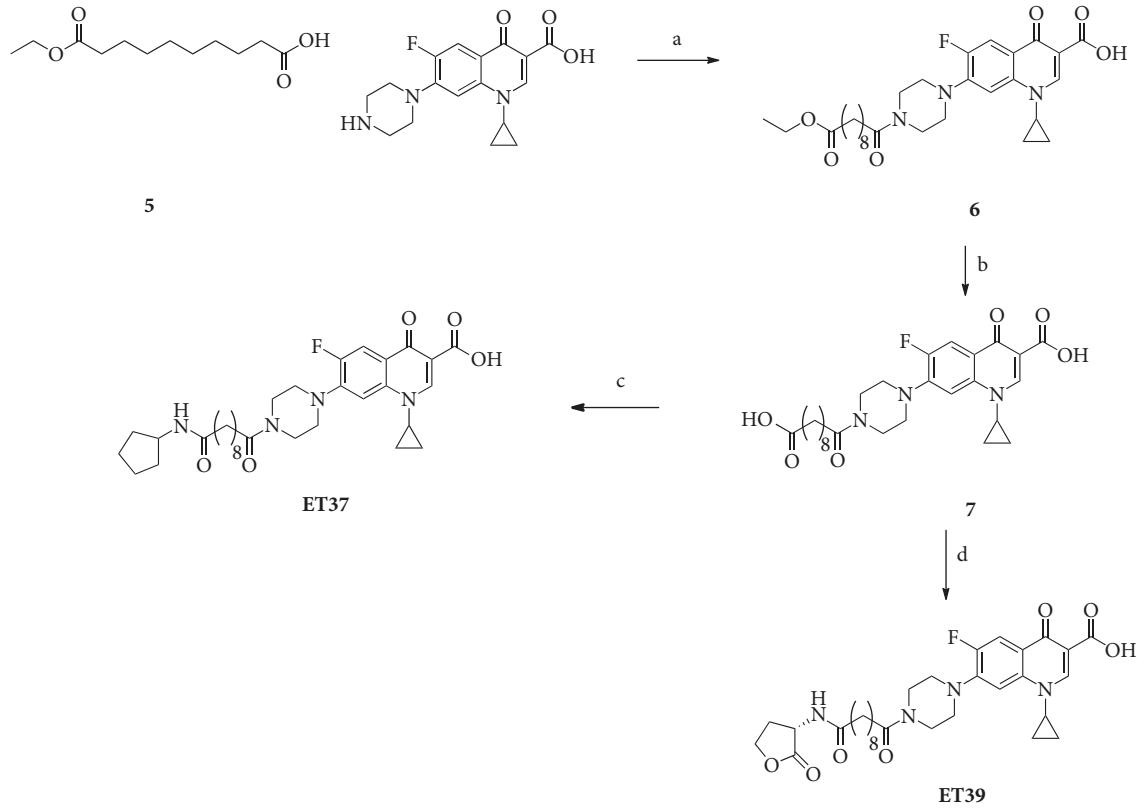

Figure 5: Schematic synthesis of **ET37** and **ET39** compounds. Conditions: (a) WSC/HOBt, DMF, 0°C, o.n., $Y = 88\%$. (b) NaOH 2N, EtOH, r.t. 4 h, Y = quant. (c) WSC/HOBt, cyclopentylamine, DMF, 0°C, 12 h, $Y = 71\%$. (d) WSC/HOBt, L-homoserine, NMO, DMF, 12°h, $Y = 63\%$.

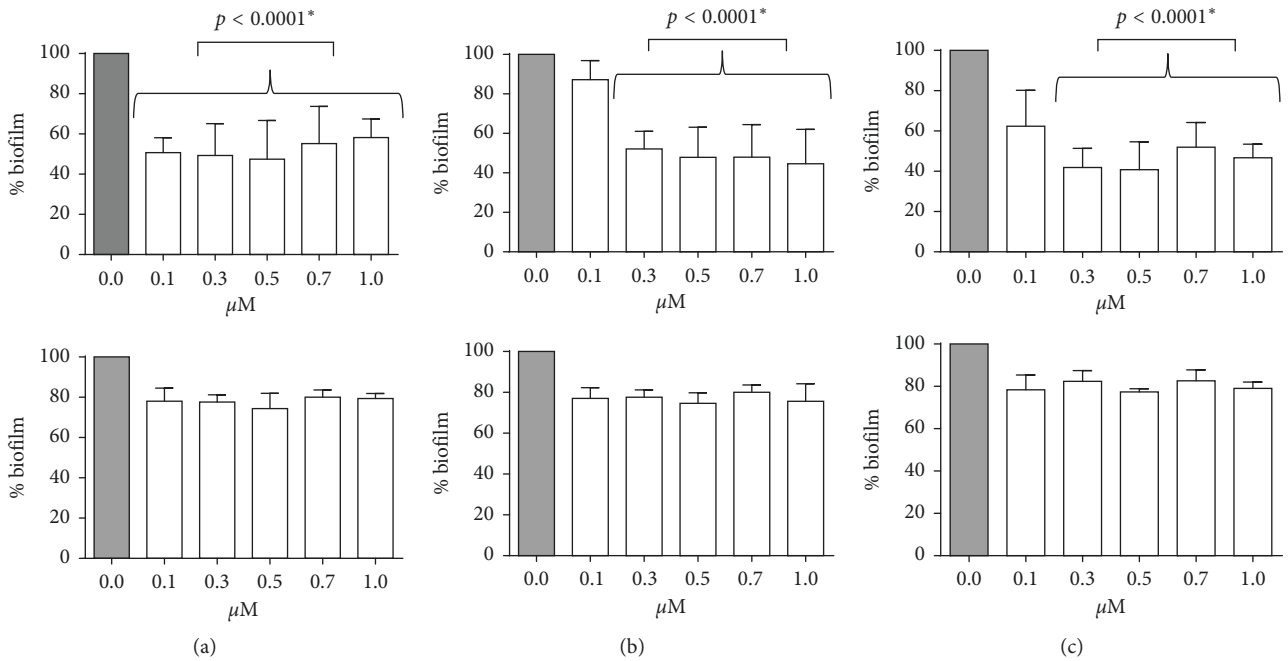

Figure 6: Evaluation of biofilm mass after **ET37** (upper panel) or **ET39** (lower panel) treatment on (a) *P. aeruginosa* PAO1, (b) wild-type, and (c) LasR mutant clinical strains, using crystal violet assay. *Student's *t*-test.

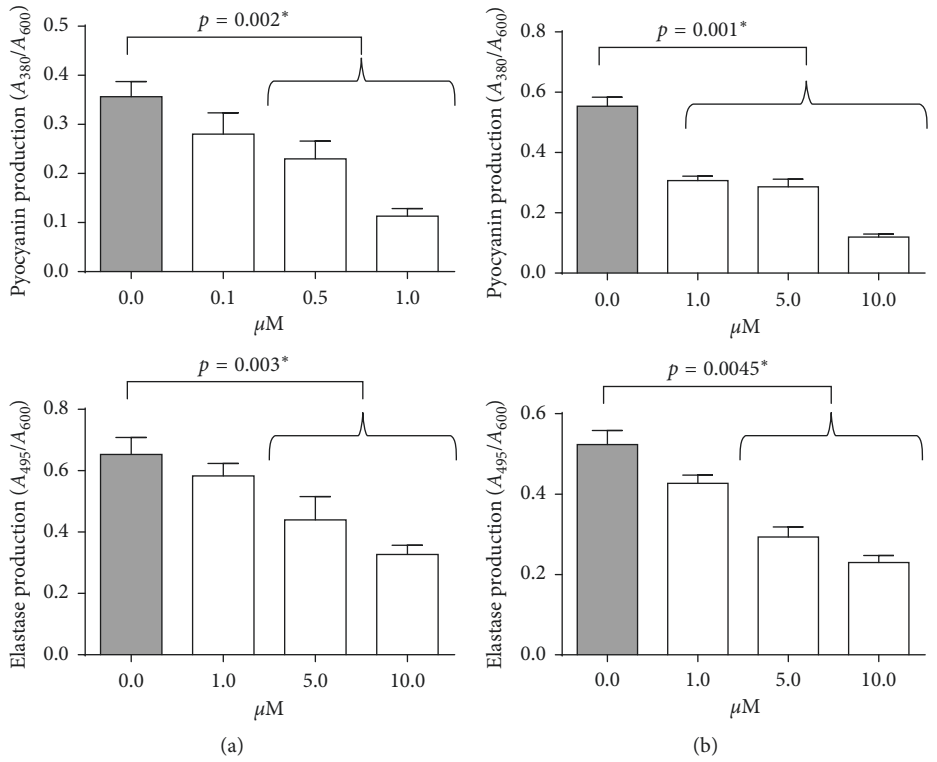

Figure 7: Evaluation of pyocyanin (upper panels) and elastin (lower panels) production after compound **ET37** treatment of (a) wild-type and (b) LasR mutant clinical strains. *Student's *t*-test.

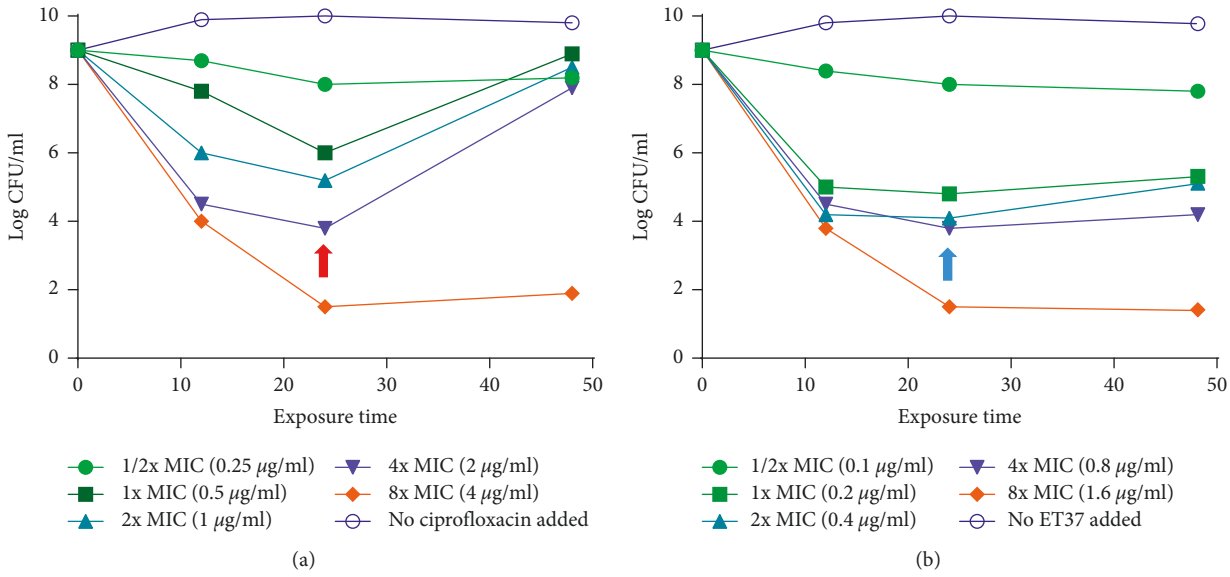

Figure 8: Time-kill studies with PAO1 with concentration ranging from 0.5 to 8x the MIC of (a) ciprofloxacin or (b) **ET37** treatment. Red arrow: "drug-tolerant" cells in ciprofloxacin-treated PAO1; blue arrow: "drug-tolerant" cells in **ET37**-treated PAO1.

and at 0.2 μg/ml ET37. The MIC_{90} and modal MIC were $\geq$32 μg/ml.

We evaluate the population dynamics in response to ciprofloxacin unconjugated and conjugated to compound number **3** (**ET37**). The exposure was at concentrations ranging from 0.5 to 8x the MIC in PAO1 strain (Figure 8). After the treatment with ciprofloxacin, a large part of the bacteria was killed by the drug (Figure 8(a)), as the expected response to an antibiotic treatment with an efficacy of 24 h, as illustrated in Figure 5(a). At lower exposure levels, there was a surviving subpopulation of "drug-tolerant" cells, that survived after ciprofloxacin treatment (Figure 8(a), red

arrow). The "drug-tolerant" cell population was not present in **ET37** treated PAO1 cultures (Figure 8(b), blue arrow).

Previous data showed that ciprofloxacin reduced virulence factors and biofilm formation decreasing the production of QS signal molecules in *P. aeruginosa* [40]. We tested the minimal biofilm inhibitory concentration (MBIC) of both ciprofloxacin and **ET37** on PAO1. Results demonstrated that **ET37** MBIC was similar to MIC_{50} (0.22 μg/ml) while ciprofloxacin MICB was eight times higher (4.0 μg/ml) than MIC_{50} (0.5 μg/ml).

3.1.3. ET37 Effect on P. aeruginosa Clinical Strains Susceptibility to Ciprofloxacin. The efficacy of ciprofloxacin was then tested also on *P. aeruginosa* clinical strains. The resulting MIC_{50} are reported in Table 2.

Three strains were resistant to ciprofloxacin (TR2, TR1, and KK72), three where intermediate (AA2, TR66, and KK1), and four were susceptible (SG1, SG58, AA12, and AA11). We evaluated the genetic background at the basis of ciprofloxacin resistance. QRDRs regions of GyrA and ParC, implicated in *P. aeruginosa* resistance to quinolone [6, 7], were amplified from resistant isolates and were sequenced for mutations involved in ciprofloxacin resistance. One strain resistant to ciprofloxacin (TR1) had a mutation in GyrA (Thr83⟶Ile) and a mutation in ParC (Ser87⟶ Leu or Trp). Two strains resistant to ciprofloxacin (TR2 and KK72) and three intermediate strains (AA2, TR66, and KK1) had a mutation in GyrA (Thr83⟶Ile).

When we evaluated the time-kill studies with the **ET37** concentration of 4 μg/ml, equal to the theoretical plasma peak [41], we observed 6-log decrease at 24 h for strain SG1, SG58, AA12, and AA11 (Figure 9), which are the most susceptible according to their MICs (Table 2). Interestingly, a bactericidal activity was observed with 3.0-log inoculum reduction at 12 h for strains AA2 and TR66, that were categorized as intermediate and TR2 and KK72, that were considered resistant to ciprofloxacin (Table 2) (Figure 9). A bacterial growth reoccurred at 24 h for strains TR1 and KK1 (Figure 9), the most resistant strains according to their MICs (Table 2).

3.1.4. Time-Kill Studies on P. aeruginosa Clinical Strains. Time-kill studies were performed in parallel, on *P. aeruginosa* clinical strains, with 1/2, 2, 4, 8, X MIC_{50}, for both ciprofloxacin and **ET37** (Table 2). Following 24 h treatment with ciprofloxacin, there was a surviving subpopulation of "drug-tolerant" cells only in low-dose ciprofloxacin-treated *P. aeruginosa* clinical strains (Figure 10(a)), while both wild-type and LasR mutant clinical strains did not change over 48 h treatment with **ET37** (Figure 10(b)).

The clinical strains were then tested for their tolerance to ciprofloxacin or ET37 treatment. The measured MDK_{99} decreased in the **ET37**-treated strains in comparison with the ciprofloxacin-treated strains (Figure 11). Both *P. aeruginosa* wild-type and LasR mutant clinical strains showed a MDK_{99} of 12 hours after treatment with **ET37** in comparison with the MDK_{99} of 24 hours observed in the same strains without **ET37** treatment ($p < 0.1$).

Table 2: MIC (μg/ml) of the *P. aeruginosa* clinical strains.

Clinical strain	LasR status	MIC_{50} cipro	Diameter inhibitory zone (mm)*	Categorization
SG1	WT	0.12	32	S
SG58	WT	0.25	33	S
AA2	WT	1.0	27	I
AA12	WT	0.008	36	S
AA11	MUT	0.5	34	S
TR2	WT	2.0	10	R
TR1	MUT	2.0	13	R
TR66	MUT	1.0	27	I
KK1	MUT	1.0	27	I
KK72	MUT	2.0	11	R

*According to the EUCAST Clinical Breakpoint Tables v. 9.0, valid from 2019 to 01-01, the zone diameter breakpoint >26 for susceptible strains (*S*) and <26 for resistant strains (*R*).

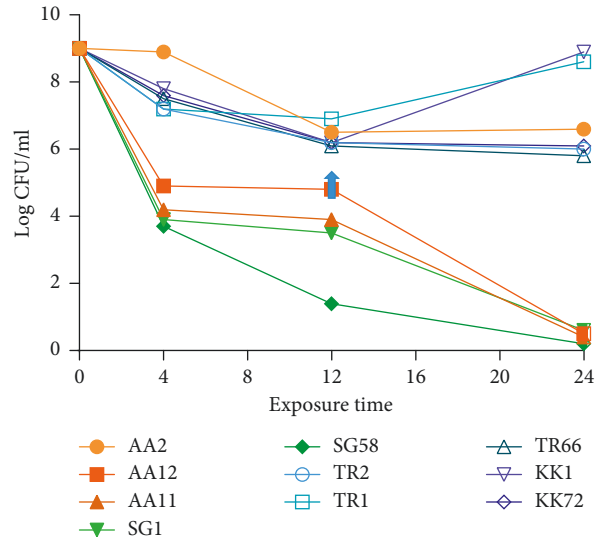

Figure 9: Time-kill studies with *P. aeruginosa* clinical strains with a concentration of **ET37** equal to the theoretical plasma peak (4 μg/ml). Blue arrow: "drug-tolerant" cells in **ET37** treated *P. aeruginosa* clinical strains.

4. Discussion

In this research, for the first time, we analysed the effect of selected LasR antagonists, on *P. aeruginosa* clinical strains, and we identified two compounds designed on 3O-C12-HSL scaffold, number **3** and number **4**, which exhibit an inhibitory effect on both LasR wild-type and mutant strains. These LasR antogonists interact with the N-terminal ligand-binding domain of LasR, blocking the binding site for QS molecules.

Since clinical *P. aeruginosa* strains are commonly LasR mutants, it is fundamental to obtain QSi with a demonstrated functionality also in LasR mutant clinical strains. Both number **3** and **4** compounds are able to counteract biofilm formation also in LasR mutant strains, possibly due to the shorter aliphatic chain that results in a lower steric hindrance that could facilitate the cross interaction with RhlR [17, 18]. In fact, it has been reported that LasR antagonist could be effective also on other QS receptors, as

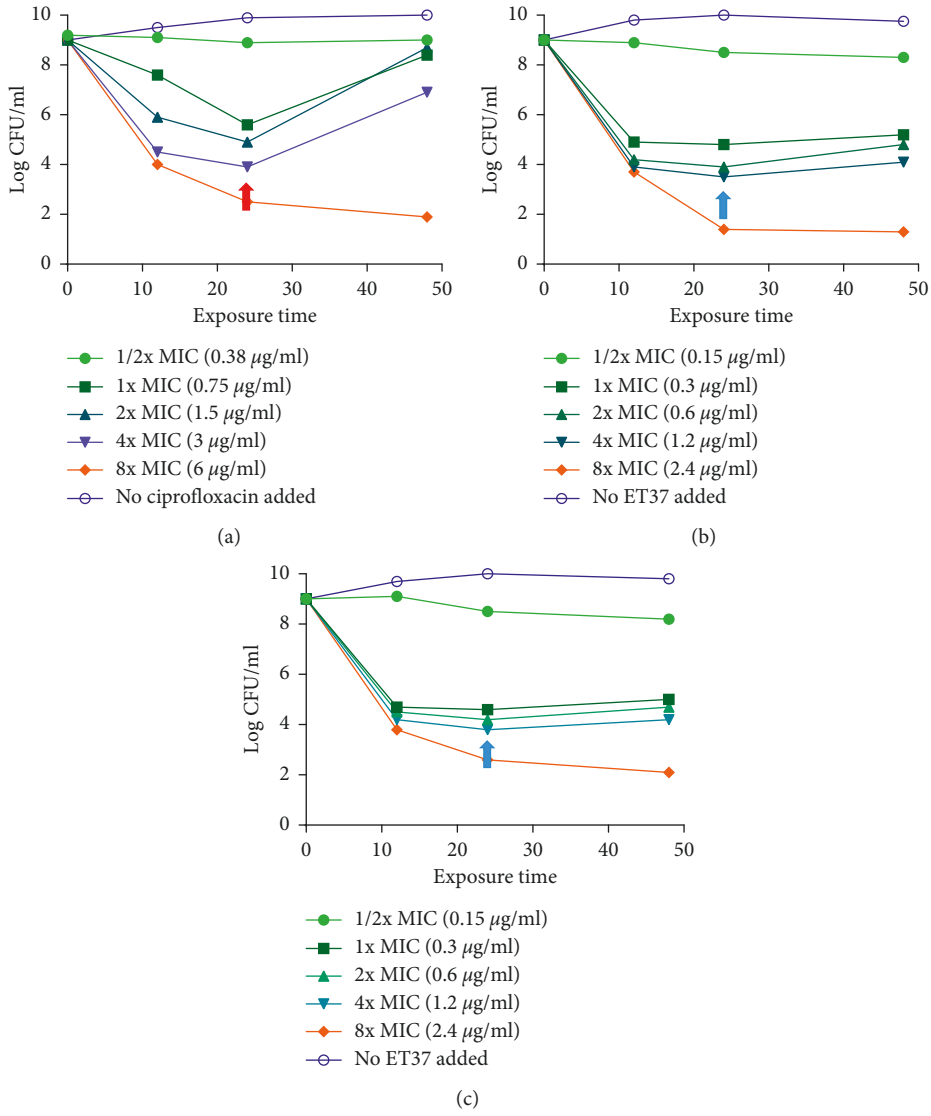

Figure 10: (a) Time-kill studies with *P. aeruginosa* clinical strains with concentration ranging from 0.5 to 8x the MIC of ciprofloxacin. (b) Time-kill studies with *P. aeruginosa* wild-type clinical strains with concentration ranging from 0.5 to 8x the MIC of **ET37** treatment. (c) Time-kill studies with *P. aeruginosa* LasR mutant clinical strains with concentration ranging from 0.5 to 8x the MIC of **ET37** treatment. Red arrow: "drug-tolerant" cells in ciprofloxacin-treated *P. aeruginosa* clinical strains; blue arrows: "drug-tolerant" cells in **ET37**-treated *P. aeruginosa* clinical strains.

RhlR [17, 19]. We speculate that, in the presence of LasR mutations, LasR antagonists numbers **3** and **4** could interact with other QS receptors that are still functional in LasR mutant strains. It has been described that some virulence factors, such as pyocyanin, are still produced in LasR mutants, confirming that QS system is still functional. This could be explained by the ability of *P. aeruginosa* to circumvent QS deficiency using rhl and Pqs systems [20]. Similarly, 3O-C12-HSL is known to bind not only its receptor LasR, but also other QS receptor, as RhlR and PqsR. This is in line with the results of Kalaiarasan and coauthors reporting the ability of two anti-QS compounds to decrease biofilm production in two *P. aeruginosa* clinical isolates, affecting both lasR and rhlR transcription [42].

The identification of new mechanisms to block the appearance of drug resistance in *P. aeruginosa* is important because it is characterized by a quick adaptation to resist to new drug compounds [1], causing chronic lung infection in individuals with CF [43] or chronic obstructive pulmonary disease (COPD) [44], and the 10% of hospital-acquired infections.

The antibiotics used to treat *P. aeruginosa* infection include ciprofloxacin, tobramycin, ceftazidime, gentamicin, and imipenem. *P. aeruginosa* bacteria present a high degree of resistance to these antibiotics. Interestingly, the response to ciprofloxacin is very effective at the beginning of the treatment, but high-level resistance is rapidly acquired by *P. aeruginosa*, making the treatment ineffective in the 30% of strains obtained from clinical isolates [1]. The mechanisms that could increase *P. aeruginosa* antibiotic susceptibility still remain unclear [2–4] and few studies tried to develop therapeutic strategies to decrease the insurgence of resistances.

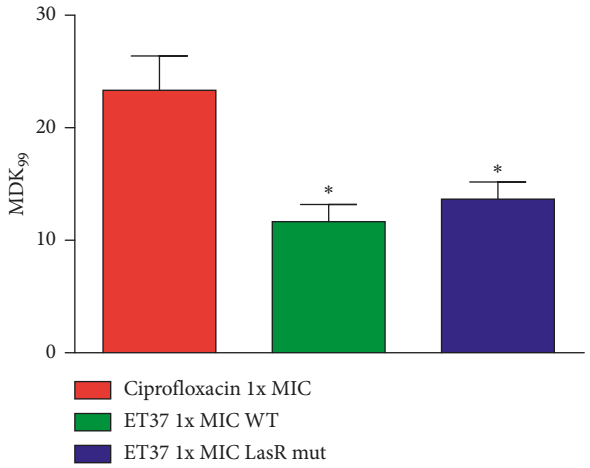

FIGURE 11: MDK_{99} was determined by measuring the time to kill 99% of the population, in *P. aeruginosa* clinical strains with concentration 1x the MIC of ciprofloxacin (red histogram), *P. aeruginosa* wild-type clinical strains (green histogram, WT) and LasR mutant clinical strains (blue histogram, LasR mut) with 1x the MIC **ET37**. $^{*}p$ value < 0.01, obtained by Student's t-test. Data are presented as the mean ± SD.

The onset of resistant mutants seems to be connected with the use of antibiotic concentrations that can select mutants, that ranges between 0.5 and 8 $\mu g\,ml^{-1}$ [45], in line with the concentrations used in our work. As drug concentrations increased, we observed the selection of particular tolerant clones ("drug-tolerant"). The use of **ET37** reduced the formation of biofilm, the expression of virulence genes (e.g., pyocyanin and elastin), and the onset of tolerant clones. This was obtained also in those strains that presented a genetic mutation in GyrA and ParC, that are now known to play an integral role in quinolone resistance in *P. aeruginosa* [6, 7].

From the time-kill curves with ciprofloxacin and **ET37**, it seems that the pharmacodynamics of ciprofloxacin changes from concentration-/dose-dependent killing, as reported in Figure 8(a), to more time-dependent killing, as we observed similar time-killing curves at different **ET37** concentrations (1x, 2x, and 4x MICs), as reported in Figure 8(b). This is also evident in the time-kill curves of the clinical isolates presented in Figure 10. This might suggest that the mechanisms involved in ciprofloxacin tolerance is quorum-sensing related. In fact, when the clinical strains were tested for their tolerance to ciprofloxacin or **ET37** treatment, we observed a decrease in MDK_{99} after **ET37** treatment in comparison with the ciprofloxacin (Figure 11). Both *P. aeruginosa* wild-type and LasR mutant clinical strains showed an MDK_{99} of 12 hours after treatment with **ET37** in comparison with the MDK_{99} of 24 hours observed in the same strains without **ET37** treatment. The reduced incidence of tolerant bacteria after **ET37** treatment could be associated with a modified gene expression [34]. In particular, ciprofloxacin treatment induces the production of hydroxyl radicals that might cause oxidation-mediated cell death [46]. The onset of drug-tolerant bacteria might be associated with the increase in the phosphorylation of two proteins, PA0265 (succinate-semialdehyde dehydrogenase (SSADH), encoded by GabD) and PA3570 (methylmalonate-semialdehyde dehydrogenase (MMSADH), encoded by MmsA) [8], that seems to induce the production of NADH as a reaction product. NADH is a reducing agent, induced in response to environmental stress [35] to prevent oxidative damage. Therefore, the upregulation of NADH by the phosphorylation of these two proteins might buffer oxidative stress induced by ciprofloxacin and facilitate the onset of tolerant bacteria. **ET37** compound could facilitate the accumulation of ciprofloxacin within the cell, increasing the intracellular superoxide anion ($O2^{-}$) concentration causing more oxidative stress that cannot be buffered by SSADH/MMSADH, than in *P. aeruginosa* strains treated with ciprofloxacin alone. The benefit of **ET37** in comparison with ciprofloxacin is its ability to reduce the formation of biofilm and consequently diminishing the physical barrier to ciprofloxacin penetration into bacterial cells. In fact, the MBIC of **ET37** was similar to MIC_{50} (0.22 μg/ml), while ciprofloxacin MBIC was eight times higher (4.0 μg/ml) than MIC_{50} (0.5 μg/ml). Moreover, the inhibition of QS could also affect the production of rhamnolipid. The production of rhamnolipid in *P. aeruginosa* is controlled by the transcriptional regulator RhlR of the QS system [47]. Even if the role of rhamnolipids in bacterial physiology is not clear, they seem to take part to the assimilation of insoluble substrates [36], to antimicrobial activities [48], to hemolytic activity [49], to the solubilization of the quinolone signal, and to the swarming motility [50]. Moreover, rhamnolipids seem to reduce the activation of host innate immunity, facilitating *P. aeruginosa* survival and colonization on compromised epithelia. The decrease in rhamnolipids production by QSi could facilitate the elimination of the infection by the host's immune system.

5. Conclusions

On the basis of our results, we reported, for the first time, the reduction of biofilm formation and ciprofloxacin tolerance in *P. aeruginosa* clinical strains treated with **ET37** compound. This molecule, generated by the conjugation of a QSi and ciprofloxacin, could have a wide application in clinical setting. The possibility to affect biofilm formation in chronically infected patients, such as CF and COPD patients, and to reduce the onset of ciprofloxacin tolerance would improve patient healing and allow to decrease antibiotic drug dosage.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

An earlier version of this study was presented as a poster in "Congresso Nazionale della Società Italiana di Microbiologia, 2018".

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Daria Bortolotti and Claudio Trapella equally contributed to the work.

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Supplementary Materials

The experimental part of the synthesis of the chemical compounds and NMR data are reported in the Supplementary Material file. (*Supplementary Materials*)

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