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Furoxan nitric oxide donors disperse *Pseudomonas aeruginosa* biofilms, accelerate growth and repress pyoverdine production

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Abstract

The use of nitric oxide (NO) as a signal for biofilm dispersal has been shown to increase the susceptibility of many biofilms to antibiotics, promoting their eradication. The delivery of NO to biofilms can be achieved by using NO-donors with different kinetics and properties of NO release that can influence their efficacy as biofilm control agents. In this study, the kinetics of three furoxan-derivatives were evaluated. The effects of these NO-donors, which have an advantageous pharmacological profile of slower onset with an extended duration of action, on *Pseudomonas aeruginosa* growth, biofilm development and dispersal were also characterized. Compound LL4254, which showed a fast rate of NO release, induced biofilm dispersal at approximately 200 μM. While LL4212 and LL4216 have a slower rate of NO release, both compounds could induce biofilm dispersal, under the same treatment conditions, when used at higher concentrations. Further, LL4212 and LL4216 were found to promote *P. aeruginosa* growth in iron-limited minimal medium, leading to a faster rate of biofilm formation and glucose utilization, and ultimately resulted in early dispersal of biofilm cells through carbon starvation. High concentrations of LL4216 also repressed production of the siderophore pyoverdine by more than 50-fold, via both NO_x-dependent and NO_x-independent mechanisms. The effects on growth and pyoverdine levels exerted by the furoxans appeared to be mediated by NO-independent mechanisms, suggesting functional activities of furoxans in addition to their release of NO and nitrite. Overall, this study reveals that secondary effects of furoxans are important considerations for their use as NO-releasing dispersal agents, and that these compounds could be potentially re-designed as pyoverdine inhibitors.

Introduction

Bacterial cells growing within biofilms can be up to a thousand times more resistant to anti-microbial agents than their planktonic counterparts, making their eradication very difficult [1, 2]. This enhanced tolerance to stress, immune defenses and antibiotics may partly explain the observation that biofilms are associated with up to 80% of all microbial infections and the majority of chronic and recurrent infections including pneumonia in cystic fibrosis patients, wound infections and medical implant-associated infections [1, 3, 4].

The insensitivity of biofilms to conventional antimicrobial treatments appears to be multifactorial and involves: (i) a protective barrier of self-produced extracellular polymeric substances (EPS) that can inactivate or reduce diffusion of bactericidal compounds [5], (ii) enhanced lateral transfer of genes including those from drug resistant and extremely drug resistant (XDR) strains [6] and (iii) the presence of dormant-like persister cells [7]. Given the central role played by biofilms in promoting antibiotic resistance and causing the failure of therapeutic treatments, there is an urgent need to develop alternative strategies specifically aimed at biofilm development processes. Towards this end, the dispersal phase of the biofilm life cycle has been targeted as a key stage that can be manipulated to control biofilms [8]. During dispersal, bacterial cells transit from a biofilm to a planktonic mode of growth, rendering them more susceptible to various antibiotics [9, 10]. Biofilms disperse in response to several environmental cues, such as changes in carbon levels and iron levels [11-13]. In addition, low nanomolar concentrations of the gaseous free radical nitric oxide (NO) were found to induce biofilm dispersal in *Pseudomonas aeruginosa*, an opportunistic pathogen and the model organism for biofilm studies [9], as well as several other bacterial species [8]. Furthermore, exposure of pre-established biofilms to the NO-donor compound sodium nitroprusside (SNP) increased the susceptibility of biofilm cells towards several antimicrobial agents, including the antibiotics tobramycin and ceftazidime in *P. aeruginosa* biofilms grown both on abiotic surfaces and in ex vivo sputum samples of CF patients [9, 10]. Recently, the use of NO gas delivered at low dose to CF patients, together with intravenous tobramycin and ceftazidime, reduced *P. aeruginosa* biofilms and improved lung function when compared to placebo-treated control patients in proof of concept clinical studies [14]. These observations suggest that the use of NO, together with conventional antibiotics, represents a promising alternative for the treatment of chronic biofilm infections.

The delivery of NO for medical purposes, in addition to the gaseous form, is typically achieved by using soluble donors. These compounds spontaneously release NO when dissolved in aqueous solutions and the kinetics of release are a function of their donor chemistry. In this way, donors can be tuned to optimize the release of NO. Furoxans are heterocyclic compounds containing a 1,2,5-oxadiazole 2-oxide ring and two substituent groups at positions 3 and 4 of the furoxan ring (Figure 1) [15]. These substituents influence several properties of the furoxan compound, such as its solubility, the rate of NO production, and whether NO-release is thiol-activated or could occur spontaneously [15-17]. This flexibility allows for the design of furoxan compounds that generate varying fluxes of NO when administered [17]. In general, furoxans have an advantageous pharmacological profile of a slow onset with an extended duration of action, as compared to other NO donors [15, 18]. In turn, furoxans as such, or used in designing NO-donor hybrid drugs, have been observed to be active against a variety of targets and have been assessed for use in cardiovascular diseases, neurological and inflammatory disorders [19-21]. More recently, a few furoxan derivatives were found to inhibit ABC transporters in MDR tumor cells [22, 23]

Despite the many applications of furoxans, they have not been tested for their activity against biofilms. In this study, we evaluated the release of nitrogen oxide species (NO_x), namely NO and nitrite (NO_2^-), from three furoxans, LL4212, LL4216 and LL4254, and studied their effect on *P. aeruginosa* biofilm development, where they induced biofilm dispersal. LL4212 and LL4216 were, additionally found to affect bacterial growth under iron-limited conditions in an NO_x-independent manner, increasing the rates of glucose utilization and in turn leading to the earlier onset of glucose-starvation-induced dispersal of *P. aeruginosa* biofilms. Further, LL4216 was found to reduce expression of the siderophore pyoverdine in an NO_x-independent manner. Taken together, the study indicated that, depending on their backbone, furoxans may have secondary effects on bacterial growth and is an important consideration for their design and use as NO-releasing agents.

Results and Discussions

Kinetics of NO_x release from LL4254, LL4212 and LL4216

The study of the kinetics of NO_x release from the furoxan compounds indicated that both NO and nitrite (NO_2^-) were spontaneously released from the furoxans under physiologically relevant conditions (pH, temperature). The results, expressed as percent mol/mol of NO_x released with respect to the concentration of furoxan in solution (Figure 2a – c), showed two types of NO_x-production behavior. For LL4254, NO was the predominant species released, accounting for 82% of the total NO_x detected (~ 95%) by 24 h (Figure 2a); the liberation occurred very fast, with 80% of the total NO_x produced within the first 30 min. In contrast, NO_2^- was the dominant species generated from LL4212 and LL4216 and liberation occurred in an almost linear fashion over a few hours. By 24 h, 83% and 32% of the NO_x species were detected as NO_2^- from LL4212 and LL4216, respectively, while only 2.6% and 1.2% were measured as NO (Figure 2b, c). An alternative chemiluminescence-based NO detection method, also confirmed the results obtained with the DAN assay (data not shown).

Previous studies showed that thiol groups such as those present in L-cysteine (L-cys) can promote NO_x liberation from furoxan compounds and increase their activity [17]. Since thiol groups are commonly produced by bacteria, in the form of metabolites or proteins, they are highly relevant in the context of bacterial infection treatments and their effects on NO_x release from LL4212, LL4216 and LL4254 were investigated. ~~Exogenous addition of L-cys reduced the extent of NO_x liberated from the fast releasing LL4254 but promoted their production from the slower releasing LL4212 and LL4216 (Figure 2d – f). At t = 1 h, the presence of L-cys reduced the amount of NO and NO_2^- generated from LL4254 to 4% and 36%, respectively, from an initial 70% and 90% in the absence of L-cys (Figure 2d). At t = 4 h, NO_x production increased from 26% to 28% in the presence of L-cys for LL4212, and from 8% to 20% for LL4216. NO production from these compounds also increased in the presence of L-cys, from 1.8% to 2.2% for LL4212 and from 0.8% to 1.5% for LL4216 (Figure 2 e, f).~~

Exogenous addition of L-cys reduced the extent of NO_x liberated from the fast releasing compound LL4254, promoted their production from the slower releasing LL4216 while did not affected the NO_x production from LL4212 (Figure 2d – f). At t = 1 h, the presence of L-cys reduced the amount of NO and NO_2^- generated from LL4254 to 4% and 36%, respectively, from the initial 70% and 90% in the absence of L-cys (Figure 2d). At t = 4 h, NO_2^- production increased from 8% to 20% in the presence of L-cys for LL4216. NO production from LL4216 and LL4212 was not influenced by L-cys (Figure 2 e, f).

Furoxan NO_x-donors can induce *P. aeruginosa* biofilm dispersal

To determine if the NO-releasing furoxans could induce biofilm dispersal, *P. aeruginosa* biofilms were treated with 100 μM, 200 μM or 500 μM of the furoxans for 1 h before quantification of biofilm biomass by CV staining. At 200 μM or above, LL4254 reduced the biofilm biomass by >70% compared to untreated control samples ($p < 0.0001$) (Figure 3a, c). This biomass reduction, which was associated with an increase in OD_{600} reading of the supernatant (data not shown), occurred after 1 h treatment, and therefore cannot be linked to growth effects but instead is a clear indication of biofilm dispersal events. These results were in agreement with previous studies, where NO was found to induce *P. aeruginosa* biofilm dispersal [9, 24]. Dispersal by LL4254 occurred in a concentration-dependent manner. In contrast, LL4212 and LL4216 were unable to induce significant biofilm dispersal at concentrations between 100 μM and 500 μM.

To assess if dispersal was caused by NO or other non-specific interactions between *P. aeruginosa* and the LL4254 backbone, the assay was repeated in the presence of the NO scavenger cPTIO. Under the conditions used, cPTIO alone did not have a significant effect on both planktonic and biofilm growth (data not shown). Addition of cPTIO reduced dispersal by LL4254 from an average of 73% to 18%, suggesting that the effect of LL4254 on dispersal was NO-dependent (Figure 3b, d).

NO release from LL4254 was established as an important factor for inducing biofilm dispersal. As the rate of both NO and NO_2^- generated from LL4212 and LL4216 was much lower than that of LL4254 under cell-free physiological conditions (Figure 2), it is likely that at the same compound concentration, both LL4212 and LL4216 produce a lower amount of NO, This may in turn influence the effective NO concentration perceived by the bacteria at a given time, as NO generated may be consumed by cellular processes, reaction with oxygen, and reactions with other chemical compounds present in the medium [25, 26], hence limiting the extent of biofilm dispersal. Therefore, the assay was repeated using 3.6 mM LL4212 and LL4216, which correspond to the amount of NO_x released from approximately 400 μM and 200 μM LL4254 within 1 h. The furoxan stocks were solubilized in DMSO, and while at low concentrations (≤ 0.5% v/v) DMSO did not significantly alter *P. aeruginosa* growth or biofilm formation in our assays (data not shown), at a higher level of 3.6% v/v (the amount present when adding 3.6 mM furoxan), DMSO reduced the amount of biofilm formed by approximately 14% (Figure 3f). Therefore, samples to which an equivalent volume of DMSO was added, were used as a control for comparison of the extent of biofilm dispersal induced with 3.6 mM furoxan treatments. At 3.6 mM, LL4212 and LL4216 dispersed 48% and 70% of the biofilm biomass respectively (Figure 3e, f). Decrease in biofilm biomass observed was concomitant with an increase in planktonic biomass, thus confirming that the observed effects correlated with dispersal of bacteria from the biofilm. Although LL4216 alone, under physiological, cell-free conditions has a slower rate of NO_x release than LL4212, in this experiment, LL4216 was found to induce a larger extent of dispersal than LL4212. This is likely due to the presence of nucleophiles, e.g. thiol groups, that are produced or secreted by the bacteria [15-17] that promoted NO_x release from LL4212, LL4216 and LL4254 to different extents, as supported in cell-free NO kinetics experiments (Figure 2).

Furoxans accelerate *P. aeruginosa* biofilm development and enhance glucose utilization

Because furoxans have a slower rate of release and can produce NO_x over longer periods of time, we then assessed whether these compounds can constantly prevent the switch for attachment of free-floating bacteria, maintain cells in a planktonic mode of growth and thus inhibit biofilm formation over time. Furoxans were added to M9GC medium together with the *P. aeruginosa* at the time of inoculation. After 6 h, wells that had been treated with LL4212 and LL4216 showed a significant concentration-dependent reduction in biofilm biomass, with increases in planktonic growth, compared to untreated wells, with 100 μM of LL4216 reducing biofilm biomass by > 70% ($p < 0.0001$) (Figure 4a, c).

To further characterize the impact of furoxans on biofilm formation, biofilm biomass of untreated control groups and groups treated with 500 μM LL4212 or 200 μM LL4216, both of which reduced the biofilm biomass at t = 6 h by > 80%, was quantified over time (Figure 4b). Surprisingly, addition of LL4212 and LL4216 promoted both biofilm formation and planktonic growth over the first 4 h and 3 h, respectively (Figure 4b). Subsequently, the biofilm dispersed while planktonic growth continued to increase. A similar sharp decrease in biofilm biomass during growth had already been observed in *P. aeruginosa* biofilms and was linked to biofilm dispersal due to the sudden depletion of the carbon source and the onset of starvation [27]. The levels of glucose in the biofilm cultures were then determined over time and the extent of biofilm reduction at t = 6 h was consistent with a reduction in glucose concentration (Figure 4d, e). The effect was the most pronounced with 500 μM LL4216, which led to complete glucose depletion by t = 6 h.

While NO is known to affect biofilm dispersal, a potential role in regulating glucose utilization and growth has not been observed before. To determine if the effects of LL4212 or LL4216 on glucose consumption were dependent on NO, biofilm prevention experiments were carried out with furoxans added together with the NO scavenger cPTIO, or using NO-depleted furoxans which had been incubated in culture medium for 24 h before inoculating with *P. aeruginosa*, thus resulting in exhaustion of NO released from the donor compounds. The data show that after 6 h, *P. aeruginosa* biofilms had dispersed under both conditions (Figure 5a – c), suggesting that the effects of LL4212 and LL4216 on biofilm formation were not due to NO.

These observations of the impact of LL4212 and LL4216 on biofilms did not correlate with a typical dispersal response to NO resulting in biofilm prevention. To further elucidate the effect of the furoxans on biofilms, we then examined their impact in our assay on a known marker of biofilm dispersal in *P. aeruginosa*, including when induced by NO, which is a decrease in the production of the iron chelating siderophore pyoverdine [28]. A similar decrease in pyoverdine levels was also previously observed upon dispersal with 200 μM of LL4254 and 3.6 mM of LL4212 or 4216 (Figure 3e). The results revealed that in the presence of 500 μM NO-exhausted LL4216, pyoverdine levels were reduced by at least 50-fold compared to untreated biofilms, even though there was no free NO present. In contrast, the use of 500 μM of NO-depleted LL4212 had no effect on the pyoverdine levels with respect to the untreated controls (Figure 5c). To control for a potential direct influence of LL4216 on the pyoverdine fluorescent signal, 500 μM of LL4216 were added to filtered, cell-free M9GC medium collected from microtiter plate cultures grown under the same conditions, which contained pyoverdine. Pyoverdine levels were found to be relatively stable in filtered medium without any compound and LL4216 at 500 μM only slightly decreased pyoverdine relative fluorescent units (RFU) by 6.3% and 6.9%, after 6 h and 24 h respectively, compared to untreated cell-free solutions. In contrast, when 500 μM LL4216 was added to a non-filtered culture inoculated with *P. aeruginosa* at t = 0 h, pyoverdine expression was > 99% lower than that of the control after both 6 h and 24 h, suggesting that LL4216 actively repressed pyoverdine expression, rather than simply affecting the fluorescence signal from pyoverdine (Figure 5d). Thus, these results suggest that the furoxans can reduce pyoverdine synthesis in *P. aeruginosa*, via both NO-independent (LL4216) and NO-dependent (LL4212) mechanisms.

Finally, because another major NO_x released from LL4212 and LL4216 is NO_2^-, its effect on biofilm formation in our assay was investigated. As opposed to NO, NO_2^- is stable in solution and can be added directly to the culture medium When 100 – 500 μM of exogenous nitrite were added at t = 0 h in place of LL4212 and LL4216, there was no significant effect on planktonic growth, biofilm formation or pyoverdine production in *P. aeruginosa* (Supplementary figure 3).

Collectively, these results suggest that LL4212 and LL4216 likely increase the growth rates of *P. aeruginosa* through increased rates of glucose metabolism and cause biofilm dispersal via a carbon or glucose starvation-induced response [11, 12]. The use of NO depleted LL4212 or LL4216 in M9GC medium induced the same effects. Further, the addition of exogenous sources of nitrite, the main NO_x species released from LL4212 and LL4216, did not affect planktonic growth, biofilm formation or pyoverdine production. This indicates that the backbone or by-product of NO_x released from these two compounds, but not NO or NO_2-, were responsible for the increased growth.

LL4212 and LL4216 are not utilized as direct carbon or nitrogen sources by *P. aeruginosa*

One possibility to explain the accelerated biofilm formation and increased growth of *P. aeruginosa* in the presence of the furoxans, is that these compounds may be used as carbon or nitrogen sources for metabolism. To determine the impact of the furoxans on metabolism, *P. aeruginosa* was inoculated in various modified M9 media with or without available carbon or nitrogen sources. In M9 medium supplemented with casamino acids and glucose as sources of carbon (M9GC), or M9 medium supplemented with glucose only (M9G), the addition of 500 μM LL4212 or LL4216 increased both the growth rates and growth yields of *P. aeruginosa* (Figure 6a, Supplementary figure 4), which agrees with our previous observations. In contrast, the addition of LL4212 and LL4216 to M9 medium with glucose but without any nitrogen source (M9-N), did not significantly alter growth rates, although there was a slight increase in total growth (Figure S2c). In M9 medium without any carbon source (M9S), *P. aeruginosa* did not grow at all whether in the absence or presence of LL4212 and LL4216 (Figure Supplementary figure 2d).

This hence suggests that the compounds are most likely not utilized as a direct source of energy for *P. aeruginosa*, and may influence growth through interfering with processes related to glucose metabolism and increases in ATP production (Supplementary figure 6). This latter point is also supported by the observation that glucose was more rapidly depleted in the presence of these compounds.

LL4216 inhibits pyoverdine production under both low and high iron conditions in *P. aeruginosa*, but under high iron conditions reduces the growth rate

Another nutrient essential for growth in *P. aeruginosa* is iron, which is typically limiting in M9. However, bacteria can acquire essential trace amounts from contaminants in the water or components used to prepare the medium, typically by producing siderophores like pyoverdine, which has high iron affinity. Since the furoxans appeared to influence both growth and pyoverdine production in *P. aeruginosa*, we assessed if these effects may be associated with iron uptake. As expected, the addition of 500 μM LL4212 and LL4216 to M9GC resulted in increased growth and reduced pyoverdine levels (Figure 6b, c). In contrast, under the same conditions, LL4254 did not affect growth or pyoverdine production. In M9GC medium, the increase in growth rate showed a clear concentration dependence for LL4212, while there was only a slight increase in growth rate for LL4216 at 500 μM. With respect to pyoverdine production, LL4212 repressed pyoverdine only when added at 500 μM, while LL4216 showed a clear concentration-dependent reduction.

In M9GCFe, *P. aeruginosa* displayed an increased growth rate of about 8-fold compared to M9GC. In M9GCFe, LL4212 addition resulted in a slight reduction in growth rate relative to the control while LL4216 reduced the growth rate in a concentration dependent fashion, with a 38% reduction when added at 500 μM (Figure 6b). The presence of added iron also generally reduced pyoverdine production. While LL4212 only slightly further reduced this production, by 12% when added at the highest concentration tested, the effects were more pronounced with LL4216, which at 500 μM induced a drastic further reduction in pyoverdine to background levels. The addition of LL4254 had no influence on growth rate and pyoverdine production in either medium (Figure Supplementary figure 5c, f). Taken together these data suggest that LL4212 and LL4216 may have opposite impacts on *P. aeruginosa* growth depending on the availability of iron, resulting in increased growth under iron limited conditions, while reducing growth when iron is replete.

LL4212 and LL4216 may affect biofilm and planktonic growth via influences on glucose utilization and pyoverdine production in *P. aeruginosa*

The decrease in pyoverdine levels upon LL4212 and LL4216 addition may be in part due to NO release, which is known to repress the expression of pyoverdine related genes [24]. Indeed, when NO-depleted LL4212 was used, no repression in pyoverdine production was observed. In contrast, the effect of LL4216 on pyoverdine appeared to be independent of NO, as the use of the NO-depleted compound still induced a decrease in pyoverdine levels (Figure 5c). Overall, the results show that decreases in pyoverdine production with LL4212 are predominantly mediated by NO while similar decreases in LL4216-treated samples were either due to the presence of NO or mediated directly by the LL4216 backbone. Despite these differences, both compounds could promote bacterial growth in *P. aeruginosa* and no correlation between growth rate and pyoverdine levels was observed (Figure 6b, c). Thus, the furoxans likely exert independent effects on growth and pyoverdine levels, especially in the absence of added iron.

We initially hypothesized that LL4216 may function as a siderophore in place of pyoverdine. Repression of pyoverdine production by LL4216, while still being able to take up iron, could then direct metabolites towards energy generation and growth away from the costly production of pyoverdine. For example, pyoverdine productionwhich has been previously estimated to require approximately 10% of additional carbon consumption per growth unit in a closely related species, *Pseudomonas putida*, grown under iron limitation [29, 30]. However, when supplemented with ferric chloride, no further increase in growth was observed with addition of 500 μM of LL4216, suggesting that LL4216 does not function as an alternative iron scavenger and further supporting that LL4216 exerts growth-related effects independent of pyoverdine production and iron acquisition.

Conclusions

The results suggest that furoxans with fast and slow NO release profiles could be used at low and high concentrations, respectively, to promote biofilm dispersal. Slow NO-releasing furoxans were also found to be potent at preventing biofilm formation over longer periods of time, in a similar manner as slow NO-releasing polymers had been previously found to inhibit biofilm formation in *P. aeruginosa* [31], although a potential role of NO in mediating the effects of furoxans on biofilm prevention could not be clearly determined here. Interestingly, slow NO-releasing furoxans may be useful at higher concentrations to, in one single treatment, induce dispersal of pre-established biofilms and prevent re-formation of a biofilm over extended times.

In addition to inducing biofilm dispersal via NO, the furoxans LL4212 and LL4216 also influenced growth and pyoverdine production of *P. aeruginosa*. The data suggest that the two furoxans exert effects on growth and pyoverdine levels independently of each other and further work is needed to elucidate these mechanisms. While the increased growth rate would not normally be advantageous from the perspective of pathogen control, if the compounds strongly repress siderophore production, they may facilitate control in the host, where iron is severely limited. In this respect, there may be interest in developing these compounds as pyoverdine inhibitors [32]. This is relevant as *P. aeruginosa* is predominantly found in the lungs of cystic fibrosis patients, with pyoverdine expression being a major factor accounting for *P. aeruginosa* virulence, antibiotic resistance and biofilm maturation [33-35]. Further studies would be required for the successful development of furoxans as dispersal agents or for their use in other applications.

Materials and methods

Nitric oxide donors and scavengers

The furoxans LL4212 (3-((2-(dimethylamino)ethyl)oxy)-4-phenylfuroxan), LL4216 (3-((2-aminoethyl)thio)-4-phenylfuroxan) and LL4254 (4-(phenylsulfonyl)-3-((2-dimethylanimo)ethyl)thio)furoxan) were synthesized as previously reported by Sorba *et al.* [17]. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-PTIO, cPTIO) was purchased from Sigma Aldrich (# C221).

Nitrite measurements by Griess reaction (total NOx evaluation)

The total release of NO_x was evaluated as nitrite (NO_2^-) by Griess reaction. Furoxan compounds were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration in the absence or in the presence of 0.5 mM L-cysteine (L-cys) (5 times mol/mol excess). At regular intervals, the presence of nitrite in the reaction mixture was determined by the Griess assay: 1 mL of the reaction mixture was treated with 250 μL of the Griess reagent (4% w/v sulphanilamide, 0.2 % w/v *N*-naphthylethylenediamine dihydrochloride, 1.47 M phosphoric acid). After 10 min at room temperature, the absorbance was measured at 540 nm. A calibration curve was obtained using standard solutions of sodium nitrite at 10 μM to 80 μM. The yield in nitrite was expressed as percent NO_2^- (mol/mol, relative to the initial compound concentration) ± SEM.

NO measurements by DAN (2,3-diaminonaphthalene) method

NO release was quantified using a 2,3-diaminonaphthalene (DAN)-based chemical assay, which is based on the immediate reaction of NO with oxygen (O_2) to form dinitrogen trioxide (N_2O_3), which then reacts with non-fluorescent DAN to form the highly fluorescent 2,3-naphthotriazole (NAT) that can be quantified by RP-HPLC. Compounds were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration with 0.2 mM DAN in the absence or presence of L-cys at 0.5 mM. At fixed time points, NAT in the reaction mixture was evaluated by HPLC analysis according to previously published protocol [36].
HPLC analyses were performed with a HP 1200 chromatograph system (Agilent Technologies) equipped with a quaternary pump (G1311A), a membrane degasser (G1322A), a multiple wavelength UV detector (G1365D) and a fluorescence detector (G1321A) integrated in the HP1200 system. Data analysis was performed using a HP ChemStation system (Agilent Technologies). The sample was eluted on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm; Agilent) with an injection volume of 20 μL. The mobile phase consisted of 65% of 15 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile at a flow rate of 1.0 mL min^{-1}. The fluorescence signals were obtained using an excitation and emission wavelength of 355 and 460 nm, respectively (gain factor = 10). Data analysis was performed by with Agilent ChemStation. The values obtained from integration of the peak of NAT were interpolated in a calibration line, prepared using $NaNO_2$ (in acidic conditions) as a standard. Briefly sodium nitrite standard solutions were acidified with HCl (pH 2) in the presence of excess DAN (0.2 mM). After 10 min, the reaction mixture was diluted in phosphate buffer at pH 7.4 (NO final concentration 1 to 80 μM) and analyzed by HPLC.

Bacterial strains and growth conditions

P. aeruginosa PAO1 (ATCC BAA-47) was maintained on agar plates of Luria-Bertani medium with 10 g/L NaCl (LB10) (644520, Difco). Cultures were grown overnight in LB10 medium at 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm). Overnight PAO1 cultures were subsequently diluted 200 times to an OD_{600} = 0.005 in various culture media depending on the assay, made of M9 salts (M9S) (48 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.0) supplemented with different carbon and nitrogen sources: M9GC (M9S, 0.04 % w/v glucose; 0.2 % w/v casamino acid), M9G (M9S, 0.4 % w/v glucose), M9-N (M9G made without any NH_4Cl), M9GCFe (M9GC, 3 µM $FeCl_3$) or $M9GCNO_2$ (M9GC, 100 μM to 500 μM KNO_2) medium.

Growth studies

Overnight cultures of *P. aeruginosa* were washed three times in M9S made without NH_4Cl and diluted to an OD_{600} of 0.005 in 200 μl of M9GC, M9G, M9-N, M9S, M9GCFe, or $M9GCNO_2$ medium, which were added to each well of a 96 well plate and incubated statically at 37°C for 24 h. Furoxans were added at the time of inoculation (t = 0 h). The growth of *P. aeruginosa* was monitored spectroscopically at 600 nm, while pyoverdine production was quantified by measurement of fluorescence intensity (excitation at 398 nm and emission at 460 nm) [37]. All measurements were carried out using a microtiter plate reader (Infinite 200 pro, Tecan). Growth rates were determined by calculating changes in OD_{600} over time during exponential growth phase while growth yields refer to OD_{600} values recorded at late stationary phase

Biofilm assays

Overnight cultures of *P. aeruginosa* were diluted 1:200 in fresh M9 medium. One mL of the diluted culture was added into a well of a 24-well polystyrene plate (142475, Nunclon), which was subsequently incubated at 37°C with 200 rpm shaking (Infors HT, orbit diameter 25 mm) for no more than 6 h. To assess the effect of furoxan on biofilm dispersal, compounds were added into each well after 5 h incubation (t = 5 h) to a final concentration of 100 μM, 200 μM, 500 μM or 3.6 mM, and the plates were incubated for a further 1 h. To assess the effect of each compound on biofilm formation, the furoxans were added into each well at the time of inoculation (t = 0 h) and biofilms were allowed to form over the next 6 h. For experiments involving NO scavengers, c-PTIO was added into each well to a final concentration of 0.5 mM at the same time as furoxans. For experiments involving NO-depleted M9GC medium containing LL4212 or LL4216, 500 μM of each furoxan were first added to M9GC medium and incubated at 37°C for 24 h prior to inoculation with *P. aeruginosa*. Biofilm biomass was quantified by crystal violet (CV) staining as described by Barraud *et al.* (2014) [38]. Planktonic growth and pyoverdine production were quantified as described above.

***P. aeruginosa* glucose utilization assay**

The utilization of glucose by *P. aeruginosa* in M9 medium was measured using the GO assay kit (GAGO20, Sigma). Experiments were carried out as described in the biofilm assays. Subsequently, the medium from each well was filtered and diluted in ultrapure water to obtain a glucose concentration of approximately between 20 to 80 μg mL^{-1}. Glucose standards were prepared per the manufacturer's instructions. Each volume of the standard or sample was mixed with two volumes of the assay reagent and incubated at 37°C statically for 30 min. The reaction was stopped by adding two volumes of 12 N H_2SO_4. Glucose concentrations were determined with a microtiter plate reader (absorbance at 540 nm) and interpolating to the standard curve.

Statistics

All statistical tests were carried out using Graphpad Prism 7.0. Results from biofilm and growth assays were analyzed with one-way ANOVA followed by Dunnett's test for multiple comparison ($\alpha = 0.05$) against a relevant control group. Geometric means were used in statistical tests involving pyoverdine measurements and analyzed using ANOVA as described above.

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Supporting information

Supporting information available: This material is available free of charge *via* the internet.

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Figures

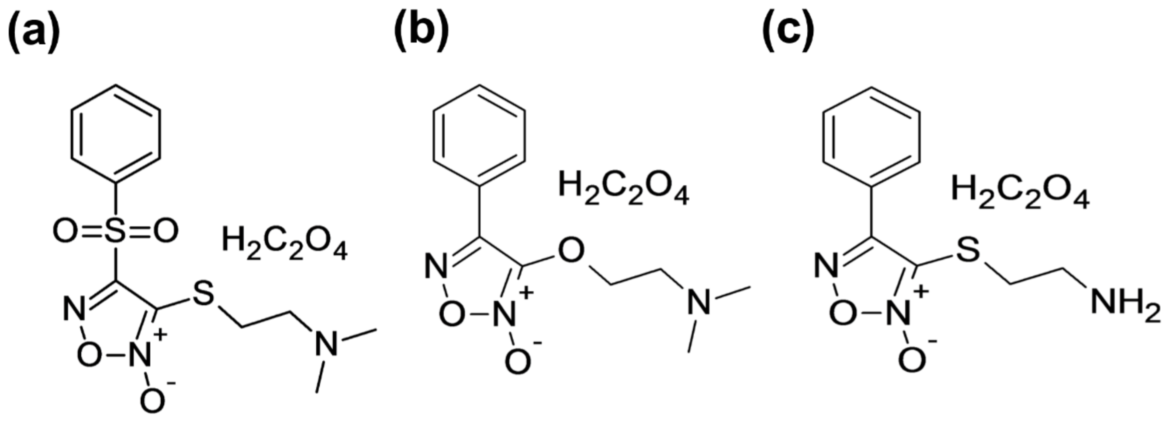

Figure 1. Structures of LL4254 (**a**), LL4212 (**b**) and LL4216 (**c**).

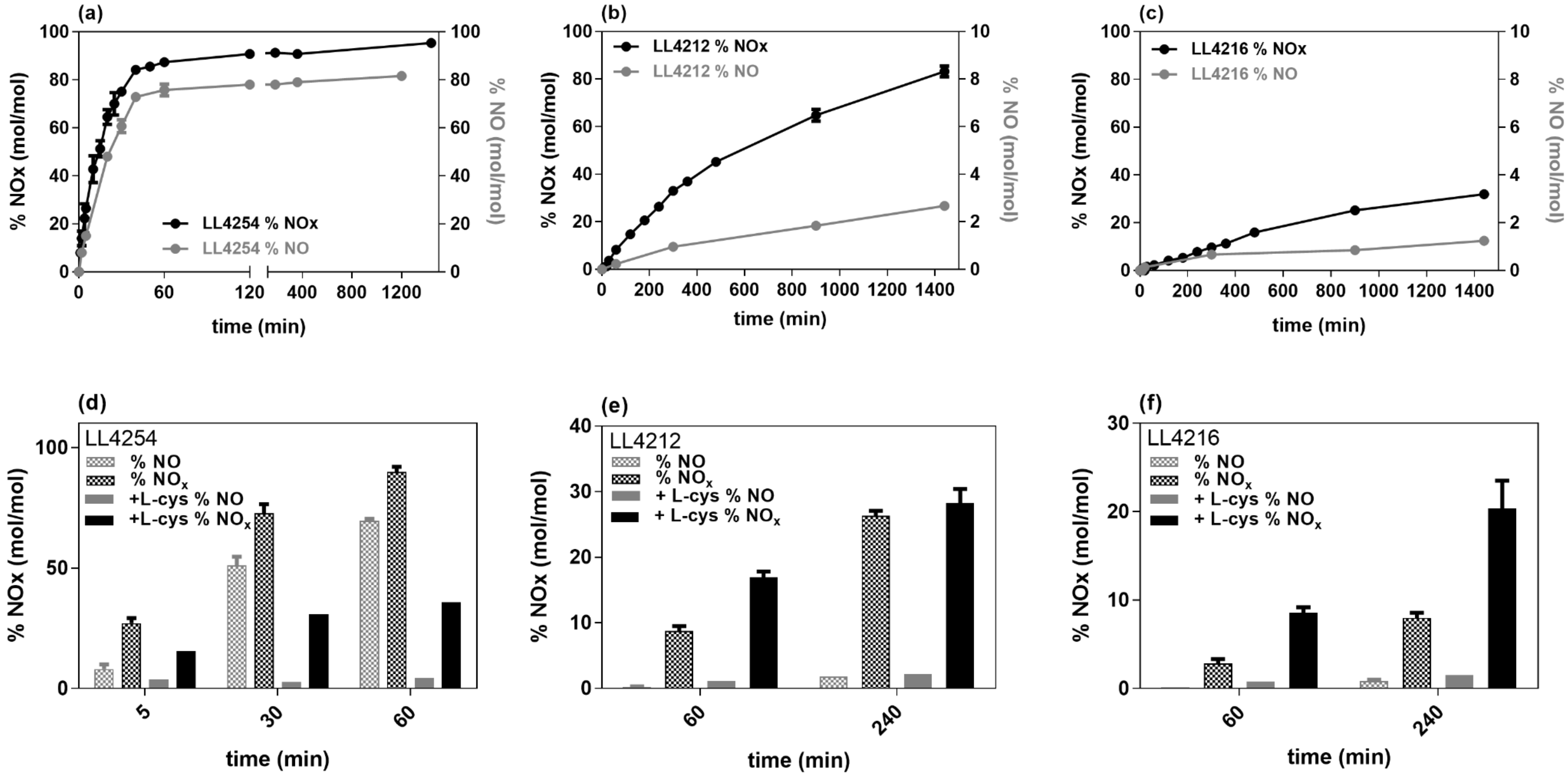

Figure 2. NO (gray circle) and NO_x (NO and NO_2^-) (black circle) release kinetics of LL4254 (a, d), LL4212 (b, e) and LL4216 (c, f) in phosphate buffer at pH 7.4 in the absence of L-cysteine over time (a – c) and in the presence or absence of L-cysteine at selected time points (d – f). The results are expressed as percent (% mol/mol) of NO or NO_x released with respect to the quantity of parent furoxan compound. Bars or symbols represent data from three or more replicates and error bars represent standard deviation from the mean.

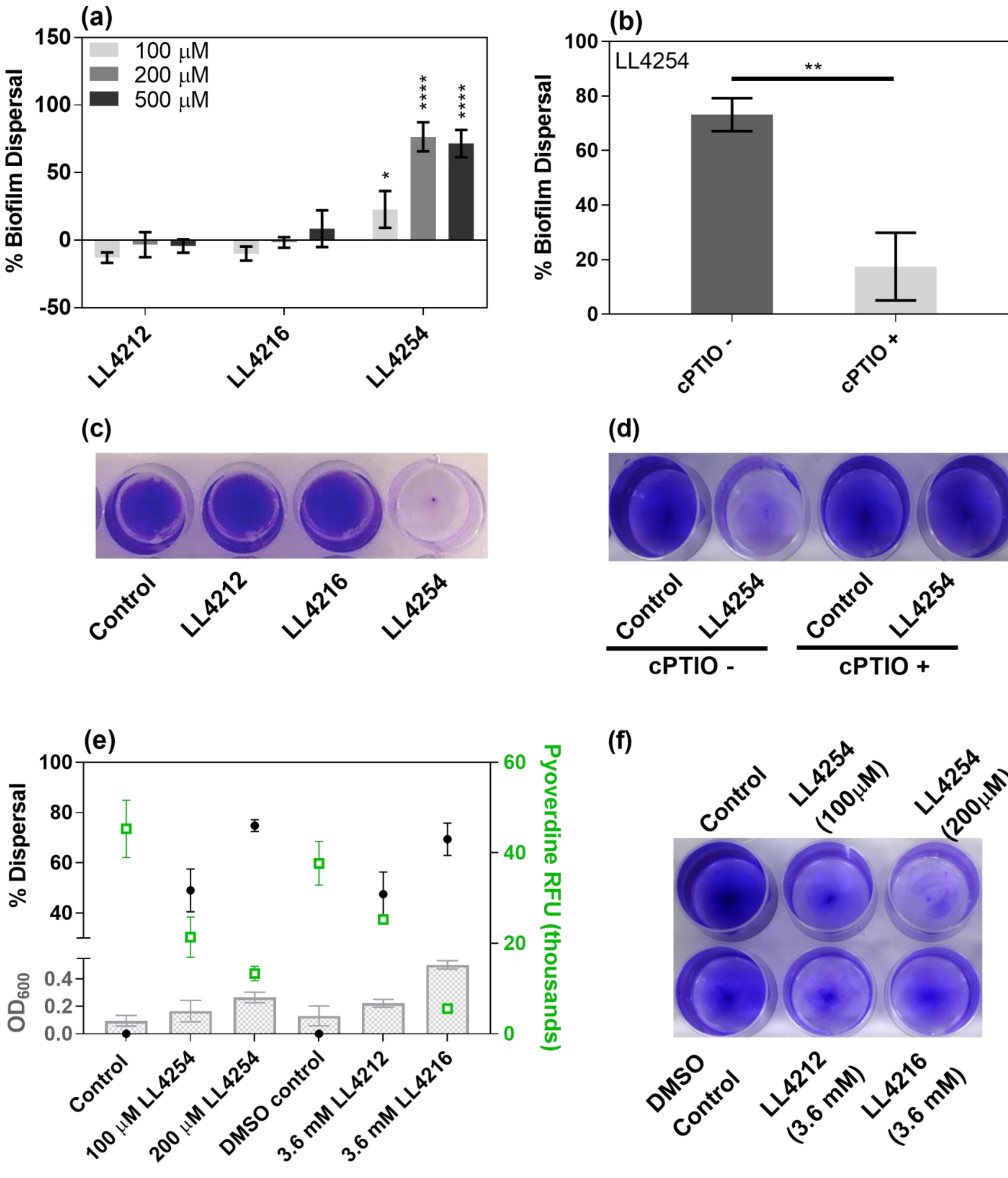

Figure 3. Dispersal of *P. aeruginosa* biofilms upon treatment with the furoxans for 1 h **(a)**, or upon treatment with 200 µM of LL4254 in the presence or absence of 0.5 mM cPTIO **(b)**.

The extent of dispersal (black circle) corresponds to an increase in OD_{600} (gray bars) and a decrease in pyoverdine fluorescence (green squares) **(e)**. Bars or symbols represent data from three **(a, b)** or two **(e)** biological replicates whereas error bars represents the standard deviation from the mean – *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of remaining biofilms following 200 μM of furoxan treatment **(c, d)** with or without cPTIO **(d)** or when treated with different concentrations of furoxans **(f)**.

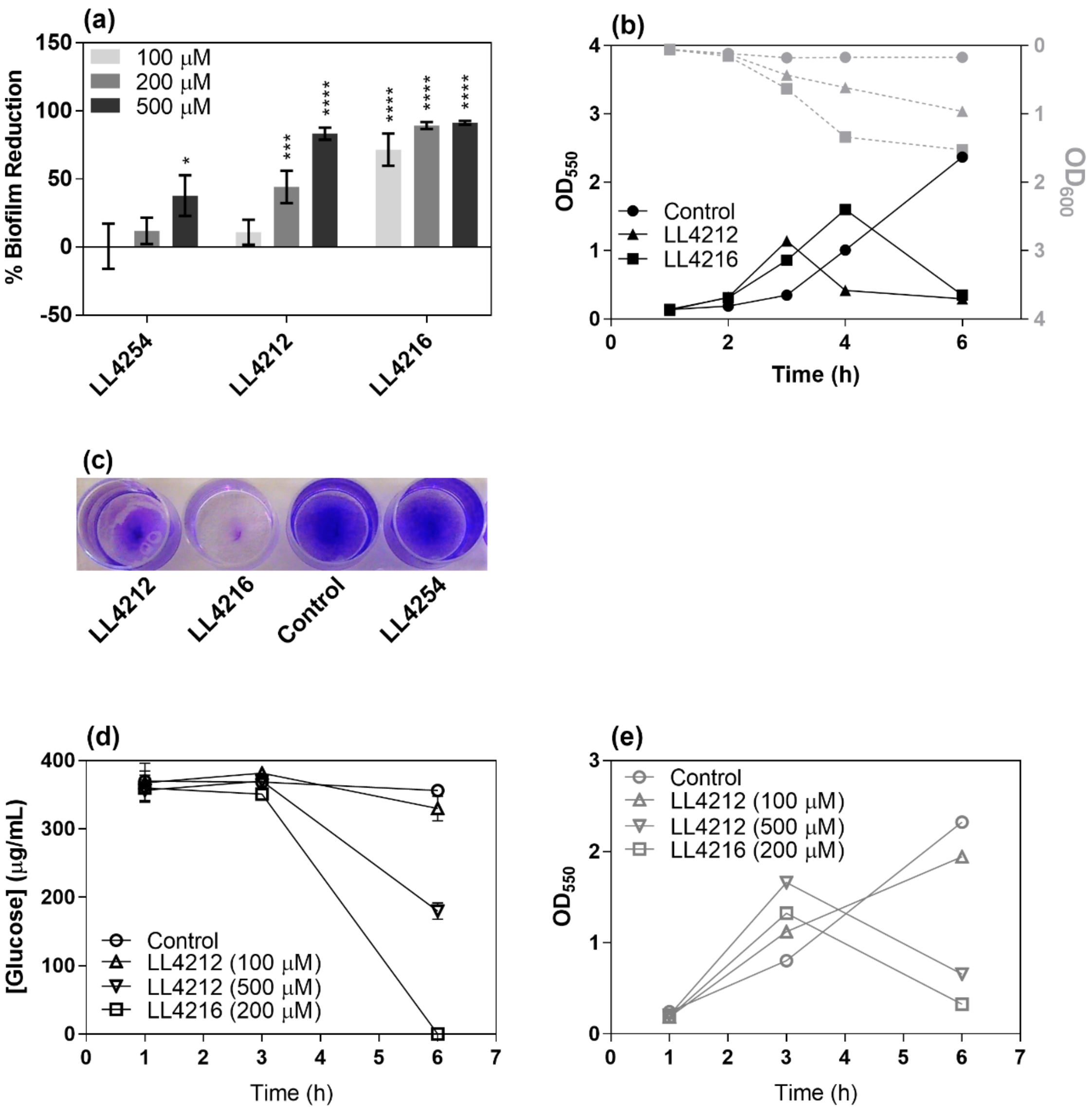

Figure 4. Reduction of *P. aeruginosa* biofilms upon treatment with furoxans for 6 h **(a)**, with bars representing data from three biological replicates and errors bars representing standard deviation of the mean – *, $p \leq 0.05$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$. Photographs show CV stains of biofilm remaining after 6 h following 200 μM furoxan treatment **(c)**. Biofilm formation and corresponding OD_{600} changes over 6 h upon addition of 200 μM of LL4216 and 500 μM LL4212 **(b)**. Glucose concentrations were quantified at t = 1 h, 3 h or 6 h after addition of furoxans **(d)**, with correponding changes in CV staining measured **(e)**. Symbols

represent data from two biological replicates with error bars showing the standard deviation of the mean in **(e)**, while one representative data set was plotted for **(b)** and **(e)**.

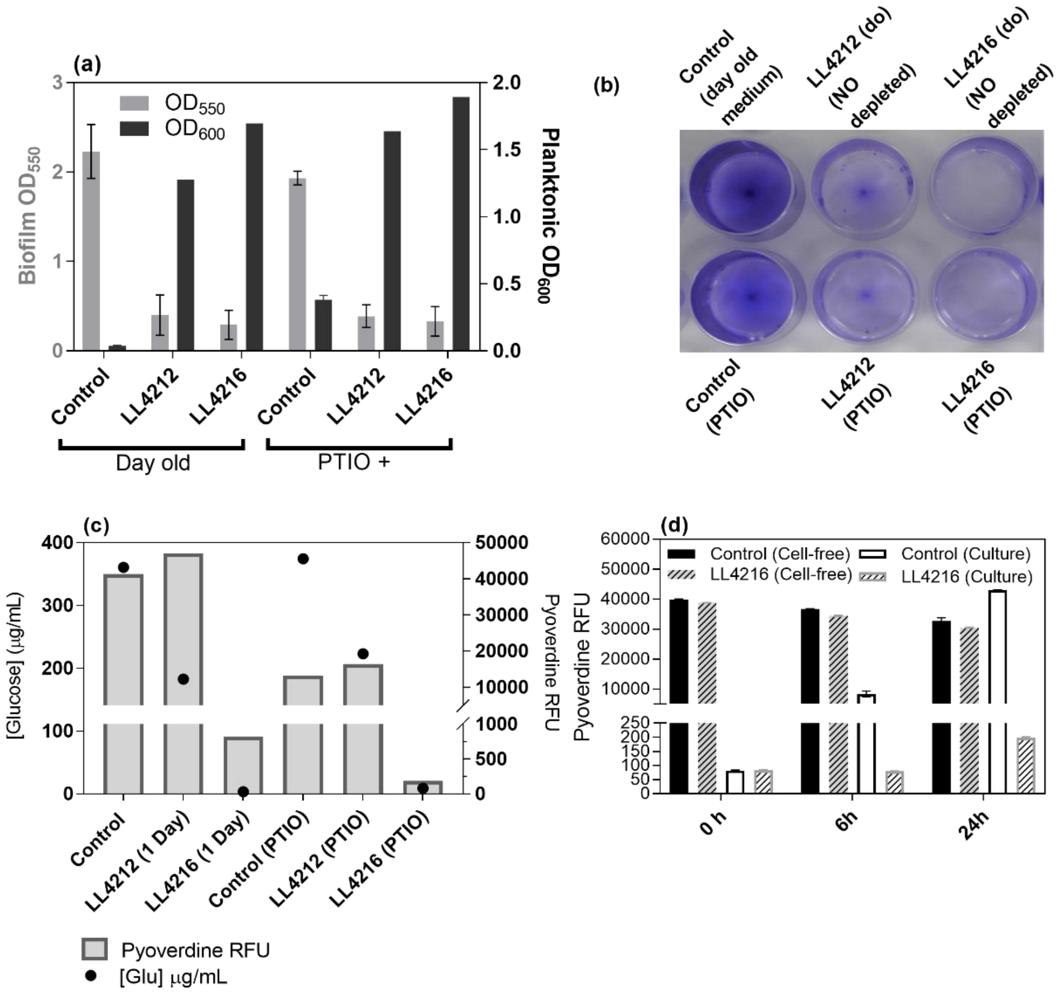

Figure 5. *P. aeruginosa* biofilm reduction upon treatment with 500 μM of furoxans in the presence of 0.5 mM cPTIO or NO-depleted furoxans in 1-day-old M9GC medium. Bars of OD_{550} measurements represent data from three biological replicates while bars of OD_{600} measurements represent data from one experiment. Errors bars represent standard deviation of the mean **(a)**. Photograph show CV stains of biofilm remaining after 6 h following furoxan treatment **(b)**. Changes in pyoverdine levels and glucose concentrations of samples treated as described in (a), with one representative data set plotted **(c)**. Pyoverdine changes over time in cell-free medium or culture in the presence or absence of 500 μM LL4216 **(d)**.

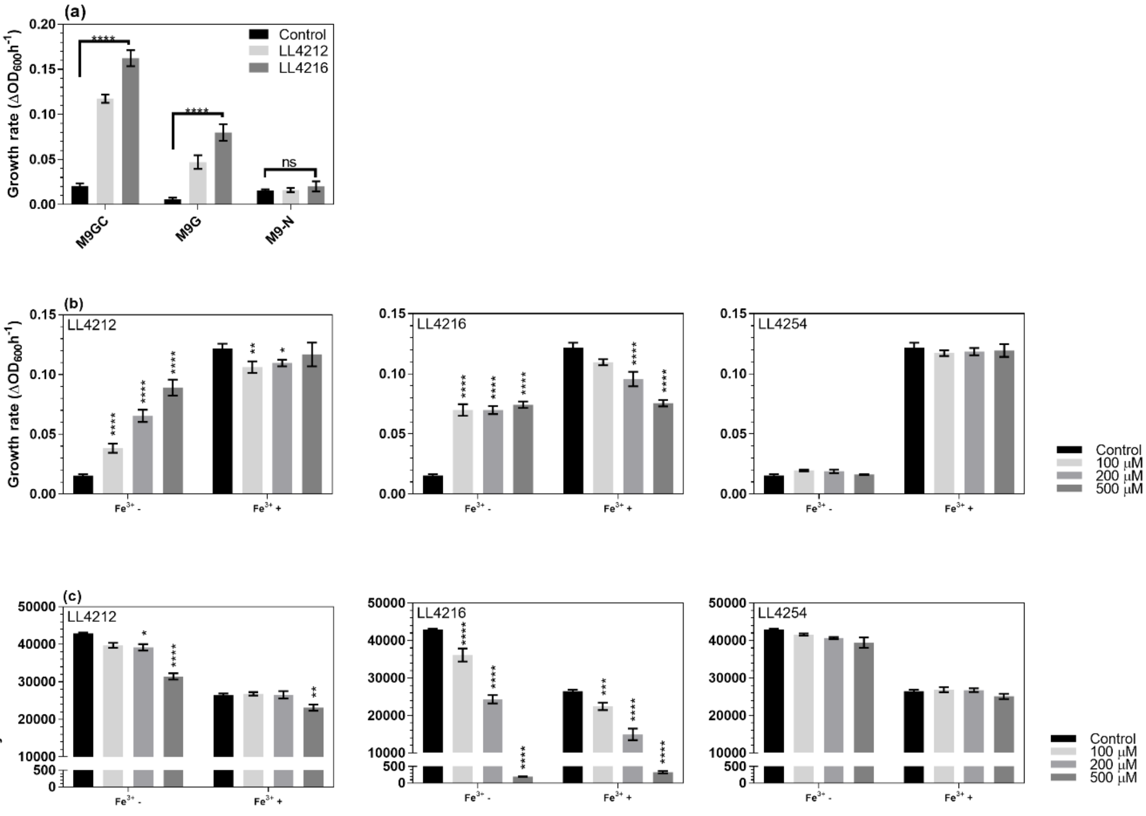

Figure 6. Growth rates in the absence or presence of 500 μM of LL4212, LL4216 and LL4254, calculated by changes in OD_{600} during the exponential growth phase, in M9 medium supplemented with different carbon or nitrogen sources **(a)**. Growth rates **(b)** and pyoverdine fluorescence **(c)** at 24 h of *P. aeruginosa* inoculated statically at 37°C in M9GC (Fe^{3+}-) or M9GCFe (Fe^{3+}+) medium in the presence of 100 – 500 μM of the furoxans. Bars represent data from three biological replicates and errors bars representing standard deviation of the mean. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$