Anti-pseudomonas activity of 3-nitro-4-phenylfuroxan

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Running title: Furoxans and *Pseudomonas aeruginosa* biofilm

Keywords: *Pseudomonas aeruginosa*, biofilm, furoxans, furazans,

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Abbreviations

MGE (mobile genetic elements), GFP (green fluorescent protein), NO (nitric oxide), MIC (minimal inhibitory concentration), CLSI (Clinical and Laboratory Standard Institute), LB (Luria Bertani), CV (crystal violet), CLSM (Confocal Laser Scanning Microscopy), UV (Ultraviolet), HPLC (High pressure Liquid Chromatography), ANOVA (Analysis of Variance), DMSO (dimethyl sulfoxide).

Abstract

Pseudomonas aeruginosa is a microorganism that is well adapted to both clinical and industrial settings, where it can form adherent communities that are difficult to eradicate. New anti-*Pseudomonas* compounds and strategies are necessary, as the current antimicrobial approaches for the inhibition of biofilm formation and, above all, the eradication of formed biofilms are ineffective.

Compounds that belong to the furoxan-family, which are well-known NO donors, have recently been shown to display anti-*Pseudomonas* activity. The present study investigates three furoxan compounds that are substituted at the heteroring with electron-withdrawing groups (NO_2, CN, $CONH_2$) for their effects on *P. aeruginosa* PAO1 growth and biofilm formation/dispersal. Of the furoxans tested, only 3-nitro-4-phenylfuroxan (KN455) inhibited the growth of suspended *P. aeruginosa* PAO1 cultures. Furthermore, KN455 inhibited the formation of both younger and older biofilms with very high yields and thus proved itself to be toxic to planktonic subpopulations. It also displayed moderate eradicating power. The activity of KN455 does not appear to be related to its capacity to release small amounts of NO. Interestingly, the isomer 4-nitro-3-phenylfuroxan (KN454), included for comparison, displayed a comparable antibiofilm rate, but did not show the same antimicrobial activity against suspended cells and planktonic subpopulations. While hypotheses as to the mechanism of action have been formulated, further investigations are necessary to shed light onto the antimicrobial activity of this furoxan.

Introduction

Pseudomonas aeruginosa is a microorganism that is optimally adapted to a variety of environmental conditions and is ubiquitously present in soil, water and sewage as well as in human, animal and plant hosts [1]. *P. aeruginosa* is also known to be a human opportunistic pathogen as it plays an important role in infections such as pneumonia in cystic fibrosis patients, chronic-wound infections and medical-device-related infections [2, 3].

Treatment of *P. aeruginosa* infections is often a challenge due to its resistance to antibiotics, which is acquired via a number of mechanisms. *P. aeruginosa* secretes enzymes, including β-lactamases and aminoglycoside modifying enzymes, that inactivate antibiotic molecules. It can also reduce antimicrobial agent accumulation in the cell producing multidrug efflux pumps (i.e. MexAB-OprM, MexEF-OprN, MexCD-OprJ, MexXY-OprM, etc.) [4]. Chromosomal mutations in DNA gyrase and topoisomerase IV genes lead to resistance towards quinolones [5]. The isolation of multidrug-resistant strains is increasing in clinical settings, where antibiotic selection is routinely practiced. Furthermore, the transfer of mobile genetic elements (MGE) contributes to the spread of resistance determinants among strains that belong to the same species and those of different species [6]. *P. aeruginosa* is also able to contaminate disinfectants such as chlorhexidine, benzalkonium and triclosan [7].

The treatment of *P. aeruginosa* infections becomes more difficult when bacteria are encased in their self-made matrix. This tolerance is caused by restricted compound penetration through the exopolysaccharide matrix and decreased metabolism, which is due to low nutrient and oxygen levels and accumulated waste [8]. The inhibition of biofilm formation and the eradication of mature biofilms are goals that are important for a wide range of antimicrobial applications because microbial biofilms are not only a significant issue in clinical infections, but also in the biofouling of industry water systems. Anti-biofilm strategies either attempt to destroy the established community in its microenvironment or target a specific step within the biofilm cycle. Biofilm disruption and/or corrosion can be achieved by metal chelators, such as EGTA and EDTA [9], by surfactants, such as SDS [10], by D-amino acids [11], and, as found more recently, by photoantimicrobials [12,13].

Nitric oxide (NO) has recently emerged as an endogenous regulator of biofilms [14]. Low levels of NO induce a transition from the biofilm mode of growth to the planktonic form [15]. A number of NO donors, products that can release NO under physiological conditions, have been found to suppress biofilm formation and elicit dispersal events in *P. aeruginosa* [15, 16, 17]. Furoxans

(1,2,5-oxadiazole N-oxides) are an interesting class of NO donors. A recent study has described the effects of three furoxan derivatives that are able to spontaneously release nitrogen oxide species (NOx), namely NO and nitrite (NO_2^-), on *P. aeruginosa* PAO1 biofilm formation and dispersion [18]. The present work extends our research into the use of furoxan derivatives as potential PAO1 antibiofilm tools, and focuses on the impairment of *P. aeruginosa* biofilms using furoxan derivatives, which release NO under the action of thiol cofactors. Phenylfuroxan derivatives with electron withdrawing substituents (NO_2, CN, $CONH_2$) at the 3 position of the heteroring (Figure 1) are investigated for their effect on *P. aeruginosa* biofilm formation in this work, as electron-withdrawing groups have been shown to facilitate the release of NO from furoxans [19, 20]. 3-Nitro-4-phenylfuroxan (KN455), 3-cyano-4-phenylfuroxan (CF357) and 4-phenyl-3-furoxancarboxyamide (CF1656) have been selected for this study. The isomer 4-nitro-3-phenylfuroxan (KN454) is included for comparison, as is the related des-NO furazan, 3-nitro-4-phenylfurazan (CF389).

Materials and methods

Synthesis of the tested compounds

Compounds KN455 [21]), CF357 [22], CF1656 [23], CF389 and KN454 [24] were synthetized as previously described.

Bacteria strains and growth conditions

Pseudomonas aeruginosa PAO1 was chosen as the model biofilm microorganism [25]. PAO1 was transformed using the pVO(GFP) expression vector; it is a pJB3 KmD derivative, in which the Green Fluorescent Protein (GFP) coding sequence was cloned under an arabinose inducible promoter (pAra). Both strains were grown overnight in LB medium at 37°C under 200 rpm shaking.

Antimicrobial assays

A modified disk diffusion assay (Kirby-Bauer test) was set up to test the sensitivity that *P. aeruginosa* PAO1 shows towards furoxans [26]. Microorganisms grown in LB overnight at 37°C were diluted 100-fold in phosphate buffer (KH_2PO_4/K_2HPO_4 10 mM, pH 7.4) and a 100 µl sample was plated onto LB Agar. Filter paper disks (bioMerieux) with 6 mm diameters were loaded with increasing volumes of furoxans/furazan (20, 15, 10 and 5 µl) at a concentration of 50 mM, and placed on LB Agar. A disk loaded with 20 µl DMSO was used as a control. The plates were incubated at 37°C for 24 h and the antibacterial effect was evaluated by measuring the growth inhibition halo.

In order to evaluate the furoxans' Minimal Inhibitory Concentration (MIC) toward *P. aeruginosa* PAO1, according to guidelines from CLSI (Clinical and Laboratory Standard Institute) [27], bacteria were grown overnight in LB at 37°C and diluted 100-fold to give a cellular concentration of 10^7 CFU ml^{-1}. Two-fold dilutions of KN455, CF357, CF1656, KN454 and CF389 (from 2 to 0.001 mM), were added to bacterial samples. The dilution series were observed for microbial growth after 24 hours of incubation at 37°C and MIC values were evaluated. MBC (Minimal Bactericidal Concentrations) values were determined by re-culturing dilutions that inhibited PAO1 growth (i.e. those at or above the MIC). Broth dilutions were streaked onto LB agar and incubated for 24 to 48 hours. The MBC was the lowest broth dilution of furoxan/furazan that prevented *P. aeruginosa* PAO1 growth on the agar plate. All experiments were performed in triplicate, at least.

Biofilm assays

Overnight-PAO1 cultures were diluted 1:200 times in M9 medium that was supplemented with glucose 10 mM and 0.2% w/v casamino acid, used as carbon and nitrogen sources respectively, to obtain homogenous and repeatable biofilms [28, 29, 30]. One ml of the diluted culture was added to each well of a 24-well polystyrene plate. The furoxans were administered at three different concentrations (5, 50 and 500 μM) and dimethylsulfoxide (DMSO) 0.5 % was used as a solvent control. L-cysteine 5 mM, NO scavenger 2-phenyl-4,4,5,5,-tetrame-thylimidazoline-3-oxide-1-oxyl (PTIO) 1 mM and sodium nitrite, at 0.01, 0.1 and 0.5 mM, were added when indicated. The cells were incubated at 37°C under 200 rpm shaking for 6 h to evaluate the inhibitory effect of furoxans. The biofilm dispersal effect of the furoxans was observed upon administering compounds for 1 h, after 5 h of untreated biofilm growth [18].

Planktonic biomass was spectrophotometrically quantified for each well via a measurement of absorbance at 600 nm, while adherent biomass was quantified via crystal violet (CV) staining. Briefly, planktonic biomass was removed and collected, and wells were washed once with one ml phosphate buffer. One ml of 0.1% CV was added to each well for approximately 20 min to stain the biofilm, after which the CV was removed and each well washed twice with 1 ml phosphate buffer. The remaining CV, which indicated the amount of biofilm present, was dissolved in acetic acid 30 % for 10 min. The amount of solubilized dye was spectroscopically measured at 595 nm.

In order to evaluate the effect of the different treatments on the cellular viability of suspended and adherent populations, the planktonic phase was axenically collected and adherent cells were recovered by scraping and suspended in 1 ml of phosphate buffer. Viable counts - expressed as colony forming units per mL, CFU ml^{-1}, in cell suspensions and as CFU per well in adherent biomass - were

estimated using a plate count technique; a volume (0.01 ml) of undiluted or serially diluted samples was plated onto LB Agar plates and incubated for 24 h at 37°C. All experiments were independently repeated at least three times.

Confocal analyses

Overnight PAO1- GFP tagged cultures were diluted 1:200 times in M9 medium that was supplemented with glucose 10 mM and 0.2% w/v casamino acid. Five ml of the diluted culture was added to a glass coverslip and placed into a well of a 6-well polystyrene plate. Volumes of the furoxans, 500 μM, and DMSO 0.5 % were added to the bacteria and subsequently incubated at 37°C. After either 24 h or 48 h of incubation, cells were induced with arabinose 1 mM to express green fluorescent protein. The coverslip was placed on a microscope glass slide for the acquisition of the adherent biofilm image. In order to observe the suspended bacteria, cells were placed between the glass and coverslip after 24 hours of treatment with furoxan/furazan 125 μM and DMSO 0.5 % as control. All microscopy observation and image acquisition was performed on a Leica TCS SP5 confocal laser scanning microscope (CLSM) (Leica Microsystems, Wetzlar, Germany) equipped with a detector and filter set for GFP monitoring. Images were obtained using a 63/1.3 objective lens. Simulated 3D images were generated using the free, open-source software ImageJ (National Institute of Healt, USA).

NO and NO_2^- detection

Nitrite measurements by Griess reaction

The total release of NO_x was evaluated as nitrite (NO_2^-) using the Griess reaction. Compounds were incubated at 37°C in 50 mM phosphate buffer, pH 7.4 at 0.1 mM concentration (thus final 1% DMSO) either in the absence or in the presence of L-cys at 0.1, 0.5 or 1 mM (1, 5 and 10 times mol/mol excess). The presence of nitrite in the reaction mixture was determined using the Griess assay at regular time intervals; 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). Absorbance was measured at 540 nm on a UV spectrophotometer after 10 min at room temperature (UV-2501PC, Shimadzu). A calibration curve was obtained using standard solutions of sodium nitrite in a concentration range of 1-80 μM ($r^2 > 0.99$). The yield in nitrite was expressed as NO_2^- percentage (mol/mol, relative to the initial compound concentration) ± SEM.

NO measurements using the DAN (2,3-diaminonaphthalene) method

NO release from furoxan compounds was quantified using a 2,3-diaminonaphthalene (DAN)-based chemical assay. This assay 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), which 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 (then final 1% DMSO) with 0.2 mM (2 times mol/mol excess) DAN, either in the absence or in the presence of L-cys at 0.5 mM. The presence of NAT in the reaction mixture was evaluated using HPLC analysis at fixed time points, according to a previously published protocol [31]. HPLC analyses were performed using a HP 1200 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) that was equipped with a quaternary pump (model G1311A), a membrane degasser (G1322A), a UV detector, MWD (model G1365D), and a fluorescence detector (model G1321A), all integrated into the system. Data analyses were performed on a HP ChemStation system (Agilent Technologies). The sample was eluted on a Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 µm; Agilent); the injection volume was 20 µl. The mobile phase consisted of 65% 15 mM potassium phosphate buffer (pH 8.0) and 35% acetonitrile at a flow rate of 1.00 ml min^{-1}. The fluorescence signals were obtained at excitation and emission wavelengths of 355 and 460 nm, respectively (gain factor = 10). Data manipulation was performed using an Agilent ChemStation. The values obtained from integrating the NAT peak were interpolated into a calibration line that was 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 an excess of DAN (0.2 mM). The reaction mixture was diluted in a phosphate buffer after 10 min (at pH = 7.4 and NO final concentration 1 to 80 µM), and analysed by HPLC.

Statistical analyses

Experiments were repeated at least three times on separate dates. Mean and SD calculations were performed using Microsoft Excel 2010. Data were analysed by means of one-way ANOVA (Origin_ 7.0 SR0; Origin lab) and the statistical significance of all treatments was estimated ($P < 0.05$ and $P < 0.01$).

Results

Effect of furoxans on P. aeruginosa PAO1 growth

The furoxans KN455, CF357 and CF1656, which bear different substituents on the 3 position of the furoxan heteroring (Figure 1), were investigated for their effect on *P. aeruginosa* PAO1 growth, both in solid and liquid media. As a first step, a Kirby-Bauer test was performed to assay the activity

of the three compounds. The DMSO used to dissolve the furoxans did not influence bacterial growth. Of the three compounds, only KN455 provided a reproducible inhibition growth halo (12.3 ± 0.57 mm). In order to investigate the possible involvement of nitric oxide (NO) in the observed inhibitory activity, the corresponding furazan CF389, which is known not to release NO, was included in the analysis and no inhibition halo was observed (Figure 2). Furthermore, the corresponding 4-nitro isomer (KN454) was assayed to evaluate whether the nitro position on the heteroring influenced antimicrobial activity. In fact, KN454 failed to show any antimicrobial activity (Figure 2) and 3-nitro-4-phenylfuroxan was the only active compound. Furthermore, its activity was dose-dependent (data not shown).

The MIC value of KN455 was 125 μM, and the MBC value 250 μM, meaning that its Killing Quotient, calculated as MBC/MIC ratio, was lower than 4. This would indicate that the effect of KN455 is bactericidal. The MIC value of its isomer KN454 was 1 mM, whereas the MIC values of furoxans CF357 and CF1656, and furazan CF389 were all higher than 2 mM.

Confocal analyses were performed to evaluate the effect of KN455 on suspended PAO1 cells. The PAO1 strain was transformed with the Green Fluorescent Protein-gene-carrying plasmid, pVO, under the control of the *ara*BAD promoter. Upon induction with arabinose, the cultures produced enough GFP to give a fluorescent signal, while cells that were not induced with arabinose were not fluorescent (data not shown). Upon 24 hours of treatment with KN455 125 μM (MIC), few survivors were detected and most showed morphological changes compared to untreated (not shown) and DMSO 0.5 % treated cells (3A). As seen in Figure 3B, the surviving bacteria are longer than the untreated cells. In fact, the binary division process seems to be impaired, septation appears to be incomplete and giant cells are present in the treated group. Moreover, some of the filamentous rods show bright fluorescence while others show less pronounced GFP fluorescence. CF389 (Figure 3C) and KN454 125 μM (data not shown) did not impaired the cellular viability as KN455, however few long cells were detectable. The administration of CF1556 (Figure 3D) and CF357 (data not shown) did not cause any change in cellular density, GFP expression and bacterial morphology.

Effect of furoxans on the inhibition of P. aeruginosa biofilm formation

As it is known that furoxans release NO, the effect of increasing furoxan concentration (5, 50 and 500 μM) on PAO1 biofilm formation was investigated. Compounds were added to minimal medium together with PAO1 and the adherent biomass was examined using crystal violet staining after 6 hours of incubation. Compounds were dissolved in DMSO 0.5 %, without significantly changing the amount of adherent biomass ($p = 0.61$) compared to untreated controls (data not shown).

The nitro substituted furoxan, KN455, provided the most significant and dose-dependent biofilm inhibition of the three different furoxans tested, giving significant inhibition rates at the highest concentrations of 50 and 500 μM, respectively (Figure 4A). The CN substituted furoxan, CF357, inhibited biofilm formation in a significant manner ($p = 0.014$) at 500 μM (Figure 4B), while the $CONH_2$ substituted analogue, CF1656, did not show any anti-biofilm effect at any tested concentration (Figure 4C). As the nitro derivative showed the highest inhibitory activity, its 4-nitro isomer KN454 and the corresponding des-NO furazan analogue, CF389, were also tested for their ability to inhibit biofilm formation. The rates of inhibition caused by KN454 and CF389 at the highest tested concentrations were statistically significant as shown in figures 4D and 4E, respectively (data not shown).

The viability of sessile biofilm cells was checked and KN455, alone among the tested furoxans, displayed a toxic effect against this sub-population (Figure 5A). A ~3-log unit decrease, compared to untreated cells, was observed. The corresponding 4-nitro isomer, KN454, and furazan, CF389, caused 2-log unit decreases when administered at 500 μM.

The planktonic counterparts of each biofilm were also evaluated as they play an important role in bacterial dissemination and equilibrium maintenance in the biofilm community. DMSO 0.5 % did not influence the cellular viability of planktonic biomass, compared to untreated cells (data not shown), while KN455 provided a much more significant decrease in viable cells ($p = 3.2 \times 10^{-7}$) than the untreated samples (Figure 5B). Furoxans, CF357 and CF1656, did not alter the viability rate of cells (Figure 5B). Furazan CF389 and furoxan KN454 only caused a 1-log unit decrease, compared to untreated control biofilms.

Confocal analyses of 24- and 48-hour-old biofilms showed that the inhibitory effect of KN455 500 μM was also reliable when used in prolonged treatment (Figure 6). The GFP signal of a furoxan-incubated biofilm was lower than those of untreated and DMSO 0.5 % v/v treated biofilms. KN455's inhibitory effect was also evident against a 48-hour-old biofilm, which was noticeably thicker than the 24-hour-old biofilm (Figure 6). The corresponding furazan CF389 was not as effective as KN455, especially against 48 hour old biofilm (figure 6).

Effect of furoxans on biofilm eradication

While the inhibition of biofilm formation is an important goal, biofilm eradication is much more difficult to achieve and antimicrobials usually fail. The compounds were administered at 500 μM for 1h to cells grown for 5h to assay biofilm eradication power. The solvent was not toxic to cells (data not shown). KN455 showed the most statistically significant ($p = 9.65 \times 10^{-4}$) eradication power, of the furoxans tested (Figure 7). The decreases in adherent biomass caused by isomer KN454 and des-

NO furazan CF389 were not significant. The solvent was not toxic to cells in tests on planktonic counterparts and the biomass changes observed upon furoxan treatment were not significant (data not shown), compared to control samples DMSO treated biofilms.

Insight into anti-Pseudomonas activity of KN455

KN455 was the most effective anti-*Pseudomonas* agent of the furoxans tested as it inhibited both cellular growth and biofilm formation, and even showed moderate biofilm eradicating power. The kinetics of NO_x release from KN455 were compared to those of the other furoxans, CF357 and CF1656, because it this thought that NO may play the role of anti-biofilm messenger. Furthermore, the isomer KN454 and corresponding furazan CF389, which were included in the antimicrobial and antibiofilm assays, are also included in Figure 8, as % mol/mol. The amounts of NO_2^- detected for 3-nitro-4-phenylfuroxan (KN455) were about 130 mol/mol after 1 hour. The amount of NO_2^- produced by KN455 is the sum of the NO_2^- formed by the nucleophilic substitution of the nitro group (see supporting information, S1, pathway a), and of the NO_2^- produced by the aerobic oxidation of the NO released following the ring opening of the tetrahedral intermediate precursors (see supporting information, S1, pathways b, R=NO_2, R = CyS). DAN (2,3–diaminonaphtalene) assays (Fang *et al.* 2009), which allow NO centre dots to be specifically detected, were carried out to shed further light onto this process. NO_2^- was the dominant species produced by KN455 after 1 h, whereas NO only corresponded to 5% of total NOx. The amount of NO_2^- detected for the 4-nitro isomer KN454 was about 80 % mol/mol after 1 hour (Figure 8). For the *des*NO-nitrofurazan CF389, which cannot release NO from the heterocycle core, the reaction rate was slower and the amount of NO_2^- detected was 25 % mol/mol after 1 hour (Figure 8). 3-cyanofuroxan, CF357, and 3-carbamoylfuroxan, CF1656, produced nitrite in good and modest amounts, respectively. However, it is worth noting that these yields derived exclusively from the oxidation of NO (DAN test).

The highest rate of NO release was displayed by CF357, which, however, showed low anti-biofilm activity. Furoxans KN455 and CF1656, which release the same very low NO amount, showed different effects on microbial growth and biofilm formation. These data seem to rule out the possible involvement of NO in KN455 toxicity.

In order to investigate this hypothesis, biofilm formation experiments were repeated in the presence of PTIO, a known NO radical scavenger, and no changes were observed (data not shown). The possible involvement of nitrite, the other species released in large amounts by KN455, was also evaluated. The biofilm inhibition effect induced by acidified sodium nitrite was checked and compared to that of equimolar increasing concentrations of KN455. Sodium nitrite was less active than KN455 at the tested concentrations (Figure 9).

Since previous studies have shown that thiol groups, such as those present in L-cysteine (L-cys), can promote the liberation of NOx (NO and NO_2^-) from furoxan compounds (Feelisch M. *et al.* 1992), PAO1 was treated with increasing concentrations of KN455 in the presence of an excess of cysteine 5mM. L-Cysteine decreased the anti-biofilm activity of 50 μM and 500 μM KN455 (Figure 10). Furthermore, L-cysteine abolished the anti-biofilm activity of KN455-related compounds, KN454 and CF389 (data not shown).

The reaction between L-cysteine and KN455 was monitored by HPLC in order to more deeply probe the role played by thiol groups. HPLC/mass data (Figure S2) indicate that, after 20 min, the compound was almost quantitatively transformed into 3-cysteinyl-4-phenylfuroxan (282 M+1, 120 M-cysteinyl), which is the product of the nucleophilic substitution of the 3-nitro group with cysteine (see supporting information S1, pathway a). Over time, this latter compound was largely transformed into unidentified products and NO_2^- (see supporting information S1, pathway b). The 4-nitro isomer, KN454, also reacted with L-cysteine giving rise to the corresponding cysteinyl derivatives (see supporting information S3). Des-NO furazan CF389, which cannot release NO from its heterocycle core, behaved similarly and afforded the cysteinyl substituted product. 3-Cyanofuroxan, CF357, and 3-carbamoylfuroxan, CF1656, produced nitrite and unidentified products (see supporting information S4).

Discussion

Previous studies have shown that thiol groups, such as those present in L-cysteine (L-cys), can promote the liberation of NOx from furoxan compounds and increase their activity [18]. In this study, three phenylfuroxan derivatives that can release varying amounts of NO_x were assayed for their antimicrobial activity against *P. aeruginosa* PAO1, and it would appear that the substituent influences furoxan antimicrobial activity; the NO_2-substituted analogue, KN455, inhibited the growth of PAO1, whereas CN- and $CONH_2$-substituted furoxans did not show any antimicrobial activity in the solid media. Furthermore, KN455 activity was bactericidal. Interestingly, the position of the nitro group on the furoxan skeleton significantly influences antimicrobial activity against *P. aeruginosa* PAO1; KN454 did not show any inhibition halo in the solid media and its MIC value was four-times higher than that of KN455.

Furthermore, the chosen furoxans showed different NOx release rates. As NO is emerging as an anti-biofilm messenger [32], the biofilm formation inhibition activity of the furoxans was investigated and correlated with NOx release. KN455, which is characterized by very low NO release, proved itself to be the most active compound in inhibiting biofilm formation, whereas CF357 and

CF1656, which can release good and very low amounts of NO, respectively, showed weak or null activity even at the highest concentrations tested. CF389, which is known not to release NO, and KN454, which releases nitrite, showed inhibitory activity. It would thus appear that nitric oxide does not play a role in furoxan biofilm inhibition. Indeed, KN455 was very toxic and no viable cells were recovered from the planktonic subpopulation of the six-hour-old biofilms. By contrast, the 4-nitro isomer KN454 was not toxic to the planktonic subpopulation, and only caused a slight decrease in cellular viability. The two furoxans showed different selectivities against biofilm cellular subpopulations; KN455 inhibited the growth of both planktonic and adherent cells, while KN454 was selectively active against adherent bacteria.

Furthermore, the furoxans release different amounts of nitrite (KN455 > CF357 > CF1656), which has been reported to inhibit staphylococcal biofilm formation [33, 34]. Under the tested conditions, sodium nitrite alone did inhibit *P. aeruginosa* PAO1 biofilm formation, but showed lower activity than equimolar concentrations of KN455. Although the nitrite-effect cannot be ruled out, other mechanisms should be considered.

L-cysteine prevented KN455 from inhibiting biofilm formation. It is reasonable to connect KN455 activity with its capacity to react with the thiol groups (or other nucleophilic centres) present on functional targets that are involved in cellular division and/or biofilm formation. This may also explain the activity of the 4-nitro isomer, KN454, and of the related des-NO furazan, CF389, which behave similarly in their reactions with cysteine.

Cysteine has also been demonstrated to act as an antioxidant, supporting the hypothesis that furoxan and furazan can elicit oxidative stress [35]. This is in agreement with the morphological changes observed upon furoxan treatment. Indeed, filamentation is a typical stress response that occurs when cell growth continues in the absence of cell division [36]. Both highly fluorescent giant cells and other cells that showed less pronounced GFP fluorescence were observed in furoxan/furazan-treated samples. GFP expression in the first group of cells was compatible with functional transcriptomic and expression machinery. The longest bacteria showed fuzzy fluorescence, which is compatible with GFP that has been broken down following decreased protein synthesis and/or permeability stress. However, the impairment of divisome machinery and cell wall septation seems to precede a pre-death phase. The low fluorescence of filamentous cells can probably be related to decreased metabolic activity before cellular death, as the formation of multinucleate giant cells may be a pre-death phase. Both nitro-furoxans, KN455 and KN454, and furazan, CF389, seem to elicit cellular stress, which induces cellular elongation.

In conclusion, nitrofuroxan KN455 quite potently inhibits *P. aeruginosa* PAO1 growth and prevents PAO1 biofilm formation. This activity does not appear to be related to its capacity to release small amounts of nitric oxide, rather than nitrite. Oxidative stress and/or the impairment of divisome machinery, which is ascribable to its strong electrophilic properties, may contribute to this anti-*Pseudomonas* activity. Additional work is necessary to investigate the intriguing differences found between furoxan KN455 and the isomer KN454, as well as to identify their target/s.

Acknowledgments

The authors thank Chiara Scanferla and Giulia Della Vedova for their assistance with experimental work, Nina Mackova for supplying KN455 and Alberto Gasco for his fruitful discussions.

Disclosure statement

The authors report no potential conflict of interest.

Funding

This research was funded by the University of Insubria (Fondo di Ateneo per la Ricerca) and by the University of Turin (Ricerca Locale ex 60%).

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CAPTIONS

Figure 1. Schematic representation of the furoxans and furazan investigated.

Figure 2. Kirby-Bauer disk diffusion tests. Effect of 20 µl of DMSO 0.5 %, KN455, KN454 and CF389 at a concentration of 50 mM on the growth of *P. aeruginosa* PAO1.

Figure 3. Confocal images of *P. aeruginosa* PAO1 that was GFP-tagged after 24 hours of treatment with DMSO 0.5 % (A) and KN455 (B), CF389 (C) and CF1656 (D) 125 µM, respectively (bar 10 µm). GFP expression was induced by arabinose 1 mM, one hour before confocal analyses.

Figure 4. Effect of phenyl-R-substituted furoxans/furazan on the inhibition of *P. aeruginosa* PAO1 biofilm formation. The effect was evaluated by CV staining OD595 values upon treatment for 6 hours with KN455 (A), CF357 (B), CF1656 (C), KN454 (D) and CF389 (E) at increasing concentrations, with bars representing data from at least three biological replicates and error bars representing standard deviation from the mean *, $p \leq 0.05$; **, $p \leq 0.001$.

Figure 5. Viable counts of the adherent (A) and planktonic (B) phases upon the inhibition of *P. aeruginosa* PAO1 biofilm formation exerted by furoxans/furazan 500 µM. The data expressed as CFU $well^{-1}$ and CFU ml^{-1} of survivors, respectively, are the means of at least three independent experiments ± SD * $p < 0.05$, ** $p < 0.001$ and *** $p < 10^{-6}$.

Figure 6. Projections and sections of CLSM images of *P. aeruginosa* PAO1 GFP-tagged biofilms that were subjected to furoxan/furazan and solvent treatments. Bacteria were treated for 24 hours (left) or 48 hours (right) with KN455 and CF389 500 µM or DMSO 0.5 %. The live cells that express GFP are green.

Figure 7. *P. aeruginosa* biofilm dispersal upon treatment with furoxans at 500 µM for 1h. The bars represent data from three biological replicates and error bars represent the standard deviation from the mean *, $p \leq 0.05$.

Figure 8. NO and total NO_2^- released by furoxans (KN455, KN454, CF357,CF1656) and furazan CF389 in the presence of L-cysteine (5:1 molar excess) at a selected time point (60 min). The results are expressed as percentage (% mol/mol) of NO or total NO_2^- released with respect to the quantity of parent compound. Bars represent data from three or more replicates and error bars represent standard deviations from the mean.

Figure 9. *P. aeruginosa* biofilm inhibition upon treatment with KN455 and Sodium Nitrite for 6 hours at increasing concentrations. The error bars represent the standard deviation from the mean of three biological replicates.

Figure 10. *P. aeruginosa* biofilm inhibition upon treatment with KN455 500 μM for 6 hours in the presence and absence of L-cysteine 5 mM. The bars represent OD595 values upon Crystal Violet staining of three biological replicates, whereas error bars represent the standard deviation from the mean.