New Life for an Old Drug: the Anthelmintic Drug Niclosamide Inhibits *Pseudomonas aeruginosa* Quorum Sensing

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The need for novel antibacterial strategies and the awareness of the importance of quorum sensing (QS) in bacterial infections have stimulated research aimed at identifying QS inhibitors (QSIs). However, clinical application of QSIs identified so far is still distant, likely due to their unsuitability for use in humans. A promising way to overcome this problem is searching for anti-QS side activity among the thousands of drugs approved for clinical use in the treatment of different diseases. Here, we applied this strategy to the search for QSIs, by screening a library of FDA-approved compounds for their ability to inhibit the QS response in the Gram-negative pathogen *Pseudomonas aeruginosa*. We found that the anthelmintic drug niclosamide strongly inhibits the *P. aeruginosa* QS response and production of acyl-homoserine lactone QS signal molecules. Microarray analysis showed that niclosamide affects the transcription of about 250 genes, with a high degree of target specificity toward the QS-dependent regulon. Phenotypic assays demonstrated that niclosamide suppresses surface motility and production of the secreted virulence factors elastase, pyocyanin, and rhamnolipids, and it reduces biofilm formation. In accordance with the strong antivirulence activity disclosed *in vitro*, niclosamide prevented *P. aeruginosa* pathogenicity in an insect model of acute infection. Besides the finding that an FDA-approved drug has a promising antivirulence activity against one of the most antibiotic-resistant bacterial pathogens, this work provides a proof of concept that a lateral anti-QS activity can be detected among drugs already used in humans, validating a new approach to identify QSIs that could easily move into clinical applications.

The introduction of antibiotics into clinical practice at the middle of the 20th century was a milestone in the history of medicine. However, the original expectation that all bacterial infections could be defeated one day by antibiotics was soon diminished by the emergence of antibiotic-resistant strains, prompting the still-ongoing race for the discovery of new antibacterial agents. While the treatment of infections sustained by antibiotic-resistant bacteria has high socio-economic costs and represents a major health problem worldwide, the pharmaceutical industry has dramatically reduced investments in antibiotics research. As traditional antibiotic research appears to be helpless in coping with the emergence of antibiotic-resistant strains, novel scientifically sound, and cost-effective approaches should be undertaken in order to identify new drugs (1).

Selective optimization of side activities of drug molecules (the SOSA approach) is a smart strategy for the identification of new potential drugs (2). A limited number of highly diverse drugs whose use in humans has already been approved are screened for side activities against unrelated diseases. Once a hit compound has been found, it can be either tested directly in clinical studies or used as the lead for drug optimization programs. This strategy has a high probability of yielding safe and bioavailable drug-like compounds, and it is thus expected to reduce the time and cost generally associated with standard drug discovery processes (2–4).

An innovative strategy to combat bacterial infections relies on specific inhibition of bacterial virulence, hence the ability to cause disease rather than bacterial growth (5). The use of "antivirulence drugs" could have the advantage of reducing bacterial adaptability to the host environment, facilitating the host immune system to combat the infection and reducing the strong selective pressure exerted by conventional antibiotics (6), although this is not yet supported by direct clinical evidence.

In many bacteria, pathogenicity is controlled and coordinated by an intercellular communication process named quorum sensing (QS). QS is based on the synthesis and secretion of a signal molecule that binds to a cognate receptor. The signal-activated receptor controls the expression of target genes. Since the production of the signal molecule is proportional to bacterial growth, QS coordinates gene expression in response to a bacterial population density (7). So far, QS is considered one of the most promising targets for antivirulence therapies (6, 8, 9).

In this study, the SOSA approach has been applied to the identification of antivirulence drugs targeting bacterial QS, using *Pseudomonas aeruginosa* as the model organism. *P. aeruginosa* is one of the most dreaded Gram-negative pathogens in developed countries, being responsible for both community- and hospital-acquired infections. In addition, *P. aeruginosa* chronic lung infection is the major cause of death in cystic fibrosis (CF) patients, a genetic disease affecting about 1/3,000 newborns in the Caucasian population (10–13). Besides being intrinsically resistant to several antibiotics, *P. aeruginosa* can easily acquire new resistance determinants, and indeed the emergence of pan-resistant strains has already been documented (14). For these reasons, *P. aeruginosa* infections are generally characterized by high morbidity and mortality rates (13, 15).

The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors (16), the majority of which are positively controlled by QS (17). The *P. aeruginosa* QS network consists of three different QS systems, based on the production of specific signal molecules: *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL), *N*-butanoyl-homoserine lactone (C_4-HSL), and 2-heptyl-3-hydroxy-4-quinolone (PQS). *P. aeruginosa* QS is hierarchically organized, since $3OC_{12}$-HSL is required for optimal production of the other QS signals (17).

QS controls the expression of nearly 10% of the *P. aeruginosa* genome, including genes for biofilm formation, secreted virulence factors, and immune-modulatory and proinflammatory agents (17). QS signal molecules can be detected in clinical samples, proof that QS is active during *P. aeruginosa* infections. Moreover, QS-defective mutants show strongly impaired virulence in several animal models of infection, corroborating the importance of QS for *P. aeruginosa* pathogenicity and its suitability as a target for the development of anti-*Pseudomonas* drugs (18, 19).

We recently developed a convenient system for the identification of compounds affecting the *P. aeruginosa* $3OC_{12}$-HSL-based QS system at multiple levels: (i) expression/activity of the signal receptor, (ii) expression/activity of the signal synthase, and (iii) activity/availability of the signal molecule (20). Here, the screening of a library of about 1,000 compounds with known pharmacological activities has validated this system.

Seven hit compounds were identified. Among these, we focused our investigation on the anthelmintic drug niclosamide, which showed high inhibitory activity against *P. aeruginosa* QS and virulence both *in vitro* and *in vivo*. To the best of our knowledge, this is the first demonstration that the SOSA approach can be successfully applied to the search for anti-QS drugs.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. Wild-type *P. aeruginosa* PA14 (21), the PA14 *lasI* mutant (22), and the $3OC_{12}$-HSL reporter strain PA14-R3 (20) were routinely grown in Luria-Bertani (LB) broth (23) supplemented with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS; pH 7.0), unless otherwise stated. AB minimal medium supplemented with 0.02% (wt/vol) glucose as carbon source was used in the biofilm assay (24). Synthetic acyl-HSLs were purchased from the University of Nottingham, United Kingdom. Stock solutions (25 mM) were prepared in ethyl acetate acidified with 0.1% (vol/vol) acetic acid. Niclosamide was purchased from Sigma-Aldrich and resuspended in dimethyl sulfoxide (DMSO) at a 10 mM final concentration. Niclosamide ethanolamine salt was purchased from Chemos and resuspended in DMSO at 1 M and then diluted in water to a 10 mM final concentration.

QSI screening assay. *P. aeruginosa* PA14 and the $3OC_{12}$-HSL reporter strain PA14-R3 were grown overnight at 37°C on LB agar plates. Bacteria were scraped from plate surfaces and diluted in LB to an absorbance at 600 nm (A_{600}) of 0.045 and 0.015 for PA14-R3 and PA14, respectively (3/1 reporter/wild type ratio) (20). Aliquots of 200 μl of the coculture were grown at 37°C in 96-well microtiter plates in the presence of 10-fold dilutions of each compound from the Prestwick Chemical Library (final concentrations of 100, 10, and 1 μg/ml). The A_{600} and light counts per second (LCPS) were measured at 4 h of growth in a Wallac 1420 Victor³V multilabel plate reader (PerkinElmer). Six wells containing the same coculture grown in the absence of any added compound were used as controls in each microtiter plate.

Quantification of QS signal molecules. Levels of QS signal molecules in *P. aeruginosa* culture supernatants were determined at different times during bacterial growth by using the previously described reporter strains specific for $3OC_{12}$-HSL (20), C_4-HSL (25), and PQS (26), according to a recently developed protocol (20). Briefly, 10 μl of culture supernatant (or appropriate dilutions) was added to 190 μl of LB inoculated with each reporter strain (final A_{600}, 0.045) in 96-well microtiter plates. Microtiter plates were incubated at 37°C with gentle shaking, and the A_{600} and LCPS were measured after 4 h of growth. Dedicated calibration curves were generated by growing each reporter strain in the presence of increasing concentrations of the corresponding synthetic signal molecule, and these curves were used to calculate the concentration of the different QS signal molecules in each culture supernatant.

Transcriptome analysis. *P. aeruginosa* PA14 was inoculated at an A_{600} of 0.01 into 20 ml of LB supplemented with 50 mM MOPS (pH 7.0), with or without 20 μM niclosamide. The cultures were grown at 37°C with vigorous shaking until they reached an A_{600} of 2.5, and then 1 ml of cells was harvested by centrifugation and resuspended in 2 ml of RNAProtect bacteria reagent (Qiagen). Transcriptome analysis was performed by using high-density oligonucleotide microarrays as previously described (27), with minor modifications. Briefly, total RNA was purified by using RNeasy minicolumns (Qiagen), including the on-column DNase I digestion described by the manufacturer. In addition, the eluted RNA samples were incubated for 1 h at 37°C with Turbo DNase (0.2 U per μg of RNA; Ambion) and SUPERase-In (0.4 U per μg of RNA; Ambion). DNase I was removed by using the RNeasy MinElute cleanup kit (Qiagen) according to the manufacturer's instructions. RNA integrity was monitored by agarose gel electrophoresis of glyoxylated samples and use of an RNA 6000 Nano LabChip in an Agilent 2100 bioanalyzer (Agilent Technologies); the RNA integrity number (RIN) was ≥9.7. Next, 10 μg of total RNA was used with random primers and Superscript II reverse transcriptase (Life Technologies) to perform cDNA synthesis. cDNA fragmentation, labeling, hybridization, staining, and washing steps were performed according to the manufacturer's protocol for the Affymetrix *P. aeruginosa* GeneChip arrays. Finally, the arrays were scanned with the Affymetrix GeneChip scanner 3000. Processing of the *P. aeruginosa* GeneChip (Affymetrix) was performed at the Genopolis Consortium for Functional Genomics (University of Milan-Bicocca, Milan, Italy). Under each condition, cultures were grown in triplicate, and RNAs from these cultures were pooled before proceeding to cDNA synthesis. In addition, biological replicates for each condition were performed on a separate day and run on a different microarray chip. The *P* value threshold was <0.05, and the cutoff for fold changes in gene expression was >2.

Assays for production of secreted virulence factors. Pyocyanin was extracted with 3 ml of chloroform from 5-ml cell-free supernatants of *P. aeruginosa* cultures grown at 37°C for 10 h in LB supplemented with different concentrations of niclosamide and then reextracted into 1 ml of 0.2 N HCl. The A_{520} of the resulting solution was measured to determine the amount of extracted pyocyanin (28). Elastase activity was determined in 100 μl of the same cell-free supernatants by the elastin-Congo red method as described previously (29).

Rhamnolipids in cell-free supernatants of *P. aeruginosa* cultures grown for 24 h in LB at 37°C were determined by the orcinol method as described previously (30), using defined concentrations of rhamnose as a standard. The rhamnolipid concentration was calculated based on the assumption that 1 μg of rhamnose corresponds to 2.5 μg of rhamnolipids (30).

Bacterial motility and biofilm assays. Swimming, swarming, and twitching motilities were assessed as described previously (31). PA14 cultures grown in LB for 14 h were either directly transferred to swimming (0.1% [wt/vol] tryptone, 0.05% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 0.3% [wt/vol] bacteriological agar) and twitching (1.0% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl, 1.0% [wt/vol] bacteriological agar) plates by using a sterile toothpick or diluted in fresh LB medium to an A_{600} of 0.1 and then spotted (2 μl) onto swarming plates (0.8% [wt/vol] nutrient broth N.2, 0.5% [wt/vol] glucose, 0.5% [wt/vol] bacteriological agar). Plates were supplemented or not with increasing concentrations of niclosamide. After 16 h of growth at 37°C, swimming

and swarming motilities were directly observed at the air-agar interface, while twitching motility was measured at the agar-plastic interface after removal of the agar layer and staining with crystal violet ([31]).

Biofilm formation was assessed using the microtiter plate biofilm assay ([32]). Bacterial cells were grown in LB for 14 h, washed twice with AB medium, and resuspended in AB medium supplemented with 0.02% (wt/vol) glucose at an A_{600} of 0.1 in the presence or absence of increasing niclosamide concentrations. Aliquots of 100 μl were transferred to a sterile 96-well polystyrene microtiter plate (3 wells per sample) and incubated at 30°C for 24 h. Planktonic cells (80 μl) were transferred to a sterile microtiter plate for A_{600} measurements in a Wallac 1420 Victor^{3}V multi-label plate reader, while the attached cells were gently washed three times with sterile phosphate-buffered saline, air dried, and stained with 1% (wt/vol) crystal violet. After washing the wells four times with distilled water, the surface-associated dye was solubilized with 200 μl of ethanol. The A_{600} of the dye solutions was measured in a Wallac 1420 Victor^{3}V multilabel plate reader.

***Galleria mellonella* killing assay.** The *G. mellonella* killing assay was performed as previously described ([33]), with minor modifications. Briefly, *G. mellonella* caterpillars in the final instar larval stage (average weight, 490 ± 90 mg) were infected with a lethal inoculum of *P. aeruginosa* (adjusted to about 10 bacterial cells in 10 μl of saline) containing or not niclosamide ethanolamine salt at 750 μM. Although *P. aeruginosa* cells were incubated in the presence of niclosamide for less than 5 min before injection, preliminary assays showed that 750 μM niclosamide treatment *in vitro* (for up to 90 min) does not significantly affect *P. aeruginosa* PA14 cell viability (data not shown). *G. mellonella* larvae were incubated at 28°C in petri dishes (five larvae per dish) and monitored for a week. Larvae were considered dead when they did not respond to gentle prodding. At least 30 larvae were inoculated per condition in three independent experiments. To rule out any growth-inhibitory effect of niclosamide due to conversion into a toxic compound(s) in the larval hemolymph, additional larvae were inoculated with 10 μl of 750 μM niclosamide or saline as a control. Five-microliter aliquots of the larval hemolymph recovered 2, 4, or 6 h after treatment were spotted on *Pseudomonas* isolation agar plates previously inoculated with *P. aeruginosa* PA14 cells to produce a lawn of confluent growth. The appearance of growth inhibition halos was checked after 14 h of incubation at 37°C. In this experiment, no inhibition halos were detected.

Statistical analysis. Statistical analysis was performed with the software GraphPad Instat, using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Survival curves for the *G. mellonella* killing assay were generated by the Kaplan-Meier method and analyzed by the log-rank test. Differences having a *P* value of <0.05 were considered statistically significant.

RESULTS

Identification of FDA-approved compounds that inhibit *P. aeruginosa* QS. We recently developed a novel screening system for the identification of *P. aeruginosa* QSI. This system is based on the cocultivation of a biosensor strain for 3OC$_{12}$-HSL detection, PA14-R3, and a wild-type *P. aeruginosa* PA14 strain. The 3OC$_{12}$-HSL signal synthesized by the wild-type PA14 induces bioluminescence emission by the biosensor ([20]). The addition of a molecule with inhibitory activity toward any process related to the 3OC$_{12}$-HSL-dependent QS system, including 3OC$_{12}$-HSL synthesis, transport, and perception, reduces the luminescence emitted by the biosensor with respect to a control coculture without any compound added ([20]).

The PA14/PA14-R3 cocultivation system was used to screen a commercial library of marketed drugs from Prestwick Chemicals (www.prestwickchemical.fr). This library contained 1,120 chemical compounds with known biological activities, selected for their high chemical and pharmacological diversities, as well as for

TABLE 1 Hit compounds identified by screening the Prestwick Chemical Library with the PA14/PA14-R3 QSI screening system

Prestwick code	Compound name	IC$_{50}$ (μM)^a	Property
01D11	Niclosamide	10	Anthelmintic
02H11	Gentamicin	20	Aminoglycoside antibiotic
05G06	Mitoxantrone dihydrochloride	150	Antineoplastic agent
07E06	Rifampin	50	Antibiotic of the rifamycin group
07H08	Dirithromycin	50	Macrolide glycopeptide antibiotic
13C08	Sanguinarine	60	Quaternary ammonium salt of the benzylisoquinoline alkaloids group
14G10	Rifabutin	10	Antibiotic of the rifamycin group

^{a} The IC$_{50}$ values were determined using the PA14/PA14-R3 coculture grown for 4 h at 37°C in the presence of 100, 80, 60, 40, 20, 10, 5 and 2.5 μg/ml of each compound.

known bioavailability and safety information for humans. Each drug was tested at three different concentrations (100, 10, and 1 μg/ml) in duplicate. Criteria used for the selection of hit compounds were (i) ≥50% inhibition of bioluminescence emission and (ii) ≤20% reduction of growth with respect to the untreated controls. The latter criterion was aimed at avoiding any unspecific effect of impaired growth on the QS response.

The screening assay allowed the identification of seven putative QSIs that reproducibly inhibited the QS response of the PA14/PA14-R3 cocultivation system, without affecting bacterial growth at the highest concentration tested. The seven hits were further tested in triplicate at 100, 80, 60, 40, 20, 10, 5, and 2.5 μg/ml final concentrations, showing a half-maximal inhibitory concentration (IC$_{50}$) in the range of 3 to 77 μg/ml (corresponding to 10 to 150 μM) ([Table 1]). Four of the identified compounds were antibiotics, in agreement with the well-known negative effect of subinhibitory concentrations of antibiotics on the *P. aeruginosa* QS response ([34], [35]). The remaining three compounds corresponded to a quaternary ammonium salt, an anticancer drug, and a teniacide for the treatment of tapeworm infections ([Table 1]). Among nonantibiotic drugs, the teniacide niclosamide showed the highest anti-QS activity (lowest IC$_{50}$) ([Table 1]) and was therefore selected for further investigations.

Niclosamide inhibits the 3OC$_{12}$-HSL-dependent QS system of *P. aeruginosa*. To verify the result of the screening assay, niclosamide was purchased from an alternative supplier (Sigma-Aldrich) and retested in the PA14/PA14-R3 cocultivation system. As expected, a strong inhibition of the 3OC$_{12}$-HSL-dependent QS response was observed, with an IC$_{50}$ even lower than that calculated for the compound from the Prestwick library ([Fig. 1A]). Notably, niclosamide was also able to inhibit luminescence emission by the PA14-R3 reporter strain grown in the presence of exogenously added synthetic 3OC$_{12}$-HSL (3 μM final concentration) ([Fig. 1B]). This result suggests that the QS-inhibitory activity of niclosamide relies on its ability to hamper the response of *P. aeruginosa* to the signal molecule rather than to inhibit its synthesis. The possibility that the observed QS-inhibitory activity of niclosamide was due to unspecific inhibition of either bioluminescence-generating enzymes or bacterial transcription was ruled out by the observation that niclosamide had no effect on the bioluminescence emitted by a *P. aeruginosa* strain in which bioluminescence genes were under the control of the promoter region of the
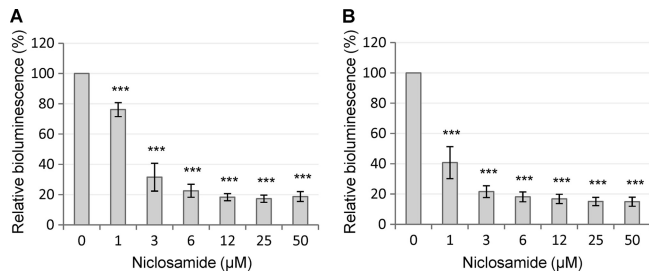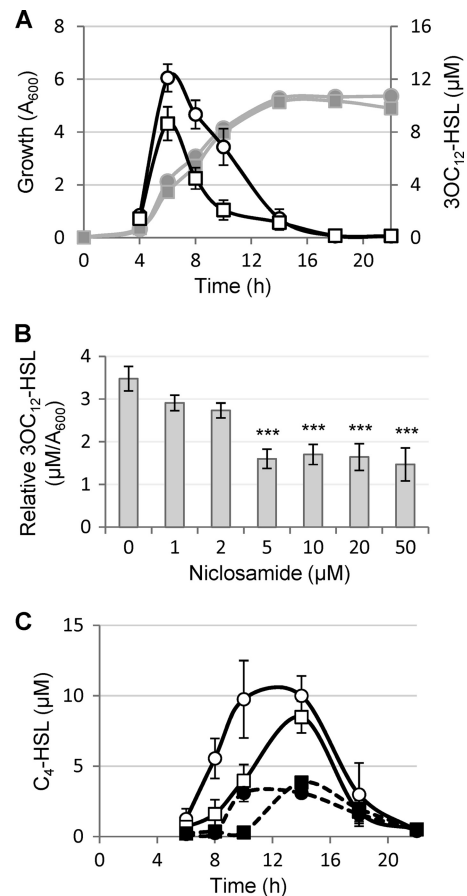
FIG 1 Effect of niclosamide on the 3OC$_{12}$-HSL-dependent QS of *P. aeruginosa*. Response to increasing concentrations of niclosamide (0 to 50 μM) of the PA14/PA14-R3 cocultivation system (A) and the PA14-R3 biosensor (B) in the presence of 3 μM exogenously provided 3OC$_{12}$-HSL. Bioluminescence emission was normalized to the cell density of the bacterial culture (relative bioluminescence, LCPS/A_{600}) and expressed as a percentage relative to untreated controls. Values are the means (± standard deviations) of at least three independent experiments. ***, $P < 0.001$ (ANOVA).

QS-independent *cysB* gene (data not shown), which is involved in cysteine metabolism and iron uptake (36).

The effect of niclosamide on the production of 3OC$_{12}$-HSL was then assessed. *P. aeruginosa* PA14 was grown in the absence or in the presence of 20 μM niclosamide, and 3OC$_{12}$-HSL levels in culture supernatants were quantitatively determined during the whole growth curve. Niclosamide caused a significant reduction (30 to 60%) of 3OC$_{12}$-HSL production from late exponential growth to the entry into the stationary phase, after which the 3OC$_{12}$-HSL concentration fell to almost-undetectable levels in both niclosamide-treated and -untreated cultures (Fig. 2A). When 3OC$_{12}$-HSL levels were determined in *P. aeruginosa* PA14 cultures grown in the presence of different niclosamide concentrations (0 to 50 μM), the maximum inhibitory effect on 3OC$_{12}$-HSL production (about 60% reduction) was observed at 5 μM, and higher concentrations did not further reduce 3OC$_{12}$-HSL production (Fig. 2B).

Since 3OC$_{12}$-HSL influences the expression of other QS systems, the effects of niclosamide on the production of C$_4$-HSL and PQS were tested. To this aim, C$_4$-HSL and PQS levels in *P. aeruginosa* PA14 cultures treated or not with 20 μM niclosamide were determined along the growth curve. While niclosamide did not significantly affect PQS levels (data not shown), it considerably delayed the production of C$_4$-HSL (Fig. 2C). By comparison with untreated cultures, C$_4$-HSL levels were significantly lower in niclosamide-treated cultures during exponential growth, while levels were comparable between treated and untreated cultures in the stationary phase (Fig. 2C). This could be due, at least in part, to the positive effect exerted by 3OC$_{12}$-HSL on C$_4$-HSL production (17). However, the niclosamide-induced delay in C$_4$-HSL production was also evident in a 3OC$_{12}$-HSL-defective mutant strain inactivated for the *lasI* gene, encoding 3OC$_{12}$-HSL synthase (Fig. 2C), indicating that niclosamide also affects C$_4$-HSL production independently of its inhibitory activity on 3OC$_{12}$-HSL production.

Niclosamide represses QS-activated genes. To investigate the global effect of niclosamide on the *P. aeruginosa* transcriptome, the transcriptional profiles of PA14 grown to an A_{600} of 2.5 in the presence or in the absence of 20 μM niclosamide were compared by Affymetrix high-density oligonucleotides microarray analysis. Niclosamide affected the transcription of 258 genes, 73.2% of which were repressed by this drug, including genes involved in the

FIG 2 Effect of niclosamide on acyl-HSL production. (A) Growth curve and 3OC$_{12}$-HSL production by *P. aeruginosa* PA14 treated (squares) or untreated (circles) with 20 μM niclosamide. Symbols: bacterial growth (left vertical axis), gray lines and filled symbols; 3OC$_{12}$-HSL levels in culture supernatants (right vertical axis), black lines and open symbols. (B) Relative 3OC$_{12}$-HSL levels (in μM/A_{600}) in culture supernatants of *P. aeruginosa* PA14 grown for 8 h in the presence of increasing concentrations of niclosamide (0 to 50 μM). (C) C$_4$-HSL levels in culture supernatants of *P. aeruginosa* PA14 (solid lines, white symbols) and PA14 *lasI* mutant (dashed lines, black symbols) grown in the presence (squares) or in the absence (circles) of 20 μM niclosamide. Growth curves of bacterial cultures were comparable to those reported in panel A. Values are the means (± standard deviations) of at least three independent experiments. ***, $P < 0.001$ (ANOVA).

production of important virulence factors, such as phospholipase C, LasA protease, pyocyanin, chitinase, rhamnolipids, and LasB elastase (Table 2; see also Table S1 in the supplemental material). Moreover, niclosamide repressed the transcription of genes involved in adhesion and biofilm formation, such as those coding for adhesins (PA0852-*cbpD* and PA2570-*pa1L*) and for cyclic di-GMP turnover or response proteins (PA1120-*tpbB*, PA2572, and PA4781). The transcription of the *mexGHI-opmD* genes, encoding an efflux pump required for full virulence in rat and plant infection models (37), was also strongly decreased in the presence of niclosamide (Table 2). Notably, among the 189 genes repressed by niclosamide, 96 have been identified as genes activated by 3OC$_{12}$-HSL and/or C$_4$-HSL in the main reference studies (38–40) (see Table S1), and an additional 25 genes have been suggested to be part of the QS network (27, 31, 41–44). Among the core components of the *P. aeruginosa* QS network (i.e., signal syntheses and

TABLE 2 List of selected genes whose transcription is affected by niclosamide[a]

Gene role and PA no.	Gene name	Fold change[b]	Gene product
Virulence factors			
PA0026	*plcB*	−4.15	Phospholipase C, PlcB
PA0070	*tagQ1*	2.73	Protein secretion apparatus, type VI secretion system
PA0085	*hcp1*	2.99	Type VI protein secretion system component Hcp
PA1871	*lasA*	−10.05	LasA protease precursor
PA1901	*phzC1, phzC2*	−2.22	Phenazine biosynthesis protein PhzC
PA1905	*phzG2*	−2.19	Probable pyridoxamine 5′-phosphate oxidase
PA2300	*chiC*	−7.24	Chitinase
PA3478	*rhlB*	−5.99	Rhamnosyltransferase chain B
PA3479	*rhlA*	−5.65	Rhamnosyltransferase chain A
PA3724	*lasB*	−2.90	Elastase LasB
PA4210	*phzA1, phzA2*	−3.06	Probable phenazine biosynthesis protein
PA4211	*phzB1, phzB2*	−2.42	Probable phenazine biosynthesis protein
Adhesion and biofilm formation			
PA0852	*cbpD*	−3.92	Chitin-binding protein CbpD precursor
PA1120	*tpbB*	−2.19	Diguanylate cyclase
PA2570	*pa1L*	−4.08	PA-I galactophilic lectin
PA2572		−3.17	Probable two-component response regulator
PA4781		−2.50	Cyclic di-GMP phosphodiesterase
Gene regulation			
PA2931	*cifR*	2.10	Transcriptional regulator
PA3477	*rhlR*	−2.56	Transcriptional regulator RhlR
PA4296	*pprB*	−3.21	Two-component response regulator
Drug efflux			
PA4205	*mexG*	−29.17	Hypothetical protein
PA4206	*mexH*	−16.94	Probable RND efflux membrane fusion protein precursor
PA4207	*mexI*	−13.87	Probable RND efflux transporter
PA4208	*opmD*	−9.12	Probable outer membrane protein precursor

[a] The PA number, gene name, and gene product are from the *Pseudomonas* Genome Database. Genes previously reported to be activated by the 3OC$_{12}$-HSL and/or C$_4$-HSL QS systems are shown in bold characters (38–40). RND, resistance-nodulation-cell division.
[b] Fold change in gene expression of *P. aeruginosa* PA14 grown in LB supplemented with 20 µM niclosamide compared to the same strain grown in LB.

signal receptor genes), only the C$_4$-HSL receptor gene *rhlR* was significantly repressed by niclosamide (Table 2). Similar results have been reported for other QSIs, such as furanone C-30 (38), iberin (45), and ajoene (46). In total, 121 out of the 189 genes repressed by niclosamide (64%) can be classified as QS regulated.

Niclosamide displayed a positive effect on the transcription of 69 genes, including two genes involved in type VI secretion pathways (PA0070-*tagQ1* and PA0085-*hcp1*) (Table 2). These genes are the only virulence-related determinants whose transcription is induced by niclosamide. Only four of the niclosamide-activated genes were previously reported to be repressed by 3OC$_{12}$-HSL and/or C$_4$-HSL (38–40) (see Table S2 in the supplemental material), suggesting that the majority of the genes induced by niclosamide are affected via a QS-independent pathway(s). This observation, together with the finding that 36% of the niclosamide-repressed genes have never been reported to be QS controlled, suggests that this drug may have additional cellular targets besides the QS network. Notably, a total of 16 putative or confirmed transcriptional regulators were identified among the genes repressed or activated by niclosamide (see Tables S1 and S2). Besides *rhlR*, niclosamide decreased the transcription of *pprB*, which encodes a transcriptional activator associated with biofilm formation (47). Conversely, it positively affected the transcription of *cifR* (Table 2), which encodes the transcriptional repressor of the Cif toxin, responsible for apical membrane downregulation of the

cystic fibrosis transmembrane conductance regulator (CFTR) in epithelial cells (48). The niclosamide-affected transcriptional factors may act as ancillary regulators, increasing the number of genes whose expression is altered by this drug beyond the QS regulon. However, a complete understanding of the niclosamide impact on *P. aeruginosa* physiology is partially hampered by the high percentage of niclosamide-controlled genes (~41%) coding for proteins still classified as hypothetical (see Tables S1 and S2 in the supplemental material).

Niclosamide strongly reduces the virulence potential of *P. aeruginosa in vitro.* In order to validate the transcriptomic data at the phenotypic level, we assessed the effect of niclosamide on the production of a set of QS-regulated virulence traits. In particular, we focused on (i) the LasB elastase, which is directly regulated by the 3OC$_{12}$-HSL receptor LasR at the transcriptional level (49), (ii) pyocyanin and rhamnolipids, which are regulated by a number of different regulatory pathways and extracellular signals (50, 51), and (iii) multifactorial phenotypes, such as motility and biofilm, which are crucial for the establishment and persistence of *P. aeruginosa* infections (52–54).

In accordance with microarray analysis, niclosamide had a marked inhibitory effect on the levels of QS-regulated secreted virulence factors of *P. aeruginosa* PA14 (Fig. 3). Production levels of both pyocyanin and elastase were dramatically reduced (85 to 90%) by 5 to 10 µM niclosamide. Likewise, the amount of rham-
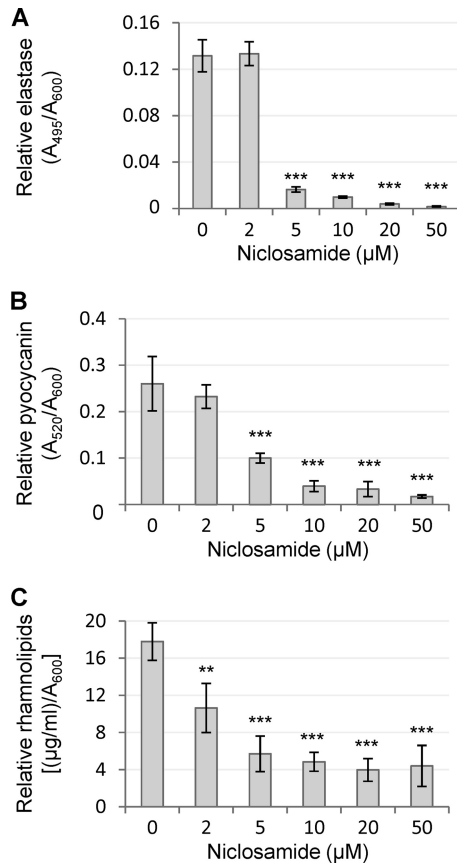
FIG 3 Effects of niclosamide on the production of QS-regulated extracellular virulence factors. LasB elastase (A), pyocyanin (B), and rhamnolipids (C) levels in culture supernatants of *P. aeruginosa* PA14 grown for 10 h (A and B) or for 24 h (C) in the presence of increasing concentrations of niclosamide (0 to 50 μM). Values were normalized to the cell density of the bacterial culture (relative values) and are the means (± standard deviations) of four independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (ANOVA).

nolipids in supernatants of niclosamide-treated cultures was about 25% of the niclosamide-untreated control level (Fig. 3).

Regarding bacterial motility, niclosamide only slightly reduced swimming and twitching motilities of *P. aeruginosa* PA14 at high concentrations (≥50 to 100 μM), while it exerted a dramatic inhibitory effect on swarming motility (Fig. 4A to C). Swarming was completely prevented at 4 μM niclosamide, although a significant reduction was also observed at lower concentrations (Fig. 4B) which, however, had no effect on the $3OC_{12}$-HSL-dependent QS system (Fig. 1 and 2).

Niclosamide was also tested for its effect on *P. aeruginosa* biofilm formation in a standard crystal violet binding assay (32). Niclosamide showed a significant biofilm-inhibitory activity, resulting in a 2-fold reduction and 3-fold increase in the number of attached and planktonic cells, respectively (Fig. 4D). However, such inhibitory activity was only observed at ≥200 μM niclosamide, i.e., at concentrations that are exceedingly higher than those active against the QS response and virulence factor production (Fig. 1 to 3), suggesting that the effect of niclosamide on biofilm formation is independent of its anti-QS activity.

Niclosamide protects *G. mellonella* from *P. aeruginosa* infection. In order to explore the suitability of niclosamide as an antivirulence drug against *P. aeruginosa* infections, we assessed the ability of this compound to inhibit the pathogenicity of *P. aeruginosa* in the *G. mellonella* insect model of infection (33).

Larvae of the wax moth *G. mellonella* are extremely sensitive to *P. aeruginosa* injected into the hemolymph, and the PA14 strain was found to be highly virulent in this model, with a 50% lethal dose of about one bacterial cell (33). In our study, *G. mellonella* larvae were inoculated with 10 μl of saline solution containing a lethal dose of *P. aeruginosa* PA14 (10 ± 4 cells from exponential cultures) and containing or not 750 μM niclosamide ethanolamine salt and then incubated at 28°C for up to 1 week. Niclosamide ethanolamine salt was used because of its higher solubility in aqueous solutions compared with niclosamide (55). Notably, the two niclosamide formulations displayed comparable inhibi-
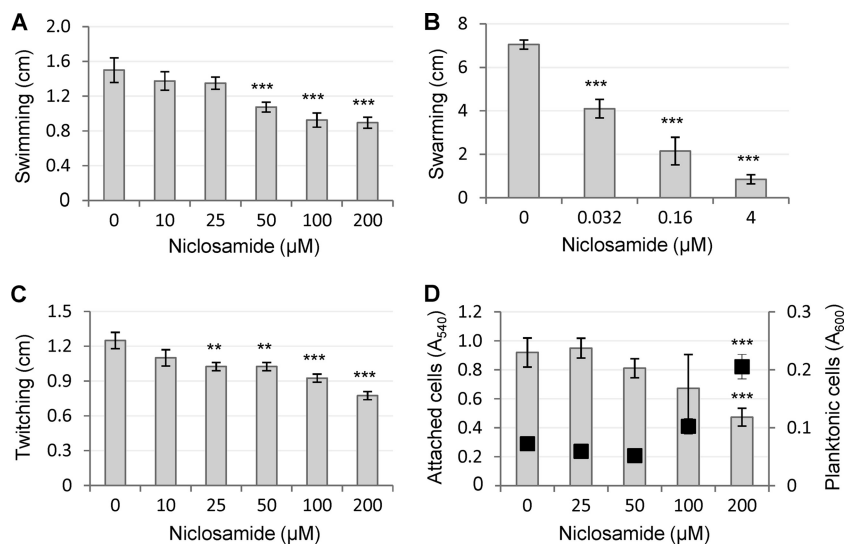
FIG 4 Effects of niclosamide on *P. aeruginosa* motility and biofilm formation. (A to C) Swimming (A), swarming (B), and twitching (C) by *P. aeruginosa* PA14 in the presence of increasing concentrations of niclosamide. (D) Biofilm formation by *P. aeruginosa* PA14 in the presence of increasing concentrations of niclosamide, assessed as the amount of attached cells (gray histograms, left axis) versus planktonic cells (black squares, right axis). Values are the means (± standard deviations) of at least three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ (ANOVA).
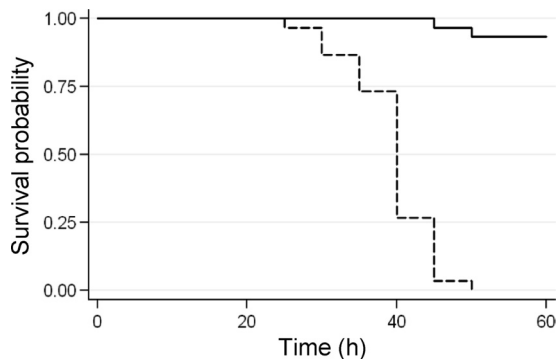
FIG 5 Efficacy of niclosamide in protecting *G. mellonella* larvae from *P. aeruginosa* killing. The Kaplan-Meier plot shows the survival of *G. mellonella* larvae inoculated with a lethal dose of *P. aeruginosa* PA14 (10 ± 4 exponentially growing cells) in 10 μl of saline supplemented or not with 750 μM niclosamide ethanolamine salt and then incubated at 28°C. All untreated larvae died within 60 h postinfection, while *G. mellonella* killing was almost completely prevented upon treatment with the niclosamide ethanolamine salt. $\chi^{2(1)} = 61.07$; $P = 0.0000$ (log-rank).

tory effects on virulence factor production, motility, and QS signal molecule production (data not shown). Considering that the average weight of the *G. mellonella* larvae was about 500 mg (see Materials and Methods), and arbitrarily assuming 500 μl as the hemolymph volume of a larva, the final concentration of niclosamide in each larva was estimated to be approximately 15 μM. We showed above that such niclosamide concentration inhibits $3OC_{12}$-HSL production and expression of $3OC_{12}$-HSL-dependent virulence factors (Fig. 2 and 3), without affecting bacterial growth (Fig. 2A). While 100% of the larvae not treated with niclosamide died within 60 h postinfection, niclosamide almost completely protected *G. mellonella* larvae from the lethal challenge with *P. aeruginosa* (Fig. 5), even if incubation was prolonged for a week (data not shown). To monitor the presence of PA14 in niclosamide-treated and -untreated larvae, at 60 h postinfection five larvae per group were homogenated in saline solution, and serial dilutions of the resulting homogenates were plated on *Pseudomonas* isolation agar. Dead larvae contained about $7 (\pm 5) \times 10^8$ *P. aeruginosa* cells per larva, while no bacterial cells were detectable in niclosamide-treated larvae. Notably, niclosamide-treated larval hemolymph had no effect on *P. aeruginosa* PA14 growth *in vitro* (see details in Materials and Methods), further confirming that the observed effect of niclosamide on *P. aeruginosa* pathogenicity is due to virulence inhibition rather than to a growth-inhibitory effect. Overall, these findings indicate that niclosamide allowed the innate immune response of *G. mellonella* to efficiently counteract the infection.

DISCUSSION

The need for new anti-infective strategies based on nonantibiotic compounds, together with the growing awareness of QS importance in bacterial infections, has raised interest toward the identification of QSIs endowed with antivirulence properties (reviewed in reference 56).

The relationships among QS, virulence regulation, and biofilm formation have most extensively been studied in *P. aeruginosa*. Therefore, it is not surprising that most of the research on QS inhibition has been centered on this bacterium as a model system (56).

Research on QS inhibition in Gram-negative bacteria has largely been focused on structural homologues of QS signal molecules, targeting the site of the signal receptor protein that is occupied by the natural ligand (56). Alternatively, promising QSIs belonging to different chemical classes have been discovered by screening random libraries of synthetic and natural compounds, and some of them have proved to be effective in preventing *P. aeruginosa* infection in animal models (38, 56, 57). Probably due to the high toxicity of the majority of the QSIs identified to date, garlic extract is the only QSI that has been tested in humans. Although the results were not statistically significant, a trend toward improvement of the clinical outcome of *P. aeruginosa*-infected CF patients after oral garlic extract administration was observed (58). A recent study identified ajoene as the most active QSI compound in garlic extract. However, synthesized ajoene was less active than the crude garlic extract *in vitro*, and it was very poorly effective in an *in vivo* murine model of infection (46). Thus, despite the huge efforts made to date in the field of anti-QS research, clinical applications remain far away (reviewed in reference 56).

The main aim of this work is to validate a new strategy for the identification of QSIs rapidly deliverable for clinical use, by proving that a lateral anti-QS activity can be identified in drugs already used in humans.

By screening a library of FDA-approved chemicals, we identified some hit compounds disclosing relevant QSI activity at concentrations that did not cause substantial inhibition of *P. aeruginosa* growth. The salicylanilide compound niclosamide, a cestocide already approved for use in humans (55), was characterized in detail for its anti-QS activity. At micromolar concentrations, niclosamide strongly inhibited both $3OC_{12}$-HSL and C_4-HSL production, as well as production of several secreted virulence factors, such as pyocyanin, elastase, and rhamnolipids (Fig. 2 and 3). As a comparative example, the previously described QSI ajoene only inhibits C_4-HSL production at very high concentrations (>300 μM), while it has no effect on $3OC_{12}$-HSL, the major QS signal produced by *P. aeruginosa* (46).

The large percentage of QS-regulated genes repressed by niclosamide (64%) highlights a high degree of target specificity toward C_4-HSL- and $3OC_{12}$-HSL-dependent regulons and is comparable to or even higher than that disclosed for other QSIs identified so far (38, 45, 46, 59). Overall, niclosamide strongly decreased the transcription of multiple genes involved in *P. aeruginosa* pathogenicity, corroborating its potential as an antivirulence drug.

Consistent with the strong antivirulence activity *in vitro*, niclosamide suppressed *P. aeruginosa* pathogenicity in an acute infection model based on *G. mellonella* larvae (Fig. 5). *P. aeruginosa* can also cause chronic infections characterized by a biofilm mode of growth. Several studies using cell flow chambers for biofilm formation, coupled with confocal scanner microscopy observations, have shown that a proficient QS system is required for optimal biofilm shaping and development (60). Accordingly, biofilms treated with QSIs show specific structural features and decreased resistance to antibiotics (19). In this work, we performed a pilot experiment using a simple biofilm model, showing that niclosamide is able to reduce cell attachment to a plastic surface, while increasing the number of planktonic cells (Fig. 4D). Although this effect was only observed at high niclosamide concentrations, this preliminary result should encourage further charac-

terization of the effect of niclosamide on biofilm development and resistance to antibiotics by using advanced biofilm models.

Concerning future developments of niclosamide as an anti-*P. aeruginosa* drug, there are some issues that need to be addressed. First of all, the effect of niclosamide on a wide panel of clinical *P. aeruginosa* strains isolated from different infection sites should be assessed, including CF patient chronically infected lungs. Moreover, even if niclosamide is currently used as an anthelmintic drug to treat intestinal infections and displays overall low toxicity (55), it is poorly soluble in water, shows low intestinal absorption, and once in the bloodstream, it is quickly cleared via the urinary tract or by enzymatic modification in the liver (55). Although these features could represent drawbacks to the systemic administration of niclosamide, they could be advantageous in the local treatment of wound infections, burns, otitis, gastrointestinal infections, and other external *P. aeruginosa* infections. It is also worth mentioning that the toxicity of inhaled niclosamide powder is quite low for mammals (55), opening new perspectives for aerosol treatment of *P. aeruginosa* lung infections. Additional studies in different mammalian models of both acute and chronic infections are required to assess the suitability of niclosamide as an anti-*P. aeruginosa* drug, prior to the move into clinical trials. However, in accordance with the SOSA approach (2), niclosamide could also be used as a promising scaffold for the design of structural analogues endowed with improved activity and pharmacokinetic properties. The hypothesis that some niclosamide derivatives may retain anti-QS activity is strengthened by our preliminary observation that other compounds belonging to the same structural class of niclosamides, i.e., the salicylanilides rafoxanide and oxyclozanide, showed *in vitro* the same anti-QS activity as niclosamide (data not shown).

The development of niclosamide-based QSIs could be pursued either by screening random chemical modifications introduced within the salicylanilide structure or by rational drug design. The latter approach requires detailed information about the anti-QS mechanism of action of niclosamide. Unfortunately, despite niclosamide's use since the 1960s, its mechanism of action remains elusive. The anthelmintic activity of niclosamide seems to rely on its ability to uncouple mitochondrial oxidative phosphorylation (61). More recently, it has been found to inhibit proliferation of some tumor cells by hampering different regulatory pathways, without relevant effects on normal nontumor cells (62–64). Notably, niclosamide has also been reported to act as an antimycobacterial agent (64), plausibly disrupting *Mycobacterium* membrane potential and pH homeostasis (65). Although the characterization of the molecular targets of niclosamide was not the aim of this study, some of our observations might be the first steps toward the comprehension of the niclosamide mechanism of action in *P. aeruginosa*. First, niclosamide is likely to target the $3OC_{12}$-HSL reception process rather than signal biosynthesis (Fig. 1). Second, the maximum inhibitory effect disclosed for niclosamide on $3OC_{12}$-HSL production (60%) is reached at 5 μM, and increases in concentration did not reduce $3OC_{12}$-HSL production (Fig. 2), ruling out the possibility that niclosamide competes with $3OC_{12}$-HSL for receptor binding. Third, microarray analysis showed that niclosamide also affects the expression of genes not controlled by QS, including some putative or confirmed transcriptional regulators. Fourth, niclosamide represses C_4-HSL production both dependently and independently from its action on $3OC_{12}$-HSL (Fig. 2). Finally, the repressive effect disclosed by

niclosamide is higher on swarming motility and on the production of virulence factors than on $3OC_{12}$-HSL production (Fig. 2, 3, and 4). Although this could be due to the combined effect of this QSI on $3OC_{12}$-HSL and C_4-HSL production, it cannot be excluded that niclosamide also influences some QS-independent cellular process involved in virulence gene regulation.

Putting together our observations and data from the available literature on the cellular processes affected by niclosamide in other organisms, a very preliminary hypothesis about the mechanism of action of niclosamide in *P. aeruginosa* is that this molecule targets some regulatory pathway(s) responsive to the energetic/metabolic status of the cell and that is required for full activity of the QS signaling network. Studies on the mechanism of action of niclosamide in *P. aeruginosa* are therefore in progress in our laboratory.

In conclusion, the major outcome of this study was the identification of a strong anti-QS activity in a compound already approved for use in humans. Our findings provide a new promising drug candidate against *P. aeruginosa* and a proof of concept that FDA-approved drugs may be endowed with antivirulence properties that are worthy of exploration.

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