Antimicrobial blue light photoinactivation of *Pseudomonas aeruginosa*: Quorum sensing signaling molecules, biofilm formation and pathogenicity

Grzegorz Fila | Marta Krychowiak | Michal Rychlowski | Krzysztof Piotr Bielawski | Mariusz Grinholc

1Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland
2Laboratory of Biologically Active Compounds, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland
3Laboratory of Virus Molecular Biology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland

*Correspondence
Mariusz Grinholc, Laboratory of Molecular Diagnostics, Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Gdansk, Poland.
Email: mariusz.grinholc@biotech.ug.edu.pl

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**Pseudomonas aeruginosa** is a common causative bacterium of acute and chronic infections that have been responsible for high mortality over the past decade. *P. aeruginosa* produces many virulence factors such as toxins, enzymes and dyes that are strongly dependent on quorum sensing (QS) signaling systems. *P. aeruginosa* has three major QS systems (las, rhl and *Pseudomonas* quinolone signal) that regulate the expression of genes encoding virulence factors as well as biofilm production and maturation. Antimicrobial blue light (aBL) is considered a therapeutic option for bacterial infections and has other benefits, such as reducing bacterial virulence. Therefore, this study investigated the efficacy of aBL to reduce *P. aeruginosa* pathogenicity. aBL treatment resulted in the reduced activity of certain QS signaling molecules in *P. aeruginosa* and inhibited biofilm formation. in vivo tests using a *Caenorhabditis elegans* infection model indicated that sublethal aBL decreased the pathogenicity of *P. aeruginosa*. aBL may be a new virulence-targeting therapeutic approach.

**KEYWORDS**
antimicrobial blue light, biofilm, *Caenorhabditis elegans*, photodynamic inactivation, photoinactivation, *Pseudomonas aeruginosa*, quorum sensing

1 | INTRODUCTION

*Pseudomonas aeruginosa* is one of the major etiological factors responsible for many infections diagnosed in both outpatient settings and hospitalized patients. This Gram-negative bacterium causes acute and chronic infections of the respiratory tract, wounds, urinary tract and other sites. Because of its high incidence and infection severity, *P. aeruginosa* is responsible for excessive mortality, especially among immunocompromised patients, those with cystic fibrosis or those with chronic wounds [1].

*Pseudomonas aeruginosa* is highly pathogenic due to the production of many virulence factors as well as rapid...
biofilm formation during colonization. Genes responsible for producing enzymes, toxins, pyocyanin and dyes are strongly connected with quorum sensing (QS) signaling systems [1–3]. *P. aeruginosa* possesses three major QS systems: rhl, las and *Pseudomonas* quinolone signal (PQS). Two of these systems involve the production of acyl-homoserine lactones (AHLs) as QS signaling molecules (QSSMs): N-3-oxo-dodecanoyl-homoserine lactone (3-O-C12-HSL) in the las system and N-butyryl-homoserine lactone (C4-HSL) in the rhl system [2]. Both systems are hierarchical, and when the LasI molecule binds to the LasR receptor, it increases the transcription of the rhl gene. In addition, the las system activates the production of virulence factors such as elastase, LasA protease and endotoxin A and induces biofilm formation, while the rhl system induces the production of elastase, LasA protease, pyocyanin, chitinase, cyanide and other molecules [4]. The third system, PQS, is also controlled by las but uses 2-heptyl-3-hydroxy-4-quinolone as a signaling molecule. This system regulates genes responsible for production of elastase, proteases and pyocyanin [2, 5].

Cell-to-cell communication systems play significant roles in biofilm formation and maturation. Biofilms, which are bacterial complexes embedded in a self-produced polymeric matrix that adheres to a surface, vary from flat, thin layers to complex mushroom-like constructs [6]. Microorganisms in a biofilm exhibit distinct features and are more resistant to immune responses, chemotherapy and other treatments than free-living cells [7]. *P. aeruginosa* forms biofilms based on many factors such as fimbriae, type IV pili, flagella, exopolysaccharides, proteins and extracellular DNA. The las QS system is required for biofilm formation. Moreover, eDNA release is controlled by all three QS systems in *P. aeruginosa* [8]. Many published papers indicate that biofilm disruption and eradication are of high priority when treating and preventing bacterial infections.

One alternative therapeutic option that potentially triggers the QS system and biofilm formation is antimicrobial photodynamic inactivation, which is based on three components: light of an appropriate wavelength, oxygen and a chemical compound known as a photosensitizer (PS). The PS absorbs a light photon, achieving an electronically excited state. Next, the electron is transferred onto oxygen molecules or biomolecules to produce singlet oxygen or reactive oxygen species (ROS), respectively [7, 9]. One widely studied version of antimicrobial photodynamic inactivation is antimicrobial blue light (aBL), which activates endogenously produced photosensitizing compounds via visible aBL irradiation (λ = 411 nm). We previously confirmed that aBL: (1) lethally inactivates both wild type and extensively drug-resistant *P. aeruginosa*; (2) inactivates numerous virulence factors (ie, elastases, proteases and pyocyanin); (3) decreases the cytotoxicity of extracellular *P. aeruginosa* fractions toward eukaryotic cells; and (4) sensitizes multidrug-resistant *P. aeruginosa* isolates to antimicrobials [10].

This study describes the effects of aBL irradiation on *P. aeruginosa* biofilm formation and pathogenicity in the absence of an exogenously administered PS. aBL primarily acts by inducing ROS production, leading to the photodestruction of target cells and various cellular components such as cellular envelopes, proteins, lipids and genetic material; thus, we hypothesized that the oxidation-associated destruction of bacterial cells significantly decreases QSSM activity and biofilm formation, leading to decreased *P. aeruginosa* pathogenicity. Using QSSM assays and real-time biofilm formation monitoring, we provide evidence that aBL triggers QS systems and influences biofilm formation. Finally, employing a *Caenorhabditis elegans* in vivo model, we describe the efficacy of sublethal aBL to combat *P. aeruginosa* pathogenicity.

2 | METHODS

2.1 | Bacterial strains and culture conditions

All experiments were performed using two reference and 12 clinical *P. aeruginosa* strains (listed in Table 1). Clinical isolates were provided by Julianna Kurlenda from the Provincial Hospital in Koszalin. Three bioreporting strains used to analyze QSSMs were kindly provided by Professor Paul Williams from the University of Nottingham (United Kingdom). Overnight bacterial cultures were incubated at 37°C and 150 rpm (Innova40 incubator, New Brunswick Scientific) in LB broth (Oxoid, United Kingdom).

2.2 | Light source and photoinactivation

A single-emitter diode lamp (411 nm, 120 W, and output light power of 11 W/12 mm², full width at half maximum of 17 nm), (SecureMedia, Gdynia, Poland) was used for illumination. Full characteristics of the light source are provided within published study by Ogonowska et al. [11] The emission spectrum of the employed light source is presented in Figure 1. Overnight (18 hours) bacterial cultures were diluted to a final concentration of approximately 10⁶ colony forming units (CFU)/mL. Cells were then illuminated with aBL (irradiance 15.7 mW/cm²; light doses 7.5-50 J/cm², irradiation time from 480 to 3200 seconds). The well contents were mixed before sampling, and aliquots were serially diluted 10-fold in PBS to achieve final dilutions of 10⁻¹ to 10⁻⁶, which were then streaked horizontally as previously described [12]. The control consisted of bacteria incubated in the dark for the same amount of time as the bacteria exposed to light. Each experiment was performed three times (three independent experiments with three repetitions per sample; the time interval between the three replicates was 24 hours). The survival fraction was expressed as the
ratio of bacterial treated with aBL to untreated bacterial. The detection limit was 10 CFU/mL.

2.3 | Fluorescence spectroscopy

Detection of *P. aeruginosa* endogenous porphyrins was performed in accordance with previously published protocol [13]. Briefly, an overnight *P. aeruginosa* culture was centrifuged, and pellet washed twice with PBS. Next, *P. aeruginosa* pellets were suspended in 1 mL of a mixture of 0.1 M NaOH/1% sodium dodecyl sulfate (SDS) and incubated for 24 hours in the dark. Fluorescence of the dissolved *P. aeruginosa* pellets in NaOH/SDS was measured on a fluorimeter (EnVision Multilabel Plate Reader, PerkinElmer, Waltham, Massachusetts), with excitation at 405 nm and emission scanned from 600 to 700 nm. In addition, as a

![Molecular Structure](source:PubChem)

**Molecular Formula:** C_{34}H_{34}N_{4}O_{4}

**Molecular Weight:** 562.67 g/mol

**FIGURE 1** Photochemistry of *Pseudomonas aeruginosa* endogenous photosensitizing molecule. (A) Chemical structure of PPIX. (B) Absorption spectrum of the extracted endogenous porphyrins, PPIX and output spectrum of the light source.
reference, the fluorescence spectrum of 0.05 μM protoporphyrin IX (PPIX) in NaOH/SDS was recorded.

2.4 | ROS detection

2.4.1 | Cell-free system (supernatants)

ROS detection was performed in accordance with previously published protocol [14]. ROS-detecting fluorescent probes 3′-p-(aminophenyl) fluorescein (APF; detects mainly hydroxyl radical [•OH]) and Singlet Oxygen Sensor Green (SOSG; detects singlet oxygen [1O2]) were both obtained from Invitrogen (Thermo Fischer Scientific, Waltham, Massachusetts) and handled according to the manufacturer’s manual. Fresh overnight bacterial cultures were diluted to optical density (OD)600 = 1. A total volume of 50 μL of this dilution was transferred into 5 mL of fresh LB in a 50-mL glass flask and incubated for 8 hours at 37°C at 150 rpm. After incubation, the cultures were transferred into Falcon tubes and centrifuged (10 000rpm for 10 minutes). Next, the supernatants were filtered through a 0.22-μm syringe filter. The supernatants were then combined to 3 μM of APF or SOSG in wells of a black flat-bottom 96-well plate and illuminated with aBL at room temperature 10 and 20 J/cm² (irradiance 15.7 mW/cm², irradiation time 640 and 1280 seconds). Fluorescence readings were taken immediately after irradiation using the EnVision Multilabel Plate Reader (PerkinElmer). Excitation/emission wavelengths for APF were 490/515 nm, and for SOSG 505/525 nm.

2.4.2 | Intracellular

A bacterial suspension of ~10⁸ cells/mL of P. aeruginosa was prepared in PBS, pelleted by centrifugation (6500rpm for 5 minutes) and suspended in 100 μL of a solution of the probe APF or SOSG (5 μM, in PBS), following by incubation in the dark, at 35°C, for 30 minutes. Cells were again pelleted, washed to remove the excess of probe and then suspended in 100 μL of PBS. After 5 minutes of incubation in the dark, suspensions were irradiated with aBL with light dose 10 and 20 J/cm² (irradiance 15.7 mW/cm², irradiation time 640 and 1280 seconds) and then transferred to a black 96-well plate for fluorescence reading. Control suspensions without APF or SOSG were used as blanks: cells were incubated in PBS for 30 minutes, pelleted, irradiated at the same light dose and had their fluorescence determined. Fluorescence readings were taken immediately after irradiation using the EnVision Multilabel Plate Reader (PerkinElmer). Excitation/emission wavelengths for APF were 490/515 nm and for SOSG were 505/525 nm.

2.5 | Determination of sublethal light dosing

A sublethal dose was defined as a dose that did not eliminate the majority of the microbial population. The lethal dose was defined as the dose that eliminated ≥99.9% of the bacterial population [15]. In this study, the lethal dose was determined under photodynamic conditions as the dose that reduced bacterial cell numbers by ≥3 log₁₀. A sublethal dose reduced bacterial numbers by 0.5 to 2 log₁₀. Sublethal doses were defined per previous publications [16–20].

2.6 | Pseudomonas aeruginosa supernatant preparation

Bacterial supernatants were prepared to determine the influence of aBL on QSSM. Fresh overnight bacterial cultures were diluted to OD₆₀₀ = 1. A total volume of 50 μL of this dilution was transferred into 5 mL of fresh LB in a 50-mL glass flask and incubated for 8 hours (for QS analysis) at 37°C at 150 rpm. After incubation, the cultures were transferred into Falcon tubes and centrifuged (10 000rpm for 10 minutes). Next, the supernatants were filtered through a 0.22-μm syringe filter. The supernatants were then transferred to a 24-well microtiter plate (600 μL per well) and illuminated with aBL at room temperature (irradiance 15.7 mW/cm²; light dose 150 J/cm², irradiation time 9600 seconds). Following irradiation, the supernatants were stored at −20°C for further AHLs and PQS detection.

2.7 | AHLs detection on soft-agar plates

QSSM detection was performed per a previously published protocol [21]. The method was modified by preparing and cooling the soft agar (LB with 0.5% agar) to 50°C. Next, an appropriate volume of an overnight culture of the bioreporter strain (listed in Table 1) was added to a final OD₆₀₀ = 0.1, plated on plastic plates and left to dry for approximately 2 hours. In addition, 10 μL of the prepared supernatants were transferred onto solid soft-agar plates and incubated overnight at 37°C. Plates were read the next day using a ChemiDoc XRS camera (Bio-Rad, Hercules, California) with an acquisition time of 600 seconds. Next, regions of interest were created, and signal density (intensity/mm²) was calculated. Each experiment was performed three times (three independent experiments; the minimal time interval between the three replicates was 24 hours).

2.8 | PQS detection in 96-well plates

PQS extraction and detection were performed per a previously published protocol [21].

2.8.1 | PQS extraction

Supernatants were mixed with fresh acidified ethyl acetate (0.01% vol/vol) at a 1:1 ratio, vortexed for at least 1 minute and centrifuged (10 000g for 10 minutes), and the organic phase for each sample was transferred into a glass vial. Extraction was repeated three times, and the organic phase was collected in the same glass vial for each sample. The organic phase was evaporated using a vacuum centrifuge. A set volume (1:10 of the supernatant) of methanol was gently added to the dry vials and mixed. The samples were transferred into new glass vials for analysis.
2.8.2 | PQS analysis
An overnight culture of the *P. aeruginosa* PAO1 Δ*qsa* CTX-*lux::qsa* bioreporter strain was diluted to a final OD₆₀₀ = 0.1, then mixed with previously prepared supernatant extract at a ratio of 1:100 in a sterile Eppendorf tube. Then, 200 μL of prepared solution was transferred into black 96-well plates with transparent bottoms; all samples were assessed in triplicate. Prepared plates were placed in an EnVision plate reader (PerkinElmer) for overnight incubation at 37°C. Every 30 minutes, the OD₆₀₀ and bioluminescence signals were measured. Each experiment was performed three times (three independent experiments with three repetitions per sample; the minimal time interval between the three replicates was 24 hours).

2.9 | *Pseudomonas aeruginosa* eGFP mutant construction
Real-time biofilm formation monitoring required the construction of an eGFP *P. aeruginosa* mutant. The first step involved the creation of *P. aeruginosa* competent cells per Bio-Rad’s protocol for *P. aeruginosa* electroporation using the pME 6032: eGFP (Tc125) plasmid. After electroporation, the culture suspension was mixed with 1 mL of fresh LB broth and incubated for 6 hours. Next, 100 μL of the culture was plated on an LA plate supplemented with 125 μg/mL tetracycline. eGFP mutants were constructed for two clinical and two reference strains.

2.10 | Biofilm formation
Biofilm formation was assessed using a previously published protocol [22]. Overnight cultures of *P. aeruginosa* expressing green fluorescence were diluted in fresh LB broth to OD₆₀₀ = 0.1 and incubated for 4 hours. Cells were washed twice in PBS (centrifuged for 5 minutes at 4000rpm) and resuspended in M9 supplemented with 2% glycerol to a final OD₆₀₀ of 0.05 to 0.1. Bacteria were treated with sublethal doses of aBL (light dose 10 J/cm²; irradiance 15.7 mW/cm²; irradiation time 640 seconds; λ = 411 nm), and samples were transferred into black 24-well plates with transparent bottoms (Greiner Bio-One, Kremsmünster, Austria). Plates were placed under a confocal laser scanning microscope Leica SP8X equipped with an incubation chamber for the live analysis (37°C, lens ×10), and two positions from every sample were set. For the next 10 hours, three-dimensional images were taken every 20 minutes during incubation. Each experiment was performed three times (three independent experiments with two repetitions per sample; the minimal time interval between the three replicates was 24 hours).

2.11 | *Caenorhabditis elegans* cultures and infection model
The *C. elegans* used in this study was wild type Bristol N2 obtained from the Caenorhabditis Genetic Center. Worms were maintained and cultured per the protocol published by Stiernagle [23]. All cultures and infection procedures were performed at 25°C. A synchronized L4 larvae suspension in S-complete medium was obtained from 3-day old cultures on NGM agar plates. First, the dose-dependent lethality of the aBL was established after light treatment of L4 worms in S-complete medium supplemented with *Escherichia coli* OP50. The *P. aeruginosa* cells used to infect the worms were cultured for 6 hours in LB medium (37°C, 150 rpm), washed twice with physiological saline and resuspended in S-complete medium. Worms in liquid medium (n = 25 ± 5) were transferred to 48-well plates, and bacterial inoculum was added to a final concentration of 1.5 × 10⁸ CFU/mL. During the 14-day experiments, animals in the wells were treated with aBL (light dose 10 J/cm²; irradiance 15.7 mW/cm²; irradiation time 640 seconds) daily, and the living worms were counted every 24 hours with a stereomicroscope (Leica MZ10F, Wetzlar, Germany). The S-complete medium was refilled every 3 days due to vaporization. Bacterial CFU were measured on days 1, 3, 5, 10 and 14 to determine the total pathogen numbers per well and control the influence of daily treatment on the surviving *P. aeruginosa* fraction. Each experiment was performed three times (three independent experiments with six repetitions per sample; the minimum time interval between the three replicates was at least 24 hours).

2.12 | Statistical methods
Statistical analyses were performed using the statistical suite STATISTICA (data analysis software system) version 10.0 (StatSoft. Inc. [2011], www.statsoft.com) and Excel. Quantitative variables were characterized using SD. The Leven (Brown-Forsythe) test was used to assess variance homogeneity. The statistical significance of differences between two groups was determined using Student's *t* test. The significance level was *P* < .05.

3 | RESULTS

3.1 | Intracellular PPIX-like derivatives are associated with the aBL treatment of *Pseudomonas aeruginosa*
The absorption and fluorescence spectrum (excitation at 405 nm) of the *P. aeruginosa* cells dissolved in NaOH/SDS is shown in Figures 1 and 2, respectively. The absorption spectrum peaked at 405 nm, and fluorescence spectrum at 635 nm, which represents the typical absorbance and fluorescence emission of PPIX [24]. As reference, the absorption and fluorescence spectrum of PPIX was recorded for comparison (Figures 1 and 2). It indicates that PPIX or PPIX-like derivatives within the *P. aeruginosa* cells were the photosensitizing molecules associated with the antimicrobial effect of aBL; thus, the absorption spectrum of these endogenous
porphyrins as well as corresponding PPIX together with the emission spectra of the employed light source and PPIX chemical structure is presented in Figure 1.

3.2 | ROS generation

To explain the mechanism of aBL as the excitation of endogenous PS that leads to the generation of ROS, the ROS detection assay in vitro was performed. *P. aeruginosa* suspensions were combined with ROS-detecting probes and irradiated with aBL. Generation of hydroxyl radical (·OH) and singlet oxygen (¹O₂) was measured by indirect fluorescence and the results are illustrated in Figure 3. Endogenous porphyrins are known to generate either singlet oxygen (type II mechanism) and radical species (type I photodynamic mechanism). Therefore, the profile of generated ROS seen in Figure 3 was expected.

3.3 | Lethal and sublethal dose determination of aBL toward *Pseudomonas aeruginosa* strains

To investigate the possible impact of aBL on biofilm formation or infection prevention, we utilized sublethal doses, as both biofilm formation and bacterial pathogenicity are characteristics of surviving cells. Conversely, when analyzing the impact of aBL on QSSMs, a lethal aBL dose was applied. The lethal and sublethal aBL doses were defined by studying a wide range of aBL fluencies. Different light doses ranging from 7.5 to 50 J/cm² were applied to three clinical isolates and two reference strains of *P. aeruginosa* (Figure 4). A light dose of 10 J/cm² reduced viable cells to no higher than 1.5 log₁₀ and was considered a sublethal dose for all analyzed strains.

3.4 | Lethal aBL affects QSSMs

To investigate the influence of aBL on secreted QSSMs, three sensor strains, *E. coli* JM109 pSB536, *E. coli* JM109...
pSB1142 and *P. aeruginosa* PAO 1 *pqxA CTX-lux::pqxA* (Table 1), were used to produce bioluminescent signals in the presence of C4-HSLs (Rhl), 3-oxo-C12-HSL (Las) or PQS molecules, respectively. The bioluminescence measurements of the bioreporter cultures in soft agar with irradiated and nonirradiated extracellular fractions revealed the decreased activity of Rhl and Las molecules in all analyzed samples upon light administration (Figure 5). These results indicate that signal molecule production was heterogeneous among all examined strains, reflecting varying response levels to aBL. Rhl molecule activity decreased in all analyzed samples by 12% to 63.7% (strains 153/K and 2284/p, respectively) (Figure 5A). Las QSSMs were even more susceptible to aBL treatment. Las signaling molecular activity decreased by 45.1% (strain 556/K) to 82.4% (strain PA 14) (Figure 5B). Conversely, lethal aBL up to 150 J/cm² did not affect PQS molecular activity (Figure 5C,D). Employing two different QSSM detection assays revealed comparable PQS molecular activity in the aBL-treated and control groups, indicating that aBL irradiation up to 150 J/cm² did not affect PQS system activity.

### 3.5 | Sublethal aBL delays biofilm formation

To investigate the influence of aBL on biofilm formation, *P. aeruginosa* strains expressing green fluorescence were treated with sublethal aBL irradiation and cultured for 18 hours at 37°C for real-time biofilm formation monitoring. To ensure that living bacterial cell numbers were the same in the aBL-treated and control groups, the plates were counted to determine CFU/mL, and cell viability did not differ between the irradiated and dark control samples (for details see Figure S1, Supporting Information). Biofilm plates were imaged automatically every 20 minutes. Three-dimensional images were analyzed, and the time required for the biofilm to completely cover the well bottom was described. As shown in Figure 3, sublethal aBL delayed biofilm formation in all examined strains (Figure 6). The parameter Δt indicates the change in time required for comparable biofilm...
formation by aBL-treated and nonilluminated *P. aeruginosa* strains. Although each *P. aeruginosa* isolate demonstrated a different biofilm formation ratio, longer times, ranging from 100 to 220 minutes, were necessary for all sublethal aBL-treated strains (Figure 6). A real-time imaging video of biofilm formation by four analyzed strains are available in the Supporting Information data (Videos S1-S4).

### 3.6 Sublethal aBL decreased *Pseudomonas aeruginosa* pathogenicity toward *Caenorhabditis elegans*

For the in vivo *C. elegans* model, the effects of sublethal aBL on *P. aeruginosa* survival were investigated over 14 days of daily application, and aBL did not affect the viability or survival fractions of any *P. aeruginosa* strains studied. Identical growth rates were observed for control, nontreated and sublethal aBL-treated *P. aeruginosa* (for details see Figure S2). Thus, the reduced pathogenicity of aBL-treated *P. aeruginosa* results from reduced virulence or inhibited biofilm formation rather than decreased numbers of live *P. aeruginosa* cells (Figure 7).

### 4 DISCUSSION

*Pseudomonas aeruginosa* is recognized as a key bacterial pathogen. It colonizes infected surfaces by forming biofilms in which bacterial cells attach to and are fixed within an extracellular matrix. *P. aeruginosa* biofilm cells are several times more resistant to antimicrobials than planktonic cells, which often leads to difficulties in eradicating them from infected patients. Virulence factor production and biofilm formation by *P. aeruginosa* are controlled by QS systems; thus, interrupting *P. aeruginosa* QS is a new potential anti-pathogen therapeutic approach [25, 26]. In the present study, we demonstrated that sublethal aBL treatment of several clinical isolates and reference *P. aeruginosa* strains inhibited *in vitro* biofilm formation. Moreover, the bacteria exposed to sublethal aBL exhibited reduced virulence in a *C. elegans* in vivo infection model.

aBL, a novel light-based approach, is gaining attention for its antimicrobial effects without involving exogenous PS [13, 27, 28]. The mechanism underlying the bactericidal activity of aBL is unclear. A common hypothesis is that visible aBL excites endogenous photosensitizing compounds that occur naturally within microbial cells, inducing the production of cytotoxic ROS [29]. Within the present work, we made an effort to detect the photosensitizing molecule within *P. aeruginosa* cells and indicated that PPIX or PPIX-like derivatives are mainly responsible for the bactericidal effect of aBL. It is in accordance with previous findings by Wang et al [30] who indicated that PPIX and PPIX-like derivatives could potentially play a key role in aBL treatment of...
P. aeruginosa. Nevertheless, further studies, such as the use of electrospray ionization tandem mass spectrometry, are warranted to identify the structures of these PPIX-like derivatives. These data support our findings. Next, to provide the mechanistic insight in the aBL process, the detection of generated ROS due to aBL excitation of endogenous porphyrins was performed within the present study. The conducted analysis showed that aBL treatment of both cell-free P. aeruginosa supernatants as well as bacterial cultures results in hydroxyl radical and singlet oxygen production, confirming the hypothesis of ROS generation upon aBL activation of endogenous porphyrins. Many studies have demonstrated that aBL is effective in fighting a wide range of pathogenic microbes regardless of their drug resistance profiles. These microbes include both Gram-negative and Gram-positive bacteria and yeasts growing in planktonic and biofilm cultures [13, 17, 27, 30–32]. In addition, aBL does not significantly damage host cells or impair wound healing [28, 30, 33].

In our previous study, we investigated the efficacy of aBL toward P. aeruginosa using a panel of wild type and clinical strains, including multidrug-resistant and extensively drug-resistant strains. aBL exerted significant antimicrobial effects on all P. aeruginosa strains tested, resulting in 5.2 to 8-log10 reductions in CFU. Importantly, lethal aBL treatment inactivated multiple P. aeruginosa virulence factors, including both cell-associated determinants and secreted factors [10]. The ability of microbes to cause disease is determined by multiple virulence factors acting at different stages of infection [34]. Even after inactivating a pathogenic microbe, virulence factors that cause significant damage to host tissues may remain. In addition, several studies have shown increased virulence factor production and activity after exposure to different antibiotics [35]. Thus, approaches that inhibit virulence factor production and/or activity are of significant interest. A small number of studies in P. aeruginosa have proposed the photoinactivation of secreted proteases and phospholipase C via photodynamic treatment with exogenously administered sensitizers. [36, 37] Proteases were significantly reduced by irradiation with red light in the presence of the PS toluidine blue O (TBO). Similarly, only a few studies have described the inactivating effects of aPDT in combination with exogenously administered PSs in other bacterial species. Tubby et al reported the inactivation of staphylococcal virulence factors using a light-activated antimicrobial agent. Protease, alpha-hemolysin and sphingomyelinase activities were inhibited following exposure to a laser light in the presence of methylene blue. [38] Kato et al [39] described the antimicrobial photodynamic inactivation of Candida albicans virulence factors and a reduction in the in vivo pathogenicity of this microbe. Thus, photodynamic therapy in combination with exogenously administered sensitizers reduces the harmful impacts of preformed virulence factors on the host. Finally, our previous study was the first report of virulence factor inactivation by aBL alone. [10]

To elevate aBL as an advantageous approach over conventional antibiotics, the anti-biofilm and anti-pathogenic efficacy of aBL must be determined using an in vivo infection model. Many in vivo models of bacterial infection exist, including Arabidopsis thaliana [40], Drosophila melanogaster [41] mice and rat models [42, 43]. One promising organism is C. elegans. This self-fertilizing nematode is one
of the best-understood metazoans [44], and its greatest advantage is the similarity between the *C. elegans* genome and human genes: between 60% and 80% of human genes have orthologs in *C. elegans* [45]. *C. elegans* has a rapid life cycle and requires only 3 days at 25°C to develop into egg-laying adults. In addition, its body tissues, such as the epidermis, muscle bands, ventral and dorsal nerve cords, as well as the digestive, reproductive and excretory systems, make it an attractive in vivo model [46]. The innate immune system of *C. elegans* mimics host-pathogen interactions [47]. These attributes make *C. elegans* an excellent in vivo model for studying bacterial infection or virulence factor activity.

A significant finding from this study is that sublethal aBL inhibits the activity and/or production of QSSMs, thus delaying biofilm formation in aBL-pretreated *P. aeruginosa* strains and reducing their overall pathogenicity in a *C. elegans* infection model.

QSSMs diffuse or are actively pumped out of the bacterial cell; therefore, once they appear in the extracellular environment, they are targets for destruction or inactivation. We evaluated the potential use of sublethal aBL to inhibit AHL and PQS activity. Our studies demonstrated that the activity and/or production of AHL molecules, specifically C4-HSLs (Rhl) (Figure 5A) and 3-oxo-C12-HSL (Las) (Figure 5B), were significantly inhibited by lethal aBL treatment. These experiments were performed in cell-free supernatants what could explain why the significant photoactivation of QSSMs was not observed when sublethal aBL was employed. The level of QSSMs photoinactivation increased in an aBL dose-dependent manner starting from light dose of 50 to 150 J/cm². To exemplify the obtained results, the highest studied light dose of 150 J/cm² has been chosen. However, the activity and/or production of PQS were unaffected neither by sublethal or lethal aBL treatment (Figure 5C, D). Issues concerning the functionality of *P. aeruginosa* QS systems treated only with aBL have never been addressed before and the present study is the first report indicating aBL potential in affecting the activity and/or production of QSSMs. However, studies involving the impact of antimicrobial photodynamic inactivation with the use of exogenously administered PS against *P. aeruginosa* QSSMs have already been published. Rout et al [48] extracted the lactone samples from the medium of *P. aeruginosa* cultures and studied their activity upon photodynamic inactivation with TBO. The lactone samples treated with photodynamic inactivation in the presence of TBO showed a notable decrease in the levels of lactones compared to the control group. This could be due to the generation of hydroxyl radicals following the photosensitization of TBO. Several studies recently identified the ability of lactonase enzymes cleaving the lactone ring in QSSMs to produce nonfunctional molecules that are unable to activate their transcriptional regulators. When a lactonase was expressed in *P. aeruginosa*, AHL production and virulence factor expression were significantly reduced [49]. These enzymes may not be ideal therapeutics due to the difficulty in delivering active enzymes to an infection site; however, methods that decrease QSSM activity are clearly needed. Although additional studies are required to further evaluate aBL, our data suggest that sublethal aBL inhibits QS and may represent a new tool for an anti-QS approach.

*Pseudomonas aeruginosa* uses a QS system to produce biofilm; therefore, inhibited biofilm formation was expected with sublethal aBL treatment. We obtained data confirming the anti-biofilm activity of aBL from the present study. Real-time biofilm formation monitoring of two clinical and two reference *P. aeruginosa* strains revealed significantly delayed biofilm formation upon sublethal aBL (Figure 6 and Videos S1-S4).

As most in vitro results are not easily translated to clinical applications, we used a *C. elegans* in vivo infection model to investigate the anti-pathogenic ability of sublethal aBL. Although sublethal aBL did not reduce *P. aeruginosa* numbers in *C. elegans* for the entire duration of the infection (up to day 14), it did result in an increased survival rate, potentially resulting from decreased virulence and/or biofilm production (Figure 7).

Together with previously published data [10] indicating the strong anti-virulence efficacy of aBL toward *P. aeruginosa* isolates, the results of this study suggest that sublethal aBL is a promising approach to combat *P. aeruginosa* infections.

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**AUTHOR CONTRIBUTIONS**

G.F. did the experimental work, participated in conception of the study and wrote the draft of the manuscript. M.K. performed studies concerning *Caenorhabditis elegans*. M.R. participated in conception of the study concerning microscopy-related experiments. K.P.B. participated in the ROS detection studies and helped drafting manuscript. M.G. participated in the experimental work, has been involved in the coordination, conception, and design of the
study and helped drafting manuscript. All of the authors have read and approved the final manuscript.

CONFLICT OF INTEREST
The authors declare that they have no potential conflict of interests.

AUTHOR BIOGRAPHIES
Please see Supporting Information online.

ORCID
Mariusz Grinholc http://orcid.org/0000-0003-4041-0891

REFERENCES

[1] C. Van Delden, B. H. Iglewski, Emerg. Infect. Dis. 1998, 4, 551.
[2] T. R. de Kievit, B. H. Iglewski, Infect. Immun. 2000, 68, 4839.
[3] R. S. Smith, B. H. Iglewski, J. Clin. Invest. 2003, 112, 1460.
[4] M. Whiteley, K. M. Lee, E. P. Greenberg, Proc. Natl. Acad. Sci. U.S.A. 1999, 96, 13904.
[5] J. Lee, L. Zhang, Protein Cell 2015, 6, 26.
[6] T. R. de Kievit, Environ. Microbiol. 2009, 11, 279.
[7] A. Taraszkiewicz, G. Fila, M. Grinholc, J. Nakonieczna, Biomed. Res. Int. 2013, 150653.
[8] Q. Wei, L. Z. Ma, International Journal of Molecular Sciences, Vol. 14 2013, p.20983.
[9] M. Kossakowska-Zwierucho, R. Kazmierkiewicz, K. P. Bielawski, J. Nakonieczna, Front. Microbiol. 2016, 7, 1141.
[10] G. Fila, A. Kawiak, M. S. Grinholc, Virulence 2017, 8, 938.
[11] P. Ogonowska, Wozniak, A., Pieranski, M.K., Wasylew, T., Kwiek, P., Brasel, M., Grinholc, M., Nakonieczna, J., Lighting Research & Technology. SAGE Publications Ltd, London, UK 2018, in press.
[12] D. B. Jett, K. L. Hatter, M. M. Huycke, M. S. Gilmore, Biotechniques 1997, 23, 648.
[13] T. Dai, A. Gupta, Y. Y. Huang, R. Yin, C. K. Murray, M. S. Vrahass, M. E. Sherwood, G. P. Tegos, M. R. Hamblin, Antimicrob. Agents Chemother. 2013, 57, 1238.
[14] L. M. de Freitas, E. N. Lorenzon, N. A. Santos-Filho, L. H. P. Zago, M. P. Uliana, K. T. de Oliveira, E. M. Cilli, C. R. Fontana, Sci. Rep. 2018, 8, 4212.
[15] A. L. Barry, R. A. Lasner, Am. J. Clin. Pathol. 1979, 71, 88.
[16] C. E. R. Dodd, R. L. Sharman, S. F. Bloomfield, I. R. Booth, G. S. A. B. Stewart, Trends Food Sci. Technol. 1997, 8, 238.
[17] R. M. Amin, B. Bhayana, M. R. Hamblin, Lasers Surg. Med. 2016, 48, 562.
[18] M. A. Kohanski, M. A. DePristo, Mol. Cell 2010, 37, 311.
[19] J. Latimer, S. Forbes, A. J. McIlain, Antimicrob. Agents Chemother. 2012, 56, 3092.
[20] D. I. Andersson, D. Hughes, Nat Rev Micro. 2014, 12, 465.
[21] M. P. Fletcher, S. P. Diggle, M. Camara, Nat. Protoc. 2007, 2, 1254.
[22] T. R. De Kievit, R. Gillis, S. Marx, C. Brown, B. H. Iglewski, Appl. Environ. Microbiol. 2001, 67, 1865.
[23] T. Sierengle, WormBook: The Online Review of C. elegans Biology, Oxford University Press, Oxford, UK 2006, p. 1.
[24] R. M. Valentine, S. H. Ibbotson, K. Wood, C. T. Brown, H. Moseley, Photochem. Photobiol. Sci. 2013, 12, 203.
[25] M. E. Shurtleff, J. T. Mader, A. K. Camper, Chem. Biol. 2002, 9, 859.
[26] J. E. Gonzalez, N. D. Keshavan, Microbiology and Molecular Biology Reviews : MMBR 2006, 70, 859.
[27] M. Maclean, S. J. MacGregor, J. G. Anderson, G. Woolsey, Appl. Environ. Microbiol. 2009, 75, 1932.
[28] R. McDonald, S. J. Macgregor, J. G. Anderson, M. Maclean, M. H. Grant, J. Biomed. Opt. 2011, 16, 048003.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article. Video S1-S4 Real-time monitoring for biofilm formation. The movies were made of three-dimensional images taken by Leica confocal microscope every 20 minutes of incubation. One second of movie contains two shots corresponding to 40 minutes of biofilm observation (2400x acceleration). Left screen shows biofilm formation by control samples, right screen refers to biofilm formed by aBL pretreated bacteria. The parameter Δt indicates the change in time required for comparable biofilm formation by two analyzed samples.

Figure S1 Pseudomonas aeruginosa starting inocula for real-time biofilm formation monitoring. The bar graph represents the starting P. aeruginosa inocula for biofilm formation for two hospital-acquired (3752/sz and 4190/p/A) and two reference strains (PA01 and PA 14). Samples pretreated with aBL were administered with the light dose 10 J/cm² (irradiance 15.7 mW/cm², irradiation time 640 seconds; λ = 411 nm) and plate counting was performed after aBL treatment. The detection limit was 10 CFU/mL. The values are the means of three separate experiments.
**Figure S2** *Pseudomonas aeruginosa* viability during *C. elegans* in vivo assay. Synchronized L4 larvae in S-complete medium were infected by selected *P. aeruginosa* strains (kept in the dark or aBL treated) (3752/sz—gray; 556/K—yellow; 4190/p/A—dark blue; PAO1—light blue; PA14—orange). aBL treatment (light dose 10 J/cm²; irradiance 15.7 mW/cm², irradiation time 640 seconds; \( \lambda = 411 \) nm) was applied daily for 14 days. Worm numbers were analyzed under a stereomicroscope, and bacterial numbers were analyzed by the serial dilution method on selected days. The values are the means of three separate experiments.

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