Antimicrobial and anti-biofilm effect of a novel BODIPY photosensitizer against Pseudomonas aeruginosa PAO1

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(Received 28 March 2014; accepted 27 June 2014)

Photodynamic therapy (PDT) combines the use of organic dyes (photosensitizers, PSs) and visible light in order to elicit a photo-oxidative stress which causes bacterial death. GD11, a recently synthesized PS belonging to the boron-dipyromethene (BODIPY) class, was demonstrated to be efficient against planktonic cultures of Pseudomonas aeruginosa, causing a 7 log unit reduction of viable cells when administered at 2.5 μM. The effectiveness of GD11 against P. aeruginosa biofilms grown in flow-cells and microtiter trays was also demonstrated. Confocal laser scanning microscopy of flow-cell-grown biofilms suggests that the treatment has a biocidal effect against bacterial biofilm cells.

Keywords: BODIPY; photodynamic treatment; Pseudomonas aeruginosa

Introduction

P. aeruginosa is a free living Gram-negative microorganism ubiquitous in the environment. Its catabolic versatility makes this bacterium able to metabolize different carbon sources either in an aerobic or anaerobic atmosphere and to survive in oligotrophic niches as well as in high nutrient environments. P. aeruginosa colonizes human tissues, such as skin, eyes, and lungs, and its eradication may be complicated by the presence of multidrug-resistant strains (Nordmann et al. 2007) and hypermutable variants (Oliver et al. 2000). P. aeruginosa is also able to colonize and form biofilms, eg in the lungs of cystic fibrosis patients, in diabetic wounds, and on inert surfaces such as medical devices, prosthesis and catheters (Donlan 2001; Mittal et al. 2009), in whirlpools and hot tubs (Mena & Gerba 2009), in filters (Uhl & Hartmann 2005) and in fan coil units (Liguori et al. 2010). It is well documented that biofilms develop tolerance to antibiotics and biocides (Campanac et al. 2002; Smith & Hunter 2008; Hoiby et al. 2010). The concentrations of nutrient and oxygen are low in the depth of biofilms due to bacterial consumption; bacteria located deep in biofilms therefore display low physiological activity and increased tolerance to antibiotics that target growing bacteria (Walters et al. 2003; Pamp et al. 2008). Furthermore the matrix components that interconnect bacteria in biofilms can bind and/or neutralize antimicrobial agents (Mulcahy et al. 2008; Chiang et al. 2013). Increased tolerance to antimicrobials in biofilm-growing bacteria contributes to the chronicity of infections such as those associated with medical devices (Stewart & Costerton 2001; Hall-Stoodley & Stoodley 2009) and to the failure of sanitization of colonized environments.

Due to the frequent difficulties in treating P. aeruginosa infections and fouling, development of novel effective treatments is important. Among new alternative antimicrobial strategies, photodynamic therapy (PDT) is a promising approach, potentially applicable to the treatment of localized infections easily accessible by light, as well as to the sanitization of inert devices.

When bacteria are treated with a photosensitizer (PS), irradiation with visible light favors the development of reactive species such as oxygen radicals, singlet oxygen and radicals that impair cell integrity and cause cell death (Wainwright 1998). Oxidative stress can alter the integrity of the cell envelope as well as the organization of the cytoplasmic environment (Nitzan & Kauffman 1999; Caminos et al. 2008; Pudziuvyte et al. 2011). The most studied PS families (azines, porphyrins, chlorins, and phthalocyanine) are much more active against Gram-positive bacteria compared to Gram-negative bacteria, likely due to differences in PS uptake (Malik et al. 1992; Hamblin & Hasan 2004; Demidova & Hamblin 2005; Huang et al. 2010). Thus, many efforts have been made to synthetize PSs that are effective against Gram-negative bacteria. GD11 (2,6-diiodo-1,3,5,7-tetramethyl-8-(N-methyl-4-pyridyl)-4,4′-difluoroboradiazaindacene) is a PS belonging to the family of boron-dipyromethene (BODIPY) fluorescent dyes, commonly used in biological imaging and only recently exploited as PSs against...
both eukaryotic and prokaryotic cells (Banfi et al. 2012; Caruso et al. 2012). The GD11 structure was designed in order to make it effective in PDT against Gram-negative bacteria, using E. coli as the target organism (Caruso et al. 2012). Here, the efficacy of GD11-mediated PDT was tested against P. aeruginosa PAO1, a bacterium reported to be very much less sensitive to PDT than E. coli (Philippova et al. 2003; Tegos et al. 2006). Since antimicrobial PDT could be used as an anti-biofilm or an anti-biofouling treatment, a further investigation was aimed to assay this technique against P. aeruginosa biofilms grown in microplates and continuous culture flow-cells.

Materials and methods

Photosensitizer

A stock 1 mM (water:acetone, 1:1) solution of 2,6-diodo-1,3,5,7-tetramethyl-8-(N-benzyl-4-pyridyl)-4,4′-difluoroboradiazaindace (GD11) was prepared as previously described (Caruso et al. 2012) and diluted in PBS (KH₂PO₄/K₂HPO₄ 10 mM pH 7.4) to the final working concentration (0.5–80 μM).

Strains and growth conditions

P. aeruginosa PAO1 (Stover et al. 2000) and GFP-tagged P. aeruginosa PAO1 (Yang et al. 2007) were routinely grown in Luria Bertani (LB) broth under aerobic conditions at 37°C.

Two biofilm models were set up for photokilling by PDT (photoinactivation) experiments: a microplate system and a flow-cell. For the microplate system, overnight cultures of P. aeruginosa PAO1 were diluted in AB minimal medium supplemented with 10 mM glucose (Pamp & Tolker-Nielsen 2007) to a final OD₆₀₀ = 0.1, dispensed in 96 well plates equipped with peg-lids (Nunc TSP, Thermo Fisher Scientific, Roskilde, Denmark) and incubated for 24 h at 37°C.

For the flow-cell, biofilms were grown for two days at 37°C in flow chambers with individual channel dimensions of 1 × 4 × 40 mm. The flow system was assembled and prepared as described previously (Cruz et al. 2012). Inoculation of the system was carried out by injecting 300 μl of a GFP-tagged overnight culture of P. aeruginosa PAO1 diluted to an OD₆₀₀ of 0.01 into each flow channel. Upon inoculation, the flow-cells were left upside down and undisturbed for 1 h to allow bacterial attachment to the glass cover, then the flow of medium was started by means of a Watson Marlow 205S peristaltic pump (Ismatec, Wertheim, Germany). The flow-cells were irrigated with AB minimal medium supplemented with 0.3 mM glucose. The mean flow velocity in the flow-cells was 0.2 mm s⁻¹, corresponding to laminar flow with a Reynolds number of 0.02.

Photoinactivation experiments

P. aeruginosa PAO1 cell suspensions were obtained by diluting overnight cultures in PBS to reach a cell density of ~10⁸ CFU ml⁻¹. After the addition of GD11 at the chosen concentrations the suspensions were incubated for 10 min in the dark without shaking. When microaerophilic conditions were required, a thin layer of sterile mineral oil was laid down over the cell suspension to limit gas diffusion (Monrás et al. 2012).

Biofilms grown on peg-lids were washed with PBS, transferred to new plates containing increasing concentrations of GD11 (0–80 μM) in PBS and incubated in the dark at 37°C for 30 min.

Flow-cell biofilms were treated with the photosensitizer as follows: the flow was stopped, the system clamped off just upstream of the flow chambers, and 450 μl of photosensitizer solution (40 μM) or PBS were slowly injected into the individual chambers creating a batch-like treatment regimen. Care was taken not to disturb the biofilm. The flow-cells were then wrapped in tinfoil and left for 30 min in the dark for the compound to penetrate the biofilm.

Planktonic samples and biofilms were irradiated with a 500 W halogen-tungsten lamp (fluence rate 48 mW cm⁻², considering the 400 nm of the whole width of the lamp emission spectrum) for 1 or 3 h (energy density 171 or 513 J cm⁻²). The samples were irradiated from above, thus, for the biofilms grown on pegs, the direction of the irradiation was parallel to the biofilm, whereas for the biofilms grown in flow-cells the direction of irradiation was perpendicular to the biofilm, reaching the bottom of the biofilm first (Figure S1; Supplementary material is available via a multimedia link on the online article webpage). The lamp was placed at a distance of 20 cm above the sample and a 1.5 cm thick circulating water/glass filter was interposed to avoid overheating.

The PDT efficacy was evaluated as viable counts in the planktonic cell suspensions or the peg-lid grown biofilm, and by crystal violet staining or live/dead staining and confocal laser scanning microscope (CLSM) examination in the case of static or dynamic model biofilms, respectively.

A panel of controls was set for each experiment: PS untreated and dark incubated samples (– PS, – light), PS treated and dark incubated samples (+ PS, – light), PS untreated and irradiated samples (– PS, + light). The experiments were performed at least in triplicate.

Viable counts

Viable counts, expressed as colony forming units (CFU ml⁻¹) in cell suspensions were estimated after dark incubation and after irradiation by a plate count technique: a volume (0.1 or 0.01 ml) of undiluted or serially diluted samples was plated on LB agar plates and incubated for
24 h at 37°C. Viable cells in peg lid grown biofilm were evaluated after detachment by sonication for 5 min in a water bath (Branson 2510E-DTH, Branson Ultrasonic Corporation, Danbury, CT, USA).

Crystal violet staining
The abundance of biomass on peg lid grown biofilms was measured by crystal violet (CV) staining. Two series of identical samples, subjected to PDT as described above, were used. One series was stained soon after the treatment. The second series samples were washed with PBS in order to remove the excess PS, transferred to fresh medium, and allowed to grow for an additional 24 h before CV staining. The biofilms were stained with CV 0.1% for 15 min, washed twice with PBS, dried at 37°C for 2 h and then the CV was dissolved in acetic acid 30% for 10 min. The amount of solubilized dye was spectrophotometrically measured at 590 nm.

Microscopy and image acquisition
After the PDT treatment of the flow-cell-grown biofilms, the medium was replaced to include 0.3 μM propidium iodide for the visualization of dead cells. The system was unclamped and the flow resumed for growth for an additional 24 h before image acquisition in order to assess the long-lasting effects of the treatment. All microscopic observations and image acquisitions were performed with a Zeiss LSM710 confocal laser scanning microscope (CLSM) (Carl Zeiss Microimaging GmbH, Jena, Germany) equipped with detectors and filter sets for the monitoring of GFP and propidium iodide. Images were obtained with a 63/1.3 objective. Simulated 3D images were generated using the Imaris software package (Bitplane AG, Zurich, Switzerland).

Statistical analysis
The photoinactivation experiments were repeated at least three times on separate dates. Mean and standard deviation (SD) calculations were performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Data were analyzed by means of one-way ANOVA (Origin 7.0 SR0, Originlab Corporation, Northampton, MA, USA). Significant effects of treatments (p<0.05 and p<0.01) were estimated with the following contrasts: -PS + light vs -PS - light; +PS - light vs -PS - L; +PS + light vs -PS - light.

Results
Photoinactivation of planktonic cells of P. aeruginosa PAO1 by GD11
Before assaying the effect of PDT against P. aeruginosa biofilm, the sensitivity of planktonic cells of the microorganism to GD11 was evaluated. The GD11 killing effect upon irradiation by a wide spectrum white light (171 J cm\(^{-2}\)) was dose-dependent (Figure 1) and ascribable only to the photodynamic process, as no intrinsic toxicity (+PS, −L) or light toxicity (−PS, +L) was observed (data not shown). A 7 log reduction in viable cells, the highest viability reduction detectable in the experimental conditions (detection limit ≥ 10 CFU ml\(^{-1}\)), was obtained upon irradiation of samples treated with GD11 at a concentration equal to or higher than 2.5 μM.

As microaerophilic conditions may be expected in biofilms (Borriello et al. 2004), GD11 efficacy was tested against planktonic cell suspensions under oxygen limitation. When the cells were treated with a GD11 concentration of 2.5 μM and irradiated with the highest energy dose (171 J cm\(^{-2}\)) under aerobic conditions, the expected 7 log reduction in survivors was measured. However, under oxygen limitation the decrease was approximately a 3 log reduction (Figure 2).

Photoinactivation of P. aeruginosa PAO1 biofilms
PDT assays were at first performed in a static biofilm model. The efficacy of the treatment was evaluated measuring the amount of biomass by CV staining immediately after PDT, and 24 h later in order to allow growth of any survivors.

Concentrations of GD11 in the range of 2.5–80 μM were administered to the biofilms in order to identify an effective dose. Moreover, dark incubation of PS exposed biofilms was extended from 10 to 30 min to favor PS
overcoming the matrix barrier, whereas the light-dose treatment was the same as previously used for planktonic cell treatment (171 J cm\(^{-2}\)).

As for the experiments with planktonic cells, both the exposure to the PS in the absence of irradiation (+ PS – L) and irradiation in the absence of the PS (– PS, + L) did not cause a biofilm reduction (Figure 3). Immediately after PDT no biomass depletion ascribable to the photodynamic effect (+ PS, – L vs + PS, + L) was detectable independently of the GD11 concentration administered (Figure 3A). On the contrary, 24 h after the treatment a significant biomass reduction was measured in samples exposed to GD11 (≥ 5 μM) and irradiated (Figure 3B).

Since biofilm is composed of both cells and extracellular matrix components, the cellular concentration (cfu peg\(^{-1}\)) was assessed (Figure 3C and D). With regard to cell viability, GD11 displayed a certain degree of intrinsic toxicity (+ PS, – L), especially at the highest concentration tested (80 μM) (Figure 3C). This toxicity was, however, fully overcome after additional growth for 24 h (Figure 3D). The decrease in cell viability ascribable to PDT with high GD11 doses (≥ 20 μM) observed immediately after the treatment (Figure 3C) was confirmed even after recovery for 24 h (Figure 3D).

When PDT with 40 μM GD11 and a light intensity of 171 J cm\(^{-2}\) was applied to biofilms grown in flow-cells, no effect was detectable (Figure S2). Therefore, as bacterial PDT sensitivity is correlated not only to the PS concentration, but also to the irradiated energy in a dose-dependent manner (Sabbahi et al. 2013; see also Figure 2), the light intensity was increased to 513 J cm\(^{-2}\). Compared to the controls, the increased energy density caused a substantial increase in PI stained dead cells in the flow-cell-grown biofilms (Figure 4). No differences in the overall biofilm structure were detectable, indicating that the PDT resulted in killing of the biofilm cells without an immediate alteration in the biofilm structure.

**Discussion**

Conventional cleaning and disinfection regimens are often ineffective against biofilms as the bacteria are embedded within the biofilm and more tolerant to antimicrobials than their planktonic counterpart (Hall-Stoodley & Stoodley 2009). Among the new strategies for biofilm control, PDT appears to be promising and potentially applicable to very diverse contexts where PSs and light can be easily delivered. Recent reviews suggest PDT as a possible anti-biofilm approach in wound and middle ear infections, endodontic infections, sinusitis, gingivitis, endocarditis, and infections of permanent indwelling devices such as joint prostheses, heart valves and catheters (Sevgi et al. 2013; de Melo et al. 2013), although in vivo studies are still at the preliminary stage (Dai et al. 2010).

GD11 is a novel PS belonging to the BODIPY family of dyes traditionally used for biological imaging and only recently exploited for PDT against both eukaryotic and prokaryotic cells (Banfi et al. 2012; Caruso et al. 2012). In the authors’ previous studies GD11-mediated PDT was found active against both Gram-positive and Gram-negative bacteria, although the latter were, as expected, less sensitive to the treatment (Caruso et al. 2012). Among Gram-negative bacteria, the opportunistic pathogen *P. aeruginosa* is known to be less sensitive to photoinactivation than the commonly used model bacterium *E. coli* (Philippova et al. 2003; Tegos et al. 2006), which is also the case for GD11 mediated PDT. Nevertheless, an approximately 7 log reduction in viable cells of *P. aeruginosa* was achievable with a modest concentration increase (2.5 vs 1.5 μM) (Figure S3).

A crucial factor for the success of PDT is the oxygen supply (Maisch et al. 2007). In in vivo conditions, very low oxygen tension may occur, where some pathogens may survive and persist as biofilms, such as *P. aeruginosa* in chronic wounds (De Beer et al. 1994). Thus GD11 mediated photoactivation was also tested under microaerophilic conditions and it was found that its efficiency significantly decreased, causing only a 3 log decrease in viable cells vs the 7 log decrease observed under aerobic conditions. The lower PDT efficiency observed under microaerophilic conditions compared to aerobic conditions was not unexpected as the structure of GD11 was designed in order to increase the rate of singlet oxygen generation and to decrease the high fluorescence quantum yield of the typical BODIPY
dyes used for imaging that in an antimicrobial context represents an energy waste (Caruso et al. 2012). This photo-oxidative mechanism is compatible with the results of photodynamic inactivation experiments performed in the presence of antioxidants. Sodium azide, a well-known physical quencher of singlet oxygen and inhibitor of PDT cell killing caused by singlet oxygen (Li et al. 2001), significantly protected *P. aeruginosa* cells from the photooxidation induced by GD11 even at the highest light doses tested (114 J cm\(^{-2}\)), whereas mannitol, a scavenger of free radicals (Tavares et al. 2011), did not elicit any effect (Figure S4).

GD11 administered at a concentration of 2.5 μM showed the highest level of photoinactivation in planktonic cultures, but was completely inefficient against biofilms. The higher sensitivity to PDT of the planktonic cultures in contrast to the biofilms is not surprising and has been observed with both Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative (*P. aeruginosa*) bacteria (Lee et al. 2004; Street...
et al. 2010; Mantareva et al. 2011). Indeed, biofilms display features that may represent factors constraining the efficacy of PDT, such as high cell density, calcification of the mature biofilm, oxygen deficiency within the biofilm environment and the presence of extracellular polymeric substances (Shrestha & Kishen 2012). Nevertheless, the experiments performed on biofilms grown in a microplate system to define a pilot antibiofilm PDT protocol allowed a satisfactory activity at a GD11 concentration of 40 μM to be obtained and a time of contact with the PS of 30 min incubation in the dark.

In a biofilm, PDT induced photooxidative damage does not necessarily guarantee a long-lasting effect, as the survivors might be able to re-form the biofilm in quite a short time. Garcez et al. (2007) reported that in a root-canal infection model in which a three-day-old P. aeruginosa biofilm was subjected to PDT with 10 μM PEI-ce6, good eradication was observed immediately after the irradiation, but 24 h later the biomass was comparable to that measured before the photoinactivation treatment. In the authors’ experiments such re-growth was not observed, suggesting that GD11 mediated PDT is highly efficient.

Most studies applied PDT against biofilm grown in static environments (Donnelly et al. 2007; Collins et al. 2010; Mantareva et al. 2011; Shrestha & Kishen 2012). However, the static model is not really representative of naturally occurring biofilms, which are often subjected to hydrodynamic conditions. It was thus decided to assay PDT efficacy against biofilms grown in flow-cells. When the protocol developed for the biofilm grown in a microplate system was applied to biofilm grown in flow-cells
no photoinactivation was obtained and it was necessary to increase the light intensity threefold to obtain an antimicrobial effect.

The higher tolerance of the biofilm grown under flow with respect to the biofilm grown in the microplate system could be due to the nature of the biofilm. Biofilms characterized by different age, thickness and matrix composition can show different sensitivities to PDT (Street et al. 2010). In the present experimental conditions the biofilms differed not only in age, but also in the presence or absence of flow, which in turn affects nutrient availability and the biofilm architecture. It is thus conceivable that the biofilms grown under static and hydrodynamic conditions differed in structure and physiology and that this affected the PDT efficiency.

Among anti-biofilm drugs it is important to distinguish between disrupting agents able to disaggregate the organic and complex extracellular matrix produced by bacteria (Tegos & Hamblin 2013) and the antimicrobial agents that kill bacteria without disaggregating the extracellular matrix. When 24 h old biofilms grown on lid pegs were photoinactivated, no biomass eradication was observed immediately after PDT, suggesting that an effect on the extracellular matrix is unlikely. However, 24 h later, PS treated and irradiated biofilms were significantly thinner than the controls. The viability assays suggest that photo-treatment affected the viability of biofilm forming cells rather than their ability to build the matrix. These observations are also supported by the CLSM observations that clearly demonstrate that GD11-mediated PDT caused a high cell lethality and a negligible alteration in the biofilm architecture. The absence of disaggregation of the biofilm structure may be regarded as a positive feature, as it avoids the dissemination of viable cells that could colonize other sites. Since complete eradication of the biofilm is a desirable event, the photodynamic protocol could be combined with the administration of a disaggregating agent.

In the biofilms grown under flow, the bacteria located in the depth of the biofilm were killed. This observation is compatible with the setup of the flow-cell apparatus with respect to the irradiation direction. At first, the light encounters the cells attached to the transparent glass, and it is conceivable that the extracellular biofilm matrix can interfere with the light and prevent killing of the cells further from the glass and facing the medium.

The photoinactivation of the cells placed in the deeper layers of the biofilm allows for some considerations to be put forward. Since photo-oxidative stress had little effect at the top of the biofilm where oxygen concentration should be higher than in the deeper layers, oxygen availability did not seem to be a limiting factor. Moreover, the diffusion of the cationic PS across a complex biofilm matrix barrier rich in negatively charged organic compounds such as exopolysaccharides and DNA (Friedman & Kolter 2003; Jackson et al. 2004; Allesen-Holm et al. 2006) did not seem to be a factor limiting PDT efficiency. Furthermore, bacteria deep in biofilms display a dormant phenotype and are tolerant to the activity of many antibiotics (Walters et al. 2003; Pamp et al. 2008), but they can evidently be killed by GD11-mediated PDT.

Conclusions

In conclusion, the data demonstrate that GD11 is a very promising PS to be used against *P. aeruginosa* biofilm, as the concentrations needed to achieve a substantial degree of photoinactivation (40 μM) are considerably lower than those reported for other PSs, such as the commercial δ-aminolaevulinic acid (20–40 mM) (Lee et al. 2004), methylene blue (~ 1 mM) (Biel et al. 2011) and a cationic porphyrin 5,10,15,20-tetrakis (1-methylpyridino)-21H,23H-porphine (225 μM) (Collins et al. 2010). In addition, under the experimental conditions used the treatment was effective even against bacteria in the deepest layer of the biofilms, and no re-growth of the photoinactivated bacteria was observed.

Acknowledgements

The authors thank Fabrizio Pogliana for collaboration in the experimental work. This research was funded by the University of Insubria (Fondo di Ateneo per la Ricerca), and by a grant from the Danish Council for Independent Research.

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