# Tandem dispersion and killing of bacteria from a biofilm†

**Steven A. Rogers,‡ Michael Krayer,‡ Jonathan S. Lindsey* and Christian Melander***

***Received 13th October 2008, Accepted 27th October 2008***
***First published as an Advance Article on the web 10th December 2008***
**DOI: 10.1039/b817923a**

The combined effects of biofilm dispersion with a 2-aminoimidazole–triazole conjugate and bactericidal activity with a photodynamic inactivation agent suggest a novel combination therapy for treating diverse microbial infections.

## Introduction

The emergence of antibiotic-resistant strains of microbial organisms poses severe threats to human health. As one example, *Acinetobacter baumannii* is an opportunistic γ-proteobacterium that leads to life-threatening nosocomial infections.[1–7] Recent outbreaks both in the UK and the US have led to the closing of hospital facilities due to an inability to contain bacterial spread with conventional means. In fact, *ca.* 25% of all hospital swabs are positive for *A. baumannii*,[8] underscoring its pervasiveness in the healthcare system. The persistence of *A. baumannii* stems in part from its ability to form robust biofilms.[9] Indeed, in this phenotype the bacterium is able to survive for weeks on a dry surface. Furthermore, strains of *A. baumannii* have appeared that are extremely multi-drug resistant.[2] Clearly, there exists a tremendous need to develop new approaches to control and eradicate *A. baumannii* infections as well as the broader class of antibiotic-resistant organisms.

Photodynamic inactivation (PDI) is a promising approach for remediation of microbial infections.[10–12] PDI employs light-sensitized production of singlet oxygen and other reactive oxygen species as the microbicidal agents. Thus, without illumination, PDI agents are generally innocuous to bacteria; however, upon illumination with the appropriate wavelength of light, PDI agents can rapidly destroy bacteria. There have been a limited number of reports[13–16] that detail the PDI approach towards killing *A. baumannii* in its planktonic (free-swimming) form.[17–19] Indeed, almost all reports of microbial PDI have employed bacteria (regardless of bacterial strain) in the planktonic rather than biofilm form. The limited number of studies of the latter suggests the ineffectiveness of PDI in eradicating bacteria in a biofilm state. This dearth is not surprising given that bacteria in biofilms are known to be upwards of 1000-fold more resistant to microbicides.[20]

We have been studying the effect of simple analogues of sponge-derived marine alkaloids upon biofilm development and maintenance.[21–28] In this vein, we recently described the synthesis and anti-biofilm activity of 2-aminoimidazole–triazole (2-AIT) conjugate **1**.[21] This compound is able to inhibit and disperse *Pseudomonas aeruginosa*, *A. baumannii*, *Bordetella bronchiseptica*, and *Staphylococcus aureus* biofilms through a non-microbicidal mechanism, making it the first small molecule reported with anti-biofilm activity across bacterial order, class and phylum. Given the ability of **1** to inhibit and disperse *A. baumannii* biofilms, and the power afforded by PDI agents to kill planktonic bacteria, we herein describe an investigation of the ability of **1** to work in tandem with a PDI agent towards eradicating *A. baumannii*. The PDI agents chosen for examination are the common compounds toluidine blue O (TBO)[29] and Pd(II)-*meso*-tetra(*N*-methyl-4-pyridyl)porphyrin tetrachloride (PY)[17] (Fig. 1).

*Department of Chemistry, North Carolina State University, Raleigh, NC, 27695-8204, USA. E-mail: jlindsey@ncsu.edu, christian_melander@ncsu.edu; Fax: +1-919-515-5079; Tel: +1-919-515-6406 (JSL); Tel: +1-919-513-2960 (CM)*

† Electronic supplementary information (ESI) available: Planktonic and biofilm activity data. See DOI: 10.1039/b817923a

‡ These authors contributed equally to this work.

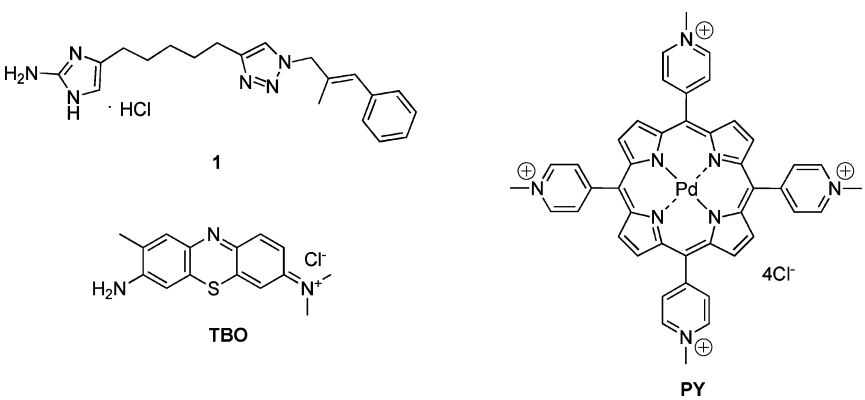


**Fig. 1** Structures of 2-AIT conjugate **1**, TBO and PY.

## Results and discussion

We first determined the $IC_{50}$ of TBO and PY towards *A. baumannii* under the PDI light source used in our lab. Using a fluence of 100 J/cm$^2$ (40 mW/cm$^2$, 40 min, $\lambda_{irr}$ = 400–700 nm), the $IC_{50}$ of TBO was 0.11 μM and the $IC_{50}$ of PY was 4.2 μM. These values correspond well to previous reports of PDI inactivation of *A. baumannii*.[13–16] As expected, each PDI agent alone, or light without the PDI agent, failed to induce bacterial death (see ESI†).

Next, the effect of each PDI agent upon biofilm mass was examined. Briefly, *A. bamaunnii* was allowed to form biofilms in a 96-well microtiter plate in the absence of TBO or PY for 24 h. Media and planktonic bacteria were then removed and the wells were washed to remove any loosely adherent bacteria. Media alone or media containing TBO or PY was added. The wells were then illuminated with 100 J/cm$^2$ (40 mW/cm$^2$, 40 min, $\lambda_{irr}$ = 400–700 nm). The wells were then washed and biofilm mass determined by crystal violet staining.[30] Spectrophotometric quantitation of the dye ($A_{540}$) indicated that both TBO and PY had no effect on biofilm mass up to 10 μM (highest concentration tested).

Next, we examined the ability of **1** to work in sequence with either TBO or PY to both disperse bacteria from the biofilm as well as kill any free-floating bacteria that result from dispersion. This was important because bacteria in biofilms are inherently insensitive to oxidative species,[20] and it was unclear if the dispersed bacteria would maintain this phenotype and be refractory to killing by a PDI agent. Again, *A. baumannii* was allowed to form biofilms for 24 h in the absence of TBO or PY. After 24 h, media was removed and the wells washed. Media containing **1** (231 μM) was then added to effect dispersion. After 6 h following addition of **1**, wells were either treated with TBO or PY. The final concentration of TBO or PY was set at its respective $IC_{50}$ value (0.11 μM or 4.2 μM). Wells were then illuminated with 100 J/cm$^2$ (80 mW/cm$^2$, 20 min, $\lambda_{irr}$ = 400–700 nm). Viable colonies were then enumerated (Fig. 2). An 88% reduction in viable colonies was observed upon comparison of the dispersed bacteria alone *versus* the dispersed bacteria treated with TBO, while an 82% reduction in viable colonies was observed for the PY-treated sample. This indicated that the dispersed bacteria are susceptible to killing by a PDI agent. Moreover, the dispersed bacteria appear to be more vulnerable to the PDI effect than bacteria grown planktonically, although this may be due to differences in the overall number of bacteria in the dispersed sample vs. the planktonic culture. Further studies are needed to delineate this effect.

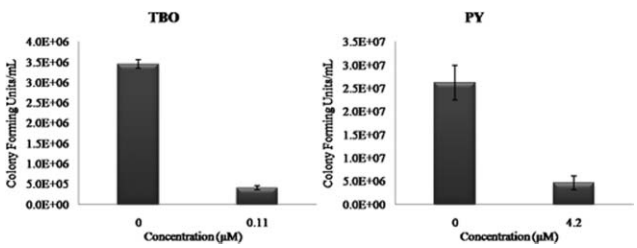


**Fig. 2** Viability of dispersed bacteria upon illumination with or without PDI agent.

Finally, we wanted to determine if **1** and a PDI agent could function in concert to eliminate biofilm mass. In this regard, it is known that the presence of bactericidal agents can induce biofilm formation (presumably as a protective mechanism).[31] Therefore, it was unclear if the compounds would act synergistically or if the presence of the PDI agent would mitigate the effects of **1**. Accordingly, we investigated the simultaneous administration of **1** with a PDI agent under illuminating conditions.

**Table 1** $EC_{50}$ of **1** in the presence and absence of PDI agent[a]

| [PDI Agent][b] | $EC_{50}$ of **1** with TBO[b] | $EC_{50}$ of **1** with PY[b] |
|---|---|---|
| 0 | 423 ± 22 | 423 ± 22 |
| 0.10 | 337 ± 10 | 322 ± 4 |
| 1.0 | 298 ± 17 | 317 ± 21 |
| 10.0 | 343 ± 7 | 345 ± 7 |

[a] 100 J/cm$^2$ illumination over 6 h. [b] Concentrations are in μM.

First, a control experiment established that **1** was stable under the illumination conditions employed above (see ESI†). A second issue concerned the different duration of the dispersion process and the PDI process as practiced here, which entailed 6 h at 37 °C *versus* 20–40 minutes at room temperature, respectively. Thus, it was necessary to determine conditions that would allow characterization of the synergistic effects of both agents. Empirically, we chose a middle ground of 6 h of intermittent irradiation (100 J/cm$^2$ total energy) at room temperature. Based upon these conditions, in the absence of PDI agent, the $EC_{50}$ of **1** was 424 ± 22 μM, which is *ca.* 4-fold higher than under the originally reported conditions (37 °C, 24 h). Given that the formation and dispersion of bacterial biofilms is a dynamic process, such slight differences in $EC_{50}$ values are not unexpected. We then determined the $EC_{50}$ of **1** toward dispersion of *A. baumannii* biofilms as a function of PDI concentration. The results are summarized in Table 1. As can be seen, the PDI agent and **1** exhibit a slight synergistic effect in reducing biofilm mass as indicated by a reduction in the $EC_{50}$ values of **1**.

## Conclusion

It is possible to use the sequential addition of a biofilm dispersal agent (**1**) followed by a PDI agent and light to effectively reduce both biofilm mass and viable *A. baumannii* bacteria. Furthermore, the observation of a slight synergistic effect by the simultaneous addition of the PDI agent and biofilm dispersal agent with continuous low level illumination indicates that these compounds may be used in concert to control *A. baumannii* colonization. The light intensity, illumination time, and PDI agent can be further tuned, hence it may be possible to achieve a more dramatic synergistic effect as well as reconcile the different temporal requirements of the biofilm dispersal agent and the PDI agents employed herein, including use of more potent photosensitizers.[32] Examination of this tandem therapy in a wide variety of real-world conditions appears meritorious.

## Experimental

### General experimental

*A. baumannii* (ATCC # 19606) was purchased from the ATCC. A LumaCare™ model LC-122 non-coherent light source for photodynamic inactivation was used with a Fiber Optic Probe model LUM 300–700, which transmitted light from 400–700 nm. A xenon lamp (OSRAM Model 64653) was employed. The light

 

intensity was measured using an Orion-PD laser power meter equipped with a PD300-UV photodiode head. All other supplies were purchased from commercially available sources.

### Colony count procedure to determine planktonic killing capacity of PDI agent

Colony counts were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into Luria-Bertani (LB) media (10 g tryptone, 5 g of yeast extract and 10 g of NaCl in 1 L of water). The compound being tested was then added to the media at a predetermined concentration (0.033–10.0 μM). The resulting stock solution was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. A sample of media without test compound served as the control. The plate was then subjected to 100 J/cm$^2$ (40 mW/cm$^2$, 40 min, $\lambda_{irr}$ = 400–700 nm) of radiation at ambient temperature. After irradiation, 50 μL was taken from each sample well and then diluted serially into LB media. Then, 10 μL was removed from each serial dilution and plated out on a square gridded Petri dish followed by 16 h of incubation at 37 °C to grow viable colonies, which were quantified through employment of the track-dilution method.[33] Dose-response data for TBO and PY against planktonic *A. baumannii* can be found in Figures S1 and S2.†

### PDI agent effects on planktonic viability without irradiation

Assays were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into LB media. The PDI agent being tested was then added to the media at a predetermined concentration. The resulting stock solution was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. Samples were then incubated at ambient temperature for 40 min. After incubation, 50 μL was taken from each sample well and then diluted serially into LB media. Then, 10 μL was removed from each serial dilution and plated out on a square gridded Petri dish followed by 16 h of incubation at 37 °C to grow viable colonies, which were quantified through employment of the track-dilution method.[33] The dose-response effects of TBO and PY on planktonic *A. baumannii* without illumination can be found in Figures S3 and S4.†

### Radiation effects on planktonic viability

Assays were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into LB media. The resulting bacterial suspension was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. Samples were then subjected to 100 J/cm$^2$ (40 mW/cm$^2$, 40 min, $\lambda_{irr}$ = 400–700 nm) of radiation at ambient temperature. A control was employed which was incubated in the dark for 40 min. After incubation, 50 μL was taken from each sample well and then diluted serially into LB media. Then, 10 μL was removed from each serial dilution and plated out on a square gridded Petri dish followed by 16 h of incubation at 37 °C to grow viable colonies, which were quantified through employment of the track-dilution method.[33] Data comparing the irradiated and non-irradiated samples are found in Figure S5.†

### Biofilm dispersal ability of the PDI agents

Dispersion assays were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into LB media. The resulting bacterial suspension was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. Plates were then wrapped in GLAD Press n' Seal® followed by incubation under stationary conditions at ambient temperature to establish the biofilms. After 24 h, the media was discarded from the wells, and the plates were washed thoroughly with water. Samples of stock solutions of predetermined concentrations of the PDI agent were then made in LB media. The stock solutions were then aliquoted (110 μL) into the wells of the 96-well PVC microtiter plate with the established biofilms. LB media alone was added to some wells to serve as a control. Plates were then subjected to 100 J/cm$^2$ (40 mW/cm$^2$, 40 min, $\lambda_{irr}$ = 400–700 nm) of radiation at ambient temperature. After this, the media was discarded from the wells and the plates were washed thoroughly with water. Plates were then stained with 100 μL of a 0.1% solution of crystal violet (CV) and then incubated at ambient temperature for 30 min. Plates were then washed with water and the remaining stain was solubilized with 200 μL of 95% ethanol. 125 μL of solubilized CV stain from each well was transferred to the corresponding wells of a polystyrene microtiter dish. Biofilm dispersion was quantitated by measuring the $OD_{540}$ of each well in which a negative control lane (wherein no biofilm was formed) served as a background and was subtracted out. Dose-response data of the PDI agent to *A. baumannii* biofilms can be found in Tables S1 and S2.†

### Stability of 1 in the presence of a PDI agent under irradiation

A solution of **1** and TBO (10 mM and 0.1 mM respectively) was subjected to 100 J/cm$^2$ (80 mW/cm$^2$, 20 min, $\lambda_{irr}$ = 400–700 nm) of radiation at ambient temperature in a well of a 96-well PVC microtiter plate. Controls were employed which contained only **1** or TBO. After irradiation no noticeable decomposition was observed either by laser-desorption mass spectrometry in the absence of a matrix (LD-MS) (Figures S6-S8†) or by TLC [silica, $CH_2Cl_2$/MeOH saturated with $NH_3$ (9:1), stained with $KMnO_4$ solution] (Figure S9†).

### PDI agents effect on dispersed planktonic bacteria viability

Assays were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into LB media. The resulting bacterial suspension was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. Plates were then wrapped in GLAD Press n' Seal® followed by incubation under stationary conditions at ambient temperature to establish the biofilms. After 24 h, the media was discarded from the wells and the plates were washed thoroughly with water. Then, a 231 μM solution of **1** in LB was prepared and aliquoted (100 μL) into the wells of the 96-well PVC microtiter plate with the established biofilms. LB alone was added to a subset of the wells to serve as a control. Samples were then incubated for 6 h at ambient temperature to disperse the preestablished biofilm. Then, the PDI agent was added directly to the wells at a predetermined concentration and then subjected to 100 J/cm$^2$ (80 mW/cm$^2$, 20 min, $\lambda_{irr}$ = 400–700 nm) of radiation at ambient temperature. Wells containing no PDI agent served as a control. After irradiation, 50 μL was taken from each sample

well and then diluted serially into LB media. Then, 10 μL was removed from each serial dilution and plated out on a square gridded Petri dish followed by 16 h of incubation at 37 °C to grow viable colonies, which were quantified through employment of the track-dilution method to determine the ability of the PDI agents to kill dispersed planktonic bacteria.[33]

**Static bacterial biofilm dispersion assay for *A. baumannii***

Dispersion assays were performed by taking an overnight culture of bacterial strain and subculturing it at an $OD_{600}$ of 0.01 into LB media. The resulting bacterial suspension was aliquoted (100 μL) into the wells of a 96-well PVC microtiter plate. Plates were then wrapped in GLAD Press n' Seal® followed by incubation under stationary conditions at ambient temperature to establish the biofilms. After 24 h, the media was discarded from the wells and the plates were washed thoroughly with water. Stock solutions of predetermined concentrations of **1** and PDI agent were then made in LB media. These stock solutions were aliquoted (110 μL) into the wells of the 96-well PVC microtiter plate with the established biofilms. LB media alone was added to a subset of the wells to serve as a control. Plates were then incubated for 6 h while subjected to 100 J/cm$^2$ (5.6 mW/cm$^2$, 6 × 50 min, $\lambda_{irr}$ = 400–700 nm; 10 min dark intervals) of radiation at ambient temperature. After incubation, the media was discarded from the wells and the plates were washed thoroughly with water. Plates were then stained with 100 μL of 0.1% solution of crystal violet (CV) and then incubated at ambient temperature for 30 min. Plates were washed with water again and the remaining stain was solubilized with 200 μL of 95% ethanol. A sample of 125 μL of solubilized CV stain from each well was transferred to the corresponding wells of a polystyrene microtiter dish. Biofilm dispersion was quantitated by measuring the $OD_{540}$ of each well in which a negative control lane (wherein no biofilm was formed) served as a background and was subtracted out. Dispersion data for the 0.10, 1.0 and 10.0 μM PDI solutions with **1** are found in Figures S10, S11 and S12.†

## Acknowledgements

Financial support is gratefully acknowledged from the UNCGA competitiveness fund (CM) and a Burroughs-Wellcome Fellowship (MK). We thank Duran H. Pardue for technical assistance.

## Notes and references

1 J. J. Schafer and J. E. Mangino, *Emerg. Infect. Dis.*, 2008, **14**, 512–514.
2 T. D. Gootz and A. Marra, *Expert Rev. Anti. Infect. Ther.*, 2008, **6**, 309–325.
3 J. Gilad and Y. Carmeli, *Drugs*, 2008, **68**, 165–189.
4 P. Scott, G. Deye, A. Srinivasan, C. Murray, K. Moran, E. Hulten, J. Fishbain, D. Craft, S. Riddell, L. Lindler, J. Mancuso, E. Milstrey, C. T. Bautista, J. Patel, A. Ewell, T. Hamilton, C. Gaddy, M. Tenney, G. Christopher, K. Petersen, T. Endy and B. Petruccelli, *Clin. Infect. Dis.*, 2007, **44**, 1577–1584.
5 A. Jones, D. Morgan, A. Walsh, J. Turton, D. Livermore, T. Pitt, A. Green, M. Gill and D. Mortiboy, *Lancet Infect. Dis.*, 2006, **6**, 317–318.
6 C. K. Murray and D. R. Hospenthal, *Curr. Opin. Infect. Dis.*, 2005, **18**, 502–506.
7 D. H. Forster and F. D. Daschner, *Eur. J. Clin. Microbiol.*, 1998, **17**, 73–77.
8 E. Bergogne-Bérézin, M. L. Joly-Guillou, and K. J. Towner, *Acinetobacter: microbiology, epidemiology, infections, management*, CRC Press, Boca Raton, 1996.
9 A. P. Tomaras, C. W. Dorsey, R. E. Edelmann and L. A. Actis, *Microbiol.-Sgm*, 2003, **149**, 3473–3484.
10 T. Maisch, R. M. Szeimies, G. Jori and C. Abels, *Photoch. Photobio. Sci.*, 2004, **3**, 907–917.
11 M. R. Hamblin and T. Hasan, *Photoch. Photobio. Sci.*, 2004, **3**, 436–450.
12 M. Wainwright, *J. Antimicrob. Chemoth.*, 1998, **42**, 13–28.
13 I. C. J. Zanin, M. M. Lobo, L. K. A. Rodrigues, L. A. F. Pimenta, J. F. Hofling and R. B. Goncalves, *Eur. J. Oral Sci.*, 2006, **114**, 64–69.
14 I. C. J. Zanin, R. B. Goncalves, A. Brugnera, C. K. Hope and J. Pratten, *J. Antimicrob. Chemoth.*, 2005, **56**, 324–330.
15 F. Gad, T. Zahra, T. Hasan and M. R. Hamblin, *Antimicrob. Agents C.h*, 2004, **48**, 2173–2178.
16 M. Sharma, L. Visai, F. Bragheri, I. Cristiani, P. K. Gupta and P. Speziale, *Antimicrob. Agents Ch.*, 2008, **52**, 299–305.
17 Y. Nitzan, A. Balzam-Sudakevitz and H. Ashkenazi, *J. Photoch. Photobio. B-Biology*, 1998, **42**, 211–218.
18 Y. Nitzan and H. Ashkenazi, *Curr. Microbiol.*, 2001, **42**, 408–414.
19 H. Ashkenazi, Y. Nitzan and D. Gal, *Photochem. Photobiol.*, 2003, **77**, 186–191.
20 D. J. Musk and P. J. Hergenrother, *Curr. Med. Chem.*, 2006, **13**, 2163–2177.
21 S. A. Rogers and C. Melander, *Angew. Chem., Int. Edit.*, 2008, **47**, 5229–5231.
22 J. J. Richards, C. S. Reed and C. Melander, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 4325–4327.
23 J. J. Richards and C. Melander, *J. Org. Chem.*, 2008, **73**, 5191–5193.
24 J. J. Richards, R. W. Huigens, T. E. Ballard, A. Basso, J. Cavanagh and C. Melander, *Chem. Commun.*, 2008, 1698–1700.
25 J. J. Richards, T. E. Ballard and C. Melander, *Org. Biomol. Chem.*, 2008, **6**, 1356–1363.
26 J. J. Richards, T. E. Ballard, R. W. Huigens and C. Melander, *ChemBioChem*, 2008, **9**, 1267–1279.
27 R. W. Huigens, L. Ma, C. Gambino, A. Basso, P. D. R. Moeller, J. Cavanagh, D. J. Wozniak and C. Melander, *Mol. Biosyst.*, 2008, **4**, 614–621.
28 R. W. Huigens, G. Parise, J. J. Richards, T. E. Ballard, W. Zeng, R. Deora and C. Melander, *J. Am. Chem. Soc.*, 2007, **129**, 6966–6967.
29 T. N. Demidova and M. R. Hamblin, *Antimicrob. Agents Ch.*, 2005, **49**, 2329–2335.
30 G. A. O'Toole and R. Kolter, *Mol. Microbiol.*, 1998, **30**, 295–304.
31 L. R. Hoffman, D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones and S. I. Miller, *Nature*, 2005, **436**, 1171–1175.
32 C. Ruzie, M. Krayer, T. Balasubramanian and J. S. Lindsey, *J. Org. Chem.*, 2008, **73**, 5806–5820.
33 B. D. Jett, K. L. Hatter, M. M. Huycke and M. S. Gilmore, *Biotechniques*, 1997, **23**, 648–650.

 