Antimicrobial Photochemistry

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Exploiting a Bacterial Drug-Resistance Mechanism: A Light-Activated Construct for the Destruction of MRSA**

*Xiang Zheng, Ulysses W. Sallum, Sarika Verma, Humra Athar, Conor L. Evans, and Tayyaba Hasan**

The prevalence of bacterial drug resistance makes it imperative to develop new targeted strategies that can be used either as monotherapies or in conjunction with existing antibiotic regimens. Photodynamic therapy (PDT) has such potential. PDT is a photochemistry-based emerging technology that relies on the wavelength-specific light activation of certain nontoxic chemicals (photosensitizers, PSs) to form active molecular species (AMSs) that are toxic to surrounding biological targets.[1] The reported effectiveness of PDT against pathogens in general, and methicillin-resistant *Staphylococcus aureus* (MRSA) in particular,[2] makes it a potentially powerful technology for the treatment of drug-resistant infections. AMSs have multiple cellular targets, in contrast to conventional antibiotics, such as β-lactams and aminoglycocides,[3] which inhibit the activity of single enzymes. This multifaceted nature of PDT action has the advantage that it decreases the probability of generating PDT-resistant strains of bacteria; however, this feature can also be a limitation, owing to nonspecific PS accumulation, which results in damage to healthy host tissue.[4]

The aim of this study was to exploit a bacterial drug-resistance mechanism to activate the PS locally, only at the site of infection, for a more specific PDT effect. The strategy involves the synthesis of a construct which can be activated by light and which recognizes a molecular target that is unique to the bacterium of interest. The advantage of this approach is that it enables much-enhanced selectivity, as the construct can only be activated by light after interaction with the molecular target. The construct can not be activated at any other site. This enhanced selectivity could enable the use of PDT more broadly for regional infections than is currently possible; at present, PDT can only be used for highly localized infections. This study focuses on the β-lactamase enzyme as the molecular target. One way in which bacteria resist the action of β-lactam antibiotics is through the production of β-lactamase, which cleaves the β-lactam ring hydrolytically.[5] In the case of cephalosporins, ring opening of the β-lactam is accompanied by the release of the substituent at the 3′-position. Zlokarnik et al. used this feature to design β-lactamase reporter systems to detect gene-promoter activation in mammalian cells.[6,7]

In this study, we targeted the β-lactamase expressed by MRSA. In designing the molecular construct (β-lactamase-enzyme-activated photosensitizer, β-LEAP), we took advantage of the photophysical phenomenon known as quenching. PSs can be quenched when in close proximity to each other. As a result, the probability of a PS excited-state transition is diminished, which leads to decreased fluorescence or AMS formation. β-LEAP is designed in such a way that, upon cleavage by β-lactamase (Scheme 1), the PS is released from homodimeric, ground-state quenching to yield an enzyme-specific, light-activated antimicrobial action. The β-lactamases were ideal targets for this proof-of-principle study owing to the prevalence of β-lactamase expression among bacteria and its high enzymatic efficiency.[8,9]

The PS 5-(4′-carboxybutylamino)-9-diethylaminobenzo[*a*]phenothiazinium chloride (EtNBS-COOH), an EtNBS derivative, was demonstrated previously to be a potent antimicrobial agent.[10] In the current study, the free terminal carboxy group of the PS was conjugated to a cephalosporin derivative, 7-amino-3-chloromethyl-3-cephem-4-carboxylic acid *p*-methoxybenzyl ester (ACLE), which contains a β-lactam ring. Thus, ACLE was modified with two primary amino groups and treated with EtNBS-COOH (Scheme 2). The final product **3** (β-LEAP) was purified and characterized by HPLC, mass spectrometry, and NMR spectroscopy (see Figures 1 and 3 in the Supporting Information). β-LEAP showed a nearly fivefold decrease in fluorescence emission (excitation at 625 nm) relative to EtNBS-COOH (Figure 1). This result indicates the quenching effect of the two PS moieties in the molecule. There are two mechanisms of PS quenching: 1) static (ground-state) quenching, such as fluorophore homo- or heterodimerization, and 2) dynamic (excited-state) quenching through Förster resonance energy transfer (FRET). The distortion of the long-wavelength absorption peak of β-LEAP (with an extra shoulder that is blue-shifted from the maximum absorption peak by 30 nm) with respect to that of EtNBS-COOH (see Figure 2 in the Supporting Information) suggested a ground-state quenching mechanism.[11] The short separation (ca. 2.4 nm) of the two PS moieties assured a high quenching efficiency. Homodimeri-

[*] Dr. X. Zheng,[+] U. W. Sallum,[+] S. Verma, H. Athar, C. L. Evans, Prof. T. Hasan
Wellman Center for Photomedicine
Harvard Medical School and Massachusetts General Hospital
40 Blossom Street, Boston, MA 02114 (USA)
Fax: (+1) 617-726-8566
E-mail: thasan@partners.org
Homepage: http://www2.massgeneral.org/wellman/people/thasan.asp

[+] X. Zheng and U. W. Sallum contributed equally to this work.

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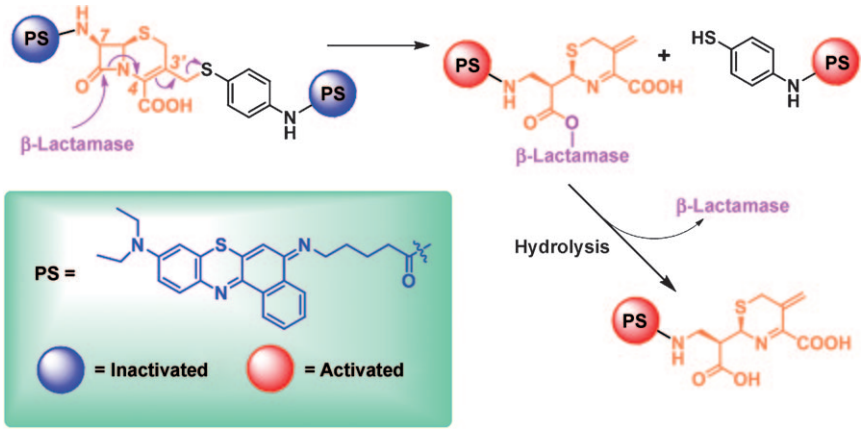
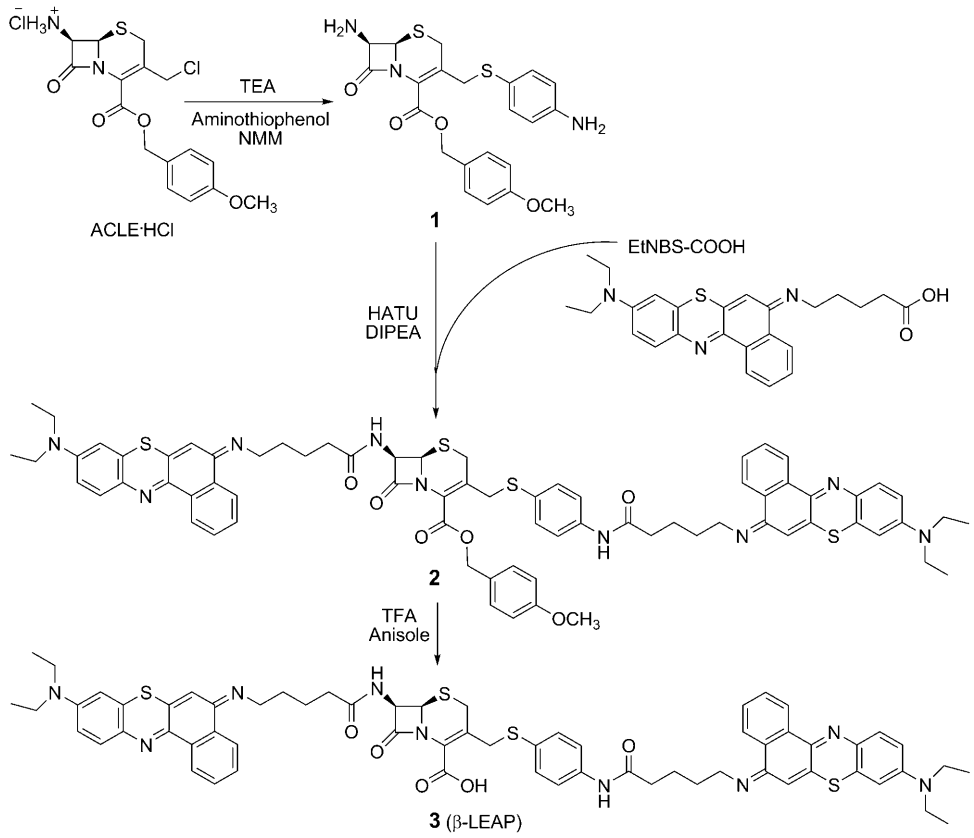


Scheme 1. Mechanism for the cleavage of β-LEAP. The blue balls represent the inactive PSs in the uncleaved construct, and the red balls represent the potentially phototoxic active PSs following the β-lactamase-mediated cleavage of β-LEAP.



Scheme 2. Synthesis of β-LEAP. DIPEA = *N*,*N*′-diisopropylethylamine, NMM = 4-methylmorpholine, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TEA = triethylamine, TFA = trifluoroacetic acid.

zation (PS pair) quenching has the advantage of releasing two active PSs after cleavage to yield twice the potential phototoxicity of a comparable heterodimer system.

The ability of *Bacillus cereus* penicillinase to cleave and activate β-LEAP was assayed, and a concentration-dependent increase in fluorescence emission as a function of time was demonstrated for both the enzyme and the substrate (Figure 2b, and Figure 4 in the Supporting Information). The Michaelis constant (K_m) and the K_{cat} value at 50 units per milliliter of *B. cereus* penicillinase were determined to be 1.648 μM and 2.79 s^{-1}, respectively, by a global fitting procedure with the program GraphPad Prism 5.0. The K_{cat}/K_m ratio of β-LEAP was comparable to those reported for penicillins and greater than those reported for cephalosporins, and thus indicated efficient cleavage (Table 1).

Four clinical blood isolates of MRSA and a *S. aureus* (non-MRSA) control strain were selected to assess β-LEAP cleavage and PDT efficacy. The five strains exhibited different levels of susceptibility to penicillin G, as determined by a minimum inhibitory concentration (MIC) assay (Figure 2a and Table 2).

Within the first 30 min of incubation, the rates of β-LEAP hydrolysis by the three strains of MRSA were relatively close (Figure 2c), whereas the control strain, 29213, exhibited a rate of hydrolysis no different from that of the buffer alone. The total amount of hydrolysis by strain 9307 during the 3-h incubation period was less than that observed for strains 8150 and 8179. The susceptibilities of all bacterial strains for penicillin G and β-LEAP PDT were determined. Bacteria were cultured overnight in brain heart infusion medium with penicillin G (10 μg mL^{-1}). Overnight cultures were then diluted 1:1000 in fresh antibiotic-free medium and cultured to mid exponential phase. The bacteria were then diluted to a final concentration of 5×10^6 CFU mL^{-1} (CFU = colony-forming unit) and incubated with β-LEAP for 90 min in the dark to enable sufficient β-LEAP activation prior to exposure to the appropriate fluence of laser light at 670 nm. The respective levels of β-LEAP hydrolysis correlated with the observed differences in the susceptibilities of the three strains to penicillin G as well as PDT with β-LEAP (compare Table 2 and Figure 2). Strain 9307 was more susceptible to penicillin G than strains 8150 and 8179 (Table 2, Figure 2a). Interestingly, at a β-LEAP concentration of 2.5 μM, strain

a)

Photosensitizer	Φ (fl)	Φ ($^1O_2$)
EtNBS-COOH	0.216	0.032
β-LEAP	0.052	0.029

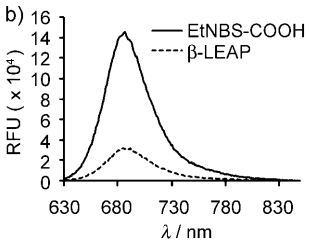


Figure 1. Physical characterization: a) Fluorescence quantum yield (Φ (fl)) and singlet-oxygen quantum yield (Φ ($^1O_2$)) of β-LEAP and EtNBS-COOH; ethanol with 0.1% acetic acid was used as the solvent. b) Fluorescence spectra of β-LEAP and EtNBS-COOH. The spectra were recorded at equimolar concentrations; methanol was used as the solvent. RFU = relative fluorescence units.

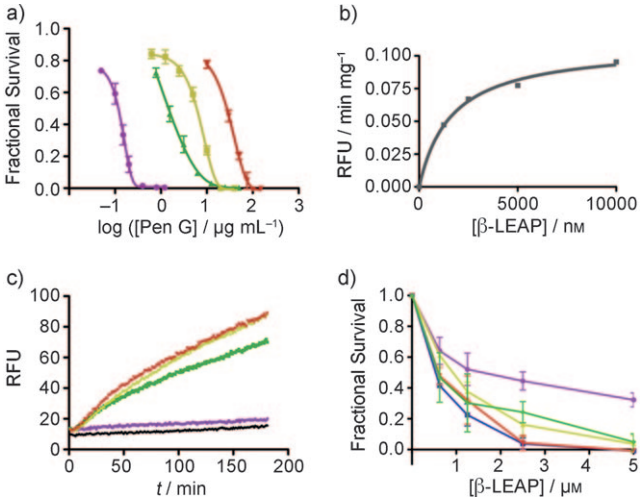


Figure 2. Specificity of β-LEAP for β-lactamase. a) Inhibition profiles for selected strains of *S. aureus* with penicillin G. b) Nonlinear regression analysis of β-LEAP hydrolysis by *B. cereus* penicillinase. c) β-LEAP hydrolysis by selected strains of *S. aureus*. d) Profiles for the inhibition of selected strains of *S. aureus* by PDT with β-LEAP. (Key for a, c, and d: purple 29213, green 9307, gold 8150, red 8179, blue 8140 (d only), black no cells (c only)).

Table 1: Rate constants for the cleavage of β-LEAP and other β-lactams by *B. cereus* penicillinase.

Substrate	K_{cat}/K_m [μM^{-1} s^{-1}]
β-LEAP	1.69 ± 0.15
phenethicillin[9]	18.7 ± 1.5
L-propicillin[9]	13.2 ± 0.7
D-propicillin[9]	15.4 ± 0.4
carbenicillin[9]	2.84 ± 0.2
phenoxymethylpenicillin[9]	38.4 ± 1.0
benzylpenicillin[12]	0.427 ± 0.06
cephaloridine[12]	0.0676 ± 0.004
cefuroxime[12]	0.774 ± 0.07

9307 was more resistant to PDT than strains 8150 and 8179 (Figure 2d). These findings demonstrate both an inverse relationship between bacterial susceptibility to penicillin G and bacterial susceptibility to β-LEAP PDT and a direct

Table 2: Susceptibility of strains of *S. aureus* to penicillin G and β-LEAP, and relative β-lactamase activity.

Strain		MIC [μg mL^{-1}][a]	β-Lactamase activity [U mL^{-1}][b]	Survival with β-LEAP (2.5 μM)[c]
29213	ATCC	0.24 ± 0.01	n.d.[d]	0.44 ± 0.06
9307	clinical MRSA	5.92 ± 1.0	0.84	0.24 ± 0.07
8150	clinical MRSA	15.7 ± 1.2	0.79	0.16 ± 0.08
8179	clinical MRSA	74.6 ± 5.3	1.23	0.04 ± 0.04
8140	clinical MRSA	n.d.[d]	n.d.[d]	0.04 ± 0.04

[a] MIC = minimum inhibitory concentration of penicillin G. [b] 1 U mL^{-1} = β-lactamase activity of 1×10^9 bacteria per mL. [c] Fraction of the bacteria that survived treatment with β-LEAP (2.5 μM). [d] n.d. = not determined.

relationship between β-lactamase activity and bacterial susceptibility to β-LEAP PDT. Taken together, these results indicate the success of our approach. All MRSA strains were inactivated effectively at a 5 μM concentration of β-LEAP with illumination at 15 J cm^{-2} (Figure 2d); this result is comparable to the observed efficacy of EtNBS-COOH (see Figure 6 in the Supporting Information). The reference strain exhibited a concentration-dependent inhibition of growth upon incubation with β-LEAP that did not differ significantly from the observed inhibition without PDT (Figure 2d, and Figure 5 in the Supporting Information).

The phototoxicity of β-LEAP to human foreskin fibroblasts (HFF-1) was compared to that of EtNBS-COOH. β-LEAP was found to be less phototoxic than the free PS to HFF-1 cells (see Figure 7 in the Supporting Information). Together, these findings indicated that β-LEAP could target MRSA selectively and produce less damage to host tissue than PDT with free PS. Cocultures of MRSA and HFF-1 were incubated with β-LEAP and examined by confocal microscopy to compare their relative levels of β-LEAP fluorescence. Although there was some nonspecific uptake of β-LEAP by the HFF-1 cells, the presence of MRSA8179, the strain most resistant to penicillin G (Figure 2a and Table 2), led to far greater fluorescence (Figure 3). The higher fluorescence levels observed in MRSA (Figure 3, insets) demonstrate quantitatively the specificity of β-LEAP in targeting the drug-resistant strain.

PDT for entirely localized infections is currently in clinical trials;[13] however, the concept of using PDT systemically for regionally localized infections is new. The successful implementation of this strategy requires the combination of an appropriate molecular target that is specific to the bacteria, such as β-lactamase, with a robust chemical construct that recognizes the target. A perceived limitation of this technology could be light delivery to the site of infection. However, with the advent of fiber optics, light delivery to complex anatomical sites, although challenging, is feasible. PDT is an FDA-approved treatment for lung cancer[14] and is in clinical studies for systemic diseases, such as mesothelioma, disseminated ovarian cancer, and other intraperitoneal diseases.[15,16]

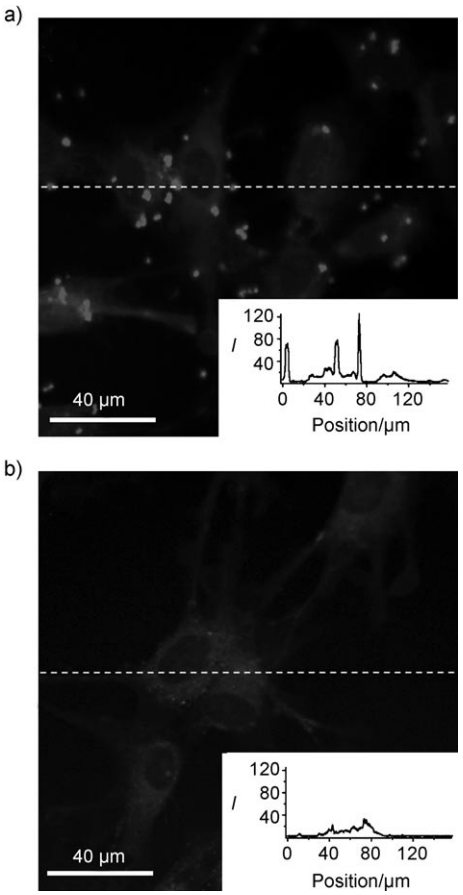


Figure 3. β-LEAP fluorescence in cell cultures. Two-dimensional projections of 90 depth-resolved confocal slices separated by 0.4 μm: a) coculture of HFF-1 cells and strain 8179; b) HFF-1 cells alone. Insets are plots of the fluorescence intensity along the dotted lines in the images. The insets show the significantly greater fluorescence intensities for MRSA than for the HFF cells and thus indicate the local cleavage of β-LEAP only at the site of the bacteria. *I* = intensity (arbitrary units).

We anticipate that the strategy developed in the current study will be able to be used in combination with standard antibiotic treatment to destroy resistant and nonresistant bacteria. This study demonstrates the successful exploitation of an antibiotic-resistance mechanism for the activation of a novel therapeutic photodynamic molecule. The significance of this study lies in the broader applicability of PDT for preseptic infections at sensitive anatomical sites. This strategy is adaptable to other mechanisms of drug resistance.

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