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Photoinactivation of Gram Positive and Gram Negative Bacteria with the Antimicrobial Peptide $(KLAKLAK)_2$ Conjugated to the Hydrophilic Photosensitizer Eosin Y

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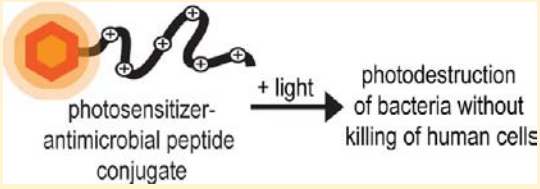
Supporting Information

ABSTRACT: We test the hypothesis that the antimicrobial peptide $(KLAKLAK)_2$ enhances the photodynamic activity of the photosensitizer eosin Y upon conjugation. The conjugate eosin-$(KLAKLAK)_2$ was obtained by solid-phase peptide synthesis. Photoinactivation assays were performed against the Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and multidrug resistant *Acinetobacter baumannii* AYE, as well as the Gram-positive bacteria *Staphylococcus aureus*, and *Staphylococcus epidermidis*. Partitioning assays were performed with *E. coli* and *S. aureus*. Photohemolysis and photokilling assays were also performed to assess the photodynamic activity of the conjugate toward mammalian cells. Eosin-$(KLAKLAK)_2$ photoinactivates 99.999% of 10^8 CFU/mL of most bacteria tested at a concentration of 1 μM or below. In contrast, neither eosin Y nor $(KLAKLAK)_2$ cause any significant photoinactivation under similar conditions. The increase in photodynamic activity of the photosensitizer conferred by the antimicrobial peptide is in part due to the fact that $(KLAKLAK)_2$ promotes the association of eosin Y to bacteria. Eosin-$(KLAKLAK)_2$ does not significantly associate with red blood cells or the cultured mammalian cell lines HaCaT, COS-7, and COLO 316. Consequently, little photodamage or photokilling is observed with these cells under conditions for which bacterial photoinactivation is achieved. The peptide $(KLAKLAK)_2$ therefore significantly enhances the photodynamic activity of eosin Y toward both Gram-positive and Gram-negative bacteria while interacting minimally with human cells. Overall, our results suggest that antimicrobial peptides such as $(KLAKLAK)_2$ might serve as attractive agents that can target photosensitizers to bacteria specifically.



INTRODUCTION

The emergence of drug-resistant infections has become a widespread problem in public health.[1,2] As a result, the development of new antimicrobial therapeutic agents has become crucial. In this context, antimicrobial photodynamic therapy (PDT) is an attractive modality for the treatment of a variety of localized infections.[3] PDT is based on combining photosensitizers (PS) with light irradiation at visible or near-infrared wavelengths to trigger cell killing. Photosensitizers are molecules that are relatively innocuous in the dark but, upon excitation with light, generate reactive oxygen species (ROS) such as singlet oxygen.[3,4] Excited PS and ROS can kill cells by damaging biomolecules such as proteins, nucleic acids, and lipids. To date, antimicrobial PDT has been applied to the inactivation of several pathogenic microbes both *in vitro* and *in vivo*.[3,5] This approach appears to kill antibiotic-resistant strains as effectively as their antibiotic-sensitive counterparts.[5,6]

Common antimicrobial PS include porphyrins, chlorins, pthalocyanines, xanthenes, or phenothiazines.[3] These compounds have historically been selected in part because of their high singlet oxygen quantum yields. Their photodynamic activity also typically correlates with their lipophilicity, and effective photosensitizers often have a high propensity to bind and damage biological membranes.[7,8] Several PS kill Gram-positive bacteria readily, but many compounds are much less effective at killing Gram-negative bacteria.[9−11] An explanation for this difference in behavior is attributed to the outer membrane of Gram-negative bacteria acting as a barrier that prevents the penetration of the PS. The photodynamic efficacy of PS toward Gram-negative bacteria has been improved by addition of cationic compounds such as poly(lysines) (pL) and poly(ethyleneimine) (PEI).[12−14] The size of the polymer conjugate is an important determinant of the efficacy with which bacterial photoinactivation is achieved.[15] For instance, *Escherichia coli* was not photoinactivated efficiently by a PS conjugated to small-molecular-weight pL (i.e., 8 lysines).[15] However, a larger pL conjugate (i.e., 37 lysines) was more phototoxic presumably because of the increased propensity of the large polycationic compound to disrupt the outer membrane of Gram-negative bacteria.[16] On the other hand, large pL complexes might not permeate the surface layer of Gram-positive bacteria as efficiently as smaller analogues because of a molecular-sieving effect.[15,17] Consequently, increasing the size of a pL-PS conjugate might increase activity against Gram-negative bacteria but reduce the photoinactivation efficiency achieved against Gram-positive strains. Recently, a LPS-binding peptide was shown to preferentially target a porphyrin conjugate to Gram-negative bacteria for inactiva-

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tion.[18] The photodynamic activity of this conjugate toward Gram-positive bacteria has not been tested but is presumably low. Because *in vivo* applications of antimicrobial PDT might involve infections containing many different types of bacteria, there is therefore still a need to identify molecules that can target a PS to both Gram-positive and Gram-negative bacteria with comparable efficiencies.

The arginine-rich cell-penetrating peptide (CPP) TAT conjugated to a porphyrin was shown to inactivate both Gram-positive and Gram-negative bacteria.[19] These results therefore suggest that a small peptide might serve as a broad spectrum targeting agent. However, a potential problem with this approach, along with approaches using pL or PEI conjugates, is related to the toxicity of polycationic PS toward human cells. Arginine-rich peptides and cationic polymers have been shown to also increase the killing activity of PS toward mammalian cells.[12,13] This phototoxicity arises in part because cationic polymers are often efficiently internalized by human cells through endocytic mechanisms.[20−22] Accumulation of pL-PS or TAT-PS conjugates within endocytic organelles can in turn cause the lysis of these organelles upon irradiation and facilitate the photokilling of human cells.[23,24] Hamblin and co-workers have shown that the binding of pL-PS conjugates to microbial cells is very rapid while the endocytic uptake of these compounds into human cells is slower.[14,15,24] In principle, it is therefore possible to target and kill bacteria selectively by controlling the time cells are exposed to the conjugates. However, moieties that can target bacteria while inducing minimum endocytic uptake in human cells might provide a better selectivity between these different cell types and be more practical in general.

In this report, our goal was to test whether an amphiphilic antimicrobial peptide (A-AMP) might serve as an attractive tool to target PS to bacterial membranes.[25] A-AMPs are typically small peptides that bind to the membranes of Gram-positive and Gram-negative bacteria. Above certain threshold concentrations, these peptides disrupt the integrity of bacterial lipid bilayers and kill both Gram-positive and Gram-negative pathogens in the dark.[25,26] We therefore reasoned that an A-AMP might target a PS to different bacteria with relatively comparable efficiencies. While A-AMPs are typically cationic, they nonetheless contain fewer positive charges than pL or CPPs. Consequently, their endocytic uptake into mammalian cells is expected to be comparatively reduced. Overall, our hypothesis was therefore that an A-AMP would improve the antimicrobial photodynamic effect of a PS while inducing little damage toward mammalian cells.

To test this hypothesis, a conjugate between the antimicrobial peptide $(KLAKLAK)_2$ and the photosensitizer eosin Y was used (eosin-$(KLAKLAK)_2$).[27,28] On one hand, $(KLAKLAK)_2$ is a prototypical A-AMP with MIC values of approximately 6 μM for *E. coli*, *P. aeruginosa*, and *S. aureus*.[28,29] Moreover, hemolytic concentrations and sublethal concentrations to 3T3 cells are 2 orders of magnitude greater than MIC values.[28] On the other hand, eosin Y is a photosensitizer that, despite a high quantum yield of singlet oxygen (Φ ~0.6), is not very phototoxic on its own.[7] This can be attributed in part to the fact that eosin Y is relatively hydrophilic and does not significantly partition into membranes.[7] We were therefore interested in testing whether $(KLAKLAK)_2$ could enhance the photodynamic activity of this singlet oxygen generator by bringing it into proximity to bacterial membranes. The choice of a PS with low intrinsic phototoxicity might seem surprising as a starting point. However, we were concerned that a more photoactive but more lipophilic PS might significantly compromise the targeting specificity of the A-AMP conjugate. Indeed, many PS are too lipophilic to distinguish between human and bacterial bilayers. With eosin-$(KLAKLAK)_2$, however, we anticipated that the A-AMP would dictate binding specificity with minimal interference by the conjugated PS.

MATERIALS AND METHODS

Cell culture reagents were purchased from Invitrogen. Peptide synthesis reagents were from Novabiochem. The compound 5,6-carboxy-eosin was purchased from Marker Gene Technologies. All other reagents were from Sigma. COS-7 and COLO 316 were obtained from ATCC. HaCaT cells were a generous gift from Joan Massagué (Memorial Sloan-Keterring Cancer Center). Whole blood was purchased from Gulf Coast Regional Blood Center (Houston, TX).

Peptide Design and Synthesis. H_2N-KLAKLAKKLAKLAK-NH_2 ("$(KLAKLAK)_2$") was synthesized by Fmoc solid-phase peptide synthesis using Rink amide MBHA resin according to previously reported protocols (Novabiochem). Eosin-$(KLAKLAK)_2$ was synthesized by coupling of 5,6-carboxy-eosin Y to the N-terminal residue of the peptide. H_2N-KLAKLAKKLAKLAK-NH_2 and eosin-$(KLAKLAK)_2$ were purified using HPLC and their mass was confirmed by MALDI-TOF. Because possible differences in the eosin Y isomers may produce different membrane affinities, the isomer of the labeled peptide that first eluted from HPLC was used for all experiments.

H_2N-KLAKLAKKLAKLAK-NH_2 expected mass: 1522.08, observed mass: 1523.18. Eosin-$(KLAKLAK)_2$ expected mass: 2198.82, observed mass: 2196.67.

Bacterial Strains. *Escherichia coli* BL21 DE3 was obtained from Agilent. *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* subsp. *aureus* (ATCC 29213), and *Staphylococcus epidermidis* (ATCC 12228) were purchased from the American Type Culture Collection. Multidrug-resistant *Acinetobacter baumannii* AYE strain (ATCC BAA-1710) was a gift from Dr. Ry Young at Texas A&M University Center for Phage Technology. *E. coli* and *S. aureus* were grown in Luria−Bertani broth (LB), *Ps. aeruginosa* and *A. baumannii* were grown in tryptic soy broth, and *S. epidermidis* in nutrient broth. Glycerol stocks were established for each strain and used to streak agar plates. Colonies from plates were used to inoculate overnight cultures, which were grown aerobically at 37 °C. Fresh cultures were inoculated the next day in a 1:1000 dilution of overnight culture and used for experiments after growth to mid log phase ($O.D._{600}$ ~0.4−0.6).

Bacterial Photoinactivation Assay. Bacteria were grown as described above in 14 mL round-bottom Falcon culture tubes in their respective media, then centrifuged at 1500 *g* for 10 min and resuspended in sterile phosphate buffer (100 mM NaCl, 10 mM Na_2HPO_4, pH 7.4). This wash procedure was repeated a second time, and this stock suspension was used to make suspensions at the OD required for the particular strain to have approximately 10^8 CFU/mL (colony forming units were determined by plating 10-fold serial dilutions of cultures on agar plates as described below). Peptide solutions (10×, 22 μL) were prepared in wells of a 96-well plate before addition of 200 μL of bacteria in phosphate buffer (~10^8 CFU/mL). Samples were allowed to incubate for approximately 3−5 min before irradiation to allow for peptide binding, and micro stir bars (2 × 2 mm, Cowie via Fisher) were added for continued

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aeration during irradiation. The lipid to peptide (L/P) ratio under these conditions is 1:1 when the peptide or PS concentration is approximately 3 μM (these calculations assume 25×10^6 lipids per bacteria).

Irradiation was achieved using a homemade setup with a 600 W halogen lamp. The lamp was suspended by clamps and air-cooled during operation. A homemade water filter was placed below the lamp to filter out IR with continuous exchange of the water supply. A stir plate was placed underneath the water filter to hold the samples for illumination. Samples were placed in a 96-well plate with a lid. A 5×7 in.^{2} green filter (Edmund Optics cat. no. NT46−624, 470−550 nm, fwhm) was placed on top of the lid for excitation of eosin. A single pane of 1/16 in. diffusing glass was placed on top of the green filter to provide an even distribution of light intensity. Experiments detecting the $^1O_2$ production from Rose Bengal via reaction with RNO demonstrated that this setup provides even distribution of light across all 96 wells (data not shown). Samples were stirred at 200 rpm and set at a distance of 20 cm from the light source. Exposure time was 30 min for all killing assays.

After samples were illuminated or kept in the dark for 30 min; 30 μL of each sample was added to 270 μL of phosphate buffer in a separate 96-well plate. Further 10-fold serial dilutions of the samples were made in phosphate buffer to give samples ranging 10^1-10^5 in dilution factor. From each dilution, 50 μL was removed and spread on an agar plate, then incubated overnight at 37 °C. Colonies were counted the next morning to determine the remaining CFU/mL. Plates without peptide treatment were included as a negative control for sample comparison to determine percent survival.

Partitioning Assay. Mixtures of bacteria and peptide or PS were prepared in the same manner as the photoinactivation assays above (222 μL total volume). Samples were then centrifuged at 1500 *g* for 10 min, and 200 μL of supernatant was transferred to a 96-well plate. Any remaining supernatant was removed, and the pellet was resuspended in 200 μL of phosphate buffer. The fluorescence intensity of the pellets was measured with a microplate reader (Promega Glomax-Multi) using the green filter set (ex 525/em 580−640 nm). Absorbance values were ≤0.100 at 525 nm to avoid the inner filter effect. To ensure that quenching was not occurring, 2-fold serial dilutions of samples were performed. A linear decrease in fluorescence was observed, indicating that no quenching occurred in the resuspended pellet. Experiments were performed in triplicate.

Photohemolysis Assay. A concentration of 0.05% by volume of RBCs was used for hemolysis experiments. This RBC concentration gives a similar L/P ratio to that used in photoinactivation assays, where the L/P ratio is 1:1 when the peptide or PS concentration is approximately 3 μM (assuming $\sim 5 \times 10^8$ lipids per RBC). RBCs (200 μL) were placed in a well of a 96-well plate, and 22 μL of 10× peptide-conjugate or PS was added and mixed. RBCs were incubated for 5 min before illuminating (or keeping in darkness) for 30 min using the halogen lamp setup described above. The extent of hemolysis was determined by centrifuging samples at 1500 *g* for 10 min, then reading the absorbance of hemoglobin in the supernatant at 450 nm. Untreated RBCs were included as a negative control for both dark and illuminated samples. RBCs treated with 0.1% Triton X-100 were used as a positive control for 100% lysis. The data represent experiments in triplicate with their respective standard deviations.

Mixed RBC and bacteria samples were prepared similarly, with 200 μL of bacteria (10^8 CFU/mL) in PBS placed in a well of a 96-well plate, then 11 μL of 20× RBCs added and mixed together. Peptide-conjugate or PS (11 μL of 20× stock solution) was added only after the RBCs and bacteria were mixed to ensure equal opportunity for binding of the peptide to either the RBCs or bacteria. Illumination by halogen lamp and measurement of hemolysis was carried out in the same manner. In order to determine the amount of bacteria killed after illumination, parallel samples were included to determine the CFU/mL remaining by performing serial dilutions on agar plates, as described for bacteria killing assays. Experiments were performed in triplicate.

For imaging of RBCs or mixed RBC and bacteria samples, a 384-well plate with a glass bottom was used to enable use of the 100× oil-immersion objective. Samples were prepared in the same manner as above, but scaled down from 220 μL to 55 μL total volume because of the well size. Cells were imaged before and after illumination for 30 min under the halogen lamp to observe binding and/or killing. SYTOX Blue was incubated for 15 min at room temperature before imaging as an indicator of bacterial cell death. Intact erythrocytes have a dark contrast in the bright field image, while lysed ghosts do not.[23]

Microscopy. Imaging was performed on an inverted epifluorescence microscope (Model IX81, Olympus, Center Valley, PA). The microscope is equipped with a heating stage maintained at 37 °C. Images were captured with a Rolera-MGI Plus back-illuminated EMCCD camera (Qimaging, Surrey, BC, Canada). Imaging was performed using bright field imaging and the following fluorescence filter sets: DAPI (ex = 360 ± 20 nm/em = 460 ± 30 nm), FITC (ex = 488 ± 10 nm/em = 520 ± 20 nm), and RFP (ex = 560 ± 20 nm/em = 630 ± 35 nm). Fluorescence excitation was achieved with a 100 W mercury lamp (Leeds Precision Instruments # L202 Osram) and with neutral density filters (ND 1, 2, 3, and 4 on the instrument, corresponding to 100%, 25%, 12.5%, and 5% transmittance). Images were captured with *SlideBook 4.2* software (Olympus, Center Valley, PA).

Cell-Based Assays. Mammalian cells were cultured in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C in a humidified environment with 5% CO_2. For viability experiments, cells were plated in sterile 96-well plate so that the cells were approximately 80% confluent after 24 or 48 h. Cells were washed twice with PBS and once with Leibovitz's L-15 medium, before addition of the desired concentration of eosin-$(KLAKLAK)_2$ in L-15. The cells were then kept in the dark or illuminated for 30 min in the same manner as the bacterial inactivation experiments. Afterward, the cells were washed out with PBS twice and once with L-15 before incubation with SYTOX Green and Hoechst in L-15 media according to the manufacturer's instructions. SYTOX Green is cell-impermeable and only stains cells with a compromised plasma membrane, while Hoechst stains all cells. Cells were imaged with a 20× objective using bright-field and fluorescence in DAPI and FITC channels. Ten to twenty images were acquired in the green and blue channels for each experiment. The total number of cells in a given image was determined from the blue channel image (Hoechst) by counting the number of blue nuclei present. The number of dead cells was determined by identifying cells containing a green fluorescent nucleus stained by SYTOX green. Cell viability was determined by establishing a ratio of dead cells/total number of cells for each

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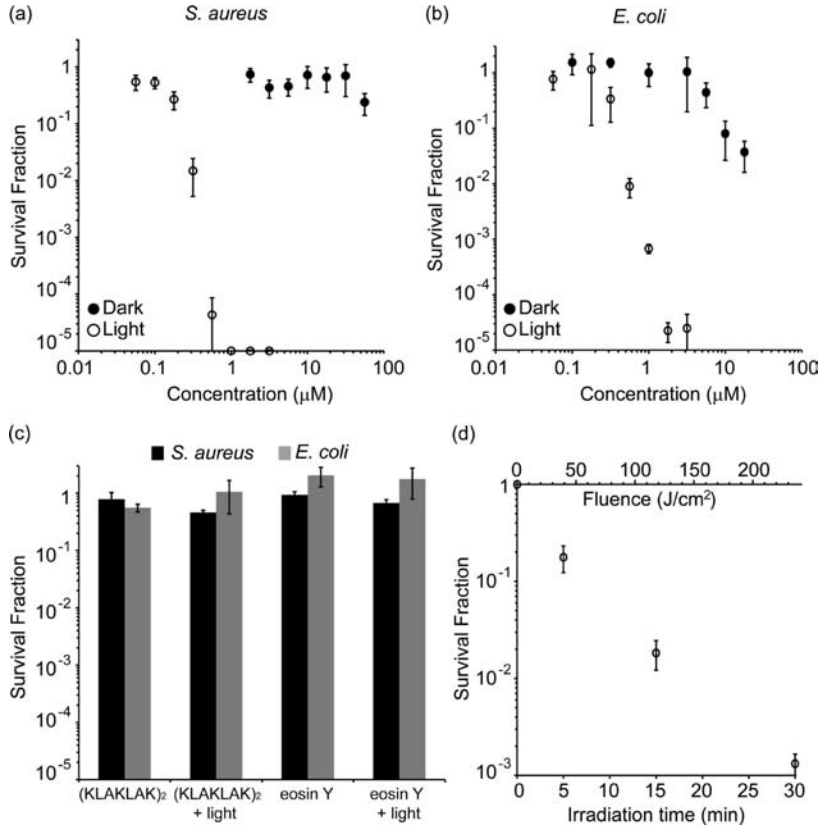

Figure 1. Eosin-$(KLAKLAK)_2$ kills bacteria upon light irradiation while eosin Y does not. (a) Survival fraction of *S. aureus* (10^8 CFU/mL) after exposure to eosin-$(KLAKLAK)_2$ in the dark or irradiated with visible light for 30 min. (b) Identical experiment performed with *E. coli* (10^8 CFU/mL). (c) Effects of $(KLAKLAK)_2$ (10 μM) or eosin Y (10 μM) on the survival of *S. aureus* or *E. coli* after 30 min incubation in the absence or presence of light. (d) Photoinactivation of *E. coli* (10^8 CFU/mL) by eosin-$(KLAKLAK)_2$ (1 μM) as a function of irradiation time and fluence.

sample (at least 1000 cells were counted in each experiment and each experiment was repeated 3 times).

For comparison of cellular uptake between eosin-TAT and eosin-$(KLAKLAK)_2$, cells were plated in 8-well glass bottom dishes (Lab-Tek) 24 or 48 h prior to experiments. Cells were washed twice with PBS and once with L-15, then incubated with 1 μM eosin-TAT or eosin-$(KLAKLAK)_2$ in L-15 for 30 min. After incubation, cells were washed twice with PBS and once with L-15. Fluorescence (IRFP channel) and bright field images were captured at 100×. For comparison of uptake, the fluorescence intensity per cell was determined with the *Slidebook* software by measuring the total fluorescence intensity present in each cell. This was performed by first creating outlines for each cell, which were converted to masks of the whole cell with the software. Background removal was performed by subtracting the highest background value from the IRFP channel, then using the software to calculate the sum intensity of the endocytic organelles that remained visible within the masks and above the background. The intensities of all endocytic organelles were then added to obtain the total fluorescence intensity per cell. Approximately 50 representative cells were imaged for each condition.

RESULTS

Eosin-$(KLAKLAK)_2$ Kills Gram-Negative and Gram-Positive Bacteria upon Light Irradiation. The photodynamic activity of eosin-$(KLAKLAK)_2$ was tested against *A. baumannii*, *P. aeruginosa*, and *E. coli* (Gram-negative), as well as *S. aureus* and *S. epidermidis* (Gram-positive). (Figure 1 and Figure S1) Eosin Y was used as an unconjugated control. The bactericidal activity of the tested compounds was determined both in the dark and after irradiation at 525 nm for 30 min (525 nm corresponds to the excitation maximum of eosin Y). As shown in Figure 1, eosin Y alone or $(KLAKLAK)_2$ alone had no significant effects on cell viability at 10 μM in the absence or presence of light. Similarly, eosin-$(KLAKLAK)_2$ had little activity toward *S. aureus* or *E. coli* in the dark below 10 μM. However, the antimicrobial activity of eosin-$(KLAKLAK)_2$ toward both strains was greatly enhanced with irradiation at 525 nm, as the peptide killed 99.9−99.999% of bacteria at 1 μM. As expected for a light-induced process, the extent of photoinactivation was dependent on the irradiation time, and increasing light exposure increased killing (Figure 1d). Similar results were obtained with *A. baumannii*, *P. aeruginosa*, and *S. epidermidis* (Figure S1). Dark toxicity of eosin-$(KLAKLAK)_2$ against the Gram-negative strains *E. coli* and *Ps. aeruginosa* occurred at 10 μM or less. Even greater dark toxicity was observed toward the Gram-negative *A. baumannii*, with 99% killing at 1 μM, a greater extent of killing than observed by $(KLAKLAK)_2$ alone for this strain.

Eosin-$(KLAKLAK)_2$ Associates with Bacteria to a Greater Extent than Eosin Y. In order to investigate a potential cause for the difference in activity between eosin Y and eosin-$(KLAKLAK)_2$, the association of these two compounds with bacteria was characterized. Eosin-$(KLAKLAK)_2$ and eosin Y were incubated with 10^8 CFU/mL of *E. coli* or *S. aureus* under conditions identical to those used for photoinactivation assays. The mixtures were centrifuged to

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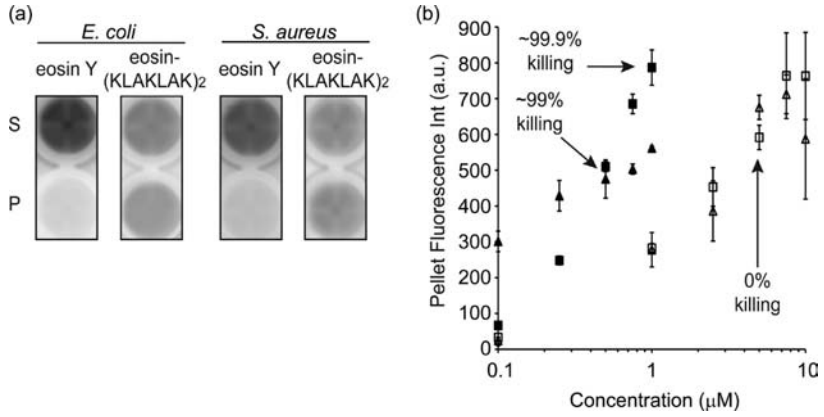

Figure 2. Eosin-$(KLAKLAK)_2$ has a higher propensity to bind to bacteria than eosin Y. (a) Partitioning of eosin Y and eosin-$(KLAKLAK)_2$ between soluble (S) and bacteria-bound (pellet, P) fractions. Bacteria (10^8 CFU/mL) were incubated with 1 μM of eosin Y or eosin-$(KLAKLAK)_2$. The samples were centrifuged to separate the S and P fractions and placed in a multiwell plate. The fluorescence of each fraction was imaged on a fluorescence scanner. The fluorescence image obtained is represented as an inverted monochrome (dark contrast = bright fluorescence). (b) Fluorescence intensity associated with bacteria (pellet fraction) as a function of eosin-$(KLAKLAK)_2$ or eosin Y concentration. *E. coli* (■ eosin-$(KLAKLAK)_2$, □ eosin Y) and *S. aureus* (▲ eosin-$(KLAKLAK)_2$, Δ eosin Y) were used at 10^8 CFU/mL and the fluorescence intensities reported are those obtained when the samples are kept in the dark (no bacterial killing is obtained under these conditions). The percentage of killing achieved when the same samples are exposed to light for 30 min are highlighted with arrows (these numbers correspond to the results obtained in Figure 1).

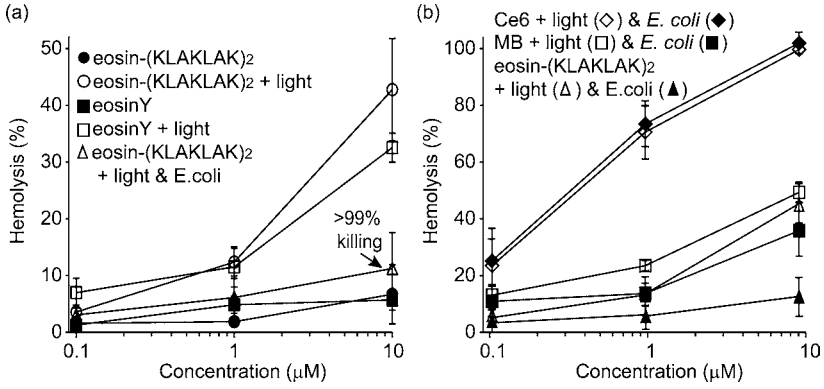

Figure 3. Photohemolysis increases with eosin-$(KLAKLAK)_2$ concentration but the presence of bacteria reduces the photodamage to RBCs. (a) Hemolytic activities of eosin-$(KLAKLAK)_2$ and eosin Y in the dark or after 30 min irradiation with light. Suspensions of RBCs (0.05% by volume) were prepared with or without *E. coli* (10^8 CFU/mL) present. (b) Comparison of eosin-$(KLAKLAK)_2$ hemolytic activiity to chlorin e6 (Ce6) and methylene blue (MB). Experiments were performed in the same way as in (a).

separate the molecules present in solution (soluble fraction) to those bound to bacteria (pellet fraction). The amount of eosin-$(KLAKLAK)_2$ or eosin Y associated with bacteria was then determined by measuring the fluorescence present in the pellet fraction. The concentrations tested were in the ranges of 0.1 to 1 μM for eosin-$(KLAKLAK)_2$ and 0.1 to 10 μM for eosin Y. These conditions correspond to peptide to bacteria ratios at which no killing is detected in the dark. The signal detected is therefore proportional to the binding of eosin-$(KLAKLAK)_2$ or eosin Y to live bacteria as opposed to the binding of these compounds to dead cells. As shown in Figure 2, eosin-$(KLAKLAK)_2$ associates with *E. coli* or *S. aureus* to a greater extent than eosin Y. For instance, eosin-$(KLAKLAK)_2$ partitions equally between soluble and bacteria-bound fractions at 1 μM, while eosin Y is mostly present in solution at this concentration (Figure 2a). These data therefore suggest that $(KLAKLAK)_2$ enhances the binding of eosin Y to bacteria. Moreover, the amount of eosin-$(KLAKLAK)_2$ bound to *E. coli* at 1 μM is equivalent to that obtained for eosin Y at 10 μM (Figure 2b). It is interesting to note that, at these respective concentrations, eosin-$(KLAKLAK)_2$ photoinactivates 99.9% of bacteria, while eosin Y has no photoinduced activity (see Figure 1). These results therefore suggest that $(KLAKLAK)_2$ enhances the photodynamic activity of the photosensitizer.

Eosin-$(KLAKLAK)_2$ Photoinactivates Bacteria without Causing Significant Photohemolysis. In order to test the specificity of the compounds between bacterial and mammalian cells, the photohemolytic activities of eosin-$(KLAKLAK)_2$ and eosin Y were assessed. For these assays, RBC suspensions containing 2.5 million cells per milliliter were used (this corresponds to a 2000-fold dilution of the concentration of RBCs present in human blood). These conditions were used because they correspond to lipid to eosin-$(KLAKLAK)_2$ ratios similar to that expected in the bacterial inactivation assays (see Discussion for details). The photosensitizers Chlorin e6 (Ce6) and Methylene Blue (MB) were also used for comparison. Ce6 (Φ_Δ ~0.65)[30] photolyses RBCs readily, and this compound was therefore used as a positive control.[31] MB (Φ_Δ ~0.52),[30] on the other hand, is not significantly photohemolytic and it has been successfully used for blood decontamination.[32] MB was therefore used to assess the stringency of our photohemolysis assay. Ce6 showed approximately 70% and 100% photo-

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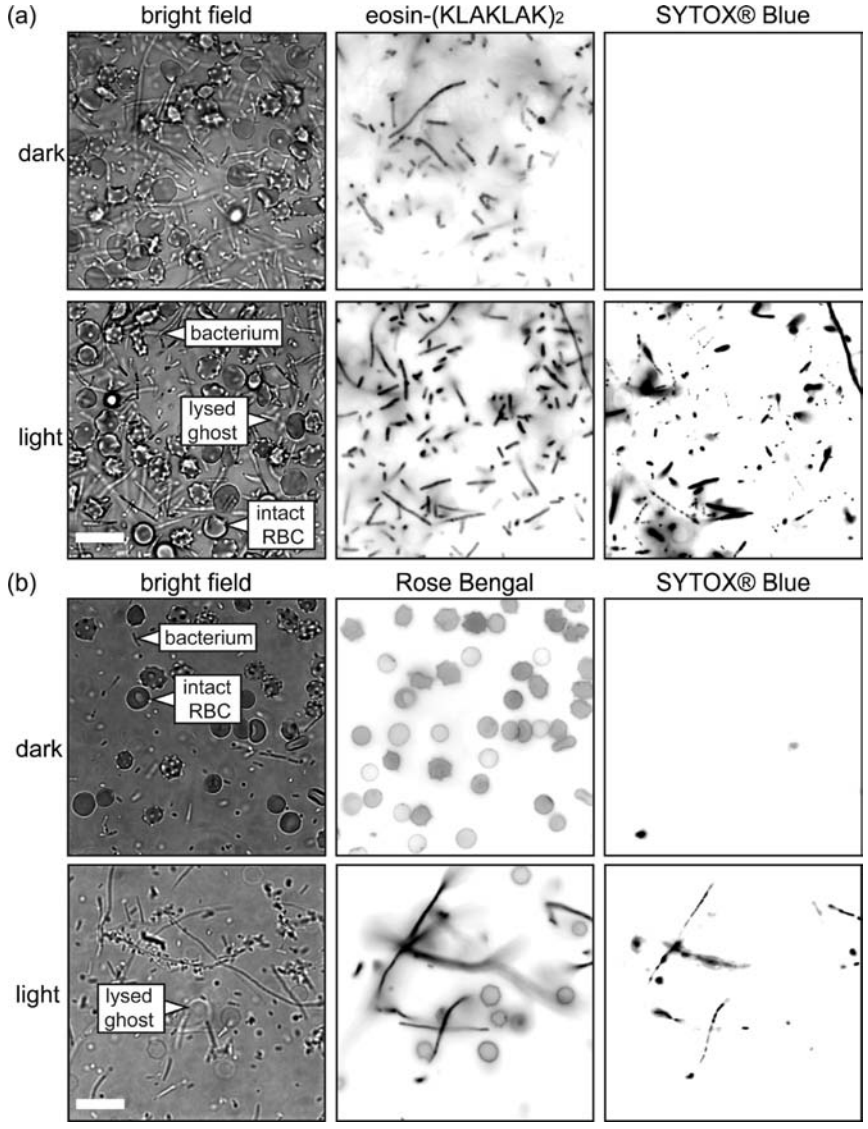

Figure 4. Eosin-$(KLAKLAK)_2$ binds more to bacteria than RBCs. Bright-field and fluorescence imaging of RBCs (0.05% by volume) mixed with *E. coli* (10^8 CFU/mL) and (a) eosin-$(KLAKLAK)_2$ (1 μM) or (b) Rose Bengal (1 μM). Images were acquired after 30 min incubation in the absence or presence of light. SYTOX Blue was added to the samples afterward to detect dead bacteria. Intact RBCs in the bright field images have a dark contrast while lysed ghosts are transparent and only visible as rings. Scale bar is 10 μm.

hemolysis at 1 μM and 10 μM, respectively, while photohemolysis by MB was 20% and 45% at these concentrations. Eosin-$(KLAKLAK)_2$ showed less photohemolysis than MB, with less than 10% photohemolysis at 1 μM or lower, and 40% at 10 μM (Figure 3b). Eosin-$(KLAKLAK)_2$ also showed the lowest hemolysis in the dark with only 5% hemolysis at 10 μM. Interestingly, eosin Y gave similar results to eosin-$(KLAKLAK)_2$. These data therefore suggest that conjugation of $(KLAKLAK)_2$ to eosin Y does not significantly increase the photolytic activity of the photosensitizer toward erythrocytes.

In order to address the issue of specificity more directly, a suspension of *E. coli* (10^8 CFU/mL) was added to the RBCs before mixing with PS or peptide conjugate for light irradiation. The photohemolytic activity and bacterial photoinactivation were measured after irradiation. As shown in Figure 3, the photohemolytic activity of eosin-$(KLAKLAK)_2$ was reduced in the presence of *E. coli*. For instance, the photohemolysis obtained at 10 μM eosin-$(KLAKLAK)_2$ was reduced from approximately 40% to 10% in the presence of bacteria. On the other hand, more than 99% bacterial photoinactivation was achieved. These results therefore suggest that eosin-$(KLAKLAK)_2$ destroys bacteria preferentially over red blood cells. In order to confirm that the reduction in photohemolysis observed was not a general phenomenon simply caused by the addition of *E. coli*, Ce6 was here again included as a control. It has been shown that *E. coli* does not take up Ce6 at the concentrations used in our assays.[15] We therefore expected that the presence of *E. coli* should not affect the photohemolytic activity of Ce6. Indeed, no significant change in the photohemolysis activity of Ce6 was observed in the presence of *E. coli* (Figure 3b).

In order to test whether the reduced hemolysis by eosin-$(KLAKLAK)_2$ in the presence of *E. coli* and *S. aureus* is caused by the association of the peptide with the bacteria, samples of eosin-$(KLAKLAK)_2$ and cells were examined by microscopy before and after irradiation (Figure 4 and Figure S2). The fluorescent photosensitizer Rose Bengal (RB) was also observed with cells in order to compare eosin-$(KLAKLAK)_2$ with a lipophilic PS known to be nonspecific in its binding.[33] As

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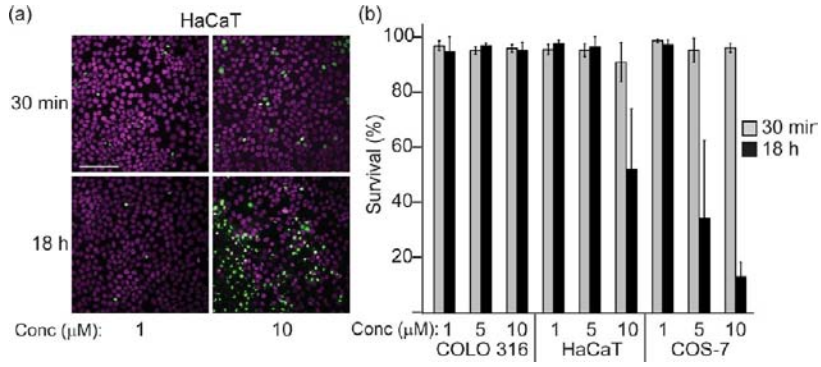

Figure 5. Eosin-$(KLAKLAK)_2$ is not significantly phototoxic toward COLO 316, HaCaT, or COS-7 cell lines. (a) Fluorescence microscopy imaging (20×) of HaCaT cells incubated with eosin-$(KLAKLAK)_2$ (1 or 10 μM) for 30 min with light exposure. Cells were coincubated with Hoescht (pseudo colored purple here) and SYTOX Green dyes immediately following (30 min) or 18 h after illumination. Percent survival of cells was determined by counting the cells with compromised plasma membranes (stained by SYTOX Green) compared to the total (all cells are stained by Hoescht). Scale bar is 100 μm. (b) Survival of COLO 316, HaCaT, and COS-7 cells exposed to eosin-$(KLAKLAK)_2$ (1, 5, and 10 μM) for 30 min with light irradiation. Cell viability was assessed 30 min and 18 h after exposure.

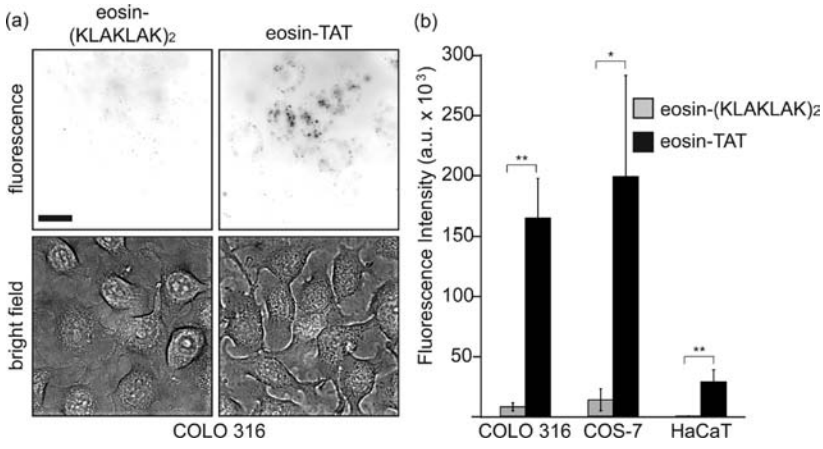

Figure 6. Eosin-$(KLAKLAK)_2$ is taken up by cells significantly less than eosin-TAT. (a) Bright-field and fluorescence microscopy imaging of COLO 316 cells (100×) incubated with eosin-$(KLAKLAK)_2$ (1 μM) or eosin-TAT (1 μM) for 30 min. Scale bar is 10 μm. (b) Total fluorescence intensity of cells incubated with eosin-$(KLAKLAK)_2$ or eosin-TAT (1 μM) for 30 min (two-tailed t test, * = $p < 0.05$, ** = $p < 0.01$).

shown in Figure 4a, the fluorescence signal of eosin-$(KLAKLAK)_2$ is associated with bacteria (also visible in bright field image) while RBCs present in the sample are not stained by eosin-$(KLAKLAK)_2$. In comparison, RB stains the plasma membrane of RBCs. This therefore indicates that eosin-$(KLAKLAK)_2$ binds to bacteria to a much greater extent than to RBCs. In addition, most of the RBCs present after irradiation have a dark contrast consistent with these cells being intact and only a few lysed ghosts can be observed (ghost are transparent but their plasma membrane remains visible). These results therefore confirm that the extent of photohemolysis of RBC in the presence of *E. coli* is limited. Moreover, bacteria were stained by SYTOX Blue after irradiation but not before. SYTOX Blue is a nuclear stain that does not penetrate live cells but can stain cells with a compromised membrane. These data therefore indicate the bacteria present were photoinactivated. A colony-forming assay after serial dilutions of the sample confirmed that more than 99% of the bacteria were killed. Overall, these data suggest that, while eosin-$(KLAKLAK)_2$ is capable of lysing the membrane of RBCs at certain peptide to cell ratios, the photolytic activity of this compound is more pronounced toward bacteria. In particular, this appears to be consistent with a preferential association of the peptide with bacterial cells over erythrocytes.

Eosin-$(KLAKLAK)_2$ is Less Phototoxic toward Mammalian Cells than Bacteria. The phototoxicity of eosin-$(KLAKLAK)_2$ was tested with HaCaT (human keratinocytes), COS-7 (monkey fibroblasts), and COLO 316 (human ovarian carcinoma). Cells were incubated with eosin Y, $(KLAKLAK)_2$, and eosin-$(KLAKLAK)_2$ and irradiated under the same conditions used for bacterial photoinactivation. The viability of cells was assessed before and after irradiation using SYTOX green exclusion assays (Figure 5a). In the dark, the compounds showed no toxicity toward the cells in the range of concentration tested (1 to 10 μM) (Figure S3). In the light, some toxicity was observed only at higher concentrations (5 or 10 μM) for COS-7 and HaCaT, while COLO 316 showed no toxicity even at 10 μM eosin-$(KLAKLAK)_2$ (Figure 5b).

The propensity of eosin-$(KLAKLAK)_2$ to be internalized by mammalian cells was evaluated by fluorescence microscopy (Figure 6a). The peptide TAT labeled with eosin-Y (eosin-TAT) was used as a positive control. TAT is an arginine-rich peptide known to be endocytosed efficiently by mammalian cells.[23] After incubation with cells for 30 min, eosin-TAT distributed in a punctate manner inside cells, indicative of the accumulation of the compound inside endocytic organelles. In contrast, a fluorescence signal at least 10-fold less than this intensity was detected for eosin-$(KLAKLAK)_2$ under identical

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conditions (Figure 6b). Together, these data suggest that eosin-$(KLAKLAK)_2$ associates minimally with mammalian cells and that the photodynamic activity of eosin-$(KLAKLAK)_2$ toward mammalian cells is significantly less than that obtained with bacteria.

DISCUSSION

Bacterial photoinactivation assays show that a moderate dose of light can reduce the lethal concentration of eosin-$(KLAKLAK)_2$ toward bacteria by more than 10-fold. The peptide $(KLAKLAK)_2$ greatly enhances the photodynamic activity of eosin Y, as this PS is not very phototoxic on its own. Moreover, eosin-$(KLAKLAK)_2$ caused the photoinactivation of Gram-positive and Gram-negative strains to a similar extent. Our results therefore suggest that, unlike what has been observed for other cationic PS, the outer membrane of the Gram-negative bacteria might not represent a significant barrier to the penetration of the peptide conjugate.[9−11] This is consistent with the reported MICs of $(KLAKLAK)_2$ being similar for Gram-negative and Gram-positive bacteria.[28] The binding of eosin-$(KLAKLAK)_2$ with *E. coli* and *S. aureus* was greater than that of eosin Y alone, indicating that the peptide promotes the association of the PS to bacteria. The peptide therefore appears to enable the photodynamic activity of the photosensitizer by acting as a targeting agent.

Based on the models proposed in the literature for $(KLAKLAK)_2$ and related antimicrobial peptides, one can envision that eosin-$(KLAKLAK)_2$ binds to bacterial lipid bilayers. At the low concentration at which photoinactivation is achieved, however, the peptide itself is not able to cause the formation of lytic pores, as no antimicrobial activity is detected in the dark. Yet, binding experiments reveal that, with equal amount of eosin-$(KLAKLAK)_2$ and eosin Y bound to bacteria, eosin-$(KLAKLAK)_2$ is able to photoinactivate bacteria but eosin Y cannot. A possible explanation for this effect might be that $(KLAKLAK)_2$ contributes to destabilizing the bacterial membrane. $(KLAKLAK)_2$ could, for example, promote lysis or enhance the damaging effect of ROS generated by the photosensitizer. We envisioned, for instance, that the A-AMP-disrupted bacterial membrane might become more susceptible to the ROS produced by the PS agent, while ROS-induced membrane damage might also facilitate membrane disruption by the A-AMP. Another possible explanation involves the idea that $(KLAKLAK)_2$ might position eosin Y in a cellular location that eosin Y alone is not otherwise able to access and that the generation of ROS at this particular location kills cells more effectively. Further studies are required to validate these models, and elucidating which of these principles can be exploited should be useful for the design of optimized PDT agents.

An important aspect of antimicrobial PDT is the specificity of the PS toward bacterial cells. Ideally, the photodynamic drug should kill bacteria without damaging host tissues. In order to compare the photodynamic activity of eosin-$(KLAKLAK)_2$ toward bacteria to that obtained with RBCs, photohemolysis assays were performed with human erythrocytes. Under the assumption that a lipid bilayer is a primary target of the photodynamic activity of eosin-$(KLAKLAK)_2$, conditions were chosen to obtain peptide to lipid ratios similar between bacterial photoinactivation and photohemolysis assays. RBCs were, for instance, diluted 2000-fold in comparison to human blood. One can therefore expect that hemolysis would be more pronounced at the low RBC concentration used than at the high concentration of human blood and that the assays used are relatively stringent. Approximately 10% hemolysis was obtained at 1 μM eosin-$(KLAKLAK)_2$, the concentration required to achieve approximately 99.99% inactivation of *E. coli* or *S. aureus*. These results therefore suggest that bacterial photoinactivation can be achieved under conditions where RBCs can be spared. Yet, a concern is raised by the observation that photohemolysis increases to 40% as the concentration of eosin-$(KLAKLAK)_2$ is increased to 10 μM. The concentration window at which bacterial killing can be achieved without adverse effects to RBCs might therefore be relatively narrow. Interestingly though, mixing experiments between bacteria and erythrocytes show that eosin-$(KLAKLAK)_2$ associates with bacteria to a greater extent than red blood cells. Eosin-$(KLAKLAK)_2$ binding to bacteria presumably reduces the concentration of compound present in solution or on the surface of RBCs. Consequently, photohemolysis with eosin-$(KLAKLAK)_2$ at 10 μM was reduced to 10% when bacteria were present. More than 99% bacterial killing could be achieved under these conditions, further confirming that eosin-$(KLAKLAK)_2$ can inactivate bacteria before significant damage is observed for a human cell.

To further address the issue of specificity, the photodynamic activity of eosin-$(KLAKLAK)_2$ was tested against epithelial cells, keratinocytes, and fibroblasts. As with RBCs, one might expect eosin-$(KLAKLAK)_2$ to possibly interact with the plasma membrane of these cells. In addition, relatively large and amphiphilic molecules like eosin-$(KLAKLAK)_2$ can be endocytosed by cells. As a matter of fact, lysine and arginine-rich peptides, previously used to improve the targeting of PS such as Chlorin e6 to bacteria, are well-known to be effectively endocytosed by human cells.[20−22] Unfortunately, PS that accumulate in the endocytic pathway can photolyze endocytic organelles such as lysosomes and this might in turn cause cell death.[23,24,34] Consistent with this idea, cells that have endocytosed PS conjugated to lysine or arginine-rich polymers can be killed readily upon irradiation.[23,24,35] Moreover, the photolysis of endocytic organelles raises a concern related to the penetration of antimicrobial peptides inside human cells. In particular, it has been shown that antimicrobial peptides that escape the endocytic pathway might gain access to mitochondria, disrupt the membrane of these organelles, and induce apoptosis.[27] For instance, $(KLAKLAK)_2$ causes cellular apoptosis when combined with agents capable of delivering this peptide in the cytoplasm of human cells, although $(KLAKLAK)_2$ is not toxic without these delivery agents.[36,37] We were therefore concerned that eosin-$(KLAKLAK)_2$ might be taken up into mammalian cells, lyse endocytic organelles upon irradiation, and possibly cause cell death. To test whether eosin-$(KLAKLAK)_2$ would be endocytosed by cells and in order to assess the phototoxicity of the compound, eosin-$(KLAKLAK)_2$ was incubated with different cell lines and uptake was examined by fluorescence microscopy. While internalization of the positive control eosin-TAT could be readily observed, eosin-$(KLAKLAK)_2$ was not significantly internalized by cells. Also, the viability of cells was not significantly affected by irradiation for 30 min with incubation at 1 μM eosin-$(KLAKLAK)_2$ (conditions at which more than 99.9% bacterial photoinactivation is achieved). These results therefore suggest that bacterial cells are more susceptible to the photodynamic activity of eosin-$(KLAKLAK)_2$ than mammalian cells. At higher concentrations (5 or 10 μM), the phototoxicity toward mammalian cells was increased in a cell-dependent manner. In order to design optimal compounds, it will be interesting to

determine in future studies what causes the differences observed in phototoxicity. Nonetheless, these results suggest that the photoinactivation of bacteria without human tissue damage might be achievable. Of course, *in vivo* experiments will be necessary to validate this idea.

Overall, our results establish that the conjugation of eosin Y to the antimicrobial peptide $(KLAKLAK)_2$ increases the photodynamic activity of eosin Y toward *E. coli* and *S. aureus* considerably. How the effectiveness of eosin-$(KLAKLAK)_2$ compares to that of other PDT agents (e.g., cationic porphyrins or 5-aminolevulinic acid) remains to be determined. Nonetheless, it is interesting to note that the activity and specificity of a PS-AMP conjugate could potentially be increased. First, one might speculate that peptides that have higher antimicrobial activity in the dark would further reduce the concentration of PS-peptide conjugate required to achieve bacterial photoinactivation. In addition, since eosin Y is a PS with poor activity on its own, it is also possible that $(KLAKLAK)_2$ might increase the effectiveness of PS with intrinsic PDT activity. Whether such improvements are possible will be the subject of further studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Eosin-$(KLAKLAK)_2$ inactivation of additional strains, inactivation of *S. aureus* in the presence of RBCs, and dark toxicity of eosin-$(KLAKLAK)_2$ toward mammalian cell lines. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PDT, photodynamic therapy; PS, photosensitizer; pL, poly(lysine); PEI, polyethyleneimine; CPP, cell-penetrating peptide; A-AMP, amphiphilic antimicrobial peptide; MIC, minimum inhibitory concentration

■ REFERENCES

(1) Morens, D. M., Folkers, G. K., and Fauci, A. S. (2004) The challenge of emerging and re-emerging infectious diseases. *Nature 430*, 242−9.

(2) Poirel, L., Menuteau, O., Agoli, N., Cattoen, C., and Nordmann, P. (2003) Outbreak of Extended-Spectrum β-Lactamase VEB-1-Producing Isolates of Acinetobacter baumannii in a French Hospital. *J. Clin. Microbiol. 41*, 3542−3547.

(3) Dai, T., Huang, Y. Y., and Hamblin, M. R. (2009) Photodynamic therapy for localized infections–state of the art. *Photodiagn. Photodyn. Ther. 6*, 170−88.

(4) Kochevar, I. E., and Redmond, R. W. (2000) Photosensitized production of singlet oxygen. *Methods Enzymol. 319*, 20−8.

(5) Maisch, T., Hackbarth, S., Regensburger, J., Felgentrager, A., Baumler, W., Landthaler, M., and Roder, B. (2011) Photodynamic inactivation of multi-resistant bacteria (PIB) - a new approach to treat superficial infections in the 21st century. *J. Dtsch. Dermatol. Ges. 9*, 360−6.

(6) Vera, D. M., Haynes, M. H., Ball, A. R., Dai, T., Astrakas, C., Kelso, M. J., Hamblin, M. R., and Tegos, G. P. (2012) Strategies to potentiate antimicrobial photoinactivation by overcoming resistant phenotypes(dagger). *J. Photochem. Photobiol. B 13*, 39−50.

(7) Valenzeno, D. P., and Pooler, J. P. (1982) Cell membrane photomodification: relative effectiveness of halogenated fluoresceins for photohemolysis. *Photochem. Photobiol. 35*, 343−50.

(8) Pooler, J. P. (1989) Photooxidation of cell membranes using eosin derivatives that locate in lipid or protein to study the role of diffusible intermediates. *Photochem. Photobiol. 50*, 55−68.

(9) Segalla, A., Borsarelli, C. D., Braslavsky, S. E., Spikes, J. D., Roncucci, G., Dei, D., Chiti, G., Jori, G., and Reddi, E. (2002) Photophysical, photochemical and antibacterial photosensitizing properties of a novel octacationic Zn(II)-phthalocyanine. *Photochem. Photobiol. Sci. 1*, 641−8.

(10) Usacheva, M. N., Teichert, M. C., and Biel, M. A. (2001) Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms. *Lasers Surg. Med. 29*, 165−73.

(11) Maisch, T., Bosl, C., Szeimies, R. M., Lehn, N., and Abels, C. (2005) Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells. *Antimicrob. Agents Chemother. 49*, 1542−52.

(12) Polo, L., Segalla, A., Bertoloni, G., Jori, G., Schaffner, K., and Reddi, E. (2000) Polylysine-porphycene conjugates as efficient photosensitizers for the inactivation of microbial pathogens. *J. Photochem. Photobiol. B 59*, 152−8.

(13) Soukos, N. S., Ximenez-Fyvie, L. A., Hamblin, M. R., Socransky, S. S., and Hasan, T. (1998) Targeted antimicrobial photochemotherapy. *Antimicrob. Agents Chemother. 42*, 2595−2601.

(14) Tegos, G. P., Anbe, M., Yang, C., Demidova, T. N., Satti, M., Mroz, P., Janjua, S., Gad, F., and Hamblin, M. R. (2006) Protease-stable polycationic photosensitizer conjugates between polyethyleneimine and chlorin(e6) for broad-spectrum antimicrobial photoinactivation. *Antimicrob. Agents Chemother. 50*, 1402−1410.

(15) Hamblin, M. R., O'Donnell, D. A., Murthy, N., Rajagopalan, K., Michaud, N., Sherwood, M. E., and Hasan, T. (2002) Polycationic photosensitizer conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria. *J. Antimicrob. Chemother. 49*, 941−951.

(16) Vaara, M., and Vaara, T. (1983) Polycations as outer membrane-disorganizing agents. *Antimicrob. Agents Chemother. 24*, 114−22.

(17) Sara, M., and Sleytr, U. B. (1987) Molecular sieving through S layers of Bacillus stearothermophilus strains. *J. Bacteriol. 169*, 4092−8.

(18) Liu, F., Soh Yan, Ni, A., Lim, Y., Mohanram, H., Bhattacharjya, S., and Xing, B. (2012) Lipopolysaccharide neutralizing peptide−porphyrin conjugates for effective photoinactivation and intracellular imaging of Gram-negative bacteria strains. *Bioconjugate Chem. 23*, 1639−1647.

(19) Bourre, L., Giuntini, F., Eggleston, I. M., Mosse, C. A., MacRobert, A. J., and Wilson, M. (2010) Effective photoinactivation of Gram-positive and Gram-negative bacterial strains using an HIV-1 Tat peptide-porphyrin conjugate. *Photochem. Photobiol. Sci. 9*, 1613−1620.

(20) Ryser, H. J., and Hancock, R. (1965) Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture. *Science 150*, 501−3.

(21) Shen, W. C., and Ryser, H. J. (1978) Conjugation of poly-L-lysine to albumin and horseradish peroxidase: a novel method of enhancing the cellular uptake of proteins. *Proc. Natl. Acad. Sci. U. S. A. 75*, 1872−6.

(22) Nakase, I., Niwa, M., Takeuchi, T., Sonomura, K., Kawabata, N., Koike, Y., Takehashi, M., Tanaka, S., Ueda, K., Simpson, J. C., Jones, A.

T., Sugiura, Y., and Futaki, S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther. 10*, 1011−22.
(23) Srinivasan, D., Muthukrishnan, N., Johnson, G. A., Erazo-Oliveras, A., Lim, J., Simanek, E. E., and Pellois, J.-P. (2011) Conjugation to the cell-penetrating peptide TAT potentiates the photodynamic effect of carboxytetramethylrhodamine. *PLoS ONE 6*, e17732.
(24) Soukos, N. S., Hamblin, M. R., and Hasan, T. (1997) The effect of charge on cellular uptake and phototoxicity of polylysine chlorin(e6) conjugates. *Photochem. Photobiol. 65*, 723−9.
(25) Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol. 3*, 238−50.
(26) Wimley, W. C., and Hristova, K. (2011) Antimicrobial peptides: successes, challenges and unanswered questions. *J. Membr. Biol. 239*, 27−34.
(27) Ellerby, H. M., Arap, W., Ellerby, L. M., Kain, R., Andrusiak, R., Rio, G. D., Krajewski, S., Lombardo, C. R., Rao, R., Ruoslahti, E., Bredesen, D. E., and Pasqualini, R. (1999) Anti-cancer activity of targeted pro-apoptotic peptides. *Nat. Med. 5*, 1032−8.
(28) Javadpour, M. M., Juban, M. M., Lo, W.-C. J., Bishop, S. M., Alberty, J. B., Cowell, S. M., Becker, C. L., and McLaughlin, M. L. (1996) De novo antimicrobial peptides with low mammalian cell toxicity. *J. Med. Chem. 39*, 3107−3113.
(29) Dathe, M., Meyer, J., Beyermann, M., Maul, B., Hoischen, C., and Bienert, M. (2002) General aspects of peptide selectivity towards lipid bilayers and cell membranes studied by variation of the structural parameters of amphipathic helical model peptides. *Biochim. Biophys. Acta 1558*, 171−186.
(30) Redmond, R. W., and Gamlin, J. N. (1999) A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem. Photobiol. 70*, 391−475.
(31) Frolov, A. A., and Gurinovich, G. P. (1992) The laws of delayed photohaemolysis sensitized by chlorin e6. *J. Photochem. Photobiol. B 13*, 39−50.
(32) Wainwright, M. (2000) Methylene blue derivatives — suitable photoantimicrobials for blood product disinfection? *Int. J. Antimicrob. Agents 16*, 381−394.
(33) Valenzeno, D. P., and Pooler, J. P. (1982) Cell membrane photomodification: Relative effectiveness of halogenated fluoresceins for photohemolysis. *Photochem. Photobiol. 35*, 343−350.
(34) Berg, K., and Moan, J. (1994) Lysosomes as photochemical targets. *Int. J. Cancer 59*, 814−822.
(35) Choi, Y., McCarthy, J. R., Weissleder, R., and Tung, C. H. (2006) Conjugation of a photosensitizer to an oligoarginine-based cell-penetrating peptide increases the efficacy of photodynamic therapy. *ChemMedChem 1*, 458−63.
(36) Takeuchi, T., Kosuge, M., Tadokoro, A., Sugiura, Y., Nishi, M., Kawata, M., Sakai, N., Matile, S., and Futaki, S. (2006) Direct and rapid cytosolic delivery using cell-penetrating peptides mediated by pyrenebutyrate. *ACS Chem. Biol. 1*, 299−303.
(37) Angeles-Boza, A. M., Erazo-Oliveras, A., Lee, Y. J., and Pellois, J. P. (2010) Generation of endosomolytic reagents by branching of cell-penetrating peptides: tools for the delivery of bioactive compounds to live cells in cis or trans. *Bioconjugate Chem. 21*, 2164−7.