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Photosensitizer–Antibiotic Conjugates: A Novel Class of Antibacterial Molecules

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ABSTRACT

Bacterial resistance to a variety of antibiotics has led to intensive research into the effect of photosensitizers as a cytotoxic agent against bacterial cells. In this study, we synthesized the following conjugates with or without a linker: rose bengal-penicillanic acid (RBPA), rose bengal-linker-penicillanic acid (RBLPA) and rose bengal-linker-kanamycin (RBLKAN). The antibacterial activity of these conjugates was examined on *Staphylococcus aureus* and *Escherichia coli*. Exposure of the cultures to 100 J cm^{-2} showed that the minimum inhibitory concentration (MIC) of RBPA, RBLPA and RBLKAN on *S. aureus* was 0.195, 0.156 and 0.004 μM, respectively. The MIC of RBPA, RBLPA and RBLKAN on *E. coli* was 1.56, 2.5 and 0.156 μM, respectively. In dark control experiments, the MIC of these conjugates was not detected until a concentration that was 16-fold that of the MIC found in the light experiments. RBPA and RBLPA as well as RBLKAN are bactericidal for both bacterial cells. Total eradication of *S. aureus* and *E. coli* was observed with RBLKAN (0.078 and 20 μM 16 J cm^{-2}, respectively). Under these conditions, scanning electron microscopic analysis showed significant damage to these bacteria. However, the photosensitizer and antibiotics individually were not effective.

INTRODUCTION

Photodynamic antimicrobial chemotherapy (PACT) combines nontoxic dyes called photosensitizers (PS) and visible light. PACT is an up and coming alternative treatment for microbial infections, especially antibiotic-resistant microbial strains (1,2). The therapy involves delivering light of appropriate wavelength to the PS that leads to a triplet excited state in the PS. The photosensitization can be divided into two groups according to its mechanism of action. The mechanism involved in Type I photosensitization, typically in a hypoxic environment, involves the electron transfer from the PS triplet state to a substrate that subsequently gives rise to radical anions (3). The mechanism of Type II photosensitization is based on the transfer of energy from the PS triplet state molecule to an oxygen molecule that leads to the formation of singlet oxygen ($^1O_2$). This mechanism shows an oxygen-dependent pattern (3,4). During PACT Type II reactions are the dominant mechanism. The cytotoxic species evolved from either Type I or Type II mechanisms ($^1O_2$ or the free radicals) can oxidize biological molecules, such as proteins, nucleic acids and lipids that consequently lead to cell death (2,5). It was shown that PS upon illumination destroyed bacteria, viruses, fungi, yeasts and parasites (5–11). PS such as the cationic tetra (4-*N*-methylpyridyl) porphine can, when illuminated, totally eradicate multiple antibiotic-resistant bacteria such as *Acinetobacter baumannii* and *Escherichia coli* (12). Gram-positive bacteria appeared to be more susceptible to PACT than gram-negative bacteria (13–17). Permeabilization of the bacterial membrane using polymyxin nonapeptide or Tris/EDTA allowed the entry of noncationic PS within the outer membrane, which consequently improved the efficacy of killing gram-positive and gram-negative bacterial cells (11,18–21). There are reports in which it is clear that the PS do not have to penetrate the bacterium to be effective or even come in contact with the cell. Singlet oxygen generated in sufficient quantities near the bacterial outer membrane diffused into the cell and destroyed it (22,23).

Antibiotics can be categorized according to their biochemical structure and by their mechanism of action. These mechanisms involve the inhibition of processes which are essential for bacterial growth and division. The major mechanisms are inhibition of cell wall, protein and nucleic acid synthesis (24). The increase in pathogenic bacterial cell resistance to antibiotics is a well-known phenomenon, with many reports describing bacterial cells that have developed the ability to survive despite antibiotic treatment (25,26). Resistance can be either an intrinsic property of the bacteria itself which is possessed by all members of the genus or it can be developed as the result of a *de novo* mutation. More commonly, it involves the presence of extrachromosomal DNA acquired from other bacteria (27–29). The emergence of resistant pathogenic bacterial cells to antibiotics has led to intensive research efforts to find alternative antimicrobial therapeutics. It has been noted that diseases caused by methicillin-resistant *Staphylococcus aureus* are ideally suited to treatment by PACT as well as periodontal microbial biofilms (30).

The aim of the present research was to investigate the antibacterial effect of the conjugates rose bengal-6-aminopenicillanic acid (RBPA), rose bengal-linker-6-aminopenicillanic acid (RBLPA) and rose bengal-linker-kanamycin (RBLKAN) on *S. aureus* as well as *E. coli*. It was found that the novel

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conjugates RBPA, RBLPA as well as RBLKAN are effective against the pathogenic bacteria *S. aureus* and *E. coli*. This antibacterial effect of the conjugate appeared to happen after a short illumination period with very low conjugate concentrations. The treatment with these conjugates or similar conjugates composed of other kinds of PS and antibiotics against bacterial cells may overcome the increasing problem of bacterial cell resistance to antibiotic treatment. In addition, the fact that the eradication of *S. aureus* and *E. coli* bacterial cells was achieved at low conjugate concentrations may reduce the occurrence of unwanted side effects in the patient.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* and *S. aureus* strains were used as representatives of gram-negative and gram-positive bacteria, respectively. These strains were obtained from Meir Hospital, Kfar Saba, Israel. The strains were grown overnight in Nutrient Broth (NB) (Difco, Detroit, MI) at 37°C with aeration. Cultures were transferred into fresh NB medium to obtain an O.D. of 0.1–0.13 at 660 nm and allowed to grow to the beginning of the exponential phase, with an O.D. of about 0.3 at 660 nm. Cultures were diluted 1:100 for minimal inhibitory concentration (MIC) determinations.

Photosensitization procedure. Photosensitization was carried out by illumination of the bacterial cultures with three 18 W white luminescent lamps with an emissions range of 400–700 nm (with total UV cutoff) placed up to 30 cm above a 96-well microtiter plate. An energy density of 1.4–1.7 mW cm^{-2} was measured by an LX-102 Light meter (Lutron, Taiwan) depending on the experimental setup. During illumination, the bacterial suspensions were agitated at 180 rpm at 37°C.

Determination of antibacterial activity. *Minimal inhibitory concentration*: The MIC of the antibacterial molecules was performed in microtiter plates. For *S. aureus* the initial concentration of RB, PA, KAN, RBPA, RBLPB, RBLKAN, RB + PA and RB + KAN was 40, 320, 20, 3.12, 1.25, 0.312 0.078 + 0.078 and 0.156 + 0.156 μM, respectively. For *E. coli* the initial concentration of RB, PA, KAN, RBPA, RBLPB, RBLKAN, RB + PA and RB + KAN was 40, 320, 25, 12.5, 12.5, 2.5, 1.25 + 1.25 and 0.625 + 0.625 μM, respectively. Each of the antibacterial molecules was suspended in NB (150 μL) and serial diluted by two-fold, followed by the addition of 50 μL *E. coli* or *S. aureus* bacterial cells (final concentration of 10^6 cells mL^{-1}) at their logarithmic growth phase. The cultures were illuminated (100 J cm^{-2}) using fluorescent lamps and agitated at 180 rpm for 20 h at 37°C. The same procedure of MIC analysis was carried out for dark control except for illumination. The initial concentrations of PA and KAN were the same as those used in the light experiments, while the initial concentrations of RB and the conjugates were at least 16-fold that of the MIC found in the light experiments. The initial concentrations of the nonconjugated combinations were the same as the initial concentrations of the antibiotics alone in the light experiments. The dark control setup was covered and incubated in the dark. The absorbance was determined by an ELISA reader at 660 nm. The minimal concentration of the antibacterial solution resulting in an absorbance of approximately O.D. 0.05 at 660 nm was considered to be the MIC.

Minimal bactericidal concentration: The minimal bactericidal concentration (MBC) was determined from the broth dilution MIC analysis. A sample of 100 μL of the MIC and several higher concentrations were serially diluted 10-fold and seeded on NB agar plates. The plates were incubated at 37°C for 20 h and the colony-forming units (CFU) were counted. The MBC is the lowest concentration of the antibacterial solution required to kill the bacterial cells. The MIC and MBC of each of the antibacterial agents was performed three times.

Bacterial survival assay: The survival of bacterial cells (approximately 0.3 O.D., 10^{-8} cells mL^{-1}, in log phase), after treatment with the antibacterial molecules and illumination with an energy density of 2–16 J cm^{-2} for 20 min at 37°C was monitored by counting the residual cells. This was performed by counting the number of CFU after pour plating of appropriate dilutions onto NB agar plates and calculating the number of viable cells per mL. Bacterial cultures grown under illumination with an energy density of 2–16 J cm^{-2} for 20 min at 37°C but not treated with the antibacterial molecules served as light controls. Bacterial cultures treated with the antibacterial molecules but not exposed to illumination and incubated in the dark served as dark controls.

Scanning electron microscopy (SEM). Cultures were fixed with 2% glutaraldehyde for 2 h, followed by 1% osmium tetroxide. The material was then dehydrated by incubations in increasing concentrations of ethanol solutions. The specimens were gold-coated using an LKB device (Stockholm, Sweden). Scanning was performed with a JEOL 840 scanning electron microscope at an accelerating voltage of 20 kV.

Maldi TOF mass spectrometric analysis of the PS-antibiotic conjugates. Intact molecular mass measurements were performed on a Bruker Reflex III matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer (Bruker, Bremen, Germany) equipped with delayed ion extraction, a reflector and a 337 nm nitrogen laser. Each mass spectrum was generated from accumulated data of 200 laser shots.

Statistics. Each experiment was performed at least in triplicate. All primary data are presented as means with standard deviations of the mean. The difference between two means was compared using Student's *t*-test. $P < 0.05$ was considered significant.

Synthesis of the conjugates. *General*: Analytical HPLC was performed on a 250 × 4.2 mm Lichroprep RP-18 column from Merck, with a 1 mL min^{-1} flow and detection at 214 nm. The eluents were triply distilled water and HPLC-grade CH3CN (containing 0.1% TFA) or MeOH. The purities of the compounds are determined by comparative "area under a peak." ^1H & ^{13}C NMR spectra were recorded at 300 MHz. All chemical shifts are reported with respect to TMS. Chromatography was carried out by standard flash chromatography and TLC on silica-gel.

The syntheses of the conjugates listed in this paper were performed in basic conditions in the presence of Na_2CO_3 that were not acidified during the work up. Therefore, the compounds show extensive incorporation of Na ions that replace the acidic phenol or carboxyl protons (31,32).

Rose bengal-6-aminopenicillanic acid (RBPA) ***(1a)***. RB (0.68 g, 0.64 mmol) was refluxed in 50 mL of 1:1 mixture of $CHCl_3$ and redistilled $SOCl_2$ for 4 h under N_2 atmosphere. The reaction mixture was evaporated under vacuum, the residue was dissolved in 5 mL of dioxane and the resultant acyl chloride solution was added dropwise to 60 mL chilled solution of 6-aminopenicillanic acid (0.22 g, 1 mmol) in 1 *N* Na_2CO_3 and dioxane (2:1). After stirring for 1 h at room temperature (TLC monitoring), the solvent was evaporated and the residue was transferred into a small amount of water. The precipitate obtained was filtered off and purified on silica gel (7% MeOH/ethyl acetate), yielding pure RBPA as a reddish oil in overall 47% (0.41 g, 93% purity by HPLC); λ_{max} (MeOH): 558 (22340); HRMS (DI, *m/z*) calcd for $C_{28}H_{14}Cl_4I_4N_2O_7S$ 1175.54 found 1199.48 (100%), ($M^+ + Na$);^1H NMR (DMSO-d_6): δ 1.43 (s, 3, CH_3), 1.58 (s, 3, CH_3), 4.62 (s, 1, H-5), 4.93 (d, 1, J = 7.2 Hz, H-4), 5.21 (d, 1, J = 7.2 Hz, H-1), 7.25 (s, 1, C<u>H</u> = C), 7.88 (s, 1, C<u>H</u> = C), 10.92 (bs, 1, CO_2<u>H</u>).

Rose bengal-linker-6-aminopenicillanic acid (RBLPA) ***(1b)***. 4-bromohexanoic acid (0.18 g, 1 mmol) was refluxed in 50 mL acetone with rose bengal (1.02 g, 1 mmol) in the presence of 2 g of K_2CO_3 overnight. After cooling and filtration of the salt, the filtrate was evaporated to give intermediate **2**, after purification (2% MeOH/ethyl acetate) on silica gel, at a reasonable yield (0.82 g, 76%). The resultant carboxylic acid (18) was transformed to the corresponding acyl chloride by refluxing with excess of redistilled thionyl chloride (10 mL) in 20 mL methylene chloride for 3 h under N_2 atmosphere. After evaporation of the solvent, the resultant acyl chloride was taken in 5 mL of dioxane and directly reacted with an excess of 6-aminopenicillanic acid (0.22 g, 1 mmol) dissolved in chilled 20 mL of 1 *N* Na_2CO_3/Dioxane (1:2) solution. After stirring for 5 h (TLC monitoring) the solvent was evaporated and the residue was chromatographed on silica gel (5% MeOH/EtAc) to yield pure rose bengal-linker-penicillanic acid conjugate **1b** (RBLPA) as a reddish oil in 53% (0.51 g, 94% purity by HPLC); λ_{max} (MeOH): 565 (14620); HRMS (DI, *m/z*) calcd for $C_{34}H_{24}Cl_4I_4N_2O_9S$ 1283.61 found 1305.71 ([M–H] + Na), (100%); ^1H NMR (DMSO-d_6): δ 1.20–1.41 (m, 6, -C<u>H</u>$_2$-), 1.49 (s, 3, CH_3), 1.62 (s, 3, CH_3), 2.05 (t, 2, J = 6.2 Hz, C<u>H</u>$_2$-C = O), 4.36 (t, 2, J = 6.3 Hz,

O-C$\underline{H_2}$-C), 4.64 (s, 1, H-5), 4.87 (d, 1, J = 7.2 Hz, H-4), 5.16 (d, 1, J = 7.2 Hz, H-1), 7.28 (s, 1, C$\underline{H}$ = C), 7.90 (s, 1, C$\underline{H}$ = C), 11.14 (bs, 1, $CO_2\underline{H}$).

Rose bengal-linker-kanamycin (RBLKAN) ***(1c)***. RBLKAN conjugate was prepared by the same procedure as conjugate **1b** using 0.8 g of intermediate **2** and 0.49 g (1 mmol) of KAN. Due to the presence of several amino groups in the antibiotic, the binding of RB to KA is not regiospecific (specific to a certain amine). **1a** was obtained after purification on silica gel (15%MeOH/EtAc) as a purple oil in 62% (0.78 g, 90% purity by HPLC); λ_{max} (MeOH): 530 (23450), 575 (34830); HRMS (DI, m/z) calcd for $C_{44}H_{48}Cl_4I_4N_4O_{17}$ 1551.79 found 1552.31 ([M–2H] + Na), (70%); ^{1}H NMR (DMSO-d_6): δ 1.20–1.60 (m, 8, -C$\underline{H_2}$-), 2.05 (t, 2, J = 6.2 Hz, C$\underline{H_2}$-C = O), 2.52–2.78 (m, 8, -C$\underline{H_2}$-NH, -C$\underline{H}$-NH), 3.43–3.82 (m, 12, -C$\underline{H_2}$-O-, -C$\underline{H}$-O-), 4.28 (t, 2, J = 6.3 Hz, O-C$\underline{H_2}$-C), 5.07 (s, 2, O-C$\underline{H}$-O), 7.23 (s, 1, C$\underline{H}$ = C), 7.92 (s, 1, C$\underline{H}$ = C).

RESULTS AND DISCUSSION

Bacterial cells can be eradicated by either antibiotics, PS, peptides or phage therapy (1,2,33–37). In this research we suggest a method to destroy bacterial cells by conjugating an antibiotic to a PS.

Conjugate synthesis

In this research we examined the effect of the photosensitizer rose bengal (RB) with either of the antibiotic penicillanic acid (PA) or kanamycin (KAN) on bacterial cells. These molecules were linked *via* an amide bond with or without a linker between the free carboxyl in the RB and the free amine in each of the antibiotics.

It was suggested that a chosen PS can be bound either directly to the antibiotic or through a linker, depending on the nature of the reagents. A facile synthetic method can be applicable to conjugate a variety of PS (porphyrines, phthalocyanines, chlorines, toluidine blue, hypericin, *etc.*) to a variety of antibiotics that bear functional groups like $-NH_2$, $-OH$ $-SH$ and $-CO_2H$. In addition, the linkage moiety between these two parts forming amide, carbonate, carbamate, ester and other connecting functionalities can be easily controlled. This may lead to an improvement in the pharmacokinetic profile of the particular PS-antibiotic substance. It has been reported that conjugating a polylysine chain to a PS leads to an increase in the antibacterial activity against a wide spectrum of bacterial cells. The antibacterial effectiveness of the polylysine-PS conjugate was dependent on the length of the polylysine that mediated the penetration of the conjugate to the bacterial cells (38–40).

The structures of the conjugates studied in this work are shown in Schemes 1 and 2. The molecular masses of RBPA (Scheme 1), RBLPA and RBLKAN (Scheme 2) were analyzed by MS, exhibiting 1199, 1305 and 1570 Da respectively, as anticipated. HPLC and MS analysis of the conjugates did not show the molecular masses of the intermediate RB, RB-Linker or antibiotics, clearly indicating the absence of these precursor molecules in the resultant conjugates. The synthetic yield of the conjugates RBPA, RBLPA and RBLKAN was 47%, 53% and 62%, respectively. The purpose of connecting the molecules *via* a 6-bromohexanoate linker is to prevent a possible steric disruption between the PS and the antibiotics that may diminish the antibacterial activity of the conjugates.

Antibacterial activity

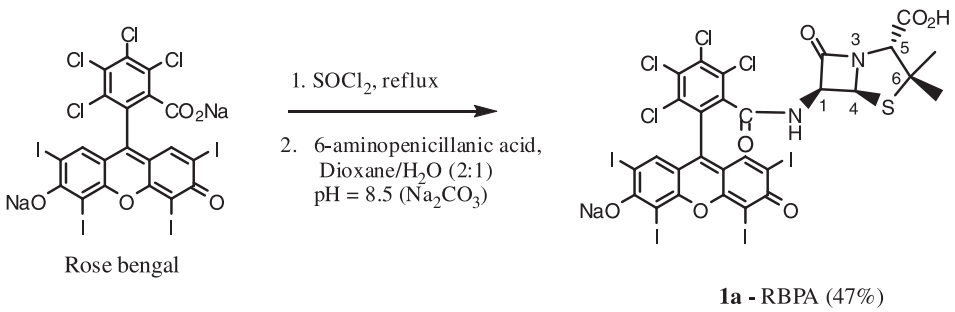
MIC determination of the PS-antibiotics conjugates (RBPA, RBLPA and RBLKAN), the combination of the PS and each of the antibiotics (RB + PA and RB + KAN), as well as each of the components alone (RB, KAN and PA) were performed by serial dilution in NB (Table 1). The bacterial cells were added to the wells in their logarithmic phase and were exposed to an energy density of 100 J cm^{-2} for 24 h. The MIC examinations were made in NB, a relatively low-protein NB, as it was found by Nitzan *et al.* (10) that the bacterial growth medium influenced the susceptibility of bacterial cells to photodynamic therapy. The high protein concentration of the Brain–Heart infusion medium may interfere with the PS's ability to get close or penetrate the bacterial cells.

The MICs of RBPA, RBLPA and RBLKAN for *S. aureus* were 0.195, 0.078 and 0.039 μL, respectively, and for *E. coli* 1.56, 1.56 and 0.312 μL, respectively.

The MICs of the conjugates for both *S. aureus* and *E. coli* were lower than each of the molecules alone (RB, PA and KAN). These results emphasize that the conjugates have significantly higher antibacterial activity compared to each of the molecules alone (for *S. aureus* 1–3 orders of magnitude while for *E. coli* three- to 25-fold).

The MIC of these three conjugates (RBPA, RBLPA and RBLKAN) for *E. coli* is higher than the nonconjugated combinations (RB + PA, RB + KAN). Similar results were obtained for RBPA and RBLPA for *S. aureus*. These results show that linking the PS and the antibiotic diminishes their antibacterial activity compared to the nonconjugated combinations. However, the MIC of RBLKAN for *S. aureus* was similar to the combination RB + KAN.

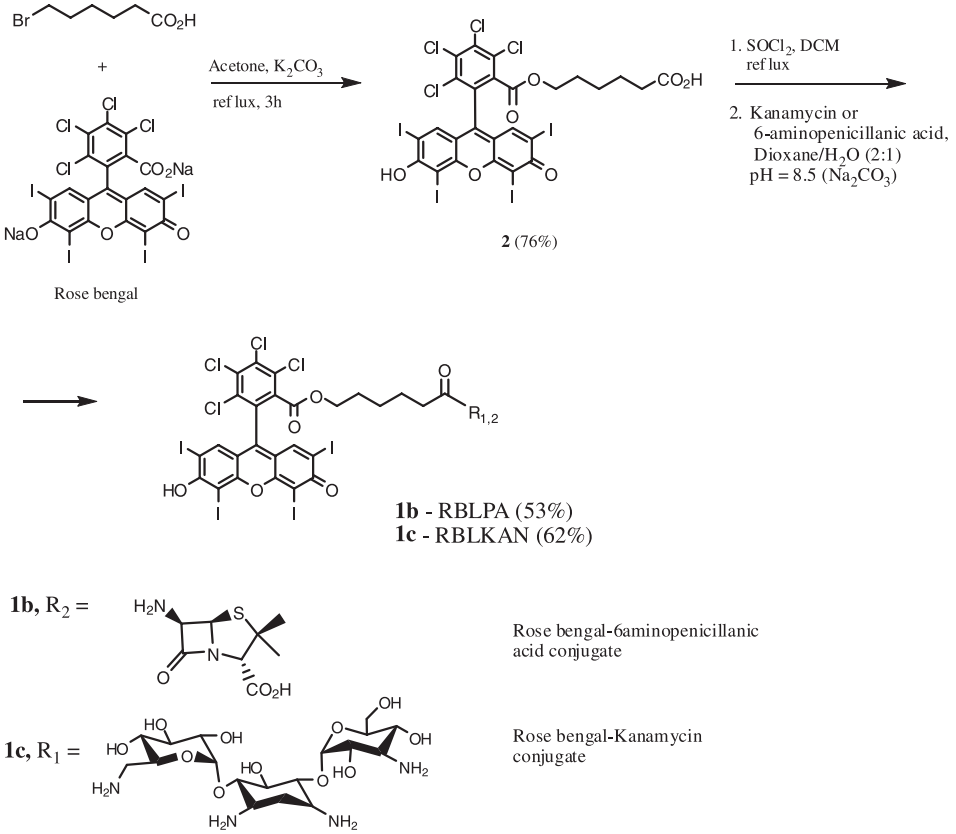
The results of the MIC analysis of the antibiotics for *S. aureus* and *E. coli* were similar in both dark and light experiments (Table 1). The MICs of the nonconjugated combinations RB + PA and RB + KAN for *S. aureus* were



Rose bengal

1a - RBPA (47%)

Scheme 1. Synthesis of RBPA (**1a**).



Scheme 2. Synthesis of RBLPA (**1b**) and RBLKAN (**1c**).

Table 1. MIC of the conjugates RBPA, RBLPA and RBLKAN as well as the combination of RB with PA or KAN, and each of the components alone.

		MIC (μM)							
	Light	RB	PA	KAN	RB + PA	RB + KAN	RBPA	RBLPA	RBLKAN
Staphylococcus aureus	+	2.5	80	1.25	0.019	0.039	0.195	0.078	0.039
	−	40 ↑	80	1.25	80	1.25	3.12 ↑	1.25 ↑	0.624 ↑
Type of action*		C	S	C	C	C	C	C	C
Escherichia coli	+	5	40	3.12	0.312	0.156	1.56	1.56	0.312
	−	80 ↑	40	3.12	40	3.12	25 ↑	25 ↑	4.5 ↑
Type of action*		C	S	C	C	C	C	C	C

MIC = minimum inhibitory concentration; RB = rose bengal; PA = penicillanic acid; KAN = kanamycin; RBPA = rose bengal-penicillanic acid; RBLPA = rose bengal-linker-penicillanic acid; RBLKAN = rose bengal-linker-kanamycin. Light+: MIC analysis performed with an energy density of 100 J cm^{-2} for 20 h at 37°C. Light−: MIC analysis in the dark. Type of action* (in light experiments): C = bactericidal; S = bacteriostatic. The arrow adjacent (right side) to the MIC result indicates that until this concentration bacterial growth was observed.

80 and 1.25 μM and for *E. coli* 40 and 3.12 μM, respectively, in dark experiments, with similar results obtained with the antibiotics PA and KAN alone in light experiments. These results show that the PS and RB did not possess antibacterial activity, and therefore can be excluded as an influence on the antibacterial activity of the nonconjugated combinations in the dark control experiments. The MIC of the PS RB as well as the conjugates RBPA, RBLPA and RBLKAN in the dark for each bacterium was examined at a concentration that was at least 16-fold that of the MIC found in the light experiments. This implies that in the dark experiments, the molecules RB, RBPA, RBLPA and RBLKAN did not possess antibacterial activity below these concentrations (emphasized with arrow, Table 1) as bacterial growth was observed.

The MIC results show that *S. aureus* cells are significantly more sensitive to these conjugates than *E. coli*. The high susceptibility of gram-positive bacterial cells may be explained by their physiology, as their cytoplasmic membrane is surrounded by a relatively porous layer of peptidoglican and lipoteichoic acids that allows the PS to enter the cell. Conversely, gram-negative bacteria are surrounded by an outer membrane functioning as a physical and chemical

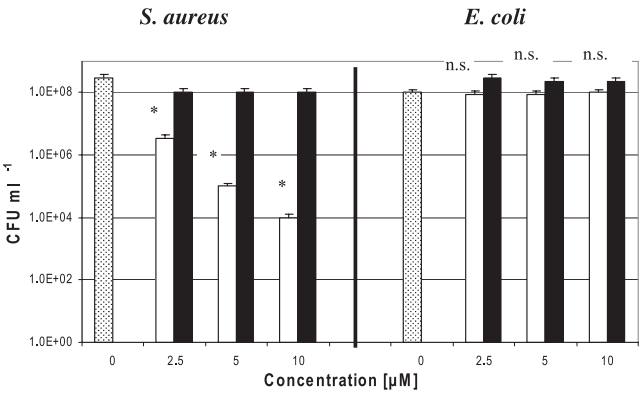


Figure 1. Effect of various concentrations of RBPA conjugate on *Staphylococcus aureus* (left panel) and *Escherichia coli* (right panel) after illumination at an energy density of 2 J cm^{-2} for 20 min (open bars), dark control—without illumination (dark bars). Light control cultures were not treated with RBPA, but illuminated at an energy density of 2 J cm^{-2} for 20 min (dotted bar). The survival of the bacterial cells was determined by the CFU assay. Each bar is the mean ± standard deviation of three experiments. $*P < 0.05$; nonsignificance (n.s.) = $P > 0.05$. Illumination results compared with dark control for each concentration.

barrier that prevents the PS or the singlet oxygen generated outside the cells to cross the cell wall and interact with vital targets, such as membrane or cytoplasmic components (22,23). It was reported that porphyrin-based PS was phototoxic against the gram-positive *S. aureus* strains, while at the same concentrations no inactivation of *E. coli* was detected (41).

It was found that the conjugates had less antibacterial activity than their relative combinations. However, it is important to indicate that a treatment with one molecule (the conjugate) is easier than a treatment with two molecules (the combination). Moreover, linking the PS to the antibiotic may reduce the appearance of bacterial resistance to the antibiotic.

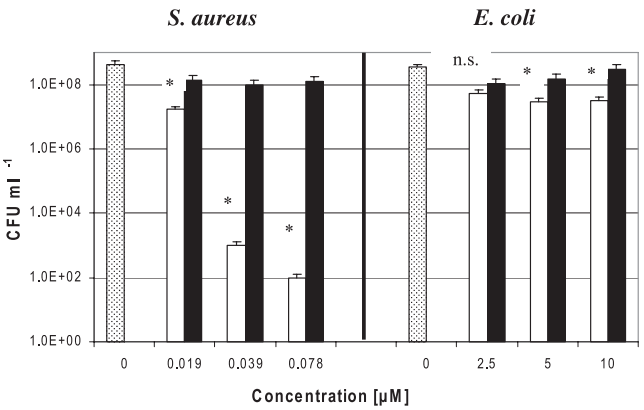


Figure 2. Effect of various concentrations of RBLPA conjugate on *Staphylococcus aureus* (left panel) and *Escherichia coli* (right panel) after illumination at an energy density of 2 J cm^{-2} for 20 min (open bars), dark control—without illumination (dark bars). Light control cultures were not treated with RBLPA, but illuminated at an energy density of 2 J cm^{-2} for 20 min (dotted bar). The survival of the bacterial cells was determined by the CFU assay. Each bar is the mean ± standard deviation of three experiments. $*P < 0.05$; nonsignificance (n.s.) = $P > 0.05$. Illumination results compared with dark control for each concentration.

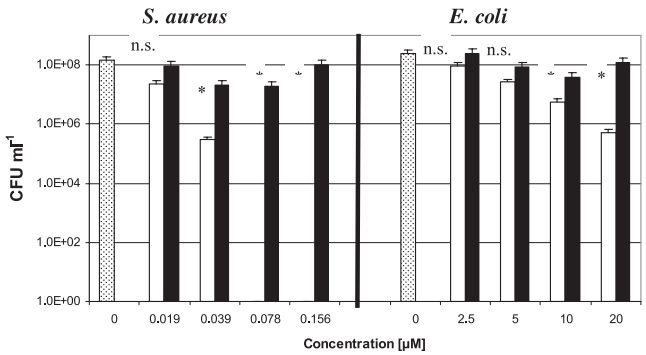


Figure 3. Effect of various concentrations of RBLKAN conjugate on *Staphylococcus aureus* (left panel) and *Escherichia coli* (right panel) after illumination at an energy density of 2 J cm^{-2} for 20 min (open bars), dark control—without illumination (dark bars). Light control cultures were not treated with RBLKAN, but illuminated at an energy density of 2 J cm^{-2} for 20 min (dotted bar). The survival of the bacterial cells was determined by the CFU assay. Each bar is the mean ± standard deviation of three experiments. $*P < 0.05$; nonsignificance (n.s.) = $P > 0.05$. Illumination results compared with dark control for each concentration.

The MBC of the conjugates, the concentration at which colony formation is inhibited, was evaluated for concentrations beyond the MIC, to test whether growth inhibition was associated with a bactericidal effect (Table 1). *E. coli* and *S. aureus* treated with the conjugates lost colony-forming ability at the same concentrations as the MIC (for each of the conjugates). Therefore, the conjugates RBPA, RBLKAN and RBLKAN can be regarded as bactericidal agents against *E. coli* and *S. aureus*.

Conjugate concentration dependence

S. aureus and *E. coli* were treated with each of the conjugates and illuminated at an energy density of 2 J cm^{-2} (Figs. 1–3). As demonstrated in Fig. 1, the conjugate RBPA shows a pattern of concentration dependence toward *S. aureus*. RBPA

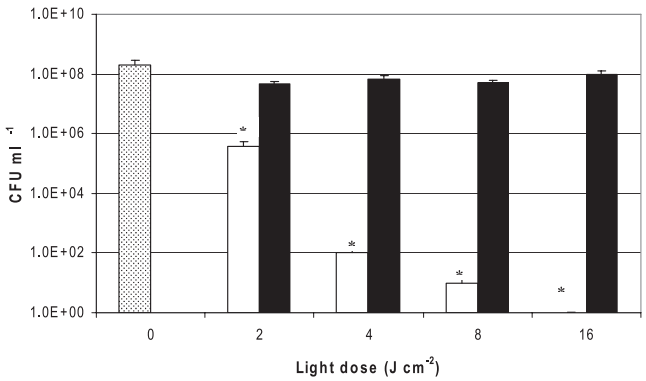


Figure 4. Effect of 20 μM RBLKAN conjugate at various energy densities on *Escherichia coli* after illumination at an energy density of 2–16 J cm^{-2} for 20 min (open bars), dark control—without illumination (dark bars). Light control cultures were not treated with RBLKAN, but illuminated at an energy density of 16 J cm^{-2} (dotted bar). The survival of the bacterial cells was determined by the CFU assay. Each bar is the mean ± standard deviation of three experiments. $*P < 0.05$. Illumination results compared with dark control for each light dose.

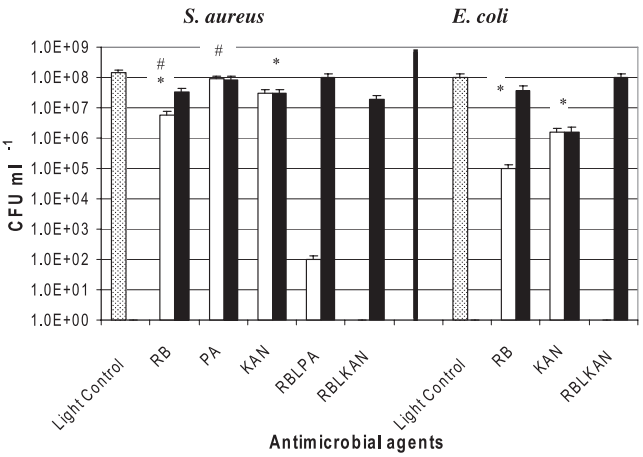


Figure 5. Effect of antimicrobial agents on *Staphylococcus aureus* (left panel) and *Escherichia coli* (right panel). *Staphylococcus aureus* bacterial cells were illuminated at an energy density of 2 J cm^{-2} for 20 min while *E. coli* cells were illuminated at 16 J cm^{-2} for 20 min (open bars); dark control—without illumination (dark bars). The concentration of each of the antimicrobial agents for *S. aureus* and *E. coli* was 0.078 and 20 μM, respectively. Light control cultures were not treated with either of the antibacterial molecules, but illuminated under the same conditions for each bacterium (dotted bar). Each bar is the mean ± standard deviation of three experiments. $*P < 0.05$; RB alone as well as KAN in light experiments compared with the conjugate RBLKAN. $\#P < 0.05$; RB alone as well as PA in light experiments compared with the conjugate RBLPA.

at concentrations of 10 and 5 μM caused CFU reduction by 5 and 4 orders of magnitude respectively compared to control cultures. This conjugate did not have a significant antibacterial activity toward *E. coli*.

Addition of a linker (5 carboxy pentyl ester) between RB and PA, conjugate RBLPA, led to an improvement in the antibacterial activity toward *S. aureus* (Fig. 2), as compared to RBPA (the conjugate without the linker). RBLPA (0.078 μM) supplied to the culture at a lower concentration than RBPA, reduced the CFU by 6 orders of magnitude. We therefore assumed that the direct conjugation of RB and PA caused a steric disruption. However, the antibacterial activity of RBLPA toward *E. coli* (Fig. 2) was similar to RBPA, meaning that the addition of the linker in this case did not improve the antibacterial activity against the gram-negative *E. coli*.

The RBLKAN conjugate at an energy density of 2 J cm^{-2} (Fig. 3) eradicated *S. aureus* bacterial cells at a concentration of 0.078 μM, and was more effective than RBLPA or RBPA. However, the RBLKAN conjugate when applied against *E. coli* at a concentration of 20 μM reduced the CFU by only 2 orders of magnitude compared to control cultures.

Energy density dependence

E. coli cultures were treated by RBLKAN at various energy densities (2–16 J cm^{-2}). As shown in Fig. 4, an *E. coli* culture

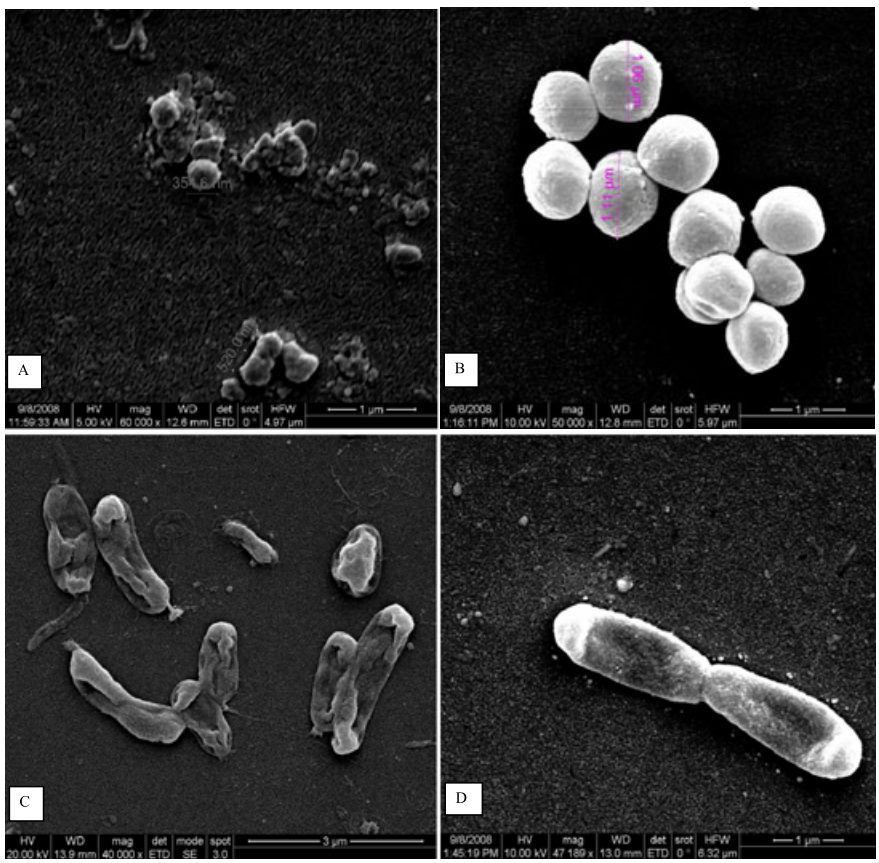


Figure 6. SEM analysis of RBLKAN conjugate on *Staphylococcus aureus* (A, B) and *Escherichia coli* cells (C, D). *S. aureus* cells treated with 0.039 μM RBLKAN (MIC) at 100 J cm^{-2} (A); untreated S. *aureus* cells (B); *E. coli* cells treated with 0.312 μM RBLKAN (MIC) at 100 J cm^{-2} (C); untreated *E. coli* (D).

treated with 20 μM at 16 J cm^{-2} was totally eradicated. We believe that the positive charge of KAN as well as the sufficient energy density led to the better penetration of RBLKAN through the *E. coli* outer membrane.

The conjugates that revealed the best antibacterial activity were compared with their related antibiotic and PS alone. This comparison was made at the best conjugate concentration and energy density for each bacterium. As shown in Fig. 5, the conjugates RBLPA and RBLKAN (both at 0.078 μM and 2 J cm^{-2}) showed significant antibacterial activity toward *S. aureus,* while at these conditions the RB, PA and KAN individually were not effective. For *E. coli* the conjugate RBLKAN (20 μM at 16 J cm^{-2}) totally eradicated the bacterial cells while under these conditions the RB and KAN individually were not effective.

Nitzan *et al.* (11) showed that the polycationic peptide polymixin B nonapeptide increased the permeability of *E. coli* and *Pseudomonas aeruginosa* gram-negative outer membranes and allowed the PS, normally excluded from the gram-negative cells, to penetrate and, upon illumination, cause fatal damage. This method was also used to kill a multi-antibiotic resistant strain of *A. baumannii* (10). It was found that the use of EDTA to release LPS or the induction of outer membrane competence with calcium chloride increased the susceptibility of *E. coli* and *Klebsiella pneumoniae* to photodynamic therapy by the PS hematoporphrin or zinc phtalocyanine (18). Phenothiazine toluidine blue O, a PS that bears a positive charge, as well as zinc phtalocyanine had a photodynamic antibacterial activity, against a large range of both gram-positive and gram-negative bacteria (42–44).

Morphological changes

SEM analysis of *S. aureus* bacterial cells treated with RBLKAN at MIC and an energy density of 100 J cm^{-2} for 20 h (Fig. 6a) showed total damage of the cells. There was not even a single normal cell in the SEM analysis sample, the cells debris were smaller than 520 nm, while untreated cells (Fig. 6b) exhibited normal cell surfaces and sizes (1.1 μm in average). SEM analysis of *E. coli* cells treated with RBLKAN at MIC and an energy density of 100 J cm^{-2} for 20 h (Fig. 6c), showed significant damage of *E. coli* cell walls, as well as a shrunken cell size. Untreated cells (Fig. 6d) exhibited normal cell surfaces and sizes (2.5 μm).

The current study focused on the effect of the conjugates RBPA, RBLPA and RBLKAN on *E. coli* and *S. aureus,* two pathogenic bacteria. MBC analysis showed that these conjugates are bactericidal for *E. coli* and *S. aureus*. Total eradication of *S. aureus* bacterial cells was observed with RBPA and RBLKAN. However, total eradication of *E. coli* was observed only with the conjugate RBLKAN. In addition, SEM analysis showed significant damage to the *E. coli* and *S. aureus* bacterial cells.

Conflict of interest—The authors hereby declare that no conflict of interest exists concerning this manuscript.

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