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Conjugation of Antimicrobial Peptide to Zinc Phthalocyanine for an Efficient Photodynamic Antimicrobial Chemotherapy

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Abstract: Photodynamic antimicrobial chemotherapy is an attractive and novel therapeutic approach to treat microbial infections. Antimicrobial peptides (AMPs) have the potential to specifically target and kill the microorganism while showing no effect toward mammalian cells. In the current study, antimicrobial peptide (GGG(RW)3), an analogue of MP-196, was conjugated to a zinc phthalocyanine (ZnPc) photosensitizer (PS) for photoinactivation assay to enhance the bacterial killing efficacy of the peptide. The AMPs showed selectivity toward the Gram-positive strain of bacteria. We observed that the conjugate ZnPc-GGG(RW)3 also displayed a photoinactivation effect against the Gram-positive strains of *S. aureus*. The results showed that ZnPc-GGG(RW)3 induced a 6-log reduction (i.e., 99.999% cell killing) in Gram-positive *S. aureus* at a light dose of 22 J/cm^2 upon illumination under red light, while the peptide did not exhibit such a significant effect when tested alone at the same concentration. The conjugate also showed 50% inhibition of the bacterial strain in the dark at a higher concentration. Furthermore, the addition of potassium iodide salt to the PS at lower concentrations also significantly killed the Gram-negative *E. coli* strain and killed the *E. coli* strain with up to a 5-log reduction at a light dose of 22 J/cm^2 under red light illumination. We demonstrated the efficacy of antimicrobial peptide (GGG(RW)3 enhanced by conjugation to a ZnPc photosensitizer.

Keywords: antimicrobial peptide; ZnPc-GGG(RW)3 conjugate; *S. aureus*; *E. coli*; photodynamic antimicrobial chemotherapy

Citation: Rukh, G.; Ullah, A.; Khattak, R.; Fazil, P.; Ali, A.; Shah, M.R.; Khan, M.S.; Alsubaie, A.S.; Mahmoud, K.H.; Ateeq, M. Conjugation of Antimicrobial Peptide to Zinc Phthalocyanine for an Efficient Photodynamic Antimicrobial Chemotherapy. *Coatings* **2022**, *12*, 200. https://doi.org/10.3390/coatings12020200

Academic Editor: Simona Liliana Iconaru

Received: 28 November 2021
Accepted: 24 January 2022
Published: 3 February 2022

1. Introduction

Bacterial infections from minor to severe complications are usually caused by microorganisms such as *Propionibacterium acne* and *Staphylococcus aureus* [1,2]. The wide-ranging applications of antibiotics have been important in curing the microbial infectious diseases caused by these microbes and other pathogens. In the last two decades, bacterial resistance to common antibiotics has become a major threat to public health and has contributed to restoring infectious diseases. The emergence of antibiotic resistance has encouraged many researchers to find better alternative antibiotic therapeutics. Many efforts are currently underway to discover new alternatives to overcome health problems. Antimicrobial peptides (AMPs) are the most important natural defense system of a living organism against invading pathogens [3,4]. The finding of these natural antimicrobial peptides provides a

new direction to combat microbial resistance. Despite broad-spectrum antibiotics, these AMPs, however, pose some difficulties in clinical applications, including toxicity to eukaryotic cells, stability in in vivo applications and high cost [5,6]. One promising approach is the development of a short antimicrobial peptide to find a potent alternative to large antimicrobial peptides. Antimicrobial peptides are often characterized by high positive charges and hydrophobic amino acids, resulting in high selectivity toward bacterial membranes [7]. In an attempt to find an arginine–tryptophan-rich short-sequence hexa-peptide, RWRWRW-NH2 (MP196) was synthesized with the objective of developing an antimicrobial peptide. MP-196 is a cationic hexa-peptide that possess good antibacterial activity against the Gram-positive strains of bacteria [8]. This synthetic peptide integrates into the peripheral membrane and delocalizes the peripheral membrane protein involved in cell wall biosynthesis and respiration [9]. Being neither cytotoxic nor hemolytic, MP-196 was found to be highly selective for bacterial cells in comparison with mammalian cells [10]. It is therefore selected as a lead structure for further derivatization approaches to find more potent analogues.

Photodynamic antimicrobial chemotherapy (PACT) is another important attractive modality to treat localized microbial infections. PACT, with a unique mode of action, has received a great deal of attention as a promising approach to eradicate drug-resistant bacteria. It is based on the combined effect of non-toxic substances, such as photosensitizers (PSs), light and cytotoxic reactive oxygen species (ROS), to trigger cell death [11]. PACT uses various photosensitizers, and, out of these, zinc phthalocyanine (ZnPc)-based PSs are of particular interest for their unique physico-chemical properties [12,13]. Antimicrobial photodynamic therapy typically correlates with the lipophilicity and high propensity of PSs to bind and damage the biological membrane [14]. Many studies have reported utilizing PACT to target the bacterial membrane using cationic PSs and cell-targeting peptides [15].

A cell-penetrating peptide (arginine-rich TAT) coupled to porphyrin was shown to photoinactivate both Gram-negative and Gram-positive bacteria. The results therefore indicate that arginine-rich small peptides can serve as a broad-spectrum therapeutic agent. Polylysine was conjugated with ZnPc to treat oral periodontitis by eradicating *Porphyromonas gingivalis*, a bacterial strain identified in periodontal plaque [16]. The conjugate (at 20 μM) significantly reduced the bacterial strain in a dog periodontal disease model after receiving a light dose of 1.5 J/cm^2 [16]. However, this approach is associated with potential problems related to the toxicity of the polycationic PS toward human cells [17]. Eosin Y conjugated with antimicrobial peptides $(KLAKALAK)_2$ enhances the binding efficiency to bacteria 10-fold. These compounds did not show antibacterial effects when tested alone; however, the conjugate at a concentration of 1 μm displayed a 99% killing efficiency of bacteria. On the basis of these results, it was stated that $(KLAKALAK)_2$ selectively targeted the bacterial membrane and improved the killing efficiency of the PS [18]. Pentalysine conjugated with phthalocyanine reported by Chen et al. showed a significant photodynamic inactivation effect against the bacterial strains [19,20]. A novel strategy using non-toxic salts in combination with photosensitizers has been considered and reported to kill both types of bacterial strains [21].

Various salts in combination with many PSs were reported by M. Hamblin, e.g., potassium iodide (KI), potassium selenocyanate, potassium thiocyanate and sodium azide, under light illumination, and their photodynamic effect against different bacterial strains was observed [22]. In another report, Wen et al. established the synergistic effect of KI in combination with rose bengal as a PS under light illumination, and they found that rose bengal as a PS in combination with KI triggered bacterial cell death with up to six extra log reductions in bacterial strains [23]. In another study, methylene blue with KI was reported by Huang et al. for urinary tract infections [24]. The potentiation of KI was further reported by Vieira et al. using non-porphyrinic and porphyrinic PSs for a significant photodynamic inactivation effect against bacterial strains [25].

In this report, we aim to enhance the antimicrobial efficacy of the antimicrobial peptide (GGG(RW)3) by conjugation to a photosensitizer, zinc phthalocyanine (ZnPc). The results

show that the ZnPc-GGG(RW)3 conjugate induced a significant photodynamic inactivation effect against Gram-positive *S. aureus* at a low concentration, while the peptide did not exhibit any significant effect at the same concentration. Additionally, the PS under red light illumination was treated with potassium iodide against the Gram-negative bacterial strains and demonstrated a significant photodynamic effect.

2. Experimental Section

2.1. Materials and Instruments

The UV/Vis spectra were recorded in 96-well plate using synergy microplate reader (BioTek Instruments, Winooski, VT, United States of America). Gram-positive *Staphylococcus aureus* Xen29 (NCTC8532) was purchased from Shanghai Biofeng Company (Shanghai, China), and bioluminescence analysis was conducted by using microplate reader (BioTek Instruments, Winooski, VT, USA). Antimicrobial peptide (GGG(RW)3) was purchased from the company Zhejiang Ontores Biotechnologies Inc. (Zhejiang, China). The light source used was a planar red light-emitting diode (LED) customized with 24 lamps, which produces light of around 660 nm with 75 mW/cm^2 power density (Uniglory Electronics, Hong Kong, China).

2.2. Synthesis of Beta-Carboxy Zinc Phthalocynine Conjugated with Peptide

Beta-carboxy phthalocyanine zinc was synthesized using our previously reported method [26]. ZnPc-COOH was conjugated with GGG(RW)3 protected via Wang resin (Zhejiang Ontores Biotechnologies Inc. Zhejiang, China). A 0.02 mmol solution of beta-carboxy phthalocyanine zinc (CPZ) was made in 2 mL DMF. HBTU (23 mg, 0.06 mmol) and DIPEA (0.012 mL) were added to the DMF solution and stirred for 30 min. The peptide (GGG(RW)3) (100 mg, 0.02 mmol) protected with Wang resin was added into the solution, and stirring continued for 24 h. After 24 h, diethyl ether was added, and the solution was centrifuged to obtain residual. The final residue was further treated with TFA (95%) for 4 h at room temperature. The reaction solution mixture was filtered off via Buchner funnel, filtrate was further concentrated, and cool absolute ether was added to obtain the precipitate. The conjugated compound was further purified on a preparative high performance liquid chromatography(HPLC) using a reverse-phase column (Sino Chrom ODS-BP, 10 mm) purchased from Dalian Elite Analytical Instruments Co. Ltd., Dalian, China, eluting at a flow rate of 5 mL/min with a linear gradient of 50–100% in DMF:Water in 0.01% TFA for a period of 30 min.

2.3. Bacterial Strain

Staphylococcus aureus Xen29 (NCTC8532) is the luminescent strain of the bacteria, and a copy of the biofilm forming *S. aureus* 12,600 was purchased from Shanghai Biofeng Company (Shanghai, China). The bacterial strain had a stable copy of the modified Photorhabdus luminescens luxABCDE operon, grown at 37 °C using Luria–Bertani (LB) broth containing kanamycin (200 μg/mL to select for resistance encoded by the plasmid) to an absorbance of 0.5 at 600 nm corresponding to 1.44×10^8 organisms per milliliter.

A bioluminescent strain of *Escherichia coli* (*E. coli*) DH5α was constructed by transformation with the plasmid pAKlux2.1, an expression vector that contains a complete bacterial luciferase operon as described in [23]. Bacteria were grown at 37 °C using Luria–Bertani broth and agar plates containing ampicillin (100 mg/mL to select for resistance encoded by the plasmid) to an absorbance of 0.6 at 600 nm corresponding to 10^8 organisms per milliliter. This suspension was centrifuged, washed with phosphate-buffered saline (PBS) and re-suspended in PBS at the same density. Luminescence was routinely measured by taking 100 μL aliquots of bacterial suspensions in 96-well black-sided plates, using a Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.4. Cellular Uptake of Photosensitizer

Microorganism suspension of *S. aureus* was grown at 37 °C and centrifuged with various concentrations of photosensitizer. Bacterial suspension was washed twice with sterile phosphate-buffered saline (PBS) with NaOH (0.1 N, 1.0 mL with 1% sodium dodecyl sulfate (SDS)) before lysis to give a homogenous solution. The fluorescence of the cell extract was measured on a microplate reader. The method of Lowry et al. was used to determine the cellular protein concentration. Standard curves were made with cell lysates treated as mentioned above with known added amounts of bovine serum albumin. Results are expressed as nanomoles of phthalocyanine per milligram of cell protein.

2.5. Measurement of Reactive Oxygen Generation (ROS)

The property of PS to generate ROS after interaction with light was studied with 2,7 dichlorofluorescein diacetate (DCFH-DA) used as a probe as per the reported method [27]. The solution of DCFH-DA (40 μm) was used for fluorescent study, and the spectra of DCF solution was obtained via synergy plate at the emission range of 528 nm and excitation of 480 nm. The activation of DCFH solutions under light irradiation in the presence of PS converts non-fluorescent DCFH into 2,7 dichlorofluorescein (DCF), a highly fluorescent product. Confirmation of DCF at the 525 nm emission was performed both in the presence and absence of PS and light illumination. The linearity between DCF fluorescence signal and PS concentration was verified. The total 200 μL of DCFH solution (40 μm) and PS (10 μm) in PBS was mixed in 96-well plate and irradiated for 30 s using LED red light. Linear response was found in fluorescent intensity after each irradiation of the plate every 30 s.

2.6. Hemolytic Activity

Human erythrocytes from a healthy donor were studied for hemolytic assay. In Eppendorf tube, an equal volume of 2% human erythrocytes was mixed with 450 μL of 0.9% NaCl, and three different concentrations of 100 μL CPZ-GGG(RW)3 were added to adjust the final concentrations up to 0.1, 0.3 and 0.5 μm. Erythrocytes were mixed with ultrapure water as a positive control experiment, while 0.9% NaCl (550 μL) was used as a negative control. Samples were then incubated for 30 min at 37 °C and illuminated with LED light for 5 min. The illuminated samples were further incubated for 30 min at 37 °C, followed by centrifugation for 10 min at 800 rpm/min. The optical density of sample solutions were measured in 96-well plate at 576 nm. The percentage of hemolysis rate was determined using the following formula:

$$Z = (A_s - A_n) / (A_c - A_n) \times 100\%$$

Here, As, An and Ac are the absorbance of the samples, negative control and positive control, respectively.

2.7. Photodynamic Antimicrobial Activity

The bacterial suspension of the luminescent *S. aureus* was diluted in PBS to 10^6 CFU/mL before incubating with PS. Stock solutions (1 mm) of CPZ-GGG(RW)3 conjugate and peptide were prepared in dimethyl sulfoxide (DMSO) and then diluted further up to 100 μm with PBS (using 0.1% Tween 20 by volume and final DMSO concentration of 0.5%). The PBS suspension of the luminescent *S. aueus* was then incubated in 96-well plate either with the conjugated CPZ-GGG(RW)3 or with the peptide (GGG(RW)3) for 10 min at room temperature (at concentrations of 10^{-10} M, $10^{-9.5}$ M, 10^{-9} M, $10^{-8.5}$ M, 10^{-8} M $10^{-7.5}$ M, 10^{-7} M, $10^{-6.5}$ M and 10^{-6} M) followed by irradiation for 5 min using the customized planar LED light (680 nm) [28] with a light dose of 22.5 J/cm^2. The plate was irradiated for 1 min and left to cool for 3 min in order to prevent heating of the plate and photodegradation of the PS. Such light illumination was repeated 5 times to give total light fluence of 22.5 J/cm^2. The percentage of viable cells was determined from the luminescence intensities of the

treated plates and compared with the luminescence intensities of the control plates. Dark toxicity was also measured while keeping the plate away from illumination for a period equal to that of the illuminated plate. Furthermore, the PS was treated with the addition of potassium iodide (KI) salt against the Gram-negative *E. coli* bacterial strain. We added potassium iodide (100 mm) with different concentrations of PS (1.0, 3.0 and 10 μm), and it was incubated with bacterial strains for 10 min and illuminated by red LED light (680 nm) for 5 min with a light dose of 22.5 J/cm^2. The photoantibacterial effect was measured from the luminescence signals by using microplate reader, and it was calculated by colony counting units.

3. Results

3.1. Synthesis of CPZ−GGG(RW)3 Conjugate

The compound (CPZ−GGG(RW)3) was designed and synthesized according to our previously reported method and then further conjugated with the antimicrobial peptide (GGG(RW)3) for photodynamic application against the microorganisms. The conjugate was characterized by electrospray ionization mass spectrometry (ESI-MS) and fourier transform infrared (FTIR) spectroscopy. The ESI-MS result was found to be m/z $(M + H)^+$ = 1820. The UV/Vis absorption spectrum of the CPZ−GGG(RW)3 conjugate was recorded to range from 550 to 800 nm in DMSO and found to have a 680 nm absorption intensity (Figure 1). The HPLC chromatogram appeared at 26.5 min on the C−18 reversed-phase HPLC system (Figure 2A).

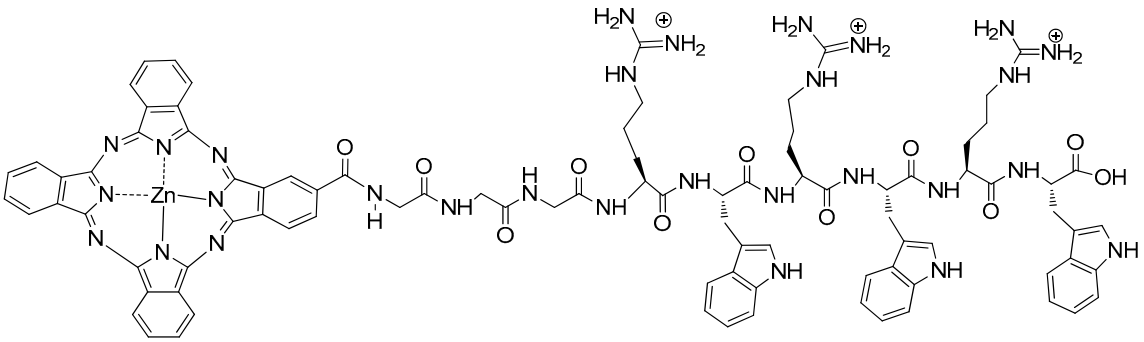

Figure 1. Chemical structure of CPZ-GGG(RW)3.

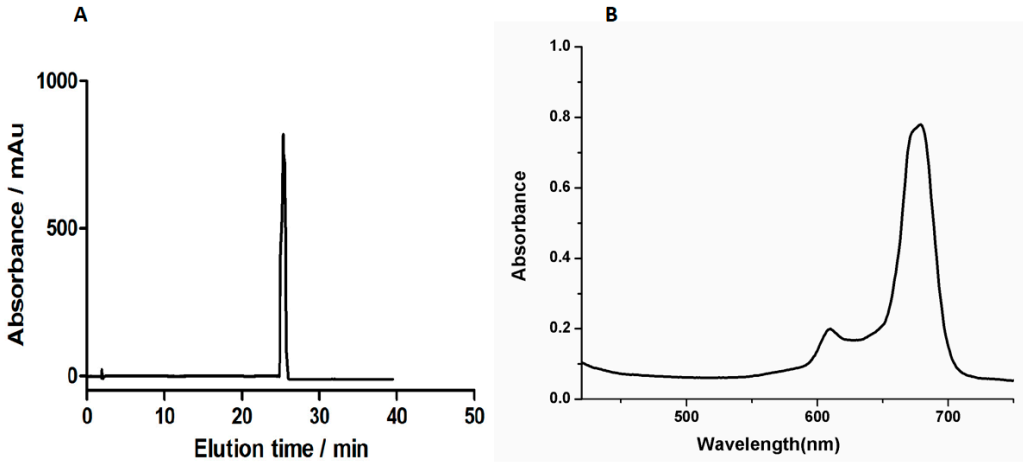

Figure 2. Characterization of CPZG-GG(RW)3. (**A**) C-18 reversed-phase analytical HPLC chromatogram for CPZG-GG(RW)3. (**B**) UV/Vis absorption spectrum of CPZ-GGG(RW)3 in DMSO in the range of 550–800 nm.

3.2. Cellular Uptake of CPZ-GGG(RW)3

The binding kinetics of the PS toward Gram-positive *S. aureus* was studied using a chemical extraction procedure. We mixed 1×10^6 CFU/mL of the bacteria with various concentrations of CPZ-GGG(RW)3 and monitored the binding kinetics using fluorescence intensity. In short, the number of PS molecules that accumulated into the bacterial cell was quantified and monitored using a fluorescent method of the collected samples. It was observed that the uptake of CPZ-GGG(RW)3 by *S. aureus* occurred in a concentration-dependent manner. Our results show that the PS exhibited a higher uptake at a higher concentration, and the uptake decreased further in parallel with the concentration gradient (Figure 3A).

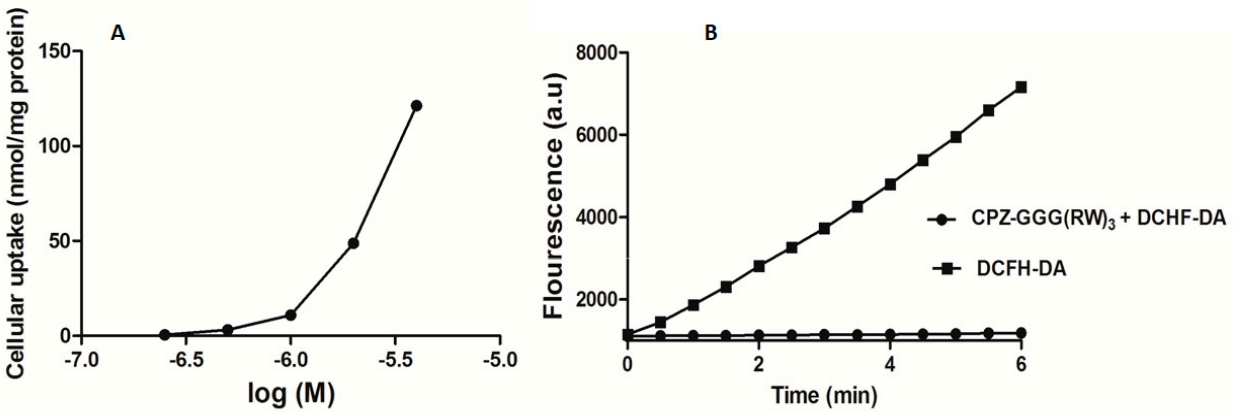

Figure 3. Binding of CPZ-GGG(RW)3 to *S. aureus* (**A**) and analysis of the ROS generated by CPZG-GG(RW)3 in the presence of probe DCFH-DA (40 µm, ex 480 nm, em. 528 nm), which is converted into highly fluorescent product (DCF) (**B**).

3.3. Mechanism for Enhanced Potency of the Conjugate ROS Measurement

The mechanism involved in the lethal damage of bacteria during photodynamic inactivation is due to the cytotoxic ROS produced by PS activation when interacting with a specific wavelength of light. ZnPc based PSs normally produce ROS under light illumination, mainly via type I and type II mechanisms. Usually, the type I process results in either an electron transfer or hydrogen atoms, yielding radicals or radical ions, whereas the type II mechanism results in the transfer of energy between the excited triplet state of a PS and the ground state of oxygen to induce singlet oxygen generation, which is critical for photodynamic therapy (PDT) applications. In this study, our PS (CPZ-GGG(RW)3) generated ROS efficiently via the type I mechanism, and DCFH-DA was converted into the highly fluorescent substance dichlorofluorescein (DCF) after the illumination of the PS from the LED red light at 680 nm (Figure 3B). DCF formation was examined with an increase in the fluorescent intensity (ex 480 nm and em 528 nm) using a microplate reader.

3.4. Antimicrobial Efficacies of CPZ-GGG(RW)3 Conjugate and GGG(RW)3 Peptide

In order to assess the antimicrobial efficacy of CPZ-GGG(RW)3 and GGG(RW)3, CPZ-GGG(RW)3 was incubated for 10 min at room temperature with 10^6 CFU/ mL of luminescent *S. aureus* before illumination, and GGG$(RW)_3$ was also treated with bacteria under the same experimental conditions to those used for the photoinactivation assay. The bioluminescent intensity (relative luminescence units, RLU) reflects the number of live bacteria. We incubated the bacterial suspension in 96-well plates with various concentrations of the CPZ-GGG$(RW)_3$ conjugate and GGG$(RW)_3$ alone, followed by illumination from the customized planar LED light (660 ± 25 nm with a power density of 75 mW/cm^2) for 5 min (22.5 J/cm^2). The LED light source irradiates all 96 wells at the same time with the same intensity, thus reducing systematic error. To prevent the photothermal effects of the light source, the plates were illuminated in a fraction of 1 min (4.5 J/cm^2), followed by

3 min intervals, and illumination was repeated for a total of 5 min (22.5 J/cm^2) to reach the maximum photoinactivation effect. After the illumination, the antibacterial rate was determined from the number of live bacteria on the plate treated with the PS compared with the luminescent intensity of the controlled plate without the PS. We observed that the CPZ-GGG(RW)3 conjugate significantly photoinactivated *S. aureus* compared to the peptide alone at the same concentration. Our results show that CPZ-GGG(RW)3 induced photoinactivation of Gram-positive *S. aureus* with an IC_{50} value of 4 nM, while the peptide induced a 50% reduction under the same experimental setup (Figure 4A). Further, the potency of the PS was also evaluated using a CFU assay against *S. aureus*, and it was found that the peptide conjugate significantly eradicated the bacterial strain; moreover, up to a 6-log reduction in CFU units was achieved (Figure 4B). The dark toxicity of the PS was studied, and it was found that the antimicrobial peptide conjugate also reduces bacterial strains to some extent at high concentrations (Figure 4C).

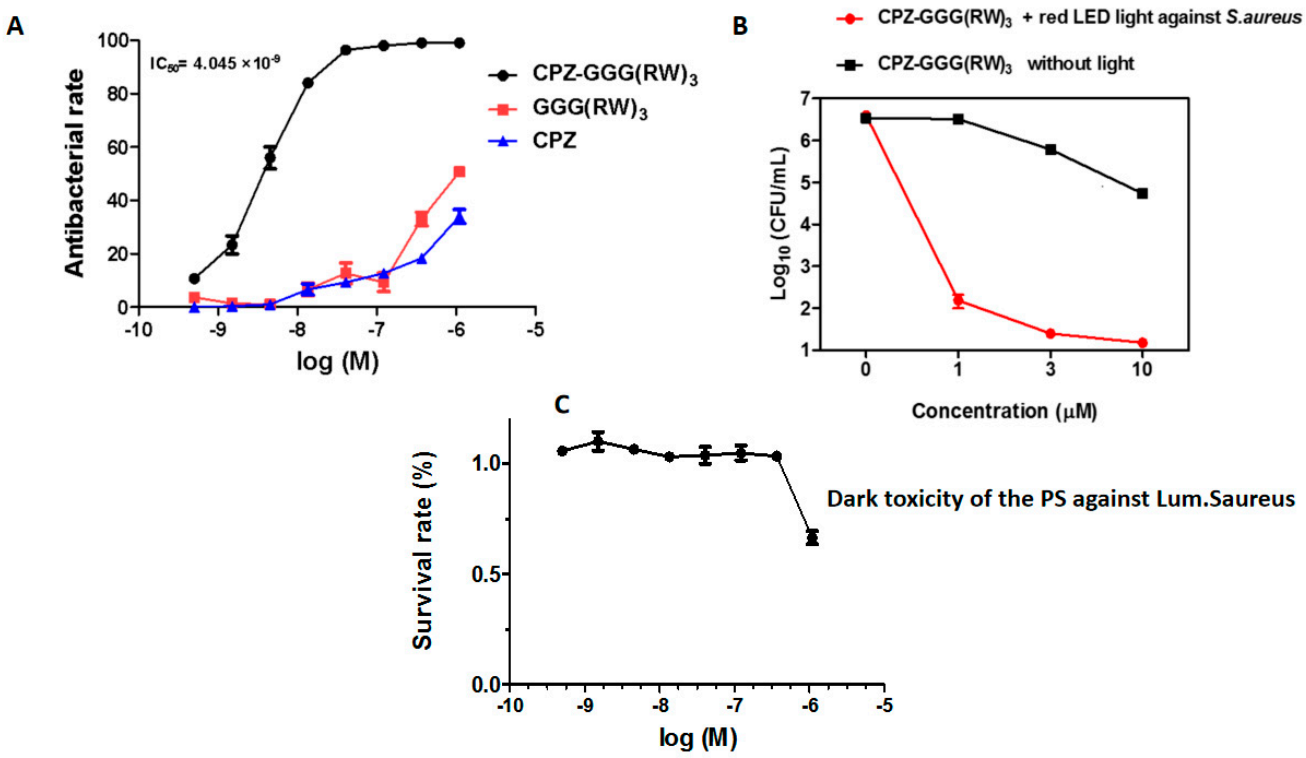

Figure 4. Phototoxicity of CPZ-GGG(RW)3 and antibacterial efficacy of GGG(RW)3 against the luminescent *S. aureus* (**A**) under light illumination using red LED light illumination (22.5 J/cm^2). Photodynamic effects of CPZ-GGG(RW)3 against *S. aureus* (**B**) were also measured using the colony counting method under light illumination from a red LED light (22.5 J/cm^2), as well as the dark toxicity of CPZ-GGG(RW)3 against the luminescent *S. aureus* under the same experimental condition (**C**).

3.5. Potentiation of CPZ-GGG(RW)3 Using Potassium Iodide

To further assess the photoinactivation effect (PDI) of the PS against the Gram-negative *E. coli* strain of bacteria, we first evaluated the effect of high concentrations of CPZ-GGG(RW)3 (10, 30 and 50 μm) against the *E. coli* strain up to high concentrations under red LED light illumination at a light dose of 22.5 J/cm^2 using the bioluminescent strain and CFU assay measurements. We observed that no photodynamic effect was achieved against Gram-negative *E. coli* under this condition. Additionally, we further added KI salt (100 mm) in combination with the PS (1.0, 3.0 and 10 μm), followed by red LED light illumination for 5 min with a light dose of 22.5 J/cm^2. Interestingly, we found that the synergistic effect of KI and the PS led to a significant killing of the *E. coli* strain, with a 5-log reduction in CFU

units, and 875 nm IC_{50} was obtained against the bioluminescent strain, demonstrating that the toxicity of KI was enhanced with an increase in PS concentration (Figure 5A,B).

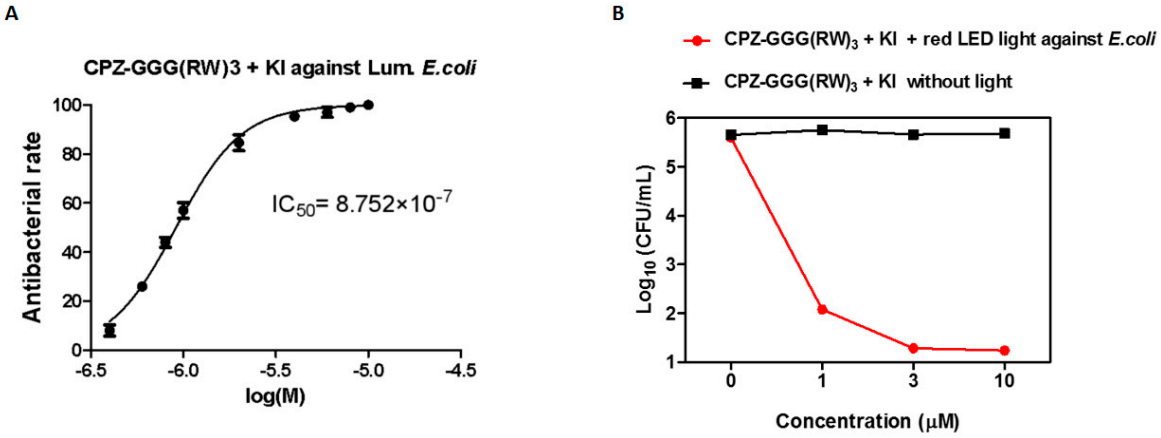

Figure 5. Phototoxicity of CPZ-GGG(RW)3 and potassium iodide against the luminescent *E. coli* strain under red LED light illumination (22.5 J/cm^2) measured by luminescent signals (**A**). *Phototoxicity of* CPZ-GGG(RW)3 and potassium iodide under illumination (22.5 J/cm^2) by red LED light against Gram-negative *E. coli* measured by colony forming units (**B**).

3.6. Safety of CPZG-GG(RW)3 toward Human Red Blood Cells

The photodynamic effect of the PS and the positive charges of a molecule may cause toxicity toward human erythrocytes. We used the hemolytic assay to evaluate the toxicity of the PS toward mammalian cells. In our experiment, we treated human red blood cells (RBCs) with different concentrations (0.1, 0.3 and 0.5 μm) of PSs, which were higher than the IC_{50} value obtained in our PACT experiment. We found that ZnPC-GGG(RW)3 showed 10% and 2% photohemolysis at higher concentrations (0.5 and 0.3 μm) compared with water hemolysis used as a positive control. The dark hemolysis of the PS showed no obvious lytic effect on human erythrocytes (Figure 6).

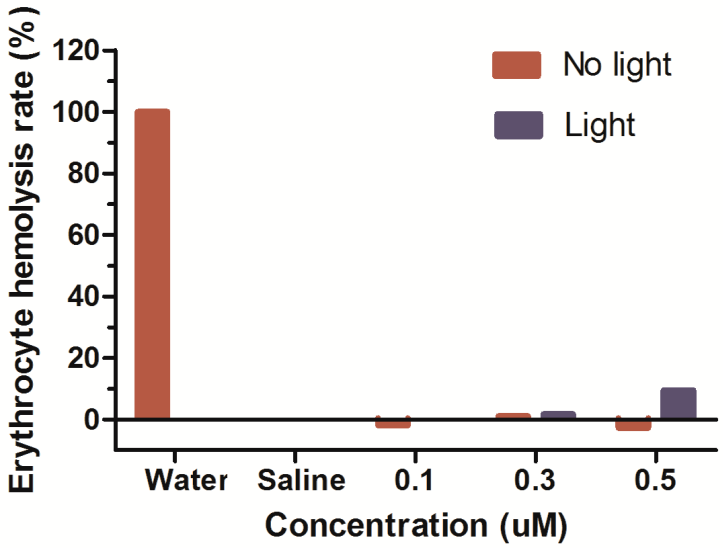

Figure 6. Study of the hemolytic effect of CPZ-GGG(RW)3 under LED red light illumination and in dark control against human erythrocytes.

4. Discussion

The increasing bacterial resistance to common antibiotics is a major threat to public health. The new promising alternative of using antimicrobial peptides is currently under

study to overcome this health crisis. Herein, we used antimicrobial peptide GGG(RW)3, an analogue of MP-196 (RWRWRW-NH_2), mimicking the hexa-peptide antimicrobial effect. The peptide sequence rich in Try and Arg was found to be the shortest antimicrobial peptide delocalized to the peripheral membrane protein of Gram-positive bacteria, which trigger bacteria cell death. We further conjugated GGG(RW)3 with either ZnPc or CPZ for photodynamic application to find a more potent therapeutic agent. The results show that a photodynamic inactivation assay using a low dose of CPZ-GGG(RW)3 is enough to trigger bacterial killing. The photodynamic activity of ZnPc is greatly enhanced by the antimicrobial peptide, as the peptide itself is not cytotoxic enough at a very low concentration. The peptide, therefore, acts as a targeting agent to enable the photodynamic activity of the PS. The photoinactivation efficacy of the conjugate is significantly higher than that of the MP-196 analogs reported earlier, for which the lethal concentration to bacterial inhibition is up to a micromolar concentration [29].

Based on the reported studies in the literature of the MP-196 peptide sequence [29,30], one can envision that ZnPc-GGG(RW)3 targets the lipid membrane of Gram-positive bacteria to induce the fast killing effect. As the photodynamic inactivation was achieved at a low concentration, the conjugates were also able to show antimicrobial effects in the dark but only at a high concentration in our setup, where 50% inhibition of *S. aureus* was attained. The potency of the PS was further examined against the Gram-negative *E. coli* strain, and it was found that the PS (CPZ-GGG(RW)3) conjugate found negligible phototoxicity against *E. coli*. The affinity of CPZ-GGG(RW)3 toward Gram-positive bacteria is accredited to the different morphologic structures of their cell membranes. The outer membrane of Gram-negative *E. coli* have a highly organized structure, which acts as a permeability barrier for the penetration of PSs into the cells. Moreover, in the synergy of the PS and KI salt, we observed a significant photodynamic effect against the *E. coli* strain. In this work, we explored whether the efficacy of PSs can be potentiated by using KI salt. The importance of inorganic salts in PACT in combination with PSs is reported by M. Hamblin to photoinactivate Gram-negative bacterial strains. They reported a Photofrin (PF) PS potentiated by KI salt for broad-spectrum therapeutics, and they established that potassium iodide at a 100 mm concentration with PF (10 μm) after illumination with a light dose of 10 J/cm^2 eradicated (>6-log reduction) several Gram-negative bacterial strains (*Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis*), while no such photoinactivation effect was seen without KI salt [22].

Based on our current study, many reports have examined the conjugation of photosensitizers with AMPs to study the synergistic and additive biological effects of the PS and AMP conjugate. An amphiphilic AMP was conjugated to a PS by Johnson et al. and killed Gram-positive and Gram-negative bacteria. The PS eosin Y was conjugated to AMP KLA $(KLAKLAK)_2$, and it was observed that the conjugate (1 μm) was able to photoinactivate 99% of the Gram-negative and Gram-positive bacteria. However, both eosin Y and AMP showed no antimicrobial effect alone under the same experimental conditions. Furthermore, the conjugate showed 10% photohemolysis, indicating that the photolytic effect toward bacteria is more pronounced than toward eukaryotic cells [18]. Porphyrin was also conjugated to apidaecin as an AMP by Doselli et al. to study the PDI against *E. coli* and *S. aureus*. The conjugate showed no dark toxicity, which probably suggests that the uptake of the conjugate was prevented by porphyrin inside the bacteria cells. Interestingly, the porphyrin–apidaecin conjugate was able to efficiently kill bacteria after light illumination, and it exhibited significant photodynamic activity against *E. coli* and *S. aureus* strains [31]. In the current study, our results show that the CPZ-GGG(RW)3 conjugate is particularly potent against Gram-positive *S. aureus* with an IC_{50} value of only 4 nm required to significantly eradicate the bacterial strain, whereas such an antibacterial effect was not found for the peptide when using the same concentrations of both compounds. Moreover, the dark experiment showed that CPZ-GGG(RW)3 also reduces the survival of *S. aureus* by 50% at a concentration of 1.1 μm. We therefore propose that, unlike what has been found for other reported PS–AMP conjugates, the CPZ-GGG(RW)3 conjugate still has an antimicrobial

effect in the dark at a low concentration, and the concentration of the conjugate would need to be further reduced to achieve the same PDI effect. These results clearly reflect that additional glycine moieties (GGG) and ZnPC do not affect the efficacy of MP-196 analogues reported in the literature.

The key importance of the antimicrobial peptide is to improve the selectivity of the PS toward microbial cells. An ideal PS kills the bacterial cell without affecting the host tissues. The reported MP-196 analogues are benign to mammalian cells, and no significant hemolytic or cytotoxic activity was observed in the literature. In previous reports, no hemolytic activity of CPZ against mammalian cells was observed [32]. Based on these previous studies, we can envision that the CPZ peptide conjugate (ZnPc-GGG(RW)3) would selectively target the bacterial membrane without affecting mammalian cells. We studied the hemolytic assay of the conjugate treated with RBCs in the dark and under illumination. Approximately 10% photohemolysis was obtained at a high concentration, where the maximum photoinactivation of the bacteria was achieved. In the dark, no noticeable hemolysis was observed at any concentration of the conjugate. The conjugate demonstrated the potential safety of the PS for human red blood cells. To further ensure the safety benefits for mammalian cells, the long-term toxicity needs to be evaluated for future clinical applications. The cellular uptake of the PS involved the incubation of the bacteria with various concentrations of PSs for a specific period. The unbound PS was subsequently removed by PBS, followed by a spectrophotometric determination of the bacterial-bound PS fluorescence intensity. The binding results indicated that the uptake of the PS occurred in a concentration-dependent manner. The uptake of the PS was comparable to that of the MP-196 derivative reported in the literature. The detailed study conducted and confirmed in vivo peptide tracing using the ruthenocene-substituted MP196 derivative (MP276). The detection of MP276 in a cytosolic fraction suggests the strong binding ability of the peptide [9]. We also showed that ZnPc-GGG(RW)3 generated ROS through the type 1 mechanism, producing OH radicals effectively, and it converted DCFH-DA into a highly fluorescent DCF product. This ROS highly produced by the PS which subsequently eradicated the bacterial strains.

5. Conclusions

In this report, we established the conjugation of the antimicrobial peptide (GGG(RW)3) to a zinc phthalocyanine-based photosensitizer. The peptide showed selectivity toward the Gram-positive strain of bacteria. The CPZ-GGG(RW)3 conjugate induced a 6-log reduction in Gram-positive *S. aureus* upon illumination, while the peptide did not exhibit such a significant effect. The effectiveness of CPZ-GGG(RW)3 in PACT is comparable to that of GGG(RW)3, which was found to be highly potent using a low concentration of PS. Nevertheless, it is fascinating that the selectivity and photoactivity of a PS-AMP conjugate could potentially be increased. Furthermore, the synergistic effect of KI salt and the PS substantially killed *E. coli* strains with a 5-log reduction in CFU. The PS-AMP conjugate also showed antimicrobial activity in the dark against Gram-positive *S. aureus*.

Author Contributions: Conceptualization, G.R., A.U. and P.F.; methodology, G.R., R.K. and A.A.; software, G.R., R.K. and A.A.; validation, G.R., R.K. and A.A.; formal analysis, A.U., P.F. and M.S.K.; investigation, M.S.K., M.R.S. and M.A.; resources, M.A., A.S.A. and K.H.M.; data curation, M.S.K., M.R.S., A.S.A. and K.H.M.; writing—original draft preparation, G.R., A.U. and P.F.; writing—review and editing, G.R., A.U. and M.A.; visualization and supervision, M.R.S., A.U. and M.A.; project administration, A.U. and M.A.; funding acquisition, A.S.A., K.H.M. and M.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors would like to acknowledge the financial support of Taif University Researchers Supporting Project number (TURSP-2020/189), Taif University, Taif, Saudi Arabia, and the Higher Education Commission (HEC) of Pakistan.

Conflicts of Interest: The authors declare no conflict of interest.

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