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Antimicrobial Photodynamic Therapy with Single Application of Phthalocyanines in *Staphylococcus aureus* and *Escherichia coli* Wounds Infections

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ABSTRACT

Phthalocyanines are considerable interest due to their general chemical and thermal stabilities. They are photochemical materials used in photodynamic therapy (PDT) and also as dyes due to their intense colors. In this study, we evaluated some phthalocyanines for their wound healing properties in mice models. Four groups of mice were tested for healing of wounds infected with *Staphylococcus aureus* and *Escherichia coli*. Animals were induced with a skin injury and infected 30 minutes later by applying *Staphylococcus aureus* into each right dorsal wound and *Escherichia coli* into each left dorsal wound. Three groups treated with phthalocyanine-containing ointments and the fourth group was used as control that was treated with ointment base only. The source of red light was applied for 5 minutes on these mice and observed them for one week. In treatment groups, the wounds had shown healing within the treated mice compared with the control group. The compound 1(PcZn) was more affective on *E coli* than *S. aureus*, compound 2($(4\text{-}NH_2)_4$PcZn.$2H_2O$) more potency on *S. aureus* than *E coli* and the compound 3($(4\text{-}NO_2)_4$PcZn) has similar potency on both, *S. aureus* and *E coli*. In conclusion, these results show that the three phthalocyanine compounds were active as bactericidal on both *S. aureus* and *E. coli in vivo*, and helpful for rapid wound healing. These compounds might be suggested for treatment of infected burns.

KEYWORDS: Photodynamic therapy; photosensitizer; PcZn; $(4\text{-}NH_2)_4$PcZn.$2H_2O$; $(4\text{-}NO_2)_4$PcZn; photo-antimicrobial; antimicrobial, wounds infections.

Introduction

Phthalocyanine compound (Pc) is one type of the porphrazinic microcyclic compounds containing a ring system of four isoindole units linked together by aza nitrogen atoms. Thus it has a porphrazine base and similar to the porphine base which has methene linkage between the isoindole units (Moser and Thomas, 1963). Most of the elements of the periodic table combine with the phthalocyanine ring different phthalocyanine complexes (PcM). Since they have been produced in the beginning of the twentieth century, they were under great interest due to their interesting properties, such as their intense colors, chemical and thermal stabilities, photo and electrical conductivities and photo dynamic therapy (PDT) (McKeown, 1998; Kadish et al., 2003).

Phthalocyanies have several unusual properties. They have low solubility in approximately most solvents and don't melt but sublime at high temperatures. They strongly absorb light in the red range of the optical spectrum (about 600 to 700 nm), thus they act as dyes and are characterised as blue or greenish pigments. Porphyrins, porphrazine and phthalocyanines act as planar tetradentated anionic ligands which bind metallic ions through four inwardly projecting nitrogen centres. Many peripherally subistituted derivatives of the phthalocyanine are known. In addition to the ring-substituted derivatives, there are also subphthalocyanines(with three isoindole units) and superphthalocyanine (with more than four isoindole units) (De Diesbach and Weid, 1927; Simon and Andre, 1985).

Photodynamic therapy (PDT) is a bimodal therapeutic strategy based on the photo-oxidative action of drug. They act as photosensitizer (PS) which is induced by visible red light. The PS activity depends on the preferential accumulation of PS on the targeted microorganism's membrane. Selective accumulation of PS on target cells, highly selective in destroying bacteria and yeast (also resistant), leaving tissues unharmed and show no skin photosensitivity (Löbbert, 2002; Leznoff and Lever, 1993; Wong et al., 2005). Phthalocyanines give a great yield of singlet oxygen than other photosesitizers, and tuneable properties by chemical modification. Several positions could be substituted, yielding from + to – charged Pc that have a particular

cell membrane binding spectrum and hence have a specific cidical activity. Phthalocyanines are used for localized infections. PS is delivered locally to the infected area topically or by subcutaneous administration. No induction of resistance by multiple PDT treatments. Photobactericidal testing of phthalocyanines has also been carried out *in vitro*. The photo-killing of *Streptococcus sanguis* in biofilms and also of methicillin-resistant *Staphylococcus aureus* by aluminium-phthalocyanine. Metal-free tetra(tert-butyl) phthalo-cyanine has been incorporated into polymer films as a photobactericidal material which is effective against *S. aureus*. That the bacteria were killed only on illumination suggests that the site of action must be the cell wall, the photosensitizer was immobilized and so could not enter the bacterial cell, thus singlet oxygen generated at the polymer-cell interface would react immediately on contact with the cell wall. The use of phthalocyanines against various forms of HIV infection has been reported example, silicon phthalocyanine bearing a cationic dialkylaminoalkylsilyloxy-residue on the central silicon was active not only against cell-free HIV but also against the actively replicating virus and latently infected red blood cells. Such an activity profile against viruses is obviously highly desirable although there remains the problem of red blood cell damage which requires the addition of an antioxidant such as vitamin E. It has also been reported that the use of high irradiance in conjunction with sulphonated aluminium phthalocyanine is less toxic to red blood cells. In addition, the synchronous use of thiol-containing species such as reduced glutathione. In terms of structure–activity relationships for the phthalocyanines, there appears little general correlation between the antiviral potency and the central atom of the phthalocyanine, although against vaccines virus the activity increased in the order Ga(III) < Al(III) < Zn(II) (Engelhardt et al., 2010; Simonetti et al., 2011; Jori et al., 2006).

In this study, we evaluated some phthalocyanines for their wound healing properties in mice models.

Materials and Methods

Synthesis of the Phthalocyanine Compounds

The used chemicals were purchased and used as received: Phthalic anhydride, urea, nitrobenzene and ammonium molybdate were purchased from BDH. Acetone, HCl, H_2SO_4 and HNO_3 from Riedel Dehaen. Benzene from Fluka, hydrated zinc sulphate and $Na_2S.9H_2O$ from Aldrich.

The measurements were done as follows: CHN, EA-1108 Erba Elemental analysis. IR, 300-SP3 IR spectrophotometer. UV-visible 4050 LKB Ultraspec.

Synthesis of Phthalocyanine Zinc (II), PcZn

In around bottomed flask fitted with condenser, a mixture of (4.73 g, 0.064 mole) of phthalic anhydride, (2.73 g, 0.019 mole) of hydrous zinc sulphate, 10 g of urea, 0.2 g of ammonium molybdate in 50 ml of nitrobenzene were refluxed for 2 hours. The reaction mixture then filtered and washed with ethanol, benzene, carbon tetrachloride and acetone and then dried at 110°C. Due to the insolubility of the product in most of the known solvents, then it was purified by refluxing it with a solution of 0.1 M of HCl for 1.5 hours, followed by refluxing it with a solution of 0.1 M of NaOH for 1.5 hours too. Filtered and washed with distilled water till it was free of base using litmus paper as indicator. The solid product then refluxed with benzene acetone respectively for ½ hour for each. Then dried at 110°C. The final product was blue crystalline solid (yield, 3.2 g, 70%) (Roger et al., 1999).

The product was identified by CHN analyses, IR, UV-visible electronic spectroscopy. The analyses was as followings: CHN, calculated ($C_{32}H_{16}N_8Zn$) C: 66.51, H: 2.08, N: 19.397; Found: C: 66.15, H: 1.86, N: 19.01; IR (KBr): (3100 w, 1615 m, 1540 s) cm^{-1}; UV-visible (λ_{max}, $CHCl_3$): (685(Q band), 350 (sort band)) nm.

Synthesis of 4-nitro-phthalic Acid

In a round bottomed flask fitted with condenser and separating funnel, a mixture of (100 g, 0.675 mole) of phthalic anhydride and 100 of concentrated H_2SO_4 was heated to 100°C. The flask then was transferred to a water bath, 42 mL of fuming HNO_3 was added (which was prepared from the distillation of 3 volumes of HNO_3 and 1 volume of concentrated H_2SO_4. The first volume was taken). The fuming nitric acid was added drop wise, using separation funnel. The mixture then heated for 2 hours with mechanical stirring and keeping the temperature below 110°C. The reaction mixture then cooled and left overnight. The reaction mixture is containing two isomers, 3-nitro and 4-nitro phthalic acid. The 3-nitro isomer was precipitated while the 4-nitro isomer is soluble. The reaction mixture then filtered and the 4-nitro isomer was separated from the filtrate by extraction with ether. The extract then dried. The product was pale yellow (melting point 165°C, literature 164°C-165°C) (Vogel, 1974).

Synthesis of $(4\text{-}NO_2)_4$ PcZn

In a round bottomed flask, a mixture of (3.375 g, 0.064 mole) of 4-nitro phthalic acid, 2.73 g, 0.019 mole) of hydrated zinc sulphate, 10 g of urea, 0.2 g of ammonium molybdate in 50 ml of nitrobenzene. The reaction procedure and purification was carried out exactly as in the preparation of PcZn. The product was a blue crystal (yield, 2.7 g, 70%). The product was identified by CHN analysis, IR, UV-visible electronic spectroscopy.

The analysis were as followings: CHN, calculated ($C_{32}H_{12}N_{12}O_8Zn$) C: 50.70, H: 1.58, N: 22.18 ; Found: C: 50.10, H: 1.48, N: 21.83; IR (KBr): (2900 w, 1525 s, 1340 s, 1230 w, 1180 m, 745) cm^{-1}; UV-visible (λ_{max}, $CHCl_3$): (685 (Q band), 355 (sort band)) nm.

Synthesis of $(4\text{-}NH_2)_4$PcZn.$2H_2O$

In a round bottomed flask, a mixture of 1 g of 4-

nitrophthalic acid and a solution 5 g hydrated sodium sulphide ($Na_2S.9H_2O$) in 25 mL of H_2O was added. The mixture then heated to 50°C with mechanical stirring for 5 hours. The blue solid product the was separated using centrifuge and then purified by reflux with a solution of 0.1 M HCl, and a solution of 0.1 M of NaOH respectively, with mechanical stirring and for 1 hour for each. The product then filled and washed with water till it was free from base using litmus paper as indicator. The product then refluxed with acetone for 10 minutes filtered and dried at 110°C. The solid product was deep green crystals (yield, 0.8 g, 86%). The product was identified by CHN analysis, IR, UV-visible electronic spectroscopy.

The analysis were as followings: CHN, calculated ($C_{32}H_{24}N_{12}Zn$) C: 57.05, H: 3.56, N: 24.96; Found: C: 56.71, H: 3.01, N: 25.72; IR (KBr):(3305 w, 2950 w, 3320 w, 1285 m, 1145 m, 775s) cm^{-1}; UV-visible (λ_{max}, $CHCl_3$): 740(Q), 360 (sort)) nm.

Study Design

This study was performed in the Laboratory of Animal Research Unit, College of Pharmacy, University of Basrah. All the mice in this study were purchased from the animal house of college of education. The sample size was estimated using the two proportions formula with an 82% confidence interval. The sample size calculation was performed using the Power and Sample Size Calculation system (PS), with a type 1 error of 0.025. The inclusion criteria included albino mice weighing between 20 g and 30 g. The mice were housed individually in cages, and fed with free access to standard commercial mice food and water throughout the study. Twenty four mice both sexes were divided into 4 groups randomly, six mice in each group. Three testing groups were used for sample treatment and the forth as control group). The back of each mouse was shaved. Immediately before the operation, the shaved areas were cleaned with povidone iodine and alcohol. The wound marked area was injured with using a surgical sterilized blade in the right and left dorsal side. The wounds were subjected to evaluation in third day. Each wound was examined and photographs were taken after wound creation until healing was complete. Clinical assessments included observations concerning the appearance.

Excision Wound Model with Infection

Animals were infected 30 minutes later by applying clinical *Staphylococcus aureus* into each right dorsal wound and clinical *Escherichia coli* into each left dorsal wound. The animals were then monitored daily for survival differences between treated groups (Chalekson et al., 2003).

The phthalocyanines samples were prepared by weighting 1mg of each compound and mixed with 2.5 ml of vaseline and 2.5 ml of liquid paraffin. Group 1 for compound 1 (PcZn), group 2 for compound 2 ($(4\text{-}NH_2)_4$ PcZn) and group 3 for compound 3 ($(4\text{-}NO_2)_4$PcZn. Application of each phthalocyanines compounds as ointment on the wounds of each mouse to the both sides, left and right, and the control group with ointment base only. All the wounds in group 1 were treated with compound 1. All the wounds in group 2 were treated with compound 2. All the wounds in group 3 were treated with compound 3. All the wounds in control group were treated with ointment base only (Khoo et al., 2010; Grocott and Campling, 2009). The source of red light was applied for 5 minutes on these mice and notified them for one week. The areas of the wounds were measured (sq.mm) immediately by placing a transparent polythene graph paper over the wound and then tracing the area of the wound on it. This was taken as the initial wound area reading. The percentage of wound contraction was calculated from the days of measurements of wound area and evaluation of the wounds as in Table 1 (Braham et al., 2011; Fletcher, 2010).

TABLE 1
Wound evaluation system.

Wound contraction	**Very contracted**	**Contracted**	**Not contracted**	
	1	2	3	
Redness area	small	medium	large	
	1	2	3	
Inflammation volume	small	medium	large	very large
	1	2	3	4

Results

Treatment shows wounds healing of test groups (1-3) in compared with the control group, after 24 hours and day after day for one week. Figure 1 shows pictures of control and tested groups treatment after 3 days.

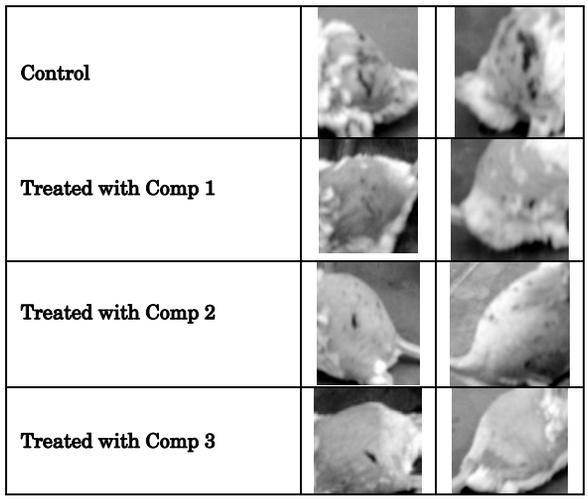

Fig. 1. Effect of pthalocyanines on experimental wound healing in mice model of bacterial infections. The wound healing was evaluated as per the rating listed in Table 1. The wounds on the back of each mouse treated with phthalocyanine test, show clear healing, especially these wounds which contaminated with *E coli* bacteria, During 5 days the wound will be healing. Drug containing PcZn is more preferred on treatment than the others.

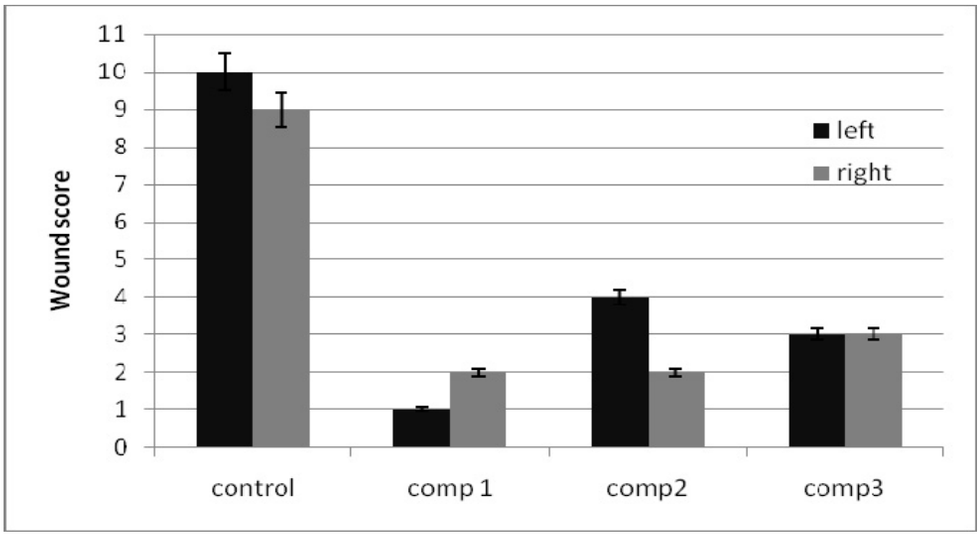

Fig. 2. The evaluation of wound treatment with phthalocyanine compounds.

The mice in control group were appeared worse wound and the activity of these mice will be decrease due to immune disease. The red light acts as facilitating factor will increase activity of phthalocyanine compounds. We prefer red light than other due to its long wave length and with low energy level, so, it will not damage the tissue. The Phthalocyanine compounds were acted on (Gram-negative) *E. coli* bacteria and (Gram-positive) *Staphylococcus aureus* bacteria, the compound 1 was more affective on *E coli* than *S. aureus*, compound 2 more potent on *S. aureus* than *E coli*. The compounds 3 have same potent on *S. aureus* and *E coli*. Hence the results of this study confirm that the compounds possess anti-bacterial activity and this will help keep the wound area sterile, thus promoting wound healing. This fact supports a faster wound healing in the treated groups compared with the control group (Bhat et al., 2007). In control group the infection convert from topical infection to local infection, and then to regional infection, the animal not survived within one week.

Discussion

Zinc (II)-phthalocyanines are lipophilic photosensitizers that show promise agent for photodynamic therapy of hyper-proliferative conditions. In order to address the treatment of skin conditions, we have developed a topical formulation of these molecules. Therefore, we incorporated the photosensitizer in an ointment formulation using paraffin. Under standard storage conditions, there was little or no degradation or aggregation of the dye and this formulation will be used to explore the potential of photodynamic therapy in various skin diseases. The phthalocyanine compounds were very potent as bactericidal effectively on both *S. aureus* and *E coli in vivo*, and help the wounds to contract and prevents another microorganism to growth on the wounds so help the wounds curing very rapidly. It may be used for treatment of infected wounds and may be used for treatment of infected burns. The red light was essential for activity of phthalocyanine compounds, and was applying for a short time (5 minutes) only to appear activity and no harm become visible on the tissue.

This study was carried out using PDT to reduce bacterial infection in topical infection. Bearing in mind that the data refer to only a single treatment, it is clear that these compounds are effective in statistically reduced bacterial load in wounds. There was a strong trend towards accelerated healing in the phthalocyanines treated mice.

Conclusions

In conclusion, these results show that the three phthalocyanine compounds were active as bactericidal on both *S. aureus* and *E coli in vivo*, and helpful for rapid wound healing. These compounds might be suggested for treatment of infected burns.

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