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Influence of formulation on photoinactivation of bacteria by lumichrome

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Lumichrome, a photodegradation product of riboflavin, is an endogenous compound in humans. The compound is more photostable and a more efficient photogenerator of singlet oxygen than riboflavin. It absorbs radiation in the UVA and blue-light region, which can be an advantage in antibacterial photodynamic therapy (aPDT) of superficial infections. The aim of this study was to investigate the *in vitro* aPDT effect of various lumichrome pharmaceutical formulations. Solutions of lumichrome (10^{-5} – 10^{-3}M) were prepared in plain phosphate buffered saline (PBS) or in PBS solutions containing cyclodextrins, DMSO, PEG 400 or polyoxamers (Pluronic®). Supersaturated solutions of lumichrome in PBS were prepared *via* the cosolvent and solvent evaporation method. Phototoxic effects of selected lumichrome preparations were studied in planktonic Gram-positive (*E. faecalis*) and Gram-negative (*E. coli*) bacteria models. The UVA/blue light source emitted mainly in the range 340-440 nm. Lumichrome was up to tenfold more phototoxic against Gram-positive than to Gram-negative bacteria. Bacterial eradication was induced after exposure of lumichrome formulations (PBS, PEG 400 and HPγCD) combined with 24 J/cm^2 UVA/blue light. Increasing the concentration of lumichrome did not enhance the phototoxic effect, probably due to radiation attenuation in the highly absorbing solution (inner filter effect). Cyclodextrins were efficient enhancers of the lumichrome solubility in aqueous solutions, but inhibited the phototoxic effect. The study demonstrates that assuming the use of an optimized formulation, lumichrome has potential as a UVA/blue light photosensitizer in aPDT.

1. Introduction

Lumichrome (Lc) (Fig. 1a) is a photodegradation product of riboflavin (Rf) and an endogenous compound in humans. Lc and Rf are also present in a wide range of food and natural products. Rf is applied as a photosensitizer for the inactivation of pathogens present in blood components (Goodrich 2000; Ruane et al. 2004; Cui et al. 2008; Mundt et al. 2014), in the treatment of infectious keratitis (Wollensak et al. 2003; Wollensak 2006; Martins et al. 2008; Wollensak 2010; Makdoumi et al. 2012) and is suggested for use in dental disinfection systems (Bouillaguet et al. 2008). Lc is nearly 100 times more photostable than Rf and is a more efficient photogenerator of singlet oxygen in aqueous media ($\Phi_{Lc} = 0.63$ vs $\Phi_{Rf} = 0.48$) (Remucal and McNeill 2011). Further, the log P value of Lc (1.27) is approximately 13 times higher than that of Rf and the polar surface area (82.9) is about half the value of that of Rf (SciFinder 2014). Thereby, Lc has a higher potential for uptake in a lipophilic membrane than Rf. The UV-Vis absorption characteristics of Lc are pH dependent (Marchena et al. 2011; Prukala et al. 2012). Lc in the ground state can undergo deprotonation in two steps leading to the formation of the mono- and dianion, respectively. The estimated pKa values for these protonation steps are 8.2 and 11.4 (Prukala et al. 2012). The compound can undergo photo-induced tautomerization to form an isoalloxazine structure (Fig. 1b). This process is strongly dependent on the microenvironment (Miskolczy and Biczók 2005). Both ground-state deprotonation and excited state tautomerization will induce spectral changes (Sikorska et al. 2004; Marchena et al. 2011; Prukala et al. 2012). At physiological pH the neutral form of the molecule is predominant. This form presents two main absorption bands in water; at 355 nm (S_0-S_2 transition) and at 400 nm (S_0-S_1 transition) (Tyagi and Penzkofer 2011). Lc can thereby be excited by UVA or blue light radiation, although the absorbance above 400 nm is limited. Most of the current antibacterial photodynamic therapy (aPDT) photosensitizers are water soluble, cationic compounds absorbing visible light above 550 nm (e.g., methylene blue, rose bengal) (Takasaki et al. 2009). Light in this wavelength range penetrates deeper into healthy tissues and carries less energy than radiation of shorter wavelengths (e.g., blue light, UVA). aPDT is intended for treatments of localized infections where a shallow light penetration depth can be advantageous to avoid harmful effects on underlying healthy tissue. Further, UVA radiation and blue light are shown to inactivate certain bacterial species even in the absence of a photosensitizer (Dai et al. 2012; McKenzie et al. 2013; St Denis et al. 2013) and can thereby increase the effect. The number of efficient aPDT photosensitizers reported that can take advantage of UVA or blue light is however, limited and new photosensitizers are sought (Bouillaguet et al. 2008). The phototoxic potential of Lc also warrants consideration because it rapidly becomes dominant when Rf is irradiated (Ahmad et al. 2006; Diakonis et al. 2012). The application of a stable compound rather than the labile Rf molecule facilitates standardization of the aPDT preparation and *in vivo* protocol as well as handling of the preparations by reducing the need for light protection.

Lc has a rather low water solubility (0.02 mM) (Lilletvedt et al. 2010). Nanocarriers (e.g., cyclodextrins, micelles) or cosolvents

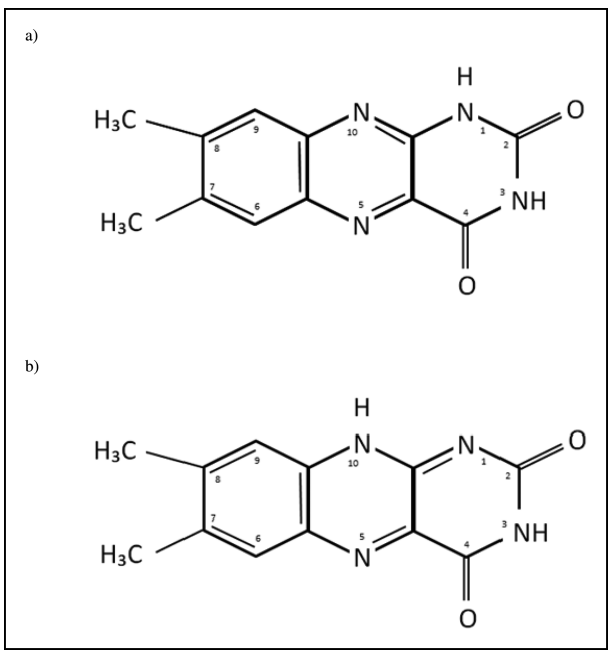

Fig. 1: Structure of Lc (a) and its tautomer (b).

can be used to increase the water solubility of Lc in order to achieve a therapeutic dose. Cyclodextrins (CDs) are reported to be very efficient solubility enhancers of Lc in aqueous solutions. A 1:1 inclusion complex has been predicted between Lc and CDs (Sarkar et al. 1995; Terekhova et al. 2011). The influence of different excipients on this complexation has previously been investigated by our group (Lilletvedt et al. 2010). Further, we have demonstrated that the phototoxic effect of another neutral photosensitizer (i.e., curcumin) could be strongly enhanced by application of a supersaturated solution (Hegge et al. 2012, 2013). The aim of the present study was to evaluate the increase in solubility and investigate the phototoxic effect of Lc in various aqueous preparations on Gram-positive (G^+) and Gram-negative (G^-) bacteria *in vitro*. Lc was dissolved in PBS at pH 7.4 in the presence of hydroxypropyl-α-cyclodextrin (HPαCD), hydroxypropyl-β-cyclodextrin (HPβCD), hydroxypropyl-γ-cyclodextrin (HPγCD), a cosolvent (DMSO) or surfactants (PEG 400 and Pluronic® F127). Supersaturated aqueous Lc solutions were prepared by the co-solvent or solvent evaporation methods.

2. Investigations and results

2.1. Solubility studies

The equilibrium concentration of Lc in eleven aqueous solutions was determined at 22 ± 1 °C (Fig. 2). A concentration of 5% PEG 400 and 5% DMSO enhanced the Lc concentration by approximately 30% compared to a plain PBS aqueous solution. Pluronic® F127 was used below the critical micelle concentration (CMC) (Batrakova et al. 1999; Sezgin et al. 2006), and no enhancement in solubility compared to plain PBS was obtained. The solubility of Lc in organic solvent such as methanol (MeOH) was approximately six times higher than in PBS. The concentration of Lc increased thirty times by addition of 10% HPβCD compared to plain PBS solution while only a four and seven times increase were obtained with 10% HPαCD and 10% HPγCD, respectively (Fig. 2). A concentration of 3% CD did also enhance the Lc concentration in the sequence β>γ>α.

In order to obtain a higher concentration of Lc in aqueous solution without using a nanocarrier (i.e., cyclodextrins), the co-solvent and solvent evaporation methods were used. With the co-solvent method a concentration of 0.31 mM was achieved. This solution was stable for at least 30 min (< 1% average decrease in absorbance at 386 nm). After 90 min < 2% average decrease in absorbance was measured and still no precipitation was observed. With the solvent evaporation method a concentration of 0.52 mM Lc was obtained. The absorbance of the solution at 386 nm remained constant for 30 min. After 60 min Lc had precipitated and a 15% average decrease in absorbance was measured. The solution obtained by the solvent evaporation method was included in the bacteria phototoxicity experiments.

2.2. Antibacterial phototoxicity studies

2.2.1. Effect of irradiation

The effect of irradiation in the absence of the photosensitizer was studied on *E. faecalis* and *E. coli*. Exposure to about 12 J/cm^2 or 24 J/cm^2 did not lead to any significant bacterial reduction of *E. faecalis.* In the case of *E. coli a* 0.25 and 1 log reduction was obtained after exposure to 12 J/cm^2 and 24 J/cm^2 respectively ($p < 0.05$).

2.2.2. Antibacterial phototoxicity of lumichrome

The phototoxic effect of combined exposure of Lc and UVA/blue light irradiation on *E. faecalis* was investigated using a 5% PEG 400 preparation at two different Lc concentrations (0.06 mM and 0.52 mM) using Option 1 lamp. The bacterial survival decreased with increasing radiant exposure (Fig. 3). The sample with the lowest Lc concentration was the most efficient, inducing 3 log reductions after 30 min exposure (~12 J/cm^2). The sample with the highest Lc concentration induced only a 2 log reduction after 30 min exposure.

The influence of various post-irradiation incubation times (0, 15, 30 and 60 min) on bacterial death of *E. coli* after treatment with Lc 0.06 mM solutions containing 5% PEG 400 was investigated. The degree of bacterial death was similar irrespective of the post-irradiation time. A 15 min post-irradiation time was chosen in the further studies to ensure completion of dark toxicity reactions following phototoxicity reactions.

The antibacterial phototoxicity of Lc was investigated with various formulations, Lc concentrations and radiant exposures (Table). The excipients used have previously been shown not to affect bacteria in the absence of the photosensitizer (Haukvik et al. 2009). Lc showed to be more effective against G^+ bacteria compared to G^- bacteria independent of formulation. With a radiant exposure of about 12 J/cm^2 (Option 1) 5% PEG 400 and 3% HPγCD were the most efficient Lc preparations against both bacterial strains, resulting in 0.3% and 0.1% mean survival of *E. faecalis* and 7% and 9% mean survival of *E. coli,* respectively (Figs. 4, 5). The least efficient Lc formulations were those containing HPβCD which gave reduced effect compared to Lc in plain PBS solution at a lower Lc concentration (0.04 mM) (Figs. 4, 5). When selected formulations were tested against *E. faecalis* and *E. coli* with twice as high radiant exposure (~24 J/cm^2) (Option 2) *E. faecalis* eradication was induced (6 log reductions) independent of the formulation (Fig. 6). All the Lc formulations also induced bacterial eradication of *E. coli*, with the exception of 0.1 mM 3% HPβCD for which a 5% survival (3 log reduction) was detected.

3. Discussion

A low aqueous solubility is a major limitation for the use of Lc as a photosensitizer. An increase in solubility of Lc was achieved in the presence of a cosolvent (DMSO), surfactant (PEG 400) or a nanocarrier (CD); the latter being clearly the most efficient

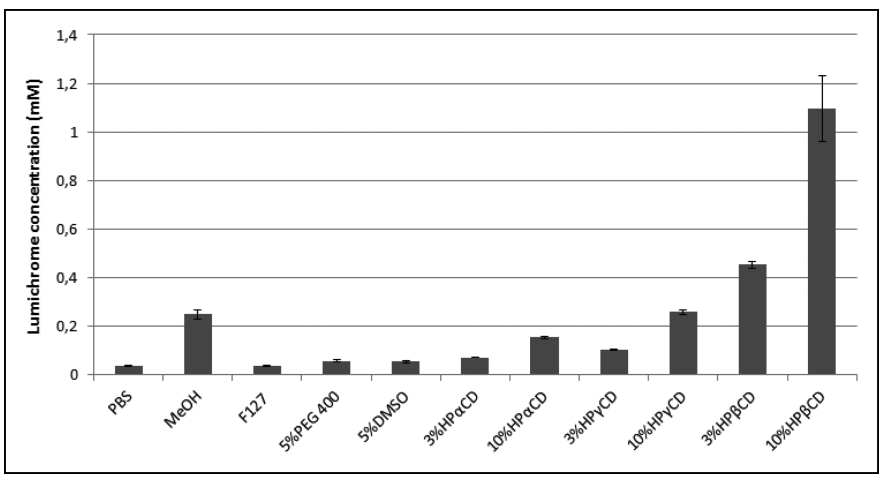

Fig. 2: Mean molar solubility (S_0) of Lc in different solvents at 22 ± 1 °C (±SD; n = 5). F127 = Pluronic® F127.

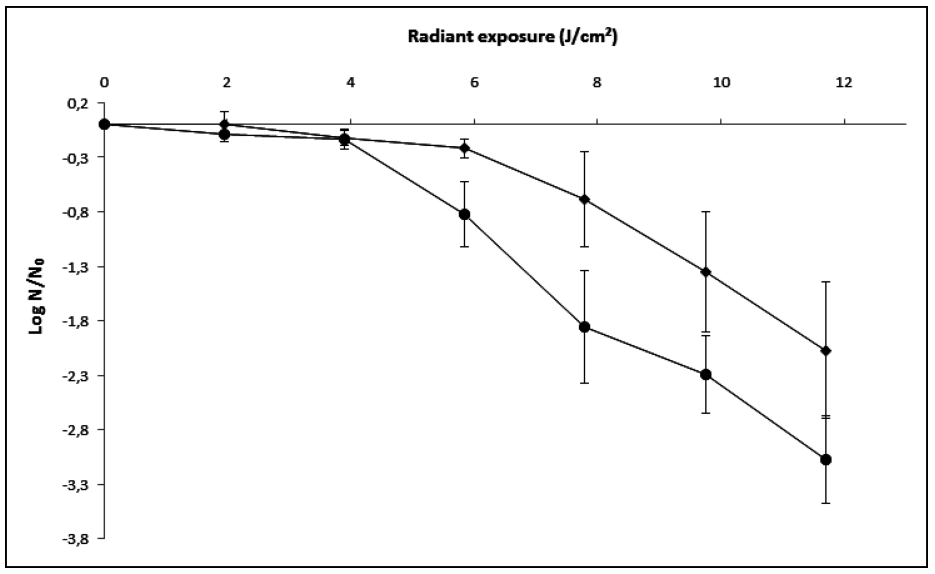

Fig. 3: Reduction in colony forming units (CFU/ml) of *E. faecalis* after exposure to two Lc concentrations in 5% PEG 400 and UVA/blue light as a function of irradiation dose. N: number of CFU/ml after exposure; N_0: number of CFU/ml in the absence of Lc formulation and irradiation (±SD; n = 6). 0.06 mM Lc (●); 0.52 mM prepared by the solvent evaporation method (⧫).

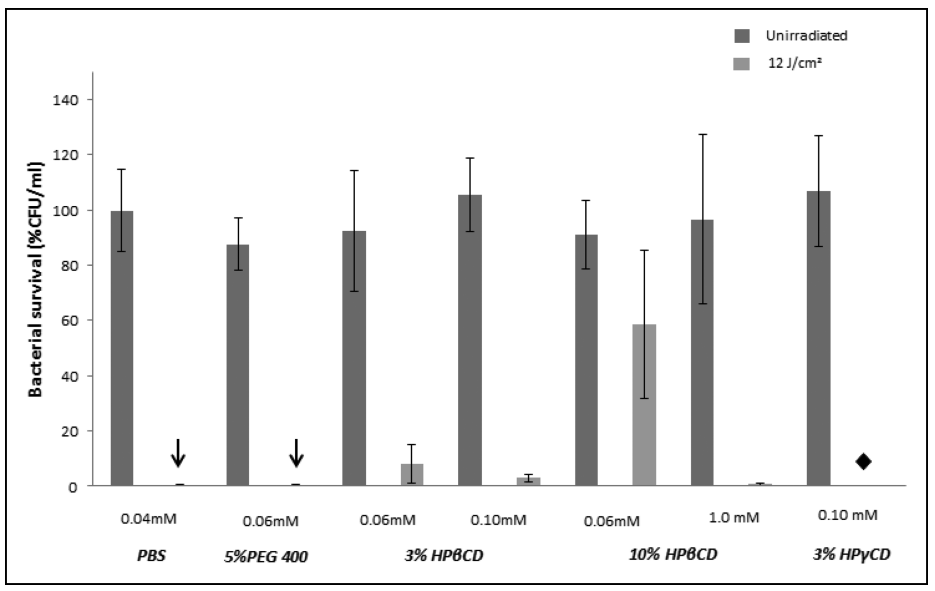

Fig. 4: Phototoxic effect against *E. faecalis* after exposure to Lc in selected PBS preparations combined with UVA/blue light (~12 J/cm²). Values represent mean CFU/ml of bacterial survival (%) compared to controls (± SD; n = 9). Arrows (↓) < 0.5% bacterial survival; diamond (⧫) < 0.1% bacterial survival.

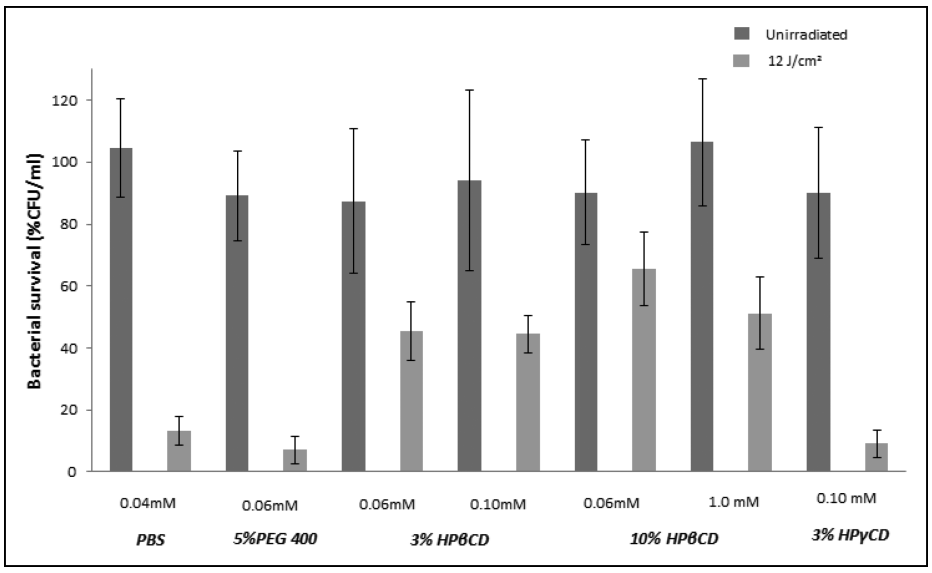

Fig. 5: Phototoxic effect against *E. coli* after exposure to Lc in selected PBS preparations combined with UVA/blue light (∼12 J/cm^2). Values represent mean CFU/ml of bacterial survival (%) compared to control (± SD; n = 9).

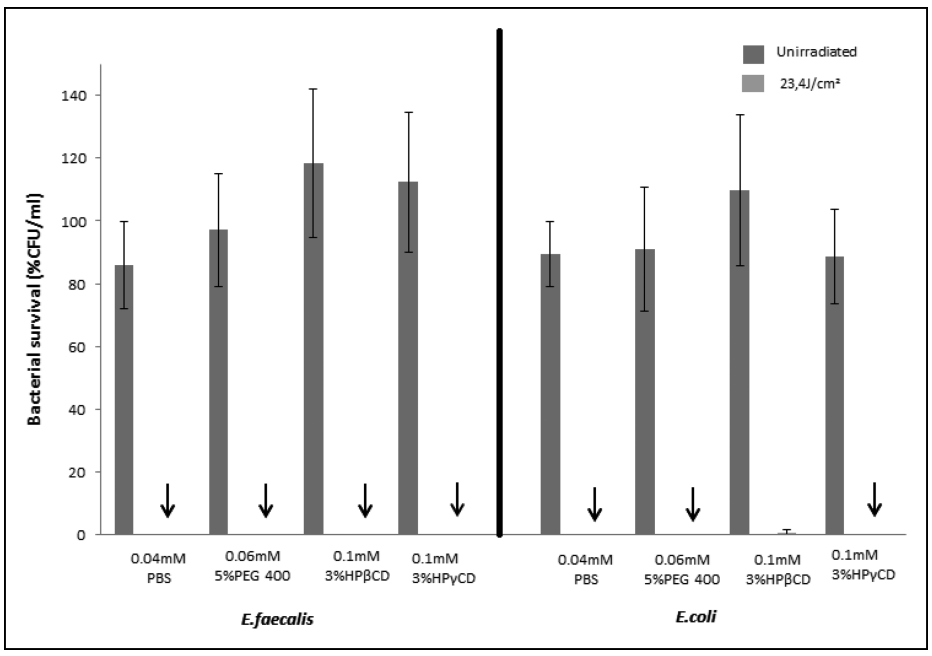

Fig. 6: Phototoxic effect against *E. faecalis* and E. coli after exposure to Lc in selected PBS preparations combined with UVA/blue light (∼24 J/cm^2). Values represent mean CFU/ml of bacterial survival (%) compared to control (± SD; n = 9). Arrows (↓) 100% bacterial death (no surviving colonies).

solubilizer (Fig. 2). The finding that HPβCD was the most efficient solubilizer among the tested CDs, followed by HPγCD and HPαCD, was consistent with a report on the interaction between Lc and αCD or βCD (Terekhova et al. 2011). The Lc solubility in organic solvents (i.e., MeOH) was higher than in all the aqueous solutions studied, with the exception of those containing 3 and 10% HPβCD or 10% HPγCD; for the latter one similar solubility of Lc was observed. An increased concentration of Lc in aqueous media in the absence of a nanocarrier was achieved by the solvent evaporation method and the co-solvent method. These preparation methods could be useful in cases where a high Lc concentration is required without addition of a nanocarrier. The Lc supersaturated solutions obtained had a physical stability sufficient for handling and phototoxicity investigations.

The phototoxicity studies showed that Lc solubilized in plain PBS or PBS containing a cosolvent, surfactant or CD was phototoxic to both G^+ and G^- model bacteria when combined with a UVA/blue light irradiation with emission maximum at 365 nm. The emission and absorption spectra of the irradiation source and of Lc, respectively, mainly overlap in the region 350-400 nm (Fig. 7). The Lc absorption spectrum shows that an emission source that includes shorter (310-350 nm) wavelengths than the lamp used could also have been absorbed by Lc. The temperature inside the light chambers was measured and maintained below 40 °C during the experiments to avoid temperature influence on the phototoxicity. Temperatures > 40 °C were recorded after longer irradiation times when using the Option 1 lamp. The design of the Option 2 lamp represented a different irradiation geometry which contributed to keeping the temperature below 40 °C although the irradiance was twice that of Option 1. The observation that UVA/blue light irradiation in the absence of Lc induced a reduction in survival of *E. coli* is consistent with the literature (Hockberger 2002; Hijnen et al. 2006; McKenzie et al. 2013). The lower reduction in G^- bacteria survival compared to G^+ after irradiation with UVA/blue light ∼12 J/cm^2 (Figs. 4, 5) is a tendency shown for several photosensitizers such as Rf

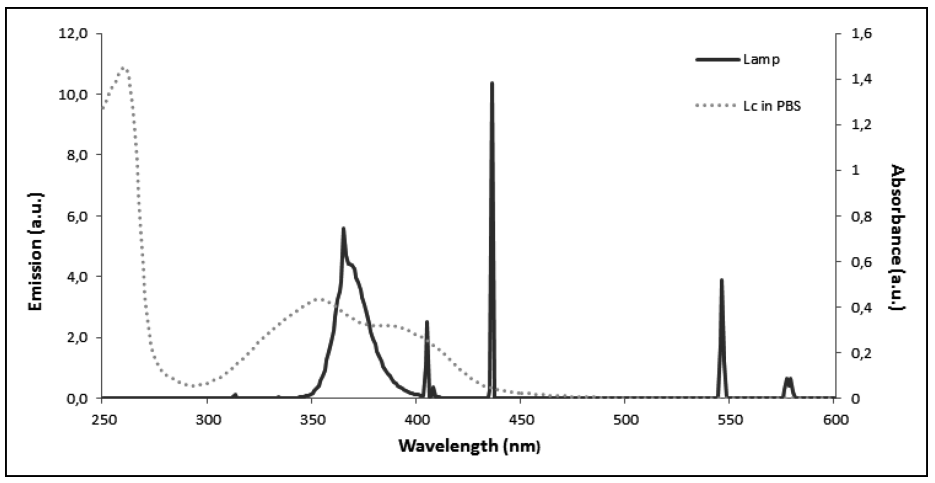

Fig. 7: Combined emission spectrum of the lamp and absorption spectrum of Lc in PBS pH = 7.4.

(Makdoumi et al. 2010), curcumin (Haukvik et al. 2011) or porphyrins (Malik et al. 1995). The main reason is assumed to be the difference in membrane structure. The G^+ bacteria present a relatively permeable and porous peptidoglycan layer surrounding the cytoplasmic membrane that offers low protection against aPDT. G^- bacteria present both an inner and outer membrane with a peptidoglycan layer between them. This structure makes the G^- bacteria more resistant not only to aPDT, but also against several antibiotics (Sperandio et al. 2013). Methods to alter the G^- bacterial membrane and thereby making them more susceptible to external attack have been studied (Malik et al. 1992; Sperandio et al. 2013).

The comparison of 5% PEG 400 preparations at two different Lc concentrations showed that an increase in Lc concentration did reduce the phototoxic effect against bacteria (Fig. 3). The concept of supersaturation did not prove favorable for Lc in the way expected from studies on other lipophilic photosensitizers (Hegge et al. 2012). Further, not only was higher bacterial death obtained at lower Lc concentrations in PEG 400 preparations, but shorter irradiation doses were needed to obtain a reduction in bacterial count (Fig. 3). This relationship is consistent with a previous report on Rf (Makdoumi et al. 2010), where they state that an increase in Rf molarity did not induce a larger phototoxic response in bacteria. We suggest that the reason could be an inner filter effect due to the high concentration of Lc associated with a high absorbance value (>2). Thus, less radiation energy would reach the bacteria and photosensitizer in the deeper layers, resulting in a lower overall phototoxic effect. Lc preparations with high absorbance should therefore be avoided in bacteria phototoxicity investigations.

Although CDs are suitable excipients to enhance the aqueous solubility of Lc, they reduce the ability of Lc to act as phototoxic agent against bacteria. This relationship is particularly valid for HPβCD due to its high complexation efficiency with Lc. A CD with sufficient, but not too high solubilizing capacity (i.e., not too high complex binding constant) should be chosen for highly lipophilic photosensitizers (Hegge et al. 2008). This property can explain why Lc in 3% HPγCD (lower complexation efficiency with Lc than HPβCD) and 5% PEG 400 (not forming an inclusion complex) gave the highest phototoxic effect (Figs. 4, 5). The phototoxicity seemed to be independent of the Lc concentration when it was maintained between certain values (high enough to produce an effect and low enough to avoid inner filter effect). Increasing the irradiation dose ($\sim$24 J/cm^2) enhanced the phototoxicity and complete bacterial eradication was reached for *E. faecalis* (independent of the formulation) and for *E. coli* (except the formulation containing 3% HPβCD).

Makdoumi et al. (2010) reported a reduction of 86-90% of (G^+) *S. epidermidis* after exposure to Rf in PBS and UVA/blue light. In comparison, in the present study we observed more than 99% reduction of (G^+) *E. faecalis*. Further, Makdoumi et al., evaluated the phototoxic effect of Rf on the G^- bacteria *P. aeruginosa* and obtained a 98-100% reduction. In our case, a slightly lower phototoxic effect was obtained with a similar radiation dose in the G^- model bacteria *E. coli*. However, the consistency between the results obtained when using the hydrophilic Rf as photosensitizer and the present results using the lipophilic Lc indicates that Rf may have been fully converted to Lc in the former case. In conclusion, our study presents an opportunity to make well-characterized, photostable formulations of Lc as an alternative to the photolabile Rf for the treatment of infectious conditions.

4. Experimental

4.1. HPLC method

The mobile phase was composed of water (Milli-Q water, Merck Millipore, USA) and methanol (MeOH for liquid chromatography, LiChrosolv®, Merck, Germany) in the ratio 65:35 (v/v). The flow rate was 0.8 ml/min, the detection wavelength was 374 nm, and the retention time of Lc (Sigma-Aldrich, Steinheim, Germany) was approximately 15 min. The calibration curve was prepared from a stock solution of 1 mg/200 ml of Lc in MeOH. The samples were diluted 1:1 in mobile phase prior to injection. The HPLC system was equipped with a silica-based column (Nova-Pak®, C18, 4 μm, Waters, Ireland) and consisted of the following: LC-9A pump; SIL-9A autoinjector; SPD 10-A UV-spectrophotometric detector; C-R5A Chromatopac integrator (Shimadzu, Kyoto, Japan).

4.2. Samples for solubility studies

Lc was used as received. The solubility of Lc was studied in eleven different aqueous solutions: phosphate buffered saline (PBS) pH 7.4 (PBS was prepared by dissolving one PBS tablet (1.90 g; Sigma-Aldrich, St. Louis, MO, USA) in 200 ml Milli-Q water); 5% poly ethylene glycol 400 (PEG 400, Fluka Analytical, Sigma-Aldrich, Steinheim, Germany) in PBS; 5% dimethyl sulfoxide (DMSO, Sigma-Aldrich) in PBS; Pluronic® F127 (Sigma-Aldrich) $3 \cdot 10^{-7}$ M in PBS; MeOH (LiChrosolv®, Merck, Germany); 3 and 10% (w/v) 2-hydroxypropyl-α-cyclodextrin (HPαCD, Fluka Chemie, Sigma-Aldrich) in PBS; 3 and 10% (w/v) hydroxypropyl-β-cyclodextrin (HPβCD, Cavasol® W7 M, Wacker Chemie AG, Germany) in PBS and 3 and 10% (w/v) hydroxypropyl-γ-cyclodextrin (HPγCD, Cavasol® W8, Wacker Chemie AG) in PBS. The water content of the cyclodextrins was taken into account.

Lc was added in excess to each of the solutions. The samples were kept under continuous agitation in a shaker-incubator (Environmental Shaker-Incubator ES-20, BioSan, Riga, Latvia) protected from light at 22 ± 1 °C. After 1 week the samples were filtered (cellulose acetate membrane 0.45 μm; VWR International, Radnor, PA, USA) and the Lc concentration was immediately determined by HPLC. The cellulose acetate filter was used in all the experiments.

Table: Combinations of preparations, Lc concentrations and radiant exposures used in phototoxicity studies of *E. faecalis* and *E. coli*.

Type of preparation	Lumichrome concentration (mM)	Radiant exposure (J/cm^2)
PBS	*0.04*	12 and 24
5% PEG400	*0.06*	12 and 24
3% HPβCD	*0.06*	*12*
	0.1	12 and 24
10% HPβCD	*0.06*	*12*
	1.0	12
3% HPγCD	0.1	12 and 24

4.3. Preparation of supersaturated solutions

The co-solvent method and solvent evaporation method (Jatwani et al. 2012) were used to obtain a higher concentration of Lc in aqueous solutions.

4.3.1. Co-solvent method

Lc was dissolved in 2 ml DMSO. After 2 h stirring in the dark, PBS was added to give a concentration of 5% (v/v) DMSO in the final solution. The physical stability of the formulation was studied spectrophotometrically (UV-VIS spectrophotometer, UV-2401 PC, Shimadzu, Kyoto, Japan) by measuring the decrease in absorbance at 386 nm after filtration at ambient temperature (22 ± 1 °C) 0, 30 and 90 min after addition of PBS. The sample concentration after filtration was quantified by HPLC.

4.3.2. Solvent evaporation method

Lc was dissolved in MeOH. The solutions were kept under stirring until all the Lc was dissolved, approximately for three days. A volume of 2 ml PEG 400 was added to the solutions. After homogenization by manual shaking, the methanol was evaporated in a rotavapor (Büchi, Switzerland) at 45 °C for approximately 15 min. PBS was then added to give a final concentration of 5% (v/v) PEG 400 in the solution. The physical stability of this formulation was studied spectrophotometrically by measuring the decrease in absorbance at 386 nm after filtration at ambient temperature (22 ± 1 °C) 0, 30 and 60 min after addition of PBS. The sample concentration after filtration was quantified by HPLC.

4.4. Lumichrome preparations for bacterial inactivation

All preparations containing Lc were protected from light. The formulations were prepared as saturated solutions by addition of an excess amount of Lc to the solvents. The same procedure as for the solubility studies was followed (section 4.2). The formulations were filtered immediately before use. The Lc concentration was adjusted by dilution with the corresponding aqueous preparation without Lc when necessary. The final concentration of Lc was determined by HPLC. The preparations used in the phototoxicity experiments are presented in the Table.

4.5. Antibacterial phototoxicity studies

4.5.1. Light source

Two irradiation geometries were used in the bacteria inactivation experiments: Option 1 and Option 2. This must not be confused with Option 1 and 2 in the ICH Guideline. The Option 1 lamp consisted of a single irradiation chamber (Polylux PT, Dreve, Unna, Germany) equipped with three compact fluorescent tubes (Ralutec 9W/78, Radium, Wipperfürth, Germany) placed in the chamber roof emitting in the wavelength range 350-435 nm (≥1% of maximum irradiance; emission maximum: 365 nm). The Option 2 lamp consisted of two light chambers (Polylux PT) mounted in an open "sandwich" formation, irradiating the samples from above and below simultaneously. The average irradiances measured across the area of the samples were 6.5 mW/cm^2 and 10.4 mW/cm^2 for Option 1 and Option 2 respectively. The irradiance values were measured with a CCD spectrometer (Ocean Optics, USB4000-UV-VIS, Duiven, The Netherlands) connected to an integrating sphere (Bentham DTM300, Bentham Instruments Ltd., Reading, UK) through an optical fiber. The instrument was equipped with a diffusor. Variation in irradiance across the chamber floor (sample position) was < 25%. Instrument (combined) irradiance measurement error was 9%. The emission spectra of the lamps were identical and overlap with parts of the absorption spectrum of Lc in the UVA/blue light region (Fig. 7). The irradiance was monitored regularly with a UDT 271 radiometer (United Detector Technologies, San Diego, CA, USA) with probes in the blue (268BLU S/N 23476, calibrated at 450 nm) and UVA (268UVA S7N 8U021, calibrated at 365 nm) range.

4.5.2. Temperature control

The temperature inside the irradiation chambers was monitored during irradiation with temperature strips (VWR International) and with a maximum temperature thermometer (Fluke 16 multimeter, Eindhoven, Netherlands). Option 1 lamp reached approximately 40 °C after 30 min (∼12 J/cm^2) continuous irradiation. The temperature of Option 2 lamp was controlled by the use of a fan and did not increase above 40 °C during the experiment (37.5 min to obtain ∼24 J/cm^2).

4.5.3. Microorganisms

Enterococcus faecalis (ATCC 19433) and *Escherichia coli* (ATCC 25922) were maintained overnight in tryptone soy broth (TSB, Oxoid Ltd., Basingstoke, England) medium at 37 °C before use.

4.5.4. Testing of phototoxicity

Aliquots of the overnight culture were diluted in PBS (0.0067 M, pH 7.4, Bio-Whittaker®, Lonza, Switzerland) to a concentration larger than 1×10^6 colony forming units (CFU)/ ml. All the Lc preparations were filtered immediately before use (See 4.4). The bacterial suspensions were diluted 1:1 (v/v) with the different samples (Table) prior to irradiation. *E. faecalis* and *E. coli* bacterial suspensions were diluted 40 × and 60 × respectively, with PBS pH 7.4 before being plated. Aliquots of the suspensions were plated onto TSB agar (Agar bacteriological No.1, Oxoid Ltd.) with an automatic spiral plater (Don Whitley Scientific LTD, Shirley, West Yorkshire, UK) to determine the survival by colony forming ability. After approximately 24 h incubation at 37 °C the colonies were counted with an Acolyte colony counter (Synbiosis, Cambridge, UK). Bacterial suspensions mixed 1:1 with plain PBS maintained in the dark are referred to as control samples in all the studies. The bacterial survival was calculated as percentage of the control samples.

4.5.4.1. Effect of UVA/blue light irradiation. The bacteria suspensions were diluted 1:1 in PBS before irradiation. The irradiation dose tested was ∼12 J/cm^2 and ∼24 J/cm^2, corresponding to 30 min with Option 1 and 37, 5 min with Option 2. The bacterial mixture was incubated for 15 min (22 ± 2 °C) protected from light after irradiation before plating.

4.5.4.2. Effect of radiant exposure and lumichrome concentration. *E. faecalis* suspensions were diluted 1:1 with Lc in 5% PEG 400 solutions at two different concentrations (0.06 mM and 0.52 mM) and irradiated 0-12 J/cm^2 (corresponding to maximum 30 min in Option 1 light chamber). Samples (100 µl) were withdrawn every 5 min.

4.5.4.3. Effect of post- irradiation time. *E. coli* suspensions were diluted 1:1 with Lc 0.06 mM in 5% PEG 400 and pre-incubated (22 ± 2 °C) for 15 min under agitation in the dark. After an irradiation with ∼12 J/cm^2 the samples were post-incubated protected from light at 22 ± 2 °C for 0, 15, 30 and 60 min prior to plating.

4.5.4.4. Effect of selected lumichrome preparations. The bacteria suspensions were diluted 1:1 with selected Lc preparations. The samples were protected from light and agitated (50 rpm) for 15 min (22 ± 2 °C) prior to irradiation. After irradiation with Option 1(∼12 J/cm^2) or Option 2 (∼24 J/cm^2) the samples were post-incubated (22 ± 2 °C) for 15 min protected from light prior to plating.

4.6. Statistics

Student's t-test for independent samples (two–tailed) and unequal analysis of variance were used to evaluate the results and estimate significant differences. A P-value less than 0.05 was chosen as statistically significant.

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