Photodynamic inactivation of antibiotic resistant strain of *Pseudomonas aeruginosa in vivo*

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ABSTRACT

Burns are frequently contamined by pathogenic microorganisms and the widespread occurrence of antibiotic resistant strains of *Pseudomonas aeruginosa* in hospitals is a matter of growing concern. Hypocrellin B (HB) is a new generation photosensitizer extracted from the fungus *Hypocrella bambusae* with absorption bands at 460, 546 and 584 nm. Lanthanide ions change the HB molecular structure and a red shift in the absorption band is observed as well as an increase in the singlet oxygen quantum yield. In this study, we report the use of HB:La^{+3} to kill resistant strain of *P. aeruginosa* infected burns. Burns were produced on the back of mice and wounds were infected subcutaneously with $1x10^9$ cfu/mL of *P. aeruginosa.* Three-hours after inoculation, the animals were divided into 4 groups: control, HB:La^{+3}, blue LED and HB:La^{+3}+blue LED. PDT was performed using 10μM HB:La^{+3} and 500mW light-emitting diode (LED) emitting at λ=470nm±20nm during 120s. The animals of all groups were killed and the infected skin was removed for bacterial counting. Mice with photosensitizer alone, light alone or untreated infected wounds presented $1x10^8$ cfu/g while mice PDT-treated showed a reduction of 2 logs compared to untreated control. These results suggest that HB:La^{+3} associated to blue LED is effective in diminishing antibiotic resistant strain *P. aeruginosa* in infected burns.

Keywords: *Pseudomonas aeruginosa,* hypocrellin B, photodynamic therapy, LED, infected burn wound.

1. INTRODUCTION

Burns are one of the most common forms of trauma and patients with serious thermal injuries require immediate specialized care in order to minimize morbidity and mortality[1]. A major problem for the patients is the burn wound infection and sepsis acquired in the hospital environment due to extent of the burn injury itself and to secondary immunosuppression resulting from the thermal injury. Infections continue to be the main concern, reportedly causing over 50% of burn deaths[2]. The destruction of the cutaneous barrier, presence of coagulated proteins and other microbial nutrients in the wound and the avascularity of the eschar that prevents delivery of immunologically active cells, humoral factors, and antibiotics to it provide an optimal environment for bacterial growth and the development of the infection[3,4]. Moreover, prolonged hospitalization and invasive diagnostic and therapeutic procedures further contribute to the infectious process.

A burn wound infection evolves into sepsis through the invasion of the bloodstream by bacteria (bacteremia). Bacteremia develops as a result of damage to the external (skin) or the internal (respiratory tract, digestive tract) barriers of the body and it is one of the criteria for the diagnostics of sepsis. Sepsis is very dangerous for burned patients, because it increases the production of inflammatory mediators and cytokines, and causes their interaction that predisposes to the development of multiple organ failure that is the main cause of mortality in burned patients [5,6].

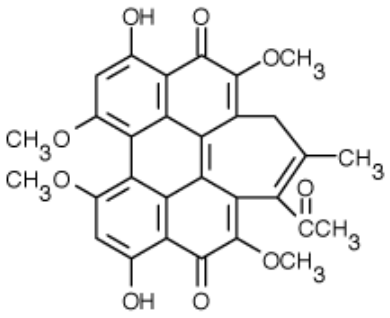
The treatment of severe burns is a long processs, and burn centers use a lot of wide-spectrum antibiotics, which result in the development of antibiotic-resistant strains. They are especially dangerous to burned patients due to their weak immune system[7].

Bacterial resistance to antibiotic therapy is subject of worldwide concern. Many human pathogens are now multiresistant to antimicrobial drugs. So, infections with such organisms may be particularly difficult to treat.

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen involved in serious infections in immunocompromised hosts, characteristic of patients with severe burn wounds. The ability of this pathogen to survive under different environment conditions, combined with its inherent resistance to several antibiotics, allows it to colonize and proliferate inside the burned tissues. This localized proliferation may lead to systemic sepsis, which is often associated with a high degree of morbidity and mortality[8,9].

Photodynamic Therapy: Back to the Future, edited by David H. Kessel, Proc. of SPIE Vol. 7380, 73803F · © 2009 SPIE · CCC code: 1605-7422/09/$18 · doi: 10.1117/12.823028

Superficial wound infections are potentially suitable for treatment by Photodynamic Therapy (PDT) because of the ready accessibility of these wounds for both topical delivery of the photosensitizer (PS) and light.
Photodynamic therapy involves the killing of organisms by light in the presence of a non-toxic photoactivable PS. Excitation of the PS by absorption of light of appropriate wavelenght in the presence of oxygen converts the PS to its photoactive triplet state, which will then generate reactive oxygen species, such as singlet oxygen and superoxide, resulting in cell death[10].
PDT has been suggested as an alternative approach for treating local infections since it has been shown that a wide range of microorganisms including bacteria, viruses and yeasts can be killed by photodynamic action[11,12]. Treating localized infections by the PDT could be a useful alternative to systemic medications, thus avoiding the development of microbial resistance to systemic drugs.
Several dyes have been proposed as antimicrobial PS, but the only used clinically for antimicrobial treatments are phenothiazinium salts like methylene blue (MB) and toluidine blue (TBO)[13]. Otherwise, previous studies identified the presence of multidrug resistance pumps (MDRs) in a wide range of microorganisms. MDRs are membrane-localized proteins that pump drugs out of cells and have become broadly recognized as major components of microbial resistance to many classes of antibiotics[14].
It has been shown that killing of *P. aeruginosa* by photodynamic therapy is more difficult than others species of bacteria, both gram-positive and gram-negative. Even greater dye concentrations and intensity of light was required for complete killing. This was reached at a toluidine blue concentration of 200μM and at a light intensity of 100mW/cm^2 [15].
Even though the ideal photosensitizer has not been discovered yet, the new generation studied chromophores, hypocrellins and naturally occurring perylenequinonoid pigments extracted from the fungus of *Hypocrella bambusae* stand out. Hypocrellin B (HB) is a new generation photosensitizer, a native pigment frequently found in Asian forests, mainly in countries like China and Sri Lanka. This compound has been target of study in last two decades due to properties such as high singlet oxygen generation quantum yield and absorption in the range of 460, 546 and 584 nm [16,17].
Properties like strong absorption bands, easy preparation, fast elimination from the body and high singlet oxygen generation quantum yield are found in perylenequinonoid pigments, such as hypocrellins and hypericin.
HB:La^{+3} in ethanol provided an enhancement of the singlet oxygen generation quantum yield of HB, from 0.47 to 0.62 (32%)[18].
So, hypocrellin B complexes with metal ions possess even more notable optical and photodynamic properties[18]. Figure 1 shows the chemical structure of Hypocrellin B and Hypocrellin B associated to lanthanide ions. Figure 2 shows the optical absorption spectra for HB and HB:La^{+3} solutions. It can be seen that HB:La^{+3} presents a very large absorption band in the visible, ranging from about 400 to 650 nm.
In this study, we report the use of HB:La^{+3} and blue LED for killing antibiotic resistant strain of *P. aeruginosa* in burn wounds. We used an antibiotic resistant strain isolated from blood catheter of a patient with septicemia. This strain presented resistance to thirteen types of antibiotic.



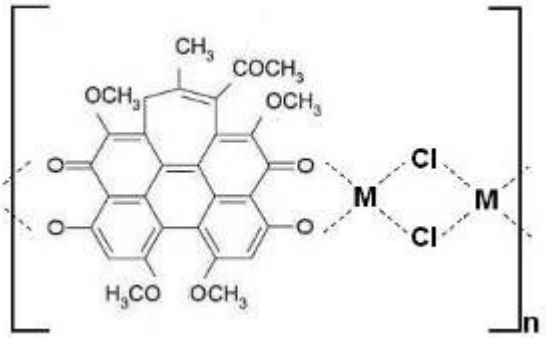


Fig.1: Hypocrellin B *($C_{30}H_{24}O_9$)* HB:La^{+3} (M corresponds to lanthanide ions)

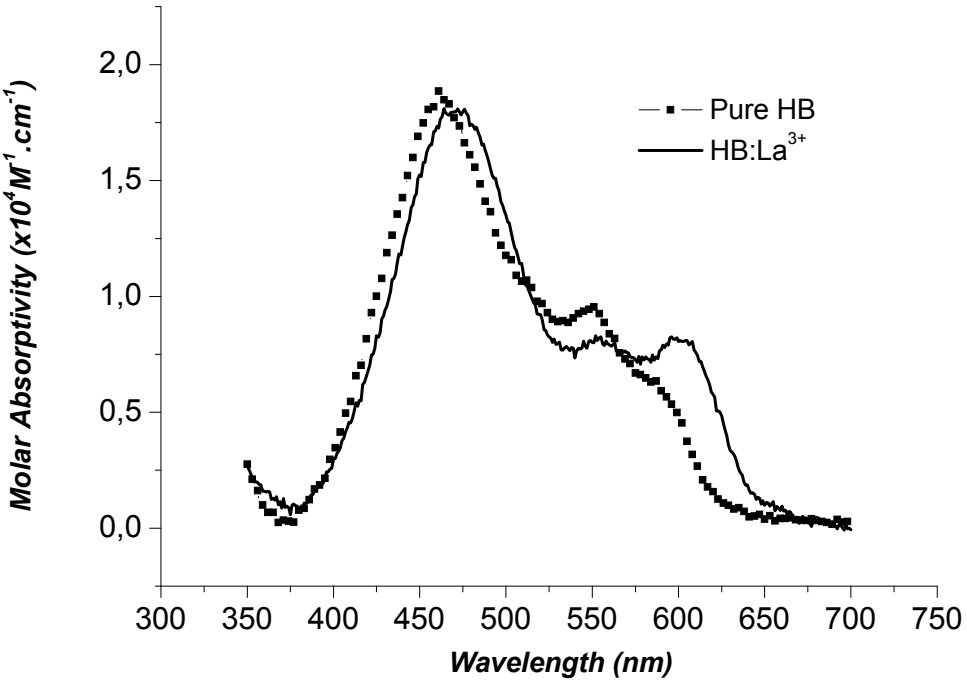


Fig.2: HB and HB:La^{+3} optical absorption spectra.

2. MATERIALS AND METHODS

Mice

Twenty eight 6-8 weeks female Balb/c (20-25 g) mice were used. All experimental procedure performed in this study was approved by the Ethic Committee on Research Animal of IPEN-CNEN (Sao Paulo/Brazil)

The animals were housed individually in ventilated cages in a central animal research facility. The facility maintained an environment of controlled temperature and relative humidity, with a 12-h light-dark cycle. The mice were supplied with sterile bedding, standard chow, and water *ad libitum*.

Thermal injury model

Mice were anesthetized by intraperitoneal injection of ketamine (90mg/kg) and xylasine (10mg/kg), and their back hair was shaved using a shaving blade. Butorfanol (2 mg/kg) was used as a postburn analgesic. Thermal injury was induced by pressing a pre-heated steel device (6cm^2 area) against the back of the animal for 10s . The steel device was pre-heated in boiling water to about 95°C and produced a third degree burn confirmed by histological analysis of 6-6.5 cm^2 area that corresponds to 9-9.5% of body surface area calculated according to Meeh's formula[19]. All mice exposed to the burn injury survived when they were not infected.

Bacterial infection

The wild-type strain of *P. aeruginosa* was isolated from a haemodynamic catheter's patient with septicemia (Emílio Ribas Hospital/ Sao Paulo-Brazil) and it is β- lactamase producer and resistant to thirteen types of antibiotic.

P. aeruginosa was grown in triptic soy agar (TSA) for 24h at 37°C. The infecting bacterial inoculum was prepared in sterile sodium phosphate buffer (PBS), transmittance of 16% at 620 nm (10^9 ufc/mL). The number of infecting bacteria was verified by plating serial dilutions of the injected inocula onto TSA plates.

The inoculum of 100μL of the bacterial suspension was injected under the burn immediately after burning.

Photosensitizer

Hypocrelin B ($C_{30}H_{24}O_9$) with lanthanide ions (HB:La^{+3}) was obtained from Optical Spectroscopy Laboratory (CLA-IPEN/CNEN). Hypocrellin B (HB) was purchased from Shaanxi Tianze Bio-Technology CO., LTD. Lanthanide chloride ($LaCl_3.7H_20$) was purchased from Sigma-Aldrich Corporation Ltd., with analytical grade. HB was diluted in ethanol as well as the lanthanide chlorides were. HB and La^{+3} solutions were mixed in 1:2 molar ratios, and stirred for 20 minutes. Then, an equilibrium mixture of HB complexes and free HB was obtained, denominated HB:La^{3+} solution. HB concentration for complexes in ethanol was 1mM. Stock solutions of 1mM were dissolved in PBS to a final concentration of 10μM.

HB:La^{+3} Toxicity

To verify if HB:La^{+3} could be lethal to animals, an experiment was peformed separately. Twelve 6-8 weeks female Balb/c (20-25 g) mice were used. All the animals were submitted to thermal injury and divided as follow:
Group I_+: mice with infected burns;
Group $I.PS_+$: burns without infection with HB:La^{+3} inoculated under the burn
Group $I.PS_+L_+$: burns without infection with HB:La^{+3} inoculated under the burn and irradiated with blue LED
All the animals were observed during 7 days, and after that they are killed by cervical dislocation.

Light source

All illuminations were carried out with 500mW light-emitting diode (LED) (Eccofibras/Sao Carlos-Brazil) emitting at λ=470nm±20nm.

Photodynamic inactivation *in vivo*

Twenty eight 6-8 weeks female Balb/c (20-25 g) mice were used. The animals were divided into 4 groups as follow:
Group I_+: mice with infected burns that received no treatment at all;
Group I_+PS_+: mice with infected burns treated with HB:La^{+3} but kept in the dark;
Group I_+L_+: mice with infected burns that were illuminated in the absence of PS;
Group $I_+PS_+ L_+$: mice with infected burn wounds that were treated with HB:La^{+3} and illuminated with blue LED.
The HB:La^{+3} concentration used was 10μM, the solvent was PBS and pre-irradiation time (PIT) was 5 minutes, 100μL of the PS was injected under the burns three hours after bacterial inoculation and burns were illuminated directly for 120s with a fluence rate of 83mW/cm^2, resulting in fluence of 10J/cm^2. In all experiments the light source was placed vertically in contact with animal skin and it was protect one by one with sterile plastic film.
To measure the quantity of bacteria in local burn tissues, as a measure of local proliferation, mice were killed by cervival dislocation immediately after treatment. Burn wound tissues were cut and homogeneized in 1 mL of PBS. Number of *P. aeruginosa* in one-g of tissue (wet weight) was determined by serial dilution plate-count in triplicate on TSA.

Tissue surrounding the burn areas

Tissues surrounding the entire burn area were excised using a sterile surgical scissor. The depth of the skin biopsy extended all the way to the the panniculus carnosus of the back such that all epidermal and dermal components were removed. Immediately following excision, the tissues were weighted and grinded with 1mL of sterile PBS, the aliquots were serially diluted in PBS to give dilutions from 10^{-1} to 10^{-5} times the original concentrations and were streaked horizontally on TSA plates [20]. Plates were incubated at 37°C overnight.

Statistics

The results obtained were expressed as means ± standard deviation and were analysed statistically using one way ANOVA test. Differences between means were tested for significance by Tukey test with Brown-Forsythe variations. Differences were considered significant at $p<0.05$. All the experiments were performed in triplicate.

3. RESULTS and DISCUSSION

HB:La^{+3} Toxicity

The mice of group $I.PS_+$ and group $I.PS_+L_+$ survived for 7 days, while all mice of the group I_+ died within 48h after bacterial inoculation. These results indicate that animals died because of local infection that evolved to sepsis. Therefore, in our model, neither HB:La^{+3} nor HB:La^{+3} associated to blue LED produced a lethal effect.

Photodynamic inactivation *in vivo*

Local bacteria proliferation in the burn area was evident in the non treated infected burn group, as well as PS alone and light alone groups. Mice with wounds infected with $1x10^9$cfu/mL of *P. aeruginosa* presented $1x10^8$cfu/g in groups untreated, PS alone and light alone. Mice PDT-treated showed reduction of 2 logs compared to other groups (Fig.3).
There was no significant difference ($p<0.05$) in the number of cfu/g per burn wound when non-treated, PS alone and light alone were compared, but there was a statistically significant difference when group PDT-treated was compared with groups untreated, PS alone and light alone.

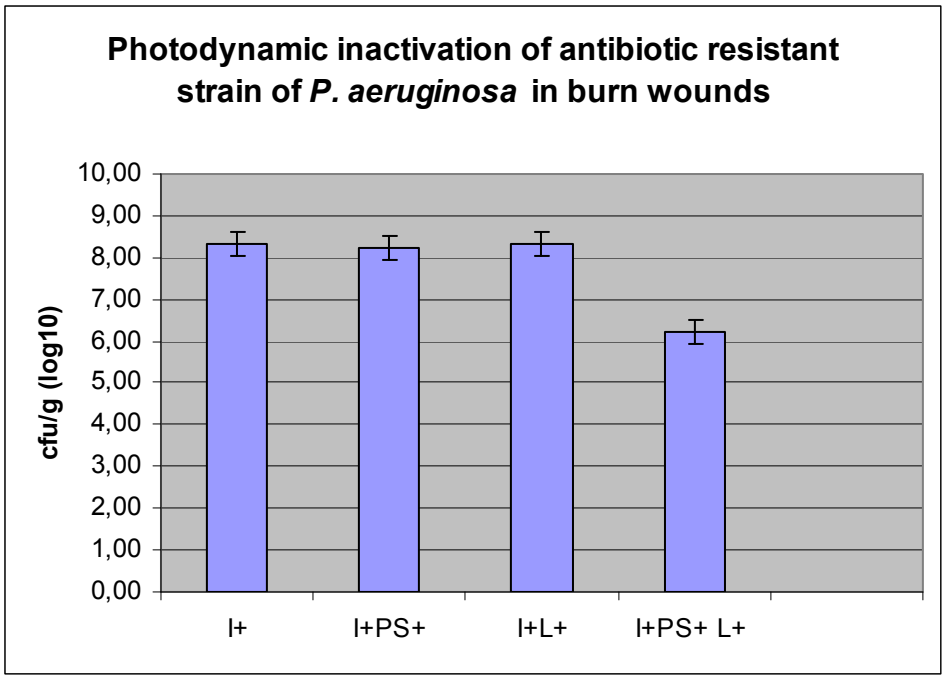


Fig. 3: Number of viable cells with combined action of HB: La^{+3} and LED light at λ=470 nm. Bars are representative of ± standard deviation. $P<0.05$. I+: mice with infected burns that received no treatment at all; I_+PS_+: mice with infected burns treated with HB:La^{+3} but kept in the dark; I_+L_+: mice with infected burns that were illuminated in the absence of PS; $I_+PS_+ L_+$: mice with infected burn wounds that were treated with HB:La^{+3} and illuminated with blue LED.

There are many studies *in vitro* showing the microbial susceptibility to PDT[11,12]. However, studies concerning PDT for infections *in vivo* are scarces. Full-thickness burns are a realistic animal infection model which provides an optimal environment for bacterial growth and the development of infection. The presence of coagulated proteins and other microbial nutrients in the wound and the avascularity of the eschar prevent delivery of immunologically active cells, humoral factors and antibiotics.
To the best of our knowledge, it is the first time that HB:La^{+3} is reported in an *in vivo* model. Our results did not show any lethal effect, since the animals that received HB:La^{+3} alone or HB:La^{+3} associated to blue LED did not die within 7 days, while all the animals of the infected group died within 48 h, indicating that the local infection evolved to sepsis.
The photosensitized inactivation of pathogenic microorganisms is a complex phenomenon and depends on many parameters, mainly *in vivo*, where conditions like presence of blood or plasma, kind of tissue, interaction between dye and bacteria into hosts, bacteria spread into tissue, dye behavior into host's tissue, and several other factors could change the initial parameters of photosensitization.
Previous studies with well-established photosensitizers have shown that *P. aeruginosa* is more difficult for killing by photodynamic therapy than others species of bacteria [16]. In addition, *P. aeruginosa* used in this study is a multi-resistant wild-strain that has been undergone mutations and became β-lactamase producer and antibiotic resistant. These changes could have an influence on PDT treatment, since that could to occur to changes in the bacterial outer membrane structure,

DNA and other cellular system[21]. Nevertheless, in this study, we observed 2 logs of bacterial reduction in PDT-treated group compared to control group.
Killing of bioluminescent *P. aeruginosa* by PDT in infected wounds have been shown in a full-thickness excisional wound. The authors used a polycationic PS conjugate and a 660 nm, 300mW diode laser and observed that luminescence had disappeared after a fluence of 240J/cm^2 [22]. In our study we used an invasive strain and a full-thickness burn model and observed 2 logs of bacterial reductions after 10J/cm^2.

5. CONCLUSION

Our experiments indicate that HB:La^{+3} associated to blue LED is effective in diminishing antibiotic resistant strain of *P. aeruginosa* in infected-burn mice. Moreover, HB:La^{+3} or HB:La^{+3} associated to blue LED shows no lethal effect for the animals in this model. However, further studies must be carried out to verify the potential of this photosensitizer to be used in clinical trials.

ACKNOWLEDGEMENTS
This work was supported in part by CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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