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Photodynamic inactivation of Bovine herpesvirus type 1 (BoHV-1) by porphyrins

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Abstract

In this work, the photodynamic efficiency of anionic *meso*-tetrakis sulfonophenyl ($TPPS_4$), cationic *meso*-tetrakis methylpyridiniumyl (TMPyP) and their zinc complexes ($ZnTPPS_4$ and ZnTMPyP) in the inactivation of Bovine herpesvirus type 1 (BoHV-1) was evaluated. At a non-cytotoxic concentration, all porphyrins showed significant antiviral activity after irradiation using a halogen lamp. The efficiency of the cationic porphyrins was higher than that of the anionic ones. Porphyrin complexation with zinc increases its lipophilicity and the number of absorbed photons, dramatically reducing the time for complete virus inactivation. The high superposition of the compound optical absorption and light source emission spectra played a key role in the virus inactivation efficiency. The results demonstrated the high effectivity of the photodynamic inactivation of BoHV-1. This method can be used as an auxiliary in the treatment of disorders attributed to BoHV-1 infection, and the porphyrins are promising photosensitizers for this application.

Bovine herpesvirus type 1 (BoHV-1) is associated with a variety of clinical manifestations such as respiratory infections and reproductive disorders in cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious pustular balanoposthitis [1] and, especially, neurological disorders [2, 3]. BoHV-1 is classified by the World Organization for Animal Health as a notifiable disease, defined as a transmissible disease considered of socio-economic and/or public health importance and therefore relevant to the international trade of animals and animal products [4].

Biosecurity protocols and vaccination programmes have been the main methods of preventing infection in herds [5]. However, new, safe and effective methodologies need to be developed to eliminate viruses from animals or animal by-products (semen or oocytes) to ensure the safety that is required for the trading of these products.

In this work, we have shown the high efficiency of *in vitro* photodynamic inactivation (PDI) of BoHV-1, demonstrating good perspectives for the practical application of this methodology for the treatment of infections caused by BoHV-1.

PDI is based on the synergistic action of a photoactive compound (photosensitizer, PS), visible or near-infrared light and molecular oxygen on the treated biological object [6]. After absorption of the light photon, the PS passes from its ground state (S_0) to the electronically excited singlet state (S_1) from which, due to inter-system crossing, it can pass to the excited triplet state (T_1) or relax to the S_0 state via fluorescence or internal conversion (Fig. 1a). Being in the S_1 or T_1 state, the PS is able to realize charge transfer reactions with participation of molecular oxygen producing reactive oxygen species (ROS) such as superoxide anions and ions radicals (type I reactions). Besides, PS molecules in the T_1 state can transfer energy to molecular oxygen, forming its singlet excited state, the singlet oxygen (type II reaction). ROS, including singlet oxygen, can react with biological structures (nucleic acids, proteins, membrane phospholipids, etc.), resulting in the destruction of these structures and cell death by oxidative stress [7, 8 and references therein]. It was demonstrated that the efficacy of PDI

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Abbreviations: BoHV-1, bovine herpesvirus type 1; MDBK, Madin–Darby bovine kidney cells; N_{abs}, number of photons absorbed per second; PDI, photodynamic inactivation; $P_{B/W}$, 1-butanol/water; $P_{O/W}$, partition coefficient; PS, photosensitizer; ROS, reactive oxygen species; S_0, singlet ground state; S_1, excited singlet state; T_1, excited triplet state; $TCID_{50}$ ml^{-1}, 50% Tissue Culture Infective Dose per ml; $TPPS_4$, *meso*-tetrakis p-sulfonophenyl porphyrin; $ZnTPPS_4$, zinc *meso*-tetrakis sulfonophenyl porphyrin; TMPyP, *meso*-tetrakis methylpyridiniumyl porphyrin; ZnTMPyP, zinc *meso*-tetrakis methylpyridiniumyl porphyrin; ε, molar absorption coefficient; $\phi\Delta$, singlet oxygen quantum yield.

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under physiological conditions is determined principally by type II reactions [6]. The scheme of PDI reactions is demonstrated in Fig. 1(a).

To increase the PDI efficiency, it is necessary that:

(1) The PS possesses a high molar absorption coefficient (ε) in the visible spectral region and its absorption spectrum overlaps effectively with the light source emission spectrum, which provides effective light energy absorption.
(2) The quantum yield of singlet oxygen production (ϕ_Δ) should preferably be higher.
(3) The PS molecules should possess high affinity with their biological target.

The last condition is dependent on the physicochemical characteristics of the PS, such as amphiphilicity and charge [9].

Despite the fact that the effectiveness of photodynamic therapy against infectious diseases has previously been demonstrated over recent decades, its use in veterinary applications is still incipient. Few authors have addressed the challenges and potential of photodynamic therapy for the control of infectious bovine diseases [10–12]. Therefore, for more effective use of this technique, new protocols for PSs applied in PDI should be created (or formulated) for each pathogen by adjusting the dose, irradiation time, light source and light intensity. Recently, our group demonstrated PDI in BoHV-1 using zinc and aluminium tetracarboxy-phthalocyanines [13].

Among different types of PS, porphyrins possess advantageous properties such as intensive absorption in the visible spectral region and high quantum yield of the T_1 state. Consequently, they exhibit a high quantum yield of singlet oxygen production, water solubility, simple charge variation, potential to form metallic complexes, and high thermal and photochemical stability.

In this study, for PDI in BoHV-1 we used synthetic water-soluble porphyrins, the anionic *meso*-tetrakis (p-sulfonatofenyl) ($TPPS_4$), cationic *meso*-tetrakis (methylpyridiniumyl) (TMPyP) [14] and their zinc complexes ($ZnTPPS_4$ and ZnTMPyP) (Fig. 1b).

Porphyrins were purchased from Porphyrin Products Inc. and solutions were prepared in phosphate-buffered saline. The porphyrin absorption spectra were monitored using a double-beam spectrophotometer (Hitachi U-2900). Porphyrin concentrations were controlled spectrophotometrically using the molar absorption coefficients $\varepsilon_{TPPS4}=1.30\times10^4$ M^{-1} cm^{-1} at 515 nm, $\varepsilon_{TMPyP}=1.39\times10^4$ M^{-1} cm^{-1} at 518 nm, $\varepsilon_{ZnTPPS4}=1.74\times10^4$ M^{-1} cm^{-1} at 556 nm and $\varepsilon_{ZnTMPyP}=1.74\times10^4$ M^{-1} cm^{-1} at 563 nm.

BoHV-1 (Los Angeles-LA reference strain) was grown on Madin–Darby bovine kidney cells (MDBK) in Eagle's minimal essential medium (MEM-Sigma) supplemented with 6 % fetal bovine serum (Gibco) and antibiotic penicillin/streptomycin (10 mg ml^{-1}). Viral titration was performed by the Spearman–Karber method [15].

PDI of the virus was carried out in the following way. An initial viral suspension containing $10^{5.75}$ $TCID_{50}$ ml^{-1} was incubated with the PS at a concentration of 5 μM for 1 h at 37 °C under agitation and protection from light. The samples were placed in microtitre plates and irradiated for 15, 30, 60 and 120 min using an irradiation system with a halogen lamp (130 mW cm^{-2}) in the spectral range 400–900 nm [16].

To evaluate virus viability, tenfold serial dilutions of treated viral and control samples were inoculated into MDBK cells in a microtitre 96-well plate (100 μl per well). The plates were incubated at 37 °C with 5 % CO_2 for 72 h, and the cytopathic effect was determined using an inverted microscope. Samples containing viruses in the dark with and without PS and samples irradiated in the absence of PS were used as controls. All data are presented as mean±standard deviation using analysis of variance followed by Tukey's test. P values <0.05 were considered statistically significant.

The results of BoHV-1 PDI for different PS are shown in Fig. 2. No control samples showed any significant changes in virus viability, demonstrating that neither light without PS nor PS without light induced virus inactivation. Irradiation in the presence of PS reduced virus viability in descending order: ZnTMPyP > TMPyP > $ZnTPPS_4$ > $TPPS_4$. The PDI effect increased with irradiation time (dose increase) so that after 120 min of irradiation, ZnTMPyP, TMPyP and $ZnTPPS_4$ induced complete virus inactivation, characterized by the observation that no cytopathic effect was caused by the virus. After 120 min of irradiation, $TPPS_4$ reduced virus concentration by 10^5. For ZnTMPyP, the virus concentration was reduced by 3×10^3 after 15 min of irradiation, and after 30 min of irradiation the virus was completely inactivated.

The ROS lifetimes were very short (10^{-6}–10^{-9} s) and the diffusion distance of the singlet oxygen in the cell was approximately 10–20 nm [17, 18]. Therefore, binding of PSs with their targets is extremely important in regard to increasing the efficacy of photodynamic treatment. Electrostatic interaction between PSs and the treated object is one of the effective mechanisms involved in this process. Negatively charged phospholipids present in the viral envelope may play a crucial role in electrostatic interactions with PSs. Since $TPPS_4$ and $ZnTPPS_4$ are negatively charged molecules, their binding with viruses is weak, probably due to electrostatic repulsion. This can explain the lower photodynamic efficiency of $TPPS_4$ and $ZnTPPS_4$, as compared to that of the positively charged TMPyP and ZnTMPyP.

In addition to electrostatic interaction with lipids, the photodynamic action of cationic PS can extend to adjacent molecules (glycoproteins) on the surface of the viral envelope. Viral replication and virulence are driven by several glycoproteins, these playing an important role in viral fusion and the penetration process into the host cell, and in cell-to-cell

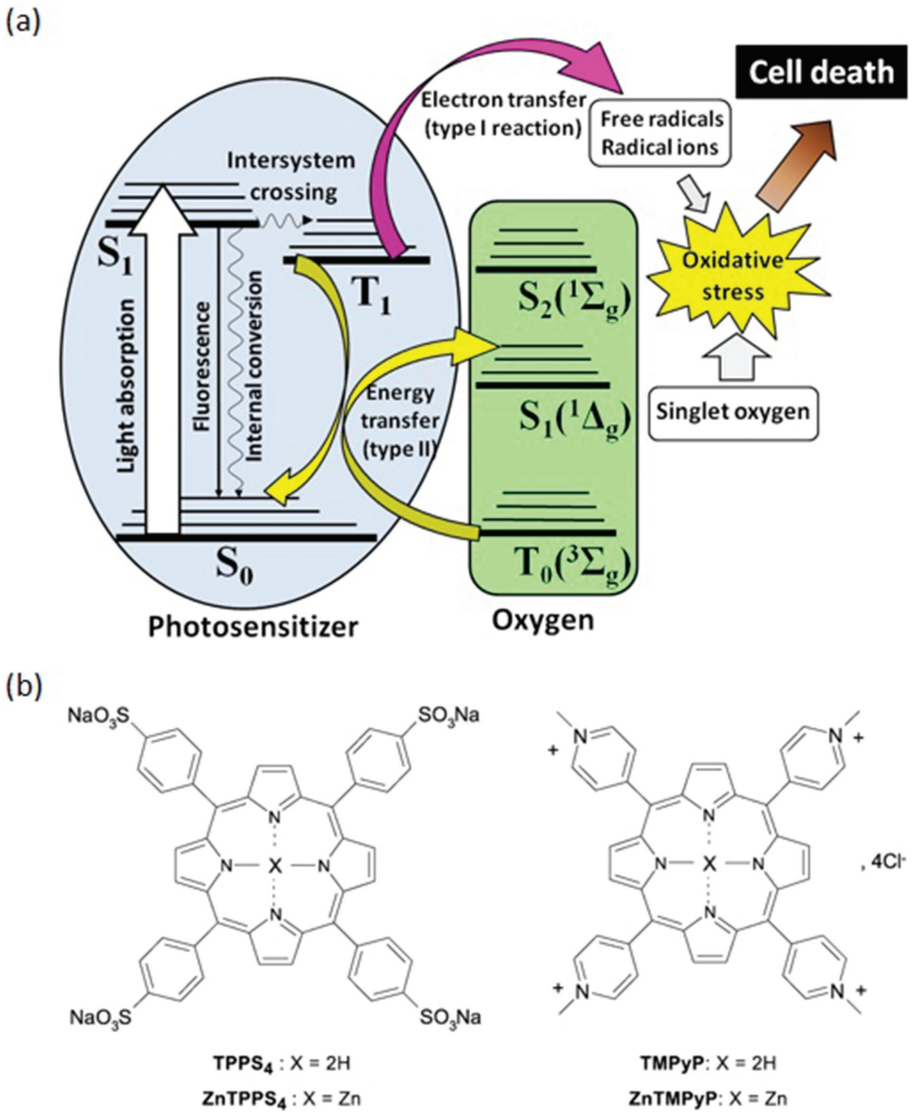

Fig. 1. (a) Energy level diagram and photophysical/photochemical process involved in photodynamic inactivation (PDI), adapted from [8, 30]. (b) Chemical structure of the porphyrins studied, adapted from [31].

spread [19]. Thus, combined with the greater electrostatic interaction observed with cationic porphyrins, the photodamage process may also act on viral glycoproteins leading to marked reduction in the time required for virus inactivation when exposed to ZnTMPyP treatment, as compared to samples incubated with $TPPS_4$ and its Zn derivative.

In general, the viral envelope is characterized by low polarity [20]. Therefore, a compound with greater lipophilicity should possess higher affinity with the viral envelope, which should increase the probability of its binding with the virus.

In order to evaluate the lipophilicity indices of the PS, the shake-flask method was used [21]. This method is based on the compound partition coefficient ($P_{O/W}$) in the n-octanol/water separated phases. Log $P_{O/W}$ is widely used to represent the hydrophilic/lipophilic nature of a molecule and to predict its tendency to become incorporated in biological membranes. It is well known that photodynamic efficiency is directly proportional to the affinity of PS and membranes [22].

Because of low PS solubility in octanol, the partition coefficients were measured in 1-butanol/water ($P_{B/W}$) and then converted to the n-octanol/water system ($P_{O/W}$) [23]. Equal volumes of 1-butanol and water were vigorously mixed for 3 days at 25 °C to obtain solvent saturation in both phases. The PS was then diluted in this mixture, which was stirred for 5 min and incubated for 2 h at room temperature. The UV-vis spectra of both phases were recorded and the partition coefficient was calculated based on the absorbance ratio of the Soret band.

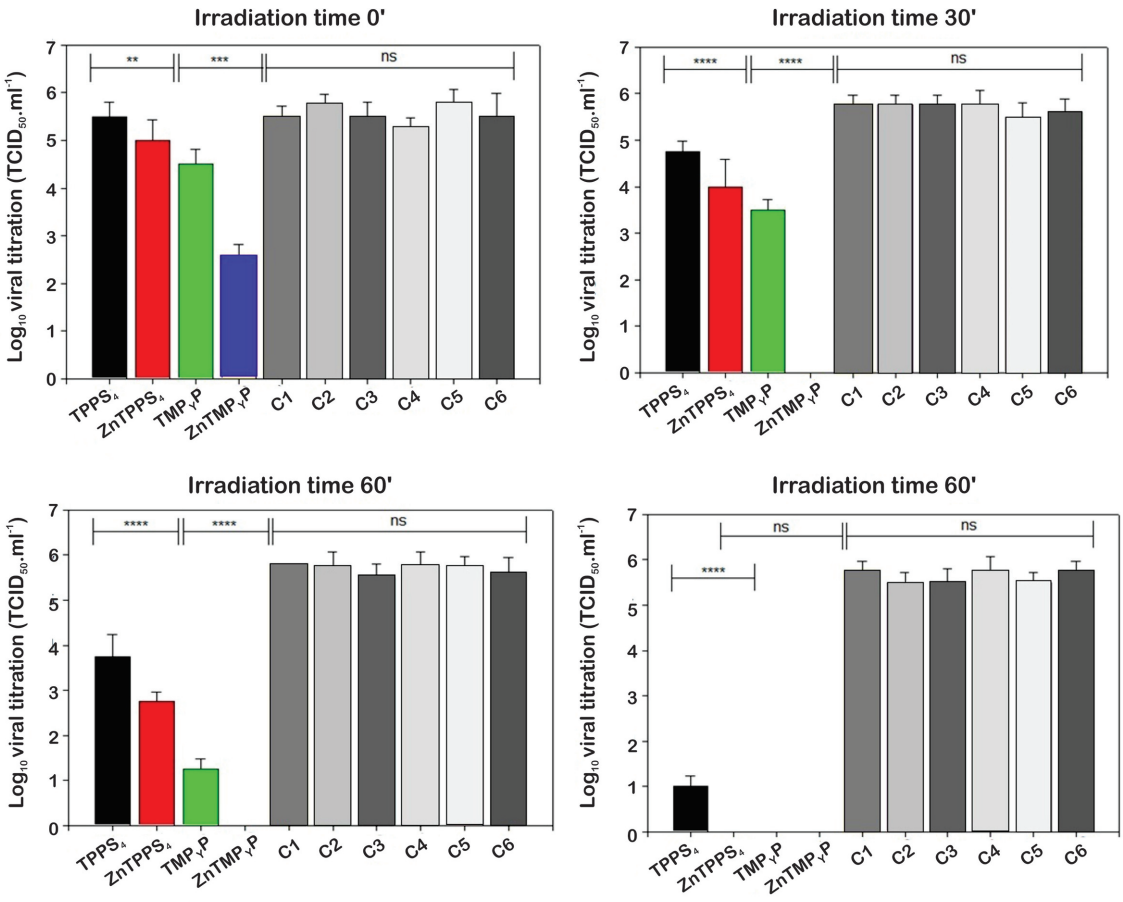

Fig. 2. Comparative analysis of viral titres under PDI using the porphyrins $TPPS_4$, $ZnTPPS_4$, TMPyP and ZnTMPyP, at irradiation times of 15, 30, 60 and 120 min. Controls: C1 – $TPPS_4$ without light; C2 – $ZnTPPS_4$ without light; C3 – TMPyP without light; C4 – ZnTMPyP without light; C5 – virus culture without PS and light; C6 – virus culture with light. NS – no significant difference (P<0.05).

The obtained Log $P_{O/W}$ values were −4.0±0.1, –2.9±0.2, –3.4±0.1 and −3.0±0.1 for $TPPS_4$, $ZnTPPS_4$, TMPyP and ZnTMPyP, respectively. In accordance with [24], introduction of the zinc atom in the central ring reduces PS hydrophilicity, which could favour its interaction with the lipoproteic viral envelope. The electrostatic attraction and lowest hydrophilicity observed for ZnTMPyP could explain its efficiency as compared with that of other PSs. However, the marked difference between the efficacy of ZnTMPyP and that of the other PSs cannot be due only to differences in their hydrophilicity, which are sufficiently weak.

As noted above, one of the principal conditions for effective PDI is high singlet oxygen quantum yield (ϕ_Δ). Higher ϕ_Δ values attributed to the presence of Zn (Fig. 3a), observed for both porphyrins, can explain their enhanced photodynamic efficiency as compared with those of free base porphyrins [22, 24].

One more parameter that should be taken into account is the number of photons absorbed per second (N_{Abs}) by the PS during irradiation, which depends on the overlapping of PS absorption and lamp emission spectra (Fig. 3b). The insertion of zinc atoms redshifts the porphyrin absorption bands toward the more intense emission region of the halogen lamp, indicating that zinc porphyrins absorb a higher number of photons than do free base porphyrins. N_{Abs} values were calculated in accordance with [25] and are shown in Fig. 3(c). The photodynamic activity of the PS can be better explained by taking into account both effects, N_{Abs} and ϕ_Δ, through their product ($N_{Abs} \times \phi_\Delta$) (Fig. 3d), which follows the sequence ZnTMPyP > TMPyP > $ZnTPPS_4$ > $TPPS_4$, similar to the photodynamic efficacy in the case of BoHV-1.

Several studies have demonstrated the efficacy and safety of PSs in the therapy of animal and human infectious diseases and neoplasias using topical and systemic formulations [26–29]. Although our study was carried out *in vitro*, we previously evaluated the effect of photodynamic inactivation on bovine cells (MDBK) to determine a non-cytotoxic concentration and ensure safety in *in vivo* therapy. We believe that the PSs used in the conditions presented here could be

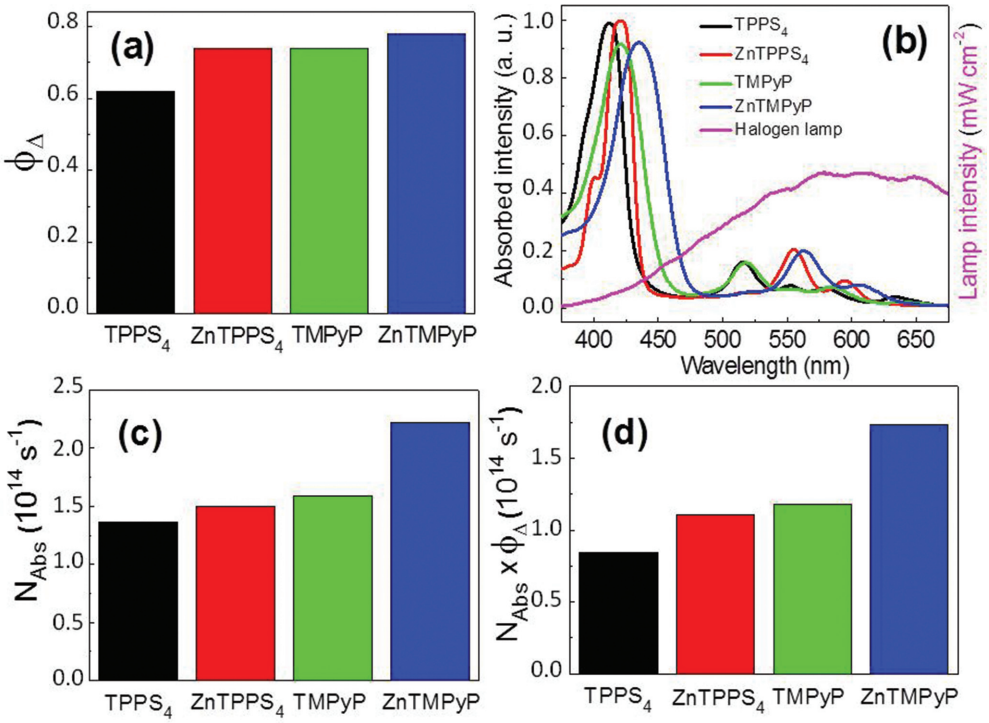

Fig. 3. (a) PS singlet oxygen quantum yields (ϕ_Δ) [22, 24]. (b) Porphyrin absorption and halogen lamp emission spectra. (c) Number of absorbed photons per second: $N_{Abs} = \frac{1}{hc}\int_{\lambda_1}^{\lambda_2} I_0(\lambda)(1-10^{-A(\lambda)})P(\lambda)d\lambda$, where A is the absorbance of the samples, $I_0(\lambda)$ is the incident light intensity, h is Planck constant (h=6.63×10^{-34} J▾s) and c is the velocity of light (c=3.0×10^8 m s^{-1}) [27]. (d) Product ($N_{Abs}\times\phi_\Delta$).

valuable in topical formulations for loco-irradiated treatment of lesions characteristic of reproductive disorders (balanoposthitis and vulvovaginitis) caused by BoHV-1.

The use of viricides for disinfection of animal by-products (semen or oocytes) has not been extensively investigated [12]. Our results demonstrate the complete inactivation of virus with a low and non-cytotoxic concentration of PS. The findings will inform further investigations on the use of PSs for this purpose. In addition, our group has investigated the photodynamic action of ZnTMPyP on several biomolecules, including proteins, lipids and DNA (manuscript in preparation). This approach is advantageous compared to that of conventional drugs because it virtually eliminates the possibility of the selection of resistant viral strains during treatment.

The results obtained demonstrate the high effectiveness of the photodynamic inactivation of BoHV-1, and porphyrins are promising photosensitizers for this application using this method as an auxiliary in the treatment of disorders attributed to BoHV-1 infection.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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