

Photodynamic inactivation of herpes viruses with phthalocyanine derivatives

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Abstract

The antiviral photosensitization capacity of 11 different phthalocyanine (Pc) derivatives was examined using herpes simplex virus-1, herpes simplex virus-2 and varicella zoster virus in the search for the most potent sensitizers for viral decontamination of blood. The kinetics of viral photoinactivation were resolved during the stages of viral adsorption and penetration into the host cells. The capacity of Pc in the photodynamic inactivation of viruses was compared with that of merocyanine 540 (MC540), another widely studied photosensitizer. Sensitivity to photoinactivation decreased progressively with time after addition of viruses to their host cells. The viruses were most sensitive to photodynamic inactivation up to 30 min from the initiation of adsorption. Cell-associated viruses, 45–60 min after the onset of adsorption, are highly resistant to photodynamic treatment by most photosensitizers, with the exception of amphiphilic Pc derivatives. Thus the mixed sulfonated Pc–naphthalocyanine derivatives AlNSB₃P and AlN₂SB₂P demonstrated a remarkable decontamination activity even 60 min after the onset of adsorption. Ultrastructural examination of these photosensitized viruses demonstrated damage to the viral envelope which prevented viral adsorption and/or penetration. The non-enveloped adenovirus was found to be resistant to all the dyes tested.

Key words: Phthalocyanines; Photosensitization; Photodynamic treatment; Herpes viruses

1. Introduction

Photodynamic inactivation of viruses in blood and blood components using various photosensitizers and light of appropriate wavelength is currently under intensive study [1–4]. This interest is due to the continued risk of the transmission of viruses by blood and blood products. Among these sensitizers, phthalocyanine (Pc) derivatives have shown great promise [4–6]. Pc are porphyrin-like second-generation sensitizers for photodynamic therapy (PDT) of cancer [7, 8]. Their intense absorption in the far red and long-lived excited triplet state are among the important attributes that make them ideally suited to PDT. In addition, by selecting appropriate metal ligands and peripheral substituents, Pc derivatives that are pow-

erful sensitizers for PDT demonstrate only minimal damage to red blood cells [9], thus making them potentially useful for viral decontamination of blood.

The mechanism by which Pc inactivates viruses is not known. Only enveloped viruses have been photoinactivated by Pc [4]. Membrane photosensitizer dyes such as porphyrin-like compounds are less mutagenic than DNA photosensitizing dyes [10]. The viral nucleic acids are therefore not an important target during Pc-induced photoinactivation. Rather, some protein(s) comprising the viral envelope is (are) probably sensitive to photoinactivation [5]. With respect to photochemistry, it appears that viral inactivation proceeds primarily by a type II singlet-oxygen-mediated mechanism [6]. However, it is not known how photochemical damage in the viral envelope causes inactivation. To answer this question we determined viral pho-

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photodynamic inactivation during the first stages of viral infection. The possibility that viral inactivation is due to impaired adsorption or penetration to the host cell was examined.

2. Materials and methods

2.1. Photosensitizers

A mixture of sulfonated aluminum Pc (AlPcSn) was obtained from Ciba-Geigy. All other Pc derivatives were synthesized as described [11–13] and purified to homogeneity by high performance liquid chromatography. The disulfonated MPC was enriched in isomers with adjacent substituents (MPcS₂a). The following compounds were used: H₂PcS₄, AlPcS₂, AlPcS₄, ZnPcS₄ and AlPcSn dissolved in phosphate-buffered saline; AlNSB₃P and GaNSB₃P in H₂O–MeOH (H₂O:MeOH = 4:1); GaN₂SB₂P and AlN₂SB₂P in MeOH–dimethylsulfoxide (DMSO) (MeOH:DMSO = 1:2); GaPcS₂ in MeOH; GaN₃SBP in DMSO; merocyanine 540 (MC540) in 50% EtOH. The solutions were stable for 1 year. For molecular structure and nomenclature of these compounds see ref. 12. MC540 was obtained from Sigma. The stable dyes were stored at 4 °C as 1 mM stock solutions and sterilized by filtration.

2.2. Viruses and cells

Herpes simplex virus-1 (HSV-1), MacIntyre strain, and herpes simplex virus-2 (HSV-2), RL strain, were propagated at a low multiplicity of infection (MOI, 0.1) on African green monkey cells (VERO cells; ATCC CCL 81; American-type culture collection) and harvested from culture supernatant when a cytopathic effect was present in 70% of the cells. After low speed (800 g; 10 min) centrifugation the virus was aliquoted and stored at –70 °C until use. Virus titers were determined by infecting VERO cell monolayer in multiwell plates (24 wells) with 200 µl of serial tenfold dilution. After 15 min each well was overlaid with 1.5 ml of Eagle's minimum essential medium supplemented with non-essential amino acids (MEM–NAA) containing 2% fetal calf serum (FCS) and 2% methyl cellulose and incubated. Plaques were scored after 4 days at 37 °C in a humidified atmosphere containing 51% CO₂ and the titers estimated on the basis of mean counts of three or more replicate wells. Standard errors were less than 10% and are not shown for clarity.

Varicella zoster virus (VZV) 10283 strain (laboratory strain) was propagated on primary human embryonic fibroblast (HEF) cells and harvested

when a cytopathic effect was observed in more than 80% of the cells. Cells were suspended in PBS and rinsed twice. They were then sonicated for 60 s and centrifuged at 1500 rev min⁻¹ for 15 min in the cold. The supernatant-containing cell-free virus was used immediately. Virus titers were determined by infecting HEF cells in multiwell plates with 200 µl of serial tenfold dilution of the virus. After 30 min adsorption, the monolayer was overlaid with 1.5 ml of MEM–NAA supplemented with 2% FCS and 2% methyl cellulose and incubated at 33 °C in a humidified atmosphere containing 5% CO₂. Plaques were scored 5 days later and the titers calculated on the basis of mean counts of three replicate wells. Standard errors were less than 10% and are not shown for clarity.

Human adenovirus-2 (laboratory strain) was propagated in human kidney cells (HK cell line established in the laboratory in 1975), harvested from culture supernatants and stored at –70 °C in 1 ml aliquots until use. Virus titer was determined by infecting HK monolayer in multiwell plates with 200 µl of serial tenfold dilution overlaid with 1.5 ml of MEM–NAA containing 3% FCS. Plaque forming units (PFU) were scored 5 days later.

2.3. Light exposure

The light source was a slide projector equipped with a 150 W quartz halogen light bulb. For exposure of Pc-containing samples the light was filtered by a cut-off filter ($\lambda > 605$ nm). Light exposure of MC540 was without a filter. The incident fluence rate was 200 W m⁻². Exposure of viral particles and cells was in 3 ml of PBS containing 2% FCS in 35 mm polystyrene plates at 23 ± 2 °C, for 15 min unless otherwise specified.

2.4. Viral photoinactivation

For photodynamic treatment, VERO, HK HA or HEF cells were infected with 200 µl of cell-free viral suspension at 2.9 × 10⁵–2 × 10⁶ (MOI, 1–2) for HSV-2, 1 × 10⁶–3 × 10⁶ (MOI, 1–2) for adenovirus-2, and 3.9 × 10³–1 × 10⁴ (MOI, 0.002–0.003) for VZV. The viruses were allowed to adsorb for various time intervals at 33 ± 2 °C. After adsorption the excess virus was removed and the cells were rinsed twice with 3 ml of PBS. Next, 5 µM of dye was added. Except for MC540, which was used at a concentration of 25 µM, the plates were then immediately exposed to light or kept in the dark for 15 min. In each experiment, infected cells without dye, at the same experimental conditions, were included and served as a control. In addition, cells exposed to neither light nor dye, or to light or dye only, were tested.

Following light exposure, the plates were rinsed three times with 3 ml of MEM–NAD and overlaid with 3 ml of MEM–NAD containing 2% FCS and 2% methylcellulose. The plates were incubated at 37 °C for HSV and adenovirus-2, and at 33 °C for VZV in a 5% CO₂ humidified incubator. Plaques were scored 5 days later and the titers calculated based on the mean count of three replicate wells.

To assess the effect of the dyes on cellular processes essential for virus replication we treated cells with the compounds for different intervals before the addition of the viruses. The dyes were added to the cells at time zero, 15 min, 30 min and 60 min. At the relevant time, the excess dye was removed. Cells were rinsed three times with PBS and then exposed to light or left in the dark for 15 min. Appropriate virus titers of HSV-1 or HSV-2 or VZV at 3×10^6 PFU ml⁻¹, 2×10^6 PFU ml⁻¹ or 1×10^4 PFU ml⁻¹ respectively were added and overlaid with 1.5 ml of MEM–NAA containing 3% FCS. PFU was scored 5 days later.

2.5. Electron microscopy

2.5.1. Preparation for transmission electron microscopy

The virus-infected cell preparations were fixed in Karnovsky's phosphate buffer pH 7.2, 0.15 M containing glutaraldehyde–paraformaldehyde 2% fixative and post-fixed in OsO₄. Dehydration was carried out with ethanol. The embedding medium was Epon–Araldite. Sections were cut on a LKB-III ultratome, stained with uranyl acetate and lead citrate and photographed using a JEOL 1200EX transmission electron microscope.

2.5.2. Negative staining

Carbon-coated Formvar grids were each flooded for 20 s with a drop of a fresh preparation of the virus. The trapped particles were negatively stained with 2% uranyl acetate. Preparations were examined with a JEOL 100 transmission electron microscope.

3. Results

The antiviral photodynamic activity of 11 different Pc compounds was examined on the enveloped viruses HSV-1, HSV-2 and VZV and on the non-enveloped adenovirus. The concentration of compounds and duration of exposure to light were chosen in order to minimize toxicity to the cells. Thus the lowest compound concentrations and the shortest times of exposure giving maximum effects were chosen (data not shown). Using these

conditions we examined various concentrations of viral preparations ranging from 10² to 10⁶ PFU ml⁻¹. In these experiments, diluted virus preparations exhibited the same pattern of adsorption to cells as the concentrated virus preparations, thus excluding the possibility of interference of defective viruses with the replication of the standard HSV [14]. We therefore performed our experiments using one concentration of viral stock for each virus as indicated in Section 2. The toxicity of each compound to the cells was determined before performing the experiments of virus inhibition. We worked only under conditions that gave less than 10% toxicity to HEF and VERO cells. Photodynamic inactivation of HSV-1 at various times after adsorption by AlPcSn and AINSB₃P (5 μM; 15 min light exposure) was compared with that of MC540 (25 μM; 15 min light exposure), as shown in Fig. 1(a). Evidently, complete (5 log₁₀ or more) HSV-1 inactivation was achieved when VERO-infected cells were exposed to the dyes at time zero up to 30 min after adsorption. Photoinactivation was reduced at 45 min for MC540 and AlPcSn but not for AINSB₃P or AlN₂SB₂P; the latter two dyes were fully effective even after 60 min. Neither light alone nor dyes in the dark were toxic, except for MC540, which reduced viral survival by about 50%. Previous reports have failed to mention a dark toxicity of MC540 [2]. This may be due to our use of a much lower FCS during treatment (2% vs. 12%). Figure 1(b) shows the photosensitization capacity of other Pc derivatives on HSV-1. Obviously all of them, except for the metal-free derivative H₂PcS₄, were fully effective at time zero, but their activity decreased rapidly with increasing time of adsorption.

The same photosensitization pattern was seen for HSV-2 (Figs. 2(a) and 2(b)). Photodynamic inactivation was efficient (5 log₁₀ or more) at time zero by all the dyes, except for H₂PcS₄. Loss of photosensitivity with adsorption time was seen here as well.

VZV was found to be photoinactivated (3.9 log₁₀ or more) at time zero by all dyes (Figs. 3(a) and 3(b)). Upon adsorption, sensitivity was gradually lost between 15 and 60 min for H₂PcS₄, GaN₃SBP, GaNSB₃P, GaPcS₄ and MC540. The efficiency of viral photodynamic inactivation was decreased between 45 and 60 min after adsorption for GaPcS₂, AlPcSn, ZnPcS₄, AlPcS₄, AINSB₃P, AlN₂SB₂P and GaN₂SB₂P (Figs. 3(a) and 3(b)). None of the dyes was toxic in the dark.

Pre-treatment of the cells with each dye before viral infection revealed that none of the dyes tested had an effect on virus yield except for MC540,

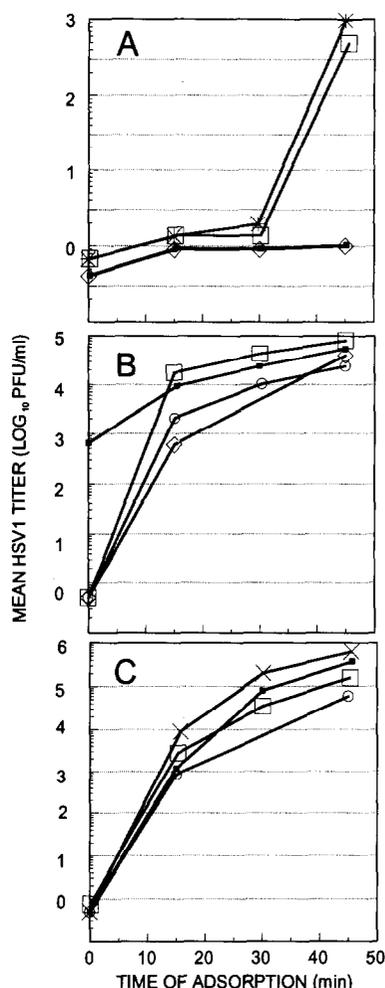


Fig. 1. Photosensitized inactivation of HSV-1 as a function of time after viral adsorption to VERO cells, where samples of HSV-1, 6×10^5 PFU per $200 \mu\text{l}$, were employed: (A) at the time indicated $5 \mu\text{M}$ of AlPcSn (\square), AlNSB₃P (\diamond), AlN₂SB₂P (\blacksquare) or MC540 ($*$) were added and exposed to light for 15 min, as described in Section 2; (B) H₂PcS₄ (\blacksquare), GaN₂SB₂P (\diamond), ZnPcS₄ (\square) and GaPcS₂ (\circ); (C) GaN₃SBP (\square), AlPcS₄ (\circ), GaNSB₃P (\blacksquare) and GaPcS₄ (\times).

which caused a 50% reduction. The ultrastructural examination of the photosensitized VZV infected human HEF cells is presented in Fig. 4. When the VZV-infected cells were treated by GaN₂SB₂P and exposed to light for 15 min, only remnants of viral debris could be detected (Fig. 4(a)), while viral particles are seen adsorbed on the control cells. When free HSV-1 particles were treated with AlN₂SB₂P followed by light for 15 min, extensive damage to the viral envelopes was detected after negative staining with uranyl acetate (Fig. 5(b)), while control particles are intact (Fig. 5(a)).

Adenovirus, a representative non-enveloped virus treated under the above standard experimental conditions, was found to be resistant to photodynamic inactivation by all tested dyes.

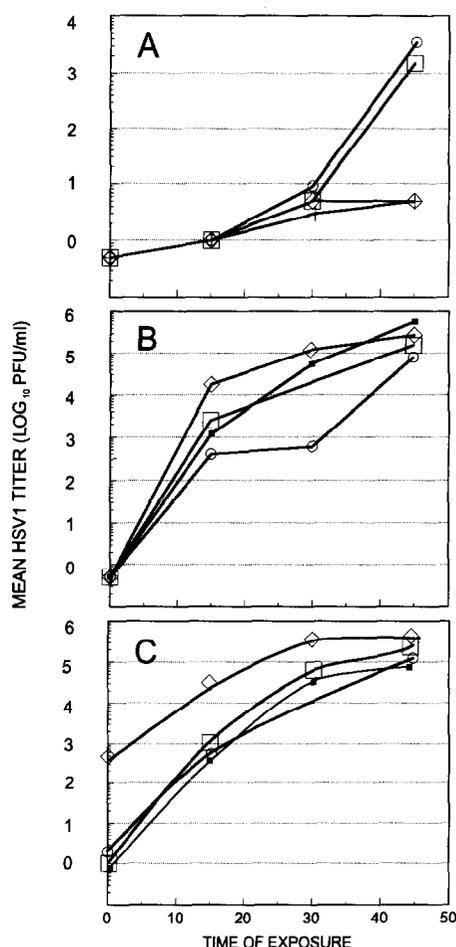


Fig. 2. Photosensitized inactivation of HSV-2 at the concentration of 4×10^5 per PFU per $200 \mu\text{l}$ were employed: (A) AlPcSn (\square), AlNSB₃P (\diamond), AlN₂SB₂P ($+$) and MC540 (\circ); (B) AlPcS₄ (\square), ZnPcS₄ (\diamond), GaPcS₂ (\circ) and GaN₃SBP (\blacksquare); (C) GaNSB₃P (\square), H₂PcS₄ (\diamond), GaN₂SB₂P (\circ) and GaPcS₄ (\blacksquare).

4. Discussion

In the present work a series of Pc derivatives containing the metal ligands Zn, Al and Ga were shown to be effective sensitizers for the photodynamic inactivation of HSV-1, HSV-2 and VZV, while H₂PcS₄ was less effective compared with MC540. Previous studies [4, 5] have shown that the efficacy of photodynamic inactivation of cell-free enveloped viruses by Pc derivatives depended upon the metal ligand and the extent of sulfonation. In this work a similar trend was observed although the differences were not in accordance with structural modification (evidenced by the kinetics of viral inactivation). The main observation that comes out of our studies is the progressive disappearance of viral sensitivity to photodynamic inactivation with time of adsorption to host cells. This implies that intracellular viruses are more resistant than the free virions. However, it is note-

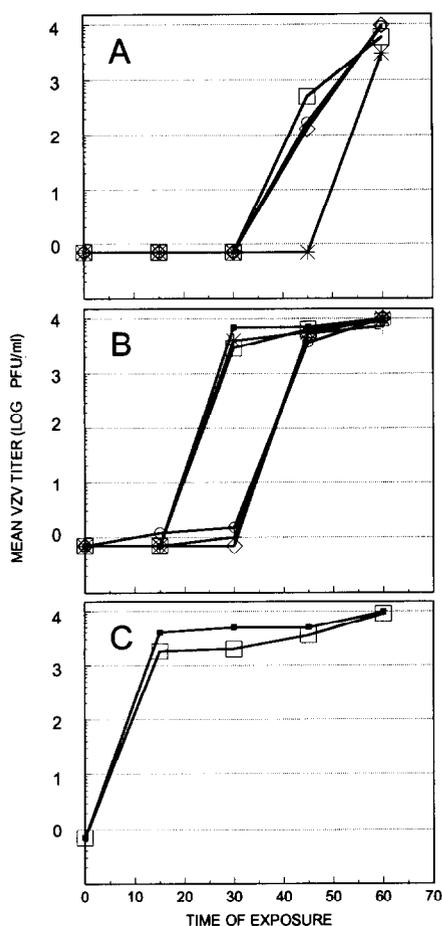


Fig. 3. Photosensitized inactivation of VZV at the concentration of 4×10^3 per PFU per $200 \mu\text{l}$: (A) AlPcSn (\square), AlNSB₃P ($*$), AlN₂SB₂P (\diamond) and GaN₂SB₂P (\circ); (B) MC540 (\square), GaPcS₂ (\diamond), GaNSB₃P ($*$), GaPcS₄ (\blacksquare), AlPcS₄ ($+$) and ZnPcS₄ (\circ); (C) GaN₃SBP (\square) and H₂PcS₄ (\blacksquare).

worthy that the loss of photosensitivity with adsorption time varies for the various dyes. Most notable is the preservation of sensitivity of HSV-1 and HSV-2 to the amphiphilic dyes AlNSB₃P and AlN₂SB₂P, up to 60 min after adsorption. For VZV these dyes also appear to be among the most effective although, by 60 min, only about 50% viral kill is observed. It is likely that the amphiphilic nature of these dyes, which enables them to penetrate cell membranes readily [12], is the basis for their ability to photosensitize inactivation of intracellular viruses.

It is not yet clear at which point in the infection process the virus becomes more resistant to photodynamic inactivation. The viral envelope is evidently a primary target for photoinactivation but, since sensitivity to some dyes persists longer than to others, it suggests that other processes associated with the virus life cycle which occur following virus penetration might also be sensitive to the pho-

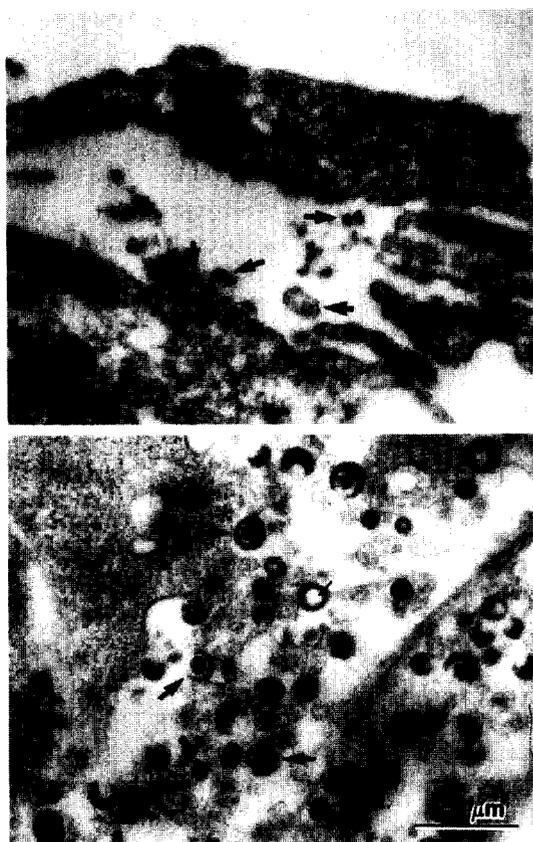


Fig. 4. The ultrastructural examination of the photosensitized VZV infected HEF treated by GaN₂SB₂P and exposed to light for 15 min: (a) only remnants of viral debris could be detected (arrow); (b) intact viral particles (arrow) are seen adsorbed on the control unilluminated cells during endocytosis (asterisk); defective virions (small arrows). (Magnification, 39 000 \times .)

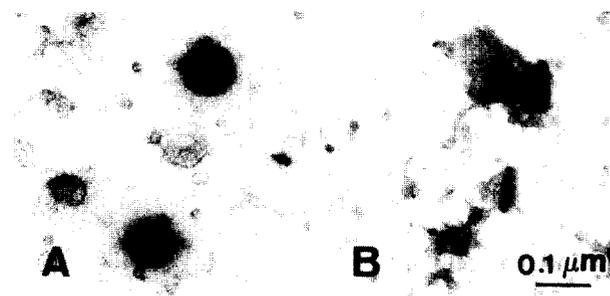


Fig. 5. Electron micrographs of uranyl acetate negatively stained HSV-1 free particles treated with AlN₂SB₂P followed by light for 15 min. (B) Extensive marked damage to the viral envelopes was detected, while (A) control particles treated similarly but unilluminated are intact. (Magnification, 83 000 \times .)

photodynamic damage. More studies are required to clarify and evaluate these points.

In vitro, the characteristics of HSV adsorption to cells vary, indicating differences in receptors or avidity of the receptors for the attachment of the HSV envelope proteins. The virus envelope

contains at least eight membrane glycoproteins. Fusion and virus penetration is promoted by these viral glycoproteins, most probably the proteins gB, gD, gH and gC [15–17]. Several factors such as the ionic strength, the pH and the presence of divalent cations influence the attachment of viruses to host cells, which are all multivalent. The rate of virus attachment is highly dependent on the cell line and on the density of cellular receptors. Both naked and enveloped viruses have multiple molecules of ligands capable of binding to cellular receptors [18]. Virions adsorb to cells more readily than the nucleocapsids, indicating a specific interaction between viral envelope and the cell surface membrane. The attachment and penetration are rapid; at 37 °C, more than 90% of the attached HSV can penetrate the host cell within 30 min [19]. Another publication demonstrated that the internalization of HSV is completed within 20–30 min after attachment [20]. The virion–cell fusion event, whether occurring at the cell surface, or after endocytosis, was shown to be sensitive to inhibition by neutralizing antibodies. The virion–cell fusion is an essential step in the pathway leading to infection by enveloped viruses, and one that could be exploited in the development of antiviral agents and vaccines [21].

Another unresolved question is exactly what stage in the penetration process is affected by the photodamage to the viral envelope. It could either prevent adherence of the virus to its receptor on the plasma membrane of the host cell and/or inhibit internalization of the adhered virus [2, 22]. The extensive structural damage to the viral envelope after photosensitization (Fig. 5) suggests the former. The use of herpes virus mutants that are capable of binding but incapable of penetration will help to resolve this question.

It is conceivable that free viruses are more sensitive to photoinactivation than cell-associated viruses such as VZV when hydrophobic photosensitizers (AIPc and benzoporphyrin derivative) have been used [4, 23]; this is in accordance with previous publications [24]. Our results showed that this is not necessarily the case for more hydrophilic sensitizers, presumably because the latter penetrate the cells less readily. This also is in accordance with previous publications [24]. It should be noted that the examined Pc derivatives have low toxicity to HEF and VERO cell cultures when tested for the indicated time of illumination. It seems that treatment with all dyes except MC540 did not inhibit cellular processes essential for viral entry and/or replication. Cell-free viruses are a minor problem in blood-banking applications, since they

are liable to photoinactivation, whereas red blood cells are relatively resistant to such a treatment with Pc derivatives [9]. Inactivation of latent and/or replicating viruses remains the main challenge, e.g. HIV in T lymphocytes. Therefore the observation that AlNSB₃P and AlN₂SB₂P are effective in inactivating HSV during prolonged interval after adsorption is encouraging. The last dye, AlN₂SB₂P, is of particular interest since it is rather ineffective in sensitizing photohemolysis of human erythrocytes [9]. Damage to red blood cells and platelets is the limiting factor during viral decontamination of blood by PDT [6].

In conclusion, some amphiphilic Pc derivatives such as AlN₂SB₂P appear to be effective sensitizers for photodynamic inactivation of free and intracellular enveloped viruses. Further studies to determine their potential for blood banking purposes and mechanisms of action are currently underway in our laboratories.

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