Photodynamic inactivation of retroviruses by phthalocyanines: the effects of sulphonation, metal ligand and fluoride

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Abstract

The photodynamic inactivation of retroviruses was investigated using aluminium and zinc phthalocyanine (Pc) derivatives. The N2 retrovirus packaged in either of the two murine cell lines, Psi2 and PA317, was used as a model for enveloped viruses. AlPc derivatives were found to be more effective photodynamically for inactivation of the viruses than the corresponding ZnPc derivatives. Sulphonation of the Pc macrocycle reduced its photodynamic activity progressively for both AlPc and ZnPc. Fluoride at 5 mM during light exposure completely protected viruses against inactivation by AlPc. In the presence of F^-, inactivation by the sulphonated derivatives $AlPcS_1$ and $AlPcS_4$ was reduced 2.5- and twofold respectively. In a biological membrane (erythrocyte ghosts), F^- had no significant effect on $AlPcS_4$-sensitized lipid peroxidation. Under similar conditions, cross-linking of spectrin monomers in ghosts is drastically inhibited (E. Ben-Hur and A. Orenstein, *Int. J. Radiat. Biol., 60* (1991) 293–301). Since Pc derivatives do not inactivate non-enveloped viruses, it is hypothesized that inactivation occurs by photodynamic damage to envelope protein(s). Substitution of sulphonic acid residues reduces the binding of Pc derivatives to the envelope protein(s), thereby diminishing their photodynamic efficacy and the ability of F^- to modify it.

Keywords: Metallophthalocyanines, retroviruses, photodynamic therapy, fluoride.

1. Introduction

Photodynamic therapy (PDT) of viral infections, mainly those of *Herpes simplex* virus (HSV), has previously used positively-charged dyes (*e.g.* proflavine or methylene blue) which interact primarily with the viral DNA [1]. This approach has been discouraged because photodynamic damage to the viral DNA unmasks the oncogenic potential of the virus, as manifested by the appearance of transformed cells in the culture [2]. Membrane-photosensitizing dyes have the advantage of inactivating the virus at a site other than the genetic material [3]. A number of lipophilic and negatively-charged

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hydrophilic photosensitizers have been investigated, both for viral infections and for sterilizing blood and blood products. These include haematoporphyrin derivative (HPD) [4, 5], merocyanine [6], pyrene dodecanoic acid [7], sapphyrins [8] and phthalocyanine (Pc) derivatives [9].

Pc derivatives are efficient photosensitizers [10, 11] that are being studied as second-generation sensitizers for PDT of cancer [12–14]. For the purpose of sterilizing blood, Pc has the advantage of an intense absorption band in the far red, beyond that of haemoglobin. Thus, light used to excite Pc can penetrate blood to a considerable depth that is limited only by scatter. Compared with HPD or Photofrin^R, zinc tetrasulphophthalocyanine ($ZnPcS_4$) is approximately ten times more effective in inactivating HSV and increases the mutation frequency only slightly (less than twofold) [15]. Interestingly, in spite of the ability of $AlPcS_4$ to induce photohaemolysis of human erythrocytes quite effectively *in vitro* [16–18], only minor damage to red blood cells is seen when whole blood is photodynamically treated with this dye [9]. The infectivity of vesicular stomatitis virus (VSV) is eliminated under these conditions.

The present work was undertaken to investigate some parameters that determine the efficacy of Pc-induced photoinactivation of enveloped viruses. Two retroviruses were used to study the role of the metal ligand, as well as the extent of sulphonation, in the photodynamic effect. Aluminium appears to potentiate the photodynamic activity of the Pc macrocycle compared with zinc and sulphonation progressively decreases viral inactivation. Both viruses display a similar sensitivity. The presence of F^- during illumination completely abolishes the photodynamic activity of AlPc and only to some degree that of $AlPcS_1$ and $AlPcS_4$.

2. Materials and methods

2.1. Photosensitizers

AlPc and ZnPc were obtained from Eastman Kodak, Rochester, NY, USA. The sulphonated derivatives of Pc were a gift from Dr. J. E. van Lier (University of Sherbrooke, Canada). The dyes were stored at 4 °C as 0.5 mM stock solution in dimethylformamide (AlPc and ZnPc), methanol ($AlPcS_1$ and $ZnPcS_1$) or phosphate-buffered saline (PBS), pH 7.4 ($AlPcS_4$ and $ZnPcS_4$). The latter solution was sterilized by passing through a 0.2 μm Millipore filter.

2.2. Light exposure

The light source was a slide projector equipped with a 150 W quartz halogen light bulb. The light was filtered by a cut-off filter ($\lambda > 605$ nm). The incident fluence rate was 200 W m^{-2}. The viral particles were exposed in 1 ml growth medium containing the appropriate dye concentration in 3.5 cm plastic Petri dishes at a titre of $(1–2) \times 10^6$ infective particles ml^{-1}.

2.3. Lipid peroxidation of red blood cell ghosts

Ghosts were prepared from human red blood cells (RBCs) as described previously [19]. Lipid peroxidation in RBC ghosts was measured in 1.2 ml of suspension (0.16 mg protein ml^{-1}) in PBS by addition of 0.3 ml 30% trichloroacetic acid for 15 min at 4 °C. The suspension was then centrifuged and 1 ml of 1% thiobarbituric acid in 0.05 N NaOH was added to 1 ml of the supernatant. The solution was heated for 15 min at 100 °C, cooled and the absorbance was measured at 532 nm.

2.4. Cell culture

All sera and tissue culture media were purchased from GIBCO Laboratories (Grand Island, NY, USA). Culture plastic dishes and other disposables were obtained from Greiner (Nürtingen, FRG). Cell lines were grown in high-glucose (4.5 g l^{-1}) Dulbecco's modified Eagle's medium supplemented with 8% foetal calf serum in a humidified atmosphere containing 5% CO_2 at 37 °C. The HPRT$^-$Balb/c 3T3 cell line B77 has been described previously [20].

2.5. Virus preparation and titration

Viral inactivation by Pc-PDT was assayed on recombinant retroviruses, a model which normally serves as retroviral vector. Retrovirus N2 was constructed by deletion of most of the sequences encoding the viral proteins and introduction of the bacterial gene for the enzyme neomycin phosphotransferase, which confers resistance to the neomycin analogue G418 in eukaryotic cells [21].

The general strategy for generating high-titre-producing cell lines has been described previously [22]. The retrovirus-packaging cell lines Psi2 [23] and PA317 [24] were transfected with N2-plasmid DNA as a retroviral vector [21] by the conventional calcium phosphate method [25]; 24 h post-transfection the growth medium was replaced by fresh medium, and after an additional 48 h the virus-containing medium was harvested. When derived from Psi2 cells, the medium was used to infect fresh PA317 cells and vice versa; 24 h after infection, the infected cell population was split 1:10 in medium containing 400 μg ml^{-1} G418 (GIBCO) to select for infected cells. From the infected Psi2 and PA317 cells, individual G418-resistant clones were isolated, expanded and assayed for virus production.

To isolate virus stocks, near-confluent cultures were grown in fresh medium for 16 h, after which the medium was harvested and filtered through a 0.45 μm filter (Gelman Sciences Inc., Ann Arbor, MI, USA) to remove detached cells, and stored at −80 °C until further use. The virus titres of the samples were determined by their ability to transfer G418 resistance to cells. The procedure involved exposure of B77 cells to dilutions of virus-containing medium in the presence of 8 μg ml^{-1} polybrene (Sigma); 24 h later the cells were split 1:10 in growth medium containing 500 μg ml^{-1} G418 to select for infected cells. Colonies were stained and counted 11 days after infection. Titres of the virus stocks were 8×10^5 and 3×10^6 $G418^R$ colony-forming units per millilitre for the Psi2-N2 and PA317-N2 lines respectively.

3. Results

Figure 1 shows the survival curves of the N2 vector enveloped in Psi2 photosensitized by AlPc derivatives with and without fluoride. AlPc is about fivefold and 15-fold more effective than $AlPcS_1$ and $AlPcS_4$ respectively. F^- completely protects against the photodynamic effect of AlPc. Protection is by a factor of 2.5 and 2.1 for $AlPcS_1$ and $AlPcS_4$ respectively, calculated as the ratio of light fluences required to inactivate 90% of the viruses. In calculating the relative potency of the dyes, an inverse reciprocity was assumed between the dye concentration and the light fluence required for inactivation [9].

Substitution of aluminium by zinc in the Pc macrocycle decreases the photodynamic potency of the dye (Fig. 2). The photoinactivation of the virus decreases by a factor of 5–10 when aluminium is replaced by zinc, depending on the extent of sulphonation. Qualitatively, sulphonation decreases the activity of ZnPc in a similar fashion to that

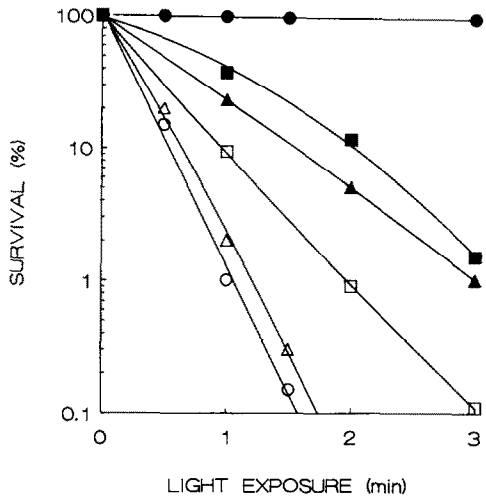

Fig. 1. Survival of the murine retrovirus N2-Psi2 photosensitized with 1 μM AlPc (circles), 5 μM $AlPcS_1$ (triangles) and 10 μM $AlPcS_4$ (squares). Open and filled symbols represent exposure in the absence and presence of 5 mM NaF respectively.

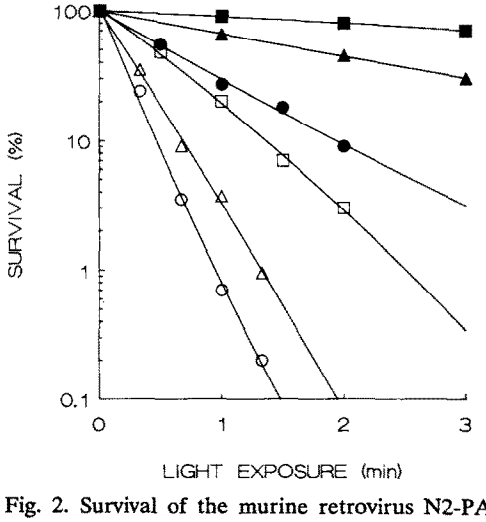

Fig. 2. Survival of the murine retrovirus N2-PA317 photosensitized with 1 μM Pc (circles), 5 μM PcS_1 (triangles) and 10 μM PcS_4 (squares). Open and filled symbols represent the aluminium and zinc derivatives respectively.

of AlPc (Fig. 2). In addition, when the activities of the AlPc derivatives against the two viruses (Fig. 1 *vs.* Fig. 2) are compared, no significant difference is observed.

Fluoride protects against the photodynamic inactivation of some proteins by AlPc derivatives [26, 27]. To understand further the protection by F^- in the case of enveloped viruses, the effect of F^- on $AlPcS_4$-photosensitized lipid peroxidation was studied in ghosts of human erythrocytes. The results (Fig. 3) show that the rate of lipid peroxidation is the same in the presence or absence of F^-.

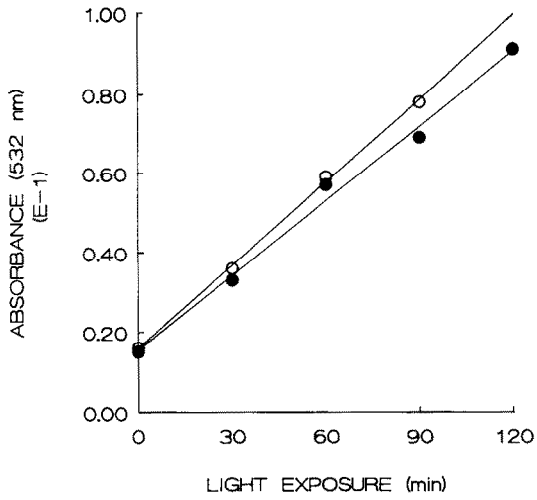

Fig. 3. Lipid peroxidation in RBC ghosts sensitized by $AlPcS_4$. Ghosts suspended in 10 ml PBS (0.16 mg ml^{-1}) were incubated for 10 min at 21 °C with 10 μM $AlPcS_4$. The ghost suspension was then irradiated with (●) or without (○) 5 mM NaF. At certain time intervals 1.2 ml samples were withdrawn and lipid peroxidation was determined as described in Section 2.3.

4. Discussion

The interest in Pc derivatives as photodynamic agents for PDT in general, and for inactivation of enveloped viruses in blood in particular, has prompted us to explore the parameters that determine the efficacy of Pc for the latter purpose. Our results show that, as found for other biological systems [11–14], both the metal ligand and the degree of sulphonation are important. Aluminium is more effective than zinc for photodynamic inactivation of retroviruses (Fig. 2), as has also been shown for Chinese hamster cells [28]. For both AlPc and ZnPc the effectiveness is reduced as the degree of sulphonation increases. In the case of Chinese hamster cells, the reduced effectiveness of the sulphonated derivatives has been shown to be due to reduced uptake by the cells and not to a reduced photodynamic activity [29, 30]. For retroviruses the reduced activity of the sulphonated Pc derivatives probably reflects reduced binding to the viral envelope. Sulphonated Pc derivatives bind less tightly to proteins than non-sulphonated Pc compounds [27] and the same is probably true in the case of viral membrane lipids.

Fluoride can protect human erythrocytes against photohaemolysis by $AlPcS_4$ [18] and Chinese hamster cells against phototoxicity of AlPc [31]. This is most probably due to complex formation between F^- and aluminium [32], leading to a modified binding of the dye to the target protein [27], and reduced photodynamic damage. Figure 3 shows that $AlPcS_4$ photosensitizes lipid peroxidation in RBC ghosts, confirming a previous report [33], and that F^- has no effect on this process. Under these conditions cross-linking of spectrin, a membrane protein of RBC, is drastically inhibited by F^- [26]. It is therefore hypothesized that the complete protection by F^- against inactivation of retroviruses by AlPc is due to its effect on binding of the dye to the target membrane protein(s). The reduced effect of F^- for the sulphonated AlPc may be due to a reduced affinity of these derivatives for the target protein. The viral nucleic acids are probably not an important target in the photoinactivation. This is deduced from the observations

that non-enveloped viruses are not inactivated [9] and $ZnPcS_3$ is a very weak mutagen for enveloped viruses [15].

It is of interest to note that, in whole blood, the sulphonated derivatives $AlPcS_2$ and $AlPcS_4$ are more effective than AlPc in inactivating VSV [9], in contrast with the trend found in our work. This is unlikely to be due to the use of a different virus, since, as shown here, when viruses are enveloped in different cells they respond similarly (compare Figs. 1 and 2). Rather, it is more probable that the difference in irradiation conditions is responsible for the observed change in trend. The sulphonated derivatives bind less tightly to serum proteins in whole blood than AlPc and thus there is more dye available for binding to the viral envelope.

Finally, our results points to a potential improvement in the sterilization of blood using $AlPcS_4$ and red light in the presence of F^-. $AlPcS_4$ is a powerful photohaemolytic agent *in vitro*. Although this effect is markedly reduced in whole blood [9], it could still pose a problem. Since 5 mM NaF can completely inhibit $AlPcS_4$-induced photohaemolysis [18], while reducing viral inactivation by a factor of two only (Fig. 1), its addition at an appropriate concentration could improve the therapeutic ratio of $AlPcS_4$-PDT. Toxicity of F^- should not be a problem. Thus 0.5 kg transfused blood containing 1 mM NaF results in less than 1 ppm tissue distribution.

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