Photodynamic inactivation of prions by disulfonated hydroxyaluminium phthalocyanine

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Sulfonated phthalocyanines (Pcs) are cyclic tetrapyroles that constitute a group of photosensitizers. In the presence of visible light and diatomic oxygen, Pcs produce singlet oxygen and other reactive oxygen species that have known degradation effects on lipids, proteins and/or nucleic acids. Pcs have been used successfully in the treatment of bacterial, yeast and fungal infections, but their use in the photodynamic inactivation of prions has never been reported. Here, we evaluated the photodynamic activity of the disodium salt of disulfonated hydroxyaluminium phthalocyanine (PcDS) against mouse-adapted scrapie RML prions in vitro. PcDS treatment of RML brain homogenate resulted in a time- and dose-dependent inactivation of prions. The photodynamic potential of Pcs offers a new way to inactivate prions using biodegradable compounds at room temperature and normal pressure, which could be useful for treating thermodabile materials and liquids.

The pathogenesis of transmissible spongiform encephalopathies (TSEs) is associated with the conversion of the protease-sensitive cellular prion protein (PrPC) to the partially protease-resistant amyloidogenic abnormal isoform of prion protein (PrP\textsuperscript{TSE}), which, upon digestion with proteasome K (PK), gives a protease-resistant fragment (PrPres) (Choi et al., 2011; Prusiner, 1998). TSEs are invariably fatal, as there are no available effective therapies. However, several different compounds were identified that inhibited the formation of PrP\textsuperscript{TSE} in vitro (Cashman & Caughey, 2004; Charvériat et al., 2009; Kocisko et al., 2003, 2005) and were partially effective in vivo using animal models (Priola et al., 2003; Trevitt & Collinge, 2006). Only a few of these compounds have been tested in the treatment of human TSEs (e.g. pentosan polysulfate, tetracycline and quinacrine) (Collinge et al., 2009; Stewart et al., 2008; Todd et al., 2005), but these compounds showed unsatisfactory results. Among the most potent classes of anti-prion inhibitors are the cyclic tetrapyroles, which include porphyrins and phthalocyanines (Pcs) (Abdel-Haq et al., 2005; Stewart et al., 2008; Todd et al., 2005), and other reactive oxygen species that have known degradation effects on lipids, proteins and nucleic acids (Sigler et al., 1999). Pcs have been used both in photodynamic therapy of dermal tissue to effectively kill malignant cells (Baron et al., 2010; Sheng et al., 2004) and in the treatment of bacterial, yeast and fungal infections (Calzavara-Pinton et al., 2005; Jori & Brown, 2004). Recently, successful photocatalytic inactivation of prions by titanium dioxide or by photo-Fenton reagent was demonstrated (Paspaltsis et al., 2006, 2009). However, the utility of the photodynamic effect of Pcs in the inactivation of prions has not been reported.

In the present study, we evaluated the photodynamic effect of the disodium salt of disulfonated hydroxyaluminium phthalocyanine (PcDS) – C\textsubscript{32}H\textsubscript{15}N\textsubscript{8}S\textsubscript{2}O\textsubscript{7}Na\textsubscript{2}Al (Fig. 1), which was synthesized by the Research Institute of Organic Synthesis (Rybitvi, Czech Republic) (Jancula et al., 2009). In addition, Pcs can also block the other types of disease-associated protein aggregation. PcTS has been shown to suppress the formation of an α-synuclein amyloid, which is a pathological factor in Parkinson’s disease (Lee et al., 2004), and inhibit the aggregation of insulin (Pasternack et al., 2006). Haemin analogues delay the fibril formation caused by the amyloid beta peptide that is associated with Alzheimer’s disease (Howlett et al., 1997), demonstrating the general propensity of tetrapyroles to interact with amyloidogenic peptides and disrupt fibril formation. As photosensitizers, phthalocyanines are capable of generating singlet oxygen and other reactive oxygen species after stimulation with visible light of a suitable wavelength (Claessens et al., 2008). While low levels of singlet oxygen can serve as signalling molecules, high levels can be fatal for cells by promoting oxidative degradation of lipids, proteins and nucleic acids (Sigler et al., 1999). Pcs have been used both in photodynamic therapy of dermal tissue to effectively kill malignant cells (Baron et al., 2010; Sheng et al., 2004) and in the treatment of bacterial, yeast and fungal infections (Calzavara-Pinton et al., 2005; Jori & Brown, 2004). Recently, successful photocatalytic inactivation of prions by titanium dioxide or by photo-Fenton reagent was demonstrated (Paspaltsis et al., 2006, 2009). However, the utility of the photodynamic effect of Pcs in the inactivation of prions has not been reported.

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First, we studied the effect of PcDS photodynamic treatment on the stability of PrP
t and PrP\text{TSE} molecules by Western blot. As a source of PrP\text{TSE}, we utilized brain homogenate of terminally ill CD1 mice infected with the mouse-adapted Rocky Mountain Laboratory strain (RML) of scrapie – RML5 (provided by Adriano Aguzzi, Institute of Neuropathology, University of Zurich, Switzerland). As controls, brain homogenates of healthy FVB mice (WT) and PrP\text{C}-overexpressing Tga20 mice (TGA) were used. To evaluate the dose dependence of the PcDS effect, RML, WT and TGA brain homogenates [50 µl, 1% (w/v)] were treated with increasing concentrations of PcDS (0, 1, 2.5, 5, 10 and 20 µg ml\textsuperscript{-1}) for 60 min at a distance of 0.6 m from a standard fluorescent visible light source (Osram Lumilux T8 L 58W/830) or incubated for 60 min in the dark with a standard fluorescent visible light source (Osram Lumilux). After demonstrating the dose- and time-dependent effect of PcDS photodynamic treatment on PrP\text{TSE}, we evaluated its impact on infectivity of RML brain homogenate utilizing a CAD5 cell-culture assay. We first tested the effect of PcDS on the viability of CAD5 cells (provided by Charles Weissmann, The Scripps Research Institute, FL, USA) (Qi \textit{et al.}, 1997), a neuronal catecholaminergic cell line sensitive to prion infection (Mahal \textit{et al.}, 2007). PcDS was added to a final concentration of 0.2, 1 or 2 µg ml\textsuperscript{-1} to the cultivation medium, and the cells were then incubated for 48 h in the dark. At a concentration of 2 µg ml\textsuperscript{-1} (two times higher than the highest concentration achieved in the medium during our study), there was no significant effect on cell proliferation, morphology or viability (Fig. S2).

RML brain homogenate was aliquotted [100 µl; 1 or 0.1% (w/v)] and treated with 5 or 20 µg PcDS ml\textsuperscript{-1} for 10, 30 or 60 min in the light or incubated for 60 min in the dark. As controls, non-treated 1% RML and WT brain homogenates were incubated for 60 min in the light. The effect of the treatment on prion infectivity was evaluated using a cell infectivity assay as described previously (Julak \textit{et al.}, 2011). CAD5 cells were cultivated in Opti-MEM (Invitrogen) supplemented with 10% bovine growth serum (BGS) (Thermo Scientific HyClone), 100 U penicillin ml\textsuperscript{-1} and 100 µg streptomycin ml\textsuperscript{-1} (PAA).

agent against cyanobacteria (Jancula \textit{et al.}, 2009). A sample of PcDS for non-commercial research can be obtained upon request from J. R. or M. K. (Centre for Organic Chemistry Ltd).

Fig. 1. Disodium salt of disulfonated hydroxyaluminium phthalo-cyanine (PcDS): C\textsubscript{32}H\textsubscript{15}Na\textsubscript{8}S\textsubscript{2}O\textsubscript{7}Na\textsubscript{2}Al. ‘Me’ represents (HO)Al.

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(a) Dose dependence

Concentration (µg ml⁻¹)

(b) Time dependence

Treatment (min)

Concentration (µg ml⁻¹)

(d) Time dependence

Treatment (min)

(e) Time dependence

Treatment (min)

(f) Time dependence

Treatment (min)
Cells were plated at a density of $4 \times 10^4$ cells in 0.5 ml in a 24-well plate format 1 day prior to infection. The cells were challenged with 25 ml RML (1% or 0.1%) or with the WT brain homogenate for 24 h. Subsequently, cells were split at a 1:10 ratio every 3 days. After the third passage, prion infection was detected using a cell blot (Bosque & Prusiner, 2000) as described previously (Julak et al., 2011). Briefly, cells were plated at a 1:10 dilution on round plastic coverslips and cultivated for 4 days. The coverslips were blotted onto a nitrocellulose membrane and treated with PK to remove PrPC. Proteins on the blot were denatured using 3 M guanidine isothiocyanate. The blot was probed with the mAb AH6 (Roslin Institute, Midlothian, UK) and then developed.

The infection of the cells with dilutions of non-treated RML homogenate resulted in decreasing densities of spots corresponding to the decreasing concentration of RML homogenate over a ~3 log$_{10}$ range (Fig. 3c). PcDS
treatment of RML homogenate with exposure to light led to significant inactivation of prions. Treatment of 0.1 % RML homogenate with 20 μg PcDS ml⁻¹ and exposure to light for 30 and 60 min led to complete inhibition of CAD5 cell infection (Fig. 3a, d). The lower concentration of PcDS (5 μg ml⁻¹) led to substantial suppression of CAD5 cell infection (~2 log₁₀) after all exposure times (Fig. 3a, d). The treatment effects on the more concentrated 1 % RML brain homogenate were less dramatic. Both concentrations of PcDS led to a >50 % decrease in spot density corresponding to a 1.5 log₁₀ decrease in infectivity after a 10 and 30 min exposure to light compared with non-treated 1 % RML homogenate (Fig. 3a, e). The longer treatment (60 min) of 1 % RML-infected cells with 5 or 20 μg PcDS ml⁻¹ led to a higher inhibition of cell infection, equivalent to a ~2.9 log₁₀ decrease of the infectivity. Incubation of RML homogenate with 20 to 200 μg PcDS ml⁻¹ led to substantial suppression of CAD5 cell infection (~2 log₁₀) after all exposure times (Fig. 3a, d). The treatment effects on the more concentrated 1 % RML brain homogenate were less dramatic. Both concentrations of PcDS led to a >50 % decrease in spot density corresponding to a 1.5 log₁₀ decrease in infectivity after a 10 and 30 min exposure to light compared with non-treated 1 % RML homogenate (Fig. 3a, e). The longer treatment (60 min) of 1 % RML-infected cells with 5 or 20 μg PcDS ml⁻¹ led to a higher inhibition of cell infection, equivalent to a ~2.9 log₁₀ decrease of the infectivity. Incubation of RML homogenate with PcDS in the dark did not prevent the infection of cells (Fig. 3b), demonstrating the necessity of photodynamic stimulation for the full inhibitory effect. The density of spots obtained from cells following infection with PcDS-treated 1 % RML in the dark was comparable to the density observed for non-treated, 1 % RML-infected cells. The density of spots obtained from cells infected with PcDS-treated 0.1 % RML in the dark was slightly lower, exhibiting approximately 65 % of the density obtained from the non-treated, 0.1 % RML-infected cells (Fig. 3b, c). This finding correlates with the decrease of PrPres band density after incubation of RML with PsDS in the dark (Fig. 2f). In accordance with the results reported by Caughey et al. (1998), this suggests that PcDS also has direct anti-prion activity unrelated to the generation of reactive species. Incubation of CAD5 cells with PcDS-treated 1 or 0.1 % WT homogenates did not produce any staining, demonstrating the specificity of our PrPTSE-detection method (Fig. 3b). The densities of the spots from three experiments were analysed by densitometry, and the results are plotted as bar graphs (Fig. 3d, e). The dependence of the effectiveness of PcDS treatment on the incubation time and concentration of the RML brain homogenate is apparent. The level of prion inactivation demonstrated in our study using PcDS is lower than that observed with established inactivation methods, which often reduce prion infectivity by 6 log₁₀ (Taylor, 2000). However, generation of reactive species by Pcs depends on the light wavelength and intensity (Ogunsipe & Nyokong, 2005), which were not optimized in our study. In addition, Pcs chemistry allows preparation of a repertoire of Pcs derivatives that were shown to differ both in the affinity for their biological targets and in the level of reactive species produced (Jancula et al., 2009).

In conclusion, Pcs have previously been shown to be potent inhibitors of PrPTSE formation in cell culture (Caughey et al., 1998) and strong prophylactic anti-scrabie compounds in vivo (Caughey et al., 2007; Priola et al., 2000, 2003). However, the photodynamic potential of Pcs is only well-documented in the treatment of cancer and of bacterial, yeast and fungal infections. Our experiments have demonstrated the photodynamic effects of PcDS on the inactivation of prion infectivity and the reduction in the PrPTSE signal. This finding opens up new avenues for the development of an effective low-cost method of prion inactivation at room temperature and normal pressure using non-toxic compounds activated with visible (solar) light (Rakusan et al., 2011). Combined with simple equipment, this method may offer environmentally friendly solutions for the inactivation of prion particles in complex biological, thermaolable liquids.

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