Photodynamic inactivation of prions by disulfonated hydroxyaluminium phthalocyanine

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Sulfonated phthalocyanines (Pcs) are cyclic tetrapyrroles 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 thermolabile materials and liquids.

The pathogenesis of transmissible spongiform encephalopathies (TSEs) is associated with the conversion of the protease-sensitive cellular prion protein (PrP⁰) to the partially protease-resistant amyloidogenic abnormal isoform of prion protein (PrPSTSE), which, upon digestion with protease 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 PrPSTSE in vitro (Cashman & Caughey, 2004; Charvériat et al., 2009; Köcisko 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 tetrapyrroles, which include porphyrins and phthalocyanines (Pcs) (Abdel-Haq et al., 2009; Caughey et al., 2006, 2007). One of the phthalocyanines, phthalocyanine tetralsulfonate (PcTS), has been shown to inhibit the formation of PrPSTSE in vitro (Caughey et al., 1998, 2007) and prolonged the survival of scrapie-infected mice substantially (Priola et al., 2000, 2003). 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 tetrapyrroles 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 (Paspaltis 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) – \( \text{C}_{32}\text{H}_{15}\text{N}_{8}\text{S}_{2}\text{O}_{7}\text{Na}_{2}\text{Al} \) (Fig. 1), which was synthesized by the Research Institute of Organic Synthesis (Rybitví, Czech Republic) (Jancula et al., 2008) – on the stability of PrP⁰ and PrPSTSE molecules and the ability of mouse RML prions to infect prion-susceptible cultured cells. PcDS was previously shown to possess algicidal activity (Jancula et al., 2008) and to be an effective
First, we studied the effect of PcDS photodynamic treatment on the stability of PrP\textsuperscript{C} and PrP\textsuperscript{TSE} molecules by Western blot. As a source of PrP\textsuperscript{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\textsuperscript{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 20 μg PcDS ml\textsuperscript{-1}. The time dependence of PcDS treatment was evaluated using 20 μg PcDS ml\textsuperscript{-1} and increasing exposure of samples to light (0, 5, 15, 30, 45 and 60 min). Controls were incubated with PcDS in the dark or without PcDS in the light for 60 min. The brain homogenates were digested with PK and analysed by Western blot with a mix of the mAbs AH6 (epitope PrP\textsubscript{159–174}, Roslin Institute, Midlothian, UK) and DC2 (epitope PrP\textsubscript{39–46}, provided by Vladka Curin-Serbec, Blood Transfusion Centre of Slovenia, Ljubljana, Slovenia) as described previously (Panigaj \textit{et al.}, 2011) (Supplementary Methods and Fig. S1, available in JGV Online). The densities of bands on blots from three experiments were quantified using a MiniLumi gel documentation system utilizing GelQuant densitometer software (DNR Bio-Imaging Systems Ltd) and the results are plotted as bar graphs (Fig. 2b, c, e, f).

PcDS treatment of 1% RML in the presence of light led to dose- and time-dependent reductions in both the PrP\textsuperscript{C}/PrP\textsuperscript{TSE} and PK-resistant PrPres signals (Fig. 2a, d). In the majority of cases, the PrPres signal was eliminated completely after 60 min treatment with 20 μg PcDS ml\textsuperscript{-1} (Fig. 2a, d), corresponding to an at least 64-fold decrease in the protein level (Fig. S1). Interestingly, the PrP signal in an undigested RML aliquot containing both PrP\textsuperscript{C} and PrP\textsuperscript{TSE} was still detectable, suggesting that PrP\textsuperscript{C} is less affected by PcDS treatment than PrP\textsuperscript{TSE} (Fig. 2b, c). In accord with this observation, the density of PrP\textsuperscript{C} bands in WT and TGA PcDS-treated brain homogenates was reduced significantly less than the PrP\textsuperscript{C}/PrP\textsuperscript{TSE} signal in undigested RML (Fig. 2b).

PcDS at a concentration of 20 μg ml\textsuperscript{-1} led to an approximately 60% decrease of the initial PrP\textsuperscript{C} band density compared with complete elimination of the PrPres signal in RML (Fig. 2b, c). The calculated concentration of PcDS needed to lower the PrP signal on Western blot to 50% of its initial value was 1.4 μg ml\textsuperscript{-1} for PrPres in RML, 9 μg ml\textsuperscript{-1} for PrP\textsuperscript{C} in WT and 8 μg ml\textsuperscript{-1} for PrP\textsuperscript{C} in TGA. Similarly, when testing the time dependence of PcDS treatment, PrPres was eliminated at a higher rate than PrP\textsuperscript{C} (Fig. 2d, e, f). The calculated time needed to lower the PrP signal using 20 μg PcDS ml\textsuperscript{-1} to 50% of its initial density was 13.5 min for PrPres in RML, 51 min for PrP\textsuperscript{C} in WT and 56.5 min for PrP\textsuperscript{C} in TGA. These results demonstrate that PcDS facilitates the destruction of PrP\textsuperscript{TSE} more efficiently than that of PrP\textsuperscript{C}. PcDS treatment of brain homogenates in the dark, as well as exposure of homogenates to light without PcDS, did not have any effect on the undigested PrP\textsuperscript{C}/PrP\textsuperscript{TSE} signals, and the densities of bands were comparable to bands from non-treated RML, WT and TGA brain homogenates (Fig. 2e). However, at the same time, treatment of RML with 20 μg PcDS ml\textsuperscript{-1} for 60 min in the dark led to a slight, but significant decrease of the PrPres band density (P<0.05; Fig. 2f). This finding probably represents a direct destabilizing effect of PcDS binding to PrP\textsuperscript{TSE} molecules, making a proportion (approx. 20%) of them PK-sensitive.

After demonstrating the dose- and time-dependent effect of PcDS photodynamic treatment on PrP\textsuperscript{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} (PA).
(a) Dose dependence

Concentration (µg ml⁻¹)

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(b) Density (%)

1 % TGA

1 % WT

1 % RML

(c) Density (%)

1 % RML -PK

1 % RML +PK

(d) Time dependence

Treatment (min)

CTRLR 0 5 15 30 45 60

CTRLw 0 5 15 30 45 60

CTRLT 0 5 15 30 45 60

(e) Density (%)

1 % TGA

1 % WT

1 % RML

(f) Density (%)

1 % RML -PK

1 % RML +PK
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 $\sim 3 \log_{10}$ range (Fig. 3c). PcDS

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**Fig. 2.** Stability of the PrP$^C$/PrP$^{TSE}$ protein signal after photodynamic treatment with PcDS. Western blots of RML, WT and Tga20 (TGA) 1% brain homogenates were treated with PcDS and developed with prion mAbs AH6 and DC2. (a) Dose dependence: brain homogenates were incubated with increasing concentration of PcDS in light for 60 min. (b) Densitometry analysis of PrP$^C$/PrP$^{TSE}$ signal of the samples without PK digestion ($n=4$). (c) Densitometry analysis of PrP$^C$/PrP$^{TSE}$ signal of the samples with PK digestion ($n=4$). (d) Time dependence: brain homogenates were treated with 20 $\mu$g PcDS ml$^{-1}$ in the light for increasing time intervals. (e) Densitometry analysis of PrP$^C$/PrP$^{TSE}$ signal of the samples without PK digestion ($n=4$). St, Molecular mass standards; PK +/-, proteinase K-digested/undigested samples; D, homogenates incubated with 20 $\mu$g PcDS ml$^{-1}$ in the dark; CTRL, untreated brain homogenates; L, homogenates incubated without PcDS in the light for 60 min. Significance level: *$P<0.05$; **$P<0.005$.

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**Fig. 3.** Inactivation of prion infectivity by PcDS treatment. Cell infectivity assay of RML mouse brain homogenates treated with the disodium salt of PcDS. (a) 1 and 0.1% RML brain homogenate was incubated with 5 or 20 $\mu$g PcDS ml$^{-1}$ in the light for 10, 30 and 60 min. (b) 1 and 0.1% RML brain homogenate was incubated with PcDS in the dark for 60 min, and 1 and 0.1% WT healthy mouse brain homogenate was incubated in the light for 10 min. (c) Serial dilutions of non-treated RML brain homogenate. Densitometry evaluation of the intensities of cell blot spots after treatment of 0.1% RML (d) and 1% RML (e) brain homogenate with 5 or 20 $\mu$g PcDS ml$^{-1}$ using three different light exposure times ($n=3$). Significance level: *$P<0.05$; **$P<0.005$. 

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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 to 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 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, thermolabile liquids.

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References


