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Photodynamic effects of 31 different phthalocyanines on a human keratinocyte cell line

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HIGHLIGHTS

- Toxicity of 31 phthalocyanines on human keratinocyte cell line HaCaT was investigated.
- Effects on skin cells were strongly light-dependent.
- Phthalocyanines substituted with heterocycle are the most toxic.
- Relevant Pcs concentrations if used as algicides or herbicides seems to be safe for human skin.

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ABSTRACT

Phthalocyanines (Pcs, colored macromolecular compounds with the ability to generate singlet oxygen) represent a promising group of photosensitizers due to their intense absorption in the red and UV portion of the spectrum which leads to their excitation. In order to characterize possible toxic effects associated with eventual practical use and application of these chemicals, we employed an *in vitro* cell culture model to evaluate cytotoxic effects of 31 different phthalocyanines using neutral red uptake assay. An immortalized human keratinocyte cell line HaCaT was exposed to the tested chemicals for 2 or 24 h, either with or without illumination in the last 60 min of the exposure period. After 2- or 24-h exposure without illumination, no cytotoxic effects or weak cytotoxic effects were induced by any Pc under the study and EC50 values could not be obtained within the tested concentration ranges (1.25–20 mg L^{-1} or 0.625–10 mg L^{-1}). On the other hand, exposure to phthalocyanines under illumination induced a significant cytotoxic effect. The most pronounced cytotoxicity was elicited by Pcs previously shown to have high positive charge densities at peripheral parts of substituent groups, which is most likely the factor responsible for the binding of Pc to negatively charged membranes on the cell surface and thus guaranteeing the tight connection necessary for the singlet oxygen attack on the cell surface.

1. Introduction

Phthalocyanines (Pcs) and porphyrins are promising chemicals for their ability to generate singlet oxygen ($^1O_2$) in the presence of water and dissolved oxygen after absorption in the red and/or UV portion of the light spectrum. Research interest in Pcs has been increasing over the last few decades (Claessens et al., 2008), which can be attributed to their architectural flexibility (Fig. 1), various coordination properties and improved spectroscopic characteristics (Piskin et al., 2011).

The great potential of Pcs can be illustrated by their numerous industrial, clinical and biological applications. In the last decades, Pcs have been used as dyes in the textile industry and in solar cells (Zarate et al., 2011), as carbon cathode catalysts for polymer electrolyte fuel cells (Kobayashi et al., 2011), as materials for the degradation of pollutants (Chen et al., 2011) and natural toxins (Jancula et al., 2010), and also as agents inhibiting proliferation of tumors (Chan et al., 1987; Sheng et al., 2004; Xue et al., 2011) and growth of bacteria (Klepac-Ceraj et al., 2011) or fungi (Jori and Brown, 2004). A new area of potential phthalocyanine application has recently been reported by Drabkova et al. (2007) and Jancula et al. (2008), who suggested the possible use of Pcs to suppress algal and cyanobacterial growth in aquatic environments. Our group reported that the best candidates for such applications seem to be Pcs substituted with a heterocycle, which are very toxic to cyanobacteria and algae, but less toxic to non-target species like daphnids, for example. EC50 values of such molecules for cyanobacteria were 0.06–0.63 mg L^{-1}, whereas for daphnids they were even higher than 10 mg L^{-1} (Jancula et al., 2008). Thus, some Pcs were demonstrated as possible agents for management of

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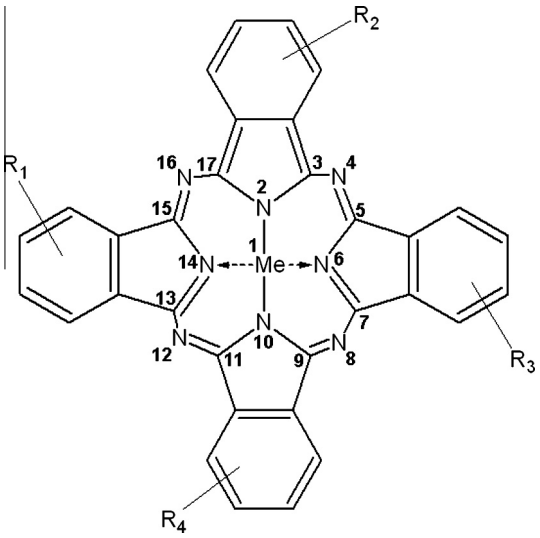

Fig. 1. General molecular structure of phthalocyanine.

cyanobacterial bloom development or weed control in lakes, reservoirs or aquaria, but the question of possible risks in cases of dermal exposure for vertebrate organisms, including humans during such treatment, has not been answered.

In spite of many current and possible future applications of Pcs, knowledge about the effects of these chemicals on human skin is mostly limited to either prototypical Pcs and phthalocyanine materials such as zinc phthalocyanine (Fabris et al., 2006) or several Pcs considered for clinical applications for photodynamic cancer treatment (Qin et al., 2012). However, with respect to their physical–chemical and photosensitizing properties, also other Pcs could eventually act as potent sensitizers of white light-induced photooxidative stress in human skin cells.

Therefore, the aim of this study was to further contribute to our understanding of health hazards associated with dermal exposure of vertebrates to structurally different Pcs in an aquatic environment. Cytotoxic effects induced by 31 Pcs were evaluated *in vitro* under different light conditions using a human skin cell line HaCaT (HaCaT cells possess a close similarity in functional competence to normal keratinocytes) and neutral red uptake assay. The results of Pcs toxicity induced in the dark and phototoxicity induced by the photodynamic treatment will be presented and discussed in this paper.

2. Materials and methods

2.1. Tested compounds

Thirty one phthalocyanine derivates were prepared by Research Institute of Organic Synthesis (VUOS PLC), Rybitvi, Czech Republic. The set of tested Pcs included three charge phthalocyanine groups (i.e., anionic, cationic, and neutral), which can be further subdivided into eleven groups according to the substituent chemical structure as shown in Table 1. Stock solutions of Pcs were always freshly prepared before the experiments by dissolving Pcs (0.4–1 g L^{-1}) in ultrapure water (18.2 MΩ cm). All tested Pcs were well soluble in water and the cell culture medium in the employed concentration range.

2.2. HaCaT cell culture and neutral red uptake assay

A human keratinocyte cell line HaCaT (immortalized skin cells) was cultured without antibiotics in DMEM medium (PAA, Pasching, Austria) containing 1 g L^{-1} glucose, 110 mg L^{-1} sodium pyruvate, phenol red, and supplemented with 2 mM glutamine and 5% fetal bovine serum (PAA). The cells were incubated in a CO_2 incubator at 37° C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were routinely cultured in T75 tissue culture flasks (Sterilin, Staffordshire, UK) and passaged once or twice per week. Cytotoxicity of Pcs was determined by neutral red uptake assay adapted from Repetto et al. (2008). For the assay, 100 μL of cell suspension containing 156×10^3 cells was seeded per flat bottom well of a 96-well microplate (Iwaki, Tokyo, Japan), resulting in a final cell seeding density of 50×10^3 cell cm^{-2}. The experiments were performed with completely confluent cultures obtained after 3–4 d of growth.

Twofold serial dilutions of Pcs were prepared by diluting Pc aqueous stock solutions aseptically with complete cell culture medium to obtain the concentration range 0.625–10 mg L^{-1} (Pcs 8.2, 8.3 and 11.2) or 1.25–20 mg L^{-1} (all other tested Pcs). The spent cell culture medium was discarded and the cells were exposed to fresh cell culture medium (100 μL $well^{-1}$) containing Pcs or a corresponding amount of solvent water (no more than 5%, v/v). The following exposure scenarios were employed: 24 h exposure to Pcs in the dark, 23 h exposure in the dark followed by 1 h illumination, 2 h exposure in the dark, 1 h exposure in the dark followed by 1 h illumination. The illumination 20.000 lux (approx. 30.6 W m^{-2}) was provided by white LEDs (GM Electronic, Czech Republic). All exposures including illumination treatments were carried out in the CO_2 incubator.

After the exposure, the culture medium was discarded and the cells were washed twice with 100 μL of phosphate buffered saline (PBS). Neutral red solution (Sigma–Aldrich, Prague, Czech Republic) dissolved in DMEM medium (Sigma–Aldrich, D5030, w/o phenol red, w/o FBS, supplemented with 1 g L^{-1} glucose, 0.5 g L^{-1} sodium bicarbonate) and filtered through a 0.45 μm polyethersulfone syringe filter (Chromservis, Prague, Czech Republic) was added to the cells (100 μL $well^{-1}$). After 60 min incubation in the CO_2 incubator, the cells were rinsed three times with 100 μL of PBS. Finally, 100 μL of 1% acetic acid–50% ethanol (v/v) was added to each well and the microplate was incubated at RT on orbital shaker for 10–15 min. Absorbance values were measured at 540 nm (690 nm reference wavelength) with a Sunrise microplate reader (TECAN, Männedorf, Switzerland). The absorbance values from experimental variants were referred to solvent control cultures to calculate the percentage of neutral red uptake. Acute toxicity was expressed as the average effective concentration (EC50) determined using linear regression analysis of neutral red uptake percentage versus experimental Pc concentrations.

3. Results and discussion

3.1. Phototoxicity

In vitro cytotoxicity of phthalocyanines on a human keratinocyte cell line HaCaT was evaluated by neutral red uptake assay after two different exposure periods (2 and 24 h respectively) and under two different irradiation regimes (with or without illumination during the last 60 min of exposure). EC50 values calculated for 31 tested Pcs are reported in Table 2. It is apparent that the effects of Pcs on HaCaT cells were strongly dependent on illumination treatment as well as on the molecular structure of tested Pc derivates. None of the investigated Pcs was able to induce greater than 50% inhibition of neutral red uptake within the tested concentration range 2- or 24-h cell exposure in the dark. These results correspond to other studies. For example, Sarrazy et al. (2011) tested phototoxicity of 6 porphyrin–polyamide conjugates and concluded that photoactivation is an absolute requirement for