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In vitro photodynamic therapy on melanoma cell lines with phthalocyanine

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Abstract

Photodynamic therapy (PDT) is a new treatment modality of tumours. The photochemical interactions of sensitizer, light, and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxid anion. Phthalocyanine $ClAlPcS_2$, belonging among the promising second generation of sensitizers, was tested as an inducer of photodamage. We report the production of reactive oxygen species (ROS) and the phototoxicity of $ClAlPcS_2$ assessed using G361 melanoma cells. A semiconductor laser ($\lambda = 675$ nm, output power 21 mW) was used as a source for evocation of the photodynamic effect. ROS generation and H_2O_2 release after PDT on G361 cells were detected using probe CM-H_2DCFDA and recorded by luminescence spectrometer. Viability studies show, that the optimum phototoxic effect tested on G361 melanoma cells was determined in the combination of laser dose of 25 J cm^{-2} and phthalocyanine $ClAlPcS_2$ concentration of 5 μg/ml. This combination of phthalocyanine concentration and corresponding radiation dose was lethal for melanoma cells.

Keywords: Phthalocyanine; Phototoxicity; Laser

1. Introduction

Photodynamic therapy is a treatment that is used for the destruction of a certain type of tumours (Kessel et al., 1997; Allison et al., 2004). The photochemical interactions of the photosensitizer, light, and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxid ion. The resulting damage to organelles within malignant cells leads to tumour ablation. Membranous organelles, including mitochondria, plasma membrane, and lysosomes, have been suggested to be the main sites of PDT damage (Rogers et al., 1991; Kessel et al., 1997). Apoptosis after PDT has been demonstrated in vitro (Moor, 2000), and in vivo (Zaidi et al., 1994). However, the mechanism of PDT induced cell death is unknown. The initiation of apoptosis after the photodynamic therapy appears to be a function of the sensitizer and the cell line, respectively (Luo and Kessel, 1997). For this therapy, dyes such as hematoporfyrin derivates are used. A disadvantage of these dyes is that they are a mixture of various mostly unidentified compounds. Moreover, they absorb light at relatively short wavelengths that do not penetrate deeply into the tissue. To circumvent these disadvantages, other dyes are tested such as chlorins and phthalocyanines. The phthalocyanines are chemically pure compounds that absorb light of longer wavelengths than hematoporphyrin derivate. Although much research has been done on a wide variety of cells, the mechanism

Abbreviations: PDT, photodynamic therapy; $ClAlPcS_2$, disulfonated chloroaluminium phthalocyanine; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; G361, human melanoma cell lines; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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responsible for cell death due to photodynamic treatment with phthalocyanines has not been elucidated yet. After photodynamic treatment of mammalian cells with phthalocyanine, several kinds of damage were observed. Damage of plasma membrane-bound functions was shown to occur in Chinese hamster cells and in mouse melanoma cells (Ben-Hur et al., 1992). Another study indicates energy depletion due to photodynamic treatment with sulfonated phthalocyanine. Yeast cells offer many advantages in model studies on photodynamically induced cellular damage. Photodynamic treatment of Kluyveromyces marxianus with chloroaluminium phthalocyanine resulted in a loss of clonogenicity. Inhibition of various plasma membrane-bound processes was observed, such as substrate transport and plasma membrane ATPase activity. Moreover, K^+ loss from the cells was observed. Photodynamic treatment also reduced the activity of various enzymes involved in energy metabolism by decreasing the cellular ATP level (Paardekooper et al., 1995).

Tumour photosensitization studies with zinc(II) octadecylphthalocyanine were carried out 24 h after administration of ZnODPc and showed that this phthalocyanine has a very high phototerapeutic efficiency. This is probably the consequence of multiple mechanisms by which the phthalocyanine induces tumour damage, involving both direct modification of malignant cells and impairment of blood flow, as well as the alteration of a variety of subcellular components, such as mitochondria, the rough endoplasmic reticulum, the perinuclear membrane and occasionally, cell nuclei. Tumour necrosis appears to be the consequence of both random cell death and apoptosis. ZnODPc showed unusually high affinity for serum low density lipoproteins (LDLs) and high efficiency and selectivity of tumour targeting. The maximum of accumulation in the tumour occurred at 24 h after injection of ZnODPc, whereas no detectable amount of phthalocyanine was recovered from the muscle, i.e. peritumoural tissue, between 1 h and 1 week after injection. At the same time, low amounts of phthalocyanine were recovered from skin and then only at short times after injection, with skin photosensitivity rapidly disappearing and the phthalocyanine present in the serum only (Ometo et al., 1996). Phthalocyanines belonging to the new generation of substances for PDT can be chelated with a variety of metals, chiefly aluminium and zinc, while these diamagnetic metals enhance their phototoxicity (Liu et al., 2004). A ring substitution in phthalocyanines with sulphonated groups will render them water soluble and affect their cellular uptake. In our present study we report the influence of the phthalocyanine concentrations in combination with laser irradiation doses on the photodamage of G361 human melanoma cells.

2. Materials and methods

Sensitizers: Chloroaluminium phthalocyanine $ClAlPcS_2$ was prepared by Jan Rakušan at the Research Institute for Organic Syntheses in Rybitví, Czech Republic. ClAlPc was prepared by the reaction of *o*-phthalodinitrile with aluminium chloride at 200 °C. The product of the reaction was purified by diluted sulfuric acid, washed and dried. The dry ClAlPc was subsequently sulfonated by fuming sulfuric acid. The reaction was drowned into the ice–water mixture. The precipitated disulfonated chloroaluminium phthalocyanine was isolated by filtration. In order to get good solubility, the product was transformed into ammonia salt and in this form used.

Spectral properties of the sensitizer: The absorption spectra were measured by spectrometer Pye-Unicam SP8-100. The used concentration of photosensitizers was 1×10^{-5} M, pH = 7.6 (buffer Tris–HCl).

Cell line: The G361 (10^4 well^{-1} of microplate) human melanoma cells (ATTC, USA) were grown in cultivation medium DMEM and incubated at 37 °C and 5% CO_2.

Measurement of reactive oxygen species: Cells were then incubated with 0.75, 7.5 and 75 μg/ml phthalocyanine $ClAlPcS_2$ and after 24 h irradiated by semiconductor laser (Medistellar, power 50 mW, wavelength 675 nm,) with light doses of 10 and 20 J cm^{-2}. The intracellular ROS production was measured using non-fluorescent compound 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Upon crossing the membrane, the compound undergoes deacetylation by intracellular esterases producing the non-fluorescent CM-H_2DCF, which quantitatively reacts with oxygen species inside the cell to produce the highly fluorescent dye CM-DCF. This compound remains trapped within the cell and can be measured to provide an index of intracellular oxidation (Jiao and Zhao, 2002; Liu et al., 2001; Rodríguez et al., 2002). After 24 h incubation, DMEM was replaced by PBS with glucose (5.5 mM) and the cells were treated with 10 μM CM-H_2DCFDA for 30 min at 37°C in darkness. The excess probe was washed out. The fluorescence of CM-DCF was recorded at 495 nm excitation and 530 nm emission (excitation/emission slit = 10 nm/5 nm) by Perkin–Elmer LS 50B luminescence spectrometer equipped with well plate reader accessory (Perkin–Elmer Corp., Norwalk, CT). The whole plate was read twice with a read time of 0.1 s for each well.

Phototoxicity measurement: G361 human melanoma cells (ATTC, USA) were cultivated with ClAlPcS2 (VÚOS Rybitví, Czech Republic) at concentration 1, 5 and 10 μg/ml. After 24 h of cultivation the cells were subsequently irradiated by a laser (wavelength 675 nm, doses of 10 and 25 J cm^{-2}. The quantum of irradiance was measured by Radiometer RK 2500. Morphological changes in the cell cultures were evaluated using inversion fluorescent microscope Olympus IX 70 and by image analysis Olympus MicroImage. The viability of cells was determined by means of molecular probes (Molecular Probes Europe BV) for fluorescence microscopy (LIVE/DEAD kit). The quantitative changes of cell viability in relation to phtalocyanine concentrations and irradiation doses were proved by fluorimetric measurements with fluoroscan Ascent Labsystems.

3. Results

We evaluated absorption spectra of $ClAlPcS_2$ (Fig. 1).

The phthalocyanines have high absorption in the UVA region of the electromagnetic spectrum with maximum at 350 nm and in the visible region of the spectrum between 650 and 700 nm with maximum at 670 nm. Phthalocyanines are the most suitable for PDT, having absorption in the long-wave range of the spectrum, as it is evident from Fig. 1.

ROS generation in photosensitized G361 cells was measured using fluorescent product CM-DCF, which is formed by oxidation and deacetylation of CM-H_2DCFDA.

Our results demonstrated, that ROS generation increases in the presence of higher concentration of sensitizer (Fig. 2). As shown in Fig. 3, irradiation of cells by laser dose of 10 J cm^{-2} induces higher rates of fluorescence in cells loaded with 7.5 and 75 μg/ml phthalocyanine compared to 20 J cm^{-2}. Cells with lower concentration of sensitizer (0.75 μg/ml) produces more ROS after irradiation by light dose of 20 J cm^{-2}. Our results demonstrated the highest ROS generation at laser dose of 10 J cm^{-2} and 75 μg/ml $ClAlPcS_2$.

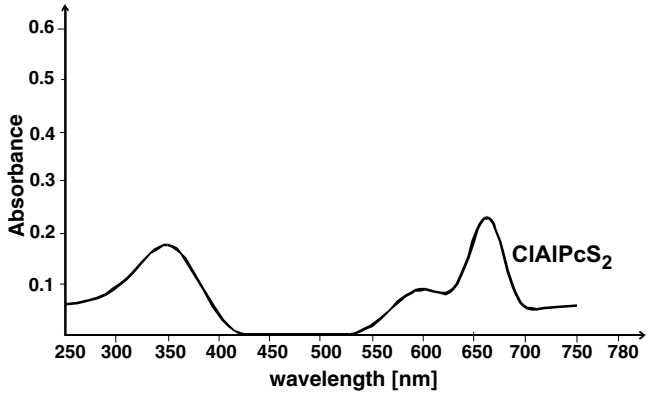

Fig. 1. Absorption spectra of $AlClPcS_2$ at concentration 1×10^{-5} M, pH = 7.6 (buffer Tris–HCl).

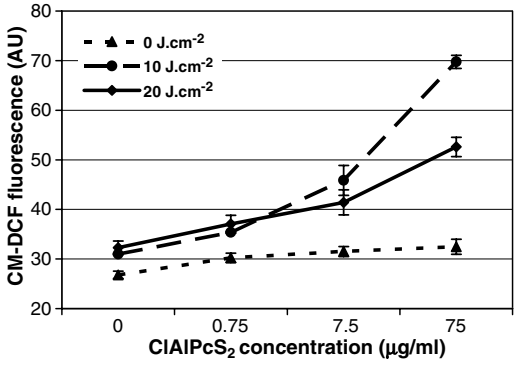

Fig. 2. Dependence of ROS production in G361 cells on the phthalocyanine concentration after irradiation with light dose of 10 and 20 J cm^{-2}. Values represent mean ± SE from six independent experiments.

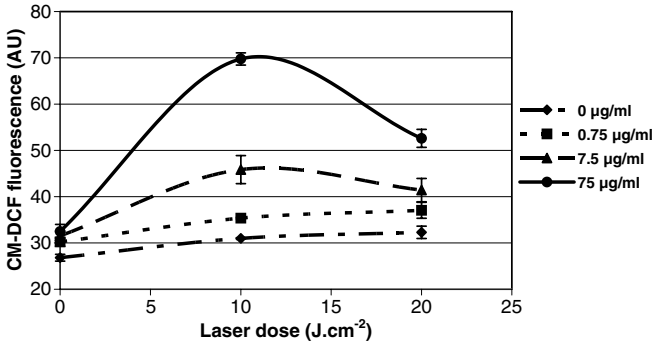

Fig. 3. Dose-dependent CM-DCF fluorescence of G361 cell line. The cells were pretreated with 0.75, 7.5 and 75 μg/ml $ClAlPcS_2$. Values represent mean ± SE from six independent experiments. The curves were fitted using software MS Excel.

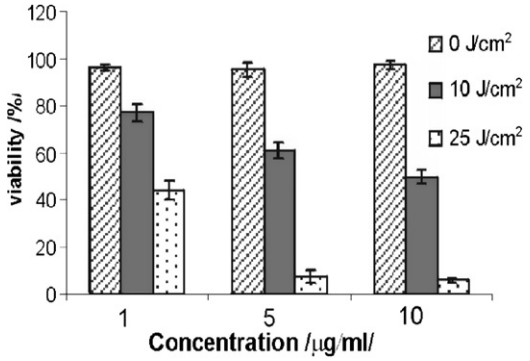

Fig. 4. Viability dependence of G361 cells on $ClAlPcS_2$ phthalocyanine concentration after irradiation with laser dose of 10 and 25 J cm^{-2}.

We report the influence of $ClAlPcS_2$ concentrations of 1, 5 and 10 μg/ml without irradiation and in combination with light irradiation doses at 10 and 25 J cm^{-2} on the photodamage of G361 human melanoma cells (Fig. 4). Viability studies have shown, that the optimum phototoxic effect observed in G361 melanoma cells was obtained in the presence of laser light dose of 25 J cm^{-2} and at the $ClAlPcS_2$ concentrations of 5 μg/ml and 10 μg/ml. There are no significant differences between these values of viability. These combinations of sensitizer concentrations and corresponding radiation doses were lethal for the melanoma cells. Viability of G361 cells decreases quickly with the increase of phthalocyanine concentration to 5 μg/ml at laser dose of 25 J cm^{-2}. The higher concentration caused already slight decrease in the G361 viability.

4. Discussion

Photodynamic therapy requires three components present simultaneously for cytotoxicity: a sensitizer, light, and oxygen. Reactive oxygen species (ROS) cause both cell death and damage to blood vessels thus contributing to tumour regression (Kessel et al., 1997).

Martins et al. investigated the formation of radical species involved in plasma low-density lipoproteins (LDL) and erythrocyte oxidation by electron paramagnetic resonance (EPR) spectroscopy. Plasma low-density lipoproteins are important carriers of phthalocyanines in the blood, but after exposure to visible light, phthalocyanine-loaded LDL undergo an oxidation process that propagates to erythrocytes. They reported that for a constant LDL/phthalocyanine ratio, the formation of oxygen free radicals shows a biphasic behavior with the concentration of LDL increasing and reaching a plateau, whereas the formation of $^1O_2$ increases linearly with LDL concentration (Martins et al., 2004). In this study, we used the probe CM-H_2DCFDA for detection of ROS. Rates of dye fluorescence correspond to the quantity of produced intracellular ROS. Liu et al. detected the existence of singlet oxygen under irradiation of red light using water-soluble sodium salt of sulfonated phthalocyanines (MPc(SO3Na)4) and organo-soluble tetrakis(2,9,16,23-*tert*-butyl) dysprosium bisphthalocyanines (Dy(TBPc)2). These sensizirers have been utilized to kill microorganism (*Escherichia coli*, *Bacillus cereus*, and *Aurebacterium* sp.) under photodynamic treatment (Liu et al., 2004). Lacey et al. investigated the lethal photosensitization of the Gram-negative bacterium *E. coli* using red laser or LED light and the anionic photosensitizer disulphonated aluminium phthalocyanine ($AlPcS_2$), that can reduce the cell viability of *E. coli*. The greatest reduction in the viable count of *E. coli* was achieved by exposure to fractionated LED light (energy dose 2.4 J; energy density 4.0 mW cm^{-2}; fractionated, illumination time 2 min; dark period 2 min) in the presence of 35 μg/ml AlPcS2 (86% reduction). High bactericidal activity was also observed when *E. coli* was exposed to red laser light (energy dose, 4.5 J; energy density, 30.0 mW cm^{-2}) in the presence of 27.5 μg/ml AlPcS2 (72% reduction) (Lacey and Phillips, 2001). The wavelength of light that activates the sensitizer used dictates the proper light spectrum as well as the depth of the treatment effect. The most suitable source of radiation used in PDT is laser. Semiconductor laser with power 50 mW and wavelength 675 nm for invocation of photodynamic effect was chosen, because its wavelength corresponds with the absorption maximum in long-wave region of the visible part of electromagnetic spectra. In this study we demonstrate photodamage after PDT by phthalocyanines. PDT of neoplastic cell lines is sometimes associated with the rapid initiation of apoptosis, a mode of cell death that results in a distinct pattern of cellular and DNA fragmentation. In this study PDT combined with chloroaluminium phtalocyanine sensitizer resulted in the death of melanoma cancer cells. However, we observed that the photodamage is also dependent on the irradiation dose used. Viability studies have shown, that the optimum phototoxic effect tested on G361 melanoma cells was determined in the combination of laser doses of 25 J cm^{-2} and concentration of phthalocyanine $ClAlPcS_2$ of 5 μg/ml or 10 μg/ml. These combinations of phthalocyanine concentration and corresponding radiation dose were lethal for melanoma cells. Interestingly, the higher phthalocyanine concentration did not caused significantly higher decrease in melanoma cell viability. This observation can be related to the pharmacokinetics of the sensitizer. In general, the effect of drugs can be expressed by a hyperbolic function when it is used a linear scale of drug concentration. Moreover, the other influences can be also involved. The experiments studying sensitizers uptake into melanoma cells show that there exists a saturation threshold in their accumulation into the cells (Kolarova et al., 2005). Production of ROS generally corresponds to phototoxic effect. Higher ROS generation causes larger cell photodamage and consequently better phototoxic effect which is manifested later. ROS are formed in live cells promptly after irradiation, thus it is necessary to detect their production immediately after irradiation when cell viability is not yet affected within our used method sensitivity.

Efficiency of PDT is affected by a number of factors including absorption spectrum of the photosensitizer, wavelength of the activation light, depth of the light penetration in the biological tissue, tissue answer on singlet oxygen.

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