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MEASUREMENT OF REACTIVE OXYGEN SPECIES AFTER PHOTODYNAMIC THERAPY IN VITRO

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Abstract

Photodynamic therapy (PDT) is an emerging modality for the treatment of neoplastic and non-neoplastic diseases. It is based on the use of a sensitiser, which is localised in target tissue, light, and molecular oxygen. Sensitisers are activated with the appropriate wavelength of light and then are excited to the long-lived triplet state. In this state they react with biomolecules via type I or II mechanism resulting in cell death and tumour necrosis. Free radicals and radical ions are formed by electron transfer reactions (type I). They rapidly react with oxygen leading to the production of reactive oxygen species (ROS). Type II reactions (energy transfer) lead to the formation of highly reactive singlet oxygen. It is very toxic to cells and tissues. In this work, we studied photodynamic effects of a free sensitiser $ZnTPPS_4$ or sensitiser bound to cyclodextrin carrier hpβCD in G361 human melanoma and MCF7 mamma carcinoma cells. The generation of ROS and hydrogen peroxide release after PDT were detected using non-fluorescent probes CM-H_2DCFDA and Amplex Red. The fluorescence of their oxidised products was recorded by a luminescence spectrometer in a time-drive measurement of cell suspension. We determined that the generation of ROS and H_2O_2 concentration in photosensitised cells were higher for free $ZnTPPS_4$ than for the sensitiser bound to hpβCD carrier.

Key words

Photodynamic therapy, Reactive oxygen species, Hydrogen peroxide, Fluorescence

INTRODUCTION

Photodynamic therapy offers an alternative, less invasive treatment for several types of cancers. It involves the use of three basic components – light-absorbing molecule (sensitiser), light with the corresponding wavelength, and molecular oxygen. A potential advantage of PDT is its dual selectivity: increased concentration of the sensitiser in target tissue and the limited irradiation to the specified volume (*1*). After irradiation, the sensitiser is excited to a higher energy state, leading to the production of cytotoxic species, resulting in cell death and tumour necrosis. In detail, the sensitiser (S) absorbs the energy of a photon of ultraviolet or visible radiation to become an excited singlet state ($^1S^*$), which rapidly converts into an

excited triplet state ($^3S^*$). The lifetime of $^3S^*$ is longer (typically microseconds) than that of $^1S^*$ (typically nanoseconds), so that energy transfer from ^3S to a dissolved oxygen molecule O_2 ($^1\Delta_g$) to form singlet oxygen is possible. $^3S^*$ is converted back to the initial ground state S, which can subsequently absorb another photon to begin a cycle again. Each molecule of the sensitiser can generate many (10^3–10^5) singlet oxygen molecules (*2*). The rate of absorption of photons is dependent on the concentration and molar absorption coefficient of the sensitiser, the overlap between the emission spectrum of the light source and the absorption spectrum of the sensitiser, and the intensity of the light.

The quenching mechanism of the triplet state of the sensitiser can occur via type I or type II. The type I mechanism involves hydrogen atom extraction or electron transfer reactions between the excited state of the sensitiser and some substrate, biological, solvent, or another sensitiser, to yield radicals and radical ions. These radical species are highly reactive and can interact with molecular oxygen to either generate reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radicals or fix the irreparable damage. These reactions cause the formation of oxidative damage and lead to cytotoxic effects during PDT. Type II results from an energy transfer from the triplet state of the sensitiser to ground state molecular oxygen, leading to the generation of an excited state of oxygen known as singlet oxygen. Due to its high reactivity, singlet oxygen can react with a large number of biological substrates, causing cell death (*3*).

MATERIALS AND METHODS

Cell culture and sensitisers: The G361 human melanoma and MCF7 mamma carcinoma cells (10^6 /dish) were grown in culture dishes, using cultivation medium DMEM. The cell cultures were stored in a thermobox at 37 °C in a humidified atmosphere containing 5 % CO_2. The cells were then incubated in DMEM growth medium containing 10μM of the sensitiser $ZnTPPS_4$ *(Fig. 1)* or sensitiser with 1mM hpβCD (2-hydroxypropyl-β-cyclodextrin) *(Fig. 2)*. $ZnTPPS_4$ is a zinc complex of $TPPS_4$ (*Meso*-tetrakis(4-sulfonatophenyl)porphyrin) *(4)*.

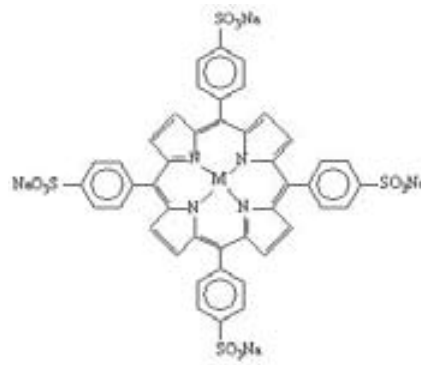

Fig. 1
Chemical structure of tetrapyrrolic sensitiser $TPPS_4$ (M = 2H), $ZnTPPS_4$ (M = Zn)

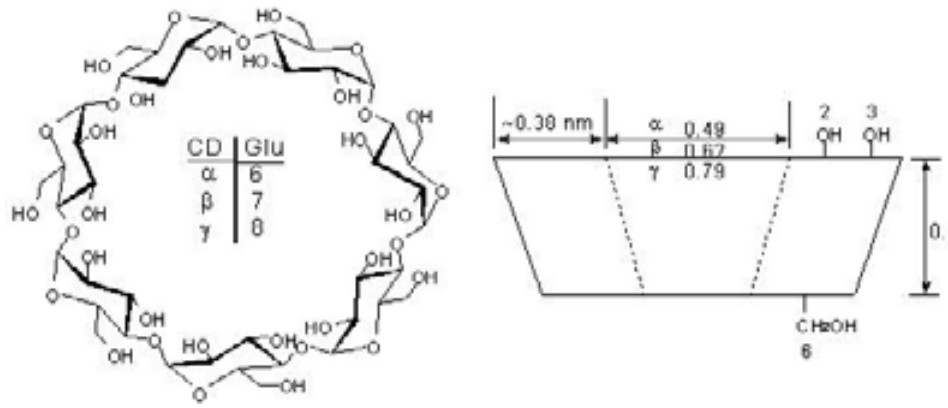

Fig. 2
Chemical structure of cyclodextrins α, β, and γ

Microscopy: The intracellular ROS production was detected using a non-fluorescent compound 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) *(Fig. 3)*. 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 (*5, 6, 7*). After 24 hours' incubation at 37 °C the growth medium was replaced with a fresh one containing 5 μM CM-H_2DCFDA without the sensitiser. After 30 min incubation in the dark, the cells were washed with glucose-enriched PBS and irradiated for 10 and 20 min by LEDs (420 nm). The production of ROS after PDT was then visualised with the help of an inverted fluorescent microscope Olympus IX 70, a DP70 digital camera Olympus, and Olympus MicroImage software.

Measurement of reactive oxygen species: After 24 h incubation, DMEM was replaced by PBS with glucose (5.5 mM) and the cells were treated with 1μM CM-H_2DCFDA for 30 min at 37 °C in darkness. After washing out the excess probe, the cells were suspended in glucose-enriched PBS and transferred to a fluorometer cuvette. The fluorescence was recorded at 495 nm excitation and 530 nm emission (excitation/emission slit = 10 nm/5 nm) by a Perkin–Elmer LS 50B luminescence spectrometer with continuous high stirring. For time-drive measurements the suspension was simultaneously irradiated by 4 LEDs with a wavelength of 420 nm, FWHM 15 nm (4.5 mA) at 37 °C for 15 or 20 minutes.

Hydrogen peroxide release measurements: The enzymatic determination of hydrogen peroxide can be accomplished with high sensitivity and specificity using *N*-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red) *(Fig. 4)*, which is colourless and non-fluorescent. Amplex Red oxidation in the presence of extramitochondrial horseradish peroxidase (HRP) bound to H_2O_2 generates resorufin, a highly fluorescent compound, with a 1:1 stoichiometry (*8, 9, 10*). The mixture of the Amplex Red reagent and HRP was added to the samples in PBS with glucose (5.5 mM). The reaction mixture of a resulting concentration of 20 μM Amplex Red and 0.04 U/ml HRP was incubated for 10 min in darkness at 37 °C and then displaced to a cuvette. Resorufin fluorescence was monitored using a Perkin–Elmer LS 50B luminescence spectrometer operating at excitation and emission wavelengths of 560 nm and 590 nm, respectively (excitation/emission slit = 2.5 nm/2.5 nm). Time-drive measurements of the samples were performed at the same conditions as the measurement of ROS.

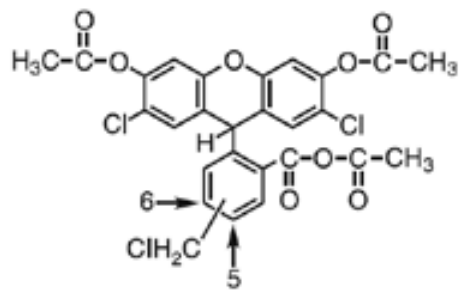

Fig. 3
The structure of CM-H_2DCFDA

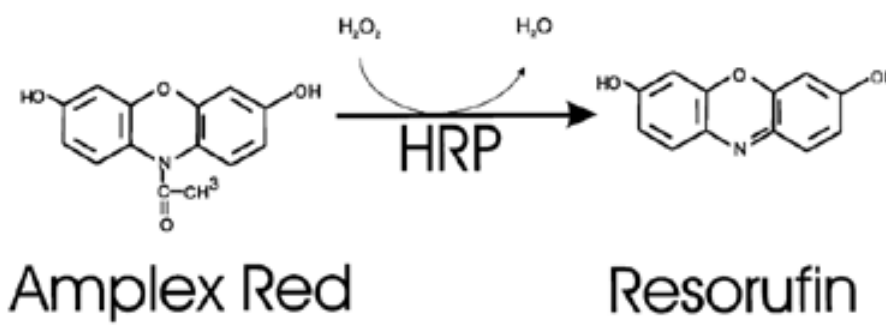

Fig. 4
Conversion of Amplex Red into resorufin

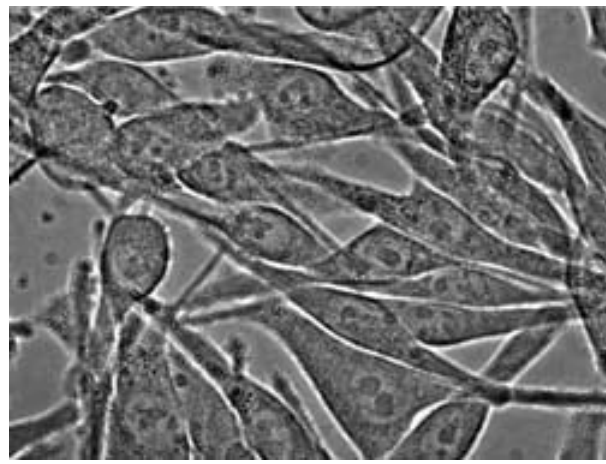

Fig. 5
Microscopic image of live G361 cells (magnification 400x)

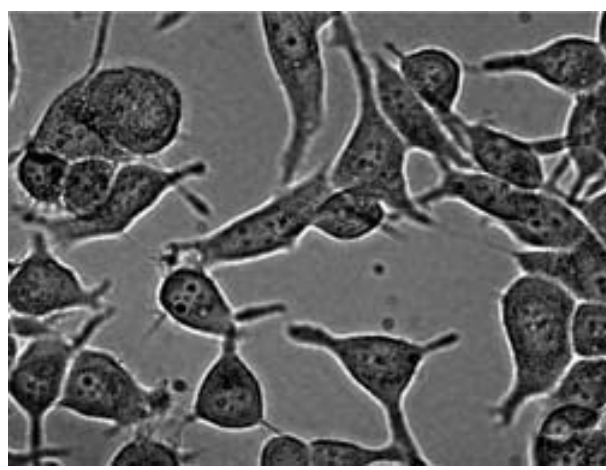

Fig. 6
Microscopic image of live MCF7 cells (magnification 400x)

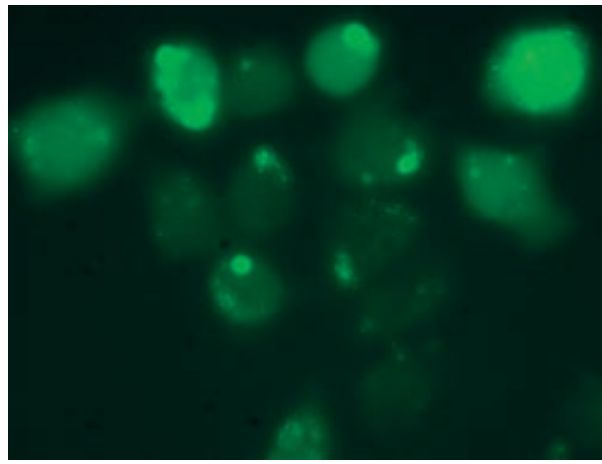

Fig. 7
Microscopic image of MCF7 cells pretreated with CM-H_2DCFDA and 10 min irradiation (magnification 400x)

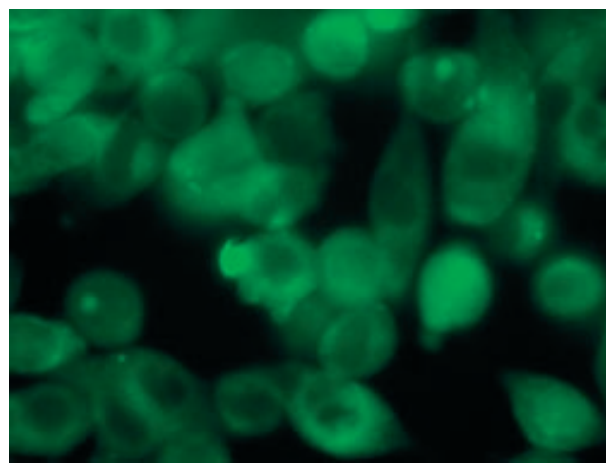

Fig. 8
Microscopic image of MCF7 cells pretreated with CM-H_2DCFDA and 20 min irradiation (magnification 400x)

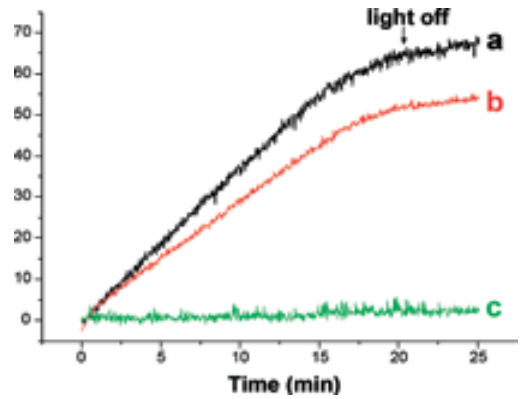

Fig. 9
Kinetic analysis of ROS reaction with CM-H_2DCFDA. (a) G361 cells loaded with $ZnTPPS_4$, (b) G361 cells loaded with $ZnTPPS_4$ and hpβCD, (c) G361 cells

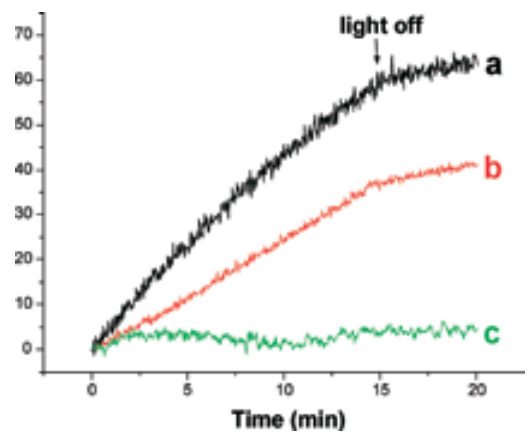

Fig. 10
Kinetic analysis of ROS reaction with CM-H_2DCFDA. (a) MCF7 cells loaded with $ZnTPPS_4$, (b) MCF7 cells loaded with $ZnTPPS_4$ and hpβCD, (c) MCF7 cells

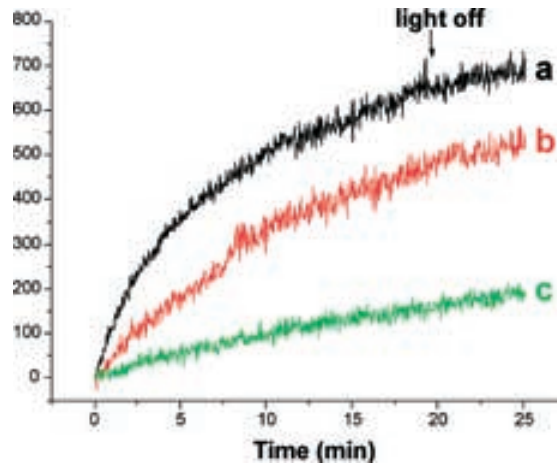

Fig. 11
Kinetic analysis of H_2O_2 reaction with Amplex Red. (a) G361 cells loaded with $ZnTPPS_4$, (b) G361 cells loaded with $ZnTPPS_4$ and hpβCD, (c) G361 cells

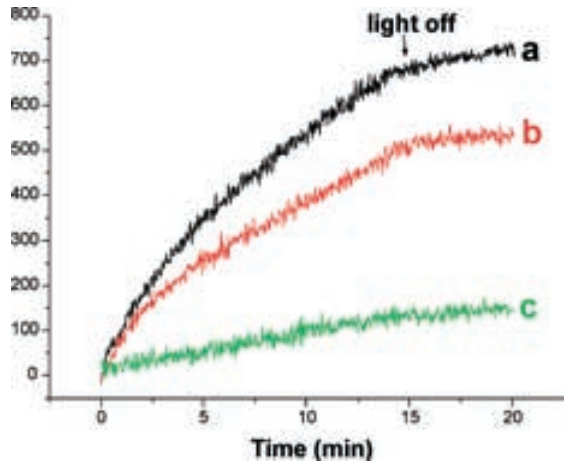

Fig. 12
Kinetic analysis of H_2O_2 reaction with Amplex Red. (a) MCF7 cells loaded with $ZnTPPS_4$, (b) MCF7 cells loaded with $ZnTPPS_4$ and hpβCD, (c) MCF7 cells

RESULTS

Demonstrative images of live G361 and MCF7 cells are shown in *Fig. 5* and *Fig. 6*. Using a molecular probe CM-H_2DCFDA in photosensitised cells we visualised production of ROS after 10 and 20 min irradiation (*Figs. 7 and 8*).

We measured ROS generation in G361 and MCF7 cell lines using a fluorescent product CM-DCF, which is formed by oxidation and deacetylation of CM-H_2DCFDA. We observed that G361 (*Fig. 9*) and MCF7 cells (*Fig. 10*) accumulated with a free sensitiser presented higher rates of ROS production than those loaded with $ZnTPPS_4$ with a cyclodextrin carrier.

Hydrogen peroxide release was measured by Amplex Red, which is oxidised specifically by HRP-bound H_2O_2 to form the fluorescent product resorufin. Similarly, we found that H_2O_2 release rates of G361 (*Fig. 11*) and MCF7 (*Fig. 12*) cells saturated with $ZnTPPS_4$ bound to hpβCD were lower than those observed in cells with free $ZnTPPS_4$.

DISCUSSION AND CONCLUSIONS

In this study, we used for ROS and hydrogen peroxide detection two fluorescent probes, CM-H_2DCFDA and Amplex Red. The rates of dye fluorescence correspond to the quantity of the intracellular ROS produced and H_2O_2 released from cells. The results obtained using both fluorescent probes were very similar. Our measurements demonstrated higher CM-DCF and resorufin fluorescence in both G361 and MCF7 cell lines loaded with a free sensitiser than for a sensitiser bound to the cyclodextrin carrier. This can be explained by a difference in the uptake of the free and bound sensitiser into the cells. Our previously presented results showed that a free sensitiser permeates the cell membrane more easily than a sensitiser bound to the cyclodextrin carrier. After 24 hours of incubation the difference in the intracellular concentration of the free and bound sensitiser was 26 % on an average *(11)*. The production of ROS after PDT was also demonstrated using an inverted fluorescent microscope.

Acknowledgements

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MĚŘENÍ REAKTIVNÍCH FOREM KYSLÍKU PO FOTODYNAMICKÉ TERAPII IN VITRO

Souhrn

Fotodynamická terapie (PDT), nově se rozvíjející léčebná metoda maligních nádorů a některých benigních procesů, nabízí selektivní odstranění patologické tkáně bez poškození okolních zdravých buněk. Fotodynamická reakce představuje kombinované použití fotosenzitizující látky (senzitizéru), lokalizované v cílové tkáni, světla a molekulárního kyslíku. Po aktivaci světlem o vhodné vlnové délce je senzitizér excitován do vyššího energetického stavu. Existují dva typy mechanizmu interakce tripletního stavu senzitizéru s biomolekulami. Při fotochemické reakci I. typu (přenos elektronu) se vytvářejí volné radikály a radikálové ionty. Ty mohou okamžitě reagovat s kyslíkem za vzniku reaktivních forem kyslíku (ROS). Mechanizmus II. typu (přenos energie) vede k tvorbě vysoce reaktivního singletního kyslíku, jehož přímý oxidační účinek poškozuje biomolekuly, včetně lipidových složek buněčných membrán. Oba mechanizmy mohou při PDT probíhat současně, jejich relativní podíl na destrukci nádoru však závisí na typu a koncentraci senzitizéru, na dávce ozáření, na koncentraci kyslíku v nádorové tkáni a na vazbě senzitizéru na substrát. Výsledným terapeutickým efektem je smrt nádorových buněk (nekróza nebo apoptóza).

V této práci jsme se zabývali fotodynamickými účinky volného senzitizéru $ZnTPPS_4$ a senzitizéru vázaného na cyklodextrinový nosič hpβCD na buněčných liniích G361 (lidský melanom) a MCF7 (adenokarcinom prsu). Tvorba ROS a peroxidu vodíku po PDT byla detekována pomocí nefluorescenčních značek CM-H_2DCFDA a Amplex Red a po jejich oxidaci byla měřena časová závislost fluorescence na luminiscenčním spektrometru. Zjistili jsme, že ve fotosenzitivních buňkách byla produkce ROS a H_2O_2 vyšší pro volný senzitizér než pro senzitizér vázaný na hpβCD nosič.

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