Comparative Studies of the Phototoxixity of Halogenated Photosensitizers- A Mechanistic Approach

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Summary

We compared the photodynamic activity of new stable chlorin and bacteriochlorin substituted with halogen atoms in the *ortho* position of the phenyl rings. The spectroscopic and photophysical properties were determined and the relative contributions of Type I and Type II photochemical processes were studied by measurement of 1270 nm luminescence, EPR spin trapping and intracellular ROS detection. The bacteriochlorin derivative characterized by low dark toxicity and high phototoxicity was much more active sensitizer than its chlorin analogue against S91 cells and induced apoptosis and necrosis after illumination. Our work shows that this higher phototoxicity does not correlate with physicochemical properties and singlet oxygen quantum yield but rather with other reactive oxygen species formed during the illumination. We conclude that halogenated bacteriochlorins have great potential as novel PDT agents and their phototoxicity may be mediated both by electron transfer (superoxide ion, hydroxyl radicals) and by energy transfer (singlet oxygen) but the photodynamic action is more effective when mechanism with superoxide ion production is also operated.

Introduction

Photodynamic therapy (PDT) employs the combination of nontoxic photosensitizers and harmless visible or near infrared light to generate reactive oxygen species (ROS) and kill cells. Critical to the success of PDT is the development of new photosensitizers with stronger light absorption in the phototherapeutic window. Most clinically studied sensitizers are based on the tetrapyrrole structure. Chlorins and bacteriochlorins, having intense absorption in the red and NIR, respectively, appear more advantageous than analogue porphyrins [1-3]. The reduction from the porphyrin to the bacteriochlorin state increases the amount of light absorbed by a factor of 50 and displaces the absorption to the near infrared, where human tissues are twice

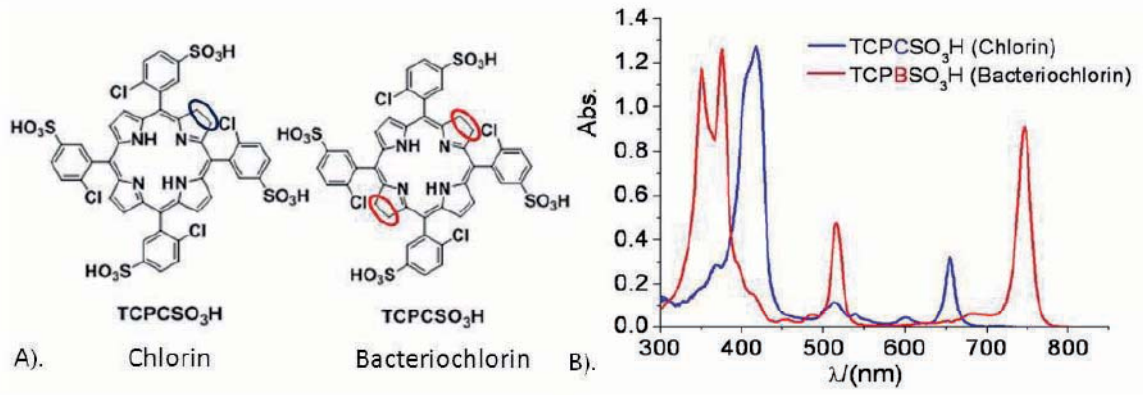

Fig. 1

as transparent as in the red. Halogenated chlorins and bacteriochlorins make use of the internal heavy-atom effect to increase the quantum yield of the triplet state, and can enhance PDT efficacy because the triplet state is the precursor of ROS. In view of the roles played by ROS in the oxidative stress, it is important to establish the mechanisms of energy and electron transfer from sensitizers to oxygen and the mechanism of cells death.

Materials and Methods

Singlet oxygen quantum yields in ethanol were obtained with a procedure described in detail elsewhere [1-2] using phenalenone as reference, for which Φ_{Δ}=0.95 in ethanol. Singlet oxygen phosphorescence at 1270 nm was detected following laser excitation of ethanol solutions containing the sensitizer at the concentration necessary to produce an absorbance of 0.2 in 1 cm quartz cuvette at the excitation wavelength. Other ROS produced by irradiation of bacteriochlorin in dimethylsulfoxide (DMSO) or PBS solutions, namely the hydroxyl radical and the superoxide ion, form adducts with 5,5-dimethylpyrroline-N-oxide (DMPO) and were identified by EPR (Bruker ESP 300 spectrometer). Phototoxicity was evaluated using MTT. The intracellular detection of ROS was studied by confocal microscopy. The cells death pathway was investigated using fluorescence microscopy.

Results

Our water-soluble chlorin and bacteriochlorin derivatives are presented in Fig. 1.A.

Fig. 1. A) Molecular structures of 5,10,15,20-tetrakis(2-chloro-5-sulfophenyl) chlorin ($TCPCSO_3H$) and 5,10,15,20-tetrakis(2-chloro-5-sulfophenyl)bacteriochlorin ($TCPBSO_3H$). B) Absorption spectra of chlorin and bacteriochlorin measured in water at room temperature.

The ground state absorption spectra of the 5,10,15,20-tetrakis(2-chloro-5-sulfophenyl) chlorin ($TCPCSO_3H$) and corresponding bacteriochlorin ($TCPBSO_3H$), recorded at room temperature in phosphate buffer water solutions (PBS, pH 7.4), are presented in Fig. 1.B. The lowest energy electronic absorption of the chlorin occurs at 655 nm

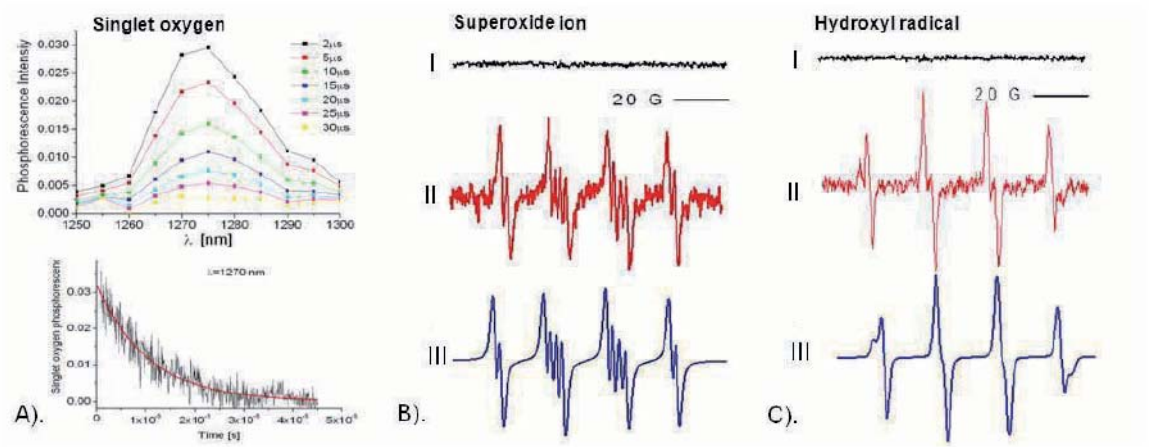

Fig. 2

with molar absorption coefficient of 7000 M^{-1} cm^{-1} and that of the bacteriochlorin is ε=24000 M^{-1} cm^{-1} around 750 nm. The longest-wavelength absorption band at 750 nm is advantageous for PDT, because near infrared light can deeply penetrate the tissue. The singlet oxygen quantum yields were obtained from its phosphorescence at 1270 nm (Fig. 2.A). The high Φ_Δ observed for the chlorin (Φ_Δ=0.64) is the result of the internal heavy atom effect coming from halogen atoms in the ortho position of the phenyl ring. The lower Φ_Δ measured for the bacteriochlorin (Φ_Δ=0.43) is related to the involvement of charge-transfer in the mechanism of energy transfer to molecular oxygen, that lead to the formation of the superoxide ion (Fig. 2.B) and hydroxyl radical (Fig. 2.C).

Irradiation of the S91 cells previously treated with different photosensitizers resulted in a pronounced inhibition of the cell metabolism, as illustrated in Fig.3.A. The enhanced phototoxicity of the bacteriochlorin is assigned to the higher toxicity of the ROS formed by the bacteriochlorin. Evidence that $TCPBSO_3H$ can also generate hydroxyl radicals in the cells is presented in Fig. 3.B). Fluorescein emission is observed in the cells after incubating this probe with $TCPBSO_3H$ and irradiating the cells with 750 nm laser light but is not observed after excitation of analogous porphyrin. This is assigned to the release of fluoresceinc after the reaction of 3'-(p-aminophenyl)fluorescein with photogenerated hydroxyl radicals.

Fig. 2. Detection of ROS: A). Singlet oxygen phosphorescence in D_2O at various laser intensities after excitation of sensitizers with 355 nm pulses, together with its decay curve. B) EPR spectrum of the spin trap adduct DMPO-OOH in DMSO: I) before illumination, II) during illumination and III) simulation. C). EPR spectrum of the spin trap adduct BMPO-OOH observed in PBS I) before illumination, II) during illumination and III) simulation.

Cell death can be induced via two mechanisms: apoptosis or necrosis. Apoptosis is characterized by changes in cellular morphology, such as shrinkage, cell surface blabbing, chromatin condensation and DNA fragmentation. This type of morphology is different from the well-known necrotic type, characterized by swelling organelles, clumping of chromatin, breakdown of plasma membrane and, finally, total cell disintegration. In our study PDT-treated and untreated controls were stained with Hoest/PI in order to verify the modes of cell death based on their morphological changes.

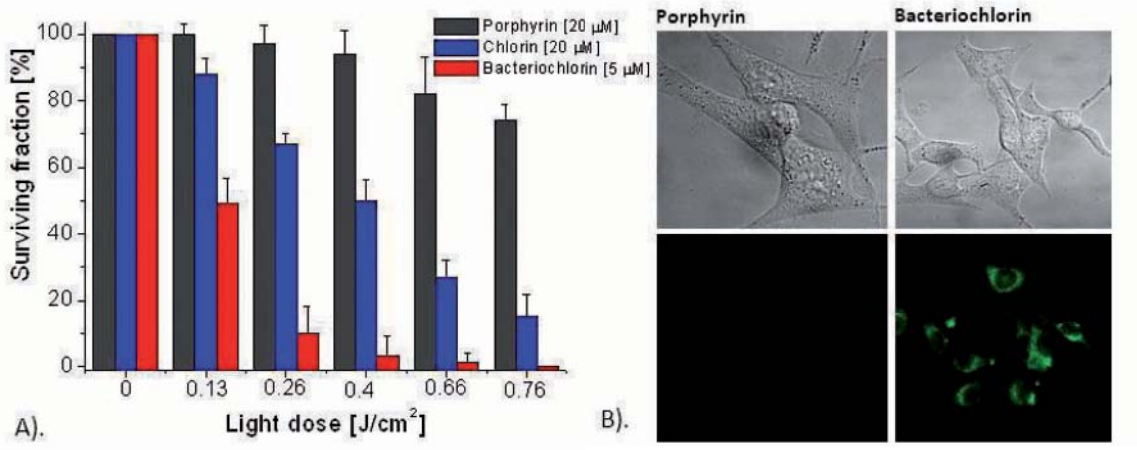

Fig. 3

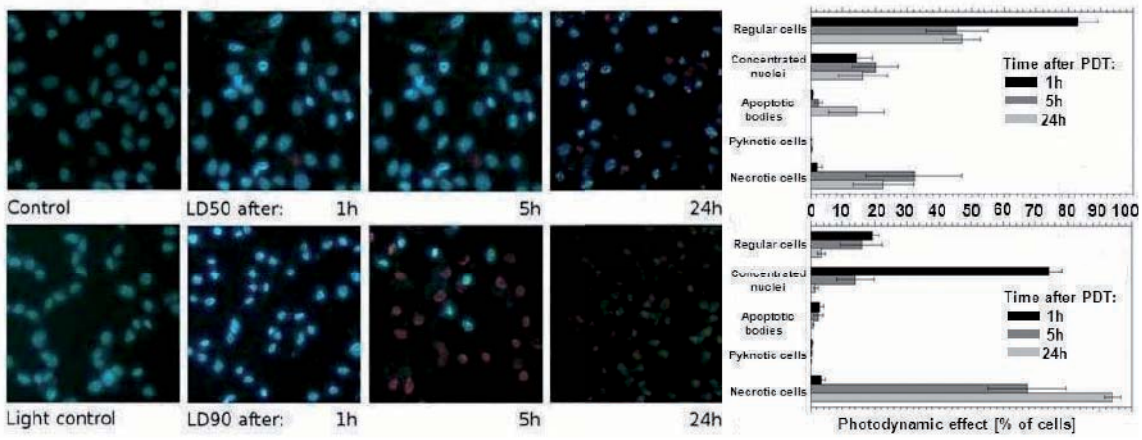

Fig. 4

The investigations were carried out using S91 cells and the results are presented in Fig. 4.

Fig. 3. A). Comparison of the photodynamic efficiency of $TCPPSO_3H$ (■), $TCPCSO_3H$ (■) and $TCPBSO_3H$ (■) in S91 cells determined by MTT assays 24 h post-irradiation: 0.13, 0.26, 0.4, 0.66, 0.76 J/cm^2. B). Contrast phase (up) and fluorescence micrographs (down) of S91 cells incubated with $TCPPSO_3H$ (porphyrin) and $TCPBSO_3H$ (bacteriochlorin) and 3'-(p-aminophenyl)fluorescein. Pictures were taken after illuminating the cells with light.

Fig. 4. Cells death pathway analysis of PDT treated S91 cells. The cells were illuminated by halogen lamp. Pictures demonstrate cells after 1 h, 5 h and 24 h after PDT. All bars are presented with SEM (N=3, n=4).

Conclusions

We compared the spectroscopic and photodynamic properties of halogenated chlorin

and bacteriochlorin and showed that their PDT potency *in vitro* exceeds the values expected from the absorbance and singlet oxygen quantum yield. A possible explanation of the enhanced phototoxicity can be assigned to the high reactivity of hydroxyl radicals. A small amount of hydroxyl radicals may initiate a chain reaction of lipids peroxidation that is significantly more toxic to cancer cells than peroxidation by a much higher concentration of singlet oxygen. Although necrosis may be the major cell death pathway, photodynamic efficacy is increased and the apoptosis is observed when light is delivered at lower intensities.

References

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