Inhibition of Taq polymerase activity by singlet oxygen generation at photodynamic therapy

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Abstract. The goal of this study was to assess the ability of singlet oxygen generated at PDT to inhibit the activity of the model enzyme - Taq polymerase. Experimentally were determined the laser irradiation dose and the singlet oxygen concentration (over 300 μM) that results to complete inhibition of Taq polymerase activity. The results show that enzymatic proteins have an order of magnitude higher susceptibility than DNA molecules to damage by singlet oxygen.

1. Introduction
Photodynamic therapy (PDT) is a clinically approved method for the treatment of cancer which can have cytotoxic activity against tumor cells [1-3]. The damage mechanism of tumor cells at PDT strongly depend on the photosensitizer (PS) localization (lipid membrane, intracellular organelles or nucleus) [4]. The chlorine and porphyrin PS drugs used in clinical practice (Radachlorin, NPe6, Photofrin, BPD and mTHPC), mainly accumulate in the cell cytoplasm (not in the nucleus), binding to protein components and localizing the damaged area. PS can accumulate in endoplasmic reticulum, Golgi apparatus, mitochondria, lysosomes, and plasma membrane. Upon the laser irradiation with specific wavelength PS can generate an excited state of the molecular oxygen - singlet oxygen which can induce further oxidation of biological objects. In the same time, it is possible to damage DNA molecules near the surface of the nuclear membrane, which can lead to mutation formation. In this study was investigated of the damage of enzymatic protein (Taq DNA polymerase) and single strand DNA (ssDNA) molecules under photodynamic treatment with Radachlorin PS. It was observed that the inhibition of Taq polymerase enzymatic activity was correlated with the laser irradiation dose and the concentration of singlet oxygen generated at PDT. The obtained results demonstrated that enzymatic proteins have an order of magnitude higher sensitivity to damage by singlet oxygen than DNA molecules.
2. Materials and methods

2.1. DNA damage by singlet oxygen

ssDNA was taken as a model system to investigate DNA oxidation at photodynamic therapy. cDNA was obtain from K562 cells using the GeneJET RNA purification Kit (Thermo Scientific, USA) and Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, USA) according to the manufacturer’s instructions. Concentration of cDNA was measured on NanoDrop 2000c (Thermo Scientific, USA). 20µl water solution of cDNA 9.6 ng/µl and 3.8 µM Radachlorin photosensitizer (Rada-Pharma Co, Ltd., Moscow, Russia) in individual tubes was prepared. This solution was irradiated with 662 nm laser «LAHTA – MILON» (MILON Laser, St. Petersburg, Russia) at power density I = 50 mW/cm² with energy doses: 2.5 J/cm², 5 J/cm², 7.5 J/cm², 10 J/cm², 15 J/cm². cDNA damage after photodynamic treatment was analyzed using polymerase chain reaction - diagnosis method (PCR). The PCR was performed in a 40 µL volume using 20 µl water solution of cDNA (9.6 ng/µl) after irradiation, 0.25 µM forward (5’-TGTTTCATCATCATTCAACGGTGG-3’) and reverse (5’-GTTCCCGTAGGTCATGAACTCAG-3’) primers [5]. Taq polymerase (1.25 units per reaction), dNTP (200 µM) and 10x Mg²⁺ Tris-HCl buffer («Sileks», Russia). Used primers were specific to the BCR-ABL gene fragment of cDNA. The PCR protocol contained initially denatured for 1 minute at 95°C followed by 35 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds, with a final extension at 72°C for 5 minutes using a Thermocycler T100 (Bio-Rad, USA). The PCR products (374 b.p.) were separated by electrophoresis in 8% polyacrylamide gel. Results were analyzed with Gel Doc XR+ (Bio-Rad, USA). The results were reported as the mean values with standard deviation from three experiments.

2.2. Inhibition of Taq polymerase at PDT

Taq polymerase («Evrogen», Russia) was added to 20µl Taq Buffer in 9 individual tubes. Taq polymerase concentration in experiments was equal 11.9 µg/ml (0.136 µM). The molar concentration of protein was measured on NanoDrop 2000c spectrophotometer. Then water solution of Radachlorin (0.5-2.0 µM) was added to each samples. This solution was mixed and irradiated with 662 nm laser at power density I = 20 mW/cm² with energy doses: 0.5, 1.25, 2.5, 3.75, 5, 7.5 and 10 J/cm². Then the 20µprepped solution of “control cDNA”, dNTP and forward and revers primers for target gene BCR-ABL [5], was added to treated samples. The PCR was performed according to the previous protocol using a Thermocycler T100. The PCR products for BCR-ABL gene (374 bp) were separated by electrophoresis in 8% polyacrylamide gel. The intensity of bands was used as a quantitative characteristic of the polymerase activity. The intensity value of bands was measured in the control and in irradiated samples. Taq polymerase replication activity was presented in percent as band intensity in control sample with PS and without irradiation.

2.3. Detection of singlet oxygen generation at PDT

Chemical trap method was used to register singlet oxygen generated in water under photodynamic interaction [6]. Chemical trap 1,3-diphenylisobenzofuran (DPBF) was prepared in 1.5 ml water (or PDS) with 0.2M SDS in 1 cm quartz cuvette. The concentration [DPBF] = 42 µM and Radachlorin concentration [PS] = 3.5 µM (extinction coefficient ε₆₅₅ = 28 300 M⁻¹cm⁻¹ in PBS). This solution was irradiated with 662 nm laser «LAHTA – MILON» at power I = 10 mW/cm² during t = 100 sec. Absorption spectra were measured each 10 second on NanoDrop 2000c. The decrease of absorbance on 414 nm wavelength corresponds to the interaction the chemical traps DPBF with singlet oxygen. Absorption change with time corresponds to the singlet oxygen generation rate, taking into account the
efficiency of singlet oxygen quenching by DPBF in solution. The efficiency of singlet oxygen quenching by DPBF depended on singlet oxygen lifetime, DPBF concentration and quenching rate constant.

3. Results

3.1. Inhibition of Taq polymerase activity at PDT
Fig.1 shows the DNA polyacrylamide gel which presented Taq polymerase activity after photodynamic interaction. The Taq polymerase samples were incubated with three concentration of Radachlorin (0.5 μM, 1 μM, 2 μM) and then irradiated under laser at 662 nm. For a low PS concentration equal 0.5 μM, there was no complete inhibition of the polymerase activity. For concentrations 1.0 and 2.0 μM were observed complete inhibition of Taq polymerase activity at irradiation doses 2.5 and 7.5 J/cm², respectively. On Fig.2 presented the relative values of Taq polymerase replication activity on the irradiation doses for 0.5, 1.0 and 2.0 μM Radachlorin. At a low PS concentration and small light dose an increase the Taq polymerase activity (PCR product) was observed. It can be suggested that this effect may be associated with the appearance of an unstable oxidized enzyme but more active for PCR product formation.

![Figure 1](image1.png)

**Figure 1.** DNA polyacrylamide gel electrophoresis after photodynamic interaction on the Taq polymerase solution. Lanes 1-7 — PDT with irradiation doses 0.5, 1.25, 2.5, 3.75, 5.0, 7.5, 10 J/cm²; Lane 8 — control PS+; Lane 9 — control PS-; Lane 10 — 300-bp DNA ladder.

![Figure 2](image2.png)

**Figure 2.** Dose dependent inhibition Taq polymerase replication activity (relative PCR product) after photodynamic treatment with 0.5, 1 and 2.0 μM of Radachlorin PS and irradiation doses 0.5-10 J/cm².

3.2. Direct ssDNA damage by singlet oxygen at PDT
The experimental results of ssDNA damage at PDT were obtained in our previous study [5]. Fig. 3 shows picture of DNA polyacrylamide gel electrophoresis after photodynamic treatment and intensity
of BCR-ABL DNA fragment. Fig. 4 shows dependence of target PCR product (374 bp) from the irradiation dose. The second fragment of DNA (about 50 b.p.) was primer (forward and reverse) dimers. This is a nonspecific product of PCR reaction and it was increased at decreased amount of target DNA. The PCR product (DNA replication activity) was presented as relative intensity I/I0, where I is intensity of the analyzed band, I0 is intensity of the control band.

Figure 3. DNA polyacrylamide gel electrophoresis after photodynamic treatment (target gene BCR-ABL). Lane 1 — control PS-; Lane 2 — control PS+; Lanes 3-7 — PDT with irradiation doses 2.5, 5, 7.5, 10, 15 J/cm²; Lane 8 — 300-bp DNA ladder.

Figure 4. Irradiation dose dependent PCR product (DNA replication activity) after photodynamic treatment with 3.8 μM of Radachlorin PS and irradiation doses 2.5-15 J/cm².

This data demonstrated that DNA replication activity depends from irradiation doses at PDT. The analysis of DNA damages can be applied to investigate the efficiency of laser irradiation modes at photodynamic therapy.

3.3. DPBF detection of singlet oxygen generation

Fig. 5 shows photodegradation of DPBF by laser irradiation with Radachlorin in water 0.2M SDS. Absorbance decrease on λ = 414 nm was measured. Fig. 6 shows the calculation concentration dependences of oxidized DPBF and calculated concentration of 1O2 from irradiation time. Using the development of the methods for the direct study of kinetics of the reaction of singlet oxygen [7, 6], equation (1) was obtained that describes the interaction of singlet oxygen with a reactive substrate DPBF:

\[
\frac{1}{\epsilon} \frac{dA}{dt} = \frac{d[1O_2]}{dt} \frac{k_{ox}[DPBF]}{\tau_A^{-1} + k_{ox}[DPBF]} 
\]

where A is absorption of DPBF at 414 nm without value of PS absorption, ε is molar absorption coefficient DPBF equal to 2.35 × 10⁴ M⁻¹ cm⁻¹ (414 nm), τA is life time of 1O2 in water equal to 4.0 μs, kox is a constant of oxidation of DPBF equal to 2.8 × 10⁹ M⁻¹ s⁻¹ in water with SDS [6], [DPBF] is concentration of active DPBF traps. The same experimental data was received in ethanol [5].
Photodegradation of DPBF by laser irradiation at 662 nm (20 mW/cm\(^2\)) in water with 3.5 \( \mu \)M Radachlorin.

Time dependences of concentration of DPBF oxidized by singlet oxygen (black line) and total singlet oxygen concentration (red line).

Using the equations of macroscopic singlet oxygen model [8, 9] the equation (2) was obtained for calculated the singlet oxygen generation rate. \( \xi \) is the parameter of singlet oxygen generation efficiency including in itself the PS extinction coefficient at certain wavelength and singlet oxygen quantum yield of PS. This parameter was calculated using linear approximations of data from Fig. 6. This parameter is the proportionality constant between the concentration of PS, the irradiation intensity, oxygen concentration and the rate of singlet oxygen generation. \( \xi \) has value of singlet oxygen generation rate per \( I = 1 \) mW/cm\(^2\) irradiation and 1 \( \mu \)M of photosensitizer per second. \( \beta \) is the ratio of the triplet photosensitizer decay rate to the rate of the triplet photosensitizer quenching by \( ^3\text{O}_2 \) (8.6 \( \mu \)M for chlorine PS).

\[
\frac{d[^3\text{O}_2]}{dt} = \xi \cdot [PS] \cdot I \cdot \left( \frac{[^3\text{O}_2]}{[^3\text{O}_2] + \beta} \right)
\]

(2)

Table 1. Photochemical parameters: singlet oxygen generation rates of Radachlorin PS and life time of \(^1\text{O}_2\) molecule in ethanol and water upon laser irradiation of at 662 nm.

| Solvent            | \( \xi \), mW\(^{-1}\)cm\(^3\)s\(^{-1}\) | \( \tau_{\text{\(3\)O}_2} \), \( \mu \)s | Ref. |
|--------------------|-----------------------------------------|--------------------------------------|-----|
| Ethanol            | 0.067 \( \pm 0.007 \)                   | 13.5                                 | [5] |
| Water + 0.2M SDS   | 0.043 \( \pm 0.003 \)                   | 4                                    | [5, 10] |
| PBS + 0.2M SDS     | 0.062 \( \pm 0.003 \)                   | 3.5                                  | [10, this study] |

This value can be used to recalculate generation of singlet oxygen in solutions with other concentrations of PS and irradiation intensity. This constant was further used in our study to calculate cumulative singlet oxygen concentration at photodynamic therapy.
3.4. Taq polymerase damage mediated by singlet oxygen

**Figure 7.** Singlet oxygen dose-dependent inhibition Taq polymerase activity after photodynamic treatment with 0.5, 1.0 and 2.0 μM of Radachlorin and irradiation doses 0.5-10 J/cm².

**Figure 8.** Singlet oxygen dose-dependent inhibition Taq polymerase activity and DNA replication activity after photodynamic treatment with Radachlorin PS and irradiation dose 0.25-15 J/cm².

The mathematical modeling of singlet oxygen generation at PDT allowed calculating singlet oxygen concentration and PS photobleaching [8-10]. This modeling was applied to calculated singlet oxygen doses which induced the inhibition of Taq polymerase activity and the damaged of ssDNA (oxidation ssDNA). Fig.7 demonstrated the dependency of Taq polymerase activity on singlet oxygen concentration after photodynamic treatment. At a low dose of the PS concentration equal 0.5 μM, there was no complete inhibition of the polymerase activity. Perhaps this effect is due to PS binding to the enzyme, and at a low PS concentration, there are free molecules of the enzyme that are not interacted with active singlet oxygen. Using the dose-response fitting were determined the IC50 values of singlet oxygen concentration which inhibited 50% Taq polymerase activity and reduced the ssDNA replication activity. The data of replication activity from singlet oxygen concentration was presented on Fig.8. The IC50 values of [¹⁰₂] were 145±7 μM for Taq polymerase and 856±50 μM for ssDNA.

4. Discussion

Experimentally were determined the laser irradiation dose and the singlet oxygen concentration (over 300 μM) that results in complete inhibition of enzymatic activity of Taq polymerase. Also were determined the irradiation doses at PDT, which reduce the replication activity of single-stranded DNA molecules [5]. The results show that enzymatic proteins have an order of magnitude higher susceptibility to damage by singlet oxygen than DNA molecules and direct damage the DNA in the nucleus of tumor cells is unlikely. Obtained data confirm that the protein have high rate constants for reaction with singlet oxygen. Using the singlet oxygen quenching rate constants by proteins [11] can be determined the concentration of molecule damages which induces complete inhibition of polymerase activity. The understanding dose dependences of molecular damages at PDT are an important step in assessing the photodynamic effect on tumor cells [4, 9].

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