ssDNA damage dependence from singlet oxygen concentration at photodynamic interaction

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Abstract. Single stranded DNA damage at photodynamic treatment with Radachlorin photosensitizer was investigated. Chemical trap method was used to evaluate generation of singlet oxygen in water solution. Interaction of singlet oxygen with ssDNA resulted into decrease of the replication activity of ssDNA. DNA stopped replicating during PCR at irradiation doses greater than 15 J/cm² and concentration of photosensitizer [PS] = 3.8 μM. The dependence of replication activity of ssDNA on generated singlet oxygen concentration was identified.

1. Introduction
Photodynamic therapy (PDT) is an innovative method for the treatment of many tumors [1,2]. The main mechanism of PDT involves the interaction between the photoactive state of the photosensitizer (PS) and molecular oxygen (O₂) to produce singlet oxygen (¹O₂). Generally, it is believed that ¹O₂ is the main cytotoxic species in PDT [3]. ¹O₂ mainly damages lipids, proteins, RNA and DNA. There was a lot of interest in how singlet oxygen affects DNA chain in recent studies, because such interaction may lead to mutations and carcinogenesis [4,5,6]. But we have not found any data presenting dependence of DNA replication activity from concentrations of singlet oxygen generated during irradiation at PDT. The analysis of DNA molecular damages can be applied to investigate the efficiency of irradiation modes at photodynamic therapy. In present study we analyzed the dependence of ssDNA damage on generated singlet oxygen concentration.

2. Materials and methods

2.1. ssDNA damage by singlet oxygen
ssDNA (cDNA) was taken as a model system to investigate DNA oxidation at photodynamic therapy. RNA was isolated from k562 cells using the GeneJET RNA purification Kit (Thermo Scientific, USA) according to the manufacturer’s instructions. cDNA was synthesized with an input of 2 μg of total RNA using Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Scientific, USA), following the manufacturer’s instructions. Concentration of cDNA was measured on NanoDrop 2000c (Thermo Scientific, USA). 20μl water solution of cDNA (9.65 ng/μl) and 3.8 μM photosensitizer Radachlorin (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 (9.65 ng/µl) after irradiation, 0.25 µM forward (5’-TGGTTTCATCATCATTCAACGGTG-3’) and reverse (5’-GTGCCGTAGGTCATGAACTCAG-3’) primers [7], 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 samples were 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. Singlet oxygen generation in solution
Chemical trap method was used to register singlet oxygen emerged in solution under photodynamic interaction [8]. Ethanol solution of chemical trap 1,3-diphenylisobenzofuran (DPBF) at concentration [DPBF] = 42 µM and Radachlorin at concentration [PS] = 3.5 µM (ε₆₅₅ = 28300 M⁻¹ cm⁻¹ in PBS) was prepared. 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 (Thermo Scientific, USA). The decrease of absorbance on 412 nm wavelength corresponds to the interaction of singlet oxygen with chemical traps DPBF.

3. Results

3.1. ssDNA damage by singlet oxygen after PDT
Fig. 1 shows UV picture of DNA polyacrylamide electrophoresis after photodynamic treatment. Fig. 2 shows dependence of PCR product from the irradiation dose. PCR product was calculated as I/I₀, where I is intensity volume of the analyzed band, I₀ is intensity volume of the control group band.

3.2. Detection of singlet oxygen generation in solution at PDT
Fig. 3 shows photodegradation of DPBF by irradiation with red laser in the presence of Radachlorin in ethanol. Absorbance decrease can be observed on λ = 412nm. Fig. 4 shows concentration dependences of oxidized DPBF and calculated concentration of ¹⁰₂ (Eq. (1)) from irradiation time.
where $A$ is absorption of DPBF at 412nm, $\varepsilon$ is molar absorption coefficient equal to $2.35 \times 10^4$ M$^{-1}$ cm$^{-1}$ (412 nm) for DPBF, $\tau$ is life time of $^1$O$_2$ in ethanol equal to 13.5 $\mu$s, $k_{\text{ox}}$ is a constant of oxidation of DPBF equal to $1.4 \times 10^9$ M$^{-1}$ s$^{-1}$ in ethanol, $[\text{DPBF}]$ is concentration of active DPBF. The same experiment was conducted in H$_2$O solution. Fig. 5 shows absorbance decrease of DPBF from irradiation time at different power densities $I = 10, 20, 40$ mW/cm$^2$. Using Eq. 1 and parameters for water solution ($^1$O$_2$ life time $\tau$ = 4$\mu$s, $k_{\text{ox}} = 2.8 \times 10^9$ M$^{-1}$ s$^{-1}$ [8]) singlet oxygen concentration was calculated. Fig. 6 shows linear dependence of $^1$O$_2$ generation rate from laser power density.

Figure 3. Photodegradation of DPBF by irradiation with red laser in the presence of Radachlorin in ethanol.

Figure 4. Time dependences of singlet oxygen and oxidized DPBF concentrations.

Figure 5. Photodegradation of DPBF by irradiation with red laser with different irradiation power in the presence of Radachlorin in H$_2$O + 0.2M SDS.

Figure 6. Singlet oxygen generation rate dependence of laser power density in H$_2$O + 0.2M SDS.

$\eta$ parameter was calculated using Eq. 2 and linear approximations of data from fig. 4 and fig. 6. It stands for the [$^1$O$_2$] generation rate per I = 1 mW/cm$^2$ irradiation and 1 $\mu$M of photosensitizer per second.
\[
\frac{d[1^O_2]}{dt} = \eta \cdot [PS] \cdot I
\]  

(2)

Table 1. Life time of \(1^O_2\) molecule and its generation rate in ethanol and water solutions upon irradiation of Radachlorin photosensitizer by 662 nm laser.

| Solvent                  | \(\tau_\Delta, \mu s\) | \(\eta, \text{mW}^{-1}\text{cm}^2\text{s}^{-1}\) |
|--------------------------|------------------------|-----------------------------------------------|
| Ethanol                  | 13.5                   | (6.7±0.7)\(\times\)10\(^{-2}\)                |
| Water + 0.2M SDS         | 4                      | (4.3±0.3)\(\times\)10\(^{-2}\)                |

This value can be used to recalculate generation of singlet oxygen in solutions with other concentrations of photosensitizer and intensities of irradiation. This constant was further used in our study to calculate cumulative singlet oxygen concentration at photodynamic therapy.

3.3. DNA damage dependence from cumulative singlet oxygen concentration

With \(\eta\) parameter we calculated cumulative singlet oxygen concentrations generated in DNA solution at PDT using macroscopic singlet oxygen model with photobleaching [9]. It allowed us to recalculate dependence of PCR product from the concentration of total singlet oxygen generated in water. Concentration of singlet oxygen that oxidized DNA \([1^O_2]_{q}\) was calculated using Eq. (3).

\[
[1^O_2]_{q} = \frac{k_{ox}[cDNA]}{\tau_\Delta + k_{ox}[cDNA]}
\]  

(3)

where \(\tau_\Delta\) is life time of \(1^O_2\) in water equal to 4 \(\mu s\), \(k_{ox}\) is a constant of oxidation of cDNA equal to 5.1\(\times\)10\(^{6}\) M\(^{-1}\) s\(^{-1}\) in water [4], \([cDNA]\) is DNA nucleotide concentration equal to 9.65\(\times\)10\(^{-3}\) (g/L) / 300 (g/mol) = 32.2 \(\mu M\). In our study \([1^O_2]_{0}/[1^O_2] = 6.6\times10^3\). Table 2 shows energy-dose dependent concentrations of cumulative singlet oxygen, singlet oxygen that oxidized cDNA and PCR product after PDT. At 15 J/cm\(^2\) the replication of cDNA terminates. For this irradiation dose the ratio of oxidized guanines to total concentration of guanines was calculated and it equals 352 8-oxodG per 10\(^5\) dG.

Table 2. Energy-dose dependent concentrations of cumulative singlet oxygen at \([PS] = 3.8\mu M\), singlet oxygen that oxidized cDNA and PCR product.

| Dose, J/cm\(^2\) | \([1^O_2]\), \mu M (±7\%) | \([1^O_2]_{q}\), \mu M | 8-oxodG per 10\(^5\) dG | PCR product, % |
|------------------|--------------------------|------------------------|--------------------------|----------------|
| 0 (control)      | 0                        | 0.000                  | 0                        | 100±0          |
| 2.5              | 370                      | 0.024                  | 76                       | 89.9±8.5       |
| 5                | 702                      | 0.046                  | 144                      | 55.8±5.9       |
| 7.5              | 999                      | 0.066                  | 205                      | 31.5±6.5       |
| 10               | 1266                     | 0.084                  | 259                      | 18.0±9.8       |
| 15               | 1719                     | 0.113                  | 352                      | 2.7±2.4        |

4. Discussion

We showed that PDT influence leads to dose-dependent decrease of ssDNA replication activity. For the first time we calculated the dependence of replication activity of ssDNA from cumulative concentration of singlet oxygen generated in solution during irradiation (Table 2). At irradiation doses
greater than 15 J/cm² and concentration of photosensitizer [PS] = 3.8 µM PCR product was not observed (Fig. 1). It matches cumulative singlet oxygen concentration [1'O₂] = 1.7 mM. Decrease in DNA replication of target gene can be explained by oxidation of ssDNA by singlet oxygen. It is known that singlet oxygen reacts with DNA molecule and generally oxidizes guanine nucleotides forming 8-oxodG [5]. There are two general mechanisms of ssDNA damage by singlet oxygen: 8-oxodG generation and single-strand breakage of ssDNA [4]. According to our PCR results single-strand breakage of DNA was not observed. We suggest that first mechanism of damage is the main reason for decrease in replication activity of DNA. In our study, PCR started from binding of reverse primers on cDNA (5'-GTTCCCGTAGGTCATGAACTCAG-3'). Most primers are synthesized so that they have high GC-content for their strong binding. If guanines in cDNA are oxidized by singlet oxygen with generation of 8-oxodG then reverse primer cannot bind to complementary DNA fragment. This leads to inhibition of replication activity of target gene and decrease of PCR product. If 8-oxodG is "inside" target gene of cDNA, complementary DNA chain generates but it has G to T mutations [5]. However, these mutations have no effect on PCR product. Finally, this experiment shows that decrease in replication activity of DNA is dependent from cumulative concentration of singlet oxygen generated during irradiation. Efficiency of photodynamic treatment strongly depends on generated singlet oxygen concentration [10]. Therefore, this simple method of registration of DNA molecular damages can be applied to investigate the biotoxic efficiency of irradiation modes at photodynamic therapy.

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