Combination of cytostatic chemotheraphy with cisplatin and photodynamic therapy with Radachlorin reduced resistance of K562 and Hela cell lines

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Abstract. In this work were study combination effect of photodynamic therapy and cisplatin on the proliferation activity of K562 human leukemia cells and Hela cervical carcinoma cells. A decrease in cell viability and an increase the fraction of apoptotic cells for combination treatment compared with single therapy were observed. It has been shown that the G2/M-phase of cell cycle decreases compared with cisplatin treatment alone, which demonstrates an increase anti-proliferative effect. The combination index of the photodynamic therapy with Radachlorin and cisplatin was calculated and indicates a synergistic effect.

1. Introduction
Photodynamic therapy (PDT) is an actively developing method for the treatment of oncological diseases of various localization. However, in clinical practice PDT is not often used as a monotherapy and is usually used in combination with chemotherapy as an additional antitumor effect. Currently, there are no unambiguous protocols for the combined use of cytostatic drugs and PDT. However, in a number of works \cite{1, 2, 3, 4}, the effectiveness of the combined use of various chemotherapeutic drugs and PDT with the photosensitizer Photofrin in vitro was shown and a synergistic effect of the combination treatment was revealed. Chlorin e6 based photosensitizers (Radachlorin and Photoditazin) have less dark toxicity than Photofrin and widely used in clinical practice in Russian Federation. The aim of this study was to investigate the combination effect of photodynamic therapy with Radachlorin and the cytostatic drug cisplatin on human erythroleukemia K562 cell line and human cervical carcinoma Hela cell line. K562 cells are cisplatin-resistant (IC\textsubscript{50} > 40 μM), whereas Hela cells are sensitive to cisplatin (IC\textsubscript{50} < 15 μM). This work were evaluated the cytotoxic and cytostatic effects in combination cisplatin treatment and PDT with the identification of the cell death mechanisms and the cell cycle changes.
2. Materials and methods

2.1. Cell cultures
The human erythroleukemia K562 cells and human cervical cancer HeLa cells were obtained from the bank of cell cultures, Institute of Cytology of the Russian Academy of Sciences, Russia. The K562 cells were cultured in RPMI-1640 and the Hela cells were cultured in DMEM medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and 40 μg/mL of gentamicin (Sigma, USA) in a humidified incubator with 5% CO₂ at 37°C.

2.2. Photosensitizer
Radachlorin (Rada-Pharma, Russia) was used as photosensitizer drag for photodynamic treatment. Radachlorin is a water soluble chlorin e6 complex with the molar extinction coefficient 28300 M⁻¹cm⁻¹ at 654 nm in dPBS and 34000 M⁻¹cm⁻¹ at 662 nm in PBS + 10% FBS. The stock solution of Radachlorin has concentration 3.5 mg/mL. The Radachlorin was diluted in dPBS and work concentration was evaluated by spectrophotometer Nanodrop 2000c (Thermo Fisher Scientific, USA).

2.3. Photodynamic therapy
The medical semiconductor laser Lakhta-MILON (Milon Lasers, Russia) were applied for photodynamic treatment at wavelength of 662 nm. The effect of single PDT on cell viability was studied for K562 cells and Hela cells. Cells were seeded in a 12-well plate at density 1x10⁵ cells per well in 1 ml cultured medium. At 24-hours photosensitizer Radachlorin was diluted in 100 ml dPBS and added to experimental wells at final concentration 2 μM and incubated for 24 hours in dark. Intracellular photosensitizer uptake was controlled by confocal microscopy and Radachlorin concentrations were equal to 8.7 ± 0.7 μM for K562 cells and 7.0 ± 0.5 μM for Hela cells [5]. Cells were treated by laser irradiation with light doses 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 J/cm² and 5 J/cm² at fluency rate 20 mW/cm². Cell viability and cell cycle analysis were obtained by flow cytometry for 24 hours after irradiation (72 hours after cell seeding).

2.4. Cisplatin treatment
Cisplatin (Kemoplart, Germany) was used as a chemotherapy drug. The effect of single cisplatin treated on cell viability K562 cells and Hela cells were studied. Cells were seeded in 12-well plate at density 1x10⁵ cells/well in cultured medium. At 24 hours the cisplatin was added to experimental wells at concentrations of 1–166 μM for K562 cells and 1–25 μM for Hela cells. Cell viability and cell cycle analysis were obtained by flow cytometry for 24 hours and 48 hours after cisplatin treatment.

2.5. Combination treatment of cisplatin and photodynamic therapy
The study the combination effect of cisplatin and PDT on cell viability was used a cisplatin at concentrations of 15 μM for K562 cells and 5 μM for Hela cells. These cisplatin concentrations were not caused the cytotoxic effect, but significantly affected on the cell cycle and inhibit cell division. The experiments of combination treatment were carried out according to the following scheme: cells were seeded in a 12-well plate at 1x10⁵ cells per well; at 0 hours (24 h after seeding) cisplatin 15 μM (K562) or cisplatin 5 μM (Hela) was added to cells; at 24 h after cisplatin addition (48 h after seeding) PDT was performed with light doses 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 J/cm² at fluency rate 20 mW/cm²; at 48 hours after cisplatin addition (72h after seeding) the cell viability and the cell cycle analysis were taken.

2.6. Cell viability by flow cytometry
Cell viability and dead cells (early apoptosis and necrosis) were detected by FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, USA) and Propidium Iodid (PI, Sigma-Aldrich, USA) for 24 h after PDT and for 24 h, 48 h after cisplatin treatment. The cell viability was evaluated by a flow cytometry EPICS XL (Beckman Coulter, USA). Cell viability was presented as presents of live cell
count in experimental groups relative to live cell count in untreated control and was named cell viability (% of control). Data of cell viability were presented as mean ± SD for three independent experiments. Apoptosis of cells was presented as presents of cells with FITC-Annexin V positive FL1 signal and PI negative FL3 signal in individual samples. Data of apoptosis were presented as mean ± SD for three independent experiments.

2.7. Cell cycle analysis
The distribution of cells between phases of the cell cycle (G0/G1-, S- and G2/M-phases) was assessed by determining the relative DNA content in cells. Cells were washed three times with phosphate-buffered saline (PBS) and then 0.05 mg/ml propidium iodid (Invitrogen, USA), 0.2 mg/ml saponin (Sigma-Aldrich, USA), 0.25 mg/mL RNase (Sigma-Aldrich, USA) were added to each sample. After incubation for 30 min at room temperature, the samples were analyzed by a flow cytometry EPICS XL (Beckman Coulter, USA). More than 20,000 cells were recorded for each sample. SubG1-phase presented in percent from total cell count and was not included in cell cycle distribution. Data were analyzed with ModFit LT 4.1 software (Verity Software, Topsham, ME, USA). Cell cycle distribution were obtained for control samples, single cisplatin treatment for 24 h and 48 h, single PDT for 24 h after PDT and combination treatment of cisplatin and PDT for 48 h.

3. Results

3.1. Cisplatin treatment of K562 cells and Hela cells
The cell viability of K562 cells and Hela cells at cisplatin treatment were investigated. Figure 1 shown the relative cell viability of K562 cells from cisplatin 10-166 μM for 24 h and 48 h treatment intervals. The cisplatin concentrations which inhibited cell viability to 50% were determined and equal LD50_{K562(24h)} = 75 μM for 24 h and LD50_{K562(48h)} = 40 μM for 48 h. Figure 2 shown the relative cell viability of Hela cells from cisplatin concentration for 24 h and 48 h treatment intervals. The cisplatin concentrations inhibited cell viability to 50% were equal to LD50_{Hela(24h)} = 14 μM for 24 h and LD50_{Hela(48h)} = 10 μM for 48 h.

![Figure 1. Cisplatin-induced cytotoxicity in human erythroleukemia K562 cells. Cell viability (% to control) in K562 cells treated with cisplatin at 10-166 mM for 24 h and 48 h.](image1)

![Figure 2. Cisplatin-induced cytotoxicity in human cervical cancer HeLa cells. Cell viability (% to control) in HeLa cells treated with cisplatin at 1-25 mM for 24 h and 48 h.](image2)

The investigation of cytostatic effect of cisplatin on the distribution cell cycle phases was carried out. On the Figure 3(A, B) shown the dependences of the cell cycle phases distribution from cisplatin concentration for K562 cells for 24 h and 48 h after cisplatin addition. The significant changes of the cell cycle were observed at cisplatin concentrations from 5 to 30 μM, in which the S-phase increased up to 90% without strongly cytotoxic effect. The maximal cytostatic effect of K562 cells was observed at cisplatin concentration about 20-30 μM due to the blocking in G0/G1- and the S-phase of the cell cycle for 48 h after treatment. The blocking of cell cycle in the G0/G1-phase is observed up to 50%, the S-phase decreases to 40% at increasing cisplatin concentration more than 30 μM. This effect of
cisplatin practically inhibited the cell division and causes an increase in the sub-G1 phase up to 40%, (DNA fragmentation in apoptotic cells), which well correlates with the cell viability data. The presence of the G2/M phase at high concentration of cisplatin (60 and 80 μM) may be associated with the rapid DNA intercalation by cisplatin and cell cycle arrest at the G2/M phase that indicates the damage of intracellular DNA is difficult to repair. It is also worth noting that at low concentrations, up to 7 μM, there is an increase in the cell population up to 40 % in the G2/M phase, followed by a decrease to 0% with increasing concentrations, that also indicates the damage DNA but is possible to repair.

On the Figure 3 (C, D) shown the dependences of the cell cycle phases distribution from cisplatin concentration for Hela cells for 24 h and 48 h after cisplatin addition. The maximal cytostatic effect of Hela cells was observed at cisplatin concentration about 7-10 μM due to the blocking in G2/M and the S-phase of the cell cycle for 48 h after treatment.

**Figure 3.** The dependences of cell cycle phases (G0/G1, S, G2/M) from cisplatin concentration of K562 cells for 24 h (A) and 48 h (B) and Hela cells for 24 h (C) and 48 h (D).

### 3.2 Cell cycle analysis Combination treatment of cisplatin and PDT

Combination effect of cisplatin treatment and PDT was investigated by analysis of cell viability in experimental groups: single “PDT” (light doses 0.125-5 J/cm², Radachlorin 2 μM) and “cisplatin + PDT” (treated with cisplatin 15 μM - K562 cells and 5 μM-Hela cells; 24 h later cells were threatened with PDT at light doses 0.25-5 J/cm², Radachlorin 2 μM). Cell viability was analyzed for 48 h after cisplatin addition and 24 h after PDT as described in material and methods.

**Figure 4.** Cell viability (% to control) of K562 cells from light dose at single PDT and combination treatment of cisplatin 15 μM with PDT.

**Figure 5.** Cell viability (% to control) of Hela cells from light dose at single PDT and combination treatment of cisplatin 5 μM with PDT.

Figure 4 shows the cell viability of K562 cells from PDT light dose under single PDT and combination treatment of cisplatin 15 μM and PDT for 48 h after cisplatin addition. The light dose leading to inhibit of cell viability to 50% were determined by exponential approximation of cell viability decay. For PDT the light dose was LD50PDT = 1.10 ± 0.02 J/cm², for the combination treatment LD50comb = 0.20 ± 0.04 J/cm². Figure 5 shows the cell viability of Hela cells from light dose under single PDT and combination treatment of cisplatin 5 μM and PDT for 48 h after cisplatin addition...
addition. The irradiation dose leading to cell viability 50% were determined. For PDT, the light dose is \(LD_{50_{\text{PDT}}} = 1.06 \pm 0.04 \text{ J/cm}^2\), for the combination treatment \(LD_{50_{\text{comb}}} = 0.25 \pm 0.05 \text{ J/cm}^2\).

Based on the obtained data was calculated combination index (CI) using the formula: \(CI = \frac{D_1}{D_{50(1)}} + \frac{D_2}{D_{50(2)}} + \frac{D_1 \times D_2}{D_{50(1)} \times D_{50(2)}}\), where 1 and 2 are the values of the cisplatin concentration and PDT light dose giving a 50% of cell viability in combination “cisplatin + PDT” treatment, and \(D_{50(1)}\) and \(D_{50(2)}\) are the values of the actions required to achieve 50% of cell viability for “single cisplatin” or “single PDT” when these treatment are applied separately (CI < 1, CI = 1 and CI > 1 show, respectively, synergism, additive effect or antagonism of the considered influences) [4,6]. The combination index was calculated and in the case of K562 cells - CI = 0.62, in the case of HeLa cells - CI = 0.87. Thus, we can talk about the synergistic effect of cisplatin and PDT under the conditions of the experiments. This data was demonstrated the presence of a significant synergistic effect with the combination treatment of cisplatin and photodynamic therapy on K562 and HeLa cells.

**Figure 6.** Effect of combination treatment cisplatin and PDT in human erythroleukemia K562 cells (A) and human cervical cancer Hela cells (B). Cell viability and % value of apoptotic cells value were obtained for 48h after treatment.

Figure 6 demonstrated cell viability in % relative to untreted control and dead cell fraction in early apoptosis (Annexin V+/PI-, %) for experimental groups: control, single cisplatin treatment, single PDT, combination treatment of cisplatin and PDT. It has been shown (Figure 6A) that the combination treatment of cisplatin 15 μM and PDT with a light dose 0.5 J/cm² on K562 cell line leads to a decreased cell viability to 29.2% and increased apoptosis percentage greater than 38.6 %, which significantly exceeded the single treatment and indicated the presence of a synergistic effect. The same study for Hela cells (Figure 6B) demonstrated that the combination effect of cisplatin 5 μM and PDT with a light dose 0.5 J/cm² which lead to decreased cell viability to 40.1% and increased apoptosis percentage up to 24.6%, which also demonstrated a synergistic effect.

### 3.3 Cell cycle distribution at combination treatment

On the Table 1 demonstrated the distribution between phases of the cell cycle at control samples, single cisplatin treatment, single PDT and combination treatment of cisplatin and PDT for K562 cells. The effect of cisplatin at 15 μM leads to blocking of the cell cycle in the S-phase. Single PDT does not lead to strong changes in the cell cycle, however, the proliferative index (G2/M+S)/(G0/G1) and G2/M phase were increased for survived cells. The percentages sub-G1 phase were increased from 1.0% to 9.6%. The combination treatment of cisplatin and PDT was lead to the accumulation of cells in the S-phase cell cycle from 93.3% to 98.9 % and a strong decrease in the G2/M-phase from 6.7% to 1.1% compere to single cisplatin treatment. The cell fraction in the sub-G1 phase for combination treatment was 21.2% (1.7% - single cisplatin; 9.6% - single PDT). The sub-G1 fraction demonstrated the percentage of cells with DNA fragmentation which occurs at later phase of apoptosis. This values have a good correlation with present apoptosis for combination treatment 38.6% (4.7% - single cisplatin; 12.1% - single PDT) obtained by flow cytometry using the Annexin V-FITC/PI test.
Table 1. Cell cycle distribution of K562 cells and Hela cells at combination treatment for 48 h.

| Cell cycle phase | K562 | Hela |
|------------------|------|------|
|                  | Control | Cisplatin | PDT | Cisplatin | Control | Cisplatin | PDT | Cisplatin |
|                  | 15 μM | 0.5 J/cm² | 15 μM | 0.5 J/cm² | 5 μM | 0.5 J/cm² | 5 μM | 0.5 J/cm² |
| G0/G1, %         | 47.1  | 28.8  | 65.4   | 6.9  | 26.0  | 15.0  | 88.6  | 1.7 |
| S, %             | 46.0  | 45.2  | 98.9   | 9.8  | 1.1  | 21.2  | 8.6  | 2.0 |
| Sub G1, %        | 1.0   | 9.6   | 2.0    | 6.9  | 6.7   | 6.9   | 12.7 |

On the table 1 demonstrated the cell cycle distribution for Hela cells. The effect of cisplatin at 5 μM leads to blocking of the cell cycle in the G2/M-phase. Single PDT does not lead to strong changes in the cell cycle compared to control cells, only increasing the sub-G1 phase was observed from 0.0% (control) to 6.9% (PDT). The combination treatment of cisplatin and PDT lead to the accumulation of cells in the S-phase cell cycle from 9.8% to 25.9% and a decrease in the G2/M-phase from 88.6% to 71.2% compared to single cisplatin treatment. The cell fraction in the sub-G1 phase (DNA fragmentation) for combination treatment was 12.7% (2.0% - single cisplatin; 6.9% - single PDT) and have a good correlation with percentage of apoptosis at combination treatment 38.6% (1.7% - single cisplatin; 12.1% - single PDT) obtained by flow cytometry using the Annexin V-FITC/PI test.

4. Conclusion

In this work, new data were obtained on the cell viability and the cell cycle distribution of K562 and Hela cells under the combination treatment of cisplatin and PDT. The obtained data were demonstrated a significant synergistic effect in the combination treatment of cisplatin and photodynamic therapy with CI=0.62 for K562 cells (cisplatin-resistant cell line) and CI=0.87 for Hela cells (cisplatin-sensitive cell line). It can be assumed that the demonstrated effect may be associated with a photodynamic effect on the mitochondria of cells [7], leading to membrane disruption, release of cytochrome C into the cytoplasm, followed by activation of caspase-3, which triggers the apoptosis cycle on the one hand, and on the other, with an increase in expression of the Fas/Fas ligand, which also activates the apoptosis mechanism, when exposed to cisplatin [8]. It was found that PDT with Radachlorin in combination with cisplatin treatment significantly increased apoptotic cell death up to 40% for K562 cells and up to 25% for Hela cells at low cisplatin concentration. PDT did not significantly affect the cell cycle distribution during treatment with low-dose cisplatin, but increases cisplatin-cytotoxic effect on proliferating cancer cells. Further investigation of the intracellular mechanisms under the combination treatment of cisplatin and PDT will possibly reveal the reasons for the synergistic effect and find more effective treatment regimens for cisplatin.

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