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Photosensitizer chlorophyllin in the treatment of oncopathologies †

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Abstract: In this article we report the antitumor activity of the derivative chlorophyllin with Cu (CuChlNa) and Chlorin e6 (Ce6). Ehrlich' s ascites tumor 1X10^6 cells/ml were incubated with Ce6 and CuChlNa at various concentrations and subjected at 15 min to the photodynamic irradiation from a semiconductor laser with 32 mW output power at the light wavelength of 650 nm, giving an irradiation dosage of 6 J/cm^2. Intracellular uptake of CuChlNa and Ce6, phototoxicity, apoptosis and necrosis of cells after incubation at 9 days was studied. Were shown, that CuChlNa is able to more intense absorption at therapeutically relevant wavelengths. After 6-days exposure to CuChlNa more than 77±6.1% of the cells apoptoses were detected, whereas in the same period after contact with Ce6 were indicated predominant cell necrosis. The therapeutic efficacy of photosensitizers has been shown in vivo by demonstrating the high survival rate of mice treated with CuChlNa and PDT.

Keywords: photosensitizer; chlorophyllin; Chlorin e6; cytotoxicity, intracellular localization; Ehrlich' s ascites tumor; photodynamic therapy; oncology

1. Introduction

Traditional cancer treatment includes surgery, radiation- and chemotherapy and other treatments. Such methods of therapy have serious side effects and patients undergoing these procedures acquire various somatic pathologies as a rule. Currently, the search for alternative treatment regimens that can provide a cure with minimal or no side effects is relevant. Photodynamic therapy (PDT) is a modern non-invasive form of therapy used in the treatment of non-cancer diseases, as well as cancers of various types and locations. This method is based on the local or systemic application of light-sensitive compounds photosensitizers (PS), which accumulate in pathological tissues. Upon absorption of the appropriate wavelength of light photosensitizer molecules initiate the conversion of oxygen molecules which leads to selective destruction through apoptosis, necrosis and autophagy of tumor cancer cells [1].

PS obtained naturally or artificially contain a chromophore is a set of conjugated unsaturated bonds that absorb energy of a certain wavelength of the visible spectrum with a high molecular absorption coefficient. More than 400 compounds have been recognized as possible candidates for using as PS nowdays [2]. Various natural compounds have been identified that have a photosensitizing potential and are safer than synthetic compounds. Many articles are devoted to the mechanisms of PDT and the biological effect of photosensitizers, however, limited attention is paid to the phototoxic activity of phytocompounds.

Thus, the phototoxicity of hematoporphyrin, namely its derivative photofrin (HpD), has been proven in breast and ovarian cancer. High efficiency has been shown in the treatment of malignant skin lesions resistant to other types of anticancer therapy with detailed description of the mechanism of cytotoxic action [3]. HpD is a mixture of nine components and enhances the light sensitivity of the skin with weak absorption at the «optical window» [4]. It has been shown that the active substances of the most effective second generation photosensitizers are insoluble in an aqueous medium and their using as a therapeutic agent is associated with the applying of special pharmacological forms. These substances include various chemical modifications of Chlorin 6 (Chl E6) associated with the esterification of side carboxyl groups.

Chlorophyll derivatives have become promising compounds in the development of new photosensitizing drugs for PDT in order to overcome the disadvantages of HpD [5]. Chlorophyllin belongs to the family of chlorophyll derivatives and is extracted from cyanobacteria and chloroplasts in algae and plants. Recently, chlorophyllin has attracted the attention of researchers because of its optical properties (600–670 nm), easy solubility in aqueous solutions, ease and low cost of the extraction process in comparison with synthetic PSs. It exhibits slight toxicity and is rapidly eliminated from the body [6]. Chlorophyll derivatives have several advantages, such as: relatively deep penetration of light into tissues due to absorption of light with a longer wavelength, selective accumulation in the target tissue and minimal side effect [7]. One of the promising directions for the creation of such theranostics is the synthesis of conjugates, for example, natural chlorophyllins with additional fragments capable of firmly incorporating ions of various metals [8].

Herein, we report the antitumor activity of the derivative chlorophyllin with Cu (CuChlNa) and Chlorin e6 (Ce6). Photophysical characteristics of CuChlNa and Ce6 were measured, and its cytotoxicity, intracellular localization, efficiency of photodynamic therapy (PDT), cytological analysis were investigated using Ehrlich' s ascites tumor (EAT) in mouse.

2. Materials and methods

2.1. Photosensitizer characterization

Chlorin e6 (Ce6; Frontier Scientific Inc., USA) and sodium copper chlorophyllin salt (CuChlNa, Sigma-Aldrich, USA) was dissolved in various solvents: water, PBS (pH 7.4), RPMI medium and bovine serum albumin (Gibco, USA) and the absorption spectra were mesured in the 400–800 nm range. The optical feature of the dyes and fluorescence spectra was determined by obtaining the molecular absorption profile by using spectrofluorometers Synergy H1 (BioTek, USA) and RF-5301 PC (Shimadzu, Japan). Slits were kept narrow to 1 nm in excitation and 1 or 2 nm in emission. Right angle detection was used. All the measurements were carried out at room temperature in quartz cuvettes with path length of 1 cm.

2.2. Ehrlich ascitic tumor

Ehrlich ascitic tumor (EAT, breast cancer, 1905; ascites variant) derived from a spontaneous murine mammary adenocarcinoma was maintained in the ascitic form by passages in syngenic on mouse (hybrids F1 CBA + C57|B6, males weighing 20–21 g) by weekly transplantation of $5x10^6$ tumor cells (ip). Ethical clearance Protocol of this study for the using of mice as animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAME BBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (225/320-IAME BBC/IBSc), Pacific State Medical University (protocol N 21 21.10.2020). The ascitic fluid was removed by opening the belly and collecting all the fluid with a sterile syringe. Ascitic tumor cell counts were carried out in TC20™ automated cell counter (Bio-Rad, USA) using the Trypan blue dye exclusion method. The animals used for the experiment received 200 μL of a suspension containing 5×10^6 tumor cells. These cells were cultured with RPMI medium containing 10% fetal bovine serum (Gibco, USA), 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all

from Thermo Scientific, USA), in a 37°C, 5% CO_2 humidified incubator. Cells in the logarithmic growth phase were used for the experiments.

2.3. PDT procedure

EAT 1×10^6 cells/ml were incubated with Ce6 and CuChlNa at various concentrations (1.25, 2.5, 3.125, 5.0, 6.25, 12.5, 25.0 and 50 μg/ml), and subjected at 15 min to the photodynamic irradiation from a semiconductor laser (PC «Light and Life», Tomsk, Russia) with 32 mW output power at the light wavelength of 650 nm, giving an irradiation dosage of 6 J/cm^2. After incubation of cells at 1 h RPMI medium containing PSs was removed and cells were washed. Then, the cells were further incubation for 1, 2, 3, 4, 5 and 8 days. For intracellular localization of Ce6 and CuChlNa the cells was analyzed on a MACSQuant 6.0 flow cytometer (Miltenyi Biotec, Germany) with determination of the number of PSs-positive cells in a two-parameter FL-1/FL-2 histogram using the Kaluza Analysis 2.1 software (Beckman Coulter, USA) and also determined the percentage of such cells by using fluorescence microscope Zeiss Axio Observer (Carl Zeiss, Germany).

After ip injected $2.5x10^6$ cells/mL intraperitoneal, two groups of mice (20 mice per group) were treated with Ce6 and CuChlNa in a dose 7.5 μg/kg and irradiated with 350 mW/cm^3 lasers for 10 min (120J/cm^2 , λ = 620nm), the negative control group (10 mice) were received saline solution and 2% Tween ip in the same final volume and positive control group (10 mice) were irradiated to the peritoneal area under the above conditions. The body weights of the mice were measured daily until their death. The only animals to survive more than 30 days were sacrificed 30 days after the beginning of the treatment.

2.4. In vitro phototoxicity assay

EAT cells ($1X10^6$ cells/well) were seeded in 96-well plates in 200 mL RPMI medium with various concentrations of Ce6 and CuChlNa and after irradiation allowed to attach for 1 h. RPMI medium containing PSs was removed and cells were washed. Then the cells were further incubation for 1, 3, 5, 7, and 9 days. The resulting dark or photoxic effects on cells were evaluated via 3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-terazolium bromide (MTT, 5 mg/mL) colorimetric assay. At the end of the treatment period 20 μL of MTT solution was added to each well. After incubation for 4 h at 37 °C added 100 μL of solubilizing buffer (10% sodium dodecyl sulfates dissolved in 0.01 N HCl). The formazan dye produced by viable cells can be quantified by measuring the absorbance at OD = 570 nm using Multiskan Sky Microplate Spectrophotometer (TermoFisher, USA). The percentage of the viable cells was calculated using the following formula: (%) = [100 × (sample abs)/(control abs)]. Data presented are averaged results of triplicate experiments.

2.5. Flow cytometric analysis of apoptosis

After incubation with Ce6 and CuChlNa cells from each treatment condition were washed once in PBS and resuspended in Annexin binding buffer and stained with FITC-conjugated Annexin V and PI as per the manufacturer's instructions (BioLeged, USA). After staining cells were washed with Annexin binding buffer, resuspended in a fixing solution of PBS containing 4% paraformaldehyde and stored at 4 °C in the dark. Annexin-FITC fluorescence (FL1) was collected through a 525/40 band pass filter, whereas PI fluorescence (FL11) was collected through a 610/20 band pass filter. Data acquisition (2×10^4 events per sample) was performed using the MACSQuantify Software (Miltenyi Biotec, Germany).

2.6. Statistical analyses

For purposes of «*in vivo*» assessment, the number of animals was limited to avoid unnecessary using of living subjects. Data are given as means ± SD and were analyzed statistically by the student's t-test (unpaired, two-tailed) using the analysis software Statistica Advanced 6.1 (StatSoft, USA) the level of significance was set at p <0.05.

3. Result and discussion

2.1. UVevisible absorption and fluorescence spectra

Due to the four conjugated pyrrole rings, porphins possess high rigidness and good coplanarity, exhibiting strong UVevisible absorption and fluorescence at room temperature [9]. The absorption spectra of Ce6 and CuChlNa with a concentration of 25 ug/ml in the range of 250-750 nm were obtained (Figure 1A (**a**)). The following absorption maxima were determined: 402, 503 and 652 nm, which is characteristic for PSs of the porphyrin class. The identity of the absorption maximum in the spectra of Ce6 and CuChlNa were shown. However, CuChlNa is able to more intense absorption at therapeutically relevant wavelengths. This provides the possibilities of CuChlNa as near infrared (NIR) fluorescence imaging agents. The absorption spectrum of CuChlNa in various solvents is shown in the Figure B (**b**). Maximum absorption noted when used PBS as a solvent.

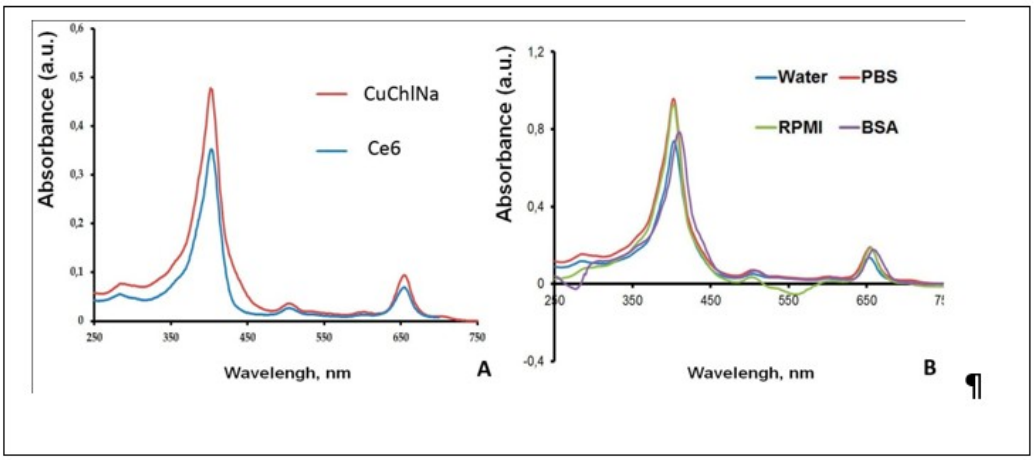

Figure 1. (A) The absorption spectra of Ce6 and CuChlNa (both 1 mg/m) (**a**) and (B) CuChlNa in various solvents (**b**).

2.2. In vitro phototoxicity assay

Figure 2 represents the viability of EAT cells after contact with PSs in different concentration. It was found that viability levels of cells incubated with CuChlNa in the MTT assay were strikingly higher than the cells after contact with Ce6. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells. Surprisingly, although the cells were totally dead at especially higher concentrations, the MTT assay still produced extremely high viability values. These viability values were false and this finding was confirmed using both phase microscope evaluation and fluorescence imaging that confirmed cell death (Figures 2 B (**b**), C (**c**)). It

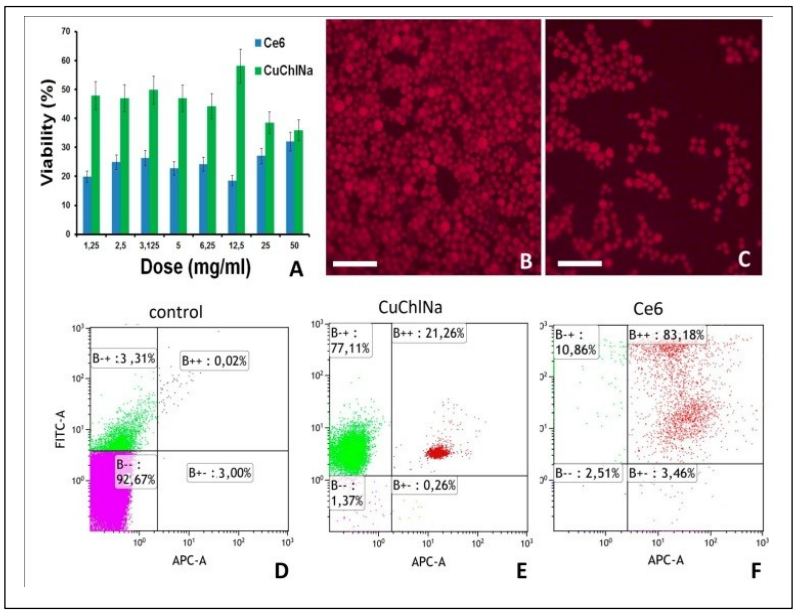

Figure 2. (A) EAT cells treated with Ce6 and CuChlNa (1.25 μg/ml) after incubated for 6 days (**a**). (B, C). The cell survival rates of EAT cells were assessed by the MTT assay (**b, c**); subcellular localization (red) of Ce6 and CuChlNa in EAT were detected with fluorescence microscope. (D-F) Scale bar, 25 μm (**d-f**). Cell apoptosis and necrosis of EAT cell was detected by the Annexin-V/PI staining and flow cytometry.

is clearly shown that cell densities decreased when cells were treated with 1.25 μg/mL CuChlNa. However, at the same concentration, the MTT assay present low cytotoxic activity. The results of a flow cytometric analysis of apoptosis were more compatible with the assay results of cell microscopy, which the reliably showed these cell deaths. Experiments were conducted to determine whether PSs and annexin V could be used simultaneously to detect PDT exposure. Prior to Ce6 and CuChlNa exposure, dot plots of staining by FITC-conjugated annexin V and PI showed that only 3.5 ± 0.2% and 3.0 ± 0.4% cells were determined, respectively (Figure 2D (**d**)). After 6-days exposure to CuChlNa more than 77 ± 6.1% of the cells were Annexin V and 21 ± 1.6% PI positive (Figure 2E (**e**)). Whereas in the same period after contact with Ce6 (Figure 2F(**f**)), the percent both Annexin V and PI positive showed that cellular population is Annexin V^+PI^+ cells, which indicated predominant cell necrosis.

2.3. In vivo therapeutic efficacy of PSs

The mice were injected EAT $2.5x10^6$ cells/mL intraperitoneal and the mice in PDT group at 3 days post-inoculation were treated with PSs, and then irradiated with 350 mW/cm^3 lasers for 10 min (120 J/cm^2 , λ=620 nm) of the tumor area. The control mice were not subjected to PSs or light. All the tumors were measured in three dimensions every other day for 14 days, the results of the tumor volume growth curves were provided on Figure 3A (**a**). The volume of tumor in control, irradiation and treated CuChlNa without irradiation groups were larger than that in PDT group at the same time. Therefore, the light irradiation group has little influence on the growth of tumor and there is no significant statistical difference among the light irradiation group, and treated CuChlNa without irradiation and the control group. After 14 days, the mean size of tumor only reached 126 mm in PDT CuChlNa group, compared to that of 785 mm in the control group. In the light irradiation has little influence on survival of animals, and there is no significant statistical difference among the light irradiation group and the treated CuChlNa without irradiation and the control group (Figure 3B (**b**)). The survival of CuChlNa and PDT treated mice was longer at comparison with another groups.

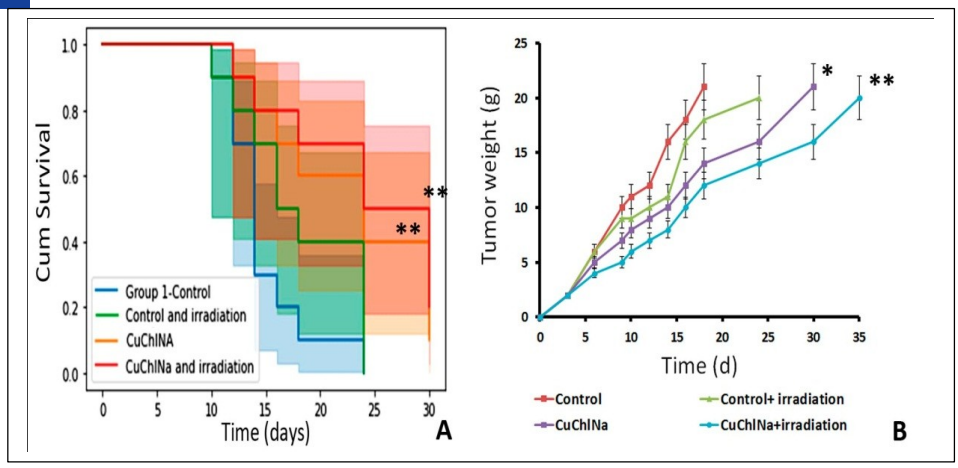

Figure 3. (A) Kaplan-Meier survival curve of mice treated with saline, saline plus Tween and CuChlNa (7.25 μg/ml) (N = 10 in each group) (**a**). (B) Effect of Ce6 and CuChlNa (7.25 μg/ml) on tumor weight in mice. *p <0.05 and ** p <0.01 compared with control group.

3. Conclusion

As discussed in this article, natural PS isolated from plants can be seen as a green approach to PDT in cancer therapy. Low systemic cytotoxicity in relation to normal cells and selective action against malignant cells is one of the main advantages of natural PS for PDT. PDT in combination with other natural compounds fights malignant cells using three main components: PS, light and oxygen. All these components promote photochemical reactions that lead to the production of ROS in malignant cells and, as a consequence, to cell death due to the induction of apoptosis and / or necrosis. In addition, since natural compounds are commonly found ubiquitously, they may be more readily available compared to synthetic chemotherapeutic agents. These strategies increase the accumulation of PS in the tumor microenvironment and even inside tumor target cells. We believe that there may be many unknown natural agents with different phototoxic properties. It is sometimes criticized that it is unlikely that evolution would choose to express the very potent PS in plants, as they would then self-destruct in sunlight, on which all plants depend for their growth and survival. The answer to this criticism is that, along with developing the light-sensitive compound itself, plants can also develop a self-defense mechanism to ensure that the plant cells themselves are protected from PDT destruction, while predators (such as unwanted grazing cattle, etc.) still suffer from phototoxicity reactions). Researchers are now able to discover these photoactive plants in nature and compare the effects with more traditional PSs. In addition, loading conventional PSs or natural phototoxic agents into nanostructures can help achieve better cancer treatment with PDT.

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