Enhancement of photodynamic tumor therapy effectiveness by electroporation in vitro

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***Key words**: electroporation; photodynamic tumor therapy; chlorin e6; aluminum phthalocyanine tetrasulfonate.*

***Summary.** The aim of our study was to determine if electroporation could improve the efficacy of photodynamic tumor therapy. A disadvantage of photodynamic therapy is a slow and in some cases insufficient accumulation of photosensitizer in tumor tissue, which could restrict the achievement of an efficient dose. Under the action of electric pulses, cells undergo membrane electroporation, which results in an increased permeability to various exogenous molecules. In this study, murine hepatoma MH22A cells were exposed to light in vitro in the presence of a photosensitizer, either chlorin e6 or aluminum phthalocyanine tetrasulfonate, following electroporation. Accumulation of the photosensitizers was registered by fluorescence microscopy. Cell viability was determined by the MTT assay. Our results demonstrate that electroporation improves an access of chlorin e6 and aluminum phthalocyanine tetrasulfonate to MH22A cells. Electroporation in combination with photosensitization significantly reduces viability of the treated cells even at low doses of photosensitizers.*

Introduction

When a cell is exposed to short and strong external electric pulses, the anode-facing side becomes hyperpolarized, and the cathode-facing side becomes depolarized, and cell membrane undergoes a remodeling process characterized by the occurrence of transient permeation structures – "electropores" (1, 2). In case if the strength of the external electric field is properly chosen, it is possible to permeabilize most of the pulsed cells while preserving the viability of the cell population. Electroporation (EP) is under scientific interest due to insight it affords into membrane behavior and its potential application in biology, biotechnology, and medicine (3–6).

Many chemotherapeutic drugs used in cancer therapy have limited access to the tumor cells. New drug formulations or specific carriers are therefore needed to target the drug into the tumor cell. Membrane electroporation offers an approach for enhanced drug delivery into the cells and better antitumor effectiveness. This new approach, termed electrochemotherapy (ECT), was introduced by Okino and Mir (7). Indeed, it has been shown that in vitro cytotoxicity of some chemotherapeutic drugs can be potentiated several hundred-fold by exposing cells to short intense electric pulses (8, 9). It has been proposed in many instances that ECT can be very efficient even with highly reduced doses of chemotherapeutic drug, which in turn almost completely eliminate adverse side effects of the drug. For example, antitumor effectiveness of bleomycin can be greatly potentiated with electric pulses, inducing partial and complete responses of the tumors (10, 11). Indeed, ECT with bleomycin requires such low concentration of bleomycin that the treatment itself as a consequence does not induce any side effects. Bleomycin at these concentrations is ineffective without application of electric pulses.

New possibilities for expanding the application of this phenomenon are explored. Recently a new cancer treatment method, photodynamic tumor therapy (PDT), has been introduced into oncology clinics (12, 13). It is based on the phenomenon of photosensitization: a special chemical compound, photosensitizer, is excited by visible light of an appropriate wavelength, and its ground singlet state is transformed to an excited singlet state. It then undergoes intersystem crossing to a longer-lived excited triplet state. One of the few chemical species present in tissue with a ground triplet state is molecular oxygen. When the photosensitizer and an oxygen molecule are in proximity, an energy transfer can take place that allows the photosensitizer to relax to its ground singlet state and create an excited singlet state of oxygen molecule. Singlet oxygen is a very aggressive chemical compound that very rapidly reacts with any nearby biomolecule. Ultimately, these destructive reactions induce

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oxidative damage to the cellular organelles. This damage triggers a combination of molecular events, which result in cancer cell death. Adjacent normal tissue does not accumulate the photosensitizer and does not suffer damage. PDT can be used as individual treatment method and as adjuvant therapy after surgical tumor resection and as palliative treatment method.

The amount of photosensitizer and its localization within the tumor tissues can affect the efficacy of PDT. One of the main disadvantages of PDT is the slow uptake of photosensitizers by tumor tissues. To enhance treatment efficiency, PDT has been successfully combined with hyperthermia, ionizing radiation, and anticancer drugs (14–16).

In this study, we have raised a hypothesis that electric impulses can improve access of photosensitizers to tumor cells and thus enhance selective accumulation of photosensitizers in cancer tissue. In that way, electroporation could help: a) to reduce accumulation duration of a photosensitizer, b) to minimize the effective dose of the photodrug, c) to reduce the phototoxic effect of photosensitized damage on the organism. Few recent studies performed in vitro and in vivo have shown that electroporation could increase the effectiveness of PDT (17–20).

We have applied two photosensitizers in the experiments. Chlorin e6 (C e6) is a second-generation photosensitizer of great potential in PDT. It has a strong absorption in a transmission window of tissues in the red spectrum of light; its coefficient of molar extinction of absorption maximum is 53 000 at 664 nm. Aluminum phthalocyanine tetrasulfonate (AlPcS4) belongs to phthalocyanines, which have even longer maximum absorption wavelengths (approximately 690 nm) as compared to porphyrins. The incorporated metal ion (aluminum) enhances triplet yield and the lifetime of a photosensitizer; this is important for increasing the activity of photosensitization.

Our experiments were carried out in vitro in murine hepatoma cell line MH22A.

Materials and methods

Cell culture

Tissue culture products were obtained from Sigma. Fetal calf serum (FCS) was from Gibco BRL, Australia. Culture flasks and Petri dishes were Primaria™ from BD Falcon.

MH22A cells were obtained from the Institute of Cytology, Sankt-Petersburg, Russian Federation. Cells were cultured in monolayer in 25 cm^2 Falcon flasks in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS, 100 IU mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, and 2 mM glutamine at 37°C in 5% CO_2 atmosphere. Cells were subcultured by dispersal with 0.025% trypsin in 0.02% ethylenediaminetetraacetate (EDTA) and replated at 1:3 dilution twice a week.

Photosensitizers

C e6 was obtained from the Institute of Physics, Byelorussian Academy of Science, Minsk, Byelorussia. AlPcS4 was purchased from Porphyrin Products, USA. The stock solution of 1 mg/mL of C e6 was prepared in ethanol, and the stock solution of 5 mg/mL of AlPcS4 was prepared in Dulbecco's phosphate-buffered saline (DPBS). Stock solutions were stored at –20˚C in the dark. All experiments in vitro were performed diluting the stock solutions to get the appropriate concentration: 0.3, 1, and 3 μg/mL of C e6 and 1, 10, and 50 μg/mL of AlPcS4.

Electroporation

For manipulation with the MH22A cell line, a high intensity impulse generator constructed at Vytautas Magnus University was applied. Distance between the stainless steel electrodes was 2 mm. Generator provided electric impulses at an intensity of 1200 V/cm, 0.1-ms duration, 1-Hz frequency, and repetition of 8 impulses.

Fluorescence microscopy

Cells were visualized with an Olympus AX70 fluorescence microscope equipped with ×60 NA 1.25 oil immersion lens. The images were recorded with a CCD camera Orca (Hamamatsu) and analyzed with Micro-Image v. 4.0 software (Media Cybernetics). The differential interference contrast (DIC) images were registered using an IR filter. A specially produced filter cube (400–410 nm for excitation and beyond 590 nm for emission) was used for visualization of photosensitizers.

PDT procedure

The cells were exposed to light from LED array UNIMELA-1 (λ=660±20 nm), (Laser Research Centre, VU, Lithuania), the fluence rate at the level of the cells was 7.6 W/m^2, as measured using an irradiation power meter IMO (Russian Federation). After PDT, the cells were incubated in the dark for 24 h, and the cell viability was assessed.

Scheme of combined treatment of PDT with electroporation

There were seven groups in each experiment: *control*, untreated and sham exposed cells; e*lect-*

roporation, 1200 V/cm, 0.1 ms, 1 Hz, 8 electric impulses; *photosensitizers,* C e6 (0.3, 1, and 3 μg/mL) or AlPcS4 (1, 10, and 50 μg/mL); *light exposure,* intensity of 7.6 W/m^2 at 1 min illumination time for C e6 and 20 minutes for AlPcS4; *combination of electroporation and light illumination*; *PDT*, after application of photosensitizers, cells were illuminated by light; *PDT and electroporation combined action*, after adding photosensitizers, cells were electroporated and exposed to light illumination. The scheme of combination of PDT and electroporation is shown in Fig. 1.

Cell viability assay

For cell viability assessment, the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was used. The incubation medium was replaced with a volume of 1 mL of MTT solution at 0.2 mg/mL in DPBS, and the cells were incubated for 2 h at 37˚C. After incubation, the MTT solution was discarded, and 2 mL of 2-propanol was added. The extraction process was performed during 20 min at room temperature. The optical density (OD) was then recorded at 570 nm using a Perkin Elmer Lambda20 spectrophotometer. The mean OD_{570} of the control cells exposed to test-compound-free culture medium was set to represent 100% of viability, and the results were expressed as percentage of these controls (21).

Results

To evaluate the influence of electroporation on the access of photosensitizers to tumor cells, the murine hepatoma MH22A cells were incubated with the photosensitizers AlPcS4 and C e6 and exposed to electric pulses. With the help of a fluorescence microscope, the entrance of photosensitizers before and after electroporation was visualized.

A fluorescence microscopy revealed an insignificant amount of C e6 incorporated in plasma membrane of cells that were not exposed to electric pulses (Fig. 2, C e6). No fluorescence of AlPcS4 was detected inside the cells (Fig. 2, AlPcS4). For accumulation of these photosensitizers inside the cells, a long period of incubation (24 h) is needed (data not shown). Electroporation of the cells was applied straight after adding the photosensitizers. Evident fluorescence of both photosensitizers was detected inside the cells after

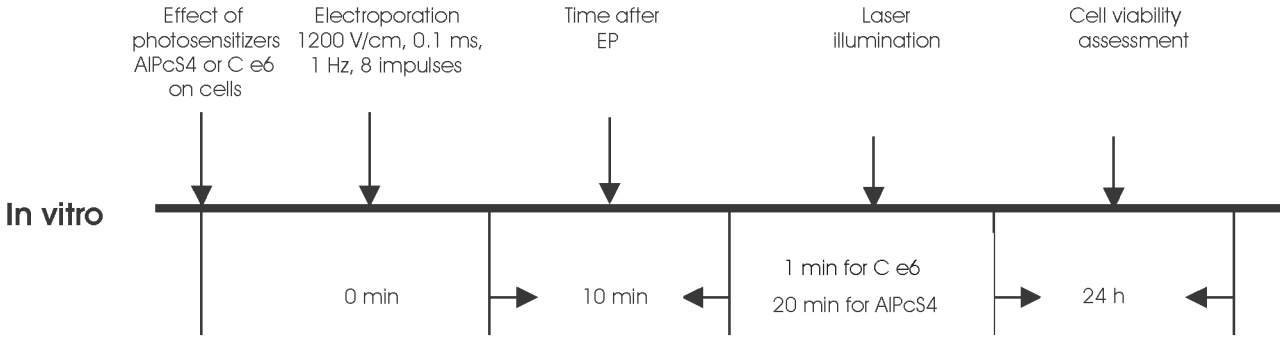

Fig. 1. **Scheme of combination of photodynamic tumor therapy and electroporation in vitro**

C e6 or AlPcS4 was added to MH22A cells, and cells were immediately exposed to eight electric pulses at an intensity of 1200 V/cm, 0.1-ms duration, and 1-Hz frequency. Cells were let reseal membrane after electroporation for 10 min and exposed to light for 1 min in the case of C e6 and 20 min in the case of AlPcS4. After following incubation for 24 h in the dark, the cell viability was assessed. C e6 – chlorin e6; AlPcS4 – aluminum phthalocyanine tetrasulfonate.

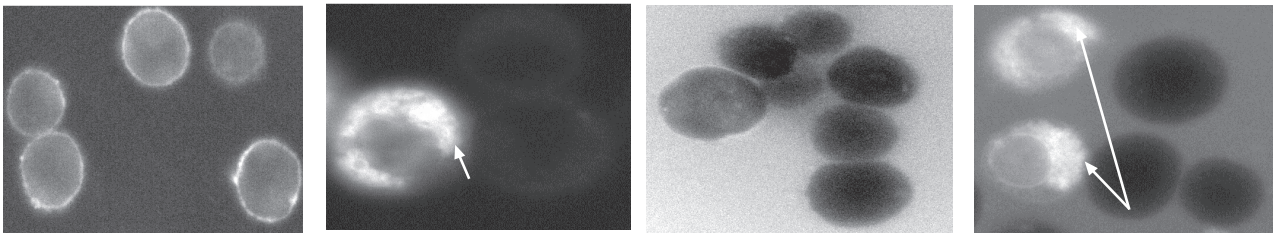

Fig. 2. **Fluorescence of AlPcS4 and C e6 in MH22A cells before and after electroporation**

Cells were affected with 10 μg/mL of C e6 or 50 μg/mL of AlPcS4, and at zero time electroporation was performed at an intensity of 1200 V/cm, 0.1-ms duration, 1-Hz frequency, and repetition of 8 impulses. C e6 – chlorin e6; AlPcS4 – aluminum phthalocyanine tetrasulfonate.

application of electric impulses (Fig. 2, C e6+EP and AlPcS4+EP). Thus, electroporation effectively introduced photosensitizers into cell cytoplasm without prolonged incubation. From these results, we have made a presumption that electroporation determined a rapid and more effective accumulation of photosensitizers inside the cells. Further experiments on cells were performed with the aim to evaluate the influence of electroporation on the effectiveness of PDT.

The impact of electroporation on the viability of photosensitized cells was evaluated. Fig. 3 reflects the influence of electroporation on PDT effectiveness at different doses of the photosensitizer C e6. Changes in MH22A cell viability in the control group (untreated cells) (group 1), treated by photosensitizer C e6 at the doses of 0.3, 1, or 3 μg/mL (group 2), were statistically insignificant. Viability of the cells affected by electric impulses or exposed to laser light was influenced slightly (data not shown). It shows that neither electric pulses nor light illumination has any effect on cell viability at the intensities used in the study. The viability of the photosensitized cells (PDT, group 3) was significantly lower comparing to the control group. A statistically significant (P<0.04) suppression of cells treated by the combination of PDT and electroporation (group 4) was observed. Electroporation was performed at zero time immediately after adding C e6, and 10 minutes later (to let the cell membrane recover), the cells were exposed to light for 1 min. The viability of the cells was suppressed twice more than that in the group 3 (just PDT). For comparison, the viability of cells in the PDT group was 85, 80, and 68% of control at 0.3, 1, or 3 μg/mL of C e6, respectively, while in the group 4, a combination of PDT and electroporation, the viability was 43, 46, and 33%, respectively. In comparison to the other groups, it was a significant suppression of cancer cell viability.

In order to check out the influence of electroporation, we have performed the same experiments with another photosensitizer, AlPcS4.

Fig. 4 shows that the electroporation-increased cellular uptake of AlPcS4 has a significant influence on viability of the photosensitized cells. The application of 1, 10, or 50 μg/mL of AlPcS4 (group 2) caused a slight but significant decrease of cell viability, which was 97, 90, and 80% of control, respectively. In the PDT group (group 3), where cells were incubated with AlPcS4 for 10 min and exposed to the light illumination, the treatment resulted in cell viability of 91, 69, and 59% at doses of 1, 10, and 50 μg/mL of AlPcS4, respectively. The most significant decrease of cell viability was obtained with the combined application of PDT and EP (group 4). Electroporation was performed at zero time after adding AlPcS4 to the cells. Ten minutes later, after the cell membrane recovered, the cells were exposed to light for 20 min. The cell viability in this group decreased to 68, 53, and 13% at the respective doses of AlPcS4.

These results substantiate the suggestion that electroporation enhances the effect of PDT even at low doses of photosensitizers.

Discussion

According to the literature data, access of 5-aminolevulinic acid (ALA) to the cells can be augmented 2 times with the help of electric impulses (17), and the production of photofrin IX can be increased by almost 3 times in comparison with passive diffusion

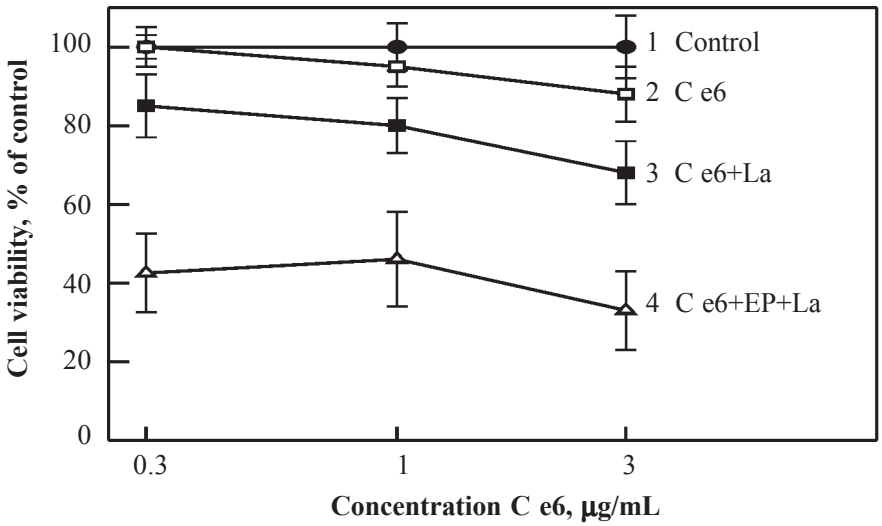

Fig. 3. **Viability of C e6-photosensitized MH22A cells following electroporation**
C e6 – chlorin e6; EP – electroporation; La – light illumination.

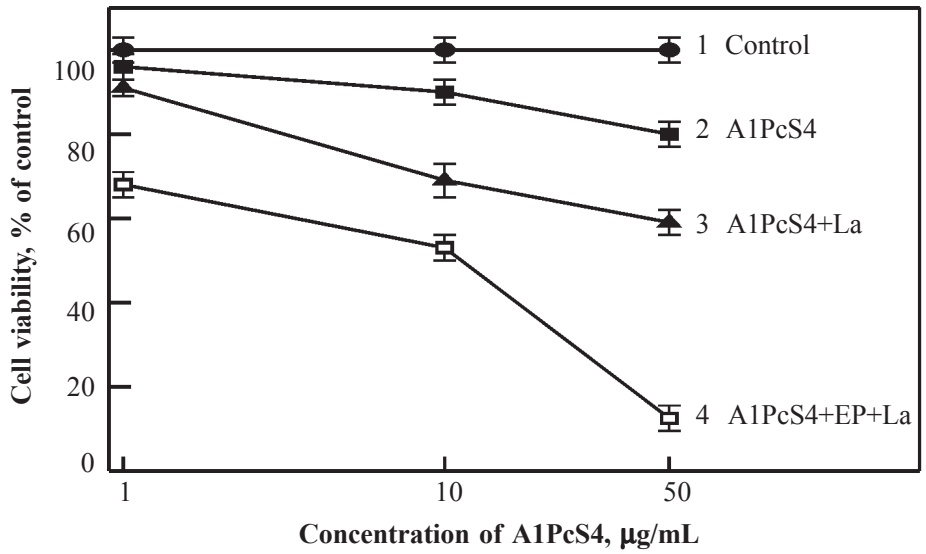

Fig. 4. **Viability of AlPcS4-photosensitized MH22A cells following electroporation**
AlPcS4 – aluminum phthalocyanine tetrasulfonate; EP – electroporation; La – light illumination.

of ALA (18). In this study, the lifetime of the pores was measured, and it was found that lifetime of electropores varied within an interval of milliseconds depending on electroporation parameters. Possibly, the increase of duration of electric pulses prolongs the lifetime of pores, and larger amount of the photosensitizer ALA can get into the cell at the same time. Lambreva et al. (2004) used electroporation to deliver thyopyronin, trypan blue, and Cibacron-dextran to U-937 lymphoma and K-562 myeloid leukemia cells and found that access of even large molecules as Cibacron-dextran can be augmented by electroporation (19).

One of the main disadvantages of PDT is the slow accumulation of photosensitizers in the tumor tissues, and concentration needed for effective antitumor effects is often insufficient. Our initial investigations showed that it was possible to improve the accumulation of photosensitizers in tumor cells by applying cell electroporation. This suggests that electroporation could be applied to improve the delivery of some photosensitizers, especially hydrophilic ones, into the tumors and consequently result in an enhancement of photodynamic tumor therapy.

In this study, we have established the dependence of the combined effect of PDT and EP on the dose of the photosensitizer. This could explain the advantage of electric impulses, i.e. to empower the application of lower doses of photosensitizers in PDT. This feature would be essential in PDT for avoiding side effects of phototoxicity applying high doses of the photosensitizers. In future, investigations of the influence of electroporation on PDT should be carried out with other photosensitizers, approved for application in clinical practice.

Investigations of electroporation influence on PDT holds out a hope of a successful application of this new method for introducing photosensitizers into cells and herewith increasing the effectiveness of PDT.

Conclusions

1. Electroporation improves an access of photosensitizers, aluminum phthalocyanine tetrasulfonate and chlorin e6, to MH22A cells.

2. Electroporation in combination with photodynamic tumor therapy significantly increases effectiveness of the latter in vitro.

Fotodinaminės navikų terapijos veiksmingumo padidinimas elektroporacijos būdu *in vitro*

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Raktažodžiai: elektroporacija, fotodinaminė navikų terapija, chlorinas e6, aliuminio ftalocianino tetrasulfonatas.

Santrauka. Tyrimo tikslas. Įvertinti elektroporacijos įtaką fotodinaminės navikų terapijos veiksmingumui. Vienas iš fotodinaminės terapijos trūkumų yra lėtas, kartais ir nepakankamas fotosensibilizatorių susikaupimas navikiniame audinyje – tai daro įtaką fotodinaminio gydymo veiksmingumui. Veikiant ląstelę elektriniais impulsais, jos membranoje susiformuoja pralaidžios struktūros, dėl to pagerėja įvairių egzogeninių molekulių patekimas į ląstelės vidų. Šio tyrimo metu pelių hepatomos ląstelės MH22A buvo paveiktos, su fotosensibilizatoriais chlorinu e6 arba aliuminio ftalocianino tetrasulfonatu arba neelektriniais impulsais, o vėliau apšvitintos lazeriu. Fotosensibilizatorių susikaupimas ląstelėse registruotas fluorescentiniu mikroskopu. Gyvybingumui įvertinti taikytas MTT metodas. Tyrimo rezultatai parodė, kad elektroporacija pagerina fotosensibilizatorių susikaupimą navikinėse ląstelėse, o dėl elektroporacijos poveikio *in vitro* fotodinaminės navikų terapijos veiksmingumas žymiai padidėja netgi taikant mažesnes fotosensibilizatorių dozes.

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