# Original Article

# The Influence of Electroporation on *in Vitro* Photodynamic Therapy of Human Breast Carcinoma Cells

(electro-photodynamic therapy / electroporation / electrochemotherapy / photodynamic therapy / breast cancer)

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**Abstract. Phototoxicity of drugs used in cancer photodynamic therapy could be augmented by increased accumulation of a photosensitizer in target cells. The intracellular delivery mode that enhances drug transportation could facilitate therapy by reducing the exposure time. Doses of the administered drug and related side effects could be lowered, whilst maintaining the same therapeutic efficiency. Electroporation supports transport of many drugs by creating electric field-induced transient nanopores in the plasma membrane. In this study, the electroporation-assisted transport of a photosensitizer was tested *in vitro* in human breast carcinoma cell lines: wild-type (MCF-7/WT) and doxorubicin-resistant (MCF-7/DOX). The efficacy of photodynamic therapy alone and in combination with electroporation was evaluated by cell viability with MTT test, using a haematoporphyrin derivative as a model. The data presented show up to 10-fold greater efficacy of the combined method, with very significantly reduced drug exposure times.**

## Introduction

Cancer of the breast is a significant health problem for women, reaching 20 % of all malignant cancers. Typical treatment involves a wide margin surgery leaving sequelae. On the other hand, systemic chemotherapy is very invasive for the patients, affecting also healthy tissue. Additionally, chemotherapy leads to primary or secondary resistance of cancer cells. Hence, investigation into other, lower-invasive therapies is anticipated. Two such therapies – electrochemotherapy and photodynamic therapy – could be considered.

Photodynamic therapy (PDT) is a low-invasive and promising anticancer strategy that involves the combination of light, a photosensitizer and oxygen. Each of these factors is not toxic in itself, but their combination triggers localized generation of cytotoxic singlet oxygen or reactive oxygen species (ROS) and tumour damage (Kessel, 2004; Plaetzer et al., 2009). Effective transport of a photosensitizer across the membrane and the intracellular accumulation of the drug are the most crucial elements in PDT. Depending on the physicochemical properties and the uptake mechanism, sensitizers can reach different intracellular concentrations and localize in different subcellular compartments.

Application of high electric field to the plasma membrane affects organization of the lipid molecules, generating transient hydrophilic electropores, which are capable of conducting various non-permeant molecules into the cell. The new pathway into the cytoplasm is non-selective and only controlled by the electric field parameters. The pores are of nanometre scale and very dynamic. Therefore, characterizing their geometry, conducting properties, and dynamic characteristics are of major interest (e.g. Koronkiewicz et al., 2002; Kotulska et al., 2004; Kotulska, 2007; Kotulska et al. 2007; Krassowska and Filev, 2007; Kanduser et al., 2008; Pucihar et al., 2008). Nevertheless, electroporation (EP) is widely used in biology and medicine. One of the most common applications of electroporation is in gene transfection into cells, where the electroporation substitutes chemical methods or viral vectors (Neumann et al., 1982). Recently, new applications of the electroporation have been developed, including electrochemotherapy (ECT) (Mir et al., 2006; Sersa, 2006; Gehl, 2008; Cemazar et al., 2008) and electro-immunogene therapy (Daud et al., 2008; Mir, 2009). Both therapies take advantage of the facilitated access into cells that are subjected to the appropriate electric fields.

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Abbreviations: CR – complete response, ECT – electrochemotherapy, EP – electroporation, EPDT – enhanced photodynamic therapy, FBS – foetal bovine serum, HpD – haematoporphyrin derivative, IR – irreversible, MTT – 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – phosphate-buffered saline, PDT – photodynamic therapy, ROS – reactive oxygen species.

In ECT, the cellular absorption of a poorly transported drug can be greatly enhanced following induction of electropores by local application of electric pulses to the tumour tissue. An important advantage of electrochemotherapy is enhanced selectivity of the treatment – the drug transportation rate is only elevated in the area of the electric field operation; unchanged elsewhere. In the case of a non-permeant cytotoxic drug, such as bleomycin that is the most frequently used drug in the electrochemotherapy of cancer, the cells exposed to electroporation exhibit 300–700-fold increased intracellular concentrations of the drug (Mir et al., 2006). Therefore, adjacent healthy tissue does not suffer the side effects of systemic chemotherapy. The efficiency of ECT with intravenously administered bleomycin can reach 53% complete response (CR) rate (Sersa, 2006). ECT has been most widely used in the therapy of melanoma (Mir et al., 2006); however, other applications, even the treatment of brain tumours (Linnert and Gehl, 2009), have been considered. There have also been applications of ECT in the treatment of breast cancer, obtaining CR of 60 % or even higher for smaller tumours (CR = 71 %, below 3 cm) (Whelan et al., 2006; Larkin et al., 2007); experiments also involved cancers with MCF-7 cell lines (Larkin et al., 2007). Based on this series of data we hypothesized that combining EP with PDT, another highly selective anti-cancer therapy, may offer greater selectivity and/or efficacy in breast cancer treatment than could be achieved without the EP component.

In the present study we investigate the therapeutic potential of combining PDT, based on haematoporphyrin derivative (HpD), with EP – termed electroporation-enhanced photodynamic therapy (EPDT). EPDT has previously been tested *in vitro* on yeast cells treated with thiopyronine (Wang et al., 1998), human histolytic lymphoma cells and human chronic myeloid leukaemia cells, using a number of different sensitizers (Pang et al., 2001; Lambreva et al., 2004; Lambreva and Berg, 2010; Traitcheva and Berg, 2010), hamster lung fibroblast cell line treated with chlorin $e_6$ (Labanauskiene et al. 2007), and human lung cancer cells treated with HpD (Saczko et al., 2010).

A positive impact for EPDT has also been reported in animal *in vivo* models (Johnson et al., 2002; Tamosiunas et al., 2005). Here we present the first study of the synergistic effect of the photodynamic effect and electroporation in human breast cancer, using wild-type breast carcinoma cells (MCF-7/WT) and doxorubicin-resistant cells (MCF-7/DOX) that are not responsive to the typical chemotherapy. As a model photosensitizer HpD was used (Saczko et al., 2009). This is one of the first photosensitizers used in PDT (Kessel, 2004; Castano et al., 2004). Although HpD is generally regarded as amphiphilic, it is a mixture of monomers and oligomers. The monomers can form an aqueous phase or self-associated aggregates and, by comparison with chemically bound oligomers, are poorly taken up by cell membranes (Wendenburg et al., 1995). The application of EP can also affect the transport efficiency of oligomers by generating very conductive pores.

The objective of this research was to establish the optimum parameters for such an experiment *in vitro* and to examine whether the efficacy, selectivity, or treatment time could be significantly affected by the new method compared to standard PDT. It would be of special interest with regard to cells resistant to conventional chemotherapeutics, such as MCF-7/DOX and alike. Such therapy could be considered for patients unresponsive to standard chemotherapy and/or radiotherapy treatment.

## Material and Methods

### *Chemicals*

Haematoporphyrin "D" (HpD) was obtained from Porphyrin Products Inc., (Logan, UT), DMEM from Lonza (Basel, Switzerland), L-glutamine and trypsin from Sigma (St. Louis, MO), foetal bovine serum (FBS) from Bio-Whittaker (Walkersville, MO); phosphate-buffered saline (PBS) was purchased from IITD (Wroclaw, Poland). Sucrose magnesium chloride and potassium phosphate came from POCH S. A. (Gliwice, Poland).

### *Cell Culture*

Breast adenocarcinoma MCF7 cells (MCF7-WT wild-type, and the doxorubicin-resistant MCF7-DOX) were used. Both tumour cell lines were a kind gift from the Department of Tumour Biology of Comprehensive Cancer Centre Maria Sklodowska-Curie Memorial Institute (Gliwice, Poland). The cells were allowed to grow in DMEM medium supplemented with 10% FBS and glutamine in a humidified $CO_2$ atmosphere at 37 °C. The cells were adherent to the culture support and detached by trypsinization (trypsin 0.025%, EDTA 0.02%). In all experiments pH was neutral.

### *Electroporation*

The electroporation was carried out using BTX ECM 830 (manufactured by Genetronics Inc. (now Inovio Biomedical Corporation), Blue Bell, PA) square-wave electroporator, generating electrical pulses with the magnitude of 0–3000 V, 10–600 μs long, in the series of 1–99 pulses separated by the time interval of 100 ms–10 s. Two thin aluminum parallel electrodes embedded in the cuvette for electroporation (Cuvettes Plus 640, 800 μl) were 4 mm apart. The voltage generator produced a uniform electric field in the cuvette. The electroporation protocol was a series of five electric pulses of 200–1300 V, 50 μs long, with the repetition frequency 1 Hz. Cells in suspension were centrifuged for 3 min at 537 *g* and resuspended in the electroporation buffer with low electrical conductivity (10 mM phosphate, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) (Teissie and Rols; 1988). After pulsation, cells were left for 20 min with addition of 1800 μl DMEM, then washed and centrifuged twice with DMEM containing 10% FBS, and seeded into 96-well microculture plates for the MTT assay.

### *Electro-Photodynamic Therapy*

HpD was dissolved according to the manufacturer's recommendations in PBS with addition of 20 µl of 10 mM NaOH. The final HpD concentration in culture was 10 µg/ml (PDT and EPDT treatments are labelled as: PDT10 or EPDT10, respectively) or 20 µg/ml (PDT20 or EPDT20). The cells were irradiated by light using a lamp (OPTEL Fibre Illuminator, Opole, Poland), with the fluency of 10 mW/cm$^2$ at the level of cell monolayer, and a red filter, λmax = 632.8 nm. Cells were incubated for 3 min in the presence of HpD in the dark. Then, the electroporation with selected parameters was applied and the cells were left for 20 min with addition of 1800 µl DMEM. Next, the cells were kept for 10 min in the light of intensity 1.9 J/cm$^2$. All irradiations were performed at room temperature. Finally, the cells were washed and centrifuged twice with DMEM containing FBS, and seeded into 96-well microculture plates for the MTT assay.

### *MTT Assay*

The 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was used for assessment of cell viability following phototoxicity of the treatment. After selecting EP or EPDT, cells were seeded into 96-well microculture plates (Nunc, Nunclon™ Surface, Thermo Fisher Scientific, Biokom, Janki k/Warszawy, Poland) at the concentration of 5 × 10$^3$ cells/well and incubated for 24 h at 37 °C and 5% $CO_2$. Then the medium of each well was replaced with 10 µl of 0.5 mg/ml MTT stock solution (dimethylthiazol-diphenyltetrazoliumbromide thiazolyl blue; Sigma-In Vitro Toxicology Assay) diluted in 90 µl PBS. After 2 h of incubation, isopropanol with 0.04 M HCl was added (100 µl/well). The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Labsystem Multiscan MS type 352, Helsinki, Finland). Mitochondrial function was expressed as a percentage of viable cells under treatment relative to control cells treated by an identical sequence of protocol steps except for PDT and EP, which excluded the influence of trypsinization, centrifugation and other procedures. Single outstanding results were not taken into account. The standard deviation within one test did not exceed 6 %. However, even slight changes in the experimental conditions, e.g. the resting time between each stage of the experiment, had a great influence on the results. The differences between separate experiments were up to 25 %. The conditions initially selected for the experiments were based on results reported for MCF-7/WT (Cemazar et al., 1998), optimized.

### *Optical Microscopy*

Images of live cells before and after electroporation were taken using an optical microscope (Nikon ECLIPSE TS100F, Nicon-Poland, Warsaw, Poland) with a phase contrast at 400× magnification.

### *Fluorescence Measurements*

The fluorescence intensity was measured by a custom-made apparatus with 405 nm super bright LED for fluorescence excitation and 630 nm interference filter (NT62-108 Edmund Optics, Barrington, NJ) for separating excitation and fluorescence light. After pulsation in the presence of HpD, the cells were left for 20 min in a dark place to prevent photobleaching and to allow the photosensitizer to enter the cells. The cells were then washed and centrifuged twice, resuspended in 2 µl PBS, and fluorescence was measured.

Since higher fluorescence would be detected from a sample exposed to less cytotoxic conditions (more cells are viable and contribute to the total fluorescence), the fluorescence $F_s$ of a sample was rescaled by the viability ratio $S_s$ at particular experimental conditions. The relative fluorescence $F_r$ is expressed as a ratio of the rescaled sample fluorescence $F_s$ and the rescaled fluorescence $F_c$ of the control cells (treated by the same protocol but with E = 0 V and HpD = 0 µg/ml):

$$F_r = 100\% \left( \frac{F_s}{S_s} \div \frac{F_s}{S_s} \right)$$

## Results

To determine the effect of EPDT on the breast adenocarcinoma cells, the cytotoxic effect on cells under treatment was tested against the control groups that were untreated or subjected either to EP or PDT alone. Electroporation parameters were selected based on the viability of the cells after EP, expressed by MTT. The selected electric field energy was high enough for the electropore formation and increased transport of the photosensitizer into the cytoplasm, but below the level leading to decay in the viability of 50 % or more, which would indicate an excessively high rate of irreversible electroporation. Our study indicated the field of intensity ca 1000 V (5 pulses, τ = 50 µs, f = 1 Hz, d = 4 mm) as optimal for EPDT (Fig. 1). Doxorubicin-resistant breast carcinoma cells MCF-7/DOX proved more sensitive to the electric field than the wild-type cells, showing that the field intensity cannot exceed 800 V in this case (Fig. 2). Therefore, the optimum electrochemotherapy should be at a lower level than for MCF-7/WT. Accordingly, comparison of the experimental results between both cell lines can only be done with regard to this difference, relating viability after treatment obtained at different field intensities.

Cytotoxicity of an excessively high electric field is due to irreversible damage to the plasma membrane. This is shown by optical microscope images of doxorubicin-resistant breast carcinoma cells MCF-7/DOX subjected to electric pulses (compare the control cells in Fig. 3A and electroporated cells in Fig. 3B and C). Electroinduced blebs of the lipid membrane and fluids accumulating around the cell membrane, which is altered by reversible

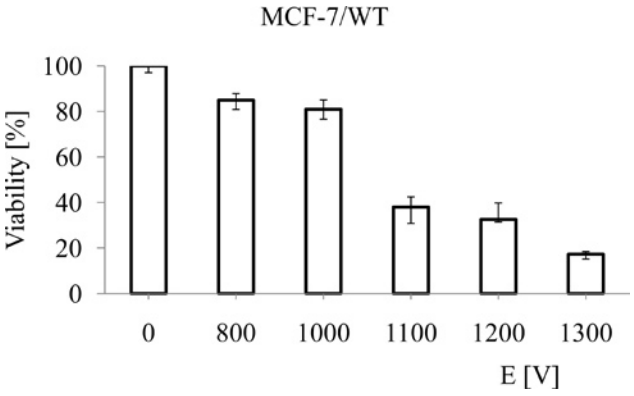


*Fig 1.* The viability ratio of wild-type human breast carcinoma cells MCF-7/WT after electroporation (MTT test, relative to the control with no electroporation, E = 0 V)

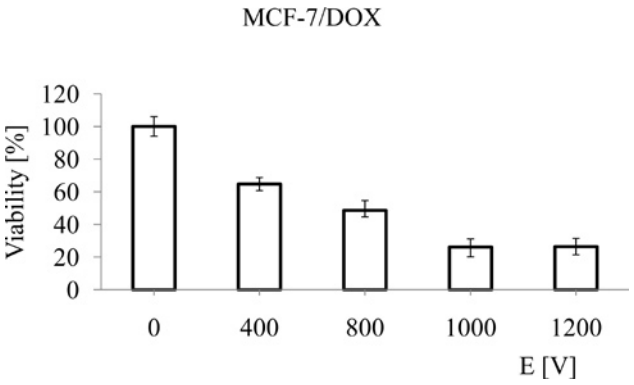


*Fig. 2.* The viability ratio of doxorubicin-resistant human breast carcinoma cells MCF-7/DOX after electroporation

electroporation at E = 1000 V, are shown in Fig. 3B. Ejection of the intracellular material could be observed after applying an excessively high and cytotoxic electric field of intensity E = 1200 V (Fig. 3C).

Supporting PDT by electroporation improved the phototoxicity even in safely low electric fields. In MCF-7/WT, electroporation at 800 V raised the photodynamic effect 4-fold in EPDT-10 and 3-fold in EPDT-20 (Fig. 4). Increasing the intensity to E = 1100 V enhanced the

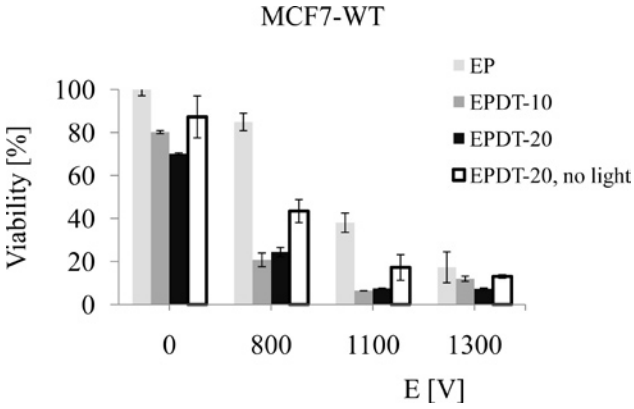


*Fig. 4.* The viability ratio of MCF-7/WT cells after a combination of EP and PDT at HpD concentration of 10 μg/ml (EPDT-10) and 20 μg/ml (EPDT-20). The therapy results can be related to the viability after PDT *in vitro* (E = 0 V) with short exposure time and cytotoxic activity of HpD not activated by light (EPDT-20, no light).

therapeutic efficiency by about 10-fold (Fig. 4). In the latter case, EPDT showed a similar effect at both concentrations of the photosensitizer (EPDT10 – 10 μg/ml, EPDT20 – 20 μg/ml), suggesting that the photosensitizer was efficiently transported into the cells during electroporation. Since the cell viability ratio may be affected by additional procedures related to the photodynamic therapy combined with electroporation, we examined the protocol at our optimal experimental conditions, demonstrating that the enhanced cytotoxic effect of EPDT is not entirely due to the photo-activity of the therapy (Fig. 4). White bars display the effect of the photosensitizer transported into cells by the electric field, without light activation. In this case the decrease in the cell viability ratio comes from a higher intracellular concentration of the drug, exceeding the concentration obtained in standard PDT, combined with EP, which always elevates the cytotoxicity. The addition of HpD and absence of EP showed higher viability ratios: 87 % in EPDT-20 without light, 70 % at the short HpD exposure time, only applied in our experiments (Fig. 4, E = 0 V, EPDT-20, no light), and 60 % when the HpD

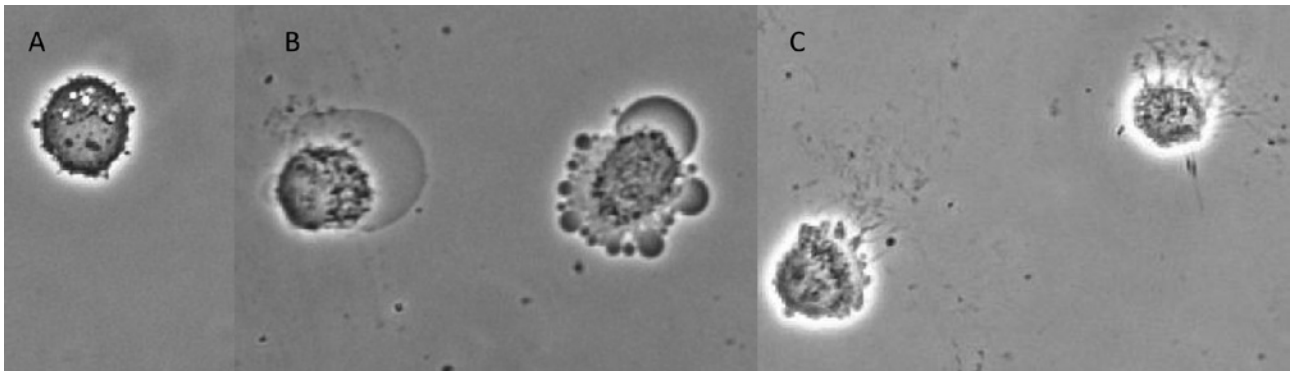


*Fig. 3.* Phase contrast images of breast carcinoma cells MCF-7/DOX. **A**. No electric field applied; **B**. after electroporation at 1000 V – blebs in the membrane indicate unsealing of the membrane; **C**. after electroporation at 1200 V – release of the intracellular material through the pores

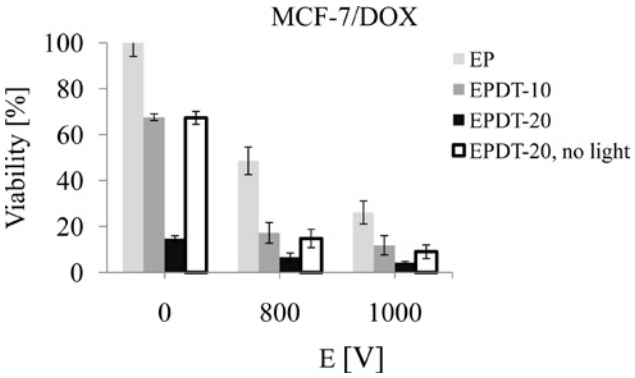


*Fig. 5.* The viability ratio of MCF-7/DOX cells after a combination of EP and PDT at HpD concentration of 10 μg/ml (EPDT-10) and 20 μg/ml (EPDT-20). The therapy results can be related to the viability after PDT *in vitro* (E = 0 V) with short exposure time and cytotoxic activity of HpD not activated by light (EPDT-20, no light).

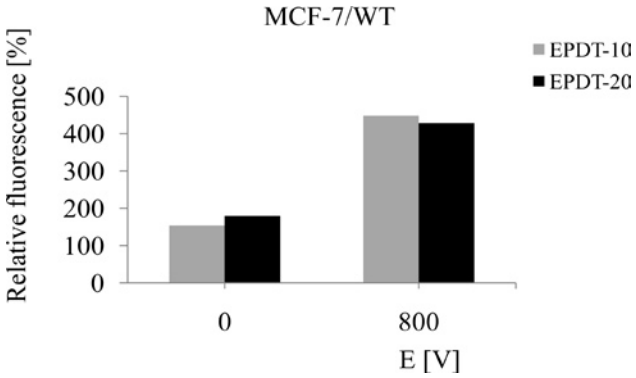


*Fig. 6.* Fluorescence in MCF-7/WT cells shows increased intracellular concentration of the photosensitizer after electroporation at 800 V; it is similar for both extracellular HpD concentrations (EPDT-10 and EPDT-20). The transport in PDT is presented at E = 0 V. Results related to control cells (100 %), for which E = 0 V and HpD = 0 μg/ml.

exposure time was increased to the more typical 24 h duration (Saczko et al., 2009). The shorter duration is obviously insufficient for the standard PDT, as shown in Fig. 4 at E = 0 V. Furthermore, a good effect of EPDT, which was carried out at a lower concentration than is typical for HpD-PDT *in vitro*, i.e. 30 μg/ml (Saczko et al., 2008), shows that EPDT may not only reduce the drug exposure time, but also the drug dose.

The doxorubicin-resistant cells MCF-7/DOX also responded to EPDT, although lower field intensities had to be applied. At 800 V and at HpD concentration of 10 μg/ml, the therapeutic efficiency was raised four times compared to PDT (E = 0 V) and twice at 20 μg/ml (Fig. 5). The tests showed that a concentration of 10 μg/ml, too low for standard *in vitro* PDT without electroporation (viability ratio of 67.6 %, EPDT-10 at E = 0 V, Fig. 5), is sufficient for effective photodynamic therapy when electroporation is also applied. Also here the HpD exposure time was short. The extent of EPDT cytotoxic effect of the therapy on MCF7-DOX that is not directly due to the photo-activity was also tested. As in the previous case, the decrease in viability of cells treated with non-activated HpD results from a higher intracellular concentration of the drug and the loss of cell viability following EP. The cell viability after 20 min exposure to 20 μg/ml non-activated HpD was 70 % (Fig. 5) and 75.6 % when the duration of exposure to HpD was increased to 24 h, in accordance with the typical PDT-HpD protocols *in vitro* (Saczko et al., 2008).

By means of fluorescence intensity, we tested the intracellular concentration of the photosensitizer in cells under treatment (Fig. 6), relative to natural fluorescence of cells (HpD = 0) that were not exposed to the electric field (E = 0 V). The fluorescence was rescaled to the viability ratio at the relevant experimental conditions (see Material and Methods). The fluorescence ratio indicated approximately 3-fold increase in HpD concentration when the cells were electroporated at E = 800 V (Fig. 6). Absorption time was 20 min, as in all our experiments.

## Discussion

Introducing electroporation to support the transport of the photosensitizer into cells increases the phototoxic effect. Combining photodynamic therapy with electroporation can potentially raise the efficiency of low-invasive and targeted cancer therapy for those tumours that show high sensitivity to PDT and weak response to other therapies. In our study, breast carcinoma wild-type cells (MCF7-WT) and doxorubicin-resistant cells (MCF-7/DOX) were treated by PDT with HpD as a model photosensitizer. We tested the effects of electroporation-mediated enhanced transport of photosensitizers on cellular phototoxicity. The electric field, when appropriately selected, significantly increases the molecular transport of a drug and its intracellular concentration, with no significant and sustained harm to the cell. Appropriate amplitude of the field (2–2.5 kV/cm, at 50 μs of impulse duration) opens transient pores that allow increased drug concentration in the cells and reduces the drug exposure time. On the other hand, as shown by MTT, such electric field alone is fairly safe for the cells, insignificantly affecting their viability. These field intensities also show moderate effects on intercellular morphology, which was observed under electron microscopy for cells after EP (Skołucka et al., 2011). Very high electric field could be irreversibly damaging to the cells, starting with undulation of the membrane, leading to leakage of the intracellular material and very high cell mortality, as was visualized by microscopic examination. The necrosis of cells triggers undesirable inflammation processes in the tissue. Cemazar et al. (1998) showed that the intensity of 800–1000 V/cm is the most appropriate for electroporation of MCF-7/WT cancer cells *in vitro*. In particular, at 800 V/cm (8 pulses, $\tau$ = 100 μs, f = 1 Hz) MCF-7/WT carcinoma cells displayed approximately 80 % of the viability ratio and 70 % of uptake ratio tested by the propidium iodide dye, which is impermeant without electroporation. A steep decrease in the viability was observed at higher intensities.

Our study showed similar energy levels necessary for the electroporation-supported therapy. The difference in absolute values of the electric field amplitude comes from the pulse duration, which is twice shorter in our case. These levels of the electric field are similar to the intensities used for ECT *in vivo*. For successive clinical electrochemotherapy of human breast cancer with bleomycin, Whelan et al. (2006) applied pulses of intensity 1400 V/cm, 100 μs duration. On the other hand, Neal et al. (2009) propose irreversible (IR) electroporation for a breast cancer ablation, with no additional cytotoxic drug. According to their results, obtained *in vitro* on cells MDA-MB-231 (Neal et al., 2009) and mouse model of breast cancer *in vivo* (Neal et al., 2010), sufficient pulse intensity for cell irreversible electroporation, adjusted to induce 95 % cell death, is 1000 V/cm (100 μs of the pulse duration), which is significantly lower than by other authors for breast cancer cells (Cemazar et al., 1998; Whelan et al., 2006; Larkin et al., 2007).

Applied to the breast carcinoma cells *in vitro*, the electro-photodynamic therapy showed a 10-fold increase of the cytotoxic effect on wild-type MCF7 cells and a four-fold increase in toxicity for the doxorubicin-resistant cells MCF7-DOX. The results are comparable to the effects of the standard HpD-PDT, which requires higher doses of the drug (typically 30 μg/ml – Saczko et al., 2009, instead of 10 μg/ml in the present study). Most importantly, very significantly shorter exposure time (minutes instead of several hours) was sufficient. The reduced exposure time is a characteristic advantage of combining photodynamic therapy with electroporation.

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### *Statement of Author Contributions*

Designed the study and supervised the experiments: M. K., J. S., J. K. Conducted the experiments: M. N., N. S. Cell culture laboratory: J. S., J. K. Electroporation laboratory: M. K. Data analysis and interpretation: all authors. Manuscript: M. K. Manuscript corrections and improvements: J. K., J. S.

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