Electrically-Enhanced Chemodrug Delivery to Human Breast Cancer Cells

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***Abstract*—Electrical pulse-mediated enhanced drug delivery (known as electroporation), is a minimally invasive treatment of chemotherapy and gene therapy that has been gaining momentum in biotechnology. Presented in this paper is our research on electrically-enhanced chemodrug delivery for breast cancer. For this purpose, we delivered Bleomycin, a chemodrug commonly used around the world, and a breast cancer chemodrug, Paclitaxel, to investigate their efficacy on breast cancer cells with and without electrical pulses. MCF-7 human breast cancer cells were used to evaluate the treatment efficacy. The electroporation parameters include 200V/cm, 20ms and 40ms pulses. Our results indicate that the chemodrug and electrical pulse combination therapy is more efficacious than the drug alone and suggest that application of electrical pulses along with chemotherapy may benefit patients by enhancing the drug transport across the plasma membranes.**

***Index Terms*—Breast cancer, Electroporation, Bleomycin, Paclitaxel (Taxol), Electrochemotherapy.**

I. Introduction

Breast cancer is the second most common cancer among women, estimated at 719,000 cases worldwide, compelling the development of alternate therapies, because conventional breast cancer treatments such as chemotherapy, radiation therapy, and surgery have several drawbacks and are not applicable to all patients [1]. They are not only expensive, but also have severe and extensive side effects, and do not effectively treat some aggressive forms of cancer. Chemotherapy drugs must enter the cancerous cell to be effective. Many potential drugs that have been developed to treat cancer have had limited success due to the lack of efficient and safe delivery mechanisms that allow the molecules to cross the cell membrane.

We and others have previously shown that under appropriate conditions, electrical pulses can drive large numbers of molecules into cells [1-7]. Both *in vitro* and *in vivo* studies and Phase II/III human clinical trials for skin cancer have demonstrated the efficacy of this technique requiring only a very small dosage of chemodrug, thus minimizing its side effects [2, 5-7]. The objective of this study is to develop an outpatient-based, efficient, economical, electrical pulse-based, physical technique with minimal side effects targeting this health care disparity.

Previously, our lab used electroporation to deliver Tamoxifan, the anti-estrogen drug, into MCF-7 breast cancer cells, which showed good drug delivery efficacy [7]. This study supports the hypothesis that electrical pulse could be used to enhance intracellular delivery and effects of chemotherapeutics in breast cancer cells (known as Electrochemotherapy (ECT)). For this purpose, the applicability of electroporation to chemotherapy drugs, such as Bleomycin and Paclitaxel, is investigated in breast cancer cells.

II. Materials & Methods

A. The Cell Line

Cytoplasmic estrogen receptor positive (ER+), malignant breast cancer MCF-7 (human, breast, 69 year old Caucasian woman, adenocarcinoma) cells were used. The cells were cultured in 90% RPMI 1640 media with 10% fetal bovine serum (ATCC) and 1% penicillin/streptomycin (Invitrogen) and incubated in a 5% CO_2 atmosphere at 37°C.

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For electroporation, MCF-7 cells were washed twice with 1x PBS whose pH was adjusted to 7.4, and left in serum-free 199 medium (Invitrogen) for 24 hours. Cells were dissociated from the incubation flask with 0.25% trypsin/EDTA (ATCC) solution. A hemocytometer was used to obtain a final concentration of 1x 10^6 cells/mL. Aliquots of 750 μL in 0.4 cm cuvettes were used for electroporation by adding RPMI 1640 medium with 10% charcoal stripped fetal bovine serum.

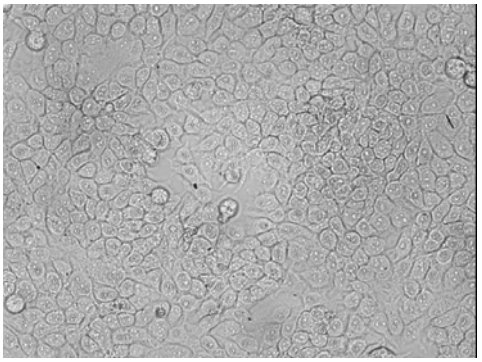


Fig. 1. MCF-7 Human ER+ Breast Adeno-carcinoma cell line.

B. The Chemodrugs

a) Bleomycin:

Bleomycin (Bleo) is a chemodrug approved by the FDA for the treatment of squamous cell carcinomas, testicular carcinoma, melanomas, sarcomas, and lymphomas. It is a DNA targeting, anticytotoxic, antitumor, antibiotic chemodrug. Common side effects include fever, chills, skin reactions, hair loss, alopecia, gastrointestinal issues, and nail thickening and banding. Its anticancer function is generated by binding to DNA, which causes DNA base strands to break. The breakage of DNA basic composition inhibits DNA replication, leading to cell death. Bleomycin is also used in the treatment of other types of cancers, such as testicular carcinoma-embryonal cell, choriocarcinoma, and teratocarinoma [8, 9]. Bleomycin is approved to treat cancer via several different routes. For instance, Bleomycin can be injected into a vein, under the skin, into a muscle, or into the pleura. Bleomycin dose depends on several factors, including: tumor size, regimen being used, cell condition being treated, height, weight, and overall health of the patient. However, Bleomycin is capable of causing lung fibrosis if the cumulative dose is as high as 300mg/m^2. About 50% of patients treated with systemic Bleomycin note some degree of cutaneous toxicity, which includes erythema, induration, hyperkeratosis and peeling of the skin [10]. In this paper, Bleomycin is used to treat human breast cancer cells, MCF-7 with only 5μM concentration, because electrochemotherapy requires less dose of drug than standard clinical doses. In addition, due to the reduced dose of the drug, there is lower degree of toxicity and side effects. Fig. 2a illustrates the structure of this drug.

b) Paclitaxel

Paclitaxel (Taxol) is approved by the FDA for the treatment of advanced breast cancer. This was first discovered from the bark of the slow-growing Pacific Yew tree and has proven to be highly effective in treating women with advanced breast cancer [11]. Recent studies have shown that adding Taxol to standard chemotherapy for breast cancer reduced the rate of recurrence by over 20% and decreased the number of deaths by approximately 25%. It inhibits disassembly and reorganization of the microtubule structures necessary for cell division. Common side effects include low white and red blood cell counts, weakness, hair loss, fatigue, nausea, vomiting, diarrhea, and muscle pain, as well as numbness, tingling, and burning sensations in the arms and legs. Thus, it is an ideal candidate for ECT, so less dose could be used and hence reduce side effects. Fig. 2b shows the structure of this drug.

C. The Electroporation Technique

Two conditions have to be met with for efficient ECT. First, sufficient amount of chemotherapeutic drug must be present in the targeted tissue/tumor when the pulses are applied. Second, electric pulses have to be of appropriate magnitude to create reversible (transient) pores in the cell plasma membranes. Thus, it is critical to choose appropriate electrical parameters to achieve pore formation in the cell membrane without cell death. In our previous work, we successfully delivered the hormone drug Tamoxifen using electropolration with 200 V/cm, 10-40ms, 8 pulses at one second intervals [7, 12, 13]. Therefore, we chose the same electric field intensity to start our initial

effort in this study. Thus, in this research, we used eight square wave pulses with 200V/cm electric field intensity, and 20 ms and 40 ms durations, at 1 Hz frequency. BTX ECM 830 (Genetronics, Inc, San Diego, CA), square wave electroporator (Fig. 3a), with 0.4cm (electrode gap) cuvettes (Fig. 3b) were used for this purpose.

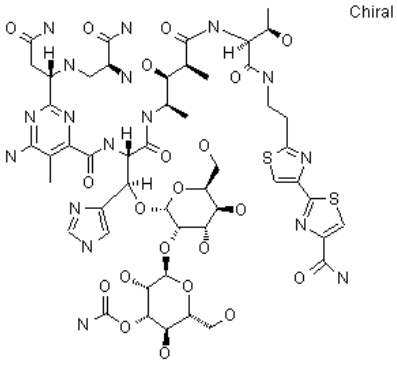


Fig. 2a. Bleomycin Structure

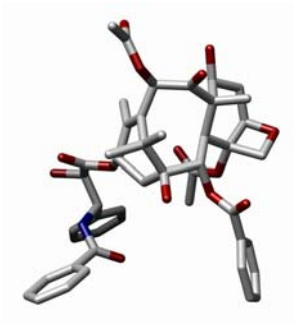
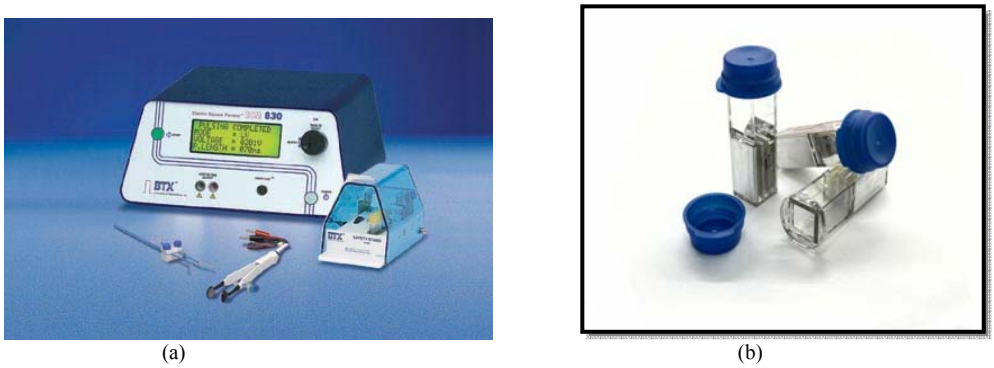


Fig. 2b. Paclitaxel Structure



(a) (b)

Fig. 3. a) BTX ECM 830 Square Wave Electroporator with the Cuvette Holder (that holds the cells), b) Cuvettes used for Electroporation Study

MCF-7 cancer cells were treated with Bleomycin and various electrical and dose parameters (Table I). Treated cells were cultured at 37 ºC, 5% CO_2 incubator for 24 hours before analyzing using various assays, such as viability, Fluorescence-Activated Cell Sorter (FACS) assay and fluorescence microscopy.

TABLE I
TREATMENT PARAMETERS

Sample	Bleomycin Dose μM (micro molar)	Pulse width (ms)	# Pulses	Electric field intensity (V/cm)
Control	0	0	0	0
Bleomycin only	5	0	0	0
EP	5	20	8	200
EP	5	40	8	200

D. Assays

a) Cell Viability and Growth Assay:

After electroporation and incubation, cells suspended in media were stained with a 1:1 concentration of Trypan Blue. The dead cells stained blue so they could be distinguished from the clear, live cells and were counted using Cellometer Auto (Nexcelom bioscience LLC. MA) (or hemocytometer).

b) Fluorescence-Activated Cell Sorter (FACS) Assay

Flow cytometry is a technique used to count, examine, and sort microscopic particles suspended in fluid. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of single cells flowing through an optical or electronic detection apparatus.

Preparation of cells: Before using flow cytometry, the electroporated cells were treated using fluorescence reagents. Two types of buffer were used as fluorescence reagents. One was Annexin V Incubation Reagent

(AVIR), while the other was a binding buffer. The AVIR was prepared using the doses of each chemical as in Table II, for each sample with approximately 1×10^6 cancer cells/mL. The AVIR was kept in ice in the dark after preparation. The binding buffer was prepared by diluting 50µL of 10x binding buffer into 450µl dH_2O, and then kept in ice. The cells were washed twice with 500µL cold 1x PBS before using these buffers. Washed cells were resuspended using AVIR gently and then incubated for 15 minutes in dark at room temperature. The binding buffer was then added into each sample after incubation. These samples were used for flow cytometery using Cell Lab Quanta™ SC flow cytometer within one hour for obtaining good data acquisition with maximal signal.

TABLE II
AVIR PREPARATION

Chemical Items	Volume (µL)
10x Binding Buffer	10
Propidium Iodide	10
Annexin V-FITC	1
dH_2O	79
Total Volume	100

c) Fluorescence Microscopy:

Fluorescence microscopy is a technique used to study biological activities in specimens. It functions based on the phenomenon that certain materials emit energy detectable as visible light when irradiated with the light of a specific wavelength. When analyzing fluorescent images, one evaluates the color intensity of the stained cells. A more intensely stained (darker) cell means that the cell is apoptotic, has ceased growth, and, in this instance is undergoing programmed cell death. A Nikon Eclipse TE2000-S was used for our cell imaging in this study.

Preparation of cells: Media was removed from the wells containing electroporated cells and they were fixed using 100% ethanol. Propidium Iodide (PI) solution was prepared at a concentration of 25µL PI per 5mL of 1xPBS. After 15 minutes, ethanol was removed and PI solution was added to stain the cells. 15 minutes later, PBS was added after removing PI, and the cells were imaged using a standard set of optical filters for this dye (530nm excitation/600nm emission).

III. Results & Discussion

A. Cell Viability and Growth Assay

Cell viability results are shown in Figs. 4-6 for Bleomycin and Paclitaxel. Fig. 4 shows the results for Bleomycin. Here, the control sample (no treatment) has more than 90% of the tumor cells are alive. The viability of cells with 5µM Bleomycin is reduced approximately 10% compared to the control. Cell viability of 5µM Bleomycin and 20ms duration electroporation decreased to 70%. The 40ms electroporated, bleomycin-treated cells had a further 40% reduction compared to the 20 ms electroporated, Bleomycin-treated cells. Cell viability with Bleomycin and 40ms electroporation showed a reduction of more than 50% compared to the control, indicating the enhanced efficacy of the combination therapy with electrical pulses and the chemo drug at concentrations as low as 5µM.

Fig. 5 shows a similar reduction in viability with Paclitaxel drug at 100mM using electroporation. Fig. 6 depicts differences in average cell viability between samples with and without electroporation at different concentrations of Paclitaxel, known as Dose Curve. Here, 40ms electrical pulses were applied. It can be seen that the number of live cells decreased as the concentration of Paclitaxel increased and the electroporated cells showed decreased viability compared to those only treated with Paclitaxel.

We demonstrate that low chemodrug doses of 9nM and 20nM are as effective as 5µM (a factor of ~1000). The nM concentration was studied for Paclitaxel compared to initial mM (Fig. 5) and µM (data not shown) concentrations, as at higher concentrations, the Paclitaxel discolored the cuvette cells, indicating the high toxicity of these molecules. This exemplifies the need for a low dose technique than current chemotherapy techniques which use high doses with substantial side effects.

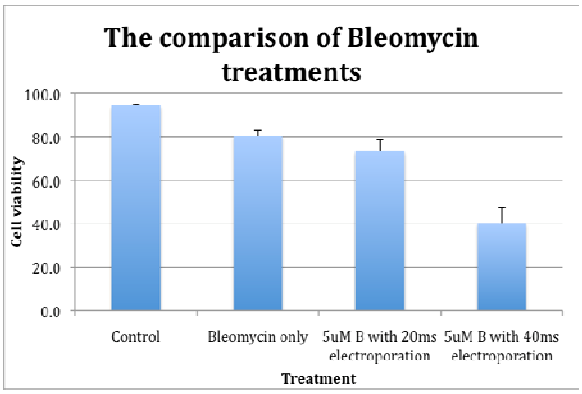


Fig. 4. Efficacy of Elecroporation + Bleomycin treatment

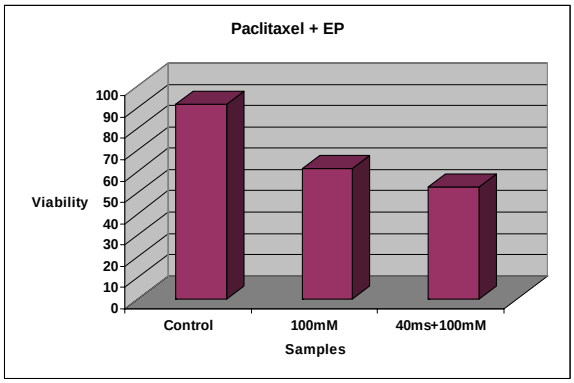


Fig. 5. Efficacy of electroporation + Paclitaxel treatment

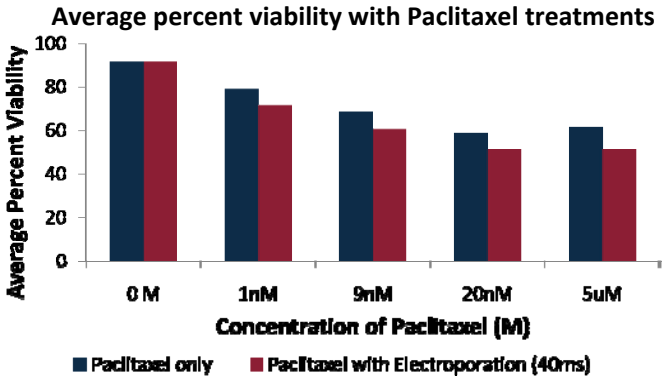
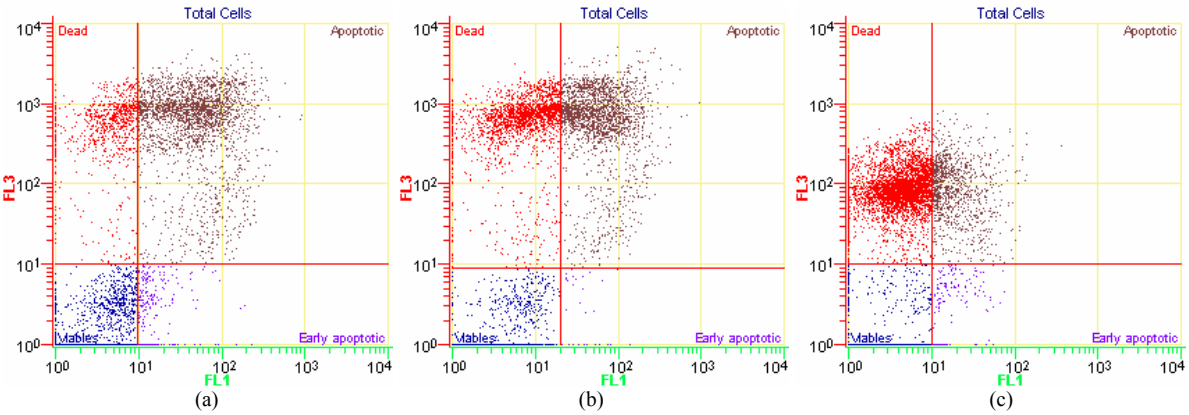


Fig. 6. Dose curve of Paclitaxel treatments, with and without electroporation.

B. FACS Assay

Figures 7a, b, and c display results obtained using flow cytometery. They indicate the various conditions of the cells, using two parameters, FL1 and FL3. There are four areas in each image showing the status of the cells, as live (**blue**), early apoptotic (**purple**), apoptotic (**brown**), and dead (**red**). When using the drug only, there are more live cells than dead cells (Fig. 7a). Due to electroporation, more cells moved from the live area to upper dead area, especially using 40ms pulses than 20ms pulses (Fig. 7b and c).

It was reported that a number of anticancer drugs induce apoptosis in cancer cells [14]. Apoptosis occurs in most, if not all solid cancers. It is preferred to have apoptotic cell death, as opposed to necrosis, which involves inflammation, swelling of organelles, clumping of chromatic, and membrane disintegration. In contrast, apoptotic cells contain chromatin compacted into sharply delineated masses, and condensation of the cytoplasm occurs. No inflammatory reaction is noted due to apoptotic cell death.

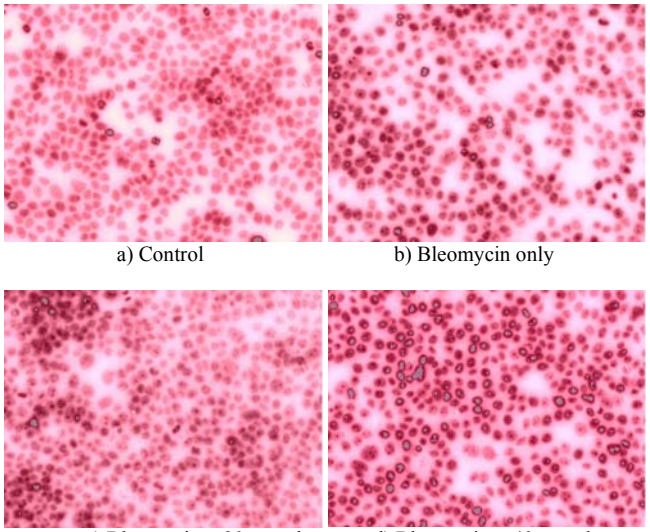


(a) (b) (c)

Fig. 7: FACS Histograms: a) Bleomycin only, b) Bleomycin and 20ms Pulses, c) Bleomycin and 40ms Pulses.

C. Fluorescence Miscoscopy

Figs. 8a, b, c, and d show the fluorescence microscopy images. Fig. 8a shows the control, 8b) Bleomycin only, 8c) Bleomycin with 20 ms electroporation, and 8d) Bleomycin with 40 ms electroporation. The dead cells are stained darkly. These images illustrate that the sample treated with 40 ms electroporation at 5μM concentration Bleomycin has the most dead cells, while control has the least dead cells, again illustrating the efficacy of the combination therapy of chemodrug and electrical pulses.



a) Control b) Bleomycin only

c) Bleomycin + 20ms pulse d) Bleomycin + 40ms pulse

Fig. 8. Fluorescence Microscopy images of MCF-7 breast cancer cell samples

IV. Summary

Once a disease of affluent Americans, Canadians, and Western Europeans, today breast cancer is everywhere including: Asia, Africa, and Eastern Europe [15]. There is a lack of safe, economical, and efficient delivery system that allows a drug to easily enter the cell through the plasma membrane. Medicines administered orally must circulate throughout the entire body before taking effect. This poses a potential threat to the patient, the drug could take action in any part of the body causing adverse effects, as well as creating an opportunity for the drug to be broken down through enzymatic degradation. An improved delivery system, such as electroporation, which will provide a more targeted pathway into the cell, will increase the cell's receptiveness to the drug and enhance its capabilities while reducing systemic side effects.

Our study assessed the prospects of using electrical voltage pulses to enhance the effect of minute doses of chemotherapeutics for breast cancer. This will particularly be beneficial to drug resistant patients as not all the

patients are responsive to conventional breast cancer treatments, and since this treatment allows targeted delivery of the therapeutic agents in a minute quantity, it would be less expensive and less toxic than the traditional treatments.

In this research, we delivered eight 200V/cm, 20 and 40ms pulses at 1Hz to enhance the uptake of chemodrugs, Bleomycin and Paclitaxel into MCF-7 breast cancer cells.

Our results indicate that the MCF-7 Cell Line is receptive to electroporation. It is also demonstrated that the combination therapy, electroporating cells in addition to the chemodrugs, bleomycin and Paclitaxel administrations is more effective than treatment with drug alone. This supports the hypothesis that electroporation and drug combination therapy will increase the cell's response to chemodrugs including Bleomycin and Paclitaxel and enhance its cancer curing capabilities.

The experiments also provide evidences that low voltage pulses with longer duration are effective towards permeabilization in MCF-7 breast cancer cell, and may be used in clinics. We find that electrochemotherapy is a promising technique for patients with cutaneous or subcutaneous tumors, in any tissue, which are recurrent, inoperable, or progressive and/or metastatic and unresponsive to traditional chemotherapy and radiation treatments. The procedure is simple (could be performed as an outpatient treatment), fast, and has no apparent cytotoxic or systemic effects.

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