Enhanced Doxorubicin Uptake in Malignant Breast Cells using Electrical Pulses

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***Abstract*—Conventional breast cancer treatments such as chemotherapy, radiation therapy, and surgery have several drawbacks. These are expensive, have severe and extensive side effects, and do not effectively treat some aggressive forms of cancer. In this research, we propose the use of electroporation as an alternate treatment to enhance the uptake of therapeutic drugs into breast tumors. To build upon previous research using the chemotherapeutic drugs Tamoxifan, Bleomycin, and Paclitaxel, we have conducted comparative experiments using the chemodrug Doxorubicin. To investigate its efficacy, we used MCF-7 human breast cancer cells. The electroporation parameters used were 200V/cm 40ms pulses and 1200V/cm 100µs pulses. The results indicate that Doxorubicin and electroporation combination therapy is more effective than treatment with the drug alone. This further suggests that electrochemotherapy is a viable treatment for breast cancer patients.**

INTRODUCTION

Breast cancer is by far the most common cancer among women worldwide with more than a million new cases diagnosed each year. In the United States as many as 1 in 8 women will face breast cancer in her lifetime [1]. Conventional breast cancer treatments include chemotherapy, radiation therapy, and surgery. These treatments are expensive and have severe and extensive side effects. In addition, conventional treatments are not effective in treating some aggressive forms of cancer.

Chemotherapy drugs must gain entry into the tumor cells to be effective in treating the malignancy. Many drugs have been developed to treat cancer but some have had limited success due to the lack of efficient in-vivo delivery mechanisms that allow the molecules to cross the cell membrane and enter the cell to induce cell death. Experi-

ments in our lab and others have previously shown that under appropriate conditions and particular parameters, electrical pulses of high intensity and short duration can allow large numbers of molecules to enter cells [2-8]. In vivo and vitro studies of electroporation treatments in addition to Phase II and III clinical trials of electroporation of skin cancer lesions have demonstrated the efficacy of this technique using low doses of chemotherapeutic drugs, thus minimizing the unpleasant side effects and cost [3, 6-8]. The objective of this study is to continue to document the evidence that electrochemotherapy (ECT) has the potential to be an outstanding outpatient-based, efficient, effective, economical, electrical pulse-based, physical treatment technique with minimal side effects.

Previously, our lab has used electroporation to deliver Tamoxifan, Bleomycin, and Paclitaxel into MCF-7 breast cancer cells, showing adequate efficacy [8,9]. These studies support the hypothesis that electroporation can be used to enhance intracellular drug delivery and efficacy of chemotherapeutic drugs in breast cancer. In this project, the effectiveness of Doxorubicin electrochemotherapy on MCF-7 breast cancer cells is investigated.

MATERIALS & METHODS

A. Cell Line

Cytoplasmic estrogen receptor positive (ER+), malignant breast cancer MCF-7 human adenocarcinoma cells were used. MCF-7 cells were isolated from the breast of a 69-year-old female Caucasian woman in 1970. The cells were cultured in 90% RPMI 1640 media with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) and incubated at 37°C in a 5% CO_2 atmosphere.

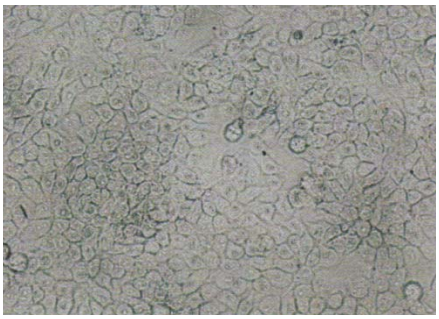

Fig. 1. MCF-7 Breast Cancer Cell Morphology.

To prepare the cells for electroporation, the MCF-7 cells were washed twice with 1x PBS at pH 7.4 and incubated in serum-free 199 medium (Invitrogen) for 24 hours. Cells were dissociated from the incubation flask with 0.25% trypsin/EDTA (ATCC) solution. The cell density was measured using a Cellometer Auto T4 from Nexcelom Bioscience, LLC. The cell solution was centrigued at 1500 rpm for 5 min and resuspended in RPMI 1640 media with 10% charcoal stripped fetal bovine serum to a final concentration of approximately 1 x 10^6 cells/mL. Aliquots of 750 µL of cell suspension were added to electroporation cuvettes with a 0.4 cm gap.

B. Doxorubicin

Doxorubicin is a widely used cytotoxic drug in cancer chemotherapy. It is a natural product of a species of soil fungus, *Streptomyces*. Doxorubicin is classified as an anthracycline antibiotic that works by binding to nucleic acids and intercalating DNA [10]. These drugs are cell cycle-specific. It was approved by the FDA in 2007 for the treatment of some leukemias, lymphoma, soft tissue sarcoma, multiple myeloma, and cancers of the bladder, breast, stomach, head and neck, lung, ovaries, pancreas, prostate, uterus, and thyroid. Common side effects include: pain, nausea, vomiting, diarrhea, low blood counts, mouth sores, and hair loss. An uncommon but very serious side effect is cardiotoxicity that can interfere with the pumping of the heart. For this reason there is a limit to the amount of Doxorubicin you can receive in your lifetime [11]. This makes Doxorubicin an ideal candidate for electrochemotherapy. Fig. 2 shows the structure of this drug [12].

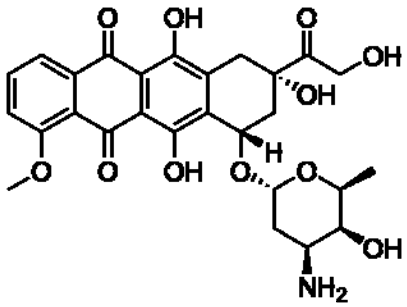

Fig. 2. Atomic Structure of Doxorubicin [12].

C. Electroporation Parameters

There are two essential conditions for successful and efficient electrochemotherapy. First, a sufficient amount of chemotherapeutic drug must be present in the interstitial space around the tumor or targeted area when the pulses are applied. Second, the electric pulses must be of the appropriate length and magnitude to induce the formation of transient pores in the cell plasma membrane. The choice of electrical parameters is the most critical component because pore formation must be achieved without inducing cell death. In previous experiments we have successfully delivered the chemotherapeutic drugs Tamoxifen, Bleomycin, and Paclitaxel using eight electric pulses, one second apart, with a field strength of 200 V/cm, and a duration of 10-40 ms [8, 9, 13, 14]. Therefore, we used these same parameters to begin the initial trials with Doxorubicin. Eight square wave pulses with a frequency of 1 Hz, an electric field intensity of 200 V/cm, and a duration of 40 ms. Later trials used eight square wave pulses with an frequency of 1 Hz, an electric field intensity of 1200 V/cm, and a duration of 100 µs. In addition, we also studied the efficacy of 450V/cm, 30ms and 500V/cm, 20ms pulses. The BTX ECM 830 square wave electroporator from Genetronics, Inc. was used for all these experiments (Fig. 3).

Fig. 3. BTX ECM 830 Square Wave Electroporator and Cuvette Holder and Cuvettes.

D. Cell Viability Count

After electroporation cells must remain in cuvettes for 30 minutes before being transferred to well plates for incubation. After 24 hours media and dead, unadhered cells were removed from the wells. Live, adhered, cells were dissociated from the incubation flask with 0.25% trypsin/EDTA (ATCC) solution. Live cells were counted and recorded with the Cellometer Auto T4 from Nexcelom Bioscience, LLC.

RESULTS & ANALYSES

A. Dose Curve

The dose curve for Doxorubicin is shown in Fig. 4. A dose curve was developed to determine the appropriate (the least, but effective) concentration of Doxorubicin for our electroporation experiments. The cell viabilities shown indicate the survival rate for cells treated with different concentrations of Doxorubicin only. No electroporation was used.

Fig. 5 shows the dose curve using electroporation at various doses of 0.1, 1 and 5µM respectively.

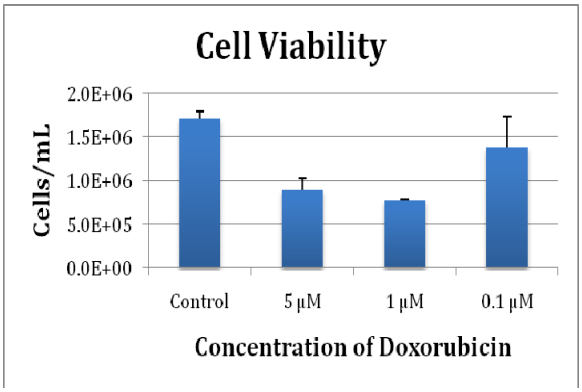

Fig. 4. Doxorubicin Dose Curve without Electroporation.

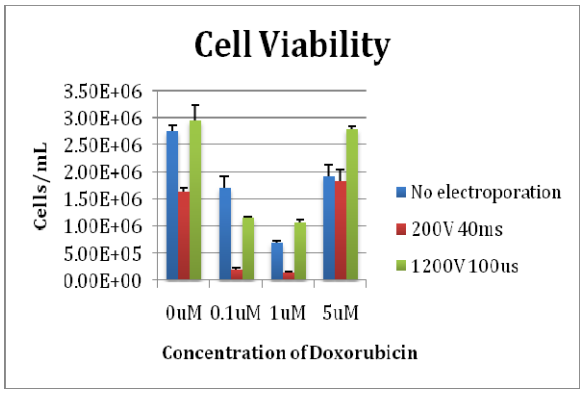

Fig. 5. Doxorubicin Dose Curve with Electroporation.

B. Viability Study

Shown in Fig. 6 are the results of the effect of various voltage parameters. These values were tried in order to study the efficacy of lower intensity, longer duration pulses, compared to the most commonly used 1200V/cm, 100µs pulse set. For this purpose, 200V/cm, 40ms, 450V/cm, 30ms, and 500V/cm, 20ms pulses were studied. The results indicate that indeed the lower intensity, longer duration pulses very effective, owing to their higher energy content due to longer durations. We used 8 pulses in each of these. Reducing the number of pulses to 2 or 4 and studying their efficacy will be interesting as the energy content is relatively higher with eight millisecond pulses whose durations are in the 10s of ms.

The treatment of Doxorubicin in combination with the electroporation parameters shows promise. The cell viability shows a reduction of nearly 90% compared to treatment with Doxorubicin alone, even with a very low dose of 0.1µM. An increase of dosage to 1µM showed slightly more cell death.

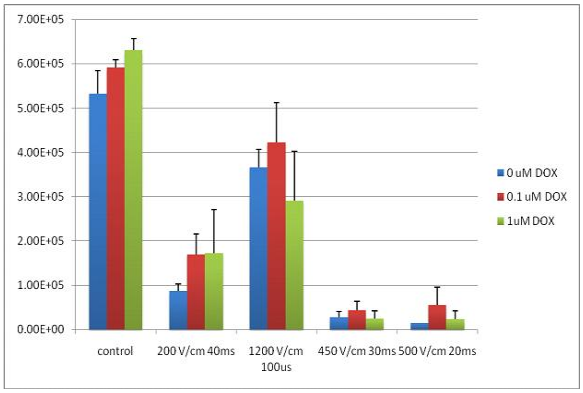

Fig. 6. Viability Study using various Electroporartion Pulse Parameters.

DISCUSSION & SUMMARY

Doxorubicin is part of a group of medications called anthracyclines. Anthracyclines kill cells (including cancer cells and normal cells) by working in several ways [15]. Doxorubicin binds to DNA in cells, changing the shape of the DNA by injecting itself into some of the empty spaces in the DNA molecule. This damages the DNA, causing a bit of unwinding of their spirals, making it impossible to form new cancer cells.

Doxorubicin can damage the membranes (outer coating) of cells and may damage other parts of cells as well [15].

Doxorubicin is an anthracycline glycoside antibiotic produced by *Streptomyces peucetius* var. *caesius*. The drug is structurally related to daunorubicin and epirubicin. Doxorubicin differs structurally from daunorubicin in that doxorubicin contains a hydroxyacetyl group instead of an acetyl group in the 8-position. Epirubicin is the 4′-epimer of doxorubicin (Fig. 7 [16]).

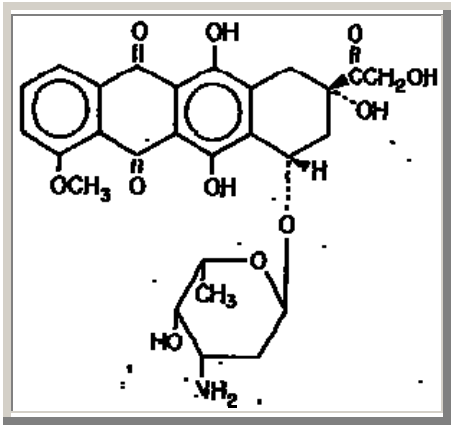

Fig. 7. Doxorubicin Structure [16]

Doxorubicin is commercially available as the hydrochloride salt. Commercially available doxorubicin hydrochloride powder for injection occurs as a sterile, lyophilized, crystalline, red-orange or red powder; the powder for injection also may contain lactose and methylparaben to enhance dissolution. Doxorubicin hydrochloride is freely soluble in water, slightly soluble in 0.9% sodium chloride solution, and very slightly soluble in alcohol. When doxorubicin hydrochloride powder for injection is reconstituted with 0.9% sodium chloride injection, the pH of the resulting solution is 3.8–6.5 [16].

Doxorubicin hydrochloride also is commercially available as a sterile, isotonic, aqueous solution of the drug. Hydrochloric acid is added during manufacture of the injection to adjust the pH to approximately 3 (range: 2.5–3.5); the injection also contains 0.9% sodium chloride.

While doxorubicin can kill both healthy and cancerous cells, it has a greater effect on cells that are multiplying rapidly. Generally, cancer cells multiply more rapidly than healthy cells, and are therefore more affected by doxorubicin. Doxorubicin is prescribed for treating several types of cancer, including certain types of leukemia, breast cancer, Hodgkin's disease, and lung cancer. While doxorubicin can kill both healthy and cancerous cells, it has a greater effect on the cancer cells because they multiply more rapidly.

Doxorubicin is a highly protein-bound drug. Its penetration in multilayerd solid tumors depend up on a number of factors including its concentration, kinetics of drug penetration, effects of tumor cell density and tissue composition. Thus drug-resistance, the inability of the drug to penetrate is possible. It was observed that penetration of Doxorubicin in 3-dimensioned tumor cell spheroids after 1 to 2 hours is limited to periphery. Similarly, a steep concentration gradient in breast tumors has been observed in patients. Hence, better methods to enhance drug delivery are needed and electroporation is one such vehicle to enhance impermeable drug uptake using electrical pulses [17]. It is an attractive alternative to treat patients with cutaneous or subcutaneous tumors which are recurrent, inoperable, or progressive and were refractory to systemic chemotherapy and/or radiotherapy [18, 19]. Our results indicated the effectiveness of electroporation using Doxorubicin. We tested both high intensity, short duration (1200V/cm, 100μs) pulses as well as other low intensity, long duration (200V/cm, 40ms and 450V/cm, 25ms) pulses. These results indicating the versatility of the pulses used and their efficacy, are useful for taking this technique to the next level.

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