# Enhanced Drug Uptake in Normal and Malignant Breast Cell Lines Using Electrical Pulses

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*Abstract-* **Conventional breast cancer treatments, such as surgery (the Gold Standard), radiation therapy and chemotherapy serve most of the patients while they do not serve all of them. They are expensive and have extensive side effects, both near term and long term. Affordable, alternate treatments are needed to treat aggressive and drug resistant breast cancer tumors. We propose the use of electrical pulses to enhance the therapeutic molecule uptake in breast cancer patients. In order to better understand uptake processes of malignant tissues, it is necessary to investigate uptake in normal tissues as a reference. In this research, we studied the different electroporation characteristics of malignant breast cancer cell lines, MCF-7 and CRl 1743 compared to the non-malignant breast cancer cell lines MCF-10. We applied 200V/cm, 10-40ms pulses and studied their effect on viability and other phenomena. Considering that approximately one million incidences of breast cancers are estimated in the world, with ~200,000 in the US and ~320,000 in the Europe, and the rest in the rest of the world, inexpensive, less toxic, existing alternative treatments need to be developed and implemented for clinical practice.**

## I. INTRODUCTION

In our research, we attempt to compare the electroporation [1-4] characteristics of mailignant breast cancer cell lines, MCF-7 (human) and CRL 1743 (rat) along with non-malignant cell line MCF-10A (human). Understanding the distinction between cancer

and normal cells is a fundamental step of diagnosis and further biological analyses of cancer [5].  Carcinogen-induced, mammary tumorigenesis in rats has long been an accepted model system for eleucidation of mechanisms involved in human human breast cancer development and treatment [6].  For this purpose, the uptake of tamoxifen [7, 8], the classical hormone drug for breast cancer in these cells have been studied.  The advantages of Tamoxifen could be found in [7].  However, for up to 60% of patients, tamoxifen is not an effective treatment.  Patients receiving tamoxifen experience significant side effects that include hot flashes, increased risk of thromboembolic events and increased risk of endometrial cancer [7].  These side effects result from estrogenic affects of orally delivered tamoxifen that travels via the blood stream and acts on estrogen receptor containing tissues other than the breast, such as the uterus.  Our work focuses on testing strategies for improving response to Tamoxifen in patients that are resistant to hormonal treatment.  We are especially interested in improving hormonal therapy in aggressive breast tumors in obese subjects.  Towards reducing toxicity and enhancing drug delivery we propose the development of a novel technique that couples low volume electroporation with tamoxifen.

Electroporation or Electropermeabilization (EP) is a physical, non-viral technique utilizing precisely controlled electric fields of short duration and high intensity to open up transient aqueous pathways through semi-permeable membranes, allowing targeted delivery of therapeutic molecules including drugs, antibodies, and nucleotides [1-4, 9]. EP offers several fold improved therapeutic benefit compared to using a drug alone.  It is a very efficient technique to enhance the efficacy of drug delivery for cancer treatment, gene transfer and other similar applications in biology, biotechnology and medicine [1-4]. EP is a local, site-specific, physical technique with minimal side effects, if any.  We believe that an improved delivery system, which will provide a more targeted pathway into the cell via electroporation, will increase the cell's receptiveness to the drug and enhance its capabilities (Fig. 1).
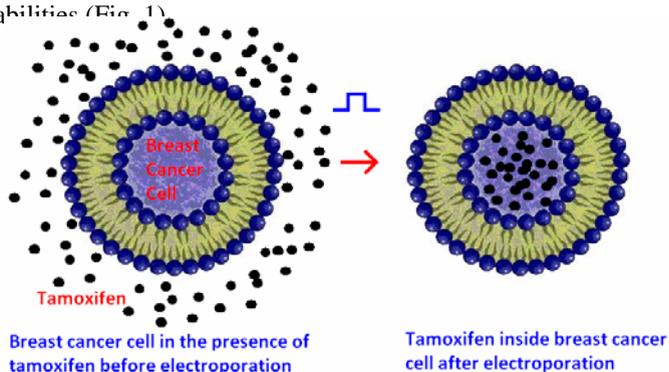


Fig.1 Uptake of Molecules using Electroporation or electrpermeabilization

Based on the potential usefulness of EP methods towards breast cancer therapy, the main objectives of our current research are to identify optimal EP pulse parameters and to investigate the translational effectiveness of the same reduced low electrical field strengths on MCF-7 human breast cancer cells and CRL 1743 rat breast cancer cells. It is hypothesized that low field intensity electro-endocrine therapy will improve therapeutic outcome and reduce systemic toxicity of Tamoxifen by increasing permeability of cell membranes exposed to electrical pulses.

## II. MATERIALS & METHODS

### A. MCF-7 Cancer Cell line
Estrogen receptor positive MCF-7 (human, Caucasian, breast adenocarcinoma) cells were used. Cells were cultured in 90% RPMI 1640 media + 10% FBS serum (ATTC, Manassas, VA) and 1 % Penicillin/Streptomysin (Invitrogen, Carlsbad, CA). Cells were grown in an incubator at 37°C at 95% humidity and 5% $CO_2$.

### B. MCF-10A Non-Cancer Cell line
Estrogen receptor positive MCF-10A (human, Caucasian, spontaneously immortalized but non-transformed, non-cancerous) cells (ATCC, VA) were used as a negative control to show the effects of electroporation on non-cancerous cells. Cells were cultured in 95% DMEM/F-12 (ATTC, Manassas, VA) modified with 20ng/ml EGF (Peprotect, Rock Hill, NJ) 10  g Insulin (Sigma-Aldrich,), 0.5 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO) + 10% FBS serum (ATTC, Manassas, VA) and 1 % Penicillin/Streptomysin (Invitrogen, Carlsbad, CA). Cells were grown in an incubator at 37°C at 95% humidity and 5% $CO_2$. Fig. 2 shows an illustration of the morphology of the MCF-7 and MCF-10A cells.

### C. CRL 1743 Cancer Cell line
Estrogen receptor positive CRL 1743 (rat, Sprague Dawley, breast adenocarcinoma) cells were used. Cells were cultured in 90% EMEM media + 10% FBS serum (ATTC, Manassas, VA) and 1 % Penicillin/Streptomysin (Invitrogen, Carlsbad, CA). Cells were grown in an incubator at 37°C at 95% humidity and 5% $CO_2$.

### D. Electroporation of MCF-7, MCF-10A and CRL 1743 cells
Cells were dissociated from flask by treatment with 0.25% trypsin/EDTA solution (Invitrogen, Carlsbad, CA). Cells were counted using a hemocytometer and resuspended in either RPMI 1640 or EMEM culture media + 10% charcoal stripped FBS serum for MCF-7 and CRL 1743 or modified DMEM/f-12 for MCF-10A to a final concentration of $1 \times 10^6$ cells/ml. Aliquots of 750 µL were used for electroporation.

A BTX 830 square wave electroporator (Genetronics, San Diego, CA) along with 0.4cm cuvettes were used for electroporation (Fig. 3). Various voltages, pulse durations and number of pulses at one-second intervals were studied (details in Table 1). A

reduced voltage magnitude but increased duration compared to skin cancer clinical trials [1-4, 9] was used in this study to reduce the tingling feeling or any other sensation that could be felt while applying pulses. Also, it is always preferred to use a lower voltage if it works. Cells were pulsed in media without tamoxifen or in media containing 1, 5 or 10μM tamoxifen. Cells were undisturbed for thirty minutes after pulsing, and then removed from cuvettes. MCF-7A and CRL 1743 cells were seeded in 12 well plates. MCF-10A cells were seeded in 6 well plates. All cells were then incubated at 37°C in a 5% $CO_2$ atmosphere at 95% humidity for 2, 24 or 48 hours.
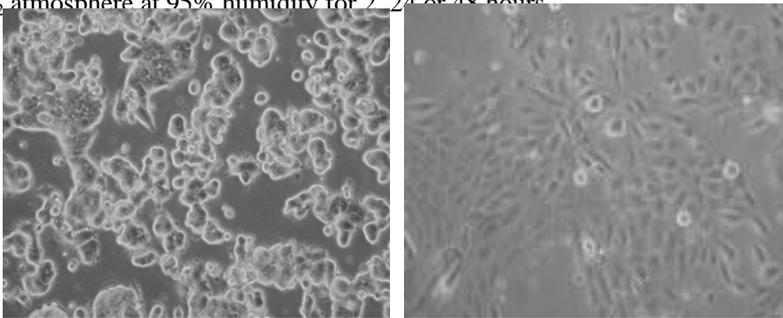


(a) MCF-& Malignant cells            (b) MCF-10A Non-malignant Cells
Fig. 2. MCF Cells used in this study



Fig. 3 Square wave electroporator used for Electro-Endocrine Therapy.

*E. Cell Viability and Growth Assay*

Following electroporation and incubation, cells suspended in media were stained with a 1:1 ratio of Trypan Blue and counted using a hemocytometer comprised of a five grid counting system. Counts for live and dead cells (viability), and total numbers of cells (growth) were done for each electroporation treatment. Three replicates for each treatment were counted 4 times and the averages calculated.

TABLE 1: ELECTROPORATION PARAMETERS USED ON MCF-7 CELLS

| Treatment | Electric Field, V/cm | Pulse Length | # pulses |
|---|---|---|---|
| Control-No Electroporation (None) | 0 | 0 | 0 |
| Low V Electroporation 1 (LP1) | 200 | 10 ms | 8 |
| Low V Electroporation 2 (LP2) | 200 | 20 ms | 8 |
| Low V Electroporation 3 (LP3) | 200 | 30 ms | 8 |
| Low V Electroporation 4 (LP4) | 200 | 40 ms | 8 |
|  |  |  |  |
| Tamoxifen only, No Electroporation | 0 | 0 | 0 |
| Low V Electroporation 1+Tamoxifen (LP1) | 200 | 10 ms | 2 |
| Low V Electroporation 2 +Tamoxifen (LP2) | 200 | 20 ms | 8 |
| Low V Electroporation 3 +Tamoxifen (LP3) | 200 | 30 ms | 8 |
| Low V Electroporation 4+Tamoxifen (LP4) | 200 | 1 ms | 8 |

## F. Effect of Tamoxifen on MCF-7 Cells

The effect of tamoxifen alone, without electroporation, on cell growth and viability was determined. Cells suspended in 750 µl aliquots ($1x10^6$ cells/ml) containing RPMI 1640, modified DMEM/F-12 media as described above. Control cells were treated with media devoid of tamoxifen. Cells were cultured at $37^0$ C for either 2 -24 hours, stained with Trypan Blue (1:1 ratio) and counted via hemocytometer.

## G. Fluorescence-activated cell sorter (FACS) analysis of MCF-7 cells

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

After electroporation, the MCF-7 cells were allowed to sit for 30 minutes so that they could recover. After this, they were plated with about 500 microliters of charcoal stripped media and were placed in the incubator for 24 hours. At the end of 24h time point, the cells were fixed at -20degC in absolute ethanol. For FACS analysis, the cells were centrifuged and resuspended in 1 ml PBS containing 100µl of 200µg/ml RNase A and incubated for 30min at 37degC. The RNase A is boiled for 5 min prior to use. Then 10 µl of 1 mg/ml propidium iodide was added to the cells and they were taken over for FACS analysis using Cytomics™ FC 500 by Beckman Coulter

## H. Statistical Analysis

Results are expressed as mean ± SEM. Analyses were performed using one way and repeated measures of ANOVA with Tukey's posttest using Proc Mixed (SAS Institute 1994) [10].

## III. RESULTS & DISCUSSION

*A. Effect of Electrical Pulses on MCF-7, MCF-10A and CRL 1743 Cell Death and Cell Growth After 2 Hours Incubation,*

Optimal EP parameters that do not compromise viability were determined for MCF-10A (non-malignant MCF-7 human breast epithelial), MCF-7 (malignant human breast epithelial) and CRL-1743 (malignant rat epithelial) cells after 2 hour of incubation. Voltage and number of pulses remained constant in all treatments. Only the duration of pulses were varied. No tamoxifen was used in this set of experiments. Resulting percentage of dead cells with varying pulse duration are illustrated in Fig. 4. Increasing percentage of dead cells was observed in both human and rat cancer cell lines with increasing duration of pulses. For both cancer cell lines increased cell death was first observed at 20 ms (5.5%) with maximum cell death at 40 ms (8%). In the normal human breast cancer cell line the effect of EP remain relatively constant over duration times from 10-40 ms. The increase in cell death was less than observed in cancer cell lines (4-5%) from 10 to 40 ms. These results indicate that low EP parameters affect cell viability in cancer cells more than normal cells. Structural differences in cell membrane of normal and cancer cells may be responsible for the lower EP effect in normal cells at 2 hours.



a) MCF-10A Normal Human Breast Cell Line          (b) MCF-7 Human Breast Cancer Cell Line



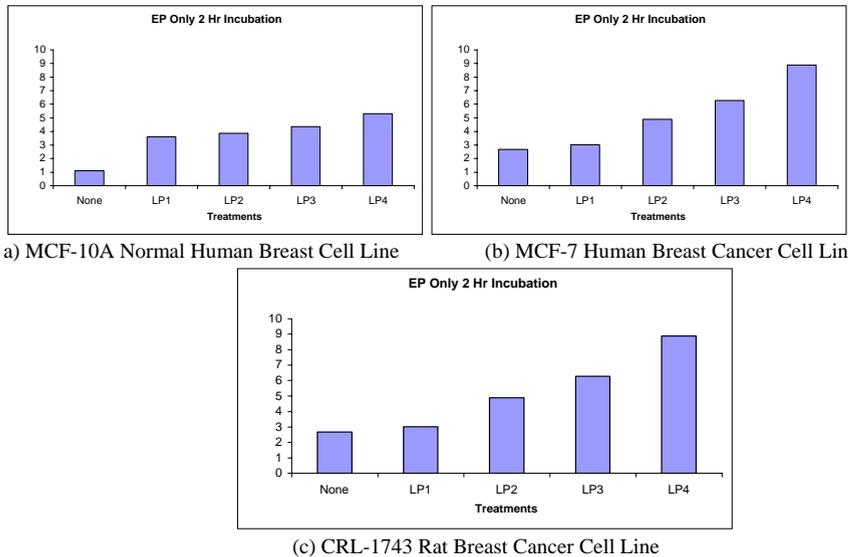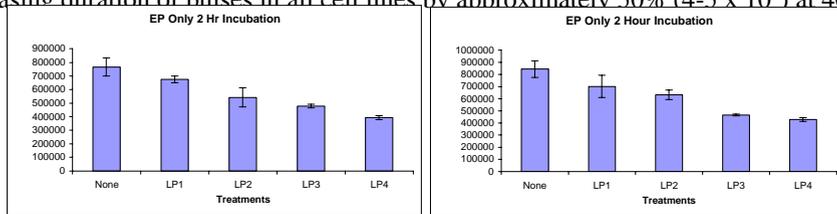(c) CRL-1743 Rat Breast Cancer Cell Line

Fig. 4. Effects of Electroporation Only on Cell Death After 2 hours of incubation. Cells MCF-10A (a), MCF-7 (b) and CRL-1743(c) were electroporated and cultured for 2 hours. The percent dead cells were determined as described in *Materials & Methods*. Bars represent average $\pm$ SEM, n=3.
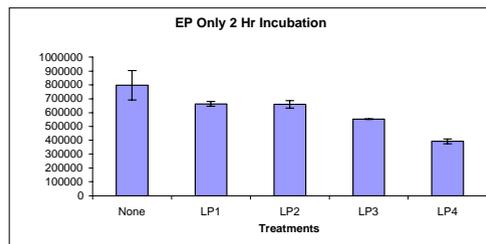
Cell growth was determined in MCF-10A (non-malignant MCF-7 human breast epithelial), MCF-7 (malignant human breast epithelial) and CRL-1743 (malignant rat

epithelial) cells after 24 hours of incubation. Voltage and number of pulses remained constant in all treatments. Only the duration of pulses were varied. No Tamoxifen was used in this set of experiments. Resulting total number of cells alive at 24 hours with varying pulse duration are illustrated in Fig. 5. After 24 hours, all cell lines receiving no EP exhibited similar cell growth ($8 \times 10^5$ cells/well). Growth was suppressed with increasing duration of pulses in all cell lines by approximately 50% ($4-5 \times 10^5$) at 40 ms.



(a) MCF-10A Normal Human Breast Cells   (b) MCF-7 Human Breast Cancer Cells



(c) CRL-1743 Rat Breast Cancer Cells

Fig. 5.  Effects of Electroporation Only on Cell Growth After 2-Hour Incubation.   Cell lines MCF-10A (a), MCF-7 (b) and CRL-1743(c) were electroporated then incubated for 2 hours.  The total number of cells was counted using a hemocytometer as described in Materials & Methods.  Tamoxifen was not used in this set of experiments.  Bars represent average $\pm$ SEM, n=3.

*B. Effect of Electrical Pulses on MCF-7 and CRL 1743 Cell Death and Cell Growth After 24 Hr Incubation.*

Cell death was determined for MCF-7 (malignant human breast epithelial) and CRL-1743 (malignant rat epithelial) cells after 24 hour of incubation.  Voltage and number of pulses remained constant in all treatments.  Only the duration of pulses were varied. No Tamoxifen was used in this set of experiments.  Resulting percentage of dead cells with varying pulse duration are illustrated in Fig. 6.  Percentage of cell death increased form 0.5% (no EP) to 8 % with increasing duration of EP pulses in MCF-7 human cancer cell line.  Lower percentage of dead cells were observed in the rat cancer cell line with an increase from 1% (no EP) to 3% with 30 ms pulse duration.

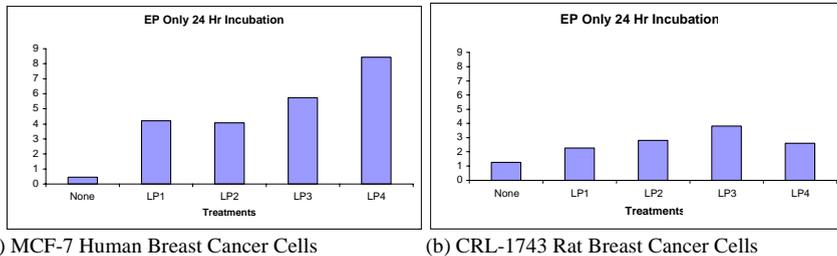(a) MCF-7 Human Breast Cancer Cells          (b) CRL-1743 Rat Breast Cancer Cells

Fig. 6. Effects of EP Only on Cell Death After 24-Hour Incubation. Cells MCF-7 (a) and CRL-1743 (b) were electroporated and cultured for 24 hours. The percent dead cells were determined as described in *Materials & Methods*. Bars represent average $\pm$ SEM, n=3.

Cell growth was determined in MCF-7 (malignant human breast epithelial) and CRL-1743 (malignant rat epithelial) cells after 24 hours of incubation. Resulting total number of cells alive at 24 hours with varying pulse duration are illustrated in Fig. 7. After 24 hours, all cell lines receiving no EP exhibited similar cell growth ($8 \times 10^5$ cells/well). Growth was suppressed with increasing duration of pulses in both CRL-1743 lines, whereas, in MCF-7's the amount of growth suppression remained relatively constant over 10-40 ms duration times. The degree of suppression differed between the two cell lines. Suppression of cell growth at 40 ms in MCF-7 cells was approximately 30%. In CRL-1743 cells growth was suppressed by approximately 60%.



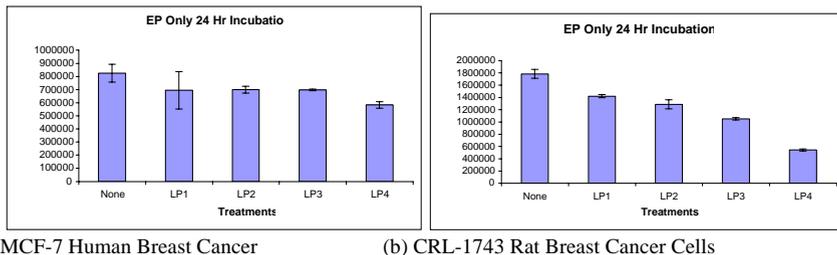(a) MCF-7 Human Breast Cancer          (b) CRL-1743 Rat Breast Cancer Cells

Fig. 7. Effects of Electroporation Only on Cell Growth After 24-Hour Incubation. Cell lines MCF-7 (a) and CRL-1743 (b) were electroporated then incubated for 2 hours. The total number of cells was counted using a hemocytometer as described in Materials & Methods. Tamoxifen was not used in this set of experiments. Bars represent average $\pm$ SEM, n=3.

*C. Effect of 1 µM Tamoxifen on MCF-7 and CRL-1743 cell growth After 24 Incubation*
Cells were incubated without or with 1µM tamoxifen. Fig. 8` shows the total number of live cells for each treatment after 24 incubation. Combined electo-endocrone therapy using 1 µM Tamoxifen in the human cell line produced a 35% decrease in live cell number with pulse duration of 40 ms at 24 hours (Fig. 7). No difference in the number of MCF-7 cells was observed between 10-30 ms pulse duration. CRL-1743 cells showed a decrease in number of live cells with increasing pulse duration. No difference between No treatment (none) and Tamoxifen only was observed (data not shown).
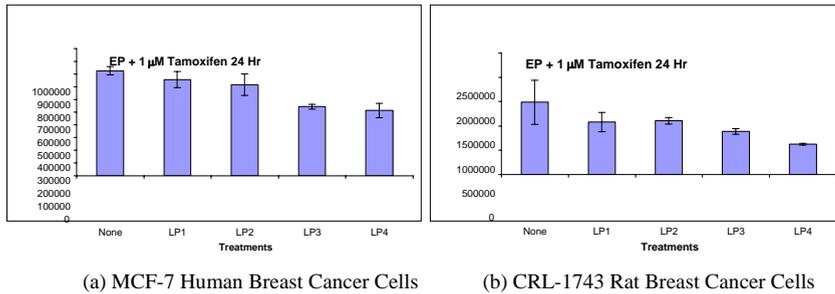
(a) MCF-7 Human Breast Cancer Cells          (b) CRL-1743 Rat Breast Cancer Cells

Fig. 8.  Effect of Electroporation + 1 µM Tamoxifen on MCF-7 and CRL-1743 Cells. MCF-7 (a) and CRL-1743 (b) cell lines were cultured and cell counts for live, dead and total numbers of cells were done for each experimental group as described in Materials & Methods.  Data represents number of live cells.  Cells exposed to different pulse durations received either no treatment (none) or 1 µM Tamoxifen and incubated and incubated for 24 hours.  Bars represent average + SEM, n=3.

*D.  Effect of 5 µM Tamoxifen on MCF-7 and CRL-1743 cell growth after 24 hours incubation.*

Cells were incubated without or with 5 µM Tamoxifen following EP. Fig. 9 shows the total number of live cells for each treatment after 24 hours.  In both rat and human cell lines combined electro-endocrine therapy produced a maximum 40% decrease in live cell number with pulse duration of 40 ms.  No significant difference was observed between no treatment and 10, 20 or 30 ms pulse duration in MCF-7 or CRL cells. Both cell lines showed a decrease in number of live cells with increasing pulse duration. No difference between No treatment (none) and Tamoxifen only was observed.
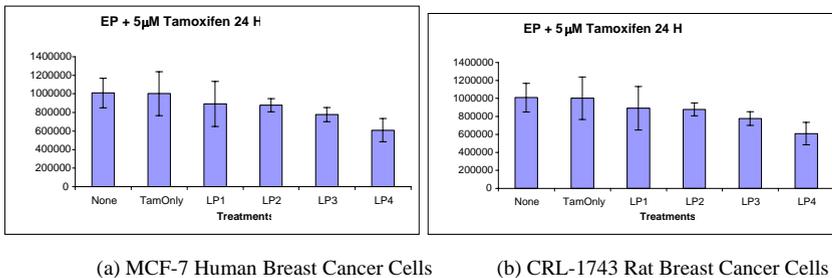


(a) MCF-7 Human Breast Cancer Cells          (b) CRL-1743 Rat Breast Cancer Cells

Fig. 9.  Effect of Electroporation + 5µM Tamoxifen on MCF-7 and CRL-1743 Cells. MCF-7 (a) and CRL-1743 (b) cell lines were cultured and cell counts for live, dead and total numbers of cells were done for each experimental group as described in Materials & Methods.  Data represents number of live cells.  Cells exposed to different pulse durations received either no treatment (none), 5µM Tamoxifen or EP + 5µM Tamoxifen and incubated for 24 hours.   Bars represent average $\pm$ SEM, n=3.

*E. Effect of 10 μM Tamoxifen on CRL-1743 cell growth after 24 hours incubation*

Cells were incubated without or with 10 μM Tamoxifen following EP. Fig. 10 shows the total number of live cells for each treatment after 24 hours. In the rat cell line combined electro-endocrine therapy using 10 μM Tamoxifen produced a decrease in live cell number with increasing pulse duration. A maximum 80% decrease in live cell number was observed with pulse duration of 40 ms. Significant differences were observed at each pulse duration used. A difference between No treatment (none) and 10μM Tamoxifen only was observed. Similar results have been observed in our lab with MCF-7 cells (data not shown).
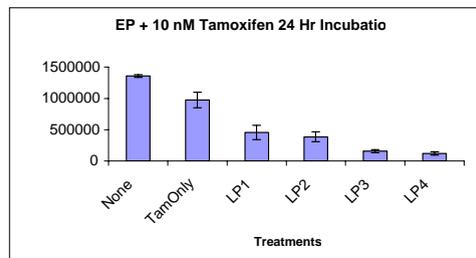


Fig. 10. Effect of Electroporation + 10 M Tamoxifen on CRL-1743 Cells. CRL-1743 cell lines were cultured and cell counts for live, dead and total numbers of cells were done for each experimental group as described in Materials & Methods. Data represents number of live cells. Cells exposed to different pulse durations received either no treatment (none), 5 M Tamoxifen or EP + 5 M Tamoxifen and incubated for 24 hours. Bars represent average $\pm$ SEM, n=3.

*F. FACS analyses*

Fig. 11 illustrates the flow cytometry results for MCF-7 cells for control, electroporation only (10ms duration) and electroporation + 5μM Tamoxifen. In these figures, "G" denotes apoptotic cells, 'c" or "G0_G1" denotes cells in the G0 or g1 phase, "D" denotes cells in the S phase, "E" peak or "G2_M" denotes cells in the M or G2 phase, and "E" denotes debris. Fig. 12 shows a comparison of the quantitative data for various durations for control, electroporation only and electroporation + 5μM Tamoxifen concentration conditions. An analysis of the data shows that there is more number of cells in the G0_G1 phase when electroporated with Tamoxifen at 5μM than with electropporation only (Fig. 12a and b). Referring to Fig. 13, the illustration showing the various stages in a cell cycle, it can be seen that in this phase, the cells are undergoing pause, which reveals that the tamoxifen is taking effect inside of the cell. However, more cells are also in S phase under electroporation + Tamoxifen condition that with electroporation only. More work is required on this and is in progress.
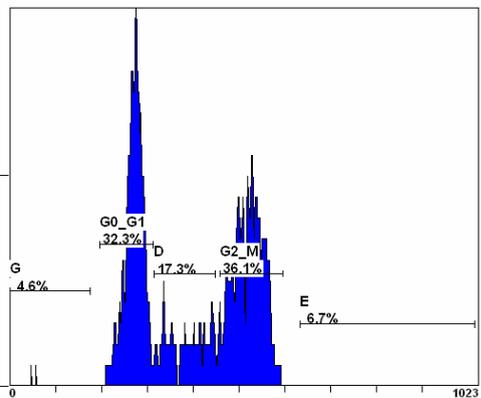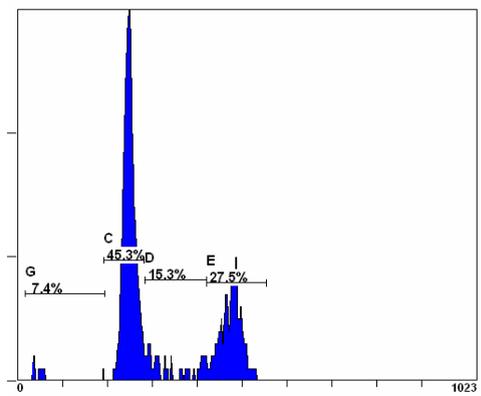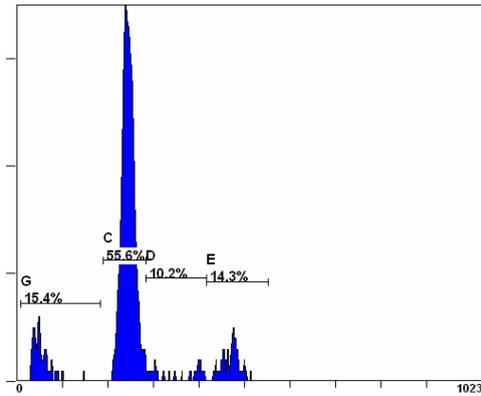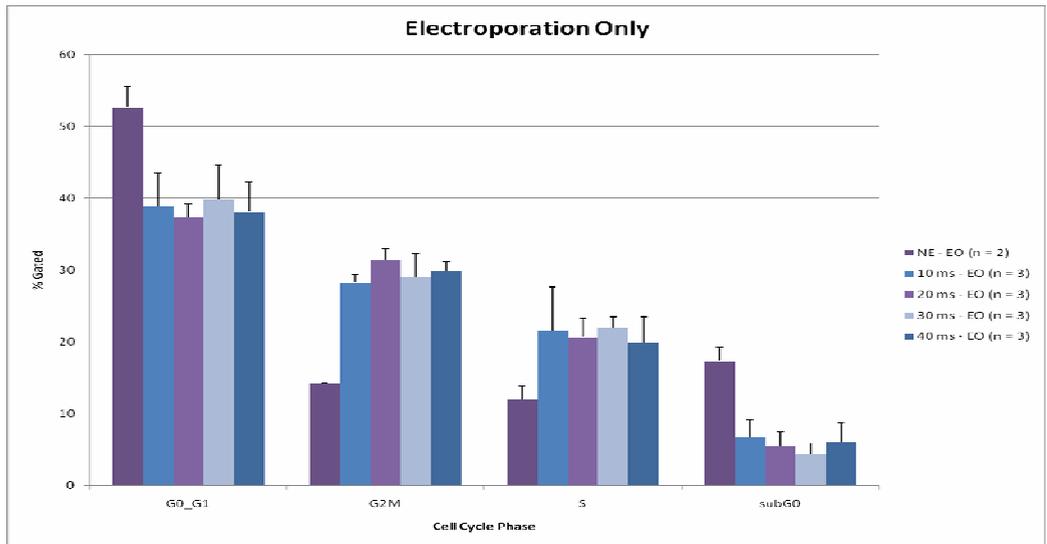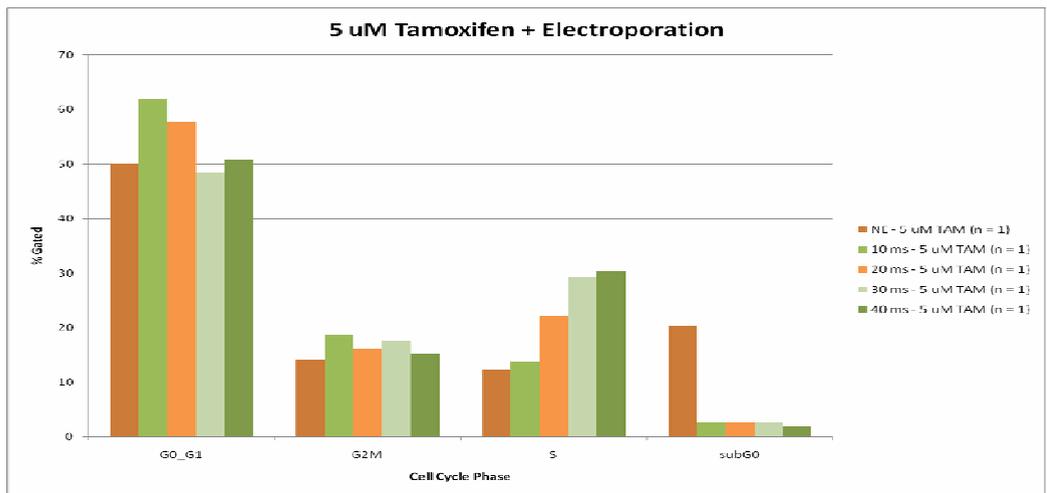
Fig. 11. Flow cytometry diagrams illustrating the percent cells in each phase of the cell cycle. (top) control (no electroporation and no Tamoxifen), (middle) electroporated without Tamoxifen – 200V/cm, 10ms pulses, 8 pulses at 1s interval (bottom) electroporated with Tamoxifen – 200V/cm, 10ms pulses, 8 pulses at 1s interval.



(a)     Electropoartion Only



(b) Electropoartion+5uM Tamoxifen Only

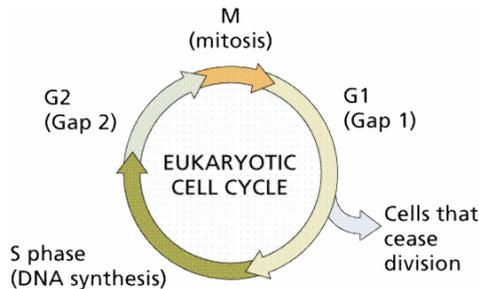Fig. 12. Influence of Electroporation+Tamoxifen on Cell Cycle Stages

Fig. 13. Eukaryotic Cell Cycle

## IV. Summary

The MCF-7 cell line is the most widely used and best characterized of all the human breast cancer cell lines [11]. It was first isolated from the pleural effusion of a postmenopausal 69 years old adult Caucasian woman. The patient had received radiotherapy and endocrine therapy before the appearance of effusion. The estradiol-dependence for growth, antiestrogen sensitivity, and low metastaic potential of MCF-7 cells has led to the hypothesis that they represent an early epithelial carcinoma of the breast [12]. The MCF-7 line retails several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. The MCF-7 cells used in this study are ER+.

The MCF-10A cell line is described as a spontaneously immortalized "normal" human breast epithelial cell line [13]. These cells were isolated from a 36 years old Caucasian patient with fibrocystic disease. After 849 days in culture, it was designated as the MCF-10A and this cell line has been established since then. They resemble luminal epithelial cells rather than myoepithelial cells, and express antigens for several keratins and epithelial sialomucins [14]. Its hormonal status is ERα negative.

CRL 1743 cells are a rat mammary epithelial cell line from an N-nitromethylurea (MNU)-induced tumor. These cells are ER+ and are comparable to MCF-7s. As the rat model is an excellent model fort human cancer, the CRL 1743 cells were studied in this research along with MCF-7 and MCF-10A cells.

Our initial study results indicate that the three cell lines are receptive to electroporation, and, that there is a difference in the sensitivity of these cell lines to the application of electrical pulses. Cancer, like many other diseases accompanied by strong metabolic disorders, shows characteristic effects on cell lines. It is of practical interest to compare non-malignant cells and malignant cells. Our results demonstrate that electroporating cells in conjunction with tamoxifen is a more effective treatment than with tamoxifen alone. Electroporation is effective in increasing membrane permeability because it enhances the uptake in the cells. These results support the theory that electroporation will increase the cell's response to tamoxifen and enhance its capabilities.

Electrochemotherapy is ideal for patients with cutaneous or subcutaneous tumors, of any histologic type, which are recurrent, inoperable, or progressive or metastatic and unresponsive to standard chemotherapy and/or radiotherapy regimens [9]. Sometimes, surgery is not an option [2]. In these cases, alternate treatments are necessary and electrical pulse-mediated enhanced drug uptake is a simple, quick (it only takes a few minutes per nodule) and out-patient-based [2]. No costly equipment similar to those for radiation therapy is not needed. The applied electric pulses (short and intense square-wave pulses) have no apparent cytotoxic or systemic effects. Unlike radiation, the same spot can be treated more than once, if needed. Extension of this to electro-endocrine therapy is our goal as Tamoxifen is not always received well by all the ER+ women [7].

In summary, this translational study begins to provide the conceptual foundation for the clinical use of EP towards breast cancer treatment. This method can be used to substantially increase the local concentrations of tamoxifen, or several other therapeutic molecules. We also provide evidence that low voltage pulses are effective towards permeabilization, and may be able to abolish clinical symptoms caused by high voltage pulses (low level pain, muscle contractions, small burn marks). Our data validate the use of cancer cell lines as models for the development and testing of novel therapeutics aimed at curing breast cancer in a better way.

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