ELECTROCHEMOTHERAPY WITH GALLOFLAVIN FOR EFFECTIVE TRIPLE NEGATIVE BREAST CANCER TREATMENT: AN IN VITRO MODEL STUDY

by

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Dedication

To my Amma, Daddy, Shru, Ammuchi, Dhiya and Macha.

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First and foremost, I would like to thank major advisor, Dr. Raji Sundrarajan, for accepting me, guiding me and supporting me in my every step.

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LIST OF ABBREVIATIONS

ECT	Electrochemotherapy
LDH	Lactase Dehydrogenase
GF	Galloflavin
EP	Electroporation
CTRL	Control
LA	Lactate
ROS	Reactive Oxygen Species

GLOSSARY

Electroporation	Electroporation is the process by which drugs are introduced into the cells by application of electric pulses to the tumor area (Haltiwanger, 2014).
Electrochemotherapy	Electrochemotherapy is chemotherapy followed by electroporation, based on administration of electric pulses (Sersa et al., 2008).
Viability	Ability of the cells to continue normal metabolic functioning and the amount of healthy cells (Mittal, et at, 2021).
Dosage Curve	The process of evaluating the performance of the drug on various dosages and the live cell count (Majiduddin et al., 2002).

ABSTRACT

One in eight woman develop breast cancer in the United States of America and is the most common type of cancer in the world. Breast cancer has the highest rate of death compared to any other form of cancer. Triple Negative Breast Cancer (TNBC) is the most lethal type of breast cancer, which is the most fatal of all breast cancer types. TNBC is onerous to treat since it lacks all the three most commonly targeted hormones and receptors. Current patients afflicted with TNBC are treated with platinum core chemotherapeutics, namely Cisplatin. Despite the anticancer effects shown by Cisplatin, TNBC attenuates its effect and develops a resistance eventually, which results in reoccurrence of TNBC after few years. Hence there is a demand for effective and alternative ways to treat TNBC. To inhibit the TNBC cell proliferation, blocking the key glycolytic enzyme Lactase Dehydrogenase B (LDHB) is studied and validated. Galloflavin (GF), a proven LDHB inhibitor is utilized in this series of studies and analysis. In addition, Electrochemotherapy, which involves the application of electrical pulses (EP) were utilized to enhance the uptake of GF. The combination of Electrochemotherapy (ECT) with LDHB is a novel way to treat TNBC to produce an alternative to traditional chemotherapy. EP+GF will be subjected onto TNBC cells at various concentrations and pulse parameters. The purpose of this study is to test the effect of alternative chemotherapeutic drug delivery methods for TNBC patients for decrease in mortality rate and improve quality of life. Results indicate TNBC cell viability is the least for EP+GF treatments and the maximum Reactive Oxygen Species (ROS) levels and a maximum decrease in Glucose and Lactate uptake for EP+GF treatments relative to control. Immunoblotting studies indicate the inhibition of LDHB is the most on EP+GF treatments, indicating that this could be a novel modality to treat TNBC.

CHAPTER 1. INTRODUCTION

1.1 Background

More than 2,099,600 new cases of breast cancer were reported in the world in 2021 by World Health Organization (*Cancer,2021*). Out of which 15 – 20% are TNBC. TNBC lacks all the three most commonly targeted hormones and receptors, namely the estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2/EGFR2) amplification. Triple Negative Breast Cancer is difficult to prognose; hence, the high recurrence among breast cancer types (Giri et al., 2021). The rate of survival for TNBC patients is 30% compared to 66% for the other breast cancer phenotypes (Dent et al., 2007). Hence, effective and alternative ways to treat TNBC demands serious attention. Triple Negative Breast Cancer cells are characterized to have Lactase Dehydrogenase (LDH) enzyme, which increase their Lactate production in both aerobic and anaerobic respiration during ATP production. Lactase dehydrogenase is classified into various subunits, among LDH subunits, LDHA and LDHB are seen to be significantly upregulated during TNBC proliferation (Valvona et al., 2016). Otto Heinrich Warburg, a Nobel Laureate identified an abnormality which was later termed as Warburg effect (Otto, 2016). Production of Lactate is commonly seen in anaerobic respiration, when lack of Oxygen is delivered to mitochondria. But after glycolysis Lactate was produced even when abundant Oxygen is delivered to mitochondria during aerobic respiration which is not normal and classified as Warburg effect. It was found that during Warburg effect LDHB was expression was significantly high TNBC cell line with glycolytic, basal-like phenotypes. (JB Dennison, 2013). LDHB was significantly upregulated in TNBC cell line (Urbańska & Orzechowski, 2019). Hence, LDHB, a key gene in glycolysis is reported as an essential gene for TNBC (McCleland et al., 2012). Therefore, we are specifically interested in studying the effects of electrochemotherapy on the LDHB expression in TNBC in vitro models.

The downregulation of LDHB is an apt method to inhibit tumour cells and administering drugs into the cells followed by controlled local administration of electric pulses to the tumour area generates to pores present in the cell membrane to expand and permeabilize. A temporary permeability was caused and allowed the drug to better reach the tumour cells; such therapy is termed Electrochemotherapy (ECT) (Sersa et al., 2008). Electrochemotherapy does not depend

upon the cell receptors to target the treatment, as the drug molecules no longer depend upon the receptors to influence the cellular signalling. A combination of LDH and Electrochemotherapy is studied for the first time to provide a better alternative to traditional chemotherapy.

Galloflavin (3,8,9,10-tetrahydroxypyrano benzopyran-2,6-dione), a proven inhibitor of LDHB is a natural compound identified in 1897 by German chemist Bohn and Graeb, by synthesizing gallic acid with potassium hydroxide at 0°C (Ierzig, 2012). Galloflavin Molecular formulae is C12H6O8 and formal name "3,8,9,10-tetrahydroxy-pyrano[3,2-c][2] benzopyran-2,6-dione". Figure 1.1 illustrates the structure of Galloflavin. Galloflavin was originally used in chromium dyeing of wool and silk. Researchers from the University of Bologna have found that this substance is a very potent inhibitor of both forms of LDH. Lactase Dehydrogenase B catalyses the change of pyruvate to lactate in the final stage of glycolysis (Manerba et al., 2012). Adenosine triphosphate (ATP) production in tumour cells is dependent on glycolysis and one way to stop tumour growth is to inhibit glycolysis by inhibiting LDHB and prevent tumour cells from metabolizing glucose without affecting the energy balance in normal tissues. Galloflavin mainly binds to the free enzyme in the cytoplasm, but does not compete with pyruvate or NADH molecules. The binding to the free enzyme is with the help of Hydrogen bonds present in the Galloflavin.

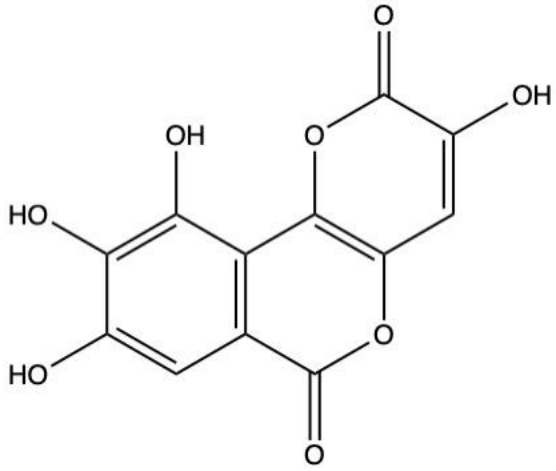


Figure 1.1. Chemical Structure of Galloflavin

Galloflavin, an inhibitor of both LDHB isoenzymes cultured in tumour cells, blocked aerobic glycolysis and ATP production already at micromolar concentrations (Manerba et al., 2012). The inhibition of LDHB is by binding of galloflavin to the NADH. Galloflavin blocks both aerobic

glycolysis where high differences in their energy metabolism were found in some human cancer cell lines and also blocked the growth of cell lines that were resistant to other chemotherapeutics. The predominant form of action against cancer cells has been the induction of apoptosis (Farabegoli et al., 2012). Galloflavin is shown to be effective in LDHB inhibition and blocks aerobic glycolysis without interfering with cell air intake and inducing cell death. Galloflavin is found to be less toxic and has sole function of LDHB inhibition. Lethal side effects were not observed in Galloflavin; some of the rare side effects may include irritation to respiratory membranes, harmful when in direct contact with skin and eye (Calvaresi et al., 2013).

1.2 Problem

Triple Negative Breast Cancer cells are difficult to treat without a quality-of-life compromise (Dent et al., 2007). Breast cancer leads in mortality and incidence worldwide (*Globocan, 2012*). The 5-year survival rate is the least for Triple Negative Brest Cancer among various classifications of breast cancers (*Global Cancer Observatory, 2021*). One woman every eight minutes is diagnosed with TNBC. The problem addressed by this study is on lack of effective therapies to tackle TNBC and an immediate requirement for alternative therapies.

1.3 Significance

This research aims to study and investigate an effective, alternate therapy for TNBC patients, utilizing the synergy of electrical pulses and Galloflavin. First, the optimal electrical pulse parameters and Galloflavin concentration were identified for the maximum cell death in TNBC cells, while minimizing the cell death in non-cancerous cells, using the cell viability assays. This research aims to study the enhanced effects observed under the electro-Galloflavin-therapy, employing a high-throughput mass spectroscopy-based proteomics approach. The proteomics profile provided information of thousands of cellular proteins involved in the cellular processes, enables a comprehensive understanding of the electro-galloflavin-therapy mechanism. The results obtained from this research is expected to indicate the suppression of LDHB and that electro-galloflavin-therapy can specifically target TNBC cells by influencing multiple pathways and would be antiproliferative towards TNBC.

1.4 Purpose

With the rise of the breast cancer cases and the high recurrence and mortality rates for the TNBC patients, it is important to develop effective, alternate therapies against TNBC. The absence of the three main receptors and thus the lack of targeted therapies for TNBC, create a gap in the TNBC treatment. The purpose of this research is to examine if the electro-galloflavin therapy can be an effective and alternate therapy for TNBC and gaining insights as how the electro-galloflavin therapy affects the LDHB and other cellular pathways in TNBC cells to have enhanced effects. This work establishes a first step towards the clinical application of the electro-galloflavin therapy to benefit TNBC patients.

1.5 Research Questions

The research study answered the following research questions:

1. Is the electro-galloflavin therapy effective in targeting TNBC in vitro models?
2. What are the optimal electrical pulse parameters and the galloflavin dosage to inhibit LDHB effectively?
3. Can electro-galloflavin-therapy, which utilizes an electro-physical phenomenon, suppress LDHB and manipulate multiple signalling pathways in TNBC cells, when coupled with the Galloflavin?

1.6 Limitations

The limitation of this research is that this study is limited to *in vitro* model. Therapies can be tested on preclinical models after proteomics validation and on drug toxicity validation.

1.7 Delimitations

The scope of this study is limited TNBC Cell line. The voltages applied will be limited to the range of 800V/cm to 1200 V/cm, and the drug concentrations up to 150µM.

CHAPTER 2. REVIEW OF LITERATURE

2.1 Methodology

The sources used in this Literature Review are research papers, published in scholarly journals and conferences including “IEEE, Journal of Clinical Oncology, Journal of the National Cancer Institute, A Cancer Journal for Clinicians” and various others. Some sources were chosen on the recommendations of dissertations from reputed Universities. The cited sources are of high quality because more than 50% of the sources are from Purdue library. The remaining are from World Health Organization website and articles referenced by World Health Organization. Several sources were chosen based on the recommendations of the Graduate Committee members for understanding concepts pertaining to the research. Figure 2.1 shows a concept map connecting the key concepts.

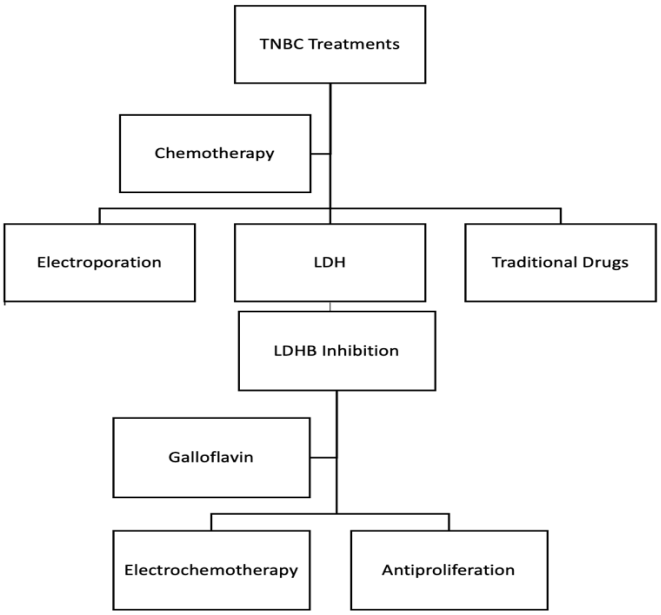


Figure 2.1. Concept map connecting the key concepts.

2.2 Databases and Tools

The databases and tools used for this study are mentioned below:

1. Purdue Libraries: Purdue libraries website has an array of databases. Provided access to

multiple databases and websites including Engineering Village and ProQuest. ProQuest provided a massive collection of journals, research articles and dissertations/theses related to search terms.

2. Google scholar: Google scholar also has peer reviewed scholarly articles and research papers published in all major conferences and journals

3. ACM: ACM is a research library which maintains publications from high impact journals and conferences.

2.3 Strategy

For all the described databases, users have the option of accessing the citations, which was used to trace other relevant papers, journals and theses using similar concepts. Figure 2.2 shows a Venn diagram on the search strategy used for finding the required research papers.

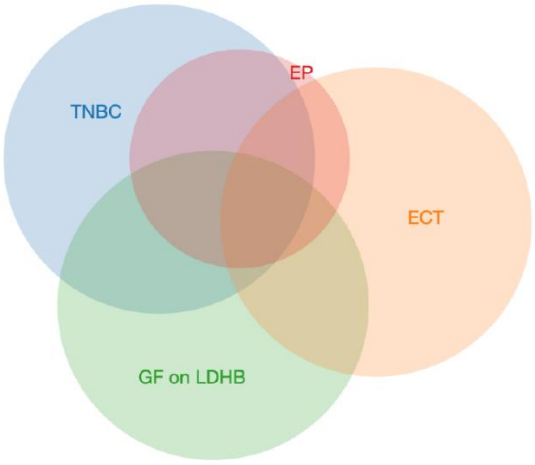


Figure 2.2. Venn diagram on the search strategy

2.4 Current Treatments for TNBC patients

Triple Negative Breast Cancer is classified under grade 3 tumor (Lips et al., 2013). Patients afflicted by TNBC will test negative for the commonly hormonal targets, such as ER, PR, and HER2. Triple Negative Breast Cancer patients are currently being treated by chemotherapeutics, surgical procedures and radiation therapies. Some treatments include a combination of more than one of the above listed procedures. The quality of life of TNBC patients are very poor (Shen et al., 2020).

Despite the excellent anticancer effects shown by conventional chemo drugs, their severe side-effects cannot be ignored. Therefore, the natural compounds or chemotherapeutics derived from natural compounds have received much needed attention for their anticancer potential over the last three decades. A total of 85% in the present world population rely on natural products for some part of essential health support (Ekor, 2014). Recent studies have shown that TNBC also damages the metabolic regulation in the rate of glycolysis, lactate production and Glutamine production (Poggio et al., 2018). Therefore, the interest has been growing in the advancing of unique therapeutic methods beyond conventional chemotherapy TNBC patients. Increasing evidence suggest that the platinum salts (cisplatin and carboplatin) based single agent or neoadjuvant therapy may be particularly effective for sporadic and BRCA1-deficient metastatic TNBC (Cabezón-Gutiérrez et al., 2017). Studies have demonstrated an improved overall response rate of 23-35% as the single agent and significantly increased pathological complete response from 37% to 52% in neoadjuvant setting for cisplatin and carboplatin in TNBC, renewing the interest towards platinum salts (Foulkes et al., 2010). However, the use of platinum-based therapies come at the cost of increased toxicity and increased anti-drug resistance (Greenman et al., 2007).

2.5 Novel Approach, Treatment and Analysis towards TNBC

Inhibiting one of the stages of energy production will act as an antiproliferative mechanism. Galloflavin derived from natural gallic acid shows potential in blocking the energy cell cycle energy production by inhibiting both forms of LDH. The intracellular ripple effects should be studies to substantiate the intricacies involved in the process which play a vital role in inducing apoptosis in TNBC cell line. The toxicity were analyzed for on non-cancerous human epithelial cell line to validate the less effect to no effect in viability of noncancerous cells, which would promote the quality of life for patients. The viability studies were studied to understand on the effect of Galloflavin inducing permanent damage. The study of up and down regulated proteins is done with the effect of various treatments to understand the key metabolic pathways. Despite the number of pathways and processes involved in cancer is large, their count is finite (Patterson & Aebersold, 2003). This makes it theoretically possible to visualize all the possible pathway involved in cancer development and treatment, if enough observations are recorded on all the elements that create the system (Patterson & Aebersold, 2003). Since most treatments targets

proteins, a method which can effectively analyze the cell wide protein levels can contribute directly to the rapid drug development (Majiduddin et al., 2002).

Electrochemotherapy is a clinically tested and proven technique to address TNBC, the combination of electroporation and chemotherapy can target multiple factors. Electrochemotherapy, which involves the electrical pulse application can be effective against TNBC, without causing severe toxicity and drug-resistance as ECT does not rely on the receptors or hormones which target cancer cells (Probst et al., 2018). The controlled local administration of electric field opens up porous layers in the cells, which temporarily converts the membrane permeable into otherwise hydrophilic membrane, in a phenomenon called Electroporation (Son et al., 2014). The biological cells contain multiple membranes, from the plasma membrane at the outer boundary of cells to the membranes of nucleus, mitochondria, and other subcellular organelles (James C. Weaver & Schoenbach, 2011). The cell membranes made up of phospholipids and proteins and are selectively permeable to external molecules (Kotnik et al., 2012). The primary role of these membranes is to separate the regions of different materials, while facilitating a controlled flow to maintain the distribution of selected ions and molecules between the regions to ensure a proper cellular function (James C. Weaver & Schoenbach, 2011). Electrical properties to the cells, as the ion distribution on both sides of the membrane makes it an electrical capacitor, and the ionic movement through the specialized transmembrane ion channels creates a flow of electric current through the membrane (Haltiwanger, 2014). These capacitive and conductive properties of the cell membrane make it an excellent leaky dielectric with dielectric constant of approximately 5 and resistivity of more than 109Ωcm (Xiao et al., 2011). The cell membranes are phospholipid bilayers, due to the nonpolar-hydrophobic and polar-hydrophilic ends of the phospholipids in the bilayer, which selectively allow the flow of sodium, potassium, chlorine, and other charged mineral ions. These ions accumulate on both sides of the membrane in different concentrations, creating an imbalance in charge distribution (Weaver, 2003). This imbalance causes the outer cell membrane surface to be highly positive than the inner membrane surface to create a potential, called membrane potential (Haltiwanger, 2014). This administration of electric pulses will increase the potential across the membrane and during high intensity of 0.5V or more pore formation takes place (Zimmermann et al., 1974).

In addition, to identify the multiple signaling effects induced by ECT with Galloflavin, proteomics profile of thousands of proteins expression at a time, providing a holistic understanding

of the phenomenon, was used as an effective strategy. The mechanistic understanding gained for the effects induced by ECT help to improve this technique further in clinical applications, by eliminating the off targets. However, the application of proteomic technologies is not yet evaluated for ECT applications. More research is needed to prove a significant correlation between identified biomarkers and ECT action mechanism using the proteomics and the subsequent success of the treatment in cell and animal models to open the way to enhance this electrical pulse-based technique to clinical practices. The results from the proteomics findings is validated by multifarious assays.

CHAPTER 3. RESEARCH METHODOLOGY

3.1 The Cell Lines

Triple Negative Breast Cancer cell line MDA-MB-231 was studied in detail. To support the various findings, selected studies were also performed using MCF7 and MCF10A cell lines.

MDA-MD-231 is a TNBC cell line, first deduced from an afflicted 51-year-old Caucasian female with the condition. The MCF-7 cells cell line is a breast cancer cell line which tests positive for estrogen receptor positive hormone. The MDA-MB-231and MCF-7 cell lines were incubated and cultured in DMEM containing 10% FBS along with 1% Penicillin-Streptomycin (PS) at suitable humidity and optimal growth conditions. They were cultured in optimal growth conditions with presence of DMEM along with 10% FBS and 1% PS

The MCF10A cells (non-cancerous epithelial) were cultured in optimal growth conditions with presence of DMEM:Ham's "F12 supplemented with 5% horse serum 20ng/ml human epidermal growth factor (Sigma-Aldrich, USA), 0.5mg/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10mg/ml bovine insulin (Sigma-Aldrich), 100 IU/ml penicillin and 100mg/ml streptomycin. The cells were incubated at 70%–80% humidity, 5% CO2, and 37°C." as before (Mittal et al., 2020)

3.2 The Drug

Galloflavin's (Cayman Chemical) stock solution with 35mM was prepared by dissolving Galloflavin in Dimethyl sulfoxide. The calculated volume of Galloflavin stock were diluted and exposed in the cells to obtain 20-150μM Galloflavin treatment concentrations.

3.3 Electric Pulse Application

The MDA-MB-231 cells were collected for electroporation in a concentration of 1×10^6 cells/ml in appropriate cell culture media and suspended at 600μl per cuvette with 4mm gap between the cuvette walls. The experiments were conducted in triplicates. Galloflavin replicates and electroporation only replicates were subjected to multifarious voltages utilizing BTX-ECM830 electroporator. Galloflavin replicates and electroporation only replicates was exposed to 700-1200V/. The parameters used were eight electrical pulses (EP) with unipolar,

square pulses, of duration 100µs, and 1s (1Hz) interval. Galloflavin only replicates and Control replicates were not subjected to electric pulse treatment.

3.4.1 MT Real Time Viability Assay

Samples include control (no treatment), GF only, EP only and EP+GF. They were seeded into 96-well plates. In the case of MDA-MB231, 20,000 cells were used as per Promega's protocol. The cells are supplied with 55µl of prepared media in each well. The cells are then incubated with optimal growth conditions for 12 h to 72h time periods. To study the viability and metabolic activities, "RealTime-Glo MT Cell Viability Assay (Promega, USA)" were used. "Synergy LX Multi-Mode Reader (BioTek Instruments, USA)" was utilized for recording the luminescence (Lum). All of the samples were normalized with respect to control and Equation 1 was used to calculate the viability % as previously(Giri et al., 2021).

$$Cell\ Viability\ (\%) = \frac{\text{Treated cell Luminescence value}}{\text{Control Luminescense}} \times 100 \qquad (1)$$

Figure 3.1 illustrates the workflow for carrying out the initial stages of viability measurements as reported previously (Giri et al., 2021).

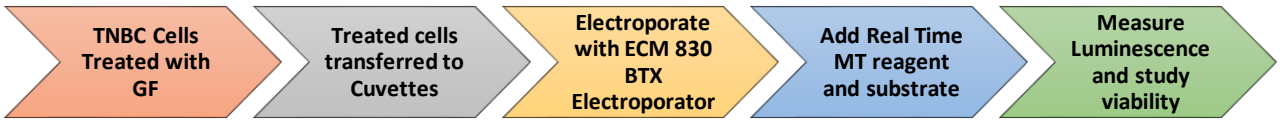


Figure 3.1. Workflow for Viability Measurements

3.4.2 Colony Forming Assay

MDA-MB-231 cells after Electroporation and drug treatments were incubated with optimal growth conditions for 12h to 72h with appropriate media in 100ml Petri dishes with 1000 cells on each Petri dish for a period of 9 to14 days. After the colonies are formed they were stained with 1.5ml of "crystal violet (Sigma-Aldrich)" and were counted using a custom MATLAB code

(provided in Appendix), which thresholds, segments and counts the number of colonies. A collection of cells of >50 cells was counted as 1 colony.

3.5 Western Blotting

The cultured, MDA-MB-231 cells treated with control (Ctrl), EP only and EP+GF were incubated for 12h period and all the cells were scraped from the Petri dish surface and washed with 4℃ PBS. The treated cell pellet was collected in "RIPA lysis buffer (Thermo Fisher Scientific, USA)" and sonicated for total of 40s with 10s on and 10s off time interval at 15% amplitude intensity. The debris were avoided by centrifugation at 14000 rpm for 20 minutes and collecting only the supernatant. "The Pierce BCA Assay (Thermo Fisher Scientific, USA)" was utilized to quantify the protein using albumin as standard protein. The 4 × Laemelli buffer added into protein 17μg and ensured equal concentration of proteins were seeded into the wells. After sample buffer addition the samples were heated at 100℃ to denature the proteins and later cooled before seeding. Biorad Precast Gels were utilised samples were run at 200 V for 1.3h. SDS-polyacrylamide gel electrophoresis were processed to isolate the proteins and passed onto a polyvinyl difluoride (PVDF) membrane.. The PVDF membrane was incubated at 4℃ overnight in the cold room without light with 3% BSA Blocker. The blocked membrane is treated primary antibodies for "LDHB (rabbit, PA5-35365; Thermo Fisher Scientific)" and "β-tubulin (mouse, E7; DSHB, University of Iowa)". The blocked membrane is washed with PBST three times and subjected to "Alexa Fluor antibody (anti-rabbit, A-21109 and anti-mouse, A11375; Thermo Fisher Scientific)". The blots were washed again and collected in PBST before imaging, Blots were imaged by infrared imaging analysed using ImageJ.

3.6 Reactive Oxygen Species Assessment Assay

Triple Negative Breast Cancer cells were treated and seeded back to 96-well plates with 60μL of DMEM with 10% FBS and 1% PS and set to rest for 12h. At 7h time point the substrate solution from "ROS-Glo H2O2 assay kit (Promega)" was supplied to the cells and left in suitable growth conditions for 5h. During 12h time point ROS-Glo detection solution was added and the luminescence was recorded. Synergy LX Multi-Mode Reader (BioTek Instruments, USA)" was

utilized for recording the luminescence (Lum). All of the samples were normalized with respect to control.

3.7 Metabolites Uptake and Detection assays

After treatments cells were incubated for 12h period in 100mm petri dishes all the cells were scraped and washed with cold PBS stored at 4 °C. The treated cells were collected and counted to ensure each well of is supplied with 35,000 cells. Which include "glucose uptake (Glucose Uptake-Glo Assay, Promega), lactate (Lactate-Glo™ Assay, Promega), glutamine, and glutamate (Glutamine/Glutamate-Glo Assay, Promega)" (Mittal, Camarillo, et al., 2020).

3.8 Statistical Analysis and Validation

All experiments were done in biological and experimental triplicates. Standard error of the replicates with respect to the same treatments were computed. Experiments with standard error higher than 10% were repeated until the repeatability and standard error less than 10% was obtained consistently. To establish the significance between the different treatment data, analysis of variance was utilized. The level of Confidence was set to 95% along with constant variance, data following a normal curve was confirmed prior to Analysis of Variance tests. Data from Assays were also subjected to Tukey's comparison test to establish normality and homoscedasticity on TNBC cell treatments and activities.

3.9 Proteomics Study

Triple Negative Breast Cancer cultured cells were treated with Drug only and EP+GF and were incubated for 12 hour period and all the cells were scraped from the Petri dish surface and washed with 4°C PBS. The treated cell pellet was collected in cold PBS.

The LC-MS/MS data was collected using a reverse-phase HPLC-ESI-MS/MS system (Thermo Fisher Scientific) consistingof UltiMate™ 3000 RSLCnano and a Q-Exactive (QE) High Field (HF) Hybrid Quadrupole Orbitrap™ MS and with a Nano-spray Flex ion source. The samples with various treatments in multiple replicates was analysed on a standard data-dependent acquisition mode. Mobile phase solvent A was 98% purified water/2% acetonitrile (ACN)/0.01% formic acid (FA), and solvent B was 80% ACN/20% purified water/0.1% FA. Peptides (1 µg) were

loaded onto a trap column (300 µm ID × 5 mm, 5 µm 100 Å PepMap C18 medium) at 5 µL/min flow rate. After 5 min, trap column toggled to in-line with Acclaim™ PepMap™ RSLC C18 (75 µm × 15 cm, 3 µm 100 Å PepMap C18 medium, Thermo Fisher Scientific) analytical column and peptide was separated using 120 min LC gradient method at 35 °C. A 5–30% linear gradient of solvent B was run for 80 min, followed by 11 min of 45% solvent B and 2 min of 100% solvent B with an additional 7 min of isocratic flow. Solvent A was applied at 95% for 20 min for column equilibration. A significantly regulated data-dependent MS/MS scan method was used to recieve MS data. Injection timing was set to 100 milliseconds, resolution to 120,000 at 200 m/z, spray voltage of 2 eV and an AGC target of 1 × 106 for a full MS spectrum scan with a range of 400–1650 m/z. The MS/MS scans were obtained with a resolution of 15,000 at 200 m/z. The dynamic exclusion was set at 30 sec and the above procedure was performed as before (Mittal, 2019). MaxQuant (v1.6.1.0)71, 72 was utilized to process MS/MS data against the Uniprot Homo sapiens fasta. Cleavage enzymes was established as Trypsin/P and LysC. Mass errors were left at 10 ppm and 20 ppm for precursor and fragment ions, respectively. The LC-MS/MS data and LFQ intensities were recorded as before (Mittal, 2019). MaxQuant (v1.6.1.0)71 software was used to analyze MS/MS data with respect to Uniprot Homo sapiens. The analyzed data with containing intensity information about the regulated proteins were gathered and segregated based of the LFQ intensity and MS/MS data points. The proteins with null values of LFQ and MS/MS were avoided along with proteins containing MS/MS less than 2 were removed. The significantly regulated were processed upon deducing the average of the log2 values between proteins from GF vs EP+GF. Proteins with fold-change of $|\Delta \log 2| > 0.4$ was established as significantly regulated as before.

3.10 Enrichment and string interaction

The significantly up and down regulated proteins were sorted and compared to the total obtained proteins to establish background of the process. “Proteins from KEGG pathway analysis were uploaded to the Cytoscape 3.6.1 software and matched using the WikiPathway app (beta), with the degree of shading representing the fold change” (Mittal, Camarillo, et al., 2020). Pathways and grouped proteins were cross-referenced with KEGG database using DAVID 6.8 (Wei Huang et al., 2008). The Gene Ontology of for cellular localization, molecular functions, and biological processes of differentially expressed proteins were studied using GeneCodis Bioinformatics tool (Carmona-Saez et al., 2007).

CHAPTER 4. RESULTS

4.1 Dosage Curve

Galloflavin causes a dose dependent reduction of MDA-MB-231 TNBC cell viability. Figure 4.1 displays how cell viability relative to control (normalized to 100%) changes with respective to the different dosages of Galloflavin at 24h time point. The MDA-MB-231 cell viability drops to 80% at 25μM and ranges between 75% to 85% for 25μM to 40μM. It is observed that there is a steep drop-in viability after 40μM saturates after 100μM drug concentration.

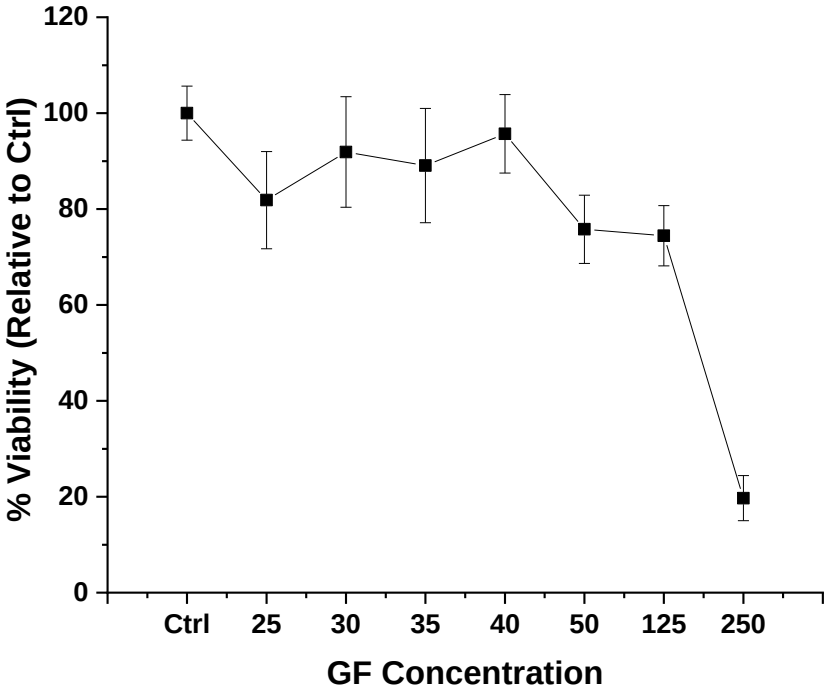


Figure 4.1. Dosage Curve of Galloflavin

4.2 Effect of EP only on TNBC

Figure 4.2 on the following page displays the effect of electrical pulses, at 24h after treatment. As the electric field strength increases, the cell viability decreases. The electric field strength was seen to be inversely proportional to the viability of the cells-higher the field strength, lower the

viability. There is a drop off after 600V/cm and saturates after 1000V/cm. At 800V/cm, the viability was 67% relative to Control at 24h point.

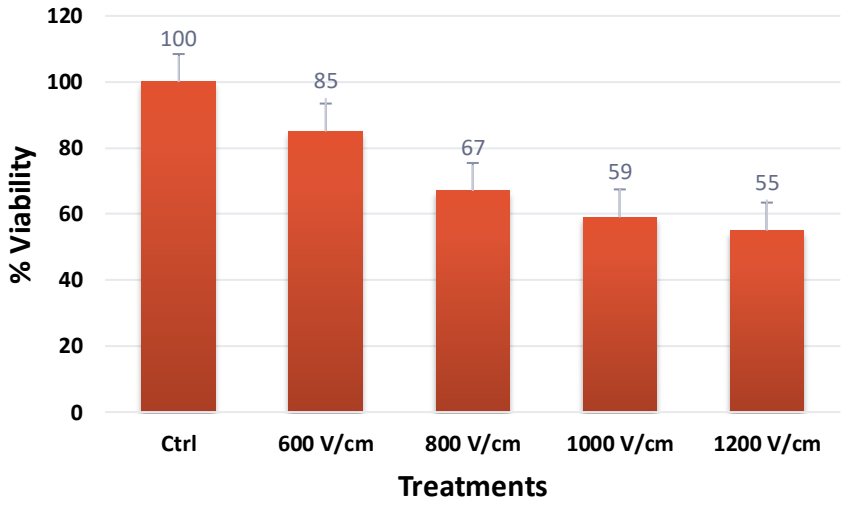


Figure 4.2. Viability of MDA-MB-231 at various Voltages

4.3 Cell Viability Studies using EP+GF

Electroporation at 800V/cm only or GF at 100µM alone did not produce a steep decrease in viability; this indicates that electric field and Galloflavin alone are less effective in comparison, necessitating the combination of the drug with electric field. Figure 4.3 shows the viability decrease caused by combination of Galloflavin of concentration at 10 and 25µM with 800V/cm and 1000V/cm pulses, for up to 72h. The viability of MDA-MB-231 cells reduced at 12 and 24h initially, but they increased eventually with time, indicating that these concentration do not cause cell death over prolonged period at the reduced doses of 10 and 25µM. Hence a higher dose of 100µM was used for further experiments.

Figure 4.4 in the following page shows the viability decrease caused by combination of Galloflavin of concentration at 100µM with 800V/cm and 1000V/cm. The viabilities were monitored at 24h, 48h and 72h time points.

The viability of MDA-MB-231 cells reduced at 24h standpoint the most for combination of Galloflavin and 1000V/cm. The viability drop of for combination of Galloflavin and 1000V/cm was not significantly different when compared to combination of 800V/cm with Galloflavin.

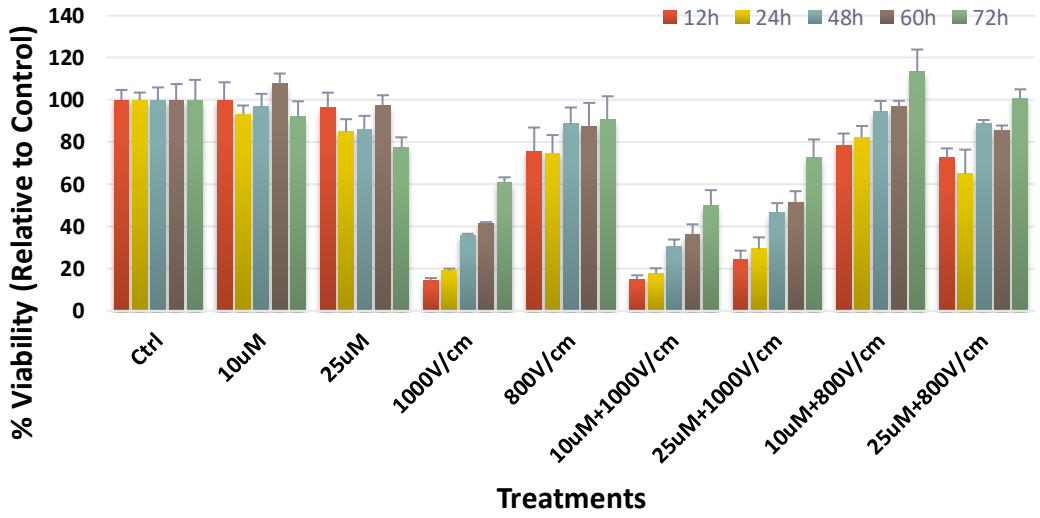


Figure 4.3. Viability of MDA-MB-231 cells 10µM and 25µM along with 800V/cm and 1000V/cm compared to control and drug only treatments

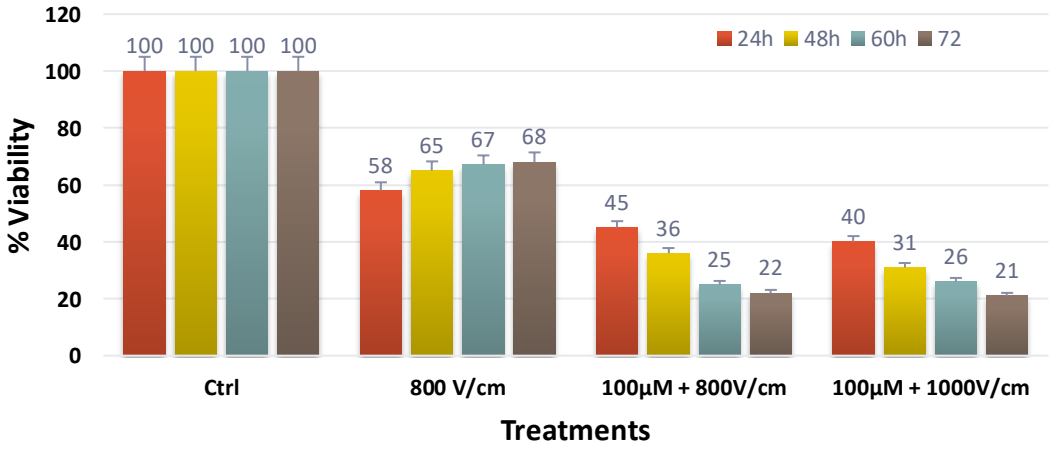


Figure 4.4. Viability of MDA-MB-231 cells at 100µM+EP compared with Drug only and Control treatments

Figure 4.5 shows the viability of MCF10A cells at 12, 24, and 36h. At 24h, the viability for Galloflavin only treatments reduced only 13% relative to control. A combination of 100µM and EP reduces the viability by 15%. The results also show the regaining of MCF10A cell viability after 36h. This indicates the nonimpact of Galloflavin and EP on non-cancerous cells.

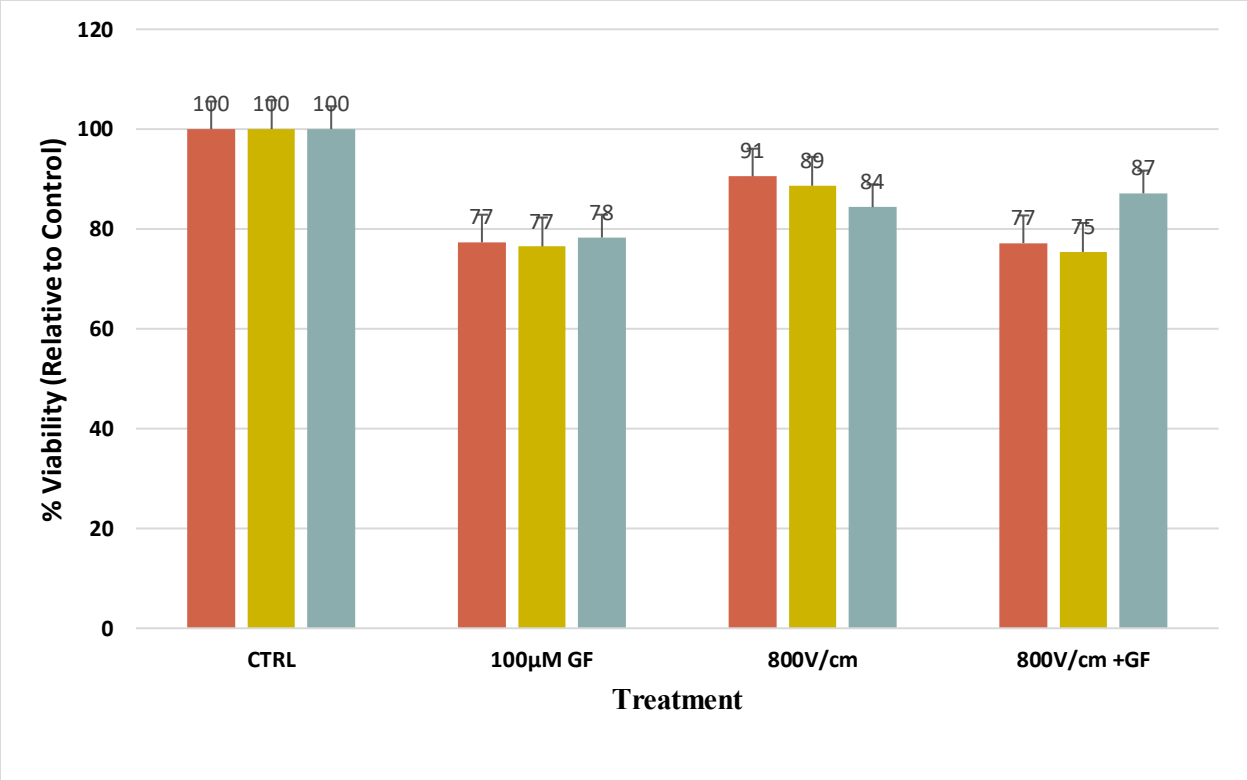


Figure 4.5. Viability of MCF10A at combination of 100µM Galloflavin and 800V/cm

4.4 Colony Formation Assay

Figure 4.6 shows that the combination of 100µM Galloflavin and 800V/cm produced the least colonies at 601 colonies compared to control at 1089 colonies. 800V/cm alone and 100µM Galloflavin alone did not manage to reduce the number of colonies significantly, as they produced 847 and 780 colonies respectively. The highest reduction of colonies were seen with the combination of drug and electroporation.

Here, the letters indicate the statistical analysis of the results. Same letter means that they are not statistically significantly different and different letters means that they are statistically

significantly different. In this case, control and EP only have the same letter, while drug only and EP+GF have the same letter.

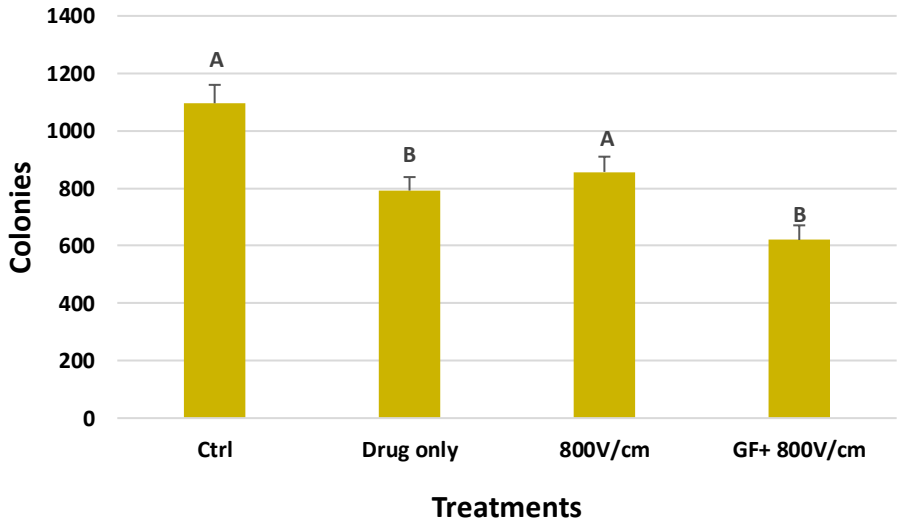


Figure 4.6. MDA-MB231 Colony formation for various treatments

4.5 Reactive Oxygen Species Assessment Assay

The Reactive Oxygen Species (ROS) levels in MDA-MB-231 cell line was observed and studied. Figure 4.7 displays results of ROS levels, from various treatments. EP at 800V/cm along with GF at 100 µM produced the maximum ROS production with more than 4 times of control. GF only treatment produced similar results but lower than EP+GF. While EP only produced similar results as of control. All the treatments were done in triplicates and the letters A and B denote that they are significantly different from each other. This analysis provided us an insight about EP only, GF only and EP+GF compared with Control. Electroporation only treatment does not alter the ROS levels as expected. While the combination of 800V/cm and Galloflavin treatments increased ROS production the best. This causes oxidative stress in the MDA-MB-231 cells which promote controlled cell death (apoptosis).

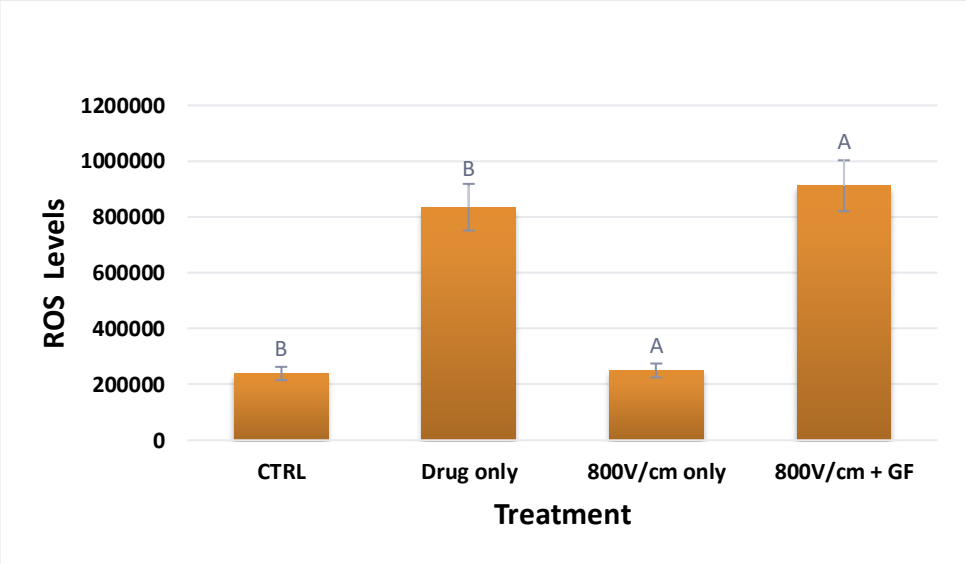


Figure 4.7. ROS levels for various treatments in TNBC

4.6 Glucose Uptake Assays

The Glucose uptake assay was performed to understand the difference in the uptake of Glucose to provide validation to the pathways, which are upregulated and down regulated by the various treatments. The Glucose uptake are shown in Figure 4.8, where the levels of glucose with respect to control (normalized at 100%) are 40% for EP+GF treatments; with EP only treatments there are no significant changes seen. All the treatments were done in triplicates and the letters A and B denote that they are significantly different from each other. This analysis provided an insight about EP only, GF only and EP+GF compared with Control. The EP only treatment does not alter the Glucose uptake in the cell cycle pathways and EP+GF treatments is effective in reducing the Glucose uptake, which aid in MDA-MB-231 proliferation.

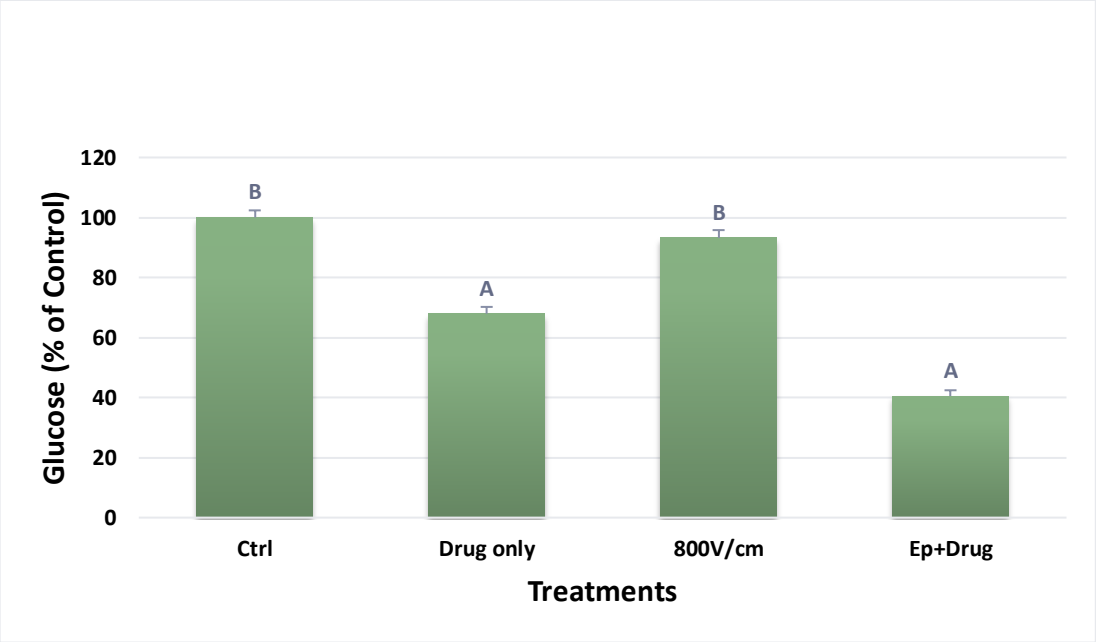


Figure 4.8. Intracellular Glucose levels for various treatments in MDA-MB-231

4.7 Lactate Uptake Assay

The Lactate uptake is shown in Figure 4.9, where the control is normalized at 100%. The levels of lactate production of the EP+GF treatment is only 26% with respect to control. For the Drug only treatment, the level of Lactate production reduces to 56%, which confirms the inhibition of LDHB proteins, which play a vital role in Lactate production. All the treatments were done in triplicates and the letters A and B denote that they are significantly different from each other. This analysis provided us an insight about EP only, GF only and EP+GF compared with Control. With EP only treatment, no significant changes was seen. This establishes that the EP only treatment does not alter the Lactate uptake in the cell cycle pathway, and EP+GF treatment reduces the lactate production the maximum.

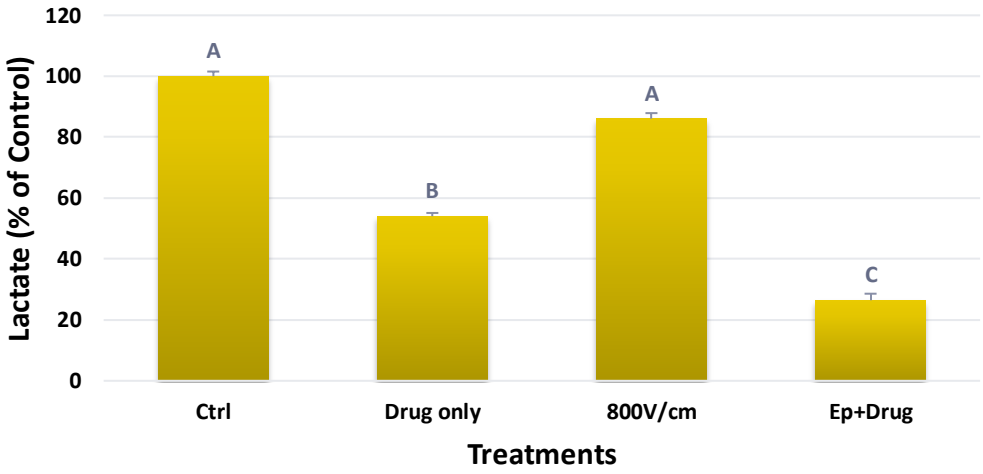


Figure 4.9. Intracellular Lactate levels for various treatments in MDA-MB-231

4.8 Glutamine Uptake Assay

The intracellular glutamine levels were studied, and the results are shown in Figure 4.10. The levels showed that the combination of EP+GF were significantly different from the other treatments and the lowered levels of intracellular Glutamine supports the proteomics findings. All the treatments were done in triplicates and the letters A and B denote that they are significantly different from each other. The analysis provided us an insight about EP only, GF only and GF+EP compared with Control.

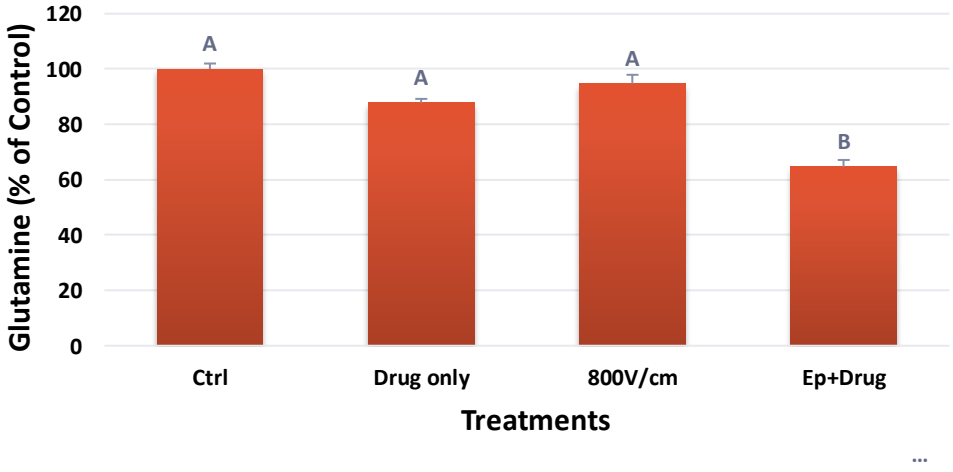


Figure 4.10. Intracellular Glutamine levels for various treatments in MDA-MB-231

4.9 Proteomics Analysis

Figure 4.11 shows the Venn diagram of the protein distribution of the GF only and EP+GF samples. There were total 2400 proteins, of which 1923 are common for both samples. There are 229 unique proteins for GF only, while the unique proteins for EP+GF are 248. Table 4.1 displays the top 20 upregulated and downregulated proteins and genes obtained from the proteomics study. The upregulated proteins comprise of membrane proteins, OXPOS proteins, TCA cycle proteins, which indicate the need for energy production in the cells due to the inhibition of glycolysis by inhibiting LDHB. The downregulated proteins include Ubiquitin proteins, DNA replication proteins, which are antiproliferative and pro-apoptotic in nature.

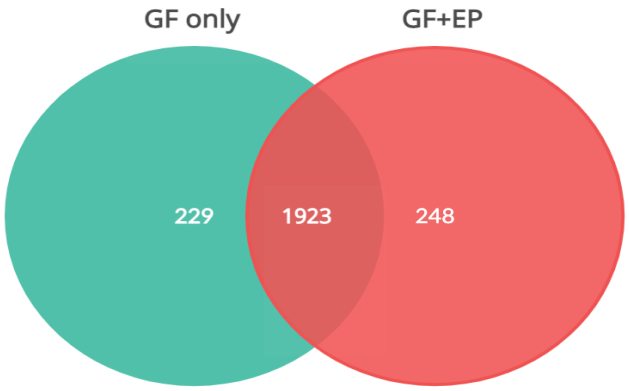


Figure 4.11. Venn diagram of the protein distribution

Table 4.1. Top 20 Upregulated Proteins and Genes

Upregulated Proteins	Upregulated Genes	Average Log EP+GF	ΔLog2
E9PHI4	SUN1	16.99131129	8.991390434
O15230	LAMA5	24.09939459	2.132389358
A8K607	XPO7	24.85567191	0.882623369
P33527	ABCC1	24.23682334	0.824241282
Q9P035	HACD3	26.46239101	0.779914098
Q14XT3	COX2	27.28375906	0.774277511
Q6NX51	EXOC4	24.28228036	0.771003017
B3KTQ2	MFGE8	26.48150516	0.716230427
B2R4R9	RPS28	29.7621172	0.692686826
Q14789	GOLGB1	25.2759861	0.665280086
Q8TC07	TBC1D15	24.66351662	0.648742362
P09914	IFIT1	25.97764922	0.62973872
A6PVN5	PPP2R4	26.01581357	0.607616546
F8W9J4	DST	27.23280144	0.607584316
B4DI61	CYR61	26.93782331	0.597806605
Q92538	GBF1	26.07181492	0.596227144
A8K3S0	RFC5	25.31055434	0.571655874
E9PF10	NUP155	26.998371	0.559031616
Q9HAB8	PPCS	24.05327593	0.540508624
A0A024R179	NCBP1	25.86733878	0.539977731

Table 4.2. Top 20 Downregulated Proteins and Genes

Downregulated Proteins	Downregulated Genes	Average Log EP+GF	ΔLog2
Q9BTC0	DIDO1	26.30366116	-0.73386812
Q5RHS7	S100A2	28.71754677	-0.708976272
A0A024R525	FTH1	27.77541684	-0.690754725
Q6PJ77	BTF3L4	26.53700676	-0.670285235
P63218	GNG5	27.10248118	-0.642878287
Q8NI27	THOC2	28.21459527	-0.559100893
B8ZZQ6	PTMA;PTMAP7	28.0598403	-0.551616356
P41208	CETN2	26.46960574	-0.535760981
A0A024R5U3	ARPP-19	25.80894773	-0.52244792
E9PQY2	PFDN4	29.53997949	-0.500414235
A0A024R0Q0	FLJ12886	26.4407709	-0.499184358
A0A024R2K4	LRRFIP2	24.8758278	-0.497523217
A0A0C4DGB0	SCOC	27.4687659	-0.494236711
A0A024R7S3	CLTB	25.2720125	-0.481103955
Q4KMP7	TBC1D10B	28.65912944	-0.479844112
Q8WW12	PCNP	26.59781984	-0.477376456
Q96AB3	ISOC2	26.15856296	-0.473253098
P05161	ISG15	28.10840195	-0.471562369
A0A024R4S0	CHMP2A	25.47211499	-0.45920888
K7EKI8	PPL	30.51760911	-0.452380223

4.9.1 Upregulated Pathways

In Figure 4.12, mitochondrial ribosomal proteins (RP) were seen to be significantly upregulated by EP+GF treatments. The upregulated ribosomal proteins include ribosomal protein L17 (RPL17), ribosomal protein L18 (RPL18), ribosomal protein L9 (RPL9), ribosomal protein S24 (RPS24), ribosomal protein S28 (RPS28) and ribosomal protein S9 (RPS9). The significant upregulation of ribosomal proteins indicates possible DNA damage to the TNBC cells by the EP+GF treatment. The red starts represent the significantly regulated protein due to the treatment.

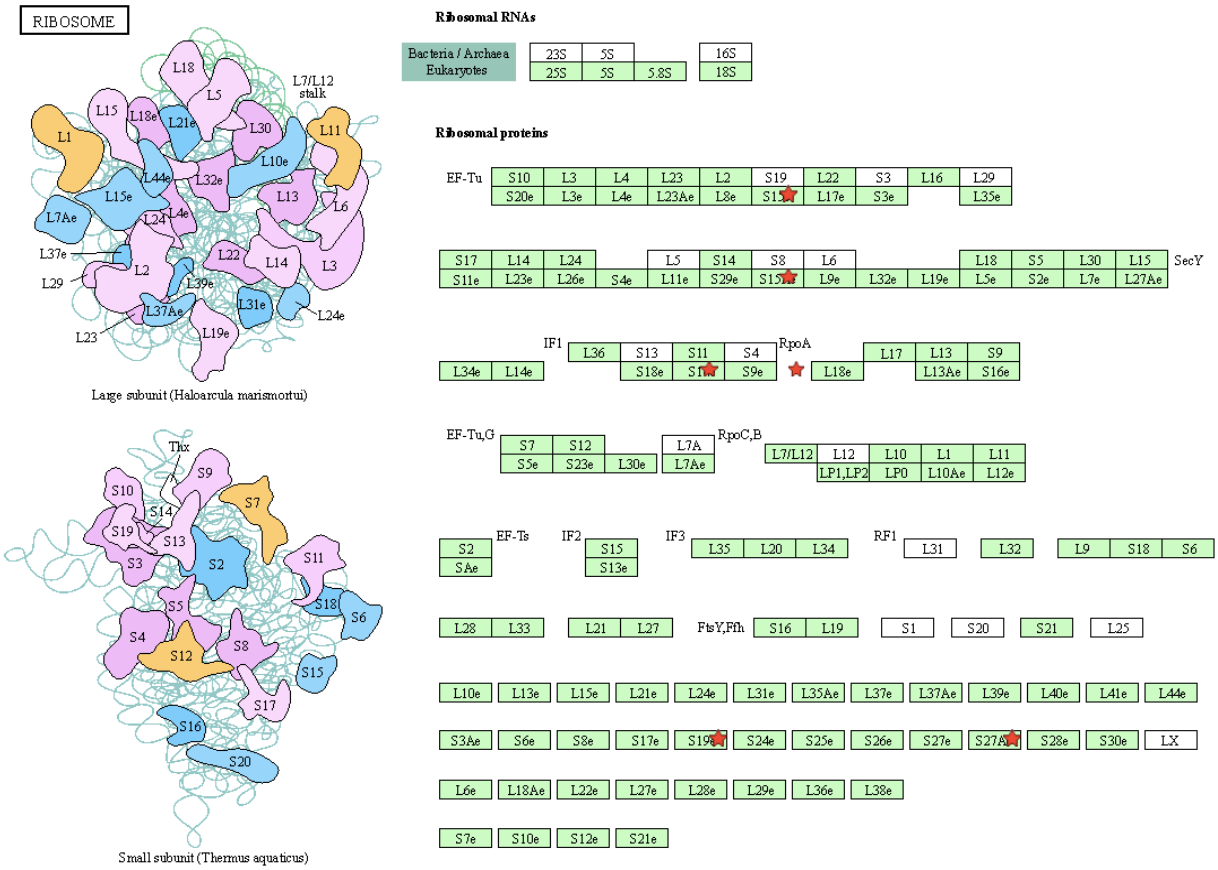


Figure 4.12. Upregulated Mitochondrial Ribosome protein pathways, indicating DNA damage

Endocytic pathway in Figure 4.13 was significantly upregulated. Endocytosis is one of the mechanistic pathways, which are responsible for cell regulation. They involve processes like pinocytosis cell drinking and phagocytosis (cell eating). EH domain containing 4 (EHD4), retromer complex component (VPS35), adaptor related protein complex 2 alpha 1 subunit (AP2A1), Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1), transferrin receptor (TFRC) were upregulated by the treatments. The upregulation of endocytosis pathways helps in cell cycle regulation since “endocytosis is an attenuator of signalling, and therefore a potential candidate as a tumour suppressor pathway/System” (Lanzetti & di Fiore, 2008).

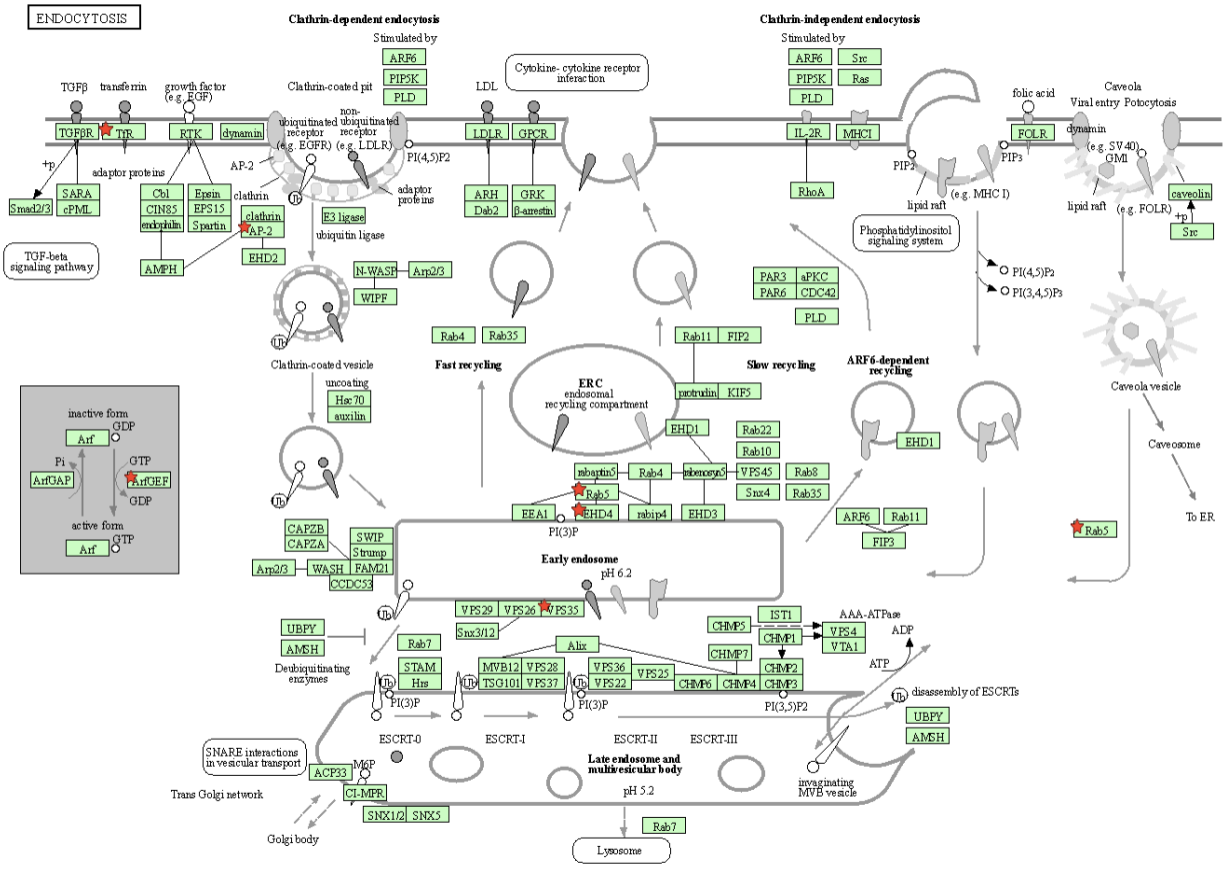


Figure 4.13. Upregulated Endocytosis Pathway

Along with endocytic pathway, phagosomes and phagocytosis were also upregulated significantly by EP+GF treatments, as shown in Figure 4.14. Antigen presenting cells with the presence of Natural Killer cells constitute immune response and fight against malignant cells. This response triggers the adaptive immune system (Iwasaki & Medzhitov, 2010). Antigen presenting cells possess the ability to engulf tumour cells by phagocytosis (Feng et al., 2019). RAB5C, member of RAS oncogene family (RAB5C), dynein cytoplasmic 1 heavy chain 1 (DYNC1H1), integrin subunit alpha 2 (ITGA2), thrombospondin 1 (THBS1), transferrin receptor (TFRC) were some of the upregulated genes in this pathway.

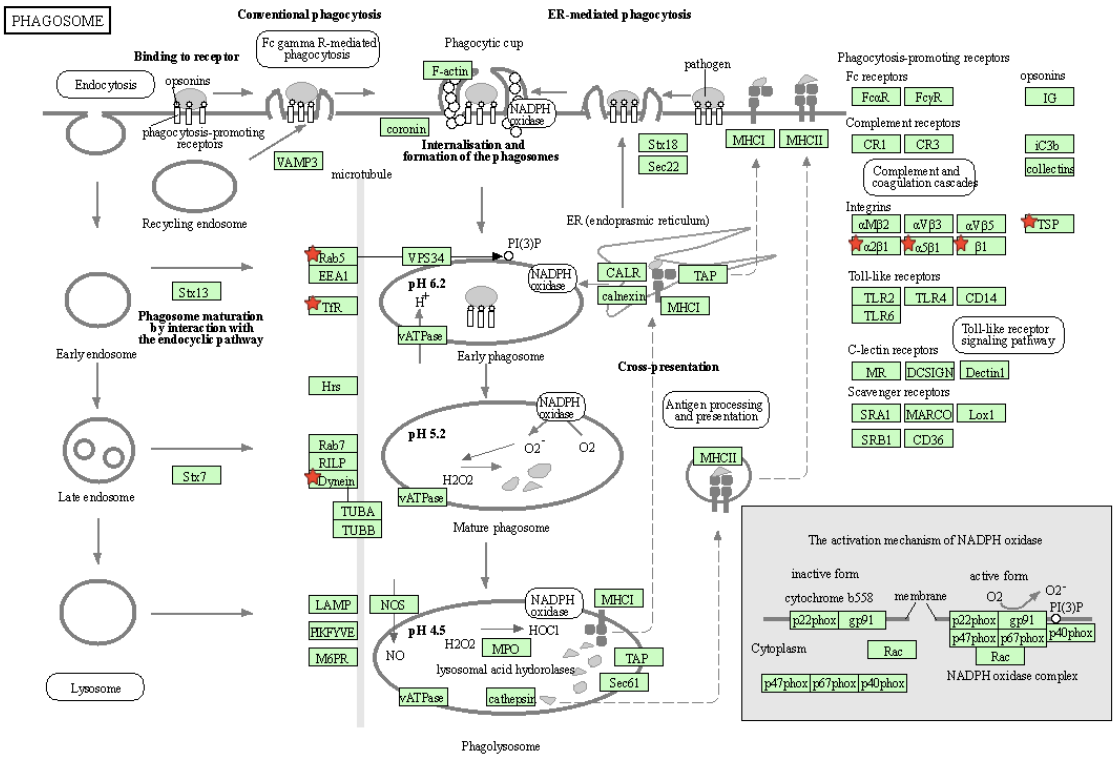


Figure 4.14. Upregulated Phagosome Pathway

4.9.2 Gene Ontology Enrichment-upregulation

Gene ontology (GO) analysis of significantly upregulated proteins was done to find the various proteins and their numbers involved in a given Biological Process, Cellular Component or Molecular Function. The Upregulated genes in EP+GF treatments were identified and categorized are shown in Figures 4.15, 4.16 and 4.17. The highest number of genes were found in membrane, cytoplasm, protein and RNA binding, which expresses the significance of electroporation and upregulation of the effects of Galloflavin due to electroporation this also supports prior results and analysis in the Biological Processes, with more than 153 genes present in these processes.

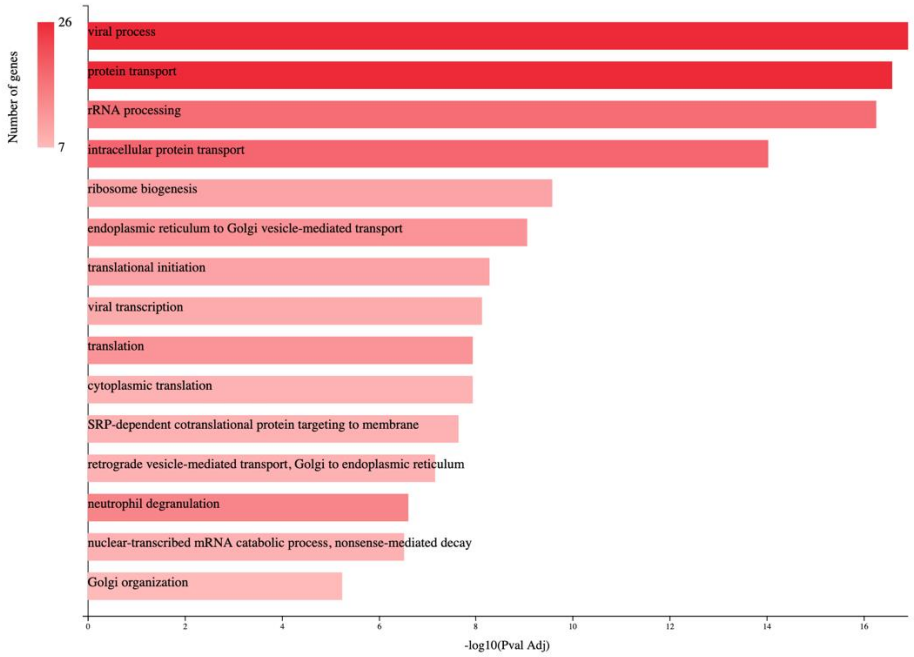


Figure 4.15. Upregulated Gene Biological Functions

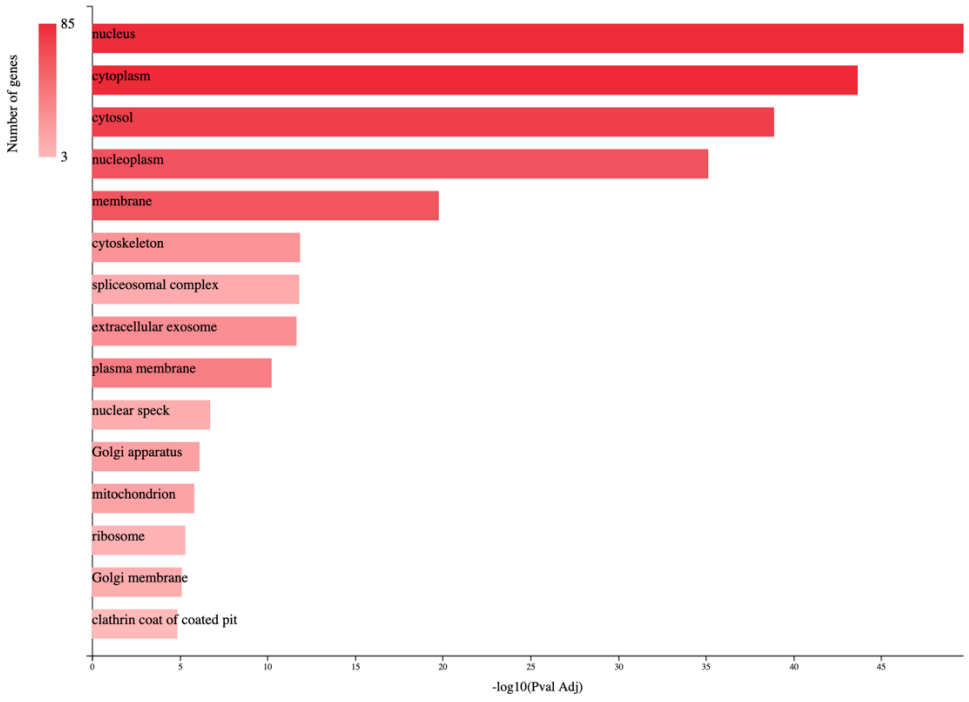


Figure 4.16. Upregulated Gene Cellular Components

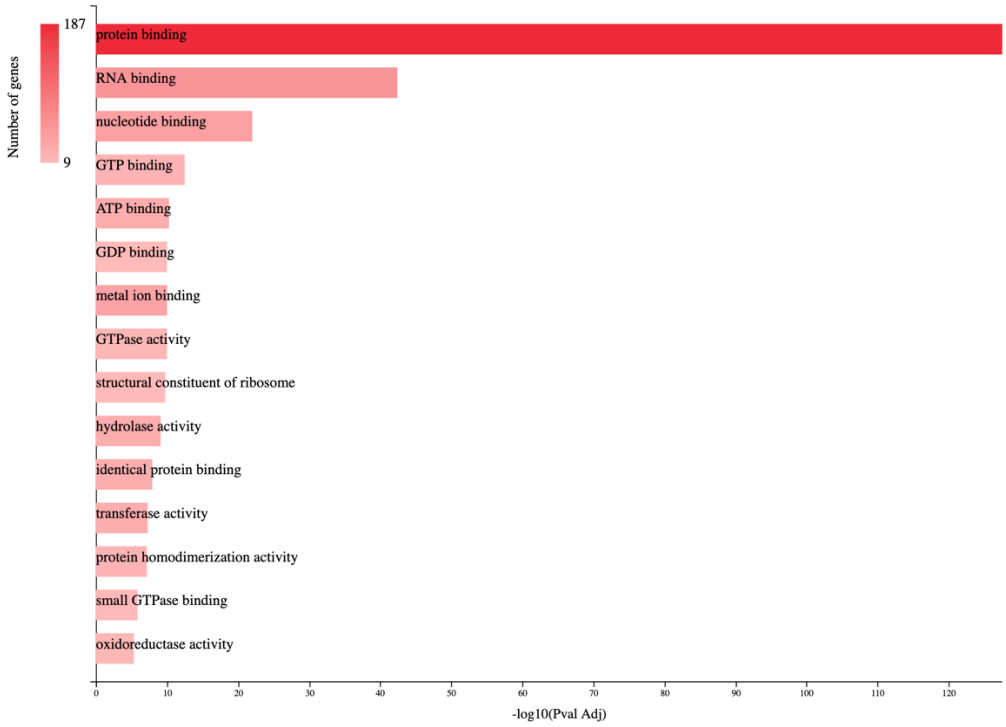


Figure 4.17. Upregulated Gene Molecular Functions

4.9.3 Downregulated Pathways

Spliceosome is a ribonucleoprotein particle found predominantly in cells, with nucleus surrounded by a nuclear envelope, which barricades nucleus from cytoplasmic protoplasm. Spliceosomes are responsible and plays a vital part in tumorigenesis (van Alphen et al., 2009). The downregulation of splicing caused by spliceosome was seen in EP+GF treatment. The key proteins, which are significantly downregulated include spliceosome associated protein homolog (CWC15), LSM5 homolog, U6 small nuclear RNA and mRNA degradation associated (LSM5), RNA binding motif protein 17(RBM17), U2 snRNP associated SURP domain containing (U2SURP), nuclear cap binding protein subunit 2 (NCBP2), small nuclear ribonucleoprotein D2 polypeptide (SNRPD2) and survival motor neuron domain containing 1 (SMNDC1). Figure 4.18 indicates this pathway.

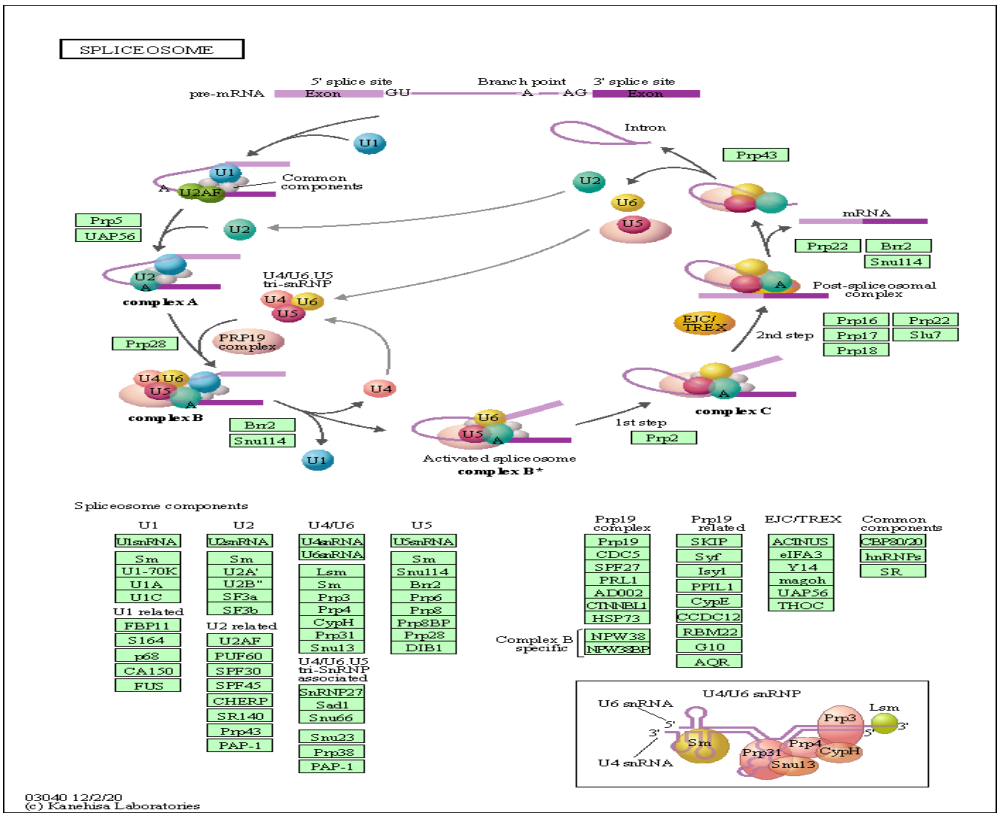


Figure 4.18. Downregulated Spliceosome Pathway

Ubiquitin-mediated Proteolysis pathways are downregulated shown in Figure 4.19. The salient specifically downregulated proteins including Cbl proto-oncogene (CBL), cell division cycle 34 (CDC34), cullin 4A (CUL4A), kelch like ECH associated protein 1 (KEAP1) are highlighted with red stars in Figure 4.19. The inhibition of the ubiquitin-proteosome pathway effectively halts the cell cycle.

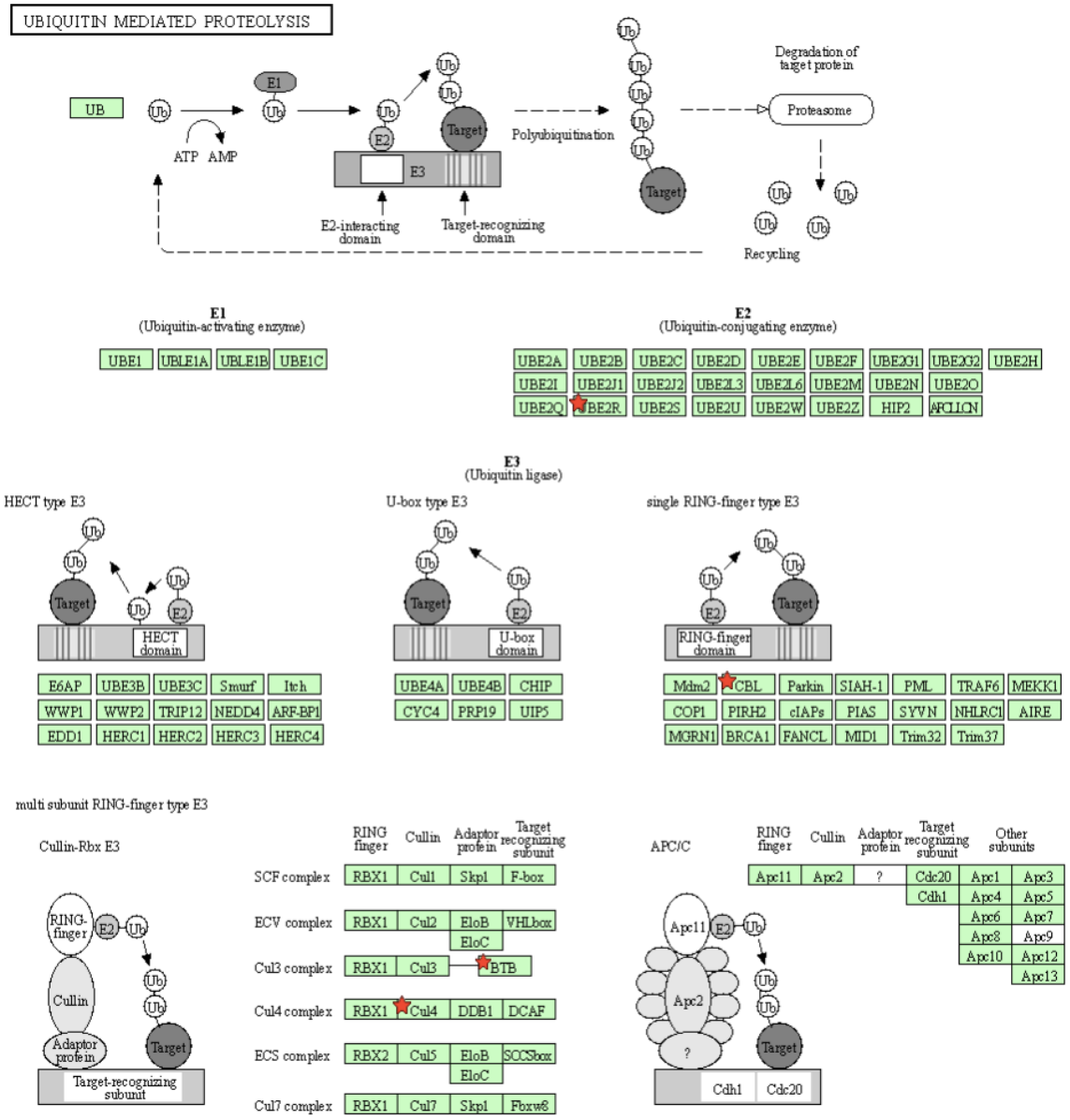


Figure 4.19. Downregulated Ubiquitin Mediated Proteolysis Pathway

4.9.4 Gene Ontology Enrichment-downregulation

Analysis of GO enrichment with significantly downregulated genes were done to find the number of genes involved in these. The downregulated genes in EP+GF treatments were studied and categorized based into Biological Process, Cellular Component and Molecular Function. A total of 19 genes, which is the highest number of genes, were found in mRNA processing, RNA

splicing, followed by splicing via Spliceosome. Cell cycle and Cell Division is also significantly downregulated with 20 genes in total. Mitochondrial translations is also downregulated. This supports and provided evidence to our findings in the downregulated Spliceosome pathway and the downregulation of cell cycle and cell division is pro-apoptotic are shown in Figures 4.20, 4.21 and 4.22.

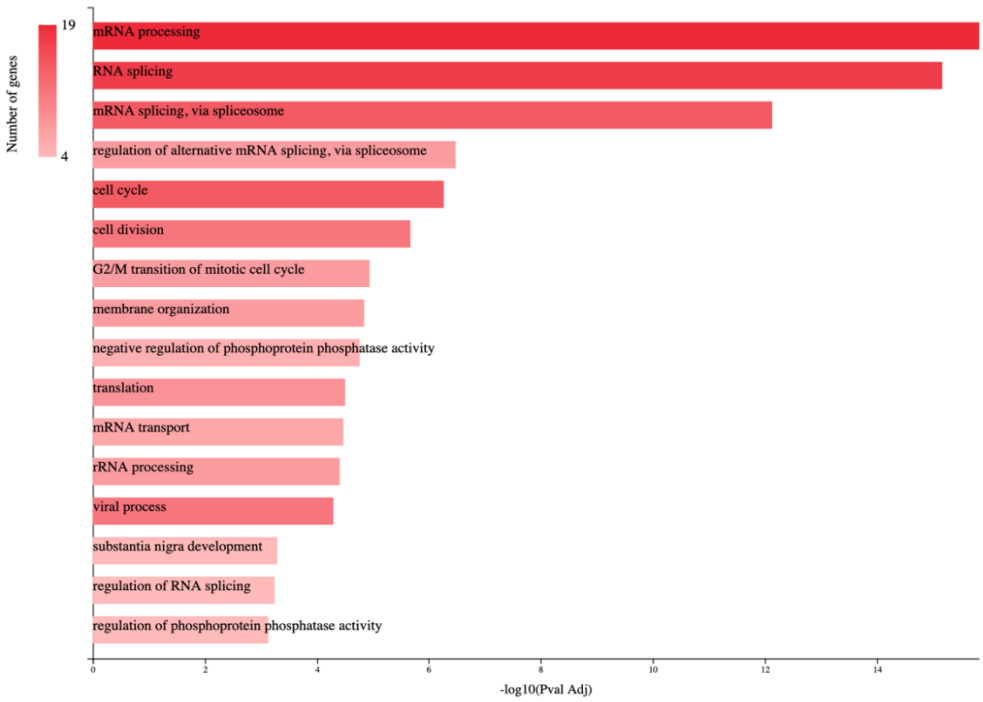


Figure 4.20. Downregulated Gene Biological Processes

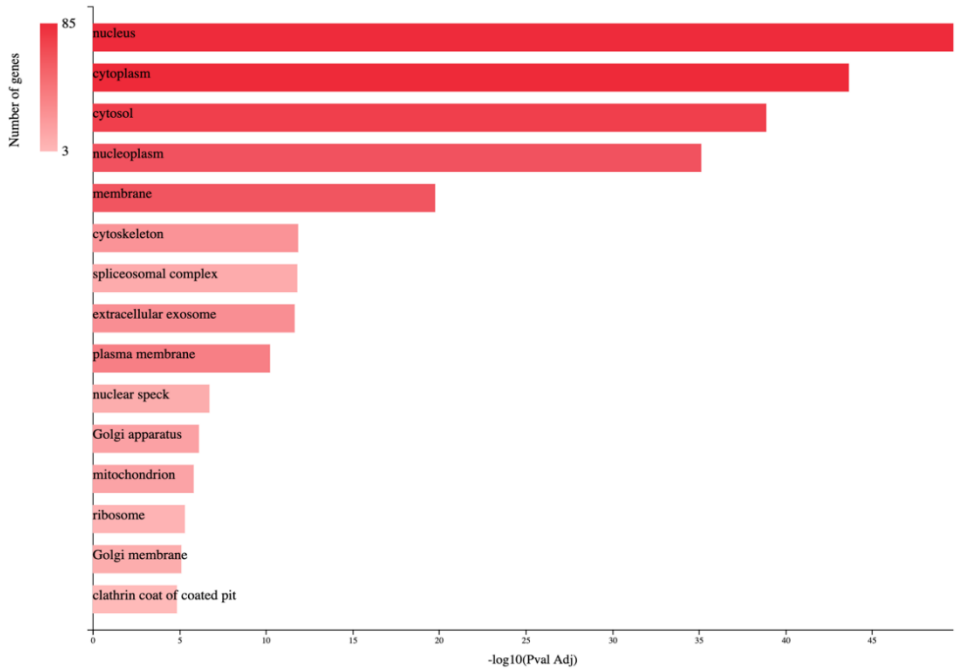


Figure 4.21. Downregulated Gene Cellular Component

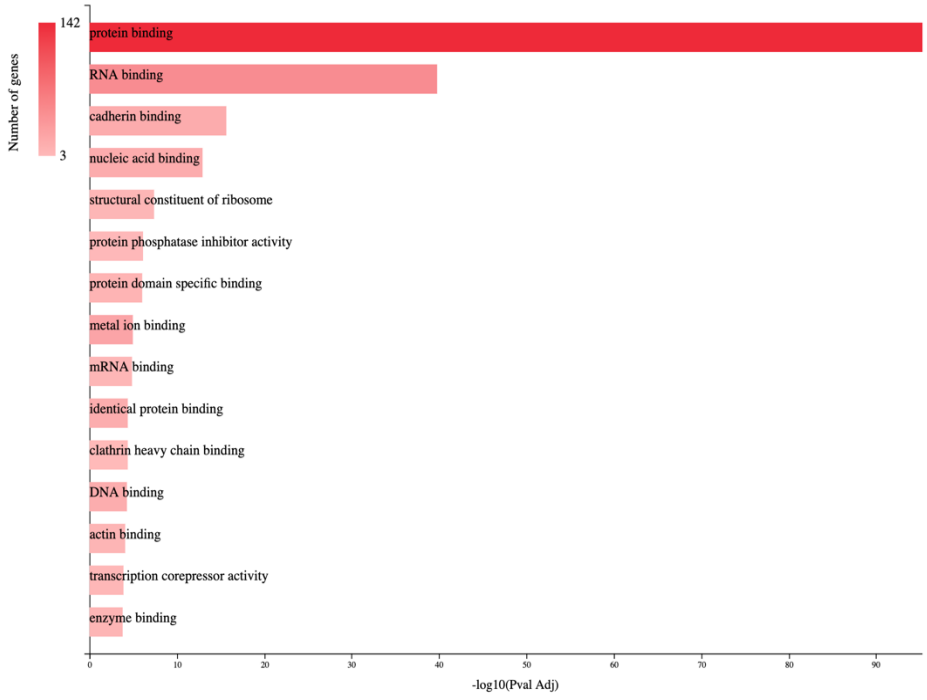
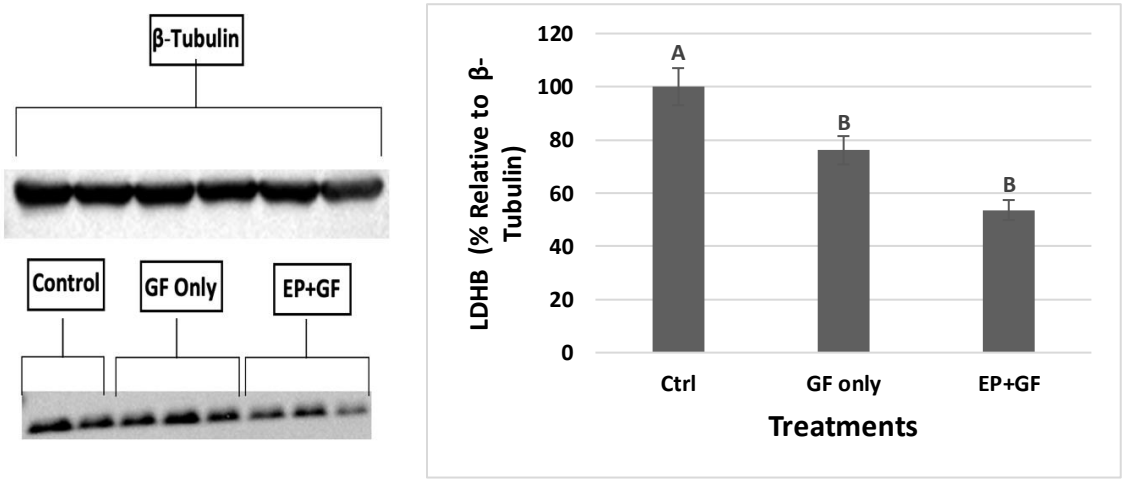


Figure 4.22. Downregulated Gene Molecular Function

4.10 Western Blotting

Immunoblots in Figure 4.23 (a) shows the individual protein expression of LDHB for the MDA-MB-231 cultured cells, treated with Control, EP only and EP+GF. Figure 4.23(b) shows the quantification of LDHB normalized with β-tubulin (McCleland et al., 2012). Western Blotting protein quantification provided us evidence of the inhibition of LDHB due to the treatments. The treatments, EP+GF inhibits LDHB protein the most to 55% relative to control (normalized at 100%) and GF only inhibits LDHB close to 77% relative to control. The facilitation of electric pulses and GF targets and inhibits LDHB with more intensity on the MDA-MB-231 cell line. The letters A and B indicate that they are significantly different from each other.



(a) (b)

Figure 4.23. (a) Western Blots and (b) Quantification relative to β-tubulin

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CHAPTER 5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In conclusion, these studies support that idea that the combination of the agent GF with EP is able to substantially reduce MDA-MB-231 cell viability, to a much greater degree than either of these treatments alone. Our subsequent experiments were able to provide a critical landscape of mechanistic insights that may be responsible for the anticancer actions of this treatment combination. With the results from proteomics studies, we can conclude that a combinational effect of EP+GF initiates various landmark pathways that ultimately lead to a cellular apoptotic endpoint. In support of our findings, it is well documented that apoptosis is triggered by the inhibition of LDHB which supresses the glycolysis pathways, which provides the key source of energy for TNBC proliferation. These major observations are important mechanistic clues that suggest how, EP+GF is an efficacious antiproliferative treatment for TNBC MDA-231 cells. Collectively, these studies provide justification for further work, particularly in vivo studies, to define and characterize the potential utility of this combination treatment. Successful studies will be important to prompt the development of valuable clinical options based on novel therapeutic targets centered in LDHB and EP mechanisms.

The major outcomes of each of the experiments described herein can be summarized as follows:

1. GF at 100µM reduced the TNBC cell viability to 80% at 24h time point.
2. EP at 800V/cm, 100µs, 8 pulses at 1Hz reduced the TNBC cell viability to 70% at 24h time point.
3. Combination of EP+GF reduced the TNBC cell viability to 30% at 24h time point.
4. Proteomics analysis of GF only vs EP+GF treatments identified more than 2000 proteins, of which 248 were downregulated and 229 were upregulated. The pathways identified based on the proteomics findings indicate EP+GF act as antiproliferative and pro-apoptotic.
5. Western Blotting validates the proteomics analysis by quantifying the downregulation of LDHB.

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APPENDIX A. CODE

Automatic Colonies count Matlab code

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Cell Colony Segmentation
%    “ The goal of the assignment is to count the number of black cells in the input image”.
%     “The procedure is to invert the image, followed by thresholding and finally”
% “counting the white blobs in the resulting binary image”.
%
% “Input: Original image”
% “Output: Count of number of cells, output image indicating the cells”.
% Author: Praga Giri
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%


clc;
clear all;
close all;
fprintf('Cell colony counting\n')
fprintf('Author: Praga Giri\n')
%% Initialization

img_rgb = imread('xxx->CFA.png');     % Load the image

img=rgb2gray(img_rgb);
% Plot the original image
figure
imshow(img, 'InitialMagnification','fit')
title('The original image')
```

```
inv_img = imcomplement(img);                % Invert the image
% Plot the inverted image
figure
imshow(inv_img, 'InitialMagnification','fit')
title('The original image')

% Break the image into blocks and apply thresholds individually
%    and combine them.
nquad = 2;  % Number of subdivisions into quadrants.
fun = @(block_struct) otsu(block_struct.data);
th_img = blockproc(inv_img, floor(size(img)/(2^nquad)), fun);

%% Additional operations
% Uncomment the below two lines to apply morphological opening
% se = strel('disk',1);      % Create structuring element and size
% th_img = imopen(th_img, se);     % Close to eliminate broken regions
% Uncomment below line to apply median filtering
% th_img = medfilt2(th_img, [3,3]);

% Plot the thresholded image
figure
imshow(th_img, 'InitialMagnification','fit')
title('The thresholded binary image')

% Get area of regions and save it in an array
lab_img = bwlabel(th_img);  % Labelled image

blob_areas = zeros(max(lab_img(:)),1);  % Array to store areas
for i=1:max(lab_img(:))
   % Compute area of regions
```

```
    blob_areas(i) = size(find(lab_img==i),1);
end

min_area = min(blob_areas);     % Find minimum area
beta = 1.15;                    % Area scaling threshold
reg_img = zeros(size(lab_img));

%% Cell counting
% Remove regions with area less than beta*min_area
for i=1:size(blob_areas,1)
    % Check if area is greater than threshold
    if blob_areas(i) > beta*min_area
        tmp = lab_img==i;
        reg_img = reg_img | tmp;    % Add to image
    end
end

[bdry, lab, nreg, A] = bwboundaries(reg_img,8);
fprintf('The cell count is %d\n',nreg)

%% Plot image with boundaries
figure
imshow(reg_img)
hold on
for i=1:size(bdry,1)
    plot(bdry{i}(:,2),bdry{i}(:,1),'m-')
end
title('The image with cells highlighted')
```

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