Applied Biochemistry and Biotechnology (2022) 194:18–36
https://doi.org/10.1007/s12010-021-03723-5

ORIGINAL ARTICLE

# Enhanced Antiproliferation Potency of Electrical Pulse-Mediated Metformin and Cisplatin Combination Therapy on MDA-MB-231 Cells

**Praveen Sahu[1] · Ignacio G. Camarillo[2,3] · Raji Sundararajan[1]**

Received: 22 July 2021 / Accepted: 8 October 2021 /
Published online: 6 November 2021


## Abstract

We investigated the combined potency of metformin and cisplatin on the MDA-MB-231, triple-negative breast cancer (TNBC) cells with the application of electrical pulses. There are no targeted therapies for this subset of breast cancer because of the absence of specific biomarkers. Cytotoxic chemotherapy is the mainstream mode of treatment for TNBC, and cisplatin is the most commonly used chemotherapeutic drug. While there is a good response initially, TNBC cells develop drug resistance eventually. Thus, there is a need for alternate therapies. Toward this, we studied the antiproliferation characteristics of electrical pulse-mediated combination therapy using metformin, the commonly used Type-2 diabetes drug, along with cisplatin. We used metformin, as it has various anticancer properties caused by repressing energy pathways in a cancer cell. Application of 8 pulses of 1000 V/cm, 100 μs, at 1 Hz frequency, enhanced the drug uptake leading to cell viability as low as 25.86% at 30 μM cisplatin and 5 mM metformin in a 24 h study. Also, the same studies were conducted on MCF10A, a non-cancerous human epithelial cell. It aided in comparing the result for both MDA-MB-231 and MCF10A cell lines while establishing a better understanding of the experimental outcomes. Overall, the various experimental results from colony-forming assay, reactive oxidative analysis, and the intracellular glucose metabolic assay indicate the possibility of the electrical pulses-based cisplatin and metformin drug combination as a potential alternative to TNBC treatment.

**Keywords** Electrical pulses · Metformin · Cisplatin · Triple-negative breast cancer · MDA-MB-231 cells

✉ Raji Sundararajan
raji@purdue.edu

[1] School of Engineering Technology, Purdue University, West Lafayette, IN, USA

[2] Department of Biological Sciences, Purdue University, West Lafayette, IN, USA

[3] Purdue University Center for Cancer Research, West Lafayette, IN, USA

## Introduction

Metformin has traditionally been used as the first line of treatment in type 2 diabetes mellitus (T2DM) patients [1, 2]. However, more recent studies indicated expanded roles for metformin in cancer treatment. For instance, a population-based observational study shows an association between the reduced risk of various cancers in patients with type 2 diabetes and metformin usage [1, 2]. In another related study, statistical examination of the record-linkage databases from Tayside, Scotland, U.K., on a larger cohort sample, supports the same with a 37% reduced risk of cancer [3]. Compared to other antidiabetic drugs, metformin offers a 31% reduction in overall relative risk [4], as shown by a comprehensive literature search and meta-analysis of epidemiologic studies [5, 6]. The improved survival was primarily observed in cancer types, such as liver [7], pancreatic [8], lung [9], colorectal [10], and breast [11] cancer. Metformin acts through multiple signaling pathways. One such pathway includes AMPK (AMP-activated protein kinase), whose activation requires LKB1, a well-known tumorigenesis suppressor [12].

In the case of TNBC, which accounts for 10 to 15% of all breast cancer cases and having ER -ve, PR -ve, and no overexpression of HER2 [13, 14], has no standard therapy [15] with lower survival outcome. Chemotherapy drugs, such as cisplatin, exhibit cytotoxicity, causing cancer cell death by damaging the DNA [16]. Still, over the course of treatment, the chances of developing drug resistance are immense [17]. In contrast, high-energy X-rays kill cancer cells or shrink tumors using radiation therapy which comes with additional complexities [18, 18]. One of the possibilities is to examine the combination of metformin with cisplatin to tackle multiple pathways contributing to the proliferation of triple-negative breast cancer. The evidence of metformin promoting growth inhibition and sensitizing resistant cancer cell lines to cisplatin is available in case of bone cancer [20], lung cancer [21], including breast cancer [22, 23].

In this study, we tested a combinational therapeutic approach by coupling oral antidiabetic agent metformin and standard chemotherapeutic drug cisplatin in the presence of electric pulses (EP) on MDA-MB-231 TNBC cells. The effectiveness of electrical pulses along with chemo drugs, known as electrochemotherapy, is widely reported [24–27] (Fig. 1).

The concept of how the electroporation technique enhances the uptake of drug molecules is illustrated in Fig. 1. Due to the application of an external electric field, the charges inside and outside the cells are displaced. Thus, it gives rise to an accumulated transmembrane potential difference, $\Delta V_m$, on the cell surface [28]. The transmembrane potential difference equation can be represented by:

$$V_m = \frac{1}{2} \times r \times E_{applied} \times \cos \phi \tag{1}$$

where $r$ is the radius of the cell, $E_{applied}$ is the externally applied electric field, and $\phi$ is the polar angle concerning the electric field direction. The change in the hydrophobic cell membrane appears when the transmembrane potential difference exceeds a threshold value ($\Delta V_m > V_{th}$).

In addition, since in a real-time in vivo ECT treatment, both healthy and cancerous cells are involved, we evaluated the cell viability of MCF10A, a non-cancerous human epithelial cell utilizing the same combination of the drugs with and without EP.

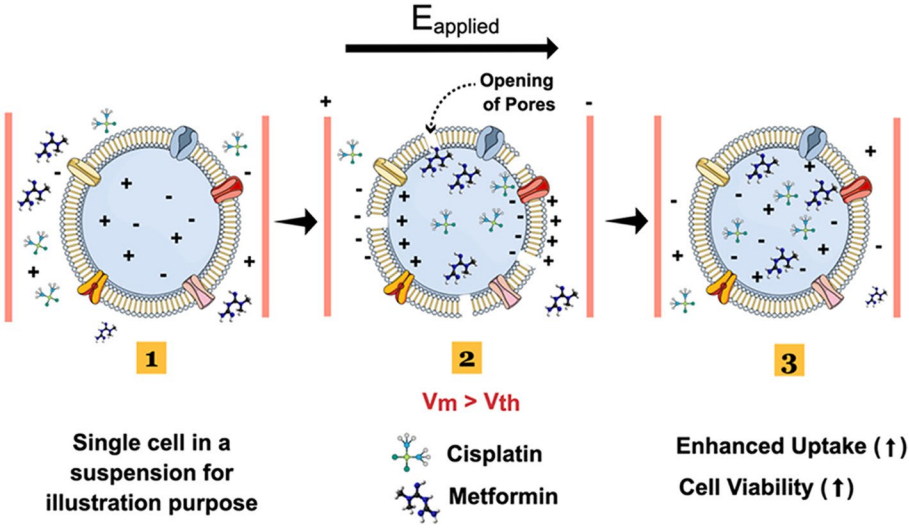


**Fig. 1** An illustration of enhanced drug delivery in the presence of an electric field and the proposed drug combination

## Materials and Methods

### MDA-MB-231 Cell Line

We considered epithelial TNBC cells, the MDA-MB-231 (ATCC® HTB-26™) obtained from the human metastatic tumor site. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco™) media accompanied by 10% Fetal Bovine Serum (FBS) and 1% Penicillin–Streptomycin (PS). It is incubated in a controlled microenvironment of 37 °C with 5% $CO_2$ and 83% humidity. After 90–95% confluency, the cell in the flask is trypsinized and centrifuged at 900RPM for 5 min duration. The supernatant solution is aspirated, and the remaining cell pellet is resuspended in 1 ml of fresh culture media. In Fig. 2, we see the MDA-MB-231 cell count and viability of untreated TNBC cells with 96% cell viability using the Countess™ II FL automated cell counter (Thermo Fisher Scientific™). Trypan blue (0.4%, Gibco™) staining was used for cell viability, as shown in Fig. 2a, where the brightfield image reveals the live and dead cells in green and red circles, respectively. Figure 2b shows the optimal cell size profile for accurate viability assessment. In the end, an appropriate amount of dilution was added to achieve a cell concentration of $1 \times 10^6$ cells/ml.

### MCF10A Cell Line

MCF10A is a non-tumorigenic, cuboidal-shaped human mammary epithelial cell line that does not express estrogen receptor (ER) [29]. The selection of MCF10A is vital to assess and compare the viability of a non-cancerous cell line under the same drug combination of metformin and cisplatin with or without EP with the MDA-MB-231 cancerous cell

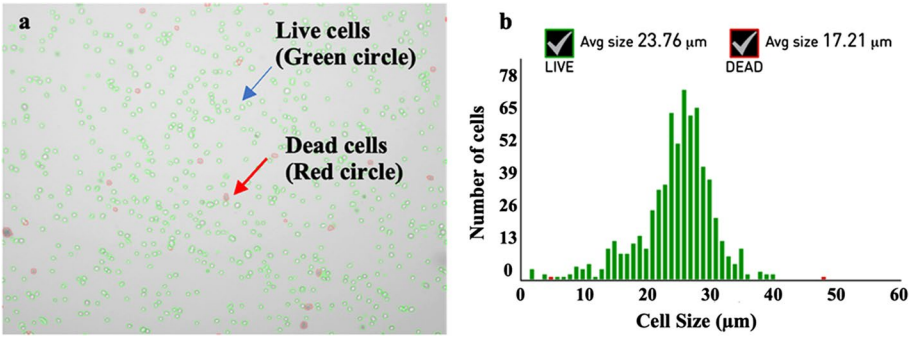


**Fig. 2** MDA-MB-231 cell count and viability using Countess™ II FL automated cell counter. **a** Brightfield image of untreated TNBC cells stained with Trypan blue showing cell viability of 96% with the live cells as green and dead cells as red circles and **b** optimal cell size profile for accurate viability assessment

line. Unlike in MDA-MB-231, a 500 ml growth media for MCF10A is formed by mixing DMEM and F12 in an equal ratio (275 ml each), including 25 ml horse serum (Sigma-Aldrich™), 5 ml PS, 100 µl (H)EGF (Human-Epidermal growth factor), 20 µl Cholera toxin (Sigma-Aldrich™), 5 µl Hydrocortisone (Sigma-Aldrich™), and 1 ml bovine insulin (Sigma-Aldrich). The incubation and resuspension procedure to achieve 1 million cells per ml is the same as for MDA-MB-231. In Fig. 3, like for MDA-MB-231, we performed the cell count and viability of untreated MCF10A cells using the Countess™ II FL automated cell counter. Figure 3a shows cell viability of 90% with the live and dead cells in green and red circles, respectively, after Trypan blue staining. In contrast, Fig. 3b shows the optimal cell size profile for accurate viability assessment.

## Metformin and Cisplatin Drugs

Metformin hydrochloride (1,1-Dimethylbiguanide hydrochloride) and cisplatin (*cis*-Diaminedichloroplatinum) were both obtained from Sigma-Aldrich™. Figures 4a and b show the chemical structures of Metformin and Cisplatin, respectively. Metformin

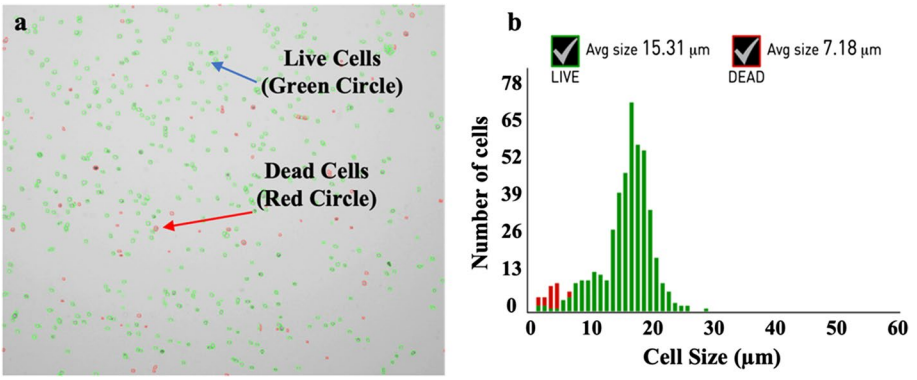


**Fig. 3** MCF10A cell count and viability using Countess™ II FL automated cell counter. **a** Brightfield image of untreated TNBC cells stained with Trypan blue showing cell viability of 90% with the live cells as green and dead cells as red circles and **b** optimal cell size profile for accurate viability assessment

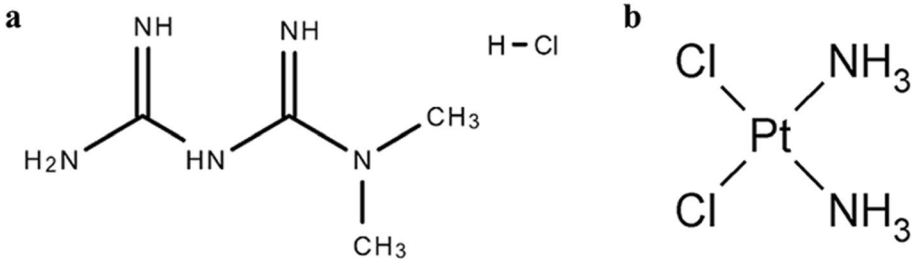


**Fig. 4** The chemical structural formulas for **a** Metformin Hydrochloric and **b** Cisplatin

was dissolved in de-ionized sterile water to make a stock solution of 500 mM and further diluted to 5 mM as the final concentration for treatment. Cisplatin stock solution of 1000 µM was prepared using sterile Di-ionized (DI) water and stored in a black centrifuge tube at room temperature to shield it from external light. Subsequent, a final concentration of 5 µM, 10 µM, 15 µM, and 30 µM was prepared for treatments.

## Delivery of Electric Pulses

A BTX ECM 830 square wave pulse generator (Genetronics Inc. San Diego, CA, USA) was used in our study. Eight unipolar, square wave pulses of 1000 V/cm at 100 µs; 1 s intervals were applied to the sterile cuvettes having an aluminum electrode separated by a gap of 4 mm. $6 \times 10^5$ (600 µl) of MDA-MB-231 cells were used for each sample. The experimental setup has been demonstrated in Fig. 5. Treated samples were dispensed to either a 96-well plate or a 6-well plate, depending upon the assay type.

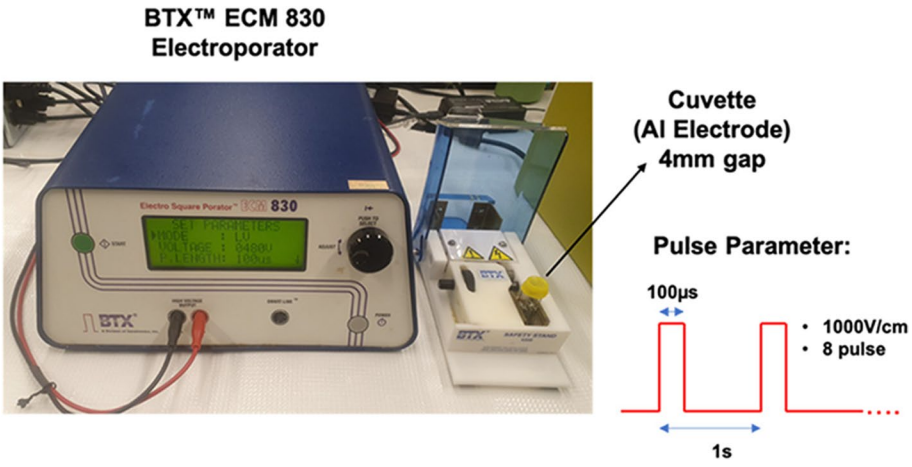


**Fig. 5** Electroporation setup with BTX™ ECM 830 electroporator square pulse generator and cuvette with aluminum electrodes having 4 mm gap and 600 µl cell suspension

### MT Cell Viability Assay

The time- and dose-dependent cytotoxicity for the combined treatment was studied using a nonlytic assay to monitor cell viability at 24 h. This assay measures light emission when an added substrate is reduced to a NanoLuc substrate by only metabolically active cells [30]. Here, for the MT cell viability assay, 20 µl (20,000 cells/well) treatment samples were added in a 96-well plate with a supplementary 55 µl of cell media. Then, 1.5 µl each enzyme and substrate from RealTime-Glo™ (Promega Inc.) was mixed with 373.5 µl of culture media. Subsequently, 25 µl of freshly prepared MT assay reagent mix was added to each sample treatment and incubated for 24 h. This protocol allows us to monitor cell viability in real-time. Finally, the luminescence as a relative light unit (RLU) is measured using Synergy HTX multi-mode microplate reader at a fixed interval of 24 h. Each mean luminescence value was normalized with respect to the control.

$$\%\ \text{Vaibility} = \frac{\text{Luminescence value for Samples}}{\text{Luminescence value of Control}} \times 100 \tag{2}$$

### Colony-Forming Assay (CFA)

The colony-forming assay is a measure of the ability of cancer cells to form colonies [31]. We can evaluate the percentage of treated cells that can produce colonies, hence, determining the drug's effectiveness in terms of cytotoxicity. The treatment samples (500 cells per well) were plated in a 6-well plate with 2 ml of fresh culture media. For the plating efficiency, 500 cells per well were seeded from the untreated cells (control). After an incubation period of 15 days, the culture media was discarded accompanied by Phosphate Buffer Saline (PBS) wash. Next, the colonies formed were subjected to 1 ml of fixing agent (methanol:glacial acetic acid = 1:7) for 30 min. Subsequently, the fixing mixture was aspirated and washed with double-distilled water before introducing the staining reagent (0.1% crystal violet in methanol) then allowed to rest for 2 h. Finally, the staining reagent was aspirated, and the colony image was capture using I-Bright Imaging Systems (Thermo Fisher Scientific™) before utilizing ImageJ software to compute the number of colonies formed [32].

$$\text{PE}_{(\text{Untreated cells})} = \frac{\text{Number of colonies formed}}{\text{Total cells seeded}} \times 100 \tag{3}$$

$$\text{Survivalpercentage}_{(\text{Treatedcells})} = \frac{\text{Numberofcoloniesformed}}{\text{Totalcellsseeded} \times \text{PE}} \times 100 \tag{4}$$

### Reactive Oxygen Species

Reactive species, such as free radicals and molecules from oxygen, are produced in cancer cells can cause oxidative stress and cell death [33]. Here, the reactive oxygen species (ROS) assessment at 24 h was done. 20 µl (20,000 cells) of treatment samples were added in a 96-well plate, with an additional 60 µl of cell media. After 18 h incubation, 20 µl of hydrogen peroxide ($H_2O_2$) substrate from ROS-Glo™ (Promega) was added and further

incubated for 6 h. The Luciferin Precursor is formed by the direct reaction between the $H_2O_2$ present in the media and the $H_2O_2$ substrate added externally from the kit. Subsequently, a 100 µl luciferase detection reagent is introduced to each well, and luminescence (RLU) measurement using Synergy HTX multi-mode microplate reader is taken after 20 min of incubation time. During the 20 min period, the luciferin precursor is converted to luciferin in the presence of the ROS-Glo™ substrate detection solution. The $H_2O_2$ in the sample is proportional to the light signal [34].

### Glucose Metabolite Assay

One of the essential metabolites in many living cells is glucose. We used the bioluminescence-based Glucose-Glo™ assay from Promega [35] to study the effect of the combination treatment on the intracellular glucose level at 24 h. For this, 20 µl of treatment samples were added to a 96-well plate, and after 24 h, the culture media was removed, followed by cold PBS wash, to avoid any misrepresentation due to glucose present in the media. Next, 12.5 µl each of the inactivation solution (0.6 N HCl) and neutralization solution (1 M Tris base) was introduced to stop the metabolism rapidly and lyses the cells upon mixing. Then, 50 µl of glucose detection reagent is added and incubated at room temperature for 1 h. During this period, nicotinamide adenine dinucleotide (NAD) + hydrogen (H) (NADH) is produced by glucose dehydrogenase added to the sample, which consumes glucose and NAD+. With NADH, luciferin is obtained by reducing reductase substrate to produce light proportional to the amount of glucose. Finally, luminescence (RLU) using Synergy HTX multi-mode microplate reader was taken after the incubation period.

### Statistical Analysis

All the sample treatments were performed in triplicates, and the results are indicated as average(µ) ± standard error (SE). The R-studio software was used for statistical experimental data [36]. In the analysis of experimental data to find the statistically significant difference between the sample treatment, we performed multiple comparison tests (MCTs) using analysis of variance (ANOVA) [37, 38] then Tukey's honestly significant difference (HSD) test [39]. Also, we completed the Jarque–Bera test to check the normality distribution norm, independence, and homogeneity of variance of the variables [40]. Based on the critical value from the Tukey significance test, samples are assigned letter grades, where the same letter (s) means no statistical difference, and different letters imply that they are statistically different [41, 42].

## Results and Discussion

### MT Cell Viability Assay

We performed the cell viability in a nonlytic manner, i.e., without disrupting the cell membranes. The MBD-MB-231 cells and non-cancerous MCF10A cells were subject to EP only; 5 mM metformin only; 5 µM, 10 µM, 15 µM, 30 µM concentrations of cisplatin only; and then with cisplatin in combination with 5 mM metformin, with and without EP. Figure 6 shows the dose-dependency in cell cytotoxicity for various combinations of drugs for both the cell lines.

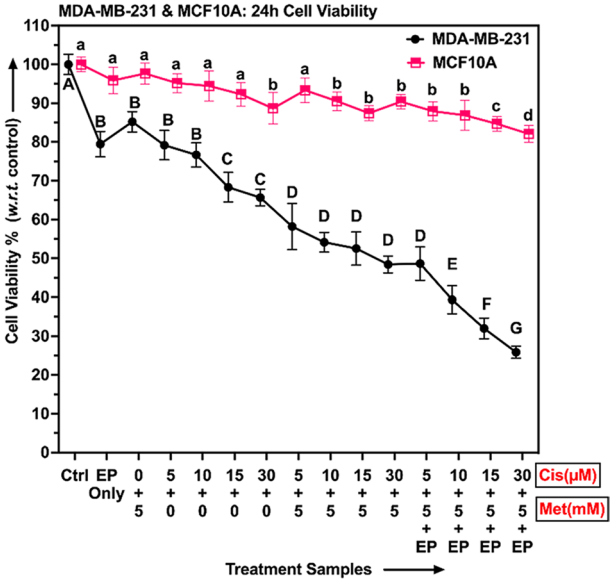


**Fig. 6** The dose-dependent cell viability of MDA MB 231 and MCF10A cells with EP only; metformin (5 mM); different concentrations of cisplatin (5 μM, 10 μM, 15 μM, 30 μM) and cisplatin + 5 mM metformin with and without EP at 24 h. The EP parameters were 8 pulses of 1000 V/cm, 100 μs at 1 s interval. The same letters indicate no significant difference between the treatments, whereas different letters indicate a statistically significant difference ($P < 0.05$). The values represent mean ± standard error ($N = 3$)

Firstly, for MDA-MB-231 at 24 h, the lowest viability of 25.87% was obtained using the combinations of electroporation with 30 μM cisplatin + 5 mM metformin. The results indicate a significant twofold drop in cell viability from 48.40 to 25.87% for 30 μM cisplatin + 5 mM metformin in the presence of electric field than drug only counterpart, i.e., for the 30 μM cisplatin + 5 mM metformin. Whereas for EP only and 5 mM metformin only treatments, cell viability was 79.45% and 85.20%, respectively, which are three times significantly higher than for 30 μM cisplatin + 5 mM metformin + EP. For the treatment with cisplatin alone, the maximum concentration of 30 μM yielded a drop in viability to 65.66% that is still higher than what was finally achieved with 30 μM cisplatin + 5 mM metformin + EP combination or with 30 μM cisplatin + 5 mM metformin (48.40%).

We performed ANOVA analysis to understand better the significance level, followed by Tukey's multiple comparisons test. As shown in Table 1, the $P$ value is less than 0.05, and the null hypothesis can be rejected. It implies that the means between the nine treatment groups are not equal. Furthermore, Tukey's HSD test reveals group means which are different. The control is significantly different ($P < 0.05$) from EP only and hence depicted by the different letters 'A' and 'B.' While EP only, 5 mM metformin, 5 μM, and 10 μM cisplatin only are represented by letter same letter 'B.' Similarly, 15 μM and 30 μM cisplatin

**Table 1** Summary of ANOVA analysis on the MT cell viability assay for MDA-MB-231 with all the 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between Treatments | 18,241 | 14 | 1303 | F (14, 30) = 37.65 | $P < 0.0001$ |
| Within treatments | 1038 | 30 | 34.60 | | |
| Total | 19,279 | 44 | | | |

treatment are represented by the same letter 'C.' For drug combination without EP, the 5 μM cisplatin + 5 mM metformin shows no comparable difference with subsequent doses till 30 μM cisplatin + 5 mM metformin, including for lower dose of 5 μM cisplatin + 5 mM metformin + EP, so these are depicted by the same letter 'D' and ranges from 58.20 to 48.65%. However, with EP, we see a significant difference from 10 μM cisplatin + 5 mM metformin at 39.34% indicated by 'E;' 15 μM cisplatin + 5 mM metformin at 32% indicated by 'F' and 30 μM cisplatin + 5 mM metformin at 25.86% indicated by 'G.' Thus, it confers a more prominent inhibiting of TNBC cell proliferation for drug combination in the presence of EP. Also, it should be noted that the same cell death can be achieved at one lower-step dosage with EP, leading to a lower side effect of the drug and delay in drug resistance with a better prognosis.

Unlike MBA-MB-231, the MCF10A shows much higher cell viability of 95.89% and 97.64% for treatment with EP only and 5 mM metformin alone, respectively, and no significant difference from control thus represented by the same letter 'a.' Furthermore, with cisplatin treatment, the viability unlike MBA-MB-231, the MCF10A shows much higher cell viability of 95.89% and 97.64% for treatment with EP only and 5 mM metformin alone, respectively, and no significant difference from control thus represented by the same letter 'a.' Furthermore, with cisplatin treatment, the viability drops to a meager 88.68% ('b') compared to 49.56% for MDA-MB-231 cells for the same 30 μM cisplatin treatment. The combination of cisplatin and metformin without EP also shows marginal cell viability variation, represented by the letter 'c.' Even for the highest configuration of 30 μM cisplatin + 5 mM metformin + EP ('d'), the cell viability is only 82%, equivalent to the effect observed at only 5 μM cisplatin alone for MDA-MB-231 cells. This overall suggests that the normal mammary epithelial MCF10A cells are not affected by the treatment type or combination, maintaining higher cell viability throughout drops to a meager 88.68% ('b') compared to 49.56% for MDA-MB-231 cells for the same 30 μM cisplatin treatment. The combination of cisplatin and metformin without EP also shows marginal cell viability variation, represented by the letter 'c.' Even for the highest configuration of 30 μM cisplatin + 5 mM metformin + EP ('d'), the cell viability is only 82%, equivalent to the effect observed at only 5 μM cisplatin alone for MDA-MB-231 cells. This overall suggests that the normal mammary epithelial MCF10A cells are not affected by the treatment type or combination, maintaining higher cell viability throughout (Table 2).

## Colony-Forming Assay

Clonogenic assay was performed to assess the cytotoxic effects at various concentrations of cisplatin in the presence of 5 mM metformin with and without the application

**Table 2** Summary of ANOVA analysis on the MT cell viability assay for MCF10A with all the 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between treatments | 1048 | 14 | 74.88 | $F$ (14, 30) = 3.087 | $P$ = 0.0047 |
| Within treatments | 727.6 | 30 | 24.25 | | |
| Total | 1776 | 44 | | | |

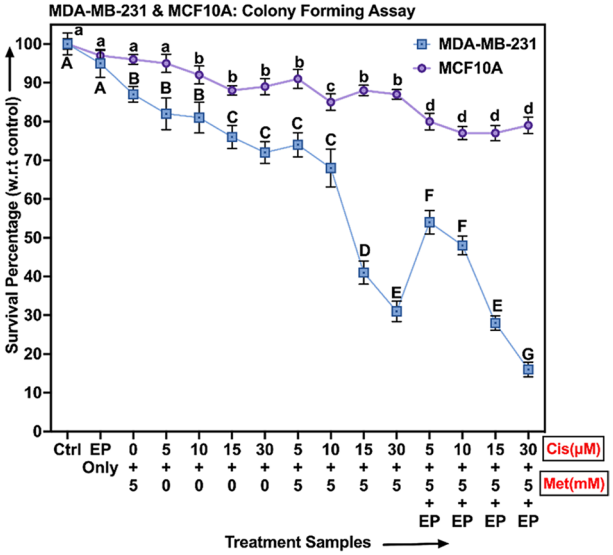


**Fig. 7** The survival percentage of MDA MB 231 cells with different concentrations of cisplatin and 5 mM metformin combination with and without EP. The same letters indicate no significant difference between the treatments, whereas different letters indicate a statistically significant difference ($P < 0.05$). All the values represent mean ± standard error ($N = 3$)

**Table 3** Survival percentage for all the sample treatment with and without electroporation for both MDA-MB-231 and MCF10A using digital imaging and cell per plate = 500

| Sr no | Drug combination | MDA-MB-231 Survival percentage (PE = 0.72 or 72%) | MCF10A Survival percentage (PE = 0.84 or 84%) |
|---|---|---|---|
| 1 | Control | 100% (reference) | 100% (reference) |
| 2 | EP only | 95% | 97% |
| 3 | 5 mM Met | 87% | 96% |
| 4 | 5 μM Cis | 82% | 95% |
| 5 | 10 μM Cis | 81% | 92% |
| 6 | 15 μM Cis | 77% | 88% |
| 7 | 30 μM Cis | 72% | 89% |
| 8 | 5 μM Cis + 5 mM Met | 74% | 91% |
| 9 | 10 μM Cis + 5 mM Met | 68% | 85% |
| 10 | 15 μM Cis + 5 mM Met | 41% | 88% |
| 11 | 30 μM Cis + 5 mM Met | 31% | 87% |
| 12 | 5 μM Cis + 5 mM Met + EP | 54% | 80% |
| 13 | 10 μM Cis + 5 mM Met + EP | 48% | 77% |
| 14 | 15 μM Cis + 5 mM Met + EP | 28% | 77% |
| 15 | 30 μM Cis + 5 mM Met + EP | 16% | 79% |

of electroporation. Figure 7 shows the results for both MDA-MB-231 and MCF10A cell lines. The colony survival capability of MDA-MB-231 decreases as the concentration of cisplatin + metformin increases, especially in the presence of an electric pulse. The triplicates from the untreated cell (control) were used to estimate plating efficiency in Eq. 3 and survival percentage for each of the treatment samples from Eq. 4. Table 3 summarizes the outcomes of this assay. In the case of MDA-MB-231, the survival percentage is 95%

**Table 4** Summary of ANOVA analysis on the colony-forming assay for MDA-MB-231 with a total of 15 treatment

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between treatments | 27,881 | 14 | 1992 | $F$ (14,30) = 71.68 | $P < 0.0001$ |
| Within treatments | 833.5 | 30 | 27.78 | | |
| Total | 28,715 | 44 | | | |

**Table 5** Summary of ANOVA analysis on the colony-forming assay for MCF10A with a total of 15 treatment

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between treatments | 2283 | 14 | 163.1 | F (14, 30) = 15.82 | $P < 0.0001$ |
| Within treatments | 309.2 | 30 | 10.31 | | |
| Total | 2592 | 44 | | | |

with EP only and 87% with 5 mM metformin. For various concentrations of cisplatin alone treatments, the survival ranges from 82 to 72%, whereas the survival percentage is 74%, 68%, 41%, 31% for 5 µM, 10 µM, 15 µM, 30 µM cisplatin + 5 mM metformin respectively with no EP treatment. With EP application, the survival percentage is 54%, 48%, 28%, 16% for 5 µM, 10 µM, 15 µM, 30 µM cisplatin + 5 mM metformin in each respectively, indicating the enhanced cell death due to EP application.

From ANOVA analysis, as shown in Table 4, the $P$ value < 0.05, we can reject the null hypothesis ($\mu_{control} = \mu_1 = \mu_2 = \mu_3 \ldots = \mu_8$). Thus, we inspect the difference between the means of the 15 treatment groups for colony-forming assay using Tukey multiple comparisons for the average percentage of survived colonies [43]. The test shows close to a twofold reduction in the survival percentage, which is notable, indicating more prominent cytotoxic effects of 30 µM cisplatin + 5 mM metformin ('G') in the presence of electric pulses on MDA-MB-231 cells compared to the corresponding drug combination only. The treatment for which there is no significant difference, such as 5 µM cisplatin + 5 mM metformin and 10 µM cisplatin + 5 mM metformin, is denoted by the letter 'C.' Similarly, 5 µM cisplatin + 5 mM metformin + EP and 10 µM cisplatin + 5 mM metformin + EP have the same letter 'F.' At the same time, 15 µM cisplatin + 5 mM metformin and 15 µM cisplatin + 5 mM metformin + EP shows significant difference represented by the letter 'D' and 'E,' respectively. A higher dose of 30 µM cisplatin + 5 mM metformin drug without EP has the same effect as a lower dose of 15 µM cisplatin + 5 mM metformin with EP. It shows that EP can considerably reduce the drug requirement for treatment with 15 µM cisplatin + 5 mM metformin significant differences of one order.

In comparison, the MCF10A cell line displays a higher ability to form colonies and low-variability *w.r.t* the variation in the drug combination. It is evident from Table 5 as the $F$ value for MCF10A (15.82) is 5 times lower than that of MBA-MB-231 ($F$ value – 71.68). The percentage of survival is 97% with EP only and 96% with 5 mM metformin. For cisplatin alone treatments, the survival is 95%, 92%, 88%, 89% for 5 µM, 10 µM, 15 µM, and

30 µM respectively, whereas for 5 µM, 10 µM, 15 µM, 30 µM cisplatin + 5 mM metformin without EP, it is 91%, 85%, 88%, 87% respectively. Furthermore, high survival of 79% for even the maximum dose of 5 µM cisplatin + 5 mM metformin + EP. Therefore, it is apparent that there is not much difference in the cytotoxicity with an increase in drug combination with or without EP for MCF10A.

## Reactive Oxygen Species Assessment

The hydrogen peroxide ($H_2O_2$) induced in the cell culture medium in the presence of TNBC cells under various drug combinations was measured using ROS-Glo™ (Promega). It has been reported that the excessive increment in intracellular ROS indicates enhanced cellular stress and can lead to drug-mediated cancer cell death [33, 44]. Therefore, it was vital to assess $H_2O_2$ directly into culture media. Figure 8a and b show the bioluminescent reading corresponding to the hydrogen peroxide levels for MDA-MB-231 and the non-cancerous MCF10A cell line. Firstly, we observe that MDA-MB-231 cells are considerably affected by the electric field application and follow a dose-dependent pattern compared to the MCF10A. For MDA-MB-231, the highest ROS level (224,639 RLU) was produced when the TNBC cells were exposed to 30 µM + 5 mM metformin in the presence of an electric field, compared to 30 µM + 5 mM metformin alone at 24 h. It indicates that EP enhances the uptake of the drug combination and is capable of generating added oxidative stress in the MBA-MB-231 cells, leading to an increase in cell death. In contrast, for MCF10A, the ROS levels remain almost 5.9 times lower (37,879 RLU) even for the highest combination of 30 µM cisplatin + 5 mM metformin + EP.

With ANOVA analysis, as shown in Table 6, the null hypothesis is rejected because of $P$ value < 0.05. It confirms that the difference between the means of the 15 treatment groups for reactive oxygen species. Furthermore, based on Tukey multiple comparisons test, we could differentiate between the significant change amongst various treatment samples. The same letter assigned to two or more samples conveys that they hold no significant difference, such as in the case of MDA-MB-231 control (36,009 RLU); EP only (36,909 RLU);

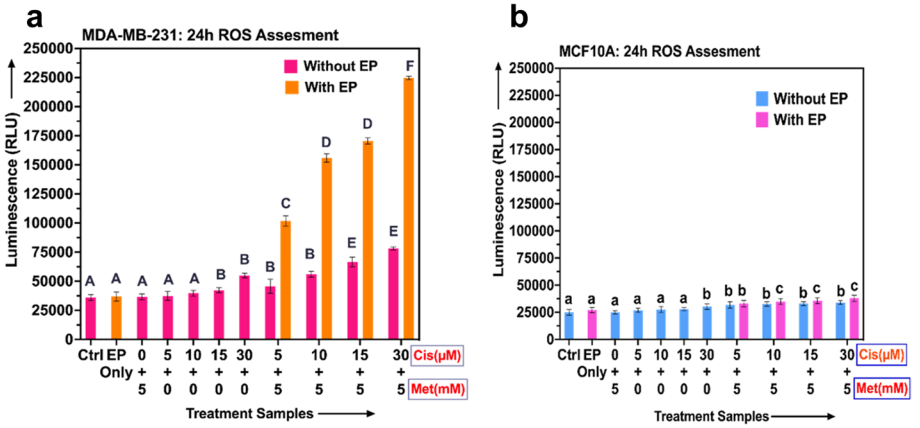


**Fig. 8** The reactive oxygen species levels using ROS-Glo™ (Promega). **a** MDA MB 231 cells, **b** MCF10A cells with EP only, metformin(5 mM), and different concentrations of cisplatin and cisplatin + 5 mM metformin with and without EP at 24 h. The same letters indicate no significant difference between the treatments, whereas different letters indicate a significant difference ($P < 0.05$). The values are represented in the form of mean ± standard error ($N = 3$)

**Table 6** Summary of ANOVA analysis on the reactive oxygen species assessment for MDA-MB-231 with a total of 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between treatments | 145,361,188,636 | 14 | 10,382,942,045 | $F$ (14, 30) = 321.1 | $P < 0.0001$ |
| Within treatments | 969,920,292 | 30 | 32,330,676 | | |
| Total | 146,331,108,928 | 44 | | | |

5 mM metformin alone (36,509 RLU); cisplatin 5 μM (37,289 RLU), 10 μM (39,673) are marked with a letter 'A,' whereas cisplatin 15 μM (42,295 RLU), 30 μM (54,980); and 5 μM cisplatin + 5 mM metformin (45,717 RLU), and 10 μM cisplatin + 5 mM metformin (56,009 RLU), without EP and are indicated by the letter 'B.' However, the electroporation leads to a significant increase in ROS for all the combinations of drug doses. For instance, 5 μM cisplatin + 5 mM metformin + EP (101,817 RLU), 10 μM cisplatin + 5 mM metformin + EP (155,883 RLU), 30 μM cisplatin + 5 mM metformin + EP (224,639 RLU) indicated by letter 'C,' 'D,' 'F' respectively. One exception to is 15 μM cisplatin + 5 mM metformin + EP (170,559 RLU) denoted by letter 'D' same as 10 μM cisplatin + 5 mM metformin + EP because it does not show significant increment.

Compared to control and the drug only, the EP counterpart for 30 μM cisplatin + 5 mM metformin shows a 6× and 3× increment in ROS levels, respectively. In the case of MCF10A, we do not see any dose dependency *w.r.t* the treatment combination, maintaining a low level of ROS value ranging from 35,009 RLU ('a') for 5 mM metformin to 47,879 RLU for 30 μM cisplatin + 5 mM metformin + EP ('c'). Moreover, from Table 7, we can observe that the $F$ value of 2.982 is much lower in the case of MCF10A than MDA-MB-231 ($F$ value = 321.1), thus leading to very low variability between the treatment samples, as apparent from Fig. 8b. Therefore, it supplements the sign that metformin can also sensitize MDA-MB-231 TNBC cells to cytotoxicity induced by cisplatin through oxidative stress [45] and insignificant change for MCF10A.

## Glucose Metabolite Assay

For a Type 2 diabetic patient, the lack of glucose uptake is linked to insulin resistance. In contrast, cancer cells are equated with higher glycolytic rates [46]. Therefore, measuring glucose will help us to analyze the specific effect of metformin on cancer cells. The intracellular glucose levels are measured in the MDA-MB-231 TNBC cells and non-cancerous MCF10A cells using a Glucose-Glo™ (Promega) bioluminescent assay without

**Table 7** Summary of ANOVA analysis on the reactive oxygen species assessment for MCF10A with a total of 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | ***P* value** |
|---|---|---|---|---|---|
| Between treatments | 694,102,635 | 14 | 49,578,760 | $F$ (8, 18) = 2.982 | $P = 0.0059$ |
| Within treatments | 499,019,820 | 30 | 16,633,994 | | |
| Total | 1,193,122,455 | 44 | | | |

deproteinization. This assay is critical to identifying the variation in glucose consumption due to glucose production alterations through gluconeogenesis resulting from metformin combined with cisplatin and electric pulses. In MDA-MB-231, we see that at the higher drug concentrations of 30 μM cisplatin + 5 mM metformin combination in the presence of EP, the glucose levels are at the lowest (222,570 RLU), which can lead to ATP (Energy) deficiency causing cell death. In contrast, for the MCF10A it is 3.4 times higher (764,947 RLU). Furthermore, it corroborates the decrease in ATP levels in MDA-MB-231 cells as reported earlier by metformin treatment [47].

With ANOVA analysis, as shown in Table 8, the null hypothesis can be rejected as the *P* value is less than 0.05. It implies that the means between the 15 treatment groups for glucose metabolic assay are not equal, so we observed a significant difference from Tukey's multiple comparison test ($P < 0.001$) in glucose levels in samples with and without electroporation. The control treatment has the highest glucose levels at 869,990 RLU compared to all other treatments and is marked by the letter 'A,' like 5 mM metformin treatment. EP only (779,990 RLU) up to 15 μM cisplatin (698,960 RLU) is represented with 'B.' The subsequent treatment of 30 μM cisplatin (601,875 RLU) and 5 μM cisplatin + 5 mM metformin (567,313 RLU) without EP are not significantly different, hence, the same letter 'C.' The 10 μM cisplatin + 5 mM (443,906 RLU) and 15 μM cisplatin + 5 mM (343,697 RLU) without EP are significantly different and indicated by letters 'D' and 'E,' respectively. The 5 μM cisplatin + 5 mM metformin + EP (343,310 RLU) and 10 μM cisplatin + 5 mM metformin + EP (315,423 RLU) does not significantly vary and are represented by letter 'E.' Moreover, interestingly, the same reduced level of glucose could be achieved at a lower dose of 10 μM cisplatin + 5 mM metformin with EP (315,423 RLU) compared to 30 μM cisplatin + 5 mM metformin without EP (312,880 RLU). Both are denoted by the letter 'E.' Therefore, we can say that it strengthens the fact that EP enhances the uptake of the drug where metformin promotes energy stress [48] and cytotoxicity at a lower dose compared to drug only. The higher concentration, i.e., 15 μM cisplatin + 5 mM metformin + EP (269,708 RLU) and 30 μM cisplatin + 5 mM metformin + EP (222,570 RLU) are significantly different and are represented by letters 'F' and 'G' respectively.

In Table 9, the lower *F* value (8.28) for MFC10A than MDA-MB-231 (*F* value of 193.5) indicates a low variability amongst the group means between the 15 treatment samples. The same is evident from Fig. 9, where the glucose levels are relatively close together (low variability). Besides, there is no substantial drop in glucose levels, even for 5 mM metformin + 30 μM cisplatin + EP drug combination (764,947 RLU for MCF10A compared to 222,570 RLU for MBA-MB-231). Therefore, we can state that the cancer cells are susceptible to the proposed drug combination with EP compared to non-cancerous MCF10A.

In Fig. 10, we outline a comparison between the mechanism of metformin + cisplatin working in synergy based on the experimental observation, with and without EP.

**Table 8** Summary of ANOVA analysis on the glucose metabolic assay for MBA-MB-231 with a total of 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | *F* (DFn, DFd) | *P* value |
|---|---|---|---|---|---|
| Between treatments | 2,245,625,669,547 | 14 | 160,401,833,539 | $\boldsymbol{F}(14, 30) = 193.5$ | $P < 0.0001$ |
| Within treatments | 24,874,579,470 | 30 | 829,152,649 | | |
| Total | 2,270,500,249,017 | 44 | | | |

**Table 9** Summary of ANOVA analysis on the glucose metabolic assay for MCF10A with a total of 15 treatments

| ANOVA table | Sum-of-squares (SS) | Degree of freedom (DF) | Mean square (MS) | $F$ ($DF_n$, $DF_d$) | $P$ value |
|---|---|---|---|---|---|
| Between treatments | 70,007,760,389 | 14 | 5,000,554,313 | $F$ (14, 30) = 8.28 | $P < 0.0001$ |
| Within treatments | 18,117,447,264 | 30 | 603,914,909 | | |
| Total | 88,125,207,653 | 44 | | | |

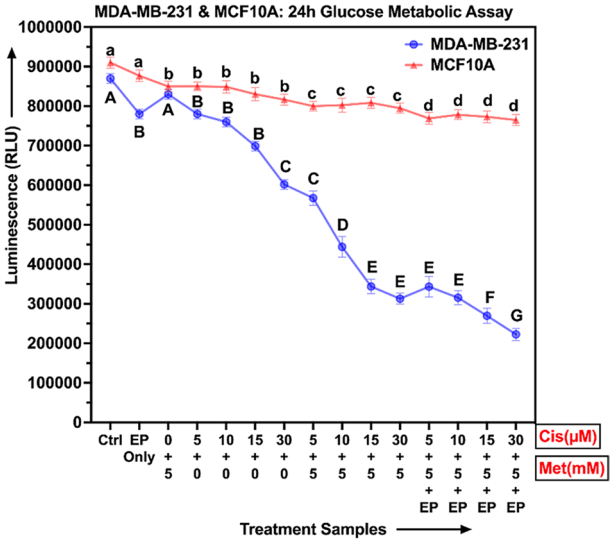


**Fig. 9** The intracellular glucose metabolite levels after cell lysis and using Glucose-Glo™ (Promega) for MDA-MB-231 and MCF10A cell for metformin (5 mM), EP only and various concentrations of cisplatin and cisplatin + 5 mM metformin with and without EP at 24 h. The same letters indicate no significant difference between the treatments, whereas different letters indicate a significant difference ($P < 0.05$). The values represent as mean ± standard error ($N = 3$)

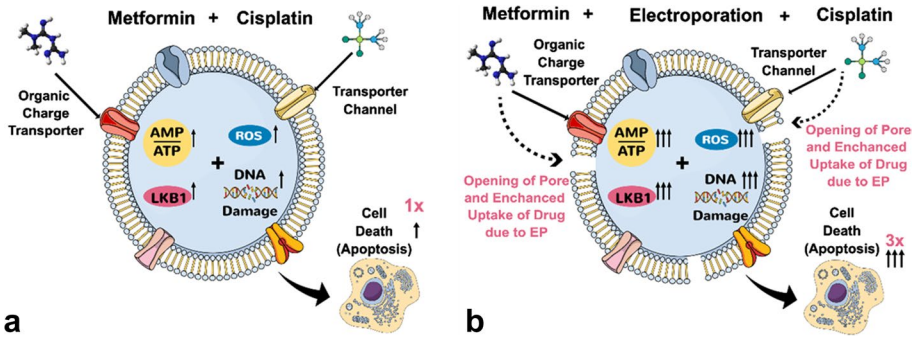


**Fig. 10** An illustration of a plausible mechanism. **a** With only metformin + cisplatin **b** metformin + cisplatin in the presence of electric pulse shows synergy based on the enhanced uptake, as can be seen from experimental observation

Figure 10a shows without EP, where cisplatin causes cell apoptosis by damaging the DNA under increased levels of ROS while assisted by metformin to lower the AMP:ATP creating energy stress and expression of LKB1. In contrast, Fig. 10b shows that with EP, the opening of the cell pore leads to enhancement in the amount of drug delivered. It results in a ROS suppressor [49] by further lowering the AMP:ATP ratio.

## Conclusion

Our results demonstrate the synergy of the combination of metformin and cisplatin, along with electrical pulses to decrease cell viability to 25.86% at 24 h for MDA-MB-231 TNBC cells. The resulted cell death with EP is 2× less than drug only at 30 μM cisplatin + 5 mM metformin at 24 h. The 6× increase in oxidative stress and lowering of glucose creating ATP deficiency of up to 4× times reveals that electrical pulses reduce the proliferation of cancer cells. Furthermore, the experimental data suggest that the equivalent cell death can be achieved at a lower concentration of drug dose combination in the presence of an electric field than drug combination alone. For instance, cell survival percentage at the 30 μM cisplatin + 5 mM metformin can be attained at 15 μM cisplatin + 5 mM metformin + EP. Furthermore, based on the experimental observations, we can infer that the metformin + cisplatin + EP combination has a specific effect on the MDA-MB-231 TNBC cells as the non-cancerous MCF10A cells did not show any significant variation *w.r.t* the cell viability and other assays. The results present preliminary signs of the potential of this combination therapy to clinical applications. The growing interest in alternative treatment and electrochemotherapy can benefit cancer survival rates and lesser side effects.

**Acknowledgements** One of the authors (P Sahu) is extremely grateful to the Ross Fellowship and is also thankful to Dr. L. Mittal and Mr. P. Giri for their assistance, guidance in conducting the experiments, analyzing the results, and procuring supplies and samples. All the authors are grateful to the reviewers for their insightful comments.

**Author Contribution** Concept and design: R Sundararajan, P Sahu. Experiments: P Sahu, IG Camarillo, R Sundararajan. Data analysis and draft: P Sahu, R Sundararajan, IG Camarillo. Manuscript: all.

**Data Availability** Data available on request.

## Declarations

**Ethical Approval** Not applicable.

**Consent for Publication** Yes.

**Conflict of Interest** The authors declare no competing interests.

## References

1. Evans, J. M. M., Donnelly, L. A., Emslie-Smith, A. M., Alessi, D. R., & Morris, A. D. (2005). Metformin and reduced risk of cancer in diabetic patients. *BMJ, 330*(7503), 1304–1305. https://doi.org/10.1136/bmj.38415.708634.F7.
2. Noto, H., Goto, A., Tsujimoto, T., & Noda, M. (2012). Cancer risk in diabetic patients treated with metformin: A systematic review and meta-analysis. *PLoS ONE, 7*(3), e33411. https://doi.org/10.1371/journal.pone.0033411.
3. Libby, G., Donnelly, L. A., Donnan, P. T., Alessi, D. R., Morris, A. D., & Evans, J. M. M. (2009). New users of metformin are at low risk of incident cancer. *Diabetes Care, 32*(9), 1620–1625. https://doi.org/10.2337/dc08-2175.
4. Mekuria, A. N., Ayele, Y., Tola, A., & Mishore, K. M. (2019). Monotherapy with metformin versus sulfonylureas and risk of cancer in type 2 diabetic patients: A systematic review and meta-analysis. *Journal of Diabetes Research, 2019*, 7676909. https://doi.org/10.1155/2019/7676909.
5. Gandini, S., Puntoni, M., Heckman-Stoddard, B. M., Dunn, B. K., Ford, L., DeCensi, A., & Szabo, E. (2014). Metformin and cancer risk and mortality: A systematic review and meta-analysis taking

into account biases and confounders. *Cancer Prevention Research (Philadelphia, Pa.), 7*(9), 867–885. https://doi.org/10.1158/1940-6207.CAPR-13-0424.
6. Decensi, A., Puntoni, M., Goodwin, P., Cazzaniga, M., Gennari, A., Bonanni, B., & Gandini, S. (2010). Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prevention Research (Philadelphia, Pa.), 3*(11), 1451–1461. https://doi.org/10.1158/1940-6207.CAPR-10-0157.
7. Ma, S.-J., Zheng, Y.-X., Zhou, P.-C., Xiao, Y.-N., & Tan, H.-Z. (2016). Metformin use improves survival of diabetic liver cancer patients: Systematic review and meta-analysis. *Oncotarget, 7*(40), 66202–66211. https://doi.org/10.18632/oncotarget.11033.
8. Wang, Z., Lai, S.-T., Xie, L., Zhao, J.-D., Ma, N.-Y., Zhu, J., … Jiang, G.-L. (2014). Metformin is associated with reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus: A systematic review and meta-analysis. *Diabetes Research and Clinical Practice*, *106*(1), 19–26. https://doi.org/10.1016/j.diabres.2014.04.007.
9. Yao, L., Liu, M., Huang, Y., Wu, K., Huang, X., Zhao, Y., … Zhang, R. (2019). Metformin use and lung cancer risk in diabetic patients: A systematic review and meta-analysis. *Disease Markers*, *2019*, 6230162. https://doi.org/10.1155/2019/6230162.
10. Col, N. F., Ochs, L., Springmann, V., Aragaki, A. K., & Chlebowski, R. T. (2012). Metformin and breast cancer risk: A meta-analysis and critical literature review. *Breast Cancer Research and Treatment, 135*(3), 639–646. https://doi.org/10.1007/s10549-012-2170-x.
11. Xu, H., Chen, K., Jia, X., Tian, Y., Dai, Y., Li, D., … Mao, Y. (2015). Metformin use is associated with better survival of breast cancer patients with diabetes: A meta-analysis. *The Oncologist*, *20*(11), 1236–1244. https://doi.org/10.1634/theoncologist.2015-0096.
12. Hardie, D. G., & Alessi, D. R. (2013). LKB1 and AMPK and the cancer-metabolism link - Ten years after. *BMC Biology, 11*(1), 36. https://doi.org/10.1186/1741-7007-11-36.
13. Irvin, W. J., & Carey, L. A. (2008). What is triple-negative breast cancer? *European Journal of Cancer, 44*(18), 2799–2805. https://doi.org/10.1016/j.ejca.2008.09.034.
14. Thike, A. A., Cheok, P. Y., Jara-Lazaro, A. R., Tan, B., Tan, P., & Tan, P. H. (2010). Triple-negative breast cancer: Clinicopathological characteristics and relationship with basal-like breast cancer. *Modern Pathology, 23*(1), 123–133. https://doi.org/10.1038/modpathol.2009.145.
15. de Ruijter, T. C., Veeck, J., de Hoon, J. P. J., van Engeland, M., & Tjan-Heijnen, V. C. (2011). Characteristics of triple-negative breast cancer. *Journal of Cancer Research and Clinical Oncology, 137*(2), 183–192. https://doi.org/10.1007/s00432-010-0957-x.
16. Hu, J., Lieb, J. D., Sancar, A., & Adar, S. (2016). Cisplatin DNA damage and repair maps of the human genome at single-nucleotide resolution. *Proceedings of the National Academy of Sciences, 113*(41), 11507–11512.
17. Basu, A., & Krishnamurthy, S. (2010). Cellular responses to cisplatin-induced DNA damage. *Journal of Nucleic Acids, 2010*, e201367. https://doi.org/10.4061/2010/201367.
18. Chen, H. H. W., & Kuo, M. T. (2017). Improving radiotherapy in cancer treatment: Promises and challenges. *Oncotarget, 8*(37), 62742–62758. https://doi.org/10.18632/oncotarget.18409.
19. Senkus-Konefka, E., & Jassem, J. (2006). Complications of breast-cancer radiotherapy. *Clinical Oncology, 18*(3), 229–235. https://doi.org/10.1016/j.clon.2005.11.004.
20. Quattrini, I., Conti, A., Pazzaglia, L., Novello, C., Ferrari, S., Picci, P., & Benassi, M. S. (2014). Metformin inhibits growth and sensitizes osteosarcoma cell lines to cisplatin through cell cycle modulation. *Oncology Reports, 31*(1), 370–375. https://doi.org/10.3892/or.2013.2862.
21. Teixeira, S. F., Guimarães, I. dos S., Madeira, K. P., Daltoé, R. D., Silva, I. V., & Rangel, L. B. A. (2013). Metformin synergistically enhances antiproliferative effects of cisplatin and etoposide in NCI-H460 human lung cancer cells. *Jornal Brasileiro de Pneumologia*, *39*, 644–649. https://doi.org/10.1590/S1806-37132013000600002.
22. Cioce, M., Valerio, M., Casadei, L., Pulito, C., Sacconi, A., Mori, F., … Blandino, G. (2014). Metformin-induced metabolic reprogramming of chemoresistant ALDHbright breast cancer cells. *Oncotarget*, *5*(12), 4129–4143.
23. Hwang, S.-Y., Park, S., & Kwon, Y. (2019). Recent therapeutic trends and promising targets in triple negative breast cancer. *Pharmacology & Therapeutics, 199*, 30–57. https://doi.org/10.1016/j.pharmthera.2019.02.006.
24. Lin, W.-Y., Cooper, C., Camarillo, I., Reece, L. M., Clah, L., Natarajan, A., … Sundararajan, R. (2014). The Effectiveness of Electroporation- based Nanocurcumin and Curcumin Treatments on Human Breast Cancer Cells, 7.
25. Mittal, L., Raman, V., Camarillo, I. G., & Sundararajan, R. (2017). Ultra-microsecond pulsed curcumin for effective treatment of triple negative breast cancers. *Biochemical and Biophysical Research Communications, 491*(4), 1015–1020. https://doi.org/10.1016/j.bbrc.2017.08.002.

26. Mittal, L., Aryal, U. K., Camarillo, I. G., Ferreira, R. M., & Sundararajan, R. (2019). Quantitative proteomic analysis of enhanced cellular effects of electrochemotherapy with Cisplatin in triple-negative breast cancer cells. *Scientific Reports, 9*(1), 13916. https://doi.org/10.1038/s41598-019-50048-9.
27. Mittal, L., Aryal, U. K., Camarillo, I. G., Raman, V., & Sundararajan, R. (2020). Effective electrochemotherapy with curcumin in MDA-MB-231-human, triple negative breast cancer cells: A global proteomics study. *Bioelectrochemistry, 131*, 107350. https://doi.org/10.1016/j.bioelechem.2019.107350.
28. Li, S. (Ed.). (2008). *Electroporation Protocols: Preclinical and Clinical Gene Medicine*. Humana Press. https://doi.org/10.1007/978-1-59745-194-9.
29. Qu, Y., Han, B., Yu, Y., Yao, W., Bose, S., Karlan, B. Y., … Cui, X. (2015). Evaluation of MCF10A as a Reliable Model for Normal Human Mammary Epithelial Cells. *PLOS ONE*, *10*(7), e0131285. https://doi.org/10.1371/journal.pone.0131285.
30. RealTime-Glo™ MT Cell Viability Assay | Live Dead Assay. (n.d.). Retrieved July 7, 2021, from https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/realtime_glo-mt-cell-viability-assay/.
31. Franken, N. A. P., Rodermond, H. M., Stap, J., Haveman, J., & van Bree, C. (2006). Clonogenic assay of cells in vitro. *Nature Protocols, 1*(5), 2315–2319. https://doi.org/10.1038/nprot.2006.339.
32. Rafehi, H., Orlowski, C., Georgiadis, G. T., Ververis, K., El-Osta, A., & Karagiannis, T. C. (2011). Clonogenic Assay: Adherent Cells. *Journal of Visualized Experiments : JoVE, 49*, 2573. https://doi.org/10.3791/2573.
33. (2018). Myricetin-induced apoptosis of triple-negative breast cancer cells is mediated by the iron-dependent generation of reactive oxygen species from hydrogen peroxide. Food and Chemical Toxicology, *118*, 154–167. https://doi.org/10.1016/j.fct.2018.05.005.
34. ROS-Glo™ H2O2 Assay. (n.d.). Retrieved July 7, 2021, from https://www.promega.com/products/cell-health-assays/oxidative-stress-assays/ros_glo-h2o2-assay/.
35. Glucose-Glo™ Assay | Glucose Assay Kit | Glucose Detection. (n.d.). Retrieved July 7, 2021, from https://www.promega.com/products/energy-metabolism/metabolite-detection-assays/glucose-glo-assay/.
36. Pallmann, P., & Hothorn, L. A. (2016). Analysis of means: A generalized approach using R. *Journal of Applied Statistics, 43*(8), 1541–1560. https://doi.org/10.1080/02664763.2015.1117584.
37. Lee, S., & Lee, D. K. (2018). What is the proper way to apply the multiple comparison test? *Korean Journal of Anesthesiology, 71*(5), 353–360. https://doi.org/10.4097/kja.d.18.00242.
38. McHugh, M. L. (2011). Multiple comparison analysis testing in ANOVA. *Biochemia Medica, 21*(3), 203–209. https://doi.org/10.11613/BM.2011.029.
39. Seaman, M. A., Levin, J. R., & Serlin, R. C. (1991). New developments in pairwise multiple comparisons: Some powerful and practicable procedures. *Psychological Bulletin, 110*(3), 577–586. https://doi.org/10.1037/0033-2909.110.3.577.
40. Thadewald, T., & Büning, H. (2007). Jarque-Bera test and its competitors for testing normality – A power comparison. *Journal of Applied Statistics, 34*(1), 87–105. https://doi.org/10.1080/02664760600994539.
41. Piepho, H.-P. (2004). An algorithm for a letter-based representation of all-pairwise comparisons. *Journal of Computational and Graphical Statistics, 13*(2), 456–466. https://doi.org/10.1198/1061860043515.
42. Piepho, H.-P. (2018). Letters in mean comparisons: What they do and don't mean. *Agronomy Journal, 110*(2), 431–434. https://doi.org/10.2134/agronj2017.10.0580.
43. Braselmann, H., Michna, A., Heß, J., & Unger, K. (2015). CFAssay: Statistical analysis of the colony formation assay. *Radiation Oncology, 10*(1), 223. https://doi.org/10.1186/s13014-015-0529-y.
44. Pattarawat, P., Wallace, S., Pfisterer, B., Odoi, A., & Wang, H.-C.R. (2020). Formulation of a triple combination gemcitabine plus romidepsin + cisplatin regimen to efficaciously and safely control triple-negative breast cancer tumor development. *Cancer Chemotherapy and Pharmacology, 85*(1), 141–152. https://doi.org/10.1007/s00280-019-04013-y.
45. Wandee, J., Prawan, A., Senggunprai, L., Kongpetch, S., & Kukongviriyapan, V. (2019). Metformin sensitizes cholangiocarcinoma cell to cisplatin-induced cytotoxicity through oxidative stress mediated mitochondrial pathway. *Life Sciences, 217*, 155–163. https://doi.org/10.1016/j.lfs.2018.12.007.
46. Argilés, J. M., & López-Soriano, F. J. (1990). Why do cancer cells have such a high glycolytic rate? *Medical Hypotheses, 32*(2), 151–155. https://doi.org/10.1016/0306-9877(90)90039-H.
47. Zhuang, Y., Chan, D. K., Haugrud, A. B., & Miskimins, W. K. (2014). Mechanisms by which low glucose enhances the cytotoxicity of metformin to cancer cells both in vitro and in vivo. *PLoS ONE, 9*(9), e108444. https://doi.org/10.1371/journal.pone.0108444.

48. Griss, T., Vincent, E. E., Egnatchik, R., Chen, J., Ma, E. H., Faubert, B., … Jones, R. G. (2015). Metformin antagonizes cancer cell proliferation by suppressing mitochondrial-dependent biosynthesis. *PLOS Biology*, *13*(12), e1002309. https://doi.org/10.1371/journal.pbio.1002309.
49. Vallianou, N. G., Evangelopoulos, A., & Kazazis, C. (2013). Metformin and cancer. *The Review of Diabetic Studies : RDS, 10*(4), 228–235. https://doi.org/10.1900/RDS.2013.10.228.