# Electrical Pulse-Mediated Veliparib for Effective Treatment of Triple Negative Breast Cancer: An *in vitro* Model Study

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## Abstract

Use of Poly (ADP-ribose) polymerase (PARP) inhibitors has received major attention as therapeutic agents for the treatment of breast cancers with or without mutations in BRCA1 or BRCA2. Thus, it can be effectively used for triple negative breast cancer (TNBC), which lacks the conventional receptors that many chemo drugs attack. Veliparib, a PARP agent is used as 2nd line of treatment along with platinum compounds. It is of practical interest to study the efficacy of Veliparib alone as an anti-cancer drug for TNBC. Towards this, in this research, we studied the efficacy of Veliparib on MDA-MB-231, human triple negative breast cancer cells. A concentration of 330μM was used for this purpose. In addition, to enhance the uptake of Veliparib against the plasma membranes of the cells, electroporation technique is used, which involves the local application of electrical pulses to open pores, which enables easy drug passage across the cell membranes. Thus, the objective of this study is to identify the potential of Veliparib and Electroporation as an alternate combinational therapy for TNBCs. Electrical pulses of high intensity, low duration 1200V/cm, 100μs, 8 pulses and low intensity high duration 500V/cm, 20ms, 8 pulses are used in this study. Cell viabilities were measured immediately, as well as after 24, 48 and 72 hours of treatment. The results indicate cell viabilities of 70% immediately after Veliparib+electrical pulses treatment, compared to 94% with drug only, indicating the potential of the synergy of electrical pulses+Veliparib. The viabilities were lower by 11 to 13 times after 72 hours. This promising treatment is transferrable to clinical practice.

## Introduction

With 1.7 million new cases (11.9%) in 2012, breast cancer was the second most common cancer and ranked 5th with 522,000 of deaths (6.4%) (Ferlay *et al.,* 2014). Triple Negative Breast Cancer (TNBC) contributes to about 12% to 17% of all invasive breast cancers in Western populations (Foulkes *et al.,* 2010). Studies indicate that TNBC is more common among Black and Hispanic women compared to White women (Desantis *et al.,* 2013). In India, TNBC is more prevalent with as many as one in three women with breast cancer could have triple negative (Sandhu *et al.,* 2016). Breast cancer patients are usually screened for an expression of

estrogen receptor (ER), progesterone receptor (PR) and evaluated for the amplification of HER-2/Neu. TNBCs do not display these expressions; hence it is more difficult to treat (Chavez *et al.,* 2011). Conventional therapies which target any of these receptors to treat breast cancers are not helpful for TNBC. Keam, B. *et al.* indicate that the probability of overall survival rate of TNBC patients is a poor 2.1%, while the relapse survival rate is mere 0.1% (Keam *et al.,* 2007). Thus, there is a critical need of alternate techniques to treat TNBC.

In clinical trials, Gemcitabine + Cisplatin is used as a first line treatment for metastatic TNBC, and Veliparib and carboplatin is also used to treat TNBC (Zhang *et al.,* 2014; Stover *et al.,* 2016). Veliparib is a Poly (ADP Ribose) Polymerase (PARP) inhibitor that stops the repairing of DNA of cancer cells, and hence serves as an anticancer drug (Velic *et al.,* 2015). The PARP function is critical in restoring the genome following the accidental single strand breaks in replication fork, which are equivalent to double stranded breaks in double helix (Weinberg, 2007). PARP inhibitors inhibit PARP enzyme to undermine DNA repair and improve the effect of DNA-damaging agents (Figure 1; Sonnenblick *et al.,* 2014).

Recently Murai *et al.,* (2012) have suggested an additional mode of actions of PARP inhibitors. They have suggested that PARP inhibitors cause the localization and trapping of PARP proteins at DNA repair site, which blocks the DNA replication and thus increasing toxicity in cells. Thus the reduced efficiency of DNA repair by PARP inhibitors in cancer cells can be attributed to the inhibition and trapping of PARP enzyme.

**Fig.1** Mechanism of action of PARP to cause cell death (Sonnenblick *et al.,* 2014)
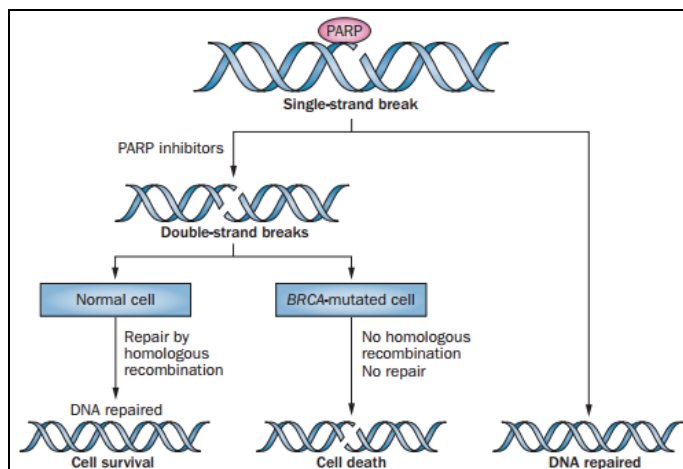


A study by Rozensal *et al.,* (2009) suggests that the phenanthridine derived PARP inhibitors (for example, PJ-34) are effective in breast cancer cells without BRCA1 or BRCA2 mutation. They have demonstrated that PARP inhibitors promote cell cycle arrest at G2/M and cell death in MCF-7 and MDA-MB-231 breast cancer cell lines, which lack BRCA1 and BRCA2 mutation. Therefore, PARP inhibitors are sensitive to MDA-MB-231 TNBC cell line as well. Thus, interest has been growing towards PARP inhibitors in combination with DNA damaging drugs (Carboplatin, Paclitaxel and Cisplatin etc.) to treat cancers with or without BRCA mutations. However, the efficacy of various PARP inhibitors in TNBC without BRCA mutations is not yet fully established.

Veliparib is a potent PARP inhibitor very effective in the treating variety of cancers with poor prognoses (www.onclive.com). Veliparib has been shown to improve the antitumor action of various DNA damaging agents such as temozolomide, cyclophosphamide, platinum, and radiation in preclinical models of melanoma, and breast and colon cancer (Donawho *et al.,* 2007). It has been used with platinum chemo drug, carboplatin in triple negative breast cancer treatment in several Phase I and II clinical trials and have demonstrated the efficacy and safety of Veliparib in patients with triple negative breast cancer (Pahuja *et al.,* 2015). Considering this, Veliparib has been used in present study as an anticancer drug against non BRCA mutated, Triple Negative breast cancer cell line MDA-MB-231. Which is a spindle shaped invasive adherent type epithelial cell (www.cellbiolabs.com). This cell line was derived at M.D. Anderson Cancer Centre in 1973 from a Caucasian women through metastatic pleural effusion (Sonnenblick *et al.,* 2014). MDA-MB-231 is classified as Claudin-low type among the five types of molecular classification in breast carcinoma. It exhibits intermediate response to chemotherapy (Holliday *et al.,* 2011).

Electric potentials are not only limited to inorganic process but they can be found in living organism as well. Cells, the basic unit of life are known to possess ions and other charged molecules and thus exists a voltage difference between the cytoplasm and extracellular matrix, known as membrane potential ($V_m$). Due to their electrical properties cells elicit a response to the external electric field. The membrane potential in cancerous cells has been observed to be depolarized than their normal counterparts (Redmann *et al.,* 1972). The depolarization serves as signal for mitosis and DNA synthesis in normal

and tumorous cells (Binggeli *et al.,* 1986; Orr *et al.,* 1972). Thus, considering the distinct bioelectric properties of cancer cells, it makes sense to use electrical pulse to treat cancers. Towards this, a novel physical technique, using electrical pulses and Veliparib is studied in this research for their efficacy on human TNBC cell line, MDA-MB-231. This technique involves the application of high intensity, short duration electric pulses which improve the permeability of cell membrane to facilitate increased uptake of the chemo drug (Teissie and Tsong, 1981). This therapy is particularly useful when big-3 (radiotherapy, chemotherapy and surgery) fail to treat cancers (Campana *et al.,* 2009; Weaver, 2000). In the present study electrical pulses of varied magnitude and duration are used to enhance the uptake of Veliparib.

## Materials and Methods

### The cells

In present study, triple negative, basal type human adenocarcinoma epithelial breast cancer cell line, MDA-MB-231 is used. This cell line is negative to ER, PR, and HER2 receptors. This cell line is low in Ki67, E-cadherin, claudin-3, clau-dinin-4 and claudinin-7 (Holliday *et al.,* 2011).

### The drug

Veliparib di-hydrochloride (ABT-888, Medchemexpress LLC, NJ) is used for this study. It is a 2-((R)–2-methylpyrrolidin-2-yl)-1H-benzimidazole-4-carboxamide di-hydrochloride with chemical formula $C_{13}H_{18}Cl_2N_4O$ and molecular weight of 317.21 g/mol. The structure is shown in figure 2. Veliparib inhibits both PARP-1 and PARP-2 with Kis (inhibitory constants) of 5.2 and 2.9nM/L, respectively (Wagner, 2015).

**Fig.2** Chemical Structure of Veliparib (di-hydrochloride) (www.medchemexpress.com)
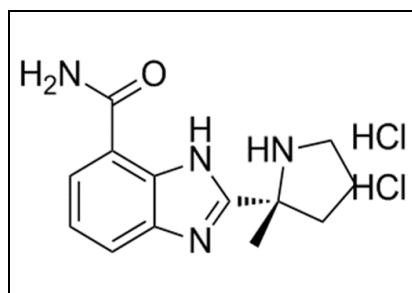


As seen with many PARP inhibitors, inhibition by Veliparib is quite selective and pharmacologically

relevant concentration of Veliparib doesn't produce substantial effects on other receptors or ion channels. Veliparib is used to treat ovarian cancer, oral cancer, basal like breast cancer, pancreatic cancer, prostate cancer (Pahuja *et al.,* 2015; Wagner, 2015).

The various side effects include gastrointestinal toxicity, nausea, vomiting, secondary leukemia, myelodysplastic syndrome, diarrhea, constipation, stomach pain, fatigue (Pahuja *et al.,* 2015). These side effects can be effectively reduced if the concentration of Veliparib used in the treatment is reduced.

Veliparib, solubilized in DMSO at 10mM/mL was used at to study the dose curve and a concentration of 330µM was used for the viability study.

### The electroporator

BTX ECM 830 electroporator (Genetronics Inc., San Diego, CA) is used to generate unipolar square wave pulses with 1 Hz frequency. Table 1 shows the applied pulse parameters. The pulse parameters are based on previous research (Sundararajan *et al.,* 2012; Gehl and Geertsen, 2000).

### The viability assays

### Trypan blue assay

20µL of treated samples and 20µL of trypan blue were mixed together. From this mixture, 20µL was used to count both live and dead cells using the Nexcelom Bioscience Cellometer®. The percentage viability was also directly measured using the Cellometer®.

### MTT viability assay

Colorimetric assay was performed using Thiazolyl Blue Tetrazolium Bromide (MTT- Methylthiazolyldiphenyl-tetrazolium bromide). This assay is useful for assessing cytotoxicity, cell viability and cell proliferation (Scott, 2003; Gerlier and Thomasset1986; Meerloo *et al.,* 2011; Stockert *et al.,* 2012).

MTT is a yellowish aqueous solution, and produces violet-blue formazan (Figure 3), on reduction by dehydrogenases and reducing agents present in metabolically active cells (Stockert *et al.,* 2012). Formed MTT formazan can be estimated with the help of spectrophotometry after the extraction with organic solvents. The amount of MTT Formazan directly

correlates with the number of living cells (Meerloo *et al.*, 2011).

In this study, 4.8mM MTT stock solution was prepared in PBS solvent. 50µL of MTT stock solution was added into each well sometime (24 hours for studying dosage curve, and 24, 48, or 72 hours for measuring proliferation) after the treatment, which was further incubated at 37°C for 24 hours.

**Fig.3** Chemical structure of MTT and its reduced product MTT formazan (Stockert *et al.,* 2012)
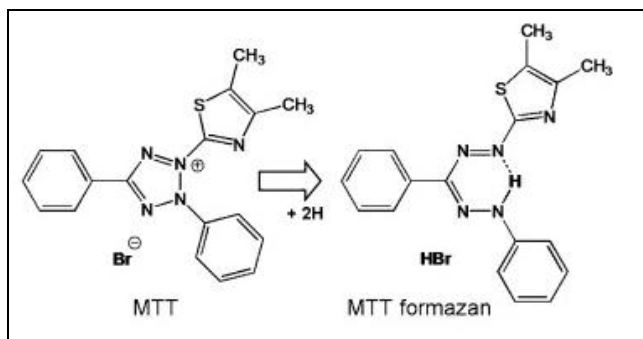


The media was discarded after 24 hours of incubation and 80µL of DMSO was added into each well to dissolve MTT formazan crystals. The 96 well plate was stirred for 15 minutes at 37°C to fully dissolve the formazan crystals. Spectrophotometer was used to study absorbance at the wavelengths of 570 and 630 nm. The difference of absorbance at 570 and 630 nm was calculated for each cell and the resulting is subtracted by the difference in absorbance for the blank cell. The final viability was obtained after normalizing with control.

**Statistical analysis**

Analysis of variance (ANOVA) is performed on the data to find statistical difference. Constant variance and normality assumptions are verified for the data before performing ANOVA. Either one way or two way ANOVA has been performed, depending upon the number of factors. One way ANOVA is performed for dose curve study, where veliparib concentration is the single factor. The layout of the model is shown in table 2.

The observations can be described with the effect model, as in equation 1. This equation is called the one way ANOVA model. In this model, $\mu$ is a constant overall mean which is common to all treatments, $C_i$ is the effect of $i^{th}$ level of concentration (treatment) which is unique

to the $i^{th}$ treatment. The effect model is a linear statistical model, with response variable $Y_{ij}$ as linear function of model parameters. $\varepsilon_{ij}$ represents the error component, which is assumed to be independently and normally distributed random variable with mean 0 and variance $\sigma^2$ (Montgomery, 2013).

$$Y_{ij} = \mu + C_i + \varepsilon_{ij} \begin{cases} i = 1,2 \dots 7 \\ j = 1,2,3; \end{cases} \quad (1)$$

where, $\sum_{i=1}^{7} C_i = 0$; $\varepsilon_{ij} \approx NID(0, \sigma_D^2)$

Two way ANOVA is performed for viability study, with two factors: Treatment and Day. Viability in triplicates was measured for each combination of treatment and day. The Layout of the resulting two factor model is shown in table 3.

An effect model can be used to describe observation in two factorial experiments, as in equation 2 (Montgomery, 2013). Equation 2 is called two-factor or two way ANOVA model. $Y_{ijk}$ is the cell viability for treatment level i, Day level j, and $k^{th}$ replicate. Also, $\mu$ is overall mean, $T_i$ is the effect of $i^{th}$ level of Treatment, $D_j$ is the effect of $j^{th}$ level of day, $TD_{ij}$ the effect of interaction between treatment and day, and $\varepsilon_{ijk}$ is a random error component. The Treatment is a fixed effect while Day is random effect, making interaction as random effect. Taking the day as random effect represents the overall population of days.

$$Y_{ijk} = \mu + T_i + D_j + TD_{ij} + \varepsilon_{ijk} \begin{cases} i = 1,2,3,4 \\ j = 1,2,3 \\ k = 1,2,3 \end{cases} \quad (2)$$

where, $\sum_{i=1}^{4} T_i = 0$; $D_j \approx NID(0, \sigma_D^2)$; $TD_{ij} \approx NID(0, \sigma_{TD}^2)$; $\sum_{i=1}^{4} TD_{ij} = 0 \ \forall j$; $\varepsilon_{ijk} \approx NID(0, \sigma^2)$

Equation 3 represents the hypothesis of interest in ANOVA (Montgomery, D. C. (2013).

$$\begin{aligned} H0: \ & \mu 1 = \mu 2 = \mu 3 \dots = \mu n \\ H1: \ & \text{Not all means are equal} \end{aligned} \quad (3)$$

where, n = number of independent comparison groups.
F test was done to test hypothesis, as described (Montgomery, D. C. (2013).

When we reject null hypothesis of equal treatment means in ANOVA, all pair wise mean comparisons were tested using Tukey's test (Tukey, 1953; Abdi, 2010), as post F test analysis. JMP® software is used for statistical analysis.

### The protocol

Figure 4 shows the protocol used in this study. All experiments were performed in triplicate.

### Results and Discussion

### Dose curve

Figure 5 shows the dose curve obtained using MTT assay for the MDA-MB-231 cells treated with various doses of Veliparib. This dose curve indicates the effect of veliparib alone as an anti-tumor drug. The viabilities are normalized with control. The viability dropped from 88% at 1μM to 65.43% at 10μM and reduced to 43.99% at 50μM. However, it increased to 54.22% at 100μM and reduces to 34.89 for 300μM. The lowest viability of 23.57% is obtained for 330μM. An increase in viability is observed with increase in dosage of Veliparib with 30.39% viability for 500μM. This increase in viability could be attributed to the saturation of the drug, which correlates with the results obtained in another study by Lee, J.*et al.,* where they report the 11% of cell kill at 50μM concentration of Veliparib. In the present study, we have chosen 330μM concentration of Veliparib to treat MDA-MB-231 cells with/out electrical pulses.

One way ANOVA was performed to detect the statistical significance difference among the various drug concentration. P-value ($p < 0.0001$) obtained from F-test indicates the existence of significance difference among treatments. As a post F-test, comparison of means was done using Tukey's HSD test. Letter report obtained from test is indicated in the table 4. The difference in the letters reported for two treatment levels, indicates that there isstatistically significantdifference between two treatments. Control with letter 'A' is significantly different from treatment levels 10, 50, 100, 300,330 and 500μM, as none of them contain letter 'A' in their report. On the other hand, control is not significantly different from 1μM treatment, as they both share letter 'A' (Table 5).

### Viability study using cellometer

Immediately after the treatment, Trypan Blue assay was used to determine the viability. Figure 6 shows the viabilities of the cell line without any treatment (control), Veliparib only, and then the combination of drug and electric pulses at 1200V/cm, 100μs, 8pulses, and 500V/cm, 20ms, 8pulses. The control has a viability of 94.14%, while the drug only has a viability of 93.8%, indicating just a cell-kill of 6%. This could be attributed to the aggressiveness of the TNBC cell line.

It can be seen that with the synergy of the electrical pulses and the PARP inhibitor, there is increased cell kill. With the high intensity, short duration electric pulses of 1200V /cm, 100μs, 8pulses, the cell-kill is about 13%, with a viability of 87%, while it is 30% with a viability of 70%, for 500V/cm, 20ms, 8pulses.

**Table.1** Pulse parameters studied

| S. No | Electric Field Intensity V/cm | Pulse Duration | No. of Pulses |
|---|---|---|---|
| 1 | 1200 | 100μs | 8 |
| 2 | 500 | 20ms | 8 |

**Table.2** The Layout of the statistical model for dosage curve

| Veliparib Concentration (μM) | Viability |
|---|---|
| Control (0μM) | xxx |
| 1 | xxx |
| 10 | xxx |
| 50 | xxx |
| 100 | xxx |
| 300 | xxx |
| 330 | xxx |
| 500 | xxx |

**Table.3** The Layout of the two factors statistical model for viability study

| Treatment | Day | | |
|---|---|---|---|
| | **Day 1** | **Day 2** | **Day 3** |
| Control | xxx | xxx | xxx |
| Veliparib only | xxx | xxx | xxx |
| Veliparib + 1200V/cm, 100μs, 8 pulses | xxx | xxx | xxx |
| Veliparib + 500V/cm, 20ms, 8 pulses | xxx | xxx | xxx |

**Table.4** Connecting Letter Report from Tukey's HSD test

| Level | Letter Report | Least Square Mean |
|---|---|---|
| Control | A | 100 |
| 1μM | AB | 88.06 |
| 10μM | BC | 65.42 |
| 100μM | CD | 54.22 |
| 50μM | CD | 43.99 |
| 300μM | CD | 34.89 |
| 330μM | D | 23.57 |
| 500μM | D | 30.39 |

**Table.5** Connecting Letter Report from Tukey's HSD test on treatments

| Level | Letter | Least Square Mean |
|---|---|---|
| Control | A | 94.136667 |
| Drug only | A | 93.800000 |
| 1200V/cm, 100μs, 8 pulses | A | 87.027778 |
| 500V/cm, 20ms, 8 pulses | B | 69.575556 |

**Fig.4** Procedure for *in-vitro* electroporation
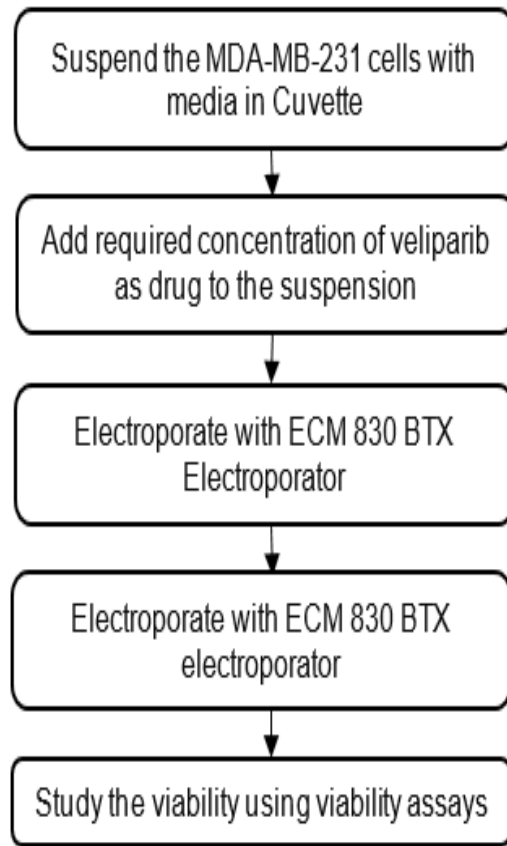


**Fig.5** Dose curve of Veliparib on MDA-MB-231 cells without electroporation
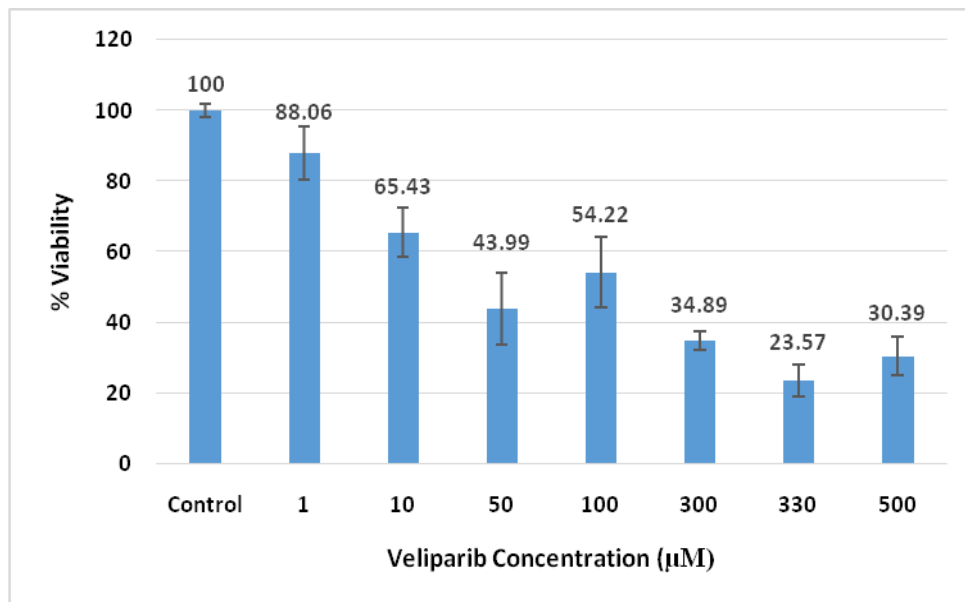
**Fig.6** Viabilities of MDA-MB-231 cells without any treatment, with Veliparib alone (330µM) and combination of Veliparib (330µM) and electroporation
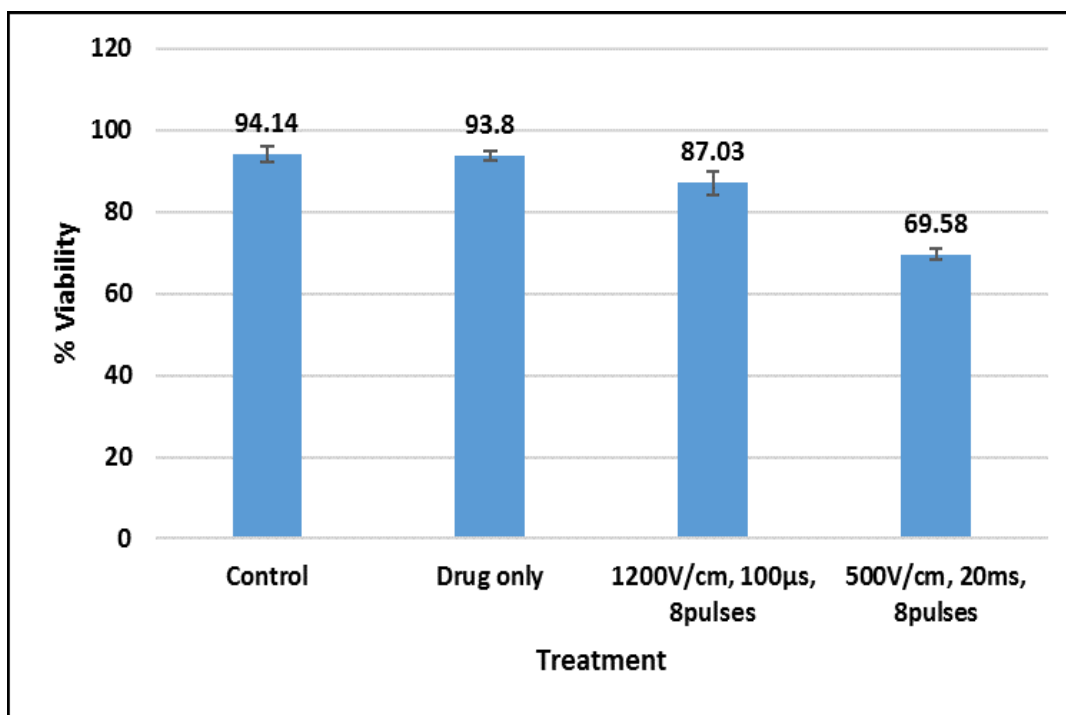


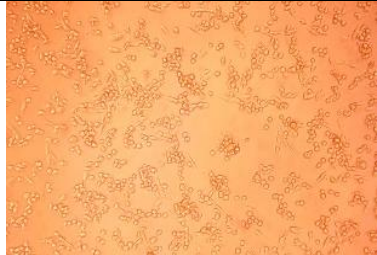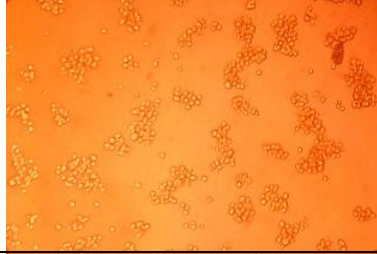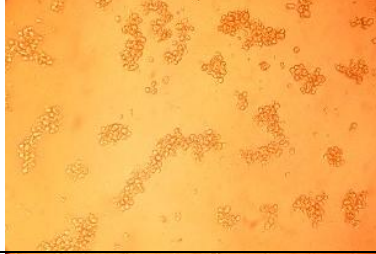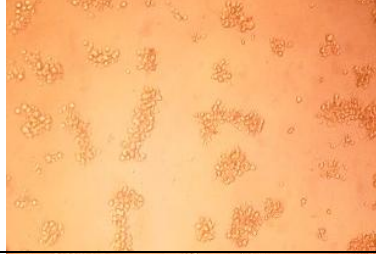**Fig.7** Viabilities of MDA-MB-231 cells after 24, 48 and 72 hours: without any treatment, with Veliparib alone (330µM), and combination of Veliparib (330µM) and electroporation
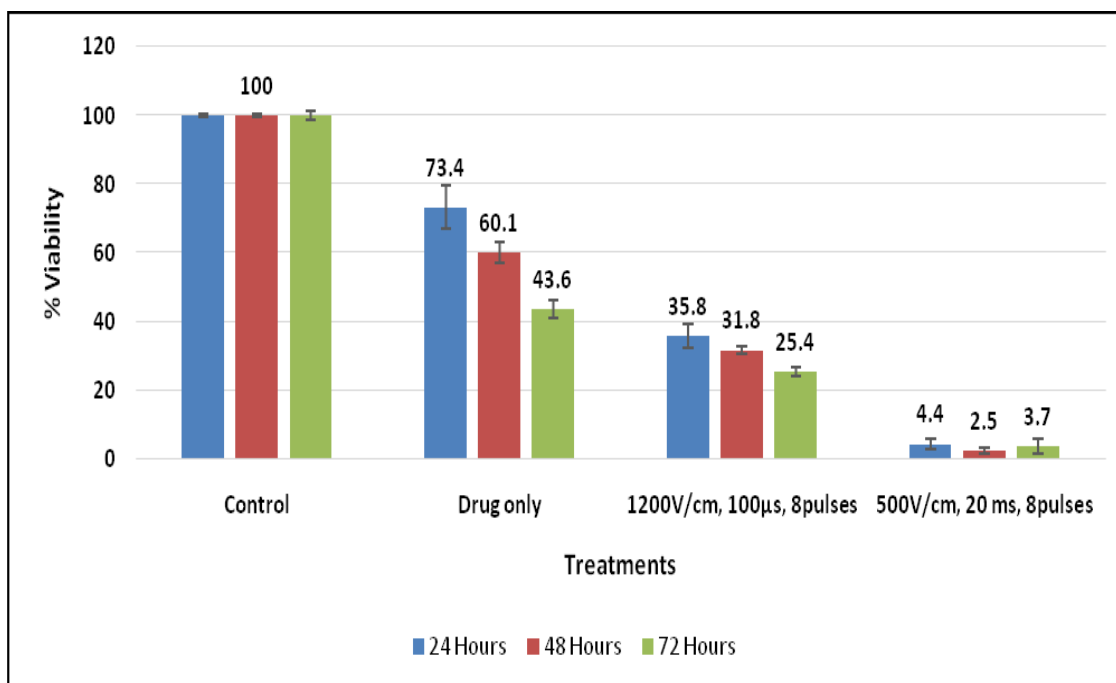
**Fig.8** Typical microscopic images of MDA-MB-231 cells after 24, 48 and 72 hours: without any treatment, with Veliparib alone (330µM), and combination of Veliparib (330µM) and electroporation

| Treatment | 24 Hours | 48 Hours | 72 Hours |
|---|---|---|---|
| **Control** | | | |
| **Drug only** | | | |
| **1200V/cm, 100µs, 8pulses** | | | |
| **500V/cm, 20ms, 8pulses** | | | |



Veliparib in combination with low duration electrical pulses of 1200V/cm, 100µs, 8 pulses also shares the same letter 'A' with control and drug only treatments, indicating the inefficiency of these low duration pulses. Treatment with long duration pulses of 500V/cm, 20ms, 8pulses has the letter B, different from those of control, and drug only, and electroporation treatment using 1200V/cm pulses, thus significantly different.

**Viability study using MTT assay**

The viabilities of MDA-MB-231 cells 24, 48 and 72 hours following the treatment were measured with MTT assay. Figure 7 illustrates the viabilities at the various conditions, including, control, drug only, and the two conditions of Veliparib and electroporation. All

viabilities are normalized with respect to the viability of control. The drug only samples had a viability of 73.4% after 24 hours, which dropped down by ~18% to reach ~60% after 48 hours, which further dropped by ~27% to reach ~44% after 72 hours of treatment. These reductions indicate the long term efficacy of the drug. Similar results were also obtained for the Veliparib+electrical pulses treated samples.

Treatment with higher intensity, lower duration electrical pulses of 1200V/cm, 100µs, 8 pulses yielded the viability of ~36% after 24 hours and a drop of 11% and 20% was observed after 48 and 72 hours of the treatment. The treatment with 500V/cm, 20ms, 8 pulses with Veliparib yielded viability of 4.4%, after 24 hours, which dropped to 2.5%, after 48 hours and increased to 3.7% after 72

hours. This low viability or high cell kill may be due to the high energy content of the 500V/cm, 20ms, 8 pulses, which is sufficient to cause cell death. It can be observed that by varying the pulse intensity, duration, it is possible to obtain desired cell kill.

Figure 8 shows the typical microscopic images of MDA-MB-231 cells after 24, 48 and 72 hours of the treatment. It can be observed that for control, the cell confluency increases with time. In cells treated with Veliparib, the cells form clusters which may be due to the alterations in cell to cell and cell-substratum interactions. Previously, Masiello *et al* have also observed such alterations in MDA-MB-231 cell line exposed to microgravity (Masiello *et al.,* 2014). In the cells treated with 1200V/cm, 100µs, 8pulses, the cells appear to lose their adhesive properties, but unlike the drug only treatment, they do not form clusters. This highlights the differential mechanism when MDA-MBA-231 cells were treated with the combination of drug and electrical pulses. Further study is required to understand the exact mechanism. Another study conducted by Pehlivanova, *et al*. also indicate that electroporation can reduce the cell adhesion and replication (Pehlivanova *et al.,* 2012), which is in line with our study. Microscopic pictures obtained under low intensity, high duration electrical pulses of 500V/cm, 20ms, 8pulses indicate the absence of live cells after 24, 48 and 72 hours of treatment. This is in agreement with viability count obtained with MTT assay (Figure 7), where 4.4%, 2.5%, and 3.7% of live cells were observed after 24, 48 and 72 hours.

## Conclusion

Triple Negative Breast Cancers are hard to be treated, due to the lack of the three receptors, and hence majority of therapies fail to work. In this study, the feasibility of Veliparib and electroporation as alternate therapy has been explored. Towards this, In this study Veliparib at a concentration of 330µM was used in combination with electrical pulses on MDA-MB-231 cell line. Various pulses of different intensity and pulse duration were used to enhance the Veliparib uptake against the cell membrane.

Results indicate that the treatment with Veliparib and electrical pulses is effective for treating TNBCs. Immediately after the treatment, low intensity, high duration electrical pulses of 500V/cm, 20ms, 8 pulses yielded cell kill of ~30% compared to ~13% with high intensity, low duration pulses of 1200V/cm, 100µs, 8 pulses and ~6% with drug only and control. After 72

hours of treatment ~6 times lower viability is obtained with 500V/cm, 20ms, 8pulses compared to 1200V/cm, 100µs, 8pulses, and ~11 times lower compared to viability of drug only treatment. Thus, it is concluded that low intensity, high duration electrical pulses are more effective in combination with Veliparib to treat TNBCs.

Optimal efficiency and desired amount of cell kill could be achieved optimizing the parameters of electrical pulses. This treatment could be transferred to clinical practice quickly as an alternate therapy against triple negative breast cancers.

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