Anti-Cancer Drugs 1998, 9, pp. 319–325

Research paper

Enhancement of cytotoxicity by electropermeabilization: an improved method for screening drugs

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Electropermeabilization (EPN), also termed electroporation, is a physical method to overcome the barrier of the cell membrane by applying short and intense electric pulses. It is the basis for a new cancer treatment modality, electrochemotherapy, where uptake of chemotherapeutics is enhanced by EPN. Preclinical and clinical trials have shown that application of electric pulses *in vivo* is feasible and that electrochemotherapy is highly efficient. The aim of this study was to develop an improved method of screening drugs on electropermeabilized versus non-electropermeabilized cells. In this study we describe an easy protocol which gives high cell viability, good reproducibility and a high rate of cell permeabilization. Cell cytotoxicity is simply determined by the MTT assay. Cell death due to the EPN procedure was less than 4% and more than 90% of cells were permeabilized. For daunorubicin, doxorubicin, etoposide and paclitaxel, no effect of EPN was found. For carboplatin and cisplatin the effect of EPN was a factor 3 and 2.3, respectively, on the IC_{50} (inhibitory concentration 50%). For bleomycin we found a dramatic effect of EPN of the magnitude of a factor 300 on the IC_{50}. In conclusion, we have established a new, easy and reliable protocol to test new drugs for cytotoxicity with or without the limitations of the cell membrane. Our data support the role of bleomycin as the drug of choice for electrochemotherapy. [© 1998 Lippincott-Raven Publishers.]

Key words: Cytotoxicity, electrochemotherapy, electropermeabilization, electroporation, lucifer yellow, MTT.

Introduction

Electropermeabilization (EPN), also termed electroporation, is a physical method to make cell membranes permeable by applying short and intense electric pulses. EPN is used for introducing various molecules directly into the cytosol, e.g. DNA, dyes and various marker molecules.[1,2] Electrochemotherapy—the use of *in vivo* EPN combined with chemotherapy—has proven very efficient in the local treatment of tumors both in animal studies[3–9] and in phase I-II studies.[10–13] This new treatment modality in cancer therapy can be used both in the curative and palliative settings for localized tumors. It is simple, inexpensive and requires one or few treatments. Optimization of the various parameters involved in electrochemotherapy is warranted, including a screening of drugs to be used in electrochemotherapy. Previous studies have tested the effect of EPN using 5 min drug exposure.[9,14] We felt that a 1 h exposure at 37°C would be more relevant when compared with the clinical setting and would perhaps reduce mortality from the EPN procedure. Therefore, we developed a new protocol combining EPN and the MTT assay using 1 h drug exposure. The level of cell permeabilization was tested with the fluorescent dye lucifer yellow examined by flow cytometry and appropriate parameters were determined. Under these improved experimental conditions, seven drugs commonly used in the clinical setting were tested: bleomycin, cisplatin, carboplatin, etoposide, daunorubicin, doxorubicin and paclitaxel.

Materials and methods

Cell line and drugs

The DC-3F cell line, a Chinese hamster lung fibroblast cell line, was kindly provided by Dr Jean Belehradek Jr, Institut Gustave-Roussy, Paris. Cells were maintained

We thank Fabrikant Einer Willumsens Mindelegat for financial support.

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in MEM culture medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) and penicillin-streptomycin at 37°C and 5% CO_2.

The following drugs were used: bleomycin (Lundbeck, Copenhagen, Denmark), cisplatin (Bristol-Myers Squibb, Princeton, NJ), carboplatin (Bristol-Myers Squibb), etoposide (Bristol-Myers Squibb), daunorubicin (Rhône-Poulenc Rorer, Birkerød, Denmark), doxorubicin (Pharmacia & Upjohn, London, UK) and paclitaxel (Bristol-Myers Squibb).

EPN

Cells were harvested using trypsin-EDTA, washed once, resuspended in SMEM medium (Gibco) and counted. Viability was estimated using the Trypan blue exclusion test. Cells were chilled on ice and 400 μl of cell suspension (2.2×10^6 cells/400 μl) was put in each of two 4 mm wide cuvettes (Equi-Bio, Kent, UK). Then 40 μl of drug, or in the case of controls isotonic saline, was added to reach the final concentration of drug. One cuvette was left unpulsed and the other exposed to eight electric pulses at an electric field intensity of 1.2 kV/cm, with a pulse duration of 99 μs, using a BTX T820 square wave electroporator (BTX, San Diego, CA). Then the pulsed and unpulsed cuvettes, which were covered with lids to maintain pH and sterility, were placed in a heat block at 37°C for 60 min. Cells were then diluted by a factor of 100 in RPMI 1640 culture medium (Gibco) with 10% FCS and penicillin-streptomycin, and seeded in 96-well plates.

MTT assay

For cell viability determination we used the MTT assay.[15,16] Briefly, after 96 h of culture, 10 μl of 5 mg/ml MTT (dimethylthiazol-diphenyltetrazoliumbromide thiazolyl blue; Sigma, St Louis, MO) was added to each well. Four hours later, the reaction was stopped by addition of 10% sodium dodecylsulfate in 0.01 M HCl. After 24 h of incubation at 37°C, optical density (OD) was measured in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The IC_{50}, defined as the drug concentration which inhibited dye formation by 50% compared to controls, was determined directly from semi-logarithmic dose-response curves.

Flow cytometry

Cells were trypsinized, harvested, counted and then chilled on ice. Lucifer yellow (Sigma) was added to a final concentration of 1 mM. Then cells were exposed to electric pulses in cuvettes and left at 37°C for 5 min. Cells were then chilled on ice again, washed twice in PBS buffer without calcium and magnesium, and examined for fluorescence emission in a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon ion laser emitting at 488 nm. Fluorescence was detected at 530 nm with a 30 nm bandwidth. Median fluorescence intensity (arbitrary units) was determined and cells were grouped by fluorescence intensity in two groups, showing up to or over 100 units, respectively.

Calculations and statistics

Pulsed and unpulsed controls were compared to estimate the cytotoxic effect of EPN alone. The pulsed control sample was set as 100% for the pulsed samples and likewise for the unpulsed samples. Mean ± SEM and *t*-test for paired data were calculated using SAS software.

Results

Cell EPN and viability

For convenient pharmacological testing, it was necessary to determine optimal conditions for achieving high cell survival and efficient permeabilization. Cells were submitted to eight square wave pulses of 99 μs duration, delivered at a frequency of 1 pulse per second (1 Hz), in correspondence with previously published work.[14] Viability of cells exposed to various field strengths and incubated for 60 min before any further cell manipulation is shown in Figure 1. Less than 4% reduction in viability was observed up to 1.2 kV/cm and no dramatic decrease in cell viability was observed at field strengths up to 1.6 kV/cm. Cell permeabilization was determined by flow cytometry after simultaneous exposure of cells to electric pulses and lucifer yellow (Figure 2). Median fluorescence intensity increased steeply after 1 kV/cm and at a field strength of 1.2 kV/cm more than 90% of cells were permeabilized (Figure 2).

At the optimal electrical conditions, defined as 1.2 kV/cm, eight pulses of 99 μs duration, the mean value of viability of the pulsed controls was 96.5% of that of the unpulsed controls (range 88-106%), when comparing all pulsed and unpulsed controls (32 samples each). This corresponds to a mean loss of viability of 3.5% as a result of the EPN procedure itself.

The importance of the incubation period was tested in an experiment comparing 5 and 60 min incubation. When comparing viability of pulsed in relation to unpulsed samples after 5 and 60 min, the mean viability after 5 min incubation was 4.6% lower than after 60 min incubation ($p < 0.05$ by t-test for paired samples).

Toxicity of anticancer drugs on electropermeabilized cells

The cytotoxicity of bleomycin, carboplatin, cisplatin, daunorubicin, doxorubicin, etoposide and paclitaxel on pulsed and unpulsed cells was determined using the optimal permeabilization conditions, and the IC_{50} values are shown in Table 1. There was a marked augmentation of the cytotoxicity of bleomycin of a factor 300 after EPN (Figure 3 and Table 1). For carboplatin and cisplatin, the effect of EPN was an increase in cytotoxicity of a factor 3 and 2.3, respectively (Figure 4 and Table 1). For daunorubicin, doxorubicin, etoposide and paclitaxel, there was no difference in cytotoxicity (Figure 5 and Table 1). As an example, only data on daunorubicin and etoposide are shown in Figure 5.

Discussion

Previous studies have already dealt with the toxicity of anticancer drugs on electropermeabilized and non-

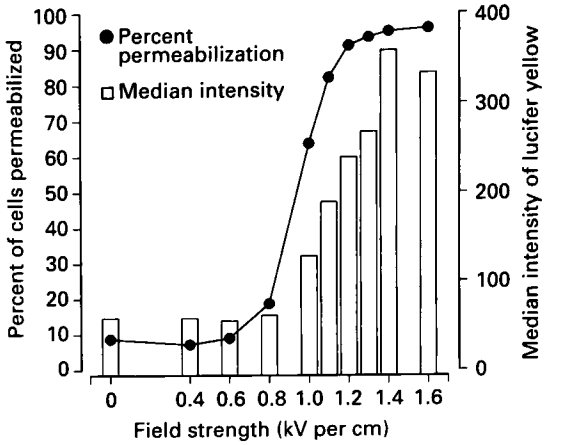


Figure 2. Percent of cells permeabilized, as determined by uptake of the fluorescent dye lucifer yellow (solid circle symbols). Median intensity of fluorescence in cells exposed to electric pulses at increasing field strengths in the presence of lucifer yellow (bars). Results from one experiment, representative of two independent experiments giving the same results.

Table 1. IC_{50} with and without EPN

Drug	IC_{50} (μM)	IC_{50} with EPN (μM)	Fold difference
Bleomycin	27	0.09	300
Carboplatin	450	150	3
Cisplatin	24	10.5	2.3
Daunorubicin	3	3	no difference
Doxorubicin	2	2	no difference
Etoposide	12	12	no difference
Paclitaxel	8	8	no difference

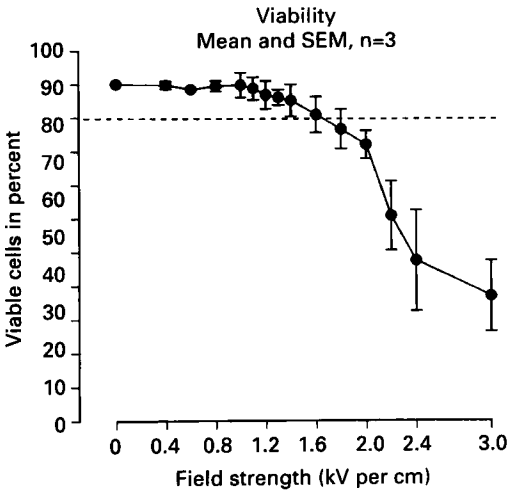


Figure 1. Viability as a function of field strength, expressed as percent of control. Means ± SEM of n=3 independent experiments.

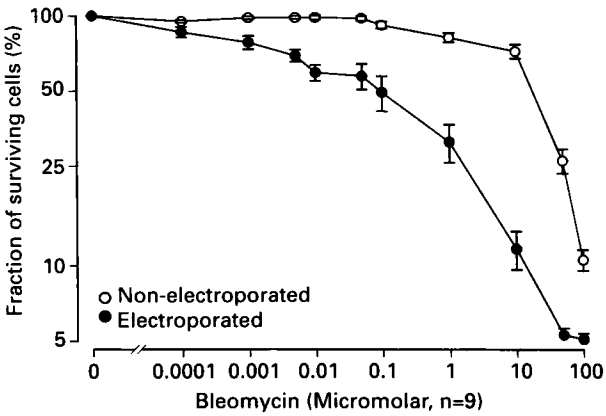
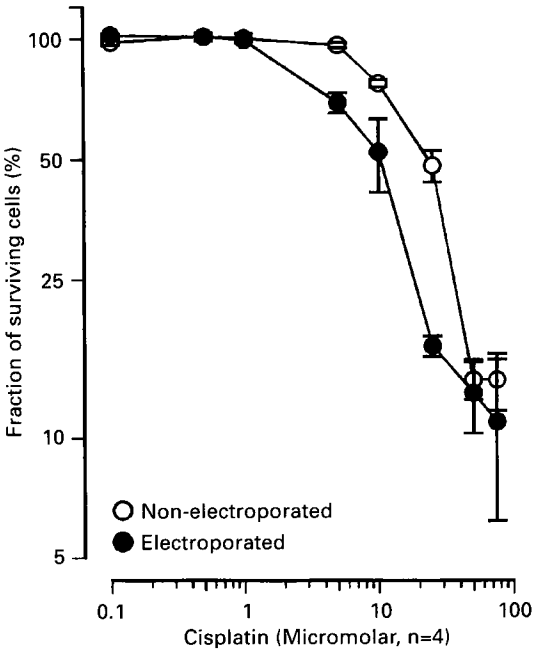


Figure 3. Cytotoxicity of bleomycin with or without electroporation. Means ± SEM of n=9 independent experiments.

electropermeabilized cells. In most of these studies,[9,14,17] cells were exposed to similar electric pulses as in the present study, but were suspended in 50 μl and incubated for only 5 min at room temperature before further manipulation. Moreover, all these authors used clonogenic assays to determine cell viability.

Here we describe a new protocol that introduces several improvements to the methods used in the previous studies. First, cells are pulsed in 400 μl, in a



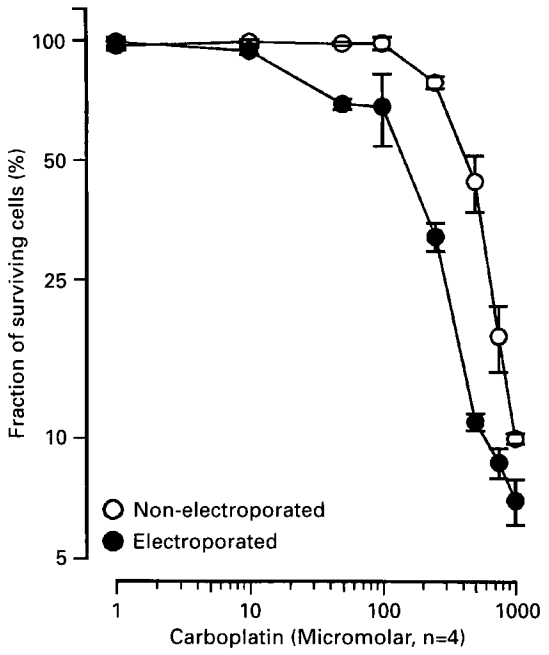
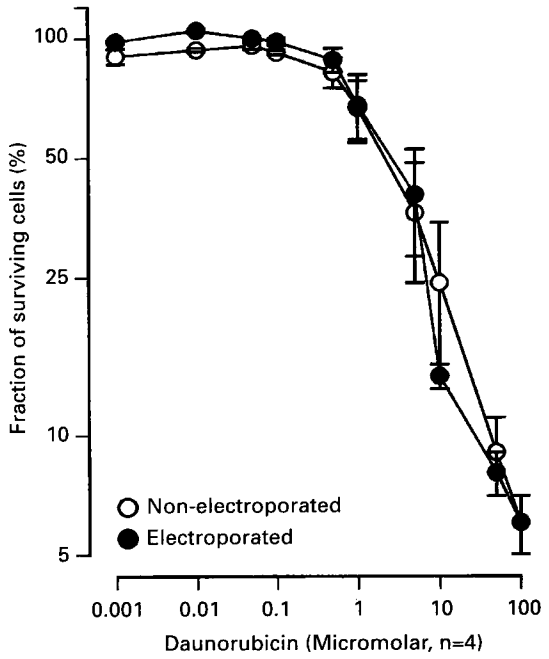


Figure 4. Cytotoxicity of cisplatin (top) and carboplatin (bottom) with and without electroporation. In both cases, means ± SEM of n=4 independent experiments.



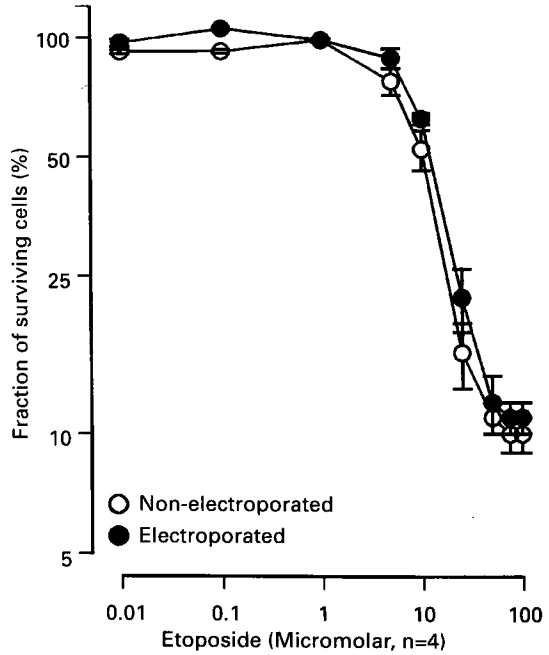


Figure 5. Cytotoxicity of daunorubicin (top) and etoposide (bottom) with and without eletroporation. In both cases, means ± SEM of n=4 independent experiments.

closed cuvette. This opens possibilities to incubate cells for a longer time. Indeed with only 50 μl in a well, water evaporation can lead to a reduction in volume and medium concentration that could potentially be harmful to the cells. Therefore, we could incubate the cells for 60 min at 37°C before further manipulations. As a consequence, the mean difference in viability between pulsed and unpulsed cells was less than 4%. Other studies report a higher mortality from the electroporation procedure, under 10%,[14] or 12%[9] using 1.25 kV/cm, but these studies all employ 5 min incubation after electroporation. Under our conditions, prolonging the incubation time from 5 to 60 min resulted in a significant gain in cell viability of approximately 5%. In other words, minimal handling after the EPN procedure seems to be important for obtaining low mortality. Furthermore, a longer incubation time at a set temperature, in this case 37°C, makes it easier to control both incubation time and temperature from experiment to experiment. Finally, in this study we utilized the MTT assay, which is widely used as an easy and reliable test when screening for cytotoxicity.

These advantages make this new protocol an interesting alternative for the screening of new cytotoxic non-permeating drugs. Moreover, excellent electrical parameters for obtaining high cell survival and cell permeabilization were obtained. The mean difference between pulsed and unpulsed controls was 3.5%, indicating very low cytotoxicity of the EPN procedure itself, and at the applied field strength more than 90% of cells were permeabilized as determined by loading with the dye lucifer yellow. Lucifer yellow is a non-toxic organic anion fluorescent dye, which does not cross the cell membrane. Mir *et al.*[18] showed that lucifer yellow could be used to distinguish permeabilized from non-permeabilized cells by fluorescence microscopy. Later, Dinchuk *et al.* used EPN to load lymphocytes with lucifer yellow[19] and flow cytometry to quantitate lucifer yellow content over time as a model for studying organic anion transport. We found lucifer yellow loading and flow cytometry to be a fast, easy and reliable way to determine the fraction of cells permeabilized by EPN. Furthermore, quantitative information can be obtained by looking at the median intensity of fluorescence. We found a very sharp increase in both fraction of permeabilized cells and median fluorescence intensity when field strengths exceeded 0.8 kV/cm, indicating a threshold value. This is in line with what has previously been reported.[20] At 1.2 kV/cm and higher, more than 90% of cells were permeabilized, but there was still an increase in median fluorescence intensity up to 1.4 kV/cm. However, since viability started declining after 1.4 kV/cm, we chose 1.2 kV/cm to test drug effects on the DC-3F cells. The use of this EPN procedure means that drugs can be tested for their cytotoxic effect *per se*, i.e. for the cytotoxic effect when the cell membrane barrier is eliminated.

For bleomycin the IC_{50} was a factor of 300 lower when using EPN, which is expected as bleomycin is a hydrophilic, charged molecule with a molecular weight of 1400. Orlowski *et al.*[14] found a reduction of the IC_{50} of a factor 700 using clonogenic assay and 5 min exposure to the drug after eight pulses of 100 μs each at 1.5 kV/cm. The higher electric field would give rise to a somewhat larger degree of permeabilization, which would in turn give a lower IC_{50} for samples exposed to electric pulses. Indeed the IC_{50} found was 1.5 nm, where in this study we found an IC_{50} of 90 nM. There is also a difference between the two studies on the samples not exposed to electric pulses, as the Orlowski study finds an IC_{50} of 1 μM where we find one of 27 μM. This is most likely due to differences in the study set-up, e.g. one study used a clonogenic assay and the other study used a MTT assay. However, the general picture remains clear: combining bleomycin with EPN increases the cytotoxicity of this drug dramatically. In a later study,[17,20] bleomycin uptake was quantitated using ^{57}Co-labeled bleomycin, confirming that the increase of bleomycin uptake was indeed the basis for the described increase in cytotoxicity. Bleomycin is regarded the drug of choice for electrochemotherapy and has been used in phase I–II studies.[11–13]

In the present study, for carboplatin and cisplatin the IC_{50} was a factor 3 and 2.3 lower with EPN. This is in accordance with a paper by Melvik *et al.*[21] from 1986 where cisplatin cytotoxicity and uptake, measured by atomic absorption spectroscopy, were described. In that study, exponential wave pulses were used and the cells were exposed to drugs for 2 h. An increase of both uptake and cytotoxicity of cisplatin of a factor of 3 when combined with EPN was reported. In 1995, Sersa *et al.*[9] found a factor of 8 increase of cytotoxicity of cisplatin after EPN, using a clonogenic assay. However, in this study cells were only exposed to cisplatin for 5 min, and it is likely that increasing the incubation to 1 h will lessen the difference between electropermeabilized and non-electropermeabilized cells. Again, the new protocol gives results in accordance with previously published data. The effect of EPN on carboplatin cytotoxicity has to our knowledge not yet been described. The similarity of the results obtained is in agreement with the chemical similarity of these two compounds.

For the drugs daunorubicin, doxorubicin, etoposide and paclitaxel, no effect of EPN was found, which is what would be expected[14] for these amphi- or

lipophilic drugs, respectively. It was, however, important to determine that this test will reliably show which drugs migrate easily across the cell membrane without the assistance of the transient permeation structures created by EPN. A previous study on uptake of daunorubicin and doxorubicin[22] showed that equilibrium was reached for daunorubicin within 30 min and that for doxorubicin there was some, but very little, additional uptake after 60 min. With short incubation an effect of EPN on doxorubicin toxicity is likely to be found, but in the present study, as expected, with incubation over 1 h there is no difference.

Conclusion

We have established a new protocol to easily and reliably test drugs for cytotoxicity with or without the limitations of the cell membrane. With this test, the loss of cell viability due to EPN is marginal since minimal handling after EPN increases cell viability. We have validated this procedure using previously tested drugs and we have also tested new drugs amongst those in general use in cancer chemotherapy.

Our results show, as would be expected, that amphi- or lipophilic drugs like daunorubicin, doxorubicin, etoposide and paclitaxel enter the cells readily and no effect of EPN was found. The cytotoxicity of carboplatin and cisplatin was augmented by EPN with a factor of 3 and 2.3, respectively. The large, charged and hydrophilic molecule bleomycin showed a dramatic increase in the IC_{50} of a factor 300 when combined with EPN, in agreement with previous reports.

In conclusion, the new protocol established here could be a method of reference for the future testing of new drugs suitable for electrochemotherapy. Our data support the role of bleomycin as the drug of choice for electrochemotherapy[11-13] and points out that electrochemotherapy can also be used in conjunction with cisplatin or carboplatin.

Acknowledgments

We thank Marianne Friis Knudsen for excellent technical assistance.

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(Received 20 January 1998; accepted 29 January 1998)