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COMBINED EFFECT OF ELECTROPORATION AND MILTEFOSINE ON KERATINOCYTE CELL LINE HaCaT

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Abstract. In this study we investigated the effect of combined treatment of HaCaT keratinocyte cells with electrical pulses (200–500V/cm) and the alkylphosholipid (ALP) miltefosine. The data show that electroporation in combination with miltefosine induces cytoskeleton disruption and increases the permeability of cell monolayers due to interruption of cell-cell junctions', as documented by fluorescent imaging of ZO-1 and actin integrity. This was accompanied with reduction of cell viability. The combination of these conditions could be considered as a method for treating several types of skin cancer or other pathological conditions affecting the skin integrity.

Key words: electroporation, drug delivery, miltefosine, fluorescent imaging, ZO-1, keratinocytes.

1. INTRODUCTION

The permeability of a cell membrane can be controlled by application of external electrical pulses. Changes in the intensity and/or duration of pulses over a threshold result in electroporation or electropermeabilization of the membrane [1]. After the first application of electrical pulses for delivery of plasmid DNA into cells, this approach became routinely used in cell and molecular biology. Recently, electrochemotherapy was developed. This method allows enhanced delivery of antineoplastic drugs such as bleomycin and cisplatin into cancer cells, thus reducing the side effects of conventional chemotherapy [2–7]. Electrochemotherapy could be considered as a very effective method for treating skin cancer. In this regard, epithelial cells (keratinocytes), which are part of the skin barrier, are a main subject of electrochemotherapy [8].

Recently, the new branch of synthetic alkylphosholipids (ALPs) was introduced [9]. Unlike conventional anticancer agents, which act predominantly at

the DNA level, ALPs act on the cellular membrane, because of their similarity to the endogenous phospholipids. At low concentrations ALPs are inserted into the plasma membrane and subsequently cause a wide range of biological responses that ultimately lead to cell death. Important features of ALPs are their amphiphilic properties, which allow them to interact with cell membranes and to influence cellular metabolism at different levels. Like other ALPs, miltefosine exhibits substantial antiproliferative activity [10, 11].

The cytoskeleton is essential for cell functions as maintaining the cell's shape and participates in intercellular interactions. Application of electrical pulses can affect the integrity of the cytoskeleton, thus affecting cell survival, adhesion and intercellular contacts [8].

The purpose of this *in vitro* study was to determine whether miltefosine, alone or in combination with electroporation affects the survival of cells of the keratinocyte cell line HaCaT, their closed cell-cell contacts and the re-organization of the intracellular actin cytoskeleton.

The results of this study contribute to elucidating the effect of the combination of miltefosine and electrical pulses on healthy keratinocyte cells, as this combination is a treatment, which can be administered locally to certain skin diseases.

2. MATERIALS AND METHODS

2.1. CELL LINES

The cell line HaCaT was used, which arose *in vitro* from spontaneously transformed keratinocytes from histologically normal human skin. HaCaT cells were grown as monolayer [DMEM medium high glucose, supplemented with 2 mM L-glytamine, 10% fetal calf serum (FCS), and 1% antibiotic] at 37 ^{o}C in an incubator with humidified atmosphere and 5% CO_2. Cells were passaged two times weekly by trypsinization. All media and chemicals were from Lonza, Belgium.

Miltefosine was synthesized in the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, and was most graciously provided by Prof. M.R. Berger. It was dissolved in 4% ethanol at a volume of 10 ml and stored at 4°C.

2.2. MTS TEST FOR CELL SURVIVAL AND PROLIFERATION

To analyse the cytotoxic effect of miltefosine on non-cancer HaCaT cells, the MTS test (Promega) was performed as previously described [12]. Briefly, the adherent cells were treated as described above and incubated additionally for 24h. Then, 50 μL of MTS reagent was added directly to the adherent cells. They were incubated for 2 h at 37°C and the absorbance at 490 nm was recorded with a 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). Three independent experiments were performed for each treatment. To evaluate

the statistical significance of the cell viability reduction, a comparison between exposed and control probes was performed by Student's t-test. P-values lower than 0.05 were considered statistically significant.

2.3. ELECTROTREATMENT PROTOCOL

For electrotreatment a new electroporator (Chemopulse IV developed in the Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria) was used, which generates bipolar pulses. The instrument is equipped with a large voltage control in the limits of 100-2200V, simplified operations, locking against illegal manipulations, enhanced protection against electrical hazards, a battery supply, providing autonomy for more than 200 electroporations with one battery charge, and a recharging time for a depleted battery of less than 10 hours. The electrotreatment was done by 16 biphasic pulses, each of which lasted for 50+50μs with 20 ms pause between both phases and a pause between bipolar pulses of 880μs [6]. In each experiment, electrodes with an inter-electrode distance of 1cm were used. The intensity of applied electric fields was 200V/cm and 500V/cm. One hundred μl with 1.5×10^5 cells were seeded 24h before electroporation. Immediately before pulse delivery miltefosine at different concentrations was added. For immunofluorescence staining experiments, cells were cultivated on cover glasses.

After the electrical treatment, 900 μl DMEM, supplemented with 10% FCS, was added to each sample. The controls were treated under the same conditions but without electric pulse application and/or miltefosine.

2.4. ACTIN STAINING

HaCaT at a cell density of 1.5×10^5cells/ml were cultivated on cover glasses (18/18 mm) that had been placed in 6 well plates. After 24-hour incubation, the cells were electroporated in a basal cell medium and were cultivated for an additional 24 hours in full cell medium. After the incubation period, non-adherent cells were removed by rinsing threefold with PBS, pH 7.4. The adherent cells were fixed with 1 ml 3% solution of PFA for 15 minutes at room temperature. The fixed cells were permeabilised using 1 ml 0.5% solution of Triton X-100 for 5 minutes and then incubated with 1 ml 1% solution of bovine serum albumin (BSA) for 15 minutes. The samples were washed three times with PBS, pH 7.4 and then incubated for 30 minutes at room temperature with BODIPY 558/568 phalloidin. Again, the samples were washed three times with PBS and once with distilled water, and then were installed on objective glasses by Mowiol. Preparations were

analysed using an inverted fluorescent microscope (Leica DMI3000 B, Leica Microsystems GmbH, Germany) with object HCX PL FLUOTAR 63×/1.25 oil.

2.5. ZONULA OCCLUDENS (ZO-1) STAINING

HaCaT cells were plated onto sterile glass coverslips and processed for indirect immunofluorescence as follows: coverslips were rinsed with PBS, fixed, and permeabilized with ice-cold –20°C methanol for 3 min. Coverslips were rinsed in PBS. After rinsing in PBS, coverslips were incubated with an anti-ZO-1 rat polyclonal antibody (Novex®) 1:1000 dilution for 1h. After three times rinsing with PBS, the cells were incubated with a second antibody, *i.e.* fluorescein-conjugated rabbit anti-rat IgG – Alexa 594, 1:250 (Boehringer Mannheim Biochemicals) for 1h. Coverslips were washed in PBS, mounted in a solution containing PBS, 50% glycerol, 0.4% n-propyl gallate (Sigma Chemical Co.), and viewed with a 63× objective on a Zeiss microscope.

3. RESULTS

3.1. MTS ASSAY

The cell viability was determined by MTS assay after treatment with high voltage electric pulses alone or in combination with miltefosine. The aim of the experiment was to clarify whether miltefosine would additionally decrease the cell viability.

The cell viability of HaCaT keratinocytes is given in Fig. 1. There was a statistically significant reduction of cell viability at 24 hour after pulse application. More than 20% reduction of cell viability was achieved after electrotreatment with pulses of 500V/cm. The application of miltefosine alone at 10–50 μM concentrations decreased cell viability by 50–70%. When miltefosine was given immediately before electroporation, this co-treatment led to an additional decrease of the cell viability in all electroporated samples. The highest cytotoxic effect was observed at concentrations of 50 μM miltefosine and electric pulses of 500 V/cm. This suggests an additive effect of combined treatment with electroporation and miltefosine on the cell viability.

Our results show that the cell viability decreased additionally from co-treatment with miltefosine and electroporation when compared to control cells treated only with miltefosine. In our further experiments we confirmed that the

combination of electroporation and miltefosine leads to structural changes that reduce the cell survival of HaCaT keratinocytes.

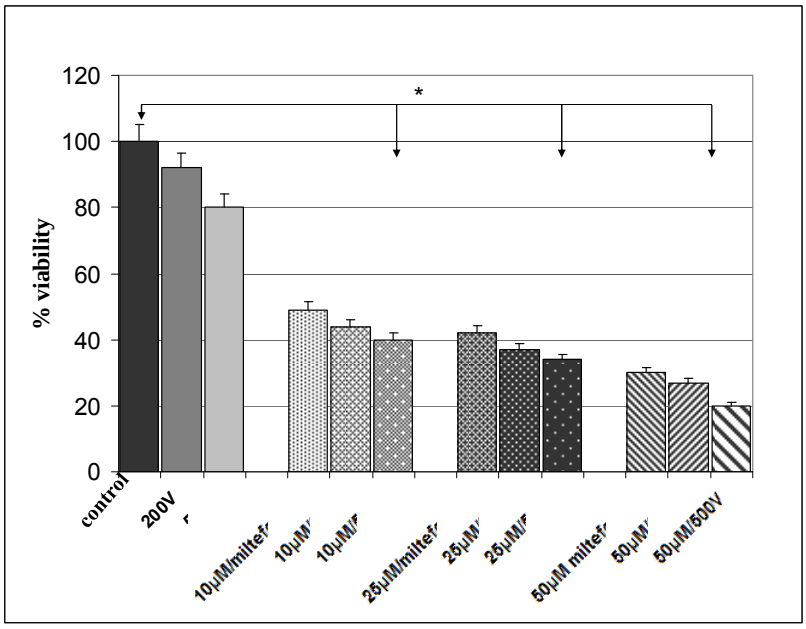


Fig. 1 – Cell viability of HaCaT keratinocytes. The cells were treated with high voltage electric pulses or miltefosine alone or by the combination of miltefosine and electrical pulses.
Bars – SD (Standard Deviation); *p < 0.0 *versus* untreated control.

3.2. IMMUNOFLUORESCENCE OF ZO-1 AT COMBINED TREATMENT WITH MILTEFOSINE AND ELECTRIC PULSES

Tight junctions (TJ) are essential for maintenance of the cellular interactions. ZO-1 is a periphery-associated transmembrane protein. In our studies we examined the expression of ZO-1. The absence of ZO-1 proteins disrupts TJ formation. The lack of TJ is associated with disruption of the cell monolayer and reduces the number and viability of the cells.

ZO-1 was visualized after 24 hours of treatment. Control cells without treatment with pulses retained cell-cell adhesion and exhibited discontinuous punctate localization of ZO-1 along the cytoplasmic surface (Fig. 2a). When HaCaT cells were cultured in a medium containing a high level of calcium or in the presence of SP600125 (one of the JNK inhibitors), the cells displayed a string-like localization pattern of ZO-1. With increasing intensity of the applied pulses we observed reduced expression of ZO-1 at 200 and 500 V/cm (Fig. 2b). ZO-1 exhibited punctate localization.

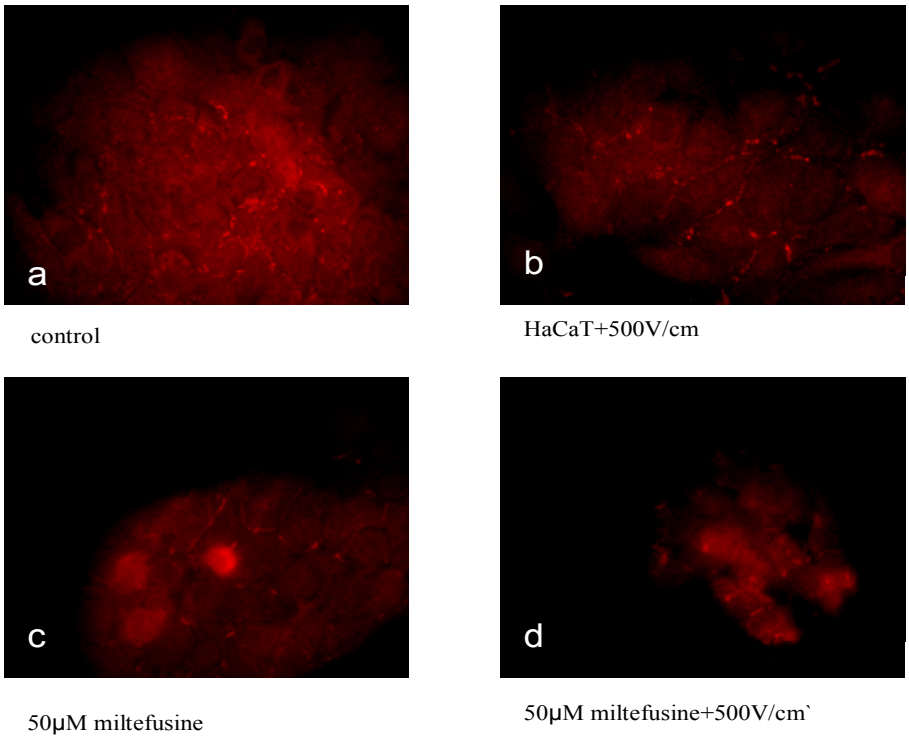


Fig. 2 – Immunofluorescence of ZO-1 after single and combined treatment with miltefosine and electrical pulses (Magnification 63.0×).

Similarly, decreased ZO-1 expression (Fig. 2c) was observed after addition of miltefosine. After treatment with 50 μM miltefosine and pulses with different intensity, a lower expression of ZO-1 was observed than in control cells (Fig. 2a). Treatment with 50 μM miltefosine and 200 and 500V/cm pulses destroyed the cell monolayer more strongly, respectively, and cell contacts could not be visualized (Fig. 2d). Obviously, a strong electric field (500 V/cm) and a high concentration of miltefosine (50 μM) showed an additive effect resulting from the two treatment modes.

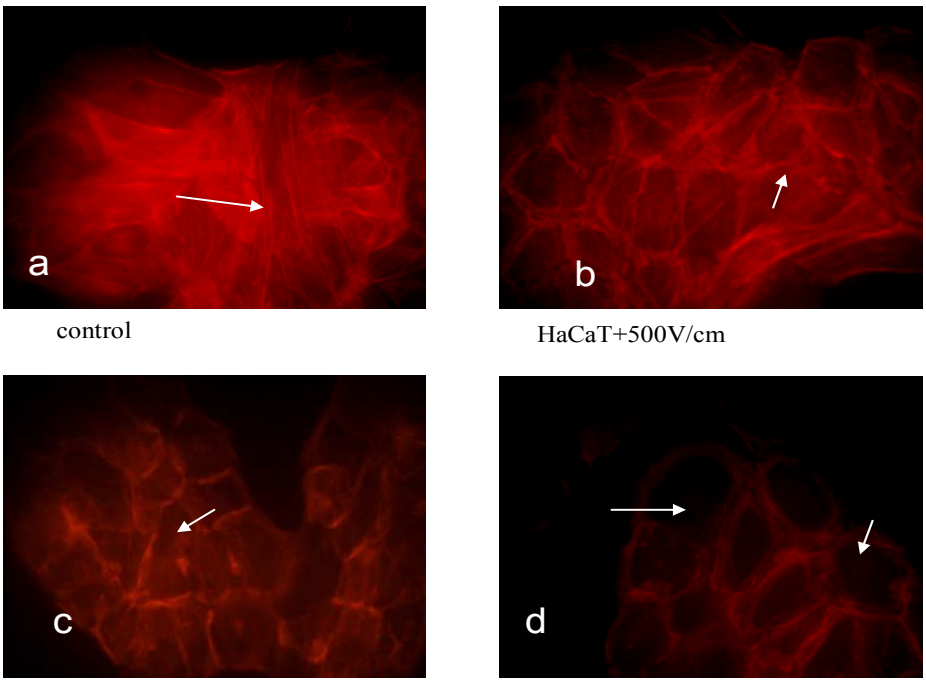
3.3. IMMUNOFLUORESCENCE OF ACTIN FOLLOWING COMBINED TREATMENT WITH MILTEFOSINE AND ELECTRICAL PULSES

We also examined the changes of the actin cytoskeleton after treatment with miltefosine and electric pulses. Untreated (control) cells displayed intact actin filaments with a lot of stress fibres and typical shape (pointed by white arrow Fig. 3a).

At 500V/cm pulses, the actin cytoskeleton organisation was changed. It was disposed near the cell membrane and actin stress fibres could not be visualised (pointed by white arrow Fig. 3b).

Dissociation of actin filaments correlated with increasing concentrations of miltefosine. At a concentration of 25 μM miltefosine, the stress fibres disappeared and a diffuse organization of actin filaments was observed (data not shown), 50 μM miltefosine destroyed the actin cytoskeleton and modified the shape of HaCaT cells (pointed by white arrow Fig. 3c). There is evidence that miltefosine changes the PLC-β activity and modulates the tight junction permeability by causing changes in the organisation of the actin filament network [13].

The increase in the concentration of miltefosine led to a disruption of the actin cytoskeleton, 50 μM miltefosine and 200V/cm pulses led to the disorganisation of actin filaments at 24 hour after treatment. In response to a combined treatment of 50 μM miltefosine and 500 V/cm pulses, an organisation of actin filaments could no longer be detected (pointed by white arrow Fig. 3d). This means that the 50 μM concentration of miltefosine impaired the cell monolayer irreversibly. Even 24 hours after electroporation, cells could not be restored. Miltefosine at concentrations of 25 μM or higher plus electrical pulses of 200 and 500 V/cm can lead to the destruction of cell mass.



control HaCaT+500V/cm

50 μM Miltefosine 50 μM Miltefosine+500V/cm

Fig. 3 – Immunofluorescence of actin after single and combined treatment with miltefosine and electrical pulses. (Magnification 63.0×).

4. DISCUSSION

In our study we analyzed how electroporation alone or in combination with miltefosine affects the viability and adhesion properties of HaCaT keratinocytes *in vitro*. Applying a combination of miltefosine at concentrations of 10μM, 25μM, and 50μM plus electrical impulses of 200 and 500V/cm, a significant reduction in cell viability was achieved. To follow the morphological changes caused by the aforementioned conditions, we analyzed the re-organization of actin filaments and the loss of ZO-1 protein from intercellular contacts. By the electrical pulses applied, we probably enhanced miltefosine delivery into the cells, and thus this ALP affected not only the external cell membrane but also any intracellular membrane structures (mitochondria, endoplasmic reticulum etc.). It is known that ALPs act on cell membrane level by a lysis-like effect [14].

It is also known that electroporation leads to changes in the cytoskeleton, which decreases the epithelial barrier function, and in turn is the cause of increased permeability of the epithelial monolayer [15]. A good correlation between cell viability measured by MTS test and the results from immuno-staining was achieved.

The results of this work indicate new opportunities for effective treatment of skin lesions, by topical treatment with miltefosine and electroporation which increases the penetration of the substance and its effect on skin lesions, without the need to take it for a long time and in large doses as is known in the literature [16]. The latter mode of administration can lead to a number of side effects and adverse interactions with other drugs.

In conclusion, we claim that the electroporation of cells of the keratinocyte line HaCaT in combination with miltefosine resulted in reduced proliferation of the treated cells, disruption of their cytoskeleton and increased permeability of the respective monolayer. The increase in permeability is due to the degradation of intercellular contacts with subsequent reduction of cell viability.

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