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Calcium electroporation in three cell lines; a comparison of bleomycin and calcium, calcium compounds, and pulsing conditions

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ABSTRACT

Background: Electroporation with calcium (calcium electroporation) can induce ATP depletion-associated cellular death. In the clinical setting, the cytotoxic drug bleomycin is currently used with electroporation (electrochemotherapy) for palliative treatment of tumors. Calcium electroporation offers several advantages over standard treatment options: calcium is inexpensive and may readily be applied without special precautions, as is the case with cytostatic drugs. Therefore, details on the use of calcium electroporation are essential for carrying out clinical trials comparing calcium electroporation and electrochemotherapy.
Methods: The effects of calcium electroporation and bleomycin electroporation (alone or in combination) were compared in three different cancer cell lines (DC-3F, transformed Chinese hamster lung fibroblast; K-562, human leukemia; and murine Lewis Lung Carcinoma). Furthermore, the effects of electrical pulsing parameters and calcium compound on treatment efficacy were determined.
Results: Electroporation with either calcium or bleomycin significantly reduced cell survival ($p < 0.0001$), without evidence of a synergistic effect. Cellular death following calcium or bleomycin treatment occurred at similar applied voltages, suggesting that similar parameters should be applied. At equimolar concentrations, calcium chloride and calcium glubionate resulted in comparable decreases in cell viability.
Conclusions: Calcium electroporation and bleomycin electroporation significantly reduce cell survival at similar applied voltage parameters. The effect of calcium electroporation is independent of calcium compound.
General significance: This study strongly supports the use of calcium electroporation as a potential cancer therapy and the results may aid in future clinical trials.

1. Introduction

Electroporation, where short, high voltage electric pulses induce permeabilisation of the cell membrane, is used in combination with chemotherapeutic drugs (electrochemotherapy) for local treatment of malignant tumors both in the clinical management of cancer [1–11] and in veterinarian medicine [12–14]. Over the years several novel agents have been applied in combination with electroporation [15–19]. Recently, it was shown that calcium, in combination with electroporation (calcium electroporation), could eradicate tumors by inducing cellular death and tumor necrosis [20]. This finding suggests that calcium is a possible new agent for electroporation-based cancer therapy.

Numerous existing anti-cancer therapies affect calcium signaling, supporting the idea of calcium pathways as a target in cancer treatment [21,22]. Calcium is a ubiquitous second messenger and depending on time, place, amplitude, frequency, and duration, it is involved in several cellular processes, including cell death. Accordingly, calcium is tightly regulated within cells [23–27]. In eukaryotic cells, the cytosolic concentration of free calcium is approximately 10^{-7} M, a concentration that is very low compared to the extracellular concentration of approximately 10^{-3} M. This suggests that even a small influx of calcium will lead to a dramatic increase in intracellular calcium concentration [28]. By electroporating cells or tumors in the presence of calcium, the intracellular calcium concentration increases greatly, resulting in energy depletion [20], likely caused by direct ATP loss, increased ATP expenditure due to activity of the Ca^{2+}-ATPase and other ATPases, as well as loss of ATP production.

Calcium electroporation has shown great efficacy when using reversible electroporation parameters (microsecond pulses) [20]. Alternative electroporation methods, such as irreversible electroporation or nanosecond pulse electroporation, have yet to be tested with calcium. Irreversible electroporation has been tested as an anti-cancer treatment

Abbreviations: ANOVA, Analysis of Variance; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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[29] and its efficacy might be increased when combined with calcium injection. Nanosecond pulse electroporation has been shown to cause a transient increase in the intracellular calcium concentration by influx of calcium from the extracellular space, as well as calcium release from the endoplasmic reticulum [30,31]. Therefore, nanosecond pulse electroporation, which also has been tested as an anti-cancer treatment [32,33], might be potentiated by addition of calcium injection.

In the field of gene therapy, calcium electroporation may have yet another interesting perspective. When using electroporation-based gene electrotransfer, a particular tissue area (e.g. muscle) is used as host tissue for a transgene, which may encode a therapeutic protein. This protein can be excreted to the bloodstream to exert a systemic effect [34–39]. Several clinical trials have tested gene therapy in both humans [38] and animals [40–42]. As a method to terminate transgene expression, due to unexpected side effects or other reasons, calcium electroporation may be applied to the relevant tissues [43].

Before calcium electroporation can be implemented in the clinical setting, several things must be investigated: 1) the efficacy of calcium electroporation must be directly compared to bleomycin-based electrochemotherapy. Furthermore, a possible synergy between the effects of calcium and bleomycin must be explored. 2) Electroporation depends on electrical field induced membrane permeabilisation. As calcium ions are significantly smaller than bleomycin molecules, it would be of interest to test whether different electroporation parameters should be applied. Calcium electroporation has previously been tested using identical electric field strengths as those applied in electrochemotherapy [20,43]. 3) Calcium intended for injection is commercially available and routinely used at most hospitals. Two different calcium compounds, namely calcium chloride and calcium glubionate, are approved for clinical use. We therefore aim to compare both compounds in calcium electroporation.

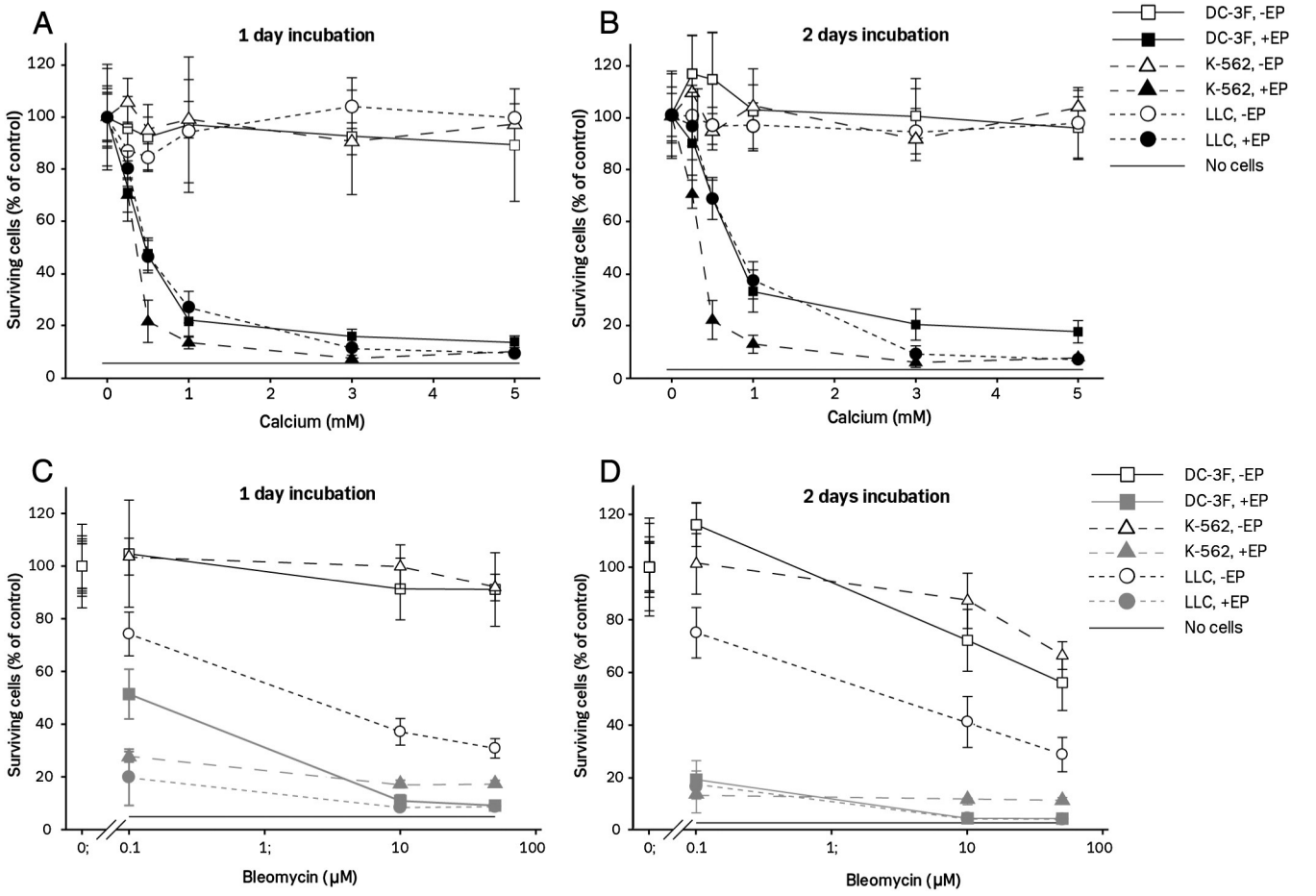
2. Material and methods

2.1. Cell culturing

Three cell lines were used for the experiments, DC-3F, a transformed Chinese hamster lung fibroblast cell line; K-562, a human leukemia cell line; and Lewis Lung Carcinoma, a murine lung carcinoma cell line. The cell lines were mycoplasma-negative, tested using MycoAlert Mycoplasma Detection Kit (Lonza) prior to experiments. Cells were maintained in RPMI 1640 culture medium (Gibco, Invitrogen) with 10% fetal calf serum (Gibco, Invitrogen), penicillin, and streptomycin at 37 °C and 5% CO_2.

2.2. Electroporation protocol

Following harvesting, cells were diluted in HEPES buffer (10 mM HEPES (Lonza), 250 mM sucrose and 1 mM $MgCl_2$ in sterile water). This buffer not containing phosphate was used since calcium and



Q2 **Fig. 1.** Calcium electroporation and bleomycin electroporation. Cell viability measured by MTT assay 1 and 2 days after treatment of three different cell lines (DC-3F, a transformed Chinese hamster lung fibroblast cell line; K-562, a human leukemia cell line; and Lewis Lung Carcinoma (LLC), a murine lung carcinoma cell line) with increasing calcium concentrations either electroporated or not (A–B) and with increasing bleomycin concentrations either electroporated or not (C–D). Note that a log scale is employed on the bleomycin data. Results are illustrated as percentage of control (electroporated or non-electroporated cells without added drug), mean ± S.D., $n \geq 6$. Viability decreases significantly ($p < 0.01$) starting from 0.5 mM calcium for all cell lines treated with calcium electroporation (A–B) and viability decreases significantly ($p < 0.0001$) starting from 0.1 μM bleomycin for all cell lines treated with bleomycin electroporation (C–D).
Panels A–B are reproduced from [17].

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