



29 **Abstract**

30 Electrochemotherapy (ECT) enhances responsiveness to cytotoxic drugs in numerous cell lines *in*  
31 *vitro*. Clinically ECT is widely applied for skin tumor ablation and has shown efficacy in treating non-  
32 resectable colorectal liver metastases. There is limited experience of ECT for ocular tumor therapy.  
33 We investigated the cytotoxic effect of bleomycin and cisplatin in combination with electroporation  
34 on chemoresistant human uveal melanoma (UM) cell lines *in vitro*. Four UM cell lines (Mel 270, 92-1,  
35 OMM-1, OMM-2.5) were treated with electroporation (pulse amplitude 300-1000 V/cm, 8-80 pulses,  
36 100 $\mu$ s, 5 Hz) and increasing concentrations of bleomycin and cisplatin (0-7.5 $\mu$ g/ml). Cell survival was  
37 analyzed by MTT viability assay after 36 hours. UM cell lines were resistant to both bleomycin and  
38 cisplatin. In combination with electroporation, the effects of bleomycin and cisplatin were  
39 increased 8–70 fold and 3–15 fold, respectively, in all UM cell lines. At the lowest concentration of  
40 bleomycin tested (1 $\mu$ g/ml), viability was maximally reduced in all UM cell lines by  $\geq$ 69% with  
41 electroporation conditions of 750 Volts/cm and 20 pulses. All UM cell lines were more resistant to  
42 cisplatin; however, electroporation of 1000 Volts/cm and 8 pulses resulted in similar reductions in cell  
43 viability of 92-1, Mel270 with 2.5 $\mu$ g/ml cisplatin, OMM2-5 cells with 5 $\mu$ g/ml cisplatin and OMM1 cells  
44 with 1 $\mu$ g/ml cisplatin. *In vitro* ECT with bleomycin or cisplatin is more effective than the highest  
45 concentration of the antineoplastic drug or electroporation alone, opening new perspectives in  
46 primary and metastatic UM treatment.

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**53 Introduction**

54 Disseminated uveal melanoma (UM) is clinically resistant to many chemotherapy drugs, and indeed  
55 the current standard of care, dacarbazine, is effective in <8% of individuals with metastatic UM [1,2].  
56 The mechanisms for the relative innate chemoresistance of UM cells are unclear. Those  
57 chemoresistance mechanisms previously described in cancer include: decreased drug accumulation;  
58 enhanced anti-apoptotic mechanisms; and increased/altered DNA repair pathways. Electroporation,  
59 which is based on the local application of short and intense electric pulses that transiently  
60 permeabilise cells, has been used to enhance drug entry into otherwise chemoresistant cancer cells  
61 and resulted in their death [3-10]. This process of electrochemotherapy (ECT) is also used currently in  
62 clinical practice to treat cutaneous and subcutaneous tumor nodules in patients with progressive  
63 disease of different malignancies, e.g. soft tissue sarcomas and carcinomas, cutaneous melanoma  
64 [11,12], as well as colorectal liver metastases, located in the vicinity of major hepatic vessels, not  
65 amenable to surgery or radiofrequency ablation [13]. The treatment can result in complete  
66 responses of the tumors with very limited side effects [11] with drug doses that by themselves have  
67 minimal or no antitumor activity.

68 Amongst the several clinically-approved drugs that have been tested in pre-clinical studies of ECT,  
69 bleomycin and cisplatin have been shown to be highly effective [4,8]; exposure of cells to electric  
70 pulses increases the cytotoxicity of bleomycin and cisplatin given either intravenously or intra-  
71 tumorally [14-16]. Previous studies examining the efficacy of cisplatin in UM cells isolated from  
72 primary tumors demonstrated no effect of the drug to reduce cell number in nine cultures tested  
73 [17].

74 In order to determine whether chemoresistance in UM is due to an inability to accumulate drug  
75 inside the cancer cell, this study evaluated the cytotoxic effect of cisplatin or bleomycin after  
76 electroporation of four UM cell lines; Mel 270, 92-1, OMM-1 and OMM-2.5. **The initial**  
77 **electroporation conditions were selected according to the ESOPE protocol [12]. The aim of the study**

78 was to examine the effect of ECT on cell viability after reduction of the voltage/pulses combined with  
79 different concentrations of the drug. These parameters would support the hypothesis that ECT could  
80 be applied on the eye with minor side effects.

81

82 **Materials and Methods**

83 *Cell lines and culture*

84 The human UM cell lines 92-1 and Mel270, derived from primary tumor and the OMM-1 as well as  
 85 OMM-2.5, derived from subcutaneous and liver metastasis respectively, were kindly provided by  
 86 Prof. Dr. Martine Jager, Leiden University Medical Centre (LUMC), The Netherlands. All cell lines have  
 87 been STR profiled and mycoplasma tested. They were grown in Roswell Park Memorial Institute  
 88 (RPMI) 1640 medium containing 10% fetal bovine serum, 1% L-Glutamine (all from Invitrogen, GIBCO,  
 89 USA) and 2% Penicillin Streptomycin (Thermo Fisher Scientific, USA). All cell lines were maintained as  
 90 monolayers in 175 cm<sup>2</sup> tissue culture flasks (Thermo Fisher Scientific, USA) at 37 °C in a humidified  
 91 atmosphere containing 5% CO<sub>2</sub>.

92 *In vitro Electrochemotherapy (ECT)*

93 When cells reached 70% confluence they were harvested with 0.05% trypsin, counted and 1x10<sup>6</sup> cells  
 94 were re-suspended in 400µl of RPMI, with or without bleomycin or cisplatin, in a 4mm gap  
 95 electroporation cuvette with parallel aluminum plate electrodes (Geneflow, UK). A range of  
 96 electroporation conditions were applied to the cell suspensions using the voltage pulse generator  
 97 (Cliniporator™) designed by Igea S.p.A. (Capri, Modena, Italy). Details of all experimental conditions  
 98 are given below.

99 All cells were treated with 0, 1 µg/ml, 2.5 µg/ml, 5 µg/ml and 7.5 µg/ml bleomycin or cisplatin  
 100 combined with all following electroporation settings:

- 101 (A) No electroporation;
- 102 (B) 80 square wave electric pulses of 300 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz  
 103 repetition frequency;
- 104 (C) 40 square wave electric pulses of 300 Volts/cm pulse strength, 100 µs pulse duration, 5 Hz  
 105 repetition frequency;

106 (D) 40 square wave electric pulses of 500 Volts/cm pulse strength, 100  $\mu$ s pulse duration, 5 Hz  
107 repetition frequency;

108 (E) 20 square wave electric pulses of 500 Volts/cm pulse strength, 100  $\mu$ s pulse duration, 5 Hz  
109 repetition frequency;

110 (F) 20 square wave electric pulses of 750 Volts/cm pulse strength, 100  $\mu$ s pulse duration, 5 Hz  
111 repetition frequency;

112 (G) 8 square wave electric pulses of 750 Volts/cm pulse strength, 100  $\mu$ s pulse duration, 5 Hz  
113 repetition frequency;

114 (H) 8 square wave electric pulses of 1000 Volts/cm pulse strength, 100  $\mu$ s pulse duration, 5 Hz  
115 repetition frequency.

116 Following treatment,  $2 \times 10^4$  cells were pipetted into 6 wells of a 96-well plate for each treatment  
117 condition and RPMI was added up to a maximum volume of 100  $\mu$ l. The plates were then incubated  
118 for 36 hours.

119 The protocol was conducted for all four UM cell lines. Each experiment was performed in triplicate on  
120 different dates, giving a total of 18 biological replicates for each ECT setting.

#### 121 *MTT viability assay*

122 RPMI-1640 medium was aspirated from each well after 36 hours and 3-(4,5-dimethylthiazol-2-yl)-2,5-  
123 diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) stock solution (5 mg/ml) was added to each  
124 well, equal to one-tenth the original culture volume following the protocol provided by Sigma-Aldrich  
125 (90  $\mu$ l media and 10  $\mu$ l MTT). All plates were then incubated at 37 °C for 4 hours. Following this, the  
126 solution was removed and the formazan formed in the cells was dissolved using 100  $\mu$ l of a 1:1  
127 solution of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and 2-propanol (isopropanol, Sigma-  
128 Aldrich, USA). Absorbance of converted dye was measured with a SPECTRAFLUOR (Tecan, Austria)  
129 spectrometer at a wavelength of 570 nm.

130

**131 Results**

132 Each of the four UM cell lines were exposed to eight different electrical fields. The duration of 100 $\mu$ s  
133 and the pulse frequency of 5Hz remained stable whereas the amplitude and the number of pulses  
134 varied. Electroporation alone, reduced cell viability in all cell lines at amplitudes of 500 Volts/cm or  
135 higher and this effect was augmented with increasing number of pulses. The greatest reduction in  
136 cell viability was noted at 1000 Volts/cm for 8 pulses across all four cell lines ranging from a 29.5%  
137 reduction in the most sensitive 92.1 cell line to a 25.0% reduction in the least sensitive OMM-2.5 cell  
138 line (Figure 1).

139 Bleomycin alone had no effect on cell viability in the OMM-1 and OMM-2.5 cell lines and reduced cell  
140 viability in the 92.1 and Mel270 cell lines by <10% at the maximum concentration tested (7.5 $\mu$ g/ml;  
141 (Figure 2A). When electroporation conditions  $\geq$ 750 Volts/cm were administered to the UM cells,  
142 however, bleomycin cytotoxicity was maximally increased by 8-fold in the 92.1 cell line, 25-fold in the  
143 Mel270 cell line and by more than 70-fold in the OMM-1 and OMM-2.5 cell lines (Figure 3). In order  
144 to minimize systemic toxicity of bleomycin, we were interested in the electroporation conditions that  
145 in combination with the lowest dose of bleomycin tested (1 $\mu$ g/ml) had the maximal effect to reduce  
146 cell viability. In the 92.1 and Mel270 cell lines this was achieved at 750 Volts/cm for 20 pulses,  
147 reducing cell viability by 74% and 69%, respectively (Figure 3A and 3B). In the OMM-1 and OMM-2.5  
148 cell lines there was little difference between the effectiveness of 1 $\mu$ g/ml bleomycin when combined  
149 with electroporation conditions of either 750 Volts/cm for 20 pulses or 1000 Volts/cm for 8 pulses,  
150 with a reduction in cell viability of between 76% and 89% (Figure 3C and 3D).

151 Similar to bleomycin, cisplatin alone had little effect on cell viability at the concentrations tested  
152 (Figure 2B), with a maximum 15% reduction in viability of the 92.1 cell line at 7.5 $\mu$ g/ml cisplatin.  
153 When electroporation conditions  $\geq$ 500 Volts/cm were administered to the UM cells, however,  
154 cisplatin cytotoxicity was maximally increased by 3, 6, 10 and 15-fold in the 92.1, Mel270, OMM-1  
155 and OMM-2.5 UM cell lines respectively (Figure 4). In combination with electroporation the most

156 sensitive UM cell line was OMM-1, which showed an 80% reduction in cell viability with 1000  
157 Volts/cm for 8 pulses and 1 $\mu$ g/ml cisplatin (Figure 4C). In the 92.1, Mel270 and OMM-2.5 cell lines,  
158 higher concentrations of cisplatin in combination with electroporation conditions of 1000 Volts/cm  
159 for 8 pulses were necessary to achieve similar reductions in viability as noted for the OMM-1 cells.  
160 For example, 1000 Volts/cm for 8 pulses with 2.5 $\mu$ g/ml cisplatin was necessary to reduce viability of  
161 the 92.1 and Mel270 cell lines by 77% and 70% respectively (Figure 4A and 4B); whilst 1000 Volts/cm  
162 for 8 pulses with 5.0 $\mu$ g/ml cisplatin was necessary to reduce viability of the OMM-2.5 cell line by 75%  
163 (Figure 4D).

164

165 **Discussion**

166 In this novel study we investigated the efficiency of electroporation with bleomycin and cisplatin in  
167 four human UM cell lines that demonstrate resistance to these chemotherapeutic drugs at their  
168 commonly achieved peak plasma concentrations of 0.5 – 5.0 µg/ml and 0.5 – 2.0 µg/ml, respectively.  
169 We show for the first time that electroporation sensitizes UM cells to doses of either drug within  
170 these ranges.

171 Bleomycin is an anti-tumor antibiotic that causes single and double strand DNA breaks in tumor cells  
172 resulting in cell death. It is used to treat a range of malignancies, including head and neck cancer,  
173 testicular carcinomas and lymphomas [18-22]. In UM it has been used in the metastatic setting as  
174 part of a multicentre study of bleomycin, vincristine, lomustine and dacarbazine (BOLD) in  
175 combination with recombinant interferon alpha-2b, although only a modest effect of this regimen  
176 against UM at hepatic sites was reported [23]. Cisplatin is another commonly used anti-cancer agent  
177 that causes DNA crosslinks resulting in DNA damage, and subsequently inducing apoptosis in cancer  
178 cells. It is commonly used in the treatment of lung-, ovarian-, and head-and-neck carcinomas, but has  
179 been shown to have little effect in combination chemotherapy for metastatic UM [24].

180 Bleomycin is a large non-permeant drug, a characteristic that contributes to the resistance of many  
181 cell types to this agent [25]. Studies on the Chinese hamster lung cell line (DC-3F) have shown that if  
182 bleomycin can enter the cell, <500 molecules of the drug are needed to cause cell death [25,26].  
183 Although resistance to cisplatin is considered to be multifactorial, evidence suggests that plasma  
184 membrane transporters resulting in the extrusion of cisplatin play a major role in the resistance  
185 mechanism(s) [27].

186 In this study we have shown that by applying an electrical field to UM cells above a threshold  
187 amplitude of 500 Volts/cm, sensitivity to bleomycin and cisplatin are greatly increased, and that this  
188 is further enhanced by an increased number of pulses as has previously been reported [28,29].  
189 Electroporation creates transient permeable pores in the cell membrane thus enhancing drug entry

190 and accumulation in the cell [30,31], and indeed ECT has been shown to be effective in a variety of  
191 other tumor cell types *in vitro*[3-10]. Furthermore, ECT for skin metastases from tumors of non-  
192 cutaneous origin as well as for skin melanoma is currently part of the NICE interventional procedure  
193 guidance for these lesions [32].

194 Small differences in the sensitivity of the cell lines to ECT with both bleomycin and cisplatin were also  
195 noted. In particular, the OMM-1 cell line was more sensitive to ECT with cisplatin than the 92.1,  
196 Mel270 and OMM-2.5 cell lines. OMM-1 cells are derived from a subcutaneous metastatic UM; whilst  
197 92.1 and Mel270 cells, are derived from primary tumors, and OMM-2.5 is from a hepatic UM  
198 metastasis. Previous studies have reported that cell size, shape, membrane structure, composition  
199 and transmembrane potential can affect electroporation [33,34]. In the current study no differences  
200 were observed in the response of the four UM cell lines to electroporation despite striking  
201 differences in the size and shape of these cells. We did not examine, however, other membrane  
202 features, but this will be pursued in primary and metastatic UM cell cultures in the near future.  
203 Various preclinical models are available for the study of primary and metastatic UM, and would lend  
204 themselves to the examination of new and older chemotherapeutic agents in combination with ECT  
205 [35].

206 In summary, electroporation provides a more targeted pathway into UM cells for bleomycin and  
207 cisplatin. The application of this treatment could lead to the shrinkage of large, non-treatable UM in  
208 order to enable a further surgical intervention and avoid enucleation as primary treatment.  
209 Furthermore the application of ECT could allow a lower drug doses and a reduction of systemic side  
210 effects in the treatment of large non-resectable UM hepatic metastases, as has been demonstrated  
211 in colorectal liver metastases located close to major hepatic vessels, not amenable to other  
212 treatments [13]. The combination of various chemotherapy agents and ECT thus requires further  
213 investigation *in vitro* and *in vivo* to investigate the challenges of a clinical application of the protocol  
214 in disseminated UM.



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301 **Figure legends**

302 **Figure 1** – Effects of electroporation on cell viability 36 hours following exposure. Data are the mean  
303  $\pm$  SEM of 6 individual experiments for the 92.1 (black bars), Mel270 (Dark grey bars), OMM-1 (white  
304 bars) and the OMM-2.5 (hashed bars) cell lines.

305 **Figure 2** – Effects of (A) bleomycin and (B) cisplatin on viability of the 92.1 (solid black line), Mel270  
306 (dotted black line), OMM-1 (solid grey line) and OMM-2.5 (dashed black line) UM cell lines 36 hours  
307 after exposure to the drugs. Data are the mean  $\pm$  SEM of 3 separate experiments.

308 **Figure 3** – Cytotoxic effects of increasing doses of bleomycin on the viability of (A) 92.1, (B) Mel270,  
309 (C) OMM-1 and (D) OMM-2.5 UM cell lines following electroporation. Data are the mean of 18  
310 replicates across three separate experiments for the effect of electroporation alone (black bars),  
311 1 $\mu$ g/ml (dotted bars), 2.5 $\mu$ g/ml (grey bars), 5 $\mu$ g/ml (striped bars) and 7.5 $\mu$ g/ml (white bars)  
312 bleomycin to reduce cell viability.

313 **Figure 4** – Cytotoxic effects of increasing doses of cisplatin on the viability of (A) 92.1, (B) Mel270, (C)  
314 OMM-1 and (D) OMM-2.5 UM cell lines following electroporation. Data are the mean of 18 replicates  
315 across three separate experiments for the effect of electroporation alone (black bars), 1 $\mu$ g/ml  
316 (dotted bars), 2.5 $\mu$ g/ml (grey bars), 5 $\mu$ g/ml (striped bars) and 7.5 $\mu$ g/ml (white bars) cisplatin to  
317 reduce cell viability.