

Electrochemotherapy with Cisplatin in the Treatment of Tumor Cells Resistant to Cisplatin

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Abstract. The aim of our study was to investigate the role of electroporation in the treatment of cisplatin resistant tumor cells *in vitro*. For this purpose we used well characterized human ovarian carcinoma IGROV 1 cells and their resistant subclone IGROV 1/DDP. The cells were either continuously exposed to cisplatin or treated with electrochemotherapy (exposure time: 5 minutes). After chronic exposure of cells to cisplatin, IGROV 1/DDP cells exhibited 8-fold resistance to cisplatin. Cisplatin cytotoxicity was greatly potentiated by treatment with electric pulses in both cell lines. However, the IGROV 1/DDP cells still exhibited a 50-fold resistance. Our results demonstrate that electroporation treatment potentiates cytotoxicity in both human ovarian carcinoma IGROV 1 cells as well as in their resistant subclone IGROV 1/DDP.

Cisplatin has demonstrated activity against several tumors and is currently used in clinical chemotherapy for the treatment of testicular, ovarian, head and neck and small cell lung cancer (1). The cytotoxicity of cisplatin is thought to be mediated by the binding of platinum to DNA and inducing the formation of various types of inter and intra-strand cross links. Natural resistance of tumor cells to cisplatin, as well as development of acquired resistance, is a major problem in clinical chemotherapy. Several cisplatin resistant cell lines and tumors were developed in order to study which mechanisms were responsible for the observed resistance of tumors to cisplatin (2). So far, several mechanisms have been reported to be responsible for the resistance of tumor cells to cisplatin, including decreased intracellular accumulation, increased levels of intracellular glutathione and activity of glutathione-S-transferase, increased levels of intracellular metallothioneins and enhanced DNA repair (3).

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Key Words: Ovarian carcinoma, cisplatin resistance, electroporation, electrochemotherapy.

Discovery of the resistance mechanisms initiated several studies where different approaches were tested in order to overcome the resistance. These approaches have included modulation of plasma membrane permeability, thiol content modulators, chromatin conformation modulators and DNA repair inhibitors (3-6).

Electroporation of cells *in vitro* and tumors *in vivo* has already proved that it increases cisplatin cytotoxicity. In preclinical studies on various tumor models, it was demonstrated that, in comparison to cisplatin-based treatment alone, the combined use of electroporation and cisplatin (electrochemotherapy) resulted in increased antitumor effectiveness inducing also tumor cures (7-11). Electrochemotherapy with cisplatin was also entered into clinical trial and was applied in the treatment of subcutaneous tumor nodules of malignant melanoma, basal cell carcinoma and squamous cell carcinoma (12). After electrochemotherapy, 100% of tumor nodules regressed completely.

The aim of our study was to investigate the role of electroporation in the treatment of resistant tumor cells *in vitro* with cisplatin. For this purpose, we used well characterized human ovarian carcinoma IGROV 1 cells and their cisplatin resistant subclone IGROV 1/DDP.

Materials and Methods

Chemicals. Cisplatin (Platinol, Bristol Myers Squibb, Austria) was dissolved in sterile water at a concentration of 4 mg/ml. Further dilutions were prepared in RPMI 1640 medium (Sigma, USA). Propidium iodide (Sigma, USA) was dissolved in sterile water at a concentration of 100 μ M.

Cells. In our study, human ovarian adenocarcinoma IGROV 1 cells and their resistant subclone IGROV 1/DDP were used. The cells were grown as monolayer in humidified incubator at 37°C and 5% CO₂ in RPMI 1640 medium (Sigma, USA), supplemented with 15% fetal calf serum (FCS, Sigma, USA). The IGROV 1/DDP cells were continuously exposed to 1 μ g/ml cisplatin.

Study design. To test whether electrochemotherapy could be beneficially employed in the treatment of cells resistant to cisplatin, we first determined the electric pulses amplitude at which cells were permeabilized (electropermeabilization of plasma membrane), but remained viable (electrosensitivity of cells). Second, we tested the survival of cells continuously exposed to cisplatin, and third, we

performed electrochemotherapy on parental IGROV 1 and resistant IGROV 1/DDP cells. The results were reported by means of IC_{50} value: the electric field intensity or cisplatin concentration that causes a 50% inhibition of colony formation. All experiments were performed in triplicates and repeated 3-times.

Determination of electroporation and electrosensitivity. Electroporation and electrosensitivity of IGROV 1 and IGROV 1/DDP cells was determined as described previously (13). Briefly, electroporation of plasma membrane was measured by means of propidium iodide uptake and electrosensitivity (survival of cells treated with electric pulses) by colony forming assay. The cells were prepared from exponential growth phase, trypsinized and washed twice at 4°C, first in the medium supplemented with 10% FCS for inactivation of trypsin (Sigma, USA), and then in the serum-free medium supplemented with 0.5 mM $CaCl_2$. Cell suspension (2.2×10^7 cells/ml in 90 μ l) was mixed with 10 μ l propidium iodide (100 μ M) for measurement of propidium iodide uptake, or with medium supplemented with 0.5 mM $CaCl_2$ for colony forming assay. Each of these mixtures (50 μ l) was placed between two flat parallel stainless-steel electrodes (length 6 mm, width 6 mm, distance 2 mm) and subjected to 8 square wave electric pulses (pulse width 100 μ s, repetition frequency 1 Hz) of different electric field intensities, ranging from 100 to 1800 V/cm. After exposure of cells to electric pulses, the cells were incubated for 5 minutes at room temperature (24°C). To measure the propidium iodide uptake, 25 μ l of cell suspension was resuspended in 1 ml of 0.01 M phosphate buffered saline (pH 7.4) and analyzed immediately by FACSsort (Becton Dickinson, Mountain View, CA, USA). The percentage of stained cells was determined in comparison to the control cells that were not subjected to electric pulses.

Electrosensitivity of cells was determined by means of colony forming assay. The cells exposed to electric pulses were diluted and seeded in quadruplicate in 60 mm Petri dishes (Costar, Badhoevedorp, The Netherlands). After 14 days, the colonies were fixed, stained with Crystal violet (Kemika, Croatia) and counted. The colonies containing less than 50 cells were disregarded. The survival of cells treated with electric pulses was presented as a percentage of the colonies obtained from the control untreated cells.

Cytotoxicity assay for continuous exposure of cells to cisplatin and electrochemotherapy. To determine the sensitivity of IGROV 1 and IGROV 1/DDP cells to continuous exposure to cisplatin, the cells were plated in Petri dishes in 4 ml of medium containing different cisplatin concentrations ranging from 0.01 to 10 μ g/ml. The sensitivity of the cells to combined treatment with cisplatin and electric pulses (electrochemotherapy) was determined as described above for electrosensitivity, except that the cells were mixed with cisplatin instead of the medium supplemented with $CaCl_2$. One half of this mixture was exposed to electric pulses (800 V/cm) and the other half served as a control for cisplatin treatment alone. The survival of cells treated with electrochemotherapy was normalized to electric pulses treatment alone.

Results

Electroporation and electrosensitivity. Both, IGROV 1 and IGROV 1/DDP cells were permeabilized at 800 V/cm, but had different electrosensitivity, with IGROV 1 cells being more electrosensitive than the resistant ones (Figure 1, 2). The IC_{50} value of the IGROV 1 and of the IGROV 1/DDP cells were 740 V/cm and 1640 V/cm, respectively. In electrochemotherapy experiments, we used the electric field intensity of 800 V/cm in both cells lines. At this field intensity, both, the parental and resistant cells were permeabilized to the same extent, but had different survival. Therefore, in

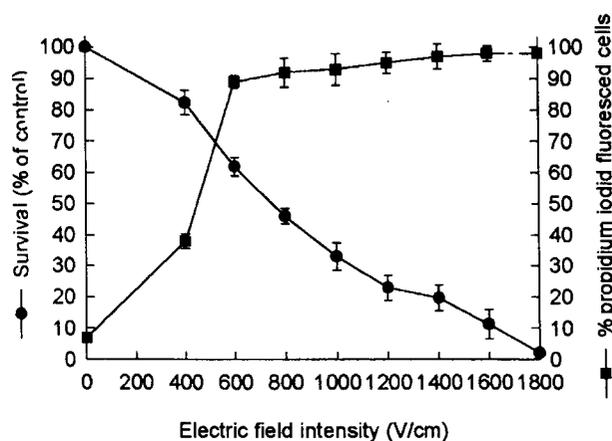


Figure 1. Electroporation and electrosensitivity of IGROV 1 cells. Electrosensitivity was measured by means of propidium iodide uptake and electrosensitivity by means of clonogenic assay. Data are mean \pm standard error of the mean.

order to compare the cell survival after electrochemotherapy, the survival of electrochemotherapy treated cells was normalized to the electric pulses treatment alone.

Survival of cells continuously exposed to cisplatin. The IC_{50} values of continuously treated IGROV 1 and IGROV 1/DDP cells were 0.1 μ g/ml and 0.8 μ g/ml, respectively. Thus, IGROV 1/DDP cells exhibited 8-fold resistance compared to IGROV 1 cells to cisplatin (Figure 3).

Electrochemotherapy. Cisplatin cytotoxicity was greatly potentiated by exposing the cells, both, parental and resistant cells, to electric pulses (Figure 4). The survival curves of electrochemotherapy treated cells were shifted to the left compared to the cells treated with cisplatin only. The IC_{50} values of IGROV 1 cells treated by electrochemotherapy and resistant ones were 0.6 μ g/ml and 32 μ g/ml, respectively. Thus, in the treatment with electrochemotherapy, IGROV 1/DDP cells exhibited 50-fold resistance to cisplatin.

The treatment with cisplatin alone resulted in IC_{50} value of 12 μ g/ml for IGROV 1 cells. The IC_{50} value of resistant subclone could not be determined due to short exposure of cells to cisplatin (5 minutes). Therefore, it was not possible to calculate the dose enhancing ratio for resistant subclone. The dose enhancement ratio (DER) of the parental cells was approximately 20 at all levels of cell survival.

Discussion

Our study has shown that electroporation of IGROV 1/DDP tumor cells, resistant to cisplatin, increases cytotoxicity of cisplatin.

The resistance of tumor cells to chemotherapeutic drugs is a major problem in clinical chemotherapy. Therefore, several

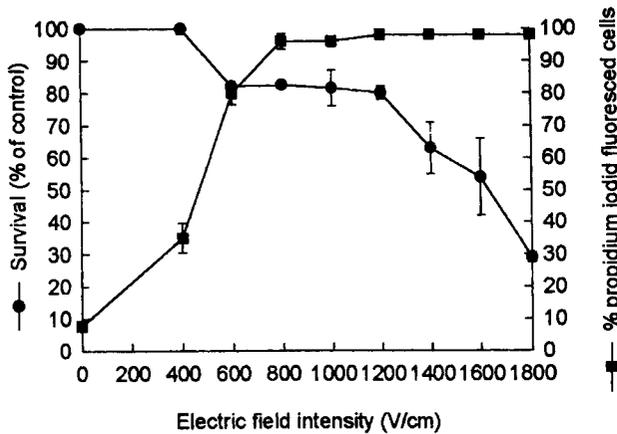


Figure 2. Electroporation and electrosensitivity of IGROV 1/DDP cells. Electrosensitivity was measured by means of propidium iodide uptake and electroporation by means of clonogenic assay. Data are mean \pm standard error of the mean.

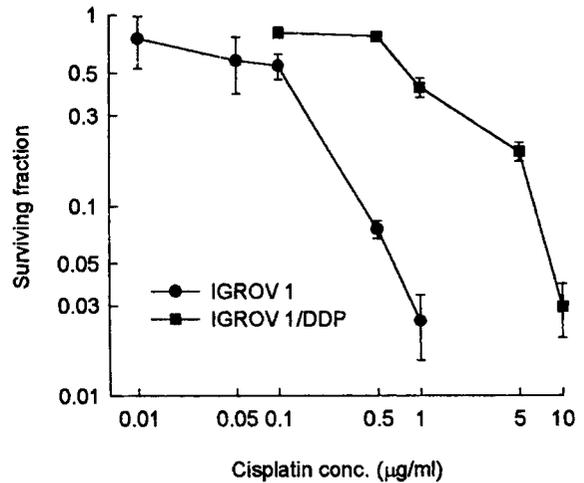


Figure 3. Cell survival after continuous exposure of cells to cisplatin. Data are mean \pm standard error of the mean.

attempts have been made to overcome the resistance of tumors to cisplatin. These attempts include the agents which act on glutathione and metallothioneins in cytoplasm, the agents with the activity on DNA, and the agents that influence cisplatin accumulation (3). Among the agents that modulate cisplatin accumulation in the cells, hyperthermia, forskolin, dipyridamole, digitonin, and spermine have already proved their usefulness (4-6, 14,15).

Electroporation is also one of the methods that affects cell membrane (16). Exposure of cells to high intensity electric pulses causes a transient increase in plasma membrane permeability and, consequently, also an increase in the uptake of cisplatin into the cells. We have demonstrated that in EAT tumors treated with electrochemotherapy, 2-times higher amount of platinum was detected in both whole tumors and DNA, than in the cisplatin treated tumors (9). In the present study, which aimed to determine the role of electrochemotherapy in the treatment of cells resistant to cisplatin, we have used well characterized human ovarian adenocarcinoma IGROV 1 cells and their resistant subclone IGROV 1/DDP (17,18). In a recent study of Fajac *et al* it was demonstrated that, in comparison to parental IGROV 1 cells, resistant IGROV 1/DDP cells exhibit enhanced drug efflux, higher glutathione content, a 2-fold increase of p53 mRNA and p53 protein, overexpressed mdm-2 protein and a 5-fold decrease in the number of platinum atoms bound per nucleotide (18).

Our results, as had been expected, demonstrated that electroporation of plasma membrane increased cisplatin induced cell killing in both, parental and resistant cells. However, due to the various resistance mechanisms existing in resistant IGROV 1/DDP cells, the degree of cell killing was not the same for both cell lines. The cell killing of IGROV 1 cells by electrochemotherapy was approximately 50-fold the

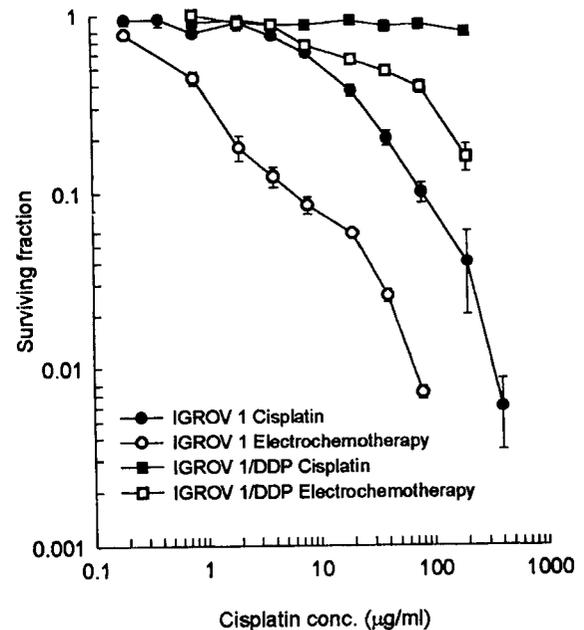


Figure 4. Cell survival after electrochemotherapy and cisplatin treatment (exposure time: 5 min). Data are mean \pm standard error of the mean.

killing of the resistant cells. It is clearly established that the binding of platinum to DNA is responsible for cisplatin induced cell death (2). Therefore, although by electroporation an equal amount of platinum should enter the cells, in this particular cell line IGROV 1/DDP the contribution of other mechanisms of resistance, which are not

confined to the plasma membrane, probably prevented cisplatin to bind to DNA in higher amounts and was thus responsible for the observed cell killing. Nevertheless, compared to cisplatin treatment alone, a great potentiation of cell killing was obtained for this short exposure (5 minutes) of cells to cisplatin.

Electrochemotherapy with cisplatin proved its effectiveness in several preclinical studies and one clinical study (7-10, 12). In this clinical study on malignant melanoma, squamous cell carcinoma and basal cell carcinoma, we performed several electrochemotherapy sessions on the same tumor nodule due to its big size, and also several sessions in one patient due to the large number of the tumor nodules. The response to electrochemotherapy was good after each session in both cases and we did not observe the development of acquired resistance of these tumors to cisplatin (12).

In conclusion, our results demonstrate that electrochemotherapy potentiates cisplatin cytotoxicity on IGROV 1 and IGROV 1/DDP cells *in vitro*. In the future, studies on other tumor cells *in vitro* and tumors *in vivo* resistant to cisplatin are planned in order to explore the role of electrochemotherapy in the treatment of tumors resistant to cisplatin.

Acknowledgements

The IGROV 1 and IGROV 1/DDP cells were generously provided by Dr. J. Bernard, Institut Gustave Roussy, Villejuif, France. This work was supported by the Ministry of Science and Technology of the Republic of Slovenia and by PROTEUS Programme for scientific, technological and cultural cooperation between the Republic of France and the Republic of Slovenia.

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Received May 25, 1998

Accepted July 28, 1998