

Antitumor Effectiveness of Electrochemotherapy with *cis*-Diamminedichloroplatinum(II) in Mice¹

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ABSTRACT

One of the ways to increase drug delivery into cells and tissues is by a local application of short, intense electric pulses, *i.e.*, electroporation. This approach is used in electrochemotherapy to potentiate antitumor effectiveness of chemotherapeutic drugs. To determine whether electroporation can potentiate antitumor effectiveness of *cis*-diamminedichloroplatinum(II) (CDDP), electrochemotherapy with CDDP was tested *in vitro* and *in vivo* on s.c. SA-1, EAT, and melanoma B16 tumors in mice. Electric pulses were applied to the tumors by percutaneously placed electrodes after *i.v.* injection of CDDP. Severalfold potentiation of CDDP antitumor effectiveness with electric pulses was obtained, inducing partial or complete responses in tumor growth. Electrochemotherapy was CDDP dose dependent, as well as dependent upon the amplitude of electric pulses. Also important was the sequencing and the interval of CDDP administration, relative to application of electric pulses. Specifically, a good antitumor effect without side effects was obtained with eight electric pulses (electric pulse amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μ s; electrode distance, 8 mm; 1300 V/cm) applied 3 min after *i.v.* injection of 4 mg/kg CDDP. With a higher CDDP dose (8 mg/kg), some long-term complete responses were obtained (14%) on melanoma B16 tumors. Thus, electrochemotherapy with CDDP offers an approach to making chemotherapy with CDDP more effective.

INTRODUCTION

Although CDDP³ is an effective chemotherapeutic drug in the treatment of many human malignancies, the search continues for a way to potentiate its antitumor effectiveness so that lower doses can be used and side effects avoided. One of the ways to potentiate its effectiveness is to increase cellular accumulation of CDDP and thus, to potentiate CDDP cytotoxicity (1). This can be done by manipulation of the plasma membrane, because it is a barrier through which CDDP must enter the cells (2-6).

Specifically, the plasma membrane can be permeabilized by exposure of cells to short, intense EP (7-11). The application of EP transiently and reversibly increases plasma membrane permeability without impairing cell viability (8, 12, 13). Thus, increased plasma membrane permeability enables hydrophilic drugs to diffuse into the cells and reach their intracellular targets. For example, the *in vitro* cytotoxicity of bleomycin, netropsin, actinomycin D, and CDDP can be potentiated severalfold by exposing cells to short, intense EP (3, 4, 12, 14).

Tissues can also be electroporated; thus, the antitumor effectiveness of chemotherapeutic drugs is potentiated by increasing drug delivery into the cells (15). This novel approach, termed electrochemotherapy, was introduced by Okino and Mohri (16) and Mir *et al.* (17). For example, the antitumor effectiveness of bleomycin can

be greatly potentiated with EP, inducing partial and complete responses of the tumors. Furthermore, the treatment requires such a low amount of bleomycin that it is ineffective without EP and does not induce side effects (18-23).

Whether electroporation of the tumors *in vivo* also potentiates the antitumor effectiveness of CDDP is not known. If electrochemotherapy with CDDP is effective in the treatment of tumors, it is not known how the antitumor effect depends upon the electric field intensity, the sequencing and timing of CDDP administration, and the CDDP dose. To answer these questions, we studied the antitumor effects of electrochemotherapy with CDDP on different s.c. tumors in mice.

MATERIALS AND METHODS

Chemicals. CDDP (Pliva, Zagreb, Croatia) was prepared in sterile H₂O to obtain a concentration of 1 mg/ml. The final concentration was prepared in EMEM (Sigma Chemical Co., St. Louis, MO) for *in vitro* experiments or in 0.9% NaCl solution for *in vivo* experiments. For each experiment, a fresh solution of CDDP was prepared. Propidium iodide (Sigma) was dissolved in sterile H₂O at a concentration of 100 μ M.

***In Vitro* Electrochemotherapy Protocol.** Melanoma B16 cells (Royal Marsden Hospital, Cancer Research Institute, Sutton, United Kingdom) were grown as a monolayer in EMEM supplemented with 10% FCS (GIBCO, Grand Island, NY), 10 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 11 μ g/ml gentamicin. The cells were routinely subcultured every 5 days and incubated at 37°C in humidified air with 5% CO₂.

The optimal EP amplitude for permeabilization of melanoma B16 cells was determined. The permeabilization of the plasma membrane was measured by means of propidium iodide uptake and cell survival after exposure to EP by a colony-forming assay. Cells were prepared from the exponential growth phase, trypsinized, and washed twice at 4°C in EMEM supplemented with 10% FCS for inactivation of trypsin and then in serum-free EMEM supplemented with 0.5 mM CaCl₂ and then resuspended. The cell suspension (2.2 \times 10⁷ cells/ml in 67.5 μ l) was mixed with 7.5 μ l propidium iodide (100 μ M) for measurement of propidium iodide uptake, or EMEM supplemented with 0.5 mM CaCl₂ for the colony-forming assay. This mixture (50 μ l) was placed between two flat, parallel stainless steel electrodes (length, 6 mm; width, 6 mm; distance, 2 mm) and subjected to eight square-wave EP, with a pulse width of 100 μ s and a repetition frequency 1 Hz of different amplitudes, ranging from 80-360 V (14). After pulsing, cells were incubated for 5 min at room temperature (24°C). To measure the propidium iodide uptake, 25 μ l of pulsed cells were resuspended in 1 ml of 0.01 M PBS (pH 7.4) and analyzed immediately by FACSsort (Becton Dickinson, Mountain View, CA). The percentage of stained cells was determined in comparison to control cells that were not subjected to EP.

For the colony-forming assay, 5 μ l of pulsed cells were diluted 250 times and seeded in quadruplicates in 60-mm Petri dishes (Costar, Badhoevedorp, the Netherlands; 400 cells/dish). After 10 days, colonies were fixed, stained with crystal violet (Kemika, Zagreb, Croatia), and counted. Colonies containing less than 50 cells were disregarded. The results were expressed as the percentage of the colonies obtained with the untreated control cells. The plating efficiency of control cells was above 70%.

The electrochemotherapy protocol was the same as described above with some differences; 7.5 μ l of different CDDP concentrations (from 2.7 to 675 μ M) were added to 67.5 μ l of cell suspension. An EP amplitude of 250 V was applied to cells in suspension, keeping the remaining variables constant.

Animals. In these experiments, inbred strains of mice of both sexes were used. They were maintained at a constant room temperature (24°C) with a

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³ The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); EP, electric pulses; EMEM, Eagle minimal essential medium; EAT, Ehrlich-Lettre Ascites carcinoma; DT, tumor doubling time.

natural day/night light cycle in a conventional animal colony. A/J and C57Bl/6 mice were purchased from Rudjer Bošković Institute (Zagreb, Croatia), and CBA mice were purchased from the Institute of Pathology, University of Ljubljana. Before the experiments, the mice were subjected to an adaptation period of at least 10 days. Mice in good condition, without fungal or other infections and 10–12 weeks of age, were included in the experiments.

Tumors. Three different tumor models were used in the study: fibrosarcoma SA-1 cells (The Jackson Laboratory, Bar Harbor, ME) syngeneic to A/J mice; Ehrlich-Lette Ascites carcinoma cells (EAT; American Type Culture Collection, Rockville, MD) syngeneic to CBA mice; and melanoma B16 cells (Royal Marsden Hospital) syngeneic to C57Bl/6 mice. SA-1 and EAT tumor cells were obtained from the ascitic form of the tumors in mice, serially transplanted every 7 days. Melanoma B16 cells were obtained from *in vitro* cell cultures. Solid s.c. tumors, located dorsolaterally in mice, were initiated by an injection of 5×10^5 SA-1 cells, 1×10^6 melanoma B16 cells, or 5×10^6 EAT cells in 0.1 ml 0.9% NaCl solution. The viability of the cells, as determined by a trypan blue dye exclusion test, was over 95% for all three tumor models. Six to 8 days after transplantation when the tumors reached approximately 40 mm³ in volume, mice were randomly divided into experimental groups, consisting of 8–10 mice each and subjected to a specific experimental protocol on day 0.

In Vivo Electrochemotherapy Protocol. CDDP was injected i.v. in bolus into the mice. The doses used for CDDP treatment (1, 4, and 8 mg/kg) were sublethal and well tolerated by the mice. For i.v. tail injections, mice were preheated under infrared light for two minutes to dilate the veins.

EP were delivered by two flat, parallel stainless steel electrodes 8 or 10 mm apart (two stainless steel strips: length, 35 mm; width, 7 mm with rounded corners) and placed at the opposite margins of the tumor. Good contact between the electrodes and the skin was assured by means of conductive gel. Eight square-wave EP of different amplitudes, with a pulse width of 100 μ s and repetition frequency of 1 Hz, were generated by Electropulsator Jouan GHT 1287 (Saint Herblain, France). The EP amplitude and electrode distance are given as an EP amplitude:electrode distance ratio (V/cm ratio; in most reports, referred as “electric field intensity”) to enable comparison and easier presentation of the results. Treatment with EP was performed without anesthesia and was well tolerated by the mice.

In the electrochemotherapy protocol, mice were treated with EP 3 min after CDDP injection. In the experiments for optimal timing of CDDP treatment in combination with EP, different time intervals were tested: i.v. CDDP injections 30, 15, 9, 6, and 3 min before EP treatment; EP treatment immediately after CDDP treatment (< 30 s); and CDDP injections 3, 9, 15, and 30 min after EP treatment. Mice in the control and EP groups were injected with 0.01 M PBS (pH 7.4) instead of CDDP.

Assessment of Response and Statistical Analysis. Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1 , e_2 , and e_3) with Vernier calipers on each consecutive day. Tumor volumes were calculated by the formula $\pi \times e_1 \times e_2 \times e_3/6$. From these measurements, the arithmetic mean and SE were calculated for each experimental group comprising at least 16 mice pooled from two separate experiments, including all of the pertinent control groups. The DT was determined for each individual tumor, and tumor growth delay from the mean DT of experimental groups was calculated by:

$$\text{Tumor growth delay}_x = \overline{DT}_x - \overline{DT}_c$$

$$SD^2 = \frac{(n_x - 1) \times SD_x^2 + (n_c - 1) \times SD_c^2}{n_x + n_c - 2} \times \left(\frac{1}{n_x} + \frac{1}{n_c} \right)$$

$$v = n_x + n_c - 2$$

where \overline{DT} is the mean doubling time, n is the number of mice within the group; v , is the degree of freedom; subscript x , experimental groups; and subscript c , control group.

The response to electrochemotherapy was scored according to WHO guidelines as: (a) progressive disease if tumors increased in size; (b) no change if tumors reduced in size by less than 50%; (c) partial response if the size of the tumor was reduced by more than 50%; and (d) complete response if they became unpalpable. In the tumor growth curves and tumor growth delay curves, only mice with tumor recurrence after the treatment were included; the mice that were tumor free 100 days after the treatment were excluded.

Each mouse was also weighed 2–3 times/week. The percentage of body weight loss from pretreatment values was calculated. The general condition of the mice was followed throughout the experiments, and mortality was recorded.

The significance of the differences between the mean values of the DT and tumor growth delay of the experimental groups was evaluated by modified t test (Bonferroni t test) after one-way ANOVA was performed and fulfilled. Survival distribution functions were analyzed using the BMDP statistical software (Los Angeles, CA). Survival curves were plotted using the Kaplan-Meier method. Homogeneity of the survival distribution functions was tested using Cox's regression model.

RESULTS

Effect of Electroporation on CDDP Cytotoxicity *in Vitro*. In the preliminary experiments, we wanted to determine whether EP increase CDDP cytotoxicity in the melanoma B16 cell line, which forms colonies; therefore, a colony-forming assay for cell survival was performed. The optimal EP amplitude for plasma membrane permeabilization was chosen, which maximally permeabilized the plasma membrane and did not reduce the reproductive potential of the cells (EP amplitude, 250 V; electrode distance, 2 mm; 1250 V/cm). Cells exposed to these EP were permeabilized in $92.4 \pm 0.6\%$ measured by propidium iodide uptake, and their surviving fraction was 0.88 ± 0.09 , measured by colony-forming assay. Exposure of the cells to EP resulted in a marked increase in CDDP cytotoxicity. Throughout the range of CDDP concentrations investigated, cells exposed to EP were more sensitive to CDDP than those which were not (Fig. 1). The cells exposed to EP were 8-fold more sensitive to CDDP, as determined by the concentration causing 50% inhibition of colony formation. The potentiating effect of electroporation was even more pronounced at a concentration causing 80% inhibition of colony formation, where a 70-fold dose-enhancing effect was observed.

Antitumor Effect of Electrochemotherapy with CDDP *in Vivo*. To determine whether short, intense EP *in vivo* can potentiate the antitumor effectiveness of CDDP, treatment was performed on EAT s.c. tumors 40 mm³ in volume. Combined treatment with CDDP (4 mg/kg) and EP (EP amplitude, 1040 V; electrode distance, 8 mm;

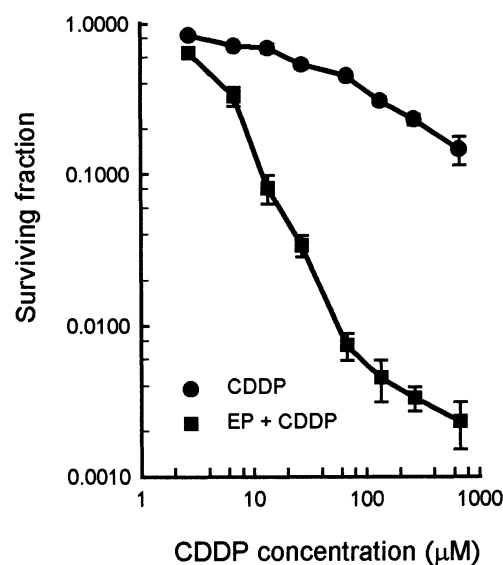


Fig. 1. Potentiation of CDDP cytotoxicity *in vitro* by electroporation of melanoma B16 cells. The clonogenicity of the cells was determined after CDDP treatment for 5 min, EP treatment, or a combination of both. The survival curve for electrochemotherapy-treated cells is normalized for the cytotoxicity of EP treatment alone (surviving fraction, 0.88 ± 0.09). Bars, SE.

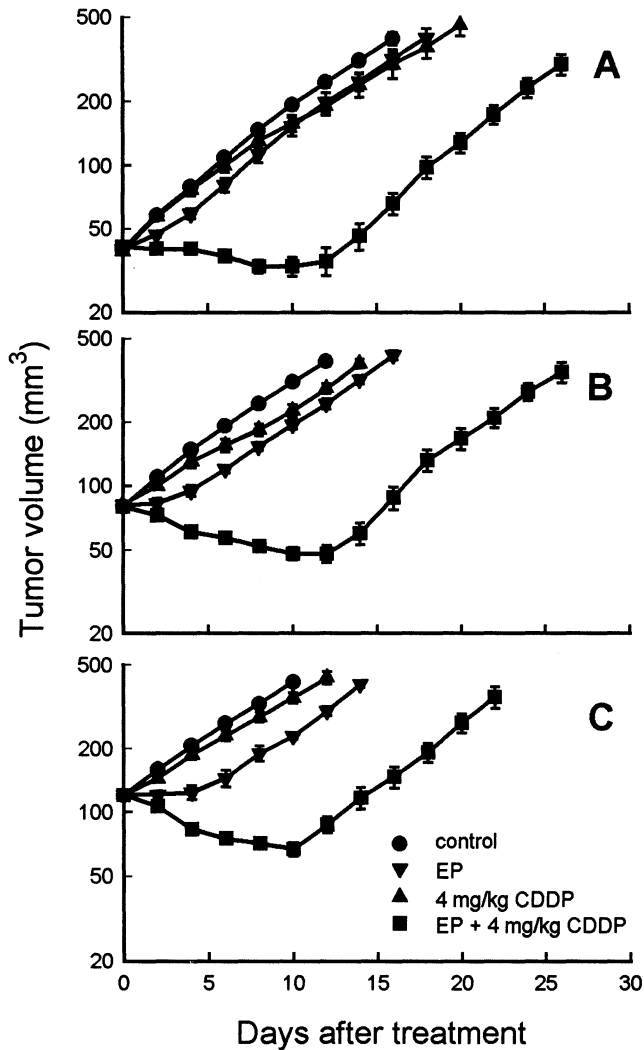


Fig. 2. The antitumor effectiveness of electrochemotherapy with CDDP on EAT s.c. tumors of different volumes at the beginning of treatment. Mice were treated with CDDP (4 mg/kg) i.v. and/or with eight EP 3 min after CDDP treatment. Tumors that were 40 mm³ (A) and 80 mm³ (B) in volume were treated with electrodes that had 8 mm space between them, whereas 120-mm³ (C) tumors were treated with electrodes that were 10 mm apart. All tumors were treated with the same EP amplitude:electrode distance ratio of 1300 V/cm; therefore, 40- and 80-mm³ tumors were subjected to EP of amplitude 1040 V, and 120-mm³ tumors were subjected to EP of amplitude 1300 V (repetition frequency, 1 Hz; pulse width, 100 μ s). Tumor growth curves represent the arithmetic mean \pm SE of the tumor volumes measured every second day.

1300 V/cm ratio) had a marked antitumor effect (Fig. 2A). In the first days after electrochemotherapy, tumors ceased to grow, but tumor volume was not noticeably reduced. Superficial scabs appeared on almost all of the tumors, but they progressively disappeared in 10–15 days. No change in tumor growth was observed until 13 days after electrochemotherapy, when the tumors started to regrow. The tumor growth delay of the electrochemotherapy-treated tumors (13.1 ± 0.7 days) was significant compared to EP- and CDDP-treated groups ($P < 0.05$). Electrochemotherapy, as well as single treatments with CDDP and EP, was well tolerated by the mice; no body weight loss was observed, and no treatment-related mortality was recorded.

Electrochemotherapy effectiveness was also tested on EAT tumors that were twice (80 mm³) and three times larger (120 mm³) at the beginning of treatment (Fig. 2, B and C). Tumors of different volumes were treated with the same EP amplitude:electrode distance ratio of 1300 V/cm (EP amplitude: 1040 V at an electrode distance 8 mm and 1300 V at an electrode distance of 10 mm). The antitumor effect of

electrochemotherapy with CDDP was equally effective on 40-mm³ tumors (tumor growth delay, 13.1 ± 0.7 days), 80-mm³ tumors (tumor growth delay, 15.0 ± 0.9 days), and 120-mm³ tumors (tumor growth delay, 15.4 ± 0.8 days). The antitumor effect of CDDP alone as a single treatment was equally effective on small and big tumors (tumor growth delay, 0.1 ± 0.5 days on 40-mm³ tumors, 1.9 ± 0.8 days on 80-mm³ tumors, and 1.2 ± 0.4 days on 120-mm³ tumors). Treatment with EP alone as a single treatment resulted in 2.1 ± 0.5 days on 40-mm³, 3.8 ± 0.4 days on 80-mm³, and 4.7 ± 0.6 days tumor growth delay on 120-mm³ tumors. These results indicate that electrochemotherapy with CDDP is effective in reducing the tumor burden, regardless of the treated tumor volume, under the condition that the whole tumor mass is encompassed between the electrodes.

In search for optimal electrochemotherapy conditions, we determined how the antitumor effectiveness of electrochemotherapy with CDDP is dependent upon EP amplitude. We also determined the importance of the sequence and interval of CDDP administration with respect to EP application.

The dependence of EP amplitude used for electrochemotherapy on antitumor effectiveness of CDDP (4 mg/kg) was evaluated (Fig. 3). Electrochemotherapy treatment of tumors with EP of different amplitudes resulted in significant interaction between both treatments in the whole range of EP amplitudes used for the treatment. The antitumor effect was increased from 10.0 ± 0.9 days tumor growth delay at EP amplitude 720 V (900 V/cm ratio) to 16.7 ± 0.6 days at EP amplitude 1200 V (1500 V/cm ratio). Furthermore, the linear relationship between the antitumor effectiveness of electrochemotherapy with CDDP and EP amplitudes used for combined treatment was found. However, EP treatment only moderately affected tumor growth, even at the highest EP amplitude (1200 V; tumor growth delay, 3.5 ± 0.5 days).

The importance of the sequence and interval of CDDP administration with respect to EP application in electrochemotherapy was evaluated. In these experiments, CDDP (4 mg/kg) was injected either before or after EP treatment (EP amplitude, 1040 V; 1300 V/cm ratio)

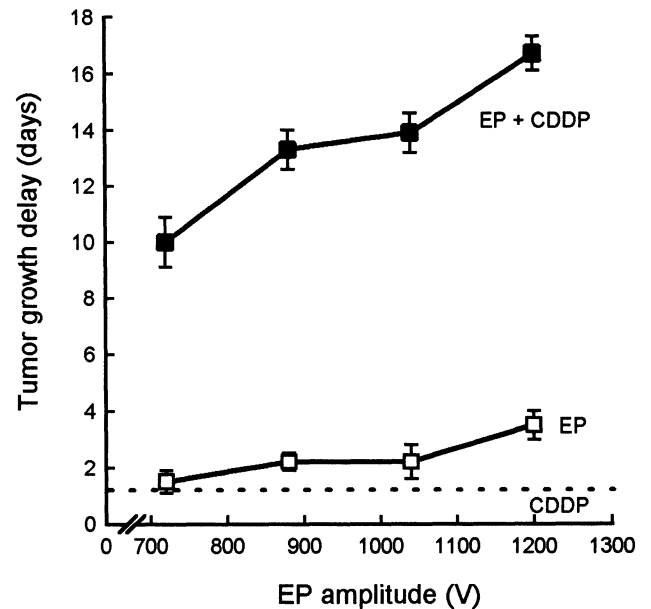


Fig. 3. Tumor growth delay as a function of EP amplitudes used in electrochemotherapy with CDDP. Mice bearing s.c. EAT tumors (40 mm³) were treated with CDDP (4 mg/kg) i.v. and/or with eight EP 3 min after CDDP treatment. Amplitudes used for EP treatment were 720, 880, 1040, or 1200 V (900, 1100, 1300, and 1500 V/cm ratio), whereas the remaining variables were the same for all experimental groups (repetition frequency, 1 Hz; pulse width, 100 μ s; electrode distance, 8 mm). Tumor growth delays were calculated from the DT of the experimental groups in relation to the DT of the untreated control group (arithmetic mean); bars, SE.

with different intervals between both treatments (Fig. 4). The antitumor effect of electrochemotherapy with CDDP was greatly dependent on the timing of CDDP administration. Specifically, the best interaction was achieved when CDDP was injected i.v. 3 min before EP treatment, whereas with prolonged intervals between the treatments, electrochemotherapy was less effective. Nevertheless, even the injection of CDDP 30 min before EP treatment resulted in significant tumor growth delay (5.1 ± 1.2 days; $P < 0.05$). However, if CDDP treatment was performed immediately before (<30 s) or after EP treatment, the antitumor effectiveness of electrochemotherapy was less pronounced. According to the shape of the curve, the antitumor effectiveness of electrochemotherapy dissipated very quickly if CDDP was injected after EP treatment.

Antitumor Effect on Different Tumor Models. The importance of the CDDP dose on the antitumor effectiveness of electrochemotherapy was evaluated on three tumor models: fibrosarcoma SA-1; EAT; and melanoma B16 tumors (Fig. 5). The dose-response relationship between the electrochemotherapy antitumor effect and CDDP dose (1, 4, and 8 mg/kg) was obtained on all three tumor models. However, differences in responsiveness to electrochemotherapy were found among the three tumors tested. Specifically, the highest CDDP dose induced a 4.9-fold increase in the antitumor effect on SA-1, a 5.6-fold increase on EAT, and a 7.9-fold increase on melanoma B16 tumors, compared to the lowest CDDP dose tested.

Although tumor growth delay on electrochemotherapy-treated SA-1 and EAT tumors was pronounced and some partial responses were observed (5% on SA-1 and 33% on EAT tumors) for up to 13 days, no long-term complete responses were observed, since all tumors eventually regrew. In contrast, on melanoma B16, a high percentage of partial and complete responses were observed (86%) within 14 days after the treatment, and 100 days after the treatment, 14% of the mice were still tumor free. Also, the median survival time of the melanoma B16-bearing mice after electrochemotherapy with CDDP was significantly prolonged, from 41 days in CDDP and 44

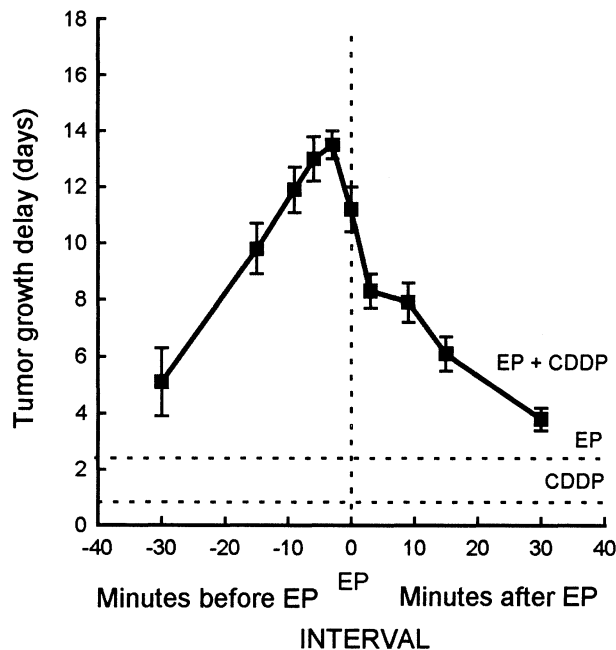


Fig. 4. Tumor growth delay as a function of the sequence and interval between CDDP and EP treatment. Mice bearing 40-mm³ s.c. EAT tumors were treated with CDDP (4 mg/kg) i.v. and/or with eight EP (EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μ s; electrode distance, 8 mm; 1300 V/cm ratio). Tumor growth delays were calculated from the DT of the experimental groups in relation to the DT of the untreated control group (arithmetic mean); bars, SE.

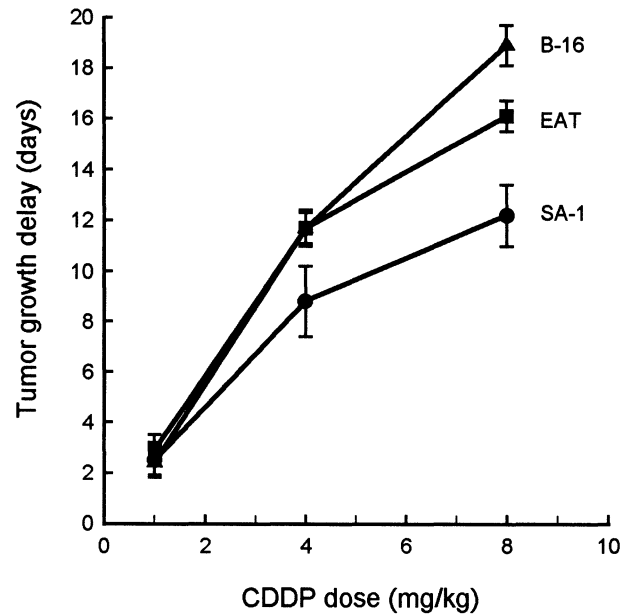


Fig. 5. Tumor growth delay as a function of CDDP dose used in electrochemotherapy on three different tumors: melanoma B16, EAT, and fibrosarcoma SA-1. Mice bearing 40-mm³ tumors were treated with different CDDP doses (1, 4, or 8 mg/kg) i.v. and/or with eight EP (EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μ s; electrode distance, 8 mm; 1300 V/cm ratio) 3 min after CDDP treatment. Tumor growth delay was calculated from the DT of the experimental groups in relation to the DT of the corresponding CDDP-treated group (arithmetic mean); bars, SE. Tumor growth delays of the experimental groups treated with CDDP as a single treatment did not exceed 0.9 days in the lowest dose and 2.9 days in the highest CDDP dose. Treatment with EP alone resulted in up to 2.0 days of tumor growth delay.

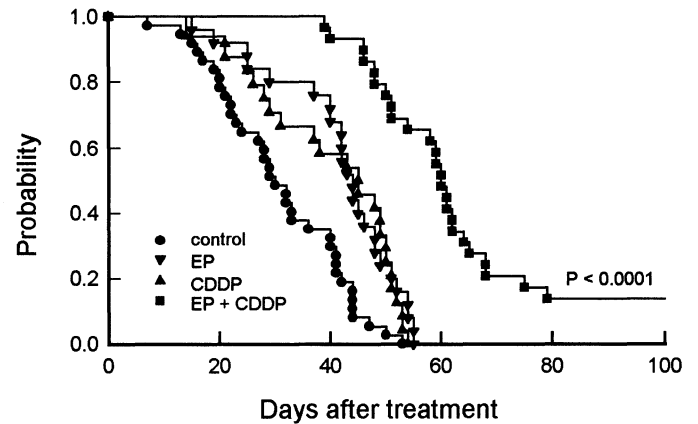


Fig. 6. The survival of melanoma B16-bearing mice after electrochemotherapy treatment with CDDP. Mice bearing 40-mm³ s.c. melanoma B16 tumors were treated with CDDP (8 mg/kg) i.v. and/or with eight EP (EP amplitude, 1040 V; repetition frequency, 1 Hz; pulse width, 100 μ s; electrode distance, 8 mm; 1300 V/cm ratio).

days in EP-treated mice to 59 days in mice treated with electrochemotherapy (Fig. 6).

DISCUSSION

This study shows that CDDP cytotoxicity can be potentiated by treatment with short, intense EP. We found that electropermeabilization of melanoma B16 cells *in vitro* as well as electropermeabilization of tumors *in vivo* increased the antitumor effectiveness of CDDP severalfold.

Our *in vitro* experiments demonstrated that permeabilization by EP of melanoma B16 cells potentiated CDDP cytotoxicity up to 70 times. There are limited data addressing the use of electropermeabilization of

plasma membranes to increase cell sensitivity to CDDP. On cultured NHIK 3025 cells, it was demonstrated that exponentially decaying EP increase permeability of the plasma membrane for CDDP and that the process is entirely reversible, without affecting cell viability (3). Electroporation of NHIK 3025 cells immediately before or during exposure to CDDP potentiated CDDP cytotoxicity 3-fold (3). Similar results were obtained on CDDP-sensitive and -resistant RIF-1 tumor cells, where electrochemotherapy with CDDP increased cell killing 1.9-fold in sensitive and 2.3-fold in CDDP-resistant cells (4). These studies demonstrated that the increased cell killing was associated with higher intracellular CDDP accumulation (3, 4). The difference in potentiating factors between our and other studies could be explained by the shape of the EP used for permeabilization as well as intrinsic cell sensitivity to CDDP and EP (1, 24). In our study, square-wave EP were used, which was demonstrated to be suitable for permeabilization of the cells, and its use was optimized on different cell lines *in vitro* (13, 24). All of these data confirm the notion that electroporation is effective in predisposing cells to the cytotoxic action of CDDP.

Also, tumors can be electroporated for more effective drug delivery (15–23). This was demonstrated on tumors and tissue sections, qualitatively and quantitatively for electrochemotherapy, with bleomycin (15). The results of our study show that electrochemotherapy with CDDP is also effective in reducing tumor burden. We found that the antitumor effectiveness of electrochemotherapy with CDDP was dependent upon the EP amplitude, sequencing, and timing of CDDP treatment, as well as on the CDDP dose applied. According to the results on electrochemotherapy with bleomycin, an EP amplitude:electrode distance ratio of approximately 900 V/cm must be exceeded to obtain a long-lasting effect on tumor growth (15, 17). Therefore, we tested the antitumor effectiveness of electrochemotherapy with CDDP, using different EP amplitudes exceeding 900 V/cm ratio. As demonstrated, the antitumor effect increased from 10.0 days of tumor growth delay at 900 V/cm ratio to 16.7 days at 1500 V/cm ratio. Probably with higher EP amplitudes, even better antitumor effects can be achieved, but amplitudes of these EP can result in severe side effects as a result of tumor lysis syndrome, due to massive tumor destruction, if the chemotherapeutic drug used for electrochemotherapy is effective (15, 17). Nevertheless, long-lasting antitumor effects can be achieved also at 1300 V/cm ratio, where the minimal antitumor effect of EP itself is observed and most of the tumor cells are permeabilized. Evidently, also by using high EP amplitudes, all clonogenic tumor cells cannot be sterilized. Therefore, for better antitumor effect, higher CDDP doses must be used or some other adjuvant treatment added to eradicate the last clonogenic cell (25, 26).

The importance of subjecting the whole tumor to an electric field was demonstrated by the same antitumor effectiveness of electrochemotherapy with CDDP on different tumor volumes. The same treatment procedure can be equally effective in reducing different tumor volumes as long as most of the tumor cells are permeabilized and the whole tumor mass is encompassed between the electrodes. This indicates that, under the same treatment conditions, the same fraction of tumor cells is permeabilized and sterilized with electrochemotherapy, regardless of the tumor volume at the time of treatment. In addition, a way to subject the whole tumor mass to EP that permeabilizes all clonogenic tumor cells is needed.

The antitumor effectiveness of electrochemotherapy was dependent also upon the sequencing and timing of CDDP administration with respect to EP application. The best interaction was achieved when EP were delivered 3 min after CDDP treatment, whereas prolongation or shortening of the time intervals between the treatments was less effective. Obviously, an optimal CDDP concentration in the tumor

was achieved within 3 min, when electrochemotherapy was the most effective. It is likely that after rapid accumulation of CDDP in the tumor, by prolongation of the interval, more CDDP is washed out from the tumor. Prolongation of the interval between CDDP and EP treatment linearly decreased antitumor effectiveness of electrochemotherapy. On the other hand, because permeabilization is a short-lived process (27), CDDP was less effective when administered after EP treatment, as demonstrated by the steep decrease in the response curve (Fig. 4). Besides, some long-lasting effect seemed to be present until 30 min after EP treatment. Similar experimental data are available on electrochemotherapy with bleomycin, where the best antitumor effect was also achieved when bleomycin was injected i.v. 3 min before EP treatment (22). It seems that both chemotherapeutic drugs have similar accumulation properties in the tumors of mice, but for other chemotherapeutic drugs, a new time-response relationship must be determined.

The antitumor effectiveness of electrochemotherapy was also CDDP dose dependent. This study demonstrates that for maximal antitumor effect, a sufficient CDDP concentration must be achieved in the tumor. A CDDP dose of 1 mg/kg (a corresponding dose in humans would be 3.1 mg/m²; Ref. 28) was moderately effective and seems to be suboptimal. With 4 mg/kg (a corresponding dose in humans would be 12.3 mg/m²), electrochemotherapy was very effective and did not induce side effects, whereas with the dose of 8 mg/kg (a corresponding dose in humans would be 24.7 mg/m²), a nonlinear increase in antitumor effect was achieved. From these results, we can conclude that electrochemotherapy with the 4-mg/kg CDDP dose in the treatment of experimental tumors is sufficient, and this dose is below the dose that is usually used in the treatment of patients with CDDP, either in bolus or in continuous infusion (29).

The CDDP dose relationship in the antitumor effectiveness of electrochemotherapy was achieved in the three tumor models tested, *i.e.*, sarcoma SA-1, EAT, and melanoma B16. The antitumor effectiveness of electrochemotherapy with the lowest CDDP dose (1 mg/kg) was equally effective in all three tumors. However, with higher CDDP doses, some variability in responsiveness of the tumors was observed. SA-1 was the least and melanoma B16 the most responsive. Electrochemotherapy with 8 mg/kg CDDP was 1.5-fold more effective on melanoma B16 than on SA-1 tumor. The variability in the responsiveness of tumors to electrochemotherapy with CDDP presently cannot be explained but can be attributed to a different intrinsic susceptibility of the cells to CDDP or EP (1, 24). However, a variability in responsiveness of different tumor types was demonstrated by electrochemotherapy with bleomycin. In our previous experiments, we determined the antitumor effectiveness of electrochemotherapy with bleomycin on the same tumor models as in this study (22). A reverse degree in response was observed; the least responsive was melanoma B16 and the most responsive was fibrosarcoma SA-1. On melanoma B16, 5% of the mice after electrochemotherapy with bleomycin were tumor free 100 days after treatment, whereas on SA-1 tumor, 62% of the mice were tumor free. In this study on electrochemotherapy with CDDP, the best results were obtained on melanoma B16, but only 14% of the mice were tumor free 100 days after the treatment, whereas no SA-1 or EAT-bearing mice were tumor free.

Evidently, electrochemotherapy with bleomycin is more effective than with CDDP, but nevertheless, a significant antitumor effect was achieved, demonstrated by the tumor growth delay and median survival time. Electrochemotherapy with bleomycin is very effective and can be used as a treatment modality, whereas electrochemotherapy with CDDP can be used as an adjunct to on-going CDDP treatment in patients in whom the antitumor effect needs to be potentiated locally. Many patients who are on CDDP-based chemotherapy have tumor lesions that are accessible to *in vivo* application of short, intense EP.

Such tumor nodules can be treated to achieve a better antitumor effect locally. Thus, electrochemotherapy with CDDP offers an approach to making chemotherapy with CDDP more effective. The development of electric generators for a more suitable application of EP in clinics may pave the way to a broader clinical use of electrochemotherapy.

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