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Electrochemotherapy of Mouse Sarcoma Tumors Using Electric Pulse Trains with Repetition Frequencies of 1 Hz and 5 kHz

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Abstract Electrochemotherapy is an efficient local treatment of tumors that combines administration of a chemotherapeutic drug with the subsequent application of electric pulses to the tumor. Although no difference in clinical response of the treated tumors to the electrochemotherapy when using 1 Hz or 5 kHz repetition frequency was observed, it is mandatory to be aware of possible differences in the effectiveness of electrochemotherapy when using suboptimal doses of the drugs. Therefore, this study compares the antitumor effectiveness of electrochemotherapy using electric pulse trains with repetition frequencies of 1 Hz and 5 kHz at suboptimal drug doses of bleomycin or cisplatin. Electrochemotherapy of fibrosarcoma SA-1 subcutaneous tumors transplanted in A/J mice resulted in good antitumor effectiveness, but antitumor effectiveness was significantly better at 1 Hz repetition frequency than at 5 kHz. The platinum content was higher in tumors treated with a 1 Hz repetition frequency. The application of electric pulses to the tumors at a 5 kHz repetition frequency induced an immediate reduction in tumor perfusion, comparable to the reduction at 1 Hz but with faster reperfusion. The greater effectiveness of electrochemotherapy using electric pulse trains of 1 Hz compared to 5 kHz is due to the greater electroporative effect and longer time in which electroporated tumors are exposed to the two chemotherapeutic drugs. These differences are observed at suboptimal drug doses, whereas at optimal drug doses of bleomycin or cisplatin the antitumor effectiveness is the same, as demonstrated in clinical trials.

Keywords Electroporation · Electrochemotherapy · Bleomycin · Cisplatin · Experimental tumor

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Introduction

Electrochemotherapy is based on the principle of functional electroporation, using electric pulses and biogenic substances (Neumann et al. 1982). It consists of local treatment of tumors, which combines local or systemic administration of nonpermeant or poorly permeant chemotherapeutic drugs—bleomycin (BLM) or *cis*-diamminedichloroplatinum (II) (CDDP)—with electroporation. Subsequent to drug administration, trains of short but high electric pulses are directly applied to the tumors (electroporation) in order to electroporate and thereby permeabilize the cell membranes for efficient delivery of these drugs into the cells (Mir 2006; Teissie et al. 2008). The results of preclinical and clinical studies demonstrated excellent efficacy of electrochemotherapy on cutaneous and subcutaneous tumors, irrespective of their histology (Gehl 2003; Sersa 2006; Byrne and Thompson 2006; Larkin et al. 2007; Campana et al. 2009; Quaglino et al. 2008).

All clinical studies of electrochemotherapy on cancer patients, except for the European Standard Operating Procedures of Electrochemotherapy (ESOPE) clinical study, were performed using a train of eight electric pulses. Each rectangular pulse lasts 100 μs with a pulse repetition frequency of 1 Hz (Gehl 2003; Sersa 2006; Miklavcic et al.

2006; Marty et al. 2006; Sersa et al. 2008a, b). In the ESOPE study, patients were treated using either 1 Hz or 5 kHz electric pulse repetition frequencies (Marty et al. 2006). Each electric pulse in the train induces contraction of the underlying muscles, evoking a certain degree of discomfort and pain due to application of these electric pulses (Sersa 2006; Miklavcic et al. 2006). In order to reduce discomfort and avoid the sensation of pain from the electric pulses, application of an increased frequency of electric pulses has been suggested (Miklavcic et al. 2005).

In a recent report on a fibrosarcoma SA-1 tumor model, we demonstrated that the antitumor effectiveness of a pulse train of 5 kHz is comparable to a pulse train of 1 Hz (Miklavcic et al. 2005). The higher electric pulse repetition frequency, besides shortening the duration of the treatment, also reduces the number of induced muscle contractions. The latest study on volunteers demonstrated that application of electric pulses at 5 kHz is better tolerated by the subjects and that significantly more of them would choose 5 kHz over 1 Hz electric pulses as treatment (Zupanic et al. 2007).

In a project funded by the European Union (EU), the CLINIPORATOR^{TM} electric pulse generator was developed, which can also generate electric pulses with a 5 kHz pulse repetition frequency and was used for treatment of patients in the EU-funded clinical project ESOPE (Marty et al. 2006). Evaluation of responses of different types of tumors to electrochemotherapy with BLM or CDDP using 1 Hz or 5 kHz pulse trains showed that there was no difference (Marty et al. 2006; Snoj et al. 2007). However, there is still a lack of preclinical data that would clearly demonstrate equal responsiveness of tumors to electrochemotherapy.

Therefore, our aim was to determine the antitumor effectiveness of electrochemotherapy of mouse sarcoma tumors using pulse trains with 1 Hz and 5 kHz repetition frequencies at suboptimal drug doses of BLM or CDDP, which do not produce tumor cures. At such doses, possible differences in tumor responsiveness can be observed, which are due to differences in the electroporative effect of the two repetition frequencies used. Based on this approach, we found that the higher pulse repetition frequency (5 kHz) was less effective than the most widely used standard electroporation with a 1 Hz electric pulse repetition frequency at suboptimal drug doses (CDDP or BLM), as judged from tumor growth delay, platinum content in tumors and tumor perfusion.

Materials and Methods

Drugs

CDDP was obtained from Pharmacia & Upjohn (Milan, Italy). The crystalline powder was dissolved in sterile H_2O at a concentration of 2 mg/ml (6.7×10^{-3} mol/l). A stock solution (3 mg/ml, 2.1×10^{-3} mol/l) of BLM (Blenoxane; Bristol Myers Squibb, Princeton, NJ) was prepared in phosphate-buffered saline. Further dilutions of the drugs were done in phosphate-buffered saline. A fresh stock solution was prepared for each experiment.

Animals and Tumors

An inbred strain of A/J mice of both sexes was used in the experiments (purchased from the Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia). Mice were kept at a constant temperature of 21°C with a 12 h light cycle in a specific pathogen-free animal colony. Before the experiments, mice were subjected to an adaptation period of at least 10 days. Mice in good condition, 10–12 weeks of age, were included in the experiments.

A fibrosarcoma SA-1 tumor (Jackson Laboratory, Bar Harbor, ME) syngenic to A/J mice was used. SA-1 tumor cells were obtained from the ascitic form of the tumors in mice, serially transplanted every 3 days. Solid subcutaneous tumors, located on the right flank of the mice, were initiated by injection of 5×10^5 SA-1 cells in 0.1 ml 0.9% NaCl (1.5×10^{-5} mol/l) solution. Mice were marked, divided randomly into different experimental groups and subjected to a specific experimental protocol when the tumors reached a diameter of 5–6 mm (6–8 days).

Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission 323-02-170/2004/2) and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

Electrochemotherapy Protocol

Tumors were treated by electrochemotherapy, a combined treatment of BLM or CDDP, followed by application of electric pulses. BLM (0.5 mg/kg) or CDDP (4 mg/kg) was injected intravenously in a volume of 7.5 ml/kg body weight. Electric pulses were applied to the tumors through two parallel stainless-steel plate electrodes 6 mm apart (two stainless-steel strips, width 8 mm, length 15 mm, with rounded corners), 3 min after drug administration. Good contact between the electrodes, which were placed at the opposing margins of the tumors and the overlying skin, was assured by means of ultrasound conductive gel. A train of eight square-wave electric pulses of 1,300 V/cm amplitude-over-distance ratio and duration of 100 μs, generated by the CLINIPORATOR^{TM} (IGEA, Carpi, Italy), was delivered in the cranial–caudal direction at two different repetition frequencies of 1 Hz and 5 kHz.

Assessment of Tumor Response

Tumor growth was followed by measuring three orthogonal tumor diameters with a Vernier caliper (Fig. 1). From these measurements, tumor volumes were calculated according to the formula $V = \pi/6 \times a \times b \times c$. The tumor doubling time (DT), i.e., the time in which the tumor has doubled its volume from the beginning of treatment, was determined for each individual tumor. Tumor growth delay (GD) was calculated for each individual tumor by subtracting the DT of each tumor from the mean DT of the control group and then averaged for each experimental group. Tumor growth was monitored until the tumors reached approximately 350 mm^3 in volume, at which point the animals were killed. Animals in which tumors were cured were checked for possible regrowth of tumors up to 100 days, after which the animals were killed according to standard procedures. A 100 days DT was used for calculation of average DT of the experimental group and of GD (Table 1).

Platinum Determination in Tumors

Platinum (Pt) content in SA-1 tumors was determined after intravenous injection of CDDP alone (4 mg/kg) and after electrochemotherapy with CDDP. Tumors (4–10 tumors/group) were excised at different time points after injection

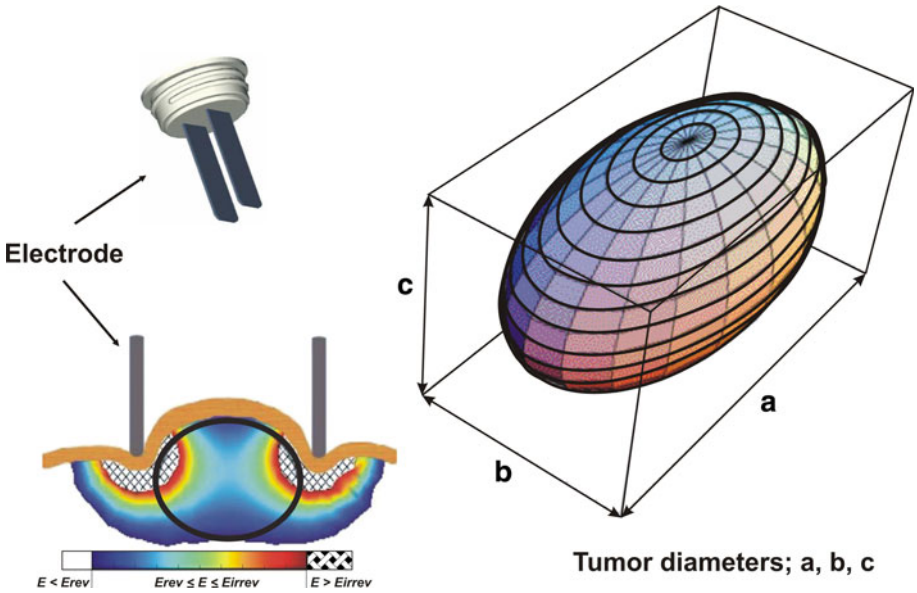

Fig. 1 Electric pulses were applied by plate electrodes with a 6-mm gap between them to the tumors. The tumor was embraced between the electrodes so that the electroporative electric field was applied to the whole tumor mass. Tumor measurements were made in three perpendicular directions: the longest (*a*) and shortest (*b*) diameters and height (*c*)

Table 1 Antitumor effectiveness of electrochemotherapy with BLM or CDDP: comparison of the effects of treatment with pulse trains at 1 Hz or 5 kHz repetition frequency

Group	*n*	DT[a] (days)	GD[b] (days)	*P*-$DT_{control}$	*P*-$DT_{1\ Hz/5\ kHz}$
Control	18	1.7 ± 0.2			
BLM[c]	12	3.4 ± 0.5	1.7	0.706	
CDDP[d]	12	2.3 ± 0.3	0.6	0.729	
EP[e], 1 Hz	20	4.4 ± 0.5	2.7	0.076	
EP, 5 kHz	20	3.0 ± 0.4	1.3	0.390	
ECT[f]-BLM, 1 Hz	17	42.3 ± 7.0	40.6	0.001	0.001
ECT-BLM, 5 kHz	18	15.5 ± 1.7	13.8	0.001	
ECT-CDDP, 1 Hz	26	13.1 ± 1.7	11.4	0.001	0.001
ECT-CDDP, 5 kHz	20	7.7 ± 0.8	6.0	0.001	

[a] Tumor doubling time for those tumors that regrew after treatment (mean ± SEM). A DT of 100 days was assigned for tumors that were cured

[b] Growth delay

[c] Bleomycin (0.5 mg/kg)

[d] Cisplatin (4 mg/kg)

[e] Electric pulses

[f] Electrochemotherapy with BLM or CDDP

of CDDP and removed from the overlying skin. Each tumor was then weighed, placed in a 15 ml graduated polyethylene tube and digested in 1 ml of 65% nitric acid by incubation at room temperature for at least 2 days to obtain a clear solution. Samples were diluted with water up to 10 ml before analysis. Pt content in the samples was determined by electrothermal atomic absorption spectrometry on a Hitachi Z-8270 Polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi, Tokyo, Japan), adjusted to a wavelength of 265.9 nm (Milacic et al. 1997).

Calculations of Pt Elimination

Pt elimination was calculated by fitting the experimental data to the equation

$$C_t = C_0\, e^{-kt}, \quad (1)$$

where C_0 is the initial quantity of Pt (mg/kg), C_t is the quantity of Pt at a given time t, k is the elimination rate constant (h^{-1}). Prism GraphPad, version 5.0b (GraphPad Software, San Diego, CA), was used.

Assessment of Tumor Perfusion by Patent Blue Staining

Tumor perfusion was assessed by the Patent blue (Byk Gulden, Kreuzlingen, Switzerland) staining technique, which was found to correlate well with the pharmacological method of measuring relative tumor blood flow (Sersa et al. 1999a, b). Patent blue (1.25%) was diluted at 1:2 with 0.9% NaCl, and 100 µl were injected intravenously at different time points after application of electric pulses to the tumors. One minute after dye injection, which allows the dye to distribute evenly throughout the tissue, the animals were killed and the tumors carefully excised. Tumors were cut in half along their largest diameter, and immediately thereafter the percentage of the stained area was visually estimated by two individuals independently in a blind fashion (Fig. 2). The percentage of the stained area of the tumor cross section was recorded as the perfused area and the nonstained area as nonperfused. The results of the two independent evaluations were then pooled for each tumor.

Statistical Analysis

All data were tested for normality of distribution. Statistical differences between the treatment groups were assessed by a t test after one-way ANOVA was performed and fulfilled. SigmaStat statistical software (Systat Software Inc., San Jose, CA) was used for statistical analysis. $P < 0.05$ was considered significant. However, exact P values are reported.

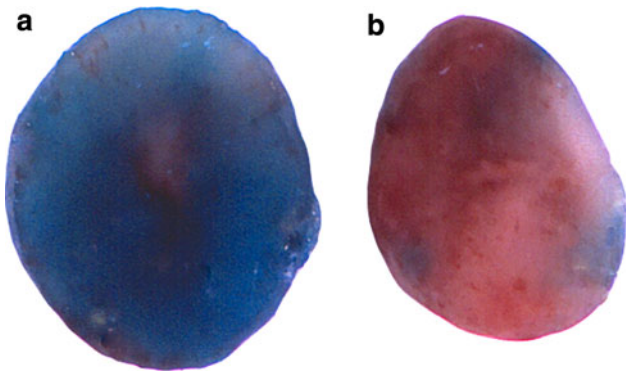

Fig. 2 Patent blue staining of tumors cut along the longest diameter. **a** Untreated tumor is stained blue, indicating good Patent blue distribution as a measure of good vascular perfusion of the tumor. **b** Tumor treated with application of electric pulses. The tumor is not stained, indicating abrogation of tumor perfusion

Results

Antitumor Effectiveness of Electrochemotherapy

Electrochemotherapy of SA-1 tumors with BLM or CDDP resulted in significant antitumor effectiveness, using either of the two electric pulse repetition frequencies (1 Hz or 5 kHz) (Table 1). Suboptimal doses of the drugs were used in order to investigate the effect of electrochemotherapy when only a small number of treated tumors were cured and the tumors regrew after a certain period of time. It was found that treatment with BLM using a drug dosage of 0.5 mg/kg resulted in better antitumor effectiveness than CDDP at 4 mg/kg.

Treatment with either BLM or CDDP was, however, more effective at an electric pulse repetition frequency of 1 Hz than 5 kHz (Table 1). Treatment with BLM at 1 Hz resulted in a longer tumor GD (40.6 days). Among these, there were three tumor cures (DT 100 days) compared to a 5 kHz electric pulse train (13.8 days). Similarly, CDDP and a 1 Hz train resulted in significantly longer tumor GD (11.4 days) than a train of 5 kHz (6.0 days) and no tumor cures (Fig. 3).

Application of electric pulses alone to the tumors, as well as BLM or CDDP treatment alone, did not affect tumor growth significantly (Table 1).

Pt Content in Tumors

The amount of CDDP accumulated in the tumors was measured by the Pt content in the tumors using electrothermal atomic absorption spectrometry at different times after CDDP injection alone and after pulse train with CDDP. The amount of CDDP decreased with time in all cases (Fig. 4). Four minutes after CDDP injection in

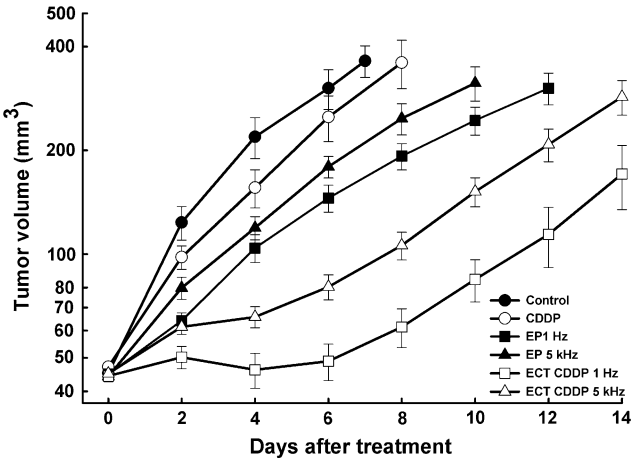

Fig. 3 Growth curves of tumors exposed to treatment with CDDP, trains of electric pulses (*EP*) and the combination of both (*ECT* electrochemotherapy)

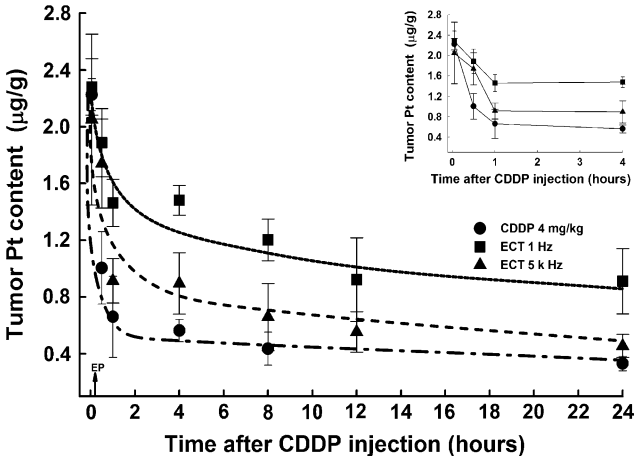

Fig. 4 Kinetics of CDDP elimination measured as Pt content by atomic absorption spectrometry, as a function of time (h), after injection of CDDP at $t_0 = 0$. Electric pulses were applied at $t_1 = 3$ min and the first measurements of Pt content in tumors were done at $t_2 = 4$ min. Pt content was measured in tumors treated with CDDP and in electrochemotherapy-treated tumors using electric pulse repetition frequencies of either 1 Hz or 5 kHz. Each point is mean ± SE of four to 10 measurements

tumors, the initial Pt content was 2.22 ± 0.11 μg Pt/g, and this was the same as in tumors that were exposed to electroporative pulses of 1 Hz (2.28 ± 0.20 μg Pt/g) or 5 kHz (2.05 ± 0.60 μg Pt/g). At 24 h after CDDP injection, Pt content in the tumors was 0.33 ± 0.05 μg Pt/g.

Analysis of kinetic data shown in Fig. 4 reveals that Pt was removed from the tumor by a one-phase elimination process according to Eq. 1 with an elimination rate constant (k) of 2.52 ± 0.32 h^{-1} and a half-time ($t_{1/2}$) of 0.27 h. Treatment with electric pulses prolonged the $t_{1/2}$ of Pt from 0.27 h (no treatment with electric pulses) to 0.59 h (1 Hz) and 0.53 h (5 kHz) since the elimination rate constants (k) decreased from 2.52 ± 0.32 h^{-1} (CDDP) to 1.17 ± 0.47 h^{-1} (1 Hz) and 1.30 ± 0.40 h^{-1} (5 kHz), thus indicating that elimination was faster after 5 kHz pulses than after 1 Hz. This decrease in k values was not statistically significant. Regardless of this, the concentrations of Pt remaining within the tumors after 24 h were significantly higher when electroporation treatment was used. The 1 Hz treatment was shown to be the most effective, leaving 0.91 ± 0.23 μg Pt/g within the tumor, which was statistically significant ($P < 0.05$), followed by 5 kHz, which resulted in 0.45 ± 0.08 μg/g of Pt within the tumor, thus indicating the lower permeabilizing potential of the 5 kHz pulse train.

Tumor Perfusion

Time-dependent tumor perfusion changes after application of electric pulses alone to the tumors were determined by Patent blue staining (Fig. 5). Untreated tumors were well perfused; the fraction of stained tumor area (0.90 ± 0.60) was constant with time. Immediately after treatment with pulse trains of either 1 Hz or 5 kHz, tumor perfusion was almost completely reduced. However, perfusion started to recover very quickly thereafter, being approximately on the same level as before the application of trains of pulses at 24 h of 0.67 ± 0.09 at 1 Hz and 0.77 ± 0.07 at 5 kHz. The recovery of tumor perfusion was faster after treatment with the 5 kHz train of pulses than with 1 Hz. These data indicate that the higher frequency of pulse trains has less effect on tumor perfusion.

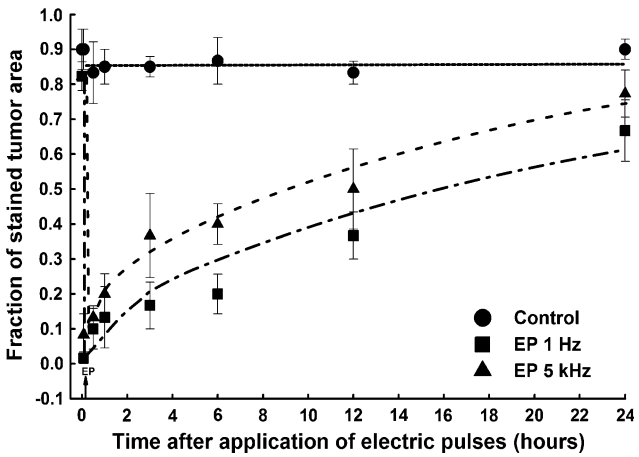

Fig. 5 Kinetics of tumor perfusion in SA-1 tumors measured by fraction of Patent blue staining area after application of either 1 Hz or 5 kHz electric pulse repetition frequency (*EP*) to the tumors alone. No statistical difference was observed between the effects of 1 Hz and 5 kHz. Each point is mean ± SE of four to seven measurements

Discussion

This study shows that electrochemotherapy with an electric pulse repetition frequency of 5 kHz is an effective treatment; however, its effectiveness is lower than that with a 1 Hz electric pulse repetition frequency when suboptimal doses of BLM or CDDP are used. These results on antitumor effectiveness are also supported by the differences in Pt content in the tumors and tumor perfusion. Both parameters were less affected after electrochemotherapy with a 5 kHz electric pulse repetition frequency.

Most of the studies on electrochemotherapy performed so far, with either BLM or CDDP, have used a "standard" set of electrical parameters—amplitude-over-distance ratio 1,300 V/cm, eight electric pulses and pulse duration of 100 μs, with a repetition frequency 1 Hz. This set of electrical parameters was based on preclinical studies elaborating the effect of amplitude and the number of electric pulses applied (Mir et al. 1991; Sersa et al. 1995; Miklavcic et al. 1998; Satkauskas et al. 2005). Based on the fact that this set of electrical parameters has good antitumor effectiveness, there was no need to further elaborate the electrical parameters for optimization of electrochemotherapy, until the idea emerged to shorten the duration of the electric pulses applied and, hence, reduce the sensation of pain by delivering the electric pulses at a higher repetition frequency (Zupanic et al. 2007).

A few in vitro studies have indicated that uptake of molecules into cells by electroporation is possible at increased electric pulse repetition frequencies (from 100 Hz to 8 kHz); however, uptake was lower than at a 1 Hz repetition frequency (Belehradek et al. 1994; Vernhes et al. 1999; Macek Lebar et al. 2002; Pucihar et al. 2002). A higher electric pulse repetition frequency (6 Hz) has been used in in vivo studies, using multiple needle electrodes in order to cover a larger tumor area (Gilbert et al. 1997; Ramirez et al. 1998; Pucihar et al. 2002). A study on rats measuring muscle torque has shown that by increasing the electric pulse repetition frequency above the frequency of tetanic muscle contraction (>100 Hz), the number of individual contractions was reduced to a single muscle contraction (Pucihar et al. 2002; Miklavcic et al. 2005). Furthermore, a preliminary study on an SA-1 fibrosarcoma tumor model showed that the in vivo antitumor efficacy of electrochemotherapy with BLM at higher electric pulse repetition frequencies (10 Hz, 100 Hz, 1 kHz, 5 kHz) is evident but comparable to that at 1 Hz (Miklavcic et al. 2005). Although the data in that study were preliminary, it was demonstrated that the use of a 5 kHz electric pulse repetition frequency resulted in a lower percentage of tumor cures (22.2%) than at a repetition frequency of 1 Hz (36.8%). It is also evident in our present study that electrochemotherapy with either BLM or CDDP is effective using a higher electric pulse repetition frequency (5 kHz); however, its effectiveness is lower than at 1 Hz repetition frequency. Furthermore, after electrochemotherapy with BLM, some tumor cures were obtained at a 1 Hz electric pulse repetition frequency, whereas there were no tumor cures at 5 kHz.

Electroporation of tumors increases permeability of the cell membrane, thus increasing drug uptake and consequently cytotoxicity, which is the major determinant of the antitumor effectiveness of electrochemotherapy. As has already been shown, electroporation of tumors increases BLM or CDDP accumulation in tumors (Belehradek et al. 1994; Cemazar et al. 1998, 1999; Engstrom et al. 1999). In our study, we obtained similar results to prior published studies on Pt content of tumors after treatment with CDDP alone or after electrochemotherapy with CDDP (Cemazar et al. 1999). The present results demonstrate that electrochemotherapy with a 5 kHz electric pulse repetition frequency increases Pt content in tumors, though to a lesser degree than with a 1 Hz electric pulse repetition frequency. The reason could be that with a higher electric pulse repetition frequency (5 kHz) less electropermeabilization of the cell membrane is obtained, which is reflected in lower drug accumulation in the tumor (Macek Lebar et al. 2002; Pucihar et al. 2002). Our data indicate that immediately after application of electric pulses to the tumors, a relatively high drug accumulation is obtained, presumably by drug entrapment in the tumor; nevertheless, after 60 min there is increased washout of the drug. This could be due to fact that the drug had not accumulated in the cells but, rather, in the interstitial compartment, as suggested by the rapid washout afterward. Therefore, the higher electric pulse repetition frequency at 5 kHz did not induce electropermeabilization of the cells to the same extent as an electric pulse repetition frequency of 1 Hz, which is supported by our in vivo data on the antitumor effectiveness of electrochemotherapy, in which the higher electric pulse repetition frequency was less effective than the lower. In addition, our data show that after the initial rapid reduction of tumor perfusion, as already reported for the 1 Hz repetition frequency, restoration was faster in tumors exposed to electric pulses at a 5 kHz repetition frequency (Sersa et al. 1999a, b, 2008a, b; Gehl et al. 2002). This is in accordance with an increased washout of CDDP from tumors treated with electrochemotherapy at 5 kHz as the entrapment of drug in the tumor is of shorter duration due to the quicker restoration of tumor perfusion. The drug accumulation and consequently antitumor effectiveness of electrochemotherapy at a higher electric pulse repetition frequency could, however, be increased by modification of other electrical parameters that are known to increase permeabilization of the cells, either with an increased number or

a longer duration of electric pulses, or by an increase in drug dosage.

In the ESOPE clinical study, a 5 kHz electric pulse repetition frequency was used based on preliminary data published on the same tumor model as in this study, demonstrating that a high electric pulse repetition frequency has a comparable effect to a low electric pulse repetition frequency in electrochemotherapy (Marty et al. 2006). However, the clinical study of different types of tumors, with either BLM or CDDP, given either locally or systemically, demonstrated no difference in the antitumor effectiveness of electrochemotherapy at either 1 Hz or at 5 kHz. The same percentage of objective responses was obtained in the treated tumors regardless of the electric pulse repetition frequency used. This fact is difficult to explain, but we can presume that in spite of lower cell membrane permeabilization, the concentration of chemotherapeutic drugs was high enough in the tumor cells to exert their cytotoxic action. This was a consequence of the optimal doses of chemotherapeutic drugs used in the clinical study compared to this study, where suboptimal doses were selected to evaluate the effect of different electric pulse repetition frequencies on the antitumor effectiveness of electrochemotherapy.

In conclusion, this study demonstrates that electrochemotherapy using a higher electric pulse repetition frequency (5 kHz) is an effective treatment; however, in the SA-1 tumor model it is less effective than electrochemotherapy at 1 Hz when using suboptimal BLM or CDDP doses. On the other hand, electrochemotherapy with a higher electric pulse repetition frequency has certain advantages over the use of a 1 Hz electric pulse repetition frequency—namely, the shorter duration of electroporation, the sensation of only one application of electric pulses and the important feature that muscle contraction is obtained only after the electric pulses have already been delivered (Zupanic et al. 2007), which prevents electrode displacement due to muscle contraction during pulse delivery. Specifically, these properties of 5 kHz electric pulse repetition frequency are beneficial for patients because less pain is associated with electric pulse delivery. Therefore, when the optimal drug dose is used in electrochemotherapy, a higher pulse repetition frequency can be securely used. This treatment leads to equal antitumor effectiveness as lower pulse repetition frequency, as demonstrated in the ESOPE clinical study (Marty et al. 2006).

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