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Electrochemotherapy of tumours resistant to cisplatin: a study in a murine tumour model

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Abstract

The aim of the study was to determine whether electrochemotherapy with cisplatin could be implemented in treatment of cisplatin-resistant solid tumours. For this purpose, we used cisplatin-sensitive TBL.Cl2 cells and their cisplatin-resistant subclone TBL.Cl2 Pt, which can be grown as *in vitro* cell cultures and as solid subcutaneous tumours in C57Bl/6 mice. Cytotoxicity of cisplatin alone and combined with electroporation was determined by colony forming assay. Treatment effects of electrochemotherapy *in vivo* were assessed by tumour growth delay and tumour curability. Platinum content in the cells and tumours was determined by atomic absorption spectroscopy. *In vitro,* TBL.Cl2 Pt cells were equally sensitive to electrochemotherapy as their cisplatin-sensitive counterparts. *In vivo,* electrochemotherapy was effective on both tumour types, resulting in a prolonged tumour growth delay and tumour cures. However, electrochemotherapy was more effective on parental than cisplatin-resistant tumours, in which platinum content was significantly lower compared with parental tumours. In conclusion, electrochemotherapy is an effective treatment of cisplatin-resistant solid tumours and may prove useful in clinical chemotherapy for the treatment of tumours with intrinsic or acquired resistance to cisplatin.

Keywords: Electroporation; Cisplatin; Drug resistance; Sarcoma; Mice

1. Introduction

The use of electroporation to enhance the delivery of chemotherapeutic agents and DNA into cells *in vivo* has gained a lot of attention during the past few years. Electroporation is a physical method that is performed by application of high voltage direct current electric pulses to the cells *in vitro* or tissues *in vivo* [1]. It causes non-selective plasma membrane permeabilisation by induction of structural changes in the plasma membrane, the precise nature of which has yet to be defined. Electroporation combined with the chemotherapeutic drugs bleomycin and cisplatin (electrochemotherapy) is a very effective therapy and has proved its antitumour efficiency on cells *in vitro*, experimental tumours *in vivo* and in treatment of cutaneous tumour nodules in cancer patients [1–3].

Cisplatin is a chemotherapeutic drug active against a wide spectrum of tumours [4]. Platinum-based chemotherapy is a curative therapy for most testicular cancer patients and cisplatin is a component of chemotherapy schedules for ovarian, head and neck, cervical, bladder and small-cell and non-small cell lung cancers. However, the tumours can become refractory to chemotherapy after the initial response and some of the cancer types such as colorectal and pancreatic cancer show minimal responsiveness to cisplatin [5,6]. Therefore, the presence or acquisition of resistance to cisplatin represents an obstacle in chemotherapy treatments for cancer. Most of the knowledge about resistance to cisplatin came from preclinical studies. Several cisplatin-resistant cell lines and tumours were developed in order to study which mechanisms were responsible for the observed resistance of tumours to cisplatin. So far,

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several mechanisms have been reported to be responsible for the resistance of tumour 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 capacity for DNA repair or increased ability to tolerate DNA damage with a concomitant failure to trigger apoptosis [7–10]. Discovery of the resistance mechanisms initiated several studies testing different approaches in order to overcome this resistance. These approaches included modulations of plasma membrane permeability, of thiol content, of chromatin conformation, as well as use of platinum-based compounds and DNA repair inhibitors [11–20]. In our previous study on cells *in vitro,* we tested whether electroporation could be used to overcome cisplatin resistance. We showed that electroporation potentiates cisplatin cytotoxicity in cisplatin-resistant IGROV 1/CDDP cells, despite the fact that these cells are resistant to cisplatin due to several mechanisms of resistance not confined to the plasma membrane [21]. In addition, platinum accumulation and the enhancement factor for cell killing was increased in cisplatin-resistant cells RIF/Ptr1 compared with the parental cisplatin-sensitive RIF cells after *in vitro* electroporation using exponential decaying electric pulses [22].

The aim of this study was to further study the use of electrochemotherapy with cisplatin in the treatment of cisplatin-resistant solid tumours. For this purpose, we used cisplatin-sensitive TBL.Cl2 cells and their cisplatin-resistant subclone TBL.Cl2 Pt, which can be grown as *in vitro* cell cultures and as solid subcutaneous (s.c.) tumours in mice. Survival of cells continuously exposed to cisplatin and treated by electrochemotherapy *in vitro* was measured by means of clonogenic assay and the response of tumours *in vivo* by means of growth delay assay. In addition, platinum content was measured in cells and tumours after all treatments to elucidate, in part, the underlying mechanisms involved in treatment-response.

2. Materials and methods

2.1. Chemicals

cis-Diamminedichloroplatinum (II) (cisplatin; Platinol, Bristol-Myers Squibb, Austria) was dissolved in sterile water at a concentration of 4 mg/ml. Further dilutions were prepared in Eagle's Essential minimum medium (EMEM; Sigma, USA) for *in vitro* and in 0.9% (w/v) NaCl for *in vivo* experiments.

2.2. Mice

In the experiments, the inbred strain of C57Bl/6 mice was used, purchased from the University of Ljubljana Medical Faculty (Ljubljana, Slovenia). They were kept at a constant room temperature (22°C) with a natural day/night light cycle in a conventional animal colony. Before the experiments, the mice were subjected to an adaptation period of at least 14 days. Mice of both sexes in good condition, without fungal or other infections, weighing 20–25 g and 10–12 weeks of age, were included in experiments.

2.3. Cells and tumours

In the study, murine sarcoma TBL.Cl2 cells and their resistant subclone TBL.Cl2.Pt were used [23]. The mechanisms of resistance in the TBL.Cl2.Pt cells have not yet been determined. The cells were grown as a monolayer in a humidified incubator at 37°C and 5% CO_2 in EMEM, supplemented with 10% fetal calf serum (FCS, Sigma, USA). Solid s.c. tumours, located dorsolaterally in mice, were initiated by an injection of 1×10^6 TBL.Cl2 or TBL Cl.2 Pt cells in 0.1 ml EMEM supplemented with 2% FCS prepared from cell culture *in vitro*. The viability of the cells was over 95% as determined by the Trypan blue dye exclusion test. Ten to 14 days after implantation when the tumours reached approximately 40 mm^3 in volume, mice were randomly divided into experimental groups and subjected to a specific experimental protocol. The experiments were performed twice.

2.4. Cytotoxicity assay for continuous exposure of cells to cisplatin and electrochemotherapy in vitro

To determine the sensitivity of TBL.Cl2 and TBL.Cl2.Pt cells to continuous exposure to cisplatin, the cells (300–5000 cells per petri dish) were plated in 60 mm diameter petri dishes (Costar, Badhoevedorp, The Netherlands) in 4 ml of EMEM containing different cisplatin concentrations ranging from 0.05 to 1.6 μg/ml. The sensitivity of the cells to combined treatment with cisplatin and electric pulses (electrochemotherapy) was determined as previously described [24]. Briefly, cells were prepared from the exponential growth phase, trypsinised and washed twice at 4°C, first in the EMEM supplemented with 10% FCS for inactivation of trypsin (Sigma, USA), and then in the serum-free medium. Cell suspension (2.2×10^7 cells/ml in 90 μl) was mixed with 10 μl of different cisplatin stock solutions ranging from 8–4000 μg/ml). One half of this mixture was placed between two flat parallel stainless-steel electrodes (length 6 mm, width 6 mm, distance 2 mm) and subjected to eight square-wave electric pulses (pulse width 100 μs, repetition frequency 1 Hz, electric field intensity 1000 V/cm, distance between the electrodes 2 mm) and the other half served as a control for cisplatin treatment alone. After exposure of cells to electric pulses, the cells were incubated for 5 min at room temperature (22°C)

and then seeded in 60 mm diameter petri dishes. After 14 days, the colonies were fixed, stained with Crystal violet (Sigma) and counted. The colonies containing less than 50 cells were disregarded. The survival of cells treated with electrochemotherapy was normalised to electric pulses treatment alone (surviving fraction (Sf) = 0.8 ± 0.05). The results were reported by means of IC_{50} value: the cisplatin concentration that causes a 50% inhibition of colony formation. All experiments were performed in triplicate and repeated three times.

2.5. *Electrochemotherapy protocol* in vivo

Cisplatin was injected intravenously (i.v.) into the lateral tail vein. Injection volume was 0.02 ml/g body weight. Eight square-wave electric pulses of 1040 V amplitude (amplitude/distance ratio: 1300 V/cm), with a pulse width of 100 μs and repetition frequency 1 Hz were delivered by two flat, parallel stainless steel electrodes 8 mm apart (two stainless steel strips: length 35 mm, width 7 mm with rounded corners) which were placed percutaneously at the opposite margins of the tumour. Good contact between the electrodes and the skin was assured by means of conductive gel (Parker Laboratories Inc, NY, USA). Electric pulses were generated by an electropulsator Jouan GHT 1287 (Saint Herblaine, France). In the electrochemotherapy protocol, mice were treated with electric pulses 3 min after cisplatin injection. All treatments were well tolerated by the animals.

2.6. *Assessment of response*

Tumour growth was followed by measuring three mutually orthogonal tumour diameters (e_1, e_2 and e_3) with a vernier calliper every day. Tumour volumes were calculated by the formula $V = \Pi \times e_1 \times e_2 \times e_3/6$. Tumour volume doubling time was determined from the growth curve of individual tumours. Tumour growth delay was calculated from the mean tumour volume doubling times of each treatment group compared with the mean tumour volume doubling time of the control, untreated tumours ($GD = DT_{\text{treated group}} - DT_{\text{control group}}$).

2.7. *Platinum determination in cells and tumours*

To determine the platinum content in the cells, cells were treated in the same manner as described above for cytotoxicity assay. At the end of the treatment, cells were centrifuged and washed with EMEM to remove the traces of cisplatin and centrifuged again. The pellet was resuspended in 200 μl of 65% (w/w) nitric acid. To determine platinum content in the tumours, mice were sacrificed 1 h after treatment with cisplatin alone or after electrochemotherapy. Tumours (six per group) were excised and removed from the overlying skin. Each tumour was then weighed (tumour weights were approximately 100 mg), placed into a 15 ml graduated polyethylene tube and digested in 1 ml of 65% nitric acid by incubation at 37°C for at least 2 days to obtain a clear solution. Samples were diluted with ultra pure water (1:10) before analysis. Platinum content in the samples was determined by flameless atomic absorption spectroscopy on a Perkin-Elmer Analyst 300 Atomic Absorption Spectrophotometer, adjusted to a wavelength of 265.9 nm.

2.8. *Statistical analysis*

Data were tested for normality of distribution using the Kolmogorov–Smirnov normality test. The significance of differences between the mean values of tumour growth delays and IC_{50} values of the experimental groups as well as platinum concentration in cells *in vitro* treated with IC_{50} cisplatin concentration and in tumours *in vivo* was evaluated by a modified *t*-test (Bonferroni test) after a one way analysis of variance was performed and fulfilled. The significance of difference between platinum concentrations in cells after *in vitro* exposure to cisplatin alone or electrochemotherapy was determined by Dunn's method after Kruskal–Wallis Anova on ranks, since these data were not normally distributed. The data were analysed using SigmaStat statistical software (SPSS Inc., USA). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Cell survival and platinum content after continuous exposure of cells to cisplatin and electrochemotherapy* in vitro

To determine the sensitivity of TBL.Cl2 and TBL.Cl2 Pt cells to cisplatin alone, cells were continuously exposed to the drug and cell survival determined (Fig. 1). The IC_{50} value for TBL.Cl2 cells was 0.05 μg/ml of cisplatin and for the resistant subclone 0.46 μg/ml. Thus, TBL.Cl2 Pt cells exhibited an approximately 9-fold resistance to cisplatin compared with TBL.Cl2 cells. The measurement of platinum content in both cell lines at IC_{50} concentrations 1 and 4 h after the beginning of incubation (and subsequent statistical analysis) revealed that the platinum content in the cells was equal indicating that the same amount of cisplatin is needed to produce the same amount of cell killing (Fig. 2).

In *in vitro* electrochemotherapy experiments, the cells were exposed to cisplatin for only 5 min. The IC_{50} value for TBL.Cl2 cells treated with cisplatin alone for 5 min was 60 μg/ml and for TBL.Cl2 Pt 300 μg/ml (Fig. 3). Exposure of both cell lines to electric pulses combined with cisplatin resulted in non-significantly different IC_{50}

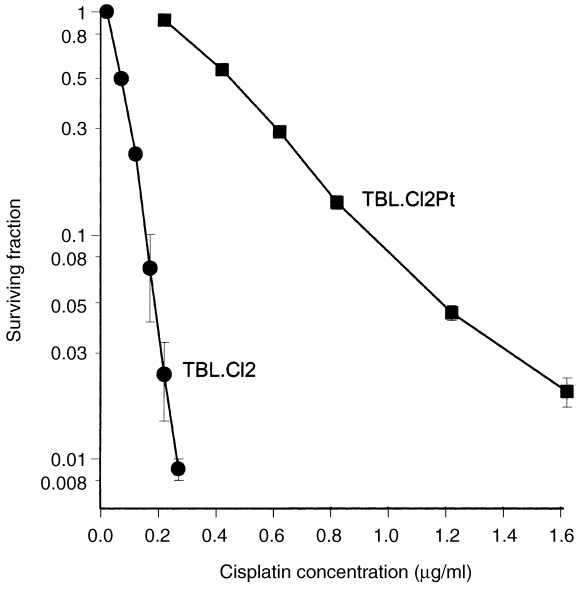

Fig. 1. Cell survival after continuous exposure of TBL.Cl2 and TBL.Cl2 Pt cells to cisplatin. Data are means ±1 standard error of the mean pooled from three independent experiments. The error bars in the TBL.Cl2.Pt survival curve do not exceed size of the symbol.

values (2.5 μg/ml for parental and 3.8 μg/ml for cisplatin-resistant cells), suggesting that the membrane restriction might be the major obstacle for cisplatin cytotoxicity in these particular cell lines. Furthermore, the measurement of platinum content in these cells after *in vitro* electrochemotherapy with different concentrations of cisplatin demonstrated that there was no statistically significant difference between platinum content in parental TBL.Cl2 compared with the resistant TBL.Cl2 Pt cell line (Fig. 4). In addition, there was no difference in the platinum content in both cell lines treated with cisplatin alone for 5 min, which is in agreement with the survival curves showing no difference in the

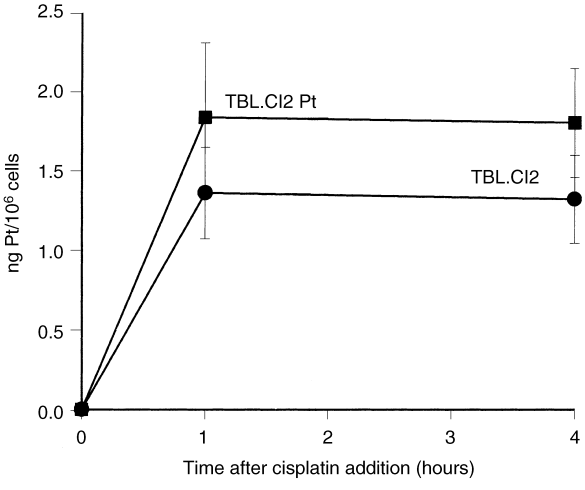

Fig. 2. Platinum content in the parental TBL.Cl2 and cisplatin-resistant TBL.Cl2 Pt cells after exposure of cells to equitoxic (IC_{50}) doses of cisplatin. Data are means ±1 standard error of the mean pooled from three independent experiments.

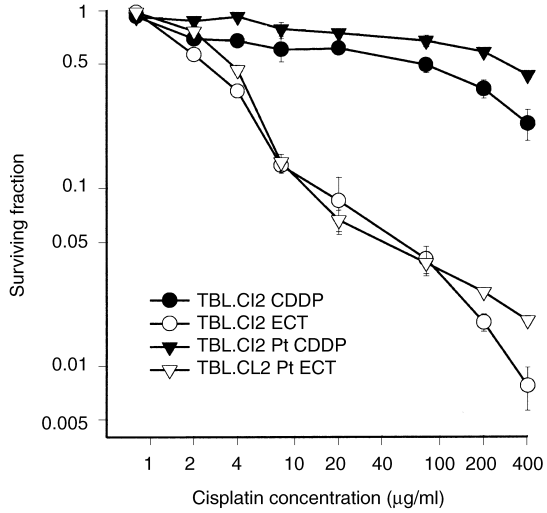

Fig. 3. Cell survival of TBL.Cl2 and TBL.Cl2 Pt cells after treatment with cisplatin (CDDP) or electrochemotherapy with cisplatin (ECT). Data are means ±1 standard error of the mean pooled from three independent experiments.

survival of the two cells lines at this very short incubation time.

3.2. *Tumour response and platinum content after treatment with electrochemotherapy* in vivo

Treatment of animals with cisplatin as a single treatment was ineffective up to the 6 mg/kg in both the parental and cisplatin-resistant tumours. (Table 1, Fig. 5a and b). Only treatment of parental TBL.Cl2 tumours with the highest dose tested (8 mg/kg) produced a significant delay in tumour growth compared with the control untreated tumours (Table 1, Fig. 5a). Electric

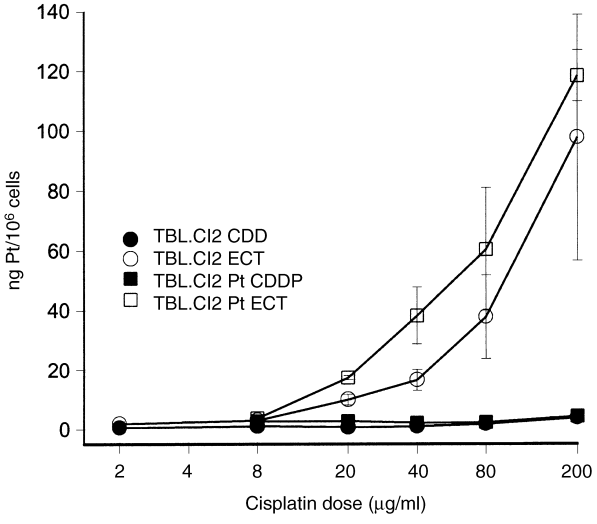

Fig. 4. Platinum content in the parental TBL.Cl2 and cisplatin-resistant TBL.Cl2 Pt cells after exposure of cell to cisplatin (CDDP) alone and electrochemotherapy (ECT) using different cisplatin doses. Cells were exposed to cisplatin for 5 min. Data are means ±1 standard error of the mean pooled from three independent experiments.

Table 1
Antitumour effectiveness of electrochemotherapy (ECT) with cisplatin (CDDP) in TBL.Cl2 and TBL.Cl2 Pt tumours[a]

Group	TBL Cl.2				TBL Cl.2 Pt			
	n	DT (days) (AM±SEM)	GD (days)	CR (%)	*n*	DT (days) (AM±SEM)	GD (days)	CR (%)
Control	19	3.82±0.39			14	3.96±0.33		
Electric pulses	15	4.60±0.54	0.78		12	5.44±0.75	1.48	
CDDP 1 mg/kg	12	3.61±0.26	−0.21		9	4.06±0.28	0.10	
CDDP 4 mg/kg	12	3.73±0.22	−0.09		8	4.51±0.41	0.55	
CDDP 6 mg/kg	14	3.80±0.25	−0.02		11	5.68±0.49	1.72	
CDDP 8 mg/kg	14	5.14±0.24*	1.32		11	5.41±0.48	1.45	
ECT 1 mg/kg	21	3.83±0.26	0.01		17	5.46±0.40	1.50	
ECT 4 mg/kg	21	12.66±1.89*	8.84	4 (19)	19	12.16±1.35*	8.20	
ECT 6 mg/kg	21	17.38±2.25*	13.56	5 (24)	18	18.34±1.03*	14.38	
ECT 8 mg/kg	20	18.99±1.54*	15.17	17 (85)	17	23.46±1.40*	19.50	1 (6)

DT, doubling time; GD, growth delay; CR, complete response; SEM, standard error of the mean; AM, mean.
[a] *$P<0.05$.

pulses as a single treatment did not result in significantly prolonged tumour growth delay (Table 1, Fig. 5a and b). The application of electric pulses had no side-effects and no treatment-related mortality was observed (data not shown). Electrochemotherapy, combining intravenously (i.v.) injected cisplatin with an application of electric pulses to the tumour 3 min after the injection had a dose-dependent effect on both the parental and cisplatin-resistant tumours (Table 1, Fig. 5a and b). The antitumour effectiveness of electrochemotherapy with 1 mg/kg did not result in increased tumour growth delay, while higher doses showed a significant antitumour effect. Tumours treated with electrochemotherapy were not exulcerated and had no superficial scabs. Their growth was significantly delayed compared with control tumours or those treated with cisplatin and electric pulses as a single treatment. The tumour growth delay was similar for both parental and resistant tumours. However, electrochemotherapy of parental cisplatin-sensitive tumours resulted in 19% cured animals at the 4 mg/kg cisplatin dose and at 8 mg/kg 85% curability rate was achieved. In contrast, electrochemotherapy was not so effective in cisplatin-resistant tumours in terms of the curability rate, since only 6% of tumours (one mouse) were cured after electrochemotherapy with the highest cisplatin dose (8 mg/kg).

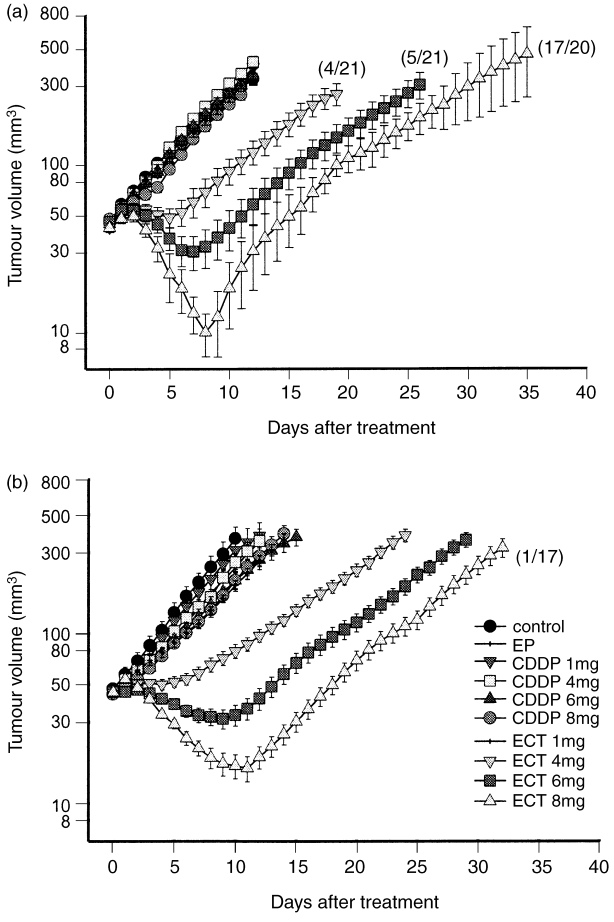

Fig. 5. Tumour growth curves of subcutaneous (s.c.) TBL.Cl2 (a) and TBL.Cl2 Pt (b) tumours treated with cisplatin (CDDP), electric pulses (EP) and electrochemotherapy (ECT). Data are means 1 standard error of the mean pooled from the two independent experiments. For the number of animals used, see Table 1. Numbers in parentheses mean number of cured animals/number of animals in the group.

To determine whether both tumours have the same platinum content following treatments, measurement of the platinum content in the tumours was performed 1 h after electrochemotherapy and cisplatin treatment with 4 mg/kg. The platinum content in the parental tumours treated by electrochemotherapy (2.67±0.13 μg/g) was almost 3 times higher than in cisplatin-treated tumours (0.98±0.1 μg/g), while in cisplatin-resistant tumours platinum content in electrochemotherapy-treated tumours (1.93±0.03 μg/g) was approximately 2 times higher compared with cisplatin-treated tumours (0.81±0.23 μg/g). Furthermore, the platinum content in the parental tumour treated with electrochemotherapy (2.67±0.13 μg/g) was significantly higher compared with cisplatin-resistant tumours (1.93±0.03 μg/g), which could explain the smaller antitumour effectiveness of electrochemotherapy observed in the cisplatin-resistant tumours.

4. Discussion

This study shows that electrochemotherapy with cisplatin can be used in the treatment of solid tumours resistant to cisplatin. The results showed that TBL.Cl2 Pt cells *in vitro* were equally sensitive to electrochemotherapy with cisplatin as their cisplatin-sensitive counterparts suggesting that for these cells the predominant mechanism of resistance might be due to the membrane restriction of cisplatin uptake. In support of this assumption were the measurements of platinum accumulation in cells after electrochemotherapy and after incubation with equitoxic doses (IC_{50}), which showed that the same amount of platinum present in the cell produced an equal cell kill. *In vivo,* on solid s.c. tumours, electroporation of tumours highly potentiated the antitumour effectiveness of i.v. injected cisplatin in both cisplatin-sensitive and cisplatin-resistant tumours, resulting in a significantly prolonged tumour growth delay for doses higher than 1 mg/kg and also tumour cures at these higher doses. However, electrochemotherapy with cisplatin was more effective on the parental TBL.Cl2 tumours resulting in an 85% cure rate at 8 mg/kg dose, while only 6% of the cisplatin-resistant TBL.Cl2 Pt tumours were cured at this dose. In contrast to the platinum measurement in cells *in vitro*, the content of platinum 1 h after electrochemotherapy was significantly higher in parental tumours compared with cisplatin-resistant tumours. This difference in platinum content in tumours most likely account for the observed difference in the antitumour effectiveness of electrochemotherapy between these two tumour types. However, the reasons for the different accumulation of cisplatin in the tumours *in vivo* remains to be elucidated. One might hypothesise that, although both tumours grow with approximately the same growth rate, other factors, such as differences in vascularisation and all other pertinent conditions in the tumours can contribute to the accessibility of cisplatin in the tumours and concomitant entrance into the cells.

Cisplatin resistance is a multifactorial phenomenon and therefore different attempts, acting at different levels of the cisplatin resistance mechanism, have been evaluated with the aim of overcoming resistance. These attempts have been made mainly using agents that increase cisplatin accumulation, deplete glutathione levels, inhibit DNA repair and modulate signal transduction pathways. Most of the studies investigating the effects of cisplatin resistance modulation at the membrane level have been done on cells *in vitro*. Jekunen and colleagues demonstrated that uptake of ^{3}H-dichloro(ethylendiamine)platinum II, an analogue of cisplatin, could be increased into human ovarian carcinoma 2008 cells by treating the cells with the plasma membrane-selective detergent digitonin [11]. In addition, stimulation of cisplatin accumulation was also achieved by treating 2008 cells and their resistant subclone C13* with the natural polycationic amine spermine [13]. Amphotericin B, an antifungal agent, increased cisplatin accumulation in human lung cancer cell lines resistant to cisplatin more than in cisplatin-sensitive cells [17]. Resistance to cisplatin at a membrane level can also be reversed by using platinum analogues. Ammine/amine platinum (IV) carboxylates were capable of circumventing acquired cisplatin resistance, which was due to decreased intracellular accumulation; however, they could not overcome resistance at the level of DNA platination and removal [18]. Several other agents, such as dipyridamole, a nucleoside membrane transport inhibitor, forskolin, an adenyl cyclase agonist, hyperthermia and thromboxane A2 receptor antagonists were reported to restore or increase cisplatin accumulation in cisplatin-resistant cell lines [12,14,16,19,20,25]. In the present study, we tested a physical approach, electroporation, which also showed the restoration of cisplatin accumulation in the cells and increased accumulation of cisplatin in the tumours, both of which resulted in increased cytotoxicity of the drug. Among the approaches used to overcome cisplatin resistance, only hyperthermia and electrochemotherapy are already in clinical use [3,20]. Electrochemotherapy with cisplatin was tested in clinical trials on several different s.c. tumours including malignant melanoma, basal cell carcinoma, squamous cell carcinoma and adenocarcinoma of ovary. Intratumoral cisplatin injection followed immediately by the application of electric pulses to the tumour nodules resulted in up to 100% complete responses 4 weeks after the therapy. In addition, in these clinical studies, we performed several electrochemotherapy sessions on the same tumour nodules of squamous cell carcinoma due to their large size, and several sessions in 1 patient presenting malignant melanoma due to the large number of tumour nodules [3,26–28]. The response to electrochemotherapy was good after each session in both cases and we did not observe the development of acquired resistance of these tumours to cisplatin.

In conclusion, electrochemotherapy highly potentiated cisplatin antitumour effectiveness in cisplatin-resistant solid tumours. This may prove useful in clinical chemotherapy for the treatment of tumours with intrinsic or acquired resistance to cisplatin. Specifically, electrochemotherapy is clinically applicable in the treatment of cutaneous and s.c. lesions of various tumour types, such as melanoma and basal cell carcinoma, recurrent head and neck squamous cell carcinoma or adenocarcinoma of the breast.

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