The Influence of Electroporation on Cytotoxicity of Anticancer Ruthenium(III) Complex KP1339 *In Vitro* and *In Vivo*

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Abstract. *In our study, the ruthenium-based anticancer agent KP1339 was tested in combination with electroporation for its cytotoxic effect on CHO and SA-1 cell lines in vitro and on SA-1 murine tumour model in vivo. Cells were treated with different doses of KP1339 for 15 or 60 min with or without electroporation in vitro. Cell viability was measured with the MTS assay. In vivo, mice bearing SA-1 tumours were treated with different doses of KP1339 with or without electroporation. Tumour growth was measured at various time points after treatment. Intratumoural ruthenium content was analysed as a measure of KP1339 accumulation to correlate it with antitumour effectiveness. Our results show that electroporation does not potentiate the cytotoxicity of KP1339 in vitro, but significantly potentiates antitumour effectiveness in vivo. Electroporation enhanced ruthenium uptake immediately after treatment, consequently causing persistently higher intratumoural ruthenium content throughout the whole observation period (48 h). In addition, ruthenium content rose continuously in electroporated and intact tumours throughout the whole observation period. The observed antitumour effectiveness is the result of both the direct cytotoxicity of KP1339 and an antivascular effect of electroporation.*

Chemotherapy is one of the standard clinical anticancer treatments. Clinically established chemotherapeutic drugs are not fully selective against cancer cells, hence they act cytotoxically on healthy cells too and, as such, cause undesired side-effects. In addition, the response rate of tumours to cytotoxic drugs is in many instances low because of the intracellular sites of action of the drugs and hindered transmembrane transport. To overcome drawbacks of chemotherapeutic drugs, new drug delivery systems are being developed which enable chemotherapeutic drug delivery into tumour cells while preserving the surrounding tissue. Several approaches for selective or local drug delivery have been developed, one of them exploiting the features of electroporation (1-4).

Electroporation is an electrical phenomenon, entailing an increase of membrane permeability when cells or vesicular membranes are exposed to an external electric field (5-8). Although the exact molecular mechanism of electroporation is yet not fully understood, the most widely accepted theory describes electroporation as a multistep process, consisting of induction, expansion, stabilization and, in cases of reversible electroporation, resealing of hydrophilic pores (9-11). All these steps depend on the electrical parameters applied, hence they must be carefully chosen in order to achieve electroporation of cell membranes and preserve cell viability at the same time. Reversible electroporation is the basis for a variety of biotechnological and medical methods, one of them being introduction of cytotoxic drugs into tumour cells. *In vitro* data have demonstrated that electroporation potentiates the cytotoxicity of a drug, when its efficacy is otherwise limited by its low ability to cross cell membranes (12). *In vivo* and clinical application of this method, termed electrochemotherapy, is performed by using local or systemic administration of a chemotherapeutic drug, such as cisplatin or bleomycin in combination with local administration of electric pulses (2-4, 13-18). In addition to electroporation of tumour cells, electrochemotherapy causes also an antivascular effect which results in drug entrapment in tumours and increased cytotoxicity (19-23).

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Key Words: KP1339, anticancer ruthenium(III) compounds, electroporation, electrochemotherapy, tumour models, sarcoma, mice.

The drug with the highest potentiation of cytotoxicity induced by electroporation tested so far is bleomycin, with a 100- to 5000-fold lower IC_{50}, *i.e.* concentration of the drug required to reduce cell viability by 50% (14, 24). Extensive *in vivo* studies on animal tumour models and in clinical trials have been performed only with two drugs, namely bleomycin and cisplatin. Both drugs are now routinely used in many cancer centres for treatment of cutaneous and subcutaneous tumours of various histologies (4, 15, 17).

Among new metal-based drugs, some ruthenium compounds have been tested for antitumour activity due to the ability of ruthenium to mimic iron in the binding to biological molecules and due to the range of oxidation states ruthenium can adopt under physiological conditions (25). A few of these compounds have shown promising results *in vitro*, and two of them, [ImH] *trans*-[RuCl4(Im)(DMSO-S)] (where Im=imidazole), NAMI-A, and [IndH] *trans*-[RuCl4(Ind)2] (where Ind=indazole), KP1019 (Figure 1), have already successfully completed phase I clinical trials as anticancer drugs (26, 27). NAMI-A shows no significant cytotoxicity *in vitro*, but surprisingly possesses selective activity against solid tumour metastases. On the basis of these discoveries, Dyson and Sava emphasize that cell viability as a first screen for anticancer drugs should be viewed with caution (28). On the other hand, the second ruthenium complex, KP1019, shows significant cytotoxicity both *in vitro* and *in vivo*, particularly in colorectal tumour cells and carcinomas in rats (29-32). As KP1019 is only sparingly soluble in water, its sodium salt KP1339 has been synthesized, possessing improved solubility, which is of crucial importance, especially when performing *in vivo* experiments (33). Studies *in vitro* showed that cytotoxicity of KP1339 is significant (30). Neither the mechanisms of action of ruthenium anticancer drugs nor their potential targets are, however, well established.

To date, potentiation of cytotoxicity by electroporation has been studied only with one ruthenium compound, namely NAMI-A. In our previous study, we demonstrated that electroporation significantly potentiates the cytotoxicity of NAMI-A *in vitro* (34); therefore, we continued our studies with another promising ruthenium drug candidate.

The aim of our study was to determine whether electroporation influences cytotoxicity of KP1339 on two cell lines (Chinese hamster ovary cells CHO and murine fibrosarcoma cells SA-1) *in vitro*, and the antitumour effectiveness of electrochemotherapy with KP1339 in the murine fibrosarcoma model SA-1 *in vivo*. In addition, the ruthenium content in tumours was determined, in order to correlate antitumour effectiveness of electrochemotherapy with increased KP1339 concentration in tumours.

Materials and Methods

Drugs. KP1339 was stored under argon atmosphere at –20°C. A saturated solution of KP1339 in 0.9% NaCl (19 mM) was prepared directly (max. 10 min) before application. To completely dissolve KP1339, the solution had to be mixed by vortex for 5-10 min. It was then filtered and different concentrations of KP1339 in 0.9% NaCl (0.2, 2, 20, 100, 200, 400 μM) were prepared. For experiments *in vivo*, different doses of KP1339 were prepared in sterile distilled water (10.5, 21.0, 42.0 mg/kg).

Cell lines. Two different cell lines, CHO (Chinese hamster ovary cells; European Collection of Cell Cultures, UK) and SA-1 (murine fibrosarcoma cells; Jackson Laboratory, Bar Harbor, ME, USA) were used in our experiments. CHO cells were grown in F12-HAM growth medium (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) with 10% foetal calf serum (FCS; Sigma), 0.5% L-glutamine (Sigma) and the antibiotics benzyl-penicillin (Crystacillin; Pliva, Zagreb, Croatia) and gentamicin sulfate (Gentamicin; Lek, Ljubljana, Slovenia). SA-1 cells were grown in Advanced Eagle's Minimum Essential Medium (Advanced MEM Reduced Serum Medium; Gibco, Grand Island, NY, USA) with 2.4% FCS (Gibco). Cells were incubated in humidified atmosphere with 5% CO_2 at 37°C. Cell suspension was prepared from cell cultures in exponential growth phase by trypsinization. From the obtained cell suspension, trypsin and growth medium were removed by centrifugation at 270 RCF and 4°C for 5 min. The cell pellet was resuspended in 0.9% NaCl to obtain a final cell density of 2×10^7 cells/ml.

Treatment protocol in vitro. Aliquots of 150 μl of a freshly prepared cell suspension (2×10^7 cells) were added to 150 μl of freshly prepared KP1339 solutions of different concentrations. The final concentrations of KP1339 solutions were 0, 0.1, 1, 10, 50, 100 and 200 μM. Cells were incubated in KP1339 solutions at room temperature. After 15 min of incubation, they were mixed by vortex and 55 μl droplets of cell suspension with different concentrations of KP1339 were placed between flat parallel stainless-steel electrodes that were 2 mm apart. A train of eight square-wave electric pulses with an amplitude of 160 V (800 V/cm), duration of 100 μs and a repetition frequency of 1 Hz was applied with a Cliniporator electroporator (Igea, Carpi, Italy). After electroporation, 50 μl droplets containing 5×10^5 cells and different concentrations of KP1339 were placed into each well of a 96-well microtitre plate (TPP, Trasadingen, Switzerland). Cells were incubated for 10 min at room temperature, allowing KP1339 molecules to pass the electroporated cell membranes. After this, 125 μl of Spinner's Minimum Essential Medium (SMEM; Sigma) were added to each well and cells were incubated at room temperature for additional 10 min for cell membranes to reseal. After incubation, 5000 cells in 200 μl of suitable growth medium were placed into each well of a 96-well microtitre plate. Cells were then incubated in humidified atmosphere with 5% CO_2 at 37°C for 48 h. The same procedure without electric pulses was used for cells exposed to different KP1339 concentrations for 15 and 60 min.

After 48 h of incubation, a cell viability test was performed using the MTS-based Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). A volume of 20 μl of reagent per well were added directly to each well. After 2 h of incubation at 37°C, the absorption at 490 nm wavelength (A_{490}) was measured with a Tecan infinite M200 spectrophotometer (Tecan, Switzerland). Cell viability (C.V.) of treated cells (tr) was calculated using the formula: $C.V.=(A_{490})_{tr}/(A_{490})_c\times100$ (%), taking the cell viability of the control (c) as 100%. The IC_{50} values were graphically determined from dose–response curves. Experiments *in vitro* were repeated three times and every time at least seven parallel measurements were made for each parameter.

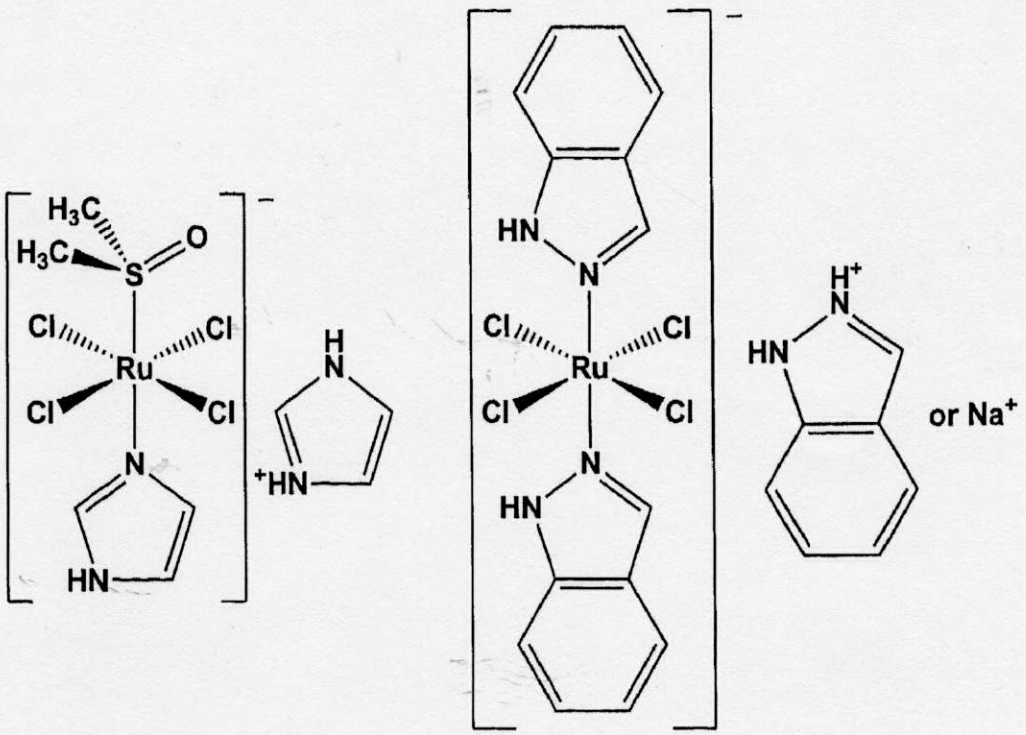

Figure 1. *Anionic part of trans-[$RuCl_4$(Im)(DMSO-S)]–(where Im=imidazole), NAMI-A, on the left and of two compounds trans-[$RuCl_4$(where Ind)$_2$]$^-$ (where Ind=indazole), KP1019 and KP1339, on the right. In NAMI-A, the corresponding cation is imidazolium [ImH], in KP1019 indazolium [IndH] and in KP1339 sodium.*

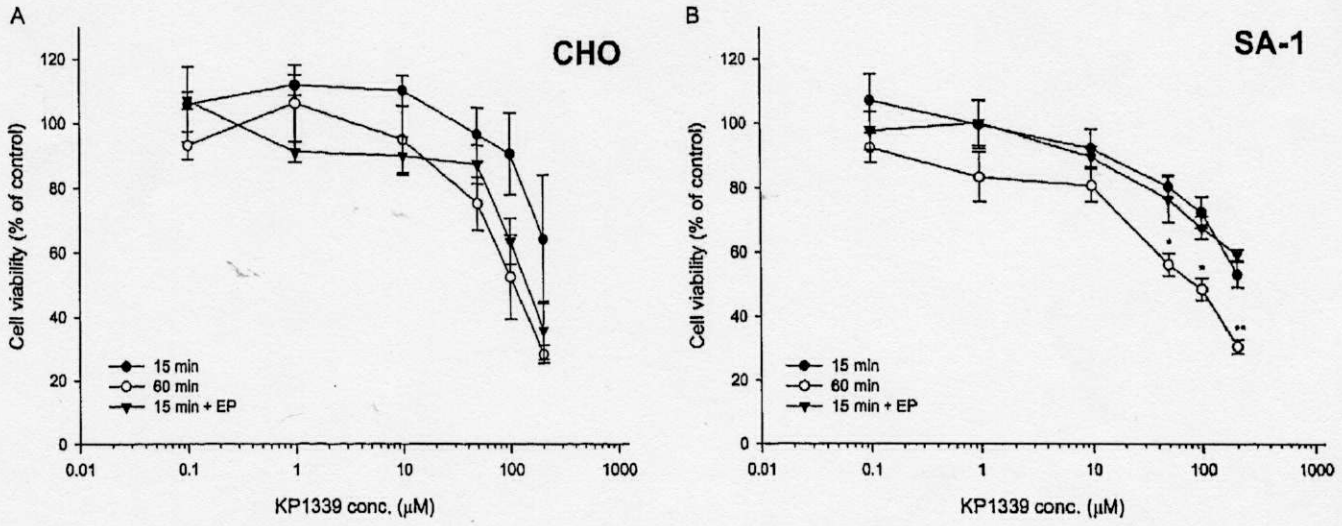

Figure 2. *KP1339 cytotoxicity towards the cell lines CHO (A) and SA-1 (B) in vitro. Dose–response curves for KP1339 treatment with exposure time of 15 min and 60 min, and for KP1339 treatment in combination with electroporation (exposure time: 15 min; electroporation parameters: 800 V/cm, 8×100 μs, 1 Hz). Data points represent the mean values±SE of three independent experiments. *p<0.05 and **p<0.001 in the case of 60 min vs. 15 min, and 60 min vs. 15 min+electroporation (EP) (one-way ANOVA).*

Animals and tumours. Inbred A/J mice were purchased from the Institute of Pathology, Faculty of Medicine, University of Ljubljana (Slovenia). Mice were kept at 21°C with a controlled 12 h light/dark cycle in a specific pathogen-free animal colony. Mice of both sexes, 12-14 weeks old, weighing 20-25 g, were included in experiments.

Solid subcutaneous tumours were induced dorsolaterally by the injection of 5×10^5 viable SA-1 cells obtained from the donor mouse, in which the SA-1 cells were grown as ascites. The tumours reached approximately 6 mm in diameter in 7-10 days, corresponding to a volume of approximately 40 mm^3. The mice were then marked,

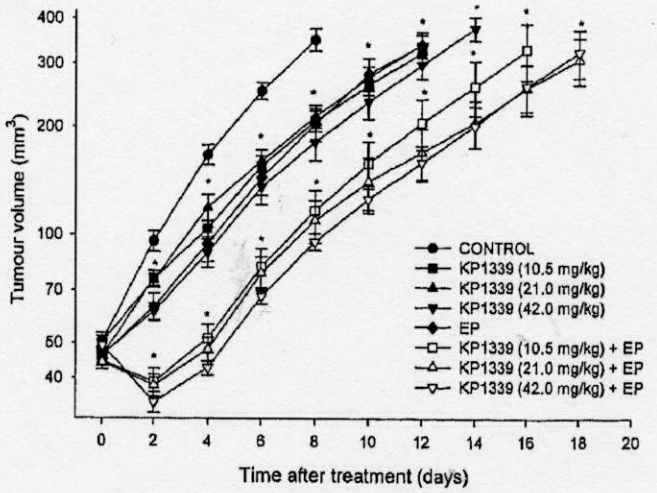

Figure 3. *The antitumour effectiveness of electrochemotherapy with KP1339 in SA-1 murine fibrosarcoma. Experimental groups consisted of 10 mice. Tumour growth curves represent the mean±SE of the tumour volumes; *p<0.05 compared to control (one-way ANOVA).*

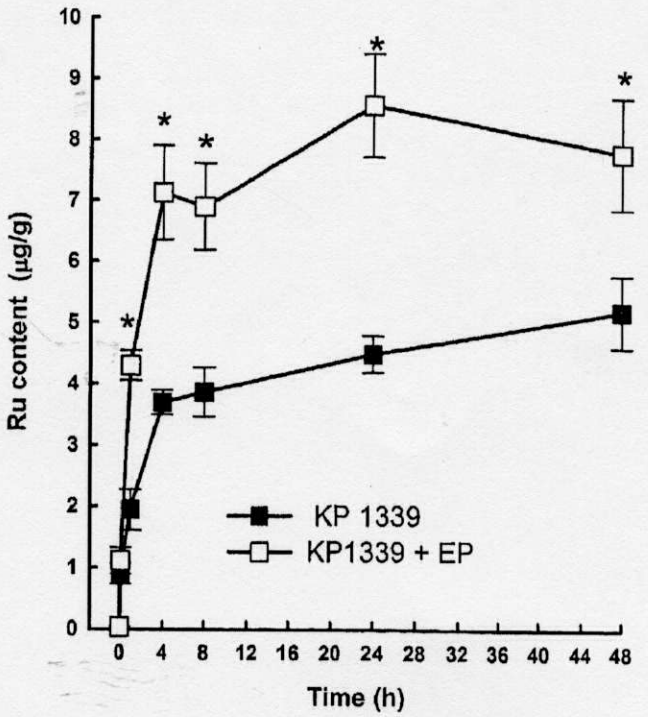

Figure 4. *Ruthenium content in electroporation (KP1339+EP) and KP1339-treated tumours. KP1339 dose used was 21.0 mg/kg. Experimental groups consisted of 6 mice. Data points represent the mean±SE of ruthenium content; *p<0.05 (one-way ANOVA).*

divided randomly into experimental groups and subjected to the specific experimental protocol.

Treatment protocol in vivo. Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission #: 34401-36/2008/6), and in compliance with the EU directive 86/609/EEC. The SA-1 tumours were treated with KP1339 injected intravenously in the orbital sinus. KP1339 was injected at doses of 10.5, 21.0, 42.0 mg/kg. The injection volume was 0.150 ml. Three minutes after injection, electric pulses were locally applied to the tumour. Electroporation of the tumours was performed by application of eight square-wave electric pulses, delivered in two sets of four pulses in perpendicular directions with an amplitude of 1040 V (1300 V/cm), duration of 100 µs and a repetition frequency of 1 Hz. The electric pulses were delivered to the tumours by two flat parallel stainless-steel electrodes (length 15 mm, width 7 mm, with rounded corners), which were placed percutaneously at opposite margins of the tumour. Interelectrode distance was 8 mm. A good contact between the electrodes and the skin was assured by means of ultrasonographic conductive gel. The electric pulses were generated by a Jouan GHT 1287 electroporator (Saint Herblain, France). All treatments were well tolerated by animals and were performed without anaesthesia.

Tumour growth was followed by measuring three mutually orthogonal tumour diameters (e_1, e_2 and e_3) with a vernier caliper, every second day. The tumour volumes were calculated by the formula $V=\pi \times e_1 \times e_2 \times e_3/6$. The arithmetic mean of the tumour volumes and the standard error of the mean (SE) were calculated for each experimental group for each measurement day. The tumour growth delay was determined for each individual tumour by subtracting the average doubling-time of the control group from the doubling-time of each individual tumour. All animals were monitored for possible systemic side-effects with physical examination every two days from the beginning of the experiment. This included monitoring each animal's body weight and evaluation of the general health status with observation of the animal's appetite, locomotion, coat and general appearance.

To determine ruthenium content in the tumours, mice were sacrificed at different time points (4 min, 1, 4, 8, 24 and 48 h) after treatment with KP1339 alone or in combination with electroporation. The dose of KP1339 was 21.0 mg/kg. Tumours were excised and removed from the overlying skin. Each tumour was then weighed into a PTFE vessel; 4 ml of 65% HNO_3 supra pure and 0.5 ml of 30% H_2O_2 supra pure were added into each vessel and samples were subjected to closed vessel microwave digestion (CEM, MARS 5, Matthews, NC, USA) at maximal power of 1200 W: ramp to temperature 20 min, 180°C, pressure 10 bar, hold 20 min, cooling 20 min. Clear solutions were transferred into 25 ml polyethylene graduated tubes and filled with water up to a mark. Ruthenium content in the samples was determined by inductively coupled plasma mass spectrometry (Agilent 7500ce; Tokyo, Japan) monitoring m 101 under optimized instrument operating conditions.

Statistical analysis. Statistical analysis was performed using one-way ANOVA test and SigmaStat statistical software (SPSS, Chicago, IL, USA).

Results

In vitro cell viability. In the first part of our study, we determined the cytotoxicity of KP1339 and the effect of electroporation on cytotoxicity of KP1339 at different concentrations *in vitro*. In addition, we determined time dependence of KP1339 cytotoxicity by measuring viability of cells exposed to different concentrations of KP1339 for 15

and 60 min. Experiments were performed on two cell lines, CHO and SA-1. Cell viability was measured by means of the MTS assay. Cell viability of each treatment was normalized to a corresponding control treated identically, but without exposure to KP1339. To fully understand our results, we performed some preliminary tests concerning the influence of 0.9% NaCl on cell viability. Viability of both cell lines prepared and incubated in 0.9% NaCl was not affected after 15 min of incubation compared to the control cells that were incubated for less than 0.5 min. Viability of CHO and SA-1 cells after electroporation in 0.9% NaCl was reduced to 87.6%±8.9 and 86.7%±2.3, respectively. Viability of CHO and SA-1 cells after incubation in 0.9% NaCl for 60 min was reduced to 81.6%±10.5 and 88.0%±9.2, respectively.

Dose-response to KP1339 treatment was determined on both cell lines. A significant decrease in viability of CHO cells was achieved above 100 μM KP1339 ($p<0.001$), while in the case of SA-1 cells, it was achieved above 50 μM KP1339 ($p<0.001$).

The effect of electroporation on KP1339 cytotoxicity and its time dependence was different on different cell lines. The IC_{50} value for CHO cells exposed to KP1339 for 15 min was approximately 200 μM. In the case of cells treated with KP1339 in combination with electroporation, the IC_{50} was lower, being approximately 130 μM. Thus, electroporation increased cytotoxicity of KP1339 1.54-fold. Exposure of cells to KP1339 for 60 min resulted in a similar IC_{50} value (~100 μM) to the combination of KP1339 with electroporation. However, statistical analysis of cell viability at any concentration showed that neither combination of KP1339 with electroporation nor exposure for 60 min caused a significant decrease of cell viability compared to cell viability after 15 min of exposure to KP1339. On the other hand, SA-1 cells were more resistant to short-term exposure to KP1339 (15 min), alone or combined with electroporation. Thus, IC_{50} values could not be determined in these cases. However, cytotoxicity of KP1339 after longer exposure time (60 min) was more pronounced, resulting in an IC_{50} value of 100 μM. At the highest concentrations tested (50, 100 and 200 μM KP1339), cell viability after 60 min exposure was significantly reduced compared to 15 min exposure at equimolar concentration ($p<0.05$). KP1339 cytotoxicity was not significantly potentiated by electroporation, but was significantly time dependent in the case of SA-1 cells (Figure 2).

In vivo antitumour effectiveness. The SA-1 murine tumour model was used to determine the effect of electroporation on KP1339 antitumour effectiveness by measuring tumour growth and intratumoural ruthenium content. The doubling time of untreated SA-1 tumours was 2.2±0.2 days. Treatment of tumours with electroporation alone resulted in non-significant 1.9 days of tumour growth delay. Tumour growth curves showed that treatment with KP1339 at different doses resulted in minimal, but dose-dependent antitumour effectiveness with tumour growth delays of 1.0, 1.5 and 2.6 days. The antitumour effectiveness of KP1339 was significantly potentiated by electroporation at all doses tested. Tumour growth was delayed for 5.2 days at the lowest dose tested (10.5 mg/kg; $p<0.05$) and for 7.3 days at the highest (42 mg/kg; $p<0.05$).

Possible systemic side-effects of experimental treatments were evaluated by following the animals' weights and their general health status. All animals were euthanized when their tumour reached approximately 350 mm^3 and no deaths from other causes occurred. Animals treated with KP1339 alone or in combination with electroporation lost up to 10% of their body weight in the first 2 days after treatment. The body weight loss was not dependent on the dose of KP1339 nor combination with electroporation. After 2 days, the animals started to regain weight, which returned to pre-treatment values after 4 days, except in the group of animals that received the highest dose of KP1339 (42.0 mg/kg) in combination with electroporation, which regained the pre-treatment level 10 days after treatment (Figure 3).

The intratumoural ruthenium content at different time points after treatment with KP1339 alone and in combination with electroporation was measured to determine if there was a correlation between intratumoural KP1339 content and tumour growth delay. Electroporation increased intratumoural ruthenium content at all time points. As early as 4 min after KP1339 injection (1 min after electroporation of tumours), the ruthenium content was determined to be 1.3 times higher in electroporated tumours. At 1 h post-treatment, this difference significantly increased to 2.2 times ($p<0.05$) and remained at this level throughout the observation period (48 h). The ruthenium content in both groups of tumours continuously rose. Electroporation enhanced ruthenium uptake immediately after treatment, consequently causing persistent higher intratumoural ruthenium content with respect to tumours treated with KP1339 only (Figure 4).

Discussion

In our present study, we demonstrate that the cytotoxicity of KP1339 is not potentiated by electroporation in either of the two cell lines CHO and SA-1 *in vitro*. However, results from experiments on murine SA-1 tumours *in vivo* demonstrate that the antitumour effectiveness of KP1339 is potentiated by electroporation of solid tumours. Furthermore, in accordance with these results, the ruthenium content in tumours was higher in tumours treated with KP1339 in combination with electroporation than in those treated with KP1339 alone. This indicates that the antitumour effectiveness of electrochemotherapy could be at least partially explained by increased KP1339 content in electroporated tumours.

In the first part of our study, the effect of electroporation on cytotoxicity of KP1339 *in vitro* was tested. Electroporation exerted no potentiation of cytotoxicity of KP1339 on CHO and

SA-1 cells *in vitro*. It was shown in previous studies that electroporation significantly potentiates the cytotoxicity of the chemotherapeutics bleomycin and cisplatin, which have intracellular target sites, on SA-1 cells *in vitro*. At the level of the IC_{50}, the cytotoxicity of bleomycin was increased 50000-fold and that of cisplatin 10-fold (20, 35). The different extent to which electroporation potentiates the cytotoxicity of KP1339 on one hand, and some chemotherapeutic drugs on the other, may be ascribed to the differences in their intrinsic cytotoxicity, *i.e.* cytotoxicity of a drug once inside the cell, and to their mode and rate of transmembrane transport. Bleomycin is intrinsically more cytotoxic than KP1339 as it has lower IC_{50} values, even though its transmembrane transport is hampered to a higher extent (24, 36). Bleomycin cannot pass cell membranes by diffusion, only by receptor-mediated endocytosis, which is relatively slow and limited by the number of receptors on the cell membranes (37). Cisplatin undergoes partly restricted transmembrane transport as only ~50% of cisplatin can cross the plasma membrane by passive diffusion (38). In the case of KP1339, it seems that free molecules can enter cells readily. As electroporation potentiates the cytotoxicity of a drug by increasing cell membrane permeability, the lack of potentiation observed in the case of KP1339 *in vitro* could be ascribed to its ability to readily pass cell membranes by itself. On the other hand, the time-dependent cytotoxicity of KP1339 *in vitro* shows that not all KP1339 molecules can diffuse through the cell membrane immediately.

In other studies, the antitumour effectiveness of other ruthenium complexes was not ascribed solely to direct cytotoxicity on tumour cells, but it was proposed that they also interact with pathways associated with the formation of metastases (39). Therefore, subsequent experiments *in vivo* were performed in our study, although preliminary *in vitro* results were not promising. We considered that a cell viability assay *in vitro* as a first screening of antitumour drugs does not always reflect the results of tests *in vivo*, as exemplified by the case of NAMI-A (28). In our experiments *in vivo*, tumour growth and intratumoural ruthenium content were measured at different time points after treatment. We demonstrated that electroporation potentiates antitumour effectiveness and increases intratumoural accumulation of KP1339 in the SA-1 murine tumour model. Growth of tumours treated with KP1339 and electroporation was delayed in comparison to growth of tumours that were treated with KP1339 alone. In the case of combined treatment, a reduction of tumour size was observed already immediately after treatment. This indicates direct cytotoxicity of KP1339 on tumour cells. The effect could be result of the increased ruthenium content in tumours immediately after electroporation. The regrowth of these tumours followed the same growth kinetics as of tumours treated with KP1339 alone, resulting in a gentle (less steep) slope of the growth curves compared to the control, indicating on multiple mechanisms involved in antitumor effectivness. Other chemotherapeutics, namely bleomycin and cisplatin, were also tested in electrochemotherapy on the SA-1 tumour model (20, 21, 35). Comparison of the antitumour effectiveness of electrochemotherapy with bleomycin, cisplatin, and KP1339 reveals a similar pattern as in the *in vitro* studies. The highest potentiation was observed for bleomycin, followed by cisplatin and KP1339.

Our measurements of intratumoural ruthenium content showed a pronounced increase in ruthenium content, and hence KP1339 in electroporated tumours compared to nonelectroporated tumours. As a higher intratumoural ruthenium content was observed immediately after electroporation, it seems that the increase is a consequence of a direct effect of electroporation on tumour cells, which causes an increased permeability of cell membranes, although this explanation is not in accordance with our results obtained in tests *in vitro*. Ruthenium content in electroporated tumours remained higher than in nonelectroporated tumours throughout the whole observation period (48 h). Intratumoural ruthenium content continuously rose throughout the whole observation period in both groups of tumours (*i.e.* electroporated and nonelectroporated), which indicates continuous accumulation of KP1339. Tissue accumulation of the complex anion of KP1339 has been proposed by Kratz *et al.* (40). Thus, results obtained with KP1339 are not in accordance with previous results of electrochemotherapy with cisplatin, where intratumoural platinum concentration decreased as soon as 1 h after electroporation to approximately 80%, and to 30% after 48 h (41). Since the cytotoxicity of KP1339 was not potentiated by electroporation *in vitro*, while significantly increased antitumor effectiveness of KP1339 and increased ruthenium content was achieved by electroporation *in vivo*, other mechanisms than those in electrochemotherapy with bleomycin and cisplatin seem to be involved.

We tried to explain our results on the basis of known biochemical features and the mode of action of the anionic part of KP1339, which differ greatly from those of cisplatin and bleomycin, and on the basis of electroporation effects. We took into consideration the following critical aspects: binding kinetics of a drug to serum proteins, reversibility of binding, biological activity of the released drug, as well as cell membrane permeabilization and antivascular effect achieved by electroporation. All mentioned aspects influence bioavailability of the drug and consequently its cytotoxicity.

The binding kinetics of a drug to serum proteins, which is common to metal-based anticancer drugs, determines the amount of free drug which is active and capable of diffusing through cell membranes. Electroporation under conditions used in our experiment enables increased diffusion of the free drug molecules, but not of proteins. As the majority of KP1339 molecules bind to serum proteins within a few minutes (42)

and as electroporation was performed 3 minutes after *i.v.* injection of KP1339, the proportion of free KP1339 molecules capable of diffusing through electroporated membranes was probably diminished in our experiment. In the case of cisplatin, it takes 3 h for 95% of cisplatin to bind to serum proteins (43). Thus, there is probably still a high concentration of molecules of the free drug during electroporation (3 minutes after injection). Even though only ~50% of cisplatin is capable of diffusing through cell membranes (38, 44), the percentage of cisplatin capable of diffusing through electroporated membranes should be higher than the percentage of KP1339 capable of diffusing through electroporated membranes in our experiment. Bleomycin, which is not a metal-based drug, does not bind to serum proteins. Thus, the amount of bleomycin capable of diffusing through electroporated membranes should be even higher than that in the case of cisplatin, as it should not be diminished due to binding to serum proteins at all. Additionally, the fact that bleomycin cannot diffuse through intact membranes contributes to the even higher potentiation of its cytotoxicity induced by electroporation (24, 36, 37). These aspects are in accordance with the antitumour effectiveness of electrochemotherapy with all three mentioned antitumour drugs *in vivo*, as bleomycin is the most and KP1339 the least effective one.

It is common that binding of metal-based drugs (*e.g.* cisplatin) to serum proteins is irreversible and causes deactivation of the drug (45). In contrast, the binding of KP1339 is reversible and the released drug retains its biological activity, as was demonstrated previously (40, 42). In addition, KP1339 bound to the serum protein transferrin is capable of transmembrane transport by transferrin receptor-mediated endocytosis, which enables continuous uptake of KP1339 (40). Considering these facts, we presume that prolonged uptake of KP1339 (48 h) is mediated by transferrin receptor, contrary to cisplatin. Additionally, the low pH inside endosomes enables the release of biologically active KP1339 (42). Hence, the gentle slope of the tumour growth curves could be a result of constant KP1339 delivery from serum proteins.

As neither of the mentioned aspects seems to contribute to explain the increased antitumour effectiveness and accumulation of KP1339 induced by electroporation, we propose an alternative explanation involving the antivascular effect of electroporation. An antivascular effect has already been described in the case of electrochemotherapy with bleomycin and cisplatin in the SA-1 murine tumour model (22). The effect was explained by nonselective electroporation of cells nearby tumour cells, specifically endothelial cells (23), which resulted in reduced tumour blood flow and vascular damage. Sersa *et al.* observed reduced tumour blood flow in the case of electrochemotherapy with bleomycin and cisplatin that lasted for 24 h and 8 h, respectively (20, 21). Consequently, the chemotherapeutic drug remains entrapped in tumours at a higher concentration for a longer period of time.

We propose that in the case of KP1339, entrapment of the drug leads to an increased rate of uptake from serum proteins for the duration of the antivascular effect, resulting in increased intratumoural KP1339 content for at least 48 h and increased antitumour effectiveness. In addition, an antiangiogenic effect was already demonstrated for KP1339 and other ruthenium complexes *in vitro*. Therefore, we can assume that KP1339 can inhibit angiogenesis also *in vivo*, similarly as was shown for NAMI-A (46).

In conclusion, electroporation does not influence the cytotoxicity of the ruthenium(III) compound KP1339 *in vitro*, but significantly potentiates its antitumour effectiveness *in vivo*. The antitumour effectiveness of electrochemotherapy with KP1339 is most probably a result of a combination of both direct cytotoxicity of KP1339 on tumour cells and of the antivascular effect of electroporation. However, further studies on the mechanisms of antitumour activity of KP1339, including studies on antiangiogenic effects *in vivo*, are needed to fully elucidate its antitumour effectiveness.

Acknowledgements

The Authors acknowledge the financial support received from the State budget by the Slovenian Research Agency (programmes No. P1-0175, P2-0249 and P3-0003; projects No. J1-0200-0103-008 and BI-AT/09-10-016). This work was performed within the framework of COST D 39 Action. R.H. is grateful to ARRS for funding through a junior researcher grant. The Authors would also like to thank Duša Hodžić and Mira Lavrič for their help and useful comments on the laboratory work.

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Received October 19, 2009
Revised April 16, 2010
Accepted April 22, 2010