J Membrane Biol (2014) 247:1239–1251
DOI 10.1007/s00232-014-9696-2

Modulation of Activity of Known Cytotoxic Ruthenium(III) Compound (KP418) with Hampered Transmembrane Transport in Electrochemotherapy In Vitro and In Vivo

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Received: 19 January 2014 / Accepted: 29 May 2014 / Published online: 24 June 2014

Abstract To increase electrochemotherapy (ECT) applicability, the effectiveness of new drugs is being tested in combination with electroporation. Among them two ruthenium(III) compounds, (imH)[*trans*-$RuCl_4$(im)(DMSO-S)] (NAMI-A) and Na[*trans*-$RuCl_4(ind)_2$] (KP1339), proved to possess increased antitumor effectiveness when combined with electroporation. The objective of our experimental work was to determine influence of electroporation on the cytotoxic and antitumor effect of a ruthenium(III) compound with hampered transmembrane transport, (imH)[*trans*-$RuCl_4(im)_2$] (KP418) in vitro and in vivo and to determine changes in metastatic potential of cells after ECT with KP418 in vitro. In addition, platinum compound cisplatin (CDDP) and ruthenium(III) compound NAMI-A were included in the experiments as reference compounds. Our results show that electroporation leads to increased cellular accumulation and cytotoxicity of KP418 in murine melanoma cell lines with low and high metastatic potential, B16-F1 and B16-F10, but not in murine fibrosarcoma cell line SA-1 in vitro which is probably due to variable effectiveness of ECT in different cell lines and tumors. Electroporation does not potentiate the cytotoxicity of KP418 as prominently as the cytotoxicity of CDDP. We also showed that the metastatic potential of cells which survived ECT with KP418 or NAMI-A does not change in vitro: resistance to detachment, invasiveness, and re-adhesion of cells after ECT is not affected. Experiments in murine tumor models B16-F1 and SA-1 showed that ECT with KP418 does not have any antitumor effect while ECT with CDDP induces significant dose-dependent tumor growth delay in the two tumor models used in vivo.

Keywords KP418 · Electrochemotherapy · Ruthenium · Metastatic potential · In vitro · In vivo

Introduction

Electrochemotherapy (ECT) is one of the applications of electroporation in which pulsed electric field is used to

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improve delivery of non-permeant molecules into the cell (Sersa et al. 2008). Today ECT is successfully used in clinical practice for treatment of cutaneous and subcutaneous tumors, especially melanoma nodules, and is being developed for treatment of deep-seated tumors and chest-wall breast cancer recurrences (Haberl et al. 2013). In 2013 over 130 hospitals around the world implemented ECT treatment in clinics. However, complete tumor eradication after ECT treatment was obtained in 73.7 % according to the results of the European Standard Operating Procedures for Electrochemotherapy and Electrogenetherapy (ESOPE) study (Marty et al. 2006). In order to increase ECT applicability, research and development are focused on ECT treatment for deep-seated tumors (Miklavcic et al. 2010; Miklavcic et al. 2012; Edhemovic et al. 2011), new medical devices with electrodes optimization and computer-assisted simulations of field distribution (Spugnini et al. 2005; Corovic et al. 2013), treatment planning and suitable software for clinicians (Pavliha et al. 2013a; 2013b), and also on drug discovery adjusted for ECT (Jaroszeski et al. 2000; Hudej et al. 2010).

A drug effective in ECT treatment is a hydrophilic molecule with hampered cellular transmembrane transport and intracellular site of activity. The more pronounced these properties are the more effective electroporation is in increasing drug cytotoxicity (Orlowski et al. 1988). Only two drugs are used in ECT in clinics, namely bleomycin and cisplatin (CDDP) (Fig. 1). Although many chemotherapeutics have been tested, a significant increase in antitumor effectiveness in vitro and in vivo was only obtained with the two mentioned compounds (Heller et al. 2000). Electroporation in vitro potentiates bleomycin cytotoxicity by up to 100 000 times and CDDP cytotoxicity by up to 70 times (Orlowski et al. 1988; Sersa et al. 1995; Jaroszeski et al. 2000; Miklavcic et al. 2014). It is also effective in resistant cell lines (Cemazar et al. 1998) and it does not increase metastatic potential of the cells that survived the ECT treatment (Todorovic et al. 2011; Todorovic et al. 2012). The effectiveness of both drugs in ECT was demonstrated in several tumor models and for different tumor histologies in preclinical studies in vivo and later on in clinical trials (Sersa et al. 2008). Bioavailability of CDDP is reduced due to its fast irreversible binding to the serum protein albumin and as such when applied intravenously, its efficacy is reduced in comparison to intratumoral application in ECT (Mir et al. 2006; Hudej et al. 2010).

Until recently screening of drug, candidates for effective ECT treatment has only included drugs that are classic anticancer chemotherapeutics and that can be transported by passive or active mechanisms across the cell membrane (Orlowski et al. 1988; Jaroszeski et al. 2000; Miklavcic et al. 2014). The search for new effective drugs in ECT should also include screening of drugs which have shown too low effect for classic chemotherapy due to their hydrophilic nature and intracellular site of action and as such have never entered clinical phase I/II trials.

Ruthenium(III) compounds are an interesting group of metallotherapeutics whose anticancer activity is related to some prominent properties (Hartinger et al. 2008). Ruthenium(III) compounds which have entered clinical trials are (imH)[*trans*-$RuCl_4$(im)(DMSO-S)] (im = imidazole) (NAMI-A) as an antimetastatic drug, and (indH)[*trans*-$RuCl_4(ind)_2$](ind = indazole) (KP1019) and its sodium salt analog Na[*trans*-$RuCl_4(ind)_2$] (KP1339) as antitumor drugs effective against a variety of solid tumors including resistant colorectal tumors (Fig. 1) (Antonarakis and Emadi 2010). NAMI-A has a unique mechanism of activity which is not fully understood yet. Its high antimetastatic properties are accompanied with low antitumor effect for primary tumors in vivo and no cytotoxic effect in vitro (Gava et al. 2006; Antonarakis and Emadi 2010). Among other investigated ruthenium(III) compounds, (imH)[*trans*-$RuCl_4(im)_2$] (KP418) had significant antitumor activity; however, it did not reach clinical trials (Fig. 1). KP418 at equimolar concentrations was more effective than KP1019 against chemically induced autochthonous colorectal tumors resistant to other chemotherapeutics, though systemic toxicity accompanied its antitumor effect (Berger et al. 1989; Seelig et al. 1992). The nephrotoxicity of KP418 in rats was, however, still lower than that of CDDP (Kersten et al. 1998). It has been shown that cytotoxicity of all three KP compounds is related to their transmembrane transport, with KP418 being at least 10 times less efficiently taken up into cells than KP1019 and KP1339 (Kapitza et al. 2005; Hartinger et al. 2008). KP418 never entered clinical trials due to its hampered transmembrane transport and consequently systemic toxicity at effective doses in vivo (Seelig et al. 1992). However, it was never proven that lack of activity of KP418 at low doses is actually due to the lack of drug penetration into cells. Thus, the intrinsic cytotoxicity of KP418 and KP1019 was never compared.

Ruthenium(III) compounds have already been tested in combination with electroporation in vitro and in vivo in our previous studies (Bicek et al. 2007; Kljun et al. 2010; Hudej et al. 2010; 2012). The experiments in vivo have shown that mechanisms of activity in ECT with ruthenium compound KP1339 are significantly different from those with CDDP (Hudej et al. 2010).

The aim of our present study was to evaluate whether reversible electroporation would increase KP418 intracellular content and its cytotoxicity. In addition to this we investigated the applicability of the ruthenium compound KP418 in ECT treatment. We treated the cells with KP418 alone or in combination with electroporation and measured cellular accumulation of KP418 and cytotoxic effect in vitro. In addition, we studied metastatic potential of cells

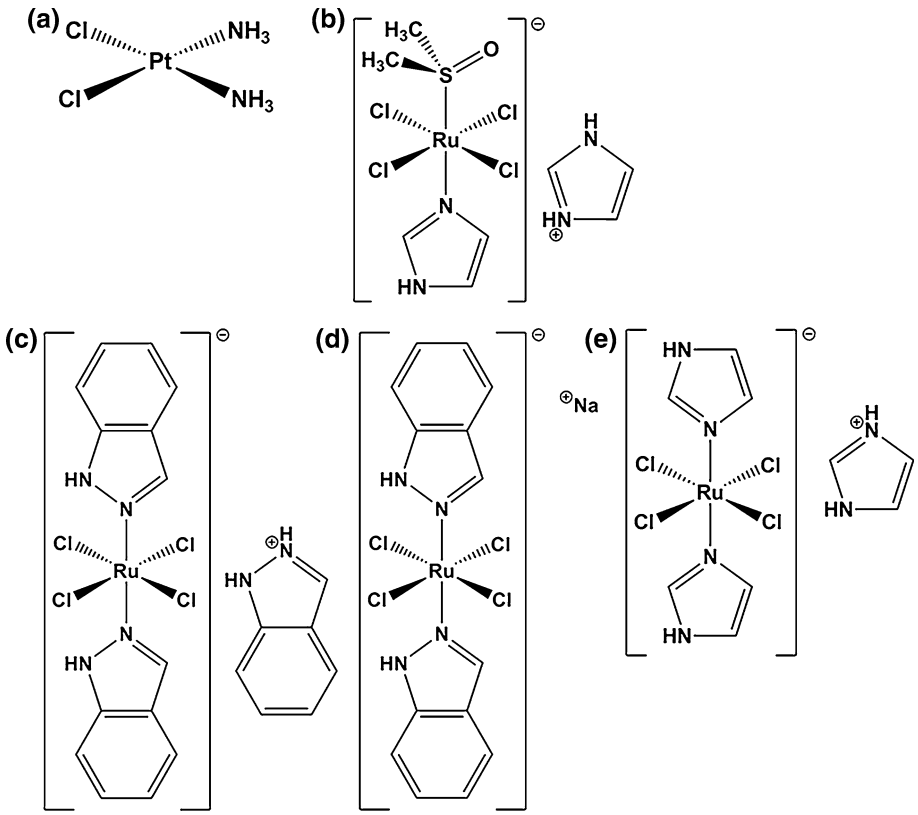

Fig. 1 Chemical structures of CDDP (**a**), NAMI-A (**b**), KP1019 (**c**), KP1339 (**d**) and KP418 (**e**)

after treatment by measuring cell resistance to detachment, migration, invasion, and re-attachment of cells in vitro. Finally, we determined the antitumor activity of ECT with KP418 in two mouse tumor models in vivo and compared it to antitumor activity of ECT with CDDP.

Materials and Methods

Compound Solutions

A 10 mM solution of each of KP418 and NAMI-A in 0.9 % NaCl were prepared directly before application. To dissolve the compound, the solution was mixed on vortex for 10 min. It was then sterile filtered through 0.22 μm pores filter (TPP, Trasadingen, Switzerland) and different concentrations of KP418 and NAMI-A were prepared in 0.9 % NaCl. For experiments in vitro the concentrations prepared were 0.1, 1, 10 mM and for experiments in vivo, the concentrations were 2.6, 5.2, 10.4 ,and 20.8 mM. CDDP solutions were prepared in a similar way. The concentrations prepared were 0.05, 0.5, and 5 mM for in vitro and 2.6 and 5.2 mM for in vivo experiments. For in vitro experiments we used 99.9 % pure CDDP (Sigma-Aldrich, St. Louis, MO, USA) and for in vivo experiments the formulation of CDDP supplemented with D-mannitol, NaCl, and HCl which is used in the clinics (CDDP, 50 mg/1000 mg, Medac, Hamburg, Germany). The compounds were dissolved whether in low conductive iso-osmolar electroporation buffer NaPB (10 mM Na_2HPO_4/NaH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose; pH 7.4; Sigma-Aldrich) whether in physiological solution 150 mM NaCl (0.9 % NaCl; pH 7.0; B. Braun, Melsungen, Germany). After being thoroughly mixed on vortex they were sterile filtered through 0.22 μm pores filter (TPP, Trasadingen, Switzerland) and different concentrations were prepared. Each solution was prepared directly before its application in cells or in tumors.

Cell Lines

Three different cell lines were used in our experiments where cytotoxicity of tested compounds in ECT in vitro was determined: SA-1 (murine fibrosarcoma cells; Jackson Laboratory, Bar Harbor, ME, USA), B16-F1, and B16-F10 (murine malignant melanoma cells with low (F1) and high (F10) metastatic potential; European Collection of Cell Cultures, Porton, UK). Cells were incubated in humidified atmosphere with 5 % CO_2 at 37 °C. SA-1 cells were grown in Advanced Eagle's Minimum Essential Medium (Gibco, Grand Island, NY, USA) supplemented with 5 % FBS (Gibco), 200 mM Glutamax (100×; Gibco), 50,000 U Penicillin (PANPHARMA S.A., Fougeres, France) and 50 mg/l Gentamicin (Krka, Novo mesto, Slovenia). B16-F1 cells were grown in Dulbecco's Modified Eagle's Medium

with high glucose (4.5 g/l; PAA, Pasching, Austria), 10 % Fetal Bovine Serum (FBS; PAA), 1 % 200 mM L-glutamin (Sigma-Aldrich), 0.01 % Penicillin/Streptomycin (100×; PAA) and 0.1 % Gentamicin (50 mg/ml; PAA).

The experiments for evaluation of metastatic potential of cells in vitro were performed in the laboratories of Callerio Foundation, Trieste, Italy. The cell lines B16-F1 and B16-F10 (American Type Culture Collection, Manassas, VA, USA) were used. Cells were grown in Minimal Essential Medium (EuroClone, Wetherby, UK) supplemented with 10 % FBS (Gibco), 2 % $NaHCO_3$ (Sigma-Aldrich), 1 % sodium pyruvate (Sigma-Aldrich), 1 % glucose (Sigma-Aldrich), 1 % 2 mM L-glutamine (EuroClone), 1 % nonessential amino acids (Sigma-Aldrich), Penicillin (100 IU/ml), and Streptomycin (100 μg/ml).

Cell suspension was prepared from cell cultures in exponential growth phase by trypsinization using trypsin–EDTA (5 g trypsin/2 g EDTA in 0.9 % NaCl; Sigma-Aldrich) 10 × diluted in Hanks' Balanced Salt solution (Sigma-Aldrich). From the obtained cell suspension, trypsin and growth medium were removed by centrifugation at 270 RCF for 5 min at 4 °C (Sigma 3-15 K, UK). The cell pellet was resuspended to obtain a final cell density of 2.2×10^7 cells/ml. The solution used for cell resuspension was whether NaPB whether NaCl, according to the solution used for the tested compound.

Electrochemotherapy In Vitro

Aliquots of freshly prepared KP418 or CDDP solutions of different concentrations were added to freshly prepared cell suspension (2.2×10^7 cells/ml) in volume proportion 1:9. The final concentrations of KP418 solutions were 0, 10, 100, and 1000 μM. Immediately after incubation (<30 s) a 60 μl droplet of cell suspension was placed between flat parallel stainless-steel electrodes 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, cells were incubated for 5 min at room temperature, allowing KP418 molecules to pass through the electroporated cell membranes. Cells were then diluted 40 times with the appropriate cell growth medium, and 5×10^3 cells were placed into each well of a 96 well-microtiter plate (TPP, Trasadingen, Switzerland) and incubated in humidified atmosphere with 5 % CO_2 at 37 °C for 72 h. The same procedure without electric pulses was used for cells exposed to KP418 alone for 5 min or 60 min. After the incubation time (72 h) a cell viability test was performed using the MTS-based Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). A volume of 10 μl of reagent per well was added directly to each well. After 2 h of incubation at 37 °C, the absorption at 490 nm was measured with a Tecan infinite M200 spectrophotometer (Tecan, Switzerland). The percentage of viable cells 72 h after the therapy was determined as follows:

$$\text{viable cells} = \frac{Abs_{(490)\text{ect}}}{Abs_{(490)0}} \times 100\ [\%],$$

where $Abs_{(490)\text{ect}}$ is the absorbance of treated cells and $Abs_{(490)0}$ is the absorbance of control cells at 490 nm.

The IC_{50} values (Inhibitory Concentration 50 is the concentration where 50 % of cells are viable) were graphically determined from dose–response curves. Experiments in vitro were repeated three times independently with six parallel measurements for each parameter.

Cellular Accumulation of Ruthenium After Electrochemotherapy In Vitro

To determine the ruthenium or platinum intracellular concentration after ECT treatment, cells after ECT in vitro were immediately centrifuged at 270 RCF and 4 °C for 5 min (Sigma 3–15 K, UK). Supernatant was carefully discarded and cell debris digested with incubation in 100 μl HNO_3 (Merck, s.p., KGaA, Darmstadt, Germany) and 100 μl H_2O_2 (Merck, s.p.) at 80 °C for 12 h. Clear solution was obtained. After that 50 μl HCl (Merck, s.p.) and deionized water were added. The content of platinum or ruthenium in the samples analyzed was determined by inductively coupled plasma—mass spectroscopy (ICP-MS 7700x, Agilent Technologies, Tokyo, Japan). An aliquot of cells after ECT treatment was used also for determination of viable cells in these sets of experiments.

Metastatic Potential of Cells In Vitro

Resistance to Detachment

Immediately after the treatment (described under section » Electrochemotherapy in vitro «) the cells were diluted, and 2×10^4 cells per well were seeded in 96-well microtiter plate. Cells were incubated at 37 °C and 5 % CO_2 for 24 h. Medium was then removed, cells were washed and incubated in 0.008 % trypsin/EDTA for 30 min with gentle shaking of the plate. Thereafter, trypsin was removed. Non-adherent cells were washed and adherent cells were detected by sulphorhodamin B assay (SRB). Cells were first fixed with 10 % trichloroacetic acid (TCA; Sigma-Aldrich) for 1 h at 4 °C. TCA was then removed, cells were washed, dried, and stained with 0.4 % SRB and 1 % acetic acid (Sigma-Aldrich). The dye was then dissolved with 10 mM Tris base (tris-hydroxymethyl-aminomethane) with pH 10.5 (Sigma-Aldrich). Absorbance was measured at 570 nm with

a spectrophotometer (SpectraCount, Packard, Meriden, Conn, USA). The percentage of cells resistant to detachment after the therapy was determined as follows:

$$\text{cells resistance to detachment} = \frac{Abs_{(570)ect}}{Abs_{(570)0}} \times 100\,[\%],$$

where $Abs_{(570)ect}$ is the absorbance of treated cells and $Abs_{(570)0}$ is the absorbance of control cells at 570 nm.

Invasion Assay

One day before the experiment the inserts for 24-well microtiter plates with polycarbonate membrane with 8 μm pores (Greiner bio-one, Frickenhausen, Germany) were coated with 50 μl of Matrigel (600 μg/ml) (BD Bioscience, Palo Alto, CA, USA) and incubated for 24 h at room temperature to allow Matrigel polymerization. Next day inserts were filled with DMEM and gently shaken for 90 min. In the meanwhile cells were treated as described under section » Electrochemotherapy in vitro «. Cell suspension was diluted 200 times with DMEM with 0.1 % BSA (Sigma-Aldrich), and 10^5 cells have been seeded per insert. Complete growth medium (DMEM with all supplements as described under » Cell lines «) was added to the wells with FBS as a chemoattractant. The cells were incubated for 24 h at 37 °C and 5 % CO_2 to allow cell invasion through Matrigel layer and porous membrane. In positive control group the cells were not treated while in negative control group the cells were not treated and were seeded in the inserts without the chemoattractant in the growth medium. After the incubation, the medium was removed and cells were fixed with 1.1 % glutaraldehyde (Sigma-Aldrich) and stained with crystal violet (Sigma-Aldrich). The stained cells on the membranes and in the wells were dissolved in 10 % acetic acid and the absorbance was measured at 590 nm with a spectrophotometer (SpectraCount, Packard). The percentage of invasive cells after the therapy was determined as follows:

$$\text{invasiveness of cells} = \frac{Abs_{(590)ect}}{Abs_{(590)0}} \times 100\,[\%\,],$$

where $Abs_{(590)ect}$ is the absorbance of treated cells and $Abs_{(590)0}$ is the absorbance of control cells at 590 nm.

Re-adhesion

Immediately after the treatment (described under section » Electrochemotherapy in vitro «) the cells were diluted, and 2×10^4 cells per well were seeded in 96-well microtiter plate. Cells were incubated at 37 °C and 5 % CO_2 for 1 h. Medium was removed, cells were washed, and fixed with 10 % trichloroacetic acid for 1 h at 4 °C. The amount of cells was determined with the SRB assay (described under section » Resistance to detachment «). The percentage of adherent cells after the therapy was determined as follows:

$$\text{readhesion of cells} = \frac{Abs_{(570)ect}}{Abs_{(570)0}} \times 100\,[\%],$$

where $Abs_{(570)ect}$ is the absorbance of treated cells and $Abs_{(570)0}$ is the absorbance of control cells at 570 nm.

Animals and Tumors

Animal studies were carried out according to the guidelines of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permissions #:34401-36/2008/6 and 34401-1/2011/3) and the EU directive 86/609/EEC.

Inbred C57BL/6 and A/J mice were purchased from the Institute of Pathology, Faculty of Medicine, University of Ljubljana (Ljubljana, Slovenia) and kept at the Institute of Oncology Ljubljana, Department of Experimental Oncology. Mice were kept at 18–22 °C at 55 ± 10 % humidity with a controlled 12 h light/dark cycle in a specific pathogen-free animal colony. Healthy mice of both sexes, 8–10 weeks old, weighing 20–25 g, were included in the experiments. Solid subcutaneous tumors were induced dorsolaterally by the injection of 5×10^5 viable SA-1 cells to A/J mice and B16-F1 cells to C57BL/6 mice. SA-1 cells were obtained from the ascitic form of tumor, while B16-F1 cells were obtained from cell culture. When tumors reached 6 mm in diameter (approximately 40–50 mm^3), the mice were randomly divided into experimental groups (6–10 and 6–7 animals per group in experiments in SA-1 and B16-F1 tumor model, respectively) and subjected to the specific experimental protocol. The confirmatory second experiment was performed in SA-1 tumor model.

Electrochemotherapy In Vivo

The tumors were treated with KP418 and CDDP injected intravenously (V = 100 μl) in the orbital sinus. For SA-1 tumor treatment KP418 was injected at equimolar concentrations to KP1339 in previous experiments on SA-1 tumor model (2.6, 5.2 and 10.4 mM) (Hudej et al. 2010). As animals tolerated well, the highest concentration of KP418, we decided to proceed with 2-times higher concentration of KP418 on B16-F1 tumors (10.4, 20.8 mM). CDDP was injected at concentrations 2.6 and 5.2 mM for SA-1 tumors and 5.2 mM for B16-F1 tumors. Higher concentrations of CDDP are lethal for mice and were not prepared. Animals in control group were treated with 0.9 % NaCl solution. Three minutes after injection, electric pulses were locally applied to the tumor. Electroporation of the tumors was performed by application of eight square-wave electric pulses, delivered in two sets of four pulses in

perpendicular directions with an amplitude of 780 V (1300 V/cm), duration of 100 μs and a repetition frequency of 1 Hz. The electric pulses were delivered to the tumors 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 tumor. Inter-electrode distance was 6 mm. A good contact between the electrodes and the skin was assured by means of ultrasonographic conductive gel (Kameleon d.o.o., Maribor, Slovenia). The electric pulses were generated by a Cliniporator electroporator (IGEA, Italy). All treatments were well-tolerated by animals and were performed without anesthesia.

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (a, b, and c) with a vernier caliper, every second day. The tumor volumes were calculated as follows: $V = \pi \times a \times b \times c/6$. The arithmetic mean of the tumor volumes and the standard error of the mean (SE) were calculated for each experimental group for each measurement day. The tumor growth delay was determined for each individual tumor by subtracting the average doubling time of the control group from the doubling time of each individual tumor. Animals with tumors in regression were followed up to 100 days after the treatment. After that, if no tumor regrowth was observed, animals were considered to be in complete remission.

All animals were monitored for possible systemic side-effects with physical examination every second day from the beginning of the experiment. This included monitoring animal's body weight and evaluation of the general health status with observation of the animal's appetite, locomotion, coat, and general appearance.

Statistical Analysis

Statistical analysis was performed using One–Way ANOVA test and SigmaStat statistical software (SPSS, Chicago, USA).

Results and Discussion

Cellular Accumulation of Ruthenium After Electrochemotherapy In Vitro

To get insight into the transmembrane transport of KP418 and its intrinsic cytotoxicity we correlated intracellular accumulation of ruthenium with viability of cells after electroporation alone (EP) or ECT with 1000 μM KP418. B16-F1 cells were treated with KP418 and electroporated at different electric field strengths (400–1,200 V/cm) to achieve different amounts of cell membrane permeabilization. In addition, we answered the question whether 0.9 % NaCl can be used in

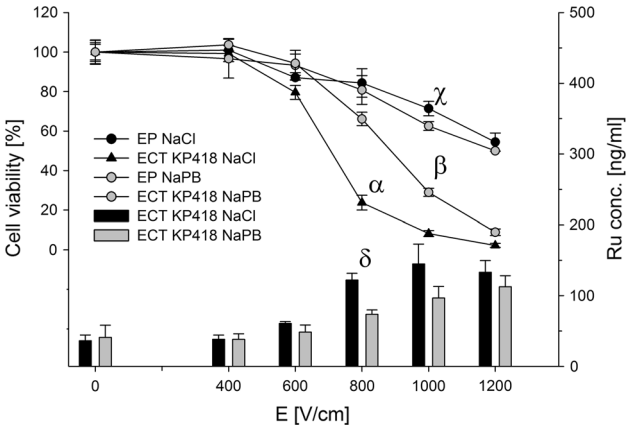

Fig. 2 The influence of electroporation buffer (NaCl, NaPB) and electric field strength on ruthenium (Ru) intracellular accumulation (histogram) and cell viability (plots) after ECT with 1000 μM KP418 (ECT KP418) in vitro. 100 % cell viability represents the viability of the untreated control group (C). Data points represent the mean values ± SD; αßχδ indicates data point significantly different from defined groups ($p < 0.05$): α – ECT KP418 NaCl versus C, EP NaCl and ECT KP418 NaPB; ß – ECT KP418 NaPB versus C and EP NaPB; χ – EP NaPB and EP NaCl versus C; δ – ECT KP418 NaCl vs. C

ECT experiments instead of commonly used phosphate electroporation buffers due to the fact that ruthenium KP compounds are unstable in phosphate buffers. Two sets of experiments were performed in two different electroporation solutions: low conductivity phosphate buffer (NaPB) and high conductivity NaCl solution (NaCl). Intracellular accumulation of ruthenium and viability of cells were measured (Fig. 2). EP caused a decrease in cell viability which is due to irreversible electroporation at electric fields above 1,000 V/cm with approximately 20 and 40 % of non-viable cells at 1,000 V/cm and 1,200 V/cm, respectively. We determined that 1,000 V/cm is a threshold for irreversible electroporation of B16F1 cells in suspension regardless of electroporation solution used in vitro meaning that reversible electroporation for effective ECT should be performed at lower field strength. The electric field strength threshold at which significantly higher decrease of cell viability was achieved for ECT than for EP was 800 V/cm in NaCl solution and 1,000 V/cm in NaPB buffer. Intracellular accumulation of ruthenium after ECT with KP418 was dependent on electric field strength and correlated well with decrease in cell viability. Similar results were obtained with CDDP (Fig. 3). Taken all together, we assume that higher cytotoxicity of ECT in NaCl could be due to the known fact that both compounds are more stable in NaCl than in NaPB. It was shown that low concentration of chloride ions leads to formation of reactive hydrolyzed CDDP products which bind promptly and irreversibly to cell membrane phospholipids (Speelmans et al. 1996). These molecules do not exert cytotoxic effect, but are anyway measured with ICP-MS as cellular CDDP which fully explain the

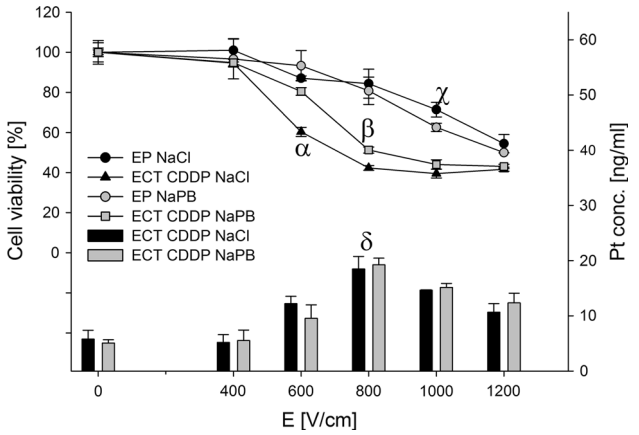

Fig. 3 The influence of electroporation buffer (NaCl, NaPB) and electric field strength on platinum (Pt) intracellular accumulation (histogram) and cell viability (plots) after ECT with 50 μM CDDP (ECT CDDP) in vitro. 100 % cell viability represents the viability of the untreated control group (C). Data points represent the mean values ± SD; αßχδ indicates data point significantly different from defined groups ($p < 0.05$): α – ECT CDDP NaCl versus C, EP NaCl, and ECT CDDP NaPB; ß – ECT CDDP NaPB versus C and EP NaPB; χ – EP NaPB and EP NaCl versus C; δ – ECT CDDP NaCl and ECT CDDP NaPB vs. C

results we obtained. In ECT CDDP at electric fields above 1,000 V/cm cell viability reaches its plateau which is probably due to the low subcytotoxic CDDP concentration and the fact that only 5–10 % of covalently bound CDDP in cells binds to DNA exerting its cytotoxic effect (Cepeda et al. 2007). Another interesting observation at electric fields above 1,000 V/cm is decrease in intracellular Pt. Irreversible electroporation causes leaking of unbound CDDP which is not observed in case of KP418 due to different pattern of binding to cellular proteins already observed in vitro and also in ECT in vivo (Hudej et al. 2010). Detailed study by Heffeter et al. also revealed that majority of ruthenium KP compounds bind to high molecular weight cytosolic proteins while majority of cisplatin bind to low molecular weight cytosolic proteins (Heffeter et al. 2010).

Our results show that 0.9 % NaCl can be used as an electroporation solution as it does not affect the electrical field threshold for irreversible electroporation but it increases the cytotoxicity of ECT with both tested compounds. We showed that KP418 is a compound with hampered transmembrane transport and that its cytotoxic effect can be potentiated by reversible electroporation achieved at 800 V/cm in NaCl solution. In addition, we showed that cytotoxicity of a compound alone exposed to electric field at 800 V/cm does not change (data not shown) which is an additional confirmation that the electroporation-enhanced drug cytotoxicity is due to its effect on cells and not on KP418. Based on these results next experiments in vitro were performed with electroporation solution NaCl and electric field strength applied at 800 V/cm.

Electrochemotherapy with KP418 In Vitro

The applicability of KP418 for ECT was first evaluated by determining its cytotoxic effect in three different tumor cell lines in vitro. Cells were exposed to different concentrations of KP418 alone or in a combination with reversible electroporation. Eight rectangular unipolar pulses with 100 μs duration were applied with the repetitive frequency 1 Hz. The optimal electrical field strength for reversible electroporation in 0.9 % NaCl was determined to be 800 V/cm for B16-F1 cells and we used the same electric field strength for the other two cell lines (B16–F10, SA–1) as it was shown previously that electropermeabilization of these cells is achieved at electrical fields already above 600 V/cm (Cemazar et al. 1998). However, ECT and EP effects are dependent on cell size and cell type, as well as on intrinsic sensitivity of cells to the chemotherapeutic drug (Cemazar et al. 1998; 2001; Pucihar et al. 2006), thus the effectiveness of ECT is not dependent only on electrical parameters, but it depends also on types of tumor cells used. The difference in sensitivity of B16 and SA-1 cells to ECT with CDDP has already been shown using clonogenic assay as a measure of ECT cytotoxicity (Cemazar et al. 2001). CDDP was used in our experiments in order to compare the effect of KP418 with relevant chemotherapeutic agent used for ECT in the clinics.

Our results demonstrated that KP418 itself is not cytotoxic up to 1000 μM for the three cell lines tested. On the other hand, statistically significant increase of KP418 cytotoxicity was achieved after only 5 min incubation time with 1,000 μM KP418 in combination with electroporation (ECT KP418) (Fig. 4a, c, e). Electroporation did not increase the cytotoxicity of KP418 in SA-1 cells (Fig. 4a) but it did increase it in B16–F1 and B16–F10 cells ($IC_{50} = 600$ μM) (Fig. 4c, e). In case of CDDP electroporation increased its cytotoxicity in SA–1 cells ($IC_{50} = 200$ μM), proving that electropermeabilization of SA–1 cells was indeed achieved, however, the increase was more prominent in B16–F1 cells ($IC_{50} = 70$ μM) (Fig. 4b, d). Comparison of cytotoxicity of both tested compounds in B16–F1 cells revealed that KP418 is less cytotoxic than CDDP whether in combination with or without electroporation ($IC_{50\ ECT\ KP418} = 600$ μM vs. $IC_{50\ ECT\ CDDP} = 70$ μM; Fig. 4c, d). The B16F1 cell survival at 1,000 μM of KP418 differed between the experiments, which is most probably due to the different experimental protocols.

Metastatic Potential of Cells In Vitro

In anticancer treatment there is a certain possibility that not all treated cancer cells are successfully eliminated. For a treatment to be safe the remaining cells after the treatment must not metastasize. A combination of three assays

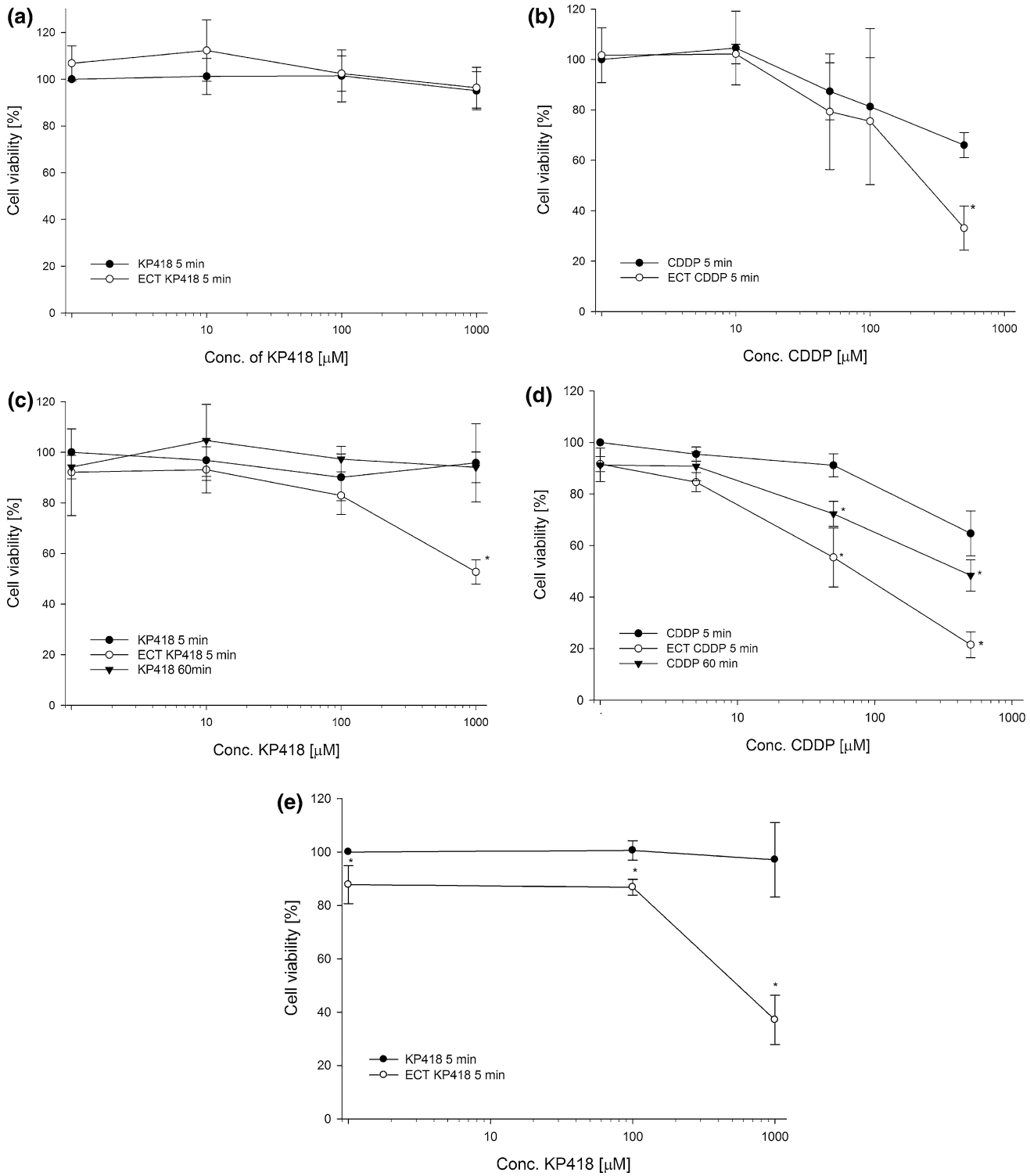

Fig. 4 KP418 (**a**, **c**, **e**) and CDDP (**b**, **d**) cytotoxicity in the cell lines SA-1 (**a**, **b**), B16-F1 (**c**, **d**) and B16-F10 (**e**) in vitro. Dose–response curves for KP418 or CDDP treatment with exposure time of 5 min and 60 min, and for ECT treatment and 5 min exposure time (ECT KP418 5 min, ECT CDDP 5 min; electroporation parameters: 800 V/cm, 8 × 100 μs, 1 Hz). Cell viability was determined 72 h after the treatment by the MTS assay. Data points represent mean values ± SD of three independent experiments; $*p < 0.05$ versus control group

in vitro, namely invasion, resistance to detachment, and re-adhesion, can be used to evaluate the metastatic properties of tumor cells in vivo (Bergamo et al. 2009). We performed all three assays using cells that survived treatment with the compound alone, the electroporation alone or a combination of both. In addition to KP418, the ruthenium(III)

compound NAMI-A was tested, as it is known to have antimetastatic effect (Sava et al. 2003). The compounds tested were applied at 100 μM concentration. This is the subcytotoxic concentration of KP418 when in combination with electroporation. In addition, this concentration of NAMI-A was used in previous studies where it was shown that antimetastatic properties are absent at lower concentrations of NAMI-A in vitro (Zorzet et al. 2000; Gava et al. 2006).

With the three assays, we determined the effect of ECT with ruthenium compounds KP418 and NAMI-A on cell metastatic potential in vitro. The results confirm the observations from Todorovic et al. (2011), (2012) showing that electroporation has no significant influence on metastatic potential of cells. We observed slight, however, not statistically significant decrease in cells resistance to detachment after EP, while no influence on re-adhesion and invasion of cells after EP was observed. Similarly, no effect was observed after NAMI-A treatment alone (NAMI-A) or in combination with electroporation (ECT NAMI-A). Gava et al. reported that incubation of adherent B16-F10 cells with 100 μM NAMI-A for 1 h significantly reduced invasion up to 85.8 % (Gava et al. 2006). However, the results should not be compared directly as the incubation time in our experiments was much lower (5 min) and cells were not adherent. The latter might be an important factor as one of the main active sites of NAMI-A is supposed to be collagen in extracellular matrix which is not present on the trypsinized cells in suspension (Sava et al. 2003).

It was already shown that KP418 is virtually devoid of effects in similar assays in vitro (Bergamo et al. 2009). Our results from the three assays performed are in accordance with results from Bergamo. In addition, we showed that subcytotoxic concentrations of KP418 in combination with electroporation (ECT KP418) also did not affect the invasive potential of cells as there was no significant difference between ECT KP418-treated cells and EP treated cells in any of the three assays performed (Fig. 5). Overall, our results showed that neither EP alone nor ECT with NAMI-A or KP418 affected metastatic potential of cells in vitro.

Electrochemotherapy In Vivo

To determine the antitumor effect of electrochemotherapy with KP418 we continued the study in two different tumor models in vivo, namely murine fibrosarcoma SA-1 and murine melanoma B16-F1, by measuring tumor growth after the treatment. In addition to KP418 we performed the

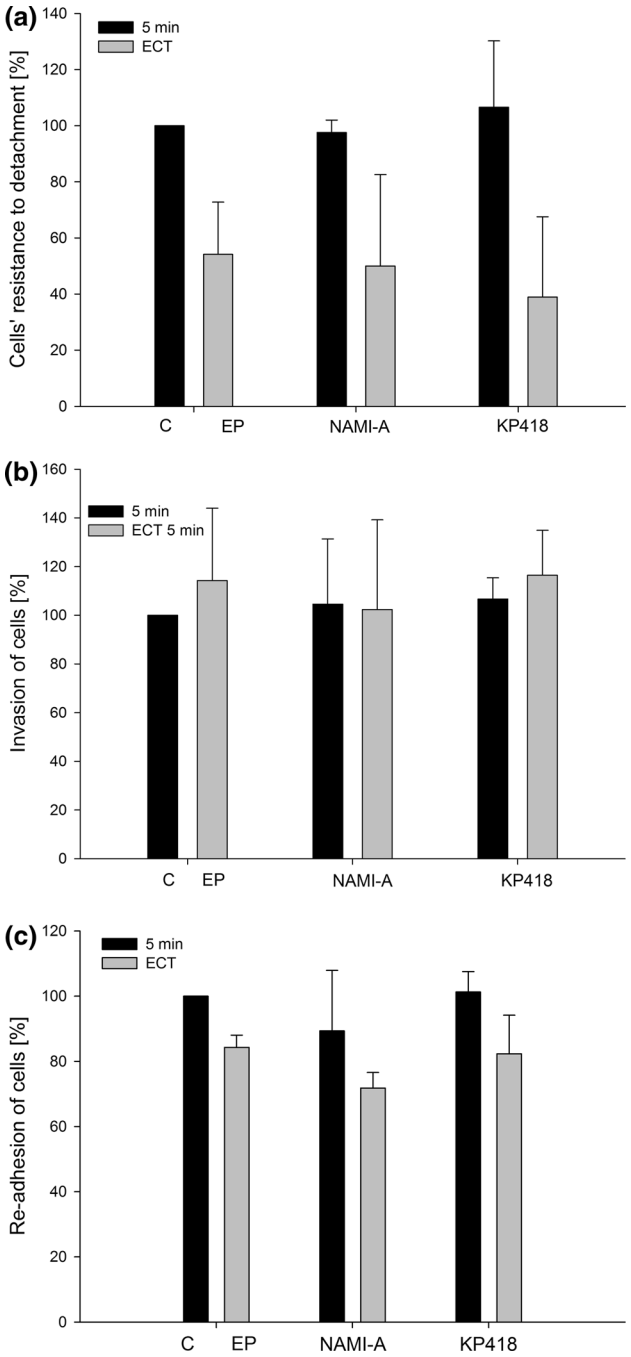

Fig. 5 The change in cell resistance to detachment (**a**), invasion ability (**b**) and re-adhesion ability (**c**) of B16-F10 cells that survived treatment with a tested compound alone (5 min: 100 μM NAMI-A, 100 μM KP418), electroporation alone (EP: 800 V/cm, 8 × 100 μs, 1 Hz) or combined treatment (ECT 5min) was determined in relation to control group of untreated cells (C) for which invasion was defined as 100 %. The cells were incubated for 24 h to allow invasion through Matrigel and porous membrane and the amount of invaded cells was determined spectrophotometrically after crystal violet staining. Data points represent the mean values of three independently repeated experiments ± SD; $^{*}p < 0.05$. versus control group (C)

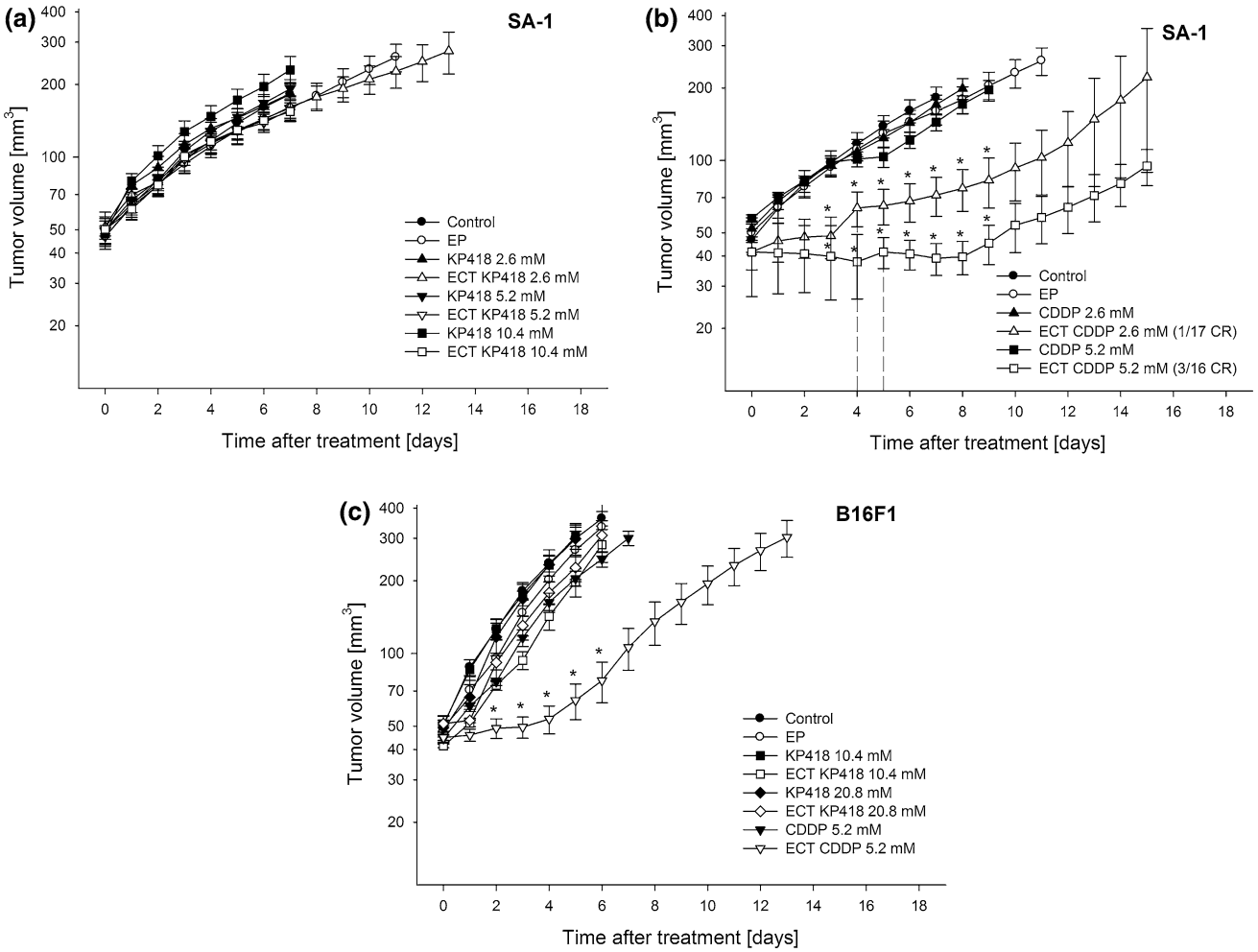

Fig. 6 Tumor growth curves representing antitumor effect of ECT with KP418 (ECT KP418) in comparison with ECT with CDDP (ECT CDDP) in two murine tumor models in vivo, SA-1 (**a**, **b**) and B16-F1 (**c**). Data points represent the mean values of all animal tumors ± SD; $^*p < 0.05$ versus control group. *Dashed lines* represent the complete responses (CR)—complete tumor eradication with no recurrence 100 days after the treatment

experiment also with equimolar concentrations of CDDP to compare and evaluate the results of the tested compound with a drug already used in the clinics. Both compounds were applied systemically (i.v.). Tumors were measured three times weekly using a digital caliper until tumors reached 300 mm^3 whereupon mice were humanely euthanized.

Treatments with KP418 alone or in combination with electroporation (ECT KP418) did not influence tumor growth kinetics (Fig. 6a, c). On the other hand, ECT with CDDP treatment caused dose-dependent tumor growth delay in both tumor models used (Fig. 6b, c). Our results are in good correlation with previous study. Cemazar et al. obtained 10.3 days of growth delay after ECT with CDDP (4 mg/kg) in SA-1 tumors (Cemazar et al. 1999) while we obtained 7.5 days of growth delay and additional 5.9 % complete regression after ECT with CDDP (3.9 mg/kg = 2.6 mM CDDP, 100 µl). Comparing the response of ECT CDDP treatment in two different tumor models revealed that ECT CDDP is more effective in SA-1 than in B16-F1 tumors. ECT with the highest dose of CDDP used (5.2 mM = 7.8 mg/kg) resulted in a statistically significant tumor growth delay calculated from tumor doubling time: 11.5 days and 5.5 days in SA-1 and B16-F1, respectively. Additionally, three mice out of sixteen (18.8 %) were in complete regression in case of SA-1 tumors (Fig. 6b). However, in previous study Sersa et al. already compared ECT CDDP tumor response between SA-1 and B16-F1 tumor models and observed no significant difference. Similarly to experiments in vitro different effect of ECT on different tumor models is observed often in experiments in vivo (Sersa et al. 1994). The clinical data also support the differential sensitivity of tumors to ECT, according to their histology and tumor size (Mali et al. 2013a; 2013b). Taking into account, the result from in vitro study where IC_{50} for ECT KP418 was as high as 1,000 µM there is a

possibility that the effective concentration of KP418 in tumors in vivo was not even reached. Comparison of these results with our results from previous similar studies shows distinct difference between KP418 and KP1339 effectiveness in ECT (Hudej et al. 2010). However, this can be explained with the difference in intrinsic properties of the two compounds. The increased accumulation of KP1339 found in SA-1 tumors as long as 48 h after the treatment with KP1339 alone and even more pronounced when in combination with electroporation was supposed to be due to synergistic effect of KP1339 ability to cross cell membrane itself and its intrinsic cytotoxicity after 1 h incubation time on SA-1 cells in vitro ($IC_{50} = 100$ μM), its fast and reversible binding to serum albumin, EPR effect (Enhanced Permeability and Retention effect) and vascular lock caused by electroporation (Sersa et al. 2002). All mentioned leads to prolonged transmembrane transport of compound during vascular-lock effect (Hudej et al. 2010). On the contrary, KP418 binds to serum proteins slowly (in hours) and as such cannot accumulate in tumors due to EPR effect. It also cannot pass cell membrane itself (Kapitza et al. 2005). Consequently it cannot exert its activity during vascular lock and when the reversibly electroporated cells reseal.

Negative effects of serum protein binding and vascular lock can be overcome by local intratumoral administration of a drug (Brincker 1993). In this way higher antitumor activity is achieved in ECT with CDDP (Cemazar et al. 1995) and we suppose it might increase also the effectiveness of ECT with KP418. However, in accordance with EU 3R strategy in animal experimentation and the lack of significant effect of KP418 in vitro and in vivo compared to effects caused by CDDP, further experiments with *i.t.* administration of KP418 were not anticipated.

As KP418 was shown to be particularly active in colorectal cancers this result might suggest that KP418 targets some specific molecule diferentialy expressed in different cell types. Electroporation might be useful to increase the anticancer activity of drugs provided that the tested tumors express the target(s) for these drugs. In this context it would be interesting to repeate experiments on colorectal tumor models in vitro and in vivo where KP418 was shown to be extremely potent (Berger et al. 1989; Seelig et al. 1992).

Conclusion

Ruthenium(III) compound KP418 cannot pass intact cell membranes readily. We showed that higher intracellular concentration of KP418 can be achieved by means of reversible electroporation in vitro and this correlates well with increased cytotoxicity of the compound in B16-F1 cell line in vitro. ECT with KP418 is cytotoxic for B16-F1 and B16-F10 cells but not for SA-1 cells in vitro. Similarly, ECT with CDDP was more cytotoxic for B16-F1 cells than SA-1 cells. The difference in ECT effectiveness among cell lines observed is a consequence of variable effectiveness of ECT on different cell types (Cemazar et al. 1998). We also showed that metastatic potential of cells that survived ECT with KP418 or NAMI-A was not affected. Their ability to resist detachment, their invasiveness and re-attachment were not affected by ECT with KP418 nor NAMI-A in vitro. However, these results are not sufficient to prove that any of the treatments tested is devoid of metastasis promotion in in vivo models.

ECT with up to 20.8 mM KP418 applied i.v. had no antitumor effect on B16-F1 and SA-1 murine tumor models in vivo. Based on the results in vitro, where IC_{50} for ECT KP418 was as high as 1,000 μM, we speculate that the effective concentration of KP418 was not achieved in tumor cells in vivo. ECT with CDDP i.v. caused tumor growth delay for both tumor models and also 18.8 % complete responses in case of SA-1 tumors, which is in accordance with the previous studies (Cemazar et al. 1999).

Taken all together, electroporation can increase in vitro cytotoxicity of KP418 but its effectiveness in vitro and in vivo is still lower than the effectiveness of chemotherapeutic already used in ECT in clinics, namely CDDP.

Acknowledgments The authors acknowledge the financial support received from the State budget by the Slovenian Research Agency (ARRS) for programmes No. P1-0175, P2-0249, P3-0003, project J1-4131 and junior researcher grants for R.H. The authors would also like to acknowledge that all the experimental work related to metastatic potential of cells was performed at the Callerio Fundatione in Trieste under the supervision of dr. Gianni Sava and dr. Alberta Bergamo and with the help of their researches. The authors are also thankful to Dr. M. Jakupec (University of Vienna) for critical reading of the manuscript. This work was supported by COST D39 and COST CM1105, in particular by a short-term scientific mission for R.H.

References

Antonarakis ES, Emadi A (2010) Ruthenium-based chemotherapeutics: are they ready for prime time? Cancer Chemoth Pharm 66:1–9

Bergamo A, Masi A, Jakupec MA, Keppler BK, Sava G (2009) Inhibitory effect of the ruthenium complex KP1019 in models of mammary cancer cell migration and invasion. Met-Based Drugs. doi:10.1155/2009/681270

Berger MR, Garzon FT, Keppler BK, Schmähl D (1989) Efficacy of new ruthenium complexes against chemically induced autochthonous colorectal carcinoma in rats. Anticancer Res 9:761–766

Bicek A, Turel I, Kanduser M, Miklavcic D (2007) Combined therapy of the antimetastatic compound NAMI-A and electroporation on B16F1 tumour cells in vitro. Bioelectrochemistry 71:113–117

Brincker H (1993) Direct intratumoral chemotherapy. Crit Rev Oncol Hemat 15:91–98

Cemazar M, Miklavcic D, Vodovnik L, Jarm T, Rudolf Z, Stabuc B, Cufer T, Sersa G (1995) Improved therapeutic effect of

electrochemotherapy with cisplatin by intratumoral drug administration and changing of electrode orientation for electropermeabilization on EAT tumor model in mice. Radiol Oncol 29:121–127

Cemazar M, Jarm T, Miklavcic D, Macek Lebar A, Ihan A, Kopitar NA, Sersa G (1998) Effect of electric field intensity on electropermeabilization and electrosensitivity of various tumor-cell lines in vitro. Electro Magnetobiol 17:263–272

Cemazar M, Miklavcic D, Scancar J, Dolzan V, Golouh R, Sersa G (1999) Increased platinum accumulation in SA-1 tumor cells after in vivo electrochemotherapy with cisplatin. Br J Cancer 79:1386–1391

Cemazar M, Parkins CS, Holder AL, Chaplin DJ, Tozer GM, Sersa G (2001) Electroporation of human microvascular endothelial cells: evidence for an anti-vascular mechanism of electrochemotherapy. Brit J Cancer 84:565–570

Cepeda V, Fuertes MA, Castilla J, Alonso C, Quevedo C, Pérez JM (2007) Biochemical machanisms of cisplatin cytotoxicity. Anticancer Agents Med Chem 7:3–18

Corovic S, Lackovic I, Sustaric P, Sustar T, Rodic T, Miklavcic D (2013) Modeling of electric field distribution in tissues during electroporation. Biomed Eng Online 12:16

Edhemovic I, Gadzijev EM, Brecelj E, Miklavcic D, Kos B, Zupanic A, Mali B, Jarm T, Pavliha D, Marcan M, Gasljevic G, Gorjup V, Music M, Pecnik Vavpotic T, Cemazar M, Snoj M, Sersa G (2011) Electrochemotherapy: a new technological approach in treatment of metastases in the liver. Technol Cancer Res Treat 10:475–485

Gava B, Zorzet P, Spessotto P, Cocchietto M, Sava G (2006) Inhibition of B16 melanoma metastases with the ruthenium complex imidazolium trans-imidazoledimethylsulfoxide-tetrachlororuthenate and down-regulation of tumor cell invasion. J Pharmacol Exp Ther 317(1):284–291

Haberl S, Miklavcic D, Sersa G, Frey W, Rubinsky B (2013) Cell membrane electroporation—Part 2: The applications. IEEE Electr Insul M 29(1):29–37

Hartinger CG, Jakupec MA, Zorbas-Seifried S, Groessl M, Egger A, Berger W, Zorbas H, Dyson PJ, Keppler BK (2008) KP1019, a new redox-active anticancer agent-preclinical development and results of a clinical phase I study in tumor patients. Chem Biodivers 5:2140–2155

Heffeter P, Böck K, Atil B, Hoda MAR, Körner W, Bartel C, Jungwirth U, Keppler BK, Micksche M, Berger W, Koellensperger G (2010) Intracellular protein binding patterns of the anticancer ruthenium drugs KP1019 and KP1339. J Biol Inorg Chem 15:737–748

Heller R, Gilbert R, Jaroszeski MJ (2000) Clinical trials for solid tumors using electrochemotherapy. In: Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery. Electrically Mediated Delivery of Molecules to Cells. Jaroszeski MJ, Heller R and Gilbert R (eds.). New Jersey: Humana Press, pp. 137-156

Hudej R, Turel I, Kanduser M, Scancar J, Kranjc S, Sersa G, Miklavcic D, Jakupec MA, Keppler BK, Cemazar M (2010) The influence of electroporation on cytotoxicity of anticancer ruthenium(III) complex KP1339 in vitro and in vivo. Anticancer Res 30:2055–2064

Hudej R, Kljun J, Kandioller W, Repnik U, Turk B, Hartinger CG, Keppler BK, Miklavcic D, Turel I (2012) Synthesis and Biological Evaluation of the Thionated Antibacterial Agent Nalidixic Acid and its Organoruthenium(II) Complex. Organometallics 31:5867–5874

Jaroszeski MJ, Dang V, Pottinger C, Hickey J, Gilbert R, Heller R (2000) Toxicity of anticancer agents mediated by electroporation in vitro. Anti-Cancer Drug 11:201–208

Kapitza S, Pongratz M, Jakupec MA, Heffeter P, Berger W, Lackinger L, Keppler BK, Marian B (2005) Heterocyclic complexes of ruthenium(III) induce apoptosis in colorectal carcinoma cells. J Cancer Res Clin Oncol 131:101–110

Kersten L, Bräunlich H, Keppler BK, Gliesing C, Wendelin M, Westphal J (1998) Comparative nephrotoxicity of some antitumour-active platinum and ruthenium complexes in rats. J Appl Toxicol 18:93–101

Kljun J, Petricek S, Zigon D, Hudej R, Miklavcic D, Turel I (2010) Synthesis and Characterization of Novel Ruthenium(III) Complexes with Histamine. Bioinorg Chem Appl 183097:1–6

Mali B, Jarm T, Snoj M, Sersa G, Miklavcic D (2013a) Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. Eur J SurgOncol 39:4–16

Mali B, Miklavcic D, Campana LG, Cemazar M, Sersa G, Snoj M, Jarm T (2013b) Tumor size and effectiveness of electrochemotherapy. Radiol Oncol 47:32–41

Marty M, Sersa G, Garbay JR, Gehl J, Collins CG, Snoj M, Billard V, Geertsen PF, Larkin JO, Miklavcic D (2006) Electrochemotherapy—an easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. Eur J Cancer (Suppl 4): 3-13

Miklavcic D, Snoj M, Zupanic A, Kos B, Cemazar M, Kropivnik M, Bracko M, Pecnik T, Gadzijev E, Sersa G (2010) Towards treatment planning and treatment of deep-seated solid tumors by electrochemotherapy. Biomed Eng Online 9:10

Miklavcic D, Sersa G, Brecelj E, Gehl J, Soden D, Bianchi G, Ruggieri P, Rossi CR, Campana LG, Jarm T (2012) Electrochemotherapy: technological advancements for efficient electroporation-based treatment of internal tumors. Med Biol Eng Comput 50:1213–1225

Miklavcic D, Mali B, Kos B, Heller R, Sersa G (2014) Electrochemotherapy: from the drawing board into medical practice. Biomed Eng Online 13:29

Mir LM, Gehl J, Sersa G, Collins CG, Garbay JR, Billard V, Geertsen PF, Rudolf Z, O'Sullivan GC, Marty M (2006) Standard operating procedures of the electrochemotherapy: instructions for the use of bleomycin or CDDP administered either systemically or locally and electric pulses delivered by the CliniporatorTM by means of invasive or non-invasive electrodes. Eur J Cancer 4:14–25

Orlowski S, Belehradek J Jr, Paoletti C, Mir LM (1988) Transient electropermeabilization of cells in culture: increase of the cytotoxicity of anticancer drugs. Biochem Pharmacol 37(24):4727–4733

Pavliha D, Kos B, Marcan M, Zupanic A, Sersa G, Miklavcic D (2013a) Planning of electroporation-based treatments using web-based treatment-planning software. J Membrane Biol 246:833–842

Pavliha D, Music MM, Sersa G, Miklavcic D (2013b) Electroporation-based treatment planning for deep-seated tumors based on automatic liver segmentation of MRI images. PLoS ONE 8(8):e69068

Pucihar G, Kotnik T, Valic B, Miklavcic D (2006) Numerical determination of transmembrane voltage induced on irregularly shaped cells. Annals Biomed Eng 34:642–652

Sava G, Zorzet S, Turrin C, Vita F, Soranzo MR, Zabucchi G, Cocchietto M, Bergamo A, DiGiovine S, Pezzoni G, Sartor L, Garbisa S (2003) Dual action of NAMI-A in inhibition of solid tumor metastasis: selective targeting of metastatic cells and binding to collagen. Clin Cancer Res 9:1898–1905

Seelig MH, Berger MR, Keppler BK (1992) Antineoplastic activity of three ruthenium derivatives against chemically induced colorectal carcinoma in rats. J Cancer Res Clin Oncol 118:195–200

Sersa G, Cemazar M, Miklavcic D, Mir LM (1994) Electrochemotherapy: variable anti-tumor effect on different tumor models. Bioelectrochem Bioenerg 35:23–27

Sersa G, Cemazar M, Miklavcic D (1995) Antitumor effectiveness of electrochemotherapy with cis-diamminedichloroplatinum(II) in mice. Cancer Res 55:3450–3455

Sersa G, Krzic M, Sentjurc M, Ivanusa T, Beravs K, Kotnik V, Coer A, Swartz HM, Cemazar M (2002) Reduced blood flow and oxygenation in SA-1 tumours after electrochemotherapy with cisplatin. Brit J Cancer. 87:1047–1054

Sersa G, Miklavcic D, Cemazar M, Rudolf Z, Pucihar G, Snoj M (2008) Electrochemotherapy in treatment of tumours. Eur J Surg Oncol 34:232–240

Speelmans G, Sips WHHM, Grisel RJH, Staffhorst RWHM, Fichtinger-Schepman AMJ, Reedijk J, de Kruijff B (1996) The interaction of the anti-cancer drug cisplatin with phospholipids is specific for negatively charged phospholipids and takes place at low chloride ion concentration. Biochim Biophys Acta 1283:60–66

Spugnini EP, Citro G, Porrello A (2005) Rational design of new electrodes for electrochemotherapy. J ExpClin Cancer Res 24(2):245–254

Todorovic V, Sersa G, Mlakar V, Glavac D, Flisar K, Cemazar M (2011) Metastatic potential of melanoma cells is not affected by electrochemotherapy. Melanoma Res 21:196–205

Todorovic V, Sersa G, Mlakar V, Glavac D, Cemazar M (2012) Assessment of the tumourigenic and metastatic properties of SK-MEL28 melanoma cells surviving electrochemotherapy with bleomycin. Radiol Oncol 46(1):32–45

Zorzet S, Bergamo A, Cocchietto M, Sorc A, Gava B, Alessio E, Iengo E, Sava G (2000) Lack of in vitro cytotoxicity, associated to increased G2-M cell fraction and inhibition of matrigel invasion, may predict in vivo-selective antimetastasis activity of ruthenium complexes. JPET 295(3):927–933