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Short communication

Combined therapy of the antimetastatic compound NAMI-A and electroporation on B16F1 tumour cells in vitro

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Abstract

Ruthenium complex NAMI-A [ImH][*trans*-$RuCl_4$(DMSO-S)Im] (Im = imidazole) is a potential chemotherapeutic drug in cancer treatment. Electroporation can be used to facilitate delivery of NAMI-A into cells. Suspension of B16F1 tumour cells from mouse melanoma in NAMI-A solution was exposed to a train of electric pulses. The effect of NAMI-A was determined by examining cell viability in clonogenic test. Our results show that electroporation increases the otherwise scarce in vitro effects of NAMI-A, i.e. reduces cell viability. At the conditions chosen for experiments 90% of cells survived in the presence of 1 μM NAMI-A, whereas in a combined treatment with 1 μM NAMI-A and electroporation only about 10% of cells survived.

Keywords: NAMI-A; Electroporation; In vitro; Electrochemotherapy; B16F1

1. Introduction

It is known that short and sufficiently strong electric field pulses temporarily increase permeability of the cell membranes providing facilitated access of exogenous molecules into cells and tissues. Exposure of cells to electric pulses presumably results in formation of hydrophilic pores in cell membrane. This phenomenon is termed electroporation or electropermeabilization. Therefore membrane permeability is increased and non-selective transport of otherwise non-permeant molecules trough cell membrane is enabled. Electroporation is a new drug delivery approach and it is used in electrochemotherapy [1,2]. The efficiency of electroporation depends on the parameters of the external electric field: the shape and the number of pulses delivered, pulse duration, pulse amplitude and electric field strength [3]. When all these parameters are properly chosen electroporation causes transient state where cell membranes after electric treatment reseal and cells return to their normal physiological state. This is the so-called reversible electroporation. In case of irreversible electroporation cell membrane cannot reseal and cell death due to membrane's damages is observed [2].

Different chemotherapeutic agents have been tested in combination with permeabilizing electric pulses [4,5]. Bleomycin and cisplatin proved to be the most effective in this combination. Bleomycin is clinically used in the treatment of head and neck squamous carcinoma, Hodgkin and non-Hodgkin lymphoma, and testicular carcinoma. The major limiting effect of bleomycin is lung fibrosis that develops when a certain cumulated dose is reached. Bleomycin does not diffuse across plasma membrane and some authors suggested that the cell endocytic activity plays a role in cellular uptake of this drug [6]. Since bleomycin induces DNA double-strand cleavages it has to enter the cell to reach the target. The use of cell electropermeabilization proved to be a way to bypass the restriction imposed by plasma membrane to cell uptake of non-

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permeant cytotoxic drugs. It was also demonstrated that electroporation increases radiosensitising effect of cisplatin [7] and bleomycin [8].

Cisplatin and other platinum-based compounds are known as the most efficient metal containing anticancer drugs used in the treatment of many different human cancers. However, since cisplatin therapy is usually accompanied by rather strong side effects, and considering that several tumours are – or become – resistant to cisplatin, new non-platinum antitumour drugs (containing ruthenium [9] but also tin, gold, titanium and other metals [10] have been developed with the aim of obtaining a broader spectrum of activity and reduced side effects. Ruthenium compounds have gained a particular interest for their potential application on tumours which developed resistance to cisplatin or in which cisplatin is inactive [11]. Moreover, some ruthenium complexes bear an interesting property, showing a strong activity against metastases of solid metastasising tumours [12] with a mechanism related to the modulation of cell invasion processes [13]. The most active of these compounds, namely imidazolium *trans*-imidazoledimethylsulfoxidetetrachlororuthenate, [ImH][*trans*-$RuCl_4$(DMSO-S)Im] (Im = imidazole) nicknamed NAMI-A, has already completed a phase I clinical trial showing also some activity on non-small cell lung cancer (NSCLC) cell line [14]. In general, however, NAMI-A is scarcely cytotoxic on cell cultures in in vitro tests.

The behaviour of NAMI-A under physiological conditions was studied extensively but the final targets and the mechanism of action of NAMI-A have not yet been defined. It is known that, under physiological conditions, the complex is transformed into more reactive, aquated species by stepwise chloride dissociation [15,16]. The resulting species may bind various biomolecular targets (e.g. albumin) already in plasma [17]. Although extracellular cell membrane components have been proposed as binding sites [17,18], NAMI-A was shown to activate intracellular pathways leading to G2M cell cycle arrest, this effect being attributed to the reaching of an appropriate intracellular threshold (A. Bergamo, Callerio Foundation Onlus, personal communication to G. Sava). It is not clear how NAMI-A enters the cell, but it was hypothesized that this can be possible either by passive diffusion and/or by an active transport [19].

We therefore wanted to test whether electroporation, which is known to increase transmembrane transport, may modify cell cytotoxicity of NAMI-A, as determined by a clonogenic assay. To do that, we chose the B16 melanoma line on which previous studies in vitro have shown lack of meaningful direct cytotoxicity of NAMI-A. On the other hand, in vivo NAMI-A shows a selective activity against solid metastases that develop in the lungs of mice bearing B16 melanoma [20].

2. Experimentals

2.1. Compound and cell line

NAMI-A was prepared according to a patented procedure [21] and was dissolved in electroporation buffer that consisted of 10 mM potassium phosphate buffer (PB), 1 mM $MgCl_2$ with added 250 mM sucrose until isoosmolarity was reached (EP-buffer). The solution was sterilized by filtration through 0.22 μm filter. For each experiment a fresh solution of NAMI-A was prepared immediately before its use.

2.1.1. Tumour cell line

An established B16F1 cell line was cultured in Eagle's minimum essential medium with Earles salts (EMEM) (Life Technologies Ltd, Paisley, UK), supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Diesenhofen, Germany), 2 mM L-glutamine (Sigma-Aldrich Chemie GmbH), benzyl penicillin (Crystacillin, Pliva d.d., Zagreb, Croatia) and gentamicin sulphate (Lek d.d., Ljubljana, Slovenia). Cells were grown in monolayers at 37 °C in humidified atmosphere with 5% CO_2 for 3–4 days to obtain confluent culture from which cell suspension in electroporation buffer was prepared.

2.2. Effects of NAMI-A on cell clonogenic activity

The cell suspension of confluent culture was mixed with different concentrations of NAMI-A (1 μM, 10 μM and 100 μM) and incubated for 1 h at 37 °C. After the incubation, cells were diluted in EMEM growth medium and plated in Petri dishes for the clonogenic test (200 cells per Petri dish). NAMI-A free control cell suspensions were handled in the same way. Colonies grown in Petri dish for 6 days were then fixed with methanol for 4 min (Merck KgaA, Darmstadt, Germany) and stained with crystal violet (Sigma-Aldrich Chemie GmbH). Finally, the stain was rinsed with water, and plates were air-dried. All colonies in Petri dishes were counted. Cytotoxicity of NAMI-A at different concentrations was given as NAMI-A free control. Each experiment in which all concentrations of NAMI-A were tested was repeated three times in triplicates.

2.3. Electroporation

Prior to electroporation, the cell suspension of confluent culture (2×10^7 cells/ml) was incubated in EP-buffer or in NAMI-A of chosen concentration for 15 min at room temperature.

2.3.1. Determination of working pulse amplitude

To determine the voltage that would not essentially reduce cell survival a 50 μl droplet of cell suspension containing 10^6 cells was placed between stainless steel plate electrodes 2 mm apart. Before each experiment electrodes were cleaned with alcohol, rinsed with sterile physiological solution and dried with sterile gauze. A train of eight square electric pulses and a voltage from 80 V to 240 V in 40 V steps was applied. Pulses of 100 μs duration were delivered at 1 s interval (i.e. repetition frequency of 1 Hz). Electric pulses were generated by a medical device Cliniporator™ (Igea S.r.l., Carpi (MO) Italy). Control drops of cell suspensions were put between plate electrodes without any voltage application. When electroporation was completed, each drop of cell suspensions was put in a single well on a 24-well plate. After incubation at room temperature for 15 min, cells were diluted in 950 μl of Spinner modification

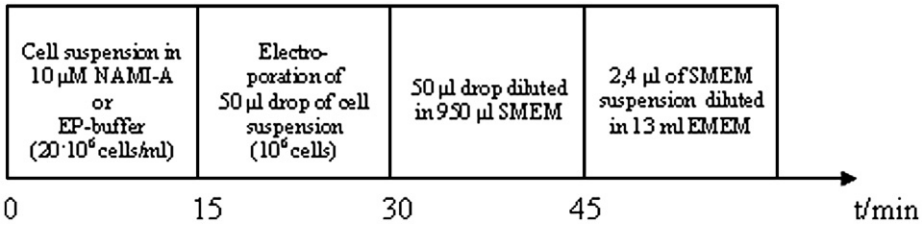

Fig. 1. Treatment time schedule. Cells were first incubated in EP-buffer or NAMI-A for 15 min. One drop of cell suspensions was then electroporated and 15 min after pulse delivery this 50 μl drop was diluted in 950 μl of SMEM and another 15 min later in 13 ml of EMEM with 10% fetal bovine serum. Finally EMEM with pulsed cells was distributed in 3 Petri dishes–4 ml per Petri dish.

of Eagle's minimum essential medium (SMEM), incubated for 15 min before they were finally diluted in 13 ml EMEM with 10% fetal bovine serum and plated in Petri dishes for the clonogenic test. Treatment schedule is presented in Fig. 1 and it is similar to the procedure already employed in the study of electroporation with bleomycin [22]. Non-pulsed cell suspensions were used to control the effect of electroporation on cell survival. Cells were treated in the absence or presence of 10 μM NAMI-A.

2.3.2. Combined treatment: electroporation and NAMI-A

To determine the survival of cells in a combined treatment with NAMI-A and electroporation, the cell suspension was mixed with different concentrations (from 0.01 to 50 μM) of NAMI-A. Pulse voltage applied during electroporation was 160 V as determined in experiment of working electric pulse amplitude (Fig. 2). Other steps of the treatment protocol were the same as described above (Fig. 1).

The cell suspension of non-pulsed cells diluted only in EP-buffer and placed between the electrodes served as the control. Survival of cells in this suspension represented 100% of survival. Treated cells were: exposed to NAMI-A for 15 min, exposed to NAMI-A for 60 min and exposed to NAMI-A for 15 min and electroporation pulses (combined treatment). Each experiment for each chosen parameter was performed in triplicate and repeated three times.

3. Results

3.1. Cytotoxicity of NAMI-A

The clonogenic test was carried out to determine the sensitivity of B16F1 cells to 60 min exposure to NAMI-A. In this test we assumed that in a single cell suspension only a living cell represents a colony forming unit and it will retain the ability to divide. When, after exposure to a cytotoxic compound, cells are incubated in Petri dishes, in 6 days each survived cell gives rise to a visible colony.

B16F1 cells were exposed to three different concentrations of NAMI-A from 1 to 100 μM. When cells were incubated in 1 μM and 10 μM NAMI-A solutions, only a small decrease of cell survival was observed; however, no colonies were observed in Petri dishes with cells exposed to 100 μM NAMI-A (Table 1).

B16F1 cells were exposed to 1 μM, 10 μM and 100 μM NAMI-A for 60 min before the colony forming test was performed. Each value is the mean of three separate experiments ± standard error (S.E.). For further examination where we wanted to determine the effect of different electric pulse amplitudes on cell survival in the presence and in the absence of NAMI-A 10 μM concentration of NAMI-A was chosen.

For experiments with electroporation (see below — combined treatment), NAMI-A concentration ranged from 0.01 μM to 100 μM.

Table 1
B16F1 cell survival after 60 min exposure to different concentrations of NAMI-A

NAMI-A concentration	Cell survival (% of control ± S.E.)
0 mM	100
1 mM	87 ± 6
10 mM	81 ± 3
100 mM	0

3.2. Determination of working electric pulse amplitude

Electropermeabilization of membrane depends on the amplitude of electric pulses. Amplitudes in range from 0 V to 240 V showed to preserve reproductive potential of cells. Survival of non-treated cells was around 90% (Fig. 2, empty triangles). Higher pulse amplitudes increase cell permeability. Therefore in combination of electroporation with the cytotoxic compound NAMI-A the survival of cells is decreasing with increasing the pulse amplitude (Fig. 2, filled circles).

The survival of cells, treated with 10 μM NAMI-A for 15 min and electric pulses (filled circle), was normalized to the survival of the cells without any treatment. The amplitudes of the applied electric pulses were investigated and for each pulse amplitude an equal train of pulses was applied. Based on these results, we chose for further testing of the combined treatment (see below) the pulse amplitude of 160 V (0.8 kV/cm) as it is high enough to cause cell membrane permeabilization of about 90% of cells followed by cell resealing and almost 100% cell survival which is seen after electroporation in the absence of NAMI-A in Fig. 2. Under these conditions, the application of electroporation significantly modified cell cytotoxicity of NAMI-A.

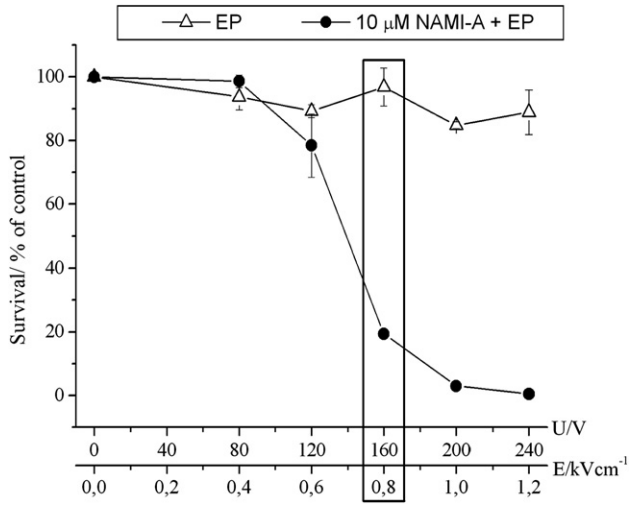

Fig. 2. Effect of different electric pulse amplitudes on cell survival in the presence or in the absence of 10 μM NAMI-A. A train of eight rectangular pulses with repetition frequency of 1 Hz and duration of 100 μs, was delivered. Different pulse amplitudes with 80 V, 120 V, 160 V, 200 V or 240 V were applied. Control cell suspensions were only put between electrodes and further handled in the same way as drops of pulsed cell suspensions. Values are means of three experiments ± S.E.

3.3. Combined treatment: electroporation and NAMI-A

The effect of electroporation on B16F1 cells was already described by Kanduser et al. [23]. We determined 160 V (0.8 kV/cm) as our working pulse amplitude and explored the effect of electroporation in combination with NAMI-A on B16F1 cells.

The data reported in Fig. 3 show the survival of B16F1 cells in 3 treatments as a function of NAMI-A concentration. In the combined treatment with NAMI-A and electroporation (filled squares), in which electric pulses were delivered after incubation of cells with NAMI-A for 15 min, and irrespectively of the working concentration of NAMI-A, the lowest survival fraction was thus obtained in combination with electroporation as compared to the other two treatments i.e. treatment with NAMI-A alone (filled circles and empty triangles).

4. Discussion

The application of NAMI-A with electroporation significantly reduces viability of B16F1 cells in vitro. Pulse voltage applied during electroporation was 160 V (0.8 kV/cm; electrode distance, 2 mm) and caused cell membrane permeabilization without seriously affecting reproductive potential of the cells. Therefore, in these conditions in combined treatment with electroporation and the antimetastatic drug NAMI-A we observed a marked decrease in cell survival as compared to that of cells that were only electroporated or incubated with NAMI-A (Fig. 3).

In fact, when a concentration of NAMI-A as low as 0.1 μM was used in combination with electroporation, only about 45% of cells survived the combined treatment. When NAMI-A concentration was raised to 1 μM cell clonogenicity further decreased to 12%, which became 5% at 50 μM, that is a killing efficiency 20-times greater than that observed after cell challenges with NAMI-A alone for up to 60 min. A possible explanation for this activity is that electroporation might allow an increased transfer of NAMI-A from the incubation mixture into the cells, giving rise to the observed increase of cell cytotoxicity.

Pluim et al. [24], comparing in vitro cytotoxicity, intracellular accumulation and DNA binding of cisplatin and NAMI-A, showed that NAMI-A was about 1000 times less cytotoxic than cisplatin, and DNA binding and intracellular accumulation of NAMI-A were lower than those of cisplatin. Since binding of NAMI-A to plasma proteins decreases NAMI-A bioavailability for cells and therefore reduces its biological activity [25], increasing the intracellular concentration of NAMI-A should increase its cytotoxicity. Provided that the reduced cell clonogenicity here observed might not be the simple consequence of a cytotoxic effect but it might be the result of a modified cell behaviour, already shown for NAMI-A [18,19], leading to the normalization of B16F1 cells with a reduced dividing ability, electroporation might be a tool to potentiate the effects of NAMI-A also in vivo. Considering the basal responsiveness of B16 melanoma metastases to NAMI-A [20], the in vivo study of the combination of this drug with electroporation should be expected to give interesting results, particularly considering the pattern of metastasis formation of melanoma tumours in humans where very often they are easily accessible in the skin of the patients.

The results obtained in the present study seem to stress the validity of the hypothesis that the application of electric pulses, already shown to increase cell membrane permeability to other cytotoxic drugs [7,8], allows even micromolar concentrations of the selective antimetastatic ruthenium drug NAMI-A to markedly reduce viability [26].

In order to further confirm this hypothesis, studies are in progress to evaluate the effects in vivo by applying electroporation

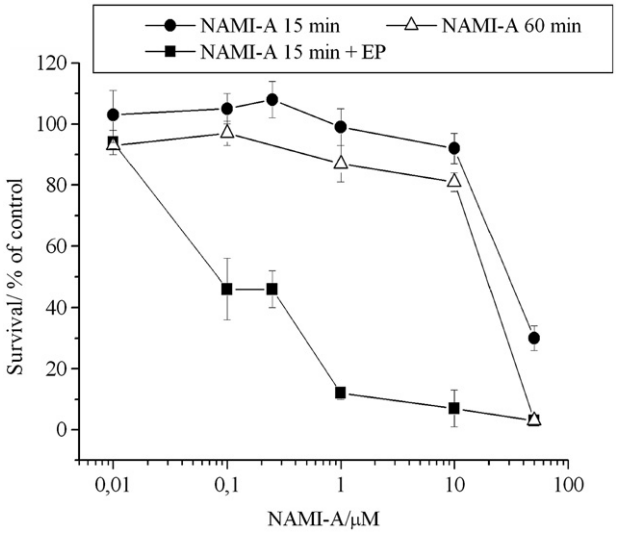

Fig. 3. Survival curves of B16F1 cells after combined treatment with different NAMI-A concentrations (μM) and electroporation (EP) (8 pulses, duration 100 μs, repetition frequency 1 Hz), and after incubation in NAMI-A for 15 or 60 min.

to the primary tumour of mice receiving systemic administrations of NAMI-A.

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References

[1] L.M. Mir, S. Orlowski, Mechanisms of electrochemotherapy, Adv. Drug Deliv. Rev. 35 (1999) 107–118.
[2] E. Neumann, The relaxation hysteresis of membrane electroporation, in: E. Neumann, A.E. Sowers, C.A. Jordan (Eds.), Electroporation and Electrofusion in Cell Biology, Plenium Press, New York, 1989, pp. 61–82.
[3] M.P. Rols, J. Teissié, Electropermeabilization of mammalian cells. Quantitative analysis of the phenomenon, Biophys. J. 58 (1990) 1089–1098.
[4] G. Sersa, M. Cemazar, D. Miklavcic, Antitumor effectiveness of electrochemotherapy with *cis*-diamminedichloroplatinum(II) in mice, Cancer Res. 55 (1995) 3450–3455.
[5] S. Pendas, M.J. Jaroszeski, R. Gilbert, M. Hyacinthe, V. Dang, J. Hickey, C. Pottinger, P. Illingworth, R. Heller, Direct delivery of chemotherapeutic agents for the treatment of Hepatomas and sarcomas in rat models, Radiol. Oncol. 32 (1998) 53–64.
[6] L.M. Mir, O. Tounekti, S. Orlowski, Bleomycin: revival of an old drug, Gen. Pharmacol. 27 (1996) 745–748.
[7] S. Kranjc, M. Cemazar, A. Grosel, J. Scancar, G. Sersa, Electroporation of LPB sarcoma cells in vitro and tumors in vivo increases the radiosensitizing effect of cisplatin, Anticancer Res. 23 (2003) 275–282.
[8] S. Kranjc, M. Cemazar, A. Grosel, M. Sentjurc, G. Sersa, Radiosensitising effect of electrochemotherapy with bleomycin in LPB sarcoma cells and tumors in mice, BMC Cancer 5 (2005) 115.
[9] E. Alessio, G. Mestroni, A. Bergamo, G. Sava, Ruthenium anticancer drugs, in: H. Siegl (Ed.), Metal ions in Biological Systems, Metal Complexes in Tumor Diagnosis and as Anticancer Agents, Marcel Dekker, New York, 2004, pp. 323–351, (0).
[10] B.K. Keppler (Ed.), Metal Complexes in Cancer Chemotherapy, Wiley-VCH Verlag GmbH, Weinheim, 1993.
[11] M. Galanski, V.B. Arion, M.A. Jakupec, B.K. Keppler, Recent developments in the field of tumor-inhibiting metal complexes, Curr. Pharm. Des. 25 (2003) 2078–2089.
[12] G. Sava, E. Alessio, A. Bergamo, G. Mestroni, Sulfoxide ruthenium complexes: non-toxic tool for the selective treatment of solid tumour metastases, in: P.J. Sadler (Ed.), Metallopharmaceuticals I: DNA Interactions, Springer, Berlin, 1999, pp. 154–169.
[13] E. Alessio, G. Mestroni, A. Bergamo, G. Sava, Ruthenium antimetastatic agents, Curr. Top. Med. Chem. 4 (2004) 1525–1535.
[14] J.M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J.H. Beijnen, J.H. Schellens, A phase I and pharmacological study with imidazolium-*trans*-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent, Clin. Cancer Res. 10 (2004) 3717–3727.
[15] G. Mestroni, E. Alessio, G. Sava, S. Pacor, M. Coluccia, A. Boccarelli, Water-soluble ruthenium(III)-dimethyl sulfoxide complexes: chemical behaviour and pharmaceutical properties, Met.-Based Drug. 1 (1994) 41–63.
[16] L. Messori, F. Kratz, E. Alessio, The interaction of the antitumor complexes Na[*trans*-$RuCl_4$ (DMSO) (Im)] and Na[*trans*-$RuCl_4$ (DMSO) (Ind)] with apotransferrin: a spectroscopic study, Met.-Based Drug. 3 (1996) 1–63.
[17] L. Messori, P. Orioli, D. Vullo, A. Enzo, E. Iengo, A spectroscopic study of the reaction of NAMI-A, a novel ruthenium(III) anti-neoplastic complex, with bovine serum albumin, Eur. J. Biochem. 267 (2000) 1206–1213.
[18] P.J. Dyson, G. Sava, Metal-based antitumour drugs in the post genomic era, Dalton Trans. 16 (2006) 1929–1933.
[19] F. Frausin, M. Cocchietto, A. Bergamo, V. Scarcia, A. Furlani, G. Sava, Tumor cell uptake of metastasis inhibitor ruthenium complex NAMI-A and its in vitro effects on KB cells, Cancer Chemother. Pharmacol. 50 (2002) 405–411.
[20] B. Gava, S. Zorzet, P. Spessotto, M. Cocchietto, G. Sava, 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 (2006) 284–291.
[21] G. Mestroni, E. Alessio, G. Sava, New salts of anionic complexes of Ru (III) as antimetastatic and antineoplastic agents, International Patent PCT C 07F 15/00, A61K 31/28, WO 98/00431.
[22] T. Kotnik, A. Macek-Lebar, D. Miklavcic, L.M. Mir, Evaluation of cell membrane electropermeabilization by means of a nonpermeant cytotoxic agent, BioTechniques 28 (2000) 921–926.
[23] M. Kanduser, M. Sentjurc, D. Miklavcic, Cell membrane fluidity related to electroporation and resealing, Eur. Biophys. J. 35 (2006) 196–204.
[24] D. Pluim, R.C. van Waardenbur, J.H. Beijnen, J.H. Schellens, Cytotoxicity of the organic ruthenium anticancer drug NAMI-A is correlated with DNA binding in four different human tumor cell lines, Cancer Chemother. Pharmacol. 54 (2004) 71–78.
[25] A. Bergamo, L. Messori, F. Piccioli, M. Cocchietto, G. Sava, Biological role of adduct formation of the ruthenium(III) complex NAMI-A with serum albumin and serum transferrin, Invest. New Drugs 21 (2003) 401–411.
[26] A. Bergamo, B. Gava, E. Alessio, G. Mestroni, B. Serli, M. Cocchietto, S. Zorzet, G. Sava, Ruthenium-based NAMI-A type complexes with in vivo selective metastasis reduction and in vitro invasion inhibition unrelated to cell cytotoxicity, Int. J. Oncol. 21 (2002) 1331–1338.