The role of cisplatin and NAMI-A plasma-protein interactions in relation to combination therapy

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Received September 9, 2005; Accepted November 2, 2005

Abstract. The aim of the study is to evaluate the differences of protein binding of NAMI-A, a new ruthenium drug endowed with selective antimetastatic properties, and of cisplatin and to ascertain the possibility to use two drugs based on heavy metals in combination to treat solid tumour metastases. For this purpose, we have developed a technique that allows the proteins, to which metal drugs bind, to be identified from real protein mixtures. Following incubation with the drugs, the bands containing platinum and/or ruthenium are separated by native PAGE, SDS-PAGE and 2D gel electrophoresis, and identified using laser ablation inductively coupled plasma mass spectrometry. Both drugs interact with essentially the same proteins which, characterised by proteomics, are human serum albumin precursor, macroglobulin α2 and human serotransferrin precursor. The interactions of NAMI-A are largely reversible whereas cisplatin forms stronger interactions that are less reversible. These data correlate well with the MCa mammary carcinoma model on which full doses of NAMI-A combined with cisplatin show additive effects as compared to each treatment taken alone, independently of whether NAMI-A precedes or follows cisplatin. Furthermore, the implication from this study is that the significantly lower toxicity of NAMI-A, compared to cisplatin, could be a consequence of differences in the mode of binding to plasma proteins, involving weaker interactions compared to cisplatin.

Introduction

Cisplatin and the second-generation platinum drug, carboplatin, are among the most successfully used anticancer drugs available today, being used for treating a number of different cancers, including testicular, ovarian, oropharyngeal and bronchogenic cancers, cervical and bladder carcinomas, lymphoma, osteosarcoma, melanoma and neuroblastoma (1). However, platinum drugs have a high general toxicity which leads to a number of side effects, among which nephrotoxicity is particularly severe (2,3). Numerous complexes based on metals other than platinum have been synthesised in order to ameliorate the therapeutic properties of cisplatin and to reduce its adverse effects, and some of them reached early clinical evaluation (4), but failed to reach the clinic because almost all showed relevant toxicity, even more severe than that of cisplatin (5). Ruthenium-based anticancer drugs are proving to be the exception (6) and one of them, ImH[Ru(Im)(Me_2SO)Cl_4] (NAMI-A), shows a strong efficacy against solid tumour metastases (7,8) with a general toxicity lower than that of cisplatin, particularly on the kidney, which completely recovers from mild epithelial damage after drug withdrawal (2). NAMI-A has recently concluded a phase I clinical evaluation, showing a relatively low toxicity and the absence of renal toxicities at the maximum tolerated dose (9).

The most common method of cisplatin administration is intravenous injection. However, other administration methods have been evaluated in order to reinforce its effect, and also to reduce the side effects. The interaction of cisplatin with proteins has been investigated in some detail (10,11) and it would appear that non-specific binding to thiol residues on protein surfaces could take place. A large body of information describing how platinum drugs induce cytotoxicity by cross-linking DNA, causing structural changes that inhibit replication and protein synthesis has also accumulated (12-14).

In general, data on the mechanism of action of NAMI-A seem to exclude DNA as the primary target for its activity on metastases (15-17), and binding to DNA is far weaker than that of platinum complexes (18). Conversely, NAMI-A was shown to bind tightly to serum albumin and serum transferrin, two representative plasma proteins (19,20), suggesting that binding to specific proteins may represent the molecular basis for its peculiar biological activity. It was also suggested that binding of ruthenium(III) complexes to serum transferrin might help to target cancer cells taking advantage of the specific receptor-binding mechanism of transferrin, but these results remain controversial (21,22).

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Key words: cisplatin, ruthenium, cancer, combination therapy

Comparing the plasma proteins' interactions with the platinum compound, cisplatin, and the newly developed ruthenium compounds should contribute significantly to the understanding of the delivery, mode of action, toxicity and side effects of the investigated drugs. We therefore decided to use a protocol developed in our laboratory (23) to study these systems *in vitro* but under the complex environment of the real system, and to discuss the results in the light of the pharmacological effects of cisplatin and NAMI-A, used alone or in combination, in a mouse model of solid metastasising tumour.

Materials and methods

In vitro experiments. Human plasma, human serum albumin (HSA) and transferrin were purchased from Sigma (St. Louis, MO, USA), bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). A montage albumin deplete kit was purchased from Millipore (Volketswil, Switzerland) and trypsin was purchased from Promega (Wallisellen, Switzerland). Cis-diamineplatinum(II) (cisplatin) was purchased from Lancaster (UK) and NAMI-A was prepared using a procedure from the literature (24). All other chemicals were purchased from Sigma unless otherwise stated.

Incubation and electrophoresis. HSA in human plasma was depleted using a montage albumin deplete kit. Human plasma (20 μl) was diluted using the equilibration buffer (180 μl) supplied with the kit and the albumin was depleted according to the kit procedure. The total protein concentration was measured using a quick start Bradford protein assay (Bio-Rad, Reinach, Switzerland). The depleted plasma was separated into 4 eppendorf vials (150 μl per vial), one vial was used as control, the others were supplemented with cisplatin (90 μM), NAMI-A (90 μM), or cisplatin (90 μM) and NAMI-A (90 μM). Albumin-depleted human plasma was incubated with the drugs for 24 h at 37˚C in a thermomixer (eppendorf). After incubation, the samples were stored at -20˚C prior to separation by electrophoresis. The samples were mixed with sample buffer (1:4 ratio), either denaturizing sample buffer containing SDS and DTT for SDS-PAGE, or with non-denaturing sample buffer for native PAGE. Each sample (20 μl, approximately 1.8 μg/μl total proteins) was applied to a Bio-Rad Tris-HCl 4-20% gel, and separated either with separating buffer containing SDS for denaturing SDS-PAGE, or buffer without SDS for native PAGE. Prior to LA-ICP-MS, the gels were dried overnight between two cellophane sheets. The sample of human plasma incubated overnight with cisplatin was also subjected to two-dimensional electrophoresis. The sample was mixed with rehydration buffer (9 M urea, 2% CHAPS, 0.8% Pharmalytes 3-10, 15 mM DTT) and applied to an immobilized pH gradient strip (IPG, Amersham). The sample was allowed to rehydrate to the IPG strip overnight and then isoelectric focusing (IEF) was performed using the following program: 14 h at 30 V for rehydration, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 4 h at 8000 V (34,120 Vh total). At the end of IEF, the strip was equilibrated with SDS in two 15-min equilibration stages (25) and the proteins were resolved on vertical 8% SDS-PAGE for second dimension. Proteins resolved on SDS-PAGE were stained with Coomassie brilliant blue R (Sigma).

For the quantification of platinum and ruthenium bound to certain proteins, 76 μm of HSA and BSA and 64 μm transferrin were incubated with a 10-fold concentration of cisplatin or NAMI-A, or with both cisplatin and NAMI-A. After incubation overnight, 5 μg of proteins from each sample were separated with 4-20% SDS-PAGE and stained with Coomassie blue. The gel was dehydrated with 100% acetonitrile and dried in air, the dried gel was soaked evenly with rhodium solution at 10 ppm/g gel, used as an internal standard. A linear curve was prepared, with 1, 10 and 50 ppm/g gel of platinum and ruthenium (cisplatin and NAMI-A) soaked in gel pieces simultaneously with rhodium at 10 ppm/g gel, then the gel pieces were dried between two cellophane sheets were used for the preparation of a linear standard curve.

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). A PE SCIEX Elan 6100 DRC ICP-MS coupled with Geolas laser system equipped with lambda physic laser technology of a 193-nm UV laser was used. The instrument was operated at 10 Hz/sec, flounce of 19 J/CM^2, and pits diameter size of 120 microns. Helium was used as the carrier gas with a flow rate of 1.1 l/min, and nebulizer gas at a 0.99 l/min flow rate.

Tryptic digestion of protein PAGE bands. Protein bands excised from the gel were rehydrated with buffer and destained with acetonitrile (50 mM ammonium bicarbonate, 1:1). The destained bands were dehydrated and reswilled with DTT (10 mM) for reduction (30 min, 56˚C) and alkylated with iodoacetamide (100 mM) in the dark at room temperature (30 min). Following further washes with bicarbonate buffer, the proteins were digested overnight at 37˚C with Promega trypsin (12.5 ng/μl in 25 mM bicarbonate buffer). Peptides were extracted with 5% formic acid, followed by acetonitrile, dried and resuspended in 0.1% formic acid (10 μl) prior to analysis by ESI-MS.

Mass spectrometry. 1D HPLC-MS/MS was conducted on an LCQ DECA XP Surveyor LC-MS system (ThermoFinnigan, San Jose, CA). Chromatographic separation was performed on a C18 column (5 μm, 100 μm i.d, 100 mm) with a flow rate of 200 nl/min. The mobile phase consisted of water and MeCN, and both contained 0.05% (v/v) formic acid. The HPLC column eluent was eluted directly into the electrospray ionization source of the LCQ-Deca ion trap mass spectrometer. Automated peak recognition, dynamic exclusion, and daughter ion scanning of the top three most intense ions were performed using Xcalibur software as described previously (26,27). Spectra were scanned over a range of 500-1800 mass units. The excitation energy for the precursor ions selected for collision-induced dissociation was set at 35% (using the operational parameter '% relative collision energy') with a 1-sec activation time. Spectra were interpreted by searches of product ion mass against the Swissprot database using the Sequest algorithm.

In vivo experiments. The MCa mammary carcinoma line of CBA mouse was originally obtained from the Rudjer Boskovic Institute, Zagreb, Croatia (28). The tumour lines were locally maintained by serial biweekly passages of 10^6 viable tumour

cells, obtained from donors similarly inoculated two weeks earlier, and injected i.m. (intra-muscularly) into the calves of the left hind legs of CBA adult mice. Tumour propagation for experimental purposes was similarly made using CBA mice obtained from a locally established breeding colony, grown according to the standard procedure for inbred strains. Briefly, 2.5 g of freshly removed tumour was minced with scissors, finely dispersed in Dulbecco's phosphate buffer saline, Ca^{2+} and Mg^{2+}-free (PBS), and filtered through a double layer of sterile gauze. After centrifugation at 250 x g for 10 min, pellets were re-suspended in an equal volume of PBS and cell viability was checked by the trypan-blue exclusion test: only cell suspensions with at least 55-60% viable cells were used.

In vivo treatments. For *in vivo* studies, both compounds were dissolved in isotonic non-pyrogenic physiological saline and given to mice by i.p. (intraperitoneal) administration. NAMI-A was used at 35 mg/kg/day on days 7-12 (experiment 1) or on days 12-17 (experiment 2) after tumour implant. Cisplatin was given at 8 mg/kg on day 16 (experiment 1) or on day 8 (experiment 2). Compounds were dissolved in an appropriate volume of physiological saline calculated in order to administer 0.1 ml/10 g of body weight.

Primary tumour growth and lung metastasis evaluation. Primary tumour growth was determined by calliper measurements, by determining two orthogonal axes and calculating the tumour volume using the following formula: $(\pi/6)xa^2xb$, (a<b) and assuming tumour density equal to one. The evaluation of the number and weight of lung metastases, spontaneously formed from the i.m. tumour implants, was performed at day 21 from tumour implantation, after sacrificing the animals by cervical dislocation. The number of lung colonies were counted by carefully examining the surface of the lungs, dissected into the five lobes, washed with PBS and examined under a low power microscope equipped with a calibrated grid. The weight of each metastatic nodule was calculated by applying the same formula used for primary tumours; the sum of these individual weights gives the total weight of the metastatic tumour per animal.

Animal studies. Animal studies were carried out according to the guidelines in force in Italy (DDL 116 of 21/2/1992) and in compliance with the Policy on Human Care and Use of Laboratory Animals, National Institutes of Health, Department of Health and Human Services, Rockledge Drive, Bethesda, MD, USA.

Statistical analysis. Data were submitted to computer-assisted statistical analysis using ANOVA analysis of variance and Tukey-Kramer post-test.

Results

Pt and Ru protein interaction. Platinum and ruthenium profiles were similar in SDS- and native PAGE, although there was a more intense signal for ruthenium binding to transferrin (Fig. 1A and C). However, there were notable differences in the profiles and intensities between the SDS- and native PAGE gels (Fig. 1). In the native gel, dimers of albumin and transferrin were observed and corresponding intense signals for platinum and ruthenium were detected (Fig. 1A, C and E). Taking into account this observation, it is possible to say, overall, that the signal for platinum and ruthenium is more intense in the native gel compared to the SDS-PAGE.

Table I. Concentration of platinum and ruthenium bound to serotransferrin, human serum albumin and bovine serum albumin.

			Cisplatin + NAMI-A ppm/g gel	
	Cisplatin Pt ppm/g gel	NAMI-A Ru ppm/g gel	Cisplatin	NAMI-A
Transferrin	9.6±0.71	1.4±0.08	7.8±0.66	1.2±0.06
HSA	9.0±0.68	1.6±0.08	10.0±0.58	1.7±0.01
BSA	10.6±0.60	1.4±0.10	7.8±0.36	1.3±0.02

Concentration of platinum and ruthenium in ppm/g gel. The drugs cisplatin and NAMI-A incubated separately as in column 2 and 3 with transferrin, HSA, or BSA, or together as in column 4/5. The difference between cisplatin incubated separately or with NAMI-A and the difference between NAMI-A incubated separately or with cisplatin are not significant.

Preferential binding of cisplatin and NAMI-A to plasma proteins was studied by adding both drugs simultaneously to plasma proteins (Fig. 1E and F). It is evident that the peak intensity of the platinum corresponding to HSA is higher than the intensity of the ruthenium peak in SDS-PAGE (Fig. 1F). Such a difference is absent from the spectrum of native PAGE (Fig. 1E). On the contrary, the ruthenium peak intensity is higher in the area of the serotransferrin and macroglobulin $\alpha 2$ of native PAGE compared to the intensity of platinum.

Since the bands shown in Fig. 1 are broad and could potentially contain more than one type of protein, two-dimensional electrophoresis was performed which confirmed that the identified proteins, serotransferrin and HSA, are indeed the major proteins that bind platinum; also demonstrating that treatment of proteins toward two-dimensional electrophoresis does not have a significant effect on the binding of the different drugs (Fig. 2).

In order to quantify the binding level of cisplatin and NAMI-A to transferrin, HSA and BSA, a linear curve with r^2=0.999 was prepared from 1, 10 and 50 ppm of cisplatin and NAMI-A (see Materials and methods). Subsequently, the amount of platinum and ruthenium corresponding to cisplatin and NAMI-A, was quantified and we found that the platinum concentration was approximately five times higher than the ruthenium concentration in the proteins (Table I). Competitive binding of cisplatin and NAMI-A has been tested by incubating the different proteins with both cisplatin and NAMI-A, which shows that the binding levels are similar to the binding level of the drugs incubated separately with the proteins, probably indicating that the drugs bind to the proteins at different sites or in different ways.

Antitumour and antimetastatic activity of the combination therapy. Treatment of mice bearing MCa mammary carcinoma

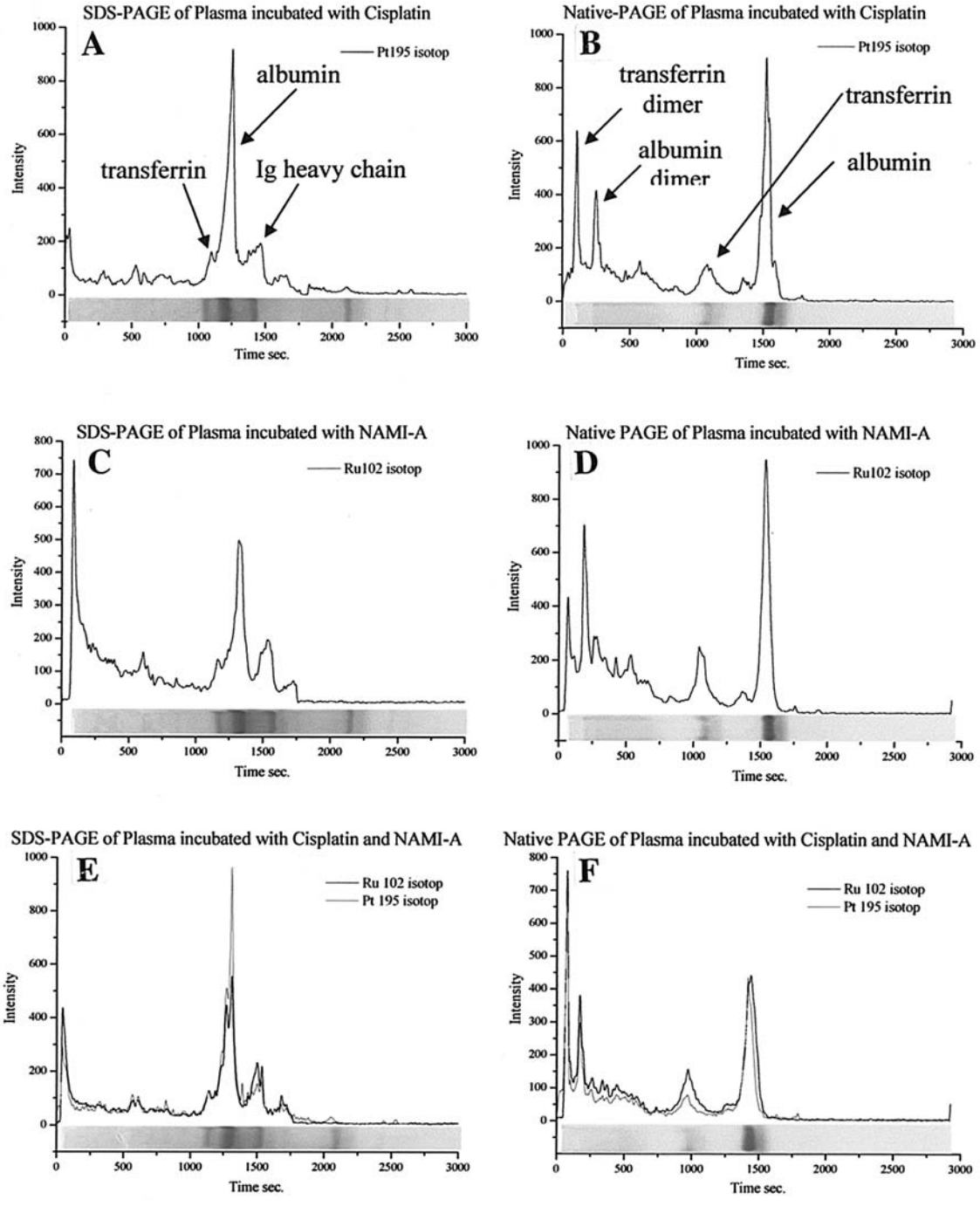

Figure 1. LA-ICP mass spectra/gel profiles of plasma incubated with cisplatin, SDS-PAGE (A); cisplatin, native-PAGE (B); NAMI-A, SDS-PAGE (C); NAMI-A, native-PAGE; (D); cisplatin/NAMI-A, SDS PAGE; (E) cisplatin/NAMI-A, native PAGE (F).

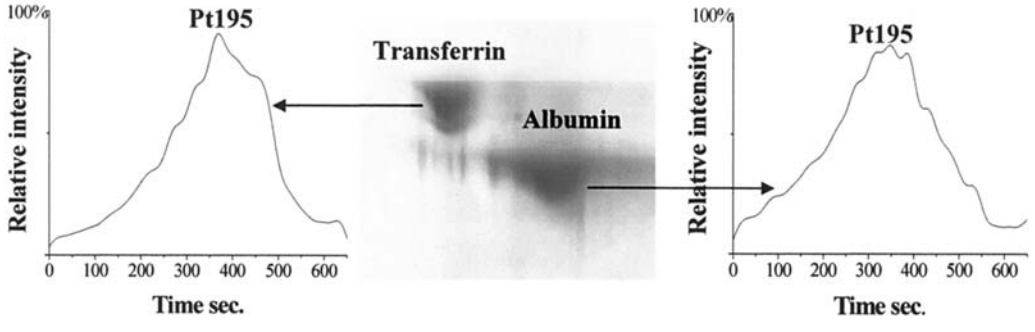

Figure 2. 2D SDS-PAGE of human plasma incubated with cisplatin; section showing serotransferrin and human serum albumin and the corresponding LA-ICP-MS of platinum.

Table II. Variation of body weight and of primary tumour weight following the combination treatments with cisplatin and NAMI-A.

Treatment group	Body weight (%)	Primary tumour weight (%)
1st experiment		
NAMI-A alone (35 mg/kg/day on days 7-12)	-10.0	-19.5
Cisplatin alone (8 mg/kg on day 16)	-13.1	-25.3
NAMI-A (35 mg/kg/day on day 7) + cisplatin (8 mg/kg on day 16)	-21.9	-28.6
2nd experiment		
Cisplatin alone (8 mg/kg on day 8)	-8.00	-50.0
NAMI-A alone (35 mg/kg/day on days 12-17)	-4.90	-34.4
Cisplatin (8 mg/kg on day 8) + NAMI-A (35 mg/kg/day on days 12-17)	-15.1	-59.0

48 CBA female mice, implanted i.m. with $1x10^6$ MCa mammary carcinoma cells on day 0, were randomly divided into 4 groups and given i.p. 35 mg/kg/day NAMI-A on days 7-12, or 8 mg/kg cisplatin on day 16, or both drugs (experiment 1). Control mice were given the vehicle (NaCl 0.9%) on days 7-12 and on day 16. In the 2nd experiment, the treatment with cisplatin, on day 8, preceded the treatment with NAMI-A on days 12-17. Data are expressed as percent of variation between before and after treatment versus untreated controls.

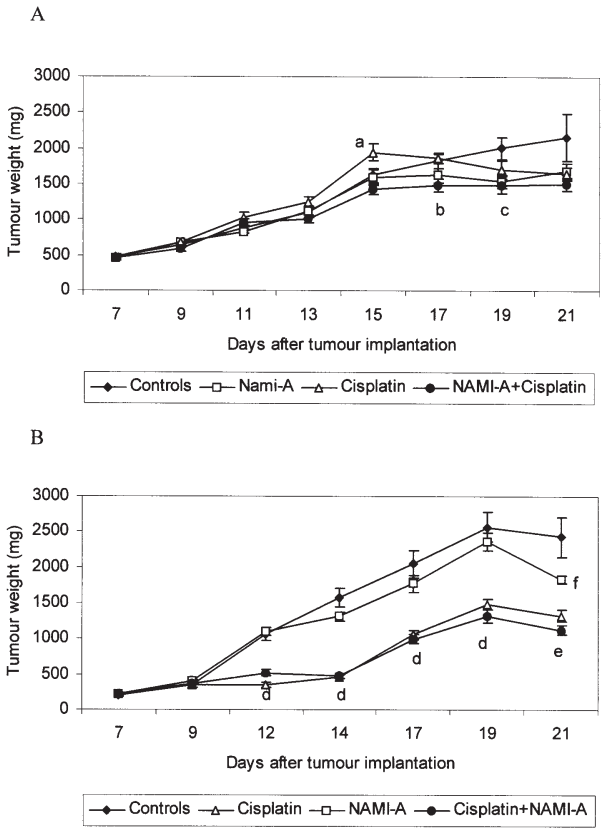

Figure 3. Primary tumour growth. CBA female mice (48), implanted i.m. with $1x10^6$ MCa mammary carcinoma cells on day 0, were randomly divided into 4 groups and given i.p. 35 mg/kg/day NAMI-A on days 7-12, or 8 mg/kg cisplatin on day 16, or both drugs (A, experiment 1). Control mice were given the vehicle (NaCl 0.9%) on days 7-12 and on day 16. In a 2nd analogous experiment (B), the treatment with cisplatin on day 8 preceded the treatment with NAMI-A on days 12-17. [Statistics: One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test: a, cisplatin $p<0.01$ vs NAMI-A + cisplatin; b, NAMI-A + cisplatin $p<0.05$ vs controls and vs cisplatin; c, NAMI-A + cisplatin $p<0.05$ vs controls; d, cisplatin and cisplatin + NAMI-A $p<0.001$ vs controls and vs NAMI-A; e, cisplatin and cisplatin + NAMI-A $p<0.001$ vs controls; f, NAMI-A $p<0.05$ vs controls and vs cisplatin + NAMI-A].

with cisplatin or NAMI-A, or with a combination of the two drugs with NAMI-A preceding cisplatin or vice-versa, showed different degrees of toxicity, in terms of lack of body weight gain during the experiment (Table II). In general, each treatment alone shows only a low toxicity as expected by the use of maximally tolerated doses. When combined, the toxicity increases and is essentially equal to the sum of the individual toxicity shown by each single agent. Considering the effects on the primary tumour, determined by comparing the tumour progression in the treated groups with that of the untreated controls, it is possible to say that, as expected, NAMI-A is only weakly active while cisplatin showed a more pronounced antitumour effect when treatment was performed on early-stage tumours rather than on advanced tumours.

The effect on the primary tumour can be more fully appreciated from Fig. 3, which summarises the time curves of primary tumour growth. The data plotted in Fig. 3 show that cisplatin stops tumour growth after administration, independently of whether it was given on early tumours (B) or on advanced tumours (A). In addition, these data show that the administration of NAMI-A prior or after cisplatin-treatment did not modify the MCa mammary carcinoma response to cisplatin.

Conversely, the combination treatment showed significant effects compared to the results obtained with each drug, used as a single agent, on lung metastasis formation and growth (Table III). In particular, NAMI-A alone showed remarkable antimetastatic activity, causing approximately 30% of animals to be free of macroscopically detectable lung metastasis, independently of whether it was given to mice with early or advanced tumours. Cisplatin also showed reasonable anti-metastatic activity although slightly less pronounced than that of NAMI-A. However, the antimetastatic effect of cisplatin differs to that of NAMI-A, as can be appreciated by considering the ratio between metastasis weight and metastasis number, showing NAMI-A to be much more effective than cisplatin in reducing metastasis dimensions (0.07-0.065 mg for NAMI-A and 0.41-0.35 mg for cisplatin). When treatments with these two drugs were combined in the same animals, a significantly higher effect was observed in terms of the number of animals free of macroscopically detectable lung metastases, which increased to approximately 60% of the treated animals. If NAMI-A precedes cisplatin, the result

Table III. Lung metastasis evaluation.

	Metastases		
	Number	Weight (mg)	Animals free (%)
1st experiment			
Controls (NaCl 0.9% on days 7-12 and 16)	22.1±5.63	12.2±5.87	0
NAMI-A alone (35 mg/kg/day on days 7-12)	7.83±2.49[a]	0.580±0.140[a]	33.3
Cisplatin alone (8 mg/kg on day 16)	9.33±2.88	3.84±1.28	0
NAMI-A (35 mg/kg/day on day 7) + cisplatin (8 mg/kg on day 16)	7.75±2.13[a]	0.500±0.140[a]	60
2nd experiment			
Controls (NaCl 0.9% on days 8 and 12-17)	58.7±11.0	42.2±9.80	11.1
Cisplatin alone (8 mg/kg on day 8)	11.0±2.20[b]	3.90±1.60[b]	33.3
NAMI-A alone (35 mg/kg/day on days 12-17)	15.3±4.60[b]	1.00±0.300[b]	30.0
Cisplatin (8 mg/kg on day 8) + NAMI-A (35 mg/kg/day on days 12-17)	7.70±5.20[b]	0.500±0.300[b]	62.5

CBA female mice (48), implanted i.m. with $1x10^6$ MCa mammary carcinoma cells on day 0, were randomly divided into 4 groups and given i.p. 35 mg/kg/day NAMI-A, or 8 mg/kg cisplatin on the days indicated, or both drugs. Control mice were given the vehicle (NaCl 0.9%) on the same days. Lung metastases were determined on day 21. [Statistics: One-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison test: [a]$p<0.05$ vs controls; [b]$p<0.001$ vs controls].

shows a pronounced increase as compared to the single treatment (60.0% vs 33.3%) and, when cisplatin preceded NAMI-A, the number of animals free of macroscopically detectable lung metastasis was equal to the sum of those observed in the groups treated with each single agent alone (62.5% vs 63.3%).

Discussion

The need for general screening methods to test metal drug-protein interactions in the biological system is increasing in order to cover the wide and diverse range of compounds and for high throughput analysis and evaluation of newly developed drugs or existing drugs in novel combinations. Since the function of metal drugs is highly influenced by plasma proteins, we decided to examine the plasma protein-drug interactions for two important drugs, cisplatin and NAMI-A, and compare the results to pharmacological data in order to see if a connection could be made between *in vitro* and *in vivo* systems. Such a goal is particularly important since NAMI-A failed *in vitro* evaluation and could easily have been omitted from further evaluation (negative data from the NCI protocols for the screening of chemical agents on the 60-panel cell lines). In order to directly compare the interaction of cisplatin and NAMI-A with plasma proteins, samples of the two drugs, and a mixture of the two, were incubated with human plasma proteins (in which the albumin had been depleted) for 24 h at 37˚C. Following incubation, the plasma protein-drug samples were partially separated using native PAGE and SDS-PAGE gel electrophoresis in order to delineate differences between covalent and non-covalent binding of the drugs with the plasma proteins. The gels were then analysed by laser ablation inductively coupled plasma mass spectrometry, in which the gel is scanned with a laser, which ablates the sample, and the resulting desorption plume is extracted under vacuum, converted to a plasma, and the elemental composition of the plasma is determined by mass spectrometry. ICP-MS is a powerful and highly sensitive technique for elemental analysis, and has been widely used when interfaced with HPLC for the analysis of metals in biological samples (30,31). Combined with laser ablation, ICP-MS has been used to analyse metallo-, seleno- or phospho-proteins resolved by PAGE (26,27,32,33).

Accordingly, elements such as platinum and ruthenium, which are not naturally present in living systems are readily identified and quantified. The spectra obtained from the LA-ICP-MS experiments, superimposed with their corresponding gels, are shown in Fig. 1, from which a global view of the binding of the drugs to the plasma proteins can be immediately appreciated, thereby allowing a direct comparison between the two drugs.

In order to identify the chief proteins that interact with cisplatin and NAMI-A, the protein bands corresponding to the most intense platinum and ruthenium peaks were excised from the gels and the proteins were identified using proteomics methods (see below). Three major platinum and ruthenium peaks are present in the profile of plasma separated with SDS-PAGE, which correspond to three different proteins, viz. human serum albumin (HSA) precursor, human serotransferrin precursor and macroglobulin α2. These proteins were characterised using LC-MS-MS with the Sequest algorithm search of the spectra against the Swissprot database, and the identity of the proteins was confirmed with 49.3% amino acid coverage of HSA precursor, 32.7% of human serotransferrin and 34.7% of macroglobulin α2.

Four major platinum and ruthenium peaks are present in the profile of the plasma separated on native PAGE which correspond to HSA precursor (observed as monomer and dimer) and human serotransferrin precursor (observed as monomer and dimer), and characterised with essentially the same % amino acid coverage.

Analysing and comparing the differential binding of cisplatin and NAMI-A drugs to plasma proteins resolved on PAGE, adopting the methodology that had been employed previously to analyse platinum binding plasma proteins and platinum binding *E. coli* whole cell proteins (23,34), we obtained spectra remarkably similar for both drugs, although the level of cisplatin binding is approximately 5-fold higher than that of NAMI-A. The most intense and prominent signals come from HSA and transferrin, and the results are consistent with pharmaceutical and plasma protein studies which show that albumin and transferrin correspond to the proteins that bind platinum and ruthenium anti-cancer drugs (14,19,35). The native binding of both drugs to the plasma proteins is similar. However, using a reducing agent, such as DTT, and SDS for the denaturising of proteins, the drug binding is interrupted, only partially but to a greater extent for the ruthenium compound. Dual incubation of plasma with cisplatin and NAMI-A appears to exhibit no competition between the platinum and ruthenium drugs as it is possible to observe similar signals either with the drugs incubated alone or in combination. This implies that the different drugs bind to different sites on the proteins, at least in the case of transferrin and albumin.

This study represents the first report comparing the quantitative binding of cisplatin and NAMI-A, using LA-ICP-MS. The data reported in this study are consistent with reports in the literature that the binding level of NAMI-A is similar either to transferrin or to albumin (35). With the results here, it is possible to see that cisplatin also binds to transferrin and albumin in approximately equal quantities. It has been proposed that albumin is able to tightly bind at least five ruthenium equivalents when BSA is incubated with NAMI-A (19). The above assumption could be true for binding quantification under native conditions. However, the covalent binding quantification in this study shows that NAMI-A bind almost ten times less than cisplatin to either transferrin or albumin.

Combination therapy represents one of the most successful ways to treat cancer and cisplatin is usually administered together with other drugs for increased effect. It is likely that once NAMI-A progresses further through clinical trials it may prove to be more effective in preventing metastases when administered with cisplatin, which induces apoptosis. Since binding to plasma proteins is implicated in the transport mechanism for both drugs (9,29), it is interesting to see whether the simultaneous addition of both drugs to plasma results in significant differences in binding, i.e. cisplatin could block NAMI-A binding to certain proteins or *vice versa*, even if in any potential combination therapy the drugs are administered at different times. Concerning the relevance of plasma protein binding of cisplatin and NAMI-A for their activity against tumour growth, the combined experiments performed in mice with MCa mammary carcinoma seem to suggest the lack of any negative influence, independently of whether NAMI-A is given prior to or after cisplatin, and the overall result is globally better than that of each treatment taken alone. The increase of host toxicity, in terms of lack of body weight gain, is probably due to the intrinsic nature of these compounds, both being based on a heavy metal and thus being toxic to the kidneys. Although NAMI-A toxicity on kidneys is reversible upon a sufficient wash-out, we cannot exclude the possibility that the irreversible damage caused by cisplatin could be more severe and long lasting after combined treatment with NAMI-A. However, the pharmacological data reported here demonstrate the compatibility of the concomitant treatment with two metal-based drugs (the platinum drug, cisplatin, and the ruthenium compound, NAMI-A) for treating malignant carcinomas. The overall effect observed is in fact the sum of the combination of the cytotoxic efficacy of cisplatin and of the metastasis inhibition of NAMI-A in both experiments performed, independently of the sequence of administration of the two drugs. Thus, they would appear to maintain their individual binding sites on plasma proteins without any interference from each other (as shown by the LA-ICP-MS study), and they also maintain their individual targets and mechanisms of action on cancer cells. These results highlight the potential usefulness for a compound such as NAMI-A for the treatment of disseminated tumours in combination with conventional cytotoxic drugs.

Acknowledgements

This study was supported by contributions from the Swiss Cancer League (ligue Suisse contre le cancer), EPFL, LINFA laboratory and the COST D20 Action. We thank Enzo Alessio for the generous donation of NAMI-A.

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