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Influence of chemical stability on the activity of the antimetastasis ruthenium compound NAMI-A

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Abstract

The influence of chemical stability on the antimetastatic ruthenium(III) compound imidazolium *trans*-imidazoletetrachlorodimethylsulphoxideruthenium(III) (NAMI-A) in aqueous solution was studied both *in vitro* and *in vivo*. The loss of dimethylsulphoxide (DMSO) ligand from the compound was tested by using a NAMI-A solution acidified with HCl at pH 3.0 and aged for 0, 4, 8 and 24 h prior to intraperitoneal (i.p.) injection into CBA mice bearing advanced MCa mammary carcinoma. The activity of NAMI-A on lung metastases showed no change even after the loss of DMSO ligand from up to 50% of the molecules. The reduction of NAMI-A did not modify the number of KB cells blocked in the $S + G_2M$ phases, independent of whether the reduction occurred outside the cells or after loading the cells with the compound prior to treatment with the reductants (ascorbic acid, glutathione or cysteine). *In vivo*, the complete reduction of NAMI-A with equivalent amounts of ascorbic acid, glutathione or cysteine prior to administration to mice bearing advanced MCa mammary carcinoma was more active than NAMI-A alone. The data show that NAMI-A, although undergoing a series of chemical modifications, maintains its antimetastatic activity in a broad range of experimental conditions.

Keywords: Ruthenium; Metabolism; Tumour; Metastasis; Experimental

1. Introduction

trans-imidazolete-trachlorodimethylsuphoxideruthenium(III) (NAMI-A) recently entered phase I clinical trials as an antimetastatic drug [1,2]. To implement these studies, a series of chemical and biological investigations aimed at establishing the best administration mode of the compound and understanding the nature of its active species were undertaken.

NAMI-A is in fact known to undergo a series of hydrolytic processes in aqueous solution, the nature and rate of which is strongly pH-dependent [3]. In physiological conditions (phosphate buffer, pH = 7.4, NaCl 0.9%), NAMI-A is relatively labile and undergoes stepwise hydrolysis of up to two chlorides in two well-separated steps (Fig. 1); hydrolysis of the first chloride occurs in approximately 40 min at 25 °C, while that of the second chloride is slower and occurs in approximately 2 h at 25 °C. We have clear experimental evidence that the rate of the first hydrolytic step is catalysed by the Ru(II) species. The second chloride hydrolysis is also accompanied by the slow partial dissociation of the neutral dimethylsulphoxide (DMSO) ligand, and by a progressive darkening of the solution which is attributed to the formation of the oxo-bridged polymeric species. The rate of chloride hydrolysis is markedly pH-dependent and becomes almost negligible in moderately acidic solutions (pH range 5–3), such as those obtained by dissolving NAMI-A in distilled water. Under these conditions, NAMI-A is quite inert and the slow hydrolysis of DMSO is the main reaction occurring at a rate of approximately 2% per hour (Fig. 1); acidification of the solution to pH 3.0 and the addition of 5% DMSO reduces that loss to approximately 0.8% per hour (data kindly supplied by POLYtech, Area Science Park, Trieste, Italy).

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Moreover, biological reductants might also reduce *in vivo* NAMI-A to Ru(II) species [3]. Owing to the presence of *S*-bound DMSO, which is a moderate π-acceptor of electron density, NAMI-A has a relatively high reduction potential (+235 mV versus standard saturated calomel electrode (SSCE)) [4]. Accordingly, when stoichiometric amounts of biological reductants are added to a physiological solution of NAMI-A, rapid and complete reduction to the Ru(II) species occurs. The reduction of NAMI-A is accompanied by a dramatic change in the colour of the solution, which turns from the original deep yellow-orange colour to pale yellow (Fig. 2). The nature of the reduced species and their evolution with time was determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. NAMI-A is reduced to the corresponding dianionic Ru(II) species [*trans*-$RuCl_4$(DMSO-*S*)Im]$^{2-}$ which then undergoes stepwise hydrolysis of the chloride ligands. The monoaquo complex [*mer*-$RuCl_3(H_2O)$(DMSO-*S*)Im]$^-$ is the main species in the first 2 h after reduction. During this time, no loss of either DMSO or imidazole was detected, which indicated that reduction of the metal centre caused a reinforcement of the (DMSO-*S*)-Ru-Im axis. The reduced species are also stable with respect to air oxidation.

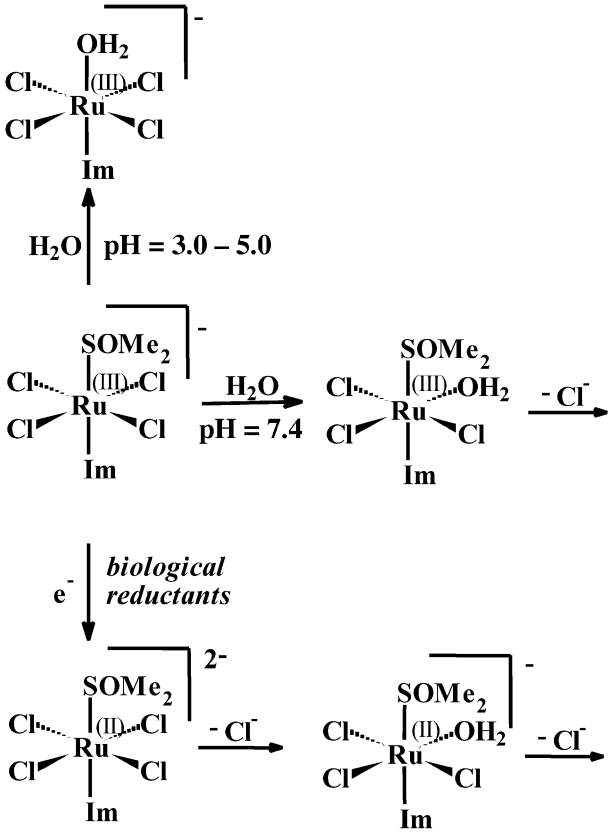

Fig. 1. Chemical behaviour of NAMI-A in aqueous solution. Top: in a moderately acidic solution; middle: at physiological pH; bottom: after complete reduction to the Ru(II) species.

Therefore, NAMI-A is a coordinately saturated species and, as such, probably inactive since the active species must be one (or more) of its metabolites. Thus, depending on the mode of administration, the drug *in vivo* might be the sum of a series of '*in vitro* generated metabolites' which might affect or be responsible for its activity.

In our work, we verified that, at physiological pH and at the concentration of NAMI-A used for biological experiments, the addition of 0.5 equivalents of the two-electron reductant, ascorbic acid or one equivalent of cysteine or glutathione were equally effective in inducing the full and immediate reduction of the complex.

The chemical transformations that NAMI-A undergoes in aqueous solution prior to administration are relevant in establishing the number and nature of the species that are actually administered; however, the chemical transformations occurring under physiological conditions are relevant in understanding the nature of the active species.

We believed that a detailed evaluation would help in determining whether the selective antimetastatic effects of NAMI-A were caused either totally or partially by its reduction, and whether the chemical transformations occurring in aqueous solution might influence the pharmacological activity [1,5] and/or toxicity [6] of this compound. The study was conducted *in vitro* on KB cells and *in vivo* on the MCa mammary carcinoma tumour. The thorough NAMI-A reduction prior to administration was obtained by using three different, biologically common reductants, ascorbic acid, glutathione and cysteine.

2. Materials and methods

2.1. Compounds

Imidazolium *trans*-imidazoledimethylsulphoxidetetrachlororuthenate, ImH[*trans*-$RuCl_4$(DMSO-*S*)Im] (NAMI-A) was prepared according to procedures already reported in Ref. [7]. The NAMI-A dose required for the *in vivo* daily administration was dissolved in isotonic apyrogenic physiological saline; when necessary, an equivalent amount of the reductant was added to the solution immediately prior to administration. NAMI-A was given to mice by intraperitoneal (i.p.) administrations of 35 mg/kg/day. NAMI-A was studied *in vitro* at a 0.1 mM concentration dissolved in calcium- and magnesium-free phosphate-buffered saline (PBS) and sterilised by filtration with a 0.2 μm filter. Ascorbic acid, glutathione and cysteine (Sigma Chemical Co, Milan, Italy) were used at equimolar doses with NAMI-A and dissolved either in PBS or in culture medium.

2.2. *Tumour lines for* in vitro *testing*

KB established cell lines (ECACC No. 86103004) were cultured according to standard procedures [8]. Vials of the original line maintained in liquid N_2 provided cells that were serially sub-cultured and used for the experiments reported.

The KB cell line was maintained in Eagle's Minimum Essential Medium with 1% Non Essential Amino Acids (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% new-born calf serum (Gibco BRL, Gaithersburg, MD, USA) and buffered with tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid (3 mM), *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid (3 mM), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (3 mM) and *N*-tris[hydroxymethyl]methylglycine (3 mM) (Sigma Chemical Co., Milan, Italy). The cell population doubling time was approximately 24 h. The culture medium contained penicillin–streptomycin solution (Sigma Chemical Co., Milan, Italy) (100 units/ml penicillin G and 100 μg/ml streptomycin). Cells from confluent monolayers were removed by 0.05% Trypsin solution (Sigma Chemical Co., Milan, Italy). Cell viability was determined by the trypan blue exclusion test.

2.2.1. In vitro *treatment*

Cells were sown in multi-well cell culture clusters. After 96 h, cells were treated with NAMI-A alone or with NAMI-A plus reductants simultaneously for 1 h at 37 °C with 5% CO_2 and 100% relative humidity. Test compounds were dissolved in isotonic saline and diluted to the required concentration immediately before use. A further test group was prepared with cells treated with reductants dissolved in the culture medium for 24 h (following the 1 h exposure to NAMI-A). NAMI-A and the reductants were used at equivalent concentrations corresponding to 0.1 mM. After the drug challenge, the incubation solutions were removed and, after washing with isotonic saline, cells were cultured in a complete medium until the analysis, which was performed 24 h later. Each experiment was done in quadruplicate and repeated at least twice.

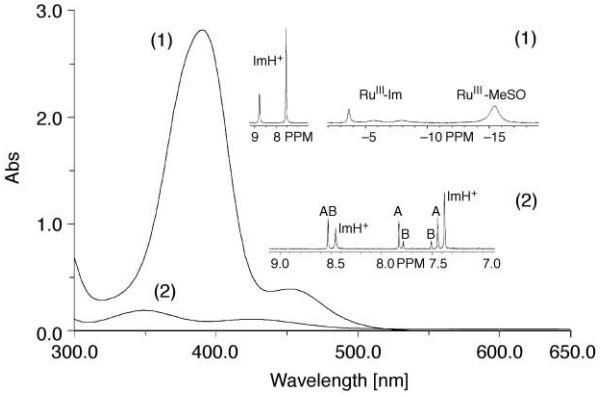

Fig. 2. Complete reduction of NAMI-A in solution by the addition of ascorbic acid. (1) Electronic spectrum and ^{1}H nuclear magnetic resonance (NMR) spectrum of NAMI-A before reduction with ascorbic acid. (2) Electronic spectrum and ^{1}H NMR spectrum of NAMI-A (region of imidazole resonance; $A = [Ru^{II}Cl_4(Me_2SO)(Im)]^{2-}$, $B = [Ru^{II}Cl_3(Me_2SO)(H_2O)(Im)]^{-}$) immediately after reduction with ascorbic acid. Conditions: 0.1 mol dm^{-3} phosphate buffer, pH 7.4, 0.14 mol dm^{-3} NaCl; the concentration of ruthenium is the same as in the pharmacological experiments.

2.2.2. *Propidium iodide (PI) test*

1×10^6 viable cells of a single cell suspension, as determined by a trypan blue exclusion test, were fixed in 70% ethanol at 4 °C for at least 1 h. Prior to the analysis, ethanol was removed by centrifugation and cells were washed twice with PBS. Cells were re-suspended in PBS containing 1 mg/ml RNase (Sigma Chemical Co., Milan, Italy) at 37 °C for 30 min and further stained with PI (40 μg/ml) (Sigma Chemical Co., Milan, Italy) for at least 30 min at room temperature in the dark (modified from Ref. [9]). Red fluorescence (610 nm) was analysed using the peak fluorescence gate to discriminate aggregates. Each analysis consisted of 10 000 counted events. Flow cytometry analyses were carried out with an EPICS® XL flow cytometer (Coulter Electronics, Miami, FL, USA). The cell cycle distribution of the cells was determined using Multicycle® software, (Phoenix Flow Systems, San Diego, CA, USA).

2.3. *Tumour line for* in vivo *testing*

MCa mammary carcinoma cells (grown in CBA mice) were used for *in vivo* testing. CBA mice were obtained from a locally established breeding colony grown according to the standard procedure for inbred strains. The tumour graft procedure for MCa mammary carcinomas was the same as that described in detail in Ref. [10]. Briefly, 10^6 tumour cells of a single cell suspension, prepared from mincing with scissors the primary tumour masses obtained from donors that had been similarly implanted 2 weeks before, were injected intramuscularly (i.m.) into the left hind leg calves of mice in the experimental groups.

2.4. In vivo *treatment*

Groups of female CBA mice implanted i.m. with 10^6 MCa mammary carcinoma cells on day 0 were given i.p. the test substances. Controls received 0.1 ml of apyrogenic sterile saline/10 g of body weight. The combined treatment with NAMI-A and reductants was carried out by simultaneously dissolving equivalent amounts of both compounds in a tube with apyrogenic sterile saline and by administering the solution to mice within the next 20 min. Lung metastases were examined on days 21 and 23 for experiments 1 and 2, respectively. In order to evaluate the influence of degradation processes on the

compound, NAMI-A was kept in solution with acidic isotonic saline (sterile, apyrogenic and acidified at pH 3.0 with HCl) with or without 5% DMSO, and maintained at room temperature and in the dark for 0, 4, 8 and 24 h prior to injection.

Swiss CD1 female mice, weighing 25–30 g and purchased from Harlan-Nossan, (S. Pietro al Natisone, Italy) were used in the toxicity studies. The doses to cause 50% death (LD50 values) were calculated according to the Litchfield and Wilcoxon method [11] in groups of 10–15 mice treated i.p. at doses increasing by a constant factor.

2.4.1. Primary tumour growth and lung metastasis evaluation

Primary tumour growth was determined by measurements of two orthogonal axes and the tumour volume was calculated by the formula: $(\pi/6)\times a^2\times b$, where a is the shorter and b is the longer axis; the tumour density was assumed to be equal to 1. Lung metastases were counted by carefully examining the surface of the lungs immediately after killing of the animals by cervical dislocation. Lungs were dissected into the five lobes, washed with PBS and examined under a low-power microscope equipped with a calibrated grid. The weight of each metastasis was calculated by applying the same formula as for the primary tumours and the sum of each individual weight gave the total weight of the metastatic tumour per animal.

2.5. Measurement of creatinine concentration

The analytical method used for the determination of creatinine concentration in the plasma was that currently in use in the hospital's routine assays for human samples. Specifically, for the colorimetric assay of Heinegard and Tiderstrom [12], appropriate kits purchased from Sigma Diagnostics (St. Louis, MO, USA) were used. Blood collection was performed by intracardiac puncture into the open chest of mice previously anaesthetised with 1.5 g/kg ethyl urethane. Nine volumes of blood were mixed directly in the syringe with 1 volume of 0.16 M trisodium citrate. Blood was immediately transferred to a glass tube and allowed to clot, centrifuged at 3000 rpm for 10 min, blood serum was removed and stored at 4 °C up until use.

2.6. Measurement of ruthenium

Ruthenium was measured in triplicate by atomic absorption spectroscopy (AAS) using a Varian SpectrAA-300 instrumentation supplied with a graphite furnace mod GTA-96, an autosampler mod PSD-96, and a specific ruthenium emission lamp (Hollow cathod lamp Varian P/N 56-101447-00). Ruthenium was measured in samples of 10 μl at 349.9 nm with an atomising temperature of 2500 °C, using argon as a purge gas at the flow rate of 3.0 l/min. Before daily analysis, a five-point calibration curve was performed by Ruthenium Custom-Grade Standard 998 μg/ml in 3.3% HCl (Inorganic Ventures Inc., Lakewood, NJ, USA).

2.7. Light transmission histology

Sections for light microscopy were prepared from paraffin-embedded kidneys, which were removed, washed in water and fixed in 10% formalin, and processed according to the standard procedure for inclusion and following rehydration (xylene, alcohol, water), with 6-μm sections being cut. Sections were stained with Cajal-Gallego, mounted in Canada Balsam and were observed with a Leitz-Orthoplan microscope. Single blind examinations were made on three different slides, each containing three slices for each sample.

2.8. Statistical analysis

The data were submitted to a computer-assisted statistical analysis using ANOVA analysis of variance and Tukey–Kramer post-test.

3. Results

3.1. Biological effects of the hydrolytic processes of NAMI-A

Data in Table 1 show that the acute toxicity of NAMI-A, dissolved in 0.9% NaCl, acidified with 1 N HCl to pH 3.0 and added with 5% DMSO, was significantly reduced in the Swiss CD1 model, compared with the solution in 0.9% NaCl alone. The similar ratio between LD_{50}, LD_{10} and $LD_{0.05}$ of the two solutions (1.3–1.6) indicated that the slope of the curve of lethality did not significantly change in the two experimental groups.

We also found that the administration of aqueous solutions of NAMI-A (dosage of 200 mg/kg), aged up to 24 h prior to injection, induced kidney toxicity, as revealed by the serum measurement of creatinine levels. Toxicity was completely prevented by the addition of 5% DMSO to the fluid used to dissolve the NAMI-A (Fig. 3).

The influence of DMSO loss from the compound on its antimetastatic activity was tested by using a NAMI-A solution acidified with HCl to pH 3.0 and aged for 0, 4, 8 and 24 h prior to i.p. injection in CBA mice bearing advanced MCa mammary carcinoma (Table 2). Solutions of NAMI-A aged up to 8 h showed no change of activity if compared with fresh solutions; in all examples, we observed no or marginal effect on the primary tumour growth and a marked reduction of metastasis

weight. A 25% decrease of activity on metastasis weight was instead obtained with solutions aged 24 h, also when 5% DMSO was added to the solution in order to reduce the hydrolysis of *S*-bound DMSO approximately from 48 to 19% during the time considered. In these conditions, no marked change of the ruthenium distribution in the tissues was detected in the organs tested (Fig. 4).

3.2. Biological effects of the reduction of NAMI-A

The *in vitro* effects of the biological reductants on the NAMI-A-induced cell cycle distribution of KB cells are reported in Fig. 5a–c. NAMI-A caused a significant increase of cells in the S phase and an even larger increase of cells in the G_2-M phase, and a corresponding decrease of cells in the G_0/G_1 phase. The addition of ascorbic acid, glutathione or cysteine to NAMI-A did not substantially modify this behaviour, either when the reductant was given to cells at the same time as NAMI-A or when it was given for 24 h following a 1-h treatment with NAMI-A.

The results of the combined treatment with NAMI-A and biological reductants *in vivo* are reported in Table 3 and pertain to two separate experiments. Regardless of the experiment and number of metastases in the control untreated group, NAMI-A caused a marked reduction in the number of metastases and an even more marked reduction in the weight of the metastases. Ascorbic acid, glutathione and cysteine used in doses equimolar to NAMI-A had no significant effects on both the number and weight of lung metastases. The treatment with NAMI-A when reduced immediately prior to administration with any of the three bio-reductants caused an

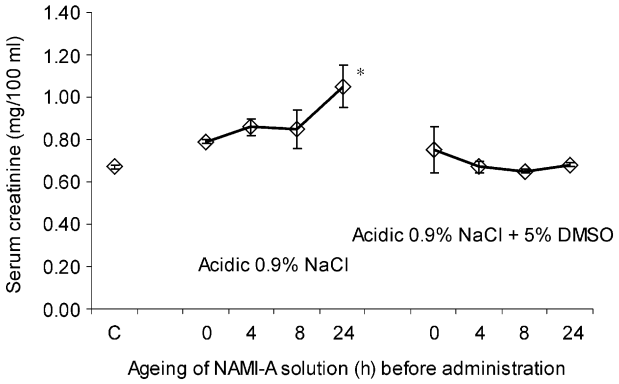

Fig. 3. Effects of NAMI-A on serum creatinine levels following acute treatment with aged formulations. Groups of three Swiss CD1 female mice of approximately 25 g were given intraperitoneally (i.p.) a solution of 200 mg/kg of body weight NAMI-A previously aged for 0, 4, 8 and 24 h in the dark and at room temperature. NAMI-A solutions were prepared with or without 5% dimethylsulphoxide (DMSO) and acidified at pH3 with 1 N HCl. Blood samples for analyses were taken 24 h after treatment. Statistical analysis: ANOVA analysis of variance and Tukey–Kramer post-test. * $P<0.05$ from untreated controls.

Table 1
Acute toxicity of NAMI-A in Swiss CD1 mice[a]

Dose (mg/kg)	Lethality (deaths/treated)	LD_{50} (mg/kg)	LD_{10} (mg/kg)	$LD_{0.05}$ (mg/kg)
NAMI-A dissolved in acidic 0.9% NaCl (pH 3.0)				
100	0/10			
141	0/10			
200	2/15			
282	14/15			
		230	193	163
NAMI-A dissolved in acidic 0.9% NaCl (pH 3.0) with 5% DMSO				
100	0/10			
141	0/10			
200	0/15			
282	1/15			
		370[b]	282	208

DMSO, dimethylsulphoxide; LD_{50}, dose causing 50% death; LD_{10}, dose causing 10% death; $LD_{0.05}$, dose causing 0.05% death.

[a] Groups of 10 Swiss CD1 female mice were treated intraperitoneally (i.p.) with freshly prepared solutions of NAMI-A at the reported doses. The experiment was repeated at the two higher doses with five animals per group. Deaths for toxicity were recorded for up to 14 days after the last animal died. The statistical values (including confidence limits at 95%) were calculated according to the Litchfield and Wilcoxon method.

[b] Considering 100% lethality at the immediately superior dose of 400 mg/kg.

Table 2
Effects of *in vivo* treatment with aged solutions of NAMI-A on primary tumour growth and on lung metastasis formation in mice bearing MCa mammary carcinoma

Treatment group	Primary tumour (mg)	Lung metastases			
		Number	%T/C	Weight (mg)	%T/C
NAMI-A dissolved in acidic 0.9% NaCl (pH 3.0)					
Controls	2573±172	30.0±5.7	–	163±30	–
0	2152±161	12.1±3.0*	40	26±7**	16
4	1954±178	12.2±3.1*	41	23±5**	14
8	2072±184	16.9±2.8	56	32±5**	19
24	2184±92	22.2±2.6	74	50±4**	31
NAMI-A dissolved in acidic 0.9% NaCl (pH 3.0) with 5% DMSO					
Controls	2201±201	26.5±3.7	–	151±32	–
0	2205±122	15.1±3.7*	57	29±8**	19
4	2202±201	13.4±3.9*	51	29±10**	19
8	2098±141	17.4±3.9	66	34±8**	22
24	2586±175	23.5±3.7	86	57±11*	37

T/C, treated/control. Groups of eight female CBA mice, implanted intramuscularly (i.m.) with MCa mammary carcinoma on day 0 were treated intraperitoneally (i.p.) with NAMI-A on days 9–14; the primary tumour growth and lung metastasis formation were determined on days 15 and 19, respectively. Controls received 0.1 ml/10 g body weight apyrogenic sterile acidic saline (with or without 5% dimethylsulphoxide (DMSO); NAMI-A was given at 35 mg/kg/day, with solutions aged in the dark and at room temperature for 0, 4, 8 or 24 h prior to injection. Statistical analysis: ANOVA analysis of variance and Tukey–Kramer post-test. *$P<0.05$ and **$P<0.01$ from untreated controls.

even higher reduction in the metastasis growth, which was particularly evident with ascorbic acid (five of the six treated mice were free of macroscopically detectable lung metastases), but which showed a significant reduction of the metastasis weight, superior than that of NAMI-A alone, also with glutathione and cysteine (Table 3).

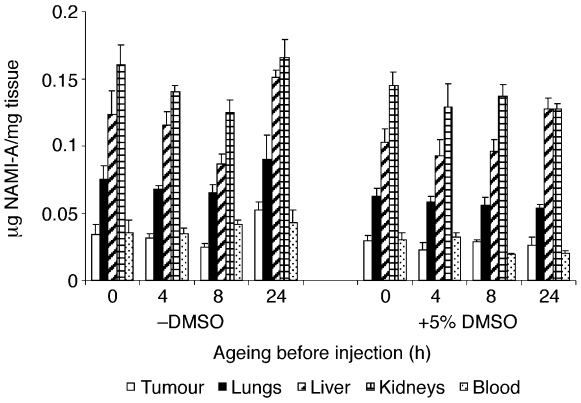

Fig. 4. Ruthenium uptake by tumour and host tissues following *in vivo* treatment with aged solutions of NAMI-A. CBA female tumour-bearing mice, weighing 20–22 g, were treated for 6 days with 35 mg/kg/day of NAMI-A kept in solution with acidic isotonic saline (sterile, apyrogenic and acidified at pH3.0 with HCl) with or without 5% dimethylsulphoxide (DMSO), and maintained at room temperature and in the dark for 0, 4, 8 and 24 h prior to injection. Blood samples were obtained by cardiac puncture from animals injected with a sub-lethal dose of ethyl urethane; tumours and organ samples were taken shortly after sacrifice of the animals. All samples mentioned above were collected 24 h after the last treatment.

The ruthenium uptake in tumour cells treated *in vitro* or in some host tissues, following *in vivo* treatment, is reported in Fig. 6. The ruthenium uptake by KB cells either treated with NAMI-A alone or combined with the biological reductant did not markedly change and the ratio of NAMI-A concentration inside and outside the cells ranged from 4.4 (NAMI-A alone) to 3.5 (NAMI-A plus ascorbic acid). Ruthenium levels in the blood and ruthenium uptake in the primary tumour and the kidneys following *in vivo* treatment for 6 consecutive days showed no difference between NAMI-A alone or NAMI-A plus glutathione or cysteine. Conversely, treatment with NAMI-A alone resulted in a higher concentration in the lungs compared with the combined treatments, whereas in the liver, the association of glutathione and NAMI-A significantly reduced the ruthenium concentration per mg of wet tissue. A pilot study aimed at evaluating the damage to the kidney glomeruli in the treated mice showed that the combination of NAMI-A with glutathione significantly reduced ($P<0.05$) the number of damaged glomeruli ($8.50\pm0.50\%$) if compared with both NAMI-A alone ($12.03\pm1.04\%$) and NAMI-A plus cysteine ($10.03\pm2.01\%$). An example of the damage to the glomeruli is given in Fig. 7.

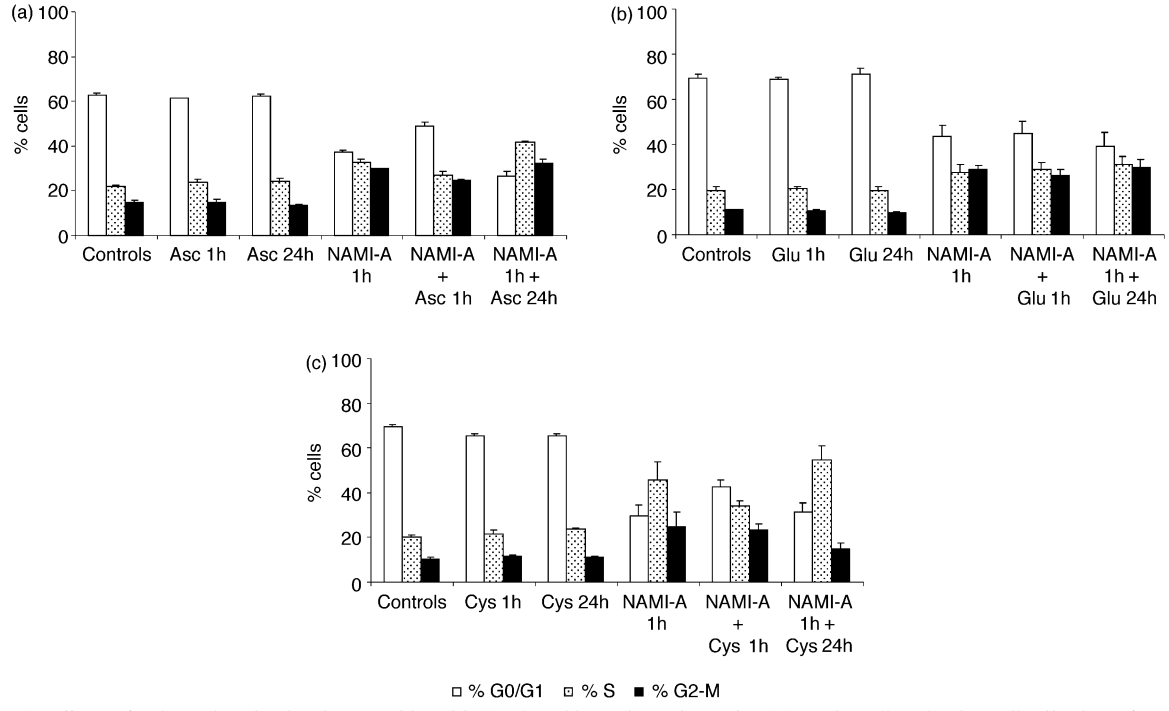

Fig. 5. (a–c). Effects of NAMI-A reduction by ascorbic acid (a), glutathione (b) and cysteine (c) on the cell cycle phase distribution of KB cells. KB cells, sown on plastic plates 96 h before, were challenged with NAMI-A 0.1 mM alone (NAMI-A 1 h), NAMI-A plus reductant simultaneously (i.e. NAMI-A + Asc 1 h), or for 24 h following the 1 h NAMI-A treatment (i.e. NAMI-A 1 h + Asc 24 h). Reductants were used at equimolar doses. Analyses were performed 120 h after cell sowing. Statistical analysis: ANOVA analysis of variance and Tukey–Kramer post-test: all groups treated with NAMI-A are statistically significant versus relevant controls.

4. Discussion

The experiments on the ageing of aqueous solutions of NAMI-A showed that only when the loss of coordinated DMSO exceeded 50% was the activity of the complex significantly affected (for solutions of NAMI-A in acidified saline aged at least 24 h before use); however, a similar significant loss of antimetastasis activity was also found after 24 h when only 20% of coordinated DMSO had dissociated by virtue of the addition of 5% DMSO to the solution. The decrease of NAMI-A activity with ageing might therefore be attributed to the subtraction of the compound by the formation of dark polymers, which also occurs in the presence of 5% DMSO; indirectly, we might also infer that both species (with and without DMSO-*S*) are probably capable of recognising the target for antimetastasis activity.

Both the marked reduction potential of NAMI-A [4] and the abundance of biological reducing agents *in vivo* (for example, the cell concentration of glutathione in its reduced form (GSH) is quite high, ranging from 1 to 8 mM) [13] imply that either the complex or its metabolites with bound DMSO-*S* might very likely undergo reduction soon after *in vivo* administration. Thus, we decided to investigate the biological effects in solutions of NAMI-A fully reduced to the corresponding Ru(II) species immediately prior to administration. It should be noted that this procedure offers the possibility to generate Ru(II) species that might be difficult to prepare from common Ru(II) precursors either as isolated species or as their hydrolytic derivatives.

We showed that treatment of tumours with the reduced form of NAMI-A appeared to be slightly more active than NAMI-A against metastasis growth, without changes affecting the toxicity in the host. Thus very likely NAMI-A is normally reduced *in vivo,* although we can not conclude that this event represents the so-called 'activation by reduction' since it seems independent of the generation of cytotoxic species *in situ*. In fact, 1 h *in vitro* exposure of tumour cells to the reduced form of NAMI-A showed no cytotoxicity as measured by the SRB test or by cell counting by the trypan blue exclusion test (data not shown). Rather, *in vitro* data evidence that, except for some qualitative changes, NAMI-A does not significantly change the cell distribution

Table 3
Effects of NAMI-A alone or combined with biological reductants on lung metastasis formation in mice bearing MCa mammary carcinoma[a]

Treatment group	Effect on lung metastases		
	Number	Weight (mg)	%T/C[b]
Experiment 1			
Controls	24.2±7.4ab	92.0±17.0cd	100
Ascorbic acid	18.3±8.6	81.5±14.0ef	86
Cysteine	35.0±13.5	94.6±26.0gh	103
NAMI-A	7.0±1.9a	15.2±2.3ceg	16
NAMI-A + ascorbic acid	4.0[c]	15.2	16
NAMI-A + cysteine	3.5±1.0b[d]	9.2±2.0dfh	10
Experiment 2			
Controls	44.2±6.6il	184.5±26.9opq	100
Glutathione	47.0±3.9mn	138.8±20.5rs	75
Cysteine	30.5±7.2	141.2±15.1[tu]	76
NAMI-A	27.5±3.0	83.6±4.5o	45
NAMI-A + glutathione	23.1±1.8im	47.1±4.6prt	26
NAMI-A + cysteine	19.2±3.4ln	44.2±7.5qsu	24

[a] Groups of six (experiment 1) or seven (experiment 2) female CBA mice, implanted intramuscularly (i.m.) with 10^6 MCa mammary carcinoma cells on day 0, were given intraperitoneally (i.p.) the test substances on days 12–17 (experiment 1) or on days 10–15 (experiment 2). Lung metastases were examined on days 21 and 23, respectively. Controls received 0.1 ml of apyrogenic sterile saline/10 g of body weight, NAMI-A was given at 35 mg/kg/day; cysteine, ascorbic acid and glutathione were given at the same molar dose as NAMI-A. The combined treatment with NAMI-A and the reductants was carried out by dissolving both compounds with apyrogenic sterile saline in a tube and by administering the solution to the mice within the next 20 minutes.

[b] Metastasis weight, treated over controls.

[c] Five out of six animals free of metastases.

[d] Two out of six animals free of metastases.

Statistical analysis: ANOVA analysis of variance and Tukey–Kramer post test. Groups with the same letter are statistically different ($P<0.05$).

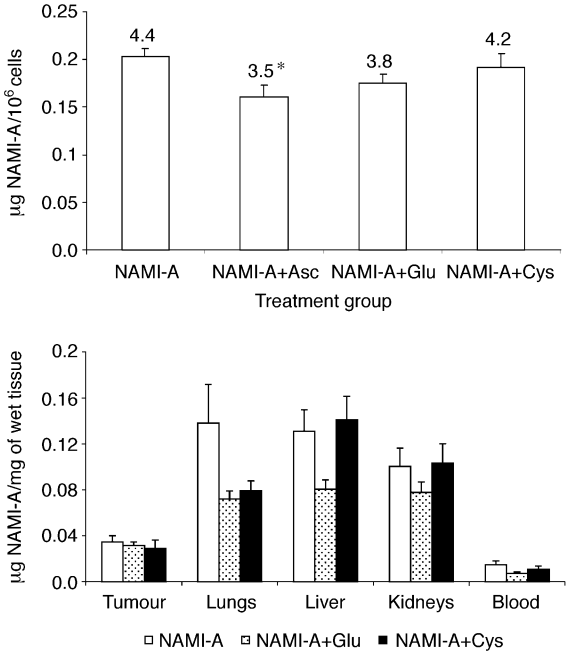

Fig. 6. Ruthenium uptake by tumour cells *in vitro* (upper panel) and by tumour and host tissues *in vivo* (lower panel). Upper panel: *in vitro* ruthenium uptake in the KB tumour cell treated as described in Fig. 5a–c was determined by atomic adsorption spectroscopy (AAS) 120 h after cell sowing. The number at the top of the histograms indicates approximately the ratio between the concentration of NAMI-A outside and inside the cells. *$P<0.05$ versus NAMI-A. Lower panel: Three mice per group treated as described in Table 1 (experiment 2) were killed 24 h after the last drug administration. The ruthenium distribution in blood and *ex vivo* tissues was detected by graphite furnace atomic absorption spectroscopy (AAS).

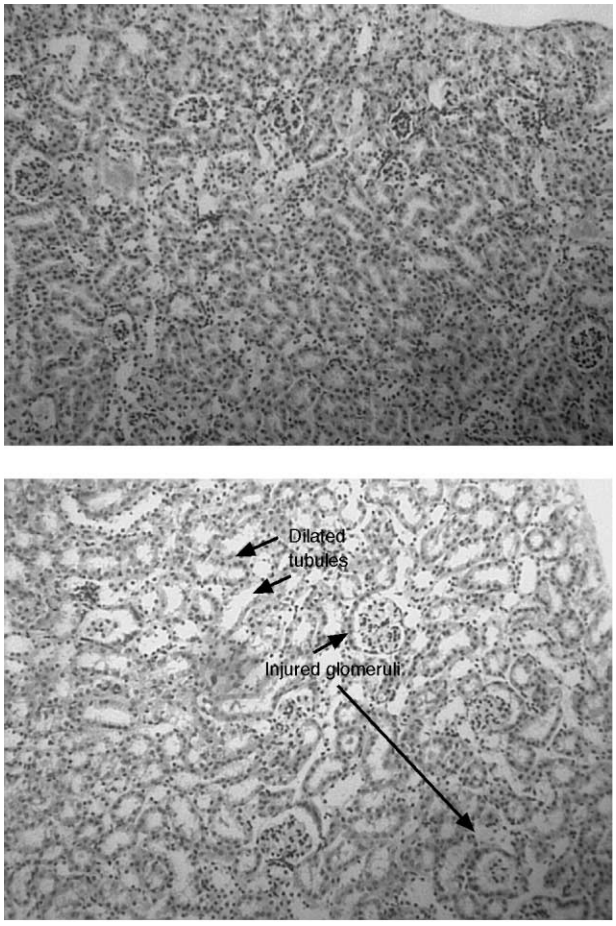

Fig. 7. Example of NAMI-A-related renal damage shown by light microscopy. Upper panel: kidney parenchyma of a control animal; lower panel: kidney parenchyma of a NAMI-A-treated animal.

between resting (G_0/G_1) and dividing ($S+G_2$-M) cells, irrespective of whether its reduction takes place outside or inside the cells.

In vitro uptake of NAMI-A provides a cell concentration in the range 0.35–0.44 mM, i.e. 4 times higher than that in the perfusion medium and comparable to that obtained *in vivo* in the lungs (0.22 mM) at the end of treatment cycles at doses that are active against metastases. This was independent of either its possible reduction or the freshness of the solution before use.

Our data suggest that NAMI-A is quite easy to handle for the treatment of metastases in advanced solid tumours. It is not clear whether metastases are inhibited by the reduced form or by a hydrolytic product of NAMI-A; what is probable is that no selective intratumour or intra-metastatic reduction is needed to generate the active species. Most probably, NAMI-A interacts with its target, and thereby inhibits metastasis, even when 50% of the molecules have lost their axial DMSO. One important consequence of these observations is that the clinical use of this compound will be facilitated. Although the compound is subject to chemical changes when kept in solution, it is likely that NAMI-A can also be used when these solutions are aged up to 8 h, since it maintains entirely its pharmacological efficacy against lung metastases.

Another important finding of this study concerns the effects of NAMI-A on the toxicity in the host. Apparently, the structure modifications occurring on NAMI-A following reduction to Ru(II), as well as those following the addition of 5% DMSO and HCl to the solution, reduce this toxicity. If proven further, this finding suggests that the reduction of NAMI-A before use might be a useful procedure which could provide particular benefit for prolonged treatments, like those presumably necessary for control of metastasis, where the toxicity in the host might be a limiting factor.

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