

In Vitro Cell Cycle Arrest, In Vivo Action on Solid Metastasizing Tumors, and Host Toxicity of the Antimetastatic Drug NAMI-A and Cisplatin¹

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ABSTRACT

The effects of NAMI-A (imidazolium *trans*-imidazoledimethyl sulfoxide-tetrachlororuthenate) are compared with cisplatin on tumor cells cultured in vitro at doses of 1 to 100 μ M and on tumor metastases in vivo at maximum tolerated doses. Using mouse tumors that metastasize to the lungs, NAMI-A given i.p. for 6 consecutive days at 35 mg/kg/day, was effective independently of the tumor line being treated and of the stage of metastasis growth. Conversely, cisplatin (2 mg/kg/day for 6 days) was as effective as NAMI-A on MCA mammary carcinoma and TS/A adenocarcinoma and less effective than NAMI-A on Lewis lung carcinoma. Cisplatin reduced body weight gain and spleen weight during treatment and was much more toxic than NAMI-A on liver sinusoids, kidney tubules, and lung epithelium.

In vitro NAMI-A caused a transient cell cycle arrest of tumor cells in the premitotic G₂/M phase, whereas cisplatin caused a progressive dose-dependent disruption of cell cycle phases. Correspondingly, NAMI-A did not modify cell growth, whereas cisplatin caused a dose-dependent reduction of cell proliferation, as determined by sulforhodamine B test. Thus, NAMI-A, unlike cisplatin, is a potent agent for the treatment of solid tumor metastases as well as when these tumor lesions are in an advanced stage of growth. NAMI-A is endowed with a mechanism of action unrelated to direct tumor cell cytotoxicity, and such mechanism of action is responsible for a reduced host toxicity.

Ruthenium complexes originally were synthesized as compounds selectively toxic for solid tumors, because of the selective activation to cytotoxic species into these tissues (Clarke et al., 1988). A wide series of investigations, performed on sulfoxide-ruthenium complexes, pointed out a more specific activity of these compounds on solid-tumor metastases, putting light on the pharmacological possibilities of such drugs (Sava, 1994; Sava and Bergamo, 1997). The property that renders ruthenium complexes unique among anticancer agents is principally the lack of evident direct cell cytotoxicity at doses that increase lifetime expectancy in tumor-bearing hosts (Sava et al., 1994, 1995; Capozzi et al., 1998). Rather than being a limitation, the lack of direct cell cytotoxicity is the leading aspect of these complexes in that it indirectly means a low or absent bone marrow or epithelial toxicity at active dosages (Giraldi et al., 1977; Sava et al., 1984; Gagliardi et al., 1994).

Although many studies point out the capacity of ruthenium

complexes to bind to DNA of isolated plasmids or eukaryotic cells (Clarke and Stubbs, 1996), many others seem to suggest a certain difficulty of these complexes to penetrate cell membrane, preferring extracellular components as binding sites (Ghosh et al., 1981; Deinum et al., 1985). These characteristics may contribute to the understanding of the mechanism of antitumor activity in in vivo systems, where ruthenium interactions may deprive tumor cells of normal cell-cell and cell-matrix contacts, which are essential for cell growth, division, and metastasis formation (Fox et al., 1995; Schaden-dorf et al., 1995; Umansky et al., 1996).

The aim of the present investigation, therefore, was to examine the difference between the effects of NAMI-A [imidazolium *trans*-imidazole-dimethyl sulfoxide-tetrachlororuthenate, ImH[*trans*-RuCl₄(DMSO)Im]] on tumor cells cultured in vitro and the effects on tumor metastases in vivo, as determined by a direct count of lung metastatic tumor or indirectly by measuring the prolongation of lifetime expectancy of tumor-bearing mice. The study was conducted by comparing the effects of NAMI-A with those of *cis*-dichlorodiammine platinum(II) (cisplatin), to which the ruthenium complex is often referred because it contains a heavy metal of

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ABBREVIATIONS: NAMI-A, imidazolium *trans*-imidazoledimethyl sulfoxide-tetrachlororuthenate; NAMI, sodium *trans*-imidazoledimethyl sulfoxide-tetrachlororuthenate; cisplatin, *cis*-dichlorodiammine platinum(II); SRB, sulphorhodamine B.

group VIII transition metals. In this context, cisplatin represents a drug of particular interest. It is myelosuppressive, emetic, and nephrotoxic (Tognella, 1990; Rozenweig et al., 1977); nevertheless, it must be considered a unique agent, and, since its introduction into clinical trials, it is a drug that has completely changed the prognosis of some tumors and significantly ameliorated that of others (AHFS Drug Information 1994, American Society of Hospital Pharmacists, 94, 572). In particular, the work will focus on the relevance of in vitro cell toxicity, as determined by flow cytometry analysis of cell cycle, and the correspondent in vivo effects, including organ toxicity.

Materials and Methods

Compounds. NAMI-A was prepared according to a patented procedure (Mestroni et al., 1998). Cisplatin was obtained by Sigma Chemical Co. (St. Louis, MO). Each compound was administered as a solution in isotonic saline, in volumes of 10 ml/kg b.wt.

Tumor Lines for In Vivo Test. Lewis lung carcinoma (grown in C57Bl mice and propagated for experimental purposes in BD2F1 hybrids), MCa mammary carcinoma (grown in CBA mice), and TS/A adenocarcinoma (grown in Balb/c mice) were used for in vivo testing. C57Bl, BD2F1, and Balb/c mice were obtained from Harlan Nossan, and CBA mice were obtained from a locally established breeding colony grown according to the standard procedure for inbred strains. The procedure of tumor graft was identical for all tumor lines. Briefly, for Lewis lung carcinoma and MCa mammary carcinoma, 10^6 cells of a single cell suspension, prepared from mincing with scissors the primary tumor masses obtained from donors similarly implanted 2 weeks before, were injected i.m. into the calf of the left hind leg of experimental-group mice; for TS/A adenocarcinoma (generously supplied by G. Forni, Consiglio Nazionale delle Ricerche Centro di Immunogenetica ed Oncologia Sperimentale, Torino, Italy), 10^5 cells were obtained from an in vitro confluent culture of the same cells. The TS/A cell line was maintained in complete medium consisting of RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (HyClone Europe, Holland), 2 mM L-glutamine (HyClone Europe), and 50 μ g/ml gentamycin sulfate solution (Irvine Scientific, Santa Ana, CA). Cells from confluent monolayers were removed from flasks by 0.25% trypsin solution (Sigma Chemical Co.), washed twice, and pellet diluted with complete medium or PBS.

Primary Tumor Growth and Lung Metastasis Evaluation. Primary tumor growth was determined by caliper measurements, by determining two orthogonal axes and calculating tumor weight with the formula: $\pi/6 \times a^2 \times b$, where a is the shorter and b is the longer axis. 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 used for primary tumors, and the sum of each individual weight gives the total weight of the metastatic tumor per animal.

Animal Studies. Animal studies were carried out according to the guidelines enforced in Italy (DDL 116 of February 21, 1992) and in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

Tumor Lines for In Vitro Test. An established KB cell line (ECACC no. 86103004) was cultured according to standard procedure (Craciunescu et al., 1987). Vials of the original line were maintained in liquid N_2 ; from them, cells were obtained, serially subcultured, and used for the experiments reported in the present work.

The KB cell line was maintained in Eagle's minimum essential medium (Eagle, 1959) with 1% nonessential amino acids (GIBCO BRL), supplemented with 10% newborn calf serum (GIBCO BRL),

and buffered with 3 mM tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid, 3 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid, 3 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 3 mM Tricine (Sigma Chemical Co.). The cell population doubling time was ca. 24 h. Culture medium was added with penicillin-streptomycin solution (Sigma Chemical Co.) (100 U/ml penicillin G and 100 μ g/ml streptomycin). Cells from confluent monolayers were removed by 0.05% trypsin solution (Sigma Chemical Co.). Cell viability was determined with trypan blue dye exclusion test.

In Vitro Cytotoxicity Test. Cell cytotoxicity was evaluated against the KB cell line in 24-well cell culture clusters (Costar) according to previously described procedures (Alvarez et al., 1997). Test compounds were dissolved in saline immediately before use, and the solution was diluted with the growth medium to the desired concentrations. Cells were incubated with the test compounds at 37°C with 5% CO_2 and 100% relative humidity. The cytotoxic effect was evaluated with the test compound dissolved in PBS. Three concentrations (1, 10, and 100 μ M) of NAMI-A and cisplatin and six wells per concentration were used. One hour after drug challenge, unreacted drug was removed and cells were cultured in complete medium for a further 24, 48, and 72 h. Each experiment was repeated at least twice.

Sulforhodamine B (SRB) Test To Evaluate In Vitro Cytotoxicity. Cell growth was determined by staining with the protein-binding dye SRB (Sigma Chemical Co.) (Skehan et al., 1990). Briefly, adherent cell cultures were fixed in situ by the addition of 250 μ l of cold 50% (wt/vol) trichloroacetic acid and were kept for 60 min at 4°C. The supernatant was then discarded and the plates were washed three times with deionized and distilled water and dried. SRB solution was added and the cells were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid. The plates then were air-dried. Bound stain was dissolved with unbuffered Tris base (Sigma Chemical Co.), and the optical density was read at 565 nm on a Perkin-Elmer 550 SE spectrophotometer. Cytotoxicity was evaluated from the cell-growth inhibition in the treated cultures versus untreated controls. The statistical significance of these results was estimated by Student's t test ($p < .01$). IC_{50} , the micromolar concentration of compound at which cell proliferation was 50% of that observed in control cultures, was determined by linear regression analysis.

Propidium Iodide Test. Viable cells (1×10^6) of a single cell suspension, as determined by trypan blue exclusion test, were fixed in 70% ethanol at 4°C for at least 1 h. Before analysis the ethanol was removed by centrifugation and cells were washed twice with PBS. Cells were resuspended in PBS containing 1 mg/ml RNase at 37°C for 30 min and stained further for at least 30 min at room temperature in the dark with propidium iodide (40 μ g/ml) (Sigma Chemical Co.). Red fluorescence (610 nm) was analyzed, using peak fluorescence gate to discriminate aggregates. Each analysis consisted of 10,000 events counted. The flow cytometric analyses were done either at Fondazione Callerio with an EPICS XL-MCL flow cytometer (Coulter Electronics, Miami, FL) or at the Center for Flow Cytometry (section of CISP of the University of Trieste) with an EPICS ELITE ESP cytometer (Coulter Electronics). Cell cycle distribution of the cells was determined by analysis with Multicycle software (Phoenix Flow Systems).

Histological Examination. Sections for light microscopy were prepared from paraffin-embedded lungs, which were removed, washed in water, fixed in 10% formaline, and processed according to the standard procedure for inclusion and after rehydration (xylene/alcohol/water), with sections cut at 6 μ m. Sections were stained with Cajal-Gallego mounted in Canada Balsam and were observed with a Leitz-Orthoplan microscope. Examinations were made on three different slides, each containing three slices for each sample.

Statistical Analysis. Each experiment was subjected to statistical analysis by the Student-Newmann-Keuls ANOVA and by Student's t test for grouped data. Significance was accepted with $p \leq .05$.

Results

On in vitro cultured KB cells, NAMI-A is virtually devoid of cytotoxicity up to 100 μM concentration, after 1-h exposure of confluent cells in PBS, as determined by the SRB test (Fig. 1). In a dose-dependent way, NAMI-A caused a transient increase of protein content versus controls at 24 h, which is completely abolished from 48 h onward. In the same experimental conditions, cisplatin caused a significant reduction of protein content at the maximum dose used; this effect increased from 24 to 72 h after drug exposure.

After DNA analysis of KB cells by flow cytometry, 24 h after treatment, NAMI-A showed a significant increase of cells in G_2/M phase at 100 μM and 10 μM concentration, and an increase of cells in S and G_2/M phase, with a corresponding reduction of those in G_0/G_1 , at 100 μM concentration. Such alterations are still evident at 48 h with 100 μM NAMI-A and are completely abolished at 72 h after drug exposure (Table 1). Cisplatin caused a marked alteration of cell cycle distribution of KB cells at 100 μM concentration. A statistically significant alteration of the distribution of KB cells within the cell cycle phases also was caused at 10 μM and 1 μM concentrations; at the lowest dose used, these alterations were abolished 72 h after drug challenge. Data reported in Fig. 2 show an example of flow cytometry histograms of DNA distribution of control KB cells and of those treated with 100 μM NAMI-A and cisplatin. In particular, data on cisplatin show the complete alteration of DNA distribution that made impossible the calculation of the percentage of cells in the cell cycle phases.

NAMI-A caused a marked reduction of lung metastasis formation in mice carrying i.m. implants of Lewis lung carcinoma and TS/A adenocarcinoma and significantly increased the postsurgical lifetime expectancy of mice bearing MCa mammary carcinoma (Fig. 3). The reduction of the weight of lung metastases on TS/A adenocarcinoma was equal to that caused by cisplatin, despite the fact that only cisplatin caused a significant reduction of primary tumor growth by 32%. When tumor-bearing mice were treated after surgical removal of primary tumors, the effect of NAMI-A on Lewis lung carcinoma was markedly greater than that of cisplatin. In mice bearing MCa mammary carcinoma, a similar postsurgical treatment with NAMI-A caused a statisti-

cally significant increase of the lifetime expectancy that is comparable to that caused by cisplatin.

The analysis of histological sections of kidneys of mice treated with daily doses of 35 mg/kg/day NAMI-A and 2 mg/kg/day cisplatin for 6 consecutive days showed a significant increase of glomeruli that resulted as atrophic or damaged as compared with untreated controls (Table 2). The compared analysis of this effect, done 24 h after the last dose, showed a significant difference between cisplatin and NAMI-A as far as atrophic glomeruli are concerned, whereas no statistically significant changes were observed on those identified as damaged, although cisplatin tended to increase the values more than NAMI-A. When examining the whole parenchyma of kidney, liver, and lungs (Table 3), cisplatin always showed effects greater than those of NAMI-A. In general, the epithelial tissue of these organs appeared edematous and altered compared with untreated controls and also with mice treated with NAMI-A, either in terms of number of damages per field of microscopy examination or in terms of severity of the damage observed. On lungs, cisplatin also caused an increase of inflammatory sites. Such inflammatory process, present in the kidney of controls, is virtually negligible in the same organ of mice treated with NAMI-A and cisplatin; a dramatic reduction of the number and dimensions of lung metastases is observed on these slices as well. Cisplatin caused an intermediate grade of inflammation of liver that was more pronounced than that of NAMI-A.

Discussion

Typically, it is expected that a compound such as cisplatin that shows antitumor action on experimental tumors also would be cytotoxic against tumor cells in vitro. Therefore, a compound like NAMI-A, which often is compared with cisplatin because it similarly is based to a heavy metal of the same group, is totally atypical if it shows the same action as cisplatin (or even better) in vivo on solid metastasizing tumor but is virtually devoid of cytotoxicity against tumor cells in vitro. Data reported in the present investigation show this discrepancy and show further that NAMI-A is much less toxic than cisplatin for healthy tissues at equieffective doses. In vivo doses are optimal for both compounds, considering the schedule of administration used and the ratio between host toxicity and antitumor effect (i.e., optimal doses). Furthermore, atomic absorption spectroscopy studies of tissue disposition showed that NAMI-A, at the in vivo dose of 35 mg/kg given for 6 consecutive days, reaches a 100- μM order concentration in tumor, liver, and lungs (Cocchietto, 1999). Therefore, in vitro comparison of both drugs using a top dose of 100 μM appears to be appropriate.

With the experimental conditions presently adopted, i.e., mouse tumors that metastasize to the lungs, NAMI-A resulted as effective independently of the tumor line being treated, either considering the reduction of the number and weight of lung tumor or considering the prolongation of lifetime expectancy of tumor-bearing mice. Conversely, cisplatin was as effective as NAMI-A in mice bearing MCa mammary carcinoma, which is slightly less effective than NAMI-A in mice bearing TS/A adenocarcinoma and markedly less effective than NAMI-A in mice bearing Lewis lung carcinoma. Cisplatin, at the dose and treatment schedule used, was more toxic than NAMI-A in terms of reduction of body weight gain

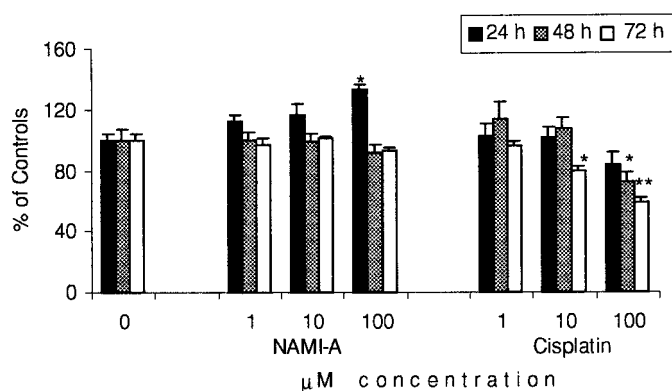


Fig. 1. Effects of NAMI-A and cisplatin on in vitro cell proliferation. KB cells, seeded on 24-well plastic plates 96 h before, were challenged with NAMI-A and cisplatin for 1 h at 1, 10, and 100 μM concentration. SRB test was performed after 24, 48, and 72 h of further cultivation in the appropriate medium.

TABLE 1

Effects of NAMI-A and cisplatin on cell distribution among cell cycle phases after 24-, 48-, and 72-h drug exposure
KB cells were exposed to drug concentrations for 1 h; then drug was removed and cells were added with fresh medium.

Drug Concentration	% Cells in Cycle Phase								
	24 h			48 h			72 h		
	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M	G ₀ /G ₁	S	G ₂ /M
μM 0	72.5 \pm 0.6	20.9 \pm 0.4	6.6 \pm 0.5	70.0 \pm 2.0	22.2 \pm 1.3	7.8 \pm 0.7	72.5 \pm 0.4	19.3 \pm 0.3	8.2 \pm 0.2
NAMI-A (1)	70.7 \pm 0.9	21.6 \pm 0.4	7.7 \pm 0.6	67.9 \pm 1.0	21.3 \pm 2.0	8.5 \pm 0.7	72.2 \pm 1.8	19.4 \pm 1.0	8.4 \pm 1.2
NAMI-A (10)	69.5 \pm 1.1	20.4 \pm 0.8	10.2 \pm 0.4**	69.2 \pm 1.5	22.5 \pm 1.2	8.4 \pm 0.4	68.4 \pm 0.9	21.8 \pm 0.6	9.8 \pm 0.7
NAMI-A (100)	39.5 \pm 2.2**	30.9 \pm 1.8**	29.6 \pm 0.8**	61.5 \pm 1.8**	24.6 \pm 1.1	13.9 \pm 0.8*	71.2 \pm 1.0	20.4 \pm 0.7	8.4 \pm 0.4
Cisplatin (1)	51.4 \pm 1.9**	33.2 \pm 1.0**	15.5 \pm 1.1**	58.1 \pm 1.2**	28.6 \pm 0.2**	13.3 \pm 1.0*	70.6 \pm 0.8	20.6 \pm 0.7	8.8 \pm 0.3
Cisplatin (10)	23.6 \pm 0.6**	76.4 \pm 0.6**	0 \pm 0**	16.0 \pm 1.6**	30.7 \pm 1.5**	53.3 \pm 2.9**	42.4 \pm 2.5**	27.0 \pm 0.7**	30.4 \pm 3.1**
Cisplatin (100)	79.4 \pm 0.5**	18.9 \pm 0.6	1.7 \pm 0.6**	ND	ND	ND	ND	ND	ND

ND, not detectable. * $p < .05$, ** $p < .01$ versus 0 concentration, Student-Neumann-Keuls ANOVA.

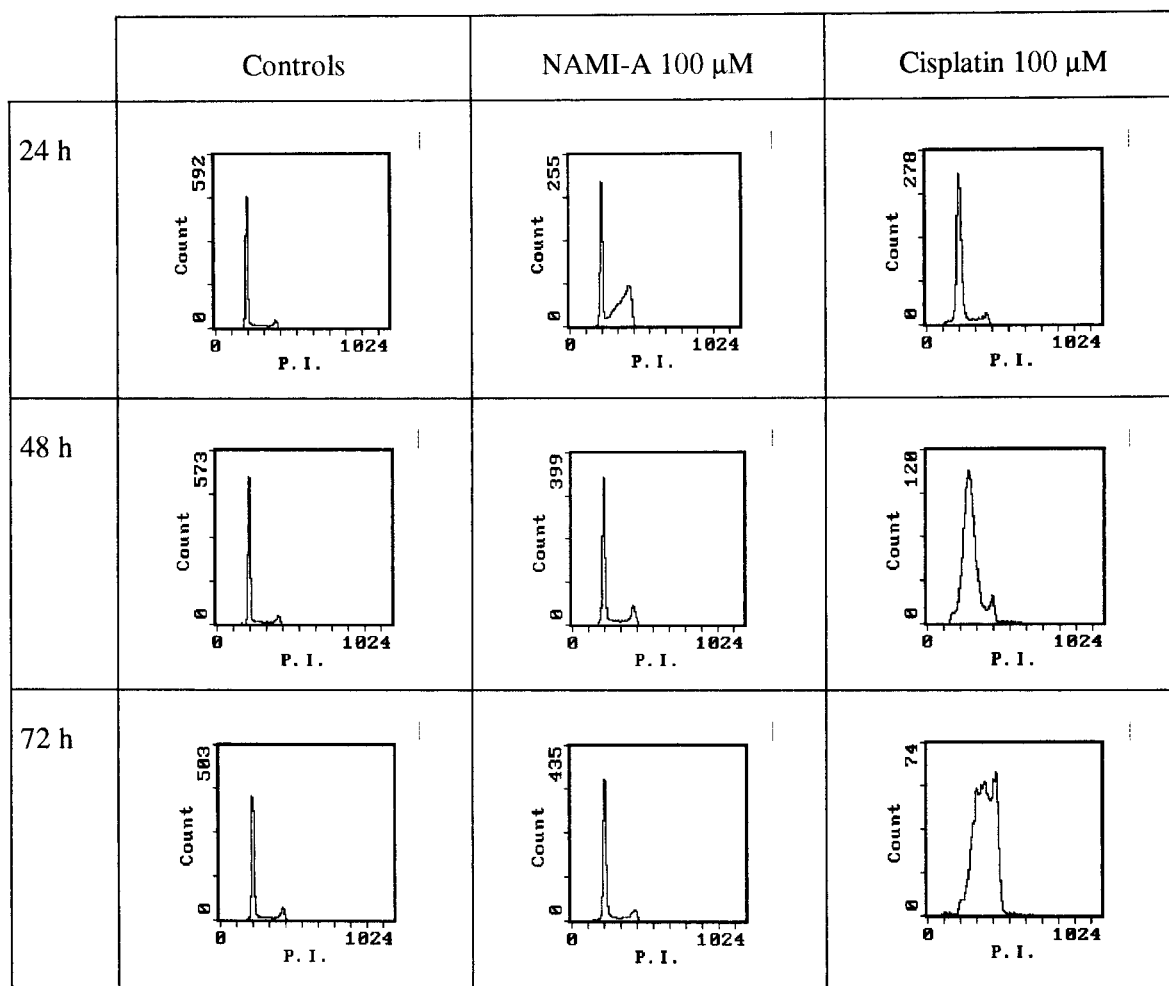


Fig. 2. Individual flow cytometry histograms of cell cycle distribution of KB cells of untreated controls and of NAMI-A- and cisplatin-treated groups. KB cells, seeded on six-well plastic plates 96 h before, were challenged with NAMI-A and cisplatin for 1 h at 100 μM concentration. Cell cycles were obtained at 24, 48, and 72 h after drug challenge.

in the period of drug treatment (up to -11% versus controls) and in terms of reduction of spleen weight (up to -50% versus controls). These data, added to those related to the toxicity for healthy epithelia, stress the more favorable therapeutic properties of NAMI-A versus cisplatin.

Tumor cells, challenged in vitro with NAMI-A, do not show a significant alteration of cell cycles. NAMI-A causes a mild and transient arrest of cell cycle in the premitotic phase, which, besides that reported for KB cells, also was demonstrated with TS/A cells cultured in vitro, with the same doses

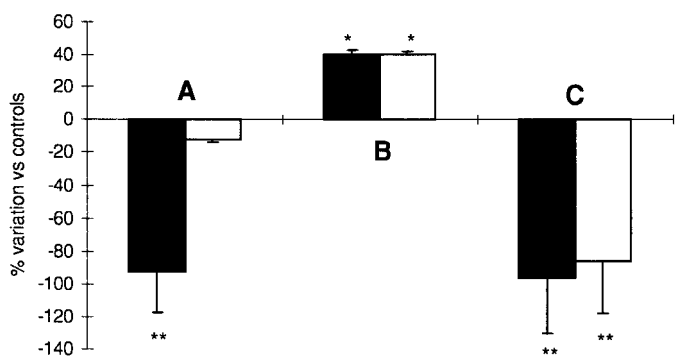


Fig. 3. Effects of NAMI-A and cisplatin on the reduction of lung metastasis and on the increase of survival time versus controls. Groups of seven mice, implanted i.m. on day 0, underwent surgical ablation of primary tumor on days 11 (Lewis lung carcinoma, A), 13 (MCA mammary carcinoma, B), and 15 (TS/A adenocarcinoma, C). NAMI-A (35 mg/kg/day) or 2 mg/kg/day cisplatin was given i.p. on days 12–17 (Lewis lung carcinoma, A), 14–19 (MCA mammary carcinoma, B), and 9–14 (TS/A adenocarcinoma, C). The effects of drug treatment were determined as an increase of the lifetime expectancy (MCA mammary carcinoma, B) or by measuring the weight of the metastatic tumor in the lungs after killing the mice on day 23 (Lewis lung carcinoma, A) or day 30 (TS/A adenocarcinoma, C). **p* < .05; ***p* < .01, statistically different from controls using the Student-Newmann-Keuls test.

and timings (Fnd Callerio, unpublished data on file). The correspondent mean increase of total protein content, as determined by the SRB test, should be regarded as a consequence of this effect. This effect is a property of NAMI-A that is not shared with NAMI (sodium *trans*-imidazole-dimethyl sulfoxide-tetrachlororuthenate, Na[*trans*-RuCl₄(DMSO)Im]), which was totally ineffective in vitro on tumor cells (Sava et al., 1995; Capozzi et al., 1998), probably associated with the advantages given by a molecule endowed with better pharmaceutical characteristics (Mestroni et al., 1998). The relevance of these mild effects of NAMI-A on in vitro tumor cells

for the selective action on in vivo metastases is not clear yet. It could be speculated that NAMI-A has a receptor-mediated effect, which might explain both its selectivity for tumor metastases, which behave differently from the primary tumor counterparts, and the reversibility of its in vitro (present paper) as well as in vivo (Sava et al., 1994) effects. Furthermore, such a mechanism might also help to explain the effects of NAMI on mRNAs for metalloproteinases and their inhibitors (Sava et al., 1996), the selection of tumor cell populations that NAMI caused with vitro-vivo and vivo-vivo bioassays of treated tumor cells (Sava et al., 1995), and, particularly, the irrelevance of intracellular migration of NAMI for its effects on TLX5 lymphoma (Capozzi et al., 1998). That in vitro treated tumor cells do not show a reduced rate of growth as compared with in vivo treated lung metastases might be attributed to the cross-talk interactions that in vivo growing cells have with other healthy cells and with extracellular matrix constituents that might accentuate growth arrest or apoptosis (Fox et al., 1995; Schadendorf et al., 1995; Umanski et al., 1996).

Considering that the main target for antitumor chemotherapy is represented very often by distant metastases, which are present, although not always diagnosable, at the time of eradication of the primary lesion and invariably are responsible for the failure of most of the available antitumor therapies (Poste, 1986; Fidler and Balch, 1987), this study presents NAMI-A as a reliable candidate for such work. NAMI-A is devoid of any link with cisplatin, presently used as a reference compound, because it is free of direct cytotoxicity for tumor cells. The lack or low toxicity of NAMI-A for host tissues, here compared with that exhibited by active doses of cisplatin, is a support for this new compound, which also should be regarded as a novel and potent agent for the treatment of solid tumor metastases when these tumor lesions are already present and in an advanced stage of growth.

TABLE 2
Histological analysis of glomeruli in mice treated with NAMI-A and cisplatin

Kidneys of three different mice, implanted with MCA mammary carcinoma on day 0 and dosed i.p. with NAMI-A or cisplatin daily on days 14–19, were removed from animals 24 h after last dose, fixed with formalin, and examined with light microscopy. For each animal, three different glasses, each mounted with three different slices, were read completely in a single blind procedure.

Treatment Group (dose)	Atrophic Glomeruli		Altered Glomeruli	
	Number/total counted	% of total, mean ± S.E.	Number/total counted	% of total, mean ± S.E.
Controls	15/3259	0.46 ± 0.06 ^{a,b}	66/3259	2.04 ± 0.29 ^{a,b}
NAMI-A (35 mg/kg)	233/4485	5.27 ± 0.36 ^{a,c}	1207/4485	27.20 ± 3.40 ^a
Cisplatin (2 mg/kg)	278/4484	6.31 ± 0.71 ^{b,c}	1594/4484	35.54 ± 1.61 ^b

Means with the same letter differ statistically; ^{a,b} *p* < .01 and ^c *p* < .05, Student-Newmann-Keuls ANOVA.

TABLE 3
Histological analysis of kidney, liver, and lung parenchyma after treatment with NAMI-A and cisplatin

Kidneys, livers, and lung of three different mice, implanted with MCA mammary carcinoma on day 0 and dosed i.p. with NAMI-A or cisplatin daily on days 14–19, were removed from animals 24 h after last dose, fixed with formalin, and examined with light microscopy. For each animal, three different glasses, each mounted with four different slices, were read completely in a single blind procedure.

Treatment Group (dose)	Kidney Swelling of proximal tubules and edema	Liver Swelling of hepatic sinusoids and damage of lobular architecture	Liver Compression of alveoli and increased thickness of epithelium
NAMI-A (35 mg/kg)	+++	++	+
Cisplatin (2 mg/kg)	++++	++++	+++

+, Focal; ++, moderate; +++, diffuse; +++++, marked and generalized.

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