

ARTICLE

Comparison of the Effects of the Antimetastatic Compound ImH[*trans*-RuCl₄(DMSO)Im] (NAMI-A) on the Arthritic Rat and on MCa Mammary Carcinoma in Mice

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The effects of the new molecule ImH[*trans*-RuCl₄(DMSO)Im] (NAMI-A), administered orally or intraperitoneally to adjuvant-arthritic rats or orally to mice bearing s.c. or i.m. implants of MCa mammary carcinoma, were studied. NAMI-A was not able to modify the progression of chronic inflammation in the complete Freund-adjuvant injected animals. Histology indicated a significant worsening of the inflammatory process, characterised by an increased infiltration of inflammatory cells, as well as by a remarkable deposition of connective tissue fibres around the blood vessels and alveolar walls. NAMI-A had no effect on primary i.m. implanted MCa mammary carcinoma growth and its lung metastasis formation, but significantly interfered with the cell cycle of primary

tumor cells following bolus oral administration. On the contrary, NAMI-A caused a significant inhibition of lung metastasis accompanied by a dramatic deposition of connective tissue fibres around the primary tumor mass, when given as medicated food to mice implanted s.c. with MCa tumor. These data indicated that NAMI-A is well absorbed after oral administration although there is no connection between lung concentration and the antimetastatic activity. Conversely, the marked deposition of connective tissues in NAMI-A treated animals is in agreement with the reported effects of the compound on extracellular matrix and tumor blood vessels. (Pathology Oncology Research Vol 4, No 1, 30–36, 1998)

Key words: ruthenium, inflammation, tumor, metastasis, treatment

Introduction

Ruthenium complexes, characterised by the metal atom in the +3 oxidation state and by the presence of sulfoxide ligands, have been shown to selectively interfere with the mechanism of metastasis formation of solid tumors in mice.^{5,9,15} In particular, their anti-metastatic effect is not related to a direct cytotoxic effect on tumor cells nor to a significant reduction of primary tumor growth.^{13,14,16} Yet,

Na[*trans*-RuCl₄(DMSO)Im] (NAMI), the most widely studied compound of this series, was found to remarkably reduce the proteolytic activity of the tumor, to increase the thickness of the capsule surrounding tumor mass and to dramatically enhance the deposition of connective tissue around tumor blood vessels; thus inhibiting malignant cells to invade the host's circulation.^{3,4,10,11} Moreover, in doses which reduced lung metastasis formation and increased survival, NAMI seems to be virtually non-toxic for epithelial cells, e. g. in the lung, liver and kidney, which is characteristic for cisplatin compounds and other anticancer drugs.⁵

The above mentioned biological properties make it theoretically interesting to examine the behaviour of these

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types of ruthenium complexes in models of inflammation where the rearrangement of the extracellular matrix and connective tissue play important role in the development and progression of inflammatory process.

The aim of our work was therefore to evaluate the effects of a new compound belonging to this class of ruthenium complexes, i.e. ImH[*trans*-RuCl₄(DMSO)Im] (NAMI-A), on a classical model of chronic experimental inflammation, the adjuvant arthritis of the rat.^{1,7,8,18} It should be noted that NAMI-A is virtually identical to NAMI as far as anti-metastatic activity is concerned¹¹, but is much more stable on air and there is no free sulfoxide in its crystalline structure.⁶ The present study was mainly focused on evaluating the antiarthritic effects of NAMI-A compared to its antimetastatic potential in mice transplanted with MCa mammary carcinoma.

Materials and Methods

Compounds – ImH[*trans*-RuCl₄(DMSO)Im], i.e. imidazolium *trans*-imidazole-dimethylsulfoxidetetrachlororuthenate] (NAMI-A), was prepared according to already reported procedures.⁶ A commercial preparation of indomethacin (Merck Sharp & Dome) was used as the reference compound to check the reliability of the adjuvant arthritis experiment.

Treatment of rats was performed by i.p. injections, or gastric gavage, by dissolving the test compounds in 5 ml/kg body weight of isotonic sterile saline. Using CBA mice, the compound was administered by gastric gavage in volumes of 0.05 ml/10 g body weight of isotonic sterile saline. Alternatively, medicated food was prepared by adding the test compound to powdered food, assuming a daily food consumption of 3.5 g/mouse; the preparation was renewed every second day. The loss of compound due to discarded food was lower than 10% of the administered dose.

Animal studies – were carried out according to the guidelines in force in Italy (DDL 116 of 21/2/1992) and in

compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. No (NIH)86-23. Bethesda, Md: NIH, 1985.

Adjuvant arthritis of the rat – Ninety female Sprague-Dawley rats were used (CD-COBS from Charles River, Italia) weighing 175 ± 15 g at the beginning of the experiment. The animals were housed in groups of 5 to 10, kept on a 12 h light-dark cycle, at constant temperature (20 ± 1°C) and humidity (55 ± 5%). They were fed *ad libitum* on tap water and a purified semi-synthetic diet (AIN-76™, purchased from Dottori Piccioni, Brescia, Italy).

Following a modification of the method described by Pearson,⁸ adjuvant arthritis was produced in all rats by intradermal injection of 0.6 mg of heat killed *Mycobacterium butyricum* finely suspended in 0.1 ml of liquid paraffin into the tail base of each rat. Fourteen days later, animals were weighed and subjectively scored on the basis of the following scale: left and right hind feet each 0–7, left and right fore feet each 0–4.5, tail 0–5, ears 0–2 and nose each 0–1 (maximum score equal to 32). The animals, reaching the scores higher than 25, were divided into 5 groups, to be treated (and then examined for progression of the disease at day 21 and 28) as described in Table 1.

MCa mammary carcinoma – was grown in CBA mice obtained from a locally established breeding colony grown according to standard procedures for inbred strains. 10⁶ viable tumor cells (trypan blue excluding cells) of a single cell suspension – prepared by mincing with scissors primary tumor masses obtained from donors similarly implanted 2-weeks earlier – were injected i.m. into the calf of the left hind leg (or s.c. in the flank) of experimental groups of mice of (at least 7 animals per group), unless differently stated.

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

Table 1. Adjuvant arthritis of the rat: scheme of treatments and basal conditions (at day 14 after complete adjuvant injection) of the animals used to evaluate the activity of NAMI-A.

Compounds	Route of administration [^]	Body weight g ± SD	Arthritic conditions score ± SD	Days of treatment
Sterile saline	oral (10)	168 ± 9.7	25.6 ± 3.9	daily on days 14-27
NAMI-A, 100 mg/kg	oral (10)	171 ± 10.1	25.3 ± 2.1	daily on days 14-27
Sterile saline	i.p. (10)	169 ± 9.1	25.4 ± 3.2	on days 14,16,18,20,22,24,26
NAMI-A, 100 mg/kg	i.p. (10)	168 ± 9.3	25.7 ± 3.1	on days 14,16,18,20,22,24,26
Indomethacin, 2 mg/kg*	oral (10)	170 ± 10.3	25.5 ± 2.9	daily on days 14-27

[^] Ten rats per group

* Given suspended in 1% acacia gum

Table 2. Effect of orally or intraperitoneally administered NAMI-A on the development of adjuvant arthritis of the rat

Treatment	21 st day		28 th day	
	Body weight g $\pm$ SD	Score $\pm$ SD	Body weight g $\pm$ SD	Score $\pm$ SD
Vehicle os	164 $\pm$ 7.1	27.3 $\pm$ 2.1	170 $\pm$ 9.2	26.7 $\pm$ 3.3
NAMI-A os	173 $\pm$ 6.8*	29.3 $\pm$ 0.8	181 $\pm$ 8.7*	28.5 $\pm$ 1.6
Vehicle i.p.	163 $\pm$ 7.9	28.1 $\pm$ 2.1	167 $\pm$ 8.3	28.4 $\pm$ 2.9
NAMI-A i.p.	166 $\pm$ 8.1	27.7 $\pm$ 3.1	176 $\pm$ 7.8*	27.1 $\pm$ 4.0
Indomethacin os	180 $\pm$ 5.6***	23.2 $\pm$ 2.2***	197 $\pm$ 6.1***	16.3 $\pm$ 1.7***

Ten rats per group.

* $p < 0.050$; *** $p < 0.001$; Students "t" test.

counted by carefully examining the lung surfaces, immediately after terminating the animals. 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 gave the total weight of metastatic tumor per animal.

Flow cytometric analysis – Small pieces of about 10 mm³, harvested from three different sites of the primary tumor mass and frozen at -20°C until analysis, were minced in PBS by Medimachine® (Coulter Electronics, Ltd) to obtain a single cell suspension. An aliquot of 10^6 cells was stained for at least 30 min at room temperature in the dark, with a solution containing 50 μg propidium iodide (Sigma Chemical Co.), 0.1% Nonidet P-40 in bi-distilled water. Red fluorescence (610 nm) was analysed, using peak fluorescence gate to discriminate aggregates. Each analysis consisted of 10,000 events counted.

Flow cytometric analyses were carried out at the facility laboratory organised by the University of Trieste at the Tumor Center, via Della Piet 19, Trieste, Italy, using an EPICS ELITE ESP flow cytometer (Coulter Electronics, Miami, FL, USA).

Atomic Absorption Spectroscopy – Aliquots of whole blood were dried at 60°C for 1 week and then treated with an aliquot of 25% tetramethylammonium hydroxide (TMAH, Aldrich Chemical Company) in water according to a procedure adapted from that described by Tamura *et al.*¹⁷ Volumes were adjusted to 500 μl with distilled water. For solid tissue analysis, a fragment of each organ – after careful weighing – was put in a cryovial and heated at 105°C until complete dryness. Weights were taken continuously, considering the entirely dry when no further changes of weight occurred. Each fragment was then completely solubilised in its cryovial (closed) by adding 0.5 ml of 25% TMAH in water at room temperature. After digestion, each volume was adjusted to 1 ml with Milli-Q water.

Ruthenium was determined by atomic absorption spectroscopy using a Varian SpectraAA-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 $2,500^{\circ}\text{C}$, using argon gas as carrier 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 $\mu\text{g}/\text{ml}$ in 3.3% HCl. Inorganic Ventures Inc.

Histological examinations – Twenty-four hours after the last drug administration, 3 rats were killed and blood, lungs, liver, kidney and small bowel were removed, washed in water and fixed in 10% formalin. Sections for light microscopy were prepared from paraffin embedded organs, and processed according to standard procedures for inclusion and rehydration (xylene, alcohol, water), with sections cut at 6 μm . Sections, stained with Cajal-Gallego mounted in Canada Balsam, were observed in single blind with a Leitz-Orthoplan microscope.

Statistical analysis – Each experiment was subjected to statistical analysis using the appropriate test of the Pharmacological Calculation System. Significance was accepted when p was equal to or below 0.05.

Results

Anti-inflammatory activity of NAMI-A

The effects on the adjuvant arthritis model of the rat are reported in Table 2. Unlike indomethacin which exhibits its usual, and widely documented, anti-inflammatory action in this model of chronic inflammation,^{1,7,18} NAMI-A was not able to show any influence on the arthritic score and the progression of the experimental disease, compared to control animals. At autopsy, 9 out of 10 rats treated i.p. with NAMI-A showed the presence of ascites, the amount of which was estimated to range between 1 and 2.5 ml.

Effect of NAMI-A on the histology of liver, lungs, kidney and intestine

Freunds complete adjuvant promoted a systemic inflammatory response which, besides causing major damages in the joints, affected all the other tissues and organs examined. For example, in the liver of arthritic rats, we found inflammatory cells scattered or grouped around blood vessels, as well as small aggregates of lymphocyte. Inflammatory cells also invaded sinusoids and spaces between parenchyma cells. Dilatation of sinusoids was sometimes observed. Treatment with NAMI-A dramatically enhanced inflammatory cell number in all liver areas and caused an increase in the thickness of the liver capsule, as well as the appearance of portal connective tissue fibres. The above effects were clearly more pronounced when NAMI-A was given i.p. rather than by oral route. Moreover, i.p. treatment caused the appearance of isolated necrotic areas.

Lungs of arthritic rats showed an extremely high density of inflammatory cells, especially neutrophils. Moreover, interalveolar walls were swollen, while alveoli were often heavily infiltrated by both lymphocytes and neutrophils. Finally, bronchi were surrounded by islets of inflammatory cells. Treatment with NAMI-A promoted a significant worsening of the inflammatory response. Lymphocytosis as well as neutrophil infiltration were dramatically enhanced, and connective tissue fibres accumulated in the alveolar walls. As for the liver, these effects were significantly enhanced when NAMI-A was administered intraperitoneally, and, in this latter

case, particularly heavy infiltrates of inflammatory cells surrounded the bronchi.

As far as the kidneys of arthritic animals are concerned, many inflammatory cells were observed scattered in the cortex or concentrated around the blood vessels. The medulla was less infiltrated by these cells, which rarely appeared as aggregates. Tubules showed swelling, and glomerules usually maintained their normal morphology, although some of them had partially lost the definition of the Bowmann capsule, while others showed signs of atrophy. Once again, NAMI-A, especially when given i.p., resulted in a remarkable enhancement of the inflammation, producing necrotic areas in the cortex, as well as damages at the glomerular level.

The hosts inflammatory status was also evident in the intestines. In the small bowel, numerous inflammatory cells were found invading intestinal villi and under the mucosa, while an increase in proliferating nodules appeared to affect the GALT and, especially, the Peyer patches. Treatment with NAMI-A increased all components of this inflammatory response, which effect was significantly more pronounced when i.p. route of administration was used.

Effects of NAMI-A in mice with MCa mammary carcinoma.

NAMI-A was given orally to mice with MCa mammary carcinoma by means of two distinct procedures: a) mixed with the powdered food (assuming an average daily food consumption of 3.5 g/mouse), from day 10 to day 21, after

Table 3. Effects of oral treatment with NAMI-A on primary tumor growth and lung metastasis formation

Treatment dose mg/kg/day	Body weight % variation [^]	Primary tumor g $\pm$ SE	Metastasis mg $\pm$ SE
<i>Gastric gavage administered on days 1–12 to i.m. implanted mice</i>			
Vehicle	–	1.23 $\pm$ 0.05	58.8 $\pm$ 12.5
25	–12.4	1.06 $\pm$ 0.08	43.2 $\pm$ 17.5
50	–11.4	1.05 $\pm$ 0.07	78.1 $\pm$ 18.8
100	–9.5	1.09 $\pm$ 0.08	95.5 $\pm$ 9.65
<i>Medicated food given on days 10–21 to s.c. implanted mice</i>			
Vehicle	–	1.82 $\pm$ 0.19	7.93 $\pm$ 2.69
50	2.06	1.84 $\pm$ 0.17	11.5 $\pm$ 4.44
100	3.70	1.93 $\pm$ 0.17	6.55 $\pm$ 2.93
200	2.47	1.90 $\pm$ 0.22	3.13 $\pm$ 1.16*

[^] The percent variations of body weight were calculated *versus* the vehicle-treated controls considering the time interval between the first and the last treatment.

Groups of 10 CBA mice, implanted either i.m. or s.c. with 10⁶ MCa mammary carcinoma cells on day 0, were given NAMI-A as indicated. Primary tumor weight was determined 24 hr after last day of treatment, whilst the lung metastasis weight was recorded on day 28. * $p < 0.050$, Student-Newmann-Keuls test

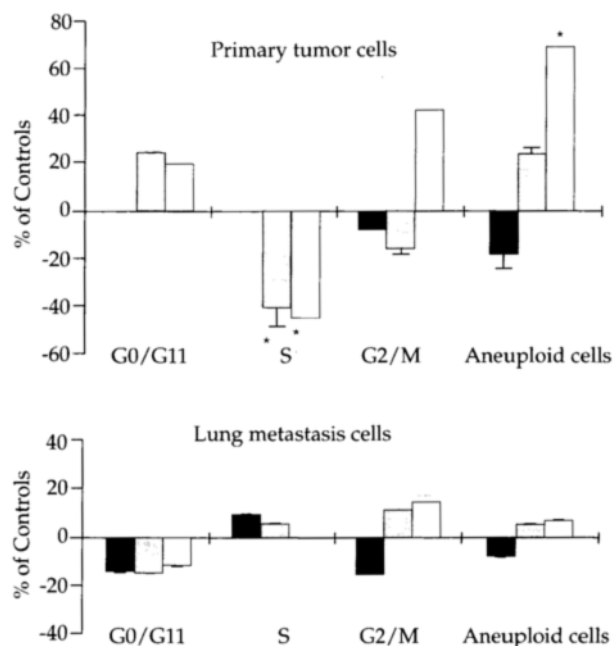


Figure 1. Cell cycle analysis of tumor cells of MCA mammary carcinoma. A piece from each of three tumors per group of the experiment shown on Table 3 (treatment by powdered food), harvested 24 hr after the last day of treatment and lung metastases obtained on day 28, were processed for cell cycle analysis by PI DNA staining. Actual values in Controls are: 62.8 ± 4.9 (%G₀/G₁), 27.9 ± 4.6 (%S), 9.2 ± 0.9 (%G₂/M), 34.8 ± 11.4 (%aneuploid cells) for primary tumor cells; 58.2 ± 0.6 (%G₀/G₁), 39.4 ± 1.6 (%S), 7.8 ± 1.1 (%G₂/M), 67.4 ± 2.2 (%aneuploid cells) for lung metastasis cells. *: means statistically different from controls, Student-Newmann-Keuls test, $p < 0.05$.

s.c. tumor implantation; b) by gastric gavage, from day 1 to day 12, after i.m. tumor implantation. Note that the i.p. route of administration was not used in these experiments.

Antitumor effects

Primary tumor grown at different kinetics following s.c. or i.m. transplantation. As a matter of fact, in our experiments, 2 weeks after implantation, tumor weight was about 0.5 g/animal in case of s.c. and 1.2 g/animal in case of i.m. implanted mice. Furthermore, lung metastasis formation was about 8 mg/mouse after s.c and 60 mg/mouse after i.m. grafts. NAMI-A administered by gastric gavage to i.m. implanted mice had no effect on the primary tumor and its lung metastases (Table 3). Conversely, NAMI-A significantly inhibited metastasis formation (~60%) following administration as medicated food to animals implanted s.c. by MCA tumor, though this effect was only significant at the highest dose used (Table 3).

Histological analysis of primary tumors in animals bearing s.c. tumors and treated with NAMI-A showed an

increase of intratumor necrotic areas as well as a dramatic deposition of connective tissue around the tumor mass; these effects were clearly dose-related (data not shown).

Effects on cell cycle

Flow cytometric analysis of cell distribution within the cell cycle phases of tumor cells (derived from both primary tumor and lung metastases) harvested from i.m. implanted mice is seen on Figure 1. At primary tumor level, NAMI-A (given by gastric gavage) caused a significant reduction in the percentage of cells in the S phase, which was greater than the increase of cells in the G₀/G₁ or G₂/M phases. At the same time, NAMI-A remarkably increased the number of aneuploid cells in a dose-dependent manner (see Figure 1, upper panel). On the contrary, no appreciable change of the same parameters was observed by cell cycle examination of lung metastases (Figure 1, lower panel). Given by means of medicated food to s.c. implanted mice, NAMI-A had no significant effect on the cell cycle of either primary tumor or lung metastasis derived cells.

Ruthenium distribution in the mouse

Metal distribution in organs such as kidney, liver, lungs, small intestine, spleen and primary tumor mass, was determined in animals coming from either s.c. (administration of medicated food) or i.m. (treatment by gastric gavage) graft experiments ($n=3$ mice randomly selected per group). The amount of ruthenium found in the organs examined was clearly greater when NAMI-A was given by gastric gavage than after treatment as medicated food (Figure 2). In this latter case (Figure 2, upper panel), an accumulation of the metal could be detected in the kidney, liver and the primary tumor, although only at the high doses (i.e. 100 or 200 mg/kg/day).

Following intragastric administration (Figure 2, lower panel), ruthenium concentrations were greater in the kidney and lung as compared to those measured in small intestine, spleen and the liver. Moreover, with the sole exception of the lungs, the metal concentration in all tissues studied was found to be strictly dependent on the administered dose.

Discussion

On adjuvant arthritic rats, treatment with NAMI-A (100 mg/kg), administered by either oral or i.p. route, did not modify the progression of the chronic inflammation, as judged by the "arthritic score", which mainly takes into account systemic reactions at articular level. However, at histologic level, the drug seemed to have a pro-inflammatory effect since it significantly increased the number of

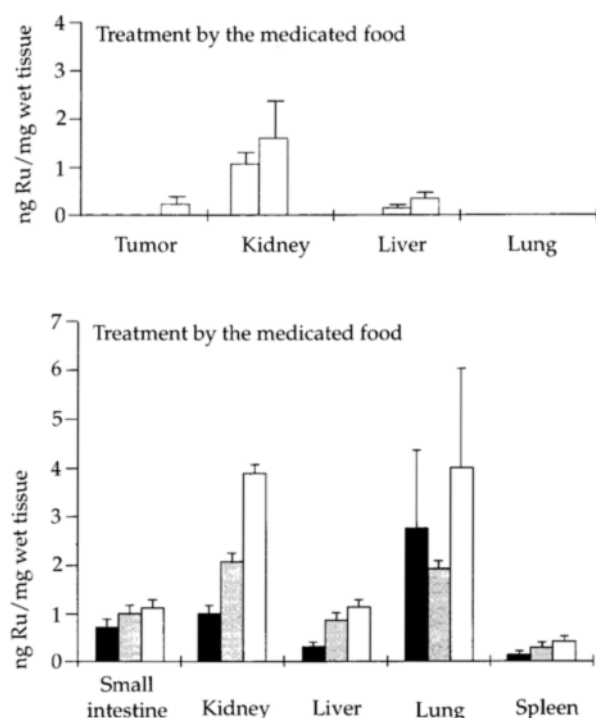


Figure 2. Ruthenium content in lung, kidney, liver, spleen, small bowel and primary tumor of CBA mice with MCa mammary carcinoma. Three mice per group from the experiment shown on Table 3 were terminated 24 hr after the last day of treatment and the above tissues were processed for determining the ruthenium content by AAS.

inflammatory cells present in other tissues (such as liver, lungs, kidneys and small intestine) which were also involved in the systemic response primed by injection of Freund's complete adjuvant. In particular, treatment with NAMI-A promoted deposition of connective tissue fibres, especially in the liver and lungs of arthritic animals. Although the above effects were clearly more pronounced when the compound was given i.p., they were also evident following treatment by gastric gavage, suggestive of the fact that a *bolus* of NAMI-A can be efficiently absorbed after oral administration.

NAMI-A had different effects depending on the site of tumor implantation (s.c. or i.m.), as well as on the route of administration (medicated food or *bolus*).

Bolus administration of NAMI-A into mice implanted i.m. by MCa tumor had no significant effect on the primary tumor or its lung metastases, in spite of the fact that relatively high levels of ruthenium (10^{-5} M) were measured in the lungs of the treated animals. This result seems to suggest that NAMI-A is devoid of any direct cytotoxic effect on tumor cells lodged in the lungs, as already shown by studies on the antimetastatic action of NAMI given to mice prior to tumor cell implantation.²

Concerning the effects of oral treatment, it must be stressed that when NAMI-A is given by gastric gavage, although the effects on cell cycle distribution are similar to those observed after i.p. administration,^{11,12} this treatment has no significant antitumor effects. These data seem to confirm that the antimetastatic effect of NAMI-A is not (or only marginally) related to the effects on tumor cells of the primary tumor mass. Moreover, the lack of the effects on the cell cycle distribution of cells harvested from lung metastases, was associated to a relatively high concentration of ruthenium in the lung tissue suggesting that metastasis reduction by NAMI-A is related to other factors.

Data obtained on NAMI-A treatment of s.c. tumor-bearing mice are more interesting. This treatment caused a significant reduction of leukocyte infiltration of the primary tumor, accompanied by a dramatic deposition of peritumoral connective tissue. Moreover, as already noted by examining tissues of adjuvant-challenged rats, NAMI-A increased connective tissue fibre deposition around both liver blood vessels and in the pulmonary alveoli. These observations may account for the significant inhibition of lung metastasis formation observed following treatment with 200 mg/kg given as medicated food. The results are consistent with those showing that NAMI-A is antimetastatic at doses able to reduce the balance between the matrix-metalloproteinase MMP2 and its specific tissue inhibitor TIMP2, by significantly modifying their respective mRNAs.¹⁰ Last but not least, treatment by powdered food containing the drug, given over 12 consecutive days, did not show signs of toxicity and similar results were obtained when NAMI-A was administered by gastric gavage to arthritic rats.

Collectively, these evidences may suggest the modulatory role of the ruthenium complex, NAMI-A, on the inflammatory processes – either associated with tumor growth or induced by the complete Freund-adjuvant. This also could explain its inhibitory effect on lung metastasis formation. Although the target(s) of the anti-metastatic action of NAMI-A is still unknown, our presented data could serve as useful guidelines for further studies.

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