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Preclinical report

Effects of NAMI-A and some related ruthenium complexes on cell viability after short exposure of tumor cells

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A series of three ruthenium complexes, i.e. *trans*-dichlorotetrakisdimethyl-sulfoxide ruthenium(II) (*trans*-Ru), imidazolium *trans*-imidazoletetra-chlororuthenate (ICR) and sodium *trans*-tetramethylensulfoxideisoquinoline-tetrachlororuthenate (TEQU), were studied *in vitro* in comparison to NAMI-A, a potent ruthenium-based antimetastasis agent. *In vitro* challenge of TS/A adenocarcinoma or KB oral carcinoma tumor cells with 10^{-4} M concentration for 1 h evidenced the lack of cytotoxicity of NAMI-A, ICR and *trans*-Ru, the accumulation of cells in the G_2/M pre-mitotic cell phase by NAMI-A and the attachment of tumor cells to the plastic substrate was significantly greater for NAMI-A than for ICR. These data stress that *in vitro* cytotoxicity is not necessary for *in vivo* activity of ruthenium antitumor complexes: NAMI-A, ICR and *trans*-Ru, are in fact known to be active against murine tumors in the mouse system. Rather, TEQU, the compound free of *in vivo* activity, was the only one to reduce cell growth of *in vitro* cultured cells. In conclusion, the data on the effects of NAMI-A on *in vitro* cultured cells show that the increase of cell adhesion properties and the transient cell cycle arrest in the G_2/M phase are much more relevant than the effects on cell properties relevant to cell growth (i.e. on CD44, CD54 or CD71 antigens) for determining *in vivo* anti-metastasis activity. [© 2000 Lippincott Williams & Wilkins.]

Key words: Cytotoxicity, ruthenium.

Introduction

The last decade has shown the development of some classes of ruthenium complexes endowed with interesting chemical properties.[1–3] Some of them, such as those characterized by sulfoxide ligands, showed a pronounced antitumor activity in experimental models of murine tumors.[1] Among these complexes, one, i.e. imidazolium *trans*-imidazoledimethylsulfoxidetetrachlororuthenate (NAMI-A), evidenced a selective action against lung metastases of solid experimental tumors, irrespective of the reduction of primary tumor growth.[4] The pharmacological characterization of the favorable ratio between anti-metastasis action and host toxicity of NAMI-A has recently prompted the start of a phase I clinical trial as an anti-metastasis agent at the Netherlands Cancer Institute, Amsterdam. The evolution of NAMI-A is based upon the chemical and pharmacological information obtained from the preclinical studies of a number of other ruthenium compounds. Among these, *trans*-dichlorotetrakisdimethylsulfoxide ruthenium(II) (*trans*-Ru), originating from the original studies on the *cis* isomer,[5] provided evidence of the role of sulfoxide ligands for metastasis reduction; imidazolium *trans*-imidazoletetrachlororuthenate (ICR),[6] a ruthenium(III) complex, showed the favorable role of imidazole for antitumor activity; and sodium *trans*-tetramethylensulfoxideisoquinoline-tetrachlororuthenate (TEQU),[7] a highly liposoluble ruthenium(III), showed a marked tumor cell cytotoxicity, not different from that of cisplatin.

We therefore thought it worthwhile to compare the effects of these four ruthenium complexes *in vitro* on the human KB cell line and on the murine TS/A adenocarcinoma cell line. The aim of the study was to examine the relative capacity to alter cell growth following short exposure of tumor cells at a concentration comparable to that obtained *in vivo* following treatment at the maximum tolerated dose.

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Materials and methods

Compounds and treatment

NAMI-A,[4] TEQU,[7] *trans*-Ru[5] and ICR[6] were prepared according to already reported procedures. For *in vitro* studies NAMI-A, TEQU, *trans*-Ru and ICR were dissolved in PBS-Ca^{2+}-Mg^{2+} and sterilized by filtration with a 0.2 μm filter.

Tumor cell lines

An established KB cell line (ECACC no. 86103004) was cultured according to standard procedure.[8] Vials of the original line were maintained in liquid N_2. The KB cell line was maintained in Eagle's minimum essential medium[9] with 1% non-essential amino acids (Serva, Heidelberg, Germany), supplemented with 10% newborn calf serum (EuroClone, Paignton, UK), 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, St Louis, MO). Culture medium was added with penicillin–streptomycin solution (Sigma) (100 U/ml penicillin G and 100 μg/ml streptomycin). Cells from confluent monolayers were removed by 0.05% trypsin solution (Sigma). Cell viability was determined by the Trypan blue dye exclusion test.

TS/A adenocarcinoma cell line was kindly supplied by the group of Dr G Forni (CNR Centro di Immunogenetica ed Oncologia Sperimentale, Torino, Italy), cultured according to standard procedure,[10] and maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS (Hyclone Europe, Aalst, Belgium), 2 mM L-glutamine (Hyclone Europe) and 50 μg/ml gentamycin sulfate solution (Irvine Scientific, Santa Ana, CA).

Cells from confluent monolayers were removed from flasks by 0.25% trypsin solution (Sigma). Cell viability was determined by the Trypan blue dye exclusion test.

MTT test to evaluate *in vitro* cytotoxicity

Cell growth was determined by the MTT viability test.[11] Briefly, KB cells (5000/well) were seeded in 96-well cell culture clusters (Costar, New York, NY) in culture medium and grown for 96 h. Test compounds were dissolved in PBS containing Ca^{2+} and Mg^{2+} immediately before use, and diluted to 10^{-4} M concentration; cells were incubated for 1 h with the test compounds, at 37°C with 5% CO_2 and 100% relative humidity. At the end of incubation, drug solutions were removed and replaced with complete medium. The cytotoxic effect was evaluated 24 h after drug challenge: 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS, was added to each well of the 96-well culture plate containing 100 μl of medium and incubated for 4 h at 37°C with 5% CO_2 and 100% relative humidity. At this time the medium was discarded and 100 μl of acidified isopropanol (0.2 ml of 0.04 N HCl in 10 ml isopropanol) was added to each well accordingly to the modification utilized by Galeano *et al.*[12] Optical density was measured at 540 nm on a spectrophotometer Multiskan MCC/340 (Labsystems, Helsinki, Finland).

Sulforhodamine B (SRB) test to evaluate *in vitro* cytotoxicity

Cell growth was determined by staining with the protein-binding dye SRB.[13] Briefly, KB cells (5000/well) were seeded in 96-well cell culture clusters (Costar) in culture medium and grown for 96 h. Test compounds were dissolved in PBS containing Ca^{2+} and Mg^{2+} immediately before use, and diluted to 10^{-4} M concentration; cells were incubated for 1 h with the test compounds, at 37°C with 5% CO_2 and 100% relative humidity. At the end of incubation, drugs solutions were removed and replaced with complete medium. The cytotoxic effect was evaluated 24 h after drug challenge: briefly, adherent cell cultures were fixed *in situ* by addition of 50 μl of cold 50% (w/v) trichloroacetic acid (TCA) and were kept for 60 min at 4°C. The supernatant was then discarded, and the plates were washed 5 times with distilled water and air dried. SRB solution (0.4% w/v in 1% acetic acid) was added and the cells were allowed to stain for 30 min at room temperature. Unbound SRB was removed by washing 3 times with 1% acetic acid. Then the plates were air dried. Bound stain was dissolved with unbuffered 10 mM Tris base (tris-hydroxymethyl-aminomethane) (Sigma) and the optical density was read at 540 nm on a spectrophotometer Multiskan MCC/340 (Labsystems).

Flow cytometry measurements

Phenotypic analysis Monolayers of TS/A cells were detached with 0.2% EDTA dissolved in HBSS and buffered to pH 6.7 with HEPES (Sigma). Cells of a single-cell suspension prepared as above were washed with PBS containing 0.5% bovine serum albumin (BSA; Sigma) and 0.1% NaN_3 (Sigma), and adjusted to 1×10^7 cells/ml. Cell viability, as determined by the Trypan blue dye exclusion test, was above 90%. Antibody

staining of single-cell suspensions was performed using the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: anti-CD44 (CD44–FITC; Southern Biotechnology Associates, Birmingham, AL; clone KM201, 4 μg/10^6 cells), anti-CD54 (CD54–FITC, Southern Biotechnology Associates; clone KAT-1, 4 μg/10^6 cells) and anti-CD71 (CD71–FITC; Sigma; clone RI7217, 1 μg/10^6 cells). Controls were run with cells stained with an irrelevant isotype control (IgG2a,k–FITC, Sigma; clone UPC-10). After 30 min staining at 4°C, unbound monoclonal antibody was discarded by washing cells twice with PBS (0.5 % BSA/0.1% NaN_3). The resulting pellet was diluted with PBS, added with 1% HCHO and analyzed within 5 days. Flow cytometry was performed with an Epics XL (Coulter Electronics, Miami, FL), acquiring at least 10 000 events per sample. Electronic gates were set to exclude 98% of the cells in the isotype control.

Propidium iodide test Viable cells (1×10^6) of a single-cell suspension, as determined by the 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 re-suspended 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 40 μg/ml propidium iodide (Sigma) (modified from Crissman and Steinkamp[14]). Red fluorescence (610 nm) was analyzed, using the peak fluorescence gate to discriminate aggregates. Each analysis consisted of 10 000 events counted. The flow cytometric analyses were done at Fondazione Callerio with an Epics XL flow cytometer. Cell cycle distribution of the cells was determined by analysis with Multicycle software (Phoenix Flow Systems).

Assay for detachment to grown substrate

TS/A cells (5×10^4/well) were plated onto 12-well culture plates and incubated in complete medium for 48 h in 5% CO_2 humidified atmosphere at 37°C. The cells were treated with 10^{-4} M NAMI-A or ICR, dissolved in PBS-Ca^{2+}-Mg^{2+}, for 1 or 4 h, at 37°C. At the end of treatment the cell layer was washed with PBS and then rotated on an orbital shaker in PBS containing 0.002% (w/v) trypsin for 5 min and repeating the procedure for up to 3 times.[15] The percentage of cells detached after one, two or three procedures was calculated considering as 100% the cells determined in a separate group of three wells, sown in the same manner and kept for 1 or 4 h in PBS, which were completely harvested with 0.25% trypsin solution.

Statistical analysis

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

Results

The short 1 h exposure of TS/A adenocarcinoma cells to 10^{-4} M of NAMI-A, ICR and *trans*-Ru did not influence cell growth: 24 h after a further cultivation in the absence of the drug, the number of cells harvested from the cultures is not different from that of untreated controls (Figure 1). Conversely, TEQU completely blocked cell growth: cell number did not differ from that found in the well before treatment (Controls-0).

Similarly, also on KB cells, the short 1 h exposure of cells to 10^{-4} M concentration showed only TEQU to markedly reduce the number of cells harvested from the well 24 h after a further cultivation in the absence of the drug (Figure 2). While ICR and *trans*-Ru showed the same behavior as with TS/A cells, NAMI-A, on this cell line, caused a marked and significant increase of the number of cells harvested 24 h after challenge. The analysis of cell viability by the SRB or by the MTT test confirmed the cytotoxic effects of TEQU and the lack of activity on cell proliferation by the other three test compounds (Figure 2).

The study of some morphological changes of TS/A tumor cells following 1 h exposure to the test compounds and a further 24 h cultivation in drug-free medium showed effects randomly distributed among the parameters examined (Table 1). NAMI-A increased the number of $CD54^+$ small cells; ICR increased the number of $CD54^+$ cells independently of the cell subset, increased the number of $CD71^+$ small cells and reduced CD44 expression in the small cell population; *trans*-Ru reduced CD44 expression in the large and particularly in the small cell population. The study with TEQU was not possible because the cells harvested from the wells did not allow the flow cytometry analysis with the respective monoclonal antibodies.

TS/A and KB cell distribution among cell cycle phases, following 1 h exposure to the test compounds and a further 24 h cultivation in drug-free medium, showed NAMI-A to reduce the fraction of G_0/G_1 cells and to increase those of G_2/M, independently of the cell line being treated (Figure 3). TEQU, for which it

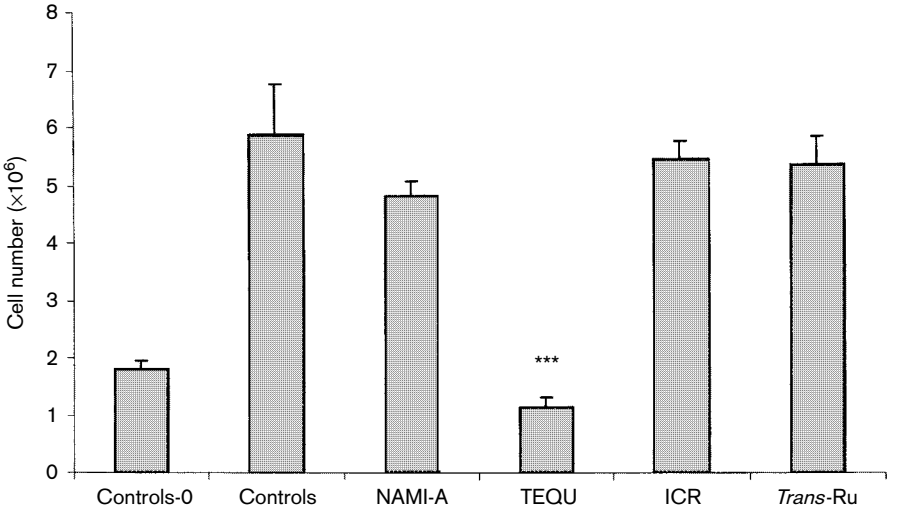

Figure 1. Effects of NAMI-A, TEQU, ICR and *trans*-Ru on cell proliferation of TS/A adenocarcinoma cells. TS/A adenocarcinoma cells, cultured *in vitro* in the presence of 10^{-4} M NAMI-A, TEQU, ICR and *trans*-Ru for 1 h, were washed at the end of drug challenge, resuspended in complete medium and further cultured for 24 h prior to analysis of cell number in each well. Controls-0 is the number of cells measured immediately before drug addition to cell cultures. Each value is the mean ± SE obtained with three separate determinations. ***$p < 0.001$ versus controls, ANOVA and Tukey–Kramer test.

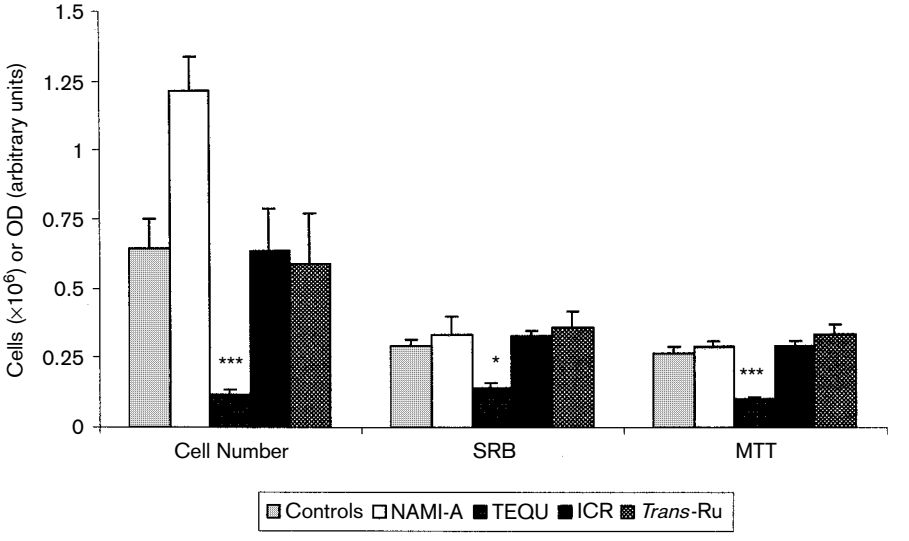

Figure 2. Cytotoxicity of NAMI-A, TEQU, ICR and *trans*-Ru on KB cells as determined by cell number, SRB and MTT test. KB carcinoma cells, cultured *in vitro* in the presence of 10^{-4} M NAMI-A, TEQU, ICR and *trans*-Ru for 1 h, were washed at the end of drug challenge, resuspended in complete medium and further cultured for 24 h prior to analysis of cell number in each well by the Trypan blue exclusion test, by the SRB or MTT test. Each value is the mean ± SE obtained from three separate determinations. ***$p < 0.001$, *$p < 0.05$ versus controls, ANOVA and Tukey–Kramer test.

was possible to examine only the cells of the TS/A cell line, showed a marked inhibition of G_0/G_1 fraction and a significant increase of the S fraction, whereas ICR and *trans*-Ru are completely free of effects on cell cycle distribution.

The number of TS/A cells detachable, with a dilute trypsin solution, from the well after treatment with NAMI-A or ICR for 1 or 4 h at 10^{-4} M concentration is markedly reduced (Figure 4). Plots in Figure 4 represent the cumulative number of cells harvested from the plastic well by a 5–15 min washing with a diluted trypsin solution. NAMI-A, much more than ICR and independently of the length of drug challenge (4 or 1 h), inhibited the recovery of TS/A cells by the

Table 1. Morphological changes on TS/A cells

Treatment	$CD44^+$ cells		$CD54^+$ cells		$CD71^+$ cells	
	%	MnX	%	MnX	%	MnX
Large cells						
controls	96.0±0.4	9.9±0.2	6.7±1.2	5.4±0.1	90.8±0.9	1.4±0.1
NAMI-A	96.5±0.8	8.6±0.5	7.3±0.5	5.0±0.2	90.9±1.1	1.5±0.1
ICR	95.5±0.5	10.2±1.1	14.1±0.4[c]	5.4±0.5	90.7±0.2	1.4±0.0
trans-Ru	96.0±0.4	6.1±0.2[a]	6.3±0.4	4.7±0.4	90.6±1.4	1.4±0.1
Small cells						
controls	61.7±3.2	6.3±0.2	37.7±1.8	3.3±0.2	61.1±3.3	1.3±0.0
NAMI-A	69.4±2.7	5.4±0.3	48.9±3.2[a]	2.7±0.1	70.8±1.5	1.2±0.1
ICR	79.6±1.4[b]	4.6±0.4[b]	70.2±0.6[c]	3.1±0.2	78.8±2.1[a]	1.5±0.2
trans-Ru	67.5±1.0	4.1±0.1[b]	45.4±1.7	2.6±0.2	70.4±4.0	1.3±0.0

TS/A adenocarcinoma cells, cultured *in vitro* in the presence of 10^{-4} M NAMI-A, ICR and *trans*-Ru for 1 h were washed at the end of drug challenge, resuspended in complete medium and further cultured for 24 h prior to analysis with monoclonal antibodies. Results are the mean value of triplicate. MnX represent the mean channel of fluorescence peak. [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ versus controls, ANOVA and Tukey–Kramer test.

trypsin solution and less than 20% of the treated cells were recovered as compared to untreated controls (60 or 40% in the case of ICR, respectively, after 1 h or 4 h exposure).

Discussion

The challenge of the last 10 years on new antitumor agents based on transition metals has shown the emergence of some ruthenium-based compounds. Besides some common features, the different ruthenium compounds synthesized appear to show rather different characteristics either considering the chemical behavior in solution or their biological effects: some being capable of cytotoxic effects[16,17] and some others completely devoid of cytotoxicity but with a potent antimetastatic activity *in vivo* in experimental models of solid metastasizing tumors.[18,19]

Data from the present investigation also support these view and point out that *in vitro* cytotoxicity does not predict *in vivo* antitumor effects, at least not the antimetastatic action which the sulfoxide ruthenium complex NAMI-A showed in experimental models. Apart from TEQU, which is free of *in vivo* activity,[16,20] none of the other three ruthenium complexes showed any appreciable *in vitro* cytotoxicity, neither measuring the growth capacity of the treated cells nor examining their effects on protein synthesis (SRB test) or on oxidative metabolism (MTT test). Rather, the only effect relevant for antimetastasis action seems the G_2-M cell accumulation, an alteration of cell cycle distribution which we know to be completely reversed in a further 24 h time[21] (unpublished work) and which does not lead to cell death. This effect suggests the pharmacological interaction of NAMI-A, but not of the other complexes, with the fine mechanisms that regulate cell cycle progression.

A large part of the present study was performed with the mouse TS/A adenocarcinoma. TS/A is a cell line in which cells with high proliferation capacity (large cells with an epithelial-like shape) are in equilibrium with more resting, small (fibroblast-like) cells.[22] This model therefore allows us to discriminate between the effects on the highly malignant cells from those on the low proliferating cell subtype. Ruthenium complexes cause modifications of this cell line, independently of the lack of effects on cell proliferation. NAMI-A, similarly to ICR and *trans*-Ru, increases the $CD54^+$ cell population of small cells. If, on the one hand, this effect might account for the increased interaction of tumor cells with host lymphocytes in mice treated with NAMI-A (unpublished work), on the other hand, it tells us that this effect is not sufficient for the *in vivo* activity since ICR, which causes effects even superior to those of NAMI-A on $CD54^+$ cells, is not antimetastatic *in vivo* in comparison experiments (unpublished work). Besides the effects on ICAM-1 (CD54), NAMI-A seems to have more effectiveness on the stimulation of cell adhesion. In fact, cells treated with NAMI-A show a much greater adhesion to the plastic substrate as compared to ICR and this effect might be ascribed to the stimulation of the integrin system, as already shown in a study in which leukocytes treated with NAMI adhered much strongly to their substrate because of the β_2-integrin activation.[23]

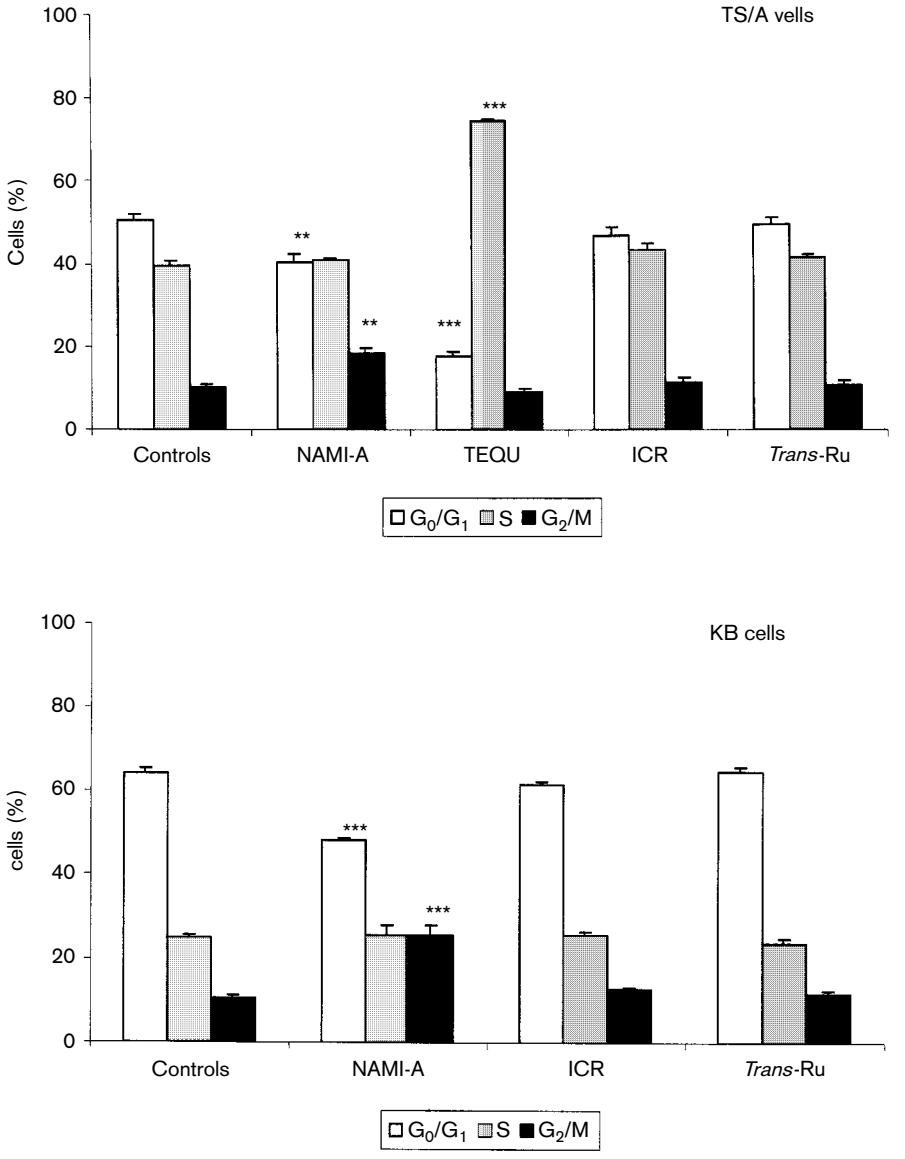

Figure 3. Cell cycle distribution of TS/A and KB cells following treatment with NAMI-A, TEQU, ICR and *trans*-Ru complexes. Histograms represent mean ± SE obtained with three separate determinations of cells taken from the experiment reported in Figures 1 and 2. ***$p < 0.001$, **$p < 0.01$ versus controls, ANOVA and Tukey–Kramer test.

These data stress that *in vitro* cytotoxicity is not necessary for *in vivo* activity of ruthenium antitumor complexes. NAMI-A, ICR and *trans*-Ru were in fact active against murine tumors in the mouse system. Rather, TEQU, the compound free of *in vivo* activity, was the only one to reduce cell growth of *in vitro* cultured cells. In conclusion, the data on the effects of NAMI-A on *in vitro* cultured cells show that the increase of cell adhesion properties and the transient cell cycle arrest in the G_2/M phase are much more relevant than the effects on cell properties relevant to cell growth (i.e. on CD44, CD54 or CD71 antigens), for determining *in vivo* anti-metastasis activity.

Acknowledgments

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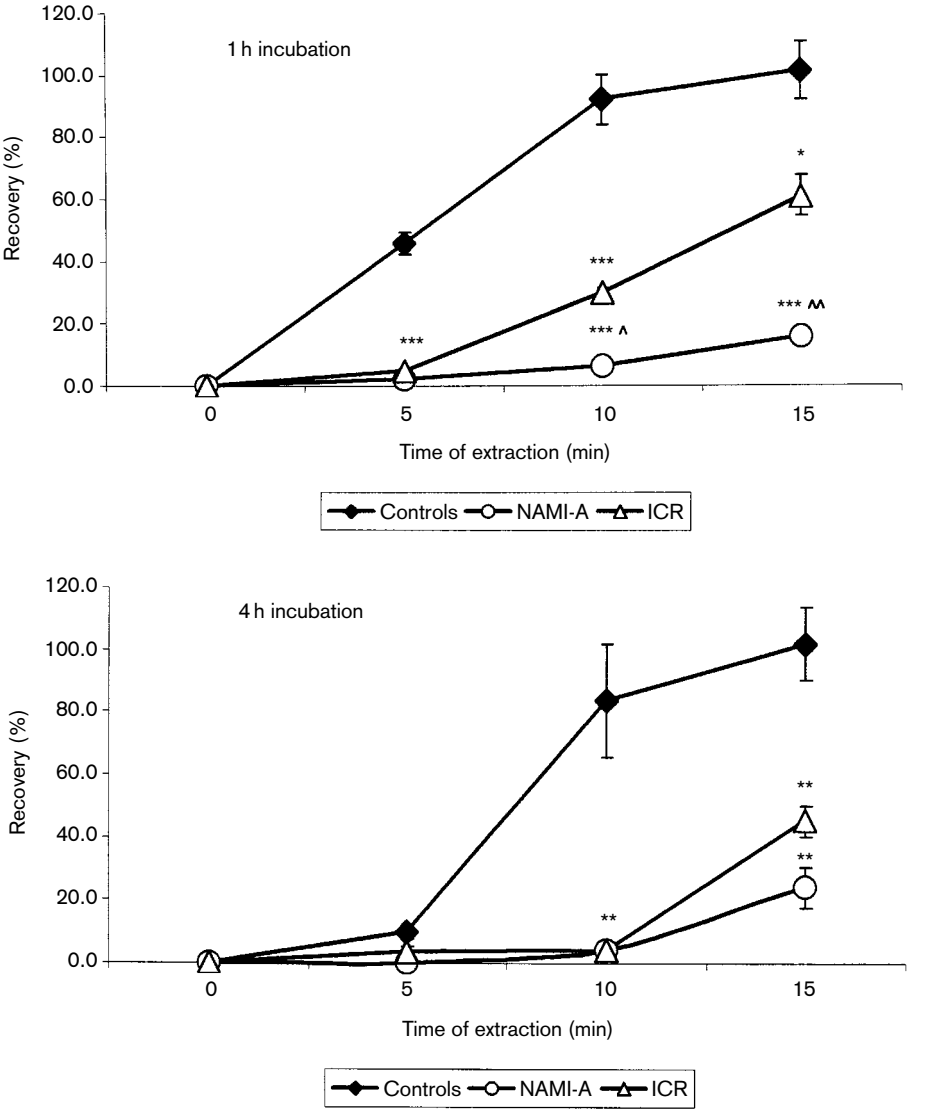

Figure 4. Cell detachment from plastic substrate of TS/A cells following treatment with NAMI-A and ICR. TS/A cells were treated with 10^{-4} M NAMI-A or ICR for 1 or 4 h, at 37°C. At the end of treatment the cell layer was removed by a trypsin solution for the indicated times. Results are the mean ± SE obtained from three separate determinations. ***$p<0.001$, **$p<0.01$, *$p<0.05$ versus controls; ^^$p<0.01$, ^$p<0.05$ versus ICR, ANOVA and Tukey–Kramer test.

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