

Modulation of the metastatic progression of breast cancer with an organometallic ruthenium compound

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Abstract. The modulation of the metastatic progression of breast cancer has been evaluated *in vitro* and *in vivo* with RAPTA-T, $[\text{Ru}(\eta^6\text{-toluene})\text{Cl}_2(\text{PTA})]$, an organometallic ruthenium compound. *In vitro* RAPTA-T inhibits some steps of the metastatic process such as the detachment from the primary tumour, the migration/invasion and the re-adhesion to a new growth substrate. All these effects are boosted when cells grow on components of the extra cellular matrix such as collagen IV and fibronectin and minimized on the non-specific substrate poly-L-lysine and are more pronounced when experiments are performed with the highly invasive MDA-MB-231 cells than with the non-invasive MCF-7 or the non-tumourigenic HBL-100. *In vivo* RAPTA-T selectively reduces the growth of lung metastases, an effect that might be explained by the *in vitro* activity. The effect on tests requiring the interaction of the tumour cells with extra cellular matrix components, might suggest an interaction with cell surface molecules in the activity of this ruthenium compound.

Introduction

In cancer chemotherapy, research on platinum-based compounds was stimulated by the serendipitous discovery of cisplatin and by its enormous impact in the treatment of several cancer types (1). Previously, the development of different metal compounds has been facilitated by the rich coordination chemistry and redox properties of metal ions and driven by the necessity to fill the unmet needs of chemotherapy, in particular to minimise undesirable side-effects, to overcome drug resistance and to enlarge the

spectrum of activity to more tumour types and to metastatic (secondary) cancers (2). Notably, ruthenium complexes have been found to be effective against cancers that cannot be treated with platinum drugs and it was also found that they exhibit a lower general toxicity compared to platinum compounds (3-5). Among them, imidazolium *trans*-imidazole-dimethylsulfoxide-tetrachlororuthenate, NAMI-A, shows high and peculiar selectivity for solid tumour metastases and low toxicity at pharmacologically active doses (6) and has successfully completed phase I clinical trials (7). NAMI-A metastasis control is associated with a series of biological activities that influence cell function such as adhesion, motility and invasion (8,9).

Previously, increasing interest has focused on organometallic compounds, specifically on ruthenium(II)-arene compounds which show excellent antiproliferative properties *in vitro* and *in vivo*, also in cisplatin-resistant cell lines (10). Our attention has focused on ruthenium(II)-arene complexes combined with the 1,3,5-triaza-7-phosphaadamantane (PTA) ligand. The prototype $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2(\text{PTA})]$, termed RAPTA-C (Fig. 1), was found to exhibit pH-dependent DNA damage: at the pH typical of hypoxic tumour cells DNA was damaged, whereas at the pH characteristic of healthy cells, little or no damage was detected (11,12). Several structurally diverse RAPTA derivatives have been studied and their *in vitro* cytotoxicity has been evaluated (13,14). The *in vivo* effect on the growth of lung metastases was also established for RAPTA-C and RAPTA-B, i.e. $[\text{Ru}(\eta^6\text{-benzene})\text{Cl}_2(\text{PTA})]$ (14). *In vivo* RAPTA-C shows diverse behaviour depending on the schedule of administration: it was found to reduce the number of lung metastases when a cumulative dose of 400 mg/kg is given fractionated in 2 or 4 treatments. Notably, 200 mg/kg/day on days 5 and 9 after primary tumour implant, appear to be the most efficient in reducing lung metastases formation. RAPTA-B, in the same experimental mouse model, administered with a total dose of 400 mg/kg at two different frequencies has the same effect on the metastases number. Moreover, both RAPTA-C and RAPTA-B do not influence the evolution of growth of the primary tumour.

From a structural and chemical viewpoint NAMI-A and RAPTAs are very different compounds, albeit based on ruthenium. Their oxidation states differ as do their ligand sets, RAPTAs being organometallics and NAMI-A a traditional

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coordination compound. In any case, the anti-metastatic activity of NAMI-A, RAPTA-B and RAPTA-C is unique although NAMI-A is considerably more potent than the two RAPTA compounds. Nevertheless, the signals of activity on metastases of the RAPTA family of compounds suggest the need to know more about their ability to interact with this important phenomenon of cancer growth as current approaches in clinical cancer therapy of most solid tumours have only limited success. Despite earlier tumour detection and better therapeutic regimens having brought significant advances, the presence of metastases remains the most significant poor prognostic factor implying a shortened survival and reduced quality of life. Effective novel therapeutic strategies targeting selectively metastasis development and growth should provide the greatest clinical benefit and RAPTA compounds offer potential as they can easily be systematically modified to exhibit desirable physical, chemical and biological properties.

We studied the effects of RAPTA-T in a series of experiments that simulate *in vitro* the main steps of the metastatic progression, i.e. the detachment from the primary tumour, the degradation of the extracellular matrix, the ability to migrate, to invade and to adhere to a new organ, comparing the behaviour of MDA-MB-231 highly invasive breast cancer cells to that of MCF-7, tumourigenic but not invasive and of HBL-100 non-tumourigenic mammary epithelial cells. The *in vitro* behaviour is then compared with the *in vivo* anti-metastatic efficacy of the compound in a mouse experimental model. RAPTA-T was selected for this study despite being unproven *in vivo*, since it showed *in vitro* the greatest selectivity towards cancer cells compared to non-tumourigenic cells (14).

Materials and methods

Materials. RAPTA-T was prepared according to the published procedure (14). All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Tumour cell lines for *in vitro* tests. The MDA-MB-231 human highly invasive breast cancer cell line was kindly supplied by Dr P. Spessotto (Cro, Aviano, Italy) and maintained in Dulbecco's modified Eagle's medium (EuroClone®, Devon, UK) supplemented with 10% FBS (Gibco, Invitrogen™, Paisley, Scotland, UK), 2 mM L-glutamine (EuroClone), 1% nonessential amino acids and 100 IU/ml penicillin and 100 µg/ml streptomycin (EuroClone).

The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA; catalog number HTB-22) and maintained in Dulbecco's modified Eagle's medium/F12 medium 1:1 v/v (EuroClone) supplemented with 10% FBS, 2 mM L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin.

The HBL-100 human non-tumourigenic epithelial cell line was kindly supplied by Dr G. Decorti (Department of Biomedical Sciences, University of Trieste, Italy) and maintained in McCoy's 5A medium supplemented with 10% FBS, 2 mM L-glutamine and 100 IU/ml penicillin and 100 µg/ml streptomycin.

All cell lines were kept in a CO₂ incubator with 5% CO₂ and 100% relative humidity at 37°C. Cells from a confluent

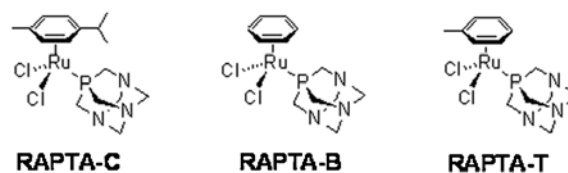


Figure 1. Structure of the ruthenium(II)-arene PTA (RAPTA) compounds.

monolayer were removed from flasks by a trypsin-EDTA solution. Cell viability was determined by the trypan blue dye exclusion test. For experimental purposes cells were sown in multiwell culture clusters.

Resistance to detachment assay. The ability of cells to resist detachment after treatment with RAPTA-T was measured as follows: 96-well plastic plates (Corning Costar, Milano, Italy) were coated with the following substrates: 10 µg/ml polylysine, 20 µg/ml fibronectin from human plasma and 20 µg/ml collagen IV from human placenta and left in a humidified cell culture chamber at 37°C for 4 h. Before cell seeding, plates were washed with CMF-DPBS (Calcium and Magnesium Free Dulbecco's phosphate-buffered saline), then 6x10³ cells in 0.2 ml complete medium were sown in each well. After 2 days at 37°C, complete medium was replaced with serum-starved medium, containing 0.1% w/v BSA. After 24 h, the medium was removed and the plates washed with CMF-DPBS, before the treatment with RAPTA-T 10⁻⁴ M, dissolved in DPBS (Dulbecco's phosphate-buffered saline), was added to the wells and incubated for 1 h. At the end of the treatment the RAPTA-T containing solution was removed, the plates were washed twice with CMF-DPBS and trypsin solution, 0.008% w/v, was added to each well. Plates were kept in agitation for 30 min at room temperature then trypsin solution was removed and wells washed with CMF-DPBS. Cells that still adherent to the plates were detected by the sulforhodamine B (SRB) test. Resistance to detachment is expressed as arbitrary units, calculated by dividing the mean absorbance of treated cells by the mean absorbance of control cells.

Re-adhesion assay. The effect of the ability of the cells to re-adhere after RAPTA-T treatment, was studied in cells maintained for 24 h in serum-starved medium and then treated for 1 h with RAPTA-T 10⁻⁴ M in DPBS. At the end of the treatment cells were removed from flasks by a trypsin-EDTA solution, collected by centrifugation, re-suspended in serum-starved medium supplemented with 0.1% w/v BSA and kept for 30 min at room temperature to allow surface receptor reconstitution. The cells were then seeded at a density of 1x10⁴ cells in 0.1 ml/well on 96-well plastic plates previously coated as described above with polylysine, fibronectin, collagen IV or 20 µg/ml Matrigel® (BD, Biosciences, San José, CA). Cells were left to adhere for 30 and 60 min at 37°C with 5% CO₂ and 100% relative humidity, then the medium containing the non-adherent cells was removed and wells were gently washed with CMF-DPBS. Cells that in 30 or 60 min have adhered to the substrates were detected by the sulforhodamine B (SRB) test.

Sulforhodamine B assay. Adherent cells were detected with the SRB test described by Skehan *et al* (15). Briefly, adherent cells were fixed with 10% v/v cold trichloroacetic acid (TCA) at 4°C for 1 h. After fixation TCA was discarded and wells washed five times with distilled water and air-dried. SRB solution (0.4%, w/v, in 1% acetic acid) was added to the wells and plates were kept for 30 min at room temperature. Unbound SRB was removed by washing three times with 1% acetic acid. Plates were air dried, then bound stain was dissolved with un-buffered 10 mM Tris base (*tris*-hydroxymethyl-aminomethane) at pH 10.5 and the optical density was read at 570 nm with an automatic computerised spectrophotometer (SpectraCount; Packard, Meriden, CT).

Migration assays. Migratory ability resulting from a haptotactic or a chemotactic stimulus was measured in Transwell® cell culture chambers (Costar, Milano, Italy). In the haptotaxis assay the lower surface of a polyvinylpyrrolidone-free polycarbonate filter (8- μ m pore size) was coated with 10 μ g/ml fibronectin and left in a humidified cell culture chamber at 37°C for 2 h, then washed with CMF-DPBS before cell seeding. In the chemotaxis assay, inserts were used without coating. Cells were treated for 1 h with RAPTA-T 10^{-4} M in DPBS. After treatment cells were removed with a trypsin-EDTA solution, collected by centrifugation, re-suspended in serum-starved medium supplemented with 0.1% w/v BSA and 1×10^5 cells in 0.2 ml were sown in the upper compartment of each chamber. The lower compartment was filled with serum-starved medium supplemented with 0.1% w/v BSA and with complete medium for the haptotaxis and the chemotaxis assay, respectively.

Cells were left to migrate for 24 h, then the cells on the upper surface of the filters were removed with a cotton swab and migrating cells, present in the lower surface, were detected by the crystal violet assay.

Invasion assay. Invasive ability was measured in a Transwell cell culture chamber according to the method of Albini *et al* (16). Briefly, the upper surface of the polycarbonate filter (8- μ m pore size) of Transwell cell culture chambers was coated with 50 μ l of a 600 μ g/ml Matrigel solution and air dried overnight at room temperature. The filters were re-constituted with DMEM medium for 90 min under gently shaking immediately before use. Cells were treated as described for the migration assays and 0.5×10^5 cells in 0.2 ml were sown in each chamber. Cells were left to invade for 96 h, then the cells on the upper surface of the filters were removed with a cotton swab and invading cells, present in the lower surface, were detected by the crystal violet assay.

Crystal violet assay. The crystal violet assay was performed according to the method described by Kueng *et al* (17). Briefly, the cells present on the lower surface of the filter were fixed with a 1.1% w/v glutaraldehyde solution for 15 min. After fixation the wells were washed three times with distilled water and air dried. Cells were stained for 20 min with 0.1% w/v crystal violet prepared in 200 mM boric acid, pH 9.0, then washed three times with distilled water and air-dried prior to dissolve the dye with 10% acetic acid

solution. The optical density was read at 590 nm with an automatic computerised spectrophotometer (SpectraCount).

Cell viability. To evaluate if treatment with RAPTA-T, in the experimental conditions adopted, can affect cell viability cells were treated as described above for migration tests, except they were seeded on 96-well plates. After 24 h, the cell viability was measured by the MTT assay: a solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dissolved in CMF-DPBS (5 mg/ml) was added to each well (10 μ l per 100 μ l of medium) and the plates were incubated at 37°C with 5% CO₂ and 100% relative humidity for 4 h. After this time, the medium was discarded and 200 μ l of DMSO was added to each well to dissolve the formazan crystals (18). The optical density was measured at 570 nm with an automatic computerised spectrophotometer (Spectra Count).

Zymography. To visualise the direct effect of RAPTA-T on the activity and/or production of MMP-2 and MMP-9 enzymes, sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) zymography was carried out with conditioned medium of MDA-MB-231 and HBL-100 cells. Cells at 70% confluence were incubated for 24 h in serum-starved medium containing 0.1% BSA, before being treated with RAPTA-T 10^{-4} M for 1 h. At the end of the treatment, the RAPTA-T solution was discarded and complete serum-free medium containing 0.1% BSA was added for a further 24 h, when culture media were collected, centrifuged to remove cellular debris, then concentrated ~15 times using Amicon® Ultra-15 30,000 nominal molecular weight limit centrifugal filter devices (Millipore Corporation, Bedford, MA). The conditioned media obtained were stored at -80°C until use. Equal amounts of proteins, as determined by the Bradford method (19), for each sample were eluted with Laemmli non-reducing sample buffer and analysed by SDS-PAGE on a 7% polyacrylamide gel containing 0.1% (w/v) gelatine. At the end of electrophoresis in a dual-laboratory system (Protean II, Bio-Rad Laboratories, Hercules, CA), the gels were washed two times for 30 min at 4°C in 2.5% Triton X-100 to remove SDS. After additional washing in water (three times for 5 min), the gels were incubated at 37°C overnight in collagenase buffer [200 mM NaCl, 50 mM *tris*(hydroxymethyl) aminomethane, 5 mM CaCl₂, adjusted to pH 7.4] to reactivate enzyme activity. The gels were then stained with 0.5% Coomassie brilliant blue. The gelatinolytic regions were observed as white bands against a blue background. Quantitative evaluation of the band intensity, on the basis of grey levels, was performed using Image Master 2D version 4.01 and Magic Scan 32 version 4.3 software.

In vivo tests. The *in vivo* experiments were carried out with the murine mammary carcinoma (MCa), originally obtained from the Department of Biology, Rudjer Boskovich Institute (Zagreb, Croatia), grown in CBA female mice (20), obtained from a local breeding colony grown according to the standard procedures for inbred strains. The tumour line was locally maintained by serial biweekly passages of 10^6 viable tumour cells, of a cell suspension prepared from mincing (with scissors) the primary tumour masses obtained from donors

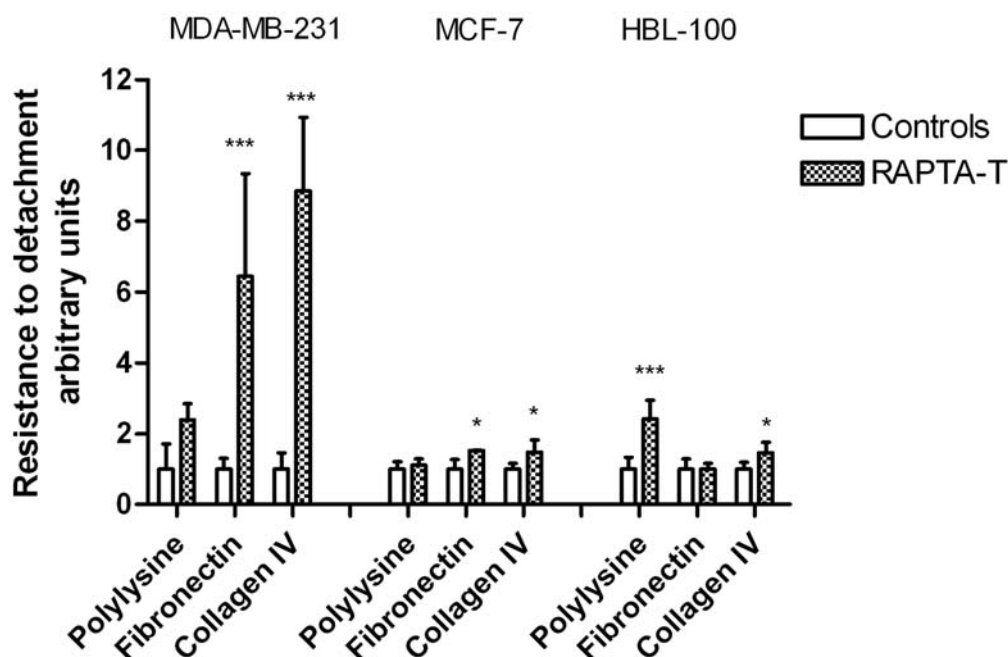


Figure 2. Effect of RAPTA-T on resistance to detachment. MDA-MB-231, MCF-7 and HBL-100 cells seeded on 96-well plastic plates previously coated with polylysine, fibronectin and collagen IV, exposed for 1 h to RAPTA-T 10^{-4} M and then to a diluted trypsin solution for 30 min, before detecting cells still attached to the growth substrate by the SRB test. Arbitrary units are calculated from the mean \pm SD of two experiments, each performed in quadruplicate. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. controls, ANOVA and Tukey-Kramer post test.

similarly implanted 2 weeks before. The minced tissue was filtered through a double layer of sterile gauze, centrifuged at $250 \times g$ for 10 min and re-suspended in an equal volume of CMF-DPBS; viable cells were counted by the trypan blue exclusion test. Viable tumour cells (10^6) were injected i.m. into the left hind calf of experimental groups. RAPTA-T was dissolved in physiological saline (0.9% NaCl) and given to mice by i.p. (intra peritoneal) administrations of 80 mg/kg/day on days 8, 9 and 12 after tumour implant.

Primary tumour growth was determined by calliper measurements, by measuring two orthogonal axes and the tumour volume was calculated with the formula: $(\pi/6) \times a^2 \times b$, where a is the shorter axis and b the longer axis, assuming tumour density equal to 1 g/ml. The evaluation of the number and weight of lung metastases was performed by examining the surface of the lungs immediately after sacrificing the animals by cervical dislocation. Lungs were dissected into five lobes, washed with CMF-DPBS 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 tumours and the sum of each individual weight gave the total weight of metastatic tumour per animal.

Animal studies. Animal studies were carried out according to guidelines enforced in Italy (DDL 116 of 21/2/1992 and subsequent addenda) and in compliance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publ. No. 86-23, Bethesda, MD, NIH, 1985).

Statistical analysis. Results were subjected to computer-assisted statistical analysis using the one-way analysis of

variance ANOVA and the Tukey-Kramer post-test. Differences of $p < 0.05$ were considered to be significantly different from the controls.

Results

The effects of RAPTA-T treatment were studied using three cell lines coming from the mammary epithelium, characterized by different degrees of malignancy (MDA-MB-231 > MCF-7 > HBL-100).

Resistance to detachment. When seeded on different Extra Cellular Matrix (ECM) components, or on poly-L-lysine as a control substrate, 1 h treatment with 10^{-4} M RAPTA-T of the highly invasive MDA-MB-231 cells leads to an 8-fold statistically significantly increase of the number of cells that remained adherent to fibronectin or collagen IV after 30 min exposure to a very diluted trypsin solution (Fig. 2). On the non-specific substrate poly-L-lysine, RAPTA-T was unable to induce the same effect. Also, no such effects are detected on MCF-7, tumourigenic but not invasive cells and on HBL-100, non-tumourigenic cells, in response to RAPTA-T.

Re-adhesion after treatment. The effects on cell ability to re-adhere after RAPTA-T treatment were studied exposing cells to the compound while they were adherent to the growth substrate.

Under these conditions MDA-MB-231 cells, after treatment, are not more able to adhere to proteins of ECM (e.g. fibronectin, collagen IV or Matrigel), or to the non-specific substrate poly-L-lysine. The reduction of adhesion is quantitatively comparable in all the reported substrates. The prolongation of the time allowed for adhesion, from 30 min to

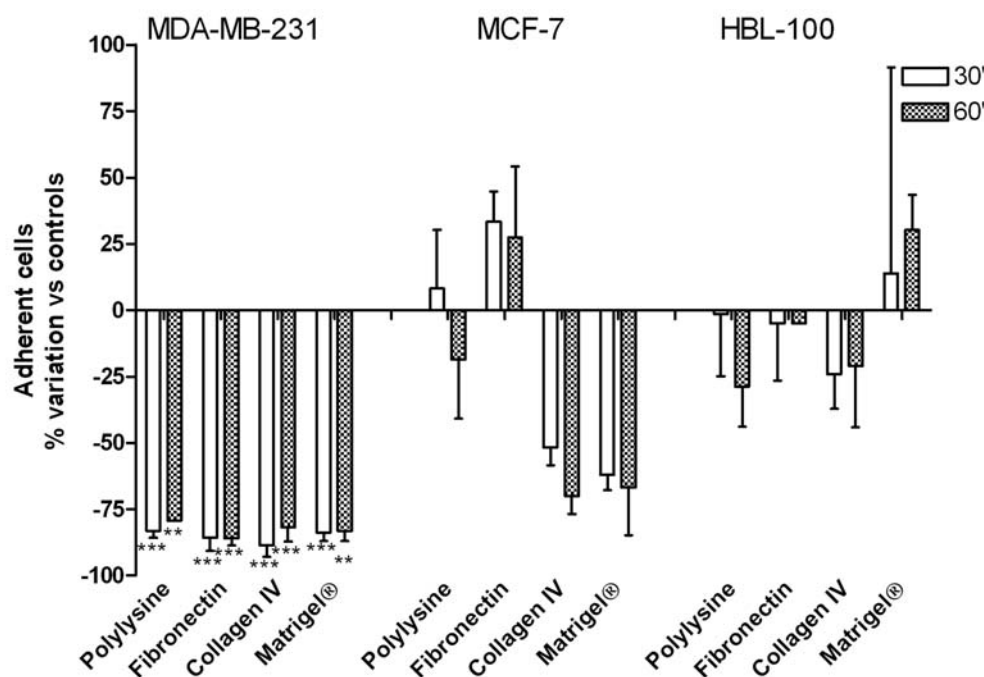


Figure 3. Effect on ability of cells to re-adhere after RAPTA-T treatment. MDA-MB-231, MCF-7 and HBL-100 cells were treated for 1 h with RAPTA-T 10^{-4} M, then the cells were removed from the flasks, collected, re-suspended and seeded on 96-well plastic plates previously coated with polylysine, fibronectin, collagen IV and Matrigel. After 30 and 60 min of incubation cells that adhered to the substrates were detected by the SRB test. Data are the percent of variation vs. controls calculated from the mean \pm SD of two experiments, each performed in triplicate. ** $P < 0.01$; *** $p < 0.001$ vs. controls, ANOVA and Tukey-Kramer post test.

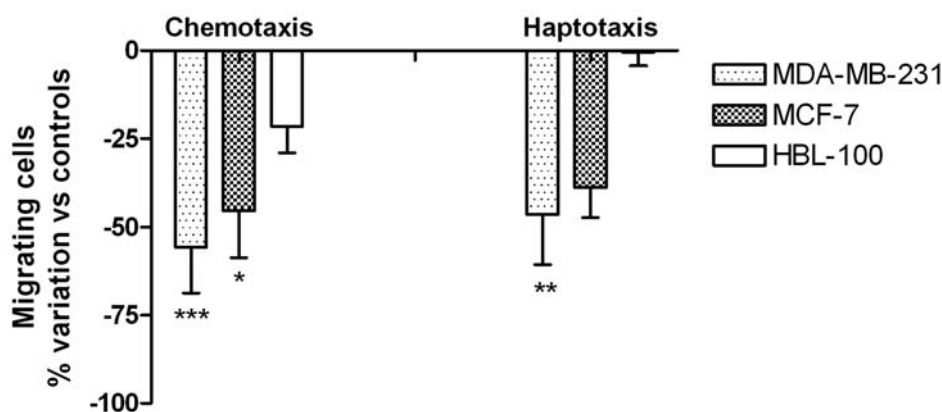


Figure 4. Effect of RAPTA-T on migration of cells through polycarbonate filters. MDA-MB-231, MCF-7 and HBL-100 cells were treated for 1 h with RAPTA-T 10^{-4} M, then the cells were removed from the flasks, collected, re-suspended and seeded on the inserts of Transwell cell culture chambers. Data represent cells that after 24 h have migrated and are present on the lower surface of the filter. Data are the percent of variation vs. controls calculated from the mean \pm SD of two experiments each performed in triplicate. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. controls, ANOVA and Tukey-Kramer post test.

60 min, does not increase the number of treated cells ability to adhere (Fig. 3). Under the same experimental conditions RAPTA-T does not alter the ability of HBL-100 cells to re-adhere, in comparison to untreated controls and reduces the tendency to adhere to collagen IV and to Matrigel of MCF-7-treated cells, although this trend is not statistically significant.

Effects on migration and invasion. The influence of RAPTA-T on the migration process of the three test cell lines is qualitatively similar when a chemical (chemotaxis) or a contact (haptotaxis) stimulus is applied to promote cell movement (Fig. 4). In both cases, the greatest inhibition is achieved with MDA-MB-231 cells that reduce, in a statistically significant

manner, their migration ability to ~50% of controls. MCF-7 cells also show a reduced migratory ability, although this effect is not always significant, whereas HBL-100 maintain, practically unaltered, their movement properties. A similar trend is found also in the invasion test: the invasive ability of MDA-MB-231 cells is reduced by ~50%, whereas it is only slightly affected with MCF-7 cells and it remains unaltered in HBL-100 cells (Fig. 5).

Effect on cell viability. To exclude the interference of RAPTA-T on cell viability on the cell invasion and migration processes an MTT test was performed using the same experimental conditions adopted in the functional assays

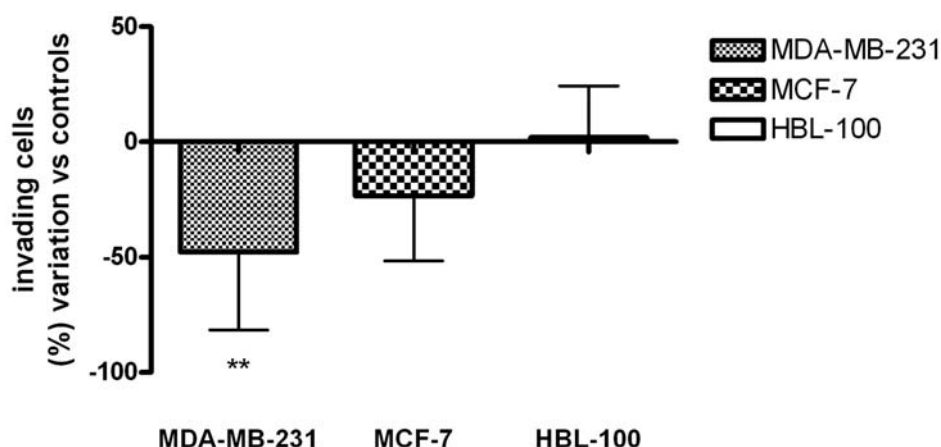


Figure 5. Effect of RAPTA-T on invasion of cells through Matrigel®. MDA-MB-231, MCF-7 and HBL-100 cells were treated for 1 h with RAPTA-T 10^{-4} M, then the cells were removed from the flasks, collected, re-suspended and seeded on inserts. Data represent cells that after 96 h have invaded and are present on the lower surface of the filter. Data are the percent of variation vs. controls calculated from the mean \pm SD of two experiments, each performed in triplicate. ** $P < 0.01$; vs. controls, ANOVA and Tukey-Kramer post test.

Table I. Effect of RAPTA-T on cell viability.

	MDA-MB-231	MCF-7	HBL-100
Controls	2.276 \pm 0.071	2.412 \pm 0.150	2.483 \pm 0.199
RAPTA-T	2.596 \pm 0.070 (114%)	2.478 \pm 0.130 (103%)	2.061 \pm 0.151 (83.0%)

MDA-MB-231, MCF-7 and HBL-100 cells were treated for 1 h with RAPTA-T 10^{-4} M, then the cells were removed from the flasks, collected, re-suspended and seeded on 96-well plates. After 24 h, cell viability was determined by the MTT assay. Data are the mean optical density \pm SD of two experiments each performed in quadruplicate. Data in parentheses represent the percentage of each treated group vs. the relevant controls (T/C%).

Table II. Effect of RAPTA-T on lung metastases formation in mice with MCA mammary carcinoma.

	Primary tumour weight (mg)		Lung metastases ^a	
	Day 13	Day 20	Number	Weight (mg)
Controls	884 \pm 202	2455 \pm 376	24.4 \pm 15.8	8.99 \pm 6.50
RAPTA-T	682 \pm 170 (77.1%)	2443 \pm 389 (99.5%)	19.4 \pm 11.9 (79.5%)	3.19 \pm 4.10 (35.5%)

Groups of 9 CBA mice, inoculated i.m. with 10^6 MCA tumour cells on day 0 were treated i.p. with RAPTA-T at 80 mg/kg/day on days 8, 9 and 12 after tumour implant. Data in parentheses are expressed as the percentage of the treated vs. controls (T/C %). ^aLung metastases were determined on day 20 after tumour implant.

(Table I). Cell viability, 24 h after the 1 h treatment with RAPTA-T, shows that the ruthenium compound induces only slight and non-significant oscillations ($\pm 10\%$ depending on the cell line being treated) as compared to the relevant controls.

Effect on MMPs production and activity. The effect of RAPTA-T on the matrix metalloproteinases (MMPs) production and/or activity was studied by a gelatine zymography test (Fig. 6). MDA-MB-231 cells produce the 92 kDa MMP-9 in appreciable amounts; RAPTA-T treatment statistically reduces the production/activity of this gelatinase by $\sim 30\%$ vs. controls. In the conditioned medium harvested from HBL-100 cells, on which both the 92 kDa MMP-9 and the 72 kDa MMP-2 are present, RAPTA-T treatment moderately increases the production/activity of both MMP-2 and MMP-9, although the variations are not statistically significant.

Effect on lung metastases in vivo. The effect of RAPTA-T treatment on primary tumour growth and lung metastasis formation were studied in the model of MCA mammary carcinoma, a murine transplantable tumour that spontaneously metastasise to the lungs (Table II). RAPTA-T, given at 80 mg/kg/day on days 8, 9 and 12 after tumour implant, only marginally affects primary tumour growth, showing the maximum effect (23% inhibition vs. controls) on day 13 (i.e., 24 h after the last compound injection). The modest control of the primary tumour growth is not maintained after the end of RAPTA-T administration and it is lost on day 20. Despite the negligible reduction of the primary tumour, RAPTA-T is able to selectively counteract the development and growth of lung metastases particularly as regards the weight of these tumours that is reduced to 35% of controls. The pronounced inhibition of metastasis weight is caused by the decrease of the number of metastases of large dimensions (diameter ≥ 2 mm) in the treated group where only 2 out of 8 mice showed them in comparison to 7 out of 9 mice in the control group.

Discussion

In the present study, we evaluated the interference of RAPTA-T with some steps of the metastatic progression by *in vitro* tests that mimic the *in vivo* processes, i.e. detachment, motility, invasion and the adaptation/adhesion to a new environment (21). RAPTA-T was chosen among other

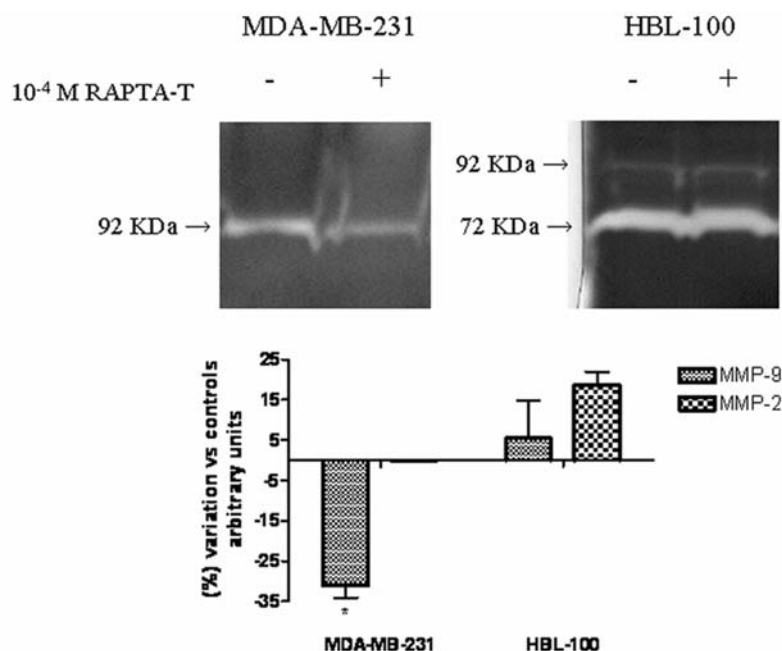


Figure 6. Effect of RAPTA-T on MMPs production and/or activity. MDA-MB-231 and HBL-100 cells were treated for 1 h with RAPTA-T 10⁻⁴ M, then incubated for an additional 24 h in serum-starved complete medium containing 0.1% BSA. Supernatants containing MMPs were collected and concentrated and equal protein amounts (100 μ g) subjected to SDS-PAGE. Gelatine digestion by proteases is detected as white bands against a blue background (upper panel). Band digestion is quantified by using Image Master 2D version 4.01 and Magic Scan 32 version 4.3 software (lower panel). *P<0.05; vs. controls, ANOVA and Tukey-Kramer post test.

members of the RAPTA family, because of its selective cytotoxicity against the TS/A adenocarcinoma cells (IC₅₀ = 74 μ M, 72 h), whereas it is essentially non-toxic to the HBL-100 non-tumourigenic cells (no cytotoxicity observed at the maximum dose of 300 μ M) under analogous conditions (14). In the functional tests adopted in our experimental protocol, RAPTA-T also shows a clear cell type specificity, characterized by a more pronounced effect on tumour cells, rather than on the non-tumourigenic HBL-100. Moreover, these effects are stronger when the highly invasive MDA-MB-231 cells are used, in comparison to MCF-7 non-invasive cells. It would seem therefore, that RAPTA-T is able to interfere more selectively with tumour cells with the highest inclination to invade and metastasise. Moreover, it should be considered that RAPTA-T displays such properties in a series of tests chosen and designed to highlight activities in one or more steps of the metastatic progression (22). The higher activity of RAPTA-T towards the invasive MDA-MB-231 is a common trait, although quantitatively different as a function of the experiment type. In particular, the ruthenium compound has the greatest effect on cell detachment from, and on cell re-adhesion on growth substrates, two tests that would mimic the de-adhesion of the tumour cells from the primary site of growth and the adhesion to a new organ of cells that begin the metastatic colonization, after surviving the stresses of the bloodstream. In the detachment assay, after treatment of MDA-MB-231 cells with RAPTA-T the cells remain much more attached to the substrate, ~7-9-fold in comparison to control cells, when they are seeded on proteins of the extracellular matrix such as fibronectin and collagen IV, but not when cells grow on poly-L-lysine, a substrate on which cells adhere by electrostatic interactions only (23). On

the contrary, in the re-adhesion test RAPTA-T prevents the adhesion of treated cells on all the used substrates exactly to the same extent. The effects found in the detachment test and in the re-adhesion test could be caused by the same modification induced by RAPTA-T and consisting in a stiffening of the cell body, that results in the loss of the plasticity required both for the detachment and for the re-adhesion processes. In both these assays the effects, similarly investigated, on MCF-7 or HBL-100 are virtually negligible or less pronounced. The same cell specificity emerges in the migration/invasion tests, in which MDA-MB-231 is the only cell line that is always inhibited, in a statistically significant manner, in both chemotaxis and haptotaxis, as well as in the invasion tests. These results also support the hypothesis that the modulation of the cytoskeleton is involved in RAPTA-T activity, that is the loss of cell body plasticity that hampers cell detachment and re-adhesion, also prevents cell motility, a cyclic repetition of these two processes (24). It is worth noting that the reduction of migrating MDA-MB-231 cells is always 50%, irrespectively of the type of the applied stimulus, chemical in chemotaxis or associated to the extracellular matrix as in haptotaxis, as if the role of this component is not relevant. Moreover, in the invasion test the reduction caused by RAPTA-T is also 50% indicating that the presence of the Matrigel barrier to be digested is indifferent to RAPTA-T-treated cells. The zymography confirms the independence of the invasion inhibition from the down-regulation of the MMPs activity and/or production. Again the results are cell line-dependent in that MMP-9 production and/or activity by MDA-MB-231 cells is reduced by RAPTA-T, whereas the same protein is not affected if it comes from HBL-100 subjected to analogous conditions, neither is the production

and/or activation of MMP-2, also present in the supernatant of this cell line. Although the reduction of MMP-9 in MDA-MB-231 is probably not strong enough to have a biological significance, the fact that it is affected by RAPTA-T treatment, otherwise MMP-9 in HBL-100, allows us to hypothesise the ruthenium compound acts on a target expressed by the highly invasive cells, but not shared by the non-tumourigenic ones. Moreover, this target is not related to a conventional cytotoxic mechanism as highlighted by the lack of significant variations of cell growth in the adopted experimental conditions. The propensity of RAPTA-T to interfere with cells endowed with invasive ability is suggested from the *in vivo* experiments, where despite a negligible reduction of the primary tumour growth it interferes with the growth of lung metastases. The analysis of the anti-metastatic effects points out a reduction of the weight, probably due to the reduction of metastases of large dimensions. On a pure speculative basis, we might suppose that the *in vivo* effect reflects a fast pharmacokinetics of RAPTA-T which is released from the target and eliminated from the body rapidly after drug withdrawal. Then, metastasis formation is inhibited only during drug treatment and the overall effect is affected by those formed after the end of the treatment. Anyhow, it should be said that this result puts RAPTA-T among the substances of great therapeutic impact, given that metastasis growth is considered the most promising stage of the metastasis process for therapeutic intervention (25,26).

The *in vivo* selective anti-metastatic activity displayed by RAPTA-T might be justified by the *in vitro* activity, particularly relevant on the highly invasive cells and in the detachment- and adhesion-tests, both requiring the interaction of the tumour cells with extra cellular matrix components and suggests the involvement of cell surface molecules in the activity of this ruthenium compound.

It is worth noting that RAPTA-T is unlikely to be the most active/effective compound in the RAPTA family. Based on these experiments it is now possible to screen compounds for antimetastatic activity prior to testing the compounds *in vivo*. This screening protocol is particularly important as NAMI-A and most RAPTA compounds are only weakly active in traditional *in vitro* cytotoxicity studies and would not normally be considered for *in vivo* studies, although the correlation with *in vivo* antimetastatic activity is poor. The correlation between our *in vitro* findings and the *in vivo* anti-metastatic activity is in good agreement with similar results reporting Salvicine as a selectively anti-metastatic substance, able to affect the integrin-mediated tumour cell adhesion (27,28). These data point out that the search of new targets, unrelated to the DNA interactions that drove medicinal metal-based research in the past decades (29,30) can provide new tools for therapy and significant clinical advancements.

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