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Treatment of residual metastases with Na[*trans*-$RuCl_4$(DMSO)Im] and ruthenium uptake by tumor cells

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Treatment of MCa mammary carcinoma metastases by i.p. administration of a total dose of 450 mg/kg Na[*trans*-$RuCl_4$(DMSO)Im], after successful surgical removal of primary tumor mass, causes a significant prolongation of the host's life-time expectancy. This effect, related to lung metastasis inhibition, seems not attributable to a direct inhibition of tumor cells since antimetastatic effects can be achieved also when drug treatment occurs before tumor cell injection into the host. Also, the activity of Na[*trans*-$RuCl_4$(DMSO)Im] seems independent of its concentration in tumor cells. Rather it must be stressed that the fate of this compound in the blood, following i.v. administration, is fast and only a very low percent of the total dose reaches the tumor target in the lungs. These data emphasize the possibility that Na[*trans*-$RuCl_4$(DMSO)Im] increases the resistance of the host against metastasis formation, possibly by the already shown mechanism of potentiation of the extracellular matrix and reduction of blood stream invasion by tumor cells.

Key words: Antimetastatic, residual metastasis, ruthenium, ruthenium determination.

Introduction

In recent years we have provided evidence of the capacity of a new generation ruthenium complex, i.e. sodium *trans*-rutheniumtetrachloroimidazole-dimethylsulfoxide (Na[*trans*-$RuCl_4$(DMSO)Im]), for reducing the formation of solid tumor metastases in experimental systems.[1–4] In particular, unlike many so-called antimetastatic agents such as dacarbazine and related benzenoid triazenes, anticoagulants, proteinase inhibitors and Razoxane,[5–10] that were able to prevent the formation of the metastatic tumors, the major novelty granted by Na[*trans*-$RuCl_4$(DMSO)Im] consists of the fact that it is equally effective either in preventing metastasis formation or in reducing their growth when already present at the beginning of drug treatment.[11] A point of particular interest is represented by the fact that, unlike anticancer agents in common use, the antimetastatic effects of Na[*trans*-$RuCl_4$(DMSO)Im] are completely disconnected from any cytotoxic action of the compound since a significant reduction of lung metastases can be observed at doses that do not alter cell growth and clonogenic capacity.[11,12] In particular it differs from cisplatin, to which Na[*trans*-$RuCl_4$(DMSO)Im] may be compared because of its heavy metal origin, either because of the mechanism of antitumor action or in terms of host toxicity because it works at doses that give lower typical side-effects on normal cells of host tissues such as liver, kidney and lung epithelia.[13] Taken together, all these data thus indicate Na[*trans*-$RuCl_4$(DMSO)Im] as a promising agent for treating solid tumor metastases.[14]

The aim of the present investigation was that of examining the effects of postsurgical treatment with Na[*trans*-$RuCl_4$(DMSO)Im] on the survival time of mice with MCa mammary carcinoma that underwent surgical removal of the primary tumor prior to drug administration at doses and treatment schedules greater than those used up-to-date. Furthermore, the effects of the treatment of the host, previous to tumor cell inoculation, on lung metastasis formation was also investigated. Finally, these effects will be examined in the light of the fate of the compound in the blood and in metastatic tumor cells following i.p. or i.v. administration to mice with MCa mammary carcinoma.

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Materials and methods

Drugs and animal treatment

Na[*trans*-$RuCl_4$(DMSO)Im] was prepared according to already reported procedures.[15] For animal treatment, solutions of Na[*trans*-$RuCl_4$(DMSO)Im] in isotonic saline were prepared immediately before use and injected in volumes of 0.1 ml/10 g animal weight (for i.p. treatments) and of 0.05 ml/10 g animal weight (for i.v. administrations).

Tumor model

MCa mammary carcinoma is a solid metastatizing tumor of the CBA mouse growing i.m. into the calf of the left hind leg.[16] For experimental purposes, single cell suspensions were prepared with tumor masses harvested from animals carrying 2-week-old implants. Tumor masses were mechanically disrupted in about 20 volumes of phosphate buffered saline (PBS) calcium- and magnesium-free, filtered through a double layer of sterile gauze, centrifuged at 250 *g*, and resuspended in an equal volume of PBS. Cell viability was checked by the Trypan blue exclusion test. Primary tumors were obtained by i.m. or s.c. inoculation of 10^6 viable tumor cells, respectively, into the calf of the left hind leg or in the flank. Artificial lung metastases (lung colonies) were obtained by i.v. inoculation of 10^5 viable tumor cells through a lateral tail vein.

Lung metastases (or lung colonies) were counted on the surface of the lungs by means of a low power stereo microscope, equipped with a calibration grid, immediately after lung removal from the animals killed by cervival dislocation. Lung metastasis (or lung colony) weight was determined as the sum of the weight of each single nodule by the formula $(\pi/6) \times a^2 \times b$, where a and b are two perpendicular axis ($a < b$).

Ruthenium measurement

Ruthenium was determined by atomic absorption spectroscopy using a Perkin Elmer model 1100 instrument with a graphite furnace and with a temperature programmer HGA500, using a 30 mA PE lamp Intensitron model 2260. Ruthenium was measured in samples of 20 μl at 349.9 nm with an atomizing temperature of 2600°C, using argon gas as a carrier. Excess ruthenium concentration in samples was reduced by dilution before measurement with 0.2% HCl.

Blood samples in 3.8% citrate were prepared by digestion in 37% HCl (4 volumes of HCl per volume of blood). Plasma and deproteinized plasma were used directly. Plasma was obtained by centrifuging whole blood at 13 000 *g* for 1 min at room temperature. Deproteinized plasma was obtained by treatment of plasma samples with an equal volume of 15% trichloroacetic acid, followed by centrifugation at 13 000 *g* for 1 min at room temperature.

Determination of ruthenium content of tumor cells was made after homogeneization of tumor tissue, previously weighed, immediately after removal from the host, with a potter at room temperature. Treatment of homogenates was the same of that of whole blood samples.

Animal studies

Animal studies were carried out according to the guidelines in force in Italy (DDL 116 of 21/2/1992) and in compliance to the *Guide for the Care and Use of Laboratory Animals*, DHHS publ. no (NIH)86-23, Bethesda, MD (1985).

Statistical analysis

Each experiment was subjected to a statistical analysis. The analysis of variance test of Student–Newmann–Keuls was used to compare means whereas the Kaplan–Meyer test was used to evaluate the significance of the survival curves. Significance was accepted with p equal or lower than 0.05.

Results

Postsurgical treatment of mice with MCa mammary carcinoma with Na[*trans*-$RuCl_4$(DMSO)Im] causes a similar and statistically significant prolongation of the life-time expectancy of the hosts with residual metastases, with daily doses of 44 mg/kg/day given for five consecutive days for two cycles and of 30 mg/kg/day given for five consecutive days for three cycles; the higher daily doses of 66 and 88 mg/kg/day, given for five consecutive days for one cycle, are much less effective (Figure 1). In this experiment, the schedule of 44 mg/kg/day for five consecutive days for two cycles given after surgical removal of the primary tumor was compared with other schedules in which the same total amount of

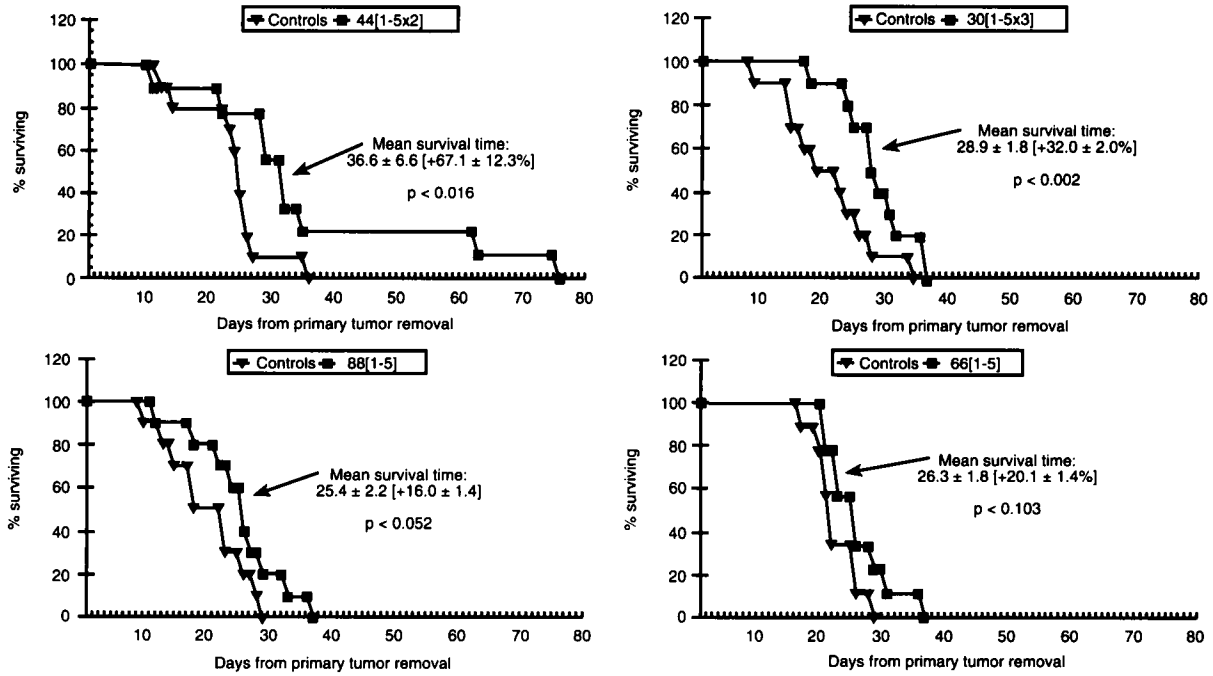

Figure 1. Effects of postsurgical treatment with Na[*trans*-$RuCl_4$(DMSO)Im] on the survival time of mice with MCa mammary carcinoma. Groups of 10 CBA mice, implanted i.m. with 10^6 MCa mammary carcinoma cells on day 0, underwent surgical removal of the primary tumor on days 10–14. Na[*trans*-$RuCl_4$(DMSO)Im] was given i.p. for five consecutive days at the indicated doses and cycles. The Kaplan–Meyer test was applied to evaluate the statistical significance of the modification of the survival time.

this compound was given for a longer (33 mg/kg/day for five consecutive days for three cycles) or for a shorter period (88 mg/kg/day for five consecutive days) periods. Controls of the four experiments were very similar, independent of the different times at which surgical amputation was perfomed. Details about the weight of the tumor removed, the beginning of drug treatment, the weight of the tumored hosts at the beginning of the drug treatment as well as the variation of body weight gain during the treatment versus the respective controls are given in Table 1. The effect of Na[*trans*-$RuCl_4$(DMSO)Im] on the loss of body weight gain is lower when treatment is started on hosts of about 20 g and when drug treatment is made for more than one cycle of administration.

Na[*trans*-$RuCl_4$(DMSO)Im] causes a statistically significant reduction of lung metastases either following i.v. administration to mice with i.m. implants of MCa mammary carcinoma or when hosts are

Table 1. Parameters of the treatment groups whose survival curves are reported in Figure 1

Treatment schedule	Day of surgery[a]	Weight of tumor removed (g)	Delay from chemotherapy (days)	Body weight at the beginning of treatment (g)	Body weight loss variation versus controls (%)
44 mg/kg/day 5 days for two cycles	10	1.34 ± 0.09	4	17.8 ± 1.0	−12.75
30 mg/kg/day 5 days for three cycles	14	1.60 ± 0.10	1	18.2 ± 0.5	−12.90
66 mg/kg/day 5 days for one cycle	13	1.39 ± 0.09	6	20.4 ± 0.6	−3.95
88 mg/kg/day 5 days for one cycle	14	1.93 ± 0.17	5	20.4 ± 0.9	−5.57

[a]Removal of primary tumor from i.m. implantation of 10^6 Mca mammary carcinoma cells.

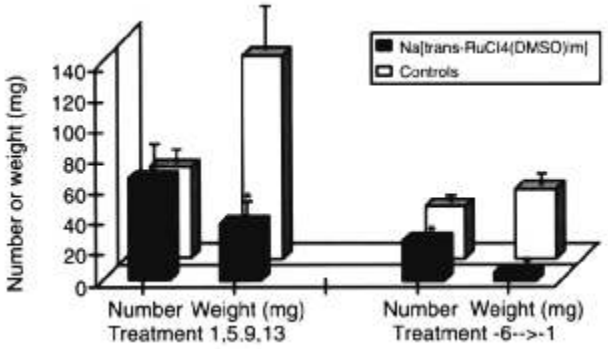

Figure 2. Effects of Na[*trans*-$RuCl_4$(DMSO)Im] on lung metastasis formation in mice bearing i.m. MCa mammary carcinoma or in hosts treated with Na[*trans*-$RuCl_4$(DMSO)Im] prior to inoculation of MCa mammary carcinoma. Groups of 10 CBA mice, implanted i.m. with 10^6 MCa mammary carcinoma cells on day 0, were given i.v. 60 mg/kg/day Na[*trans*-$RuCl_4$(DMSO)Im] on days 1, 5, 9 and 13. Alternatively, groups of 10 CBA mice, treated i.p. for six consecutive days with 44 mg/kg/day Na[*trans*-$RuCl_4$(DMSO)Im] (control groups were similarly treated with isotonic saline for the same period) were injected i.v. with 10^5 MCa mammary carcinoma cells 24 h after the last drug administration. *Statistically different from controls, Student–Newmann–Keuls test, $p < 0.05$.

treated i.p. with 44 mg/kg/day for six consecutive days before injection of tumor cells (Figure 2).

The ruthenium content of MCa tumor cells of spontaneous lung metastases or of artificially induced lung colonies following a single i.p. injection of 250 mg/kg Na[*trans*-$RuCl_4$(DMSO)Im] is reported in Figure 3. The amount of ruthenium in tumor cells is approximately 5–10% of the injected dose and does not appreciably change from 1 to 24 h after

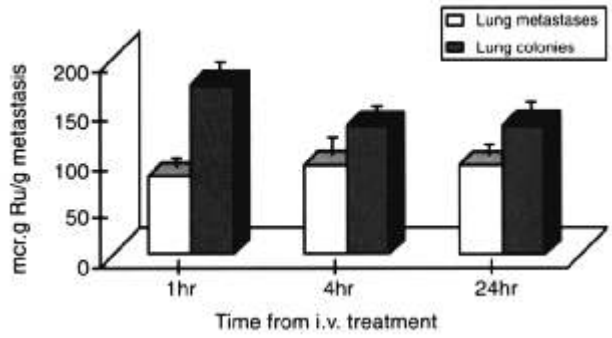

Figure 3. Ruthenium concentration in tumor cells of lung metastases or lung colonies after i.p. treatment with Na[*trans*-$RuCl_4$(DMSO)Im]. Groups of five CBA mice, implanted i.v. with 10^5 or i.m. with 10^6 MCa mammary carcinoma cells, respectively, for lung colony or lung metastasis formation, were given i.p. 250 mg/kg Na[*trans*-$RuCl_4$(DMSO)Im] on day 18 (lung colonies) or on day 25 (lung metastases). Ruthenium in tumor cells was determined by atomic absorption spectroscopy.

treatment. This experiment was performed with lung tumors of roughly the same extent of total weight per mouse (120 ± 20 and 99 ± 14 mg/mouse, respectively, for lung metastases and lung colonies).

The fate of ruthenium in the blood of CBA mice, following a single i.v. injection of 250 mg/kg Na[*trans*-$RuCl_4$(DMSO)Im], is reported in Figure 4. The amount of ruthenium in the whole blood rapidly drops from that expected by the amount injected and, 5 min after treatment, the ruthenium concentration per ml is about 10% of the injected dose. From this point, the decay is slower and the relative ratios between ruthenium content in whole blood, in plasma or in deproteinized plasma is constant.

When Na[*trans*-$RuCl_4$(DMSO)Im] is kept *in vitro* in a sample of whole blood for up to 120 min, the

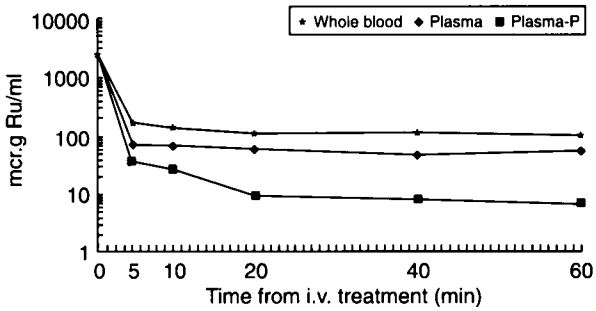

Figure 4. Clearance of Na[*trans*-$RuCl_4$(DMSO)Im] from the blood stream after i.v. injection, as determined by atomic absorption spectroscopy. Groups of three mice were given i.v. a single dose of 100 mg/kg Na[*trans*-$RuCl_4$(DMSO)Im]. Ruthenium in the blood (whole blood, plasma or deproteinized plasma) was evaluated from 5 to 120 min after treatment.

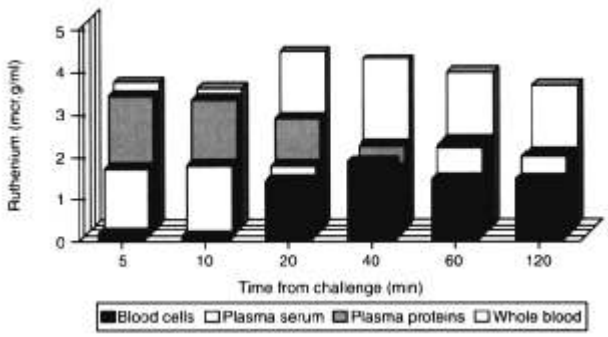

Figure 5. Interactions of Na[*trans*-$RuCl_4$(DMSO)Im] with blood. Aliquots of 3 mg/ml Na[*trans*-$RuCl_4$(DMSO)Im] were incubated *in vitro* at 37°C with whole blood in 3.8% citrate, obtained from a rabbit immediately before the experiment. Each point is the mean of three different measurements. The reaction was stopped by immediately treating each sample at the end of each time of incubation.

association of ruthenium with blood cells, plasma or deproteinized plasma is reported in Figure 5. Data reported in Figure 5 show that the ruthenium associated to plasma proteins drops after 10 min in favor of that associated to blood cells; the ruthenium measured in the samples of deproteinized plasma remains constant along the 120 min of observation.

Discussion

The study of the efficacy of Na[*trans*-$RuCl_4$(DMSO)Im] against lung metastases of solid metastasizing tumors requires more information on the mechanism of metastasis reduction. In particular, provided that much evidence excludes a direct cytotoxicity of this compound for tumor cells, the capacity of this compound to reduce the formation and the growth of lung metastases but moreover to significantly prolong the life-time expectancy of hosts with lung metastasizing tumors remains of great importance.

The results of the present investigation point out that the survival time of mice with residual lung metastases, after successful surgical removal of the primary tumor, is similarly increased by two different schedules of administration of Na[*trans*-$RuCl_4$(DMSO)Im] which have in common the total cumulative dose of about 450 mg/kg body weight. The reduction by one-third of this dose or the use of daily doses greater than 44 mg/kg, but for a shorter period, causes a decay of the efficacy of the compound. Compared with previous studies and particularly to those in which Na[*trans*-$RuCl_4$(DMSO)Im] was given for six consecutive days at 44 mg/kg/day, these results indicate that the increase of the total dose does not increase the overall efficacy in terms of prolongation of the life-span.[11] Indeed, in the present investigation we adopted a considerable delay of 4–6 days between surgery and the beginning of treatment with the aim to help the animals to recover better from surgical amputation. Considering that the fractionation of the dose of 44 mg/kg/day given for 6 days into a smaller dose of 22 mg/kg/day given for 12 days showed a reduction of the activity of Na[*trans*-$RuCl_4$(DMSO)Im],[11] the actual finding that 30 mg/kg/day for 5 days for three cycles is as effective as that of 44 mg/kg/day for 5 days for two cycles (same total amount of drug) might be attributed to the fact that, with the three-cycle experiment, drug treatment started immediately after tumor removal.

The observation that lung metastasis formation can be reduced also when no contact occurs between tumor cells and Na[*trans*-$RuCl_4$(DMSO)Im] stresses the fact that this drug does not act by some cytotoxic mechanism directed to cancer cells. Conversely, this finding is in agreement with previous observations on the reduction of basement membrane degradation activity in tumor tissues after treatment with Na[*trans*-$RuCl_4$(DMSO)Im] and with the preservation of a barrier against tumor cell invasion via the blood stream.[17] Thus, the administration of compounds such as Na[*trans*-$RuCl_4$(DMSO)Im] could make the host stronger against aggression by metastasizing cancer cells.

The lack of a direct cytotoxicity for tumor cells may also be explained by the rather low amount of ruthenium that reaches tumor cells after treatment (less than 10% of the administered dose). There is apparently no difference of entry into tumor cells between spontaneous and artificially induced lung metastases, although it has been described that these two types of lung tumors respond quite differently to the antimetastatic effects of Na[*trans*-$RuCl_4$(DMSO)Im], the latter being less affected by Na[*trans*-$RuCl_4$(DMSO)Im] treatment.[2] The penetration of Na[*trans*-$RuCl_4$(DMSO)Im] into cells seems to be a rather slow process, compared with the rapid clearance from the blood stream of the compound itself. In fact, following an i.v. administration, the residual amount of ruthenium in the blood stream after 5 min is markedly low (about 10%) and this compound, which is mainly associated with the plasma compartment, requires longer times (at least 10 min) to enter blood cells in appreciable amounts.

The rapid clearance of Na[*trans*-$RuCl_4$(DMSO)Im] from the blood stream might be responsible for the lack of significant toxicity for normal host tissues, already shown by histological examination,[3] and could be the target of further studies aimed at evaluating the relationship between body clearance of ruthenium, host toxicity and antitumor activity.

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