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Anti-leukaemic Action of $RuCl_2(DMSO)_4$ Isomers and Prevention of Brain Involvement on P388 Leukaemia and on P388/DDP Subline

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Two ruthenium(II) complexes, characterised by the presence of dimethylsulphoxide ligands, were investigated in comparison to cisplatin on mouse P388 leukaemia and on a subline made resistant to cisplatin (P388/DDP). Both *cis*- and *trans*-$RuCl_2(DMSO)_4$ significantly prolonged the survival time of leukaemic mice, independently of the tumour line used. Unlike cisplatin, the prolongation of life-span of tumour-bearing hosts caused by ruthenium complexes was not supported by a parallel inhibition of the number of tumour cells in the treated hosts, as evidenced by tumour cell count in the peritoneal cavity and by *vivo–vivo* bioassays of blood samples and of whole brains. Thus, *cis*- and *trans*-$RuCl_2(DMSO)_4$ appear capable of preventing leukaemic spread into the central nervous system also when the number of tumour cells in the peritoneal cavity and in the blood stream is as high as in untreated controls. When the drug-induced DNA damage was investigated by modifying double stranded DNA and identifying the lesions able to inhibit DNA synthesis *in vitro*, *trans*-$RuCl_2(DMSO)_4$ and, to a lesser extent, *cis*-$RuCl_2(DMSO)_4$ formed blocking lesions at the same sites of cisplatin; nevertheless, the mechanism of antitumour activity of ruthenium complexes appears to be different from that of cisplatin for the absence of any relationship between cytotoxicity and prevention of leukaemic dissemination into the central nervous system. These data indicate that the activity of *cis*- and *trans*-$RuCl_2(DMSO)_4$ on the P388 leukaemia is characterised by the lack of cross-resistance with cisplatin and by the alteration of the metastasising behaviour of leukaemic cells which lose their natural capacity to invade the central nervous system.
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INTRODUCTION

SINCE THE discovery of the powerful antitumour activity of *cis*-$[PtCl_2(NH_3)_2]$ (cisplatin), several thousand metal complexes have been synthesised and evaluated in a variety of preclinical antitumour screens in hopes of expanding the clinical indications of cisplatin. The massive effort of synthesising new platinum complexes with modified spectrum of activity on the basis of structure-antitumoral activity relationships valid for cisplatin [1, 2] has been rather disappointing; nevertheless, in studying the structural aspects of DNA–platinum interactions and their biological consequences, the cisplatin analogues have been essential tools of investigation, and the growing interest of the last years for new active platinum(II) complexes structurally different from the 'classical' analogues derives ultimately also from this strategy [3].

The research on the antitumoral activity of non-platinum compounds has also been influenced by the structure–activity relations of cisplatin, as in the case of a well-known ruthenium complex, *cis*-dichlorotetrakis(dimethylsulphoxide)-ruthenium (II), *cis*-$RuCl_2(DMSO)_4$ and of its *trans* isomer [4].

cis-$RuCl_2(DMSO)_4$, like cisplatin, is easy to dissolve in water, has two chloride ligands in a planar *cis* position and shows a pronounced affinity for nitrogen donor ligands. Both *cis*- and *trans*-$RuCl_2(DMSO)_4$, although to a different extent, share with cisplatin some microbiological properties [5, 6] and the capability to form covalent bonds at N7 of guanine in the DNA major groove [7–9]. Besides *cis*- and *trans*-$RuCl_2(DMSO)_4$, whose activity in a number of solid metastasising tumours has been often equal or even greater than that of cisplatin [10–12], other ruthenium complexes have been tested with success in models of both transplantable and chemically induced autochotonous tumours of the experimental animals [13].

Overall evidence accumulated so far indicates that ruthenium complexes may have antitumoral properties different from those of cisplatin, thus stimulating deeper investigations on their chemico-biological interactions and on the mechanism of antitumour activity. A mechanism of action different from that of cisplatin could contribute to explaining the different spectrum of activity of ruthenium complexes.

In this study we have examined the antitumoral activity of *cis*- and *trans*-$RuCl_2(DMSO)_4$ in the murine P388 leukaemia, and, in addition, in a cisplatin-resistant subline (P388/DDP), whose *in vivo* cross-resistance profile is in agreement to that reported by Waud *et al.* [14]. The effects of *cis*- and *trans*-$RuCl_2(DMSO)_4$, and of cisplatin, on host survival time have been compared to those on tumour cells surviving in the peritoneal cavity and in samples of blood or whole brains, evaluated by means of *vivo–vivo* bioassays in intact syngenic hosts performed at the end of treatment. The experimental models of blood and brain bioassays were chosen in that they provide direct evidence of leukaemic spread and of central nervous system (CNS) involvement by tumour cells in the treated groups.

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Moreover, since the DNA might represent one of the cellular targets responsible for the antitumour activity, we have investigated the sequence specificity of DNA modification by ruthenium complexes and their capability to inhibit the action of DNA polymerase.

MATERIALS AND METHODS

Chemicals

Ruthenium complexes were prepared according to published procedures [4]; cisplatin was purchased from the Sigma Chemical Co. (U.S.A.).

Animals and tumours

Adult female B6D2F1 mice (Charles River, Italy) weighing 18–20 g were used in all experiments. The P388 leukaemia, obtained as frozen stock from the National Cancer Institute (U.S.A.), was maintained by weekly intraperitoneal (i.p.) injection of 10^6 cells in DBA/2J female mice. Cisplatin-resistant subline of P388 (P388/DDP) was established *in vivo* in our laboratory by i.p. treatment with a single dose of cisplatin (6 mg/kg) given 2 days after the passage of 10^6 leukaemic cells over successive generations in B6D2F1 mice.

Life extension assays

After the recovery of cells from carrier mice by peritoneal washing with Dulbecco's phosphate buffered saline (PBS), B6D2F1 mice were inoculated i.p. with 10^6 cells on day 0. All complexes were dissolved in water just before use and administered i.p. (0.1 ml per 10 g body weight) on days 1–7 at equitoxic dosages (cisplatin, 0.6 mg/kg; *cis*-$RuCl_2(DMSO)_4$, 800 mg/kg; *trans*-$RuCl_2(DMSO)_4$, 48 mg/kg) [10, 15]. Antitumour activity was expressed as (T/C) × 100, with T the mean survival time of treated mice and C that of untreated control ones.

The effect of ruthenium complexes and cisplatin was also evaluated in mice implanted intracerebrally with 10^5 leukaemic cells; in this case, a single i.p. administration of the maximum tolerated doses (cisplatin, 6 mg/kg; *cis*-$RuCl_2(DMSO)_4$, 2000 mg/kg; *trans*-$RuCl_2(DMSO)_4$, 200 mg/kg) [15] was performed on day 1 after tumour implantation and the mean survival time of treated animals and controls was recorded.

Evaluation of leukaemic brain dissemination

The relationship between growth of i.p. implanted P388 leukaemia and appearance of leukaemic cells in blood and brains of mice was evaluated by means of bioassays performed at different times after the inoculation. Brains and samples of whole blood (250 μl collected by cardiac puncture) of P388-bearing mice were harvested and i.p. implanted under aseptic conditions into normal mice of the same strain which were then observed for survival time.

The number of P388 cells in the transplanted organs was calculated from the survival time of the recipient mice, using the regression curve obtained by plotting the mean survival days of mice inoculated i.p. with 10^2–10^6 P388 cells against the number of inoculated cells [16].

At the same time as bioassays, the increase of leukaemic cell number in host mice was evaluated as follows. After killing, the mice were weighed, then 3 ml of PBS were injected i.p. and drawn off. Harvested cells were counted using a haemocytometer, and the ascitic mass was determined from the difference in weight after several washes of the peritoneal cavity.

Chemotherapeutic effect on leukaemic brain dissemination

The effect of ruthenium complexes and cisplatin on leukaemic spreading to the CNS of P388- and P388/DDP-bearing mice was evaluated after a daily (days 1–7) i.p. treatment with the maximum tolerated doses of the drugs or vehicle. On day 8 the animals were sacrificed by cervical dislocation and the cytocidal effect of treatment and brain involvement were evaluated as described above. Since in this case the brains of mice were subcutaneously (s.c.) implanted in the axillary region of recipients, the correlation between s.c. inoculum size and survival time was also established to evaluate the cell number in the brains from the survival time of recipient mice.

In the *cis*- and *trans*-$RuCl_2(DMSO)_4$-treated mice with P388 leukaemia, a blood bioassay was also performed at the end of treatment, to evaluate the presence of leukaemic cells in circulating blood in comparison to untreated mice.

Primer extension footprinting assay

PBR 322 double stranded DNA (New England Biolabs) (1.5×10^{-8} mol nucleotides) was allowed to react with cisplatin at drug/nucleotide (D/N) molar concentration ratio of 0.02, *cis*- and *trans*-$RuCl_2(DMSO)_4$ (D/N = 0.1) in a total volume of 10 μl of 10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 8, for 1 h at 37°C. All complexes were dissolved just prior to the reactions. At the end of the incubation period excess drug was removed by centrifugation through Sephadex G-50 columns. Drug-treated DNA was denatured in 0.2 mol/l NaOH, 0.2 mmol/l EDTA for 30 min at 37°C. After neutralisation with 3 mol/l sodium acetate and ethanol precipitation, 9 pmol 16-mer Pst(+) primer (New England Biolabs) were added and the annealing reaction was performed for 2 min at 65 °C in a total volume of 10 μl of 40 mmol/l Tris–HCl, pH 7.5, 20 mmol/ $MgCl_2$, 50 mmol/l NaCl. Replication was initiated by addition of Sequenase 2 enzyme (United States Biochemicals) in the presence of [α-^{32}P]dATP (370 KBq, 111 TBq/mmol) (Amersham) and unlabelled dGTP, dCTP and dTTP (0.2 μmol/l) in a total volume of 16 μl of 25 mmol/l Tris–HCl, pH 7.5, 12.5 mmol/l $MgCl_2$, 31 mmol/l NaCl, 6.25 mmol/l dithiothreitol. This mixture was incubated for 10 min at room temperature, then an excess of all four dNTP was added to a total of dNTP concentration of 150 μmol/l, and the incubation was continued for 15 min at 37 °C. The reaction was stopped by the addition of 95% formamide, 20 mmol/l EDTA-dye mixture; the samples were then denatured for 3 min at 90°C and the products analysed by electrophoresis on 6% polyacrylamide/7 mol/l urea gels. Sequencing reactions were carried out following the Sequenase protocol and electrophoresed in parallel. The autoradiography was performed overnight with Kodak Ektamat G film.

Statistical analysis

All data are expressed as mean ± S.D. Difference among groups was evaluated by the analysis of variance; when appropriate, multiple comparisons were performed by the Student–Newman–Keuls test.

RESULTS

Life extension assay

The antitumour activity of ruthenium complexes and cisplatin administered at equitoxic dosages in mice bearing i.p. implants of P388 and P388/DDP lines or in mice carrying an intracerebral implant of P388 line, are reported in Tables 1 and 2, respectively. *cis*- and *trans*-$RuCl_2(DMSO)_4$ showed a comparable and significant activity against P388 leukaemia (T/C% = 152 and 156,

Table 1. Effects of cis-*$RuCl_2(DMSO)_4$*,* trans-*$RuCl_2(DMSO)_4$ and cisplatin on mice bearing P388 or P388/DDP leukaemia*

Compound	Dose (mg/kg)	P388 MST (Days ± S.D.)	P388 Wt (g)	P388 T/C%	P388/DDP MST (Days ± S.D.)	P388/DDP Wt (g)	P388/DDP T/C%
Vehicle	—	8.6 ± 0.5	+2	—	11.8 ± 1.4	+3	—
cis-$RuCl_2(DMSO)_4$	800	13.1 ± 1	−1.4	152	18.9 ± 1.3	−1.5	160
trans-$RuCl_2(DMSO)_4$	48	13.4 ± 1	+0.3	156	21.0 ± 1	+1.6	178*
Cisplatin	0.6	17.5 ± 2	−1	203	10.6 ± 1.5	+3	90
Cisplatin	1	—	—	—	11.5 ± 1	+3	97

P388 leukaemia cells (10^6/mouse) were implanted i.p. into B6D2F1 mice on day 0 (eight animals/group, 10 controls). The treatment was performed i.p. on days 1–7 from tumour inoculation. MST = median survival time. Wt = variation of body weight at the end of treatment. T/C% = survival time as per cent of controls. *$P < 0.01$ as compared to the same treatment in P388-bearing mice.

Table 2. Effect of cis-*$RuCl_2(DMSO)_4$*, trans-*$RuCl_2(DMSO)_4$ and cisplatin on intracerebrally implanted P388 leukaemia*

Compound	Dose (mg/kg)	MST (Days ± S.D.)	T/C%
Vehicle	—	8.5 ± 0.54	—
cis-$RuCl_2(DMSO)_4$	2000	8.8 ± 1	103
trans-$RuCl_2(DMSO)_4$	200	8.5 ± 1	100
Cisplatin	6	9.3 ± 0.5	109

P388 leukaemia cells (10^5/mice) implanted intracerebrally into B6D2F1 mice (six animals/group, eight controls) on day 0. The treatment was performed i.p. on day 1.

respectively) but cisplatin produced the highest increase in survival time (T/C% = 203). When tested against P388/DDP subline both *cis*- and *trans*-$RuCl_2(DMSO)_4$ showed good activity, while cisplatin was completely inactive also at a dose higher than that normally used. Moreover, *trans*-$RuCl_2(DMSO)_4$ was more effective against P388/DDP than against the platinum-sensitive line.

In contrast, none of the tested compounds significantly modified the survival time of mice bearing intracerebrally implanted P388 leukaemia (Table 2); as to cisplatin these results are in agreement with those already reported on brain-injected L1210 leukaemia [17] and altogether indicate that the complexes under examination are ineffective when there is a high concentration of leukaemic cells in the brain.

Spreading pattern of P388 leukaemia to the CNS

The relationship between growth of i.p. implanted leukaemia and time of appearance of leukaemic cells in blood and brains of P388-bearing mice is reported in Table 3. The cell number in the peritoneal cavity increased with time, and the bioassay of brains became positive on day 5, while the blood bioassay was already positive on day 4. Moreover, the survival time of recipient mice was shortened with time, according to an increasing number of leukaemic cells in the transplanted organs. The number of P388 cells in blood and brains was estimated by using the linear relationship drawn in Fig. 1 between the i.p. inoculum size and the survival time of P388-bearing mice.

Chemotherapeutic effect on leukaemic spreading to the CNS

The effects of cisplatin and of the tested ruthenium complexes on peritoneal tumour growth and on blood and brain involvement are reported in Table 4. Cisplatin significantly reduced by 99% the number of peritoneal tumour cells in the treated mice with no ascites formation. The effects of *cis*-$RuCl_2(DMSO)_4$ are marked, although less pronounced, and consist of a reduction

*Table 3. Relationship between growth of i.p. implanted leukaemia and appearance of leukaemic cells in blood and brains of P388-bearing mice as evaluated by means of bioassays**

Days from tumour implantation	Ascites g	Ascites Cell no. ($\times 10^6$)	Blood bioassay Dead	Blood bioassay MST	Blood bioassay Cell no.	Brain bioassay Dead	Brain bioassay MST	Brain bioassay Cell no.
−1	0	0	0/6	Infinite	—	0/6	Infinite	0
4	0	ND	6/6	34.4 ± 4.4	< 10	0/6	Infinite	0
5	0	64.00 ± 11.71	NE	—	—	6/6	19.40 ± 1.29	5.7×10^3
6	0.5 ± 0.2	239.50 ± 94.53	NE	—	—	NE	—	—
7	2.2 ± 0.4	855.86 ± 202.2	6/6	20.0 ± 2.0	4.2×10^3	6/6	16.78 ± 0.83	2.3×10^4
8	2.2 ± 0.2	1061.5 ± 173.76	6/6	14.0 ± 1.6	1×10^5	6/6	14.09 ± 0.73	1×10^5

*P388 leukaemia cells (10^6/mouse) implanted i.p. on day 0 (six animals/group). Dead, number of animals dead after i.p. implantation of blood (250 μl) or whole brain. MST, mean survival time of deceased mice (± S.D.). Cell no, calculated from the regression curve reported in Fig. 1. ND, not detectable. NE, not evaluated.

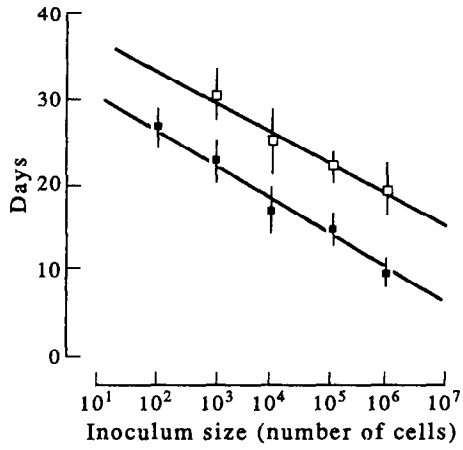

Fig. 1. Inoculum size–survival time relationship of P388 leukaemia-bearing mice (■ i.p. inocula; □ s.c. inocula).

by 62%, while the effects of *trans*-$RuCl_2(DMSO)_4$ are much less evident and show only a 30% reduction of the number of tumour cells present in the peritoneal cavity.

The brain bioassay showed that all recipients of control group developed the leukaemia in the form of s.c. tumoral mass and ascites and died within 16.5 ± 2.2 days, because of a tumoral burden of about 4×10^6 cells in the transplanted brains, as calculated from the s.c. inoculum size-survival time regression curve (Fig. 1).

On the contrary, all recipients from the cisplatin-treated group survived without signs of disease and were ascertained to be tumour-free by autopsy on day 90. Both ruthenium complexes showed a similar and significant efficacy ($P < 0.01$, χ^2 test) against leukaemic brain dissemination with six out of eight survivors.

Blood bioassays, with blood samples obtained from tumour-bearing mice treated with ruthenium complexes, showed survival times comparable to those observed in controls, indicating the lack of effects of *cis*- and *trans*-$RuCl_2(DMSO)_4$ on the leukaemic cell population present in the blood stream. A complementary *vivo–vivo* bioassay was performed on P388-bearing mice treated with ruthenium complexes or cisplatin. In this case, the leukaemic cells were harvested at the end of treatment from the peritoneal cavity of treated and control mice and 10^6 aliquots of viable cells (trypan blue) were injected into healthy recipients which were observed for survival time and, by means of a further bioassay performed 7 days later, for the brain involvement. No difference was found either in the survival time of P388 cell recipients or in the survival time of brain recipients (data not shown).

Bioassays with P388/DDP leukaemia line (Table 5) showed that the cisplatin treatment of the donor mice led to a 40% inhibition of P388 cells without appreciable variation in the ascites formation; seven out of eight brain recipients developed the leukaemia and their survival time was significantly prolonged in comparison with control mice, indicating that a partial reduction of tumour burden in the brain district had been obtained.

Cis-$RuCl_2(DMSO)_4$ showed a pronounced activity with no ascites formation, and 72% inhibition of P388 cells; all brain recipients from this group survived without signs of disease, indicating total prevention of brain dissemination. On the other hand, *trans*-$RuCl_2(DMSO)_2$ treatment produced a slight inhibition (34%) of P388 cells, but showed an efficacy similar to that of the *cis*-$RuCl_2(DMSO)_4$ in the brain bioassay with seven out of eight survivors.

Primer extension footprinting assay

The primer extension footprinting assay was performed to investigate the DNA binding specificity of *cis*- and *trans*-$RuCl_2(DMSO)_4$ in comparison with cisplatin. Native pBR 322 DNA was modified by the drugs, denatured and reannealed with pst(+) primer, and then used as a template for second strand synthesis by Sequenase 2 enzyme (Fig. 2). In agreement with analogous experiments [17, 18], cisplatin inhibited the DNA synthesis by forming blocking lesions mainly at G_n ($n \geq 2$). *trans*-$RuCl_2(DMSO)_4$ showed a similar pattern of stop sites, even though approximately 10-fold more complex was required. On the contrary, in the lane containing *cis*-$RuCl_2(DMSO)_4$-treated DNA, only faint bands were visible in the G rich regions.

DISCUSSION

This study demonstrates that *cis*- and *trans*-$RuCl_2(DMSO)_4$ exert antileukaemic effects on P388 leukaemia and maintain such activity also on the cisplatin-resistant subline P388/DDP, *trans*-$RuCl_2(DMSO)_4$ being even more active on the latter tumour than on the parental line.

The antileukaemic activity of these ruthenium complexes does not depend on a cytotoxic action on tumour cells located i.p., i.v. or intracerebrally. More likely other mechanisms, different from cytotoxicity, are responsible for the prolongation of life-span of the treated hosts. *cis*- and *trans*-$RuCl_2(DMSO)_4$ prevent leukaemic dissemination into the brain of mice bearing i.p. growing P388 or P388/DDP leukaemia lines. Unlike cisplatin,

Table 4. Effects of cis-*$RuCl_2(DMSO)_4$*, trans-*$RuCl_2(DMSO)_4$ and cisplatin on leukaemic spread to the central nervous system in P388-bearing mice**

Compound	Dose (mg/kg)	Ascites g	Ascites Cell no. (×10^6)	Ascites % I	Brain bioassay Dead	Brain bioassay MST	Brain bioassay Cell no.†	Blood bioassay Dead	Blood bioassay MST	Blood bioassay Cell no.†
Vehicle	—	2.0 ± 0.4	728 ± 110	—	8/8	16.5 ± 2.2	4×10^6	8/8	14.2 ± 1.7	1×10^5
cis-$RuCl_2(DMSO)_4$	800	0.4 ± 0.2	276 ± 55	62	2/8	22.37*	—	8/8	17.5 ± 3.7	1.6×10^4
trans-$RuCl_2(DMSO)_4$	48	1.3 ± 0.4	509 ± 74	30	2/8	20 ± 1.7	5×10^5	8/8	15.4 ± 2	5 ± 10^4
Cisplatin	0.6	0	6.4 ± 2	99	0/8	—	—	NE	—	—

*P388 leukaemia cells (10^6/mouse) implanted i.p. on day 0 (eight animals/group, eight controls). The effect of treatment performed on days 1–7 with the tested compounds was evaluated as described in Materials and Methods. Dead, number of animals dead after implant of brain (s.c.) or blood (250 μl, i.p.) obtained from treated and control mice. MST, mean survival time (±S.D.) of decreased mice. * Single values. NE, not evaluated. † Calculated from the regression curves reported in Fig. 1. %I, cytocidal effect, per cent inhibition as compared to the controls.

Table 5. Effects of cis-$RuCl_2(DMSO)_4$, trans-$RuCl_2(DMSO)_4$ *and cisplatin on leukaemic spread to the central nervous system in P388/DDP-bearing mice**

Compound	Dose (mg/kg)	Ascites g	Ascites Cell. no. ($\times 10^6$)	Ascites %I	Brain bioassay Dead	Brain bioassay MST
Vehicle	—	2.3 ± 0.7	612 ± 87	—	8/8	25.3 ± 5‡
cis-$RuCl_2(DMSO)_4$	800	< 0.4	170 ± 47	72	0/8	—
trans-$RuCl_2(DMSO)_4$	48	2.1 ± 0.2	406 ± 71	34	1/8	48†
Cisplatin	0.6	2.1 ± 0.3	365 ± 76	40	7/8	35.0 ± 10‡

*P388/DDP leukaemia cells (10^6/mouse) implanted i.p. on day 0 (eight animals/group, eight controls). The effect of treatment performed on days 1–7 with the tested compounds was evaluated as described in Materials and Methods. Dead, number of animals dead after s.c. implantation of brains obtained from treated and control mice. MST, mean survival time of deceased mice. † Single value. %I, cytocidal effect, per cent inhibition as compared to the controls. ‡$P < 0.05$.

which is effective only on the parental P388 line, *cis*- and *trans*-$RuCl_2(DMSO)_4$ prevent brain colonisation by tumour cells with the same effectiveness on both tumour lines and in the absence of any significant reduction of i.p. tumour growth or i.v. leukaemic involvement. Thus, it appears that *cis*- and particularly *trans*-$RuCl_2(DMSO)_4$ can control the dissemination of tumour cells to the brains by a non-cytotoxic mechanism. It is well known that DMSO is a potent *in vitro* differentiation inducer [20], and several antitumour metal complexes containing DMSO have been reported [21]. Nevertheless, a direct role for the DMSO ligand in the *in vivo* antileukaemic activity of Ru(II)DMSO isomers appears to be unlikely. In fact, no differences were found in survival time of P388-bearing mice treated with 0.1 ml/10 g body weight of 0, 20, 40 and 60% DMSO in water (data not shown).

The model of leukaemic brain metastases presently adopted was found useful in that it mimics the pathological behaviour of human cerebral leukaemia that occurs mainly in patients with high peripheral blast count [22]. In fact, brain bioassays were negative until tumour growth reached a population size of $64 \pm 11 \times 10^6$ cells in the peritoneal cavity (corresponding to day 5 from tumour inoculation) and after spreading into the blood stream (positive since day 4 from tumour inoculation), indicating the need of the presence of a high number of tumour cells in the host. The effects of the ruthenium complexes tested on leukaemic brain involvement by tumour cells suggest that a therapeutic control of the cerebral dissemination in the P388 system, besides by a cytotoxic mechanism that reduces the number of leukaemic cells in the body below the threshold required (mechanism operative for cisplatin), may be reached also in the absence of any significant cytotoxicity for tumour cells. Because of the effectiveness on P388/DDP and of the mechanism of action different from that of cisplatin, *trans*-$RuCl_2(DMSO)_4$ might be supposed capable of synergising with cisplatin in the combined treatment of such tumours.

The low cytotoxicity of *cis*- and *trans*-$RuCl_2(DMSO)_4$ in the P388 system is in agreeement with data obtained on other murine tumours [10–12] and with *in vitro* experiments performed on some human tumour-derived cell lines [23]. This behaviour could reflect, at least in part, the chemical properties of ruthenium isomers: in particular, the extracellular reactivity of the intermediate species might determine, on one hand, the higher host toxicity of the *trans* isomer in comparison with the *cis* one and, on the other, a reduced targeting of the active species to the cellular DNA. As to the drug-induced damage, the cisplatin-DNA major adduct has been shown to be an intrastrand cross-link between N7 atoms of adjacent guanines [24], a lesion very likely responsible for the inhibition of DNA replication and transcription [25]. *trans*-$RuCl_2(DMSO)_4$ also forms blocking lesions at adjacent guanines, as indicated by footprinting assay, thus suggesting the formation of an analogous adduct. On the contrary, *cis*-$RuCl_2(DMSO)_4$ produced no strong stop sites; this behaviour indicates a lower reaction rate of the two ruthenium complexes with native DNA in comparison with cisplatin and reflects the higher stearic hindrance of *cis*-$RuCl_2(DMSO)_4$ in comparison to the *trans* isomer. These results suggest that it may well be possible to improve the ability of ruthenium complexes to attack DNA by appropriate modifications of the leaving groups. Therefore, although *trans*-$RuCl_2(DMSO)_4$ shares with cisplatin the DNA binding pattern and the capability of inhibiting the DNA polymerase *in vitro*, the mechanism of action operating *in vivo* for both ruthenium complexes appears to be different from that of cisplatin, particularly for the absence of any relationship between cytotoxicity and prevention of leukaemic dissemination into the CNS.

The alteration of leukaemic arrival to the CNS might represent the counterpart pertaining to a lymphoproliferative disease of the antimetastatic activity of *trans*-$RuCl_2(DMSO)_4$ on solid metastasising tumours. In the case of solid tumour metastases the need for the integrity of host immune responses for the reduction of the formation of lung metastases has been stressed [26]. Moreover, both isomers were found to be capable of reducing the fibrinolytic potential of Lewis lung carcinoma cells [27]. In the case of P388 leukaemia, the treatment with ruthenium complexes did not modify the cell clonogenic capability, as indicated by the bioassay performed with peritoneal cells harvested at the end of treatment, thus suggesting a relevant role for the host in the antileukaemic action of $RuCl_2(DMSO)_4$ isomers.

Although the spread of cancer cells to the CNS in the case of leukaemias and the formation of lung metastases of solid tumours might have different behaviours, both phenomena show mechanistic analogies such as the passage of tumour cells through the vascular endothelium and the susceptibility of low burden disseminated cells to the host-mediated cytolytic effect that are greater than for large burden tumours.

In conclusion, the effects of *cis*- and *trans*-$RuCl_2(DMSO)_4$ on P388 leukaemia provide further evidence for a different

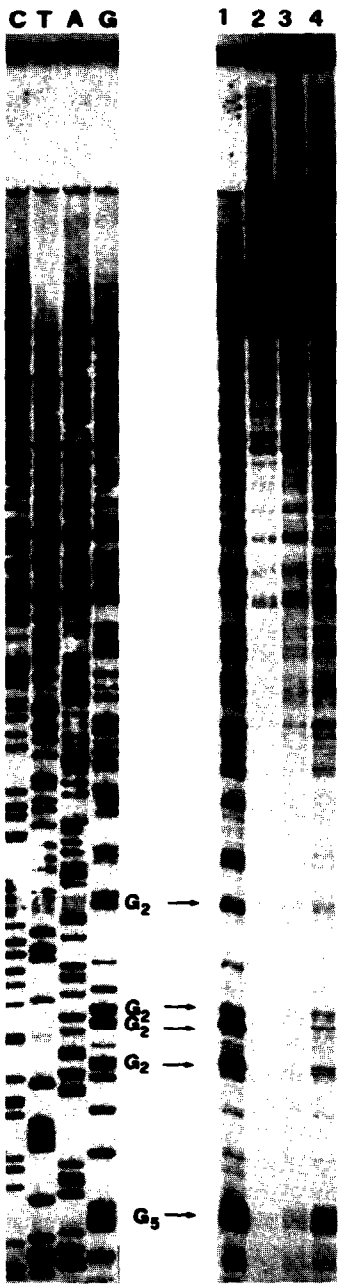

Fig. 2. Autoradiogram of an 6% polyacrylamide/7 mol/l urea sequencing gel showing inhibition of DNA synthesis on drug-treated pBR 322 DNA. (Left) Lanes designed C, T, A and G refer to the base positions on the copied strand. (Right) Lanes: 1, cisplatin-modified DNA (D/N = 0.02); 2, no reagent; 3, *cis*-$RuCl_2(DMSO)_4$-modified DNA (D/N = 0.1);4, *trans*-$RuCl_2(DMSO)_4$-modified DNA (D/N = 0.1). The positions of adjacent guanines are also indicated, and arrows correspond to blocking lesions.

mechanism of action of ruthenium–dimethylsulphoxide complexes in comparison to cisplatin. In other words, it is further predicted that other metal-containing compounds should not necessarily behave like cisplatin. This consideration is further stressed by the effects of other ruthenium complexes, such as those characterised by heterocycle ligands prepared by Keppler *et al.* [13], that exhibit activity on tumours of the colorectal district on which cisplatin is virtually ineffective. It is further concluded that the evidence on the lack of correlation between antitumour and antimetastatic activity, now valid also in the case of leukaemic tumours, is a peculiar characteristic of ruthenium–dimethylsulphoxide complexes that should be better emphasised in terms of relevance for possible clinical use.

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The Effect of α and γ Interferon on Cell Growth and Histocompatibility Antigen Expression by Human Renal Carcinoma Cells *in vitro*

Rachel Angus, C.M.P. Collins and M.O. Symes

Tumour cells were separated from 19 renal carcinomas and cultured *in vitro*. The effect of interferon (IFN) α and γ on cell proliferation was measured and compared to the effect of IFN on the expression of class I and class II major histocompatibility complex (MHC) antigens. When tested within the first 14 days of culture, IFN-α inhibited protein synthesis in 12 of 15 and IFN-γ in four of nine tumours. Reduction in cell counts was in parallel. In six tumours the culture period was extended and in all six the effect of IFN-α was lost. Exposure to IFN-α induced or enhanced class I antigen expression in eight of 19 tumours and class II expression in two of 19. The analogous figures for IFN-γ were five and three tumours. In four of five cases where a comparison could be made there was a correlation between the effects of IFN-α on cell proliferation and class I antigen expression. The efficacy of IFN in the treatment of renal carcinomas may thus, in part, result from inhibition of cell proliferation and enhancement of antigen expression.
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INTRODUCTION

INTERFERONS HAVE a variety of effects on cells which mitigate against the growth of neoplasms. For example, interferon α (IFN-α) inhibits cell proliferation [1], is cytotoxic [2] and amplifies or induces the expression or re-expression of major histocompatibility complex (MHC) antigens [3, 4]. Interferon-γ (IFN-γ) activates immune cells, for example, mononuclear phagocytes [5, 6], T-lymphocytes [7] and natural killer cells [8]. It is particularly effective in amplifying or inducing the expression of class II MHC antigen (Ag) [9]. MHC Ag molecules play an important role in the presentation of self or non-self Ag leading to the initiation of an immune response and thus their increased expression may be important in potentiating host reactivity against tumour cells. IFN-α and IFN-γ have been used to treat metastatic human renal carcinoma with a response rate of approximately 15% [10–13].

We have recently described a method for the establishment of human renal carcinoma cells (RCC) in long-term culture [14]. As the identification of a subset of RCC likely to respond to IFN therapy could be clinically useful it seemed germane to study the effect of interferons on the growth and histocompatibility antigen expression by RCC cells cultured from the primary biopsy.

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MATERIALS AND METHODS

General plan of the experiments

Carcinoma cells from 15 RCC were tested *in vitro* for sensitivity to IFN-α and -γ, using the uptake of [^{75}Se]selenomethionine [^{75}SeM], as a measure of protein synthesis, and by cell counting. Cells from each tumour were tested within the first 14 days of culture and in some cases were re-examined after longer periods of *in vitro* growth. At the same time the effect of the interferons on the expression of class I and II MHC Ag was examined. An association was sought between these two effects of interferons.

Culture of renal carcinoma cells

The methods for separation of carcinoma cells from the RCC [14, 15], the purity of the tumour cell suspensions obtained [15] and their culture [14] have previously been described. A cytospin preparation was made of the cells from the top band on the Nycodenz column for 11 tumours. The cells were stained with