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MODE OF ACTION OF *CIS*-DICHLORO-DIAMMINE PLATINUM(II) ON MOUSE EHRLICH ASCITES TUMOUR CELLS

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Abstract—Mice bearing an Ehrlich ascites tumour received one intraperitoneal injection of *cis*-dichloro-diammine platinum (II), *cis*-Pt (II). The tumour cells were analysed with cytological and quantitative cytochemical methods. *Cis*-Pt (II) strongly inhibits cell multiplication. When mitosis begins, a block in metaphase usually occurs. This platinum compound blocks the cells in G2 (post-synthesis as to DNA) but, RNA and protein synthesis remaining very active, the cells become giant and contain prominent nucleoli. Later on, in the majority of cases, they slowly degenerate and the ascites tumour tends to disappear. However, tumour growth can sometimes resume.

Cis-dichloro-diammine platinum (II) (*cis*-Pt (II); [1, 2]) is a potent inhibitor of various experimental tumours [3–7]. In various mammalian cells, this platinum compound reacts with DNA (cross-linking) and can inhibit DNA synthesis, but also RNA and protein synthesis [3, 8–13]. According to Drewinko *et al.* [14], lymphoma cells are killed in all stages of the cycle by *cis*-Pt (II). Under the action of the latter [15], the cAMP content increases in Walker carcinoma cells; the same effect is observed after a treatment with alkylating agents.

In a previous work [16], we have analysed the effects of *cis*-Pt (II) on chick embryo fibroblasts cultivated *in vitro*. These cells are very sensitive to the drug. Cell multiplication is stopped, DNA synthesis is inhibited or the cells are blocked in G2, cells degenerate and abnormal mitoses are noted. Under these conditions, nucleoli are condensed and altered but, after two days, they are again large and very active if the concentration of the drug is relatively low. We study here the effects of *cis*-Pt (II) on a tumour cell line (*Ehrlich ascites* tumour cells) growing in the mouse. We show, among other facts, that *cis*-Pt (II) stops cell multiplication, dissociates DNA synthesis from RNA and protein synthesis and can kill the tumour cells.

MATERIAL AND METHODS

1. C 57 Bl mice (females, 20 g) received one intraperitoneal (i.p.) injection of 14 million *Ehrlich ascites* tumour cells (ELT; hypertetraploid line, 90 chromosomes). Four days later, they received one i.p. injection of *cis*-dichloro-diammine platinum (II) (4.55; 9.1 or 18.2 mg/kg fresh wt). Control mice received Ringer saline solution. Tumour cells were studied 2 6 days and in some cases up to 1 month after the treatment.

2. Living tumour cells in suspension were analysed with an electronic Coulter counter (total number of *ascites* cells per mouse and volume of the cells).

3. Tumour cells were fixed, after smearing, with ethanol–acetone (1/1) at 4°.

4. Cytochemical stainings: (a) *Feulgen reaction for DNA*; Hydrolysis by 3.5 N HCl, at 37° for 20 min (b) *Naphthol yellow S staining* for total proteins at pH 2.7 according to Deitch [17] (c) *Gallocyanin staining* [18] after neutral deoxyribonuclease digestion, for total cellular RNA [19] and for the analysis of the nucleoli.

5. Quantitative cytochemical measurements. The DNA content (after Feulgen reaction), the total protein content (after naphthol yellow S staining) and the total RNA content (after gallocyanin staining) were measured in individual cells with a scanning and integrating microdensitometer (GN 2 from Barr and Stroud) at 543 nm, 435 nm and 550 nm respectively. In some cases, DNA and proteins were successively measured in the same cells after double staining (Feulgen reaction and naphthol yellow S staining).

6. Electron microscope observations. Tumour cells were centrifuged (5 min at 1000 rev/min). Fragments of the pellets were fixed with glutaraldehyde and post-fixed with osmium tetroxide. They were embedded in Epon 812. Ultrathin sections (Reichert OMU 3 microtome) were contrasted with uranyl acetate and lead citrate. The observations were performed with a Siemens Elmiscope 101 at 80 kV.

Other cells, fixed with glutaraldehyde–osmium, were dehydrated by the critical point method (with CO_2). They were observed after gold–paladium metallization, with a Cambridge Stereoscan microscope at 20 kV.

RESULTS

1. Number and volume of the cells

As shown in Fig. 1, the number of tumour cells was much lower than in controls after *cis*-Pt (II) treatment. For the three doses (4.55; 9.1 and 18.2 mg/kg), this effect is observable after 2 days and is maintained for 6 days. In most cases, the tumour tends to disappear after 6 days.

For the controls, the modal cell volume was around 1400 cubic microns. Two days after *cis*-Pt (II) injection, the modal volume of the treated cells was increased to around 4000 μm^3. After 4 days, this value

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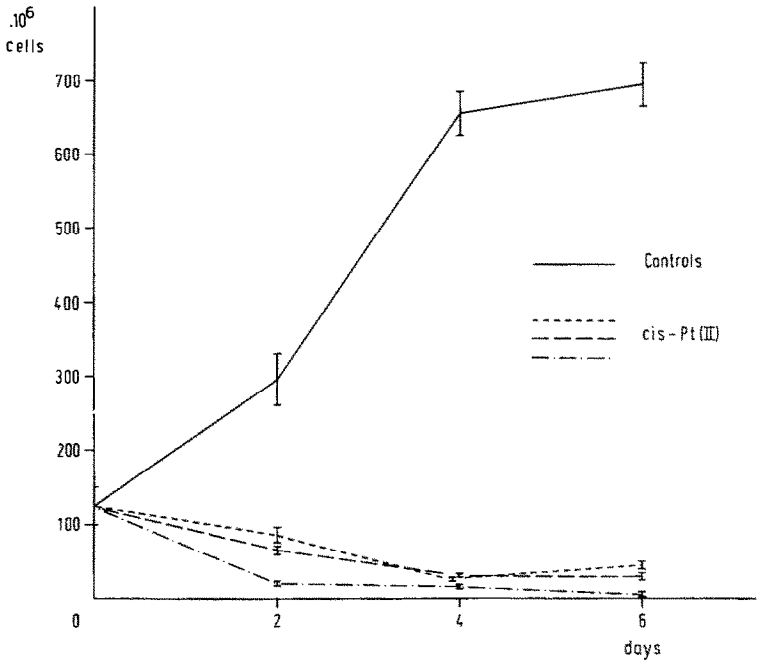

Fig. 1. Number of *Ehrlich ascites* tumour cells, measured with an electronic counter, in mice treated with *cis*-Pt (II) and in controls. One i.p. injection of *cis*-Pt (II) at day 0: ——— 4.55 mg/kg; — — — — 9.1 mg/kg; -·-·-·- 18.2 mg/kg. For each value, the standard deviation is indicated. The numbers are expressed in millions of cells.

remained at this level for the highest dosage, but for the low and mean doses, the cell volume increased further after 4 days (6500 μm^3) and after 6 days (10,000 μm^3).

II. Morphological analysis

(a) *Light microscopy.* The treated cells were much larger than the controls. They were strongly basophilic. Many cells contained micronuclei and were pycnotic and degenerated. Numerous treated cells were in metaphase; some of the latter were also degenerating. Two and six days after the treatment, nuclei and nucleoli were enlarged and in many nucleoli, a clear zone was detectable. The number of nucleoli per nucleus was the same as in controls (2–4). The leukocytes were much more numerous than in control smears.

(b) *Electron microscopy.* Many treated cells were degenerating and contained cytoplasmic vacuoles filled with some dense material.

When observed with the scanning electron microscope, the control cells were spherical and their surface was occupied by numerous microvilli. After *cis*-Pt (II) treatment, the microvilli were transformed into microvesicles, the diameter of which tended to increase during cell degeneration. Leukocytes and macrophages were often situated close to the tumour cells.

III. Mitotic activity

In the controls, the mitotic index was around 25‰. It was strongly lowered after *cis*-Pt (II) treatment and for 2–6 days afterwards: zero ‰ for the highest dosage; three and zero ‰ for the mean dosage; 7 and 21‰ for the low dosage. In the last case, 100 per cent of the mitotic cells were blocked in metaphase and slowly degenerated. DNA measurements (see the next paragraph) have shown that these metaphases have a normal DNA content.

This inhibition of cell multiplication in the tumour increases the survival of mice bearing an *Ehrlich ascites* tumour. Out of 40 treated mice [*cis*-Pt (II), 4.55 mg/kg], 33 were still alive after 1 month and bore no *ascites* any more. Only one survived among the controls. Seven treated mice died with a large amount of ascitic tumour. In these mice, tumour growth was inhibited for 11 to 15 days but then resumed. Giant tumour cells, numerous inflammatory cells and mitotic tumour cells were observed in these smears. Many mitoses were undisturbed but some of them were pycnotic or contained lagging chromosomes (mitotic index: approx 20‰).

IV. DNA, RNA and total protein content of the cells

(a) *DNA content.* As shown in Fig. 2, the post-mitotic DNA content in the control cells was tetraploid (4 DNA, hypertetraploid line with 90 chromosomes). This value is twice as high as in leukocytes of the mouse. In many control cells (around 40 per cent of the cells), the DNA content was doubled; premitotic DNA synthesis had occurred. They were thus in G2 phase or post-synthesis as to DNA and were ready to divide. The percentage of cells in G2 is much higher after *cis*-Pt (II) treatment: around 90 per cent after 2 and 6 days for the mean and low doses. This percentage is about 50 for the highest dosage, after 2 days. These results show that *cis*-Pt (II) blocks the cells in G2, but some inhibition of DNA synthesis occurs under the action of the high doses.

(b) *RNA content.* In cells treated with *cis*-Pt (II), the total RNA content was usually much higher than in controls (Fig. 3 and Table 1). This effect appears less clearly after treatment at the high dosage.

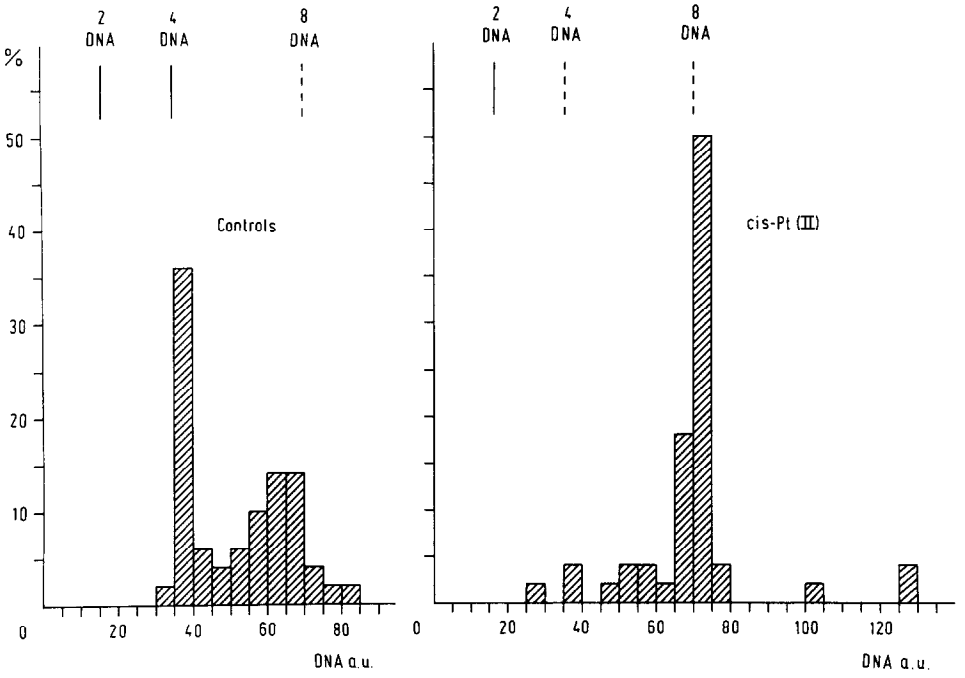

Fig. 2. DNA content measured by cytophotometry in *Ehrlich* tumour cells treated with *cis*-Pt (II) and in controls. On the abscissa, DNA content in arbitrary units (A.U.) measured in cells in interphase. On the ordinate, percentage of cells in each class. 2 DNA: mean diploid amount measured in leukocytes of the same animal. 4 DNA: mean post-mitotic content measured in ELT cells (hypertetraploid line). 8 DNA: premitotic content in ELT cells calculated by doubling the mean post-mitotic value. The treated cells have been analysed 6 days after the injection of *cis*-Pt (II), 4.55 mg/kg. Fifty cells were measured in each case.

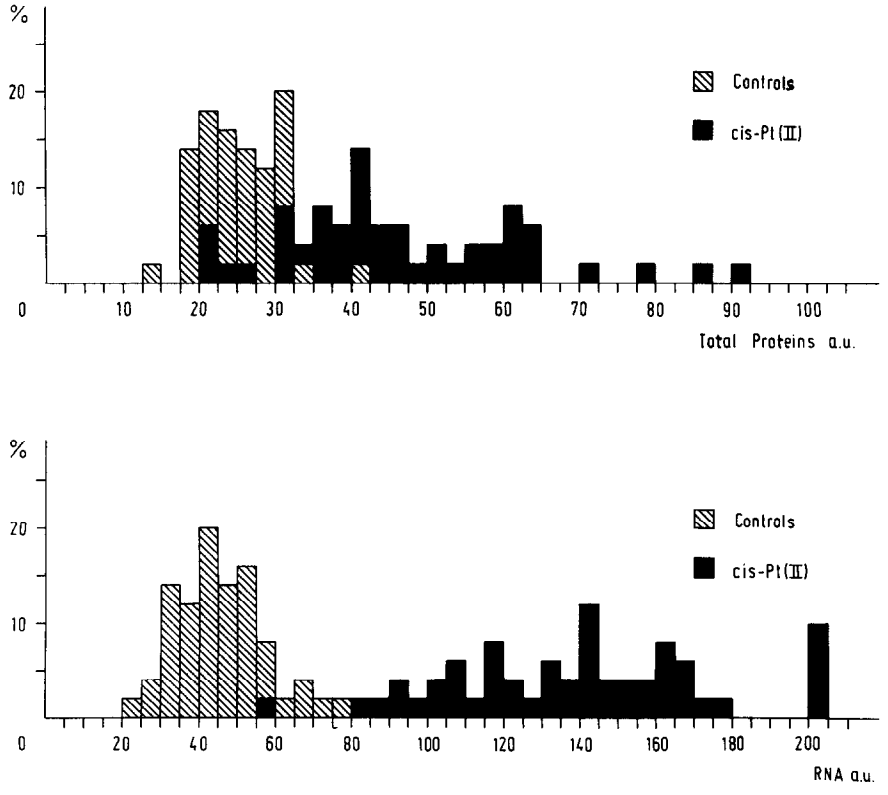

Fig. 3. Total protein content and total RNA content measured by cytophotometry in Ehrlich tumour cells treated with *cis*-Pt (II) and in controls. The construction of these histograms and the experimental conditions are the same as in Fig. 2.

Table 1. RNA content (A.U.)

	Controls	*cis*-Pt (II) 4.55 mg/kg	*cis*-Pt (II) 18.2 mg/kg
Second day	42.04 ± 9.68	77.0 ± 21.92	54.24 ± 19.90
Sixth day	45.06 ± 11.44	137.88 ± 33.91	no tumour

Mean cellular RNA or protein content measured by cytophotometry in ELT cells, 2 and 6 days after the beginning of the experiment [mean values in arbitrary units (A.U.) and standard deviation]. The observed differences between control and treated cells are statistically significant ($P < 0.01$; test of Kolmogorov–Smirnov). Fifty cells were measured in each case.

(c) *Total protein content.* As shown in Fig. 3 and Table 2, the total cellular protein content is also strongly increased after a treatment with *cis*-Pt (II). In most treated cells, it is much higher than in control cells that are ready to divide and in which the protein content has doubled.

From the results of this quantitative cytochemical analysis, we conclude that *cis*-Pt (II) prevents the cells from entering into mitosis (block in G2) but RNA and protein synthesis remains quite unaltered and the cells become very large. However, some inhibition of DNA and RNA synthesis occurs if a high dosage of *cis*-Pt (II) is injected.

(d) *DNA and protein measurements* were also performed in tumour cells during the reversal of growth inhibition, as it occurs in a few cases after *cis*-Pt (II) treatment. Eleven days after the injection of the drug (4.55 mg/kg), some tumour cells were very large and their protein and DNA content was 4 or even 8 times as high (16p/16 DNA, 32 p/32 DNA) as in post-mitotic control ELT cells (4p/4 DNA, hypertetraploid line). Many treated cells divide and their DNA and protein content is at the usual level (8p/8 DNA) or, more frequently, is higher (16p/16 DNA, 32 p/32 DNA). In other cells, the ratio p/DNA is abnormal: 16p/8 DNA; 32p/16 DNA; however, some of them are able to divide. Probably due to the fact that the cells fail to divide for more than 6 days, endopolyploidisation occurs as regards DNA and total proteins. Unbalanced growth is observed in many cells but cells multiply again during the next day and the tumour kills the animal.

DISCUSSION

According to our results, *cis*-Pt (II) strongly inhibits the multiplication of *Ehrlich ascites* tumour cells in the mouse. The survival of the latter is clearly increased by this treatment. In our material, the mode of action of *cis*-Pt (II) at the cell level seems to be as follows during the first 6 days after the injection. A high dosage of *cis*-Pt (II) provokes some inhibition of premitotic DNA synthesis. After the injection of a lower dosage, DNA synthesis takes place and is completed, but the majority of the cells cannot enter into prophase (premitotic or G2 block). Some cells succeed in beginning a mitosis but they are blocked during metaphase and degenerate. When cells treated with *cis*-Pt (II) are blocked in G2 and cannot divide, RNA and protein syntheses are, however, quite unaffected. So, the cell volume increases progressively. These cells have an abnormally high RNA and protein content but their DNA content remains at the normal premitotic level (unbalanced growth). In these giant and strongly basophilic cells, the nucleoli are remarkably large but their number is the same as in control cells. The increase of the nucleolar volume is clearly related to active RNA synthesis in the cell. These abnormally large cells slowly degenerate and the *ascites* tumour tends to disappear.

An increase of the cell volume has also been described when Ehrlich tumour cells [3] or human amnion cells [20] are treated with *cis*-Pt (II). According to Howle and Gale [3], *cis*-Pt (II) strongly inhibits the incorporation of thymidine into DNA in Ehrlich tumour cells; incorporation of uridine into RNA and of leucine into proteins is also somewhat inhibited but the synthesis of RNA and proteins resumes after 12–24 hr while DNA synthesis is irreversibly stopped, even after 96 hr. These results agree very well with those we have obtained with completely different methods. However, it should be stressed that the inhibition of thymidine incorporation into DNA described by Howle and Gale [3] could be related, at least in part, to the fact that the tumour cells are blocked in G2 after completion of premitotic DNA synthesis. So, this inhibition of incorporation does not necessarily

Table 2. Protein content (A.U.)

	Controls	*cis*-Pt (II) 4.55 mg/kg	*cis*-Pt (II) 18.2 mg/kg
Second day	27.62 ± 7.19	31.77 ± 7.11	35.90 ± 9.77
Sixth day	25.12 ± 5.34	46.65 ± 15.65	no tumour

Mean cellular RNA or protein content measured by cytophotometry in ELT cells, 2 and 6 days after the beginning of the experiment [mean values in arbitrary units (A.U.) and standard deviation]. The observed differences between control and treated cells are statistically significant ($P < 0.01$; test of Kolmogorov–Smirnov). Fifty cells were measured in each case.

demonstrate only a selective inhibition of DNA synthesis.

It is possible that active RNA synthesis occurs in the cells after treatment with *cis*-Pt (II) because the DNA content is doubled (premitotic value). This fact could also explain why the nucleoli are so large (duplication of DNA in nucleolar organizers). Very large nucleoli occur in ELT cells treated with sarcolysine or with daunomycin [21]. These agents also block the cells in G2 as to DNA but cell growth (protein synthesis) is not stopped. Such a dissociation between DNA and protein synthesis can be demonstrated in fibroblasts or in Ehrlich tumour cells after a treatment with myleran, melphalan, daunomycin, mitomycin C [21–24]. Interestingly, all these agents react with DNA either by cross-linking or intercalation. Usually, agents of this type inhibit DNA synthesis if the concentration of the drug is high or block the cells in G2 if the concentration is relatively low [25].

In a small number of cases, tumour growth can resume after a period of strong inhibition due to *cis*-Pt (II) action, as shown in the present work. Several cytological alterations (giant cells, mitotic anomalies) are present in these smears. It is difficult to state with precision if this reversal of the effect of *cis*-Pt (II) is due to the selection of resistant cells and (or) to DNA repair, as demonstrated in other materials [26]. Reversibility of mitotic inhibition in the same tumour can occur after a treatment with sarcolysine or daunomycin [24, 27], an alkylating agent and an antibiotic which also react with DNA. *Cis*-Pt (II) cross-links DNA molecules in a similar way as bifunctional alkylating agents do [13]. It is worthwhile noting that when cell multiplication resumes after *cis*-Pt (II) treatment, the DNA and protein content is abnormally high in many tumour cells. Indeed, when ELT cells treated with *cis*-Pt (II) fail to divide for more than 6 days, endopolyploidisation can occur as far as DNA and total proteins are concerned. In many cells, unbalanced growth is observed: the protein content is increased much more than the DNA content. However these polyploid and abnormal cells can divide later on. The same fact was observed in ELT cells treated with daunomycin [24].

Cis-Pt (II) is thus toxic to *Ehrlich tumour* cells. It is also very toxic to chick embryo fibroblasts cultivated *in vitro* as shown before [16]. Interestingly, the effects of this drug are quite similar in these two cell lines.

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