Effect of Selenium in Combination with cis-Diamminedichloroplatinum(II) in the Treatment of Murine Fibrosarcoma

Jean-Pierre Berry,1 Catherine Pauwells, Sylvie Tlouzeau, and Geneviève Lespinats

ABSTRACT

cis-Diamminedichloroplatinum(II) (cis-DDP) is a well-known anticancer agent the use of which is limited by its toxicity. Since it has been demonstrated that selenium is able to combine with metals like cadmium and mercury and to reduce their toxicity, we decided to investigate whether it could reduce the toxicity of platinum. We treated fibrosarcoma-bearing mice with a combination of cis-DDP and selenium. The dose of 2 or 4 μg selenium/g animal weight had no effect on tumor growth. The i.p. injection of 16 μg cis-DDP/g led to early death of animals. The i.p. treatment of tumor-bearing animals with 2 or 4 μg of selenium reduced the early mortality induced by cis-DDP at a dose of 16 μg/g. Therefore, the addition of selenium allowed the administration of high doses of cis-DDP, which resulted in an improved antitumor effect.

Clonogenic assays following drug exposure showed that selenium had no direct effect on tumor cells and did not modify the antitumor activity of cis-DDP. Electron microscopy showed reduced changes in renal cells when selenium was added to the cis-DDP treatment. Microanalysis showed no accumulation of either selenium or platinum within renal cells.

These results suggest that the addition of selenium decreases the nephrotoxicity of cis-DDP.

INTRODUCTION

Since Rosenberg first established the antitumor activity of cis-DDP (25), this effect has been widely confirmed for the treatment of tumors of various origins (7, 13). However, its use is limited by its toxicity to different organs, particularly kidneys and bone marrow (18, 24). It has been demonstrated that, during experimental intoxications, a platinum element is accumulated in the lysosomes of renal cells, where it is bound to sulfur (2). Conversely, selenium has been described as counteracting the toxic activity of cadmium and mercury (16, 19, 22). It also appears to favor the precipitation of silver in renal tissue (3); however, selenium itself, if used at high doses, is not deprived of toxic capacity to provoke hepatic and renal lesions (5). The chemical properties of selenium closely resemble the properties of sulfur; these 2 elements both belong to Group VI A, in the periodic classification of chemicals (10). This may be the reason selenium is able to substitute for sulfur in numerous mineral or organic compounds and to favor the in vivo precipitation of some metals (8). Since the sulfur-platinum association has been detected in renal cells (2), we sought to investigate whether selenium could be substituted for sulfur and thus counteract the toxic effect of platinum in a reaction similar to that already known for other metals.

On an experimental tumor model, we tested the effect of cis-DDP2 combined with selenium both for general toxicity and for chemotherapeutic activity on tumor growth, as compared with the effect of each compound individually. The in vitro sensitivity of the tumor cells in soft agar was studied with each drug alone or associated in a stem cell assay in order to determine the mechanism of protective activity of selenium. The ultrastructure of various organs of in vivo-treated animals was observed, and the presence of mineral elements (platinum and selenium) in intracellular inclusions was looked for using microanalysis by electron probe.

MATERIALS AND METHODS

Mice. Male and female C57BL/6 × C3H/He F1 mice were obtained from the production colonies of the Institut de Recherches Scientifiques sur le Cancer, Villejuif, France. Animals were used at the age of 8 to 12 weeks.

Tumors. The MC B6-1 fibrosarcoma was originally induced by s.c. injection of 2 mg of methylcholanthrene in a female C57BL/6 mouse and serially transplanted in syngeneic mice. It was used between the 19th and 24th passages. Tumor cells (5 × 10⁴) were injected s.c., and the animals were treated 12 to 14 days later, when all animals had palpable tumors. Groups of 10 mice were used in all experiments.

Treatment. Selenous acid (selenium) (BDH Chemicals, Ltd.) was dissolved in Eagle’s minimum essential medium, neutralized with sodium bicarbonate, and adjusted to the desired concentration. It was injected i.p. in a volume of 0.1 ml. cis-DDP (cisplaty, Laboratoire Roger Bellori) was injected i.p. 4 hr later.

Mice were checked twice a week. For each tumor, 2 diameters perpendicular to each other were recorded, and the tumor volumes were calculated by the formula

\[ V = 0.4ab^2 \]

where \( a \) is the maximum diameter of the tumor and \( b \) is the diameter at right angles to \( a \). The curves were analyzed for significant differences using analysis of variance.

Cell Culture. MC B6-1 tumor cells were dissociated from solid tumor by trypsinization at 37°C for 90 min. They were cultivated in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (penicillin (100 units/ml) and streptomycin (100 μg/ml)).

Clonogenic Assays. Plating efficiencies were calculated using the techniques described by Hamburger and Salmon (12), Van Hoff et al. (26), and Pavelic et al. (23) using a bilayer soft-agar system. The top layer consisted of 0.3% agar in RPMI 1640 enriched with fetal calf serum (10%), antibiotics, and glutamine. The cell concentration in this layer was 2 × 10⁶ cells/ml. One ml of this mixture was pipetted and placed on the overlay of 0.5% agar in the same culture medium. These layers were poured into 35-mm Petri dishes.

After preparation of both bottom and top layers, the plates were incubated 8 days at 37°C in a 5% CO₂-humidified atmosphere.

Drug Sensitivity. Cell suspensions with a final concentration of 2 × 10⁶ were added to the top layer and incubated for 7 days at 37°C. The plates were then fixed in 2% (w/v) paraformaldehyde in PBS for 1 hr at 4°C, and stained with Giemsa for 30 min. Clonogenic assays were performed on the bottom layer using a bisulphite-specific protocol.

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2 The abbreviation used is: cis-DDP, cis-diamminedichloroplatinum(II).

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10⁴ cells/ml were transferred to tubes in the presence of appropriate cis-DDP and/or selenium. Cells were incubated at 37° for 60 min in RPMI 1640 in a 5% CO₂-humidified atmosphere.

Cells were washed and centrifuged (1500 rpm) twice with RPMI 1640 and prepared for culture as described above. After 8 days in culture, the number of colonies on the 4 control plates and on the 4 plates containing the concentration to be tested were counted using an inverted-phase microscope. Groups of 60 or more cells were considered as colonies, whereas groups of fewer cells were not.

The results were expressed as percentage of control, i.e., the number of colonies with the drug divided by the number of colonies without the drug. Each experiment was performed 3 times. Statistical analysis was performed by Student’s t test.

Electron Microscopy and Microprobe Analysis. Samples from tumor, kidney, liver, and bone marrow were collected from killed mice 10 days after drug injection. The samples were fixed in a 1.5% glutaraldehyde solution in cacodylate buffer, pH 7.3; some also were postfixed in a 1% solution of osmic acid in the same buffer. The samples then were washed in the cacodylate buffer, dehydrated, and mounted in Epon. Ultrathin sections of approximately 100 nm were cut using an LKB Ultrotome III and mounted on aluminum or copper grids. None of the sections was stained.

Instrumental Methods. A Philips EM-300 electron microscope was used to study the ultrastructural morphology of the sections. A MBX electron microprobe with camera was used to reveal the presence of individual elements in the structure within thin sections (9). The latter instrument was equipped with 2 wave length-dispersive spectrometers. The transmission electron microscope accessory on the electron microprobe was magnified on the order of X50.000.

An ASR 33 teletype unit was used to record X-ray signals automatically for preset counting times.

The experimental conditions used in these analyses were accelerating voltage at 30 kV, beam current at 50 na, and electron probe diameter at 500 nm.

### RESULTS

#### In Vivo Toxicity

**Normal Mice.** Preliminary experiments with normal mice determined the toxicity of selenium and cis-DDP separately. Early death, within 7 days after i.p. injection, was observed for selenium at doses higher than 4 µg per g of animal weight and for cis-DDP at doses of 12 µg or more per g of animal weight.

Table 1 indicates the results of 2 experiments combining administration of selenium and cis-DDP. The sum of 2 experiments shows that 14 days after treatment, the survival of control animals was 100%, that of selenium-treated (4 µg) was 95%, that of cis-DDP-treated animals (16 µg) was 10%, and that of selenium plus cis-DDP-treated animals was 75%.

Table 2 shows the results of another experiment in which animals receiving different treatments were weighed biweekly (groups of 10 mice). cis-DDP (8 µg) led to no mortality and to a moderate loss of weight, which was regained within 2 weeks. With cis-DDP (12 µg), 50% mortality was observed; surviving mice showed a weight loss, which was regained within the same length of time. With cis-DDP (16 µg), 100% mortality was observed within the first week after treatment. With selenium (4 µg), there was no mortality and only a slight loss of weight. Selenium (6 µg) led to 80% mortality, and for surviving mice a severe weight loss which was only partly regained 3 weeks after treatment. With cis-DDP (12 µg) and selenium (4 µg), there was no mortality and no loss of weight. With cis-DDP (16 µg) and selenium (4 µg), 30% mortality was observed; surviving mice showed a severe loss of weight, which was partly regained 3 weeks after treatment.

**Tumor-bearing Mice.** Experiments were carried out with tumor-bearing animals. Table 3 groups 3 different experiments with comparable results. The effect on early mortality by treatment with selenium at 2 and 4 µg, alone or in combination with cis-DDP (16 µg), was determined. Treatment was performed 10 days after tumor inoculation. Survival of mice 14 days after treatment indicated early toxicity of drugs and not of tumor mortality, which occurs much later. Survival of mice which received 2 µg selenium was better (100%) than with selenium (4 µg) (75%), and survival with 2 µg selenium plus 16 µg cis-DDP was better (93%) than with selenium (4 µg) plus cis-DDP (16 µg) (80%).

These experiments primarily demonstrated that survival of animals treated with cis-DDP (16 µg) was 20%, whereas the
addition of selenium (4 μg) increased survival to 80%, and the addition of selenium (2 μg) increased it to 93%.

In order to evaluate the activity of these different treatments, tumor growth was followed. Table 4 indicates the results of a typical experiment. Selenium, at a dose of 2 or 4 μg/mg of animal weight had no effect on tumor growth (p > 0.05). cis-DDP had a dose-dependent effect; treatment with cis-DDP (16 μg) was more efficient than was cis-DDP (8 μg) and significantly reduced tumor growth (p < 0.01). Therefore, the addition of selenium to cis-DDP did not improve the efficiency of the latter but prevented early death; it thereby allowed the use of high doses of cis-DDP, which when used alone, were highly toxic.

Clonogenic Assay of MCB6-1

**Test of cis-DDP.** Results (Table 5) clearly show that cis-DDP incubated 1 hr with the tumor cells reduced their plating efficiencies. Cloning efficiencies decreased when drug concentrations increased, indicating direct toxicity for cloning capacity of the MCB6-1 cells (Chart 1). This drug acted directly upon tumor cells in this test in vitro, and the plating efficiency was significantly decreased using 0.1 μg/ml (p < 0.05).

**Table 3**

<table>
<thead>
<tr>
<th>No. of surviving mice/group</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
</tr>
<tr>
<td>14 days</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>29/30 (97)</td>
</tr>
<tr>
<td>Selenium (4 μg/g)</td>
</tr>
<tr>
<td>18/20 (90)</td>
</tr>
<tr>
<td>Selenium (2 μg/g)</td>
</tr>
<tr>
<td>20/20 (100)</td>
</tr>
<tr>
<td>cis-DDP (16 μg/g)</td>
</tr>
<tr>
<td>0/20 (0)</td>
</tr>
<tr>
<td>Selenium (4 μg/g) + cis-DDP (16 μg/g)</td>
</tr>
<tr>
<td>17/20 (85)</td>
</tr>
<tr>
<td>Selenium (2 μg/g) + cis-DDP (16 μg/g)</td>
</tr>
<tr>
<td>29/30 (96)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentage.

**Table 4**

<table>
<thead>
<tr>
<th>Tumor volume at following times after tumor injection (μm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Selenium</td>
</tr>
<tr>
<td>cis-DDP</td>
</tr>
<tr>
<td>+ selenium</td>
</tr>
<tr>
<td>cis-DDP + selenium</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of 10 animals/group.

**Test of Selenium.** In the range of concentration utilized here (Table 6), colony formation was not reduced by selenium incubated for 1 hr with tumor cells; selenium did not influence the plating efficiency of the MCB6-1 cells, showing that it was not directly toxic for tumor cells.

**Test of Selenium and cis-DDP.** The simultaneous exposure of the tumor cells to these 2 drugs (selenium concentration chosen, 0.4 μg/ml) showed no significant difference (p > 0.05) in the reduction of the cloning capacity as compared to cis-DDP alone.

These results show that cis-DDP had a direct toxicity on the capacity of MCB6-1 cells to produce clones, but this action was not amplified by selenium acid at high concentration.
Electron Microscopy and Microanalysis

Kidney

Electron Microscopy. Animals Treated with Selenium. Major changes occurred in the proximal convoluted tubules. These changes were characterized by intracytoplasmic vacuoles, dispersion of intercellular spaces, the presence of clusters of small granules in the lysosomes, and lipidic inclusions.

Animals Treated with cis-DDP. Numerous proximal convoluted tubules and distal tubules were altered. In convoluted tubules, we observed the presence of large intracytoplasmic vacuoles, the disappearance of the apical part of the cells, the distension of mitochondria, numerous lysosomes containing clusters of dense granules, and lipidic inclusions. Distal tubules showed considerable cellular necrosis, with destruction of some tubular cells in which only the nucleus and basal membrane were still visible.

Animals Treated with Selenium plus cis-DDP. Results obtained at both dose levels (cis-DDP, 8 and 16 µg) were essentially identical and differed from results obtained with animals treated with cis-DDP alone; proximal tubular cells were not altered, and clusters of small granules were present in numerous lysosomes. In the mitochondria, small round inclusions made up of clusters of dense granules were observed. These inclusions were larger than those usually observed in mitochondria. Distal tubular cells were not altered, and the ultrastructure was comparable to the structure of controls.

Microanalysis. Iron exclusively detected was in dense granules in the lysosomes of renal tubular cells. Selenium and platinum were not detected in any of the dense inclusions present in the lysosomes or in the mitochondria. The same observation was made for all samples of kidney.

Liver

Selenium induced zones of cellular rarefaction. These lesions were not observed in animals receiving cis-DDP or selenium plus cis-DDP.

Bone Marrow

No significant differences were observed with the different treatments.

DISCUSSION

Several attempts have been made to reduce the toxicity of cis-DDP. This toxicity is a major limitation of the potential effectiveness of the drug (4, 6, 14). We combined administration of cis-DDP treatment with selenium, since previous studies had demonstrated that selenium can protect against the toxic action of certain metals, e.g., mercury and cadmium (21). This protective role has been interpreted as being a consequence of the precipitation of these metals by selenium, forming insoluble or very slightly soluble complexes. This precipitation results in the abruption of the toxic elements from the internal medium. Microscopic studies have shown that selenium precipitates other metals in tissues, leading to their storage inside cells (3).

We had previously demonstrated that, after administration of cis-DDP, inclusions of iron, platinum, and sulfur in association were detected in renal tubular cells (2). It was hypothesized that selenium may substitute for sulfur, interact with platinum, and protect against the toxic action of cis-DDP.

Selenium proved to be useful in that it allowed administration to mice of doses of cis-DDP which, when used alone, would be toxic. The highest dose of cis-DDP which was not toxic, 8 µg/g was incapable of inducing an antitumor effect. Doses higher than 8 µg, and which were effective, could be used only in association with selenium.

Selenium used alone had no effect on tumor growth and, when associated with cis-DDP, neither enhanced nor abolished the antitumor activity of cis-DDP. Clonogenic assays showed that cis-DDP was toxic for tumor cells, and that the addition of selenium did not enhance or decrease this toxicity. These results were in good agreement with in vivo data.

Many reports indicate that selenium may have an effect on tumors; it is capable of reducing the incidence of carcinogen-induced tumors (15–17) and mammary tumors in virus-positive mice (19), and of suppressing the growth of Ehrlich ascites tumor cells (10) and L1210 leukemic cells (21). The discrepancy between these and our results could be related to the use of different models. It would be interesting to test the effects of the association of selenium and cis-DDP on several tumors. If, in some tumors, it were possible to combine the beneficial effects of both agents, the association could be even more effective.

Observations with electron microscopy have shown that renal cells from mice which received selenium and cis-DDP were altered less than were renal cells from mice which received either selenium or cis-DDP alone. In these cells, intramitochondrial inclusions could be seen, but neither platinum nor selenium could be detected by microanalysis methods.

Thus, the mechanism of action of selenium on platinum is different from that of selenium on other metals. Selenium and platinum could associate in the circulation and be eliminated directly without being reabsorbed by renal cells, but if complexes were formed in the extracellular compartment, selenium should then decrease the penetration of cis-DDP in tumor cells and therefore the antitumor activity of cis-DDP. This is not the case, and our results do not favor this mechanism of action.

We must assume that there exists an interaction between selenium and the molecule of cis-DDP which prevents reabsorption by renal cells but does not impede antitumor activity. Selenium could fix on groups of the molecule of cis-DDP which do not intervene in its antitumor activity. Whatever the mechanism, the reduction of nephrotoxicity without a concomitant loss of antitumor activity improves the therapeutic index of cis-DDP.

REFERENCES

J-P. Berry et al.


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