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Original Paper

In Vitro and *In Vivo* Characterisation of Low-resistant Mouse Reticulosarcoma (M5076) Sublines Obtained After Pulse and Continuous Exposure to Cisplatin

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In order to simulate drug resistance observed in the clinic, two cisplatin-resistant cell lines were produced from a murine ovarian reticulosarcoma, M5076 (M5), by pulse (M5/CDDP) and continuous (M5/CDDPc) treatment with *cis*-diamminedichloroplatinum(II)(CDDP). These cell lines showed a similar stable low level of resistance (approximately 3-fold) to CDDP and cross-resistance to carboplatin, iproplatin and the new alkylating agent tallimustine, but not to L-PAM (L-phenylalanine mustard) and BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea). Collateral sensitivity to two inhibitors of topoisomerase II, VP16 (etoposide) and doxorubicin (Dox), but cross-resistance to the topoisomerase I inhibitor, camptothecin, were observed. The two cell lines were also sensitive to 5-fluorouracil. No increase in the level of glutathione or activity of glutathione S-transferase could be observed in resistant cells compared with the parental M5 cells. Total DNA platination immediately after treatment was similar in the parental and resistant cell lines. Repair of total DNA platination, measured after 24 h of recovery, was undetectable in M5 and M5/CDDP cells, but was 33% in M5/CDDPc cells. Initial DNA-interstrand cross-links (DNA-ISC) were six times higher in M5 than in M5/CDDP cells, but 24 h after treatment, both lines had completely repaired this damage. M5/CDDPc cells did not show formation of DNA-ISC at any time after treatment. The two resistant cell lines were tumorigenic when implanted in mice and resistant to CDDP treatment *in vivo*. The CDDP resistant tumours were not cross-resistant *in vivo* to L-PAM, BCNU and Dox, which had been active *in vitro*, nor to tallimustine, which had been cross-resistant *in vitro*. Mechanisms of resistance in M5/CDDP and M5-CDDPc seem to be based on a lower formation of DNA-ISC combined, for the latter cell line, with a higher repair capacity for total DNA platination.

Key words: cisplatin, resistance, DNA damage, *in vivo*

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INTRODUCTION

cis-DIAMMINEDICHLOROPLATINUM(II) (CDDP) is one of the most effective drugs used in the treatment of human ovarian, testicular, bladder and head and neck cancers [1]. However, as with many other cytotoxic drugs, the development of acquired resistance is a common consequence of therapy, limiting its clinical efficacy [2, 3]. The biochemical and genetic mechanisms of CDDP resistance are not yet fully understood. A number of cell lines with acquired resistance to CDDP have been developed and they have provided model systems for the study of the mechanisms of CDDP resistance [4]. In most cases, resistance mechanisms have been studied in highly resistant (>10-fold) mammalian tumour cell lines in which resistance has been induced by exposing cells continuously to increasing concentrations of CDDP for extensive periods of time [4]. In the clinic, how-

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ever, patients may develop drug resistance at relapse after only a few courses of chemotherapy. Moreover, the tumour resistance observed in patients is not very high (approximately 3- to 6-fold) [5, 6]. Therefore, in terms of the method of induction, the drug resistance induced by a short-term exposure to relatively high doses of antitumour agents may have more clinical relevance than that produced by long-term exposure to low concentrations. Furthermore, study of the mechanisms involved in low level resistance seems more relevant for *in vivo* studies and thereby clinical practice.

In order to simulate the resistance observed in the clinic, we obtained a low CDDP-resistant subline from the ovarian murine reticulosarcoma M5076 (M5) after pulse exposure of the cells to cytotoxic concentrations of CDDP. We compared the level and the supporting mechanism(s) of resistance of this cell line (M5/CDDP) with that of a CDDP-resistant subline (M5/CDDPc) obtained after continuous treatment with non-cytotoxic concentrations of CDDP. We chose this murine tumour cell line because we had already obtained a transplantable tumour resistant to CDDP from this cell line after *in vivo* treatment [7] and characterised the growth and sensitivity to CDDP of this tumour in primary culture [8]. In this report, we describe the cross-resistance pattern of the two independently selected cell lines to other chemotherapeutic agents *in vivo* and *in vitro*.

Glutathione (GSH) levels and the activity of glutathione-S-transferase (GST) were measured and the formation and repair of total DNA platination and DNA-interstrand cross-links (DNA-ISC) were evaluated.

MATERIALS AND METHODS

Chemicals and reagents

RPMI-1640 medium was obtained from Biowhittaker (Walkersville, Maryland, U.S.A.). Glutamine, pyruvate and horse serum were obtained from GIBCO Europe (Paisley, U.K.). DNase I and Nuclease P1 were from Calbiochem Behring (San Diego, California, U.S.A.). NADPH, DTNB (5-5'-dithiobis-2-nitrobenzoic acid), CDNB (1-chloro-2,4-dinitrobenzene), GSH and GSH reductase were from Sigma (St Louis, Missouri, U.S.A.) and metaphosphoric acid from Merck (Darmstadt, Germany). CDDP, carboplatin and VP16 (etoposide) were obtained from Bristol-Myers Squibb Int. Corp. (Syracuse, New York, U.S.A.). L-PAM (L-phenylalanine mustard), iproplatin, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), camptothecin and 5-FU (5-fluorouracil) were provided by the National Cancer Institute (Drug Synthesis and Chemistry Branch, DCT, NIH, Bethesda, Maryland, U.S.A.). Dox (doxorubicin) and tallimustine were obtained from Pharmacia Farmitalia-Carlo Erba (Nerviano, Italy). For cell culture treatment, CDDP and iproplatin were dissolved in the medium and incubated at 37°C for 30 min, before addition to the cells, to equilibrate free and serum protein-bound platinum. L-PAM and BCNU were dissolved in 0.5 ml of 0.3 N HCl and then diluted with the medium, and the other drugs were dissolved directly in the medium. For animal treatment, CDDP and Dox were dissolved in 0.9% NaCl and water, respectively, L-PAM was dissolved in 2% 3 N HCl and buffered with 3 N NaOH. BCNU was dissolved in dimethyl sulphoxide and diluted with 0.9% NaCl, 5-FU was dissolved in water.

Cell lines

The M5 cell line was obtained from Talmadge and colleagues [9] and was propagated normally as a monolayer culture in RPMI-1640 medium, supplemented with 15% heat-inactivated horse serum, 1% glutamine and 1% pyruvate at 37°C in an atmosphere with 5% CO_2. Two drug schedules were used to develop resistant cell lines: intermittent and continuous exposure to CDDP (Figure 1). For the intermittent schedule, the M5 cells (3×10^5) were plated in

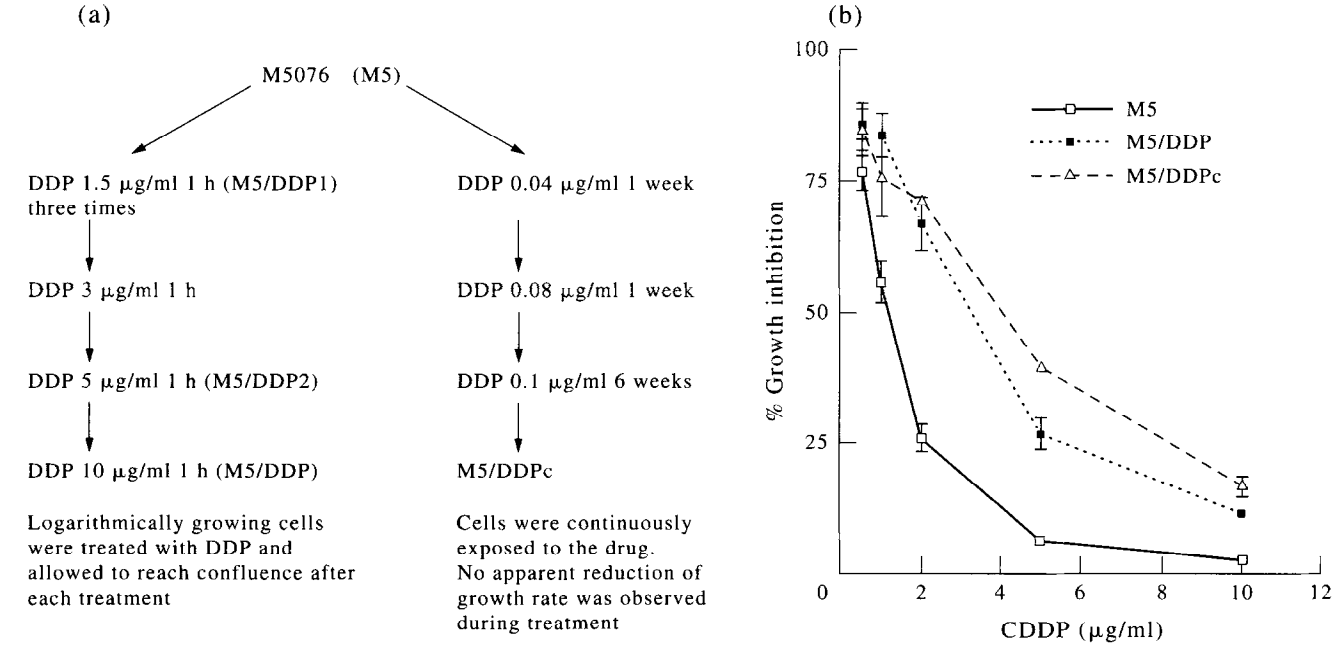

Figure 1. (a) Cisplatin (CDDP) treatment schedules used *in vitro* to obtain the resistant cell lines and (b) growth inhibition caused by CDDP in M5 sublines progressively resistant to CDDP.

25 cm^2 plastic flasks (Nunc, Roskilde, Denmark), and after 24 h the cells were treated with increasing concentrations of CDDP from 1.5 to 10 μg/ml for 1 h. In the continuous schedule, M5 cells were maintained in the presence of a low concentration of CDDP, increasing stepwise from 0.04 to 0.1 μg/ml CDDP. Experiments were performed on cells that were propagated in the absence of CDDP.

Animals

Groups of 10 female C57BI/6 mice (20 ± 2 g) obtained from Charles River (Calco, Italy) received an intramuscular inoculum, in the leg, of 5×10^5 viable cells of reticular cell sarcoma M5 or of the resistant sublines at day 0. Tumour size in treated and untreated mice was determined by recording maximum and minimum diameters with a Vernier caliper every 2 or 4 days. Tumour volume (V) was calculated by the formula $V = LW^2/2$, where L is the average length in millimetres and W the width of the tumour; the volume was converted to weight in grams, assuming unit density.

Growth kinetics

Doubling times (T_d) of the cell lines were determined by seeding 3×10^5 cells into 25 cm^2 plastic flasks preloaded with 5 ml of medium. Two cell counts for each replicate from each cell line were made every 24 h for 5 days. Cell counting was performed with a Model ZM Culture Counter (Coulter Electronics, Inc., Hialeah, Florida, U.S.A.). The data were subjected to linear regression analysis, in which the T_d was calculated from the formula $T_d = \ln 2/\text{slope}$.

Dose-dependent survival analysis for drug cytotoxicity

The growth inhibition assay was used in all studies concerning the survival response to antitumour agents. Briefly, cells ($1.5–3 \times 10^5$) were seeded in 25 cm^2 plastic flasks and treated 24 h later with antitumour drugs for 1 h, washed with PBS (phosphate-buffered saline) and incubated for 72 h with a medium change after 48 h. The cells were then harvested by shaking and an aliquot was counted with the Coulter Counter. The degree of resistance was expressed as the ratio of the IC_{50} (concentration which inhibits cell growth by 50%) of the resistant cells to that of the parental line.

GSH and GST determination

Cultures of the three cell lines were analysed for GSH and GST during the exponential phase of growth. For the enzymatic assay of GSH, cells were gently harvested, counted, centrifuged and resuspended in 300 μl of cold metaphosphoric acid (10% w/v), and a crude cytosolic fraction was obtained by centrifugation at 2600 g for 5 min in a Eppendorf microcentrifuge. Then, 120 μl of Na_3PO_4 (1.3 M) was added and, after centrifugation at 5800 g for 5 min, the supernatant was collected and stored for GSH assay at −20°C.

Total GSH content was measured as described by Tietze [10] mixing, in the spectrometer cuvette, 700 μl of 0.3 mM NADPH suspended in Na_2EDTA buffer at pH 7.5, 100 μl of DTNB (6 mM), 140 μl of sample and adding 10 μl of GSH reductase. The rate of formation of TNB (thionitrobenzoic acid) was followed using a spectrophotometer set at 412 nm at 30°C and the GSH concentration was determined with a calibration curve made with each experiment.

For GST determination, exponentially growing cells (10^6) were lysed by sonication in distilled water, the cell lysate was centrifuged (2600 g for 15 min) and the supernatant was used for GST activity, according to the method of Habig and associates [11]. The reaction mixture contained 1 mM GSH, 1 mM CDNB, and 100 μl of sample in a final volume of 1 ml (pH 6.5). The rate of increase in absorbance at 340 nm was measured at 30°C with a spectrophotometer.

Formation of DNA-ISC

After thawing, cells were passaged three times ($1–2 \times 10^5$) and then seeded in 25 cm^2 flasks. Twenty-four hours later DNA was labelled with ^{3}H-thymidine (0.5 μCi/ml, 30 mCi/mmol) (New England Nuclear, Boston, Massachusetts, U.S.A.). After 24 h, tritiated thymidine was removed, and chasing with fresh medium was performed for approximately 24 h. Cells were treated for 1 h with 2 μg/ml CDDP, washed twice with PBS and re-incubated with 5 ml of medium for different periods of time. At the end of recovery, cells were washed with cold PBS, shaken and resuspended in 10 ml of PBS.

DNA-ISC were detected by the alkaline elution technique described by Kohn and colleagues [12]. Treated and untreated cells in 10 ml PBS were irradiated with 300 rads. The cells ($1–2 \times 10^6$) were loaded on to 25 mm diameter polycarbonate filters (0.8 μm pore size) (Nucleopore Co, Pleasanton, California, U.S.A.) and lysed on the filters at room temperature with 5 ml of lysis solution containing 0.1 M glycine, 0.025 M EDTA and 2% sodium dodecylsulphate (pH 10). The lysis solution was allowed to elute and the funnels were then connected to the pumps and 2 ml of lysis solution containing proteinase K (Merck, Darmstadt, Germany) (0.5 mg/ml) was added. The funnels were filled with about 40 ml of a solution containing 0.02 M acid EDTA, 0.1% sodium dodecylsulphate and 10% tetrapropylammonium hydroxide (Fluka, Buchs', Switzerland) in water to give a pH of 12.2. Fractions (6 ml) were collected at 3 h intervals for 15 h. Before counting, fractions were mixed with Aquasure (New England Nuclear, Boston, Massachusetts, U.S.A.) containing 0.7% acetic acid to reduce the chemiluminescence produced by the alkali. The fraction of DNA remaining on the filters was plotted against time, after normalising the fractions collected to the first fraction to reduce differences from variations in volume between fractions. DNA-ISC calculations were made according to Kohn and colleagues [12].

Pt–DNA

For the assay of total Pt–DNA adducts, approximately 30×10^6 of logarithmically growing cells were treated in 175 cm^2 flasks with 10 μg/ml of CDDP for 4 h. After treatment, the cells were washed twice with PBS and vital counting with Erytrosine B staining was performed. DNA was isolated according to Fichtinger–Schepman and colleagues [13]. Briefly, a phenol extraction and ethanol precipitation were followed by RNAse treatment. The remaining proteins were extracted by trichloromethane(chloroform)/isoamylic alcohol. To 74 μl of DNA solution in distilled water, 12 μl of buffer (100 mM Tris–HCl, 40 mM $MgCl_2$, 1 mM Na_2–

EDTA) was added, supplemented with 2.5 µl 10 mM $ZnSO_4$, 8.4 µl DNAse I (3000 IU/ml) and 23.4 µl Nuclease P1 (1 mg/ml) and, after overnight digestion, the DNA content was estimated by absorption at 260 nm and the amount of platinum was determined with a Varian model 300/400 atomic absorption spectrophotometer (AAS) equipped with a Zeeman correction [14].

RESULTS

Production of resistant cell lines

Two CDDP-resistant M5 sublines were obtained after intermittent and continuous treatment schedules (Figure 1a). With the intermittent schedule, M5 cells were exposed once a week for 3 weeks, for 1 h each time, to a concentration of CDDP (1.5 µg/ml) close to the IC_{50} of CDDP for this cell line. The cells were allowed to grow until they reached the initial density. After sequential single treatments with 3, 5 and 10 µg/ml of CDDP a 2.7-fold resistant cell line, M5/CDDP, was obtained (Figure 1b). The cells were frozen after five passages following the final treatment, and the experiments were performed on cells that were propagated in the absence of further CDDP treatments.

In the continuous schedule, M5 cells were exposed initially to 0.04 µg/ml of CDDP freshly added to the medium every 2 days and the dose was increased to 0.1 µg/ml (Figure 1a). After 6 weeks of continuous treatment with 0.1 µg/ml, the sensitivity to CDDP of the derived cell line, M5/CDDPc, was tested and the cell line M5/CDDPc was found to be 3.4-fold resistant (Figure 1b). The cells were frozen after five passages and, after thawing, grown in the absence of CDDP.

The stability of the resistance of the two cell lines M5/CDDP and M5/CDDPc was tested, in the absence of drug treatment, 45, 90 and 180 days after establishment of the line and did not change during this time (data not shown).

Characterisation of the cell lines

The M5 and M5/CDDPc cells showed a mixed population of round and bipolar spindle-shaped cells, while the M5/CDDP line was mostly spindle-shaped cells (Figure 2). The cells of the three lines grew as individual cells with very little intercellular contacts.

Table 1 shows the characteristics of the three cell lines. Population doubling time was slightly higher in the resistant cell lines compared with the parental cells. Cell volume of both resistant cell lines was smaller compared with the parental cell line, whereas cellular protein content was higher in the M5/CDDP cells, but lower in the M5/CDDPc cells compared with the M5 cells. All three lines formed colonies on plastic although these were not very compact; clonogenicity was approximately 30% for all cell lines.

GSH content in the resistant M5/CDDP line was half that of the M5 cells, while in the M5/CDDPc cells it was similar to the levels in the parental line. The ratio of the levels of GSH beween the parental and the resistant cell lines was the same, expressing the values per milligram of protein or per number of cells. Similar results were observed for GST activity.

Cross-resistance

Table 2 shows the sensitivity of the M5 cell line and cross-resistance of the resistant cell lines to several drugs. Both CDDP-resistant cell lines were cross-resistant to iproplatin and carboplatin, but sensitive to two other alkylating agents, L-PAM and BCNU. The only alkylating agent that was cross-resistant, besides the Pt-derivatives, was the new drug tallimustine, a derivative of distamycin with two chloroethyl groups. The topoisomerase II inhibitors, Dox and VP16, showed collateral sensitivity in the resistant cell lines, but the topoisomerase I inhibitor, camptothecin, was cross-resistant and half as active in the M5/CDDP than in the M5/CDDPc cell line. This drug, together with iproplatin, were the only two agents showing different levels of resistance in the two resistant cell lines. The antimetabolite 5-FU was active in the CDDP-resistant cell lines.

Induction and repair of platinum–DNA adducts

Immediately after treatment, no difference in DNA platination could be observed between the three cell lines (Table 3). No repair of Pt–DNA adducts, measured for up to 24 h, could be observed in the M5 and M5/CDDP cell

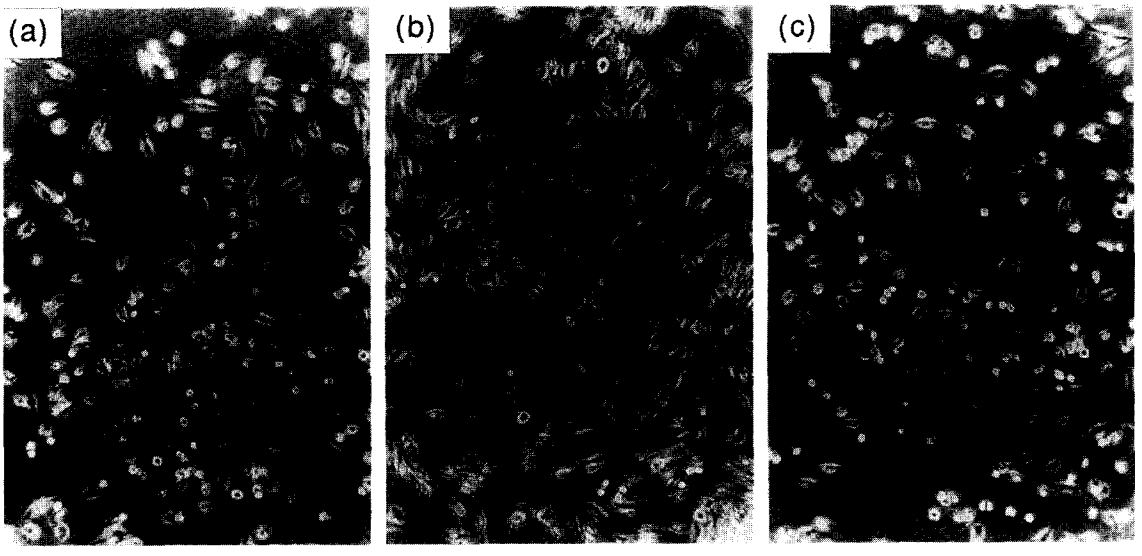

Figure 2. Phase contrast micrographs of cell cultures in logarithmic growth: M5 (a); M5/CDDP (b); M5/CDDPc (c).

Table 1. Characteristics of M5 and resistant cell lines

	Cell lines		
	M5	M5/CDDP	M5/CDDPc
Doubling time (h)	17 ± 1	20 ± 1	20 ± 1
Cell volume (fl)	1121	925	982
μg protein/10^6 cells	370 ± 6	430 ± 14	327 ± 20
% Clonogenicity	30 ± 2	24 ± 4	24 ± 4
GSH (nmol/10^6 cells)	12 ± 0.9	6.9 ± 0.7	11.4 ± 0.9
GST (nmol/10^6 cells)	0.12 ± 0.05	0.043 ± 0.05	0.09 ± 0.05

M5, murine ovarian reticulosarcoma M5076; M5/CDDP, M5/CDDPc, cisplatin-resistant cell lines from pulse and continuous treatment, respectively; GSH, glutathione; GST, glutathione-S-transferase.

lines, but M5/CDDPc cells showed approximately 35% repair capacity. The formation of DNA-ISC was assayed after incubation of the three cell lines with 2 μg/ml of CDDP for 1 h. In the M5 and M5/CDDP cells, a similar level of DNA-ISC was observed immediately after treatment. Six hours after cell washing, the level of DNA-ISC was 6-fold higher in the M5 cells compared with the resistant line (Figure 3). However, 24 h later DNA-ISC were almost completely repaired in both cell lines. M5/CDDPc cells did not show formation of DNA-ISC at any time after treatment (data not shown).

In vivo cross-resistance of M5/CDDP and M5/CDDPc cell lines

The effects of CDDP and several different anticancer agents were tested *in vivo* on M5 (Figure 4a,d), M5/CDDP (Figure 4b,e) and M5/CDDPc (Figure 4c,f). CDDP treatments (either 3 or 6 mg/kg) were active in M5-bearing animals and inactive in animals inoculated with M5/CDDP and M5/CDDPc cancer cells. As observed *in vitro*, no cross-resistance was observed when treating the parental and resistant tumours with L-PAM. BCNU, a well-known active nitrosourea in murine tumours, was very active on all of the three tumours tested (Figure 4d–f). Tallimustine treatment at the dose of 2.5 mg/kg i.p. was very active in M5- and M5/CDDPc-bearing mice, but it was too toxic in M5/CDDP-bearing mice (9/10 animals died from toxic effects). Dox was equally effective on the three tumours. The antimetabolite 5-FU was inactive on all of the tumours tested.

DISCUSSION

We have obtained two M5 ovarian reticulosarcoma sublines with a stable low level resistance to CDDP with continuous and pulse treatment schedules. These cell lines were tumorigenic in mice and completely resistant to CDDP *in vivo*.

In most of the described studies on resistance mechanisms to CDDP, high-resistant (>10-fold) cell lines have been obtained after low-dose continual treatment [4]. However, experimental studies, wherein resistance was induced in L1210 and P388 murine leukaemia by serial transplantation and progressive dose escalation of alkylating agents, have established that, after three to five transplant generations, resistance was 'clinically complete', even though the tumours were only 5-fold resistant *in vitro* [15]. It has also been observed that 2.2-fold resistant cell lines could be isolated from a xenograft of human ovarian tumours subjected to 12 doses of CDDP [16]. In clinical practice, 3 of 6 untreated patients with ovarian cancer with low level resistance to CDDP (2- to 4-fold) showed disease progression, whereas none of 11 patients with sensitive tumours progressed [6].

The schedule of administration of an alkylating agent can markedly influence the therapeutic index and duration of remission *in vivo*. The aim of our project was, therefore, to understand whether different dose schedules of CDDP *in vitro* could lead to the production of cell lines with the same low level, but different mechanisms and stability of resist-

Table 2. Resistance and cross-resistance of M5 and resistant cell lines to other antitumour agents

Drugs	IC_{50}(μg/ml)	Relative resistance	
		M5/CDDP	M5/CDDPc
Cisplatin	1.16 ± 0.09	2.7	3.4
Iproplatin	8.2 ± 0.32	2.4	5.2
Carboplatin	26.5 ± 0.01	2.6	2.6
L-PAM	4.0 ± 0.3	0.8	0.9
BCNU	0.78 ± 0.09	1.5	1.7
Tallimustine	0.2 ± 0.04	2.0	2.2
Doxorubicin	0.56 ± 0.03	0.3	0.6
VP16	2.0 ± 0.2	0.5	1.1
Camptothecin	0.31 ± 0.02	5.6	2.2
5-FU	0.92 ± 0.04	0.5	1.2

Resistance was determined by growth inhibition and reported as the ratio of IC_{50}s.
M5, murine ovarian reticulosarcoma M5076; M5/CDDP, M5/CDDPc, cisplatin-resistant cell lines from pulse and continuous treatment, respectively; L-PAM, L-phenylalanine mustard; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; VP16, etoposide; 5-FU, 5-fluorouracil.

Table 3. Repair of total Pt–DNA adducts measured at different times after cell treatment with 10 μg/ml of CDDP for 4 h

Time after treatment (h)	% of total platination		
	M5	M5/CDDP	M5/CDDPc
0	100	100	100
6	108	90	62
24	98	102	67

M5, murine ovarian reticulosarcoma M5076; M5/CDDP, M5/CDDPc, cis-platin-resistant cell lines from pulse and continuous treatment, respectively; CDDP, *cis*-diamminedichloroplatinum (II).
DNA-Pt adducts measured by atomic absorption at 0 h and expressed as ng Pt/mg DNA ± standard deviation ($n = 3$) were respectively: 28.5 ± 0.1 (M5); 29.2 ± 11.9 (M5/CDDP) and 28.2 ± 5.2 (M5/CDDPc).

ance. Continuous drug exposure is considered to be more effective in producing methotrexate resistance than is intermittent treatment [17]. However, conflicting results were obtained in the induction of resistance to CDDP and BCNU in a cell line derived from a human Burkitt lymphoma. Whereas continuous daily exposure was less effective than pulse treatment for CDDP, the opposite was observed with BCNU [18], and these cell lines lost resistance in a 3-month period to the alkylating agent. In our case, pulse treatment was equally effective as continuous exposure in inducing CDDP resistance in M5 cells. Moreover M5/CDDP and M5/CDDPc cells both showed stable resistance over 6 months.

While the same level of cross-resistance was observed among some analogues of CDDP and tallimustine, it was not a general phenomenon because L-PAM and BCNU were not cross-resistant in both lines. This suggests qualitative differences in the mechanism of drug resistance to the platinating or alkylating agent, but not between the two resistant lines. We found no evidence of pleiotropic resistance

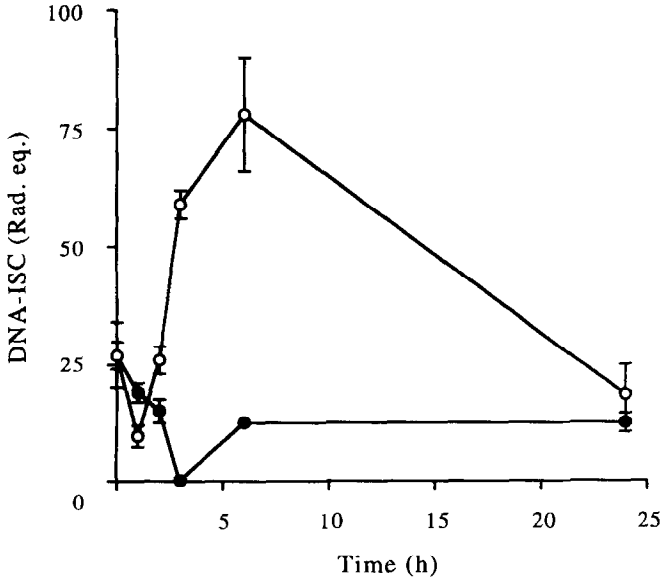

Figure 3. Time course of the formation and repair of DNA interstrand cross-links (DNA-ISC) caused by CDDP in M5 (○) and M5/CDDP (●) cell lines. The cells were incubated for 1 h with 2 μg/ml of CDDP and DNA-ISC were measured at different times after drug withdrawal.

to non-alkylating drugs and, in the case of Dox and VP16, we observed collateral sensitivity. These findings are in agreement with what has been observed for other cell lines resistant to alkylating agents [19]. Higher levels of topoisomerase II have been found in some cells resistant to CDDP compared with parental cells and, as a consequence, collateral sensitivity to inhibitors of this enzyme has been observed [20].

Detoxification mechanisms may have a role in modulating CDDP resistance, but the relevance of GSH levels in mediating resistance is at present controversial [4, 21]. In cell lines in which resistance to CDDP has been induced by acute and chronic treatments, an increase in GSH content has been observed only in the resistant cell lines obtained after chronic treatment, but this increase seems to be related to the much higher resistance (2- to 5-fold) observed in these lines compared with that of the cell lines obtained after acute treatment [4]. The similar or lower content of GSH in the two M5-resistant lines compared with the parental cells and the similar levels of GST activity does exclude a role of GSH or related enzymes in the resistance of these cell lines to CDDP. Moreover, after *in vivo* induction of CDDP resistance in the M5 tumour no elevations in GSH levels were observed [7].

Although it is generally accepted that DNA is the major target for the cytotoxic action of CDDP [22], the Pt–DNA adducts responsible for the antitumour or cytotoxic action of CDDP remain to be identified. The major adducts are DNA-intrastrand cross-links, but the DNA-ISC that represent less than 1% of total platination have more often been implicated as the most toxic lesions [23]. Deficiencies in cellular repair processes in mammalian cells can also contribute to the sensitivity to DNA damaging agents [24], but this does not hold for every cell line [25]. In fact, for CDDP, it has been observed that some resistant cell lines have similar or higher levels of DNA platination and DNA-ISC formation than the parental cells, implying an increased tolerance to DNA damage or even similar rates of repair in both sensitive and resistant cell lines [26, 27]. In our case, a similar level of DNA platination immediately after treatment was observed in the three lines. No repair of Pt–DNA adducts was observed after a 24 h recovery in the M5 and M5/CDDP cell line, but M5/CDDPc cells showed a 33% repair capacity. In our investigation, rather high CDDP incubation concentrations, exceeding the IC_{50} values of the cell lines by about 3-fold, were needed for reliable AAS measurements. Under these circumstances, a lack of a repair capacity was also observed in some germ cell tumour cell lines [28, 29]. The difference between the cell lines in the formation and repair of DNA-ISC was completely different compared with total Pt–DNA adducts. In the time course of DNA-ISC formation, a six times higher level of DNA-ISC was observed 6 h after cell washing in the parental cell line compared with M5/CDDP cells. The high toxicity of these DNA adducts [23], and the possibility of the occurrence of a different formation or repair of DNA-ISC at the level of selected genes [30], could explain the different sensitivity of these cell lines to CDDP.

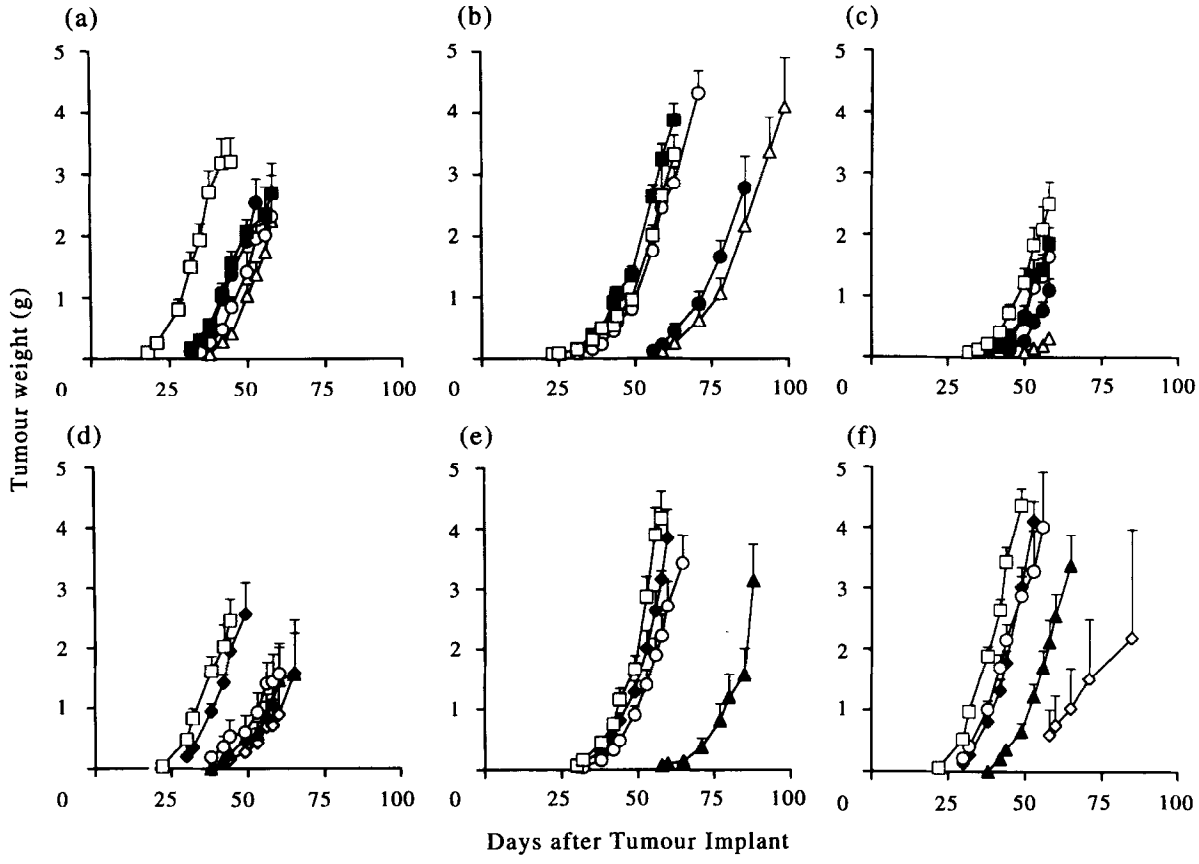

Figure 4. Effect of CDDP, L-PAM, BCNU, tallimustine, Dox and 5-FU on the growth of M5 (a, d), M5/CDDP (b, e) and M5/CDDPc (c, f) tumours in mice. Controls (□); CDDP, 3 mg/kg (days 3, 7, 11, 15) (■); CDDP, 6 mg/kg (days 7, 15) (○); L-PAM, 8 mg/kg (days 3, 7, 11) (●); Dox, 7 mg/kg (days 1, 8, 15) (△); BCNU, 40 mg/kg (day 4) (▲); tallimustine 2.5 mg/kg (days 3, 7, 11) (◇); 5-FU, 98 mg/kg (days 3, 10, 17, 24) (◆). All drugs were administered i.p.

Both resistant cell lines were tumorigenic when injected into mice, but a slightly greater lag in the growth of the resistant tumours was observed compared with the parental tumour. This behaviour has also been observed in a human ovarian cancer, resistant to CDDP, transplanted into nude mice [31]. As observed with the CDDP-resistant M5 tumour obtained after *in vivo* treatment [7], mice inoculated with M5/CDDP and M5/CDDPc cells survived longer (mean survival time 45, 60 and 90 days in M5, M5/CDDPc and M5/CDDP tumour-bearing mice, respectively, data not shown) with a large tumour burden. L-PAM was active *in vitro* and *in vivo* in both resistant tumours, although, in the M5 tumour made resistant *in vivo*, L-PAM was found to be cross-resistant [7]. The sensitivity to L-PAM in cell lines made resistant to different agents has been shown to depend either on the cell line or the agent used to induce the resistance [18]. In this case, the sensitivity to L-PAM also appears to depend on the method used to induce the resistance, *in vitro* or *in vivo*. According to our previous observations [7], BCNU and Dox were active in both resistant tumours (Figure 4). For Dox, this activity is in agreement with the collateral sensitivity observed in cell lines resistant to alkylating agents [18]. Tallimustine was the most active drug *in vitro* in M5 cells, but it was cross-resistant by 2-fold in M5/CDDP and M5/CDDPc cell lines. *In vivo*, however, this drug was very active in the resistant tumour M5/CDDPc. This activity could be due to mechanisms operating only *in vivo*, affecting the formation of active metabolites or effects on the immune system [32]. The inactivity of the antimetabolite 5-FU in the parental and resistant tumours, despite its sensitivity in the resistant cell lines *in vitro*, could be explained by the high thymidine levels observed in mice. Therefore, the inhibition of the enzyme thymidylate synthase [33] produced by 5-FU is less important *in vivo* than *in vitro*. We have shown that the two CDDP-resistant cell lines obtained, despite the low level of CDDP resistance *in vitro*, are completely resistant when they are inoculated into mice and show a pattern of sensitivity to different anticancer agents *in vivo* similar to that observed for the M5 tumour made resistant to CDDP *in vivo* [7]. *In vitro* cultivation of the latter tumour coincided with progressive loss of resistance to CDDP after some passages [8]. Primary cultures of the resistant M5/CDDP and M5/CDDPc tumours would allow the study of whether the loss of resistance, observed *in vitro* for the CDDP-resistant tumour previously obtained, is due to a progressive selection of sensitive cells or to resistance mechanisms operating only *in vivo*, because we know that these resistant cell lines showed stable resistance after several months of culture. The mechanisms of resistance of the

M5/CDDP anbd M5/CDDPc cell lines seem to be based on a lower formation of DNA-ISC, combined, for the latter cell line, with a higher repair capacity for total DNA platination.

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