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Effects of Prolonged Exposure to Cisplatin on Cytotoxicity and Intracellular Drug Concentration

V. Troger, J.L. Fischel, P. Formento J. Gioanni and G. Milano

The present study was designed to analyse the cytotoxic effect of cisplatin *in vitro* as a function of various exposure times (up to 120 h), keeping constant the parameter $C \times T$ (product of the drug concentration per time). Intracellular drug concentrations were measured in parallel following analysis of cisplatin influx and efflux characteristics. A head and neck cancer cell line was selected to represent the spectrum of cisplatin antitumour activity. The IC_{50} values (μg/ml) for 1, 2, 11 and 121 h were, respectively 4.51, 2.73, 0.27 and 0.151. Reduction of the IC_{50} was clearly not linearly related to prolongation of the cisplatin exposure time. The kinetics of cisplatin incorporation into CAL 27 cells was investigated as a function of different cisplatin concentrations. A plateau was reached after 16 h of contact. For the extracellular cisplatin concentrations of 1, 2.5, 5 and 10 μg/ml, the average intracellular Pt concentrations at the plateau were, respectively (ng/10^6 cells): [mean (S.D.)] 12.8 (0.98), 31.11 (5.12), 71.38 (6.03) and 136.7 (16.5). Intracellular Pt concentrations were linearly related to the extracellular drug concentration ($r = 0.99$). The drug left the cells following a two-slope kinetics pattern with an α half-life of 1.29 h and a β half-life of 94.4 h. The cytotoxic effect for a given $C \times T$ clearly differed for the different cisplatin exposure times. The longest exposure time (121 h) gave the least pronounced cytotoxicity. The intracellular Pt concentrations were linearly related to the $C \times T$ values. Cisplatin levels were much lower after the 121 h exposure. These data may prove valuable in establishing a rationale which can aid in selection of optimal modes of clinical cisplatin administration.
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INTRODUCTION

CISPLATIN IS one of the most active drugs for the treatment of cancer, and particularly testicular, ovarian, head and neck, and bladder carcinomas [1]. However, use of this drug is often accompanied by numerous and frequently severe toxicities, such as nausea and vomiting, nephrotoxicity, neurotoxicity, and ototoxicity [2]. Attempts to avoid such adverse effects include use of various analogues [3], association with neutralising agents [4], and, recently, the development of efficient antiemetics [5]. Another approach is based on modification of the duration of cisplatin infusion. Alternatives to short administration durations, such as continuous 5-day infusions, dramatically reduce acute nausea and vomiting without loss of efficacy [6]. A pharmacological rationale serving as a guideline for selection of optimal cisplatin administration schedules would be helpful. Such information can be obtained from both pharmacokinetic explorations and experimental studies. The pharmacokinetic investigations conducted so far on various cisplatin infusion durations represent a good basis of knowledge [7–13]. By

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contrast, few experimental studies have compared different cisplatin exposure durations; the cisplatin exposure times in these studies are limited to 24 h for *in vitro* experiments [14, 15] and 72 h for an investigation in laboratory animals [16].

The present study was designed to analyse the cytotoxic effect of cisplatin *in vitro* as a function of various exposure times (up to 120 h), keeping constant the parameter $C \times T$ (product of the drug concentration per time). Intracelluar drug concentrations were measured in parallel following analysis of cisplatin influx and efflux characteristics. A head and neck cancer cell line was selected to represent the spectrum of cisplatin antitumour activity.

MATERIALS AND METHODS

Drugs and chemicals

Cisplatin (powder, ref L7-2585) was obtained from R. Bellon Laboratories (Neuilly/Seine, France). The stock solution in 0.9% NaCl was stored at + 4°C and working solutions were prepared before use by dilutions in the culture medium. Dulbecco's modified Eagles medium (DMEM) medium, L-glutamine and fetal bovine serum (FBS) were from Gibco (Paisley, UK). Penicillin and streptomycin were from Merieux (Lyons, France). The MTT test was performed with 3-(4-5 dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO), both from the Sigma Chemical Co. (St Louis, Missouri).

Cell cultures

The human tumour cell line (CAL 27) used was isolated at our institute from a patient with a squamous cell carcinoma of the head and neck [17]. Cells were routinely cultured in a humidified incubator (Sanyo) at 37°C with an atmosphere of 8% CO_2 in air. Cells were grown in DMEM medium supplemented with 10% FBS, penicillin (50.000 IU/l), streptomycin (86 μmol/l) and L-glutamine (2 mM). Cells were grown in 96-well microtitration plates for experiments concerning cellular toxicity; cells were grown in Falcon cell culture flasks (175 cm^2 or 75 cm^2) for experiments concerning cellular platinum (Pt) concentrations.

Kinetics of cellular cisplatin incorporation

The initial cisplatin concentrations were 1, 2.5, 5 and 10 μg/ml, with a cell density of 7×10^4 cells per ml. Cells were collected at set times after the start of cisplatin exposure (TO): 15 and 30 min, and 1, 2, 4, 8, 16, 32 and 64 h. Cells were washed three times with phosphate buffered saline (PBS), trypsinised, and centrifuged at room temperature for 5 min at 1000 rpm. The supernatant was discarded, and the cellular pellet was recovered with 3–5 ml of culture medium. The cells were counted, then recentrifuged; the supernatant was discarded, and the final pellet was used for Pt measurement. Experiments were performed in triplicate.

Kinetics of cellular cisplatin efflux

Cells were incubated for 36 h at a set cisplatin concentration (2.5 μg/ml, 7×10^4 cells per ml). Cisplatin efflux was measured at 0, 15, and 30 min, and 1, 2, 4, 8, 16, 32, and 64 h. All incubation plates were run in parallel. At the end of the 36 h incubation period, the culture medium was removed. Cells were washed with 1.5 ml PBS, and 15 ml of culture medium was added to all plates except the "time 0" plates where cells were directly trypsinised after the washing step. For the other time periods, cells were maintained in culture medium during the corresponding durations; after the corresponding time had elapsed, the medium was removed, and was followed by three washes with PBS at 37°C; the cells were then trypsinised. The next steps were identical to those described in the section on cellular cisplatin incorporation. Experiments were performed in triplicate.

Cytotoxicity as a function of $C \times T$

Four different $C \times T$ values were selected (μg/ml.min): 240, 360, 600 and 1200. Three cisplatin exposure durations were studied: 1, 11 and 121 h; the corresponding cisplatin concentrations ranged between 0.033 and 20 μg/ml. The initial cell density was 3500 cells/well (96-well plates). All plates were started together. After the preset incubation times, cells were washed with fresh medium; MTT was added 121 h after the start of cisplatin exposure. The cellular Pt concentration was determined from cells incubated in parallel in Falcon cell culture flasks (initial cell concentration 7×10^4 cells/ml). Cells were recovered after the corresponding times (1, 11 and 121 h) and were treated for cellular cisplatin measurement as described previously.

Dose response curves

The initial cell concentration was 2500 cells/well (96-well plates). Three drug exposure durations were investigated: 1, 11 and 121 h. The CDDP concentrations ranged between 10^{-2} and 10^2 μg/ml. All plates were started together; cells were washed after the preset incubation periods and MTT was added to all plates 121 h after the start of drug exposure.

Evaluation of toxicity

The cytotoxic effects of cisplatin were assessed by the MTT semi-automated test [18] in the 96-well incubating plates. Results were expressed as the relative percentage of absorbance compared with the controls without drugs. Absorbance was set at 540 nm and measured on a Titertek twin reader. Each experimental point was evaluated in sextuplicate. For all experiments, the coefficients of variation ranged between 3 and 10%. The IC_{50} was defined as the cisplatin concentration inhibiting 50% of the cell growth compared to controls without the drug.

Measurement of intracellular Pt

Cells were treated by 1 ml of 50% HNO_3 for 8 h. Treated cells were homogenised in a glass–glass potter which was rinsed twice with 250 μl of 10% HNO_3; rinsing solutions were collected with the homogenate and dried under a nitrogen stream (50–60°C). The drug residue was reconstituted with 200–250 μl H_2O, and 20 μl of this solution was used for Pt measurement by flameless atomic absorption spectrometry (Perkin Elmer 3030 Zeeman). The limit of sensitivity was 0.3 ng Pt/10^6 cells.

RESULTS

Figure 1 shows the dose response curves of CAL 27 cell for the various cisplatin exposure times. The IC_{50} values (μg/ml) for 1, 2, 11 and 121 h were, respectively 4.51, 2.73, 0.27 and 0.151. Reduction of the IC_{50} was clearly not linearly related to prolongation of the cisplatin exposure time. For example, much more drug was required to achieve the same cytotoxic effect after 121 h as after 11 h than could have been predicted merely by the prolongation of the cisplatin contact period from 11 to 121 h. For the prolonged contact time of 121 h, a parallel experiment was performed where cisplatin was renewed at 24,

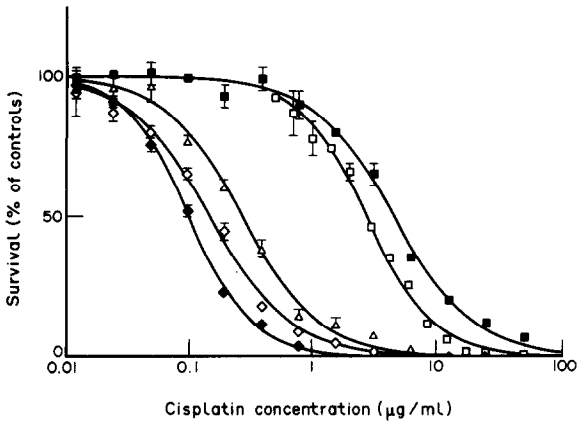

Fig. 1. Dose response curves of CAL 27 cells to cisplatin for different drug exposure durations. ■ = 1 h; □: 2 h; △ = 11 h; ◇ = 121 h; ◆ = 121 h with drug renewal at 24, 48 and 72 h.

48 and 72 h; there was only a limited benefit in the cisplatin IC_{50} changing from 0.15 to 0.10 μg/ml.

Figure 2 illustrates the kinetics of cisplatin incorporation into CAL 27 cells as a function of different cisplatin concentrations. A plateau was reached after 16 h of contact. For the extracellular cisplatin concentrations of 1, 2.5, 5 and 10 μg/ml, the average intracellular Pt concentrations at the plateau were respectively (ng/10^6 cells): [mean (S.D.)] 12.8 (0.98), 31.11 (5.12), 71.38 (6.03) and 136.7 (16.5). Total cellular Pt accumulation was linearly related to the extracellular drug concentration (r = 0.99).

Figure 3 illustrates the kinetics of cisplatin efflux after 36 h of incubation with an extracellular cisplatin concentration of 2.5 μg/ml. The drug leaves the cells following a two-slope kinetics pattern with an α half-life of 1.29 h and a β half-life of 94.4 h.

Figure 4 shows the evolution of cell survival as a function of the intensity of drug exposure ($C \times T$). The cytotoxic effect for a given $C \times T$ clearly differed for the different cisplatin exposure times. The longest exposure time (121 h) gave the least pronounced cytotoxicity; inversely, the greatest effects were observed for 11 h contact period; cell survival after the 1 h exposure period was similar to that after 11 h.

The intracellular Pt concentrations obtained for the various $C \times T$ exposures are given in Fig. 5. The intracellular Pt

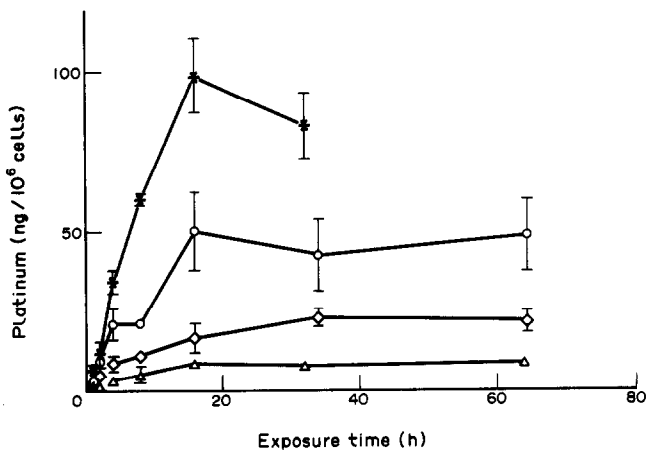

Fig. 2. Kinetics of cellular Pt accumulation in CAL 27 cells. △ = 1 μg/ml; ◇ = 2.5 μg/ml; ○ = 5 μg/ml; * = 10 μg/ml.

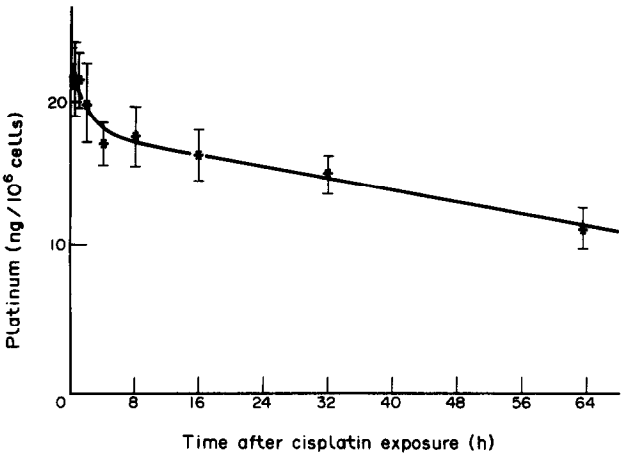

Fig. 3. Kinetics of cellular Pt efflux in CAL 27 cells. Time–concentration points were best fitted by a two exponential model (r = 0.9890, df = 8, $P < 0.001$) with an α half-life of 1.29 h and a β half-life of 94.4 h.

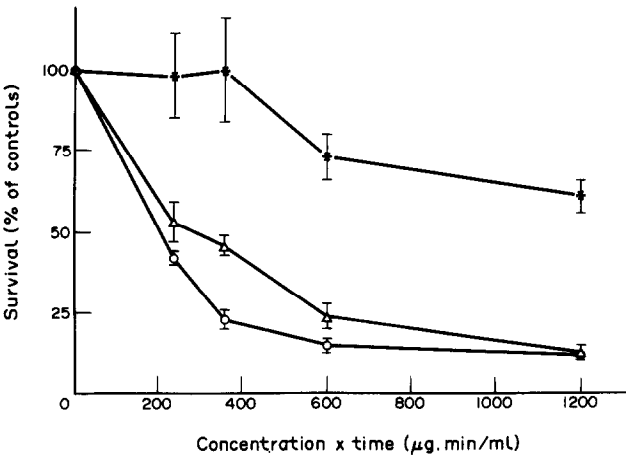

Fig. 4. Cell survival as a function of the $C \times T$ values for different cisplatin exposure durations. △ = 1 h; ○ = 11 h; * = 121 h.

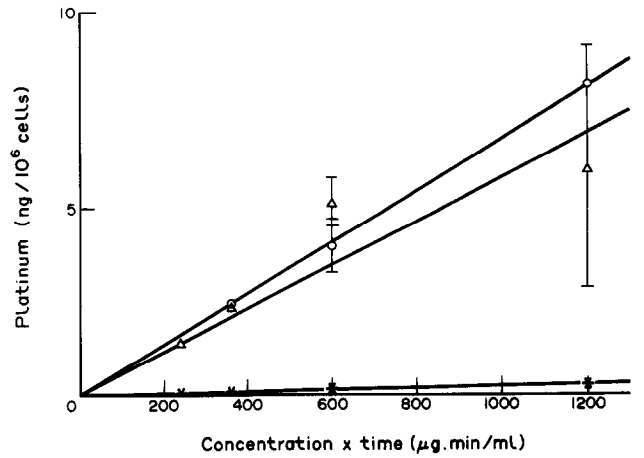

Fig. 5. Evolution of the Pt concentration as a function of the $C \times T$ for different cisplatin exposure durations. △: 1 h; ○ = 11 h; * = 121 h. The x–y points were best fitted by linear regressions: for 1 h, r = 0.9085; for 11 h, r: 0.9998; for 121 h, r = 0.9847.

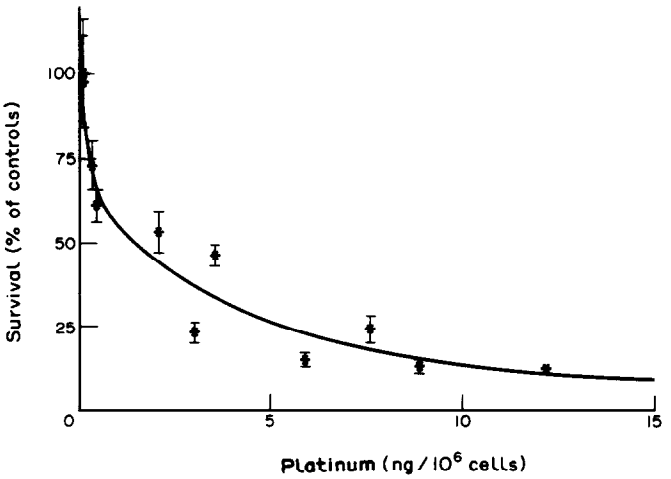

Fig. 6. Evolution of cell survival as a function of the intracellular Pt concentration. *x–y* points were best fitted by a two-exponential model ($r^2 = 0.943$, df = 6).

concentrations were linearly related to the $C \times T$ values. Comparable intracellular drug concentrations were observed for cisplatin exposure periods of 1 and 11 h; by contrast, cisplatin levels were much lower after the 121 h exposure.

The relationship between cell survival and the intracellular Pt concentration, all experimental conditions taken together, is shown in Fig. 6. The curve is well fitted by a two-exponential decay, first a rapid decay then a slower decay. Cell death was thus not linearly proportional to the intracellular Pt level; the most pronounced increase in cytotoxicity was observed with the first increase in the intracellular Pt concentration (0–5 ng/ml).

DISCUSSION

The present study was designed to analyse the influence of the cisplatin exposure time on cytotoxicity and intracellular drug concentrations *in vitro*. Experiments were performed on a cell line derived from a human squamous cell carcinoma [17], a model representative of the spectrum of cisplatin antitumour activity [1]. As a preliminary and necessary step, the cellular kinetics of both drug influx and efflux were characterised. Like other investigators [19–21], we found that cellular Pt accumulation is a linear function of the extracellular drug concentration. In addition, intracellular Pt concentrations were shown to reach a plateau after approximately 16 h of drug exposure. This interesting kinetic characteristic was not revealed in previous studies using shorter incubation durations [19–21]. Intracellular Pt uptake increased linearly as a function of the extracellular drug concentration (Fig. 2); this finding and the notion of a plateau both suggest that a certain amount of drug exchange can occur between the inside and the outside of the cell. This was confirmed by cellular drug release experiments; like others [21], we identified a biphasic drug efflux pattern. The percentage of exchangeable cisplatin is not negligible, because the maximum amount of drug released was 49%. This percentage concurs with the report by Hecquet *et al.* [19], who incubated tumour fragments *in vitro*, and found an average 36% of released drug.

The range of $C \times T$ values was selected so as to be clinically relevant [8–11, 22]. The mechanism of action of cisplatin is mainly related to its cross-linking with DNA; it is thus similar to the action of alkylating agents. The cytotoxic action of cell cycle phase-non-specific agents, such as alkylating agents, is dependent on the concentration–time product [23]. Rupniak *et al.* [24] reported that the cytotoxicity of cisplatin *in vitro* increases when the drug concentration is increased or when the exposure time is extended. In our study, cytotoxicity was enhanced when the concentration–time product was increased. However, the most important finding was the fact that effects were not identical for a given $C \times T$: the longest exposure time (121 h) generated the least cell kill. In another study (results not shown) with a 36 h exposure period using, respectively 1, 2.5 and 5 μg/ml cisplatin, with renewal of the medium containing the drug every 12 hours, to more closely simulate continuous drug infusion, the final cytotoxic effect was not significantly different from experiments without renewal of the medium. Intracellular drug concentrations were not identical for a given $C \times T$; the lowest intracellular Pt levels were obtained with the longest exposure time. As a corollary, cell survival was found related to the intracellular Pt content; this concurs with similar reports concerning hamster cells [25]. The only difference was the bi-exponential relationship we found between cell survival and cellular Pt content compared to the mono-exponential relationship reported by other investigators. Studies on the murine L1210 leukaemia model failed to identify a marked advantage for either bolus or infusion therapy with cisplatin. However, with continuous infusion, total cisplatin doses 2-fold higher were required to achieve equivalent responses [16]. Our findings corroborate this observation because, when the total drug exposure ($C \times T$) was kept constant, prolonging the contact with low cisplatin concentrations reduced drug efficacy. This new pharmacological data concerning cisplatin may prove valuable in establishing a rationale which can aid in selection of optimal modes of clinical cisplatin administration.

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POMB/ACE Chemotherapy in Non-seminomatous Germ Cell Tumours: Outcome and Importance of Dose Intensity

David J. Husband and John A. Green

This study reports the outcome of POMB/ACE (cisplatin, vincristine, methotrexate, bleomycin, actinomycin D, cyclophosphamide, etoposide) chemotherapy in 53 male patients with metastatic non-seminomatous germ cell tumour (NSGCT) treated between 1983 and 1989 in one centre. The overall complete response (CR) rate was 62% [95% confidence interval (CI) 49–75%), and for patients with large or very large volume disease (L/VL, MRC criteria), the CR rate was 56% (95% CI 41–71%). The overall 5 year survival was 61%, and for L/VL volume disease 67%. Comparison with previous studies suggests that POMB/ACE chemotherapy is not superior to BEP, even in patients with adverse prognostic factors. Increased average relative dose intensity and increased relative dose intensity of cisplatin over the first seven courses were not associated with improved survival. However, in patients receiving a relative dose intensity of etoposide ≥ 0.75, survival at 5 years was significantly improved compared with those in whom this parameter was < 0.75 (79% vs. 44%, $P < 0.05$), suggesting that dose intensity of etoposide may be an important determinant of outcome in the chemotherapy of metastatic NSGCT.
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INTRODUCTION

IT IS now well recognised that a majority of patients with non-seminomatous germ cell tumours (NSGCT) who have advanced metastatic disease at diagnosis will survive if treated with cisplatin-containing combination chemotherapy. After the demonstration by Einhorn and Donohue that the combination of cisplatin, vinblastine and bleomycin (PVB) was associated with an 85% complete response (CR) rate, and a 64% long term survival rate [1], subsequent developments have been along two main lines. At the Royal Marsden Hospital, vinblastine was replaced by etoposide, resulting in equivalent survival but decreased toxicity [2, 3], and the BEP regimen has become the standard chemotherapy for metastatic NSGCT [4]. At other centres more complex protocols were developed incorporating