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The relative effectiveness of analogues of cisplatin in the experimental chemotherapy of human non-small-cell lung cancer and neuroblastoma grown as multicellular spheroids

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Summary. We compared cisplatin (*cis*-DDP) and two of its analogues, carboplatin (JM8, CBDCA) and iproplatin (JM9, CHIP) for their ability to retard the growth of multicellular tumour spheroids. The spheroids were derived from two human tumours, a neuroblastoma and a non-small-cell lung cancer. To produce a given level of regrowth delay in lung cancer spheroids, carboplatin and iproplatin were required at concentrations approximately 10 times that of *cis*-DDP. In the neuroblastoma spheroid experiments, iproplatin and *cis*-DDP produced the same level of regrowth delay when iproplatin was present at a concentration > 10 times that of *cis*-DDP. Carboplatin also required much higher concentrations than *cis*-DDP to produce equivalent regrowth delay in neuroblastoma. The dose-response curve produced by carboplatin on neuroblastoma spheroids displayed a pronounced shoulder in the low-dose region; this phenonemon was not seen with *cis*-DDP. These findings may have implications for the clinical use of these drugs and in particular would support a role for carboplatin in the treatment of lung cancer, since total free-drug exposure of patients to carboplatin may be up to 16-fold greater than with *cis*-DDP. However, one must be cautious about generalising on the basis of results from only two cell lines as well as applying in vitro data to clinical situations.

Introduction

cis-Diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) has become an agent of major importance in cancer chemotherapy. However, its usefulness is limited by severe side effects, principally renal toxicity, nausea and vomiting and ototoxicity. For this reason, several analogues of *cis*-DDP have been developed in the hope that they would retain the antitumour activity of the parent compound while showing reduced toxicity to normal tissues. Two analogues that have shown particular promise are carboplatin (CBDCA or JM8) and iproplatin (CHIP or JM9). Clinical trials have shown carboplatin to be active in the treatment of ovarian and small-cell lung cancer [7, 14], and it has recently been suggested for use in neuroblastoma [12]. Iproplatin has also shown activity against ovarian cancer [1, 13].

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We compared the relative effectiveness of *cis*-DDP, JM8 and JM9 in the experimental chemotherapy of two human tumour cell lines (derived from neuroblastoma and non-small-cell lung cancer) and grown as multicellular tumour spheroids. In many respects, spheroids provide a more realistic in vitro tumour model than do monolayer cultures [16]. Spheroids resemble micrometastases during the avascular phase of their growth and display forms of cellular resistance ("contact resistance") to drug and radiation treatment [6, 15] that are not seen in monolayer cultures. Most importantly for chemotherapy studies, the three-dimensional structure of spheroids may present diffusion barriers to some drugs. Limited drug penetration into spheroids has been demonstrated for several agents [9, 18, 20]. The development of an increasing proportion of non-cycling cells during spheroid growth is another feature that is common to many solid tumours [16]. Spheroids may therefore provide an appropriate in vitro model for experimental chemotherapeutic studies on the relative efficacies of different agents.

Materials and methods

Cell lines. Two cell lines were used: a neuroblastoma, NB1-G, whose properties have been described elsewhere [3], and a non-small-cell lung cancer, L Dan [9]. Both cell lines were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 15% foetal calf serum, L-glutamine (2 m*M*), penicillin/streptomycin (100 IU/ml) and amphotericin B (2.5 μg/ml). The cell lines were grown in an atmosphere of 5% CO_2 at 37°C; both were shown to be free from mycoplasma contamination by the method of Chen [4]. Both cell lines had been multiply passaged independently of each other. All medium ingredients were supplied by Gibco (Paisley, Scotland).

Spheroids. NB1 spheroids were formed by growing cells in 25-cm^2 flasks that had been base-coated with 1% agar (Difco, Detroit). Cells were added at a concentration of 10^5/ml in 5 ml medium. After 4–5 days of growth, spheroids were used for experiments. L Dan spheroids were produced by adding 10^6 cells to a spinner flask (Techne, Cambridge) containing 50 ml medium. After 10 days of growth the spheroids were taken for experiments. The NB1 spheroids used in these experiments were typically 250 μm in diameter, and the L Dan spheroids had a diameter of approximately 150 μm. Radiation studies on NB1 [19] and L Dan

spheroids (unpublished data), suggest that at this size spheroids contain no significant hypoxic population.

Drugs. All drugs were obtained from Bristol-Myers, Syracuse, New York. JM8 and JM9 were obtained as powders and were stored in dark bottles at 4°C. Prior to their use they were dissolved in 5% dextrose to give a stock solution of 1 mg drug/ml. *cis*-DDP was obtained as a 1 mg/ml solution and was stored at room temperature in a dark environment. All drugs were diluted to the desired concentrations in MEM. Spheroids were exposed to drugs for 1 h in bacteriological Petri dishes at 37°C; subsequently they were transferred to universals and the drug was rinsed off.

Spheroid growth. After treatment, spheroids were placed singly in 0.5 ml medium in multiwell plates base-coated with 1% agar. There were 24 spheroids per experimental group. Spheroids were fed once a week by adding 0.5 ml medium to each well. Their growth rate was followed by measuring their cross-sectional area on an image analysis system linked by a video camera to an inverted microscope. Measurements were made two or three times per week. Assuming spherical geometry, the spheroid areas were converted to volume.

Regrowth delay was obtained by measuring the time taken by each spheroid in an experimental group to reach 10 times its original volume; the median regrowth delay was then calculated. We calculated the results by subtracting the regrowth delay of the controls from that of the treated spheroids. In some cases treated spheroids failed to regrow. For all experimental groups, these "cured" spheroids were included in the data analysis, provided that the proportion cured did not exceed 50% (i.e. to allow the definition of a finite median).

Results

Figures 1 and 2 show typical regrowth patterns of L Dan and NB1 spheroids. In preliminary experiments (unpublished data) we determined that our procedure of feeding spheroids every 7 days produced maximal growth for both cell lines. The regrowth delay observed in treated spheroids could only be ascribed to the effect of the drug and did not contain any artefact due to inadequate medium conditions. We expressed drug concentration in μg Pt/ml (as opposed to μg drug/ml) to make our units consistent with the pharmacokinetic data presented later.

Both cell lines were more sensitive to *cis*-DDP than to either of its analogues. The L Dan line required a concentration of carboplatin or iproplatin approximately 10 times that of *cis*-DDP to produce an equivalent growth delay (Fig. 3). NB1 was more sensitive than L Dan to *cis*-DDP. Its response to the three drugs (Fig. 4) shows that iproplatin and *cis*-DDP produce the same degree of regrowth delay when iproplatin is present at a concentration more than 10 times that of *cis*-DDP. Carboplatin differs from both drugs in displaying an appreciably "shouldered" response curve, i.e. the initial slope on the growth delay curve is shallow compared with the final slope.

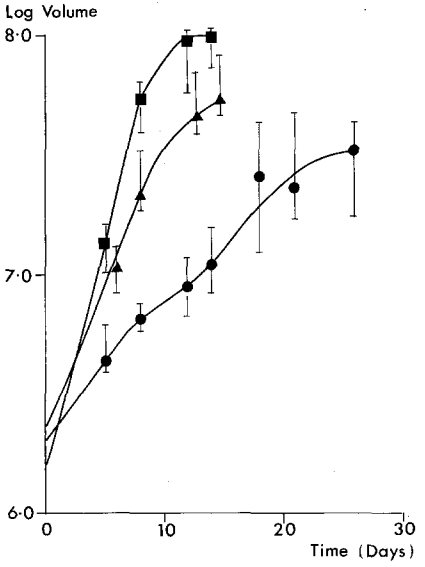

Fig. 1. Regrowth of L Dan spheroids after exposure to *cis*-DDP: ■, control; ▲, 2 μg Pt/ml; ●, 8 μg Pt/ml. Error bars represent 95% confidence limits

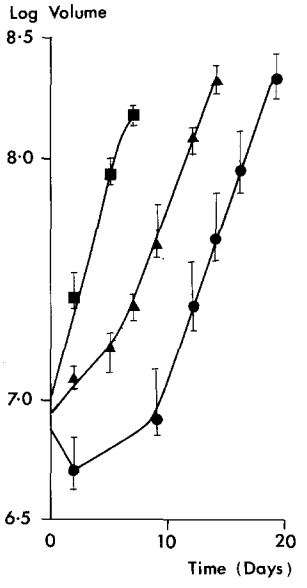

Fig. 2. Regrowth of NB1 spheroids after exposure to *cis*-DDP: ■, control; ▲, 2 μg Pt/ml; ●, 4 μg Pt/ml

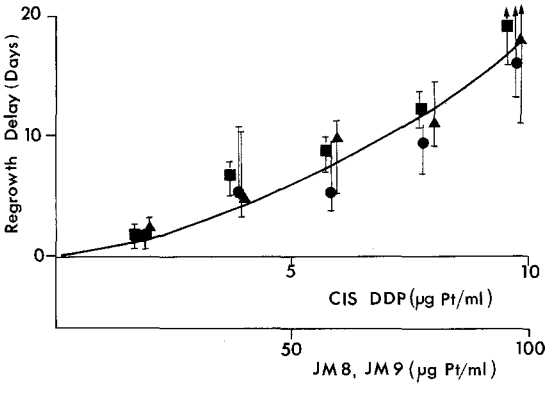

Fig. 3. Regrowth delay of L Dan spheroids after treatment with: ▲, *cis*-DDP: ●, JM8; ■, JM9. Error bars represent 95% confidence limits. *Arrows* indicate that an upper confidence limit could not be determined due to the number of cured spheroids present

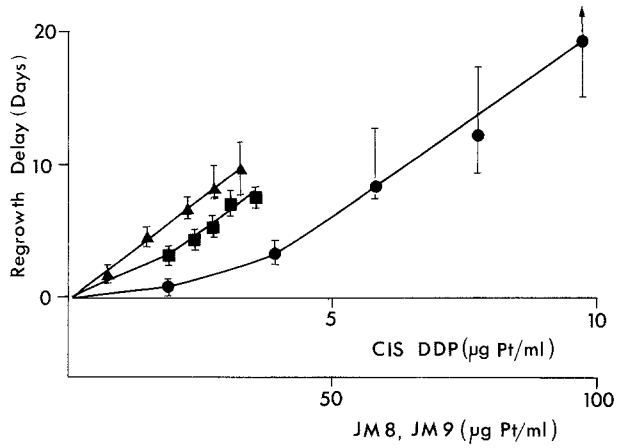

Fig. 4. Regrowth delay of NB1 spheroids after treatment with: ▲, *cis*-DDP; ●, JM8; ■, JM9

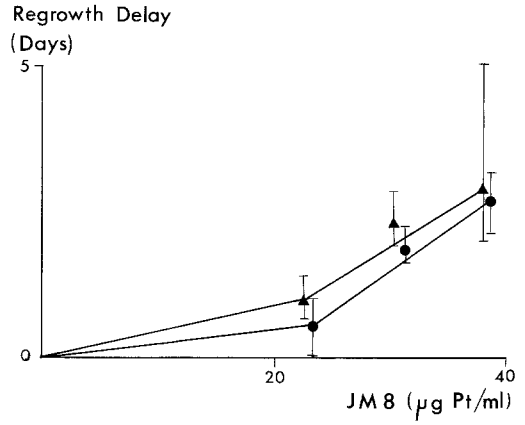

Fig. 5. Regrowth delay of NB1 spheroids of different diameters after treatment with JM8: ▲, diameter 260 μm; ●, diameter 180 μm

To examine whether the response of NB1 spheroids to carboplatin depend on spheroid size, the growth delay for different-sized spheroids was examined (Fig. 5). The smaller spheroids had a diameter of approximately 180 μm compared with larger spheroids of 260 μm diameter. The change in spheroid size did not affect the shoulder of the dose-response curve.

In certain conditions it is possible to estimate the clonogenic survival of cells in spheroids from regrowth delay data. To do this one must assume that the only significant component of regrowth delay in treated spheroids is due to the loss of clonogenic cells. This assumption can only be justified if the regrowth slopes of spheroids recovering from treatment are parallel or nearly parallel to those of the controls. Full details of this calculation are set out elsewhere [19]. As can be seen from Figs. 1 and 2, only the NB1 spheroids satisfy the requirement of parallel regrowth slopes. Figure 6 shows the relationship of clonogenic cell survival to drug concentration that is obtained by this method.

Discussion

The results obtained from the L Dan line suggest that at equivalent concentrations carboplatin and iproplatin are both about 10 times less toxic than cisplatin to tumour

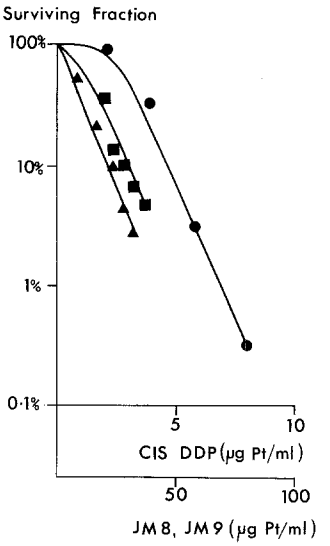

Fig. 6. Cell survival derived from regrowth plots of NB1 spheroids after treatment with: ▲, *cis*-DDP; ●, JM8; ■, JM9

cells in spheroids. Predictions of the relative therapeutic effectiveness of these drugs can only be made if information on their relative toxicity for normal tissue in clinically tolerable doses is taken into account. The recommended maximal single-agent doses for *cis*-DDP and carboplatin are 100 and 400 mg/m^2, respectively [2]. For iproplatin the value has been quoted as 300 and 350 mg/m^2 [5, 7]. Together with pharmacokinetic differences between the drug, e.g. rates of clearance and protein binding, these doses result in the following AUC (area under the concentration-time curve) values for the three drugs: *cis*-DDP, 5 μg Pt h/ml; carboplatin, 80 μg Pt h/ml; iproplatin, 30 μg Pt h/ml [2, 11, 17]. These values are for free platinum. The iproplatin data were obtained from patients treated at a dose of 350 mg/m^2. Carboplatin has an AUC 16 times that of *cis*-DDP, whereas the AUC of iproplatin was 6 times that of *cis*-DDP.

Our results with the L Dan line showed that carboplatin and *cis*-DDP were iso-effective only when carboplatin was present at concentrations 10 times that of *cis*-DDP. However, the pharmacokinetic data already quoted suggest that patients may receive exposures of carboplatin (in terms of concentration x time) 16 times that of *cis*-DDP. Taken together, these facts suggest that carboplatin might be a promising replacement for *cis*-DDP in non-small-cell lung cancer. To be an equally effective antitumour agent iproplatin would need to possess greater toxicity than carboplatin. We did not observe this in our experiments with the L Dan line.

The regrowth of L Dan spheroids (Fig. 1) after treatment failed to recover to a rate comparable to that of the controls, which might compromise the value of regrowth delay data. However, we had previously determined that this slowed regrowth was not due to inadequate medium conditions in the experiment but could be ascribed to the effects of treatment. Other factors beyond simple cell killing may contribute to the regrowth pattern: for instance the presence of doomed cells with a limited proliferative capacity.

It is more difficult to interpret the NB1-G data because of the different shapes of the survival curves for the three

drugs. However, one can compare the levels of cell survival produced by drug exposures equivalent to the reported AUCs for each of the three drugs. At this level, both cisplatin and carboplatin reduced cell survival to < 1%. Iproplatin was less effective, bringing survival down to only 10%. However, neuroblastoma is a childhood cancer and pharmacokinetic data obtained from adults may not provide a reliable guide to drug behaviour in children.

The most interesting feature of the NB1 results is the shoulder that appears on the carboplatin dose-response curve. There are three obvious explanations as to why the carboplatin and cisplatin dose-response curves might differ with regard to the shoulder region. The shoulder may reflect some difference in the mechanism of cell killing at a biochemical level. However, both drugs have been shown to inflict identical damage in terms of both quantity and type on DNA [10].

The shoulder may be an artefact produced by a resting (G_0) population of cells in the spheroid. At low levels of cell kill no growth delay would be seen, as spheroids could maintain the number of actively cycling cells by recruiting cells from the G_0 population to replace dead cells. However, there is no reason why this should be seen only in spheroids treated with carboplatin.

There may be some difference in the ability of the drugs to penetrate into the spheroids. Using spheroids of different sizes, we failed to observe any difference in the response of NB1 to carboplatin; however, the difference in spheroid diameters was not great (180 μm vs 260 μm). Further experiments are warranted, using spheroids of widely differing size and monolayer cultures and, if possible, measuring drug penetration directly.

Other investigators [8, 12] have found that carboplatin displays sufficient cytotoxicity at tolerable drug concentrations to be a promising replacement for cisplatin. Our results with the L Dan line support this conclusion. The results from the NB1-G line cannot be interpreted with such clarity; however, they suggest that carboplatin may have significant activity at clinically achievable doses, especially if even higher concentrations could be used in neuroblastoma treatment in conjunction with autologous marrow rescue [12]. Further studies on both the sensitivity of cultured neuroblastoma cells to carboplatin and relevant pharmacokinetics in children are clearly merited. The results we obtained using iproplatin showed less promise. However, it would be dangerous to generalise on the basis of two cell lines; as with carboplatin, further studies on iproplatin seem worthwile.

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