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Cell survival in four ovarian carcinoma xenografts following in vitro exposure to melphalan, cisplatin and *cis*-diammine-1,1-cyclobutane dicarboxylate platinum II (CBDCA, JM8)

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Summary. *Four human ovarian carcinoma xenografts were established and maintained in immune-suppressed mice. Cells obtained from these xenografts were exposed in vitro to melphalan, JM8, and cisplatin; cell survival following a 1-h exposure was measured using a soft-agar colony assay.*

A similar dose-response curve was obtained with melphalan for each of the four xenografts, despite previous treatment with an alkylating agent in two of the patients from whom the xenografts originated. Cell survival was also compared after JM8 and cisplatin exposure in each individual xenograft. It was found to be similar for each tumour when the concentrations of JM8 used were 10-fold greater than those of cisplatin. Early clinical studies in which JM8 has been shown to be effective in the treatment of ovarian carcinoma support the view that xenograft tumours may have a role in phase-II screening of new cytotoxic agents.

Introduction

The majority of patients who present with ovarian carcinoma have advanced disease. Treatment for these patients, when possible, consists of maximal surgical removal of tumour followed by cytotoxic drug therapy. Alkylating agents, in particular, have been used for many years to treat this disease and continue to be widely employed both as single agents and in drug combinations [18].

Melphalan has been given in high doses with autologous marrow rescue in a number of tumours, with encouraging results [5, 6]. Since ovarian carcinoma is recognized as being sensitive to melphalan, and does not significantly metastasize to bone marrow [2], we have considered using the drug in high doses in patients with very advanced disease. Prior to embarking on a pilot study in these patients, we obtained dose-response data in vitro for four established ovarian xenografts of human origin.

Cisplatin is now known to be an active drug in the treatment of ovarian carcinoma, both as a single agent and in drug combinations [3, 16]. The severe side-effects and the progressive nephrotoxicity and neurotoxicity associated with its administration have prompted a search for less toxic analogues. JM8, one such analogue, was found to have significant antitumour activity in experimental test systems, while producing much less nephrotoxicity than cisplatin [9]. This led to its introduction into the clinic at our Institute. In the second part of this report we present the results of a comparative study of cisplatin and JM8. These experiments were undertaken to identify whether JM8 was cytotoxic to human ovarian carcinoma cells at drug concentrations equivalent to those of cisplatin known to be clinically cytotoxic.

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Materials and methods

Xenograft material. Four ovarian xenografts were established from human tumour material and maintained by serial passage in immune-suppressed mice as described previously [13]. In Table 1 the FIGO clinical stage of patients at presentation, the drug treatment, and the histology of the tumour prior to the establishment of the xenograft are shown. Also included in Table 1 is the range of tumour passages used in the experiments. Each tumour has been confirmed to contain human chromosomes.

Tumours measuring approximately 1 cm in diameter were excised aseptically from the mouse following cervical dislocation. The excised tumours were washed twice in PBS at 4° C, and representative pieces were taken for histology. The remainder of the tumour was used to prepare a single-cell suspension.

Tumour disaggregation. The washed tumour pieces were finely chopped and then incubated in filter-sterilized collagenase (Sigma type II) at a concentration of 2 mg/ml in full medium (Ham's F12 (Gibco) + 15% special Bobby calf serum (SBCS) (Gibco) + penicillin 50 U/ml, streptomycin 50 μg/ml, and neomycin 10 μg/ml) for 1 h at 37° C. We used 10 ml collagenase per gram of tissue. At the completion of incubation the tumour fragments and cells were centrifuged, washed twice in PBS, and then exposed to prewarmed 0.25% trypsin (Bacto) in PBS for 15 min at 37° C. The action of the trypsin was stopped by the addition of SBCS to give a final serum concentration of 15%. Two further washes in PBS with recentrifugation were carried out, followed by the addition of full medium. The resulting suspension was agitated for a further 2–3 min, the large fragments allowed to settle, and the supernatant filtered through a 25-μm polyester mesh filter. This procedure produced a satisfactory single-cell suspension. The cell yield per gram of tissue was usually in the range of $1-6 \times 10^7$ cells/g, or slightly lower in the case of HX61 ($\sim 8 \times 10^6$/g).

Cell clumps of up to four usually constituted $< 5\%$ of the overall cell number. Viable nucleated cell counts were performed in a haemocytometer after lissamine green (1%) staining.

Table 1. Characteristics of the tumours used to establish the four ovarian xenografts

Xenograft designation	Source of tumour	FIGO[c] stage	Drug exposure prior to xenograft establishment	Histological appearance	Passage range	Morphology of chromosomes and modal number
HX61	Secondary[a], from 2nd laparotomy	III	Cisplatin, chlorambucil, adriamycin (7 courses)	Poorly differentiated papillary cystadenocarcinoma	8–10	Human 85
HX62	Primary, untreated	III	None	Moderately differentiated papillary cystadenocarcinoma	3–8	Human 83
HX109	Secondary[b], from 2nd laparotomy	III	Cisplatin, chlorambucil, adriamycin (8 courses)	Poorly differentiated papillary cystadenocarcinoma	3–4	Human 65
HX110	Primary, untreated	IV	None	Moderately differentiated papillary cystadenocarcinoma	5	Human 56

[a] Clinically in complete remission after chemotherapy; second-look laparotomy following chemotherapy revealed residual disease
[b] Clinically minimal response to chemotherapy; extensive disease confirmed at second laparotomy performed following chemotherapy
[c] International Federation of Gynaecology and Obstetrics

Treatment with cytotoxic agents and plating. Prior to their exposure to cytotoxic drugs, an appropriate concentration of tumour cells was prepared by dilution in full medium. Drug was then added to each 0.9-ml aliquot of tumour cells to give a final volume of 1.0 ml at a cell concentration of 1×10^6/ml contained in 10-ml plastic tubes. Following the addition of drug the cells were vigorously agitated and then gassed with a sterile 90% N_2, 5% O_2, 5% CO_2 mixture, sealed, and incubated for 1 h at 37° C. Throughout the incubation the tubes were agitated regularly.

At the completion of the incubation the cells were washed twice with PBS at 4° C and then resuspended in 1.0 ml full medium.

Cell survival was assessed using a twin-layer soft-agar method similar to the one previously described [7]. The agar overlayer was 0.25% agar (Agar Noble, Difco) in full medium, and the underlayer 0.5% agar made up in full medium. Washed August rat red blood cells treated for 1 h at 44° C were added to give a final concentration of one in 40 in the agar overlayer. In addition, heavily irradiated non-drug-treated tumour cells were employed when necessary to achieve a final plated cell number of 10^4 tumour cells per dish. Treated and control tumour cells were diluted prior to plating to provide approximately 50–100 colonies per 35-mm dish (Corning). Incubation was carried out in sealed humidified polystyrene boxes, after gassing for 15 min with a sterile 90% N_2, 5% O_2, 5% CO_2 mixture, at a flow rate of 2 l/min. Cultures were regassed twice weekly. Dishes were counted on day 21 using an Olympus binocular microscope. Cells giving rise to colonies of > 50 cells were scored as survivors.

Drugs. Stock solutions of drugs were made up freshly prior to each experiment at a concentration of 1 mg/ml. Melphalan, supplied by the Drug Synthesis and Development Branch, NCI, was dissolved in 2% HCl in ethanol prior to dilution in PBS.

Cisplatin (Drug Development and Synthesis Branch, NCI) and JM8, supplied by Dr A. H. Calvert, Institute of Cancer Research, were both dissolved and subsequently diluted in preservative-free PBS. Sterilization was achieved by filtration through a 0.2-μm filter (Millipore).

Drug concentrations for tumour cell exposure were based on plasma concentrations achievable in man for melphalan (peak plasma level of 3.38 μg/ml with a range of CXT of 0.87–5.50 μg · h/ml following an IV dose of 0.6 mg/kg) and cisplatin (peak plasma level of 2.49 μg/ml with a range of CXT of 1.45–2.52 μg · h/ml following an IV dose of 100 mg/m^2) [1].

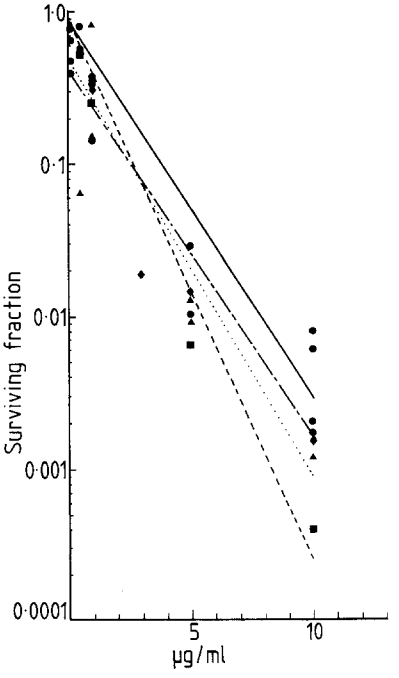

Fig. 1. Cell survival results for four ovarian xenografts (■– – –■, HX61; ●———●, HX62; ▲· · · · ·▲ HX109; ◆———◆, HX110) following exposure to varying concentrations of melphalan for 1 h. In this and subsequent figures individual *points* represent the mean of five dishes and when drawn at the same drug concentrations represent data from separate experiments

For melphalan, pharmacokinetic data obtained after IV high-dose administration were also used (after 140 mg/m^2 a mean level of 8.2 ± 2.7 μg/ml at 5 min and 3.3 μg/ml at 1 h after injection has been reported) [10]. The drug concentrations for JM8 were 10-fold greater than those for cisplatin and were based on LD_{10} ratios obtained in mice for JM8 and cisplatin (A. H. Calvert, personal communication).

Results

For each of the drugs studied in vitro, calculation of D_{10} (the dose of drug which reduces a population of cells on the exponential part of the cell survival curve to one-tenth of their initial number) and tests of significance have been performed using a computer program written by Dr J. L. Millar, Institute of Cancer Research. Exponential survival curves not constrained through the origin were fitted to the data by linear regression analysis.

Table 2. The cell survival parameters of the ovarian xenografts following melphalan exposure in vitro

Xenograft	n[a]	D_{10} (μg/ml)	95% confidence limits	Significance of difference from HX62
HX61	0.68	3.0	2.4–3.8	Not significant
HX62	0.77	4.2	3.7–4.7	
HX109	0.35	3.9	2.5–8.9	Not significant
HX110	0.30	4.1	2.6–9.6	Not significant

[a] Extrapolation number

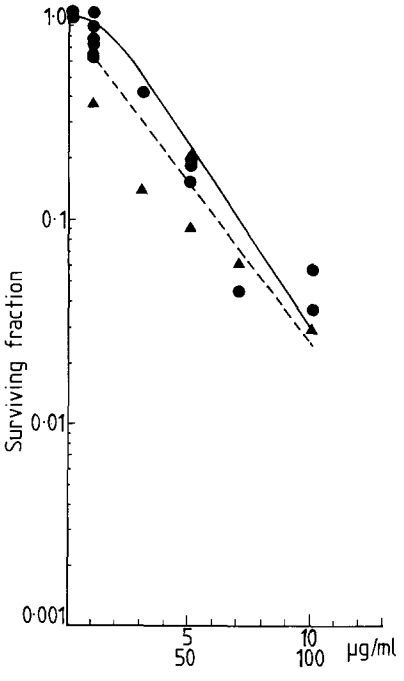

Fig. 2. Cell survival curves for the xenograft HX62 following a 1-h exposure to varying concentrations of cisplatin (●———●) and JM8 (▲– – –▲). Drug concentrations of JM8 are 10-fold greater than those of cisplatin

Melphalan response

In Fig. 1 the cell survival curves of four ovarian xenografts following melphalan exposure in vitro are shown. From these data it can be seen that there is a dose-response effect with apparently little difference in sensitivity among tumours. Table 2 gives the derived parameters.

JM8 and cisplatin

Cell survival curves comparing the response of HX62 to JM8 and cisplatin are shown in Fig. 2. In Fig. 3a the results following exposure to cisplatin for the four xenografts are

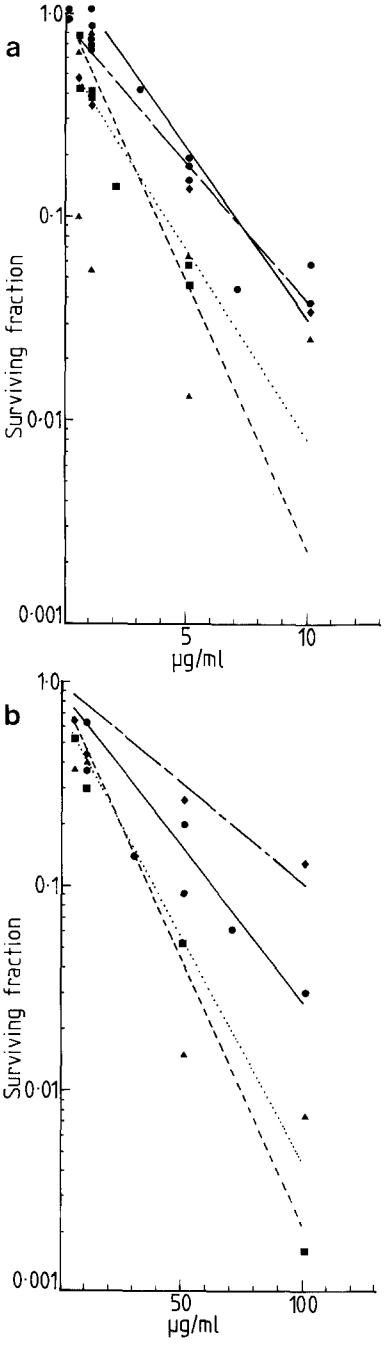

Fig. 3a, b. Cell survival curves following 1 h exposure to cisplatin (**a**) and JM8 (**b**). ■– – –■, HX61; ●———●, HX62; ▲· · · · ·▲, HX109; ◆– · – · –◆, HX110

Table 3. Cell survival parameters of ovarian xenografts exposed to cisplatin or JM8 in vitro

Xenograft	*n*	Cisplatin		*n*	CBDCA		Ratio	CBDCA D_{10} / cisplatin D_{10}
		D_{10} (μg/ml)	(95% confidence limits)		D_{10} (μg/ml)	(95% confidence limits)		
HX61	0.97	3.8	(2.9– 5.6)	0.89	37	(32.8– 42.9)	9.7	
HX62	2.7	5.0	(4.2– 6.2)	0.76	68	(50 –105.8)	13.6	
HX109	0.33	7.0	(3.4–22.6)	0.53	48.2	(31.3–103.9)	6.9	
HX110	0.7	7.7	(7.2– 8.2)	0.86	123.3	(83 –240)	16.0	

shown; in Fig. 3b cell survival following exposure to JM8 for the xenografts can be seen.

The results for HX62, on which the majority of experiments were performed, show that at a drug-concentration ratio of 1 : 10 (cisplatin : JM8) the cell survival curves were very similar. A comparison of the cell survival of the four xenografts after cisplatin exposure shows a broadly similar response; this was also seen with JM8 when the xenografts were compared. (Computer analysis had suggested some statistically significant differences in response between the xenograft for a given drug, but we have not attached great importance to these differences due to the limited data points for some of the xenograft responses.)

Table 3 includes the sensitivity data for both JM8 and cisplatin and the ratio of the D_{10} values of each xenograft for the two drugs. The ratios of the D_{10} values for the two drugs in each of the xenografts ranged from 6.9 to 16.0, with a mean of 11.6, which is close to the drug concentration ratio of 1.10 for cisplatin : JM8 selected for in vitro drug exposure.

Discussion

In this study the sensitivity of human ovarian carcinoma xenograft cells exposed to melphalan in vitro has been evaluated. No obvious differences in sensitivity have been demonstrated for the four tumours that were examined. Such a similarity was not expected, especially as two of the xenografts (HX61, HX109) had been derived from patients who at a second laparotomy had persistent disease following six or more courses of combination chemotherapy containing an alkylating agent. One might have expected that clinical drug resistance would be reflected in a reduced xenograft responsiveness, as was found in small cell lung carcinomas [12], but this was not the case. Unpublished results that we have obtained on testicular teratoma xenografts have also shown no difference in sensitivity between grafts taken from treated and untreated patients. Such results imply that in these cases clinical resistance was not reflected in a reduced drug sensitivity of the tumour cells.

Heterogeneity of response to in vitro alkylating agent exposure and apparent resistance to melphalan (as shown by a terminal plateau in the cell survival curves) have been reported for human tumour cells assayed direct from the patient [11] and from an ovarian xenograft [14]. Variations in technique and criteria for colony counting could be responsible in part for these differences, although other factors, as yet unidentified, may also have a role in producing these differences.

On the basis that a dose-response relationship for human ovarian carcinoma cells exposed to melphalan in vitro was demonstrated by these data, a clinical pilot study has now been initiated at the Royal Marsden Hospital. Selected patients with advanced, inoperable ovarian carcinoma are now receiving high-dose melphalan and autologous marrow rescue. At present four patients have been treated. One patient, who presented with very bulky stage IV (FIGO) disease, attained a partial remission of 8 months' duration. A second patient who had progressive disease despite high-dose cisplatin stabilized following high-dose melphalan. Of the remaining two patients, one with a very aggressive tumour had a short-lived minimal response to melphalan, and the final patient had no measurable disease following surgery for recurrent pelvic disease after first-line chemotherapy.

JM8 in vitro at concentrations 10-fold greater than cisplatin achieved broadly comparable levels of cell kill in each of the ovarian tumours examined. These data were used to predict that JM8 would be an active drug in the treatment of ovarian carcinoma if a similar in vivo concentration × time ratio to that seen with cisplatin could be obtained for JM8. The early clinical studies performed with JM8 have indicated that the dose of drug should be in the range of 300–400 mg/m^2 [4]. When used in this dose range the drug was found to have definite activity against ovarian carcinoma even in pretreated patients [4].

Recent pharmacokinetic studies of JM8 in patients [A. H. Calvert, personal communication; 8] and cisplatin [15] have revealed that in a number of patients the area under the time-concentration curve for free JM8 is approximately 30 times greater than for free cisplatin. This suggests that JM8 may have a therapeutic advantage over cisplatin in patients, if only 10-fold differences in concentration achieve similar levels of cell kill in vitro.

This is clearly a simplification of quite complex pharmacokinetics. However, a prospective randomized trial has been initiated to compare JM8 and cisplatin in advanced, previously untreated ovarian carcinoma. An interim analysis has shown that the overall response rates for the two drugs are identical [17].

In conclusion, it appears that the in vitro assay prediction that JM8 would be broadly comparable to cisplatin in activity against ovarian carcinoma is at present substantiated by current clinical studies. This experimental study thus adds further support to the view that xenografts may be of value in phase-II screening of new cytotoxic agents.

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