In vitro testing of platinum-based drugs on a panel of human ovarian tumour cell lines

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ABSTRACT

Much improvement in the treatment of ovarian cancer has been achieved since the introduction of platinum compounds in the 1980s, with the result that single-agent platinum-based therapy following primary surgery is now the standard treatment for advanced ovarian cancer. The main therapeutic effect of chemotherapy is based on the sensitivity of the patient's tumour to the drug. However, testing a new chemical compound on humans requires much care, time and resources, whereas prior testing of drugs on cancer cell lines may indicate those drugs particularly suited to treatment of a specific disease. This study investigates the actions of two established platinum-based chemotherapeutic agents (cisplatin and carboplatin) on a panel of 10 human ovarian cancer cell lines. Each cell line was plated onto 96-well tissue culture plates, incubated for 72 hours with the drug, formalin-fixed and then assessed using the methylene blue colorimetric microassay to detect viable cells. The IC_{50} values for each cell line were calculated in order to assess the toxicity of each drug, and a wide range of responses were observed across the 10 cell lines investigated. This suggests that the panel reflected the heterogeneous nature of ovarian cancer, a malignancy in which a huge range of drug sensitivities can be seen even among tumours of the same histological type. The results indicate that the panel could be of use either as a primary screen to test new drugs against ovarian cancer or to investigate the drug resistance that is so common in this disease.

KEY WORDS: Carboplatin. Cisplatin. Drug therapy. Ovarian neoplasms. Platinum compounds.

Introduction

Ovarian cancer is the most common gynaecological malignancy and the fourth most common cause of death from cancer in women. Annual incidence is approximately 20 per 100 000 women, resulting in some 6000 new cases each year in the UK.[1] Much improvement in the treatment of ovarian cancer has been achieved since the introduction of platinum compounds in the 1980s, and current standard chemotherapy worldwide relies on platinum-based agents (cisplatin or carboplatin), either alone or in combination with an alkylating agent (commonly cyclophosphamide).[1]

In previously untreated patients with ovarian cancer, high response rates of 60% to 80% are seen at first, although relapse occurs frequently and further therapy using alternative drugs (such as paclitaxel) is largely ineffective.[2] This is probably due to the survival of drug-resistant tumour cells which may be responsible for disease recurrence in many cases.[3]

The pharmacokinetics of cisplatin and carboplatin are very different. Cisplatin demonstrates a triphasic disappearance curve with an α half-life of 20 min, a ß half-life of 48-70 min and a γ half-life of 24 h.[4] The first two phases of disappearance represent clearance of free drug from the plasma, the third phase is probably removal of the drug from the plasma proteins. Some 90% of the drug is removed by renal mechanisms (glomerular filtration and tubular secretion), whereas <10% is removed by biliary excretion.

In contrast, carboplatin clearance from plasma is slower, a much higher percentage being excreted in the urine. Very little carboplatin binds to plasma proteins, and its half-life is five days. Elimination is mainly by glomerular filtration and tubular secretion, with little if any true metabolism of the drug. Carboplatin is widely distributed in body fluids and achieves good penetration into pleural effusions and ascites.[4]

Both cisplatin and carboplatin carry a pair of chloride atoms that interact with various components of DNA, especially with the nitrogen at the N7 position of guanine. Thus, these platinum-based drugs are able to bind to DNA to form DNA adducts (both interstrand and intrastrand DNA cross-linkages).[5] DNA adducts formed by cisplatin are thought to mediate its cytotoxic effects by inhibiting DNA replication and transcription, and finally by inducing programmed cell death (apoptosis).[6] Cells that are resistant to these drugs either have the capacity to limit the formation of platinum-DNA adducts or are able to repair or tolerate these lesions once they are formed.[2]

Clinical chemotherapy depends for its main therapeutic effect on the sensitivity of a patient's tumour to the drug. However, *in vitro* chemosensitivity testing using cancer cell lines to evaluate the tumour response before therapy has yet to find widespread clinical use. Testing a new chemical compound on humans requires much care, time and resources, whereas prior testing of drugs on cancer cell lines may indicate those that are particularly suited to the treatment of a specific disease. This strategy of drug testing, which is disease-related, has been used by the National Cancer Institute (NCI) in the USA which, since 1990, has changed from *in vivo* screening using a murine panel to

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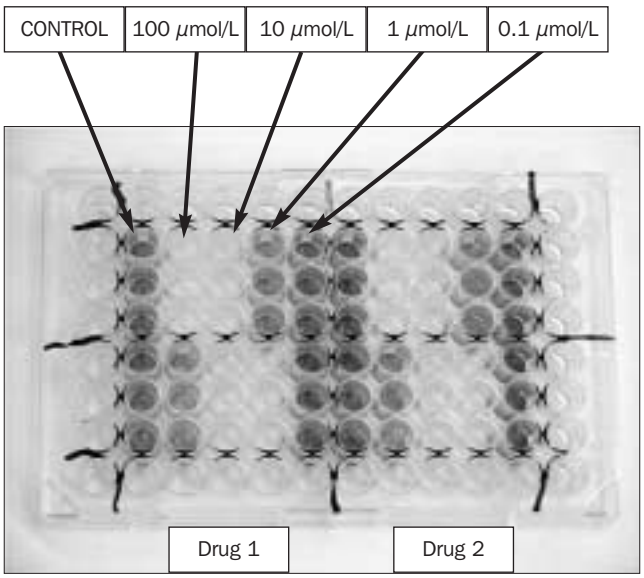
Table 1. Clinical information about cell lines used in this study

Cell line	Histology	Source	Treatment
138D	Bilateral serous cystadenocarcinoma	Ascites	Carboplatin (x6) Melphalan
180D	Metastatic papillary serous adenocarcinoma	Ascites	Cisplatin (x6) Carboplatin (x6)
200D	Serous papillary adenocarcinoma with clear cell areas	Solid tumour	None
253D	Pseudochylous serous carcinoma	Ascites	Cyclophosphamide Warfarin Tamoxifen Medroxyprogesterone acetate
59M	Endometrioid ovarian cancer with clear cell areas	Ovary	Ifosulphamide Melphalan
D013	Papillary serous adenocarcinoma	Peritoneal fluid	None
D016	Papillary serous adenocarcinoma	Omentum	None
D206	Papillary serous adenocarcinoma	Ascites	CAP (x3) (cyclophosphamide, doxorubicin, cisplatin) Carboplatin (x2) Cyclophosphamide Warfarin Tamoxifen
OAW 28	Adenocarcinoma	Ascites	Cisplatin Melphalan
OAW 42	Papillary serous cystadenocarcinoma	Ascites	Cisplatin (x6)

in vitro testing with human tumour cell lines from various sites.[7] So far, the NCI has screened more than 60 000 chemical compounds against a panel of 60 human cancer cell lines.

While the IC_{50} value (the mean drug concentration that produces a 50% growth inhibition in the cells) is only an index of the cytotoxicity of drugs, the NCI has noticed that patterns of drug activity observed in its cell screening have proved predictive of the action of the drug at a molecular level.[7]

Fig. 1 A stained 96-well plate showing the central 60 wells being used.



This study investigates the action of two platinum-based drugs (cisplatin and carboplatin) on a panel of 10 human ovarian cancer cell lines. The cytotoxic effect is assessed using the methylene blue colorimetric microassay, an assay previously validated in our laboratory as a simple, reliable and sensitive method with low variability.[8] In this assay, dead (or non-viable) cells will round up, detach from the tissue culture plate and be removed during the rinsing procedure. Methylene blue will bind to any cellular protein present, which, in this case, means only the remaining viable cells.

It is hoped that a panel of ovarian cell lines with a range of activities to various chemotherapeutic agents can be established, which might then be of use either as a primary screen in the testing of new drugs against ovarian cancer or to further investigate the drug resistance that is so common in ovarian cancer.

Materials and methods

Ten ovarian cell lines were developed from ascites and from solid tumour tissue (Table 1), all of which proved to be epithelial and to reflect the clinical and biological diversity of ovarian cancer. Seven of the human ovarian cell lines used in this study (OAW 42, OAW 28, 59M, 138D, 180D, 200D and 253D) have already been documented for various characteristics (including morphology, ultrastructure, karyotype and expression of OC125, HMFG2, vimentin, pan-keratin and keratin7);[9] the others were characterised as epithelial by morphological criteria and using epithelial markers to pan-cytokeratin and Ber-EP4 (Dako Ltd, Ely, Cambridgeshire, UK). All were checked routinely for the presence of mycoplasma by enzyme-linked immunosorbent assay (ELISA) and found to be negative.[10]

All cell lines were grown as monolayers at 37°C in an atmosphere of 5% CO_2 in tissue culture flasks (Nunc, Life Technologies, Paisley, Scotland) and subcultured when confluent at split ratios of 1:2 to 1:9, using standard trypsinisation methods. Culture media used are shown in Table 2. DME, F12, glutamine, penicillin/streptomycin, sodium bicarbonate and sodium pyruvate were all purchased from Life Technologies. Human Actrapid insulin (neutral insulin injection) was purchased from Novo Industries, Bagsvaerd, Denmark, and McCoys 5A was purchased from Sigma Chemical Company, Poole, Dorset, UK.

Cell lines were plated onto 96-well tissue culture plates, using the appropriate culture medium and cell concentration for each cell line (Table 2). The cell concentration for each cell line was determined according to the growth rate and size of individual cells. To allow for the edge effect seen in 96-well plates, where the cells do not adhere or grow so well in the outside wells of the plate, a 10 x 6 matrix was devised.[11] This excluded the outer wells and divided the control (untreated wells) between the middle and the outer rows of the plate (Figure 1). The plates were incubated overnight at 37°C in a 5% CO_2-humidified incubator to allow the cells to adhere, and then the drugs were added.

The drugs (cisplatinum and carboplatinum – both purchased from Sigma) were dissolved initially in dimethylsulphoxide (DMSO; Sigma) at 10 mmol, and subsequent dilutions were prepared using HBSS (Life Technologies) to 1, 10, 100 and 1000 µmol/L. Samples (20µL) of these dilutions were added to each well containing 200µL to give final dilutions of 0.1, 1, 10 and 100 µmol/L, respectively.

Experimentally, DMSO alone did not have a toxic effect on the cell lines. A row of untreated wells (controls) was included in each assay and was used as a reference to calculate the percentage of cells killed at each concentration.

The plates were then incubated for 72 hours, rinsed twice with 200 µL phosphate-buffered saline (PBS; pH 7.3) (Oxoid; supplied by Scientific Laboratory Supplies, Nottingham) and fixed for 10 min with 10% formalin in PBS (200 µL; Western Solvents Medical, Warminster, Wilts). After two washes with 0.1 mol/L borate solution (pH 8.4) (200 µL; Sigma) they were stained for 10 min with 1% methylene blue (Sigma) in 0.1 mol/L borate solution (100 µL). The plates were then washed (x6) with 0.1 mol/L borate solution (200 µL) and air-dried for 3 h at room temperature.

The dye was solubilised with 0.1N hydrochloric acid (200 µL; Merck Eurolab, Lutterworth, Leics, UK) followed by 15 min agitation on a Dynatech plate-shaker. Absorbance was measured at 650 nm using a microplate reader (UV max; Molecular Devices).

Only the central 60 wells of the plate were used for the assay and the plate plan shown allowed two drugs to be

Table 2. Cultural information about cell lines

Cell line	Cells/well	Medium
138D	9 X 10^3	P/F12 5% FCS
180D	9 X 10^3	P/F12 5% FCS
200D	9 X 10^3	P/F12 5% FCS
253D	8 X 10^3	P/F12 10% FCS
59M	6 X 10^3	PPIGSS
D013	9 X 10^3	P/F12 5% FCS +ITS
D016	12 X 10^3	P/F12 5% FCS +ITS
D206	9 X 10^3	P/F12 5% FCS +ITS
OAW 28	9 X 10^3	PPIGSS
OAW 42	1.5 X 10^3	PPIGSS

PPIGSS: Dulbecco's modification of Eagle's medium (DMEM) with 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 000 u/L penicillin, 100,000 µg/L streptomycin, 0.03 u/mL human Actrapid insulin and 10% fetal calf serum (FCS).

P/F12 5%: (10%) PPIGSS : F12 (Equal parts of DMEM and F12with 2 mmol/ L glutamine, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 3.7 g/L sodium bicarbonate, 100 000 u/L penicillin, 100 000 µg/L streptomycin, 0.03 u/mL human Actrapid insulin and 5% (10%) FCS.

P/F12 5% + ITS: P/F12 5% with ITS (5 µg/mL insulin, 5 µg/mL transferrin and 5 µg/mL sodium selenite)

Fig. 2. Example of graph used to calculate IC_{50} values.

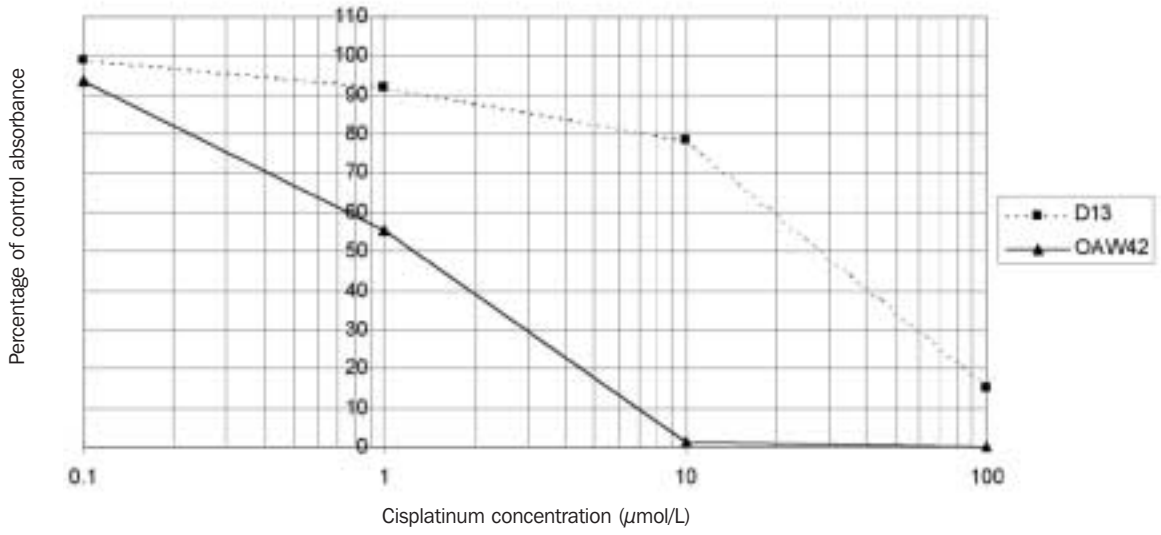
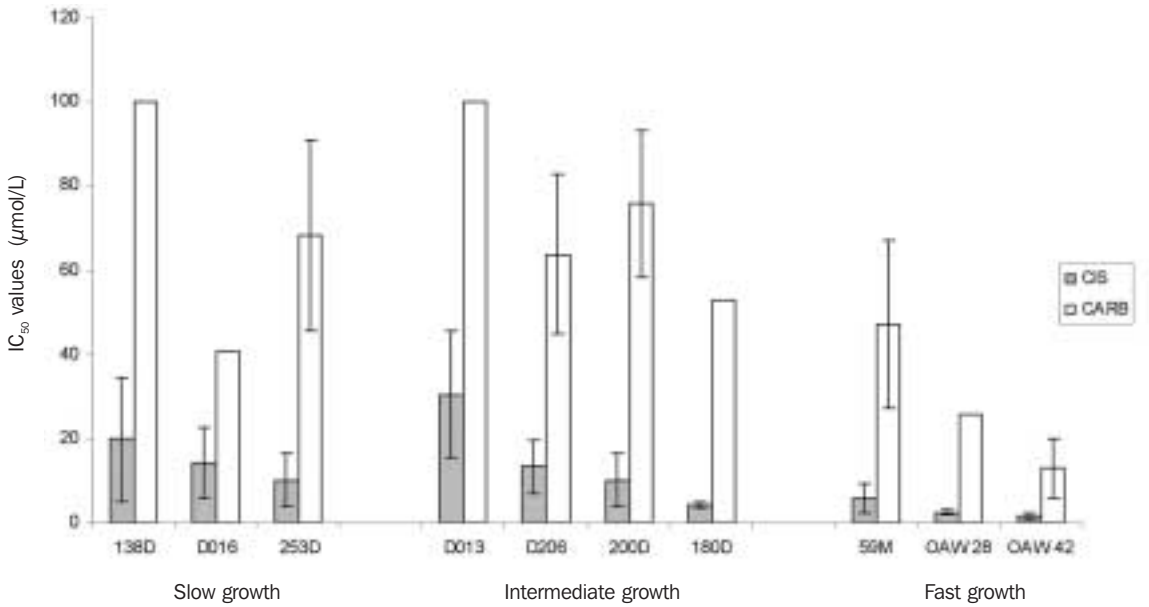


Fig. 3. IC_{50} values of cell lines treated with either cisplatin or carboplatin and grouped according to growth rate.



tested per plate (Figure 1). Three replicates were performed in the top half of the plate and three in the bottom half. This gave a total of six replicates for each drug concentration and permitted assessment of intra- and inter-assay variability. Two coefficients of variation were calculated: the intra-assay variability was 6.7% and the inter-assay variability was 6.35% (n=24).

Results

The assay was repeated at least twice for each drug, dose response curves were plotted and IC_{50} values calculated as shown by the example in Figure 2. For each cell line, the IC_{50} value corresponded to the cisplatinum concentration at the point where control absorbance was 50%. The mean and standard deviation of each set of IC_{50} values were calculated and a two-sample, unequal variance Student's t-test was used to compare each sample mean with that of the most sensitive cell line (OAW 42). Cell lines with less than four experimental values were omitted from the statistical analysis. Table 3 shows these significant differences in drug sensitivity scored as: $P \leq 0.05$ (5% significant), $P \leq 0.01$ (1% highly significant), or $P \leq 0.001$ (0.1% very highly significant).

Mean IC_{50} values obtained after treating the cell lines with carboplatinum were usually some ten times higher than those achieved with cisplatin, and was seen across the cell lines. This is consistent with the reduced level of toxicity seen when carboplatin is used *in vivo*.[12,13] Large variation was seen in the response of the various cell lines to both cisplatin and carboplatin (Table 3), the most sensitive cell line being OAW 42 (1.56 ± 0.82; 12.93 ± 7.04) and the most resistant being D013 (30.41 ± 15.22; >100).

In order to confirm that the variation seen in the reaction of the various cell lines to these drugs was not due to a similar variation in growth rate, the cell lines were grouped according to their growth rate (Figure 3). Cell lines with a slow growth rate were split once every seven days (split ratio 1:2), whereas those with a fast growth rate were split every two/three days (split ratio 1:2 or more). Cell lines with an intermediate growth rate were split approximately every four days (split ratio 1:2). Within each group, a range of IC_{50} values was seen: for example, in the intermediate growth rate group, D013 was resistant to the action of cisplatin but 180D proved to be very sensitive.

Discussion

The use of platinum-based drugs may result in prolonged remissions and improved survival for patients with ovarian cancer.[12] However, cisplatinum is associated with both nephrotoxicity and neurotoxicity, and new analogues of platinum have been developed in order to reduce these effects. Carboplatin is one such example, showing less nephrotoxicity and neurotoxicity but more myelosuppression.[12-14] Although there has been limited *in vitro* investigation of the cytotoxicity of these two platinum-based drugs in human ovarian cancer cell lines, the results of the study reported here confirm that there is considerable difference between them. During the course of the experiments it became clear that the IC_{50} values after carboplatinum treatment were much higher than after cisplatinum treatment (Table 3), and we believe that this may be related to the different pharmacokinetic mechanisms associated with these two drugs.

Cell lines D013, D016 and D206 were obtained from the same patient at different times during treatment. D013 and D016 were both taken before commencement of chemotherapy, while D206 was taken after several courses had been administered. Clearly, D013 showed resistance to platinum-based drugs prior to the start of treatment. The variation in response to the drugs cannot be explained by the site of origin of the cell line – both D013 and D206 were obtained from the same source (peritoneal fluid/ascites),

Table 3. Effect of drug treatment on the cell lines (IC_{50} values)

Cisplatin				
Cell line	Number of experiments	Mean ± SD (μmol/L)	*P* value	Range (μmol/L)
OAW 42	30	1.56± 0.82		0.25-3.0
59M	11	5.86 ± 3.53	**	3.1-15.0
200D	10	10.04 ± 6.33	**	3.8-20
OAW28	6	2.47 ± 0.62	*	1.5-3.0
138D	4	19.88 ± 14.80		4.5- 33.0
D206	7	13.26 ± 6.37	**	8.8- 23.0
D013	27	30.41 ± 15.22	***	17-100
253D	9	10.01 ± 6.32	**	3.7-19.0
180D	7	4.36 ± 0.79	***	2.8-5.2
D016	6	14.15 ± 8.43	*	2.9-22.0
Carboplatin				
Cell line	Number of experiments	Mean ± SD (μmol/L)	*P* value	Range (μmol/L)
OAW 42	9	12.93 ± 7.04		3.5-22.0
59M	7	47.14 ± 19.80	**	31.0-84.0
200D	5	75.80 ± 17.50	***	52.0-100
OAW28	3	25.67	ND	23.0-28.0
138D	2	>100	ND	
D206	4	63.75 ± 19.0	*	52.0-92.0
D013	6	>100	ND	
253D	5	68.20 ± 22.54	**	33.0-84.0
180D	3	53.00	ND	29.0-70.0
D016	2	40.50	ND	35.0-46.0

* $P \leq 0.05$ (5% significant).

** $P \leq 0.01$ (1% highly significant).

*** $P \leq 0.001$ (0.1% very highly significant).

ND Statistical analysis not done due to insufficient experimental data.

whereas D016 was obtained from tissue (omentum).

When IC_{50} values for cisplatin treatment were compared statistically to that of the most sensitive cell line (OAW 42), all except 138D were seen to be significantly different (Table 3). A very highly significant difference was seen with two of the cell lines (180D and D013), indicating that these were the most resistant to cisplatinum treatment. The other six cell lines exhibit a range of sensitivities to this drug.

Owing to the smaller number of experiments performed using carboplatinum, only four cell lines could be compared statistically to OAW 42; however, this notwithstanding, a range of sensitivity to this drug was observed, with 200D proving to be the most resistant.

When the cell lines were grouped according to growth rate (Figure 3), no obvious within-group correlation was seen; however, cells in the faster growing group appeared to show increased toxicity to these drugs. This may have been because they were dividing rapidly and thus able to metabolise the drugs more quickly, resulting in the increased toxicity. However, one particularly slow growing cell line (D016) showed increased sensitivity to these drugs, suggesting that growth rate and drug toxicity were not related in this case and that the sensitivity was due to some other mechanism.

The main biochemical alterations that contribute to cisplatinum resistance in tumour cell lines are decreased drug accumulation, increased levels of glutathione and metallothioneins, and enhanced DNA repair.[3] As yet, none of these parameters have been measured in any of the cell lines used in this study, and it may be of interest to do so in the future.

In conclusion, a panel of human ovarian tumour cell lines, showing different responses to drugs, may be a useful preliminary test to screen for potential cytotoxic agents against ovarian cancer, and in investigating further the many factors involved in drug resistance. □

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