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In Vitro Chemosensitivity of Four New Carcinoma of the Cervix Cell Lines: Relationship to Radiosensitivity

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Abstract—*Using a clonogenic cell survival assay, the chemosensitivity of four recently established cervix carcinoma cell lines to four drugs used in the treatment of this disease (bleomycin, cisplatin, etoposide and methotrexate) has been determined. Exposure was for* 1 h *except for methotrexate where* 24 h *was used. Results showed that, on a molar basis, bleomycin was most cytotoxic against each cell line (doses to produce* 10% *cell survival from* 10 *to* 40 μM). *However, if the clinical area under the curve (AUC) values are taken into consideration, the cell lines appeared to be relatively resistant to cisplatin (surviving fraction range of* 0.9–0.6) *and methotrexate but sensitive to bleomycin and etoposide (surviving fraction range of* 0.18–0.012). *Among this small group of lines, there was some evidence to suggest a positive correlation between chemo- and radiosensitivity; the HX/*151*c line was significantly more radiosensitive than the other three lines* (P = 0.03) *and clearly more chemosensitive, particularly against bleomycin (correlation coefficient of* 0.81) *and etoposide (correlation coefficient of* 0.80).

INTRODUCTION

CARCINOMA OF THE CERVIX represents a major public health threat, being responsible for around 2500 deaths in the U.K. each year [1]. Overall 5-year survival is approx. 60%. While surgery and/or radiotherapy offer the main choice of treatment for early stage disease, there is increasing clinical opinion that chemotherapy may have a role to play in the treatment of advanced local disease or metastasis, particularly in an adjuvant setting [2–4]. Furthermore, recent clinical evidence suggests that cervical carcinoma may be a chemotherapy-responsive tumour; greater than 60% objective response has been reported for a combination of cisplatin, vinblastine and bleomycin (PVB) [5, 6]. The PVB combination has also been used in an adjuvant setting prior to radical radiotherapy for stage III and IV disease [7].

There are few *in vitro* cell lines of this common tumour available for study [8–11], and none to our knowledge are recently established and at low passage number. As a result, there is little information as to the chemosensitivity of cervix carcinoma cell lines. In this investigation, we have used four recently established tumour cell lines, taken from previously untreated biopsies, and determined their chemosensitivity to four drugs commonly used in the treatment of this disease; bleomycin, cisplatin, etoposide and methotrexate. In addition, to help answer the important question of whether chemoresistant tumours are also radioresistant, we have compared the responses obtained with previously derived radiobiological information which used the same clonogenic assay methodology [12, 13].

MATERIALS AND METHODS

Cell lines

The four lines used in this study, HX/151c, HX/155c, HX/156c and HX/171c, were all established from previously untreated biopsy specimens collected between January 1985 and February 1987 from patients presenting at the Radiotherapy Department of the Royal Marsden Hospital, London. A comprehensive biological characterization of three of the lines (HX/151c, HX/155c and HX/156c) has been described previously [14]. HX/171c was established from a 25-year-old woman presenting with a poorly differentiated stage IIb squamous cell carcinoma. The line was established

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using the same 3T3 feeder layer technique as described for the other lines [14]. HX/171c exhibited epithelial cell morphological features similar to the above lines and possessed a mean of 70 chromosomes per cell. All four lines grew as monolayer cultures in Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 15% foetal calf serum (Imperial Laboratories), 10^5 U/l of penicillin, 100 mg/l of streptomycin, 2 mM glutamine, 0.4 mg/l hydrocortisone sodium succinate and 5 mg/l insulin in a 5% CO_2, 5% O_2, 90% N_2 atmosphere. Cells were used from passage 5 to 15. Further *in vitro* properties of the lines relevant to this investigation are shown in Table 1. Cells were routinely found to be free of mycoplasma contamination by staining with Hoechst 33528 dye and examining under a fluorescent microscope.

Assessment of chemosensitivity

Chemosensitivity to four drugs [bleomycin, cisplatin (*cis*-platinum(II) diamminedichloride), etoposide and methotrexate] was investigated using a monolayer clonogenic assay developed for these lines and described previously [12, 13]. Briefly, a single cell suspension was prepared from a subconfluent monolayer by dissagregation with 0.02% EDTA in 0.05% trypsin. Cells were then plated at the appropriate number on three replicate 60 mm tissue culture dishes, each containing 4.5 ml of growth medium plus 2×10^5 heavily irradiated (200 Gy) Swiss mouse 3T3 feeder layer cells. As described previously [14], the feeder layer is essential for achieving workable cloning efficiencies in these cell lines. Cells were then gassed with 5% CO_2, 5% O_2, 90% N_2 mixture and allowed to attach at 37°C for 18 h.

Drugs were dissolved at 1 mM in either 0.9% saline or water (for bleomycin) immediately before use. Etoposide was obtained as a formulated pharmaceutical product dissolved in vehicle. Serial dilutions were then made in growth medium and drug added as a 10 times concentrate (0.5–5 ml) to triplicate dishes. Drug exposure was for 1 h at 37°C, except for methotrexate, where 24 h was used. For etoposide, control dishes were exposed to the drug vehicle alone at the maximum concentration of etoposide used. After exposure, the cells were washed three times [once with phosphate buffered saline (PBS), twice with growth medium] to remove all drug.

The concentrations of drugs employed and the time of exposure were chosen according to previously published pharmacological considerations [15, 16]. The 1 h exposure time has been routinely used in the 'human tumor stem cell assay' [17] in order to standardize assay conditions. In addition, clinical pharmacokinetic data suggest that tumour cell exposure to most drugs is greatest during the first hour of administration [16]. The most important parameter in considering the range in drug concentrations for use *in vitro* assays is the concentrationtime product (CXT). This equates to the area under the plasma concentration time curve [15, 16]. In addition, the peak plasma drug concentration is also of importance. We have chosen drug doses to reflect approximate area under the curve (AUC) values for these drugs in their clinical setting [18]. AUC values for intravenous administration may be calculated according to the following equation:

$$\text{AUC } (\mu M \times h) = \text{total dose administered } (\mu M)/\text{clearance } (1/h).$$

Using published data for drug clearance and commonly used patient schedules the following approximate AUC values were calculated: bleomycin, 24 $\mu M \times h$; cisplatin, 17 $\mu M \times h$; etoposide, 80 $\mu M \times h$; methotrexate, 100 $\mu M \times h$.

Cells were then incubated in a gassing incubator (same gaseous environment as above) for 16–19 days. Dishes were then washed with PBS, stained for 15 min using 0.5% methylene blue in 50% methanol–water, and colonies containing greater than 50 cells were counted by eye.

Assessment of radiosensitivity

Cells were plated as above and exposed to ^{60}Co gamma rays at a dose rate of 100 cGy/min using a

Table 1. In vitro *properties of the cell lines*

Line designation	Clinical stage of disease	Age of patient (years)	*In vitro* doubling time (h)	Cloning efficiency ± S.E. (%)
HX/151c	IB	30	42	4.8 ± 2.0
HX/155c	IB	44	48	24.0 ± 6.9
HX/156c	IIB	31	30	31.8 ± 7.4
HX/171c	IIB	25	36	29.6 ± 4.5

*Cloning efficiency = $\frac{\text{No of colonies}}{\text{No of viable cells seeded}} \times 100$.

74TBq source. During irradiation (maximum length of time was 12 min) cells were held at 37°C. Post-irradiation incubation and assessment of survival was as above.

Statistical analysis

All experiments presented in Figs. 1–3 represent the mean ± standard error of three independent determinations. Radiation cell survival curves were fitted using a computer program based on the 'incomplete' repair model for survival under continuous irradiation [19]. Drug survival curves were fitted by eye. Significance testing was performed using Student's *t*-test and regression analysis performed to determine correlation coefficients for drug and radiation sensitivity.

RESULTS

Chemosensitivity data are shown in Figs. 1 and 2. The results are illustrated in two ways: Fig. 1 shows the results for individual drugs (1a, bleomycin; 1b, cisplatin; 1c, etoposide and 1d, methotrexate) while Fig. 2 shows the sensitivity of the individual lines to the four drugs (2a, HX/151c; 2b, HX/155c; 2c, HX/156c; and 2d, HX/171c). In this way one may visualize both individual sensitivities of each cell line to the range of drugs used and the effect of each drug on the four lines evaluated.

Several points of interest are evident from these data. Firstly, from Fig. 1 (individual drug sensitivity) it is apparent that there is a range in sensitivity observed with bleomycin and etoposide, while for cisplatin all four lines showed similar surviving fractions at all concentrations investigated. For both bleomycin and etoposide, HX/151c was clearly more sensitive than the other three lines. For methotrexate, experiments were initially performed with a 1 h exposure time but very little cell kill was observed. As a result, a 24 h exposure time was used. As a consequence, AUC values up to 10-fold higher than those achievable with a clinical dose of 500 mg have been used. This suggests a low sensitivity of these lines to methotrexate, although as discussed below, assay factors may, in part, be

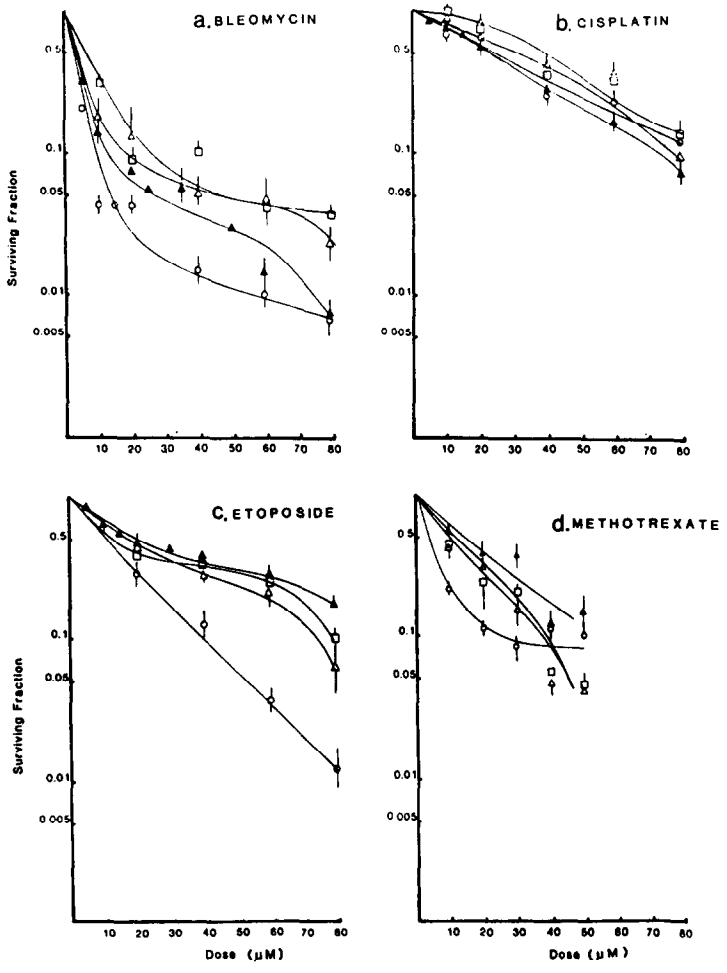

Fig. 1. Survival curves for the four cervix carcinoma cell lines: HX/151c (⊙), HX/155c (△), HX/156c (▲) and HX/171c (□) to each drug investigated. a, Bleomycin; b, cisplatin; c, etoposide; d, methotrexate.

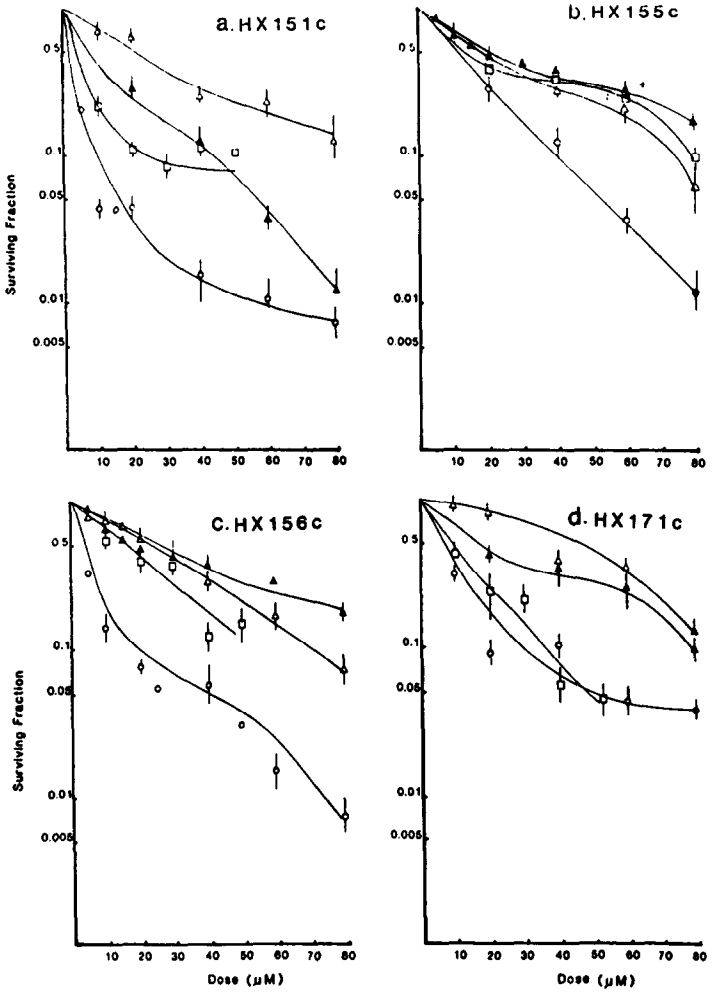

Fig. 2. Survival curves for the four drugs investigated; bleomycin (○), cisplatin (△), etoposide (▲), and methotrexate (□) against each cell line. a, HX/151c, b, HX/155c; c, HX/156c; d, HX/171c.

responsible for this. At the AUC value there was a range in surviving fraction from only 0.85 to 0.5. As with bleomycin and etoposide, HX/151c appeared to be most sensitive.

In order to obtain a better comparison of cell line sensitivity, survival has been calculated at the AUC value for each drug. These surviving fractions are shown in Table 2, and are shown in rank order (resistant to sensitive) for each drug. Also included in Table 2 is the fold difference in sensitivity across the lines for each drug. This is seen to vary from only 1.5 for cisplatin (surviving fraction 0.9–0.6; HX/171c to HX/151c) to 15 for etoposide (surviving fraction range 0.18–0.012; HX/156c to HX/151c).

Figure 2 shows the sensitivity of each cell line to the four drugs. For each cell line, on a molar basis, bleomcyin appears to be the most cytotoxic agent.

Table 2. The correlation between chemosensitivity (surviving fraction at AUC value) and radiosensitivity (SF_2 value) for the four cell lines*

	Chemosensitivity								Radiosensitivity	
	Bleomycin		Cisplatin		Etoposide		Methotrexate			
1	HX/171c	0.12	HX/171c	0.9	HX/156c	0.18	HX/156c	0.9	HX/171c	0.60
2	HX/155c	0.08	HX/155c	0.9	HX/171c	0.10	HX/171c	0.9	HX/156c	0.56
3	HX/156c	0.055	HX/156c	0.6	HX/155c	0.06	HX/155c	0.9	HX/155c	0.45
4	HX/151c	0.020	HX/151c	0.6	HX/151c	0.012	HX/151c	0.5	HX/151c	0.23
Fold difference	6		1.5		15		1.8		2.6	

Ranking 1–4; resistant to sensitive.
*SF_2 = surviving fraction at a radiation dose of 2 Gy.

However, if the clinical AUC value is taken into consideration, the lines appear to be relatively resistant to cisplatin and methotrexate, and sensitive to bleomycin and etoposide. To obtain a ranking of sensitivity for the four drugs, the doses to cause 1 log of cell kill (D_{10} value) have been extrapolated. These values plus the rank effectiveness (most cytotoxic to least) for each cell line are shown in Table 3. These data reveal that, although bleomycin is always most cytotoxic, the ranking of the other three agents is cell line dependent. For example, while cisplatin is at least cytotoxic against HX/171c and HX/151c, it ranks second in activity against HX/155c and third against HX/156c.

Figure 3 shows ^{60}Co gamma ray survival curves for the four cell lines using a dose rate of 100 cGy/min. A more detailed analysis of the radiobiology of these lines including investigations of the effect of varying the dose rate [12] and measuring recovery during split dose experiments [13] has been published. Figure 3 shows that three of the lines (HX/155c, HX/156c and HX/171c) exhibit similar radiosensitivity while HX/151c is significantly more radiosensitive ($P = 0.03$). As a measure of radiosensitivity we have used the surviving fraction at 2 Gy (the SF_2 value). As shown previously [20, 21] this parameter shows a positive correlation with clinical radioresponsiveness and gives a good discrimination between resistant and sensitive tumour types. SF_2 values are shown in Table 2.

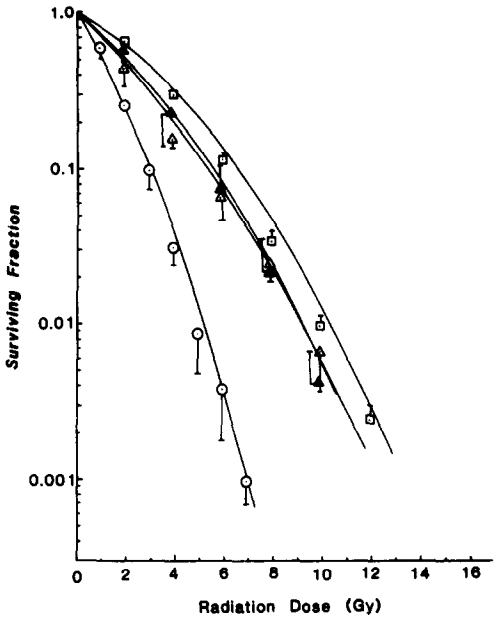

Fig. 3. Radiation cell survival curves at a dose rate of 100 cGy/min. *HX*/151*c* (⊙), *HX*/155*c* (△), *HX*/156*c* (▲) *and HX*/171*c* (⊡). *Lines are calculated by fitting according to the incomplete repair model* [18].

DISCUSSION

As far as we are aware, this is the first occasion that a detailed chemosensitivity profile of a panel of recently established cervix carcinoma cell lines has been reported. This is probably, in part, due to the general scarcity of continuous cell lines representative of this disease. We have chosen to investigate four agents, bleomycin and cisplatin, as they form part of the PVB regime undergoing trial in cervical cancer at the moment [6], etoposide, as it has, to some extent, replaed vinblastine in the PBV regimen for the treatment of testicular teratomas in the U.K. [2], and methotrexate. One of the key questions we set out to ask was 'are tumour cells resistant to chemotherapy also radioresistant and *vice versa*?'

From Table 2 and Fig. 1, there does appear to be a similar pattern of response, within this small number of cervix carcinoma cell lines, between radiosensitivity and chemosensitivity (especially bleomycin and etoposide). Moreover, recent clinical experience with advanced cervical carcinoma (using cisplatin, vinblastine and bleomycin) lends support to the view that a positive correlation may exist between response to chemotherapy and subsequent response to radiotherapy [23]. Of particular interest is the HX/151c cell line. It is clearly more radiosensitive than the other three lines and appears to be most sensitive (at their AUC values) to bleomycin, etoposide and, to some extent, methotrexate. Regression analysis reveals a positive correlation for cell line response between radiation and (a) bleomcyin (correlation coefficient of 0.81), (b) etoposide (correlation coefficient of 0.80), and (c) methotrexate (correlation coefficient of 0.92). The relationship was less obvious for cisplatin (correlation coefficient of only 0.45).

Table 3. Doses required to reduce cell survival to 10% (D_{10}, μM) *and drug ranking for the four cell lines*

Rank	HX/151c		HX/155c		HX/156c		HX/171c	
1	Bleomycin	10	Bleomycin	40	Bleomycin	20	Bleomycin	28
2	Methotrexate*	25	Cisplatin	75	Methotrexate*	54	Methotrexate*	35
3	Etoposide	45	Methotrexate*	80	Cisplatin	72	Etoposide	78
4	Cisplatin	98	Etoposide	106	Etoposide	110	Cisplatin	85

*Methotrexate = 24 h exposure.

The patients from which these tumour lines were established were largely treated by radiotherapy and surgery (as they were early stage tumours). However, the tumour which was most resistant *in vitro* to bleomycin, cisplatin and radiation (HX/171c) exhibited progressive disease in the patient during treatment with radiotherapy, and three courses of cisplatin and ifosfamide. Conversely, the HX/151c tumour showed an initial complete response to radiotherapy: the patient, however, was then lost to any further follow-up. Therefore, it is not possible to speculate to any length as to possible correlations between the *in vitro* chemosensitivity profiles observed in this study and clinical responses.

In terms of mechanisms of cell killing, the general correlation between response to radiation and bleomycin, etoposide and methotrexate are perhaps not that surprising. Radiation [24], bleomycin [25] and etoposide [26] are all thought to exert their activity primarily via induction of DNA single- and double-strand breaks, etoposide as a consequence of its inhibitory effects on DNA topoisomerase II [27 for a review]. Furthermore, recent evidence [28] has shown that irradiated HX/151c cells incur more initial DNA double-strand breaks per Gray and repair these breaks at a slower rate than the other three lines used in this study.

In addition to the induction and repair of lesions in DNA, an elevation in cellular glutathione (GSH) levels has been associated with both radio- and chemoresistance [29] (for a review). This has been particularly the case for cisplatin where some cisplatin-resistant L1210 murine leukaemia and human ovarian carcinoma cell lines have been shown to possess increased levels of glutathione [30, for a review]. Therefore, it is perhaps surprising that a more positive correlation between radiation and cisplatin sensitivity was not found in this study (correlation coefficient of 0.45). However, no direct correlation has yet been observed between GSH levels and the degree of resistance to radiation or cisplatin, and moreover, it appears that severe depletion of GSH is necessary to reverse resistance [30].

As well as the correlations between chemosensitivity and radiosensitivity, these data revealed interesting chemosensitivity patterns. Whereas the lines exhibited a range in sensitivity against bleomycin and etoposide, they were all (perhaps surprisingly in view of the above GSH discussion) relatively resistant to cisplatin. Indeed, clinically, cisplatin is considered to be the most active single chemotherapeutic agent against cervical carcinoma [31]. This finding for cisplatin may, in part, be attributable to the short (1 h) exposure time. Other data with cisplatin and human ovarian tumour cells *in vitro* [32] have shown a clear time-dependency for increased cytotoxicity up to 18 h exposure time. It is thought that cisplatin has to undergo aquation reactions before exerting its cytotoxic effect through interaction with DNA, primarily via intra- and interstrand DNA–DNA crosslinks [33]. However, it is clear from a comparison of cisplatin data involving other human tumour cell types, that these cervix lines are at the resistant end of the spectrum (D_{10} values from 72 to 98 μM). For instance, human embryonal carcinoma cells exposed for 2 h showed D_{10} values of around only 5 μM [34]. Moreover, other data using continuous exposure, and bladder and testicular carcinoma cell lines [35] have shown mean D_{10} values of around 0.7 and 0.2 μM respectively. Whereas these cervix lines showed only a narrow range in their survival after cisplatin treatment (Fig. 1b), it is interesting that, across 10 human ovarian cell lines (a disease where cisplatin provides about a 50% initial response rate), an 86-fold range in cytotoxicity has been demonstrated [36].

The relative resistance of the lines to methotrexate is probably not surprising when one considers the fact that undialysed foetal calf serum at 15%, which is known to contain high concentrations of protecting nucleosides, had to be used in the assay. This aspect of assessing methotrexate cytotoxicity *in vitro* has been highlighted previously [e.g. 37].

What are the clinical implications of the findings of this study? It is encouraging that at least some cervix carcinoma cells appear to be chemosensitive (e.g. HX/151c) although this line also appears to be radiosensitive. As mentioned above, recent clinical data lend support to the view that chemosensitive cervix tumours may also be radiosensitive [23]. This observation combined with some evidence of differences in drug sensitivity ranking (Table 3) within such a small number of lines and drugs investigated does suggest that rapid predictive testing of chemosensitivity might be advantageous in this disease. However, it must be remembered that *in vitro* chemosensitivity may not equate with *in vivo* activity where metabolism and pharmacokinetic parameters operate. Indeed, while etoposide has been shown to possess activity in this study, it had insignificant activity against carcinoma of the cervix in phase II studies and the Gynecological Oncology Group do not recommend the use of this agent [38, 39]. In addition, in terms of single agent clinical activity in cervical carcinoma, a typical rank order of effectiveness has been found to be cisplatin (most active, 40% overall response), then methotrexate, bleomycin and etoposide [31]. Therefore, rather than determining rank orders of effectiveness for agents, predictive testing may be better utilized to identify 'chemosensitive' tumours such as HX/151c.

Further to the above predictive testing considerations, these continuous cell lines may be of value

in an *in vitro* drug screening context in the light of the recent adoption of a panel of human tumour cell lines as an anticancer screen at the National Cancer Institute [40]. Indeed we anticipate using the lines as a secondary screen for novel 'third-generation' platinum compounds of interest that emerge from the ovarian carcinoma primary *in vitro* panel [36].

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